Enantioselectivity in Clinical Pharmacology: Theoretical Considerations and Studies with Ibuprofen

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Science of the University of Adelaide.

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Declaration

I declare that the work described in this thesis was carried out in the Department of Clinical and Experimental Pharmacology, University of Adelaide, and the Pharmacy School, South Australian Institute of Technology, under the joint supervision of Prof. F. Bochner, and Dr. R.L. Nation. This thesis contains no material which has been submitted for a degree at any other University. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference is given.

Allan Mark Evans

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Abstract

1. Ibuprofen is a chiral drug which is used clinically as a racemic mixture of its pharmacologically active (S(+)-ibuprofen; S-I) and inactive (R(-)-ibuprofen; R-I) enantiomers.

2. By theoretical analysis of plasma concentration-time profiles, generated for a model chiral drug, it was shown that pharmacokinetic and concentration-effect studies on racemic drugs, such as ibuprofen, can not be relied upon to produce accurate results unless the individual enantiomers are measured in the reference biological fluids.

3. To examine various aspects of the clinical pharmacology of ibuprofen, methods for quantifying total and unbound R-I and S-I in plasma, and ibuprofen and its metabolites in urine, were developed.

4. After oral administration of a range of doses of RS-I (200 to 1200mg) to healthy volunteers, ibuprofen absorption (as assessed by urinary recovery of ibuprofen and its metabolites) was extensive, and dose-independent. There was, for each enantiomer, a linear relationship between the area under the plasma unbound concentration-time curve (AUC_u) and dose, which indicated that the intrinsic clearance of each enantiomer was dose-independent. Each enantiomer exhibited concentration-dependent plasma protein binding which led to a non-linear relationship between the area under the total plasma concentration-time curve (AUC) and dose.

5. The intensity and duration of ibuprofens anti-platelet effects (assessed by measuring thromboxane generation during blood clotting) were dose-dependent. There was a close relationship between the concentration of plasma unbound S-I, and the degree of inhibition of cyclo-oxygenase, according to a sigmoidal concentration-effect model.

6. In healthy volunteers, cimetidine had no effect on the extent of absorption of ibuprofen or the total and unbound plasma concentration-time profiles for each enantiomer. It was concluded that cimetidine had no effect on the metabolism of ibuprofen, providing further evidence to support the concept that cimetidine has variable inhibitory effects on the various isozymes of cytochrome P-450.

7. Both enantiomers of ibuprofen were extensively (>99%) bound to plasma proteins. R-I was more extensively bound, such that the plasma unbound fraction of S-I exceeded that of R-I, by an average of 50 to 70%. Controlled *in vitro* studies indicated that the presence of one ibuprofen enantiomer could decrease the plasma protein binding of its mirror-image form.

List of publications in support of this thesis.

1. Evans AM, Nation RL, Sansom, LN, Bochner F, Somogyi AA. Stereoselective drug disposition: potential for misinterpretation of drug disposition data. British Journal of Clinical Pharmacology 26, 771-780, 1988.

2. Evans AM, Nation RL, Sansom LN, Bochner F, Somogyi AA. Stereoselective plasma protein binding of ibuprofen enantiomers. European Journal of Clinical Pharmacology 36, 283-290, 1989

3. Evans AM, Nation RL, Sansom LN. Lack of effect of cimetidine on the pharmacokinetics of ibuprofen enantiomers in humans. British Journal of Clinical Pharmacology, In Press, 1989.

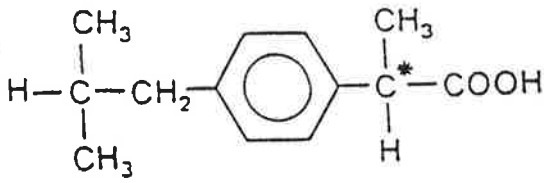
4. Evans AM, Nation RL, Sansom LN, Bochner F, Somogyi AA. The relationship between the pharmacokinetics of ibuprofen enantiomers and the dose of racemic ibuprofen in humans. Biopharmaceutics and Drug Disposition, Submitted for publication, April 1989.

5. Evans AM, Nation RL, Bochner F, Sansom LN, Somogyi AA. The effect of ibuprofen dose on the magnitude and duration of platelet cyclo-oxygenase inhibition in humans: Relationship between inhibition of thromboxane production and plasma concentration of unbound S(+)-ibuprofen. In preparation.

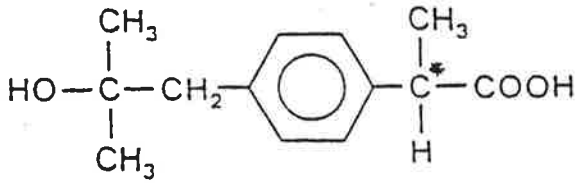
6. Evans AM, Nation RL, Bochner F, Sansom LN, Somogyi AA. Studies on the enantioselective plasma protein binding of ibuprofen enantiomers. In preparation.

Nomenclature

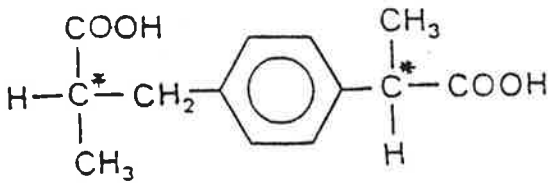
The chemical structure of ibuprofen and its major metabolites are presented below.
A chiral carbon atom is denoted by the symbol *



2-(4-isobutylphenyl)propionic acid; "ibuprofen"



2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid
"hydroxy-ibuprofen"



2-[4-(2-carboxypropyl)phenyl]propionic acid
"carboxy-ibuprofen"

The following standard symbols have been used within this thesis. Attempts have been made to comply with the guidelines established by the American College of Clinical Pharmacology (Aronson et al 1988).

AUC	Area under the plasma total (bound plus unbound) concentration-time curve from zero to infinity
AUC(0-t)	Area under the plasma total (bound plus unbound) concentration-time curve from zero to time t.
AUC_u	Area under the plasma unbound concentration-time curve from zero to infinity
$AUC(0\text{-}t)_u$	Area under the plasma unbound concentration-time curve from zero to time t.
ß	Rate constant for the terminal portion of a plasma log concentration-time profile after extravascular drug administration
C	Concentration of drug in plasma
C_{max}	Maximum plasma drug concentration
C_{min}	Plasma concentration at the end of a dosing interval
C(0)	Initial (or extrapolated) drug concentration after rapid intravenous injection
C_{ss}^{av}	Average steady-state drug concentration in plasma
C_u	Concentration of unbound drug in plasma
CL	Clearance of total drug from plasma
CL_H	Hepatic clearance of drug from plasma
CL_{int}	Intrinsic clearance of unbound drug from plasma
CL_M	Clearance of drug from plasma by metabolism
CL_o	Apparent oral clearance of drug from plasma
D	Dose of drug
E	Extent of effect produced by a drug
EC_{50}	Concentration of drug required to produce 50% of maximum effect
EC_{80}	Concentration of drug required to produce 80% of maximum effect

E_{max}	Maximum effect produced by a drug
f	Fraction of an orally administered dose which is systemically available
f_a	Fraction of the administered dose which is absorbed from the gastrointestinal tract
f_{fp}	Fraction of an absorbed dose reaching the systemic circulation in the presence of hepatic first-pass elimination
f_u	Unbound fraction of drug in plasma
$\bar{f}_u$	Time-averaged unbound fraction of drug in plasma
f_{ut}	Unbound fraction of drug in tissue
k	Rate constant for the elimination of drug from plasma
K_m	Michalis-Menten constant of an enzyme-mediated reaction
n	Slope-factor of the Hill equation
Q	Organ blood flow
Q_H	Hepatic blood flow
t	Time after drug administration
$t_{1/2}$	Disposition half-life of drug
t_{max}	Time to reach maximum concentration after drug administration
T	Dosing interval
Vd	Volume of distribution with reference to plasma
V_p	Plasma volume
V_t	Tissue volume
V_{ss}	Volume of distribution at steady-state
V_{max}	The maximum rate of an enzyme-mediated reaction

The following abbreviations have been used throughout this thesis.

AA	Arachidonic acid
AGP	Alpha 1-acid glycoprotein
AI	The amount of S(+)-ibuprofen formed from the metabolic inversion of R(-)-ibuprofen
ANOVA	Analysis of variance
AUFS	Absorbance units full scale
Buff	Pertaining to the buffer compartment of an equilibrium dialysis cell
°C	Degrees Celsius
CDA	Chiral derivatizing agent
^{14}C-I	^{14}C-radiolabelled ibuprofen
Ci	Curie
cm	Centimetre
CNS	Central nervous system
cpm	Counts per minute
CSP	Chiral stationary phase
CV	Coefficient of variation
d	Deuterium
dpm	Disintegrations per minute
FI	Fraction of a dose of R(-)-ibuprofen which is metabolically inverted to S(+)-ibuprofen
GFR	Glomerular filtration rate
GIT	Gastrointestinal tract
GLC	Gas-liquid chromatography
GLU	Glucuronidation
h	Hours
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
ID	Internal diameter
INV	Inversion
i.v.	Intravenous
kPa	Kilopascal
L	Litre
M	Moles per litre
mg	Milligram

min	Minutes
ml	Millilitre
mmol	Millimole
NA	Sample not available for analysis
ND	Not detectable
ng	Nanogram
NSAIA	Non-steroidal anti-inflammatory agent
OX	Oxidation
pg	Picogram
PG	Prostaglandin
Plas	Pertaining to the plasma compartment of an equilibrium dialysis cell
2-PPA	2-Phenylpropionic acid
r	Regression correlation coefficient
R-I	R(-)-ibuprofen
RIA	Radio-immunoassay
rpm	Revolutions per minute
RS-I	Racemic ibuprofen; RS-ibuprofen
SD	Standard deviation
S-I	S(+)-ibuprofen
SE	Standard error
TLC	Thin layer chromatography
TXA2	Thromboxane A2
TXB2	Thromboxane B2
µl	Microlitre
UV	Ultraviolet
UNR-I	Ibuprofen of undefined enantiomeric composition

Chapter 1
Introduction: Enantioselectivity in Clinical Pharmacology

It has been estimated that 25% of all drugs intended for human use are administered as two (or more) optical isomers, usually as a racemic mixture of two enantiomers (Simonyi 1984). Because of the enantio-discriminatory capacity of biological molecules, a pair of drug enantiomers may differ considerably, both in the effect that they have upon, and the manner in which they are handled by, the body (Ariens 1984). It is unfortunate, that this important concept is commonly over-looked by investigators studying aspects of the clinical pharmacology of racemic drugs.

It is the principal objective of this thesis, to convey the importance of using enantioselective analytical techniques when examining the pharmacokinetics and concentration-effect relationships of chiral drugs which are administered as racemates. This objective is achieved, firstly, by describing a theoretical pharmacokinetic analysis which was performed on concentration-time data simulated for the enantiomers of a model chiral drug. This theoretical analysis permitted a critical evaluation of the interpretative errors which may be made if the concentrations of a racemic drug, in reference biological fluids, are measured using a non-enantioselective assay.

The primary goal will also be achieved by describing investigations into various aspects of the clinical pharmacology of a racemic drug, ibuprofen. These studies were performed using a number of developed analytical techniques, including methods for measuring the total and unbound concentrations of the individual enantiomers of ibuprofen in plasma. Ibuprofen is a particulary interesting drug, firstly because only one enantiomer appears to be pharmacologically active, and secondly, because one of the metabolic pathways of the inactive enantiomer is conversion to its active, mirror-image form. The studies described include: an investigation of the effect of the magnitude of ibuprofen dose on the pharmacokinetics of the individual enantiomers; an investigation of the effect of ibuprofen dose on the intensity and duration of inhibition of platelet thromboxane synthesis; and a study performed to examine the effect of cimetidine, a potent inhibitor of drug metabolism, on the pharmacokinetics of ibuprofen enantiomers.

1.1. INTRODUCTION TO OPTICAL ISOMERISM

Before considering fully the consequences of enantioselectivity in clinical pharmacology, it is instructive to retrace, briefly, some of the important historical events which have led to todays understanding of stereochemical principles.

1.1.1. Historical Aspects

Stereochemistry owes its origins to the experiments of James Biot, who discovered, between 1813 and 1818, that certain biological substances were able to rotate the plane of polarized light (Ramsay 1982), that is, light which has passed through a crystal, or a film, which permits that component of light travelling in one direction only to pass. In later years, Biot examined the effect of plane polarized light on two compounds obtained from the fermentation of grapes. The first, tartaric acid rotated plane polarized light to the left i.e. tartaric acid was optically active. The second compound, known at the time as racemic acid, was found to be optically inactive. It was here, however, that any difference between these two substances ended, for in all other ways, including chemical composition, they were identical (Ramsay 1982).

In 1848, Louis Pasteur examined the crystal structures of the sodium ammonium salts of tartaric acid and racemic acid (also referred to as para-tartaric acid). Pasteur discovered, as had Eilhardt Mitscherlich four years previously, that the crystals of both compounds displayed hemihedral facets. In addition, however, Pasteur detected a subtle difference between the two compounds. In the crystals of tartrate, all of the hemihedral facets were orientated in one direction, to the left. However, with the sodium ammonium salt of racemic acid, some of the crystals had the hemihedral facets directed to the right and some of the crystals had the facets directed to the left. Pasteur manually separated these two crystal forms, and found that a solution of the "left-handed" crystals rotated plane polarized light to the left, in an identical manner to the tartaric acid salt, while the "right-handed" molecule rotated plane polarized light to a similar extent, but in the opposite direction. A solution containing equal quantities of the right- and left-handed crystals had no effect on plane polarized light (Drayer 1988a; Ramsay 1982). Pasteur had actually achieved the first manual resolution of a pair of enantiomers. Consequences of this work and subsequent investigations on the optical activity of various other molecules, including lactic acid, led Van't Hoff and LeBel, in 1874 to independently propose theoretical concepts for the spacial arrangement of atoms about the saturated carbon atom, and stereochemical principles which still hold true today. The revolutionary ideas of these workers, which were at the time described by an

eminent chemist as fanciful, and not far removed from a belief in witches (Ramsay 1982), heralded a new era in the understanding of molecular and stereochemical principles.

1.1.2. Chirality, Enantiomers and Diastereomers

A chiral (from the Greek word *cheir*, meaning hand) molecule is one which is not superimposable upon its mirror-image. Such a molecule possesses no element (plane, axis or alternating axis) of symmetry, and is said to be asymmetric. The different non-superimposable forms of such a molecule are known as optical isomers, because they exhibit optical activity. A molecule containing a saturated carbon atom covalently bonded to four different substituents represents the most common form of asymmetric molecule. An example of a chiral molecule, and its mirror-image, is depicted in Figure 1.1.

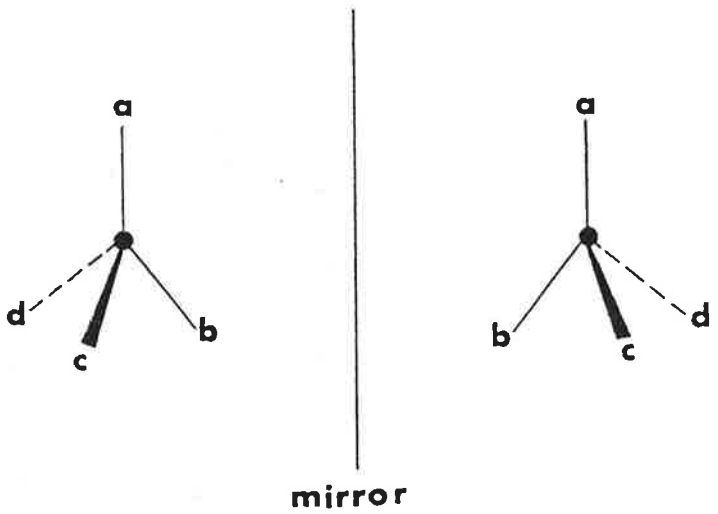


Figure 1.1. The mirror-images (enantiomers) of a chiral molecule. The molecule is depicted using the "flying-wedge" format. If the central atom is considered to be lying on the plane of the page, the atomic groupings a and b are on the same plane while c and d are above and below the plane of the page, respectively.

Two molecules related to one another as non-superimposable mirror-images are referred to as enantiomers (also referred to as optical antipodes, or enantiomorphs). The atom to which the four different groups are attached is referred to as an asymmetric centre, or a chiral centre. In a field of plane polarized light, one enantiomer will rotate the plane to the left, the other to the right. An equal mixture of both enantiomers (a racemate) will have no overall effect on plane polarized light.

Enantiomers possess identical physical and chemical properties in a symmetrical environment. They have the same melting point, boiling point, octanol-water partition coefficient, UV spectrum etc. It is only when they are exposed to an optically discriminating environment, that they may differ from one another. This important concept was discovered by Pasteur, who found that while in the presence of non-asymmetric compounds with superimposable mirror-images, tartaric and racemic acids (referring to the two crystal forms), behaved identically. However, in the presence of compounds with non-superimposable images, such as quinine, albumin and sugar, "all is changed in an instant" (Drayer 1988a). Figure 1.2 depicts an interaction of two enantiomers (A and B) with an asymmetric molecule (C). Molecule A can be aligned with molecule C in a manner such that the groups a, b and c are adjacent to atoms a', b' and c', respectively, i.e., a three-point interaction is possible. However, for molecule B, no such alignment can be made, and so the three-point interaction is not possible. Hence, it follows that two enantiomers (A and B) may differ in the manner in which they interact with one enantiomer of another chiral molecule (C).

The number of optical isomers (stereoisomers) of a molecule is related to how many asymmetric centres it contains. For each asymmetric centre one pair of enantiomers is possible, and so for a molecule containing i asymmetric centres there are a maximum of 2^i optical isomers. Optical isomers which are not mirror-images are termed diastereoisomers, or diastereomers. Unlike enantiomers, diastereomers may have completely different physical and chemical properties. An example of a molecule containing 2 chiral carbon atoms is presented in Figure 1.3. Isomers 1 and 2 are related to one another as enantiomers, as are 3 and 4. Isomers 1 and 2 are diastereomers of 3 and 4, respectively. Hence, while the physico-chemical properties of 1 will be identical to those of 2, and those of 3 identical to 4, the properties of 1 and 2 may differ greatly from those of 3 and 4.

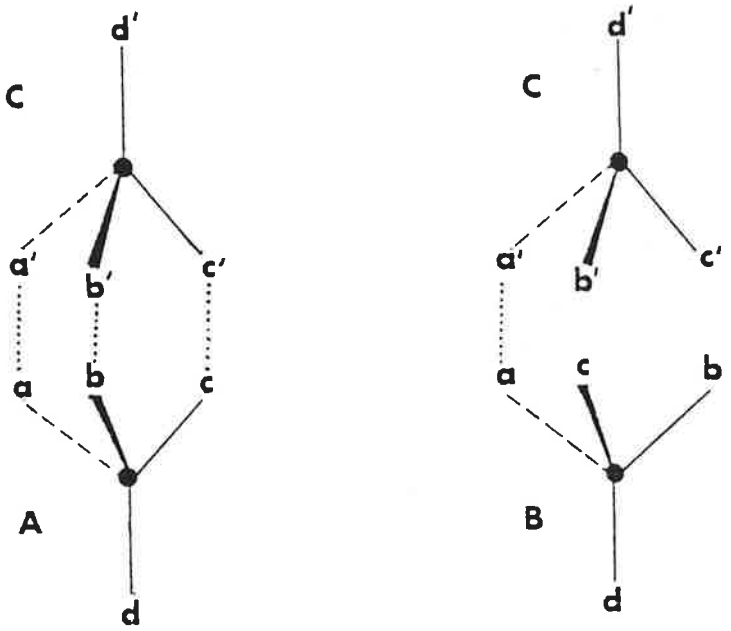


Figure 1.2. The interaction of a pair of enantiomers (A and B) with an optically active molecule, C. While A can be aligned with C so as to permit a three-point interaction, no such interaction is possible for B.

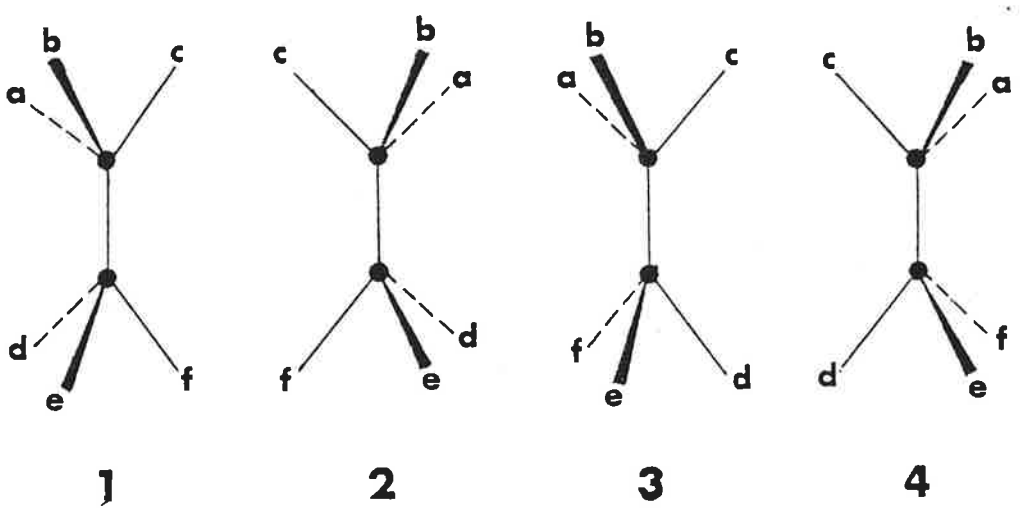


Figure 1.3. The four possible optical isomers (stereoisomers) of a molecule containing two chiral centres.

1.1.3. Nomenclature

The enantiomer which rotates plane-polarized light to the left is ascribed the prefix (-)-, and that which rotates it to the right is (+)-. These two symbols are sometimes replaced by the prefixes *laevo-* and *dextro-*, respectively (occasionally shortened to *l-* and *d-*). The degree of optical rotation is dependent on the number of molecules through which the light must pass, and therefore the concentration of the substance in the chosen solvent. It is worth noting, also, that the nature of the solvent can modify the effects of a compound on the rotation of plane polarized light.

Optical rotation gives no information about the configuration of the individual enantiomers, and two conventions have been adopted for this purpose. The first, introduced by Emile Fischer in 1919, requires that the asymmetric centre of the molecule under investigation be related to the asymmetric centre of glyceraldehyde (Ramsay 1982). If determined to be the same as (+)-glyceraldehyde, it is given the prefix D, while if it is the same as (-)-glyceraldehyde, the symbol L is used. The convention, which has been used most commonly in carbohydrate and amino acid chemistry, has a number of limitations, not the least of which is that the compound of interest must be chemically transformed to a molecule of known configuration.

In recent years, the Fischer convention has been largely superseded by the Cahn-Ingold-Prelog convention (Ramsay 1982). This convention uses a number of "sequence rules", for assigning priority to the atomic groupings attached to a chiral centre (usually a carbon atom). The molecule is oriented so that the group of lowest priority is directed away from the viewer. If the direction of assigned sequence of the other three substituents, moving from the group of highest to lowest priority, is clockwise, the molecule is given the prefix R- (Latin, *rectus* right); if the order of this sequence is anti-clockwise, the prefix S- is applied (Latin, *sinister* left). Because there is no relationship between the absolute configuration of an enantiomer and its effect on plane polarized light, both systems of identification are used. A racemic mixture of two enantiomers may be ascribed the prefix RS, (±) or DL, depending on the adopted convention.

Where possible, the Cahn-Ingold-Prelog convention (R,S), together with the optical rotation (+,-), will be used to identify chiral molecules within this thesis.

1.1.4. Summary

A molecule which contains no element of symmetry, such as one possessing a chiral carbon atom, can exist as two mirror- image forms which are not superimposable

(enantiomers). Because enantiomers possess identical physico-chemical properties, they are virtually indistinguishable in the test-tube. However, the interaction of a pair of enantiomers with another chiral molecule may result in the transient or permanent formation of diastereomeric relationships. Hence, under such conditions a pair of enantiomers may behave quite differently.

Within this thesis, the word "enantioselective" is used to describe any process which can distinguish between two enantiomers. For example, an enantioselective assay is one which can measure each enantiomer when present together; enantioselective protein binding occurs if two enantiomers bind differentially to a given protein. Where a process is specific for one enantiomer, it is referred to as "enantiospecific". For example, an enantiospecific assay is one which measures one enantiomer only. Whereas "enantioselective" is a relative term, "enantiospecific" is absolute. Because biological macromolecules are flexible structures, it is rare that a biological process is enantiospecific, in the true sense of the word. Stereoselective and stereospecific are much broader terms, which are taken to describe a process which can distinguish between stereoisomers (i.e. enantioselectivity is a form of stereoselectivity).

1.2. OPTICAL ACTIVITY AND CLINICAL PHARMACOLOGY

Drugs which are derived from biological sources are usually chiral, and in most cases are obtained, and subsequently used clinically, as one particular enantiomer. Between 1959 and 1980, drugs obtained from biological sources represented 25% of all prescriptions dispensed from community pharmacies in the U.S.A. (Balandrin et al 1985). The majority of drugs, however, are produced by chemical synthesis, starting with achiral precursors and using procedures which are not enantioselective. Hence, the chemical synthesis of a drug which contains a chiral centre invariably yields an optically inactive racemate. While there are notable exceptions (e.g. synthetic steroid preparations; Simonyi 1984), synthetically produced chiral drugs are generally used clinically in their racemic form. Of the 486 synthetically produced chiral pharmaceuticals which were listed in the 1980 U.S.A. *Pharmacopeal Dictionary of Names*, 398 were being used clinically as racemates (Mason 1984). Overall, it appears that one in every four drugs is used as a racemate (Ariens & Wuis 1987; Simonyi 1984). In Australia in 1988, 20% of the 200 most frequently dispensed pharmaceuticals listed on the National Health Schedule were chiral molecules which were being used as racemates.

The human body is a chiral environment of the highest complexity. Amino-acids, exclusively of the L-configuration and carbohydrates of the D-configuration are selectively arranged in an ordered manner to comprise the elementary biological

macromolecules (proteins, polysaccharides, glycoproteins, RNA and DNA, etc). The pharmacological properties of a drug are governed by the manner in which it interacts with these various chiral macromolecules. The interaction of the enantiomers of a chiral drug with these biological macromolecules results in the formation, albeit transient in most cases, of diastereomeric complexes of differing stability and conformation. Enantioselectivity, in pharmacological terms, is essentially a measure of the extent to which the biological macromolecules interact with one enantiomer relative to the other.

Differences between enantiomers in the pharmacological response elicited by a given dose, may arise if the enantiomers interact differently with the various receptor site(s), and/or if the unbound concentration of the enantiomers in the immediate vicinity of the receptor site(s) (i.e. the biophase) differ; in other words, from differences between enantiomers in their pharmacodynamic and/or pharmacokinetic properties. With respect to enantioselectivity in the pharmacology of chiral drugs in humans, both factors are important and, often, their relative contributions are difficult to delineate.

1.2.1. Enantioselective Pharmacological Activity.

The general theme of enantioselectivity in the pharmacological activity of chiral drugs has been the topic of a number of recent reviews (Ariens 1983; Ariens et al 1988; Lehmann 1983; Powell et al 1988; Simonyi 1984; Soudijn 1983). Because of its relevance to hundreds of drugs, it is not within the scope of this review to comprehensively discuss enantioselectivity in the pharmacological activities of all chiral drugs. Therefore, a brief outline only of enantioselective pharmacological activities of chiral drugs is given.

Rarely will a pair of enantiomers have pharmacological properties which are identical in all aspects, both qualitatively and quantitatively (Lehmann 1983). The enantiomers of the chiral drug primaquine appear to be equally effective antimalarials (Schmidt et al 1977) and equally potent inhibitors of drug metabolism in the isolated perfused rat liver (Mihaly et al 1985). However, this lack of enantioselectivity does not extend to the toxic effects of the drug, since, in experimental animals, (+)-primaquine is the more toxic enantiomer (Schmidt et al 1977).

For most chiral drugs, one enantiomer will be more potent than the other in eliciting a given pharmacological response; the more potent enantiomer is termed the eutomer, and the less active enantiomer the distomer. The ratio of the activity of the eutomer to the distomer (eudismic ratio) is a mathematical measure of the degree of enantioselectivity (Lehmann 1983, 1986). A reliable measure of the pharmacodynamic eudismic ratio is best obtained *in vitro*, where dispositional factors are less important

and less likely to influence the concentration of each enantiomer in the immediate vicinity of the receptor(s). Under such conditions, the eudismic ratio is a useful tool for investigating the structural requirements of pharmacological receptors (Ariens 1983; Lehman 1983, 1986). Eudismic ratios vary considerably. For the coumarin anticoagulant warfarin, the eudismic ratio (ratio of activity of S(-)-warfarin to R(+)-warfarin) with respect to anticoagulant activity is approximately 5 to 6 (Eble et al 1966; Hewick & McEwen 1973), whereas for terbutaline, a ß2-adrenoreceptor agonist, activity is confined almost exclusively to the (-)-enantiomer, and the eudismic ratio exceeds 3000 (Jeppsson et al 1984).

Commonly, a chiral drug will elicit a variety of pharmacological responses and enantioselectivity may, or may not, be confined to certain effects. In the case of propranolol, the eudismic ratio for ß-adrenoreceptor blocking activity [S(-)/R(+)] is about 100, while both enantiomers are equally potent as membrane stabilizers (Ariens 1983). Furthermore, for some racemic drugs, enantioselectivity may be reversed for the various actions. In the case of the anti-arrhythmic agent disopyramide, for example, the S(+)-enantiomer is the eutomer with respect to type I anti-arrhythmic activity, while R(-)-disopyramide is the eutomer with respect to negative inotropic effect (Lima et al 1985), a property which can limit the usefulnes of disopyramide as an anti-arrhythmic.

Enantioselectivity may be so appreciable for some chiral drugs that the enantiomers will elicit completely different pharmacological responses. An example of particular interest exists in the case of fenfluramine, which is used in its racemic form as an anorectic agent and for the treatment of autistic children. Recently, it has been discovered that while (+)-fenfluramine is more potent as an appetite suppressant (Invernizzi et al 1986), its optical antipode has significant antipsychotic activity (Bettini et al 1987). As a result of these enantioselective pharmacological investigations, (+)-fenfluramine has been developed as a more specific anorectic agent, and (-)-fenfluramine is under investigation as a neuroleptic agent (Spinelli et al 1988).

The enantiomers of a chiral drug may also elicit opposing effects. For ketamine, the S(+)-enantiomer appears to be the more potent anaesthetic agent (CNS depressant activity), whilst the R(-)-enantiomer is associated with a higher incidence of the unpleasant CNS-excitatory effects associated with recovery from ketamine-induced anaesthesia (White et al 1980).

Just as a pair of enantiomers may elicit various activities with differing potency, their therapeutic index may also differ. In such cases it is clearly more favourable to use the enantiomer with the more favourable therapeutic index. It should be noted that the enantiomer which is more potent with respect to the desired effect(s), may not be the one which is better tolerated.

There are many chiral drugs for which most of the pharmacological activity resides with one enantiomer. For many of the chiral drugs which act on the sympathetic nervous system, such as the ß-adrenoreceptor agonists, one enantiomer possesses the majority of the pharmacological activity (Simonyi 1984). However, even if one enantiomer appears to be "inactive", it should be remembered that our knowledge of drug effect is limited by the pharmacological tests which have been performed, and it is virtually impossible to prove that the "inactive" enantiomer is totally without effect on the body.

In summary, the pharmacological activities of the enantiomers of a chiral drug can be classified as follows:

(i) Both enantiomers possess qualitatively, and quantitatively, similar pharmacological activity.

(ii) The phamacological effects of the enantiomers are qualitatively similar, but differ quantitatively.

(iii) The enantiomers differ from one another both qualitatively and quantitatively.

(iv) Only one enantiomer is pharmacologically active.

For many of the racemic drugs which are used clinically, little is known of the relative pharmacological activities of the individual enantiomers, but most would be expected to fit into either the second or third classification.

1.2.2. Enantioselective Pharmacokinetics.

The absorption of a drug from its administration site, its binding to tissue and plasma proteins, its distribution into and out of the various physiological compartments, its metabolic transformation and its renal and biliary excretion, all potentially involve close interactions between the drug molecule and chiral biological components. Hence, all of these processes have the capacity to elicit selectivity towards one of a pair of enantiomers. It follows that after dosing with a racemic drug, the concentration-time profiles of the individual enantiomers in plasma and, in particular, at the site of action (i.e. the biophase) may differ appreciably. These pharmacokinetic differences may accentuate or suppress pre-existing differences at the pharmacodynamic level. Hence, the eudismic ratio of a drug determined *in vivo* may differ considerably from the eudismic ratio *in vitro*, where distributional factors are minimal. It should also be noted that inter- and intra-individual variability in the degree of enantioselectivity may introduce, or contribute further, to the variability in the overall pharmacological response elicited by a racemic drug. A brief discussion of enantioselectivity in each of

the aforementioned pharmacokinetic processes is presented below.

1.2.2.1. Absorption

The absorption of most drugs from the gastrointestinal tract (GIT) is a passive process, governed mainly by the physico-chemical properties of the drug (Rowland & Tozer 1989). In such cases, the absorption of chiral drugs from the gastrointestinal tract is non-enantioselective. Certain endogenous substances, such as mono-saccharides, amino-acids and some vitamins, are absorbed from the GIT by carrier-mediated processes, which may elicit a high degree of structural selectivity (Gibaldi 1977). The carrier-mediated absorption of glucose (Inui et al 1980) and certain amino-acids (Jervis & Smyth 1959) exhibits enantioselectivity. Accordingly, the gastrointestinal absorption of chiral drugs which structurally resemble these endogenous molecules may also be carrier-mediated, and therefore potentially enantioselective. The absorption of dopa from the gastrointestinal tract is carrier-mediated and enantioselective, favouring the enantiomer with the L-configuration (Wade et al 1973). For methotrexate, gastrointestinal absorption appears to be much more rapid for the L-enantiomer (Hendel & Brodthagen 1984) which suggests enantioselectivity in the carrier-mediated absorption of this agent. Because both dopa and methotrexate are used clinically as their L-isomers, enantioselective gastrointestinal absorption is of minor importance in these cases. For the amino acid analogue, penicillamine, carrier-mediated gastrointestinal absorption in the rat is selective for the L-enantiomer, and is inhibited by other L-amino acids (Schanker & Jeffrey 1961). Clinically, this anti-rheumatic agent is used as the less toxic D-enantiomer, which has an oral bioavailability of about 50 to 70% (Kukovetz et al 1983).

The bioavailability of a drug after oral administration (f) is governed by the fraction of the administered dose which is absorbed into the hepatic portal vein (f_a) and the fraction which escapes extraction during the first pass through the liver (f_{fp}), in accordance with equation 1.1.

$$f = f_a . f_{fp} \qquad (1.1)$$

Hence, a difference in the bioavailablity of enantiomers may arise from enantioselectivity in either of these two processes. Enantioselective first pass hepatic metabolism will be discussed in section 1.2.2.5.

It should be emphasised that the extent to which a drug is absorbed from the GIT can be influenced by competing reactions within the GIT itself, such as complexation

and metabolism. The possibility that these competing processes may exhibit enantioselectivity should not be overlooked.

1.2.2.2. Distributional Processes

The plasma protein binding of a drug is a primary determinant of its clearance, volume of distribution and elimination half-life (Rowland & Tozer 1989). The influence of enantioselective plasma protein binding on hepatic and renal clearance will be discussed in sections 1.2.2.3 and 1.2.2.4, respectively. Also, it is generally accepted that the pharmacological effect of most drugs is related to the unbound drug concentration in plasma.

In humans, the major plasma proteins responsible for the binding of xenobiotics are human serum albumin (HSA) and alpha-1-acid glycoprotein (AGP). Both of these proteins can bind a wide range of chemical entities and because of this diversity, there are rarely large differences between a pair of enantiomers in the extent to which they are bound to plasma proteins.

Enantioselective plasma protein binding has been documented for a number of chiral drugs which are bound predominantly to albumin, including the chiral anticoagulants warfarin (Yacobi & Levy 1977) and phenprocoumon (Brown et al 1977). Cases of extreme enantioselectivity in the binding of chiral xenobiotics to albumin has been reported for the enantiomers of tryptophan (Jahnchen & Muller 1983) and various esters of the chiral benzodiazepine, oxazepam (Jahnchen & Muller 1983; Muller & Wollert 1975).

The acute-phase reactant protein, AGP (orosomucoid), has been found to selectively bind the enantiomers of the cardiovascular drug propranolol (Walle et al 1983). Other chiral drugs which are bound predominantly to AGP, and for which enantioselective plasma protein binding in humans has been documented, include disopyramide (Valdivieso et al 1988) verapamil (Eichelbaum et al 1984) and methadone (Romach et al 1981). There have been no reports of extreme enantioselectivity in drug binding to AGP.

Just as some chiral drugs are bound to differing extents to plasma proteins, it is to be expected that the binding of chiral drugs to tissue sites would also exhibit enantioselectivity. In fact, since 60% of albumin is located outside the vascular compartment (Rowland & Tozer 1989), this protein alone may give rise to enantioselective tissue binding for some drugs. It should also be remembered that pharmacodynamic differences between enantiomers arise, in many cases, from enantioselective binding to specific tissue sites (i.e. receptors; Lehmann 1986). There

are some interesting examples of enantioselectivity in the uptake of chiral drugs into tissue stores. In the case of propranolol, it appears that the S(-)-enantiomer (the active ß-adrenoreceptor blocker) is enantioselectively stored in adrenergic nerve endings, and may be released during sympathetic nerve stimulation (Walle et al 1988). In fact, it has been suggested that this storage and release process may explain, at least in part, why the cardiovascular effects of propranolol may persist even when the plasma levels of the drug have fallen below recognised effective concentrations (Walle et al 1988). Although the R(-)-enantiomers of some 2-phenylpropionic acid derivatives are enantioselectively taken up into adipose tissue, this phenomenon appears to be the consequence of enantioselectivity at a metabolic, rather than distributional, level (Caldwell & Marsh 1984; see section 3.5.2).

A number of physiological membranes can influence the distribution of xenobiotics. Many of these membranes, for example the blood-brain barrier (Joo 1985), contain carrier-mediated transport systems which control the movement of certain molecules between the physiological compartments. For some endogenous chiral compounds, these transport processes exhibit enantioselectivity (Simonyi et al 1986). Although few data are presently available, it would be expected that enantioselectivity may be important for chiral drugs which may be candidates for these carrier-mediated transport processes.

The extent of plasma protein binding, tissue binding, and distribution into, and out of, the various physiological compartments, all influence the volume of distribution of a drug. Differences in volume of distribution have been reported for the enantiomers of several chiral drugs, including verapamil (Eichelbaum et al 1984), propranolol (Olanoff et al 1984) and warfarin (Toon et al 1986)

1.2.2.3. Metabolism

Stereochemical factors play an important role in the metabolic transformation of xenobiotics, and the topic of enantioselectivity in drug metabolism has been comprehensively reviewed (Caldwell et al 1988; Eichelbaum 1988; Low & Castagnoli 1978; Testa 1988; Testa & Mayer 1988; Vermeulen & Breimer 1983). Enantioselectivity in drug metabolism may arise from differences between enantiomers in their binding to the metabolizing enzymes (i.e. different affinities) and/or from enantioselectivity in the catalytic processes (Testa & Mayer 1988). Enantioselectivity in drug metabolism can be broadly divided into two categories; substrate enantioselectivity and product enantioselectivity.

Substrate enantioselectivity occurs when a pair of enantiomers are biotransformed

by a given metabolic route at different rates (Testa & Mayer 1988). An interesting example of substrate enantioselectivity is provided in the case of warfarin. After oral administration of pseudoracemic warfarin (see section 1.3) to 6 healthy men, Toon et al (1986) examined the percentage of each enantiomer excreted in urine as the various warfarin metabolites. While R(+)-warfarin was recovered in the urine mainly as products of ketone reduction and of 6- and 7-hydroxylation, the S(-)-enantiomer was recovered mainly as the 7-hydroxy-metabolite, with products of ketone reduction being minimal. In one volunteer, given pseudoracemic warfarin along with a tracer quantity of ^{14}C-warfarin, and from whom urine and faecal samples were collected, the intrinsic clearance of formation of 7-hydroxy-warfarin was about 14 times greater for S(-)-warfarin than for R(+)-warfarin (Toon et al 1986). A similar pattern of warfarin metabolism was found *in vitro* using human hepatic microsomal enzymes. The rate of formation of 7-hydroxy-warfarin from S(-)-warfarin greatly exceeded that for its optical antipode (Kaminsky et al 1980).

The metabolism of the enantiomers of the chiral ß-adrenoreceptor blocker, metoprolol, exhibits interesting features. Lennard et al (1983) found that metoprolol metabolism cosegregated with debrisoquine phenotypes. In subjects with high metoprolol clearance (extensive metabolizers of debrisoquine), given an oral dose of racemic metoprolol, the area under the plasma concentration-time profile of S(-)-metoprolol exceeded that of R(+)-metoprolol by an average of 35%, possibly due to enantioselective metabolism (oxidation). However, in subjects who were poor metabolizers of metoprolol (and of debrisoquine), there was no difference between the enantiomers in the area under the plasma concentration-time curve, suggesting that oxidation phenotype was a major source of the enantioselective pharmacokinetics of the drug (Lennard et al 1983). This example is clinically important because only S(-)-metoprolol exhibits ß-adrenoreceptor blocking activity. The chiral anticonvulsant agent, mephenytoin, is another compound whose enantioselective metabolism is phenotype-dependent. In extensive metabolizers of mephenytoin, the S(+)-enantiomer is biotransformed almost exclusively by para-hydroxylation while R(-)-mephenytoin is metabolised much more slowly, by para-hydroxylation and N-demethylation. However, in poor metabolizers, the ability to para-hydroxylate the S(+)-enantiomer is virtually absent (Wedlund et al 1987).

Not only do the phase I biotransformations exhibit substrate enantioselectivity, but also the various phase II metabolic processes. For example, there is evidence for the substrate enantioselective glúcuronidation of the chiral drugs, bufuralol (Dayer et al 1986), propranolol (Silber et al 1982) and ibuprofen (Lee et al 1985) in humans, and for the enantioselective sulphate conjugation of 4-hydroxy-propranolol *in vitro* (Christ

& Walle 1985). It should be remembered that the glucuronide conjugates of two enantiomers are actually diastereomers, which may have different physico-chemical properties. Interestingly, Quon et al (1988) have reported that the hydrolysis of the short acting ß-adrenoreceptor blocker, esmolol (an ester), by blood esterases, is enantioselective in certain animal species.

If, during the metabolism of a molecule, a chiral centre is created, then that metabolic process is said to be product enantioselective if the two possible configurations of the metabolite are produced at different rates (Testa & Mayer 1988). The aromatic hydroxylation of the achiral anticonvulsant phenytoin, generates a metabolite with a chiral centre (5-para-hydroxy-diphenylhydantoin). The process exhibits product enantioselectivity, since the major product of metabolism is the para-hydroxy metabolite possessing the S-configuration (Maguire & McClanahan 1986). For the achiral molecule debrisoquine, 4-hydroxylation constitutes a major route of elimination, and results in the formation of a chiral metabolite. Eichelbaum et al (1988) reported that in extensive metabolizers of debrisoquine, 4-hydroxylation resulted almost exclusively in the formation of the metabolite with the S(+)-configuration. In poor metabolizers of debrisoquine, this high degree of enantioselectivity was lost, and between 5 and 36% of 4-hydroxy-debrisoquine consisted of the R(-)-enantiomer.

The hepatic clearance of a drug (CL_H)is a function of: its unbound fraction (f_u); the hepatic blood flow (Q_H); and the intrinsic clearance (CL_{int}) of the drug, which is a measure of the ability of the metabolizing organ to clear the drug (in most cases approximated by V_{max}/K_m), according to equation 1.2 (Wilkinson & Shand 1975).

$$CL_H = Q_H \times \frac{f_u\, CL_{int}}{Q_H + f_u\, CL_{int}} \qquad (1.2)$$

Hence, differences between enantiomers in hepatic clearance does not necessarily imply a difference in V_{max}/K_m, i.e. it is not always an indicator of enantioselective metabolism *per se*. For propranolol, there is evidence of enantioselectivity in f_u (Walle et al 1983) and CL_{int} (Olanoff et al 1984). Furthermore, because of enantioselectivity at the pharmacodynamic level (Ariens 1983), the two enantiomers may have a differential effect on Q_H, which may lead to a difference in the clearance of the two enantiomers

when administered alone. The inter- relationship between f_u, CL_{int} and Q_H is considered in section 1.2.2.5.

1.2.2.4. Renal and Biliary Excretion

Renal elimination involves three processes: filtration at the glomerulus of plasma water into the renal tubular lumen; carrier-mediated secretion of selected molecules from blood into the proximal tubule; and reabsorption (passive and active) from the proximal and distal and portions of the renal tubule (Rowland & Tozer 1989). The kidney may also be a site of drug metabolism for some drugs. Renal filtration is dependent exclusively on the glomerular filtration rate (GFR) and f_u. Hence, differences between enantiomers in renal filtration will normally arise from differences in their plasma protein binding. An exception may exist in the case of a chiral drug whose individual enantiomers have a differential effect on GFR, via an alteration in renal blood flow.

The carrier-mediated sytems in operation in the renal tubule are responsible for the active secretion of certain endogenous substances and toxins, and for the active reabsorption of various nutrients such as glucose and amino-acids (Rowland & Tozer 1989). Analogous to hepatic clearance, the clearance of a drug by renal secretion can be influenced by renal blood flow, the intrinsic ability of the carrier-mediated systems to transport the drug into the renal tubular lumen, and the plasma protein binding of the drug.

Very few data are available on enantioselectivity in the processes of renal clearance. Hsyu & Giacomini (1985) found that for pindolol, a drug which is actively secreted, the renal clearance of the (-)-enantiomer slightly exceeded that of (+)-pindolol. Because there was no evidence of enantioselective plasma protein binding, these workers concluded that this difference was caused either by enantioselective renal transport, or enantioselective renal metabolism (Hsyu & Giacomini 1985). Although Lennard et al (1983) reported differences in the renal clearance of metoprolol enantiomers, this clearance mechanism contributes only minimally to the elimination of this drug. In 1986, Notterman et al reported a significant difference in the renal clearance of quinine and quinidine, claimed to be a result of a difference between the compounds in renal secretion. Quinine and quinidine are related to one another as diastereomers, and hence although an example of stereoselectivity, it does not represent an example of enantioselectivity.

Because biliary excretion is an active, capacity-limited process, (Gregus & Klaassen 1987), it is potentially enantioselective for chiral drugs, although the topic has

received very little attention to date.

1.2.2.5. The Clinical Implications of Enantioselective Pharmacokinetics

The implications of differences between enantiomers in their pharmacokinetic properties are extremely diverse. Enantioselective gastrointestinal absorption is believed to be of minor importance, given that the drugs for which this phenomenon is suspected, are being used currently as pure enantiomers (see section 1.2.2.1). For a chiral drug which is cleared mainly by renal mechanisms, enantioselective metabolism will usually be of little consequence. Similarly, enantioselective renal clearance will be of minor importance for an extensively metabolized drug.

In most cases, enantioselective plasma protein binding and/or metabolism are responsible for pharmacokinetic differences between enantiomers. For a drug which is predominantly cleared by hepatic metabolism, the influence of enantioselectivity in plasma protein binding and intrinsic clearance will depend on whether it is a drug of high, intermediate or low extraction ratio. It is instructive to consider the case of a drug at each end of this spectrum. For the discussion presented below, it is assumed that both enantiomers are completely absorbed into the hepatic portal vein after oral administration and both are cleared exclusively by hepatic metabolism.

For a drug of high extraction, hepatic clearance (CL_H) is dependent largely upon hepatic blood flow, Q_H (perfusion rate limited; Wilkinson & Shand 1975), and therefore a difference between the enantiomers in f_u or CL_{int} will not affect the average total (bound plus unbound) concentrations of the individual enantiomers in plasma after multiple i.v. administration of the racemate. However, the concentrations of the unbound enantiomers, although independent of CL_{int}, will be influenced by enantioselective plasma protein binding. For example, if the enantiomers have the same volume of distribution with respect to total species, the unbound concentrations of the enantiomer with the highest f_u will exceed those of its mirror-image form, while the total plasma concentration of each enantiomer will be equal. After oral administration of a racemate of high hepatic extraction, the fraction of the dose of each enantiomer which escapes first-pass hepatic extraction (1-extraction ratio) is small and very sensitive to differences in f_u and CL_{int}. The enantiomer for which the product of f_u and CL_{int} is of the highest magnitude, will have the highest first-pass extraction. Consequently, the total plasma concentration of the enantiomers after multiple oral dosing will be

dependent on differences in f_u and CL_{int}. Unbound levels of the individual enantiomers, on the other hand, will only be sensitive to differences in CL_{int}. Propranolol is a high extraction drug; after i.v. administration, the plasma concentration-time profiles of the two enantiomers follow similar paths (Olanoff et al 1984; Von Bahr et al 1982) while after oral dosing, the plasma concentrations of S(-)-propranolol significantly exceed those of R(+)-propranolol (Silber et al 1982; Von Bahr et al 1982). This is consistant with a higher intrinsic clearance for the R(+)-enantiomer (Olanoff et al 1984; Sibler et al 1982; Von Bahr et al 1982; Walle et al 1984), although enantioselective plasma protein binding (Walle et al 1983) may also be involved. For verapamil, another high extraction drug, the difference between the plasma concentrations of the individual enantiomers is considerably greater after oral administration compared to when the drug is administered parenterally (Vogelgesang et al 1984), a consequence of enantioselective first-pass hepatic metabolism.

For a drug whose hepatic metabolism is capacity-limited (low extraction ratio), such as warfarin, the plasma concentrations of total drug after i.v. or oral dosing are depedent upon f_u and CL_{int}, while the unbound concentrations are sensitive to differences in CL_{int} only (Wilkinson & Shand 1975). Hence, for a pair of enantiomers with identical volumes of distribution with respect to total (bound plus unbound) drug; identical intrinsic clearances; and enantioselective plasma protein binding, the total plasma concentrations after administration of the racemate will differ, whilst the plasma unbound concentrations of the enantiomers will be similar.

1.2.3. Comment

The enantiomers of a chiral drug may differ from one another in the effects that they have upon, and the manner in which they are dealt with by, the body. These differences arise because of the enantio-discriminatory capacity of biological macromolecules. Clearly, when a racemate is administered, the patient receives a fixed-ratio combination of two "drugs". When pharmacodynamic or pharmacokinetic studies are performed on racemic drugs, this concept must be borne in mind, and analytical methods for measuring the concentration of such drugs in biological fluids should be enantioselective.

1.3. MEASUREMENT OF ENANTIOMERS IN BIOLOGICAL FLUIDS

Because enantiomers demonstrate identical physical and chemical properties in a non-chiral environment, specialized techniques are needed to measure enantiomers in biological fluids after the racemate has been administered. The approaches which have been used for such purposes include enantioselective immunoassay, the use of pseudoracemates, and enantioselective chromatography.

Enantioselective immunoassays exploit the chirality of antibodies. Promising results have been obtained when enantiomerically pure immunogens (a pure enantiomer 'hapten' coupled to a protein 'carrier') have been used to generate enantioselective antisera (Cook 1983, 1988). If both enantiomers need to be detected, antisera to each enantiomer may be produced, each with a low cross-reactivity for the opposite enantiomer. This technique has been used to measure the individual enantiomers of methadone, warfarin, and some chiral barbiturates (Cook 1983, 1988).

In the case of pseudoracemates, the chemical nature of one of the enantiomers is altered. Typically, the enantiomers are resolved prior to administration and one is isotopically labelled. The labelled enantiomer is then re-mixed with an equal quantity of its non-labelled optical antipode, and this "pseudoracemate" is administered. Because the two "enantiomers" are now chemically different, (i.e. different molecular weight) they can be distinguished from one another by mass spectrometry. It is important that the isotope should be chemically stable, and that there should be no physiological "isotope-effect" (Cook 1983, 1988).

In recent years, chromatography, in particular high-performance liquid chromatography (HPLC) has become the most utilized technique for the resolution of enantiomers for analytical and preparative purposes.

The chromatographic resolution of enantiomers is achieved either (i) by forming transient non-covalent diastereomeric relationships between the analyte enantiomers and a chiral agent which is associated with the chromatographic system (the direct approach) or, (ii) by reacting the enantiomers with an optically pure chiral derivatizing agent, and resolving the so-formed diastereomers using conventional (achiral) chromatographic techniques (the indirect approach)

In the direct approach, the chiral discriminant, or chiral source, may be a chiral substance which acts as the stationary phase, or, in the case of HPLC, is incorporated into the mobile phase.

Chiral mobile phases additives may take the form of a chiral counter-ion such as the basic compounds quinine, quinidine and cinchonine, which have been used to resolve many anionic racemates; and (+)- and (-)-camphorsulphonic acid, for the

resolution of certain cationic racemates (Lindner & Pettersson 1985; Pettersson & Schill 1988). Chiral macromolecules such as ß-cyclodextrin, albumin, and alpha 1-acid glycoprotein have also been used as mobile phase additives for the chromatographic resolution of some racemic compounds (Lindner & Pettersson 1985). The main advantages of using the chiral mobile phase method of direct enantiomeric resolution are that the mobile phase additives are usually inexpensive, and resolution may be achieved using conventional, achiral, HPLC stationary phases. To date, chiral mobile phases have not been used with great success for the analysis of chiral compounds in biological fluids.

Over recent years, a range of chiral stationary phases (CSPs) have been developed which allow the direct resolution of racemic chemicals. The Pirkle-type CSPs (Finn 1988; Lindner & Pettersson 1985) were designed to optimize interactions (π-π, dipole-dipole, hydrogen bonding, and Van der Waals interactions) between chiral molecules bonded to a silica support, and the individual enantiomers of a racemic analyte. Macromolecular CSPs, based on ß-cyclodextrin, albumin and AGP, have all been used successfully for a variety of pharmaceutical applications (Lindner & Pettersson 1985).

At the present time, the chiral CSPs appear to have limited capacity, and it is common that additional chromatographic steps are required to resolve the compound of interest from background contamination prior to enantiomeric resolution.

The majority of enantioselective bio-assays have relied on the indirect approach of chromatographic analysis. This approach exploits the differences in the physical and chemical properties of diastereomers formed by the derivatization of the analyte enantiomers with a chiral derivatizing agent (CDA) of high optical purity (Lindner 1988). Advantages with this technique are that: a wide range of CDAs are available, mostly inexpensive; conventional chromatographic columns may be used, avoiding the need for expensive CSPs; and, for enhanced sensitivity, a CDA with favourable UV absorption or fluorescent properties may be selected. Limitations include: the need for a suitable CDA of high optical purity, and an analyte with a potentially derivatizable moiety; there must be no racemization of the analyte or the CDA during the preparative and chromatographic procedures; and, for preparative purposes, reconversion of the resolved diastereomers should be possible. The use of an indirect technique for the quantification of ibuprofen enantiomers in human plasma will be described in Chapter4.

In summary, several approaches are available to distinguish between enantiomers for analytical purposes. However, the quantification of the individual enantiomers of many chiral drugs, in biological fluids, is difficult, particularly at low analyte concentrations. Because of these difficulties, many pharmacokinetic and

concentration-effect studies on chiral drugs which are administered as racemates, continue to rely on assays which quantify unresolved drug in the reference biological fluid(s), i.e. non-enantioselective assays. The concentration data generated for unresolved drug is usually interpreted as if one compound only was administered. A recent survey (Ariens & Wuis 1987) of research articles appearing in the journal "Clinical Pharmacology & Therapeutics" indicated the urgent need for an increased appreciation of enantioselectivity in the field of clinical pharmacology.

The theoretical analysis described in the next chapter is aimed at elaborating on some of the problems which may arise if studies on racemic drugs are performed non-enantioselectively.

Chapter 2
The Consequences of Neglecting Enantioselectivity in Studies on Racemic Drugs: Some Theoretical Considerations.

2.1 INTRODUCTION

This chapter examines some limitations of using non-enantioselective methods for analysing chiral drugs when they are administered in their racemic form. Three main aspects are emphasized, and evidence for each will be provided by hypothetical analysis of simulated plasma concentration-time data generated for a model chiral drug. Firstly, it will be demonstrated that the pharmacokinetic properties of a chiral drug, determined using the results of non-enantioselective drug analysis, may not reflect the true pharmacokinetic characteristics of the individual enantiomers. For example, the pharmacokinetics of a chiral drug may be seen to demonstrate concentration- and/or time-dependence, even though for each enantiomer the drug disposition processes are concentration- and time-independent. Secondly, the limited value of non-enantioselective methods, in particular certain radiochemical techniques, used for determining the plasma protein binding of chiral drugs will be illustrated. Finally, some of the limitations associated with the use of non-enantioselective analysis in concentration-effect correlations and in therapeutic drug monitoring will be considered.

2.2 METHODS

2.2.1. Pharmacokinetic Properties of the Model Chiral Drug

The model chiral drug X, posesses a single chiral carbon atom and exists as two enantiomers, R and S. Clinically X is employed as an equal mixture of R and S (i.e. as the racemate).

X undergoes enantioselective disposition which originates solely from the enantioselective binding of R and S to both plasma and tissue proteins. The fraction unbound in plasma (f_u) of R (f_u^R) is 0.100 while that of S (f_u^S) is 0.200. The ratio of the unbound fraction in plasma to the unbound fraction in tissue (f_u / f_{ut}) is identical for

both R and S. Accordingly, given that plasma volume (V_p) and tissue volume (V_t) are the same for R and S, equation 2.1 (Rowland & Tozer, 1989) predicts that the volume of distribution (Vd) of R and S will be identical.

$$Vd = V_p + V_t . f_u / f_{ut} \qquad (2.1)$$

For the purposes of this investigation Vd^R and Vd^S have been arbitrarily set at 20.8L. Both R and S are cleared from the body by glomerular filtration, with no tubular secretion or reabsorption, and so the total clearance (CL) of each enantiomer is given by the equation,

$$CL = f_u . GFR \qquad (2.2)$$

Assuming a glomerular filtration rate (GFR) of 120 ml/min, the total clearance of R (CL^R) is 12.0 ml/min and that of S (CL^S) is 24.0 ml/min. The elimination half-lives ($t_{1/2}$) of R (20.0h) and S (10.0h) have been calculated using equation 2.3.

$$t_{1/2} = 0.693 \, Vd / CL \qquad (2.3.)$$

Accordingly, the elimination rate constant (k) of R (kR) is 0.0347 h^{-1} and that of S (kS) is 0.0693 h^{-1}. Following intravenous administration of the racemate each enantiomer undergoes instantaneous distribution and mono-exponential disposition which is both time- and concentration-independent.

2.2.2 Dosing

The consequences of administering racemic X in two ways will be examined;

Single Dose

Racemic X is administered as a rapid intravenous (i.v.) bolus dose of 208 mg (i.e. 104 mg of each enantiomer).

Chronic Dose

Racemic X is again administered as a rapid i.v. bolus dose of 208 mg but, in addition, an i.v. infusion of racemic X is administered at a rate of 9.61 mg/h. The infusion is commenced at the same time as the bolus dose is administered and is

continued for 160 hours. This dosage regimen has been designed so as to achieve and maintain a total concentration of X in plasma of 10 mg/L. The infusion rate has been calculated using a clearance value for X of 16.02 ml/min (see later).

2.2.3. Simulations

The disposition data for R, S and X resulting from the two dosage regimens were generated using the pharmacokinetic parameters of R and S and standard pharmacokinetic equations.

Single dose

In the single dose simulation, the total, that is bound plus unbound, plasma concentration(C) -time(t) data for R and S were calculated by substituting their respective parameters into equation 2.4.

$$C = \frac{\text{Dose}\ e^{-k.t}}{Vd} \qquad (2.4.)$$

The concentrations of unbound R and S in plasma were determined by multiplying the total plasma concentrations by their respective unbound fractions. The total and unbound concentrations of unresolved X in plasma were determined by summing the corresponding plasma levels of R and S. These data for unresolved drug represent those which would be determined if non-enantioselective techniques were used for measuring X in plasma.

Chronic dose

In this case, the plasma concentration-time data of total R and total S were simulated using equation 2.5.

$$C = \frac{\text{i.v. bolus dose}\ e^{-k.t}}{Vd} + \frac{\text{infusion rate}\ (1 - e^{-k.t})}{CL} \qquad (2.5.)$$

The plasma concentrations of unbound R and S were determined as described previously. The total and unbound plasma concentrations for X were determined by summing the corresponding values for R and S.

2.3. RESULTS AND DISCUSSION

2.3.1. Single dose

The simulated log plasma concentration-time profiles of total (bound + unbound) R and total S following single dose i.v. administration of racemic X are presented in Figure 2.1. The profile of total unresolved X is presented on the same plot. The volume of distribution of both enantiomers is the same (20.8L) and accordingly, following the administration of 208mg of racemic X, both R and S attain the same initial plasma concentration ($C(0)^R$ and $C(0)^S$, respectively) of 5mg/L. Subsequently, the plasma concentrations of R and S decline mono-exponentially, but at different rates. Accordingly, the plasma concentration of X (C^X) declines bi-exponentially, in accordance with equation 2.6.

$$C^X = C(0)^R e^{-kR.t} + C(0)^S e^{-kS.t} \qquad (2.6)$$

This differential disposition of R and S results in a noticable curvature of the log concentration-time profile for total unresolved X (Figure 2.1).

The disposition of X at any time-point is dependent upon the disposition of R and S, and accordingly, as the enantiomeric composition in plasma changes with time, so too will the pharmacokinetic behaviour of X. For example, at any time following the administration of racemic X the unbound fraction of X in plasma (f_u^X) is dependent upon the unbound fractions of the individual enantiomers and their relative plasma concentrations, in accordance with equation 2.7.

$$f_u^X = \frac{C^R}{C^R + C^S} f_u^R + \frac{C^S}{C^R + C^S} f_u^S \qquad (2.7).$$

Hence, although f_u^R and f_u^S remain constant, as the enantiomeric composition of X in plasma changes, so too does its unbound fraction. Because the clearance of X is directly proportional to its unbound fraction ($CL^X = f_u^X . GFR$) the change with time in f_u^X is reflected in corresponding changes in CL^X. These changes are presented graphically in Figure 2.2. which shows that the unbound fraction and clearance of X change from 0.150 and 18.0 ml/min, respectively (the arithmetic means of the corresponding values for the two enantiomers), and approach 0.100 and 12.0ml/min,

respectively (the values of the more slowly cleared enantiomer).

It should be noted that if the total clearance of X is calculated by dividing the i.v. dose by the area under the plasma concentration-time curve from zero to infinite time, a value of 16.02 ml/min is obtained which represents a weighted average of CL^R (12.0 ml/min) and CL^S (24.0 ml/min).Mathematically, the clearance of X can be calculated (see Appendix A for derivation) according to the equation

$$CL^X = \frac{2\,CL^R\,CL^S}{CL^R + CL^S} \qquad (2.8)$$

The plasma concentration-time profiles of unbound R, unbound S and unbound X are presented in Figure 2.3. In contrast to that which occurs with total drug, the initial plasma concentration of unbound R is half that of unbound S. This difference arises because of the difference between the enantiomers in their volumes of distribution with respect to unbound drug. As with total X, the plasma concentration-time profile of unbound X displays a distinct curvature (Figure 2.3) which reflects, in this case, a change with time in the volume of distribution of unbound X. The unbound clearances of R and S are identical and so the unbound clearance of X is independent of its enantiomeric composition and remains constant at 120ml/min. However, it should be emphasized that for some chiral drugs, the possibility exists that the unbound clearances of the individual enantiomers may differ significantly (e.g. as a result of enantioselective hepatic metabolism or renal secretion; see section 1.2.2). In such a case, in contrast to that which occurs for X, there exists a potential for both the total and unbound clearance of the unresolved species to change with time.

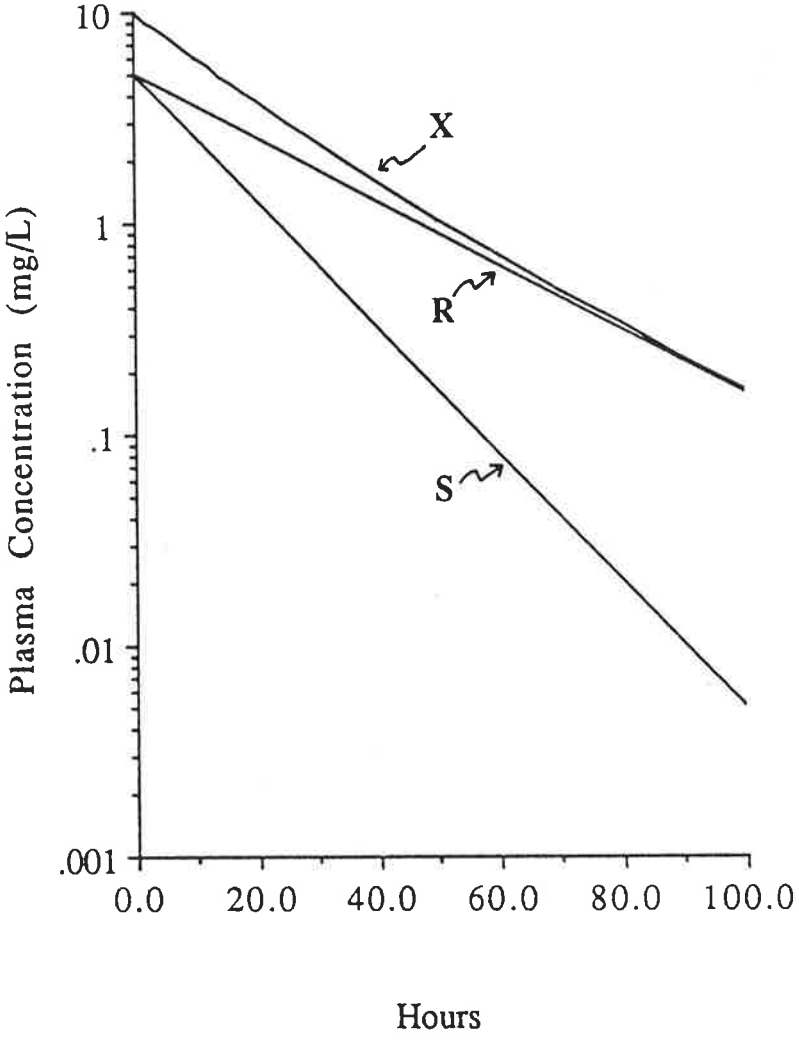


Figure 2.1. Plasma concentration-time profiles of total R, total S and total X following the single i.v. bolus administration of racemic X (208mg).

