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An examination of the metabolism and pharmacokinetics of methadone with respect to stereoselectivity

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February, 2001

A thesis submitted for the degree of Doctor of Philosophy

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Abstract

- Methadone is a chiral molecule, almost exclusively administered as a racemic mixture and widely used for the pharmacological treatment of opioid addiction and also for the treatment of pain.

- *In vitro* studies in human liver microsomes identified that CYP3A4 mediates the N-demethylation of methadone to form the major metabolite EDDP. There was no difference in the affinity of (R)- and (S)-methadone for the enzyme(s) mediating the formation of EDDP or for the intrinsic clearance of this pathway. The lack of stereoselectivity indicates that stereoselectivity due to metabolic activity in this metabolic pathway *in vivo* is unlikely.

- The steady-state pharmacokinetics of rac-methadone and the individual enantiomers were examined in 18 methadone maintenance patients. There was considerable inter-individual variability in all methadone pharmacokinetic parameters, whether estimated from the individual enantiomers or the racemic compound. However, the extent of inter-individual variation in metabolic activity did not prevent a dose-plasma concentration relationship for the racemic compound or the methadone enantiomers.

- The sum of renal clearance and partial apparent clearance to EDDP accounted for less than 40% of methadone oral clearance, indicating that approximately two thirds of methadone clearance from plasma occurs via non-renal elimination of methadone and EDDP and/or further metabolism of these compounds. However, the urinary excretion of several other known methadone metabolites in unconjugated form did not account for a substantial proportion of the dose.

- The steady-state pharmacokinetics of methadone are stereoselective. (R)-methadone had a significantly greater unbound fraction (173%) and total renal clearance (182%) compared to (S)-methadone, while maximum plasma concentrations (83%) and apparent

partial clearance of methadone to EDDP (76%) were lower. In contrast, there were no significant differences between the methadone enantiomers for AUC_{τ}, oral plasma clearance, trough plasma concentrations and unbound renal clearance.

- When protein binding was considered, (R)-methadone oral clearance of the unbound fraction (59%) and apparent partial intrinsic clearance to EDDP (44%) were lower than for (S)-methadone. Patients excreted significantly more (R)-methadone and (S)-EDDP than the corresponding enantiomers. These data are in contrast to those predicted from the *in vitro* liver metabolism studies. Reasons for this lack of agreement include the elimination of EDDP by other stereoselective metabolic pathways and/or that there is stereoselectivity in the binding of methadone to proteins in the *in vitro* liver microsomal fraction.

- Stereoselective differences in the pharmacokinetics of methadone may have important implications for pharmacokinetic-pharmacodynamic modelling but is unlikely to be important for therapeutic drug monitoring of methadone, in the setting of opioid dependence.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

16/2/2001

David J R Foster

Acknowledgments

I would like to thank my supervisors Associate Professor Andrew Somogyi and Professor Felix Bochner. Your unerring guidance, mentoring and encouragement was invaluable over the course of my studies, as was your understanding and patience over the years.

Thank you to all the staff and students of the Department of Clinical and Experimental Pharmacology of the University of Adelaide. In particular, I would like to thank Associate Professor Anne Tonkin for her advice and assistance as Postgraduate Coordinator and Gordon Crabb for his computing assistance ("Bloody Macs!"). Special thanks go to Andrew Menelaou for the years of fellowship he provided, Sue Porter for the cheerful conversations, and to Dr Janet Coller for going through this whole process with me.

I would also like to thank the IMVS (Adelaide) and Dr Janet Coller for genotyping of donor liver DNA for CYP2D6 and CYP2C19, respectively; Dr Kyle Dyer for his invaluable work recruiting subjects and collecting samples; and Professor J White and the staff at the Warinilla Clinic of the South Australian Methadone Maintenance Programme for their invaluable assistance in this project.

Thanks must be made to the Royal Adelaide Hospital for providing a Dawes Postgraduate Scholarship, without which my studies would never have began, and to the National Health and Medical Research Council for providing a Dora Lush (Biomedical) Postgraduate Scholarship, without which my studies would have been difficult to complete. Thanks go to ASCEPT for providing travel grants that enabled me to attend national and international scientific meetings, and to the University of Adelaide Alumni Association for providing an RC Heddle travel grant, which made it possible for me to attend the 1998 IUPHAR meeting in Munich, Germany.

I would like to thank my parents Shirley and Dave Foster, and Margaret and Richard Green. Mum and Dad, I can't thank you enough for your unending love, support and belief in me throughout my whole life. 'Course, you know, the thesis is finished now Richard.

Words cannot express how much I value the companionship, love and support given to me by my wife Helen over the years. Thank you.

I spent four years prostrate to the higher mind, got my paper
And I was free

The Indigo Girls "Closer to Fine"

Publications in support of this thesis

Foster, D. J. R., Somogyi, A. A. & Bochner, F. (1999). Methadone N-demethylation in human liver microsomes: lack of stereoselectivity and involvement of CYP3A4. *British Journal of Clinical Pharmacology* **47**: 403-12.

Foster, D. J. R., Somogyi, A. A. & Bochner, F. (2000). Stereoselective quantification of methadone and its major oxidative metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, in human urine using high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Applications* **744**: 165-76.

Foster, D. J. R., Somogyi, A. A., Dyer, K. R., White, J. M. & Bochner, F. (2000). Steady-state pharmacokinetics of (R)- and (S)-methadone in methadone maintenance patients. *British Journal of Clinical Pharmacology* **50**: 427-40.

Additional publications associated with the work contained in this thesis

Dyer, K. R., Foster, D. J. R., White, J. M., Somogyi, A. A., Menelaou, A. & Bochner, F. (1999). Steady-state pharmacokinetics and pharmacodynamics in methadone maintenance patients: comparison of those who do and do not experience withdrawal and concentration-effect relationships. *Clinical Pharmacology and Therapeutics* **65**: 685-94.

Dyer, K. R., White, J. M., Foster, D. J. R., Bochner, F., Menelaou, A. & Somogyi, A. A. (2001). The relationship between mood state and plasma methadone concentration in methadone maintenance patients. *Journal of Clinical Psychopharmacology* **21**: 78-84.

Abbreviations, prefixes and symbols

AUC, AUC_{τ}, AUC_{y-z}	Area under the plasma concentration-time curve (AUC) during the dosing interval (AUC_{τ}) and the y to z time interval (AUC_{y-z})
B/F	Ratio of bound/unbound plasma concentration
CE	Capillary electrophoresis
C_{av}^{ss}	Steady-state plasma concentration, superscript "ss" also indicates that a pharmacokinetic parameter was calculated during steady-state dosing
C_{max}	Maximum measured plasma concentration
C_{min}	Minimum plasma concentration
$C_{min(first)}$	Minimum plasma concentration pre-study dose
$C_{min(last)}$	Minimum plasma concentration post-dose
CL	Total systemic plasma clearance
CL_{int}	Intrinsic clearance
CL/F	Apparent oral plasma clearance
CL_R	Renal clearance
$CL_{MD \rightarrow EDDP}$	Apparent partial clearance of methadone to EDDP *in vivo*
$CL_{MD \rightarrow EDDP\,u}$	Apparent partial clearance of unbound methadone to EDDP *in vivo*, the *in vivo* correlate of *in vitro* CL_{int} for the metabolism of methadone to EDDP
$CL_{MD \rightarrow EDDP}^{pred}$	The *in vitro* correlate of *in vivo* $CL_{MD \rightarrow EDDP}$, predicted from scaling up of microsomal incubation CL_{int} for the metabolism of methadone to EDDP incorporating plasma protein binding.
CI	Confidence interval(s)
CV	Coefficient of variation, expressed as a percentage
EC_{50}	Concentration eliciting 50% of the maximum effect
EDDP	Mono N-demethylated methadone metabolite
EMDP	Di-N-demethylated methadone metabolite
E_{max}	Maximum effect
f_e	Percent dose recovered
f_u	Unbound fraction in plasma, subscript "u" also indicates that a pharmacokinetic parameter is corrected for the unbound concentration of the drug
GC	Gas chromatography
HPLC	High performance liquid chromatography
γ	Sigmoidicity or slope factor (sigmoid E_{max} pharmacodynamic model)
IC_{50}	Concentration inhibiting 50% of ligand binding

i.m.	Intramuscular administration
i.t.	Intrathecal administration
i.v.	Intravenous administration
Ka	Binding association constant
k_{eo}	Rate constant for the removal of drug from effect site
K_i	Inhibition constant
K_m	Affinity constant of CYP450 enzyme for substrate, concentration at which the reaction rate is half V_{max}
MS	Mass spectroscopy
n	Number of binding sites per binding protein molecule
N	Molar binding site concentration
P	Statistical significance *P*-value
P	Molar concentration of the binding protein
p-	Para position on a benzene (aromatic) ring
P*	Apparent partition coefficient
pKa	Acidity constant $\log_{10}$ transformed
PM	Poor metaboliser
p.o.	Oral administration
P/T	Peak to trough plasma concentration ratio
r	Correlation coefficient
r^2	Coefficient of determination
(R)/(S)	Ratio of (R)/(S) enantiomer concentration
S	Substrate concentration
s.c.	Subcutaneous administration
SD	Standard deviation
SEM	Standard error of the mean
$t_{1/2}$, $t_{1/2\alpha}$, $t_{1/2\beta}$	Half-life ($t_{1/2}$) during the distribution ($t_{1/2\alpha}$) and terminal elimination ($t_{1/2\beta}$) phase
t_{max}	Time to reach maximum measured plasma concentration (C_{max})
U	Unbound molar concentration of ligand
V_d, V_c, $V_{d\beta}$, V_{dss}	Apparent volume of distribution (V_d) of the central compartment (V_c), during the terminal elimination phase ($V_{d\beta}$), and at steady-state (V_{dss})
V	Reaction velocity
V_{max}	Maximum reaction velocity

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1. Methadone: a review of the literature

The purposes of this review are manyfold: to introduce the concept of stereochemistry and its biological relevance; to review the historical development and current uses of the chiral compound methadone; to review the physicochemical and pharmacological properties of methadone and its many metabolites; to review the analytical methods that have been employed in the detection of methadone and its metabolites in biological fluids necessary for a comprehensive review of the complex metabolism and pharmacokinetics of methadone. This review will pay particular attention to stereochemistry, and will highlight its importance throughout.

With respect to the metabolism and disposition of methadone, this introduction will review the literature until the commencement of the work contained in this thesis in 1995-96. The results presented in this thesis will then be with compared with an up-to-date review of the literature in the relevant individual chapters of this thesis. Other, more general research, published after 1995-96 and not specific to the metabolism and disposition of methadone will be reviewed in the introduction.

1.1. History of methadone

The use of opium, derived from the dried juice of the unripe seed capsule of the opium poppy *Papaver somniferum*, has been documented for thousands of years. It is generally considered that the Sumerians cultivated the poppy and isolated opium at the end of the third century B.C. and that opium use spread from Sumeria to the remainder of the world (Brownstein, 1993). Opium was used medicinally for thousands of years. Indeed its analgesic and anti-diarrhoeal effects were known to the Sumerians and early Egyptians, and the therapeutic uses of opium were discussed by Hippocrates, Dioscorides and Galen (Brownstein, 1993; Dhawan et al., 1996). However, its euphoric effects were known even to the Sumerians, as they called "gil" ("joy") what is now known as opium, and the poppy plant "hul gil" ("plant of joy" Brownstein, 1993). As early as the eighth century A.D. Arab traders introduced opium to India and China, and by the tenth to thirteenth centuries opium

use had spread from Asia Minor to all parts of Europe (Brownstein, 1993, and references therein). Due to the well known mood-altering effects produced by opium, in particular euphoria, and increased availability, opium dependence became prevalent. Manuscripts dating back to the sixth century A.D. describe opium abuse and tolerance in Turkey, Egypt, Germany, England and China (Brownstein, 1993).

In 1806, the German chemist Sertürner reported the isolation of the principal active component of opium, morphine, which was then administered as a surgical premedication, as an analgesic post-surgically and for chronic pain (Brownstein, 1993; Dhawan et al., 1996; Van Ree et al., 1999). Not surprisingly, morphine was found to be as addictive as opium. Efforts were made to develop safer, more efficacious opioids that lacked the potential for dependence and abuse, and it was for this reason that heroin was synthesised in 1898. Unfortunately, history has shown that heroin did not realise this aim and has since became a widely abused drug. Since that time, research aiming to develop new opioid compounds has continued. However, morphine remains the standard against which new analgesics are compared.

In addition to the opiate alkaloids, and their semi-synthetic derivatives, which are grouped into the phenanthrene (morphine, codeine, thebaine) class of opioid compounds, a variety of other structurally distinct chemical classes of compounds have been found to possess pharmacological actions similar to morphine. These include the morphinans, benzomorphans, phenylpiperidines, propionilides, and the diphenylpropylamines.

At the end of World War II, the United States State Department sent the Technical Industrial Intelligence Committee to investigate the research activities at the I. G. Farbenindustrie at Hoechst-am-Main (Chen, 1948). The report published in 1945 examined 25 projects, among which the research involving new analgesics was identified as the most outstanding (Chen, 1948). The investigations revealed that chemists at the I. G. Farbenindustrie had discovered that derivatives of 3,3-diphenylpropylamine possessed

significant analgesic action (Chen, 1948). Similar reports were also made by the British Objectives Sub-Committee (Scott et al., 1948). One member of this group of compounds, with the I. G. serial number 10820, code named "amidon" for clinical testing by the German chemists, was subsequently the subject of much research in both the United States and the United Kingdom. Workers in the United States and the United Kingdom quickly confirmed the analgesic activity of 10820. In 1947, 10820 was introduced into therapeutic use and given the non-proprietary name methadone, although the names "miadone", and "amidone" or "methadon" were popular with many researchers in the United Kingdom and the United States, respectively (Chen, 1948; Isbell et al., 1948).

Methadone possesses a methyl group α to the basic nitrogen resulting in a stereocentre at the 6-carbon atom, having four different substituents attached to it (Figure 1-2). The German chemists considered that the (-)-enantiomer possessed four times the activity of the (+)-enantiomer (Scott et al., 1948). A sample labelled "l-amidon" obtained from the chemists at the I. G. Farbenindustrie possessed optical activity in agreement with that of the recently resolved (-)-methadone enantiomer (Small, 1948), and was found to possess twice the analgesic potency of the racemate (Thorp et al., 1947a). A second sample labelled "partly resolved d-amidon" obtained by United States agents in Frankfurt possessed no optical activity, containing impurities which probably included (-)-methadone (Small, 1948).

1.2. Stereochemical concepts

A molecule that possesses at least one atom with four different substituents attached to it is said to be chiral (Greek: *cheir* "hand") with the central atom referred to as the stereocentre. Chiral molecules are therefore asymmetrical and the spatial arrangement of the four substituents about a single stereocentre can be such that two non superimposable mirror images are possible (see Figure 1-1).

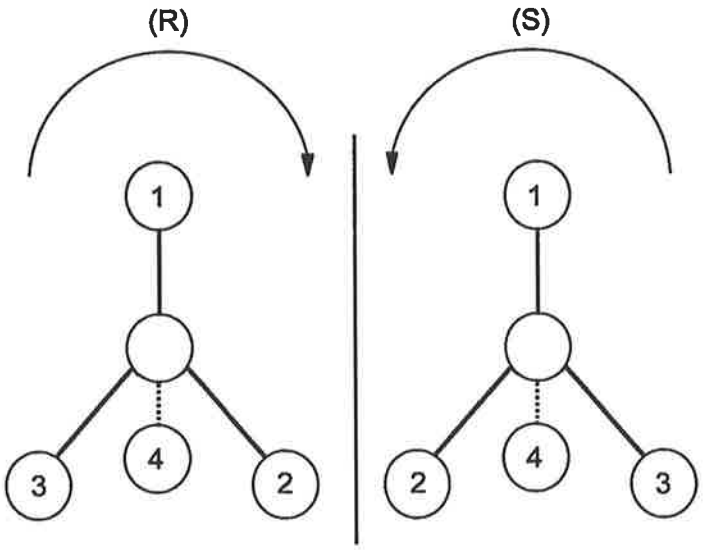

Figure 1-1: Schematic representation of the Cahn-Ingold-Prelog convention.
Notes: Order of priority: 1>2>3>4. Dotted lines indicate projection into the page.

The term enantiomer (Greek: *enantios* and *meros* "opposite parts") is used to refer to each of the non superimposable mirror images. In a non-chiral environment, enantiomers possess identical physical properties such as lipophilicity, pKa, and melting and boiling points, with one notable exception -the ability to rotate the plane of polarised light. This phenomenon is commonly used to distinguish between enantiomers, as each enantiomer will rotate the plane of polarised light in an equal but opposite direction under the same conditions. One enantiomer will rotate the plane of polarised light to the right (clockwise), and is termed the dextrorotatory (Latin: *dexter* "on the right side") enantiomer while the other enantiomer will rotate the plane to the left (anticlockwise) and is referred to as the levorotatory (Latin: *lævus* "on the left side"). The dextrorotatory enantiomer is denoted by a d- or (+)- prefix while the levorotatory enantiomer is assigned a l- or (-)- prefix. For example, d-(+)-methadone is the dextrorotatory mirror image of the levorotatory l-(-)-methadone enantiomer. An equimolar mixture of two enantiomers is optically inactive and is referred to as a racemic mixture or racemate, and is denoted by the rac- prefix.

The current system used to describe the absolute stereochemistry of a stereocentre is the Cahn-Ingold-Prelog convention (Cahn et al., 1966). This convention assigns an order of priority, principally according to atomic number, to each of the four substituents about the

stereocentre. When viewed from the side opposite the substituent of lowest priority, the absolute configuration is designated (R)- (Latin: *rectus* "right handed") if the order from highest to lowest priority describes a right-handed (clockwise) turn. If the order of the groups is left-handed (anti-clockwise), then the absolute configuration is (S)- (Latin: *sinister* "left handed") (see Figure 1-1).

The absolute configuration of a stereocentre can be determined by x-ray crystallography techniques or other indirect methods such as conversion of the compound to one of known stereochemistry via stereospecific chemical reactions. Although the most readily observed difference between enantiomers is their effect on the plane of polarised light, there is no simple relationship between the sign of optical rotation and the absolute configuration about the stereocentre. For example, an enantiomer may have an (S)- absolute configuration and be dextrorotatory [(+)-(S)-methadone] or levorotatory [(-)-(S)-warfarin]. The current recommended nomenclature system requires that chiral compounds be identified using both the optical rotation and absolute configuration (Chirality, 2001). However, for brevity only the Cahn-Ingold-Prelog (R)- and (S)- nomenclature will be used throughout this thesis when the absolute configuration of a compound is known, as this system provides structural information. When the absolute configuration of a compound is not known, the prefixes (+)- and (-)- will be applied to distinguish between enantiomers.

Molecules that contain two or more inequivalent stereocentres can exist as 2^n different stereoisomers, where n is the number of stereocentres. The stereoisomers consist of enantiomeric pairs, with a structure from one pair defined as diastereomeric when compared to a structure from another pair. The enantiomers have the opposite configuration at each stereocentre, while diastereomers do not. Diastereomers lack the mirror image relationship of enantiomers and possess different physicochemical properties.

A basic principle in the current understanding of the interactions between biologically active molecules requires that they interact in a "lock and key" fashion, and is termed

stereocomplimentarity. The principle of stereocomplimentarity originated mainly from the hypothesis proposed by Easson and Stedman in 1933 (for commentary see Gilbert & Greenberg, 1984; Smith, 1989). Their work on molecular asymmetry and physiological activity led them to propose the three-point interaction hypothesis, in which they explained how the spatial configuration of a molecule might play a critical role in its effects. They noted that the mirror image relationship of the atoms about a stereocentre precludes enantiomers from interacting in an identical fashion with three points on a receptor surface. This theory is not limited to drug-receptor interactions, but can be extended to interactions between any two chiral molecules. Although there are other important factors for drug-receptor site interactions, such as gross structural requirements, many cases of stereoselective interactions can be cited (for reviews see Nation, 1994; Tucker & Lennard, 1990).

Living organisms are dependent on a multitude of stereoselective processes and are consequently highly asymmetrical. For example, biosynthetic proteins and carbohydrates consist of optically pure amino acids and monomeric sugar rings; endogenous hormones and neurotransmitters are also optically pure. It is therefore not surprising that many chiral drugs exhibit stereoselective differences in metabolic, pharmacokinetic, pharmacodynamic and toxicological profiles (for reviews see Nation, 1994; Tucker & Lennard, 1990).

When comparing the relative pharmacological effects of enantiomers, it is possible that one enantiomer may be more potent than the other. In this case, the more potent enantiomer is referred to as the eutomer, while the enantiomer with lower potency is referred to as the distomer. This terminology can only be used when referring to a specific pharmacological effect, as the eutomer for one effect may be the distomer for a different effect, due to divergent receptor affinities between the enantiomers (Nation, 1994; Tucker & Lennard, 1990).

1.3. Current clinical uses of methadone

1.3.1. Treatment of pain

Methadone is effective in the treatment of acute pain, while its long term use is associated with the control of chronic pain and cancer pain that is non-responsive to or has lost responsiveness to high doses of morphine or other μ-opioid receptor agonists (oxycodone, fentanyl, hydromorphone), due to disease progression or the development of tolerance (Ayonrinde & Bridge, 2000). Methadone is regarded by the World Health Organisation as an alternative opioid to morphine in the management of moderate to severe cancer pain (WHO, 1996). In comparison to other opioid analgesics, the relatively low cost and long duration of action of methadone makes it an attractive alternative opioid analgesic (Bruera & Neumann, 1999; Bruera et al., 1995; Fainsinger et al., 1993; Watanabe et al., 1996). Many authors have found methadone to be equivalent to morphine in both potency and duration of analgesia after a single dose (for reviews see Fainsinger et al., 1993; Foley & Houde, 1998; Gannon, 1997; Ripamonti et al., 1997; Säwe, 1986). However, upon repeated administration, methadone produces prolonged analgesia in comparison to morphine postoperatively (Gourlay et al., 1986b), and in patients with severe cancer pain (Gourlay et al., 1986a), with fewer and less frequent supplementary doses required to maintain adequate pain relief (for reviews see Fainsinger et al., 1993; Foley & Houde, 1998; Gannon, 1997; Ripamonti et al., 1997; Säwe, 1986). The observed prolongation of analgesia can be attributed to its long elimination half-life, which results in accumulation of the drug upon multiple dosing if dosing intervals are inappropriately short (Gourlay et al., 1986b), producing undesirable effects such as respiratory depression (for reviews see Fainsinger et al., 1993; Foley & Houde, 1998; Gannon, 1997; Ripamonti et al., 1997; Säwe, 1986). Until recently, the lack of accurate estimates of equianalgesic doses on conversion from other opioid analgesics, combined with the risk of cumulative toxicity during chronic dosing, have prevented the more widespread use of methadone in this setting (Ayonrinde & Bridge, 2000). However, recent reports of more accurate estimates of equianalgesic doses (Ayonrinde & Bridge, 2000; Foley & Houde, 1998; Ripamonti et al., 1998) have led to a rediscovery of methadone for the treatment of chronic pain (Ayonrinde & Bridge, 2000;

Fainsinger et al., 1993; Gannon, 1997; Ripamonti et al., 1997). Despite this, large inter-individual variations in pharmacokinetics have decreased the enthusiasm for methadone among clinicians, and increased knowledge in this area is desirable (Bruera & Neumann, 1999; Fainsinger et al., 1993; Gannon, 1997; Ripamonti et al., 1997).

1.3.2. Treatment of opioid addiction

People addicted to opioids, primarily heroin, are at a high risk of suffering numerous medical and non-medical disorders, including psychiatric disorders, other substance abuse, infection through needle sharing (HIV, hepatitis) or sexually transmitted diseases, accidents and injuries, family dysfunction and domestic violence, unemployment, and criminal behavior (for commentary see O'Connor & Fiellin, 2000).

Since its introduction in 1964 (Dole & Nyswander, 1965), methadone maintenance has become the primary form of pharmacological treatment for opioid dependence, with about a quarter of a million patients treated globally (Farrell et al., 1994). Recent pharmacological treatment options include the use of (-)-α-acetylmethadol (an opioid receptor agonist), buprenorphine (a partial opioid receptor agonist), clonidine or lofexidine (both α_2-agonists), for the treatment of withdrawal symptoms; and detoxification using opioid receptor antagonists (naloxone, naltrexone) to precipitate a rapid withdrawal syndrome and antagonise the pharmacological effect of illicitly administered opioids post detoxification. However, these alternative pharmacological treatment options have yet to gain widespread use, or to consistently demonstrate improved patient outcomes compared to methadone maintenance therapy (for review see O'Connor & Fiellin, 2000).

Methadone has particular advantages over other opioid agonists in the treatment of opioid addiction because of its high oral bioavailability and relatively long elimination half-life (Kreek, 1979). These attributes form the basis of the current orthodox once daily dosage regimen. The main aims of methadone maintenance programs are directed (i) to the individual to reduce (and hopefully eliminate) illicit drug consumption, concomitant

criminal behavior and to improve medical and psychological status and social functioning; and (ii) to society to reduce the spread of potentially lethal infectious diseases associated with intravenous drug use, such as HIV and hepatitis B and C (Farrell et al., 1994). Randomised controlled studies of methadone maintenance, over substantial periods of time, in the United States (Dole et al., 1969; Strain et al., 1993b), Hong Kong (Newman & Whitehill, 1979) and Sweden (Gunne & Grönbladh, 1981) have shown it to be effective in realising these aims in widely varying cultural contexts. The study in Sweden (Gunne & Grönbladh, 1981) and one in the United States (Dole et al., 1969) compared methadone maintenance with no treatment, while the remaining studies were double-blind, placebo controlled comparisons of methadone maintenance and placebo, with comprehensive support services available to participants in both treatment groups (Newman & Whitehill, 1979; Strain et al., 1993b). Taken together these studies have provided consistent evidence of the effectiveness of methadone maintenance in retaining patients in treatment, reducing heroin use, and reducing the rate of incarceration while they remain in treatment. Further evidence of the effectiveness of methadone maintenance therapy for opioid dependence is provided by a series of observational studies which have surveyed a large number of opioid dependent individuals who have enrolled in methadone maintenance treatment programmes (for reviews see Farrell et al., 1994; O'Connor & Fiellin, 2000; Ward et al., 1992). However, the ability of methadone maintenance programs to achieve these aims is governed by certain individual patient characteristics and program practices. While motivation and compliance of patients are important (Gerstein & Harwood, 1990), so are the quality and quantity of counselor care (McLellan et al., 1988) and general clinic policy (Ward et al., 1992). Nevertheless, it appears that methadone dose is a critical determinant of patient compliance and retention in a program, with doses above 50 $mg.day^{-1}$ achieving greater success (Caplehorn & Bell, 1991; Caplehorn et al., 1993; Farrell et al., 1994; O'Connor & Fiellin, 2000; Strain et al., 1993b; Swensen et al., 1993; Ward et al., 1992).

Isbell and Eisenmann (1948a; 1948b) observed no objective (blood pressure, respiratory rate, heart rate, miosis) or subjective (euphoria, sedation) morphine-like effects after the

administration of 15 to 90 mg *s.c.* and *i.v.* of (S)-methadone to 12 non-tolerant former opioid dependent subjects. In contrast, (R)-methadone was very potent in inducing both subjective and objective morphine-like effects in non-tolerant subjects in doses ranging 5 mg to 15 mg. No amelioration of withdrawal symptoms was observed when 30 to 90 mg of (S)-methadone was administered to 10 opioid dependent subjects after abrupt cessation of chronic morphine administration (240-480 $mg.day^{-1}$), while (R)-methadone produced a marked diminution at doses ranging 6 to 23 mg. These authors found that (R)-methadone was approximately twice as potent as rac-methadone in all experiments, and concluded that the activity of the racemic compound was due solely to the (R)- enantiomer. Later, Fraser and Isbell (1962) reported that large doses of (S)-methadone produced opioid-like effects in some, but not all, non-tolerant former opioid dependent subjects (200 mg: 2 of 7 subjects, 300 mg: 7 of 12 subjects; 400 mg: 4 of 6 subjects). These authors also compared very large single doses of (S)-methadone (400-750 $mg.day^{-1}$) with morphine (18-90 $mg.day^{-1}$) for the suppression of withdrawal symptoms in subjects (n=10) dependent on 240 $mg.day^{-1}$ morphine. Dose response curves were constructed, and these authors calculated that approximately 15 mg of (S)-methadone was equivalent to 1 mg of morphine for the suppression of withdrawal symptoms. Despite this, all subjects exposed to the high doses of (S)-methadone experienced severe side-effects (nausea, vomiting, insomnia, nervousness, vision impairment), that were not present during morphine administration, and none reported the effects of (S)-methadone as enjoyable. The results of these studies indicate that (S)-methadone may elicit some opioid-like and non-opioid-like effects when administered in large doses. However, compared to (R)-methadone or morphine, (S)-methadone is markedly less potent, consistent with the relative opioid receptor binding affinities of these compounds (see section 1.5.2.1 below).

Noting the evidence of Isbell and coworkers (Fraser & Isbell, 1962; 1948a; 1948b) for the dysphoric effects of (S)-methadone, Judson and co-workers (1976) compared the rate of adverse events after administration of rac- and (R)-methadone in 66 methadone maintenance patients in a randomised double-blind study. Subjects received a 4 week treatment with

(R)-methadone at a dose containing an equivalent amount of (R)-methadone to their pre-study rac-methadone maintenance dose. Twenty subjective symptom measures, and five objective measures of program compliance, were rated at weekly clinic consultations using a structured checklist. Mean differences between the treatment groups were less than 10% for all measures, and did not reach statistical significance ($P>0.05$). Similarly, Scherbaum and co-workers (1996) studied a group of former opioid dependent patients maintained with (R)-methadone. At the time, only (R)-methadone was licensed for the treatment of opioid addiction in Germany, where the study was conducted. Using a randomised double-blind study design, subjects (n=26) either continued to receive (R)-methadone or were treated with rac-methadone containing an equivalent (R)-methadone content, for two weeks. These authors found no significant differences between the groups in dosage requirements, patient- or clinician-rated withdrawal symptoms, or rates of illicit drug use. Using a similar experimental protocol, de Vos and co-workers (1998) later confirmed the lack of difference between with rac- and (R)-methadone treatment in dosage requirements and rates of illicit drug use reported earlier, and extended this to include desire ("craving") for opioid use.

The results of the studies presented above indicate that, although (S)-methadone may elicit some opioid-like and non-opioid-like effects when administered in large doses, it is unlikely that it contributes to the pharmacological effects of rac-methadone when used in therapeutic doses. Despite this high eudismic ratio, methadone is used as the racemate in most countries.

1.4. Chemistry

1.4.1. Structure

1.4.1.1. Methadone

As an introduction to the following sections on the chemistry and pharmacology of methadone and its metabolites, the scheme in Figure 1-2 shows the pathways leading to the formation of identified methadone metabolites.

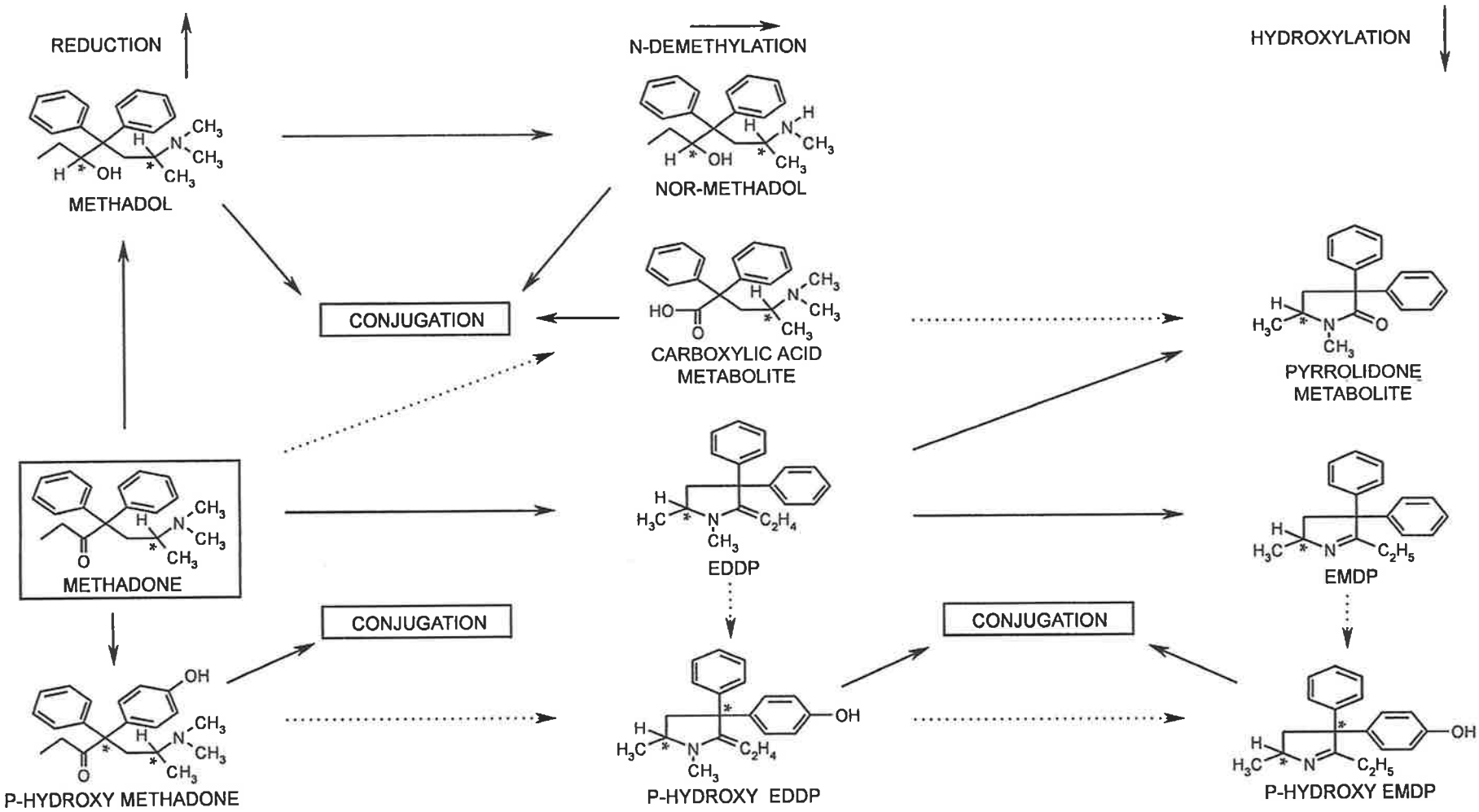

Figure 1-2: Metabolic pathways of methadone.

Notes: * Indicates a stereocentre. Dotted arrows indicate more than one possible pathway of formation.

The various synthetic approaches and structural determination of methadone have been adequately reviewed elsewhere (see Hardy & Howell, 1965; Small, 1948). Briefly, diphenylacetonitrile is treated with sodium amide and 2-chloro-N,N-dimethylpropylamide producing a mixture of two aminonitriles in nearly equal amounts. The appropriate aminonitrile is then purified and converted to methadone via a Grignard reaction with ethylmagnesium bromide and subsequent hydrolysis of the resulting ketimine (Hardy & Howell, 1965).

Methadone (6-dimethylamino-4,4-diphenyl-heptan-3-one, Figure 1-2) possesses a methyl group α to the basic nitrogen resulting in a stereocentre at the 6 position carbon atom. The individual methadone enantiomers were first resolved by Thorp and co-workers (1947b). Unable to resolve the methadone enantiomers directly, they successfully resolved the enantiomers of the pre-cursor aminonitriles, which were then readily converted to (+)- and (-)-methadone by the procedure outlined above (Thorp et al., 1947b). Later, Brode & Hill (1948) and Larsen and co-workers (1948) also resolved the methadone enantiomers using alternative techniques.

It was not until 1955 that the absolute configuration of the methadone enantiomers was examined. Beckett & Casy (1955) chemically converted D-(-)-alanine to the aminonitrile methadone pre-cursor via a series of reactions not involving the stereocentre. The optical rotation of the resultant aminonitrile corresponded to that of the (-)-methadone enantiomer, thus relating (-)-methadone to D-(-)-alanine (Beckett & Casy, 1955). As the absolute stereochemistry of D-(-)-alanine is known to be of the (R)- configuration, this result indicated that (-)-methadone was also of the (R)- configuration. The absolute configuration of the methadone enantiomers was later confirmed by Hanson & Ahmed (1958) using X-ray crystallographic techniques. These results confirm the absolute configuration of the enantiomers as (R)-(-)- and (S)-(+)-methadone.

The methadone molecule possesses a high degree of conformational flexibility due to the ability of the carbon skeleton to rotate about itself (Bürgi et al., 1973; Froimowitz, 1982; Portoghese et al., 1982). Based upon apparent acid dissociation constants (pKa) of methadone and structurally related compounds, it has been proposed that the protonated form in solution exists preferentially in a cyclic conformation due to intra-molecular folding which allows N(+)-H···O hydrogen bonding, thus stabilising this cyclic conformation (Beckett, 1956; Portoghese & Williams, 1969). Similar conclusions have been reached, based upon nuclear magnetic resonance (NMR) studies (Smith, 1966), and computer generated theoretical energy calculations, which indicated the cyclic conformation to be more stable than the acyclic form (Froimowitz, 1982). In contrast, a crystallographic study reported that the hydrochloride salt (the protonated form) prefers an extended acyclic conformation, due to inter-molecular hydrogen bonding, at least in the solid state (Hanson & Ahmed, 1958). Crystallographic techniques have shown that the methadone base in the solid state exists preferentially in a cyclic form, stabilised by an intra-molecular N:···C=O interaction (Bürgi et al., 1973). Due displacement of the C atom out of the plane of its neighbours by 6 pico metres towards the N atom, the N:···C=O distance (291 pico metres) is approximately 30 pico metres less than the Van der Waals radii of C and N (Bürgi et al., 1973).

1.4.1.2. Methadone metabolites

Early evidence suggested that methadone was metabolised by N-demethylation in animals and humans. Attempts to isolate or chemically synthesise N-desmethyl methadone were unsuccessful. In an attempt to synthesise N-desmethyl methadone, Pohland and co-workers (1959) found that it was a highly unstable compound that spontaneously cyclised and dehydrated, forming 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP, Figure 1-2). Subsequent studies have confirmed this finding by comparison with authentic compounds (Harper et al., 1966; Sullivan & Due, 1973), and have identified EDDP in urine and other biological fluids of both humans and animals administered methadone using chromatographic, NMR and mass spectrometry (MS) techniques (see sections 1.6 and 1.7).

Synthesis of the individual enantiomers of EDDP confirmed that the stereochemistry of the stereocentre is unchanged by the N-demethylation and cyclisation reactions, such that (R)-EDDP is formed from (R)-methadone (Beckett et al., 1975).

Noting evidence of both primary and secondary amine metabolites in rat urine following a methadone dose, Pohland and co-workers (1971) synthesised the N-desmethyl derivative of EDDP, 5-ethyl-2-methyl-4,4-diphenyl-3,4-dihydro-2*H*-pyrrole (EMDP, Figure 1-2). Using gas chromatography-mass spectroscopy (GC-MS) techniques and authentic compounds, EDDP and EMDP were detected in the urine of a volunteer after a single 10 mg oral dose of methadone (Pohland et al., 1971). Since that time EMDP has been identified in the urine of humans and animals by comparison with authentic compound using a variety of analytical techniques (see sections 1.6 and 1.7).

Reduction of either methadone enantiomer may lead to the formation of two diastereomeric alcohols (6-dimethylamino-4,4-diphenyl-heptan-3-ol), due to the creation of a second stereocentre at the 3 carbon position, such that reduction of rac-methadone produces a mixture of two pairs of diastereomeric enantiomers (methadol, Figure 1-2). The resultant pair of enantiomers are designated as α-methadol if the stereochemistry is the same at each of the two stereocentres, or β-methadol if the stereochemistry of each of the stereocentres is opposite (Pohland et al., 1949). It was observed that the direction of optical rotation of the α-methadol enantiomers was opposite to that of the parent methadone enantiomer (Pohland et al., 1949). In contrast, the β-methadol enantiomers retain the same direction of optical rotation as the parent methadone enantiomer (Eddy et al., 1952). Using a series of stereoselective chemical reactions, and subsequent NMR spectroscopy, Portoghese & Williams (1966; 1969) determined the absolute stereochemistry at the 3 carbon of α-(-)- and β-(-)-methadol to be of the (S)- configuration. As the stereochemistry of the methadone enantiomers was known at this time, they could assign the following absolute configurations to the four methadol stereoisomers: α-(-)-(3S,6S)-methadol, α-(+)-(3R,6R)-methadol, β-(-)-(3S,6R)-methadol and β-(+)-(3R,6S)-methadol. To date, both methadol and its N-demethylated metabolite, 6-methylamino-4,4-diphenyl-heptan-3-ol (N-desmethyl methadol,

Figure 1-2), have been identified (Änggård et al., 1975; Sullivan & Due, 1973) and quantitated (see section 1.7.3) in the urine of human methadone maintenance subjects, although confirmation of the absolute stereochemistry was lacking in most studies. However, Sullivan and co-workers (1972b) reported only the α- stereoisomers of N-desmethyl methadol in the urine of a patient receiving oral rac-methadone (80 $mg.day^{-1}$) for maintenance therapy, indicting that only the α- stereoisomers are formed in humans. These investigators derivatised the extracted samples, which allowed them to separate the α- and β-methadol compounds using GC-MS comparison with authentic compounds. However, this technique is unable to differentiate between the α-methadol enantiomers, therefore no conclusions can be made with respect to the absolute stereochemistry of the α-methadol formed. Thus, it is unknown whether this metabolic pathway is stereoselective.

During the course of their studies investigating the metabolism of methadone in maintenance subjects, Sullivan and co-workers (1972a) reported that urine samples contained relatively polar metabolites which were hydrolysable to less polar compounds by a mixture of β-glucuronidase and aryl sulfatase. Two compounds were isolated, and their structures were examined by GC-MS and chemical methods, producing results consistent with phenolic derivatives of EDDP, 4-(2-ethylidene-1,5-dimethyl-3-phenyl-pyrrolidin-3-yl)-phenol (p-hydroxy EDDP, Figure 1-2), and EMDP, 4-(2-ethyl-5-methyl-3-phenyl-4,5-dihydro-3*H*-pyrrol-3-yl)-phenol (p-hydroxy EMDP, Figure 1-2). The position of the phenolic hydroxyl group on the aromatic ring was tentatively assigned as para (p-), in accordance with other aromatic hydroxylation reactions, but was not confirmed. Baselt and co-workers (Baselt & Bickel, 1973; Baselt & Casarett, 1972a) reported that the mass fragmentation pattern of this metabolite was consistent with phenolic derivatives of EDDP and EMDP in human urine, and rat bile and urine. Although they could not state unequivocally that the GC conditions could distinguish between para- or meta- methoxy derivatives, they stated that based upon previous work, the meta- derivative would have a greater retention time than that observed. Other studies using GC-MS techniques have also identified a phenolic derivatives of EDDP and EMDP in rat bile (Abbott et al., 1985; Gerber

et al., 1977; Lynn et al., 1976a; Roerig et al., 1976) and human urine (see section 1.7.3). Due to the creation of a second stereocentre, hydroxylation of one aromatic ring may lead to the formation of two pairs of diastereomeric compounds. However, the absolute stereochemistry of this metabolic pathway remains unknown.

A conjugated phenolic metabolite of methadone has been identified in the bile and urine of rats, and the urine of humans. After hydrolysis of the urine sample, Sullivan & Due (1973) reported a methadone metabolite with a mass spectrum indicative of a phenolic derivative of methadone, and again tentatively assigned the para position to the hydroxyl substituent. Änggård and co-workers (1975) confirmed this phenolic metabolite, 6-dimethylamino-4-(4-hydroxy-phenyl)-4-phenyl-heptan-3-one (p-hydroxy methadone, Figure 1-2), in the urine of six methadone maintenance subjects by GC-MS of the methoxy derivatised compound. The mass fragmentation pattern was identical to that of methadone with the exception that all fragments containing the diphenyl group produced ions that were increased by 30 mass units, corresponding to the mass of the methoxy substituent. Again the para position was tentatively assigned to the phenolic substitution, awaiting confirmation by authentic compound. Later, Gérardy and co-workers (1986) isolated p-hydroxy methadone from the bile and urine of rats, and confirmed the para position of the phenolic hydroxyl substituent using ^{13}C NMR in comparison to authentic p-hydroxy methadone. Due to the creation of a second stereocentre, hydroxylation of one aromatic ring may lead to the formation of two pairs of diastereomeric compounds. However, the absolute stereochemistry of this metabolic pathway remains unknown.

A metabolite containing a carboxylic acid functional group, 4-dimethylamino-2,2-diphenyl-pentanoic acid (also commonly referred to as 4-dimethylamino-2,2-diphenylvaleric acid in the literature; carboxylic acid metabolite, Figure 1-2), has been detected in the urine of methadone maintenance subjects, and its structure confirmed by GC-MS comparison with the authentic compound (Sullivan & Due, 1973; Sullivan et al., 1972a). However, other

investigators have been unable to isolate or identify this compound as a metabolite in humans using GC-MS (Kang & Abbott, 1982).

A pyrrolidone metabolite (1,5-dimethyl-3,3-diphenyl-pyrrolidin-2-one); pyrrolidone metabolite, Figure 1-2), has been reported to be a methadone metabolite in humans and animals (Änggård et al., 1975; Beckett et al., 1968; Kreek et al., 1983; Kreek et al., 1980a; Sullivan & Due, 1973). The pathway leading to the formation of this metabolite is unclear, with conflicting hypotheses proposed by several authors. Sullivan & Due (1973) considered this metabolite to be formed via N-demethylation and subsequent spontaneous cyclisation of the carboxylic acid metabolite, analogous to the formation of EDDP, although no empirical evidence was provided. An alternative mechanism has also been proposed in which the pyrrolidone metabolite is the product of chemical oxidation of EDDP, a process that is not enzymatically mediated (Bowen et al., 1978; Kreek et al., 1983; Kreek et al., 1980a; Kreek et al., 1976b). In support of this hypothesis, Kang & Abbott (1982) reported a stoichiometric non-enzymatic decomposition of EDDP to the pyrrolidone metabolite using GC-MS. The first synthesis of EDDP was reported by Beckett and co-workers (1968). Two approaches were used, the most interesting of which used the pyrrolidone metabolite as the starting compound in the synthesis. Interestingly, in this and a subsequent report (Sullivan & Due, 1973), the authors noted that EDDP decomposed in contact with air and heat, but failed to investigate the process further. The weight of evidence favours the formation of the pyrrolidone metabolite via a non-enzymatic chemical oxidation process, which may occur *in vivo* or *ex vivo*. In summary, although the pyrrolidone compound is not a metabolite *per se*, it should be considered in mass-balance studies, as significant concentrations have been reported by several independent groups of investigators despite careful sample handling procedures.

Using GC-MS techniques and comparison with authentic material, methadone N-oxide (4,4-diphenyl-2-butenyl-ethylketone) was reported to be a major metabolite in humans (Beckett et al., 1972). The following year, methadone N-oxide was firmly established as an

experimental artefact caused by poor sample storage and/or handling procedures resulting in *ex vivo* oxidation of methadone to the N-oxide (Sullivan & Due, 1973; Sullivan et al., 1973). Measurable concentrations of the compound were only obtained after storage methadone solutions at 30°C for 5 days, while no methadone N-oxide was detected in samples that had been stored at -10°C and assayed after thawing (Sullivan & Due, 1973; Sullivan et al., 1973).

For a detailed review of the quantitative recovery of methadone and metabolites after methadone administration to human subjects, the reader is referred to section 1.7.3.1.

1.4.2. Physical and chemical properties

1.4.2.1. Methadone

The molecular weight of methadone as the free-base is 309.4. The pKa of the amine functional group of methadone has been reported to be 8.62 in 100% methanol at 23°C (Portoghese & Williams, 1969) and 8.99 in 100% water at 25°C (Beckett, 1956). Other investigators have used solvents comprising 50% ethanol in water, reporting values corresponding to a 100% water solvent of 9.64 at 20°C, and 9.26 at 37°C, when the data were corrected to for the presence of ethanol (Kaufmann et al., 1975). These values may appear to be conflicting, but when one considers the correction factor needed to adjust the value of 8.62 obtained in 100% methanol (approximately 1 unit) these values agree well (Kaufmann et al., 1975).

The optical rotation of the methadone enantiomers has been thoroughly reviewed by Janssen (1960). Typical $[\alpha]_D^{25}$ values for the hydrochloride salts in water are between +125° (Larsen et al., 1948) and +127° (Pohland et al., 1949) for (S)-methadone; and between -125° (Larsen et al., 1948) and -127° (Pohland et al., 1949) for (R)-methadone.

The apparent partition coefficients (P*) reported for methadone in various organic and aqueous (pH 7.4) phases, are given in Table 1-1.

Table 1-1: Apparent partition coefficients (P*) of methadone.

P*	Solvent System[a]	Temperature (°C)	Source
55.5	Octanol/67 mM phosphate buffer	25	Misra et al., 1974
43.2	Octanol/water	20	Kaufmann et al., 1975
116.3	Octanol/water	37	Kaufmann et al., 1975
37.0	Octanol/1 mM tris-HCl buffer	N/R	Mulé et al., 1974
38.4[b]	Octanol/200 mM phosphate buffer	N/R	Lynn et al., 1977
38.3[c]	Octanol/200 mM phosphate buffer	N/R	Lynn et al., 1977
44.7	Heptane/phosphate buffer[d]	N/R	Kutter et al., 1970
44.9	Heptane/200 mM phosphate buffer	N/R	von Cube et al., 1970

Notes: rac-methadone unless otherwise indicated; N/R=not reported; [a]pH of aqueous phase adjusted to 7.4; [b](R)-methadone; [c](S)-methadone; [d]molarity of buffer not reported.

Kaufmann and co-workers (1975) reported an increase in P* (octanol/water) from 58.7 to 229.0 as pH increased from 7.10 to 7.70 at 37°C; and from 43.2 at 20°C to 116.3 at 37°C at pH 7.4. Unexpectedly, Misra & Mulé (1973) reported markedly greater partitioning (octanol/pH 7.4 phosphate buffer) of (R)-methadone (P*=57.3) compared to (S)-methadone (P*=28.3). Lynn and co-workers (1977) examined the partitioning of methadone enantiomers, and reported no significant differences between the calculated P* values of (R)-, (S)- and rac-methadone using 2,2,4-trimethylpentane or 1-chlorobutane with aqueous phase pH values ranging 0-9. In addition, when the work of Misra & Mulé (1973) was re-examined by Lynn and co-workers (1977), the partitioning of (R)- and (S)-methadone in to octanol was not significantly different (Table 1-1). The apparent stereoselective partitioning of methadone reported by Misra & Mulé (1973) was probably due to the presence of radioactive impurities of the radiolabelled compounds used by these investigators, a factor circumvented by Lynn and co-workers (1977) who used unlabelled compounds. The true partition coefficient (ratio of the concentrations of the unionised species) between octanol and water increased from 7545 to 8621 as temperature was increased from 20°C to 37°C (Kaufmann et al., 1975). From inspection of the reported pKa values for methadone it can be calculated that methadone is less than 1% unionised at pH 7.4, which combined with the data presented above, indicates that unionised methadone is highly partitioned into non-polar organic solvents.

1.4.2.2. Methadone metabolites

Few studies have examined the pKa or apparent partition coefficients of any methadone metabolite. Baselt and co-workers (1973; 1972b) reported the pKa of the basic nitrogen atom of EDDP and EMDP to be 10.42 and 5.88, respectively. Portoghese & Williams (1969) reported a pKa of 7.86 and 7.59 for racemic α- and β-methadol, respectively, in 100% methanol at 23 °C, which are approximately 0.8-1.0 units lower than they reported for methadone under identical conditions. The pKa values reported were not corrected for aqueous conditions, which would be approximately 1 unit higher as noted in section 1.4.2.1. It is also unclear as to whether the reported pKa corresponds to the either the amino or hydroxyl functional group, or is a composite of the two.

Baselt & Bickel (1973) reported the "partition coefficient" (heptane/phosphate buffer pH 7.4, 23°C) of methadone, EDDP and EMDP to be 5.0, 0.04 and 13.4, respectively. The "partition coefficient" reported for methadone (5.0) is markedly different to that reported by other investigators (see Table 1-1). However, it is not clear how this was calculated. Despite this, comparison of the values reported for all three compounds is possible, as the same method was applied for all three compounds. As noted by the investigators, EMDP is an unusual metabolite, being considerably more lipophilic than the precursor compounds methadone and EDDP.

Garrett and co-workers (1985) examined the partitioning of methadone and several of its metabolites between different organic phases and buffers of varying pH in order to establish extraction procedures for analytical assays. Unfortunately, specific details of temperature or buffers employed were not provided. The partitioning was quantified using a "partition coefficient", presumably the apparent partition coefficient, although this was not clear. For simplicity, only the results with heptane as the organic phase will be reported here and are summarised in Table 1-2.

Table 1-2: Partitioning of methadone and metabolites between heptane and buffers of various pH.

Compound	P*	Buffer pH[a]
rac-Methadone	>7	>7
rac-EDDP	Negligible	<5
rac-EMDP	1.5	12.6
	0.6	7
	Negligible	<5
p-hydroxy methadone	0.4	9
α-(3S,6S)-methadol	>11	>7
	Negligible	<5
β-(3S,6S)-N-desmethyl methadol	1.4	12.6
	1	9
	Negligible	<5
rac-pyrrolidone	8-12	"almost all pH values"

Notes: [a]details of buffer composition not provided.
Source: Garrett and co-workers (1985).

The apparent partition coefficients reported by Garrett and co-workers (1985) for methadone and EDDP are in agreement with those of Baselt & Bickel (1973), while the values for EMDP vary markedly, being between 0.6 and 1.5 at pH 5 to 12.6 (Garrett et al., 1985) and 13.4 at pH 7.4 (Baselt & Bickel, 1973). From inspection of the pKa values reported for methadone (approximately 9.6), EDDP (approximately 10.4) and EMDP (approximately 5.9) it becomes apparent that methadone is less than 1% unionised at pH 7.4, EDDP is about 0.1% unionised, while EMDP is greater than 96% unionised. These calculations indicate EMDP would be highly partitioned into organic phases at pH 7.4, in comparison with methadone and EDDP. The stereochemistry of the p-hydroxy methadone compound at the two chiral centres was not reported by these authors.

Recently, Moody and co-workers (1999) examined the stability of methadone, EDDP, EMDP in urine samples. Aliquots of drug-free urine fortified at three concentrations (25 $ng.ml^{-1}$, 100 $ng.ml^{-1}$ and 300 $ng.ml^{-1}$) with methadone, EDDP and EMDP were stored at -20°C and assayed by GC-MS over intervals of 384 days (methadone), 272-280 days (EDDP) and 254-280 days (EMDP). Stability was assessed by the slope obtained from linear regression analysis of concentration versus time data. For methadone, the estimated slope values were not significantly ($P>0.05$) different to zero at any concentration. For

EDDP and EMDP, estimated slope values were not significantly ($P>0.05$) negative at any concentration. However, estimated slope values were significantly ($P<0.05$) positive for EDDP at 25 $ng.ml^{-1}$ and 300 $ng.ml^{-1}$, and at 100 $ng.ml^{-1}$ for EMDP. In summary, methadone, EDDP and EMDP concentrations in urine were shown not to significantly decrease when stored at -20°C for prolonged periods of time. However, the authors were not able to explain the observed increase in concentrations of EDDP and EMDP over time as sample dehydration was deemed unlikely due to the nature of the storage vessels.

1.5. Basic pharmacology of methadone and its metabolites

1.5.1. Overview of opioid receptors

A detailed review of opioid receptor pharmacology is beyond the scope of this thesis. However, a brief overview is necessary. In the mid 1960's, it was proposed that the actions of opioid agonists, antagonists and mixed agonist-antagonists could be best explained by the existence of more than one class of opioid receptor (Martin, 1967; Portoghese, 1965). In 1973, three independent groups demonstrated the existence of stereospecific opioid binding sites in mammalian brain (Pert & Snyder, 1973; Simon et al., 1973; Terenius, 1973). Martin and co-workers' (1976) studies with the chronic spinal dog led them to propose the existence of three classes of opioid receptor: μ (for morphine), κ (for ketocyclazocine) and σ (for SKF 10,047). Later, a fourth opioid class of receptor, the δ (for deferens) was proposed by Kosterlitz's group following their work with endogenous opioid peptides (Lord et al., 1977). Subsequently, the σ receptor has been shown to be non-opioid in nature, and the existence of other opioid receptor classes (ε, ζ, and λ) remains controversial (Connor & Christie, 1999; Dhawan et al., 1996; Simon & Gioannini, 1993).

In 1996, the Opioid Receptor Sub Committee of the International Union of Pharmacology (IUPHAR) proposed a new nomenclature consistent with the IUPHAR Nomenclature Committee's recommendations for mammalian receptors (Dhawan et al., 1996). This system designates the opioid receptors as OP (for opioid peptide) with numeric subscripts indicating the chronological cloning of the receptor types. Thus OP_1, OP_2, and OP_3

correspond to the δ-, κ-, and μ-opioid receptor types, respectively. However, this nomenclature has not been well adopted, and a revised system is expected in the near future. Therefore, this thesis will use the established Greek lettering nomenclature (δ, κ, and μ).

The δ receptor was the first opioid receptor to be cloned and was followed shortly after by the κ and μ receptors in a variety of species including humans (for recent reviews see Connor & Christie, 1999; Dhawan et al., 1996; Quock et al., 1999). Cloning of the δ, κ, and μ receptors established them as members of the superfamily of G-protein coupled receptors and act preferentially, although not exclusively, with the pertussis toxin sensitive G_i/G_o G-proteins (for review see Connor & Christie, 1999). The sequence of events that occur between the interaction of an agonist with the opioid receptor and the electrophysiological response of the neuron are complex. A detailed description is beyond the scope of the present thesis, and the reader is therefore directed to a series of recent reviews and commentaries: Connor & Christie, 1999; Dhawan et al., 1996; Koob & Nestler, 1997; Law et al., 2000; Quock et al., 1999. Briefly, opioid receptor activation results in recruitment of G_i/G_o G-proteins, leading to activation of inwardly rectifying K^+ channels and inhibition of voltage-gated Ca^{2+} channels, inhibition of adenylyl cyclase and the cAMP-protein phosphorylation cascade. These mechanisms result in inhibition of electrical excitability and decreased neurotransmitter release.

Opioid receptors are widely distributed throughout the central nervous system, while the myenteric nervous system of the gut also expresses μ-opioid receptors (for review see Dhawan et al., 1996; see also Madar et al., 1996; Pilapil et al., 1987; Quirion et al., 1987). Subtypes of δ (δ_1 and δ_2), κ (κ_1, κ_2 and κ_3), and μ (μ_1 and μ_2) receptor have been proposed based on *in vivo* and *in vitro* studies. However, only one example of each of the δ, κ, and μ receptors has been cloned from any one species. Alternative explanations for the evidence of these subtypes have been proposed; such the existence of alternative splice variants or post translational modifications; or a single receptor with multiple affinity states, coupled to different G-proteins or uncoupled in the plasma membrane (for recent reviews see Connor

& Christie, 1999; Dhawan et al., 1996; Quock et al., 1999). Despite this, it is currently considered that the δ, κ and μ recombinant receptors correspond to the δ_2, κ_1 and μ_1 subtypes (Dhawan et al., 1996).

1.5.2. Pharmacology of methadone

1.5.2.1. Receptor binding

1.5.2.1.1. Opioid receptors

Studies of the displacement by rac-methadone of a range of ligands specific for the respective opioid receptor sub-types located in homogenates prepared from bovine, guinea pig, mouse and rat brain have resulted in inhibition constants (K_i) for racemic methadone in the order of 4-30 nM (Chen et al., 1991; Codd et al., 1995; Kristensen et al., 1995; Magnan et al., 1982; Raynor et al., 1995), 15-750 nM (Codd et al., 1995; Kristensen et al., 1995; Magnan et al., 1982) and 76-1800 nM (Codd et al., 1995; Kristensen et al., 1995; Magnan et al., 1982) for the μ, δ and κ opioid receptors respectively. Studies examining the receptor binding of the individual methadone enantiomers have predominantly used the poorly selective μ opioid receptor antagonist naloxone. Reported ranges for the concentrations inhibiting 50% of ligand binding (IC_{50}) are 4.5-200 nM (Horng et al., 1976; Neil, 1984; Pert & Snyder, 1973; Wong & Horng, 1977) and 200-1000 nM (Horng et al., 1976; Pert & Snyder, 1973; Wong & Horng, 1977) for (R)- and (S)-methadone, respectively. Although there appears to be some overlap in the range of reported IC_{50} values, it should be noted that in all comparative studies, (R)-methadone was found to be 5-50 times more potent than (S)-methadone in inhibiting ligand binding (Horng et al., 1976; Pert & Snyder, 1973; Wong & Horng, 1977).

Kristensen and co-workers (1995) examined the binding of racemic, (R)- and (S)-methadone to μ_1 and μ_2, δ and κ opioid receptors in bovine caudate using ligands specific to these receptors. At the μ_1 (3 nM versus 26 nM), μ_2 (7 nM versus 88 nM), and δ (371 nM versus 9532 nM) receptors, (R)-methadone IC_{50} was one order of magnitude lower than for (S)-methadone. At κ receptors, both (R)- and (S)-methadone IC_{50} values (1332 nM

and 2137 nM, respectively) were similar. Codd and co-workers (1995) reported a similar opioid receptor specificity and stereoselectivity of methadone in rat forebrain membrane fragments using receptor-specific ligands. They found K_i values for (R)-methadone to be 20-fold lower than for (S)-methadone at μ receptors (1 nM versus 20 nM). (R)- and (S)-methadone K_i values for δ (371 nM and 960 nM, respectively) and κ (1860 nM and 1370 nM, respectively) receptors were similar for the two enantiomers.

1.5.2.1.2. NMDA receptors

A detailed discussion of the central nervous system distribution and intra-cellular second messenger systems N-methyl-D-aspartate (NMDA) receptors is beyond the scope of this thesis. However, the recent reviews by Mao (1999), Mao and co-workers (1995b), and Fundytus & Coderre (1999) are summarised briefly. NMDA receptors are ioinotropic receptors, gated in a voltage-dependent manner by Mg^{2+}. Receptor activation results in opening of the associated ion channel and influx of Ca^{2+} and Na^{+}, with a concurrent efflux of K^{+}. The influx of Ca^{2+} results in the activation of several intra-cellular messengers, including protein kinase C and nitric oxide. In general, NMDA receptors are located both spinally and supraspinally, with minimal variation in distribution between species (for review see Mao, 1999). Recently, there has been much interest in the interaction between NMDA and opioid receptors. Considerable evidence demonstrates that opioid receptors may modulate NMDA receptor-mediated electrophysiological events, and that there are interactions in the intra-cellular events mediated by the two receptors. The involvement of such interactions have been shown in the neural mechanisms of nociceptive transmission, antinociception, hyperalgesia, opioid tolerance/dependence, and neuroplasticity (for reviews see Fundytus & Coderre, 1999; Mao, 1999; Mayer & Mao, 1999; Mayer et al., 1999; Price et al., 2000; Trujillo, 1999). Briefly, NMDA receptor antagonism (MK-801, LY274614, dextromethorphan, ketamine) has been demonstrated to attenuate or reverse the development of tolerance to morphine induced antinociception and provide analgesia in some settings.

Ebert and co-workers (1995) examined the ability of rac-methadone to inhibit the binding of ^{3}H-labelled MK-801 (a selective non-competitive NMDA receptor antagonist) in rat cortical membrane preparations. They found that rac-methadone was similar in potency to dextromethorphan (a known antagonist for the non-competitive NMDA receptor site), with mean±SD K_i value of 0.85±0.31 μM. These authors confirmed the functional activity of NMDA receptor antagonism by rac-methadone in rat cortical wedge and neonatal spinal cord preparations, as a dose-dependent blockade of NMDA induced depolarisation was observed. In contrast, no effect was observed on kainate or AMPA receptor-mediated activities. Subsequently, Gorman and co-workers (1997) confirmed the binding of rac-methadone and the individual methadone enantiomers to the non-competitive site of the NMDA receptor in rat forebrain and spinal cord synaptic membrane preparations. They reported K_i values of 8.3±1.2 μM, 7.4±1.2 μM and 3.4±0.3 μM for rac-, (R)- and (S)-methadone, respectively, in the forebrain, while this was 2.5±0.0 μM, 2.6±1.4 μM and 2.8±0.9 μM in the spinal cord. There was no selective displacement of CGS-19755 indicating that methadone does not bind to the competitive NMDA receptor site.

Shimoyama and co-workers (1997) examined the antinociceptive effects of intrathecal (*i.t.*) (S)-methadone to determine whether the observed NMDA receptor antagonism *in vitro* results in functional *in vivo* activity. They found that *i.t.* (S)-methadone mediated its pharmacological effects via non-opioid receptor mechanisms, specifically from NMDA receptor antagonism. These authors speculated that although (S)-methadone is unlikely to contribute to the opioid-receptor mediated pharmacological effects of racemic methadone, it may contribute to the antinociceptive effects and attenuate the development of tolerance after administration of rac-methadone, via NMDA receptor antagonism.

The *in vivo* antagonism of NMDA-mediated hyperalgesia observed above was confirmed by Davis & Inturrisi (1999). These authors also examined the effect of spinal (*i.t.*) and systemic (*s.c.*) administration of (S)-methadone on the development of tolerance to the antinociceptive effect of morphine, using the rat and mouse tail flick assays. They

demonstrated that systemically co-administered (S)-methadone prevents systemically induced morphine tolerance; *i.t.* (S)-methadone attenuates the tolerance produced by *i.t.* morphine; *i.t.* (S)-methadone antagonises NMDA-induced hyperalgesia at the same dose which prevents the development of tolerance produced by *i.t.* morphine; providing further evidence that (S)-methadone affects the development of morphine tolerance and NMDA-induced hyperalgesia via NMDA receptor antagonism.

When taken together, these studies provide convincing evidence that (S)-methadone affects the development of morphine tolerance and NMDA-induced hyperalgesia via antagonism of the non-competitive NMDA receptor site. However, evidence in humans for this property of (S)-methadone is lacking. In the case of (R)-methadone, which is both a μ-opioid receptor agonist and NMDA receptor antagonist, the role of NMDA receptor antagonism in modulating the development of tolerance to its opioid receptor mediated pharmacological effects would be complex, and is unknown at present.

1.5.2.1.3. Serotonin re-uptake

Central serotonergic pathways are well established as being involved in pain regulation, and tricyclic antidepressants have long been used for the treatment of severe pain in non-depressed patients. Despite this, the antinociceptive mechanism(s) remain poorly understood, with evidence for a direct central potentiation of the endogenous opioid system, activation of mixed serotonergic and/or noradrenergic pathways, or various combinations of these mechanisms (for commentary see Bardin et al., 2000; Codd et al., 1995; Schreiber et al., 2000, and references therein).

Early reports indicted that rac-methadone elicited inhibition of 5-HT re-uptake *in vitro* (Donzanti & Warwick, 1979; Larsen & Hyttel, 1985; Slotkin et al., 1978). Noting the evidence for the differential inhibition of monoamine re-uptake and opioid receptor binding of the tramadol enantiomers, Codd and co-workers (1995) found that (R)- and (S)-methadone inhibited the re-uptake of both 5-HT and noradrenaline in rat brain

homogenates containing cerebral cortex and medulla-pons. (R)-methadone was 50-fold more selective for 5-HT compared to noradrenaline re-uptake (14 nM versus 702 nM), while this was 13-fold for (S)-methadone (992 nM versus 12700 nM). These data demonstrate that (R)-methadone is 70-fold and 18-fold more potent than (S)-methadone for the inhibition of 5-HT and noradrenaline re-uptake, respectively. Interestingly, morphine and naloxone did not inhibit either 5-HT or noradrenaline re-uptake, even at concentrations of 100 μM.

These authors concluded that monoamine re-uptake may modulate opioid-induced antinociception for compounds possessing both opioid receptor agonist and monoamine re-uptake inhibitory activity, although the contribution of noradrenaline re-uptake is likely to be minor. However, others have reported no effect of rac-methadone administration on brain 5-HT or noradrenaline concentrations *in vivo* after both acute and chronic (Middaugh & Zemp, 1976; Slotkin et al., 1978) administration, despite finding inhibition of this process *in vitro* (Slotkin et al., 1978). Therefore, the role of monoamine re-uptake inhibition in methadone elicited antinociception remains unclear.

1.5.2.2. Analgesia

Several groups investigated the analgesic effects of methadone in animals soon after the publication of its discovery at the end of World War II. Some of these results are summarised in Table 1-3 (see section 1.5.3). In general, the (R)- enantiomer is consistently reported as being several fold more potent in terms of analgesia, although there is species variation. In rats and mice it can be seen from Table 1-3 that (R)-methadone is 3-50 times more potent than the (S)- enantiomer, while in dogs (R)-methadone has been shown to be approximately 25 times more potent (Scott et al., 1948). In humans, a dosage of 160 mg *i.v.* of (S)-methadone was required to produce the same level of analgesia elicited by only 3 mg *i.v.* of (R)-methadone, resulting in a corresponding potency ratio of approximately 50 in favour of the (R)- enantiomer (Scott et al., 1948). When the potency of the racemic mixture is compared to that of the (R)- enantiomer, ratios of 1.6 (Scott et al., 1948) and 2.0 (Thorp et

al., 1947a) have been reported in rats. These potency ratios are consistent with the expected ratio (2.0) if (S)-methadone is assumed to contribute very little to the observed analgesic activity of the racemate.

The opioid receptor binding data above support the view that methadone elicits its analgesic action predominantly via an interaction with μ receptors, and that the majority of the observed analgesia produced by the racemate is elicited by the (R)- enantiomer. Pasternak and Wood (Pasternak & Wood, 1986) proposed that there are two μ opioid receptor subtypes, and that it is the μ_1 receptor that mediates analgesia. However, this remains controversial. In any case, Kristensen and co-workers (1995) have shown (R)-methadone to possess comparable affinities at each of the putative μ_1 and μ_2 receptor subtypes, and to have a 10-fold greater affinity than (S)-methadone at these receptors. However, the role of NMDA receptor antagonism in the analgesia mediated by methadone further complicates this issue.

1.5.2.3. Respiration

For a detailed review of the effects of opioids on respiration the reader is directed to the reviews of Flórez and Hulé (Flórez & Hurlé, 1993), and White & Irvine (1999), and references therein. Opioid agonists act on receptors in the medulla to produce a dose-dependent reduction in minute volume leading to an increase in the partial pressure of carbon dioxide, and in particular, depression of the ventilatory response to an increase in the partial pressure of carbon dioxide. There is general agreement amongst investigators that the respiratory depression observed after opioid administration is mediated via the activation of δ and μ receptors. Ling and co-workers (1985) have suggested that it is the purported μ_2 receptor which mediates the respiratory effects of opioids, rather than the μ_1 receptor proposed by Pasternak and Wood (1986) to mediate analgesia. However, this remains controversial.

Potentially the most serious medical complication associated with methadone use is respiratory depression, especially after repeated administration due to accumulation of the drug. The development of tolerance to the respiratory depressant activity of opioid compounds appears to be slow, and may not fully develop despite long-term opioid treatment. Several studies have shown that patients in long-term methadone maintenance treatment programmes exhibit significant respiratory depression after administration of their daily rac-methadone dose (Dyer et al., 1999; Marks & Goldring, 1973; Olsen et al., 1970; Olsen et al., 1981; Santiago et al., 1977), although others have found no effect (McCaul et al., 1982). Marks & Goldring (1973) suggested that the tolerance to the carbon dioxide-sensitive chemoreceptor mechanism was essentially complete, while tolerance to hypoxia-sensitive chemoreceptor mechanism was incomplete. In healthy normal subjects, Olsen and co-workers (1977) compared the effects of placebo and acute oral administration of doses of 15 mg rac-, 7.5 mg (R)- and 7.5 mg (S)-methadone (per 1.79 m^2 of body surface area) on carbon-dioxide induced respiratory depression. These authors reported that the dose-response curve and the area under the dose-effect curve for (S)-methadone did not differ from placebo. There was marked respiratory depression after rac- and (R)-methadone, that were not different (P>0.5) between the two compounds. At doses of 50 mg and 100 mg of (S)-methadone, the area under the dose-effect curves were <20% that of the mean values for rac- and (R)-methadone. Similarly, Scott and co-workers (1948) reported that (R)-methadone was 25-fold more potent in inducing respiratory depression, as measured by respiratory minute volume, in anaesthetised dogs.

In summary, these data demonstrate that tolerance to the respiratory depressant effects of methadone does not fully develop, even after long-term chronic treatment, and that these effects are likely to be due to the (R)-enantiomer.

1.5.2.4. Gastrointestinal effects

Opioid compounds alter autonomic outflow to the gut from within the central nervous system, and act directly on the myenteric nervous system to affect gastrointestinal motility.

The effects of opioids on the gastrointestinal tract of animals and humans has been reviewed by Kromer (1993), which will be briefly summarised. The gastrointestinal opioid system serves a neuromodulatory function, physiologically interacting with smooth muscle cells and several gastrointestinal neurotransmitters (such as acetylcholine), with evidence for the involvement of μ, δ and κ receptors. Opioid agonists have been shown to delay gastric emptying and/or inhibit gastric contractions in sheep, goat, cat, dog, rhesus monkey and humans. Opioid agonists appear to both switch the intestinal motility pattern from peristalsis to segmentation, and to cause constipation.

Constipation is one of the major complications associated with methadone use, particularly in maintenance therapy (Dole & Nyswander, 1965; Kreek, 1973a; Kreek, 1979). Of 214 methadone maintenance subjects, 59% reported laxative use for the treatment of constipation, which continued for an average of 8 months, while this reduced to 17% after three or more years of maintenance treatment (Kreek, 1973a). More recently, a similar incidence (60%) of constipation was found, albeit in a smaller sample (n=22) of subjects (Yuan et al., 1998). The tonus of the sigmoid and defecation reflex remains depressed even in patients with a high degree of tolerance to other methadone related opioid effects (Dole & Nyswander, 1965). Over a period of one week, daily X-ray examinations of patients given a barium sulfate meal showed that the motility of the upper gastrointestinal tract and small intestine was normal, but that of the colon was abnormally slow (Dole & Nyswander, 1965).

Yuan and co-workers (1998) examined the oral-caecal transit time in 17 methadone maintenance subjects without evidence of significant gastrointestinal disorder, using the lactulose hydrogen breath test. Oral-caecal transit time in the methadone maintenance subjects (mean±SD; 159±49 minutes) was significantly ($P<0.01$) longer than in healthy normal subjects from this groups previous studies (105±31 minutes, Yuan et al., 1996; 114±37 minutes, Yuan et al., 1997), indicating that tolerance to this opioid action of methadone was incomplete. Furthermore, in a subsequent study this group demonstrated that *i.v.* methylnaltrexone, an opioid receptor antagonist considered not to cross the blood

brain barrier, was able to reduce oral-caecal transit time by 78±37 minutes, while placebo treatment was ineffective (Yuan et al., 2000b). Additionally, an immediate laxation response was obtained in all methylnaltrexone treated subjects, but not after placebo, and subjects in either group did not experience withdrawal symptoms (Yuan et al., 2000b; Yuan et al., 1999). These data agree with this group's previous observations that methylnaltrexone prevents morphine induced delay of oral-caecal transit in healthy subjects, without affecting analgesia (Yuan et al., 2000a; Yuan et al., 1996; Yuan et al., 1997), and provide evidence that the constipating effect of opioid treatment is predominantly mediated by opioid receptors located peripherally in the gastrointestinal tract, rather than in the central nervous system.

1.5.2.5. Nausea and vomiting

Nausea and vomiting is sometimes associated with methadone administration (Chen, 1948; Dole & Nyswander, 1965; Kreek, 1973a; Kreek, 1979). This effect most likely arises out of stimulation of opioid receptors in the chemoreceptor trigger zone on the dorsal medullary surface. Stimulation of this zone activates a "vomiting centre" deeper within the medulla which the initiates and coordinates the vomiting reflex (Flórez & Hurlé, 1993). As vomiting also occurs after parenteral administration of methadone, it is unlikely that this effect is mediated by local irritation of the gastric mucosa (Chen, 1948).

1.5.2.6. Tolerance and dependence

Despite limiting the usefulness of opioid analgesics, the underlying mechanisms of the development of analgesic tolerance and physical dependence after repeated administration remain poorly understood. Tolerance to a drug effect is characterised by decreased efficacy of the drug for a given response, with a corresponding increase in the dosage required to elicit an adequate response. Physical dependence is manifested as a need for continued administration of the drug in order to prevent the occurrence of an undesirable withdrawal syndrome. In the case of opioid withdrawal syndrome, this consists of signs and symptoms which generally reflect a rebound of the organ systems affected by opioids (O'Brien, 1993;

Way, 1993). Signs and symptoms of opioid withdrawal can include the central nervous system (pupillary dilatation, dysphoria, restlessness, irritability, insomnia, drug craving), gastrointestinal system (nausea, vomiting, diarrhoea), skin (piloerection), and mucous membranes (rhinorrhea, lacrimation) (Gossop & Strang, 1991; Kanof et al., 1992; O'Brien, 1993; Strain et al., 1993a; Way, 1993). While psychosocial and cultural influences are undoubtedly important factors in determining continued drug use, the unpleasantness and severity of this syndrome may be such that sufferers continue drug use as a preventative measure, in effect acting as negative reinforcement of continued drug use (Koob & Nestler, 1997).

Tolerance does not necessarily occur over a specific time-frame, which may depend on the compound, dose and route of administration; and develop at different rates and/or extents to the various pharmacological effects elicited by the compound (for review see Simon, 1992). Tolerance can be divided into several broad classes (O'Brien, 1996). Innate tolerance is associated with reduced sensitivity to the pharmacological effects of a drug that are genetically determined. Learned or conditioned behavioral tolerance describes the development or adaptation of compensatory behavioral skills which diminish the magnitude of the drug-induced pharmacological effect (see Redmond & Krystal, 1984; Trujillo & Akil, 1991). Pharmacokinetic tolerance is mediated by a decrease in concentration of the drug at the effect site, and may be due to increased elimination. With respect to methadone, it is unlikely that pharmacokinetic tolerance is of significant importance, as the evidence is weak (see sections 1.7.3 and 1.7.5). Pharmacodynamic tolerance is associated with adaptive changes within the systems affected by the drug, and may involve alteration in the function and number of receptors and their associated effector mechanisms. Some authors have suggested that receptor down regulation is important, although this remains controversial (for reviews and commentaries see Bhargava, 1991; Koob & Bloom, 1988; Law & Loh, 1999; Simon, 1992; Trujillo & Akil, 1991; Werling et al., 1989). Others have implicated up-regulation of cyclic AMP pathways and a functional de-coupling of opioid receptors from the G-protein-regulated intra-cellular mechanisms (for reviews and commentaries see

Nestler & Aghajanian, 1997; Trujillo & Akil, 1991; Werling et al., 1989). A model incorporating observed increases in protein kinase C translocation, resulting in phosphorylation of opioid receptors or their associated G_i-protein, and phosphorylation of the NMDA receptor-Ca^{2+} channel has recently been proposed by Mao and coworkers (see Mao, 1999; Mao et al., 1995a; Mayer & Mao, 1999; Trujillo, 1999).

In summary, although it has been the subject of much research producing significant advances in the understanding underlying mechanisms, the pathophysiology of tolerance, with respect to opioid compounds, remains incompletely understood.

1.5.3. Pharmacology of methadone metabolites

In the rat tail flick assay, EDDP and EMDP have been shown to be devoid of analgesic activity at 30 and 60 minutes after doses of up to 200 $mg.kg^{-1}$ via oral and subcutaneous routes of administration (Pohland et al., 1971). The same doses of EDDP did not antagonise the analgesia produced by 1 $mg.kg^{-1}$ subcutaneous morphine in the rat tail flick assay (Pohland et al., 1971). Similarly the analgesia produced by 0.05 $mg.kg^{-1}$ of racemic methadone administered subcutaneously was unaffected by EMDP at doses of 100 $mg.kg^{-1}$ via both the oral and subcutaneous routes of administration (Pohland et al., 1971).

The analgesic activity of p-hydroxy methadone has been assessed in mice using the hot-plate assay and was found to be inactive (Gérardy et al., 1986). Unfortunately the details of this investigation were not reported in any detail.

As the methadol diastereomers were first synthesised in an attempt to find more potent analgesics as an alternative to methadone, the pharmacological activity of the methadol diastereomers has been extensively studied with regard to analgesia (for review see Janssen, 1960). The analgesic activity of the methadol stereoisomers presented something of a conundrum to investigators, due to an observed "inversion" of activity in comparison to the parent methadone enantiomers. It was quickly established that the more potent methadol stereoisomer was derived from the less potent (S)-methadone enantiomer (Eddy et al., 1952;

Pohland et al., 1949), while the methadol stereoisomers obtained from (R)-methadone were comparatively less potent, especially with respect to the parent methadone enantiomer (Eddy et al., 1952; Pohland et al., 1949). These results are summarised in Table 1-3.

Table 1-3: Antinociceptive activity of the methadol diastereomers in comparison to that of the methadone enantiomers.

Compound	Method[a]	Route	ED_{50} ($mg.kg^{-1}$)	Potency Ratio[b]	Source
(6S)-methadone	M	*p.o.*	89.3	0.09	Eddy et al., 1952
	M	*s.c.*	25.7	0.03	Eddy et al., 1952
	RF	*s.c.*	24[c]	0.05	Pohland et al., 1949
	RP	*i.p.*	30[d]	0.13	Scott et al., 1948
α-(3S,6S)-methadol	M	*p.o.*	3.8	2.11	Eddy et al., 1952
	M	*s.c.*	3.5	0.23	Eddy et al., 1952
	RF	*s.c.*	5[c]	0.25	Pohland et al., 1949
β-(3R,6S)-methadol	M	*p.o.*	70.0	0.11	Eddy et al., 1952
	M	*s.c.*	63.7	0.01	Eddy et al., 1952
(6R)-methadone	M	*p.o.*	8.0	1	Eddy et al., 1952
	M	*s.c.*	0.8	1	Eddy et al., 1952
	RF	*s.c.*	1.3[c]	1	Pohland et al., 1949
	RP	*i.p.*	4.0[d]	1	Scott et al., 1948
α-(3R,6R)-methadol	M	*p.o.*	61.8	0.01	Eddy et al., 1952
	M	*s.c.*	24.7	0.03	Eddy et al., 1952
	RF	*s.c.*	80[c]	0.02	Pohland et al., 1949
β-(3S,6R)-methadol	M	*p.o.*	36.7	0.22	Eddy et al., 1952
	M	*s.c.*	7.6	0.11	Eddy et al., 1952

Notes: All compounds tested as the hydrochloride salts. [a]M=Mouse hot plate assay; RF=Rat tail flick assay; RP=Rat tail pinch assay; [b]Ratio of the ED_{50} of (R)-methadone divided by that of the compound for the same route of administration within the same study; [c]"Threshold dose" shown to elicit an analgesic response; [d]"Dose of equal activity" defined as the doses of (R)- and (S)-methadone which resulted in an equianalgesic response.
Source: Adapted from Janssen (Janssen, 1960).

From Table 1-3 it can be seen that while the (6R)- configuration is important for analgesic potency of the methadone enantiomers, this is less important in the methadol compounds where the (3S)- configuration is more important. Unexpectedly, β-(3S,6R)-methadol, possessing both the (6R)- and (3S)- configurations, is less potent than α-(3S,6S)-methadol which possesses only one of the important configurations (3S). This anomaly may be explained in part by examining the work of Sullivan and co-workers (1972b) with the N-desmethyl derivatives of the α-methadol enantiomers. This study reported that α-(3S,6S)-N-desmethyl methadol was 2-3 times more potent than its precursor

α-(3S,6S)-methadol in both the rat tail flick and mouse writhing assays for analgesia (Sullivan et al., 1972b). The analgesic activity of α-(3R,6R)-N-desmethyl methadol was also studied and found to be inactive in the rat tail flick assay and $1/40^{th}$ as potent as the α-(3S,6S)- enantiomer in the mouse writhing assay (Sullivan et al., 1972b). As the authors suggested at the time, the analgesia observed after administration of α-(3S,6S)-methadol or (S)-methadone may be elicited by formation of the more potent α-(3S,6S)-N-desmethyl metabolite. There are lines of evidence in support of this theory: the presence of α-(3S,6S)-N-desmethyl methadol in concentrations greater than that of α-(3S,6S)-methadol in the bile and urine of rats after administration of (S)-methadone (Sullivan et al., 1972b), and both α-(3S,6S)-methadol and (3S,6S)-N-desmethyl methadol are formed *in vitro* from (S)-methadone in rat liver homogenate (Sullivan et al., 1972b). While the analgesic activity of β-(3S,6R)-N-desmethyl methadol has not been determined, it would seem unlikely that it contributes to the activity of its precursor β-(3S,6R)-methadol, as the ED_{50} of β-(3S,6R)-methadol is 5-fold greater after oral as compared to *s.c.* administration (Eddy et al., 1952). If the activity of β-(3S,6R)-N-desmethyl methadol was significant, then bioactivation of β-(3S,6R)-methadol via first-pass metabolism would be expected to result in a lower ED_{50} for the oral route of administration, due to a greater formation of the putative β-(3S,6R)-N-desmethyl methadol active metabolite.

This review of the literature revealed only two studies investigating the receptor binding affinity of any methadone metabolite. Using authentic compounds, Horng and co-workers (1976) examined the opioid receptor binding of the methadone enantiomers, and the α-methadol and α-N-desmethyl methadol enantiomers in rat brain homogenate using the poorly selective μ opioid receptor antagonist naloxone. The results of this study are summarised in Table 1-4. It is worth noting that the IC_{50} values of the methadol compounds are increased by one to two orders of magnitude in comparison to their respective parent methadone enantiomers, as is the α-(3R,6R)-N-desmethyl methadol derived from the more potent (R)-methadone. In contrast, the α-(3S,6S)-N-desmethyl methadol, derived from the less potent (S)-methadone enantiomer, has an IC_{50} 3- and 4- orders of magnitude lower than that of its parent (S)-methadone and α-(3S,6S)-methadol enantiomers, respectively, and one

order of magnitude lower than the more potent (R)-methadone enantiomer. This is in agreement with the earlier *in vivo* analgesia data, and is consistent with the proposal that α-(3S,6S)-N-desmethyl methadol is mediating some, if not all, of the analgesia observed after administration of α-(3S,6S)-methadol. Additionally, α-(3S,6S)-N-desmethyl methadol may possibly contribute to the *in vivo* opioid-like effects of (S)-methadone in the rat, thus adding further complexity to the interpretation of such experiments.

Table 1-4: IC_{50} values for the displacement of ^{3}H-labelled naloxone by the individual enantiomers of methadone, α-methadol and α-N-desmethyl methadol in rat brain homogenate.

Compound	IC_{50} (nM)
(6S)-methadone	250
α-(3S,6S)-methadol	2500
α-(3S,6S)-N-desmethyl methadol	0.35
(6R)-methadone	5
α-(3R,6R)-methadol	500
α-(3R,6R)-N-desmethyl methadol	380

Source: Adapted from Horng and co-workers (1976).

Using authentic p-hydroxy methadone, Gérardy and co-workers (1986) reported a "poor affinity" for opioid receptors in rat brain preparations. Unfortunately, these authors provided no details of the concentrations or methods employed, or stereochemistry of the compounds used.

In summary, of the known methadone metabolites examined, only those of the methadol series have been shown to exhibit significant opioid receptor binding or analgesic activity. Of particular interest are α-(3S,6S)-methadol and α-(3S,6S)-N-desmethyl methadol metabolites derived from the relatively inactive (S)-methadone enantiomer. These two metabolites possess analgesic activity comparable to that of the more potent (R)-methadone enantiomer, and may contribute to the activity of (S)-methadone the rat (Smits & Myers, 1974; Sullivan et al., 1972b). However, it is unlikely that the methadol metabolites contribute to the activity of (S)-methadone in humans, as they account for very little of the administered dose (see section 1.7.3.1).

1.6. Methods for the quantification of methadone and metabolite concentrations in biological fluids

The brief outline of the chemistry and metabolic pathways of methadone given in section 1.4 is sufficient for a concise discussion of the methods employed by investigators for the determination of methadone and its metabolites in biological fluids; this is necessary for a review of the metabolic profile and pharmacokinetics of methadone to follow. For a detailed review of the methods available to date, the reader is directed to Chapter 2. Additionally, summary tables are provided for comparison of the non-stereoselective (Appendix 1) and stereoselective (Appendix 2) assays that have provided validation data (precision, accuracy, extraction efficiency, selectivity assessments). When viewing the table in Appendix 2, the readers attention is drawn to the fact that before the commencement of the present thesis in 1995-96, there were few published stereoselective assays for the quantification of enantiomers of methadone. Indeed, none of the published assays was applied to the investigation of the disposition of methadone in more than a single subject prior to 1996.

