Targeting advantage of pulmonary delivery of colistin for treatment of respiratory infections

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Doctor of Philosophy

2013

Amendments and Typographical Errors

Abbreviation List
p. xvi, line 20 **Insert after "CYP":** colistin sulphate: the salt form of colistin colistin: the base form of colistin formed colistin: *in vivo* formed colistin from CMS sodium colistin methanesulphonate: the salt form of CMS colistin methanesulphonate: the base form of CMS

Chapter 1
p. 4, line 16: "evolved" for "designed"
p. 25, line 6: "have" for "has"
p. 25, line 10: "vary" for "varies"
p. 28, line 6: "are" for "is"
p. 31, line 3: "eliminating" for "elimination"
p. 35, line 12: "defined" for "define"
p. 38, line 20: a comma following "antibiotic"
p. 39, line 3: "plays" for "play"
p. 39, line 17: a comma following "colistin" and following "antibiotics"

Chapter 2
p. 82, line 19: a comma following "non-linearity"
p. 85, line 6: "were" for "was"
p. 96, line 8: insert "acceptable" following "demonstrated"

Chapter 3
p. 98, line 16: insert "delivery" following "pulmonary"
p. 100, line 4: remove comma following "colistin"
p. 103-104, Section 3.4.3.1 **Comment:** Intratracheal instillation was used to deliver colistin into the lungs since our research group did not have access to a micro-sprayer at the time these PK studies were conducted. We do acknowledge that a better lung exposure and increased absorption would be expected with a micro-sprayer when compared to intratracheal instillation. However tracheal instillation is still a valid method as it delivers an accurate and reproducible volume of dosing solution into the lungs and this delivery method was utilised for both the colistin and CMS pulmonary studies. *In vitro* studies to characterise the instilled colistin solution (i.e. droplet size) was not undertaken for these studies.
p. 109, lines 21-22 **Comment:** The target dose (mg/kg) was the nominal dose and the actual dose (mg/kg) was the dose administered to each rat. The latter dose can vary depending on the volume of dosing solution that was retained in the dosing syringe (in the void space of the needle and syringe tip) Therefore the dosing syringe was weighed pre- and post-administration to calculate the volume of solution that was delivered to each rat. The dosing solution concentration and the volume administered were then used to estimate the actual dose delivered to each rat.
p. 112, lines 9-12 **Comment:** A "tukey post-hoc test" was conducted after one-way ANOVA analysis to determine which dosing groups differed from each other.
p. 120-121, Figure 3-7B and Table 3-7 **Comment:** Colistin AUC_{0-t} (mg·h/L) in ELF and plasma that are presented in Table 3-7 are shown in Figure 3-7B.
p. 123, Table 3-8 **Comment:** To estimate therapeutic availability (TA) and drug targeting index (DTI) the same dose of colistin needs to be administered via the IV and pulmonary route. The colistin ELF exposure was estimated following an IT dose of 0.62 mg/kg however this dose could not be administered via the IV route. As outlined in p. 121, lines 11-14, a lower IV colistin dose of 0.41 mg/kg was administered due to observation of transient adverse effects at the 0.62 mg/kg dose. Therefore the AUC of colistin in ELF and plasma after IT 0.62 mg/kg was dose-normalised to an IT dose of 0.41 mg/kg (linear pharmacokinetic relationship for the two doses, Figure 3-5, p. 117) which enabled the estimation of the TA and DTI for colistin at the 0.41 mg/kg dose.
p. 125, lines 1-13 **Comment:** The IV and pulmonary dosing groups referred to in the Marchand *et al* study are following administration of CMS. The authors only reported the PK of colistin following subcutaneous administration of colistin and hence these results are compared to the PK of colistin in the present IV dose-ranging studies.
p. 125, lines 23-25, and p.126, line 1 **Delete:** "During IT instillation an unknown fraction of the dose may have been delivered into the gastrointestinal tract...." **Comment:** As the dosing cannula was placed at the trachea-bronchus bifurcation and 200 µL of air was administered after the solution it is unlikely that a proportion of the dose would have been delivered to the gastrointestinal tract.
p. 126, lines 9-10 **Following the sentence:** "This suggests that colistin is slowly absorbed from the lungs which is in contrast to the rapid appearance of colistin in plasma in the current study." **Insert:** "These observations are likely to be due to the difference in the surface area of the two systems (4.7 cm² in Transwell systems versus several meters in the rat's lungs".
p. 127, line 24-25 and p.128, line 1 **Comment:** The overall disposition of colistin in ELF would be influenced by distribution (binding), absorption and clearance mechanisms in the lungs. As cross-referenced in p. 127, line 25 and p. 128, line 1, the presence of CYP enzymes, peptidases, mucociliary clearance and phagocytosis (by alveolar macrophages) may contribute to the pulmonary clearance of colistin.
p. 128, line 9: "effluxed" for "efflux"

p. 128, line 2: "clearance" for "loss"
Chapter 4
p. 135, Section 4.4.3 Comment: The selection of CMS doses for the IV and IT dose-ranging studies were based on i) the limit of quantification of the analytical method for formed colistin and ii) to minimise adverse effects.
p. 144, lines 10-12 Comment: A "tukey post-hoc test" was conducted after one-way ANOVA analysis to determine which dosing groups differed from each other.
p. 145, line 21 and p. 146, lines 1-2 Comment: Only two CMS doses were administered for the IT dose-ranging studies as analytical sensitivity limits and potential adverse effects limited a lower and higher IT dose from being delivered, respectively. Since two doses were administered, CMS and formed colistin AUC versus CMS dose plots and associated correlations (R^2) are not shown. The pharmacokinetic properties of CMS and formed colistin are presented in Table 4-3 (p.151). Linear pharmacokinetic properties for both entities was reported due to i) a proportional increase in CMS and formed colistin AUC with CMS dose, ii) no significant difference in the terminal $t_{1/2}$ for CMS and formed colistin for the two doses, and iii) the systemic bioavailability for CMS of 80 – 89% for both doses (Table 4-3).
p.148, Table 4-2 and p. 149, Figure 4-2B Comment: The positive y-intercept observed for formed colistin AUC versus CMS dose plot (Figure 4-2B) is reflective of a higher than expected formed colistin AUC for the CMS dose of 14 mg/kg (Table 4-2). These observations are likely to be due to the sensitivity limit of the colistin analytical method as mentioned in p. 161, lines 7-10. The limit of quantification (LOQ) for the colistin assay was 0.10 mg/L and following administration of CMS 14 mg/kg formed colistin plasma concentrations were within a narrow range around the LOQ for the duration of the sampling period (4 h). This can influence the estimation of AUC especially the extrapolated AUC.
p. 157, Figure 4-5A Figure title: Delete (-■-) and (··■··) and replace with (-▲-) and (··▲··), respectively.
p. 161, Table 4-8 Delete: "Formed colistin $AUC_{0\ldots}$ estimate dose-normalised for a colistin 0.62 mg/kg dose". And Insert: "Estimation of $AUC_{dose-normalised 0\ldots}$ for formed colistin: The CMS 14 mg/kg dose was converted to a equimolar dose of colistin (10.01 mg/kg), and then the formed colistin $AUC_{0\ldots}$ of 7,564 mg·h/L was dose-normalised to a 0.62 mg/kg colistin dose."
p. 163, lines 20-22 Delete Line: "During IT instillation an unknown fraction of the CMS dose may have been delivered into the gastrointestinal tract... " Comment: As the dosing cannula was placed at the trachea-bronchus bifurcation and 200 µL of air was administered after the solution it is unlikely that a proportion of the dose would have been delivered to the gastrointestinal tract.
p.164, lines 12-25 and p. 165, lines 1-6 Comment: The PKs of CMS and formed colistin in plasma following IT instillation of CMS was compared to the Marchand *et al* study. Similarities and differences were noted between the two studies. The differences observed in the absorption kinetics of CMS and formed colistin could be due to CMS administered by instillation in the current study and via microspray in the Marchand *et al* study. This can influence the regions of the lung that the drug is delivered to and hence the extent of absorption. This has been acknowledged in p. 165, lines 3-6.
p. 168 Comment: The discussion on this page alludes to the higher binding affinity of colistin to lung tissue/constituents when compared to CMS. The relatively higher binding affinity of colistin may explain i) potential displacement of CMS from lung binding sites with formation of colistin (paragraph 1), and ii) unquantifiable formed colistin concentrations in ELF following IV CMS administration (paragraph 2).
p. 169, line 21: "therapeutically" for "therapeutic".
p. 170, line 1: "administration" for "administering"
p. 171, line 5: "conclusions" for "conclusion"
Chapter 5
p. 175, lines 14-15 Comment: One of the primary reasons for the development of the population pharmacokinetic model was to better understand the CMS-to-colistin conversion kinetics in ELF. The model demonstrated that conversion of CMS to colistin occurred in BAL fluid₁ (trachea, upper airways, some peripheral airways) and BAL fluid₂ (remaining peripheral lung regions) compartments with the conversion kinetics slower in the former compartment. Such in-depth interpretation of CMS conversion kinetics could not be undertaken in the noncompartmental analysis in Chapter 4. The statements made in relation to "quantification of formed colistin in ELF at the first sampling time (5 min post-dose)" (p. 166, lines 7-8) may be reflective of the relatively faster conversion that occurred in BAL fluid₂ compartment. The outcomes from the modeling work support the findings in Chapter 4.
p. 177-183, Section 5.3 and p. 183-192, Section 5.4 Comment: For the development of the population pharmacokinetic model for CMS and colistin the concentrations are expressed in molar units (µmol·l.) to facilitate accurate estimation of CMS to colistin conversion kinetics (1 molecule of CMS is hydrolysed into 1 molecule of colistin).
p. 194-196 Comment: It is unclear as to whether the plasma protein binding characteristics of CMS contributes to the conversion kinetics in plasma. This is due to the unbound fraction of CMS in rat plasma not being known (p. 236, lines 8-9). There is only limited knowledge pertaining to the plasma protein binding of colistin (as outlined in p. 235, lines 5-10) with approximately 55 – 57% of colistin bound to plasma protein in rats.
Chapter 6
p. 204, lines 15-22 and p.205, lines 1-7 Comment: Data pertaining to the pathophysiological status of the airways due to bacteria colonisation was not collated for this study. The reason for this was that the pilot study was conducted with the primary objective of defining the pharmacokinetics of CMS and colistin in the lungs and systemic circulation following inhalation and IV administration to CF subjects. However, future follow-up studies will incorporate airway characterisation and microbiological assessments and the influence of these factors on drug deposition in the lungs following inhalation.
p. 205, line 22 and p.206, lines 1-2 Comment: *In vitro* studies to characterise the solution droplet size, distribution of the nebulised CMS solution and estimation of the fine particle fraction was not conducted in this pilot study. However the device information for the Salter Nebu Tech® HDN® Disposable Nebulizer provided by Salter Labs' state that 80% of the generated particles are within the respirable range (less than 5 microns) [http://www.salterlabs.com/index.cfm?fuseaction=products.product&product_id=56].

p. 205, lines 8-20
Comment: The selected inhaled and IV doses of CMS were based on current clinical dosing regimens and therefore provide an explanation for the different doses for the two routes of administration. Inhaled CMS dosing regimens vary between different CF centers (1 million IU of CMS twice daily to 2 million IU of CMS three times daily). The decision to initially administer a single inhaled CMS dose of 2 million IU was based on the current inhaled dosing regimen at the Alfred hospital (1 million IU of CMS twice a day) since the pilot study was conducted at this site. The inhaled dose was then doubled (a single dose of 4 million IU of CMS) to assess for linear pharmacokinetic behaviour and tolerability. For IV CMS, the recommended daily dose is 2.5-5 mg/kg expressed as CBA. Therefore a single dose of 150 mg CBA was selected for a subject weighing 60 kg.
The sequence of administration was based on monitoring for adverse effects and minimising the carry over effect. Therefore subjects received the lowest dose in the first treatment period (inhaled 2 million IU CMS) and the highest dose (IV 5 million IU CMS (equivalent to 150 mg CBA)) in the last treatment period.

p. 212, lines 10-13, and p. 215, lines 5-9
Comment: The reported sputum drug concentrations are those that were quantified in the pre-dose and post-dose samples without any further manipulation. Pre-dose drug concentrations were not subtracted from post-dose concentrations. The justification for this approach was that for a particular subject the region of the lungs from which sputum samples originate from can vary over the sampling period (12 h). Therefore subtracting the pre-dose concentrations (from one lung region) from the post-dose concentrations (from another lung region) may not be an accurate approach to minimise the carry over effect. For this reason the concentration of CMS and colistin in pre-dose and post-dose samples are specifically reported in each section (p. 212, lines 10-11 and p. 215, line 6). In future studies implementation of a longer washout period is warranted.

p. 216, para 1, lines 2-5
Comment: The nominal CMS dose (specified on p. 210, line 10) was utilised to estimate the systemic CMS availability. The actual dose administered (by weighing the nebuliser container pre- and post-inhalation) could not be calculated due to safety issues relating to the dosing solution remaining at room temperature which could increase the risk of *in vitro* colistin formation. *In vitro* studies were not conducted to determine the efficiency of delivery of the Salter Ultra-Mist nebuliser. For future PK studies such procedures need to be incorporated as systemic CMS availability will alter accordingly.

p. 216, lines 5-7
Comment: Urine samples were collected up to 24 h post-dose from each subject. This sampling interval was based on previously published studies in rats and humans (healthy volunteers) that have shown that the majority of CMS is recovered in urine within the first 12 h following dosing. An explanation for the lower systemic availability of CMS when using the urinary recovery data when compared to the plasma data is as a result of colistin formed from CMS in the renal tubules being reabsorbed back into the blood stream. Acknowledging this limitation, urinary data was still useful as a secondary measure of the systemic exposure of CMS

p. 218
On line 4, insert after "... CMS nebulised dose.": "It should be noted that subject 5 had the third highest formed colistin sputum AUC despite samples collected up to 8 h when compared to 12 h post-dose in the remaining subjects (Table 4). Similarly, subject 6 who experienced cough and chest tightness had the second highest formed colistin sputum exposure (Table 4)."
On line 11, delete: "Subject 2 reported transient dizziness 4 h after administration of IV CMS"
And insert: "Subject 2, who was the youngest (20 years) and smallest in weight (56 kg) reported transient dizziness 4 h after administration of IV CMS. It should be noted that subject 2 showed the highest formed colistin C_{max}, the second highest AUC for CMS and the second smallest CL for CMS (Table 2)."

p. 219-225
Comment: This section makes comparisons to the concentrations of CMS/colistin in sputum, BAL and ELF from previously published studies. The reasons for comparing drug concentrations in varying lung matrices is as a result of a small number of studies having been conducted following IV and inhalation administration of CMS. However it is important to emphasise that the drug concentrations will vary depending on the CMS dose administered, the delivery method and the lung matrix sampled. Therefore when comparisons were made between the current findings and those of citied papers explanations as to why these differences were observed were clearly outlined (refer to p. 221, lines 4-11, p. 222, lines 7-11 and p. 224, lines 24-25 to p. 225, lines 1-4).

p. 222, lines 7-11
Delete lines 7-11 and insert: "The variability in the reported colistin concentrations in sputum and ELF across the studies will likely be a function of the efficiency of nebulised delivery, the solution droplet size and distribution in the lungs, whether the study involved single or multiple dosing, the different biological matrices (sputum versus ELF), analytical issues as discussed above and variation in the pathophysiological status of the respective patient population."

p. 222, line 17: "sputum" for "the lung"

Chapter 7

p. 228, line 7: "routes" for "route"

p. 228, line 9: move "following IV CMS administration" to line 10 after "site"

p. 230, line 9: insert "being" following "not"

Targeting advantage of pulmonary delivery of colistin for treatment of respiratory infections

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

from

Monash Institute of Pharmaceutical Sciences

Monash University

by

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Bachelor of Pharmacy (Hons)

May, 2013

Drug Delivery, Disposition and Dynamics

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This thesis is dedicated to

my parents for their continual love and support.

TABLE OF CONTENTS

Abstract

Colistin has undergone resurgence in clinical use due to the emergence of multidrug-resistant (MDR) Gram-negative bacteria. The inactive prodrug of colistin, colistin methanesulphonate (CMS), has increasingly been administered via the pulmonary and intravenous (IV) route for the management of respiratory infections in cystic fibrosis (CF) and critically-ill patients. Despite this, there is a dearth of information on the pharmacokinetics of CMS and formed colistin following pulmonary and IV administration of CMS which limits the optimisation of inhalational CMS dosing regimens. Therefore the aim of the current project was to characterise the pharmacokinetics of CMS and formed colistin in the lungs and plasma following IV and pulmonary administration of CMS, and in turn, determine the targeting advantage that can be achieved following pulmonary delivery, both in the pre-clinical and clinical setting. Linear pharmacokinetic behaviour was observed for CMS and formed colistin in plasma following IV and intratracheal (IT) instillation of CMS in Sprague-Dawley rats. Pulmonary administration of CMS 14 mg/kg resulted in extensive and prolonged exposure (12 h) to CMS and formed colistin in lung epithelial lining fluid (ELF) when compared to exposure in plasma. Formed colistin concentrations in ELF were maintained well above the minimum inhibitory concentration (MIC) for *Pseudomonas aeruginosa* and *Acinetobacter* spp of 1.0 mg/L for the 12 h sampling period. In contrast, ELF exposure of CMS was 1,200-fold lower and formed colistin concentrations unquantifiable following IV administration of the same CMS dose. The extensive exposure of CMS and formed colistin in the ELF following IT instillation was proposed to be due to drug residing in a small volume of lung lining fluid (~82 – 84 µL), potential binding to lung tissue, slow absorption of CMS from the lungs and CMS not available for renal clearance which leads to a greater fraction of the IT CMS dose converted to colistin in the lungs compared to fractional conversion of CMS to colistin in plasma. Building of a population pharmacokinetic model for CMS and colistin confirmed that

a greater fractional conversion of CMS to colistin was occurring in the lungs (23%) following IT dosing when compared to in plasma (2.6%) after IV administration. In comparison to plasma, the conversion kinetics of CMS was slower in the lungs which contributed to the extensive exposure of formed colistin in ELF. The greater systemic exposure of formed colistin following IT CMS dosing (2.5- to 3.8-fold higher) when compared to IV administration of CMS was due to the absorption of pre-systemically formed colistin into plasma. Since formed colistin concentrations in ELF were maintained well above the MIC, a significant reduction in the IT CMS dose can be implemented to minimise the systemic exposure to colistin. Similar pharmacokinetics for colistin in ELF and plasma was observed after IT and IV dosing of the active antibacterial moiety, colistin. In CF subjects, pulmonary administration of 2 and 4 million international units of CMS resulted in extensive CMS and formed colistin sputum concentrations that remained above the MIC (1.0 mg/L) for the 12 h sampling period. A proportional increase in formed colistin sputum exposure was evident in the majority of CF subjects with doubling of the nebulised CMS dose. Despite high formed colistin concentrations in the lungs, unquantifiable colistin concentrations in plasma were evident with less than 2% of the CMS nebulised dose recovered in urine. In contrast, following IV infusion of CMS (150 mg of colistin base activity), formed colistin concentrations in sputum were below the MIC throughout the 12 h sampling time. Both nebulised CMS doses were well tolerated in the majority of CF subjects. This thesis demonstrates for the first time, in both pre-clinical and clinical studies the targeting advantage that can be achieved by administering CMS directly into the lungs when compared to after IV administration. In rats and CF subjects, the therapeutic availability and drug targeting index of CMS and formed colistin were magnitudes higher than unity which indicates a greater exposure of CMS and formed colistin in the lungs (ELF, sputum) which represents an effective increase in targeting to the lungs while minimising systemic exposure following

pulmonary administration when compared to IV dosing. Therefore the studies undertaken in this thesis have characterised the pharmacokinetics of CMS and formed colistin in the lungs and plasma following pulmonary and IV administration of CMS to Sprague-Dawley rats and CF subjects. The targeting benefit that can be achieved following administration via the pulmonary when compared to the IV route has been demonstrated both in the pre-clinical and clinical setting.