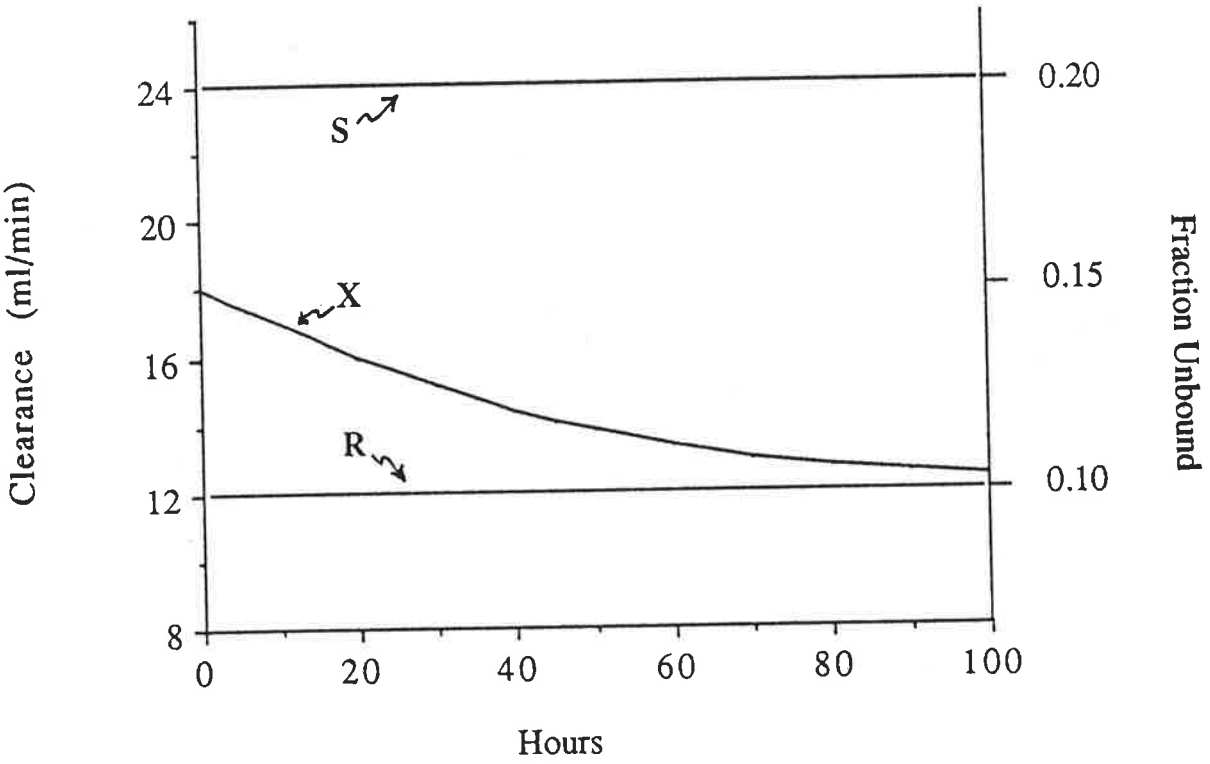


Figure 2.2. The clearance and unbound fraction of R, S and X plotted against time following the single i.v. bolus administration of racemic X.

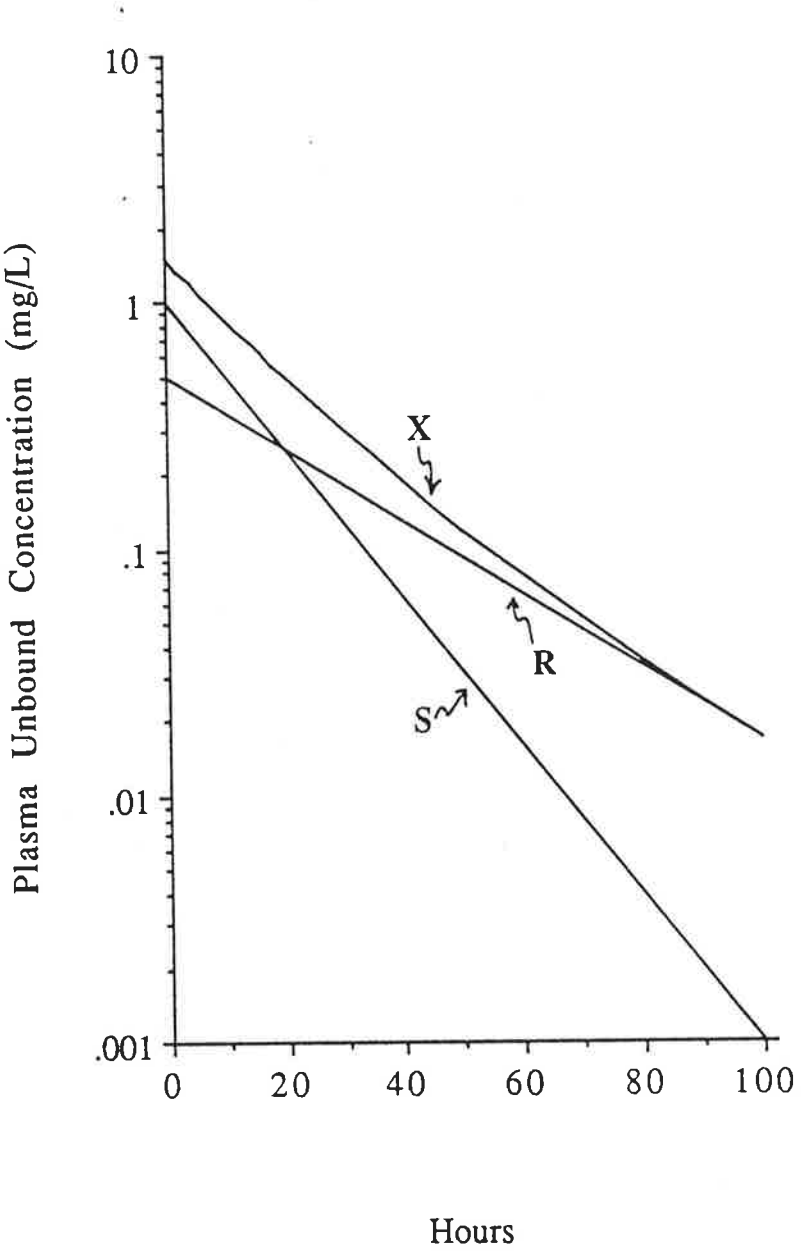


Figure 2.3. Plasma concentration-time profiles of unbound R, unbound S and unbound X following the single i.v. bolus administration of racemic X (208mg).

Potential Limitations of Non-enantioselective Analysis of Total X

The use of non-enantioselective analytical techniques for studying the disposition of X may lead to a number of erroneous conclusions regarding its pharmacokinetic behaviour. The curvature of the total plasma log concentration-time profile (Figure 2.1) may be interpreted as "multicompartmental" disposition. Indeed, for a drug such as X, which undergoes enantioselective disposition, data generated for unresolved drug alone add additional complications to the application and interpretation of compartmental model analysis (Ariens 1984). The estimation of a half-life for X would also prove difficult because of the constant curvature of the profile.

The accepted method for determining the renal clearance of a drug is to relate the total amount of unchanged drug eliminated in the urine over a discrete time interval to the levels of the drug in the plasma over that same period of time (Rowland & Tozer 1989). If this method was used to determine serial estimates of the total renal clearance of X, the values would be found to decrease with time (Figure 2.2). For example, the value obtained over the time interval 0 to 4 hours would be about 17.8 ml/min while that obtained over the interval 78 to 82 hours would be about 12.7 ml/min. Purely on the basis of the data for total X, a variety of postulates, such as concentration-dependent plasma protein binding, may be proposed to explain such a change.

There are a number of examples in the literature where potentially erroneous conclusions regarding the renal clearance of racemic drugs have been reached on the basis of disposition data for unresolved drug only. When the renal excretion rate of the chiral ß-adrenergic blocking drug, pindolol, was plotted against plasma concentration (Balant et al 1981), the slope of the resulting line (renal clearance) decreased as the pindolol concentrations decreased. To explain this phenomenon a saturable tubular reabsorption process was postulated. A similar mechanism was proposed to explain the decrease with time in the renal clearance of hydroxychloroquine (Cutler et al 1987), another chiral compound which is administered as a racemate. However, in both of these cases the mechanism was proposed by the authors solely on the basis of unresolved drug disposition data. Clearly, for a racemic drug the possibility must not be overlooked that a change with time in the renal clearance of the unresolved species may be due to changes in enantiomeric composition, as shown for drug X (Figure 2.2).

Potential Limitations of Non-enantioselective Analysis of Unbound X.

Because the unbound fraction of X in plasma changes with time (Figure 2.2) one may falsely conclude, purely on the basis of unresolved disposition data, that the plasma binding of X demonstrates concentration-dependence, possibly as a result of saturation of protein binding sites. In addition, the reason for the decrease with time in

the renal clearance of X may be attributed to this proposed concentration-dependent binding.

There are a large number of methods available for studying the plasma protein binding of drugs. However, these approaches have important potential limitations when applied non-enantioselectively to racemic drugs. One of the most common and convenient methods for measuring the unbound fraction of a drug in a post-dose *ex vivo* plasma sample involves radiochemical analysis. In general, this methodology involves the addition of a tracer quantity of the radiolabelled form of the drug to the plasma sample under investigation. Following a suitable separation technique, such as equilibrium dialysis or ultrafiltration, radiochemical analysis is used to obtain an estimate of the unbound fraction of the drug. In many cases this approach has been utilised for investigating the plasma protein binding of drugs which are administered as their racemates. However, if the plasma binding of the two enantiomers differs, then for a binding estimate obtained in such a manner to be accurate the enantiomeric composition of the spiked radiolabelled drug must be identical to that of the unlabelled drug present within the plasma sample. Generally, the radiolabelled material used in such studies is racemic and the radiochemical analysis is performed non-enantioselectively. Accordingly, if the plasma protein binding of the individual enantiomers differ and the drug undergoes enantioselective disposition such that the enantiomers are not present in the plasma sample in equal concentrations, then the result obtained will be inaccurate.

Evidence of such inaccuracies is exemplified in the case of the model drug. Consider a sample collected 80 hours after the single dose i.v. administration of X. The true unbound fraction of unresolved X is 0.106. If racemic radiolabelled X is added to the sample and the unbound fraction determined using non-enantioselective radiochemical analysis, the result obtained would be 0.150, which is the arithmetic mean of f_u^R (0.100) and f_u^S(0.200). The difference between the true unbound fraction of X and the unbound fraction determined radiochemically arises because the spiked material is racemic while the unlabelled X present within the plasma sample consists predominantly of R. If this methodology was used to monitor the plasma protein binding of X after its single dose administration, no time-dependence would be detected, and quantitative errors of up to 50% would be made. Furthermore, it would not be possible to explain the decreasing renal clearance of X in terms of a changing unbound fraction. Thus, the use of racemic radiolabelled drug added into post-dose plasma samples for determining plasma protein binding is potentially misleading. This factor should be considered when assessing the results and conclusions of studies

which have used such techniques for assessing the unbound fraction of a drug in post-dose plasma samples, for example, in the case of the non-steroidal anti-inflammatory agent ibuprofen (Aarons et al 1983a; Albert et al 1984a; Gallo et al 1986; Lockwood et al 1983a, 1983b) a racemic drug which undergoes enantioselective disposition (Lee et al 1985) and plasma protein binding (Hansen et al 1985; see also subsequent chapters).

Another approach commonly used for examining plasma protein binding in drug disposition studies is to generate a binding curve by spiking varying concentrations of the drug under investigation into drug-free plasma. Such binding curves are used to infer the unbound fraction of the drug in post-dose plasma samples where the total drug concentration is known. In the case of racemic drugs there are potential problems associated with this approach. For example, if the plasma protein binding of X was determined using this method the results would indicate that the unbound fraction is constant (0.150). This result does not take into account the enantioselective disposition of X and once again may lead to confusion as to the cause of its changing renal clearance. Such a technique should not be used to determine the unbound concentration of X in post-dose plasma samples. A binding curve approach has been used by a number of workers for studying the disposition of the racemic antiarrhythmic drug disopyramide (Cunningham et al 1977; Giacomini et al 1982; Meffin et al 1979). The results of these studies may be inaccurate, because of the enantioselective disposition and plasma protein binding of disopyramide in humans (Giacomini et al 1986; Lima et al 1985; Valdivieso et al 1988)

2.3.2. Chronic Dose

The simulated plasma concentration-time profiles of total X, total R and total S over the time interval 0 to 160 hours are presented in the upper panel of Figure 2.4. Because R and S have identical volumes of distribution their initial plasma concentrations are the same. However, because of the two-fold difference between the enantiomers in their half-lives and clearances, their rates of approach to steady-state and steady-state concentrations, respectively, differ by a factor of two. In this particular case the total plasma concentration of S changes with time from 5.00 mg/L, immediately after the bolus loading dose to 3.33 mg/L at steady state, while that of R changes from 5.00 mg/L to 6.67 mg/L. Over the same time period the concentration of unresolved X remains virtually constant. The transient fall in the total plasma concentration of unresolved X, a phenomenon usually associated with drugs which display multicompartmental characteristics (Gibaldi & Perrier, 1982) results from the

differential disposition of R and S. Because of the change in its enantiomeric composition, the unbound fraction of X and consequently its renal clearance, decrease from 0.150 to 0.133 and 18.0 ml/min to 16.0 ml/min, respectively.

The plasma concentration-time profiles of unbound X, unbound R and unbound S are presented in the upper panel of Figure 2.5. In contrast to that which occurs for total R and S, the initial concentrations of unbound R and S differ by a factor of two (because of the difference in the volume of distribution of R and S with respect to unbound drug) while their steady-state concentrations are identical (because of identical clearances of unbound R and unbound S). The unbound concentrations of R and S change from 0.500 mg/L to 0.667 mg/L and 1.00 mg/L to 0.667 mg/L, respectively, and the unbound concentration of X changes from 1.50mg/L to 1.33mg/L. It should be noted that if non-enantioselective radiochemical analysis was used to determine the unbound fraction of unresolved X in a steady-state plasma sample, a value of 0.150 would be obtained. A similar result would be obtained if the binding was investigated using a binding curve approach. In either case, if the unbound fraction was multiplied by the total plasma concentration of X an incorrect answer of 1.50mg/L for the unbound concentration would result.

The enantiomeric composition (expressed as the concentration of R divided by the concentration of S) of total and unbound X in plasma versus time are presented in the lower panels of Figure 2.4 and 2.5, respectively. The enantiomeric composition of total X increases from 1.00 at the commencement of therapy to 2.00 at steady-state, while that of unbound X increases from 0.500 to 1.00. A change similar to that which occurs for total X has been documented for the chiral antiarrhythmic drug tocainide (Thomson et al 1986). Following an infusion of racemic tocainide to twelve patients, the ratio of total S(+)-tocainide to total R(-)-tocainide in plasma increased steadily from an average of 1.03 shortly after the commencement of the infusion to an average of 1.76 after 48.5 hours.

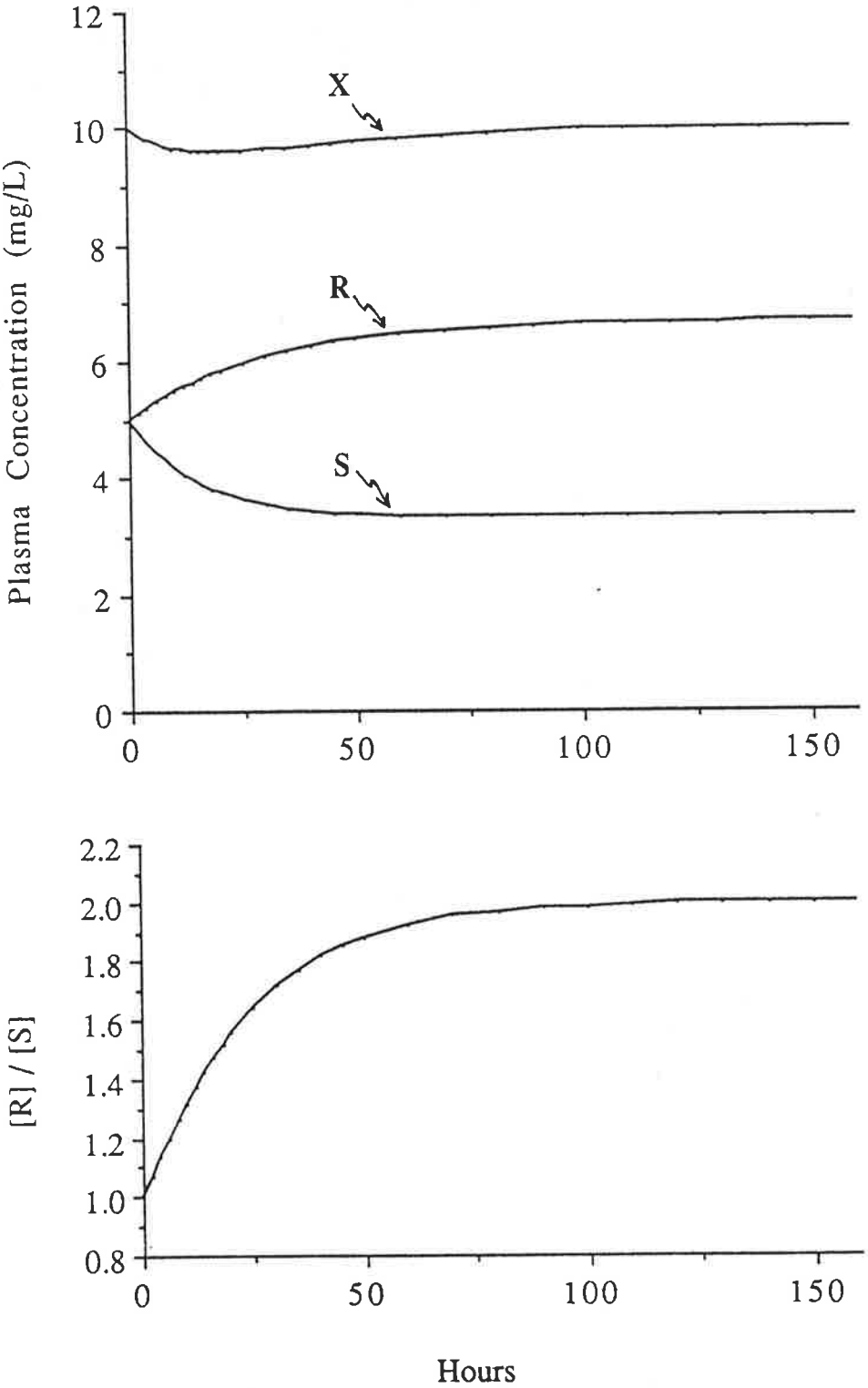


Figure 2.4. Upper panel: Plasma concentration-time profiles of total R, total S and total X following chronic i.v. dosing (bolus + infusion) with racemic X. Lower panel: The enantiomeric composition of total X versus time following chronic i.v. dosing with racemic X.

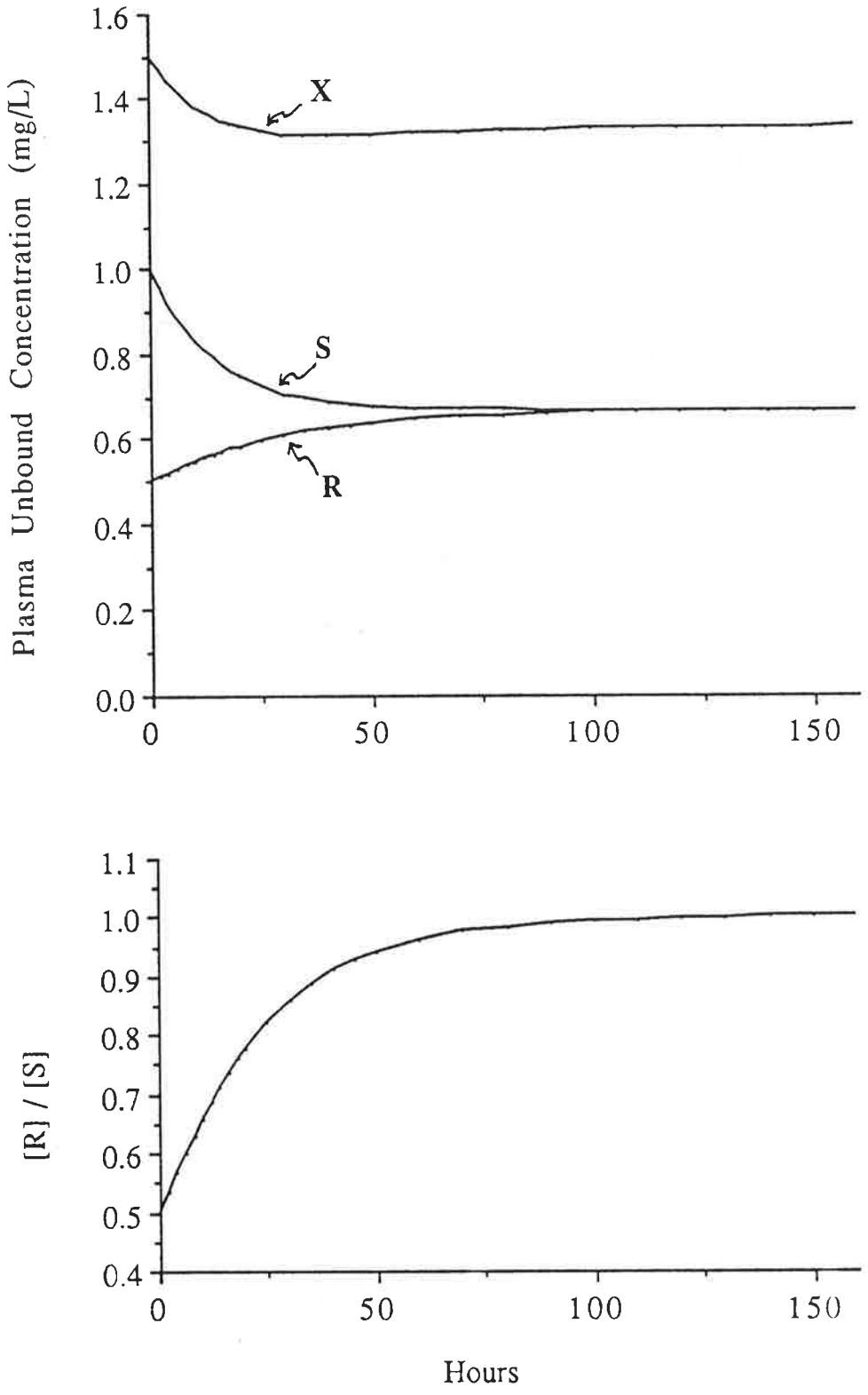


Figure 2.5. Upper panel: Plasma concentration-time profiles of unbound R, unbound S and unbound X following chronic i.v. dosing (bolus + infusion) with racemic X. Lower panel: The enantiomeric composition of unbound X versus time following chronic i.v. dosing with racemic X.

Potential Limitations of Non-Enantioselective Analysis of X

The enantiomeric composition of a chiral drug in plasma can differ between routes of administration, as in the case of verapamil (Vogelgesang et al 1984) and between individuals, as with metoprolol (Lennard et al 1983) and tocainide (Sedman et al 1984). Although some of the potential problems associated with such differences with respect to therapeutic drug monitoring have been discussed (Drayer 1986) very little attention has been focused on the consequences of intra-individual variability in enantiomeric composition. Lima et al (1985) postulated that the enantioselective clearance of disopyramide may lead to a change with time in its enantiomeric composition in plasma upon chronic administration and that this change may be important clinically. Although changes in the enantiomeric composition of chiral drugs in plasma with respect to time have been described for other drugs such as tocainide (Thomson et al 1986) the theoretical aspects relating to such changes occurring during concentration-effect studies and therapeutic drug monitoring do not appear to have been considered.

For drug X, depending on the relative pharmacological properties of R and S, there are a variety of pharmacological outcomes which may result from a chronic dosage schedule such as that described. Many chiral drugs used clinically as the racemate derive the majority of their primary pharmacological activity from one enantiomer only (see section 1.2.1). Examples include the 2-phenylpropionic acid non-steroidal anti-inflammatory agents, such as ibuprofen (Adams et al 1976) and the ß-adrenergic blocking drugs (Ariens 1983). In the case of the model drug X, if unbound R alone correlates with therapeutic and/or toxic effects then the increase in the plasma concentration of unbound R over the period of chronic dosing may result in an enhanced response. Such an enhancement could not logically be explained on the basis of results from non-enantioselective analysis, because not only does the total plasma concentration of unresolved X remain almost constant (Figure 2.4), but the unbound plasma concentration of unresolved X actually decreases (Figure 2.5).

Conversely, if unbound S alone is the active entity, then a significant reduction in the response may occur over the period of dosing, since the unbound concentration of S decreases by 33.3% (Figure 2.5) . On the basis of non-enantioselective analysis of total X in plasma such a decrease could not be explained and one may erroneously conclude that a degree of tolerence to the effect of X develops upon chronic administration. Alternatively, a decrease in the clinical response may be interpreted as a reflection of the reduced unbound concentration of unresolved X (assuming that the binding of X was not measured using one of the problematical approaches outlined previously).

With many racemic drugs, both enantiomers contribute significantly to the overall pharmacological profile of the drug. In some cases, as with ketamine (White et al

1980), and the optically active barbiturates (Ho & Harris 1981), the individual enantiomers may elicit qualitatively different responses. If such was the case with drug X, then the alteration in the enantiomeric composition of the unbound drug with time may result in a change in the nature of the pharmacological response which again would be difficult to explain on the basis of unresolved plasma concentration data.

The chiral antiarrhythmic agents disopyramide, tocainide, and mexiletine are all administered in their racemic form and are subject to therapeutic drug monitoring. For all three drugs there is evidence of enantioselective pharmacokinetics (Edgar et al 1984; Giacomini et al 1986; Grech-Belanger et al 1986; Hoffman et al 1984; Thomson et al 1986) and for disopyramide (Giacomini et al 1980) and tocainide (Byrnes et al 1979) there is also evidence of enantioselective pharmacodynamics. However non-enantioselective analytical techniques are used in routine therapeutic drug monitoring to measure the concentrations of these drugs in plasma. For both old and new drugs where therapeutic drug monitoring is indicated, one would anticipate that the use of enantioselective drug analysis may enable the establishment of a more relevant and clinically useful relationship between concentration and effect.

2.4 CONCLUSION

The work described in this chapter has highlighted some of the limitations arising from non-enantioselective analysis of racemic drugs in pharmacokinetic studies, concentration-effect correlations and therapeutic drug monitoring. Additionally, the limitations of some plasma protein binding techniques in dealing with racemic drugs have been outlined.

The properties of the model drug were selected so as to simplify its theoretical analysis. However the two-fold difference between R and S in their unbound fractions and clearances is in accordance with enantiomeric differences reported for drugs used clinically (Drayer 1988b). Although the unbound clearances of R and S were identical, more complex scenarios may result when the unbound clearances differ, as for example in the case of a racemic drug which undergoes enantioselective hepatic metabolism or renal secretion. Finally, although attention has been focused on chiral drugs which are administered in their racemic form, in some cases the biotransformation of a non-chiral drug may result in the generation of a metabolite with a chiral centre (see section 1.2.2.3). In such cases, where one is interested in measuring metabolite levels in biological fluids, the interpretation of results obtained using non-enantioselective analysis should be approached cautiously.

It is hoped that the work described in this chapter will promote awareness of

enantioselectivity in the field of clinical pharmacology. This awareness should be applied to re-evaluate the results of previous studies on chiral drugs which have failed to consider enantioselective drug disposition, and to anticipate potential complications of using non-enantioselective drug analysis in future studies.

The remainder of this thesis will describe investigations performed on various aspects of the enantioselective clinical pharmacology of ibuprofen, a chiral drug which is administered as a racemate, and for which a great number of pharmacokinetic studies have been performed using non-enantioselective analytical techniques.

Chapter 3
Ibuprofen: A Review of the Literature

Ibuprofen, RS-2-(4-isobutylphenyl)propionic acid, is a non-steroidal anti-inflammatory agent (NSAIA) which possesses anti-inflammatory, analgesic and antipyretic properties (Adams et al 1969b). For the past 20 years, ibuprofen has been used with considerable success for the treatment of rheumatic disorders, such as rheumatoid arthritis and osteoarthritis, and more recently as an analgesic for the relief of various other forms of pain (Kantor 1984).

Ibuprofen belongs to the 2-phenylpropionic acid (2-PPA) class of NSAIAs, known also as the "profens". With the exception of naproxen , which is marketed as its S(+)-enantiomer, all of the profens are currently used clinically as racemates. Enantioselectivity plays a significant role in the clinical pharmacology of ibuprofen. While its desired pharmacological effects reside almost exclusively with the S(+)-enantiomer (Adams et al 1976), one of the metabolic pathways of the distomer, R(-)-ibuprofen, is chiral inversion to its pharmacologically active mirror-image (Hutt & Caldwell 1983).

3.1 PHYSICO-CHEMICAL PROPERTIES OF IBUPROFEN

Ibuprofen ($C_{13}H_{18}O_2$,molecular weight 206.3) is a white, crystalline, stable solid which has a melting point of 76°C (Ibuprofen safety sheet, Boots Co). It is a weak acid (pKa ≈ 5) which is insoluble in water, but readily soluble in chloroform, methanol and most other organic solvents (Monograph on ibuprofen; Martindale, The Extra Pharmacopoeia, 28th Edition). The propionic acid side chain of ibuprofen possesses a chiral centre, and therefore, ibuprofen exists as two enantiomers, R(-)-ibuprofen (R-I) and S(+)-ibuprofen (S-I), the structures of which are presented in Figure 3.1. Currently, ibuprofen is used clinically in its racemic form (RS-I).

3.2 THE PHARMACOLOGY OF IBUPROFEN

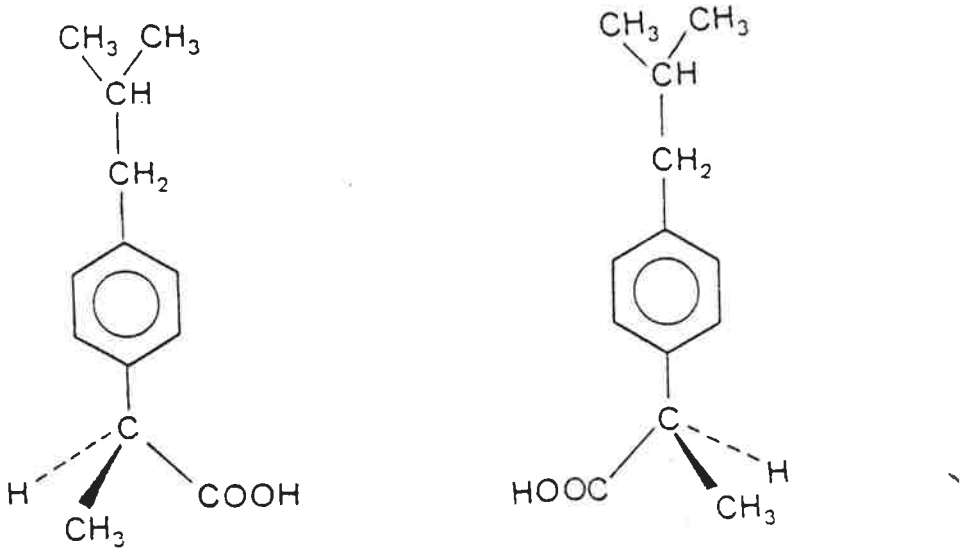
3.2.1. Historical Development.

In the 1950s and 60s, the Boots Company Ltd, under the guidance of Stewart S. Adams, was in the search for anti-rheumatic compounds of lower toxicity than aspirin

(Adams 1987b). After extensive pharmacological and toxicological testing of hundreds of chemicals (mostly weak organic acids), in a variety of animal models, ibuprofen was selected for further investigation and was shown, ultimately, to be a well-tolerated and effective anti-rheumatic in humans. In 1969, after two to three years of clinical trials, ibuprofen was introduced in the United Kingdom as an anti-inflammatory agent for the treatment of rheumatoid arthritis and osteoarthritis (Adams 1987b). The development of ibuprofen by the Boots company was important historically because it heralded the arrival of a new family of NSAIAs, the profens. By 1985, no less than 23 profens had been either marketed or used in clinical trials (Lombardino 1985).

3.2.2. Pharmacological Properties

At the time of the discovery of ibuprofen, the desired pharmacological actions of a drug which was to be used in rheumatic conditions were three-fold; anti-inflammatory, analgesic and antipyretic. Ibuprofen proved to be active in all three aspects. In 1968 it was also discovered that aspirin-like drugs, including ibuprofen, could prevent the aggregation of platelets (O'Brien 1968). As will be discussed in section 3.2.4, all of these actions appear to share a common mechanism.



S(+)-IBUPROFEN R(-)-IBUPROFEN

Figure 3.1. Flying-wedge representations of the enantiomers of ibuprofen.

In a variety of animal models ibuprofen was shown to possess anti-inflammatory activity at non-toxic doses (Adams & Buckler 1979; Adams et al 1967, 1969b; Orzalesi et al 1977). Ibuprofen was found to reduce the erythema produced in depilitated guinea-pig skin upon exposure to UV light; reduce the inflammation of adjuvant-induced arthritis in the rat; and reduce the inflammation produced by the subcutaneous injection of carrageenan into the hind paw of the rat (Adams & Buckler 1979; Adams et al 1967, 1969b). In humans, orally administered ibuprofen reduced the degree of erythema resulting from topical exposure to the irritant chemical, thurfyl nitrate (Adams et al 1969b).

Ibuprofen effectively reduced the writhing induced in mice by an intraperitoneal injection of acetylcholine - a model of analgesia in the absence of inflammation (Adams et al 1967; 1969b). Ibuprofen also reduced the response to pressure on the inflammed rat foot - another test for analgesic activity (Adams et al 1969b). However, the drug had no effect on the response to pain in the non-inflammed rat foot and had no effect on the reaction time of mice placed onto a hot-plate (Adams et al 1969b). Both of these latter findings suggested to the investigators that the analgesic properties of ibuprofen were mediated peripherally, rather than centrally as with narcotic analgesics.

As an antipyretic, ibuprofen effectively reduced the fever induced in rats by the subcutaneous injection of a yeast suspension (Adams et al 1967; 1969b).

3.2.3. Toxicity

The major toxic effects of acute ibuprofen administration in experimental animals were gastrointestinal. In the rat, the LD_{50} of ibuprofen was 1600mg/kg orally, and 1300mg/kg subcutaneously. Irrespective of the route of administration, death in rats resulted from intestinal ulceration, although lethal doses also caused central nervous system depression (Adams et al 1969b). After chronic oral dosing (180mg/kg daily for 6 months) rats were anaemic and had lower body weights than controls, and a number had intestinal ulceration. Although there was reversible enlargement of the liver, there were no signs of hepatotoxicity, an important finding given the untoward effects of ibufenac (a NSAIA released by the Boots Company a few years prior to ibuprofen) on the liver (Adams 1987b). Enlargement of the kidney and spleen was also noted.

3.2.4. Mechanism of Action

At the time of development of ibuprofen, little was known of the mechanism by which the NSAIAs, a group of agents extremely diverse in their chemical structures yet strikingly similar in their pharmacological properties, exerted their effects. The effects of NSAIAs on numerous enzymatic pathways have been examined and although significant progress has been made, the true mechanism of action remains poorly understood (Day 1988; Goodwin 1984).

Widely contrasting theories have been proposed over the last 30 years to explain the mechanism of action of NSAIAs. One of the earliest, that "aspirin-like" drugs acted by uncoupling oxidative phosphorylation (Adams & Cobb 1958), was based on a good correlation between the activity of a range of drugs in inhibiting UV erythema in the guinea-pig and their ability to uncouple oxidative phosphorylation *in vitro*. The theory has been largely dismissed, however, because many potent uncouplers of phosphorylation possesse no anti-inflammatory activity (Brune et al 1976). Convincing arguments opposing many other theories have been reviewed by Brune et al (1976).

The most satisfactory and widely held theory, proposed by John Vane in 1971, was that the biological effects of aspirin-like drugs may be due to their ability to inhibit the synthesis of prostaglandins. Evidence supporting this theory was, and still remains to a large extent, circumstantial. The evidence was based upon the observation that, firstly, aspirin-like drugs could all inhibit the synthesis of prostaglandins in various models, and secondly, prostaglandins could be implicated as playing important roles in the generation of fever, inflammation and posssibly pain (Vane 1971). It was also suggested that prostaglandins may perform protective functions in the gastrointestinal tract and, by inhibiting prostaglandin synthesis, aspirin-like drugs could induce mucosal damage (Vane 1971). By 1974, inhibition of prostaglandin synthesis by NSAIAs had been demonstrated in over 30 different systems, including tissue homogenates and subcellular fractions, isolated organs and in whole animals (Flower 1974). The currently accepted pattern of synthesis of prostaglandins, and associated pathways, is summarised below.

Arachidonic acid (AA), derived from dietary linoleic acid, is stored as a component of phospholipids in the mammalian cell membrane. In response to stimuli (hormonal, neuronal, immunological or mechanical), AA is liberated from phospholipids via the action of the enzyme phospholipase A2. Released AA is metabolized by two distinct enzyme systems (cyclo-oxygenase pathway and lipoxygenase pathway), to a range of oxygenated metabolites, known collectively as the eicosanoids (Higgs et al 1980; Lands 1985; Moncada et al 1980; Salmon 1986).

Cyclo-oxygenase (prostaglandin synthetase) cyclates and oxygenates AA to the unstable intermediate prostaglandin G2 (PGG2), which is then metabolized by a peroxidase enzyme to prostaglandin H2 (PGH2). PGH2 is a pivotal intermediate which is transformed by: 1) prostacyclin synthetase to prostacyclin (PGI2); 2) thromboxane synthetase to thromboxane A2 (TXA2); and 3) by a variety of enzymatic and non-enzymatic processes to a range of prostaglandins including PGE2, PGD2 and PGF2∝ The roles of the prostaglandins are both complex and multiple. Different tissues synthesize different ranges of prostaglandins. Platelets, for example, synthesize large quantities of TXA2, which is a potent inducer of platelet aggregation. Prostacyclin, a potent vasodilator and inhibitor of platelet aggregation, is the major cyclo-oxygenase product of the endothelial cells of blood vessel walls. Prostaglandins are important mediators of erythema, hyperalgesia and hyperthermia (some of the cardinal signs of inflammation), and play significant roles in the functioning of the kidney, vascular smooth muscle, endocrine and reproductive systems and in the generation of the immune response (Higgs et al 1980; Lands 1985; Moncada et al 1980; Salmon 1986).

The more recently discovered 5-lipoxygenase pathway converts AA to hydroperoxyeicosatetraenoic acids (HPETEs), which are reduced by peroxidase to the corresponding hydroxy-acids. One of the hydroperoxy derivatives, 5-HPETE, is a precursor of a series of biologically active compounds known as the leukotrienes. Of these, leukotriene B4 is a potent chemotactic factor for many cell types, notably polymorphonuclear leukocytes, and may have an important function in inflammation (Salmon 1986). Whereas the cyclo-oxygenase enzyme system is distributed widely amongst the various mammalian cell types, the 5-lipoxygenase system appears to be restricted primarily to cells originating from bone-marrow, i.e. neutrophils, eosinophils, monocytes, macrophages and mast cells (Salmon 1986).

It is widely accepted that NSAIAs act by specifically inhibiting cyclo-oxygenase, thereby preventing the formation of the prostacyclins, thromboxanes and the various other prostaglandins. (Flower 1974; Higgs et al 1980; Salmon 1986). It should be noted, however, that Vane's theory has not gone totally unchallenged (Goodwin 1984) and there is increasing evidence to suggest that certain NSAIAs may elicit important pharmacological effects which are mediated via alternative mechanisms (Day 1988; Goodwin 1984), such as altering leukotriene biosynthesis. Although there is at present no unifying theory to explain the mechanism by which NSAIAs exert their effects, Vane's hypothesis has withstood the test of time and remains popular. Convincing supportive evidence includes a good rank-order correlation between inhibition of prostaglandin synthesis and anti-inflammatory effect (Higgs et al 1980; Salmon 1986)

and the fact that most of the pharmacological effects of the drugs can be explained as being mediated via inhibition of cyclo-oxygenase (Table 3.1)

Table 3.1. The pharmacological properties of NSAIAs, related to the effects of prostaglandins (PGs): Data from Higgs et al 1980; Moncada et al 1980; Vane & Ferriera 1979, unless otherwise stated.

1. ANTIPYRETIC	
All NSAIAs are antipyretic in febrile states	Most PGs are pyrogenic. Fever is a common side-effect of systemically administered prostaglandins
2. ANALGESIC	
All NSAIAs are used to relieve various forms of pain.	Prostaglandins can cause severe pain when injected intramuscularly and headache after intravenous dosing. Intradermal injection of PGE2 causes a long-lasting hyperalgesia.
3. ANTI-INFLAMMATORY	
All NSAIAs are anti-inflammatory in experimental animals and humans	PGE2 and PGI2 cause erythema, increase local blood flow and vascular permeability. PG release has been detected in a range of inflammatory states including rheumatoid arthritis.
4. ANTIPLATELET	
NSAIAs inhibit the adhesiveness of platelets, leading to an increased bleeding time	TXA2 is a pro-aggregatory agent involved in platelet aggregation and thrombus formation
5. GASTROINTESTINAL	
NSAIAs are gastrointestinal irritants and can induce peptic ulceration and perforation	PGs protect against gastric and duodenal mucosal damage (Russell 1986)

Table 3.1. (continued)

6. RENAL	
NSAIAs cause fluid retention and can increase blood pressure. Chronic therapy is associated with a low incidence of renal toxicity	PGs increase renal blood flow and promote diuresis. PGI2 mediates the release of renin from the renal cortex
7. GESTATION	
NSAIAs can prolong gestation in animals and humans	PGs produce strong contractions of uterine smooth muscle
8. DUCTUS ARTERIOSUS	
Various NSAIAs have been used to promote closure of the ductus in neonates.	PGs are involved in maintaining the patency of the ductus arteriosus.
9.FERTILITY	
In rare cases, NSAIAs may have undesirable effects on male fertility	Seminal fluid is rich in PGs, which may facilitate conception. A correlation exists between amounts of PG in semen and some cases of male infertility.
10. BRONCHIAL CONSTRICTION	
In predisposed individuals, NSAIAs can precipitate bronchoconstriction.	PGs released in the lung relax smooth muscle and cause bronchodilatation

Ibuprofen is a potent inhibitor of prostaglandin synthesis in microsomal preparations, intact cell preparations (including platelets), in perfused organs and in the living organism (Adams & Buckler 1979). The majority of NSAIAs, including ibuprofen, are "competitive, reversible" inhibitors of cyclo-oxygenase (Lands 1985; Salmon 1986). Aspirin, on the other hand, irreversibly acetylates the enzyme, thereby leading to a more prolonged inhibition (Lands 1985; Salmon 1986).

In addition to its ability to inhibit cyclo-oxygenase, ibuprofen has been found to

exhibit a number of other potentially important pharmacological actions which may or may not be related to cyclo-oxygenase inhibition. *In vitro*, ibuprofen causes variable degrees of depression of sulphated glycosaminoglycan synthesis in articular cartilage (Brandt & Palmoski 1984; McKenzie et al 1976;). The consequences of this potentially adverse-effect, which is shared by many other NSAIAs (Brandt & Palmoski 1984), is uncertain. Ibuprofen uncouples oxidative phosphorylation (energy metabolism) in rat liver mitochondria (Tokumitsu et al 1977); can suppress neutrophil aggregation and degranulation (Kaplan et al 1984); and has been found to suppress inflammatory oedema by an action on polymorphonuclear leukocytes which is seemingly independent of cyclo-oxygenase inhibition (Rampart & Williams 1986). The size of experimentally induced myocardial infarction is reduced by ibuprofen, a property which may be related to its ability to inhibit neutrophil aggregation and degranulation (Flynn et al 1984). In experimental animals, ibuprofen also has been found to promote vascular patency after burns injury. This effect, which appears to be unrelated to cyclo-oxygenase inhibition, may be a result of fibrinolytic activity (Ehrlich 1984).

In conclusion, it appears that inhibition of the cyclo-oxygenase cascade of phospholipid metabolism is the principal mechanism by which ibuprofen exerts its anti-inflammatory, analgesic and antipyretic activity. This same mechanism is also involved in the adverse effects of ibuprofen on the gastrointestinal, renal and blood coagulation systems. There is, however, accumulating evidence that ibuprofen has certain other biological properties, which may be independent of the cyclo-oxygenase system, that contribute to its overall pharmacological profile

3.3. THERAPEUTIC INDICATIONS OF IBUPROFEN

Ibuprofen was developed primarily as an anti-rheumatic agent which would be less toxic than aspirin (Adams 1987b). A large number of controlled clinical trials, conducted in thousands of individuals, and carefully reviewed by Altman (1984), Davies & Avery (1971), Huskisson et al (1971), Kantor (1979) and Ward (1984), clearly demonstrate ibuprofen to be superior to placebo, and in most instances at least equally effective as aspirin and other NSAIAs, in the treatment of rheumatic conditions.

Since the release of ibuprofen in 1967, it has become increasingly apparent that the initial dosage recommendations were suboptimal (Ward 1984). In 1971, the recommended adult dose of ibuprofen was 600mg daily, in three divided doses (Davies & Avery 1971). At present, the daily dose recommended by the manufacturer is 1200 to 1600mg per day, but up to 2400mg daily can be used in the initial treatment and during acute exacerbations of rheumatic illness (BRUFEN®, product information,

Australian Prescription Products Guide, 1988). Clinical trials continue to examine the maximum tolerated dose of ibuprofen.

Not only is the optimum and the maximum therapeutic daily dose of ibuprofen unknown (Ward 1984), but there is also uncertainty regarding the most efficacious dosing frequency. Because of its short elimination half-life of about 2 hours (see section 3.6.5), ibuprofen has traditionally been administered three to four times daily. However, certain studies (Brugueras et al 1978; Pownall & Pickvance 1986) have found no significant difference in the clinical efficacy of ibuprofen whether a standard daily dose of the drug (1600mg) was divided into two or four doses. This may be partly due to the kinetics of the drug in synovial fluid (see section 3.6.2).

In addition to its established use in the treatment of rheumatic conditions, ibuprofen has been shown to be effective in the treatment of dysmenorrhoea (Dawood 1984; Shapiro 1988). As an analgesic, ibuprofen has also been exploited for the relief of post-partum pain (Sunshine et al 1983); post-operative pain (Slavic-Svircev et al 1984); the pain of dental extraction (Cooper 1984); and for the relief of cancer pain (Ferrer-Brechner & Ganz 1984; Stambaugh & Drew 1988).

3.4. ADVERSE EFFECTS.

The most frequently reported adverse effects of ibuprofen in humans are those on the gastrointestinal tract. In most cases, the gastrointestinal effects are minor and include nausea and vomiting, epigastric pain, abdominal cramps and distress, constipation, diarrhoea and indigestion (Royer et al 1984; Semble & Wu 1987). Ibuprofen is irritant to the gastointestinal tract, causing a small degree of blood loss after single and chronic administration (Brooks et al 1973; Carson et al 1987; Semble & Wu 1987). In a small proportion of patients receiving chronic therapy with ibuprofen, more serious gastrointestinal side-effects may develop, including gastric and duodenal ulceration and perforation. Compared to most other NSAIAs, ibuprofen generally appears to cause these serious reactions less frequently, especially compared to aspirin and the NSAIAs with long half-lives, such as naproxen, phenylbutazone and piroxicam (Adams 1987a; Brooks 1988; O'Brien & Burnham 1985; Paulus 1985; Semble & Wu 1987).

Adverse effects on the central nervous system, usually mild, have been reported to occur with ibuprofen administration. Headache, dizziness, somnolence and tinnitus have all been described (Royer et al 1984). Ibuprofen-induced depression has also been reported (Spittle 1982). Ibuprofen has also been associated with aseptic meningitis in patients with systemic lupus erythematosus (Durback et al 1988; Wasner 1978).

A very small proportion of patients will develop dermatological side-effects from

ibuprofen. Symptoms may range from a mild rash, pruritis and urticaria (Royer 1984) to a more severe and potentially fatal reaction, such as erythema multiforme (Johnson et al 1985). Cases of severe systemic hypersensitivity to ibuprofen have also been reported (Mandell & Raps 1987).

The renal side-effects of ibuprofen range from mild fluid retention, which may result in peripheral oedema and elevated blood pressure in predisposed individuals (Clive & Stoff 1984), through to serious renal toxicity (Marasco et al 1987; Royer et al 1984). Acute renal failure, renal papillary necrosis, interstitial nephritis and nephrotic syndrome have all been associated with ibuprofen use (Marasco et al 1987). However, the overall incidence of ibuprofen-induced renal toxicity is very low (Fox & Jick 1984).

It is well appreciated that certain individuals may suffer bronchoconstriction when exposed to NSAIAs, and for ibuprofen, the importance of this reaction is highlighted by a recent report of ibuprofen-induced death due to bronchoconstriction (Ayres et al 1987). Individuals with 'aspirin-sensitive' asthma are likely to be sensitive to other NSAIAs including ibuprofen (Szczeklik & Gryglewski 1983).

Additional rare but potentially important adverse reactions include hepatic toxicity in adults and children (Royer et al 1984; Stempel & Miller 1977), Stevens-Johnson syndrome, haemolytic anaemia, thrombocytopaenic and thrombotic purpura (Marasco et al 1987) and ophthalmic disturbances (Kantor 1979).

Overall, with long-term use, ibuprofen appears to have a very low incidence of serious adverse effects and most reviewers agree it to be one of the better tolerated NSAIAs. It should be noted, however, that as the recommended dose of ibuprofen increases, so may the incidence of ibuprofen related adverse effects.

Even after extremely high doses, ibuprofen appears to be a relatively safe drug (Barry 1984). In addition, exposure *in utero* appears to be associated with negligible or an extremely low incidence of abnormalities and complications (Barry 1984).

3.5. THE METABOLISM OF IBUPROFEN

In humans, ibuprofen is predominantly cleared by metabolism (Mills et al 1973). The two major metabolites, formed by the oxidation of the isobutyl group, are 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid, which is also referred to in the literature as metabolite A (Mills et al 1973) or hydroxy-ibuprofen (Lockwood & Wagner 1982), and 2-[4-(2-carboxypropyl)phenyl]propionic acid, referred to as metabolite B or carboxy-ibuprofen. Both of these major metabolites are excreted in the urine, either unchanged or as acyl-glucuronide conjugates (Lockwood & Wagner 1982; Mills et al 1973). Ibuprofen is also metabolized, to a minor extent, to an acyl glucuronide

(Lockwood & Wagner 1982; Mills et al 1973). After oral administration of a 400mg tablet of RS-I to 15 healthy volunteers, Lockwood et al (1983a) reported that a mean (±SD) of 84.9 ± 3.02% of the administered dose was recovered in urine. This total recovery consisted of 28.2% of hydroxy-ibuprofen (11.2% unconjugated; 17% conjugated), 43% of carboxy-ibuprofen (30.1% unconjugated; 12.9% conjugated) and 13.7% of ibuprofen glucuronide. No unchanged ibuprofen was detected in the urine. Lockwood et al (1983a) also found that there was no significant change in the partial recoveries of these individual species at higher ibuprofen doses (800mg and 1200mg). The hydroxy- and carboxy-metabolites of ibuprofen do not possess significant analgesic or anti-inflammatory potency (Adams et al 1969b). In healthy subjects, the plasma levels of the ibuprofen matabolites are low, relative to those of ibuprofen, after single (Lockwood & Wagner 1982; Mills et al 1973) and repeated dosing (Mills et al 1973).

Brooks & Gilbert (1974) and Pettersen et al (1978) isolated two additional ibuprofen metabolites from human urine. These metabolites were 2-[4-(1-hydroxy-2-methylpropyl)phenyl]propionic acid and 2-[4-(2-hydroxymethyl-propyl)phenyl]propionic acid. In the dialysis fluid of a nephrectomized female patient receiving ibuprofen, Pettersen et al (1978) also identified a new metabolite, 2-(4-carboxy)phenylpropionic acid. It was postulated that this compound arose from the oxidation of 2-[4-(2-carboxypropyl)phenyl]propionic acid. In individuals with normal renal function this precursor is rapidly cleared, whereas in the nephrectomized patient it may have been retained and therefore was available for further biotransformation to an end-metabolite (Pettersen et al 1978). An additional "metabolic route" of ibuprofen has also been identified; incorporation into natural triglycerides (Caldwell & Marsh 1983), which will be detailed in section 3.5.2.

The metabolism of the individual ibuprofen enantiomers is more complex. In 1967, Adams et al reported that after oral dosing with racemic ibuprofen, the two major metabolites (referring to carboxy- and hydroxy-ibuprofen), when isolated from urine, were dextrorotatory. A possible explanation for this phenomenon was that S-I was being metabolized by oxidation to a greater extent than its optical antipode. Mills et al (1973) administered the individual enantiomers of ibuprofen to a single volunteer and examined the optical rotation of the metabolites isolated from urine. Both ibuprofen enantiomers gave rise to oxidized metabolites which were dextrorotatory. The authors stressed that carboxy-ibuprofen contains two chiral centres, and the dextrorotatory property of this metabolite may have arisen from the introduction of this second chiral centre. However, such was not the case for hydroxy-ibuprofen. It was therefore postulated that for R-I there must be a structural inversion of the chiral carbon atom during the formation of the hydroxy-metabolite (Mills et al 1973).

By 1974, it was known that the enantiomers of ibuprofen appeared to be biologically equivalent *in vivo* (Adams et al 1967) whereas the biological activity of many other 2-phenylpropionic acid derivatives, was restricted to the dextrorotatory enantiomer (Fried et al 1973; Harrison et al 1970; Kuzuna et al 1974; Tomlinson et al 1972, Vincent & Remond 1972). This difference between ibuprofen and other analogues was consistent with the concept that R-I was being inverted *in vivo*, giving rise to the active enantiomer.

That inversion of the chiral centre of ibuprofen was occurring, at least in part, prior to oxidation was conclusively demonstrated by Wechter et al (1974). These workers administered, R-I, S-I and racemic ibuprofen, on separate occasions, to healthy humans. The enantiomeric composition of ibuprofen and hydroxy-ibuprofen in urine were determined using an enantioselective GLC method (Vangiessen & Kaiser 1975). After dosing with S-I, both ibuprofen and hydroxy-ibuprofen in the urine were of the S(+)-configuration. However, when R-I was administered, 80% of unchanged ibuprofen, and 54% of hydroxy-ibuprofen in the urine, were of the S(+)-configuration. Aware of the evidence for this novel metabolic inversion pathway, Adams et al (1975, 1976) examined the relationship between the *in vivo* biological activity of R-I and S-I, and their activity against prostaglandin synthesis *in vitro*. S-I was found to be 160 times more potent than R-I at inhibiting the synthesis of prostaglandins from arachidonic acid in the *in vitro* bovine seminal vesicle preparation. *In vivo*, however, both enantiomers were equally effective anti-inflammatory and analgesic agents in experimental animals (Adams et al 1975, 1976). These observations, which would have otherwise discredited Vane's hypothesis (1971), were attributed to extensive *in vivo* metabolic chiral inversion of R-I (Adams et al 1976).

Further support for the chiral inversion of ibuprofen was provided by the studies of Kaiser et al (1976) and Lee et al (1985). The chiral inversion process has been documented for a number of 2-phenylpropionic acid derivatives (Hutt & Caldwell 1983). The mechanism of chiral inversion and its pharmacolgical importance will be examined in section 3.5.1.

There is very little data on the degree of enantioselectivity of the non-inversion metabolic routes for ibuprofen. Kaiser et al (1976) administered 800mg of R-I, S-I and RS-I to three healthy volunteers, after which ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen were isolated from urine. The enantiomeric composition of each species was determined using a method which involved derivatization of the compounds with S(+)-phenylethylamine followed by gas-liquid chromatography. For the carboxy-metabolite, which contains two chiral centres, four optical isomers are possible (two pairs of enantiomers). Complete differentiation of all four isomers was not

achieved. It was discovered that in humans there was no net inversion of S-I to R-I. This was evidenced by the absence of R-I or metabolites of R-I after dosing with S-I. In addition, both enantiomers of ibuprofen were found to be metabolized to the corresponding hydroxy- and carboxy-metabolites. It was not possible to establish whether or not the oxidation of the individual ibuprofen enantiomers was enantioselective - this would have required the estimation of the individual unbound partial clearances.

3.5.1.The Chiral Inversion of Ibuprofen and Other 2-PPA Derivatives

Chiral inversion has been documented for a number of 2-PPA derivatives. Compounds for which there is evidence of chiral inversion in at least one animal species include ibuprofen (Kaiser et al 1976; Lee et al 1985; Wechter et al 1974) fenoprofen (Hayball & Meffin 1987; Rubin et al 1985), cicloprofen (Kripalani et al 1976; Lan et al 1976), benoxaprofen (Bopp et al 1979; Simmonds et al 1980), clidanac (Tamura et al 1981), loxaprofen and its hydroxylated metabolite (Nagashima et al 1984), naproxen (Goto et al 1982), 2-(2-isopropylindan-5-yl)-propionic acid (Tanaka & Hayashi 1980), ketoprofen (Abas & Meffin 1987), and 2-PPA itself (Fournel & Caldwell 1986; Meffin et al 1986; Yamaguchi & Nakamura 1985). Substances for which chiral inversion is thought to be negligible include indoprofen (Tamassia et al 1984), carprofen (Iwakawa et al 1987), and flurbiprofen (Jamali et al 1988a).

In all cases where inversion has been documented, the process has involved the transformation of the R(-)-configuration of the 2-PPA derivative to the S(+)-configuration. There is no substantial evidence to suggest that the reverse reaction (i.e. inversion of the S(+)- to R(-)-configuration), occurs to any appreciable extent in humans after therapeutic doses. Studies which have detected relatively low concentrations of the S(+)-enantiomer in plasma or urine after administration of the R(-)-enantiomer (Lee et al 1985) may have been confounded by optical contamination of the administered samples of the S(+)-enantiomer. However, Fournel & Caldwell (1986) administered high doses (150mg/kg and 300mg/kg) of S(+)-2-PPA (99% optical purity) to rats and found that about 10% of the drug recovered in urine (which was about 50% of the administered dose) consisted of the R(-)-enantiomer, providing conclusive proof that S(+)- to R(-)- inversion is possible. It should be noted that it would be difficult to demonstrate metabolic inversion of the S(+)-enantiomer to the R(-)-enantiomer if the reaction proceeded at a substantially slower rate than the reverse process.

The majority of research which has examined the mechanism of chiral inversion of

2-PPA derivatives has been performed using analogues in which various hydrogen atoms surrounding the chiral centre have been substituted with deuterium. Wechter et al (1974) administered R-I in which the three protons of the alpha-methyl carbon, and the sole proton attached to the chiral carbon (methine substituent) were replaced with deuterium (Figure 3.2).

After the administration of this tetra-deuterated analogue (d4-R-I), mass spectrometry was used to identify the deuterium content of the individual enantiomers of ibuprofen and its hydroxy-metabolite. Both S-I and the S(+)-enantiomer of hydroxy-ibuprofen contained, predominantly, 2 deuterium atoms, both located on the alpha-methyl carbon. For the R(-)-enantiomer of hydroxy-ibuprofen, all four deuterium atoms were retained. Interestingly, the rate of elimination of d4-R-I from plasma was about half that of the non-deuterated compound, indicating a significant, and potentially important isotope effect (Wechter et al 1974). In response to their findings the group proposed a possible mechanism for the inversion process, suggesting that it takes place via the formation of a thioester between R-I and coenzyme A, with a 2,3-dehydro-ibuprofen intermediate.

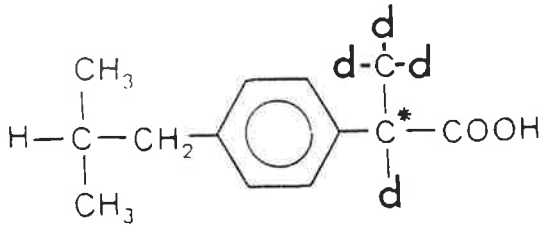


Figure 3.2. Ibuprofen molecule in which the alpha-methine and three alpha-methyl protons have been replaced with deuterium.

In 1980, Tanaka & Hayashi added speculation to the mechanism of inversion, when they reported that when 2-(2-isopropylindan-5-yl)propionic acid, in which the three methyl protons had been replaced with deuterium, was administered to rats, the urinary hydroxy-metabolite, which was predominantly of the S(+)-configuration, retained all three deuterium atoms. When racemic 2-(2-isopropylindan-5-yl)propionic acid containing a single deuterium atom in the alpha-methine position was administered, the recovered hydroxy- metabolite was an equal mixture of the deuterated and non-deuterated forms. Hence, in this case, inversion proceeded with the loss of the alpha-methine proton only. Although a different compound and a different species was used in this study, the data clearly conflicted with those of Wechter et al (1974).

Support for the results of Tanaka & Hayashi (1980) was reported in 1981 by Nakamura and coworkers. After the administration of alpha-methine deuterated R-I (d1-R-I) to rats, the deuterium content of the derived S-I in plasma was negligible. However, after dosing with alpha-methyl trideuterated R-I (d3-R-I), the deuterium content of S-I was unaltered (i.e. all three deuterium atoms were retained). Similar results were obtained when the deuterated compounds were perfused through an isolated rat liver (Nakamura et al 1981).

In 1988, Baillie et al investigated the inversion mechanism by administering 300mg each of R-I and alpha-methyl trideuterated R-I, to four adult male volunteers. By measuring the deuterium content of each ibuprofen enantiomer as a function of time, it was shown that the chiral inversion proceeded with the retention of all three deuterium atoms. It was also found that the inversion of deuterated R-I was not subject to an isotope effect. This study confirmed that 2,3-dehydro-ibuprofen does not serve as an intermediate in the chiral inversion of ibuprofen in man. Hence, three independent studies have cast doubt on the validity of some aspects of the early work of Wechter et al (1974).

The concept that coenzyme A played an important role in the inversion process, as proposed by Wechter et al (1974), was strengthened by the research of Nakamura & coworkers (1981). When the synthetically prepared coenzyme A (Co A) thioesters of R-I and S-I were incubated with rat liver homogenate, there was, in each case, the formation of both ibuprofen enantiomers. An enzyme responsible for the formation of ibuprofen CoA thioester *in vivo*, was isolated from rat liver mitochondria and microsomes. However, only R-I was a substrate for this enzyme. Nakamura et al (1981) proposed a mechanism for the chiral inversion process which, to date remains widely accepted (see Figure 3.3). Recently, Knihinicki et al (1988), repeated the experiments of Nakamura and coworkers, and confirmed their results. An enzyme responsible for the formation of the CoA thioesters of R-I has been identified as

long-chain acyl-CoA synthetase (Knights et al 1988). Although this initial reaction in the chiral inversion process is well characterized, much less is known about the subsequent steps.

In 1983, Hutt & Caldwell comprehensively reviewed the literature on the metabolic chiral inversion of 2-PPAs. It was suggested, that while the enzymatic formation of the acyl-CoA thioester of the R(-)-2-PPA analogue appears to be essential for inversion, the possibility exists that the subsequent steps may proceed non-enzymatically. This idea was based on the concept that if the methine proton of the thioester was sufficiently acidic to render it labile, then detachment and subsequent reattachment of this proton could result in chiral inversion. In addition, the subsequent hydrolysis of the thioester may also be spontaneous (Hutt & Caldwell 1983). In testing this hypothesis, Knihinicki et al (1987) found that when the CoA thioester of R-I was incubated in plasma at 37°C for 4 hours, there was 32% hydrolysis of the thioester, although no chiral inversion of the ibuprofen moiety was detected. Mayer et al (1988) examined the lability of the methine proton of 2-dimethylaminoethanethiol-2-phenylpropionate (DEPP), on the basis that it would have similar properties to the methine proton of a 2-PPA-CoA thioester. DEPP was incubated in various deuterated solvents, and proton/deuterium exchange was monitored. No exchange was detected in deuterated methanol, water and methanol/water. However, in solvents of lower polarity, such as deuterated acetonitrile/water, proton/deuterium exchange occurred. Hence, the lability of the alpha-methine proton of DEPP (and potentially 2PPA-CoA thioesters) is dependent on the polarity of the environment. These results, and those of Knihinicki et al (1987), neither support nor refute the concept that racemization and hydrolysis of 2-PPA-CoA thioesters, *in vivo*, may proceed, at least in part, non-enzymatically. The failure to detect inversion of R-I, in plasma for example (Knihinicki et al 1987), may have been due to the absence of an environment of suitable polarity. It is possible that such an environment is present in the intact cell or in certain cellular preparations.

The site of inversion of the 2-PPA derivatives is unknown. Isolated organ experiments (Cox et al 1985; Nakamura et al 1981) indicate, that in rats, the liver is an important site of chiral inversion of ibuprofen. Knihinicki et al (1988) reported chiral inversion of R-I in rat liver homogenate, but not in the homogenate of rat kidney or rat intestine. Similarly, Knadler & Hall (1989) reported that R-I, but not S-I, formed a CoA thioester upon incubation with rat liver homogenate, ATP and coenzyme A. R(-)-Fenoprofen also formed CoA thioesters, although none were formed from R(-)- or S(+)-flurbiprofen. For R(-)-fenoprofen, CoA thioester formation was detected in liver, lung, intestinal and kidney tissue, although the extent of formation was by far the greatest for liver tissue. Fournell & Caldwell (1986) found that the inversion of 2-PPA

in rats and rabbits was unaffected by the route of administration, suggesting that little, or no inversion occurred in the gastrointestinal tract, prior to absorption. Nakamura & Yamaguchi (1987) found that chiral inversion of 2-PPA proceeded in organ slices from rat liver, kidney and intestine. Further experiments by these workers (Yamaguchi & Nakamura 1987) on the disposition of 2-PPA in nephrectomised and bile-duct ligated rats, and eviscerated rats with non-functional livers, suggested that both the kidney and liver contributed to the inversion process. However, the interpretation of the results of this later experiment are complicated by enantioselectivity in the non-inversion metabolic (e.g. glucuronidation) and distributional processes of 2-PPA. In 1986, Meffin et al found that the clearance by inversion of unbound R(-)-2-PPA, administered intravenously to rabbits, was unaffected by renal failure. Simmonds et al (1980) found that R(-)-benoxaprofen was inverted when incubated with the everted rat gut.

In recent years Jamali and coworkers have suggested that ibuprofen, administered orally, is subjected to inversion to a major extent pre-systemically, and that the extent of inversion may be dependent on the rate of absorption (Jamali et al 1988b; Mehvar & Jamali 1988). This suggestion was made solely on the basis of compartmental model analysis of plasma concentration data for total (bound plus unbound) R-I and S-I after oral dosing with racemic ibuprofen. However, a recent report by Cox (1988, in abstract form) suggests that inversion does not proceed to any significant extent in the gastro-intestinal tract prior to, or during, gastrointestinal absorption. This conclusion was based on the finding that the inversion of ibuprofen (as guaged by enantioselective GLC analysis of ibuprofen and its metabolites in urine) proceeded to the same extent, irrespective of whether R-I was administered orally or intravenously, to humans.

The extent of inversion in man varies considerably among substrates. For the 2-PPA derivatives used clinically, inversion in humans is most extensive and clinically important for fenoprofen (Rubin et al 1985) and ibuprofen (Cox 1988; Lee et al 1985; see section 3.6.4). In addition to this variability among substrates, there is also evidence that the rate and extent of inversion of particular analogues may vary considerably among species. For example, R(-)-2-PPA is inverted to S(+)-2-PPA in rats and rabbits, but not in the mouse (Fournel & Caldwell 1986). A more clinically relevant example is provided by the 2-PPA derivative, benoxaprofen. The extent of inversion of benoxaprofen was substantially greater in rats than in humans (Simmonds et al 1980). This variability among species has important implications regarding toxicity testing of these compounds in experimental animals. For example, the toxicity of R(-)-benoxaprofen in rats may not have been adequately evaluated since it was rapidly inverted to its mirror-image. In humans, however, the relative exposure to R(-)-benoxaprofen may be more appreciable.

Whether the inversion process is physiologically regulated is unknown. It has been documented that the activity of acyl CoA ligase (synthetase) is under physiological control (Jason et al 1976). Insulin, for example, can increase the activity of fatty acid CoA ligase. In periods of carbohydrate excess, where insulin levels are elevated, an increased activity of acyl CoA synthetase promotes the rate of incorporation of free fatty acids into triglycerides for storage. The increased activity of this enzyme may be reflected in an increase in the rate of formation of the CoA thioesters of 2-PPA derivatives and perhaps the rate of inversion.