1.6.1. Non-stereoselective assays

1.6.1.1. Radioimmunoassay

Methadone plasma concentrations are typically less than 50 $ng.ml^{-1}$ after acute administration in humans. The search for simple, highly sensitive methods for the determination of methadone in biological fluids led to the development of radioimmunoassay (RIA) techniques. Due to the availability of radiolabels of high specific activity, RIA potentially offers simple assay procedures of high sensitivity. Cross-reactivity of antisera with compounds of similar structure to the analyte, such as metabolites and other commonly co-administered drugs, must be assessed if an RIA assay is to considered valid.

RIA assays for methadone in plasma have developed by two independent groups (Bartos et al., 1977; Ling et al., 1981). However, only the assay developed by Ling and co-workers (1981) has been applied in human pharmacokinetic studies. Cross-reactivity with several

methadone metabolites and other opioid analgesics, including those structurally related to methadone, was adequately addressed by both groups, and found to be <0.5%. Plasma concentrations of the major metabolite EDDP have been shown to be approximately 10% those of methadone, while concentrations of this metabolite in urine are comparable to those of methadone. Other methadone metabolites have not been detected in plasma, and are present in concentrations several-fold lower than unchanged methadone in urine (see section 1.7). These data indicate that cross-reactivity with methadone metabolites is unlikely to result in significant (>1%) over-estimation of methadone concentrations by RIA assays. Acceptable intra- and inter-assay precision and accuracy data were provided by Ling and co-workers (Ling et al., 1981), while this was not addressed by Bartos and co-workers (1977).

More recently, commercially available solid-phase RIA techniques for the quantification of rac-methadone have become available. Although generally considered semi-quantitative compared to more sophisticated GC-MS and high performance liquid chromatography techniques, several authors have successfully modified these assays to be used for pharmacokinetic-pharmacodynamic studies. These assays are reported by the manufacturers not to cross-react with the methadone metabolites EDDP and EMDP, or with a variety of morphine-like opioid compounds.

In summary, despite good sensitivity from small sample volumes, the immunoassay techniques reported in the literature for the quantification of rac-methadone are generally not suited for use in pharmacokinetic studies, as they use small calibration curve concentration ranges unlikely to span the range of methadone concentrations present over a inter-dosing interval thus requiring routine dilution and re-analysis of patient samples. Despite this, immunoassay techniques are useful for therapeutic drug monitoring of patient compliance, and may be employed in pharmacokinetic studies.

1.6.1.2. Gas chromatography

Gas chromatography (GC) has been used to detect methadone and its metabolites, predominantly EDDP and EMDP, after solvent extraction following alkalinisation of the matrix (plasma, urine, saliva, gastric fluid, bile, semen) to a pH greater than 9. Detection of the analytes was most commonly performed using hydrogen flame-ionisation or nitrogen-phosphorus detection. Many authors have also employed mass spectroscopy (GC-MS) detection techniques to provide qualitative structural confirmation of methadone and many of its metabolites in addition to quantitative analysis. Many of these authors reported limits of detection rather than quantification, and some do not report assay validation data or differentiate between assays in different biological matrices where more than one matrix has been analysed.

In summary, methods employing conventional GC and GC-MS analysis after extraction of biofluid samples have been successfully developed and applied, although thorough validation data were not always provided. Despite this, it is likely that the methods were sufficiently precise, accurate and robust for their intended application in studies examining the disposition of methadone.

1.6.1.3. High-performance liquid chromatography

Due to its relatively lower cost and ease of use, investigators have more recently used high-performance liquid chromatography (HPLC) as an alternative to GC methods after prior solid-phase or solvent-solvent extraction of the matrix (plasma, urine) alkalinised to a pH >9. These authors have utilised UV detection, most commonly at wavelengths below 235 nm. Few investigators have reported HPLC methods for the quantification of methadone and metabolites other than EDDP and EMDP.

In summary HPLC has provided investigators with simple, robust and sensitive methods for the determination of methadone and its metabolites in biological fluids. Sensitivities reported for the GC and immunoassay techniques do not offer appreciable gains especially

when the complexity of assay development, specificity and expense of apparatus are taken into account.

1.6.2. Stereoselective assays

Before the commencement of the present thesis in 1995-96, there were few published stereoselective assays for the quantification of enantiomers of methadone, none which quantitated the enantiomers of the major metabolite EDDP. Indeed none of the published assays were applied to the investigation of the disposition of methadone in more than a single subject prior to 1996. Kristensen and Angelo (1992) developed a stereoselective GC assay for the quantification of the methadone enantiomers. This assay employed derivatisation of the methadone enantiomers with (-)-methyl chloroformate, resulting in the formation of diastereomers, which were then separated chromatographically using a conventional GC column. However, the complex series of extraction-derivatisation-extraction processes make this assay of limited practical value.

More recently, techniques for the stereoselective quantification of methadone became available with the advent of robust chiral chromatography columns for HPLC. These columns rely upon chiral molecules covalently bonded to the silica stationary phase which then adsorb and desorb the individual enantiomers of a mixture at different rates. Sample preparation and the HPLC system employed (with the exception of the HPLC column) are essentially the same as conventional non-chiral HPLC methods mentioned above. The most widely used chiral chromatography columns are the α_1-acid glycoprotein- or cyclodextrin-derivatised type. Assays employing these columns have been shown to be reproducible, however, several authors have not provided detailed accuracy data.

In summary, recent advances in chiral HPLC techniques have provided investigators with methods to investigate the disposition and metabolism of methadone with respect to stereoselectivity.

1.7. Pharmacokinetics of methadone

A summary of the pharmacokinetic (terminal elimination half-life ($t_{1/2\beta}$), clearance, volume of distribution) and excretion (urinary, faecal) data available in the literature to date for methadone and its metabolites is provided in Appendix 3 and Appendix 4, respectively. These studies have all employed assays for the quantification of methadone that are deemed to be adequately precise, accurate and selective (see section 1.6). The pharmacokinetics of methadone have, most often, been studied in patients undergoing methadone maintenance therapy. However, many of these authors have only reported values for $t_{1/2\beta}$ (Änggård et al., 1979; Inturrisi & Verebely, 1972c; Verebely et al., 1975a) or clearance (Cobb et al., 1998; Pond et al., 1985). Others have provided more comprehensive data in methadone maintenance (de Vos et al., 1995; Meresaar et al., 1981; Nilsson et al., 1982a; Nilsson et al., 1983; Novick et al., 1985; Rostami-Hodjegan et al., 1999; Wolff et al., 1993; Wolff et al., 1997) and pain patients (Denson et al., 1990; Gourlay et al., 1986a; Gourlay et al., 1982; Inturrisi et al., 1987b). Studies in healthy normal volunteers are less common, presumably due the wide availability of patients in the former two groups and the potential for adverse reactions in opioid naive subjects, and have only reported limited pharmacokinetic data (Inturrisi & Verebely, 1972a; Olsen et al., 1977; Wissel et al., 1987), with few exceptions (Nilsson et al., 1982b; Wolff et al., 1997). In all studies, blood sampling protocols have rarely exceeded 48 hours -much less than the necessary 3- to 5-times the calculated $t_{1/2\beta}$ (approximately 24 hours, see Appendix 3). Therefore, it is likely that absorption and distribution processes may affect the calculation of $t_{1/2\beta}$, as absorption and distribution may occupy a substantial part of the sampling period (see section 1.7.2). Indeed, Wolff and co-workers (1997) estimated that 20% of the total area under the concentration-time curve (AUC) would reside in the distribution phase during a 24 hour collection period. Thus, total systemic clearance values are likely to be over-estimates if a mono-exponential, rather than bi-exponential, disposition function is applied to such limited sampling protocols. In contrast, collection of blood samples over an entire 24 hour inter-dosing interval during chronic administration and employing non-compartmental analyses obviates this phenomenon in the estimation of clearance, but not $t_{1/2\beta}$.

Most authors have employed a bi-exponential disposition function for the elimination of methadone (see Appendix 3). However, authors often do not report whether the model chosen resulted in the best possible fit of the data in comparison to other models. After chronic oral dosing, De Vos and co-workers (1995) compared the fitting of one and two compartment models to 0-24 hour plasma concentrations. The best fit was the two compartment model. Other authors have reported that a bi-exponential disposition function resulted in a significantly better fit of the data, using a population pharmacokinetic approach after single and chronic administration with blood sampling up to 57 hours and 27 hours, respectively (Wolff et al., 1997).

Plasma concentrations of EDDP have only been quantitated in a single study to date (de Vos et al., 1995). These authors found that the inter-dosing interval AUC, and hence steady-state plasma concentration, of EDDP was a mean±SD (range) of 8±4% (2-18%) that of methadone in a group of 20 methadone maintenance patients receiving chronic oral methadone. Similarly, the maximum plasma concentration (C_{max}) of EDDP was 10±5% (4-24%) that of methadone. Interestingly, the T_{max} of rac-methadone (2.3±0.7 hours) occurred significantly (P=0.01) later than that of rac-EDDP (1.9±0.9 hours), which the authors suggested may be due to first pass metabolism. Other pharmacokinetic parameters of EDDP were not reported, and EDDP and methadone concentrations were not quantified in urine.

Studies examining the disposition of the (R)- and (S)-methadone enantiomers are limited. Indeed there was only a single study published prior to the commencement of the work presented in this thesis in 1995-1996 which involved more than three subjects (Olsen et al., 1977). However, that study only reported $t_{1/2\beta}$ values, and not other pharmacokinetic parameters, after separate acute administration of the individual enantiomers to healthy subjects. The disposition of the methadone enantiomers after chronic dosing has been investigated in three studies only. However, these studies suffered from low subject

numbers (n=1, Beck et al., 1991; n=3, Kreek et al., 1979; n=2, Nakamura et al., 1982) and only reported $t_{1/2\beta}$ values.

Since 1996, the disposition of the individual methadone enantiomers has been published as a single report in seven chronic pain patients after acute administration of the racemate (Kristensen et al., 1996). These authors reported that the disposition of the (R)-enantiomer is different to that of the (S)-enantiomer, having a larger volume of distribution, longer $t_{1/2\beta}$ and higher total body clearance, although this has only been examined after single dose administration in pain patients. Recent studies, using a population pharmacokinetic approach, have reported an apparent difference in the disposition of rac-methadone between healthy volunteers and former opioid dependent subjects entering methadone maintenance treatment (Wolff et al., 1997), and time-related changes in the disposition of rac-methadone in methadone maintenance patients (Rostami-Hodjegan et al., 1999). These studies will not be discussed in further detail in this chapter, rather, they will be included in Chapters 4 and 5 of this thesis. However, for the readers convenience, the relevant pharmacokinetic parameters reported by these authors have been included in Appendix 3.

1.7.1. Absorption and bioavailability

1.7.1.1. Oral administration

The absorption of methadone from the gastrointestinal tract is rapid, with measurable plasma concentrations occurring 30-45 minutes after acute oral administration. Times (T_{max}) to reach maximum plasma concentrations (C_{max}) of approximately 1.5 hours (Gourlay et al., 1986a) in cancer patients; 4 hours (Inturrisi & Verebely, 1972a), 2 hours (Olsen et al., 1977), and 2 hours (range:1-4 hours, Wolff et al., 1997) in healthy volunteers, after a single dose. In methadone maintenance patients undergoing chronic administration mean±SD values for T_{max} of 3.0±1.4 hours (Meresaar et al., 1981), 2.5±0.7 hours (Wolff et al., 1993), 3.8 hours (range:1-7.5 hours, Wolff et al., 1997), 2.3±0.7 hours (de Vos et al., 1995), approximately 3 hours (Lynn et al., 1976b), and 2 hours (Schall et al., 1996) have been reported. No changes in T_{max} were reported postpartum or during the individual trimesters

in pregnant methadone maintenance patients, with means ranging 2.0-2.1 hours (Pond et al., 1985). These data demonstrating relatively T_{max} values and long lag-times are consistent with the known inhibitory effect methadone on gastric emptying time (see section 1.5.2.4). Interestingly, tolerance to this effect does not seem to be fully developed during chronic dosing, as prolonged T_{max} values also were observed in long-term methadone maintenance patients.

Studies examining the absolute oral bioavailability of methadone are surprisingly limited. In summary, these studies have found that the bioavailability of oral methadone is high, although there is relatively large inter-subject variability. Gourlay and co-workers (1986a) reported a mean±SD (range) bioavailability of 79±12% (60-95%) in 9 patients with cancer after separate administration of 15-40 mg oral and *i.v.* doses of unlabelled rac-methadone. Similar values of 79±21% (41-93%) (Meresaar et al., 1981) and 92±21% (36-119%) (Nilsson et al., 1982a) in former opiate addicts have been reported after single doses using a stable isotope technique allowing the simultaneous administration of the oral and *i.v.* rac-methadone doses. Nilsson and co-workers (1982a) also re-examined bioavailability in the same subjects after 25 days of once daily oral dosing, in which half of the 12 subjects continued with the 30 $mg.day^{-1}$ dosage, while the other half had a dosage increase (60 $mg.day^{-1}$) at day 10 which continued until day 25. As their statistical analysis was unclear and appeared inadequate (they removed subjects with bioavailability >110%), the following analysis has been performed by this author. They found that the oral bioavailability was unchanged (P=0.45, paired t-test) at day 25 (92±21%, range:36-106%) when compared to day 1 (84±26%, range:67-118%) in the six subjects who received constant 30 $mg.day^{-1}$ dosing. A similar result was obtained for six subjects who received the dose increase, day 25 (88±18%, range:65-129%) was not significantly different (P=0.31, paired t-test) compared to day 1 (99±12%, range:84-119%). The lack of statistical differences remained when all of the subjects were combined for day 1 and compared with the six subjects at day 25 receiving 60 $mg.day^{-1}$ (P=0.72, unpaired t-test) or all 12 subjects at day 25 regardless of dose (P=0.81, unpaired t-test). Additionally, there were no

significant differences between the dose groups at day 25 (P=0.70, unpaired t-test) or day 1 (P=0.25, unpaired t-test). Although the power of the study is likely to be low, these data demonstrate that significant differences in the oral bioavailability of rac-methadone after acute compared to chronic administration are unlikely, even with a doubling of dose.

Steady-state plasma concentrations of rac-methadone measured immediately before the next daily dose (trough) have been shown to linearly correlate (r^2=0.79, P<0.001) with daily dosage in 31 subjects over a wide range (approximately 5-100 mg.day^{-1}) of doses (Wolff et al., 1991d). When these data were combined with similar data from six independent studies in the literature, the relationship was similarly strong (r^2=0.85, P<0.001) (Wolff et al., 1991d). The steady-state plasma AUC_{τ}^{ss} of rac-methadone has also been shown to be highly correlated (r^2=0.96; P<0.001) with the daily dose over 10-60 mg.day^{-1} range in five methadone maintenance subjects, three of which were studied on two occasions after dose increases of 5-10 mg.day^{-1} (Wolff et al., 1993). A case report in a single subject showed that the dose-trough plasma concentration relationship for rac-methadone was also demonstrable within a single subject over a 80-360 mg.day^{-1} dose range (Wolff et al., 1991a). These data demonstrate that the pharmacokinetics of methadone are linear over a wide dose range, and that inter-individual variation in metabolic activity does not prevent a strong dose-plasma concentration relationship between individuals.

1.7.1.1.1. Factors affecting gastrointestinal absorption

1.7.1.1.1.1. P-glycoprotein

In humans, P-glycoprotein is the *MDR1* gene product, which functions as a transmembrane efflux pump, and its role in the development of multidrug resistance by tumour cells is well recognised. P-glycoprotein is also expressed in normal tissues, such as the intestine, blood-brain barrier, liver and kidney. P-glycoprotein has recently been demonstrated to influence the absorption and pharmacokinetics of some drugs by impairing drug absorption and distribution, and enhancing drug elimination (for reviews see Kusuhara et al., 1998; Yu, 1999). However, its exact physiological role is still under investigation. In the intestine,

P-glycoprotein is generally considered to remove drugs from the blood, or decrease absorption, via countertransport. There is increasing evidence that P-glycoprotein is co-localised to cells in which CYP3A is extensively expressed, considerable overlap of substrate specificity exists between the two proteins, and there is increasing evidence that they may function in concert to reduce intracellular concentrations of xenobiotics (Kusuhara et al., 1998; Wacher et al., 1998; Yu, 1999; Zhang et al., 1998).

Recently, evidence has been established using the rat everted intestinal sac technique that rac-methadone is a substrate for P-glycoprotein (Bouër et al., 1999) confirming earlier reports that rac-methadone has an affinity for this transporter in drug resistant CaCo2-cells (Callaghan & Riordan, 1993). Briefly, rac-methadone was shown to increase the accumulation of vinblastine in drug resistant CaCo2-cells in a concentration-dependent manner over a 15-75 μM range, and displaced the binding of vinblastine and iodomycin to P-glycoprotein in membranes of drug resistant CaCo2-cells (Callaghan & Riordan, 1993). The transport of methadone across the mucosa into the everted rat intestinal sac was linear with respect to time, while transport was non-linear with concentration over a 15-1500 μM range, consistent with an active transport process (Bouër et al., 1999). Verapamil and quinidine, both known P-glycoprotein inhibitors, caused a concentration-dependent inhibition of rac-methadone transport. In addition, formation of EDDP was observed in the sac contents and tissue, the concentrations of which were unaffected by co-incubation with verapamil or quinidine, indicating that EDDP is unlikely to have significant affinity for P-glycoprotein, at least in this model, or that the formation of EDDP occurred at a site remote to P-glycoprotein (Bouër et al., 1999). Although these data provide evidence that rac-methadone is a substrate for P-glycoprotein, the high bioavailability observed after oral administration indicates that this may be relatively unimportant in humans, as does the very low recoveries (<2%) of unchanged methadone in faeces (see section 1.7.3). However, P-glycoprotein may contribute to the observed variability in the gastrointestinal absorption of methadone reported above. Further studies are required to clarify the role of P-glycoprotein in the disposition of methadone in humans.

1.7.1.1.1.2. Effect of gastrointestinal pH

The effect of gastrointestinal pH has not been the primary aim of any pharmacokinetic study in humans. De Castro and co-workers (1996) examined the effect of changes in gastric pH on the *in vivo* absorption of 5 $mg.kg^{-1}$ rac-methadone administered via gastric intubation in rats. Groups of five rats were administered either (a) 1 M sodium bicarbonate and methadone (*p.o.*); (b) an acidic (pH 2) solution of methadone (*p.o.*); (c) pre-treated with 2 $mg.kg^{-1}$ omeprazole *i.v.* (a proton pump inhibitor) and methadone (*p.o.*); or (d) oral methadone alone. Compared to controls (mean±SEM; 3.6±0.2) gastric pH was significantly ($P<0.01$) higher in rats administered omeprazole (6.4±0.1) and sodium bicarbonate (8.8±0.06), while in pH 2 treated rats, gastric pH (2.2±0.04) was significantly lower ($P<0.01$). Compared to controls (51±6 $ng.ml^{-1}$; 6.8 $\mu g.min.ml^{-1}$), plasma methadone C_{max} and AUC_{0-180} minutes were significantly ($P<0.05$) increased by sodium bicarbonate (167±13 $ng.ml^{-1}$; 23.0±2.5 $\mu g.min.ml^{-1}$) and omeprazole (156±7 $ng.ml^{-1}$; 18.6±1.4 $\mu g.min.ml^{-1}$) treatment, but not pH 2 treatment (33±5 $mg.ml^{-1}$; 3.6±0.9 $\mu g.min.ml^{-1}$). T_{max} values for all groups were between 1-1.5 hours. A significant correlation ($r=0.92$; $P<0.001$) between intra-gastric pH at 120 minutes, and methadone plasma AUC_{0-180} minutes was also observed when the four groups were combined. One cannot rule out a pharmacokinetic drug-drug interaction with omeprazole as both drugs are CYP3A4 substrates. However, the results with sodium bicarbonate would suggest that alteration of gastric pH has a significant effect on the extent of rac-methadone absorption in rats. Extrapolation of this data to humans is difficult, although it does indicate that high intra-gastric pH may increase methadone absorption, presumably by increasing the extent and/or rate of passive diffusion, secondary to an increase in the fraction of unionised drug.

1.7.1.1.1.3. Gastrointestinal motility

The effect of gastrointestinal motility on the pharmacokinetics of methadone has not been examined in humans. Walsh and co-workers (1975a; 1975b) investigated the absorption of methadone *in vivo* in rats using a closed segment technique. Absorption was found to follow first order kinetics, consistent with passive diffusion, with a mean absorption $t_{1/2}$

from the duodenum of 15.6 minutes. Similar values were obtained for the jejunum, ileum and caecum. In contrast, the absorption $t_{1/2}$ from the stomach was 10 hours, indicating that stomach emptying is the rate limiting factor in the gastrointestinal absorption of methadone. Extrapolation of this study to humans is difficult, although gastric emptying time is likely to delay the absorption of methadone. Support for this view can be found in the relatively long T_{max} values (1.5-4 hours) reported for methadone after oral administration of the drug (see section 1.7.1.1), consistent with the known inhibitory effect methadone on gastric emptying time (see section 1.5.2.4).

1.7.1.2. Other routes of administration

Although the oral and intravenous routes of administration are most commonly employed, some authors have investigated alternative routes. Nilsson and co-workers (1982b) and Inturrisi & Verebely (1972b) administered a single intramuscular (*i.m.*) injection of 10 mg rac-methadone dose healthy volunteers. Inspection of the plasma concentration-time curves resembled *i.v.* administration, and indicated that T_{max} occurred within 1 hour.

Using human cadaver forearm skin in an *in vitro* study, Fullerton and co-workers (1991) demonstrated rac-methadone to be absorbed transdermally. The calculated 24 hour steady-state delivery rate was maximal at 0.49 $mg.cm^{-2}$ when the patch contained an azone® (5% w/v) and propylene glycol (18% w/v) mixture. The authors calculated that patch sizes of 40-96 cm^2 could be used to provide steady-state plasma concentrations equivalent to the daily administration of 20 mg methadone, although this would be of limited practical value.

Methadone is well absorbed from the buccal cavity in a pH- and time-dependent manner (Beckett & Triggs, 1967; Weinberg et al., 1988) that is not stereoselective (Beckett & Triggs, 1967), consistent with a passive diffusion process. The loss of drug from buffered solutions was less than 35% at pH values less than 7, but there was a marked increase in absorption at higher pHs with approximately 70% of the methadone absorbed at pH 9 after a 5 minute application (Beckett & Triggs, 1967) to approximately 75% at pH 8.5 after a 10

minute application (Weinberg et al., 1988). Christrup and co-workers (1990) compared the buccal absorption of 25 mg rac-methadone from a chewing gum preparation they had developed, to a tablet formulation. Analysis of the gum for remaining (unabsorbed) methadone showed that 77±7% of the methadone had been released from the gum after 15 minutes of chewing. The analysis of plasma samples for methadone showed that the plasma AUC/(dose released) was not statistically different (P>0.05) for the gum compared to the plasma AUC/(dose administered) for the tablet preparation, resulting in a mean relative bioavailability of 84±17%. Unfortunately, values for other pharmacokinetic parameters were not given, despite an adequate sampling protocol. All of these authors may have overestimated bioavailability, as removal of the drug from the solution or gum does not necessarily imply buccal absorption. Gastrointestinal absorption of methadone, via inadvertent swallowing, cannot be ruled out as a contributing factor to the absorption of methadone after buccal administration, although attempts were made to prevent incidental swallowing in all three studies. Nevertheless, it appears that this route of administration results in bioavailability comparable to oral administration.

Despite many reports of its clinical use (Bruera et al., 1992; Bruera et al., 1995; Moolenaar et al., 1984; Ripamonti et al., 1995; Watanabe et al., 1996), this review of the literature found only three reports (Bruera et al., 1995; Moolenaar et al., 1984; Ripamonti et al., 1995) on the pharmacokinetics of methadone administered as a rectal suppository.

Methadone is absorbed from both aqueous (Moolenaar et al., 1984; Ripamonti et al., 1995) and oil-based (Bruera et al., 1995; Moolenaar et al., 1984) suppositories. However, this there is large variability (coefficients of variation >50%) in the resulting trough plasma concentrations, even when corrected for the dosage administered. Moolenaar and co-workers (1984) compared the pharmacokinetics of 10 mg rac-methadone administered as an oral solution with two different rectal formulations in five chronic cancer pain patients. Compared to oral administration, the relative bioavailability of a aqueous suppository was almost 80% at 8 hours. In contrast, the fatty suppository resulted in a more variable 8 hour

relative bioavailability, ranging between 35% and 58% compared to oral administration. No marked differences in T_{max} (approximately 3 hours) were observed between oral administration and either suppository formulation. The authors suggested that the decrease in absorption from the fatty suppository may be due to poor solubility of the methadone hydrochloride salt at physiological pH.

1.7.2. Distribution

Bullingham and co-workers (1982) examined the disposition of rac-methadone in patients undergoing surgery. Subjects were divided into two groups: in group I, methadone was administered approximately 15 minutes after induction of anaesthesia (n=14); in group P, methadone was administered postoperatively (n=12). After a 1 minute *i.v.* infusion of 10 mg rac-methadone, multiple blood samples were taken for up to 6 hours. C_{max} values were not statistically different (P>0.05), and occurred at the first sampling time (2 minutes). The data were fitted to a tri-exponential equation, but the short sampling period did not allow for accurate description of the terminal elimination rate constant. In both groups, distribution was found to have an initial very rapid component with a $t_{1/2}$ of approximately 2 minutes, followed by a slower component with a $t_{1/2}$ approximately 30 minutes. Similar results were obtained in eight patients with chronic pain administered a 10-30 mg *i.v.* dose of rac-methadone (Inturrisi et al., 1987b). When the data were fitted to a tri-exponential equation, an initial rapid distribution $t_{1/2}$ (mean±SD) of 1.7±1.1 minutes was reported, followed by a slower component with a $t_{1/2}$ of 42±36 minutes (Inturrisi et al., 1987b). Similarly, in 23 subjects administered a 20 mg *i.v.* dose of rac-methadone during surgery, a mean±SD distribution $t_{1/2}$ of 6.1±5.7 minutes was reported when the data were fitted to a bi-exponential equation (Gourlay et al., 1982). The ability of these authors to detect the very rapid distribution phase is due to the frequent blood sampling (n=5-7) during the first 30 minutes post dose.

In contrast, other authors employing less intensive early sampling protocols (1-2 samples in the first 30 minutes post dose) have reported relatively longer distribution $t_{1/2}$ values.

Meresaar and co-workers (1981) administered 20 mg of rac-methadone orally, and 20 mg *i.v.* of stable isotope labelled rac-methadone (d_3) simultaneously, to eight opioid tolerant volunteers commencing methadone maintenance therapy. Multiple blood samples (n=11) were taken up to 48 hours, and plasma methadone concentrations were fitted to a two compartment model. Calculated from the *i.v.* dose, the mean±SD values for the distribution $t_{1/2}$ was 2.95±0.93 hours. Similarly, after oral administration distribution $t_{1/2}$ was found to be 1.78±0.80 hours (n=20, de Vos et al., 1995) for rac-methadone in methadone maintenance patients during chronic oral dosing.

Volumes of distribution, with respect to plasma, for methadone have been reported to be several-fold greater than total body water in studies examining this parameter in humans. Authors have reported values for V_c (the volume distribution of the central compartment), a measure of the distribution of the drug in blood and into well-perfused tissues that reach equilibrium rapidly; V_{dss} (volume distribution at steady-state), a measure of the distribution of the drug once distribution into tissues has reached equilibrium); $V_{d\beta}$ (volume distribution calculated during the terminal elimination phase), a measure of the distribution of the drug in blood and into tissues that reach equilibrium very slowly (Rowland & Tozer, 1995b). Accurate estimation of $V_{d\beta}$, and to some extent V_{dss}, is dependent on a well characterised terminal elimination phase.

In an open cross-over study, a significant ($P<0.01$) increase in mean±SD of $V_{d\beta}$ of rac-methadone from 3.5±0.4 $l.kg^{-1}$ to 5.2±0.8 $l.kg^{-1}$ in healthy volunteers has been reported when urinary pH was modified to below 5.3 or above 7.3, respectively, (Nilsson et al., 1982b). This study used intramuscular administration of rac-methadone so differences could not be attributable to alteration of intestinal absorption, and the authors suggested that this unexpected result may be due to lower binding of methadone during acidosis. Other subject populations have been studied and mean±SD V_c values for rac-methadone of 2.5±0.8 $l.kg^{-1}$ (Denson et al., 1990) in burns patients, while values of 6.1±2.4 $l.kg^{-1}$ and 1.1±0.7 $l.kg^{-1}$ for V_{dss} and V_c, respectively, in the perioperative period (Gourlay et al., 1982),

and 3.5±1.2 l.kg^{-1} and 0.2±0.1 l.kg^{-1} for V_{dss} and V_c, respectively, in chronic pain patients (Inturrisi et al., 1987b). The markedly low V_c value reported by Inturrisi and co-workers (1987b) could be expected, as methadone is extensively bound to α_1-acid-glycoprotein (see section 1.7.2.1), concentrations of which are likely to be elevated in chronic pain patients, possibly resulting in a lower unbound fraction of the drug able to distribute out of the central compartment. Furthermore, the estimates of V_{dss} and V_c reported by Inturrisi and co-workers (1987b) were based on concentrations of methadone in blood. Methadone is known to distribute into red blood cells with a mean blood-to-plasma concentration ratio of 0.75, indicating that the corresponding values for V_{dss} and V_c in plasma would be approximately 25% lower (see section 1.7.2.2). Studies in methadone maintenance patients have reported $V_{d\beta}$ mean±SD values for rac-methadone of 3.9±1.0 l.kg^{-1} (Meresaar et al., 1981), 4.1±1.9 l.kg^{-1} (de Vos et al., 1995), 6.7±2.9 l.kg^{-1} (Wolff et al., 1993), and 3.8±0.6 l.kg^{-1} with a minor increase from the first dose to steady state when the dosage increased 2-fold (4.3±0.8 l.kg^{-1}) or 4-fold (4.5±0.7 l.kg^{-1}) over the duration of the 25 day study (Nilsson et al., 1982a). Mean±SD V_c values for rac-methadone of 2.2±0.4 l.kg^{-1} (Meresaar et al., 1981), 2.1±1.3 l.kg^{-1} (de Vos et al., 1995) have also been reported.

Novick and co-workers (1985) reported no statistically significant difference between the $V_{d\beta}$ of rac-methadone in maintenance patients with a history of alcoholism with (716±100 l) or without (438±94 l) concurrent liver disease. However, the difference was close to statistical significance (P=0.06). The sampling protocol of this study (six blood samples taken over the 24 hour inter-dosing interval) combined with the relatively flat concentration-time profiles in many of the subjects decreases the confidence that can be placed in the interpretation of these data.

Nilsson and co-workers (1983) examined the pharmacokinetics of rac-methadone in maintenance patients divided into two groups; "therapeutic failures" (n=8) who complained of recurrent withdrawal symptoms and demonstrated insufficient progress in social rehabilitation and who had urinalysis results positive for illicit drugs, and a comparison

group of unselected patients (n=12). All subjects in both groups were healthy and had normal haematological clinical chemistry laboratory results. Using *i.v.* administration of stable-isotope labelled rac-methadone, they showed that mean±SD V_{dss} (2.7±1.0 $l.kg^{-1}$), V_c (1.4±0.3 $l.kg^{-1}$) and $V_{d\beta}$ (3.1±1.0 $l.kg^{-1}$) values for the therapeutic failure group were lower than for the comparison group (4.2±0.8 $l.kg^{-1}$, 2.7±0.4 $l.kg^{-1}$ and 4.6±1.0 $l.kg^{-1}$, respectively). The difference reached statistical significance ($P<0.01$) for $V_{d\beta}$, however, statistical analysis was not reported for V_{dss} or V_c. This difference in volume of distribution resulted in a significantly ($P<0.001$) shorter $t_{1/2\beta}$ in the therapeutic failure group (24.5±2.6 hours) compared to the comparison group (34.0±7.0 hours), as plasma clearances were similar in the two groups (104±36 $ml.min^{-1}$ versus 111±36 $ml.min^{-1}$, respectively). The authors suggested that the smaller volume of distribution (and half-life) found in the therapeutic failure group may result in a greater degree of fluctuation in plasma concentrations, and hence opioid effects, compared to the comparison group. This could result in the therapeutic failure patients experiencing relative "highs" shortly after receiving a dose, followed by withdrawal as plasma concentrations rapidly decreased, ultimately resulting in poor treatment success.

The reported values for V_c (>100 l) greatly exceed plasma volume (approximately 3 l in a 70 kg person) by over 30-fold, indicating that the drug rapidly distributes out of the plasma into well-perfused tissues, such as the liver and kidneys (Rowland & Tozer, 1995b), although distribution into the lungs is also likely, given the large values for V_c, and the evidence that methadone is avidly concentrated in the lungs of animals (see section 1.7.2.3). Most of the studies described in this section have collected blood samples for only 48 hours or less -a time period comparable to the half-life of methadone (see section 1.7). Longer sampling times after acute administration, or when using stable isotopes during chronic dosing, would allow more accurate determination $V_{d\beta}$ and V_{dss}. Despite this, the similarity of the magnitude of V_{dss} and $V_{d\beta}$ indicates that little elimination of methadone occurs before distribution equilibrium in achieved (Rowland & Tozer, 1995b). However, the estimates of both V_{dss} and $V_{d\beta}$ are typically 2-fold greater than V_c, indicating that methadone is

extensively distributed into and binds extensively with tissues other than those which comprise the central rapidly equilibrated compartment. Furthermore, the data indicate that the amount of methadone in plasma is small when compared to the total amount in the body. Comparison of the volume of distribution of the individual methadone enantiomers has not been reported in any study prior to the commencement of this thesis.

1.7.2.1. Binding in plasma

The binding of methadone in human plasma has been reported to be high, with bound fractions in the range of 80-97% (see section 1.7.2.1.5). Maximum binding of rac-methadone to purified human albumin of 43.8% (Olsen, 1972), 43.5% (Horns et al., 1975), 39% (Tocque et al., 1980) and 36.6% (Romach et al., 1981) and purified gamma globulins of 17.4% (Olsen, 1973) and 8.26% (Judis, 1977) have been reported. From these data it was obvious to investigators that methadone bound to other plasma proteins as the sum of binding to albumin and gamma globulins only accounts for a maximum of 60% binding.

Alpha$_1$-acid glycoprotein has been shown to be the main plasma protein for binding basic drugs (Kremer et al., 1988; Piafsky & Borga, 1977). Methadone is well established as binding to purified α_1-acid glycoprotein (Abramson, 1982; Eap et al., 1990; Romach et al., 1981). Methadone binding in human plasma has been shown to linearly correlate with plasma α_1-acid glycoprotein concentrations (but not albumin) in healthy volunteers, methadone maintenance patients, rheumatoid arthritis patients and cancer patients (Abramson, 1982; Garrido et al., 2000; Romach et al., 1981). In contrast, no relationship between methadone binding and either albumin or α_1-acid glycoprotein was reported in burns patients (Denson et al., 1990), which the authors attributed to the large fluid shifts that occur after severe burn injuries. These data indicate a primary role of α_1-acid glycoprotein, rather than albumin, in the binding of methadone in human plasma, although albumin (Horns et al., 1975; Olsen, 1972; Romach et al., 1981; Tocque et al., 1980) and lipoproteins (Romach et al., 1981) may play a role. However, the importance of each individual protein

appears to be minor compared to that of α_1-acid glycoprotein, as plasma albumin (Abramson, 1982; Eap et al., 1990; Garrido et al., 2000; Romach et al., 1981), cholesterol (Eap et al., 1990), triglyceride (Eap et al., 1990) and total protein concentrations (Eap et al., 1990) have been shown not to contribute significantly to the binding of methadone.

1.7.2.1.1. Regulation of α_1-acid glycoprotein

Alpha$_1$-acid glycoprotein is an acute phase reactant protein. Plasma concentrations may vary considerably, even in healthy subjects, and are affected by many physiological and pathological conditions (Kremer et al., 1988; Routledge, 1986). Environmental factors have been suggested as playing an important role in the regulation of α_1-acid glycoprotein, because plasma concentrations of α_1-acid glycoprotein are better correlated between spouses than parent and child (Blain et al., 1985), and there is a poor correlation between α_1-acid glycoprotein plasma concentrations in identical twins (Störiko, 1968). Healthy subjects show variability in α_1-acid glycoprotein plasma concentrations, with reported values (mg.dl^{-1}; mean±SD, range) of 67±19 (n=14, Barré et al., 1984), 66 (range: 50-107, n=25, Piafsky et al., 1978), 63±17 (range: 28-120, n=29, Romach et al., 1981), 78±22 (n=10, Abramson et al., 1982), 65±16 (range: 33-98, n=55, Eap et al., 1990), 63±19 (n=8, Benedek et al., 1983), 77±15 (range: 36-146, n=200, Blain et al., 1985), and 69 (range: 36-113, n=23, Piafsky & Borga, 1977). Healthy males (60±13 mg.dl^{-1}, n=6) and females (52±10 mg.dl^{-1}) do not have significantly different (P>0.05) plasma α_1-acid glycoprotein concentrations (Yost & DeVane, 1985). The finding was later confirmed by Eap and co-workers (1990), who found no significant difference (P>0.05) between males (71±15 mg.dl^{-1}, n=23) and females (63±13 mg.dl^{-1}, n=17) once the data from five women who were taking oral contraceptives had been removed. In contrast, others (Blain et al., 1985) have reported plasma α_1-acid glycoprotein concentrations in healthy males (81±18 mg.dl^{-1}, n=100) were significantly higher (P>0.01) than in healthy females (74±17 mg.dl^{-1}, n=100). A significant proportion (24%) of the females in this study were taking oral contraceptives, and re-analysis of the data without these subjects was not performed. Blain and co-workers (1985) compared plasma α_1-acid glycoprotein

concentrations in females taking oral contraceptives (67±15 $mg.dl^{-1}$, n=24) and found that although the mean was lower than in age matched females who were not taking oral contraceptives (72±13 $mg.dl^{-1}$, n=24), the difference did not reach statistical significance (P>0.05). Plasma α_1-acid glycoprotein concentrations are decreased during pregnancy and by use of oral contraceptives, most likely because circulating oestrogens appear to reduce α_1-acid glycoprotein concentrations (Routledge, 1986). During the menstrual cycle, plasma concentrations of α_1-acid glycoprotein have been shown to be significantly decreased (P=0.005, n=18) in the luteal phase (median 71 $mg.dl^{-1}$) when compared to midcycle (median 68 $mg.dl^{-1}$), but not (P>0.05) during the follicular phase (median 73 $mg.dl^{-1}$). No other significant differences were observed (Jilma et al., 1997). Although these authors reported similar mean values, there is an approximate 2- to 4-fold range within studies, indicating marked inter-subject variability.

Changes in α_1-acid glycoprotein plasma concentrations may result from various physiological and pathological conditions characterised by physiological stress (for reviews see Kremer et al., 1988; Piafsky, 1980; Routledge, 1986). A review of the literature until 1988, noted that approximately half the data in ill subjects represented a situation where average α_1-acid glycoprotein concentrations were twice that of healthy control subjects, while about a third gave values three times higher (Kremer et al., 1988). Elevated plasma concentrations (approximate fold increase compared to healthy subjects) of α_1-acid glycoprotein are found in patients with myocardial infarction (1- to 3-fold), malignancy (2- to 3-fold), ulcerative colitis (3- to 4-fold), obesity (2-fold), burns (2- to 4-fold), trauma (2- to 3-fold), rheumatoid arthritis (2-to 3-fold), bacterial and viral infection (2- to3-fold) and after surgery (2- to3-fold) (taken from reviews by Kremer et al., 1988; Piafsky, 1980; Routledge, 1986). Two independent studies (Barré et al., 1984; Barry et al., 1990) have shown that cirrhosis (20±8 $mg.dl^{-1}$, Barré et al., 1984; 37±3 $mg.dl^{-1}$, Barry et al., 1990) significantly reduces plasma α_1-acid glycoprotein concentrations compared to healthy subjects (67±19 $mg.dl^{-1}$, P<0.001, Barré et al., 1984; 77±7 $mg.dl^{-1}$, P<0.01, Barry et al., 1990). Other groups have reported similar findings (Arima et al., 1976; Arima et al., 1977;

Fraeyman et al., 1988). In contrast, some investigators have found that α_1-acid glycoprotein concentrations in cirrhotic patients are comparable to healthy subjects (Chio & Oon, 1979; Pacifici et al., 1986; Viani et al., 1992). The variability of α_1-acid glycoprotein concentrations in cirrhotic patients has been reported to be high, with values ranging 23-178 $mg.dl^{-1}$ (n=30, Chio & Oon, 1979). Others have reported that the coefficient of variation of α_1-acid glycoprotein concentrations in patients with liver disease is twice that found in healthy subjects (Viani et al., 1992). Similar results have also been reported in hepatitis, with some studies reporting decreased plasma α_1-acid glycoprotein concentrations (Arima et al., 1976; Arima et al., 1977), while others have reported normal concentrations (Chio & Oon, 1979; Ozeki et al., 1987).

Before the commencement of this thesis in 1995-96, only a single study reported plasma α_1-acid glycoprotein concentrations in methadone maintenance patients. Romach and co-workers (1981) reported that mean±SD plasma α_1-acid glycoprotein concentrations were comparable in patients enrolled in methadone maintenance therapy for methadone opioid addiction (53±20 $mg.dl^{-1}$) and healthy volunteers (63±17 $mg.dl^{-1}$). In contrast, Garrido and co-workers (2000) recently reported that plasma α_1-acid glycoprotein concentrations were significantly greater ($P<0.005$) in heroin dependant individuals (112 $mg.dl^{-1}$; range: 58-239 $mg.dl^{-1}$; n=27 subjects) experiencing withdrawal symptoms 12-24 hours after their last heroin use, compared to healthy volunteers (73 $mg.dl^{-1}$; range: 40-142 $mg.dl^{-1}$; n=21 subjects). Furthermore, there was a significant, albeit weak relationship between severity of withdrawal and plasma α_1-acid glycoprotein concentration ($r=-0.48$, $P<0.005$). These authors did not monitor plasma α_1-acid glycoprotein concentrations in the heroin dependent subjects after commencement of methadone maintenance therapy. However, no significant change in plasma α_1-acid glycoprotein concentrations was found for up to 31 days in seven patients after commencing methadone maintenance treatment (Rostami-Hodjegan et al., 1999). In this study, plasma samples were obtained on 8-22 different days, and linear regression analysis found no significant

(r^2=0.054; *P*=0.17) time related changes in plasma α_1-acid glycoprotein concentrations, which ranged from 50 $mg.dl^{-1}$ to 120 $mg.dl^{-1}$.

1.7.2.1.2. Variants of α_1-acid glycoprotein

Human α_1-acid glycoprotein is normally composed of several genetic variants, broadly grouped as F-α_1-acid glycoprotein (a mixture of two variants: ORM1 F1 (major) and ORM1 F2 (minor)) and S-α_1-acid glycoprotein (a mixture of two variants: ORM1 S and ORM2 A) (Eap et al., 1990). This results in three main phenotypes, F1/S/A, F1/A and S/A, dependent upon the presence of two or three of the variants, which have relative frequencies of 50%, 35% and 15% (Eap et al., 1988; Eap et al., 1990). The relative concentrations of these variants are highly variable, not only dependent on phenotype, but also between subjects of the same phenotype (Eap et al., 1990). These authors reported that the relative concentrations (% of total α_1-acid glycoprotein) of the α_1-acid glycoprotein variants have been reported to be 36±7% (ORM2 A, range: 17-48%), 26±20% (ORM1 S, range: 0-65%) and 38±22% (ORM1 F1, range: 0-79%).

1.7.2.1.3. Binding to α_1-acid glycoprotein

The binding of methadone to α_1-acid glycoprotein appears to involve two sites, similar to other drugs known to bind to α_1-acid glycoprotein, one site of low affinity while the second site demonstrates much higher affinity and is quantitatively more important (Abramson, 1982). Analysis of binding (high affinity site) data yielded a half saturating concentration of 46 $\mu g.ml^{-1}$ for methadone (Abramson, 1982). The authors concluded that it would be unlikely that methadone would reach saturating concentrations, or be significantly displaced from α_1-acid glycoprotein by the binding of other drugs to α_1-acid glycoprotein, even during usual chronic dosing maintenance schedules (Abramson, 1982). These authors did not consider the role of α_1-acid glycoprotein variants. More recently Hervé and co-workers (1996) examined the binding of rac-methadone to purified α_1-acid glycoprotein variants. These authors found that rac-methadone bound selectively to ORM2 A at two sites, with approximately one high affinity site per α_1-acid glycoprotein molecule. These authors

commented that it is reasonable to assume that methadone binding is unlikely to be saturated, or to be significantly displaced from α_1-acid glycoprotein by the binding of other drugs, as the concentration of binding sites on α_1-acid glycoprotein (approximately 20 μM at one site per α_1-acid glycoprotein molecule) in the plasma greatly exceeds that of plasma concentrations for many drugs (approximately 2 μM for methadone). This is subject to the caveat that concentrations of the ORM2 A variant may be much lower (17-18%, see section 1.7.2.1.2 above) than total α_1-acid glycoprotein. In the case of methadone, with only one high affinity binding site on a single α_1-acid glycoprotein variant, this results in lowering of the binding site concentration to methadone concentration ratio, and thus in an increased risk of binding saturation and drug-drug interactions at the level of binding to α_1-acid glycoprotein.

1.7.2.1.4. Binding to α_1-acid glycoprotein variants

Rac-methadone binding (ratio of bound/free, at 600 $ng.ml^{-1}$ total methadone concentration) in plasma obtained from 45 healthy volunteers was shown to correlate strongly with total α_1-acid glycoprotein (r^2=0.52; P<0.001) and ORM2 A (r^2=0.51; P<0.001), poorly with ORM1 S (r^2=0.24; P<0.001), but no correlation was found with ORM1 F1 (r^2<0.01; P>0.05) concentrations (Eap et al., 1990). Almost identical results were obtained for the individual enantiomers of methadone by this group (see section 1.7.2.1.7). These results are in agreement with a previous report of rac-methadone binding to S-α_1-acid glycoprotein (which comprises both ORM1 S and ORM2 A) in preference to F-α_1-acid glycoprotein with a 7-fold selectivity (Eap et al., 1988). More recently, Hervé and co-workers (1996) confirmed that rac-methadone is bound selectively to the ORM2 A variant, and has no significant affinity for the ORM1 F1 and ORM1 S variants.

1.7.2.1.5. Variability of binding

Mean±SD values of rac-methadone unbound fractions in plasma of 12.7±3.3% (Eap et al., 1990), 15.6±0.04% (Abramson, 1982) and 10.62±1.43% (range: 7.56-12.99%; n=29) (Romach et al., 1981) for healthy volunteers; 19.4±0.44% for cancer patients (Abramson,

1982); 13.31±2.51% (range: 10.59-18.08%; n=12) (Romach et al., 1981), 10.1±3.4% (range:3.4-19.4%; n=85 samples from 48 patients) (Wilkins et al., 1997) for methadone maintenance patients; 9.12±2.51% (range: 4.55-14.24%; n=21) for rheumatoid arthritis patients (Romach et al., 1981); and 12.13±3.91% (range: 8.01-20.82%; n=9) for hypoalbuminemic patients (Romach et al., 1981) have been reported. The unbound fraction in the plasma of healthy men (10.25±1.64%; range 7.56-12.99; n=14) and women (10.96±1.16%; range 9.70-12.91%; n=15) has been shown not to be significantly different (P>0.05), and the use of oral contraceptives did not affect methadone binding (Romach et al., 1981). Wilkins and co-workers (1997) reported similar unbound fractions for men (9.8±3.3%; range 3.4-16.6%; n=71 samples from 38 patients) and women (11.9±3.8%; range 5.6-19.4%; n=14 samples from 10 patients) in a methadone maintenance program, although statistical analysis was not performed. Recently, Garrido and co-workers (2000) reported that unbound fraction of methadone was significantly lower (P<0.005) in heroin dependant individuals (8.8%; range: 5.1-14.9%; n=27 subjects) experiencing withdrawal symptoms 12-24 hours after their last heroin use, compared to healthy volunteers (10.7%; range: 8.0-14.3%; n=21 subjects), presumably due to increased plasma α_1-acid glycoprotein concentrations in the former group (see section 1.7.2.1.1). These authors did not monitor methadone unbound fractions in the heroin dependent subjects after commencement of methadone maintenance therapy. The unbound fraction in the plasma of pregnant methadone maintenance patients was not significantly (P>0.05) altered during pregnancy or up to nine weeks postpartum (Pond et al., 1985). Romach and co-workers (1981) reported no marked effect on the binding of methadone following an overnight fast, compared to two hours after a meal, in 10 healthy volunteers. In this study, rac-methadone unbound fractions increased in five patients, and decreased in five, while the mean relative percent change, irrespective of direction, in all subjects was 8.1±5.3% (range: 0.6-14.6%; n=10).

These data indicate that there is marked variability in the protein binding of methadone, up to 5-fold within a single study, and that states of altered physiological condition, including

the occurrence of opioid withdrawal symptoms, may affect protein binding and hence pharmacokinetic parameters based upon measurement of total (bound plus unbound) drug.

The binding of rac-methadone at a concentration of 2 $\mu g.ml^{-1}$ in dog plasma was unaffected by the presence of EDDP at concentrations ranging 0.1-10 times that of methadone, however the binding of EDDP itself was not reported (Derendorf & Garrett, 1983). The bound fraction of methadone decreased from 72.8% at 100 $ng.ml^{-1}$ to 62.3% at 9090 $ng.ml^{-1}$, indicating that methadone is markedly less bound to plasma proteins in the dog in comparison to humans (see above). Extrapolation of these data to humans is difficult, although it seems unlikely that EDDP would affect methadone protein binding since plasma concentrations of EDDP are approximately one tenth those of methadone in humans (see section 1.7).

1.7.2.1.6. Concentration dependency

Over a therapeutic concentration range Horns and co-workers (1975) reported an apparent concentration dependency of rac-methadone unbound fraction, increasing from 63.3±2.1% bound (36.7% unbound) at 0.1 μM (35 $ng.ml^{-1}$) to 58.6±2.5% bound (41.4% unbound) at 1 μM (350 $ng.ml^{-1}$) to 52.8±2.1% bound (47.2% unbound) at 10 μM (3500 $ng.ml^{-1}$) based upon *in vitro* results from 11 different plasma samples from healthy volunteers. Relatively minor concentration dependency has been reported by others, with values increasing from 10.9% unbound at 100 $ng.ml^{-1}$ to 14.4% unbound at 800 $ng.ml^{-1}$ (based upon results from one healthy volunteer's plasma, Eap et al., 1990). Similarly, Inturrisi and co-workers (1987b) reported little change in binding with 89.4±2.9% bound (10.6% unbound; range: 6-15% unbound) to 85.8±2.0% bound (14.2% unbound; range: 12-18% unbound) at 73 $ng.ml^{-1}$ and 661 $ng.ml^{-1}$ in the plasma of patients (n=6 patients, multiple plasma samples, assayed in duplicate) receiving methadone for chronic pain (cancer, postoperative, osteoporosis) control. Binding of methadone in the plasma obtained from methadone maintenance patients showed a relatively minor change in binding from 25% unbound to 31% unbound over the concentration range of 150-340 $ng.ml^{-1}$ (Horns et al., 1975), based

upon six samples from patients receiving methadone maintenance therapy. All of the studies mentioned above used equilibrium dialysis techniques, and corrected for non-specific binding of the drug to the apparatus. More recently, using an ultra-filtration technique the unbound fraction of rac-methadone was reported to be independent of the total (bound plus unbound) plasma concentration over a 100-1000 $ng.ml^{-1}$ range (Wilkins et al., 1997). Although the authors only presented the results using single individual plasma sample at 500 $ng.ml^{-1}$ (mean±SD; 15.6±0.1% unbound) and 750 $ng.ml^{-1}$ (15.5±0.2% unbound).

1.7.2.1.7. Stereoselectivity

In the plasma of 45 healthy volunteers, unbound fractions of (S)-methadone (10.0±2.9% unbound; range: 5.7-18.4% unbound) have been reported to be significantly ($P<0.001$) lower than those of (R)-methadone (14.2±3.2% unbound; range: 7.8-21.6% unbound) at a concentration of 600 $ng.ml^{-1}$ total (bound plus unbound) methadone (Eap et al., 1990). In the same study, the ratio of bound/free concentrations of (R)- and (S)-methadone correlated with total α_1-acid glycoprotein ($r^2=0.43$; $P<0.001$ and $r^2=0.48$; $P<0.001$, respectively) and ORM2 A variant ($r^2=0.55$; $P<0.001$ and $r^2=0.51$; $P<0.001$, respectively) concentrations, while a weak correlation was found with ORM1 S variant ($r^2=0.26$; $P<0.001$ and $r^2=0.23$; $P<0.001$, respectively) concentrations. No correlation was found for either enantiomer with ORM1 ($r^2<0.01$; $P>0.05$ and $r^2<0.01$; $P>0.05$, respectively) concentrations (Eap et al., 1990). These data confirm an earlier report of stereoselective methadone binding in the plasma of healthy volunteers, with unbound fractions of 9.2±1.6% and 12.4±1.5% reported for (R)- and (S)-methadone, respectively (Romach et al., 1981).

The unbound fractions of the individual methadone enantiomers have also demonstrated to be somewhat concentration dependent (Eap et al., 1990). Unbound fractions increased from 14.4±1.0% to 16.8±0.5% for (R)-methadone and 9.2±0.3% to 13.9±0.7% for (S)-methadone over a 100-800 $ng.ml^{-1}$ concentration range (Eap et al., 1990). Samples were prepared in duplicate and five concentrations were employed over the concentration range examined,

although care must be employed in the interpretation of these data as they were only preformed using the plasma from a single subject.

There are no data available on the plasma protein binding of the individual methadone enantiomers in methadone maintenance patients.

1.7.2.2. Distribution into red blood-cells

The distribution of rac-methadone into red blood cells from plasma appears to be relatively minor in humans, most likely due extensive binding to plasma proteins (section 1.7.2.1). Nilsson and co-workers (1982b) reported rac-methadone concentrations in plasma were approximately 2.3-fold higher than in red blood cells in the blood of methadone maintenance patients. Inturrisi and co-workers (1987b) reported mean±SD red blood cell and blood to plasma rac-methadone concentration ratios of 0.32±0.01 and 0.75±0.03, indicating 3.1- and 1.3-fold higher concentrations in the plasma, respectively, and these were independent of concentration in the range of 0.1-0.4 $\mu g.ml^{-1}$.

1.7.2.3. Distribution into other tissues

From the large values reported for the volume of distribution in section 1.7.2, it is apparent that only a small proportion of methadone in the body resides in the blood, implying that methadone is highly distributed into extravascular sites. The volume of distribution at steady-state (V_{dss}) of a drug can be expressed in terms of the unbound fractions in the plasma (f_u) and tissues (f_{ut}), and the total body water (TBW) and plasma (V_p) volume. With the assumption that only the unbound drug is able to distribute throughout the body, these parameters can be related by Equation 1-1 (Rowland & Tozer, 1995a):

Equation 1-1: Relationship of plasma and tissue unbound fractions.

$$V_{dss} = V_p + \left(\frac{f_u}{f_{ut}}\right) \times TBW$$

Assuming f_u=0.1, V_d=300 l, Vp=3 l and TBW=40 l (Rowland & Tozer, 1995a) allows the estimation of the unbound fraction in tissues to be approximately 0.01, thus indicating that methadone is more highly bound to extravascular sites within the tissues compared to plasma proteins. The high degree of tissue binding results in relatively low peak and high trough plasma concentrations, due to a relatively long $t_{1/2\beta}$, characteristic of a compound with a large volume of distribution and a low to intermediate clearance.

Studies of methadone tissue distribution in animals have produced data that support the empirically derived high degree of tissue binding outlined above. Methadone has been shown to be present, and persist in various tissues for several weeks after concentrations in the blood were undetectable. In rats, detectable radioactivity in liver, adrenal, testes, spleen, kidney, lung and brain tissue for 10 weeks after a single 1.5 mg *s.c.* dose of ^{14}C methadone (Harte et al., 1974). At all times after the first week, concentrations in these tissues were greater than in the blood. Similarly, after individual administration of tritiated (R)- and (S)-methadone to separate groups of rats, detectable radioactivity of both enantiomers persisted in the brain for up to 3 weeks, while plasma concentrations were undetectable after 48 hours (Misra & Mulé, 1973). This study noted comparatively higher concentrations of (R)-methadone in the rat brain at all time points. Using a relatively non-specific colorimetric method, concentrations of (R)-methadone were higher than (S)-methadone in the lung, liver, spleen, kidney, heart, muscle, brain and blood of rats administered single 20 $mg.kg^{-1}$ *i.p.* doses of the individual enantiomers at 2 and 4 hours after administration (Sung & Way, 1953). Additionally, concentrations in each tissue studied were higher than in the blood at both time points, in agreement with previous reports. Concentrations in the liver and kidney at 4 hours were similar, with concentrations in the range of 22-33 $\mu g.g^{-1}$ tissue and 4-14 $\mu g.g^{-1}$ tissue for (R)- and (S)-methadone respectively, and were generally 2- to 3-fold higher than in muscle, with concentrations in the range of 5-8 $\mu g.g^{-1}$ tissue and 4-

7 $\mu g.g^{-1}$ tissue for (R)- and (S)-methadone respectively. Only trace concentrations in the blood, brain and heart were detectable at this time point. Although concentrations in muscle are much lower than in the liver and kidney, in terms of the total amount present in the whole tissue the muscle becomes far more important containing 20-30% and 16-28%, for (R)- and (S)-methadone respectively, of the administered dose as compared to approximately 5% in the liver or kidney (Sung & Way, 1953). A similar distribution of radiolabelled (R)-methadone has been observed in male and female mice, indicating a lack of sex differences in that species (Shah et al., 1976). Wallace and co-workers (1972), also employing a colorimetric method, reported similar concentrations for rac-methadone rat liver (33.6-49.6 $\mu g.g^{-1}$ tissue), kidney (68.2-87.3 $\mu g.g^{-1}$ tissue) and lung (250.8-364.5 $\mu g.g^{-1}$ tissue) 1 hour after a 20 $mg.kg^{-1}$ *s.c.* dose. These results are difficult to interpret as all of these studies may have quantified not only methadone, but also metabolites, due to the relatively non-specific methods employed for the detection of methadone in the samples.

Using a specific GC assay, Swanson and co-workers (1978) measured concentrations of methadone in the tissues of rabbits at 130 minutes after *i.m.* administration of 40 mg radiolabelled (S)-methadone. Accumulation of (S)-methadone in tissues, expressed as tissue to blood concentration ratio, in the lung (80), spleen (73), kidney (36), heart (14), prostate (11), testes (10), seminal vesicle (8), and liver (4) were reported. The reported accumulation of (S)-methadone in the liver of rabbits in this study was the lowest of all tissues studied in contrast to reports in other species.

The distribution of methadone in human tissues has been limited to post mortem examinations of methadone overdose fatalities. Drummer and co-workers (1992) reported concentrations of rac-methadone in the liver of nine maintenance patients ranging from 2.6-18 $\mu mol.kg^{-1}$ (approximately 0.9-6.3 $\mu g.g^{-1}$ tissue) which were equal to or greater than the corresponding concentrations in blood by a factor of 1- to 19-fold. Others have reported a similar rac-methadone concentration (8 $\mu g.g^{-1}$ tissue) in the liver of a single overdose victim (Dickson & Palmer, 1975). Robinson and Williams (1971) investigated the tissue

concentrations of rac-methadone of 11 overdose victims and found large variations between subjects, which was attributed to the differing degrees of drug use and time of death after taking the fatal dose. They compared the ratio of concentrations in other tissues to that in the liver for each subject. Concentrations in the kidney and spleen were similar to those in the liver with a mean±SD liver to tissue ratio of 0.87±0.28 and 0.83±0.40 respectively; concentrations in the lung were higher than in the liver (2.62±1.21), while concentrations in the brain were approximately one fifth those in the liver (0.21±0.17). In agreement with findings in animals, blood concentrations were lower than in the tissues being 0.66±1.06 times those in the liver.

The extensive accumulation of methadone in the rat lung has been examined in detail using isolated organs and tissue slices. Roerig's group (1984) examined the uptake of 25 μM rac-methadone by rat lung slices and reported that within 1 hour 40% of the methadone was removed from the incubation medium. Chi & Dixit (1977) reported that the uptake of rac-methadone increased rapidly over the first 60 minutes and reached a plateau by 180 minutes with a tissue to incubation medium concentration ratio (T/M, mean±SD) of 24±3 at an initial methadone concentration of 10 μM. Saturation of uptake occurred above 500 μM, and was not due to an increase in the incubation medium osmolarity. Uptake of methadone at low concentrations was shown to be indicative of an active transport process, while at concentrations above 100 μM passive diffusion became more important. Uptake of the individual methadone enantiomers appeared to be stereoselective as the T/M ratio for (S)-methadone was higher than for (R)-methadone over the 30-180 minutes period studied.

Racemic methadone was accumulated by the isolated perfused rabbit lung to a steady state concentration, which accounted for 81±2% of the 3 μmol methadone dose added to the perfusion media. Later, a biphasic uptake of methadone was described (Anderson et al., 1974); at low concentrations, an uptake component was observed that became saturable at concentrations above 0.1-0.2 mM, while the second linear component became quantitatively more important at higher concentrations. Law and co-workers (1975) reported that the

efflux profile of rac-methadone from the rabbit lung was characterised by three velocity components (E_1, E_2 and E_3) with mean half-lives of 22 seconds, 1.65 minutes and 8.9 minutes respectively. In addition, a saturable "non-effluxable pool" was also detected, and the authors commented that this pool is likely to be responsible for the persistence of methadone in the lungs of overdose victims. These authors reported that rac-methadone accumulated by the linear component is sequestered in either E_1 or E_2 whereas methadone accumulated by the saturable component is stored in E_3 and the "non-effluxable pool" from which the removal methadone is extremely slow. Consistent with these results, and using the isolated perfused rat lung, Roerig's group reported a saturable component and a second linear component, while efflux was characterised by three components and the presence of a "slowly effluxable fraction" (Roerig et al., 1984; Roerig et al., 1982).

The uptake of methadone by the isolated perfused rat liver has also been examined. In a preliminary report, an initial rapid decline of the perfusate methadone concentration in the first 30 minutes, followed by a second slower phase with a half-life of 114 minutes was observed (Lynn et al., 1975). Later, this group investigated the disposition of rac-, (R)- and (S)-methadone in the isolated perfused rat liver, reporting a rapid decline in perfusate concentrations of 70% within the first 10 minutes, similar to their earlier findings (Gerber et al., 1977). When compared to rac- or (R)-methadone, the concentrations of (S)-methadone in the perfusate were consistently lower during the perfusion. The mean perfusate concentration ratio of (S)-methadone to (R)-methadone decreased from near unity at the start of perfusion to 0.68-0.74 at 30-120 minutes indicating stereoselectivity in the uptake of the methadone enantiomers. This study did not use whole blood, so differences in uptake are related to the affinities of the methadone enantiomers to sites or uptake processes in the rat liver and not to plasma protein binding.

Methadone has been quantified in the semen of rabbits and humans. Concentrations of (S)-methadone in rabbit semen were an average of 6.8 times higher than those in blood at the time of peak semen concentration (Swanson et al., 1978). Gerber and Lynn (1976)

reported that concentrations of rac-methadone in semen were an average of 1.8 times those in blood in six methadone maintenance patients; semen samples were collected 1-4 hours after administration of the daily methadone dose and blood samples obtained within 15 minutes. There was a large variation in the ratios reported (range: 0.73-4.72), with equal numbers recording ratios less or greater than unity. The authors estimated that a 3.5 ml ejaculate contained less than 0.001% of the daily rac-methadone maintenance dose. It is likely that methadone is accumulated in the prostatic fluid as it would be expected to be "ion trapped" in this slightly acidic environment, a situation less likely in the seminal vesicular fluid which is distinctly more alkaline (Gerber & Lynn, 1976; Swanson et al., 1978).

Methadone, EDDP and EMDP have been detected in the sweat of maintenance patients (Henderson & Wilson, 1973). Concentrations of the racemic compounds were comparable to those found in the urine, however there were large variations in both urinary and sweat concentrations with no obvious pattern to the distribution, although methadone concentrations were generally higher in sweat. EDDP was generally present in highest concentrations in the urine, while the lowest concentrations were observed for EMDP which was only present in three of the five patients, with similar concentrations in urine and sweat. Assuming a perspiration rate of 500 $ml.day^{-1}$, the authors estimated that approximately 2% of the daily rac-methadone maintenance dose would be eliminated in the sweat, while this was less than 1% each for EDDP and EMDP.

Using a specific GC assay, Lynn and co-workers (1977; 1976b) quantified rac-methadone in the saliva and gastric juice of healthy volunteers and methadone maintenance patients. At 8 hours after the daily dose, the mean concentration of rac-methadone in gastric juice was 100-fold, and in saliva up to 10-fold, greater than the corresponding concentration found in the blood. In contrast, others have reported a mean±SD saliva to plasma rac-methadone concentration ratio of 0.5±0.13 in two maintenance patients (Kang & Abbott, 1982). More recently, saliva has been investigated as an alternative biological fluid to plasma for therapeutic drug monitoring of methadone maintenance patients. In multiple samples taken

from 21 methadone maintenance patients over a period of 30 months, a significant relationship between the concentration of rac-methadone in stimulated saliva samples taken immediately before administration of the daily dose and paired plasma samples (r=0.81, $P<0.001$) and daily rac-methadone dose (r=0.80, $P<0.001$) has been reported (Wolff et al., 1991b). A mean saliva to plasma rac-methadone concentration ratio of 1.3 was reported in this study. In single samples taken from 10 methadone maintenance patients, others have also reported a relationship between the concentration of rac-methadone in unstimulated saliva samples taken immediately before administration of the daily dose and paired plasma samples (Bermejo et al., 2000a). In this study, the relationship did not reach statistical significance (r=0.54, $P<0.2$), however, sample size was low. Saliva pH and concentrations of rac-EDDP in plasma and saliva were also measured in this study, and a relationship (r=-0.63, $P<0.1$) was found between the concentration of rac-methadone in saliva and saliva pH for rac-methadone, but not rac-EDDP, consistent with the results expected from the pKa of these compounds given that the pH range of saliva samples was 5.6-7.0. Saliva to plasma concentration ratios were highly variable, with mean±SD (range) values of 3.7±2.1 (0.6-7.2) and 0.9±0.6 (0.2-1.8), for methadone and EDDP, respectively. Additionally, these investigators examined the use of a commercial device for the collection of stimulated saliva samples, which consisted of a cotton swab in a plastic tube. Concentrations of rac-methadone and rac-EDDP were reduced in samples exposed to the device by 40% and 30%, respectively, presumably via non-specific binding to the device. Differences in the results of these studies may be explained in part by differences in sampling times after administration of the daily dose; differences in salivary pH; or whether samples stimulated or not, as increased saliva pH occurs in stimulated saliva samples, which would result in decreased concentrations of methadone (Bermejo et al., 2000a).

The reported high concentrations of methadone in the tissues of animals and humans, with respect to blood, and evidence of active transport processes, are consistent with the reported large volumes of distribution and relatively low blood concentrations observed after single doses of methadone.

1.7.3. Metabolism and excretion

1.7.3.1. Metabolites and excretory profile

While many authors have examined the metabolism of methadone in animals and humans, most of the studies have been qualitative, and a few semi-quantitative. Many authors have reported experimental evidence for the structure of certain metabolites (see sections 1.4 and 1.6), such as mass spectra fragmentation patterns. However the lack of pure standards has hindered the quantification of some metabolites. Investigators have overcome this problem to some extent by administering a ^{14}C radio-labelled dose of methadone and then chromatographically separating the metabolites, quantifying the relative proportions of the administered radioactivity found in each fraction. Although this method allows the quantification of the metabolites, it makes the assumption that the chromatographically purified metabolite is indeed pure and does not contain impurities such as other structurally similar metabolites. For the remainder of this section, results from studies will be reported where pure standards were available, and if not, only when sufficient data are available to allow confidence in the reported results. In some cases metabolite structures have been tentatively assigned and further proof provided in subsequent studies.

Most quantitative studies have reported only the recovery of unchanged methadone, EDDP and occasionally EMDP in urine, while few studies have examined the excretion of other metabolites or routes of elimination. Appendix 4 summarises the recovery of methadone and metabolites reported in the literature, expressed as a percentage of the dose administered. It is noted that many of the studies presented in Appendix 4 did not correct the recovery of the metabolites for molecular weight differences when reporting recovery values as a percentage of the dose administered. Rather, the authors calculated the percent recovery as the weight (in milligrams) recovered divided by the weight (in milligrams) of methadone administered, and expressed this as a percentage. This miscalculation results in an underestimation of the percent recovery by approximately 13% and 20% for EDDP and EMDP, respectively. Therefore, I have re-calculated the percent recovery values reported in the literature which have not considered molecular weight differences, either by

recalculation of individual subjects' data where possible, or recalculation of reported mean values. Furthermore, the readers attention is drawn the sampling duration employed in the studies reported in Appendix 4. After acute methadone administration, several studies collected biofluid samples for only 24 hours, a duration likely to be comparable to the half-life of the drug in most subjects (see section 1.7.5). Such studies would be unable to obtain complete recovery of the administered dose, and therefore unable to achieve mass-balance. In contrast, studies employing a sampling period of at least 96 hours after acute administration (approximately hour times the half-life), or 24 hours during chronic once-daily dosing, would be likely to achieve a full recovery of the study dose.

As can be seen from Appendix 4, most studies have been conducted in methadone maintenance patients during steady-state dosing conditions. This has allowed the investigators to collect urine and faecal samples over a single 24 hour inter-dosing interval, thus providing the potential to achieve mass-balance. The mean urinary recovery of unchanged methadone has been reported to range from 11% to 17% of the daily maintenance dose across studies in maintenance patients (see Appendix 4). Similar recoveries of unchanged methadone have been reported after acute administration when sampling times were sufficiently long (96 hours Inturrisi & Verebely, 1972a; Inturrisi & Verebely, 1972b; Nilsson et al., 1982b), although some studies have reported comparatively lower recoveries due to an insufficient (24 hours, Inturrisi et al., 1987b; Pohland et al., 1971; Verebely et al., 1975a) sampling duration (see Appendix 4) as noted above. Deliberate urinary acidification has been shown to increase the recovery of unchanged methadone to 34% (Nilsson et al., 1982b) by decreasing renal reabsorption, while urinary alkalinisation resulted in complete renal reabsorption of unchanged methadone (see Appendix 4 and section 1.7.5.1.1).

The excretion of EDDP has most often been demonstrated to account for a greater percentage of the administered dose than unchanged methadone, with mean recoveries ranging from 4% to 36% during steady-state dosing conditions (see Appendix 4). In

contrast, few studies have reported on the excretion of EMDP. However, this metabolite appears to account for a maximum of 4% of the administered dose in urine (Kreek et al., 1980a), although most estimates are less than 2% in both urine and faecal samples (see Appendix 4).

Surprisingly, only two studies have quantified the recovery of metabolites other than EDDP and EMDP (see Appendix 4). Pond and co-workers (1985) examined the disposition of rac-methadone in methadone maintained pregnant women. Four inter-dosing interval pharmacokinetic studies were performed in at least eight patients; two intervals during pregnancy (20-34 and 35-40 weeks gestation), and two intervals after delivery (1-4 weeks 8-9 weeks post partum). Minor changes in dose were allowed, however, there were no significant differences ($P>0.05$) during the study. The recoveries of methadone, α-methadol, and the sum of EDDP and EMDP were not significantly ($P>0.05$) altered throughout the study, and are similar to those reported in non-pregnant methadone maintenance patients (see Appendix 4 and section 1.7.5.1.3).

From the data presented in Appendix 4, and as discussed above, it is apparent that only a maximum of 60% of the administered dose is recovered as the sum of unchanged methadone, EDDP and EMDP. To date, two groups have examined the renal and faecal routes of elimination in an attempt to achieve complete recovery of the administered dose (Kreek et al., 1983; Kreek et al., 1980a; Verebely et al., 1975a). Verebely and co-workers (1975a) quantified unchanged methadone, EDDP and EMDP in 24 hour collections of urine and faeces from six methadone maintenance patients at the commencement of treatment (days 3-4 of treatment) and during two intervals at steady-state (days 12-14 and 22-24 of treatment). The daily methadone dose was increased from an initial 20-25 $mg.day^{-1}$ to 40-80 $mg.day^{-1}$ at steady-state. The mean recovery of unchanged methadone in urine and faeces ranged from 12% to 17% and 0.6% to 2.3%, respectively, and was 7% to 34% and 3% to 23%, respectively, for EDDP. EMDP was detected in urine and faecal samples, but the concentrations were below the limit of quantification. The total recovery of methadone

and EDDP in the combined urine and faecal samples increased from 23±9% during initiation of treatment, to 75±17% at steady-state (days 22-24). The greater total recovery at steady-state was due to EDDP, which the authors attributed to "auto induction" of the metabolism to EDDP by methadone, as the ratio of recovered EDDP/methadone increased by 3-fold. However, the subjects were not at steady-state during the initial phase of the study as the doses administered increased from 10 mg to 25 mg (a 2.5-fold increase) during the preceding 3-4 days, thus preventing quantitative recovery during a single inter-dosing interval. In contrast, all subjects had been receiving a fixed dose for at least five days during the final steady-state phase. In comparison to other estimates in the literature, the recovery of EDDP during initiation of treatment is low (see Appendix 4), possibly due to the lack of steady-state conditions while samples were collected during a single inter-dosing interval. It is therefore possible that the study design contributed to the reported "auto induction" of the metabolism to EDDP by methadone. Alternatively, the liver function of the subjects may have improved, possibly due to the increased medical attention and nutrition provided by the staff of the closed ward in which they were housed throughout duration of the study. Improved liver function is likely to result in an increased intrinsic clearance of methadone to EDDP, thus resembling "auto-induction". However, this was not monitored in the subjects after commencement of treatment. Similarly, Nilsson and co-workers (1982a) provided some evidence for the development of "dispositional tolerance" in methadone maintenance patients. These authors reported that total recovery of methadone and EDDP in the combined urine and faecal samples increased from 38±26% after the first dose to 48±11% at steady-state. As 0-24 hour urine collections were employed for both phases of this study, it is not surprising that a lower recovery was obtained after the first dose.

Kreek and co-workers (1983; 1980a) examined the urinary and faecal excretion of methadone and several metabolites (EDDP, EMDP, methadol, p-hydroxy methadone and pyrrolidone metabolite) in a large number (n=19) of methadone maintenance patients. The total 24 hour urinary excretion (mean±SD) of methadone and metabolites was significantly

($P<0.05$) lower in methadone maintenance patients with chronic liver disease (n=14, 5 female; 35±12%) compared to otherwise healthy male patients (n=5; 52±4%). The observed difference in total amounts excreted was predominantly due to lower recoveries of methadone (10±5% of the dose) and EDDP (23±11%) in the patients with liver disease, compared to otherwise healthy subjects (17±6% and 30±4%, respectively). Excretion of the other metabolites was similar in the two groups, with mean values ranging from 0.1-4% of the dose. Female (n=5; 34±17%) and male (n=9; 35±10% of the dose) subjects with liver disease excreted similar total amounts in urine. Despite this finding, there appeared to be a greater proportion of the dose eliminated as EDDP in females (n=5; 25±16%) compared to males (n=9; 21±7%) with liver disease, while the opposite was found for methadone (6±4% and 12±4% of the dose, respectively). The urinary excretion of the other metabolites was similar in males and females, with mean values ranging from 0.1-2% of the dose. In the same patients, the mean faecal excretion of methadone (<1%) and EDDP (7-8%) was similar in both patient groups. The total faecal excretion of methadone and metabolites was not significantly ($P>0.05$) different in otherwise healthy male patients (9±2%) compared to male (10±4%) or female (13±5%) patients with chronic liver disease. The faecal excretion of the individual compounds was quantitatively similar to that found in otherwise healthy males and in males and females with liver disease. The total recovery of methadone metabolites in the combined urine and faecal samples was lower in patients with liver disease (46±11%) compared to otherwise healthy subjects (61±5%). These investigators used mass-spectroscopy for quantification using authentic compounds, and scanned the entire mass range in which fragments might arise resulting from other metabolites. Hydrolysis (β-glucuronidase and sulfatase) of urine samples from three healthy patients yielded only small amounts of p-hydroxy EMDP (<1%) and p-hydroxy EDDP (<0.1%) in urine samples, and was not investigated in faeces. The presence of other metabolites, or increased concentrations of metabolites detected prior to hydrolysis were not found. These authors concluded that examination of the faecal excretion of conjugated metabolites, in particular p-hydroxy EDDP, p-hydroxy EMDP, and methadol would be necessary to achieve complete mass-balance.

In support of the low faecal excretion of methadone reported above, Lynn and co-workers (1976b) reported that only 8±4% of the daily maintenance methadone dose was recovered in 0-8 hour samples of gastric fluid. As *i.m.* administration was employed in this study, these data indicate that methadone is secreted into the gastrointestinal lumen to a limited extent, although it is unclear whether this is mediated via active transport and/or passive diffusion. In contrast, EDDP was detected in gastric fluid at concentrations too low to be quantified. Kreek and co-workers (1980b) collected bile from T-tube drainage in a single methadone maintenance patient for 24 hours after administration of the 100 mg once daily oral dose. They recovered 0.1% of the dose as unchanged methadone, 39% as EDDP and 0.1% as EMDP. These data, albeit limited, indicate that methadone and EMDP are unlikely to be secreted into the gastrointestinal tract via the bile, and subsequently be eliminated in the faeces, in agreement with the low faecal recoveries of these compounds reported above. Recently Bouër and co-workers (1999) have demonstrated that methadone is a substrate for P-glycoprotein, while EDDP is not (see section 1.7.1.1.1). These data suggest that the secretion of methadone into the gastrointestinal lumen may be mediated, at least in part, by this active transport process. In contrast, it would appear that the reported faecal excretion of EDDP results from biliary secretion.

Sullivan & Due (1973) reported that methadone (16%), EDDP (18%), EMDP (1.4%) and the pyrrolidone metabolite (0.8%) in the urine of three methadone maintenance patients accounted for a mean of 36±23% of the daily methadone dose. They also detected a "small amount" of the carboxylic acid metabolite, but failed to detect any other metabolites in unhydrolysed urine samples. Further investigations using GC-MS analysis of hydrolysed (β-glucuronidase and sulfatase) urine samples identified the structure of several metabolites: N-desmethyl methadol, p-hydroxy EMDP, p-hydroxy EDDP, p-hydroxy methadone, and the carboxylic acid metabolite. Quantification was not performed, with the exception that approximately 3% of the dose was recovered as N-desmethyl methadol. However, the methadol metabolite was not detected, supporting their earlier observations (Sullivan et al.,

1972b). The rank order of the relative proportion of other identified metabolites was p-hydroxy EMDP > p-hydroxy EDDP > p-hydroxy methadone, although a lack of authentic standards prevented quantification and amounts of these metabolites were not related to that of methadone, EDDP or EMDP. The carboxylic acid metabolite was present in both conjugated and unconjugated forms. In a closely related study also using GC-MS analysis, this group reported similar results (Sullivan et al., 1972a). Despite the lack of quantitative results, they reported that the relative proportion of the observed metabolites were: EDDP ~ p-hydroxy EMDP > methadone > p-hydroxy EDDP > EMDP ~ carboxylic acid metabolite.

Änggård and co-workers (1975) administered a ^{14}C-labelled rac-methadone oral dose to four methadone maintenance patients at the commencement of maintenance therapy with oral unlabelled rac-methadone (10 mg), and 24 days later at a higher maintenance dose (80 mg). They collected urine and faecal samples for 96 hours, and analysed these samples for ^{14}C radioactivity in all four subjects. There was no obvious dose or time dependency in the radioactivity excreted in either urine or faeces. The total mean±SD recovery of ^{14}C radioactivity in the combined biofluids for both dosages was 82±12% (range: 66-97%) of the administered dose, with 51±16% and 32±11% recovered in urine and faeces, respectively. The combined amounts of methadone and EDDP accounted for 34% of the dose in urine, but were not quantified in faeces. When subtracting these values from the urinary excretion of ^{14}C radioactivity, the excretion of metabolites other than EDDP accounted for 23±7% (range 7-49%) of the dose in urine. The recovery of methadone, EDDP and EMDP in faecal samples collected from methadone maintenance subjects in other studies generally accounts for approximately 10% of the dose (Kreek et al., 1980a; Verebely et al., 1975a), although this has been reported to be up to 25% (Verebely et al., 1975a). These data indicate that a significant proportion of the dose is eliminated as metabolites other than EDDP and EMDP or unchanged methadone. The above authors were able to identify several other metabolites in extracts of unhydrolysed urine samples (pyrrolidone metabolite) and after hydrolysis of the conjugated compounds (methadol, N-desmethyl methadol, p-hydroxy EDDP, p-hydroxy EMDP, p-hydroxy methadone).

Unfortunately, these metabolites were not quantified. In faeces, methadone, EDDP, and the pyrrolidone metabolite were identified but not quantified in unhydrolysed samples, while the amounts of compounds present in extracts of hydrolysed faecal samples did not permit identification. Interestingly, these authors noted a decrease in the urinary excretion of methadone from 20±9% of the initial 10 mg dose on days 1-6, to 12±7% 24 days later on chronic dosing with 80 mg. Conversely, the excretion of EDDP increased from 13±5% to 23±12% over the same interval, resulting in a 3-fold increase in the ratio of EDDP/methadone excretion. The authors postulated that this phenomenon was due to dose-dependent metabolism, or induction of the enzymes mediating the N-demethylation reaction. Dose-dependent metabolism seems an unlikely explanation due to the strong linear relationship between dose AUC_{τ}^{ss} discussed in section 1.7.1. Alternatively, as discussed above regarding Verebely and co-workers' (1975a) suggestion of methadone "auto-induction" of metabolism, the liver function of the subjects may have improved, as these subjects were also housed in a closed ward throughout the duration of the study. However, this was not monitored in the subjects after commencement of treatment.

1.7.3.2. Drug-drug interactions *in vivo*

A drug-drug interaction occurs when the effect of one drug is altered when another drug is co-administered. The clinical outcome of the interaction may be an increased or decreased pharmacological effect of the index drug in the presence of the interacting drug. The clinical significance of the interaction will depend on the therapeutic index and the steepness of the concentration-effect relationship of the drug undergoing the interaction. Broadly speaking, drug-drug interactions can be divided into two mechanistic categories: pharmacodynamic -those which act at the site of action of the drug, and pharmacokinetic- those where the interacting drug alters the pharmacokinetics of the drug undergoing interaction. Both types of interaction may result in and altered drug effect, and may occur simultaneously in some instances. This review will concentrate on reports of pharmacokinetic drug-drug interactions of methadone from *in vivo* human studies which may provide some insight into the enzymes involved in the metabolism of methadone.

Pharmacokinetic drug-drug interactions may result an increase or a decrease in the metabolism of the index drug. In the case of methadone for the treatment of opioid dependency, several drugs that are well established inducers of CYP3A4 expression (see Ketter et al., 1995) have been implicated in the occurrence of withdrawal symptoms (carbamazepine, phenytoin, Saxon et al., 1989) accompanied by increased methadone metabolism (carbamazepine, Halikas et al., 1990; rifampin, Kreek et al., 1976a; carbamazepine, Kuhn et al., 1989; phenobarbital, Liu & Wang, 1984; phenytoin, Tong et al., 1981).

Inhibition of methadone metabolism in methadone maintenance patients has been observed, as measured by an increased plasma methadone concentrations immediately before the administration of the daily maintenance dose. Bertschy and Eap's group (Bertschy et al., 1994; Bertschy et al., 1996; Eap et al., 1997) observed increases in the plasma methadone concentration/dose ratio after commencement of co-administration with fluoxetine and fluvoxamine. These authors suggested the involvement of CYP2D6 and CYP1A2 in the metabolism of methadone. Furthermore, Eap and co-workers (1997) suggested that the drug-drug interaction between fluoxetine and methadone was stereoselective, with the plasma concentration/dose ratio of the (R)-enantiomer only significantly ($P<0.05$) increased by about 30% after chronic treatment with 20 $mg.day^{-1}$ fluoxetine. In contrast, the effect of fluvoxamine was similar on both enantiomers, with plasma concentration/dose ratios increasing by approximately 40% ($P<0.05$). Recent case reports have confirmed the fluvoxamine-mediated increase in plasma methadone concentrations (Alderman & Frith, 1999; DeMaria & Serota, 1999). In contrast, others have reported no change in plasma methadone concentrations in 16 methadone maintenance patients after co-administration with fluoxetine for 9 weeks (Batki et al., 1993). Fluoxetine and its N-desmethyl metabolite (von Moltke et al., 1995a), and fluvoxamine (Brøsen et al., 1993; von Moltke et al., 1995a) are known to inhibit CYP3A4 mediated reactions, in addition to CYP2D6 and CYP1A2. Therefore, the observations of Bertschy and Eap (Bertschy et al., 1994; Bertschy et al.,

1996; Eap et al., 1997) could be interpreted as indicating the involvement of CYP3A4 in the metabolism of methadone, in addition to, or in place of, CYP2D6 or CYP1A2. More recently, Cobb and co-workers (1998) demonstrated that co-administration of fluconazole (200 $mg.day^{-1}$ for 14 days) in methadone maintenance subjects resulted in a 24% decrease in the apparent oral clearance of methadone (see Appendix 3), while the excretion of unchanged methadone increased by 52%. Fluconazole is known to potently inhibit the catalytic activity of both CYP3A4 (von Moltke et al., 1996) and CYP2C9 (Kunze et al., 1996; Miners & Birkett, 1998) *in vitro* and *in vivo*.

Methadone has been shown to alter the metabolism of CYP2D6 substrates *in vivo* (Kosten et al., 1990; Maany et al., 1989; Wu et al., 1993) and *in vitro* (Kerry et al., 1994; Wu et al., 1993). However from these data, it is difficult to determine whether methadone is metabolised by CYP2D6. It is possible for drug metabolising enzymes to have a high affinity for a substrate but not metabolise the substrate, for example quinidine potently inhibits CYP2D6, but is not metabolised by this isoform (Otton et al., 1988).

In summary, the available literature reports of *in vivo* human drug-drug interactions involving methadone strongly suggest that CYP3A4 is involved in the metabolism of methadone. The involvement of CYP1A2 and/or CYP2D6 in the metabolism of methadone is also possible. However, caution is advised when interpreting these data. The possibility of inhibition of multiple CYP450 isoforms by the interacting drug cannot be excluded in most cases, due to a lack of isoform specificity.

1.7.4. Cytochrome P450

1.7.4.1. Overview of the CYP450 system

Before being eliminated from the body, most xenobiotics undergo metabolic alteration, which occurs predominantly in the liver. Enzymatic modification of a xenobiotic usually abolishes its pharmacological activity, although there are exceptions to this such as morphine 6-glucuronidation. The enzymes mediating the metabolism of compounds can be

broadly classified into two categories: phase I and phase II. Phase I reactions result in functionalisation of xenobiotics by either introduction of a polar functional group, or exposure of a polar functionality, and include hydrolysis, reduction and oxidation. Phase II reactions are responsible for conjugation of a xenobiotic with a polar substrate, and may act in concert with phase I metabolic pathways. The increased polarity of the xenobiotic after metabolism results in an increased rate of elimination of the xenobiotic from the body, usually via renal excretion (Rang & Dale, 1991).

Phase I metabolic reactions are most often mediated by the cytochrome P450 (CYP450) superfamily of enzymes. The ancestral gene of this superfamily has existed since before the prokaryote/eukaryote divergence occurred 3.5 billion years ago (Nelson et al., 1996). In 1996, Nelson and co-workers (1996) published the latest update summarising the current knowledge of the CYP450 superfamily. They reported that, at that time, 74 gene families had been discovered, of which 14 families exist in all mammals examined to date. The 14 families comprise 26 mammalian subfamilies, of which 20 had been mapped in the human genome.

The CYP450 enzymes display distinct substrate specificities, although overlapping substrate specificities are not uncommon. In humans, subfamilies 1-4 are important for xenobiotic metabolism, while other subfamilies are involved in the metabolism of endogenous compounds, such as thromboxane or steroid synthesis and cholesterol side chain cleavage (Price Evans, 1993).