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes no original papers published in peer reviewed journals and two submitted publications. The core theme of the thesis is to characterise the pharmacokinetics of colistin methanesulphonate and colistin following pulmonary administration in the pre-clinical and clinical setting to determine the targeting advantage that can be achieved following direct delivery into the lungs. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Drug Delivery, Disposition and Dynamics Theme of the Monash Institute of Pharmaceutical Sciences under the supervision of Dr Michelle P. McIntosh, Prof Roger L. Nation, Prof Christopher J.H. Porter and Prof Jian Li.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 5 and 6 my contribution to the work included all the experimental work, all data analysis and interpretation, the concept and design of all studies, the preparation of initial drafts of all manuscripts and the subsequent revision and formulation of conclusions and hypotheses resulting from the relevant studies.

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
5	Population pharmacokinetics of colistin methanesulfonate in rats: achieving sustained lung concentrations of colistin for targeting respiratory infections	In submission	Planning and execution of experimental work, data evaluation, drafting and revision of manuscript.
6	Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulphonate in cystic fibrosis patients: targeting advantage of inhalational administration	In submission	Planning and execution of experimental work, data evaluation, drafting and revision of manuscript.

I have renumbered sections of submitted papers in order to generate a consistent presentation within the thesis.

Signed: ……………………………   **Date:………………………………**

Acknowledgments

Firstly, I would like to thank my supervisors Dr Michelle McIntosh, Prof Roger Nation, Prof Christopher Porter and Prof Jian Li for their guidance and support. Thank you for the opportunities that were provided to me during my PhD. I will forever be grateful to you and your contribution to my professional development.

To Kashyap, thank you for your patience and all the help that you have given me in the last year. I couldn't have asked for a better person to teach me the skills and concepts when it came to pharmacokinetic modelling.

To all my friends in D4 and FADDI, thank you for being there through the good and especially through the challenging times. It definitely would have been a more difficult journey without you all in it. Thank you for the support, friendship, laughter and entertainment over the years – I will truly miss you all.

To my many offices mates (Line, Tri, Kathy, Yan, Tomas, Gemma, Orlagh, Matt, Nathania and Enyuan) I could not have asked for a better bunch of people to be around on a day to day basis. Thank you for always taking care of me, the emotional support, and the chats and laughter over the years. To Line, my first PhD buddy ("twin") I couldn't imagine getting through my first year without you – you made my transition into PhD life so much easier and for that I am forever grateful.

To Yan, one of my closest friends, thank you for the endless conversions and advice over the years. You have been there and helped me through some of the toughest periods of my life (PhD and personal).

To Tri, thank you for your friendship, I still remember the day I met you (outside the South Lab) and over the years we built a good friendship. I always know that you have my back no matter the situation.

To Joe, thank you for the countless advice that you have given me. You have helped me through some of my toughest challenges during my PhD. And on top of all this you have become a close friend, you always keep me entertained with your humour and remarks.

To Khay, Danielle and Ian thank you for the lunch time conversions and ensuring that Yan and I were sane when we were writing up. To Soon-Ee, you always made me look at life from

a different perspective. Thank you for all the help and advice you have given me in the short time I have known you.

To my girlfriends, Cat and Dotty, you both kept me "normal' over the years. I am truly blessed to have you both in my life.

Finally, those closest to me, ammi, thaththi, Shaq and Jess, I could not have gone through this journey without your love and support. To ammi and thaththi, I am truly grateful for all that you have done for me. I am the person that I am today because of the way you raised me and for that I am blessed and forever grateful.

Publications and communications

<u>Publications</u>

1. Yapa SWS, Li J, Patel K, Wilson JW, Dooley MJ, George J, Clark D, Poole S, Williams E, Porter CJH, Nation RL, McIntosh MP. Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulphonate in cystic fibrosis patients: targeting advantage of inhalational administration. *Manuscript in submission.*

2. Yapa SWS, Li J, Porter CJH, Nation RL, Patel K, McIntosh MP. Population pharmacokinetics of colistin methanesulfonate in rats: achieving sustained lung concentrations of colistin for targeting respiratory infections**.** *Manuscript in submission.*

Communications

1. Yapa SWS, Li J, Porter CJH, Nation RL, McIntosh MP. Systemic bioavailability of colistin following pulmonary delivery. Poster presentation. Australasian Pharmaceutical Science Association, Manly, December 2007.*

2. Yapa SWS, Li J, Porter CJH, Nation RL, McIntosh MP. Systemic bioavailability of colistin following pulmonary delivery. Podium presentation. Globalisation of Pharmaceutics Education Network, Leuven, Belgium, September 2008.

3. Yapa SWS, Li J, Porter CJH, Nation RL, McIntosh MP. Systemic bioavailability of the prodrug colistin methanesulphonate and formed colistin following pulmonary administration of the prodrug. Poster presentation. Australasian Pharmaceutical Science Association, Hobart, December 2009.

4. Yapa SWS, Li J, Porter CJH, Nation RL, McIntosh MP. Pharmacokinetic properties of the prodrug colistin methanesulphonate and colistin following pulmonary administration of colistin methanesulphonate. Poster presentation. European Congress of Clinical Microbiology and Infectious Diseases, Vienna, Austria, March 2010.

5. Yapa SWS, Li J, Porter CJH, Nation RL, McIntosh MP. Pharmacokinetic properties of the prodrug colistin methanesulphonate and colistin following pulmonary administration of colistin methanesulphonate. Poster presentation. Monash Parkville Postgraduate Symposium, September 2011.

* Award winning.

List of abbreviations

ABC	ATP-binding cassette
ACE	acetone
Ace	acepromazine maleate
ACN	acetonitrile
AE	adverse effect
AUC	area under the curve
AUMC	area under the first moment curve
BAL	bronchoalveolar lavage
BCRP	breast cancer resistance protein
BSV	between-subject variability
CBA	colistin base activity
CF	cystic fibrosis
C_{max}	maximum concentration
CMS	colistin methanesulphonate
% CV	co-efficient of variation
r^2	co-efficient of determination
CL	systemic clearance
C_{last}	last concentration
CYP	cytochrome
Da	dalton
Dab	diaminobutyric acid
dL	deciliter
DTI	drug targeting index
EH	epoxide hydrolase
ELF	epithelial lining fluid
FDA	Food and Drug Administration
$FEV_{(1)}$	forced expiratory volume in 1 second
FMO	flavine mono-oxygenase
FMOC-Cl	9-fluor-enylmethyl chloroformate
FVC	forced vital capacity
F%	bioavailability
G	gauge
h	hour
HPLC	high-performance liquid chromatography
H_2SO_4	sulphuric acid
IT	intratracheal
IU	international units
IV	intravenous
k_a	absorption rate constant
KCl	potassium chloride
Ke	ketamine hydrochloride
KH_2PO_4	potassium dihydrogen phosphate
kg	kilogram
L	liter
Log P	octanol-water partition coefficient
LOQ	limit of quantification
LPS	lipopolysaccharide

M	molar
MC	mucociliary clearance
MDR	multidrug-resistant
MeOH	methanol
MEP	maximal expiratory pressure
mg	milligram
MIC	minimum inhibitory concentration
min	minute
MIP	minimum inspiratory pressure
mM	millimolar
MRP	multidrug-resistance-associated protein
mTorr	millitorr
M_w	molecular weight
NaCl	sodium chloride
$NaC_{12}H_{25}SO_4$	sodium dodecyl sulphate
$NaHCO_3$	sodium hydrogen carbonate
Na_2HPO_4	disodium hydrogen phosphate
NaOH	sodium hydroxide
NCA	non-compartmental analysis
NH_2CONH_2	urea
nm	nanometer
OAT	organic anionic transporter
OBJ	objective function
OCT	organic cation transporter
o.d × i.d	outer diameter × inner diameter
OD	optical density
PBS	phosphate buffered saline
PD	pharmacodynamics
PE	polyethylene
PEPT	peptide transporter
P-gp	P-glycoprotein
PK	pharmacokinetic
QC	quality control
RUV	residual unexplained variability
SC	subcutaneous
S.D.	standard deviation
sec	second
SEP	slowly-effluxable-pools
SLC	solute carrier
SPE	solid-phase extraction
SULT	sulfotransferase
TA	therapeutic availability
TFA	trifluoroacetic acid
THF	tetrahydrofuran
T_{max}	time to reach maximum concentration
TNF	tumor necrosis factor
$t_{1/2}$	terminal half-life
$t_{1/2,ab}$	absorption half-life
UGT	UDP glucuronosyl transferase
UV	ultraviolet

VAP	ventilator-associated pneumonia
V_{ELF}	volume of epithelial lining fluid
V_{ss}	volume of distribution at steady state
Xyl	xylazine hydrochloride
λ_z	terminal rate constant

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Amendments and Typographical Errors

Abbreviation List
p. xvi, line 20   **Insert after "CYP":**   colistin sulphate: the salt form of colistin   colistin: the base form of colistin   formed colistin: *in vivo* formed colistin from CMS   sodium colistin methanesulphonate: the salt form of CMS   colistin methanesulphonate: the base form of CMS
Chapter 1
p. 4, line 16: "evolved" for "designed"
p. 25, line 6: "have" for "has"
p. 25, line 10: "vary" for "varies"
p. 28, line 6: "are" for "is"
p. 31, line 3: "eliminating" for "elimination"
p. 35, line 12: "defined" for "define"
p. 38, line 20: a comma following "antibiotic"
p. 39, line 3: "plays" for "play"
p. 39, line 17: a comma following "colistin" and following "antibiotics"
Chapter 2
p. 82, line 19: a comma following "non-linearity"
p. 85, line 6: "were" for "was"
p. 96, line 8: insert "acceptable" following "demonstrated"
Chapter 3
p. 98, line 16: insert "delivery" following "pulmonary"
p. 100, line 4: remove comma following "colistin"
p. 103-104, Section 3.4.3.1   **Comment:** Intratracheal instillation was used to deliver colistin into the lungs since our research group did not have access to a micro-sprayer at the time these PK studies were conducted. We do acknowledge that a better lung exposure and increased absorption would be expected with a micro-sprayer when compared to intratracheal instillation. However tracheal instillation is still a valid method as it delivers an accurate and reproducible volume of dosing solution into the lungs and this delivery method was utilised for both the colistin and CMS pulmonary studies. *In vitro* studies to characterise the instilled colistin solution (i.e. droplet size) was not undertaken for these studies.
p. 109, lines 21-22   **Comment:** The target dose (mg/kg) was the nominal dose and the actual dose (mg/kg) was the dose administered to each rat. The latter dose can vary depending on the volume of dosing solution that was retained in the dosing syringe (in the void space of the needle and syringe tip). Therefore the dosing syringe was weighed pre- and post-administration to calculate the volume of solution that was delivered to each rat. The dosing solution concentration and the volume administered were then used to estimate the actual dose delivered to each rat.
p. 112, lines 9-12   **Comment:** A "tukey post-hoc test" was conducted after one-way ANOVA analysis to determine which dosing groups differed from each other.
p. 120-121, Figure 3-7B and Table 3-7   **Comment:** Colistin $AUC_{0-\infty}$ (mg·h/L) in ELF and plasma that are presented in Table 3-7 are shown in Figure 3-7B.
p. 123, Table 3-8   **Comment:** To estimate therapeutic availability (TA) and drug targeting index (DTI) the same dose of colistin needs to be administered via the IV and pulmonary route. The colistin ELF exposure was estimated following an IT dose of 0.62 mg/kg however this dose could not be administered via the IV route. As outlined in p. 121, lines 11-14, a lower IV colistin dose of 0.41 mg/kg was administered due to observation of transient adverse effects at the 0.62 mg/kg dose. Therefore the AUC of colistin in ELF and plasma after IT 0.62 mg/kg was dose-normalised to an IT dose of 0.41 mg/kg (linear pharmacokinetic relationship for the two doses, Figure 3-5, p. 117) which enabled the estimation of the TA and DTI for colistin at the 0.41 mg/kg dose.
p. 125, lines 1-13   **Comment:** The IV and pulmonary dosing groups referred to in the Marchand *et al* study are following administration of CMS. The authors only reported the PK of colistin following subcutaneous administration of colistin and hence these results are compared to the PK of colistin in the present IV dose-ranging studies.
p. 125, lines 23-25, and p.126, line 1   **Delete:** "During IT instillation an unknown fraction of the dose may have been delivered into the gastrointestinal tract…."   **Comment:** As the dosing cannula was placed at the trachea-bronchus bifurcation and 200 µL of air was administered after the solution it is unlikely that a proportion of the dose would have been delivered to the gastrointestinal tract.
p. 126, lines 9-10   **Following the sentence:** "This suggests that colistin is slowly absorbed from the lungs which is in contrast to the rapid appearance of colistin in plasma in the current study."   **Insert:** "These observations are likely to be due to the difference in the surface area of the two systems (4.7 cm^2 in Transwell systems versus several meters in the rat's lungs".
p. 127, line 24-25 and p.128, line 1   **Comment:** The overall disposition of colistin in ELF would be influenced by distribution (binding), absorption and clearance mechanisms in the lungs. As cross-referenced in p. 127, line 25 and p. 128, line 1, the presence of CYP enzymes, peptidases, mucociliary clearance and phagocytosis (by alveolar macrophages) may contribute to the pulmonary clearance of colistin.
p. 128, line 9: "effluxed" for "efflux"
p. 128, line 2: "clearance" for "loss"

Chapter 4

p. 135, Section 4.4.3
Comment: The selection of CMS doses for the IV and IT dose-ranging studies were based on i) the limit of quantification of the analytical method for formed colistin and ii) to minimise adverse effects.

p. 144, lines 10-12
Comment: A "tukey post-hoc test" was conducted after one-way ANOVA analysis to determine which dosing groups differed from each other.

p. 145, line 21 and p. 146, lines 1-2
Comment: Only two CMS doses were administered for the IT dose-ranging studies as analytical sensitivity limits and potential adverse effects limited a lower and higher IT dose from being delivered, respectively. Since two doses were administered, CMS and formed colistin AUC versus CMS dose plots and associated correlations (R^2) are not shown. The pharmacokinetic properties of CMS and formed colistin are presented in Table 4-3 (p.151). Linear pharmacokinetic properties for both entities was reported due to i) a proportional increase in CMS and formed colistin AUC with CMS dose, ii) no significant difference in the terminal $t_{1/2}$ for CMS and formed colistin for the two doses, and iii) the systemic bioavailability for CMS of 80 – 89% for both doses (Table 4-3).

p.148, Table 4-2 and p. 149, Figure 4-2B
Comment: The positive y-intercept observed for formed colistin AUC versus CMS dose plot (Figure 4-2B) is reflective of a higher than expected formed colistin AUC for the CMS dose of 14 mg/kg (Table 4-2). These observations are likely to be due to the sensitivity limit of the colistin analytical method as mentioned in p. 161, lines 7-10. The limit of quantification (LOQ) for the colistin assay was 0.10 mg/L and following administration of CMS 14 mg/kg formed colistin plasma concentrations were within a narrow range around the LOQ for the duration of the sampling period (4 h). This can influence the estimation of AUC especially the extrapolated AUC.

p. 157, Figure 4-5A
Figure title: Delete (-■-) and (··■··) and replace with (-▲-) and (··▲··), respectively.

p. 161, Table 4-8
Delete: "¥ Formed colistin $AUC_{0-\infty}$ estimate dose-normalised for a colistin 0.62 mg/kg dose".
And Insert: "¥ Estimation of $AUC_{\text{dose-normalised},0-\infty}$ for formed colistin: The CMS 14 mg/kg dose was converted to a equimolar dose of colistin (10.01 mg/kg), and then the formed colistin $AUC_{0-\infty}$ of 7,564 mg·h/L was dose-normalised to a 0.62 mg/kg colistin dose."

p. 163, lines 20-22
Delete Line: "During IT instillation an unknown fraction of the CMS dose may have been delivered into the gastrointestinal tract…."
Comment: As the dosing cannula was placed at the trachea-bronchus bifurcation and 200 µL of air was administered after the solution it is unlikely that a proportion of the dose would have been delivered to the gastrointestinal tract.

p.164, lines 12-25 and p. 165, lines 1-6
Comment: The PKs of CMS and formed colistin in plasma following IT instillation of CMS was compared to the Marchand *et al* study. Similarities and differences were noted between the two studies. The differences observed in the absorption kinetics of CMS and formed colistin could be due to CMS administered by instillation in the current study and via microspray in the Marchand *et al* study. This can influence the regions of the lung that the drug is delivered to and hence the extent of absorption. This has been acknowledged in p. 165, lines 3-6.

p. 168
Comment: The discussion on this page alludes to the higher binding affinity of colistin to lung tissue/constituents when compared to CMS. The relatively higher binding affinity of colistin may explain i) potential displacement of CMS from lung binding sites with formation of colistin (paragraph 1), and ii) unquantifiable formed colistin concentrations in ELF following IV CMS administration (paragraph 2).

p. 169, line 21: "therapeutically" for "therapeutic"

p. 170, line 1: "administration" for "administering"

p. 171, line 5: "conclusions" for "conclusion"

Chapter 5

p. 175, lines 14-15
Comment: One of the primary reasons for the development of the population pharmacokinetic model was to better understand the CMS-to-colistin conversion kinetics in ELF. The model demonstrated that conversion of CMS to colistin occurred in BAL fluid$_1$ (trachea, upper airways, some peripheral airways) and BAL fluid$_2$ (remaining peripheral lung regions) compartments with the conversion kinetics slower in the former compartment. Such in-depth interpretation of CMS conversion kinetics could not be undertaken in the noncompartmental analysis in Chapter 4. The statements made in relation to "quantification of formed colistin in ELF at the first sampling time (5 min post-dose)" (p. 166, lines 7-8) may be reflective of the relatively faster conversion that occurred in BAL fluid$_2$ compartment. The outcomes from the modeling work support the findings in Chapter 4.

p. 177-183, Section 5.3 and p. 183-192, Section 5.4
Comment: For the development of the population pharmacokinetic model for CMS and colistin the concentrations are expressed in molar units (µmol/L) to facilitate accurate estimation of CMS to colistin conversion kinetics (1 molecule of CMS is hydrolysed into 1 molecule of colistin).

p. 194-196
Comment: It is unclear as to whether the plasma protein binding characteristics of CMS contributes to the conversion kinetics in plasma. This is due to the unbound fraction of CMS in rat plasma not being known (p. 236, lines 8-9). There is only limited knowledge pertaining to the plasma protein binding of colistin (as outlined in p. 235, lines 5-10) with approximately 55 – 57% of colistin bound to plasma protein in rats.