The inversion process assumes considerable clinical importance, because, with the exception of naproxen, which is used clinically as its S(+)-enantiomer, the profens are currently marketed as racemic mixtures. (Note: it appears from an investigational report in the literature, that flunoxaprofen is also used as its S(+)-enantiomer; Pedrazzini et al 1987). For each of the profens, the desired pharmacological effects reside almost exclusively with the S(+)-enantiomer (Hutt & Caldwell 1983). Hence, for ibuprofen and fenoprofen, at least, a significant proportion of the therapeutic and/or toxic effects arise from the S(+)-enantiomer generated from its inactive precursor. Variability in the extent of inversion may contribute to the variability, both within and between patients, in clinical responses to these drugs (Williams & Day 1988).

3.5.2. Incorporation of Ibuprofen into Triglycerides

In 1978, Fears et al discovered that the free acid of 4-benzyloxybenzoate (BRL 10894), an experimental hypolipidaemic agent, participated in glycerolipid metabolism in rats. The agent could be incorporated into triglycerides and inhibit fatty acid synthesis from acetyl CoA. Subsequently, other compounds containing carboxylic acid moieties, including ibuprofen, fenoprofen and ketoprofen, were found to be incorporated into triglycerides in a similar manner to BRL 10894 (Fears et al 1978). This finding may explain, in part, why Adams et al (1971) found that adipose tissue in rats represented a long-lasting store of ibuprofen, an acidic compound which is extensively ionized at physiological pH. Fears et al (1978) proposed that a CoA thioester was a pivotal intermediate for the incorporation process. On this point, it is of interest to note that the rank order of the ability of the profens tested to be incorporated into triglycerides (fenoprofen > ibuprofen > ketoprofen >> flurbiprofen; Fears et al 1978) correlates well with the extent of chiral inversion of these compounds (Hutt & Caldwell 1983). Ibuprofen was also found to inhibit cholesterolgenesis from acetate, and fatty acid synthesis, and it was presumed that these effects were due to an ibuprofen-mediated depletion of coenzyme A pools (Fears & Richards 1981)

By 1981, it was suspected that only the R(-)-enantiomer of ibuprofen could form a thioester with coenzyme A (Nakamura et al 1981) Therefore, if Fears and coworkers' postulate was correct (i.e. that incorporation of these compounds into triglycerides proceeded via a CoA thioester intermediate), the incorporation of ibuprofen into triglycerides should be specific for the R(-)-enantiomer. That this was the case was demonstrated by Williams et al (1986). Racemic ibuprofen, R-I and S-I were fed to rats for seven days, after which the concentration of ibuprofen enantiomers in adipose tissue was assessed. After administration of R-I, and of racemic ibuprofen, both ibuprofen enantiomers were found in adipose tissue. However, after administration of S-I, the ibuprofen content of adipose tissue was negligible. Hence, the incorporation into adipose tissue was specific for R-I. It would appear, therefore, that enantioselectivity is exhibited at the CoA thioester formation step. Once formed, the CoA thioester of R-I could either undergo hydrolysis; be incorporated into triglycerides; or undergo chiral inversion to the CoA thioester of S-I. This thioester of S-I could then undergo hydrolysis or incorporation into triglycerides. *In vitro* experiments in both rat hepatocytes and adipocytes, indicated the presence of a high affinity metabolic process for the synthesis of triglycerides containing fenoprofen, a process that was specific for R(-)-fenoprofen (Sallustio et al 1988). Hence, the findings of Williams et al (1986) and Sallustio et al (1988), are consistent with Fears and coworkers' postulate.

The clinical and toxicological significance of the incorporation of 2-PPAs into triglycerides is unknown. Caldwell & Marsh (1983) speculated that some potential consequences included: "establishment of tissue residues of xenobiotics; disturbances of lipid biochemistry; and altered membrane function." Certainly, by using the S(+)-enantiomer only, this incorporation process could be avoided, and the potential for related toxic effects may be abated.

3.5.3. Summary

The metabolic pattern of racemic ibuprofen, based on current knowledge reviewed in this section, is summarized in Figure 3.3. Both enantiomers of ibuprofen are metabolized by oxidation of the isobutyl function and, to a lesser extent, by glucuronidation. The majority of an orally administered dose of ibuprofen can be recovered in the urine in the form of these metabolites. The R(-)-enantiomer of ibuprofen is a substrate for acyl CoA synthetase which catalyses the formation of a thioester between it and coenzyme A. It is thought that through this pivotal intermediate, R-I can undergo chiral inversion to its pharmacologically active mirror-image form, or can be combined with naturally occurring glycero-lipids to form "hybrid-triglycerides".

These hybrid-triglycerides, stored mainly in adipose tissue, may act as long-lasting tissue stores of R-I and, therefore, S-I. Hence, after administration of racemic ibuprofen, the plasma concentration of the pharmacologically active enantiomer (S-I) at any particular time, is dependent upon a large number of integrated processes.

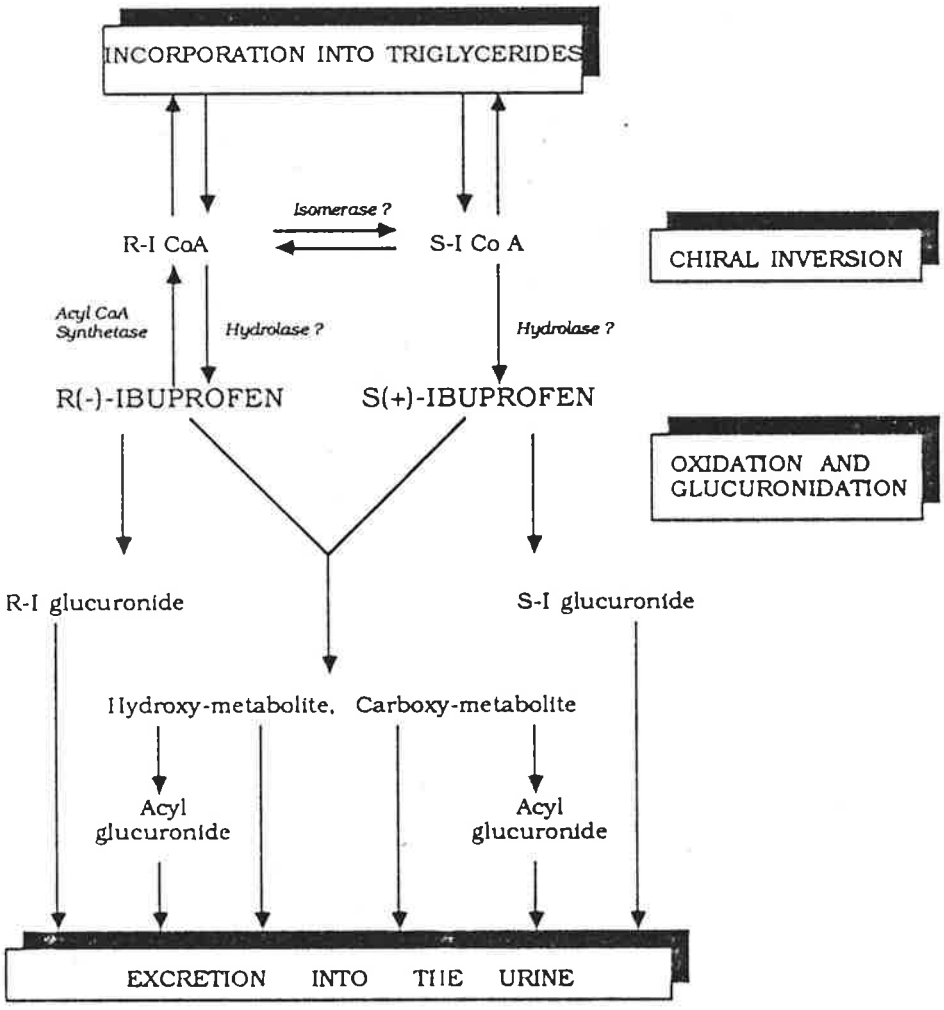


Figure 3.3 Schematic representation of the metabolic profile of R-I and S-I in humans, as discussed in section 3.5.

3.6. THE PHARMACOKINETICS OF IBUPROFEN

The majority of pharmacokinetic studies on ibuprofen have been performed after the administration of the racemate, and have relied on non-enantioselective methods for measuring the concentration of ibuprofen in biological samples. Clearly, since S-I is the pharmacologically active enantiomer, studies conducted in such a manner are of extremely limited use. In the following review of the pharmacokinetics of ibuprofen, greater emphasis will be placed, where possible, on pharmacokinetic data which have been generated for the individual enantiomers.

3.6.1. Gastrointestinal Absorption

Ibuprofen is administered, almost exclusively, by the oral route. After oral administration of an alkaline solution of racemic ibuprofen to four healthy male volunteers, Lee et al (1985) found that the absorption of both enantiomers was rapid, with peak plasma concentrations being achieved within 30 minutes. The plasma concentrations of the individual enantiomers attained during this period were almost identical, suggesting that there was no enantioselectivity in the rate of absorption (Lee et al 1985). When conventional high quality tablets of ibuprofen are administered to fasting individuals, the peak plasma concentration (C_{max}) of unresolved ibuprofen (UNR-I) is usually achieved (t_{max}) within 2 to 3 hours of ingestion (Collier et al 1978; Gillespie et al 1982; Grennan et al 1983; Kaiser & Vangiessen 1974; Kallstrom et al 1988; Lockwood et al 1983a; Muller et al 1986).

The most commonly used method for determining the oral bioavailability of a drug is to compare the area under the plasma drug concentration-time profile from time zero to infinity (AUC) following oral dosing with that after intravenous dosing (Rowland & Tozer 1989). Recently, Cox (1988) reported, in a preliminary communication, that the AUC of R-I and that of S-I after oral administration of RS-I (600mg) were identical to the corresponding values found after the same dose was administered intravenously. Hence, the bioavailability of ibuprofen was complete, although the form in which the oral ibuprofen was administered was not specified. The data of Cox (1988) indicate also that there is negligible metabolism of R-I and S-I during their first-pass through the liver, suggesting that the hepatic clearance of both enantiomers is "capacity-limited". There are no other reports on the absolute bioavailability of ibuprofen enantiomers.

Because the majority of an orally administered dose of ibuprofen can be recovered in the urine, as unchanged drug plus metabolites (Lockwood et al 1983a; see section

3.5), the total urinary recovery serves as a useful index of the extent to which the drug is absorbed into the hepatic portal vein after oral dosing. Lockwood et al (1983a) found that after oral administration of 400mg of ibuprofen in solution (20mg/ml) to 15 healthy males, an average of 75.7 ± 5.03% of the administered dose was recovered in the urine within 48 hours, mainly as oxidized metabolites and their conjugates. After oral dosing with one, two and three 400mg strength ibuprofen tablets (MOTRIN ®), the mean total recoveries were 84.9 ± 3.02%, 77.7 ±3.92% and 77.4 ± 3.46%, respectively. The data indicate that after oral administration ibuprofen absorption is extensive, and independent of the administered dose. The high recovery also suggests that the absorption of both ibuprofen enantiomers is extensive, and dose-independent. It is likely that the small amount of ibuprofen which was unaccounted for, was eliminated as minor metabolites in the urine, or in the faeces, or stored in the form of hybrid triglycerides (section 3.5).

Variability has been found in the rate and/or estimated extent of absorption of ibuprofen from commercial and experimental products (Gillespie et al 1982; Kallstrom et al 1988; Luan et al 1987; Muller et al 1986; Stead et al 1983). In all of these cases, ibuprofen absorption was assessed by measuring ibuprofen concentrations in plasma using non-enantioselective analytical methods. Two recent studies have used enantioselective techniques to examine the relative bioavailability of R-I and S-I after oral administration of various preparations of racemic ibuprofen (Cox et al 1988; Jamali et al 1988b). Cox et al (1988), for example, compared the pharmacokinetics of R-I and S-I after oral dosing with MOTRIN® 400mg and a 400mg generic ibuprofen preparation. For MOTRIN®, the absorption of both enantiomers was rapid, while for the generic product absorption of both enantiomers was delayed. Estimates of the relative extent of absorption of the compounds could not be made because the time over which plasma concentrations were collected was not suitable for the generic product, owing to its slower absorption rate. It should be stressed that the method of assessing bioavailability by comparison of AUC data for total (bound plus unbound) drug assumes that drug clearance remains constant. The clearance of a restrictively cleared drug, such as ibuprofen, is dependent upon the unbound fraction of the drug in plasma as well as its intrinsic clearance (Wilkinson & Shand 1975). Because of the non-linear plasma protein binding of ibuprofen over the therapeutic range (see section 3.6.2), the possibility exists that the time-averaged unbound fraction of ibuprofen (and of its enantiomers) after administration of a rapid-release product, may be greater than after administration of a product of similar strength, but for which drug absorption is more gradual. Therefore, the clearance of ibuprofen may not be constant for two products having different release characteristics.

The available evidence indicates that after oral administration of conventional

dosage forms of racemic ibuprofen, both enantiomers are rapidly and extensively absorbed. Although erratic absorption has been encountered with some generic products, the gastrointestinal absorption of ibuprofen from the popular proprietary preparations, MOTRIN® and BRUFEN®, is very reliable (Gillespie et al 1982; Kallstrom et al 1988; Lockwood et al 1983a; Luan et al 1987; Muller et al 1986).

3.6.2 Distributional Processes

Plasma Protein Binding

All of the 2-phenylpropionic acid drugs are extensively bound to plasma proteins, with the the major binding protein being albumin (Lin et al 1987). A number of groups have assessed the binding of ibuprofen to human plasma. In those studies where the binding of ibuprofen has been investigated in plasma obtained from normal volunteers, ibuprofen has been found to be 98 to 99% bound (Aarons et al 1983b; Abernathy & Greenblatt 1985; Greenblatt et al 1984; Grennan et al 1983; Lockwood et al 1983a,1983b; Mills et al 1973). The studies of Aarons et al 1983b and Lockwood et al 1983a,b also suggest that over the therapeutic concentration range, the plasma protein binding of ibuprofen is non-linear. There are, however, a number of important methodological problems with the techniques used to determine the plasma protein binding of ibuprofen in these studies, as discussed in Chapter 2.

Ibuprofen binds predominantly to a single high-affinity site and a number of low affinity sites on the human serum albumin (HSA) molecule (Whitlam et al 1979). The binding of ibuprofen to both classes of binding site was reduced considerably by palmitate, a representative fatty acid, and to a lesser extent by uric acid, bilirubin and cholesterol. The albumin molecule has a number of identified binding sites (Lin et al 1987). Using purified albumin, immobilized in microparticles, Kober & Sjoholm (1980), and Sjoholm et al (1979) found that ibuprofen was bound primarily to the diazepam site (site II). However, ibuprofen was also able to displace digitoxin and warfarin (site I) from HSA, although the extent of displacement was not great.

The high degree of plasma protein binding of ibuprofen indicates that any difference in the extent of binding of the individual enantiomers is minor. Not surprisingly, Hansen et al (1985), in a preliminary communication, reported that there was no significant difference between R-I and S-I in their displacement of site I and site II specific albumin probes. However, even a small difference between R-I and S-I in fraction bound to plasma proteins may be reflected in a substantial difference in their respective unbound fractions. Interestingly, Hansen et al (1985) also found that between plasma concentrations of 2 and 100mg/L, the binding of both enantiomers was

concentration-dependent and, on average, the unbound concentration of S-I over this concentration range was 70% greater than for R-I, although binding values were not given.

It is clear that very little is known about the plasma protein binding of the individual ibuprofen enantiomers, the main reason being the difficulty involved in developing a reproducible method which is both enantioselective, and highly sensitive. The development and application of such a method will be discussed in subsequent chapters.

Distribution into Synovial Fluid

It is generally considered that the synovium is the site of analgesic and anti-inflammatory action of non-steroidal anti-inflammatory drugs in the treatment of articular rheumatic conditions (Day 1988; Day et al 1987; Graham 1988). It is also widely accepted that only unbound drug is available for interaction with the biological receptors. It therefore follows that the antirheumatic activity of NSAIAs should be closely related to their unbound concentrations within synovial fluid. A number of studies have investigated the distribution of ibuprofen into synovial fluid.

Glass & Swannell (1979) administered 400mg of ibuprofen to 21 arthritic patients who required diagnostic or therapeutic knee aspirations. The concentration-time profile of ibuprofen in plasma and synovial fluid, constructed by pooling data from 26 samples obtained from the 21 patients, indicated that ibuprofen entered synovial fluid, but the concentrations achieved were less than in plasma. In 1981 Whitlam et al examined the distribution of ibuprofen into synovial fluid. Ibuprofen (400mg) was given orally three times a day for two days to patients with rheumatic disorders. After the final dose a single sample of serum and synovial fluid was collected almost simultaneously, at unspecified times. The total concentration of ibuprofen in joint fluid was, in most cases, about half of that in serum, while the unbound concentrations of ibuprofen in both compartments were similar. Because the concentration of albumin in synovial fluid was, on average, one third of that in serum, the authors postulated that albumin binding in serum and synovial fluid is an important determinant of the trans-synovial distribution of ibuprofen and suggested, furthermore, that at steady-state, the unbound serum concentration provides a reasonably good estimate of unbound concentration in synovial fluid (Whitlam et al 1981).

Wanwimolruk et al (1983) also found that the binding of ibuprofen in synovial fluid was significantly lower than in plasma, and again the difference in binding was attributed, in part, to the lower albumin concentrations within the synovium.

In patients receiving multiple oral dosing with ibuprofen, Gallo et al (1986), and

Makela et al (1981) found that the total concentrations of the drug in synovial fluid, over a dosage interval, fluctuated far less than in plasma. In both cases, the peak level in synovial fluid was less than, and occurred later than, the peak level in plasma.

In a recent study, Day et al (1988) used an enantioselective assay to monitor the time course of total R-I and total S-I in plasma and synovial fluid in 8 rheumatic patients after oral dosing with racemic ibuprofen. The following findings were reported: both enantiomers entered synovial fluid, although at all time points the synovial fluid concentrations of S-I exceeded those of R-I; the concentrations of both enantiomers in synovial fluid fluctuated far less than in plasma; and, there was no difference in the mean equilibration time (the time at which plasma and synovial fluid concentrations are identical) of R-I and S-I (5.4 ± 0.3 hours and 5.5 ± 0.6 hours, respectively). After compartmental model analysis of the data, the authors calculated rate constants (and half-lives) for the movement of R-I and S-I into, and out of, synovial fluid. It was found that S-I entered synovial fluid at a greater rate than R-I, and it was speculated that this was due to differences in the unbound fraction of R-I and S-I in plasma. The mean elimination half-lives of R-I and S-I from synovial fluid into plasma were 2.6 and 2.1 hours, respectively, and were, in all but one case, shorter than the respective half-lives of the transfer from plasma into synovial fluid. The authors suggested that R-I and S-I diffused into synovial fluid primarily in the unbound form. The mechanism of movement of R-I and S-I out of the synovial fluid was less clear. The faster rate of transfer out of, than into, synovial fluid suggested that the outward movement of the enantiomers may, in part, be in the protein bound form (Day et al 1988).

The relatively minor fluctuations in the synovial fluid concentrations of ibuprofen after oral dosing (Day et al 1988; Gallo et al 1986; Makela et al 1981) may contribute to the clinical finding that a fixed daily dose of ibuprofen, divided into two or four doses, produces equivalent antirheumatic efficacy (Brugueras et al 1978; Pownall & Pickvance 1986).

Distribution into Adipose Tissue

From a pharmacokinetic viewpoint, the contribution of distribution of R-I and S-I into adipose tissue, in the form of hybrid triglycerides, is unknown. Since the majority of an administered dose of the drug can be recovered in the urine as oxidized and conjugated metabolites within 24 hours of dosing (Lockwood & Wagner 1982), the total quantity of the drug which enters adipose tissue is probably minor, at least after single-dose administration. However, during chronic ibuprofen administration, accumulation of the drug in adipose tissue could be appreciable and may generate a long-lasting reservoir of R-I and S-I. It would be expected that the elimination of

ibuprofen from these adipose stores would be slow, which may be detected as slow terminal elimination rates of the enantiomers. That these slow elimination rates have not been detected may be due to the limited sensitivity of the analytical methods used to measure R-I and S-I in plasma, and the short duration of blood sample collection after ibuprofen dosing.

Volume of Distribution

Assuming complete bioavailability of ibuprofen enantiomers, Lee et al (1985) used statistical moment analysis to determine the volume of distribution at steady state (V_{ss}) of R-I and S-I. After oral dosing with R-I, the mean V_{ss} of this enantiomer in four healthy volunteers was 9.9 ± 0.62 L. After dosing with S-I, the mean V_{ss} of S-I was 10.5 ± 1.49 L. After dosing with racemic ibuprofen V_{ss} of R-I was 11.5 ± 2.25 L. However, because of the complication of chiral inversion, V_{ss} of S-I could not be determined after dosing with either the racemate or R-I. It appears, from this study at least, that the volumes of distribution of both enantiomers are small and similar in magnitude.

Numerous studies have estimated the volume of distribution of unresolved ibuprofen after oral dosing with the racemate. In all of these cases the apparent oral clearance (Dose/AUC) was divided by the terminal elimination rate constant, to determine an apparent volume of distribution. The majority of estimates, not normalized to body weight, fall between 10 and 12 L (Albert et al 1984; Greenblatt et al 1984; Ochs et al 1985b). In healthy adults, Lockwood et al (1983a) found the apparent volume of distribution of ibuprofen to be independent of dose between 400 and 1200mg. However, because unresolved ibuprofen was measured in all of these studies, the methods used to derive these volume estimates were invalid.

In summary, the available data indicate that ibuprofen, like other NSAIAs (Lin et al 1987), has a relatively small volume of distribution, and it appears that there are no substantial differences between the distribution volumes of the individual enantiomers.

3.6.3. Clearance

Less than 1% of an orally administered dose of ibuprofen is eliminated unchanged in the urine, whereas a large portion is eliminated as ibuprofen metabolites (see section 3.5). Hence, the drug is eliminated mainly by non-renal routes; i.e. total clearance (CL) is approximated by metabolic clearance (CL_M). After oral administration, drug clearance

can be estimated using the following equation (Rowland & Tozer 1989).

$$CL = \frac{f\,D}{AUC} \qquad (3.1)$$

Where f is the fraction of the administered dose (D) which is systemically available and AUC is the area under the plasma concentration time curve from time zero to infinity. In most cases f is not known, and therefore an apparent oral clearance (CL_O), assuming f to be unity, is usually reported.

Lee et al (1985) estimated CL_O of R-I, after the oral administration of R-I (400mg) to four healthy volunteers, to be 67.9 ±12.3 ml/min. The CL_O of S-I, after oral administration of S-I (400mg) was 74.4 ± 16.7 ml/min. After oral dosing with racemic ibuprofen (800mg) the mean CL_O of R-I (87.6 ± 26.9 ml/min) exceeded that determined after dosing with R-I. This was explained as being due to the fact that when the racemate was administered, the higher total dose of ibuprofen may have led to a higher time-averaged unbound fraction of R-I. After dosing with the racemate, the fraction of S-I which arose from inversion of R-I could not be determined and, therefore, it was not possible to estimate CL_O of S-I.

Cox (1988) reported the mean AUC of R-I in 12 healthy males, after i.v. dosing with 600mg of RS-I, to be 59 mg.h/L. This corresponds to a CL of about 85 ml/min, which is in good agreement with the CL_O reported by Lee and coworkers after oral dosing with 800mg of RS-I. Cox (1988) also reported that the fraction of R-I inverted to S-I was 0.62 after the 600mg dose of RS-I. Using these data, together with the mean AUC for S-I (89 mg.h/L), the CL of S-I can be calculated to be 91 ml/min.

Data on the clearance of R-I and S-I in humans via the glucuronidation route have been provided by the study of Lee et al (1985). After dosing with R-I, the mean clearance of R-I by glucuronidation, determined by multiplying the apparent oral clearance of R-I by the fractional amount of ibuprofen recovered in urine as its glucuronide conjugate, was 1.1 ± 0.37 ml/min. After dosing with S-I, the mean clearance of S-I by glucuronidation was substantially greater (9.1 ± 1.9 ml/min). The magnitude of the difference of the two clearance values was too great to be accounted for by enantioselective plasma protein binding. As alluded to by the authors, an apparent enantioselectivity in glucuronidation clearance may arise from a difference between the enantiomers in either the rate of formation of their glucuronide conjugates, and/or from differences in the *in vivo* stability of the diastereomeric acyl-conjugates,

once formed. Interestingly, El Mouelhi et al (1987) found that when racemic ibuprofen was incubated with human liver microsomes, the production of the S-I glucuronide, exceeded that of R-I by an average of 64%.

The apparent oral clearance of unresolved ibuprofen in healthy volunteers, after dosing with the racemate, has been reported to be in the range of 50 to 70 ml/min (Abernethy & Greenblatt 1985; Greenblatt et al 1984; Lockwood et al 1983a; Ochs et al 1985b).

3.6.4. Extent of Inversion of R(-)-Ibuprofen

The fraction of R-I which was metabolically inverted to S-I (FI) after the administration of R-I alone, was estimated by comparing the AUC of S-I after oral dosing with R-I, to the AUC of S-I after oral dosing with S-I, using the following equation (Lee et al 1985),

$$FI = \frac{\text{AUC of S-I after dosing with R-I} \times \text{Dose of S-I}}{\text{AUC of S-I after dosing with S-I} \times \text{Dose of R-I}} \qquad (3.2)$$

In the four healthy subjects studied the mean FI was 0.63 ± 0.06. However, one of the assumptions of this approach is that the clearance of S-I remains constant between the two doses. The plasma concentrations of S-I after dosing with S-I were greater than those achieved after dosing with R-I. Since the plasma binding of ibuprofen appears to be concentration-dependent (section 3.6.2) it follows that the time-averaged unbound fraction of S-I, (and therefore its clearance) may have been greater after dosing with S-I. Alternatively, the concurrent presence of R-I and S-I in plasma after dosing with R-I could also have increased the time-averaged unbound fraction of S-I. Hence, it should be recognised that in the absence of plasma protein binding data, equation 3.2 only provides an approximate estimate of FI.

Cox (1988) reported, in an abstract, that when 12 healthy volunteers were given an oral dose of RS-I (600mg), an average of 62% of R-I was inverted to S-I. It was stated that the fraction inverted was calculated after enantioselective analysis of ibuprofen and its metabolites in the urine. This approach may be complicated, firstly, by the fact that 100% of the administered dose of ibuprofen can not be recovered from urine, and secondly, by the possible chiral inversion of ibuprofen metabolites after their formation. For example, it is unknown whether all of the S(+)-hydroxy-metabolite of ibuprofen isolated from urine was formed solely by oxidation of S-I or, in part, from the chiral inversion of the R(-)-hydroxy-ibuprofen. Interestingly, chiral inversion has

been reported for the monohydroxylated metabolite of loxaprofen (Nagashima et al 1984)

3.6.5. Terminal Half-Life

After oral administration of racemic ibuprofen to humans, the $t_{1/2}$ of unresolved ibuprofen has been estimated, in most cases, to be between 1 and 3 hours, representing a terminal rate constant of 0.231 to 0.693 h^{-1}(Abernethy & Greenblatt 1985; Collier et al 1978; Greenblatt et al 1984; Juhl et al 1983; Lockwood et al 1983a; Ochs et al 1985b).

Very few studies have reported the elimination half-lives of the individual ibuprofen enantiomers. In the study of Lee et al (1985), the $t_{1/2}$ of R-I and S-I, after oral dosing with racemic ibuprofen, was 1.67 ± 0.35 and 2.5 ± 0.2 hours, respectively. Jamali et al (1988b) reported the $t_{1/2}$ of R-I and S-I in healthy volunteers, after the oral administration of four racemic ibuprofen products having different absorption characteristics. The mean $t_{1/2}$ for the four products ranged from 2.02 to 2.84 hours for R-I and from 2.21 to 2.63 hours for S-I, with no significant differences being detected between the enantiomers. After oral administration of a drug it is not possible to positiveley identify the terminal portion of the plasma concentration-time profile as an absorption or elimination phase (Rowland & Tozer 1989). However, Cox (1988) administered an i.v. dose of RS-I to healthy volunteers and reported elimination rate constants, for R-I and S-I, of 0.58 and 0.42 h^{-1}, respectively. These values correspond to elimination half-lifes of 1.19 and 1.65 hours, respectively, which are similar in magnitude to the terminal half-lives reported by Lee et al (1985) after oral administration of racemic ibuprofen.

3.6.6. Factors Affecting the Pharmacokinetics of Ibuprofen

Hepatic and Renal Disease

It is well known that alterations in hepatic and renal function can influence the pharmacokinetics of xenobiotics. Ibuprofen is cleared predominantly by metabolism, the assumed site of which is the liver, while its metabolites are predominantly excreted by the renal route (see section 3.5). One may anticipate, therefore, that hepatic disease may alter the pharmacokinetics of ibuprofen while renal disease may lead to the accumulation of ibuprofen metabolites.

Juhl et al (1983) examined the disposition of oral ibuprofen (400mg) in 15 patients with alcoholic liver disease, and in 29 control subjects. On the basis that no substantial

difference was detected in the AUC of unresolved ibuprofen between the two groups, it was concluded that alcoholic liver disease had little effect on ibuprofen pharmacokinetics. A major limitation of this study was that the effect of hepatic disease on ibuprofen plasma protein binding, or on the AUC of unbound ibuprofen, was not examined. The plasma binding of many drugs is decreased markedly in individuals with liver disease, due to either a decreased synthesis of binding proteins (hypoalbuminaemia), or accumulation of endogenous binding inhibitors (Lin et al 1987; Wilkinson 1986). More importantly, however, the effect of liver disease on the pharmacokinetics of the individual ibuprofen enantiomers was not examined.

The major metabolites of ibuprofen are reported to be inactive (Adams et al 1969a,b). However, this is only with respect to anti-inflammatory and analgesic activity, and clearly, they may possess alternative pharmacological properties such that in patients with renal disease, the accumulation of ibuprofen metabolites may be clinically important. Even if they are pharmacologically inactive, their presence may influence the binding of R-I and S-I to plasma and tissue binding sites.

Ibuprofen is not significantly removed from the body by haemodialysis (Antal et al 1986; Ochs et al 1985b), which is consistent with its high degree of plasma protein binding. Ochs et al (1985b) found that the $t_{1/2}$ and CL_O of unresolved ibuprofen, given as a single oral dose (600mg), did not differ between 8 male patients with renal insufficiency requiring long-term maintenance haemodialysis, and 10 age-matched healthy volunteers. The renal patients had a lower peak ibuprofen level and a longer time to peak than the controls and the authors suggested that ibuprofen was more slowly absorbed in patients with renal failure. However, the renal patients received haemodialysis shortly after ibuprofen dosing. The haemodynamic consequences of haemodialysis may have led to a reduced gastrointestinal blood flow, and therefore a slower rate of absorption. The unbound fraction of ibuprofen, determined in a pooled serum sample from each subject, was significantly higher in the renal patients ($3.08 \pm 0.67\%$ versus $1.07 \pm 0.04\%$), which is consistent with other reports indicating that the plasma protein binding of NSAIAs is reduced in patients with renal failure (Lin et al 1987). This decreased binding may be due to the build up of high-affinity, non-dialysable endogenous inhibitors of binding and/or from the altered primary structure of the albumin molecule in uraemic patients (Lin et al 1987). The apparent oral clearance of unbound ibuprofen in each subject was determined using that subject's apparent oral clearance for total drug and the single estimate of unbound fraction. Although the renal patients had a lower unbound CL_O, the difference was not significant. Nevertheless, it was cautiously stated by the authors that the lowered

unbound clearance of ibuprofen in renal patients may lead to excess accumulation of the unbound drug upon multiple dosing and, as a result, a reduced ibuprofen dosage (25 to 50%) may be required. However, it is not valid, for a drug which demonstrates non-linear plasma protein binding, to estimate unbound clearance using one binding estimate only. This limitation, together with the complete failure to consider enantioselectivity, casts doubt on the scientific validity of the results.

Antal et al (1986) examined the pharmacokinetics of ibuprofen and its hydroxy- and carboxy-metabolite after multiple dosing with ibuprofen in seven functionally anephric individuals with normal liver function. Haemodialysis was performed during ibuprofen treatment. After 14 days of ibuprofen therapy (800mg three times daily) there was no accumulation of ibuprofen in plasma, although there was substantial accumulation of both metabolites. The mean plasma levels of hydroxy-ibuprofen and carboxy-ibuprofen on day 15 were 57 mg/L and 249 mg/L, respectively. Haemodialysis efficiently removed both metabolites and no adverse effects were experienced by the subjects.

The effect of renal disease on the pharmacokinetics of R-I and S-I is unknown. In experimental animals, the kidney has been found to be a potential site of inversion of some 2-PPA derivatives (see section 3.5.1), although its contribution to the overall inversion of R-I in humans remains to be determined. The extent of chiral inversion of 2-PPA derivatives for which acyl-glucuronidation is a major metabolic pathway, could theoretically be influenced by impaired renal function (Meffin et al 1986). For such a drug, decreased renal function may lead to the accumulation of the acyl-glucuronide conjugates. If the acyl-glucuronide of the R(-)-2-PPA derivative is relatively unstable *in vivo*, it may act as a constant source of the parent molecule, and, as inversion may continue, this will lead, eventually to a greater total extent of inversion of the R(-)-enantiomer.

Rheumatic Disease.

Day et al (1988) found that the mean (± SE) CL_O of R-I in 8 arthritic patients who had taken 800mg of racemic ibuprofen was 78 ± 6 ml/min., which is within the range of values determined in healthy subjects (Lee et al 1985). The apparent oral clearance of S-I could not be determined accurately because the extent of inversion of R-I to S-I was unknown. However, based on an assumed 60% inversion, the mean estimated CL_O of S-I in the arthritic patients, (99 ± 16 ml/min) was similar to that in healthy volunteers. The terminal half-lives of R-I (1.6 ± 0.3 hours) and S-I (2.3 ± 0.2 hours) and the times to reach C_{max} also compared well with the data generated for the enantiomers in healthy

subjects (Jamali et al 1988b; Lee et al 1985).

In a study which examined the pharmacokinetics of unresolved ibuprofen in arthritic patients (Aarons et al 1983a), the reported values for AUC, C_{max}, t_{max}, and $t_{1/2}$ did not differ in magnitude from those values which have been reported for healthy subjects. However, there is some evidence that the plasma protein binding of ibuprofen in patients with rheumatoid arthritis is less than in healthy subjects (Aarons et al 1983b). For example, at a concentration of 51 mg/L the mean unbound fraction of unresolved ibuprofen in normal plasma was 1.64 ± 0.07%, while in rheumatoid plasma the fraction unbound was 1.86 ± 0.05%. The difference was attributed to the lower albumin concentration in the patients with rheumatoid arthritis (Aarons et al 1983b). An alternative or additional mechanism could be the altered amino-acid composition of albumin in patients with rheumatoid arthritis (Lin et al 1987). A previous study (Wanwimolruk et al 1983) found no difference in the plasma binding of ibuprofen between patients with rheumatoid arthritis and healthy controls.

The Effect of Age on Ibuprofen Pharmacokinetics.

The majority of patients who receive ibuprofen are elderly, while most pharmacokinetic studies have been conducted in healthy young adult volunteers. Two studies have examined the influence of age on the pharmacokinetics of ibuprofen. Greenblatt et al (1984) administered a single oral dose of ibuprofen (600mg) to healthy young (20 to 44 years, 7 male and 10 female) and elderly (60 to 88 years, 11 male, 9 female) volunteers. Differences between young and elderly subjects of the same sex were examined. There was no difference between the young and elderly males in ibuprofen t_{max}, C_{max} or apparent volume of distribution. However, the mean (± SE) CL_O of ibuprofen in elderly males (0.86 ± 0.04 ml/min/kg) was marginally, but significantly, lower than in the young males (1.03 ± 0.05 ml/min/kg). The $t_{1/2}$ of ibuprofen in the elderly (2.43 ± 0.12 hours) was significantly longer than in the young males (1.92 ± 0.13 hours). For females there was no difference between age groups in any of the pharmacokinetic parameters determined. For every subject, a single plasma sample was obtained to determine the plasma protein binding of radiolabelled ibuprofen. The overall percentage unbound averaged 1.05%, and was unaffected by sex or age. Upon examination of the data it is apparent that for CL_O and $t_{1/2}$, at least, there is no difference between males and females of either age group.

Albert et al (1984a) compared the pharmacokinetics of ibuprofen in 17 healthy males and females between the ages of 65 and 78 years, with those in 15 healthy young

adult males. All volunteers were given three single oral doses of ibuprofen (400mg, 800mg and 1200mg) on separate occasions. No statistically significant age-related differences were found in the pharmacokinetic parameters of (unresolved) ibuprofen. Both the young and elderly groups demonstrated highly significant dose-dependent changes in CL_O and apparent volume of distribution for total ibuprofen which were claimed to result from non-linearity in the plasma protein binding of ibuprofen. In addition, the CL_O of unbound ibuprofen, for the elderly group, increased significantly between the 400mg and 1200mg doses.

Both Greenblatt et al (1984) and Albert et al (1984a) concluded that ibuprofen pharmacokinetics were only minimally affected by age, and Albert et al (1984a) suggested that, from a pharmacokinetic viewpoint, there is no need to adjust the dose of ibuprofen in elderly patients. However, once again, neither of these two studies (Albert et al 1984a; Greenblatt et al 1984) considered the pharmacokinetics of the individual enantiomers of ibuprofen and the complications which may arise from only measuring unresolved drug.

Although limited details were given, Hageman & Borin (1988), in a recent abstract, stated that there were no differences in the pharmacokinetics of either ibuprofen enantiomer between young and elderly volunteers during chronic oral administration of RS-I. The only exception was that the C_{min} (the plasma concentration at the end of a given dosage interval) tended to be higher for the elderly individuals.

3.6.7. Comment

Despite over 20 years of continual clinical use, very little data are available on the pharmacokinetics of the active species of ibuprofen, S-I, and the effect thereon of factors such as dose size, age, disease state and concurrent drug therapy. This is because most studies on the pharmacokinetics of ibuprofen have failed to consider the implications of the enantioselective pharmacodynamic and pharmacokinetic characteristics of the drug, even though most of these studies were performed well after the metabolic inversion pathway was described.

The remainder of this thesis will describe methods which were developed for measuring total and unbound plasma concentrations of R-I and S-I, and the application of these and other methods, to studies on various aspects of the clinical pharmacology of ibuprofen.

Chapter 4.
Analysis of Ibuprofen Enantiomers in Human Plasma and of Ibuprofen and its Major Metabolites in Human Urine.

4.1. ANALYSIS OF TOTAL R(-)- AND S(+)-IBUPROFEN IN HUMAN PLASMA.

4.1.1. Introduction

There is an abundance of assays in the literature for quantifying ibuprofen in plasma or serum. The two techniques which have been used most commonly are gas-liquid chromatography (GLC) (Heikkinen 1984; Hoffman 1977; Kaiser & Vangiessen 1974) and high-performance liquid chromatography (HPLC) (Albert et al 1984b; Ali et al 1981; Aravind et al 1984; Greenblatt et al 1983; Litowitz et al 1984; Lockwood & Wagner 1982; Minkler & Hoppel 1988; Shimek et al 1981). However, all of these assays are non-enantioselective, i.e. they measure the sum of the plasma concentrations of R-I and S-I (unresolved ibuprofen). For the reasons discussed in chapters 2 and 3, the pharmacokinetics of ibuprofen cannot be comprehensively examined unless the concentrations of the individual enantiomers in plasma are measured.

Because R-I and S-I have identical physico-chemical properties in a symmetrical environment, their chromatographic resolution and subsequent analysis requires the use of a chiral discriminant (see section 1.3). Various direct techniques have been used for the HPLC resolution of certain 2-PPA derivatives. For example, chiral stationary phases based on alpha$_1$-acid glycoprotein (Schill et al 1986), and ß-cyclodextrin (Armstrong et al 1986) have been found to resolve the enantiomers of ibuprofen and ketoprofen, respectively. Although Pirkle-type chiral HPLC columns have been used with success for resolving ibuprofen enantiomers (Nicoll-Griffith 1987; Wainer & Doyle 1984) adequate resolution has been achieved only after ibuprofen was derivatized with a secondary or tertiary amine, prior to chromatography. Recently, Pettersson & Gioeli (1988) reported a method for the resolution of naproxen enantiomers involving the use of a chiral mobile-phase additive (quinine or quinidine) together with a chiral

stationary phase (acetylquinidine-silica). While chiral counter-ion mobile-phase additives have permitted the resolution of certain chiral carboxylic acids, such as 2-phenoxypropionic acid, attempts to resolve 2-PPA derivatives have been less succesful (Pettersson 1984; Pettersson & No 1983; Pettersson & Schill 1986).

Overall, the application of the direct approach for the enantioselective determination of 2-PPA derivatives in biological fluid has been unsuccessful. The one exception, is a method for the analysis of ibuprofen enantiomers in human urine samples using a Pirkle-type chiral HPLC column (Nicoll-Griffith et al 1988). The ibuprofen enantiomers in this case were resolved as 4-methoxyanilides. The workers were able to quantify R-I and S-I in urine over the concentration range 6.25 to 100 mg/L. It appears, from the published chromatograms, that enantiomeric resolution was very marginal (Nicoll-Griffith et al 1988).

Because of the currently limited usefulness of the direct approach for the analysis of chiral compounds in biological fluids, the indirect technique has more commonly been used for such purposes. Chiral derivatizing agents which have been used for the chromatographic resolution of various 2-PPA derivatives are listed in Table 4.1.

Table 4.1. Chiral derivatizing agents which have been used for the indirect resolution of 2-PPA derivatives.

Chiral Derivatizing Agent	Reference
1. S(+)- or R(-)-amphetamine	Singh et al 1986
2. S-1-(4-dimethylamino-naphthalen-1-yl)ethylamine	Goto et al 1982; Nagashima et al 1985
3. L-leucinamide	Berry & Jamali 1988; Bjorkman 1985; Foster & Jamali 1987; Mehvar et al 1988b; Spahn 1987
4. L-leucine	Lan et al 1976
5. S(-)-1-(naphthen-1-yl)-ethylamine	Avgerinos & Hutt 1987; Hutt et al 1986; Mehvar et al 1988a
6. S(+)-2-octanol	Johnson et al 1979; Lee et al 1984
7. R(+)- or S(-)-1-phenylethylamine	Bopp et al 1979; Kemmerer et al 1979; Maitre et al 1984; Rossetti et al 1986;Sallustio et al 1986; Simmonds et al 1980; Stoltenborg et al 1981; Tamasia et al 1984; Tamura et al 1981; Tossolini et al 1974; Vangiessen & Kaiser 1975

The first method for the quantification of ibuprofen enantiomers in human plasma was reported in 1975 by Vangiessen & Kaiser. After benzene extraction of plasma, and a thin-layer chromatography (TLC) clean-up procedure, the ibuprofen enantiomers were reacted with S(-)-1-phenylethylamine to form diastereomeric amides. These diastereomers were then resolved by GLC and quantified by flame-ionization detection. In 1986, Singh et al reported a method which involved GLC resolution and nitrogen-phosphorus detection of the diastereomers formed from the derivatization of extracted ibuprofen with S(+)- or R(-)-amphetamine.

At the commencement of assay development in the present work, only one HPLC method had been documented for the determination of ibuprofen enantiomers in plasma. Lee et al (1984) described a method in which the diastereomeric S(+)-octyl esters of R-I and S-I were resolved using two silica columns in series, and quantified by UV detection. Subsequently, two other methods were reported in the literature. Avgerinos & Hutt (1987) and Mehvar et al (1988a) each detailed a HPLC method for quantifying ibuprofen enantiomers based on their derivatization with S(-)-1-(naphthen-1-yl)-ethylamine.

The method developed in the present work involved two separate assays. The first (Assay 1A) was a rapid method for determining the concentration of unresolved ibuprofen in a given sample of plasma, while the second (Assay 1B) was used to define the enantiomeric composition of ibuprofen in that same sample. The two methods, used in combination, provided a sensitive, specific and reproducible method for determining the concentration of R-I and S-I in plasma.

4.1.2. Materials and HPLC Equipment

R(-)-ibuprofen, S(+)-ibuprofen, 2-[4-(2-hydroxy-2-methylpropyl)phenyl]-propionic acid (i.e. "hydroxy-ibuprofen"), 2-[4-(2-carboxypropyl)phenyl]propionic acid (i.e. "carboxy-ibuprofen") and racemic radiolabelled ibuprofen (^{14}C-ibuprofen, specific activity 25μCi/mg) were all kindly donated by Boots Co. (Nottingham, United Kingdom), and S(+)-naproxen, was supplied by F.H.Faulding Pty. Ltd. (Adelaide, Australia). Ketoprofen and phenylacetic acid were kind gifts of Flinders University (Bedford Park, South Australia). S(-)-1-Phenylethylamine was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and thionyl chloride from Fluka AG (Switzerland). HPLC grade heptane, acetonitrile and methanol were purchased from Waters Associates (Milford, MA, U.S.A.) and dichloromethane (G.R.) and 1,1'-carbonyldiimidazole from Merck (Darmstadt, F.R.Germany). Isopropanol,

sulphuric acid (B.D.H. Chemicals, Port Fairy, Australia) and phosphoric acid (Fluka AG) were AR grade.

For all HPLC procedures a Waters Associates 501 HPLC pump, connected to a Waters U6K Universal HPLC injector was used to deliver mobile phase to a chromatographic column. When large sample numbers were being analysed, the manual injector was replaced with a Waters Intelligent Sample Processor (WISP model 712). The column eluent was monitored using a Waters Lamda Max 481 LC spectrophotometric detector, set at a wavelength of 216nm, the signal from which was plotted on a BBC SE 120 recorder. Reversed-phase chromatography was performed using an Alltech C_{18} column (250mm x 4.6 mm I.D., 10 micron particle size; Alltech, Homebush, Australia). Normal-phase HPLC was performed using a Hibar Lichrosorb Si60 column (250mm x 4mm I.D., 5 micron particle size; Merck, Darmstadt, F.R.Germany).

4.1.3 Methods

HPLC Conditions

Assay 1A: The mobile phase, which consisted of acetonitrile:water adjusted to pH 2.4 with phosphoric acid, in a ratio of 60:40, was pumped at a constant rate of 1.5 ml/min to an Alltech C_{18} chromatographic column producing a typical backpressure of 1.3×10^4kPa.

Assay 1B: The mobile phase (isopropanol: heptane, 2.5 : 97.5) was delivered to a normal-phase silica column (Hibar Lichrosorb Si60) at a constant rate of 2.0 ml/min and produced a typical backpressure of 1.4×10^4kPa.

Sample Preparation

A 500µl aliquot of plasma was placed into a 20ml screw-topped test tube along with 50µl of the internal standard solution (S(+)-naproxen, 50mg/L in methanol) and 200µl of 2M sulphuric acid. After the addition of 5ml of extracting solvent (isopropanol: heptane, 5:95) the tube was sealed with a PTFE-lined screw cap, and after a 30 sec vortex-mix the contents were rotary-mixed at 30 rpm for 10 min. After centrifugation (1000g for 5 min), the upper organic phase was removed and divided into two aliquots.

For Assay 1A, approximately 2ml of the organic phase was placed into a clean, dry 10ml tapered test tube and evaporated to dryness in a 45°C water bath under a

steady flow of purified nitrogen. The dried residue was reconstituted in 200µl of methanol and after brief vortex-mixing an aliquot (20-50µl) was injected onto the reversed-phase column for determination of unresolved ibuprofen.

For Assay 1B, approximately 2ml of the organic phase extract of plasma was placed into a 20ml screw-topped test tube and evaporated to dryness under the conditions outlined above. A 100µl aliquot of freshly prepared thionyl chloride solution (1% in dichloromethane) was added to the tube which was then sealed with a PTFE-lined screw cap, briefly vortex-mixed, and heated for 1 hour in a thermostatically controlled heating block set at 70°C. Subsequently, after 15 min at room temperature, a 500µl aliquot of a freshly prepared solution of S(-)-1-phenylethylamine (1% in dichloromethane) was added, after which the tube was recapped, briefly vortex-mixed, and left to stand at room temperature. After 20 min, 500µl of 2M sulphuric acid and 5 ml of heptane were added. The contents of the tube were vortex-mixed for 30 sec, rotary-mixed at 30 rpm for a further 10 min and then centrifuged (1000g, 5min). Most of the heptane phase was removed, placed into a clean, dry 10ml tapered test tube and evaporated to dryness as described previously. The dried residue was reconstituted in 200µl of Assay 1B mobile phase and, after brief vortex-mixing, an aliquot (20-50µl) was injected onto the normal-phase silica column for the determination of the enantiomeric composition of ibuprofen.

Calibration Procedures

Stock solutions of R-I (25mg/50ml) and S-I (25mg/50ml) were prepared in methanol. Calibration standards were prepared using appropriate dilutions of these methanolic solutions. Varying amounts of these solutions were added to a series of 20ml screw-topped test tubes such that when the methanol was evaporated, and 500µl of drug-free human plasma added, the total ibuprofen concentration ranged from 0.50mg/L to 100mg/L and the enantiomeric composition of ibuprofen ranged from 10% to 60% R-I. The calibration standards were carried through the preparative and chromatographic procedures together with the unknown plasma samples.

For Assay 1A, the ratio of the height of the peak representing ibuprofen to that of the internal standard was plotted against the concentration of ibuprofen to produce a calibration curve which was used to define the concentration of unresolved ibuprofen in unknown plasma samples. For Assay 1B, the height of the peak representing the diastereomeric amide of R-I was divided by the sum of the heights of the two peaks representing the amides of R-I and S-I, and this ratio [R-I/(R-I + S-I)] was plotted against the known enantiomeric composition of ibuprofen in the plasma sample

(expressed as the fraction of R-I). The resulting calibration curve was used to determine the enantiomeric composition of ibuprofen in unknown plasma samples.

The total concentration of unresolved ibuprofen, determined from assay 1A, was multiplied by the fraction of R-I in that same sample, determined from Assay 1B, to derive the plasma concentration of R-I. The difference between the total concentration of unresolved ibuprofen and the concentration of R-I was taken to represent the plasma concentration of S-I.

Assay Accuracy and Precision

Quality control specimens were prepared by, firstly, making methanolic solutions of R-I (25mg/50ml) and of S-I (25mg/50ml) from weighings independent of those used for preparing the calibration standards. Subsequently, appropriate amounts of these quality control methanolic solutions were spiked into drug-free human plasma to produce samples of known ibuprofen concentration and enantiomeric composition. Aliquots (500µl) of these plasma samples were analysed using the outlined methodology to assess the accuracy and precision of the assay.

4.1.4. Performance of the Method

A schematic summary of the method used to determine the concentration of ibuprofen enantiomers in human plasma, is presented in Figure 4.1.

Chromatography

Under the chromatographic conditions described for Assay 1A, naproxen and ibuprofen had approximate retention times of 4 and 7 min, respectively. Both peaks eluted well clear of normal plasma constituents (Figure 4.2). Both major metabolites of ibuprofen (hydroxy- and carboxy-ibuprofen) eluted well before the naproxen peak. The sensitivity of the system was such that a plasma ibuprofen concentration of 0.50 mg/L could be measured easily (signal to noise ratio in excess of 10:1) with the detector set on 0.10 A.U.F.S.

For Assay 1B, the diastereomeric amides of R-I and S-I had approximate retention times of 3 and 5 min, respectively. The individual diastereomers were positively identified by derivatizing and chromatographing the authentic samples of R-I and S-I and by subjecting the purified column eluents to mass spectrometry (mass-spectrometry was performed by Dr. Alan Duffield of The University of New South Wales, Australia). Both amides eluted well clear of normal plasma constituents (Figure 4.3.).

In addition, there was no interference caused by the Assay 1A internal standard, S(+)-naproxen. When plasma containing hydroxy-ibuprofen (25 mg/L) or carboxy-ibuprofen (25 mg/L) was placed through the preparative and chromatographic procedures outlined, there were no interfering peaks at the retention time of either ibuprofen amide. It should be noted that the three documented HPLC methods for the determination of ibuprofen enantiomers in human plasma (Avgerinos & Hutt 1987; Lee et al 1984; Mehvar et al 1988a) do not exclude the possibility of interference from the metabolites of ibuprofen. The carboxy-metabolite contains two chiral carbon atoms, both of which are covalently bonded to a carboxy group which may be derivatized. Therefore, this metabolite alone, when derivatized, may give rise to twelve different compounds. Interference by ibuprofen metabolites may be important in pharmacodynamic and pharmacokinetic studies involving ibuprofen, especially given the degree of metabolite accumulation which can occur in anephric patients (Antal et al 1986; see section 3.6.6). The sensitivity of Assay IB was such that at a plasma concentration of 0.25 mg/L, the peak representing each diastereomer could be measured (signal to noise ratio in excess of 10:1) with the detector set on 0.05 or 0.10 A.U.F.S.

The HPLC systems described for Assay 1A and Assay 1B were in operation for over three years using the original columns. Over this time hundreds of plasma samples were analysed and there was no deterioration in column performance and therefore no need for column regeneration, a problem encountered in an earlier HPLC method for measuring ibuprofen enantiomers (Lee et al 1984). Evidence of the long term stability of the normal-phase system is provided by the fact that the mean peak height ratio [R-I/(S-I+R-I)] of derivatized racemic ibuprofen, determined on ten separate occasions, over a two year period, was 0.648 with a coefficient of variation (CV) of 2.1%.

Calibration

A representative calibration curve for Assay 1A is presented in Figure 4.4. Calibration curves were linear over the range of concentrations examined and passed through the origin. A typical set of calibration data for Assay 1B, together with the corresponding calibration curve, are presented in Table 4.2 and Figure 4.5, respectively. The manner in which the Assay IB calibration data are plotted results in a curved calibration line. For routine analysis of R-I and S-I in plasma, a bivariate curve fitting programme was used to fit a power function (of the form $y = bx^m$) to the calibration data, and in all cases there was excellent correlation between predicted and experimental data (Table 4.2). For example, the calibration data depicted in Figure 4.5. fit the power equation $y = 1.08x^{0.760}$ with a correlation coefficient greater than 0.999.

For five calibration curves constructed independently over a four week period the mean values of b and m were 1.07 (CV = 1.8%) and 0.757 (CV=0.38%), respectively, and in all cases the correlation coefficients were 0.999.

Table 4.2. Representative Assay 1B calibration data.

Enantiomeric Composition R-I/(R-I + S-I)	Peak Height Ratio R-I/(R-I + S-I)	Predicted Enantiomeric Composition*	% Error
0.10	0.183	0.097	-3.0
0.20	0.328	0.209	+4.5
0.30	0.433	0.301	+0.3
0.40	0.546	0.408	+2.0
0.50	0.634	0.497	-0.6
0.60	0.716	0.583	-2.8

* Calculated from the equation $y = 1.08 x^{0.760}$

An alternative method of plotting the Assay 1B calibration data was to divide the height of the peak representing the amide of R-I by the height of the peak representing the amide of S-I, and plot this against the ratio of the concentrations of the two enantiomers (R-I divided by S-I). When the calibration data in Table 4.2 are plotted in such a manner, a linear calibration line results (Figure 4.6). It should be noted that the slope of this calibration line differed from unity because peak heights were measured. On one occasion, when an integrator was used to determine the area under the respective amide peaks, the slope of the calibration line relating peak area ratio (R-I/S-I) to enantiomeric ratio (R-I/S-I) was close to unity (1.02, r= 0.996).

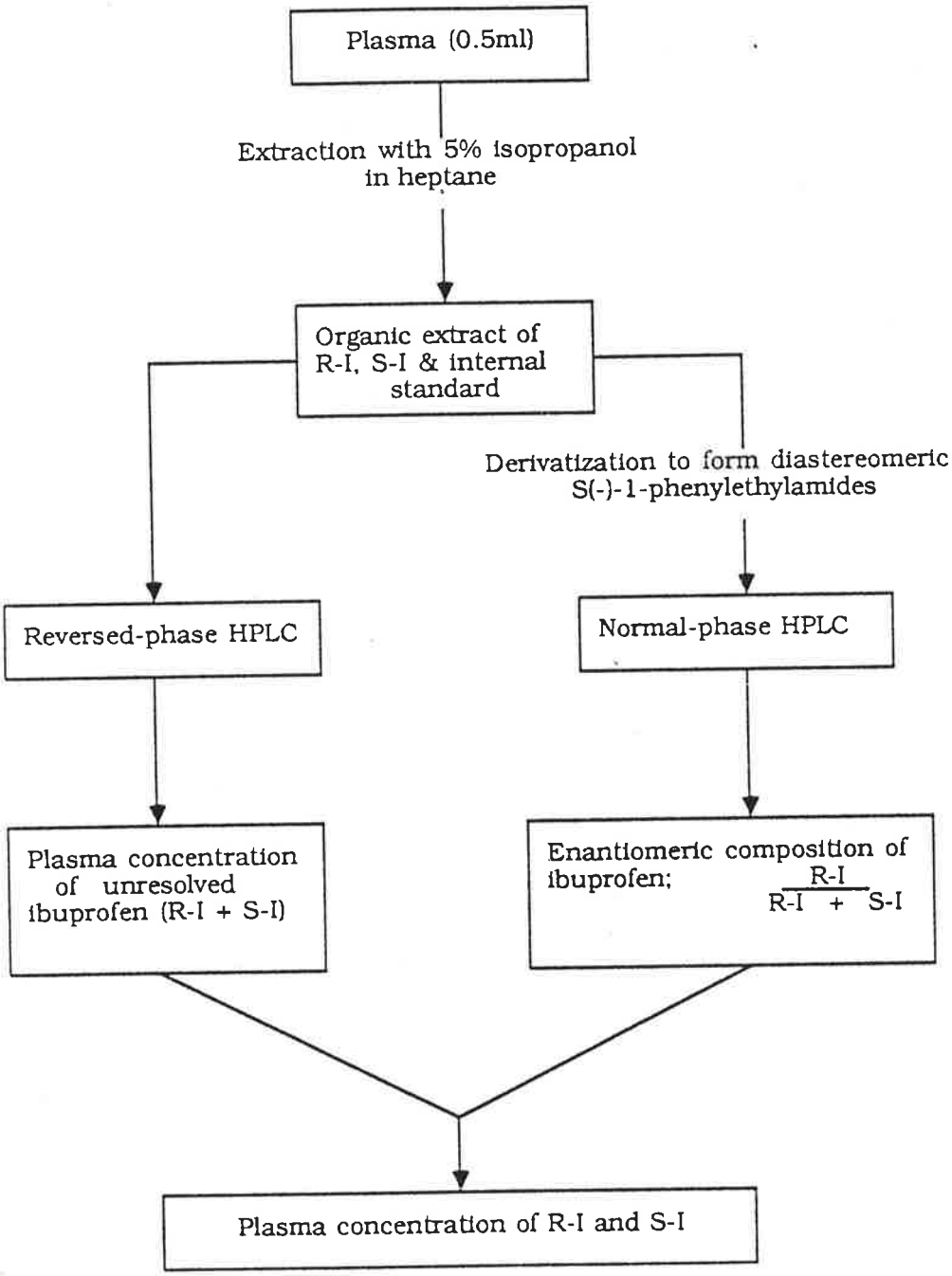


Figure 4.1. Schematic representation of the method used to determine the total (bound plus unbound) concentration of R(-)-ibuprofen, and of S(+)-ibuprofen in human plasma.

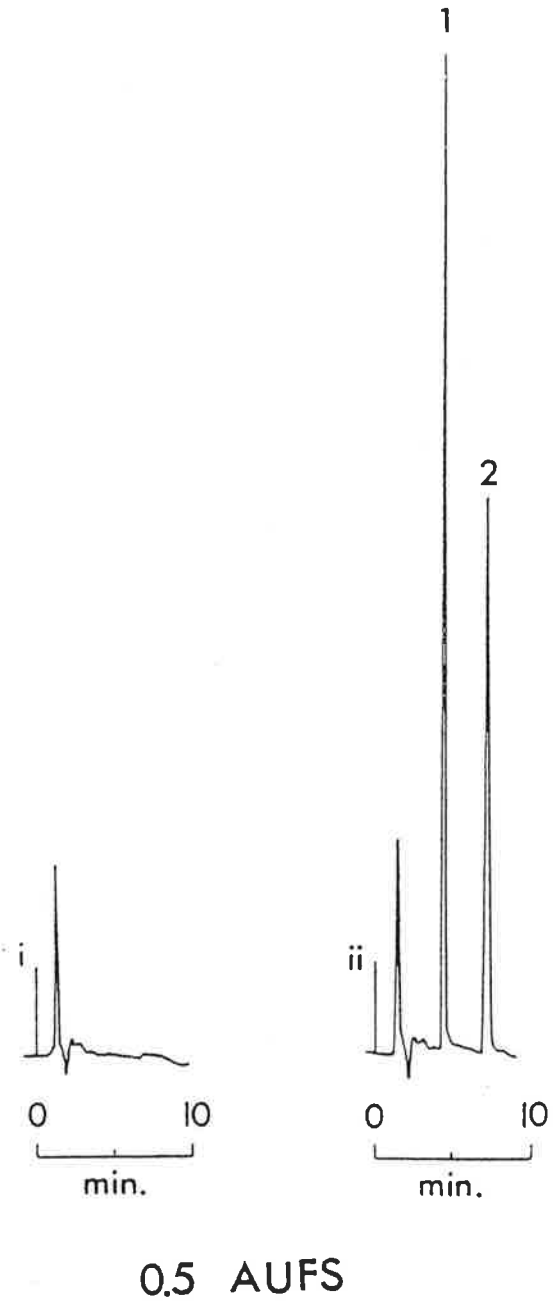


Figure 4.2. Assay 1A chromatograms of human plasma extracts: (i) drug free plasma; (ii) plasma containing 25mg/L of ibuprofen. Peak 1 represents the internal standard (naproxen), and peak 2 represents ibuprofen.

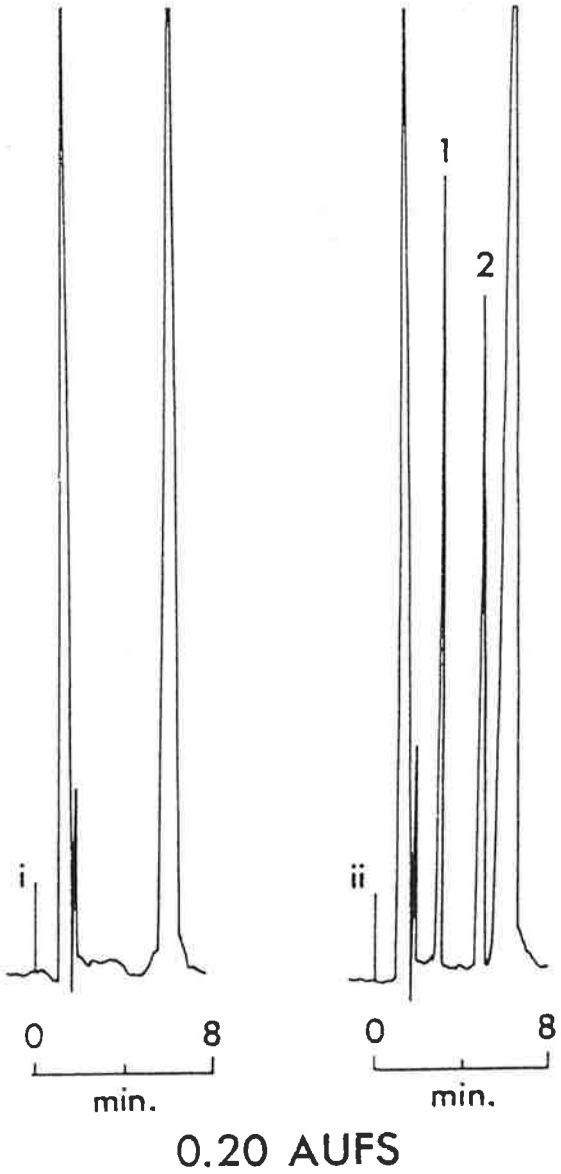


Figure 4.3. Assay 1B chromatograms of derivatized human plasma extracts: (i) drug-free plasma; (ii) plasma containing 8mg/L of R(-)-ibuprofen and 12mg/L of S(+)-ibuprofen. Peaks 1 and 2 represent the diastereomeric amides of R(-)-ibuprofen and of S(+)-ibuprofen, respectively.

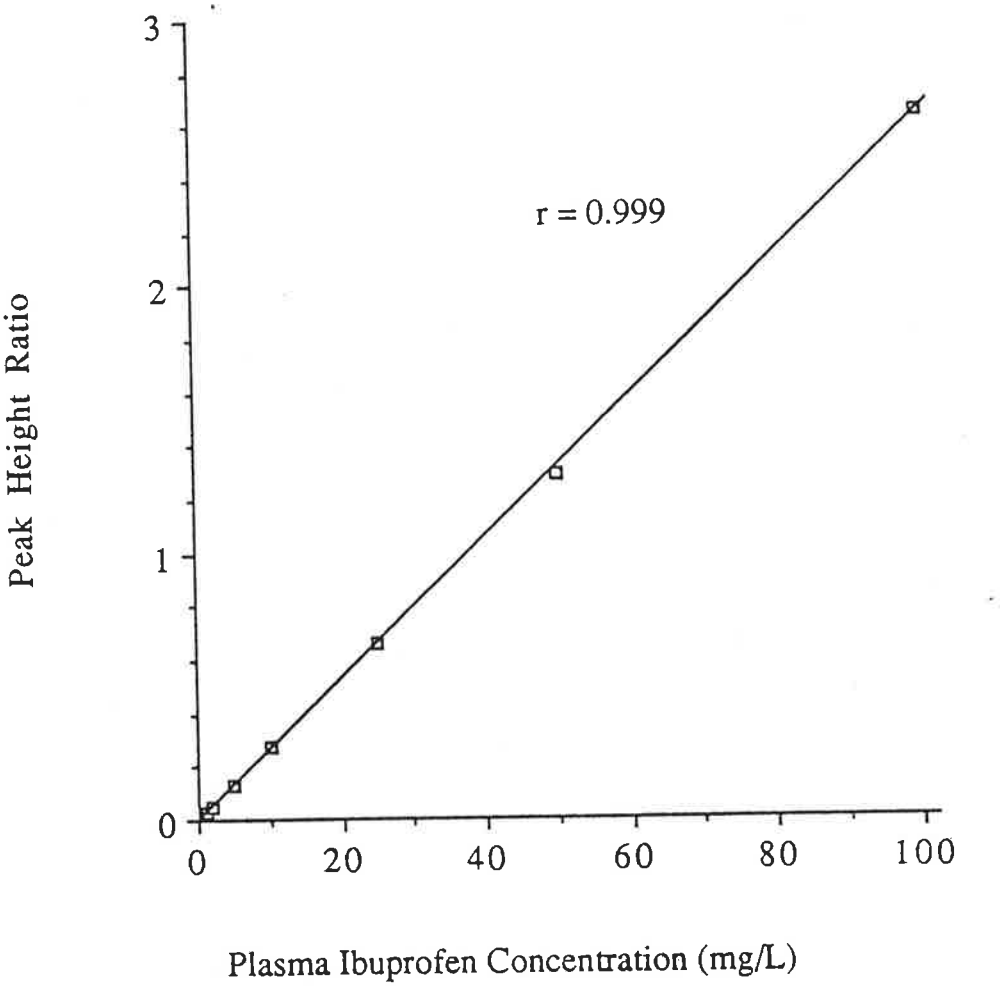


Figure 4.4. A representative Assay 1A calibration curve for determining the concentration of unresolved ibuprofen in human plasma, in which the peak height ratio (ibuprofen/internal standard; y-axis) is plotted against the known plasma concentration of ibuprofen (x-axis).