1.7.4.2. CYP3A4

CYP3A is the most abundant CYP subfamily in the human liver, constituting approximately 30% to 50% of total CYP450 (Ketter et al., 1995; Thummel & Wilkinson, 1998; von Moltke et al., 1995b; Watkins, 1994). Significant amounts of CYP3A have been measured in various regions of the intestine (Klotz et al., 1998; Kolars et al., 1994; Lown et al., 1994; Paine et al., 1997), with a progressive decline in microsomal CYP3A content from the

duodenum to the ileum (Paine et al., 1997). CYP3A3 is very closely related to CYP3A4 (>98% cDNA sequence homology), but it is unknown whether it is a separate gene product or an allelic variant (Thummel & Wilkinson, 1998). The term CYP3A4 is generally taken to indicate a collective contribution of the two isoforms (Thummel & Wilkinson, 1998). In contrast, CYP3A5 is a structurally distinct protein and has different substrate specificity.

The expression of CYP3A4 is highly variable and is not solely under genetic control, but also under environmental control and is highly inducible by synthetic glucocorticoids, macrolide antibiotics and phenobarbitone (Ketter et al., 1995; Thummel & Wilkinson, 1998; von Moltke et al., 1995b). In contrast to CYP2D6 and CYP2C19, the expression of CYP3A4 is not considered to be subject to a genetic polymorphism (Ketter et al., 1995; Thummel & Wilkinson, 1998; von Moltke et al., 1995b). Indeed, as noted by some authors, there have been no reports to date of a samples completely lacking in CYP3A4 protein or activity (Sata et al., 2000). To date, only three CYP3A4 genetic variants have been reported (Ball et al., 1999; Rebbeck et al., 1998; Sata et al., 2000). *CYP3A4*1B* is a point mutation in the 5'-flanking region of the human *CYP3A4* gene (Rebbeck et al., 1998) has a higher frequency in Black (55-67% Ball et al., 1999; Sata et al., 2000) and Hispanic (9% Ball et al., 1999) than in Caucasian subjects (3-4% Ball et al., 1999; Sata et al., 2000), and appears to be absent in Japanese (Ball et al., 1999) and Chinese subjects (Ball et al., 1999; Sata et al., 2000). The presence of this mutation has been shown not to have an effect on CYP3A4 mediated metabolism, as there were no significant phenotypic differences between subjects genotyped as homozygote for the wild-type (*CYP3A4*1A*) allele or the *CYP3A4*1B* variant allele using two separate CYP3A4 activity probe drugs *in vivo* (Ball et al., 1999). A second allelic variant, *CYP3A4*2*, has been reported with a frequency of 2.7% in Caucasian subjects, but was absent in Black and Chinese subjects (Sata et al., 2000). Preliminary evidence suggests that this variant may display a different substrate selectivity compared to the wild-type protein (Sata et al., 2000). A third, rare allelic variant (*CYP3A4*3*) has also been reported in a single Chinese subject (Sata et al., 2000), but not in Caucasian or Black subjects. However, only 20 Caucasian, 20 Black and 32 Chinese subjects have been

examined for this variant, and its metabolic activity has not yet been examined (Sata et al., 2000).

Testosterone 6β-hydroxylation is commonly used as an index of CYP3A enzymatic activity *in vitro*. In human liver microsomes, the metabolic activity of this pathway has been shown to vary 7-fold (54% CV, Forrester et al., 1992) and 5-fold (40% CV) with no marked effect of gender (Chauret et al., 1997). Forrester and co-workers (1992) reported that CYP3A4-mediated catalytic activities varied between 4- and 18-fold, dependent upon the substrate used. Others have reported similar variability in hepatic CYP3A content (53% CV, Shimada et al., 1994; 10-fold range, 60% CV, Thummel et al., 1994a). Shimada and co-workers (1994) reported no sex related differences in hepatic CYP3A4 content, or differences between samples obtained from Caucasian and Japanese subjects. Lown and co-workers (1992) found that the CYP3A content in microsomal preparations from diseased liver samples varied over a 12-fold range, and was not significantly (P>0.05) different compared to samples taken from patients without liver disease.

At present, the erythromycin breath test and plasma clearance of midazolam are considered to be the most reliable methods of estimating CYP3A activity *in vivo* (Thummel & Wilkinson, 1998; Watkins, 1994), and have been demonstrated to correlate (Lown et al., 1995). Following oral administration of the probe drug, the relative contribution of CYP3A in the intestinal epithelium cannot be distinguished from that in the liver (Thummel & Wilkinson, 1998; Watkins, 1994). The enzymatic activity observed after oral administration is therefore a function of the activity at both sites. Conversely, after *i.v.* administration, intestinal CYP3A activity is presumably not assessed (Thummel & Wilkinson, 1998; Watkins, 1994). Given that CYP3A is expressed in significant amounts in the intestinal epithelium, it is important to consider CYP3A activity in the intestine when examining the variability in CYP3A activity *in vivo*, if these data are to be extrapolated to other orally administered compounds.

In 17 healthy volunteers, Lown and co-workers (1995) demonstrated that erythromycin breath test results and midazolam unbound plasma clearance corrected for body weight showed similar inter-subject variability, in the order of 6- to 8-fold. Similarly, Watkins and co-workers (1992) reported a 14-fold range in CYP3A activity, after *i.v.* erythromycin administration to 47 volunteers. Thummel and co-workers (1996) reported midazolam clearance to vary 3-fold (30% CV) after *i.v.* administration, and 6-fold (60% CV) after oral administration to 20 healthy volunteers. This study measured the absolute oral bioavailability in each subject, and then compared this to the oral bioavailability predicted from the data obtained after *i.v.* administration only, assuming no extra-hepatic metabolism. Thus, the relative contribution of hepatic and extra-hepatic (assumed to be gastrointestinal) first-pass metabolism could be estimated. The relative contributions (mean±SD) of the gastrointestinal epithelium and liver to the overall first-pass effect were estimated to be 43±24% (range: 0 to 77%) and 44±14% (range: 22 to 76%), respectively. These data indicate a wider variability in metabolism by the intestinal epithelium when compared to the liver, although considerable variation in hepatic metabolism also exists.

1.7.4.3. CYP450 mediated methadone metabolism

At the time the present work was conducted, there were no reports examining the metabolism of methadone in any human tissue preparation. Early examinations of the metabolism of methadone *in vitro* were restricted to crude homogenates or microsomal fractions prepared from animal tissues. The techniques employed in the majority of these studies utilised the loss of methadone from samples (Sung & Way, 1950) or the measurement of formaldehyde formation as a measure of N-demethylation (Alvares & Kappas, 1972; Axelrod, 1956; Dawson & Vestal, 1984; Liu & Wang, 1975; Masten et al., 1975; Roerig et al., 1975; Sullivan et al., 1975a). These are relatively non-specific techniques which cannot differentiate between the formation of individual metabolites. In contrast, only Beckett and co-workers (1971b) employed an assay selective for EDDP.

Only four reports are available which examined the metabolism of methadone with respect to stereoselectivity (Alvares & Kappas, 1972; Axelrod, 1956; Beckett et al., 1971b; Sullivan et al., 1975a). Three of these groups employed the liberation of formaldehyde as a measure of N-demethylation in rat liver homogenates, while Beckett and co-workers (1971b) employed an assay selective for EDDP in both rat and guinea pig liver homogenates. All of these groups employed a NADP re-generating system. Although the effect of omission of the NADP re-generating system was not reported in any study, Sullivan and co-workers (1975a) reported that they optimised the co-factor concentration to provide "maximum formaldehyde production". Despite this, it would seem reasonable that all of these authors had observed an absolute requirement for an NADP re-generating system in preliminary experiments. Additionally, Axelrod (1956) reported that the greatest activity was located in the microsomal fraction of homogenate samples. While others employed this subcellular fraction, they did not make comparisons with other fractions (Alvares & Kappas, 1972; Beckett et al., 1971b).

Axelrod (Axelrod, 1956) reported that (R)-methadone was metabolised at a rate approximately twice that of (S)-methadone in rat liver homogenate. In contrast, Beckett and co-workers (1971b) found a 1.5-fold selective metabolism of (S)-methadone in rat liver homogenate, while this was almost 2-fold selective for (R)-methadone in the guinea pig. Both groups suggested that the N-demethylation of methadone was stereoselective, although no statistical analysis was performed. Sullivan and co-workers (1975a) and Alvares & Kappas (1972) reported the rate of formaldehyde production from (R)- and (S)-methadone differed by less than 10% in rat liver homogenates. Interestingly, Alvares & Kappas (1972) reported that pre-treatment of rats with phenobarbital ($38mg.kg^{-1}$) twice daily for 5 days resulted in a 4-fold increase in methadone N-demethylation, while pre-treatment of rats with rac-methadone ($20mg.kg^{-1}$) for 14 days had no effect.

In summary, although the data are limited, they indicate that the metabolism of methadone is likely to be mediated by CYP450 enzyme(s), as the greatest rate of methadone

N-demethylation occurs in the microsomal sub-cellular fraction and NADP regenerating systems were employed. The role of stereoselectivity in the metabolism of methadone remains unclear as conflicting results have been obtained within a single species. Furthermore, no study to date has investigated the metabolism of methadone in any human tissue preparation.

1.7.5. Elimination

Terminal elimination half-life values for rac-methadone have been estimated from blood sampling protocols that have rarely exceeded 48 hours -much less than the necessary 3- to 5-times the calculated $t_{1/2\beta}$ (see Appendix 3). Sampling protocols which extend longer than 48 hours after acute administration have been restricted to healthy normal subjects, with mean±SD values of 41±21 hours (0-57 hours, Wolff et al., 1997), 20±4 hours during urinary acidification (pH 4.7-7.1) and 40±9 hours during urinary alkalinisation (pH 7.0-8.8, 0-72 hours, Nilsson et al., 1982b). During chronic dosing in methadone maintenance patients, the 24 hour inter-dosing interval prevents accurate estimation of half-life values from data obtained over longer periods. Substitution of a single daily dose with stable isotope-labelled compound allowed Kreek and co-workers (1979) to follow the elimination of a single dose for up to 10 days during chronic administration of unlabelled methadone. However, these authors only enrolled three subjects and the $t_{1/2\beta}$ of rac-methadone (53±6 hours) was estimated from urinary excretion data only. In larger groups of patients using stable isotope-labelled compound, mean $t_{1/2\beta}$ values of 28±11 hours (Meresaar et al., 1981) and 35±12 hours (Nilsson et al., 1982a) after acute and 31±8 hours (Nilsson et al., 1982a), and 36±6 hours (Nilsson et al., 1982a) after chronic administration, have been estimated using 0-48 hour blood sampling in former opioid dependent methadone maintenance patients.

As can be seen from Appendix 3, mean values of rac-methadone clearance generally range from 50-200 $ml.min^{-1}$, indicating that methadone is a low hepatic extraction ratio drug. In support of this, Inturrisi and co-workers (1987b) estimated the hepatic extraction ratio of

rac-methadone to be 0.09±0.05 in chronic pain patients after acute *i.v.* administration of rac-methadone. Methadone clearance values within various patient groups (healthy subjects, former opioid dependent subjects, pain patients) demonstrate a degree of overlap. Unfortunately, comparison of clearance values between studies and patient groups is complicated. In pain patients, estimates of clearance are for total systemic clearance only, after acute parenteral administration of the drug. In contrast, in former opioid dependent subjects receiving rac-methadone maintenance therapy, estimates of apparent oral clearance (CL/F), and total systemic clearance after acute parenteral administration stable isotope-labelled drug ($^2H_3$-methadone) at the commencement of treatment and at steady-state dosing are available. The contribution of "isotope effects" appears to be minimal, as Meresaar and co-workers (1981) and Nilsson and co-workers (1982a) demonstrated that the concentration-time profiles of stable isotope-labelled and unlabelled methadone were almost identical when administered concomitantly in equal doses.

Total systemic clearance [mean±SD (range)] values have been remarkably similar between studies in pain patients after parenteral administration: 178±100 $ml.min^{-1}$ (Gourlay et al., 1982), 190±130 $ml.min^{-1}$ (Gourlay et al., 1986a), 146±68 $ml.min^{-1}$ (Inturrisi et al., 1987b), 186 (23-850) $ml.min^{-1}$ (Plummer et al., 1988). The estimates of total systemic clearance reported by Gourlay and co-workers (1986a; 1982) and Inturrisi and co-workers (1987b) were based on concentrations of methadone in blood. Methadone is known to distribute into red blood cells with a mean blood-to-plasma concentration ratio of 0.75, indicating that the corresponding plasma clearance values would be approximately 25% lower (see section 1.7.2.2) than those reported by these authors for blood. However, in patients with severe burns, total systemic clearance of methadone was markedly higher (883±317 $ml.min^{-1}$), which the authors attributed to loss of methadone into the wounds (Denson et al., 1990).

In healthy volunteers, only three authors have reported CL/F values, all of which were obtained after a single rac-methadone dose. A comparison of the CL/F values reported by these authors highlights the likelihood of overestimating clearance when a 24 hour blood

sampling protocol is employed after single dose administration, as noted in the introduction of this section (section 1.7). Mean clearance values of 134±21 ml.min^{-1} and 92±9 ml.min^{-1} have been reported when the sampling duration extended to 72 hours (Nilsson et al., 1982b). In contrast, Wissel and co-workers reported markedly higher values (223±71 ml.min^{-1} and 204±78 ml.min^{-1}) using 24 hour data only. Importantly, values reported by Nilsson and co-workers (1982b) represent the range of values when the contribution of renal clearance was maximised and made negligible, via alteration of urinary pH (see section 1.7.5.1.1).

In otherwise healthy former opioid dependent subjects receiving chronic oral dosing of rac-methadone, CL/F (mean±SD) values range from 107±55 ml.min^{-1} (de Vos et al., 1995) to 184±30 ml.min^{-1} (Wolff et al., 1993), with the exception of two studies which reported markedly higher values of 308±44 ml.min^{-1} (Novick et al., 1981) and 250±36 ml.min^{-1} (Novick et al., 1985), which will be discussed later. Using parenteral administration of stable-isotope labelled rac-methadone, similar estimates have been reported for total systemic clearance during chronic administration of the unlabelled drug, ranging from 100±55 ml.min^{-1} (Nilsson et al., 1982a) to 137±92 ml.min^{-1} (Meresaar et al., 1981). The similarity of the values for total systemic clearance and CL/F are consistent with the high oral bioavailability of methadone (see section 1.7.1).

Based upon urinary and faecal excretion studies, some authors have reported that the rate methadone metabolism increases during chronic administration of methadone (Änggård et al., 1975; Nilsson et al., 1982a; Verebely et al., 1975b). However, the evidence presented by these authors was weak, as discussed in section 1.7.3.1. Verebely and co-workers (1975b) provided some supportive pharmacokinetic evidence of this phenomenon, as $t_{1/2\beta}$ values were markedly shorter during chronic (22±7 hours) compared to acute administration (55±27 hours) in the same subjects. Half-life values were estimated from 0-24 hour data, and other pharmacokinetic parameters were not provided. As discussed previously (section 1.7), half-life values estimated from 0-24 hour data are unlikely to provide an accurate description of the disposition of methadone, and are influenced by volume of distribution in

addition to clearance. In contrast, the conclusion of Nilsson and co-workers (1982a) that the rate methadone metabolism increases over time, based on urinary excretion data (see section 1.7.3.1), is hard to reconcile with the pharmacokinetic data they presented. These authors found no consistent time-related changes in total systemic clearance, $t_{1/2\beta}$ or $V_{d\beta}$, indicting no alteration of methadone elimination over time.

Nilsson and co-workers (1983) reported that the systemic clearance of rac-methadone in methadone maintenance treatment "therapeutic failures" (104±36 $ml.min^{-1}$) was not difference to that in a group of unselected patients (111±36 $ml.min^{-1}$). However, they showed that the volume of distribution, and hence half-life, in the former group was only approximately 75% that of the unselected patients (see section 1.7.2). The authors suggested that therapeutic failure group may be subject to a greater degree of fluctuation in plasma concentrations, and hence opioid effects, compared to the comparison group.

In methadone maintenance patients, Novick and co-workers compared the effect of liver disease of increasing severity on the steady-state disposition of rac-methadone (Novick et al., 1981), and the effect of severe alcoholic liver disease in patients "abusing" alcohol, but without concurrent liver disease (Novick et al., 1985). In both studies, liver disease had no significant effect on apparent oral clearance. For a detailed discussion of this study see section 1.7.5.1.2. However, the readers attention is drawn to the high mean clearance values reported by these authors in both studies (250-393 $ml.min^{-1}$) compared to those reported by other authors in healthy methadone maintenance patients without evidence of liver disease (107-184 $ml.min^{-1}$, see Appendix 3). It is possible that the clearance values are overestimates in the former studies, due to an inadequate sampling protocol, as only six blood samples were taken over the 24 hour inter-dosing interval.

There is wide inter-subject variation within studies as evidenced by coefficients of variation generally ranging 30% to 40% (see Appendix 3). However, some authors have found much greater variability of up to 50% to 70% coefficient of variation in methadone maintenance

patients after chronic oral administration (51%, de Vos et al., 1995; 67%, Meresaar et al., 1981; 55%, Nilsson et al., 1982a), and in patients with chronic pain administered methadone as a single intravenous dose (68% Gourlay et al., 1986a; 56%, Gourlay et al., 1982). In contrast, few studies have reported low inter-subject variability in patients receiving oral methadone maintenance treatment for opioid dependence (13% Cobb et al., 1998; 16%, Wolff et al., 1993; 9%, Wolff et al., 1997). While differences in the intensity and duration of blood sampling protocols may contribute to the observed inter-subject variability in clearance between studies, it appears that the clearance of rac-methadone is somewhat variable.

As noted previously in section 1.7.3.1, the recovery of methadone and known metabolites has failed to result in complete mass-balance, although EDDP appears to be quantitatively the major metabolite. Plasma concentrations of EDDP have only been quantitated in a simple study, and were less than 10% that of methadone during chronic oral dosing (de Vos et al., 1995), although this was highly variable, with values ranging 2-18% over the 20 subjects studied. However, the excretion of EDDP or methadone was not examined by these authors. Therefore, the contribution of variability in the partial clearance to EDDP to the observed variability in methadone total body clearance remains unknown.

Prior to the commencement of the work presented in this thesis in 1995-1996, there was only single study examining stereoselectivity in the disposition of methadone in humans in which the authors recruited more than three subjects (Olsen et al., 1977). However, this study only reported $t_{1/2\beta}$ values, and not other pharmacokinetic parameters. On separate occasions, these authors administered 7.5 mg of each enantiomer orally, or 15 mg of rac-methadone, to six healthy male subjects, and collected blood samples for up to 72 hours. However, calculation of half-life values was performed using the concentration-time data up to 48 hours only, and was not performed in one subject for rac- and (S)-methadone, as plasma methadone concentrations were below the limit of quantification after 30 hours. The (mean; range) $t_{1/2\beta}$ for (R)-methadone (24 hours; 19-31 hours), (S)-methadone (25 hours;

21-28 hours) or rac-methadone (22 hours; 13-28 hours) were not significantly different (P>0.05). Whether the difference in $t_{1/2\beta}$ values observed by these authors was due to differences in CL/F and/or volume of distribution or bioavailability is unknown. In addition, the contribution of inter-occasion variability further decreases confidence in these data, as the individual enantiomers were administered on separate occasions.

The disposition of the methadone enantiomers after chronic dosing has been investigated in three studies only. However, these studies suffered from low subject numbers (n=1, Beck et al., 1991; n=3, Kreek et al., 1979; n=2, Nakamura et al., 1982) and only reported $t_{1/2\beta}$ values. In the first study, Kreek and co-workers (1979) synthesised the individual methadone enantiomers incorporating five deuterium atoms on one aromatic ring ($^2H_5$-methadone). On separate occasions, half of the patients daily oral maintenance rac-methadone dose was administered as a single isotope-labelled methadone enantiomer, or both enantiomers together as the entire methadone dose. Multiple blood and urine samples were collected on the day of administration, and daily thereafter for 10 days. Using mass-spectroscopy, these authors were able to quantitate the stable-isotope labelled compounds in the presence of steady-state concentrations of unlabelled drug. Based upon urinary elimination data, the mean±SD $t_{1/2\beta}$ for (R)-methadone (57±3 hours) was longer than (S)-methadone (34±2 hours) and rac-methadone (53±6 hours). However, when calculated from concentrations in plasma, the data from two subjects were reported for (R)- and (S)-methadone only. Plasma $t_{1/2\beta}$ values for (R)-methadone (43 hours and 53 hours) were longer than for (S)-methadone (38 hours and 41 hours, respectively) in both subjects. Nakamura and co-workers (1982) employed a similar technique using stable-isotope labelled enantiomers of (R)-methadone ($^2H_3$-methadone) and (S)-methadone ($^2H_5$-methadone) containing five and three deuterium atoms, respectively. This technique allowed the authors to administer both enantiomers together in equal proportions as a racemic mixture in substitution for a daily oral dose in two methadone maintenance patients. Multiple blood samples were collected for up to 5 days (119 hours), and a tri-exponential disposition function was fitted to the data. Terminal elimination half-life values for

(R)-methadone (38 and 59 hours) were longer than that of (S)-methadone (28 and 35 hours) in both subjects. The data presented by Kreek and co-workers (1979) and Nakamura and co-workers (1982) indicate that (R)-methadone has a longer $t_{1/2\beta}$ than that of (S)-methadone during chronic administration. However, despite employing a very powerful technique combined with an acceptable blood sampling protocol, these authors enrolled too few subjects for definitive conclusions to be made. In contrast, Beck and co-workers (1991) using a stereoselective HPLC assay, reported similar $t_{1/2\beta}$ values only for (R)-methadone and (S)-methadone (14 hours and 16 hours, respectively), in a single methadone maintenance patient.

In summary, methadone is a compound of intermediate clearance, with mean values generally in the 50-200 $ml.min^{-1}$ range. However, large inter-individual variability in both total systemic clearance and apparent oral clearance has been demonstrated both within and between studies. No study to date has examined the contribution of variability in the metabolism to the major metabolite EDDP to the observed variability in methadone clearance. There are limited data available prior to the commencement of the work presented in this thesis in 1995-96, which indicates that the $t_{1/2\beta}$ of the (R)- enantiomer is greater than that of the (S)- enantiomer during chronic administration of the racemic compound. More recently, this has been confirmed after acute administration in chronic pain patients, although whether this was due to stereoselective intrinsic clearance or plasma protein binding was not identified. No study to date has examined the disposition of methadone in methadone maintenance patients with respect to stereoselectivity in detail, or examined the role of metabolism or plasma protein binding.

1.7.5.1. Disease and other states which alter methadone metabolism and clearance

1.7.5.1.1. Urine pH

Nilsson and co-workers (1982b) examined the effect of altering urine pH, by the oral administration of ammonium chloride (acidic urine) and sodium hydrogen carbonate

(alkaline urine) on the pharmacokinetics of rac-methadone. This study employed a cross-over design involving five healthy male volunteers with no previous history of alcohol or drug abuse. Treatment with ammonium chloride or sodium hydrogen carbonate resulted in a urine pH ranges of 4.7-7.1 and 7.0-8.8, respectively. Methadone (10 mg) was administered as an *i.m.* injection to avoid the effects of altered gastrointestinal pH on absorption, and plasma and urine samples were collected for up to 96 hours. The renal clearance of methadone was not able to be calculated after alkaline urine treatment because methadone concentrations in urine were below the limit of quantification. The renal clearance (mean±SD) of methadone was 47±11 $ml.min^{-1}$ for the acidic urine treatment. Inspection of the data reveals that renal clearance accounts for 35±4% of total body clearance in this group. Elimination $t_{1/2}$ was found to be significantly ($P<0.001$) shorter when the urine was acidic (19.5±3.6 hours) compared to alkaline urine (42.1±8.8 hours). This agrees with the observation that total body clearance was significantly ($P<0.01$) higher in the acidic urine treatment group (134±21 $ml.min^{-1}$; range: 111-159 $ml.min^{-1}$) compared to the alkaline urine treatment group (92±9 $ml.min^{-1}$; range: 84-106 $ml.min^{-1}$), due to the increased contribution of renal clearance. The observation of a 50% increase in total body clearance accompanied by a 100% increase in $t_{1/2}$ for the acidic compared to the alkaline urine treatment group may be explained by the observed greater $V_{d\beta}$ of the latter group (see section 1.7.2).

In methadone maintenance patients, Bellward and co-workers (1977) reported that the renal clearance of rac-methadone was increased when urine pH was below 6. However, their statistical analysis appears somewhat flawed. They allocated patients to either a "high" (mean±SD; 35.2±6.7 $ml.min^{-1}$; n=6) or "low" (12.3±3.6 $ml.min^{-1}$; n=6) renal clearance group, and noted that urinary pH was lower in the former group. As would be expected, a highly significant ($P<0.001$) difference for renal clearance was observed between the two groups, but was not reported for urinary pH. However, comparison of the data by the present author revealed that the urinary pH (mean±SD; range) of the "high" renal clearance group (6.1±0.38) was statistically significantly (un-paired t-test; $P=0.02$) lower than the

"low" (6.6±0.28) renal clearance group. These authors calculated the theoretical percent ionisation of methadone (based upon a pKa of 8.6) in the urine samples, and plotted this against the measured renal clearance for each subject. The plot clearly demonstrated an exponential increase in renal clearance to a plateau at 100% ionisation which ranged from 60-100 $ml.min^{-1}$, while the most dramatic change was seen in the region between 96% and 99.5% ionisation (approximately pH 7.2-6.3).

These data confirm earlier preliminary reports of an increase in methadone renal clearance with urinary pH below 6 (Baselt & Casarett, 1972b; Inturrisi & Verebely, 1972a; Inturrisi & Verebely, 1972c), and highlight the need to consider renal clearance when examining the pharmacokinetics of methadone, as this pathway may account for 30-40% of total body clearance in some subjects. In contrast, other subjects may not eliminate methadone via this route due to the alkalinity of their urine. This may become more important when subjects cease or commence the use of urinary alkalisers for the treatment of medical conditions unrelated to their prescription of methadone, resulting in sudden changes of total body clearance of methadone.

1.7.5.1.2. Hepatic dysfunction

This review of the literature found surprisingly few reports examining the effect of liver disease on the pharmacokinetics and metabolism of methadone. The studies were all undertaken by essentially the same group, and comprised two pharmacokinetic studies (Novick et al., 1985; Novick et al., 1981), one urinary excretion (Kreek et al., 1980a) and one faecal (Kreek et al., 1983) excretion study.

Novick and co-workers (1981) divided subjects into three groups, based upon the severity of liver disease. A control group was included, and consisted of patients with no clinical or biochemical evidence of liver disease. All subjects were currently enrolled in a methadone maintenance program. Severity of liver disease was based on clinical and biochemical data, biopsy diagnosis, and overall clinical impression. Group 1 consisted of five patients with

severe chronic liver disease -all had evidence of cirrhosis that was secondary to alcoholic, viral or combined liver injury. Group 2 consisted of five patients with moderate liver disease, all of whom had compensated micronodular cirrhosis. Group 3 consisted of four patients with either viral hepatitis, cholestasis, alcoholic hepatitis or fatty infiltration. All four patients had evidence of chronic hepatic dysfunction, but pathologic findings indicated the condition was potentially reversible. Blood sampling was limited, with only six samples taken over the 24 hour inter-dosing interval. Daily rac-methadone maintenance doses were not statistically (P>0.05) different between groups. There were no significant (P>0.05) differences observed between the groups for plasma AUC_{0-24}, apparent oral clearance, or average plasma concentration at steady-state. Dose correction of plasma AUC_{0-24} and average plasma concentration did not change these results. Elimination $t_{1/2}$ (mean±SEM) was significantly longer in group 1 (35.5±7.6 hours) compared to group 2 (13.0±1.6 hours; P<0.01), group 3 (11.3 ±1.9 hours; P<0.01) and the contrast group (18.8±3.0 hour; P<0.05). Using essentially the same experimental protocol, Novick and co-workers (1985) later compared the pharmacokinetics of rac-methadone in patients with severe alcoholic liver disease (n=11) to those currently "abusing" alcohol, but without concurrent liver disease (n=9). Similar to the earlier study, liver disease had no significant (P>0.05) effect on AUC_{0-24}, apparent oral clearance, average plasma concentration or T_{max}. Plasma $t_{1/2}$ (mean±SEM) was found to be significantly (P=0.04) longer in the patients with liver disease (32.0±5.1 hours) compared to the control group (19.7±2.2 hours). The estimated $V_{d\beta}$ of methadone was greater in patients with liver disease (716±100 l) compared to the control group (438±94 l), but this did not reach statistical significance. The results of these studies are difficult to interpret, as the blood sampling protocol was inadequate to accurately calculate elimination $t_{1/2}$. Despite this, it is likely that the reported prolonged elimination $t_{1/2}$ in severe liver disease is due increased volume of distribution, as oral clearance differed by less than 15% between the two groups. In both studies, plasma albumin concentrations were low to normal in all subjects, while neither study reported plasma concentrations of α_1-acid glycoprotein. Liver disease is known to have variable effects on plasma concentrations of α_1-acid glycoprotein, and the preponderance of the data indicates a

lowering of concentrations (see section 1.7.2.1.1). Low plasma α_1-acid glycoprotein concentrations are likely to result in increased plasma unbound fraction of methadone, which is supported by the finding of a larger V_d in liver disease (Novick et al., 1985). This may offset any decrease in hepatic intrinsic clearance caused by liver disease, resulting in no net effect of liver disease on oral clearance when calculations are based on measurement of total (bound plus unbound) drug. Unfortunately the authors did not measure the plasma binding of methadone, and these mechanisms require further examination.

Using mass spectroscopy, Kreek and co-workers (1980a) reported that the total 24 hour urinary excretion (mean±SD) of methadone and five metabolites (combined p-hydroxy methadone and pyrrolidone metabolite, EDDP, EMDP, and methadol) was significantly ($P<0.05$) lower in methadone maintenance patients with chronic liver disease (n=14, 5 female; 35±12% of the dose) compared to otherwise healthy male patients (n=5; 52±4% of the dose). The observed difference in total amounts excreted was predominantly due to lower recoveries of methadone (10±5% of the dose) and EDDP (23±11% of the dose) in the patients with liver disease, compared to otherwise healthy subjects (17±6% and 30±4% of the dose, respectively). Excretion of the other metabolites were similar in the two patient groups, with mean values ranging 0.1-4% of the dose. Female (n=5; 34±17% of the dose) and male (n=9; 35±10% of the dose) subjects with liver disease excreted similar total amounts. Despite this finding, there appeared to be a greater proportion of the dose eliminated as EDDP in females (n=5; 25±16% of the dose) compared to males (n=9; 21±7% of the dose) with liver disease, while the opposite was found for methadone (6±4% and 12±4% of the dose, respectively). The urinary excretion of the other metabolites was similar in males and females, with mean values ranging 0.1-2% of the dose. In the same patients, the faecal excretion of methadone and metabolites was not significantly ($P>0.05$) different in otherwise healthy male patients (9±2% of the dose) compared to male (10±4% of the dose) or female (13±5% of the dose) patients with chronic liver disease. The faecal excretion of the individual compounds was quantitatively similar to that found in otherwise healthy males and in males and females with liver disease.

1.7.5.1.3. Pregnancy

Pond and co-workers (1985) examined the disposition of rac-methadone in methadone maintained pregnant women. Four inter-dosing interval pharmacokinetic studies were performed in at least eight patients; two intervals during pregnancy (interval I 20-34 and interval II 35-40 weeks gestation), and two intervals after delivery (interval II 1-4 weeks and interval IV 8-9 weeks post partum). Minor changes in dose were allowed, however, there were no significant differences ($P>0.05$) throughout the study. Creatinine clearance, methadone unbound fraction (range of means: 16-21% unbound) and T_{max}, renal clearance of total (bound plus unbound) and unbound methadone were not found to be significantly ($P>0.05$) different during any of the four intervals. The percentage of the dose recovered in urine as methadone was not significantly ($P>0.05$) altered throughout the study, and similar results were obtained for the recovery of three methadone metabolites; sum of EDDP and EMDP, and α-methadol (see Appendix 4). There were no significant differences ($P>0.05$) in pre-dose plasma methadone concentrations or oral clearance between the two intervals studied during pregnancy, or after delivery (see Appendix 3). However, the oral clearance of methadone was significantly ($P<0.05$) increased by 1.5- to 2-fold at both intervals during pregnancy, when compared to the intervals after delivery (see Appendix 3). The results for oral clearance remained essentially unchanged when corrections for body weight and/or protein binding were made.

1.7.5.1.4. Other disease states

The effect on methadone disposition of other altered physiological or disease states that may be common in methadone maintenance patients (such as HIV infection, viral hepatitis, lung disease, gastrointestinal disorders, obesity) have not been examined.

1.8. Pharmacokinetic-pharmacodynamic relationships of methadone

For many drugs there is a much better relationship between plasma concentration and effect than with drug dose and effect. Pharmacokinetic-pharmacodynamic modelling allows the characterisation of the drug concentration-effect relationship. However, certain

fundamental assumptions must be made depending on the choice of model employed (for reviews see Breimer & Danhof, 1997; Drayer, 1986; Holford & Sheiner, 1981a; Holford & Sheiner, 1981b; Holford & Sheiner, 1982; Leemann & Blaschke, 1990). These models and assumptions will not be discussed in their entirety here, rather the reader is directed to the series of excellent reviews listed above. For opioid agonists, the effect (for example analgesia) is mediated at a site remote from plasma (the central nervous system). The implication of this phenomenon is that there is likely to be a delay between changes in the concentration of the drug in the plasma and the effect site. It is possible to employ an "effect compartment" model which relates plasma drug concentration to that at the effect site incorporating a rate constant (k_{eo}) for the removal of the drug from the effect site. Incorporating the effect compartment model (for the prediction of effect site concentration from plasma concentrations) and an appropriate pharmacodynamic model (relating effect site concentration to effect) thus allows the estimation of pharmacodynamic parameters expressed in terms of the plasma concentrations.

In humans, Gourlay's group reported a relationship between plasma rac-methadone concentration and analgesia in patients with postoperative (Gourlay et al., 1986b; Gourlay et al., 1984; Gourlay et al., 1982) and cancer (Gourlay et al., 1986a) pain. The "minimum effective concentration" of methadone for analgesia was defined as the plasma concentration of rac-methadone in a blood sample collected at the time of patient's request for supplementary pain relief. Estimates (mean±SD) of the minimum effective concentration of rac-methadone were 32±11 $ng.ml^{-1}$ (n=11 subjects, Gourlay et al., 1982), 58±15 $ng.ml^{-1}$ (n=16 subjects, Gourlay et al., 1984) and 59±24 $ng.ml^{-1}$ (n=10 subjects, Gourlay et al., 1986b) postoperatively, and 29±15 $ng.ml^{-1}$ in cancer patients (n=9 subjects, Gourlay et al., 1986a) after *i.v.* administration. As can be seen from the above data, the estimated "minimum effective concentration" of methadone for analgesia was found to have large inter-subject variability. In contrast, postoperative intra-patient variability (expressed as the mean coefficient of variation of replicate determinations in each subject) was found to be lower when patients were studied on more than one occasion: 21±10% (n=54

determinations in 16 subjects, Gourlay et al., 1984) and 18±10% (n=23 determinations in 8 subjects, Gourlay et al., 1986b).

Others have utilised more sophisticated techniques for exploring pharmacokinetic-pharmacodynamic relationship of rac-methadone for analgesia (Inturrisi et al., 1987b; Inturrisi et al., 1990) and sedation (Inturrisi et al., 1990) in chronic pain patients. These authors employed an "effect compartment" pharmacokinetic-pharmacodynamic model for rac-methadone mediated analgesia using a tri-exponential disposition function and the sigmoid E_{max} pharmacodynamic model. After a single 10-30 mg *i.v.* bolus administration in eight chronic pain patients who had previous exposure to opioid analgesics (Inturrisi et al., 1987b), mean±SD values for EC_{50} (plasma concentration eliciting 50% of the maximum effect, E_{max}) and γ (the sigmoidicity or slope factor, which determines the steepness of the concentration-effect relationship) were 290±380 $ng.ml^{-1}$ and 2.0±1.0, respectively. Estimates of k_{eo} were not provided, however, the mean±SD half-life calculated from this parameter was 7.3±7.7 minutes, indicating that the plasma and effect site compartments are rapidly equilibrated. In a later study, using the same pharmacokinetic-pharmacodynamic analysis, this group studied the analgesic and sedative effects of methadone during a 3 to 4.5 hour *i.v.* infusion; data were also collected for 4 to 5 hours after cessation of the infusion. Mean EC_{50} values for analgesia (359±158 $ng.ml^{-1}$) and sedation (336±205 $ng.ml^{-1}$) were comparable to those obtained earlier for analgesia (Inturrisi et al., 1987a). Estimates of γ indicted a very steep concentration-effect relationship for both analgesia (4.4±3.8) and sedation (5.8±5.4), in agreement with their earlier study. In contrast to the earlier study, estimates of k_{eo} were could not be obtained in over 50% of subjects for both analgesia and sedation. The authors inferred from this that the plasma and effect site compartment (presumably the central nervous system) were very rapidly equilibrated in these patients, in agreement with their earlier study (Inturrisi et al., 1987a). However, it is possible that the experimental protocol did not allow for an accurate estimation of this parameter. As noted by the authors, the collection of data both during and after cessation of the infusion allowed the development of tolerance to be observed as an increase in effect. This phenomenon

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would result in clockwise hysteresis of the concentration-effect plots, which was not observed. The large inter-patient variability observed in both studies was attributed to different degrees of cross-tolerance, as all subjects had previous exposure to opioid analgesics other than methadone (Inturrisi et al., 1987a; Inturrisi et al., 1990). The effect of stereoselectivity or protein binding was not examined by the above mentioned authors.

As discussed earlier, doses of rac-methadone exceeding 50 $mg.day^{-1}$ have been associated with better treatment outcomes in patients receiving methadone for the treatment of opioid dependence (see section 1.3.2). However, there is conflicting evidence regarding the relationship of therapeutic outcome with plasma methadone concentration in methadone maintenance patients. In the case of supporters of measuring plasma methadone concentrations, trough concentrations only have been used to guide dosing. For example, Dole (1994) commented that the critical minimum plasma methadone concentration is 100 $ng.ml^{-1}$, and that 50 $ng.ml^{-1}$ is clearly insufficient; steady state plasma concentrations of below 200 $ng.ml^{-1}$ were associated with increased patient complaints of "doses not holding" and a higher frequency of urinalysis tests containing illicit drugs and poorer psychosocial rehabilitation (Holmstrand et al., 1978). These authors argued that optimising the methadone dosage regimen to achieve target trough plasma methadone concentrations of 100-200 $ng.ml^{-1}$ (an implied minimum effective concentration) would improve the effectiveness of methadone maintenance therapy. Other workers, however, have reported no such relationship of trough plasma concentrations (ranging from 100-400 $ng.ml^{-1}$) and withdrawal symptoms (Bell et al., 1990; Bell et al., 1988; de Vos et al., 1996; Horns et al., 1975; Loimer & Schmid, 1992; Loimer et al., 1991).

The lack of concordance between authors reported above is possibly not surprising, given that withdrawal severity is a subjective measure, and that other factors are likely to play a role in the success of treatment as outlined above. However, it is important to note that all of the above mentioned authors have based their conclusions on the measurement of plasma concentrations of rac-methadone only. While quantification of racemic compounds is easier

analytically, many authors have reported on the need to acknowledge stereoselective differences in drug disposition to avoid misinterpretation of data from unresolved drug due to stereoselective differences in both pharmacokinetics and pharmacodynamics (Drayer, 1986; Eichelbaum, 1988; Evans et al., 1988; Jamali et al., 1989; Lee & Williams, 1990; Nation, 1994; Tucker & Lennard, 1990). Methadone is a chiral compound that displays stereoselectivity in pharmacodynamic effects (see section 1.5), and the (R)- enantiomer prevents the occurrence of opioid withdrawal symptoms, while (S)-methadone is ineffective (see section 1.3.2). Additionally, there is evidence, albeit limited, suggesting that this compound also displays stereoselective pharmacokinetics (see section 1.7). Despite the well established importance of stereoselectivity in the examination of pharmacokinetic-pharmacodynamic relationships, it is surprising that this issue has not been examined with respect to methadone maintenance therapy for opioid dependence. Indeed, at the time that the present PhD studies were conducted, there were no studies available in the literature addressing this issue. Furthermore, since 1995 only two studies have examined relationship between stereoselective differences in the plasma concentrations of methadone and therapeutic outcome in methadone maintenance patients.

Hiltunen and co-workers (1999) examined the relationship of subjective and objective measures of withdrawal symptoms (modified Objective Opiate Withdrawal Scale (OOWS) and Subjective Opiate Withdrawal Scale (SOWS), respectively) and plasma concentrations of (R)-, (S)- and rac-methadone. Subjects were recruited, and divided into two groups, based on their satisfaction of prescribed methadone dose: "satisfied" (n=25) and "dissatisfied" (n=25). Ratings of withdrawal and blood samples for the quantification of the enantiomers of methadone were taken immediately before the study dose, and at 2 and 8 hours post-administration. Satisfied patients scored lower in the negative and higher in the positive aggregate scores of the SOWS, compared to dissatisfied patients. Similarly, satisfied patients had lower OOWS scores compared to dissatisfied patients. Mean pre-dose plasma concentrations of (R)- and rac-methadone differed by less than 10% between the two groups ($P<0.05$), indicating that the use of trough plasma concentrations of (R)-methadone

is unlikely to be a useful diagnostic indicator of withdrawal symptomatology. Plasma concentrations of (S)-methadone were not reported, although the ratio of (R)/(S) pre-dose plasma concentrations were not statistically significantly different (<10% different, $P<0.05$) between the two groups. Using a simple linear correlation analysis (Pearson correlation), they examined the correlation of plasma (R)-, (S)- and rac-methadone concentration (0, 2 and 8 hours) with positive and negative SOWS score, and OOWS score, in dissatisfied patients only. Despite finding some statistically significant ($P<0.05$) correlations at some time-points with negative aggregate SOWS score for rac-methadone (2 hours), (R)-methadone (2 and 8 hours), but not (S)-methadone. However, coefficients of determination were less than 0.2 in all cases, indicting weak relationships, most likely due to the complexity of the aggregate scores. There was a higher number of individual item SOWS scores statistically significantly ($P<0.05$) correlated with trough plasma concentrations of (R)-methadone (n=7), than for rac-methadone (n=4), although details of which items and the strength of the relationships (r values) were not reported. Multiple regression analysis revealed that plasma (R)-methadone concentrations explained 27% and 17% of variation in the negative aggregate of SOWS score and the OOWS score ($P<0.02$), respectively, while other factors (time in treatment, degree of satisfaction, and various socioeconomic variables) contributed to lesser extents. However, the relationship was not examined for (S)- or rac-methadone. The authors concluded that measurement of (R)-methadone should be further examined for its role in therapeutic drug monitoring in the setting of methadone maintenance therapy.

In a very recent study, Eap and co-workers (2000) examined whether plasma concentrations of (R)-methadone can be associated with therapeutic response in methadone maintenance patients in treatment. Trough plasma concentrations of rac-, (R)- and (S)-methadone were measured in 180 patients in maintenance treatment on stable doses (109±57 $mg.day^{-1}$; range: 10-350 $mg.day^{-1}$) and urine samples were collected during a two month period prior to blood sampling for the urinalysis of opioids other than methadone. Measures of withdrawal symptoms were not recorded. Patients was classified into "responders" (no urinalysis

results positive for opioids other than methadone in the previous two months) or "non-responders" (one or more urinalysis results positive for opioids other than methadone in the previous two months). Plasma (R)- and (S)-methadone concentrations ranging from 100 $ng.ml^{-1}$ to 500 $ng.ml^{-1}$ (100-1000 $ng.ml^{-1}$ for rac-methadone) were examined in 50 $ng.ml^{-1}$ increments to determine values that separated "responders" and "non-responders". Specificity (percentage of true negatives below the nominal concentration) and sensitivity (percentage of true positives above the nominal concentration) were assessed. Plasma concentrations of (R)-methadone ranging from 200 $ng.ml^{-1}$ to 300 $ng.ml^{-1}$ (but not other concentrations) separated the two groups (χ^2, $P<0.05$), while this only occurred at 400 $ng.ml^{-1}$ for rac-methadone. No relationship was seen with (S)-methadone concentrations. The greatest separation of the two groups was achieved at 250$ng.ml^{-1}$ for (R)-methadone and 400 $ng.ml^{-1}$ for rac-methadone, with specificity's of 93% and 81%, respectively. However, sensitivity to detect true positives was low: 25% for (R)-methadone and 32% for rac-methadone. The authors concluded that these results indicate that therapeutic drug monitoring of (R)-methadone plasma concentrations could be useful in patients who continue to use illicit opioids, thus assisting clinicians in deciding whether a dosage increase is appropriate. In contrast, this would be of limited value for other patients. It should be noted that these authors did not relate plasma concentrations of methadone to the occurrence of withdrawal symptoms, which may be present in patients who do not return to illicit opioid use as they are committed to rehabilitation. These patients would be defined as "responders", indicating successful treatment, despite experiencing inadequate control of opioid withdrawal symptoms.

The above studies provide some evidence that therapeutic drug monitoring of (R)-methadone concentrations is likely to be more appropriate than those of (S)- or rac-methadone for monitoring of treatment success in the setting of methadone maintenance therapy. However, neither study performed detailed pharmacokinetic-pharmacodynamic modelling techniques, and both utilised limited outcome measures and blood sampling protocols. Despite this, these studies provide further support for the theory that plasma

(R)-methadone concentrations are likely to be more appropriate than those of (S)- or rac-methadone when examining pharmacokinetic-pharmacodynamic relationships of withdrawal in methadone maintenance patients. A second important consideration that these studies have not examined is the concentration of unbound methadone in the plasma of patients, as it is generally acknowledged that only the unbound drug is available to occupy receptors. Indeed, Garrido and co-workers' (1996) study in rats made dependent on morphine demonstrated that the analgesic effect (area under the analgesic effect-time curve) of rac-methadone is decreased during withdrawal from morphine (401±37 sec.min) compared to control rats not dependent on morphine (288 sec.min; $P<0.05$). Plasma α_1-acid glycoprotein concentrations increased 2-fold ($P<0.001$), while unbound fraction of rac-methadone was also significantly lower (41%, $P<0.0001$) compared to control rats not dependent on morphine. In a subsequent study, this group demonstrated that the unbound fraction, clearance and volume of distribution of total (bound plus unbound) rac-methadone were significantly ($P<0.05$) lower (67%, 40% and 42%, respectively), while these parameters were not different when calculations were based on the unbound drug, compared to control rats not dependent on morphine (Garrido et al., 1999). Plasma α_1-acid glycoprotein concentrations were increased by 3.5-fold in the abstinent rats. Using an "effect compartment" pharmacokinetic-pharmacodynamic model (a bi-exponential disposition function and the sigmoid E_{max} pharmacodynamic model) for rac-methadone mediated analgesia, this study demonstrated that the mean E_{max} was decreased by 65% , while the mean EC_{50} (plasma concentration eliciting 50% of E_{max}) was increased by 38% in morphine abstinent rats, compared to control rats not dependent on morphine. Interestingly, EC_{50} values corrected for differences in methadone unbound fraction between the two groups differed by less than 10%. Not withstanding the lack of a control group of rats dependent on morphine but not subjected to withdrawal of the drug, these findings indicate that opioid dependence and/or withdrawal results in a decreased analgesic effect of rac-methadone. Furthermore, this phenomenon is likely to increase the plasma protein binding and subsequently alter of the disposition of rac-methadone, at least in rats. Recently, this group (Garrido et al., 2000) reported that unbound fraction of rac-methadone

was significantly ($P<0.005$) greater in heroin dependant human subjects experiencing withdrawal symptoms 12-24 hours after their last heroin use, compared to healthy volunteers, presumably due to increased plasma α_1-acid glycoprotein concentrations in the former group (see sections 1.7.2.1.1 and 1.7.2.1.5). However, these authors did not examine the effect of alteration in plasma protein binding on the pharmacokinetics or pharmacological or therapeutic effects of methadone.

In summary, no study to date has examined the role of stereoselectivity or protein binding on pharmacokinetic-pharmacodynamic relationships of the occurrence or severity of withdrawal symptoms in methadone maintenance patients. Furthermore, similar studies are also lacking for other pharmacological actions of methadone, such as analgesia, sedation or respiratory depression, in humans. Limited data in rats indicate that opioid dependence and/or withdrawal may result in alteration of the pharmacokinetics of rac-methadone, resulting in reduced analgesic effect, adding further complexity to this issue However, this has yet to be determined in humans. Future studies aimed at examining pharmacokinetic-pharmacodynamic relationships of methadone would therefore benefit from quantifying the unbound concentration of the individual methadone enantiomers, and may result in improved patient treatment.

1.9. Thesis overview and specific aims

Methadone is a chiral molecule, almost exclusively administered as a racemic mixture and widely used for the pharmacological treatment of opioid addiction and also for the treatment of pain. Substantial evidence exists that the pharmacodynamic effects of racemic methadone are mediated by the (R)-enantiomer. Until the commencement of this thesis in 1995-96, there was a relative dearth of literature reports on the pharmacokinetics of the individual methadone enantiomers. Limited evidence suggested the $t_{1/2\beta}$ of the two enantiomers is similar after acute administration, although this was only examined after separate administration of each enantiomer in healthy subjects. The disposition of the individual methadone enantiomers after chronic dosing has been investigated only three studies in methadone maintenance patients. However, these studies suffered from low subject numbers (n=1-3), and only reported $t_{1/2\beta}$ values. These authors suggested that the $t_{1/2\beta}$ of (R)-methadone was greater than that of the (S)- enantiomer. Since 1996, a single study reported that the disposition of the (R)-enantiomer is different to that of the (S)-enantiomer, having a larger volume of distribution, longer $t_{1/2\beta}$ and higher total body clearance, although this has only been examined after single dose administration in pain patients. Stereoselective differences in the magnitude of fluctuation of plasma methadone concentrations may have important implications for pharmacokinetic-pharmacodynamic modelling of methadone, as measurement of rac-methadone may not provide an accurate reflection of plasma concentrations of the active (R)-methadone enantiomer.

Compared to the (S)-enantiomer, (R)-methadone has lower plasma protein binding, with α_1-acid glycoprotein being the predominant binding protein. No study to date has examined the role of variability in protein binding or metabolism in the pharmacokinetics of total (R)- and (S)-methadone. It is not known whether there is stereoselectivity in the clearance of methadone during chronic oral dosing, and whether or not this is due to metabolism (intrinsic clearance) and/or protein binding.

To date, complete mass balance of known methadone metabolites has not been achieved. However, N-demethylation to form EDDP is believed to be the major metabolic pathway. The contribution of variability in this metabolic pathway to the observed variability in the clearance of methadone has not been examined. Additionally, prior to 1996, there were no reports examining the metabolism of methadone in human tissues *in vitro*, and consequently there was no understanding of the enzymes mediating its metabolism or whether the process was stereoselective. For the aims of this thesis to be achieved several assays had to be developed and validated, as methods available in the literature were lacking.

Aim 1: To develop and validate high performance liquid chromatography techniques for the analysis of methadone and its main metabolite (EDDP) in human plasma, plasma ultra-filtrate, urine and liver microsomal fractions.

This aim will be addressed in Chapter 2, and includes both stereoselective and non-stereoselective HPLC techniques, in order to provide thorough cross-validation of the assays and to allow quantification of both the racemic compounds and the individual enantiomers.

Aim 2: To investigate the kinetics of CYP-mediated N-demethylation of methadone in human liver microsomes, to examine the role of stereoselectivity and to identify the CYP isoforms involved.

This aim will be addressed in Chapter 3, and will include a prediction of the *in vivo* clearance of methadone to EDDP using an *in vitro-in vivo* scaling model.

Aim 3: To investigate the steady-state pharmacokinetics of rac-methadone in a methadone maintenance population, and to examine inter-subject variability.

This aim will be addressed in Chapter 4, and will include urinary excretion data, a comparison of the observed clearance of methadone to EDDP with that predicted using an

in vitro-in vivo scaling model in Chapter 3, and its contribution to the total oral clearance of methadone.

Aim 4: To investigate the steady-state pharmacokinetics of (R)- and (S)-methadone in a methadone maintenance population, and to examine factors which might contribute to their variability.

This aim will be addressed in Chapter 5, and will include urinary excretion data, a comparison of the observed clearance of methadone to EDDP with that predicted using an *in vitro-in vivo* scaling model in Chapter 3, and its contribution to the total oral clearance of methadone. Additionally, the plasma protein binding of the individual enantiomers will be examined in patient samples and solutions containing purified α_1-acid glycoprotein in order to determine its role in the disposition of (R)- and (S)-methadone.

Aim 5: To develop a sensitive and robust HPLC method for the quantification of methadone and eight of its metabolites in human urine.

This aim will be addressed in Chapter 6, and will allow a better understanding of the metabolism and renal elimination of methadone.

In summary, the overall aims of this thesis are to increase understanding of the metabolism and disposition of methadone in humans, in particular in subjects receiving methadone maintenance treatment for opioid dependence, and to identify factors which may contribute to inter-subject variability in these processes, with respect to stereoselectivity. The knowledge gained through this research might improve patient treatment and improve our understanding of pharmacokinetic-pharmacodynamic relationships of methadone by identifying factors which contribute to the variability in the pharmacokinetics of methadone.

2. High-performance liquid chromatographic determination of methadone and metabolites in biological fluids

2.1. Introduction

At the time the present PhD studies were commenced in 1995-96 there were few assays available for the stereoselective quantification of methadone in biological fluids, or for the quantification of methadone metabolites in *in vitro* drug metabolism systems. Furthermore, there were no assays available for the stereoselective quantification of methadone metabolites. Due to the lack of published work in these fields, the development and validation of several quantitative assays was necessary to fulfil the aims of my PhD thesis. Subsequent to the commencement of this work, and prior to the completion of this thesis, several authors have published work which described various methods which parallel those described in this thesis, and these will be discussed in the introductory section to this chapter.

The specific aims of the work contained in this Chapter are:

Aim 1: To develop and validate high performance liquid chromatography techniques for the analysis of methadone and its main metabolite (EDDP) in human plasma, plasma ultra-filtrate, urine and liver microsomal fractions. Both stereoselective and non-stereoselective HPLC techniques will be developed, in order to provide thorough cross-validation of the assays and to allow quantification of both the racemic compounds and the individual enantiomers.

2.1.1. Non-stereoselective assays

The following section will briefly discuss the non-stereoselective methods used for the quantification of methadone and metabolites in biological fluids that have been published in the literature to date. A summary table is provided in Appendix 1 for comparison of the various assays that have provided assay validation data (precision, accuracy, extraction efficiency, selectivity assessments). A comparison of these assays with those developed

during the course of the present PhD studies will be made later in the relevant sections of this chapter.

2.1.1.1. Radioimmunoassay

The search for simple, highly sensitive methods for the determination of methadone in biological fluids led to the development of radioimmunoassay (RIA) techniques. Unlike conventional chromatographic techniques, RIA often does not require extraction of analytes from the biological matrix. Due to the availability of radiolabels of high specific activity, RIA potentially offers simple assay procedures of high sensitivity. Cross-reactivity of antisera with compounds of similar structure to the analyte, such as metabolites and other commonly co-administered drugs, must be assessed if an RIA assay is to considered valid.

RIA assays for methadone in plasma have developed by two independent groups (Bartos et al., 1977; Ling et al., 1981). However, only the assay developed by Ling and co-workers (Ling et al., 1981) has been applied in human pharmacokinetic studies of methadone (Bruera et al., 1995; Bullingham et al., 1982; Inturrisi et al., 1987b; Inturrisi et al., 1990; Schwartz et al., 1992; Wissel et al., 1987). Both assays employed sample volumes of less than 100 µl, and were capable of quantifying methadone over 0.5-10 $ng.ml^{-1}$ (Bartos et al., 1977) and 3-40 $ng.ml^{-1}$ (Ling et al., 1981) ranges. The relatively low upper limits of quantification of both assays are below the lowest plasma methadone concentrations present in human subjects during chronic administration (see sections 1.7 and 1.8). Thus re-analysis of samples would be necessary after an appropriate dilution step. Cross-reactivity with several methadone metabolites and other opioid analgesics, including those structurally related to methadone, was adequately addressed by both groups, and found to be <0.5%: EDDP, EMDP and α-(3S,6S)-methadol, morphine, dextropropoxyphene and pethidine (Bartos et al., 1977; Ling et al., 1981), and α-(3S,6S)-N-desmethyl methadol, α-(3S,6S)-di-N-desmethyl methadol, levorphanol, codeine, N-desmethyl pethidine, N-desmethyl dextropropoxyphene, (-)-α-acetylmethadol, N-desmethyl (-)-α-acetylmethadol and di-N-desmethyl (-)-α-acetylmethadol (Ling et al., 1981). Acceptable intra- and inter-

assay precision and accuracy data were provided by Ling and co-workers (Ling et al., 1981), while this was not addressed by Bartos and co-workers (1977).

More recently other investigators have adapted commercially available solid-phase RIA techniques for the quantification of rac-methadone. These assays are reported by the manufacturers not to cross-react with a variety of commonly abused drugs, such as morphine-like opioid compounds. These methods are generally considered semi-quantitative, but have been shown to be useful as screening procedures when compared to more sophisticated GC-MS (Caplan & Levine, 1989) and high performance liquid chromatography (HPLC) (Loimer et al., 1992). Although designed for use in urine samples, Beck and co-workers (1990) adapted a commercially available fluorescence polarisation immunoassay (FPIA) kit for the analysis of rac-methadone in human plasma samples. Calibration curves ranged from 150-1000 $ng.ml^{-1}$ from a 4 µl sample with acceptable precision and accuracy data. Others have applied this immunoassay in methadone pharmacokinetic-pharmacodynamic studies (de Castro et al., 1996; Hiltunen et al., 1999; Hiltunen et al., 1995). Cheever and co-workers (1999) recently reported on the cross reactivity of this FPIA methadone assay. Cross-reactivity with EDDP and EMDP was found to be <0.2%. Cross-reactivity was significant with (-)-α-acetyl methadol (LAAM, 23-36%) and methadol (19-55%), but minimal with N-desmethyl LAAM (<3%) and di-N-desmethyl LAAM (0.3%), N-desmethyl methadol (<4%) and di-N-desmethyl methadol (<0.4%). Unfortunately, the stereochemistry of methadol, N-desmethyl methadol and di-N-desmethyl methadol was not reported, although it would seem likely that the compounds were of the (-)-α-(3S,6S) configuration.

In summary, despite good sensitivity from small sample volumes, the immunoassay techniques reported in the literature for the quantification of rac-methadone are generally not suited for use in pharmacokinetic studies, as they used small calibration curve concentration ranges unlikely to span the range of methadone concentrations present over a inter-dosing interval. Some assays have been demonstrated to have significant cross-

reactivity with methadone metabolites or structurally related compounds, either licitly or illicitly, by methadone maintenance patients. Despite this, immunoassay techniques are useful for therapeutic drug monitoring of patient compliance, and may be employed in pharmacokinetic studies provided that the investigators have accurate knowledge of licit and illicit co-administered medications.

2.1.1.2. Gas chromatography

Gas chromatography has been used to detect methadone and its metabolites after solvent extraction following alkalinisation of the matrix to a pH greater than 9. Detection of the analytes was most commonly performed using hydrogen flame-ionisation or nitrogen-phosphorus detection (Änggård et al., 1975; Baselt & Bickel, 1973; Baselt & Casarett, 1972a; Baselt & Casarett, 1972b; Beckett et al., 1968; Beckett & Triggs, 1967; Bell et al., 1988; Bellward et al., 1977; Chikhi-Chorfi et al., 1998; Choulis & Papadopoulos, 1975; Cooper & Oliver, 1998; Dickson & Palmer, 1975; Dole & Kreek, 1973; George & Braithwaite, 1999; Gerber et al., 1977; Gourlay et al., 1986a; Gourlay et al., 1986b; Gourlay et al., 1984; Gourlay et al., 1982; Hachey et al., 1977; Hamilton et al., 1974; Holmstrand et al., 1978; Inturrisi & Verebely, 1972a; Inturrisi & Verebely, 1972b; Inturrisi & Verebely, 1972c; Kreek, 1973b; Kreek et al., 1976b; Kreek et al., 1980b; Kreek et al., 1978; Lynn et al., 1977; Lynn et al., 1976b; Magora et al., 1987; Nilsson et al., 1982a; Novick et al., 1985; Novick et al., 1981; Pak & Ecobichon, 1981; Pak & Ecobichon, 1982; Pohland et al., 1971; Pond et al., 1985; Raitano & McMillan, 1983; Schall et al., 1996; Schmidt et al., 1993; Sullivan & Blake, 1972; Sullivan & Due, 1973; Sullivan et al., 1975a; Tennant, 1987; Torrens et al., 1998; Verebely & Kutt, 1975; Verebely et al., 1975b; Wilkins et al., 1997). Many of these assays are based upon previously reported methods, mention limits of detection rather than quantification, do not report assay validation data or differentiate between assays in different biological matrices where more than one matrix has been analysed. Although it is beyond the scope of this review to comment on all of the methods employed, the most commonly used or adapted methods will be briefly discussed.

The majority of reports have used the methods of Inturrisi & Verebely (Inturrisi & Verebely, 1972a; 1972b; Inturrisi & Verebely, 1972c; Pak & Ecobichon, 1981; Pak & Ecobichon, 1982; Raitano & McMillan, 1983; Verebely & Kutt, 1975; Verebely et al., 1975b) or Sullivan & Blake (Holmstrand et al., 1978; Nilsson et al., 1982a; 1972; Sullivan & Due, 1973; Sullivan et al., 1975a; Tennant, 1987), with minor or no modifications. In general, these methods were similar, using a 2-4 ml sample volume, alkalinisation to pH>9.5, and two subsequent extractions with n-butyl chloride. Both methods have been successfully applied to the analysis of plasma and urine samples, were capable of separating methadone, EDDP and EMDP, and used hydrogen flame ionisation detection. Unfortunately, few assay validation data were provided for either method, with the exception of percent extraction recovery for methadone, but not EDDP and EMDP. Limits of quantification were lower for the method of Sullivan & Blake (Sullivan & Blake, 1972) for methadone in plasma (50 $ng.ml^{-1}$) and urine (100 $ng.ml^{-1}$), and EDDP (10 $ng.ml^{-1}$) and EMDP (10 $ng.ml^{-1}$) in urine, compared to 100 $ng.ml^{-1}$, 250 $ng.ml^{-1}$, 200 $ng.ml^{-1}$ and 100 $ng.ml^{-1}$ for the corresponding analytes, respectively, reported by Inturrisi & Verebely (Inturrisi & Verebely, 1972b). It is unclear if the lower limits of quantification reported by either group were the lowest achievable with acceptable precision and accuracy, or an arbitrary concentration chosen as the lower limit by the investigators.

Several groups have reported developing their own quantitative GC assay for methadone, EDDP and often EMDP, in biological fluids (plasma, urine, gastric fluid, bile, saliva), however, with few exceptions they have not differed substantially from the methods of Inturrisi & Verebely (1972b) or Sullivan & Blake (1972), and have not reported detailed assay validation data (Änggård et al., 1975; Baselt & Bickel, 1973; Baselt & Casarett, 1972a; Baselt & Casarett, 1972b; Beckett et al., 1968; Beckett & Triggs, 1967; Bell et al., 1988; Bellward et al., 1977; Choulis & Papadopoulos, 1975; Cooper & Oliver, 1998; Dickson & Palmer, 1975; Gerber et al., 1977; Gourlay et al., 1986a; Gourlay et al., 1986b; Gourlay et al., 1984; Gourlay et al., 1982; Hamilton et al., 1974; Kreek et al., 1980b; Lynn et al., 1977; Lynn et al., 1976b; Pohland et al., 1971; Schall et al., 1996).

Kreek and co-workers (1976b) reported an isotope dilution technique which combined scintillation counting (to determine extraction efficiency) and hydrogen flame ionisation for the determination of methadone in plasma and faecal samples, and methadone and EDDP in urine. This method has been used in its original or slightly modified form in many reports by Kreek's groups (Dole & Kreek, 1973; Kreek, 1973b; Kreek et al., 1980b; Kreek et al., 1978; Novick et al., 1985; Novick et al., 1981; Pond et al., 1985). Calibration curves ranged from 20-2000 $ng.ml^{-1}$ and from 500-4000 $ng.ml^{-1}$ for methadone in plasma (2.0 ml) and faeces (1.0 ml of a $3g.l^{-1}$ homogenate in water) and from 1-50 $\mu g.ml^{-1}$ for both methadone and EDDP in urine (0.5 ml) samples. The methods were demonstrated to have good precision and accuracy. However, this was calculated using data from all standards in each calibration curve, rather than presented at several different concentrations. Concentration dependency, and interference by other compounds, including methadone metabolites, was not examined.

More recently, several groups have reported methods for the quantification of methadone in plasma and other biological fluids, and included detailed assay validation data. Magora and co-workers (1987) reported a method for the quantification of methadone in human plasma using GC with nitrogen-phosphorus detection, using a pentane/isoamylalcohol (95/5, v/v) extraction of the alkalinised (sodium tetra borate/sodium hydroxide, pH 10) sample, extraction of the separated organic phase with sulphuric acid, followed by alkalinisation (sodium hydroxide) and a final extraction of the separated aqueous phase with 20 µl of toluene. Inter-assay precision decreased markedly at the lower limit of quantification (1 $ng.ml^{-1}$, 22%) compared to the upper limit of quantification (50 $ng.ml^{-1}$, 4%). Accuracy was poorly addressed, as only mean concentrations were reported for the inter-assay validation results, however, the values were within 11% of the nominal concentration for all six calibration standards. Other compounds, including methadone metabolites, were not examined for potential chromatographic interference.

Schmidt and co-workers (1993) reported a method for the quantification of methadone in human plasma, urine and cerebrospinal fluid using GC with nitrogen-phosphorus detection,

using a hexane/isoamylalcohol (99/1, v/v) extraction of the alkalinised (potassium carbonate) sample, followed by re-constitution of the dried hexane extract with isopropanol. In plasma, inter-assay coefficients of variation and mean percent bias ranged from 4-13% and 1-4%, respectively, over the calibration range. Similar data were provided for the analysis of methadone in urine and cerebrospinal fluid. Although not quantified by this assay, EDDP was reported not to interfere with the chromatography, as was morphine, tramadol, and several arylpropionic acid analgesics.

Green and Wilson (Green & Wilson, 1996b) reported a butanol/hexane (2/98 v/v) extraction of alkalinised (0.5 ml 5 M potassium carbonate) rat plasma or hair digest samples (1 ml) for the quantification of methadone and EDDP. Details of accuracy and selectivity of the method were not provided, and there was no distinction made between the assay in plasma or hair for extraction efficiency or other assay validation data. Inter-assay coefficient of variation for methadone was 11% at 5 $ng.ml^{-1}$, while this was 11% at 19 $ng.ml^{-1}$ for EDDP.

Chikhi-Chorfi and co-workers (1998) reported the quantification of methadone and EDDP in human plasma, urine and saliva using GC with nitrogen-phosphorus detection. The assay used a simple hexane extraction of the alkalinised (sodium carbonate) sample, followed by re-constitution of the dried hexane extract with methanol. Calibration curves ranged from 50-2000 $ng.ml^{-1}$ for both analytes from a 1 ml sample of biofluid. Inter-assay (n=6 assays) and intra- assay (n=6 replicates) coefficients of variation were less than 6% for both analytes at concentrations of 100 $ng.ml^{-1}$ and 500 $ng.ml^{-1}$ in all three biofluids. Extraction recoveries, calculated from the intra-assay validation samples, were reported to be >90% for both analytes and the internal standard (lignocaine) in all three biofluids at concentrations of 100 $ng.ml^{-1}$ and 500 $ng.ml^{-1}$, with coefficients of variation <5%. The accuracy of the method was poorly addressed. After direct injection, or after alkaline extraction of standard solutions of morphine, codeine, dionine, narcotine, nalorphine, cocaine, benzoylecgonine, cocaethylene, dextropropoxyphene, phenytoin, valproic acid, salicylic acid, acetylsalicylic acid, diazepam, nitrazepam, and oxazepam were shown not to interfere with the assay.

Torrens and co-workers (1998) reported a method for the quantification of methadone and EDDP in human plasma using GC with nitrogen-phosphorus detection, using a tert-butyl methyl ether extraction of the alkalinised (potassium hydroxide) sample (1 ml). Details of the accuracy of the method and the calibration curve concentration range were not provided. Inter- and intra-assay coefficients of variation from 2% to 8% for methadone (100 $ng.ml^{-1}$ and 500 $ng.ml^{-1}$) and from 5% to 13% for EDDP (25 $ng.ml^{-1}$ and 75 $ng.ml^{-1}$). Extraction efficiency (mean±SD) was reported to be 90±12% for methadone, however, this was markedly lower for EDDP (40±9%). Selectivity of the method was not addressed, as potential chromatographic interference from other compounds, including methadone metabolites, was not examined.

George and Braithwaite (1999) reported a simple butyl-acetate (0.15 ml) extraction of alkalinised (sodium hydroxide) urine samples (0.7 ml) for the quantification of methadone and EDDP. Calibration curves ranged from 1-20 $\mu g.ml^{-1}$ for both compounds, with mean extraction efficiencies of 85% and 86% for methadone and EDDP, respectively. Accuracy, intra-assay coefficients of variation, and selectivity of the method were not provided. Inter-assay coefficients of variation at 1 $\mu g.ml^{-1}$ for methadone and EDDP were 9.2% and 11.0%, respectively, while this was 8.8% and 11.0%, respectively, at 10 $\mu g.ml^{-1}$.

2.1.1.3. Gas chromatography-mass spectroscopy

Gas chromatography-mass spectroscopy has been used by many investigators, although this has often been used to provide qualitative structural confirmation (Abbott et al., 1985; Änggård et al., 1975; Baselt & Bickel, 1973; Baselt & Casarett, 1972b; Bellward et al., 1977; Gerber et al., 1977; Kreek et al., 1976b; Kreek et al., 1978; Lynn et al., 1977; Lynn et al., 1976b; Molteni et al., 1994; Pohland et al., 1971; Ripamonti et al., 1995; Sullivan & Due, 1973; Sullivan et al., 1972a; Sullivan et al., 1975a) rather than for quantitative analysis (Alburges et al., 1996; Änggård et al., 1979; Baugh et al., 1991; Beck et al., 1990; Bermejo et al., 2000b; Cooper & Oliver, 1998; Galloway & Bellet, 1999; Goldberger et al., 1998;

Hachey et al., 1977; Kang & Abbott, 1982; Kreek et al., 1983; Kreek et al., 1980a; Meresaar et al., 1981; Moody et al., 1997; Moody et al., 1999; Nilsson et al., 1982a; Nilsson et al., 1983; Nilsson et al., 1982b; Skopp et al., 1996; Sullivan et al., 1975b; Wilkins et al., 1996; Wilkins et al., 1998) of methadone and metabolites. Of the quantitative GC-MS methods, most are based upon previously reported methods, do not report precision and accuracy data or differentiate between assays in different biological matrices. Although it is beyond the scope of this review to comment on all of the methods employed, the most commonly used or adapted methods will be briefly discussed.

The GC-MS method for the quantification of methadone in plasma developed by Sullivan and co-workers (1975b) has been applied in a slightly modified form (Änggård et al., 1979; Beck et al., 1990; Meresaar et al., 1981; Nilsson et al., 1982a; Nilsson et al., 1983; Nilsson et al., 1982b) and expanded to include the quantification of methadone in saliva (Nilsson et al., 1982b) and methadone and EDDP in urine (Nilsson et al., 1982a; Nilsson et al., 1983; Nilsson et al., 1982b). Briefly, the method involves extraction of alkalinised (pH>9) samples with n-butyl chloride or hexane, evaporation of the separated organic phase to dryness and reconstitution of the residue in a small volume of toluene or ethyl acetate. Plasma sample volumes ranged from 0.5 ml (Beck et al., 1990), 2 ml (Meresaar et al., 1981) to 4 ml (Änggård et al., 1979; Nilsson et al., 1982a; Nilsson et al., 1983; Nilsson et al., 1982b; Sullivan et al., 1975b), while it was presumably 4 ml for saliva (Nilsson et al., 1982b) and urine (Nilsson et al., 1982a; Nilsson et al., 1983; Nilsson et al., 1982b). Sullivan and co-workers (1975b) reported that calibration curves for methadone in plasma ranged from 16-1600 $pmol.ml^{-1}$ (approximately 5-500 $ng.ml^{-1}$) and intra-assay precision (CV, n=6-8) and accuracy (%bias, n=6-8) were both less than 4% at concentrations of approximately 10 $ng.ml^{-1}$, 50 $ng.ml^{-1}$, 100 $ng.ml^{-1}$. Other investigators have only reported on the intra-assay precision at the limit of quantification: 2% at 100 $ng.ml^{-1}$ (Beck et al., 1990), 5% at approximately 10 $ng.ml^{-1}$ (Änggård et al., 1979; Meresaar et al., 1981) for methadone in plasma, 9% at approximately 4 $ng.ml^{-1}$ for methadone in plasma (Nilsson et al., 1982a; Nilsson et al., 1983; Nilsson et al., 1982b) and saliva (Nilsson et al., 1982b), and 5% at

approximately 0.1 $ng.ml^{-1}$ for both methadone and EDDP in urine (Nilsson et al., 1982a; Nilsson et al., 1983; Nilsson et al., 1982b). Similarly, Baugh and co-workers (1991) and Bermejo co-workers (2000b) reported limited validation of their GC-MS assays for methadone and EDDP in plasma and urine, respectively.

The method for the quantification of methadone, EDDP and EMDP in plasma, urine and liver microsomes developed by Alburges and co-workers (1996) has been applied in its original or slightly modified form (Moody et al., 1997; Moody et al., 1999) and expanded to the analysis of hair samples (Wilkins et al., 1996; Wilkins et al., 1998). This method involved a solid-phase extraction of samples (1 ml) adjusted to pH 6.0, followed by a reconstitution of the dried residue in n-butyl chloride, and provided detailed assay validation data. Calibration curves ranged from 10-600 $ng.ml^{-1}$ for all three analytes in the three biofluids. Inter-assay (n=3-5 assays) coefficients of variation ranged from 2-15% for all three analytes at concentrations of 25 $ng.ml^{-1}$, 100 $ng.ml^{-1}$ and 300 $ng.ml^{-1}$ in all three biofluids, however, this was generally below 9%, while accuracy (%bias) ranged from -16% to +7%, but was generally less than ±10%. Similarly, intra-assay coefficients of variation (n=5 replicates) ranged from 1-12% for all three analytes at concentrations of 25 $ng.ml^{-1}$, 100 $ng.ml^{-1}$ and 300 $ng.ml^{-1}$ in all three biofluids, while accuracy (%bias) was generally less than ±10%. Extraction recoveries ranged from 94-105%, 85-106% and 95-107% for methadone, EDDP and EMDP, respectively, in plasma and urine at concentrations of 25 $ng.ml^{-1}$, 100 $ng.ml^{-1}$ and 300 $ng.ml^{-1}$, while this was 99%, 94% and 85% in liver microsomes at 100 $ng.ml^{-1}$. Wilkins' group (1996; 1998) reported similar excellent validation of the performance of their adaptation of this method for the analysis of hair samples, however, they used a solvent-solvent extraction, and the calibration curves ranged from 0.3-100 $ng.mg^{-1}$ of hair from a 20 mg sample.

Several groups have reported developing their own quantitative GC-MS assay for methadone, and often several metabolites, in biological fluids. However, they have not reported detailed assay validation data (Cooper & Oliver, 1998; Galloway & Bellet, 1999;

Goldberger et al., 1998; Hachey et al., 1977; Kang & Abbott, 1982; Kreek et al., 1983; Kreek et al., 1980a; Skopp et al., 1996).

Interestingly, Galloway and Bellet (1999) demonstrated the thermal conversion of methadone to EDDP in the injection port during GC-MS analysis. This confirmed an earlier report, using LC-MS-MS, that methadone and structurally related compounds are thermolabile (Verweij et al., 1995). EDDP and methadone were each quantified by selective ion monitoring of three specific ions, and authentic compounds were used to confirm the identity of the extracted samples and for quantification. In prepared samples containing methadone only, concentrations of EDDP were found to be up to 2.5% of methadone concentrations. Analysis of the same extracts by HPLC did not detect any EDDP, and lowering of the injection port temperature from 260 °C to 180°C reduced the formation of EDDP to a maximum of 0.7% at a methadone concentration of 5 $\mu g.ml^{-1}$. The authors concluded that alternative techniques, such as HPLC or capillary electrophoresis, should be considered for the analysis samples suspected of "methadone spiking" when monitoring of compliance with methadone maintenance therapy.

In summary, methods employing GC analysis after extraction of biofluid samples have been successfully developed and applied, although thorough validation data were not always provided. Despite this, it is likely that the methods were sufficiently selective, accurate and precise for their intended application in studies examining the disposition of methadone.

2.1.1.4. High-performance liquid chromatography

Due to its relatively lower cost and ease of use, investigators have more recently used high-performance liquid chromatography (HPLC) with UV detection as an alternative to GC methods after prior solid-phase or solvent-solvent extraction of the matrix alkalinised to a pH >9.

Wolff and co-workers (1991d) quantified methadone in blood, urine and saliva using a normal phase HPLC system and UV detection at 215 nm. This method used an n-butyl

chloride extraction of alkalinised sample (pH 10) which was evaporated to dryness and reconstituted in methanol. The limit of quantification from a 2 ml sample volume was 5 $ng.ml^{-1}$, with inter- and intra-assay precision within 10% at this concentration. Unfortunately, details of the calibration curve concentration range, and accuracy of the method were ill defined. This method has been applied in several studies examining the disposition of rac-methadone in humans (Rostami-Hodjegan et al., 1999; Wolff et al., 1991a; Wolff et al., 1991b; Wolff et al., 1991c; Wolff et al., 1992; Wolff et al., 1993; Wolff et al., 1997; Wolff et al., 1991d; Wolff et al., 1990), and rats (Garrido et al., 1999).

Recently, de Vos and co-workers (1995) reported a method for the quantification of methadone and EDDP in plasma. Plasma was alkalinised with potassium carbonate and extracted with hexane which was then evaporated to dryness and reconstituted in pH 2.5 phosphate buffer. Separation was achieved using reversed phase HPLC with UV detection at 206 nm. Calibration curve concentration ranges for both methadone (10-800 $ng.ml^{-1}$) and EDDP (5-400 $ng.ml^{-1}$) were broad, with inter-assay precision of 7.7% and 6.7%, respectively, from a 0.5 ml sample at the lower limits of quantification. However, the accuracy of the method not reported.

Wojnar-Horton and co-workers (1997) quantified methadone in plasma and breast milk using a reversed phase HPLC assay with UV detection. Similar to other workers, samples were alkalinised with sodium hydroxide and extracted with 1% isoamyl alcohol in hexane, with a final back extraction into dilute HCl. The calibration curve concentration range was identical (5-800 $ng.ml^{-1}$) in plasma and breast milk, with intra-assay precision <8% in both biofluids from a 1.0 ml sample at 50 $ng.ml^{-1}$ and 40 $ng.ml^{-1}$, respectively. However, accuracy and inter-assay precision data were not reported. Others have employed similar assays for methadone in plasma (Cobb et al., 1998) and urine (Cobb et al., 1998; Stolk et al., 1997) samples.

Several other groups have reported developing their own quantitative HPLC assay for methadone in biological fluids (plasma, urine). However, they have not reported detailed assay validation data (Cheng et al., 1999; Derendorf & Garrett, 1983; Fullerton et al., 1991; Galloway & Bellet, 1999; Loimer & Schmid, 1992; Loimer et al., 1991; Loimer et al., 1992; Roerig et al., 1982).

Few investigators have reported HPLC methods for the quantification of methadone and metabolites other than EDDP and EMDP. Garrett and co-workers (1985) developed HPLC conditions with UV detection (210 nm) to measure methadone and several metabolites (EDDP, EMDP, p-hydroxy methadone, α-methadol, α-N-desmethyl methadol, pyrrolidone, valeric acid metabolite) in dog plasma, urine and bile after alkaline extraction with hexane. Limits of detection were between 20ng.ml^{-1} and 30 ng.ml^{-1} for all compounds from a 0.5-1.0 ml sample, however, no assay validation data were provided. Although a cyano column was used for all chromatography, each compound was assayed separately using an optimised extraction pH and mobile phase.

Similarly, Pond and co-workers (1985) quantified urinary concentrations of methadone, EDDP, EMDP and α-(3S,6S)-methadol, using HPLC. However details of this method were not reported, but were available upon request at the time. This assay was not stereoselective, and therefore could not unequivocally differentiate between (3S,6S)-α-methadol and (3R,6R)-α-methadol. Gérardy and co-workers (1986) separated methadone, EDDP, EMDP and p-hydroxy methadone by HPLC with UV detection at 235 nm. Details of assay sensitivity or validation data were not reported in either study.

Pierce and co-workers (1992) developed a solid phase extraction for methadone, EDDP and EMDP using C_{18} extraction cartridges. The three analytes were quantified by HPLC with UV detection at 210 nm. HPLC separation was performed on a C_{18} analytical column maintained at 28°C, with a mobile phase of 25% acetonitrile in 0.08% diethylamine, pH 2.3. From a 1 ml sample, limits of quantification were 25 ng.ml^{-1} for methadone and 5 ng.ml^{-1}

for EDDP and EMDP. For methadone, intra- and inter-assay precision were reported as 1.3% and 2.9% respectively, although it is unclear as to which concentrations these results were obtained.

More recently, Iribarne and co-workers (1996) developed a reversed phase HPLC method for the quantification of EDDP and EMDP in human liver microsomes for use in their investigations of the *in vitro* metabolism of methadone (Iribarne et al., 1996; Iribarne et al., 1998a; Iribarne et al., 1997; Iribarne et al., 1998b). Samples (1 ml) were alkalinised with sodium hydroxide and extracted twice with diethyl ether which was then evaporated to dryness and reconstituted in dilute HCl. This assay did not quantitate methadone. However, adequate separation of all three compounds, and the internal standard, was demonstrated. Unfortunately, calibration curve ranges, precision and accuracy data were ill defined.

In summary HPLC has provided investigators with simple, robust and sensitive methods for the determination of methadone and its metabolites in biological fluids. Sensitivities reported for the GC and immunoassay techniques do not offer appreciable gains, especially when the added complexity of assay development, selectivity and expense of apparatus is taken into consideration. However, this author is surprised at limited extent to which assay validation data, particularly accuracy, have been presented in the more recent publications using HPLC techniques. Although many of these methods were presented as part of a pharmacokinetic study, and a detailed discussion of assay validation is inappropriate, it is necessary to provide confidence in the authors findings.

2.1.2. Stereoselective assays

The following section will briefly discuss the stereoselective methods used for the quantification of methadone and metabolites in biological fluids that have been published in the literature to date. A summary table is provided in Appendix 2 for comparison of the various assays that have provided assay validation data. A comparison of these assays with

those developed during the course of the present PhD studies will be made later in the relevant sections of this chapter.

The earliest published method for the quantification of the individual enantiomers utilised RIA with stereoselective antibodies, although these authors did not provided assay validation data and the assay was not employed in any study of the disposition of methadone in humans (Bartos et al., 1978; McGilliard et al., 1979). Later, Kreek's group quantified (R)- and (S)-methadone either alone, as 50% of the pseudo racemic methadone dose, or together, each comprising 50% of the dose administered (Kreek et al., 1979; Nakamura et al., 1982) by labelling the (R)- and (S)-methadone enantiomers with either five or three deuterium atoms, respectively. Employing a non-chiral GC-MS assay (Hachey et al., 1977), the enantiomers were individually quantified based upon the mass unit difference in their respective ion spectra, which also allowed quantification of the enantiomers in the presence of unlabelled methadone. This method allowed limits of quantification of 5 $ng.ml^{-1}$ and 100 $ng.ml^{-1}$ for each enantiomer in plasma and urine respectively, although details of assay validation were not provided. Despite providing a powerful tool, this technique would appear to be of limited practical value, and was only applied in two studies only involving a total of five subjects (Kreek et al., 1979; Nakamura et al., 1982). The administration of stable isotope-labelled compounds is not readily applicable in clinical investigations, as there is the possibility that the labelled drug will not have the same disposition as unlabelled drug, although effects can be minimised by incorporation of the label in a position where no isotope effects would be expected, and the expense of the compounds would also be prohibitive for most investigators.

Kristensen and Angelo (1992) developed a stereoselective GC assay for the quantification of the methadone enantiomers. This assay employed the derivatisation the methadone enantiomers with (-)-methyl chloroformate, resulting in the formation of diastereomers which were then separated chromatographically using a conventional GC column. However, the complex series of extraction-derivatisation-extraction processes would make

this assay of limited practical value in comparison to more recent HPLC and capillary electrophoresis (CE) techniques discussed below.

More recently, techniques for the stereoselective quantification of methadone became available with the advent of robust chiral chromatography columns for HPLC. These columns rely upon chiral molecules covalently bonded to the silica stationary phase which then adsorb and desorb the individual enantiomers of a mixture at different rates. These methods provide significant advantages when compared to stable-isotope labelling methods, as they physically separate enantiomers, hence obviating the need for costly stable-isotope labelling and mass-spectrum analysis. Further advantages are that sample preparation and the HPLC system (with the exception of the HPLC column) are essentially the same as conventional non-chiral HPLC methods, and that the compounds of interest do not need to be specially prepared, as is the case for stable-isotope labelling techniques, or derivatised before analysis.

The most widely used chiral chromatography column used for the development of stereoselective assays for methadone is the α_1-acid glycoprotein bonded (Chiral AGP, Chromtech) type. Mobile phases for these columns have used 10 mM phosphate buffer containing 9.5% isopropanol (Schmidt et al., 1992), 9.5% isopropanol and 0.1% dimethyloctylamine (Rudaz & Veuthey, 1996), 16% acetonitrile (Beck et al., 1991), 10% acetonitrile and 0.05% dimethyloctylamine (Kristensen et al., 1994), 12.7% acetonitrile and 0.05% dimethyloctylamine (de Vos et al., 1998). All of these methods used a simple solvent-solvent hexane (Beck et al., 1991; de Vos et al., 1998; Kristensen & Angelo, 1992; Schmidt et al., 1992) or solid phase (Rudaz & Veuthey, 1996) extraction of alkalinised sample and evaporation to dryness and reconstitution of sample for injection, with UV detection. Rudaz and co-workers (1999) compared solvent-solvent and solid phase extraction methods, and found them to provide similar precision and accuracy, although the extraction recovery was greater for the solvent-solvent extraction technique. Other authors have used mass-spectrometry detection in combination with a mobile phase gradient of 8%-

20% isopropanol in 2 mM ammonium acetate buffer, for the determination of the enantiomers of methadone in hair samples for forensic analysis.

A second type of chiral HPLC column that has been used successfully to separate the methadone enantiomers is the cyclodextrin-derivatised (Cyclobond I 2000 RSP, Astec) type (Eap et al., 1996; Norris et al., 1994) with UV detection. Mobile phases consisted of 1% triethylamine containing 10% acetonitrile (pH 3.0, Eap et al., 1996), or 20% acetonitrile and 5% methanol (pH 6.0, Norris et al., 1994). Both assays utilised solvent-solvent extraction with heptane containing 2% butanol (Norris et al., 1994) or ethyl acetate (Eap et al., 1996) and incorporated a back-extraction into a weak acid phase. Using the same analytical column, and a similar solvent-solvent extraction method, but without a back extraction, Pham-Huy and co-workers (1997) were able to quantitate the methadone enantiomers from EDDP using a mobile phase comprised of acetonitrile/1% triethylamine acetate buffer pH 4.5/water (18/8/73). However, the enantiomers of EDDP were not separated under the chromatography conditions described.

At the time the present PhD studies were commenced in 1995-96 there were no assays available for the stereoselective quantification of methadone metabolites. Subsequent to the commencement of this work, and prior to the completion of this thesis, several authors have published work which described various methods for the quantification of the enantiomers of EDDP. At present, there are no methods available for the stereoselective quantification of any methadone metabolite other than EDDP.

Lanz & Thormann (1996), Frost and co-workers (1997), and Ramseier and co-workers (1999) adaptation of the Lanz & Thormann (1996) method employed capillary electrophoresis (CE) for the simultaneous quantification of the enantiomers of methadone and EDDP. These authors used solvent-solvent (Frost et al., 1997; Ramseier et al., 1999) or solid-phase extractions (Lanz & Thormann, 1996) of urine samples at pH >9, with subsequent evaporation of the organic solvent to dryness, and reconstitution of the residue

prior to CE analysis. Lanz & Thormann (1996) attempted direct injection of urine samples, but this was unsuccessful as less than half of the samples tested produced acceptable electropherograms. Limits of detection for each enantiomer were reported to be 10 $ng.ml^{-1}$ (Frost et al., 1997) and approximately 100 $ng.ml^{-1}$ (Lanz & Thormann, 1996) for both methadone and EDDP from a 1 ml urine sample. Ramseier and co-workers (1999) did not report a calibration range, and the limit of detection of the assay was ill defined.