Chapter 6

p. 204, lines 15-22 and p.205, lines 1-7
Comment: Data pertaining to the pathophysiological status of the airways due to bacteria colonisation was not collated for this study. The reason for this was that the pilot study was conducted with the primary objective of defining the pharmacokinetics of CMS and colistin in the lungs and systemic circulation following inhalation and IV administration to CF subjects. However, future follow-up studies will incorporate airway characterisation and microbiological assessments and the influence of these factors on drug deposition in the lungs following inhalation.

p. 205, line 22 and p.206, lines 1-2
Comment: *In vitro* studies to characterise the solution droplet size, distribution of the nebulised CMS solution and estimation of the fine particle fraction was not conducted in this pilot study. However the device information for the Salter Nebu Tech® HDN® Disposable Nebulizer provided by Salter Labs® state that 80% of the generated particles are within the respirable range (less than 5 microns) [http://www.salterlabs.com/index.cfm?fuseaction=products.product&product_id=56].

p. 205, lines 8-20
Comment: The selected inhaled and IV doses of CMS were based on current clinical dosing regimens and therefore provide an explanation for the different doses for the two routes of administration. Inhaled CMS dosing regimens vary between different CF centers (1 million IU of CMS twice daily to 2 million IU of CMS three times daily). The decision to initially administer a single inhaled CMS dose of 2 million IU was based on the current inhaled dosing regimen at the Alfred hospital (1 million IU of CMS twice a day) since the pilot study was conducted at this site. The inhaled dose was then doubled (a single dose of 4 million IU of CMS) to assess for linear pharmacokinetic behaviour and tolerability. For IV CMS, the recommended daily dose is 2.5-5 mg/kg expressed as CBA. Therefore a single dose of 150 mg CBA was selected for a subject weighing 60 kg.

The sequence of administration was based on monitoring for adverse effects and minimising the carry over effect. Therefore subjects received the lowest dose in the first treatment period (inhaled 2 million IU CMS) and the highest dose (IV 5 million IU CMS (equivalent to 150 mg CBA)) in the last treatment period.

p. 212, lines 10-13, and p. 215, lines 5-9
Comment: The reported sputum drug concentrations are those that were quantified in the pre-dose and post-dose samples without any further manipulation. Pre-dose drug concentrations were not subtracted from post-dose concentrations. The justification for this approach was that for a particular subject the region of the lungs from which sputum samples originate from can vary over the sampling period (12 h). Therefore subtracting the pre-dose concentrations (from one lung region) from the post-dose concentrations (from another lung region) may not be an accurate approach to minimise the carry over effect. For this reason the concentration of CMS and colistin in pre-dose and post-dose samples are specifically reported in each section (p. 212, lines 10-11 and p. 215, line 6). In future studies implementation of a longer washout period is warranted.

p. 216, para 1, lines 2-5
Comment: The nominal CMS dose (specified on p. 210, line 10) was utilised to estimate the systemic CMS availability. The actual dose administered (by weighing the nebuliser container pre- and post-inhalation) could not be calculated due to safety issues relating to the dosing solution remaining at room temperature which could increase the risk of *in vitro* colistin formation. *In vitro* studies were not conducted to determine the efficiency of delivery of the Salter Ultra-Mist nebuliser. For future PK studies such procedures need to be incorporated as systemic CMS availability will alter accordingly.

p. 216, lines 5-7
Comment: Urine samples were collected up to 24 h post-dose from each subject. This sampling interval was based on previously published studies in rats and humans (healthy volunteers) that have shown that the majority of CMS is recovered in urine within the first 12 h following dosing. An explanation for the lower systemic availability of CMS when using the urinary recovery data when compared to the plasma data is as a result of colistin formed from CMS in the renal tubules being reabsorbed back into the blood stream. Acknowledging this limitation, urinary data was still useful as a secondary measure of the systemic exposure of CMS.

p. 218
On line 4, insert after "… CMS nebulised dose.": "It should be noted that subject 5 had the third highest formed colistin sputum AUC despite samples collected up to 8 h when compared to 12 h post-dose in the remaining subjects (Table 4). Similarly, subject 6 who experienced cough and chest tightness had the second highest formed colistin sputum exposure (Table 4)."
On line 11, delete: "Subject 2 reported transient dizziness 4 h after administration of IV CMS"
And insert: "Subject 2, who was the youngest (20 years) and smallest in weight (56 kg) reported transient dizziness 4 h after administration of IV CMS. It should be noted that subject 2 showed the highest formed colistin C_{max}, the second highest AUC for CMS and the second smallest CL for CMS (Table 2)."

p. 219-225
Comment: This section makes comparisons to the concentrations of CMS/colistin in sputum, BAL and ELF from previously published studies. The reasons for comparing drug concentrations in varying lung matrices is as a result of a small number of studies having been conducted following IV and inhalation administration of CMS. However it is important to emphasise that the drug concentrations will vary depending on the CMS dose administered, the delivery method and the lung matrix sampled. Therefore when comparisons were made between the current findings and those of citied papers explanations as to why these differences were observed were clearly outlined (refer to p. 221, lines 4-11, p. 222, lines 7-11 and p. 224, lines 24-25 to p. 225, lines 1-4).

p. 222, lines 7-11
Delete lines 7-11 and insert: "The variability in the reported colistin concentrations in sputum and ELF across the studies will likely be a function of the efficiency of nebulised delivery, the solution droplet size and distribution in the lungs, whether the study involved single or multiple dosing, the different biological matrices (sputum versus ELF), analytical issues as discussed above and variation in the pathophysiological status of the respective patient population."

p. 222, line 17: "sputum" for "the lung"

Chapter 7

p. 228, line 7: "routes" for "route"

p. 228, line 9: move "following IV CMS administration" to line 10 after "site"

p. 230, line 9: insert "being" following "not"

Chapter 1: Introduction

1.1 Statement of the problem

Colistin is an old antibiotic and is undergoing resurgence in clinical use as a last line of defence against multidrug-resistant (MDR) Gram-negative bacteria. Over the last two decades colistin methanesulphonate (CMS), the inactive prodrug of colistin, has been administered via the pulmonary route for management of MDR Gram-negative respiratory infections in cystic fibrosis (CF) and critically-ill patients with ventilator-associated pneumonia (VAP). Patients receiving inhaled CMS may also be treated simultaneously with intravenous (IV) CMS or other antibiotics for the management of these respiratory infections. Despite the increase in CMS usage, there is limited information about the pulmonary pharmacokinetics of CMS and formed colistin, following inhalation or IV delivery. The main reason for this is, prior to release onto the market over 50 years ago colistin and CMS were never subjected to the drug development procedures that are now mandated by international drug regulatory agencies. With limited availability of pharmacokinetic data, clinicians are constrained in making evidence-based decision in relation to dosage and route of delivery to achieve optimum therapeutic efficacy. If such problems are not rectified, there is an increased risk of therapeutic failure, increase in adverse effects and the development of resistance to an antibiotic that is already a last line of defence against MDR Gram-negative bacterial infections. Therefore, there is a significant need for more pre-clinical and clinical pharmacokinetic studies to better understand the disposition of CMS and formed colistin following pulmonary and IV administration. These studies will enable an understanding of the targeting advantage that may be achieved following direct delivery into the site of respiratory infection when compared to after IV delivery and thereby intensifying the treatment of MDR Gram-negative pulmonary infections.

1.2 Cystic fibrosis and critically-ill patients

Chronic respiratory infections most commonly caused by *Pseudomonas aeruginosa* results in much of the morbidity and the majority of the mortality in cystic fibrosis (CF) patients [1, 2]. Initial colonisation of *P. aeruginosa* in the airways followed by establishment of chronic airway infections is characteristic of CF respiratory infections [2-4]. Approximately eighty percent of adult CF patients have chronic *P. aeruginosa* infections [1]. Establishment of chronic airway infections results in a vicious cycle of infection and inflammation which leads to permanent lung damage, pulmonary insufficiency and eventual mortality [3-5]. Ventilator-associated pneumonia (VAP) is a serious complication that can occur in mechanically ventilated patients in intensive care unit settings [6-8]. In critically-ill patients, VAP is most commonly caused by multidrug-resistant (MDR) *P. aeruginosa* and *Acinetobactor baumannii* and is associated with an increase in morbidity, mortality and hospital care costs [6-8].

Effective antibiotic therapy is crucial for the management of these MDR Gram-negative respiratory infections in CF and critically-ill patients. In clinical use, colistin, in the form of its prodrug, CMS [9], is delivered via inhalation and intravenous (IV) injections for the management of these respiratory infections. In CF patients, over the last 20 years, inhaled CMS has been used at both stages of lung infection, 1) at the initial colonisation stage to delay the development of chronic infections and 2) as maintenance therapy in chronic infections to prevent and reduce the severity of acute exacerbation of lung infections [3, 4, 10, 11]. Tobramycin and aztreonam are the only other antibiotics that have been approved for use via the pulmonary route in CF patients for the management of chronic lung infections [12]. In CF patients, acute exacerbation of chronic respiratory infections is treated with a combination of IV antibiotics, including CMS [13-15]. With the therapeutic efficacy observed in CF patients following pulmonary delivery of CMS, it is increasingly being used as an effective adjunctive therapy with IV antibiotics (of which one is CMS) in patients with VAP [7, 16-19].

Inhalational delivery of antibiotics for the treatment of respiratory infections has gained significant interest over the past years due to the advantages that can be achieved with local delivery into the lungs. Achievement of high antibiotic concentrations at the site of infection, rapid onset of action and minimised systemic exposure and thereby systemic adverse effects are some of the potential benefits that can result from pulmonary delivery [20, 21]. When compared to systemic delivery of antibiotics, lower total doses could potentially be administered as antibiotics are directly delivered into the lungs and thereby do not need to cross biological membranes to reach the site of infection [20, 21]. Despite this, there is limited information in the literature about lung exposure *versus* systemic exposure following inhalational delivery of antibiotics; this is especially true for colistin, the last line of defence against MDR Gram-negative bacteria. To date there has been no attempt to quantitate the targeting advantage that may be achieved following pulmonary delivery when compared to IV administration of CMS. This information is urgently needed in order to preserve the therapeutic efficacy and reduce the development of resistance to colistin.

1.3 Human respiratory tract

1.3.1 Pulmonary physiology

The human respiratory tract is a heterogeneous organ situated in the thoracic cavity with a complex integral network of blood vessels and airway passages specifically designed to enable gas exchange of oxygen and carbon dioxide between blood and air [22]. The respiratory tract can be divided into two distinct sections, the conducting airways consisting of the trachea, bronchi, bronchioles and terminal bronchioles and the respiratory airways consisting of the respiratory bronchioles, alveolar ducts and alveolar sacs (Figure 1-1) [23]. The 23 airway generations of the human respiratory tract are structured in a dichotomous manner [23, 24]. The conducting airways bifurcate approximately 16 − 17 times and with

each sequential bifurcation the airways become smaller in diameter and length [23-25]. The process of gas exchange occurs from the alveolar surface and in an adult human the surface area of the alveolar surface (43 - 102 m^2) [26] is considerably larger when compared to that of the conducting airways (~2.5 m^2) [27].

The lung epithelium is made up of a single monolayer of cells and a distinct characteristic of the conducting *versus* respiratory airways is the structure and composition of these cells (>40 different cell types present in the lungs) [22, 23]. The epithelium of the conducting airways is composed of pseudo-stratified columnar epithelia [28] and are populated with ciliated cells, secretory cells (goblet, mucous, serous and Clara cells) and basal cells [23, 25, 28]. The different cells have distinctive roles in maintaining the integrity and homeostasis of the respiratory tract; for example the basal cells are the progenitor cells for the ciliated and secretory cells while the role of ciliated cells is the propulsion of mucus *via* mucociliary clearance [24, 25, 28]. In the alveolar epithelium, two major cell are present, Type I and Type II cells, of which the broad and extremely thin Type I cells predominantly (>95%) occupy the epithelia [23, 28]. Type II cells (small and cuboidal) also known as the progenitor cells for the Type I cells are distributed in the alveolar sacs and have the additional role of producing lung surfactants and regulating alveolar ion and solute transport [25, 28, 29]. With a gradual decline into the distal airways the lung epithelium decreases in diameter and thickness [25].
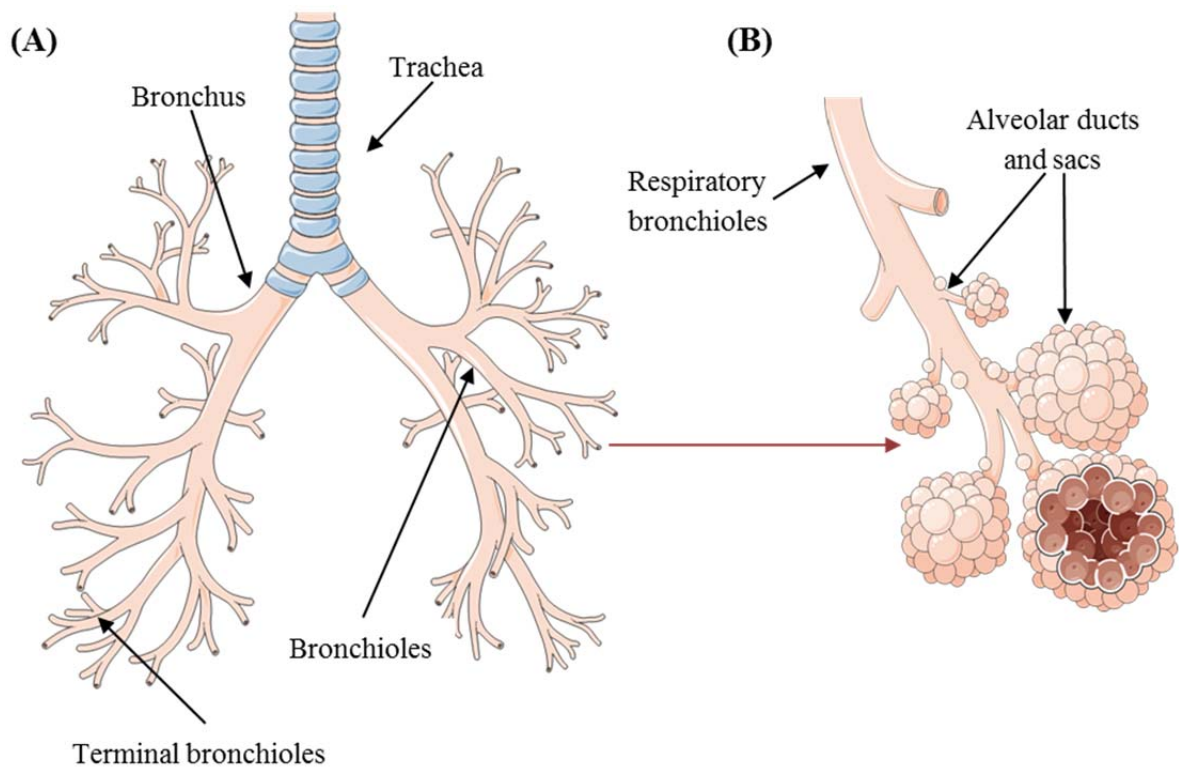
Figure 1-1: Structure of the human respiratory tract, (A) conducting airways consisting of the trachea, bronchus, bronchioles and terminal bronchioles and (B) respiratory airways consisting of the respiratory bronchioles and alveolar ducts and sacs. The figure was produced using Servier Medical Art (www.servier.com).

On the apical surface of the conducting and respiratory airways is a layer of lining fluid, known as the epithelial lining fluid (ELF, also known as epithelial surface fluid, surface lining fluid) [23, 25]. A monolayer of surfactants coat the surface of the ELF, with the hydrophobic fatty acid tail of the surfactants projected up to the airway lumen [23]. The primary role of surfactants is to reduce the surface tension of the air-lining fluid interface [23, 30]. In the conducting airways, mucus concentrates near the surface of the ELF which is in stark contrast to the alveolar region where no mucus is found [23]. As the airways gradually decrease distally the thickness of the ELF reduces from $5 - 10$ μm in the conducting airways to $0.05 - 0.08$ μm in the alveoli [23, 25]. Studies conducted in animals have suggested that the lung lining fluid is either isotonic or slightly hypotonic and slightly acidic (pH 6.9) when compared to plasma [23]. Plasma proteins such as albumin [31] and α₁-antitrypsin [32] are present in

6

lining fluid but at significantly lower concentrations than that available in plasma. Other proteins such as the immunoglobulins (IgG, IgA) and nonimmunoglobulin protein are also present in the ELF [23, 33]. The lung interstitium (comprising of the extracellular and extravascular space between cells in the lung tissue) have within it interstitial fluid, connective fibers (collagen fibers, basement membrane) and a variety of cells types (fibroblasts, monocytes, lymphocytes, pericytes and plasma cells) [22, 23]. In the conducting airways, the epithelial and endothelial (capillary) cells are attached to a structure known as the basement membrane, which is a highly specialised form of extracellular matrix composed of specific proteins and glycoconjugates [23, 34, 35]. In the conducting airways two separate basement membranes are present, however in the alveolar region a common basement membrane is present as the epithelial and endothelial cells are in close contact [23, 35]. A monolayer of capillary endothelial cells, which comprise 30% of all lung cells, makes up the vascular endothelium [24]. The lung endothelium layer has been shown to be a minor barrier to the absorption of solutes and proteins when compared to that of the epithelium layer [23, 36]. The lymphatic system of the lungs are a delicate and complex system of vessels that arise from the tissue spaces (interstitium) and are involved in the clearance of endogenous substances and toxic substances that penetrate the epithelium, keeping the lungs dry and regulating the pulmonary immune system [23, 37]. In the lungs, lymphatic vessels originate in the interstitium near the small airways and blood vessels but are absent in the alveoli region [23, 37].