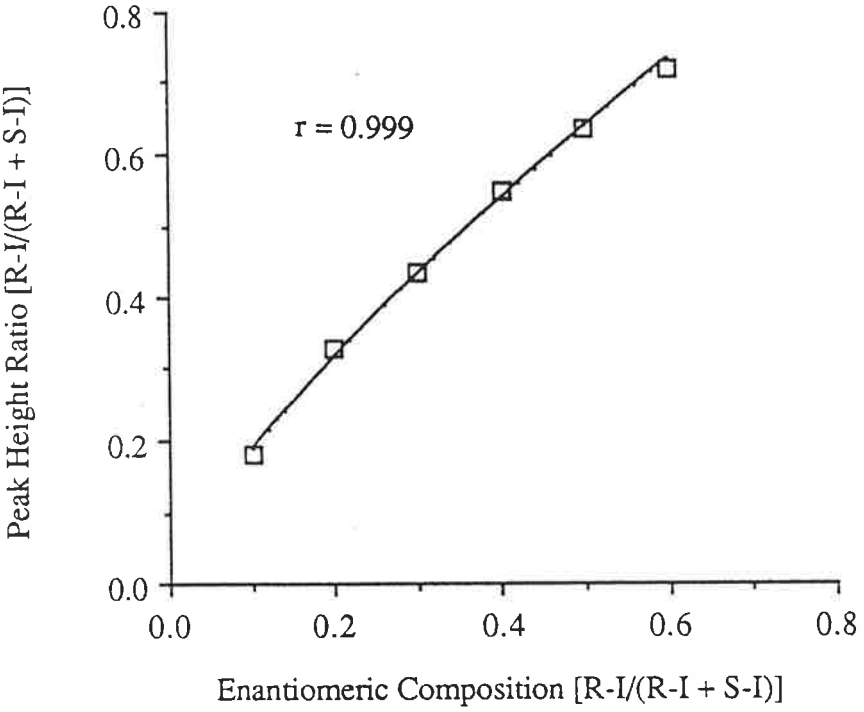


Figure 4.5. Representative Assay 1B calibration curve for determining the enantiomeric composition of ibuprofen in human plasma.

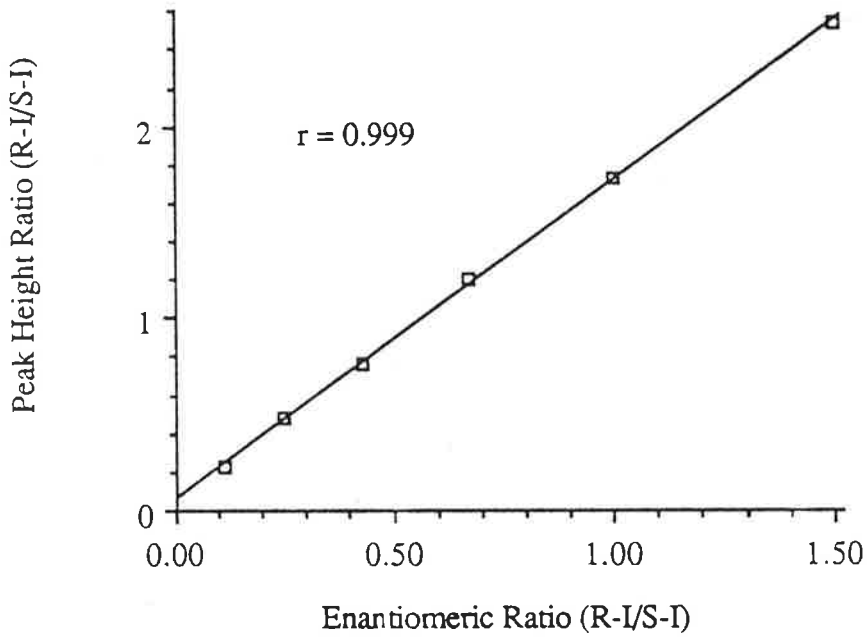


Figure 4.6. Alternative method of plotting the Assay 1B calibration data, in which the ratio of the heights of the diastereomeric peaks (R-I/S-I; y-axis) is plotted against the ratio of the plasma concentration of R-I to that of S-I (R-I/S-I; x-axis).

When aliquots of the methanolic standards of ibuprofen were evaporated to dryness, derivatized and chromatographed on the Assay 1B system, the resultant calibration curve was identical to that obtained when the standards were spiked into, and extracted from, human plasma. This confirms that the extraction of ibuprofen enantiomers from plasma was not enantioselective.

When selecting the enantiomeric composition of ibuprofen in the Assay 1B calibration standards, the enantioselective disposition of ibuprofen was carefully considered. After the administration of racemic ibuprofen to humans, the plasma concentrations of R-I, shortly after the commencement of absorption, are of a smiliar magnitude to those of S-I. Subsequently, the plasma concentrations of S-I exceed those of R-I (Lee et al 1985). For these reasons, the enantiomeric composition of ibuprofen in the calibration standards ranged from 10% to 60% R-I.

Accuracy and Precision

The intra-day accuracy and precision of the combined assay was assessed by analysing 5 separate aliquots of two independently prepared plasma samples. The first sample contained 20.0 mg/L of each enantiomer. Using the methodology described, the mean concentration of R-I and of S-I were determined to be 19.5 mg/L (CV = 0.93%) and 20.6 mg/L (CV = 0.77%), respectively. The second sample contained 0.50 mg/L of R-I and 1.50 mg/L of S-I. The concentrations determined were 0.50 mg/L (CV = 2.2%) and 1.53 mg/L (CV = 1.3%), respectively.

The inter-day accuracy and precision were determined by analysing a third plasma sample containing 5.00 mg/L of each enantiomer. Individual 500µl aliquots of this plasma sample were analysed over a 3 month period. The mean (n = 8) concentrations of R-I and S-I were 5.01 mg/L (CV= 5.9%) and 5.13 mg/L (CV = 5.2%), respectively.

Additional Validation Considerations for an Indirect Assay

The calibration, accuracy and precision data presented above confirm the suitability of the developed methodology for the analysis of ibuprofen enantiomers in human plasma. However, there are a number of potential limitations with the indirect approach of enantioselective chromatographic analysis (Caldwell & Testa 1988; Gal 1988) and in the validation of such a method a number of additional factors must be considered.

Most importantly, the chiral derivatizing agent (in this case S(-)-1-phenyl-ethylamine) and the pure standards of the enantiomers of the analyte (in this case R-I and S-I), must be free of optical contamination. In addition, there must be no racemization of either of these compounds during the derivatization procedures. These

considerations were addressed by derivatizing the authentic samples of R-I and S-I under the conditions described. When a derivatized sample of R-I was chromatographed under the conditions of Assay 1B, a small peak appeared at the retention time of the S-I amide. Similarly, for a sample of S-I, a small peak appeared at the retention time of the R-I amide. By comparing peak heights, it was estimated that in both cases, the sum total of impurities was minimal, and amounted to less than 2%. There was no tendency for this level of optical impurity to change with time. A similar finding was reported by Lee et al (1984) for the contamination of ibuprofen enantiomers and/or S(+)-2-octanol. Impurities of this level (i.e. less than 2%) are of little consequence for most pharmacokinetic applications (Gal 1988), where the ratio of the concentrations of the individual enantiomers is appreciably greater than 1 in 50. On the contrary, for the purposes of measuring small levels of one enantiomer in the presence of the other, a 2% contamination would be unacceptable (Gal 1988).

An additional requirement of an indirect assay is that both enantiomers must be derivatized to the same extent, and once derivatized, the diastereomers must be stable under the assay conditions. This was confirmed by derivatizing and chromatographing samples of racemic radiolabelled ibuprofen, collecting the column eluate fractions representing the diastereomeric amide peaks, and measuring the radioactivity associated with each amide peak. The mean (± SD) ratio of radioactivity associated with the amide of R-I to that of S-I was 1.00 ± 0.028 (n=6). This was taken to indicate that under the conditions described, R-I and S-I were derivatized to equal extents. Evidence for the equal extent of derivatization of ibuprofen enantiomers throughout the entire range of enantiomeric compositions examined, is provided by the linear nature of the Assay 1B calibration curve when expressed as peak height ratio (R-I/S-I) versus enantiomeric ratio (R-I/S-I)(Figure 4.6). There was no tendency for the peak heights of the diastereomers to diminish when derivatized samples were left at room temperature overnight.

Stability

In 1984, Aravind et al reported that ibuprofen stored in plasma at -10°C was stable for at least 3 months. This is consistent with the finding in the present work that over a 3 month period there was no change in the concentration of R-I and S-I stored in plasma at -18°C. To ensure that there was no interconversion of R-I to S-I upon storage, a plasma sample containing 10mg/L of R-I was prepared, and stored at -18°C. After two months, this sample was thawed, and placed through the preparative and chromatographic procedures, along with an authentic, fresh sample of R-I. The level of

optical contamination of the respective samples were identical (in the order of 2%). Similarly, it was found that there was no change in the level of contamination of S-I upon storage in plasma.

4.1.5. Attempts to Develop Alternative Methods

At the time the present assay was developed, only one HPLC method had been described for the analysis of ibuprofen enantiomers in human plasma (Lee et al 1984). This method suffered from a number of limitations, including the need to use two silica columns in series, together with prolonged retention times, low selectivity factor for the diastereomeric esters of R-I and S-I, and long column equilibration times. Additionally, after every 12 injections a 30 minute washout period was necessary and at regular intervals (after about 150 injections), extensive column regeneration and re-equilibration procedures were required (Lee et al 1984, Williams KM, personal communication 1985).

The method developed in the present work was originally based on an dual-assay method for analysing the enantiomers of 2-PPA, ketoprofen and fenoprofen in plasma (Meffin PJ, personal communication 1985; Sallustio et al 1986). After extraction of the analyte and a suitable internal standard from plasma, a reversed-phase HPLC procedure with UV detection was used to quantify the plasma concentration of the unresolved (i.e. the sum of both enantiomers) 2-PPA derivative. By collecting the column eluate fraction corresponding to the retention time of the analyte, this chromatographic procedure also served as a purification step. Subsequently, after a further extraction procedure, the purified analyte was derivatized with S(-)-1-phenylethylamine to form diasteromeric amides. These amides were then resolved by normal-phase HPLC and the relative peak heights of the two amides was used as an index of the enantiomeric composition of the 2-PPA derivative in the original plasma sample. Because it was suitable for 2-PPA, ketoprofen and fenoprofen, it was decided to adopt this approach for measuring ibuprofen enantiomers in human plasma. During assay development, considerable modifications were made to the preparative and chromatographic steps, although the derivatization conditions used for ibuprofen were identical to those used by Sallustio and coworkers. Most importantly, the reversed-phase HPLC method was used only for the quantification of unresolved ibuprofen, rather than for both purification and quantification. This negated the need for time consuming collection of HPLC eluate fractions, and avoided an additional extraction step prior to derivatization.

Because there was no need for chromatographic purification of the ibuprofen prior

to derivatization and normal-phase HPLC, quantification of R-I and S-I, using a single assay only, was attempted using the the conditions of Assay 1B. Initially, a variety of propionic acid derivatives were tested as possible internal standards. It was found that the derivatized amides of RS-ketoprofen, produced well defined chromatographic peaks (retention times of 8 and 14 minutes) which eluted well clear of both ibuprofen amides. Since the later eluting ketoprofen amide was well separated from endogenous U.V. absorbing material, RS-ketoprofen was examined as to its suitability as an internal standard spiked into each plasma sample. Unfortunately, the method suffered intermittently from poor reproducibility (e.g. coefficients of variation of 10 to 15% for replicate samples). Numerous attempts were made to isolate the cause of this variability. Such steps included varying the conditions (time and temperature) of the derivatization procedure, substituting 1,1'-carbonyldiimidazole for thionyl chloride as the derivatization mediator, as described by Rubin et al 1985, and close examination of extraction conditions. However, all modifications were to no avail. Similar variability was encountered when an alternative compound, phenylacetic acid, was examined as an internal standard candidate. Although the reason for the lack of success remains unknown, it may have been related to variable derivatization reactivity of the different compounds.

The quantification of R-I and S-I using one assay, by incorporating a tracer amount of radiolabelled ibuprofen, as a novel internal standard, was also examined. Racemic radiolabelled ibuprofen (400ng) was spiked into each plasma sample. After extraction and derivatization, the sample was injected onto the normal-phase system. The eluate fraction corresponding to each amide peak was collected, and the radioactivity (dpm) measured. For each enantiomer, a calibration curve was established by plotting the plasma concentration (x-axis) versus the ratio determined by dividing the height of the corresponding diastereomer peak (from the recorder tracing of the UV detector output) by the radioactivity of the eluate fraction. A calibration curve generated using this approach is presented in Figure 4.7. Although the method was moderately successful, it proved to be time consuming and assay reproducibility (coefficients of variation in the order of 10% for the analysis of replicate plasma samples) was inferior to that of the established dual-assay method.

Recently, two indirect assays have been described for the analysis of R-I and S-I in human plasma (Avgerinos & Hutt 1987, Mehvar et al 1988a). Both report similar accuracy, precision and sensitivity to the dual-assay method described herein, and offer the advantage of using one assay only. The method of Mehvar et al (1988a) has the additional advantage of favourable derivatization conditions (room temperature for 5

minutes) and the convenience of reversed-phase HPLC for the resolution of ibuprofen diastereomers.

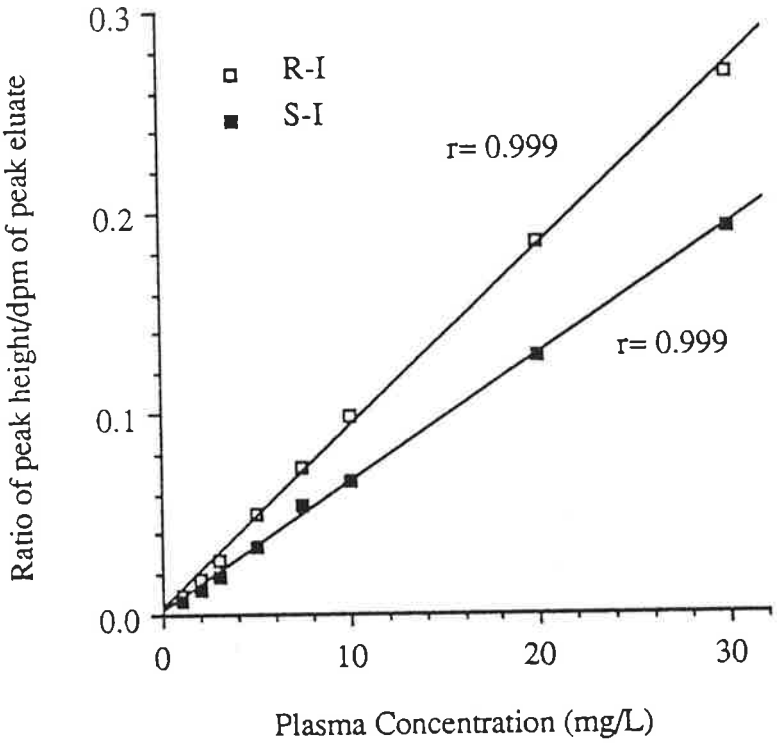


Figure 4.7. Calibration curves for the quantification of R-I and S-I in human plasma, whereby, for each enantiomer, the ratio of the height of the chromatographic peak for the diastereomeric amide to the radioactivity of the HPLC eluate fraction (y-axis) is plotted against plasma concentration (x-axis).

4.2. ANALYSIS OF IBUPROFEN AND ITS METABOLITES IN URINE.

4.2.1. Introduction

To assess the extent of ibuprofen absorption after oral dosing, it was necessary to measure the urinary excretion of ibuprofen and its metabolites. The majority of a dose of ibuprofen can be accounted for by the urinary recovery of the conjugated and unconjugated forms of hydroxy- and carboxy-ibuprofen, and in the form of ibuprofen-glucuronide (see section 3.5). Lockwood & Wagner (1982) reported on a method for measuring each of these species in human urine. The method involved sample extraction with dichloromethane, reversed-phase HPLC with a C_{18} chromatographic column, and UV detection. Because of the differences in polarity between ibuprofen and its oxidised metabolites, it was necessary for these workers to use a gradient elution system whereby the composition of the mobile phase was modified during the 20 minute run time. Each sample of urine was assayed for ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen both before (unconjugated) and after (conjugated plus unconjugated, ie 'total') alkaline hydrolysis of acyl glucuronide conjugates. A very similar method to that of Lockwood & Wagner (1982) for measuring ibuprofen and its metabolites in urine was recently described by Chai et al (1988). Once again a reversed-phase HPLC system with a gradient elution was used except, in this case, an additional extraction step was necessary prior to chromatography.

To avoid the need for a gradient elution program in the present work it was decided that two assays would be used to measure the three species in urine. The first (Assay 2), to measure hydroxy-ibuprofen and carboxy-ibuprofen in urine, was based upon the method described by Lockwood & Wagner (1982). The second (Assay 3), to measure ibuprofen in urine, was based on the method for quantifying unresolved ibuprofen in plasma (Assay 1A) as described in section 4.1.

For the studies described in this thesis, it was not necessary to determine the percent of each analyte which was excreted as the corresponding glucuronide conjugate, and for this reason only the 'total' urinary recovery of each species was measured. For a number of reasons, no attempts were made to determine the enantiomeric composition of ibuprofen and its metabolites in urine. Firstly, and primarily, it was considered unnecessary because urinary recovery was being monitored only to obtain an index of the extent of gastrointestinal absorption of ibuprofen. Secondly, using an enantioselective method for measuring ibuprofen and its metabolites in urine, Kaiser et

al (1976) reported difficulty in fully characterising and quantifying the four optical isomers of the carboxy-metabolite. Using a Pirkle-type chiral HPLC column, Nicoll-Griffith et al (1988) were unable to resolve the 4-methoxyaniline derivatives of hydroxy- and carboxy-ibuprofen. Thirdly, unless all of the administered dose is recovered, it is difficult to draw conclusions as to the overall pattern of metabolism of the individual ibuprofen enantiomers. In addition, authentic analytical samples of the optical isomers of the ibuprofen metabolites were not available.

4.2.2. Methods

HPLC Instrumentation and Conditions

Assay 2: The chemicals, and chromatographic and recording equipment employed for Assay 2 was identical to that described in section 4.1.2. The mobile phase, which consisted of acetonitrile: water adjusted to pH 2.4 with phosphoric acid in a ratio of 32: 68 was delivered to an Alltech C_{18} chromatographic column at a constant rate of 1.5ml/min, producing a typical backpressure of 1.3×10^4kPa.

Assay 3: The chromatographic analysis of ibuprofen in urine was performed using identical instrumentation and conditions described for the measurement of unresolved ibuprofen in plasma (section 4.1.2)

Sample Preparation

Assay 2: A 200µl aliquot of urine, or urine diluted 1 in 4 with purified water, was placed into a 10ml screw-topped test tube. The sample was left to stand at room temperature for 20 min with 200µl of 0.5M sodium hydroxide in order to hydrolize glucuronide conjugates. Subsequently, a 100µl aliquot of internal standard solution, containing 200mg/L of p-chlorophenoxyacetic acid (a gift of Flinders University) in methanol, was added, and the sample was acidified with 200µl of 2M sulphuric acid. After the addition of 5ml of dichloromethane, the tube was sealed with a PTFE-lined screw cap and the contents rotary-mixed at a rate of 30 r.p.m. for 10 minutes. More vigorous agitation of dichloromethane/aqueous samples was avoided because the resulting foam interface was partially resistant to centrifugation. After centrifugation (1000g for 10 minutes), the upper aqueous phase was removed by suction and discarded to waste. The organic phase (≈3ml) was removed, placed into a clean 10ml tapered test tube and evaporated to dryness in a 45°C waterbath under a gentle stream of purified nitrogen. The dried residue was reconstituted in 500µl of methanol and an aliquot (10 to 50µl) was injected into the Assay 2 HPLC system.

Assay 3: A 500µl aliquot of urine was diluted with an equal volume of 0.5M sodium hydroxide in a 10mL test tube and left to stand at room temperature. After 20 min, 200µl of 2M sulphuric acid, 50µl of internal standard solution (S(+)-naproxen, 50mg/L in methanol) and 5ml of dichloromethane were added. The subsequent extraction was carried out as for hydroxy- and carboxy-ibuprofen. The dried extract residue was reconstituted in methanol (500µl) and a portion (10 to 50µl) was injected into the Assay 3 HPLC system.

Calibration Procedures

Assay 2: Stock standards were prepared by dissolving hydroxy-ibuprofen and carboxy-ibuprofen in methanol to produce solutions of 1000 mg/L and 100 mg/L. Appropriate aliquots of these solutions were added to a series of 10ml screw-topped test tubes such that when the methanol was evaporated and 200µl of drug-free human urine added, the total concentration of each metabolite ranged from 5.0 mg/L to 250 mg/L. The calibration standards were carried through the preparative and chromatographic procedures together with the unknown urine samples. For each metabolite, the ratio of the height of its chromatographic peak to that of the internal standard was plotted against the metabolite concentration in urine to produce a calibration curve which was used to define the concentration of hydroxy- and carboxy-ibuprofen in unknown urine samples.

Assay 3: Calibration standards were prepared using methanolic stock solutions of racemic ibuprofen (1000 mg/L and 100 mg/L in methanol). Aliquots of these solutions were added to a series of 20ml screw-topped test tubes such that when the methanol was evaporated and 500µl of drug-free human urine added, the total concentration of ibuprofen ranged from 2.0 mg/L to 100 mg/L. Standards were carried through the preparative and chromatographic procedures together with the unknown urine samples. The ratio of the height of the chromatographic peak representing ibuprofen to that of the internal standard was plotted against the concentration of ibuprofen to produce a calibration curve which was used to define the concentration of ibuprofen in unknown urine samples.

4.2.3. Assay Development

Chromatography

Assay 2: Under the chromatographic conditions of Assay 2, the retention times of hydroxy-ibuprofen, carboxy-ibuprofen and p-chlorophenoxyacetic acid were 9, 10.5

and 13.5 min, respectively. All three compounds were well resolved and eluted clear of the normal constituents of human urine (Figure 4.8). As the column life progressed, it became necessary to modify the chromatographic conditions to ensure adequate resolution of the two ibuprofen metabolites. For example, for the final chromatographic runs, the acetonitrile content of the mobile phase was reduced from 32% to 25%, and the mobile phase flow-rate was increased from 1.5 to 2.0 ml/min. Peak retention times were affected only minimally when these modifications were made. The sensitivity of the system was such that a concentration of 2 mg/L of each metabolite in urine could be measured easily (signal to noise ratio in excess of 10:1) with the detector set on 0.05 or 0.10 A.U.F.S.

Assay 3: As for the plasma ibuprofen assay, the retention times of ibuprofen and S(+)-naproxen, under the conditions outlined, were in the order of 4 and 7 min, respectively. Both peaks eluted clear of normal urinary constituents (Figure 4.9.). Ibuprofen concentrations of 2 mg/L could be measured easily (signal to noise ratio in excess of 10:1) with the detector set on 0.10 A.U.F.S.

Calibration

Typical Assay 2 calibration curves for hydroxy- and carboxy-ibuprofen are presented in Figure 4.10. and an Assay 3 calibration curve for ibuprofen in urine is shown in Figure 4.11. For all three species, calibration lines over the ranges of concentrations examined were linear and passed through the origin.

Accuracy and Precision

The accuracy and precision of Assay 2 was assessed by analysing 5 separate aliquots of two independently prepared urine samples. The first sample contained 50.0 mg/L of each metabolite. Using the methodology described, the mean concentrations of hydroxy-ibuprofen and of carboxy-ibuprofen were determined to be 50.4 mg/L (CV=0.71%) and 49.4 mg/L (CV=0.64%), respectively. The second sample contained 10mg/L of each metabolite. The mean concentrations determined were 10.3 mg/L (CV = 3.74%) and 9.88 mg/L (CV = 5.00%), respectively. In two urine samples containing approximately 4 mg/L and 50 mg/L of racemic ibuprofen, the coefficients of variation (n = 5) for the peak height ratios were 4.3% and 1.4%, respectively.

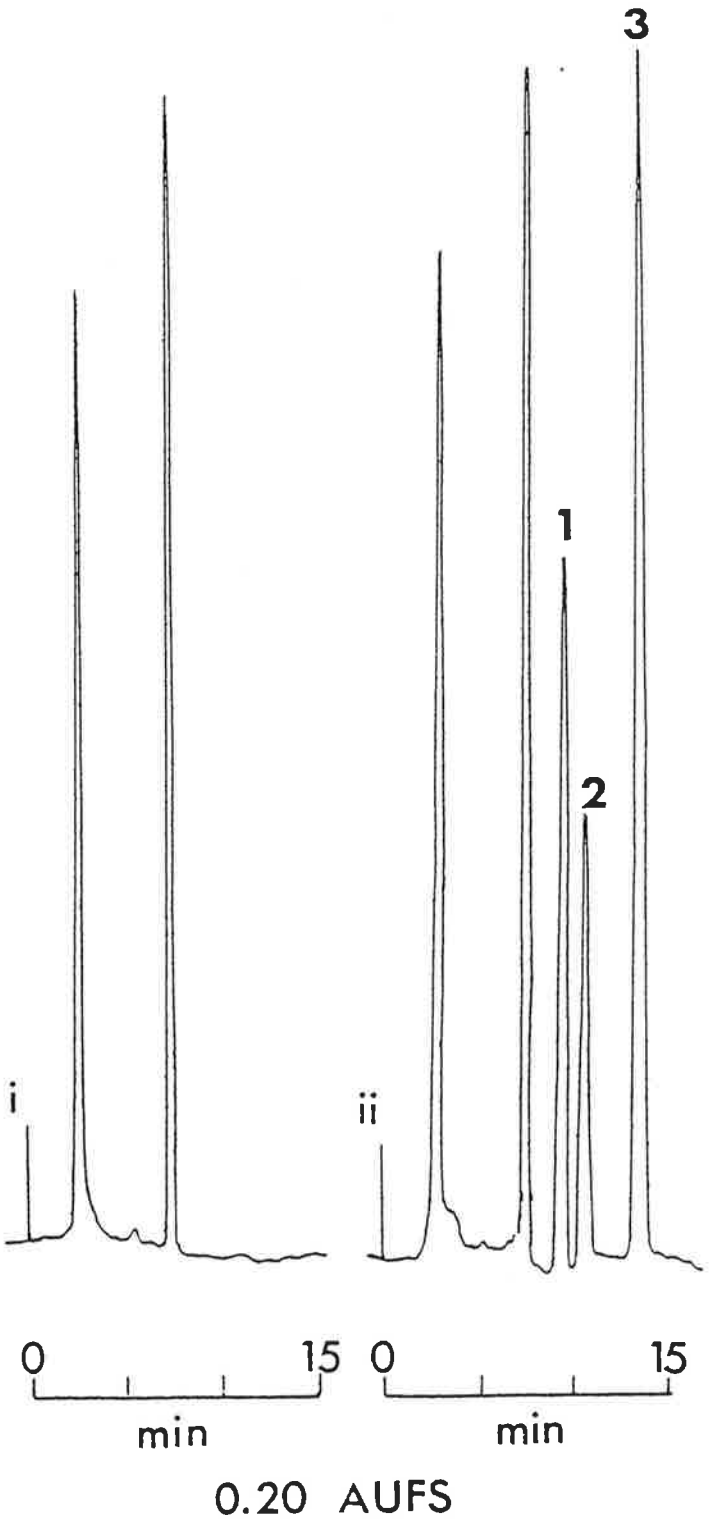


Figure 4.8. Representative Assay 2 chromatograms of human urine extracts (i) drug-free urine; (ii) urine containing 50 mg/L of hydroxy-ibuprofen and carboxy-ibuprofen. Peaks 1, 2 and 3 represent hydroxy-ibuprofen, carboxy-ibuprofen and the internal standard (p-chlorophenoxyacetic acid), respectively.

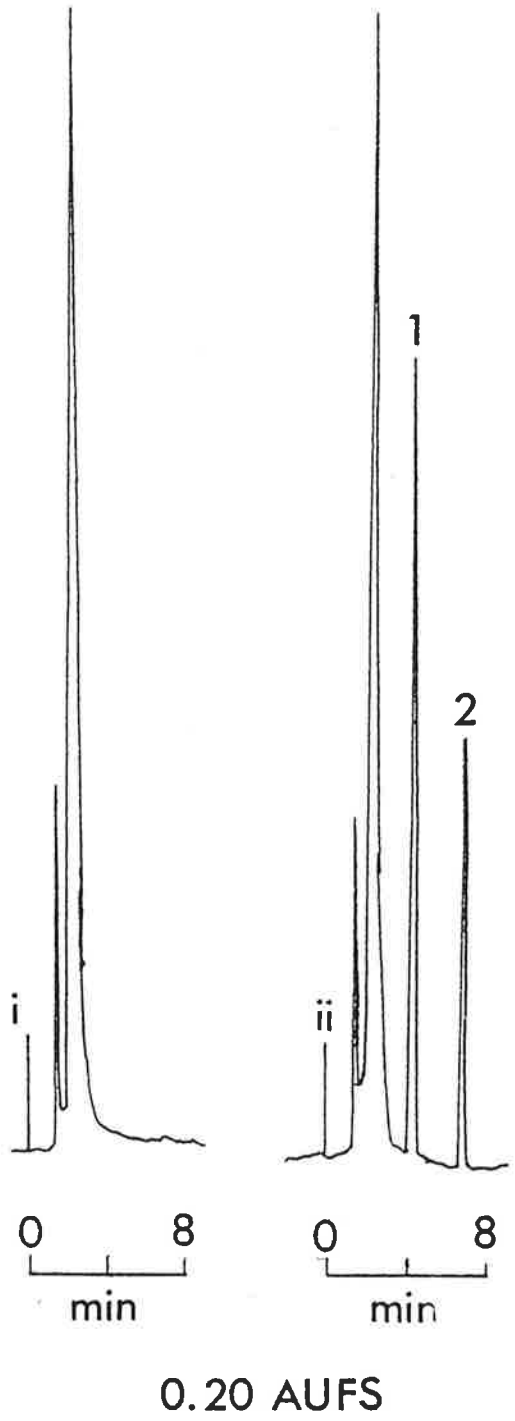


Figure 4.9. Representative Assay 3 chromatograms of human urine extracts (i) drug-free urine; (ii) urine containing 20 mg/L of ibuprofen. Peak 1 represents the internal standard (naproxen) and peak 2 represents ibuprofen.

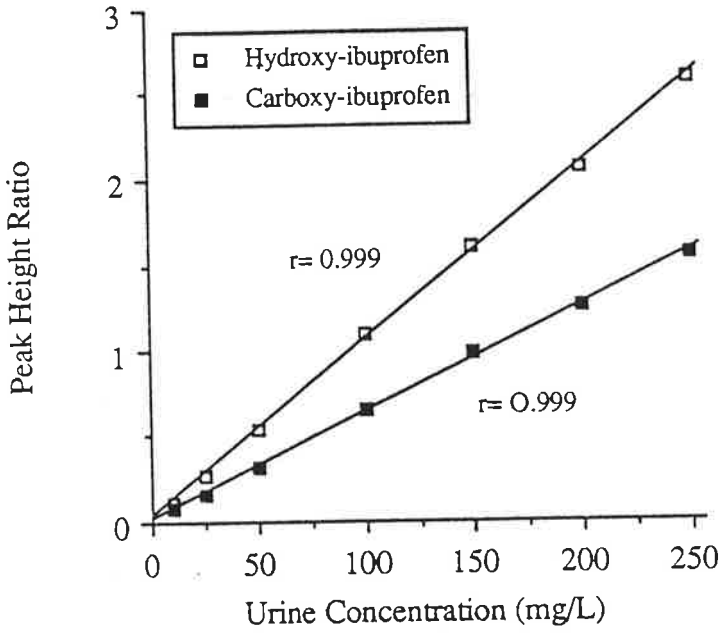


Figure 4.10. Representative Assay 2 calibration curve for the quantification of hydroxy-ibuprofen and carboxy-ibuprofen in human urine, in which peak height ratio (y-axis) is plotted against urine metabolite concentration (x-axis).

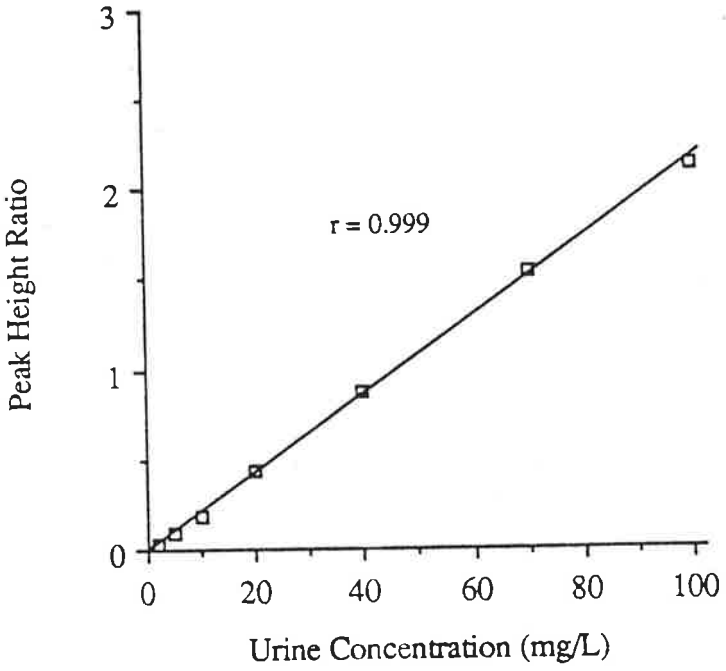


Figure 4.11. Representative Assay 3 calibration curve for the quantification of ibuprofen in human urine, in which peak height ratio (y-axis) is plotted against urine ibuprofen concentration (x-axis).

Stability

Previously, Lockwood & Wagner (1982) reported that ibuprofen and its metabolites were stable for "several months" in frozen urine. It was decided, therefore, that elaborate experiments examining the stability of these compounds were unnecessary. Nevertheless, upon repeated analysis (n=5) of frozen urine samples, containing 50 mg/L of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen, the concentration of each species was found to be constant over a 2 month period (for each species, the coefficient of variation for the determined concentration was below 5%).

Hydrolysis of Glucuronide Conjugates

There are potential problems associated with enzymatic methods of hydrolysing glucuronide conjugates for estimating the 'total' urinary concentrations of compounds excreted as acyl glucuronides. The use of ß-glucuronidase, for example, can be confounded by the intramolecular rearrangement of acyl glucuronides which occurs in alkaline media (Faed 1984). The resulting glucuronide isomers may not be substrates for ß-glucuronidase and therefore "total" urinary concentrations may be under-estimated (Faed 1984). This phenomenon can be partly avoided by acidification of collected urine, although, the possibility of *in vivo* glucuronide rearrangement can never be fully discounted. Eggers & Newman, in a personal communication to Faed (1984) reported that it was not possible to detect any significant rearrangement in the purified conjugates of ibuprofen. However, this does not discount the possibility of intramolecular rearrangement within the glucuronide conjugates of hydroxy- and carboxy-ibuprofen. It was therefore decided to hydrolyse the glucuronides of the three species by incubation with sodium hydroxide.

To examine the kinetics of alkaline hydrolysis of the glucuronide conjugates of the oxidized metabolites of ibuprofen, a healthy male volunteer took a single oral dose of ibuprofen (800mg), after which a urine sample was collected. Aliquots of this urine sample were incubated for various times (0 to 120 min) with an equal volume of 0.5M sodium hydroxide and the concentrations of each unconjugated metabolite was measured. The concentration of hydroxy- and carboxy-ibuprofen in the non-alkalinized urine sample were 38.7 mg/L and 107 mg/L, respectively. It had been established previously that exposure of the glucuronides to acid during the sample preparation procedure did not result in hydrolysis of the glucuronides. After 2 min incubation of the urine sample with sodium hydroxide, the corresponding concentrations of the two metabolites were 85.0 mg/L and 141 mg/L, respectively. Incubation for more prolonged times did not result in an increased yield. For example, the mean (±SD) concentrations

of hydroxy-ibuprofen and carboxy-ibuprofen in 5 samples analysed after various incubation times between 2 and 120 minutes were 84.2 ± 2.25 mg/L and 145 ± 4.84 mg/L, respectively. From these data, it was concluded that hydrolysis was rapid and complete within 2 min. A convenient incubation time of 20 min was chosen for routine analysis.

On another occasion, a healthy male volunteer took a 800mg dose of ibuprofen and collected a fresh urine sample. The ibuprofen concentration in an aliquot of urine which had not been exposed to alkali was 1.95 mg/L. Upon incubation for various lengths of time with equal volumes of 0.5M sodium hydroxide, the ibuprofen concentration rose steadily up to a maximum of 96.9 mg/L after 10 min and remained constant thereafter. The mean concentration determined in four aliquots incubated for between 10 and 60 min was 96.6 ± 0.68 mg/L. Hence, in agreement with previous reports (Chai et al 1988, Lockwood & Wagner 1982, Mills et al 1973) negligible ibuprofen was found to be excreted in the unchanged form. The hydrolysis of ibuprofen glucuronide was complete within 10 min, and an incubation time of 20 min was chosen for routine analysis.

In summary, Assays 2 and 3 represent reproducible and convenient methods for determining the total urinary excretion of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen, and were used for such a purpose to investigate ibuprofen pharmacokinetics.

4.3. VERIFICATION OF IBUPROFEN CONTENT IN BRUFEN® 200mg AND 400mg TABLETS.

In studies on the pharmacokinetics of ibuprofen, the drug has been administered orally either as a dilute alkaline solution or as a solid dosage form (see section 3.6.1). For the human volunteer studies reported in this thesis, ibuprofen was administered as commercially available tablets (BRUFEN® 200mg and BRUFEN®400mg), obtained from the Boots Company (Australia) Pty. Ltd., (North Rocks, New South Wales). The main advantages with this approach are that tablets are more acceptable to volunteers than alkaline solutions; the alkaline solution itself may alter the absorption characteristics of ibuprofen; and, results using a popular commercial product are more clinically relevant.

The content accuracy and variability of the commercial tablets of ibuprofen used in the pharmacokinetic studies was assessed.

Six BRUFEN® 200mg tablets (Batch number 603J) and six BRUFEN® 400mg tablets (Batch number 562J) were randomly selected from the batches used in the human studies. The ibuprofen content of each tablet was assessed according to the following method.

> The tablet was crushed into a fine powder, using a glass mortar and pestle. The crushed material was placed into a 100ml volumetric flask, with the assistance of methanol rinsing. Methanol was added up to 100ml. The solution was left for 2 hours to allow sedimentation of insoluble tablet excipients, after which an accurate aliquot (20µl) of the methanolic solution was injected onto the Assay 1A HPLC system described in section 4.1.3. The height of the ibuprofen peak was compared to the height of the peaks produced after injecting accurate aliquots of a methanolic solution of ibuprofen (20mg/10ml; prepared from the authentic material provided by the manufacturer).

The mean content of the BRUFEN® 200mg tablets was determined to be 197mg (C.V. = 1.8%) and that of the 400mg tablets was 398mg (C.V. = 1.9%). For the human studies, the ibuprofen content of the tablet batches was taken to be that stated by the manufacturer.

Chapter 5
Methodology for Measuring the Plasma Protein Binding of Ibuprofen Enantiomers.

5.1 INTRODUCTION

For ibuprofen, and other NSAIAs, the fraction bound in plasma is an important determinant of its primary pharmacokinetic properties (Lin et al 1987). As discussed in section 3.6.2, data from non-enantioselective plasma protein binding studies indicate that ibuprofen is about 98 to 99% bound at therapeutic concentrations and suggest, therefore, that any difference between the enantiomers in the fraction bound is minor, although there may be a substantial percentage difference in their respective unbound fractions.

There are very few methods available for studying the plasma binding of the enantiomers of 2-phenylpropionic acid derivatives when present together in the same sample. Jones et al (1986) studied the binding of R(-)- and S(+)-2-PPA in rabbit plasma utilizing the radiolabelled individual enantiomers. The major limitation in applying this approach to other chiral drugs is that in general, the radiolabelled material made available to investigators is racemic, and there are numerous problems associated with the optical resolution of milligram quantities of radiolabelled racemate, whilst maintaining sufficiently high purity and specific activity. A novel method which permits the reproducible, simultaneous determination of the plasma unbound fractions of R-I and S-I is described below.

5.2. METHODS

Materials

Sodium acid phosphate, disodium hydrogen phosphate and sodium chloride were all of analytical reagent grade. Isotonic phosphate buffer (pH 7.4) was prepared with 0.067M disodium hydrogen phosphate, 0.067M sodium acid phosphate, and sodium chloride. The dialysis membrane (Spectropor 2®, molecular weight cut-off 12,000-14,000; Spectrum Medical Industries Inc., U.S.A.) was prepared for use by washing three times with purified water and then soaking for 24 hours in the isotonic phosphate buffer. Human serum albumin (Calbiochem®, fraction V, 12666; Behring

Diagnostics, U.S.A.) was dissolved in isotonic phosphate buffer to produce a final concentration of 40 g/L (4% HSA). Liquid scintillation counting was performed with either aqueous (Amersham ACS II®) or non-aqueous (Beckman Ready-Solv NA®) counting scintillant, depending on the nature of the samples being counted. Plasma pH measurements were made using an Orion Research model 811 pH/millivolt meter fitted to an Orion Ross combination pH electrode. All other materials and equipment used were identical to those described previously (section 4.1.2.)

Purification of Radiolabelled Ibuprofen

Prior to use, the racemic radiolabelled ibuprofen (^{14}C-I) was purified by reversed-phase HPLC. On each occasion, purification was performed no more than 2 days prior to the commencement of a binding investigation. Aliquots (100µl) of a methanolic solution of ^{14}C-I (2mg/5ml) were injected onto an Alltech C_{18} HPLC column under the conditions described for Assay 1A (section 4.1.3). Even with these relatively large quantities, the analytical column performed well, and efficiency was maintained. The eluate fraction (2-3ml) corresponding to the peak representing ibuprofen (retention time 7 min) was collected into a tube and placed in a water bath, set at 45°C, under a steady flow of purified nitrogen, to reduce the volume to about 500µl. After the addition of 200µl of 2M sulphuric acid and 5ml of 5% isopropanol in heptane, the tube was vortex-mixed for 1 min and centrifuged at 1000g for 5 min. Subsequently, the organic phase was removed and evaporated to dryness at 45°C under a steady flow of nitrogen. The residue of ^{14}C-I was reconstituted in 4% HSA to produce a final ibuprofen concentration of approximately 7mcg/20µl. The purification process yielded an overall recovery of 96% and this figure was used to determine the amount of the methanolic ^{14}C-I solution which needed to be purified for a given set of plasma binding experiments.

Equilibrium Dialysis

The equilibrium dialysis unit (total capacity of about 8ml), constructed of clear perspex, consisted of two identical compartments separated by a single layer of dialysis membrane. Into one compartment was placed 3.5ml of the plasma sample under investigation, containing known concentrations of non-radiolabelled R-I and/or non-radiolabelled S-I, together with a 20µl aliquot of the solution of purified ^{14}C-I in 4% HSA. Into the opposing compartment was placed 3.5ml of isotonic pH 7.4 phosphate buffer. The access ports to the two compartments were tightly sealed and the dialysis unit was agitated (one 6cm oscillation per second) at 37^{0}C for an

experimentally determined equilibration time of 16 to 17 hours (see section 5.3). Subsequently, the contents of each compartment were removed and placed into separate collection vials.

Determination of the Distribution of Radiolabelled Ibuprofen Between the Equilibrated Buffer and Plasma

After equilibration, a 400µl aliquot of the plasma and of the buffer was placed into separate liquid scintillation vials along with 10ml of aqueous scintillation fluid. The concentration of radioactivity [expressed as disintegrations per min (dpm) per ml of fluid counted] within the buffer (dpm/ml_{Buff}) and plasma (dpm/ml_{Plas}) was determined with a Packard Model 2003 Tricarb Scintillation Spectrometer. The channels ratio method was used for quench correction and appropriate blanks for background subtraction.

Determination of the Enantiomeric Composition of Radiolabelled Ibuprofen in Equilibrated Buffer and Plasma

Another portion (≈2ml) of the equilibrated buffer was placed into a 20ml screw-topped test tube along with 500µl of 2M sulphuric acid, 10ml of 5% isopropanol in heptane and a 100µl aliquot of racemic non-radiolabelled ibuprofen (100mg/L in methanol), the latter to serve as a carrier. After vortex-mixing for 1 min and centrifugation at 1000g for 5 min, the organic phase was removed, placed into a clean 20ml screw-topped test tube and evaporated to dryness as described previously. The sample was then placed through the Assay 1B derivatization and extraction procedures described previously (section 4.1.3.). The dried residue was reconstituted in 100µl of 2.5% isopropanol in heptane, and after a brief vortex-mix, an aliquot (≈90µl) was injected onto the Assay 1B normal phase chromatographic system (section 4.1.3.).

As discussed previously for Assay 1B (section 4.1.4), the diastereomeric amides of R-I and S-I were well resolved and had approximate retention times of 3 and 5 min, respectively. The addition of carrier non-radiolabelled racemic ibuprofen in a previous step ensured visualization of the amide peaks and served to minimise potential sorptive losses of the radiolabelled material. The eluate fractions corresponding to the amides of R-I and S-I were collected directly into separate liquid scintillation vials. After adding 10mL of non-aqueous liquid scintillation fluid, the radioactivity associated with each HPLC fraction (dpm $R\text{-}I_{Buff}$ and dpm $S\text{-}I_{Buff}$, respectively) was determined.

A 200µl aliquot of the equilibrated plasma was placed through the extraction,

derivatization and chromatographic procedures along with the corresponding buffer sample. Once again, eluate fractions were collected and the radioactivity associated with each diastereomeric amide (dpm $R\text{-}I_{Plas}$ and dpm $S\text{-}I_{Plas}$, respectively) was determined.

Calculation of the Unbound Fraction of R(-)-Ibuprofen and S(+)-Ibuprofen.

The unbound fraction of R-I ($f_u^{R\text{-}I}$) in the plasma sample under investigation was determined from equation 5.1.

$$f_u^{R\text{-}I} = \frac{dpm/ml_{Buff} \times dpm\ R\text{-}I_{Buff}/(dpm\ R\text{-}I_{Buff} + dpm\ S\text{-}I_{Buff})}{dpm/ml_{Plas} \times dpm\ R\text{-}I_{Plas}/(dpm\ R\text{-}I_{Plas} + dpm\ S\text{-}I_{Plas})} \qquad (5.1)$$

The unbound fraction of S-I ($f_u^{S\text{-}I}$) in the plasma sample was determined in a similar manner from equation 5.2.

$$f_u^{S\text{-}I} = \frac{dpm/ml_{Buff} \times dpm\ S\text{-}I_{Buff}/(dpm\ S\text{-}I_{Buff} + dpm\ R\text{-}I_{Buff})}{dpm/ml_{Plas} \times dpm\ S\text{-}I_{Plas}/(dpm\ S\text{-}I_{Plas} + dpm\ R\text{-}I_{Plas})} \qquad (5.2)$$

The concentrations of R-I and S-I unbound in the plasma sample ($C_u^{R\text{-}I}$ and $C_u^{S\text{-}I}$, respectively) were determined from equations 5.3 and 5.4, respectively.

$$C_u^{R\text{-}I} = f_u^{R\text{-}I} \times C^{R\text{-}I} \qquad (5.3)$$

$$C_u^{S\text{-}I} = f_u^{S\text{-}I} \times C^{S\text{-}I} \qquad (5.4)$$

where $C^{R\text{-}I}$ and $C^{S\text{-}I}$ represent the predialysis total plasma concentrations of R-I and S-I, respectively.

Reproducibility

Human plasma obtained from a healthy drug-free male was spiked with non-radiolabelled racemic ibuprofen to produce concentrations of 5.0 and 50mg/L. Five aliquots (3.5ml) of each plasma sample were analysed in replicate to assess method reproducibility.

5.3. METHOD DEVELOPMENT AND PERFORMANCE OF THE METHOD

A schematic summary of the method used to determine the plasma protein binding of R-I and S-I in human plasma samples is presented in Figure 5.1. It appears that this is the first time such an approach has been used to determine the plasma protein binding of drug enantiomers when present in the same sample. One of the prerequisites of the approach is that the derivatization procedure must not be enantioselective i.e. for equations 5.1 and 5.2 to be valid, the relative quantities of the formed diastereomers must reflect, precisely, the ratio of the quantities of the individual ibuprofen enantiomers in the sample under investigation. That this was the case was confirmed in section 4.1. Because, on most occasions, samples were analysed in batches, it was also important to ensure that when one derivatized sample was injected onto the column, there was no late-eluting radiolabelled material which could interfere with the analysis of subsequent samples. A plasma sample containing 5mg/L of racemic ibuprofen together with 2mg/L of ^{14}C-I was extracted, derivatized and chromatographed according to the method described. At the first sign of the peak representing the diastereomeric amide of R-I, collection of eluate fractions into liquid scintillation vials was commenced. A total of twenty successive 4 min fractions were collected. With the exception of the fractions associated with the ibuprofen amide peaks, the radioactivity associated with subsequent eluate fractions was no greater than background. This result confirms that there was no late-eluting radiolabelled material.

The dialysis conditions employed were selected to approximate physiological conditions. Therefore, plasma binding experiments were all conducted in a thermostatically controlled room maintained at a temperature of 37°C. Some workers have advocated that in equilibrium dialysis experiments, plasma should be dialysed against ultrafiltrate of drug-free plasma (i.e. "plasma water") to best mimic physiological conditions (van der Giesen & Wilting 1982). However, in our case large dialysate volumes were required to ensure assay sensitivity, and such an approach was not practical. Plasma was therefore dialysed against 0.067 M phosphate buffer (pH 7.4) which was made isotonic by adding sodium chloride.

An experiment was performed to determine the time needed for the plasma and buffer compartments to equilibrate. To the plasma compartments of 10 dialysis cells was placed 7mcg of purified ^{14}C-I in 10µl of 4% HSA and 3.5ml of human plasma containing 5 mg/L of non-radiolabelled racemic ibuprofen. An equal volume of isotonic phosphate buffer, pH 7.4, was placed into the buffer compartment of each cell, and the dialysis cells were incubated at 37°C under the conditions described. At various times,

individual cells were removed, and the distribution of radioactivity between the buffer and plasma was determined. Because it was unnecessary for the purposes of this experiment, the plasma protein binding of the individual ibuprofen enantiomers was not determined. The distribution of radioactivity between the two compartments, as a function of time, is presented in Figure 5.2. The ratio of radioactivity in buffer to that in plasma increased steadily to 0.0046 (i.e. 0.46%) after 14 hours of dialysis, and remained essentially constant thereafter. Twenty four hours after the commencement of dialysis the ratio of radioactivity was 0.0048. An equilibration time of 16 to 17 hours was chosen for routine plasma protein binding studies, permitting the convenience of overnight dialysis.

Prior to the development of the present method, the radiochemical purity (the fraction of the radionucleotide in the form of the stated chemical) of the ^{14}C-I, as received, was determined. A 100µl aliquot of a methanolic solution containing 500ng of ^{14}C-I was injected onto the Assay 1A HPLC system (section 4.1.3), and 15 sec eluate fractions were collected directly into liquid scintillation vials for a total of 15 minutes. After adding liquid scintillation fluid, the radioactivity of each eluate fraction was measured. A total of 98% of the total radioactivity collected was associated with the ibuprofen peak; the other 2% eluted prior to the ibuprofen peak. The major disadvantage of using HPLC (as compared to thin-layer chromatography, for example) for determining radiochemical purity, is that some impurities may be retained on the column for protracted time periods, thereby not eluting during the selected period of collection. It is important, therefore, to ensure that all of the radioactivity injected onto the column is recovered. When 5 samples of ^{14}C-I were injected onto the column, and the HPLC eluate fraction corresponding to ibuprofen was collected and extracted, the overall recovery of radioactivity was 96.4 ± 1.44%, suggesting that negligible amounts of radio-contaminants were retained on the column. Hence, these experiments indicated that the level of contamination of the radiolabelled sample of ibuprofen was in the order of 2%.

For drugs which are very highly protein-bound, such as the 2-PPA derivatives, even small amounts of poorly protein bound radiochemical impurities can lead to spurious estimates of the unbound fraction (Bjornsson et al 1981; Yacobi & Levy 1975). To assess the potential for this artefact in the present work, the plasma protein binding of racemic ibuprofen was measured non-enantioselectively by radiochemical analysis with non-purified ^{14}C-I (i.e. as received), and the results were compared with those obtained by HPLC analysis. Drug-free plasma obtained from a healthy male was spiked with non-radiolabelled racemic ibuprofen to produce a final concentration of

50mg/L, a sufficiently high concentration to measure the protein binding of ibuprofen non-enantioselectively using HPLC method 1A (section 4.1). After the addition of non-purified ^{14}C-I, and equilibrium dialysis, the mean (n=3) percentage unbound of ibuprofen, determined radiochemically, was 1.68 ± 0.038%, and that determined by HPLC was 0.739 ± 0.055%, with the difference between the two methods being highly significant ($p < 0.002$, Student's paired t-test). The experiment was repeated with ^{14}C-I that had been purified by the method described in section 5.2. On this occasion, the unbound percentages, determined radiochemically and by HPLC, were 0.725 ± 0.034% and 0.714 ± 0.035%, respectively, and were not significantly different ($p= 0.82$). The results of these experiments suggested that the small (≈2%) level of radio-contamination in the non-purified sample of radiolabelled ibuprofen caused spurious estimates of the percentage of ibuprofen unbound in plasma, and therefore, confirmed the need for routine purification of the ^{14}C-I prior to use.

A number of workers have used racemic radiolabelled ibuprofen to determine the binding of the drug to human plasma proteins. Aarons et al (1983b) added racemic radiolabelled ibuprofen to drug-free plasma obtained from healthy volunteers and found that at concentrations of 1.25 and 51.4 mg/L, the mean percentage unbound of ibuprofen was 1.25% and 1.64%, respectively. Gallo et al (1986) investigated the kinetics of ibuprofen in the plasma and synovial fluid of arthritic patients. Racemic radiolabelled ibuprofen was added to the plasma and synovial fluid collected from patients who had received an oral dose of racemic ibuprofen. The percent of ibuprofen unbound in the plasma of these patients ranged from 1.54% to 2.53%. In both of these studies (Aarons et al 1983b; Gallo et al 1986), the values reported are higher than those obtained in our laboratory (see Chapter 6, 8 and 9); although, in the case of the data presented by Gallo et al (1986), the higher values may have been related to the patient disease state. However, both groups of investigators seemingly overlooked the possibility of radiochemical contamination of the radiolabelled sample. Using radiochemically pure ^{14}C-I, Lockwood et al (1983b) found that the percentage unbound of unresolved ibuprofen in healthy subjects ranged from 0.419% to 0.933% after the oral administration of a range of doses of the racemate; these values are similar to those obtained in the present work for unresolved ibuprofen (see Chapters 6, 8 and 9).

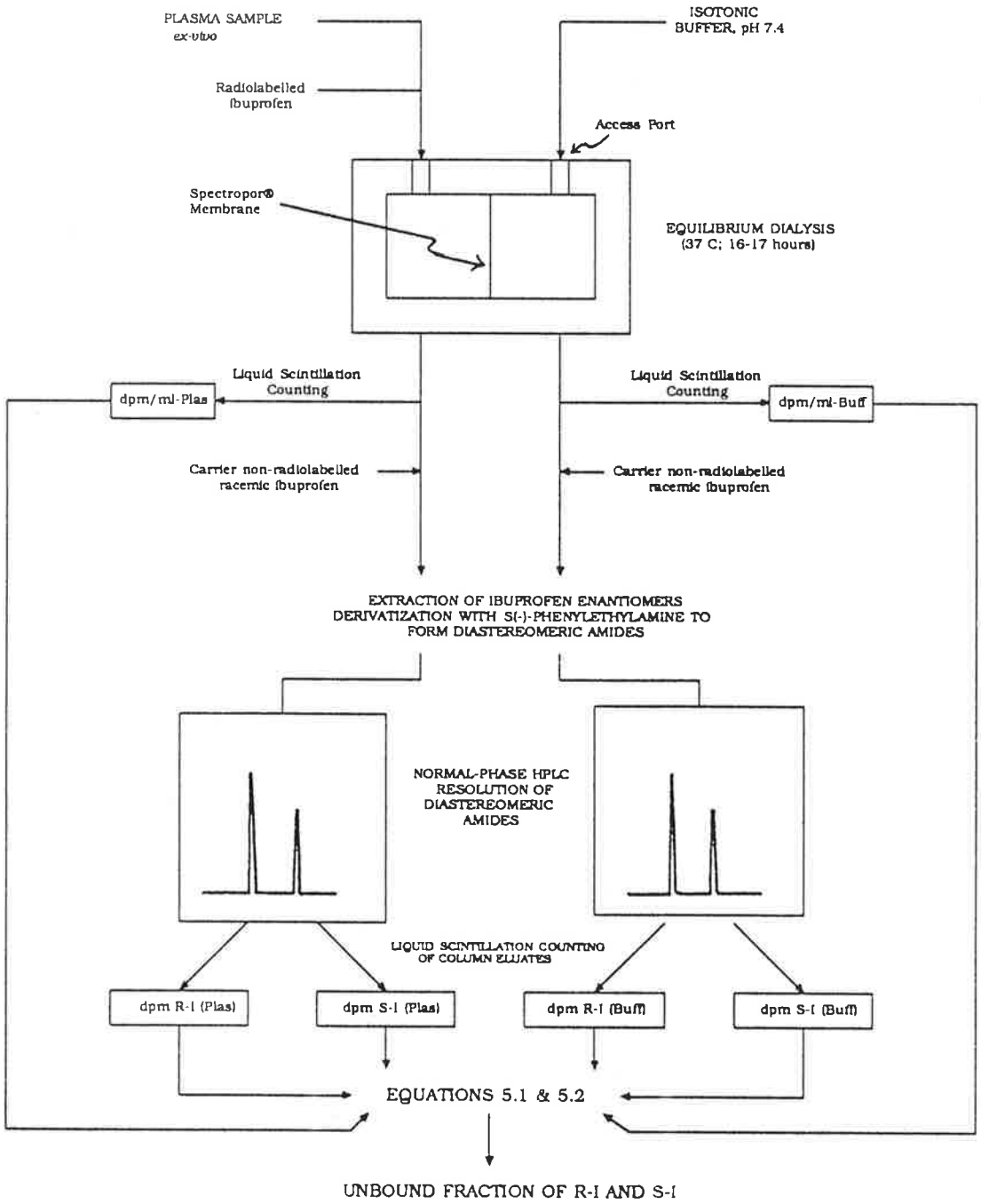


Figure 5.1. Schematic representation of the method used to determine the plasma protein binding of ibuprofen enantiomers.

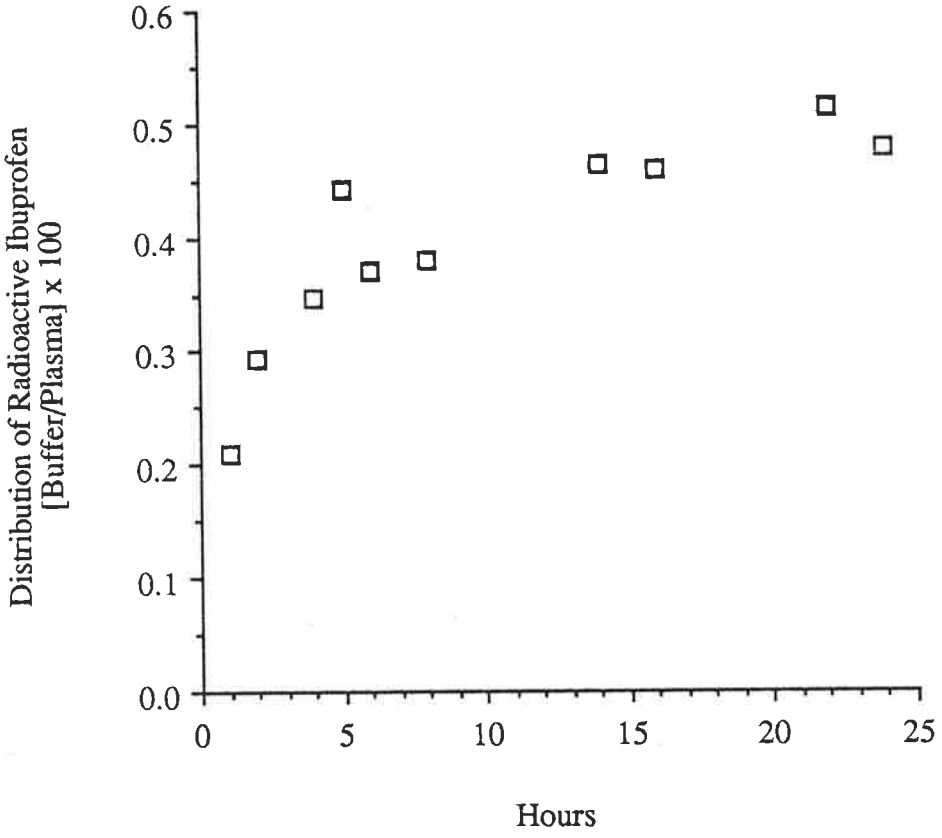


Figure 5.2. The results of an experiment performed to determine the time taken for unbound ibuprofen to equilibrate between the buffer and plasma compartments of a dialysis cell. The distribution of radiolabelled ibuprofen between buffer and plasma (y-axis) is plotted against the time of dialysis at 37°C

The concentration-dependent plasma protein binding of ibuprofen (Aarons et al 1983b; Grennan et al 1983; Lockwood et al 1983a, 1983b) was a major factor influencing the choice of the quantity of ^{14}C-I to spike into each dialysis cell. Using drug-free plasma from a healthy male, the magnitude of the concentration-dependence was assessed. This was achieved by determining the plasma unbound fraction of unresolved ibuprofen in 10 cells containing a constant concentration of ^{14}C-I, and varying concentrations of non-radiolabelled racemic ibuprofen (up to 100mg/L). The unbound fraction of ibuprofen as a function of concentration is presented in Figure 5.3. Between the concentrations of 1 and 11 mg/L, the unbound fraction of ibuprofen was relatively constant; the mean (±SD) percentage unbound determined in these cells being 0.39 ± 0.02%. As the concentration increased from 11 to 101mg/L, the percentage unbound increased steadily to 0.86%. On the basis of these results it was decided to limit the concentration of radiolabelled tracer to 2.0 mg/L, (i.e. 1.0 mg/L of each enantiomer). This was sufficient for assay sensitivity, yet represented a small percentage increase in the concentration of each enantiomer at the levels at which ibuprofen exhibited concentration-dependent plasma protein binding.

Because of the colloid osmotic pressure of plasma proteins, equilibrium dialysis is normally associated with a net flux of fluid from the buffer compartment to the plasma compartment (volume-shift). In some cases, variable volume shifts of up to 80% have been reported (Tozer et al 1983). The degree of volume shift can be controlled by using buffer which is isotonic or hypertonic with respect to plasma, although this will not completely prevent the phenomenon. Because the osmotic pressure of the plasma proteins can not be fully satisfied, volume shift will continue until the protein osmotic pressure is fully offset by the hydraulic pressure of the distended dialysis membrane (Bowers et al 1984). The dilution of plasma resulting from the volume shift can influence the estimated unbound fraction of the ligand under investigation, particularly for extensively bound drugs (Giacomini et al 1984; Hu & Curry 1986; Huang 1983; Lockwood & Wagner 1983; Tozer et al 1983). Various techniques for estimating binding parameters in the presence of volume shift have been described (Giacomini et al 1984, Huang 1983, Tozer et al 1983). These techniques usually rely on estimating the extent of volume-shift by measuring pre- and post-equilibration compartmental volumes, or by determining the concentration of protein in the plasma compartment both before and after dialysis. In the development of a protein binding method employing equilibrium dialysis it is imperative to establish the extent of volume shift and to evaluate whether or not correction techniques are needed. The degree of dilution of plasma during dialysis with the method described herein was assessed by measuring the

total recovery of fluid from each compartment following the dialysis of 20 plasma samples. Knowing the volume of fluid placed into each cell, and by carefully measuring the post-dialysis recovery of fluid from the plasma and buffer compartments, respectively, volume shift was estimated. The net movement of fluid was minimal and amounted to an average of 8.9 ± 3.1%. Hence, since routine correction of unbound fraction for volume shift involves additional measurements, which themselves are a potential source of error, no correction factors were applied (Huang 1983). The low degree of volume shift in the present work contrasts with the higher values reported by some other workers using equilibrium dialysis. It is believed that volume shift was minimal because the buffer was isotonic, and because extreme care was taken to ensure that the membrane of each dialysis cell was firmly fixed, therefore optimising the initial hydraulic pressure of the membrane and, as a result, the resistance to the volume change.

Because albumin carries a net negative charge at physiological pH, the unbound concentration of small ionic molecules may differ between the plasma and buffer compartment of an equilibrium dialysis cell. This so-called 'Donnan effect' may also influence the distribution of a drug which is ionized at pH 7.4 (Bowers et al 1984; Keen 1966). For such a molecule, the unbound fraction determined by equilibrium dialysis may differ from the unbound fraction *in vivo*. Although ibuprofen is extensively ionized at physiological pH, the Donnan-effect would have been minimised by the use of isotonic buffer.

Nonspecific adsorption of a ligand to the dialysis apparatus during equilibration, can introduce error into binding estimations, particularly if the protein binding of the ligand is non-linear within the investigated concentration range, since adsorption will lower the concentration of the drug, relative to that of protein, on the plasma side of the dialysis cell (Hu & Curry 1986). An experiment was performed to examine the extent of non-specific binding of ibuprofen to the components of the dialysis cell. Four dialysis cells were prepared, and into both compartments of each cell was placed 3.5ml of isotonic phosphate buffer containing a known concentration (0.2 mg/L) of radiolabelled ibuprofen (no protein was added to either compartment). After 16 hours at 37°C, the concentration of radioactivity of the cell contents was determined. The post-dialysis concentration of radioactivity was 95.5 ± 1.5% of that determined for the undialysed solution. This result indicated that any binding of ibuprofen to the components of the dialysis cell was negligible.

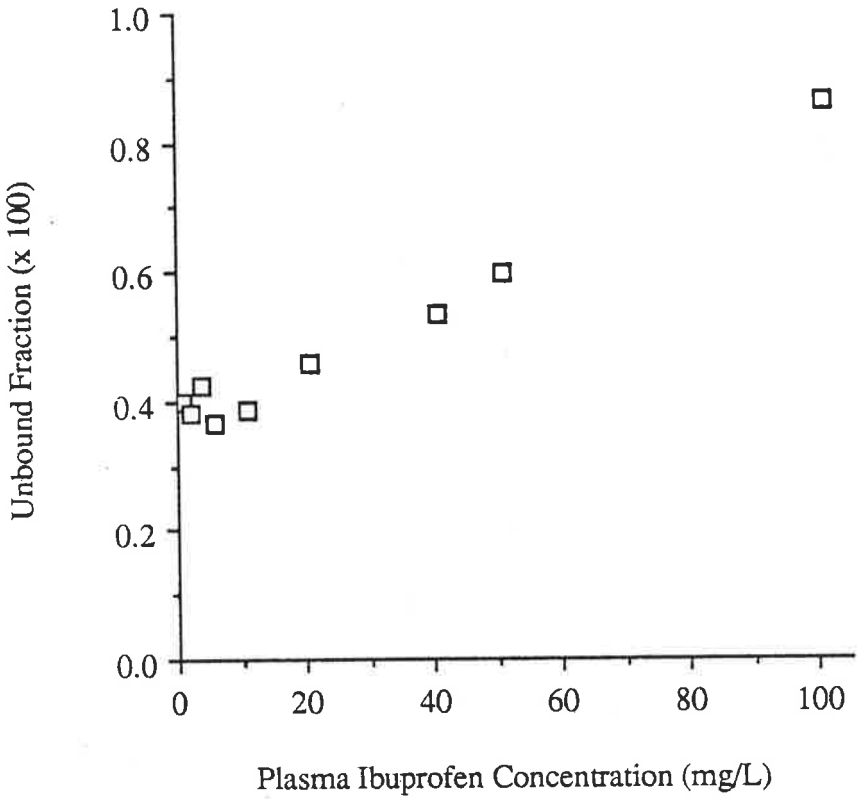


Figure 5.3. The results of an experiment performed to assess the concentration-dependence of ibuprofen plasma protein binding. The unbound fraction of unresolved ibuprofen (y-axis) is plotted against the total concentration of racemic ibuprofen in human plasma (x-axis).