Lanz & Thormann (1996) reported intra-assay precision values to be <10%, although the concentration at which this was determined is unclear. This was only assessed at 1000 $ng.ml^{-1}$ by Ramseier and co-workers (1999) who reported values of <6% for methadone and <2% for EDDP. Frost and co-workers (1997) reported intra-assay precision for each enantiomer to be <12% at the higher concentrations, but increased up to 17% at 50 $ng.ml^{-1}$. Mean concentrations were within 5% of the nominal concentration at the higher concentrations, but increased to 12-24% at 50 $ng.ml^{-1}$. Accuracy data were not reported for the other two assay methods (Lanz & Thormann, 1996; Ramseier et al., 1999), while inter-day precision or accuracy data were not presented in any of the three papers (Frost et al., 1997; Lanz & Thormann, 1996; Ramseier et al., 1999).

Separation of the methadone and EDDP enantiomers was demonstrated to be adequate in all three CE assays (Frost et al., 1997; Lanz & Thormann, 1996; Ramseier et al., 1999). Ramseier and co-workers (1999) demonstrated excellent selectivity of their assay for the methadone and EDDP enantiomers, as no interference was seen with drugs of abuse and their metabolites (13 compounds), as did Frost and co-workers (1997) (18 compounds). Lanz & Thormann did not address this issue (Lanz & Thormann, 1996).

Recently, Angelo and co-workers (1999) reported a method for the simultaneous quantification of the enantiomers of methadone and EDDP in urine using stereoselective HPLC. This method used a short non-chiral C_8 analytical column coupled in series with an α_1-acid glycoprotein bonded (Chiral AGP, Chromtech) type analytical column. The mobile

phase was relatively unchanged in comparison to their earlier report for the quantification of the methadone enantiomers; the acetonitrile content was increased to 14%. The assay used a simple solvent-solvent hexane extraction of alkalinised samples, evaporation of the organic phase to dryness and reconstitution of the residue with mobile phase. Rudaz & Veuthey (1999) recently reported their detailed chiral HPLC investigations with methadone, noting that EDDP did not interfere with methadone using a α_1-acid glycoprotein bonded (Chiral AGP, Chromtech) type column. However, they did not attempt to quantitate EDDP. Using the same a α_1-acid glycoprotein bonded HPLC column, Kintz and co-workers (1997) simultaneously quantified the enantiomers of methadone and EDDP in hair samples with mass-spectroscopy for detection. However, these authors did report detailed assay validation data.

These methods have all been shown to be reproducible, however, several authors have not provided detailed accuracy data (see Appendix 2). Limits of quantification appear to be a function of detection wavelength and sample volume, as would be expected. Highest sensitivities reported at 200 nm and a 1 ml sample volume (Kristensen et al., 1994) or 215 nm with a 2 ml sample volume (Schmidt et al., 1992) resulting in comparable limits of quantification of approximately 2 $ng.ml^{-1}$ for each enantiomer. When the sum of (R)- and (S)-methadone, or (R)- and (S)-EDDP, concentrations in samples were compared to that of total rac-methadone concentrations obtained by non-chiral GC techniques, these methods produced excellent correlations, with slope and intercept values close to 1 and 0, respectively (Angelo et al., 1999; Beck et al., 1991; Eap et al., 1997; Kristensen et al., 1994; Pham-Huy et al., 1997; Schmidt et al., 1992).

In summary, recent advances in chiral HPLC and CE techniques have provided investigators with methods to investigate the disposition and metabolism of methadone with respect to stereoselectivity. Similar sample preparation is required for both types of methods. However, it is likely that the initial set up of a stereoselective HPLC assay would be more readily achieved by many laboratories which already have a conventional HLPC system as

only a specialised chromatography column is required. In contrast, the ongoing costs of the CE methods are likely to be much less expensive compared to a stereoselective HPLC method, as the columns employed are cheaper than chiral HPLC columns, and consume much less running/rinsing solutions compared to HPLC mobile-phase consumption.

2.2. Chemicals

Racemic methadone as the hydrochloride salt, (R)- and (S)-methadone as the free bases, (R)- and (S)-EDDP as the perchlorate salts, (4R,6S)- and (4S,6S)-para hydroxy methadone as the hydrochloride salts, β-(+)-(3R,6S)-methadol, racemic α-methadol, α-(-)-(3S,6S)-methadol, α-(+)-(3R,6R)-methadol, α-(-)-(3S,6S)-N-desmethyl methadol, and racemic pyrrolidone were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). Racemic EDDP as the hydroiodide salt and rac-EMDP as the hydrochloride salt were purchased from Alltech-Applied Science Labs (State College, PA, USA). Other pharmaceutical compounds were obtained from the following sources: furafylline and (S)-mephenytoin were from Ultrafine Chemicals (Manchester, UK); caffeine, chlorzoxazone, coumarin, dextropropoxyphene, N-desmethyl dextropropoxyphene, diethyldithiocarbamic acid sodium salt, hydromorphone, naloxone, oxycodone, quinidine sulphate, sulphaphenazole, clomipramine hydrochloride, desipramine hydrochloride, doxepin hydrochloride and troleandomycin were from Sigma Chemical Company (St. Louis, Mo, USA); omeprazole was supplied by Astra Pharmaceuticals (Sydney, Australia); racemic fluoxetine was a gift from Dr. W. Hooper (Department of Medicine, Royal Brisbane Hospital, Brisbane, Australia); diazepam and ketoconazole were gifts from Prof. J. Miners (Department of Clinical Pharmacology, Flinders Medical Centre, Bedford Park, Adelaide, Australia); 3-methoxymorphinan as the hydrobromide salt and flunitrazepam were obtained from Roche Products Pty Ltd. (Sydney, Australia); morphine as the hydrochloride salt was from McFarlane Smith (Edinburgh, UK); ethylmorphine was from Merck (Darmstadt, Germany); dextromoramide as the tartrate salt and codeine phosphate were provided by FH Faulding and Co Ltd. (Adelaide, Australia); norcodeine was from Eli Lilly (Indianapolis, IN, USA); temazepam was from Wyeth Pharmaceuticals (GmBH, Munster, Germany);

dihydromorphone and 6β-oxycodol were from ICN Alkaloida Company Ltd (Tiszavasvari, Hungary); amitriptyline, nortriptyline and imipramine as the hydrochloride salts were kindly supplied by Dr. Benedetta Sallustio (Department of Clinical Pharmacology, The Queen Elizabeth Hospital, Adelaide, Australia); citalopram hydrobromide, fluvoxamine maleate, paroxetine hydrobromide hemihydrate, nefazodone hydrochloride and venlafaxine hydrochloride were kindly supplied by Mr. Noel Sims (Forensic Science Centre, Adelaide, Australia); ketamine hydrochloride was purchased from Parke-Davis Pharmaceutical Research (MI, USA); rac-ketorolac tromethamine was kindly supplied by Dr. Peter Hayball (School of Pharmacy, University of South Australia, Adelaide, Australia); sertraline hydrochloride was from Pfizer/Mack Pharm. Dev. (Illertissen, Germany). HPLC grade acetonitrile, dimethyloctylamine, hexane, methanol and triethylamine, and analytical grade diethyl ether and triethylamine were from BDH Laboratory Supplies (Poole, UK). Nitrogen gas was from CIG (Adelaide, Australia). All other reagents and chemicals were of analytical grade quality obtained from commercial sources.

Using chromatography system for the simultaneous quantification of (R)- and (S)-EDDP, and (R)- and (S)-methadone (see section 2.6), replicate injections (n=8) of aqueous solutions of rac-EDDP and rac-methadone produced mean±SD (R)/(S) enantiomer peak area ratios of 0.99±0.02 and 1.01±0.04 for EDDP and methadone, respectively, and injection of separate aqueous solutions containing (R)- or (S)-methadone, or (R)- or (S)-EDDP produced single chromatographic peaks. These data indicate that the racemic mixtures comprised equal amounts of the individual enantiomers, and that there individual enantiomers were sufficiently enantiomerically pure.

2.3. Quantification of rac-EDDP in microsomal incubations

Early methods for examining the metabolism of methadone *in vitro* used the loss of methadone from samples (Sung & Way, 1950) or the measurement of formaldehyde formation as a measure of N-demethylation (Alvares & Kappas, 1972; Axelrod, 1956; Dawson & Vestal, 1984; Liu & Wang, 1975; Masten et al., 1975; Roerig et al., 1975;

Sullivan et al., 1975a) in crude homogenates or microsomal fractions prepared from animal tissues. These are relatively non-specific techniques which cannot differentiate between the formation of individual metabolites. Additionally, the latter technique would be of questionable validity when chemical inhibitors are used to examine isoform selectivity, due to the unknown contribution of metabolism of the inhibitor to the formation of formaldehyde. Some investigators have reported the development of specific assays for rac-methadone and rac-EDDP, however, calibration curve concentration ranges and assay validation data were ill defined, all methods involved time consuming solvent-solvent extraction, and utilised GC analysis (Beckett et al., 1971a; Beckett et al., 1971b; Lynn et al., 1977) which was not readily available in my laboratory. At the time the present work was conducted, these were the only methods available in the published literature. More recently, two independent groups have reported GC-MS (Alburges et al., 1996) and HPLC (Iribarne et al., 1996) methods for the quantification of methadone metabolites in microsomal incubations, which were applied in subsequent investigations (Iribarne et al., 1998a; Iribarne et al., 1997; Iribarne et al., 1998b; Moody et al., 1997).

2.3.1. HPLC instrumentation and chromatography conditions

The HPLC system comprised a LC-6A pump (Shimadzu, Kyoto, Japan), a Wisp 710B autoinjector (Waters, Milford, MA, USA), an UVIDEC-100-V spectrophotometer (Jasco, Tokyo, Japan) set at 210 nm (0.005 aufs) and a C-R6A Chromatopac integrator with the attenuation set at 2 (Shimadzu). The analytical column was a 100x5 mm Nova-Pak C_{18} 4 µm cartridge (Waters) contained in an 8x10 Radial Compression Module (Waters) protected by a 2 µm in-line filter (Scientific Instruments, State College, PA, USA) and a 15x4.6 mm pre-column packed with 30 µm pellicular ODS C_{18} material (Whatman Laboratories, Clinton, NJ, USA). Optimal separation of the compounds of interest was achieved with a mobile phase of 30% (v/v) acetonitrile and 0.2% (v/v) triethylamine in 50 mM NaH_2PO_4 with final pH adjusted to 4.3 with ortho-phosphoric acid and pumped through the system at 1.0 $ml.min^{-1}$ at room temperature.

2.3.2. Sample preparation

Incubations were performed in duplicate at 37°C in a shaking water bath for 45 minutes. The incubates of 200 µl final volume contained 50 mM sodium phosphate buffer (pH 7.4), NADPH generating system (1mM NADP, 1 unit ml^{-1} isocitrate dehydrogenase, 5 mM magnesium chloride), substrate (minimum of 10 concentrations, final concentration range of 1-1500 µM for rac-methadone and 1-1250 µM for (R)- and (S)-methadone) and were prepared in 1.5 ml Eppendorf tubes (see Chapter 3). Microsomal protein precipitation, and cessation of EDDP formation was achieved by the addition of 100 µl ice cold acetonitrile, followed by vortexing briefly and centrifugation (10 000*g*, 10 min). A 100 µl aliquot of the supernatant was injected on to the HPLC system.

2.3.3. Calibration, precision and accuracy

Retention times of the compounds of interest were confirmed by direct injection of aqueous solutions of pure compounds, and rac-EDDP was used to prepare calibration standards and QC samples. Quantification of rac-EDDP was performed with calibration curves consisting of twelve standards over the concentration range 0.25-50 µM. Low (L), medium (M) and high (H) quality control (QC) samples were prepared in duplicate, with final concentrations of 0.62 µM (LQC), 1.74 µM (MQC) and 12.45 µM (HQC). Calibration standards and QC samples were prepared identically to the microsomal incubations, but with the substitution of substrate with an identical volume (50 µl) of calibration standard and the exclusion of NADPH generating system which was replaced by the addition of an identical volume (20 µl) of sodium phosphate buffer (0.1 M pH 7.4), and were processed as described above (section 2.3.2).

The robustness of the analytical method was assessed by assaying replicates of each QC sample on a single day to determine the intra-assay accuracy and precision. Inter-assay performance was determined using inter-assay accuracy and precision determined by the analysis of duplicates of each QC sample, and the lowest calibration standard, on different assay days. Similarly, the on-going performance of the assay was monitored using inter-

assay accuracy and precision determined by analysis of duplicates of each QC sample, and the lowest calibration standard, on each different assay day and is reported in Chapter 3.

2.3.4. Data analysis

Raw data were entered into Excel spreadsheets (Version 5.0, Microsoft Corporation, WA, USA). Linear regression analysis (Excel, Microsoft) of unweighted log transformed peak area against log transformed nominal concentration provided an estimate of slope, intercept and coefficient of determination (r^2). The estimated concentration divided by the nominal concentration multiplied by 100% was calculated for each individual sample, and accuracy was calculated as the mean of these values, and the residual standard deviation of the mean was taken as the precision.

2.3.5. Results and discussion

The chromatograms obtained from samples contained two peaks with identical retention times to rac-EDDP and rac-methadone. Under the chromatography conditions described, the retention times of (4R,6S)-p-hydroxy methadone, (4S,6S)-p-hydroxy methadone, EDDP, methadone, and EMDP were 4.2, 4.6, 8.5, 13.5, and 25 minutes, respectively, with a total run-time of 35 min. The retention time of α-(3S,6S)-N-desmethyl methadol, the α-methadol enantiomers, β-(3R,6S)-methadol and pyrrolidone were 6.4, 8.0, 11.5 and 55 minutes, respectively. All compounds were baseline resolved, with the exception of the two p-hydroxy methadone diastereomers, and α-methadol and EDDP, however, these compounds were visibly separated. No decrease in resolution was observed after over 500 injections. Increasing mobile phase pH over the range of 4.3-6.0 increased the retention of all compounds without affecting resolution. In preliminary experiments, EMDP, the p-hydroxy methadone diastereomers, α-N-desmethyl methadol, α-methadol, β-methadol and pyrrolidone were not observed following microsomal incubation (see Chapter 3) and no further modifications to the HPLC system were attempted. There were no interfering peaks in the chromatography of several different blank microsomal samples or in the incubation samples. Direct injection of chlorzoxazone, coumarin, diazepam, diethyldithiocarbamic

acid, flunitrazepam, fluoxetine, furafylline, ketoconazole, omeprazole, quinidine, (S)-mephenytoin, sulphaphenazole, troleandomycin, codeine, norcodeine, morphine, dextromoramide, ethylmorphine, hydromorphone, dihydromorphone, naloxone, oxycodone, 6β-oxycodol, temazepam, ketamine, clomipramine, sertraline, amitriptyline, citalopram, doxepin, ketorolac, venlafaxine, nefazodone produced chromatographic peaks that were adequately resolved from EDDP. Aqueous solutions containing nortriptyline, imipramine, and paroxetine produced chromatographic peaks that not well resolved from EDDP, however, the peaks were visibly separated. In contrast, aqueous solutions containing desipramine and fluvoxamine produced peaks which were poorly resolved from EDDP, and would have prevented accurate quantification. A representative chromatogram resulting from a blank microsomal incubation and a incubation containing 25 μM rac-methadone as substrate is shown in Figure 2-1.

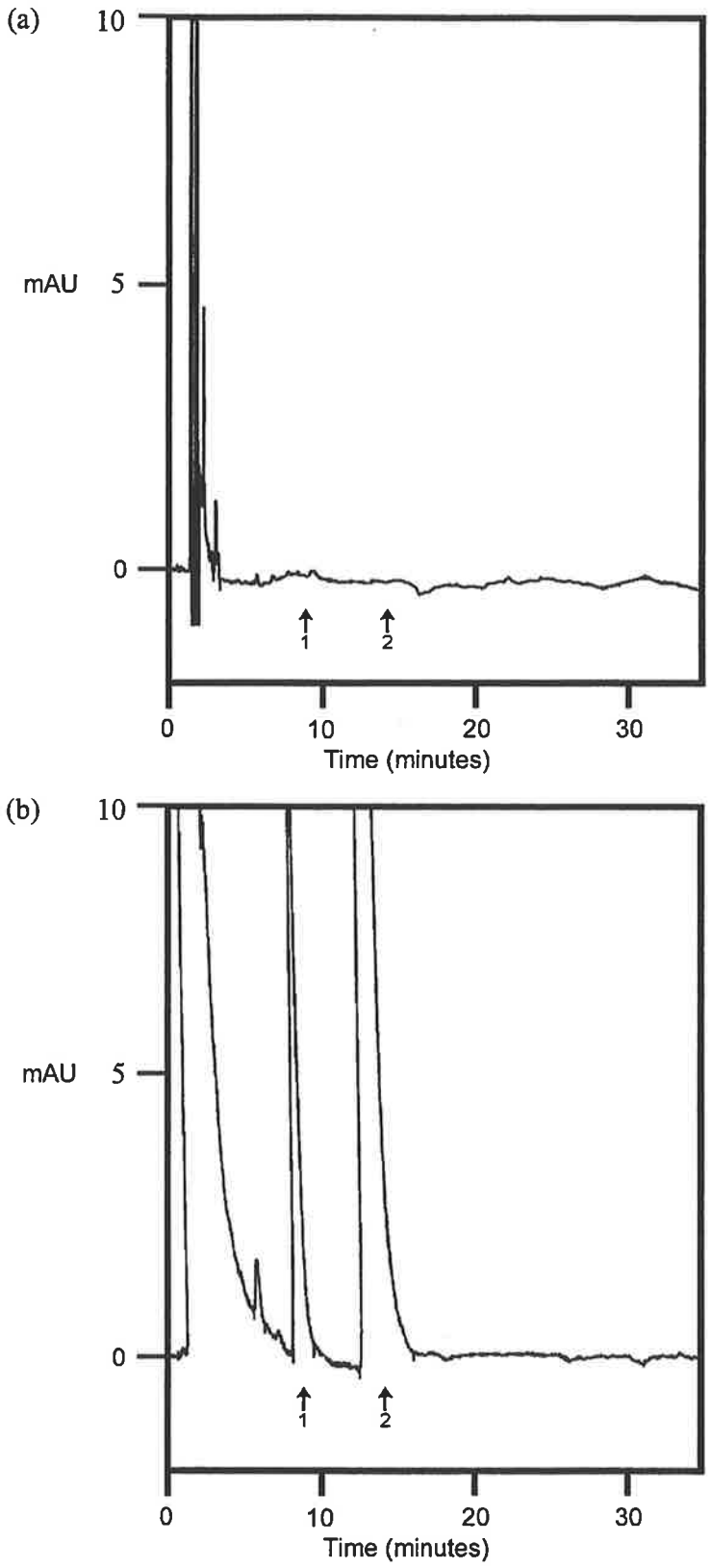


Figure 2-1: Representative chromatogram resulting from (a) blank microsomal incubation and (b) microsomal incubation containing rac-methadone as substrate.
Notes: Sample contained 25 μM rac-methadone as substrate and 2.4 μM rac-EDDP. 1=EDDP, 2=methadone.

Calibration curves for rac-EDDP were linear over the 0.25-50 μM concentration range, with r^2 values greater than 0.99 for all analytical runs, resulting in a mean±SD (n=6 assays) value of 0.9984±0.0019. Estimates of slope demonstrated no consistent time-related changes, with a mean±SD value of 1.117±0.040. The inter- and intra-assay accuracy and precision of the method at the three QC concentrations, and the lowest calibration standard are shown in Table 2-1.

Table 2-1: Inter- and intra-assay accuracy and precision for the quantification of rac-EDDP in human liver microsomes.

	Nominal concentration (μM)	Accuracy (%)	Precision (%)	n
Inter-assay (n=6 assay days)				
LOQ	0.25	99.7	5.9	6
LQC	0.62	105.3	9.7	12
MQC	1.74	107.8	4.0	12
HQC	12.45	97.3	2.0	12
Intra-assay				
LQC	0.62	91.4	6.4	6
MQC	1.74	99.8	6.2	6
HQC	12.45	96.8	3.9	6

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

Based upon the assay validation results presented above, the following assay acceptance criteria were formulated: at least ten calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of ten or more calibration standards and five or more QC samples must be less than ±15%.

The method developed by Alburges and co-workers (1996) for the quantification of methadone, EDDP and EMDP in liver microsomes employed GC-MS analysis, and was therefore able to selectively quantitate the analytes by selective ion monitoring. In contrast, the HPLC method of Iribarne and co-workers (1996) was shown to provide baseline separation of methadone, EDDP and EMDP, however EMDP and the internal standard were only adequately resolved. In comparison, the present assay demonstrated baseline resolution of methadone, EDDP and EMDP. Additionally, the p-hydroxy methadone

diastereomers, α-N-desmethyl methadol, α-methadol, β-methadol and pyrrolidone metabolites were baseline resolved, with the exception of the two p-hydroxy methadone diastereomers, and α-methadol and EDDP. However, these compounds were visibly separated and were not found to be formed in subsequent experiments with human liver microsomes, and were therefore not important for the intended assay application (see Chapter 3).

The present assay demonstrated excellent selectivity, as no interference was found for a number of CYP450 isoform selective inhibitors, and adequate separation of several oxidative metabolites of methadone was achieved. In contrast, the methods reported by Alburges and co-workers (1996) and Iribarne and co-workers (1996) did not address this issue. It is likely that the use of a selective ion monitoring GC-MS analysis provided adequate selectivity (Alburges et al., 1996). However, it is difficult to determine if this is also true for the HPLC assay (Iribarne et al., 1996), although neither group reported difficulties associated with the use of a variety of chemical inhibitors.

Alburges and co-workers' (1996) method involved solid-phase extraction of samples (1 ml) adjusted to pH 6.0, followed by reconstitution of the dried residue with n-butyl chloride and analysed by GC-MS. Extraction recoveries were 99%, 94% and 85% for methadone, EDDP and EMDP, respectively, at a concentration of 100 $ng.ml^{-1}$, although an estimate of variability was not provided. In contrast, Iribarne and co-workers (1996) employed solvent-solvent extraction of alkalinised samples (1 ml) with two aliquots of diethyl ether followed by reconstitution of the dried residue with 5 mM HCl. The present assay did not require sample extraction, but incorporated a simple protein precipitation procedure which did not necessitate the use of an internal standard, while maintaining linearity and reproducibility, and required a markedly smaller sample volume (200 µl). In comparison to the published methods, the present assay offers significant advantages in terms of sample preparation, and the sample volume required for analysis.

The assay range employed by Alburges and co-workers (1996) employed a much lower (10-600 $ng.ml^{-1}$) range of concentrations in comparison to the present method (0.25-50 μM; approximately 75-3000 $ng.ml^{-1}$). From the results of experiments investigating the metabolism of methadone to EDDP (see Chapter 3), a 600 $ng.ml^{-1}$ upper limit of quantification (Alburges et al., 1996) would be well below that necessary to prevent re-analysis of samples after dilution in a full enzyme kinetic study, due to concentrations of EDDP exceeding 600 $ng.ml^{-1}$. The calibration curve concentration range employed in the present assay spanned the range of concentrations measured in the majority of samples. No samples contained concentrations of EDDP above the limit of quantification, while very few were below the limit of quantification all of which occurred at substrate concentrations <10 μM, which would be unlikely to have resulted in a marked effect on the results of the experiments (see Chapter 3). Alburges and co-workers' (1996) method reported that inter-assay (n=3-5 assays) coefficients of variation ranged from 3-15% for methadone, EDDP and EMDP at concentrations of 25 $ng.ml^{-1}$, 100 $ng.ml^{-1}$ and 300 $ng.ml^{-1}$. However, this was generally below 10%, while accuracy (%bias) ranged from -16% to +3%, but was generally less than ±10%. Similarly, intra-assay coefficients of variation (n=5 replicates) ranged from 1-11% for all three analytes at concentrations of 25 $ng.ml^{-1}$, 100 $ng.ml^{-1}$ and 300 $ng.ml^{-1}$, while accuracy (%bias) was generally less than ±10% (Alburges et al., 1996). Iribarne and co-workers (1996) did not report assay validation data separately for EDDP and EMDP, and the calibration range and limit of detection of the assay were ill defined. In comparison, the present assay demonstrated excellent inaccuracy (range: -9% to +8%) and precision (range: 2% to 10%), which were maintained for both intra- and inter-assay analysis. The present assay did not quantitate methadone, or metabolites other than EDDP as they were not formed from methadone in preliminary experiments using human liver microsomes, however, adequate chromatographic separation was achieved. No extraction procedure was employed, thus poor or inconsistent recovery of the analytes is unlikely. Taken together, these data would indicate that the present assay could be expanded to include the quantification of other methadone metabolites if required, with minimal difficulty. In comparison to the published methods, this assay offers better precision and accuracy over a

similar concentration range. The present assay provides a significant improvement compared to previous assays, in terms of calibration range, for the quantification of rac-EDDP in human liver microsomal incubations.

In summary, the assay presented here provides a simple, accurate, precise and selective method to investigate the metabolism of methadone in human liver microsomes, and offers significant improvements over published methods.

2.4. Quantification of rac-methadone in human plasma

The following assay was initially developed in the Department of Clinical and Experimental Pharmacology at Adelaide University by Mr Andrew Menelaou. However, inter- and intra-assay performance of the assay were repeated by this author to ensure the validity of the method.

2.4.1. HPLC instrumentation and chromatography conditions

The HPLC system which comprised a LC-6A pump (Shimadzu, Kyoto, Japan), a Sil-9A autoinjector (Shimadzu), an UVIDEC-100-V spectrophotometer (Jasco, Tokyo, Japan) set at 210 nm (0.0025 aufs) and a C-R6A Chromatopac integrator with the attenuation set at 2 (Shimadzu). The analytical column was a 100x5 mm Nova-Pak C_{18} 4 μm cartridge (Waters, Milford, MA, USA) contained in an 8x10 Radial Compression Module (Waters) protected by a 2 μm in-line filter (Scientific Instruments, State College, PA, USA) and a 15x4.6 mm pre-column packed with 30 μm pellicular ODS C_{18} material (Whatman Laboratories, Clinton, NJ, USA). Optimal separation of the compounds of interest was achieved with a mobile phase of 35% (v/v) acetonitrile and 0.2% (v/v) triethylamine in 50 mM NaH_2PO_4 with final pH adjusted to 5.5 with ortho-phosphoric acid and pumped through the system at 1.0 $ml.min^{-1}$ at room temperature.

2.4.2. Sample preparation

Plasma samples (0.5 ml) and internal standard (50 µl 10 $\mu g.ml^{-1}$ N-desmethyl dextropropoxyphene in 50 mM phosphate buffer pH 2.0) were aliquoted into 10 ml tapered bottom plastic tubes, alkalinised (0.2 ml 0.1 M Na_2CO_3 pH 10) and extracted with 5 ml of 30:70 (v/v) diethyl ether:hexane for 15 minutes on a rotary mixer. Samples were then centrifuged (2000*g*, 10 minutes) and the organic phase transferred to a clean 10 ml tapered bottom plastic tube containing 0.25 ml 50 mM phosphate buffer pH 2.0 and vortexed for 30 seconds. Samples were then centrifuged (2000*g*, 10 minutes), the organic phase aspirated to waste and 100 µl of the acid phase was injected onto the chromatography system. The use of 50 mM phosphate buffer pH 2.0 for the final back-extraction and storage of N-desmethyl dextropropoxyphene was necessary to prevent the degradation of this compound. EDDP is not back-extracted into the 50 mM pH 2.0 phosphate buffer.

2.4.3. Calibration, precision, accuracy and extraction efficiency

Retention times of the compounds of interest were confirmed by direct injection of aqueous solutions of pure compounds. Quantification of rac-methadone was performed with calibration curves consisting of eight standards over the concentration range 30-1200 $ng.ml^{-1}$ of the free base. Low (L), medium (M) and high (H) QC samples were prepared in duplicate, with final concentrations of 107.3 $ng.ml^{-1}$, 178.9 $ng.ml^{-1}$ and 715.6 $ng.ml^{-1}$ of the free base. Calibration standards and QC samples were prepared by diluting 50 µl of aqueous rac-methadone stock solution with 450 µl drug-free plasma, and analysed identically to the patient samples (section 2.4.2). Patient samples which contained rac-methadone at concentrations above the limit of quantification were diluted with drug-free matrix and re-assayed.

The robustness of the analytical method was assessed by assaying replicates of each QC sample on a single day to determine the intra-assay accuracy and precision. Inter-assay performance was determined using inter-assay accuracy and precision determined by the analysis of duplicates of each QC sample, and the lowest calibration standard, on different

assay days. Similarly, the on-going performance of the assay was monitored using inter-assay accuracy and precision determined by analysis of duplicates of each QC sample, and the lowest calibration standard, on each different assay day and is reported in Chapter 4. Extraction efficiency was analysed at each QC concentration and for the internal standard using the intra-assay validation samples. The peak areas of all compounds after injection of the extracted samples were compared to those obtained after direct injection of the aqueous stock solution.

2.4.4. Data analysis

Raw data were entered into Excel spreadsheets (Version 4.0, Microsoft Corporation, WA, USA). Peak areas were converted into peak area ratios using the peak area of the internal standard. Linear regression analysis (Excel, Microsoft) unweighted peak area ratio against nominal concentration provided an estimate of slope, intercept and coefficient of determination (r^2). The estimated concentration divided by the nominal concentration multiplied by 100% was calculated for each individual sample, and accuracy was calculated as the mean of these values, and the residual standard deviation of the mean was taken as the precision.

2.4.5. Results and discussion

The chromatograms obtained from patient samples contained two peaks with identical retention times to N-desmethyl dextropropoxyphene and rac-methadone. Retention times for N-desmethyl dextropropoxyphene and rac-methadone were 9.8 and 12.5 min, respectively, with a total run time of 15 min. Under the chromatography conditions described, N-desmethyl dextropropoxyphene and rac-methadone were baseline resolved and no decrease in resolution was seen after over 300 injections. The present assay did not quantify (4R,6S)-p-hydroxy methadone, (4S,6S)-p-hydroxy methadone, EMDP, α-N-desmethyl methadol, α-methadol, β-methadol or pyrrolidone as these compounds have not been detected in the plasma of methadone maintenance patients. Similarly, rac-EDDP was not quantified as plasma concentrations were expected to be extremely low (see section

1.7). The retention time of (4R,6S)-p-hydroxy methadone, (4S,6S)-p-hydroxy methadone, α-(3S,6S)-N-desmethyl methadol, the α-methadol enantiomers, EDDP, β-(3R,6S)-methadol, EMDP and pyrrolidone were 3.9, 4.3, 6.0, 7.8, 8.6, 10.4, >60 and >60 minutes, respectively. All compounds were adequately resolved from methadone and N-desmethyl dextropropoxyphene, and did not interfere with the chromatography, with the exception of β-methadol which is not believed to be a methadone metabolite in humans and has not been detected in the plasma of methadone maintenance patients (see section 1.7). No further modifications to the HPLC system were attempted. There were no interfering peaks in the chromatography in several different drug-free plasma samples or in the patient samples. Direct injection of aqueous solutions containing codeine, norcodeine, morphine, ethylmorphine, hydromorphone, dihydromorphone, naloxone, oxycodone, 6β-oxycodol, diazepam, temazepam, ketamine, citalopram, doxepin, clomipramine, imipramine, desipramine, ketorolac, fluvoxamine, paroxetine, sertraline, venlafaxine, nefazodone produced chromatographic peaks that were adequately resolved from methadone and N-desmethyl dextropropoxyphene. In contrast, aqueous solutions containing fluoxetine, amitriptyline, nortriptyline and imipramine produced chromatographic peaks that were not well resolved from either methadone or N-desmethyl dextropropoxyphene. However, all peaks were visibly separated.

A representative chromatogram resulting from the analysis for rac-methadone of a drug-free plasma sample and a plasma sample obtained from a methadone maintenance patient 1.5 hours after administration of the 130 mg daily dose of rac-methadone is shown in Figure 2-2.

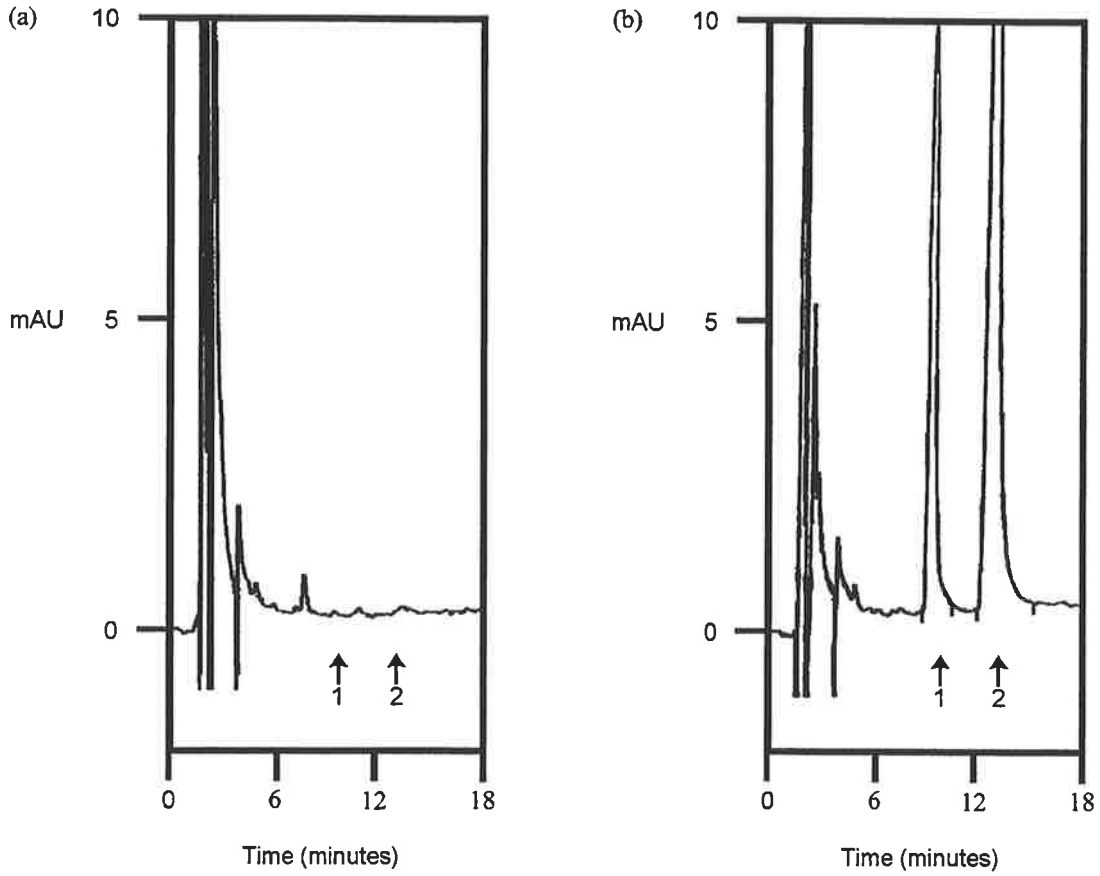


Figure 2-2: Representative chromatogram for the analysis of (a) drug-free plasma sample and (b) a plasma sample obtained from a methadone maintenance patient assayed for rac-methadone.

Notes: Sample contained 646 ng.ml^{-1} rac-methadone and was obtained 1.5 hours after the administration of the patients 130 mg daily rac-methadone dose. 1=N-desmethyl dextropropoxyphene, 2=rac-methadone.

Calibration curves for rac-methadone were linear over the 30-1200 ng.ml^{-1} range, with r^2 values greater than 0.99 for all analytical runs, and mean±SD values are presented in Table 2-2. Estimates of slope demonstrated no consistent time-related changes, and mean±SD values are presented in Table 2-2. The inter- and intra-assay accuracy and precision of the method at the three QC concentrations, and the lowest calibration standard are shown in Table 2-2.

Based upon the assay validation results, the following assay acceptance criteria were formulated: at least seven calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of seven or more calibration standards and five or more QC samples must be less than ±15%.

Table 2-2: Inter- and intra-assay accuracy and precision for the quantification of rac-methadone in human plasma.

	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
Inter-assay (n=3 assay days)				0.9977 ±0.0012	0.0071 ±0.0002	3
LOQ	30.0	100.4	6.1			8
LQC	107.3	98.2	6.2			14
MQC	178.9	96.9	5.0			14
HQC	715.6	100.5	6.3			14
Intra-assay	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Extraction efficiency (%±SD)		
LQC	107.3	98.7	6.8	81±6		10
MQC	178.9	99.2	3.0	89±4		10
HQC	715.6	104.0	2.8	82±10		10
N-desmethyl dextropropoxyphene (internal standard)				73±10		30

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

This assay for the quantification of rac-methadone in plasma compares favourably with the non-stereoselective assays published in the literature (see Appendix 1 and section 2.1.1). In terms of sample preparation, previously reported methods have employed solid-phase or solvent-solvent extraction methods similar to that employed in the present assay. However, the methods employing GC analysis have often required multiple extraction steps in order to ensure adequate sample purification. Reported extraction efficiencies for methadone have generally ranged from 80-100%, and are quantitatively similar to those obtained with the present method (see Table 2-2), as would be expected from the similarity of extraction techniques employed. Similarly, the 0.5 ml sample volume employed to achieve a calibration curve concentration range of 30-1200 $ng.ml^{-1}$ compared favourably with other methods. The lower limit of quantification was set at 30 $ng.ml^{-1}$, which could have been decreased to 15 $ng.ml^{-1}$ by utilising a 1.0 ml sample volume. When one considers the sample volume employed, other authors utilising HPLC, or GC-MS, techniques have reported similar calibration curve concentration ranges: 10-100 $ng.ml^{-1}$ (2.0 ml Wolff et al., 1990), 25-1000 $ng.ml^{-1}$ (0.9 ml Pierce et al., 1992), 10-800 $ng.ml^{-1}$ (0.5 ml de Vos et al., 1995), 10-600 $ng.ml^{-1}$ (1.0 ml Alburges et al., 1996), 5-800 $ng.ml^{-1}$ (1.0 ml Wojnar-Horton

et al., 1997), 10-500 ng.ml^{-1} (1.0 ml Cobb et al., 1998), 50-2000 ng.ml^{-1} (1.0 ml Bermejo et al., 2000b). In contrast, authors employing GC techniques have often employed much larger sample volumes to obtain comparable calibration curve concentration ranges: 5-500 ng.ml^{-1} (4.0 ml Sullivan & Blake, 1972), 20-2000 ng.ml^{-1} (2.0 ml Kreek et al., 1976b). This is most likely due to the poor sensitivity of the detectors employed at the time, in comparison to modern UV detectors currently available for use with HPLC. In addition, these authors employed comparatively low lower limits of limits quantification which may have necessitated a larger sample volume.

Despite often obtaining very low lower limits of quantification (0.5-3 ng.ml^{-1}) from small sample volumes (0.01-0.25 ml Bartos et al., 1977; Ling et al., 1981), authors using RIA techniques have reported relatively small ranges in calibration curves, with upper limits of quantification below 50 ng.ml^{-1}. These assays would be unlikely to be of widespread use in investigating the pharmacokinetics of rac-methadone in methadone maintenance patients, as many samples would contain methadone at concentrations far exceeding the upper limit of quantification. In contrast, others have employed more appropriate calibration curve concentration ranges in their RIA assays. However, the lower limits of quantification have then increased to values similar to the HPLC and GC assays, presumably due to non-linearity of antibody binding (Beck et al., 1990).

This author is surprised at the limited extent to which other authors have presented assay validation data, particularly accuracy, of their methods for the quantification of rac-methadone in plasma samples (see Appendix 1 and section 2.1.1). This is especially surprising for the more recent publications using HPLC techniques. Although many of these methods were presented as part of a pharmacokinetic study, and a highly detailed discussion of assay validation is inappropriate, it is necessary to provide confidence in the findings of the study in which the assay was employed. Several authors have not reported on the accuracy of their methods (Bermejo et al., 2000b; Chikhi-Chorfi et al., 1998; Cobb et al., 1998; de Vos et al., 1995; George & Braithwaite, 1999; Gourlay et al., 1982; Green &

Wilson, 1996b; Pierce et al., 1992; Sullivan & Blake, 1972; Torrens et al., 1998; Wojnar-Horton et al., 1997), whilst others have provided limited data (Kreek et al., 1976b) or have reported some accuracy data when applying an adapted method (Garrido et al., 1999; for Wolff et al., 1990) at unspecified concentrations. In contrast, several authors have provided intra-assay accuracy data (Sullivan et al., 1975a), while few have included detailed inter-assay data (Alburges et al., 1996; Magora et al., 1987; Schmidt et al., 1993). Despite the limitations of the data in some cases, it is likely that all methods were sufficiently accurate for the intended application, as the majority of reports which have provided accuracy data have reported values $\leq \pm 15\%$ at the lowest concentration examined (see Appendix 1).

Many authors have reported intra-assay precision data only (Baugh et al., 1991; Bermejo et al., 2000b; Gourlay et al., 1982; Kreek et al., 1976b; Sullivan et al., 1975a; Wojnar-Horton et al., 1997), while others have provided inter-assay data (Alburges et al., 1996; Chikhi-Chorfi et al., 1998; Cobb et al., 1998; de Vos et al., 1995; George & Braithwaite, 1999; Green & Wilson, 1996b; Magora et al., 1987; Pierce et al., 1992; Schmidt et al., 1993; Torrens et al., 1998; Wolff et al., 1990). Irrespective of whether the values were calculated from intra- or inter-assay data, reported values were generally <10% at the lowest concentration examined (see Appendix 1), indicating good reproducibility of all methods.

In comparison to the above mentioned assays, the present assay demonstrated excellent inaccuracy (%bias, range: -3% to +4%) and precision (range: 3% to 7%) for the three QC samples and at the limit of quantification, which was maintained for both intra- and inter-assay analysis.

Selectivity is an important component of assay validation, and should be demonstrated to ensure confidence in the results obtained and avoid errors in the interpretation of the derived data. The issue of assay selectivity becomes increasingly important when the subjects under investigation are likely to be taking multiple medications, both licit and illicit, as methadone maintenance patients do. The present assay demonstrated excellent selectivity, as no

interference was found in several different drug-free plasma samples or in the patient samples, and direct injection of aqueous solutions containing a range of opioid, anxiolytic and anti-depressant compounds produced chromatographic peaks that were adequately resolved from methadone and N-desmethyl dextropropoxyphene. However, aqueous solutions containing fluoxetine, amitriptyline, nortriptyline and imipramine produced chromatographic peaks that were not well resolved from either methadone or N-desmethyl dextropropoxyphene, although all peaks were visibly separated. Adequate separation of several metabolites of methadone was also achieved. The present assay did not quantify (4R,6S)-p-hydroxy methadone, (4S,6S)-p-hydroxy methadone, EMDP, α-N-desmethyl methadol, α-methadol, β-methadol or pyrrolidone as these compounds have not been detected in the plasma of methadone maintenance patients. The selectivity of this method has been further addressed in section 2.8. Other authors have quantified EDDP (Alburges et al., 1996; Bermejo et al., 2000b; Chikhi-Chorfi et al., 1998; de Vos et al., 1995; Green & Wilson, 1996b; Pierce et al., 1992; Torrens et al., 1998) and EMDP (Alburges et al., 1996; Pierce et al., 1992) in plasma, in addition to methadone. While quantification of EDDP in plasma may have been desirable, as this would provide additional pharmacokinetic data, EDDP was not quantified as plasma concentrations were expected to be extremely low (see section 1.7).

In contrast, the methods reported by many authors have not addressed assay selectivity (Alburges et al., 1996; Bartos et al., 1977; Baugh et al., 1991; Bermejo et al., 2000b; Cobb et al., 1998; George & Braithwaite, 1999; Gourlay et al., 1982; Green & Wilson, 1996b; Kreek et al., 1972; Magora et al., 1987; Pierce et al., 1992; Sullivan et al., 1975b; Torrens et al., 1998; Wojnar-Horton et al., 1997), although it is likely that the GC-MS methods (Alburges et al., 1996; Baugh et al., 1991; Bermejo et al., 2000b; Kreek et al., 1972; Sullivan et al., 1975b) were adequately selective through the use of a selective ion monitoring. Other authors addressed this issue to varying extents (Beck et al., 1990; Chikhi-Chorfi et al., 1998; de Vos et al., 1995; Ling et al., 1981; Schmidt et al., 1993; Wolff et al., 1990).

In summary, this assay provides a simple, accurate, precise and selective method to investigate the disposition of rac-methadone in methadone maintenance patients.

2.5. Quantification of (R)- and (S)-methadone in human plasma, plasma ultra-filtrate and urine

The method was based upon that of Norris and co-workers (1994), with major modifications, and expanded to include the analysis of plasma, plasma ultra-filtrate and urine.

2.5.1. HPLC instrumentation and chromatography conditions

The HPLC system comprised a LC-10AT pump (Shimadzu, Kyoto, Japan), a Sil-10A autoinjector (Shimadzu) and a SPD-M10A photo-diode array detector (Shimadzu) set at 210 nm. The system was controlled using Class-LC10 software (version 1, Shimadzu) running under Windows 3.11 (Microsoft Corporation, WA, USA) on a 486 DX IBM compatible computer. The analytical column was a Cyclobond I 2000 RSP column (250x4.6 mm, Astec, Whippany, NJ, USA) protected by a 2 μm in-line filter (Scientific Instruments, State College, PA, USA) and a Cyclobond I 2000 RSP pre-column (20x4.0 mm, Astec). Optimal separation of the compounds of interest was achieved with a mobile phase of 9:11:80 (v/v) methanol:acetonitrile:1% triethylamine (v/v) in water with the final pH adjusted to 6.0 with ortho-phosphoric acid and pumped through the system at 1.0 $ml.min^{-1}$ at room temperature.

2.5.2. Sample preparation

2.5.2.1. Plasma samples

Plasma samples (1 ml) and internal standard (100 μl 5 $\mu g.ml^{-1}$ 3-methoxymorphinan in water) were aliquoted into 10 ml tapered bottom plastic tubes, alkalinised (0.4 ml 0.1 M Na_2CO_3 pH 10) and extracted with 6 ml of 30:70 (v/v) diethyl ether:hexane for 20 minutes on a rotary mixer. Samples were then centrifuged (2000*g*, 10 minutes) and the organic phase transferred to a clean 10 ml tapered bottom plastic tube containing 0.25 ml 5mM HCl

and vortexed for 1 minute. Samples were then centrifuged (2000*g*, 10 minutes), the organic phase aspirated to waste and 100 µl of the acid phase was injected onto the chromatography system.

2.5.2.2. Plasma ultra-filtrate samples

The analysis of (R)- and (S)-methadone in plasma ultra-filtrate (see Chapter 5) was performed as for plasma, with the following modifications: only 0.35 ml plasma ultra-filtrate was assayed, and 50 µl of 5 $\mu g.ml^{-1}$ 3-methoxymorphinan was used as the internal standard.

2.5.2.3. Urine samples

The analysis of (R)- and (S)-methadone in urine (1.0 ml) was performed as for plasma, with the following modifications: 50 µl of 15 $\mu g.ml^{-1}$ 3-methoxymorphinan was used as the internal standard.

2.5.3. Calibration, precision, accuracy and extraction efficiency

2.5.3.1. Plasma samples

Retention times of the compounds of interest were confirmed by direct injection of aqueous solutions of pure compounds, and of the individual enantiomers of methadone by direct injection of aqueous solutions of enantiomerically pure compounds. Quantification of (R)- and (S)-methadone was performed with calibration curves consisting of eight standards over the concentration range 15-600 $ng.ml^{-1}$ of the free base. Low (L), medium (M) and high (H) QC samples were prepared in duplicate, with final concentrations of 54 $ng.ml^{-1}$, 90 $ng.ml^{-1}$ and 350 $ng.ml^{-1}$ of the free base. Calibration standards and QC samples were prepared by diluting 100 µl of aqueous rac-methadone stock solution with 900 µl drug-free plasma, and analysed identically to the patient samples (section 2.5.2.1). Patient samples which contained analytes at concentrations above the limit of quantification were diluted with drug-free matrix and re-assayed.

The robustness of the analytical method was assessed by assaying replicates of each QC sample on a single day to determine the intra-assay accuracy and precision. Inter-assay performance was determined using inter-assay accuracy and precision determined by the analysis of duplicates of each QC sample, and the lowest calibration standard, on different assay days. Similarly, the on-going performance of the assay was monitored using inter-assay accuracy and precision determined by analysis of duplicates of each QC sample, and the lowest calibration standard, on each different assay day and is reported in Chapter 5. Extraction efficiency was analysed at each QC concentration and for the internal standard using the intra-assay validation samples. The peak areas of all compounds after injection of the extracted samples were compared to those obtained after direct injection of the aqueous stock solution.

2.5.3.2. Plasma ultra-filtrate samples

The analysis of (R)- and (S)-methadone in plasma ultra-filtrate (see Chapter 5) was performed as for plasma, with the following modifications: calibration curves consisted of eight standards over the concentration range 12.5-500 $ng.ml^{-1}$ of (R)- and (S)-methadone of the free base; low (L), medium (M) and high (H) QC samples were prepared in duplicate, with final concentrations of 34 $ng.ml^{-1}$, 67 $ng.ml^{-1}$ and 366 $ng.ml^{-1}$ of the free base; calibration standards and QC samples were prepared by diluting 40 µl of aqueous rac-methadone stock solution with 360 µl isotonic phosphate buffer (67 mM Na_2HPO_4/NaH_2PO_4 in 0.9% NaCl, final pH 7.4), and 350 µl analysed identically to the patient samples (section 2.5.2.2). Patient samples which contained analytes at concentrations above the limit of quantification were diluted with drug-free matrix and re-assayed.

2.5.3.3. Urine samples

The analysis of (R)- and (S)-methadone in urine was performed as for plasma, with the following modifications: calibration curves consisted of eight standards over the concentration range 50-2000 $ng.ml^{-1}$ of (R)- and (S)-methadone of the free base; low (L),

medium (M) and high (H) QC samples were prepared in duplicate, with final concentrations of 112 $ng.ml^{-1}$, 403 $ng.ml^{-1}$ and 1120 $ng.ml^{-1}$ of the free base; calibration standards and QC samples were prepared by diluting 100 µl of aqueous rac-methadone stock solution with 900 µl of drug-free urine and analysed identically to the patient samples (section 2.5.2.3). Patient samples which contained analytes at concentrations above the limit of quantification were diluted with drug-free matrix and re-assayed.

2.5.4. Data analysis

Raw data were entered into Excel spreadsheets (Version 5.0, Microsoft Corporation, WA, USA). Peak areas were converted into peak area ratios using the peak area of the internal standard. Linear regression analysis (GraphPad Prism v2.01, GraphPad Software, CA, USA) of $1/y^2$ weighted peak area ratio against nominal concentration provided an estimate of slope, intercept and coefficient of determination (r^2). The estimated concentration divided by the nominal concentration multiplied by 100% was calculated for each individual sample, and accuracy was calculated as the mean of these values, and the residual standard deviation of the mean was taken as the precision. Comparison of extraction efficiency values for (R)- and (S)-methadone was performed using paired t-tests, comparison of extraction efficiency from plasma ultra-filtrate and isotonic phosphate buffer was performed using an unpaired t-test (GraphPad Prism v2.01, GraphPad Software).

2.5.5. Results and discussion

The chromatograms obtained from patient samples contained three peaks with identical retention times to (R)-methadone, (S)-methadone and 3-methoxymorphinan. Retention times for (R)-methadone, (S)-methadone and 3-methoxymorphinan were 8.3, 9.6 and 21 minutes, respectively, with a total run time of 30 minutes. Under the chromatography conditions described, (R)-methadone, (S)-methadone and 3-methoxymorphinan were baseline resolved (see Figure 2-3), and no decrease in resolution was observed for up to 400 injections. The present assay did not quantify (4R,6S)-p-hydroxy methadone, (4S,6S)-p-hydroxy methadone, EMDP, α-(3S,6S)-N-desmethyl methadol, the α-methadol

enantiomers, β-(3R,6S)-methadol or pyrrolidone as these compounds have not been detected in the plasma of methadone maintenance patients. Similarly, EDDP was not quantified as plasma concentrations were expected to be extremely low (see section 1.6). The enantiomers of EDDP eluted as a broad peak that was not baseline resolved from the (R)-methadone peak under these chromatography conditions, although there was visible separation of the (R)-methadone and EDDP peaks. Recently, Rudaz & Veuthey (1999) reported similar inability to resolve EDDP from methadone with the Cyclobond I 2000 RSP column. However, EDDP is not back-extracted into 5mM HCl. Patient urine samples contained EDDP in concentrations up to 80μM (see section 2.8 and Chapters 4 and 5), but no chromatogram obtained from the analysis of any patient sample contained a peak corresponding to EDDP. Therefore EDDP did not interfere with the analysis of (R)- and (S)-methadone in this assay. No further modifications to the HPLC system were attempted. Urinary concentrations of EDDP have been reported to be similar to that of methadone (see section 1.7.3), and therefore quantification of the enantiomers of metabolite in urine is desirable. A method was subsequently developed to meet this need (see section 2.6). There were no interfering peaks in the chromatography in several different drug-free plasma samples or in the patient samples. Direct injection of aqueous solutions containing diazepam, caffeine, morphine, codeine, norcodeine and naloxone did not produce interfering peaks, while N-desmethyl dextropropoxyphene interfered with (R)-methadone, although this was visibly resolved. In contrast, dextropropoxyphene co-eluted with (R)-methadone.

2.5.5.1. Plasma samples

A representative chromatogram from the analysis of a drug-free plasma sample and a sample obtained from a methadone maintenance patient 1.5 hours after administration of the 130 mg daily dose of rac-methadone assayed for (R)-methadone and (S)-methadone is shown in Figure 2-3.

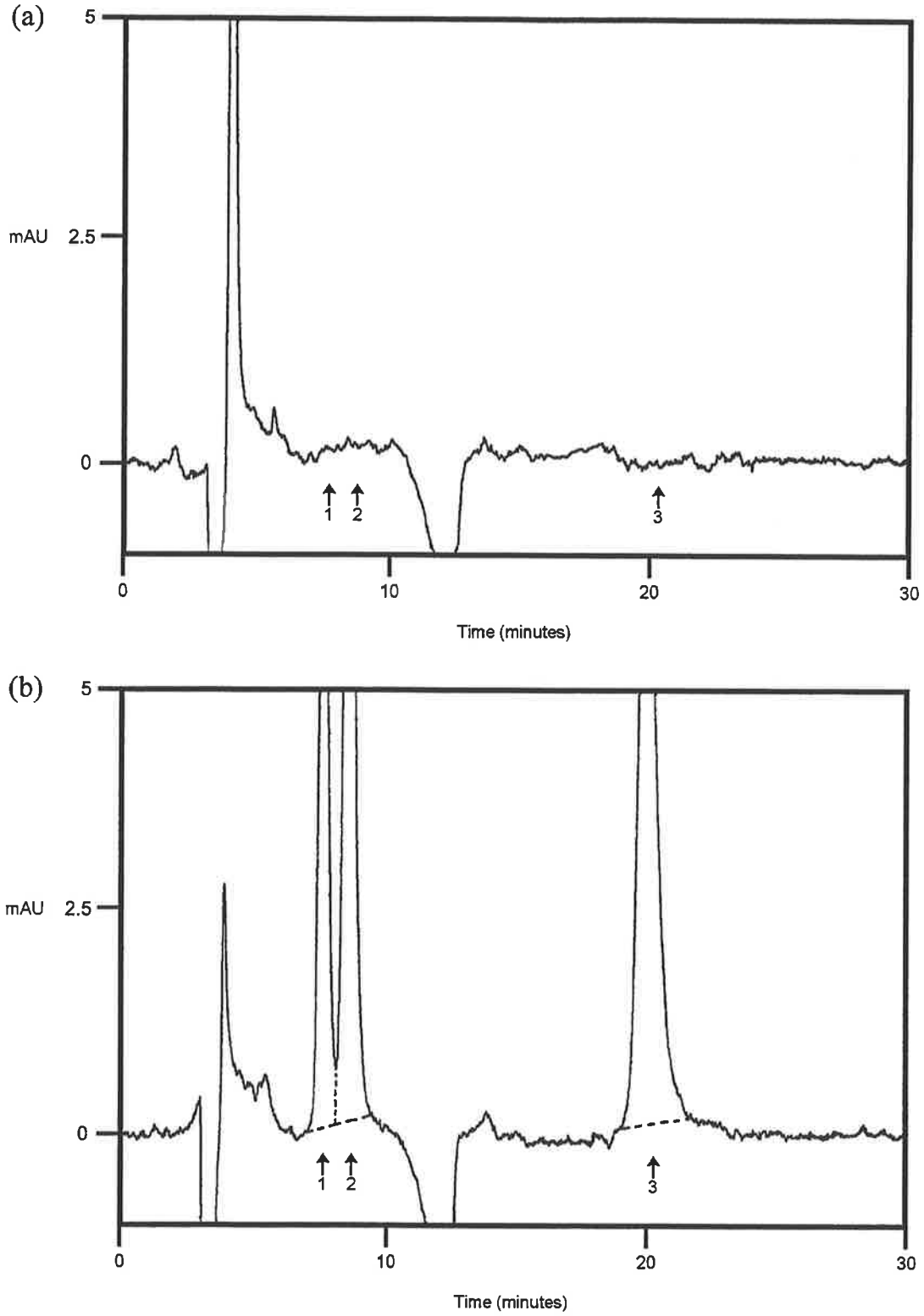


Figure 2-3: Representative chromatogram for the analysis of (a) drug-free plasma sample and (b) a plasma sample obtained from a methadone maintenance patient assayed for (R)-methadone and (S)-methadone.

Notes: Sample contained 265 $ng.ml^{-1}$ (R)-methadone and 378 $ng.ml^{-1}$ (S)-methadone and was obtained 1.5 hours after the administration of the patients 130 mg daily rac-methadone dose. 1=(R)-methadone, 2=(S)-methadone, 3=3-methoxymorphinan.

Calibration curves for (R)- and (S)-methadone were linear over the 15-600 $ng.ml^{-1}$ concentration range, with r^2 values greater than 0.99 for all analytical runs for both (R)- and (S)-methadone, and mean±SD values are presented in Table 2-3. Estimates of slope demonstrated no consistent time-related changes for either (R)- or (S)-methadone, and mean±SD values are presented in Table 2-3. The inter- and intra-assay accuracy and precision of the method at the three QC concentrations, and the lowest calibration standard are shown in Table 2-3.

Based upon the assay validation results, the following assay acceptance criteria were formulated: at least seven calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of seven or more calibration standards and five or more QC samples must be less than ±15%.

Table 2-3: Inter- and intra-assay accuracy and precision for the quantification of (R)-methadone and (S)-methadone in human plasma.

Inter-assay (n=5 assays)	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(R)-methadone				0.9985 ±0.0006	0.0030 ±0.0002	5
LOQ	15	98.9	1.5			5
LQC	54	108.0	8.9			13
MQC	90	104.8	5.2			14
HQC	350	102.9	4.8			14
(S)-methadone				0.9952 ±0.0021	0.0031 ±0.0002	5
LOQ	15	107.3	3.9			5
LQC	54	104.8	6.6			13
MQC	90	106.9	5.7			14
HQC	350	104.5	7.7			14

Intra-assay	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Extraction efficiency (%±SD)	n
(R)-methadone					
LQC	54	108.0	7.8	101±6	6
MQC	90	105.9	6.8	101±4	6
HQC	350	107.2	3.9	100±3	6
(S)-methadone					
LQC	54	103.7	7.5	99±7	6
MQC	90	106.2	5.7	100±3	6
HQC	350	111.7	4.6	103±2	6
3-methoxymorphinan (internal standard)				84±1	18

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

This assay utilised 100% hexane as the extraction solvent and 5 mM HCl for the final back extraction, rather than the 70:30 (v/v) hexane:diethyl ether and 50 mM pH 2.0 phosphate buffer combination employed in the assay for the quantification of rac-methadone in plasma (see section 2.4). These modifications to the sample preparation procedure were necessary, as the use of the latter reagents resulted in poor chromatograms in preliminary investigations. As can be seen from Table 2-3, Table 2-4 and Table 2-5 this modification provided excellent extraction recoveries of methadone and the internal standard. The extraction efficiency of (R)- and (S)-methadone from plasma was high, reproducible (coefficients of variation <10%), and did not demonstrate concentration-dependency with mean values determined from LQC, MQC and HQC intra-assay validation samples ranging

99-103% (Table 2-3). Additionally, extraction efficiency was not statistically (*P* value; mean difference, 95% confidence intervals of the mean differences) significantly different (*P*=0.99; -0.01%, -2.51 to 2.49%) between the enantiomers, indicating no stereoselectivity of the extraction of methadone from plasma samples. Extraction efficiency of the internal standard was lower than that of methadone, however, this was also very reproducible (Table 2-3).

2.5.5.2. Plasma ultra-filtrate samples

The extraction procedure was similar and chromatography conditions identical to those employed for the analysis of plasma samples, which resulted in almost identical chromatograms to that shown in Figure 2-3 for the analysis of plasma samples.

As this assay in plasma ultra-filtrate samples comprised only minor modifications of the assay for (R)- and (S)-methadone in plasma, inter-assay validation data were only performed over two assay days, while intra-assay variability was assessed using six replicate samples of each QC sample. Calibration curves for (R)- and (S)-methadone were linear over the 12.5-500 $ng.ml^{-1}$ concentration range, with r^2 values greater than 0.99 for each analytical run for both (R)- and (S)-methadone, and mean±SD values are presented in Table 2-4. Estimates of slope were similar for (R)- or (S)-methadone over both analytical runs, and mean±SD values presented in Table 2-4. The inter- and intra-assay accuracy and precision of the method at the three QC concentrations, and the lowest calibration standard are shown in Table 2-4.

Based upon the assay validation results, the following assay acceptance criteria were formulated: at least seven calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of seven or more calibration standards and five or more QC samples must be less than ±15%.

Table 2-4: Inter- and intra-assay accuracy and precision for the quantification of (R)-methadone and (S)-methadone in human plasma ultra-filtrate.

Inter-assay (n=2 assays)	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(R)-methadone				0.9931 ±0.0016	0.0012 ±0.0001	2
LOQ	12.5	103.9	4.3			2
LQC	34	102.2	5.3			8
MQC	67	99.9	2.0			8
HQC	366	100.9	1.9			8
(S)-methadone				0.9941 ±0.0021	0.0012 ±0.0002	2
LOQ	12.5	110.3	2.8			2
LQC	34	99.4	6.9			8
MQC	67	96.8	3.5			8
HQC	366	99.8	2.6			8

Intra-assay	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Extraction efficiency (%±SD)	n
(R)-methadone					
LQC	34	103.7	5.2	86±3	6
MQC	67	99.7	2.3	84±3	6
HQC	366	100.0	1.3	87±4	6
(S)-methadone					
LQC	34	102.3	4.7	84±4	6
MQC	67	98.4	2.2	83±2	6
HQC	366	98.6	1.5	86±4	6
3-methoxymorphinan (internal standard)				85±5	18

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

Extraction efficiency of (R)- and (S)-methadone was not statistically significantly different (P=0.17; -0.68%, -0.32 to 1.68%), indicating no stereoselectivity of the extraction of methadone from plasma ultra-filtrate samples. Unexpectedly, the extraction efficiency of methadone from plasma ultra-filtrate was markedly lower (range of means 83-87%, see Table 2-4) than that observed from plasma samples (approximately 100%, see Table 2-3), while that of the internal standard was essentially unchanged (approximately 85%). Reasons for this difference are not readily apparent, as the extraction efficiency of methadone from calibration standards was not affected by the use of isotonic phosphate buffer rather than plasma ultra-filtrate. Samples of ultra-filtrate prepared from drug-free plasma and isotonic phosphate buffer were spiked to contain 200 $ng.ml^{-1}$ of rac-methadone

and the extraction efficiency was not significantly different between the two matrices for both (R)-methadone (P=0.47; -3.7%, -7.2 to 14.7%) and (S)-methadone (P=0.46; -5.5%, -10.4 to 21.3%).

2.5.5.3. Urine samples

The extraction procedure was similar and chromatography conditions identical to those employed for the analysis of plasma samples, which resulted in almost identical chromatograms to that shown in Figure 2-3 for the analysis of plasma samples.

As this assay in urine samples comprised only minor modifications of the assay for (R)- and (S)-methadone in plasma, inter-assay validation data were only performed over four assay days, while intra-assay variability was assessed using ten replicate samples of each QC sample. Calibration curves for (R)- and (S)-methadone were linear over the 100-4000 $ng.ml^{-1}$ concentration range, with r^2 values greater than 0.99 for all analytical runs for both (R)- and (S)-methadone, and mean±SD values are presented in Table 2-5. Estimates of slope demonstrated no consistent time-related changes for either (R)- or (S)-methadone, and mean±SD values presented in Table 2-5. The inter- and intra-assay accuracy and precision of the method at the three QC concentrations, and the lowest calibration standard are shown in Table 2-5.

Based upon the assay validation results, the following assay acceptance criteria were formulated: at least seven calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of seven or more calibration standards and five or more QC samples must be less than ±15%.

Table 2-5: Inter- and intra-assay accuracy and precision for the quantification of (R)-methadone and (S)-methadone in human urine.

Inter-assay (n=4 assays)	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(R)-methadone				0.9959 ±0.0039	0.0012 ±0.0002	4
LOQ	50	101.3	1.2			4
LQC	112	102.6	6.6			14
MQC	403	98.1	5.5			14
HQC	1120	102.9	5.2			14
(S)-methadone				0.9970 ±0.0031	0.0014 ±0.0002	4
LOQ	50	103.0	5.8			4
LQC	112	101.8	6.3			14
MQC	403	98.2	6.0			14
HQC	1120	98.1	5.8			14

Intra-assay	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Extraction efficiency (%±SD)	N
(R)-methadone					
LQC	112	103.5	4.3	97±6	10
MQC	403	96.3	4.3	104±2	10
HQC	1120	103.5	5.5	105±2	10
(S)-methadone					
LQC	112	102.0	5.8	92±7	10
MQC	403	96.9	4.8	105±3	10
HQC	1120	96.1	4.8	108±3	10
3-methoxymorphinan (internal standard)				107±5	30

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

Extraction efficiency of (R)- and (S)-methadone was not statistically significantly different (P=0.78; -0.25%, -1.52 to 2.02%), indicating no stereoselectivity of the extraction of methadone from urine samples. Unexpectedly, in comparison to the result obtained for this assay for plasma, extraction efficiency of the internal standard from urine was markedly higher (107±5%) than observed from plasma samples (84±1%, see Table 2-3), while that of the methadone enantiomers was essentially unchanged (approximately 100%). Reasons for this difference are not readily apparent, although the two matrices are markedly different in composition.

2.5.5.4. Overall discussion

This assay did not quantitate the concentrations of EDDP either as the racemic compound, or as the individual enantiomers, in plasma, urine or plasma ultra-filtrate. The enantiomers of EDDP eluted as a broad peak that was not baseline resolved from the (R)-methadone peak. However, EDDP did not interfere with the analysis of (R)- and (S)-methadone in this assay. Therefore, this discussion will not compare the present method with those reported to quantitate the enantiomers of methadone and EDDP. Urinary concentrations of EDDP have been reported to be similar to that of methadone (see section 1.7.3), and therefore quantification of the EDDP enantiomers in urine is desirable. Subsequently, a method was developed to meet this need (see section 2.6), consequently a comparison of this new method to those alternatives available in the literature will be made in section 2.6.

Selectivity is an important component of assay validation. The issue of assay selectivity becomes increasingly complex when stereoselective analysis is required. Chiral phases often demonstrate poor separation of different chemical structures, as they are optimised for stereoselective recognition. Direct injection of aqueous solutions containing diazepam, caffeine, morphine, codeine, norcodeine and naloxone did not produce interfering peaks, while N-desmethyl dextropropoxyphene interfered with (R)-methadone, although this was visibly resolved. In contrast, dextropropoxyphene co-eluted with (R)-methadone, a phenomenon that has been previously described, and is likely to be due to the very similar structures of the two compounds, and can be monitored by dual-wavelength monitoring (Norris et al., 1994). The selectivity of this method has been further addressed in section 2.8. Many authors have not examined the selectivity of their assay in detail. To demonstrate adequate selectivity, these authors have rather monitored samples for the presence of commonly used drugs and reported no interference in the samples that have tested positive (de Vos et al., 1998; Eap et al., 1996; Kristensen et al., 1994) and/or compared the concentrations of the sum of the methadone enantiomer concentrations with those obtained using non-stereoselective assays, relying upon the marked difference in the selectivity of each method (Beck et al., 1991; Eap et al., 1996; Kintz et al., 1997; Kristensen

& Angelo, 1992; Kristensen et al., 1994; Schmidt et al., 1992). Relatively few authors have compared the retention time of the methadone enantiomers with that of other compounds, after direct injection and/or prior extraction, such as methadone metabolites (Rudaz & Veuthey, 1996), and opioid (Pham-Huy et al., 1997; Rudaz & Veuthey, 1996), tricyclic antidepressant (Norris et al., 1994; Pham-Huy et al., 1997; Rudaz & Veuthey, 1996), barbiturate (Pham-Huy et al., 1997), benzodiazepine (Pham-Huy et al., 1997; Rudaz & Veuthey, 1996), sympathomimetic (Pham-Huy et al., 1997), antiepileptic (Pham-Huy et al., 1997) and salicylic acid (Pham-Huy et al., 1997) related compounds.

This assay for the quantification of (R)- and (S)-methadone in plasma (and plasma ultra-filtrate) and urine compares favourably to the non-stereoselective assays published in the literature (see Appendix 2 and section 2.1.2). In terms of sample preparation, the majority of previously reported methods have employed solvent-solvent extraction, after prior alkalinisation of samples to pH >9 (Beck et al., 1990; de Vos et al., 1998; Eap et al., 1996; Kristensen & Angelo, 1992; Kristensen et al., 1994; Norris et al., 1994; Pham-Huy et al., 1997; Schmidt et al., 1992), while a few have utilised solid-phase extraction (Kintz et al., 1997; Rudaz & Veuthey, 1996). However, the only method employing GC analysis required an additional stereoselective derivatisation reaction in order to effect the enantiomer resolution (Kristensen & Angelo, 1992). Reported extraction efficiencies for methadone have generally ranged from 80-100%, and are quantitatively similar to those obtained with the present method (see Table 2-3, Table 2-4, Table 2-5), as would be expected from the similarity of extraction techniques employed. Although the calibration curve concentration range was 15-600 $ng.ml^{-1}$ from a 1.0 ml plasma sample, the lower limit of quantification was improved 12.5 $ng.ml^{-1}$ from a 0.35 ml sample volume in plasma ultra-filtrate, and the upper limit extended to 2000 $ng.ml^{-1}$ from a 1.0 ml urine sample. Although not attempted, the lower limit of quantification from a 1.0 ml sample volume could therefore realistically be in the order of 5 $ng.ml^{-1}$. These data indicate the present assay to offer comparable limit of quantification to those available from other methods (see Appendix 2).

In terms of precision and accuracy, the present assay compares very favourably with those developed by other workers. Most authors have provided inter-assay (Eap et al., 1996; Kristensen & Angelo, 1992; Norris et al., 1994; Rudaz & Veuthey, 1996; Schmidt et al., 1992) or intra-assay (Beck et al., 1991; Kristensen et al., 1994) accuracy data. In contrast, few authors have not reported on the accuracy of their methods (de Vos et al., 1998; Pham-Huy et al., 1997). Despite the limitations of the data in some cases, it is likely that all methods were sufficiently accurate for the intended application, as the majority of reports which have provided accuracy data have reported values $\leq \pm 10\%$ at the lowest concentration examined (see Appendix 2).

Some authors have reported intra-assay precision data only (Beck et al., 1991; Kristensen et al., 1994), while most have provided inter-assay data (Eap et al., 1996; Kristensen & Angelo, 1992; Norris et al., 1994; Pham-Huy et al., 1997; Rudaz & Veuthey, 1996; Schmidt et al., 1992). Irrespective of whether the values were calculated from intra- or inter-assay data, reported values were generally <10% at the lowest concentration examined (see Appendix 2), indicating good reproducibility of all methods.

In comparison to the above mentioned assays, the present assay demonstrated excellent inaccuracy (%bias, range: -4% to +12%) and precision (range: 1% to 9%) for the three QC samples and at the limit of quantification, which was maintained for both intra- and inter-assay analysis for both (R)- and (S)-methadone in all three biofluids.

In summary, this assay provides a simple, accurate, precise and selective method for the quantification of the individual methadone enantiomers in plasma, plasma ultra-filtrate and urine necessary for detailed investigation of the disposition of methadone with respect to stereoselectivity.

2.6. Simultaneous quantification of (R)- and (S)-methadone and (R)- and (S)-EDDP in human urine

At the time the present work was conducted, there were no methods for the quantification of the individual enantiomers of EDDP available in the published literature. However, three groups have recently used capillary electrophoresis (CE) for the simultaneous quantification of the methadone and EDDP enantiomers in serum (Frost et al., 1997), urine (Frost et al., 1997; Lanz & Thormann, 1996; Ramseier et al., 1999) and hair (Frost et al., 1997). These methods used a non-chiral silica stationary phase in combination with a cyclodextrin chiral selector present in the mobile phase. Although CE methods do provide a relatively simple and inexpensive alternative to HPLC, many laboratories, including my own, do not yet use this technique routinely. In contrast, the use of a chiral column in a conventional HPLC system is more accessible to many laboratories. Kintz and co-workers (1997) quantified the enantiomers of methadone and EDDP in hair using a liquid chromatography-mass spectrometry (LC-MS) technique. Although LC-MS assays are sensitive, they are expensive and complex. Using stereoselective HPLC, Pham-Huy and co-workers (1997) were able to simultaneously quantify methadone and EDDP. However, this method only resolved the methadone enantiomers but not those of EDDP. During the publication of the present method, Angelo and co-workers (1999) reported a method for the simultaneous quantification of the enantiomers of methadone and EDDP in urine using stereoselective HPLC. This method used a non-chiral analytical column coupled in series with a chiral analytical column to achieve resolution.

2.6.1. HPLC instrumentation and chromatography conditions

The HPLC system comprised a LC-10AT pump (Shimadzu, Kyoto, Japan), a Sil-10A autoinjector (Shimadzu) and a SPD-M10A photo-diode array detector (Shimadzu) set at 210 nm. The system was controlled using Class-LC10 software (version 1, Shimadzu) running under Windows 3.11 (Microsoft Corporation, WA, USA) on a 486 DX IBM compatible computer. The analytical column was a Chiral AGP column (100x4.0 mm, 5 μm; Chromtech AB, Hägersten, Sweden) protected by a 2 μm in-line filter (Scientific

Instruments, State College, PA, USA) and a Chiral AGP pre-column (10x3.0 mm, 5 μm; Chromtech AB). Optimal separation of the compounds of interest was achieved with a mobile phase of 20 mM NaH_2PO_4 in water containing 2 mM dimethyloctylamine and 9% acetonitrile final pH adjusted to 5.5 with ortho-phosphoric acid and pumped through the system at 0.4 $ml.min^{-1}$ at room temperature.

2.6.2. Sample preparation

Urine samples (0.5 ml) and internal standard (50 μl 10 μM dextromoramide in water) were aliquoted into 10 ml screw capped borosilicate glass tubes, alkalinised (0.4 ml 0.1 M Na_2CO_3 pH 10) and extracted with 5 ml of 100 % hexane for 20 min on a rotary mixer. Samples were then centrifuged (2000*g*, 10 min) and the organic phase transferred to a clean 5 ml borosilicate glass tube and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted in 0.5 ml mobile phase and separate 100 μl aliquots injected onto the chromatography systems for analysis of (R)- and (S)-methadone and (R)- and (S)-EDDP, and rac-methadone and rac-EDDP (see section 2.7). The extraction procedure used 100% hexane as the extraction solvent as the use of 30:70 (v/v) diethyl ether:hexane produced interfering peaks in the chromatography.

2.6.3. Calibration, precision, accuracy and extraction efficiency

Retention times of the compounds of interest were confirmed by direct injection of aqueous solutions of pure compounds, and of the individual enantiomers of methadone and EDDP by direct injection of aqueous solutions of enantiomerically pure compounds. Quantification of (R)- and (S)-methadone and (R)- and (S)-EDDP was performed with calibration curves consisting of seven standards over the concentration range 0.125-12.5 μM of (R)- and (S)-methadone and (R)- and (S)-EDDP. Low (L), medium (M) and high (H) QC samples were prepared in duplicate, with final concentrations of 0.4 μM, 1.0 μM and 6.3 μM for (R)-EDDP and (S)-EDDP, and 0.4 μM, 1.0 μM 8 μM for (R)-methadone and (S)-methadone. Calibration standards and QC samples were prepared by diluting 50 μl of an aqueous stock solution containing rac-methadone and rac-EDDP with 450 μl drug-free urine, and analysed

identically to the patient samples (section 2.6.2). Patient samples which contained analytes at concentrations above the limit of quantification were diluted with drug-free matrix and re-assayed.

The robustness of the analytical method was assessed by assaying replicates of each QC sample on a single day to determine the intra-assay accuracy and precision. Inter-assay performance was determined using inter-assay accuracy and precision determined by the analysis of duplicates of each QC sample, and the lowest calibration standard, on different assay days. Similarly, the on-going performance of the assay was monitored using inter-assay accuracy and precision determined by analysis of duplicates of each QC sample, and the lowest calibration standard, on each different assay day and is reported in Chapter 5. Extraction efficiency was analysed at each QC concentration and for the internal standard using the intra-assay validation samples. The peak areas of all compounds after injection of the extracted samples were compared to those obtained after direct injection of the aqueous stock solution.

2.6.4. Data analysis

Raw data were entered into Excel spreadsheets (Version 4.0, Microsoft Corporation, WA, USA). Peak areas were converted into peak area ratios using the peak area of the internal standard. Linear regression analysis (GraphPad Prism v2.01, GraphPad Software, CA, USA) of $1/y^2$ weighted peak area ratio against nominal concentration provided an estimate of slope, intercept and coefficient of determination (r^2). The estimated concentration divided by the nominal concentration multiplied by 100% was calculated for each individual sample, and accuracy was calculated as the mean of these values, and the residual standard deviation of the mean was taken as the precision. Comparison of extraction efficiency values for (R)- and (S)-methadone, and (R)- and (S)-EDDP was performed using a paired t-test (GraphPad Prism v2.01, GraphPad Software).

2.6.5. Results and discussion

The chromatograms obtained from patient samples contained five peaks with identical retention times to (R)-EDDP, (S)-EDDP, (R)-methadone, (S)-methadone and the internal standard (dextromoramide). Retention times for (R)-EDDP, (S)-EDDP, (R)-methadone, (S)-methadone and the internal standard (dextromoramide) were 10, 12, 13, 16 and 27 min, respectively, with a total run-time of 35 min. Under these conditions all compounds of interest were adequately resolved and no decrease in resolution was observed after over 200 injections. The present assay did not quantify (4R,6S)-p-hydroxy methadone, (4S,6S)-p-hydroxy methadone, EMDP, α-(3S,6S)-N-desmethyl methadol, the α-methadol enantiomers, β-(3R,6S)-methadol or pyrrolidone. None of these compounds interfered with the chromatography of (R)-EDDP, (S)-EDDP, (R)-methadone, (S)-methadone or the internal standard (dextromoramide). No further modifications to the HPLC system were attempted. There were no interfering peaks in the chromatography in several blank urine samples, and in the patient urine samples. Direct injection of morphine and diazepam solutions (drugs which are commonly used by former heroin addicts, especially morphine as it is also a metabolite of heroin) did not produce interfering peaks.

A representative chromatogram from the analysis of a drug-free urine sample and an inter-dosing interval 0-24 hour pooled urine sample assayed for (R)-EDDP, (S)-EDDP, (R)-methadone and (S)-methadone obtained from a methadone maintenance patient after administration of the 130 mg daily dose of rac-methadone are shown in Figure 2-4.

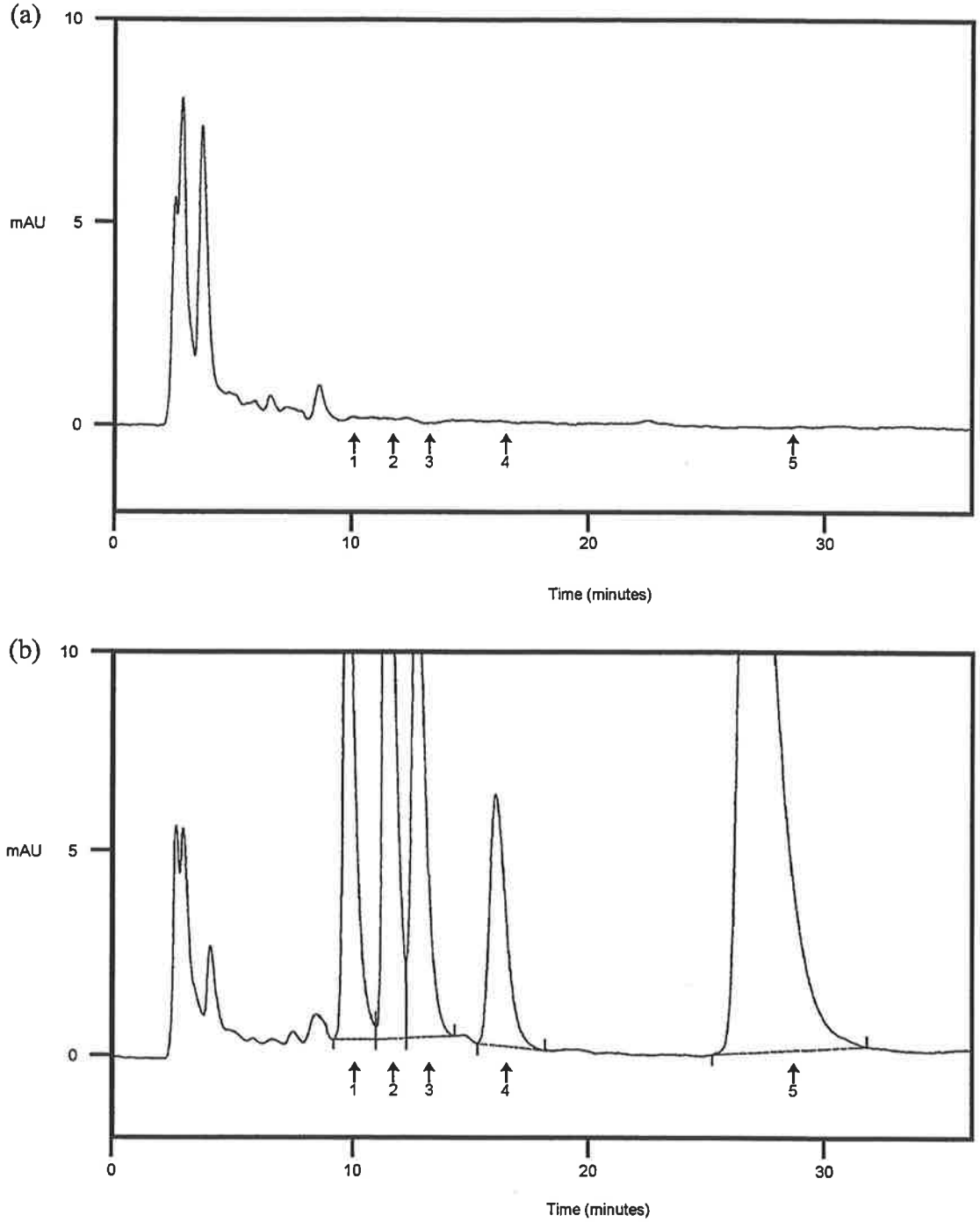


Figure 2-4: Representative chromatogram for the analysis of (a) drug-free urine sample and (b) a urine sample obtained from a methadone maintenance patient assayed for (R)-EDDP, (S)-EDDP, (R)-methadone and (S)-methadone.

Notes: Patient sample contained 1.3 μM, 1.8 μM, 1.1 μM and 0.7 μM (R)-EDDP, (S)-EDDP, (R)-methadone and (S)-methadone and was obtained as a pooled 0-24 hour inter-dosing interval sample after the administration of the patients 130 mg daily rac-methadone dose, and was diluted 1:19 with drug free urine. 1=(R)-EDDP, 2=(S)-EDDP, 3=(R)-methadone, 4=(S)-methadone and 5=dextromoramide.

Calibration curves for (R)-EDDP, (S)-EDDP, (R)-methadone and (S)-methadone were linear over the 0.125-12.5 μM concentration range, with r^2 values greater than 0.99 for all analytes in all analytical runs, and mean±SD values (R)- and (S)-EDDP, and (R)- and (S)-methadone are presented in Table 2-6 and Table 2-7, respectively. Estimates of slope demonstrated no consistent time-related changes for either (R)- or (S)-EDDP or (R)- or (S)-methadone, and mean±SD values for (R)- and (S)-EDDP, and (R)- and (S)-methadone are presented in Table 2-6 and Table 2-7, respectively. The inter- and intra-assay accuracy and precision of the method at the three QC concentrations, and the lowest calibration standard for (R)- and (S)-EDDP, and (R)- and (S)-methadone are shown in Table 2-6 and Table 2-7, respectively.

Based upon the assay validation results, the following assay acceptance criteria were formulated: at least six calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of six or more calibration standards and five or more QC samples must be less than ±15%.

Table 2-6: Inter- and intra-assay accuracy and precision for the quantification of (R)-EDDP and (S)-EDDP in human urine.

Inter-assay (n=3 assays)	Nominal concentration (μM)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(R)-EDDP				0.9942 ±0.0035	0.2699 ±0.0550	3
LOQ	0.125	95.3	3.2			3
LQC	0.4	102.4	7.3			10
MQC	1.0	101.6	7.9			10
HQC	6.3	97.6	5.7			9
(S)-EDDP				0.9940 ±0.0039	0.2582 ±0.0555	3
LOQ	0.125	100.0	2.6			3
LQC	0.4	104.0	9.7			10
MQC	1.0	100.4	8.2			10
HQC	6.3	97.8	7.1			9

Intra-assay	Nominal concentration (μM)	Accuracy (%)	Precision (%)	Extraction efficiency (%±SD)	N
(R)-EDDP					
LQC	0.4	104.5	6.8	71±9	6
MQC	1.0	101.9	9.0	73±9	6
HQC	6.3	95.3	10.5	72±6	6
(S)-EDDP					
LQC	0.4	105.1	4.6	73±6	6
MQC	1.0	102.3	8.9	74±9	6
HQC	6.3	96.5	10.3	73±7	6
dextromoramide (internal standard)				80±3	18

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

Table 2-7: Inter- and intra-assay accuracy and precision for the quantification of (R)-methadone and (S)-methadone in human urine.

Inter-assay (n=3 assays)	Nominal concentration (μM)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(R)-methadone				0.9978 ±0.0023	0.3243 ±0.0636	3
LOQ	0.125	103.8	0.6			3
LQC	0.4	96.9	6.4			10
MQC	1.0	97.4	5.3			10
HQC	8	97.3	3.9			9
(S)-methadone				0.9984 ±0.0019	0.3182 ±0.0629	3
LOQ	0.125	103.3	1.6			3
LQC	0.4	99.7	4.3			10
MQC	1.0	98.1	4.2			10
HQC	8	96.4	3.6			9

Intra-assay	Nominal concentration (μM)	Accuracy (%)	Precision (%)	Extraction efficiency (%±SD)	n
(R)-methadone					
LQC	0.4	97.8	1.7	85±3	6
MQC	1.0	100.6	3.6	93±4	6
HQC	8	94.8	2.6	89±4	6
(S)-methadone					
LQC	0.4	97.5	2.0	88±4	6
MQC	1.0	100.7	2.2	96±3	6
HQC	8	94.0	2.1	90±3	6
dextromoramide (internal standard)				80±3	18

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

The extraction efficiency of (R)- and (S)-EDDP, and (R)- and (S)-methadone was high, reproducible (CV <5%), and did not demonstrate concentration-dependency with mean±SD values determined from LQC, MQC and HQC intra-assay validation samples for EDDP and methadone shown in Table 2-6 and Table 2-7, respectively. Extraction efficiency was not statistically significantly different between the enantiomers of EDDP (P=0.50; -1.29%, -2.60 to 5.18%) or methadone (P=0.20; 2.21%, -1.08 to 5.49%), indicating no stereoselectivity of the extraction of methadone from plasma samples. Extraction efficiency of the internal standard was also very reproducible (CV <5%) and similar to that obtained for the enantiomers of EDDP and methadone (Table 2-6 and Table 2-7). EDDP showed a somewhat lower recovery than methadone and the internal standard. This may be due to its

substantially higher pKa (10.4 versus 8.6, respectively) (Baselt & Bickel, 1973) when compared to methadone. However, the calibration curves were linear, inter- and intra-assay validation data were acceptable, and the LOQ of the assay was well below the lowest concentration observed in the patient samples (see Chapter 5).