1.3.2 Pulmonary absorption, distribution, metabolism and elimination

1.3.2.1 Absorption

Following inhalation of drugs into the respiratory tract, in order for drug molecules to be absorbed from the lung lumen into the systemic circulation, the molecules need to transit through the pulmonary lining fluid, epithelium, interstitium and capillary endothelium.

Depending on the physicochemical properties of drug molecules, the rate and extent of absorption can be influenced by interactions with the surfactant layer, with an increase in solubility observed for small, hydrophobic drugs while drug aggregation and thereby macrophage uptake can occur with large molecules (peptides, proteins) [22, 23, 38, 39]. The mechanism of drug transport across the lung epithelium occurs via passive and active transport mechanisms by paracellular or transcellular transport, pore formation, vesicular transport and via the lymphatic system [22, 38].

Hydrophilic drug molecules are predominantly absorbed by passive paracellular diffusion through aqueous pores in the intercellular junction [23, 38]. In the rat lung epithelium, Schanker and colleagues reported at least three populations of pores which differ in their dimensions [40]. Passage of hydrophilic molecules are facilitated by numerous small pores (molecular weight (M_w) less than 122 g/moL), a few medium size pores (M_w less than 5,250 g/moL) or a very few large pores (M_w in the range of 5,250-20,000 g/moL) [40]. Between species the porosity of the lung epithelium varies, with greatest porosity seen in mouse and guinea pigs, followed by rats and dogs and the lowest in rabbits [41, 42]. For hydrophilic drug molecules, the rate of absorption is determined by the M_w and degree of ionisation, with molecules that are less ionised having fewer interactions with the proteins and lipids that line the aqueous pores and thereby a faster rate of absorption [22, 43]. For compounds in the M_w range of 100 – 1,000 g/moL, the degree of ionisation predominately affects the rate of absorption with absorption occurring via passive paracellular transport [22, 43]. While for macromolecules (M_w range of 1,000 – 500,000 g/moL) an inverse relationship between rate of absorption and M_w has been reported with the precise mechanism of absorption unknown but thought to occur via both passive paracellular and endocytic vesicles by processes that are diffusion-limited [23, 38]. Transcytosis or vesicular movement is thought to be involved in macromolecule transport across alveolar epithelial and endothelial cells [23, 44]. Endocytosis

is facilitated through non-coated vesicles known as caveolae, which are evident in endothelial cells [23, 44] and the presence of invaginations on the apical and basolateral membranes of Type I cells suggests vesicular transport mechanisms [22, 44]. Endocytosis facilitated by receptors which produce clathrin-coated pits on epithelial Type I cells and endothelial cells are also involved in macromolecule transport [23]. For hydrophobic drug molecules within the M_w range of 100 – 1,000 g/moL rapid absorption from the lungs is evident when compared to hydrophilic molecules, with the lipophilicity (Log P) determining the rate of absorption [22, 43]. For these hydrophobic drugs, the transport across the epithelium is by passive transcellular mechanism through integration into lipid bilayer of surrounding cells which results in rapid absorption [38, 43].

Additionally absorption of drug molecules from the lungs can be facilitated or hindered by the presence of drug transporters of the solute carrier (SLC) family and ATP-binding cassette family (ABC transporters) present in the lung epithelium and endothelium [38, 45]. Organic cation transporters (OCT) and organic anionic transporters (OAT, OATP) belong to the SLC drug transporter family and are involved predominately in the transportation of low passively permeable drugs [38, 45]. Most commonly these transporters facilitate the uptake of molecules into cells (intracellular space) [38]. Peptide transporter (PEPT2) also belonging to the SLC transport family are expressed in lung tissues and are involved in transporting di- and tripeptides and peptidomimetic drugs [45, 46]. Multidrug-resistance proteins (MDR) such as P-glycoprotein (P-gp/MDR1), multidrug-resistance-associated proteins (MRP) and breast cancer resistance protein (BCRP) belonging to the ABC transporter family are involved in the efflux of drug molecules out of cells via an ATP-dependent mechanism [45, 47]. Of the ABC transporters, MRP1 and P-gp are localised in several regions of the lungs (bronchial epithelium, alveolar Type 1 cells, goblet cells, endothelium) and MRP1 is highly expressed in the lungs [45, 47].

1.3.2.2 Distribution

The pharmacokinetics of intravenous (IV) and inhaled drugs are significantly influenced by the distribution and accumulation characteristics of the human respiratory tract. Several studies have reported on the extensive binding nature of lipophilic basic amines (pKa > 8 .5) to human lung [48], rabbit perfused lung [49-51] and rat perfused lung [52, 53]. In comparison, nonbasic amines (pKa < 7.0) have been shown to accumulate to a lesser extent in the rabbit lungs with binding occurring via non-saturable mechanisms such as through lipid partitioning [50]. Accumulation of lipophilic basic amines is thought to occur at two binding sites through saturable and non-saturable mechanisms [49, 50, 53]. Intracellular binding sites (mitochondria [52], lysosome [50]) which require transport of amines from extra- to intracellular environments facilitated by carrier-mediated transport system may represent the saturable mechanisms [49]. While accumulation of amines in the extracellular fluid following transport by diffusion may represent non-saturable mechanisms [49]. Additionally, partitioning of amines within the intercellular space may contribute to lung accumulation as has been demonstrated in the rabbit lung [51]. Following uptake into alveolar macrophages (a temperature sensitive, energy and pH dependent, saturable process) binding of amines to lysosomes structures has been proposed as a site for intracellular accumulation [50]. Following binding to alveolar macrophages some drugs have been shown to be slowly released over time (characteristic of slowly-effluxable-pools (SEP)) and this has been proposed as one of the main reasons for the persistence of amines in the lungs [51]. The accumulation of lipophilic basic amines is reversible and can be displaced by other amine drugs [50, 53]. The physicochemical properties that determine the extent of accumulation in the lungs are the degree of protonation of the nitrogen atom (degree of ionisation) and lipophilicity [48]. Between studies there is some discrepancy as to whether the protonation

and therefore the cationic nature [48-50] or the lipophilicity [52, 53] of a drug molecule is the predominant factor driving this extensive binding.

The macrolide antibiotics, azithromycin, clarithromycin, roxithromycin and erythromycin [54-57], and a ketolide antibiotic, telithromycin (semisynthetic derivative of erythromycin) [58, 59] have been shown to accumulate in alveolar macrophages. Uptake into the intracellular regions of macrophages is mediated by an active transport system (carrier system not identified) which is facilitated by the presence of Ca^{2+} and protein kinase A-dependent phosphorylation [54, 57, 59, 60]. These weak organic bases are transported across the cell membrane in the unprotonated form and following exposure to the acidic organelle compartments (cytosol and lysosomes) are protonated and become trapped (to a greater extent in lysosomes) [54, 55, 58]. This accumulation is reversible [55], with studies reporting that a slow release of azithromycin from macrophages occurs which may be a direct consequence of trapping within the lysosomes [54].

1.3.2.3 Metabolism

In the human respiratory tract there are several cell types such as the Clara cells, alveolar Type II cells and macrophages that are involved in the metabolism of inhaled drugs [61]. The cytochrome P450 (CYP) family of enzymes are expressed in the lungs, which provide an additional line of defence against inhaled drugs [38]. In comparison to the liver and intestines, the expression levels and patterns of the CYP enzymes in the lungs are different [38]. Some of the most common Phase I drug metabolising enzymes expressed in the lungs are CYP1A1, CYP1B1, CYP2A6, CYP2B6/7, CYP2E1, CYP2J2, CYP3A4, CYP3A5, epoxide hydrolase (EH), flavine mono-oxygenases (FMO) and phase II metabolising enzymes such as sulfotransferases (SULT), UDP glucuronosyl transferases (UGT), esterases, peptidases and cyclo-oxygenases [61-63]. These enzymes are distributed in different regions of the lungs, the bronchial/bronchioles/alveolar epithelium, capillary endothelium, alveolar macrophages,

Clara cells and bronchial mucosa and tissues [62]. In the lungs, the main CYP3 isomer is CYP3A5, and unlike in the liver, CYP3A4 is not the most abundant CYP enzyme and is expressed in only 20% of individuals [62]. The contribution of lung drug metabolism to the disposition of inhaled drugs is relatively low when compared to the metabolism activity in the liver [61, 63]. Somers *et al* reported that in the lungs when compared to the liver, a significantly lower expression of most of the phase I metabolising enzymes was evident, for EH and esterases approximately 20% expression when compared to that of the liver, while the expression for SULT was similar to that of the liver [63]. Similar to the rest of the body, the peptidase are abundant in the lungs [38, 43]. Therefore for drugs that are peptides, in order to evade metabolism by peptidases the chemical structures are slightly modified (blocking chemistry) [43].

1.3.2.4 Elimination

Other mechanisms of clearance of inhaled drugs from the human respiratory tract are via mucociliary clearance (MC) and phagocytosis by alveolar macrophages. Mucociliary clearance predominantly occurs in the conducting airways where ciliated epithelium cells and mucus are present [64]. Mucus is a mixture of secretions from the secretory cells (mucus glands, goblet cells, epithelial cells) and submucosal glands and forms a non-aqueous or gel-phase on top of the aqueous layer of ELF [20, 38, 64, 65]. In healthy individuals, mucus (and any insoluble drug particles trapped in mucus) is cleared from the lungs via the rapid stroking movement of cilia present on ciliated lung epithelium cells which facilitate the transport of mucus in a proximal direction into the trachea and is swallowed into the gastrointestinal tract or expectorated [20, 22, 38]. The flow rate of mucus increases from the respiratory airways to the conducting airways [64, 66]. In the alveolar epithelium, the main mechanism of clearance of slowly dissolving drugs is via internalisation by alveolar macrophages [22, 38]. Following uptake, the drug laden macrophage is slowly transported to the conducting airways via the

ELF and/or undergoes internal enzymatic degradation and/or is drained into the lymphatic system [20, 38, 67]. In some cases, engulfed drug particles within alveolar macrophages can reside in the lungs for many years [25].

1.3.2.5 Summary

Section 1.3 has provided an insight into the lung physiology and the absorption, distribution, metabolism and elimination processes in the respiratory tract that may influence the pharmacokinetics of antibiotics in the lungs following pulmonary delivery. Section 1.4 describes the pharmacokinetics in the lungs and systemic circulation following pulmonary administration of the commonly used antibiotics in CF and critically-ill patients. Such information will enable a more comprehensive analysis of the kinetics of CMS and formed colistin following CMS delivery via the pulmonary route.

1.4 Inhaled antibiotics

For the management of respiratory infections in cystic fibrosis (CF) patients a number of antibiotics have been approved for use, or are in the development pipeline, for delivery via the pulmonary route [12]. The two most commonly used inhaled antibiotics in CF patients are CMS and tobramycin, and in the last 10 years inhaled tobramycin as a dry powder formulation and nebulised aztreonam have been licensed for use in this patient population [12]. In more recent times, there has been an increase in use of inhaled polymyxin B [68] and CMS [7, 17] in critically-ill patients with ventilator-associated pneumonia (VAP).

1.4.1 Tobramycin

Tobramycin, formulated as tobramycin solution for inhalation (TSI, TOBI®, Novartis Pharmaceuticals Corp., New Jersey, USA; 300 mg of tobramycin per 5 mL ampoule [69]) is approved in the United Kingdom (UK) and United States of America (USA) for the treatment of early colonisation and chronic infection stage of *P. aeruginosa* lung infections in CF

patients [12]. For many years, nebulised tobramycin has had a pivotal role in the management of CF lung infection however the main disadvantage of the solution formulation is poor patient adherence due to the lengthy nebulisation time, multiple daily dosing and the inconvenience of cleaning/disinfecting of the nebuliser device. Therefore a novel dry powder formulation of tobramycin, tobramycin inhalation powder (TOBI PODHALER, Novartis Pharmaceuticals Canada Inc., Quebec, Canada; 28 mg of tobramycin per capsule, four capsules per dose [70]) delivered via a dry powder inhaler, Podhaler, has been approved in the UK (in 2011) and in the USA (in 2013) for use in CF patients with chronic *P. aeruginosa* lung infections [70].

Tobramycin, as the solution formulation, was the first inhalational antibiotic to be used in CF patients and therefore several studies have reported on the lung exposure [71] and lung *versus* systemic exposure [72-78] following nebulised delivery. In these studies, the majority of patients are greater than 6 years of age, although two studies covered an age range of 6 months – 6 years and included nebulised tobramycin dosing regimens varying from a single dose of 30 mg, 60 mg, 80 mg, 90 mg, 180 mg, 300 mg or 600 mg to the current clinical dosing regimen of 300 mg twice a day for 28 days [71-78]. Following nebulised delivery, maximum tobramycin concentrations in sputum are achieved at 0.2 – 2 h after a single dose and under steady-state conditions [73, 78, 79], with the observed variability likely to be due to the use of different nebuliser devices, patient populations and breathing techniques. Following nebulisation of 300 mg of tobramycin, Geller and colleagues, reported a terminal half-life for tobramycin in sputum of approximately 2 h [78, 79]. One of the major findings from all of these studies is that the pulmonary exposure (sputum, ELF, bronchoalveolar (BAL) fluid) is greater than the respective systemic exposure and that tobramycin concentrations in the lungs are maintained above the minimum inhibitory concentration (MIC) of the respective pathogenic microorganism [72-79]. The large variability in tobramycin sputum concentrations

observed in the studies are suggested to be due to the expectoration of sputum from different regions of the conducting airways and the complexities associated with aerosol drug delivery [71, 73, 74, 78].

Following nebulisation of tobramycin, maximum serum concentrations are observed $0.5 - 2$ h post-dose [73, 74, 76, 78-80]. The terminal half-life of tobramycin in plasma following inhaled delivery ranged from $2.5 - 4.5$ h [78, 79] and is broadly consistent with the terminal half-life in plasma after IV delivery ($1.4 - 2.9$ h) [81]. The exception to this is a study by Touw *et al* who reported a relatively longer terminal half-life (13.0 ± 5.2 h, n=6) and proposed the slower systemic clearance of tobramycin following nebulisation is due to a delay in absorption of tobramycin from the lungs [80]. A low systemic bioavailability for tobramycin of $17.5 \pm 8.8\%$ [80] and 11.5% [74] are reported following inhalation of tobramycin. Slight variations in the serum exposure of tobramycin are observed amongst the different studies which is likely to be a function of the actual dose deposited in the lungs, efficiency of the different nebuliser devices, the use of additional attachments such as spacers and the inhalation technique. Additionally, the recruitment of CF patients with varying degrees of CF lung infection (different extent of lung function deterioration, sputum production and inflammation) can directly affect the systemic bioavailability of inhaled tobramycin.

In the literature there are conflicting reports on whether the kinetics of tobramycin is influenced by accumulation or retention in the lungs. With the exception of a small number of studies [71, 74], most reports indicate accumulation of tobramycin in the lungs (sputum, ELF) and serum following chronic inhalational therapy [73, 75]. However, whether this accumulation is due to binding of tobramycin to lung tissue or due to the extent of CF lung infection is unclear. The longer terminal half-life of tobramycin (13.0 ± 5.2 h) in serum following nebulisation in the Touw *et al* study is suggested to be due to a slower release of

tobramycin from the lungs due to formation of a depot of antibiotic in the lungs [80]. While other studies have indicated that the slower clearance from the lungs in specific patients populations such as in young children (< 6 years of age) is as a result of the extent of CF lung infection (i.e. reduce cough clearance, decrease in central airway purulent secretions and an increase in drug deposition in the distal airways and subsequently binding to macromolecules) [75]. With such conflicting reports in the current literature more investigations to determine the kinetics of tobramycin in the lungs are needed.

Since the development of tobramycin dry powder inhalation formulation, limited pharmacokinetic studies have been conducted to define the pharmacokinetics of tobramycin in the lungs and in serum. One of the main reasons for formulating tobramycin into a dry powder formulation was to increase patient compliance by minimising the time needed for dosing and reducing the complexity of devices needed for drug administration [82, 83], but the pharmacokinetics of tobramycin needed to remain comparable to that of the solution formulation [79]. Geller and colleagues conducted a study to investigate this, with a range of tobramycin doses (28 mg, 56 mg, 84 mg and 112 mg) administered via dry powder inhalation and nebulisation of a control dose of 300 mg of tobramycin solution [79]. The kinetics of tobramycin following absorption into serum are similar for both formulations – maximal serum concentrations (T_{max}) are observed at 0.5 – 2 h and the terminal half-life ranged from 1.7 to 4.3 h [79]. The T_{max} and terminal half-life are consistent with that reported in another study following inhalation of tobramycin dry powder formulation (tobramycin PulmoSphere formulation) [83]. Similarly, the sputum kinetics for both the dry powder and solution formulations are comparable with peak concentrations observed at 0.5 – 2 h and terminal half-life of 0.1 – 3.9 h [79]. Between patients the variability in the sputum concentrations is high [79], with possible reasons for such observations previously mentioned in this discussion. The authors concluded that the major difference between the dry powder and solution formulation

of tobramycin is that the former is a more efficient and a rapid technique for inhalational delivery [79].

1.4.2 Aztreonam

Aztreonam a synthetic mono-cyclic β-lactam (monobactam) formulated as aztreonam lysinate (Cayston, Gilead Sciences) is approved in the UK and USA for delivery via the pulmonary route for the management of chronic *P. aeruginosa* respiratory infections in CF patients [12, 84, 85]. Several studies have been conducted to characterise the pharmacokinetics of aztreonam in the lungs and plasma following nebulised delivery. Children, adolescent and adult patients with documented diagnosis of CF were recruited for the different studies [85-87]. The study designs consisted of a dose-escalating study where a single dose of 75 mg, 150 mg and 225 mg are nebulised [85] and a single 75 mg dose administered via nebulisation two to three times a day for a 4 week treatment period [86, 87]. Multiple sputum and plasma sample are collected only in the dose-escalating study [85]. Following nebulisation of aztreonam, maximum sputum concentrations are achieved 0.16 h post-dose which corresponds to the initial sampling time [85-87]. Subsequently a decline in sputum concentrations over the 4 h sampling period in the dose-escalating studies is reported by Gibson and colleagues [85]. In the same study, a proportional increase in sputum concentrations with dose is evident in the adult cohort (>18 years), but not in the adolescent cohort [85]. Adolescent patients had relatively low sputum concentrations of aztreonam when compared to adults, with the authors suggesting that this may be due to an increase in clearance from the lungs, less deposition of the antibiotic in the central airways and potential increase in salivary dilution [85]. The major outcome from these studies is that following inhalation delivery of aztreonam, the sputum concentrations are higher than the respective plasma concentrations [85-87]. In the Gibson *et al* study, sputum concentrations of aztreonam are maintained above the MIC_{50} (minimum concentration inhibiting 50% of the bacteria

isolates) for *P. aeruginosa* for at least 4 h post-dose following administration of all inhalational doses [85].

