Pharmacokinetics and pharmacodynamics of colistin and colistin methanesulphonate

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Dedicated to my much-loved wife Qingwei, my parents and parents-in-law

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Glossary of abbreviations

The abbreviations and prefixes of the International System of Units have been used in this thesis, except for the alternatives listed below. Also listed are additional abbreviations and terminology, and pharmacokinetic and pharmacodynamic symbols.

Ae (0-24)	Amount of the dose recovered in urine in 24 hr
AUC_0^t	Area under the concentration versus time curve to t_{last}
AUC_0^∞	Area under the concentration versus time curve to infinite time
AUC_τ^{ss}	Area under the concentration versus time curve during a dosage interval at steady-state
$AUMC_0^t$	Area under the first moment curve to t_{last}
$AUMC_0^\infty$	Area under the first moment curve to infinite time
C_{max}^{ss}	Maximum drug concentration at steady-state in the plasma during a dosage interval
C_{min}^{ss}	Minimum drug concentration at steady-state in the plasma during a dosage interval
C_{last}	Last quantifiable plasma concentration
CL	Total body clearance
CL_R	Renal clearance
CV	Coefficient of variation
D	Dose
Dab	2, 4-Diaminobutyric acid
Dansyl-Cl	1-Dimethylaminonaphthalene-5-sulphonyl chloride
ESI-MS	Electrospray ionization mass spectrometry
f_e	Fraction of the dose recovered in urine in 24 hr
FMOC-Cl	9-Fluorenylmethyl chloroformate
GFR	Glomerular filtration rate

HPLC	High-performance liquid chromatography
hr	Hours
λ_z	Disposition rate constant during the terminal phase
MIC	Minimum inhibition concentration
MBC	Minimum bactericidal concentration
min	Minutes
MRT	Mean residence time
MST	Minimum survival time
n	Number within a sample
OPA	*ortho*-Phthalaldehyde
PAE	Postantibiotic effect
PITC	Phenyl isothiocyanate
pK_a	Acid dissociation constant
RP-HPLC	Reverse-phase high-performance liquid chromatography
SAX	Strong anion exchange
SD	Standard deviation
sec	Seconds
SPE	Solid-phase extraction
$t_{1/2}$	Half-life
t_{last}	Time of the last quantifiable concentration
TEA	Triethylamine
TLC	Thin-layer chromatography
UV	Ultraviolet
V_{ss}	Volume of distribution at steady-state

ABSTRACT

Colistin, also known as polymyxin E, is a multi-component polypeptide antimicrobial. The two major components are colistin A and colistin B, which usually constitute more than 85% of the content of colistin. It is distinguished by its rapid bactericidal activity against *Pseudomonas aeruginosa* and for the slow development of resistance against its activity. Its use against infections by *P. aeruginosa* in cystic fibrosis (CF) patients is becoming more popular as resistance to other antibiotics increases. However, there are very little data available on its activity against *P. aeruginosa* and on its pharmacokinetics in humans.

The *in vitro* pharmacodynamics of colistin and colistin methanesulphonate were investigated by studying the minimum inhibitory concentrations (MICs), time-kill kinetics, and postantibiotic effect (PAE) against mucoid and nonmucoid strains of *P. aeruginosa* isolated from patients with CF. For the susceptible strains, MICs of colistin ranged from 1 mg/L to 4 mg/L, while the MICs of colistin methanesulphonate were significantly higher and ranged from 4 mg/L to 16 mg/L. The time-kill kinetics were investigated with four strains at drug concentrations ranging from 0.5 to 64 times the MIC. Colistin showed extremely rapid killing, resulting in complete elimination at the highest concentrations within 5 min, while colistin methanesulphonate killed more slowly. Colistin exhibited a significant PAE of 2 to 3 hours after 15 min of exposure against the four strains tested at 16 times the MIC. Shorter PAEs were observed at the concentrations tested for colistin methanesulphonate. Colistin methanesulphonate had lower overall bactericidal and postantibiotic activities than colistin, even when adjusted for differences in MICs.

A simple, specific and sensitive high-performance liquid chromatographic (HPLC) method was developed for the determination of colistin in plasma and urine. Derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) was performed in the same solid-phase extraction (SPE) C_{18} cartridge used for sample pre-treatment, followed by reverse-phase HPLC with fluorimetric detection. Quantification was achieved using

the ratio of the summed peak areas of colistin A and colistin B derivatives to that of the derivative of netilmicin (internal standard). The method was validated for linearity in response, accuracy and reproducibility, and had a limit of quantification of 0.10 mg/L in plasma. This method was superior to any methods published previously.

A simple and sensitive HPLC method was described for the determination of colistin methanesulphonate in plasma and urine. The method involved hydrolysis of colistin methanesulphonate to colistin with sulphuric acid followed by derivatization using FMOC-Cl. The method was validated for linearity in response, accuracy and reproducibility. Eight commonly co-administered antimicrobials do not interfere with the assay.

Prior to performing the pharmacokinetic studies *in vivo*, the stability of colistin and colistin methanesulphonate was examined in different aqueous media. Colistin was stable in water at 4°C and 37°C for up to 60 days and 120 hr, respectively. However, degradation was observed during storage in isotonic phosphate buffer (0.067 M, pH 7.4) and human plasma at 37°C. The stability of colistin methanesulphonate from three different sources was explored in water using strong anion exchange (SAX) HPLC, and by measuring the concentrations of colistin formed by the hydrolysis of colistin methanesulphonate. The peaks of colistin methanesulphonate in SAX-HPLC disappeared almost completely after 12 hr at 37°C, but appeared to remain intact for up to 2 days at 4°C. Over the same period, no formation of colistin was detected at 4°C. In water, phosphate buffer and plasma, there was rapid formation of colistin within 24 to 48 hr at 37°C from the three sources of colistin methanesulphonate. Colistin methanesulphonate hydrolysed more rapidly at 37°C than at ambient temperature in rat urine. The stability of colistin methanesulphonate in Mueller-Hinton broth, examined for 30 min at 37°C, revealed no formation of colistin. Combined with the results from the *in vitro* pharmacodynamic studies that showed a marked reduction in bacterial counts after 30 min of incubation with colistin methanesulphonate at high concentrations, the study in broth suggests that various sulphomethylated colistin derivatives possess intrinsic antibacterial activity.

Colistin is administered parenterally to humans as colistin methanesulphonate, because of the lower toxicity of the latter compared to colistin sulphate. Prior to examining the pharmacokinetics of colistin and its methanesulphonate in humans, studies were performed in rats to investigate the pharmacokinetics of colistin after a dose of colistin sulphate, and of colistin methanesulphonate and colistin after a dose of the methanesulphonate. The concentrations of both substances in plasma and urine were measured by HPLC.

The pharmacokinetics of colistin was studied in five rats after an intravenous bolus of 1 mg/kg colistin sulphate. The pharmacokinetic parameters for colistin, colistin A and colistin B were calculated using non-compartmental analysis. The total body clearance (CL), steady-state volume of distribution (V_{ss}) and terminal phase half-life ($t_{1/2}$) of colistin averaged 5.2 mL/min/kg, 496 mL/kg and 74.6 min, respectively. Only 0.18 ± 0.14 % of the dose was excreted in urine during the first 12 hr. There were no significant differences in the CL, V_{ss}, $t_{1/2}$ and renal recovery of colistin A and colistin B. However, while the fraction of colistin unbound in plasma was approximately 0.44, the values for colistin A and colistin B differed markedly, being 0.36 and 0.52, respectively. Therefore, the pharmacokinetic behaviours of unbound colistin A and colistin B were quite different, and are in keeping with the marked differences in chromatographic behaviour of the two components.

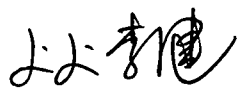
The pharmacokinetics of colistin methanesulphonate was studied in five rats after the intravenous administration of 15 mg/kg colistin methanesulphonate. Pharmacokinetic parameters of colistin methanesulphonate and colistin formed by hydrolysis were calculated using non-compartmental analysis. The CL, V_{ss}, and $t_{1/2}$ of colistin methanesulphonate averaged 11.7 mL/min/kg, 299 mL/kg and 23.6 min, respectively. The average $t_{1/2}$ of colistin was 55.7 min. Most of the dose (61.1 ± 14.4%) was recovered in urine in 24 hr. The findings from these two studies in rats show that colistin is poorly excreted by the renal route and that other unknown routes of elimination are involved; in contrast colistin methanesulphonate is excreted mainly by the kidney. It is proposed that there is extensive reabsorption of colistin by the kidney, but net secretion of colistin methanesulphonate.

The pharmacokinetics of colistin methanesulphonate at steady-state was studied in 12 patients with CF after an intravenous infusion. Four blood samples were collected from each patient after the infusion and concentrations of colistin methanesulphonate and colistin determined by HPLC. At steady-state, colistin methanesulphonate had a mean CL, V and $t_{1/2}$ of 2.01 mL/min/kg, 340 mL/kg and 124 min, respectively. Colistin formed by hydrolysis of colistin methanesulphonate had a mean $t_{1/2}$ of 251 min. Concentrations of the two compounds in plasma suggest that doses of colistin methanesulphonate higher than the recommended 2 to 3 mg/kg every 8 hr may be required for the effective treatment of *P. aeruginosa* infections in CF patients.

The results in the current thesis were the first detailed and systematic reports on the *in vitro* pharmacodynamics and *in vivo* pharmacokinetics of colistin methanesulphonate and colistin. While further work is required in humans, the results will contribute to the safe and efficacious use of this drug in patients with CF.

DECLARATION

I declare that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text.



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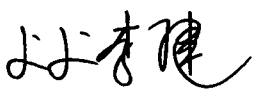

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Publications in Support of this Thesis

1. **Jian Li**, John Turnidge, Robert Milne, Roger L. Nation and Kingsley Coulthard. In vitro pharmacodynamic properties of colistin and colistin methanesulphonate against *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. ***Antimicrobial Agents and Chemotherapy***. 2001, 45(3):781-785.

2. **Jian Li**, Robert W. Milne, Roger L. Nation, John D. Turnidge, Kingsley Coulthard and David W. Johnson. A simple method for the assay of colistin in human plasma, using pre-column derivatization with 9-fluorenylmethyl chloroformate in solid-phase extraction cartridges and reverse-phase high-performance liquid chromatography. ***Journal of Chromatography, Biomedical Applications.*** 2001, 761(2):167-175.

3. **Jian Li**, Robert W. Milne, Roger L. Nation, John D. Turnidge and Kingsley Coulthard. Stability of colistin and colistin methanesulfonate in aqueous media and plasma studied by high-performance liquid chromatography. (Accepted, Antimicrobial Agents and Chemotherapy)

4. **Jian Li**, Robert W. Milne, Roger L. Nation, John D. Turnidge, Kingsley Coulthard and Jason Valentine. Simple method for the assay of colistin methanesulfonate in plasma and urine using high-performance liquid chromatography. ***Antimicrobial Agents and Chemotherapy***. 2002, 46(10): 3304-3307.

5. **Jian Li**, Robert W. Milne, Roger L. Nation, John D. Turnidge, Timothy C. Smeaton and Kingsley Coulthard. Use of high-performance liquid chromatography to study the pharmacokinetics of colistin sulfate in rats following intravenous administration. (Submitted to Antimicrobial Agents and Chemotherapy)

6. **Jian Li**, Robert W. Milne, Roger L. Nation, John D. Turnidge, Timothy C. and Kingsley Coulthard. Pharmacokinetics of colistin methanesulfonate in rats following intravenous administration studied by high-performance liquid chromatography. (In preparation)

7. **Jian Li**, Robert W. Milne, Roger L. Nation, Kingsley Coulthard, and John D. Turnidge. Pharmacokinetics of colistin methanesulfonate in patients with cystic fibrosis following intravenous administration. (In preparation)

Chapter 1 Literature review

Are antibiotics still miracle drugs, as penicillin was considered in the 1940s and 1950s? Widespread antibiotic resistance, particularly caused by multidrug-resistant microorganisms, has put human beings on the verge of a medical disaster (Travis, 1994). The extensive use of antimicrobial agents has tended to accelerate the development of antibiotic-resistant bacteria. Have we reached the end of an "antibiotic era" (Hancock, 1997c)?

Pseudomonas aeruginosa is one of the current difficult-to-treat multidrug-resistant organisms, especially in those patients at risk of chronic or persistent infections, such as those with cystic fibrosis (CF) (Bisgaard et al., 1997a; Hutchison et al., 1999; Tummler et al., 1999). However, there has been no new antipseudomonal agent released since meropenem in 1995 and none are anticipated within the current drug development pipeline (Beringer, 2001). Unfortunately, a high percentage of resistance to meropenem has already been reported in clinical isolates of *P. aeruginosa* (Beringer, 2001). Colistin is an old antibiotic with novel *in vitro* activity against *P. aeruginosa*. The low reported level of resistance of *P. aeruginosa* to colistin has provided clinicians with an alternative treatment for infections with multidrug-resistant *P. aeruginosa* (Beringer, 2001).

Although colistin has been commercially available for more than 40 years, there is limited information on its pharmacokinetics and pharmacodynamics. This thesis focuses on the *in vitro* pharmacodynamics of colistin and colistin methanesulphonate with respect to the multidrug-resistant *P. aeruginosa*, and on their pharmacokinetics. The purpose of this review is to provide a brief background knowledge of CF and *P. aeruginosa*; to introduce the reader to the historical and current uses of colistin and colistin methanesulphonate, and to give a brief outline of the relevant physicochemical properties and mechanism of action; to outline its role as an efficacious antibiotic against gram-negative bacteria, especially *P. aeruginosa*; and to review the pharmacokinetics and pharmacodynamics of colistin and colistin methanesulphonate.

1.1 Cystic fibrosis (CF)

Cystic fibrosis (CF) is one of the most common fatal genetic disorders in populations of European origin. According to reports from the Cystic Fibrosis Foundation, one in every 3,300 newborns will be diagnosed with CF and 1000 new cases are diagnosed each year worldwide (http://www.cff.org). Two decades ago, the majority of children with CF died in their teens. With advances in diagnosis and treatment, many patients with CF now can live until their third, fourth and fifth decades (FitzSimmons, 1993). The most common symptoms of CF are salty-tasting skin, persistent cough, poor weight gain despite excessive appetite, greasy and odorous stools and pneumonia. Furthermore, chronic pulmonary infections, particularly with *P. aeruginosa*, are the major clinical problems for CF patients (Conese et al., 2001; Davis *et al.*, 1996; Gilligan, 1991; Govan et al., 1992; Hutchison et al., 1999; Lyczak *et al.*, 2002; Tummler et al., 1999).

CF is caused by mutations in the gene of cystic fibrosis transmembrane conductance regulator (CFTR) (Kerem et al., 1989; Rommens et al., 1989; Kunzelmann, 1999; Riordan et al., 1989). The CFTR functions as a cAMP-regulated chloride channel in the apical membrane of epithelial cells, regulating the movement of salts and water. The mutation results in defective cAMP-dependent chloride ion conductance (Riordan et al., 1989; Rommens et al., 1989), which leads to an imbalance in the flow of fluid across epithelia and thickened mucus in the affected tissues, such as pancreas, intestine and lung. Currently there are two main models to explain the pathogenesis of CF – the hypotonic model (Goldman et al., 1997; Smith et al., 1996) and the isotonic model (Matsui et al., 1998).

Characteristically, the disorder results in the production of unusually thick and sticky mucus that obstructs the airways of the lungs, the bronchial tubes and bronchioles. In the disorders associated with CF, approximately 90% are related to the respiratory system (Stern, 1996). It is currently believed that CF lung diseases are caused by chronic pulmonary colonization with opportunistic pathogens (Hutchison et al., 1999; Stutman et al., 1987).

Chronic lung infections are prevalent in most CF patients. The spectrum of bacteria routinely isolated from CF sputum is surprisingly narrow. There appears to be a

characteristic pattern of infections in most CF patients – beginning with *Staphylococcus aureus* and/or *Haemophilus influenzae* in infancy followed by gradual colonization with *P. aeruginosa* in adolescence (Ratjen, 2001; Stutman et al., 1987). Currently, most of the bacterial infections in CF lungs, except those caused by *P. aeruginosa*, can be controlled by antibiotics, at least moderately (Bisgaard et al., 1997a; Smith, 1986), and the infections do not become chronic. However, the emergence of multidrug-resistant bacteria during chronic infections with Pseudomonads and *Burkholderia* spp. increases the morbidity and mortality.

Recurrent airway infections by *S. aureus* are the major respiratory problem during infancy and early childhood of CF patients (Davis et al., 1996; Ulrich et al., 1998). Nowadays most morbidity caused by *S. aureus* is controlled by antibiotics (Bisgaard et al., 1997a). *S. aureus* is characteristically the first pathogen to colonize the upper respiratory tract of CF patients (Bisgaard et al., 1997a; Gilligan, 1991). There are three morphological types isolated from the sputa: mucoid, non-mucoid and small colony variant (SCV) (Hutchison, 1999).

While *H. influenzae*, a gram-negative bacterium, has been isolated from CF patients, the numbers are low and its role in CF lung diseases is not well known. Generally, infections by *H. influenzae* can be controlled effectively by fluoquinolones (Ratjen, 2001). Lipopolysaccharide and toxins have been isolated from culture filtrates of *H. influenzae* and proven to slow or disrupt epithelial ciliary beat, resulting in damage to the respiratory epithelia (Johnson et al., 1986; Tsang et al., 1995).

Initially known as a plant pathogen, *Burkholderia cepacia* has been isolated over the last decades with increasing frequency in CF patients (Gilligan, 1991; Gladman et al., 1992; Henskens et al., 2000; Isles et al., 1984). A recent study isolated *B. cepacia* from the sputa of approximately 20% of CF patients (Hutchison et al., 1998). *B. cepacia* produces some classical virulence determinants which damage the function of the lung. These include haemolysin (Abe et al., 1994; Hutchison et al., 1998), lipase (Lonon et al., 1988), protease (Abe et al., 1996), catalase (Speert et al., 1994), and endotoxin (Hughes et al., 1997; Shaw et al., 1995).

P. aeruginosa is the predominant pathogen in 80% of CF patients, particularly in adults (FitzSimmons, 1993). The main contributors to morbidity and mortality are

chronic colonization by *P. aeruginosa* of CF airways and the subsequent pulmonary exacerbations (Frederiksen et al., 1999; Tummler et al., 1997). Detailed information on *P. aeruginosa* is summarized in Section 1.2.

Although the pathogens isolated from CF patients are mostly restricted to the microorganisms described above, the microbiology of the CF lung is not static. CF patients are constantly under threat from emerging pathogens. There are numerous other microorganisms being isolated from CF patients, and most are gram-negative bacteria, in particular *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxidans*, *Ralstonia pickettii*, *Xanthomonas maltophilia* (formerly *P. maltophilia*), *Achromobacter* spp. (Beringer et al., 2000). Other known respiratory pathogens, such as *Bordetella* spp., have shown an increase in their incidence of infections in CF patients (Hutchison et al., 1999). Atypical mycobacteria have also been identified (Cullen et al., 2000; Hodson, 1995; Oliver et al., 2001; Olivier et al., 1996; Smith et al., 1984; Torrens et al., 1998). However, limited information is available concerning the precise role of their colonization in CF patients.

Although many new treatments have appeared, including gene therapy, protein-repair therapy and physiotherapy, there is still no cure for CF. Fundamental problems have limited the application of gene therapy both in terms of safety (viral vector gene therapy) and efficiency (liposomal gene therapy) in the past several years of trials (Cockett, 1999; Flotte, 1999; Rosenecker et al., 1998). Relief of airway obstruction and control of infections, particularly those caused by multidrug-resistant *P. aeruginosa*, is the standard therapy for treating lung diseases in CF patients.

1.2 *P. aeruginosa*

While there have been improvements in the control of infections caused by multidrug-resistant gram-positive pathogens, such as the use of novel chemical classes of antibiotics in recent years against the methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Entercoccus* spp. (VRE), the multidrug-resistant *P. aeruginosa* (MDR-PA) is becoming an increasing clinical problem and a public health threat (Troillet et al., 1997). Reports from the National Nosocomial Infections Surveillance (NNIS) in 1998 indicate that *P. aeruginosa* ranks second among the gram-negative pathogens in the incidence of infections (NNIS, 1998).

1.2.1 Microbiology of *P. aeruginosa*

P. aeruginosa is a member of the family Pseudomonadaceae and the premier pathogen of the *Pseudomonas* species. It is a non-capsulate and non-sporing gram-negative bacillus measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm. Most strains are motile by a single polar flagellum but some strains have two or three flagella. *P. aeruginosa* is nonfermentative, usually obligately aerobic, and oxidase and catalase positive. It grows in soil, water, air, sewage, marshes and coastal marine habitats, as well as on plants, animal tissues and the human body (Hardalo et al., 1997). Clinical isolates, in general, yield one of two smooth colony types. One has a fried-egg appearance that is large, smooth, with flat edges and an elevated appearance. The other, frequently obtained from respiratory and urinary tract secretions, has a mucoid appearance. The cultures have a characteristic odor and a metallic sheen.

P. aeruginosa is an opportunistic pathogen of humans. It can cause urinary tract infections, respiratory system infections, soft tissue infections, bacteremia, dermatitis, and a variety of systemic infections, particularly in victims of severe burns, and in AIDS and cancer patients who are immunosuppressed (Buttery et al., 1998; Rodriguez et al., 1973).

One of the survival strategies of *P. aeruginosa* in a hostile environment is the biofilm mode of growth. Such biofilms also occur in the lungs of CF patients. The thickness of biofilms is variable and uneven, generally 13 - 60 μm, and is determined by the equilibrium between growth of the biofilm and detachment of biomass to the environment (Hoiby et al., 2001). Only 10 - 20% of the volume of biofilms is bacteria, the rest being polysaccharides penetrated by minute anastomozing water channels (Costerton et al., 1994). *P. aeruginosa* growing in biofilms are much more resistant to antibiotics and disinfectants than planktonic growing cells: MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) can be 100 - 1000 times higher in old biofilms (Hoiby et al., 2001). However, the cells liberated from the antibiotic-resistant biofilm have the same sensitivity as the planktonic cells (Spoering et al., 2001). The antibiotic resistance of biofilm-growing *P. aeruginosa* may be due to several factors, such as prolonged generation time, reduced oxygen concentrations at the base of the biofilm, and the binding of antibiotic to the negatively charged alginate comprising the film (Nichols et al., 1988). Ordinary techniques used in the

laboratory to determine the susceptibility of planktonic growing bacteria can not predict the possibility of eradicating bacteria growing in biofilms (Hoiby et al., 2001). The higher resistance of biofilm-growing *P. aeruginosa* can lead to failure of antibiotic therapy with respect to eradication of the bacteria in the biofilms (Costerton et al., 1994; Hoiby et al., 2001).

There are at least thirteen groups of plasmids, based on compatibility in *P. aeruginosa*, with genes conferring resistance to various antibiotics as well as chemicals like mercury, borate, tellurite and tellurate. Most *P. aeruginosa* isolated from CF patients produce large mounts of extracellular polysaccharides (Hentzer et al., 2001). Although the role of the polysaccharides in the pathogenesis of *P. aeruginosa* remains unclear, they may impede phagocytosis and diffusion of antibiotics, thereby facilitating its colonization and persistence. Furthermore, approximately 90% of *P. aeruginosa* produce extracellular proteases (Tummler et al., 1997). These proteases can cause rapid tissue destruction and may aid its invasion of tissue.

1.2.2 *P. aeruginosa* in CF patients

When *P. aeruginosa* was first isolated from CF patients, only 8% of the patients were colonized, whereas *S. aureus* was present in 92% (Di Sant'Agnese et al., 1946). With effective antistaphylococcal chemotherapy, *P. aeruginosa* emerged as the most common bacterial pathogen in airway and lung infections of these patients (Bauernfeind et al., 1987; Brett et al., 1988; Fick, 1989; Krilov et al., 1985; Mearns et al., 1972; Pedersen et al., 1992; Thomassen et al., 1987). A unique feature of CF patients chronically infected with *P. aeruginosa* is the association of mucoid strains with the CF lung (Doggett, 1969; Doggett et al., 1964; Bauernfeind et al., 1987; Brett et al., 1988; Fick, 1989; Govan et al., 1983; Krilov et al., 1985; Mearns et al., 1972; Penketh et al., 1983; Pier, 1985; Thomassen et al., 1987). .

1.2.2.1 Role of *P. aeruginosa* in pathogenesis of CF

There are several defense mechanisms employed by the lung against bacterial colonization, including mucociliary clearance, polymorphonuclear neutrophil phagocytosis and local production of antibacterial cationic peptides (Quie, 1986).

However, these defenses are weakened under the combined influence of the cystic fibrosis disorder and chronic infection with *P. aeruginosa*.

An additional function of CFTR is as a cellular receptor for binding, endocytosing, and clearing *P. aeruginosa* from the normal lung (Pier et al., 1997). The lack of functional CFTR protein could contribute significantly to respiratory infections by *P. aeruginosa*. Furthermore, mucins released from the respiratory epithelium of CF patients by the action of pseudomonal proteases are highly sulphonated and, in the presence of calcium ions can form a very viscous gel with mucoid exopolysaccharide (Cheng et al., 1989; Govan et al., 1985; Klinger et al., 1984). This may also contribute to the poor ciliary clearance of *P. aeruginosa*.

Toxins from *P. aeruginosa*, including exoenzyme S, exotoxin A and lipopolysaccharide, play a role during establishment of the initial persistent colonization of the CF respiratory tract by interfering with the non-specific (eg. phagocyte) and specific (eg. T cells, NK cells, immunoglobulins) defence mechanisms (Kharazmi, 1989; Pedersen et al., 1992). Lipopolysaccharide is the major antigenic component of immune complexes in the sputum of CF patients with chronic *P. aeruginosa* infection (Kronborg et al., 1992).

Early colonization of the CF airways by *P. aeruginosa* is often with non-mucoid forms (Penketh et al., 1983; Ramphal et al., 1987; Wood et al., 1976). However, mucoid *P. aeruginosa* soon becomes the most common morphotype, converted from the non-mucoid form under some undefined environmental pressure (Brett et al., 1988; Deretic et al., 1989; Pugashetti et al., 1982; Speert et al., 1990). Other morphotypes of *P. aeruginosa* may also be recovered frequently (Govan et al., 1983; Penketh et al., 1983; Pier, 1985). However, the mucoid isolates predominate during chronic lung infections in CF patients (Brett et al., 1988; Deretic et al., 1989; Pugashetti et al., 1982; Speert et al., 1990). The mucoid morphotype is due to the production of large amounts of polysaccharide around the cell that was designated by Pier as "mucoid exopolysaccharide" (Pier, 1985). Mucoid exopolysaccharide, as mentioned in section 1.2.1, is a alginate polymer composed of acetylated D-mannuronic acid and L-guluronic acid (Banerjee et al., 1983; Banerjee et al., 1985; Darzins et al., 1984; Flynn et al., 1988a; Flynn et al., 1988b; Ohman et al., 1981; Russell et al., 1988). Alginate has an antiphagocytic effect which may allow *P.*

aeruginosa to evade immune clearance in the lung (Baltimore et al., 1980; Pier et al., 1987; Speert et al., 1984).

1.2.2.2 Immunity to mucoid *P. aeruginosa* in CF

In the 1970s, it was found that many antibodies were produced to *P. aeruginosa* in CF patients (Hoiby et al., 1977) and the pathogenesis of lung tissue damage during chronic *P. aeruginosa* infection was described (Schiotz et al., 1977). In the 1980s, it became clear that immune complex-mediated inflammation was the major cause of the tissue damage (Hoiby et al., 1986).

Alginate is the only antigen that is clinically correlated with a poor prognosis in CF patients (Pedersen, 1992). In most patients, the transition from the non-mucoid to mucoid variant correlates with the development of a pronounced antibody response against virtually all antigens and toxins of *P. aeruginosa*. The correlation between the antibody response and poor prognosis is due to immune complex-mediated chronic inflammation in the lungs of CF patients, including complement activation and high local production of proinflammatory cytokines such as TNF (Tumor Necrosis Factor) and IL-8 (Interleukin-8) (Konstan et al., 1997). The released leucocyte proteases, myeloperoxidase and oxygen radicals are the main causative factors of damage to lung tissue (Hoiby et al., 1986).

1.2.2.3 Chemotherapy and antimicrobial resistance

With aggressive chemotherapy, it is possible to prevent or at least delay the onset of chronic infections with *P. aeruginosa*. Intensive antibiotic treatment could improve significantly the survival of CF patients (Frederiksen et al., 1996). Also anti-inflammatory treatment is effective in maintaining lung function (Bisgaard et al., 1997b).

Numerous antipseudomonal antibiotics are used currently for the treatment of bronchial infections caused by *P. aeruginosa*, including ticarcillin, carbenicillin, piperacillin, tazobactam, tobramycin, gentamicin, amikacin, ciprofloxacin, ceftazidime, imipenem, cilastatin and aztreonam. However, resistance to these agents is becoming more prevalent (Banerjee et al., 2000; Doring et al., 2000; Waterer et al.,

2001). Outbreaks of *P. aeruginosa* resistant to most available β-lactams, aminoglycosides and fluoroquinolones have been reported in burns units, cancer centres, and among patients with CF (Armstrong et al., 1995; Hsueh et al., 1998; Krcmery et al., 1994; Mouton et al., 1993). In the USA, 15% and 20% of *P. aeruginosa* isolates from intensive care unit (ICU) patients were resistant to ceftazidime and imipenem, respectively (Archibald et al., 1997). However, resistance to colistin is seldom seen in spite of a daily selective pressure in patients receiving colistin by inhalation (Evans et al., 1999; Jensen et al., 1987; Littlewood et al., 2000).

The pharmacokinetics of many antibiotics is altered in CF patients due to enhanced drug clearance by the kidney and liver (Lietman, 1988). Treatment of chronic *P. aeruginosa* infection in these patients is controversial. In contrast to most other infections, eradication of *P. aeruginosa* from chronically infected CF patients is virtually never complete (Szaff et al., 1983). Presently, antibiotic chemotherapy against chronic *P. aeruginosa* infection can only reduce and slow down, but not totally prevent, the destruction of pulmonary tissue.

From its natural habitat of living in association with bacilli, actinomycetes and fungi in soil, it is not surprising that *P. aeruginosa* is resistant to a variety of naturally-occurring antibiotics. Intrinsic resistance involves the collaboration of restricted uptake through the outer membrane and secondary resistance mechanisms such as energy-dependent efflux and β-lactamases (Hancock, 1997b). However, over-expression of four separate RND (resistance-nodulation-division) efflux systems, MexAB-OprM, MexCD-OprJ MexEF-OprN and MexXY-OprM, may be caused by regulatory mutations *nalB* (*mexB*), *nfxB* or *nfxC* (*mexT*), which leads to mutational multi-drug resistance (Germ et al., 1999). Many *P. aeruginosa* stains from CF patients are hyper-mutatible and develop their antibiotic resistance during antimicrobial therapy (Oliver et al., 2000).

Resistance can also be transferred by conjugation and transduction. This is clinically important because exchange of genetic information is involved in the rapid development of resistance to antibiotics (Vasil, 1986). The whole genome sequence of *P. aeruginosa* PAO1 contains 6.3 million base pairs (Stover et al., 2000). Studies of its genome will provide a wealth of information for new insights into how these bacteria cause diseases and defend themselves against antibiotics, and for exploitation

of new antibiotic targets. This will suggest directions for novel drug development and new therapeutic strategies for the treatment of infections.

Given their growth and ability to develop resistance, and the compromised defences of the patient, as described in this section and section 1.1, it is easy to appreciate that pulmonary infections in CF patients with *P. aeruginosa* present considerable challenges for treatment. However, there is still cause for reasonable optimism in the therapy of infections with *P. aeruginosa*. Recently, a combination of synergistic antibiotics has been recommended (Hancock et al., 2000); there are several novel combinations of agents, such as β-lactamase inhibitors with β-lactams (Livermore et al., 1997), and inhibitors of efflux pumps with fluoroquinolones (Renau et al., 1999). Moreover, colistin and other cationic peptides have shown considerable promise for treating infections with strains resistant to currently used antibiotics (Hancock, 1997a; Hancock, 1997c; Hancock et al., 1999; Hancock et al., 1998). Presently, prevention of infection and appropriate use of antibiotics, such as colistin, are the two basic strategies to control the infections caused by multidrug-resistant *P. aeruginosa*.

1.3 Colistin

1.3.1 History – discovery and clinical experience

Colistin [1066-17-7, CAS Registry Number], also known as polymyxin E, is one of the polymyxin antibiotics. It is produced as a secondary metabolite by the soil bacterium *Bacillus polymyxa* subsp. *colistinus*, now reclassified as *B. colistinus*. Three independent research groups first discovered polymyxins in 1947 (Ainsworth et al., 1947; Benedict et al., 1947; Stansly et al., 1947). Colistin was first reported by Koyama and coworkers (Koyama et al., 1950) and was originally thought to be distinct from polymyxins. However, its chemical structure was later proven to be identical to polymyxin E (Suzuki et al., 1965). Colistin has been available since 1959 for the treatment of infections caused by gram-negative organisms (Ross et al., 1960). Two forms of colistin are available commercially: colistin sulphate [1264-72-8], used topically and orally, and sodium colistin methanesulphonate [8068-28-8] (synonyms colistimethate sodium, colistin sulphomethate, sodium colistimethate and sulphomethyl colistin), used parenterally and by aerosol. The chemical structure and

physicochemical and biochemical properties of these compounds are reviewed in Section 1.3.2.

Since early clinical reports suggested a high incidence of toxicity with colistin methanesulphonate (Koch-Weser et al., 1970; Ryan et al., 1969), its use was reduced by the later availability of potentially less toxic aminoglycosides and other anti-pseudomonal agents. Colistin methanesulphonate is less toxic than colistin. The LD_{50} value for colistin methanesulphonate (222.3 mg/kg) was much higher than that for colistin (5.46 mg/kg) after intravenous administration in mice (Schwartz et al., 1960). The potential toxicity includes nephrotoxicity, neurotoxicity and hypersensitivity (Beringer, 2001; Bosso et al., 1991; Koch-Weser et al., 1970; Ledson et al., 1998; Perkins, 1964; Ryan et al., 1969; Wolinsky et al., 1962). Unfortunately, the mechanism of toxicity at a molecular level still remains unknown.

The most serious toxicity that can occur following administration of colistin methanesulphonate is nephrotoxicity (Evans et al., 1999). It is almost invariably associated with higher than recommended doses in patients with normal renal function, or failure to reduce dosage in patients with renal impairment, or when used in association with other nephrotoxic drugs (Wolinsky et al., 1962). The nephrotoxicity is characterized by a spectrum of tubular changes from slight degeneration to acute necrosis, leading to acute oliguric renal failure (Koch-Weser et al., 1970). Proteinuria, haematuria and casts in the urine may occur, but increased blood urea nitrogen and creatinine are the most invariable features (Brown et al., 1970a; Price et al., 1970; Wolinsky et al., 1962). These usually occur within the first four days of therapy and may remain highly elevated for one to two weeks after ceasing therapy. Renal function usually returns to baseline in three to nine weeks (Price et al., 1970). Nephrotoxicity is reversible if the therapy is discontinued at the first sign of developing renal impairment (Bosso et al., 1991; Evans et al., 1999; Koch-Weser et al., 1970). Monitoring of urinary output and the levels of urea or creatinine in plasma can give an early warning of nephrotoxicity.

Neurotoxicity occurs most frequently after administration of colistin methanesulphonate in patients with renal impairment (Evans et al., 1999). It occurs in both genders, all ages and with either intravenous or intramuscular administration (Koch-Weser et al., 1970). The neurotoxicity may manifest as giddiness; disturbance

of sensation such as dizziness; numbness and paresthesiae, mainly affecting the face; nausea and vomiting; muscle weakness; and peripheral neuropathy (Koch-Weser et al., 1970). It occurs unpredictably, within one hour of the first dose or after several days of therapy (Evans et al., 1999). More severe neurotoxic disturbances such as mental confusion, coma, psychosis, convulsions and ataxia may also occur. These may be more frequent in patients receiving large doses or with renal dysfunction (Wolinsky et al., 1962). Another serious neurotoxic effect is neuromuscular blockade, which may result in respiratory paralysis (Lindesmith et al., 1968). The blockade can appear quickly without premonitory minor side-effects of neurotoxicity (Koch-Weser et al., 1970), and occur principally in patients also receiving anaesthetics or other drugs which impair neuromuscular transmission (O'Grady et al., 1997). Colistin-induced neuromuscular blockade differs from that induced by the aminoglycosides; the effect from the latter is reversible by neostigmine (Lindesmith et al., 1968). Commencing with a low dose followed by subsequent upward titration has been successful in decreasing such severe symptoms (Reed et al., 2001).

Hypersensitivity reactions, such as rashes, pruritus, and drug fever have been observed during therapy with colistin methanesulphonate (Hoeprich, 1970). In addition, inhaled nebulized colistin sulphate in adults with CF can cause bronchial hyperreactivity with tightness in the chest (O'Grady et al., 1997). The application of colistin sulphate eardrops may lead to ototoxicity (O'Grady et al., 1997).

While the early clinical reports suggesting a high incidence of toxicity with colistin methanesulphonate (Koch-Weser et al., 1970; Perkins, 1964; Wolinsky et al., 1962), closer examination of these reports has revealed an exaggerated risk. The greater risk has been attributed to an inappropriate selection of patients, higher than currently recommended doses and inappropriate monitoring (Beringer, 2001; Bosso et al., 1991; Conway et al., 1997). Early reports indicate variability in the incidence and severity of adverse reactions to colistin methanesulphonate (Cox et al., 1970; Koch-Weser *et al.*, 1970; Olesen at al., 1967). Some patients had underlying illnesses (Koch-Weser *et al.*, 1970). Therefore, attributing the adverse clinical effects to colistin methanesulphonate alone may have been incorrect (Littlewood *et al.*, 2000).

Recently, the increased prevalence of infections with multidrug-resistant *P. aeruginosa* and the lack of novel agents in development has led to the need for a re-

examination of the role of colistin, especially in patients with CF (Catchpole et al., 1997; Conway et al., 2000; Doring et al., 2000; Evans et al., 1999; Hanberger et al., 2001; Littlewood et al., 2000). There has been increased support for the intravenous use of colistin methanesulphonate in the treatment of acute pulmonary infections caused by *P. aeruginosa* and for its administration by inhalation for initial colonization in the respiratory tract (Beringer, 2001; Catchpole et al., 1997; Conway et al., 2000; Doring et al., 2000; Littlewood et al., 2000). The frequency of nephrotoxicity and severity of neurotoxicity are substantially less than previously reported (Koch-Weser et al., 1970; Ryan et al., 1969). Other recent reports have confirmed the safety and enhanced efficacy of intravenous colistin methanesulphonate (Conway *et al.*, 1997; Ledson *et al.*, 1998; Reed *et al.*, 2001). With more rational use, colistin has been proven to be a useful agent in the treatment of *P. aeruginosa* infections in patients with CF (Beringer, 2001; Jensen et al., 1987; Reed et al., 2001).

1.3.2 Chemical structure, physicochemical and biochemical properties

Colistin is a cationic cyclic polypeptide. Like most other peptide antibiotics, it contains a mixture of D- and L-amino acids and is characterized by a high percentage of 2,4-diaminobutyric acid (Dab) [1758-80-1]. Its basic structure is a cyclic heptapeptide ring and a tripeptide side chain covalently bound to a fatty acid at the N-terminus via an acyl group (Figure 1.1).

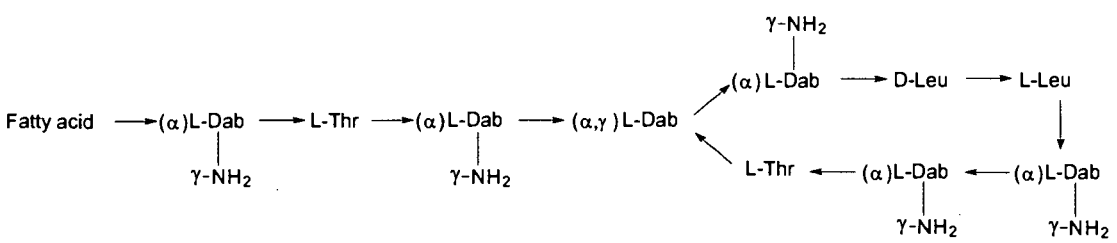

Figure 1.1 Chemical structures of colistin A and B. Fatty acid = 6-methyloctanoic acid [504-99-4] for colistin A and 6-methylheptanoic acid [929-10-2] for colistin B. Thr: threonine; Leu: leucine. Dab: α,γ-Diaminobutyric acid. α and γ indicate the respective $-NH_2$ involved in the peptide linkage.

Colistin has many different components. At least thirty have been isolated and thirteen identified (Orwa et al., 2000; Orwa et al., 2001). They differ from each other by the

composition of amino acids and fatty acids (Elverdam et al., 1981; Orwa et al., 2001). The two major components are colistin A (polymyxin E_1, [7722-44-3], N2-(6-methyl-1-oxooctyl)-L-2,4-diaminobutanoyl-L-threonyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-D-leucyl-L-leucyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-L-threonyl-, cyclic (10→4)-peptide, $C_{53}H_{100}N_{16}O_{13}$) and colistin B (polymyxin E_2, [7239-48-7], N2-(6-methyl-1-oxoheptyl)-L-2,4-diaminobutanoyl-L-threonyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-D-leucyl-L-leucyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-L-threonyl-, cyclic (10→4)-peptide, $C_{52}H_{98}N_{16}O_{13}$) (Figure 1.1). Minor components have also been reported, including polymyxin E_3 and E_4 (Thomas et al., 1980), norvaline-polymyxin E_1, valine-polymyxin E_1 (Elverdam et al., 1981), valine-polymyxin E_2, isoleucine-polymyxin E_1, isoleucine-polymyxin E_1 (Ikai et al., 1998) and polymyxin E_7 and isoleucine-polymyxin E_8 (Orwa et al., 2001). In fact, the amino acid sequences of polymyxins are all similar. The proportion of colistin A and colistin B in commercial material varies between pharmaceutical suppliers and batches, but is generally between 4.5:1 to 0.9:1 (Decolin et al., 1997). Colistin A has recently been synthesized by two different schemes, a linear strategy and a branched strategy (Kline et al., 2001). Furthermore, it was proven that the characteristic lariat structure of colistin is necessary for the antimicrobial activity (Kline et al., 2001).

Colistin exhibits hydrophobicity attributable to the fatty acid moiety, and basic properties ($pKa \approx 10$) of the five unmasked γ-amino groups of the Dab residues. The positively charged Dab and the fatty acid tail make it amphipathic, and allow colistin to distribute well in both polar and non-polar environments, such as in water and in prokaryotic and eukaryotic lipid membranes. Colistin is resistant to pepsin (pH 2.2 - 4.8), trypsin (pH 4.4 - 7.5), pancreatin (pH 4.4 - 7.5), and erepsin (pH 6.1 - 7.8), but is inactivated by lipase (Bergan et al., 1982).

Colistin sulphate is a white to cream-coloured hygroscopic crystalline compound melting at about 210°C with decomposition. It is very soluble in water and glycerin, slightly soluble in alcohol and propylene glycol, practically insoluble in acetone and in ether. A 1% solution in water has a pH of 4 to 5.8. Colistin is relatively stable in water at pH 2 to 6, but becomes less stable above pH 6 (Schwartz et al., 1960). Sulphate accounts for 16.0% - 18.0% of the total composition by weight. Calculated

with reference to the dried substance, the potency of colistin sulphate is not less than 19,000 units per milligram. For clinical purposes, one unit is contained in 0.00004878 mg of the first International Standard Preparation (1968) of colistin sulphate, which contains approximately 20,500 units per milligram (Martindale Extra Pharmacopoeia, 1977). However, this may vary a little with different batches or producers. In the dry state, colistin is very stable at room temperature, for up to 18 months (Ninger et al., 1962). Unfortunately, little information is available concerning the stability of colistin in aqueous solutions, even though the stability of polymyxin B, regarded as the model compound of polymyxins, has been well studied (Taylor *et al.*, 1994).

Sodium colistin methanesulphonate (Figure 1.2) is prepared from colistin by the reaction of sulphomethylation, in which the five free γ-amino groups of the Dab residues are treated with formaldehyde followed by sodium bisulphite. The reactions are expressed as follows:

$$R\text{-}NH_2 + HCHO \rightarrow R\text{-}N{=}CH_2 + H_2O$$

$$R\text{-}N{=}CH_2 + NaHSO_3 \rightarrow R\text{-}NH\text{-}CH\text{-}SO_3^-Na^+$$

These reactions were first reported by Schiff (Schiff, 1866) for converting primary amines to labile alkane sulphonic acids. At the beginning of the last century, the reactions were introduced to modify the properties of drugs, such as solubility, or to reduce undesirable adverse effects of some drugs possessing free amino groups (Lepetit, 1908). Polymyxin methanesulphonate was first reported in 1947 as an agent that was less toxic but retained the activity of polymyxin (Stansly et al., 1947). Colistin methanesulphonate is usually used in the form of the sodium salt. Coly-Mycin M Injectable® (Warner-Chilcott Laboratories) was the first commercial formulation of sodium colistin methanesulphonate, and was available in combination with a local anaesthetic (dibucaine HCl), buffer and thimerosal. In the 1960s, a new formulation for intravenous administration, Coly-Mycin M Parenteral®, was introduced (Cox et al., 1970), and is currently available from Parke-Davis (Morris Plains, NJ). Coly-Mycin M Parenteral® is prepared without a local anaesthetic, buffer or thimerosal and is suitable for intravenous and intramuscular administration.

Sodium colistin methanesulphonate is a white to cream-coloured amorphous compound. Its solubility is similar to that of colistin. A 1% solution in water has a pH of 6.2 to 7.5 (Martindale Extra Pharmacopoeia, 1977). Calculated with reference to the dried substance, the potency of colistin methanesulphonate is not less than 11,500 units per milligram. For practical purposes, one unit of colistin methanesulphonate is contained in 0.00007874 mg of the first International Reference Preparation (1966) of colistin methanesulphonate that contains approximately 12,700 units per milligram (Martindale Extra Pharmacopoeia, 1977).

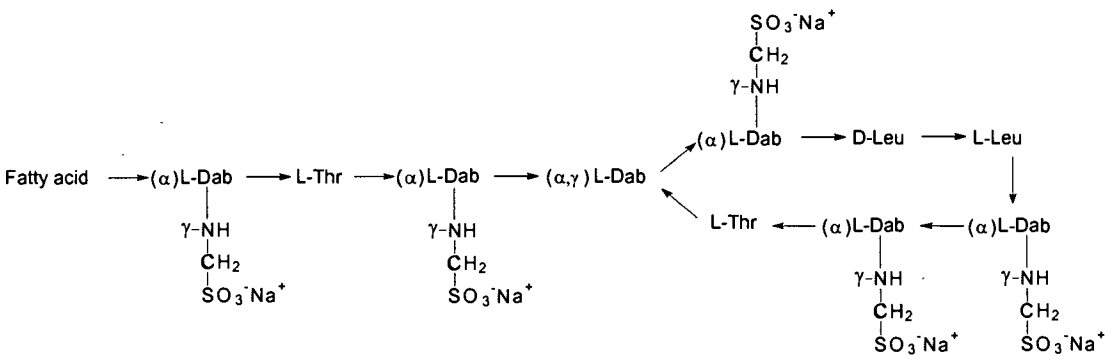

Figure 1.2 Chemical structures of sodium colistin A methanesulphonate and sodium colistin B methanesulphonate. Fatty acid = 6-methyloctanoic acid for colistin A methanesulphonate and 6-methylheptanoic acid for colistin B methanesulphonate. Thr: threonine; Leu: leucine. Dab: α,γ-Diaminobutyric acid. α and γ indicate the respective $-NH_2$ involved in the peptide linkage.

Colistin methanesulphonate, with five methanesulphonate groups, has the potential to hydrolyse in aqueous solution and form a complex mixture of partially sulphomethylated derivatives, including colistin. Two research groups showed there were various fractions after hydrolysis of colistin methanesulphonate in acetate buffer (Barnett et al., 1964) or in human plasma and urine (Beveridge et al., 1967) by electrophoresis and bioassay. In order to obviate complications caused by the presence of five methanesulphonate groups in colistin methanesulphonate, McMillan and his colleagues used NMR, IR, and electrophoresis to explore the hydrolysis of the methanesulphonate group in a simple model compound, *n*-butylaminomethane-

sulphonic acid (McMillan et al., 1969). Unfortunately, the complexity introduced by the presence of five primary amine sites per molecule of colistin limited the investigation of colistin methanesulphonate. However, it was shown that the aminomethane sulphonate solutions were involved in complex equilibria. It was concluded that the probability of the simultaneous existence of five unreacted amino groups in one molecule of colistin methanesulphonate was very small (McMillan et al., 1969).

Interestingly, there has been a report of differences in the incidence of toxicity between the two most common commercial products of colistin methanesulphonate: Colomycin® Injection (Pharmax, UK) and Coly-Mycin M Parenteral® (Parke-Davis, USA) (Barnett et al., 1964). However, the reason remains unknown.

1.3.3 Antimicrobial activities

Colistin exhibits a narrow spectrum of antimicrobial activity, mostly against common gram-negative clinical isolates. Of particular importance is its activity towards *P. aeruginosa*. Like other polymyxins, it is highly active against most of the important gram-negative bacteria that are normally inhabitants of the human bowel. *E. coli*, *Enterobacter*, *Salmonella*, *Shigella* and *Klebsiella* are highly susceptible, but all *Proteus* spp. (Schwartz et al., 1960), *Stenotrophomonas maltophilia* (Catchpole et al., 1997; Chan et al., 1987), and usually *Serratia marcescens* (Greenfield et al., 1970) are resistant. For the other gram-negative bacteria, colistin is active against *H. influenzae*, *Bordetella pertussis*, *Salmonellae*, *Shigellae* (Schwartz et al., 1960), *Acinetobacter* spp. (Kuck, 1976), and *Legionella pneumophila* (Thornsberry et al., 1978). The pathogenic *Neisseria* spp. (meningococci and gonococci), and the *Brucella* spp. are resistant (Schwartz et al., 1960). Most commonly occurring gram-positive bacteria of clinical importance are resistant to colistin. The antimicrobial activity of colistin is summarized in Table 1.1. Generally, colistin is more active than colistin methanesulphonate (Bergan et al., 1982; Goodwin, 1970; Schwartz et al., 1960). For *P. aeruginosa*, addition of colistin to other antipseudomonal agents, such as ceftazidime, imipenem, aztreonam, meropenem, gentamicin, piperacillin and ciprofloxacin, produced greater killing than monotherapy (Chan et al., 1987; Rynn et al., 1999).

Compared with colistin, colistin methanesulphonate has reduced toxicity, but inferior antibacterial activity (Nord et al., 1964). The reports on the antibacterial activity of fully and the various partially sulphomethylated derivatives of colistin are controversial. Bergan et al. believed that only colistin base was antibacterially active and that the activity of colistin methanesulphonate arose from the colistin formed by hydrolysis (Bergan et al., 1982). However, Beveridge et al. suggested that sulphomethylatation did not inactivate colistin even though it markedly reduced the antibacterial activity (Beveridge et al., 1967). Because of the heterogeneity and instability of fully and the various partially sulphomethylated derivatives of colistin, it has not been proven whether the penta-methanesulphonate and various partially sulphomethylated derivatives of colistin have intrinsic antibacterial activity.

Table 1.1 Antimicrobial activities of colistin and colistin methanesulphonate against common pathogenic bacteria, reported as minimum inhibitory concentration (MIC) (mg/L) (O'Grady et al., 1997).

	Colistin sulphate	**Colistin methanesulphonate**
Staphylococcus aureus	64 - R [a]	R
Streptococcus pyogenes	32	32
Viridans streptococci	32 - R	32 - R
E. faecalis	R	R
H. influenzae	0.5 - 1	- [b]
E. coli	0.01 - 32	0.05 - R
K. pneumoniae	0.01 - 1	0.01 - 4
Enterobacter spp.	0.03 - 32	0.5 - R
Proteus spp.	R	R
Salmonella spp.	0.01 - 1	0.03 - 0.5
Shigella spp.	0.01 - 1	0.1 - 0.25
P. aeruginosa	0.03 - 4	2 - 32

[a] R, resistant; [b] not available

The National Committee on Clinical Laboratory Standards (NCCLS) does not provide sensitivity data for the testing of colistin (Evans et al., 1999). However, Eickhoff et al.

demonstrated that the minimum concentration of colistin methanesulphonate that inhibited 90% of 225 strains of *P. aeruginosa* tested (MIC_{90}) was 6.3 mg/L, with the MIC_{50} being 1.6 mg/L (Eickhoff et al., 1965). In a recent study, the *in vitro* activity of colistin methanesulphonate was compared with that of other commonly used antimicrobial agents against 377 recent clinical isolates of gram-negative bacteria (including 94 strains of *P. aeruginosa* from patients with CF) and 16 organisms with defined resistance patterns (Catchpole et al., 1997). Colistin methanesulphonate was active against most strains of *P. aeruginosa* (MIC_{90} 4 mg/L), *Shigella* spp. (MIC_{90} 0.5 mg/L), *Salmonella* spp. (MIC_{90} 1 mg/L), *Acinetobacter* spp. (MIC_{90} 2 mg/L), *Citrobacter* spp. (MIC_{90} 1 mg/L), *E. coli* (MIC_{90} 1 mg/L), *Klebsiella* spp. (MIC_{90} 8 mg/L) and *Enterobacter* spp. (MIC_{90} 1 mg/L). No activity was demonstrated against *Providentia* spp. or *Serratia* spp. The results showed that colistin methanesulphonate remains a useful antimicrobial agent against gram-negative bacteria, particularly those multidrug-resistant strains (Catchpole et al., 1997).

Colistin is attractive because of its notable *in vitro* activity against multidrug-resistant *P. aeruginosa*. It has a number of characteristics that favour its use in the treatment of infections with *P. aeruginosa*, especially the isolates from CF patients.

In particular, it has rapid bactericidal activity and a narrower spectrum compared with most other routinely used antipseudomonal antibiotics. Clinical resistance of *P. aeruginosa* to colistin is reportedly low (Catchpole et al., 1997). The development and transference of resistance are apparently slow (Banerjee et al., 2000; Fish et al., 1995). The percent susceptibility of *P. aeruginosa* for colistin methanesulphonate was as high as 97% (MIC breakpoint susceptibility $\leq$ 4 mg/L) (Catchpole et al., 1997). In a recent quantitative susceptibility testing study of multidrug-resistant isolates (n = 47) of *P. aeruginosa* from adult CF patients, the susceptibility for commonly used antipseudomonal agents was approximately 28% for tobramycin, 17% for piperacillin, 20% for ceftazidime, 20% for ciprofloxacin, and 49% for meropenem. Fortunately, the MICs of colistin sulphate for 98% of these multidrug-resistant *P. aeruginosa* were less than 2 mg/L (Beringer, 2001).

Resistance to colistin methanesulphonate develops more slowly than to tobramycin (Littlewood et al., 2000). Long-term clinical experience with colistin and colistin methanesulphonate has shown that resistant *P. aeruginosa* did not emerge in hospitals

despite its use for many years. Colistin methanesulphonate has continued to demonstrate high efficacy and low resistance after more than 10 years of use in the Danish CF centre (Littlewood et al., 2000). Fortunately, some strains of *P. aeruginosa*, while developing resistance to colistin, have shown increased sensitivity to other antibiotics, such as chloramphenicol and in particular tetracycline, to which these organisms are normally resistant (Brown et al., 1972).

Another potential beneficial characteristic of colistin is its unique anti-endotoxin activity, being able to neutralize bacterial lipopolysaccharides (Escartin et al., 1982; Gardiner et al., 1995; Warren et al., 1985).

1.3.4 Mechanism of antibacterial action and resistance

Most investigations into the mechanism of antibacterial action of polymyxins have been conducted with polymyxin B, regarded as a model compound of polymyxins, with similar structure to colistin. Polymyxin B interacts electrostatically with the outer membrane of bacteria by competitively displacing divalent cations (calcium and magnesium) from the negatively charged phosphate groups of membrane lipids (Dixon et al., 1986). Binding between polymyxin B and the membranes can be antagonized by high concentrations of divalent cations (Chen et al., 1972; Schindler et al., 1979). The outer membranes are disrupted by insertion of polymyxin B and lipopolysaccharide is released (Peterson et al., 1985). Electron microscopic results demonstrated that membrane vesicles emerged from the surface of gram-negative bacteria in the presence of polymyxin B (Koike et al., 1969; Lopes et al., 1969; Lounatmaa et al., 1976).

The detailed bactericidal mechanism of colistin has not been studied comprehensively. Colistin is believed to be bactericidal to gram-negative bacteria with a similar mechanism to polymyxin B (Storm et al., 1977). It has been proven that high concentrations of divalent cations can antagonize the binding of colistin to the outer leaflet of the bacterial outer membrane (Davis et al., 1971; Newton, 1954). Hancock and coworkers (Hancock et al., 1999) presented a self-promoted uptake model to explain the detailed antibacterial mechanism of cationic peptides (Figure 1.3). The bactericidal effect of these peptides was believed to be extremely rapid, making it difficult to quantify the killing process (Hancock et al., 1999).

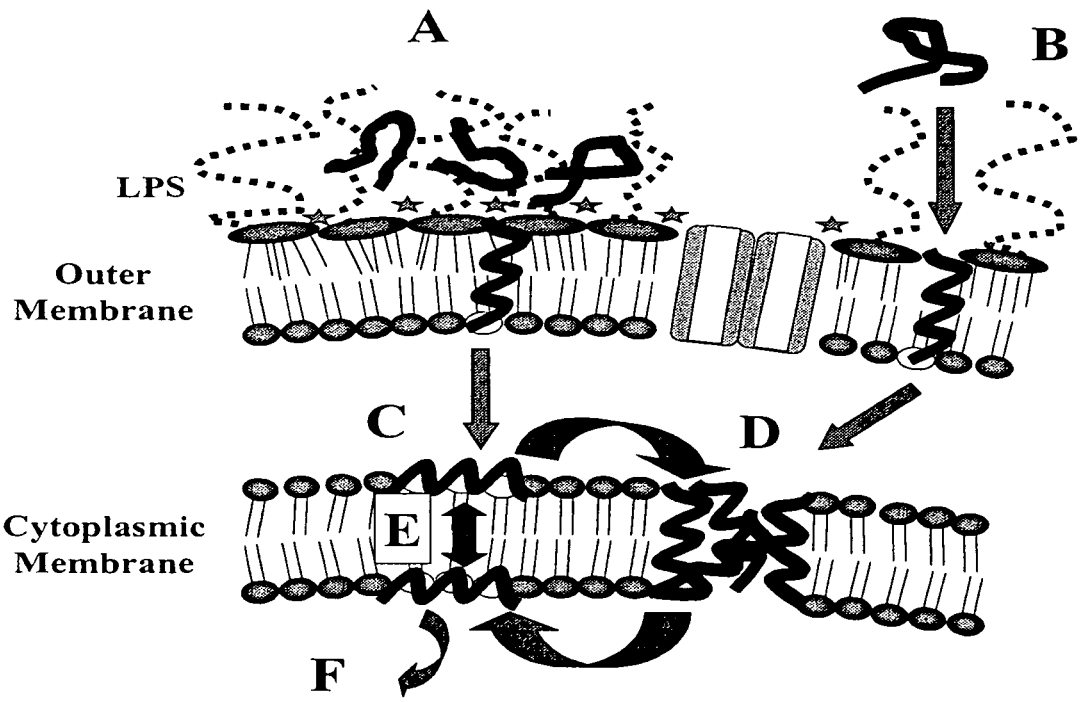

Figure 1.3 Proposed mechanism of the interaction of cationic antimicrobial peptides with the cell envelope of gram-negative bacteria (Hancock et al., 1999). LPS, lipopolysaccharide. These peptides bind to the negatively charged surface of the membrane and create cracks through which they cross the outer membrane (A), or actually bind to the divalent cation binding sites on LPS and disrupt the membrane (B). Once the peptides have passed through the outer membrane, they will bind to the negatively charged surface of the cytoplasmic membrane formed by the headgroups of phosphatidylglycerol and cardiolipin. The amphipathic peptides insert into the membrane surface, at the point where the phospholipid headgroups meet the fatty acyl chains of the phospholipid membranes (C). However, it remains unclear at which point in this process, whether during transit across the outer membrane or insertion into the cytoplasmic membrane, the peptides fold into their amphipathic structures. Many peptides are believed to insert into the membrane bilayer. Under the influence of the large transmembrane electrical potential gradient (E), it is proposed that they either aggregate into a micelle-like complex (D) or flip-flop across the membrane. The net effect of the processes D and E is that some monomers will be translocated into the cytoplasm following dissociation from the membrane and bind to cellular polyanions such as DNA and RNA (F). Ultimately, cell death is caused by the leakage of cytoplasmic constituents. (Adapted from Hancock et al., 1999).