This assay for the simultaneous quantification of the enantiomers of methadone and EDDP compares favourably with the CE techniques of Lanz & Thormann (1996), Frost and co-workers (1997), and that of Ramseier and co-workers (1999) adaptation of the Lanz & Thormann method (1996). Solvent-solvent (Frost et al., 1997; Ramseier et al., 1999) or solid-phase extractions (Lanz & Thormann, 1996) of urine samples at pH >9, with subsequent evaporation of the organic solvent to dryness, and reconstitution of the residue, were employed in the CE assays. Lanz & Thormann (1996) attempted direct injection of urine samples, but this was unsuccessful as less than half of the samples tested produced acceptable electropherograms. My assay employed a solvent-solvent extraction of alkalinised samples. In comparison to the present assay, the published CE methods (Frost et al., 1997; Lanz & Thormann, 1996; Ramseier et al., 1999) offer no advantages in terms of sample preparation.

The assay range employed by Frost and co-workers (1997) for both methadone and EDDP (10-2500 $ng.ml^{-1}$ each enantiomer) from a 1 ml urine sample, was similar to the present method (methadone: 39-3900 $ng.ml^{-1}$ each enantiomer; EDDP: 35-3500 $ng.ml^{-1}$ each enantiomer) whereas Lanz & Thormann (Lanz & Thormann, 1996) employed a much higher concentration range (methadone: 1.5-26.7 $\mu g.ml^{-1}$ each enantiomer; EDDP: 1.1-21.1 $\mu g.ml^{-1}$ each enantiomer). Limits of detection for each enantiomer were reported to be 10 $ng.ml^{-1}$ (Frost et al., 1997) and approximately 100 $ng.ml^{-1}$ (Lanz & Thormann, 1996) for both methadone and EDDP from a 1 ml urine sample. Ramseier and co-workers (1999) did not report a calibration range, and the limit of detection of the assay was ill defined. The calibration curve concentration range employed in the present assay spanned the range of concentrations measured in the majority of patient samples, and is comparable to that

reported in the CE methods. No samples contained concentrations of methadone and/or EDDP below the limit of quantification.

Lanz and co-workers (1996) reported intra-assay precision values to be <10%, this was only assessed at 1000 $ng.ml^{-1}$ by Ramseier and co-workers (1999) who reported values of <6% for methadone and <2% for EDDP. Frost and co-workers (1997) reported intra-assay precision at 50, 500 and 2500 $ng.ml^{-1}$ urine for each enantiomer to be <12% at the higher concentrations, but increased up to 17% at 50 $ng.ml^{-1}$. Mean concentrations were within 5% of the nominal concentration at the higher concentrations, but increased to 12-24% at 50 $ng.ml^{-1}$. Accuracy data were not reported for the other two assay methods (Lanz & Thormann, 1996; Ramseier et al., 1999), while inter-day precision or accuracy data were not presented in any of the three papers (Frost et al., 1997; Lanz & Thormann, 1996; Ramseier et al., 1999). The present assay demonstrated excellent inaccuracy (<6%) and good precision (<13% precision) even at the limit of quantification for all four analytes. This was maintained for both intra- and inter-assay analysis. In comparison to the published CE methods, the present assay offers better precision and accuracy over a similar concentration range.

It is difficult to compare the cost of the present HPLC method with that of the CE methods. Similar sample preparation is required for both types of methods and analysis times were similar. However, it is likely that the initial set up of the present validated assay would be more readily achieved, as many laboratories already have a conventional HLPC system and would only require a specialised chromatography column. In contrast, the ongoing costs of the CE methods are likely to be much less expensive compared the present chiral HPLC method, as the columns employed are cheaper than chiral HPLC columns, more easily maintained, and consume much less running/rinsing solutions compared to HPLC mobile-phase consumption.

During the publication of the present method, Angelo and co-workers (1999) reported a method for the simultaneous quantification of the enantiomers of methadone and EDDP in urine using stereoselective HPLC (Angelo et al., 1999). Rudaz & Veuthey (Rudaz & Veuthey, 1999) also reported their detailed investigations with methadone using chiral HPLC. While they noted that EDDP did not interfere with methadone using the Chiral AGP column, they did not attempt to quantitate EDDP. This group also noted that the separation of the EDDP enantiomers was unsuccessful using a variety of alternative commercially available chiral HPLC columns. Similarly, Pham-Huy and co-workers (1997) were able to simultaneously quantify (R)- and (S)-methadone and rac-EDDP. However, this method only resolved the methadone enantiomers but not those of EDDP using a cyclodextrin derivatised HPLC column (Cyclobond I 2000 RSP), despite considerable investigations.

The sample preparation employed by Angelo and co-workers (1999) was very similar to that used here, as both involved a simple solvent-solvent extraction with hexane of alkalinised samples. Consequently, similar extraction efficiencies were obtained, in the order of 90% for methadone and 70-80% for EDDP. However, their method was optimised for quantitating low concentrations of the analytes (methadone: 9-770 $ng.ml^{-1}$, 0.03-2.5 μM each enantiomer; EDDP: 8-690 $ng.ml^{-1}$, 0.03-2.5 μM each enantiomer) from a larger sample volume (3 ml), compared to the present assay. They reported that these concentration ranges were increased (methadone: 60-1540 $ng.ml^{-1}$, 0.2-5.0 μM each enantiomer; EDDP: 140-3450 $ng.ml^{-1}$, 0.5-12.5 μM each enantiomer), and the sample volume decreased to 1 ml, in order to prevent the re-analysis after dilution of samples obtained from methadone maintenance patients, which contained concentrations of methadone and/or EDDP above the upper limit. In terms of the precision and accuracy data presented by this group, only intra-assay data presented . At all concentrations examined, the accuracy (%bias, range: -13% to +13%) for the methadone and EDDP enantiomers was acceptable, as was the precision (range: 2-9%) for methadone. The precision for both EDDP enantiomers was good at the intermediate concentrations (range: 2-9%), however, this increased to 16-20% at

the lower limit of quantification for both enantiomers, and to 20% for (R)-EDDP at the highest concentration reported (2500 $ng.ml^{-1}$, 9.0 μM), while this was 6% for the (S)-EDDP at the same concentration. In comparison, the present assay offers somewhat improved assay validation data, that were maintained for both intra- and inter-assay analyses.

Angelo and co-workers' (1999) method used a non-chiral C_8 (30x2 mm) analytical column coupled in series with the same chiral analytical column (Chiral AGP) used in the present assay. They reported that the use of the non-chiral C_8 column provided better selectivity than the non-chiral analytical cyano column (10x3 mm) previously reported by them (Kristensen et al., 1994). In both of these assays, they also used a non-chiral pre-column, but did not use a Chiral AGP pre-column (Angelo et al., 1999; Kristensen et al., 1994). In my hands, a short cyano analytical column (50x4.6 mm) retained methadone and EDDP for a long time, resulting in unacceptable peak broadening using mobile phases containing low concentrations (<15%) of acetonitrile, even when the Chiral AGP column manufacturer's (Chromtech) highest mobile phase flow-rate (0.9 $ml.min^{-1}$) was used (results not shown). I found that using an appropriate mobile phase in combination with a low mobile phase flow-rate, it was possible to resolve the enantiomers of both methadone and EDDP simultaneously using a Chiral AGP column in conjunction with a Chiral AGP pre-column, without the need for a non-chiral analytical column or a non-chiral pre-column connected in series with the Chiral AGP column. The use of a lower mobile phase flow rate (0.4 $ml.min^{-1}$) compared to that used by Angelo and co-workers (1999) (0.9 $ml.min^{-1}$), while still obtaining very similar run-times (Angelo et al., 1999), results in considerably less mobile phase usage. The use of a non-chiral analytical column was also reported to extend the working life of the Chiral AGP column (Kristensen et al., 1994). I noticed no decrease in resolution of the Chiral AGP column after more than 200 injections. However, the present assay utilised a Chiral AGP pre-column to protect the analytical column, which was not attempted by Kristensen and co-workers (1994).

Separation of the methadone and EDDP enantiomers was demonstrated to be adequate in all three CE assays (Frost et al., 1997; Lanz & Thormann, 1996; Ramseier et al., 1999) and the HPLC assay of Angelo and co-workers (1999). Separation of the second EDDP peak and first methadone peak was less successful for Lanz and co-workers (1996) and Ramseier and co-workers (1999), although Lanz and co-workers (1996) were able to achieve baseline separation of all compounds by increasing capillary length to 100 cm with resultant long run times (>40 min). In contrast, Frost and co-workers (1997) obtained excellent separation of the methadone and EDDP enantiomers while maintaining complete baseline separation of the second EDDP peak and first methadone peak. In comparison, the present assay demonstrated adequate resolution of all compounds of interest but with run times of 35 minutes, as did Angelo and co-workers (1999). Ramseier and co-workers (1999) demonstrated excellent selectivity of their assay for the methadone and EDDP enantiomers, as no interference was seen with drugs of abuse and their metabolites (13 compounds), as did Frost and co-workers (1997) (18 compounds). Although not as thoroughly investigated, the present assay demonstrated adequate selectivity, which has been further addressed in section 2.8. Lanz & Thormann did not address this issue (Lanz & Thormann, 1996). Angelo and co-workers (1999) examined the selectivity of their assay by the comparison of the sum of the individual enantiomers of methadone and EDDP obtained by their stereoselective assay with the values obtained after analysis using a non-chiral GC assay. The samples were monitored for the presence of commonly used drugs and reported no interference in the samples that have tested positive for morphine and benzodiazepines, however, samples positive for ketobemidone produced interfering peaks in their chromatograms.

In summary, this assay provides a simple, accurate and precise assay method to investigate the role of stereoselectivity in the pharmacokinetics and metabolism of methadone. This assay offers lower mobile phase consumption than the alternative HPLC method and avoids the complication of using both a non-chiral analytical column and pre-column connected in

series with the chiral analytical column without sacrificing accuracy, precision or robustness.

2.7. Quantification of rac-methadone and rac-EDDP in human urine

Although there are many assays reported in the literature for the non-stereoselective quantification of methadone and EDDP (see section 2.1.1 and Appendix 1), the present assay was developed in order to provide a further cross-validation of the assays for the stereoselective quantification of methadone and EDDP. The chromatography conditions were previously established for the separation of methadone and EDDP, and shown to be selective for these two analytes, as there was no interference from a variety of opioid, anxiolytic and anti-depressant compounds (see section 2.3). Similarly, the sample extraction procedure for methadone and EDDP in urine was also previously developed, and shown to be reproducible (see section 2.6).

2.7.1. HPLC instrumentation and chromatography conditions

The HPLC system comprised a LC-6A pump (Shimadzu, Kyoto, Japan), a Sil-10A autoinjector (Shimadzu) and a UVIDEC-100-V spectrophotometer (Jasco, Tokyo, Japan) set at 210 nm (0.005 aufs) and a DP800 Data Station (ICI Instruments, Melbourne, Australia) running under Windows 3.11 (Microsoft Corporation, WA, USA) on a 286 SX IBM compatible computer. The analytical column was a 100x5 mm Nova-Pak C_{18} 4 μm cartridge (Waters, Milford, MA, USA) contained in an 8x10 Radial Compression Module (Waters) protected by a 2 μm in-line filter (Scientific Instruments, State College, PA, USA) and an Alltima 10x4.6 mm 5 μm C_{18} pre-column (Alltech, Deerfield, IL, USA). Optimal separation of the compounds of interest was achieved with a mobile phase of 30% (v/v) acetonitrile and 0.2% (v/v) triethylamine in 50 mM NaH_2PO_4 with final pH adjusted to 4.3 with ortho-phosphoric acid and pumped through the system at 1.0 $ml.min^{-1}$ at room temperature.

2.7.2. Sample preparation

The sample preparation was identical to that outlined in section 2.6.2. Separate 100 µl aliquots injected onto the chromatography systems for analysis of (R)- and (S)-methadone and (R)- and (S)-EDDP (see section 2.6), and rac-methadone and rac-EDDP.

2.7.3. Calibration, precision, accuracy and extraction efficiency

Retention times of the compounds of interest were confirmed by direct injection of aqueous solutions of pure compounds. Quantification of rac-EDDP and rac-methadone was performed with calibration curves consisting of seven standards over the concentration range 0.25-25 µM of rac-EDDP and rac-methadone. Low (L), medium (M) and high (H) QC samples were prepared in duplicate, with final concentrations of 0.8 µM, 2.0 µM and 12.6 µM for rac-EDDP, and 0.8 µM, 2.0 µM 16 µM for rac-methadone. Calibration standards and QC samples were prepared by diluting 50 µl of an aqueous stock solution containing rac-methadone and rac-EDDP with 450 µl drug-free urine, and analysed identically to the patient samples (section 2.7.2). Patient samples which contained analytes at concentrations above the limit of quantification were diluted with drug-free matrix and re-assayed.

The robustness of the analytical method was assessed by assaying replicates of each QC sample on a single day to determine the intra-assay accuracy and precision. Inter-assay performance was determined using inter-assay accuracy and precision determined by the analysis of duplicates of each QC sample, and the lowest calibration standard, on different assay days. Similarly, the on-going performance of the assay was monitored using inter-assay accuracy and precision determined by analysis of duplicates of each QC sample, and the lowest calibration standard, on each different assay day and is reported in Chapter 4. Extraction efficiency was analysed at each QC concentration and for the internal standard using the intra-assay validation samples. The peak areas of all compounds after injection of the extracted samples were compared to those obtained after direct injection of the aqueous stock solution.

2.7.4. Data analysis

Raw data were entered into Excel spreadsheets (Version 4.0, Microsoft Corporation, WA, USA). Peak areas were converted into peak area ratios using the peak area of the internal standard. Linear regression analysis of $1/y^2$ weighted peak area ratio against nominal concentration provided an estimate of slope, intercept and coefficient of determination (r^2). The estimated concentration divided by the nominal concentration multiplied by 100% was calculated for each individual sample, and accuracy was calculated as the mean of these values, and the residual standard deviation of the mean was taken as the precision.

2.7.5. Results and discussion

The chromatograms obtained from patient samples contained 3 peaks with identical retention times to rac-EDDP, rac-methadone and the internal standard (dextromoramide). Retention times for rac-EDDP, rac-methadone and dextromoramide were 8.4, 10.9 and 13.3 min, respectively, with a total run-time of 20 min. Under these conditions all compounds of interest were adequately resolved and no decrease in resolution was observed after over 200 injections. The present assay did not quantify (4R,6S)-para hydroxy methadone, (4S,6S)-para hydroxy methadone, EMDP, α-N-desmethyl methadol, α-methadol, β-methadol or pyrrolidone. Under the chromatography conditions described, the retention times of (4R,6S)-p-hydroxy methadone, (4S,6S)-p-hydroxy methadone, α-(3S,6S)-N-desmethyl methadol, α-methadol enantiomers, β-(3R,6S)-methadol, EMDP and pyrrolidone were 4.2, 4.6, 6.4, 8.0, 11.5, 25 and 55 minutes, respectively. All compounds were baseline resolved, with the exception of the p-hydroxy methadone diastereomers, the α-methadol enantiomers and EDDP, and β-methadol and dextromoramide. However, these compounds were visibly separated. Increasing mobile phase pH over the range of 4.3-6.0 increased the retention of all compounds without affecting resolution. No further modifications to the HPLC system were attempted. There were no interfering peaks in the chromatography in several blank urine samples, and in the patient urine samples. Direct injection of chlorzoxazone, coumarin, diethyldithiocarbamic acid, flunitrazepam, fluoxetine, furafylline, ketoconazole, omeprazole, quinidine, (S)-

mephenytoin, sulphaphenazole, troleandomycin, codeine, norcodeine, morphine, ethylmorphine, hydromorphone, dihydromorphone, naloxone, oxycodone, 6β-oxycodol, diazepam, temazepam, ketamine, clomipramine, sertraline, citalopram, doxepin, ketorolac, venlafaxine, nefazodone produced chromatographic peaks that were adequately resolved from methadone, EDDP and dextromoramide. Aqueous solutions containing amitriptyline, nortriptyline and imipramine produced chromatographic peaks that were not well resolved from dextromoramide, and paroxetine produced a chromatographic peak that was not well resolved from EDDP. However, the peaks were visibly separated. In contrast, aqueous solutions containing desipramine and fluvoxamine produced peaks which were poorly resolved from EDDP, and would have prevented accurate quantification.

A representative chromatogram from the analysis of a drug-free urine sample and an inter-dosing interval 0-24 hour pooled urine sample assayed for rac-EDDP and rac-methadone obtained from a methadone maintenance patient after administration of the 130 mg daily dose of rac-methadone are shown in Figure 2-5.

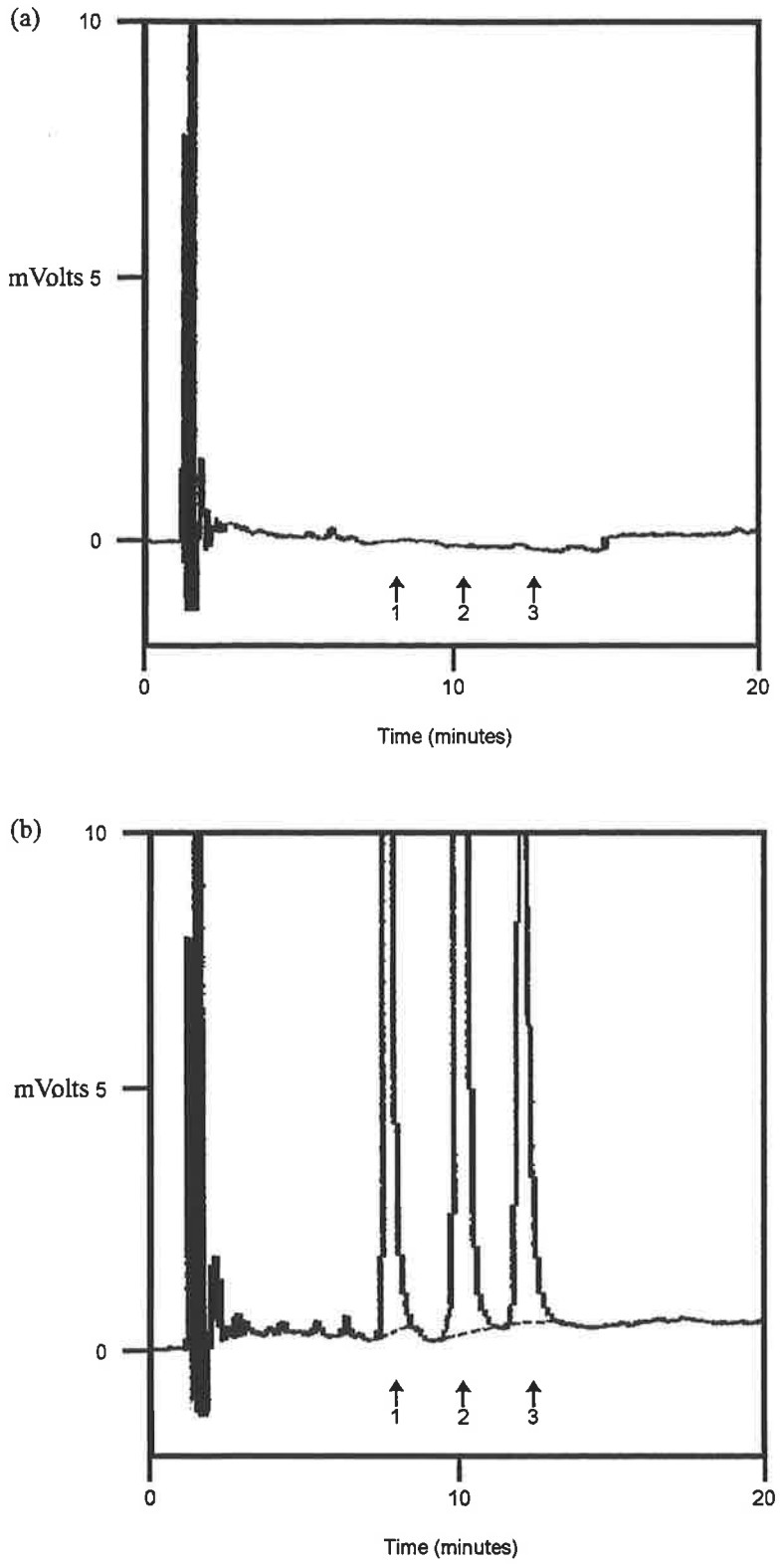


Figure 2-5: Representative chromatogram for the analysis of (a) drug-free urine sample and (b) a urine sample obtained from a methadone maintenance patient assayed for rac-EDDP and rac-methadone.

Notes: Sample contained 3.0 μM and 1.8 μM rac-EDDP and rac-methadone and was obtained as a pooled 0-24 hour inter-dosing interval sample after the administration of the patients 130 mg daily rac-methadone dose, and was diluted 1:19 with drug free urine. 1=rac-EDDP, 2=rac-methadone and 3=dextromoramide.

Calibration curves for rac-EDDP and rac-methadone were linear over the 0.25-25 μM concentration range, with r^2 values greater than 0.99 for both analytes in all analytical runs, and mean±SD values are presented in Table 2-8. Estimates of slope demonstrated no consistent time-related changes for either analyte, and mean±SD values are presented in Table 2-8. The inter- and intra-assay accuracy and precision of the method at the three QC concentrations, and the lowest calibration standard for rac-EDDP and rac-methadone are shown in Table 2-8.

Based upon the assay validation results, the following assay acceptance criteria were formulated: at least six calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of six or more calibration standards and five or more QC samples must be less than ±15%.

Table 2-8: Inter- and intra-assay accuracy and precision for the quantification of rac-EDDP and rac-methadone in human urine.

Inter-assay (n=3 assays)	Nominal concentration (μM)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
Rac-EDDP				0.9959 ±0.0040	0.2980 ±0.0535	3
LOQ	0.25	96.6	1.4			3
LQC	0.8	100.4	7.9			10
MQC	2.0	102.3	7.5			10
HQC	12.6	97.6	6.7			9
Rac-methadone				0.9981 ±0.0026	0.3310 ±0.0759	3
LOQ	0.25	100.1	0.9			3
LQC	0.8	100.7	3.5			10
MQC	2.0	100.9	7.4			10
HQC	16.0	96.8	2.5			10
Intra-assay	Nominal concentration (μM)	Accuracy (%)	Precision (%)	Extraction efficiency (%±SD)		n
Rac-EDDP						
LQC	0.8	103.0	4.8	76±6		6
MQC	2.0	105.3	8.3	81±9		6
HQC	12.6	96.8	11.3	79±6		6
Rac-methadone						
LQC	0.8	101.0	3.2	97±3		6
MQC	2.0	105.9	3.0	99±4		6
HQC	16.0	96.1	2.9	103±2		6
dextromoramide (internal standard)				85±2		18

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

This assay utilised the same chromatography system as that described earlier (see section 2.3), and the selectivity of this method will be further addressed in section 2.8. Briefly, the chromatography conditions has been shown to be very selective for methadone and EDDP, as they are identical to those described for the quantification of EDDP in microsomal incubations. However, these chromatography conditions were subjected to a larger number of potentially interfering compounds compared to the plasma assay, and incorporated an extraction procedure which was not incorporated in the assay of EDDP in microsomal incubations. These factors both indicate this assay to be more selective than either of the previously mentioned assays.

The present assay demonstrated excellent inaccuracy (%bias, range: -4% to +6%) and precision (range: 1% to 11%) for the three QC samples and at the limit of quantification, which was maintained for both intra- and inter-assay analysis for both rac-methadone and rac-EDDP.

In comparison to other available methods in the literature, similar comments to those made earlier in section 2.4.5 in discussion of the assay of rac-methadone in plasma are possible regarding selectivity, accuracy and precision (as many of the methods used for the analysis of plasma were also used for urine samples), and will not be repeated here. In summary, this assay provides a simple, accurate, precise and selective method to investigate the disposition of rac-methadone in methadone maintenance patients.

2.8. Comparison of assay results

As a validation procedure, ordinary least products linear regression analysis (Brace, 1977; Ludbrook, 1997) was used to compare: the concentrations (R)- and (S)-methadone in patient urine samples obtained with the two stereoselective assays (assays 4 and 5); the sum of plasma (R)- and (S)-methadone concentrations (assay 3) with rac-methadone concentrations (assay 2), the sum of urine (R)- and (S)-methadone concentrations (assays 4 and 5) with rac-methadone concentrations (assay 6); and the sum of (R)- and (S)-EDDP concentrations (assay 5) with rac-EDDP concentrations (assay 6). Analyses were performed using Excel (Excel v7.0a, Microsoft). Linear regression analysis was performed using GraphPad Prism (GraphPad Prism v2.01, GraphPad Software, CA, USA). Ordinary least products linear regression analysis is sensitive to both fixed and proportional bias, unlike conventional linear-regression analysis, as it does not assume that one axis is error-free (Brace, 1977; Ludbrook, 1997).

During preliminary investigations, it was noted that the plasma concentrations of rac-methadone determined by the racemic assay (assay 2) were consistently greater (>50 ng.ml^{-1}) in three subjects compared to those estimated from the sum of the individual

enantiomers (assay 3) throughout the entire inter-dosing interval (mean±SD): patient #4 82±47 $ng.ml^{-1}$, patient #6 132±22 $ng.ml^{-1}$, patient #16 131±51 $ng.ml^{-1}$. Further investigation of this phenomenon revealed an extra peak which occurred 1.6 minutes after the (R)-methadone peak (baseline resolved) in the chromatograms obtained in these subjects' plasma samples analysed for (R)- and (S)-methadone (assay 3). This peak was not present in the chromatograms of the other subjects. Methadone and internal standard peak shapes in the chromatograms of these subjects samples assayed for rac-methadone were symmetrical, indicating that the interfering compound(s) co-eluted with rac-methadone in this assay. Detailed comparison of urinalysis and self-reported co-medication use did not identify a probable cause. Interestingly, ordinary least products linear regression analysis comparing urinary concentrations of both methadone and EDDP did not reveal patient #16 as an outlier in the three analyses performed. Patients #4 and #6 did not have a urine sample collected. The plasma rac-methadone concentration data were retained in Chapter 4. However, the ordinary least products linear regression analysis comparing these two assays was repeated excluding these three patients (#4, #6, #16), although the data are still included on the graphical plot (see Figure 2-6). The publications based upon this work (Dyer et al., 1999; Dyer et al., 2001) used the sum of (R)- and (S)-methadone for these patients to avoid misinterpretation of the pharmacokinetic-pharmacodynamic data obtained from these patients. This result highlights the importance of assay selectivity assessments, although it is unusual, as it indicates a greater selectivity of the stereoselective assay compared to conventional non-chiral assay.

The results of the ordinary least products linear regression analyses comparing the concentrations of methadone and EDDP quantitated in patient samples are presented in Table 2-9, and graphically represented in Figure 2-6.

Table 2-9: Ordinary least products linear regression comparison of HPLC assays.

Assay	Assay	r^2	Slope (95% CI)	Intercept (95% CI)	n
Methadone in plasma					
Assay 2 (rac)	Assay 3 (R+S)	0.9976	1.010 (0.988, 1.052)	-8.3 (-18.4, 1.5)	185
Methadone in urine					
Assay 6 (rac)	Assay 4 (R+S)	0.9976	0.995 (0.938, 1.054)	1.1 (-0.4, 2.5)	10
Assay 6 (rac)	Assay 5 (R+S)	0.9996	0.981 (0.959, 1.030)	0.2 (-0.5, 0.9)	10
Assay 5 (R)	Assay 4 (R)	0.9957	0.970 (0.898, 1.048)	-1.1 (-2.3, 0.1)	10
Assay 5 (S)	Assay 4 (S)	0.9957	1.028 (0.951, 1.110)	-0.3 (-1.2, 0.5)	10
EDDP in urine					
Assay 6 (rac)	Assay 5 (R+S)	0.9999	0.987 (0.973, 1.001)	-1.2 (-0.6, 0.3)	10

Notes: assay 2=assay for the quantification of rac-methadone in plasma, assay 3=assay for the quantification of (R)- and (S)-methadone in plasma, assay 4=assay for the quantification of (R)- and (S)-methadone in urine, assay 5=assay for the simultaneous quantification of (R)- and (S)-methadone and (R)- and (S)-EDDP in urine, assay 6=assay for the quantification of rac-methadone and rac-EDDP in urine; rac=racemic compound, R=(R)-enantiomer, S=(S)-enantiomer, (R+S)=sum of (R)- and (S)-enantiomers.

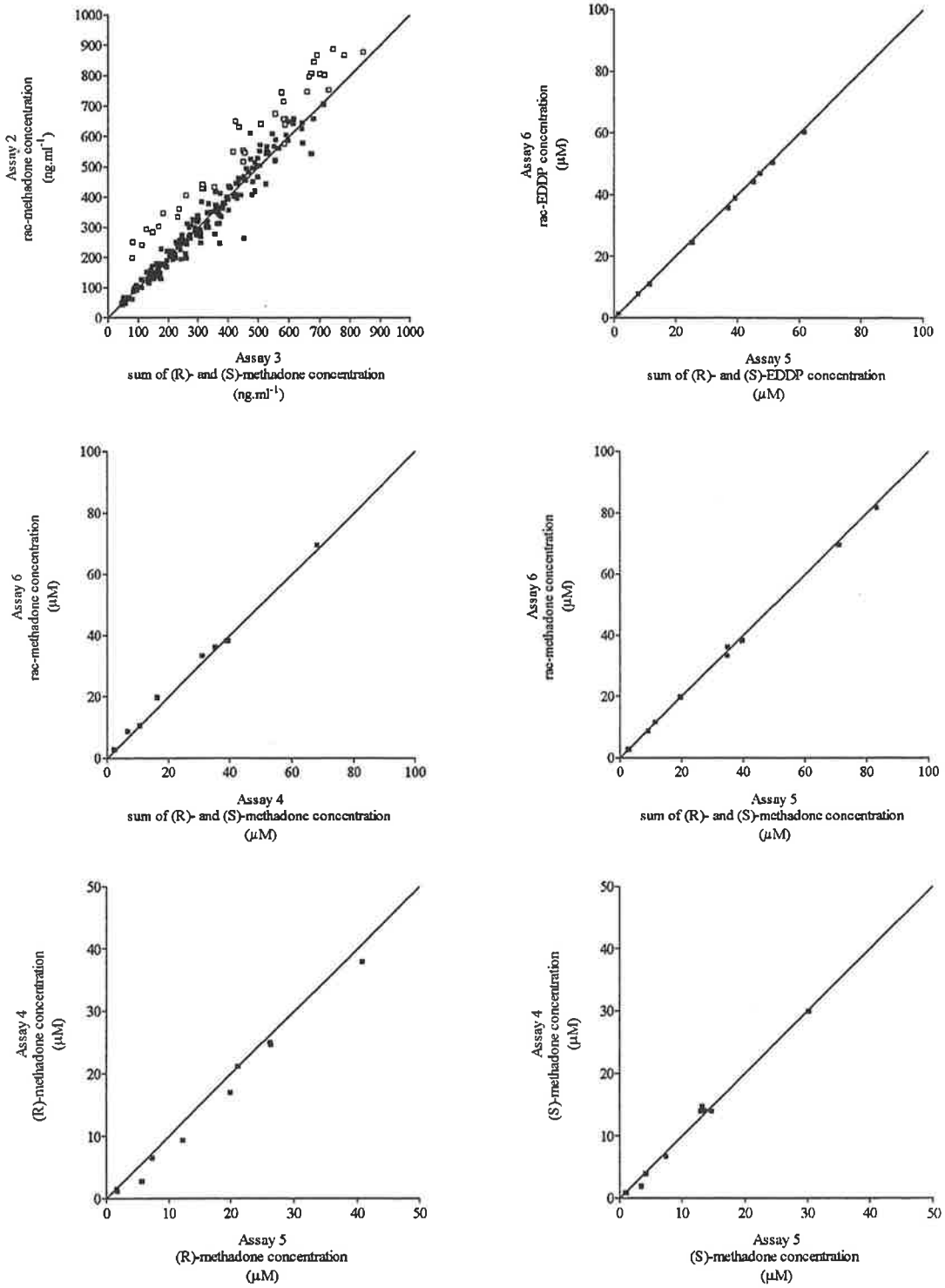


Figure 2-6: Comparison of concentrations of methadone and EDDP obtained with different HPLC assays.

Notes: assay 2=assay for the quantification of rac-methadone in plasma, assay 3=assay for the quantification of (R)- and (S)-methadone in plasma, assay 4=assay for the quantification of (R)- and (S)-methadone in urine, assay 5=assay for the simultaneous quantification of (R)- and (S)-methadone and (R)- and (S)-EDDP in urine, assay 6=assay for the quantification of rac-methadone and rac-EDDP in urine. Solid line is the y=x line of identity. Open squares (□) indicate samples obtained from patients #4, #6 and #16 that were not included in ordinary least products regression analysis.

All analyses yielded strong and significant correlations (r^2>0.97, P<0.05) for all comparisons (see Table 2-9). The 95% confidence intervals of the slope included 1 for all comparisons, indicating no proportional bias, and the 95% confidence intervals of the intercept included 0 for all comparisons, indicating no fixed bias. These analyses demonstrated an excellent performance of all stereoselective HPLC assays, and indicate that it is unlikely that there was interference by other licit and illicit drugs (with the exception of the rac-methadone assay in plasma). By using ordinary least products linear regression analysis to compare the concentrations of methadone and EDDP in urine samples using three chromatography systems with distinct selectivity characteristics, I have demonstrated these assays to be adequately selective, and most importantly, all stereoselective assays produced excellent results. Combined with the intra- and inter- assay precision and accuracy of quality control samples, these data indicate that the assays were selective, precise and accurate.

3. Methadone N-demethylation in human liver microsomes: examination of stereoselectivity and involvement of cytochrome P450 isoforms

3.1. Introduction

After single-dose administration, the two enantiomers differ significantly in their disposition in humans, with (R)-methadone having a greater volume of distribution, longer half-life, higher total systemic clearance, and lower plasma protein binding (see section 1.7). It is not known whether the stereoselectivity in systemic clearance is due to metabolism (intrinsic clearance) and/or protein binding. Methadone metabolism in humans is complex, with nine metabolites identified (see section 1.7.3) although of much the data have been qualitative rather than quantitative. Mono N-demethylation, a primary metabolic pathway, results in the formation of a highly unstable compound which then undergoes spontaneous cyclisation and dehydration to form EDDP, which may then be further N-demethylated to EMDP. Several other oxidative and reductive metabolites have also been reported *in vivo*. However, their relative importance remains ill defined, since complete mass balance has not been achieved (see section 1.7.3).

At the time the present work was conducted, there were no reports examining the metabolism of methadone in any human tissue preparation. Early examinations of the metabolism of methadone *in vitro* were restricted to crude homogenates or microsomal fractions prepared from animal tissues, and employed relatively non-specific techniques which cannot differentiate between the formation of individual metabolites. Although these authors provided limited data, they indicated that the metabolism of methadone is likely to be mediated by CYP450 enzyme(s). The role of stereoselectivity in the metabolism of methadone is unclear as conflicting results have been obtained within a single species.

With respect to knowledge of the CYP450 isoforms likely to mediate the metabolism of methadone, the involvement of CYP3A4 can be inferred from reports of either decreased rac-methadone plasma concentrations and/or concurrent complaints of opioid withdrawal symptoms by patients soon after commencement of therapy with known CYP3A inducers

(Halikas et al., 1990; Kreek et al., 1976a; Kuhn et al., 1989; Liu & Wang, 1984; Saxon et al., 1989; Tong et al., 1981). Some authors have suggested the involvement of CYP2D6 and CYP1A2 in the metabolism of methadone, based upon drug-drug interaction studies *in vivo* (Bertschy et al., 1994; Bertschy et al., 1996; Eap et al., 1997). Furthermore, Eap and co-workers (1997) suggested that metabolism of methadone mediated by CYP2D6 was stereoselective, involving only the (R)- enantiomer. In contrast, others have reported no change in plasma methadone concentrations in 16 methadone maintenance patients after co-administration with fluoxetine for 9 weeks (Batki et al., 1993). More recently, Cobb and co-workers (1998) demonstrated that co-administration of fluconazole inhibited the metabolism of rac-methadone. Fluconazole is known to potently inhibit the catalytic activity of both CYP3A4 (von Moltke et al., 1996) and CYP2C9 (Kunze et al., 1996; Miners & Birkett, 1998) *in vitro* and *in vivo*. Methadone has also been shown to alter the metabolism of CYP2D6 substrates *in vivo* (Kosten et al., 1990; Maany et al., 1989; Wu et al., 1993) and *in vitro* (Kerry et al., 1994; Wu et al., 1993). However from these data, it is difficult determine whether methadone is metabolised by CYP2D6.

In summary, the available literature reports of *in vivo* human drug-drug interactions involving methadone strongly suggest CYP3A4 involvement in the metabolism of methadone. The involvement of CYP1A2 and/or CYP2D6 in the metabolism of methadone may also be inferred from reports of inhibition of methadone metabolism. However, the relative importance of multiple CYP450 isoforms cannot be elucidated, due to a lack of isoform specificity of the interacting drugs.

The specific aims of the work contained in this Chapter are:

Aim 2: To investigate the kinetics of CYP-mediated N-demethylation of methadone in human liver microsomes, to examine the role of stereoselectivity and to identify the CYP isoforms involved. A prediction of the *in vivo* clearance of methadone to EDDP using an *in vitro-in vivo* scaling model will also be performed.

3.2. Methods

3.2.1. Chemicals

Pharmaceutical compounds were obtained from the sources reported in section 2.2 of Chapter 2. Other materials were obtained from the following sources: bovine serum albumin (fraction V), DL-isocitric acid tri-sodium salt, Folin-Ciocalteau reagent and isocitrate dehydrogenase (NADP, type IV) were obtained from Sigma Chemical Company (St. Louis, Mo, USA); nicotinamide adenine dinucleotide phosphate di-sodium salt (NADP-Na_2) was from Merck (Darmstadt, Germany); dimethylsulfoxide was from Ajax Chemicals (Auburn, Australia). Monoclonal antibodies to CYP3A4 and CYP2E1, and microsomes from human lymphoblastoid cells containing expressed CYP3A4, CYP2D6 and CYP2C19 were purchased from Gentest Corporation (Woburn, MA, USA). All other reagents and chemicals were obtained from commercial sources and were of analytical grade quality.

3.2.2. Human liver microsomes

3.2.2.1. Human liver samples

Ethical approval was obtained from the Committee on the Ethics of Human Experimentation of the University of Adelaide and the Human Ethics Committee of the Royal Adelaide Hospital. All patients gave written informed consent for their liver tissue to be used. Liver tissue (HLS #5, #16, #21, #22, #23, #31 and #24) was obtained from seven patients undergoing partial hepatectomy for hepatic tumors. Donors ranged in age from 25-73 years, four were female, non were smokers, and all had normal clinical chemistry and haematology prior to surgery except donor #31 with hypoalbuminaemia (33 $g.l^{-1}$) and anaemia (haemoglobin 9.7 $g.l^{-1}$). Refer to Appendix 5 for patient demographic information. All tissue samples used were normal on gross morphology. Liver samples dissected into small pieces, snap frozen in liquid nitrogen and stored at -80°C until used.

3.2.2.2. Genotype

DNA was extracted from the liver tissue for the determination of *CYP2D6* and *CYP2C19* genotypes. *CYP2D6* alleles screened were *CYP2D6*1A*, *CYP2D6*4A*, *CYP2D6*4B*,

*CYP2D6*4C*, *CYP2D6*4D* and *CYP2D6*5* (Heim & Meyer, 1990; Steen et al., 1995), and were conducted by the Institute of Medical and Veterinary Science (Adelaide, Australia). CYP2C19 alleles screened were *CYP2C19*1*, *CYP2C19*2* and *CYP2C19*3* (Coller et al., 1997), and were conducted by Dr Janet Coller of the Department of Clinical and Experimental Pharmacology, Adelaide University. Results of the genotyping corresponded to the extensive metaboliser (EM) phenotype for both CYP isoforms in HLS #5, #16, #21, #22, #23 and #31. Liver sample #24 was a CYP2C19 genotypic EM, and a CYP2D6 genotypic poor metaboliser (PM). Genotyping of HLS #16 was not possible due to insufficient tissue.

3.2.2.3. Preparation of microsomal samples

Microsomes were prepared by differential centrifugation of liver homogenates (Zanger et al., 1988). Briefly, a known weight of liver tissue was dissected into fine pieces and homogenised in 2-4 ml per gram of microsome preparation buffer (1 mM EDTA and 0.15 M KCl, pH 7.3) using a mechanical homogeniser (Thyristor Regler, John Morris Scientific Instruments Pty Ltd, Sydney, Australia), filtered through gauze pads, then homogensied with a teflon plunger (0.2 mm clearance) in a glass potter. Aliquots of the homogenate were then centrifuged (12000*g*, 15 min) at 4°C (J2-21 centrifuge, JA 20 rotor, Beckman, CA, USA). The pellets were discarded and the supernatant re-centrifuged (27000*g*, 15 min) at 4°C. The pellets were again discarded and the supernatant re-centrifuged (105000*g*, 60 min) at 4°C (L7-55 Ultracentrifuge, 70.1 Ti rotor, Beckman). The supernatant cytosolic fraction was discarded, and the pellets rehomogenised in 2-3 ml of washing buffer (1 mM EDTA, 0.1 M sodium pyrophosphate decahydrate, pH 7.25) in a glass-glass potter. Aliquots were then centrifuged (105000*g*, 60 min) at 4°C (L7-55 Ultracentrifuge, 70.1 Ti rotor, Beckman). The supernatant was discarded, and the pellets rehomogenised in a glass-glass potter with a volume of microsomal storage solution (1 mM EDTA, 0.1 M di-sodium orthophosphate, pH 7.4) corresponding to 1 ml per gram of original liver sample weight. Aliquots (200 µl) were place in 1.5 ml Eppendorf tubes and stored at -80°C until used.

3.2.2.4. Microsomal protein content

Total microsomal protein content of the microsome samples was quantified using the method of Lowry and co-workers (1951). Microsomal samples were diluted (1/25 and 1/50) with microsomal storage solution, and assayed together with six calibration standards (0, 50, 100, 200, 400 and 800 $mg.ml^{-1}$) of bovine serum albumin (fraction V) prepared in duplicate. Samples were then mixed with 2 ml of Solution 1 (0.02% $CuSO_4$, 0.04% K^+-Na^+-tartrate, 2.9% Na_2CO_3 and 0.39% NaOH) by briefly vortexing, allowed to stand for 10 min, 200 µl of Solution 2 (1:2 dilution of Folin-Ciocalteau reagent in water) was then added. After briefly mixing, the samples were then left to stand in darkness for 20-30 min. The absorbance at 550 nm was measured using a double-beam spectrophotometer (U-2000, Hitachi, Tokyo, Japan). Calibration standards were used to calibrate the spectrophotometer, and the total protein content of the microsomal samples was calculated.

3.2.2.5. Total cytochrome 450 content

Total microsomal cytochrome P450 content of the microsome samples was quantified using the method of Omura and Sato (1964). Carbon monoxide gas was bubbled through samples that had been diluted 1 in 10 using microsomal storage solution, for 30 seconds at a rate of approximately 1 $bubble.sec^{-1}$. The resulting solution was then divided equally into two cuvettes, placed in a double-beam spectrophotometer (U-2000, Hitachi) and the absorbance was measured from 400 to 500 nm. The sample cuvette was removed, 1-2 mg of $Na_2S_2O_4$ was added and the cuvette inverted 3 times, and the reduced carbon monoxide versus oxidised carbon monoxide difference spectrum was obtained by re-measuring the absorbance from 400 to 500 nm. The total cytochrome P450 concentration was then calculated by Equation 3-1:

Equation 3-1: Calculation of total CYP450 content in liver microsomal samples.

$$\text{Total CYP450 content} = \frac{(A_{450} - A_{490})_{\text{after } Na_2S_2O_4} - (A_{450} - A_{490})_{\text{before } Na_2S_2O_4}}{\varepsilon}$$

The extinction coefficient (ε) was assumed to be 0.106 absorbance units.$cm^{-1}.M^{-1}$. After correcting for the dilution factor and the total protein content, the final cytochrome P450 content was expressed in units of pmol P450.mg $protein^{-1}$.

3.2.3. Microsomal incubations

Incubations were performed in duplicate at 37°C in a shaking water bath for 45 minutes. The incubates of 200 µl final volume contained 50 mM sodium phosphate buffer (pH 7.4), NADPH generating system (1 mM NADP, 1 unit.ml^{-1} isocitrate dehydrogenase, 5 mM magnesium chloride), substrate (minimum of 10 concentrations, final concentration range of 1-1500 µM for rac-methadone and 1-1250 µM for (R)- and (S)-methadone) and 100 µg microsomal protein. The formation of EDDP was stopped by the addition of 100 µl ice cold acetonitrile, samples were then vortexed briefly, centrifuged (10 000*g*, 10 min) and a 100 µl aliquot injected on to the HPLC system. The rate of EDDP formation from 100 µM rac-methadone, (R)-methadone and (S)-methadone was observed to be linear up to at least 120 min (Figure 3-1) and 200 µg microsomal protein (Figure 3-2).

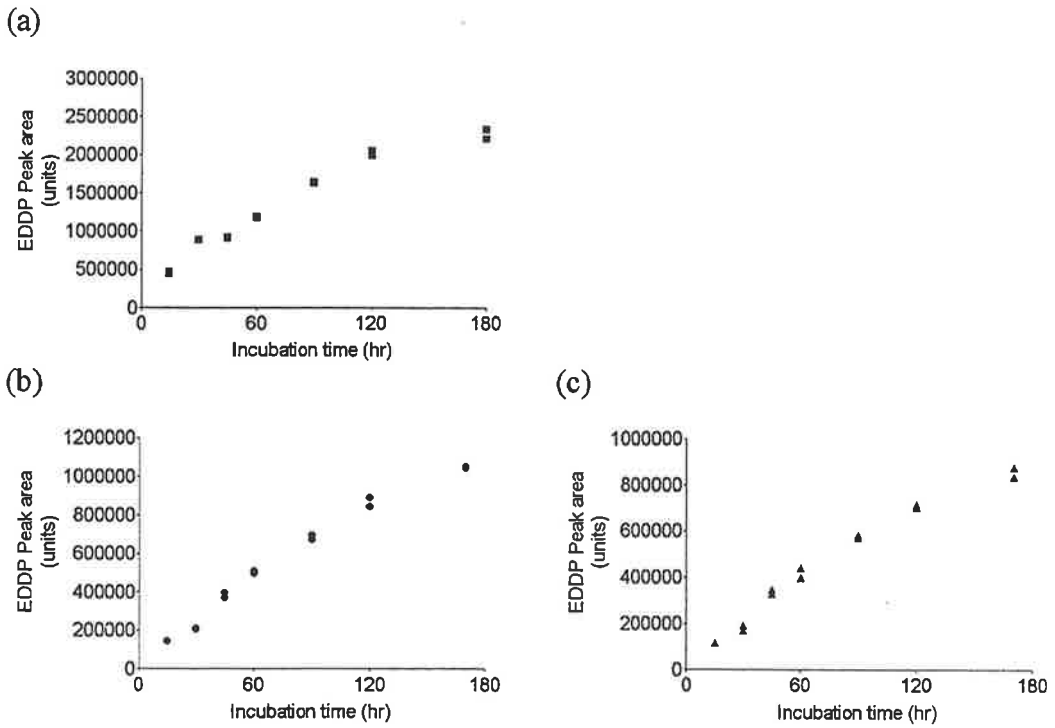


Figure 3-1: Relationship of EDDP formation from 100 μM of (a) rac-methadone, (b) (R)-methadone and (c) (S)-methadone and incubation time in human liver microsomes.
Notes: n=2 at each time point, performed in HLS #21.

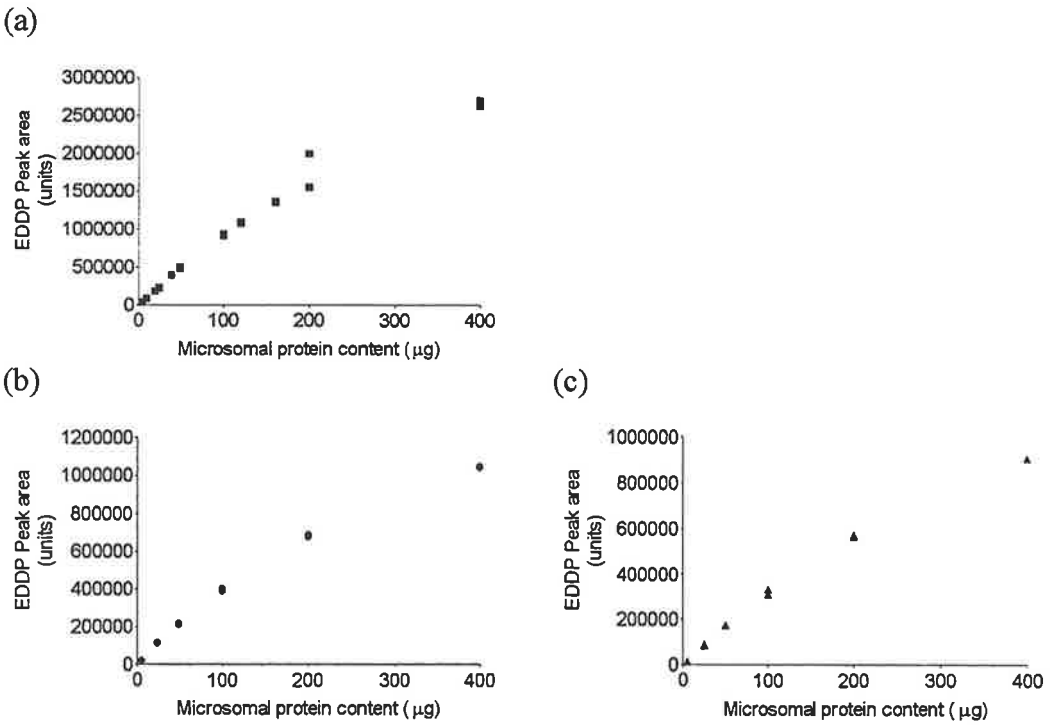


Figure 3-2: Relationship of EDDP formation from 100 μM of (a) rac-methadone, (b) (R)-methadone and (c) (S)-methadone and microsomal protein content in human liver microsomes.
Notes: n=2 at each protein concentration, performed in HLS #21.

3.2.4. EDDP Quantification

EDDP was quantified using a validated reversed phase HPLC assay as described in section 2.3. The ongoing performance of the assay was monitored with quality control (QC) samples prepared in duplicate at three concentrations; low (LQC, 0.62 μM), medium (MQC, 1.74 μM) and high (HQC, 12.45 μM). Assay acceptance criteria were: at least ten calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of ten or more calibration standards and five or more QC samples must be less than ±15%.

3.2.5. Inhibition studies with chemical inhibitors

Microsomes from three human liver samples (HLS #5, #16 and #21) were used in duplicate to examine the inhibition of EDDP formation from rac-, (R)- and (S)-methadone. Substrates were incubated at a concentration equal to their respective K_m values for that particular liver sample. Chemical inhibitors considered to be specific for various isoforms (Newton et al., 1995) were coumarin (CYP2A6, 100 μM), diethyldithiocarbamic acid (DDC, CYP2E1, 100 μM and 10 μM), furafylline (CYP1A2, 100 μM), quinidine (CYP2D6, 1 μM), sulphaphenazole (CYP2C9, 100 μM), troleandomycin (CYP3A, 100 μM and 10 μM and 1 μM), ketoconazole (CYP3A, 100 μM, 1 μM and 0.1 μM), and (S)-mephenytoin (CYP2C19, 100 μM). In addition, 100 μM diazepam (CYP3A/2C19 Andersson et al., 1994a) and fluoxetine (CYP2D6/3A von Moltke et al., 1995a) were also tested as they are often co-administered to individuals in methadone maintenance therapy. Chlorzoxazone (100 μM) and omeprazole (100 μM) were also tested. Incubation conditions did not alter from the kinetic study, except that the mechanism-based inhibitors DDC, furafylline and troleandomycin required 20 minutes pre-incubation prior to the addition of substrate. Inhibitors were dissolved in various solvents due to differences in solubility. Of those not dissolved in water, dimethylsulfoxide was used to dissolve furafylline and sulphaphenazole with final dimethylsulfoxide concentrations of 1.7% and 0.5% respectively; methanol (<0.5% final concentration) was used to dissolve chlorzoxazone, diazepam, fluoxetine, ketoconazole and troleandomycin. Omeprazole was prepared in a mixture of water with pH

adjusted to 10.5 with sodium hydroxide, and methanol (final methanol concentration 0.5%). Incubations containing equivalent amounts of diluent were always used as controls. When compared to control reactions containing no diluent, control reactions containing methanol or 0.5% dimethylsulfoxide showed greater than 90% activity, as did the pH 10.5 water containing 0.5% methanol, while those containing 1.7% dimethylsulfoxide showed greater than 80% activity (results not shown). Control incubations containing inhibitors alone did not produce any chromatographic peaks that interfered with the quantification of EDDP.

3.2.6. Inhibition studies with CYP specific antibodies

Microsomes from a single human liver sample (HLS #5) were used in duplicate to examine the inhibition of EDDP formation from (R)- and (S)-methadone. Substrates were incubated at a concentration equal to their respective K_m values for that particular liver sample. Antibodies specific for CYP3A4 and CYP2E1 were used at two titres (2 µl and 8 µl per 100 µg microsomal protein) in duplicate. Antibodies were used according to the manufacturers directions. Incubation conditions did not alter from the kinetic study.

3.2.7. Metabolism studies with expressed CYP isoforms

Microsomes from human lymphoblastoid cells containing expressed CYP3A4, CYP2D6 and CYP2C19 were used in duplicate to examine the formation of EDDP from (R)- and (S)-methadone. Microsomes containing expressed CYP isoforms were used according to the manufacturers directions. Incubation conditions did not alter from the kinetic study, except that the CYP enzymes were kept ice cold until addition to the reaction mixture. Amounts of CYP enzyme used were kept constant (6 pmol P450 per incubation), which resulted in amounts of protein similar to that used for the human liver microsomes (100 µg). Reaction velocities were compared to that obtained from a single human liver sample (HLS #5), which was incubated at the same time as the expressed enzyme. Substrate concentrations were fixed at 200 µM.

3.2.8. Secondary metabolism of EDDP

The formation of EMDP from EDDP via a second N-demethylation reaction was also examined. EDDP was incubated at a concentration of 1 mM in a single microsomal preparation under conditions identical to those used for the methadone incubations. Analysis of the sample for EMDP was performed as for the methadone incubations.

3.2.9. Data analysis

Concentrations of EDDP in microsomal incubation samples from the kinetic studies were transcribed into Excel spreadsheets (Version 5.0, Microsoft Corporation, WA, USA) and the rate of EDDP formation (V, nmol.mg protein^{-1}.hr^{-1}) were calculated. Eadie-Hofstee (V/Substrate concentration versus V) plots were constructed, and a single enzyme Michaelis-Menten kinetics equation was fitted to the unweighted data using non-linear least-squares regression analysis (Regression, Blackwell Scientific Publications, Oxford, UK) giving estimates of V_{max} and K_m, where V_{max} is the maximum reaction velocity and K_m is the substrate concentration at which the reaction rate is half V_{max}. Intrinsic clearance (CL_{int}) was calculated as V_{max}/K_m. Rates of EDDP formation obtained from the studies with expressed CYP isoforms were expressed as pmol EDDP formed.pmol P450^{-1}.hr^{-1}. Scaling up CL_{int} values to predict the hepatic *in vivo* clearance of methadone to EDDP ($CL^{pred}_{MD\rightarrow EDDP}$) was performed using the well-stirred model (Equation 3-2), without correction for non-specific binding to the microsomal protein (Obach, 1997; Obach et al., 1997):

Equation 3-2: Well stirred model for in vitro-in vivo scale up of hepatic clearance.

$$CL^{pred}_{MD\rightarrow EDDP} = \frac{Q_h \times f_u \times Cl_{int\ (whole\ organ)}}{Q_h + f_u \times Cl_{int\ (whole\ organ)}}$$

Q_h is hepatic blood flow, f_u is the unbound fraction of the drug in the blood, and $CL_{int\ (whole\ organ)}$ is the mean CL_{int} value scaled up to the whole liver. Predictions were performed for a 75 kg individual, assuming Q_h=21 ml.min^{-1}.kg^{-1}, 20 g liver.kg body weight^{-1} and 45 mg microsomal protein.g liver^{-1} (Obach, 1997; Obach et al., 1997). The unbound

fraction of rac-, (R)- and (S)-methadone were assumed to be 12%, 14% and 10%, respectively (Eap et al., 1990).

All data are presented as mean±SD. Statistically significant differences in V_{max}, K_m, CL_{int} and percent inhibition by the chemical inhibitors between rac-, (R)-, and (S)-methadone were assessed using paired t-tests. Statistically significant differences in percent inhibition of the chemical inhibitors compared to control reactions was assessed with one-tailed t-tests. Differences were considered significant at the $P<0.05$ level. For monitoring assay performance, the estimated concentration divided by the nominal concentration multiplied by 100% was calculated for each individual sample, and accuracy was calculated as the mean of these values, and the residual standard deviation of the mean was taken as the precision.

3.3. Results

3.3.1. Ongoing assay performance

Calibration curves for rac-EDDP were linear over the 0.25-50 μM concentration range, with r^2 values greater than 0.99 for all analytical runs, resulting in a mean±SD (n=20 assays) value of 0.9985±0.0018. Estimates of slope demonstrated no consistent time-related changes, with a mean±SD value of 1.151±0.040, consistent with that obtained during assay validation (see section 2.3). All analytical runs performed during the course of the experiments reported in this chapter met the acceptance criteria. The ongoing inter-assay accuracy and precision of the method at the three QC concentrations, and the lowest calibration standard are presented in Table 3-1.

Table 3-1: Ongoing performance of the assay for rac-EDDP in microsomal incubation samples.

	Nominal concentration (μM)	Accuracy (%)	Precision (%)	n
Inter-assay (n=20 assay days)				
LOQ	0.25	99.5	5.8	20
LQC	0.62	111.4	9.3	38
MQC	1.74	108.2	6.1	39
HQC	12.45	98.8	6.0	40

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

These data demonstrate that the assay continued to provide an accurate and precise method for the quantification of rac-EDDP in human liver microsomal samples.

3.3.2. Metabolic profile

No chromatographic peaks corresponding to metabolites other than EDDP were observed in any incubation containing rac-, (R)- or (S)-methadone. The only metabolite observed was EDDP, whose formation was NADP-dependent.

3.3.3. Kinetics of EDDP formation

Eadie-Hofstee plots were found to be linear (see Appendix 6) and a single enzyme Michaelis-Menten kinetic equation was fitted to the data (see Appendix 6). As can be seen from the data in Table 3-2, the V_{max}, K_m and CL_{int} values for all three substrates in the genotypic CYP2D6 PM sample are within the range found in the genotypic CYP2D6 EM samples. Statistical comparisons, therefore, included the data from the genotypic CYP2D6 PM sample. For V_{max} there was a statistically significant difference (*P* value, mean difference; 95% CI) between (S)-methadone and both (R)-methadone (*P*=0.0269, 8.2 nmol.mg protein^{-1}.h^{-1}; 1.3, 15.1 nmol.mg protein^{-1}.h^{-1}) and rac-methadone (*P*=0.0199, 4.5 nmol.mg protein^{-1}.h^{-1}; 1.0, 8.0 nmol.mg protein^{-1}.h^{-1}). However, there was no significant difference between (R)-methadone and rac-methadone (*P*=0.18, 3.8 nmol.mg protein^{-1}.h^{-1}; 2.2, -9.7 nmol.mg protein^{-1}.h^{-1}). For K_m there was no statistically significant difference between (R)-methadone and (S)-methadone (*P*=0.11, 25.5 μM; -8.1, 59.2 μM), or (S)-methadone and rac-methadone (*P*=0.48, -9.6 μM; -40.6, 21.5 μM). However,

(R)-methadone K_m was statistically significantly greater than rac-methadone (P=0.0131, 35.1 μM; 10.5, 59.7 μM). For CL_{int} there was no statistically significant difference between (R)-methadone and (S)-methadone (P=0.60, 9.2 ml.g protein^{-1}.h^{-1}; -30.9, 49.2 ml.g protein^{-1}.h^{-1}), or (S)-methadone and rac-methadone (P=0.14, -42.5 ml.g protein^{-1}.h^{-1}; -103.5, 18.5 ml.g protein^{-1}.h^{-1}). However, (R)-methadone CL_{int} was statistically significantly lower than for rac-methadone (P=0.0212, -33.32 ml.g protein^{-1}.h^{-1}; -59.7, 7.0 ml.g protein^{-1}.h^{-1}).

The *in vitro-in vivo* scaling calculations resulted in $CL^{pred}_{MD \rightarrow EDDP}$ values of 38 ml.min^{-1}, 39 ml.min^{-1} and 27 ml.min^{-1} for rac-, (R)- and (S)-methadone, respectively.

Table 3-2: Michaelis-Menten enzyme kinetic parameters (V_{max} and K_m) and intrinsic clearance CL_{int} for EDDP formation from rac-, (R)- and (S)-methadone by human liver microsome samples.

HLS #	V_{max} (nmol.mg protein^{-1}.h^{-1})			K_m (μM)			CL_{int} (ml.g protein^{-1}.h^{-1})		
	rac-	(R)-	(S)-	rac-	(R)-	(S)-	rac-	(R)-	(S)-
5 (CYP2D6 EM)	66	77	66	200	252	185	332	305	354
16 (CYP2D6 EM)	63	62	52	144	169	180	437	365	289
21 (CYP2D6 EM)	27	24	26	187	170	170	143	142	155
22 (CYP2D6 EM)	62	70	58	126	152	141	494	462	410
23 (CYP2D6 EM)	29	28	24	138	202	200	211	141	120
31 (CYP2D6 EM)	23	22	20	196	241	215	115	91	93
Mean±SD	45±21	47±25	41±20	165±33	198±41	182±25	289±157	251±148	237±132
24 (CYP2D6 PM)	55	68	47	194	244	160	282	277	297
Mean±SD	46±19**	50±24**	42±18	169±32*	204±32	179±25	288±143*	255±136	245±123

Notes: $CL_{int}=V_{max}/K_m$. Statistically significant ($P<0.05$) differences compared to *(R)-methadone and **(S)-methadone.

3.3.4. Inhibition with chemical and immunological inhibitors

EDDP formation from rac-, (R)-, and (S)-methadone was significantly ($P<0.05$) inhibited by ketoconazole at 100 μM (75±18%, 83±11%, 78±4% inhibition), 1 μM (43±12%, 43±10%, 46±14% inhibition) and 0.1 μM (8±2%, 8±3%, 7±5% inhibition); troleandomycin at 100 μM (45±7%, 44±11%, 43±10% inhibition), 10 μM (22±11%, 19±10%, 21±12% inhibition); sulphaphenazole at 100 μM (18±6%, 26±4%, 14±7% inhibition); DDC at 100 μM (44±12%, 44±11%, 45±11% inhibition); omeprazole (29±13%, 29±7.3%, 23±11 inhibition) and fluoxetine at 100 μM (56±10%, 53±6%, 50±13% inhibition) compared to control reactions (Figure 3-3). In addition, EDDP formation from (S)-methadone was significantly ($P=0.006$) inhibited by chlorzoxazone (15±2% inhibition) (Figure 3-3). The inhibition of EDDP formation was not statistically significantly different between the three substrates in all cases ($P>0.05$, Figure 3-3). The other chemical inhibition experiments did not result in significant ($P>0.05$) inhibition of the formation of EDDP from rac-, (R)-, and (S)-methadone compared to control reactions (Figure 3-3).

EDDP formation from (R)- and (S)-methadone was inhibited by both the 8 μl (43±2% and 49±1%, respectively) and 2 μl (20±3% and 19±3%, respectively) titres of the CYP3A4 specific antibodies (Figure 3-3). CYP2E1 specific antibodies did not inhibit this reaction (Figure 3-3).

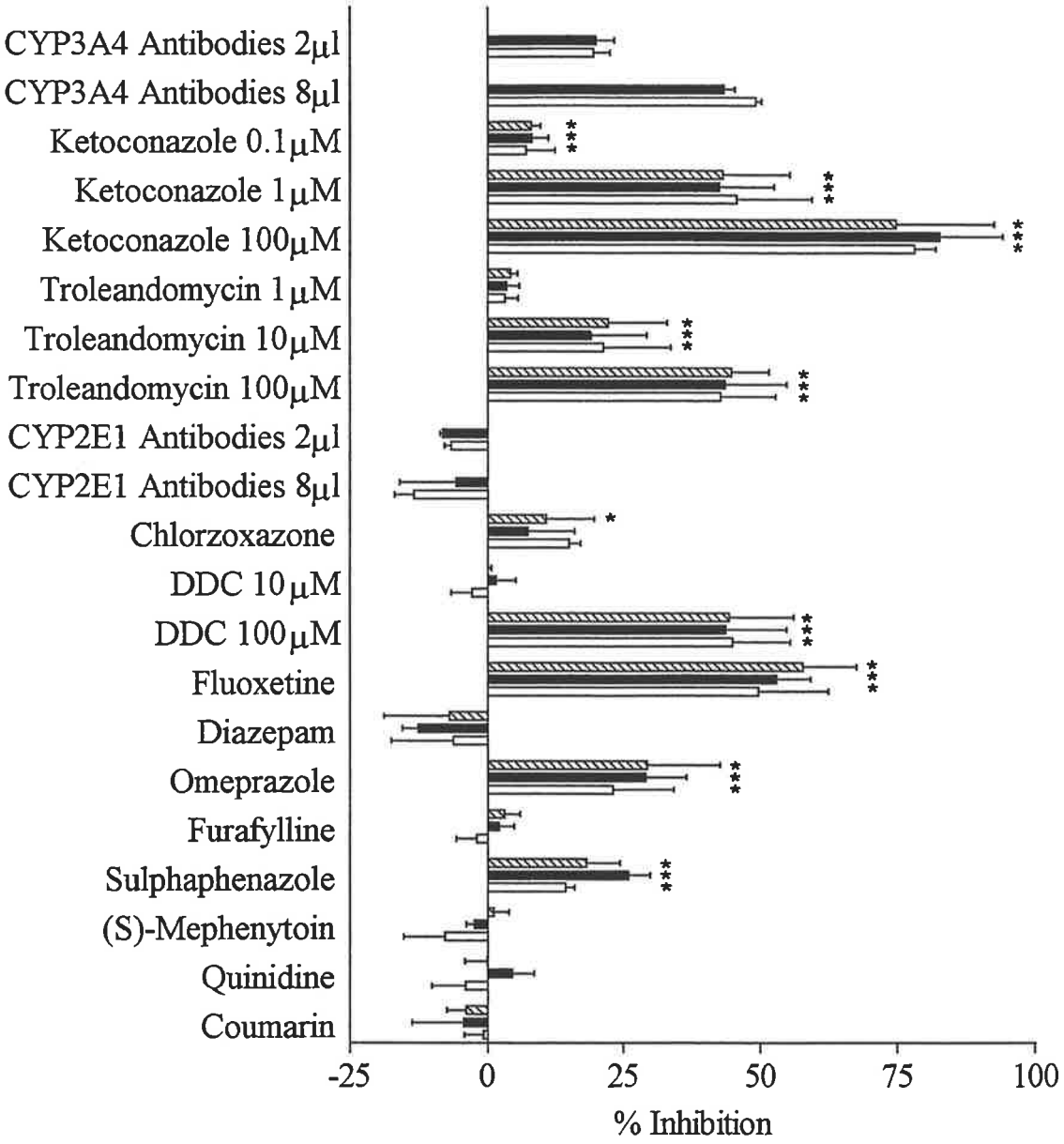


Figure 3-3: Effect of chemical and immunological inhibitors on EDDP formation from rac-, (R)- and (S)-methadone by human liver microsomes.

Notes: Error bars indicate SD (n=3 liver samples in duplicate, HLS #5, #16, #21); (▨) rac-methadone, (■) (R)-methadone, (□) (S)-methadone; *indicates statistically significant inhibition compared to control ($P<0.05$). No statistically significant differences ($P>0.05$) were observed between rac-, (R)-, and (S)-methadone; n=1 liver sample in duplicate (HLS #5) for CYP2E1 and CYP3A4 antibody experiments (no statistical analysis performed). Concentration of inhibitors was 100 μM, unless otherwise indicated.

3.3.5. Metabolism by expressed CYP isoforms

When corrected for CYP450 content, expressed CYP3A4 and CYP2C19 catalysed the N-demethylation of (R)- and (S)-methadone at similar rates, while the formation of EDDP with expressed CYP2D6 was below the limit of quantification (Figure 3-4).

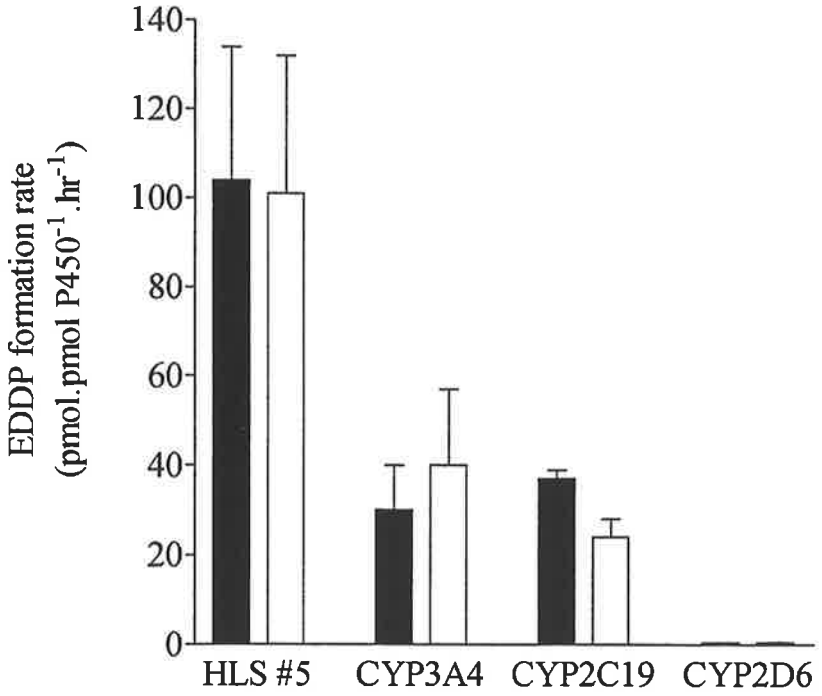


Figure 3-4: Comparison of EDDP formation from (R)-methadone and (S)-methadone by microsomes from a human liver, and human lymphoblastoid cells containing expressed CYP3A4 and CYP2C19.

Notes: Error bars indicate SD (n=2); (■) (R)-methadone, (□) (S)-methadone; HLS #5=human liver sample #5, CYP3A4=human lymphoblastoid cells containing expressed CYP3A4, CYP2C19=human lymphoblastoid cells containing expressed CYP2C19, CYP2D6=human lymphoblastoid cells containing expressed CYP2D6.

3.3.6. Secondary metabolism of EDDP

Incubation of EDDP at a single high concentration (1mM) resulted in <1% formation of EMDP.

3.4. Discussion

Substantial inter-individual variability in rac-methadone disposition has hindered its more widespread use in pain management (Ripamonti et al., 1997) and can pose difficulties in dosage regimen design in opioid dependence treatment. The 4-5 fold range in CL_{int} observed for the formation of EDDP from rac-, (R)- and (S)- methadone seen in the present study was mainly a consequence of the variability in V_{max} values since the K_m values showed little inter-subject variability. This variability is consistent with reported *in vitro* (Chauret et al., 1997; Forrester et al., 1992; Shimada et al., 1994) estimates of variability in hepatic CYP3A4 metabolic activity (see also section 1.7.4.2).

The *in vitro* oxidative metabolism of rac-methadone in human liver microsomes has only recently been described (Iribarne et al., 1996; Iribarne et al., 1998a; Iribarne et al., 1997; Iribarne et al., 1998b; Moody et al., 1997). To date the present study is the first report examining the stereoselectivity in the N-demethylation of methadone in human liver microsomes. The V_{max} for the formation of EDDP from (S)-methadone was significantly lower than for (R)-methadone, however the magnitude of the difference was small (mean difference: 8.2 nmol.mg protein^{-1}.h^{-1}) and less than 16%. There were no significant differences between (R)- and (S)-methadone observed for K_m, even though the chiral carbon is in close proximity to the site of oxidation. Similarly, there was no significant difference between (R)- and (S)-methadone for CL_{int}. These data are supported by the expressed enzyme and inhibition data, which also did not indicate a clear difference between the two substrates. Rac-methadone K_m and CL_{int} values were significantly different (P<0.05) to those of (R)-methadone, but not (S)-methadone, although the magnitude of the differences was small (mean differences approximately 15%). Interpretation of these data is difficult, as rac-methadone is a 1:1 mixture of the two enantiomers. As the two individual enantiomers do not differ in their affinity (K_m) then one would expect the K_m of the racemate to be comparable. The observed greater CL_{int} for rac-methadone compared to (R)-methadone appears to be due to the lower K_m, as V_{max} values were similar. These data indicate that it is unlikely that there would be stereoselectivity in this metabolic pathway *in vivo* due to metabolism only. In contrast, the *in vitro-in vivo* scaling calculations resulted in an estimated $CL^{pred}_{MD\rightarrow EDDP}$ value for (R)-methadone of 38 ml.min^{-1} compared to 27 ml.min^{-1} for (S)-methadone, although statistical comparison of these values is not possible. However, the stereoselective difference in this parameter arose from the correction for protein binding, which is known to be statistically significantly different between the two enantiomers (Eap et al., 1990), but not intrinsic metabolic activity. The observed stereoselectivity is in agreement with the finding that the total clearance of (R)-methadone is 20% greater than for (S)-methadone (Kristensen et al., 1996), while reported values *in vivo* for total clearance generally of methadone range 100-200 ml.min^{-1} (see section 1.7.5). The estimates of

$CL^{pred}_{MD \rightarrow EDDP}$ for all three substrates in this study are well are below the *in vivo* of methadone total oral clearance, even if one considers the minor contribution of renal clearance (<50 $ml.min^{-1}$, see section 1.7.5). Possible explanations for this under estimation include extra-hepatic metabolism or elimination via other metabolic pathways. The formation of oxidative metabolites other than EDDP was not observed in the present study, suggesting that they are likely to be minor metabolites in humans. Despite this, it is possible that the combination of several minor oxidative metabolic pathways, and the unknown contribution of reductive pathways, may amount to an elimination mechanism of significant magnitude. A further possible explanation includes non-specific binding of methadone to microsomal proteins (McLure et al., 2000; Obach, 1996; Obach, 1997) which was not examined in this study. Recently, McLure and co-workers (2000) examined the impact of non-specific protein binding on the kinetics of metabolite formation in microsomal incubations. They found that for the lipophilic weak bases amitriptyline and nortriptyline saturable non-specific binding was likely to occur. Although methadone was not examined, it is possible that a similar phenomenon would also occur. As the ratio of K_m to K_d (binding dissociation constant) increases, Eadie-Hofstee plots demonstrate marked clockwise hysteresis and Michaelis-Menten plots become increasingly sigmoidal, due to concentration dependency of the unbound fraction. In contrast, when $K_m > K_d$ the unbound fraction is concentration independent. In this case, the measured apparent K_m can be corrected to the "true" K_m by multiplying by the unbound fraction of the substrate. In the present study, Eadie-Hofstee plots were linear, resulting in typical one-enzyme Michaelis-Menten enzyme kinetics (see Appendix 6). This would seem to indicate that if binding of methadone to microsomal proteins occurs, then the K_d for non-specific binding is much lower than the K_m of the metabolism reaction.

The V_{max} values are comparable to those reported by Iribarne and co-workers (Iribarne et al., 1996) in their study of rac-methadone in a panel of 20 human liver microsomal preparations. However, they estimated the K_m for rac-methadone in three livers to be 545±258 μM, a result indicating a much lower and more variable affinity for the CYP450

isoform(s) mediating the formation of EDDP, compared to the present study. The results from the present study indicate very little variability in six human liver preparations with K_m values for rac-, (R)- and (S)-methadone almost 3-fold lower than those reported by Iribarne and co-workers (Iribarne et al., 1996). Reasons for this difference are not apparent.

CYP3A4 has been reported to be involved in the N-demethylation of rac-methadone, although the contribution of other isoforms could not be ruled out (Iribarne et al., 1996; Iribarne et al., 1997; Moody et al., 1997). The results presented here confirm the role of CYP3A4 in this metabolic pathway as troleandomycin, ketoconazole and a selective CYP3A4 antibody caused concentration dependent inhibition. Approximately 20% inhibition with sulphaphenazole (100 μM) was observed indicating that CYP2C9 may also partially mediate this reaction, in support of Moody and co-workers (1997) who reported a mean of 57% inhibition of this pathway by the same concentration of sulphaphenazole. However, Iribarne and co-workers (1996) found no inhibition with sulphaphenazole at 5 μM. The inhibition of methadone metabolism in vivo by fluconazole provides some support for the involvement of CYP2C9 (Cobb et al., 1998), although this compound potently inhibits both CYP3A4 (von Moltke et al., 1996) and CYP2C9 (Kunze et al., 1996; Miners & Birkett, 1998). The selective chemical inhibitors of CYP1A2, CYP2A6, CYP2C19, CYP2D6 and CYP2E1 had no effect, indicating that these isoforms are unlikely to contribute substantially to EDDP formation.

Heterologously expressed human CYP450 proteins have also been examined for their ability to N-demethylate rac-methadone, and only CYP3A4 was considered to be important (Iribarne et al., 1996; Moody et al., 1997). The current results agree with those above since formation of EDDP from (R)- and (S)-methadone by microsomes from human lymphoblastoid cells containing expressed CYP3A4 occurred at velocities comparable to those in human liver microsomes were observed. Microsomes containing expressed CYP2C19 were also found to catalyse the N-demethylation of (R)- and (S)-methadone at similar rates to expressed CYP3A4. It is unlikely that CYP2C19 plays a major role in this

reaction as, total CYP2C19 is expressed in the human liver in amounts approximately 1/30th that of CYP3A (Inoue et al., 1997; Yamazaki et al., 1997), and no correlation was found between (S)-mephenytoin hydroxylation and rac-methadone N-demethylation reaction rates (Iribarne et al., 1996) and 100 μM (S)-mephenytoin, a CYP2C19 inhibitor, caused no significant inhibition of EDDP formation.

There are several reports of either decreased rac-methadone plasma concentrations and/or concurrent complaints of opioid withdrawal symptoms by patients soon after commencement of therapy with known CYP3A inducers (Kreek et al., 1976a; Kuhn et al., 1989; Liu & Wang, 1984; Saxon et al., 1989; Tong et al., 1981). The present findings confirm the involvement of CYP3A4 in the metabolism of methadone and the potential for clinically important drug-drug interactions.

Methadone has been shown to alter the metabolism of several CYP2D6 substrates *in vivo* and *in vitro* (Kerry et al., 1994; Kosten et al., 1990; Maany et al., 1989; Wu et al., 1993). Furthermore, Bertschy and Eap's group (Bertschy et al., 1994; Bertschy et al., 1996; Eap et al., 1997) have suggested the involvement of CYP2D6 and CYP1A2 in the metabolism of methadone, based upon *in vivo* drug-drug interaction studies with fluoxetine and fluvoxamine. However, others have reported no change in plasma methadone concentrations in 16 methadone maintenance patients after co-administration with fluoxetine for 9 weeks (Batki et al., 1993). Taken collectively, these results would appear to suggest that methadone is metabolised by CYP2D6. However, the results of other workers and the present study have demonstrated a lack of effect of high concentrations of CYP2D6 inhibitors *in vitro* (Iribarne et al., 1996; Iribarne et al., 1997; Moody et al., 1997), extremely low metabolism of methadone to EDDP by expressed CYP2D6 (Iribarne et al., 1996; Moody et al., 1997), and a lack of correlation of methadone metabolism with CYP2D6 activity using dextromethorphan O-demethylation (Iribarne et al., 1997). Additionally, the present study reported V_{max}, K_m and CL_{int} values for rac-, (R)- and (S)-methadone obtained using microsomes from a genotypic CYP2D6 poor metaboliser to be within the range found

in the extensive metaboliser samples. These data indicate that, although methadone is a potent inhibitor of CYP2D6, the metabolism of methadone to EDDP is not mediated by this CYP isoform in humans. CYP isoforms can have high affinity for a substrate but not metabolise the substrate (for example quinidine, Otton et al., 1988). However, the contribution of CYP2D6 to alternative metabolic pathways remains unknown.

The observations of Bertschy's (1994; 1996) and Eap's groups (1997) could easily be explained by the inhibition of CYP3A4, rather than CYP2D6 by fluoxetine, or CYP2D6 and/or CYP1A2 in the case of fluvoxamine. Reported K_i values for fluoxetine are approximately 80 μM (von Moltke et al., 1995a) and 3 μM (von Moltke et al., 1995a) for CYP3A4 and CYP2D6, respectively; and 10 μM (von Moltke et al., 1995a), 20 μM (von Moltke et al., 1995a) and <0.5 μM (Brøsen et al., 1993) for CYP3A4, CYP2D6 and CYP1A2, respectively, for fluvoxamine. The major fluoxetine metabolite nor-fluoxetine has also been shown to inhibit CYP3A4 and CYP2D6 with K_i values of 11 μM and 4 μM, respectively (von Moltke et al., 1995a). Since the mean K_m values range 150-200 μM for the metabolism to EDDP of rac-methadone and the individual enantiomers, one would expect possible inhibition of this metabolism by fluvoxamine or fluoxetine via CYP3A4. Indeed, Iribarne and co-workers (1997) calculated the K_i value to be 7 μM for fluvoxamine, 55 μM for fluoxetine and 13 μM for nor-fluoxetine mediated inhibition of the metabolism of rac-methadone to EDDP. This value for fluvoxamine is remarkably similar to the known CYP3A4 K_i value for this compound (10 μM), and over 2-fold lower and 10-fold greater than that for CYP2D6 and CYP1A2, respectively. Similarly, the values are very similar to the known CYP3A4 K_i value for fluoxetine (80 μM) and nor-fluoxetine (11 μM), and 18- and 3-fold greater, respectively, than that for CYP2D6. Additionally, von Moltke and co-workers (1995a) cite several cases of pharmacokinetic drug-drug interactions involving fluvoxamine and CYP3A substrates. Further evidence against CYP1A2 involvement is provided by a lack of effect of high concentrations of a CYP1A2 inhibitor *in vitro* observed in this study (furafylline 100 μM), and a lack of correlation of methadone metabolism to EDDP with CYP1A2 activity using two different markers (Iribarne et al., 1996). However,

the contribution of CYP1A2 and/or CYP2D6 to alternative metabolic pathways remains unknown.

Eap and co-workers (1997) have suggested that the *in vivo* drug-drug interaction between fluoxetine and methadone was stereoselective, with the plasma concentration/dose ratio of the (R)-enantiomer only significantly ($P<0.05$) increased by about 30% after chronic treatment with 20 $mg.day^{-1}$ fluoxetine. In contrast, the effect of fluvoxamine was similar on both enantiomers, with plasma concentration/dose ratios increasing by approximately 40% ($P<0.05$). Interestingly, in the fluoxetine treated group, methadone dose changes ranged from -50% to +50%, while dosages remained constant in the fluvoxamine treatment group. The present study did not observe any stereoselective differences in the inhibitory effect of fluoxetine on the formation of EDDP. However, the contribution of stereoselective metabolism to alternative metabolites is unknown.

There was no inhibition of EDDP formation observed when DDC was used at 10 μM, or with a CYP2E1 selective antibody. DDC inhibited the formation of EDDP from rac-, (R)- and (S)-methadone by greater than 56% at 100 μM, however this concentration is not selective for CYP2E1, and probably reflects inhibition of CYP3A4 (Chang et al., 1994). Iribarne and co-workers (Iribarne et al., 1996) reported that catalytic activities of CYP2E1-mediated reactions did not correlate with rac-methadone N-demethylation, and more recently, rac-methadone did not inhibit CYP2E1-mediated reactions (Iribarne et al., 1997). Chlorzoxazone (100 μM) had a minimal effect on (S)-methadone N-demethylation, and no effect on rac- or (R)-methadone. These data indicate that CYP2E1 is not involved in methadone N-demethylation, and that care should be taken when selecting the concentrations of chemical inhibitors; the use of antibodies and expressed CYP isoforms are important in determining CYP isoform involvement.

Although diazepam has been reported to competitively inhibit the metabolism of rac-methadone with a K_i of 50 μM in human liver microsomes (Iribarne et al., 1996), the

present study found no inhibitory effect at a concentration of 100 μM. This difference in results is difficult to reconcile. However, Iribarne and co-workers (1996) appeared to base their results on a single liver preparation, and the influence of variation between tissue samples is one possible explanation. These *in vitro* findings are consistent with two reports of no effect of diazepam on the disposition of rac-methadone *in vivo* (Pond et al., 1982; Preston et al., 1986).

Fluoxetine inhibited the formation of EDDP from rac-, (R)- and (S)-methadone by approximately 50% which is consistent with its known CYP3A4 inhibitory action discussed above (Nemeroff et al., 1996; von Moltke et al., 1994; von Moltke et al., 1995a). Omeprazole was found to inhibit EDDP formation from rac-, (R)- and (S)-methadone by approximately 30%. This is consistent with its known affinity for CYP3A4 and CYP2C19 (Andersson et al., 1994b; Andersson et al., 1993; Ko et al., 1997).

In conclusion, the N-demethylation of methadone in human liver microsomes is not stereoselective, and is mediated predominantly by CYP3A, and possibly CYP2C9 and CYP2C19 to a minor extent. Thus, the large inter-individual variation reported for the pharmacokinetics of methadone may be due to variability in the expression of these CYP isoforms. The contribution of variability in the metabolism of methadone to EDDP *in vivo* will be examined in Chapter 4. The reported stereoselectivity in the clearance of methadone in humans is unlikely to be due to the partial intrinsic clearance of the N-demethylation reaction. However, the *in vitro-in vivo* prediction of the partial clearance of methadone to EDDP reported here indicate that stereoselectivity in plasma protein binding is likely to play a role. The role of stereoselectivity in the metabolism of methadone to EDDP *in vivo* will be examined in Chapter 5, which will also examine the role of stereoselectivity in plasma protein binding in the pharmacokinetics of methadone.

4. An examination of the pharmacokinetics of rac-methadone in a methadone maintenance population

4.1. Introduction

Methadone is a low hepatic extraction ratio drug of intermediate clearance, with mean values generally in the 50-200 ml.min^{-1} range. Large inter-individual variability in the clearance of methadone has been demonstrated within studies, with reported coefficients of variation of up to 70%. In otherwise healthy former opioid dependent subjects receiving chronic oral dosing of rac-methadone, apparent oral clearance (CL/F, mean±SD) values range from 107±55 ml.min^{-1} (de Vos et al., 1995) to 184±30 ml.min^{-1} (Wolff et al., 1993). Some authors have previously reported that the rate methadone metabolism increases during chronic administration (Änggård et al., 1975; Nilsson et al., 1982a; Verebely et al., 1975b). However, the evidence presented by these authors was weak, as discussed previously in sections 1.7.3.1 and 1.7.5. However, recent studies using a population pharmacokinetic approach have demonstrated that the CL/F of rac-methadone is markedly lower after acute administration in former opioid dependent subjects compared healthy subjects (Wolff et al., 1997). These authors reported that population mean±SD rac-methadone CL/F values in former opioid addicts commencing methadone maintenance treatment (53±5 ml.min^{-1}) calculated over the first 24 hour inter-dosing interval were markedly lower than that in healthy subjects (115±55 ml.min^{-1}). In contrast, population mean(±SD) apparent volume of distribution (V_c/F) values in former opioid addicts commencing methadone maintenance treatment (239±121 l) calculated over the first rac-methadone 24 hours inter-dosing interval were similar to that in healthy subjects (212±27 l). Of the covariables examined, only weight was significantly related to V_c/F in former opioid addicts, but not in healthy subjects. Furthermore, this group reported that the steady-state disposition of rac-methadone in former opioid dependent subjects is poorly predicted from data obtained after a single dose, and that there are time dependant changes in rac-methadone CL/F and V_c/F (Rostami-Hodjegan et al., 1999). Using a population pharmacokinetic approach that allowed V_c/F and CL/F to increase or decrease exponentially, the population mean V_c/F value at steady state was 123 l. The average increase in V_c/F was only 1.1-fold, however, the values in some

subjects decreased. The authors hypothesised that differential regulation of α_1-acid glycoprotein may explain this observation, such that up-regulation (causing V_c/F to decrease) or down-regulation (causing V_c/F to increase) of α_1-acid glycoprotein may explain this observation. In contrast, the population mean CL/F value at steady state (171 $ml.min^{-1}$) was over 3-fold greater than at the commencement of treatment (52 $ml.min^{-1}$). The mean half-life for the increase in CL/F was 94 hours (range: 85-206 hours), which the authors suggested was consistent with that of other compounds known cause auto-induction of metabolism, primarily via CYP3A4. Whether this phenomenon is due to "auto-induction" of its own metabolism by methadone, or increased liver function, possibly due to the increase in health status afforded by methadone maintenance therapy. However, all subjects were healthy and had normal haematological and clinical chemistry laboratory results at the commencement of treatment (Wolff et al., 1997), although this was not monitored at later time-points in either study (Rostami-Hodjegan et al., 1999; Wolff et al., 1997).