Following nebulisation, aztreonam is rapidly absorbed into the systemic circulation with peak concentrations observed at 1 h post-dose [85-87] with drug concentrations in plasma reported until 8 h in the Gibson *et al* study [85]. The plasma terminal half-life of 2.1 ± 0.32 h is comparable to that following IV administration of aztreonam in healthy volunteers (1.9 ± 0.27 h) which indicates that absorption is not a rate-limiting step in the clearance of aztreonam from plasma following inhalation [85, 88]. Accumulation in sputum and plasma are not observed following chronic aztreonam therapy for 28 days [84, 86, 87].

1.4.3 Polymyxins

Over the last 20 years, colistin (also known as polymyxin E) and polymyxin B has been administered via the pulmonary route for the treatment of multidrug-resistant (MDR) Gram-negative respiratory infections. The inactive prodrug of colistin, CMS, is administered via the pulmonary route in CF and critically-ill patients with VAP, discussed in more detail in Section 1.5, while polymyxin B is administered in critically-ill patients with VAP [68, 89-91]. Inhaled polymyxin B is used in combination with the IV form of this antibiotic for effective therapeutic outcomes in this patient population [68, 89]. Despite the clinical use of polymyxin B there has been no published studies (pre-clinical or clinical) investigating the pharmacokinetic of polymyxin B in the lung and systemic circulation following pulmonary delivery [90-92]. The limited knowledge of the pulmonary and systemic pharmacokinetics of polymyxin B is consistent with that of CMS and formed colistin following inhalation of CMS, as discussed in more detail in Section 1.5. If the therapeutic efficacy of CMS and polymyxin B against MDR Gram-negative bacteria is to be preserved, investigations into kinetics of these antibiotics are urgently needed. Therefore the main focus of the current thesis was to

increase the knowledge of the pharmacokinetics of colistin and CMS in the lungs following inhaled administration of both compounds.

1.4.4 Inhaled antibiotics in development

Several other inhaled antibiotics for use in CF patients are at different stages of the drug development pipeline. Levofloxacin and liposomal amikacin (Arikace, Insmed) are currently undergoing Phase III clinical trials, while liposomal ciprofloxacin and fosfomycin/tobramycin combination therapy are undergoing Phase II clinical trials [12].

1.5 Colistin

1.5.1 Background

Colistin produced by *Bacillus polymyxa* subspecies *colitinus* was discovered in 1949 and available for treatment of infections caused by Gram-negative bacteria since the 1950s [93-95]. Despite therapeutic efficacy, the use of colistin and CMS was associated with an increase incidence of adverse effects (AEs) such as nephrotoxicity and neurotoxicity, and for this reason in the early 1970s was replaced by other safer antibiotics such as the aminoglycosides [94, 96]. However, in the late 1980s to early 1990s there was a resurgence in the use of CMS due to an escalating prevalence of infections caused by multidrug-resistant (MDR) Gram-negative bacteria *P. aeruginosa*, *A. baumannii* and *Klebsiella pneumoniae* which are showing resistance to most commercially available antibiotics [93, 94, 97-99]. Management of these 'super bugs' with newer antibiotics is not a viable option as few antibiotics are in the drug development pipeline as a consequence of industry consolidation, challenging regulatory guidelines and an increase in research and development (R&D) costs [100-102]. This has been highlighted in the USA over a 20 year period from 1983 to 2002 with a drastic decrease in the newly approved antibiotics by the Food and Drug Administration (FDA) regulatory body; from 1998 – 2002 approximately seven new antibiotic agents were approved compared

to 16 agents in the years ranging from 1983 – 1987 [100]. Therefore, as mentioned previously, clinicians are having to reappraise the clinical value of colistin which maintains significant *in vitro* activity against these MDR Gram-negative bacteria [94]. Currently, colistin is used as one of the last lines of defence against MDR Gram-negative bacterial infections in particular for respiratory infections in cystic fibrosis (CF) patients and in critically-ill patients with ventilator-associated pneumonia (VAP).

1.5.2 Chemistry and physicochemical properties

Colistin, also known as polymyxin E, belongs to the polymyxin class of antibiotics [94]. Of the polymyxin antibiotics (polymyxin A, B, C, D and E), colistin and polymyxin B are in clinical use [99]. Colistin is a cationic lipopeptide antibiotic comprised of at least thirty different components, of which, thirteen have been identified [103, 104]. Colistin A (polymyxin E_1) and colistin B (polymyxin E_2) are the main components (> 80% of the total components) [104-106]. The minor components include norvaline-polymyxin E_1, valine-polymyxin E_1 [103], polymyxin E_3, polymyxin E_4 [107], valine-polymyxin E_2, isoleucine-polymyxin E_1, isoleucine-polymyxin E_2 [108], polymyxin E_7 and isoleucine-polymyxin E_8 [105]. The proportion of colistin A and B in commercially available products vary between different manufactures [106].

The structure of colistin consists of a cyclic heptapeptide ring with a tripeptide side chain covalently bound at the N-terminus to a fatty acid as shown in Figure 1-2 [94, 95, 104, 109]. The polypeptide contains a mixture of D- and L- amino acids comprised of threonine (Thr), leucine (Leu) and α,γ-diaminobutyric acid (Dab) (Figure 1-2) [93-95]. Free γ-amino groups are bound to five Dab residues on the polypeptide structure (Figure 1-2) and at physiological pH colistin has cationic characteristics due to the protonation of these amino groups [94]. Colistin A and B differ in the composition of the fatty acid side chain, with colistin A and B

containing 6-methyloctanoic acid and 6-methylheptanoic acid, respectively (Figure 1-2) [95, 104].
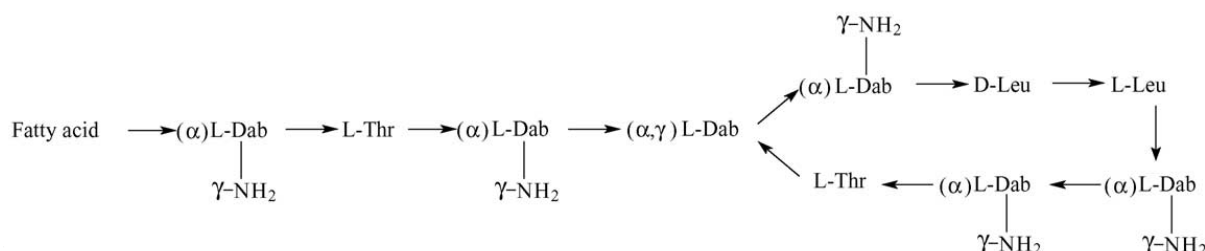
Figure 1-2: Chemical structure of colistin. Fatty acid: 6-methyloctanoic acid for colistin A and 6-methylheptanoic acid for colistin B. (Adapted from Li *et al*., 2005) [94]).

Colistin is amphipathic due to the fatty acid side chain and five free γ-amino groups which gives colistin both hydrophobic and basic characteristics, respectively; this enables colistin to distribute into polar and non-polar environments in the body i.e. water, blood and eukaryotic, prokaryotic lipid membranes [94]. Colistin is resistant to pepsin (pH range of 2.2 − 4.8), trypsin (pH 4.4 − 7.5), pancreatin (pH 4.4 − 7.5), erepsin (pH 6.1 − 7.8) and is inactivated by lipase [94]. Colistin (sulphate) is stable in aqueous solutions below pH 6 with degradation occurring *via* (pseudo) first order kinetics at neutral and basic pH conditions [110, 111]. The molecular weight (M_w) of colistin is 1,163 Dalton (Da), calculated from the average individual M_w of colistin A (1,170 Da) and colistin B (1,156 Da) [110]. Colistin A and B have partition coefficients (Log P) of -3.15 and -3.68, respectively and a polar surface area (PSA) of 490 Å for both components (predicted by ACD LogD Version 9, Toronto, Canada). The M_w of the commercially available form of colistin, colistin sulphate is 1,403 Da [110].

The inactive prodrug of colistin, CMS was introduced onto the market due to high incidence of colistin induced nephrotoxicity, neurotoxicity and discomfort at the injection site [9, 112]. The structure of CMS is shown in Figure 1-3 and differs to colistin by the presence of sulphomethyl groups on the five γ-amino groups.
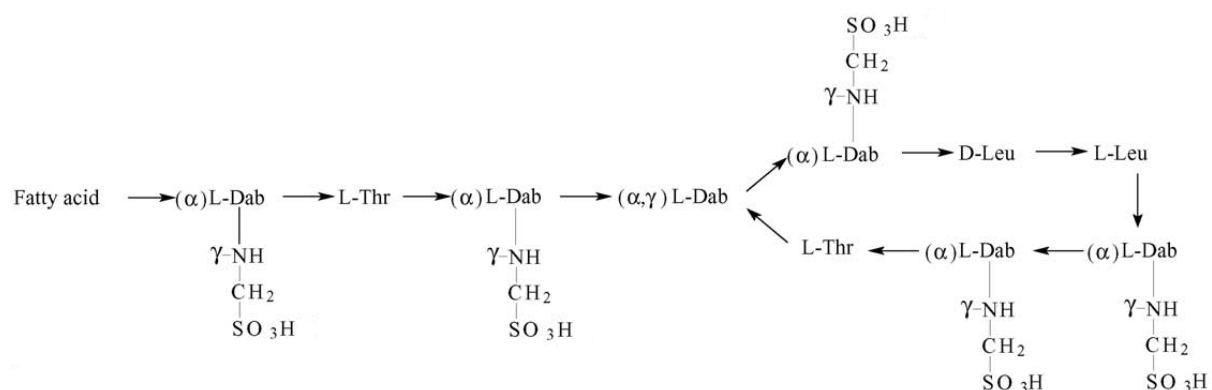
Figure 1-3: Chemical structure of CMS. Fatty acid: 6-methyloctanoic acid for CMS A and 6-methylheptanoic acid for CMS B. (Adapted from Li *et al.*, 2005 [94]).

Formation of CMS involves reaction of colistin with formaldehyde followed by sodium bisulfite (depicted by Equation 1.1 and 1.2), a process known as sulphomethylation, which facilitates the addition of sulphomethyl groups to the five free γ-amino group on colistin (Figure 1-3) [112, 113].

$$R - NH_2 + HCHO \rightarrow R - N = CH_2 + H_2O \qquad\qquad \textbf{Equation 1.1}$$

$$R - N = CH_2 + NaHSO_3^- \rightarrow R - NH - CH_2 - SO_3^- Na^+ \qquad\qquad \textbf{Equation 1.2}$$
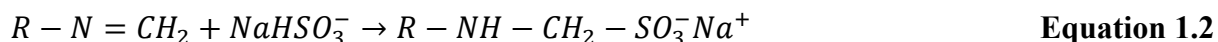
When compared to colistin, the presence of sulphomethyl groups gives CMS anionic characteristics at physiological pH [9]. Sulphomethylation reaction was a process employed as a means of modifying the toxicity of CMS [9, 112, 113]. Loss of antibacterial activity has been demonstrated with sulphomethylation of more than one amino group on polymyxin antibiotics and thereby formation of the parent moiety is required for antibacterial activity [112, 113]. In more recent times, studies by Bergen *et al* have shown that formation of colistin following CMS dosing is a pre-requisite for antibacterial activity [9]. In aqueous solutions (acetate buffer, human plasma and urine), CMS undergoes conversion to form a complex mixture of colistin and partially sulphomethylated derivatives, with up to 32 possible products creating a complex mixture [94, 110, 112, 113]. The M_w of CMS is 1,633 Da,

calculated from the average individual M_w of CMS A and B. The Log P values for CMS A and B are -11.82 and -12.35, respectively, and PSA of 734 Å and 748 Å, respectively (predicted by ACD LogD Version 9, Toronto, Canada). The commercially available form of CMS is sodium CMS also known as colistimethate sodium, colistin methanesulfate, pentasodium colistimethanesulfate, colistin sulfomethate sodium and colistin sulfonyl methate [93, 97]. The M_w of sodium CMS is 1,743 Da, calculated from the average individual M_w of sodium CMS A (1,750 Da) and sodium CMS B (1,736 Da) [110].

1.5.3 Clinical indications

1.5.3.1 Colistin

Commercially colistin (sulphate) is available in a range for formulations including Coly-Mycin®S Otic with Neomycin and Hydrocortisone suspension (JHP Pharmaceuticals, Michigan, USA; 3 mg of colistin base activity (as sulphate) per mL [114]) and Colomycin tablet (Forest Laboratories UK Ltd., Kent, UK; 1,500,000 Units of colistin sulphate per tablet [115]) and syrup (Forest Laboratories UK Ltd., Kent, UK; 250,000 Units of colistin sulphate per 5mL [116]). Coly-Mycin®S Otic with Neomycin and Hydrocortisone suspension is indicated for the treatment of superficial bacterial infections of the external auditory canal [114]. Colomycin® tablets and syrup are used for treatment of gastrointestinal infections and for bowel preparation [115, 116].

1.5.3.2 Colistin methanesulphonate

The two most common commercially available parenteral formulations of CMS (sodium) are Colomycin® (Dumex-Alpharma A/S, Copenhagen, Denmark) and Coly-Mycin M Parenteral® (Parkedale Pharmaceuticals, Rochester, USA) [97, 99]. Both products are formulated as CMS dry powder for reconstitution prior to administration [97]. The two products are labelled differently with respect to the content of CMS (sodium) where Colomycin® is presented as

international units (IU) and Coly-Mycin M Parenteral® labelled with colistin base activity (CBA) [97]. For Colomycin® the recommended dose for a patient over 60 kg with normal renal function is 1 – 2 million IU every 8 h, giving a total daily dose of 240 – 480 mg of sodium CMS [97, 117]. For Coly-Mycin M Parenteral® the recommended dose is 2.5 – 5 mg/kg of CBA per day, which is a daily dose of 400 – 800 mg of sodium CMS for a 60 kg patient with normal renal function [97, 118]. The non-uniformity in the labelling of the CMS content in the two products has resulted in inconsistency in the total recommended daily dose, with the manufactures of Coly-Mycin M Parenteral® recommending almost double the daily dose when compared to Colomycin® [97]. This has major implications for therapeutic efficacy, adverse effects and development of resistance. The current problem will only escalate with more generic products of CMS (sodium) being manufactured, non-uniformity in the labelling of CMS (sodium) content and publication of clinical studies which lack crucial information such as brand names and dosing units.

Colistin methanesulphonate has been administered via the IV route to effectively treat a variety of Gram-negative infections such as bacteraemia [18, 19, 119-123], urinary tract infections [18, 121-123], wound and surgical site infections [18, 19, 121, 122] and meningitis [18, 122-125]. Over the past two decades, CMS has been extensively used via the pulmonary and IV route the treatment of respiratory infections in CF patients and critically-ill patients with VAP. To ensure consistency, dosing regimens have been described using CBA units when referring to IV dosing and IU units when referring to inhalation dosing. For parenteral administration, manufacturers of Colomycin® recommends 1.50 – 2.25 mg/kg of CBA daily in three divided doses for children and adults weighing up to 60 kg with normal renal function [117]. The recommended dose for adults weighing more than 60 kg with normal renal function is 30 – 60 mg of CBA in three divided doses, with a maximum daily dose of 180 mg CBA [117]. Manufacturers of Coly-Mycin M Parenteral® recommends a dose of 2.5 – 5

mg/kg of CBA daily in 2 to 4 divided doses for patients with normal renal function [118]. Despite manufacturers recommending a maximum daily dose [117, 118], doses as high as 270 mg of CBA per day given every 8 h have been reported [7, 121, 123, 126]. The manufacturers recommend modification of the daily dose with renal impairment and the use of ideal body weight in obese patients [117, 118].

For nebulised delivery, CMS (sodium) dry powder formulations for reconstitution has been marketed in Australia (Tadim®, Phebra Pty Ltd, NSW, Australia; 1 million IU per vial [127]) and in UK (Promixin®, Profile Pharma Ltd, West Sussex, UK; 1 million IU per vial [128]). Prior to this, parenteral formulations were used for nebulisation. In CF patients the inhaled CMS dosage regimens varies depending on the progression of *P. aeruginosa* respiratory infection. At the initial colonisation stage, a three week course of 1 – 2 million IU of CMS administered twice to three times a day with oral ciprofloxacin is recommended [10, 127]. At the intermittent infection stage, the inhaled CMS dose is increased to 2 million IU three times a day in combination with oral ciprofloxacin for a period of three months [10, 127]. Once chronic colonisation has been established, long-term therapy with an inhaled CMS dose of 1 – 2 million IU twice a day is recommended [3, 127]. For the treatment of VAP in critically-ill patients, the inhaled CMS dosing regimens vary between hospitals from 1 million IU daily [16, 17] to 0.5 – 2 million IU every 8 h [6, 7, 129]. More recently, a dry powder inhalation formulation of CMS (sodium) (Colobreathe, Forest Laboratories UK Ltd, Dartford, UK; 1.6625 million IU (125 mg) micronised CMS (sodium) per capsule) has been approved in the UK for treatment of chronic *P. aeruginosa* lung infections [12, 130]. Schuster *et al* conducted a safety and efficacy comparative study of CMS dry powder inhalation formulation (1.6625 million IU twice a day, for 24 weeks) and tobramycin inhalation solution (300 mg twice a day, three-28 day cycles for 24 weeks) in CF patients greater the 6 years of age with chronic

P. aeruginosa infection, and reported that the CMS treatment was non-inferior to the tobramycin treatment [130].

1.5.4 Mechanism of action

Investigations into the mechanism of antibacterial activity for the polymyxin antibiotics have been conducted with polymyxin B, which is considered to be the 'model' polymyxin antibiotic [94]. Due to the similarities in the chemical structure of polymyxin B to colistin (a D-phenylalanine residue instead of a D-leucine residue on the heptapeptide ring for polymyxin B [91]) the mechanism of antibacterial activity of colistin is thought to be identical to the model compound [94, 97-99, 131]. Colistin demonstrates rapid concentration-dependent bacterial killing [132, 133] against Gram-negative bacteria, however there is limited information about the mechanism of antibacterial activity at a molecular level [98, 112, 134].