The *in vitro* interaction of colistin with lipids in liposomes and monolayers has been well studied by Mestres and coworkers using physicochemical methods (Mestres et al., 1998). The surface activity of colistin is highly dependent upon the pH of the medium. Colistin is able to form mixed monolayers with phospholipids. Furthermore, colistin was shown by fluorescent probes to coexist with mixed micelles composed of colistin/phospholipids (Mestres et al., 1998).

The killing process with colistin is not dependent upon bacterial metabolic activity (Lorian, 1971), and this may be a significant contributing factor towards the slow development of resistance to the antibiotic (Catchpole et al., 1997). Nevertheless, there may be development of resistance to colistin by either mutation or adaptation, but stable acquired resistance in normally susceptible species is very rare. Resistance by mutation is usually at a low level (Makela et al., 1978). However, it has been shown that high-level resistance was produced by adaptation in the presence of colistin *in vitro* (Brown et al., 1972). Usually there is cross-resistance between polymyxins (Storm et al., 1977).

At a molecular level, resistance may be due to different lipid compositions of lipopolysaccharides (Conrad et al., 1989; Vaara et al., 1981; Vaara et al., 1979) or substitution of protein H1 for magnesium in the outer membrane (Brown et al., 1970b; Nicas et al., 1980; Shand et al., 1988). It is unlikely that the H1 protein, rather than divalent cations, both of which bridge adjacent lipopolysaccharide molecules in the outer membranes, could be replaced by polymyxins (Brown et al., 1970b; Nicas et al., 1980).

Another major contributor to the intrinsic multi-drug resistance of *P. aeruginosa* is probably a number of chromosomally encoded multi-drug efflux systems of RND-MFP-OMF type (RND, Resistance-Nodulation-Division; MFP: membrane fusion protein; OMF: outer membrane factor) (Germ et al., 1999; Li et al., 2000). Four types of RND-MFP-OMF MDR efflux systems have been reported in *P. aeruginosa*, including MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM (Poole, 2001). They expel a remarkably broad range of antibiotics from the cells, including aztreonam, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, piperacillin, tetracycline, and tobramycin (Poole, 2001). It is not known if colistin is a substrate for these efflux systems.

1.3.5 Clinical uses

Colistin sulphate and colistin methanesulphonate are the two commercial forms of colistin used clinically. Colistin sulphate is more toxic than colistin methanesulphonate and limited to topical and oral use (Goodwin, 1970). Usually there are two preparations available commercially: Coly-Mycin S Oral Suspension and Coly-Mycin S Otic. Neither is available in Australia. The oral preparation is used primarily in the treatment of bacterial diarrhoeas of infancy and childhood. There have been no reports of systemic toxicity from the oral use of colistin sulphate alone because negligible quantities are absorbed from the gastrointestinal tract when given in recommended doses (Goodwin, 1970). The usual dosage is 3 to 5 mg/kg/day colistin base given in three divided doses. Coly-Mycin S Otic has been very effective in the treatment of otitis externa (Goodwin, 1970). Recently, an effective inhalation system for colistin sulphate dry powder has been developed (de Boer et al., 2002; Le Brun et al., 2000a; Le Brun et al., 2002). This system has overcome the foaming and reduced activity following inhalation of the intravenous formulation of colistin methanesulphonate and resulted in a shorter administration time (less than 1 min). However, the safety and tolerance of this new inhalation system for colistin dry powder inhalation is yet to be extensively investigated (Beringer, 2001).

Colistin methanesulphonate is available for parenteral use. It is one of the effective antimicrobial agents used in the treatment of *P. aeruginosa* infections, including septicemias, wound infections, urinary tract infections, and infections of the respiratory system (Carroll et al., 1961; Evans et al., 1999; Levin et al., 1999; Yow et al., 1961). Furthermore, colistin methanesulphonate is particularly efficacious in the treatment of infections with gram-negative bacteria resistant to other antibiotics (O'Grady et al., 1997). Usually there are two commercial preparations, Colomycin® Injection (Pharmax, UK) and Coly-Mycin M Parenteral® (Parke-Davis), and only the latter is available in Australia. The recent clinical successes with intravenous colistin methanesulphonate for the treatment of CF respiratory exacerbations have confirmed its efficacy and safety (Conway *et al.*, 2000; Conway *et al.*, 1997; Ledson *et al.*, 1998; Reed *et al.*, 2001). The usual recommended dose is 1.5 to 3 mg/kg (in 10 or 50 mL saline) administered three times daily as an intravenous infusion over 10 to 30 min (Conway *et al.*, 1997). Slow upward titration of the dose is suggested, to minimize the

incidence and severity of toxicity (Reed et al., 2001). Inhalation of colistin methanesulphonate is widely used for infections by *P. aeruginosa* in patients with CF (Frederiksen *et al.*, 1997; Jensen *et al.*, 1987; Littlewood *et al.*, 1985; Valerius *et al.*, 1991; Vazquez *et al.*, 1993). The dose is usually 1 to 2 million units per day. This approach has been evaluated for the management of chronic colonization and for the treatment of initial colonization with *P. aeruginosa* to prevent or delay the onset of chronic colonization and its associated effects on deterioration in lung function (Beringer, 2001).

1.3.6 Pharmacokinetics of colistin and colistin methanesulphonate

With a greater understanding of the pharmacokinetics, there is the potential for further improvements in the use of colistin for infections in humans. However, there are no published modern pharmacokinetic studies of colistin or colistin methanesulphonate in humans except for a recent report on the latter (Reed et al., 2001). Most data on concentrations of these drugs in plasma and urine and on tissue distribution have come from studies before the 1980s using microbiological assays (Evans et al., 1999). Therefore, unless specified, all the concentrations below were measured by microbiological assays.

Before reviewing the pharmacokinetics of colistin and colistin methanesulphonate, the methods that have been used for measuring their concentrations in biological fluids will be summarized.

1.3.6.1 Methods for the quantification of colistin and colistin methanesulphonate in biological fluids

1.3.6.1.1 Assays for colistin

Numerous methods for the assay of colistin have been developed, based on microbiological (Leroy et al., 1989), thin-layer chromatographic (TLC) (Thomas et al., 1978; Thomas et al., 1980), immunological (Kitagawa et al., 1985), capillary electrophoretic (Kristensen et al., 1993) and HPLC methods (Decolin et al., 1997; Elverdam et al., 1981; Kimura et al., 1981; Le Brun et al., 2000b; Orwa et al., 2000; Wall, 1981).

Microbiological methods have been widely used for clinical monitoring of colistin concentrations. *E. coli* 95 ISM, *Bordetella bronchiseptica* ATCC 4617 and other sensitive strains have been used as the test strain. *B. bronchiseptica* ATCC 4617 is particularly sensitive to colistin, more so than *E. coli* 95 ISM (Bergan et al., 1982). Unfortunately, microbiological assays lack specificity, particularly if samples contain any other co-administered antibiotics that are active against the test strain. Furthermore, microbiological methods require considerable time for incubation (up to 21 hr) (Leroy et al., 1989).

In almost all instances, TLC (Thomas et al., 1978; Thomas et al., 1980), capillary electrophoresis (Kristensen et al., 1993) and HPLC (Elverdam et al., 1981; Kimura et al., 1981; Orwa et al., 2000; Wall, 1981) have been applied to the separation of the components of colistin in batches of the raw material. Although the immunological method could be applied to biological samples, preparation of colistin immunogen was complex and the assay could not quantify colistin A and colistin B separately (Kitagawa et al., 1985).

The analysis of colistin in biological fluids by HPLC poses problems because it has very weak ultraviolet absorption and no native fluorescence. Hence, without derivatization with UV-absorbing or fluorescent reagents, it can not be quantified in biological fluids with high sensitivity by conventional techniques of detection. When this thesis was commenced, and in the time since, only two HPLC methods have been reported for the assay of colistin in biological material, and both used *ortho*-phthalaldehyde (OPA) [643-79-8] as the derivatizing reagent (Decolin et al., 1997; Le Brun et al., 2000b). Unfortunately, the OPA derivatives are not overly stable. Therefore, reaction conditions need to be carefully controlled and automated on-line derivatization is usually required (Furst et al., 1990). To improve reliability, Decolin et al. employed a switching HPLC system for the sequential derivatization and assay of colistin isolated from bovine milk, muscle, kidney, liver and fat tissues (Decolin et al., 1997). Le Brun et al. (Le Brun et al., 2000b) reported a method, based on that of Decolin et al. (Decolin et al., 1997), for the analysis of colistin in serum, urine and sputum using colistin methanesulphonate as the reference standard. It should be noted that OPA only reacts with primary amine groups and there are no primary amine groups in colistin methanesulphonate (Figure 1.2). Therefore, in the HPLC method

reported by Le Brun et al. (Le Brun et al., 2000b), it is probable that the derivatives with OPA were formed with the primary amine groups of the hydrolysis products of colistin methanesulphonate.

1.3.6.1.2 Assays for colistin methanesulphonate

While there are several HPLC methods for the assay of colistin in biological samples (see section 1.3.6.1.1), no HPLC method has been developed for the assay of colistin methanesulphonate in biological fluids. A recent report on the pharmacokinetics of colistin in patients with CF applied HPLC to measure the concentrations following derivatization with dansyl chloride (Reed et al., 2001). However, as reviewed in section 1.3.2, colistin methanesulphonate has the potential to hydrolyse in aqueous media and form a complex mixture of partially sulphomethylated derivatives plus colistin. Therefore, it was not clear, in the HPLC method reported by Reed et al. (Reed et al., 2001), which form was quantified, colistin or colistin methanesulphonate or a combination of the two.

Microbiological assay is the most common method currently available for measuring concentrations of colistin methanesulphonate in biological fluids (Bergan et al., 1982; Leroy et al., 1989). However, the product arising from complete hydrolysis of colistin methanesulphonate, colistin, is more microbiologically active than colistin methanesulphonate (Barnett et al., 1964; Beveridge et al., 1967). Furthermore, colistin methanesulphonate and colistin have different diffusion characteristics in agar, with colistin diffusing more slowly (Sande et al., 1970). Therefore, the accuracy of previously reported data on concentrations of colistin methanesulphonate measured by microbiological methods is compromised by the presence of colistin methanesulphonate as well as colistin and its partially sulphomethylated derivatives in the biological samples when collected and formed during incubation (Sande et al., 1970).

1.3.6.2 Pharmacokinetics of colistin

Colistin sulphate is only used topically and orally for clinical purposes, and there have been few reports on its pharmacokinetics in humans. The serum concentrations of colistin have recently been determined in six healthy volunteers and five CF patients

administered a single dose (25 mg) of dry colistin sulphate powder by inhalation (Le Brun et al., 2002). The peak serum concentration measured by HPLC (Le Brun et al., 2000b) ranged from 77 to 159 μg/L. Most pharmacokinetic information after a dose of colistin sulphate has come from studies in animals, such as dogs (Al-Khayyat et al., 1973) and calves (Renard et al., 1991; Ziv et al., 1982).

1.3.6.2.1 Absorption

Colistin is rapidly absorbed after intramuscular injection in humans (Goodwin, 1970) but, as with other polymyxins, colistin is poorly absorbed from the adult gastro-intestinal tract, mucosal surfaces or even inflamed surfaces or burns (O'Grady et al., 1997). Oral doses of colistin sulphate in rabbits showed poor and variable absorption (Schwartz et al., 1960). However, absorption from the gastro-intestinal tract was observed in newborn cows (less than twelve hours old) (Brownlee *et al.*, 1952) and infants (Ross *et al.*, 1960).

1.3.6.2.2 Distribution

The volume of distribution of colistin in the calf was 1.30 ± 0.29 L/kg (Ziv *et al.*, 1982) or 1.02 ± 0.29 L/kg (Renard *et al.*, 1991). Protein binding of colistin in plasma in the dog and cattle is of the order of 50% when determined using equilibrium dialysis (Al-Khayyat et al., 1973; Ziv et al., 1982; Ziv et al., 1972). There is very little information on the protein binding of colistin in plasma from other species. Binding of colistin to tissue components may persist for as long as five days after a single intramuscular injection in rabbits (Kunin, 1970).

1.3.6.2.3 Elimination

The elimination half-life of colistin in calves was 269 ± 58 min after intravenous administration of 5.0 mg/kg colistin sulphate (Ziv *et al.*, 1982). A similar value (4.52 ± 1.81 hr) was reported by Renard et al. after a single intravenous injection of colistin sulphate (25000 U/kg) in calves (Renard et al., 1991). However, the half-life was shorter in dogs (2.50 ± 0.30 hr) after an intramuscular injection of colistin sulphate (2.2 mg/kg) (Al-Khayyat et al., 1973).

There have been very few reports on the clearance of colistin. In the calf, the total body clearance was 3.4 ± 0.5 mL/min/kg after intravenous administration of 5.0 mg/kg colistin sulphate (Ziv *et al.*, 1982). The metabolic fate of colistin is not well defined. Al-Khayyat and his colleagues reported that 0.13% of a 1.1 mg/kg dose, 7.5% of a 2.2 mg/kg dose and 18.5% of a 4.4 mg/kg dose of colistin sulphate were eliminated in urine collected for up to 12 hours after an intramuscular injection in dogs (Al-Khayyat et al., 1973). However, the main elimination route of colistin remains unknown and the fate of the large percentage of the dose not accounted for in urine is obscure.

1.3.6.3 Pharmacokinetics of colistin methanesulphonate

As mentioned in section 1.3.6.1.2, in the recent report on the pharmacokinetics of colistin methanesulphonate administered to patients with CF, it is questionable whether the concentrations of drug measured in plasma and urine by HPLC were for colistin methanesulphonate or for colistin (Reed et al., 2001). Most data on the concentrations of the drug in biological fluids have come from studies using microbiological assays (Evans et al., 1999). Considering that colistin methanesulphonate has the potential to hydrolyse *in vivo* (and partially *in vitro* during sample preparation), the pharmacokinetics of 'colistin methanesulphonate' discussed below is most likely to be representative of a complex mixture of colistin methanesulphonate, various partial sulphomethylated derivatives and colistin present in the biological fluids. As discussed in section 1.3.6.1.2, there are further limitations for microbiological assays.

1.3.6.3.1 Absorption

Following an intramuscular injection of colistin methanesulphonate in humans, it is rapidly absorbed, with maximum concentrations in plasma usually reached after one to two hours (Goodwin et al., 1968). Other workers have observed considerable individual variation in the rate of absorption from intramuscular injection in humans (Bergan et al., 1982; Wright et al., 1960).

Colistin methanesulphonate is very poorly absorbed from the adult gastro-intestinal tract, urinary bladder (Chamberlain et al., 1976), mucosal surfaces or even inflamed

surfaces or burns (O'Grady et al., 1997). There was poor and variable absorption after oral doses of colistin methanesulphonate in rabbits (Schwartz et al., 1960). Ross et al. noted small and varying absorption after oral doses of colistin methanesulphonate to a few small infants, in contrast to the lack of absorption by human adults (Ross et al., 1960).

1.3.6.3.2 Distribution

Information on the volume of distribution of colistin methanesulphonate is very limited. Its volume of distribution in humans was 0.09 ± 0.02 L/kg (Reed et al., 2001). The poor tissue-permeability of colistin methanesulphonate may be a consequence of its relatively high molecular weight and polarity (Goodwin, 1970). Normal anatomical barriers or barriers caused by inflammation have a considerable inhibitory effect on its efficacy (Bergan et al., 1982). Colistin methanesulphonate was not detected (< 0.1 mg/L) in cerebrospinal fluid in dogs two hours after two intramuscular doses (4 mg/kg) in 4.5 hr (Barnett et al., 1964). While it crosses the placenta, the concentrations in the foetal circulation are very low. Following intravenous administration of 150 mg colistin methanesulphonate to pregnant women, it could not be detected in the amniotic fluid three hours later. Very low plasma levels (approximately 0.45 mg/L) were found in both mothers and infants born 6 to 20 hours after a dose (MacAulay et al., 1967). After intramuscular administration of colistin methanesulphonate (33 mg), detectable subretinal fluid levels were shown in 7 of 18 patients 3 to 4.5 hours after the dose (Schwartz et al., 1960). Disposition of colistin methanesulphonate in fluid of the airway after inhalation has not been systematically evaluated (Beringer, 2001).

Tissue binding via the positively charged amino groups is a feature of polymyxins (O'Grady et al., 1997). While colistin methanesulphonate has a limited capacity to diffuse into tissues, binding to tissues, probably by localizing on cell membranes, exerts a profound effect on its distribution and persistence in the body. However, colistin methanesulphonate binds to tissue components to a lesser degree than colistin. The lesser binding may arise from masking of the amino groups by the methanesulphonate moieties (Craig et al., 1973; Kunin et al., 1971); this parallels the lower toxicity and antibacterial activity of colistin methanesulphonate.

After a single intramuscular injection of colistin methanesulphonate (2.5 mg/kg) into the rabbit, free colistin methanesulphonate was detectable in the liver, kidney, lung, muscle and heart, but not the brain (Kunin et al., 1971). Bound drug persisted in all of these tissues, including brain, for 72 hr. After administering seven daily injections (2.5 mg/kg/day), colistin methanesulphonate had accumulated in all of these tissues, but there was no observable accumulation in plasma (Kunin et al., 1971). Five days after the last dose, the concentration of bound drug in the brain (~11 mg/kg) was higher than those in the lung (~8 mg/kg), kidney (~3.5 mg/kg), liver (~2.5 mg/kg) and muscle (~2.2 mg/kg) (Kunin et al., 1971). The high concentrations of bound drug in the brain may be one of the reasons for its neurotoxicity. Free drug present in the kidney, liver, and muscle declined very slowly and was present at concentrations of more than 5 mg/kg up to five days after the last dose, while the free drug present in the brain and lung was relatively constant over this period at approximately 1 mg/kg (Kunin et al., 1971). The bound colistin methanesulphonate was probably located on cell membranes, which may explain not only its cumulative toxicity, but also the persistence of toxic effects for many days (Kunin et al., 1971). Although direct extrapolation of the results in rabbits to humans may be difficult, the results are probably helpful for understanding the mechanisms of toxicity (Kunin et al., 1971).

The protein binding of colistin methanesulphonate has been measured in plasma from the dog and ewe. In plasma from the dog, 2% was bound at 9.6 mg/L when conducted using equilibrium dialysis at 4°C for 24 hr (Al-Khayyat et al., 1973). Using plasma from the ewe, 34.0 ± 4.2% was bound at 6.2 and 12.5 mg/L, again using equilibrium dialysis at 4°C for 24 hr, while a bound percentage of 42.8 ± 3.8% was found at the same concentrations using ultrafiltration (Ziv et al., 1972).

1.3.6.3.3 Elimination

Reported serum concentrations of colistin methanesulphonate varied with the administration routes and doses. After an intravenous injection of 150 mg colistin methanesulphonate to adults, approximately 18 mg/L was measured in serum after 10 min and 0.4 mg/L at 12 hr. After the same dose intramuscularly, peak serum levels of 5 to 6 mg/L were obtained at 1 to 2 hr and 1 mg/L at 12 hr (Froman et al., 1970). Conway and coworkers reported mean serum concentrations in the range from 4.0

mg/L to 9.4 mg/L after the first dose of 160 mg colistin methanesulphonate administered three times daily as an intravenous infusion over 30 minutes (n = 12), with corresponding values of 10.6 mg/L to 20.8 mg/L at steady-state (n = 10). However, if it was given in a bolus, higher maximum concentrations (10 min after intravenous administration) were achieved, 11.2 mg/L for the first dose (n = 12) and 28.4 mg/L at steady-state (n = 11) (Conway et al., 2000).

Colistin methanesulphonate was reported to have a shorter half-life than colistin in the dog (Al-Khayyat et al., 1973). The half-life of colistin was 2.50 ± 0.30 hr after an intramuscular injection of colistin sulphate (2.2 mg/kg), while with a dose of 2.2 mg/kg colistin methanesulphonate, the half-life was 1.37 ± 0.18 hr (Al-Khayyat et al., 1973). In humans, there is considerable variation in the values of reported half-life for colistin methanesulphonate. Generally, after intravenous administration, the half-life is 2 to 3 hr (O'Grady et al., 1997). After a single intramuscular injection of colistin methanesulphonate (150 mg), the half-life was 2.75 to 3.0 hr (Froman et al., 1970). In another study, a half-life of 6 hr was reported for subjects with normal renal function after an intramuscular injection (Kunin, 1967). In the most recent report on the pharmacokinetics of colistin methanesulphonate, the half-life was 3.4 ± 1.4 hr after a first dose and 3.5 ± 1.0 hr at steady state (Reed et al., 2001). Concentrations in this study were measured by HPLC. Considering the limitation of both microbiological assays and this HPLC method that they could not measure the concentrations of colistin and colistin methanesulphonate separately (see section 1.3.6.1.2), the half-lives of colistin methanesulphonate in the literature may be composite values representing the elimination half-lives of both colistin methanesulphonate and colistin formed by hydrolysis.

Elimination of colistin methanesulphonate occurs mainly by the renal route (Bergan et al., 1982). It is much more rapidly eliminated via urine than is colistin (O'Grady et al., 1997). The urinary recovery of colistin methanesulphonate was recently studied after mean doses of 63 ± 13 mg were administered intravenously to 13 patients with CF (Reed et al., 2001). The average renal clearance was 0.24 ± 0.15 mL/min/kg and the mean total body clearance was 0.35 ± 0.09 mL/min/kg. Overall, 62.5% of the dose was excreted in the urine during the first 8 hr after administration. The majority of the renal excretion (~50%) occurred during the first 4 hr (Reed et al., 2001). Similarly,

approximately 75% of an intravenous dosage of colistin methanesulphonate (150 mg) in ten patients was excreted in urine in 24 hr, while 65% was excreted after an intramuscular dose (150 mg) (Froman et al., 1970). Yamada et al. reported that 76% of the dose of colistin methanesulphonate was excreted in the urine in 24 hr after intravenous or intramuscular injections in humans (Yamada *et al.*, 1974).

The fate of the remaining 25% to 40% of colistin methanesulphonate not eliminated by the kidney in humans was not clear (Bergan et al., 1982). Only a small part (1% to 10%) of colistin methanesulphonate after intramuscular dosage was eliminated into the faeces via bile (Bergan et al., 1982; Pulaski et al., 1955). In contrast, another report has indicated that colistin methanesulphonate after intramuscular administration was not excreted in bile in humans, suggesting that biliary excretion is minor (Kunin, 1967).

There have been no reports on the metabolism of colistin methanesulphonate in the liver or kidney. Abe and coworkers (Abe et al., 1976) administered colistin methanesulphonate (100 mg/kg) intravenously to rabbits and used thin-layer chromatography and a reference standard to tentatively identify a metabolite of colistin, colistin-N-glucuronide [12643-15-1], in the urine (1.7% of the dose) and bile (6.7% of the dose).

1.3.7 Pharmacodynamics of colistin and colistin methanesulphonate

The aim of antibacterial chemotherapy is to achieve antimicrobial concentrations at the site of infection sufficient and persistent to eradicate infective bacteria. The discipline of pharmacodynamics of antimicrobials is to integrate both microbiological and pharmacokinetic data to predict clinical outcomes. Usually, pharmacodynamic parameters for antimicrobials include the MICs against the target pathogens, time-kill kinetics and postantibiotic effect (PAE) (Turnidge, 1990). However, besides the considerable data on the MICs of colistin and colistin methanesulphonate (see section 1.3.3), very little work has been conducted on other aspects of the pharmacodynamics of colistin and colistin methanesulphonate, particularly against the multidrug-resistant *P. aeruginosa*. Data on time-kill kinetics and PAE for colistin and colistin methanesulphonate may prove useful for the rational use of this 'problem' antibiotic. Work on such aspects is one of the aims of this thesis (see Chapter 2).

1.4 Summary

The increasing incidence of infections by microorganisms resistant to the commonly prescribed antimicrobial agents has prompted the search for new antibiotics, but also provides the opportunity to re-examine the value of older infrequently used antibiotics. Emergence of multidrug-resistant strains of *P. aeruginosa*, particularly in patients with CF, has posed significant problems for treatment. Colistin is believed to be a useful antimicrobial agent against gram-negative bacteria, particularly those strains resistant to the common antibiotics. However, there is limited information on its pharmacodynamics against *P. aeruginosa*, and its pharmacokinetics.

Potential adverse effects from the clinical use of colistin in previous decades, and the advent of newer and safer antibiotics precluded its development as a useful antibiotic for systematic treatment. Furthermore, the lack of specific assays for colistin and colistin methanesulphonate has restricted proper research on their pharmacokinetics and pharmacodynamics. Information on the pharmacokinetics and pharmacodynamics would permit more rational clinical use of this 'problem' antibiotic. This thesis is intended to provide data that will contribute to more appropriate use of colistin and colistin methanesulphonate in patients, especially those with CF.

To understand further the pharmacokinetics and pharmacodynamics of colistin and colistin methanesulphonate, the objectives of this thesis were:

1. To explore the *in vitro* pharmacodynamics of colistin and colistin methanesulphonate (Chapter 2);

2. To develop accurate and sensitive analytical methods for measuring the concentrations of colistin and colistin methanesulphonate in plasma and urine (Chapters 3 and 4);

3. To examine the stability of colistin and conversion of colistin methanesulphonate to colistin in water, saline, buffer, microbiological culture media, plasma and urine (Chapter 5);

4. To describe the pharmacokinetics of colistin (Chapter 6) and colistin methanesulphonate (Chapter 7) following intravenous administration in the rat; and

5. To describe the pharmacokinetics of colistin methanesulphonate and colistin following intravenous administration of colistin methanesulphonate in patients with CF (Chapter 8).

Chapter 2 *In vitro* pharmacodynamic properties of colistin and colistin methanesulphonate against *P. aeruginosa* from patients with cystic fibrosis

2.1 Introduction

P. aeruginosa is the predominant respiratory pathogen in patients with CF. It is isolated from about 80% of patients over their lives (FitzSimmons, 1993). *P. aeruginosa* plays an important role in the progressive lung destruction and subsequent respiratory failure that occurs in CF patients (FitzSimmons, 1993). Since the development of more frequent resistance of *P. aeruginosa* to the commonly used antimicrobials, the use of colistin has begun to attract more interest. The antibacterial activity and mechanism of antibacterial action of colistin have been reviewed in detail in Chapter 1.

The aim of antibacterial chemotherapy is to achieve antimicrobial concentrations at the site of infection which are sufficient and persistent enough to eradicate bacteria. The likelihood of clinical success depends on pathogen-, drug- and patient-related factors (Nicolau, 2001). The discipline of pharmacodynamics of antimicrobials is to integrate both microbiological and pharmacokinetic data to predict clinical outcomes. Optimized antibacterial chemotherapy is important for minimizing the risks of emerging resistance to the currently used antimicrobials (Nicolau, 2001). Usually, pharmacodynamic parameters of antimicrobials include the MICs against the target pathogens, time-kill kinetics and postantibiotic effect (PAE) (Turnidge, 1990).

The MIC is the lowest concentration of antimicrobial agent at which growth of the test microorganism can not be visually or photometrically detected after overnight incubation of a previously clear suspension of between 10^5 to 10^6 colony-forming units (CFU) per mL in the log phase of growth (Levison, 1995). The observed MIC values are considered to be accurate to a plus or minus two-fold dilution. MICs obtained by serial dilution do not represent continuous data. For example, an MIC of 4 mg/L may actually represent a value between 2 and 8 mg/L. The MIC for an antimicrobial is not usually determined routinely in laboratories. For some antimicrobial agents and microorganisms, MICs vary widely within the susceptible

range, while for others, MICs fall into a narrow range. MIC is used to predict the bacteriologic response to antimicrobial chemotherapy. The time that the concentration of antimicrobial agent exceeds the MIC in vivo (T > MIC) can be predicted once the pharmacokinetics of the antimicrobial agent is described and the MIC against the targeted pathogen is known. Analysis of many antimicrobial agent/microorganism relationships have shown that the optimal dosing interval is related to T > MIC (Turnidge, 1990).

MIC determination fails to assess the kinetics of bactericidal activity, as well as the effects of changing antimicrobial concentrations on chemotherapy. The time-kill curve is able to evaluate the time-course and extent of bactericidal action by quantitative subculturing at various times after exposure to pharmacologic concentrations of antimicrobials (Levison, 1995). The time-kill curve is more relevant for clinical situations in which bactericidal therapy is desirable, and is believed to be one of the most reliable methods for determining the bactericidal activities of antimicrobial agents (NCCLS, 1992). Based on serial colony counts, it provides a dynamic picture of antimicrobial action over time. However, it has the disadvantages of being tedious and time-consuming.

The PAE is used to describe the suppression of bacterial growth which persists after a limited exposure of microorganisms to an antimicrobial agent (Vogelman *et al.*, 1988b). It is defined by the following equation:

$$PAE = T - C \qquad \text{(Equation 2.1)}$$

where T is the time required for the count of CFU in the culture to increase by 1 $\log_{10}$ unit above the count observed immediately after removal of the drug and C is the time required for the count of CFU in an untreated control culture to increase by 1 $\log_{10}$ unit above the count obtained immediately after completion of the same procedure for drug removal which was applied to the test culture (Craig *et al.*, 1996). Since the growth rate of the previously exposed microorganism is similar to that of untreated controls beyond the first 1 $\log_{10}$ unit increase in CFU/mL, the duration for the first $\log_{10}$ unit increase is chosen for quantitation of PAE (McDonald *et al.*, 1977).

The PAE is a phenomenon that has been observed since the beginning of the antibiotic era and was first reported as delayed development in turbidity after penicillinase was

added to cultures of staphylococci and streptococci which had been exposed previously to penicillin G (Bigger, 1944). While the PAE in staphylococci induced by a 5 - 30 min exposure to penicillin G was studied in detail soon after (Parker *et al.*, 1948), such effect in gram-negative bacilli induced by other antimicrobial agents was not investigated until the middle to late 1970s (Vogelman *et al.*, 1988b). The term "postantibiotic effect" (PAE) was first introduced by McDonald et al in 1977 to describe persistent suppression of bacterial growth after a short exposure to an antimicrobial agent (McDonald *et al.*, 1977). It should be emphasized that the PAE describes the effect due to prior antimicrobial exposure, rather than to persistent subinhibitory concentrations of antimicrobial agents. Most investigations on PAE have been conducted in the past 20 years.

PAE has been an important pharmacodynamic characteristic of virtually all antimicrobial agents, particularly in the immunocompromised host. All of the antimicrobial agents tested so far are able to induce PAEs against aerobic Gram-positive cocci. However, while penicillins, cephalosporins and trimethoprin induce minimal or no PAEs against Gram-negative bacilli, PAEs induced by aminoglycosides, tetracycline, chloramphenicol and rifampin have been reported (Vogelman *et al.*, 1988b). Determination of PAEs provides important information on the interaction between microorganism and antimicrobial agent. However, such information is unable to be obtained by simple *in vitro* susceptibility testing and pharmacokinetic studies.

Many factors can affect the PAE of an antimicrobial agent, including type of microorganism and antimicrobial agent, concentration of the antimicrobial agent, duration of the exposure, size of inoculum, growth phase (exponential or stationary phase) of the microorganism at time of exposure, medium, pH of medium, aerobic or anaerobic environments, concentration of electrolyte in media and combination of antimicrobial agents (Craig *et al.*, 1996). However, the precise mechanism of the PAE remains unclear. A wide variety of antimicrobial agents have been studied for their PAE to provide a theoretical rationale for dosing. Studies on PAE provide much more information on the action of antimicrobial agents than can be obtained solely from standard *in vitro* sensitivity tests (Craig *et al.*, 1996).

Interactions between antimicrobial agent and the bacterium are usually classified as concentration-dependent (time-independent), and concentration-independent (time-dependent) (Craig *et al.*, 1990; Levison, 1995; Levison *et al.*, 1989). For concentration-dependent antimicrobial agents, the rate and extent of killing and PAE increase as the concentrations of antimicrobial agents increase. Antimicrobial agents with concentration-dependent killing include aminoglycosides, fluoroquinolones and metronidazole (Craig *et al.*, 1990; Levison, 1995; Levison *et al.*, 1989). For concentration-independent antimicrobial agents, once a threshold concentration of the antimicrobial agent is achieved, further increases of concentrations do not increase the rate and extent of killing or extend the PAE. β-Lactam antimicrobials and vancomycin are representatives of this group (Craig *et al.*, 1990; Levison, 1995; Levison *et al.*, 1989).

Because there are significant differences in antibacterial activity between colistin sulphate and colistin methanesulphonate (O'Grady *et al.*, 1997; Schwartz *et al.*, 1960), the current study was conducted with both forms. The aim was to examine their *in vitro* pharmacodynamic properties, including MICs, time-killing and the PAE.

2.2 Materials and methods

2.2.1 Bacterial strains and antimicrobial agents

Thirty clinical isolates of *P. aeruginosa*, both mucoid and non-mucoid strains (Table 2.1), were selected from routine clinical isolates from patients with cystic fibrosis at the Women's and Children's Hospital in Adelaide, Australia. The specimen types included sputum, tracheal aspirate and cough swab. Isolates were selected sequentially from patients as they presented with acute exacerbations; both mucoid and non-mucoid strains may be isolated from the same patient on different occasions. Strains were identified by colonial morphology, characteristic pigment production and resistance to 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan (Davis *et al.*, 1983). Isolation and purification of the strains were done by the laboratory technicians at the Department of Microbiology and Infectious Diseases, Women's and Children's Hospital. All the strains were stored in Glycerol Broth (Appendix I) at -80°C before use. A type culture of *P. aeruginosa* ATCC 27853 was included as the reference.

Table 2.1 Characteristics of *P. aeruginosa* identified in 30 isolates from CF patients presenting at the Women's and Children's Hospital (Adelaide, Australia)

No	Strain [a]	Resistance to some antimicrobial agents [b]								Information on patients		
		Azt	Cef	Cip	Gen	Mer	Pip	Tic	Tob	Sex	Age	Date [c]
1	18982muc	S	R	S	R	R	S	S	R	M	15	06/04/99
2	19453 muc	R	I	S	R	S	S	R	R	M	7	12/04/99
3	19449 muc	R	R	S	R	R	S	R	R	M	10	12/04/99
4	19056 muc	-	R	S	R	I	R	R	R	M	6	07/04/99
5	19271 muc	S	S	S	R	S	S	S	R	M	15	09/04/99
6	19449 n/m	R	R	S	R	R	R	R	I	M	10	12/04/99
7	19562 (lge)n/m	S	S	S	I	-	S	S	S	M	11	13/04/99
8	19562 (sm)n/m	S	S	S	S	-	S	S	S	M	11	13/04/99
9	19271 n/m	R	R	S	R	R	R	R	R	M	15	06/04/99
10	18982 n/m	R	R	S	R	R	R	R	I	M	15	06/04/99
11	19147 n/m	S	R	S	R	-	S	R	R	M	11	19/04/99
12	19056 n/m	I	I	I	R	S	S	S	R	M	6	07/04/99
13	19814 n/m	R	R	S	R	-	R	-	R	F	16	13/04/99
14	19814 muc	S	S	S	R	-	S	S	R	F	16	13/04/99
15	19626 n/m	R	R	S	R	-	R	R	R	F	16	13/04/99
16	19626 muc	S	S	S	R	-	S	S	R	F	16	13/04/99
17	20223 n/m	S	S	S	R	-	S	S	R	M	11	19/04/99
18	20143 muc	S	S	S	R	-	S	S	S	M	17	19/04/99
19	20143 n/m	R	R	I	R	-	S	R	R	M	17	19/04/99
20	20321 n/m	S	S	S	S	-	S	S	S	M	4	20/04/99
21	20509 n/m	S	S	S	S	-	S	S	S	M	4	20/04/99
22	20675 n/m	S	S	S	R	-	S	S	I	M	11	19/04/99
23	20891 n/m	S	S	S	R	-	S	S	R	M	11	19/04/99
24	20844(sm)n/m	R	R	S	R	-	R	R	R	M	15	06/04/99
25	20844(lge)n/m	R	R	S	R	-	R	R	R	M	15	06/04/99
26	20844 muc	S	S	S	R	-	S	S	R	M	15	06/04/99
27	21346 n/m	I	S	S	R	-	S	R	R	F	7	03/05/99
28	21483 n/m	S	S	S	S	-	S	S	S	F	10	04/05/99
29	213331 n/m	R	R	S	R	-	R	R	R	M	13	03/05/99
30	213332 n/m	I	S	S	R	-	S	S	R	M	13	03/05/99

[a] muc, mucoid; n/m, non-mucoid; lge, large colony; sm, small colony.

[b] Susceptibility was examined by disk test. Azt, aztreonam; Cef, ceftazidime; Mer, meropenem; Pip, piperacillin; Tic, ticarcillin; Gen, gentamicin; Tob, tobramycin; Cip, ciprofloxacin. R, resistant; I, intermediate; S, susceptible. The standards for the definition of R, I and S are listed in Appendix I.

[c] Date on which isolates collected.

Subcultures were maintained on horse blood agar (Appendix I). Colistin sulphate was obtained from Sigma (St Louis, USA) and sodium colistin methanesulphonate was from Dumex (Denmark).

2.2.2 MIC determination

Firstly, the susceptibility of all strains of *P. aeruginosa* was tested with disks containing 10 μg colistin sulphate (Oxoid, Hampshire, UK). Isolated colonies were suspended in 2 mL sterile saline and the turbidity adjusted to 0.5 McFarland by a Viter Colorimeter (Loveland, USA). The suspension was spread evenly on Mueller-Hinton agar with a sterile cotton swab. A disk containing 10 μg colistin sulphate was placed at the centre of the plate. Results were observed after the plates were incubated at 35°C for 18 hr.

MICs were determined by both broth macrodilution and microdilution in cation-adjusted Mueller-Hinton broth (Oxoid, Hampshire, England) according to NCCLS standards (NCCLS, 1993). An inoculum (0.2 mL) of approximately 10^6 CFU/mL was added into 19.8 mL cation-adjusted Mueller-Hinton broth (CAMHB). For broth macrodilution, an aliquot (1 mL) of the suspension was mixed with 1 mL CAMHB containing colistin sulphate or colistin methanesulphonate at concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 mg/L in 10-mL disposable culture tubes (Dispens-A-Pak®, Borex, Chase Scientific Glass, Georgia, USA) and incubated at 35°C. A blank control of drug-free broth was prepared. For broth microdilution, 100 μL of a suspension of each strain was mixed with 100 μL CAMHB in a 96-well microtest plate (Sarstedt, SA, Australia) and incubated at 35°C. Each strain was tested in duplicate. A blank control of drug-free broth was incubated in the same manner but without inoculation. The MIC was defined as the lowest concentration at which there was no visible growth after incubation at 35°C for 18 hr. Strains were considered resistant to colistin and colistin methanesulphonate if their MICs were ≥ 32 mg/L.

2.2.3 Time-kill kinetics

The time-kill kinetics of four strains, ATCC 27853 and three clinical isolates (18982 muc, 19056 muc, and 20223 n/m), were examined. The clinical isolates were selected in order to have a range of MICs within the susceptible category.

Colistin sulphate or colistin methanesulphonate was added to a logarithmic phase broth culture of approximately 10^6 CFU/mL to yield concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32 and 64×MIC of the strain under study. Subcultures for viable counts were performed on nutrient agar (Oxoid, Hampshire, England) at 0, 5, 10, 15, 20, 25, 30, 45, 60 min and 2, 3, 4 and 24 hr after the addition of colistin sulphate, and at 0, 15, 30, 45, 60 min and 2, 3, 4 and 24 hr for colistin methanesulphonate. Viable counts were determined after incubation of the subcultures at 35°C for 18 hr. The time-kill curves were plotted as $\log_{10}$ CFU/mL against time. Carryover of antimicrobial agent was not deemed to be a problem in this study because serial dilution was employed to minimize the effect. Bactericidal activity is defined as a 3 $\log_{10}$ decrease in CFU/mL (99.9% kill) and bacteriostatic activity is defined as a < 2 $\log_{10}$ decrease in CFU/mL (Boswell *et al.*, 1998).

2.2.4 PAE

The *in vitro* PAE was determined by the standard *in vitro* method (Craig *et al.*, 1996) for three clinical strains noted above (18982 muc, 19056 muc, and 20223 n/m) and the ATCC strain with both agents. For each experiment, *P. aeruginosa* ($\approx 10^6$ CFU/mL) in logarithmic phase growth was exposed to colistin sulphate for 15 min or to colistin methanesulphonate for 1 hr in Mueller-Hinton broth at the concentrations of 0.5, 1, 2, 4, 8 and 16×MIC. Since colistin showed a very rapid bactericidal effect, the bacteria were exposed to the agent for fifteen minutes, to ensure adequate numbers of bacteria for sampling at the termination of exposure. Antimicrobial agent was removed by twice centrifuging at 3,000 *g* at 35°C for 10 min, decanting the supernatant and resuspending in pre-warmed broth at 35°C in a water bath. Viable counts were performed at 0, 1, 2, 3, 4, 5, 6 and 24 hr, on nutrient agar. A growth control was performed in the same fashion, but without exposure to the antimicrobial agent. The colonies were counted after incubation at 35°C for 18 hr. The PAE was determined by

comparing the re-growth of treated and growth of control cultures, using the standard formula of Equation 2.1 (Craig *et al.*, 1996). A significant PAE is defined as > 0.5 hr.

2.2.5 Statistical and mathematical analysis

Comparisons of the MICs were made using the paired *t*-test with pooled variance on log-transformed values (Systat v9.0, SPSS Inc., Chicago, IL).

The killing effect of colistin sulphate and colistin methanesulphonate at different concentrations was quantified by calculating the mean survival time over 4 hr ($MST_{240\ min}$) using Equation 2.2.

$$MST_{240\,min} = \frac{AUMC_{0-240\,mn}}{AUC_{0\text{-}240\,min}} \qquad \text{(Equation 2.2)}$$

where $MST_{240\ min}$ is the mean survival time (in minutes); $AUMC_{0\text{-}240\ min}$ is the area-under-the-curve of CFU/mL multiplied by time of sampling in minutes from 0 to 240 minutes; and $AUC_{0\text{-}240\ min}$ is the area-under-the–curve of CFU/mL from 0 to 240 minutes. Areas were calculated using the trapezoidal rule.

2.3 Results

2.3.1 Susceptibility and MICs

As shown in Table 2.1, 37% of the strains were resistant to aztreonam, 43% to ceftazidime, 56% to meropenem, 30% to piperacillin, 43% to ticarcillin, 83% to gentamicin, and 70% to tobramycin.

Table 2.2 illustrates the susceptibility and MICs obtained with colistin and colistin methanesulphonate. There was essentially no difference in results for MICs obtained by macro- versus micro-dilution methods. The susceptibility of strains to colistin and colistin methanesulphonate and the range of MICs are summarized in Table 2.3 and are based on the results from the micro- or macro-dilution methods. For all susceptible strains, colistin (geometric mean MIC = 3.2 mg/L) was approximately 3-fold more

Table 2.2 Susceptibility and MICs (mg/L) of colistin methanesulphonate and colistin against *P. aeruginosa* from CF patients and ATCC type strain

No	Strain [a]	Mucoid status	Disk test (mm) [b]	Colistin sulphate		Colistin methanesulphonate	
				Micro-dilution	Macro-dilution	Micro-dilution	Macro-dilution
1	18982	muc	20	4	4	8	8
2	19453	muc	12.5 (f)	> 128	> 128	> 128	> 128
3	19449	muc	13 (f)	> 128	> 128	> 128	> 128
4	19056	muc	22.5	1	1	8	8
5	19271	muc	21	4	4	4	4
6	19449	n/m	R	> 128	> 128	> 128	> 128
7	19562 (lge)	n/m	22	4	4	8	4
8	19562 (sm)	n/m	19	2	2	8	4
9	19271	n/m	22	2	1	4	4
10	18982	n/m	22	1	1	4	4
11	19147	n/m	R	> 128	> 128	> 128	> 128
12	19056	n/m	R	> 128	> 128	> 128	> 128
13	19814	n/m	19	4	4	4	4
14	19814	muc	24.1	1	1	4	2
15	19626	n/m	R	> 128	> 128	> 128	> 128
16	19626	muc	22.2	2	1	4	4
17	20223	n/m	18.3	4	4	16	8
18	20143	muc	22	4	4	4	4
19	20143	n/m	9 (f)	> 128	> 128	> 128	> 128
20	20321	n/m	18	4	4	8	8
21	20509	n/m	18	4	4	8	8
22	20675	n/m	16	4	4	16	16
23	20891	n/m	16	4	4	16	16
24	20844(sm)	n/m	10 (f)	> 128	> 128	> 128	> 128
25	20844(lge)	n/m	R	> 128	> 128	> 128	> 128
26	20844	muc	21	4	4	8	8
27	21346	n/m	R	> 128	> 128	> 128	> 128
28	21483	n/m	16.5	4	4	16	16
29	213331	n/m	R	> 128	> 128	> 128	> 128
30	213332	n/m	17 (f)	> 128	> 128	> 128	> 128
31	ATCC 27853	n/m	20	4	4	16	16

[a] sm, small colony; lge, large colony

[b] f, fuzzy inhibitory zone; R, resistant. For colistin, the standards for susceptibility are resistant (≤ 8 mm), intermediate (9 - 10 mm), and sensitive (≥ 11 mm).

active than colistin methanesulphonate (geometric mean MIC = 8.2 mg/L) ($P < 0.001$).

The shading in Table 2.2 indicates the four strains used in time-kill and PAE experiments for colistin and colistin methanesulphonate.

Table 2.3 Summary of the susceptibility and MICs of the 31 strains of *P. aeruginosa* based on the micro- or macro-dilution method

Mucoid status	Susceptibility pattern	No.	Colistin	MIC range (mg/L)	Geometric mean MIC (mg/L)
All strains [a]	Susceptible	19	sulphate	1 - 4	3.2
			methanesulphonate	4 - 16	8.2
	Resistant	12	sulphate	> 128	> 128
			methanesulphonate	> 128	> 128
Mucoid	Susceptible	7	sulphate	1 - 4	2.9
			methanesulphonate	4 - 8	5.7
Non-mucoid [a]	Susceptible	12	sulphate	1 - 4	3.4
			methanesulphonate	4 - 16	9.7

[a] Including the type strain of *P. aeruginosa* ATCC 27853

2.3.2 Time-kill kinetics

In time-kill studies, both colistin and colistin methanesulphonate were bactericidal in a concentration-dependent manner (Figures 2.1 and 2.2). With colistin, bacteria became undetectable after 3 hr at all concentrations except 0.5×MIC. At the highest multiples of the MIC (i.e. 64×MIC), the bactericidal rate of colistin was so high that no bacteria could be detected even within 5 to 10 minutes for the three strains, except the non-mucoid strain 20223. At the same multiples of the MIC, colistin methanesulphonate was less rapidly bactericidal than colistin. However, it was still rapidly bactericidal at the highest concentrations, with counts falling to undetectable numbers in 1 to 4 hr at 16 - 64 times the MIC. Below these concentrations colistin methanesulphonate had failed to eliminate bacteria by 24 hr. The control laboratory strain, ATCC 27853, was an exception to this, being rapidly eliminated by colistin methanesulphonate at concentrations of 2 to 64 times the MIC.

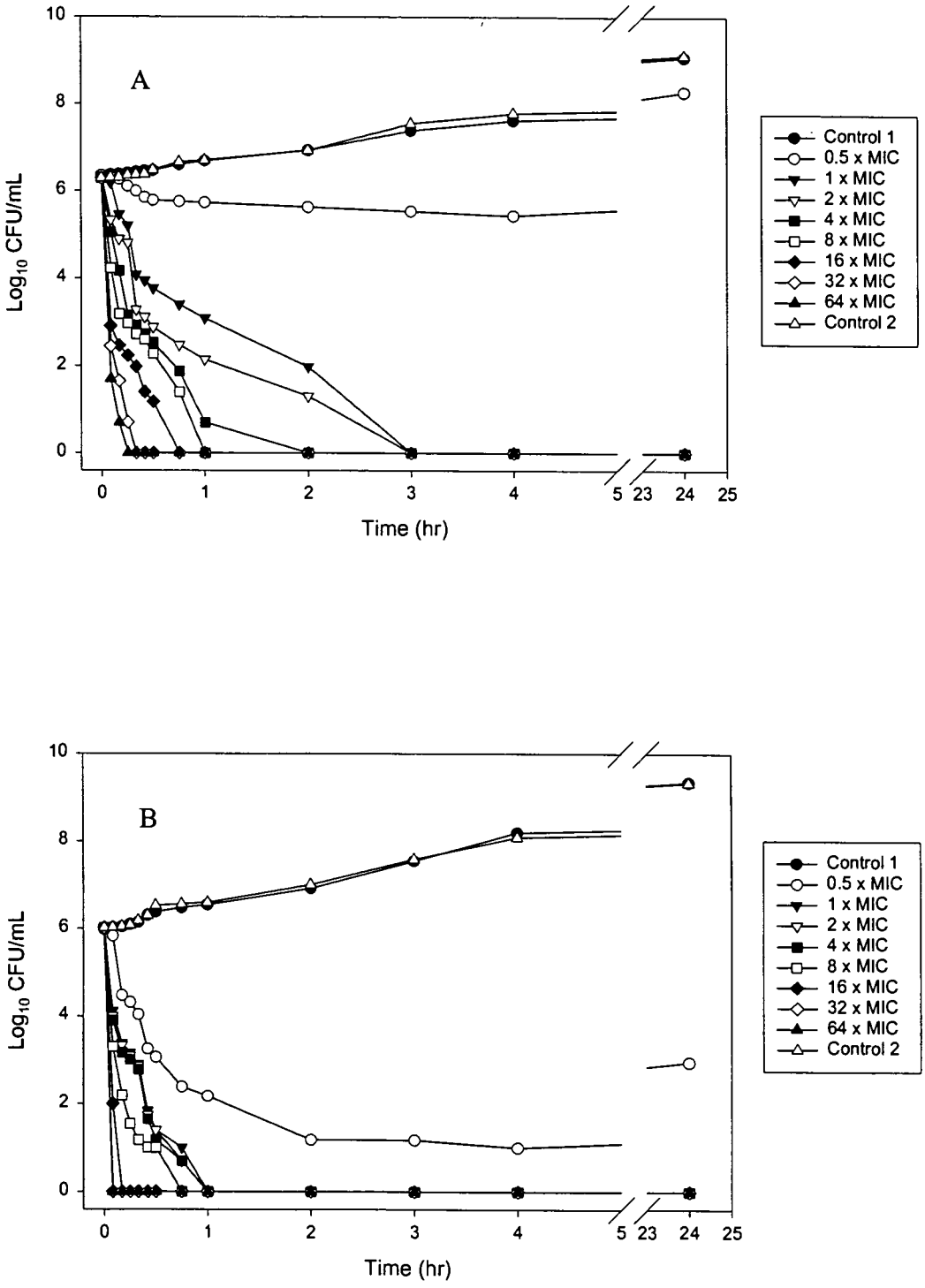

Figure 2.1 See remainder of figure and legend on following page.

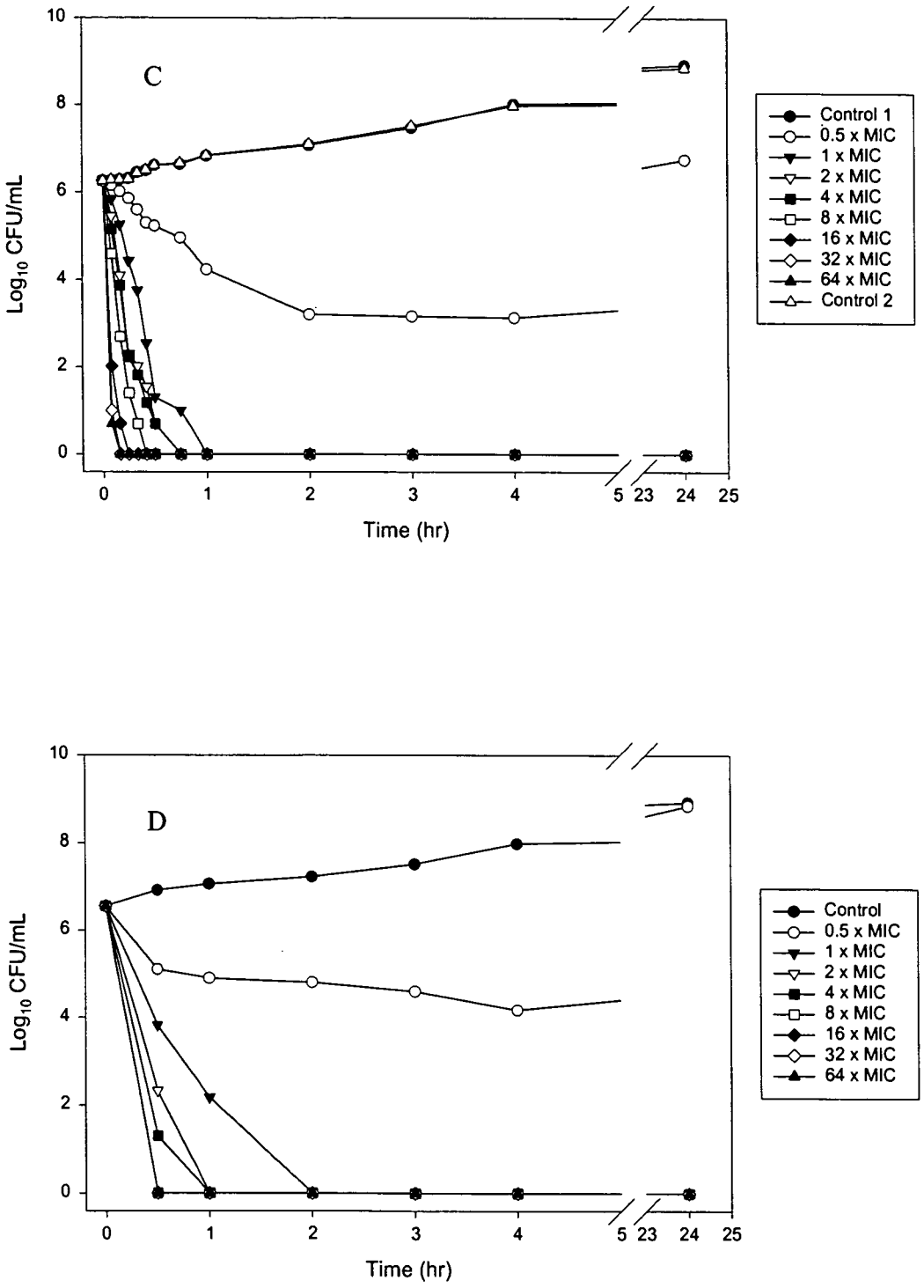

Figure 2.1 Time-kill curves of *P. aeruginosa* ATCC 27853 (A), 18982 muc (B), 19056 muc (C) and 20223 n/m (D) by colistin sulphate.

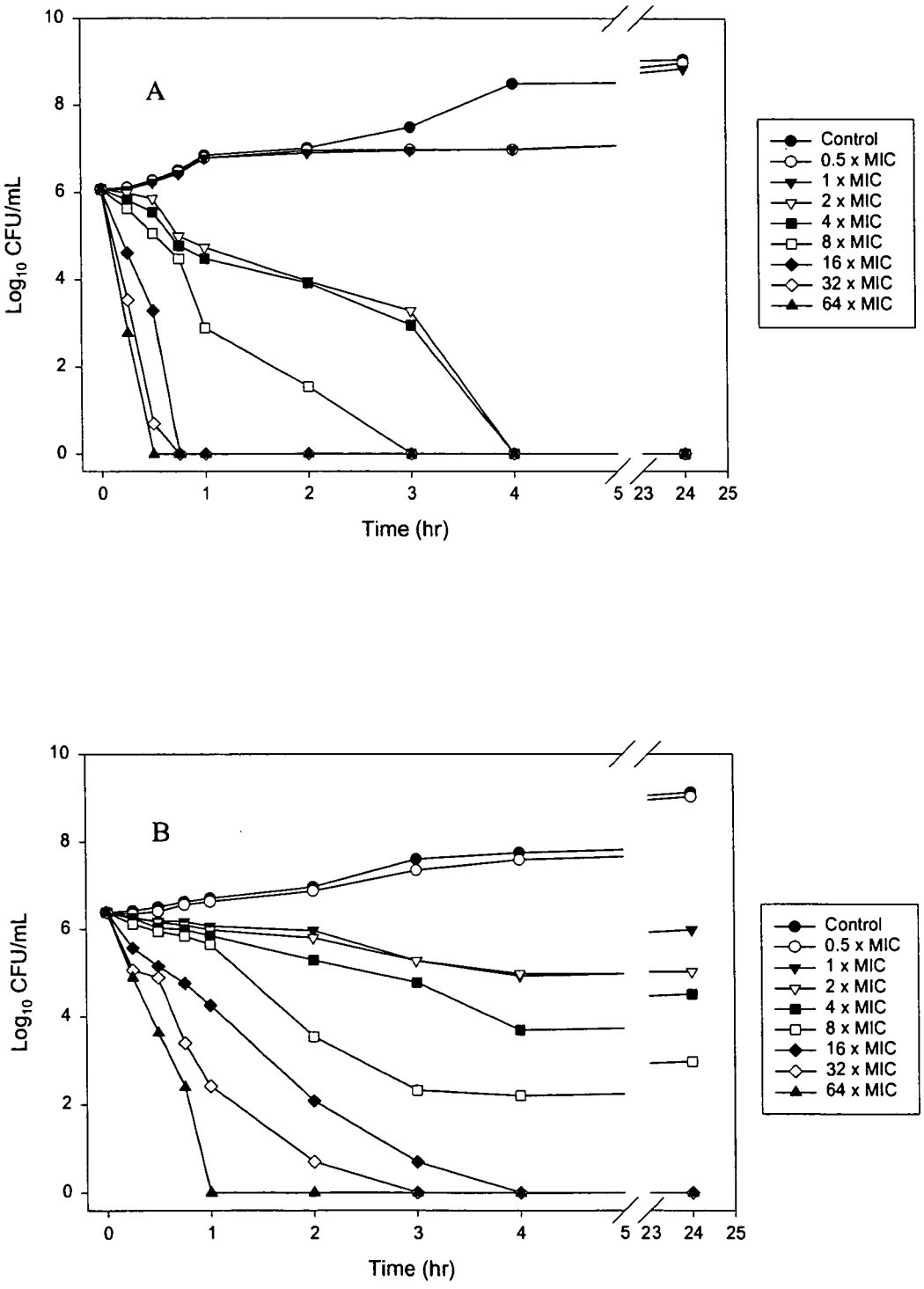

Figure 2.2 See remainder of figure and legend on following page.