It is important, when developing a plasma protein binding assay, to evaluate the effects of storage of plasma on the drug-plasma protein binding. After collection of fresh plasma, carbon dioxide is lost, and the pH tends to increase from 7.4, to within the range of 7.6 to 8.0 (Kristensen & Gram 1982). For drugs whose plasma protein binding is pH-dependent, it may be necessary to adjust the pH of the plasma prior to equilibrium dialysis (Kristensen & Gram 1982). However, even if the pH is modified, carbon dioxide may be lost from the sample during dialysis, and consequently, it can not be guaranteed that the pH of the plasma at the end of dialysis will be 7.4. An experiment was performed to examine the effect of storage of plasma at -18°C, on ibuprofen plasma protein binding. Racemic ibuprofen was spiked into a 50 ml drug-free plasma sample to produce a final concentration of 5 mg/L. This sample was then divided into 4 ml aliquots. The percentage unbound of ibuprofen (unresolved) was determined at random intervals over a 3 month period. The mean percent unbound (n = 7) of unresolved ibuprofen was determined to be 0.675 ± 0.064% and there was no trend for the values to increase or decrease with storage. The pH of each sample was measured both before and after dialysis. While the pre-equilibration pH of thawed plasma varied between 7.7 and 8.0, the pH of equilibrated plasma ranged between 7.3 and 7.4, possibly because of the effects of the phosphate buffer. Because ibuprofen unbound fraction did not change with storage, and because the post-dialysis pH was usually close to 7.4, it was considered unnecessary to modify the pH of the plasma samples prior to equilibrium dialysis.

The reproducibility of the method for determining the unbound fractions of ibuprofen enantiomers in human plasma was assessed. At a racemic ibuprofen concentration of 5.0 mg/L, the mean percentage unbound (n=5) of R-I was 0.392 ± 0.044% and that of S-I was 0.600 ± 0.070%. At 50 mg/L, the unbound percentages (n=5) were 0.595 ± 0.079% and 0.877 ± 0.078%, respectively. Thus, although several steps were involved in the performance of the enantioselective method, the overall reproducibility was good.

Because the binding of each ibuprofen enantiomer was extensive, there was negligible loss of each enantiomer to the buffer compartment during dialysis. As a result, the post-dialysis plasma concentrations of radiolabelled R-I and S-I were virtually identical. For this reason, it was standard practice, when a large number of samples were being processed, to determine dpm $R\text{-}I_{Plas}$ and dpm $S\text{-}I_{Plas}$ for a selection of the post-dialysis plasma samples only. The mean (n = 24) ratio of dpm $R\text{-}I_{Plas}$ to dpm $S\text{-}I_{Plas}$ returned by these samples was 1.008 ± 0.020. The relationships [dpm

$R\text{-}I_{Plas}/(dpm\ R\text{-}I_{Plas} + dpm\ S\text{-}I_{Plas})]$ and $[dpm\ S\text{-}I_{Plas}/(dpm\ S\text{-}I_{Plas} + dpm\ R\text{-}I_{Plas})]$ were therefore taken to be 0.50 and equations 5.1 and 5.2 were simplified accordingly.

In summary, a novel, reproducible method was developed for determining the plasma protein binding of R-I and S-I in human plasma samples. The method was used for determining the plasma protein binding of ibuprofen enantiomers in the pharmacokinetic and concentration-effect investigations which are presented in subsequent chapters. A detailed discussion of ibuprofen plasma protein binding is presented in Chapter 9.

Chapter 6
The Relationship Between the Pharmacokinetics of Ibuprofen Enantiomers and the Dose of Racemic Ibuprofen in Humans.

6.1. INTRODUCTION

For any drug, it is important to know the relationship which exists between the magnitude of the administered dose and the concentrations achieved in blood (or plasma). If the processes which govern the disposition of a particular drug (absorption, plasma protein binding, clearance etc.) are saturable within the range of concentrations achieved clinically, then the potential exists for the concentration of the drug in plasma (and at the effector sites) to change non-proportionally with dose. This non-proportionality may contribute significant variablity, both within and between individuals, to dose-response relationships. For the anticonvulsant agent phenytoin, hepatic oxidative metabolism is saturable within the range of concentrations achieved clinically, and consequently, as phenytoin concentrations increase, a given increment in dosage rate will lead to progressively greater increases in plasma phenytoin concentrations (Winter & Tozer 1986).

Several studies have examined the relationship between dose and plasma concentration for ibuprofen (Albert et al 1984a; Grennan et al 1983; Lockwood et al 1983a). Lockwood et al (1983a) administered 400, 800 and 1200mg of ibuprofen, orally, to healthy young adults and found a less than proportional increase in the area under the total (bound plus unbound) plasma ibuprofen concentration-time curve (AUC) with dose. However, the area under the plasma concentration-time curve with respect to unbound drug (AUC_u) increased in direct proportion to dose. Because the extent of absorption of ibuprofen (as gauged by the total urinary recovery of ibuprofen and its metabolites) was dose-independent, the authors suggested that ibuprofen clearance based on unbound drug was constant over the dose range examined.

The same group of workers used a similar study design to examine the effect of dose on ibuprofen pharmacokinetics in a group of volunteers aged between 65 and 78 years (Albert et al 1984a). The findings were similar to those obtained in the young

adults except, in this case, as the dose of ibuprofen increased, there was a less than proportional increase in the unbound AUC of ibuprofen. It was proposed that this non-linear relationship between dose and unbound AUC may have been due to a decrease with dose in the fraction of the oral dose absorbed; unfortunately, the urinary recovery of ibuprofen and its major metabolites was not determined.

Grennan et al (1983) examined the effect of chronic ibuprofen administration at different dosage levels in twenty rheumatic patients between the ages of 20 and 65 years. Using a Latin sequence design, each patient received three ibuprofen dosage regimens (200, 400 and 600mg, four times daily), for a period of one week. During the last dosage interval of each phase the plasma concentration-time profile of ibuprofen was determined. The dose-normalised ibuprofen AUC decreased by an average of 15% between the 200 and 600mg regimens, and this was accompanied by a 10% decrease in the dose-normalised ibuprofen C_{max}. These effects were attributed to a 20% increase in the plasma unbound fraction of ibuprofen over the range of concentrations achieved (Grennan et al 1983).

In all previous studies which examined the relationship between dose and plasma concentration for ibuprofen (Albert et al 1984a; Grennan et al 1983; Lockwood et al 1983a), the plasma concentrations of the drug were measured non-enantioselectively and the results provide no information on the effect of increasing dose of racemic ibuprofen on the pharmacokinetics of its individual enantiomers. In addition, in all of these studies, the plasma protein binding of ibuprofen was determined using racemic radiolabelled ibuprofen spiked into post-dose plasma samples (methods reported by Aarons et al 1983b and by Lockwood et al 1983b). As detailed in section 2.3.1 such an approach has important limitations when applied non-enantioselectively to chiral drugs which are used as racemates, and cannot be relied upon to produce accurate results.

The aim of the present study was to examine the relationships between the magnitude of the dose of racemic ibuprofen and the total and unbound plasma concentration-time profiles of its individual enantiomers, in humans. Major emphasis was placed on testing for any substantial change with dose in the total and unbound plasma concentration-time profiles for the active enantiomer, S-I. The study also examined the relationship between ibuprofen dose and inhibition of platelet thromboxane synthesis, the details of which are presented in Chapter 7.

6.2 METHODS

6.2.1. Subjects and Study Design

Four non-smoking male volunteers were recruited into the study. Selected details of each volunteer are presented in Table 6.1. Prior to participation, each volunteer underwent a thorough medical examination, which included haematological and biochemical analysis of a blood sample, and biochemical analysis of a urine sample. All biochemical tests (including plasma albumin) were performed at the South Australian Institute of Medical and Veterinary Science. The results of all tests were required to be within the accepted normal limits. Exclusion criteria included a recent history of drug ingestion, a history of gastrointestinal, renal, respiratory, hepatic or blood coagulation disorders, and a known allergy to ibuprofen or any other non-steroidal anti-inflammatory agent. Volunteers were instructed to abstain from all other drugs from one month prior to, until the completion of, the study. The study protocol was approved by the Ethics Committee of the Royal Adelaide Hospital and all volunteers gave written, informed consent to their participation. Volunteers were advised of their freedom to withdraw from the study at any time, and for any reason. All volunteers completed the study and no adverse effects were reported to the investigators.

Table 6.1. Details of the volunteers who participated in the dose-ranging study.

Volunteer	Age (years)	Body Weight (kg)	Plasma Albumin (g/L)
AE	24	78	46
JE	28	65	47
TS	23	73	47
SB	28	69	46

Using a four-way, balanced cross-over design, each volunteer received racemic ibuprofen (200, 400, 800 and 1200mg), orally, on four separate occasions. Ibuprofen was administered as one 200mg or one, two or three 400mg BRUFEN® tablets. The study phases were conducted on a fortnightly basis; the sequence of drug administration

is outlined in Table 6.2. The content accuracy and uniformity of the tablet batches were confirmed prior to the study (see section 4.3). Ibuprofen was administered with 200ml of water after an overnight fast of 12 hours duration. A standard snack was permitted 2 hours after dosing and a standard meal was supplied a further 2 hours later.

Table 6.2 The Sequence of Ibuprofen Administration.

	Phase 1 (Day 1)	Phase 2 (Day 15)	Phase 3 (Day 29)	Phase 4 (Day 43)
Subject	IBUPROFEN DOSE (mg)			
AE	1200	800	400	200
TS	800	200	1200	400
JE	400	1200	200	800
SB	200	400	800	1200

Blood samples were collected using an 18G Jelco® catheter which was inserted into an arm vein. Blood coagulation in the catheter was prevented by using a Jelco ® 18G stylet, which was replaced after each blood sample was collected. In addition, after the collection of each blood sample, the catheter was flushed with a small volume (≈1ml) of sterile normal saline. To ensure the removal of any residual saline from the catheter, the first 1ml of each blood sample was discarded; subsequently, 1ml of blood was collected for measuring serum thromboxane B2 levels (see Chapter 7); a further 10ml (for determining plasma R-I and S-I concentrations) was collected and placed into a heparinized blood collection tube. Blood was collected prior to drug administration and at the following times thereafter: 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 hours. Additional blood samples were collected by venepuncture after 24 and 48 hours, via a 21G needle. Immediately after collection, the blood sample for drug analysis was centrifuged and plasma was separated and retained at -18°C until assayed. A relatively large volume of blood was collected from each volunteer during the entire study (approximately 860ml over a 6 week period). For this reason, serum levels of iron, ferritin and transferrin were measured prior to the commencement of the study, and upon its completion. Serum iron and transferrin levels were unchanged during the course of the study, and remained within the normal range. Serum ferritin, however, decreased by an average of 49%, and in two volunteers was marginally below the normal range after the four phases were complete. Upon completion of the study all

volunteers were supplied with 60 ferrous sulphate tablets 350mg (FERRO-GRADUMET®) and advised to take one tablet each morning until completed.

A pre-dose urine sample was collected from each volunteer, as was all urine voided during the following time intervals after dosing: 0-12 hours, 12-24 hours and 24-36 hours. After measurement of urinary volume, an aliquot of each sample was retained at -18°C until assay.

6.2.2. Drug Analyses in Biological Fluids

Plasma samples were analysed for total concentrations of R-I and S-I, as described in section 4.1. The unbound fractions of R-I and S-I in selected plasma samples were measured using the technique detailed in Chapter 5. The unbound concentration of each enantiomer in plasma was calculated as the product of its total (bound plus unbound) plasma concentration and its unbound fraction.

After alkaline hydrolysis of glucuronide conjugates, the concentrations of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen in each urine sample were measured (see section 4.2). These concentrations, together with the recorded urinary volumes, were used to determine the amount of each species excreted during each time interval. Molecular weight conversions were used to calculate the excretion of hydroxy- and carboxy-ibuprofen as a percentage of the administered dose of ibuprofen.

6.2.3. Pharmacokinetic Analysis

The plasma concentration-time profiles of total R-I and total S-I were analysed in an identical manner. The terminal slope, determined by log-linear regression of at least the final three data points, was used to calculate the terminal rate-constant (ß). The terminal half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693/ß$. The area under the curve from time zero to infinity (AUC) was determined by summing the area from time zero to the time of the last measured concentration (determined by the linear trapezoidal method) and the extrapolated area. The extrapolated area was determined by dividing the final plasma concentration (interpolated from the log-linear regression analysis) by the terminal rate-constant, and in all cases this area accounted for no more than 10% of AUC. The AUC for unresolved ibuprofen (UNR-I), which represents that which would be determined if ibuprofen was measured using a non-enantioselective assay, was calculated by summing the AUC values of R-I and S-I. The peak plasma concentration

(C_{max}) and the time of its occurrence (t_{max}) for each enantiomer were obtained directly from the experimental observations.

For each volunteer, the unbound concentrations of R-I and S-I were determined in six plasma samples from each phase. The six samples were selected on the basis that they provided accurate representations of the plasma concentration-time profiles for total R-I and total S-I. For each enantiomer, the area under the unbound plasma concentration-time curve (AUC_u) was determined as described above except that, because of the more protracted spacing of data points, the post-C_{max} areas were determined using the logarithmic trapezoidal method. In all cases, the extrapolated area accounted for no more than 10% of AUC_u. The AUC_u of UNR-I was obtained by summing the AUC_u values of R-I and S-I. For each enantiomer, the quotient of AUC_u and AUC was taken to represent the time-averaged unbound fraction ($\bar{f}_u$).

6.2.4. Statistical Analysis

All data are presented as arithmetic mean ± standard deviation (SD). Two-tailed Student's paired t-tests were used to compare the pharmacokinetic parameters of R-I with those of S-I at each dose level. Analysis of variance (ANOVA) was used to test for changes with dose in the pharmacokinetic parameters of R-I and S-I. For both the Student's t-test and ANOVA, the *a priori* level of significance was 0.05. If ANOVA indicated a change with dose in a particular parameter, Fisher's least significant difference test was used to test for differences between the individual doses. For AUC and AUC_u, ANOVA was performed after dose-normalisation of the data. In addition, the 95% confidence limits of the slope of the line relating AUC/dose, and AUC_u/dose, to the administered dose, was determined by linear regression.

6.3. RESULTS

The plasma total and unbound concentration-time data for R-I and S-I are tabulated in Appendix B. The plasma concentration-time profiles of total R-I and total S-I, after the administration of 200, 400, 800 and 1200mg of racemic ibuprofen, in each volunteer, are presented in Figure 6.1. The corresponding profiles for unbound R-I and S-I are presented in Figure 6.2.

The AUC and AUC_u data for each enantiomer in all volunteers are presented in Table 6.3. The mean (± SD) t_{max}, C_{max}, $t_{1/2}$, $\bar{f}_u$, AUC and AUC_u of R-I and S-I, over the range of doses examined, are presented in Table 6.4.

As the dose of ibuprofen increased, there was a less than proportional increase in the mean C_{max} of both enantiomers (Table 6.4). Although the non-linearity in C_{max} for R-I was evident over the complete range of doses, the C_{max} for S-I increased proportionally between 400mg and 1200mg. While there was no significant change ($p > 0.05$) with dose in the t_{max} or $t_{1/2}$ of either enantiomer, the increase in $\bar{f}_u$ of R-I and S-I (Table 6.4) was highly significant ($p < 0.002$).

Linearity between AUC and dose was assessed by analysing the dose-normalised AUC data, presented graphically in Figure 6.3. For R-I there was a progressive decrease in AUC/dose as the dose of ibuprofen increased, while for S-I, the mean AUC/dose decreased between doses of 200 and 400mg, but remained virtually constant thereafter. The dose-normalised AUC of UNR-I, also presented in Figure 6.3, demonstrated a progressive decrease with dose. The change in AUC/dose was significant ($p< 0.05$) for R-I, but failed to reach significance for S-I ($p = 0.15$) and UNR-I ($p = 0.079$). When the dose-normalised AUC data for R-I, S-I and UNR-I were regressed against the administered dose, the slope of the linear regression line in all three cases was less than zero. For R-I and UNR-I, the 95% confidence interval of the slope excluded zero. Similar results were obtained when the regression analysis was performed using AUC data which had been normalised to volunteer body weight. Hence, both statistical tests (ANOVA and linear regression analysis) indicated a significant non-linear relationship between dose and AUC for R-I. The non-linearity between dose and AUC/dose for S-I failed to reach significance, and for UNR-I, the data were marginally significant.

The relationship between dose-normalised AUC_u and ibuprofen dose is shown for R-I, S-I and UNR-I in Figure 6.4. For each enantiomer, and for UNR-I, ANOVA

indicated no difference in AUC_u/dose across the four doses. In all three cases, the 95% confidence interval of the slope of the regression line relating AUC_u/dose, to the dose administered, included zero.

A summary of the statistical analysis performed to test for changes with dose in AUC/dose, AUC_u/dose and $\bar{f}_u$ of each enantiomer is presented in Table 6.5. The power of the study to detect a change with dose in the dose-normalised AUC and AUC_u of the pharmacologically important enantiomer, S-I, was examined. Despite the small number of subjects involved in the study, the probability of detecting a 20% change with dose in $AUC^{S\text{-}I}$/Dose and $AUC_u^{S\text{-}I}$/Dose ($\propto = 0.05$), was in the order of 95%.

The individual urinary recovery data for ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen, over the 0 to 36 hour time interval, are presented graphically in Figure 6.5, and the mean data (SD) are presented in Table 6.6. Statistical analysis indicated no significant change with dose in the 0 to 36 hour recovery of the three species. Similarly, there was no change with dose in the percentage of the administered dose of ibuprofen which was recovered in the urine (i.e. "total recovery" Table 6.6). As depicted in Figure 6.6, the major portion of each species was recovered in the first 12 hour urine collection period, indicating that the selected duration of blood sampling was suitable for defining the pharmacokinetics of ibuprofen.

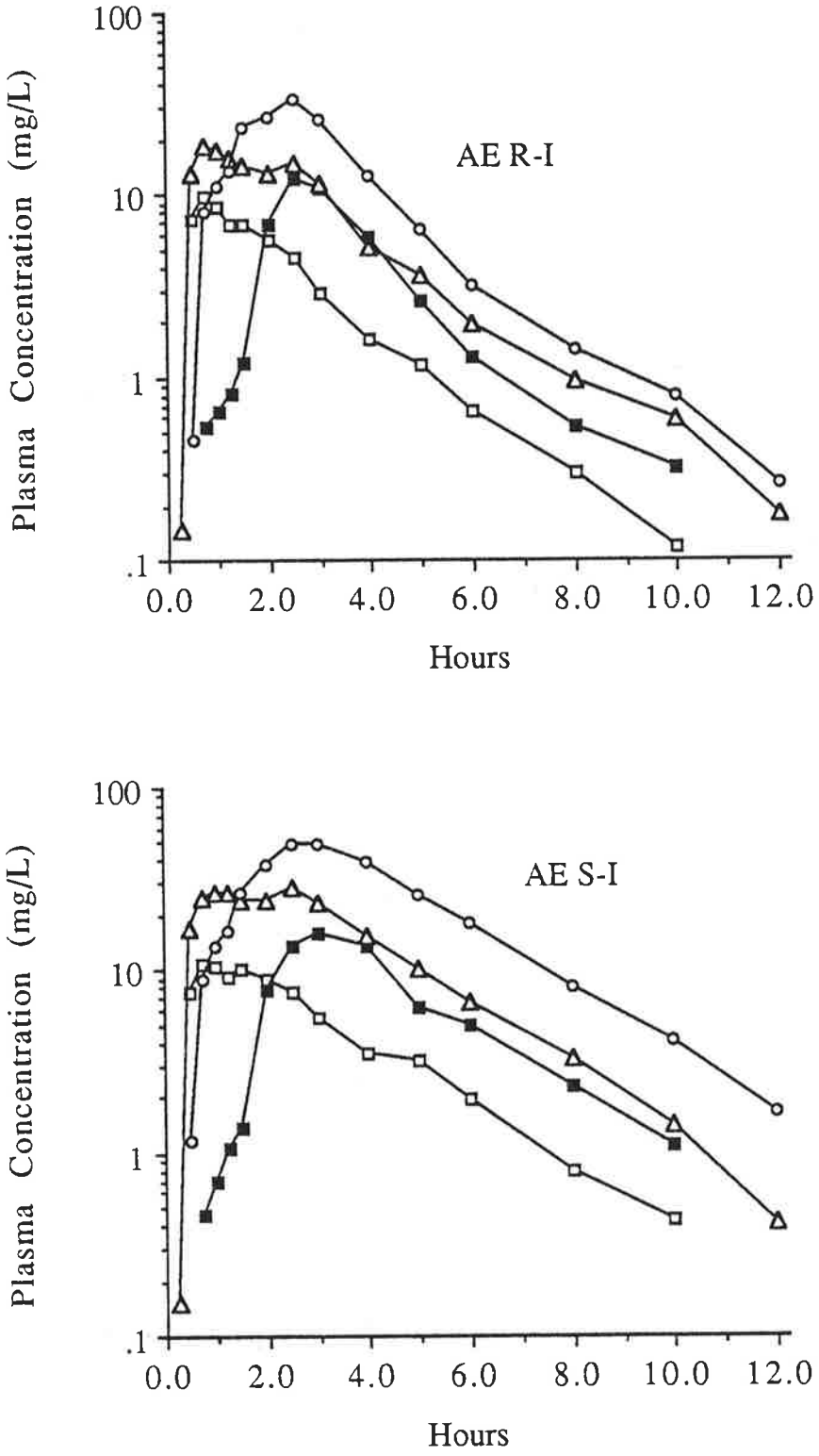


Figure 6.1.

Plasma concentration-time profiles of R-I (upper panel) and S-I (lower panel) after oral administration of RS-I (▫ 200mg, ■ 400mg, △ 800mg and ○ 1200mg).

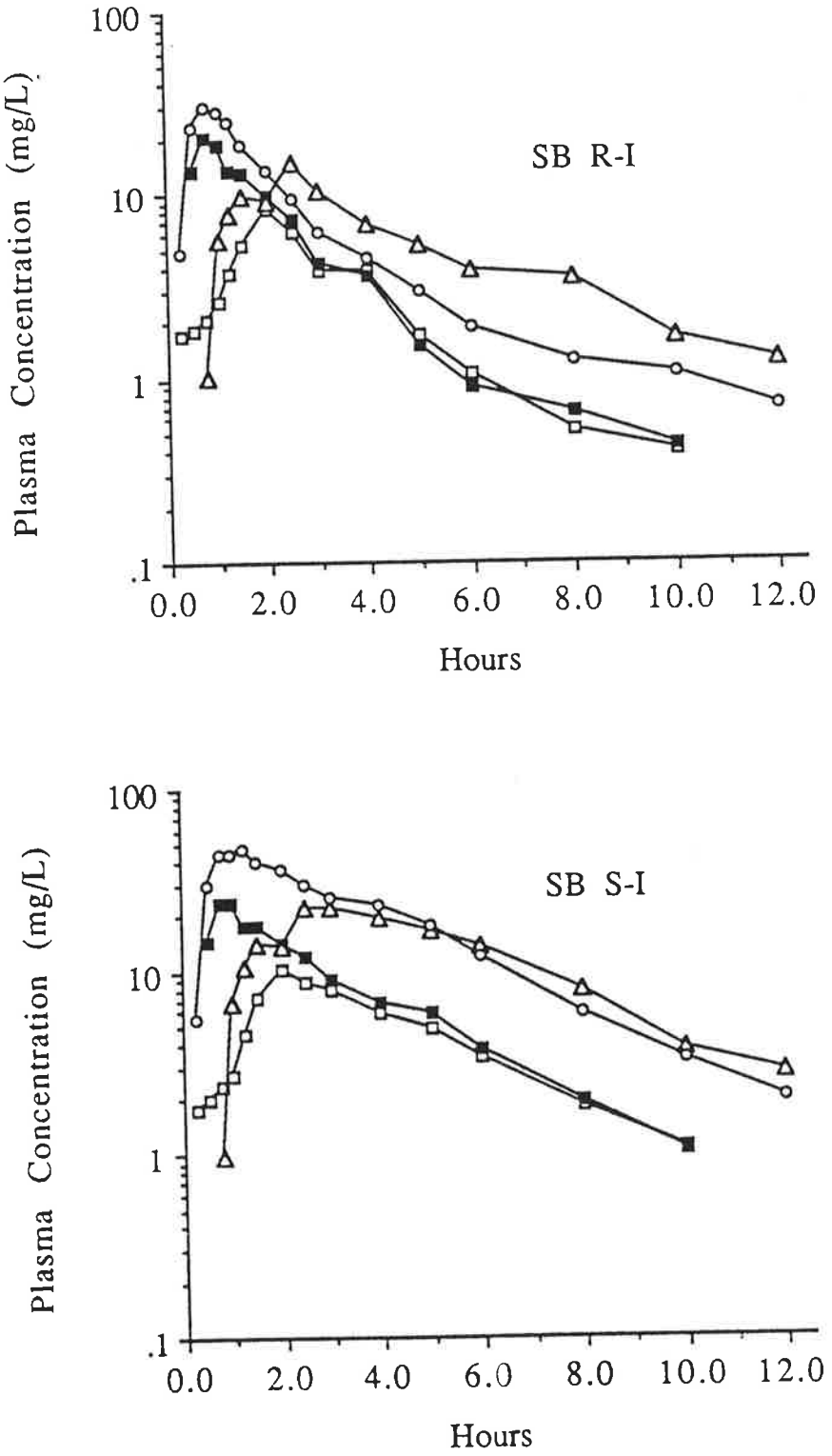


Figure 6.1 (continued).

Plasma concentration-time profiles of R-I (upper panel) and S-I (lower panel) after oral administration of racemic ibuprofen (□ 200mg, ■ 400mg, △ 800mg and ○ 1200mg).

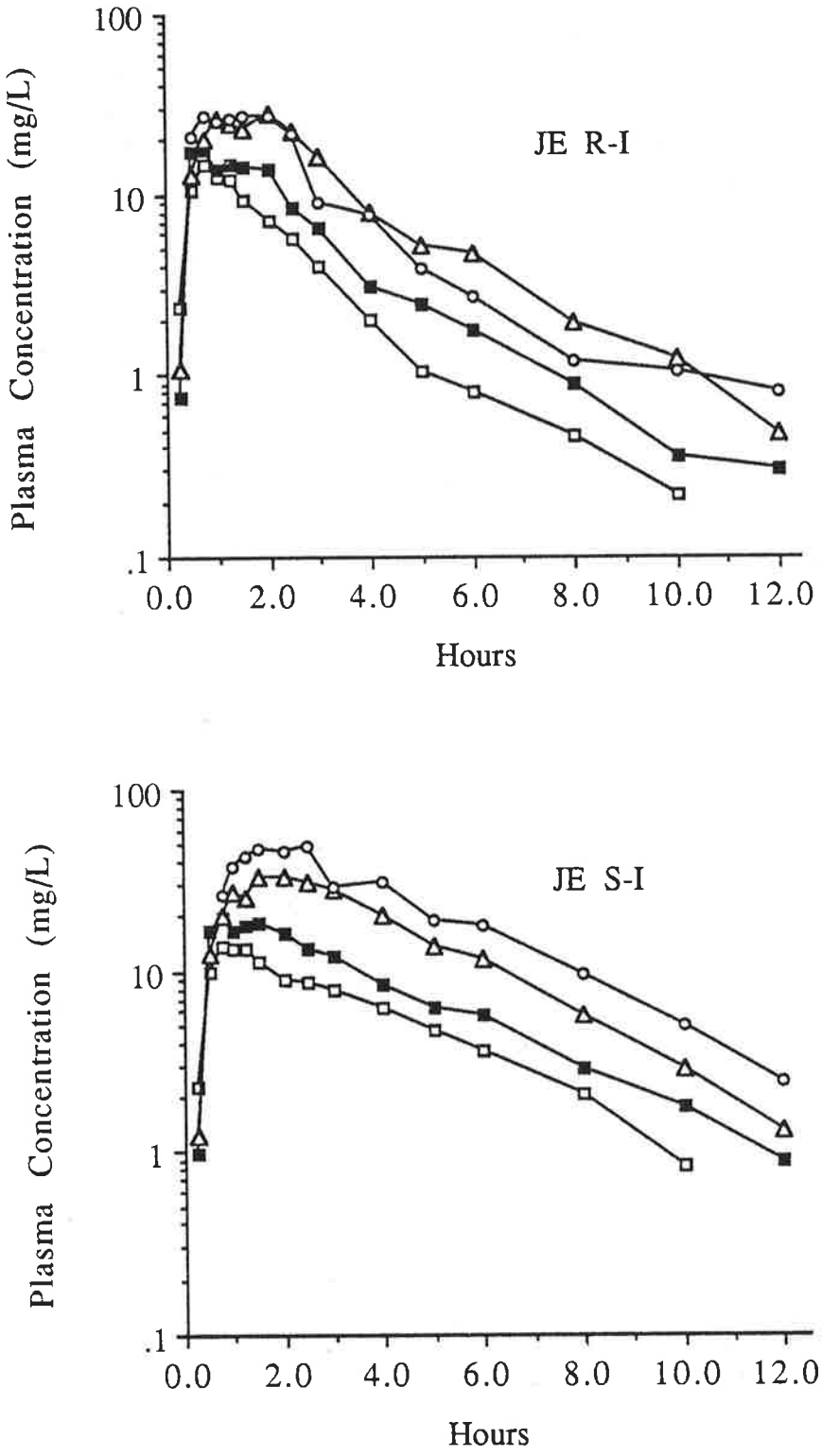


Figure 6.1 (continued).

Plasma concentration-time profiles of R-I (upper panel) and S-I (lower panel) after oral administration of RS-I (□ 200mg, ■ 400mg, △ 800mg and ○ 1200mg).

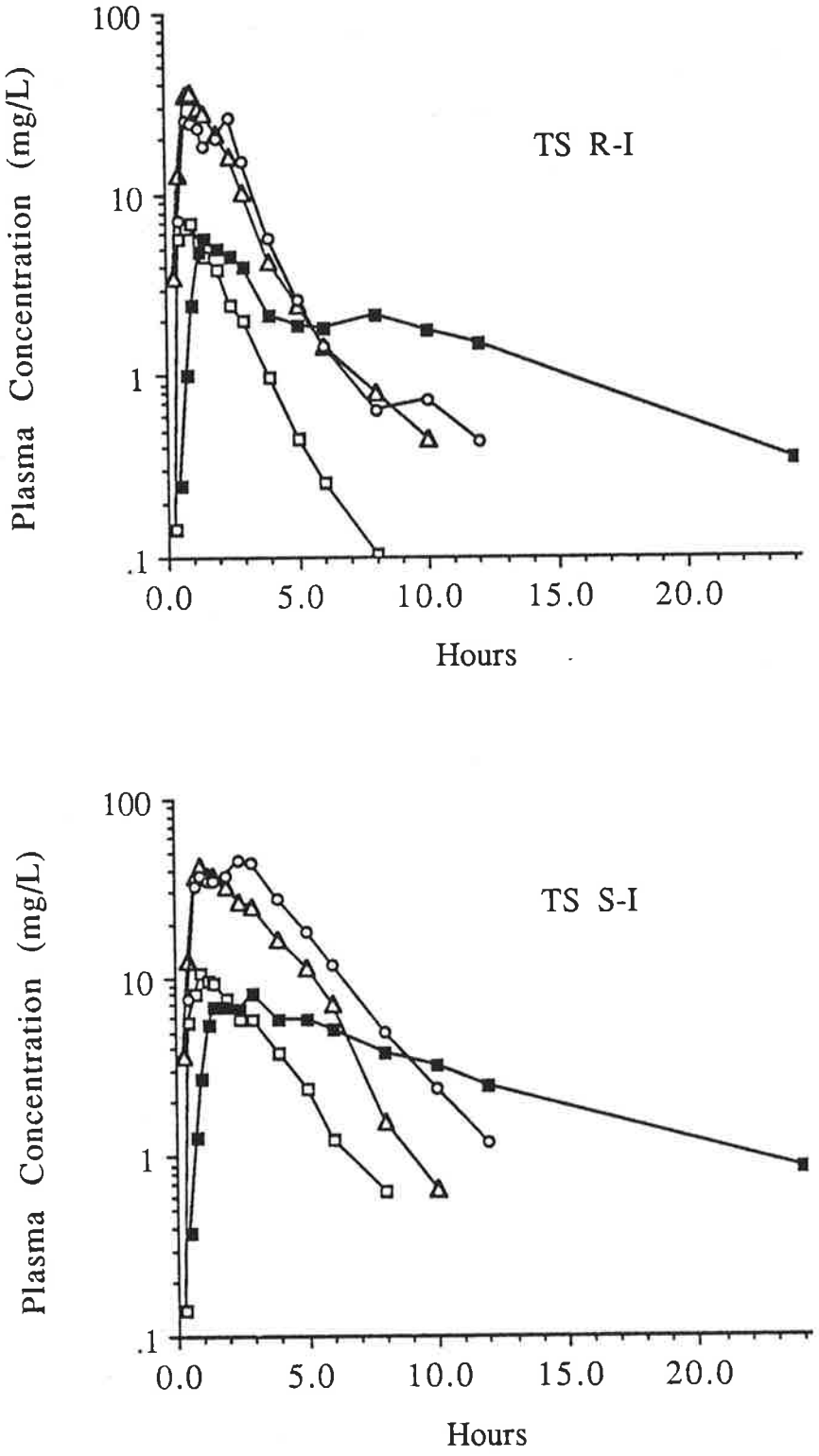


Figure 6.1 (continued).

Plasma concentration-time profiles of R-I (upper panel) and S-I (lower panel) after oral administration of RS-I (▫ 200mg, ■ 400mg, ▵ 800mg and ○ 1200mg).

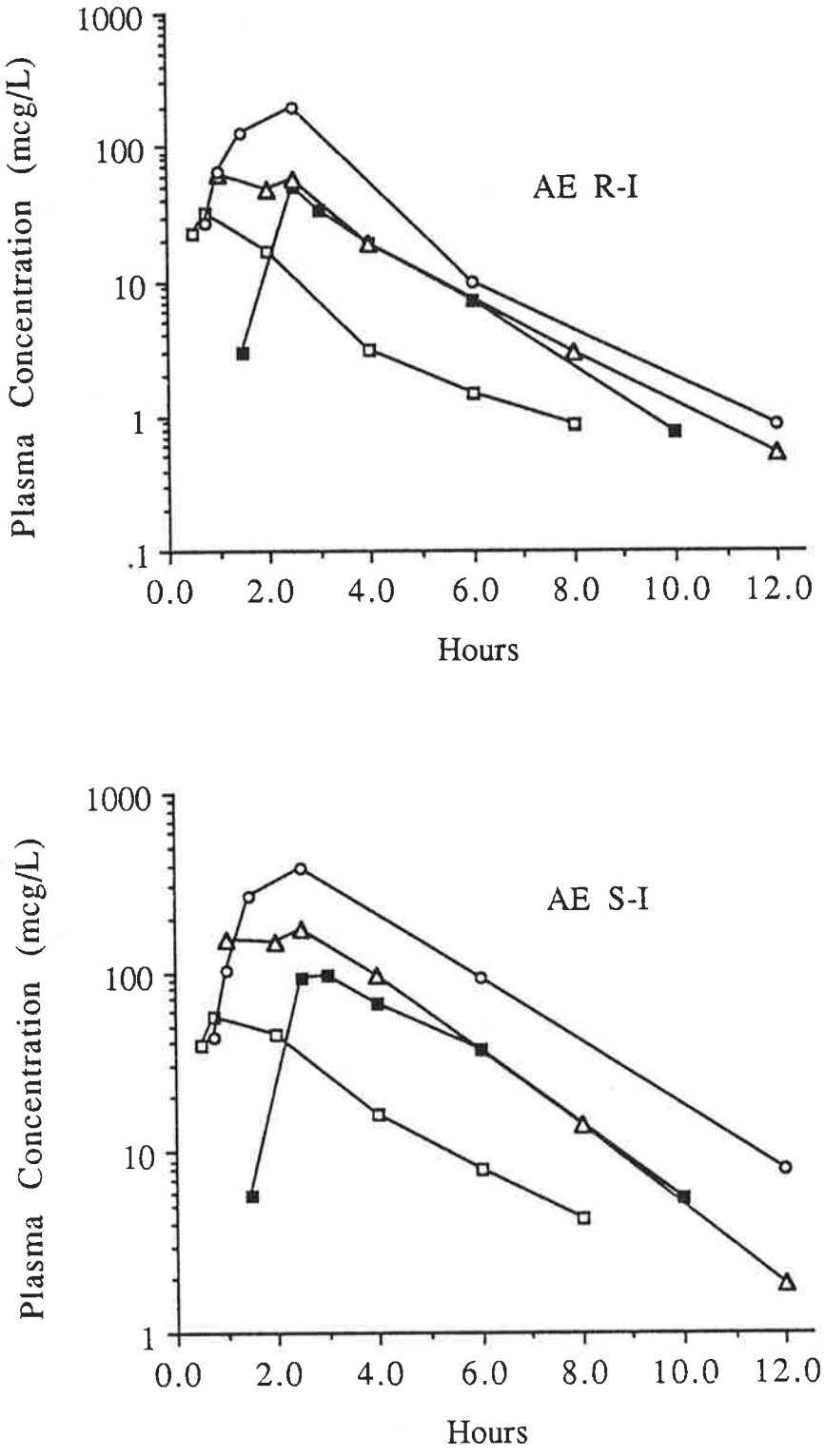


Figure 6.2.

Plasma concentration-time profiles of unbound R-I (upper panel) & unbound S-I (lower panel) after administration of RS-I (□ 200mg, ■ 400mg, △ 800mg and ○1200mg).

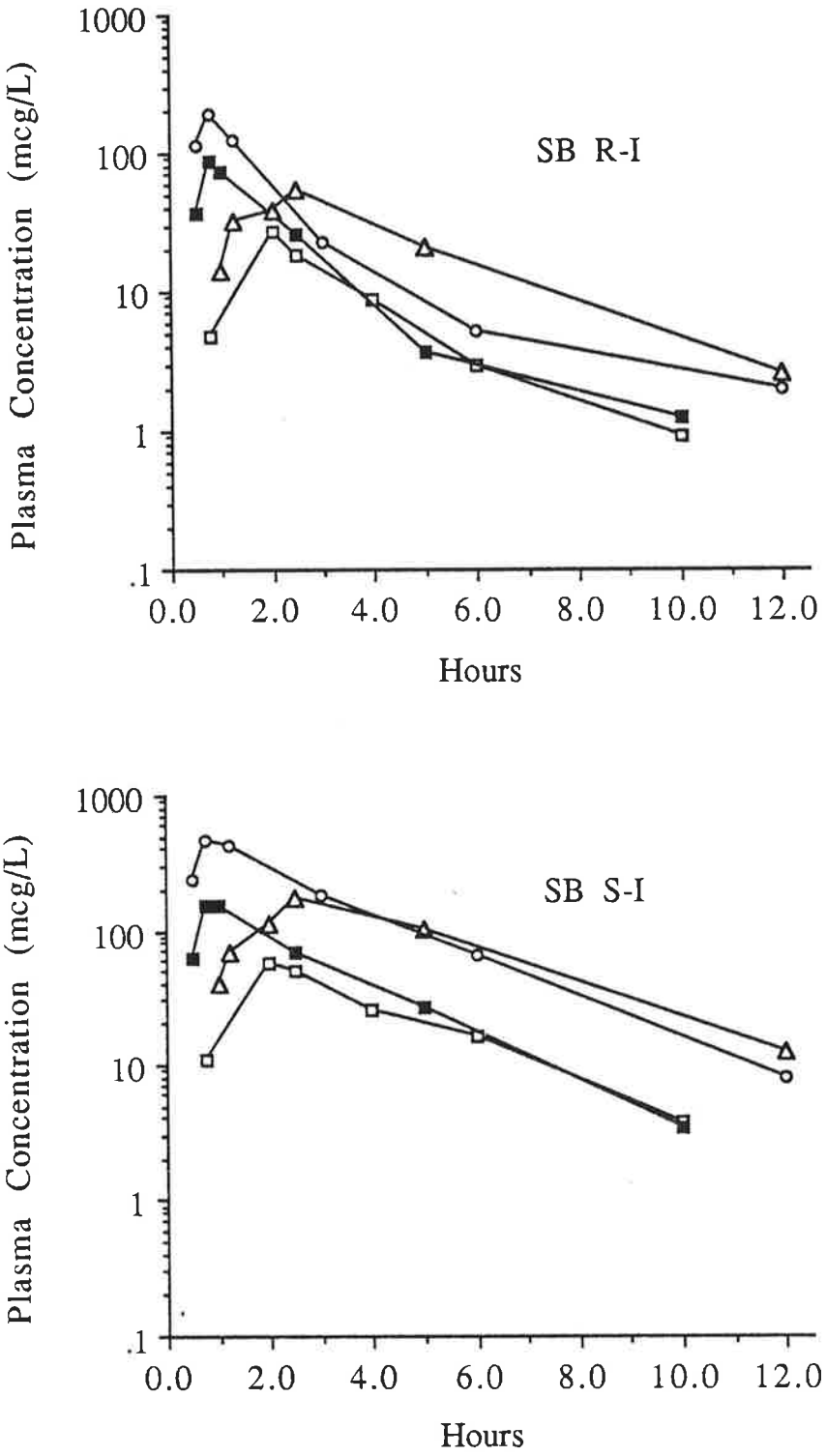


Figure 6.2 (continued).

Plasma concentration-time profiles of unbound R-I (upper panel) & unbound S-I (lower panel) after administration of RS-I (□ 200mg, ■ 400mg, △ 800mg and ○ 1200mg).

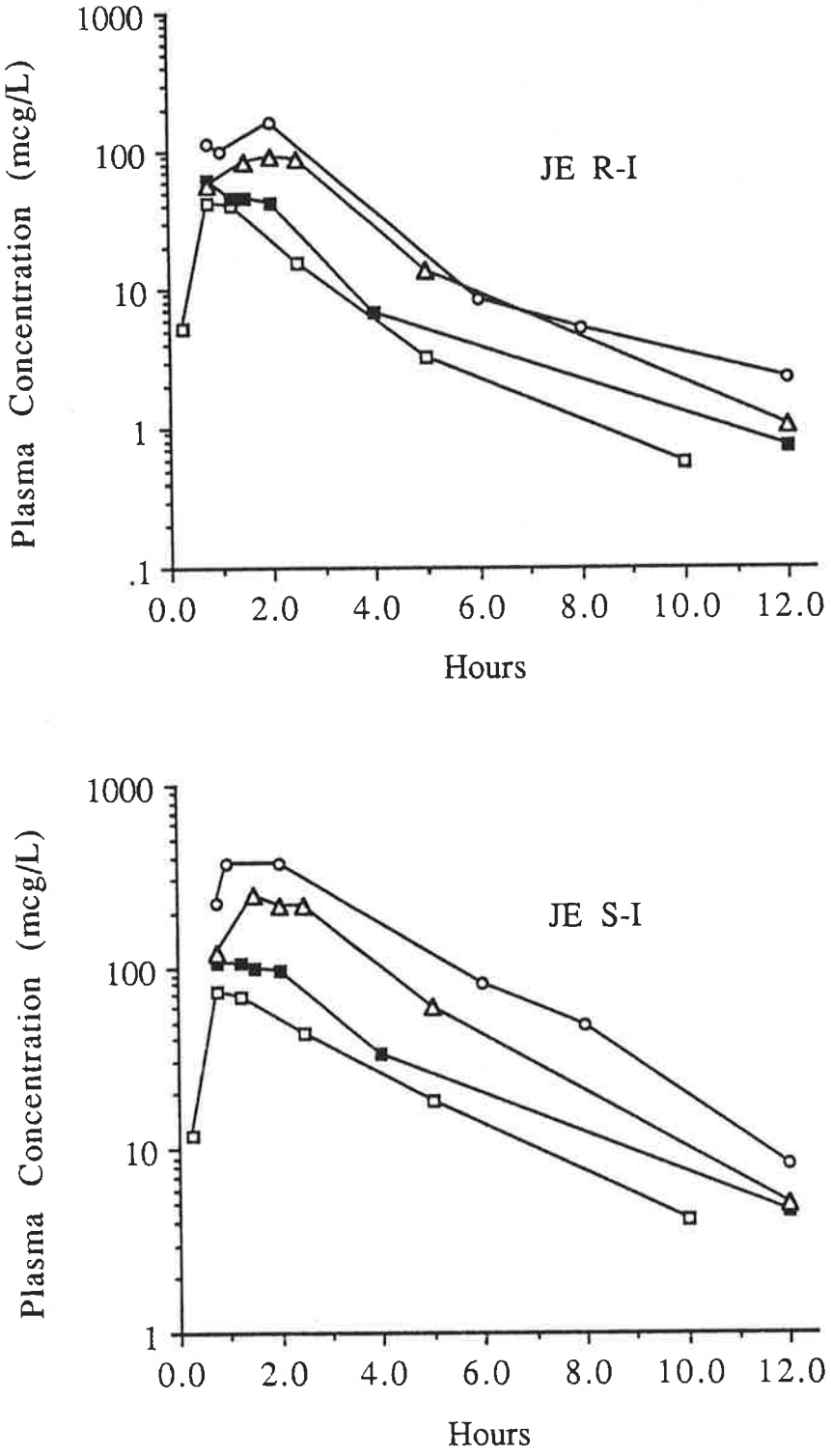


Figure 6.2 (continued).

Plasma concentration-time profiles of unbound R-I (upper panel) & unbound S-I (lower panel) after administration of RS-I (□ 200mg, ■ 400mg, △ 800mg and ○1200mg).

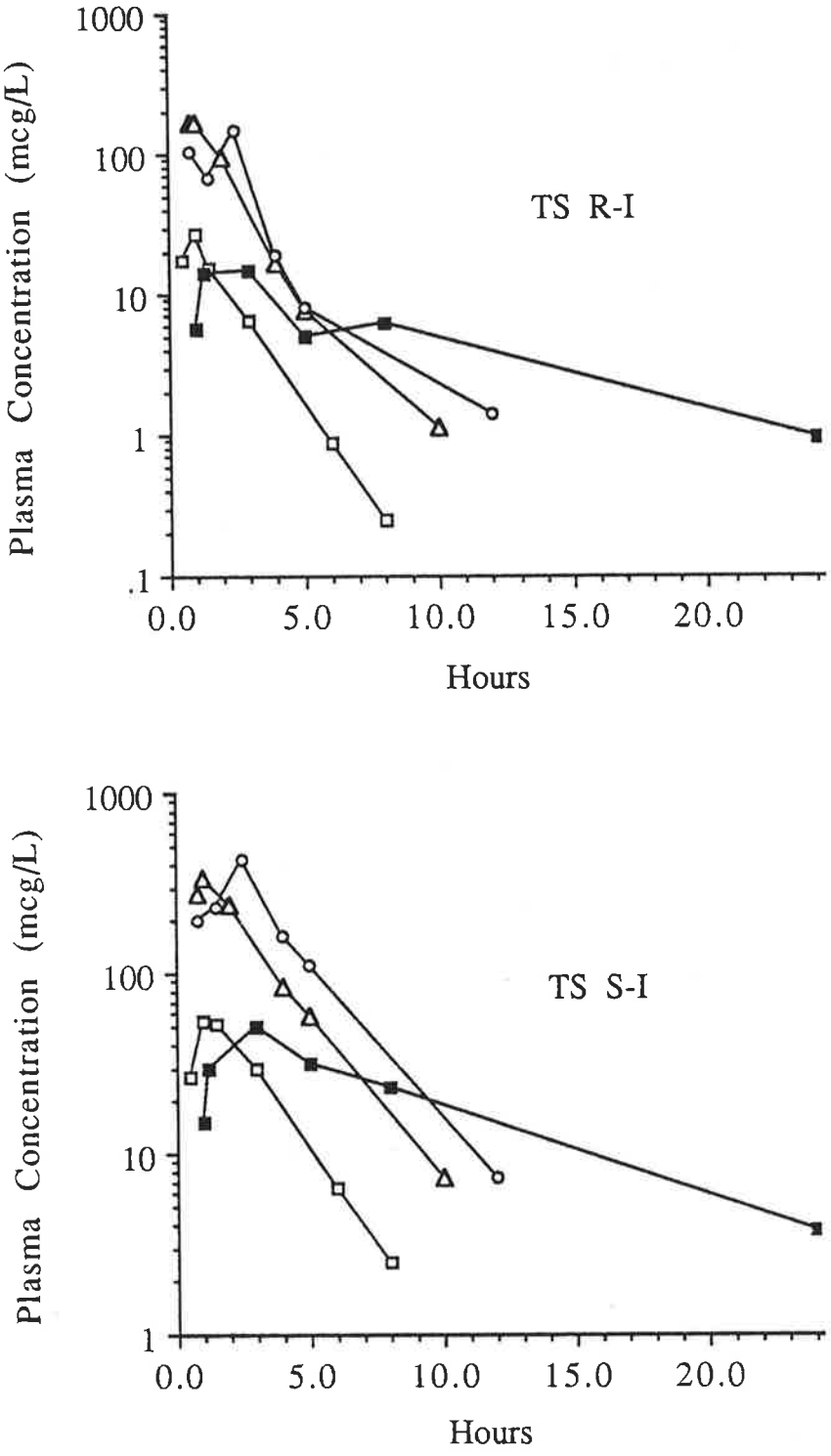


Figure 6.2 (continued).

Plasma concentration-time profiles of unbound R-I (upper panel) & unbound S-I (lower panel) after administration of RS-I (□ 200mg, ■ 400mg, △ 800mg and ○ 1200mg).

Table 6.3. Individual AUC and AUC_u data for R-I and S-I at each dose level

Volunteer		A U C (mg.min/L) 200mg	400mg	800mg	1200mg
AE	R-I	1423	1868	3624	5774
	S-I	2335	3559	7403	13381
SB	R-I	1498	2686	3660	4271
	S-I	2727	4474	8194	11757
JE	R-I	1921	2952	5624	5895
	S-I	3383	5128	9507	14685
TS	R-I	890	2535	4747	4721
	S-I	2014	4935	8836	11886

Volunteer		A U C_u (mg.min/L) 200mg	400mg	800mg	1200mg
AE	R-I	4.05	6.79	12.3	28.5
	S-I	11.4	21.8	42.3	83.5
SB	R-I	4.35	9.56	13.2	19.1
	S-I	12.8	25.1	47.8	83.8
JE	R-I	5.61	8.89	17.8	27.1
	S-I	15.7	25.3	53.6	92.5
TS	R-I	2.81	7.12	21.4	20.8
	S-I	10.4	26.9	58.8	83.5

Table 6.4. Mean (SD) pharmacokinetic parameters of R-I and S-I after oral administration of four doses of racemic ibuprofen.

	R-I				S-I			
	Dose of RS-Ibuprofen (mg)							
	200	400	800	1200	200	400	800	1200
t_{max} (hour)	1.13 (0.59)	1.38 (0.83)	1.57 (0.82)	1.81 (0.85)	1.13 (0.59)	1.87 (1.29)	1.89 (0.74)	2.19 (0.62)
C_{max} (mg/L)	10.0 (3.3)	14.1 (6.6)	24.4 (9.6)	29.5 (3.4)	11.3 (1.7)	16.9* (6.8)	31.6* (8.4)	47.7* (1.8)
$t_{1/2}$ (hour)	1.86 (0.54)	3.23 (1.95)	2.13 (0.37)	4.24 (2.19)	1.93 (0.36)	3.36 (2.43)	1.95 (0.41)	2.01 (0.26)
$\bar{f}_u$ (x100)	0.296 (0.013)	0.325 (0.040)	0.367 (0.059)	0.461 (0.024)	0.484* (0.025)	0.554* (0.049)	0.596* (0.047)	0.668* (0.047)
AUC (mg.min/L)	1433 (423)	2510 (462)	4414 (960)	5165 (796)	2620* (586)	4524* (699)	8485* (899)	12930* (1384)
AUC_u (mg.min/L)	4.21 (1.2)	8.09 (1.3)	16.2 (4.2)	23.9 (4.6)	12.6* (2.3)	24.8* (2.1)	50.6* (7.2)	85.9* (4.4)

* Statistically different ($p<0.05$) from the corresponding value for R-I at the same dose level.

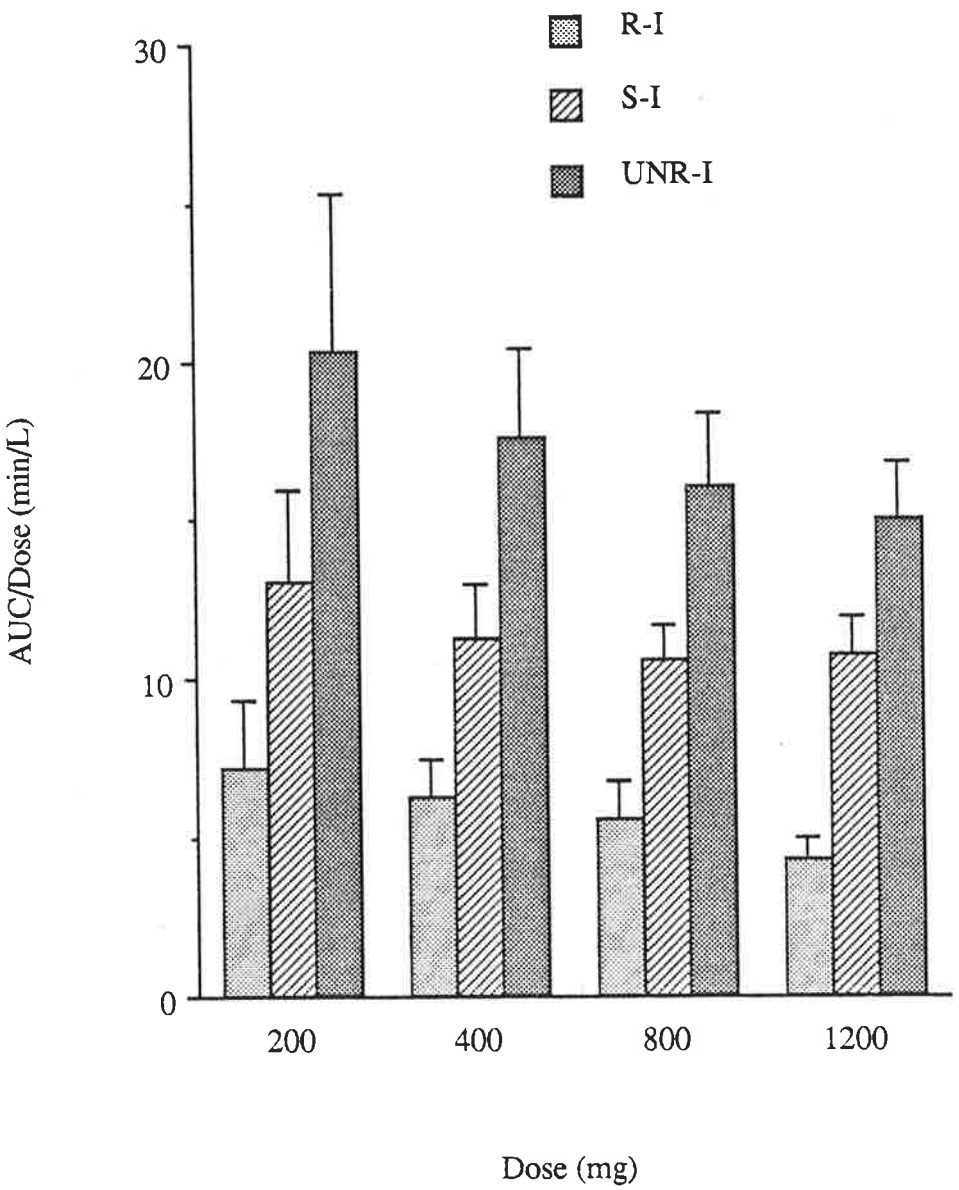


Figure 6.3. Bar graphs (mean and SD) of dose-normalised AUC of R-I, S-I and UNR-I versus the administered dose.

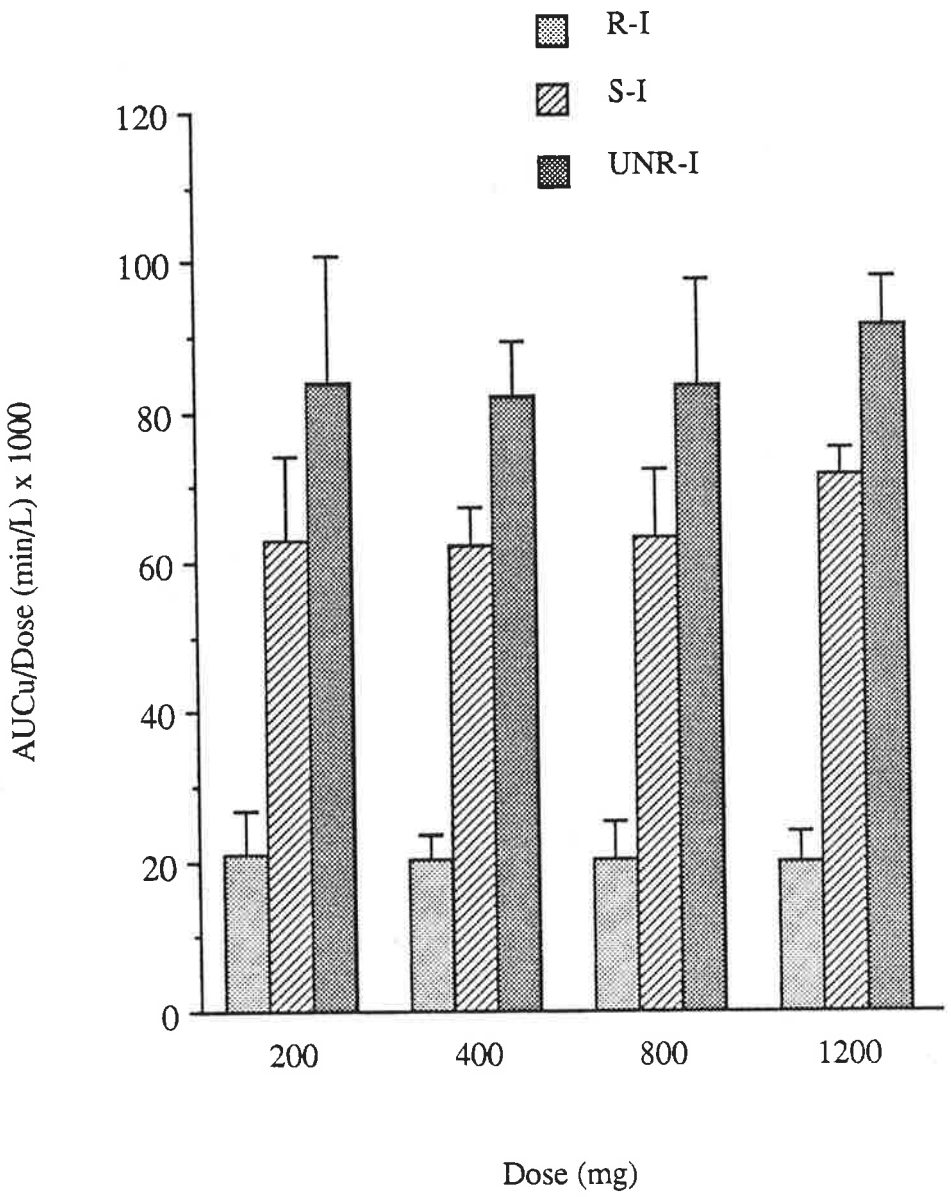


Figure 6.4. Bar graphs (mean and SD) of dose-normalised AUC_u of R-I, S-I and UNR-I versus the administered dose.

Table 6.5. Summary of the statistical analysis performed to test for changes with dose in AUC/dose, AUC_u/dose and $\bar{f}_u$ of R-I and S-I.

Parameter	ANOVA	Ibuprofen Dose*				Linear Regression Analysis**
		200	400	800	1200	
AUC^{R-I}/dose	$p < 0.05$	d	d	NS	a,b	$p < 0.05$
AUC^{S-I}/dose	NS	not performed				NS
AUC_u^{R-I}/dose	NS	not performed				NS
AUC_u^{S-I}/dose	NS	not performed				NS
$\bar{f}_u^{R-I}$	$p < 0.05$	b,c,d	a,d	a,d	a,b,c	-
$\bar{f}_u^{S-I}$	$p < 0.05$	c,d	d	a,d	a,b,c	-

*If a difference between the four doses was detected by ANOVA, Fischer's least significant difference test was used to test for differences between individual doses. A significant difference from the corresponding parameter at the 200, 400, 800 or 1200mg dose level is designated a, b, c or d, respectively.

**taken to be a significant change with dose if the 95% confidence interval of the slope of the regression line excluded zero.

NS = Not Significant

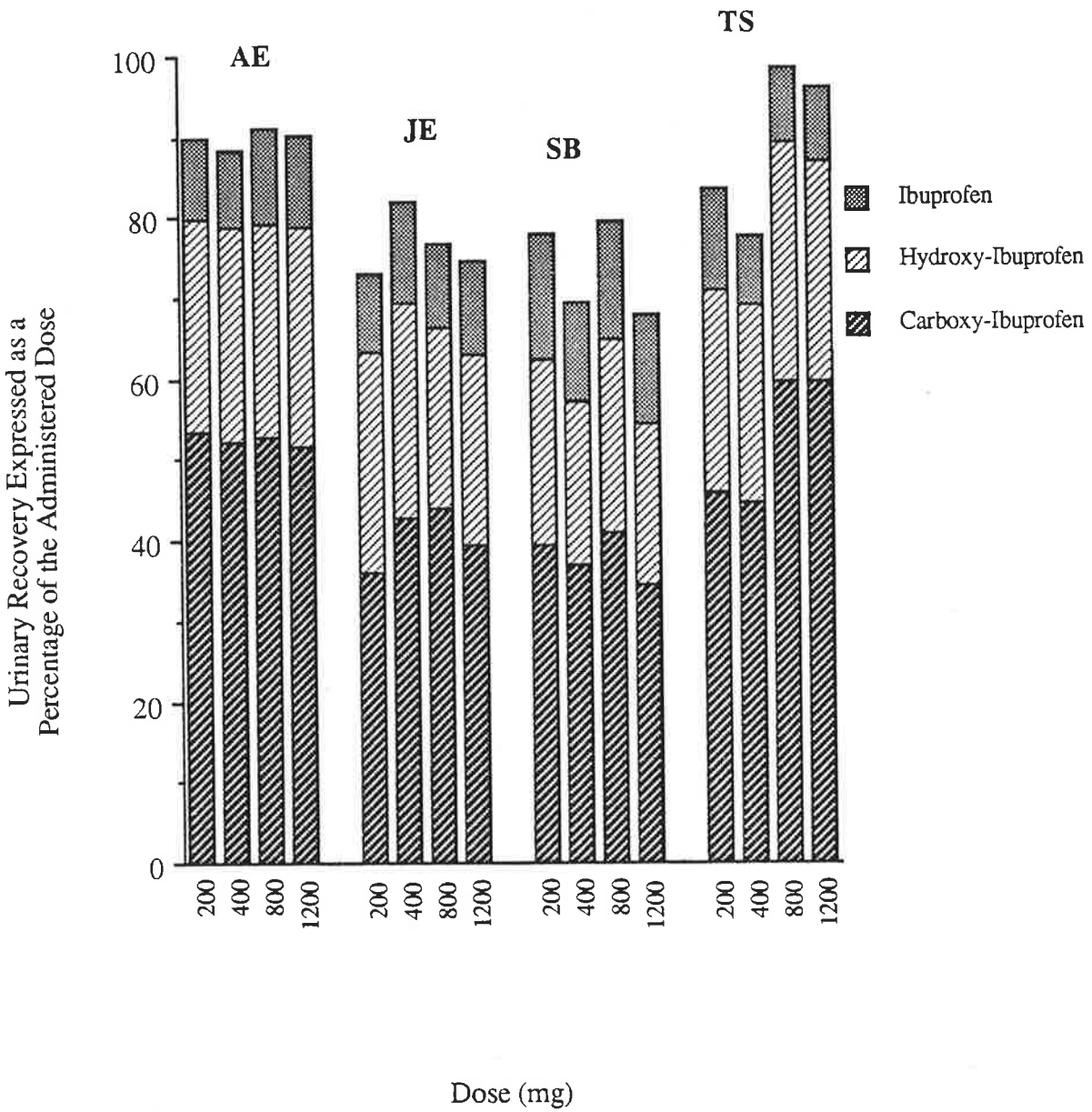


Figure 6.5. Urinary recovery (0-36 hours) of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen, as a percentage of the administered dose, for each volunteer at each dose level.

Table 6.6. Urinary recoveries (0-36 hours) of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen after hydrolysis of glucuronide conjugates. Data are expressed as mean (SD) recovery as a percentage of the administered dose.*

	DOSE OF RS-IBUPROFEN			
	200mg	400mg	800mg	1200mg
Ibuprofen	12.1 (2.6)	10.7 (2.0)	11.5 (2.5)	11.4 (1.9)
Hydroxy-ibuprofen	25.3 (1.7)	24.4 (2.9)	25.7 (3.3)	24.5 (3.7)
Carboxy-ibuprofen	43.8 (7.7)	44.2 (6.3)	49.2 (8.4)	46.3 (11.4)
Total recovery	81.2 (7.2)	79.4 (8.0)	86.5 (10.1)	82.2 (13.1)

*Analysis of variance indicated that there was no significant difference between the four doses in the urinary recovery of each species, and in the total recovery, over the four dosages.