Polymyxin B interacts with both the outer and cytoplasmic membrane of Gram-negative bacteria [135, 136]. The structural features of polymyxin B, fatty acid side chain, positively charge amine groups (at physiological pH) and ring structure are critical for interaction with the membrane constituents of bacteria [98, 135, 137, 138]; thereby the anionic nature of CMS at physiological pH may result in the absence of antibacterial activity [9]. The first step is the 'self-promoted uptake pathway' (proposed by Hancock *et al*, as a mechanism of interaction of cationic peptides with Gram-negative cell envelope [135]) which is initiated by an electrostatic interaction of cationic polymyxin B with negatively charged lipid A proportion of the acidic lipopolysaccharide (LPS) on the outer layer of Gram-negative bacteria [134, 135, 137, 139, 140]. This interaction results in the competitive displacement of native divalent cations (Mg^{2+} and Ca^{2+}) from negatively charged phosphate groups of the lipid A proportion of LPS [98, 131, 135-137, 140]. The primary role of divalent cations present on LPS is to

stabilise the outer membrane of Gram-negative bacteria by forming ionic bridges between neighbouring LPS phosphate groups [136, 140-142]. This displacement results in disruption of the normal barrier properties of the outer membrane and transient 'cracks' form on the membrane which facilitates the uptake of variety of molecules and polymyxin B itself and thereby appropriately named 'self-promoted uptake pathway' [98, 131, 135, 137, 138, 140, 142]. Following internalisation, the positive charged residues of polymyxin B bind to the negatively charged surface of the cytoplasmic membrane and subsequently insert into the membrane with the hydrophobic residues of the peptide facing the membrane interior and the hydrophilic residues facing outwards to form channels [131, 135, 138]. Formation of channels result in cytoplasmic material being released leading to cell leakage, as shown in electron microscopic studies conducted by Koike *et al*, and eventual cell death [131, 135, 143]. In addition to antibacterial activity, polymyxin B has been shown to have antiendotoxin activity [93, 94, 99, 144, 145]. Following antibiotic treatment for Gram-negative infections, bacteria cell death can result in the release of endotoxin (LPS-protein complex) which can lead to endotoxinaemia [135, 144, 145]. The presence of endotoxins instigates the release of cytokines most commonly tumor necrosis factor (TNF) which is associated with symptoms such as fever, hypertension and endotoxic shock [135, 145]. Unlike other antibiotics, polymyxin B has the ability to bind to LPS and thereby prevent the biological activities of LPS such as release of TNF [135, 139, 144, 145].

1.5.5 Adverse Effects

One of the primary reasons for delivery of CMS via the pulmonary route for the treatment of respiratory infections is to minimise the systemic exposure to CMS and formed colistin. Over the last 50 years, the two most commonly reported systemic AEs following IV administration of CMS have been nephrotoxicity and neurotoxicity, although in comparison to nephrotoxicity, the occurrence of neurotoxicity is low [97, 98, 146]. Administration of colistin

via the IV route was discontinued in the 1970s primarily due to an increase incidence of nephrotoxicity and neurotoxicity, and therefore a detailed discussion of systemic AEs following delivery of colistin has not been included in this section.

Colistin methanesulphonate is preferentially used in clinical settings as it is associated with fewer AEs when compared to colistin. However to the best of our knowledge no studies have been undertaken to determine why such variations in toxicity is evident and to investigate what the toxicity profiles would be if an equivalent colistin and CMS IV dose were administered. Colistin, the active antibacterial moiety is also responsible for the toxic side effects [96, 113], however the precise mechanism/s by which colistin induces nephrotoxicity is not known. Similar to the studies undertaken to determine the mechanism of action (Section 1.5.4), the majority of investigations to determine the mechanism of renal toxicity have been conducted with polymyxin B; however the basic molecular mechanism of both polymyxins are thought to be similar [147]. In urinary bladder epithelium of rabbits, polymyxin B induces an increase in membrane permeability, leading to an increase influx of cations, anions and water and subsequently cell swelling [147-149]. The structure of polymyxin B (fatty acid side tail and positively charged amino groups) play a key role in increasing the membrane conductance which is dependent on the concentration and duration of exposure of polymyxin B [147-149]. More recently, oxidative stress has been identified to have a key role in colistin-induced nephrotoxicity [150-152]. In rats, administration of the antioxidants, melatonin and ascorbic acid, with IV colistin has shown to have a protective effect against colistin-induced nephrotoxicity [150, 151].

In pre-clinical [151, 153] and clinical [154] studies, nephrotoxicity has been identified to be time- and cumulative-dose dependent and therefore is a dose-limiting AE following IV administration of CMS. Studies conducted by Hartzell *et al* [154], Kwon *et al* [155] and DeRyke *et al* [156] using a validated criteria for classification of CMS-associated

nephrotoxicity have reported toxicity rates of 45%, 53.5% and 33%, respectively following IV CMS delivery. Overall CMS-induced nephrotoxicity is mostly mild and reversible (following cessation of therapy) however it is a common and serious dose-limiting AE [93, 97, 99, 146].

In comparison to nephrotoxicity, colistin-induced neurotoxicity following IV CMS administration is rare [93, 97, 146, 157]. Interactions of colistin with high lipid containing neurons are associated with neurotoxicity symptoms such as paresthesia, visual disturbance, mental confusion, ataxia, seizures, myasthenia-like syndrome and respiratory muscle paralysis [93, 96, 146, 157, 158]. Impaired renal function, hypoxia, co-administration of certain medications (muscle relaxants, narcotics, sedatives) can increase the risk of neurotoxicity [146, 157].

Following pulmonary administration of colistin and CMS the most commonly reported AEs are bronchoconstriction (chest tightness), cough and transient decrease in lung function [159-163]. The studies that have evaluated tolerability following nebulised and dry powder inhalation of colistin and CMS in healthy volunteers and CF patients are summarised in Table 1-1. Overall these findings have shown that CMS is associated with fewer and a reduction in the severity of lung AEs when compared to colistin (Table 1-1). Studies conducted by Cunningham *et al* [163] and Maddsion *et al* [162] in CF patients have shown that in the majority of patients maximal bronchoconstriction occurred immediately after nebulisation of CMS. Premedication with bronchodilators such as salbutamol prior to CMS nebulisation is common practice in clinical use to prevent and/or reduce the occurrence of lung AEs [160, 161, 163, 164]. In non-CF patients, following inhalational of CMS there has been occasional reports of hypersensitivity pneumonitis [164] and neuromuscular toxicity (manifesting as apnea and respiratory failure) [165].

Table 1-1: Tolerability following nebulised and dry powder inhalation of colistin (sulphate) and CMS (sodium) to healthy volunteers and CF patients.

Drug	Formulation, Patient group	Lung adverse effects	Ref
Colistin	Dry powder inhalation of colistin to CF patients and healthy volunteers.	CF patients: Changes to lung function test (LFT), moderate-severe cough in some patients. Healthy volunteers: No significant changes to LFT.	[160]
CMS	Nebulisation of CMS to CF patients.	No significant changes to LFT.	
Colistin	Nebulisation of colistin to CF patients.	Decrease in LFT, throat irritation, severe cough (followed by perspiration and sensation of heat), chest tightness, increase in mucus production. Majority of patients (7/9) could not complete nebulisation.	[159]
CMS	Nebulisation of CMS to CF patients.	In comparison, a decrease in LFT in few patients (2/9). One patient complained of chest tightness.	
CMS	Dry powder inhalation of CMS to healthy volunteers.	No significant changes to LFT and no reports of lung AEs.	[166]
CMS	Dry powder inhalation of CMS to CF patients. Nebulisation of CMS to CF patients.	Overall both formulations were well tolerated by patients. Changes to the LFT were minimal in the dry powder inhalation group.	[167]

In the literature there is limited knowledge about whether the colistin moiety and/or the nebulised formulation (pH and tonicity) results in the observed lung AEs following pulmonary administration [159-161]. For nebulised dosing solutions, a relationship between the tonicity (hypertonic, hypotonic, isotonic) and adverse effect is evident, with an increase in tonicity resulting in a greater decline in lung function measurements [168]. This is attributed to changes in the osmotic load around mast cells resulting in histamine release following administration of non-isotonic solutions [168]. However studies by Dodd *et al* illustrated that bronchoconstriction occurred for all three tonicity solutions of CMS, with the most rapid

onset of maximal reduction in lung function occurring for the hypertonic followed by the isotonic and hypotonic solutions; this therefore eliminates tonicity as a causative factor [163, 168]. The pH for all solutions were similar and preservative free thereby elimination these factors as a cause of lung AEs [168]. In i*n vitro* studies, colistin has been shown to cause mast cell degeneration which may provide an explanation for bronchoconstriction and the resultant decrease in lung function [160, 167-169].

In the literature, not many studies have reported on the systemic AEs following inhalation of colistin and CMS [129, 159-162, 166, 167, 170, 171]. In 2011, Nakwan *et al* investigating the safety of nebulised CMS (~4.5 million IU) in VAP neonates, reported that none of the eight neonates experienced systemic or lung AEs [171].

1.5.6 Resistance

Over the last 50 years, the sparse use of colistin and CMS has limited the opportunity for the development of resistant bacterial strains to the polymyxin antibiotics [94, 172]. Nevertheless, there are several reports emerging of colistin-resistant Gram-negative bacteria in certain sub-populations of patients [173-175]. Two cases have been reported between the years 2002 to 2006; the first was colistin-resistant *K. pneumoniae* isolates in critically-ill patients hospitalised in the intensive care unit (ICU) in a Greek hospital [173] and the second was colistin-resistant *A. baumannii* isolated (certain subgroups) in hospitalised patients in two Korean hospitals [174]. Despite the increase in use of inhaled CMS in CF patients, the development of colistin-resistant *P. aeruginosa* at the initial bacterial colonisation stage is rare; with such findings most likely due to the low bacterial load in the lungs [2, 176]. In contrast, in the mid-1990s in Danish CF centres outbreaks of colistin-resistant *P. aeruginosa* isolates in patients with chronic lung infections were reported; although the number of resistant cases were still relatively low when compared to the CMS usage [175, 176]. In 2004,

a significant number (approximately 40%) of Danish CF patients who were prescribed inhaled CMS for chronic lung infection demonstrated colistin-resistant for non-mucoid *P. aeruginosa* isolates [175]. This worldwide trend of a gradual increase in resistance is of a great concern as colistin is reserved as one of the last lines of defence against MDR Gram-negative bacteria infections.

Several resistance mechanisms employed by Gram-negative bacteria have been identified; however information about the precise mechanism for colistin resistance is limited. Changes to the Mg^{2+} composition [177-179] and modifications to the LPS molecule [179, 180] are the proposed mechanisms of resistance to colistin. In polymyxin B/colistin-resistant *P. aeruginosa* isolates, an increase in the levels of outer membrane protein known as OprH (or H1) are evident [177, 178]. Protein OprH replaces Mg^{2+} at the LPS binding site on the outer membrane and thereby hinders the binding of polymyxin B and colistin [177-179, 181]. Alterations in the lipid composition of the LPS have also being proposed as a mechanism of *P. aeruginosa* resistance [179, 180]. Whether a single or combination of the above mentioned mechanisms lead to resistance is not fully understood.

The increase in the incidence of bacterial resistant strains for colistin is as a result of the intensified use of CMS in the clinical setting, however parallel to this is the sub-optimal dosing regimens that are administered which are based on limited pharmacokinetic and pharmacodynamics data [182]. Two studies have highlighted this association between the use of colistin and resistance development; 1) Matthiaou *et al* in VAP patients reported that colistin use was independently and strongly associated with colistin-resistance isolates of *K. pneumoniae, A. baumannii* and *P. aeruginosa* [182] and 2) Hawley *et al* reported that the proportion of heteroresistant *A. baumannii* isolates were significantly higher in patients previously treated with colistin when compared to no prior exposure to colistin [183]. In order to minimise resistance development to colistin, there is an urgent need for pharmacokinetic

and pharmacodynamics data following IV and pulmonary administration of CMS to define the optimal route of delivery and to design uniform dosing regimens.

1.5.7 Pharmacokinetics of colistin and colistin methanesulphonate

Despite the increase in use of inhaled CMS in clinical settings there is limited knowledge of the pharmacokinetics of CMS and formed colistin after pulmonary delivery. One of the main reasons for this is that prior to release onto the market in the 1950s, like most drugs, colistin and CMS were not subjected to the drug development procedures that now are mandatory. In more recent years, a limited number of pre-clinical and clinical studies have reported on the plasma pharmacokinetics of CMS and formed colistin following pulmonary delivery of CMS. However, characterisation of the lung pharmacokinetic of CMS and formed colistin following pulmonary delivery is limited, as discussed below. Acknowledging that an understanding of the systemic pharmacokinetics following IV delivery of colistin and CMS is needed to comprehensively analyse the pharmacokinetics in the lungs, a discussion of the pre-clinical and clinical studies following IV colistin and CMS delivery is presented in Appendix I. Additionally, Appendix I highlights the limited number of studies that have been conducted to characterise the lung pharmacokinetics of CMS and formed colistin following IV administration of CMS.

1.5.7.1 Pre-clinical studies

Pulmonary administration of colistin (sulphate)

No pre-clinical studies have been conducted to define the lung pharmacokinetics of colistin following pulmonary administration with such information essential to understand the disposition of formed colistin following administration of the prodrug, CMS.

Pulmonary administration of colistin methanesulphonate (sodium)

A recent pre-clinical study that defined the epithelial lining fluid (ELF) concentrations and systemic pharmacokinetics of CMS and formed colistin following intratracheal (IT) nebulisation of CMS 15 mg/kg in Sprague-Dawley rats was published by Marchand *et al* [184]. This study utilised robust analytical methods (liquid chromatography-mass spectrometry (LC-MS)) to quantify CMS and formed colistin concentrations in the collected biological matrices. Concentrations of CMS and formed colistin in ELF are significantly higher when compared to in plasma [184]. One of the major limitations of the Marchand *et al* study is that the disposition of CMS and formed colistin in ELF are not fully characterised, rather the primary focus is on the systemic exposure following pulmonary CMS delivery. More detailed discussion of this study is included in Chapter 4 (Section 4.8).

The only other pre-clinical studies that have quantified CMS and formed colistin concentrations in the lungs and in systemic circulation following pulmonary *versus* IV administration of CMS has been conducted by Aoki *et al* [144] and Lu *et al* [185]. However both these studies have major limitations, and therefore interpretation of these findings requires caution. In the Aoki *et al* study, following administration of intranasal CMS of 25 mg/kg (expressed as CBA) to MDR *P. aeruginosa* pneumonia infected mice, the lung exposure of CMS (concentrations in lung tissue) is approximately nine-fold higher and systemic exposure undetectable when compared to after IV CMS dose of 250 mg/kg [144]. The terminal half-life of CMS in the lungs (50.01 min) is longer after intranasal delivery when compared to in plasma (27.68 min) after IV delivery, suggesting different disposition in the two biological matrices [144]. The limitation of this study is that the quantification of CMS was undertaken using a microbiological assay and the pharmacokinetics of formed colistin is not reported, which attenuates the significance of the study, as colistin is the active antibacterial moiety [9, 144]. Lu *et al* have reported on the concentrations of formed colistin

in lung tissue and plasma of *P. aeruginosa* pneumonia infected piglets following multiple dosing of IV CMS 3.2 mg/kg (expressed as CMS) and nebulisation of CMS 4.8 mg/kg [185]. Following nebulisation formed colistin concentrations in lung segments ranged from ~2 – 25 µg/g in contrast to undetectable concentrations following IV CMS delivery [185]. The systemic bioavailability of formed colistin is estimated to be 37% [185]. A longer terminal half-life of colistin in plasma following pulmonary delivery (3.2 ± 1.1 h) when compared to after IV delivery (1.7 ± 0.4 h) suggests that absorption is a rate-limiting step in colistin clearance [185]. The limitations of this study is that the estimated pharmacokinetics of formed colistin is likely to be a combination of *in vivo* and *in vitro* formed colistin since the analytical method utilised trichloroacetic acid which can facilitate *in vitro* conversion of CMS [185, 186].

1.5.7.2 Clinical studies

Pulmonary administration of colistin (sulphate)

In the clinical setting only one study conducted by Le Brun *et al*, has define the pharmacokinetics of colistin in plasma following pulmonary delivery of a dry powder formulation of 25 mg of colistin to healthy volunteers and CF patients [160]. The absorption half-life for colistin estimated from the reported rate constants, is less than 10 min in both subject groups, with maximum colistin concentrations (C_{max}) achieved at 6 – 72 min post-dose [160]. The reported C_{max} and systemic exposure is significantly higher in CF patients when compared to in volunteers, despite the authors reporting no significant difference in the terminal half-life and apparent clearance for the two groups [160]. This difference may be due to the relatively small sample size (n= 4-6) in the two subject groups [160]. The authors concluded that different pharmacokinetics for formed colistin is observed in CF patients and healthy volunteers following inhalation of the dry powder formulation of colistin but no further explanations were included [160].

Pulmonary administration of colistin methanesulphonate (sodium)

Following pulmonary administration of CMS, most of the previous clinical studies have reported on the pharmacokinetics of formed colistin in plasma in healthy volunteers and CF patients as presented in Table 1-2. More recently, the pharmacokinetics of formed colistin in the lungs and plasma have been reported by Ratjen *et al* in CF patients [170] and Athanassa *et al* in critically-ill patients [129] following nebulisation of 2 million IU and 1 million IU of CMS, respectively. In both studies, high lung concentrations of formed colistin (in sputum [170] and ELF [129]) are observed relative to plasma following nebulisation of CMS. Maximum formed colistin concentrations in the lungs are observed at 1 h after inhalation and concentrations are maintained above the respective minimum inhibitory concentration (MIC) for the 12 h and 4 h sampling period in the Ratjen *et al* [170] and Athanassa *et al* [129] study, respectively. Following absorption from the lungs, maximum concentrations of formed colistin in plasma are observed at ~1.5 h and 2 h post-dose in the Ratjen *et al* and Athanassa *et al* study, respectively, and concentrations declined thereafter with the terminal half-life consistent between the studies as presented in Table 1-2 [129, 170]. These pharmacokinetic estimates in CF patients are broadly consistent with earlier studies by Westerman *et al* [167] and Le Brun *et al* [160] as shown in Table 1-2. The estimated terminal half-life of formed colistin in plasma following pulmonary delivery in Ratjen *et al* study (Table 1-2) is consistent with the half-life of 4.18 ± 1.32 h in Li *et al* study following IV CMS delivery in CF patients (Appendix I, Table A1-3) [187]. However, an explanation for the shorter half-life of formed colistin in plasma following inhalational delivery (Table 1-2) when compared to after IV CMS administration in critically-ill patients (Appendix I, Table A1-3) is unclear [126, 188, 189]. Despite a two-fold lower nebulised dose administered in the Athanassa *et al* study, the plasma formed colistin concentrations (~0.1 – 2.5 mg/L) are higher than that in Ratjen *et al* study (~0.03 – 0.15 mg/L) [129, 170]. This may be attributed to the use of different nebuliser

devices (vibrating-mesh nebuliser [129] *versus* PARI LC Star jet nebuliser [170]) which can influence the deposition of CMS in different areas of the lungs and determine the rate and extent of absorption of CMS and formed colistin into the systemic circulation. The limitations of both studies are that the CMS kinetics in sputum and plasma are not defined and a more thorough pharmacokinetic analysis of formed colistin in sputum is not reported. Despite this, the studies conducted by both authors are the first to have demonstrated the achievement of therapeutic relevant concentrations of formed colistin in the lungs with relatively low systemic exposure in CF and critically-ill patients [129, 170].