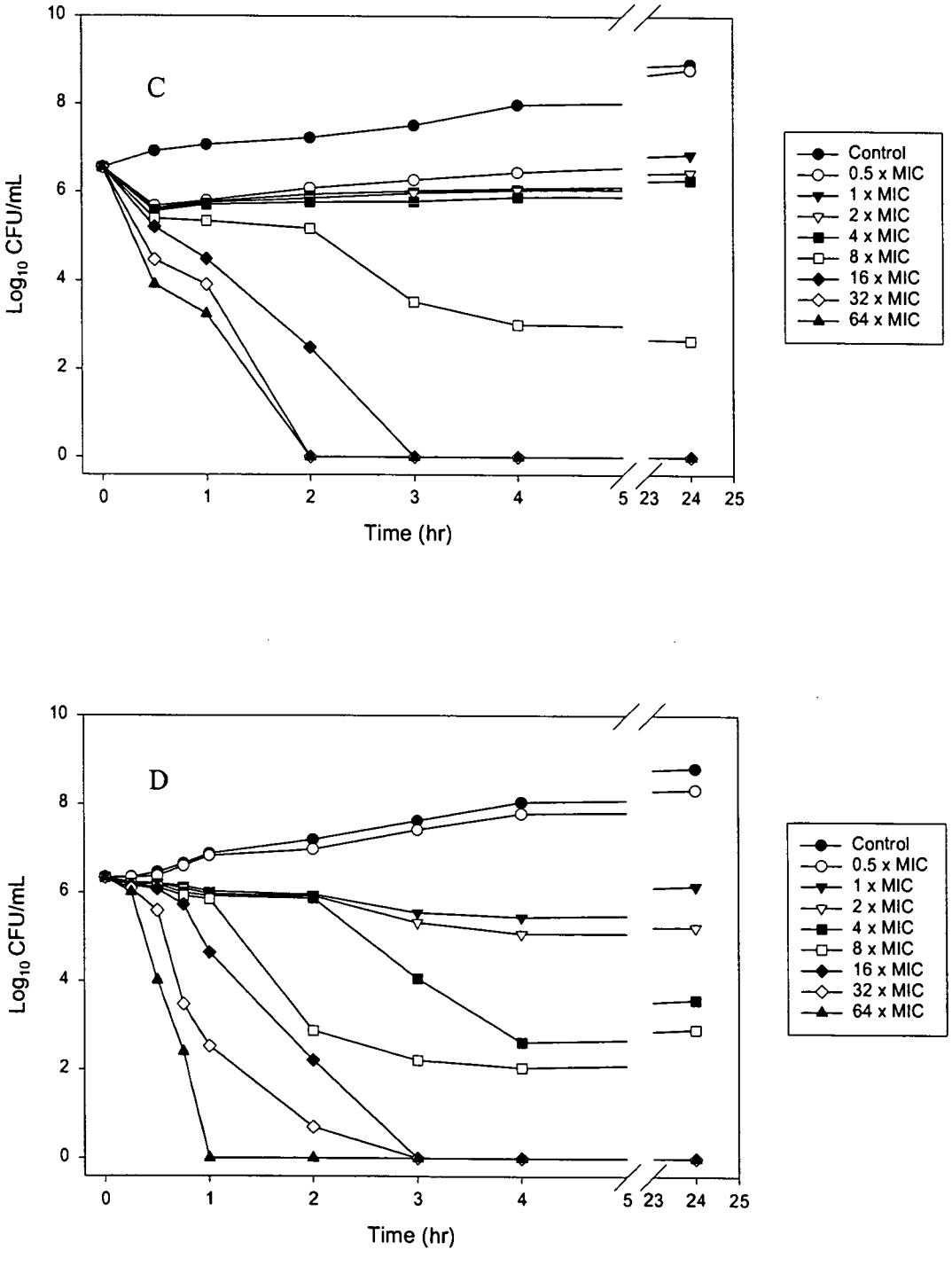

Figure 2.2 Time-kill curves of *P. aeruginosa* ATCC 27853 (A), 18982 muc (B), 19056 muc (C) and 20223 n/m (D) by colistin methanesulphonate.

Mean survival times ($MST_{240\ min}$) were calculated at different multiples of the MIC for both agents. $MST_{240\ min}$ values of the four strains for colistin ranged from 3.7 - 84 minutes at 0.5×MIC. At 64×MIC $MST_{240\ min}$ values ranged from < 0.001 to 0.003 minutes. For colistin methanesulphonate the $MST_{240\ min}$ ranges were 140 to 185 minutes at 0.5×MIC, and 0.002 to 7.4 minutes at 64×MIC. Results for all concentrations tested are shown in Figure 2.3. Both the abscissa and the ordinate of this figure were plotted on a logarithmic scale to clarify relationships between strains and drugs.

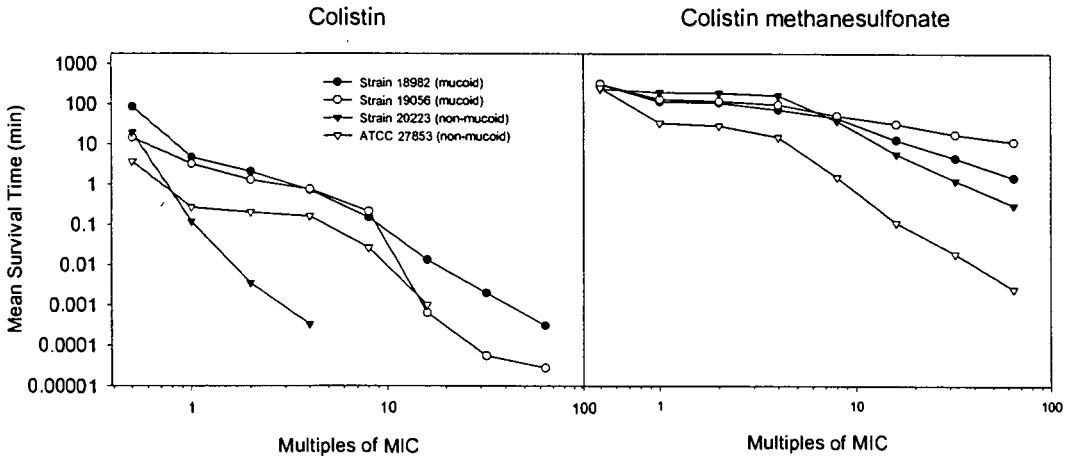

Figure 2.3 Bactericidal activity of colistin and colistin methanesulphonate against 4 strains of *Pseudomonas aeruginosa* as measured by $MST_{240\ min}$.

Again it is clear that colistin methanesulphonate is less bactericidal than colistin at the same multiples of the MIC. For instance, geometric mean $MST_{240\ min}$ values of the four strains at 4×MIC were 0.07 minutes for colistin versus 34.7 minutes for colistin methanesulphonate. Apart from a single non-mucoid strain which appeared to be highly susceptible to the bactericidal action of colistin, the three clinical strains had mean survival times similar to each other but longer than the standard laboratory strain at most concentrations of both agents. A minimum mean survival time was not observed for either agent, suggesting that higher concentrations would have resulted in even more rapid killing. There was also an apparent biphasic bactericidal action for both agents, with lower reductions in mean survival times over the 1 to 4-fold MIC range, and higher reductions at the 8 to 64-fold MIC range.

2.3.3 PAE

Figure 2.4 and Figure 2.5 illustrate the PAE of colistin and colistin methanesulphonate on the four strains.

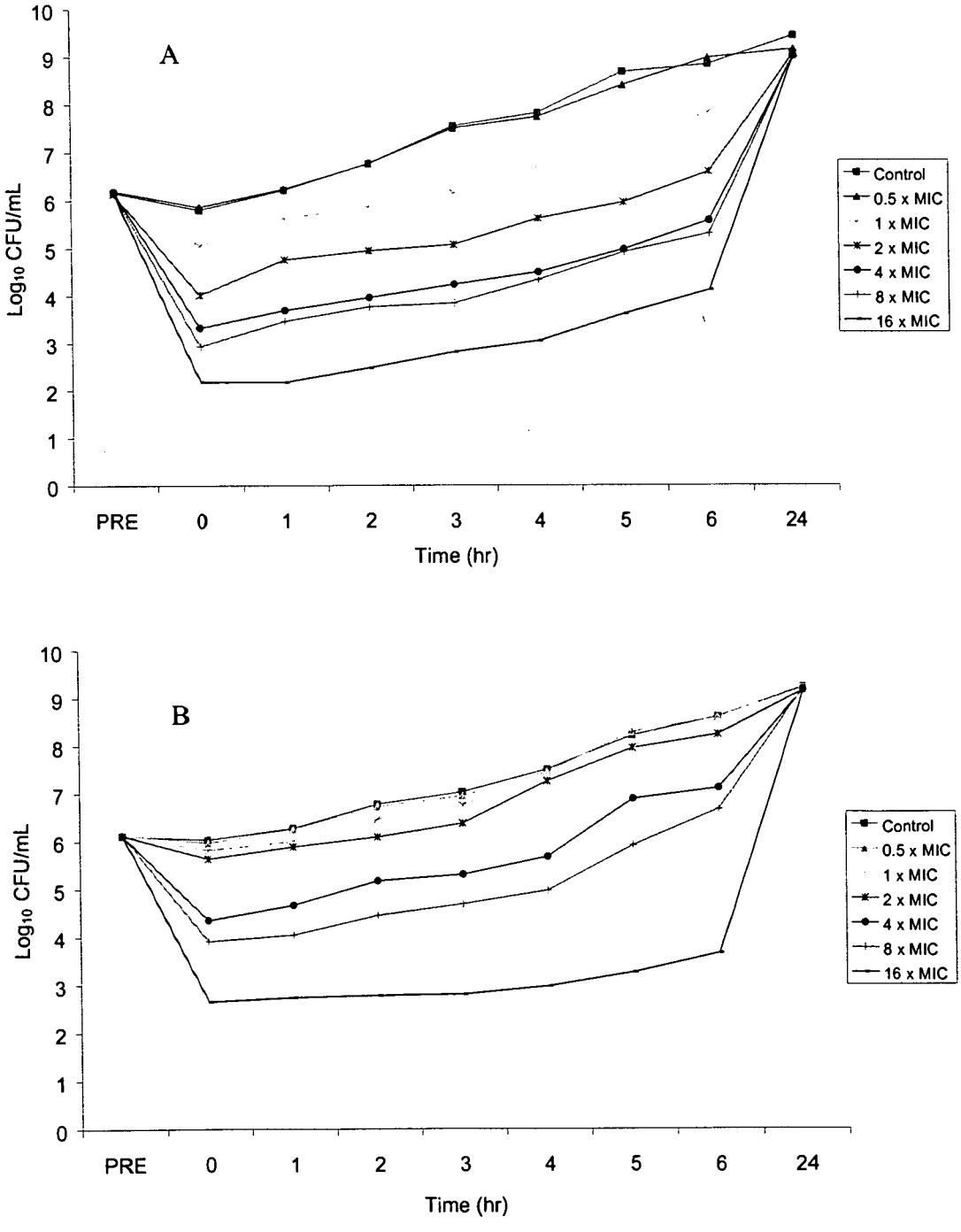

Figure 2.4 See remainder of figure and legend on following page.

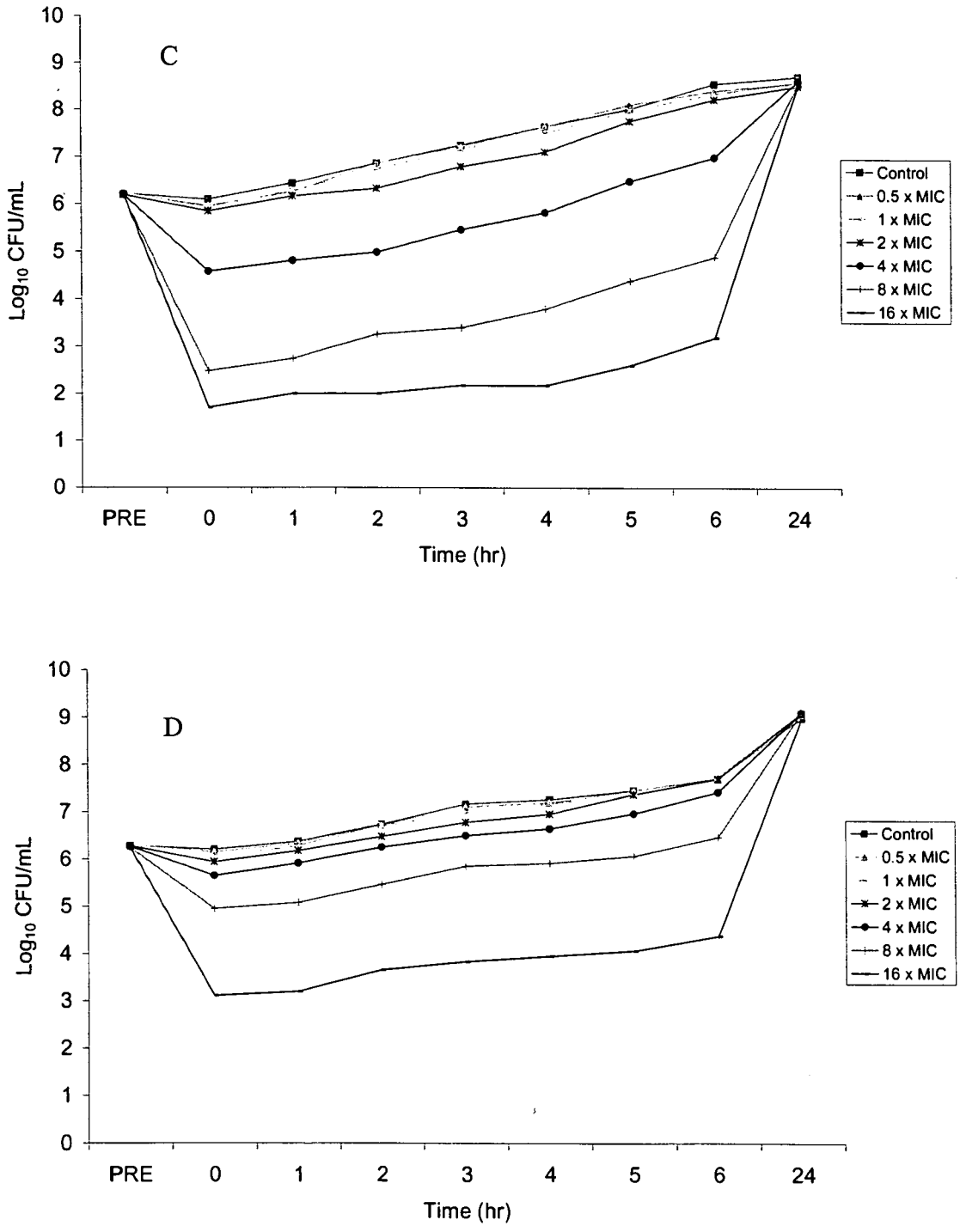

Figure 2.4 PAE of colistin against *P. aeruginosa* ATCC 27853 (A), 18982 muc (B), 19056 muc (C) and 20223 n/m (D).

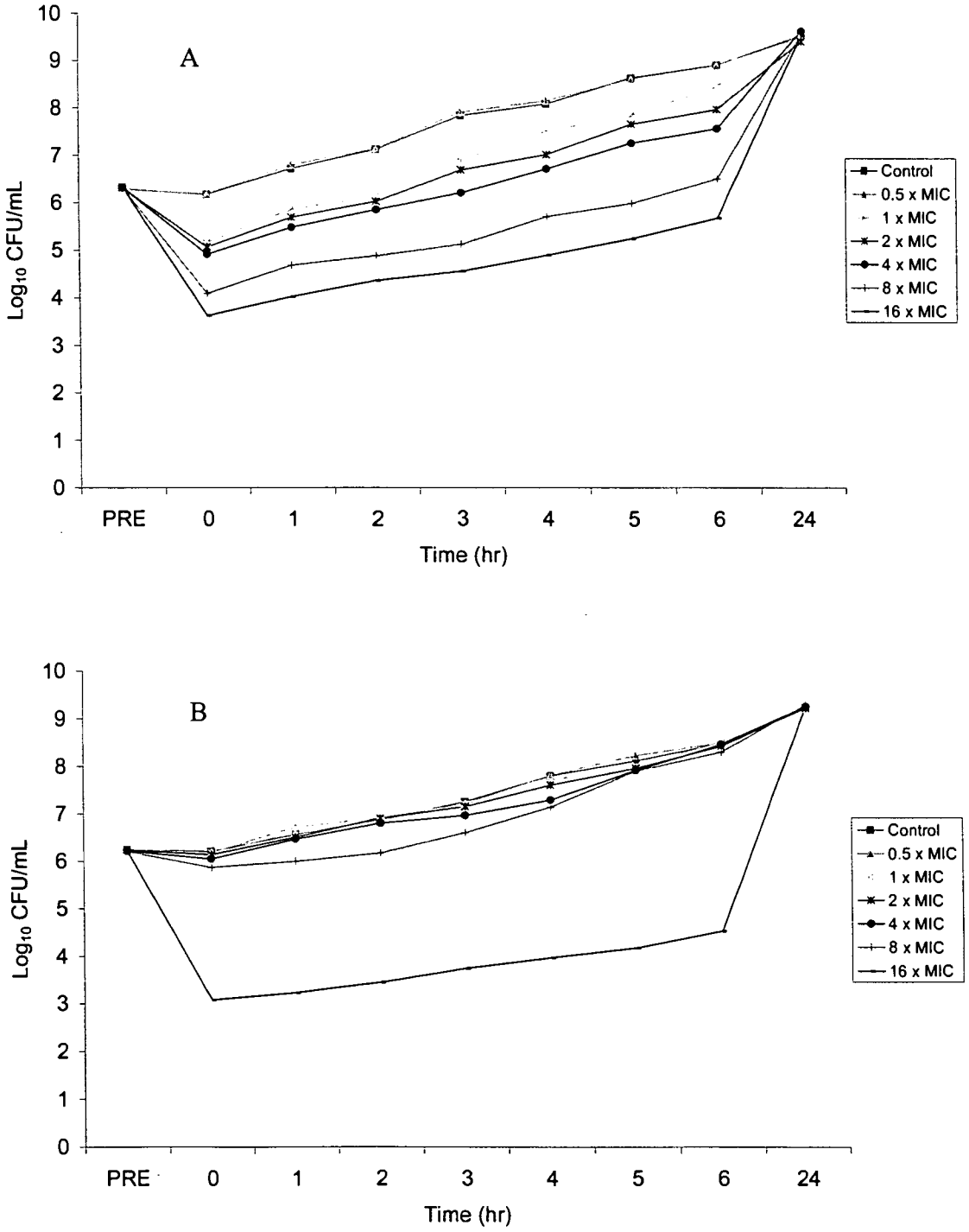

Figure 2.5 See remainder of figure and legend on following page.

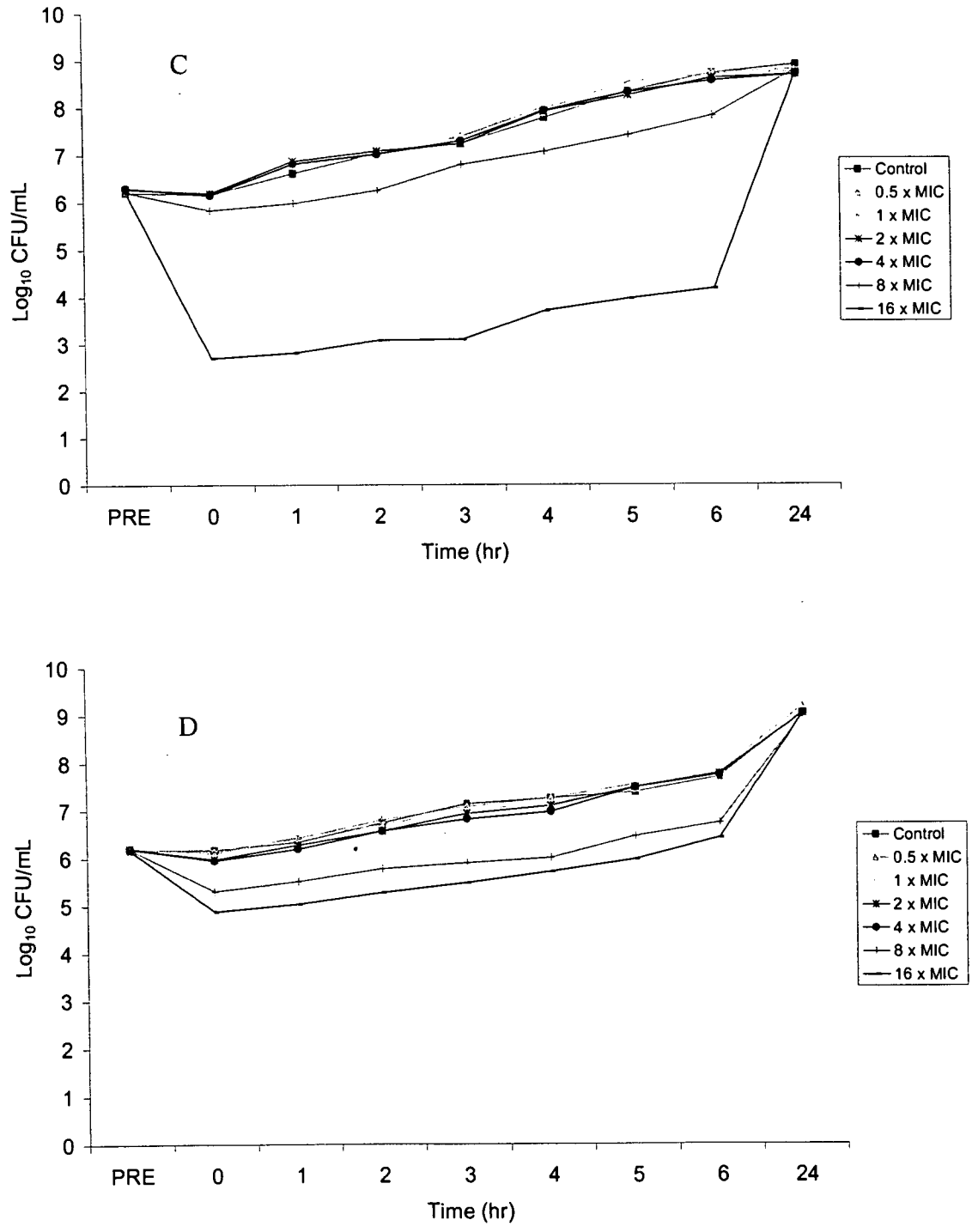

Figure 2.5 PAE of colistin methanesulphonate against *P. aeruginosa* ATCC 27853 (A), 18982 muc (B), 19056 muc (C) and 20223 n/m (D).

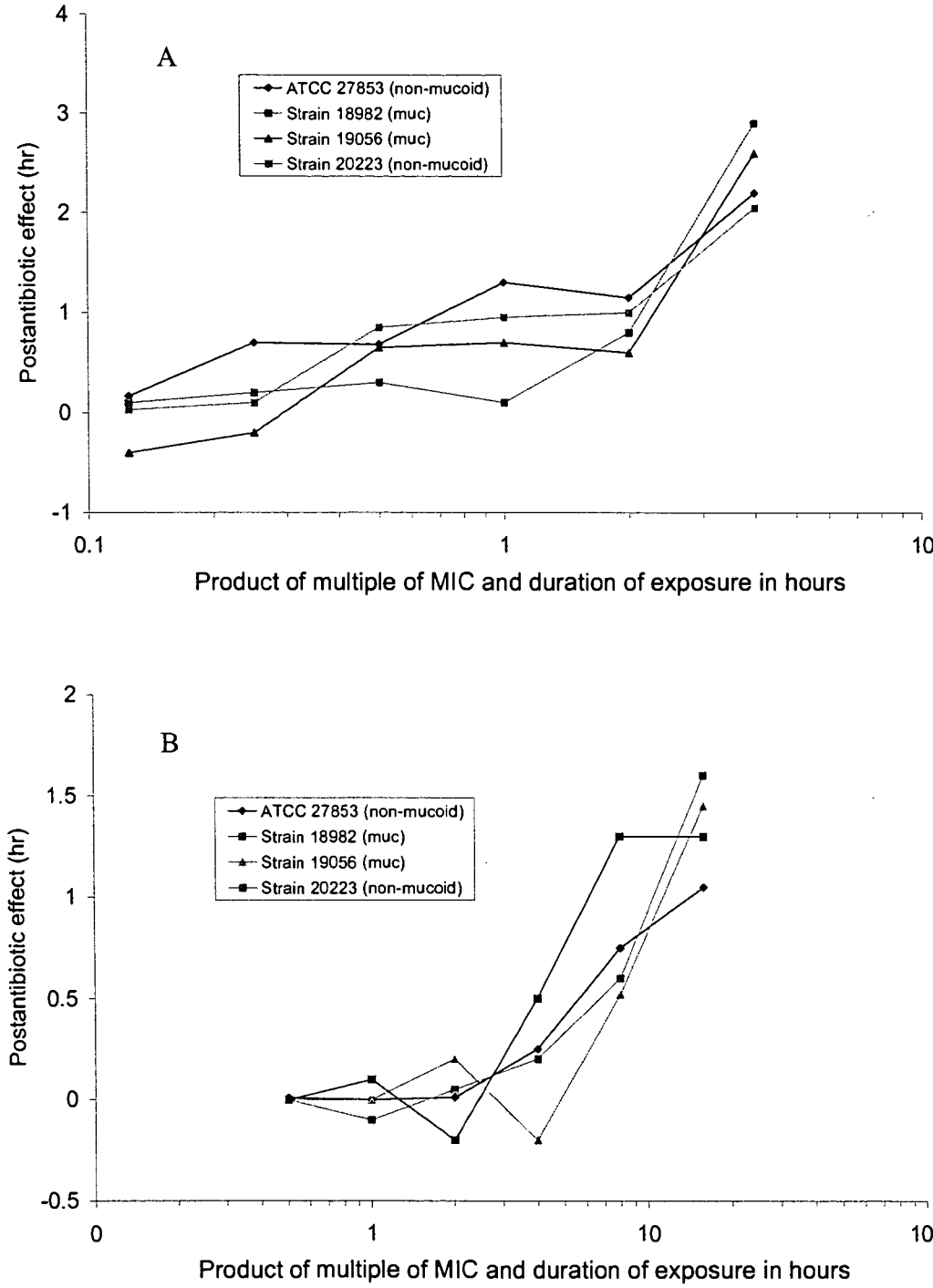

Figure 2.6 PAE of colistin (A) and colistin methanesulphonate (B) against four strains of *P. aeruginosa*.

The rapid bactericidal activity of colistin required that the exposure be limited to 15 minutes to ensure sufficient bacteria at the end of exposure for accurate determination of the PAE. For colistin methanesulphonate, a more conventional one-hour exposure was used prior to drug removal. Similar profiles were obtained with the four strains

when the effect of the overall exposure, taken as the product of concentration (as a multiple of MIC) and time of exposure on the PAE was examined (Figure 2.6).

Colistin and colistin methanesulphonate produced a significant PAE (greater than 1 hour) against the three strains only at the highest concentrations studied. However, it is clear that the maximum PAE was not achieved for either drug against any strain.

2.4 Discussion

The disk test is the most common method for examining the susceptibility of clinical isolates to antibiotics. When the susceptibility of 30 isolates of *P. aeruginosa* was tested against colistin by the disk test, five resistant strains showed fuzzy inhibitory zones. However, in the examination of MICs, the broth of these five strains became turbid without exception after 18 hr incubation irrespective of whether the macro- or micro-dilution method was used. Therefore, it was most likely that they were not pure clones.

Due to the small number of strains included in this study, the prevalence of resistance by *P. aeruginosa* was high compared to the percentage (approximately 19%) observed in the overall patient population at the Women's and Children's Hospital (unpublished observations). The higher level of resistance might result in part from the frequent use of inhaled colistin methanesulphonate in the CF clinic at the Women's and Children's Hospital. However, there was significant resistance to tobramycin (70%) and ticarcillin (44.8%), the principal antimicrobial agents used at the Women's and Children's Hospital for acute exacerbation of CF. Nevertheless, colistin remained as a promising candidate for the treatment of infections by *P. aeruginosa* in these patients.

Schwartz et al. investigated the *in vitro* antibacterial activity of colistin and colistin methanesulphonate in 112 strains representing 16 genera of bacteria (Schwartz *et al.*, 1960). For most of the strains of *Escherichia coli* (n = 17), *Klebsiella pneumoniae* (n = 5), *P. aeruginosa* (n = 3), *Salmonella* spp. (n = 11), and *Shigella* spp. (n = 5), colistin was three- to forty-times more active than colistin methanesulphonate (Schwartz *et al.*, 1960). The present study also demonstrated that colistin methanesulphonate (mean MIC = 8.2 mg/L) is less active than colistin (mean MIC = 3.2 mg/L). The difference in activity between the two compounds is important, as

colistin is often used for susceptibility testing *in vitro* whereas it is sodium colistin methanesulphonate that is most commonly used for clinical purposes.

The reported MICs of colistin and colistin methanesulphonate against different *P. aeruginosa* isolates vary in a broad range: 0.14 mg/L to 4.0 mg/L for colistin and 0.5 mg/L to 33.3 mg/L for colistin methanesulphonate (Catchpole et al., 1997; Schwartz et al., 1960). While different susceptibility of the clinical isolates is one of the main reasons for the various MICs, it should be noted that both colistin and colistin methanesulphonate are mixtures of different components. Even more problematically, colistin methanesulphonate is an undefined mixture of the mono, di-, tri-, tetra- and penta-methanesulphonate derivatives of colistin (Barnett *et al.*, 1964). The degree of sulphomethylation may well differ between manufacturers or batches, and the derivatives may completely or partially hydrolyse to colistin in solution (Barnett *et al.*, 1964; Beveridge *et al.*, 1967; McMillan *et al.*, 1969). Therefore, the antibacterial activity of colistin methanesulphonate is much more complex than colistin due to the hydrolysis products formed during the incubation.

In an attempt to quantify the bactericidal activity of colistin and colistin methanesulphonate in a way that could be used to directly compare the effects of different concentrations of antimicrobial agent, a model-independent parameter of killing, the mean survival time over 4 hr ($MST_{240\ min}$) was introduced (personal communication with Professor J. Turnidge, 1999). This parameter is based on statistical moments, and is analogous although not identical to the mean residence time (MRT) used in pharmacokinetics. It has the advantage over simple area-based methods for measuring bactericidal response in that it is not affected by variations in starting inoculum between experiments. $MST_{240\ min}$ is calculated from the bacterial concentrations (CFU/mL) observed in the time-kill curves by Equation 2.2, and was used to quantify the difference in bactericidal activity between colistin and colistin methanesulphonate. Figure 2.3 shows that MST was short for colistin sulphate, but much longer at comparative concentrations for colistin methanesulphonate.

In this study, colistin demonstrated very rapid concentration-dependent killing. Indeed, in order to obtain detailed information on the rapidity of onset of killing, sampling was required every 5 minutes in the first half-hour. For colistin methanesulphonate, killing was a little slower but still dependent on concentrations.

The rapidity of bacterial killing by colistin and colistin methanesulphonate is consistent with the report by Hancock et al. on peptide antibiotics (Hancock *et al.*, 1999). It was hypothesised that the rapid bactericidal activity was related to the permeabilising action on the cell membrane, following self-promoted uptake generated by the action of the drug on the outer membrane (Hancock *et al.*, 1999).

A 20 to 30 minute intravenous infusion of 2 mg/kg to 2.5 mg/kg of colistin methanesulphonate resulted in peak plasma concentrations of 6 - 15.5 mg/L (mean 9.6 mg/L) (Baines *et al.*, 1964). Higher values, 10 - 36 mg/L (mean 18.0 mg/L), have been observed with doses of 3 mg/kg infused over 5 minutes in patients with renal failure (Curitis et al., 1968). A recent study in patients with CF using higher doses of 1.8 mg/kg to 4.3 mg/kg (160 mg) 8-hourly (mean dose 8.8 mg/kg/day) showed a mean peak level of 12.3 mg/L (Conway *et al.*, 1997). Overall, the results from these studies and the current chapter suggest that, even without consideration of protein or tissue binding, peak concentrations were not much higher than the MICs (4 mg/L - 16 mg/L) observed in the current study with isolates of *P. aeruginosa* from patients with CF, and substantially less than the 16×MIC (64 mg/L - 256 mg/L) required for complete killing by colistin methanesulphonate within 24 hr. Precise concentrations achieved in infected sputum with inhaled colistin sulphate are unknown, but it is clear that they vary somewhat between lung regions (Gagnadoux *et al.*, 1996).

It should be noted that all the concentrations of colistin and/or colistin methanesulphonate in plasma in the previous studies mentioned above were obtained using microbiological assays. One of the main disadvantages of the microbiological assay is that the concentrations may be misleading because of the different antibacterial activities of colistin and colistin methanesulphonate present in plasma, and the continuous formation of colistin by hydrolysis of colistin methanesulphonate (as described in Chapter 5) during the 18 - 24 hr required for incubation. Therefore, to describe the pharmacokinetics of colistin and colistin methanesulphonate in humans and relate concentrations to pharmacodynamics effect, it is necessary to develop accurate and sensitive high-performance liquid chromatographic methods for their quantification separately in plasma, as described in Chapters 3 and 4.

The major objective of antimicrobial therapy is to provide an optimal amount of active antimicrobial agent at the site of infection. While MIC has hitherto been one of

the important factors determining dosing regimens of antimicrobial agents, PAE is another significant parameter in the pharmacodynamics of antimicrobials. It has a considerable influence on dosing regimens (Craig *et al.*, 1990). A theoretical rationale for intermittent or more continuous dosing of antimicrobials is based on the presence or absence of the PAE, respectively (Vogelman *et al.*, 1988b; Vogelman *et al.*, 1985). If the antimicrobial-microorganism combination exhibits a long PAE, an intermittent dosing regimen may be as efficacious as a continuous dosing regimen because growth of the microorganism is prevented, despite concentrations of antimicrobial agent falling below the MIC. Therefore, determination of the PAE does offer important information on the pharmacodynamics of antimicrobials that MICs and pharmacokinetics alone are not able to provide (Vogelman *et al.*, 1988a). The presence of a PAE is helpful in determining a dosing interval, which is equal to the T > MIC plus the PAE period. Hence, the PAE is supportive of a less frequent dosing regimen for some antimicrobials, without loss of efficacy and a potential for the reduced toxicity to the patient.

Both colistin and colistin methanesulphonate produced a moderate PAE at higher concentrations. Unfortunately, due to the rapid killing at the higher concentrations, a maximum PAE was not observed, and thus maximum values might well be substantially higher. The product of the duration of drug exposure and multiple of MIC, rather than duration of exposure or concentration alone, was shown to correlate best with the duration of the PAE with beta-lactams (Munckhof *et al.*, 1997). The relationships in Figure 2.6 were quite similar with that between the PAE of imipenem against *E. coli* ATCC 25922 and the product of the duration of drug exposure and multiple of MIC predicted by the exposure-response model (Munckhof *et al.*, 1997). Extensive data collected by these authors were used to predict the magnitude of the PAE from the product of concentration and duration of exposure, rather than either of the two alone. To make such predictions with colistin and colistin methanesulphonate will require more extensive data than have been obtained in the current study.

Colistin methanesulphonate produced a significant PAE only at concentrations higher than 16×MIC. Since the MICs of colistin methanesulphonate against *P aeruginosa* were relatively high, with current dosing schedules to produce a PAE might be difficult to achieve the necessarily high concentrations in plasma, even without

considering the effect of plasma binding. However, colistin formed by hydrolysis of colistin methanesulphonate *in vivo* might contribute to the killing of susceptible bacteria, with a much higher bactericidal activity. Nevertheless, potential toxicities with higher doses would be another limiting factor during chemotherapy with colistin methanesulphonate. More extensive trials in animals and humans will be helpful in elucidating the full significance of the PAE and its impact on dosage design (Vogelman *et al.*, 1988b).

Colistin methanesulphonate showed a concentration-dependent killing. However, it was difficult to eliminate the four examined strains of *P. aeruginosa* completely at 1×MIC. Therefore, the postantibiotic sub-MIC effect might not be an important pharmacodynamic parameter for colistin methanesulphonate. On the contrary, 1×MIC of colistin was demonstrated to have significant killing in all four tested strains. Hence, the postantibiotic sub-MIC effect of colistin against *P. aeruginosa* is worthy of further exploration.

2.5 Conclusion

It is clear from this study that colistin methanesulphonate shows lower potency than colistin, even when corrected for MIC values, as demonstrated in the time-kill and PAE experiments. Overall, the findings in this chapter suggest that doses higher than the usually recommended 2 - 3 mg/kg every 12-hr may be necessary to maximise the efficacy of colistin methanesulphonate when given intravenously for the treatment of acute exacerbations of *P. aeruginosa* infection in CF patients. Clearly, this must be weighed against the potential for increased toxicity.

Further work will be needed to ascertain the significance of MIC, killing-time and PAE to the successful therapy of infections by *P. aeruginosa* in CF patients. As a contribution to this work, subsequent chapters describe specific methods for measuring concentrations of colistin and colistin methanesulphonate in plasma and urine and an examination of the pharmacokinetics of the two compounds, firstly in rats and then humans.

Chapter 3 High-performance liquid chromatographic determination of colistin

3.1 Introduction

As reviewed in Chapter 1, numerous methods for the assay of colistin have been developed, based on microbiological (Leroy et al., 1989), thin-layer chromatographic (TLC) (Thomas et al., 1978; Thomas et al., 1980), immunological (Kitagawa et al., 1985), capillary electrophoretic (Kristensen et al., 1993) and HPLC methods (Decolin et al., 1997; Elverdam et al., 1981; Kimura et al., 1981; Le Brun et al., 2000b; Orwa et al., 2000; Wall, 1981).

The TLC (Thomas et al., 1978; Thomas et al., 1980), capillary electrophoretic method (Kristensen et al., 1993) and majority of HPLC methods (Elverdam et al., 1981; Kimura et al., 1981; Orwa et al., 2000; Wall, 1981) have been applied to the separation of the components of colistin in batches of the raw material. Although the immunological method could be applied to biological samples, preparation of colistin immunogen was complex and the assay could not quantify colistin A and colistin B separately. Microbiological methods have been widely used for clinical monitoring of colistin concentrations. Unfortunately, the methods lack specificity, particularly if there are any other co-administered antibiotics in samples that are active against the test strain.

The analysis of colistin in biological fluids by HPLC poses problems because it has very weak ultraviolet absorption and no native fluorescence. Hence, it can not be quantified in biological fluids with high sensitivity by conventional detection techniques without derivatization with UV-absorbing or fluorescent reagents. When this thesis was commenced, and in the time since, only two HPLC methods have been reported for the assay of colistin in biological samples, and both have used *ortho*-phthalaldehyde (OPA) [643-79-8] (Figure 3.1) as the derivatizing reagent (Decolin et al., 1997; Le Brun et al., 2000b). The limitations of these methods have been reviewed in Section 1.3.6.1.1.

In general, pre-column derivatization with fluorescent reagents allows for a simple and sensitive detection of compounds, such as colistin, which lack an ultraviolet

chromophore or fluorophore. Although there are many derivatizing reagents for amines, perhaps the four most commonly used are OPA, 9-fluorenylmethyl chloroformate (FMOC-Cl) [28920-43-6] (Figure 3.2), phenyl isothiocyanate (PITC) [103-72-0] and 1-dimethylaminonaphthalene-5-sulphonyl chloride (Dansyl-Cl) [605-65-2] (Furst et al., 1990).

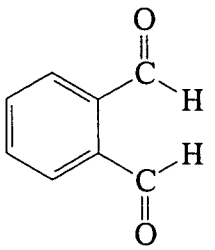

Figure 3.1 Structure of OPA

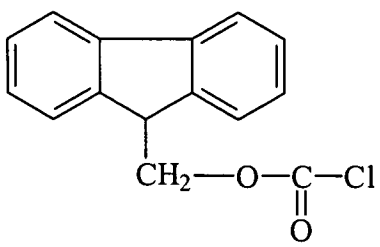

Figure 3.2 Structure of 9-fluorenylmethyl chloroformate (FMOC-Cl)

OPA is probably the most commonly used for the assay of primary amines, with derivatization followed by reversed-phase HPLC (RP-HPLC) (Roth, 1971). However, this method has several shortcomings: (a) OPA only reacts with primary amines; (b) the OPA reagent is not stable and has to be prepared freshly; and, (c) the instability of OPA derivatives limits reproducibility and proper quantification and, hence, automated on-line derivatization is usually required to ensure better control over reaction conditions (Furst et al., 1990).

PITC, also known as the Edman's reagent, is another useful derivatization reagent for the determination of primary and secondary amines with RP-HPLC (Heinrikson et al., 1984). UV detection is employed but the sensitivity of the method using PITC as the derivatizing agent is less than those with the other three fluorescent derivatizating reagents listed above. Furthermore, a noticeable reduction in column lifetime is a serious disadvantage when PITC is used (Furst et al., 1990).

Dansyl-Cl is another well-known fluorogenic reagent for the derivatization of primary and secondary amines (Seiler, 1970). The derivatization reaction requires a relatively longer time than those with the other three reagents, 35 - 50 min at ambient temperature. Furthermore, the sensitivity is lower than that with OPA and FMOC-Cl (Furst et al., 1990).

The ideal derivatization reagent should react with both primary and secondary amines rapidly and quantitatively under mild conditions, without being disturbed by sample matrix components such as salts, and produce a single stable derivative. The derivative should be detectable with high sensitivity and the reagent itself or its degradation products should not interfere chromatographically with quantification of the derivative (Bank et al., 1996).

FMOC-Cl meets most of these criteria. It has been shown to be an ideal reagent for fluorescent derivatization of primary and secondary amines (Einarsson et al., 1983). The reaction is rapid and quantitative under mild conditions (Figure 3.3), and the derivatives are stable at ambient temperature for several days (Furst et al., 1990). The resulting fluorescent carbamates may be detected at picomole level and show excellent chromatographic behavior with RP-HPLC (Furst et al., 1990). The disadvantage of derivatization with FMOC-Cl is the excess of strongly fluorescent reagent and its hydrolysis product, FMOC-OH, which may affect quantification by co-eluting with the derivatives of interest (Furst et al., 1990). However, this can often be overcome by extraction of the reagent and its hydrolysis product with organic solvents if there is a significant difference in polarity between the FMOC derivative and FMOC-Cl or FMOC-OH.

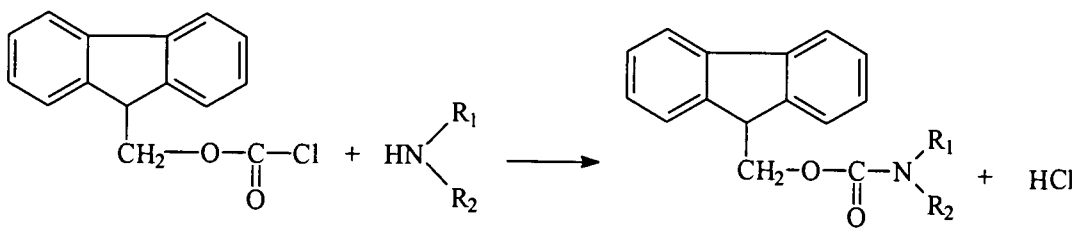

Figure 3.3 Reaction of amines with FMOC-Cl

In the present study, a new method was described in which colistin was extracted from plasma onto a solid-phase extraction (SPE) C_{18} cartridge and derivatized in the

same cartridge with FMOC-Cl. Elution from the cartridge was followed by HPLC analysis of the fluorescent derivatives of the two main components, colistin A and colistin B. The method is simple, sensitive, accurate and reproducible.

3.2 Materials and Methods

3.2.1 Apparatus

The HPLC system comprised a LC-10AS pump, a SIL-10AD*vp* autoinjector (Shimadzu, Kyoto, Japan) and either a SPD-6A UV spectrophotometric detector (Shimadzu, Kyoto, Japan) or a 470 scanning fluorescence detector (Waters, Milford, MA, USA) connected to a multi-instrument data acquisition and data process system (Maxima 820, Waters, MA, USA). Mass spectral analysis was performed with a Perkin-Elmer SCIEX API 365 tandem mass spectrometer system (MDS SCIEX, Concord, Ontario, Canada) with an ion-spray source connected to a Harvard syringe pump. All the analyses were carried out at ambient temperature (23 ± 2°C). Solid-phase extraction (SPE) cartridges (C_{18}, 100 mg) were supplied by J. T. Baker Inc. (Phillipsburg, NJ, USA) and Waters (Milford, MA, USA).

3.2.2 Chemicals and reagents

Colistin sulphate and 9-fluorenylmethyl chloroformate (FMOC-Cl) were purchased from Sigma (St. Louis, MO, USA). Netilmicin sulphate was obtained from Schering-Plough (Madison, NJ, USA), ceftazidime from Glaxo Wellcome (Boronia, Victoria, Australia), meropenem from Zeneca (Macclesfield, Cheshire, UK), aztreonam from Bristol-Myers Squibb (Noble Park, Victoria, Australia), piperacillin from Lederle Laboratories (Baulkham Hills, NSW, Australia), ciprofloxacin from Bayer AG (Pymble, NSW, Australia), tobramycin from Eli Lilly (West Ryde, NSW, Australia), ticarcillin from SmithKline Beecham (Dandenong, Victoria, Australia), and triethylamine (HPLC grade) from Prolabo (Paris, France). Trichloroacetic acid, boric acid, sodium hydrogen carbonate and sodium hydroxide of analytical grade, and acetonitrile, methanol, tetrahydrofuran and acetone of HPLC grade were all obtained from BDH Laboratory Supplies (Poole, England). Water was purified by a Millipore Milli-Q system (Millipore Corp., Bedford, MA, USA).

The carbonate buffer (1%, w/w, pH 10) and borate buffer (0.1 M, pH 8.9) were prepared by dissolving the appropriate amount of sodium hydrogen carbonate or boric acid in water and adjusting pH with sodium hydroxide (10%, w/w). All solutions were stored at 4°C.

3.2.3 Identification of the main components of colistin

3.2.3.1 Separation of the main components of colistin by HPLC

An aliquot (10 µL) of colistin sulphate in water (1 mg/mL) was chromatographed on a 250 × 4.6 mm ID steel column prepacked with 5 µm Ultrasphere C_{18} (Beckman, Berkeley, CA, USA) with a mobile phase of acetonitrile-35 mM triethylamine (pH 2.5, adjusted with 85% phosphoric acid) (17:83, v/v) (Decolin et al., 1997). The flow was 1.5 mL/min and detection was by UV absorption at 210 nm.

3.2.3.2 ESI-MS analysis of the two main components of colistin

The eluate fractions from above corresponding to the two dominant peaks were collected and freeze dried overnight (Virtis Sentry ^{TM}, NY, USA). Desalting of the collected HPLC eluates was performed on a SPE C_{18} cartridge (Waters) previously conditioned with 1 mL of methanol and equilibrated with 1 mL of water. After loading the eluate fractions reconstituted in 3 mL water, the cartridge was washed with 5 mL of water, followed by elution of the components with 1 mL of acetonitrile. Formic acid (0.25%, w/w, 1 mL) was added prior to mass spectral analysis. The electrospray mass spectrometer was operated in a positive ionization mode and data were collected and processed by Multiview 1.4 operating on a Macintosh G3 computer system.

3.2.4 Derivatization of colistin with FMOC-Cl in solution

In a 10-mL polypropylene test tube (Sarstedt, SA, Australia), colistin sulphate (1 mg/mL, 10 µL) was mixed with 500 µL borate buffer (0.1 M, pH 8.9) and 500 µL FMOC-Cl (1.0 mM in acetonitrile). After being vortex-mixed (40 rpm) for 10 min, excess FMOC-Cl was reacted with glycine (0.1 M, 30 µL) for a further 2 min. Boric acid solution (0.2 M, 600 µL) was added and the mixture vortexed for 2 min. An aliquot (10 µL) was chromatographed on the Ultrasphere C_{18} column with a mobile

phase of acetonitrile-water (87:13, v/v) at a flow rate of 1 mL/min. Fluorescence was measured at an excitation wavelength of 260 nm and an emission wavelength of 315 nm.

In experiments conducted to optimize the conditions for derivatization of colistin in solution, colistin sulphate (0.1 mg/mL, 50 µL) was reacted with FMOC-Cl as described above. The pH and reaction time were varied.

3.2.5 Adsorption of the derivatives of colistin onto the HPLC autoinjector insert

Investigations were performed to determine the suitable insert into which the solution of derivatized colistin was placed prior to injection into the HPLC. Colistin sulphate (1 mg/mL, 10 µL) was reacted with FMOC-Cl as described in Section 3.2.4. Aliquots (10 µL) of the derivatives were placed in different inserts, including a Supelco polypropylene insert (Bellefonte, PA, USA), Sun51 Glass insert (Sun International, Wilmington, NC, USA), and Alltech polypropylene insert (Baulkham Hills, NSW, Australia), and chromatographed twice consecutively (vide supra), the first injection being immediately after addition of the solution into the insert.

3.2.6 Confirmation of identity of the colistin derivatives

To identify the peaks from chromatographic analysis of the FMOC derivatives, the collected eluate, corresponding to each of the two main components of colistin (Sections 3.2.3.1 and 3.2.3.2), was reacted with FMOC-Cl. In a 10 mL polypropylene test tube (Sarstedt), 20 µL of the eluate was mixed with 500 µL carbonate buffer and 500 µL FMOC-Cl (1.0 mM in acetonitrile), and left to react for 10 min. Excess FMOC-Cl was reacted with 30 µL glycine (0.1 M) and, after a further 2 min, boric acid solution (0.2 M, 600 µL) was added. An aliquot (10 µL) of the solution was chromatographed (Section 3.2.4).

3.2.7 HPLC determination of colistin in human plasma

3.2.7.1 Sample pre-treatment

The internal standard (20 µL of 5 mg/L netilmicin sulphate in water) was mixed with 250 µL drug-free human plasma in a 1.5-mL polypropylene tube (Techno-plas,

Adelaide, SA, Australia). After adding 50 μL of methanol-10% trichloroacetic acid (50:50, v/v), the mixture was vortex-mixed for 1 min, and centrifuged (1,000 *g*, 4°C) for 10 min. The supernatant was transferred into another 1.5-mL polypropylene tube and mixed with 10 μL of 1.0 M sodium hydroxide. After being vortex-mixed for 1 min, 250 μL of methanol-0.01 M hydrochloric acid (50:50, v/v) was added followed by vortex-mixing for 1 min. The final solution was delivered to the conditioned SPE cartridge.

3.2.7.2 Derivatization in solid-phase extraction cartridges

SPE C_{18} cartridges (J. T. Baker Inc.) were conditioned with 1 mL of methanol, and equilibrated with 1 mL of carbonate buffer (pH 10.0). These steps were performed using a vacuum manifold (Supelco Visiprep 24 ^{TM}, Bellefonate, PA, USA) without allowing the cartridges to run dry. All of the final solution from the plasma pretreatment (section 3.2.7.1) was transferred to a cartridge. After washing the cartridge with 1 mL carbonate buffer, 30 μL FMOC-Cl (100 mM in acetonitrile) was added. Following 10 min of reaction, the cartridge was dried by drawing air through under vacuum. The derivatives were eluted with 900 μL acetone, and the eluate mixed with 600 μL boric acid solution (0.2 M). After being vortex-mixed for 2 min, 50 μL was chromatographed on the Ultrasphere C_{18} column (Section 3.2.3.1) with a mobile phase of acetonitrile-tetrahydrofuran-water (87:4:13, v/v) at a flow rate of 1.0 mL/min. Fluorescence was measured at an emission wavelength of 315 nm following excitation at 260 nm.

3.2.7.3 Stability of the derivatives of colistin

Spiked blank human plasma (250 μL), containing 1 mg/L colistin, was mixed with netilmicin (20 μL, 5 μg/mL) in a 1.5-mL polypropylene tube (Techno-plas). All the procedures of pre-treatment and derivatization were performed as above (Sections 3.2.7.1 and 3.2.7.2). An aliquot (50 μL) of the final solution was chromatographed (Section 3.2.7.2) immediately and after storage for 1, 2, 3, 5, 10 and 14 days at ambient temperature (23 ± 2°C).

3.2.7.4 Specificity

Seven antibiotics, ceftazidime, meropenem, aztreonam, piperacillin, ciprofloxacin, tobramycin and ticarcillin, which are commonly co-administered during therapy with colistin, were used to investigate the specificity of the HPLC method. These antibiotics (at a concentration of 8.0 mg/L) were individually reacted with FMOC-Cl and chromatographed as described for colistin in Sections 3.2.7.1 and 3.2.7.2.

3.2.7.5 Linearity, reproducibility and accuracy

Appropriate volumes of three working standards of colistin sulphate in water (1.0, 10 and 50 mg/L) were used to prepare calibration standards in drug-free human plasma containing colistin sulphate at concentrations of 0.10, 0.40, 0.80, 1.0, 2.0, 3.0, and 4.0 mg/L. A linear calibration curve was constructed from the relationship between the ratios of the summed peak areas of colistin A and colistin B to that of netilmicin (internal standard) and the concentrations of colistin sulphate. Reproducibility and accuracy were assessed by (1) the intra-day assay with six consecutive analyses of quality control (QC) samples containing 0.20 mg/L and 2.0 mg/L colistin sulphate in human plasma (the QC samples were prepared independently), and (2) the inter-day assay with three consecutive analyses of the same QC samples on separate days. The limit of quantification (LOQ) was determined by measuring the concentrations of colistin sulphate in six samples of human plasma prepared independently from the calibration standards at a concentration of 0.10 mg/L.

3.3 Results

3.3.1 Identification of the main components of colistin

When colistin was dissolved in water and an ultraviolet spectrum recorded, there was no absorption at 270 nm and above, and only one broad peak near 210 nm (Figure 3.4). Therefore, 210 nm was chosen as the detection wavelength during the separation and purification of colistin A and colistin B by HPLC.

Using an Ultrasphere C_{18} column and a mobile phase of acetonitrile-35 mM triethylamine (pH 2.5) (17:83, v/v), colistin was resolved into two main components (Figure 3.5).

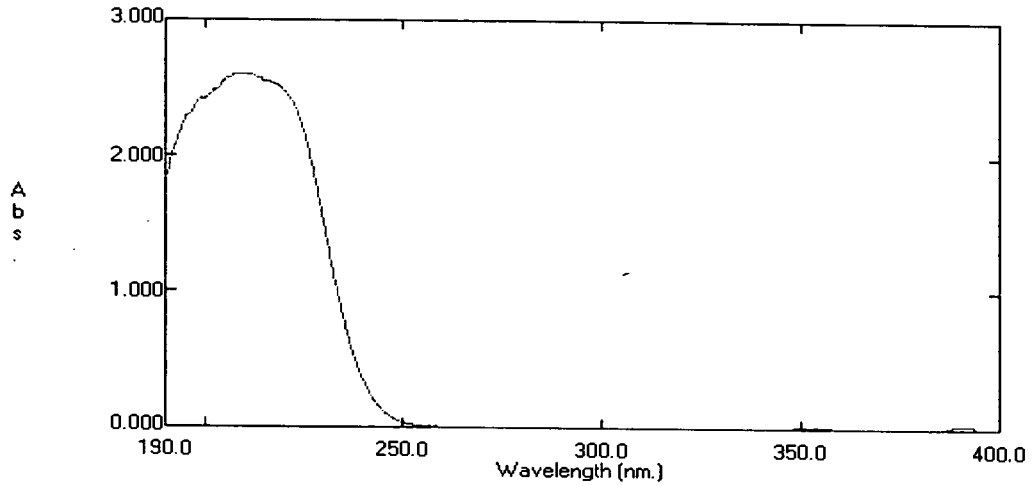

Figure 3.4 UV spectrum of colistin sulphate in water

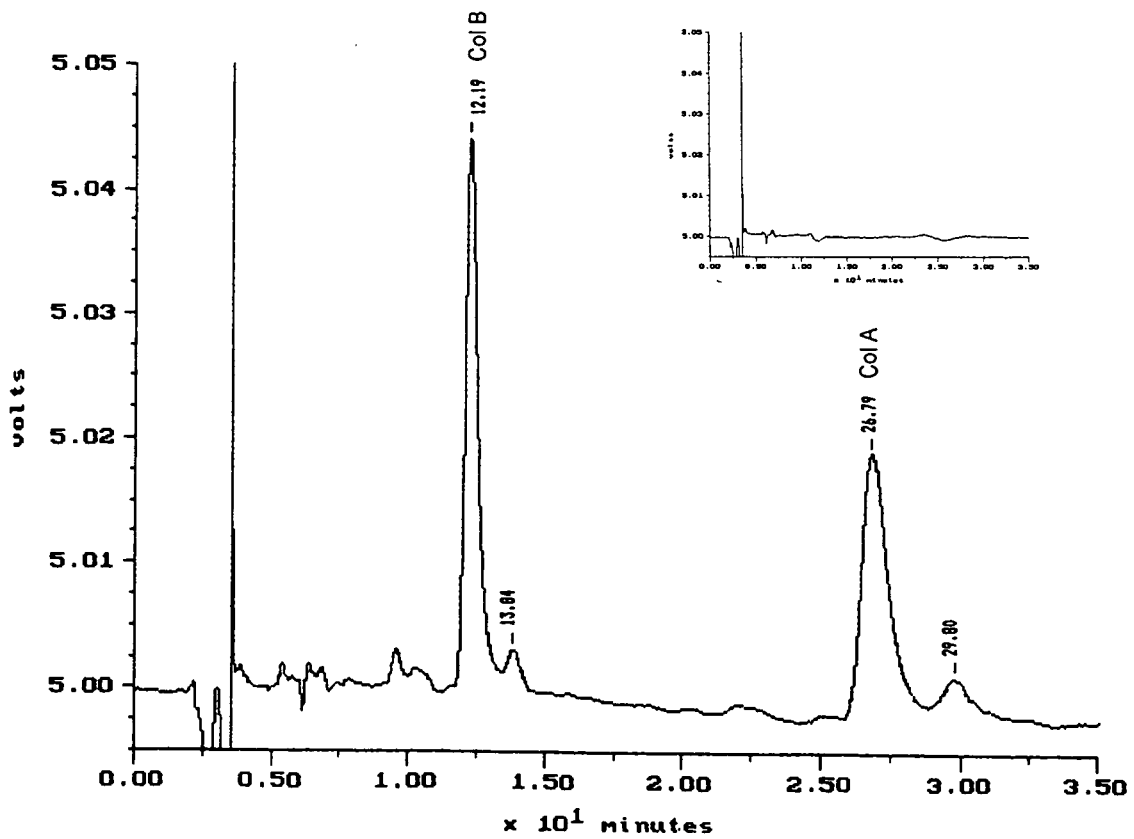

Figure 3.5 Typical chromatogram of colistin sulphate (60 µL, 0.1 mg/mL, Sigma) obtained with direct UV detection at 210 nm. The insert shows a chromatogram of a blank sample.