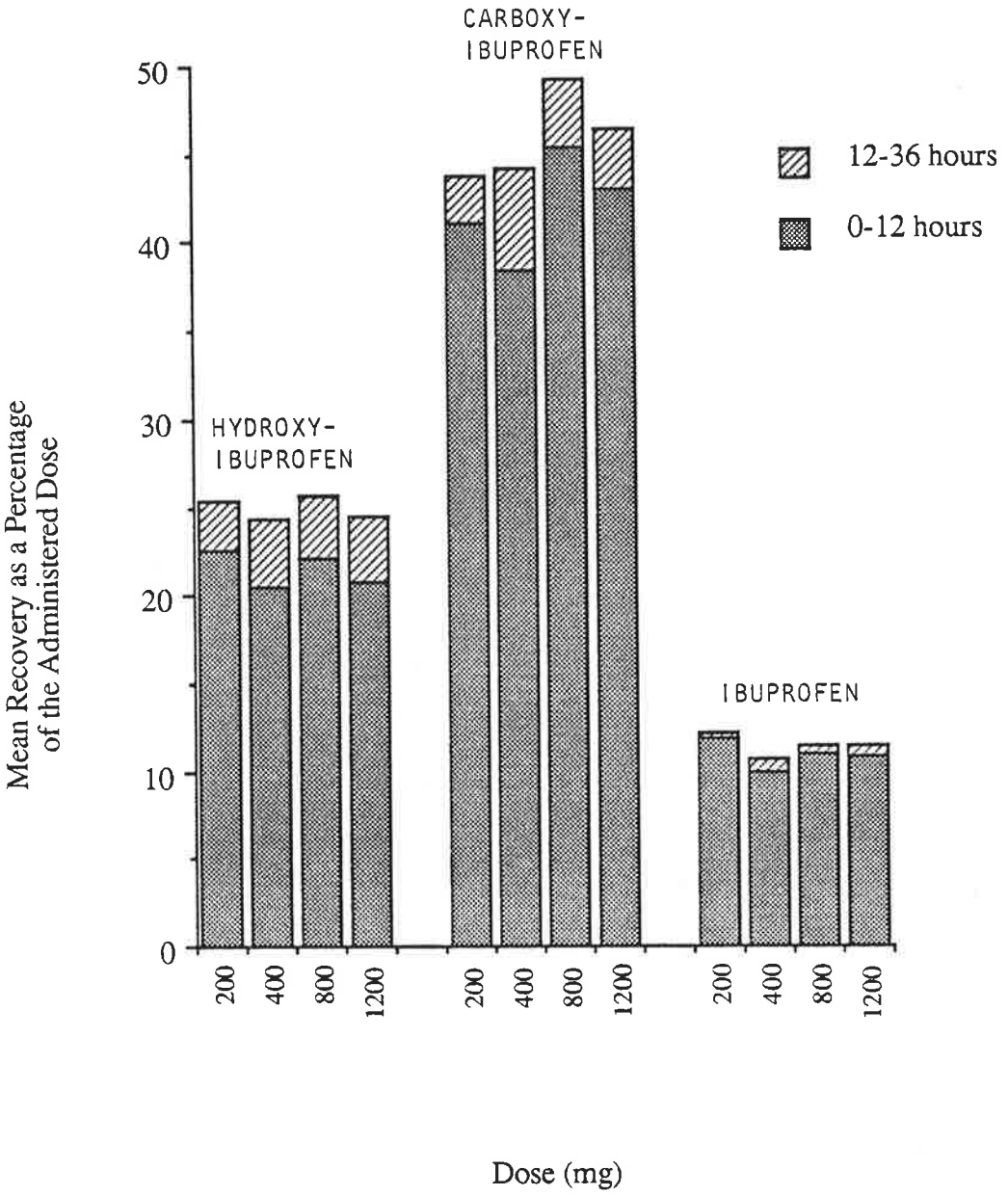


Figure 6.6. The mean recovery of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen in urine during the 0-12 hour and 12-36 hour collection intervals.

6.4. DISCUSSION

The absorption of both ibuprofen enantiomers was, in general, rapid, and in all 16 treatments the peak plasma concentrations of R-I and S-I were achieved within 3 hours of dosing. The t_{max} of R-I and S-I were not significantly different (Table 6.4), and did not change significantly with increasing ibuprofen dose. With one exception, C_{max} of S-I exceeded that of R-I, and the magnitude of the difference between the mean C_{max} of R-I and that of S-I increased with dose from 13% at 200mg through to 62% at 1200mg (Table 6.4).

At all four doses, there was no difference between the mean $t_{1/2}$ of the enantiomers (Table 6.4). In a number of cases (refer to Figure 6.1) the post-peak portion of the plasma total concentration-time profile for R-I displayed a biphasic pattern, and this added considerable variability to the $t_{1/2}$ data for this enantiomer. For one volunteer (TS), after the 400mg dose, the $t_{1/2}$ of R-I and S-I (6.04 and 6.99 hours, respectively) greatly exceeded the corresponding estimates determined in the same volunteer at the other three dose levels. This was most likely due to a protracted absorption phase, resulting in a "flip-flop" plasma concentration-time profile for the individual enantiomers, whereby the terminal slope represented drug absorption rather than elimination (Rowland & Tozer 1989). Interestingly, there was no apparent reduction in the extent of absorption of ibuprofen in this case, since 77.7% of the administered dose was recovered in the urine. The time course of urinary recovery of ibuprofen and its metabolites was, however, different. In this single case, 63% of the total dose recovered was excreted in the 0-12 hour urine collection period, while 27%, and 10% was recovered in the 12-24 and 24-36 hour periods, respectively. In contrast, for the 15 other treatments, a mean (± SD) of 92.1 ± 2.7% of the recovered material was found in the 0-12 hour urine sample, while the remainder was excreted during the 12-24 hour period.

At all four dose levels, there was a statistically significant difference ($p < 0.01$) between the $\bar{f}_u$ of R-I and S-I (Table 6.4), and, for both enantiomers, $\bar{f}_u$ increased significantly with dose (Table 6.5); indicating that the plasma protein binding of ibuprofen was both enantioselective and concentration-dependent. The finding of concentration-dependent plasma binding for the individual enantiomers is consistent with earlier studies which found that the unbound fraction of unresolved ibuprofen in plasma increased with dose (Aarons et al 1983b; Grennan et al 1983; Lockwood et al

1983a, 1983b). The plasma protein binding of R-I and S-I will be discussed in greater detail in Chapter 9.

For all 16 treatments, AUC and AUC_u for S-I were substantially greater than for R-I, and the differences were significant at all four dose levels ($p< 0.05$; Table 6.4). The mean AUC of R-I and of S-I after the 800mg dose (4414 and 8485 mg.min/L, Table 6.4) compare favourably with the mean values reported by Lee et al (1985) after oral administration of the same dose of racemic ibuprofen (4938 and 7698 mg.min/L for R-I and S-I, respectively). In addition, the range of values for the AUC of UNR-I found in the present study between the 400mg and 1200mg doses (7032 to 18095 mg.min/L; determined by summing the corresponding AUC values for R-I and S-I; Table 6.4), are similar in magnitude to those values (7560 to 17580 mg.min/L) reported by Lockwood et al (1983a) after oral administration of the same range of racemic ibuprofen doses.

The influence of dose on the pharmacokinetics of R-I and S-I was examined using the compartment-independent physiological approach described by Wilkinson & Shand (1975) which was adapted for the special circumstances encountered with ibuprofen, as described below.

After oral administration of a drug, the area under the plasma concentration-time curve from time zero to infinity (AUC) is given by equation 6.1,

$$AUC = \frac{f\,D}{CL} \qquad (6.1)$$

where f (oral bioavailability) is the fraction of the administered dose (D) which is available systemically, and CL is the total body clearance of the drug from plasma. Equation 6.1, when applied to R-I becomes,

$$AUC^{R\text{-}I} = \frac{f^{R\text{-}I}\,D^{R}}{CL^{R\text{-}I}} \qquad (6.2)$$

The total clearance of R-I is the sum of its partial clearances via the various elimination routes, mainly inversion to S-I (INV); oxidation (OX); and glucuronidation (GLU). Thus

$$CL^{R\text{-}I} = CL^{R\text{-}I,INV} + CL^{R\text{-}I,OX} + CL^{R\text{-}I,GLU} \qquad (6.3)$$

The fraction of R-I which is metabolically inverted to S-I (FI) is given by equation 6.4.

$$FI = \frac{CL^{R\text{-}I,INV}}{CL^{R\text{-}I,INV} + CL^{R\text{-}I,OX} + CL^{R\text{-}I,GLU}} \qquad (6.4)$$

Given that the oral bioavailability of ibuprofen is high (see below) the amount of S-I generated from an oral dose of R-I (AI) is given by equation 6.5.

$$AI = f^{R\text{-}I} D^{R\text{-}I} FI \qquad (6.5)$$

Hence, for S-I, after a single oral dose of racemic ibuprofen, equation 6.1 becomes,

$$AUC^{S\text{-}I} = \frac{f^{S\text{-}I} D^{S\text{-}I} + f^{R\text{-}I} D^{R\text{-}I} (FI)}{CL^{S\text{-}I}} \qquad (6.6).$$

where the total clearance of S-I is the sum of its individual partial clearances (largely by oxidation and glucuronidation), such that

$$CL^{S\text{-}I} = CL^{S\text{-}I,OX} + CL^{S\text{-}I,GLU} \qquad (6.7)$$

Oral bioavailability (f) is defined as the product of the fraction of the administered dose which is absorbed from the gastrointestinal tract (f_a) and the fraction of the absorbed dose which escapes first-pass hepatic elimination (f_{fp}). It is known, from the data of Cox (1988), that for both R-I and S-I, the AUC after oral administration is the same as that after i.v. administration (when oral and i.v. doses are equal). Therefore, the fraction of the administered dose of each enantiomer which escapes first-pass hepatic metabolism is close to unity (i.e. both enantiomers have very low hepatic extraction ratios; as discussed in section 3.6). In addition, the results of the present study (Table 6.6) suggest that after the administration of racemic ibuprofen in tablet form, the fraction of each enantiomer absorbed from the gastrointestinal tract was both extensive and independent of the magnitude of the administered dose. Hence, for the purpose of clarity in the following discussion, $f^{R\text{-}I}$ and $f^{S\text{-}I}$ have been assigned a value of unity. Accordingly, after administration of racemic ibuprofen (where $D^{R\text{-}I} = D^{S\text{-}I}$), equations 6.2 and 6.6 can be simplified to equations 6.8 and 6.9, respectively.

$$AUC^{R\text{-}I} = \frac{D^{R\text{-}I}}{CL^{R\text{-}I}} \qquad (6.8)$$

$$AUC^{S\text{-}I} = \frac{D^{S\text{-}I}(1 + FI)}{CL^{S\text{-}I}} \qquad (6.9)$$

The exact site(s) of metabolism of R-I and S-I is unknown. However, Lee et al (1985) calculated $CL^{R\text{-}I}$ and $CL^{S\text{-}I}$, to both be in the order of 70 ml/min (see section 3.6.3). Even assuming the lowest possible blood to plasma concentration ratio (1-haematocrit) for ibuprofen of about 0.55, a plasma clearance of 70 ml/min corresponds to a blood clearance of 130 ml/min, which is substantially lower than the blood flow rate to the liver (1.5 L/min) and to other organs which have been associated with xenobiotic metabolism (e.g. kidney and lung). This suggests that the metabolism of each enantiomer via each of the various processes is limited by the capacity of extracting organs involved. The unlikely exception would be if metabolism takes place across an organ of very low blood flow, in which case extraction may be perfusion rate limited.

According to the "well-stirred" physiological model of drug extraction (Gibaldi & Perrier 1982), if the metabolic clearance of a drug across an organ is substantially lower than the organ blood flow, then the clearance across that organ is a function of

(i) the unbound fraction of the drug, and

(ii) the intrinsic ability of the organ to metabolize the drug in the absence of drug binding or organ blood flow restrictions (CL_{int}) as described by equation 6.10.

$$CL = f_u\, CL_{int} \qquad (6.10)$$

Accordingly,

$$CL^{R\text{-}I} = f_u^{R\text{-}I}\, CL_{int}^{R\text{-}I} \qquad (6.11)$$

and

$$CL^{S\text{-}I} = f_u^{S\text{-}I}\, CL_{int}^{S\text{-}I} \qquad (6.12)$$

Combining equations 6.8 and 6.11, the following useful expression for describing the single dose AUC of R-I results.

$$\mathbf{AUC^{R\text{-}I} = \frac{D^{R\text{-}I}}{f_u^{R\text{-}I}\, CL_{int}^{R\text{-}I}}} \qquad \mathbf{(6.13)}$$

$$\text{where } CL_{int}^{R\text{-}I} = CL_{int}^{R\text{-}I,INV} + CL_{int}^{R\text{-}I,OX} + CL_{int}^{R\text{-}I,GLU} \qquad (6.13a)$$

Similarly, combination of equations 6.9 and 6.12, yields the following expression for the AUC of S-I.

$$AUC^{S\text{-}I} = \frac{D^{S\text{-}I}\ (1 + FI)}{fu^{S\text{-}I}\ Cl_{int}^{S\text{-}I}} \qquad (6.14)$$

$$\text{where} \qquad CL_{int}^{S\text{-}I} = CL_{int}^{S\text{-}I,OX} + CL_{int}^{S\text{-}I,GLU} \qquad (6.14a)$$

$$\text{and} \qquad FI = \frac{CL_{int}^{R\text{-}I,INV}}{CL_{int}^{R\text{-}I,INV} + CL_{int}^{R\text{-}I,OX} + CL_{int}^{R\text{-}I,GLU}} \qquad (6.14b)$$

By definition, the area under the plasma unbound concentration-time curve (AUC_u) is the product of f_u and AUC. Therefore,

$$AUC_u^{R\text{-}I} = \frac{D^{R\text{-}I}}{CL_{int}^{R\text{-}I}} \qquad (6.15)$$

$$\text{and} \qquad AUC_u^{S\text{-}I} = \frac{D^{S\text{-}I}\ (1 + FI)}{CL_{int}^{S\text{-}I}} \qquad (6.16)$$

Having derived these pertinent equations (6.13, 6.14, 6.15 and 6.16) it is possible to examine the effect of increasing dose of racemic ibuprofen on the pharmacokinetics of the individual enantiomers.

For R-I, there was a progressive decrease in the dose-normalised AUC with increasing dose, which extended to a 40% change between the 200 and 1200mg doses. Equation 6.13 predicts that such a change could arise from an increase with dose in $f_u^{R\text{-}I}$

and/or CL_{int}^{R-I}. However, there was a linear relationship between AUC_u of R-I and dose, which suggests that CL_{int}^{R-I} remained constant (equation 6.15), and that the concentration-dependent plasma protein binding of R-I (Tables 6.4 and 6.5) was the source of the non-linearity between dose and AUC^{R-I}.

Before assessing the pharmacokinetics of S-I, it is important to establish whether the fraction of R-I which was inverted to S-I (FI) was influenced by the magnitude of the administered dose. Enzyme kinetics predict that the intrinsic clearance of a metabolic route would remain unchanged or decrease in the presence of increasing concentrations of substrate. Hence, because CL_{int}^{R-I} remained constant with increasing dose, it can be concluded (equation 6.13a) that the partial intrinsic clearances of the major metabolic pathways of R-I (including inversion) also remained unchanged, and therefore, that FI (equation 6.14b) was independent of the magnitude of the administered dose. This result is interesting because the inversion pathway is, at least in part, an enzymatic process (see section 3.5), and therefore potentially saturable with increasing substrate (R-I) concentrations.

Given that FI was dose-independent, changes in the dose-normalised AUC of S-I with dose would reflect changes in f_u^{S-I} and/or CL_{int}^{S-I} (equation 6.14). However, for the pharmacologically active enantiomer, the non-linear relationship between AUC and dose was less apparent than for R-I, and failed to reach statistical significance. For S-I, there was a relative decrease in the mean dose-normalised AUC of 19% between the 200 and 800mg doses, while there was no further change between the 800 and 1200mg doses. Interestingly, while the dose-normalised AUC^{S-I} remained constant between the 800 and 1200mg doses, the mean time-averaged unbound percentage of this enantiomer increased significantly (Table 6.5), from 0.596% to 0.668%. From a clinical view-point, it is AUC_u^{S-I} which assumes greatest importance because it is unbound drug which is available for interaction with receptors in the biophase. The mean dose-normalised AUC_u^{S-I} remained constant between 200 and 800mg, and between the 800 and 1200mg dose, increased by 13% (Figure 6.4). It must be emphasized that this small change was not statistically significant. However, one can speculate that it may reflect a slight decrease in CL_{int}^{S-I} at the 1200mg level. Although a further study involving a greater number of subjects would be required to substantiate this finding, it is anticipated to be of minor clinical importance.

To emphasize the limited value of conducting pharmacokinetic studies on ibuprofen without measuring the total and unbound plasma concentrations of the

individual enantiomers, it is useful to examine the equations derived above and consider the situation which would arise if CL_{int}^{S-I} and the sum of $CL_{int}^{R-I,OX}$ and $CL_{int}^{R-I,GLU}$ (i.e. those metabolic routes of R-I not associated with the inversion to S-I; referred to subsequently as $CL_{int}^{R-I,NON-INV}$) were of similar magnitude. Under such conditions a reduction in $CL_{int}^{R-I,INV}$ (and therefore CL_{int}^{R-I}) would lead to an increase in AUC_u^{R-I} (equation 6.15). Due to a reduction in the fraction of R-I inverted to S-I (equation 6.14b), there would be a decrease in AUC_u^{S-I} (equation 6.16). As a result, the sum of AUC_u^{R-I} and AUC_u^{S-I} (i.e. AUC_u^{UNR-I}) would remain unchanged. Hence, while the unbound concentrations of the active enantiomer (S-I) would be reduced, no change would be detected in the plasma unbound concentrations of unresolved ibuprofen.

The discussion below uses the data in Table 6.4, and those of previous workers, to support the premise that $CL_{int}^{R-I,NON-INV}$ and CL_{int}^{S-I} are of similar magnitude.

(i) Using the mean AUC_u of R-I after the 800mg dose of the racemate (4414 mg.min/L; Table 6.4) and equation 6.15, CL_{int}^{R-I} can be calculated to be 24.7 L/min.

(ii) Lee et al (1985) estimated that after a 400mg oral dose of R-I to four healthy adults, an average of 63% was inverted to S-I. If it is assumed that in the present study, after the 800mg dose of racemic ibuprofen, an average of 60% of R-I was metabolised to S-I, $CL_{int}^{R-I,INV}$ and $CL_{int}^{R-I,NON-INV}$ can be estimated to be 14.8 and 9.9 L/min, respectively.

(iii) From the mean AUC_u of S-I after the 800mg dose (Table 6.4), and assuming FI to be 0.6, CL_{int}^{S-I} (equation 6.16) can be estimated to be 12.6 L/min.

Hence, $CL_{int}^{R-I,NON-INV}$ (9.9 L/min) and CL_{int}^{S-I} (12.6 L/min) are likely to be of similar magnitude. Therefore, as shown above, AUC_u of unresolved ibuprofen may be relatively insensitive to changes in $CL_{int}^{R-I,INV}$, even though this latter parameter is an important determinant of the amount of the administered dose of racemic ibuprofen which is presented to the body as the active enantiomer. It is therefore recommended that future studies which examine the influence of factors such as the magnitude of dose, disease state or interacting drugs, on ibuprofen pharmacokinetics must use enantioselective assays to measure the total and unbound plasma concentrations of the

individual ibuprofen enantiomers.

6.5 CONCLUSION

The results of the present study indicated that in healthy volunteers the extent of oral absorption of ibuprofen was independent of the magnitude of the administered dose. The disposition of ibuprofen was found to be enantioselective over the therapeutic dose range. Both enantiomers exhibited concentration-dependent plasma protein binding, which, for R-I, resulted in a significant non-linear relationship between AUC and dose. It was rationalised that the fraction of administered R-I which was inverted to its mirror-image form was dose-independent. Importantly, over the range of doses used clinically, there were no dramatic changes in the dose-normalised AUC and AUC_u of the active enantiomer, S-I. It was shown that pharmacokinetic studies on ibuprofen which rely on data for unresolved species only, are of limited usefulness.

Chapter 7
The Effect of Ibuprofen Dose on the Magnitude and Duration of Platelet Cyclo-oxygenase Inhibition.

7.1. INTRODUCTION

It is well documented that ibuprofen inhibits the aggregation of platelets in response to a number of stimuli, including adrenaline, arachidonic acid, collagen and thrombin (Brooks et al 1973; Cox et al 1987; Cronberg et al 1984; Ikeda 1977; Longenecker et al 1985; McIntyre et al 1978; O'Brien1968; Parks et al 1981). It is likely that this anti-platelet activity is related to the ability of ibuprofen to inhibit prostaglandin synthetase (cyclo-oxygenase; see section 3.2.4), and as a result, decrease the synthesis, by platelets, of thromboxane A2 (TXA2), a vasoconstrictor and powerful promoter of platelet aggregation, identified in 1975 by Hamberg and coworkers.

Although the precise physiological role of TXA2 is unknown, it appears to be involved in the positive feed-back process of blood coagulation, and in the regulation of haemostasis (Mustard et al 1980). Because of its pro-aggregatory and vasoconstrictor properties, TXA2 has been implicated, by many workers, to be a contributing factor in the aetiology of a variety of cardiovascular disorders, including myocardial ischaemia, variant angina, coronary vasospasm and myocardial infarction (Ruffulo & Nichols 1988). In addition, TXA2 may be a causative factor in hypertension and may contribute to cardio-pulmonary dysfunction associated with endotoxic shock (Ruffulo & Nichols 1988).

From a clinical viewpoint, there may be negative and positive features to the ability of ibuprofen to inhibit platelet TXA2 synthesis. On the negative side, the ibuprofen-induced decrease in platelet aggregability, and increase in bleeding time (Brooks et al 1973; Ikeda 1977; McIntyre et al 1978) limits the use of the drug in patients with pre-existing blood clotting disorders and in those who are to undergo surgical procedures. In addition, the gastrointestinal blood loss (Brooks et al 1973; Carson et al 1987; Semble & Wu 1987) and increased incidence of bruising (Ikeda 1977) experienced by some patients receiving ibuprofen, may partly be due to the effects of the drug on TXA2 synthesis.

On the positive side, inhibitors of TXA2 synthesis may prove useful for the treatment of cardiovascular disorders such as those described above. Indeed, in animal

models, ibuprofen has been found to reduce the size of experimentally induced myocardial infarcts (Lefer & Polansky 1979); diminish platelet adhesion to, and therefore promote the patency of, artificial grafts (Kaye et al 1984; Kon et al 1984; Lovaas et al 1983); protect against arachidonate-induced sudden-death (Roth et al 1983); and to decrease the cardio-pulmonary consequences of systemic toxin exposure (Almqvist et al 1984; Bone & Jacobs 1984).

The present study was designed to examine the effect of a range of single oral doses of racemic ibuprofen on the time course of inhibition of platelet TXA2 synthesis. TXA2 is chemically unstable in biological fluids, and is rapidly converted to thromboxane B2 (TXB2) (Hamberg et al 1975; Smith et al 1976), a stable compound which can be easily measured by radio-immunoassay (RIA). Hence, platelet TXA2 synthesis was monitored by measuring the serum concentration of TXB2 following the controlled clotting of whole blood. In addition, using the method for measuring the unbound plasma concentrations of ibuprofen enantiomers (described in Chapter 5), it was possible to relate the degree of inhibition of TXA2 synthesis to the unbound plasma concentrations of S-I, which was the enantiomer shown to possess the inhibitory activity.

7.2 METHODS

7.2.1. Subjects and Study Design

As described in section 6.2.1, four healthy men each received 200, 400, 800 and 1200mg of racemic ibuprofen, orally, on four separate occasions. Blood samples were collected from an arm vein immediately prior to (two samples collected), and at various times up to 48 hours after, ibuprofen dosing.

7.2.2. Assessment of Platelet Cyclo-oxygenase Inhibition

To monitor the effects of ibuprofen dosing on platelet cyclo-oxygenase, whole blood was allowed to clot under controlled conditions. The amount of TXA2 generated during clotting (an index of cyclo-oxygenase activity) was assessed by measuring the concentration of its stable metabolite, TXB2, in harvested serum. The method was based on that described by Patrono et al (1980).

Controlled Clotting of Whole Blood

Immediately after collection of each blood sample, a 1ml aliquot was placed into a

pre-calibrated Pyrex® borosilicate culture tube (12mm x 75mm) which had been pre-warmed to 37°C in a thermostatically controlled metal heating block. The tube was returned to the heating block, and maintained at 37°C for 1 hour (the generation of TXB2 during the clotting of whole blood, is a time and temperature dependent process; and at 37°C, constant levels are achieved within 15 minutes; Patrono et al 1980). After the 1 hour incubation, the tube was rimmed with a wooden spatula to detach any unretracted portions of the clot. After centrifugation (1000g for 15 minutes) a 200µl aliquot of serum was placed into a labelled 1.5 ml Eppendorf® micro test-tube and stored at -18°C until batch analysis by RIA for TXB2.

For each phase, for each volunteer, ten serum samples were selected for analysis of TXB2. These ten samples comprised: two pre-dose samples; the six serum samples corresponding to those plasma samples which had been analysed for the unbound concentrations of R-I and S-I (see section 6.3); and the 24 and 48 hour serum samples.

Radio-Immunoassay of Serum Thromboxane B2.

Measurement of serum concentrations of TXB2 was performed at the South Australian Institute of Medical and Veterinary Science by Mrs L. Tunbridge, using an established assay. The method, described in detail by Fitzpatrick (1982), is summarised below.

An extract of serum, diluted (1:10 to 1:1000) with working gelatin-tris buffer (pH 7.6), was incubated with TXB2 antisera and radiolabelled TXB2 ([5,6,8,11,12,14,15 (n)-^{3}H]-thromboxane B2, 180 Ci/mmol, Amersham, United Kingdom). The antisera was prepared by Dr M James of the Flinders Medical Centre, Bedford Park, South Australia, and had less than 0.01% cross-reactivity with other prostaglandins and arachidonic acid. Charcoal was added to sequestrate radiolabelled TXB2 which was not antibody-bound. After centrifugation, the concentration of radioactivity within the supernatant (containing radiolabelled TXB2 which remained bound to the antibody) was determined. The concentration of TXB2 was determined from a standard curve, prepared from standards containing 0.05 to 5.0 ng/ml of unlabelled TXB2 (Caymen Chemicals, Denver, USA). A representative calibration curve is presented in Figure 7.1. The sample dilution factor was then used to calculate the concentration of TXB2 in the undiluted serum sample. Each serum sample was assayed in duplicate and the values averaged, unless they differed by more than 20%, in which case, the serum sample was re-analysed.

Pharmacodynamic Analysis of TXB2 Data

For each volunteer, the pre-dose serum concentration of TXB2 in each phase was

determined by averaging the concentration of TXB2 determined in two independent pre-dose serum samples. For samples obtained after ibuprofen administration, inhibition of TXB2 production during whole blood clotting was calculated as the percentage decrease in the serum concentration of TXB2, relative to the pre-dose level.

For each volunteer, data from all four dose levels were pooled to examine the relationship between the unbound concentration of S-I in plasma, and the percentage inhibition of TXB2 generation. This was achieved by fitting a standard sigmoidal E_{max} equation to the data with an extended least squares modelling computer program (MK Model, Elsevier-Biosoft® United Kingdom). The sigmoidal E_{max} equation (Hill equation) is

$$E = \frac{E_{max}\ C^n}{EC_{50}^n + C^n} + E_O \qquad (7.1)$$

where E is the measured effect at drug concentration C; E_O is the basal effect in the absence of drug (i.e. when C=O); E_{max} is the maximum effect; EC_{50} represents the concentration of drug required to cause 50% of E_{max}; and n is a steepness factor for the log concentration-effect relationship (Colburn & Brazzell 1986). In the present case, E was expressed as a percentage of the maximal effect of the drug, and hence E_0 was set at zero, and E_{max} at 100, therefore simplifying equation 7.1 to

$$\%\ \text{inhibition} = \frac{100\ (C_u^{S\text{-}I})^n}{EC_{50}^n + (C_u^{S\text{-}I})^n} \qquad (7.2)$$

where $C_u^{S\text{-}I}$ is the unbound plasma concentration of S-I.

The computer modelling program was used to obtain an estimate of EC_{50} and n for each volunteer. These data were then used to calculate an EC_{80} (the unbound concentration of S-I required to inhibit TXB2 generation by 80%), for each volunteer.

For each volunteer in each phase, the maximum percentage inhibition of serum TXB2 was determined directly from the experimental data. Indices of the duration of TXB2 inhibition were obtained by determining the time at which the plasma unbound concentration of S-I fell below the determined EC_{80} and EC_{50} values. For each volunteer, these time values were calculated from the log-linear regression of the

terminal portion of the plasma unbound S-I versus time profiles (see section 6.2.3), using that volunteer's EC_{50} and EC_{80} values. In all cases, the EC_{80} and EC_{50} values were below the concentration at which the log-linear regression was initiated. To examine for a time lag between the observed concentration of unbound S-I in plasma, and the magnitude of inhibition of platelet cyclo-oxygenase, the percentage decrease in serum TXB2 concentration was plotted against plasma unbound S-I, according to the time sequence in which the samples were collected (hysteresis analysis)

7.2.3. *In Vitro* Determination of the Influence of R(-)-Ibuprofen, S(+)-Ibuprofen and RS-Ibuprofen on TXB2 Generation During Whole Blood Clotting.

A series of calibrated Pyrex® tubes, identical to those used for the ibuprofen dosing study above, were prepared containing a range of quantities of R-I and/or S-I, added as methanolic solutions of R-I (1mg/ml) and/or S-I (1mg/ml). Two tubes containing no R-I or S-I were also prepared. Methanol was evaporated at room temperature, under a gentle stream of purified nitrogen. A 50µl aliquot of isotonic phosphate buffer (pH 7.4) was added to each tube to redissolve the ibuprofen, and the tubes were placed into a heating block set at 37°C. Blood (20mL) was collected from an arm vein of one of the healthy male volunteers who participated in the ibuprofen dose-ranging study (AE) by simple venepuncture. With the needle removed, 1ml of blood was promptly transferred from the syringe into each tube. The final blood concentration of R-I and S-I in each tube is set out below.

Tubes	1-2	Control samples, containing no R-I or S-I
Tubes	3-7	R-I at 1, 2, 5, 10 and 20 mg/L, respectively
Tubes	8-12	S-I at 0.5, 1, 2, 5 and 10mg/L, respectively
Tubes	13-17	RS-I at 1, 2, 5, 10 and 20mg/L, respectively

Each tube was incubated at 37°C for 1 hour, and the serum concentrations of TXB2 were measured, as described in section 7.2.2. The percent inhibition of TXB2 generation, in tubes 3 through 17, was taken to be the percentage decrease in the serum concentration of TXB2 relative to the control (the mean of tubes 1 and 2).

7.2.4. Statistical Analysis

All data are presented as arithmetic mean ± SD. In the ibuprofen dose-ranging

study, analysis of variance was used to test for changes with dose in the time taken for the plasma concentration of unbound S-I to fall below EC_{80} and EC_{50}. The *a priori* level of significance was 0.05. If a difference between the four doses was detected, Fisher's least significant difference test was used to test for differences between the individual doses. Because of the discrete limit of 100% for the maximum observed inhibition of TXB2, it was necessary to use a non-parametric statistic (Kruskal-Wallis) to test for dose dependency of this parameter.

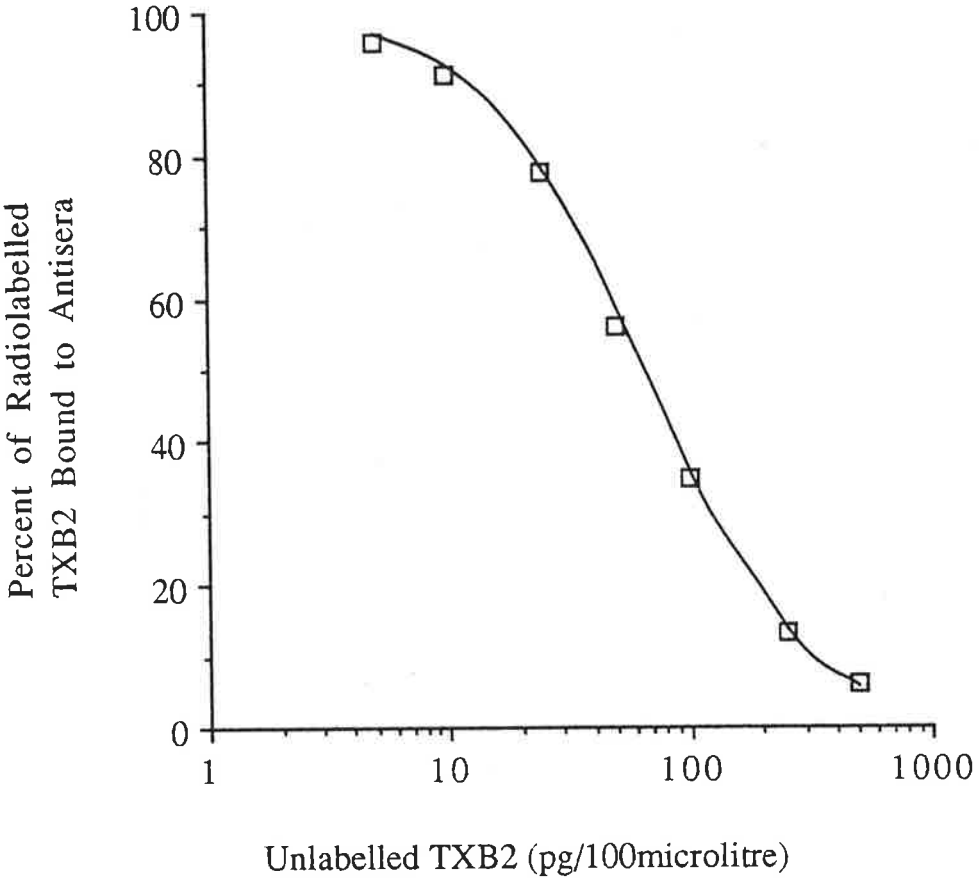


Figure 7.1 A representative calibration curve for determining the serum concentration of TXB2. The percent of radiolabelled TXB2 bound to anti-sera (y-axis) was plotted against the log of the concentration of unlabelled TXB2 in buffer (x-axis). This calibration curve was used to determine the concentration of TXB2 in a diluted sample of serum extract. The appropriate dilution factor was used to determine the serum concentration of TXB2.

7.3 RESULTS

The effect of S-I and RS-I, added *in vitro*, on TXB2 generation during the controlled clotting of whole blood, is presented in Figure 7.2. Serum TXB2 levels were unaffected by R-I over the range of blood concentrations examined (1 to 20 mg/L); the mean level of TXB2 in the five samples containing R-I was 170 ± 12 ng/ml, compared to 156 ng/ml for the control sample. In contrast, S-I reduced serum TXB2 in a concentration-dependent manner. When the effect of S-I on TXB2 generation was modelled, according to a sigmoidal E_{max} equation, the blood concentration of S-I leading to a 50% inhibition of TXB2 generation was found to be 1.1 mg/L. For racemic ibuprofen, a concentration of 2.2 mg/L (1.1mg/L of each enantiomer) corresponded to a 50% inhibition. For both S-I and RS-I, the slope factor of the log concentration-effect curve (n) was 1.6.

In the ibuprofen dose-ranging study, the mean (± SD) basal level of TXB2 in serum, determined from the pre-dose data from all volunteers, was 164 ± 82 ng/ml, which compares well with 228 ± 87 ng/ml reported by Patrignani et al (1982) as the mean level of serum TXB2 in 33 healthy volunteers who were in the drug-free state. At all dose levels, ibuprofen was found to decrease the serum concentration of TXB2. The serum TXB2 concentration data for each volunteer are tabulated in Appendix B. Plots of the percent decrease in serum TXB2 concentration versus time after ibuprofen administration (up to 12 hours), for each dose in each volunteer, are presented in Figure 7.3. The effect of ibuprofen on platelet TXA2 synthesis was transient, and in all but one case serum TXB2 concentrations returned to at least within 10% of the pre-treatment level within 24 hours. In the one exception (JE 1200mg), the serum TXB2 level (which was 32.5% below the pre-treatment value at 24 hours) had returned to the pre-treatment level by 48 hours.

The relationship between the percentage inhibition of TXB2 synthesis and the log concentration of plasma unbound S-I (includes data from all four dose levels), for each volunteer, are presented in Figure 7.4. The EC_{80}, EC_{50} and the slope factor, determined from concentration-effect modelling of the data from each volunteer, are presented in Table 7.1.

TXB2 inhibition consistently exceeded 50% within 90 minutes of dosing, which is in keeping with the rapid appearance of S-I in plasma after oral dosing with racemic ibuprofen (see Chapter 6). There was no evidence of a time lag between the appearance of unbound S-I in plasma and the decrease in serum TXB2 concentration. This feature

is well illustrated in Figure 7.5, which shows, for the 200mg and 1200mg doses in a representative volunteer, the log concentration-time profiles of both serum TXB2 and unbound plasma S-I. When drug effect is plotted against drug concentration, a hysteresis may result when the data points are joined according to time sequence. Significant hysteresis may result, for example, if there is a delay in equilibration of drug between the sampling site and the active site, or, if the metabolism of the drug gives rise to active metabolites or metabolites which modify the effect of the parent drug (Holford & Sheiner 1982). In the present work, "hysteresis analysis" was not possible in the majority of cases because of the paucity of data. However, Figure 7.6 presents two examples of the relationship between inhibition of serum TXB2 and plasma concentration of unbound S-I, in which the data have been plotted according to time sequence. It appears from these data that any equilibrium delay between plasma unbound S-I and inhibition of TXB2 generation (a measure of platelet cyclo-oxygenase inhibition), was minimal.

The peak percentage inhibition of TXB2 increased significantly ($p < 0.05$) with dose (Table 7.2) from a mean of 93.4% after the 200mg dose, to 98.8% after the 1200mg dose. In addition, the time at which the plasma unbound concentration of S-I fell below EC_{80} increased significantly ($p< 0.001$) with dose (Table 7.3). Although the time taken for unbound plasma S-I to fall below EC_{50} tended to increase with dose, the change was not statistically significant (Table 7.4). This was because, after the 400mg dose for one volunteer (TS), the time taken for unbound plasma S-I to fall below EC_{50} was 16.9 hours; as discussed in Chapter 6, the absorption of ibuprofen appeared to be delayed in this isolated case. When the data for TS were excluded from the statistical analysis, on the basis that the data for this volunteer were not representative, the change in the time for unbound S-I to fall below EC_{50} was significant ($p < 0.05$; Table 7.4).

The time-points chosen for the measurement of plasma unbound S-I (see Chapter 6) and therefore serum TXB2, differed between volunteers, and between doses. Hence, to illustrate the overall effect of dose on the average magnitude and duration of TXB2 inhibition, it was necessary to categorise the data according to the time-interval over which it was collected (samples collected up to 1 hour after dosing were included in the 0-1 hour interval; those collected after 1 hour, and up to 2 hours, were included in the 1-2 hour interval, and so on). Figure 7.7 depicts the mean percent inhibition of TXB2 generation, over each of the selected time-intervals, as a function of the magnitude of ibuprofen dose.

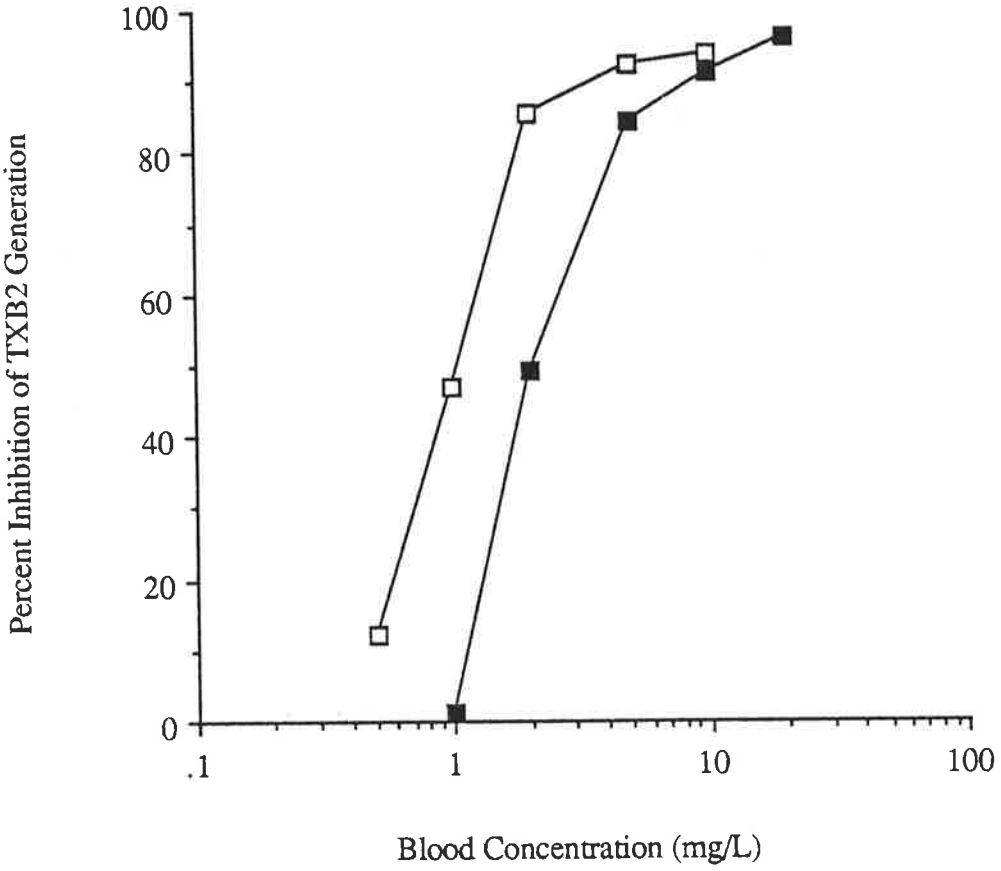


Figure 7.2. *In vitro* examination of the relationship between blood concentration of S-I (□), and RS-I (■), and the percentage inhibition of TXB2 generation (TXA2 synthesis) during the controlled clotting of whole blood.

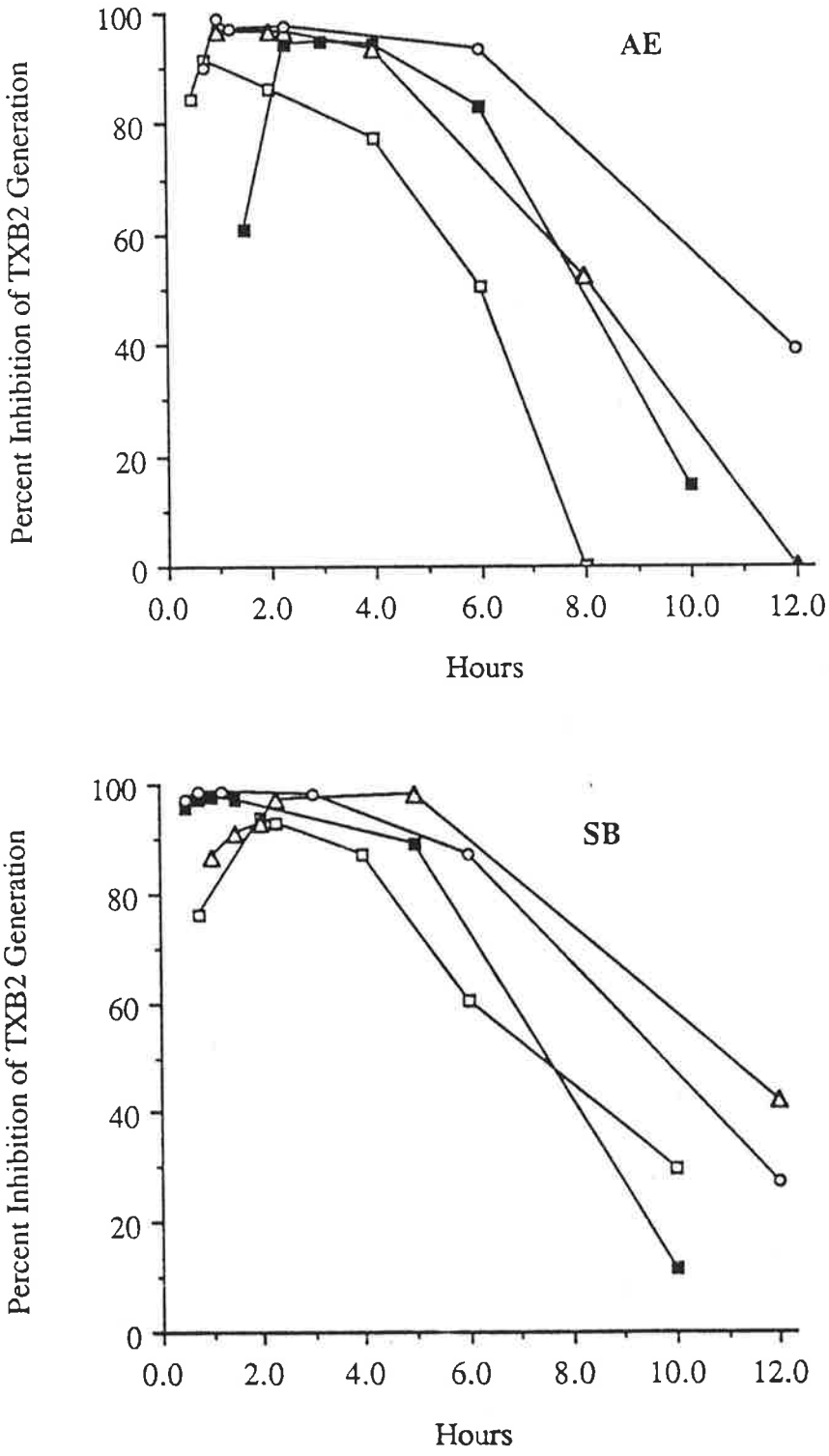


Figure 7.3. Plots of the percentage inhibition of TXB2 generation versus time after oral administration of racemic ibuprofen (□ 200mg; ■ 400mg; △ 800mg; ○ 1200mg), for each volunteer.

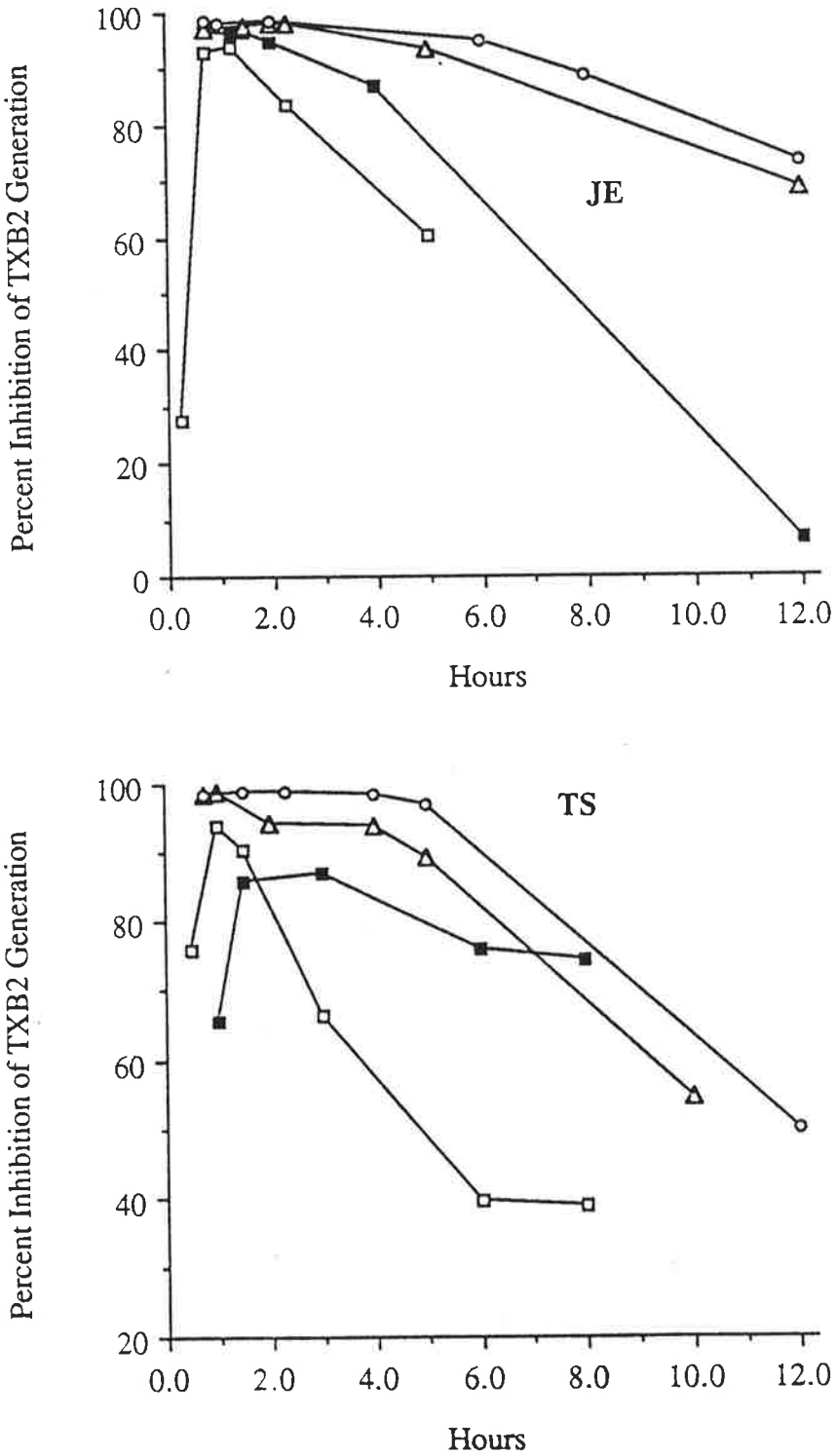


Figure 7.3. (Continued)

Plots of the percentage inhibition of TXB2 generation versus time after oral administration of racemic ibuprofen (□200mg; ■ 400mg; △ 800mg; ○ 1200mg), for each volunteer.

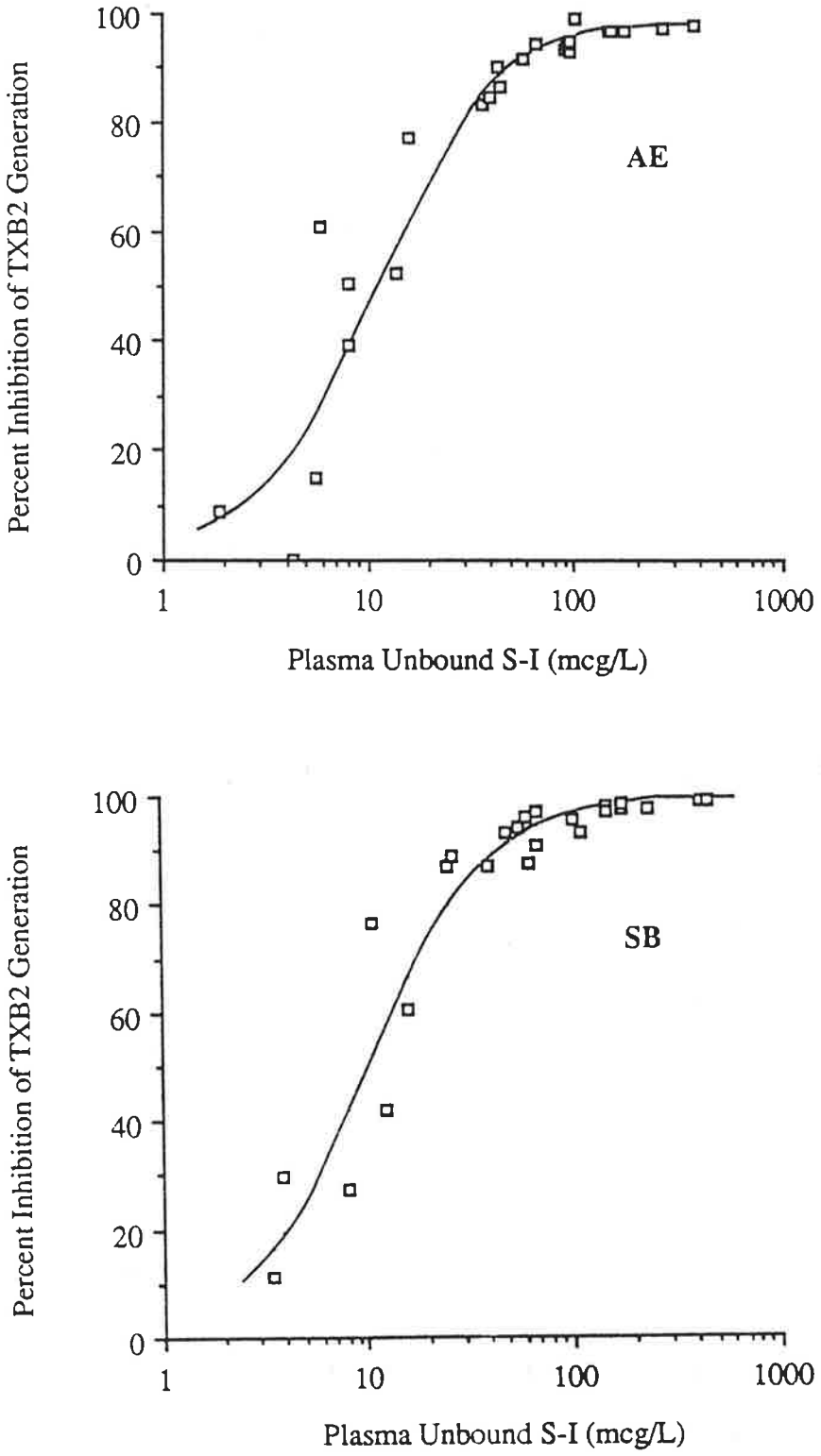


Figure 7.4. Relationship between the percentage inhibition of TXB2 generation and log plasma concentration of unbound S-I, for each volunteer. The symbols are actual data points, and the line represents the predicted relationship, according to a sigmoidal E_{max} model, from the computer generated analysis.

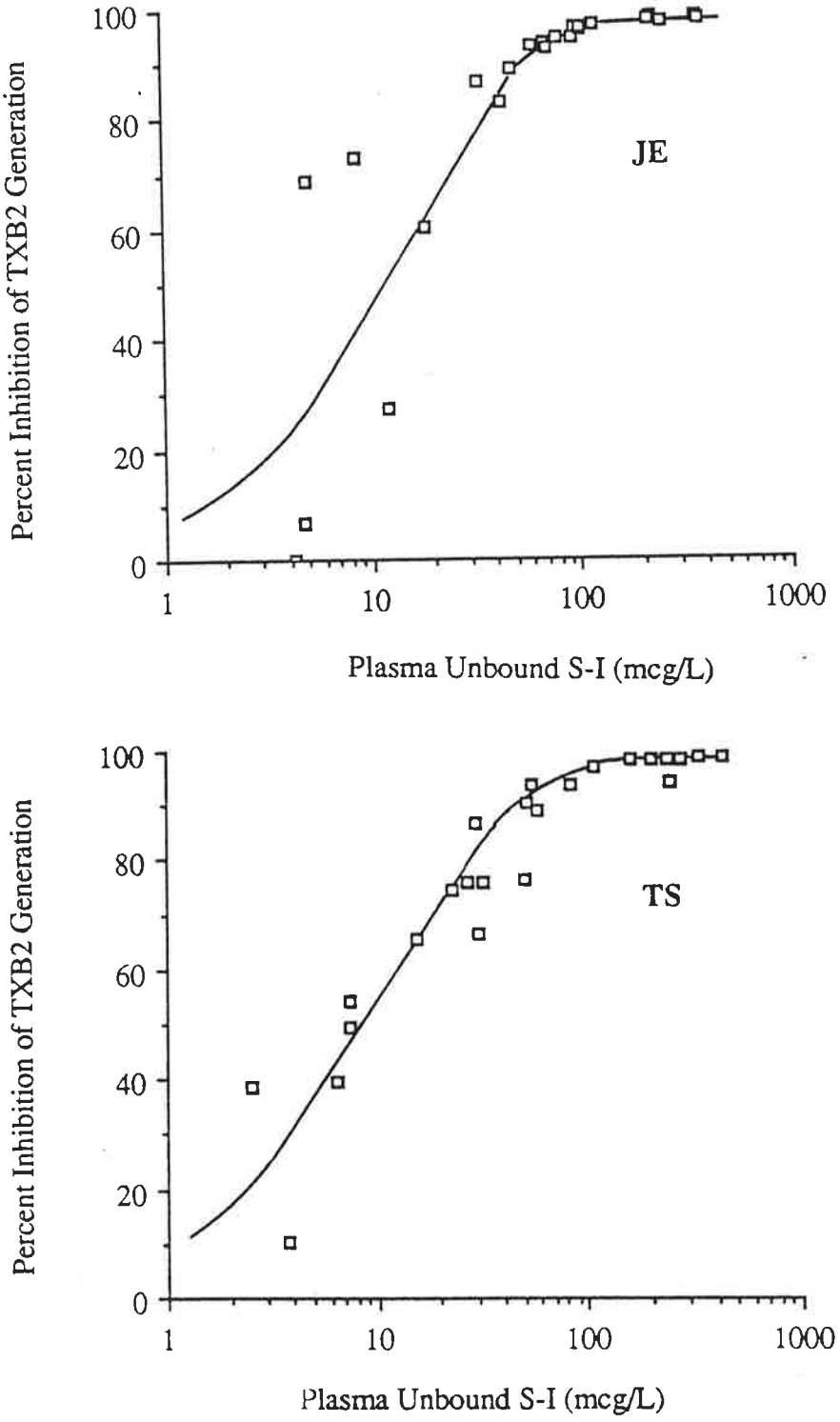


Figure 7.4 (continued).

Relationship between the percentage inhibition of TXB2 generation and log plasma concentration of unbound S-I, for each volunteer. The symbols are actual data points, and the line represents the predicted relationship, according to a sigmoidal E_{max} model, from the computer generated analysis.

Table 7.1. Computer-generated parameters (EC_{50}, EC_{80} and n) for the relationship between the unbound plasma concentration of S-I and the percentage inhibition of TXB2 generation.

Volunteer	EC_{50} (mcg/L)	EC_{80} (mcg/L)	n
AE	10.3	27.0	1.44
SB	10.1	25.7	1.48
JE	10.3	30.7	1.27
TS	8.3	30.8	1.06
Mean	9.8	28.6	1.31
SD	1.0	2.6	0.19

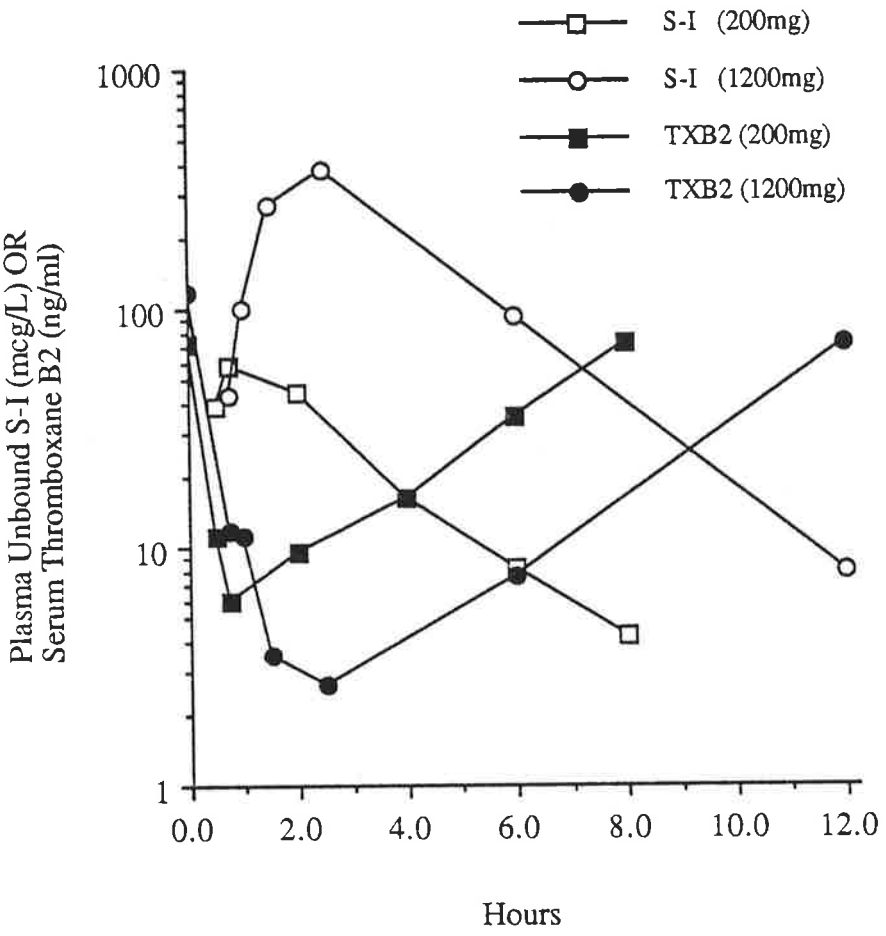


Figure 7.5. Log concentration-time profiles for both serum TXB2 and plasma unbound S-I after oral dosing with 200mg and 1200mg of racemic ibuprofen, for a representative volunteer (AE).

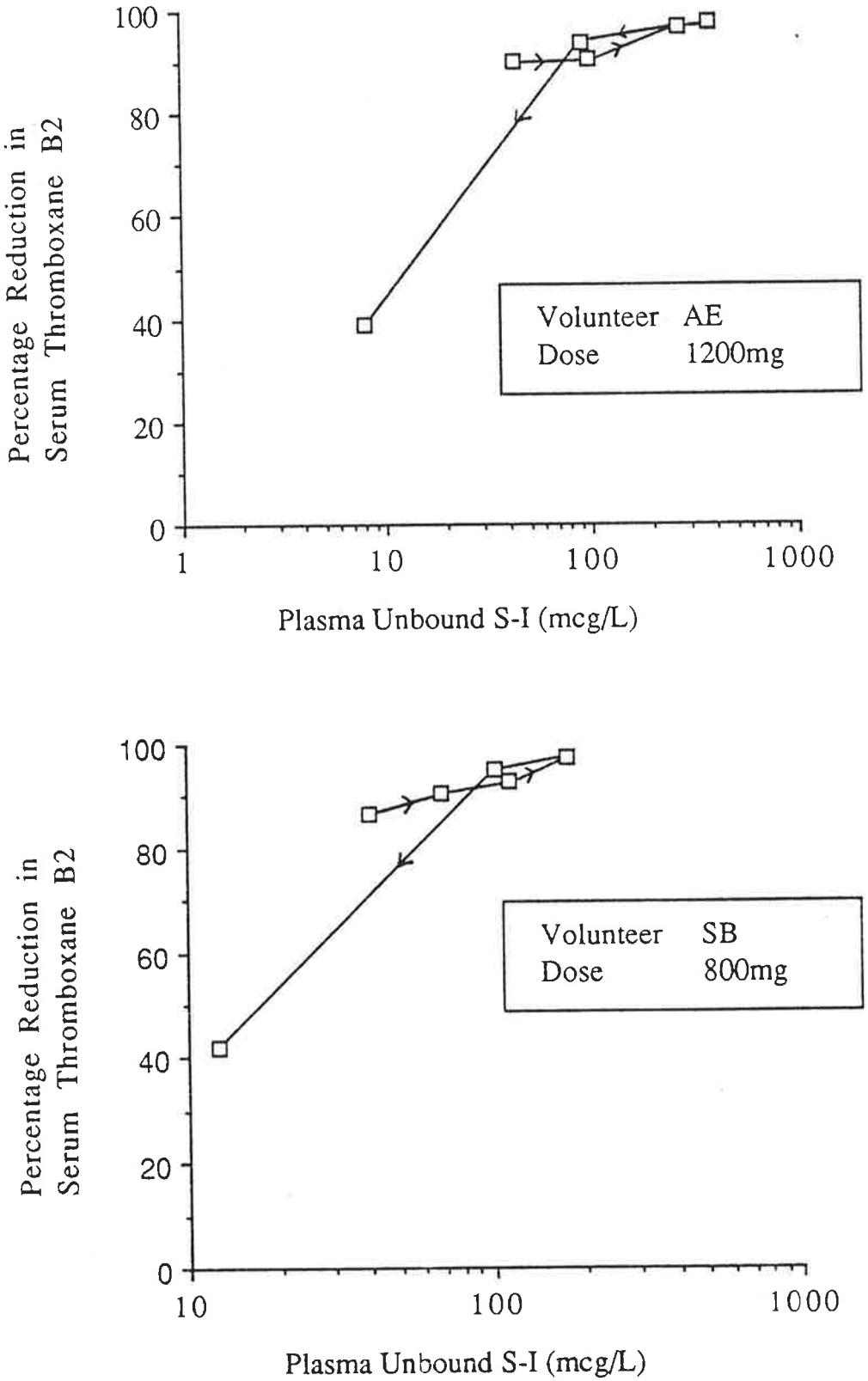


Figure 7.6 The relationship between the percentage reduction in serum TXB2 and plasma concentration of unbound S-I for volunteer AE (1200mg dose) and SB (800mg dose). Arrows show the time sequence after oral administration of racemic ibuprofen.

Table 7.2 Maximum percentage inhibition of TXB2 generation versus ibuprofen dose

	MAXIMUM % INHIBITION OF TXB2 GENERATION			
Volunteer	200mg	400mg	800mg	1200mg
AE	91.6	94.7	96.8	99.1
SB	93.8	97.5	97.1	98.6
JE	94.0	96.6	98.1	98.6
TS	94.0	86.8	99.0	99.0
Mean	93.4	93.9	97.8	98.8
SD	1.2	4.9	1.0	0.3
Significance*	c,d	c,d	a,b	a,b

* significantly different from the corresponding parameter at the (a) 200mg (b) 400mg (c) 800mg and (d) 1200mg, dose level.

Table 7.3. The effect of ibuprofen dose on the time taken for the unbound plasma concentration of S-I to fall below EC_{80}.

Volunteer	Time for C_u^{S-I} to fall below EC_{80} (hours)			
	200mg	400mg	800mg	1200mg
AE	2.44	6.38	6.63	9.00
SB	4.32	5.07	9.58	8.65
JE	3.50	4.42	7.22	8.15
TS	2.92	5.46	6.53	8.28
Mean	3.30	5.33	7.49	8.52
SD	0.81	0.82	1.43	0.38
Significance*	b,c,d	a,c,d	a,b	a,b

* significantly different from the corresponding parameter at the (a) 200mg (b) 400mg (c) 800mg and (d) 1200mg dose level.

Table 7.4. Effect of ibuprofen dose on the time taken for the unbound plasma concentration of S-I to fall below EC_{50}.

Volunteer	Time for C_u^{S-I} to fall below EC_{50} (hours)			
	200mg	400mg	800mg	1200mg
AE	5.32	8.73	8.61	11.4
SB	7.13	7.38	12.9	11.4
JE	7.06	8.80	10.0	11.4
TS	5.56	16.9	9.73	11.7
Mean	6.27	10.5	10.3	11.4
SD	0.96	4.4	1.8	0.2
Significance*				
(including TS data)	NS	NS	NS	NS
(excluding TS data)	c,d	d	a	a,b

* significantly different from the corresponding parameter at the (a) 200mg (b) 400mg (c) 800mg and (d) 1200mg, dose level.