Table 1-2: Pharmacokinetics properties of formed colistin in plasma following inhalational delivery of CMS (sodium) to healthy volunteers, CF and critically-ill patients.

CMS	Subjects	T_{max} (h)	$t_{1/2}$ (h)	Ref
Dry powder	Healthy volunteers	$1.1 \ (0.9-1.2)^{\phi}$	$2.75 \ (2.68-2.82)^{\phi}$	[166]
Nebulised	CF patients	$1.9 \pm 1.2^{\Upsilon}$	$10.4 \pm 3.6^{\Upsilon}$	[160]
Nebulised	CF patients	$1.34 \ (1.23-1.46)^{\phi}$	$3.0 \ (2.7-3.4)^{\phi}$	[167]
Dry powder	CF patients	$0.86 \ (0.80-0.93)^{\phi}$	$3.2 \ (3.1-3.3)^{\phi}$	
Nebulised*	CF patients $^{\ddot{\omega}}$	$1.47 \pm 0.16^{\pounds}$	$4.09 \pm 0.31^{\pounds}$	[170]
Nebulised	Critically-ill patients $^{\rho}$	~2	$2.7 \ (2.5-3.1)^{\S}$	[129]

$^{\phi}$ mean (95% confidence interval).
$^{\Upsilon}$ mean (±S.D.).
$^{\pounds}$ mean (±SEM).
§ mean (25 – 75% interquartile range).
*estimates following administration *via* the PARI LC star nebuliser.
$^{\ddot{\omega}}$ Ratjen *et al* study.
$^{\rho}$ Athanassa *et al* study.

No detailed studies investigating the pharmacokinetics of CMS and formed colistin following administration of the dry powder inhalation formulation of CMS (Colobreathe) have been reported in the literature. A study by Schuster *et al* reported on the sputum, serum and urine concentrations of formed colistin in CF patients collected on three separate occasions (on visits 1, 2 and 6) following pulmonary administration of CMS 1.6225 million IU twice a day for 24 weeks [130]. In the majority of collected sputum samples, formed colistin

concentrations were approximately 20 times the MIC of colistin, while concentrations in serum were just detectable or below the limit of detection (2 mg/L) [130]. In the majority of urine samples (78%) formed colistin concentrations were below 8 mg/L [130]. However these results need to be interpreted with caution as the authors provided no details of the method used to quantify formed colistin concentrations other than to make the statement that a single-reference microbiological assay was utilised which lacked sensitivity and precision [130].

1.5.7.3 Summary

Section 1.5.7 has provided an overview of the studies that have been conducted in the pre-clinical and clinical setting to define the pharmacokinetic of colistin and CMS following pulmonary delivery. It is evident that following pulmonary administration of CMS there is limited pharmacokinetic data for CMS and formed colistin, particularly in the lungs. This review has shown that the key pharmacokinetic information that are paramount for the design of optimal inhaled CMS dosing regimens such as dose-linearity, dosing intervals, exposure of both CMS and formed colistin in the lungs and plasma has not been thoroughly characterised. Furthermore there is very limited pharmacokinetic data available following inhaled administration of the antibacterial active moiety, colistin. Such information is important to enable a more comprehensive understanding of the kinetics of formed colistin following administration of CMS. Therefore further pre-clinical and clinical studies are urgently needed to define the kinetics of colistin and CMS in both the lungs and systemic circulation following delivery of both colistin and CMS via the pulmonary route.

1.6 Summary

Colistin, an old antibiotic is increasingly being used for the management of multidrug-resistant (MDR) Gram-negative respiratory infections in cystic fibrosis (CF) and critically-ill patients. Of great concern however, is despite CMS being administered via the pulmonary and

IV route for the management of these respiratory infections for over 20 years there is still limited pharmacokinetic data in the pre-clinical and clinical setting. Even though inhaled CMS play a key role in the management of these lung infections there are only a handful of pharmacokinetic studies following pulmonary CMS delivery. Of these few studies the targeting benefit that can be achieved by directly delivering into the lungs when compared to systemic delivery of CMS has not been quantified. Limited knowledge on the lung pharmacokinetics was also evident for the other commonly prescribed inhaled antibiotics (tobramycin, aztreonam, polymyxin B) in CF and critically-ill patients. Therefore for CMS and these other antibiotics, more robust studies are needed to add to the existing knowledge and to quantitatively demonstrate whether pulmonary or IV delivery will achieved optimum therapeutic efficacy and reduce adverse effects. Additionally the value of understanding the kinetics of the active antibacterial moiety, colistin, is not highlighted in the current literature with only one clinical study being conducted following colistin delivery.

Despite this, colistin still continues to exhibit antibacterial activity against MDR Gram-negative bacteria, however, reports of resistance are starting to emerge. Therefore if such practices are to continue into the future the likely outcome is a decrease in therapeutic efficacy of colistin and in an era of limited newly approved antibiotics this can have drastic consequences. Therefore this projects aims to investigate the pharmacokinetics of colistin and CMS in plasma and lungs following pulmonary and IV delivery of colistin (sulphate) and CMS (sodium) in pre-clinical and clinical environments. The main objective is to determine whether direct delivery of CMS into the lungs will achieve targeting benefits of high local concentrations and low systemic exposure when compared to IV administration.

1.7 Hypothesis and aims

The principle hypothesis of this thesis is that pulmonary administration of CMS and colistin will result in extensive exposure in the lungs when compared to plasma exposure and achieve targeted delivery when compared to after IV administration.

Specifically, that:

1. Following pulmonary administration of colistin, high colistin concentrations will be observed in the lung epithelial lining fluid (ELF) for an extended duration of time and systemic exposure will be reduced relative to that after IV delivery,

2. Relative to IV CMS administration, pulmonary delivery will result in high CMS and formed colistin ELF concentrations for an extended duration of time with a reduction in the systemic exposure,

3. Slower conversion of CMS to colistin in the lung when compared to in plasma will result in the extensive exposure of formed colistin in ELF, and that

4. Pulmonary delivery of CMS to CF subjects will be well tolerated, and high concentrations of CMS and formed colistin in sputum and a reduction in plasma exposure will be evident relative to after IV CMS administration.

In addressing these hypotheses, the thesis aims to:

1. Examine the pharmacokinetics of colistin following pulmonary and IV administration of colistin to Sprague-Dawley rats (Chapter 3),

2. Examine the pharmacokinetics of CMS and formed colistin following pulmonary and IV administration of CMS to Sprague-Dawley rats (Chapter 4),

3. Characterise the CMS conversion kinetics in the lungs and in plasma, and the absorption kinetics of colistin and CMS from the lungs in Sprague-Dawley rats using a population pharmacokinetic modelling approach (Chapter 5), and

4. Examine the tolerability and pharmacokinetics of CMS and formed colistin following pulmonary and IV administration of CMS to CF subjects (Chapter 6).

Chapter 2: Methods

2.1 Methods

This chapter provides details of the development and validation of methods for quantification of colistin and colistin methanesulphonate (CMS) concentrations in the pharmacokinetic studies undertaken in Chapters 3, 4 and 6. The present chapter is divided into two sections, the first section, Section 2.2 deals with the methods utilised for quantification of colistin and CMS concentrations in plasma and epithelial lining fluid following pharmacokinetic studies in Sprague-Dawley rats, and the second section, Section 2.3 deals with the methods utilised for quantification of colistin and CMS concentrations in plasma, urine and sputum following pharmacokinetic studies in cystic fibrosis (CF) subjects.

2.1.1 Materials

Colistin sulphate and sodium colistin methanesulphonate were purchased from Sigma-Aldrich (Missouri, USA) and Sigma-Aldrich (Brøndby, Denmark), respectively. Boric acid, sodium hydrogen carbonate ($NaHCO_3$), sodium hydroxide (NaOH), sodium dodecyl sulphate ($NaC_{12}H_{25}SO_4$), sulphuric acid (95-98%) (H_2SO_4), trifluoroacetic acid (TFA), 9-fluor-enylmethyl chloroformate (FMOC-Cl) and urea (NH_2CONH_2) were obtained from Sigma-Aldrich (New South Wales, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile (ACN), methanol (MeOH), acetone (ACE) and tetrahydrofuran (THF) were purchased from Merck (Hesse, Germany). Sterile 0.9% sodium chloride was obtained from Baxter Healthcare Pty Ltd (New South Wales, Australia). Sep-Pak® solid-phase extraction (SPE) C_{18} (100 mg) cartridges were purchased from Waters (Leinster, Ireland). Water was purified using a Milli-Q® water purification system from Millipore Corp. (Massachusetts, USA).

2.1.2 Apparatus

High-performance liquid chromatography

Shimadzu HPLC (Kyoto, Japan) was comprised of a CBM-20A controller, LC-20AD pumps, a SIL-20AC auto-injector, a CTO-20A column oven, a RF-10AXL fluorescence detector or SPD-M10A ultraviolet (UV) detector connected to a multi-instrument data acquisition and data processing system (Shimadzu LC Solution, Version 1.24).

Microplate reader

Multiskan EX microplate photometer (Thermo Scientific, Victoria, Australia) was comprised of a 400 – 750 nm wavelength range, 8-position filter wheel, quartz tungsten halogen lamp light source, eight silicon photodetectors, linear shaking (at 3 speeds) and measurement speed of 5 sec for 96-well microplates. The photometer was connected to a data acquisition and processing system (Ascent Software Version 2.6, Thermo Scientific).

2.2 Quantification of colistin and CMS following pharmacokinetic studies in rats

2.2.1 HPLC assay for quantification of colistin concentrations in dosing solutions

Quantification of colistin in normal saline was carried out using a previously developed and validated HPLC assay from our laboratory for quantification of colistin in aqueous solutions.

2.2.1.1 Method

A Phenosphere Next HPLC column (150 mm × 4.6 mm, 5 μm, Phenomenex) was used to separate colistin A and B. Colistin A and B were detected by UV absorbance at 210 nm. Mobile phase A comprised of 0.05% TFA in Milli-Q water and mobile phase B was 100% methanol. The gradient was 30% to 80% B between 0 and 6 min and decreased back to 30% between 6 and 9 min at a flow rate of 1 mL/min. Column temperature was set at 30ºC.

2.2.1.2 Normal saline samples

Preparation of calibration and quality control samples

Two independent stock solutions of colistin 20 mg/mL and 60 mg/mL were prepared in Milli-Q water and used to prepare calibration standards and quality control (QC) samples. Fresh stock solutions were stored at 4ºC and prepared every three months [190].

Colistin calibration standards ranging from 0.16 to 2.5 mg/mL were prepared in normal saline. Quality control samples at four concentration levels were included in all HPLC runs (0.50, 1.0, 2.0 and 6.0 mg/mL). Normal saline was used as the matrix to prepare calibration and QC samples as the dosing solutions for the pharmacokinetic studies in Chapter 3 were prepared in this matrix. Calibration standards and QC samples were prepared by serial dilution with appropriate volumes of normal saline. High QC samples (6.0 mg/mL) were diluted to within calibration curve range with normal saline. Following preparation no additional sample pre-treatment was needed and 25 µL of the aqueous sample was directly injected onto the HPLC column. Colistin A and B eluted at approximately 5.6 min and 5.2 min, respectively, as shown in Figure 2-1. No interfering peaks with colistin A and B were observed.
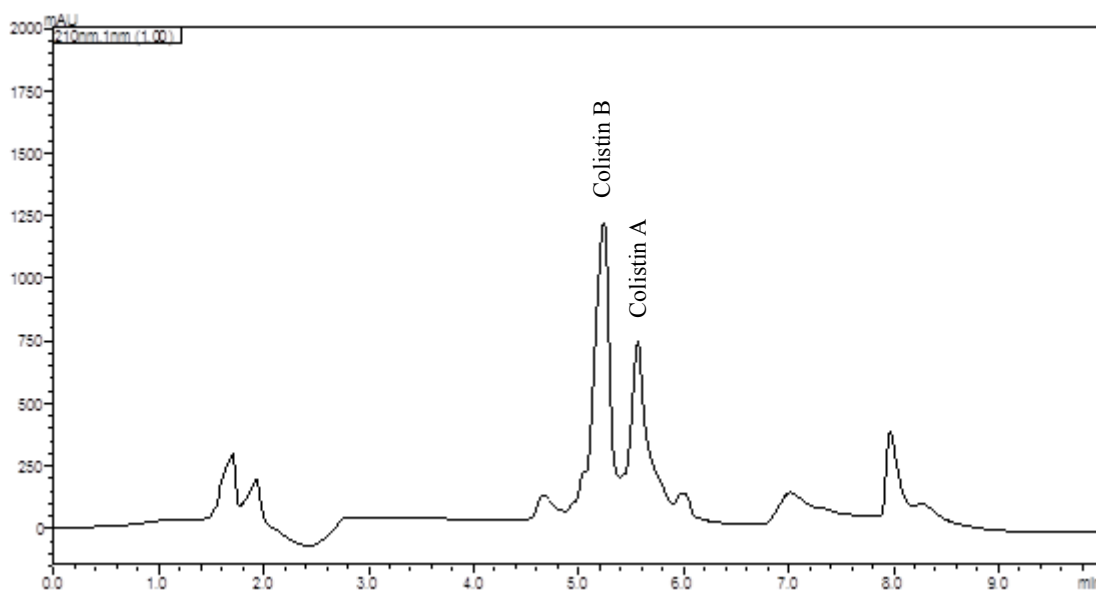
Figure 2-1: A typical UV-HPLC chromatograph of colistin A and B for colistin 2.5 mg/mL spiked in normal saline.

2.2.1.3 Validation

Intra-day (six replicates of each QC sample) and inter-day (three replicates of each QC sample run on three separate days) runs were carried out to assess the accuracy (% deviation from nominal QC concentration) and precision (coefficient of variation) of the assay. The limit of quantification (LOQ) for the assay was determined by assessing the accuracy and precision of six replicates of the lowest calibration standard. Estimation of the accuracy and precision was carried out using Equations 2.1 and 2.2, respectively. Calibration curves were constructed by summing the peak areas of colistin A and B with the nominal concentrations of colistin sulphate. To determine the slope, intercept and r^2, linear least-squares regression analysis of the calibration curves with no weighting of the data was conducted. Colistin concentrations in QC samples were determined by multiplying the colistin sulphate concentrations obtained from the assay by the ratio of molecular weights (M_w) of colistin base (1163 Dalton (Da)) and colistin sulphate (1403 Da) [187]. The performance characteristics of the colistin assay are shown in Table 2-1.

$$Accuracy~(\%) = \frac{mean~estimated~concentration - nominal~concentration}{nominal~concentration} \times 100$$

<div align="right">**Equation 2.1**</div>

$$Precision~(\%) = \frac{standard~deviation~of~estimated~concentration}{mean~estimated~concentration} \times 100$$

<div align="right">**Equation 2.2**</div>

Table 2-1: Intra-day (n=6), inter-day (n=3) and LOQ (n=6) quality control samples for colistin in normal saline.

	Intra-day				Inter-day				LOQ
Colistin (mg/mL)	**0.41**	**0.83**	**1.66**	**4.97**	**0.41**	**0.83**	**1.66**	**4.97**	**0.13**
Mean conc (mg/mL)	0.45	0.86	1.74	5.22	0.44	0.88	1.66	5.08	0.11
Accuracy (%)	8.78	3.57	4.63	5.10	8.04	6.38	-0.24	2.13	-12.4
Precision (%)	1.53	0.89	9.93	0.49	2.50	8.16	2.35	1.12	1.37

2.2.1.4 Estimation of colistin concentrations in dosing solutions

Colistin concentrations in dosing solutions from the pharmacokinetic studies in Chapter 3 were determined as described above for the QC samples, by multiplying the colistin sulphate concentrations obtained from the assay by the ratio of the M_w of colistin base and colistin sulphate, 1163/1403 [187].

2.2.1.5 Summary

The HPLC analytical method for assay of colistin in normal saline was validated and used for the quantification of colistin in dosing solutions (Chapter 3).

2.2.2 HPLC assay for quantification of colistin and CMS concentrations in rat biological samples

Colistin and CMS concentrations in rat plasma and bronchoalveolar lavage (BAL) fluid were quantified using previously developed and validated HPLC assays for quantification of colistin [191] and CMS [192] in biological matrices, with minor modifications as detailed below.

2.2.2.1 Method

An Onyx monolithic C_{18} HPLC column (50 mm × 4.6 mm, Phenomenex) was used to separate fluorescent FMOC derivatives of colistin A and B. Colistin A and B FMOC derivatives were detected at an excitation wavelength of 265 nm and emission wavelength of 315 nm. As detailed in Section 2.2.2.2, the mobile phase composition varied with the use of ACN-THF-water (50:25:25, v/v/v) and MeOH-THF-water (35:39:24, v/v/v) in different assays. This was due to a shortage in supply of ACN in 2008 which resulted in MeOH being used as an alternative organic solvent for preparation of mobile phase. Changes to the composition of the mobile phase resulted in variation of the assay run time as detailed in Section 2.2.2.2. The flow rate was set at 1 mL/min. Column temperature was set at 25ºC.

For each of the assays described below, two independent stock solutions of colistin and CMS were prepared in Milli-Q water and used to prepare calibration standards and QC samples. The stock solutions were stored at 4ºC and prepared every three months [190]. Colistin calibration standards were used to quantify colistin concentrations in the collected biological samples and CMS calibration standards to quantify CMS concentrations in the biological samples.

Quantification of CMS concentrations in biological matrices involved accelerated conversion of CMS to colistin at the sample preparation stage prior to loading onto SPE cartridges [192].

Fluorescent FMOC derivatives of colistin A and B (corresponding to CMS A and B) were detected on the HPLC system. The summated peak areas of colistin A and B were related back to the nominal sodium CMS concentrations for the construction of calibration curve and quantification of CMS concentrations in QC samples.

2.2.2.2 Rat plasma samples

Preparation of calibration and quality control samples

Two independent stock solutions of colistin 1.0 mg/mL and CMS 1.0 mg/mL were prepared in Milli-Q water.