Positive ion electrospray mass spectral analysis of the reference sample of colistin (Sigma) showed there were two main components in the sample of colistin mixture (Figure 3.6 A). The mass spectra of the individual components collected by HPLC and dissolved in formic acid solution, contained ions with *m/z* 1156.0 (Figure 3.6 B) and 1170.0 (Figure 3.6 C), respectively. These were consistent with the protonated molecular ions of colistin B (theoretical mass 1155.76) and colistin A (theoretical mass 1169.77). Therefore, in the component analysis of underivatized colistin, the first peak, eluting at approximately 12.2 min, corresponded to colistin B and the second at 26.8 min to colistin A (Figure 3.5). No attempt was made to identify the compounds corresponding to the minor peaks eluting at 13.8 and 29.8 min.

3.3.2 Identity of the peaks from chromatographic analysis of the FMOC derivatives of colistin

There were only two major peaks in the HPLC chromatograms of the FMOC derivatives of colistin, suggesting only one derivative was produced for each of the major components in colistin. Their identification was performed by reacting colistin A or colistin B in solution, separately, with FMOC-Cl. The results showed that the colistin B-FMOC derivative was eluted earlier than the colistin A-derivative (Figure 3.7).

3.3.3 Optimisation of derivatization of colistin in solution

The pH and reaction time were independently varied in the derivatization of colistin with FMOC-Cl. The effects of these parameters on the derivatization of colistin B and colistin A are shown in Figures 3.8 and 3.9.

3.3.4 Adsorption of the derivatives onto the HPLC autoinjector inserts

Table 3.1 shows that with the inserts supplied by Supelco, there was no change in fluorescent response when the derivatives were chromatographed consecutively from the same insert. However, a considerable reduction was observed with inserts from the other two suppliers.

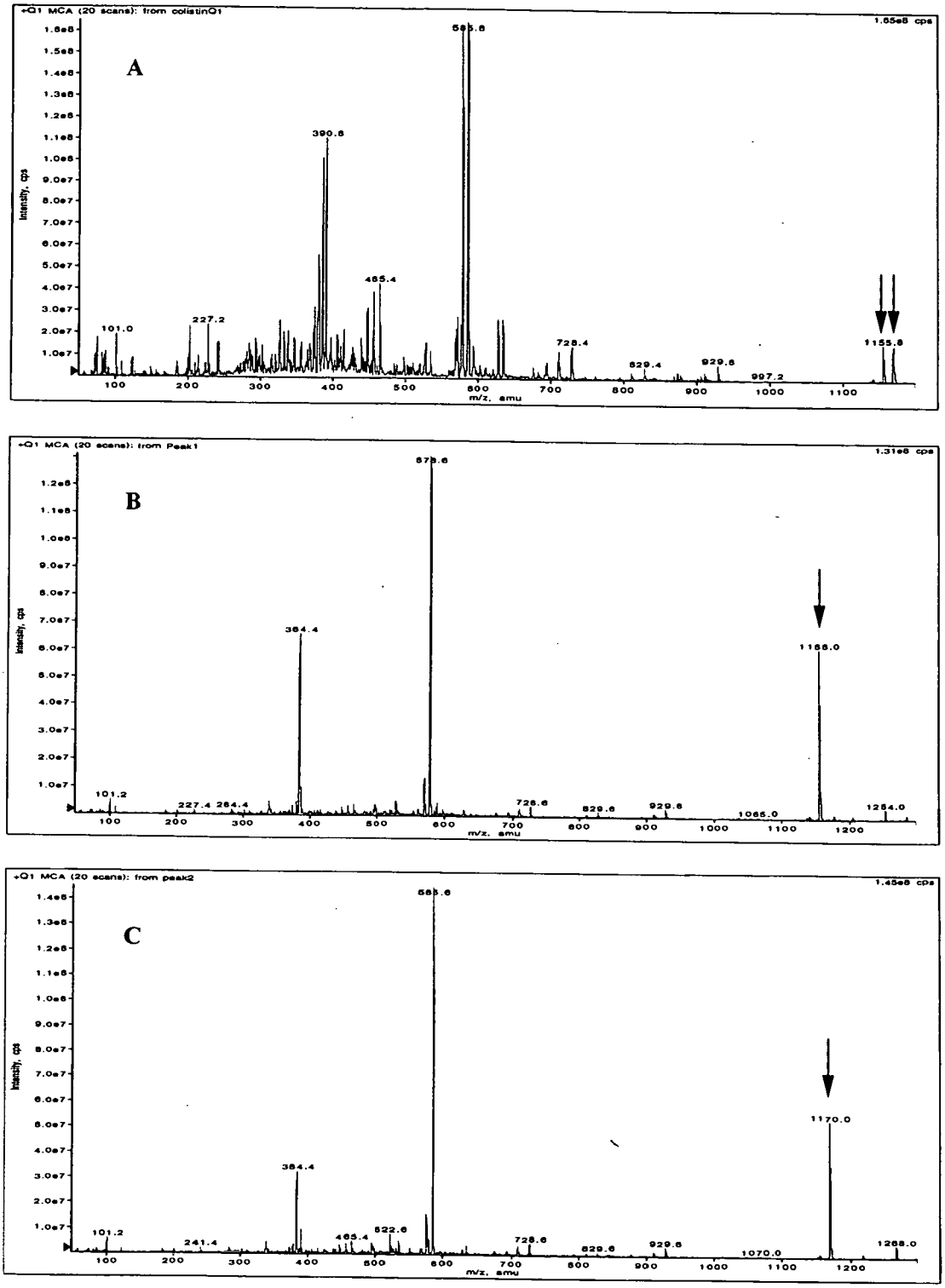

Figure 3.6 Mass spectra of colistin (A), colistin B (B), and colistin A (C) obtained by electrospray ionization MS (ESI-MS). The arrows indicate the protonated molecular ions.

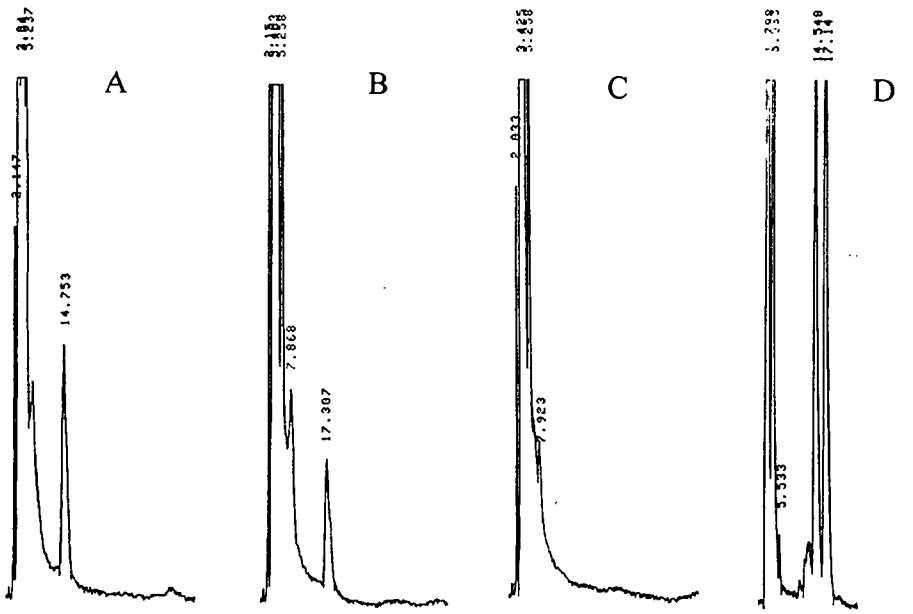

Figure 3.7 Chromatograms of FMOC derivatives of colistin B (A), colistin A (B), blank (C) and colistin (Sigma) (D).

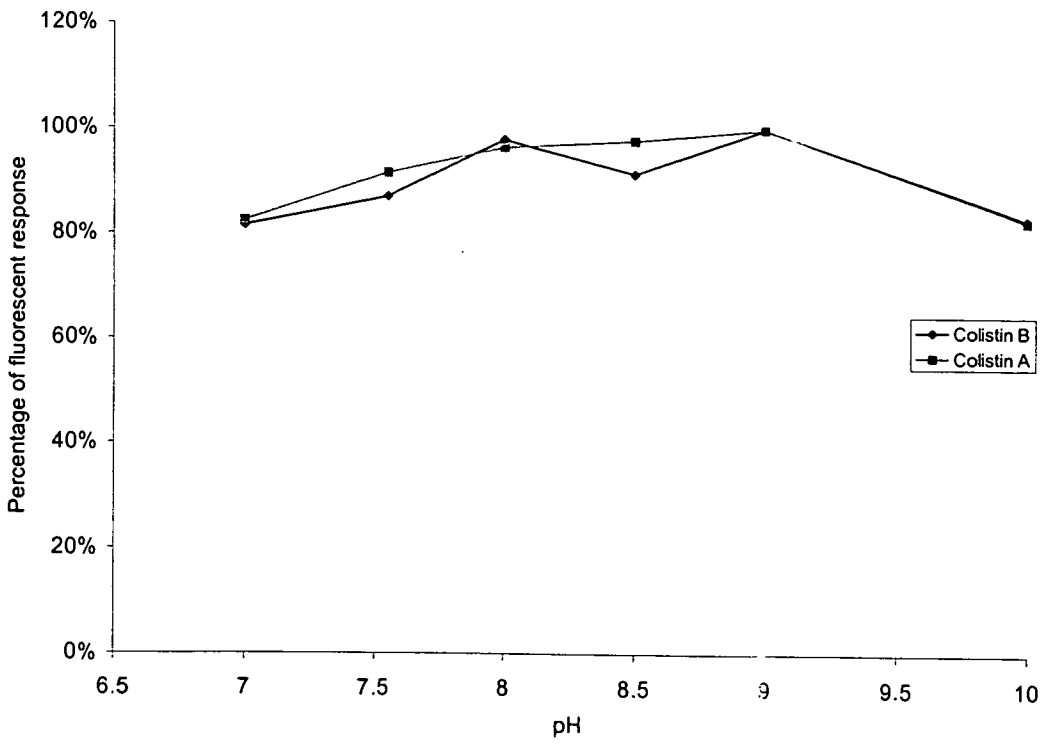

Figure 3.8 Effect of pH on the reaction (10 min) of colistin with FMOC-Cl in solution. Response is as a percentage of the maximum observed fluorescence.

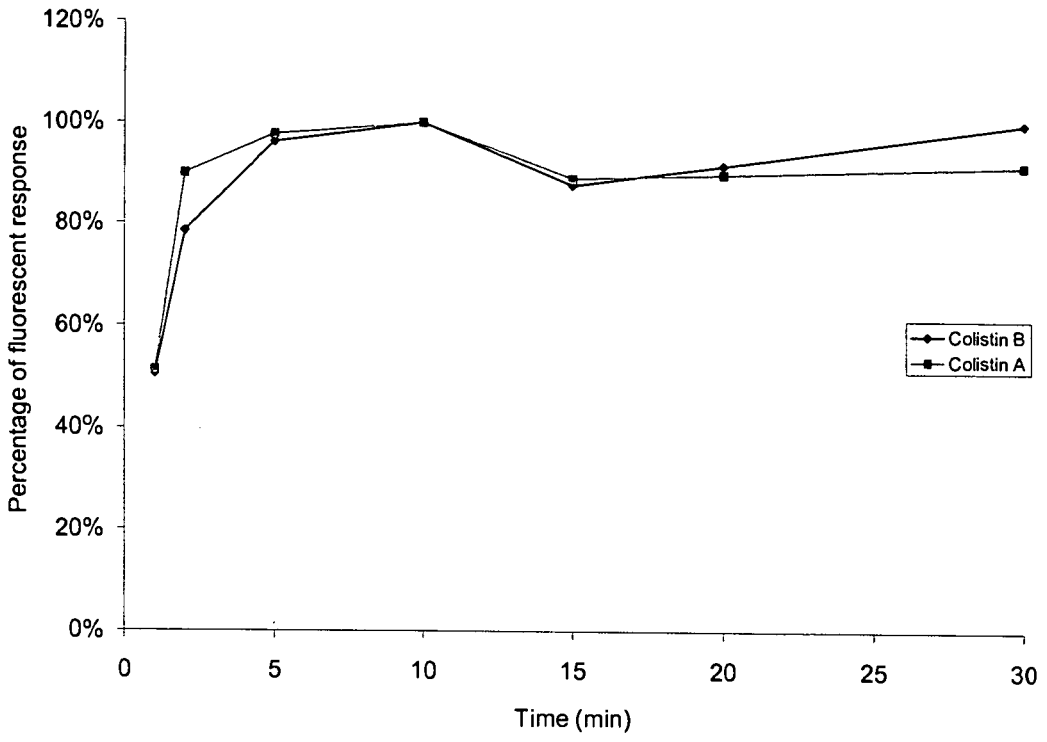

Figure 3.9 Effect of duration of reaction on the derivatization (pH 8.9) of colistin with FMOC-Cl in solution. Response is as a percentage of the maximum observed fluorescence.

Table 3.1 Examination of apparent adsorption of FMOC derivatives of colistin onto different types of HPLC autoinjector inserts

Insert	Percentage of the initial fluorescent response in the consecutive chromatography (%)	
	Colistin A	**Colistin B**
Sun51	74.9	76.7
Alltech	63.2	68.4
Supelco	106.6	104.7

3.3.5 Stability of the derivatives

FMOC derivatives of colistin were found to be stable for at least 3 days at ambient temperature (Figure 3.10). The derivatives of colistin A and colistin B displayed

almost identical stability. The derivative of netilmicin (internal standard) was quite stable even after two weeks at ambient temperature.

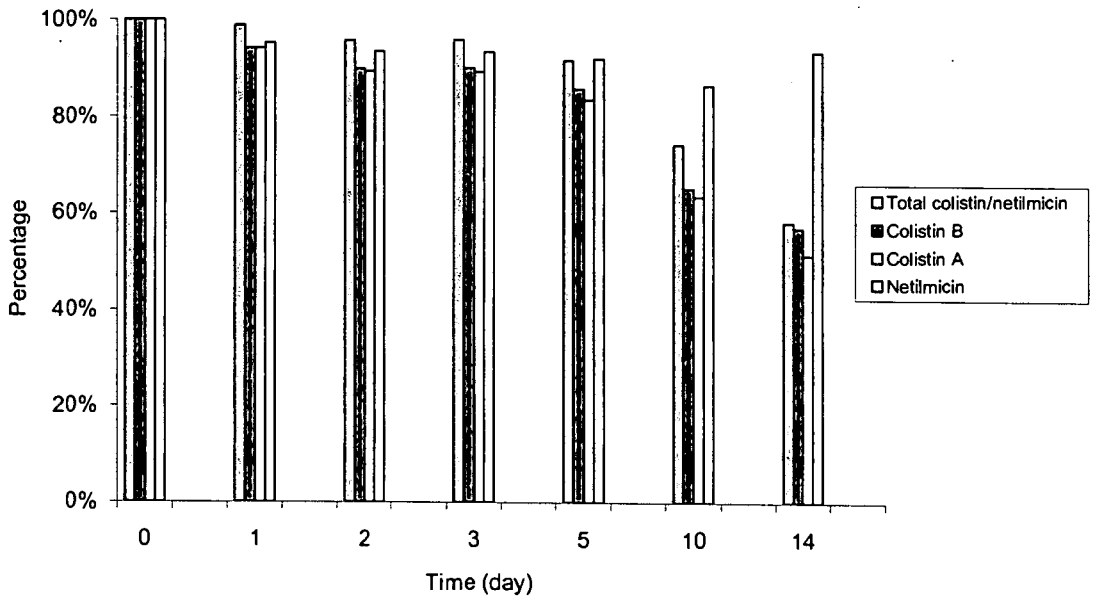

Figure 3.10 The stability of the derivatives of colistin and netilmicin in a solution of acetone:0.2 M boric acid (9:6) at ambient temperature. Values are expressed as a percentage of peak area at time zero.

3.3.6 Specificity

In patients with cystic fibrosis, ceftazidime, meropenem, aztreonam, piperacillin, ciprofloxacin, tobramycin or ticarcillin, may be co-administered with colistin to treat the infections caused by *P. aeruginosa*. None of the compounds interfered with the chromatographic analysis of the derivatives of colistin and netilmicin.

3.3.7 Validation of the HPLC method

An internal standard (netilmicin) was used in the assay to correct for potential losses of sample during sample pretreatment and errors that may be caused by instrumental variations.

Figure 3.11 shows representative chromatograms from drug-free human plasma spiked with 1.0 mg/L colistin sulphate and from blank human plasma. The derivatives of netilmicin, colistin B and colistin A were well separated. There was no

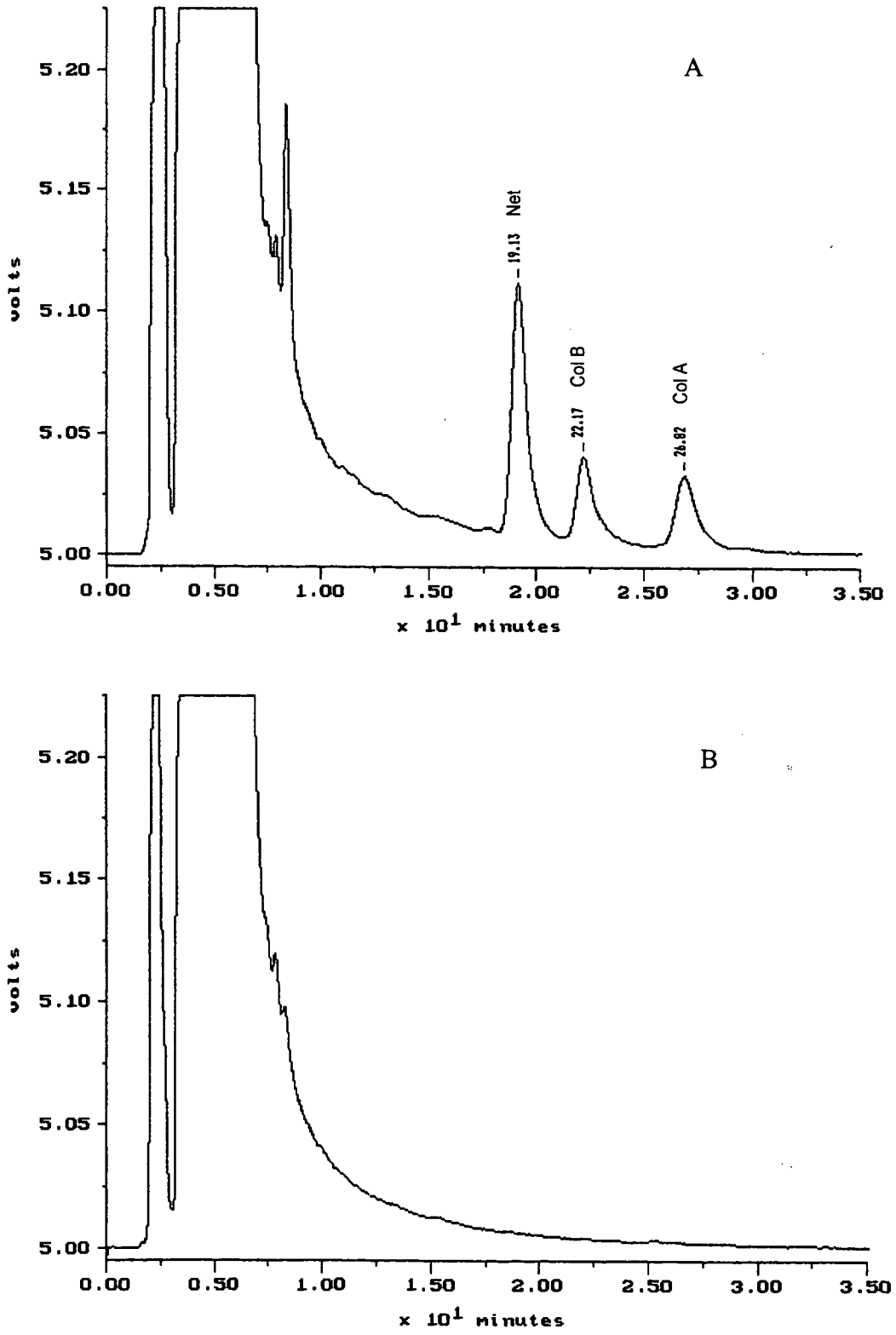

Figure 3.11 Typical chromatograms obtained with fluorescence detection for drug-free human plasma spiked with 1.0 mg/L colistin sulphate (A), and blank human plasma (B).

chromatographic interference to the derivatives of netilmicin, colistin A and colistin B from endogenous compounds or reaction by-products.

High coefficients of determination ($r^2 > 0.998$) were obtained for calibration curves with concentrations ranging from 0.10 mg/L to 4.0 mg/L. The linear regression equation for the relationship between the ratios of the summed peak areas of colistin A and colistin B (as the sulphate salt) to that of netilmicin (internal standard) and the concentrations of colistin sulphate had a mean (± SD) slope of 0.789 ± 0.032 and an intercept of -0.010 ± 0.007 (n = 6). A recovery of 107 ± 12% (n = 3) was calculated when comparing the responses from plasma with those from water spiked at 1.0 mg/L colistin sulphate. The limit of quantification was 0.10 mg/L, at which concentration the mean value measured was 0.10 mg/L and the relative standard deviation was 9.14% (n = 6). Inter-day reproducibility and accuracy were determined by conducting assays on three consecutive days of plasma containing 0.20 mg/L and 2.0 mg/L colistin sulphate as QCs; determination of intra-day reproducibility and accuracy involved six assays of the same QCs. The results are shown in Table 3.2.

Table 3.2 Reproducibility and accuracy for the assay of colistin sulphate in human plasma.

	Intra-day (n = 6)		**Inter-day (n = 3)**	
	0.20 mg/L	**2.0 mg/L**	**0.20 mg/L**	**2.0 mg/L**
Mean Concentration (mg/L)	0.18	2.0	0.18	2.1
Relative Standard Deviation (%)	4.5	3.6	8.4	2.2

Human plasma samples spiked with 1.0 mg/L colistin sulphate were assayed immediately and after storage at -20°C for three months. The percentage of the original value remaining after three month storage at -20°C was 98.9 ± 4.0% for colistin A and 101.3 ± 3.1% for colistin B (n = 3).

3.4 Discussion

When developing a method for measuring the concentrations in biological matrices of drugs lacking a chromophore or fluorophore, options available for increasing the sensitivity of the method include the formation of a UV absorbing or fluorescent derivative prior to chromatographic analysis. For multi-component drugs, formation of derivatives of the components must be efficient and quantitative. This is especially true for colistin, since it contains numerous components in proportions that may differ between suppliers (Decolin et al., 1997). Furthermore, to measure concentrations of colistin in biological fluids using chromatographic methods requires the choice of one or more of the components as suitable markers of the concentration of the drug. Previous chromatographic analysis of several commercial batches of colistin sulphate from different suppliers showed that the summed areas of the peaks from colistin A and colistin B represented more than 85% of the areas from all peaks (Decolin et al., 1997). Therefore, after having proven that FMOC-Cl was an appropriate derivatizing agent for detecting and quantifying colistin, the concentrations of colistin in plasma were calculated in the current method using the areas of the peaks arising from colistin A and colistin B.

When developing the HPLC assay for colistin, which included derivatization with FMOC-Cl, it was essential to confirm the identity of the two peaks observed following chromatography of the derivatives of colistin formed in solution. Colistin A and colistin B were isolated according to the HPLC method reported by Decolin et al. (Decolin et al., 1997). Phosphoric acid and triethylamine in the freeze-dried eluates were removed using SPE C_{18} cartridges (Waters) prior to mass spectral analysis, otherwise the signals from phosphate predominated and no peaks from colistin were observed. Confirmation of the identity of the two major peaks in the HPLC chromatograms of the FMOC derivatives of colistin was performed indirectly by reacting colistin A or colistin B separately with FMOC-Cl (Figure 3.7).

The fluorescent response decreased greatly when the derivatives from the glass insert were chromatographed consecutively. Since the FMOC-Cl derivatives of colistin are stable, they were presumed to be adsorbed onto the autoinjector inserts of some brands. Silanisation of the glass insert with 10% dichlorodimethylsilane in hexane overnight did not prevent adsorption completely. Therefore, two brands of

polypropylene inserts were examined and there was no adsorption to the inserts supplied by Supelco (Table 3.1).

A pH in the range of 7 to 10 had very little effect on the derivatization in solution (Figure 3.8), consistent with reports of the derivatization of amino acids with FMOC-Cl (Einarsson et al., 1983). Compared with amino acids (Einarsson et al., 1983), colistin required a longer time (5 to 10 min) for complete reaction. Nevertheless, the reaction was still rapid at ambient temperature, with approximately 50% of the maximal fluorescent response achieved after 1 min (Figure 3.9). Overall, optimal reaction of colistin with FMOC-Cl in solution occurred at pH 8 - 9, and was complete in approximately 10 min. The optimal conditions obtained from these data were applied successfully to derivatization of colistin in the SPE cartridge.

Solid-phase extraction is an attractive technique that reduces the consumption of, and exposure to, solvent, disposal costs and extraction time. In an effort to simplify sample pre-treatment for the assay of β-phenylethylamine in urine, Pilar et al. described a method utilizing derivatization on SPE cartridges (Pilar et al., 1997). Decolin et al. reported a method using SPE C_{18} cartridges to extract colistin from bovine tissues (Decolin et al., 1997). Therefore, a new method was developed for measuring colistin in human plasma by combining the procedures of Pilar et al. and Decolin et al. to enable efficient isolation of colistin from plasma by SPE followed by derivatization on the same cartridge.

For the conditioning and equilibration of SPE cartridges, different volumes of methanol and carbonate buffer had no effect on the recovery and derivatization of colistin. Therefore, 1 mL each of methanol and carbonate buffer was used routinely to condition and equilibrate SPE cartridges prior to loading of pre-treated plasma. Loading of pre-treated plasma was performed at low negative pressure between 0 and -10 kPa to ensure colistin was absorbed on the C_{18} matrix during its slow passage through the cartridge. This was crucial for consistency of the assay. Washing the SPE cartridge with 1 mL carbonate buffer after loading was successful in removing interfering components originating from plasma. Borate buffer had been tried, but was less successful in removing many unwanted peaks, particularly at low concentrations of colistin (< 0.5 mg/L). Deproteinization of plasma prior to application on the SPE cartridge resulted in substantially cleaner chromatograms.

The solution of FMOC-Cl in acetonitrile must be added to the SPE cartridges in as small a volume as possible, to avoid premature elution of underivatized and derivatized colistin from cartridges. In the present method, addition of FMOC-Cl (100 mM) in 30 μL acetonitrile did not cause any breakthrough loss of underivatized or derivatized colistin from cartridges.

While there was no rigorous analysis of the most suitable concentration of FMOC-Cl required for derivatization, with lower concentrations of FMOC-Cl (< 10 mM), the fluorescent responses were very low, probably because of the low reaction efficiency. With high concentrations (> 150 mM), the reaction efficiency decreased and unwanted peaks appeared near those of the colistin derivatives, possibly because the excess reagent reacted with endogenous compounds from plasma. This effect was magnified at low concentrations of colistin (< 0.20 mg/L).

The derivatization reaction generates derivatives less polar than colistin. Hence, acetone was used to ensure efficient elution of the derivatives from the SPE cartridges. The FMOC derivatives of colistin were stable for at least 3 days at ambient temperature (Figure 3.10). In the absence of boric acid, the derivatives degraded more than 40% overnight at room temperature. Boric acid should be prepared weekly and kept at 4°C before use. Phosphoric acid and hydrochloric acid were ineffective in stabilising the derivatives, suggesting their stability was not related to pH alone.

The results of intra-day and inter-day assays confirm the reliability of this method for measuring concentrations of colistin sulphate (0.10 mg/L to 4.0 mg/L) in human plasma. Similar accuracy and reproducibility was observed when this method with minor modifications was used to measure the concentrations of colistin in rat plasma and urine (Chapter 6).

3.5 Conclusion

A novel HPLC method for the assay of colistin in human plasma has been developed and validated. The method involves extraction of colistin from plasma onto SPE cartridges, followed by rapid and quantitative derivatization with FMOC-Cl under mild conditions on the same cartridge. The method is simple, specific, and avoids the need for automated equipment for derivatization and subsequent column switching.

This is the first report of an HPLC method for the assay of colistin in human plasma using FMOC-Cl as the derivatizing reagent and allows determination of the concentrations of colistin, colistin A and colistin B in biological matrices.

Chapter 4 High-performance liquid chromatographic determination of colistin methanesulphonate in plasma and urine

4.1 Introduction

While there are several HPLC methods for the assay of colistin in biological samples (Decolin et al., 1997; Le Brun et al., 2000), none has been applied to colistin methanesulphonate. A recent report on the pharmacokinetics of "colistin" in patients with CF, following an intravenous infusion of colistin methanesulphonate, applied HPLC to measure the concentrations of drug in plasma and urine following derivatization with dansyl chloride (Reed et al., 2001). However, colistin is formed by hydrolysis of colistin methanesulphonate *in vitro* and *in vivo*. Unfortunately, it was not clear from their HPLC method which form was quantified, colistin or colistin methanesulphonate or both (Reed et al., 2001). As reviewed in Chapter 1, microbiological assay is the most common method currently available for measuring concentrations of colistin methanesulphonate in biological fluids (Bergan et al., 1982; Leroy et al., 1989).

Colistin methanesulphonate has the potential to hydrolyse in aqueous matrices, releasing partially sulphomethylated derivatives and the more active colistin (Beveridge et al., 1967; see also Chapter 5). Furthermore, colistin diffused more slowly than colistin methanesulphonate in agar (Sande et al., 1970). Therefore, the accuracy of previously reported data on the concentrations of colistin methanesulphonate measured by microbiological methods may be complicated by the presence of colistin and partially sulphomethylated derivatives in the biological samples when collected, and from hydrolysis of colistin methanesulphonate during incubation (Sande et al., 1970).

The present study describes a simple and sensitive HPLC method for the assay of colistin methanesulphonate in plasma and urine. The method requires accelerated hydrolysis of colistin methanesulphonate to colistin, followed by extraction of the generated colistin, and derivatization prior to HPLC according to the method described previously in Chapter 3 for the quantification of colistin. The method was applied to the experiments

examining the pharmacokinetics of colistin methanesulphonate in rats (Chapter 7) and subsequently adapted to studies in humans (Chapter 8).

4.2 Materials and Methods

4.2.1 Apparatus

The HPLC system comprised a LC-10AS pump, a SIL-10AD*vp* autoinjector (Shimadzu, Kyoto, Japan) and a 470 scanning fluorescence detector (Waters, Milford, MA, USA) connected to a multi-instrument data acquisition and data processing system (Maxima 820, Waters, MA, USA). All the analyses were carried out at ambient temperature (23 ± 2°C). SPE cartridges (C_{18}, 100 mg) were supplied by Waters (Milford, MA, USA).

4.2.2 Chemicals and reagents

Sodium colistin methanesulphonate, colistin sulphate and 9-fluorenylmethyl chloroformate (FMOC-Cl) were purchased from Sigma (St. Louis, MO, USA), netilmicin sulphate from Schering-Plough (Madison, NJ, USA), ceftazidime from Glaxo Wellcome (Boronia, Victoria, Australia), meropenem from Zeneca (Macclesfield, Cheshire, UK), aztreonam from Bristol-Myers Squibb (Noble Park, Victoria, Australia), piperacillin from Lederle Laboratories (Baulkham Hills, NSW, Australia), ciprofloxacin from Bayer AG (Pymble, NSW, Australia), tobramycin from Eli Lilly (West Ryde, NSW, Australia), ticarcillin from Smith Kline Beecham (Dandenong, Victoria, Australia), and flucloxacillin from Rhone-Poulenc Rorer (Baulkham Hills, NSW, Australia). Trichloroacetic acid, boric acid, sulphuric acid, sodium hydrogen carbonate and sodium hydroxide of analytical grade, and acetonitrile, methanol, tetrahydrofuran and acetone of HPLC grade were all obtained from BDH Laboratory Supplies (Poole, England). Water was purified by a Millipore Milli-Q system (Millipore Corp., Bedford, MA, USA). Carbonate buffer (1%, w/w, pH 10) was prepared by dissolving the appropriate amount of sodium hydrogen carbonate in water and adjusting pH with sodium hydroxide (10%, w/w). All standard solutions of colistin methanesulphonate and colistin are as the concentrations of the sodium and sulphate salts, respectively.

4.2.3 Optimisation of conditions for the hydrolysis of colistin methanesulphonate in rat plasma

The optimal concentration of sulphuric acid for the hydrolysis of colistin methanesulphonate in rat plasma was determined by mixing 150 µL samples of drug-free rat plasma, spiked with a freshly prepared solution of sodium colistin methanesulphonate (25 mg/L) and a solution of netilmicin sulphate (5 mg/L, 30 µL, internal standard), with 20 µL aliquots of sulphuric acid solutions (0.1, 0.4, 0.8, 1.0, 1.5, 2.0 or 3.0 M). After 30 min, the reaction was stopped by adding 40 µL sodium hydroxide solution (0.1, 0.4, 0.8, 1.0, 1.5, 2.0 or 3.0 M, respectively). The fluorescent response was measured following sample preparation, derivatization and HPLC analysis (Section 4.2.4). Analyses were performed in duplicate at each concentration of sulphuric acid.

To determine the optimal time for hydrolysis, samples (150 µL) of drug-free rat plasma, spiked as above with colistin methanesulphonate and netilmicin, were mixed with 20 µL of sulphuric acid (1.0 M). The reaction was stopped at 1, 2, 5, 10, 15, 20 and 30 min by adding 40 µL sodium hydroxide (1.0 M). The fluorescent response was determined following sample preparation, derivatization and HPLC analysis (Section 4.2.4). Analyses were performed in duplicate at each time.

4.2.4 Sample pretreatment

The internal standard (30 µL of 5 mg/L netilmicin sulphate in water) was mixed with 150 µL plasma or 200 µL urine in a 1.5-mL polypropylene tube (Techno-plas, Adelaide, SA, Australia). After adding 20 µL sulphuric acid (1.0 M), colistin methanesulphonate was hydrolyzed completely for 10 min and the reaction stopped by adding 40 µL sodium hydroxide (1.0 M). Methanol-10% trichloroacetic acid (50:50, v/v) (50 µL) was added to precipitate proteins, and after being centrifuged at 1,000 *g* for 10 min at 4°C, the supernatant was transferred into another 1.5-mL polypropylene tube and mixed with 10 µL of 1.0 M sodium hydroxide. After vortex-mixing for 1 min, methanol-0.01 M hydrochloric acid (50:50, v/v) (250 µL) was added. The entire final solution was delivered to a SPE C_{18} cartridge (Sep-Pak, Waters) conditioned with 1 mL of methanol, and equilibrated with 1 mL of carbonate buffer. These steps were performed using a vacuum manifold (Supelco Visiprep 24 ^{TM}, Bellefonate, PA, USA) without allowing the

cartridge to run dry. After washing with 1 mL carbonate buffer, the cartridge was dried by drawing air through under vacuum. FMOC-Cl (30 µL, 100 mM in acetonitrile) was added followed by 80 µL methanol. After 10 min of reaction, the derivatives were eluted with 900 µL acetone, vortex-mixed for 2 min with 600 µL boric acid solution (0.2 M), and 100 µL injected onto the HPLC column.

4.2.5 HPLC analysis

HPLC was conducted on a 250 × 4.6 mm ID steel column prepacked with 5 µm Ultrasphere C_{18} (Alltech, Box Hill, Australia) using a mobile phase of acetonitrile-tetrahydrofuran-water (50:30:20, v/v) pumped at 1.0 mL/min. Fluorescence was measured at 315 nm following excitation at 260 nm, with the gain set at ×100. The run time was 18 min.

4.2.6 Specificity

With a view to later use of the method for measuring the concentrations of colistin methanesulphonate in plasma from humans (Chapter 8), the specificity of the method was evaluated using antibiotics commonly co-administered to CF patients during therapy with colistin methanesulphonate. Eight antibiotics, ceftazidime, meropenem, aztreonam, piperacillin, ciprofloxacin, tobramycin, ticarcillin and flucloxacillin, were included. Samples of rat plasma containing these antibiotics individually at a concentration of 8.0 mg/L were analysed as described above (sections 4.2.4 and 4.2.5).

4.2.7 Validation of the assay

Appropriate volumes of three working standards of colistin methanesulphonate in water (10, 100 and 1000 mg/L, prepared independently) were used to prepare calibration standards in drug-free rat plasma containing colistin methanesulphonate at concentrations of 0.33, 0.67, 2.7, 5.3, 6.7, 13.3, 26.7 and 53.3 mg/L. A linear calibration curve was constructed from the relationship between the ratios of the summed peak areas of the derivatives of colistin A and colistin B to that of netilmicin (internal standard), and concentrations of colistin methanesulphonate. Reproducibility and accuracy were assessed by (1) the intra-day assay with six consecutive analyses of QC samples containing 1.3 mg/L and 40 mg/L of colistin methanesulphonate in rat plasma, and (2) the

inter-day assay with three consecutive analyses of the QC samples on separate days. Calibration curves for colistin methanesulphonate in urine were prepared at concentrations of 0.25, 0.50, 2.0, 4.0, 5.0, 10, 20 and 40 mg/L. Validation of the HPLC method for colistin methanesulphonate in rat urine was conducted by the intra- and inter-day assays using QC samples containing colistin methanesulphonate at concentrations of 1.0 mg/L and 30 mg/L.

Rat plasma containing colistin methanesulphonate at a concentration of 26.7 mg/L was treated in triplicate as described above and the stability of the FMOC-Cl derivative was evaluated by injecting the product to HPLC immediately after elution from the cartridge and after storage for 24 hr at ambient temperature. The stability of colistin (2.0 mg/L in drug-free rat plasma) during pretreatment with sulphuric acid (1.0 M) for 30 min was tested in duplicate and compared with the results without sulphuric acid pretreatment.

4.3 Results

4.3.1 Optimisation of conditions for the hydrolysis of colistin methanesulphonate

Conditions for the hydrolysis of colistin methanesulphonate in the presence of sulphuric acid were optimized by examining the effect of the concentration of sulphuric acid and the time-course of hydrolysis. As shown in Figure 4.1, maximum hydrolysis occurred at a final concentration of sulphuric acid in the range of 0.08 to 0.1 M. The fluorescent response decreased with concentrations of sulphuric acid above 0.1 M. Using a final concentration of sulphuric acid of 0.1 M, the hydrolysis of colistin methanesulphonate in rat plasma was complete after 10 min (Figure 4.2). Therefore, the optimum condition for the hydrolysis of colistin methanesulphonate in rat plasma involved a final sulphuric acid concentration in the range of 0.08 to 0.1 M with a reaction time of 10 min.

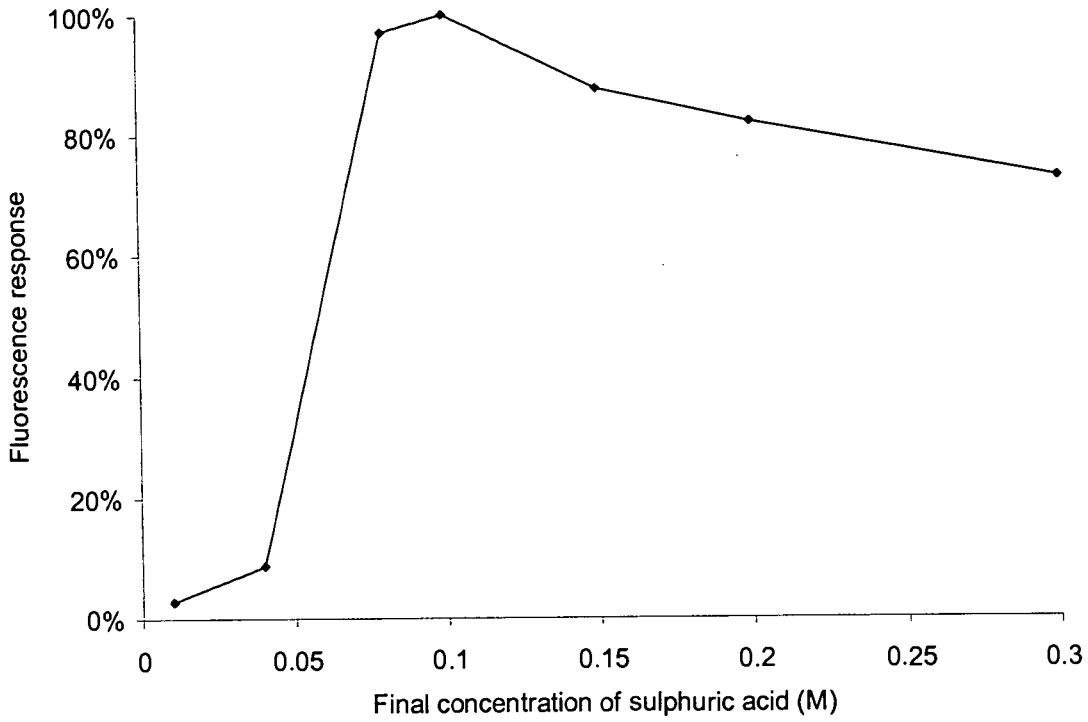

Figure 4.1 Effect of concentration of sulphuric acid on the hydrolysis of colistin methanesulphonate to colistin in rat plasma. Response is expressed as a percentage of the maximum observed response.

4.3.2 Validation of the assay

Typical chromatograms from drug-free rat plasma and plasma collected at 10 min after an intravenous bolus of colistin methanesulphonate (15 mg/kg) in a rat are shown in Figure 4.3. The derivatives were eluted in the order of netilmicin, colistin B and colistin A. Calibration curves were linear over the range from 0.33 mg/L to 53.3 mg/L in rat plasma and from 0.25 mg/L to 40 mg/L in rat urine. The linear regression equation using rat plasma had a mean (± SD) slope of 0.112 ± 0.010 and an intercept of -0.055 ± 0.038 (n = 3); corresponding values using rat urine were 0.178 ± 0.026 and -0.008 ± 0.079 (n = 3), respectively. The difference in mean slopes represents the different volumes of plasma and urine used for the assay. Data for the intra- and inter-day accuracy and reproducibility of the assay for rat plasma and urine are presented in Table 4.1. The eluted derivatives kept at ambient temperature for 24 hr gave a mean fluorescent response

of 95.1% (n = 3) of the value for the freshly eluted derivatives. Samples of plasma containing colistin (2 mg/L) and pretreated with sulphuric acid for 30 min gave a mean fluorescence response which was 102% of the response without pretreatment (n = 2).

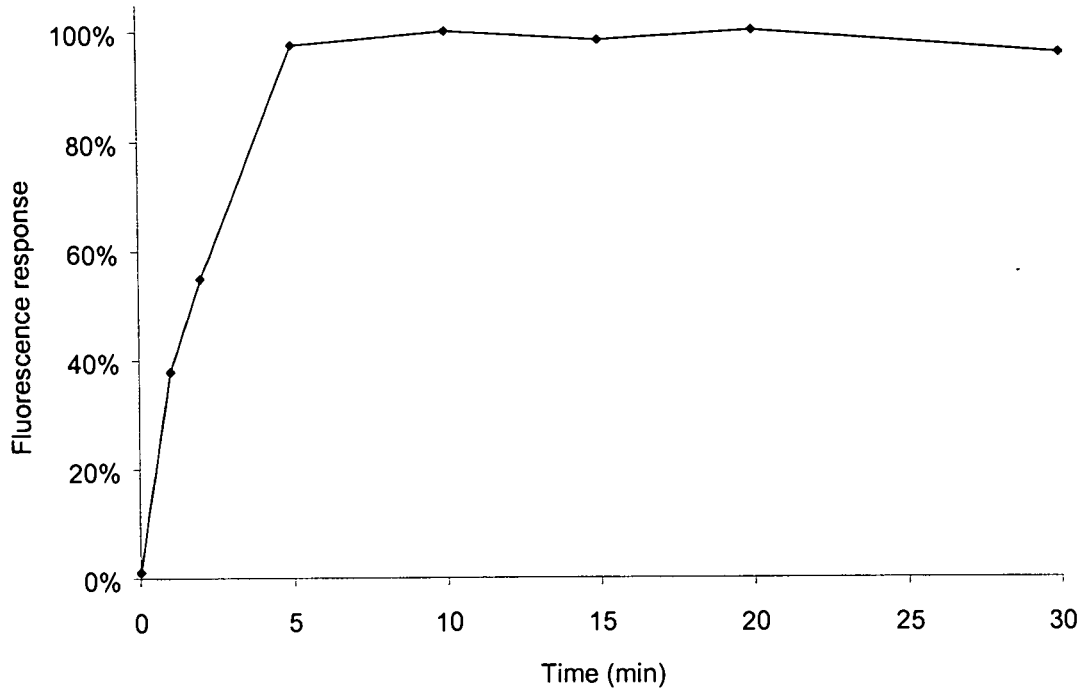

Figure 4.2 Effect of time of incubation with sulphuric acid (0.1 M) on the hydrolysis of colistin methanesulphonate in rat plasma. Response is expressed as a percentage of the maximum observed response.

4.4 Discussion

Colistin methanesulphonate is a penta-methanesulphonated derivative of colistin and, following its administration, is converted to colistin *in vivo* (Barnett et al., 1964; Beveridge et al., 1967). This process most likely occurs via intermediates with different permutations of substitution of any of the five L-Dab residues (Figure 1.2) with four, three, two and one sulphomethyl groups. Except for a recent report (Reed et al., 2001), microbiological assays are the main methods for measuring concentrations of drug in plasma and urine following dosage with colistin methanesulphonate. Previous reports

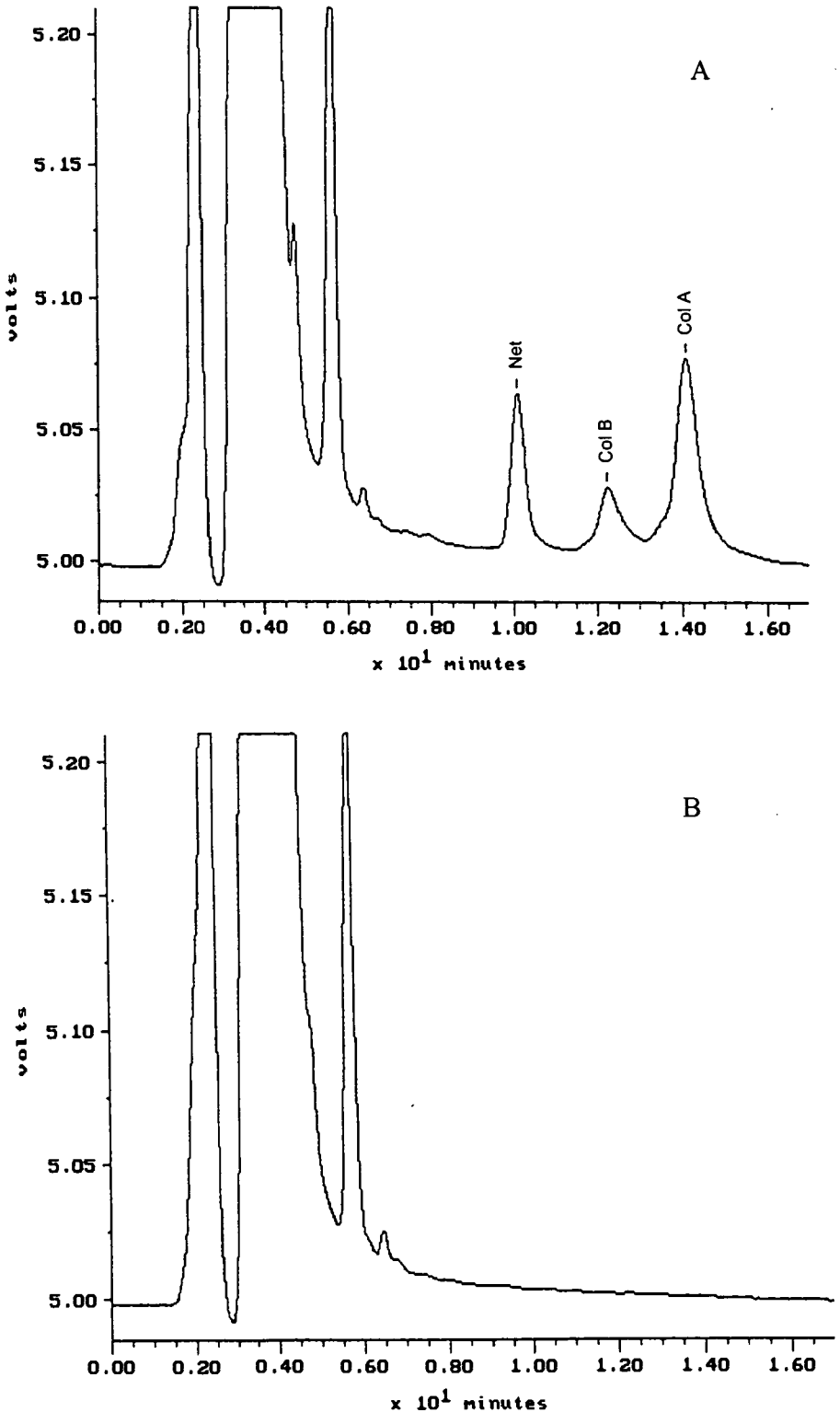

Figure 4.3 Chromatograms of a rat plasma sample collected 10 min after an intravenous bolus of 15 mg/kg colistin methanesulphonate (A) and drug-free rat plasma (B).

Table 4.1 Reproducibility and accuracy for the assay of colistin methanesulphonate in rat plasma and urine.

		Intra-day (n = 6)		**Inter-day (n = 3)**	
		1.3 mg/L	40 mg/L	1.3 mg/L	40 mg/L
Plasma	Mean Concentration (mg/L)	1.4	39	1.3	40
	Relative Standard Deviation (%)	8.8	2.4	10.1	4.1
		Intra-day (n = 6)		**Inter-day (n = 3)**	
		1.0 mg/L	30 mg/L	1.0 mg/L	30 mg/L
Urine	Mean Concentration (mg/L)	1.0	30	1.0	29
	Relative Standard Deviation (%)	4.7	6.2	11.2	7.8

have shown that the antibacterial activity of colistin against *Escherichia coli*, *P. aeruginosa*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae* ranged from three- to forty-fold the activity of colistin methanesulphonate (Chapter 2; Schwartz et al., 1960). A rapid increase in antibacterial activity was observed after storing colistin methanesulphonate at 37°C in 0.1 M phosphate buffer (pH 7.0) (Beveridge et al., 1967). Therefore, it is most likely that microbiological assays requiring overnight incubation measure the apparent concentration of colistin methanesulphonate of different degrees of sulphomethyl substitution and colistin based on their combined antibacterial activity. Irrespective of whether the agar diffusion or serial dilution methods are used (Sande et al., 1970), it is possible that there were substantial errors in measurement. A recent report described a method employing derivatization with dansyl chloride followed by HPLC to measure the concentration of drug in plasma and urine from CF patients administered colistin methanesulphonate (Reed et al., 2001). In that method, plasma and urine samples were pre-treated at 54°C or 57°C, respectively, for 2 hr (Reed et al., 2001). From the results described in Chapter 5, it is quite possible that hydrolysis of colistin methanesulphonate to colistin could have occurred within 2 hr. However, it is unknown whether hydrolysis would have been completed. Furthermore, as noted previously (Section 1.3.2), colistin could be formed *in vivo* by the hydrolysis of colistin

methanesulphonate. Therefore, it remains unclear whether total colistin, representing summed colistin and its derivatives, or only part of the total, has been measured. In addition, the limit of quantification was relatively high (5 mg/L with 1.0 mL plasma) and the method required greater than 170 min for sample pretreatment.

The method described in this chapter measures the summed concentration of all sulphomethylated derivatives of colistin, plus colistin, in plasma and urine. Combined with the previously reported method for measuring colistin in plasma (Chapter 3), the two methods have the advantage of determining the concentrations of colistin alone and the concentrations of fully and all partially sulphomethylated derivatives of colistin in plasma and urine. When plasma freshly spiked with colistin methanesulphonate (10 mg/L) was assayed using the HPLC method for colistin (Chapter 3), no FMOC derivatives of colistin appeared. Therefore, for plasma collected from humans or animals after administration of colistin methanesulphonate, the method of sample pretreatment used for colistin (Chapter 3) does not cause any apparent conversion of colistin methanesulphonate to colistin.

The efficiency of hydrolysis with acids other than sulphuric acid was examined at the same concentration of 0.1 M. However, no colistin was formed when phosphoric acid was used and there was very little hydrolysis with hydrochloric acid (6.6% of the response with sulphuric acid). As a simple HPLC assay has been described in Chapter 3 for colistin, colistin formed by the hydrolysis of colistin methanesulphonate could be assayed similarly by derivatization with FMOC-Cl followed by RP-HPLC. The stability of colistin during the pretreatment with sulphuric acid indicated that colistin formed from the hydrolysis of colistin methanesulphonate was stable under these conditions and that its derivatization with FMOC-Cl was not affected by the pretreatment with sulphuric acid. Furthermore, pretreatment with sulphuric acid did not affect the stability of colistin-FMOC derivatives once formed, since they remained stable for at least 24 hr at ambient temperature.

Sep-Pak cartridges (Waters) were preferred over SPE cartridges supplied by J. T. Baker, which were used in the HPLC method for colistin in Chapter 3, because of differences in cost. Results of the validation (Section 4.3.2) demonstrated that cartridges from Waters also gave good accuracy and reproducibility. A modification of the previous mobile phase (Section 3.2.7.2) gave a much shorter HPLC run time (18 min) which improved the

speed and economy of the assay. There were three peaks of interest in the chromatograms (Figure 4.1A), one for the FMOC derivative of netilmicin (internal standard) and the other two for the corresponding derivatives of colistin B and colistin A. High regression coefficients were obtained for calibration curves using concentrations ranging from 0.33 mg/L to 53.3 mg/L in plasma ($r^2 > 0.995$) and from 0.25 mg/L to 40.0 mg/L in urine ($r^2 > 0.983$). The results from validation of the method, as shown in Table 4.1, confirmed its reliability for measuring concentrations of colistin methanesulphonate in rat plasma and urine.

The range of concentrations used to construct the calibration curves has been found suitable for pharmacokinetic studies in rats and humans, and the results of these studies are contained in Chapters 7 (for rats) and 8 (for humans). The HPLC assay for colistin methanesulphonate in human plasma was validated in Chapter 8, and both accuracy and reproducibility (RSD) were within 10% (Section 8.2.3). Therefore, the method will be also valuable for measuring the concentrations of colistin methanesulphonate in plasma from patients with CF, during the study described in Chapter 8.

It should be noted that the concentrations of each substituted form of colistin methanesulphonate can not be determined by this method. In addition, in biological samples collected following administration of colistin methanesulphonate, the concentrations calculated from the calibration curves are apparent values with reference to those in freshly prepared standards of colistin methanesulphonate. The actual mass of colistin methanesulphonate in biological samples collected following colistin methanesulphonate administration may be less than the apparent value because of the loss of sulphomethyl groups (each with a molecular weight of 94) during the hydrolysis of colistin methanesulphonate *in vivo*. Therefore, concentrations determined in biological fluids are most appropriately considered as apparent concentrations of colistin methanesulphonate.

Once the concentrations of colistin and of total colistin methanesulphonate plus colistin in samples are measured separately by the HPLC methods described in Chapter 3 and this chapter, respectively, the concentration of colistin methanesulphonate may be calculated as the value determined from this HPLC method (Chapter 4) minus the concentration of colistin in the same sample, after correcting for differences in the average molecular

weights of colistin (1163, from the molecular weights for colistin A and colistin B of 1170 and 1156, respectively) and colistin methanesulphonate (1743, from the molecular weights for sodium colistin A methanesulphonate and sodium colistin B methanesulphonate of 1750 and 1736, respectively).

Ceftazidime, meropenem, aztreonam, piperacillin, ciprofloxacin, tobramycin or ticarcillin, may be co-administered with colistin methanesulphonate to treat infections caused by *P. aeruginosa* and flucloxacillin for infections with *Staphylococcus aureus*. None of these compounds interfered with the chromatographic analysis of the derivatives of colistin and netilmicin. Therefore, the HPLC assay for colistin methanesulphonate described in this chapter is superior to microbiological assays, particularly for the samples containing co-administered antibiotics which are antibacterial against the test strain.

4.5 Conclusion

A simple HPLC method for the assay of colistin methanesulphonate in plasma and urine has been developed and validated. The method involves hydrolysis of colistin methanesulphonate to colistin with sulphuric acid followed by derivatization with FMOC-Cl and HPLC analysis. By combining this method with the HPLC assay described in Chapter 3, it is able to determine the concentrations of both colistin and colistin methanesulphonate separately after administration of colistin methanesulphonate. This will allow a more detailed description of the pharmacokinetics of colistin methanesulphonate than has hitherto been possible. The method is simple and sensitive, and will be used to define the pharmacokinetics of colistin methanesulphonate in rats (Chapter 7) and humans (Chapter 8). To our knowledge, this is the first report of an accurate HPLC method for the analysis of colistin methanesulphonate in biological fluids.

Chapter 5 Stability of colistin and colistin methanesulphonate in aqueous media

5.1 Introduction

Colistin and colistin methanesulphonate have different antibacterial activities (Chapter 2), pharmacokinetics (Al-Khayyat et al., 1973) and pharmacodynamics (Chapter 2). Therefore, an understanding of the stability of colistin and colistin methanesulphonate in plasma, urine and aqueous solutions, including water, buffer, microbiology culture medium, and saline, will be very helpful for interpreting results from pharmacokinetic and pharmacodynamic studies. Unfortunately, as reviewed in section 1.3.2, the stability of colistin and colistin methanesulphonate has not been studied comprehensively.

Colistin methanesulphonate, with five methanesulphonate groups, has the potential to hydrolyse in aqueous solution and form a complex mixture, containing up to 32 (2^5) types of fully and partially sulphomethylated derivatives, plus colistin. Only two papers reported the chemical homogeneity of colistin methanesulphonate by separation and detection of fully and partially derivatized colistin with electrophoresis and bioassay (Barnett et al., 1964; Beveridge et al., 1967). The detection of the products was based on antibacterial activity.

In order to obviate complications caused by the presence of five methanesulphonate groups in colistin methanesulphonate, McMillan et al. explored the hydrolysis of the methanesulphonate group in a simple model compound, *n*-butylaminomethanesulphonic acid (McMillan et al., 1969). Unfortunately, the statistical factor introduced by the five free amine sites per molecule of colistin limited the investigation of colistin methanesulphonate.