NS = not significant

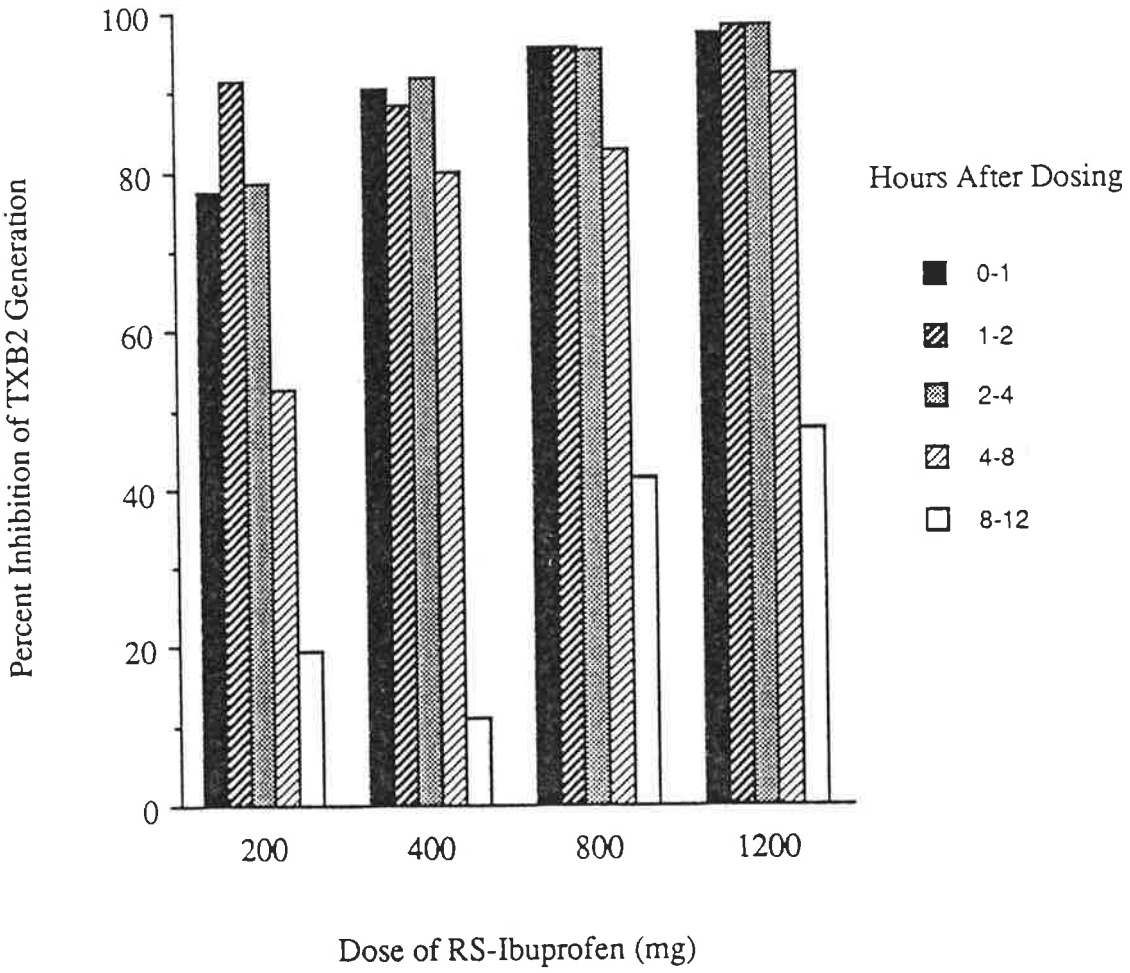


Figure 7.7 Plots of the mean percentage inhibition of TXB2 generation (TXA2 synthesis) versus time after oral dosing with a range of doses of racemic ibuprofen, using the pooled data from all four volunteers.

7.4. DISCUSSION

In the present study, the effects of ibuprofen on platelet activity was monitored by measuring TXB2 production during the controlled clotting of whole blood. In this technique, endogenous thrombin is the stimulus for TXB2 generation. Whole blood clotting TXB2 generation has been used by many other workers to monitor the anti-platelet activity of non-steroidal anti-inflammatory agents (Cerletti et al 1987; Longenecker et al 1985; Nuotto et al 1983; Patrignani et al 1982; Patrono et al 1980).

A large number of studies have shown ibuprofen to be a potent inhibitor of platelet aggregation (Brooks et al 1973; Cox et al 1987; Ikeda 1977; McIntyre et al 1978; O'Brien 1968; Parks et al 1981). Because only S-I can inhibit the synthesis of prostaglandins (Adams et al 1976), it was expected that S-I alone would be the active anti-platelet enantiomer. This was confirmed by an *in vitro* experiment (Figure 7.2.), which indicated that S-I, at a blood concentration of 1.1 mg/L, inhibited TXB2 generation, in response to whole blood clotting, by 50%. For racemic ibuprofen, the corresponding blood concentration was 2.2 mg/L (1.1 mg/L of each enantiomer). Consistent with the lack of effect of R-I on prostaglandin sythetase activity (Adams et al 1976), there was no detectable effect of this enantiomer on TXB2 generation. Hence the results of the *in vitro* experiment indicate firstly, that S-I is solely active at inhibiting TXB2 generation, and secondly, that the activity of S-I is not altered by the presence of its optical antipode, suggesting that the binding of S-I to the active site(s) on cyclo-oxygenase is not modified by the presence of R-I. The *in vitro* anti-platelet and/or anti-TXB2 activity of the chiral 2-PPA derivatives fenoprofen (Rubin et al 1985) and flurbiprofen (Kulmacz & Lands 1985) were also found to reside almost exclusively with the S(+)-enantiomer. Because of the enantioselective activity of the 2-PPA derivatives, it is essential, when relating anti-platelet effects to the concentration in plasma or plasma water, that the levels of the S(+)-enantiomer should be monitored.

Whilst there are numerous literature reports on the effect of ibuprofen dosing in humans on platelet aggregation and/or TXB2 generation (Brooks et al 1973; Cox et al 1987; Cronberg et al 1984; Ikeda 1977; Longenecker et al 1985; McIntyre et al 1978; O'Brien 1968; Parks et al 1981), relatively few studies have closely monitored the time-dependency of the anti-platelet effects of the drug. Longenecker et al (1985) administered a range of single oral doses of racemic ibuprofen (8, 10, 12 and 14 mg/kg) to healthy subjects and determined whole blood clotting TXB2 generation prior to, and 2, 4 and 6 hours after, drug administration, and again after 7 days. Inhibition of TXB2 did not differ between doses, persisted for at least 6 hours, and levels of TXB2 returned to pre-treatment values within 7 days. Cronberg et al (1984) reported ibuprofen to

inhibit platelet aggregation 1.5, 3 and 6 hours after a single 800mg oral dose of racemic ibuprofen, but not after 24 hours. These studies provide no information on the anti-platelet effects of the drug between 6 and 24 hours. In one study (Longenecker et al 1985), the mean 6 hour plasma concentration of unresolved ibuprofen was 10mg/L, which was sufficient to produce a marked inhibition of platelet TXB2 generation.

Recently, Cox et al (1987) reported on a comprehensive investigation into the relationship between TXB2 generation, platelet aggregability, and concentrations of ibuprofen and flurbiprofen. Four healthy volunteers were given 200, 400, and 800mg of ibuprofen, and the serum concentrations of the drug were determined for up to 16 hours post-dose. Platelet aggregation studies were performed both prior to, and at selected times after, ibuprofen administration. These studies took the form of an aggregation tracing, performed for 5 minutes after the addition of an aggregating agent (arachidonic acid) to platelet rich plasma; platelet TXB2 generation was also monitored over this 5 minute period. Platelet aggregability and platelet TXB2 generation were found to be closeley related processes, with plasma TXB2 levels of 40ng/ml being necessary for platelet aggregation to occur. The minimum concentration of (unresolved) ibuprofen needed to inhibit platelet aggregation was estimated to be 3 mg/L. Below this concentration, platelet aggregation proceeded, but the time which elapsed between addition of the aggregatory stimulant and the onset of aggregation was increased. After the 200mg dose of ibuprofen, platelet aggregation was inhibited for a mean (±SD) of 6 ± 2 hours, and after the 400mg and 800mg doses, the corresponding periods of inhibition were 8 ± 2 hours and 11 ± 2 hours, respectively. An elaborate model was used by Cox et al (1987) to relate platelet aggregation parameters to serum ibuprofen concentration. However, serum ibuprofen concentrations were measured as total unresolved drug, and the model assumed that the unbound concentration of S-I was a constant fraction of total unresolved ibuprofen (Cox et al 1987). Because of the enantioselective disposition of ibuprofen enantiomers (see Chapter 6) and the concentration-dependent plasma protein binding of S-I (see Chapters 6 & 9), such an assumption may not be valid.

The results of the present study indicate that in healthy volunteers, an average (± SD) of 9.8 ± 1.0 mcg/L of unbound S-I was necessary to inhibit TXA2 synthesis by 50%. The mean concentration required to inhibit TXA2 synthesis by 80% was 28.6 ±2.6 mcg/L. For all volunteers, these unbound concentrations of S-I were exceeded after oral administration of 200, 400, 800 and 1200mg of racemic ibuprofen. This suggests that the peak intensity of the inhibition of platelet cyclo-oxygenase should vary little between dose. In this study the maximum observed inhibition of TXB2 generation (Table 7.2) did increase as ibuprofen dose increased, although the magnitude of the

change with dose was minimal. With increasing ibuprofen dose, the plasma concentrations of unbound S-I remained elevated above EC_{80} and EC_{50} for a longer duration. In fact, the increase with dose in the mean time taken for plasma unbound S-I to fall below the EC_{80} was highly significant, with the mean duration increasing by about 160% between the 200 and 1200mg dose levels (Table 7.3). When the data for TS were omitted, there was also a significant increase with dose in the time taken for the plasma concentration of unbound S-I to fall below EC_{50} (Table 7.4); in this case there was a mean increase of about 75% between the 200 and 1200mg doses. It would appear, therefore, that the duration of TXB2 inhibition is influenced by the magnitude of ibuprofen dose to a greater extent than is the peak percentage inhibition of TXB2 generation, a feature which is clearly illustrated by examining the pooled data (Figure 7.7).

There was only a minimal degree of hysteresis when the relationship between plasma unbound S-I and percentage inhibition of TXB2 generation, was plotted in order of time sequence (Figure 7.6). This close temporal relationship between "concentration" and "effect" (Figure 7.5) was not unexpected, given the proximity of the biological receptor (platelet cyclo-oxygenase) to the sampling compartment (plasma water). The lack of significant hysteresis also suggests that platelet cyclo-oxygenase was unaffected by ibuprofen metabolites (Holford & Sheiner 1982).

The present study confirms the findings of other workers, that the inhibition of TXA2 synthesis after oral administration of racemic ibuprofen is relatively short-lived (Brooks et al 1973; Cox et al 1987; Ikeda 1977; Longenecker et al 1985; McIntyre et al 1978; O'Brien 1968; Parks et al 1981). The time course of inhibition, which reflects that of unbound S-I in plasma, is consistent with the fact that the binding of ibuprofen to platelet cyclo-oxygenase is a reversible process (Ikeda 1977). In contrast, the function of platelets which have been exposed to aspirin cannot be restored, since this drug irreversibly acetylates the active site on cyclo-oxygenase (Flower et al 1980). Since platelets are anuclear, they are unable to replace inactivated enzyme and the cyclo-oxygenase inactivation of an aspirin-exposed platelet persists for the life-span of that platelet (8 to 11 days; Flower et al 1980). This explains why, after a single dose of aspirin, anti-platelet effects can persist for up to 7 days (Nuotto et al 1983; Patrono et al 1980). Interestingly, ibuprofen, and other reversible inhibitors of cyclo-oxygenase, have been found to protect platelets against the irreversible inactivation by aspirin, possibly by blocking the access of aspirin to the active site on cyclo-oxygenase (Rao et al 1983).

Because of the sensitivity of platelets to S-I, it would be expected, in patients

taking ibuprofen on a chronic basis (three to four times daily), that inhibition of platelet throboxane synthesis would be vitually continual. Because of the potential for accumulation of S-I in adipose tissuen (see section 3.5.2), the time taken for the anti-platelet effects to subside after cessation of the drug is more difficult to predict. The result of the present investigation would suggest that platelet function would, in most cases, return to normal within 12 hours. However, the leaching of S-I from adipose stores may ensure more prolonged anti-platelet effects. It is unfortunate that all of the studies which have examined the time-course of the anti-platelet (and/or cyclo-oxygenase inhibition) effects of ibuprofen have administered the drug as a single dose.

Little is known regarding the degree of inhibition of TXA2 synthesis necessary to produce physiological changes which may be important clinically. In examining the pharmacokinetics and pharmacodynamics of a specific thromboxane antagonist (SQ 28, 668) in humans, Friedhoff et al (1986) found that template bleeding times were prolonged if TXA2 production was inhibited by greater than 94%. In the present study, ibuprofen, over the therapeutic dose range, was found to elicit this magnitude of inhibition, if only for a short time (Figure 7.3 & 7.7). However, it is difficult to extrapolate the present observations to platelet aggregation *in vivo*, the latter being a complex process which is controlled by numerous factors, including the anti-aggregatory and vasodilatory compound, prostaglandin I2 (PGI2; also referred to as prostacyclin). This substance is produced in large quantities by vascular endothelial cells and is believed to function in concert with platelet TXA2, to help regulate platelet aggregation (Moncada et al 1980; Mustard et al 1980). In 1981, Parks et al reported that the synthesis of PGI2 (measured as its stable break-down product, 6-keto PGF 1$\propto$) by human umbilical vein endothelial cells, and of TXA2 (measured as TXB2) by washed human platelets, were equally sensitive to the inhibitory effects of ibuprofen. It has been suggested that attempts to exploit the beneficial properties of drugs which inhibit platelet TXA2 synthesis may be confounded by concomitant inhibition of prostacyclin synthesis. Hence, a great deal of attention has been focused, in recent times, on producing drugs which selectively inhibit the synthesis of TXA2, without affecting vascular prostacyclin production. Interestingly, Longenecker et al (1985) studied the effect of orally administered ibuprofen on both platelet TXB2 production and on the basal blood levels of PGI2 (measured as 6-keto-PGF1$\propto$) in human volunteers. While ibuprofen inhibited TXB2 generation, the basal blood levels of PGI2 remained unchanged, leading the authors to suggest that ibuprofen induced anti-platelet effects while sparing endogenous anti-platelet mechanisms. The reason for the conflict between the *in vitro* data of Parks et al (1981) and the *ex vivo* data of Longenecker et al (1985)

is unknown, and warrants further investigation.

7.5. CONCLUSION

In conclusion, the present study has demonstrated a close relationship between the plasma unbound concentration of S-I and the extent of inhibition of thromboxane synthesis. There was an increase with dose in the maximum observed inhibition of platelet TXB2 generation, and in the time taken for the unbound plasma concentrations of S-I to fall below those required to inhibit TXB2 production by 80% and 50%. For the four volunteers tested, there was very little variability in the sensitivity of platelets to the actions of S-I, as gauged by the similar values of EC_{50} and EC_{80}. Because of the low levels of unbound S-I needed to inhibit platelet cyclo-oxygenase, and the potential for accumulation of S-I within adipose stores during chronic administration, there is a clear need for a close examination of the anti-platelet effects of the drug in patients who have been receiving ibuprofen for a prolonged period.

Chapter 8.
The Effect of Cimetidine on the Pharmacokinetics of Ibuprofen Enantiomers.

8.1. INTRODUCTION

As discussed in section 3.4, ibuprofen's spectrum of adverse reactions include gastrointestinal side-effects. A small percentage of patients receiving ibuprofen on a chronic basis may develop gastrointestinal ulceration (Royer et al 1984) and may therefore require treatment with anti-ulcer drugs such as antacids and/or histamine-2-antagonists. Cimetidine, which selectively antagonises the actions of histamine at H_2 receptors thereby inhibiting gastric acid secretion (Douglas et al 1980), is commonly coadministered with ulcerogenic drugs. Although relatively non-toxic in itself, cimetidine has been found to alter the pharmacokinetics of a large number of drugs (Somogyi & Muirhead 1987). These cimetidine-mediated drug interactions, many of which are important clinically, occur via a number of well-documented mechanisms.

(i) Cimetidine increases gastric pH (Douglas et al 1980) and may influence the gastrointestinal absorption of drugs for which dissolution is pH-dependent. In addition, cimetidine may alter the gastrointestinal absorption characteristics of drugs from preparations designed to release their contents under certain pH conditions, and of drugs whose physical and/or chemical stability is pH-dependent (Somogyi & Muirhead 1987). For example, cimetidine coadministration was found to lower the mean steady-state plasma concentrations of indomethacin in patients with rheumatoid arthritis, an effect which may have been due to a cimetidine-induced reduction in the extent of gastrointestinal absorption of indomethacin and may have been related to the instability of indomethacin at elevated pH (Howes et al 1983).

(ii) Cimetidine, like many other imidazole compounds, is a potent inhibitor of the cytochrome P-450 mixed function oxidase system (Nazario 1986; Somogyi & Muirhead 1987). It is thought that this action of cimetidine arises from its ability to bind to the haem binding site (also known as the O_2 binding site), of cytochrome P-450, thereby inhibiting interactions with other substrate molecules (Nazario 1986; Somogyi & Muirhead 1987). Most of the phase I metabolic pathways are inhibited by cimetidine, including N-dealkylation, sulphoxidation

and hydroxylation (Somogyi & Muirhead 1987) and, consequently many drugs are affected. For example, cimetidine can decrease the hepatic clearance of diazepam, phenytoin, quinidine, theophylline and warfarin (Nazario 1986; Somogyi & Muirhead 1987) causing increases in the plasma concentrations of these drugs which may be important clinically. The phase II conjugation metabolic processes, such as glucuronidation, sulphation and acetylation, appear to be spared from the inhibitory effects of cimetidine (Somogyi & Muirhead 1987).

(iii) Cimetidine may compete with other organic cations for the active renal tubular cation transport systems. Such a mechanism has been proposed to explain the cimetidine-induced reduction in the renal clearance of the anti-arrhthymic agent, procainamide, and its active metabolite, N-acetyl-procainamide (Christian et al 1984; Somogyi et al 1983).

(iv) Cimetidine may lower liver blood flow and therefore decrease the clearance of drugs whose hepatic metabolism is perfusion-rate limited (Nazario 1984), although much of the evidence supporting this phenomenon has been challenged (Somogyi & Muirhead 1987).

Because oxidative biotransformation plays a major role in the clearance of ibuprofen (see sections 3.5 and 3.6.3), the drug is a candidate for a pharmacokinetic interaction with cimetidine. This chapter describes an investigation into the influence of cimetidine administration on the pharmacokinetics of a single oral dose of racemic ibuprofen. Importantly, the study design and methodology employed, allowed differentiation of the possible effects of cimetidine on the absorption of ibuprofen, as well as the plasma concentration-time course and unbound fraction in plasma of the individual ibuprofen enantiomers.

8.2 METHODS

8.2.1. Subjects and Study Design

Six non-smoking male volunteers participated in the study. Selected details of each volunteer are presented in Table 8.1.

Table 8.1. Selected details for the volunteers who participated in the cimetidine-ibuprofen drug interaction study.

Volunteer	Age (y)	Body Weight (kg)	Plasma Albumin (g/L)
RN	37	75	53
CW	42	80	48
PF	21	78	48
RM	22	83	48
SW	21	95	48
RU	25	75	46

Each volunteer was assessed to be healthy on the basis of a complete medical examination. During the medical examination a sample of blood was taken for a complete blood examination and a multiple biochemical analysis. A urine sample was obtained for biochemical and micro-urine examination. The results of all tests were required to be within normal limits prior to acceptance into the study. Exclusion criteria were as described in section 6.2.1. Volunteers were instructed to refrain from taking any other medication from one month prior to, until the completion of, the study. The study protocol was approved by the Ethics Committee of the South Australian Institute of Technology, where the study was undertaken, and volunteers gave written, informed consent to their participation. Subjects were asked to report any adverse reactions to the administered medication and were advised of their freedom to withdraw from the study at any stage, and for any reason. It should be noted that eight volunteers were originally recruited for the study, but two were withdrawn prior to completion. One was a passenger in a motor vehicle accident and was unable to complete the study.

The other volunteer experienced a vaso-vagal attack during the insertion of a cannula, prior to drug administration, and was withdrawn from the study and kept under medical supervision.

All volunteers received a single 800mg oral dose of racemic ibuprofen (2x BRUFEN® 400mg tablets) on two separate occasions, once in the drug-free state (control phase) and again during cimetidine administration (treatment phase). Ibuprofen tablet content uniformity was verified prior to administration (see section 4.3). Ibuprofen was administered with 150ml of water at 8 am following an overnight fast of 12 hours duration. A standard meal was provided 3 hours after ibuprofen dosing. In the treatment phase, cimetidine (TAGAMET® 200mg tablets, Smith Kline and French, Australia) was administered as 200mg at 7 am, 12 noon and 5 pm and 400mg at 10 pm, commencing 25 hours prior to the ibuprofen dose and continuing thereafter for a further 36 hours. Two weeks elapsed between the control and treatment phases which were randomised in a balanced cross-over manner. Fortuitously, the study remained balanced after the withdrawal of the two volunteers (i.e. three volunteers took cimetidine during each phase). The sequence of drug administration is summarised below.

Day	1	2	3	.....	17	18	19
Group A (n=3)	-	RS-I	-		C	C, RS-I	C*
Group B (n=3)	C	C, RS-I	C*		-	RS-I	-

RS-I Single 800mg oral dose of RS-ibuprofen at 8.00 am

C Oral dosing with cimetidine; 200mg at 7 am, 12 noon and 5 pm and 400mg at 10 pm

C* Oral dosing with cimetidine as for C, omitting the 10 pm dose.

Blood samples (10ml), obtained via an indwelling catheter inserted into an arm vein, were collected via a three-way tap into heparinised tubes immediately prior to ibuprofen dosing and at the following times thereafter: 10, 20, 30 and 45 mins, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 10 hours. Prior to collection of the 10ml sample, a 2ml sample of blood was withdrawn and discarded. To prevent blockage, the catheter was flushed with a small volume of heparinised saline after each blood sample was collected. Plasma was separated by centrifugation and stored at -18°C until assayed. A pre-ibuprofen sample of urine was collected from each volunteer, as was all urine voided during the following time intervals after ibuprofen dosing: 0-12 hours, 12-24 hours and 24-36 hours. After measurement of urine volume an aliquot of each sample

was stored at -18°C until assayed.

8.2.2. Drug Analyses in Biological Fluids

The total (bound plus unbound) concentration of R-I and S-I in each plasma sample were determined using the method described in section 4.1. For each volunteer, the unbound fractions of R-I and S-I were measured in five plasma samples from each phase using the methodology described in Chapter 5. The unbound concentration of each enantiomer in plasma was calculated as the product of its total (bound plus unbound) plasma concentration and its unbound fraction.

After alkaline hydrolysis of glucuronide conjugates, the concentrations of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen in urine were measured by HPLC, as described in section 4.2. These concentrations were used, together with urine volumes, to determine the recovery of each species.

The concentration of cimetidine, in selected plasma samples collected during the treatment phase of each volunteer, were measured by HPLC, using the method of Cohen et al 1980. These measurements, together with supervised dosing and tablet counting, were used to confirm compliance with the cimetidine dosage regimen.

8.2.3. Pharmacokinetic Analysis

For each enantiomer, the terminal rate-constant (ß), terminal half-life ($t_{1/2}$) and the area under the plasma concentration-time curve from time zero to infinity (AUC) were calculated (see section 6.2.3). In all cases the terminal (or extrapolated area) accounted for not more than 10% of AUC. The maximum plasma concentration (C_{max}) and the time of its occurrence (t_{max}) for each enantiomer were obtained directly from the experimental observations.

Because of constraints on the availability of radiolabelled ibuprofen, the protein binding of ibuprofen enantiomers in each volunteer could be determined in only five plasma samples from each phase. From the plasma total concentration-time plots of the enantiomers, five plasma samples were selected on the basis that they provided an accurate representation of the overall profiles. The unbound concentrations of R-I and S-I in these selected plasma samples were determined as described in Chapter 5. For each enantiomer, the area under the plasma unbound concentration-time curve, from time zero to the time of the last selected plasma sample $AUC(0\text{-}t)_u$, was determined

using the linear trapezoidal rule for pre-C_{max} areas and the logarithmic trapezoidal rule for post-C_{max} areas.

The time-averaged unbound fraction ($\bar{f}_u$) of each enantiomer was determined by dividing $AUC(0\text{-}t)_u$ by the corresponding area for total plasma concentration AUC(0-t), determined in this case using the plasma concentration-time data from the five relevant data points only. When calculating AUC(0-t), the logarithmic trapezoidal rule was used when successive plasma concentrations were declining. The reason area under the plasma unbound concentration-time curve was not calculated to infinity (as for the dose-ranging study; see section 6.2.3), was due to the limitation of 5 data points per profile, which prevented a confident extrapolation of the unbound plasma concentration-time curve from the final time point to infinity.

8.2.4. Effect of Cimetidine on Ibuprofen Plasma Protein Binding *In Vitro*.

An experiment was performed to examine the effect of cimetidine *in vitro* on the plasma protein binding of ibuprofen enantiomers. Drug-free plasma, obtained from a healthy male (RN), was placed into a series of 16 test-tubes containing racemic ibuprofen (5 mg/L or 50 mg/L) with or without 3 mg/L of cimetidine. At each ibuprofen concentration 4 samples were analysed with, and 4 without, cimetidine. The unbound fraction of R-I and S-I, in each sample, was determined using the method described in Chapter 5.

8.2.5. Statistical Analysis.

All data are presented as the arithmetic mean ± SD. Comparison of the pharmacokinetic parameters of R-I and S-I between the control and treatment phases was performed using two-tailed Student's paired t-tests, and $p < 0.05$ was taken to represent significance. The same technique was used to determine if the pharmacokinetic parameters of R-I differed from those of S-I. For the *in vitro* binding study, Student's t-test for unpaired data was used to test for an effect of cimetidine on the unbound fractions of R-I and S-I.

8.3 RESULTS

The plasma concentration-time data for total and unbound R-I and S-I are tabulated in Appendix C, as are the urinary excretion data. The total plasma concentration-time profiles of R-I and S-I in the control and treatment phases of each volunteer are presented in Figure 8.1. The corresponding profiles for unbound R-I and S-I are presented in Figure 8.2.

The AUC values of each enantiomer, for each subject, in each phase, are presented in Table 8.2 and the mean (±SD) pharmacokinetic parameters of each ibuprofen enantiomer, during the control and treatment phase, are presented in Table 8.3. There was no significant difference ($p > 0.05$) between the control and treatment phases with respect to the AUC of either enantiomer (Table 8.3). The power of the study to detect a 20% change between the phases in the AUC of R-I and S-I was calculated (Bolton 1984) to be 0.91 and 0.99, respectively. The good study power was a reflection of the low degree of variability in the AUC values for each volunteer between phases (Table 8.2). There was no significant difference ($p > 0.05$) in the C_{max}, t_{max} , $t_{1/2}$, $AUC(0\text{-}t)_u$ or $\bar{f}_u$ of either enantiomer (Table 8.3) between the control and cimetidine-treatments phases. In both phases, the AUC, $AUC(0\text{-}t)_u$, $t_{1/2}$ and $\bar{f}_u$ of R-I were all significantly less ($p < 0.05$) than the corresponding parameters for S-I.

Figure 8.3. shows the mean recovery of ibuprofen, carboxy-ibuprofen, and hydroxy-ibuprofen during the 0 to 12 and 12 to 36 hour time intervals. In all volunteers, in both phases, urinary excretion of ibuprofen and its metabolites was virtually complete within the first 12 hour collection period. The recoveries of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen in the urine between 0 and 36 hours (table 8.4) were not significantly influenced ($p > 0.05$) by concurrent cimetidine treatment nor was there a difference ($p > 0.05$) between the phases in the sum of these recoveries (ie "total" recovery; Table 8.4).

Random plasma samples (2 samples from the treatment phase of each volunteer), were analysed for cimetidine by HPLC (Cohen et al 1980). The mean (± SD) cimetidine concentration was 1.33 ± 0.65 mg/L (range 0.44 to 2.67 mg/L) and was consistent with literature reports of cimetidine levels after oral dosing (Somogyi & Gugler 1983).

When assessed *in vitro*, cimetidine, at a concentration of 3mg/L, was found to have no significant effect ($p > 0.05$) on the plasma protein binding of R-I and S-I at racemic ibuprofen concentrations of 5 and 50 mg/L (Table 8.5).

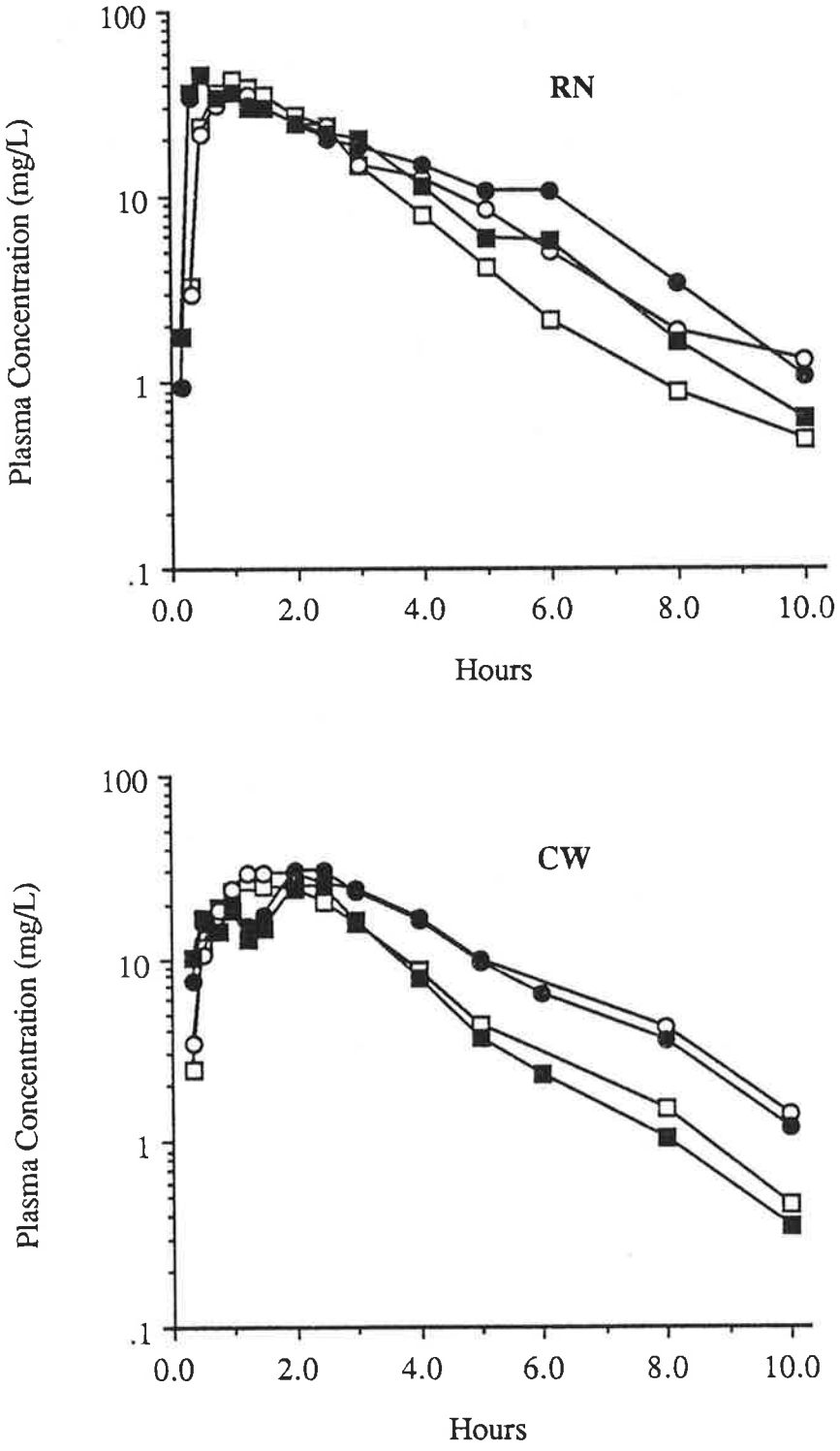


Figure 8.1

Plasma concentration-time profiles of total R-I (□ control phase ; ■ treatment phase) and total S-I (○ control phase; ● treatment phase) in each subject.

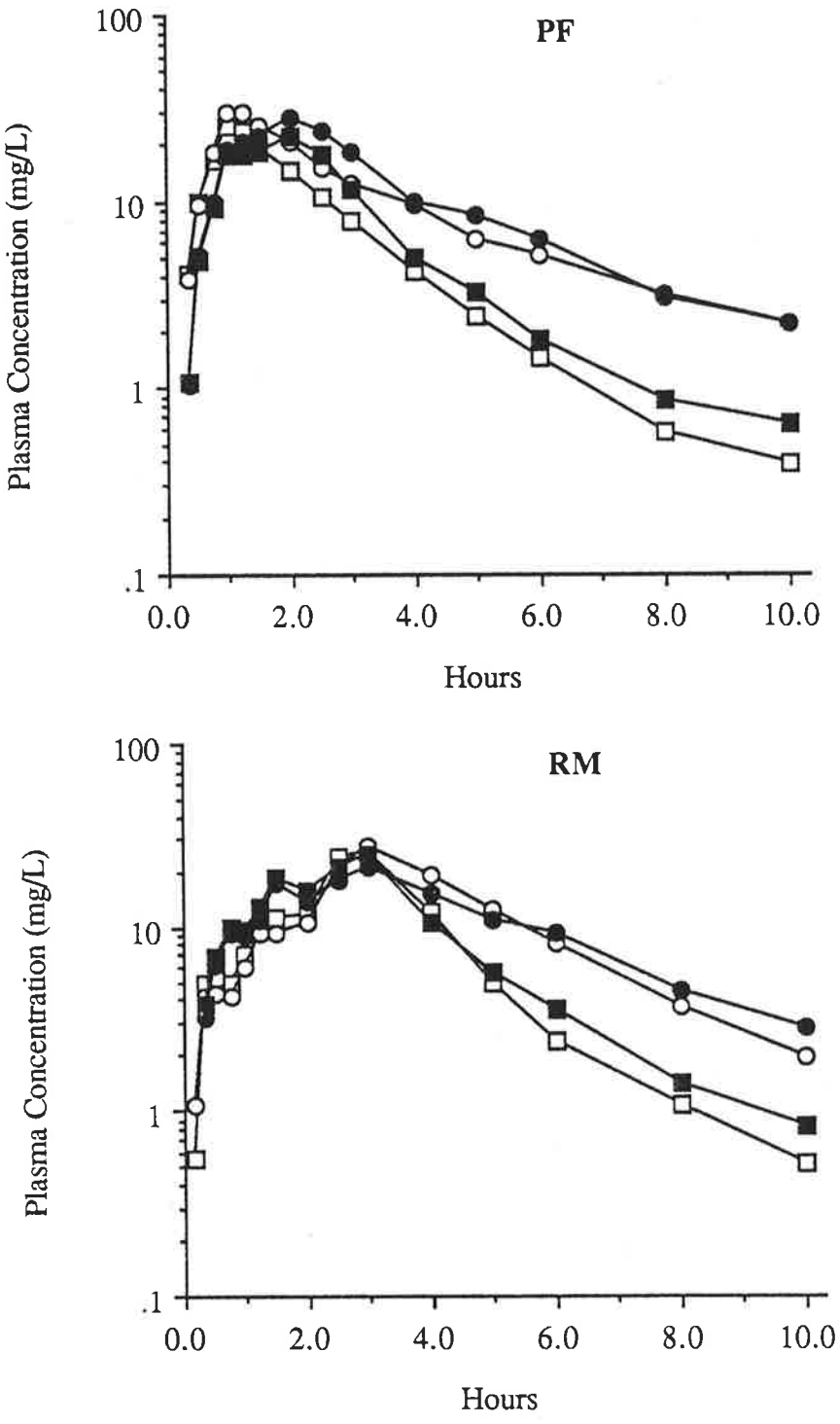


Figure 8.1 (continued)

Plasma concentration-time profiles of total R-I (□ control phase ; ■ treatment phase) and total S-I (○ control phase; ● treatment phase) in each subject.

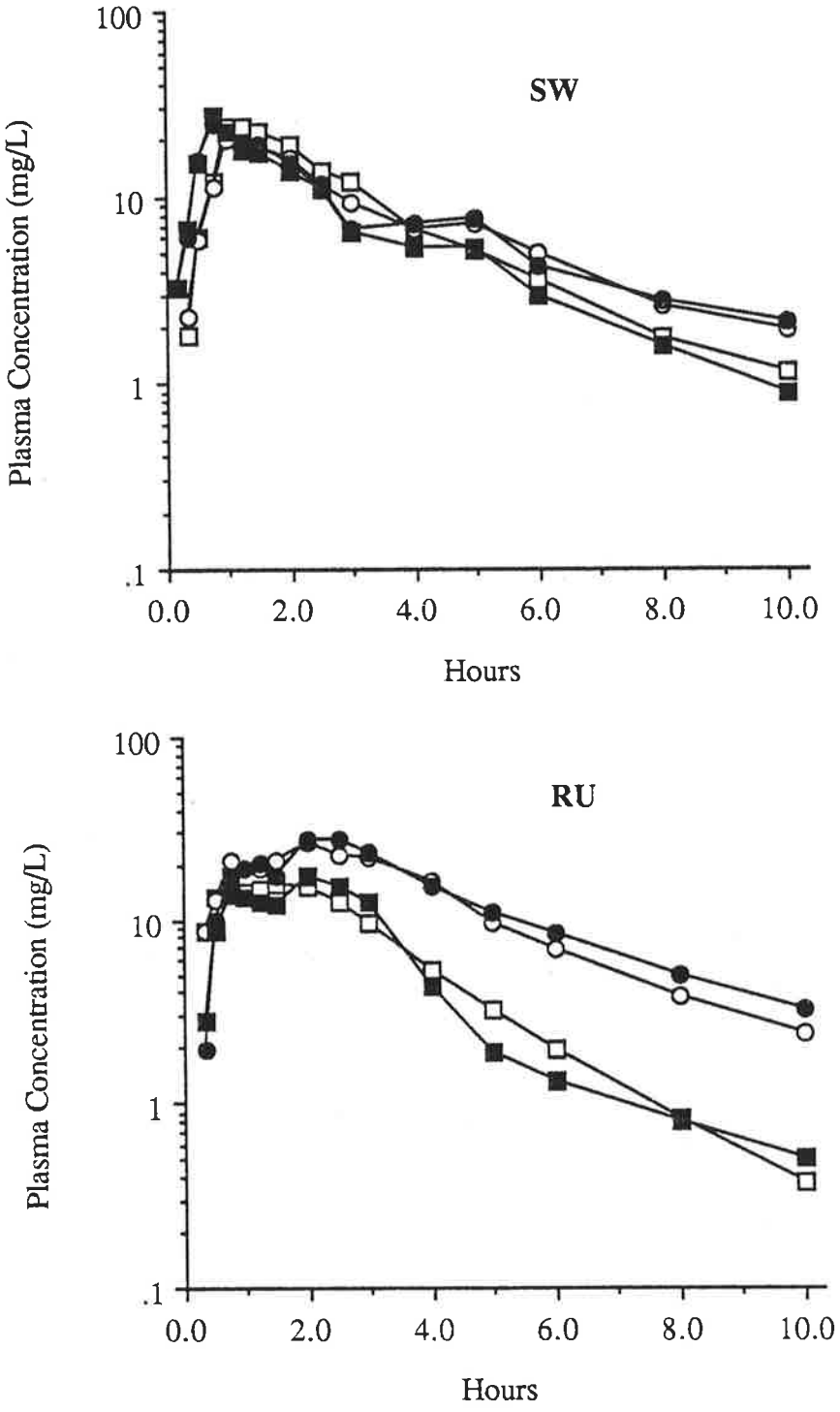


Figure 8.1 (continued)

Plasma concentration-time profiles of total R-I (□ control phase ; ■ treatment phase) and total S-I (○ control phase; ● treatment phase) in each subject.

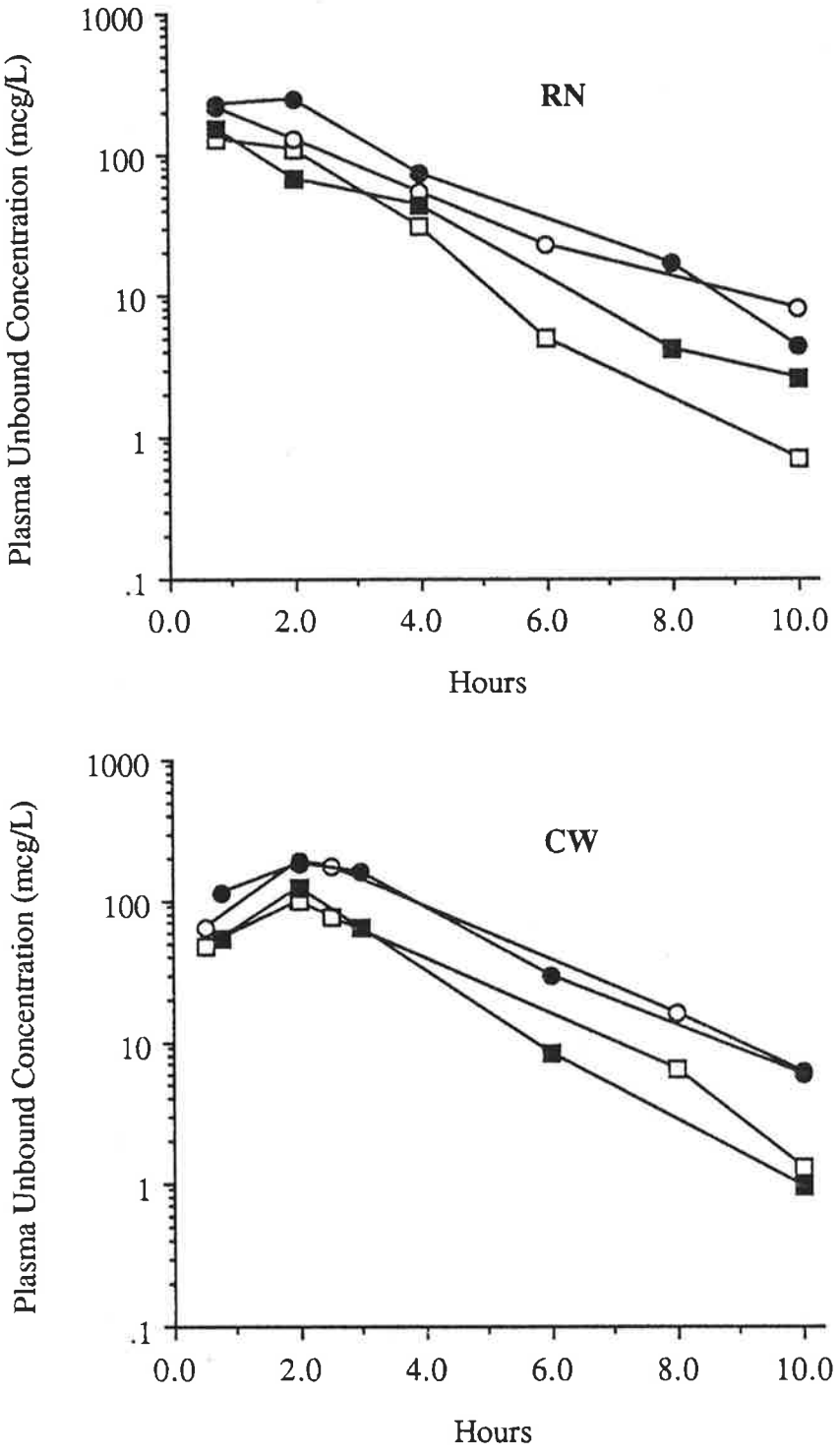


Figure 8.2.

Plasma concentration-time profiles of unbound R-I (□ control phase ; ■ treatment phase) and unbound S-I (○ control phase; ● treatment phase) in each subject.

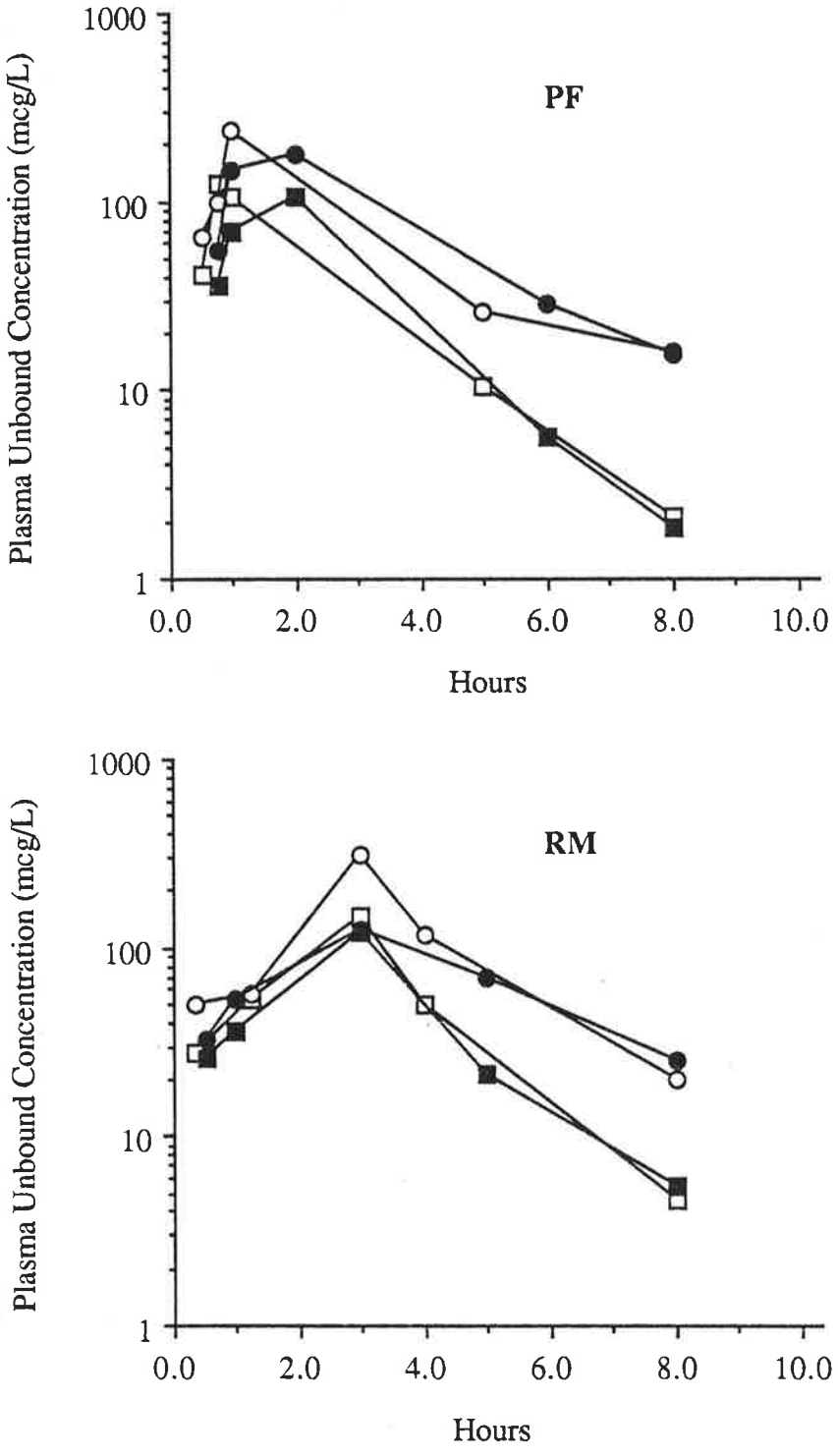


Figure 8.2 (continued)

Plasma concentration-time profiles of unbound R-I (□ control phase ; ■ treatment phase) and unbound S-I (○ control phase; ● treatment phase) in each subject.

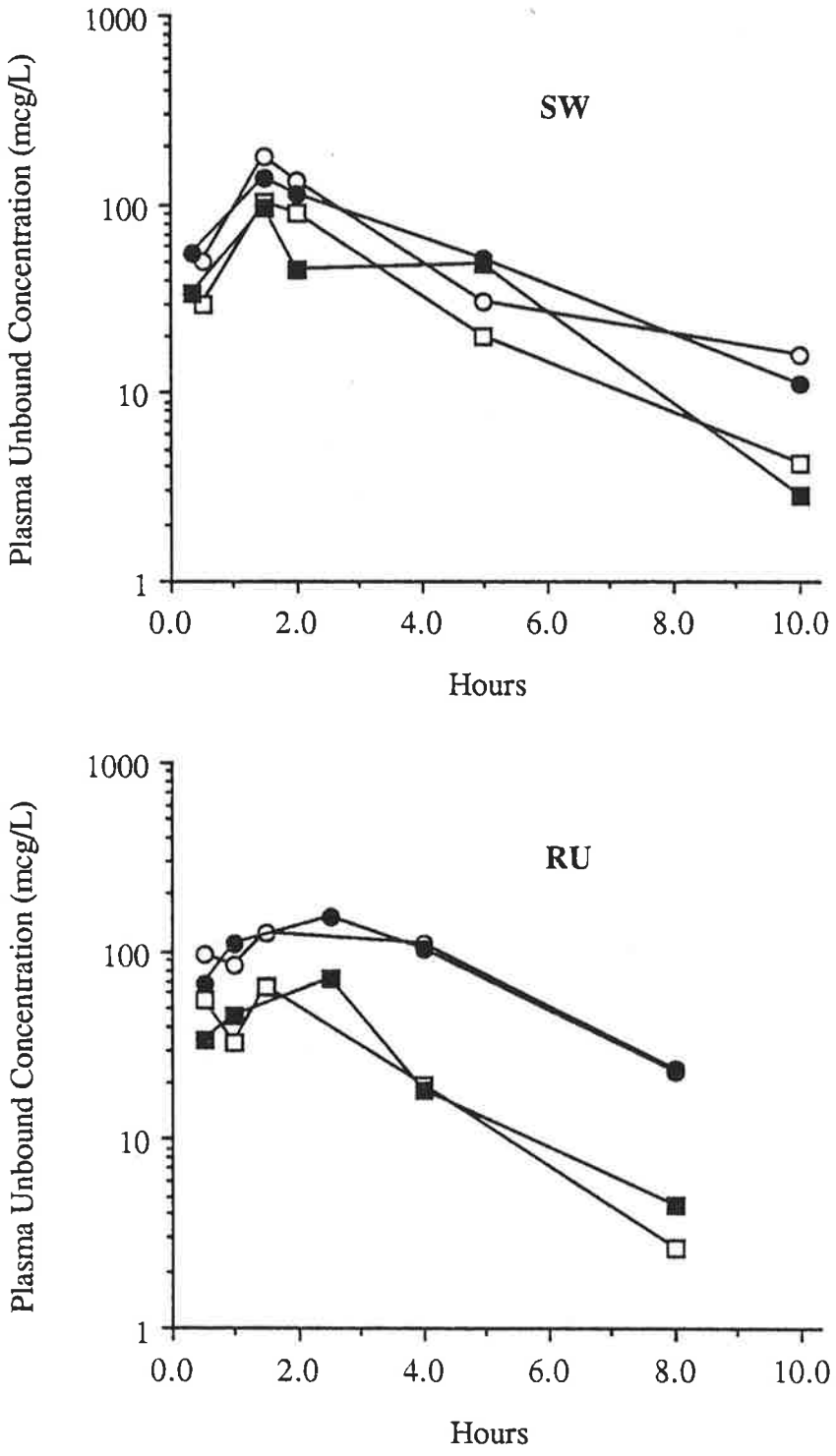


Figure 8.2 (continued)

Plasma concentration-time profiles of unbound R-I (□ control phase ; ■ treatment phase) and unbound S-I (○ control phase; ● treatment phase) in each subject.

Table 8.2. The AUC values of R(-)-ibuprofen and S(+)-ibuprofen for each subject during the control and treatment phase.

R(-)-IBUPROFEN AUC (mg.min/L)

Subject	Control	Treatment	Control/Treatment
RN	6179	7331	0.84
CW	5147	4712	1.09
PF	3368	3782	0.89
RM	4423	4887	0.91
SW	4526	3989	1.13
RU	3439	3288	1.05
Mean	4514	4665	0.99
SD	1063	1435	0.12

S(+)-IBUPROFEN AUC (mg.min/L)

Subject	Control	Treatment	Control/Treatment
RN	6807	8419	0.81
CW	7733	7144	1.08
PF	5982	6325	0.95
RM	6246	6536	0.96
SW	4729	5024	0.94
RU	7262	7870	0.92
Mean	6460	6886	0.94
SD	1063	1207	0.086

Table 8.3. Mean (SD) pharmacokinetic parameters of R(-)-ibuprofen and S(+)-ibuprofen in the control and treatment phase.

	R(-)-IBUPROFEN		S(+)-IBUPROFEN	
	CONTROL	TREATMENT	CONTROL	TREATMENT
AUC (mg.min/L)	4514 (1063)	4665* (1435)	6460 (1063)	6886* (1207)
C_{max} (mg/L)	27.2 (8.7)	27.1* (9.5)	28.5 (5.4)	29.6* (8.3)
t_{max} (hour)	1.42 (0.84)	1.78* (0.91)	1.63 (0.81)	1.87* (0.96)
$t_{1/2}$ (hours)	1.63 (0.26)	1.83* (0.48)	2.25 (0.56)	2.30* (0.54)
$AUC(0\text{-}t)_u$ (mg.min/L)	17.9 (4.31)	18.5* (3.45)	37.2 (5.76)	38.7* (7.32)
$\bar{fu}$ (x 100)	0.419 (0.051)	0.435* (0.060)	0.643 (0.093)	0.633* (0.053)

* Not significantly different ($p > 0.05$) from the corresponding data for the control phase (Student's paired t-test).

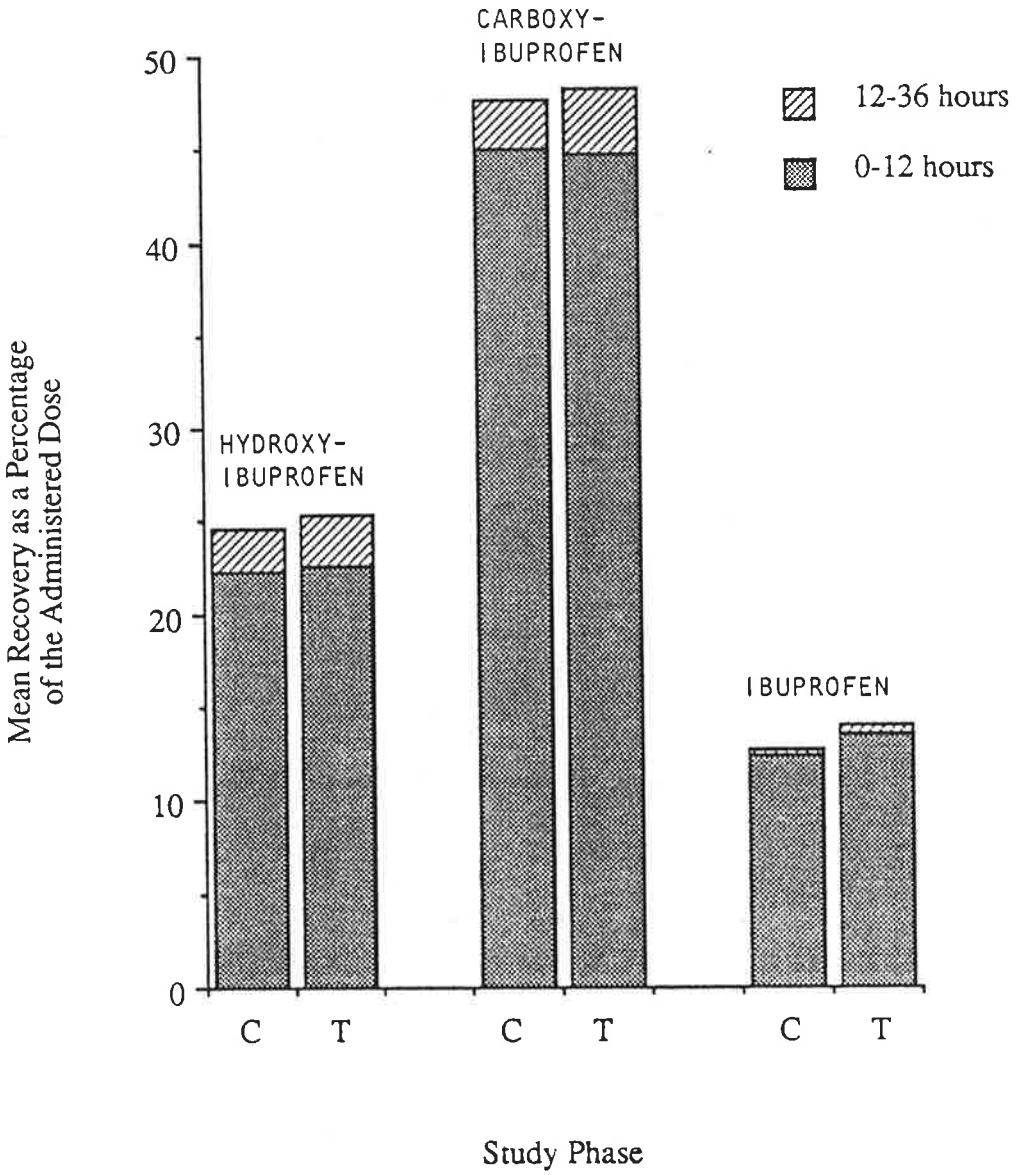


Figure 8.3.

The mean urinary recovery of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen, during the 0-12 and 12-36 hour collection periods of the control (C) and treatment (T) phases.

Table 8.4. Urinary recoveries (0-36 hours) of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen in the control and treatment phases. All data are expressed as mean (SD) recovery as a percentage of the administered dose.

	CONTROL	TREATMENT
Ibuprofen	12.6	14.1*
	(2.4)	(3.5)
Hydroxy-ibuprofen	24.7	25.3*
	(3.1)	(1.5)
Carboxy-ibuprofen	47.7	48.2*
	(3.7)	(2.4)
Total	84.9	87.6*
	(7.3)	(3.9)

* Not significantly different ($p > 0.05$) from the corresponding data for the control phase (Student's paired t-test).

Table 8.5. The effect of cimetidine on the plasma protein binding of ibuprofen enantiomers, *in vitro*.

Concentration in Plasma			
RS-Ibuprofen (mg/L)	Cimetidine (mg/L)	$f_u^{R\text{-}I}$	$f_u^{S\text{-}I}$
5	0	0.363 ± 0.024	0.651 ± 0.018
5	3	0.368 ± 0.056*	0.594 ± 0.044*
50	0	0.431 ± 0.037	0.681 ± 0.028
50	3	0.467 ± 0.059*	0.675 ± 0.122*

* Not significantly different ($p > 0.05$) from the corresponding value determined in the absence of cimetidine (Student's unpaired t-test).

8.4 DISCUSSION

In 1984, Parrott & Christensen examined the effect of cimetidine on the pharmacokinetics of ibuprofen administered orally to rats. Compared to the control phase, in which ibuprofen was given alone, the ibuprofen AUC increased by 85% during cimetidine coadministration. The terminal half-life of ibuprofen was unaltered between the two phases. While the authors concluded that cimetidine increased the extent of gastrointestinal absorption of ibuprofen, an effect of cimetidine on ibuprofen metabolism could not be excluded. In the same year, Conrad et al (1984) used a cross-over study design to examine the effect of cimetidine (300mg every 6 hours) on the pharmacokinetics of ibuprofen, given as a single oral dose to six healthy men. The ibuprofen AUC and terminal half-life were unaffected by cimetidine, and the authors concluded that cimetidine did not affect the oxidation of ibuprofen. Subsequently, there have been three similar studies investigating the effect of cimetidine on the pharmacokinetics of ibuprofen in humans. In all cases, it was concluded that ibuprofen pharmacokinetics were unaffected by cimetidine (Forsyth et al 1988; Ochs et al 1985a; Stephenson et al 1988). However, conclusions from all of these studies regarding the effect of cimetidine on the disposition of ibuprofen in humans must be drawn with caution, for the following reasons.

(i) Firstly, the urinary recovery of ibuprofen and its major metabolites was not measured. An effect of cimetidine on the metabolic clearance of ibuprofen, as assessed by determination of ibuprofen AUC, may have been masked therefore by a compensatory alteration in the extent of ibuprofen absorption. By comparing the total urinary recovery of ibuprofen and its metabolites between the phases, an index of the effect of cimetidine on the extent of absorption of ibuprofen, could have been obtained.

(ii) The plasma protein binding of ibuprofen in the control and treatment phases was not assessed. Accordingly, an effect of cimetidine on the intrinsic oxidative clearance of ibuprofen could have been masked to some degree by a change in its unbound fraction. For example, cimetidine may have inhibited the metabolic clearance of ibuprofen, thereby leading to an increase in the plasma concentrations of unbound drug. However, due to the concentration-dependent plasma protein binding of ibuprofen (section 3.6.2; Chapters 6 and 9) the increase in AUC_u may have been accompanied by a smaller increase in the AUC of total drug.

(iii) Finally, and most importantly, the analytical techniques used to measure the

plasma concentrations of ibuprofen failed to distinguish between the individual enantiomers. A differential effect of cimetidine on the oxidative clearances of R-I and S-I, or an effect of cimetidine on the metabolic inversion of R-I to S-I, would be difficult to detect solely on the basis of total plasma concentration-time data for unresolved drug. On this point, it should be noted that cimetidine has been found previously to exhibit an enantioselective effect on the pharmacokinetics of the racemic drugs, warfarin (Choonara et al 1986; Toon et al 1986) and metoprolol (Toon et al 1988).

Hence, none of the previous ibuprofen-cimetidine interaction studies (Conrad et al 1984; Forsyth et al 1988; Ochs et al 1985a; Stephenson et al 1988) have been able to positively determine whether cimetidine alters the pharmacokinetics of ibuprofen and, most importantly, the plasma concentrations of the pharmacologically important species, S-I.

As described in Chapter 6, the AUC of a drug which is cleared predominantly by hepatic metabolism is given by the equation.

$$AUC = \frac{f\,D}{CL} \qquad (6.1)$$

Upon chronic oral administration of a constant dose (D) of such a drug, at a dosing interval (T), the average steady-state plasma concentration (C^{ss}_{av}) is given by AUC/T. Hence, an alteration in the AUC of a drug after a single oral dose, may be reflected, upon chronic oral dosing, in an alteration in the average steady-state plasma drug concentration. For ibuprofen, after a single oral dose of the racemate, the AUC of R-I and S-I are described by the following equations, as derived in chapter 6.

$$AUC^{R\text{-}I} = \frac{f^{R\text{-}I}\,D^{R\text{-}I}}{CL^{R\text{-}I}} \qquad (6.2)$$

$$AUC^{S\text{-}I} = \frac{f^{S\text{-}I}\,D^{S\text{-}I} + f^{R\text{-}I}\,D^{R\text{-}I}\,(FI)}{CL^{S\text{-}I}} \qquad (6.6)$$

where $$CL^{R\text{-}I} = f_u^{R\text{-}I}\,CL_{int}^{R\text{-}I} \qquad (6.11)$$

and $$CL^{S\text{-}I} = f_u^{S\text{-}I}\,CL_{int}^{S\text{-}I} \qquad (6.12)$$

Note that for R-I and S-I, the fraction of the administered oral dose which reaches the systemic circulation (f) is equal to the fraction which is absorbed from the

gastrointestinal tract (f_a), since the fraction which escapes first-pass hepatic metabolism (f_{fp}) is close to unity (see section 3.6.1).

The pharmacokinetic parameters of R-I and S-I, obtained after oral administration of racemic ibuprofen (Table 8.3) are in excellent agreement with data described previously (Chapter 6) and with the data of other workers (Lee et al 1985). As in the dose ranging study, the AUC, AUC_u [in this case $AUC(0\text{-}t)_u$] and $\bar{f}_u$ of R-I were significantly lower than the corresponding parameters for S-I.

Importantly, the AUC of R-I and S-I were unaffected by cimetidine co-administration (Table 8.2 and 8.3). However, it is clear, from the above equations, that when investigating the effects of an interacting drug on the pharmacokinetics of ibuprofen, it is important to elucidate the effects it may have on the absorption (f), plasma protein binding (f_u), and intrinsic metabolic clearance (CL_{int}) of R-I and S-I.

In the present investigation, the total urinary recovery of ibuprofen and its metabolites was used as an index of the extent of its absorption after oral administration (f_a). The mean total recovery (84.9 and 87.6% for the control and treatment phases, respectively) was unaffected by concurrent cimetidine administration (Table 8.4), indicating that cimetidine had no significant overall effect on the extent of absorption of ibuprofen (f_a). Because ibuprofen and its metabolites in urine were analysed non-enantioselectively, the possibility that the extent of absorption of R-I and S-I may have been different between the phases can not be dismissed. However, this is unlikely in view of the extensive total recovery of ibuprofen in both phases.

Consistent with previous findings (Chapter 6), the time-averaged unbound fraction of S-I was greater than that of R-I in both phases in all volunteers. The difference between $\bar{f}_u^{R\text{-}I}$ and $\bar{f}_u^{S\text{-}I}$ was highly significant ($p < 0.001$, Student's paired t-test) in both phases. There was no significant difference ($p > 0.05$) between the two phases in the $\bar{f}_u$ of each enantiomer (Table 8.3). The *in vitro* experiment performed using plasma from a drug-free male, indicated that at racemic ibuprofen concentrations of 5.0 and 50mg/L, the presence of cimetidine at a clinically relevant concentration had no effect on the plasma protein binding of either ibuprofen enantiomer (Table 8.5). The lack of effect of cimetidine on the plasma binding of ibuprofen enantiomers is consistent with the low degree of binding of cimetidine (about 20% at concentrations encountered clinically; Somogyi & Gugler 1983) and its lack of effect on the plasma protein binding of other drugs (Somogyi & Muirhead, 1987).

Although the absorption, plasma protein binding and AUC of each ibuprofen

enantiomer were unaffected by cimetidine coadministration, conclusions regarding the effect of cimetidine on the metabolism of ibuprofen enantiomers must still be drawn with caution. There are two scenarios whereby the AUC for R-I and S-I could remain unchanged despite changes in the metabolic clearances of the enantiomers

(i) If the metabolism of R-I by non-inversion pathways was induced while that of S-I was inhibited; and the metabolic inversion of R-I was inhibited.

(ii) If the non-inversion clearance of R-I was inhibited; that of S-I was induced; and the metabolic inversion of R-I was also induced.

For the AUC of each enantiomer to remain unchanged, these changes would need to be compensatory. On the basis of our present knowledge of the actions of cimetidine (Somogyi & Muirhead 1987), induction of drug metabolism seems unlikely. Rather, if cimetidine exhibits differential effects on the various metabolic processes of a xenobiotic, it is usually varying degrees of inhibition. This is exemplified in the case of the effect of cimetidine on the metabolism of theophylline. While the 3- and 7-demethylation pathways of theophylline metabolism are inhibited by cimetidine, the 8-oxidation pathway is largely unaffected (Grygiel et al 1984). Accordingly, it is highly unlikely that cimetidine would induce the ibuprofen clearance pathways, and it is apparent, therefore, that cimetidine had no significant effect on the intrinsic metabolic clearance of R-I or S-I. Some indirect support for this conclusion is also provided by the fact that the overall profile of oxidative metabolism of ibuprofen, as assessed from the metabolic composition of ibuprofen recovered in urine (Table 8.4), was not influenced by cimetidine.

The enzyme-inhibiting properties of cimetidine are apparent within hours of administration, and the degree of inhibition does not change with time (Dossing et al 1983; Feely et al 1984). For these reasons, the cimetidine dosing protocol chosen for the present study was suitable for examining the effects of cimetidine on ibuprofen pharmacokinetics. The effect of cimetidine on oxidative drug metabolism is concentration dependent; Somogyi & Muirhead (1987) stated that the degree of inhibition increased from 10-20% at daily cimetidine doses of 300 to 400mg, to 40-50% with daily doses greater that 2000mg, depending on the substrate used. Hence, the regimen of cimetidine used in the present study (1000mg daily) was suitable for examining the potential for a pharmacokinetic interaction with ibuprofen. However, our findings do not exclude the possibility of an effect of cimetidine on ibuprofen metabolism in cases where plasma cimetidine concentrations may be elevated, such as in patients with renal failure.

In recent years, it has become increasingly apparant that there are a number of

drugs, metabolized by the cytochrome P-450 mixed function oxidase system, which are spared from the effects of cimetidine. These drugs include carbamazepine (Somogyi & Muirhead 1987), mexiletine (Klein et al 1985) and tolbutamide (Dey et al 1983; Stockley et al 1986), although the data for tolbutamide are conflicting (Cate et al 1986). Recently, Somogyi & Muirhead (1987) suggested that these variable effects of cimetidine may be related to differential binding of the enzyme inhibitor to the various cytochrome P-450 isozymes. Evidence for this notion is provided by the effects of cimetidine on theophylline metabolism. The 8-oxidation and demethylation of theophylline appear to be carried out by two distinct cytochrome P-450 isozymes (Birkett et al 1988). *In vitro* studies with human liver microsomes (Birkett et al 1988) and *in vivo* drug-interaction studies (Grygiel et al 1984) suggest that cimetidine may have differential effects on these two isozymes; selectively inhibiting the form involved in theophylline demethylation. The results of the present study, which indicate that cimetidine has no effect on the oxidative metabolism of ibuprofen, add further support to the concept that cimetidine can not be considered a universal inhibitor of phase I hepatic drug metabolism, at least at therapeutic concentrations.