The recovery of methadone and known metabolites has failed to result in complete mass-balance, although EDDP appears to be quantitatively the major metabolite (section 1.7.3.1 and see Appendix 4). The mean urinary recovery of unchanged methadone has been reported to range from 11% to 17% of the daily maintenance dose across studies in maintenance patients. The excretion of EDDP has most often been demonstrated to account for a greater percentage of the administered dose than unchanged methadone, with mean recoveries ranging from 4% to 36% during steady-state dosing conditions. In contrast, few studies have reported on the excretion other methadone metabolites, and complete mass-balance of known methadone metabolites has not been achieved in any study to date. Plasma concentrations of EDDP have only been quantitated in a single study only, and were less than 10% that of methadone during chronic oral dosing (de Vos et al., 1995), although this was highly variable, with values ranging 2-18% over the 20 subjects studied. However, the excretion of EDDP or methadone was not examined by these authors. Therefore, the

contribution of variability in the partial clearance to EDDP to the observed variability in methadone total body clearance remains unknown.

The specific aims of the work contained in this Chapter are:

Aim 3: To investigate the steady-state pharmacokinetics of rac-methadone in a methadone maintenance population, and to examine inter-subject variability. Urinary excretion data, a comparison of the observed clearance of methadone to EDDP with that predicted using an *in vitro-in vivo* scaling model in Chapter 3, and its contribution to the total oral clearance of methadone, will be also reported.

4.2. Methods

4.2.1. Patients

Ethical approval was obtained from the Royal Adelaide Hospital Research Ethics Committee. The patients had been enrolled in the South Australian Public Methadone Maintenance Program for at least 6 months (range 6 months to 10 years) and had not had a methadone dose change for at least 2 months. There were 11 males and 7 females; body weights ranged from 60 to 94 kg (mean±SD; 74±10 kg); ages ranged from 21 to 45 years (35±7 years). The once-daily methadone dose ranged from 7.5-130 $mg.day^{-1}$, which corresponded to 0.12 to 1.9 $mg.kg^{-1}$ (0.88±0.50 $mg.kg^{-1}$). The patients were allowed to take benzodiazepines in therapeutic doses. The majority smoked cigarettes, 7 showed positive urinalysis for benzodiazepines, 10 for cannabinoids, 2 for opioids other than methadone, 1 for barbiturates, 2 for sympathomimetic amines and 4 consumed alcohol regularly in quantities less than 40 grams per day. Patients were excluded from the study if they were pregnant or had positive HIV serology. Refer to Appendix 7 for patient demographic information.

Each patient was admitted to the inpatient facility of the maintenance program 1 hour before their scheduled daily dose and remained in the unit for the subsequent 24 hours. Methadone

was administered as a syrup under supervision of the study personnel. An 18 gauge indwelling venous catheter (Jelco™, Critikon Corp, Tampa, Fla, USA) was inserted into a forearm vein prior to the daily dose and kept patent with a teflon stylet (Jelco™). A 5 ml blood sample was collected before the dose and at the following times after dosing: 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 9 and 12 hours, and at the end of the inter-dosing interval (23-24 hours). Blood was centrifuged, and the plasma stored at -20°C until analysis. In 10 subjects (patients #9-#18; 9 male, 1 female), a 24 hour pooled urine sample was also obtained, volume and pH measured and an aliquot stored at -20°C until analysis.

4.2.2. Chemicals

See section 2.2 of Chapter 2 for a description of the pharmaceutical compounds and reagents used for the quantification of rac-methadone in patient plasma samples, and rac-methadone and rac-EDDP in patient urine samples.

4.2.3. Sample Analysis

4.2.3.1. Quantification of rac-methadone in plasma

Quantification of rac-methadone in plasma was achieved using a validated HPLC assay as described in section 2.4. The ongoing performance of the assay was monitored with QC samples prepared in duplicate at three concentrations (low, LQC; medium, MQC; high, HQC) of the rac-methadone free base: 107.3 $ng.ml^{-1}$ (LQC), 178.9 $ng.ml^{-1}$ (MQC) and 715.6 $ng.ml^{-1}$ (HQC). Assay acceptance criteria were: at least seven calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of seven or more calibration standards and five or more QC samples must be less than ±15%.

4.2.3.2. Quantification of rac-methadone and rac-EDDP in urine

Quantification of rac-methadone in urine was achieved using a validated HPLC assay as described in section 2.7. The ongoing performance of the assay was monitored with QC samples prepared in duplicate at three concentrations: 0.8 μM (LQC), 2.0 μM (MQC) and

12.6 μM (HQC) for rac-EDDP, and 0.8 μM (LQC), 2.0 μM (MQC) and 16 μM (HQC) for rac-methadone. Assay acceptance criteria for all four analytes were: at least six calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of six or more calibration standards and five or more QC samples must be less than ±15%.

4.2.4. Pharmacokinetic and statistical analysis

The area under the rac-methadone concentration-time curve during the dosing interval at steady-state (AUC_{τ}^{ss}) was determined by the linear trapezoidal method. Steady-state concentrations (C_{av}^{ss}) were calculated by dividing AUC_{τ}^{ss} by the dosing interval and normalised to a 70 mg rac-methadone dose by multiplication by 70 mg and dividing by dose. Time to reach (t_{max}) maximum measured steady-state plasma concentration (C_{max}^{ss}), minimum plasma concentration pre-study dose ($C_{min(first)}^{ss}$) and 24 hours post-dose ($C_{min(last)}^{ss}$) were obtained by direct observation of the data. C_{max}^{ss}, $C_{min(first)}^{ss}$ and $C_{min(last)}^{ss}$ were normalised to a 70 mg rac-methadone dose by multiplication by 70 mg and dividing by dose. Apparent plasma clearance at steady-state (CL/F) was calculated as dose/AUC_{τ}^{ss}, renal clearance (CL_R) as amount of methadone recovered in the 0-24 hour urine sample divided by methadone AUC_{τ}^{ss}, and apparent partial clearance of methadone to EDDP ($CL_{MD \rightarrow EDDP}$) as amount of EDDP recovered in the 0-24 hour urine sample divided by methadone AUC_{τ}^{ss}, taking into consideration molecular weight differences. Percent dose recovered (f_e) was expressed as the percentage of the dose administered that was recovered in the 0-24 hour urine sample, taking into consideration molecular weight differences in the case of EDDP. Peak to trough plasma concentration ratios for each patient were calculated by dividing C_{max}^{ss} by $C_{min(last)}^{ss}$. Linear regression analysis was performed to yield Pearson's r values (GraphPad Prism v2.01, GraphPad Software, CA, USA). All data are presented as mean±SD.

4.3. Results

4.3.1. Ongoing assay performance

4.3.1.1. HPLC assays for the quantification of rac-methadone and rac-EDDP in biological fluids.

Calibration curves for rac-methadone and rac-EDDP were linear over the calibration curve concentration ranges, with r^2 values greater than 0.99 for all analytical runs in plasma (n=7, rac-methadone only) and urine (n=1, rac-methadone and rac-EDDP). Estimates of slope demonstrated no consistent time-related changes, and mean values were comparable to those obtained during assay validation (see sections 2.4 and 2.7). All analytical runs performed during the course of the experiments reported in this chapter met the acceptance criteria. The ongoing inter-assay accuracy and precision of the two methods at the three QC concentrations, and the lowest calibration standard are presented in Table 4-1 and Table 4-2.

Table 4-1: Ongoing performance of the assay for the quantification of rac-methadone in human plasma.

Inter-assay (n=7 assays)	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
Rac-methadone				0.9958 ±0.0021	0.0080 ±0.0004	7
LOQ	30.0	103.6	4.7			7
LQC	107.3	92.3	8.9			14
MQC	178.9	99.2	5.6			13
HQC	715.6	106.2	3.8			13

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

Table 4-2: Ongoing performance of the assay for the quantification of rac-methadone and rac-EDDP in human urine.

Inter-assay (n=1 assay)	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
Rac-EDDP				0.9965	0.1762	1
LOQ	0.25	98.9	-			1
LQC	0.8	93.7	3.7			2
MQC	2.0	107.8	3.4			2
HQC	12.6	94.3	0.7			2
Rac-methadone				0.9990	0.2392	1
LOQ	0.25	99.2	-			1
LQC	0.8	103.8	2.5			2
MQC	2.0	106.5	3.7			2
HQC	16.0	101.1	5.9			2

Notes: No standard deviation or precision reported as n=1 assay for slope, r^2 and LOQ values; LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

These data demonstrate that both assays continued to provide accurate and precise methods for the quantification of the enantiomers of methadone and EDDP in biological fluids. However, it should be noted that the plasma concentrations of rac-methadone determined by the racemic assay were consistently greater in three patients compared to those estimated from the sum of the individual enantiomers. This was due to co-elution of an unidentified compound with the methadone peak in the chromatograms obtained from analysis of these patients plasma samples for rac-methadone. A detailed discussion of this observation has been made previously in section 2.8. This phenomenon was discovered *post hoc*, and the plasma rac-methadone concentration data for these three subjects were retained for the present pharmacokinetic analyses.

4.3.2. Pharmacokinetics of rac-methadone

Figure 4-1 shows the inter-dosing interval mean plasma rac-methadone concentration-time profiles of the 18 subjects, normalised to a 70 mg rac-methadone dose. Pre-dose ($C^{ss}_{min(first)}$) and 24 hours post-dose ($C^{ss}_{min(last)}$) plasma concentrations were not statistically significantly different (*P* value; mean difference; 95% CI) different (*P*=0.51; 8 $ng.ml^{-1}$; -17, 33 $ng.ml^{-1}$). The mean value of plasma C^{ss}_{max} was 169% that of $C^{ss}_{min(last)}$, resulting in a mean±SD peak to trough (P/T) ratio of 1.75±0.28. The time to (t_{max}) reach C^{ss}_{max} was 2.8±1.1 hours. The

mean pharmacokinetic parameters are shown in Table 4-3, and the individual profiles for each patient are presented in

Appendix 8. There was no statistically significant effect of gender on any of the pharmacokinetic parameters derived from plasma concentration-time profiles for rac-methadone ($0.95>P>0.24$).

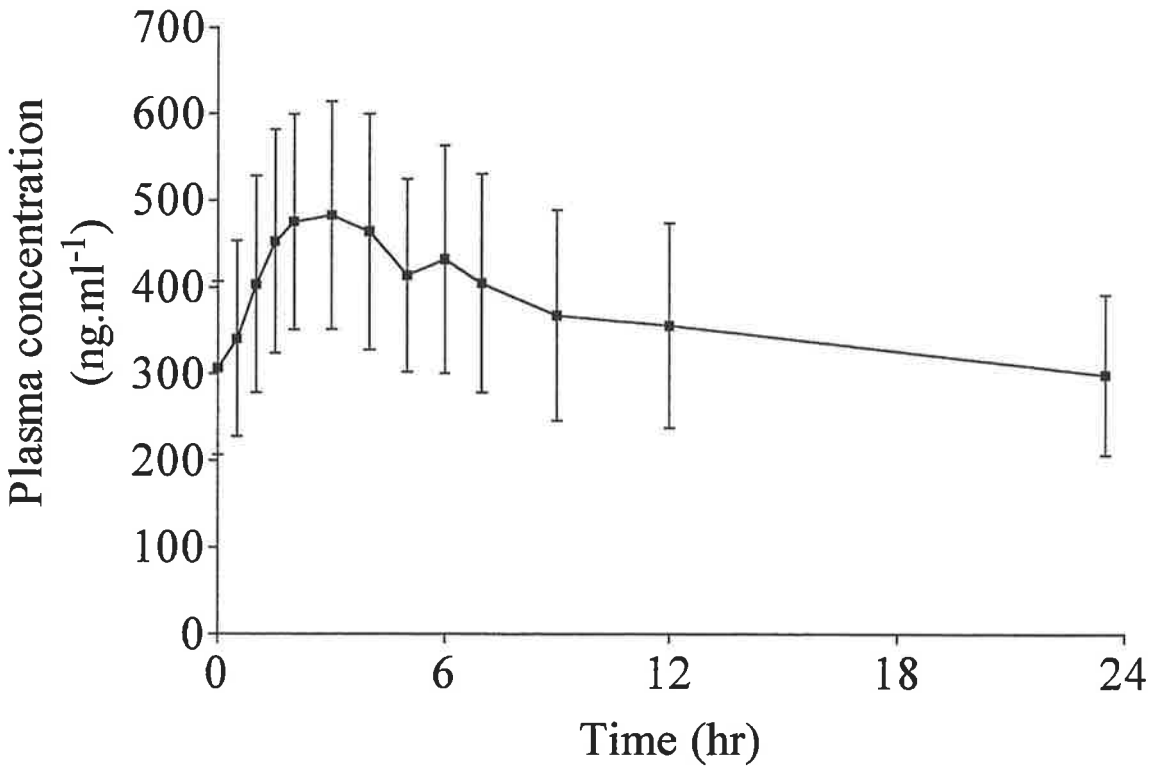


Figure 4-1: Mean plasma rac-methadone concentration-time profile for 18 methadone maintenance patients.

Notes: Data are represented as mean concentration normalised to a 70 mg rac-methadone dose. Error bars indicate SD.

Table 4-3: Disposition of total (bound plus unbound) rac-methadone following chronic dosing of between 7.5 mg and 130 mg rac-methadone once daily in 18 patients enrolled in a maintenance programme.

Parameter[1]	Mean	Range	%CV[2]
$C^{ss}_{min(first)}$ ($ng.ml^{-1}$)	307	166-499	33
$C^{ss}_{min(last)}$ ($ng.ml^{-1}$)	300	135-442	31
C^{ss}_{max} ($ng.ml^{-1}$)	510	262-735	25
C^{ss}_{av} ($ng.ml^{-1}$)	362	177-158	30
AUC^{ss}_{τ} ($ng.hr.ml^{-1}$)	7676	1210-16571	56
CL/F ($ml.min^{-1}$)	148	87-274	34
CL_R ($ml.min^{-1}$)	24.3	10.0-40.3	39
$CL_{MD\rightarrow EDDP}$ ($ml.min^{-1}$)	25.8	4.3-51.3	54
P/T	1.76	1.39-2.50	16

Notes: [1] $C^{ss}_{min(first)}$=plasma concentration, normalised to a 70 mg rac-methadone dose, pre-dose; $C^{ss}_{min(last)}$=plasma concentration, normalised to a 70 mg rac-methadone dose, 24 hours after dosing; C^{ss}_{max}=maximum measured plasma concentration, normalised to a 70 mg rac-methadone dose; C^{ss}_{av}=steady-state plasma concentration, normalised to a 70 mg rac-methadone dose; AUC^{ss}_{τ}=area under the plasma concentration-time curve during the inter-dosing interval; CL/F=apparent plasma clearance at steady-state; CL_R=renal clearance; $CL_{MD\rightarrow EDDP}$=apparent partial clearance of methadone to EDDP; P/T=peak to trough plasma concentration ratio; [2]coefficient of variation.

The excretion of unchanged rac-methadone was a mean of 77% of that obtained for rac-EDDP. However, this was not statistically significantly different (P=0.18; -4.6; -11.7, 2.6; Table 4-4), and resulted in mean±SD methadone/EDDP urinary excretion ratio of 1.01±0.7%. The mean total urinary recovery of rac-methadone and rac-EDDP was 35.3±8.4% of the dose. The sum of renal clearance and apparent partial clearance of methadone to EDDP (47.7±19.6 $ml.min^{-1}$) was a mean of 33% of CL/F for the 10 subjects from whom urine was collected.

Table 4-4: Urinary recovery during an inter-dosing interval of rac-methadone and rac-EDDP from 10 methadone maintenance patients following chronic dosing of between 7.5 mg and 130 mg rac-methadone once daily.

		% Dose recovered		
Patient #	Dose ($mg.kg^{-1}$)	rac-methadone (%)	rac-EDDP (%)	Total Dose (%)
9	1.91	14.1	23.6	37.7
10	0.33	10.0	27.3	37.3
11	0.82	17.7	19.1	36.8
12	0.30	16.6	20.9	37.4
13	0.86	19.3	25.5	44.8
14	0.77	18.9	9.0	27.9
15	0.12	9.7	4.7	14.4
16	1.41	11.5	27.5	39.0
17	0.93	23.4	14.9	38.3
18	0.68	12.8	27.1	39.8
Mean	0.88	15.4	20.0[1]	35.3
%CV[2]	57	29	40	24

Notes: [1]Not significant different (*P* value; mean difference; 95% CI) compared to rac-methadone (*P*=0.18; 4.6; -11.7, 2.6); [2]coefficient of variation.

There was a highly significant relationship between plasma rac-methadone AUC_{τ}^{ss} and dose (r^2=0.66, *P*<0.0001; Figure 4-2). The line of best fit yielded a mean (95% CI) estimate of the intercept of 1084 $ng.hr.ml^{-1}$ (-1803, 3970 $ng.hr.ml^{-1}$) with 95% confidence intervals that included zero. The estimate of the slope was 103 $ng.hr.ml^{-1}.mg^{-1}$ (64, 143 $ng.hr.ml^{-1}.mg^{-1}$).

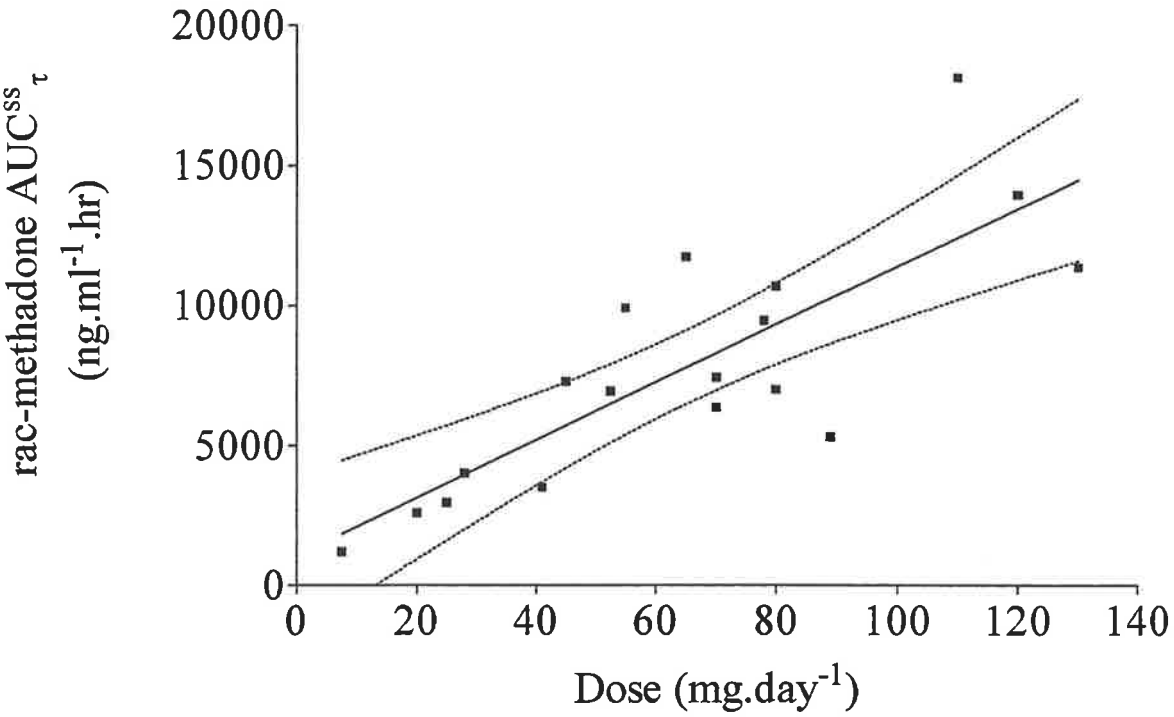


Figure 4-2: Relationship between dose and rac-methadone AUC^{ss}_{τ} for 18 methadone maintenance patients.

Notes: Dashed lines represent 95% confidence intervals of linear regression for rac-methadone (r^2=0.66, P<0.0001).

4.4. Discussion

There was substantial inter-subject variability of most rac-methadone pharmacokinetic parameters which could contribute to altered therapeutic efficacy. Recent *in vitro* studies have shown CYP3A4 to be the major CYP isoform mediating EDDP formation from rac-methadone (Iribarne et al., 1996; Iribarne et al., 1997; Moody et al., 1997) and the individual enantiomers (see Chapter 3). There was a 3- to 4-fold degree of inter-individual variability in average, trough and peak plasma rac-methadone concentrations, and renal and oral plasma clearances. The observed variability in these pharmacokinetic parameters is not solely determined by metabolic activity. However, the inter-individual variability observed in this study is consistent with reported *in vitro* (Chauret et al., 1997; Forrester et al., 1992; Shimada et al., 1994) and *in vivo* (Lown et al., 1995; Thummel et al., 1996; Thummel et al., 1994a; Thummel et al., 1994b) estimates of variability in hepatic CYP3A4 expression. Markedly higher inter-individual variability was observed for AUC^{ss}_{τ} (~12-fold) and $CL_{MD \rightarrow EDDP}$ (~10-fold). However, these parameters are also influenced by additional

factors which include in some cases dose, plasma binding, and non-renal elimination of EDDP.

As urine samples were collected over an entire inter-dosing interval, complete mass balance is possible. However, the 24 hour urinary excretion of methadone (15% of the dose) and EDDP (20%) resulted in a total recovery of only 35% of the dose administered. This finding is similar to the observations of previous investigators (see section 1.7.3), and indicates that there is significant non-renal and/or further metabolism of methadone and/or EDDP. In support of this, significant amounts of EDDP have been recovered in the faeces of methadone maintenance patients (7-10% Kreek et al., 1983; 7-23% Verebely et al., 1975a), while methadone was found to account for less than 3% (Kreek et al., 1983; Verebely et al., 1975a). N-demethylation and hydroxylation metabolites of EDDP have been reported (Änggård et al., 1975; Sullivan & Due, 1973). Although these metabolites appear to contribute very little to the metabolic profile of methadone, the metabolites identified to date have not resulted in complete mass-balance (see section 1.7.3). Indeed, Kreek and co-workers (1983; 1980a) examined the urinary and faecal excretion of methadone and several metabolites (EDDP, EMDP, methadol, p-hydroxy methadone and pyrrolidone metabolite) in five methadone maintenance patients without liver disease, and recovered 61±5% (mean±SD) of the dose. These investigators used mass-spectroscopy for quantification using authentic compounds, and scanned the entire mass range in which fragments might arise resulting from other metabolites. Hydrolysis (β-glucuronidase and sulfatase) of urine samples from three healthy patients yielded only small amounts of p-hydroxy EMDP (<1%) and p-hydroxy EDDP (<0.1%) in urine samples, and was not investigated in faeces. The presence of other metabolites, or increased concentrations of metabolites detected prior to hydrolysis were not found. These authors concluded that examination of the faecal excretion of conjugated metabolites, in particular p-hydroxy EDDP, and p-hydroxy EMDP and methadol, would be necessary to achieve complete mass-balance. Since I did not quantitate EDDP in plasma or faeces, or other possible metabolites of methadone or EDDP in urine, I am unable to examine these mechanisms further.

The predicted value of $CL_{MD \rightarrow EDDP}$ from the *in vitro* liver metabolism studies in Chapter 3 (37 ml.min^{-1}) is similar that found here *in vivo* (25 ml.min^{-1}) from urinary excretion data only. However, if one considers faecal elimination of EDDP (see section 1.7.3), *in vitro* prediction of $CL_{MD \rightarrow EDDP}$ is likely to result a marked underestimation. A possible explanation includes non-specific binding of methadone to microsomal proteins which was not examined (see Chapter 3), and may have resulted in a significant under prediction of this parameter. Other possible explanations for this under estimation include further metabolism and/or faecal elimination of methadone and EDDP as mentioned above. In support of this, the magnitude of $CL_{MD \rightarrow EDDP}$ found here is well below the total oral plasma clearance of methadone (148 ml.min^{-1}). Indeed, even if one considers the contribution of renal clearance of methadone (24 ml.min^{-1}), the sum of CL_R and $CL_{MD \rightarrow EDDP}$ (48 ml.min^{-1}) only account for a mean of 33% CL/F. These data indicate that a significant proportion of the daily dose was eliminated by alternative clearance mechanisms. However, the clearance values reported in these patients are for apparent oral clearance (CL/F), rather than total systemic clearance. Thus, the possible contribution of very low oral bioavailability values in these patients, resulting in large CL/F values, to the observed difference in the sum of CL_R and $CL_{MD \rightarrow EDDP}$ compared to CL/F cannot be excluded. However, this would appear to be an unlikely explanation, given the similarity of CL/F values reported here and those available in the literature.

Recent population pharmacokinetic modelling studies have shown that the pharmacokinetics of rac-methadone differ between single doses in healthy normal subjects and methadone maintenance patients after a single dose (Wolff et al., 1997), and that there are time-dependent changes in the clearance and volume of distribution of rac-methadone in methadone maintenance patients while steady-state is being achieved (Rostami-Hodjegan et al., 1999). The mean (range) value of 148 (87-274) ml.min^{-1} reported here is comparable to the values for the oral clearance of rac-methadone by other investigators (100-200 ml.min^{-1}, see section 1.7.3 and Appendix 3) in methadone maintenance patients at steady state.

Phenobarbital is a well established inducer of CYP3A4 expression (Ketter et al., 1995), and has been implicated in altered methadone pharmacokinetics and/or pharmacological and therapeutic effects (see section 1.7.3.2). Interestingly, a single subject (patient #5) in the present study used phenobarbital as a concomitant medication, which was confirmed by urinalysis (see Appendix 7). Surprisingly, this subject's oral clearance of rac-methadone (91 $ml.min^{-1}$) is well within the range found in the other subjects (see Table 4-3). However, this subject was not examined either before commencement or after cessation of phenobarbital use. Significant prior induction of the metabolism of rac-methadone from a low basal level may have occurred.

The highly significant relationship between plasma AUC_{τ}^{ss} and dose for rac-methadone indicates that the pharmacokinetics (extent of absorption, clearance) of rac-methadone are linear after administration of the racemate over a wide dosage range (7.5-130 $mg.day^{-1}$). This confirms previous reports of the linearity of rac-methadone pharmacokinetics (Wolff et al., 1991d). It should also be noted that this relationship was established in separate individuals taking a range of fixed doses, rather than within single individuals all given a range of dosages. This demonstrates that the extent of inter-individual variation in metabolic activity does not prevent a dose-plasma concentration relationship; indeed approximately 66% of the large inter-individual variability in the AUC_{τ}^{ss} of rac-methadone is explained by variation in dose, while the remaining variability is probably mainly due to differences in clearance.

The renal clearance of total (bound plus unbound) rac-methadone accounted for approximately 10-20% of CL/F, consistent with previous reports (Bellward et al., 1977; Inturrisi et al., 1987b; Nilsson et al., 1982b). The renal clearance of rac-methadone (range: 10-40 $ml.min^{-1}$) was between $1/12^{th}$ and $1/3^{rd}$ that of the glomerular filtration rate in a normal healthy subject, suggesting extensive net tubular reabsorption. However, the plasma unbound fraction of rac-methadone is reported to be approximately 10% (see section

1.7.2.1). Correction for plasma binding would indicate extensive net tubular secretion. The renal clearance of rac-methadone has previously been shown to be pH dependent (Bellward et al., 1977; Nilsson et al., 1982b). Examination for pH dependency of the renal clearance of rac-methadone was not possible in the present study, due to a very narrow pH range in the samples collected. Of the 10 samples, only two were below pH 6 and the remainder were within the range of 6.0-6.4 pH units.

The data presented in this study highlight that the renal clearance of methadone and the excretion of EDDP (calculated from in urine data only) do not account for a substantial proportion of the observed oral clearance, implying a significant contribution of other clearance mechanisms. The high degree of inter-individual variability observed here for the clearance of methadone is consistent with CYP3A4 mediated metabolism to the major metabolite EDDP identified in Chapter 3. However, this variability did not obscure a strong dose-plasma concentration relationship. In particular, very high inter-individual variability was observed for AUC_{τ}^{ss} (~12-fold) and $CL_{MD \rightarrow EDDP}$ (~10-fold). However, these are also influenced by additional factors which include in some cases dose, plasma binding and non-renal elimination of EDDP, which were not investigated.

Recently, a single study reported that the disposition of the (R)-enantiomer is different to that of the (S)-enantiomer, having a larger volume of distribution, longer $t_{1/2\beta}$ and higher total body clearance, although this has only been examined after single dose administration in pain patients (Kristensen et al., 1996). Limited evidence suggests that the $t_{1/2\beta}$ of (R)-methadone is greater than that of the (S)- enantiomer during chronic administration in methadone maintenance patients (Beck et al., 1991; Kreek et al., 1979; Nakamura et al., 1982). However, more detailed pharmacokinetic analyses were not performed by these authors. These data indicate that measurement of rac-methadone concentrations may not provide an accurate reflection of the relative plasma concentrations of the two enantiomers. A stereoselective difference in the magnitude of fluctuation of plasma methadone concentrations may have important implications for pharmacokinetic-pharmacodynamic

modelling of methadone, as measurement of rac-methadone may not provide an accurate reflection of plasma concentrations of the active (R)-methadone enantiomer. In contrast, therapeutic drug monitoring for patient compliance is unlikely to require stereoselective measurement of plasma methadone concentrations given the close dose-plasma concentration relationship demonstrated for rac-methadone in this study, and by others (Rostami-Hodjegan et al., 1999; Wolff et al., 1993; Wolff et al., 1991d).

The following chapter will compare the pharmacokinetics of the individual methadone enantiomers during chronic administration to methadone maintenance patients, and to examine factors which might contribute to inter-subject variability and the occurrence of stereoselectivity if observed.

5. An examination of the pharmacokinetics of (R)- and (S)-methadone in a methadone maintenance population

5.1. Introduction

Prior to the commencement of the work presented in this thesis in 1995-1996, there were only four studies available in the literature examining stereoselectivity in the disposition of methadone in humans. Limited evidence suggested the $t_{1/2\beta}$ of the two enantiomers is similar after acute administration (14-16 hours), although this was only examined after separate administration of each enantiomer in healthy subjects (Olsen et al., 1977). The disposition of the individual methadone enantiomers after chronic dosing has been investigated in only three studies in methadone maintenance patients (Beck et al., 1991; Kreek et al., 1979; Nakamura et al., 1982). The data presented by Kreek and co-workers (1979) and Nakamura and co-workers (1982) indicate that (R)-methadone (40-60 hours) has a longer $t_{1/2\beta}$ than that of (S)-methadone (30-40 hours) during chronic administration. However, these authors enrolled too few (n=2-3) subjects for definitive conclusions to be made, and did not report pharmacokinetic parameters other than $t_{1/2\beta}$. In contrast, Beck and co-workers (1991) reported similar $t_{1/2\beta}$ values only for (R)-methadone and (S)-methadone (14 hours and 16 hours, respectively), in a single methadone maintenance patient.

Since 1996, the pharmacokinetics of (R)- and (S)-methadone has been examined in detail in seven chronic pain patients after acute administration of the racemate (Kristensen et al., 1996). These authors employed a stereoselective HPLC assay for the quantification of the methadone enantiomers and collected blood samples for 48 hours after oral and intravenous administration of the racemate. A bi-exponential disposition function was fitted to the data. No differences between the bioavailability of (R)- and (S)-methadone in 10 chronic pain patients after administration of the racemate, with values in the range of 65-100% in agreement with the previous reports of racemic methadone. Unfortunately the bioavailability for three subjects was fixed at 100%, however, inspection of the data for the remaining seven subjects revealed that differences were less than 5%, and the direction of the differences was not consistent. Comparison of the absorption rate of the individual

methadone enantiomers revealed similar lag-times to the appearance of (R)-methadone (46±14 minutes) and (S)-methadone (43±14 minutes; $P>0.05$) in plasma after oral administration. Total systemic clearance of (R)-methadone (158±4 $ml.min^{-1}$) was 122% that of (S)-methadone (129±5 $ml.min^{-1}$; $P<0.008$). Similarly, $t_{1/2\beta}$ (38±8 hours) and V_{dss} (497±117 l, 6.69±1.36 $l.kg^{-1}$) values for (R)-methadone were significantly greater ($P>0.004$) than for (S)-methadone (29±11 hours; $P<0.004$ and 3.97±0.69 $l.kg^{-1}$, respectively). The influence of plasma protein binding, or metabolism to the major metabolite EDDP, in the observed stereoselective disposition of methadone was not examined by these authors.

More recently, other authors have confined their investigations to enantiomeric ratios in plasma samples taken at single time points in methadone maintenance patients, for example at the end of an inter-dosing interval. These authors have suggested that stereoselectivity in the relative concentrations of the methadone enantiomers was due to stereoselectivity in metabolism. However, other explanations are possible, such as stereoselectivity in volume of distribution. Reported ratios of (R)- to (S)-methadone concentrations have demonstrated wide inter-patient variability, with individual patients values ranging from 0.26 to 2.4 (Beck et al., 1991; de Vos et al., 1998; Eap et al., 1997; Eap et al., 1996; Kristensen & Angelo, 1992; Pham-Huy et al., 1997; Rudaz & Veuthey, 1996). Despite this wide inter-patient variability, some authors have shown that the (R)/(S) ratio is relatively constant over time within a patient during methadone maintenance treatment, with ratios not significantly different in samples collected from the same subjects one week (de Vos et al., 1998) to two weeks (Eap et al., 1996) apart in large groups (n>22) of patients.

Compared to the (S)-enantiomer, (R)-methadone has lower plasma protein binding, with α_1-acid glycoprotein being the predominant binding protein (see section 1.7.2.1). No study to date has examined the role of variability in protein binding or metabolism in the pharmacokinetics of total (R)- and (S)-methadone. It is not known whether stereoselectivity in total body clearance *in vivo* is due to metabolism (intrinsic clearance) and/or protein binding. The data presented in Chapter 3 suggest that stereoselectivity in the plasma protein

binding of methadone, but not intrinsic clearance via the N-demethylation reaction, is likely to result in a greater plasma clearance of (R)-methadone.

The specific aims of the work contained in this Chapter are:

Aim 4: To investigate the steady-state pharmacokinetics of (R)- and (S)-methadone in a methadone maintenance population, and to examine factors which might contribute to their variability. Urinary excretion data, a comparison of the observed clearance of methadone to EDDP with that predicted using an *in vitro-in vivo* scaling model in Chapter 3, and its contribution to the total oral clearance of methadone, will also be examined. Additionally, the plasma protein binding of the individual enantiomers will be examined in patient samples and solutions containing purified α_1-acid glycoprotein in order to determine its role in the disposition of (R)- and (S)-methadone.

5.2. Methods

5.2.1. Patients

Ethical approval was obtained from the Royal Adelaide Hospital Research Ethics Committee. These patients and the study protocol have been described elsewhere (see Chapter 4), in which the pharmacokinetics of rac-methadone were investigated. The work reported here used the same plasma and urine samples obtained from this cohort of patients as described in Chapter 4. Refer to Appendix 7 for patient demographic details.

5.2.2. Chemicals

Pharmaceutical compounds were obtained from the sources reported in section 2.2 of Chapter 2. Other materials were obtained from the following sources: bovine serum albumin (fraction V) and human α_1-acid glycoprotein (purified from Cohn fraction VI) were from Sigma Chemical Company (St. Louis, MO, USA). All other reagents and chemicals were obtained from commercial sources and were of analytical grade quality.

5.2.3. Sample Analysis

5.2.3.1. Quantification of (R)- and (S)-methadone in plasma, plasma ultra-filtrate and urine

Plasma, plasma ultra-filtrate and urine concentrations of (R)- and (S)-methadone were quantified using validated reversed phase HPLC assays as described in section 2.5. The ongoing performance of the assays was monitored with quality control (QC) samples prepared in duplicate at three concentrations (low, LQC; medium, MQC; high, HQC) of the (R)- and (S)-methadone free base: 54 $ng.ml^{-1}$ (LQC), 90 $ng.ml^{-1}$ (MQC) and 350 $ng.ml^{-1}$ (HQC) for plasma; 34 $ng.ml^{-1}$ (LQC), 67 $ng.ml^{-1}$ (MQC) and 366 $ng.ml^{-1}$ (HQC) for plasma ultra-filtrate; 112 $ng.ml^{-1}$ (LQC), 403 $ng.ml^{-1}$ (MQC) and 1120 $ng.ml^{-1}$ (HQC) for urine. Assay acceptance criteria for all three assays were: at least seven calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of seven or more calibration standards and five or more QC samples must be less than ±15%.

5.2.3.2. Quantification of (R)-methadone, (S)-methadone, (R)-EDDP and (S)-EDDP in urine

Quantification of the enantiomers of methadone and EDDP in urine was achieved using a validated stereoselective HPLC assay as described in section 2.6. The ongoing performance of the assay was monitored with QC samples prepared in duplicate at three concentrations: 0.4 μM (LQC), 1.0 μM (MQC) and 6.3 μM (HQC) for (R)-EDDP and (S)-EDDP, and 0.4 μM (LQC), 1.0 μM (MQC) and 8 μM (HQC) for (R)-methadone and (S)-methadone. Assay acceptance criteria for all four analytes were: at least six calibration standards must be included in the calibration curve; calibration curve r^2 value must be greater than 0.99; inaccuracy (%bias) of six or more calibration standards and five or more QC samples must be less than ±15%.

5.2.3.3. Quantification of plasma α_1-acid glycoprotein concentration

Plasma concentrations of α_1-acid glycoprotein were determined using radial immunoassay plates (Behring Diagnostics, Marburg, Germany). Measurement of the diameter of the precipitated antibody was performed using a calibrated 10x magnification eyepiece. Two mutually perpendicular measurements were taken and the average value was used for calculations. Concentrations of α_1-acid glycoprotein were calculated using the manufacturers calibration curve data sheet relating concentration to the square of the diameter of the precipitated antibody ring. Quality control samples were prepared at three concentrations (LQC: 50 $mg.dl^{-1}$, MQC: 100 $mg.dl^{-1}$, HQC: 200 $mg.dl^{-1}$) using purified human α_1-acid glycoprotein dissolved in isotonic phosphate buffer. One QC sample at each concentration was analysed on each plate concurrently with patient samples. The robustness of the analytical method was assessed by assaying replicates of each QC sample on a single plate to determine the intra-plate accuracy and precision. Inter-plate performance was determined by the analysis of each QC sample on different plates.

5.2.4. Determination of plasma (R)- and (S)-methadone unbound fraction

5.2.4.1. Ultra-filtration assay development

Determination of the unbound (non-protein bound) (R)- and (S)-methadone concentrations in plasma samples was performed using an ultra-filtration technique modified from Wilkins and co-workers (1997). Non-specific binding to the ultra-filtration device (MPS-1, Amicon, MA, USA) was investigated in order to ensure the accuracy of the results obtained using this analytical technique. One ml of a 200 $ng.ml^{-1}$ rac-methadone solution in isotonic phosphate buffer was aliquoted into the sample reservoir of assembled devices (n=2), and left to stand for 5 minutes. In addition, filtration membranes (n=2; YMT 30,000 molecular weight cut off, Amicon) were soaked in a solution of a 200 $ng.ml^{-1}$ rac-methadone solution in isotonic phosphate buffer for 5 minutes. Samples of the solutions associated with each device or membrane were collected, and 100 µl aliquots were injected onto the chromatography system for the analysis of rac-methadone in plasma (see section 2.4) without extraction. Control injections obtained after direct injection of the 200 $ng.ml^{-1}$ stock

solution were used to obtain a measure of the percent recovery of rac-methadone from each device or membrane, by comparison of peak area. Analysis of samples taken from the assembled devices (without a filtration membrane) revealed the mean±SD recovery of rac-methadone was 71±3%, indicating approximately 30% binding of rac-methadone to the device. In contrast, a 105±2% recovery of rac-methadone was found from the filtration membranes, indicating no binding had occurred.

In order to reduce the 30% non-specific binding of methadone to the device, a passivation procedure was developed based upon the manufacturer's directions. The assembled device, including the membrane, was passivated overnight with 1 ml of 60 $g.l^{-1}$ bovine serum albumin in isotonic phosphate buffer. Approximately 16 hours later, the device was rinsed three times with isotonic phosphate buffer, then re-filled with 1 ml of isotonic phosphate buffer and finally centrifuged (1500*g*) in a Beckman J2-21 centrifuge, with both the chamber and the fixed angle rotor (JA-20.1, Beckman) pre-warmed to 37°C, until the sample reservoir was devoid of buffer. The sample collection cup was then dried and replaced. One ml of a 200 $ng.ml^{-1}$ rac-methadone solution in isotonic phosphate buffer was aliquoted into the sample reservoir of the assembled devices (including filtration membrane) that had either been untreated (n=4) or passivated as outlined above (n=4) and left to stand for 5 minutes. The devices were then centrifuged (1500*g*) in a Beckman J2-21 centrifuge, with both the chamber and the fixed angle rotor (JA-20.1, Beckman) pre-warmed to 37°C, until the sample reservoir was devoid of buffer. Samples of the solutions associated with each device were collected and 100 µl aliquots were injected onto the chromatography system for the analysis of rac-methadone in plasma (see section 2.4) without extraction. Control injections obtained after direct injection of the 200 $ng.ml^{-1}$ stock solution, were used to obtain a measure of the percent recovery of rac-methadone from each device by comparison of peak area. Analysis of samples taken from the un-passivated devices revealed the mean±SD recovery of rac-methadone was 77±8.5%, indicating approximately 23% binding of rac-methadone to the device. In contrast, a 98±5.8% recovery of

rac-methadone was found from the passivated devices, indicating negligible binding had occurred.

The following technique was then used for all protein binding experiments: plasma samples were warmed to 37°C in a shaking heated water bath, and a 1 ml aliquot was then transferred to an ultra-filtration device which had been passivated and washed as described above, and pre-warmed to 37°C. Samples were then processed according to the manufacturer's directions, using a Beckman J2-21 centrifuge with both the chamber and the fixed angle rotor (JA-20.1, Beckman) pre-warmed to 37°C. Briefly, samples were centrifuged at 1500*g* for a maximum of 30 minutes or until a maximum of 400 µl of filtrate had been collected. Concentrations of (R)-and (S)-methadone in the resulting plasma ultra-filtrate were quantified using HPLC.

Initial experiments indicated unbound methadone concentrations in patient samples were below the limit of quantification of the assay for (R)- and (S)-methadone in plasma ultra-filtrate (results not shown). In order to quantify unbound methadone concentrations in these samples it was necessary to increase the concentration of total (bound plus unbound) rac-methadone in plasma to >1000 $ng.ml^{-1}$. Linearity of (R)- and (S)-methadone binding was examined in the plasma of two healthy subjects (obtained from a bank of drug-free plasma) and three methadone maintenance subjects. Due to limited sample volumes, the methadone maintenance subjects were not those reported in this thesis, rather, they were part of a cohort involved in another study within this department (Department of Clinical and Experimental Pharmacology, Adelaide University). Plasma concentrations of α_1-acid glycoprotein were comparable in all five subjects (112±35 $mg.dl^{-1}$). Plasma samples were spiked with rac-methadone at 5-6 concentrations over a 250-10000 $ng.ml^{-1}$ range of each methadone enantiomer, taking into consideration the concentration already present in the case of the methadone maintenance subjects. Unbound (R)- and (S)-methadone concentrations were determined as described above. There were no consistent concentration-related changes in the unbound fraction of (R)- or (S)-methadone over the

concentration range 250-3000 ng.ml^{-1}. However, at concentrations ranging from 3000-10000 ng.ml^{-1}, unbound fractions were consistently increased compared to the lower concentration range in each subjects plasma samples (see Figure 5-1). Linear regression analysis of the combined unbound fraction and total methadone concentration data over the 250-3000 ng.ml^{-1} range found no significant correlation for either (R)-methadone (r^2<0.001, P=0.76) or (S)-methadone (r^2<0.01, P=0.78). From these data, it is apparent that the binding of the methadone enantiomers is not saturated at total (bound plus unbound) concentrations of <3000 ng.ml^{-1} of each enantiomer in human plasma.

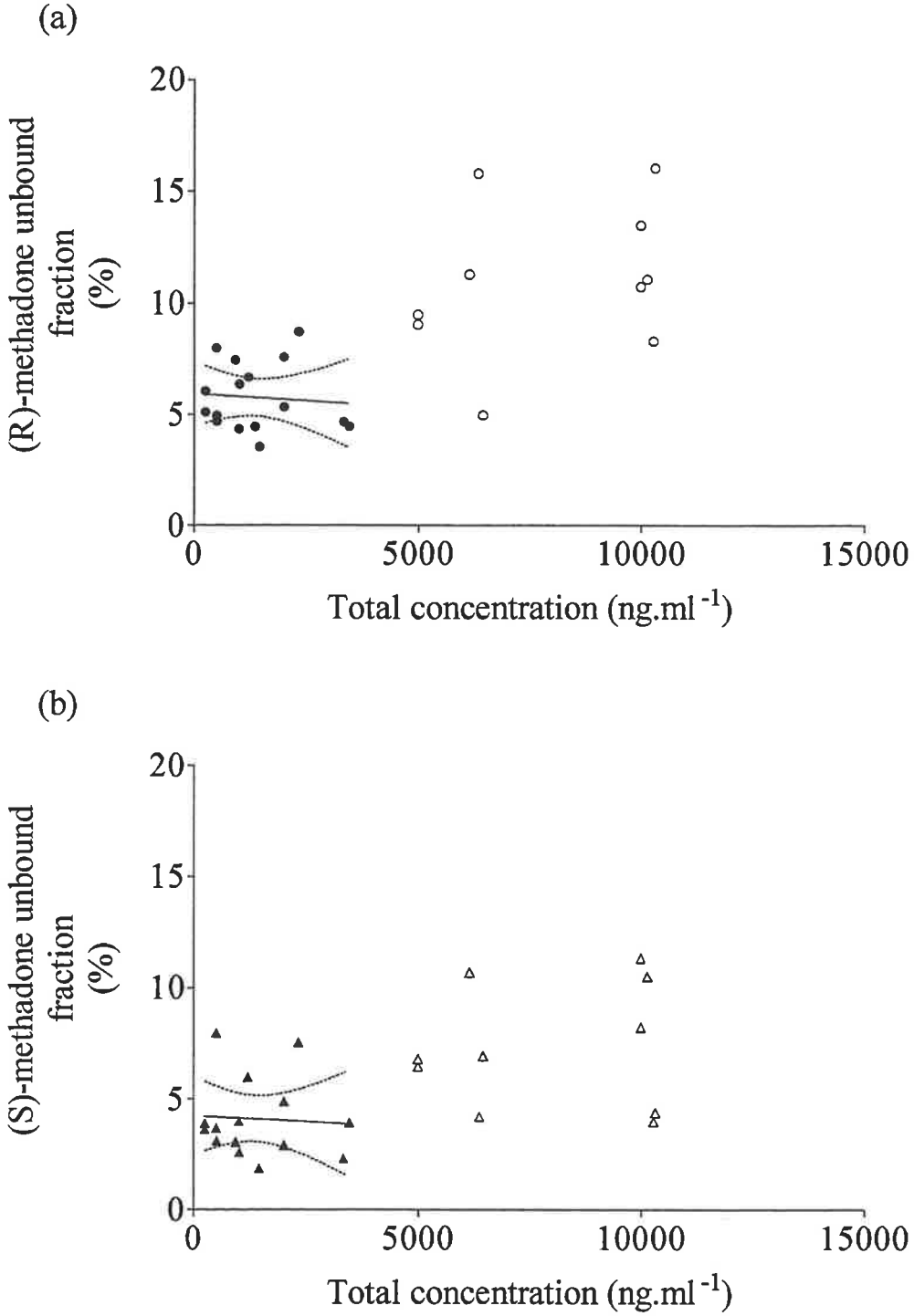


Figure 5-1: Relationship of unbound fraction and total (bound plus unbound) concentration of (R)-methadone and (S)-methadone in the plasma of two healthy subjects and three methadone maintenance patients.

Notes: Solid lines indicate line of best fit, and dashed lines are 95% confidence intervals for (a) (R)-methadone (r^2<0.001, P=0.76) and (b) (S)-methadone (r^2<0.001, P=0.78) linear regression analysis of concentration data <3000 ng.ml^{-1} (closed symbols); open symbols represent data >3000 ng.ml^{-1} which were not included in regression analysis.

5.2.4.2. Binding of (R)- and (S)-methadone to purified α_1-acid glycoprotein *in vitro*

The *in vitro* binding of (R)- and (S)-methadone to purified α_1-acid glycoprotein was also investigated. Solutions of α_1-acid glycoprotein in isotonic phosphate buffer were prepared at three concentrations (50, 100 and 200 $mg.dl^{-1}$), and spiked with concentrated rac-methadone solutions in isotonic phosphate buffer to contain 100, 250, 500, 750 and 1000 $ng.ml^{-1}$ of each enantiomer. The design of the experiment was such that the following samples were prepared: 50 $mg.dl^{-1}$ α_1-acid glycoprotein (750 $ng.ml^{-1}$ (R)- and (S)-methadone, n=2), 100 $mg.dl^{-1}$ α_1-acid glycoprotein (100, 250, 500, 750 and 1000 $ng.ml^{-1}$ (R)- and (S)-methadone, n=2 at each concentration), 200 $mg.dl^{-1}$ α_1-acid glycoprotein (750 $ng.ml^{-1}$ (R)- and (S)-methadone, n=2). Ultra-filtrate samples were collected as outlined above (see section 5.2.4.1), and the concentrations of (R)- and (S)-methadone were quantified using HPLC.

5.2.4.3. Analysis of patient samples

Quantification of the unbound concentration of (R)- and (S)-methadone in patient samples was achieved by the addition of 10-15 µl of a concentrated rac-methadone solution in isotonic phosphate buffer to a pooled 1.2 ml plasma sample obtained from each subjects' set of plasma samples. The volume of methadone solution was chosen to yield a rac-methadone concentration of 1500 $ng.ml^{-1}$, taking into consideration the original methadone concentration present in the sample. A 100 µl aliquot was taken, diluted with 900 µl of blank plasma and assayed for total (bound plus unbound) (R)- and (S)-methadone to accurately determine the concentration of total (bound plus unbound) (R)- and (S)-methadone. Plasma ultra-filtrate was collected from the remaining sample as outlined above (see section 5.2.4.1), and the concentrations of (R)- and (S)-methadone were quantified using HPLC.

5.2.5. Pharmacokinetic and statistical analysis

(R)- and (S)-methadone unbound fraction (f_u) was calculated as plasma ultra-filtrate concentration divided by total (protein bound and non-protein bound) plasma concentration, and was expressed as a percentage. Area under the (R)- and (S)-methadone concentration-time curve during the dosing interval at steady-state (AUC_τ^{ss}) was determined by the linear trapezoidal method. Steady-state concentrations (C_{av}^{ss}) were calculated by dividing AUC_τ^{ss} by the dosing interval and normalised to a 70 mg rac-methadone (35 mg each enantiomer) dose by multiplication by 35 mg and dividing by enantiomer dose. Time to reach (t_{max}) maximum measured steady-state plasma concentration (C_{max}^{ss}), minimum plasma concentration pre-study dose ($C_{min(first)}^{ss}$) and 24 hours post-dose ($C_{min(last)}^{ss}$) were obtained by direct observation of the data. C_{max}^{ss}, $C_{min(first)}^{ss}$ and $C_{min(last)}^{ss}$ were normalised to a 70 mg rac-methadone (35 mg each enantiomer) dose by multiplication by 35 mg and dividing by enantiomer dose. Apparent plasma clearance at steady-state (CL/F) was calculated as dose/AUC_τ^{ss}, renal clearance (CL_R) as amount of methadone recovered in the 0-24 hour urine sample divided by methadone AUC_τ^{ss}, and apparent partial clearance of methadone to EDDP ($CL_{MD \rightarrow EDDP}$) as amount of EDDP recovered in the 0-24 hour urine sample divided by methadone AUC_τ^{ss}, taking into consideration molecular weight differences. Percent dose recovered (f_e) was expressed as the percentage of the dose administered (assuming an equal amount of each enantiomer in the racemic dose) that was recovered in the 0-24 hour urine sample, taking into consideration molecular weight differences in the case of EDDP. These pharmacokinetic parameters were also calculated with respect to the unbound enantiomers ($C_{max_u}^{ss}$, $C_{min(first)_u}^{ss}$, $C_{min(last)_u}^{ss}$, $AUC_{\tau_u}^{ss}$, $C_{av_u}^{ss}$, CL_u/F, CL_{R_u} and $CL_{MD \rightarrow EDDP_u}$), by either multiplying or dividing the corresponding parameters by f_u. Peak to trough plasma concentration ratios for each patient were calculated by dividing C_{max}^{ss} by $C_{min(last)}^{ss}$. Ratios of (R)- to (S)-methadone were calculated for $C_{min(first)}^{ss}$ and $C_{min(last)}^{ss}$, and the resultant parameters corrected for plasma binding, by the division of (R)-methadone by (S)-methadone unbound fraction values. Additionally, ratios of total and unbound (R)- to (S)-methadone concentrations were calculated separately at the t_{max} of (R)-methadone (R_{max}^{ss}/S^{ss}, $R_{max_u}^{ss}/S_u^{ss}$) and the t_{max} of (S)-methadone (R^{ss}/S_{max}^{ss}, $R_u^{ss}/S_{max_u}^{ss}$) by the division of

(R)-methadone C^{ss}_{max} by the corresponding (S)-methadone concentration at (R)-methadone t_{max}, and the division of the (R)-methadone concentration at the (S)-methadone t_{max} by (S)-methadone C^{ss}_{max}, respectively. Ratios of plasma (R)- to (S)-methadone concentrations were also calculated at each time point. Statistically significant differences between enantiomers were assessed using paired t-tests, while other comparisons were performed using un-paired t-tests, and linear regression analysis was performed to yield Pearson's r values (GraphPad Prism v2.01, GraphPad Software, CA, USA). Mean ratios of (R)- to (S)-methadone concentrations calculated at each time point were compared to a theoretical value of 1 using a 2-tailed 1-sample t-test. Differences were considered significant at $P<0.05$. All data are presented as mean±SD.

Scatchard plots (ratio of bound/unbound concentration versus bound concentration) were constructed for the binding of 100-1000 $ng.ml^{-1}$ (R)- and (S)-methadone to a 100 $mg.dl^{-1}$ solution of purified human α_1-acid glycoprotein, and further analysed using non-linear regression analysis (GraphPad Prism v3.02) according to the technique of Hervé and co-workers (1996) and Abramson (1982) for a single binding site (Equation 5-1):

Equation 5-1: One site protein binding equation.

$$B = \frac{N \times Ka \times U}{1 + (Ka \times U)}$$

Where B is the bound concentration of the ligand, N is the molar binding site concentration, Ka is the association constant, and U is the unbound concentration of the ligand. The number of binding sites per binding protein molecule (n) is calculated by $n=N/P$, where P is the molar concentration of the binding protein.

5.3. Results

5.3.1. Ongoing assay performance

5.3.1.1. HPLC assays for the enantiomers of methadone and EDDP in biological fluids.

Calibration curves for all four assays were linear over the calibration curve concentration ranges, with r^2 values greater than 0.99 for all analytical runs. Estimates of slope demonstrated no consistent time-related changes, and mean values were comparable to those obtained during assay validation (see sections 2.5 and 2.6). All analytical runs performed during the course of the experiments reported in this chapter met the acceptance criteria. The ongoing inter-assay accuracy and precision of the four methods at the three QC concentrations, and the lowest calibration standard are presented in Table 5-1 through to Table 5-4.

Table 5-1: Ongoing performance of the assay for the quantification of (R)-methadone and (S)-methadone in human plasma.

Inter-assay (n=11 assays)	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(R)-methadone				0.9980 ±0.0019	0.0034 ±0.0005	11
LOQ	15	101.3	4.5			10
LQC	54	105.7	6.2			22
MQC	90	103.8	6.8			22
HQC	350	101.0	4.7			22
(S)-methadone				0.9973 ±0.0031	0.0035 ±0.0005	11
LOQ	15	102.4	3.2			10
LQC	54	102.0	7.0			21
MQC	90	104.2	4.9			22
HQC	350	103.3	5.6			22

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

Table 5-2: Ongoing performance of the assay for the quantification of (R)-methadone and (S)-methadone in human plasma ultra-filtrate.

Inter-assay (n=2 assays)	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(R)-methadone				0.9924 ±0.0026	0.0011 ±0.0001	2
LOQ	12.5	109.0	2.4			2
LQC	34	98.4	4.3			4
MQC	67	99.8	2.5			4
HQC	366	105.4	2.2			4
(S)-methadone				0.9947 ±0.0030	0.0012 ±0.0001	2
LOQ	12.5	108.2	0.2			2
LQC	34	101.1	12.2			4
MQC	67	95.1	3.9			4
HQC	366	103.3	0.9			4

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

Table 5-3: Ongoing performance of the assay for the quantification of (R)-methadone and (S)-methadone in human urine.

Inter-assay (n=2 assays)	Nominal concentration ($ng.ml^{-1}$)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(R)-methadone				0.9966 ±0.0008	0.0013 ±0.0001	2
LOQ	50	100.6	1.6			2
LQC	112	92.3	5.3			4
MQC	403	91.6	1.4			4
HQC	1120	88.2	3.0			4
(S)-methadone				0.9988 ±0.0006	0.0014 ±0.0001	2
LOQ	50	101.7	1.7			2
LQC	112	94.6	7.4			4
MQC	403	92.5	4.2			4
HQC	1120	88.6	2.4			4

Notes: LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

Table 5-4: Ongoing performance of the assay for the quantification of (R)-EDDP, (S)-EDDP, (R)-methadone and (S)-methadone in human urine.

Inter-assay (n=1 assay)	Nominal concentration (μM)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(R)-EDDP				0.9991	0.1720	1
LOQ	0.125	104.2	-			1
LQC	0.4	91.7	6.8			2
MQC	1.0	110.7	2.5			2
HQC	6.3	94.4	0.1			2
(S)-EDDP				0.9976	0.1646	1
LOQ	0.125	108.3	-			1
LQC	0.4	86.4	3.6			2
MQC	1.0	111.8	4.9			2
HQC	6.3	96.2	0.1			2
(R)-methadone				0.9991	0.2369	1
LOQ	0.125	102.9	-			1
LQC	0.4	100.6	1.6			2
MQC	1.0	108.7	7.7			2
HQC	8	104.2	6.9			2
(S)-methadone				0.9999	0.2318	1
LOQ	0.125	99.4	-			1
LQC	0.4	104.1	0.5			2
MQC	1.0	107.5	3.8			2
HQC	8	101.1	7.1			2

Notes: No standard deviation or precision reported as n=1 assay for slope, r^2 and LOQ values; LOQ=limit of quantification; LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

These data demonstrate that all four assays continued to provide accurate and precise methods for the quantification of the enantiomers of methadone and EDDP in biological fluids. It should be noted that the urinary concentrations of (R)- and (S)-methadone used in the pharmacokinetic analysis were obtained using the assay for the simultaneous quantification of (R)-EDDP, (S)-EDDP, (R)-methadone and (S)-methadone in human urine. The assay for (R)- and (S)-methadone only in urine samples (see Table 5-3) was utilised for assay validation only (see section 2.8), and performance data are reported here for completeness.

5.3.1.2. Quantification of α_1-acid glycoprotein

Analysis of samples containing only isotonic phosphate buffer resulted in no observable precipitate ring. The inter- and intra-assay accuracy and precision of the method at the three

QC concentrations are shown in Table 5-5, and demonstrate the assay to be precise and accurate.

Table 5-5: Inter- and intra-assay accuracy and precision for the quantification of α_1-acid glycoprotein in human plasma.

	Nominal concentration ($mg.dl^{-1}$)	Accuracy (%)	Precision (%)	n
Inter-assay (n=4 plates)				
LQC	50	-0.4	3.7	10
MQC	100	-3.4	4.2	10
HQC	200	0.8	3.8	10
Intra-assay				
LQC	50	-3.6	2.2	4
MQC	100	-7.8	1.8	4
HQC	200	-1.0	4.6	4

Notes: LQC=low quality control sample; MQC=medium quality control sample; HQC=high quality control sample.

5.3.2. Protein binding

Scatchard plots demonstrated the binding of (R)- and (S)-methadone to 100 $mg.dl^{-1}$ purified α_1-acid glycoprotein to be linear over the 100-1000 $ng.ml^{-1}$ concentration range of total (bound plus unbound) drug for (R)-methadone (r^2=0.90, P<0.0001) and (S)-methadone (r^2=0.91, P<0.0001), consistent saturable binding to a single binding site (Figure 5-2). The unbound fraction of (R)- and (S)-methadone increased from a mean±SD (n=2) of 10.6±1.9% and 6.0±0.8% at 250 $ng.ml^{-1}$ of each enantiomer, respectively, to 24.7±0.9% and 13.3±0.3% at 1000 $ng.ml^{-1}$ of each enantiomer, respectively.

Both (R)- and (S)-methadone binding was well described by the single binding site model, with r^2 values of 0.9926 and 0.9971, respectively (Figure 5-3). These analyses resulted in *n*, *N* (95% CI) and *Ka* (95% CI) values of 0.13, 3.3 μM (2.9-3.8 μM) and $2.5x10^6$ M^{-1} ($1.8-3.2x10^6$ M^{-1}) for (R)-methadone, and 0.15, 3.9 μM (3.5-4.1 μM) and $4.9x10^6$ M^{-1} ($4.1-5.7x10^6$ M^{-1}) for (S)-methadone.

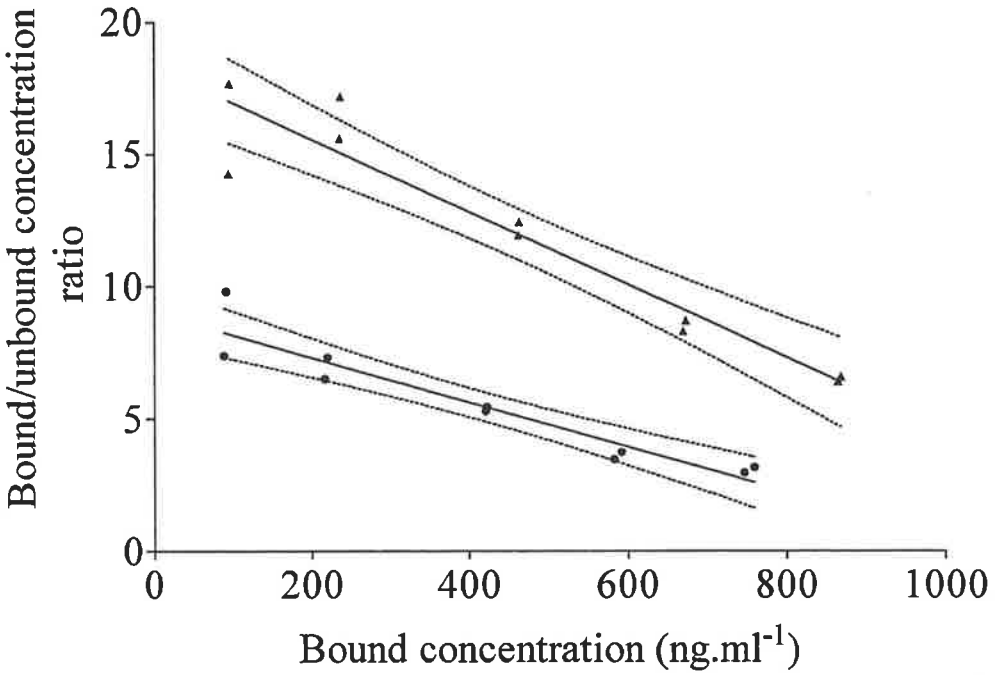


Figure 5-2: Scatchard plot of (R)- and (S)-methadone binding to 100 mg.dl^{-1} purified human α_1-acid-glycoprotein.

Notes: Dashed lines represent 95% confidence intervals of linear regression analysis for (●) (R)-methadone (r^2=0.90, P<0.0001) and (▲) (S)-methadone (r^2=0.91, P<0.001).

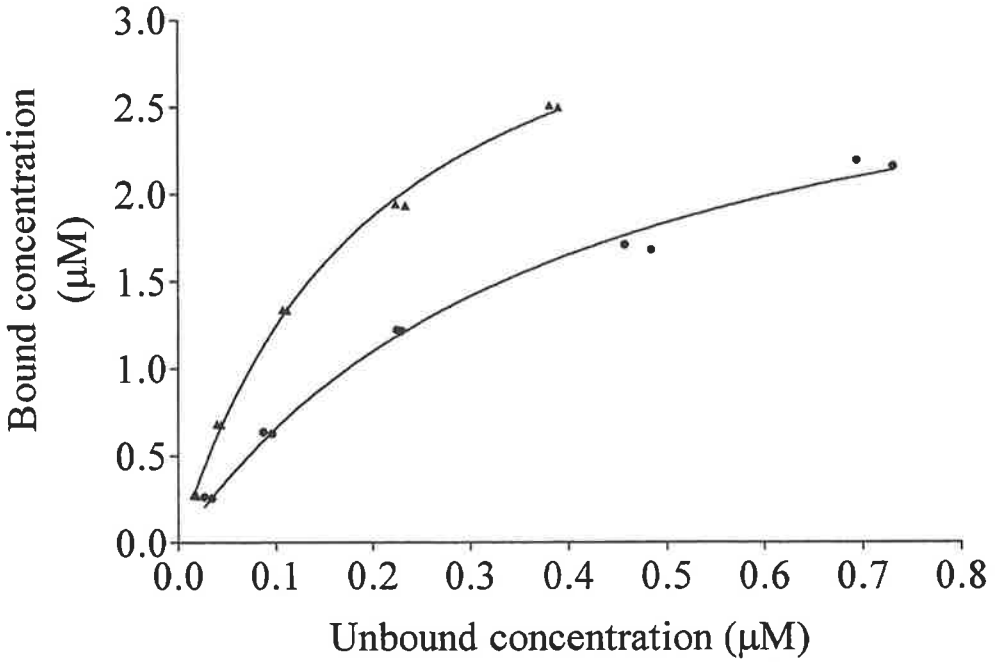


Figure 5-3: Single binding site analysis of (R)-methadone and (S)-methadone binding to 100 mg.dl^{-1} purified human α_1-acid-glycoprotein.

Notes: Solid line represents line of best fit of non-linear regression analysis for (●) (R)-methadone (r^2=0.9926) and (▲) (S)-methadone (r^2=0.9971).

Figure 5-4 shows the effect of increasing concentrations (50-200 mg.dl^{-1}) of purified human α_1-acid glycoprotein on (R)- and (S)-methadone unbound fraction at a total (bound plus unbound) concentration of 750 ng.ml^{-1} of each enantiomer.

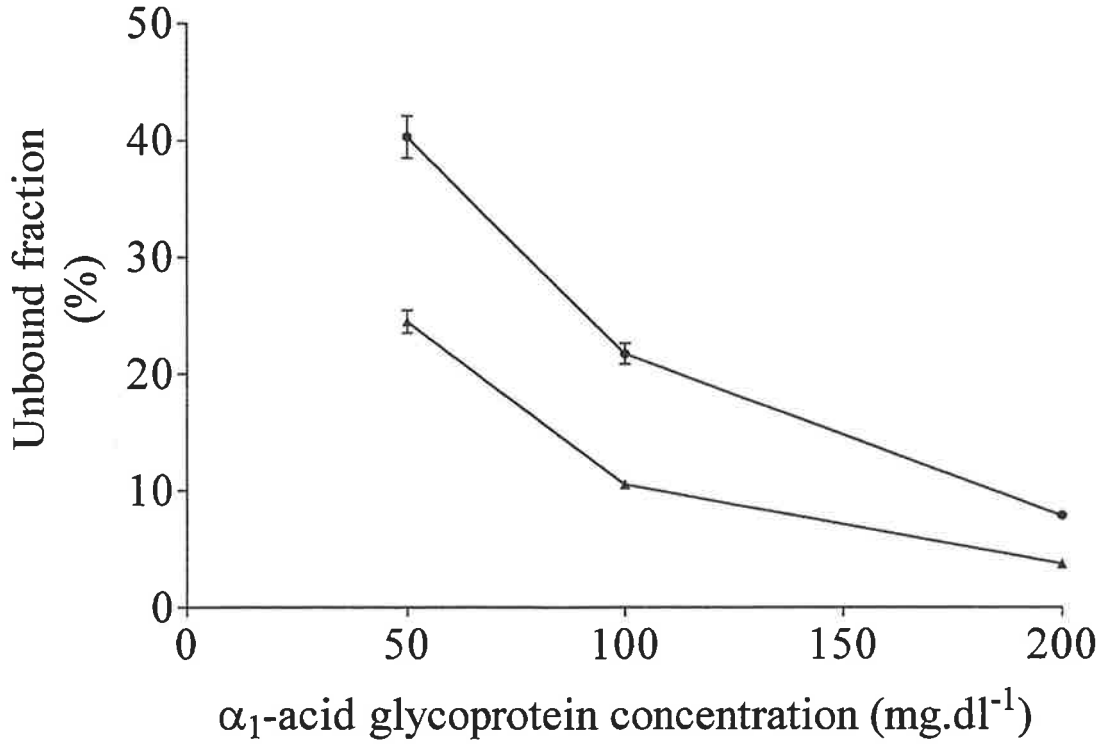


Figure 5-4: Binding of 750 ng.ml^{-1} (R)-methadone and (S)-methadone to 50-200 mg.dl^{-1} purified human α_1-acid-glycoprotein.
Notes: Data are represented mean values (n=2) and error bars indicate SD for (●) (R)-methadone and (▲) (S)-methadone.

Analysis of the patient samples spiked to contain approximately 1500 ng.ml^{-1} of total (bound plus unbound) rac-methadone revealed mean±SD concentrations of 668±27 ng.ml^{-1} and 723±37 ng.ml^{-1} for (R)- and (S)-methadone, respectively, corresponding to a rac-methadone concentration of 1391±47 ng.ml^{-1}. The difference in spiked concentrations of (R)- and (S)-methadone was statistically significant (P<0.0001; -55 ng.ml^{-1}; -78, -33 ng.ml^{-1}), although the magnitude was <10% of the mean concentration of (R)-methadone. The plasma unbound fractions of (R)-methadone were 171% those of (S)-methadone (P<0.0001; Table 5-7). There was no significant difference in unbound fractions between males and females for (R)-methadone (3.62±0.98% versus 3.69±0.65%,

respectively; *P*=0.88; -0.07%; -0.89, 1.02%) or (S)-methadone (2.15±0.58% versus 2.04±0.42%, respectively; *P*=0.69; 0.11%; -0.69, 0.47%).

The mean concentration of α_1-acid glycoprotein in plasma samples from the patients was 115±24 $mg.dl^{-1}$ (range: 73-155 $mg.dl^{-1}$). These concentrations were not significantly different (*P*=0.68; 5 $mg.dl^{-1}$; -30, 20 $mg.dl^{-1}$) between males (117±25 $mg.dl^{-1}$; range: 79-155 $mg.dl^{-1}$) and females (112±23 $mg.dl^{-1}$; range: 73-150 $mg.dl^{-1}$).

There was a weak but significant relationship between plasma α_1-acid glycoprotein concentration and ratio of bound/unbound methadone concentrations in the patient samples for (R)-methadone (r^2=0.31; *P*=0.019; Figure 5-5) and (S)-methadone (r^2=0.30; *P*=0.021; Figure 5-5). When corrected for the molecular weight of the binding protein (approximately 40000, Kremer et al., 1988; Routledge, 1986) the slope of the linear relationship of these data provide an estimate of the product of the number of binding sites per α_1-acid glycoprotein molecule (*n*) and the association constant (*Ka*) of the binding protein (Kremer et al., 1988; Routledge, 1986). These analyses resulted in *nKa* values of $7.9x10^5$ M^{-1} and $13.0x10^5$ M^{-1} for (R)- and (S)-methadone, respectively. Assuming 0.3 sites per α_1-acid glycoprotein molecule (Abramson, 1982; Eap et al., 1990; Hervé et al., 1996), the resulting *Ka* values for (R)- and (S)-methadone are $2.6x10^6$ M^{-1} and $4.3x10^6$ M^{-1}, respectively. Similarly, y-intercept values for (R)-methadone and (S)-methadone provide an estimate of the degree of protein binding in the absence of α_1-acid glycoprotein (Abramson, 1982). These analyses resulted in estimated bound fractions of 84% and 92% for (R)- and (S)-methadone, respectively, in the absence of α_1-acid glycoprotein.

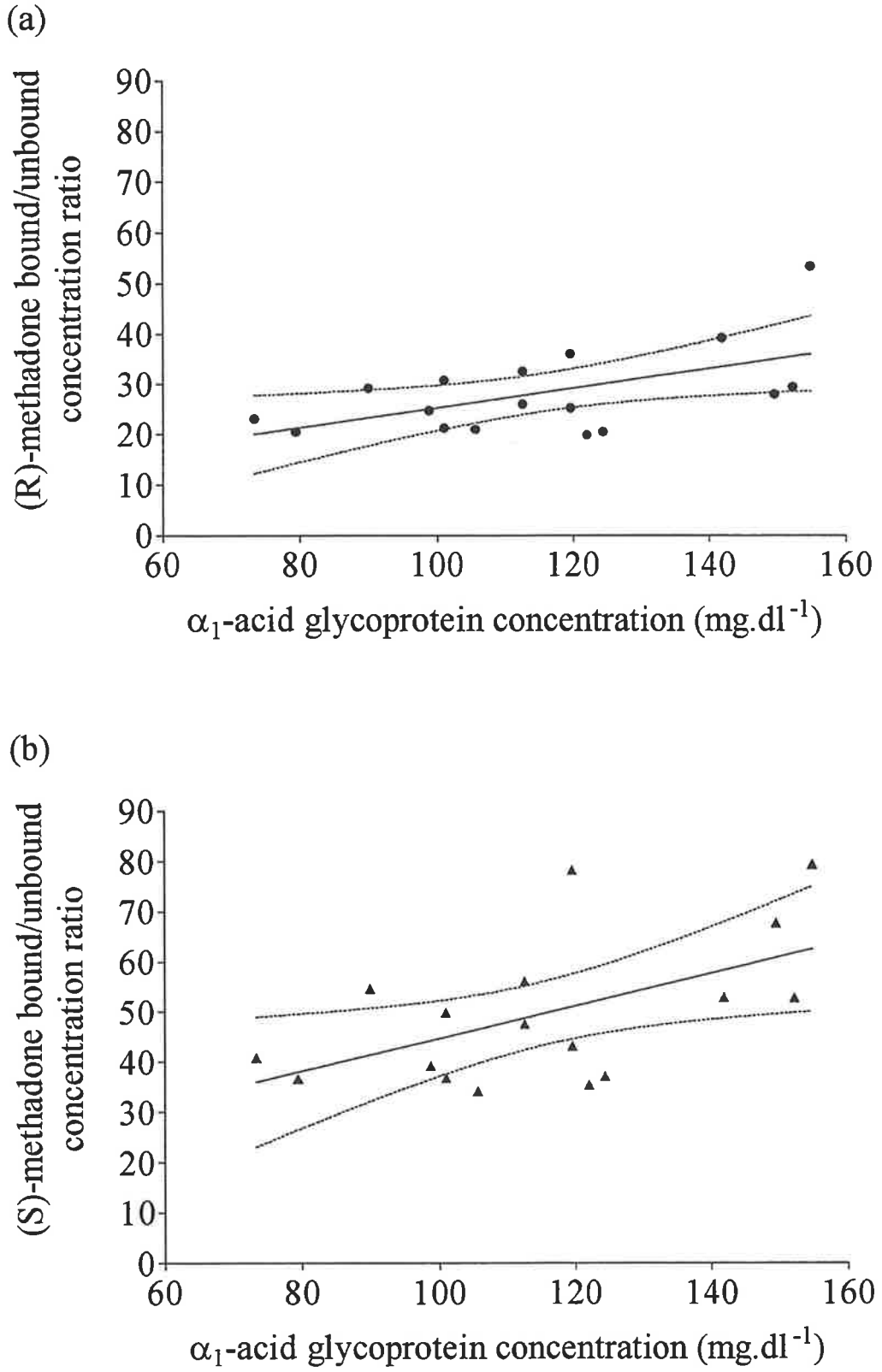


Figure 5-5: Relationship between plasma α_1-acid glycoprotein concentration and plasma (R)-methadone and (S)-methadone bound/unbound concentration ratio for 18 methadone maintenance patients.

Notes: Dashed lines represent 95% confidence intervals of linear regression for (a) (R)-methadone (r^2=0.31, *P*=0.019) and (b) (S)-methadone (r^2=0.30, *P*=0.021). The x-axis has been truncated to maintain a consistent scale.

5.3.3. Pharmacokinetics of (R)- and (S)-methadone

Figure 5-6 shows the inter-dosing interval mean plasma (R)- and (S)-methadone concentration-time profile of the 18 subjects, normalised to a 70 mg rac-methadone dose. The individual profiles for each patient are presented in Appendix 8.

Pre-dose ($C^{ss}_{min(first)}$) and 24 hours post-dose ($C^{ss}_{min(last)}$) plasma concentrations were not statistically significantly different (*P* value; mean difference; 95% CI) for (R)-methadone (*P*=0.86; -1 $ng.ml^{-1}$; -11, 9 $ng.ml^{-1}$) or (S)-methadone (*P*=0.31; 7 $ng.ml^{-1}$; -7, 21 $ng.ml^{-1}$). Similarly, $C^{ss}_{min(first)}$ (*P*=0.55; Table 5-6) and $C^{ss}_{min(last)}$ (*P*=0.95; Table 5-6) values were not statistically significantly different between the two enantiomers. In contrast, (R)-methadone C^{ss}_{max} values were on average 83% those of (S)-methadone (*P*=0.0002; Table 5-6). When corrected for protein binding, mean (R)-methadone $C^{ss}_{min(first)_u}$, $C^{ss}_{min(last)_u}$ and $C^{ss}_{max_u}$ values were between 170% and 175% those of (S)-methadone (*P*<0.0001; Table 5-7). The peak to trough plasma concentration ratio of (R)-methadone was a mean of 79% of that obtained for (S)-methadone (*P*=0.0001; Table 5-6). (R)-methadone t_{max} (3.1±1.9 hr) values were significantly (*P*=0.0072; 0.6 hr; 0.2, 1.1 hr) longer than for (S)-methadone (2.4±1.6 hr).

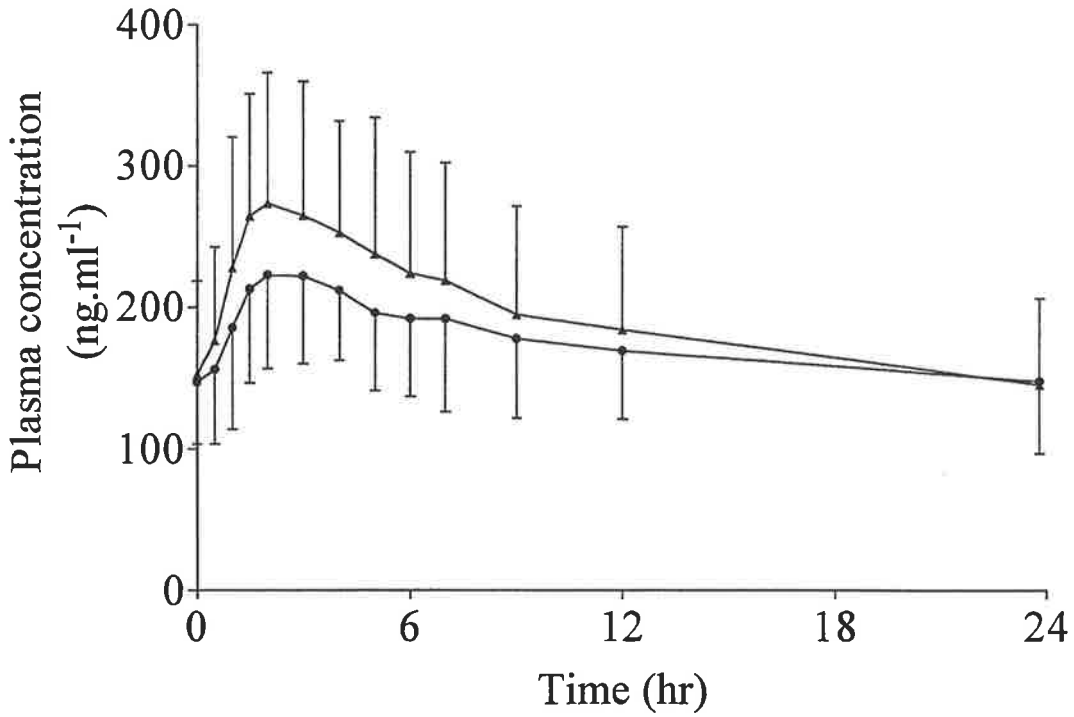


Figure 5-6: Mean plasma (R)-methadone and (S)-methadone concentration-time profiles for 18 methadone maintenance patients.

Notes: Data are represented as mean concentration normalised to a 70 mg rac-methadone dose for (●) (R)-methadone and (▲) (S)-methadone. Error bars indicate SD.

Table 5-6: Disposition of total (bound plus unbound) (R)- and (S)-methadone following chronic dosing of between 7.5 mg and 130 mg rac-methadone once daily in 18 patients enrolled in a maintenance programme.

Parameter[1]		Mean	Range	%CV[2]	*P* value[3] (mean difference; 95% CI)
$C^{ss}_{min(first)}$ (ng.ml^{-1})	(R)-	147	42 - 223	30	0.55
	(S)-	153	31 - 261	43	(-6; -24, 14)
$C^{ss}_{min(last)}$ (ng.ml^{-1})	(R)-	148	40 - 243	34	0.78
	(S)-	146	31 - 242	42	(2; -15, 20)
C^{ss}_{max} (ng.ml^{-1})	(R)-	251	120 - 362	27	0.0002
	(S)-	303	116 - 447	32	(-52; -74, -29)
C^{ss}_{av} (ng.ml^{-1})	(R)-	168	67 - 237	29	0.0571
	(S)-	186	52 - 304	37	(-18; -36, 1)
AUC^{ss}_{τ} (ng.hr.ml^{-1})	(R)-	3484	608 - 7263	53	0.21
	(S)-	3797	589 - 7229	55	(-313; -814, 188)
CL/F (ml.min^{-1})	(R)-	161	103 - 363	42	0.86
	(S)-	159	79 - 465	60	(2; -18, 21)
CL_R (ml.min^{-1})	(R)-	27.4	11.9 - 46.6	37	<0.0001
	(S)-	15.1	7.0 - 26.5	39	(12.4; 9.2, 15.5)
$CL_{MD\rightarrow EDDP}$ (ml.min^{-1})	(R)-	21.9	3.6 - 37.3	48	0.0088
	(S)-	28.8	5.5 - 58.9	55	(-6.9; -11.6, 2.2)
P/T	(R)-	1.81	1.35 - 3.58	30	<0.0001
	(S)-	2.30	1.68 - 4.49	34	(-0.49; -0.68, -0.29)

Notes: [1] $C^{ss}_{min(first)}$=plasma concentration, normalised to a 70 mg rac-methadone dose, pre-dose; $C^{ss}_{min(last)}$=plasma concentration, normalised to a 70 mg rac-methadone dose, 24 hours after dosing; C^{ss}_{max}=maximum measured plasma concentration, normalised to a 70 mg rac-methadone dose; C^{ss}_{av}=steady-state plasma concentration, normalised to a 70 mg rac-methadone dose; AUC^{ss}_{τ}=area under the plasma concentration-time curve during the inter-dosing interval; CL/F=apparent plasma clearance at steady-state; CL_R=renal clearance; $CL_{MD\rightarrow EDDP}$=apparent partial clearance of methadone to EDDP; P/T=peak to trough plasma concentration ratio; [2]coefficient of variation; [3]comparison of (R)-methadone versus (S)-methadone.

Table 5-7: Disposition of unbound (R)- and (S)-methadone following chronic dosing of between 7.5 mg and 130 mg rac-methadone once daily in 18 patients enrolled in a maintenance programme.

Parameter[1]		Mean	Range	%CV[2]	*P* value[3] (mean difference; 95% CI)
f_u (%)	(R)-	3.6	1.8 - 4.8	24	<0.0001
	(S)-	2.1	1.2 - 2.8	25	(1.5; 1.3, 1.8)
$C^{ss}_{min(first)_u}$ ($ng.ml^{-1}$)	(R)-	5.3	2.0 – 8.0	33	<0.0001
	(S)-	3.1	0.8 – 5.9	46	(2.2; 1.6, 2.8)
$C^{ss}_{min(last)_u}$ ($ng.ml^{-1}$)	(R)-	5.4	1.9 – 7.8	33	<0.0001
	(S)-	3.1	0.8 – 5.5	43	(2.3; 1.7, 2.9)
$C^{ss}_{max_u}$ ($ng.ml^{-1}$)	(R)-	8.9	4.3 – 13.4	29	<0.0001
	(S)-	6.2	2.8 – 9.2	34	(2.7; 2.0, 3.5)
$C^{ss}_{av_u}$ ($ng.ml^{-1}$)	(R)-	6.0	2.3 – 11.0	34	<0.0001
	(S)-	3.8	1.4 – 6.1	40	(2.3; 1.6, 2.9)
$AUC^{ss}_{\tau_u}$ ($ng.hr.ml^{-1}$)	(R)-	117	28 - 268	51	0.0003
	(S)-	70	16 - 126	46	(47; 26, 68)
CL_u/F ($ml.min^{-1}$)	(R)-	4611	2214 - 10590	46	0.0001
	(S)-	7845	3994 - 17750	51	(-3235; -4590, -1879)
CL_{R_u} ($ml.min^{-1}$)	(R)-	847	256 - 1832	48	0.63
	(S)-	819	265 - 1444	44	(28; -100, 155)
$CL_{MD\rightarrow EDDP_u}$ ($ml.min^{-1}$)	(R)-	727	77 - 1890	70	0.0017
	(S)-	1656	207 - 3506	66	(-930; -1406, -453)

Notes: [1]f_u=unbound fraction; $C^{ss}_{min(first)_u}$=unbound plasma concentration, normalised to a 70 mg rac-methadone dose, pre-dose; $C^{ss}_{min(last)_u}$=unbound plasma concentration, normalised to a 70 mg rac-methadone dose, 24 hours after dosing; $C^{ss}_{max_u}$=maximum measured unbound plasma concentration, normalised to a 70 mg rac-methadone dose; $C^{ss}_{av_u}$=steady-state plasma concentration of unbound drug, normalised to a 70 mg rac-methadone dose; $AUC^{ss}_{\tau_u}$=area under the plasma unbound concentration-time curve during the inter-dosing interval; CL_u/F=apparent plasma clearance at steady-state of the unbound drug; CL_{R_u}=renal clearance of the unbound drug; $CL_{MD\rightarrow EDDP_u}$=apparent partial intrinsic clearance of methadone to EDDP; [2]coefficient of variation; [3]comparison of (R)-methadone versus (S)-methadone.

The ratio of plasma (R)- to (S)-methadone concentrations pre-dose ($R^{ss}_{min(first)}/S^{ss}_{min(first)}$) was not significantly different (P=0.58; Table 5-8) to that obtained at the end of the dosing interval ($R^{ss}_{min(last)}/S^{ss}_{min(last)}$). Similarly, unbound plasma (R)- to (S)-methadone concentration ratios at pre-dose ($R^{ss}_{min(first)_u}/S^{ss}_{min(first)_u}$) and at the end of the dosing interval ($R^{ss}_{min(last)_u}/S^{ss}_{min(last)_u}$) were not significantly different (P=0.52; Table 5-8). In contrast, ratios of (R)- to (S)-methadone calculated separately at the t_{max} of (R)-methadone (R^{ss}_{max}/S^{ss}) and (S)-methadone (R^{ss}/S^{ss}_{max}) were significantly lower compared to $R^{ss}_{min(last)}/S^{ss}_{min(last)}$ (P<0.0001 and P=0.0002, respectively; Table 5-8). Similarly, ratios of unbound (R)- to (S)-methadone calculated separately at the t_{max} of (R)-methadone ($R^{ss}_{max_u}/S^{ss}_{u}$) and (S)-methadone ($R^{ss}_{u}/S^{ss}_{max_u}$) were significantly lower compared to $R^{ss}_{min(last)_u}/S^{ss}_{min(last)_u}$ (P=0.0003 and P=0.0008, respectively; Table 5-8). The ratios of (R)- to (S)-methadone concentrations were significantly lower than unity at 1 (P=0.0003; -0.16; -0.24, -0.09), 1.5 (P<0.0001; -0.18; -0.24, -0.12), 2 (P=0.0004; -0.16; -0.24, -0.08), 3 (P=0.0053; -0.12; -0.20, -0.04) and 4 (P=0.0139; -0.12; -0.22, -0.03) hours post-dose (Figure 5-7), but not at other times (0.94>P>0.14; Figure 5-7).