The toxicity of colistin methanesulphonate is much less than colistin. It was reported that the LD_{50} for intravenous colistin sulphonate in mice was 5.5 mg/kg, while that for sodium colistin methanesulphonate was 222 mg/kg when administered intravenously (both agents were from Kayaku Antibiotic Research Company, Ltd., Tokyo, Japan) (Schwartz et al.,

1960). The mechanisms for their toxicity remain unknown. The two most common commercially available products of colistin methanesulphonate, Colomycin (Pharmax, UK) and Coly-Mycin (Parke-Davis, USA), are different in the incidence of toxicity (Barnett et al., 1964). Given the significant differences in toxicity between colistin and colistin methanesulphonate, it was presumed that the difference in the toxicity between Colomycin and Coly-Mycin was caused by the generation of different concentrations of colistin by hydrolysis during storage, following reconsitution prior to administration or *in vivo*. Therefore, it will be of interest to compare the concentrations of colistin during the *in vitro* hydrolysis of the two commercial products in water, isotonic phosphate buffer (pH 7.4) and human plasma.

The purpose of this study was to investigate the stability of colistin and colistin methanesulphonate in aqueous media by specific HPLC methods.

5.2 Materials and Methods

5.2.1 Chemicals and Materials

Colistin sulphate (Lot 49H0635) was purchased from Sigma (produced in Denmark). Sodium colistin methanesulphonate was obtained from four different sources: colistin methanesulphonate (Lot 40K0601) from Sigma (produced in Denmark); Coly-Mycin from Dumex (Batch Number: A1680442, Copenhagen, Denmark); Coly-Mycin from Parke-Davis (Batch Number: 01389PZ, Caringbah, Australia), while Colomycin from Pharmax (Batch Number: 11060, Kent, UK) was a gift. All samples of colistin methanesulphonate were presumed pure and to exist as the penta(methanesulphonic acid) derivatives.

Isotonic phosphate buffer (0.067 M, pH 7.4) was prepared according to the formula in the Australian Pharmaceutical Formulary and Handbook (15th edition).

5.2.2 Analysis of colistin methanesulphonate in water by SAX-HPLC

Components in aliquots (50 μL) of the solutions of colistin methanesulphonate (1 mg/mL) prepared in water were separated on a 250 × 4.6 mm ID steel column prepacked

with 5 μm Spherisorb SAX (Alltech, Deerfield, IL, USA) through which a mobile phase consisting of acetonitrile:1.2 mM sodium sulphate (70:30, v/v) was pumped at 1.0 mL/min. The eluent was monitored for its UV absorbance at 210 nm.

5.2.3 Stability of colistin and colistin methanesulphonate in aqueous media

5.2.3.1 Stability of colistin and colistin methanesulphonate in water

Solutions of colistin sulphate and solutions of sodium colistin methanesulphonate (from Sigma, Pharmax and Parke-Davis) were prepared with Milli-Q water at a concentration of 0.1 mg/mL, and 10 mL aliquots of each transferred into 20-mL plastic bottles (Super Polyethylene Vial ™, Packard, Germany) kept at 4 ± 0.2°C and 37 ± 0.1°C. Milli-Q water was used as the control. Samples (0.5 mL) were taken at 0, 2, 4, 7, 14, 21, 28, and 60 days from the solutions kept at 4°C and at 0, 2, 4, 6, 12, 24, 48, 72, 96 and 120 hr from the solutions at 37°C, and stored immediately at -20°C pending analysis. Samples from the solutions of colistin methanesulphonate were examined by a SAX-HPLC method (Section 5.2.2). Colistin in the solutions of colistin sulphate and that formed by hydrolysis in the solutions of colistin methanesulphonate was quantified by the HPLC method for colistin (Chapter 3). The pH was monitored during the sample procedure.

5.2.3.2 Stability of colistin and colistin methanesulphonate in isotonic phosphate buffer (0.067 M, pH 7.4) at 37°C

Solutions of colistin sulphate and solutions of sodium colistin methanesulphonate (Sigma, Pharmax and Parke-Davis) were prepared in isotonic phosphate buffer (0.067 M, pH 7.4) at a concentration of 0.1 mg/mL, and 10 mL aliquots of each transferred into 20-mL plastic bottles (Packard) and kept at 37 ± 0.1°C. Isotonic phosphate buffer (0.067M, pH 7.4) was used as the control. Samples (0.5 mL) were taken from the solutions at 0, 2, 4, 6, 12, 24, 48, 72, 96 and 120 hr, and stored immediately at -20°C pending analysis. Colistin in the solutions of colistin sulphate and that formed by hydrolysis in the solutions of colistin methanesulphonate was quantified by HPLC (Chapter 3).

5.2.3.3 Stability of colistin and colistin methanesulphonate in human plasma

Blank human plasma (10 mL, Donation Number: O5881373, Australian Red Cross, Adelaide, Australia) was adjusted to pH 7.4 with phosphoric acid (85%, w/w) and spiked with colistin sulphate or sodium colistin methanesulphonate (Sigma, Pharmax and Parke-Davis) at a concentration of 20 mg/L. The spiked and control (drug-free) plasma was sterilized by passing through a 0.2 μm Minisart® filter (Sartorius, Germany) into sterile 50-mL centrifuge tubes (Greiner Labortechik, Austria) and kept at 37 ± 0.1°C. At 0, 2, 4, 6, 12, 24, 48, 72, 96 and 120 hr, samples (0.5 mL) were removed aseptically and immediately stored at -20°C pending analysis. Colistin in the solutions of colistin sulphate and that formed by hydrolysis in the solutions of colistin methanesulphonate was quantified by HPLC (Chapter 3).

5.2.3.4 Stability of colistin methanesulphonate in Mueller-Hinton broth

Sterile Mueller-Hinton broth (10 mL, Oxoid, Hampshire, England) was spiked with sodium colistin methanesulphonate (Dumex) at a concentration of 1024 μg/mL and kept at 37 ± 0.1°C in a shaking water bath (80 rpm). Samples (0.5 mL) were taken at 0, 5, 10, 15 and 30 min, and immediately stored at -20°C until assay. Colistin formed by the hydrolysis of colistin methanesulphonate was quantitated by the HPLC method for colistin (Chapter 3).

5.2.3.5 Stability of colistin methanesulphonate in rat urine

Blank rat urine (pH 6.8) was spiked with sodium colistin methanesulphonate (Sigma) at a concentration of 1.0 mg/mL. Aliquots (10 mL) were transferred into 50-mL centrifuge tubes (Greiner Labortechik, Austria) and kept at ambient temperature or at 37 ± 0.1°C in a shaking water bath (80 rpm). Samples (0.5 mL) were taken at 0, 2, 4, 6, 12 and 24 hr and immediately stored at -20°C pending analysis. Concentrations of colistin in urine were quantified by HPLC (Chapter 3).

5.2.3.6 Stability of colistin methanesulphonate in saline at 4°C

A solution of sodium colistin methanesulphonate (Sigma) was prepared in saline at a

concentration of 10 mg/mL and kept in a sterile 50-mL centrifuge tube (Greiner Labortechik, Austria) at 4°C. Samples (0.1 mL) were taken at 0, 2, 6, 12, 24, 36, 48 and 72 hr and stored at -20°C until analysis. Concentrations of colistin in the saline were quantified by HPLC (Chapter 3).

5.2.4 Data analysis

The percentages of colistin formed by the hydrolysis of colistin methanesulphonate were calculated on a molar basis. Colistin is a multi-component antibiotic and colistin A and colistin B always comprised more than 86% of the total (Decolin et al., 1997). Therefore, colistin A and colistin B were set as the markers for quantification of colistin. In the present study, the molecular weight of colistin was set at 1163, the average of the individual values for colistin A (Mw 1170) and B (Mw 1156) and that of colistin sulphate was 1408. The molecular weight of sodium colistin methanesulphonate was set at 1743, the average of the molecular weights of sodium penta(methanesulphonic acid) colistin A (Mw 1750) and B (Mw 1736). Although the ratios of colistin A to colistin B may vary in different sources of colistin sulphate and colistin methanesulphonate, the usage of average molecular weights would have little impact given the molecular weight difference of only 14.

5.3 Results

5.3.1 Stability of colistin in water, buffer and human plasma

Colistin is very stable in water at 4°C. After 60 days, the percentages of colistin A and colistin B remaining were 97.4% and 105.3%, respectively. When colistin was stored in water at 37°C for up to 120 hr, the pH varied in the range of 5.63 to 5.98 and there were no decreases in the concentrations of colistin A and colistin B (Figure 5.1). However, there were decreases in the concentrations of colistin in isotonic phosphate buffer (pH 7.4) and plasma at 37°C, and colistin A was less stable than colistin B.

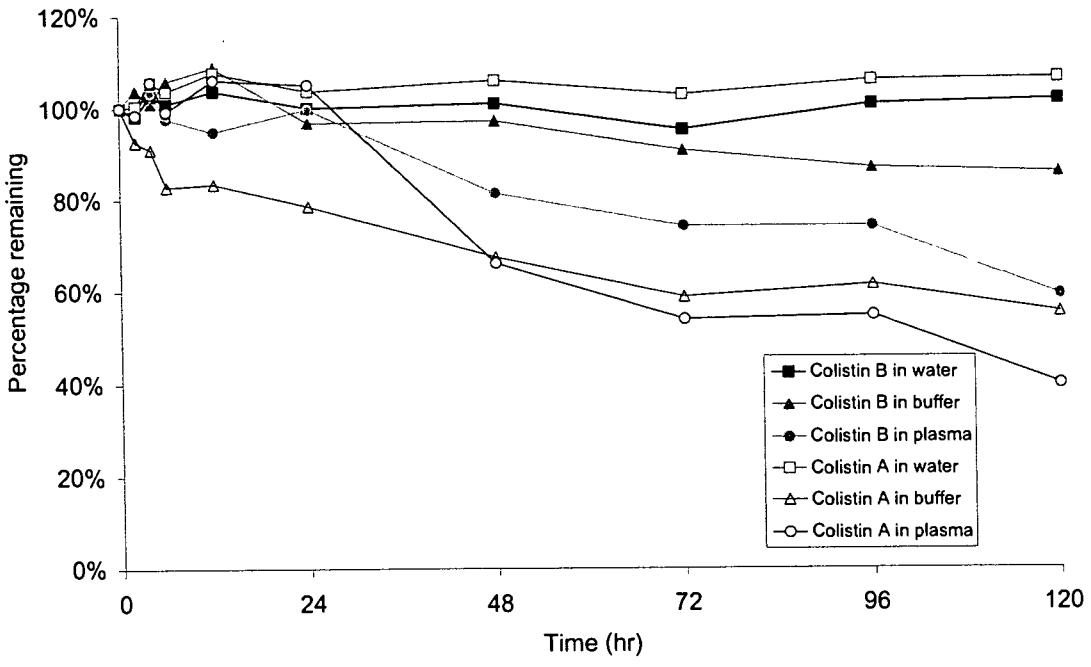

Figure 5.1 Percentage of initial concentrations of colistin A and colistin B remaining after storage at 37°C in water, isotonic phosphate buffer (0.067 M, pH 7.4) and human plasma (pH adjusted to 7.4 with phosphoric acid).

5.3.2 Development of a SAX-HPLC method for colistin methanesulphonate in water

The UV spectrum of colistin methanesulphonate in water was examined first in order to select a wavelength for its UV detection during HPLC. Figure 5.2 shows the UV spectrum of colistin methanesulphonate in water, which is similar to that of colistin (Figure 3.4). Since there was absorption around 210 nm, this wavelength was chosen.

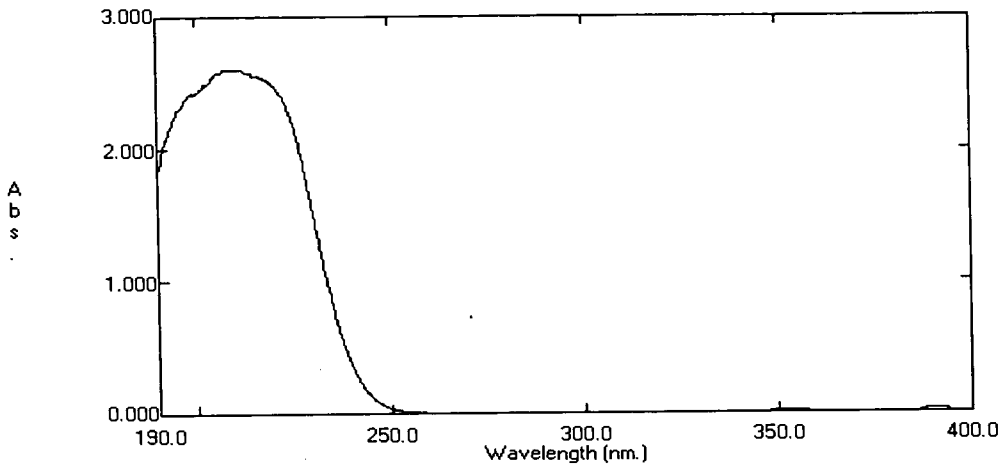

Figure 5.2 UV spectrum of colistin methanesulphonate in water

An eluent with a high organic content (acetonitrile:1.2 mM sodium sulphate, 70:30, v/v) was used in order to achieve good retention for colistin methanesulphonate. Figure 5.3 A shows a typical chromatogram of a fresh solution containing 0.1 mg/mL colistin methanesulphonate (Sigma). The chromatogram contained two dominant peaks (peaks 1 and 3) and several minor ones (peaks 2, 4, 5, 6, and 7). Several attempts using electrospray ionization mass spectrometry (ESI-MS) failed to identify peaks 1 and 3. Nevertheless, all seven peaks have been proved to be related to colistin A and/or B by collecting the elutes of each peak and adapting the specific HPLC method for the assay of colistin methanesulphonate in plasma (Chapter 4). There were no qualitative differences between the chromatograms of colistin methanesulphonate from three different sources (Sigma, Pharmax and Parke-Davis). Milli-Q water was used as the control and no peaks appeared between the retention times of peaks 1 and 7 (Figure 5.3B). When colistin was chromatographed, no peaks were apparent at the retention times of the seven peaks of colistin methanesulphonate (Figure 5.3C).

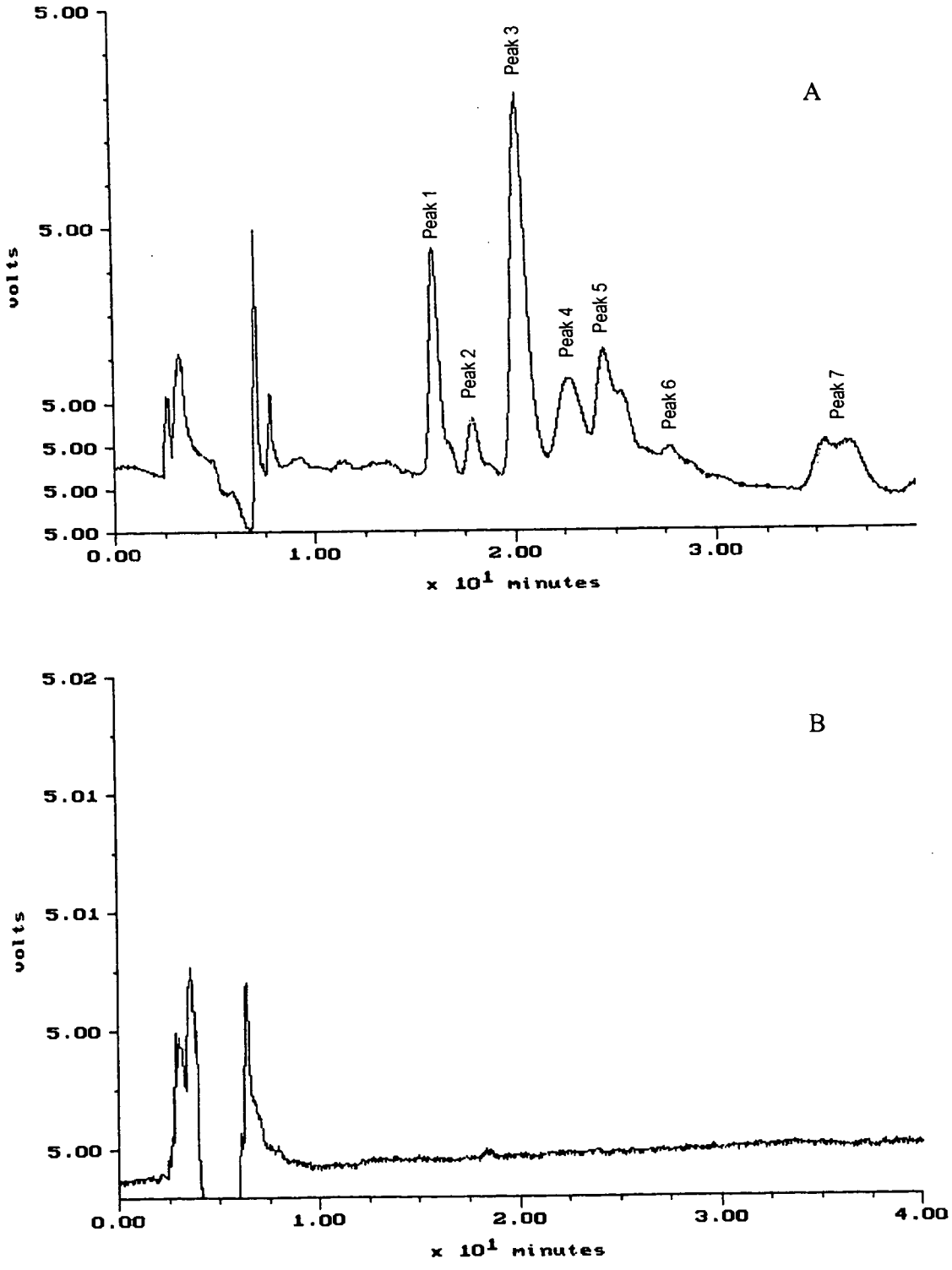

Figure 5.3 See remainder of figure and legend on following page.

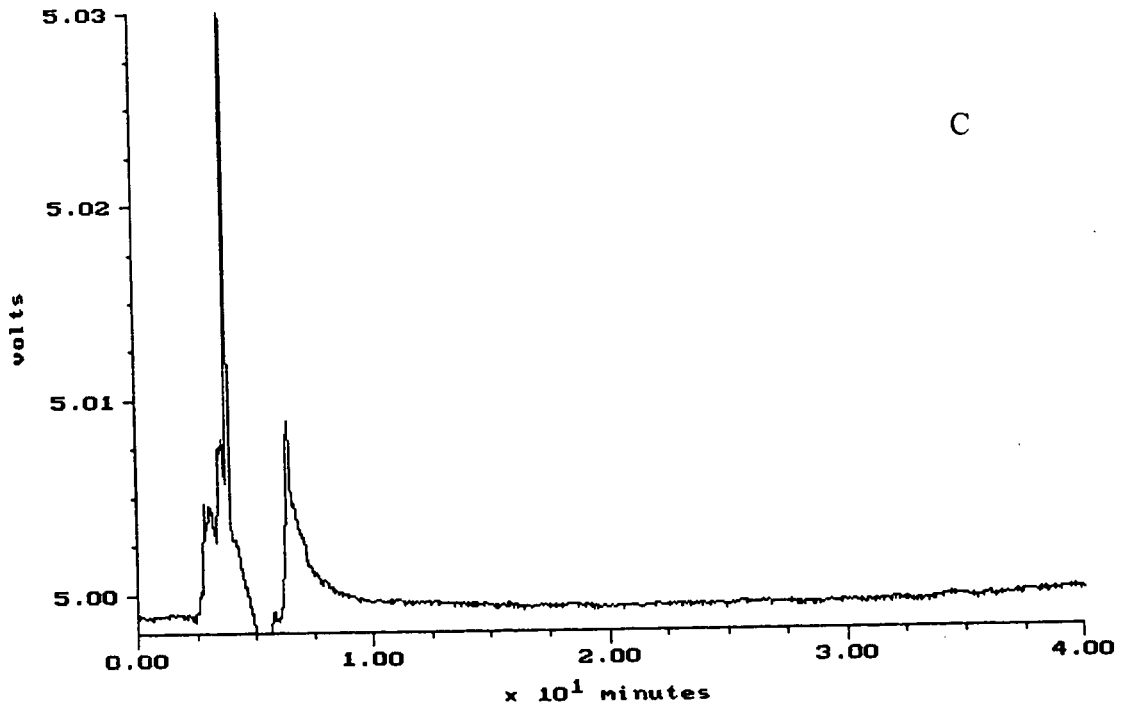

Figure 5.3 Typical chromatograms obtained for a fresh solution of colistin methanesulphonate in water (A), water (B) and colistin sulphate in water (C) examined by SAX-HPLC.

5.3.3 Stability of colistin methanesulphonate in water

Using the HPLC assay for colistin (Chapter 3), the stability of colistin methanesulphonate in water at 37°C was explored by measuring the concentrations of colistin formed by the hydrolysis of colistin methanesulphonate. There was no colistin measurable in the fresh solutions of colistin methanesulphonate prepared from the three sources. The limit of quantification was 0.4 mg/L, which is equivalent to levels of colistin in all samples of less than 0.4%. The time to reach the maximum concentration of colistin differed slightly with samples from the three sources (Figure 5.4). After 48 hr, the highest conversion to colistin was 62.8% (Sigma) and 60.3% (Pharmax), while a maximal conversion of 79.6% was observed at 72 hr for colistin methanesulphonate from Parke-Davis. No real trend in the values of pH was observed, varying in the range between 6.35 and 6.90.

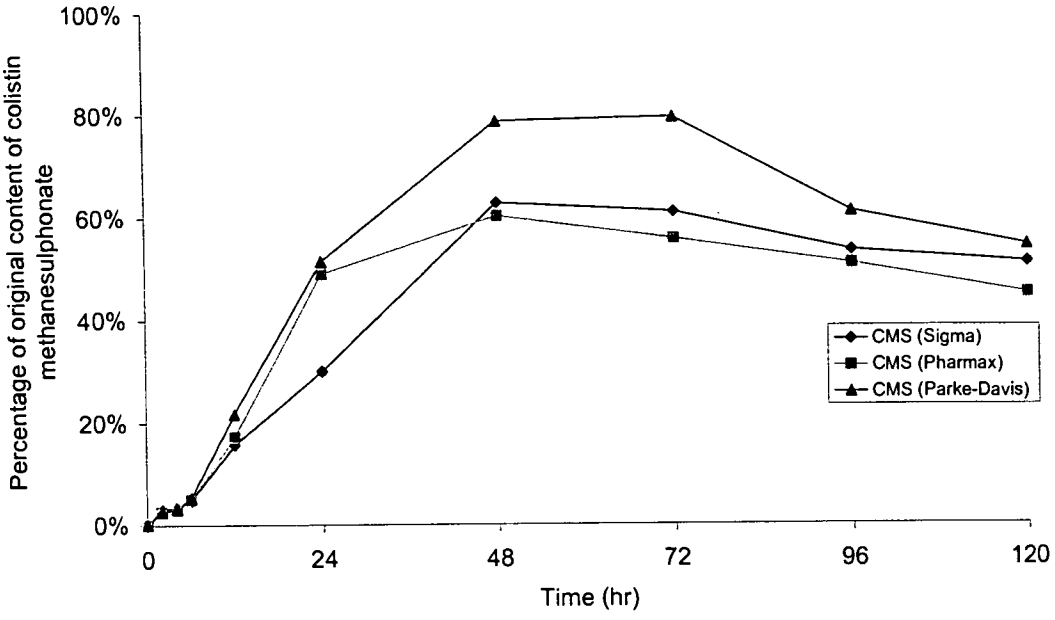

Figure 5.4 Levels of colistin in samples of colistin methanesulphonate in water kept at 37°C, expressed as a percentage of original colistin methanesulphonate on a molar basis. *CMS, colistin methanesulphonate.

When the colistin methanesulphonate samples incubated in water at 37°C were chromatographed using SAX-HPLC, the chromatograms showed decreases in the areas of all seven peaks after 2 hr; these peaks had disappeared after 12 hr of storage (Figure 5.5).

After storage of colistin methanesulphonate in water at 4°C for two days, no colistin was detected and the appearance of the chromatogram using SAX-HPLC was quantitatively identical to that of the fresh solution. After four days, the peaks 1 and 3 in SAX-HPLC began to decrease. After 60 days, approximately 30% of colistin methanesulphonate was converted to colistin. Colistin methanesulphonate from all three sources displayed similar rates of hydrolysis at 4°C. Figure 5.6 shows a typical chromatogram of colistin methanesulphonate stored in water at 4°C for 14 days.

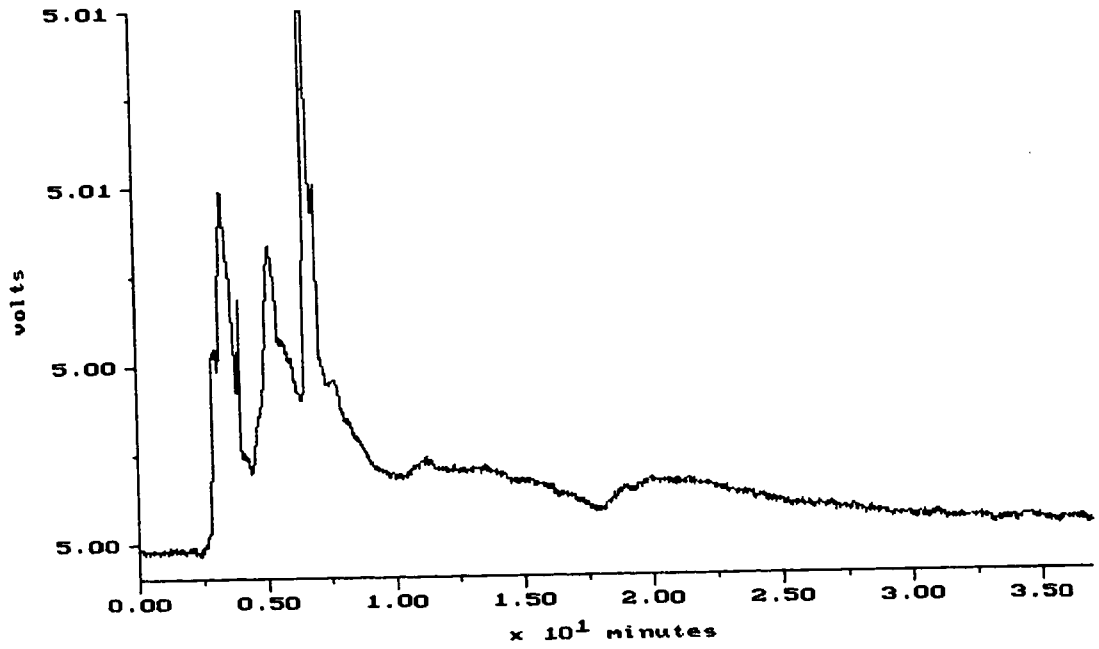

Figure 5.5 Typical chromatogram of colistin methanesulphonate in water stored at 37°C for 12 hr.

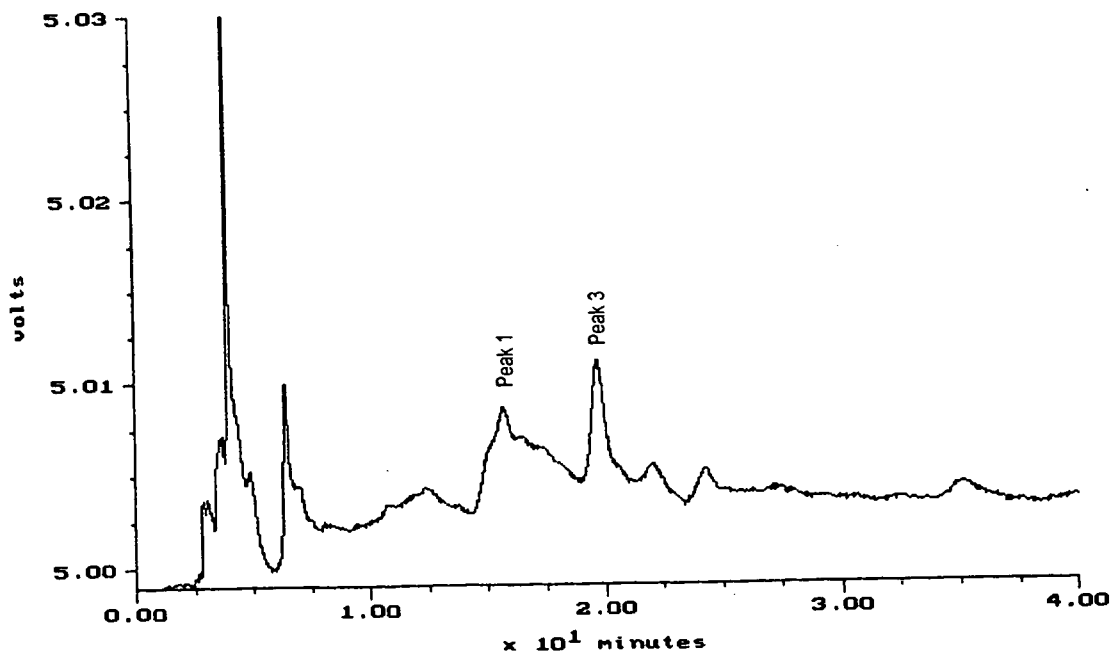

Figure 5.6 Typical chromatogram of colistin methanesulphonate in water stored at 4°C for 14 day.

5.3.4 Stability of colistin methanesulphonate in buffer

During the first 24 hr of storage in isotonic phosphate buffer (0.067 M, pH 7.4), the rates of formation of colistin by hydrolysis of colistin methanesulphonate were more rapid (Figure 5.7) compared with those in water (Figure 5.4). The concentrations of colistin increased to a maximum between 12 and 24 hr for all three samples of colistin methanesulphonate before decreasing to constant values beyond 48 hr of storage (Figure 5.7). There were no substantial differences among the three sources of colistin methanesulphonate in the time-course of formation of colistin, although colistin methanesulphonate from Parke-Davis had a higher maximum conversion than colistin methanesulphonate from Sigma and Pharmax (Figure 5.7).

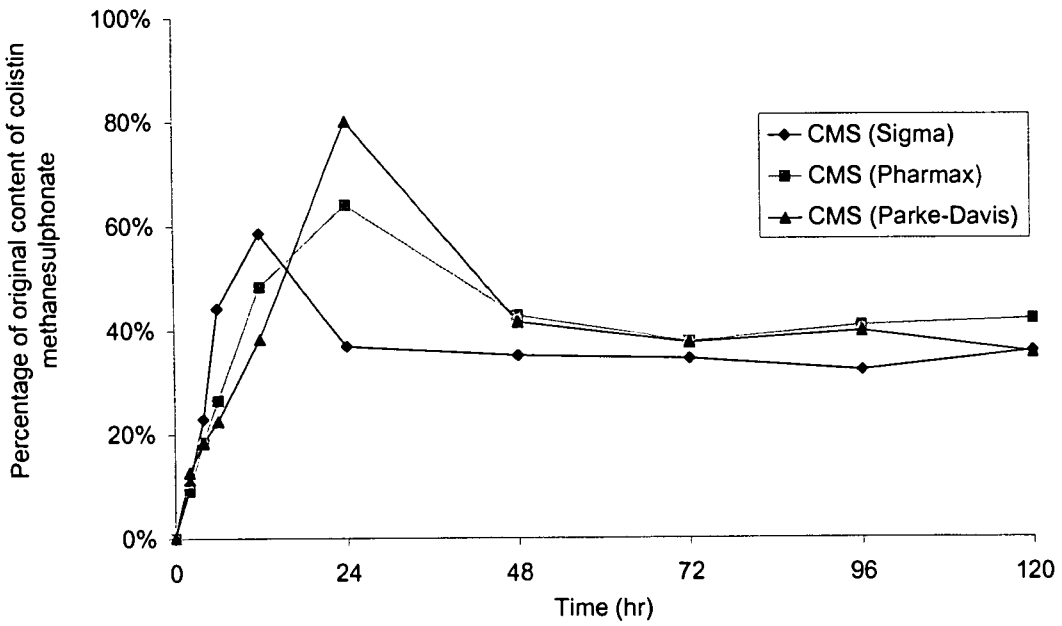

Figure 5.7 Levels of colistin in samples of colistin methanesulphonate in buffer kept at 37°C, expressed as a percentage of original colistin methanesulphonate on a molar basis. *CMS, colistin methanesulphonate.

5.3.5 Stability of colistin methanesulphonate in human plasma

The stability of colistin methanesulphonate was also investigated in human plasma at 37°C. During the first 24 hr, the rates of formation of colistin from colistin

methanesulphonate (Figure 5.8) were similar with those in buffer (Figure 5.7), but more rapid than those in water (Figure 5.4). The concentrations of colistin decreased slowly after reaching a maximum.

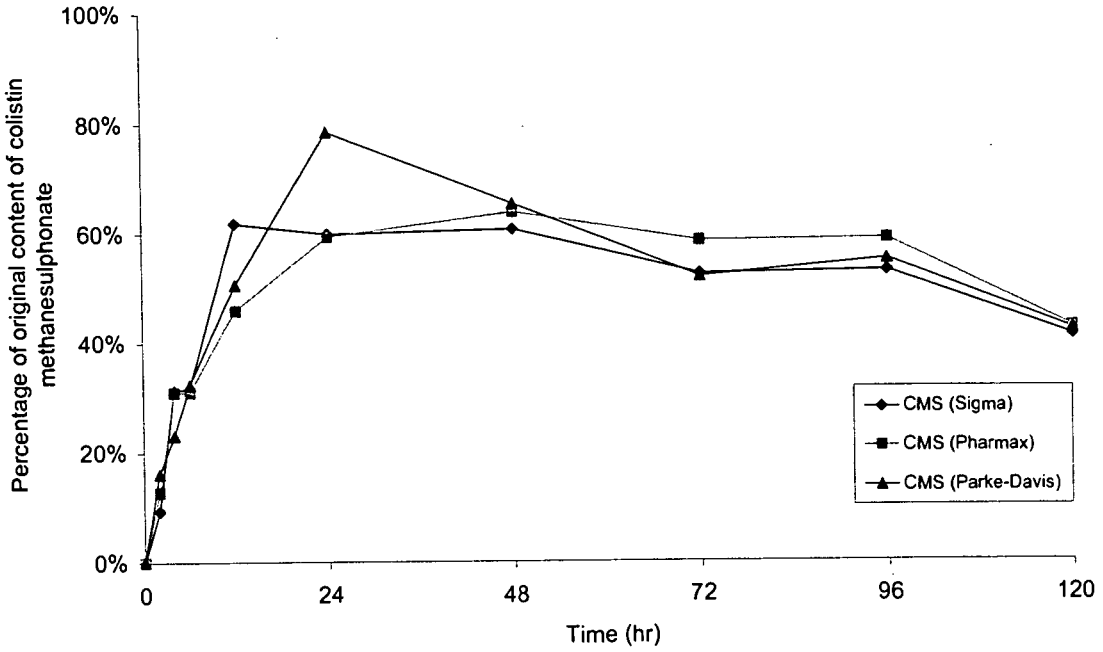

Figure 5.8 Levels of colistin in samples of colistin methanesulphonate in human plasma kept at 37°C, expressed as a percentage of original colistin methanesulphonate on a molar basis. *CMS, colistin methanesulphonate.

5.3.6 Stability of colistin methanesulphonate in rat urine

The stability of colistin methanesulphonate (Sigma) in rat urine was examined at ambient temperature and 37°C (Figure 5.9). In 4 hr, 4.4% of colistin methanesulphonate was converted to colistin at ambient temperature while 29.3% was converted at 37°C.

5.3.7 Stability of colistin methanesulphonate in saline at 4°C

Hydrolysis of colistin methanesulphonate in saline at 4°C up to 72 hr was minor, with less than 0.06% colistin methanesulphonate converted to colistin (Figure 5.10).

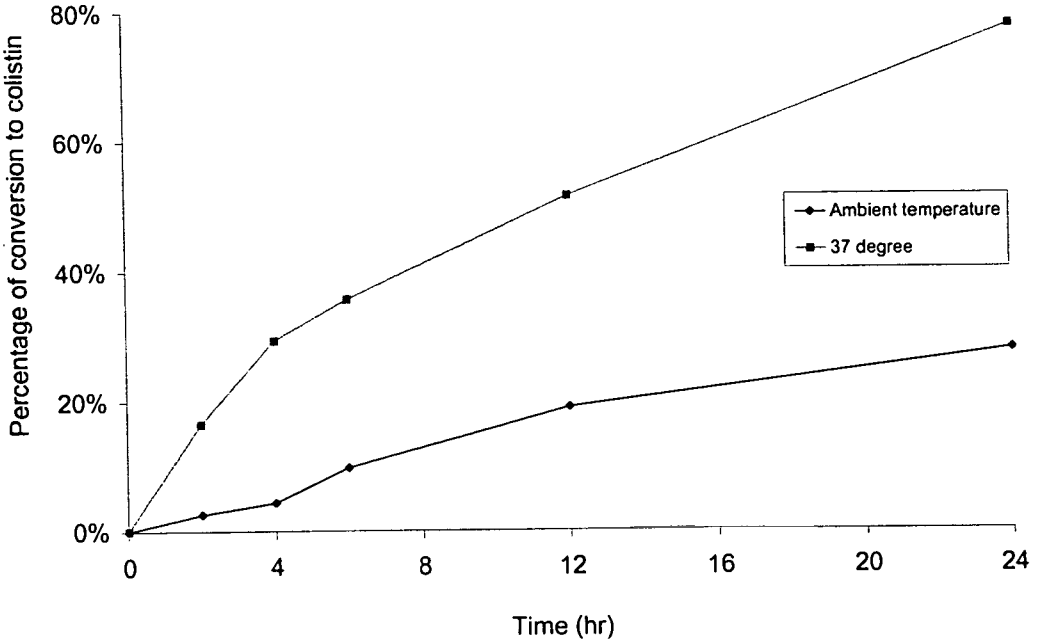

Figure 5.9 Levels of colistin in samples of colistin methanesulphonate (Sigma) in rat urine kept at ambient temperature (23 ± 2°C) and 37°C, expressed as a percentage of original colistin methanesulphonate on a molar basis.

5.3.8 Stability of colistin methanesulphonate in Mueller-Hinton broth

No colistin was detected when colistin methanesulphonate (Dumex) at a concentration of 1024 μg/mL in Mueller-Hinton broth was stored at 37°C for 30 minutes. With a limit of quantification in broth of 0.1 mg/L, this equated to a conversion to colistin of less than 0.2%.

5.4 Discussion

Barnett et al. (Barnett et al., 1964) and Beveridge et al. (Beveridge et al., 1967) observed marked increases in antibacterial activity when aqueous solutions of colistin methanesulphonate were incubated at 37°C and concluded that colistin methanesulphonate was converted to colistin by hydrolysis. Electrophoresis and detection with bioautograms were used in their studies to explore the conversion.

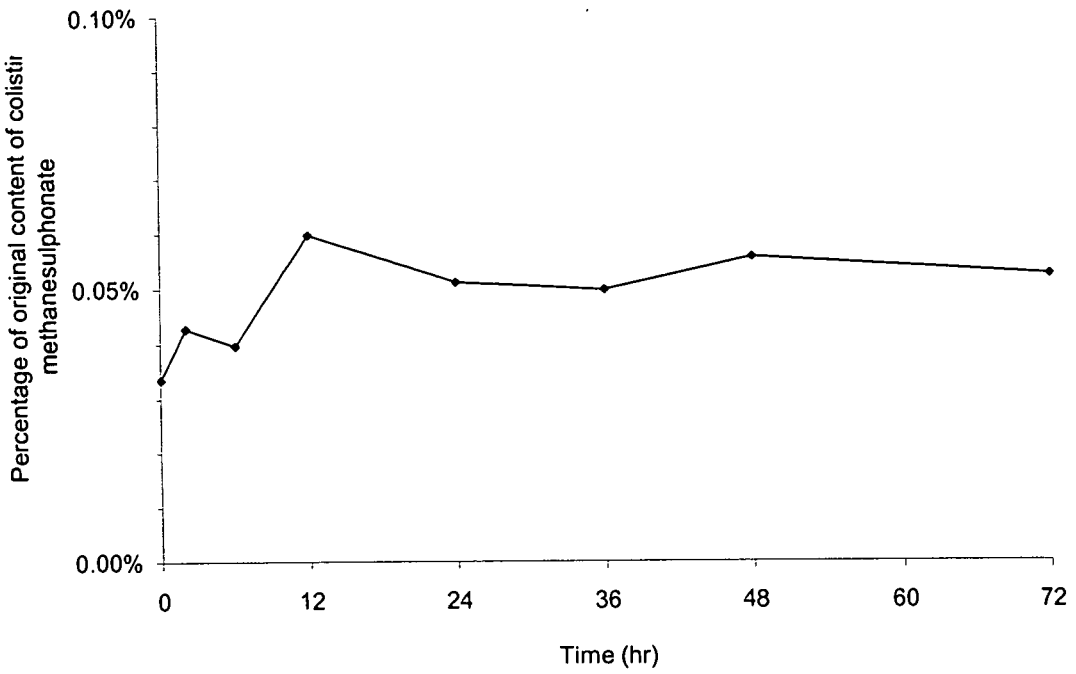

Figure 5.10 Levels of colistin in samples of colistin methanesulphonate (Sigma) in saline kept at 4°C, expressed as a percentage of original colistin methanesulphonate on a molar basis.

The HPLC method for colistin (Chapter 3) was used in the present chapter to explore the conversion of colistin methanesulphonate to colistin in several aqueous media. Barnett et al. noted that colistin methanesulphonate made in the United States had a higher toxicity in mice (LD_{50} = 220 mg/kg) than Colomycin produced in the United Kingdom (LD_{50} = 650 mg/kg) (Barnett et al., 1964). Given the known greater toxicity of colistin compared with colistin methanesulphonate (Schwartz et al., 1960), the samples from the two countries might have had different percentages of colistin or other partial hydrolysis products arising from manufacture or from hydrolysis during storage, which may have caused different toxicity. Therefore, the stability of three different sources of colistin methanesulphonate from Sigma, Parke-Davis and Pharmax was examined in water, isotonic phosphate buffer and human plasma.

Initial studies investigating the stability of colistin in water stored in glass containers at 37°C showed rapid decreases in the concentrations of colistin A and colistin B to 10% of their original concentration after 120 hr. Compared with the data obtained during storage

in polyethylene containers (Figure 5.1), it could be concluded that there is the potential for significant adsorption of colistin to glass.

Colistin was stable in water for up to 120 hr at 37°C. However, the pH values in phosphate buffer and plasma, approximately 1.5 units above the values measured in water (between 5.63 to 5.98), may account for the instability of colistin in these two media. Furthermore, in phosphate buffer and plasma at 37°C, colistin A appeared to be less stable than colistin B. The reason remained unknown, but the different fatty acid chains, the only difference in their chemical structures, may be a contributing factor.

Anion-exchange HPLC has been used widely for the analysis of biomolecules, especially acidic drugs (Law et al., 2000), since the positively charged quaternary amine groups on the resin have the potential to provide good retention for negatively charged compounds. There have not been any HPLC methods reported for separating and detecting the components of colistin methanesulphonate. In the present study, a method using SAX-HPLC was developed for the analysis of colistin methanesulphonate in water and applied to the stability study. Notwithstanding the SAX-HPLC method could not separate all the 31 types of potential hydrolysis products of colistin methanesulphonate plus itself, it is the first HPLC method to display the many different components in colistin methanesulphonate. Furthermore, hydrolysis of colistin methanesulphonate in water was shown by the disappearance of the seven major peaks in the SAX-HPLC chromatograms. It is proposed that the disappearance of the peaks was a consequence of hydrolysis of the methanesulphonate groups which led to less negative charges on the molecules of colistin methanesulphonate. Instead of electrophoresis and bioautograms, the specific HPLC method for colistin (Chapter 3) was applied to prove and quantify the formation of colistin from the hydrolysis of colistin methanesulphonate. Unfortunately, the SAX-HPLC method could not be used to examine the hydrolysis of colistin methanesulphonate in buffer and plasma, probably because of the impact of electrolytes in both systems.

The formation of colistin from colistin methanesulphonate in buffer and plasma was complicated by the observed instability of colistin in both media. The pH of the solutions of colistin methanesulphonate in buffer was from 0.5 to 1 unit above the values measured for the solutions of colistin methanesulphonate in water, which may explain the greater

rate of formation of colistin. Although colistin was stable in water at 37°C (Figure 5.1), concentrations of colistin formed by hydrolysis of colistin methanesulphonate decreased after reaching a maximum. The data in Figure 5.1 demonstrate that colistin was more stable at pH values ranging from 5.63 to 5.98 (in water) rather than pH 7.4 (in buffer and plasma). Therefore, the higher pH values (6.35 - 6.90) in the water solutions of colistin methanesulphonate, compared with colistin in water, may account for the decrease in concentrations of colistin. In addition, it is unknown whether any products from the hydrolysis of colistin methanesulphonate would affect the stability of colistin.

From measuring the appearance of colistin in the solutions of colistin methanesulphonate in water, only about 20% of colistin methanesulphonate had been hydrolyzed completely to colistin by 12 hr. This suggested that a large fraction of the colistin methanesulphonate lost from samples, as observed in the chromatogram of Figure 5.5 obtained at 12 hr, was in the form of partial methanesulphonate derivatives of colistin, which could account for the peaks appearing at retention times of less than 10 min in Figure 5.5, and not present in Figure 5.3A. Therefore, this does not mean that no hydrolysis had occurred when no or little colistin was detected in aqueous media containing colistin methanesulphonate. The end hydrolysis product, colistin, was only formed after all the five methanesulphonate groups had been cleaved from colistin methanesulphonate. While neither of the two commercial products from British or United States suppliers contained measurable amounts of colistin in freshly prepared solutions, a possible explanation for the differences in toxicity of the two products (Barnett et al., 1964) may be differences in the degree of derivatization of colistin with methanesulphonate during manufacture. Other possibilities are improper storage under different conditions or different *in vivo* hydrolysis rates, which lead to different concentrations of colistin.

The plateau in the concentrations of colistin after the highest conversion of colistin methanesulphonate to colistin in buffer (Figure 5.7) was not observed in plasma (Figure 5.8). Although it was difficult to explain the appearance of the plateau, the slow decrease in concentrations of colistin in plasma after 24 hr (Figure 5.8) is not unexpected given the steady decrease in concentrations of colistin when the substance was stored in plasma at 37°C (Figure 5.1).

Since urine would be collected in four-hour intervals until 24 hr after the administration of colistin methanesulphonate in the pharmacokinetic study (Chapter 7), the stability of colistin methanesulphonate (Sigma) was investigated in the current chapter. The hydrolysis of colistin methanesulphonate in rat urine at 37°C in 24 hr gave a similar trend with those in isotonic phosphate buffer and human plasma. However, the hydrolysis rate significantly decreased at ambient temperature.

Although very little colistin was detected in the solutions of colistin methanesulphonate (Pharmax) in saline stored at 4°C in three days, it can not be concluded that no hydrolysis occurred. The instability of colistin methanesulphonate in water at 4°C and at 37°C suggested that solutions of colistin methanesulphonate for pharmacokinetic and pharmacodynamic studies and therapeutic use should be prepared freshly to minimize the errors in interpretation of pharmacokinetic and pharmacodynamic data and potential toxicity caused by hydrolysis prior to administration. Hence, improper storage of colistin methanesulphonate solutions before administration, generating the much more toxic colistin, may have been one of the reasons for the differences in toxicity of colistin methanesulphonate from different sources reported by Barnett and coworkers (Barnett et al., 1964).

There have not been any detailed reports on the antibacterial activity of the partial methanesulphonate derivatives of colistin. The rapid formation of colistin in buffer, with an estimated 20% conversion from colistin methanesulphonate after 4 hr incubation at 37°C, corresponded well with the rapid increase in the antibacterial activity of solutions of colistin methanesulphonate, when tested against *Escherichia coli* after 4 hr of storage at 37°C in 0.1 M phosphate buffer (pH 7.0) (Beveridge et al., 1967). Other reports have shown that the antibacterial activity of colistin against *E. coli*, *P. aeruginosa*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae* was usually three- to forty-fold the activity of colistin methanesulphonate (Schwartz et al., 1960). Therefore, even a 20% conversion to colistin within 4 hr would increase the antibacterial activity considerably.

The stability of colistin methanesulphonate in Mueller-Hinton broth was examined in 30 min at 37°C in the current study. The initial concentration of colistin methanesulphonate (Dumex) was equivalent to 64×MIC of colistin methanesulphonate when the same batch

was tested against *P. aeruginosa* ATCC 27853 in the *in vitro* pharmacodynamic study of colistin and colistin methanesulphonate (Chapter 2). According to that study, when the killing effect of colistin methanesulphonate on the same strain was examined, the Log_{10} CFU/mL decreased from 6.08 to 2.78 in the first 15 min (Chapter 2). The current observation, of insignificant conversion of colistin methanesulphonate to colistin in Mueller-Hinton broth in the first 30 min, demonstrated that the sulphomethyl derivatives possess intrinsic antibacterial activity. However, the comparative antibacterial activities of the numerous partially derivatized (or hydrolyzed) forms of colistin methanesulphonate still remain unknown. Therefore, it is anticipated that the pharmacodynamics of colistin methanesulphonate *in vitro* and *in vivo* will be extremely complex.

As reviewed in section 1.3.6.2, Reed et al. recently reported on the pharmacokinetics of colistin following intravenous administration of colistin methanesulphonate to patients with CF (Reed et al., 2001). Prior to HPLC, samples of plasma were treated at 54°C for 2 hr in the presence of 3 M perchloric acid and 0.1 M hydrochloric acid. According to the data above on the stability of colistin methanesulphonate, pre-treatment at 54°C for 2 hr might accelerate the hydrolysis of colistin methanesulphonate to colistin. This was supported by the data showing considerable formation of colistin when colistin methanesulphonate was incubated in 2 M hydrochloric acid for 2 hr (Beveridge et al., 1967). Furthermore, as discussed in section 4.4, 0.1 M hydrochloric acid did cause slight hydrolysis of colistin methanesulphonate to colistin in 30 min at room temperature. Therefore, it is most likely that the concentrations of colistin measured by Reed et al. (Reed et al., 2001) were the sum of colistin originally in plasma when the samples were collected and colistin formed from the hydrolysis of colistin methanesulphonate during sample pre-treatment.

5.5 Conclusion

The stabilities of colistin and colistin methanesulphonate in aqueous media were studied by specific HPLC methods. Colistin was very stable in water, but less stable in isotonic phosphate buffer and human plasma at 37°C. The degradation of colistin

methanesulphonate in water was displayed by the disappearance of colistin methanesulphonate using SAX-HPLC, and was measured by the appearance of colistin with RP-HPLC. This is the first report using a specific HPLC method to measure the concentrations of colistin formed by the hydrolysis of colistin methanesulphonate in different aqueous solutions and a SAX-HPLC method to display the hydrolysis of colistin methanesulphonate in water. These results will be of great value for studies on the pharmacokinetics and pharmacodynamics of colistin and colistin methanesulphonate in rats and humans (Chapters 6, 7 and 8). They also show that, when concentrations of colistin are being measured in biological fluids following the administration of sodium colistin methanesulphonate, care is needed to minimize falsely elevated values arising from the hydrolysis of colistin methanesulphonate to colistin during sample storage and preparation for analysis.

Chapter 6 Pharmacokinetics of colistin after intravenous administration in rats

6.1 Introduction

As reviewed in section 1.3.6.2, there has been very limited information on the pharmacokinetics of colistin. Most reports have focused on the pharmacokinetics of colistin following a dose of colistin methanesulphonate. There are only a few reports on the pharmacokinetics of colistin, and these have come from studies following the intravenous administration of colistin sulphate in the calf (Renard et al., 1991; Ziv et al., 1982; Ziv et al., 1980) and intramuscular injection in the dog (Al-Khayyat et al., 1973). Following intravenous administration of 5 mg/kg colistin sulphate in calves, the pharmacokinetics were described by a three-compartment model with average total body clearance, volume of distribution and terminal half-life of 3.4 mL/min/kg, 1.30 L/kg and 269 min, respectively (Ziv et al., 1982). A terminal half-life of 2.7 hr was observed after a single intramuscular injection of 1.1 mg/kg colistin sulphate to dogs (Al-Khayyat et al., 1973).

Most information on the pharmacokinetics of colistin was based on microbiological assays for measuring colistin in plasma and urine. The most significant limitation of microbiological assays is the poor specificity. Since colistin is a multi-component antibiotic with two major ones (colistin A and colistin B), it is impossible to measure their concentrations separately by microbiological assays. Furthermore, microbiological assays are rendered useless in the presence of other co-administered antibiotics that are active against the test strains.

Colistin sulphate is used clinically for topical and oral purposes. It is not absorbed from the adult gastro-intestinal tract (Chamberlain et al., 1976), mucosal surfaces or even inflamed surfaces or burns (O'Grady et al., 1997). However, absorption from the gastro-intestinal tract was observed in infants (Ross *et al.*, 1960). The serum concentrations of colistin have recently been investigated in six healthy volunteers and five CF patients administered a single dose (25 mg) of dry colistin sulphate powder by inhalation (Le Brun et al., 2002). The peak serum concentration measured by HPLC (Le Brun et al.,

2000b) ranged from 77 to 159 μg/L. The shortcomings of their HPLC assay have been discussed in Chapters 1 and 3.

The current study describes the pharmacokinetics of colistin, colistin A and colistin B in rats, after an intravenous bolus of 1 mg/kg colistin sulphate, from concentrations measured by a specific HPLC method (described in Chapter 3).

6.2 Materials and Methods

6.2.1 Materials

Polyethylene tubing (medical grade, single lumen, 0.58 mm ID × 0.96 mm OD was obtained from Paton Scientific (SA, Australia). Silicone rubber tubing (0.51 mm ID × 0.94 mm OD) was purchased from Dow Corning Corporation (Midland, MI, USA). Plastipak 1-mL syringes and hypodermic needles (23G×1¼ single use needle) were from Becton Dickinson (Singapore), surgical swabs (Chux hand towel) from Johnson and Johnson (North Ryde, NSW, Australia), Medi-swab (saturated with 70% isopropyl alcohol) from Smith and Nephew (Clayton, Vic, Australia), halothane BP from Laser Animal Health (Salisbury, Qld, Australia), heparin (1000 IU/mL, sterile) from David Bull Laboratories (Mulgrave, Vic, Australia), heparinised saline (50 IU/5 mL, sterile) from Astra Pharmaceuticals (North Ryde, NSW, Australia), saline (sterile sodium chloride 0.9%) from Delta West (Bentley, WA, Australia), and surgical silk (size 3 silicone treated braid, non-sterile, nonabsorbable surgical suture) from Davis and Geck (Melbourne, Vic, Australia). All the surgical instruments, including scissors 10 cm (sharp tip), scissors 10 cm (blunt tip), vena scissors, hemostats, and forceps 10 cm (straight, curved & curved with very fine tip), were obtained from World Precision Instruments, Inc. (Glen Waverley, Vic, Australia).

6.2.2 Animals

Five male Sprague-Dawley rats (300 - 350 g) (Gilles Plains Animal House, Adelaide, South Australia), were housed in an animal care facility with a 12-hr light/dark cycle (6:00 - 18:00) and a temperature of 21 ± 1°C. Free access to food (Ridley Agriproducts Pty. Ltd., Murray Bridge, South Australia) and water was allowed at all times. Animals

were acclimated for a minimum of 5 days prior to experimentation. All experiments performed with rats were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (6th edition, 1997). The animal protocols were approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Sciences (Adelaide, South Australia).

6.2.3 Catheterization of jugular vein

At least two days prior to administration of colistin sulphate, surgery was performed for the insertion of a cannula into the right jugular vein of the rat.

The rat was placed into a desiccator and 1 or 2 mL of halothane was added to temporarily anaesthetise the rat. General anaesthesia was maintained with continuous inhalation of halothane. A 4 cm length of silicone rubber tubing was sleeved on to the end of a 30 cm length of polyethylene tubing for insertion into the right jugular vein. The cannula was filled with heparinised saline (10 IU/mL) before the cannulation. All the instruments used for surgery were cleaned and disinfected in Betadine (10% w/v povidone-iodine, Salisbury, SA, Australia) for 20 min before use. Strict aseptic technique was not necessary, but the operation area was clean.

The rat was placed on its back and the area above the right jugular vein was cleaned with a Medi-swab saturated with 70% isopropyl alcohol. A longitudinal incision of approximately 2 - 3 cm was made on the skin over the jugular vein using a pair of blunt ended scissors. The vein was exposed by two pairs of small forceps to clear surrounding tissues. Using a large needle with the piece of cannula tubing attached, a subcutaneous tunnel was formed from the site of the jugular vein to the centre of the back of the rat between the two ears. Two sutures were placed around the jugular vein, leaving approximately 0.5 - 1 cm between the two sutures. Tension was applied to the suture distal to the heart and a small incision made in the vein between the two sutures. The cannula was inserted into the jugular vein and advanced towards the heart (about 3 - 4 cm in length). Both of the sutures were tied around the cannula to hold it in position. To ensure that the cannula was in the correct position, blood was withdrawn into a syringe attached to the end of the cannula. Once blood was obtained, the cannula was flushed and filled with heparinised saline and the end of the cannula was capped with a non-bevelled 23G needle connected to a syringe filled with heparinised saline. The muscles and skin at

the site of the incision were closed by a series of approximately 5 to 6 sutures. The cannula emerging from the back of the head was secured and clamped with a pair of hemostats while the length was cut so that only approximately 2 cm protruded from the body of the rat. A pin was placed in the end of the cannula to prevent blood flowing into the tubing.

The rat was allowed to recover from the anaesthetic in a plastic tub lined with surgical swabs and the tub was placed on a heating pad set to 37°C. Recovery usually took about 10 minutes. Once recovered and mobile, the rat was transferred to a metabolic cage (Tecniplast, Buguggiate, Italy), as shown in Figure 6.1, with free access to food and water. The rat was allowed at least two days to recover before use.

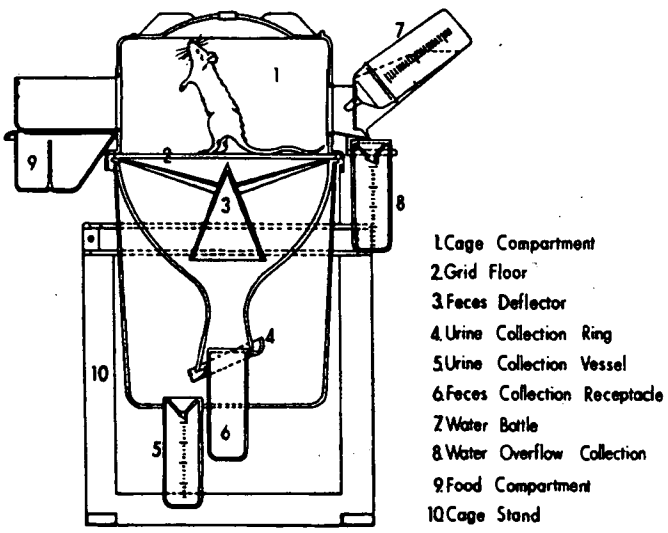

Figure 6.1 The rat free-standing metabolic cage used in the pharmacokinetic study (Kraus, 1980).