8.5 CONCLUSION

The pharmacokinetics of R-I and of the pharmacologically important S-I, after the single oral dose administration of racemic ibuprofen, were unaffected by concurrent cimetidine administration. The data suggest that the oxidative metabolism of ibuprofen is not inhibited by cimetidine and, on this basis, there would be no pharmacokinetic rationale for adjusting ibuprofen dosage when cimetidine therapy is started or stopped.

Chapter 9
The Plasma Protein Binding of Ibuprofen Enantiomers.

9.1 INTRODUCTION

In Chapters 6 and 8, significant differences were reported between the time-averaged unbound fraction of R-I and S-I in human volunteers who had received racemic ibuprofen. In addition, when a range of doses of racemic ibuprofen was administered to healthy volunteers, the time-averaged unbound fraction of both enantiomers increased significantly with dose. The present chapter examines more closely the plasma protein binding data generated in the human volunteer studies described in Chapters 6 and 8. In addition, the results of a controlled *in vitro* experiment, designed to examine the magnitude of the concentration-dependent plasma protein binding of R-I and S-I, and to determine the effect of each enantiomer on the plasma protein binding of the other, are presented.

9.2. METHODS

9.2.1. The Plasma Protein Binding of R(-)-Ibuprofen and S(+)-Ibuprofen in Humans After Oral Administration of Racemic Ibuprofen.

The data used to examine the plasma protein binding of R-I and S-I in volunteers who had ingested racemic ibuprofen, included those from all four phases of the dose-ranging study (Chapter 6), and from the control phase of the ibuprofen-cimetidine interaction study (Chapter 8).

9.2.2. *In Vitro* Binding of R(-)-Ibuprofen and S(+)-Ibuprofen in Plasma Obtained From Drug-Free Humans.

This *in vitro* study was conducted using plasma obtained from three healthy male volunteers: AE, 25 years of age, plasma albumin concentration 48 g/L; RN, 39 years, plasma albumin 48 g/L; and BM, 38 years, plasma albumin 47 g/L. In each case blood (120ml) was withdrawn from an arm vein by simple venepuncture and placed into heparinised collection tubes. After centrifugation, plasma was separated and added to a series of test-tubes, as outlined below. Equilibrium dialysis was performed within 2 to

4 hours of blood collection.

A series of 15 test-tubes containing R-I (20, 40, 80, 120 and 200 mcg, in tubes 1 to 5, respectively), S-I (20, 40, 80, 120 and 200 mcg, in tubes 6 to 10, respectively) and racemic ibuprofen (40, 80, 160, 240 and 400 mcg, in tubes 11 to 15, respectively) were prepared using appropriate aliquots of methanolic solutions of R-I (1mg/ml) and/or S-I (1mg/ml). After evaporation of methanol (using a gentle stream of nitrogen), 4 ml of drug-free plasma was added, producing final plasma concentrations of 5, 10, 20, 30 and 50 mg/L for R-I (tubes 1-5) and S-I (tubes 6-10) and 10, 20, 40, 60 and 100mg/L for RS-I (tubes 11-15). After gentle vortex-mixing, 3.5ml of each plasma sample was dialyzed against 3.5ml of isotonic pH 7.4 phosphate buffer, and the unbound fraction of R-I and S-I was determined, as described in Chapter 5. It should be noted that the racemic radiolabelled ibuprofen which was spiked into each dialysis cell (2 mg/L), contributed to the plasma concentration of R-I and S-I. However, as discussed in Chapter 5, the relatively small quantity added would have no significant influence on the measured unbound fraction of each enantiomer. Hence, the measured unbound fractions were taken to represent the plasma unbound fractions of R-I and S-I prior to equilibrium dialysis.

9.3. RESULTS

9.3.1. The Plasma Protein Binding of R(-)-Ibuprofen and S(+)-Ibuprofen in Humans After Oral Administration of Racemic Ibuprofen.

The unbound fraction of R-I and S-I were determined in a total of 96 plasma samples from the dose-ranging study. These data were used to construct a plot, for each enantiomer, of the relationship between unbound fraction and total plasma concentration (Figure 9.1). This graph highlights the enantioselective nature of the plasma protein binding of ibuprofen (with the unbound fraction of R-I being consistently less than that of S-I at equivalent total plasma concentrations); and illustrates the dependence of the plasma unbound fraction of each enantiomer on the respective total plasma concentration (particularly above 10mg/L).

In the studies described in this thesis, an 800mg dose of racemic ibuprofen was administered to ten healthy volunteers who were not receiving any other medication (to four subjects in the dose-ranging study (Chapter 6), and to six subjects in the control phase of the cimetidine-ibuprofen interaction study (Chapter 8)). The relationship between the time-averaged unbound fraction of R-I and that of S-I, determined for these ten volunteers, is presented in Figure 9.2. Linear regression analysis indicated that there was a significant correlation between the time-averaged unbound fraction of the

two enantiomers ($r = 0.84$; $p < 0.05$).

9.3.2. *In Vitro* Binding of R(-)-Ibuprofen and S(+)-Ibuprofen in Plasma Obtained From Drug-Free Humans.

For each volunteer, the plasma unbound fractions of R-I and S-I, as a function of total plasma concentration, both in the absence of, and in the presence of, an equal concentration of the optical antipode, is depicted in Figure 9.3 (mean data are also presented).

In each case, the plasma unbound fraction of R-I and S-I increased as the total concentration increased. For example, between the concentrations of 10 and 100 mg/L of RS-I (samples 11-15), the mean percentage unbound of R-I increased from 0.275 to 0.591%, and that of S-I increased from 0.576 to 0.961%; representing relative increases of 115% and 67%, respectively. The effect of increasing total plasma concentration on unbound fraction was analysed statistically by one-way analysis of variance. Irrespective of whether the statistical analysis was performed on data from the cells containing the individual enantiomers (1-5 and 6-10), or on the cells containing the racemate (11-15), there was a significant ($p< 0.05$) increase in the unbound fraction of each enantiomer as the total plasma concentration increased.

At equivalent total plasma concentrations, the unbound fraction of S-I exceeded that of R-I (Figure 9.3). For example, in the samples containing 100 mg/L of the racemate (50 mg/L of each enantiomer), the mean (n=3) percentage unbound of R-I ($0.591 \pm 0.106\%$) was significantly less (paired Student's t-test; $p < 0.05$) than that of S-I ($0.961 \pm 0.289\%$).

By comparing the unbound fractions of each enantiomer in the absence of, and in the presence of, its optical antipode, it is apparent that each ibuprofen enantiomer decreased the plasma protein binding of the other (Figure 9.3). For example, the mean ($\pm$ SD) percentage unbound of S-I, at a concentration of 50 mg/L in the absence of R-I, was $0.658 \pm 0.242\%$. In the presence of an equal concentration of R-I, the percent unbound of S-I increased significantly (paired Student's t-test; $p < 0.05$) to $0.961 \pm 0.289\%$.

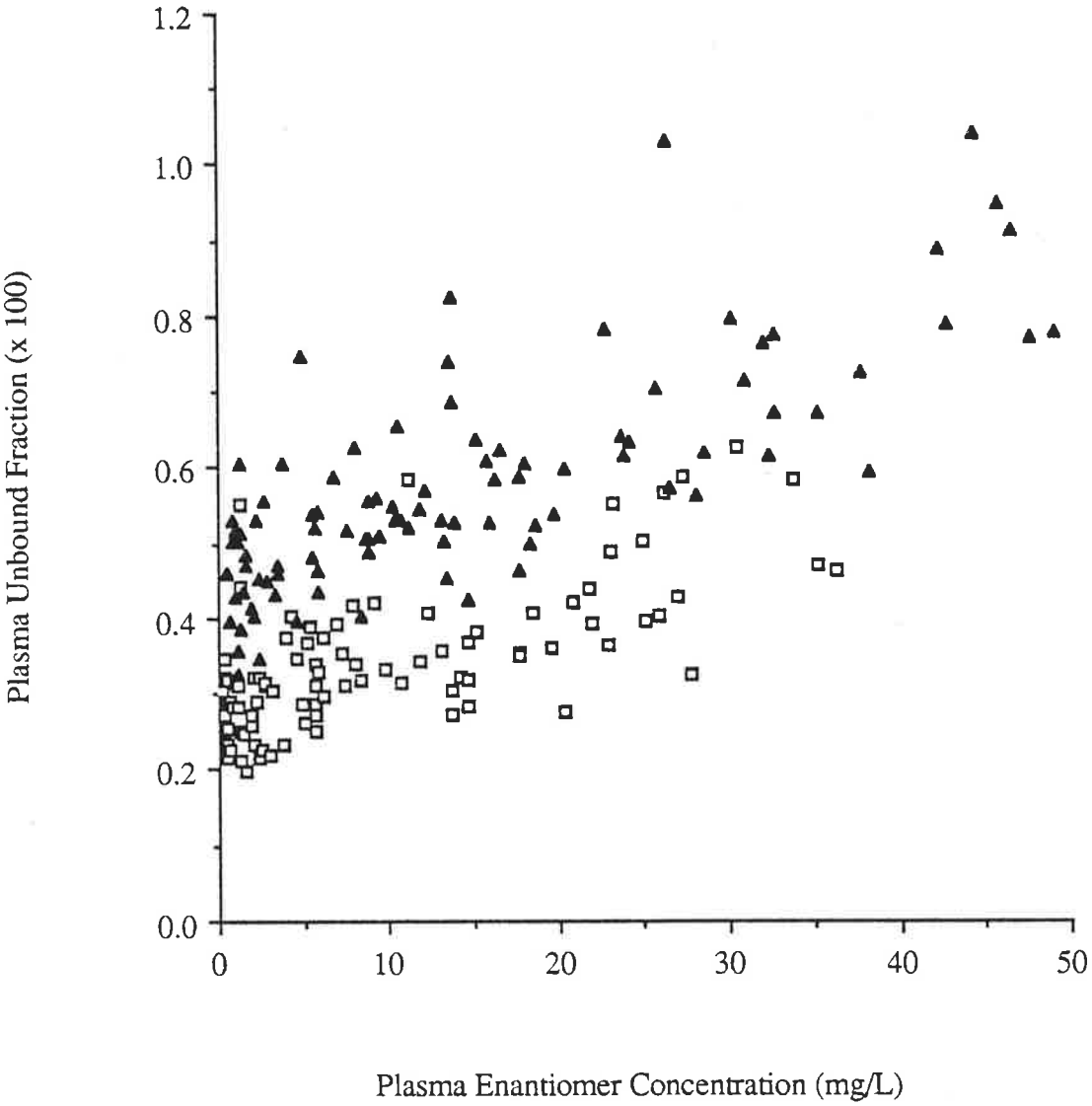


Figure 9.1.

Plots of the relationship between plasma unbound fraction and total plasma concentration for R-I (□) and S-I (▲). Data are included from all four phases of the dose ranging study (Chapter 6).

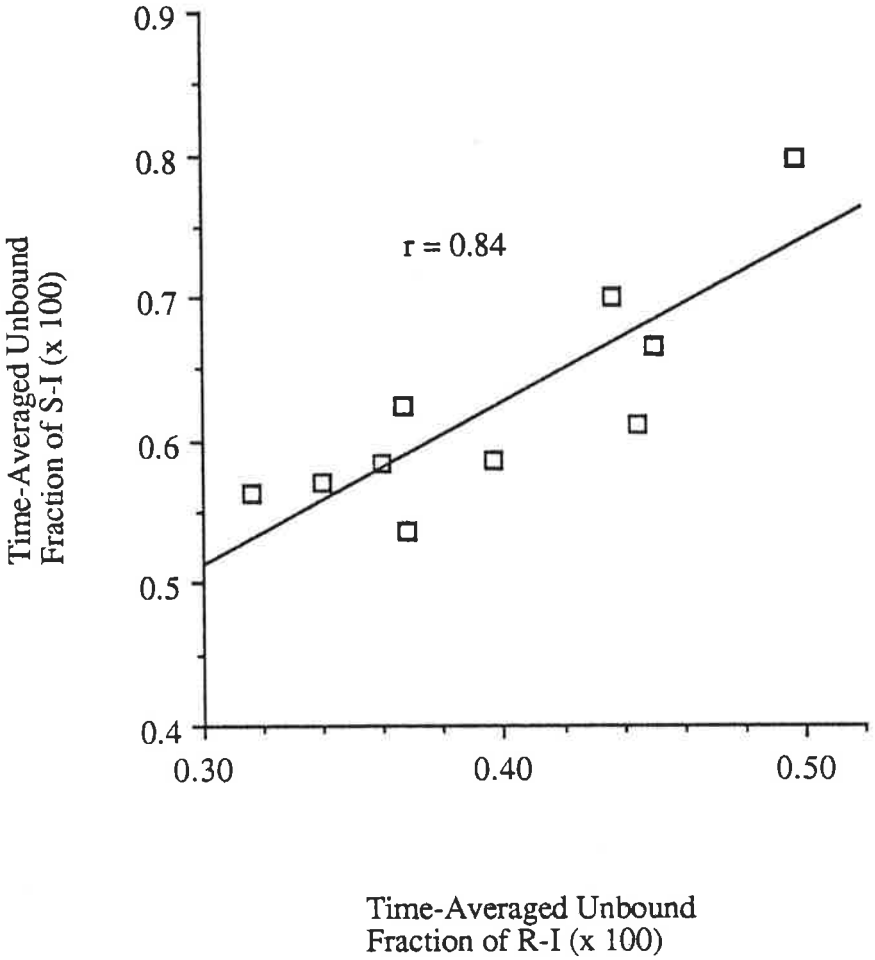


Figure 9.2. The relationship between the time-averaged unbound fraction of the individual enantiomers of ibuprofen after an 800mg oral dose of the racemate. Data are included from the dose ranging study (800mg dose level; see Chapter 6) and the ibuprofen cimetidine interaction study (Control phase only; see Chapter 8).

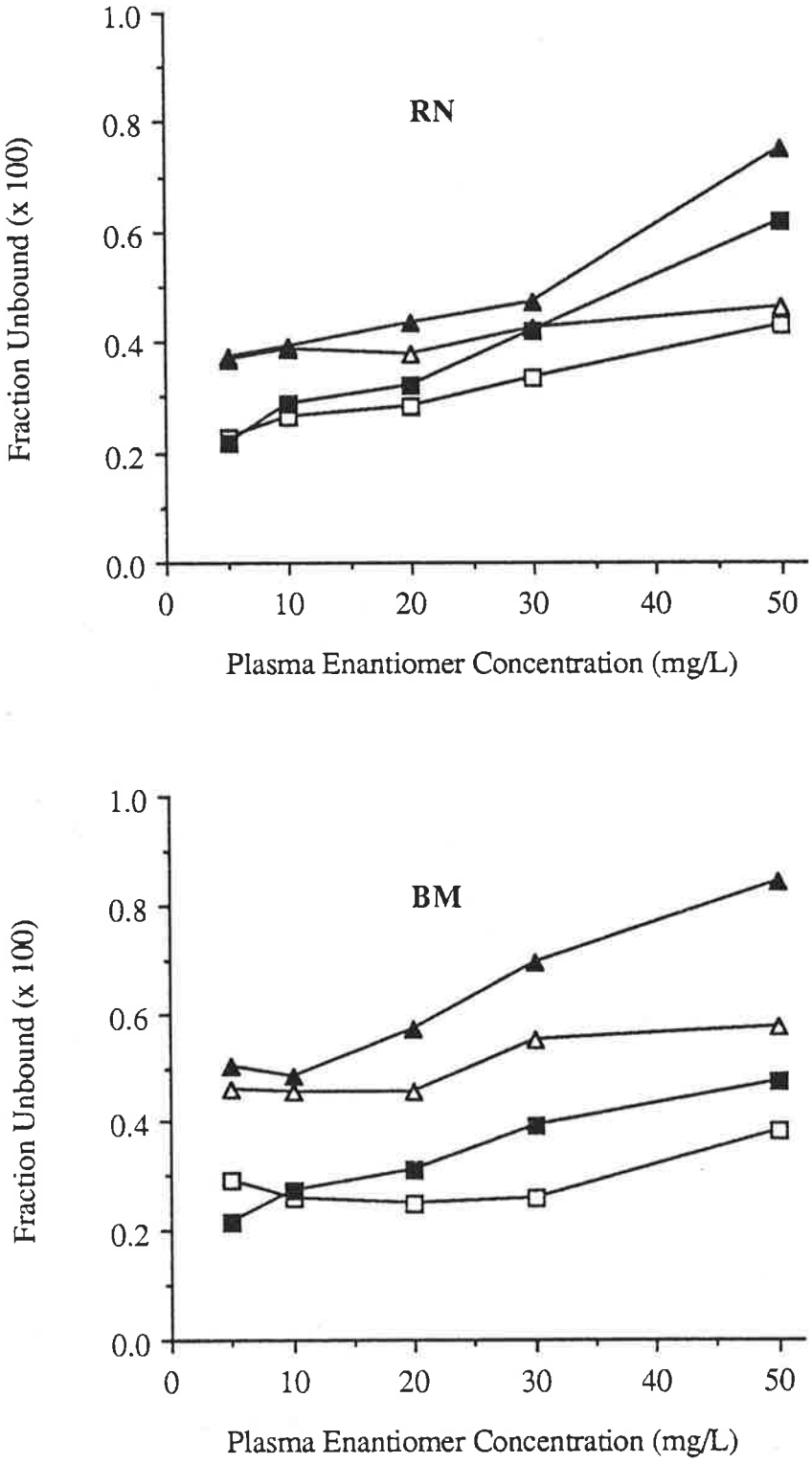


Figure 9.3. Plots of the plasma unbound fraction versus total plasma concentration (5 to 50mg/L) for each ibuprofen enantiomer, both in the absence of, and in the presence of an equal concentration of, its optical antipode (□ R-I alone; ■ R-I in the presence of S-I; △ S-I alone; ▲ S-I in the presence of R-I).

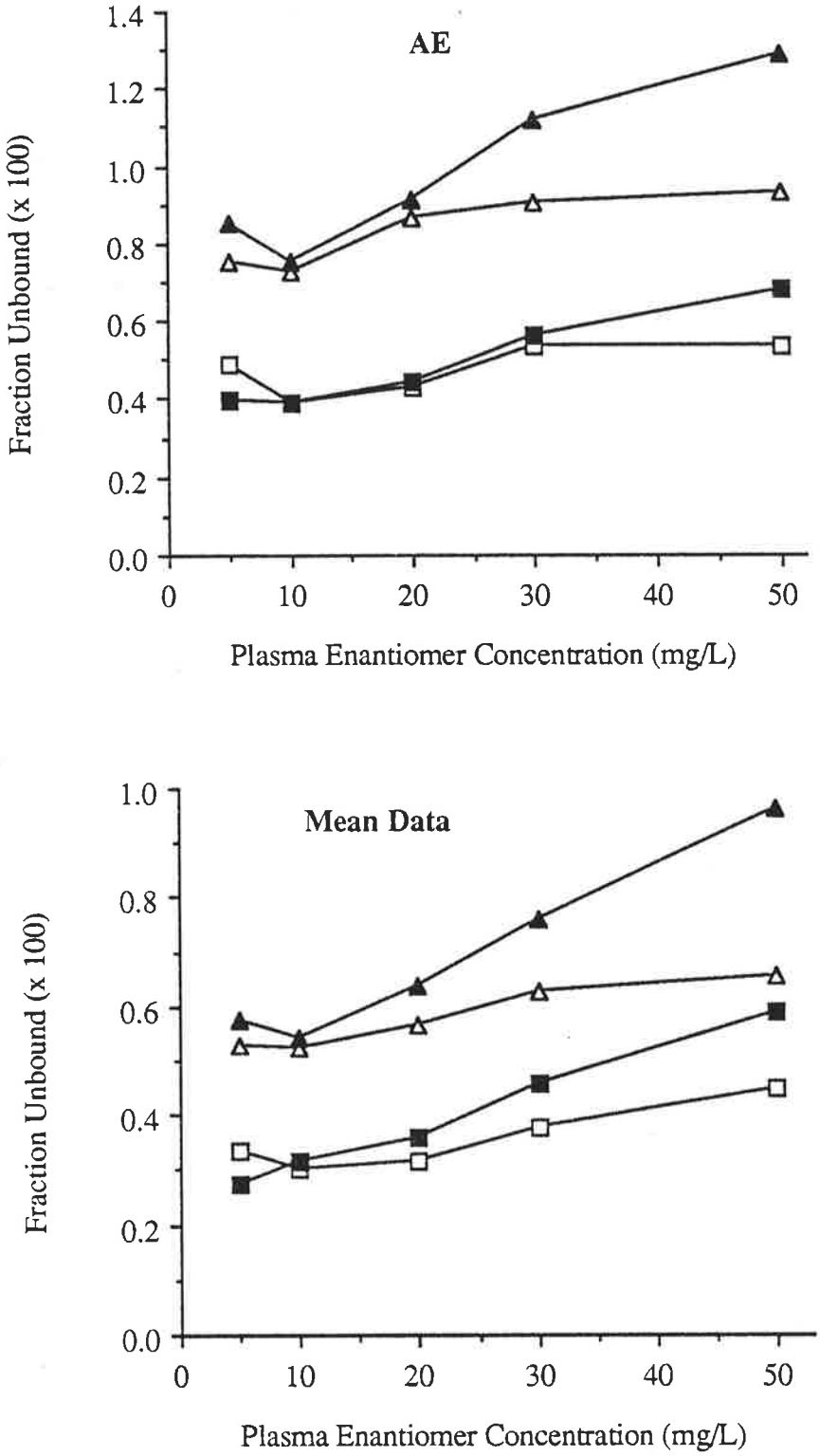


Figure 9.3 (Continued).

Plots of the plasma unbound fraction versus total plasma concentration (5 to 50mg/L) for each ibuprofen enantiomer, both in the absence of, and in the presence of an equal concentration of, its optical antipode (□ R-I alone; ■ R-I in the presence of S-I; △ S-I alone; ▲ S-I in the presence of R-I).

9.4. DISCUSSION

The data presented in this chapter unequivocally demonstrate that over the range of plasma concentrations encountered clinically the protein binding of R-I and S-I exhibit concentration-dependence. This is in keeping with the results of other workers who have found an increase in the unbound fraction of unresolved ibuprofen with increasing concentration (Aarons et al 1983a; Grennan et al 1983; Lockwood et al 1983b).

The results of an extensive investigation on the protein binding characteristics of ibuprofen were presented by Whitlam et al (1979), who used equilibrium dialysis to examine the binding of the unresolved drug to purified human serum albumin (HSA). Ibuprofen was reported to bind to a single primary site (high affinity) and six to seven secondary sites (low affinity) on the HSA molecule. Based on derived ibuprofen-albumin binding parameters, Whitlam and coworkers estimated that at a physiological concentration of HSA (4%), and an ibuprofen concentration of 20mg/L, only 17% of the available ibuprofen-binding sites would be occupied, and that the unbound fraction of ibuprofen would be 0.001. With such a low level of site occupancy, it would be unlikely that the plasma protein binding of ibuprofen would be non-linear over the concentration range encountered after therapeutic doses. However, these estimates do not take into account the possible modulating effects of endogenous molecules present in plasma. While the ibuprofen-displacing effects of uric acid, bilirubin and cholesterol were found to be negligible, palmitic acid, selected as a representative non-esterified fatty acid, significantly reduced the ibuprofen binding capacity of HSA (Whitlam et al 1979). It was estimated that the presence of palmitate, at a concentration of 118 mg/L, would increase the plasma unbound fraction of ibuprofen to 0.005 (Whitlam et al 1979). In addition to direct competition between ibuprofen and palmitate for the available binding sites on HSA, Whitlam and coworkers suggested that palmitate may induce a conformational change in the HSA molecule which may decrease the number of available ibuprofen binding sites. Hence, the concentration-dependent binding of ibuprofen enantiomers to human plasma (Figure 9.1 and 9.3) may arise from the diminished ibuprofen binding capacity of HSA in the presence of endogenous non-esterified fatty acids.

The present work indicates that over the therapeutic concentration range, the plasma protein binding of ibuprofen exhibits enantioselectivity, with R-I binding to a greater extent than S-I. In a preliminary communication, Hansen et al (1985), reported that over the concentration range of 2 to 100 mg/L, the binding of both ibuprofen enantiomers was saturable, and, on average, the mean unbound concentration of S-I exceeded that of R-I by 70%. Although no values for the unbound fractions, or unbound concentrations, were given, these data are in agreement with those presented

in this thesis.

It appears that the extent of enantioselectivity in the binding of R-I and S-I is not great, inasmuch as there was little difference between the enantiomers in percent bound. For example, at 100mg/L of RS-I, the mean percentages bound for R-I and S-I were 99.4% and 99.0%, respectively (Figure 9.3). Ibuprofen is bound with high affinity to binding site II (the benzodiazepine site) on the HSA molecule (Kober & Sjoholm 1980; Sudlow et al 1976). This site has been shown previously to exhibit extreme enantioselectivity in the binding of the enantiomers of tryptophan (Jahnchen & Muller 1983) and oxazepam hemisuccinate (Jahnchen & Muller 1983; Muller & Wollert 1975). Because HSA is the major ibuprofen binding protein (Vowles & Marchant 1980), the enantioselective plasma protein binding of the drug probably arises from differential binding of the individual enantiomers to HSA; possibly at site II.

The results of studies which have examined the enantiomeric protein binding of other 2-PPA derivatives, suggest that enantioselective plasma protein binding may be a general property of this drug class. In 1986, Jones et al used the individually radiolabelled enantiomers of 2-PPA to examine the binding of R(-)-2-PPA and S(+)-2-PPA to rabbit plasma, and to fatty acid-free rabbit albumin in the absence, and in the presence of, oleic acid. It was found that R(-)-2-PPA was bound more avidly to fatty acid-stripped rabbit albumin than was its optical antipode. For both enantiomers it was estimated that there were 2 major binding sites per albumin molecule. Upon addition of oleic acid, one of these sites disappeared, and the binding of R(-)- and of S(+)-2-PPA were reduced, although the enantioselectivity remained. The binding of the enantiomers to rabbit plasma was similar to the binding to rabbit albumin in the presence of oleic acid. An important finding was that the binding of each enantiomer to rabbit plasma was reduced by the presence of the other enantiomer. This inhibition was described by a binding model in which both enantiomers were competing for the same binding site, present once on each albumin molecule (Jones et al 1986).

Rendic et al (1980) used a gel filtration technique to examine the binding of R(-)- and S(+)-ketoprofen to HSA. Although the influence of one enantiomer on the binding of the other could not be studied, R(-)-ketoprofen was bound to a greater extent than the S(+)-enantiomer. At a HSA concentration of 1.44×10^{-4}M and a ligand concentration of 0.72 to 1.44×10^{-4}M, the bound fraction of S(+)-ketoprofen ranged between 0.979 and 0.974 (percent unbound of 2.1-2.6%), while the bound fraction of R(-)-ketoprofen ranged from 0.984 to 0.981 (percent unbound of 1.6-1.9%).

Although yet to be reported in detail, Knadler et al (1988) have presented the results of an investigation of the plasma protein binding of R(-)- and S(+)-flurbiprofen. In this case, the mean percent unbound of R(-)-flurbiprofen (0.082%) was greater than that of S(+)-flurbiprofen (0.048%) over the concentration range 1 to 26 mg/L. The

binding of each enantiomer was reported to be decreased by the presence of its optical antipode, suggesting that both enantiomers were competing for a common binding site (Knadler et al 1988).

Hence, for ibuprofen, ketoprofen, 2-PPA and flurbiprofen, enantioselective plasma protein binding has been demonstrated, suggesting that the plasma protein binding of other 2-PPA derivatives may also exhibit enantioselectivity. In 1973, Perrin reported that the binding of fenoprofen enantiomers to purified HSA was not "stereospecific", inasmuch as both enantiomers appeared to bind to the same site on HSA. In that study, circular dichroism was used to examine the extrinsic Cotton-effect resulting from the binding of the individual fenoprofen enantiomers to HSA. This technique was used largely to examine the nature of the fenoprofen binding site, and subtle differences in binding between the enantiomers could have gone undetected.

The studies of Jones et al (1986) and Knadler et al (1988) suggest that for both 2-PPA and flurbiprofen, the enantiomers compete with one another for the available sites on the plasma binding proteins. Similarly, the results of the *in vitro* plasma protein binding study, presented in this chapter (Figure 9.3), indicate that each enantiomer of ibuprofen is able to modify the plasma protein binding of the other, and suggest that the enantiomers may be competing for the same albumin binding site(s). It is interesting to note, therefore, the strong correlation between the time-averaged unbound fraction of the individual enantiomers for ten individuals who had taken 800mg of racemic ibuprofen (Figure 9.2).

As discussed in sections 2.3.1, and 3.6.2, many of the studies which have examined the disposition of ibuprofen in humans have relied on the use of racemic radiolabelled ibuprofen added to post-dose plasma to estimate the unbound fraction of the drug (Aarons et al 1983b; Abernethy & Greenblatt 1985; Albert et al 1984a; Gallo et al 1986; Greenblatt et al 1984; Grennan et al 1983; Lockwood et al 1983a, 1983b; Ochs et al 1985b). In all of these cases, the unbound fraction of ibuprofen was taken to be the ratio of the concentration of radioactivity between the buffer and plasma compartment after equilibrium dialysis or, in the case of ultrafiltration, the ratio of the concentration of radioactivity between the filtrate and unfiltered plasma. The unbound fraction of the unresolved drug, determined in this manner, simply represents the arithmetic mean of the unbound fractions of the individual enantiomers, and the approach takes no account of the enantiomeric composition of the drug in post-dose plasma samples. As discussed in Chapter 2, the true unbound fraction of unresolved drug, is a function of the relative total plasma concentrations of the individual enantiomers, and their respective unbound fractions. To predict the true unbound fraction of unresolved ibuprofen, equation 2.7 can be applied, giving equation 9.1.

$$f_u^{UNR\text{-}I} = \frac{C^{R\text{-}I}}{C^{R\text{-}I} + C^{S\text{-}I}} f_u^{R\text{-}I} + \frac{C^{S\text{-}I}}{C^{R\text{-}I} + C^{S\text{-}I}} f_u^{S\text{-}I} \qquad (9.1)$$

Listed in Table 9.1 are the total concentrations, and unbound fractions, of R-I and S-I, in a selection of samples from the dose-ranging study. These data for the individual enantiomers were used to calculate (equation 9.1) the unbound fraction of unresolved ibuprofen in each sample. It is interesting to compare this predicted unbound fraction for unresolved drug (method 1; Table 9.1) with that which would be determined if the unbound fraction had been determined non-enantioselectively, calculated, in this case, as the mean of $f_u^{R\text{-}I}$ and $f_u^{S\text{-}I}$ (method 2; Table 9.1). Clearly, where the unbound fraction and plasma concentration of R-I differ from the corresponding unbound fraction and total concentration of S-I, (as in the selected samples in Table 9.1), the true unbound fraction of unresolved ibuprofen (method 1) will not be equal to the arithmetic mean of the unbound fractions of the two enantiomers (method 2). For the data presented in Table 9.1 the percent difference between the true f_u of UNR-I, and that which would have been determined non-enantioselectively with radiolabelled RS-I, ranged from -6.8 to -19.5 %. In all cases, method 2 gave results which were less than the true unbound fraction (method 1). This is because the *ex-vivo* plasma samples were enriched with the ibuprofen enantiomer which had the highest unbound fraction (S-I). Hence, it may be concluded that much of the ibuprofen plasma protein binding data presented in the studies referenced above, contain varying margins of error.

It is instructive to briefly consider some of the dispositional consequences of the enantioselective, and concentration- dependent, plasma protein binding of ibuprofen. As discussed in Chapter 6, the clearance, referenced to total plasma concentration, of each of the ibuprofen enantiomers, is a function of both their intrinsic clearances, and the fractions unbound in plasma.

$$CL = f_u \, CL_{int} \qquad (6.10)$$

Hence, the differential plasma protein binding of R-I and S-I would be expected to contribute to a difference in their respective clearances. This concept was alluded to by Cox et al (1985). These authors found that the clearance of S-I across the isolated perfused rat liver exceeded that of R-I, and recognised that interpretation of this result was limited by the lack of protein binding data, in that the preferential clearance of S-I may have been due to a greater degree of binding to perfusate protein for the R(-)-enantiomer. Equation 6.10 also predicts that the total clearance of each enantiomer will increase as its unbound fraction increases. The findings of the ibuprofen

dose-ranging study, presented in Chapter 6, confirm this prediction. Because the presence of one enantiomer can decrease the plasma protein binding of the other (Figure 9.3), it is to be expected that the total clearance of one enantiomer may be altered by the presence of the other. Lee et al (1985) reported that the mean total apparent oral clearance of R-I, after a 400mg dose of R-I, was 67.9 ml/min. When 400mg of R-I was given along with 400mg of S-I (i.e. 800mg of RS-I), the clearance of R-I was 87.6 ml/min. The authors speculated that the presence of S-I, after dosing with RS-I, may have increased the clearance of R-I, via a perturbation of the plasma protein binding of R-I. The data presented within this chapter, suggesting an enantiomer-enantiomer plasma protein binding interaction, add substantial support to this concept.

The pharmacodynamic consequences of the enantioselective, and concentration-dependent, plasma protein binding of ibuprofen, would be expected to be minimal. This is because the area under the plasma unbound concentration-time curve for a drug eliminated by capacity-limited metabolism is independent of unbound fraction (in the case of ibuprofen, the unbound AUC of R-I and S-I is described by equations 6.15 and 6.16, respectively, derived in section 6.4). It follows that the average steady-state concentration of unbound drug in plasma, upon chronic dosing will also be insensitive to changes in plasma protein binding. However, in any attempt to establish a concentration-effect relationship for ibuprofen it would need to be recognized that the unbound concentration of S-I, the active moiety, will not be a constant fraction of total S-I, or of total UNR-I.

Table 9.1. The difference between the true unbound fraction of unresolved ibuprofen (f_u^{UNR-I}, Method 1) and that which would be found if the unbound fraction was determined by non-enantioselective analysis of the post-dialysis distribution of racemic radiolabelled ibuprofen (f_u^{UNR-I}, method 2)

Volunteer	C^{R-I}	C^{S-I}	f_u^{R-I}	f_u^{S-I}	f_u^{UNR-I}		% diff
Dose(mg)	(mg/L)				Method 1	Method 2	
AE 200	0.65	1.94	0.226	0.414	0.367	0.320	-12.8
AE 400	0.32	1.10	0.238	0.502	0.442	0.370	-16.3
AE 800	5.16	15.2	0.367	0.637	0.569	0.502	-11.8
AE 1200	3.18	18.3	0.303	0.499	0.470	0.401	-14.7
JE 200	1.04	4.61	0.313	0.395	0.380	0.354	-06.8
JE 400	0.30	0.90	0.250	0.516	0.450	0.383	-14.9
JE 800	5.12	13.4	0.263	0.453	0.400	0.358	-10.5
JE 1200	2.67	17.7	0.314	0.464	0.444	0.389	-12.4
TS 200	2.02	5.75	0.322	0.520	0.469	0.421	-10.2
TS 400	1.90	5.86	0.260	0.542	0.473	0.401	-15.2
TS 800	2.43	11.2	0.323	0.521	0.486	0.422	-13.2
TS 1200	2.62	18.0	0.311	0.607	0.569	0.459	-19.3
SB 200	1.06	3.43	0.282	0.472	0.427	0.377	-11.7
SB 400	1.49	5.90	0.249	0.463	0.420	0.356	-15.2
SB 800	5.32	16.5	0.388	0.622	0.566	0.505	-10.8
SB 1200	1.88	11.9	0.272	0.544	0.507	0.408	-19.5

9.5 CONCLUSION

From the data presented in this chapter, it can be concluded that the binding of ibuprofen to human plasma proteins exhibits enantioselectivity, with the R(-)-enantiomer binding more avidly than its mirror-image form. The plasma protein binding of both enantiomers exhibit non-linearity over the range of concentrations encountered during clinical dosing. The fact that each enantiomer was able to reduce the plasma protein binding of the other suggests that each may be competing for the common binding sites (possibly site II) on the HSA molecule. Scatchard-type binding studies, examining the binding of the enantiomers to purified HSA, would be need to be performed to confirm this.

Chapter 10
GENERAL CONCLUSION

Of the large number of chiral drugs available, the majority are used clinically in their racemic form. Because enantiomers may differ markedly in their pharmacokinetic and pharmacodynamic properties, the body is effectively exposed to two distinct compounds when a racemate is administered. Until recently, this important concept has received very little attention in the field of clinical pharmacology, and many pharmacokinetic and concentration-effect studies on racemic drugs have been performed using analytical methods which do not measure the individual enantiomers.

A theoretical analysis was performed on plasma concentration-time data, simulated for a model racemic drug. By comparing the total and unbound plasma concentration-time profiles for the individual enantiomers to those for the unresolved drug, it was possible to illustrate the potential which exists for misinterpreting drug disposition data generated for a racemic drug using a non-enantioselective assay. It was shown that pharmacokinetic data generated using such an assay may be both quantitatively and qualitatively inaccurate with respect to the true pharmacokinetic properties of the compound. Some important limitations, which appear to have been unrecognised to date, of commonly used techniques for measuring the plasma protein binding of racemic drugs, were also described.

Ibuprofen is a chiral drug which is administered clinically as a racemate. For this drug, pharmacological activity resides, almost exclusively, with the S(+)-enantiomer, although a portion (about 60%) of a dose of the inactive enantiomer, R(-)-ibuprofen (R-I), is metabolically inverted *in vivo* to S(+)-ibuprofen (S-I). A review of the available literature indicated that, despite over 20 years of clinical use, little data are available on the pharmacokinetics of the individual ibuprofen enantiomers, and the effects thereon of factors such as disease state; the magnitude of the administered dose; age; and concurrently administered drugs.

The major reason for the lack of data on the pharmacokinetics of R-I and S-I may be the difficulty involved in quantifying the total and unbound concentrations of the individual ibuprofen enantiomers in biological fluids. Therefore, to study various aspects of the clinical pharmacology of ibuprofen, assays were developed to determine the total and unbound concentrations of R-I and S-I, when present concurrently, in human plasma. Because the majority of an orally administered dose of ibuprofen can be recovered in urine (as its glucuronide conjugate, and as conjugated and unconjugated forms of the hydroxy- and carboxy-metabolites), the total urinary recovery of these

species serves as a useful index of the extent to which the drug was absorbed from the gastrointestinal tract. Hence, methods were also established for quantifying ibuprofen metabolites in human urine.

The influence of increasing dose of racemic ibuprofen on the pharmacokinetics of R-I and S-I was investigated. Four healthy males were given 200, 400, 800 and 1200mg of racemic ibuprofen, orally, in a balanced, cross-over manner. Ibuprofen absorption (as assessed by the total urinary recovery of ibuprofen and its metabolites) was extensive and independent of the magnitude of the administered dose. At all four doses, the pharmacokinetics of ibuprofen exhibited enantioselectivity: the maximum plasma concentration; the area under the total and unbound plasma concentration-time curves (AUC and AUC_u, respectively); and the time-averaged unbound fraction in plasma, were all greater for S-I. With increasing ibuprofen dose, there was a less than proportional increase in the AUC of each enantiomer, although this non-linearity was more appreciable for the inactive enantiomer (R-I). The AUC_u of R-I and S-I increased in direct proportion to the administered dose. For both enantiomers, the time-averaged plasma unbound fraction increased significantly with dose. Physiological pharmacokinetic concepts were used to derive equations relating the AUC and AUC_u of each enantiomer (after oral administration of racemic ibuprofen), to their respective intrinsic clearances via the various metabolic routes, their unbound fractions in plasma, and their administered doses. These equations were used to interpret the observed results. It was concluded that for each enantiomer, intrinsic clearance was dose-independent, and concentration-dependent plasma protein binding was the source of the non-linear relationship between AUC and dose. It was rationalised that the fraction of R-I which was metabolically inverted to S-I was independent of the magnitude of the administered dose. This latter finding was of particular interest, given that little is known of factors which influence the chiral inversion pathway, which is, at least in part, an enzymatic process. It was shown that the overall intrinsic clearance of S-I was of similar magnitude to the partial intrinsic clearance of R-I via non-inversion metabolic routes. On this basis, it was shown that the pharmacokinetics of unresolved ibuprofen would be expected to be virtually independent of the partial intrinsic clearance of R-I by inversion, even though this latter parameter is an important determinant of the percentage of the administered dose of racemic ibuprofen which is presented to the body as the active entity, S-I.

Ibuprofen diminishes the aggregability of platelets by inhibiting the ability of platelet cyclo-oxygenase to catalyse the formation of the potent pro-aggregatory agent, thromboxane A2 (TXA2). An *in vitro* experiment was performed to examine the relative effects of R-I and S-I on platelet cyclo-oxygenase activity. Antiplatelet activity

was found to reside almost exclusively with S-I, which was in keeping with the relative effects of R-I and S-I on other biochemical pathways mediated by cyclo-oxygenase. As part of the dose-ranging study outlined above, the effect of increasing dose of racemic ibuprofen on the magnitude and duration of platelet cyclo-oxygenase inhibition was examined. The percentage inhibition of TXA2 synthesis correlated with the unbound concentration of S-I in plasma, according to a sigmoidal E_{max} model. The concentration of plasma unbound S-I required to inhibit TXA2 synthesis by 50% (EC_{50}), was in the order of 10mcg/L, which was low, relative to those concentrations of plasma unbound S-I achieved after each dose of racemic ibuprofen. The maximum degree of inhibition achieved in each volunteer increased only marginally with dose. However, with increasing dose the unbound concentrations of S-I exceeded the determined EC_{50} for a progressively longer period, and therefore there was a substantial increase with dose in the duration of inhibition. Analysis of the relationship between plasma unbound S-I and TXA2 synthesis, according to time sequence after drug administration, indicated negligible hysteresis. This suggested that there was only minimal delay in the equilibration of S-I between the reference fluid (plasma water), and the active site (platelet cyclo-oxygenase), and that the effect of S-I on platelet cyclo-oxygenase was reversible. In all cases, TXA2 synthesis returned to within 20% of the pre-treatment level within 24 hours.

Ibuprofen is extensively cleared by oxidative metabolism. A balanced, cross-over study was conducted in six healthy males to examine the effect of concurrent cimetidine (a potent inhibitor of cytochrome P-450 dependent drug metabolism) administration on the pharmacokinetics of R-I and S-I, after a single oral dose of racemic ibuprofen. The extent of absorption of ibuprofen was unaffected by cimetidine coadministration. Similarly, cimetidine had no effect on the peak plasma concentration and the time of its occurrence; the plasma protein binding; and the AUC of either ibuprofen enantiomer. It was concluded that cimetidine had no effect on the metabolism of ibuprofen, providing further evidence to support the concept that cimetidine is not a universal inhibitor of oxidative drug metabolism, and may have differential inhibitory effects on the various isozymes of cytochrome P-450.

Studies were performed on the binding of ibuprofen enantiomers to human plasma proteins. *In vivo* and *in vitro* studies indicated that the plasma protein binding of ibuprofen exhibited enantioselectivity, with R-I being more extensively bound at clinically relevant concentrations. The extent of enantioselectivity was not great, in that there was only a marginal difference between the enantiomers in the percentage bound (about 99.6% and 99.4% for R-I and S-I, respectively). However, because of the magnitude of binding, the small difference, in relative terms, in percentage bound,

represented a substantial differences in percentage unbound (about 0.4% and 0.6% for R-I and S-I, respectively). Over the range of plasma concentrations achieved after oral administration of therapeutic doses of ibuprofen, both enantiomers exhibited concentration-dependent plasma protein binding. In addition, the presence of one ibuprofen enantiomer decreased the plasma protein binding of its mirror-image form, and, on this basis, one can explain an apparent enantiomer-enantiomer pharmacokinetic interaction reported previously by other workers.

Further studies are needed to determine the influence of age, disease (eg. renal, hepatic and rheumatic disease), and concurrently administered drugs, on the pharmacokinetics of ibuprofen enantiomers, and, in particular, on the extent of inversion of R-I to S-I. Studies on the relationship between the unbound concentration of S-I in plasma and inhibition of platelet cyclo-oxygenase should be extended to determine the intensity and duration of anti-platelet effects during chronic administration of the drug. In addition, the relationship between plasma unbound S-I and blood vessel prostacyclin production needs investigating. Futhermore, it would be of interest to examine the anti-rheumatic activity of the drug in relationship to the unbound concentrations of S-I achieved in plasma (or synovial fluid). Further studies are needed to investigate the binding of ibuprofen enantiomers to purified human serum albumin (the major plasma ibuprofen binding protein), and to examine the influence of endogenous (free fatty acids etc) and exogenous (other drugs, site specific probes etc) compounds, on the plasma protein binding of R-I and S-I.

The general theme of this thesis has been to examine and elaborate upon, the limitations of studies which fail to consider the capacity of biological macromolecules to discriminate between drug enantiomers. It is evident, from the work described, that it is only when pharmacokinetic and pharmacodynamic investigations on racemic drugs are conducted enantioselectively, that the data from such studies can be fully exploited, and confidently used to further our understanding of drug disposition and drug action.

Appendix A

Derivation of Equation 2.8.

By definition, after a single i.v. dose of X (a racemate of R and S), the clearance of unresolved X (CL^X) is a function of the dose of X and the area under the plasma concentration-time curve for unresolved species (AUC^X) according to equation a1.

$$CL^X = \frac{Dose^X}{AUC^X} \qquad (a.1)$$

Since AUC of unresolved species is the sum of the AUCs for the individual enantiomers,

$$CL^X = \frac{Dose^X}{AUC^R + AUC^S} \qquad (a.2)$$

By definition, after i.v. administration of a drug, AUC = Dose/CL, and therefore, equation a.2 becomes

$$CL^X = \frac{Dose^X}{Dose^R/CL^R + Dose^S/CL^S} \qquad (a.3)$$

After administration of racemic X,

$$Dose^X = 2\ Dose^R = 2\ Dose^S$$

Equation a.3 therefore can be simplified to

$$CL^X = \frac{2}{1/CL^R + 1/CL^S} \qquad (a.4)$$

which, upon rearrangement, yields equation 2.8

$$CLX = \frac{2\ CL^R\ CL^S}{CL^R + CL^S} \qquad (2.8)$$

Appendix B

This appendix contains the plasma concentration-time data for total and unbound R(-)-ibuprofen (R-I) and S(+)-ibuprofen (S-I), and the serum levels of thromboxane B2 (TXB2), for the volunteers who participated in the dose-ranging study described in Chapters 6 and 7. Also presented are the urinary recovery data for ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen during the 0-12 and 12-36 hour time intervals.

ND = Below sensitivity limit of the assay.

Volunteer AE
Dose 200mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					71
0.25	ND	ND			
0.50	7.37	7.64	22.8	39.4	11
0.75	9.85	10.8	32.8	57.3	5.9
1.00	8.65	10.4			
1.25	6.86	9.24			
1.50	6.96	10.1			
2.00	5.67	8.83	16.3	44.6	9.6
2.50	4.46	7.54			
3.00	2.86	5.54			
4.00	1.60	3.51	3.15	16.1	16
5.00	1.18	3.16			
6.00	0.65	1.94	1.48	8.03	35
8.00	0.30	0.80	0.87	4.24	72
10.00	0.12	0.43			
12.00	ND	ND			
24.00					175
48.00					173

Volunteer AE
Dose 400mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					135
0.25	ND	ND			
0.50	ND	ND			
0.75	0.54	0.46			
1.00	0.65	0.70			
1.25	0.82	1.06			
1.50	1.19	1.35	3.00	5.86	53
2.00	6.76	7.84			
2.50	12.3	13.7	50.1	93.8	7.6
3.00	10.8	15.7	34.0	95.6	7.2
4.00	5.83	13.3	19.1	66.8	7.6
5.00	2.61	6.14			
6.00	1.30	4.90	7.19	36.6	23
8.00	0.54	2.29			
10.00	0.32	1.10	0.76	5.52	115
12.00	ND	ND			
24.00					144
48.00					151

Volunteer AE
Dose 800mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					101
0.25	0.15	0.15			
0.50	13.2	16.9			
0.75	18.9	25.0			
1.00	17.6	26.5	62.3	152	3.2
1.25	16.1	26.5			
1.50	14.4	24.3			
2.00	13.1	23.8	47.0	147	3.2
2.50	15.1	28.5	57.7	176	3.4
3.00	11.5	23.3			
4.00	5.16	15.2	18.9	96.8	6.8
5.00	3.56	10.2			
6.00	1.92	6.69			
8.00	0.96	3.24	3.02	14.0	48
10.00	0.58	1.42			
12.00	0.18	0.41	0.55	1.90	108
24.00					214
48.00					158

Volunteer AE
Dose 1200mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					117
0.25	ND	ND			
0.50	0.46	1.16			
0.75	8.01	8.89	27.2	43.6	12
1.00	11.2	13.6	65.5	101	11
1.25	13.4	16.3			
1.50	23.2	26.3	128	271	3.5
2.00	27.0	38.1			
2.50	33.8	49.1	198	382	2.6
3.00	25.9	49.0			
4.00	12.5	38.8			
5.00	6.39	25.7			
6.00	3.18	18.3	9.64	91.3	7.5
8.00	1.39	7.97			
10.00	0.79	4.13			
12.00	0.27	1.64	0.86	7.94	71
24.00					191
48.00					190

Volunteer SB
Dose 200mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					177
0.25	1.70	1.77			
0.50	1.85	2.01			
0.75	2.08	2.37	4.87	10.8	42
1.00	2.59	2.67			
1.25	3.72	4.52			
1.50	5.31	7.09			
2.00	8.43	10.2	27.0	56.1	11
2.50	6.18	8.82	18.4	49.1	13
3.00	3.87	7.83			
4.00	3.78	5.83	8.88	25.4	23
5.00	1.73	4.85			
6.00	1.06	3.43	2.99	16.2	70
8.00	0.52	1.84			
10.00	0.40	1.07	0.93	3.82	124
12.00	ND	ND			
24.00					210
48.00					226

Volunteer SB
Dose 400mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					160
0.25	ND	ND			
0.50	13.7	14.6	37.1	62.1	7.0
0.75	20.8	24.1	88.0	152	5.0
1.00	18.4	23.6	74.9	151	4.0
1.25	13.5	17.7			
1.50	12.9	17.5			
2.00	9.73	14.2			
2.50	7.33	12.2	26.0	69.4	5.0
3.00	4.22	9.08			
4.00	3.56	6.74			
5.00	1.49	5.90	3.71	27.3	18
6.00	0.89	3.73			
8.00	0.65	1.95			
10.00	0.43	1.05	1.26	3.41	142
12.00	ND	ND			
24.00					197
48.00					177

Volunteer SB
Dose 800 mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					150
0.25	ND	ND			
0.50	ND	ND			
0.75	1.01	1.13			
1.00	5.60	6.80	14.1	40.1	20
1.25	7.89	10.5	32.8	68.9	14
1.50	9.83	14.0			
2.00	9.22	13.8	39.0	114	11
2.50	14.7	22.7	54.1	178	4.3
3.00	10.6	22.5			
4.00	6.76	19.6			
5.00	5.32	16.5	20.6	103	7.0
6.00	3.82	13.5			
8.00	3.52	7.88			
10.00	1.66	3.71			
12.00	1.23	2.78	2.63	12.5	87
24.00					149
48.00					189

Volunteer SB
Dose 1200 mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					124
0.25	4.84	5.56			
0.50	23.1	30.2	112	240	3.6
0.75	30.4	44.3	190	461	1.7
1.00	28.4	44.7			
1.25	24.9	46.6	125	425	1.7
1.50	18.6	39.9			
2.00	13.5	36.2			
2.50	9.50	29.6			
3.00	6.14	25.7	23.0	181	2.4
4.00	4.48	23.3			
5.00	2.95	17.7			
6.00	1.88	11.9	5.11	64.7	16
8.00	1.26	5.86			
10.00	1.06	3.32			
12.00	0.69	2.02	2.01	8.12	90
24.00					204
48.00					140

Volunteer JE
Dose 200 mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					113
0.25	2.39	2.27	5.14	12.1	82
0.50	10.5	9.87			
0.75	14.6	13.9	41.2	73.1	8.1
1.00	12.6	13.3			
1.25	11.9	13.1	40.9	69.7	6.8
1.50	9.31	11.2			
2.00	7.19	9.11			
2.50	5.73	8.67	15.7	43.9	19
3.00	3.91	7.79			
4.00	2.01	6.18			
5.00	1.04	4.61	3.26	18.2	45
6.00	0.80	3.65			
8.00	0.46	2.05			
10.00	0.22	0.83	0.57	4.18	130
12.00	ND	ND			
24.00					133
48.00					128

Volunteer JE
Dose 400 mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					120
0.25	0.74	0.99			
0.50	17.1	16.4			
0.75	17.7	19.7	62.1	106	4.0
1.00	13.9	16.4			
1.25	14.7	17.7	46.7	104	4.4
1.50	14.2	18.6	45.9	97.7	4.0
2.00	13.8	16.3	42.2	95.2	6.3
2.50	8.35	13.1			
3.00	6.49	12.1			
4.00	3.07	8.33	6.75	33.5	16
5.00	2.43	6.32			
6.00	1.76	5.76			
8.00	0.89	2.84			
10.00	0.35	1.75			
12.00	0.30	0.90	0.75	4.64	112
24.00					138
48.00					197

Volunteer JE
Dose 800 mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					100
0.25	1.08	1.21			
0.50	13.0	12.4			
0.75	20.4	20.4	56.5	122	2.7
1.00	26.2	27.2			
1.25	24.5	25.6			
1.50	22.9	32.6	83.4	253	2.2
2.00	27.8	32.6	90.1	220	1.9
2.50	22.0	30.9	86.7	221	1.9
3.00	16.1	27.8			
4.00	7.76	20.0			
5.00	5.12	13.5	13.5	60.7	6.5
6.00	4.62	11.5			
8.00	1.95	5.65			
10.00	1.23	2.83			
12.00	0.48	1.30	1.03	4.98	31
24.00					132
48.00					167

Volunteer JE
Dose 1200

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					128
0.25	ND	ND			
0.50	21.0	26.3			
0.75	26.9	38.1	115	227	1.8
1.00	25.1	42.2	99.6	375	2.3
1.25	26.2	46.9			
1.50	27.4	45.7			
2.00	27.3	47.7	160	368	1.9
2.50	21.8	49.5			
3.00	8.97	29.0			
4.00	7.53	30.6			
5.00	3.86	18.8			
6.00	2.67	17.7	8.38	82.1	6.6
8.00	1.20	9.50	5.32	48.4	14
10.00	1.04	5.06			
12.00	0.81	2.42	2.29	8.40	34
24.00					86
48.00					136

Volunteer TS
Dose 200 mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					233
0.25	0.14	0.14			
0.50	5.72	5.58	17.8	26.9	56
0.75	6.41	8.19			
1.00	6.99	10.4	27.5	55.1	14
1.25	5.43	9.57			
1.50	4.55	9.25	15.7	51.8	22
2.00	3.89	7.71			
2.50	2.47	5.92			
3.00	2.02	5.75	6.50	29.9	78
4.00	0.98	3.83			
5.00	0.45	2.39			
6.00	0.26	1.24	0.89	6.39	141
8.00	0.10	0.64	0.25	2.53	143
10.00	ND	ND			
12.00	ND	ND			
24.00					389
48.00					262

Volunteer TS
Dose 400 mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					204
0.25	ND	ND			
0.50	0.25	0.37			
0.75	1.01	1.26			
1.00	2.47	2.75	5.63	15.2	70
1.25	4.84	5.46	13.9	29.4	29
1.50	5.70	6.90			
2.00	4.98	6.82			
2.50	4.57	6.73			
3.00	4.00	8.00	15.0	50.1	27
4.00	2.13	5.81			
5.00	1.90	5.86	4.94	31.8	49
6.00	1.82	5.14			
8.00	2.15	3.81	6.21	23.1	52
10.00	1.75	3.24			
12.00	1.50	2.47			
24.00	0.35	0.87	0.95	3.72	183
48.00	ND	ND			313

Volunteer TS
Dose 800 mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					387
0.25	3.45	3.64			
0.50	13.0	12.5			
0.75	35.2	37.7	166	274	5.7
1.00	36.3	42.7	168	336	4.0
1.25	30.2	36.9			
1.50	28.0	36.6			
2.00	21.7	32.0	95.3	244	22
2.50	16.0	26.8			
3.00	10.3	25.3			
4.00	4.24	16.0	17.2	84.6	24
5.00	2.43	11.2	7.85	58.3	42
6.00	1.47	7.13			
8.00	0.81	4.11			
10.00	0.44	1.56	1.13	7.33	177
12.00	ND	0.67			
24.00					348
48.00					399

Volunteer TS
Dose 1200 mg

Time (h)	Plasma Concentration Total (mg/L) R-I	Total (mg/L) S-I	Unbound (mcg/L) R-I	Unbound (mcg/L) S-I	TXB2 (ng/ml)
0.00					298
0.25	ND	ND			
0.50	7.10	7.70			
0.75	25.8	32.3	104	199	4.4
1.00	24.5	36.6			
1.25	23.2	35.3			
1.50	18.6	35.1	67.3	237	4.0
2.00	20.4	37.1			
2.50	26.2	45.8	148	435	2.9
3.00	15.3	43.6			
4.00	5.64	28.0	19.1	158	4.6
5.00	2.62	18.0	8.15	109	8.7
6.00	1.44	11.5			
8.00	0.63	4.95			
10.00	0.73	2.38			
12.00	0.44	1.21	1.39	7.34	150
24.00					312
48.00					340

Urinary Recovery Data for the Ibuprofen Dose Ranging Study.
All data are expressed as a percentage of the administered dose recovered during the specified interval.

Volunteer	Dose (mg)	Ibuprofen 0-12h	Ibuprofen 12-36h	Hydroxy-Ibuprofen 0-12h	Hydroxy-Ibuprofen 12-36h	Carboxy-Ibuprofen 0-12h	Carboxy-Ibuprofen 12-36h
AE	200	10.2	0.0	24.6	1.7	51.1	2.4
	400	9.5	0.1	24.7	1.8	49.1	3.2
	800	11.4	0.5	24.4	2.0	49.3	3.4
	1200	10.7	0.5	22.8	4.4	48.2	3.4
SB	200	15.2	0.5	19.9	3.2	36.4	3.0
	400	11.8	0.3	18.9	1.4	35.2	1.9
	800	13.3	1.5	19.3	4.6	34.8	6.1
	1200	13.3	0.4	16.6	3.1	32.4	2.3
JE	200	9.6	0.3	23.8	3.2	32.9	3.1
	400	12.4	0.3	24.7	1.8	40.4	2.4
	800	10.2	0.1	20.1	2.3	41.3	2.6
	1200	10.6	1.0	21.4	2.2	35.9	3.6
TS	200	12.5	0.2	22.2	2.7	44.1	1.9
	400	6.0	2.6	13.8	10.6	29.3	15.5
	800	8.8	0.2	24.4	5.5	56.3	3.2
	1200	8.9	0.2	22.1	5.4	55.7	3.8

Appendix C

This appendix contains the plasma concentration-time data for total and unbound R-I and S-I for the volunteers who participated in the ibuprofen-cimetidine drug interaction study described in Chapter 8. Also presented are the urinary excretion data for ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen during the 0-12 and 12-36 hour time intervals.

ND = Not detected; Below sensitivity limit of the assay.
NA = Not available; Sample not available for analysis.

Volunteer CW
Phase Control

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	ND	ND		
0.33	2.47	3.42		
0.50	11.7	10.5	48.7	65.6
0.75	18.9	18.5		
1.00	23.0	24.0		
1.25	26.6	29.3		
1.50	25.1	29.4		
2.00	24.0	29.3	98.2	193
2.50	20.4	25.9	76.5	175
3.00	15.8	24.2		
4.00	8.67	16.8		
5.00	4.36	9.94		
6.00	NA	NA		
7.50	1.51	4.18	6.45	15.9
10.00	0.46	1.40	1.29	6.09

Volunteer CW
Phase Treatment

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	ND	ND		
0.33	10.3	7.50		
0.50	17.0	15.7		
0.75	14.2	14.2	55.4	114
1.00	18.8	19.6		
1.25	13.0	15.0		
1.50	14.5	17.1		
2.00	25.1	30.7	126	187
2.50	25.1	30.7		
3.00	16.0	23.4	64.0	164
4.00	7.77	16.1		
5.00	3.72	9.58		
6.00	2.34	6.50	8.54	29.9
8.00	1.06	3.57		
10.00	0.35	1.21	0.95	5.93

Volunteer RU
Phase Control

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	ND	ND		
0.33	8.70	8.70		
0.50	13.4	12.9	55.3	94.8
0.75	17.2	21.1		
1.00	14.1	19.0	33.1	85.3
1.25	14.9	19.0		
1.50	15.6	21.0	64.4	123
2.00	15.0	26.8		
2.50	12.6	22.9		
3.00	9.55	21.8		
4.00	5.22	16.1	19.1	110
5.00	3.20	9.60		
6.00	1.97	6.79		
8.00	0.84	3.82	2.65	23.7
10.00	0.38	2.41		

Volunteer RU
Phase Treatment

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	ND	ND		
0.33	2.80	1.99		
0.50	8.80	9.90	34.1	67.8
0.75	13.7	17.5		
1.00	13.2	18.9	45.8	108
1.25	12.5	20.4		
1.50	11.9	17.4		
2.00	17.5	27.4		
2.50	15.4	27.5	72.5	151
3.00	12.6	23.4		
4.00	4.29	15.2	18.0	102
5.00	1.92	10.9		
6.00	1.31	8.42		
8.00	0.79	4.87	4.50	22.5
10.00	0.50	3.18		

Volunteer PF
Phase Control

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	ND	ND		
0.33	4.14	3.82		
0.50	9.84	9.46	41.1	65.3
0.75	16.6	18.3	124	99.7
1.00	25.6	30.1	107	238
1.25	23.9	29.9		
1.50	19.6	25.8		
2.00	14.4	20.8		
2.50	10.5	15.2		
3.00	7.88	12.4		
4.00	4.27	9.73		
5.00	2.42	6.22	10.4	26.2
6.00	1.45	5.14		
8.00	0.58	3.13	2.13	16.1
10.00	0.39	2.20		

Volunteer PF
Phase Treatment

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	ND	ND		
0.33	1.07	1.04		
0.50	4.85	5.15		
0.75	9.22	10.0	35.8	54.5
1.00	17.5	19.0	69.5	146
1.25	17.7	20.7		
1.50	18.6	22.2		
2.00	22.2	28.2	106	178
2.50	17.6	23.7		
3.00	11.7	18.4		
4.00	4.92	9.98		
5.00	3.30	8.50		
6.00	1.82	6.28	5.7	28.3
8.00	0.85	3.02	1.87	15.6
10.00	0.64	2.21		

Volunteer RM
Phase Control

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	0.56	1.09		
0.33	4.96	4.15	28.2	49.8
0.50	5.21	4.35		
0.75	4.98	4.25		
1.00	7.14	5.96		
1.25	10.8	9.20	52.9	57.0
1.50	11.2	9.33		
2.00	11.8	10.4		
2.50	24.1	21.3		
3.00	25.9	27.4	145	310
4.00	12.0	19.2	49.6	116
5.00	4.90	12.6		
6.00	2.42	8.09		
8.00	1.07	3.62	4.68	19.9
10.00	0.52	1.94		

Volunteer RM
Phase Treatment

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	ND	ND		
0.33	3.79	3.22		
0.50	6.83	6.18	26.4	32.4
0.75	9.93	9.17		
1.00	9.44	9.07	35.5	53.5
1.25	12.7	11.1		
1.50	18.8	17.3		
2.00	15.8	13.8		
2.50	21.5	17.9		
3.00	24.9	21.2	121	126
4.00	10.5	15.2		
5.00	5.73	10.9	21.7	68.7
6.00	3.55	9.30		
8.00	1.39	4.54	5.50	25.0
10.00	0.83	2.78		

Volunteer SW
Phase Control

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	ND	ND		
0.33	1.82	2.28		
0.50	6.02	5.78	30.0	49.7
0.75	12.1	11.2		
1.00	23.8	20.3		
1.25	24.1	19.7		
1.50	22.1	18.5	103	179
2.00	19.1	16.3	88.8	133
2.50	13.7	11.7		
3.00	11.9	9.33		
4.00	6.77	6.63		
5.00	5.23	7.07	20.0	30.6
6.00	3.63	4.91		
8.00	1.75	2.63		
10.00	1.17	1.94	4.26	15.8

Volunteer SW
Phase Treatment

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	3.24	3.24		
0.33	6.78	6.02	33.8	54.8
0.50	15.3	15.7		
0.75	27.6	24.9		
1.00	22.0	22.5		
1.25	17.6	19.5		
1.50	17.1	19.0	96.6	136
2.00	13.9	15.3	44.6	112
2.50	10.8	11.3		
3.00	6.40	6.80		
4.00	5.25	7.25		
5.00	5.37	7.58	48.9	51.5
6.00	2.92	4.29		
8.00	1.60	2.78		
10.00	0.89	2.12	2.80	11.0

Volunteer RN
Phase Control

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	ND	ND		
0.33	3.22	2.92		
0.50	24.2	21.9		
0.75	36.0	31.3	131	218
1.00	43.5	37.0		
1.25	38.4	34.7		
1.50	35.0	29.8		
2.00	27.5	24.9	110	132
2.50	23.5	23.6		
3.00	14.8	14.6		
4.00	7.84	12.3	30.3	54.9
5.00	4.16	8.44		
6.00	2.15	5.02	5.03	23.0
8.00	0.88	1.86		
10.00	0.49	1.32	0.7	8.12

Volunteer RN
Phase Treatment

Time (h)	Plasma Concentration Total (mg/L)		Unbound (mcg/L)	
	R-I	S-I	R-I	S-I
0.17	1.78	0.96		
0.33	36.5	33.7		
0.50	45.2	45.2		
0.75	34.4	34.4	153	232
1.00	36.8	36.8		
1.25	30.4	31.1		
1.50	30.4	30.4		
2.00	24.3	24.3	68.0	253
2.50	21.6	20.0		
3.00	20.4	18.4		
4.00	11.4	14.6	44.5	75.2
5.00	5.94	10.6		
6.00	5.67	10.7		
8.00	1.63	3.38	4.19	16.9
10.00	0.64	1.07	2.64	4.41

Urinary recovery data for the ibuprofen-cimetidine drug interaction study: All data are expressed as a percentage of the administered dose recovered during the specified interval.

Volunteer	Phase	Ibuprofen		Hydroxy-ibuprofen		Carboxy-ibuprofen	
		0-12	12-36	0-12	12-36	0-12	12-36
RN	Control	12.4	0.1	23.6	4.3	46.5	3.0
	Treatment	14.1	0.5	24.0	3.9	46.0	4.1
RU	Control	15.1	0.4	24.7	2.5	42.7	3.3
	Treatment	17.8	0.9	24.1	2.3	42.0	3.1
SW	Control	13.8	0.1	25.3	1.5	52.4	1.9
	Treatment	17.5	0.3	23.6	1.0	46.0	1.3
PF	Control	11.1	0.1	18.0	2.0	45.3	0.6
	Treatment	9.8	0.6	20.6	4.0	47.3	4.6
RM	Control	13.1	0.6	20.5	4.0	40.9	3.6
	Treatment	10.7	0.5	21.4	2.8	43.6	4.3
CW	Control	8.6	0.2	21.6	1.6	43.0	2.8
	Treatment	11.4	0.3	22.1	2.1	43.3	3.6

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