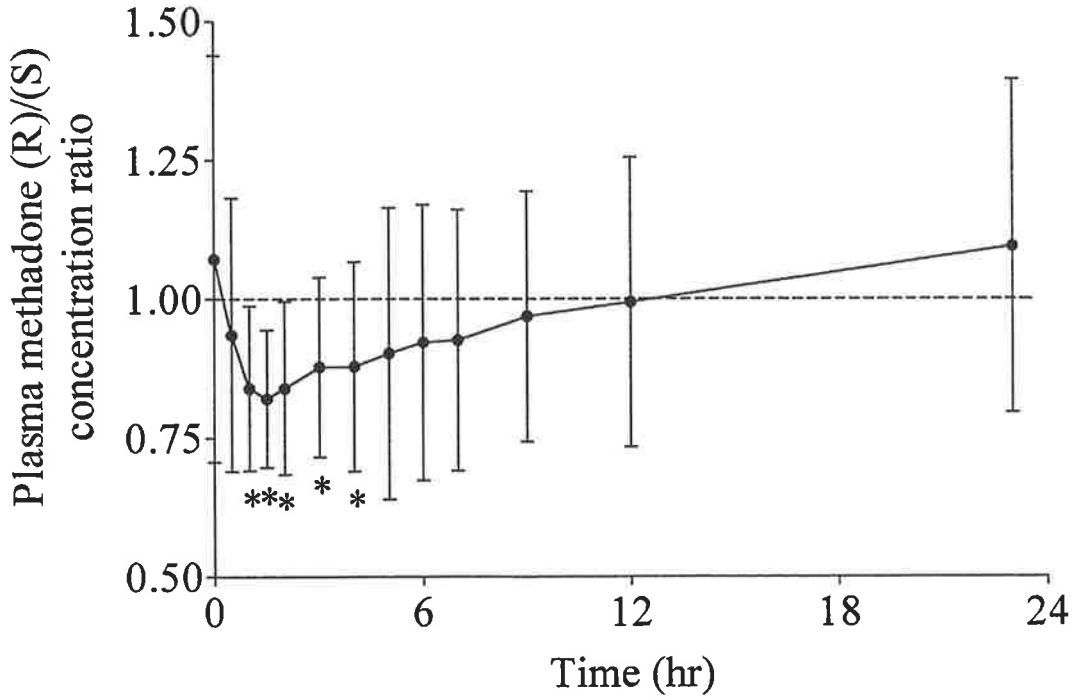


Figure 5-7: Mean plasma methadone (R)/(S) concentration ratio versus time profiles for 18 methadone maintenance patients.
Notes: *Statistically significantly (P<0.02) different to unity. Error bars indicate SD. The y-axis has been truncated to enlarge the scale.

Table 5-8: (R)/(S) enantiomeric ratio at peak and trough concentrations following chronic dosing of between 7.5 mg and 130 mg rac-methadone once daily in 18 patients enrolled in a maintenance programme.

Parameter[1]	Mean	Range	%CV[2]	*P* value[3] (mean difference; 95% CI)
$R^{ss}_{min(first)}/S^{ss}_{min(first)}$	1.07	0.68 – 2.28	34	0.57 (-0.02; -0.10, 0.06)
$R^{ss}_{min(last)}/S^{ss}_{min(last)}$	1.10	0.70 – 1.97	27	
R^{ss}_{max}/S^{ss}	0.88	0.68 – 1.40	19	<0.0001 (-0.21; -0.30, -0.13)
R^{ss}/S^{ss}_{max}	0.84[4]	0.66 – 1.07	15	0.0002 (-0.26; -0.38, -0.15)
$R^{ss}_{min(first)_u}/S^{ss}_{min(first)_u}$	1.91	1.20 – 4.11	38	0.52 (-0.05; -0.20, 0.10)
$R^{ss}_{min(last)_u}/S^{ss}_{min(last)_u}$	1.96	1.24 – 3.54	33	
$R^{ss}_{max_u}/S^{ss}_{u}$	1.55	1.09 – 2.52	26	0.0003 (-0.41; -0.60, -0.22)
$R^{ss}_{u}/S^{ss}_{max_u}$	1.45[5]	1.09 – 2.04	20	0.0008 (-0.51; -0.77, -0.25)

Notes: [1] $R^{ss}_{min(first)}/S^{ss}_{min(first)}$=ratio of (R)- to (S)-methadone plasma concentrations pre-dose; $R^{ss}_{min(last)}/S^{ss}_{min(last)}$=ratio of (R)- to (S)-methadone plasma concentrations 24 hours after dosing; R^{ss}_{max}/S^{ss}=ratio of (R)-methadone C^{ss}_{max} and the corresponding (S)-methadone concentration at (R)-methadone t_{max}; R^{ss}/S^{ss}_{max}=ratio of (R)-methadone concentration at the (S)-methadone t_{max} and (S)-methadone C^{ss}_{max}; $R^{ss}_{min(first)_u}/S^{ss}_{min(first)_u}$=ratio of (R)- to (S)-methadone plasma concentrations of the unbound drug pre-dose; $R^{ss}_{min(last)_u}/S^{ss}_{min(last)_u}$=ratio of (R)- to (S)-methadone plasma concentrations of the unbound drug 24 hours after dosing; $R^{ss}_{max_u}/S^{ss}_{u}$=ratio of (R)-methadone $C^{ss}_{max_u}$ and the corresponding (S)-methadone concentration of the unbound drug at (R)-methadone t_{max}; $R^{ss}_{u}/S^{ss}_{max_u}$=ratio of (R)-methadone concentration of the unbound drug at the (S)-methadone t_{max} and (S)-methadone $C^{ss}_{max_u}$; [2]coefficient of variation; [3]comparison within each group versus the parameter with a blank cell in the *P* value column; [4]statistically significant difference (*P* value; mean difference; 95% CI) compared to R^{ss}_{max}/S^{ss} (*P*=0.043; 0.048; 0.002, 0.094); [5]statistically significant difference compared to $R^{ss}_{max_u}/S^{ss}_{u}$ (*P*=0.047; 0.098; 0.002, 0.195).

There was a highly significant relationship between plasma AUC^{ss}_{τ} and dose for (R)-methadone (r^2=0.68, *P*<0.0001; Figure 5-8) and (S)-methadone (r^2=0.47, *P*=0.002; Figure 5-8). The line of best fit yielded a mean (95% CI) estimate of the intercept of 610 $ng.hr.ml^{-1}$ (-565, 1784 $ng.hr.ml^{-1}$) for (R)-methadone and 1132 $ng.hr.ml^{-1}$ (-571, 2834 $ng.hr.ml^{-1}$) for (S)-methadone, with 95% confidence intervals that included zero for both enantiomers. The estimate of the slope was 89 $ng.hr.ml^{-1}.mg^{-1}$ (57,

121 ng.hr.ml^{-1}.mg^{-1}) for (R)-methadone and 82 ng.hr.ml^{-1}.mg^{-1} (36, 129 ng.hr.ml^{-1}.mg^{-1}) for (S)-methadone.

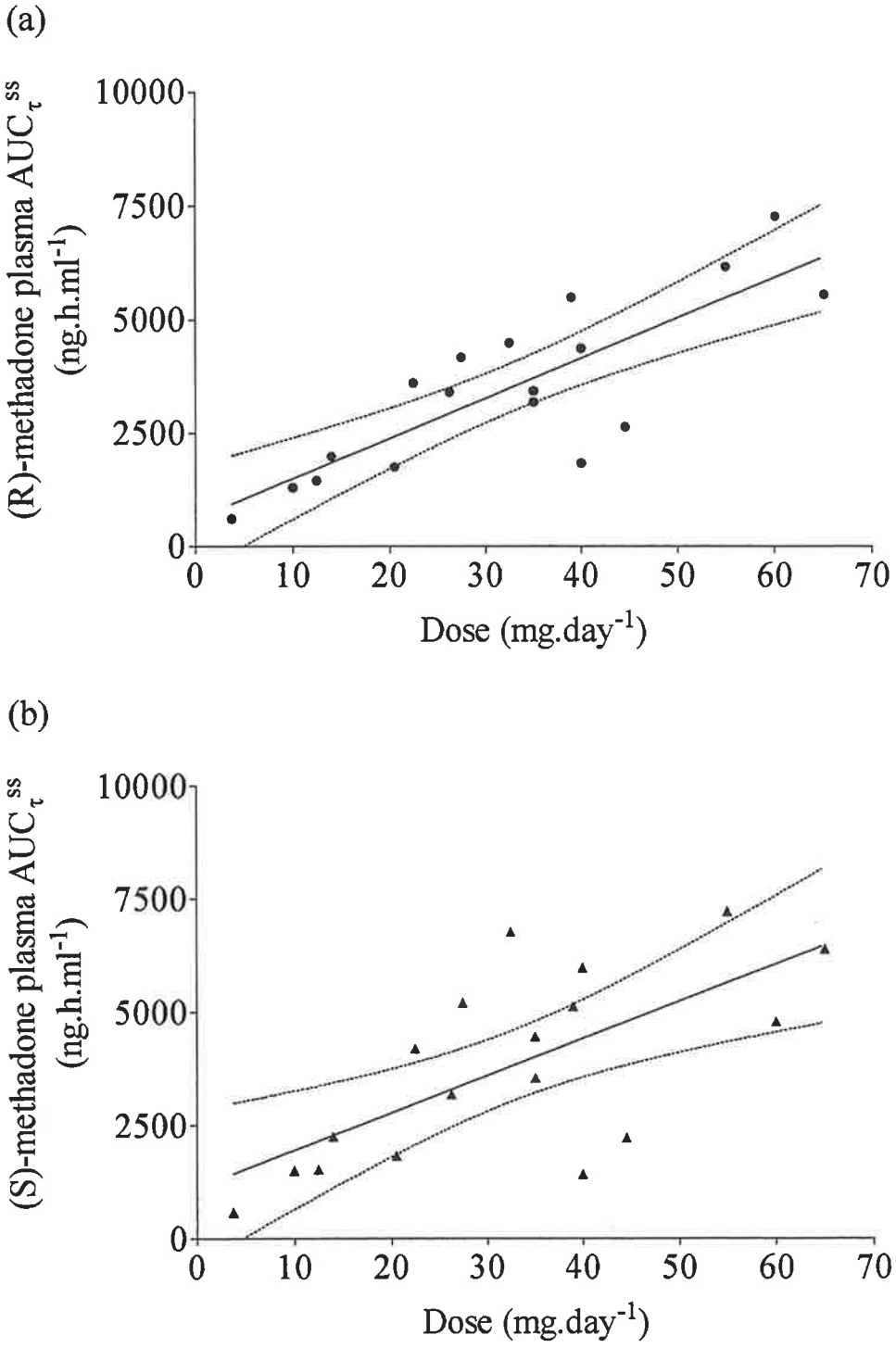


Figure 5-8: Relationship between dose and (a) (R)-methadone AUC_τ^{ss} and (b) (S)-methadone AUC_τ^{ss} for 18 methadone maintenance patients.

Notes: Dashed lines represent 95% confidence intervals of linear regression for (a) (R)-methadone (r^2=0.68, P<0.0001) and (b) (S)-methadone (r^2=0.47, P=0.002).

The plasma AUC_{τ}^{ss} of (R)-methadone was not statistically significantly different (P=0.21; Table 5-6) from the value for (S)-methadone. (R)-methadone C_{av}^{ss} was an average of 90% that obtained for (S)-methadone (P=0.0571; Table 5-6), but this was only of borderline statistical significance. However, when corrected for protein binding plasma $AUC_{\tau_u}^{ss}$ (P=0.0003; Table 5-7) and $C_{av_u}^{ss}$ (P<0.0001; Table 5-7) of (R)-methadone were on average 167% and 158% of those obtained for (S)-methadone, respectively.

The renal clearance of total (bound plus unbound) (R)-methadone was a mean of 182% that of (S)-methadone (P<0.0001; Table 5-6). After correcting for protein binding, there was no significant difference for CL_{R_u} between (R)-methadone and (S)-methadone (P=0.63; Table 5-7). The excretion of unchanged (R)-methadone was a mean of 160% that of (S)-methadone (P<0.0001; Table 5-9). Conversely, the excretion of (R)-EDDP was a mean of 68% of that obtained for (S)-EDDP (P<0.0001; Table 5-9). These differences resulted in a significantly (P=0.0008; -1.36; -1.99, -0.73) lower urinary ratio of EDDP/methadone for the (R)- enantiomer (0.93±0.50) compared to the (S)-enantiomer (2.29±1.37). However, there was no significant difference in excretion for the sum of methadone and EDDP of the (R)- enantiomers (17.8±4.2%) compared to the (S)- enantiomers (18.1±5.0%; P=0.75; -0.3%; -1.9, 1.5%), or between the sum of (R)- and (S)-methadone (15.6±4.9%) and (R)- and (S)-EDDP (20.3±8.2%; P=0.17; -4.8%, -12.0, 2.5%). The mean total urinary recovery of (R)- and (S)-methadone and (R)- and (S)-EDDP was 35.9±8.9% of the dose.

Table 5-9: Urinary recovery during an inter-dosing interval of the enantiomers of methadone and EDDP from 10 methadone maintenance patients following chronic dosing of between 7.5 mg and 130 mg rac-methadone once daily.

		% Dose recovered				
Patient #	Dose ($mg.kg^{-1}$)	(R)-methadone (%)	(S)-methadone (%)	(R)-EDDP (%)	(S)-EDDP (%)	Total Dose (%)
9	1.91	8.6	5.3	9.8	14.2	37.9
10	0.33	6.1	3.7	10.7	16.6	37.1
11	0.82	10.6	7.8	8.2	11.5	38.1
12	0.30	10.7	6.5	8.9	12.9	39.0
13	0.86	13.3	6.7	10.8	15.2	46.0
14	0.77	12.0	7.2	3.8	5.3	28.3
15	0.12	5.8	3.3	1.9	2.9	13.9
16	1.41	7.3	4.3	11.0	16.6	39.2
17	0.93	13.7	10.1	6.5	8.6	38.9
18	0.68	8.0	4.6	10.6	17.4	40.6
Mean	0.88	9.6	6.0[1]	8.2	12.1[2]	35.9
%CV[3]	57	30	35	39	41	25

Notes: [1]Statistically significant difference (*P* value; mean difference; 95% CI) compared to (R)-methadone ($P<0.0001$; -3.6; -4.6, -2.7); [2]statistically significant difference compared to (R)-EDDP ($P=0.0001$; 3.9; 2.5, 5.3); [3]coefficient of variation.

The apparent plasma clearance with respect to total (bound plus unbound) methadone was not significantly different for (R)-methadone and (S)-methadone ($P=0.86$; Table 5-6). When corrected for protein binding, (R)-methadone CL_u/F was on average 59% that of (S)-methadone ($P=0.0001$; Table 5-7). The apparent partial clearance of (R)-methadone to (R)-EDDP was on average 76% of the clearance of (S)-methadone to (S)-EDDP ($P=0.0088$; Table 5-6). When corrected for protein binding, apparent partial intrinsic clearance of (R)-methadone to (R)-EDDP was on average 44% of the clearance of (S)-methadone to (S)-EDDP ($P=0.0017$; Table 5-7). The sum of renal clearance and apparent partial clearance of methadone to EDDP for (R)-methadone (93.7±21.0 $ml.min^{-1}$) was a mean of 111% of that for (S)-methadone (84.4±20.0 $ml.min^{-1}$) for the 10 subjects from

whom urine was collected, although this was of borderline statistical significance (P=0.0522, 5.4; -0.1, 10.8).

There was no statistically significant effect of gender on any of the pharmacokinetic parameters derived from plasma concentration-time profiles for either (R)-methadone ($0.92>P>0.10$) or (S)-methadone ($0.83>P>0.10$).

5.4. Discussion

This is the first study to comprehensively describe the disposition of methadone in a large cohort of methadone maintenance patients, with respect to stereoselectivity. There was substantial stereoselectivity of most pharmacokinetic parameters, and also considerable inter-subject variability, which could contribute to altered therapeutic efficacy. The significantly lower CL_u/F for (R)-methadone, which describes the elimination of the unbound pharmacologically active methadone enantiomer, indicates that (R)-methadone has a lower intrinsic clearance when compared to (S)-methadone. Further, there was a significantly greater fraction of the dose excreted in the urine as (S)-EDDP and (R)-methadone than the corresponding enantiomers, which resulted in a stereoselective difference in the urinary ratio of EDDP/methadone concentrations, suggesting significantly less (R)-methadone than (S)-methadone was metabolised to EDDP. Recently, others have made similar observations based upon the relative concentrations of the enantiomers of methadone and EDDP in urine samples collected at single time-points only (Angelo et al., 1999; Lanz & Thormann, 1996), although they were unable to examine this further as urine samples were not collected throughout the entire inter-dosing interval. Stereoselectivity in the renal clearance of methadone is unlikely to explain this phenomenon, as there was no difference in the renal clearance of unbound methadone. In support of this, stereoselectivity was found in the apparent partial clearance to EDDP ($CL_{MD\rightarrow EDDP}$), and apparent partial intrinsic clearance to EDDP ($CL_{MD\rightarrow EDDP_u}$), with (R)-methadone values significantly lower than for (S)-methadone (44% and 76%, respectively). The reference to "apparent" is used as calculations were based on urinary excretion data only and EDDP is eliminated to some

extent in faeces (Kreek et al., 1983; Verebely et al., 1975a), and further metabolised to a limited, albeit poorly defined, extent (Änggård et al., 1975; Sullivan & Due, 1973). There was no significant difference between the enantiomers in the sum of methadone and EDDP, or between the sum of (R)- and (S)-methadone and (R)- and (S)-EDDP. These data show that important characteristics of drug metabolism would fail to be observed using non-chiral analytical techniques. These data are in contrast to the results of *in vitro* metabolism studies with methadone which found no stereoselectivity in the intrinsic clearance of methadone to EDDP (see Chapter 3). The *in vitro-in vivo* scaling calculations in Chapter 3 resulted in a predicted $CL_{MD \rightarrow EDDP}$ value for (R)-methadone of 38 ml.min^{-1} compared to 27 ml.min^{-1} for (S)-methadone. This stereoselective difference in this parameter arose from the correction for plasma protein binding, which was shown to be statistically significantly different between the two enantiomers, but not intrinsic metabolic activity ($CL_{MD \rightarrow EDDP_u}$). The predicted role of stereoselectivity is in contrast to that found here *in vivo*, in which both the $CL_{MD \rightarrow EDDP_u}$ and $CL_{MD \rightarrow EDDP}$ of (R)-methadone was significantly lower than for (S)-methadone. Stereoselectivity in the plasma protein binding of methadone is an unlikely explanation, as stereoselectivity was observed in both $CL_{MD \rightarrow EDDP}$ and $CL_{MD \rightarrow EDDP_u}$. However, reasons for this lack of agreement include the possibility that EDDP may be eliminated by other metabolic pathways which display stereoselectivity, and/or that there is stereoselectivity in the binding of methadone to proteins in the *in vitro* liver microsomal fraction, which may mask any stereoselectivity in the metabolism by the enzymes mediating the formation of EDDP. Similarly, a stereoselective difference in oral bioavailability is an alternative explanation. However, this would appear to be unlikely, given that the lack of stereoselectivity in the bioavailability of methadone after single dose administration reported previously (Kristensen et al., 1996), and in CL/F and the sum of CL_R and $CL_{MD \rightarrow EDDP}$ reported here. It is possible that there may be stereoselectivity in the renal clearance and/or in the elimination of EDDP via faeces, such that the combined urinary and faecal recovery of (R)-EDDP is greater than that of (S)-EDDP. Evidence for this can be found when the sum of CL_R and $CL_{MD \rightarrow EDDP}$ for the individual enantiomers is compared to total oral clearance values. For both enantiomers, the sum of CL_R and $CL_{MD \rightarrow EDDP}$

accounted for a mean of only 33% of CL/F and the absolute values were similar in magnitude for each enantiomer. These data indicate that a significant proportion of the daily dose was eliminated by alternative clearance mechanisms, although it does not provide information regarding possible stereoselectivity. In support of this, significant amounts of EDDP have been recovered in the faeces of methadone maintenance patients (7-10% Kreek et al., 1983; 7-23% Verebely et al., 1975a), while methadone was found to account for less than 3% (Kreek et al., 1983; Verebely et al., 1975a). N-demethylation and hydroxylation metabolites of EDDP have been reported (Änggård et al., 1975; Sullivan & Due, 1973). Although these metabolites appear to contribute very little to the metabolic profile of methadone, it is possible that there is stereoselectivity in the metabolism of EDDP to these other metabolites. I did not quantitate EDDP in plasma or faeces, or other possible EDDP metabolites in urine, so am unable to examine these mechanisms further. Despite this, if one assumes that EDDP is not eliminated by other metabolic pathways which display marked net stereoselectivity, and that there is no stereoselectivity in the microsomal protein binding of methadone, then comparison of these *in vivo* and *in vitro* data indicate that the net non-renal elimination (faecal elimination and/or further metabolism) of (R)-EDDP must be greater than that of (S)-EDDP.

It is also noteworthy that the predicted values of $CL_{MD \rightarrow EDDP}$ from the *in vitro* studies in Chapter 3 are well below those found *in vivo* here from urinary excretion data only, if one considers the faecal elimination of EDDP as discussed above. Importantly, the predicted value for $CL_{MD \rightarrow EDDP}$ from the *in vitro* assumed plasma unbound fraction of 14% and 10% for (R)- and (S)-methadone, respectively. However, the observed values in this group of patients were 4- and 5-fold lower for (R)-methadone (3.6%) and for (S)-methadone (2.1%). If these values were substituted into the *in vitro-in vivo* model, they would result in an increase in the predicted stereoselective difference discussed above, and in the magnitude of the under-prediction noted here. A possible explanation includes non-specific binding of methadone to microsomal proteins which was not examined (see Chapter 3), and may have resulted in a significant under prediction of this parameter. Similarly, the magnitude of

$CL_{MD \rightarrow EDDP}$ found here is well below the total oral plasma clearance of methadone, even if one considers the minor contribution of renal clearance. Possible explanations for this under estimation include further metabolism and/or faecal elimination of methadone and EDDP as discussed above. It is possible that the combination these processes may provide a clearance mechanism of significant magnitude, which may account for this disparity. The clearance values reported in these patients are for apparent oral clearance (CL/F), rather than total systemic clearance. Thus, the possible contribution of very low oral bioavailability values in these patients, resulting in large CL/F values, to the observed difference in the sum of CL_R and $CL_{MD \rightarrow EDDP}$ compared to CL/F cannot be excluded. However, this would appear to be an unlikely explanation, given the similarity of CL/F values reported here and those available in the literature.

Recent *in vitro* studies have shown CYP3A4 to be the major CYP isoform mediating EDDP formation from rac-methadone (Iribarne et al., 1996; Iribarne et al., 1997; Moody et al., 1997) and the individual enantiomers (see Chapter 3). There was a large degree of inter-individual variability (4- to 5-fold) in $C^{ss}_{av_u}$ and CL_u/F. These pharmacokinetic parameters are mainly determined by metabolic activity, and the inter-individual variability observed in this study is consistent with reported *in vitro* (Chauret et al., 1997; Forrester et al., 1992; Shimada et al., 1994) and *in vivo* (Lown et al., 1995; Thummel et al., 1996; Thummel et al., 1994a; Thummel et al., 1994b) estimates of variability in hepatic CYP3A4 expression. Markedly higher inter-individual variability was observed for AUC^{ss}_{τ} (~12-fold), $CL_{MD \rightarrow EDDP}$ (~10-fold) and $CL_{MD \rightarrow EDDP_u}$ (~20-fold). However, these are influenced by additional factors which include in some cases dose, plasma binding, and non-renal elimination of EDDP.

There was no difference in the steady-state plasma clearance (CL/F) between the two enantiomers. This observation is in contrast to Kristensen and co-workers (1996), who reported that the mean plasma clearance of (R)-methadone (158 $ml.min^{-1}$) was significantly greater than for (S)-methadone (129 $ml.min^{-1}$), although the magnitude of the difference was

small. In contrast, bioavailability was not found to be stereoselective (Kristensen et al., 1996). Recent population pharmacokinetic modelling studies have shown that the pharmacokinetics of rac-methadone differ between single doses in healthy normal subjects and methadone maintenance patients after a single dose (Wolff et al., 1997), and that there are time-dependent changes in the clearance and volume of distribution of rac-methadone in methadone maintenance patients while steady-state is being achieved (Rostami-Hodjegan et al., 1999). Comparison with the results of Kristensen and co-workers (1996) is difficult, as the present study involved patients at steady-state, whereas Kristensen and co-workers studied acute methadone administration. The subject populations were also markedly different, as Kristensen and co-workers' patients were receiving opioids for chronic pain control, and the patients described here were in a methadone maintenance programme. Assuming a bioavailability of 90%, results presented here for (R)- and (S)-methadone plasma clearance are in close agreement with those of Kristensen and co-workers (Kristensen et al., 1996), being 149 $ml.min^{-1}$ and 143 $ml.min^{-1}$ for (R)- and (S)-methadone, respectively.

As noted previously in Chapter 4, a single subject (patient #5) in the present study used phenobarbital as a concomitant medication, which was confirmed by urinalysis (see Appendix 7). Although phenobarbital is a well established inducer of CYP3A4 expression (Ketter et al., 1995), and has been implicated in altered methadone pharmacokinetics and/or pharmacological and therapeutic effects (see section 1.7.3.2), this subject's CL/F value for rac-methadone was comparable to the other subjects enrolled in the study. Similarly, this subject's CL/F and CL_u/F values for (R)-methadone (110 $ml.min^{-1}$ and 3499 $ml.min^{-1}$, respectively) and (S)-methadone (88 $ml.min^{-1}$ and 4476 $ml.min^{-1}$, respectively) were well within the range found in the other subjects (see Table 5-6 and Table 5-7). However as noted earlier in Chapter 4, this subject was not examined either before commencement or after cessation of phenobarbital use. Significant induction of the metabolism of methadone from a low basal level may have occurred.

The highly significant relationships between plasma AUC_{τ}^{ss} and dose for both (R)-methadone and (S)-methadone indicate that the pharmacokinetics (extent of absorption, clearance) of each enantiomer are linear after administration of the racemate over a wide dosage range (7.5-130 $mg.day^{-1}$). This is the first time that this has been demonstrated for the individual methadone enantiomers, and confirms previous reports of the linearity of rac-methadone pharmacokinetics (Wolff et al., 1991d). It should also be noted that this relationship was established in separate individuals taking a range of fixed doses, rather than within single individuals all given a range of dosages. This demonstrates that the extent of inter-individual variation in metabolic activity does not prevent a dose-plasma concentration relationship; indeed approximately 68% and 47% of the large inter-individual variability in the AUC_{τ}^{ss} of (R)-methadone and (S)-methadone, respectively, is explained by variation in dose, while the remaining variability is mainly due to differences in clearance.

Some authors have reported that ratios of (R)- to (S)-methadone concentrations in plasma samples demonstrate wide inter-patient variability in methadone maintenance patients in samples taken immediately prior to administration of the daily dose: 0.63-2.4 (Eap et al., 1996), 0.26-0.44 (mean: 0.36, Pham-Huy et al., 1997); 0.5-1.4 (Beck et al., 1991), 0.76-1.93 (mean:1.02, Eap et al., 1997); and at unspecified time points 0.55-1.4 (mean: 0.85, de Vos et al., 1998); 0.57-1.54 (mean: 1.03, Rudaz & Veuthey, 1996); 0.5-1.1 (mean: 0.8, Kristensen & Angelo, 1992). Despite this wide inter-patient variability, some authors have shown that the (R)/(S) ratio is relatively constant over time within a patient during methadone maintenance treatment, with ratios not significantly different in samples collected from the same subjects one week (de Vos et al., 1998) to two weeks (Eap et al., 1996) apart in large groups (n>22) of patients. The present study demonstrated a similar extent of inter-patient variability, with pre-dose (R)/(S) ratios ranging over 3-fold, with a mean value of 1.07. Similarly, plasma C_{max}^{ss} and peak to trough plasma concentration ratios were significantly lower for total (bound plus unbound) (R)-methadone compared to (S)-methadone. Additionally, Figure 2 shows that the ratio of (R)- to (S)-methadone plasma concentrations is not stable over an inter-dosing interval. The ratio decreases as plasma concentrations

increase, with values at 1-4 hours post-dose significantly lower than unity, while ratios at other time points are not different to unity. Indeed, (R)/(S) ratios calculated pre-dose ($R^{ss}_{min(first)}/S^{ss}_{min(first)}$) and post-dose ($R^{ss}_{min(last)}/S^{ss}_{min(last)}$) were not significantly different, but were both significantly greater than ratios calculated separately at the t_{max} of (R)-methadone (R^{ss}_{max}/S^{ss}) and (S)-methadone (R^{ss}/S^{ss}_{max}), with identical results obtained when protein binding was considered (Table 5-8). These differences may be explained by volume of distribution differences. As (R)-methadone had a lower protein binding compared to (S)-methadone, it is likely to have a greater volume of distribution, as has been previously reported (Kristensen et al., 1996). This would result in lower C^{ss}_{max} values for (R)-methadone in comparison to (S)-methadone, and hence a lower peak to trough concentration ratio. These data highlight the need for consistency and accuracy in the timing of blood sampling, if conclusions are to drawn regarding the extent of inter- and intra- patient variability in the relative plasma concentrations, and hence possible differences in clearance, between the two enantiomers.

Recently, my co-workers and I have shown in methadone maintained patients that withdrawal severity can be related to the rate of change of plasma rac-methadone concentrations from the time of peak plasma concentration, and that this relationship has a very high Hill slope factor (Dyer et al., 1999). The stereoselective difference in the magnitude of fluctuation of plasma methadone concentrations reported here may have important implications for pharmacokinetic-pharmacodynamic modelling of methadone, as measurement of rac-methadone may not provide an accurate reflection of plasma concentrations of the active (R)-methadone enantiomer. In contrast, therapeutic drug monitoring for patient compliance is unlikely to require stereoselective measurement of plasma methadone concentrations given the close dose-plasma concentration relationship demonstrated for the individual methadone enantiomers found in this study, and for rac-methadone reported in Chapter 4 and reported by others (Rostami-Hodjegan et al., 1999; Wolff et al., 1993; Wolff et al., 1991d).

The protein binding of methadone was found to be stereoselective, with unbound fractions of (R)-methadone significantly greater than for (S)-methadone in the plasma of methadone maintenance patients, and in solutions containing purified α_1-acid glycoprotein in accord with previous findings (Eap et al., 1990; Romach et al., 1981). The binding experiments using purified α_1-acid glycoprotein resulted in similar estimates of the number of binding sites per α_1-acid glycoprotein molecule (*n*) for (R)-methadone (0.13) and (S)-methadone (0.15). In contrast, others have reported approximately 0.27-0.38 binding sites per α_1-acid glycoprotein molecule. These values were obtained from *N* (molar binding site concentration) values with overlapping 95% confidence intervals for the estimate of the individual enantiomers, indicating no stereoselectivity in the number of binding sites available for the individual enantiomers. Estimated *Ka* values from the binding to purified α_1-acid glycoprotein are in excellent agreement with those calculated from the protein binding in the plasma of methadone maintenance patients (assuming 0.3 sites per molecule) in this study for both (R)-methadone (2.5×10^6 M^{-1} and 2.6×10^6 M^{-1}, respectively) and (S)-methadone (4.9×10^6 M^{-1} and 4.3×10^6 M^{-1}, respectively). These *Ka* values are very similar to those reported by other authors for rac-methadone (1.9×10^6 M^{-1}) binding to purified α_1-acid glycoprotein (Hervé et al., 1996). Others have reported *nKa* values for (R)-methadone (3.0×10^5 M^{-1}) and (S)-methadone (4.8×10^5 M^{-1}) in the plasma of volunteers (Eap et al., 1990), rac-methadone ($1.5\text{-}5.1 \times 10^5$ M^{-1}) binding to purified α_1-acid glycoprotein (Abramson, 1982; Hervé et al., 1996) and rac-methadone (1.4×10^5 M^{-1}) binding in the plasma of healthy volunteers (Abramson, 1982). The *nKa* values for the binding to purified α_1-acid glycoprotein (3.2×10^5 M^{-1} (R)-methadone, 7.3×10^5 M^{-1} (S)-methadone) and the plasma of methadone maintenance patients (7.9×10^5 M^{-1} (R)-methadone, 13.0×10^5 M^{-1} (S)-methadone) reported here compare favourably with these data. These data would suggest that the enantiomers bind to a common site, but that the (R)-enantiomer has a 2-fold greater affinity.

Previous protein binding experiments have shown that rac-methadone, and the individual enantiomers, bind predominantly to the ORM2 A variant, with very low affinity for the

ORM1 S and ORM1 F1 variants (Eap et al., 1988; Eap et al., 1990; Hervé et al., 1996). The relative concentrations of these variants are highly variable, not only dependent on phenotype, but also between subjects of the same phenotype (Eap et al., 1990). Hervé and co-workers (1996) reported that the binding of methadone to purified solutions of the ORM2 A variant resulted in similar *Ka* values to those obtained using unfractionated α_1-acid glycoprotein, while the number of binding sites increased over 2-fold. These results are consistent with the selective binding of methadone to the ORM2A variant in unfractionated α_1-acid glycoprotein mixtures, resulting in an underestimation of the binding site concentration, and hence the number of binding sites per α_1-acid glycoprotein molecule (*n*). It is possible that the purified α_1-acid glycoprotein used in the present binding experiments contained a low proportion of the ORM2 A variant, resulting in relatively low estimates of the number of binding sites per α_1-acid glycoprotein molecule. The assay used to quantitate α_1-acid glycoprotein in the patient plasma samples does not selectively measure the individual variants. Therefore, it is possible the ORM2 A variant may comprise a relatively high proportion of the total α_1-acid glycoprotein in these patients, resulting in the observed low unbound fractions of methadone. Conversely, estimated *Ka* values from the binding experiments using purified α_1-acid glycoprotein and the patient plasma were similar to those previously reported.

In contrast to other authors (Eap et al., 1988; Eap et al., 1990; Garrido et al., 2000; Hervé et al., 1996; Judis, 1977; Romach et al., 1981; Wilkins et al., 1997) and the binding to purified α_1-acid glycoprotein reported here, I observed lower unbound fractions in these patients. A possible reason for these difference may be that the pH of plasma samples was not maintained at 7.4. Although the ultra-filtration device maintains a constant sample pH during the filtration process, there is evidence that the pH of plasma samples may increase during storage due to loss of dissolved carbon dioxide, and changes in plasma pH may alter unbound fractions of other drugs (Brørs & Jacobsen, 1985; Ho Ngoc-Ta Trung & Sirois, 1987). It is possible that an increase in plasma pH would increase the binding affinity of the protein for methadone. Assuming the number of binding sites (*n*) remains constant, this is a

possible explanation for the difference between the unbound fraction values for the patient samples reported here and those in the literature. However, the estimates of the association constants (*Ka*) from the patient samples and the binding to purified α_1-acid glycoprotein (at pH 7.4) were very similar, suggesting that this is not the case. The *Ka* values obtained from the patient samples were estimated assuming 0.3 binding sites per α_1-acid glycoprotein molecule, as this value has been reported by previous investigators (Eap et al., 1990; Hervé et al., 1996). However, the *in vitro* binding to purified α_1-acid glycoprotein reported here indicated approximately 0.14 sites per molecule. If one uses 0.14 sites per molecule, then these data would indicate 2-fold higher affinity (*Ka*) of methadone for α_1-acid glycoprotein in the patient plasma samples compared to the purified α_1-acid glycoprotein, and consequently lower unbound fractions would be predicted (see Equation 5-1).

A weak relationship was found between plasma α_1-acid glycoprotein concentration and the ratio of bound/unbound concentrations, confirming other authors reports in methadone maintenance patients (Garrido et al., 2000; Romach et al., 1981) and other subject populations (Abramson, 1982; Eap et al., 1990; Romach et al., 1981). Although α_1-acid glycoprotein has been reported to be the predominant binding protein, investigators have reported that albumin (Abramson, 1982; Romach et al., 1981) and lipoproteins (Romach et al., 1981) may also play a role. However, the individual role of each protein appears to be minor compared to that of α_1-acid glycoprotein, as plasma albumin (Eap et al., 1990; Romach et al., 1981), cholesterol (Eap et al., 1990), triglyceride (Eap et al., 1990) and total protein concentrations (Eap et al., 1990) have been shown not to contribute significantly to the binding of methadone. Plasma protein concentrations, other than α_1-acid glycoprotein, were not measured and may have been elevated in the patients. Indeed the unbound fractions of the (R)- and (S)-methadone in solutions containing purified α_1-acid glycoprotein were approximately 20% and 10%, respectively, compared to 4% and 2%, respectively, in the patient samples at similar methadone (750 $ng.ml^{-1}$) and α_1-acid glycoprotein (100 $mg.dl^{-1}$) concentrations. Furthermore, estimates of *N* (maximum binding site concentration) from the binding to 100 $mg.dl^{-1}$ purified α_1-acid glycoprotein indicate

that complete saturation would occur at approximately 3.5 μM ($3.5x10^{-6}$ M; 1200 $ng.ml^{-1}$) of each enantiomer (2400 $ng.ml^{-1}$ rac-methadone). However, one can see from Figure 5-4 that higher concentrations of α_1-acid glycoprotein would markedly increase this value. In contrast, the free fraction of methadone in the plasma of healthy volunteers and methadone maintenance patients did not demonstrate any concentration dependency or saturation, below 3000 $ng.ml^{-1}$ of each enantiomer (6000 $ng.ml^{-1}$ rac-methadone), consistent with previous estimates of a half-saturating rac-methadone concentration of 4600 $ng.ml^{-1}$ in human plasma (Abramson, 1982). Additionally, it was estimated that up to 84 % and 92% of (R)- and (S)-methadone would remain bound in the patient plasma samples in the absence of α_1-acid glycoprotein, somewhat higher than other authors have reported for rac-methadone in the plasma of healthy subjects and patients with cancer (60%, Abramson, 1982). These data suggest that plasma constituents other than α_1-acid glycoprotein, such as albumin, cholesterol and triglycerides (Abramson, 1982; Eap et al., 1990; Romach et al., 1981), may be involved to a significant degree in the binding of methadone in this group of patients, and provides an alternative explanation for the observed difference between the patient binding data reported here and those in the literature. Alternatively, inter-individual variation in the ORM2A variant of α_1-acid glycoprotein may have a marked effect on binding analysis. The use of total α_1-acid glycoprotein concentrations, rather than the ORM2 A variant only, could adversely affect estimates of the y-intercept of bound/free versus α_1-acid glycoprotein concentration regression analysis, and hence the estimate of the bound fraction in the absence of α_1-acid glycoprotein. Future studies aimed at further clarifying factors contributing to inter-individual variation in methadone pharmacokinetics could therefore benefit from considering plasma concentrations of the individual α_1-acid glycoprotein variants.

The renal clearance of total (bound plus unbound) (R)- and (S)-methadone accounted for approximately 10-20% of CL/F, consistent with previous reports for rac-methadone (Bellward et al., 1977; Inturrisi et al., 1987b; Nilsson et al., 1982b). Additionally, these values are between $1/18^{th}$ and $1/4^{th}$ that of glomerular filtration rate in normal healthy

subjects, indicating extensive net tubular reabsorption. The renal clearance of rac-methadone has previously been shown to be pH dependent (Bellward et al., 1977; Nilsson et al., 1982b). Examination for pH dependency of the renal clearance of the methadone enantiomers was not possible in the present study, due to a very narrow pH range amongst samples collected. Of the 10 samples, only two were below pH 6 and the remainder were within the range of 6.0-6.4 pH units. When corrected for protein binding, the renal clearance for each enantiomer (range: 256-1832 $ml.min^{-1}$) was between 2- and 15-fold greater than glomerular filtration rate in normal healthy subjects, indicating extensive net tubular secretion. Unexpectedly, the renal clearance of total (bound plus unbound) (R)-methadone was a mean of 182% that of (S)-methadone, suggesting that the net secretion and reabsorption of methadone is not a stereoselective process. However, after correcting for protein binding there was no significant difference (P=0.63) between the enantiomers.

The data presented in this chapter highlight the importance of protein binding and stereochemical considerations when drawing conclusions on the pharmacokinetics and metabolism of compounds, and demonstrate that care should be taken when interpreting pharmacokinetic and metabolism data of chiral compounds based only upon results of non-chiral analytical techniques. In conclusion, I have shown that the pharmacokinetics of methadone are stereoselective, and that there is large inter-individual variability, consistent with CYP3A4 mediated metabolism to the major metabolite EDDP; this variability did not obscure a strong dose-plasma concentration relationship. Stereoselective differences in the pharmacokinetics of methadone may have important implications for pharmacokinetic-pharmacodynamic modelling but is unlikely to be important for therapeutic drug monitoring of compliance with methadone in the setting of opioid dependence.

6. Urinary elimination of methadone and eight of its known metabolites in a methadone maintenance population

6.1. Introduction

Several groups have reported the development of quantitative mass-spectrometry assays for methadone, and often several metabolites, in biological fluids (Alburges et al., 1996; Cooper & Oliver, 1998; Galloway & Bellet, 1999; Goldberger et al., 1998; Hachey et al., 1977; Kang & Abbott, 1982; Kreek et al., 1983; Kreek et al., 1980a; Moody et al., 1997; Moody et al., 1999; Skopp et al., 1996; Wilkins et al., 1996; Wilkins et al., 1998). Although mass-spectrometry methods provide good selectivity and may be employed quantitatively, they are an expensive alternative to HPLC. Many laboratories, including my own, do not yet use this technique routinely. In contrast, the use of a conventional HPLC system is more accessible to many laboratories. At the time the present work was conducted, there were few HPLC methods available in the published literature for the simultaneous quantification of methadone and metabolites other than EDDP and EMDP .

Garrett and co-workers (1985) developed HPLC conditions with UV detection (210 nm) to measure methadone and several metabolites (EDDP, EMDP, p-hydroxy methadone, α-methadol, α-N desmethyl methadol, pyrrolidone, valeric acid metabolite) in dog plasma, urine and bile after alkaline extraction with hexane. Limits of detection ranged from 20ng.ml^{-1} to 30 ng.ml^{-1} for all compounds from a 0.5-1.0 ml sample. However, no assay validation data were provided. Although a cyano column was used for all chromatography, each compound was assayed separately using an optimised extraction pH and mobile phase.

Others quantified urinary concentrations of methadone and some metabolites (EDDP, EMDP and p-hydroxy methadone, Gérardy et al., 1986; EDDP and EMDP, Iribarne et al., 1996; EDDP, EMDP and methadol, Pond et al., 1985) using HPLC after solvent-solvent extraction of alkalinised urine samples. However, details of the methods were not reported in some cases (Pond et al., 1985) and details of assay sensitivity or validation data were ill defined (Gérardy et al., 1986; Iribarne et al., 1996; Pond et al., 1985). Pierce and co-

workers (1992) developed a HPLC assay for methadone, EDDP and EMDP only, using solid phase extraction of alkalinised samples. From a 1 ml sample limits of quantification were 25 $ng.ml^{-1}$ for methadone and 5 $ng.ml^{-1}$ for EDDP and EMDP. For methadone only, intra- and inter-assay precision were reported as 1.3% and 2.9% respectively, although it is unclear as to which concentrations these results were obtained.

These are the only HPLC assays available in the literature to date for the quantification of methadone metabolites other than EDDP and EMDP.

The specific aim of the work contained in this Chapter are:

Aim 5: To develop a sensitive and robust HPLC method for the quantification of methadone and eight of its metabolites in human urine.

6.2. Methods

6.2.1. Patients

Ethical approval was obtained from the Royal Adelaide Hospital Research Ethics Committee. These patients have been described elsewhere, in which the pharmacokinetics of rac-methadone (Chapter 4), and (R)- and (S)-methadone (Chapter 5) were investigated. The work reported here used the same urine samples obtained from this cohort of patients as described in Chapter 4. However, the subset of 10 patients reported here are only those from which urine samples were collected (patients #9-18). There were 9 males and 1 female; body weights ranged from 65 to 91 kg (mean±SD; 73±10 kg); ages ranged from 28 to 45 years (36±8 years). The once-daily methadone dose ranged from 7.5-130 $mg.day^{-1}$, which corresponded to 0.12 to 1.9 $mg.kg^{-1}$ (0.81±0.54 $mg.kg^{-1}$). The patients were allowed to take benzodiazepines in therapeutic doses. The majority smoked cigarettes, 4 showed positive urinalysis for benzodiazepines, 5 for cannabinoids, 2 for opioids other than methadone, 1 for sympathomimetic amines, none for barbiturates and 4 consumed alcohol regularly in quantities less than 40 grams per day. Patients were excluded from the study if

they were pregnant or had positive HIV serology. Refer to Appendix 7 (patients #9-18) for patient demographic information.

Each patient was admitted to the inpatient facility of the maintenance program 1 hour before their scheduled daily dose and remained in the unit for the subsequent 24 hours. Methadone was administered as a syrup under supervision of the study personnel. A 24 hour pooled urine sample was also obtained, volume and pH measured and an aliquot stored at -20°C until analysis.

6.2.2. Chemicals

Racemic methadone as the hydrochloride salt, (4R,6S)- and (4S,6S)-para hydroxy methadone as the hydrochloride salts, β-(+)-(3R,6S)-methadol, racemic α-methadol, α-(-)-(3S,6S)-methadol, α-(+)-(3R,6R)-methadol, α-(-)-(3S,6S)-N-desmethyl methadol, and racemic pyrrolidone were obtained from the National Institute on Drug Abuse (Rockville, MD, USA). Racemic EDDP as the hydroiodide salt and rac-EMDP as the hydrochloride salt were purchased from Alltech-Applied Science Labs (State College, PA, USA), and dextropropoxyphene was purchased from Sigma Chemical Company (St. Louis, Mo, USA). HPLC grade acetonitrile and triethylamine were from BDH Laboratory Supplies (Poole, UK). All other reagents and chemicals were obtained from commercial sources and were of analytical grade quality.

6.3. Quantification of rac-methadone and eight metabolites in urine

6.3.1. HPLC instrumentation and chromatography conditions

The HPLC system comprised a LC-10AT pump (Shimadzu, Kyoto, Japan), a Sil-10A autoinjector (Shimadzu) and a SPD-M10A photo-diode array detector (Shimadzu) set at 210 nm. The system was controlled using Class-LC10 software (version 1, Shimadzu) running under Windows 3.11 (Microsoft Corporation, WA, USA) on a 486 DX IBM compatible computer. The analytical column was a C_{18} Platinum EPS (250x4.6 mm, 5 μm; Alltech) protected by a 2 μm in-line filter (Scientific Instruments, State College, PA, USA) and a C_{18}

Platinum EPS pre-column (10x4.6 mm, 5 µm; Alltech). Optimal separation of the compounds of interest was achieved with a mobile phase of 50 mM NaH_2PO_4 in water containing 0.2% triethylamine and 30% acetonitrile final pH adjusted to 4.4 with ortho-phosphoric acid. The mobile phase was pumped through the system at room temperature according to the following time-program: 0-22 minutes 1.0 $ml.min^{-1}$, increased linearly to 1.5 $ml.min^{-1}$ over a 1 minute interval, held constant at 1.5 $ml.min^{-1}$ until 56 minutes, decreased linearly to 1.0 $ml.min^{-1}$ over a 1 minute interval and held constant 1.0 $ml.min^{-1}$ until 60 minutes.

6.3.2. Sample preparation

Urine samples (1.0 ml) and internal standard (100 µl 10 µM dextropropoxyphene in water) were aliquoted into 10 ml borosilicate glass tubes, alkalinised (0.5 ml 1 M NaOH) and extracted with 5 ml of 100 % hexane for 20 min on a rotary mixer. Samples were then centrifuged (2000*g*, 10 min) and the organic phase transferred to a clean 5 ml borosilicate glass tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue was reconstituted in 0.25 ml mobile phase and a 100 µl aliquot injected onto the chromatography system.

6.3.3. Calibration, precision, accuracy and extraction efficiency

Retention times of the compounds of interest were confirmed by direct injection of aqueous solutions of pure compounds. Quantification of rac-methadone and rac-EDDP was performed with calibration curves consisting of eight standards over the concentration range 0.5-50 µM of each compound. Quantification of (4R,6S)- and (4S,6S)-para hydroxy methadone, rac-EMDP, rac-β-methadol, rac-α-methadol, rac-α-N-desmethyl methadol, and rac-pyrrolidone metabolite was performed with calibration curves consisting of eight standards over the concentration range 0.1-10 µM of each compound. Single low (L), medium (M) and high (H) QC samples were prepared, with final concentrations of 1.25 µM, 3.75 µM and 12.5 µM of each compound for methadone and EDDP, and 0.25 µM, 0.75 µM and 2.5 µM of each compound for (4R,6S)- and (4S,6S)-para hydroxy methadone, EMDP,

β-methadol, α-methadol, α-N-desmethyl methadol and the pyrrolidone metabolite. Calibration standards and QC samples were prepared by diluting 100 µl of an aqueous stock solution containing all analytes with 900 µl drug-free urine, and analysed identically to the patient samples (section 6.3.2). The robustness of the analytical method was assessed using inter-assay accuracy and precision determined by the analysis of the highest (H), medium (M) and the lowest (L) calibration standard (CS), on different assay days. Extraction efficiency was analysed for each calibration standard concentration and for the internal standard in a single analytical run. The peak areas of all compounds after injection of the extracted samples were compared to those obtained after direct injection of the aqueous stock solution.

6.3.4. Data analysis

Raw data were entered into Excel spreadsheets (Version 4.0, Microsoft Corporation, WA, USA). Peak areas were converted into peak area ratios using the peak area of the internal standard. Linear regression analysis (GraphPad Prism v2.01, GraphPad Software, CA, USA) of $1/y^2$ weighted peak area ratio against nominal concentration provided an estimate of slope, intercept and coefficient of determination (r^2). The estimated concentration divided by the nominal concentration multiplied by 100% was calculated for each individual sample, and accuracy was calculated as the mean of these values, and the residual standard deviation of the mean was taken as the precision.

As a validation procedure, ordinary least products linear regression analysis (Brace, 1977; Ludbrook, 1997) was used to compare the concentrations of rac-methadone and rac-EDDP in patient urine samples obtained with the present assay with those determined using assay 6 (see section 2.8). Analyses were performed using Excel (Excel v7.0a, Microsoft). Linear regression analysis was performed using GraphPad Prism (GraphPad Prism v2.01, GraphPad Software, CA, USA). Ordinary least products linear regression analysis is sensitive to both fixed and proportional bias, unlike conventional linear-regression analysis, as it does not assume that one axis is error-free (Brace, 1977; Ludbrook, 1997).

6.4. Results and discussion

6.4.1. Chromatography conditions

The retention times for (4R,6S)-para hydroxy methadone, (4S,6S)-para hydroxy methadone, α-N-desmethyl methadol, α-methadol, dextropropoxyphene, β-methadol, EDDP, methadone, EMDP and the pyrrolidone metabolite were 11.9, 12.7, 14.6, 21.1, 24.1, 26.7, 29.5, 32.1, 41.0, 45.3 min, respectively, with a total run-time of 60 min. Under these conditions all compounds of interest were baseline resolved, with the exception of the (4R,6S)- and (4S,6S)-para hydroxy methadone diastereomers which were adequately resolved. No further modifications to the HPLC system were attempted. There were no interfering peaks in the chromatography in several blank urine samples.

A representative chromatogram from the analysis of a drug-free urine sample and a calibration standard assayed for methadone and eight metabolites are shown in Figure 2-4.

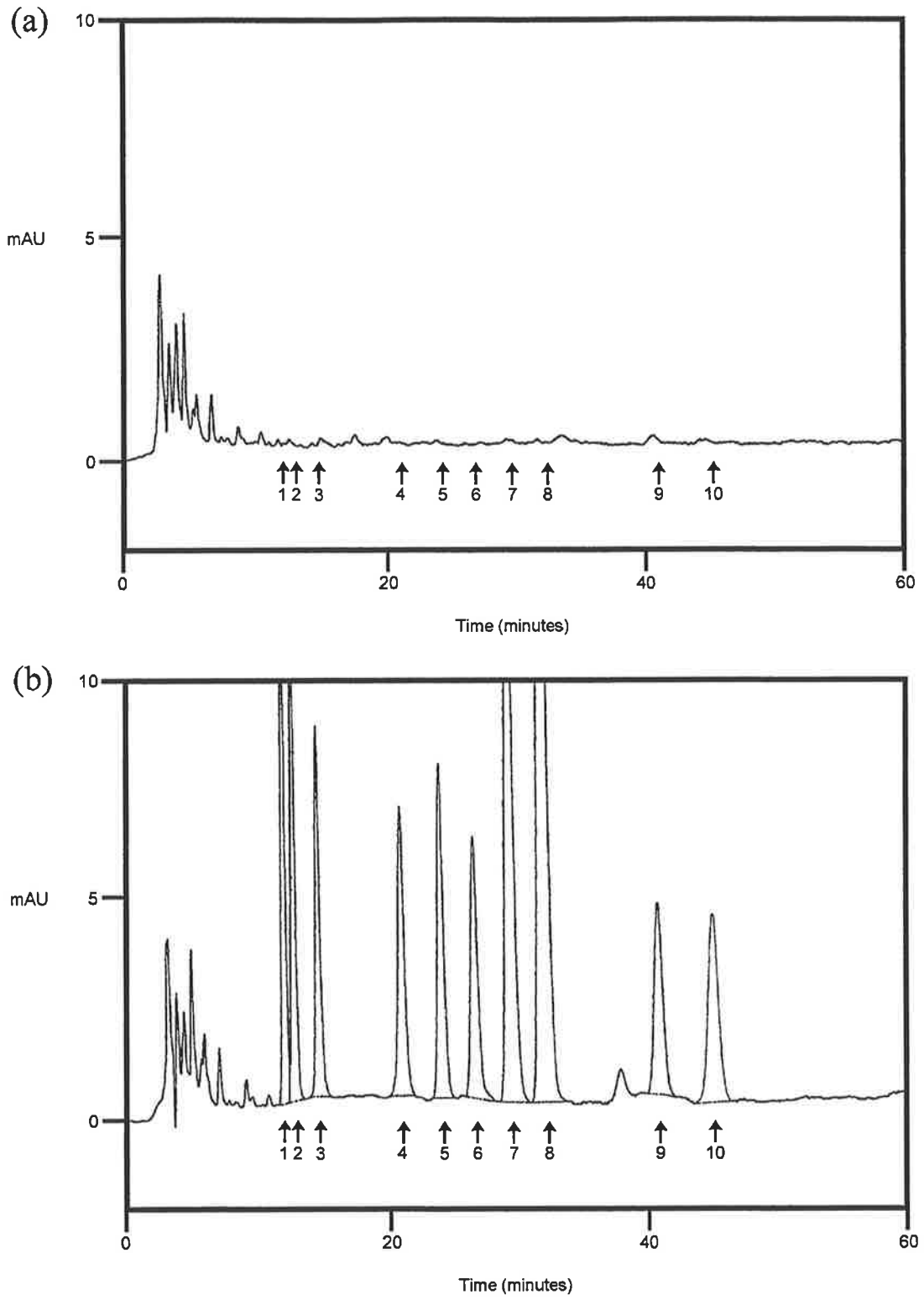


Figure 6-1: Representative chromatogram for the analysis of (a) drug-free urine sample and (b) calibration assayed for methadone and eight metabolites.

Notes: Calibration standard contained 3.75 μM methadone and EDDP, and 0.75 μM of the remaining metabolites. 1=(4R,6S)-para hydroxy methadone, 2=(4S,6S)-para hydroxy methadone, 3=α-N-desmethyl methadol, 4=α-methadol, 5=dextropropoxyphene, 6=β-methadol, 7=EDDP, 8=methadone, 9=EMDP, 10=pyrrolidone metabolite.

The extraction efficiency was greater than 80% and reproducible (CV <10%) for most analytes, and did not demonstrate concentration-dependency with mean±SD values shown in Table 6-1. Extraction efficiency of the internal standard was also very reproducible (CV <5%) and similar to that obtained for the analytes (Table 6-1). EDDP showed a lower extraction efficiency (60%) than the other analytes, while the extraction of EMDP was highly variable (Table 6-1). However, further modifications to the extraction procedure were not attempted.

Table 6-1: Extraction efficiency of the assay for methadone and eight metabolites in human urine.

	Extraction efficiency (%±SD)	n
(4R,6S)-para hydroxy methadone	82±5	8
(4S,6S)-para hydroxy methadone	88±7	8
α-N-desmethyl methadol	78±5	8
α-methadol	83±3	8
β-methadol	77±7	8
EDDP	60±10	6
methadone	85±8	7
EMDP	99±20	6
pyrrolidone metabolite	88±5	8
dextropropoxyphene (internal standard)	81±3	8

For EDDP, the observed low extraction efficiency may be due to its substantially higher pKa (10.4 versus 8.6, respectively Baselt & Bickel, 1973) when compared to methadone. However, the calibration curves were linear, inter- and intra-assay validation data were acceptable, and the LOQ of the assay was well below the lowest concentration observed in the patient samples. For EMDP, the extraction efficiency was markedly more variable compared to the other analytes. This resulted in calibration curves that were less reproducible than for the other analytes (see Table 6-2) but still satisfactory. EMDP has been reported to have relatively low pKa value of 5.9 (Baselt & Bickel, 1973). This would result in a relatively high proportion of the compound present in an unionised form after

alkalinisation of the sample. EMDP would therefore be expected to be highly partitioned into organic solvents during the solvent-solvent extraction (see section 1.4.2.2), as has been confirmed by studies which have reported EMDP to be highly partitioned into organic solvents from aqueous phases with pH values greater than 7 (Baselt & Bickel, 1973; Garrett et al., 1985), and the data reported in Table 6-1. From the favorable partitioning reported above, EMDP would be expected to demonstrate relatively consistent extraction efficiency between samples. Reasons for the observed variable partitioning of EMDP into the hexane extraction solvent employed in this study are not apparent. However, the possibility of degradation of the compound during the extraction process cannot be excluded, although this is unlikely.

In comparison to other HPLC assays available (Gérardy et al., 1986; Iribarne et al., 1996; Pierce et al., 1992; Pond et al., 1985), the present assay utilises a similar extraction procedure, while allowing the separation of an increased number of methadone metabolites (eight compared to a maximum of three) on a single chromatogram.

Due to the long analysis times of each sample (60 minutes) and resultant lengthy analytical run times, three preliminary calibration curves were assayed before a full inter- and intra-assay validation was performed: four containing calibration standards only, and one comprising calibration standards, QC samples and patient samples. This was performed in order to establish the linearity and appropriateness of the calibration curve concentration range before QC samples were prepared as the available amounts of authentic compounds were limited for the methadone metabolites other than EDDP and EMDP. Calibration curves for all analytes were linear over the calibration curve concentration ranges, with r^2 values greater than 0.99 (with the exception of EMDP: $r^2>0.97$) for all analytes in 5 analytical runs, and mean±SD values are presented in Table 6-2. Estimates of slope demonstrated no consistent time-related changes for any analyte, and mean±SD values are presented in Table 6-2. The inter-assay accuracy and precision of the method at three calibration standard concentrations for all analytes are shown Table 6-2. Quality control

samples were only assayed on a single occasion, and were within ±10% of the nominal concentration at all three QC concentrations for all analytes, with the exception of the LQC sample for α-methadol (-15%), and the LQC and MQC sample for EMDP (-21% and -22%, respectively).

In summary, with the exception of EMDP these data demonstrate that the assay procedure is likely to be robust, accurate and precise enough for a full validation of the assay to produce acceptable results for all analytes.

Table 6-2: Inter-assay accuracy and precision for the quantification of methadone and eight metabolites in human urine.

Inter-assay (n=5 assays)	Nominal concentration (μM)	Accuracy (%)	Precision (%)	Mean r^2 ±SD	Mean slope ±SD	n
(4R,6S)-para hydroxy methadone				0.9968 ±0.0011	1.2544 ±0.1614	5
LCS	0.1	99.9	1.5			4
MCS	1	99.3	6.9			5
HCS	10	98.6	2.6			5
(4S,6S)-para hydroxy methadone				0.9983 ±0.0021	1.3122 ±0.1510	5
LCS	0.1	100.3	0.6			4
MCS	1	101.7	5.8			5
HCS	10	98.3	3.0			5
α-N-desmethyl methadol				0.9993 ±0.0007	0.9867 ±0.1081	5
LCS	0.1	100.9	2.8			4
MCS	1	102.3	3.5			5
HCS	10	100.2	1.8			5
α-methadol				0.9993 ±0.0006	0.9983 ±0.1088	5
LCS	0.1	100.3	1.2			4
MCS	1	100.5	3.2			5
HCS	10	99.4	2.3			5
β-methadol				0.9994 ±0.0008	1.1428 ±0.1300	5
LCS	0.1	99.9	0.5			4
MCS	1	98.7	1.7			5
HCS	10	101.1	2.0			5
EDDP				0.9975 ±0.0020	1.001 ±0.1326	5
LCS	0.5	101.0	1.7			4
MCS	5	96.3	4.8			5
HCS	50	103.8	4.0			5
methadone				0.9995 ±0.0006	1.1259 ±0.1368	5
LCS	0.5	98.1	2.5			4
MCS	5	99.1	4.3			5
HCS	50	98.7	1.2			5
EMDP				0.9742 ±0.0279	0.5908 ±0.2454	5
LCS	0.1	100.0	9.0			4
MCS	1	97.1	5.8			5
HCS	10	134.4	13.4			5
pyrrolidone metabolite				0.9963 ±0.0044	1.2743 ±0.1786	5
LCS	0.1	103.8	6.1			4
MCS	1	99.8	6.4			5
HCS	10	103.4	4.2			3

Notes: LCS=lowest calibration standard; MCS=medium calibration standard; HCS=high quality calibration standard.

6.4.2. Analysis of patient samples

Prior to completing a full validation of the assay, patient samples were analysed in order to determine if the calibration range of all analytes was appropriate. There are few quantitative data available in the literature for metabolites other than EDDP. However, from the data available, it was considered that these metabolites were likely to be present in very low concentrations (see section 1.7.3.1). This necessitated a preliminary examination of the concentrations of the methadone metabolites present in the patient urine samples. Patient samples were analysed in an analytical assay including calibration standards and quality control samples. A representative chromatogram from the analysis of an inter-dosing interval 0-24 hour pooled urine sample assayed for methadone and eight metabolites is shown in Figure 6-2.

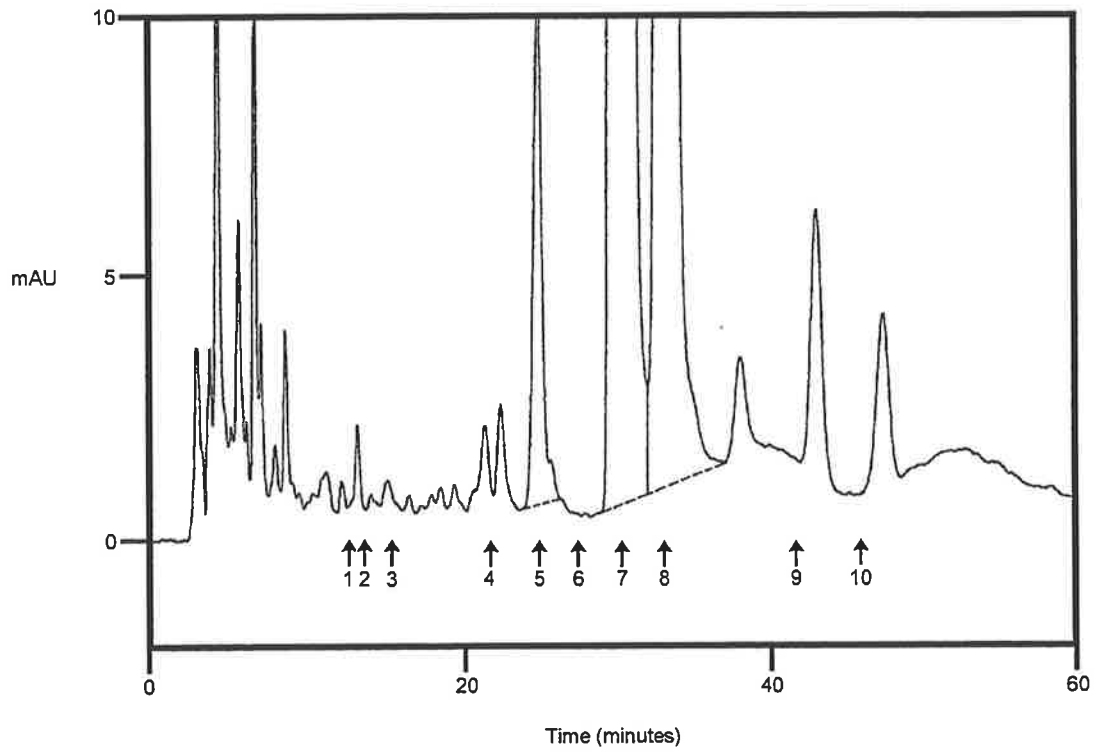


Figure 6-2: Representative chromatogram of a urine sample obtained from a methadone maintenance patient and assayed for methadone and eight metabolites.
Notes: Patient sample was obtained as a pooled 0-24 hour inter-dosing interval sample after the administration of the patients 130 mg daily rac-methadone dose. The sample was diluted 1 in 2 with blank urine and contained 16.8 μM methadone and 33.7 μM EDDP, while other metabolites were not reliably identified. 1=(4R,6S)-p-hydroxy methadone, 2=(4S,6S)-p-hydroxy methadone, 3=α-N-desmethyl methadol, 4=α-methadol, 5=dextropropoxyphene, 6=β-methadol, 7=EDDP, 8=methadone, 9=EMDP, 10=pyrrolidone metabolite.

In the patient samples, the retention times of methadone, EDDP and the internal standard (dextropropoxyphene) were similar to the calibration standards (see Figure 6-2). In contrast, the retention times of other peaks that may have corresponded to analytes other than methadone and EDDP varied considerably in the patient samples. Additionally, unstable baseline absorbances and interfering peaks from 10 minutes to 22 minutes were common (see Figure 6-2). These factors precluded positive identification of metabolites other than unchanged methadone or EDDP. Despite this, and assuming the peaks that may have corresponded to methadone metabolites possessed similar molar extinction coefficients, no single peak was present in an estimated concentration greater than 0.5 μM. This would correspond to approximately 1.5% of the daily dose in the subject receiving the lowest methadone dose (7.5 mg). An example of this phenomenon are the peaks occurring after the retention time of EMDP and the pyrrolidone metabolite in Figure 6-2. It is possible that these peaks may have corresponded to methadone metabolites which were not included in the present assay, such as p-hydroxy EDDP, p-hydroxy EMDP or the carboxylic acid metabolite. Authentic samples of these metabolites are not available. However, it is unlikely that the carboxylic acid metabolite would be recovered by the hexane extraction procedure employed in the present assay from alkalinised urine samples, as this acidic compound would be in a highly ionised form.

The excretion (mean; range) of unchanged rac-methadone (13%; 9-25%) and rac-EDDP (17%; 6-26%) accounted for 30% (12-44%) of the administered dose (Table 6-3). As noted above, chromatographic peaks corresponding to other metabolites could not be reliably identified, but were unlikely to account for a substantial proportion of the administered dose in any subject. This finding is similar to the observations of previous investigators (see section 1.7.3), and indicates that there is significant non-renal and/or further metabolism of methadone and/or EDDP. In support of this, hydroxylation metabolites of EDDP and EMDP have been reported (see section 1.7.3). Indeed, Kreek and co-workers (1983; 1980a) examined the urinary and faecal excretion of methadone and several metabolites (EDDP, EMDP, methadol, p-hydroxy methadone and pyrrolidone metabolite) in five methadone

maintenance patients, and recovered 61±5% (mean±SD) of the dose in subjects without liver disease. These investigators used mass-spectroscopy for quantification using authentic compounds, and scanned the entire mass range in which fragments might arise resulting from other metabolites. Hydrolysis (β-glucuronidase and sulfatase) of urine samples from three healthy patients yielded only small amounts of p-hydroxy EMDP (<1%) and p-hydroxy EDDP (<0.1%) in urine samples, and was not investigated in faeces. The presence of other metabolites, or increased concentrations of metabolites detected prior to hydrolysis was not found. These authors concluded that examination of the faecal excretion of conjugated metabolites, in particular p-hydroxy EDDP, and p-hydroxy EMDP and methadol, would be necessary to achieve complete mass-balance. Faecal samples were not collected from the patients reported here, and authentic samples of p-hydroxy EDDP and p-hydroxy EMDP are not available.

Table 6-3: Urinary recovery during an inter-dosing interval of rac-methadone and rac-EDDP from 10 methadone maintenance patients following chronic dosing of between 7.5 mg and 130 mg rac-methadone once daily.

		% Dose recovered		
Patient #	Dose ($mg.kg^{-1}$)	rac-methadone (%)	rac-EDDP (%)	Total Dose (%)
9	1.91	13.2	26.4	39.6
10	0.33	8.7	23.2	31.8
11	0.82	10.4	11.7	22.1
12	0.30	10.4	12.6	23.0
13	0.86	17.6	26.2	43.8
14	0.77	17.2	10.2	27.4
15	0.12	6.9	5.5	12.4
16	1.41	9.0	19.2	28.2
17	0.93	24.6	15.9	40.5
18	0.68	10.6	19.8	30.3
Mean	0.88	12.8	17.1	29.9
%CV2	57	42.3	41.5	32.0

Notes: [1]Not significantly different (*P* value; mean difference; 95% CI) compared to rac-methadone (*P*=0.18; 4.6; -11.7, 2.6); [2]coefficient of variation.

6.4.3. Comparison of the concentrations of rac-methadone and rac-EDDP obtained with a previous assay

Ordinary least products linear regression analysis was used to compare the concentrations of methadone and EDDP found in urine samples obtained with assay 6 (see section 2.7). The results of these analyses are presented graphically in Figure 6-3. Both analyses yielded a strong and significant correlation for methadone (r^2=0.97, P<0.05), while the relationship was weaker for EDDP (r^2>0.88, P<0.05). The 95% confidence intervals of the slope included 1 for both methadone (1.004; 0.872, 1.155) and EDDP (0.896; 0.684, 1.173), indicating no proportional bias. The 95% confidence intervals of the intercept included 0 for both methadone (3.298; -0.813, 6.870) and EDDP (5.434; -2.824, 11.741), indicating no fixed bias.

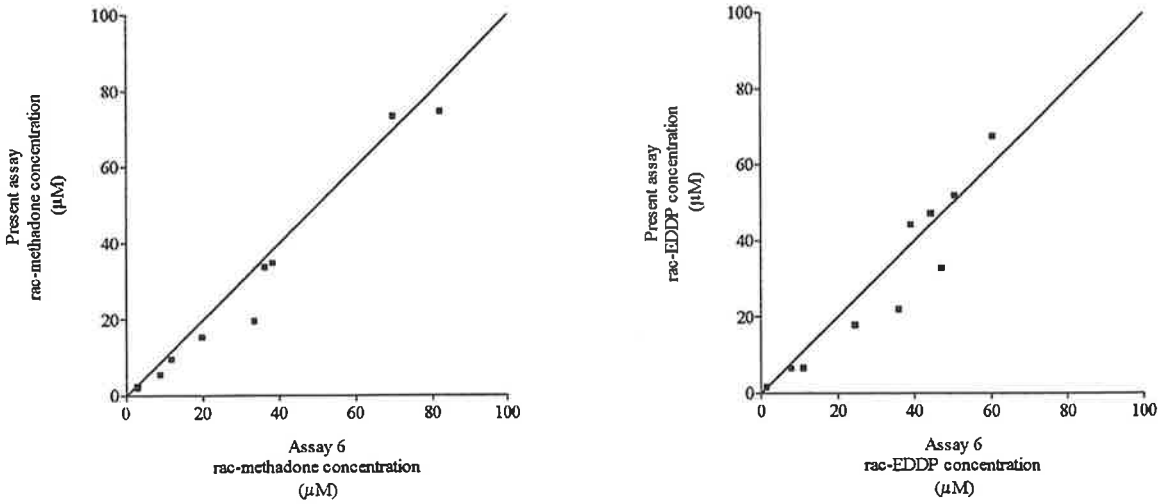


Figure 6-3: Comparison of concentrations of methadone and EDDP obtained with different HPLC assays.
Notes: assay 6=assay for the quantification of rac-methadone and rac-EDDP in urine. Solid line is the y=x line of identity.

Although these analyses demonstrate that it is unlikely that there was interference by other licit and illicit drugs, the large magnitude of the intercepts with lower 95% confidence intervals that approached 0 for both compounds does suggest the possibility of a fixed bias, especially in the case of EDDP. As noted above, there were considerable difficulties in analysing the patient samples using the present assay, which included unidentified peaks and unstable baselines. No difficulties were encountered using the previously validated

assay (assay 6, see sections 2.7 and 2.8) to which the present assay is compared. It is therefore likely that these fixed-bias errors contributed to the relatively poor correlation of the concentrations of rac-methadone and rac-EDDP obtained with the two assays.

In summary, the assay was demonstrated for an accurate and precise method for the simultaneous quantification of methadone and eight metabolites in urine. However, full inter- and intra-assay validation was not performed, as preliminary investigations in patient samples produced results that precluded positive identification of metabolites other than unchanged methadone or EDDP. Despite this, the peaks that may have corresponded to methadone metabolites were estimated to correspond to a very small proportion of the daily dose (<1.5%). This finding indicates that there is significant non-renal and/or further metabolism of methadone and/or EDDP, in agreement with the findings reported in Chapters 4 and 5. However, faecal samples were not collected from the patients and authentic samples of p-hydroxy EDDP and p-hydroxy EMDP are not available, precluding further examine these metabolic and excretory pathways.

7. Summary

The overall aims of this thesis were to increase understanding of the metabolism and disposition of methadone in humans, in particular in subjects receiving methadone maintenance treatment for opioid dependence, and to identify factors which may contribute to inter-subject variability in these processes, with respect to stereoselectivity. At the time the present PhD studies were commenced in 1995-96 there were few assays available for the stereoselective quantification of methadone or metabolites in biological fluids, or for the quantification of methadone metabolites in *in vitro* drug metabolism systems. Similarly, there were no HPLC assays available which simultaneously quantified methadone and metabolites other than EDDP and EMDP. Consequently, prior to 1996 there was no understanding of the enzymes mediating the metabolism of methadone or whether the process was stereoselective, and limited information regarding the role of stereoselectivity in the pharmacokinetics of methadone. Due to the lack of published work in these fields, the development and validation of several quantitative assays was necessary for the above mentioned aims to be achieved.

A total of seven HPLC assays, four of which stereoselective, were developed and fully validated in Chapter 2. Cross validation of the assays was performed and, with the exception of the rac-methadone assay in plasma, demonstrated that it is unlikely that there was interference by other licit and illicit drugs. Combined with the quality assurance procedures, the data indicate that all stereoselective assays were selective, precise and accurate. During the cross-validation of the assay for rac-methadone in plasma, it was noted that an unidentified compound co-eluted with rac-methadone in three subjects' plasma samples. This observation highlights the importance of assay selectivity assessments.

An eighth assay, for the quantification of methadone and eight of its known metabolites in urine was developed and partially validated (Chapter 6). This assay was developed in order to provide a quantitative assessment of the relative importance of several known methadone metabolites, as there is limited data in this field in the literature. However, when initial

investigations were conducted positive identification of most analytes was not possible, with the exception of methadone and EDDP. Despite this, the preliminary analysis of patient samples indicated that the concentrations of any metabolite other than methadone and EDDP, were likely to account for a relatively small proportion of the dose administered, and further validation was not performed. Future research should aim modify the assay for use in faecal samples and incorporate the hydrolysis of conjugated metabolites present in urine and faecal samples, in order to examine the excretion of methadone and metabolites in greater detail.

Chapter 3 describes the first study to date examining the metabolism of methadone with respect to stereoselectivity using human liver microsomes *in vitro*. The chromatography conditions employed allowed the separation of several known methadone metabolites. However, only EDDP was identified in microsomal incubations, consistent with the above mentioned observation that only EDDP was present in patient urine samples in significant concentrations. The maximum reaction velocity (V_{max}) for the formation of EDDP from (S)-methadone was significantly lower than for (R)-methadone, however, the magnitude of the difference was small (<16%). There was no significant difference in the affinity of (R)- and (S)-methadone for the enzyme (K_m) mediating the formation of EDDP, even though the chiral carbon is in close proximity to the site of oxidation. Similarly, there was no significant difference between (R)- and (S)-methadone for intrinsic clearance (CL_{int}) of this pathway. In support of this, the expressed enzyme and inhibition data did not indicate a clear difference between the two substrates. The lack of stereoselectivity observed here indicate that it is unlikely that there would be stereoselectivity in this metabolic pathway *in vivo* due to metabolism only. In contrast, the *in vitro-in vivo* scaling calculations resulted in a predicted $CL^{pred}_{MD \rightarrow EDDP}$ value for (R)-methadone greater than for (S)-methadone, which was due to the correction for *in vivo* plasma protein binding, but not intrinsic metabolic activity (CL_{int}). However, this observation cannot safely be extended to the total oral clearance of methadone, as only one metabolic pathway was examined.

The results of several different approaches identified CYP3A4 as mediating the formation of EDDP from rac-, (R)- and (S)-methadone. CYP2C9 and possibly CYP2C19 may also be involved but to a very minor extent. Thus, the large inter-individual variation reported for the pharmacokinetics of methadone may be due to variability in the expression of CYP3A4. Indeed, the *in vitro* intrinsic clearance for the formation of EDDP varied 4- to 5-fold between liver samples for the three substrates. Furthermore, it is unlikely that CYP2D6 is involved in the formation of EDDP due to a lack of effect of high concentrations of CYP2D6 inhibitors and extremely low metabolism of methadone to EDDP by expressed CYP2D6, and the Michaelis-Menten enzyme kinetic parameters for all three substrates in microsomes obtained from a genotypic CYP2D6 poor metaboliser were within the range found in CYP2D6 extensive metaboliser samples.

Future research should examine the microsomal protein binding of the individual methadone enantiomers, providing valuable information regarding the kinetics of the unbound drug. These investigations would further clarify the role of stereoselectivity in hepatic intrinsic clearance, allow for more appropriate *in vitro-in vivo* predictions of this metabolic pathway and the potential for drug-drug interactions in humans. The use of a stereoselective assay for methadone and EDDP might also be employed, thus allowing an examination of the potential interaction of the individual enantiomers at differing concentration ratios.

Metabolites other than EDDP were not found in the liver microsomal study. As reductive metabolites are not generally formed in microsomal fractions, other studies might aim to investigate the reductive metabolism of methadone in human liver cytosolic fractions. Similarly, one might quantify the loss of methadone from the microsomal incubations while simultaneously quantifying the formation of EDDP. Comparison of the molar loss of substrate and formation of known metabolite(s) would elucidate whether or not a significant degree of metabolism to unknown metabolites has occurred. Studies of this nature would be simple to perform.

The *in vitro* prediction ($CL^{pred}_{MD \rightarrow EDDP}$) of the *in vivo* partial clearance of methadone to EDDP ($CL_{MD \rightarrow EDDP}$) in Chapter 3 was substantially lower than literature estimates of total oral plasma clearance of methadone. Confirmation of this prediction was therefore desirable, as $CL_{MD \rightarrow EDDP}$ has not been reported in any study to date. Chapter 4 describes a study examining the steady-state pharmacokinetics of rac-methadone in a large cohort (n=18) of methadone maintenance patients during an inter-dosing interval, using non-compartmental pharmacokinetic analysis. The mean sum of the renal clearance and partial apparent clearance to EDDP ($CL_{MD \rightarrow EDDP}$) accounted for only 33% of total oral clearance, indicating that approximately two thirds of the clearance of methadone from plasma occurs via non-renal elimination of methadone and EDDP and/or further metabolism of these compounds. There was considerable (3- to 4-fold) inter-individual variability in all rac-methadone pharmacokinetic parameters which increased markedly to over 12-fold for $CL_{MD \rightarrow EDDP}$. However, variation in this parameter is likely to be influenced by plasma protein binding, and non-renal elimination and further metabolism of EDDP. Despite the large variability observed for the pharmacokinetic parameters, there was a highly significant relationship between plasma AUC^{ss}_{τ} and dose. This indicates that the pharmacokinetics (extent of absorption, clearance) of rac-methadone are linear after administration of the racemate over a wide dosage range (7.5-130 $mg.day^{-1}$), and confirms earlier reports in the literature.

Future studies should be aimed at the measurement of plasma EDDP concentrations, as would this would enable an estimation of the renal clearance of EDDP, thus allowing for an assessment of the non-renal elimination of this metabolite. Similarly, measurement of faecal concentrations of methadone, EDDP, and other metabolites of both compounds in both urine and faeces would improve the current understanding of the disposition of rac-methadone, and possibly provide further insight into the inter-individual variability of rac-methadone pharmacokinetics. As discussed above, the plasma protein binding of methadone is likely to play a role in the variability of rac-methadone pharmacokinetics and is likely to result in stereoselective disposition of methadone. No study to date has examined the role of variability in protein binding or metabolism in the pharmacokinetics of

(R)- and (S)-methadone. It was not known whether there is stereoselectivity in the clearance of methadone during chronic oral dosing, and whether or not this is due to metabolism (intrinsic clearance) and/or protein binding. These phenomena were examined in Chapter 5, which describes in detail the first study to date examining the pharmacokinetics of (R)- and (S)-methadone during chronic administration. In addition, α_1-acid glycoprotein concentrations, and the plasma protein binding of the individual enantiomers were examined in patient samples and solutions containing purified α_1-acid glycoprotein in order to determine its role in the disposition of (R)- and (S)-methadone.

Plasma unbound fractions of (R)-methadone were significantly greater (1.7-fold) than those of (S)-methadone. A weak but significant relationship was found between plasma α_1-acid glycoprotein concentration and the ratio of bound/unbound concentrations of each enantiomer. This is the first time that this has been demonstrated for the individual methadone enantiomers in methadone maintenance patients, and confirms earlier reports of this phenomenon in healthy volunteers. Binding experiments in the plasma of patients and purified α_1-acid glycoprotein demonstrated (R)-methadone and (S)-methadone bind to a common site, although the (R)-enantiomer has a 2-fold greater affinity.

In contrast to previous studies, and the binding to purified α_1-acid glycoprotein data reported here, I observed very low unbound fractions in the patient plasma samples. A possible reason for these difference may be that the pH of plasma samples was not maintained at 7.4. Although the ultra-filtration device maintains a constant sample pH during the filtration process, it is possible that the pH of the plasma samples may have increased during storage. This would result in an increase of the binding affinity of methadone for the plasma proteins, due to a greater proportion of the drug in an unionised state. Further possible explanations may be that the plasma concentrations of the ORM2 A variant of α_1-acid glycoprotein (to which methadone selectively binds) or binding proteins other than α_1-acid glycoprotein (albumin, cholesterol, triglycerides) may have been elevated in the patients. Future studies aimed at identifying factors that contribute to inter-individual

variation in methadone pharmacokinetics could therefore benefit from considering plasma concentrations of the individual α_1-acid glycoprotein variants and other binding proteins.

The ratio of (R)/(S) methadone concentrations was not constant over the inter-dosing interval, highlighting the need for consistency and accuracy in the timing of blood sampling if conclusions are to drawn regarding the extent of inter- and intra- patient variability in the relative plasma concentrations, and hence possible differences in clearance, between the two enantiomers. There was substantial stereoselectivity of most pharmacokinetic parameters, and also substantial inter-subject variability, which could contribute to altered therapeutic efficacy. Metabolic activity is the main determinant of $C^{ss}_{av_u}$ and CL_u/F. The extent of inter-individual variability (4- to 5-fold) observed for these parameters in this study is consistent with the reported variability in intrinsic clearance from the *in vitro* metabolism studies reported in Chapter 3. The highly significant relationship between plasma AUC^{ss}_{τ} and dose for (R)- and (S)-methadone indicate that the pharmacokinetics (extent of absorption, clearance) of the enantiomers are linear after administration of the racemate over a wide dosage range (7.5-130 $mg.day^{-1}$). This is the first report examining this relationship for the individual methadone enantiomers, and confirms previously observed linearity of rac-methadone pharmacokinetics. This demonstrates that the extent of inter-individual variation in metabolic activity does not prevent a dose-plasma concentration relationship for the individual methadone enantiomers.

There was no difference in the steady-state plasma clearance (CL/F) between the two enantiomers. However, when protein binding was considered, CL_u/F values for (R)-methadone were significantly lower than for (S)-methadone. This parameter which describes the elimination of the unbound pharmacologically active methadone enantiomer, indicates that (R)-methadone has a lower intrinsic clearance compared to (S)-methadone. In support of this observation, a significantly greater fraction of the dose was excreted in the urine as (S)-EDDP and (R)-methadone than the corresponding enantiomers. These data show that important characteristics of drug metabolism would fail to be observed using

non-stereoselective analytical techniques. There was stereoselectivity in the apparent partial clearance to EDDP ($CL_{MD \rightarrow EDDP}$, the *in vivo* correlate of *in vitro* $CL^{pred}_{MD \rightarrow EDDP}$), and apparent partial intrinsic clearance to EDDP ($CL_{MD \rightarrow EDDP_u}$ the *in vivo* correlate of *in vitro* CL_{int}), with (R)-methadone values significantly lower than for (S)-methadone. The reference to "apparent" is used as calculations were based on urinary excretion data only and EDDP is known to be eliminated to some extent in faeces, and further metabolised to a limited, albeit poorly defined, extent. These data are in contrast to those predicted from *in vitro-in vivo* scaling calculations in Chapter 3, which resulted in a predicted $CL_{MD \rightarrow EDDP}$ value for (R)-methadone greater than that of (S)-methadone. The stereoselective difference in this parameter arose from the correction for *in vivo* plasma protein binding, but not intrinsic metabolic activity (CL_{int}). The predicted stereoselectivity from the *in vitro* experiments is in contrast to that found here *in vivo*, in which the $CL_{MD \rightarrow EDDP_u}$ of (R)-methadone was significantly lower than for (S)-methadone. Reasons for this lack of agreement include the possibility that EDDP may be eliminated by other metabolic pathways which display stereoselectivity, and/or that there is stereoselectivity in the binding of methadone to proteins in the *in vitro* liver microsomal fraction, which may mask any stereoselectivity in metabolism by the enzymes mediating the formation of EDDP. It is possible that there may be stereoselectivity in the renal clearance and/or in the elimination of EDDP via faeces, such that the combined urinary and faecal recovery of (R)-EDDP is greater than that of (S)-EDDP. For both enantiomers, the sum of CL_R and $CL_{MD \rightarrow EDDP}$ accounted for a mean of only 33% of CL/F and the absolute values were similar in magnitude for each enantiomer. These data indicate that a significant proportion of the daily dose was eliminated by alternative clearance mechanisms. I did not quantitate EDDP in plasma or faeces, or other possible EDDP metabolites in urine, so are unable to examine these mechanisms further. If one assumes that there is no stereoselectivity in the microsomal protein binding of methadone, then comparison of these *in vivo* and *in vitro* data indicate that the net non-renal elimination (faecal elimination and/or further metabolism) of (R)-EDDP is greater than that of (S)-EDDP.

The predicted values of $CL_{MD \rightarrow EDDP}$ for both enantiomers from the *in vitro* studies are well below those found *in vivo* from urinary excretion data. Non-specific binding of methadone to microsomal proteins may have resulted in a significant under prediction of this parameter, providing one possible explanation. As noted above, a significant proportion of the daily dose was eliminated by clearance mechanisms other than the urinary elimination of EDDP, which may also account for this disparity.

The renal clearance of total (bound plus unbound) (R)- and (S)-methadone accounted for approximately 10-20% of CL/F, consistent with that observed for rac-methadone. However this was markedly stereoselective. In contrast, when corrected for protein binding there was no significant difference between the enantiomers. These data demonstrate that the net secretion and reabsorption of methadone is not a stereoselective process, and protein binding is an important consideration when drawing conclusions regarding the renal elimination mechanisms.