6.2.4 Dosage and collection of samples

On the day of the experiment, colistin sulphate (Sigma, St. Louis, MO) (1 mg/kg in 300 μL sterile saline) was injected in less than 20 seconds into the jugular vein and the cannula flushed with 1 mL heparinized saline. Studies conducted *in vitro* indicated that less than 0.02% of the dose of colistin sulphate remained in the cannula after the flush. Colistin used in the dosing solution was shown by HPLC analysis with UV detection (Decolin et al., 1997) to comprise 86.5% colistin A and colistin B.

Blood (400 µL) was collected prior to administration of colistin and at 10, 20, 30, 60, 90, 120, 180 and 240 min thereafter. When collecting samples, the first 100 µL blood was withdrawn and kept in the syringe. After collecting the actual sample with another syringe, the 100 µL blood in the first syringe was returned to the rat together with 400 µL of heparinized saline to ensure that the cannula was flushed and a constant intravascular volume was maintained. Blood was centrifuged (1000 *g*, 10 min) immediately and the plasma stored at -20°C pending assay. Urine was collected from the metabolic cage prior to administration of colistin and over the intervals 0 to 4, 4 to 8, and 8 to 12 hr thereafter. At the end of each interval, the metabolic cage was rinsed with 5 to 10 mL Milli-Q water and kept separately for assay. Urine samples were stored at -20°C pending assay.

6.2.5 Determination of colistin in plasma and urine

Concentrations of colistin in plasma and urine were determined by the HPLC method for colistin in plasma (Chapter 3) with minor modifications. These included changes to the volume of plasma (200 µL), the amount of internal standard (10 µL of 2.5 mg/L netilmicin sulphate), the SPE C_{18} cartridges (Sep-Pak, Waters, Milford, MA, USA), injection volume for HPLC (30 µL), and the mobile phase. The latter, consisting of acetonitrile-tetrahydrofuran-water (50:30:20, v/v), generated a shortened overall run time of 18 min with retention times for derivatized netilmicin, colistin B and colistin A, at approximately 10, 12 and 14 min, respectively. For measuring concentrations of colistin in urine, 200 µL of undiluted urine was used.

For plasma, calibration curves were constructed with concentrations of colistin sulphate ranging from 0.1 mg/L to 4.0 mg/L. The intra-day accuracy and reproducibility was 0.25 ± 0.02 mg/L and 3.74 ± 0.11 mg/L (n = 6) for colistin sulphate at 0.25 mg/L and 3.75 mg/L, respectively, and the corresponding inter-day accuracy and reproducibility was 0.26 ± 0.02 mg/L and 3.74 ± 0.12 mg/L (n = 6).

For urine, calibration curves were constructed with concentrations of colistin sulphate ranging from 0.1 mg/L to 0.8 mg/L. The intra-day accuracy and reproducibility were 0.20 ± 0.01 mg/L and 0.64 ± 0.02 mg/L (n = 6) for colistin sulphate at 0.20 mg/L and 0.60 mg/L, respectively, while the respective inter-day accuracy and reproducibility were 0.21 ± 0.01 mg/L and 0.60 ± 0.03 mg/L (n = 6).

The concentrations of colistin A and colistin B (as the sulphate salt) were calculated from the concentrations of colistin sulphate and the ratio of the area of the respective chromatographic peaks and their summed areas.

6.2.6 Protein binding

The binding of colistin in plasma *in vitro* was measured by equilibrium dialysis using a perspex dialysis cell unit containing two reservoirs (750 µL volume in each) separated by a Spectra/Por-2 membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA). Fresh plasma pooled from four drug-free rats was adjusted to pH 7.4 with 0.5 M hydrochloric acid and spiked with colistin sulphate at concentrations of 4.0, 8.0 and 12.0 mg/L. Two replicates at each concentration were incubated at 37°C for 30 min. A portion (600 µL) of the plasma (donor) was dialyzed against the same volume of isotonic phosphate buffer (pH 7.4, 0.067 M) (acceptor) at 37°C for 12 hr. Preliminary experiments showed that 12 hr was sufficient to achieve equilibrium of colistin between the two chambers. Samples of plasma and buffer were removed from each reservoir and stored at -20°C until analysis. The concentrations of colistin sulphate were determined by HPLC (vide supra). The fraction of unbound drug (f_u) was calculated as (acceptor colistin concentration)/(donor colistin concentration).

6.2.7 Pharmacokinetic analysis

Non-compartmental analysis of colistin (as the sulphate salt) in plasma was performed using WinNonlin (Version 3.0, Pharsight Corp., Cary, NC). The terminal rate constant (λ_z) was calculated by linear least-squares regression analysis using the last five log-transformed plasma concentration versus time points. The pharmacokinetic parameters were calculated with the following equations:

$$AUC_0^{\infty} = AUC_0^{t} + \frac{C_{last}}{\lambda_z} \qquad \text{(Equation 6.1)}$$

$$AUMC_0^{\infty} = AUMC_0^{t} + \frac{t_{last} \cdot C_{last}}{\lambda_z} + \frac{C_{last}}{\lambda_z^2} \qquad \text{(Equation 6.2)}$$

$$t_{1/2} = \ln 2 / \lambda_z \qquad \text{(Equation 6.3)}$$

$$CL = D / AUC_0^{\infty} \quad \text{(Equation 6.4)}$$

$$MRT = AUMC_0^{\infty} / AUC_0^{\infty} \quad \text{(Equation 6.5)}$$

$$V_{ss} = D \cdot AUMC_0^{\infty} / AUC_0^{\infty 2} = CL \cdot MRT \quad \text{(Equation 6.6)}$$

$$f_e(0\text{-}12h) = \frac{Ae(0\text{-}12\,h)}{D} \quad \text{(Equation 6.7)}$$

$$CL_R = f_e \cdot CL \quad \text{(Equation 6.8)}$$

where Ae (0-12h) is the amount of unchanged drug recovered in urine in 12 hr, AUC_0^{∞} is the area under the concentration versus time curve to infinite time, AUC_0^{t} is the area under the concentration versus time curve to t_{last}, $AUMC_0^{\infty}$ is the area under the first moment curve to infinite time, $AUMC_0^{t}$ is the area under the first moment curve to t_{last}, f_e (0-12h) is the fraction of the dose recovered in unchanged form in urine collected up to 12 hr, $t_{1/2}$ is the terminal half-life, CL is the total body clearance, CL_R is the renal clearance, D is the dose, MRT is the mean residence time, t_{last} is the time of the last quantifiable concentration, and V_{ss} is the volume of distribution at steady-state.

6.2.8 Statistical analysis

Differences between the values of the pharmacokinetic parameters for colistin A and colistin B were evaluated using a paired *t*-test. A *P*-value less than 0.05 was considered significant.

6.3 Results

The concentrations of colistin in plasma and urine (Appendix II) were measured by the HPLC method (Chapter 3) but with modifications as described in section 6.2.5. Figure 6.2 shows a typical chromatogram obtained from rat plasma. Following administration of colistin sulphate at a dose of 1 mg/kg, there were no visual signs of toxicity. Figure 6.3 shows the mean (± SD) concentrations of colistin, colistin A and colistin B (as the sulphate) in plasma as a function of time in five rats. The pharmacokinetic parameters

were calculated for colistin, colistin A and colistin B with non-compartmental analysis and are presented in Table 6.1.

There was no significant difference in the CL ($P > 0.25$), V_{ss} ($P > 0.29$) and $t_{1/2}$ ($P > 0.89$) between colistin A and colistin B. Overall, the coefficients of variation for these parameters were less than 19% (Table 6.1).

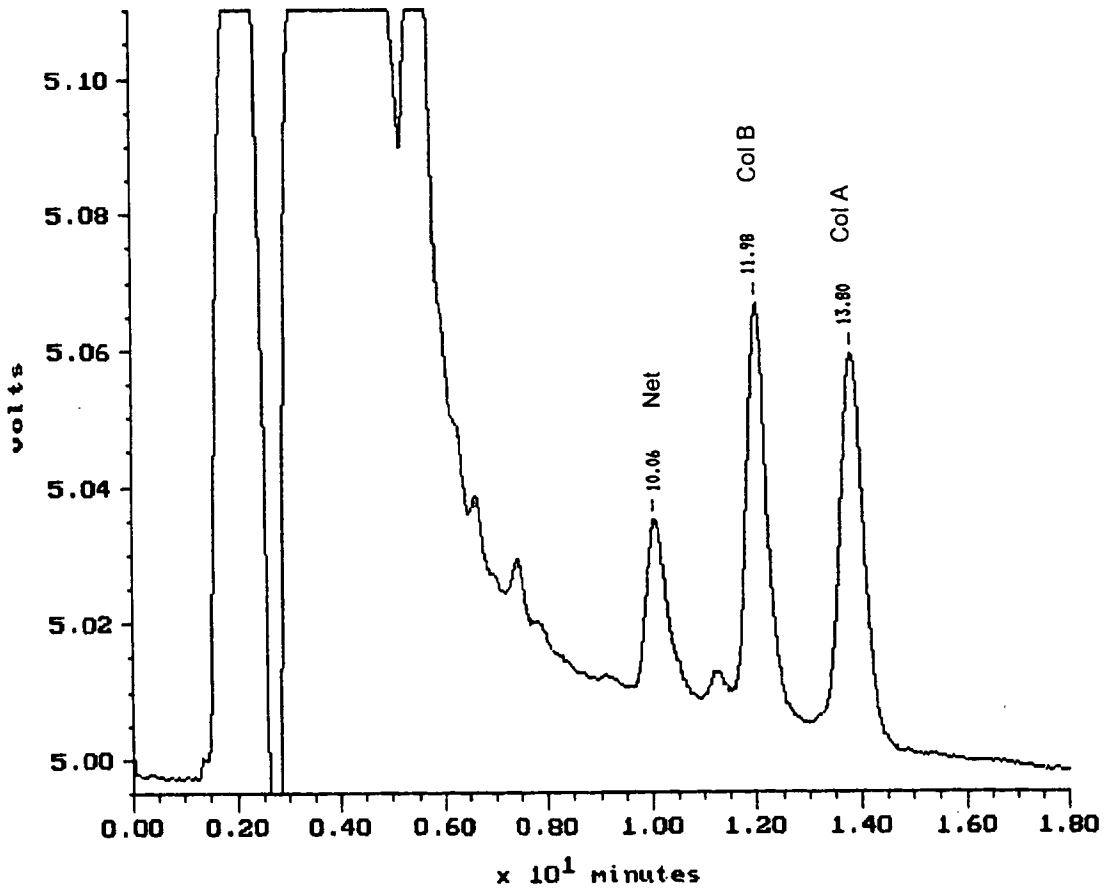

Figure 6.2 Typical chromatogram of the plasma sample from rat 1 at 10 min after intravenous administration of colistin sulphate (1 mg/kg).

During the first 12 hr after dosing, 0.18 ± 0.14% (n = 5) of the dose was excreted in urine as unchanged colistin, the majority (97.3%) of which was recovered during the first 4 hr. No colistin was detected in urine collected in the 8 - 12 hr interval. No significant difference ($P > 0.13$) was observed in the 12 hr urinary recovery of colistin A (0.13 ± 0.08%) and colistin B (0.23 ± 0.19%). The CL_R of colistin was calculated to be 0.010 ± 0.008 mL/min/kg.

The fractions of unbounded colistin, colistin A and colistin B in pooled rat plasma are shown in Table 6.2.

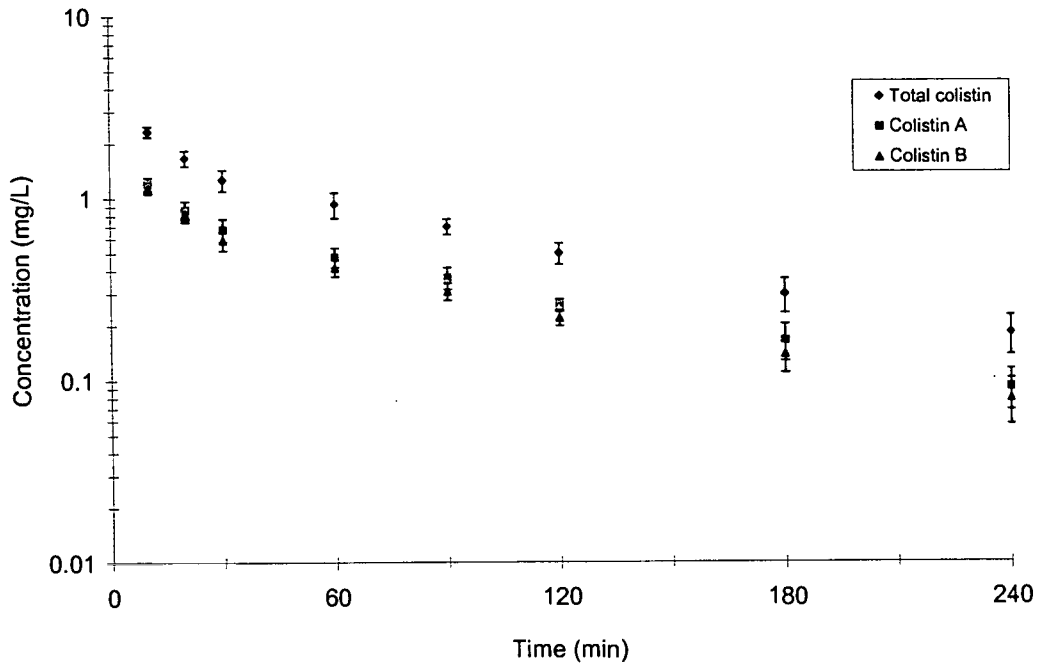

Figure 6.3 Mean (± SD) plasma concentration versus time profile for colistin, colistin A and colistin B (all as the sulphate) after intravenous administration of colistin (1 mg/kg) in rats (n = 5).

Table 6.1 Mean (± SD) pharmacokinetic parameters for colistin, colistin A and colistin B in rats (n = 5).

Parameters	Colistin	Colistin A	Colistin B
CL (mL/min/kg)	5.2 ± 0.4	5.1 ± 0.5	5.3 ± 0.5
V_{ss} (mL/kg)	496 ± 60	498 ± 70	509 ± 64
MRT (min)	94.9 ± 12.3	98.1 ± 15.9	96.1 ± 17.2
$t_{1/2}$ (min)	74.6 ± 13.2	77.1 ± 12.8	77.4 ± 14.8

Table 6.2 Fraction of unbound drug (f_u) in pooled drug-free rat plasma. Values are the mean of two replicates.

Concentration of colistin (mg/L) [a]	Colistin	Colistin A	Colistin B
1.5	0.44	0.34	0.53
3.4	0.45	0.36	0.52
6.0	0.43	0.36	0.51

[a] Concentrations in the donor compartment at equilibrium

6.4 Discussion

Recently, there has been a resurgence in the use of colistin against infections with *P. aeruginosa* in patients with CF (Beringer, 2001; Evans et al., 1999; Littlewood et al., 2000). Although colistin has been commercially available since the 1960s, pharmacokinetic data are still relatively scant (Beringer, 2001). Most reports on its pharmacokinetics were based on non-specific microbiological assays. As noted previously (Section 1.3.5), colistin is not administered parenterally to humans. Rather, it is administered as the methanesulphonate. Beveridge et al. (1967) demonstrated that the latter was converted to colistin *in vivo*, but an unknown degree. The studies described in Chapter 5 have shown that colistin methanesulphonate is converted to colistin in plasma and urine *in vitro*. Furthermore, the studies described in Chapter 2 demonstrated that colistin was approximately three-times more active than the methanesulphonate against *P. aeruginosa*. Therefore, before examining the pharmacokinetics of colistin following a dose of colistin methanesulphonate, the objective of the present investigation was to characterize the pharmacokinetics of colistin in rats measured by a specific HPLC method following the intravenous administration of colistin sulphate. An improved understanding of the pharmacokinetics of colistin should allow it to be used more safely and efficaciously as the methanesulphonate.

Colistin A and colistin B were chosen as the quantification markers of colistin. Because all the components of colistin have analogous structures (Orwa et al., 2001; Orwa et al., 2000), it was assumed that they have similar molecular UV absorbance. Therefore, the summed responses of colistin A and colistin B in the dose of colistin was evaluated by an HPLC method with UV detection (Decolin et al., 1997) and found to account for 86.5%

of the total peak areas (Figure 3.5). Hence, given that the sample of colistin sulphate administered was also used as the reference standard for quantitation, the concentrations of colistin A and colistin B (as the sulphate salt) were calculated from the concentrations of colistin and the ratios of the area of the respective chromatographic peaks and their summed areas.

The data in Figure 6.3 show a short initial distribution phase (approximately 30 min) followed by a slower log-linear decline in the concentrations over approximately one order of magnitude for colistin, colistin A and colistin B. The profiles for individual rats were similar to the mean data shown in Figure 6.3. The concentrations of colistin in plasma were of the same magnitude as those in dogs (approximately 3.5 mg/L to 0.35 mg/L in the 10 hrs after a single intramuscular injection of 2.2 mg/kg colistin sulphate) (Al-Khayyat et al., 1973) and in calves (10.5 mg/L to 0.4 mg/L in the 720 min after a single intravenous injection of 5 mg/kg colistin sulphate) (Ziv et al., 1982) when measured by microbiological assays. However, a third disposition phase in calves (Ziv et al., 1982) was not observed in the current study with rats.

Of note was that only 0.18 ± 0.14% of the administered dose was recovered in urine during the first 12 hr, and the majority occurred in the first 4 hr. The low urinary recovery of unchanged colistin indicates a very low renal clearance (0.010 ± 0.008 mL/min/kg). This value is very much lower than the anticipated clearance of colistin by glomerular filtration (2.3 mL/min/kg), which may be calculated as the product of unbound fraction of colistin (f_u = 0.44) and an approximate glomerular filtration rate in rats of 5.2 mL/min/kg (Davies et al., 1993). Therefore, it is evident that there must be very extensive net reabsorption of colistin from tubular urine back into blood, and that this accounts for its very low renal clearance. The lack of difference in urinary recovery between colistin A and colistin B is not surprising, given the variability about the mean values. The cumulative urinary recovery pattern was very similar to the results reported in dogs at a dosage of 1.1 mg/kg colistin sulphate, in which 0.13 ± 0.09% of the dose was recovered in urine in 12 hr (Al-Khayyat et al., 1973).

The total body clearance of colistin, colistin A and colistin B, ranged from 4.5 to 6.1 mL/min/kg and, as discussed above, renal clearance (0.010 ± 0.008 mL/min/kg) is only a very small component. Therefore, the majority of the total body clearance must be via

non-renal pathways. Comparison of the magnitude of the non-renal clearance (essentially the same as the total body clearance) and normal hepatic blood flow in the rat (72 - 95 mL/min/kg (Birnie et al., 1952)) indicates that colistin must have a very low hepatic extraction ratio.

Abe et al. (Abe et al., 1976) administered colistin methanesulfonate intravenously to rabbits and used thin-layer chromatography and a reference standard to tentatively identify a metabolite of colistin, colistin-N-glucuronide, in the urine (1.7% of the dose) and the bile (6.7% of the dose). However, there has not been any report on biliary excretion following a dose of colistin sulphate. Assuming that the stability of colistin in plasma from rats and humans is similar, the results described in Chapter 5 show that colistin would have been stable in plasma for the duration of the experiment. Further studies will be needed to determine the routes for the elimination and metabolism of colistin.

Protein binding of colistin was studied by equilibrium dialysis at concentrations approximating the values observed *in vivo* in rat plasma. Extensive non-specific binding (> 99% at 10 mg/L) to a commonly used membrane (Amicon regenerated cellulose YM-10) precluded the use of ultrafiltration. For colistin, a value of about 55 - 57% bound in rat plasma is similar to the results in plasma from dogs (approximately 35 to 65%) (Al-Khayyat et al., 1973) and calves (45 to 50%) (Ziv et al., 1982), both determined by equilibrium dialysis at 4°C for 24 hr (Al-Khayyat et al., 1973) and 48 hr (Ziv et al., 1982). There were significant differences between the f_u of colistin A and colistin B ($P < 0.006$) at all three concentrations investigated; the unbound fraction for colistin A was lower than that for colistin B. The higher binding for colistin A is most likely the result of its longer chain fatty acid (6-methyloctanoic acid) as compared with colistin B (6-methylheptanoic acid), since this is the only difference in the structures between the two molecules (Figure 1.1).

While the substantial difference in the unbound fractions between colistin A and colistin B is interesting and somewhat surprising, it conceals other apparent differences in the pharmacokinetic parameters for these very closely related compounds. For both total body clearance and volume of distribution, it is possible to calculate the corresponding parameters with reference to the unbound drug in plasma by dividing each parameter by

the respective f_u value (0.36 for colistin A and 0.52 for colistin B). Thus, while the total body clearances of colistin A and colistin B were comparable (mean values of 5.1 and 5.3 mL/min/kg, Table 6.1), corresponding clearances with reference to their unbound concentrations in plasma were approximately 14.2 and 10.2 mL/min/kg. The respective values for clearance of unbound colistin A and colistin B suggest marked differences in removal of the two unbound components from plasma by processes which presumably distinguish them on the basis of their differences in hydrophobicity of the fatty acid (Figure 1.1). Similarly, the volumes of distribution with reference to unbound drug in plasma were 1383 mL/kg and 979 mL/kg for colistin A and colistin B, respectively, although the corresponding values referenced to total plasma concentration were very similar (498 mL/kg and 509 mL/kg, Table 6.1). From the respective values for the volume of distribution of unbound drug, it is evident that colistin A (containing the longer chain fatty acid) must be more extensively bound in tissues than colistin B.

It is interesting to note that the modest difference in the structure of colistin A and colistin B (arising from the presence or absence of one $-CH_2-$ in the fatty acid, Figure 1.1) also leads to a substantial difference in retention time on a reversed-phase HPLC column, as observed in Chapter 3 (Figure 3.5) and by others (Decolin et al., 1997; Orwa et al., 2001; Orwa et al., 2000); colistin A is more strongly retained in such a chromatographic system. Clearly, the subtle difference in the structures of these two molecules (molecular weight difference of only 14 in a total of approximately 1163, Figure 3.6) leads to substantial differences in not only chromatographic behaviour but also disposition in the body.

6.5 Conclusion

In summary, this is the first report using an HPLC method specific for colistin (colistin A and colistin B) in plasma and urine to examine the pharmacokinetics of colistin following intravenous administration of colistin sulphate. Colistin had a relatively short terminal half-life in rats after intravenous administration (1 mg/kg) and a very low urinary recovery. While the CL and V_{ss} with reference to total drug in plasma were not significantly different between colistin A and colistin B, there were apparent differences in these parameters calculated with reference to unbound drug in plasma, which may be attributable to a difference in hydrophobicity between the two molecules.

Chapter 7 Pharmacokinetics of colistin methanesulphonate after intravenous administration in rats

7.1 Introduction

Colistin was the first antibiotic with remarkable *in vitro* activity against *P. aeruginosa* (Goodwin, 1970). The toxicity and undesirable side effects of colistin methanesulphonate are dramatically less than those of colistin sulphate, but so too is its *in vitro* antibacterial potency, as shown by previous researchers against a range of species (Barnett et al., 1964; Beveridge et al., 1967; Schwartz et al., 1960; Stansly et al., 1947) and by the work described in Chapter 2 of this thesis which examined their relative activity against numerous isolates of *P. aeruginosa* from CF patients. As reviewed in Chapter 1 and examined in detail in Chapter 4 of this thesis, colistin methanesulphonate has the potential to hydrolyse in aqueous solutions. The hydrolysis products possessed increased antibacterial activity due to the release of the more active colistin (Barnett et al., 1964; Beveridge et al., 1967).

Notwithstanding that colistin methanesulphonate has been used for more than 40 years, its pharmacokinetics has not been studied comprehensively. Most results on the pharmacokinetics of colistin methanesulphonate were obtained by using microbiological assays to measure the concentrations of 'colistin methanesulphonate' in plasma and urine. The limitations of microbiological assays for colistin methanesulphonate have been reviewed in Chapters 1 and 4. To date there is only one report on the pharmacokinetics of colistin methanesulphonate in CF patients using HPLC to quantify the drug in plasma and urine by derivatization with dansyl chloride (Reed et al., 2001). However, as noted in sections 1.3.6.1.2 and 4.1, it was not specified, in that HPLC method, which form was quantified, colistin, colistin methanesulphonate or both.

Considering that most data on the concentrations of 'colistin methanesulphonate' in biological fluids come from studies before the 1990s using microbiological assays (Evans et al., 1999), the limited pharmacokinetic information available on colistin

methanesulphonate is probably for a mixture of colistin methanesulphonate, different sulphomethylated derivatives and colistin. Al-Khayyat et al. demonstrated a clear difference in the half-life and urinary excretion of colistin and colistin methanesulphonate in dogs (Al-Khayyat et al., 1973). With an intramuscular dose of 2.2 mg/kg colistin methanesulphonate, the average half-life was 1.37 ± 0.18 hr (n = 14) and 47.9 ± 3.4% of the dose was recovered in urine in 12 hr (n = 6); following an intramuscular dose of 2.2 mg/kg colistin, the corresponding values were 2.50 ± 0.30 hr (n = 9) and 7.5 ± 2.5% (n = 3). Estimations of clearance and volume of distribution were not included in that paper (Al-Khayyat et al., 1973).

The current chapter describes a study of the pharmacokinetics of colistin methanesulphonate in rats following an intravenous bolus using two sensitive HPLC methods to quantify colistin methanesulphonate and colistin separately in plasma and urine (methods described in Chapters 4 and 3, respectively).

7.2 Materials and Methods

7.2.1 Animals

Five male Sprague-Dawley rats (340 - 380 g) (Gilles Plains Animal House, Adelaide, SA, Australia) were housed in an animal care facility with a temperature of 21 ± 1°C and a 12-hr light/dark cycle (6:00 - 18:00). Free access to food (Ridley Agriproducts Pty. Ltd., Murray Bridge, SA, Australia) and water was allowed at all times. Animals were acclimated at least 5 days prior to experimentation. The animal protocol was approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Sciences (Adelaide, SA, Australia).

7.2.2 Catheterization of jugular vein

The method was identical to that described in Section 6.2.2.

7.2.3 Dosage and collection of samples

Colistin methanesulphonate (Sigma, St. Louis, MO) (15 mg/kg, freshly prepared in sterile saline) was administered in a volume of 300 μL via the jugular vein catheter in less than 20 seconds. The catheter was flushed immediately with 1 mL heparinized saline. Preliminary studies conducted *in vitro* indicated that this method of administration resulted in less than 0.01% of the dose remaining in the catheter after the flush. In the dosing solution approximately 0.11% (on a molar basis) of the drug was present in the form of colistin, when measured by a specific HPLC method (Chapter 3). Blood (500 μL) was collected prior to administration of colistin methanesulphonate and at 5, 10, 20, 30, 60, 90, 120 and 180 min thereafter. During the sampling, 100 μL blood was drawn into a syringe before collecting the actual sample with another syringe; subsequently, the 100 μL of blood was returned to the rat. The catheter was flushed with 500 μL of heparinized saline (10 IU/mL) to maintain a constant intravascular volume. Each sample of blood was centrifuged (1000 *g*, 10 min) at 4°C immediately and the plasma stored at -20°C until analysis. Urine was collected from the metabolic cage prior to administration of colistin methanesulphonate and over the intervals 0 - 4, 4 - 8, 8 - 12 and 12 - 24 hr thereafter. After collection of each urine sample, the metabolic cage was rinsed with Milli-Q water (5 to 10 mL) and kept separately for assay. Urine samples were frozen immediately at -20°C pending assay.

7.2.4 Determination of colistin methanesulphonate and colistin in plasma and urine

Concentrations of colistin and colistin methanesulphonate in plasma and urine were measured by the HPLC methods described in Chapters 3 and 4.

Calibration curves for colistin in drug-free rat plasma were constructed with concentrations of colistin sulphate ranging from 0.20 mg/L to 8.0 mg/L. The inter-day accuracy and reproducibility were 0.86 ± 0.06 mg/L and 6.2 ± 0.3 mg/L (n = 5) for quality controls of colistin sulphate prepared independently at 0.80 mg/L and 6.0 mg/L, respectively. For urine, calibration curves were constructed with concentrations of colistin sulphate ranging from 0.10 mg/L to 5.0 mg/L. The inter-day accuracy and

reproducibility were 0.40 ± 0.01 mg/L and 3.0 ± 0.2 mg/L (n = 5) for colistin sulphate at 0.40 mg/L and 3.0 mg/L, respectively. The concentrations of colistin were calculated by multiplying the obtained concentrations of colistin sulphate by 1163/1403, where 1163 is the average molecular weight of colistin A and colistin B, and 1403 is the average molecular weight of colistin A sulphate and colistin B sulphate.

Calibration curves for total colistin species (colistin methanesulphonate, plus partially sulphomethylated derivatives plus free colistin) in drug-free rat plasma were constructed with concentrations of colistin methanesulphonate ranging from 0.33 mg/L to 53.3 mg/L. The inter-day accuracy and reproducibility was 1.40 ± 0.11 mg/L and 39.6 ± 1.10 mg/L (n = 6) for independently prepared quality controls containing colistin methanesulphonate at 1.33 mg/L and 40.0 mg/L, respectively. For urine, calibration curves were constructed with concentrations of colistin methanesulphonate ranging from 0.25 mg/L to 40.0 mg/L. The inter-day accuracy and reproducibility were 1.00 ± 0.09 mg/L and 29.6 ± 2.2 mg/L (n = 6) for colistin methanesulphonate at 1.00 mg/L and 30.0 mg/L, respectively.

The concentration of 'colistin methanesulphonate' (colistin methanesulphonate plus partial sulphomethylated derivatives) in each sample was calculated from the value obtained above minus the concentration of colistin, after correcting for differences in the average molecular weights of colistin (1163) and sodium colistin methanesulphonate (1743, from the molecular weights for sodium colistin A methanesulphonate and sodium colistin B methanesulphonate of 1750 and 1736, respectively).

7.2.5 Pharmacokinetic analysis

Non-compartmental analysis of the pharmacokinetics of colistin methanesulphonate and colistin was performed using WinNonlin (Version 3.0, Pharsight Corp., Cary, NC). The terminal rate constant (λ_z) was calculated by linear least-squares regression analysis using the last five log-transformed plasma concentration-versus-time points for colistin methanesulphonate and the last three points for colistin. Other

pharmacokinetic parameters for colistin methanesulphonate were calculated with the following equations:

$$AUC_0^{\infty} = AUC_0^{t} + \frac{C_{last}}{\lambda_z} \quad \text{(Equation 7.1)}$$

$$AUMC_0^{\infty} = AUMC_0^{t} + \frac{t_{last} \cdot C_{last}}{\lambda_z} + \frac{C_{last}}{\lambda_z^2} \quad \text{(Equation 7.2)}$$

$$t_{1/2} = \ln 2 / \lambda_z \quad \text{(Equation 7.3)}$$

$$CL = D / AUC_0^{\infty} \quad \text{(Equation 7.4)}$$

$$V_{ss} = D \cdot AUMC_0^{\infty} / AUC_0^{\infty 2} \quad \text{(Equation 7.5)}$$

$$f_e = \frac{Ae(0-24)}{D} \quad \text{(Equation 7.6)}$$

$$CL_R = f_e \cdot CL \quad \text{(Equation 7.7)}$$

where Ae(0 - 24) is the summed amount of colistin and colistin methanesulphonate recovered in urine in 24 hr and CL_R is the apparent value of renal clearance for colistin methanesulphonate. The assumption involved in the application of equations 7.6 and 7.7 for the determination of CL_R will be discussed in Section 7.4. The AUC_0^{∞} of colistin was calculated using Equation 7.1 with the presumption of the concentration at zero time being equal to zero.

7.2.6 Statistical analysis

Group data are reported as mean ± standard deviation (SD). The difference between the $t_{1/2}$ of colistin methanesulphonate and colistin was evaluated with a *t*-test. A *P*-value less than 0.05 was considered significant.

7.3 Results

Figure 7.1 shows the mean (± SD) concentrations of colistin methanesulphonate and colistin in plasma as a function of time in five rats after an intravenous dose of 15

mg/kg colistin methanesulphonate. The concentrations of colistin methanesulphonate at 180 min in two of the five rats were below the LOQ, likewise for colistin in three of the five rats. There was a short distribution phase (approximately 30 min) for both colistin methanesulphonate and colistin. In three of the five rats, maximum concentrations of colistin were observed at 5 min; maximum concentrations occurred at 10 min in the remaining two rats. Figure 7.2 shows typical chromatograms derived from the analysis of plasma.

There was no urine from one rat during the first 8 hr or from another during the interval of 4 - 8 hr after administration. Overall, 61.1 ± 14.4% (n = 5) of the dose was recovered in urine during the first 24 hr after dosing, in which 32.6 ± 15.1% (n = 5) was present in the form of colistin (Figure 7.3). The majority of the urinary recovery (60.8 ± 14.6% of the dose, n = 5) occurred during the first 12 hr. The pharmacokinetic parameters for colistin methanesulphonate are presented in Table 7.1. Colistin methanesulphonate had a significantly ($P < 0.02$) shorter terminal half-life (23.6 ± 3.9 min) than colistin (55.7 ± 19.3 min).

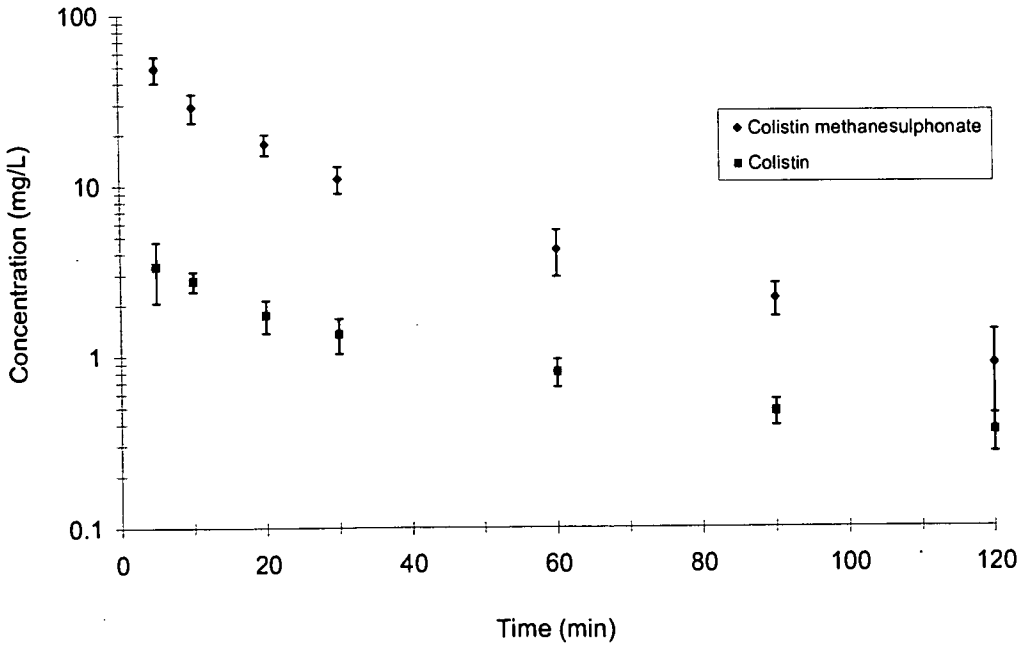

Figure 7.1 Mean (± SD) plasma concentration versus time profiles for colistin methanesulphonate and colistin in rats (n = 5).

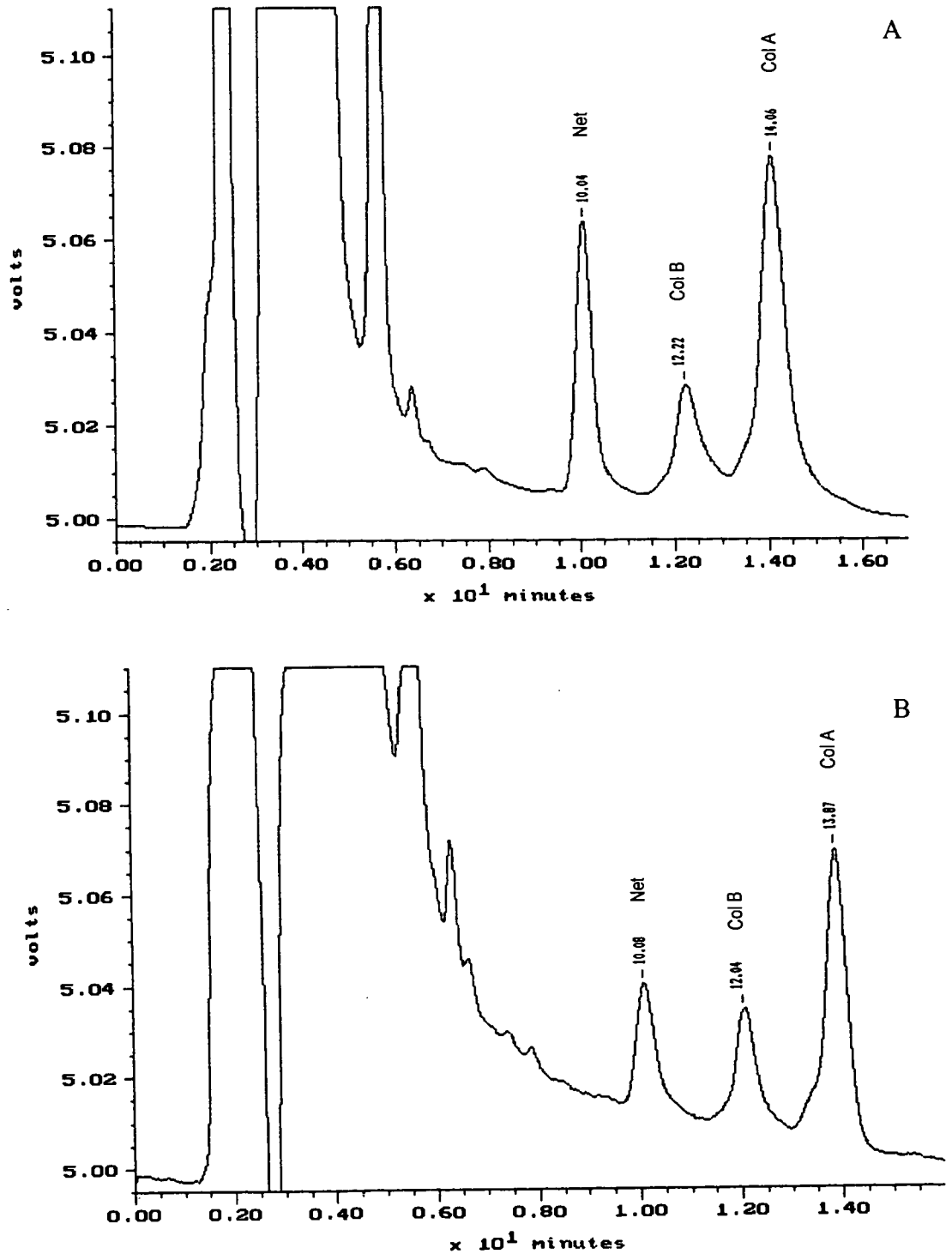

Figure 7.2 Typical chromatograms obtained with fluorescence detection for colistin methanesulphonate in the sample from Rat 9 collected at 20 min (A) and colistin in the sample from Rat 10 collected at 5 min (B) after intravenous administration of colistin methanesulphonate (15 mg/kg).

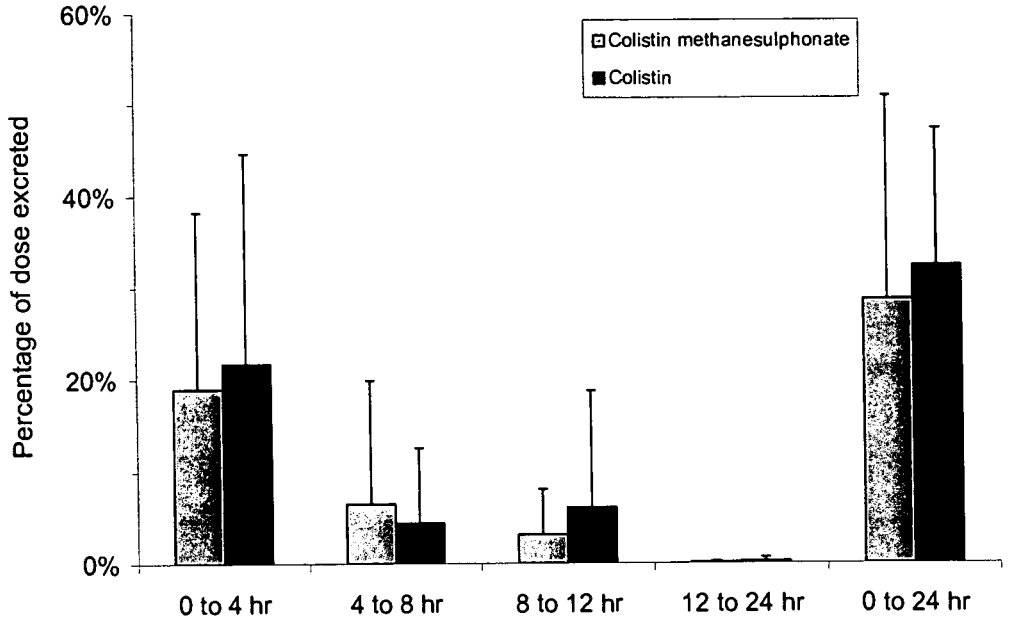

Figure 7.3 Urinary recovery of colistin methanesulphonate and colistin over the 24 hr collection.

Table 7.1 Mean (± SD) pharmacokinetic parameters for colistin methanesulphonate and colistin after a dose of colistin methanesulphonate in rats (n = 5).

Pharmacokinetic parameters	Colistin methanesulphonate	Colistin formed by hydrolysis
CL (mL/min/kg)	11.7 ± 1.8	—
V_{ss} (mL/kg)	299 ± 55	—
$t_{1/2}$ (min)	23.6 ± 3.9	55.7 ± 19.3
λ_z (1/min)	0.030 ± 0.005	0.014 ± 0.004
f_e	0.611 ± 0.144	—
CL_R (mL/min/kg)	7.2 ± 2.2	—
AUC_0^∞ (mg·min/L)	1306 ± 177	156 ± 28

7.4 Discussion

Colistin methanesulphonate and colistin differ in their chemistry, pharmacokinetics (Al-Khayyat et al., 1973) and antibacterial activity (Barnett et al., 1964; Beveridge et al., 1967; Chapter 2). Since colistin methanesulphonate has the potential to hydrolyse to colistin in aqueous media (Barnett et al., 1964; Beveridge et al., 1967), it is important in defining the pharmacokinetics of colistin methanesulphonate to determine concentrations of both colistin methanesulphonate and colistin. Unfortunately, this is impossible with microbiological assays and the only HPLC method (Reed et al., 2001) that has been described for colistin methanesulphonate. The major goal of the current chapter was to characterise the pharmacokinetics of colistin methanesulphonate, including colistin formed by hydrolysis, in rats after an intravenous bolus (15 mg/kg) using two HPLC methods to measure the concentrations of colistin methanesulphonate and colistin in plasma and urine (Chapters 3 and 4). Since the concentrations of colistin methanesulphonate quantified in plasma and urine in the current study were for the sum of the various sulphomethylated derivatives, the pharmacokinetic parameters for colistin methanesulphonate may best be considered as hybrid parameters. Nevertheless, an improved understanding of the pharmacokinetics of colistin methanesulphonate and of colistin after a dose of colistin methanesulphonate would allow it to be used more safely and efficaciously.

The data in Figure 7.1 show a fairly short initial distribution phase (approximately 30 min) followed by a slower log-linear decline in the concentrations of both colistin methanesulphonate and colistin. Although the HPLC method for colistin methanesulphonate was not able to quantify each partially sulphomethylated derivative of colistin separately (Chapter 4), the concentrations of colistin alone and the sum of all the sulphomethylated derivatives could be measured by a combination of the two HPLC methods (Chapters 3 and 4). Interestingly, substantial concentrations of colistin were observed, even within 5 min after dosing. In the dosing solution, approximately 0.11% of colistin methanesulphonate was present in the form of colistin, which indicated about 0.011 mg/kg colistin was co-administered with colistin methanesulphonate to the rats. The concentrations of colistin in the 5-min and 10-min plasma samples were 3.37 ± 1.31 mg/L and 2.76 ± 0.37 mg/L, respectively, and the

AUC_0^∞ of colistin was 156 ± 28 mg·min/L after the administration of colistin methanesulphonate. However, in the pharmacokinetic study of colistin after an intravenous bolus (1 mg/kg) in rats, there was 2.33 ± 0.16 mg/L colistin in plasma at 10 min and the AUC_0^∞ of colistin was 192 ± 14 mg·min/L (Chapter 6). Given the presumption that colistin displays linear pharmacokinetics in rats, the AUC_0^∞ of colistin in plasma after the administration of colistin methanesulphonate was much higher than would have been predicted from the administration of 0.011 mg/kg colistin. Even though the mean levels of colistin in plasma did not appear to rise to a maximum, this was observed in two of the five rats (Appendix III). Furthermore, in stability tests of colistin methanesulphonate when plasma containing colistin methanesulphonate (20 mg/L) was incubated at 37°C, only 9.3% (on a molar basis) of colistin methanesulphonate was hydrolysed to colistin *in vitro* in 2 hr (Chapter 5). Hence, it would appear that there were other mechanisms *in vivo* leading to the rapid hydrolysis of colistin methanesulphonate to colistin. While an artifact almost certainly occurred in relation to the urinary recovery of colistin methanesulphonate (underestimation of actual excretion) and colistin (overestimation of actual excretion) (vide infra), no such problem with *in vitro* hydrolysis obscures interpretation of the plasma pharmacokinetics.

The V_{ss} of colistin methanesulphonate was 299 ± 55 mL/kg, which was about 60% of the value for colistin (496 ± 62 mL/kg) (Chapter 6). The value for the $t_{1/2}$ of colistin after a dose of colistin methanesulphonate (55.7 ± 19.3 min) was comparable to the value observed after a dose of colistin in rats (74.6 ± 13.2 min, Chapter 6), but approximately double of that of colistin methanesulphonate (23.6 ± 3.9 min). The longer half-life of colistin indicated that its disposition after colistin methanesulphonate administration was rate-limited by its elimination, not by its formation from the hydrolysis of colistin methanesulphonate. The relativity in half-lives of colistin methanesulphonate and colistin in rats is similar to the report in dogs (Al-Khayyat et al., 1973). After an intramuscular injection of colistin (as the sulphate salt) at 2.2 mg/kg (n = 9) to normal dogs, the half-life of colistin was 2.50 ± 0.30 hr, while following an intramuscular injection of colistin methanesulphonate (2.2 mg/kg,

n = 14), the half-life of colistin methanesulphonate was 1.37 ± 0.18 hr (Al-Khayyat et al., 1973). The V_{ss} and CL for colistin could not be calculated in the current chapter.

In contrast to the low urinary recovery of colistin following administration of colistin in rats (Chapter 6), colistin methanesulphonate was recovered predominantly in urine (61.1 ± 14.4%, n = 5) during the first 24 hr, with 60.8 ± 14.6% of the dose eliminated in urine during the first 12 hr (Figure 7.3). The high urinary recovery in rats is consistent with reports from the administration of colistin methanesulphonate to dogs (Al-Khayyat et al., 1973) and humans (Froman et al., 1970; Reed et al., 2001; Yamada et al., 1974). The substantial difference in recovery between administration of colistin methanesulphonate and colistin suggests markedly different renal mechanisms for their removal from the body. From the point of view of treating urinary tract infections, the extremely high urinary concentrations of colistin and colistin methanesulphonate that were also reported in humans (Reed et al., 2001) after administration of colistin methanesulphonate, would be expected to be beneficial. The efficacy of colistin methanesulphonate in the treatment of urinary tract infections has been proven by several clinical reports (Brumfitt et al., 1966; Cox et al., 1970; Leruitte, 1969; Olesen et al., 1967).

Interestingly, 32.6 ± 15.1% of the dose recovered in urine in 24 hr was present as colistin and a comparable percentage as the sulphomethylated substitutes. However, it is unclear how much colistin was formed before and after the formation of urine in the kidney. Plasma levels of colistin in rats after an intravenous bolus of colistin (1 mg/kg) were similar to those in the present study, yet on average only 0.18% of the dose was recovered as unchanged drug in urine over a 12-hr collection period (Chapter 6). However, in the current study about half of the drug recovered in urine in any 4-hr interval was colistin (Figure 7.3). From an evaluation of the stability of colistin methanesulphonate in rat urine (Figure 5.9), 4.4% and 29.3% (in molar terms) of colistin methanesulphonate in urine stored for 4 hr at room temperature and at 37°C, respectively, was converted to colistin by hydrolysis. Furthermore, with the assumption that the clearance of colistin was the same in rats administered colistin methanesulphonate (present chapter) and colistin (Chapter 6), the fraction of colistin

methanesulphonate converted to colistin (f_m) could be calculated based on the respective areas under the plasma concentration versus time curves for colistin:

$$f_m = \frac{(\text{AUC}_0^\infty \text{ of colistin after CMS}) \cdot (\text{Dose of colistin}) \cdot 1743}{(\text{AUC}_0^\infty \text{ of colistin after colistin}) \cdot (\text{Dose of CMS}) \cdot 1403}$$

where 1743 and 1403 are the average molecular weights of sodium colistin methanesulphonate and colistin sulphate, respectively. The low value of f_m (0.068) based on concentrations in plasma shows only a small fractional systematic conversion of colistin methanesulphonate to colistin. In addition, from the values for the AUC_0^∞ of colistin and colistin methanesulphonate, the ratio of the respective values was about 0.12. Therefore, it is most likely that the high recovery of colistin in urine was the result of one or both of two sources: intra-renal conversion of colistin methanesulphonate to colistin within tubular cells of the kidney; and/or elimination of colistin methanesulphonate by the kidney followed by hydrolysis in the bladder and in the collection vessel. Intra-renal conversion to colistin may have implications for the renal toxicity attributed to colistin methanesulphonate.

Since the high percentage of colistin recovered in urine was assumed to be the result of hydrolysis of colistin methanesulphonate, the CL_R of colistin methanesulphonate (7.2 mL/min/kg) was calculated from the sum of colistin methanesulphonate and colistin in urine. Given that the mean total clearance of colistin methanesulphonate was 11.7 mL/min/kg, the non-renal clearance was approximately 4.5 mL/min/kg. The normal hepatic blood flow in rats is in the range of 72 - 95 mL/min/kg (Birnie et al., 1952), indicating that the hepatic extraction ratio of colistin methanesulphonate is very low, and similar to colistin (Chapter 6). The fate of the remaining proportion (approximately 40%) of the dose not eliminated by the kidney remains unknown.

There is little information on the metabolism of colistin methanesulphonate. Following intravenous administration of colistin methanesulphonate (100 mg/kg) to rabbits, a metabolite of colistin methanesulphonate, colistin-N-glucuronide, was identified tentatively in urine and in bile using thin-layer chromatography (Abe et al., 1976). However, only about 1.7% and 6.7% of the dose was recovered as this

metabolite in urine and bile, respectively. Therefore, a considerable proportion of eliminated colistin methanesulphonate remains unaccounted for.

Hydrolysis of colistin methanesulphonate in plasma at 37°C renders it almost impossible to determine its binding by equilibrium dialysis or ultracentrifugation. Unfortunately, ultrafiltration with the commonly used Amicon ultrafiltration devices could not be applied with colistin methanesulphonate since weak non-specific binding to the membrane was observed in preliminary experiments. Therefore, protein binding of colistin methanesulphonate was not measured in the current study. As reviewed in Section 1.3.6.3.2, there is very limited information on protein binding of colistin methanesulphonate in plasma. In the ewe, 42.8 ± 3.8% was bound at 6.2 and 12.5 mg/L, conducted using ultrafiltration (Ziv et al., 1972). Unfortunately, Ziv et al. did not demonstrate the absence of non-specific binding with the ultrafiltration membranes they used. Furthermore, measuring protein binding at 4°C may not reflect the true situation at 37°C.

The glomerular filtration rate (GFR) in rats is approximately 5.2 mL/min/kg (Davies et al., 1993). While the exact unbound fraction (f_u) of colistin methanesulphonate in plasma remains unknown, even allowing for an f_u at the upper limit of unity, the CL_R (7.2 mL/min/kg) is greater than the product of f_u·GFR. Therefore, it is evident that there is net renal tubular secretion of colistin methanesulphonate into the urine, which is substantially different from what occurs for colistin (Chapter 6). Clearly, derivatization of the free amine groups of colistin with the methanesulphonate groups converts the molecule from one that undergoes substantial net tubular reabsorption to one that undergoes net secretion.

7.5 Conclusion

In conclusion, the pharmacokinetics of colistin methanesulphonate in rats has been described by measuring the concentrations of both colistin methanesulphonate and colistin separately in plasma and urine after an intravenous bolus of colistin methanesulphonate. Colistin methanesulphonate has a short terminal half-life and almost two-thirds of the dose was recovered in urine during the first 24 hr; half of the

urinary recovery was present in the form of colistin. The high percentage of colistin recovered in urine was believed to be formed by hydrolysis in the bladder and in the collection vessel and/or conversion from colistin methanesulphonate in the kidney. Since colistin methanesulphonate and colistin have different antibacterial activities, the results will be valuable for further investigations on the pharmacodynamics of colistin methanesulphonate *in vivo*. Furthermore, the significant difference between the urinary recovery of colistin methanesulphonate and colistin requires further study. This may provide some clues to their nephrotoxicity.

Chapter 8 Pharmacokinetics of colistin methanesulphonate after intravenous infusion in patients with CF

8.1 Introduction

The recent increase in prevalence of multi-drug resistant *P. aeruginosa* is posing a major health concern (Hancock et al., 2000; Waterer et al., 2001), especially in patients with burns, neutropenic individuals, and in patients with cystic fibrosis (CF) (Tatterson et al., 2001). More than 90% of CF patients die of lung disease, in particular caused by infections with *P. aeruginosa* and the associated host inflammatory responses (FitzSimmons, 1993). The lack of truly novel classes of anti-pseudomonal agents in development has renewed interest in colistin (Beringer, 2001; Catchpole et al., 1997; Conway et al., 2000; Evans et al., 1999; Littlewood et al., 2000). Recent reports have assessed the safety and tolerability of intravenous colistin methanesulphonate and concluded that it is an effective treatment for *P. aeruginosa* infections associated with pulmonary exacerbations in CF patients (Conway et al., 2000; Conway et al., 1997).

As reviewed in section 1.3.6.3, most available data on the pharmacokinetics of colistin methanesulphonate in humans were obtained with concentrations of drug in plasma and urine measured by microbiological assays. The pharmacokinetics of colistin methanesulphonate in CF patients has not been comprehensively studied except for a recent report which used HPLC to measure the concentration of drug (Reed et al., 2001). The shortcomings of the HPLC method used by Reed et al. (2001) and the microbiological assays used by others have been discussed in detail in Chapters 1 and 4. Given that colistin methanesulphonate has the potential to hydrolyse, the preceding pharmacokinetic parameters estimated for colistin methanesulphonate are best regarded as apparent values for the fully and partially sulphomethylated derivatives plus colistin.

In this thesis, two sensitive HPLC methods have been described for measuring colistin (Chapter 3) and colistin methanesulphonate (Chapter 4) in biological fluids. The aim of the current study was to examine the pharmacokinetics of colistin methanesulphonate, and of colistin generated from it, at steady-state in CF patients during treatment with intravenous infusions of colistin methanesulphonate.

8.2 Materials and methods

8.2.1 Patients and ethics

Twelve CF patients, 6 males and 6 females (age range 13 to 39 years) entered this study, all with acute respiratory exacerbation caused by infections with *P. aeruginosa*. Demographic and clinical details for the patients are summarised in Table 8.1. The study protocol was approved by the Clinical Research Ethics Committee at St James' and Seacroft University Hospital, Leeds, United Kingdom, and informed consent was obtained from all patients before entry. Subjects were repeatedly questioned throughout the study and requested to report any adverse effects related to treatment with colistin methanesulphonate during the therapy; any such adverse effects were to be categorised as possible, probable, or definite in terms of their causation in relation to administration of colistin methanesulphonate. No patient had renal impairment. Administration of doses and collection of samples was conducted at the Regional Adult Cystic Fibrosis Unit of Seacroft Hospital (Leeds, United Kingdom).

In total, there were 33 isolates of *P. aeruginosa* from the patients; 44% were resistant to piperacillin, 30% to tobramycin, 25% to ciprofloxacin, 25% to ceftazidime, 47% to amikacin, 25% to meropenem, 31% to aztreonam and 3% to colistin. Susceptibility testing was conducted at the Department of Microbiology of Seacroft Hospital (Leeds, United Kingdom). In addition to colistin methanesulphonate, some patients were also receiving other antibiotics (Table 8.1).

8.2.2 Treatment

Patients were administered colistin methanesulphonate (Colomycin® Injection, Pharmax, Kent, UK, batch Number: 11860, one milligram contains approximately 12,740 units). The dosing solution was stored at 4°C and discarded if not used within 3 days. Patients weighing more than 50 kg received 2 mega-units colistin methanesulphonate (160 mg, dissolved in 50 mL of sterile saline) intravenously every eight hours while those less than 50 kg were administered 1 mega-units (80 mg, dissolved in 50 mL of sterile saline)

Table 8.1 Clinical and demographic data for patients

Patient	Age (year)	Sex	Body weight (kg)	Dose (mg/kg/8 hr)	Infusion duration (min)	Serum creatinine (μM) [a]	Sampling day [b]	Antibiotics co-administered
1	18	M	60	2.62	30	87	3	Meropenem
2	19	F	45	1.74	30	60	17	Aztreonam
3	21	F	61	2.58	30	53	7	Ceftazidime
4	13	M	68	2.32	10	77	9	Ceftazidime
5	31	F	48	1.63	15	N.A.[c]	3	-
6	22	F	52	3.02	30	50	7	Meropenem
7	22	F	39	2.01	30	53	6	Aztreonam
8	19	M	61	2.58	15	64	11	Aztreonam
9	39	F	66	2.39	60	59	3	Meropenem
10	19	M	64	2.44	20	79	6	Meropenem, tobramycin
11	19	M	60	2.64	20	53	6	-
12	18	M	51	3.11	30	96	4	Meropenem
Mean	22		56	2.42		66	6.8	
S.D.	7		9	0.45		16	4.0	

[a] Serum concentrations of creatinine on the day of admission. The normal range of concentrations for serum creatinine is 53 - 84 μM for females and 71 - 106 μM for males.

[b] The day of sampling after the first dose.

[c] Not available.

intravenously every eight hours. Colistin methanesulphonate was given as a 50-mL infusion over 10 to 60 min (Table 8.1). After at least two days of therapy had elapsed, blood samples (4 mL) were obtained with a 4-mL Vacuette container (Greiner Labortechik, Austria) at nominal times of 60, 120, 240 and 360 min from the end of the infusion. Immediately after collection, the samples were centrifuged (1,000 *g*, 10 min) at 4°C and plasma kept at -60°C. Plasma samples were packed with dry ice and transported to Adelaide by air, whereupon they were stored at -60°C until analysis.

8.2.3 Determination of colistin methanesulphonate and colistin in plasma

The concentrations of colistin methanesulphonate and colistin in plasma were measured by HPLC (Chapters 3 and 4).