Future studies should aim at the measurement of the enantiomers of EDDP in plasma, and those of methadone and EDDP in faeces. These studies would further define the role of stereoselectivity in the clearance mechanisms of methadone, and provide further insight into the reported inter-individual variability of methadone pharmacokinetics highlighted in the present study. Other studies should include an examination of the effect other plasma proteins in addition to α_1-acid glycoprotein, and of storage on the pH of plasma samples and its possible attendant effects on the plasma protein binding of methadone.

The pharmacokinetic data for rac-methadone contained in this thesis have been successfully used to examine the pharmacokinetic-pharmacodynamic relationships of rac-methadone, using the sigmoid E_{max} pharmacodynamic model. There was an inverse relationship between plasma concentrations of rac-methadone and withdrawal severity (Methadone Symptoms Checklist) and pupil diameter, and a direct relationship with subjective opioid effects (Morphine-Benzedrine Group Scale of the Addiction Research Inventory, MBG).

The plasma concentration-effect relationship was very steep for subjective effects (withdrawal severity and MBG), as mean values for γ (the sigmoidicity or slope factor) were greater than 5, indicating that small changes in plasma rac-methadone concentrations translate into relatively large changes in effect. In contrast, the values were closer to unity for the objective measure of methadone effect (pupil diameter). EC_{50} values demonstrated wide inter-individual variability for all pharmacodynamic effects, with coefficients of variation ranging from 50% to 100%.

In conclusion, the research reported in this thesis has made significant advances in the current understanding of the metabolism and disposition of methadone, particularly in methadone maintenance patients. The data presented highlight the importance of protein binding and stereochemical considerations when drawing conclusions on the pharmacokinetics and metabolism of chiral compounds, and demonstrate that care should be taken when interpreting pharmacokinetic and metabolism data of chiral compounds based only upon results of non-chiral analytical techniques. I have shown that the pharmacokinetics of methadone are stereoselective, and that there is large inter-individual variability, consistent with CYP3A4 mediated metabolism to the major metabolite EDDP. However, this variability did not obscure a strong dose-plasma concentration relationship for the individual methadone enantiomers. Therefore, therapeutic drug monitoring for patient compliance is unlikely to require stereoselective measurement of plasma methadone concentrations, or unbound concentrations of the drug. In contrast, the stereoselective difference in the magnitude of fluctuation of plasma methadone concentrations reported in this thesis is likely to have important implications for pharmacokinetic-pharmacodynamic modelling of methadone, as measurement of rac-methadone does not provide an accurate reflection of plasma concentrations of the active (R)-methadone enantiomer. Additionally, inter-individual variability in the extent of plasma protein binding reported here may also add further complexity to this issue. Future studies aimed at examining the pharmacokinetic-pharmacodynamic relationships of methadone will benefit from consideration of the unbound concentration of the individual methadone enantiomers.

Appendices

Appendix 1: Summary of precision and accuracy data of non-stereoselective assays for the quantification of rac-methadone and metabolites in biological fluids reported in the literature.

Summary of precision and accuracy data of non-stereoselective assays for the determination of rac-methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Bartos et al., 1977	Schlitt et al., 1978	P (0.01-0.1)	methadone	0.5-10 (IA)	NR	NR
Ling et al., 1981	Bruera et al., 1995; Inturrisi et al., 1987b; Inturrisi et al., 1990; Schwartz et al., 1992; Wissel et al., 1987	P (0.05)	methadone	3-40 (IA)	NR	4 (9.5%, n=10)
	Bullingham et al., 1982	P (0.1)	methadone	3-40 (IA)	50 (1.6%, n=21)[e]	50 (9.9%, n=21)[e]
Beck et al., 1990	Hiltunen et al., 1999; Hiltunen et al., 1995	P (0.05-0.25)	methadone	50-1000 (IA)	50 (18%, n=10)	50 (6.3%, n=10)
	de Castro et al., 1996	P (NR)		10-500 (IA)	NR	NR (6.3%, n=10)

Notes: NR=not reported; [a]P=plasma, U=urine, S=saliva, F=faecal homogenate (0.3 $g.ml^{-1}$), H=hair (mg), LM=liver microsomes, BM=breast milk, values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve, IA=immunoassay, GC=gas chromatography, GC-MS=gas chromatography-mass spectrometry, HPLC=high performance liquid chromatography; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]calculated using all calibration standards, [g]data not reported separately.

Summary of precision and accuracy data of non-stereoselective assays for the determination of rac-methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Kreek et al., 1976b	Dole & Kreek, 1973; Kreek, 1973b; Kreek et al., 1980b; Kreek et al., 1978; Novick et al., 1985; Novick et al., 1981; Pond et al., 1985	P (2.0)	methadone	20-2000 (GC)	NR[f] (-2.0%, n=NR)	NR[f] (3.4%, n=NR)
		U (0.5)	methadone	1000-50000 (GC)	NR[f] (1.0%, n=NR)	NR[f] (2.5%, n=NR)
			EDDP	1000-50000 (GC)	NR[f] (-5.0%, n=NR)	NR[f] (6.5%, n=NR)
		F (1.0)	methadone	500-4000 (GC)	NR[f] (-4.0%, n=NR)	NR[f] (5.0%, n=NR)
Gourlay et al., 1982	Gourlay et al., 1986a; Gourlay et al., 1986b; Gourlay et al., 1984	P (1.0)	methadone	10-NR (GC)	NR	10 (<5%, n=NR)
Magora et al., 1987		P (1.0)	methadone	1-50 (GC)	1.0 (18%, n=12)[e]	1 (22%, n=12)[e]
Schmidt et al., 1993		P (1.0)	methadone	0.5-50 (GC)	0.5 (2.0%, n=4)	0.5 (13%, n=4)[e]
		U (1.0)	methadone	0.5-50 (GC)	0.5 (0.1%, n=4)	0.5 (6.8%, n=4)[e]
		CSF (1.0)	methadone	0.5-50 (GC)	1.0 (0.1%, n=4)	0.5 (14%, n=4)[e]

Notes: NR=not reported; [a]P=plasma, U=urine, S=saliva, F=faecal homogenate (0.3 $g.ml^{-1}$), H=hair (mg), LM=liver microsomes, BM=breast milk, values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve, IA=immunoassay, GC=gas chromatography, GC-MS=gas chromatography-mass spectrometry, HPLC=high performance liquid chromatography; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]calculated using all calibration standards, [g]data not reported separately.

Summary of precision and accuracy data of non-stereoselective assays for the determination of rac-methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Green & Wilson, 1996b	Green & Wilson, 1996a	P,H (1.0,10)[g]	methadone EDDP	5-400 (GC) 19-300 (GC)	NR NR	5 (11%, n=6)[e] 19 (11%, n=6)[e]
Chikhi-Chorfi et al., 1998		P (1.0)	methadone EDDP	50-2000 (GC) 50-2000 (GC)	NR NR	100 (5.3%, n=6)[e] 100 (3.4%, n=6)[e]
		U (1.0)	methadone EDDP	50-2000 (GC) 50-2000 (GC)	NR NR	100 (3.9%, n=6)[e] 100 (3.3%, n=6)[e]
		S (1.0)	methadone EDDP	50-2000 (GC) 50-2000 (GC)	NR NR	100 (5.5%, n=6)[e] 100 (5.1%, n=6)[e]
Torrens et al., 1998		P (1.0)	methadone EDDP	27-NR (GC) 25-NR (GC)	NR NR	100 (2.4%, n=NR)[e] 25 (13%, n=NR)[e]
George & Braithwaite, 1999		U (0.7)	Methadone EDDP	1000-20000 (GC) 1000-20000 (GC)	NR NR	1000 (9.0%, n=6)[e] 1000 (11%, n=6)[e]

Notes: NR=not reported; [a]P=plasma, U=urine, S=saliva, F=faecal homogenate (0.3 $g.ml^{-1}$), H=hair (mg), LM=liver microsomes, BM=breast milk, values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve, IA=immunoassay, GC=gas chromatography, GC-MS=gas chromatography-mass spectrometry, HPLC=high performance liquid chromatography; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]calculated using all calibration standards, [g]data not reported separately.

Summary of precision and accuracy data of non-stereoselective assays for the determination of rac-methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Sullivan et al., 1975b		P (4.0)	methadone	5-500 (GC-MS)	10 (-2.2, n=8)	10 (4%, n=8)
	Änggård et al., 1979	P (4.0)	methadone	10-NR (GC-MS)	NR	10 (5.0%, n=NR)
	Meresaar et al., 1981	P (2.0)	methadone	10-NR (GC-MS)	NR	15 (5.5%, n=10)
	Nilsson et al., 1982a; Nilsson et al., 1983; Nilsson et al., 1982b	P (4.0)	methadone	4-NR (GC-MS)	NR	4 (9.3%, n=10)
		S (4.0)	methadone	4-NR (GC-MS)	NR	8 (8.5%, n=10)
		U (4.0)	Methadone	80-NR (GC-MS)	NR	80 (9.3%, n=10)
			EDDP[g]			
	Beck et al., 1990	P (0.5)	methadone	NR (GC-MS)	NR	100 (2.0%, n=NR)
Baugh et al., 1991		U (5.0)	methadone	150-1200 (GC-MS)	600 (-1.3%, n=4)	666 (15%, n=4)
			EDDP	150-1200 (GC-MS)	600 (-0.2%, n=4)	589 (1.2%, n=4)

Notes: NR=not reported; [a]P=plasma, U=urine, S=saliva, F=faecal homogenate (0.3 $g.ml^{-1}$), H=hair (mg), LM=liver microsomes, BM=breast milk, values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve, IA=immunoassay, GC=gas chromatography, GC-MS=gas chromatography-mass spectrometry, HPLC=high performance liquid chromatography; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]calculated using all calibration standards, [g]data not reported separately.

Summary of precision and accuracy data of non-stereoselective assays for the determination of rac-methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Alburges et al., 1996	Moody et al., 1997; Moody et al., 1999	P (1.0)	methadone	10-600 (GC-MS)	10 (7.0%, n=3)[e]	10 (15%, n=3)[e]
			EDDP	10-600 (GC-MS)	10 (2.0%, n=3)[e]	10 (14%, n=3)[e]
			EMDP	10-600 (GC-MS)	10 (4.0%, n=3)[e]	10 (7.7%, n=3)[e]
		U (1.0)	methadone	10-600 (GC-MS)	10 (0.0%, n=3)[e]	10 (12%, n=3)[e]
			EDDP	10-600 (GC-MS)	10 (-6.0%, n=3)[e]	10 (6.4%, n=3)[e]
			EMDP	10-600 (GC-MS)	10 (0.0%, n=3)[e]	10 (4.7%, n=3)[e]
		LM (1.0)	methadone	10-600 (GC-MS)	100 (-7.0%, n=3)[e]	100 (7.5%, n=3)[e]
			EDDP	10-600 (GC-MS)	25 (3.2%, n=3)[e]	25 (15%, n=3)[e]
			EMDP	10-600 (GC-MS)	25 (-7.2%, n=3)[e]	25 (10%, n=3)[e]
	Wilkins et al., 1996; Wilkins et al., 1998	H (20)	Methadone	0.3-100 (GC-MS)	2 (6.5%, n=6)[e]	2 (18%, n=3)[e]
			EDDP	0.3-100 (GC-MS)	2 (31%, n=6)[e]	2 (13%, n=3)[e]
			EMDP	0.3-100 (GC-MS)	2 (16%, n=6)[e]	2 (15%, n=3)[e]
Bermejo et al., 2000b	Bermejo et al., 2000a	P (1.0)	methadone	50-2000 (GC-MS)	NR	500 (2.0%, n=11)
			EDDP	50-2000 (GC-MS)	NR	500 (2.4%, n=11)

Notes: NR=not reported; [a]P=plasma, U=urine, S=saliva, F=faecal homogenate (0.3 $g.ml^{-1}$), H=hair (mg), LM=liver microsomes, BM=breast milk, values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve, IA=immunoassay, GC=gas chromatography, GC-MS=gas chromatography-mass spectrometry, HPLC=high performance liquid chromatography; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]calculated using all calibration standards, [g]data not reported separately.

Summary of precision and accuracy data of non-stereoselective assays for the determination of rac-methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Wolff et al., 1990	Wolff et al., 1991a; Wolff et al., 1991c	U (2.0)	methadone	NR (HPLC)	NR	NR
	Wolff et al., 1991d	P (2.0)	methadone	10-100 (HPLC)	NR	66 (8.2%, n=8)[e]
	Wolff et al., 1991b	P (2.0)	methadone	NR (HPLC)	NR	69 (9.8%, n=10)[e]
		S (1.0)	methadone	NR (HPLC)	NR	112 (9.0%, n=10)[e]
	Wolff et al., 1992	P (2.0)	methadone	NR (HPLC)	NR	70 (9.8%, n=10)[e]
	Wolff et al., 1993	P (2.0)	methadone	NR (HPLC)	NR	NR (9.7%, n=8)[e]
	Rostami-Hodjegan et al., 1999; Wolff et al., 1997	P (2.0)	methadone	5-NR (HPLC)	NR	5 (9.8%, n=10)[e]
	Garrido et al., 1999	P (1.0)	methadone	5-250 (HPLC)	NR (-7.6%, n=3)[e]	NR (3.2%, n=3)[e]

Notes: NR=not reported; [a]P=plasma, U=urine, S=saliva, F=faecal homogenate (0.3 $g.ml^{-1}$), H=hair (mg), LM=liver microsomes, BM=breast milk, values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve, IA=immunoassay, GC=gas chromatography, GC-MS=gas chromatography-mass spectrometry, HPLC=high performance liquid chromatography; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]calculated using all calibration standards, [g]data not reported separately.

Summary of precision and accuracy data of non-stereoselective assays for the determination of rac-methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Pierce et al., 1992		P (0.9)	Methadone	25-1000 (HPLC)	NR	NR (2.9%, n=4)[e]
			EDDP	5-75 (HPLC)		NR
			EMDP	5-75 (HPLC)		NR
de Vos et al., 1995	de Vos et al., 1996	P (0.5)	Methadone	10-800 (HPLC)	NR	NR (7.7%, n=104)[e]
			EDDP	5-400 (HPLC)	NR	NR (6.7%, n=104)[e]
Iribarne et al., 1996	Iribarne et al., 1998a; Iribarne et al., 1997; Iribarne et al., 1998b	LM (1.0)	EDDP	600-NR (HPLC)	NR	NR (11%, n=NR)[g]
			EMDP	600-NR (HPLC)		
Wojnar-Horton et al., 1997		P (1.0)	methadone	5-800 (HPLC)	NR	50 (7.3%, n=8)
		BM (1.0)	methadone	5-800 (HPLC)	NR	40 (6.6%, n=5)
Stolk et al., 1997		U (1.0)	methadone	2500-10000 (HPLC)	NR	2500 (5.6%, n=4)
			EDDP	2500-15000 (HPLC)	NR	2500 (2.4%, n=5)
Cobb et al., 1998		P,U (1.0)[g]	methadone	10-500 (HPLC)	NR	50 (9.4%, n=NR)[e]

Notes: NR=not reported; [a]P=plasma, U=urine, S=saliva, F=faecal homogenate (0.3 $g.ml^{-1}$), H=hair (mg), LM=liver microsomes, BM=breast milk, values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve, IA=immunoassay, GC=gas chromatography, GC-MS=gas chromatography-mass spectrometry, HPLC=high performance liquid chromatography; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]calculated using all calibration standards, [g]data not reported separately.

Appendix 2: Summary of precision and accuracy data of stereoselective assays for the quantification of the enantiomers of methadone and metabolites in biological fluids reported in the literature.

Summary of precision and accuracy data of assays for the determination of the enantiomers of methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Kristensen & Angelo, 1992		P (1.0)	(R)-methadone	25-400 (GC)	150 (-8.6%, n=5)[e]	150 (10%, n=5)[e]
			(S)-methadone	25-400 (GC)	150 (6.0%, n=5)[e]	150 (7.1%, n=5)[e]
Beck et al., 1991	Hiltunen et al., 1999	P (0.5)	(R)-methadone	10-1000 (HPLC-agp)	100 (-3.4%, n=7)	100 (8.3%, n=7)
			(S)-methadone	10-1000 (HPLC-agp)	100 (-2.6%, n=5)	100 (6.1%, n=5)
Schmidt et al., 1992	Schmidt et al., 1994	P (2.0)	(R)-methadone	2.5-400 (HPLC-agp)	2.5 (0.0%, n=6)[e]	2.5 (7.9%, n=6)[e]
			(S)-methadone	2.5-400 (HPLC-agp)	2.5 (0.0%, n=6)[e]	2.5 (8.0%, n=6)[e]
Kristensen et al., 1994	Kristensen et al., 1996	P (1.0)	(R)-methadone	1.7-408 (HPLC-agp)	17 (2.0%, n=8)	17 (12%, n=8)
			(S)-methadone	1.7-408 (HPLC-agp)	17 (-2.0%, n=8)	17 (12%, n=8)
Kintz et al., 1997		H (60)	(R)-methadone	0.5-20 (HPLC-MS-agp)	NR	5 (<17%, n=NR)[e,f]
			(S)-methadone	0.5-20 (HPLC-MS-agp)	NR	
			(R)-EDDP	0.2-10 (HPLC-MS-agp)	NR	5 (<14%, n=NR)[e,f]
			(S)-EDDP	0.2-10 (HPLC-MS-agp)	NR	
de Vos et al., 1998		P (0.5)	(R)-methadone	80-230 (HPLC-agp)	NR	NR
			(S)-methadone	80-230 (HPLC-agp)	NR	NR

Notes: NR=not reported; [a]P=plasma, U=urine, H=hair (mg), values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve for each enantiomer, CE=capillary electrophoresis, GC=gas chromatography, MS=mass spectrometry, HPLC=high performance liquid chromatography, agp=α_1-acid glycoprotein column, cyc=cyclodextrin column; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]data not reported separately.

Summary of precision and accuracy data of assays for the determination of the enantiomers of methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Rudaz & Veuthey, 1996	Rudaz et al., 1999	P (1.0)	(R)-methadone	25-1000 (HPLC-agp)	25 (7.5%, n=3)[e]	25 (15%, n=3)[e]
			(S)-methadone	25-1000 (HPLC-agp)	25 (22%, n=3)[e]	25 (14%, n=3)[e]
Angelo et al., 1999		U (3.0)	(R)-methadone	6-770 (HPLC-agp)	6 (6.6%, n=5)	6 (9.3%, n=5)
			(S)-methadone	6-770 (HPLC-agp)	6 (3.3%, n=5)	6 (6.1%, n=5)
			(R)-EDDP	5-690 (HPLC-agp)	5 (-13%, n=5)	5 (16%, n=5)
			(S)-EDDP	5-690 (HPLC-agp)	5 (-3.3%, n=5)	5 (20%, n=5)
Norris et al., 1994		P (1.0)	(R)-methadone	5-250 (HPLC-cyc)	5 (12%, n=16)[e]	5 (12%, n=16)[e]
			(S)-methadone	5-250 (HPLC-cyc)	5 (12%, n=16)[e]	5 (15%, n=16)[e]
Eap et al., 1996	Eap et al., 1998; Eap et al., 1997	P (1.0)	(R)-methadone	10-500 (HPLC-cyc)	100 (3.4%, n=6)[e]	100 (8.8%, n=6)[e]
			(S)-methadone	10-500 (HPLC-cyc)	100 (4.0%, n=6)[e]	100 (7.0%, n=6)[e]
Pham-Huy et al., 1997		P (0.1)	(R)-methadone	25-1000 (HPLC-cyc)	NR	50 (4.5%, n=6)[e]
			(S)-methadone	25-1000 (HPLC-cyc)		50 (4.4%, n=6)[e]
			rac-EDDP	50-2000 (HPLC-cyc)		100 (3.8%, n=6)[e]
		U (0.1)	(R)-methadone	25-1000 (HPLC-cyc)	NR	50 (2.0%, n=6)[e]
			(S)-methadone	25-1000 (HPLC-cyc)		50 (3.5%, n=6)[e]
			rac-EDDP	50-2000 (HPLC-cyc)		100 (3.8%, n=6)[e]

Notes: NR=not reported; [a]P=plasma, U=urine, H=hair (mg), values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve for each enantiomer, CE=capillary electrophoresis, GC=gas chromatography, MS=mass spectrometry, HPLC=high performance liquid chromatography, agp=α_1-acid glycoprotein column, cyc=cyclodextrin column; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]data not reported separately.

Summary of precision and accuracy data of assays for the determination of the enantiomers of methadone and metabolites in biological fluids reported in the literature.

Original assay	Other authors	Matrix[a]	Compound	Calibration range (method of analysis)[b]	Accuracy[c]	Precision[d]
Lanz & Thormann, 1996		U (1.0)	(R)-methadone	1500-26700 (CE)	NR	NR (<10%, n=3)[f]
			(S)-methadone	1500-26700 (CE)	NR	
			(R)-EDDP	1100-21100 (CE)	NR	
			(S)-EDDP	1100-21100 (CE)	NR	
Frost et al., 1997		P (1.0)	(R)-methadone	2.5-500 (CE)	5 (20%, n=3)	5 (16%, n=3)
			(S)-methadone	2.5-500 (CE)	5 (20%, n=3)	5 (15%, n=3)
			(R)-EDDP	2.5-500 (CE)	5 (-20%, n=3)	5 (19%, n=3)
			(S)-EDDP	2.5-500 (CE)	5 (20%, n=3)	5 (17%, n=3)
		U (1.0)	(R)-methadone	10-2500 (CE)	50 (16%, n=3)	50 (11%, n=3)
			(S)-methadone	10-2500 (CE)	50 (12%, n=3)	50 (13%, n=3)
			(R)-EDDP	10-2500 (CE)	50 (22%, n=3)	50 (17%, n=3)
			(S)-EDDP	10-2500 (CE)	50 (24%, n=3)	50 (15%, n=3)
Ramseier et al., 1999		U (1.0)	(R)-methadone	NR	NR	1000 (3.9%, n=4)
			(S)-methadone	NR	NR	1000 (6.3%, n=4)
			(R)-EDDP	NR	NR	1000 (1.6%, n=4)
			(S)-EDDP	NR	NR	1000 (1.8%, n=4)

Notes: NR=not reported; [a]P=plasma, U=urine, H=hair (mg), values in parentheses are the volume (ml) of matrix assayed; [b]concentration range ($ng.ml^{-1}$) of calibration curve for each enantiomer, CE=capillary electrophoresis, GC=gas chromatography, MS=mass spectrometry, HPLC=high performance liquid chromatography, agp=α_1-acid glycoprotein column, cyc=cyclodextrin column; [c]lowest concentrations ($ng.ml^{-1}$) for which intra-assay accuracy was reported, values in parentheses are accuracy presented as %bias and n value; [d]lowest concentration ($ng.ml^{-1}$) for which intra-assay precision was reported, values in parentheses are precision presented as residual standard deviation; [e]inter-assay data; [f]data not reported separately.

Appendix 3: Summary of the pharmacokinetic parameters for methadone reported in the literature.

Summary of the pharmacokinetic parameters of methadone reported in the literature.

Source	Subjects[1]	Dose (mg)	Sampling period used for calculation (hr)[2]	Number of exponential terms in decay phase[3]	Terminal elimination $t_{1/2}$ (hr)	Clearance ($ml.min^{-1}$)	Volume of distribution ($l.kg^{-1}$)
Inturrisi & Verebely, 1972a	5 HV (a)	15 *p.o.*	4-24	1	15±4	NR	NR
Nilsson et al., 1982b modification of urinary pH	5 HV (a) acidic urine alkaline urine	10 i.*m.*	0-72	2	 20±4 42±9	 134±21 92±9	 3.5±0.4[4] 5.2±0.8[4]
Wissel et al., 1987 modification of diet	7 HV (a) western diet low fat diet	15 *p.o.*	0-24	NC	NR	 223±71[7] 204±78[7]	NR
Inturrisi & Verebely, 1972c	5 MM (ss)	100-120 *p.o.*	4-24	1	25±14	NR	NR
Verebely et al., 1975a	12 MM (a) (ss)	 15 *p.o.* 40-80 *p.o.*	4-27	2	 55±27 22±7	NR	NR
Änggård et al., 1979	7 MM (ss)	54-90 $^2H_3$-labelled *i.v.* $^2H_3$-labelled *p.o.* unlabelled *i.v.*. unlabelled *p.o.*	8-24	1	 24±5 22±2 45±13 52±20	NR	NR

Notes: All data are expressed as mean±SD or (range); NR=not reported/estimated; [1]MM=methadone maintenance patients, HV=healthy volunteers, Pain=pain patients, Burn=burns patients, (ss)=steady state dosing with unlabelled methadone (daily), (a)=acute single dose; [2]peak=time of maximum plasma concentration; [3]NC=non-compartmental analysis; [4]$V_{d\beta}$; [5]V_c; [6]V_{dss}; [7]oral clearance (CL/F) or volume of distribution (V/F); [8]population mean values obtained from population pharmacokinetic modelling; [9]values for volume of distribution are expressed in litres (l) and not corrected for body weight; [10]I=20-34 weeks gestation, II=35-40 weeks gestation, III=1-4 weeks postpartum, 8-9 weeks postpartum;[11]SEM; [12]values calculated for whole blood; [13]calculated from urinary excretion data.

Summary of the pharmacokinetic parameters of methadone reported in the literature.

Source	Subjects[1]	Dose (mg)	Sampling period used for calculation (hr)[2]	Number of exponential terms in decay phase[3]	Terminal elimination $t_{1/2}$ (hr)	Clearance ($ml.min^{-1}$)	Volume of distribution ($l.kg^{-1}$)
Meresaar et al., 1981	8 MM (a)	20 $^2H_3$-labelled *i.v.*	0-48	2	28±11	137±92	2.2±0.4[5] 3.9±1.0[4]
Nilsson et al., 1982a	12 MM (a)	15 $^2H_3$-labelled *i.v.*	0-48	2	35±12	95±31	3.8±0.6[4]
	6 MM (ss)	30 $^2H_3$-labelled *i.v.*	0-48		31±8	107±45	4.3±0.8[4]
	6 MM (ss)	30 unlabelled *p.o.*	0-24		33±7	NR	NR
	6 MM (ss)	60 $^2H_3$-labelled *i.v.*	0-48		36±6	100±55	4.5±0.7[4]
	6 MM (ss)	60 unlabelled *p.o.*	0-24		34±7	NR	NR
Nilsson et al., 1983	8 MM (ss) "therapeutic failures"	50-100 $^2H_3$-labelled *i.v.*	0-24	1 or 2	25±3	104±36	1.4±0.3[5] 3.1±1.0[4] 2.7±1.0[6]
	12 MM (ss) "unselected"	30-60 $^2H_3$-labelled *i.v.*			34±7	111±36	2.7±0.4[5] 4.6±1.0[4] 4.2±0.8[6]
de Vos et al., 1995	20 MM (ss)	10-225 *p.o.*	0-24	2	31±12	107±55[7]	2.1±1.3[5,7] 4.1±1.9[4,7]

Notes: All data are expressed as mean±SD or (range); NR=not reported/estimated; [1]MM=methadone maintenance patients, HV=healthy volunteers, Pain=pain patients, Burn=burns patients, (ss)=steady state dosing with unlabelled methadone (daily), (a)=acute single dose; [2]peak=time of maximum plasma concentration; [3]NC=non-compartmental analysis; [4]$V_{d\beta}$; [5]V_c; [6]V_{dss}; [7]oral clearance (CL/F) or volume of distribution (V/F); [8]population mean values obtained from population pharmacokinetic modelling; [9]values for volume of distribution are expressed in litres (l) and not corrected for body weight; [10]I=20-34 weeks gestation, II=35-40 weeks gestation, III=1-4 weeks postpartum, 8-9 weeks postpartum;[11]SEM; [12]values calculated for whole blood; [13]calculated from urinary excretion data.

Summary of the pharmacokinetic parameters of methadone reported in the literature.

Source	Subjects[1]	Dose (mg)	Sampling period used for calculation (hr)[2]	Number of exponential terms in decay phase[3]	Terminal elimination $t_{1/2}$ (hr)	Clearance ($ml.min^{-1}$)	Volume of distribution ($l.kg^{-1}$)
Wolff et al., 1993	5 MM (ss)	10-60 *p.o.*	0-24	1	27±15	184±30[7]	6.7±2.9[4,7]
Wolff et al., 1997[8]	13 HV (a)	8-15 *p.o.*	0-57	2	41±21[8]	115±25[7,8]	212±27[5,7,8,9] 376±48[6,7,8,9]
	17 MMT (a)	15-80 *p.o.*	0-27	2	207±185[8]	53±5[7,8]	239±121[5,7,8,9] 870±444[6,7,8,9]
Rostami-Hodjegan et al., 1999[8]	17 MM (a) 35 MMT (ss)	20-80 *p.o.* 5-80 *p.o.*	0-27 0-24	2	128[8] 48[8]	52[7,8] 171[7,8]	108[5,7,8,9] 123[5,7,8,9]
Cobb et al., 1998 interaction of fluconazole	13 MM (ss) -fluconazole +fluconazole	55±6 *p.o.*	0-24	NC	NR	 138±18[7] 103±13[7]	NR
Pond et al., 1985 influence of pregnancy	9 MM (ss)[10] I II III IV	 30±8 *p.o.* 22±9 *p.o.* 28±12 *p.o.* 36±22 *p.o.*	0-24	NC	NR	 311±118[7] 256±97[7] 161±69[7] 155±62[7]	NR

Notes: All data are expressed as mean±SD or (range); NR=not reported/estimated; [1]MM=methadone maintenance patients, HV=healthy volunteers, Pain=pain patients, Burn=burns patients, (ss)=steady state dosing with unlabelled methadone (daily), (a)=acute single dose; [2]peak=time of maximum plasma concentration; [3]NC=non-compartmental analysis; [4]$V_{d\beta}$; [5]V_c; [6]V_{dss}; [7]oral clearance (CL/F) or volume of distribution (V/F); [8]population mean values obtained from population pharmacokinetic modelling; [9]values for volume of distribution are expressed in litres (l) and not corrected for body weight; [10]I=20-34 weeks gestation, II=35-40 weeks gestation, III=1-4 weeks postpartum, 8-9 weeks postpartum;[11]SEM; [12]values calculated for whole blood; [13]calculated from urinary excretion data.

Summary of the pharmacokinetic parameters of methadone reported in the literature.

Source	Subjects[1]	Dose (mg)	Sampling period used for calculation (hr)[2]	Number of exponential terms in decay phase[3]	Terminal elimination $t_{1/2}$ (hr)	Clearance ($ml.min^{-1}$)	Volume of distribution ($l.kg^{-1}$)
Novick et al., 1981	MM (ss)		0-24	2			NR
influence of liver disease	5 healthy	35-100 *p.o.*			19±3[11]	308±44[7,11]	
	4 mild	25-80 *p.o.*			11±2[11]	315±40[7,11]	
	5 moderate	25-80 *p.o.*			13±2[11]	393±92[7,11]	
	5 severe	25-80 *p.o.*			36±8[11]	237±30[7,11]	
Novick et al., 1985	MM (ss)		0-24	2			
influence of liver disease in	9 healthy	30-90 *p.o.*			20±2[11]	250±36[7,11]	438±94[4,7,9,11]
chronic alcoholism	11 liver disease	20-90 *p.o.*			32±5[11]	280±23[7,11]	716±100[4,7,9,11]
Gourlay et al., 1982	19 Pain (a)	20 *i.v.*	0-48	2	35±22[12]	178±100[12]	1.1±0.7[5,12] 6.1±2.4[6,12]
Gourlay et al., 1986a	9 Pain (a)	15-25 *i.v.*	0-36	2	30±16[12]	190±130[12]	NR
Inturrisi et al., 1987b	8 Pain (a)	10-30 *i.v.*	0-48	3	27±11	146±68[12]	0.2±0.1[5] 3.5±1.2[6]
Plummer et al., 1988	185 Pain (a)	8-67 *i.v.*	0-30	2	32 (4-130)	186 (23-850)	NR
Denson et al., 1990	14 Burn (ss)	NR *i.v.* infusion	24 post-infusion	1	2.6±1.1	883±317	2.5±0.8[5]

Notes: All data are expressed as mean±SD or (range); NR=not reported/estimated; [1]MM=methadone maintenance patients, HV=healthy volunteers, Pain=pain patients, Burn=burns patients, (ss)=steady state dosing with unlabelled methadone (daily), (a)=acute single dose; [2]peak=time of maximum plasma concentration; [3]NC=non-compartmental analysis; [4]$V_{d\beta}$; [5]V_c; [6]V_{dss}; [7]oral clearance (CL/F) or volume of distribution (V/F); [8]population mean values obtained from population pharmacokinetic modelling; [9]values for volume of distribution are expressed in litres (l) and not corrected for body weight; [10]I=20-34 weeks gestation, II=35-40 weeks gestation, III=1-4 weeks postpartum, 8-9 weeks postpartum; [11]SEM; [12]values calculated for whole blood; [13]calculated from urinary excretion data.

Summary of the pharmacokinetic parameters of methadone reported in the literature.

Source	Subjects[1]	Dose (mg)	Sampling period used for calculation (hr)[2]	Number of exponential terms in decay phase[3]	Terminal elimination $t_{1/2}$ (hr)	Clearance ($ml.min^{-1}$)	Volume of distribution ($l.kg^{-1}$)
Olsen et al., 1977	5-6 HV (a)	15 *p.o.* rac-methadone	peak-48	1	22 (13-28)	NR	NR
		7.5 *p.o.* (R)-methadone			24 (19-31)		
		(S)-methadone			25 (21-28)		
Kreek et al., 1979	3 MM (ss)	60-80 *p.o.* rac-methadone	0-240	2	53±6[13]	NR	NR
		(R)-methadone			57±3[13]		
		(S)-methadone			34±2[13]		
Nakamura et al., 1982	2 MM (ss)	80-100 *p.o.* (R)-methadone	0-119	3	38-59	NR	NR
		(S)-methadone			28-35		
Beck et al., 1991	1 MM (ss)	110 *p.o.* (R)-methadone	0-24	2	14	NR	NR
		(S)-methadone			16		
Kristensen et al., 1996	7 Pain (a)	10-60 *i.v.* (R)-methadone	0-48	2	38±8	158±4	497±117[6,9]
		(S)-methadone			29±11	129±5	289±78[6,9]

Notes: All data are expressed as mean±SD or (range); NR=not reported/estimated; [1]MM=methadone maintenance patients, HV=healthy volunteers, Pain=pain patients, Burn=burns patients, (ss)=steady state dosing with unlabelled methadone (daily), (a)=acute single dose; [2]peak=time of maximum plasma concentration; [3]NC=non-compartmental analysis; [4]$V_{d\beta}$; [5]V_c; [6]V_{dss}; [7]oral clearance (CL/F) or volume of distribution (V/F); [8]population mean values obtained from population pharmacokinetic modelling; [9]values for volume of distribution are expressed in litres (l) and not corrected for body weight; [10]I=20-34 weeks gestation, II=35-40 weeks gestation, III=1-4 weeks postpartum, 8-9 weeks postpartum;[11]SEM; [12]values calculated for whole blood; [13]calculated from urinary excretion data.

Appendix 4: Summary of the excretion of methadone and metabolites as a percent of dose administered reported in the literature.

Summary of the excretion of methadone and metabolites as a percent of dose administered reported in the literature.

Source	Subjects[1]	Dose (mg)	Sample[2]	Methadone (%dose)	EDDP (%dose)	EMDP (%dose)	Other metabolites (%dose)[3]	Total (% dose)[4]
Pohland et al., 1971[5]	1 HV (a)	10 *p.o.*	24 hr U	2.4	5.8	trace	NA	8.4
Inturrisi & Verebely, 1972b[5]	3 HV (a)	10 *p.o.*	96 hr U	32.5±5.3	12.9±7.3	0.7±0.6	NA	46.1±2.2
Inturrisi & Verebely, 1972a[5]	5 HV (a)	15 *p.o.*	96 hr U	23.7±6.5	38.0±11.1	0.9±0.3	NA	62.6±13.3
Inturrisi et al., 1987b	8 P (a)	10-30 *i.v.*	24 hr U	3.7±3.0	NA	NA	NA	3.7±3.0
Sullivan & Blake, 1972[5]	1 MM (ss)	80 *p.o.*	24 hr U	10.5	3.5	1.3	NA	15.3
Sullivan & Due, 1973[5]	3 MM (ss)	50-100 *p.o.*	24 hr U	16.0±12.4	17.7±16.3	1.4±1.8	0.8±1.4[6]	36.2±23.1
Änggård et al., 1975	6 MM							
	(a)	10 *p.o.*	96 hr U	20.1±9.1	13.3±4.6	NA	NA	33.3±11.0
	(ss)	80 *p.o.*	96 hr U	12.2±7.1	22.5±11.9	NA	NA	34.7±12.9
Bellward et al., 1977[5]	12 MM (ss)	15-110 *p.o.*	24 hr U	15.9±8.2	26.8±10.1	NA	NA	42.6±15.6

Notes: All data are expressed as mean±SD, trace=detected, but below the limit of quantification; [1]MM=methadone maintenance patients, HV=healthy volunteers, P=pain patients, (ss)=steady state dosing (daily), (a)=acute single dose; [2]U=urine, B=bile, F=faeces, GF=gastric fluid; [3]NA=not assayed; [4]total is the combined sum of all metabolites examined; [5]recalculation of reported values for metabolites with consideration of molecular weight differences; [6]pyrrolidone metabolite; [7]combined pyrrolidone and p-hydroxy methadone metabolites, [8]α-methadol metabolite; [9]SEM; [10]I=20-34 weeks gestation, II=35-40 weeks gestation, III=1-4 weeks postpartum, 8-9 weeks postpartum; [11]sum of EDDP and EMDP;[12]standard deviation not presented or calculable.

Summary of the excretion of methadone and metabolites as a percent of dose administered reported in the literature.

Source	Subjects[1]	Dose (mg)	Sample[2]	Methadone (%dose)	EDDP (%dose)	EMDP (%dose)	Other metabolites (%dose)[3]	Total (% dose)[4]
Verebely et al., 1975a[5]	6 MM (a)	20-25 *p.o.*	24 hr U	12.2±4.6	7.3±2.1	trace	NA	19.4±5.5
			24 hr F	0.6±0.9	2.8±4.1	trace	NA	3.5±5.0
			24 hr U+F	12.8±5.2	10.1±4.6			22.9±8.7
	(ss)	35-60 *p.o.*	24 hr U	16.8±8.9	24.8±11.8	trace	NA	41.6±16.7
			24 hr F	1.1±0.6	6.8±3.4	trace	NA	8.0±3.7
			24 hr U+F	17.7±9.3	30.5±9.9			48.2±15.3
	(ss)	40-80 *p.o.*	24 hr U	16.6±6.1	33.6±10.1	trace	NA	50.4±13.5
			24 hr F	2.3±1.2	22.7±10.7	trace	NA	25.0±11.7
			24 hr U+F	19.1±5.5	56.3±16.6			75.4±17.4
Kreek et al., 1983; Kreek et al., 1980a[5] influence of liver disease	5 MM (ss) healthy	15-100 *p.o.*	24 hr U	16.6±5.6	29.7±3.6	1.2±0.8	4.1±1.9[7] and 0.1±0.2[8]	51.7±3.6
			24 hr F	0.8±0.3	7.1±2.5	0.3±0.2	1.0±0.5[7] and 0.1±0.1[8]	9.2±2.2
			24 hr U+F	17.4±5.7	36.8±2.7	1.5±0.7	5.1±2.0[7] and 0.2±0.2[8]	60.9±5.4
	14 MM (ss) liver disease	15-100 *p.o.*	24 hr U	9.9±5.1	22.5±10.7	0.8±0.3	1.5±0.6[7] and 0.1±0.1[8]	34.7±12.3
			24 hr F	0.9±0.6	8.5±3.6	0.4±0.3	1.4±1.1[7] and 0.0±0.0[8]	11.2±3.9
			24 hr U+F	10.7±4.9	30.9±10.9	1.2±0.4	2.9±1.3[7] and 0.2±0.1[8]	46.0±11.8
Cobb et al., 1998	11 MM (ss)	59±7 *p.o.*	24 hr U	16.4±2.0[9]	NA	NA	NA	16.4±2.0[9]

Notes: All data are expressed as mean±SD, trace=detected, but below the limit of quantification; [1]MM=methadone maintenance patients, HV=healthy volunteers, P=pain patients, (ss)=steady state dosing (daily), (a)=acute single dose; [2]U=urine, B=bile, F=faeces, GF=gastric fluid; [3]NA=not assayed; [4]total is the combined sum of all metabolites examined; [5]recalculation of reported values for metabolites with consideration of molecular weight differences; [6]pyrrolidone metabolite; [7]combined pyrrolidone and p-hydroxy methadone metabolites, [8]α-methadol metabolite; [9]SEM; [10]I=20-34 weeks gestation, II=35-40 weeks gestation, III=1-4 weeks postpartum, 8-9 weeks postpartum; [11]sum of EDDP and EMDP;[12]standard deviation not presented or calculable.

Summary of the excretion of methadone and metabolites as a percent of dose administered reported in the literature.

Source	Subjects[1]	Dose (mg)	Sample[2]	Methadone (%dose)	EDDP (%dose)	EMDP (%dose)	Other metabolites (%dose)[3]	Total (% dose)[4]
Nilsson et al., 1982a	6 MM (ss)	30 *p.o.*	24 hr U	12.5±13.2	25.2±12.6	NA	NA	37.5±25.5
	6 MM (ss)	60 *p.o.*	24 hr U	12.8±9.1	35.5±13.9	NA	NA	48.3±11.3
Nilsson et al., 1982b	5 HV (a)							
influence of urinary pH	acidic urine	10 *i.m.*	96 hr U	33.7±3.2	18.4±4.0	NA	NA	52.1±4.4
	alkaline urine	10 *i.m.*	96 hr U	0.0±0.0	27.0±1.5			27.0±1.5
Pond et al., 1985	9 MM (ss)[10]							
influence of pregnancy	I	30±8	24 hr U	8±3	24±12[11]	NA[11]	11±6[8]	43[12]
	I	22±9	24 hr U	12±8	24±10[11]	NA[11]	7±11[8]	43[12]
	III	28±12	24 hr U	13±9	23±15[11]	NA[11]	12±10[8]	48[12]
	IV	36±22	24 hr U	9±8	21±12[11]	NA[11]	12±16[8]	42[12]
Lynn et al., 1976b	5 MM (ss)	54-81 *i.m.*	8 hr GF	7.7±4.4	trace	NA	NA	7.7±4.4
Kreek et al., 1980b	1 MM (ss)	100 *p.o.*	24 hr B	0.1	38.6	0.1	NA	38.8

Notes: All data are expressed as mean±SD, trace=detected, but below the limit of quantification; [1]MM=methadone maintenance patients, HV=healthy volunteers, P=pain patients, (ss)=steady state dosing (daily), (a)=acute single dose; [2]U=urine, B=bile, F=faeces, GF=gastric fluid; [3]NA=not assayed; [4]total is the combined sum of all metabolites examined; [5]recalculation of reported values for metabolites with consideration of molecular weight differences; [6]pyrrolidone metabolite; [7]combined pyrrolidone and p-hydroxy methadone metabolites, [8]α-methadol metabolite; [9]SEM; [10]I=20-34 weeks gestation, II=35-40 weeks gestation, III=1-4 weeks postpartum, 8-9 weeks postpartum; [11]sum of EDDP and EMDP;[12]standard deviation not presented or calculable.

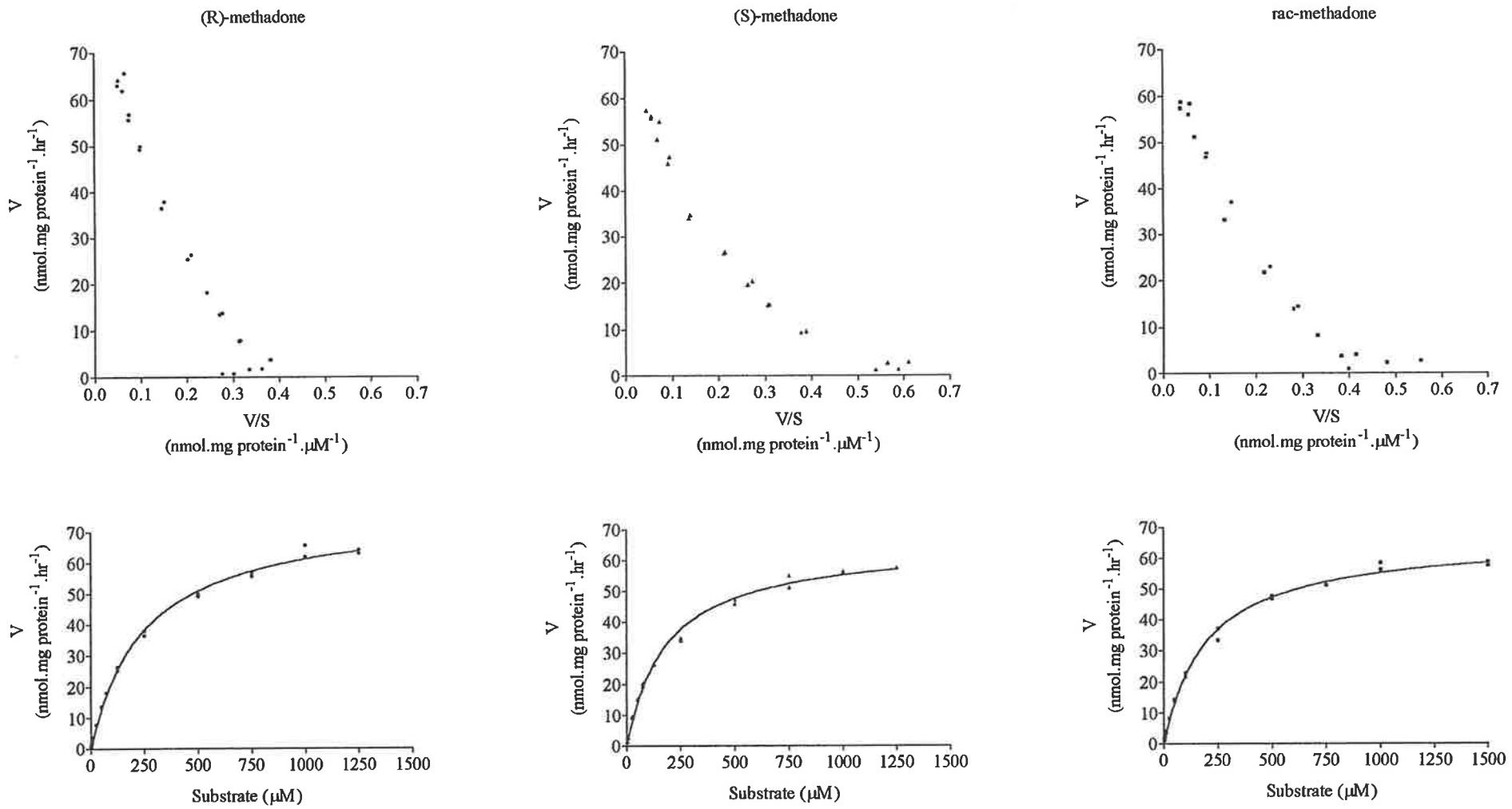
Appendix 5: Liver sample donor demographic details.

Liver sample donor demographic details.

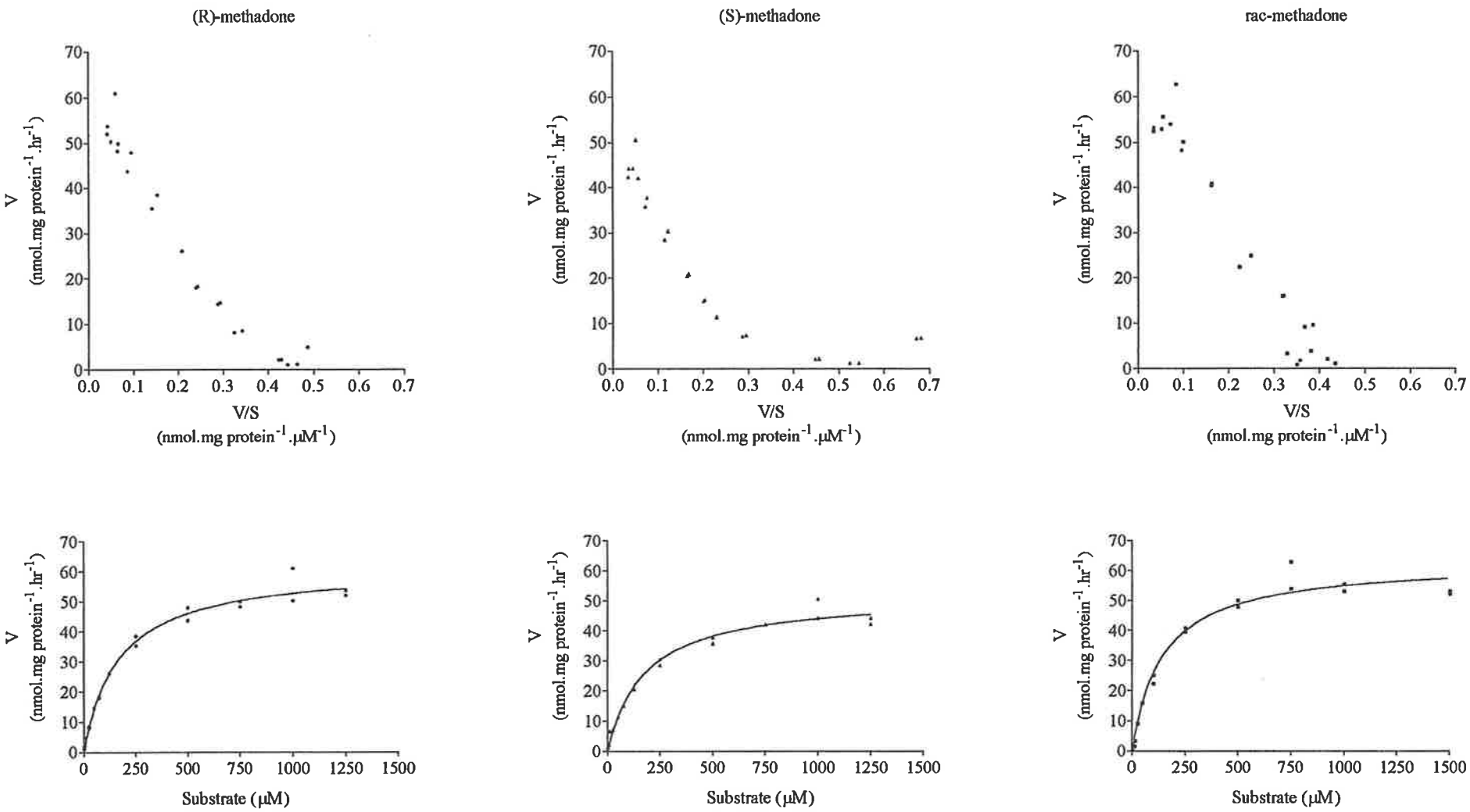
HLS	Age (years)	Sex	Alcohol (g/week)	Smoking (day^{-1})	Caffeine (day^{-1})	Total P450	Liver enzymes ($U.l^{-1}$)					Medications	
							GGT	ALP	LD	AST	ALT	Regular/ premedication	Surgical
5	66	F	NIL	NIL	1 tea 4 coffee	233	14	64	192	25	NA	NIL / pethidine, metoclopramide	gentamicin, amoxycillin, metronidazole, ephedrine, neostigmine/atropine, bupivacaine, suxamethonium, vecuronium, fentanyl, thiopentone, lignocaine
16	25	F	20	NIL	2 coffee	300	NA	NA	NA	NA	NA	diazepam, paracetamol, codeine, doxylamine / temazepam	fentanyl, pancuronium, vecuronium, propofol
21	70	M	<10	NIL	2 coffee	163	29	93	219	11	11	NIL / temazepam, heparin	thiopentone, fentanyl, vecuronium, amoxycillin, gentamicin, metronidazole
22	54	F	<10	NIL	3 coffee	482	NA	98	179	12	37	pyridoxine, microlax, metronidazole, cephalothin, gentamicin, hydrocortisone, promethazine / temazepam	thiopentone, fentanyl, atracurium, bupivacaine, adrenaline
23	62	M	300	NIL	1 coffee	201	28	76	188	45	21	prednisolone / temazepam	thiopentone, fentanyl, vecuronium, morphine, hydrocortisone, amoxycillin, gentamicin, metronidazole
24	42	F	70	NIL	3-4 tea <1 coffee	192	32	101	187	15	21	NIL / temazepam	thiopentone, fentanyl, atracurium, morphine, amoxycillin, gentamicin, metronidazole
31	73	M	<10	NIL	NIL	169	21	105	NA	NA	10	frusemide / temazepam	fentanyl, streptomycin, atracurium, bupivacaine, amoxycillin, gentamicin, metronidazole

Notes: Total P450=total P450 content of microsomes prepared from liver donor sample (pmol P450.mg $protein^{-1}$); NA=not available; normal range of liver enzymes: GGT 5-60 $U.l^{-1}$, ALP 30-95 $U.l^{-1}$, LD 110-230 $U.l^{-1}$, AST 13-45 $U.l^{-1}$, ALT 0-45 $U.l^{-1}$.

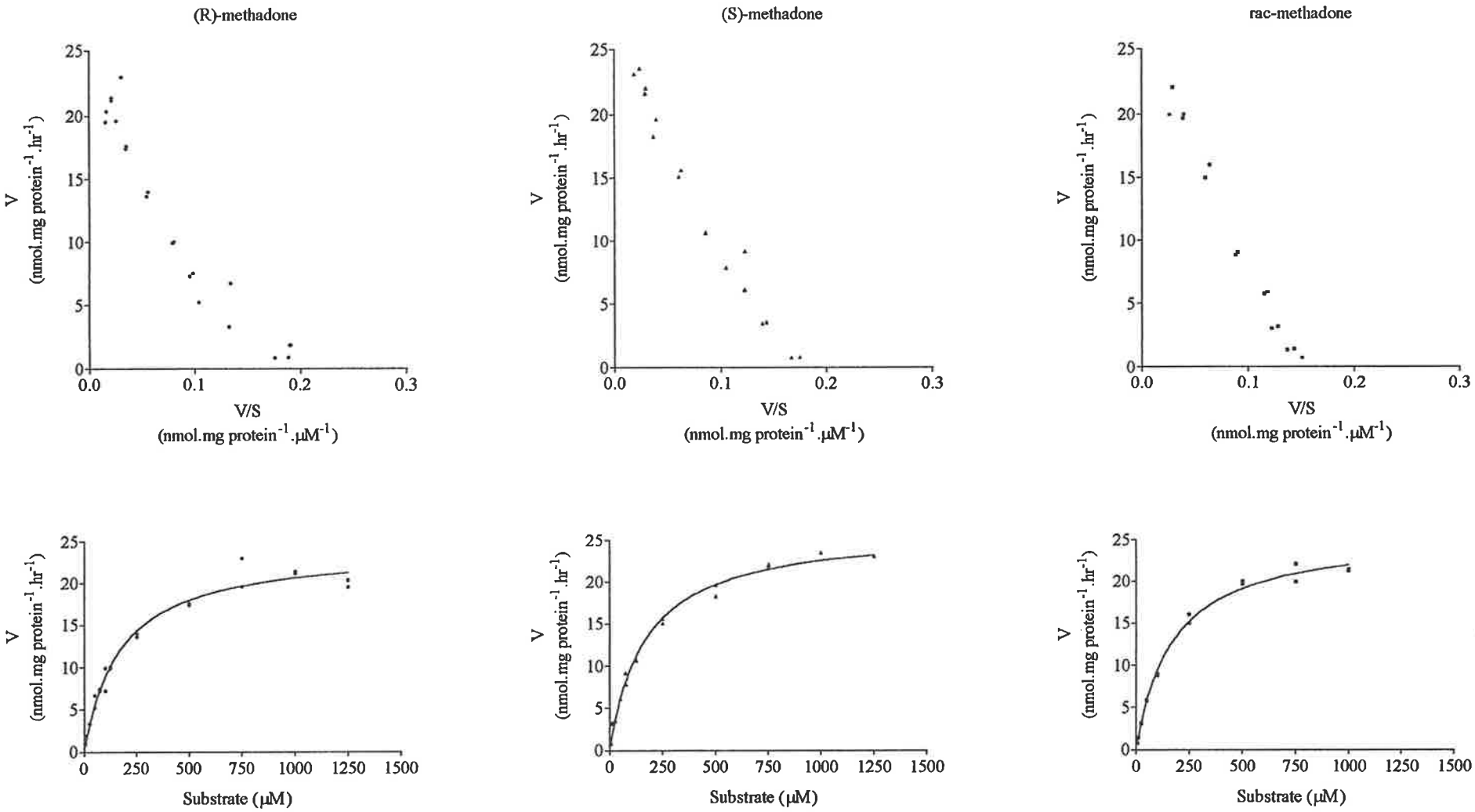
Appendix 6: Eadie-Hofstee and Michaelis-Menten enzyme kinetic plots for the formation of EDDP from rac-, (R)- and (S)-methadone in human liver microsomes.



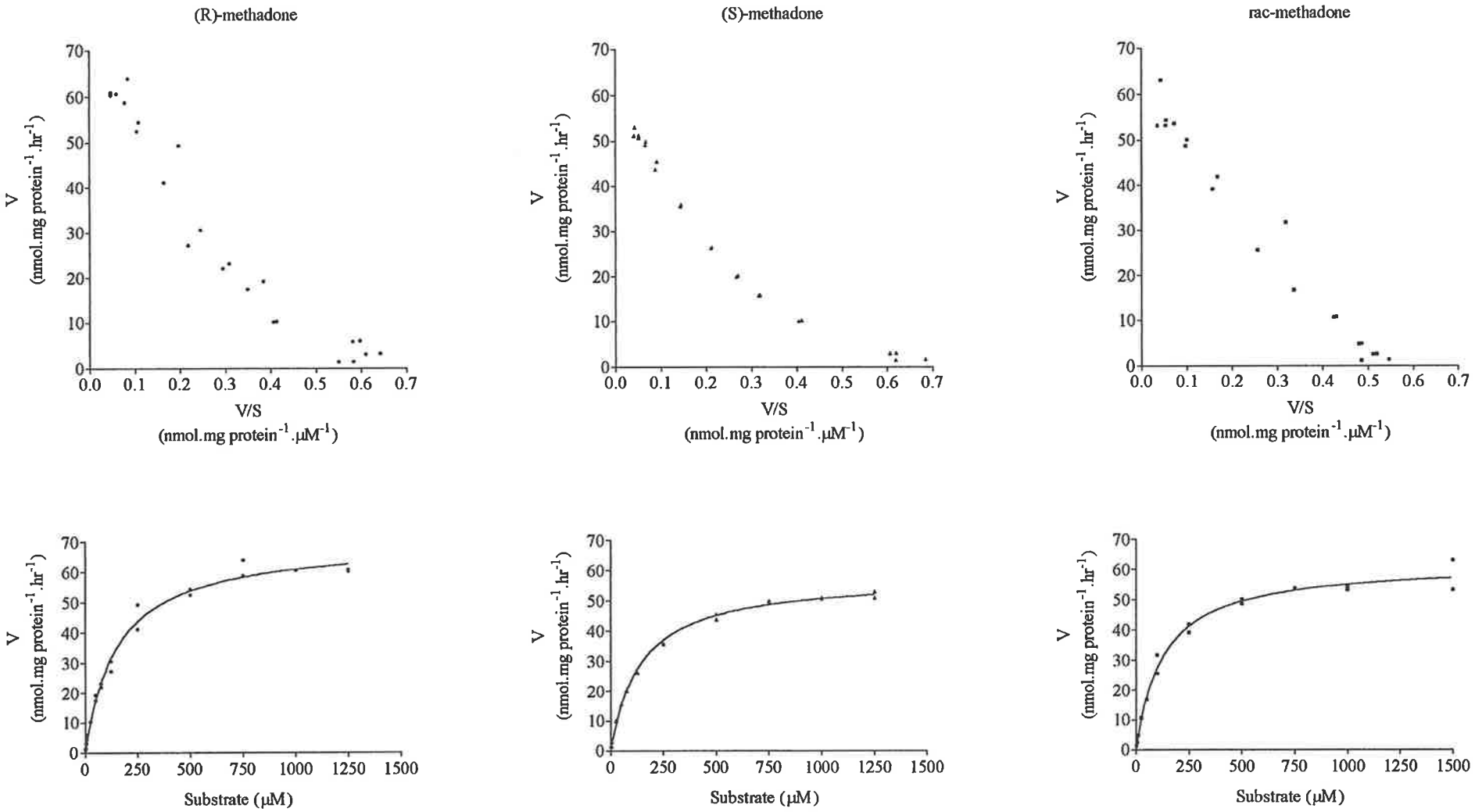
Eadie-Hofstee and Michaelis-Menten plots for the formation of EDDP from methadone in microsomes prepared from human liver sample HLS #5.



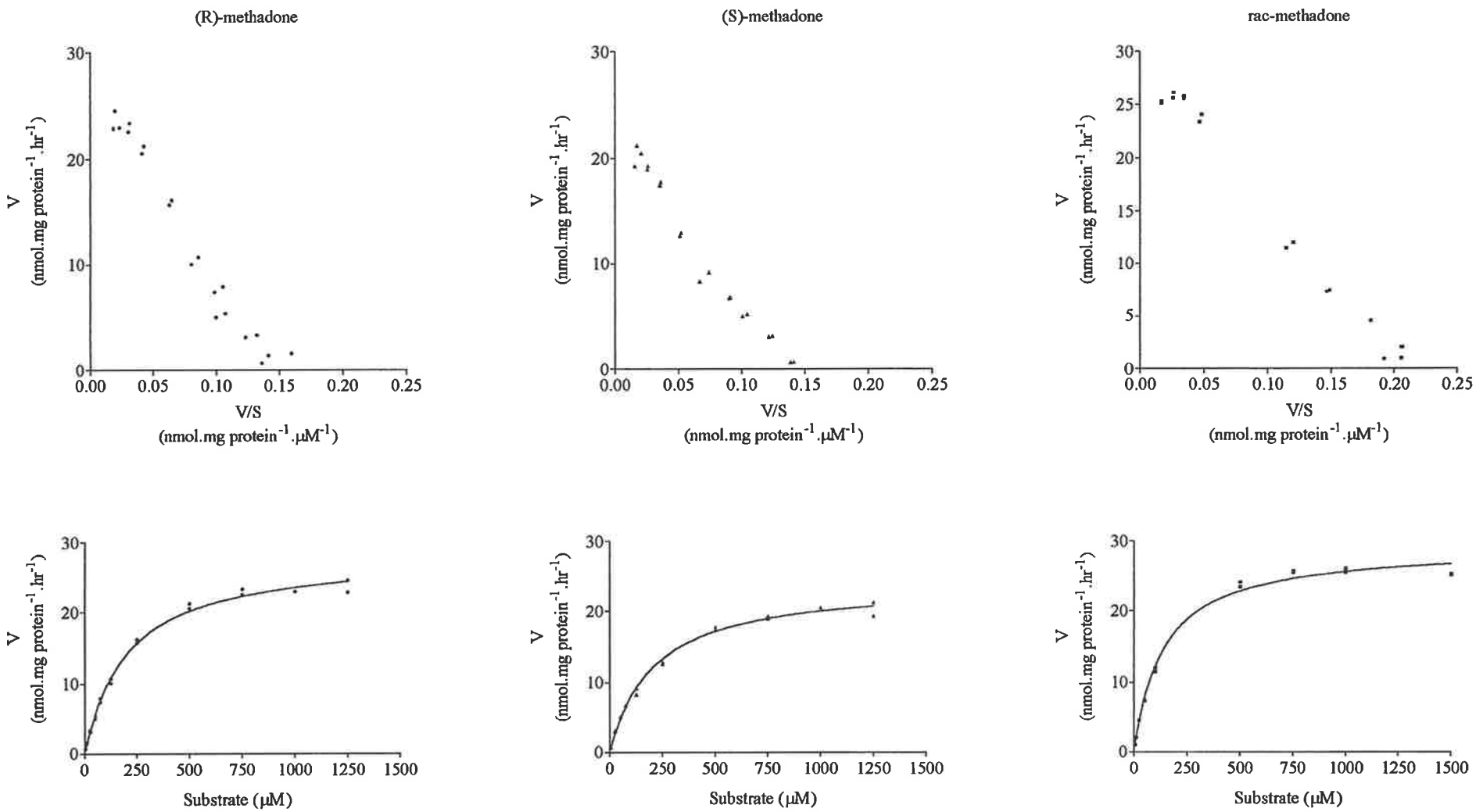
Eadie-Hofstee and Michaelis-Menten plots for the formation of EDDP from methadone in microsomes prepared from human liver sample HLS #16.



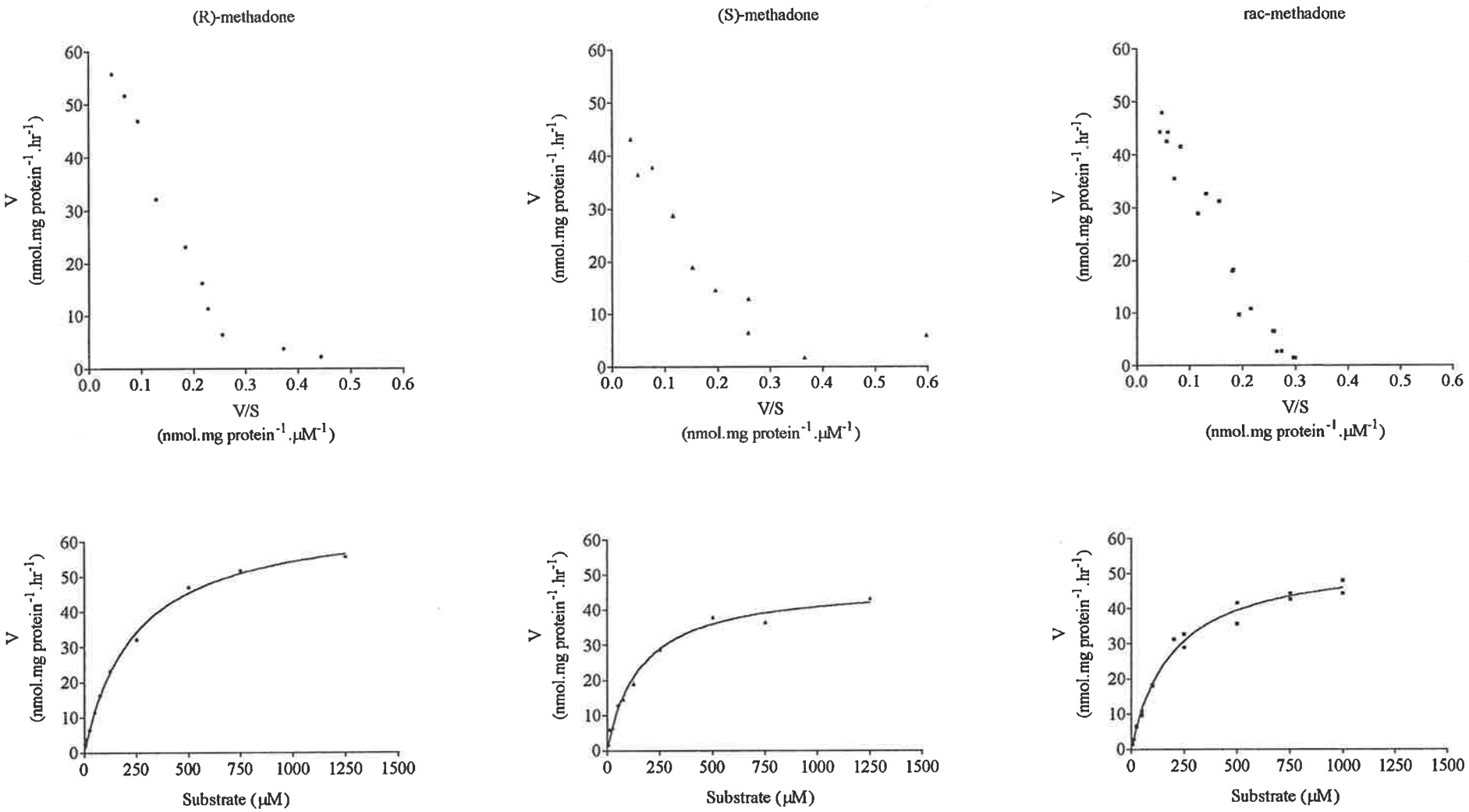
Eadie-Hofstee and Michaelis-Menten plots for the formation of EDDP from methadone in microsomes prepared from human liver sample HLS #21.



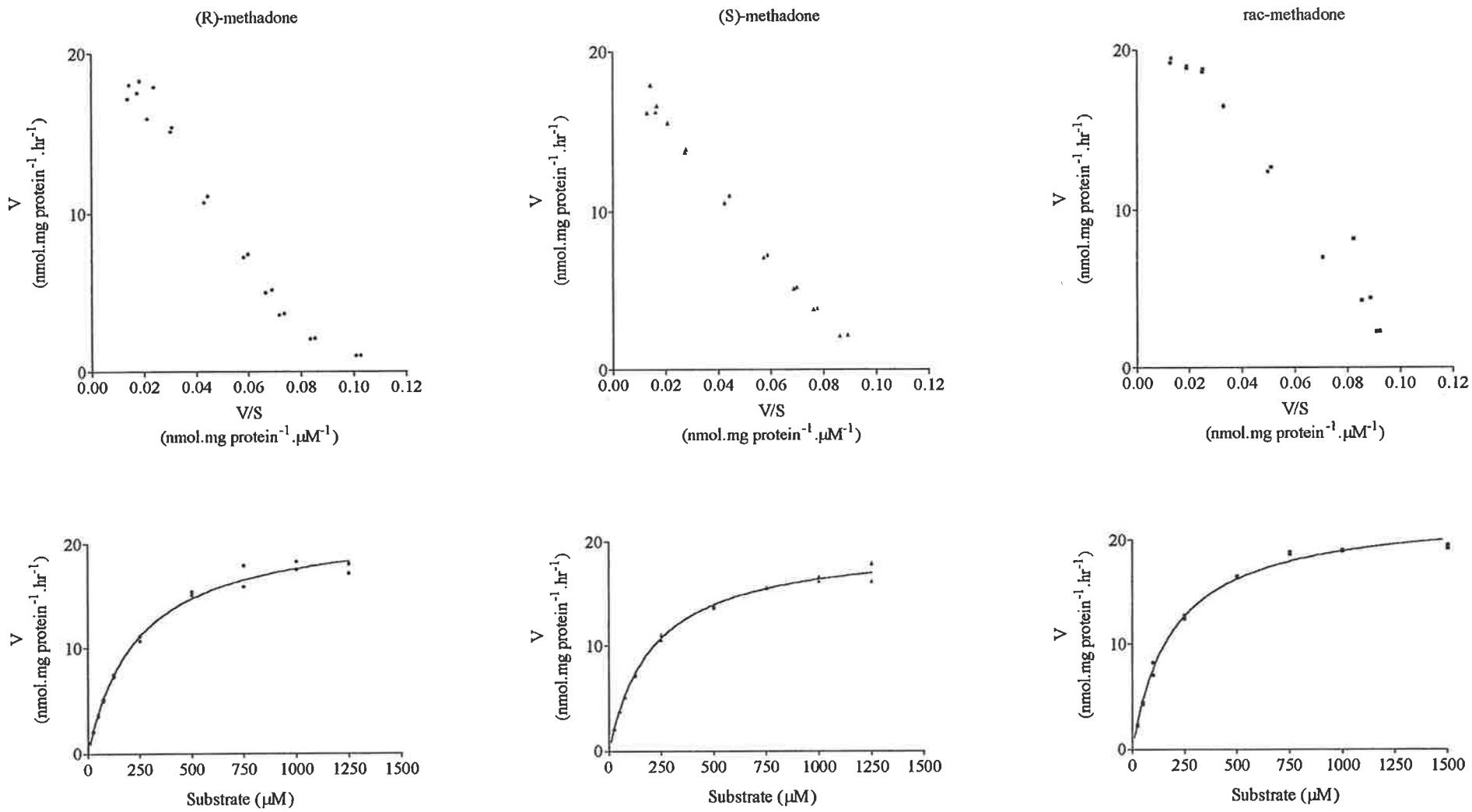
Eadie-Hofstee and Michaelis-Menten plots for the formation of EDDP from methadone in microsomes prepared from human liver sample HLS #22.



Eadie-Hofstee and Michaelis-Menten plots for the formation of EDDP from methadone in microsomes prepared from human liver sample HLS #23.



Eadie-Hofstee and Michaelis-Menten plots for the formation of EDDP from methadone in microsomes prepared from human liver sample HLS #24.



Eadie-Hofstee and Michaelis-Menten plots for the formation of EDDP from methadone in microsomes prepared from human liver sample HLS #31.

Appendix 7: Methadone maintenance patient demographic details.

Methadone maintenance patient demographic details.

#	Age (years)	Sex	Weight (kg)	Dose ($mg.day^{-1}$)[1]	Urine volume (ml)/pH	Urinalysis results					Medications
						Opioid[2]	Cannabinoid	Benzodiazepine	Sympathomimetic amine	Barbiturate	
1	40	F	65	89	NA	Negative	Negative	Negative	Negative	Negative	NIL
2	41	F	65	45	NA	Negative	Negative	Negative	Negative	Negative	NIL
3	28	M	93	70	NA	Negative	Positive	Negative	Negative	Negative	NIL
4	33	F	65	120	NA	Negative	Positive	Positive	Negative	Negative	thyroxine (150 $\mu g.day^{-1}$)
5	28	F	70	55	NA	Negative	Negative	Negative	Negative	Positive	phenobarbital (70 $mg.day^{-1}$)
6	30	F	76	80	NA	Negative	Positive	Positive	Negative	Negative	NIL
7	42	M	73	25	NA	Negative	Positive	Negative	Negative	Negative	NIL
8	37	F	94	80	NA	Negative	Positive	Positive	Positive	Negative	clonazepam (1 $mg.day^{-1}$), Sinutab (chlorpheniramine, paracetamol, pseudoephedrine)
9	37	M	68	130	1470/6.30	Negative	Positive	Positive	Negative	Negative	NIL

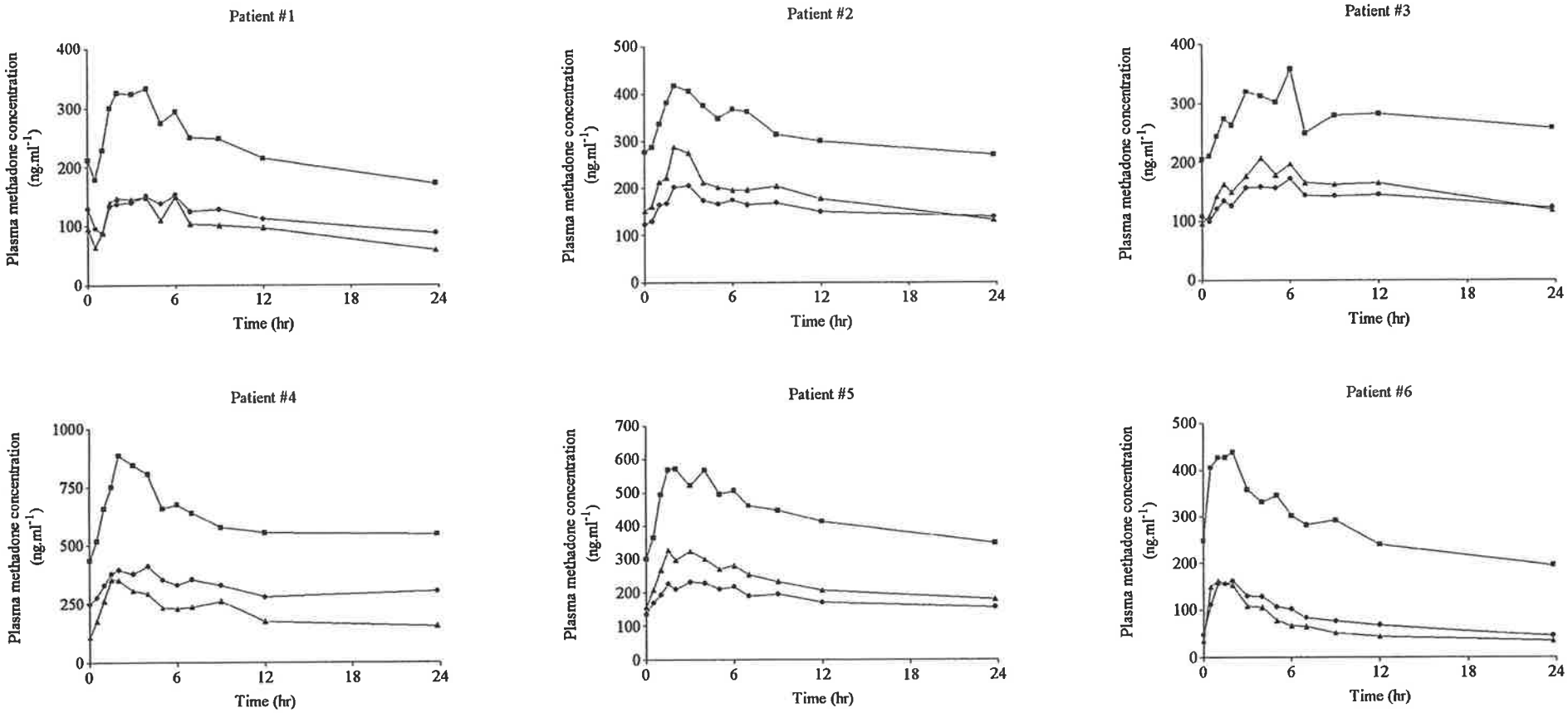
Notes: [1]dose was administered once daily as rac-methadone syrup; [2]opioids other than methadone.

Methadone maintenance patient demographic details.

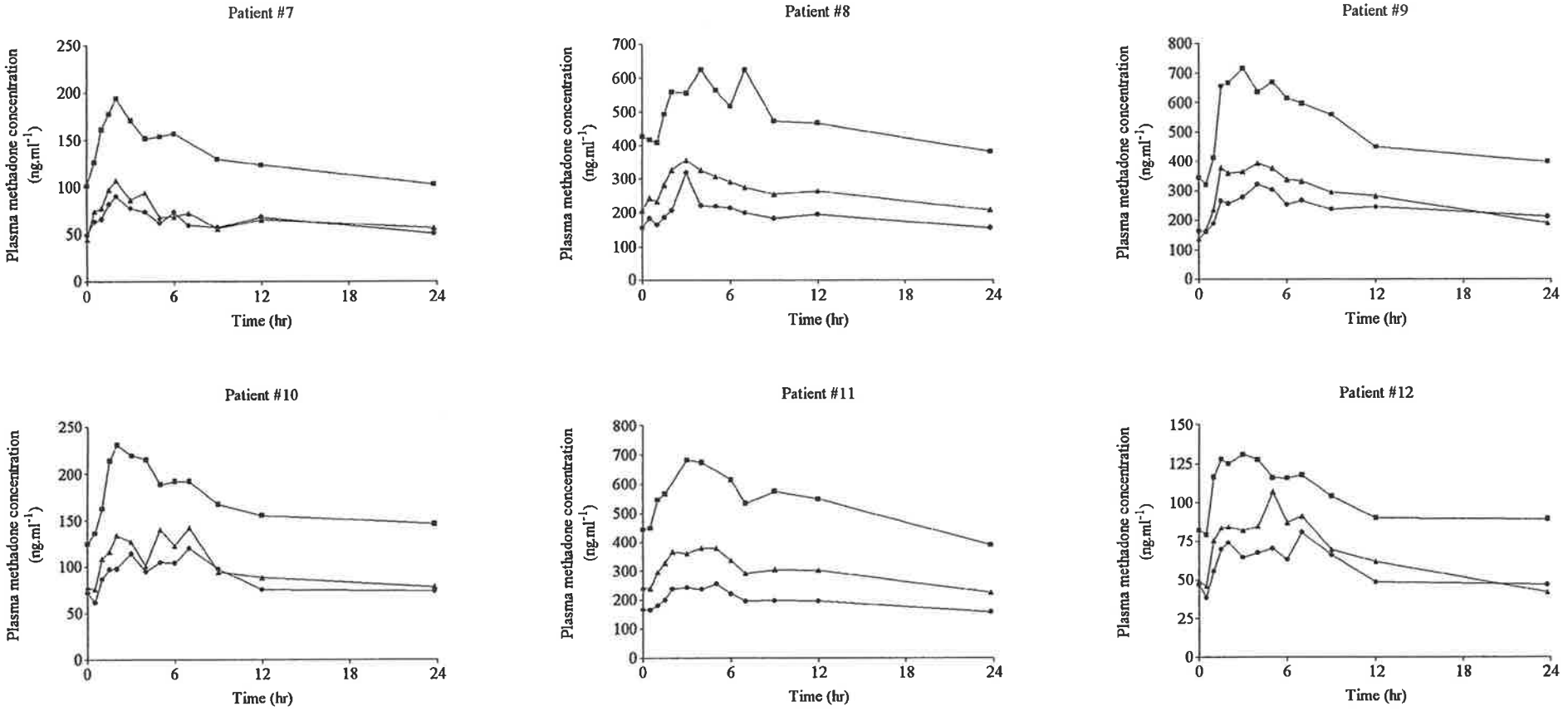
#	Age (years)	Sex	Weight (kg)	Dose ($mg.day^{-1}$)[1]	Urine volume (ml)/pH	Urinalysis results					Medications
						Opioid[2]	Cannabinoid	Benzodiazepine	Sympathomimetic amine	Barbiturate	
10	41	M	84	28	2870/6.41	Negative	Positive	Negative	Negative	Negative	NIL
11	42	M	79	65	1000/6.21	Positive	Negative	Negative	Negative	Negative	NIL
12	21	M	66	20	1100/6.20	Negative	Negative	Negative	Negative	Negative	NIL
13	45	F	91	78	1140/6.31	Negative	Negative	Negative	Positive	Negative	NIL
14	43	M	68	52.5	350/5.04	Negative	Negative	Positive	Negative	Negative	NIL
15	28	M	65	7.5	765/6.14	Negative	Positive	Negative	Negative	Negative	NIL
16	28	M	78	110	1860/6.37	Positive	Positive	Positive	Negative	Negative	clonazepam
17	37	M	75	70	680/5.29	Positive	Negative	Negative	Negative	Negative	NIL
18	35	M	60	41	1310/6.00	Negative	Positive	Positive	Negative	Negative	NIL

Notes: [1]dose was administered once daily as rac-methadone syrup; [2]opioids other than methadone.

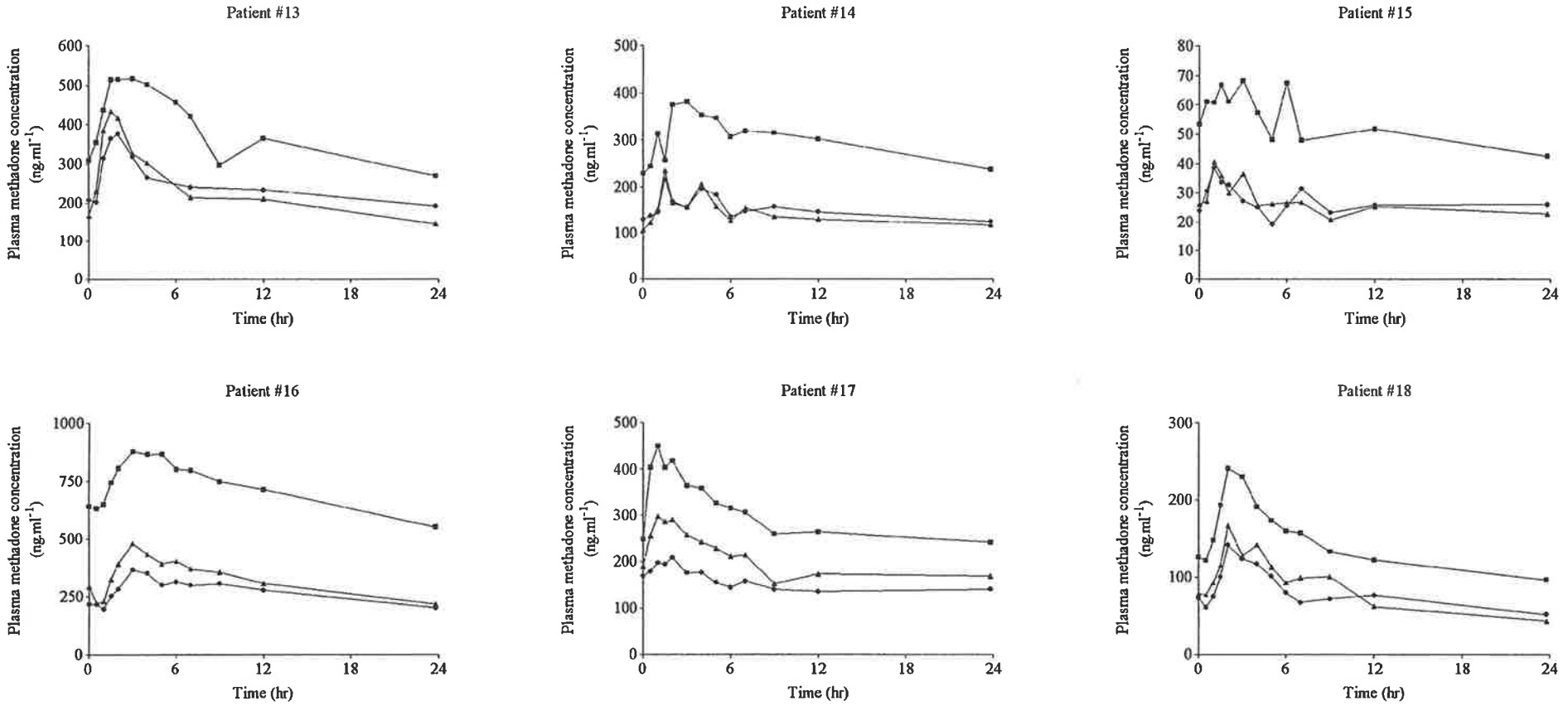
Appendix 8: Individual methadone maintenance patient rac-, (R)- and (S)-methadone concentration-time profiles.



Individual methadone maintenance patient rac-methadone (■), (R)-methadone (●) and (S)-methadone (▲) concentration-time profiles for patients #1-#6. Data are not corrected for the administered dose.



Individual methadone maintenance patient rac-methadone (■), (R)-methadone (●) and (S)-methadone (▲) concentration-time profiles for patients #7-#12. Data are not corrected for the administered dose.



Individual methadone maintenance patient rac-methadone (■), (R)-methadone (●) and (S)-methadone (▲) concentration-time profiles for patients #13-#18. Data are not corrected for the administered dose.

Appendix 9: Publications in support of this thesis.

Foster, D.J.R., Somogyi, A.A. & Bochner, F. (1999) Methadone N-demethylation in human liver microsomes: lack of stereoselectivity and involvement of CYP3A4.
British Journal of Clinical Pharmacology, v. 47(4), pp. 403-412

NOTE:
This publication is included on pages 334-343 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://doi.org/10.1046/j.1365-2125.1999.00921.x

Foster, D.J.R., Somogyi, A.A. & Bochner, F. (2000) Stereoselective quantification of methadone and its major oxidative metabolite, 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine, in human urine using high-performance liquid chromatography.
Journal of Chromatography B, v. 744(1), pp. 165-176

NOTE:
This publication is included on pages 344-345 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1016/S0378-4347(00)00246-2

Foster, D.J.R., Somogyi, A.A., Dyer, K.R., White, J.M. & Bochner, F. (2000) Steady-state pharmacokinetics of (R)- and (S)-methadone in methadone maintenance patients.
British Journal of Clinical Pharmacology, v. 50(5), pp. 427-440

NOTE:
This publication is included on pages 356-369 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://doi.org/10.1046/j.1365-2125.2000.00272.x

Dyer, K.R., Foster, D.J.R., White, J.M., Somogyi, A.A., Menelaou, A. & Bochner, F. (1999) Steady-state pharmacokinetics and pharmacodynamics in methadone maintenance patients: comparison of those who do and do not experience withdrawal and concentration-effect relationships.
Clinical Pharmacology and Therapeutics, v. 65(6), pp. 685-694

NOTE:
This publication is included on pages 370-379 in the print copy of the thesis held in the University of Adelaide Library.
It is also available online to authorised users at:

http://dx.doi.org/10.1016/S0009-9236(99)90090-5

Dyer, K.R., White, J.M., Foster, D.J.R., Bochner, F., Menelaou, A. & Somogyi, A.A. (2001) The relationship between mood state and plasma methadone concentration in maintenance patients. *Journal of Clinical Psychopharmacology, v. 21(1), pp. 78-84*

NOTE:
This publication is included on pages 380-386 in the print copy of the thesis held in the University of Adelaide Library.

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