For the assay of colistin, concentrations for the calibration curve prepared in drug-free human plasma ranged from 0.08 mg/L to 3.2 mg/L with two independently prepared quality controls (QCs) at 0.24 mg/L and 2.4 mg/L. The accuracy and reproducibility for the respective QC samples were 0.23 ± 0.08 mg/L and 2.42 ± 0.04 mg/L (n = 3).

For the assay of total colistin species (colistin methanesulphonate, plus partially sulphomethylated derivatives plus free colistin), concentrations for the calibration curve prepared in drug-free human plasma ranged from 0.2 mg/L to 32.0 mg/L with two independently prepared QCs at 0.80 mg/L and 24.0 mg/L. The accuracy and reproducibility for the respective QC samples were 0.81 ± 0.04 mg/L and 23.8 ± 0.7 mg/L (n = 3). The concentration of 'colistin methanesulphonate' (colistin methanesulphonate plus partially sulphomethylated derivatives) was the value calculated above minus the concentration of colistin in the same sample, after correcting for differences in the average molecular weights of colistin and sodium colistin methanesulphonate (1743, from the molecular weights for sodium colistin A methanesulphonate and sodium colistin B methanesulphonate of 1750 and 1736, respectively).

None of the other antibiotics administered to some patients (Table 8.1) interfered in the analysis of colistin methanesulphonate and colistin in plasma.

8.2.4 Pharmacokinetic analysis

It was assumed that the plasma concentrations of colistin methanesulphonate and colistin were at steady-state. Since comprehensive blood sampling was not possible in the current study, for the purpose of calculating pharmacokinetic parameters (vide infra), it was assumed that the concentrations of colistin methanesulphonate and colistin declined in a monoexponential manner after completion of the infusion. Therefore, the λ_z (disposition rate constant) was calculated by linear least-squares regression analysis using the four log-transformed plasma concentration versus time points for each species. C^{ss}_{max} (the maximum steady-state concentration in plasma during a dosage interval) and C^{ss}_{min} (the minimum steady-state concentration in plasma during a dosage interval) were calculated by extrapolation back to when the infusion was ceased and forward to 8 hr, respectively. The plasma concentration immediately preceding the start of the infusion was presumed equal to C^{ss}_{min}. Therefore, AUC^{ss}_{τ} (the area under the concentration versus time curve during a dosage interval at steady-state) was calculated using the linear trapezoidal rule from 0 to 8 hr; the area under the curve during the infusion was calculated as the mean of C^{ss}_{min} and C^{ss}_{max} multiplied by the duration of the infusion. Other pharmacokinetic parameters for colistin methanesulphonate were calculated using the following equations:

$$t_{1/2} = \frac{\ln 2}{\lambda_z} \qquad \text{(Equation 8.1)}$$

$$CL = \frac{D}{AUC^{ss}_{\tau}} \qquad \text{(Equation 8.2)}$$

$$V = \frac{CL}{\lambda_z} \qquad \text{(Equation 8.3)}$$

where $t_{1/2}$ is the half-life, CL is the total body clearance, D is the dose, and V is the volume of distribution.

The half-life of colistin was calculated with Equation 8.1.

8.2.5 Statistical analysis

Differences between the values of $t_{1/2}$ of colistin methanesulphonate and colistin were examined with a paired *t*-test. A *P*-value less than 0.05 was considered significant.

8.3 Results

There were no adverse events reported except for patient 9 who experienced itching of the scalp, eyebrows and face. Chromatograms typical of those from all plasma samples are shown in Figure 8.1 for patient 11. The concentrations of colistin methanesulphonate and colistin in plasma for each patient are presented in Appendix IV. A concentration in plasma versus time profile for patient 1 is shown in Figure 8.2, and is typical of the other patients. The pharmacokinetic parameters of colistin methanesulphonate and colistin are presented in Table 8.2. The half-life of colistin methanesulphonate (124 ± 52 min) was significantly shorter than that of colistin (251 ± 79 min) ($P < 0.001$).

8.4 Discussion

As reviewed in Chapter 1, for several decades colistin methansulphonate was rarely used for treatment of infections with gram-negative bacteria due to the potential for neurological and nephrological toxicity. Many clinical isolates of *P. aeruginosa*, however, have shown high resistance against the commonly used antipseudomonal antibiotics (Beringer, 2001; Oliver *et al.*, 2000). This is also borne out in the current study (Section 8.2.1) and in Chapter 2 (Table 2.1). In contrast, colistin methanesulphonate has been shown to be efficacious for treating infections with *P. aeruginosa* (Beringer, 2001; Conway et al., 1997; Littlewood et al., 2000). However, knowledge of the pharmacokinetics of colistin methanesulphonate, which would assist in its safe and efficacious use, is scanty.

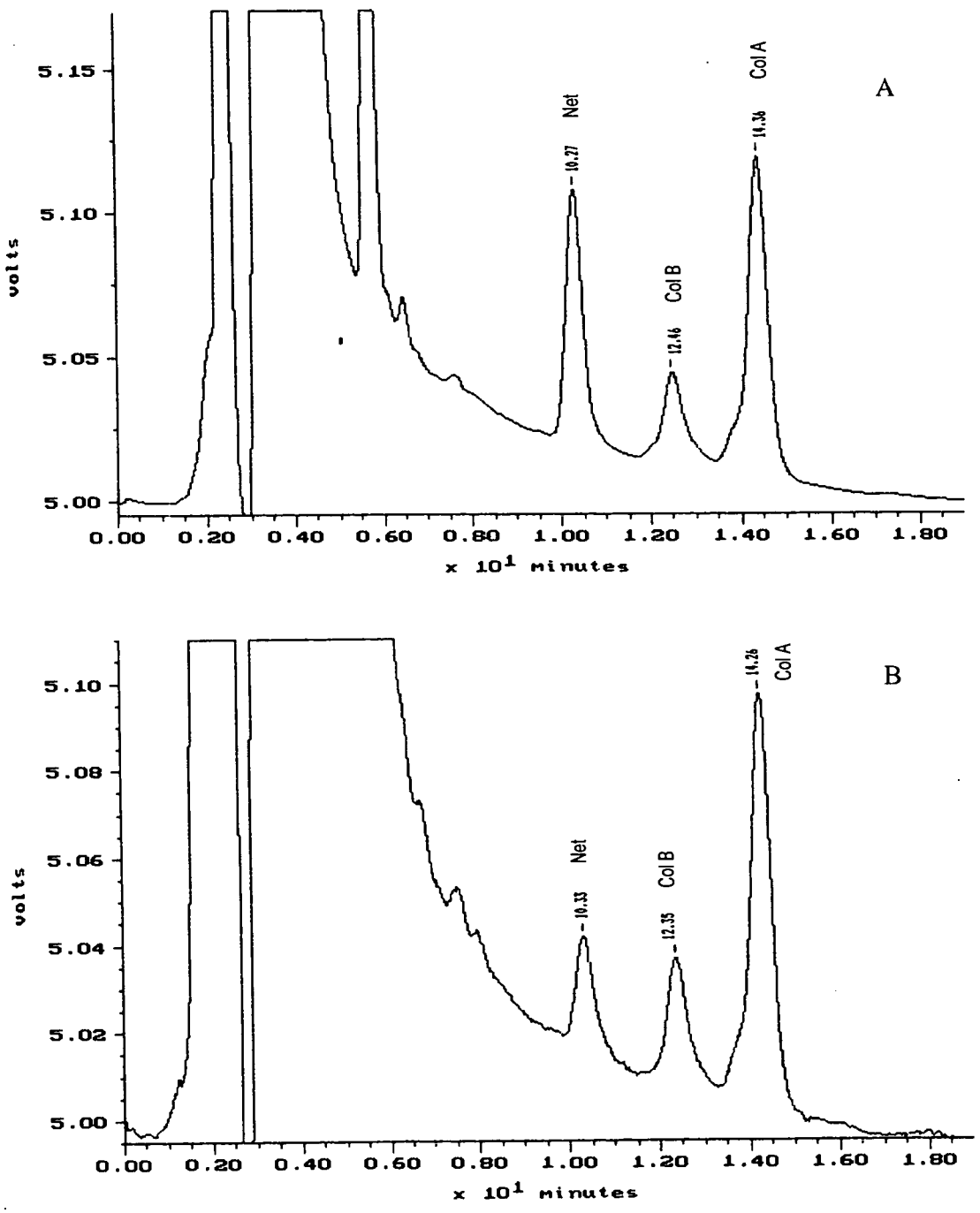

Figure 8.1 Typical chromatograms obtained with fluorescence detection for colistin methanesulphonate (A) and colistin (B) in the sample at 80 min after an intravenous infusion of 2.64 mg/kg colistin methanesulphonate in patient 11 on the sixth day of therapy.

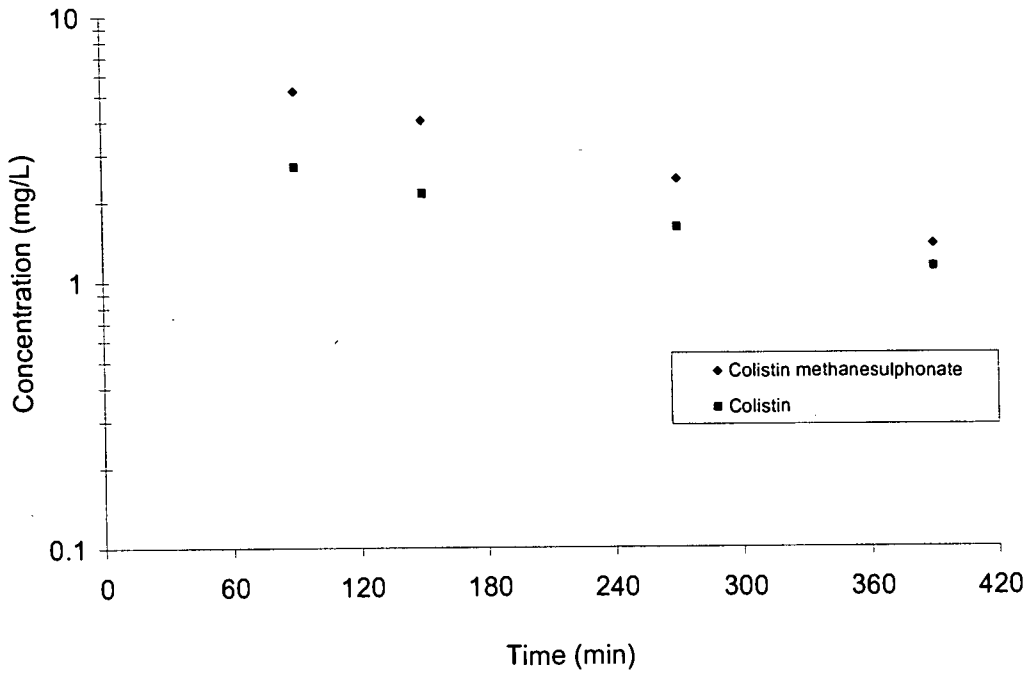

Figure 8.2 Concentrations of colistin methanesulphonate and colistin in plasma from patient 1 after an intravenous infusion of 2.62 mg/kg colistin methanesulphonate over 30 minutes administered on the third day of therapy.

Assays for colistin methanesulphonate have been reviewed in Section 1.3.6.1.2. Due to the complex mixture of products, including colistin, which arise from the hydrolysis of colistin methanesulphonate, microbiological assays and the only reported HPLC assay (Reed et al., 2001) are not specific to colistin methanesulphonate. Therefore, the concentrations of drug in plasma and urine reported in previous studies are most likely to represent a mixture of colistin methanesulphonate and colistin, and the published pharmacokinetics of colistin methanesulphonate based on these data should also be regarded as values for a mixture. Since colistin has higher overall bactericidal and postantibiotic activities than colistin methanesulphonate (Chapter 2), it will be of great value to obtain concentrations of both forms when investigating their *in vivo* pharmacodynamics. The present study was designed to measure the concentrations of both colistin methanesulphonate and colistin in plasma separately by two novel HPLC

methods (Chapters 3 and 4) after intravenous administration of colistin methanesulphonate. This has allowed a more detailed description of the pharmacokinetics of colistin methanesulphonate in humans than has hitherto been possible.

Table 8.2 Pharmacokinetic parameter estimates of colistin methanesulphonate and colistin

Patient	Colistin methanesulphonate				Colistin	
	AUC_τ^{ss} (mg·min/L)	CL (mL/min/kg)	V (mL/kg)	$t_{1/2}$ (min)	AUC_τ^{ss} (mg·min/L)	$t_{1/2}$ (min)
1	1470	1.78	404	157	859	248
2	747	2.33	324	97	313	154
3	1303	1.98	271	95	630	267
4	1939	1.20	461	268	1060	346
5	780	2.09	485	162	483	239
6	1420	2.13	332	108	500	217
7	820	2.45	323	91	258	139
8	981	2.63	462	122	376	315
9	1288	1.85	285	107	575	329
10	2156	1.13	169	103	1010	385
11	1206	2.19	296	94	686	178
12	1344	2.31	272	82	420	198
Mean	1288	2.01	340	124	597	251
S.D.	437	0.46	95	52	263	79

The half-life of colistin methanesulphonate in the 12 patients ranged from 82 min to 268 min, and that of colistin was from 139 min to 385 min (Table 8.2). Therefore, plasma

concentrations could be assumed to be at steady-state in the present study when the samples were collected at least two days after the first dose (Table 8.1).

Collection of more blood samples within the dosing interval would have allowed a more accurate determination of AUC_{τ}^{ss}. However, ethical considerations precluded a more extensive collection. In the current study, four samples were collected from each patient. The decline in concentrations of colistin methanesulphonate and colistin was assumed to be monoexponential. It is possible that the assumption of a monoexponential decline in concentrations of colistin methanesulphonate and extrapolation for calculated concentrations at the beginning and end of the infusion provided an underestimate of the true AUC_{τ}^{ss}. This would lead to the overestimation of the CL and V.

In the current study, colistin methanesulphonate had a mean CL, V and half-life of 2.01 mL/min/kg, 340 mL/kg and 124 min, respectively. While it was not possible to elucidate the relative contributions of renal and non-renal clearance to the elimination of colistin methanesulphonate because urine was not collected, comparison of the magnitude of CL with normal hepatic blood flow of 20.7 mL/min/kg in humans (Davies et al., 1993), indicates that, as in the rat (Chapter 7), the hepatic extraction ratio of colistin methanesulphonate was very low.

Comparison of the pharmacokinetics of colistin methanesulphonate in humans in the present chapter to published experience is difficult since very few early studies provided pharmacokinetic data. Most reports in humans have focused only on the plasma and urine levels of colistin methanesulphonate measured by microbiological assays (Froman *et al.*, 1970; Yamada *et al.*, 1974). The values for CL (2.01 ± 0.46 mL/min/kg) and V (340 ± 95 mL/kg) of colistin methanesulphonate in the current study were higher than the representative values of 0.35 ± 0.09 mL/min/kg and 0.09 ± 0.02 L/kg reported by Reed et al. (Reed et al., 2001). As discussed in Chapters 1, 4 and 7, it is unclear whether the concentrations measured by Reed et al. were for colistin methanesulphonate or colistin. If they were for colistin methanesulphonate, there was no evidence that colistin methanesulphonate had been hydrolysed completely to colistin before derivatization. If they were for colistin then, judging by the plasma levels of colistin in the present chapter,

it is most likely that the concentrations were overestimated and is probably one of the reasons that the values of CL and V_{ss} were lower than those in the current study. Undoubtedly, biological variation in different groups of CF patients is another important factor.

Colistin had a half-life significantly longer than that of colistin methanesulphonate (251 ± 79 min *vs.* 124 ± 52 min, $P < 0.001$). This indicated that elimination of colistin rate-limits its disposition in the body. Compared with the pharmacokinetics of colistin and colistin methanesulphonate after an intravenous bolus in rats, the half-lives in humans are longer than those observed in rats (23.6 ± 3.9 min for colistin methanesulphonate and 55.7 ± 19.3 min for colistin after the intravenous administration of colistin methanesulphonate, Chapter 7). However, the relativity in half-lives of colistin methanesulphonate and colistin is comparable between these two species. The CL and V of colistin could not be obtained in the current study.

The concentrations of colistin methanesulphonate in plasma ranged from 9.8 to 0.36 mg/L during the sampling period (approximately 1 to 6 hr after the end of the infusion) and 3.1 to 0.23 mg/L for colistin (Appendix IV). Ratios for the concentrations of colistin to colistin methanesulphonate in the first sample ranged from 0.23 to 0.61. The mean AUC_{τ}^{ss} (597 mg·min/L) of colistin was approximately 46.4% of the value for colistin methanesulphonate (1288 mg·min/L). The content of colistin in the dosing solution of colistin methanesulphonate was examined by HPLC (Chapter 3) and the maximum amount of colistin co-administered was 0.06% of colistin methanesulphonate even after storage of the dosing solution at 4°C for 72 hr (Chapter 5). It is apparent, therefore, that the hydrolysis of colistin methanesulphonate *in vivo* in humans is the major contributor to the appearance of colistin in plasma. Since previous stability work (Chapter 5) showed that about 13% of colistin methanesulphonate was converted to colistin in plasma stored at 37°C for 2 hr, the formation of colistin *in vivo* after administration of colistin methanesulphonate was more extensive and rapid than could be accounted for by hydrolysis in plasma alone.

Koch-Weser et al. (1970) reported on the common occurrence of severe adverse effects in humans after administration of colistin methanesulphonate. The dose used in that study was 4.0 - 5.4 mg/kg/day, and intramuscular injection was employed (Koch-Weser *et al.*, 1970). In the current study, no serious adverse events occurred, except that in patient 9 more rapid intravenous infusion (less than 0.5 hr) caused itching of the scalp, eyebrows and face; these side effects were overcome by slower infusion (1 hr). The doses administered in the present study, although higher than those administered by Koch-Weser et al. (1970), were generally well tolerated. In the study of Koch-Weser et al. (1970), due to the complexity of the patients' underlying illnesses, it may have been concluded erroneously that colistin was the cause of the severe adverse effects. It is also possible that the solution of colistin methanesulphonate administered by Koch-Weser et al. (1970) contained a substantial proportion of hydrolysis products (colistin and partially sulphomethylated derivatives). Another possible cause for the greater apparent toxicity observed by Koch-Weser et al. (1970) relates to the intramuscular route of administration in comparison with the intravenous route used in the current study. The safety experience of intravenous administration of colistin methanesulphonate in this study was consistent with the experience reported recently (Conway *et al.*, 2000; Conway *et al.*, 1997; Ledson *et al.*, 1998; Reed *et al.*, 2001). The adverse effects are not deemed very serious and usually transient; importantly, very few adverse effects were irreversible when the antibiotic was discontinued (Conway *et al.*, 1997).

The calculated C^{ss}_{max} of colistin methanesulphonate ranged from 3.6 mg/L to 13.2 mg/L and the corresponding C^{ss}_{min} varied between 0.18 mg/L to 2.0 mg/L. For colistin, the ranges of calculated C^{ss}_{max} and C^{ss}_{min} were 1.2 mg/L to 3.1 mg/L and 0.14 mg/L to 1.3 mg/L, respectively (Appendix IV). Considering the *in vitro* pharmacodynamics of colistin and colistin methanesulphonate against *P. aeruginosa* (Chapter 2), the C^{ss}_{max} of colistin methanesulphonate and colistin, even without consideration of protein binding, were in the same range as the MICs which were noted in the susceptible isolates of *P. aeruginosa* from CF patients (1 mg/L - 4 mg/L for colistin and 4 mg/L - 16 mg/L for colistin methanesulphonate). In addition, it should be noted that the current study was not able to provide information on the unbound concentrations in lung tissue. For colistin

methanesulphonate, the plasma concentrations were substantially less than the 16×MIC (64 mg/L - 256 mg/L) required for complete *in vitro* killing within 24 hr and significant postantibiotic effects. Similarly, the plasma concentrations of colistin were approximately 0.5×MIC (0.5 mg/L - 2 mg/L), a concentration at which not only *P. aeruginosa* could not be eradicated in 24 hr but there was no significant postantibiotic effect (Chapter 2). Therefore, doses higher than those in the current study might enhance the efficacy of colistin methanesulphonate. A dose-ranging study for intravenous colistin methanesulphonate to examine clinical and microbiological cure versus incidence of adverse effects should be considered.

Undoubtedly, MICs of colistin and colistin methanesulphonate against different *P. aeruginosa* isolates may vary over a broad range. Furthermore, care is needed in comparing the *in vitro* MICs (determined individually for the two agents in Chapter 2, and notwithstanding the potential for some hydrolysis of colistin methanesulphonate to colistin during the incubation) with the plasma concentrations for both agents occurring after administration of colistin methanesulphonate. Obviously, there is the possibility for additive antibacterial effect when these two species are present simultaneously in plasma.

8.5 Conclusions

This is the first time that the pharmacokinetics of colistin methanesulphonate has been described using concentrations of colistin methanesulphonate and colistin in plasma measured separately by two sensitive HPLC methods. Similarly to observations in the rat, colistin was formed *in vivo* from colistin methanesulphonate in humans, and colistin methanesulphonate had a shorter half-life than colistin. Because colistin methanesulphonate and colistin have different pharmacokinetics and *in vitro* pharmacodynamics, the results will assist in refining the clinical use of the drug. According to the *in vitro* pharmacodynamics of colistin and colistin methanesulphonate and the concentrations achieved in plasma (observed in this study), doses higher than 2 to 3 mg/kg every 8 hr may be necessary to maximize its efficacy when given by intravenous infusion for the treatment of *P. aeruginosa* infections in patients with CF.

Chapter 9 General conclusion

Colistin is an 'old' antimicrobial agent that is attracting increased interest because of its marked *in vitro* activity against the bacterium *P. aeruginosa*, particularly the multi-drug resistant clinical isolates. Although it was discovered half a century ago and has been clinically available since 1959, colistin had been relegated to the status of a reserve antimicrobial agent because of its potential toxicity. The rapid development of resistance to commonly used antimicrobial agents has promoted the search for novel antimicrobials. However, except for meropenem there has been no new antipseudomonal agent released since 1995. At a time when human beings are brought to the verge of a medical disaster by 'super bugs', many 'disappointed' researchers are re-examining those so-called 'old' antimicrobials, which were once abandoned or banished because of their adverse effects. Colistin is one such example.

Colistin is administered systematically as the methanesulphonate derivative, formed by reaction of the amine groups of the five diaminobutyric acid residues with formaldehyde and sodium bisulphite. Colistin has rapid bacterial activity against multi-drug resistant strains of *P. aeruginosa*, and resistance to its activity develops slowly. However, the use of colistin requires considerable care because of its neurotoxicity and nephrotoxicity. Therefore, a thorough understanding of the pharmacokinetics and pharmacodynamics is important for the safe and rational use of colistin. Unfortunately, comprehensive data are lacking. Most pharmacokinetic studies have been based on concentrations measured by microbiological assays. Such assays are not able to measure concentrations of the multiple components of colistin separately or are unreliable in the presence of other co-administered antimicrobials active against the test strain. The aims of the current thesis were to explore the *in vitro* pharmacodynamics of colistin and colistin methanesulphonate, to develop accurate and sensitive HPLC methods for measuring colistin and colistin methanesulphonate in biological fluids and to describe their pharmacokinetics.

The *in vitro* pharmacodynamic properties of colistin and colistin methanesulphonate were investigated by evaluating their MICs, time-kill kinetics and postantibiotic effects against

mucoid and nonmucoid strains of *P. aeruginosa* isolated from patients with CF at the Women's and Children's Hospital in Adelaide (Chapter 2). An ATCC typical strain of *P. aeruginosa* ATCC 27853 was used as the reference. Thirty strains were selected for the susceptibility testing. For the susceptible strains, MICs of colistin ranged from 1 mg/L to 4 mg/L, while the MICs of colistin methanesulphonate were from 4 mg/L to 16 mg/L, indicating that colistin had higher antibacterial activity. The time-kill kinetics were explored with four strains at drug concentrations ranging from 0.5 to 64 times the MIC. Colistin showed more rapid killing. Colistin exhibited a significant postantibiotic effect of 2 to 3 hr at 16×MIC against the four strains after 15 min of exposure. The postantibiotic effects were shorter at the concentrations tested for colistin methanesulphonate. Overall, colistin methanesulphonate had lower bactericidal and postantibiotic effects than colistin, but both displayed concentration dependent killing.

Pharmacokinetic studies depend on accurate assays for the drugs. Before work for this thesis was commenced, there was only one HPLC assay for colistin in biological matrices. This method required derivatization to a fluorescent product using *ortho*-phthalaldehyde. However, the instability of this reagent and its derivatives restricts its use. Because 9-fluorenylmethyl chloroformate and its derivatives are relatively more stable and the reaction is rapid at room temperature, it was chosen as a candidate for the derivatization of colistin. Colistin was extracted from plasma on a solid-phase extraction C_{18} cartridge, derivatized on the same cartridge and, after elution, was subjected to RP-HPLC with fluorescence detection (Chapter 3). The FMOC derivatives of colistin were stable and two major components, colistin A and colistin B identified by electrospray ionization mass spectra, were able to be quantitated from two well-separated peaks of their FMOC derivatives. The method was shown to be valid for measuring colistin in plasma to as low as 0.10 mg/L. This is the first report for measuring the relative concentrations of colistin A and colistin B.

Colistin methanesulphonate has the potential to hydrolyse in aqueous media to form an extremely complex mixture of partially sulphomethylated derivatives of colistin, plus colistin. It is difficult to measure the concentrations of each sulphomethylated form. However, an indirect method was developed to quantify the total of the full and partial methanesulphonate derivatives of colistin in biological matrices (Chapter 4). In the

presence of sulphuric acid, colistin methanesulphonate was hydrolysed to colistin in 10 min and the product was measured by an HPLC method similar to that developed for colistin. The apparent concentrations of colistin methanesulphonate were calculated from the values measured after hydrolysis minus the concentrations of colistin, after correcting for differences in their average molecular weights. Both HPLC methods showed no interference from the common antimicrobials co-administered with colistin or colistin methanesulphonate. Since colistin and colistin methanesulphonate have different *in vitro* pharmacodynamics, when studying their pharmacokinetics, it is important to measure their concentrations separately. This is the first report of assays for their separate measurement in biological matrices.

The stability of colistin and colistin methanesulphonate in aqueous media, including water, isotonic phosphate buffer (pH 7.4), human plasma, rat urine, saline and Muller-Hinton broth, was examined (Chapter 5) before undertaking pharmacokinetic studies. Colistin was stable in water at 37°C for up to 120 hr, while in isotonic phosphate buffer and human plasma, colistin A and colistin B showed differences in stability. Colistin methanesulphonate was found to hydrolyse to colistin in aqueous media. A new SAX-HPLC method was developed to show the hydrolysis procedure of colistin methanesulphonate in water. Although the peaks appearing in the SAX-HPLC chromatograms were not able to be identified by mass spectral analysis, they were all related to colistin (Chapter 4). Colistin methanesulphonate hydrolysed more quickly at 37°C than at 4°C and/or ambient temperature in water and rat urine. When Muller-Hinton broth containing 1024 mg/L colistin methanesulphonate (64×MIC against *P. aeruginosa* ATCC 27853) was incubated at 37°C for 30 min, no colistin was detected. However, the Log_{10} CFU of *P aeruginosa* decreased 3 orders during the same period (Chapter 2). Combined, these findings demonstrate that colistin methanesulphonate and the various partially sulphomethylated colistin derivatives have intrinsic bactericidal activity.

Colistin has been reported to be much more toxic than colistin methanesulphonate *in vivo*. Furthermore, anecdotal reports suggest that the two most commonly available commercial products of colistin methanesulphonate, Colomycin® Injection (Pharmax) and Coly-Mycin M Parenteral® (Parke-Davis) have different toxicities. The dissimilar toxicity of the two products may be caused by different levels of colistin present within

the two products, arising either from incomplete sulphomethylation during synthesis or from the hydrolysis during storage. Hence, colistin formed from the hydrolysis of colistin methanesulphonate was quantified following reconstitution of Colomycin® Injection (Pharmax) and Coly-Mycin M Parenteral® (Parke-Davis) in water, isotonic phosphate buffer (pH 7.4) and human plasma. However, there were only minor differences in the appearance of colistin (Chapter 5). Based on these findings, it remains difficult to explain the supposed differences in toxicity of the two commercial products.

Rats were used for the pharmacokinetic studies of colistin (Chapter 6) and colistin methanesulphonate (Chapter 7) after intravenous administration. Colistin (1 mg/kg, as the sulphate salt) or colistin methanesulphonate (15 mg/kg, as the sodium salt) was given as an intravenous bolus via a jugular vein cannula inserted at least two days before the experiments. Urine was collected for 12 hr after administration for colistin and 24 hr for colistin methanesulphonate. Concentrations of drugs in plasma and urine were measured by HPLC (Chapters 3 and 4). The pharmacokinetic parameters were calculated by non-compartmental analysis. Both colistin and colistin methanesulphonate had a short distribution phase followed by a longer elimination phase. However, colistin had a longer terminal half-life (74.6 ± 13.2 min) than colistin methanesulphonate (23.6 ± 3.9 min). The total body clearance of colistin (5.2 ± 0.4 mL/min/kg) was approximately half of that of colistin methanesulphonate (11.7 ± 1.8 mL/min/kg). Colistin (496 ± 62 mL/kg) had a larger volume of distribution than colistin methanesulphonate (299 ± 55 mL/kg). Interestingly, the urinary recoveries of colistin and colistin methanesulphonate were very different. An average of 0.18% of the dose of colistin was recovered in urine in the first 12 hr with no colistin recovered beyond 8 hr. In contrast, 61.1% of the dose of colistin methanesulphonate was recovered in urine in the first 24 hr, half of which was colistin. It was not possible to determine whether the high percentage of colistin was produced before or after urine was formed in the kidney. However, judging by the small fraction of colistin methanesulphonate converted to colistin, based on plasma colistin concentrations achieved after the separate administration of colistin methanesulphonate and colistin, the low urinary recovery of colistin after the administration of colistin, and the instability of colistin methanesulphonate in urine at 37°C and ambient temperature, it was most likely that the high recovery of colistin in urine came from two sources: intra-renal conversion

of colistin methanesulphonate to colistin within tubular cells of the kidney and/or excretion of colistin methanesulphonate by the kidney followed by hydrolysis in the bladder and in the collection vessel. From the markedly different renal clearances of colistin and colistin methanesulphonate, it was concluded that there was substantial net renal tubular reabsorption of colistin, while colistin methanesulphonate underwent net tubular secretion.

Since there was extensive non-specific binding of colistin to Amicon ultrafiltration membranes, protein binding of colistin in rat plasma was examined by equilibrium dialysis against isotonic phosphate buffer (pH 7.4) at 37°C for 12 hr. Approximately 44% of colistin was unbound in plasma, with the percentage less for colistin A (36%) than for colistin B (52%). Hydrolysis of colistin methanesulphonate and binding to Amicon ultrafiltration membranes prevented the investigation of its binding by equilibrium dialysis, unltracentrifugation or ultrafiltration.

While the total body clearance, volume of distribution and terminal half-life of colistin A and colistin B were not significantly different following a dose of colistin sulphate, the pharmcokinetics of unbound colistin A and colistin B were different (Chapter 6). These differences are in keeping with their substantial chromatographic differences and were probably caused by the one $-CH_2-$ difference in the fatty acid side chain.

Finally, the pharmacokinetics of colistin methanesulphonate at steady-state was explored in CF patients after an intravenous infusion (1.63 to 3.11 mg/kg) every 8 hr (Chapter 8). Concentrations of colistin methanesulphonate and colistin were measured by HPLC. Both colistin (251 ± 79 min) and colistin methanesulphonate (124 ± 52 min) had a longer terminal half-life than those in rats. However, the relativity in half-lives of colistin methanesulphonate and colistin was comparable between these two species. The ratios of the concentrations of colistin to colistin methanesulphonate increased over time within a dosage interval. Since colistin and colistin methanesulphonate had different *in vitro* pharmacodynamics, the pharmacokinetics obtained in the present thesis will be advantageous for future *in vivo* pharmacodynamic studies. The results suggest that doses of colistin methanesulphonate higher than the recommended 2 to 3 mg/kg every 8 hr may

be required to achieve concentrations effective for the treatment of *P. aeruginosa* infections in patients.

Although many potentially important findings were obtained in this thesis, there are numerous other aspects of colistin and colistin methanesulphonate requiring further investigation. Firstly, separation of the components of colistin and examination of their pharmacokinetics and pharmacodynamics may provide a compound with an improved benefit (i.e. antibacterial effect) to cost (i.e. adverse effects, development of resistance) ratio. Furthermore, chemical modification of colistin or computer assisted design of similar molecules may produce candidates with a more favorable ratio of antibacterial activity to toxicity. Secondly, description of the complex hydrolysis of colistin methanesulphonate using NMR, MS, etc., will expand the knowledge on its behaviour in biological matrices. Thirdly, employing more sensitive assays, such as HPLC-MS, to study the metabolism and urinary excretion of colistin and colistin methanesulphonate may uncover the fate of a large percentage of the dose of colistin and a significant proportion of colistin methanesulphonate that remains unaccounted for. Fourthly, investigations of their binding in tissues may provide essential information on their mechanism of toxicity. Fifthly, detailed knowledge of the mechanism of their bactericidal activity and bacterial resistance, studied particularly by molecular biological techniques, may contribute to their efficacious use with minimal low resistance. Finally, it may be helpful to conduct a double blind dose-ranging study for the optimisation of dosage. Different groups of patients would receive different size doses and the important end-points, such as microbiological cure, clinical cure and adverse effects, would be examined comprehensively. The investigation of efficacy/toxicity over a range of doses would be expected to allow colistin methanesulphonate to be used more effectively.

Overall, the current thesis provided the first detailed report on the *in vitro* pharmacodynamics, the stability in aqueous media and the pharmacokinetics in rats of colistin and colistin methanesulphonate using novel HPLC assays. The pharmacokinetics of colistin methanesulphonate at steady-state in patients with CF were presented in more detail by measuring the concentrations of both colistin and colistin methanesulphonate, which has not hitherto been possible. These observations will enhance our understanding of the pharmacokinetics and pharmacodynamics of colistin and colistin

methanesulphonate, and will be essential for the rational use of this notable antipseudomonal agent.

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Appendix I Medium formula and standards for susceptibility of *P. aeruginosa* against tested antibiotics in Chapter 2

IA Formula of the media used in the study of *in vitro* pharmacodynamics of colistin and colistin methanesulphonate against *P. aeruginosa*

IA1 Mueller-Hinton Agar (Oxoid)

	g/L
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH 7.4 ± 0.2	

Suspend 38 g in 1 L of distilled water and sterilize by autoclaving at 121°C for 15 min.

IA2 Mueller-Hinton Broth (Oxoid)

	g/L
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
pH 7.4 ± 0.2	

Suspend 21 g in 1 L of distilled water and sterilize by autoclaving at 121°C for 15 min.

IA3 Tryptone Soya Broth (Oxoid)

	g/L
Pancreatic digest of casein	17.0

Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.5
Glucose	2.5
pH 7.4 ± 0.2	

Suspend 30 g in 1 L of distilled water and sterilize by autoclaving at 121°C for 15 min.

IA4 Glycerol Media (modified by the Department of Microbiology and Infectious diseases, Women's and Children's Hospital, Adelaide)

Glycerol media is designed for the long-term storage of stock cultures at -70°C to -20°C.

Add 15 g of Tryptone Soya Broth to 400 mL of distilled water followed by mixing with 100 mL of glycerol. The media is sterilized by autoclaving at 118°C for 10 min. Dispense 1.8 mL into Nalgene Cryovials in Cryoware box and store in fridge for up to 6 months.

IA5 Blood Agar (Oxoid)

	g/L
'Lab-Lemco' powder	10.0
Peptone	10.0
Sodium chloride	5.0
Agar	15.0
pH 7.3 ± 0.2	

Suspend 40 g in 1 L of distilled water and sterilize by autoclaving at 121°C for 15 min. Add 7% of Defibrinated Horse Blood SR50 to the media above when it is at 50°C. Mix with gentle rotation and pour into petri dishes.

IA6 Nutrient Agar (Oxoid)

	g/L
'Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0

pH 7.4 ± 0.2

Suspend 28 g in 1 L of distilled water and sterilize by autoclaving at 121°C for 15 min.

IB Zoon diameter interpretive standards and equivalent minimal inhibitory concentration breakpoints for *P. aeruginosa* for the tested strains in the thesis (From the Department of Microbiology and Infectious Diseases, Women's and Children's Hospital, Adelaide)

Antimicrobial agent	Disk content	Zone diameter, nearest whole (mm) [a]			Equivalent MIC breakpoints (mg/L)	
		R	I	S	R	S
Aztreonam	30 μg	≤ 15	16 - 21	≥ 22	≥ 32	≤ 8
Ceftazidime	30 μg	≤ 14	15 - 17	≥ 18	≥ 32	≤ 8
Meropenem	10 μg	≤ 13	14 - 15	≥ 16	≥ 16	≤ 4
Piperacillin	100 μg	≤ 17	-	≥ 18	≥ 128	≤ 64
Ticarcillin	75 μg	≤ 14	-	≥ 15	≥ 128	≤ 64
Gentamicin	10 μg	≤ 12	13 - 14	≥ 15	≥ 8	≤ 4
Tobramycin	10 μg	≤ 12	13 - 14	≥ 15	≥ 8	≤ 4
Ciprofloxacin	5 μg	≤ 15	16 - 20	≥ 21	≥ 4	≤ 1

[a] R, resistant; I, intermediate; and S, susceptible.

Appendix II Data from the pharmacokinetic study of colistin in rats described in Chapter 6

Tables A2.1 to A 2.5 contain the concentrations of colistin in plasma and amount of colistin recovered in urine collected from each rat. N.Q. means non-quantifiable.

Table A2.1 Concentrations of colistin in plasma and amount of colistin recovered in urine in Rat 1

Biological fluids	Time (min)	Concentration (mg/L)		
		Colistin	Colistin A	Colistin B
Plasma	10	2.45	1.26	1.19
	20	1.91	0.99	0.92
	30	1.50	0.81	0.70
	60	1.18	0.58	0.49
	90	0.79	0.44	0.35
	120	0.50	0.28	0.21
	180	0.36	0.21	0.15
	240	0.16	0.10	0.07
	Time (hr)	**Amount (μg)**		
		Colistin	**Colistin A**	**Colistin B**
Urine	0 to 4	N.Q.	N.Q.	N.Q.
	4 to 8	N.Q.	N.Q.	N.Q.
	8 to 12	N.Q.	N.Q.	N.Q.

Table A2.2 Concentrations of colistin in plasma and amount of colistin recovered in urine in Rat 2

Biological fluids	Time (min)	Concentration (mg/L)		
		Colistin	**Colistin A**	**Colistin B**
Plasma	10	2.08	1.05	1.03
	20	1.47	0.72	0.75
	30	1.09	0.58	0.51
	60	0.91	0.44	0.41
	90	0.73	0.30	0.30
	120	0.60	0.25	0.25
	180	0.29	0.17	0.16
	240	0.20	0.07	0.08
	Time (hr)	**Amount (μg)**		
		Colistin	**Colistin A**	**Colistin B**
Urine	0 to 4	0.93	0.27	0.66
	4 to 8	N.Q.	N.Q.	N.Q.
	8 to 12	N.Q.	N.Q.	N.Q.

Table A2.3 Concentrations of colistin in plasma and amount of colistin recovered in urine in Rat 3

Biological fluids	Time (min)	Concentration (mg/L)		
		Colistin	**Colistin A**	**Colistin B**
Plasma	10	2.44	1.24	1.20
	20	1.79	0.91	0.88
	30	1.16	0.60	0.56
	60	0.83	0.44	0.39
	90	0.70	0.37	0.33
	120	0.50	0.27	0.23
	180	0.34	0.18	0.16
	240	0.23	0.12	0.11
	Time (hr)	**Amount (μg)**		
		Colistin	**Colistin A**	**Colistin B**
Urine	0 to 4	0.59	0.21	0.38
	4 to 8	N.Q.	N.Q.	N.Q.
	8 to 12	N.Q.	N.Q.	N.Q.

Table A2.4 Concentrations of colistin in plasma and amount of colistin recovered in urine in Rat 4

Biological fluids	Time (min)	Concentration (mg/L)		
		Colistin	**Colistin A**	**Colistin B**
Plasma	10	2.29	1.17	1.12
	20	1.63	0.84	0.79
	30	1.20	0.65	0.55
	60	0.84	0.45	0.39
	90	0.65	0.35	0.30
	120	0.47	0.25	0.22
	180	0.28	0.15	0.13
	240	0.19	0.10	0.09
	Time (hr)	**Amount (μg)**		
		Colistin	**Colistin A**	**Colistin B**
Urine	0 to 4	1.11	0.41	0.69
	4 to 8	N.Q.	N.Q.	N.Q.
	8 to 12	N.Q.	N.Q.	N.Q.

Table A2.5 Concentrations of colistin in plasma and amount of colistin recovered in urine in Rat 5

Biological fluids	Time (min)	Concentration (mg/L)		
		Colistin	**Colistin A**	**Colistin B**
Plasma	10	2.40	1.29	1.11
	20	1.61	0.85	0.75
	30	1.36	0.74	0.62
	60	0.85	0.46	0.39
	90	0.62	0.36	0.26
	120	0.42	0.23	0.19
	180	0.20	0.11	0.09
	240	0.11	0.06	0.05
	Time (hr)	**Amount (μg)**		
		Colistin	**Colistin A**	**Colistin B**
Urine	0 to 4	0.22	0.07	0.15
	4 to 8	N.Q.	N.Q.	N.Q.
	8 to 12	N.Q.	N.Q.	N.Q.

Table A2.6 Pharmacokinetic parameters of colistin, colistin A and colistin B in five rats

	Rat	Pharmacokinetic parameters			
		CL (mL/min/kg)	V_{ss} (mL/kg)	MRT (min)	$t_{1/2}$ (min)
Colistin	1	4.8	406	83.9	60.1
	2	5.7	514	89.7	64.5
	3	4.9	529	108	86.6
	4	5.1	566	110	90.0
	5	5.6	466	83.3	71.7
Colistin A	1	4.5	429	95.8	73.0
	2	5.7	546	95.0	73.8
	3	4.7	561	118	94.8
	4	5.1	541	106	83.3
	5	5.5	415	75.4	60.5
Colistin B	1	5.1	423	83.5	65.3
	2	5.4	543	99.7	77.8
	3	4.8	569	119	98.4
	4	5.3	549	103	83.8
	5	6.1	458	75.2	61.6

Appendix III Data from the pharmacokinetic study of colistin methanesulphonate in rats described in Chapter 7

Tables A3.1 to A3.5 contain the concentrations of colistin methanesulphonate and colistin formed by hydrolysis in plasma and the amount in urine collected from each rat. N.Q. means non-quantifiable.

Table A3.1 Concentrations of colistin methanesulphonate and colistin in plasma and the amount recovered in urine in Rat 6

Biological fluids	Time (min)	Concentration (mg/L)	
		Colistin methanesulphonate	Colistin
Plasma	5	36.9	2.4
	10	24.9	2.4
	20	15.2	1.7
	30	9.3	0.99
	60	3.3	0.63
	90	1.5	0.44
	120	0.45	0.39
	Time (hr)	**Amount (μg)**	
		Colistin methanesulphonate	**Colistin**
Urine	0 to 4	2516	606
	4 to 8	84.1	62.7
	8 to 12	3.2	5.7
	12 to 24	1.2	4.9

Table A3.2 Concentrations of colistin methanesulphonate and colistin in plasma and the amount recovered in urine in Rat 7

Biological fluids	Time (min)	Concentration (mg/L)	
		Colistin methanesulphonate	**Colistin**
Plasma	5	47.6	2.8
	10	24.9	3.1
	20	16.6	1.7
	30	12.0	1.4
	60	2.8	0.76
	90	2.5	0.42
	120	1.6	0.23
	Time (hr)	**Amount (μg)**	
		Colistin methanesulphonate	**Colistin**
Urine	0 to 4	1555	1216
	4 to 8	21.6	50.2
	8 to 12	36.5	5.6
	12 to 24	15.7	3.8

Table A3.3 Concentrations of colistin methanesulphonate and colistin in plasma and the amount recovered in urine in Rat 8

Biological fluids	Time (min)	Concentration (mg/L)	
		Colistin methanesulphonate	**Colistin**
Plasma	5	56.8	2.4
	10	33.2	2.6
	20	19.9	1.2
	30	13.0	1.1
	60	4.0	0.73
	90	2.2	0.39
	120	1.0	0.32
	Time (hr)	**Amount (μg)**	
		Colistin methanesulphonate	**Colistin**
Urine	0 to 4	146.8	2517
	4 to 8	Voided	Voided
	8 to 12	91.1	6.9
	12 to 24	11.4	1.2

Table A3.4 Concentrations of colistin methanesulphonate and colistin in plasma and the amount recovered in urine in Rat 9

Biological fluids	Time (min)	Concentration (mg/L)	
		Colistin methanesulphonate	**Colistin**
Plasma	5	45.1	5.4
	10	36.6	3.2
	20	20.4	2.2
	30	12.0	1.6
	60	6.0	0.94
	90	2.7	0.58
	120	0.97	0.42
	Time (hr)	**Amount (μg)**	
		Colistin methanesulphonate	**Colistin**
Urine	0 to 4	1454	255
	4 to 8	2069	864
	8 to 12	141.1	45.8
	12 to 24	0.0	5.3

Table A3.5 Concentrations of colistin methanesulphonate and colistin in plasma and the amount recovered in urine in Rat 10

Biological fluids	Time (min)	Concentration (mg/L)	
		Colistin methanesulphonate	**Colistin**
Plasma	5	56.9	3.9
	10	25.5	2.5
	20	15.6	1.9
	30	8.5	1.7
	60	4.9	0.99
	90	2.0	0.54
	120	0.36	0.46
	Time (hr)	**Amount (μg)**	
		Colistin methanesulphonate	**Colistin**
Urine	0 to 4	Voided	Voided
	4 to 8	Voided	Voided
	8 to 12	763	1236
	12 to 24	N.Q.	37.3

Table A3.6 Pharmacokinetic parameters of colistin methanesulphonate and colistin in five rats

	Rat	Pharmacokinetic parameters			
		CL (mL/min/kg)	V_{ss} (mL/kg)	AUC_0^∞ (mg·min/L)	$t_{1/2}$ (min)
Colistin methanesulphonate	6	14.9	355	1009	20.4
	7	11.4	346	1311	29.7
	8	10.2	251	1473	23.8
	9	10.8	311	1394	24.0
	10	11.2	233	1342	20.0
Colistin	6	-	-	153	87.1
	7	-	-	132	34.1
	8	-	-	127	50.8
	9	-	-	190	52.5
	10	-	-	179	53.9

Appendix IV Data from the pharmacokinetic study of colistin methanesulphonate in patients with cystic fibrosis described in Chapter 8

Tables A4.1 to A4.12 contain the concentrations of colistin methanesulphonate and colistin, in plasma collected from each patient. Concentrations shown in shading were not measured directly (see Section 8.2.4).

Table A4.1 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 1

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.94	0.90
30	6.8	3.1
90	5.2	2.7
150	4.0	2.1
270	2.4	1.6
390	1.4	1.1
480	0.94	0.90

Table A4.2 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 2

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.19	0.20
30	4.8	1.5
90	2.9	1.2
150	2.3	0.79
270	0.85	0.51
390	0.36	0.29
480	0.19	0.20

Table A4.3 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 3

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.31	0.69
30	8.4	2.2
90	7.4	2.1
150	2.4	1.5
270	1.4	1.1
390	0.67	1.0
480	0.31	0.69

Table A4.4 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 4

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	2.0	1.3
10	6.8	3.4
70	5.4	3.1
130	5.2	2.6
250	4.2	2.1
370	2.5	1.7
480	2.0	1.3

Table A4.5 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 5

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.49	0.47
15	3.6	1.8
65	2.6	1.6
135	2.5	1.3
255	1.3	0.93
365	0.77	0.64
480	0.49	0.47

Table A4.6 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 6

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.48	0.45
30	8.5	1.9
90	6.6	1.5
150	3.5	1.3
270	1.6	1.0
390	0.94	0.54
480	0.48	0.45

Table A4.7 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 7

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.18	0.14
30	5.6	1.3
95	3.5	1.0
150	2.3	0.67
270	0.82	0.38
390	0.38	0.23
480	0.18	0.14

Table A4.8 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 8

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.39	0.45
15	5.6	1.2
75	4.1	1.2
135	2.5	0.90
250	1.7	0.68
375	0.67	0.61
480	0.39	0.45

Table A4.9 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 9

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.47	0.73
60	7.2	1.8
120	5.4	1.6
180	3.0	1.3
300	1.3	1.2
390	0.94	0.85
480	0.47	0.73

Table A4.10 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 10

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.61	1.3
20	13.2	3.1
80	9.8	2.9
155	4.4	2.3
215	4.0	2.1
425	0.88	1.5
480	0.61	1.3

Table A4.11 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 11

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.27	0.50
20	8.0	3.0
80	7.7	2.3
140	2.1	2.0
260	1.2	1.1
380	0.66	0.77
480	0.27	0.50

Table A4.12 Concentrations of colistin methanesulphonate and colistin in plasma of Patient 12

Time (min)	Concentration (mg/L)	
	Colistin methanesulphonate	Colistin
0	0.22	0.35
30	9.9	1.7
90	6.2	1.5
150	3.4	1.0
270	1.3	0.72
390	0.47	0.51
480	0.22	0.35

Appendix V Publications in support of this thesis

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In Vitro Pharmacodynamic Properties of Colistin and Colistin Methanesulfonate against *Pseudomonas aeruginosa* Isolates from Patients with Cystic Fibrosis

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The in vitro pharmacodynamic properties of colistin and colistin methanesulfonate were investigated by studying the MICs, time-kill kinetics, and postantibiotic effect (PAE) against mucoid and nonmucoid strains of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis. Twenty-three clinical strains, including multiresistant strains, and one type strain were selected for MIC determination. Eleven strains were resistant; MICs for these strains were >128 mg/liter. For the susceptible strains, MICs of colistin ranged from 1 to 4 mg/liter, while the MICs of colistin methanesulfonate were significantly higher and ranged from 4 to 16 mg/liter. The time-kill kinetics were investigated with three strains at drug concentrations ranging from 0.5 to 64 times the MIC. Colistin showed extremely rapid killing, resulting in complete elimination at the highest concentrations within 5 min, while colistin methanesulfonate killed more slowly, requiring a concentration of 16 times the MIC to achieve complete killing within 24 h. Colistin exhibited a significant PAE of 2 to 3 h at 16 times the MIC against the three strains after 15 min of exposure. For colistin methanesulfonate, PAEs were shorter at the concentrations tested. Colistin methanesulfonate had lower overall bactericidal and postantibiotic activities than colistin, even when adjusted for differences in MICs. Our data suggest that doses of colistin methanesulfonate higher than the recommended 2 to 3 mg/kg of body weight every 12 h may be required for the effective treatment of *P. aeruginosa* infections in cystic fibrosis patients.

Cystic fibrosis (CF) is one of the most common inherited diseases in western countries and is characterized by recurrent lower respiratory tract infections. *Pseudomonas aeruginosa* is the predominant respiratory pathogen and is isolated from about 80% of patients over their life times (9). *P. aeruginosa* plays an important role in the progressive lung destruction and subsequent respiratory failure that occurs in CF patients (9).

Colistin (or polymyxin E), a polypeptide antibiotic, was first isolated in Japan from *Bacillus polymyxa* subsp. *colistinus* in 1947 and became available for clinical use in 1959. Colistin is a multicomponent antibiotic consisting of several closely related decapeptides with a general structure composed of a cyclic heptapeptide moiety and a side chain acetylated at the N terminus by a fatty acid. The two main components, which have been identified by the composition of their amino acids and fatty acids (1), are colistin A (polymyxin E1) and colistin B (polymyxin E2). They have the same amino acids but a different fatty acid (6-methyloctanoic acid and 6-methylheptanoic acid, respectively). Colistins are bactericidal to gram-negative bacteria by a detergent-like mechanism, interfering with the structure and function of the outer and cytoplasmic membranes of bacteria. This mechanism involves interaction with lipopolysaccharides and phospholipids of the outer membrane and electrostatic interference with the outer membrane by competitively displacing divalent cations (calcium and magnesium) from the negatively charged phosphate groups of membrane lipids (13). The resultant damage to the osmotic barrier leads to leakage of intracellular contents.

Colistin has many characteristics which favor its use in the treatment of multiresistant *P. aeruginosa* isolates from patients with CF, including rapid bactericidal activity, purportedly rare development of resistance, and a narrow spectrum of activity. It is currently enjoying a resurgence, with use via both the inhalational and intravenous routes, in the treatment of chronic infection and acute exacerbations of CF due to multiresistance to other agents. It is used therapeutically as colistin methanesulfonate, which has sulfomethyl moieties attached to the five amine functional groups.

Early clinical reports showed a high incidence of toxicity with colistin, but further studies suggested that this conclusion resulted from inappropriate patient selection, higher-than-recommended doses, and inappropriate monitoring (4). The most appropriate dosing schedule of an antibiotic depends on its pharmacodynamic parameters, including the MICs for the target pathogens, time-kill kinetics, and postantibiotic effect (PAE). Because there are potency differences between colistin and colistin methanesulfonate (11), our studies were carried out with both forms. The aims of our study were to examine the in vitro pharmacodynamic properties, namely, bacterial killing and PAE, of colistin and colistin methanesulfonate and to compare the magnitudes of pharmacodynamic properties with levels achieved in vivo.

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TABLE 1. MICs of colistin and colistin methanesulfonate against *P. aeruginosa* isolates from CF patients

Mucoid status	Susceptibility pattern	No.of strains	Colistin form	MIC (mg/liter)	
				Range	Geometric mean
All strains[a]	Susceptible	13	Sulfate	1–4	3.1
			Na methanesulfonate	4–16	7.1
	Resistant	11	Sulfate	>128	>128
			Na methanesulfonate	>128	>128
Mucoid	Susceptible	5	Sulfate	1–4	2.8
			Na methanesulfonate	4–8	5.6
Nonmucoid[a]	Susceptible	8	Sulfate	1–4	3.3
			Na methanesulfonate	4–16	8.0

[a] Includes type strain ATCC 27853.

MATERIALS AND METHODS

Bacterial strains and antibiotics. Twenty-three clinical isolates of *P. aeruginosa*, both mucoid and nonmucoid strains, were selected from routine clinical isolates from patients with acute exacerbations of CF. Isolates were selected sequentially from different patients as they presented with acute exacerbations; both mucoid and nonmucoid strains may have come from the same patient. Strains were identified by colonial morphology, characteristic pigment production, and resistance to C-390 (7). Sixty-one percent of these strains were resistant to at least three of the following agents: aztreonam, ceftazidime, meropenem, piperacillin, ticarcillin, gentamicin, tobramycin, and ciprofloxacin. In addition, 74% were resistant to tobramycin and 52% were resistant to ticarcillin, the principal agents used in our hospital for acute exacerbations of CF. A type culture of *P. aeruginosa* (ATCC 27853) was also studied. Subcultures were maintained on horse blood agar. Colistin (sulfate) was obtained from Sigma (St. Louis, Mo.). Colistin methanesulfonate (sodium) was obtained from Dumex (Copenhagen, Denmark).

MIC determination. MICs were determined by both broth macrodilution and microdilution in cation-adjusted Mueller-Hinton broth (Oxoid, Hampshire, England) according to NCCLS standards (16). Strains were considered resistant to colistin and colistin methanesulfonate if the MICs were ≥32 mg/liter.

Time-kill kinetics. The time-kill kinetics of four strains, ATCC 27853 and three clinical isolates, two of which were mucoid, were examined. The clinical isolates were selected in order to have a range of MICs within the susceptible category. The MICs of colistin and colistin methanesulfonate, respectively, for the four strains were as follows: ATCC 27853, 4 and 16 mg/liter; 18982, 4 and 8 mg/liter; 19056, 1 and 8 mg/liter; and 20223, 4 and 16 mg/liter. Colistin and colistin methanesulfonate were added to a logarithmic-phase broth culture of approximately 10^6 CFU/ml to yield concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32, and 64 times the MIC for the strain under study. Subcultures for viable counts were performed on nutrient agar (Oxoid) at 0, 5, 10, 15, 20, 25, 30, 45, and 60 min and 2, 3, 4, and 24 h after antibiotic addition. Viable counts were determined after 24 h of incubation of subcultures at 37°C.

PAE. The in vitro PAE was determined by the standard in vitro method (5) for two of the three clinical strains noted above and the ATCC strain with both agents. For each experiment, *P. aeruginosa* (≈10^6 CFU/ml) in logarithmic phase growth was exposed for 15 min (for colistin) or 1 h (for colistin methanesulfonate) in Mueller-Hinton broth (Oxoid) to the antibiotics at concentrations of 0.5, 1, 2, 4, 8, and 16 times the MIC. Fifteen minutes of exposure was used for colistin due to its very rapid bactericidal effect, to ensure that there were adequate numbers of bacteria for sampling at the end of the exposure interval. Antibiotic was removed by twice centrifuging at 3,000 × *g* for 10 min, decanting the supernatant, and resuspending in prewarmed broth. Viable counts were performed at 0, 1, 2, 3, 4, 5, 6, and 24 h on nutrient agar (Oxoid). A growth control was performed in the same fashion but without exposure to antibiotic. The colonies were counted after 24 h of incubation at 37°C. PAE was determined by comparing regrowth of treated and growth control cultures, using the standard formula of the time for the control culture to increase 10-fold subtracted from the time for the treated culture to do the same (5).

Statistical and mathematical analysis. Comparisons of MICs were done using the unpaired *t* test with pooled variance on log-transformed values in Systat version 9.0 (SPSS Inc., Chicago, Ill.). The killing effects of colistin and colistin methanesulfonate at different concentrations were quantified by calculation of the mean survival time over 4 h ($MST_{240\ min}$) using the equation $MST_{240\ min} = AUMC_{0\text{–}240\ min}/AUC_{0\text{–}240\ min}$, where $MST_{240\ min}$ is in minutes, $AUMC_{0\text{–}240\ min}$ is the area under the curve of CFU per milliliter multiplied by time of sampling in minutes from 0 to 240 min, and $AUC_{0\text{–}240\ min}$ is the area under the curve of CFU per milliliter from 0 to 240 min. Areas were determined using the trapezoidal rule.

RESULTS

MICs. Table 1 illustrates the MICs obtained with colistin and colistin methanesulfonate. There was essentially no difference in results obtained by macro- versus microdilution testing. For all susceptible strains, colistin was threefold more active (geometric mean MIC, 3.1 mg/liter) than colistin methanesulfonate (geometric mean MIC, 7.1 mg/liter) ($P = 0.004$). The colistin and colistin methanesulfonate MICs, respectively, for the four strains used in time-kill and PAE experiments were as follows: 18982 (mucoid), 4 and 8 mg/liter; 19056 (mucoid), 1 and 8 mg/liter; 20223 (nonmucoid), 4 and 16 mg/liter; and ATCC 27853 (standard nonmucoid strain), 4 and 16 mg/liter.

Time-kill kinetics. In time-kill studies, both colistin and colistin methanesulfonate were bactericidal in a concentration-dependent manner (Fig. 1). With colistin, bacteria became undetectable after 4 h at all concentrations except 0.5 times the MIC. At the highest multiples of the MIC, the bactericidal rate of colistin was so high that at 64 times the MIC no bacteria could be detected within 5 to 10 min for the three strains where sampling was done at these early times. At the same multiples of the MIC, colistin methanesulfonate was less rapidly bactericidal than colistin. It was still rapidly bactericidal at the highest concentrations, with counts falling to undetectable numbers in 1 to 4 h at 16 to 64 times the MIC. Below these concentrations, colistin methanesulfonate failed to eliminate bacteria at 24 h. The control laboratory strain, ATCC 27853, was an exception to this, being rapidly eliminated by concentrations of colistin methanesulfonate of 1 to 64 times the MIC.

$MST_{240\ min}$ values were calculated at different multiples of the MIC for both agents. $MST_{240\ min}$ values of the four strains with colistin ranged from 3.7 to 84 min at 0.5 times the MIC. At 64 times the MIC, $MST_{240\ min}$ values ranged from <0.001 to 0.003 min. With colistin methanesulfonate the $MST_{240\ min}$ ranges were 140 to 185 minutes at 0.5 times the MIC, and 0.002 to 7.4 min at 64 times the MIC. Results for all concentrations tested are plotted in Fig. 2. Both the abscissa and the ordinate of Fig. 2 are plotted on a logarithmic scale to clarify relationships between strains and drugs. Again it is clear that colistin

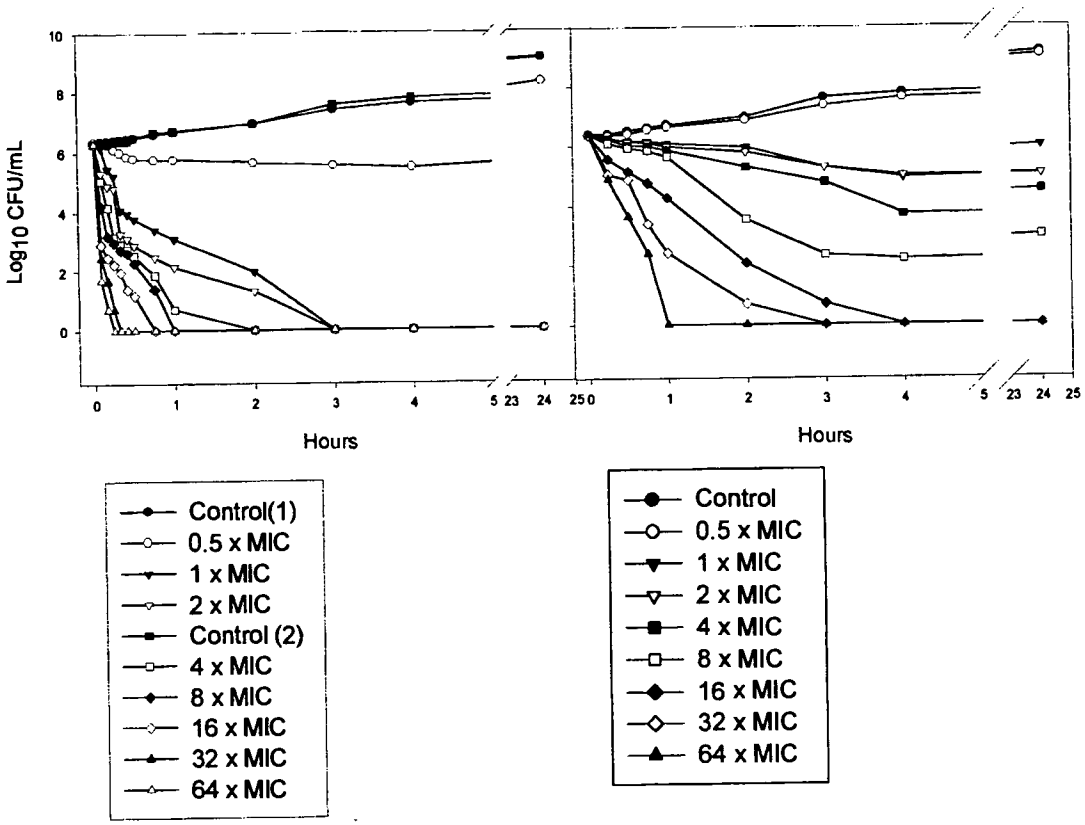

FIG. 1 Killing curves for a mucoid strain of *P. aeruginosa* (18982) by colistin (left) and colistin methanesulfonate (right).

methanesulfonate is less bactericidal than colistin at the same multiples of the MIC. For instance, geometric $MST_{240\ min}$ values of the four strains at 4 times the MIC were 0.07 min for colistin versus 34.7 min for colistin methanesulfonate. Apart from a single nonmucoid strain which appeared to be highly susceptible to the bactericidal action of colistin, the three clinical strains had MSTs similar to those of each other but longer than those of the standard laboratory strain at most concentrations of both agents. A minimum MST was not observed for either agent, suggesting that higher concentrations would have resulted in even more rapid killing. There was also an apparent biphasic bactericidal action for both agents, with lower reductions in MSTs over the 1- to 4-fold MIC range and higher reductions at the 8- to 64-fold MIC range.

PAE. Figure 3 illustrates the PAE of colistin and colistin methanesulfonate on three strains. The rapid bactericidal ac-

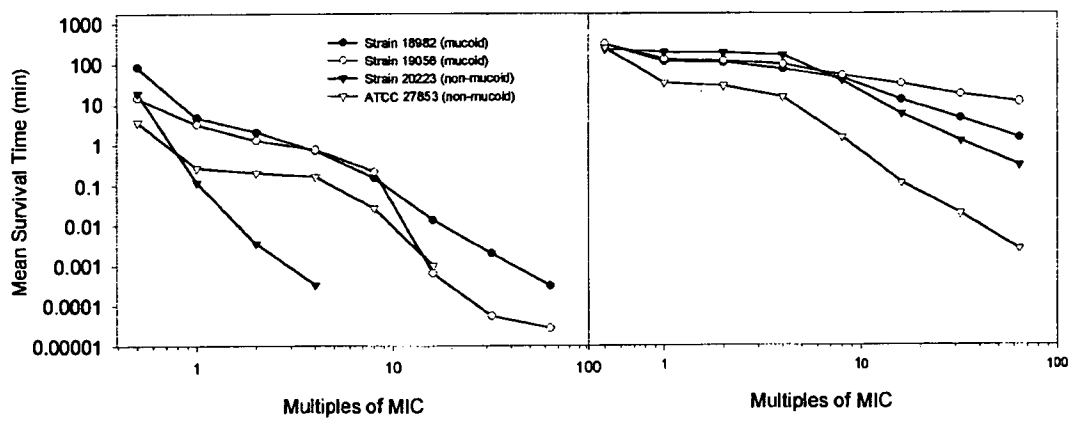

FIG. 2. Bactericidal activities of colistin (left) and colistin methanesulfonate (right) against four strains of *P. aeruginosa* as measured by MST.

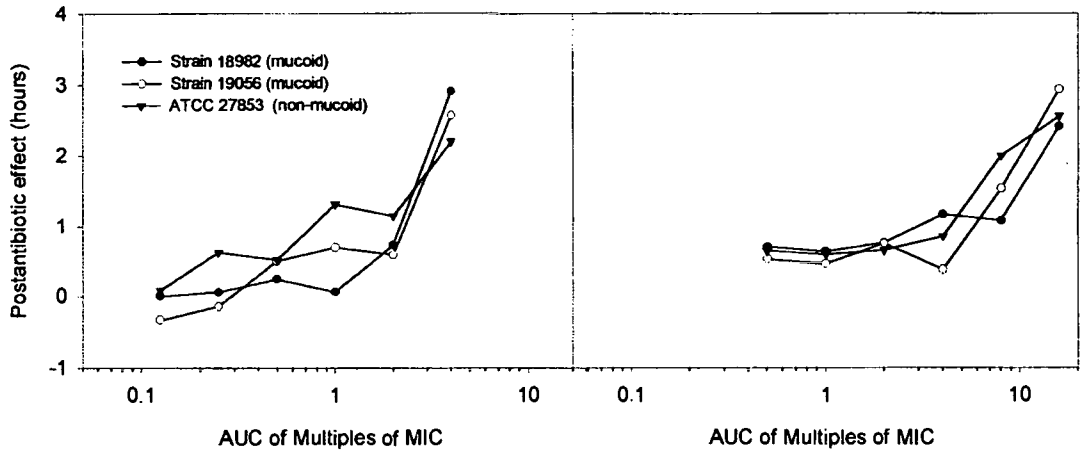

FIG. 3. PAE of colistin (left) and colistin methanesulfonate (right) against three strains of *P. aeruginosa*. AUC of multiple of MIC, product of multiple of MIC and duration of exposure in hours.

tivity of colistin required that exposure be limited to 15 min to ensure sufficient bacteria at the end of exposure for accurate determination of the PAE. For colistin methanesulfonate a more conventional 1-h exposure prior to drug removal was used. Similar profiles were obtained with the three strains examined when compared for drug exposure by multiples of the MIC. In order to make a meaningful comparison between drugs, the product of concentration and time of exposure (area under the curve of drug exposure) was used as the exposure variable. Colistin and colistin methanesulfonate produced a significant PAE (greater than 1 h) against the three strains only at the highest concentrations studied. However, it is clear that the maximum PAE was not achieved for either drug against any strain. Based on drug exposure, colistin was about four times more active than colistin methanesulfonate.

DISCUSSION

Due to the small number of strains included in our study, the prevalence of resistance to colistin in these strains is high compared to the overall rate observed in our patient population. Colistin resistance is prevalent in *P. aeruginosa* strains from our patients (~19%). This level of resistance may result in part from the frequent use of inhaled colistin methanesulfonate in our clinic. Previous suggestions that there is a low risk of resistance emergence with *P. aeruginosa* appear to precede the current widespread use of long-term inhalational therapy with colistin in some CF clinics.

As noted in the past, there is a significant difference between the in vitro activities of colistin and colistin methanesulfonate, with colistin methanesulfonate having lower activity than colistin (8). This difference is important, as colistin may often be used for susceptibility testing in vitro, whereas it is the sodium salt of colistin methanesulfonate that is used clinically. Furthermore, colistin and colistin methanesulfonate are both unequal mixtures of the salts of two components, colistins A and B (1), which may well have different in vitro activities. Even more problematically, the colistin methanesulfonate derivatives are undefined mixtures of the mono-, di-, tri-, tetra-, and pentasubstituted compounds, which may well differ between manufacturers and may completely or partially hydrolyze to colistin in solution (11). That there may be a difference between manufacturers' preparations is supported by a recent study which examined the activity of a colistin methanesulfonate preparation manufactured in the United Kingdom against *P. aeruginosa*, and found lower MICs overall than our study (3) and lower than would be expected by normal variation between strains or laboratories.

In an attempt to quantify the bactericidal activity of colistin in a way that would allow us to directly compare the effects of different antibiotic concentrations, we developed a model-independent parameter of killing, the $MST_{240\ min}$. This parameter is based on statistical moments and is analogous although not identical to the mean residence time parameter used in pharmacokinetics (12). It has the advantage over simple area-based methods for measuring bactericidal response (14) that it is not affected by variations in starting inoculum between experiments. $MST_{240\ min}$ is calculated from the bacterial concentrations (CFU per milliliter) observed in the time-kill curves. It uses the area under the first moment of the curve (i.e. plot of CFU per milliliter times time versus time) divided by the area under the time-kill curve (plot of CFU per milliliter versus time), with both areas calculated by the trapezoidal rule for the 4 h of the experiment. Use of the $MST_{240\ min}$ also allowed us to quantitate the difference in bactericidal activities between the colistin and colistin methanesulfonate.

In our study, colistin demonstrated very rapid concentration-dependent killing. Indeed, to get detailed information on the rapidity of onset of killing, sampling was required every 5 min in the first half-hour. For colistin methanesulfonate, killing was a little slower but there was still had significant concentration-dependent killing. The rapid bactericidal activities of both colistin and colistin methanesulfonate are presumably related to their permeabilizing action on the cell membrane following self-promoted uptake generated by the action of the drug on the outer membrane (13). Others have recently examined the bactericidal activities of colistin methanesulfonate at two con-

centrations (0.5 and 5 mg/liter) and have demonstrated more rapid killing with the higher concentration (14).

Intravenous administration of 2 to 2.5 mg of colistin methanesulfonate per kg of body weight results in peak concentrations in plasma of 6 to 15.5 mg/liter (mean, 9.6) following a 20- to 30-min infusion (2). Higher values (10 to 36 mg/liter [mean, 18.0]) have been seen with doses of 3 mg/kg infused over 5 min in patients with renal failure (7). A recent study in patients with CF using higher doses of 1.8 to 4.3 mg/kg (160 mg) every 8 h (mean dose, 8.8 mg/kg/day) showed a mean peak level of 12.3 mg/liter (4). Peak levels were lower than those anticipated from previous studies and were attributed to larger volumes of distribution frequently noted in patients with CF. Overall, studies have found peak concentrations that are not much higher than the MICs that we noted for isolates of *P. aeruginosa* from CF patients (4 to 16 mg/liter) and substantially less than the 16 times the MIC (64 to 256 mg/liter) required for complete in vitro killing within 24 h by colistin methanesulfonate. The precise concentrations achieved in infected sputum with inhaled colistin are unknown, but they are known to vary somewhat between lung regions (10).

Both colistin and colistin methanesulfonate produced a moderate PAE at higher concentrations. Unfortunately, due to the rapid killing at higher concentrations, a maximum PAE was not observed, and thus maximum values may well be substantially higher. The area under the curve of drug exposure, rather than time of exposure or concentration alone, has been shown to correlate best with the duration of the PAE with beta-lactams (15), and this is likely to be true also for colistin methanesulfonate.

It is clear from our studies that colistin methanesulfonate shows lower potency than colistin, even when corrected for MICs, as demonstrated in our time-kill and PAE experiments. Overall, our findings suggest that doses higher than the usually recommended 2 to 3 mg/kg every 12 h may be necessary, at least for the preparation we were examining, to maximize efficacy of colistin methanesulfonate when given intravenously for the treatment of acute exacerbations of *P. aeruginosa* infection in CF patients. Clearly, this must be weighed against the potential for increased toxicity.

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A simple method for the assay of colistin in human plasma, using pre-column derivatization with 9-fluorenylmethyl chloroformate in solid-phase extraction cartridges and reversed-phase high-performance liquid chromatography

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Abstract

A simple, selective and sensitive high-performance liquid chromatographic (HPLC) method is described for the determination of colistin in human plasma. Derivatization with 9-fluorenylmethyl chloroformate was performed in the same solid-phase extraction C_{18} cartridge used for sample pre-treatment, followed by reversed-phase HPLC with fluorimetric detection. Quantification was achieved using the ratio of the summed peak areas of colistin A and B derivatives to that of the derivative of netilmicin (internal standard). Linear calibration curves were obtained within the concentrations of colistin sulfate from 0.10 to 4.0 mg/l in plasma. Accuracy was within 10% and reproducibility (RSD) was less than 10%. Crown copyright © 2001 Published by Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, LC; Colistin; 9-Fluorenylmethyl chloroformate

1. Introduction

Colistin (polymyxin E), an antibiotic produced by *Bacillus polymyxa* subsp. *colistinus* [1], consists of a cyclic heptapeptide and a side-chain of three amino acids acylated at the N-terminus by a fatty acid (Fig. 1). It is a complex mixture of at least 30 different components [2]. The two main components are colistin A (polymyxin E1) and colistin B (polymyxin E2), which differ only in the fatty acid side chain.

Early clinical experience with the use of colistin in the 1960s for the treatment of serious infections with Gram-negative bacteria was characterized by adverse effects [3]. Consequently, it was superseded by potentially less toxic antibiotics. Recently, however, there has been a resurgence of interest in its use for the treatment of infections caused by some Gram-negative bacteria, especially *Pseudomonas aerugin-*

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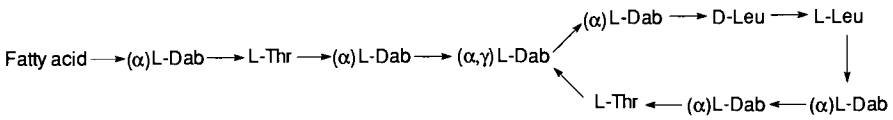

Fig. 1. Chemical structures of colistins A and B. Fatty acid=6-methyloctanoic acid for colistin A and 6-methylheptanoic acid for colistin B. Thr: Threonine; Leu: leucine. Dab: α,γ-Diaminobutyric acid. α and γ indicate the respective $-NH_2$ involved in the peptide linkage.

osa, which possess resistance to many of the more commonly prescribed antibiotics [4]. For patients with cystic fibrosis, one of the most common life-shortening genetic disorders in populations of European origin, respiratory infections with *P. aeruginosa* present a significant problem as many strains of this organism are resistant to aminoglycosides and other anti-Pseudomonal antibiotics. Two different compounds for clinical use are produced from colistin, colistin sulfate for oral and topical use, and colistin methanesulfonate (also known as colistin sulfomethate) for parenteral and aerosol therapy. To form colistin methanesulfonate, the primary amine groups on five of the Dab residues (Fig. 1) are derivatized with methane sulfonate moieties. Careful monitoring of plasma colistin levels is recommended because of its potential toxicity [5].

Numerous methods for the assay of colistin have been developed, based on microbiological [6], thin-layer chromatographic (TLC) [7], immunological [8], capillary electrophoretic [9] and high-performance liquid chromatography (HPLC) methods [2,10–14]. The microbiological method has been widely used for clinical monitoring of colistin concentration. Unfortunately, the microbiological method lacks sensitivity, selectivity and requires considerable time (21 h) [6]. The majority of HPLC methods [2,10–12] have been applied to the separation of the components of colistin in batches of raw material. The analysis of colistin in biological fluids by HPLC poses problems because it has very weak ultraviolet absorption and no native fluorescence. Hence, it cannot be quantified in biological fluids with high sensitivity by conventional techniques without derivatization with UV-absorbing or fluorescent reagents.

To the best of our knowledge, only two HPLC methods for the assay of colistin in biological material have been reported, and both used orthophthalaldehyde (OPA) as the derivatizing reagent [13,14]. Because of the instability of OPA derivatives, reaction conditions need to be carefully controlled and automated on-line derivatization is usually required [15]. To minimize those problems, Decolin et al. employed a switching HPLC system for the sequential derivatization and assay of colistin isolated from bovine milk and four different tissues [13]. Le Brun et al. [14] reported a method, based on the method of Decolin et al. [13], for the analysis of colistin in serum, urine and sputum using colistin methanesulfonate as the reference standard. It should be noted that OPA only reacts with primary amine groups and there are no primary amine groups in colistin methanesulfonate. Therefore, in the method reported by Le Brun et al. [14], it is possible that the derivatives with OPA were formed with the primary amine groups of the hydrolysis products of colistin methanesulfonate.

In the present report, a new method is described in which colistin was extracted from human plasma onto a solid-phase extraction (SPE) C_{18} cartridge and derivatized in the same cartridge with 9-fluorenylmethyl chloroformate (FMOC-Cl). Elution from the cartridge was followed by HPLC analysis of the fluorescent derivatives. The method was accurate, reproducible and sensitive.

2. Experimental

2.1. Apparatus

The HPLC system comprised a LC-10AS pump, an SIL-10AD*vp* autoinjector (Shimadzu, Kyoto, Japan) and either an SPD-6A UV spectrophotometric detector (Shimadzu) or a 470 scanning fluorescence detector (Waters, Milford, MA, USA) connected to a multi-instrument data acquisition and data process system (Maxima 820, Waters). Mass spectral analysis was performed with a Perkin-Elmer Sciex API 365 tandem mass spectrometer system (MDS Sciex, Concord, Canada) with an ion-spray source con-

nected to a Harvard syringe pump. All the analyses were carried out at ambient temperature. SPE cartridges (C_{18}, 100 mg) were supplied by J.T. Baker (Phillipsburg, NJ, USA) and Waters.

2.2. Chemicals and reagents

Colistin sulfate and FMOC-Cl were purchased from Sigma (St. Louis, MO, USA), netilmicin sulfate from Schering-Plough (Madison, NJ, USA), ceftazidime from Glaxo Wellcome (Boronia, Australia), meropenem from Zeneca (Macclesfield, UK), aztreonam from Bristol-Myers Squibb (Noble Park, Australia), piperacillin from Lederle Labs. (Baulkham Hills, Australia), ciprofloxacin from AG Bayer (Pymble, Australia), tobramycin from Eli Lilly (West Ryde, Australia), ticarcillin from SmithKline Beecham (Dandenong, Australia), and triethylamine (HPLC grade) from Prolabo (Paris, France). Trichloroacetic acid, boric acid, sodium hydrogen carbonate and sodium hydroxide of analytical grade, and acetonitrile, methanol, tetrahydrofuran and acetone of HPLC grade were all obtained from BDH Laboratory Supplies (Poole, UK). Water was purified by a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

The carbonate buffer (1%, w/w, pH 10) was prepared by dissolving in water the appropriate amount of sodium hydrogencarbonate and adjusting pH with sodium hydroxide (10%, w/w). All solutions were stored at 4°C.

2.3. Plasma sample pre-treatment

The internal standard (20 μl of 5 mg/l netilmicin sulfate in water) was mixed with 250 μl plasma in a 1.5-ml polypropylene tube (Techno-plas, Adelaide, Australia). After adding 50 μl of methanol–10% trichloroacetic acid (50:50, v/v), the mixture was vortex-mixed for 1 min, and centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was transferred to another 1.5-ml polypropylene tube and mixed with 10 μl of 1 *M* sodium hydroxide. After being vortex-mixed for 1 min, 250 μl of methanol–0.01 *M* hydrochloric acid (50:50, v/v) was added followed by vortex-mixing for 1 min. The final solution was delivered to the conditioned SPE cartridge.

2.4. Derivatization in solid-phase extraction cartridge and HPLC analysis

SPE C_{18} cartridges (J.T. Baker) were conditioned with 1 ml of methanol, and equilibrated with 1 ml of carbonate buffer. These steps were performed using a vacuum manifold (Supelco Visiprep 24, Bellefonte, PA, USA) without allowing the cartridges to run dry. All of the final solution from the plasma pretreatment was transferred to a cartridge. After washing the cartridge with 1 ml carbonate buffer, 30 μl FMOC-Cl (100 m*M* in acetonitrile) was added. Following 10 min of reaction, the cartridge was dried by drawing air through under vacuum. The derivatives were eluted with 900 μl acetone, and the eluate mixed with 600 μl boric acid solution (0.2 *M*). After vortex-mixing for 2 min, 50 μl was injected onto a 250×4.6 mm I.D. steel column prepacked with 5 μm Ultrasphere C_{18} (Beckman, Berkeley, CA, USA). A mobile phase of acetonitrile–tetrahydrofuran–water (87:4:13, v/v) was degassed by sonication prior to use and pumped at a flow-rate of 1 ml/min. Fluorescence detection was performed at an excitation wavelength of 260 nm and an emission wavelength of 315 nm.

2.5. Validation of the colistin assay

2.5.1. Confirmation of identity of the colistin derivatives

An aliquot of colistin sulfate in water (1 g/l) was chromatographed on the Ultrasphere C_{18} column with a mobile phase of acetonitrile–35 m*M* triethylamine (17:83, v/v), adjusted to pH 2.5 with 85% phosphoric acid [13]. The flow-rate was set at 1.5 ml/min and detection was by UV absorption at 210 nm. The eluate fractions corresponding to the two dominant peaks were collected and freeze–dried overnight (Virtis Sentry, New York, NY, USA).

Desalting of the collected HPLC eluates was performed on an SPE C_{18} cartridge (Waters) previously activated with 1 ml of methanol and 1 ml of water. After loading the eluate fractions reconstituted in 3 ml water, the cartridge was washed with 5 ml of water, followed by elution of the components with 1 ml of acetonitrile. Formic acid (0.25%, w/w, 1 ml) was added prior to mass spectral analysis. The electrospray mass spectrometer was operated in the

positive ionization mode and data were collected and processed by Multiview 1.4 operating on a Macintosh G3 computer system.

To identify the peaks from chromatographic analysis of the FMOC derivatives, the collected eluate, corresponding to each of the two main components of colistin, was reacted with FMOC-Cl. In a 10-ml polypropylene test tube (Sarstedt, Adelaide, Australia), 20 μl of the eluate was mixed with 500 μl carbonate buffer and 500 μl FMOC-Cl (1.0 m*M* in acetonitrile), and left to react for 10 min. Excess FMOC-Cl was reacted with 30 μl glycine (0.1 *M*) and, after a further 2 min, boric acid solution (0.2 *M*, 600 μl) was added. An aliquot (10 μl) of the solution was chromatographed on the Ultrasphere C_{18} column with a mobile phase of acetonitrile–tetrahydrofuran–water (87:4:13, v/v) at a flow-rate of 1 ml/min. The derivatives were detected using fluorescence, as described above.

2.5.2. Specificity

Seven antibiotics, ceftazidime, meropenem, aztreonam, piperacillin, ciprofloxacin, tobramycin and ticarcillin, which are commonly co-administered during therapy with colistin, were used to investigate the specificity of the method. These antibiotics (at a concentration of 8.0 mg/l) were individually derivatized and chromatographed as described for colistin in Section 2.4 above.

2.5.3. Linearity, reproducibility and accuracy

Appropriate volumes of three working standards of colistin sulfate in water (1.0, 10 and 50 mg/l) were used to prepare calibration standards in drug-free plasma (obtained from the Red Cross, Adelaide, Australia) containing colistin sulfate at concentrations of 0.10, 0.40, 0.80, 1.0, 2.0, 3.0, and 4.0 mg/l. A linear calibration curve was constructed from the relationship between the ratios of the summed peak areas of colistins A and B to that of netilmicin (internal standard) and concentrations of colistin sulfate. Reproducibility and accuracy were assessed by (1) the intra-day assay with six consecutive analyses of quality control samples of 0.20 mg/l and 2.0 mg/l colistin sulfate in human plasma (the quality control samples were prepared independently), and (2) the inter-day assay with three consecutive analyses of the same quality control samples on separate occasions. The limit of quantification was determined by measuring the concentration of colistin sulfate in six samples of human plasma prepared independently from the calibration standards at a concentration of 0.10 mg/l.

3. Results and discussion

In general, pre-column derivatization with fluorescent reagents provides a simple and sensitive analysis for compounds with poor or no UV absorbance or native fluorescence. FMOC-Cl has been shown to be an ideal reagent for fluorescent derivatization of primary and secondary amines [16]. It reacts rapidly and quantitatively under mild conditions and the resulting derivatives are stable at room temperature for several days. Also the fluorescent derivatives could be detected at the low picomole level [15]. We have developed a new simple, efficient and sensitive method for measuring the concentrations of colistin in human plasma using derivatization with FMOC-Cl, followed by reversed-phase HPLC.

3.1. Derivatization of colistin isolated from human plasma in an SPE cartridge

SPE is an attractive technique that reduces the consumption of, and exposure to, solvent, disposal costs and extraction time. In an effort to simplify sample pre-treatment for the assay of β-phenylethylamine in urine, Pilar et al. described a method utilizing derivatization on solid-phase extraction supports [17]. Decolin et al. reported a method using C_{18} SPE cartridges to extract colistin from bovine tissues [13]. We modified the method for the isolation of colistin from human plasma using an SPE C_{18} cartridge and developed a new method for the efficient derivatization of colistin with FMOC-Cl in the same cartridge.

For the conditioning and equilibration of cartridges, it was found that different volumes of methanol and carbonate buffer had no effect on the recovery and derivatization of colistin. Therefore, 1 ml methanol and carbonate buffer were used routinely to pre-treat the cartridges prior to the addition of plasma. Washing the cartridges with 1 ml carbonate buffer after loading the sample was successful in

removing interfering components originating from plasma. Deproteinization of plasma prior to application on the SPE cartridge resulted in substantially cleaner chromatograms.

FMOC-Cl must be added to the SPE cartridges in as small as possible volume of acetonitrile, to avoid elution of underivatized and derivatized colistin from the cartridge. In our method, addition of FMOC-Cl (100 m*M*) in 30 μl acetonitrile did not cause any breakthrough of underivatized or derivatized colistin from the cartridges. Superior results were obtained with SPE cartridges having a small internal diameter and particle size for the packing. This is probably due to the greater retentive capacity of the packing for the analytes, trapping them in a very narrow zone at the top of the cartridges and allowing the small volume of the derivatizing reagent to react with the majority of the adsorbed colistin.

Within the range of concentrations of colistin sulfate used to construct a calibration curve, different concentrations of FMOC-Cl in acetonitrile were tested. With lower concentrations (<10 m*M*) the fluorescence responses were very low, probably because of the low reaction efficiency. With high concentrations (>150 m*M*), the reaction efficiency decreased and unwanted peaks appeared near those of the colistin derivatives, possibly because the excess reagent reacted with endogenous compounds from plasma. The effect was magnified at low concentrations of colistin sulfate (<0.20 mg/l).

The derivatization reaction generates derivatives less polar than colistin and, hence, acetone was used to ensure efficient elution of the derivatives from the cartridge. The collected extracts were acidified by dilution with 0.2 *M* boric acid, in which the FMOC derivatives of colistin were found to be stable for at least 3 days at ambient temperature (Fig. 2). The derivatives of colistins A and B displayed almost identical stability. The derivative of netilmicin (internal standard) was quite stable even after 2 weeks at room temperature. In the absence of boric acid, the derivatives degraded more than 40% overnight at room temperature. Phosphoric acid and hydrochloric acid were ineffective in stabilising the derivatives, suggesting their stability was not related to pH alone.

Fig. 3 shows representative chromatograms of blank human plasma, the same plasma spiked with 0.10 mg/l colistin sulfate and a sample taken from a

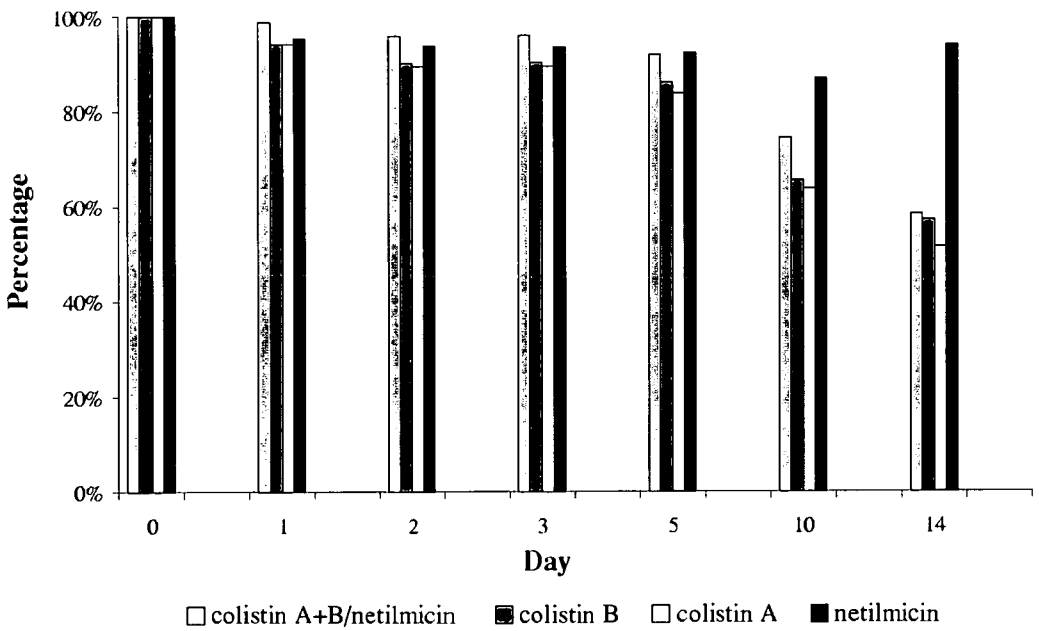

Fig. 2. The stability of the derivatives of colistin and netilmicin in a solution of acetone–0.2 *M* boric acid (9:6) at room temperature. Values are expressed as a percentage of peak area at time zero.

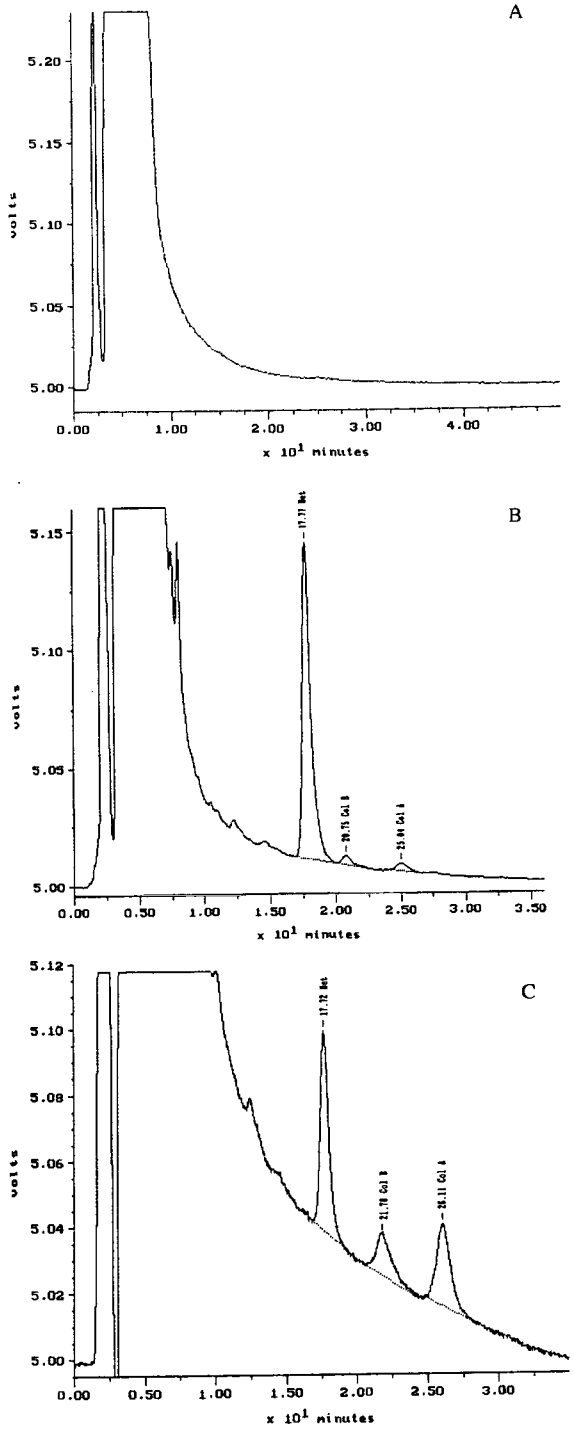

Fig. 3. Typical chromatograms obtained with fluorescence detection for blank human plasma (A), the same plasma spiked with 0.10 mg/l colistin sulfate (B), and a sample from a patient with cystic fibrosis (C). For experimental details, see text.

patient with cystic fibrosis 1 h after intravenous bolus administration of 1.54 mg/kg colistin methanesulfonate (Parke-Davis, Australia). The derivatives of netilmicin, colistin B and colistin A were well separated. The identity of the derivatives of colistin B and A was confirmed as described in the following section. There was no chromatographic interference to the derivatives of netilmicin, colistin A and B from endogenous compounds or reaction by-products.

3.2. Validation of the colistin assay

3.2.1. Confirmation of identity of the colistin derivatives

The two major derivative peaks were identified indirectly. With UV detection at 210 nm, two main components of colistin were well resolved on the Ultrasphere C_{18} column with a mobile phase of acetonitrile–35 m*M* triethylamine (17:83, v/v), adjusted to pH 2.5. Phosphoric acid in the freeze–dried eluates was removed by SPE C_{18} cartridges (Waters) prior to mass spectral analysis, otherwise the signals from phosphate predominated and no peaks from the derivatives of colistin were observed.

The positive ion electrospray mass spectral analysis of the reference sample of colistin (Sigma) showed there are two main components in the sample of colistin mixture (Fig. 4A). The mass spectra of the individual components collected by HPLC and dissolved in formic acid solution, contained ions with *m/z* 1156.0 (Fig. 4B) and 1170.0 (Fig. 4C), respectively. These were consistent with the protonated molecular ions of colistin B (theoretical mass 1155.76) and colistin A (theoretical mass 1169.77). Hence, in the component analysis of underivatized colistin, the first peak corresponded to colistin B and the second to colistin A.

There were also only two major peaks in the HPLC chromatograms of the FMOC derivatives of colistin, suggesting only one derivative was produced for each component. Their identification was performed by reacting colistin A or B with FMOC-Cl separately. The first peak corresponds to the derivative of colistin B and the second to that of colistin A (Fig. 3).

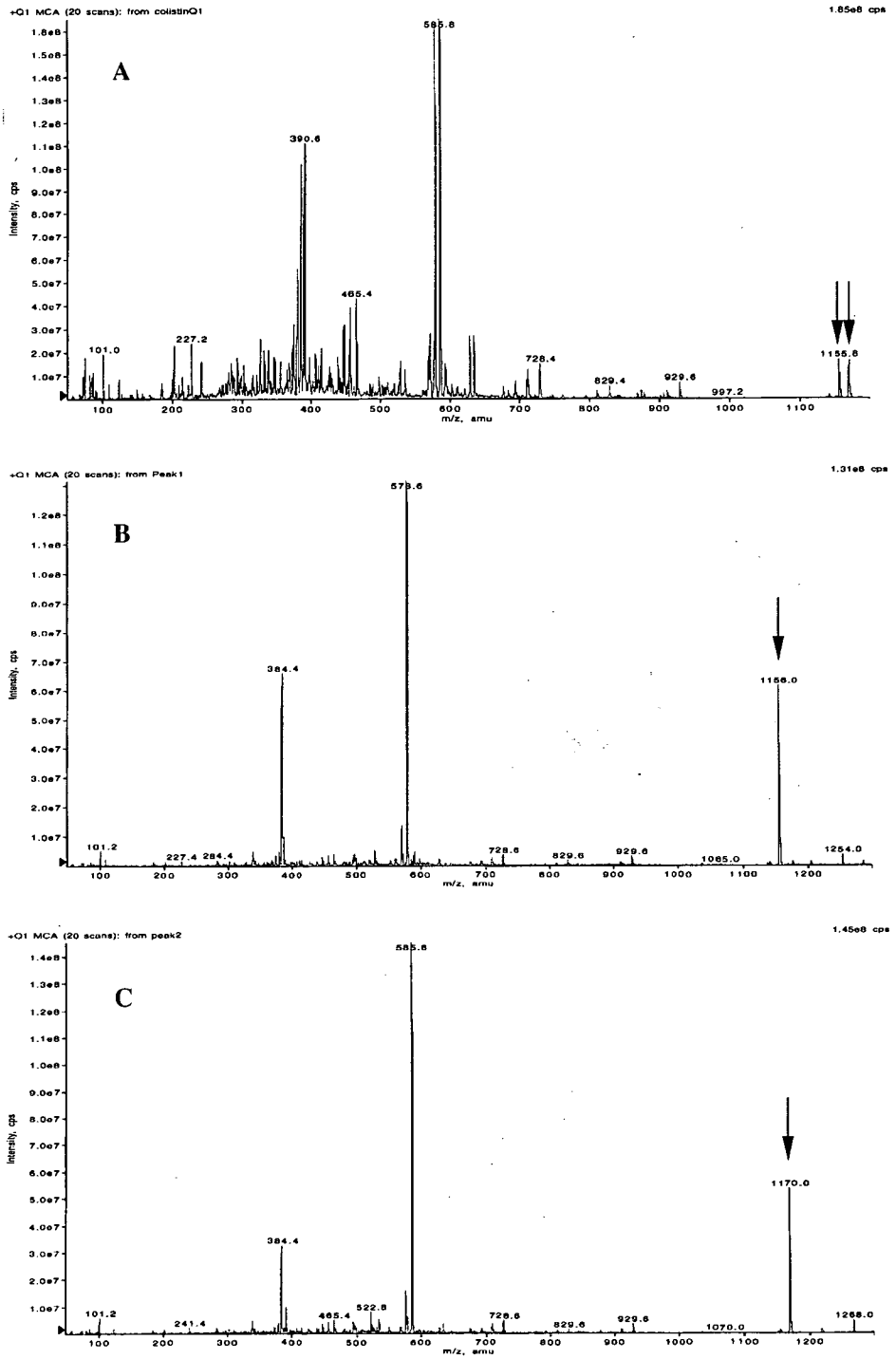

Fig. 4. Mass spectra of the colistin sulfate mixture (A), colistin B (B), and colistin A (C) obtained by electrospray ionization MS (ESI-MS). Arrows show the protonated molecular ion $[M+H]^+$ of colistin A and B.

3.2.2. Specificity

In patients with cystic fibrosis, ceftazidime, meropenem, aztreonam, piperacillin, ciprofloxacin, tobramycin or ticarcillin, may be co-administered with colistin to treat infections caused by *P. aeruginosa*. None of the compounds interfered with the chromatographic analysis of the derivatives of colistin and netilmicin.

3.2.3. Linearity, reproducibility, and accuracy

An internal standard (netilmicin) was used in our method to correct for potential losses of sample during sample pretreatment and errors that may be caused by instrumental variations. This is the first report employing an internal standard for the assay of colistin in biological matrices.

High coefficients of determination ($r^2>0.998$) were obtained for calibration curves with concentrations ranging from 0.10 to 4.0 mg/l. The linear regression equation for the relationship between the ratios of the summed peak areas of colistins A and B to that of netilmicin (internal standard) and the concentrations of colistin sulfate had a mean ($\pm$SD) slope of 0.789 ± 0.032 and an intercept of -0.010 ± 0.007 ($n=6$). A recovery of $107\pm12\%$ ($n=3$) was calculated when comparing the responses from plasma with those from water spiked at 1.0 mg/l colistin sulfate. The limit of quantification was 0.10 mg/l, at which concentration the mean value measured was 0.10 mg/l and the relative standard deviation (RSD) 9.14% ($n=6$). Inter-day reproducibility and accuracy were determined by conducting assays on 3 consecutive days of plasma containing 0.20 and 2.0 mg/l colistin sulfate as quality controls on separate occasions; determination of intra-day reproducibility and accuracy involved six assays of the same quality controls. The results, shown in Table 1, confirm the reliability of the method for measuring concentrations of colistin sulfate (0.10–4.0 mg/l) in human plasma.

4. Conclusion

We have developed and validated a novel HPLC method for the assay of colistin in human plasma. The method involves extraction of colistin from plasma onto SPE cartridges, followed by rapid and quantitative derivatization under mild conditions on the same cartridge with FMOC-Cl. The method is simple, convenient, and avoids the need for automatic equipment for derivatization and subsequent column switching. To our knowledge, this is the first report of an HPLC method for the analysis of colistin in human plasma using FMOC-Cl as the derivatizing reagent.

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Table 1
Reproducibility and accuracy for the assay of colistin sulfate in human plasma

	Intra-day ($n=6$)		Inter-day ($n=3$)	
	0.20 mg/l	2.0 mg/l	0.20 mg/l	2.0 mg/l
Mean concentration (mg/l)	0.18	2.0	0.18	2.1
RSD (%)	4.5	3.6	8.4	2.2

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Simple Method for Assaying Colistin Methanesulfonate in Plasma and Urine Using High-Performance Liquid Chromatography

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A simple and sensitive high-performance liquid chromatographic method is described for the determination of colistimethate sodium in plasma and urine. The accuracy and reproducibility was within 10.1 and 11.2% with rat plasma and urine, respectively. Several commonly coadministered antibacterial agents do not interfere with the assay.

Serious infections caused by the multidrug-resistant *Pseudomonas aeruginosa* are a significant clinical problem (18) and result in high morbidity and mortality among cystic fibrosis patients and immunocompromised hosts (6, 17). Colistin, also known as polymyxin E, was the first antibiotic with notable in vitro activity against *P. aeruginosa* (1). Although the availability of less toxic antipseudomonal antibiotics relegated colistin to the status of a reserve agent, the subsequent development of multidrug resistance in *P. aeruginosa* has made colistin of interest once more, as it possesses the advantage of rapid bactericidal activity and only slowly leads to the development of resistance (5, 11). Colistin is a multicomponent polypeptide antibiotic composed mainly of colistin A (polymyxin E1) and colistin B (polymyxin E2) (12, 13). There are two different forms of colistin available commercially, colistin sulfate for oral and topical use and colistimethate sodium (CMS) for parenteral and aerosol therapy. CMS is produced by treating the primary amine groups of the α,γ-diaminobutyric acid residues in colistin with formaldehyde followed by sodium bisulfite.

Although in vitro antibacterial potency is reduced by sulfomethylation (2, 3, 10, 15, 16), toxicity is dramatically decreased and some undesirable side effects, such as painful irritation at subcutaneous or intramuscular injection sites, are avoided (2, 3). CMS has the potential to hydrolyze in aqueous solutions and form an extremely complex mixture of partially sulfomethylated derivatives as well as colistin. These hydrolysis products possess increased antibacterial activities (2, 3).

There are several high-performance liquid chromatography (HPLC) methods for the assay of colistin in biological samples (4, 7, 9), but none has been reported for CMS. Microbiological assay is currently the common analytical method for measuring CMS in biological fluids (8). The present report describes a simple and sensitive HPLC method for the assay of CMS in plasma and urine. It involves accelerated hydrolysis of CMS to colistin in biological fluids, extraction of the generated colistin, and derivatization prior to HPLC analysis according to a previously described method for the quantification of colistin (9).

The optimal concentration of sulfuric acid for the hydrolysis of CMS (Sigma, St. Louis, Mo.) in rat plasma was determined by mixing 150-μl samples of drug-free rat plasma, freshly spiked with CMS (25 mg/liter of plasma), with 30 μl of netilmicin sulfate solution (5.0 mg/liter, internal standard; Schering-Plough, Kenilworth, N.J.) and 20 μl of sulfuric acid solution (0.1, 0.4, 0.8, 1.0, 1.5, 2.0, or 3.0 M). After 30 min at room temperature, the reactions were stopped by the addition of 40 μl of sodium hydroxide solution (at a concentration of 0.1, 0.4, 0.8, 1.0, 1.5, 2.0, or 3.0 M, respectively). To determine the optimal time for hydrolysis, samples (150 μl each) of drug-free rat plasma freshly spiked with CMS (25 mg/liter) were mixed with 30 μl of netilmicin sulfate solution (5.0 mg/liter) and 20 μl of sulfuric acid solution (1.0 M). The reactions were stopped at 1, 2, 5, 10, 15, 20, and 30 min by the addition of 40 μl of sodium hydroxide (1.0 M). The fluorescent responses arising from the sample preparation, derivatization, and HPLC analysis (see below) were determined. Analyses were performed in duplicate for each concentration or time point. Mean data for the responses from derivatized colistin under different conditions for hydrolysis of CMS are shown in Fig. 1. The optimum conditions for the hydrolysis of CMS in rat plasma involved a final sulfuric acid concentration of 0.08 to 0.1 M, with a reaction time of 10 min.

Sample pretreatment included mixing netilmicin sulfate solution (30 μl, 5 mg/liter) with 150 μl of plasma or 200 μl of urine. After the addition of 20 μl of sulfuric acid (1.0 M), CMS was hydrolyzed for 10 min and the reactions were stopped by the addition of 40 μl of sodium hydroxide (1.0 M). The entire mixture was pretreated and transferred to a cartridge (Sep-Pak C_{18}, 100 mg; Waters, Milford, Mass.), and colistin was derivatized by procedures described for a previously reported HPLC method for colistin (9). An aliquot (100 μl) of the final solution was chromatographed on the HPLC column (steel, 5-μm-particle-size Ultrasphere C_{18}, 250- by 4.6-mm inside diameter;

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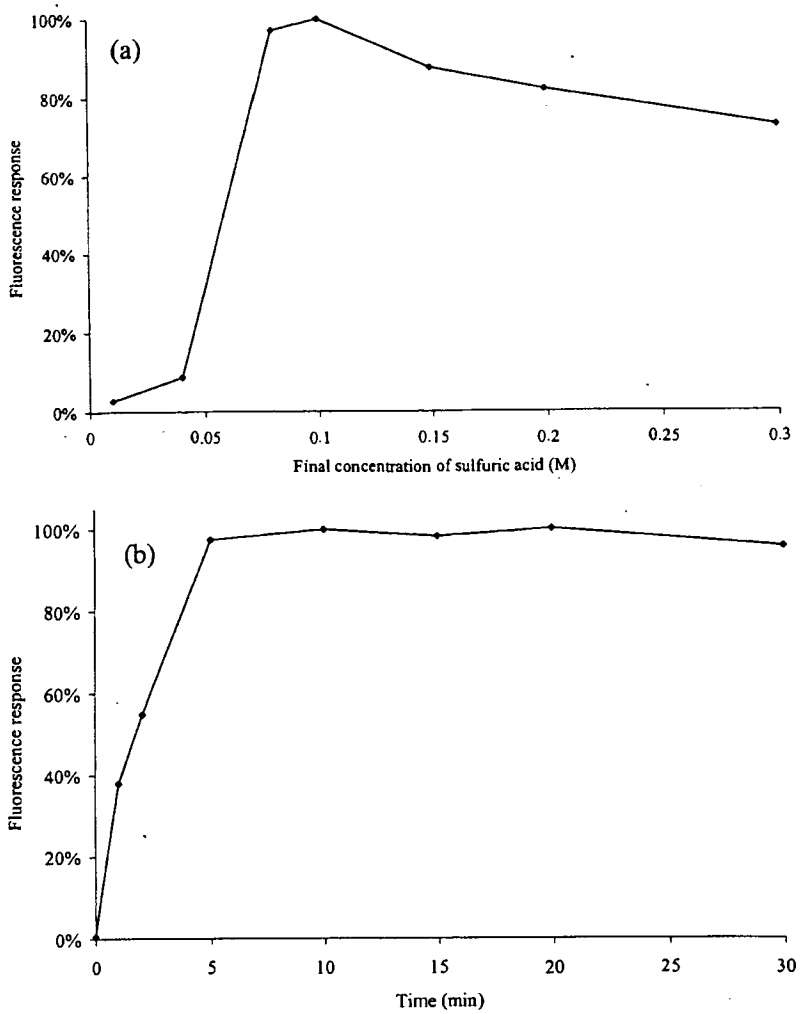

FIG. 1. Effects of the concentration of sulfuric acid (a) and time (b) on the hydrolysis of CMS to colistin in rat plasma. Response is expressed as a percentage of the maximum observed response.

Alltech, Dandenong, Australia) with a mobile phase of acetonitrile-tetrahydrofuran-water (50:30:20, vol/vol/vol) pumped at 1 ml/min. Modification of the previous mobile phase (9) gave a much shorter HPLC run time (18 min), which improved the speed and the economy of the assay. Fluorescence was measured at 315 nm following excitation at 260 nm, with the gain set at ×100. A typical chromatogram from plasma collected from a rat at 10 min after an intravenous bolus of CMS (15 mg/kg of body weight) is shown in Fig. 2. The derivatives were eluted in the order of netilmicin, colistin B, and colistin A. Analysis of drug-free rat plasma showed that there were no peaks at the corresponding retention times of the peaks of interest.

Calibration curves for CMS in rat plasma were prepared at concentrations of 0.33, 0.67, 2.7, 5.3, 6.7, 13.3, 26.7, and 53.3 mg/liter and in rat urine at concentrations of 0.25, 0.5, 2.0, 4.0, 5.0, 10, 20, and 40 mg/liter. Linear calibration curves were constructed from the relationship between the ratios of the summed peak areas of the derivatives of colistin A and B to

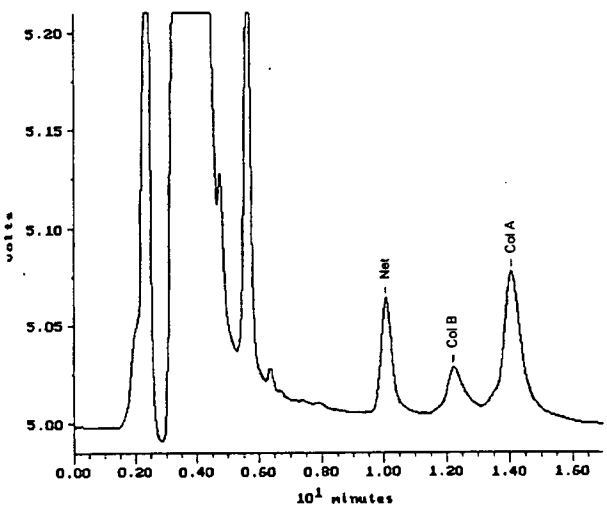

FIG. 2. Chromatogram of a rat plasma sample collected 10 min after an intravenous bolus of 15 mg of CMS/kg. Net, Col B, Col A, peaks of netilmicin, colistin A, and colistin B, respectively.

TABLE 1. Reproducibility and accuracy of assay of CMS in rat plasma and urine

Time course (no. of samples)	Concn of sample (mg/liter)	Plasma		Urine	
		Mean concn (mg/liter)	Relative SD (%)	Mean concn (mg/liter)	Relative SD (%)
Intraday (6)	1.3	1.4	8.8		
	40	39	2.4		
	1.0			1.0	4.7
	30			30	6.2
Interday (3)	1.3	1.3	10.1		
	40	40	4.1		
	1.0			1.0	11.2
	30			29	7.8

that of netilmicin and concentrations of CMS. Reproducibility and accuracy were assessed by the intraday and interday assays with quality control samples containing 1.3 and 40.0 mg of CMS per liter of rat plasma or 1.0 and 30 mg/liter of rat urine. Data on the accuracy and reproducibility of the assay for plasma and urine are presented in Table 1. Regression coefficients for calibration curves for plasma and urine were greater than 0.995 and 0.983, respectively. The linear-regression equation for rat plasma had a mean slope ± standard deviation of 0.112 ± 0.010 and an intercept of −0.055 ± 0.038 (n = 3); corresponding values for rat urine were 0.178 ± 0.026 and −0.008 ± 0.079 (n = 3), respectively. The different volumes of plasma and urine account for the difference in mean slopes. Rat plasma containing CMS at a concentration of 26.7 mg/liter was assayed in triplicate as described above. Derivatives eluted from the cartridge and kept at an ambient temperature for 24 h gave a mean fluorescent response of 95% (n = 3) of the value for the freshly eluted derivatives. This demonstrated that pretreatment with sulfuric acid did not affect the stability of the colistin-9-fluorenylmethoxy carbonyl (FMOC) derivatives, which remained stable for at least 24 h at an ambient temperature. Duplicate samples of drug-free rat plasma containing colistin (2.0 mg/liter) and pretreated with sulfuric acid (1.0 M) for 30 min gave a mean fluorescence response which was 102% of the response from samples without pretreatment (n = 2). This indicated that colistin formed from hydrolysis of CMS was stable under these conditions and that its derivatization with FMOC-Cl was not affected by pretreatment with sulfuric acid. The validation confirmed the reliability of the HPLC method for measuring concentrations of CMS in rat plasma and urine. Furthermore, this method has been found to give similar accuracy and reproducibility when it is applied to human plasma (0.97 ± 0.03 and 29.2 ± 1.4 mg/liter for quality control samples containing 1.0 and 30 mg of CMS per liter, respectively; n = 6). The HPLC method has been used for pharmacokinetic studies of rats and humans, and the results will be reported separately.

In patients with cystic fibrosis, ceftazidime (Glaxo Wellcome, Boronia, Australia), meropenem (Zeneca, Cheshire, United Kingdom), aztreonam (Bristol-Myers Squibb, Noble Park, Australia), piperacillin (Lederle Laboratories, Baulkham Hills, Australia), ciprofloxacin (Bayer AG, Pymble, Australia), tobramycin (Eli Lilly, West Ryde, Australia), or ticarcillin (SmithKline Beecham, Ermington, Australia) may be coadministered with CMS to treat infections caused by *P. aeruginosa* and flucloxacillin (Rhone-Poulenc Roger, Australia) may be coadministered for infections caused by *Staphylococcus aureus*. Samples of rat plasma containing these antibiotics at a concentration of 8.0 mg/liter were assayed as described above for CMS. None of these compounds interfered with the chromatographic analysis of the derivatives of colistin and netilmicin.

The method described in this paper measures the summed concentrations of all sulfomethyl derivatives of colistin, including colistin, that are present in samples. When combined with the previously reported method for measuring colistin in plasma (9), it has the advantage of being able to determine both the concentrations of colistin alone and the concentrations of full and partial sulfomethyl derivatives of colistin in samples. The lack of response from plasma freshly spiked with CMS (10 mg/liter) to the HPLC method for colistin (9) proved that no FMOC derivatives from CMS were eluted at the same retention times as those of the FMOC derivatives of colistin. Furthermore, this showed that, following collection of plasma from humans or animals to whom CMS has been administered, sample pretreatment by the method for colistin (9) does not cause any conversion of CMS to colistin.

It should be noted that the concentration of each substituted form of CMS cannot be determined by the method. In addition, in biological samples collected following administration of CMS, the concentrations calculated from the calibration curves are apparent values with reference to those for fresh standard preparations of CMS. The actual mass of CMS in biological samples collected following CMS administration may be less than the apparent value due to a random loss of sulfomethyl groups (each with a molecular weight of 94) during the hydrolysis of CMS in vivo. Therefore, measured values for concentrations of CMS are most appropriately considered as apparent concentrations of CMS, reflecting fully and partially sulfomethylated colistin.

Previous reports have shown that the activity of colistin against *Escherichia coli*, *P. aeruginosa*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae* ranged from 3- to 10-fold the activity of CMS (10, 15). A rapid increase in antibacterial activity was observed after CMS was stored at 37°C in 0.1 M phosphate buffer (pH 7.0) (3). Work in our laboratory has also shown hydrolysis of CMS to colistin during storage in isotonic phosphate buffer (pH 7.4) and in Mueller-Hinton broth at 37°C. Therefore, with microbiological assays requiring overnight incubation, the antimicrobial activity of the sample increases with time. Our preliminary findings from using the present HPLC method to measure concentrations of fully and partially sulfomethylated colistin (CMS) in the plasma of humans after a dose of CMS and to measure concentrations of colistin alone (9) suggest that the ratio of CMS to colistin changes with time. Hence, any comparison of the concentrations of CMS measured by HPLC and by microbiological methods is rendered meaningless. Furthermore, microbiological assays with samples containing any other antibiotics active against the test strains are likely to be unreliable. A recent report employed derivatization with dansyl chloride and HPLC to measure the concentrations of "colistin" in human plasma and urine after an intravenous dose of CMS (14). However, it was not clear which form, colistin, CMS, or both, was quantified. Addition-

ally, the limit of quantification was relatively high (5 mg/liter with 1 ml of plasma) and the method required greater than 170 min for sample pretreatment.

In summary, a simple HPLC method for the assay of CMS in plasma and urine has been developed and validated. To our knowledge, this is the first report of an HPLC method for the analysis of CMS in biological fluids. The use of the present method to measure apparent concentrations of CMS and of a separate HPLC method to measure the more microbiologically active colistin (9) is substantially better than using the only other HPLC method reported to date (14) or similarly less specific microbiological methods. For those performing pharmacokinetic studies with CMS, the combined use of two HPLC methods will be of far greater value than previous assays and will allow a more detailed description of the pharmacokinetics of CMS than has hitherto been possible.

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