2.2.2.2.1 Colistin

Two different colistin calibration standards in drug-free rat plasma were used to quantify colistin concentrations in rat plasma following pharmacokinetic studies in Chapters 3 and 4. In Chapter 3, calibration standards were prepared in drug-free rat plasma in a concentration range of 0.10 to 4.0 mg/L. Colistin concentrations of 0.19, 0.75, 3.0 and 10 mg/L were incorporated as QC samples. Above the calibration curve QC samples (10 mg/L) were diluted to within calibration curve range with drug-free rat plasma prior to loading onto SPE cartridges. Calibration standards and QC samples were prepared by serial dilution with appropriate volumes of drug-free rat plasma. An aliquot (100 µL) of each calibration and QC sample was mixed with 100 µL of ACN for protein precipitation and centrifuged at 10,621 × *g* for 10 min (Eppendorf centrifuge 5430®). The volume of supernatant transferred onto a SPE cartridge was 170 µL. Following sample pre-treatment as per Section 2.2.2.2.2, 30 µL of supernatant was injected onto the HPLC column. The composition of the mobile phase was ACN-THF-water (50:25:25, v/v/v) and a run time of 8 min. Colistin A and B eluted at approximately 6.6 min and 5.6 min, respectively. No interfering peaks with colistin A and B were observed. In Chapter 4, calibration standards in drug-free rat plasma covered the

concentration range of 0.13 to 4.0 mg/L. Colistin concentrations of 0.25, 1.0 and 4.0 mg/L were used as QC samples. Calibration standard and QC samples were prepared by serial dilution with appropriate volumes of drug-free rat plasma. A 100 µL aliquot of each calibration and QC sample was mixed with 100 µL of ACN, vortex mixed and centrifuged at 10,621 × *g* for 10 min. The volume of supernatant transferred onto a SPE cartridge was 165 µL. Following sample pre-treatment as per Section 2.2.2.2.2, 50 µL of supernatant was injected onto the HPLC column. The composition of the mobile phase was MeOH-THF-water (35:39:24, v/v/v) and a run time of 9 min. Colistin A and B eluted at approximately 6.6 min and 5.6 min, respectively as shown in Figure 2-2. No interfering peaks with colistin A and B were observed.

2.2.2.2.2 Sample pre-treatment

Calibration standards and QC samples were loaded onto SPE cartridges for the formation of fluorescent FMOC derivatives of colistin A and B. The SPE cartridge was pre-conditioned with 1 mL ACE, 1 mL MeOH and 1 mL of 1% w/w $NaHCO_3$ (adjusted to pH of 10 with 10% w/w NaOH) on a vacuum manifold. Specified volumes of supernatant were transferred onto the cartridge and following addition of 1% (w/w) $NaHCO_3$ allowed to slowly drain under a low vacuum pressure. This process facilitated attachment of colistin A and B onto the C_{18} cartridge. The cartridge was further washed with 1 mL of 1% $NaHCO_3$ and allowed to dry for 5 min under high vacuum pressure. An aliquot (110 µL) composed of 30 µL of 100 mM FMOC-Cl in ACN and 80 µL of MeOH was transferred onto the cartridge and allowed 10 min reaction time to enable fluorescent FMOC derivatives of colistin A and B to be formed. The derivatives were eluted out with 900 µL of ACE and collected into tubes containing 600 µL of 0.20 M boric acid and 500 µL of ACN, capped and vortex mixed. Samples were allowed to equilibrate to room temperature for up to 5 h, centrifuged at 3,219 × *g* for 5 min

(Eppendorf centrifuge 5810R®) and the specified supernatant volumes injected onto the

HPLC column.
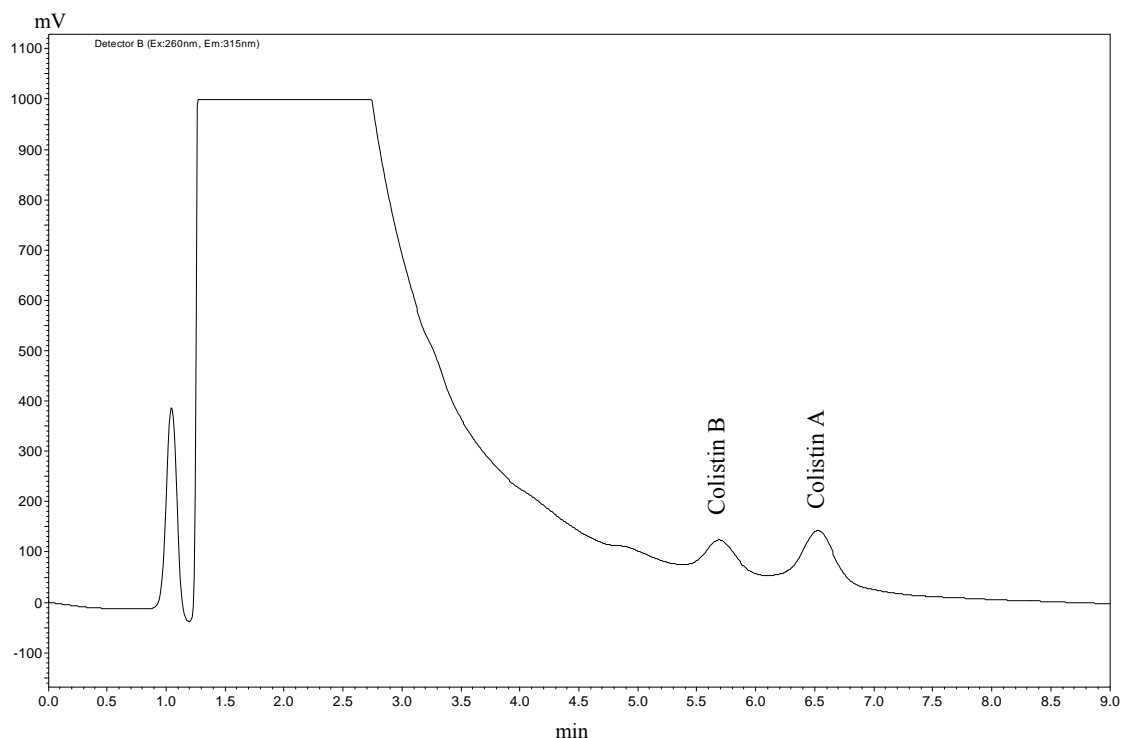
Figure 2-2: A typical HPLC chromatograph of fluorescent derivatives of colistin A and B for colistin 4.0 mg/L spiked in rat plasma.

2.2.2.2.3 Colistin methanesulphonate

CMS calibration standards were prepared in drug-free rat plasma at a concentration range of

0.78 to 50 mg/L with QC sample concentrations of 1.3, 20, 40 and 300 mg/L. Above the

calibration curve QC samples (300 mg/L) were diluted to within calibration curve range with

drug-free rat plasma prior to loading onto the SPE cartridges. Calibration standards and QC

samples were prepared by serial dilution with appropriate volumes of drug-free rat plasma. To

50 μL of calibration and QC samples a 20 μL aliquot of 1.0 M H_2SO_4 was added, vortex

mixed and allowed 10 min reaction time to enable accelerated conversion of CMS to colistin.

The reaction was stopped by mixing 40 μL of 1.0 M NaOH and thereafter protein

precipitation was facilitated by the addition of 110 μL of ACN. Following centrifugation at

$10,621 \times g$ for 10 min, 110 µL of supernatant was transferred onto a SPE cartridge. Following sample pre-treatment as per Section 2.2.2.2.2, 40 µL of supernatant was injected onto the HPLC column. The composition of the mobile phase was MeOH-THF-water (35:39:24, v/v/v) and a run time of 9 min. Colistin A and B (corresponding to CMS A and B, respectively) eluted at approximately 6.4 min and 5.6 min, respectively as shown in Figure 2-3. No interfering peaks with colistin A and B were observed.
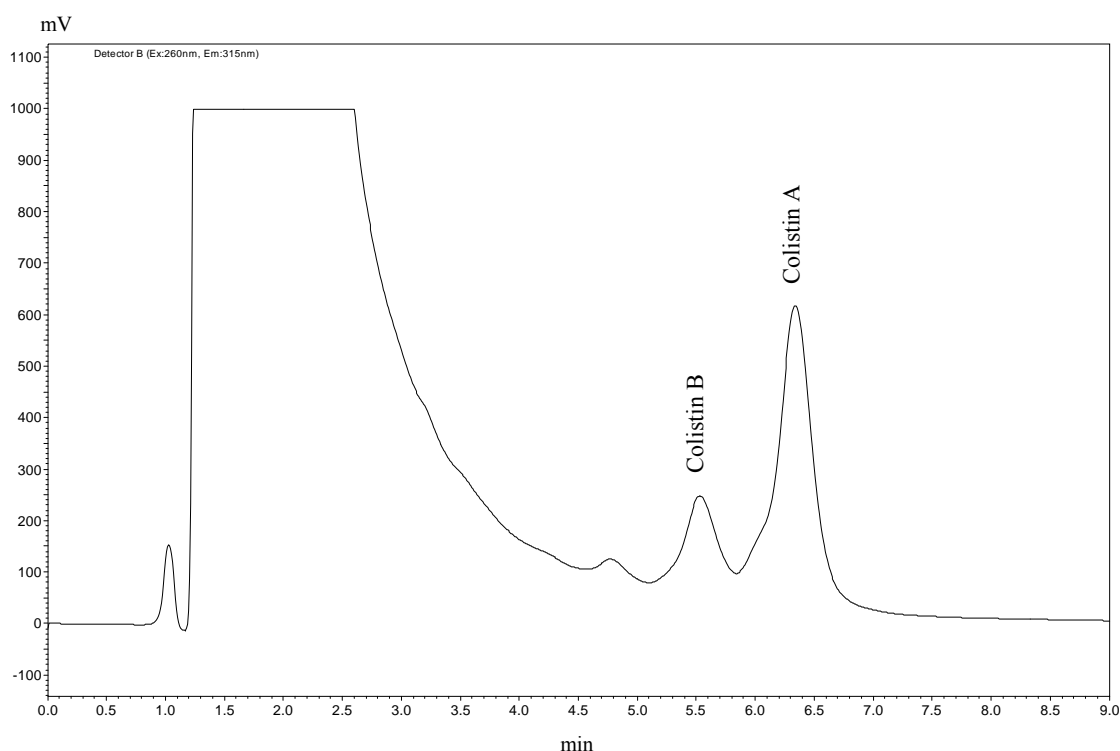
Figure 2-3: A typical HPLC chromatograph of fluorescent derivatives of colistin A and B for CMS 50 mg/L spiked in rat plasma. Note that colistin A and B corresponds to CMS A and B, respectively.

2.2.2.3 Rat bronchoalveolar lavage fluid samples

Bronchoalveolar lavaging (BAL) was a procedure conducted to sample the lung epithelial lining fluid (ELF) and thereby quantify colistin and CMS concentrations in ELF following IV and pulmonary administration. Bronchoalveolar lavaging was undertaken as detailed out in Chapter 3, Section 3.4.3.2. The procedure of BAL involved instillation of a large volume of fluid into the rat lung (15 mL, Chapter 3, Section 3.4.3.2) which resulted in extensive dilution

of the ELF volume and subsequently dilution of the ELF colistin and CMS concentrations [31]. Therefore to determine the concentration of colistin and CMS in ELF, first the concentration in BAL fluid was quantified as outlined below. Secondly, the apparent ELF volume was estimated using urea as an endogenous dilution marker as discussed in Section 2.2.3 [31]. Urea can be used as a endogenous dilution marker as the concentration of urea in ELF and plasma are in equilibrium since urea freely diffuses between the two biological fluids due to the low M_w (60.06 Da), unionised state at physiological pH and not being produced or consumed by respiratory cells [31]. Lastly, the concentration of colistin/CMS in ELF was estimated as discussed in Section 2.2.3.

Preparation of calibration and quality control samples

Two independent stock solutions of colistin 1.0 mg/mL and CMS 1.0 mg/mL were prepared in Milli-Q water. Two colistin and CMS calibration standards were required to encompass the wide range of colistin and CMS concentrations encountered in BAL fluid samples following pulmonary and intravenous (IV) administration.

2.2.2.3.1 Pulmonary administration

Colistin

Colistin calibration standards ranging from 0.13 to 4.0 mg/L were initially prepared in a matrix composed of 100% drug-free BAL fluid. Calibration standards were prepared by serial dilution with drug-free BAL fluid and protein precipitation was facilitated by addition of a 1:1 ratio of ACN. The generated calibration curve showed non-linearity at the lower concentration range of 0.13 to 1.0 mg/L, as shown in Figure 2-4A. Similar non-linearity at the lower concentration range has been observed by other laboratory colleagues following preparation of colistin calibration curves in 100% dialysate fluid. In BAL fluid and dialysate

fluid the source of non-linearity was speculated to be due to adsorption of colistin via non-specific binding to microcentrifuge tubes. In contrast, the absence of non-linearity in calibration standards prepared in rat plasma was thought to be due to colistin binding to plasma proteins and thereby hindrance of the potential non-specific binding of colistin to tubes. To overcome the observed non-linearity, colistin calibration standards ranging from 0.25 to 8.0 mg/L were prepared in a matrix composed of BAL fluid-ACN (50:50, v/v). As shown in Figure 2-4B the inclusion of ACN in the matrix overcame the non-linearity as the generated calibration curve displays a linear relationship between concentration and total peak area.
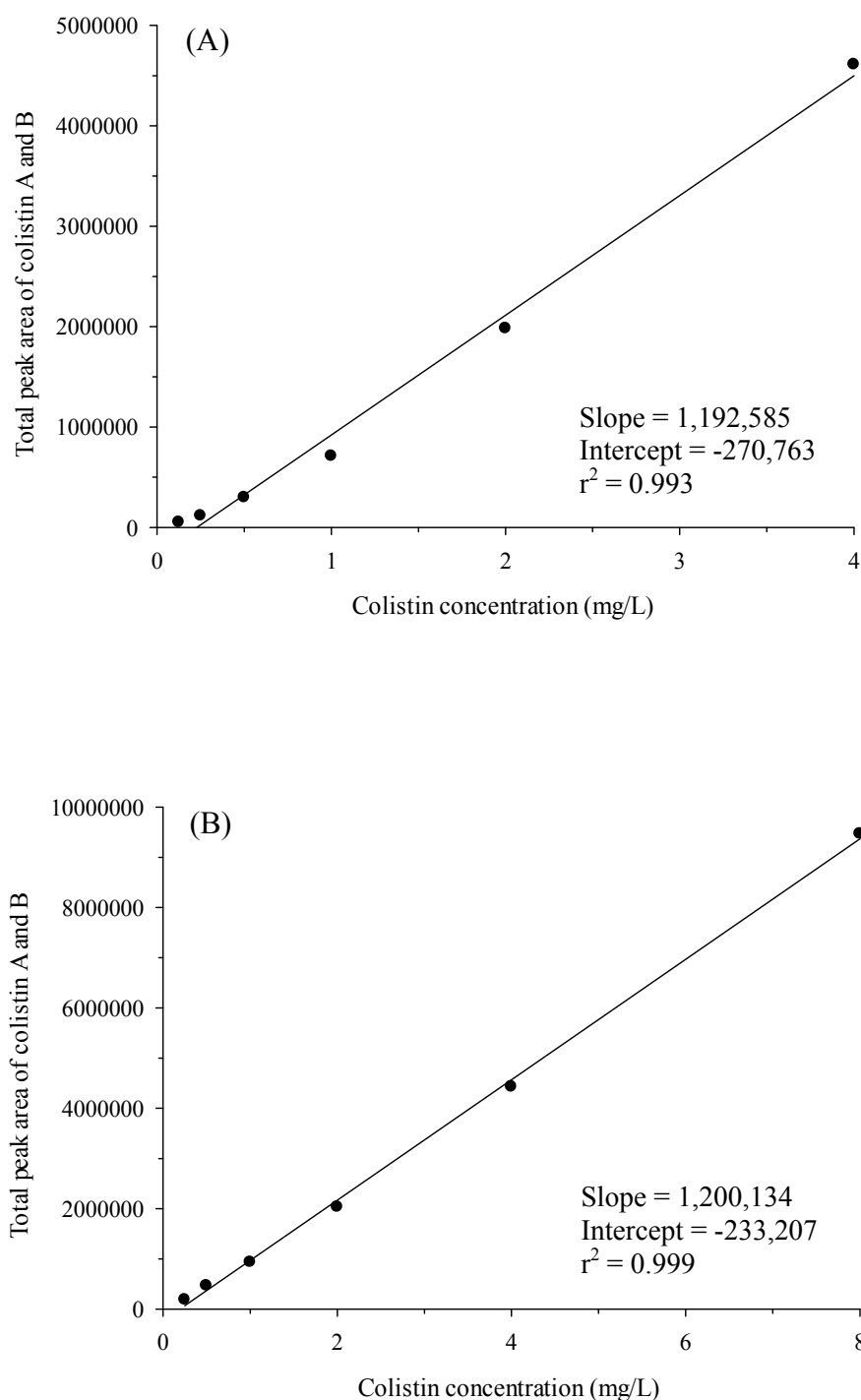
Figure 2-4: A typical calibration curve of colistin prepared in (A) drug-free BAL fluid and (B) BAL fluid-ACN (50:50, v/v). Note that colistin concentrations in the x-axis refer to colistin sulphate concentrations and that panel B has a different concentration range.

For validation, colistin calibration standards ranging from 0.25 to 8.0 mg/L with incorporation

of 0.75, 3.0 and 6.0 mg/L concentrations as QC samples prepared in a matrix composed of

BAL fluid-ACN (50:50, v/v) was used. Calibration standard and QC samples were prepared by serial dilution with appropriate volumes of drug-free BAL fluid-ACN (50:50, v/v). A 1:1 ratio of drug-free BAL fluid-ACN was mixed with 100 µL aliquot of sample and centrifuged at 10,621 × *g* for 10 min. The volume of supernatant transferred onto a SPE cartridge was 180 µL. Following sample pre-treatment as per Section 2.2.2.2.2, 50 µL of supernatant was injected onto the HPLC column. For the BAL fluid samples (100 µL aliquot) collected from the pharmacokinetic studies (Chapters 3 and 4), a 1:1 ratio of 100% ACN was added to ensure that the same composition of BAL fluid-ACN in rat samples to that of calibration and QC samples was achieved. Thereafter the rat BAL fluid samples were processed in the same manner as described above. The composition of the mobile phase was MeOH-THF-water (35:39:24, v/v/v) and a run time of 10 min. Colistin A and B eluted at approximately 6.3 min and 5.3 min, respectively, with the generated HPLC chromatograph similar to that of the colistin plasma assay (Figure 2-2). No interfering peaks with colistin peak A and B were observed.

Colistin methanesulphonate

Similar to the colistin BAL fluid assay, preparation of CMS calibration standards ranging from 0.78 to 50 mg/L in 100% drug-free BAL fluid resulted in non-linearity as shown in Figure 2-5A, although the non-linearity was not as pronounced as for the colistin assay. Preparation of calibration standards in drug-free BAL fluid-ACN (50:50, v/v) improved the linearity of the calibration curve as shown in Figure 2-5B.
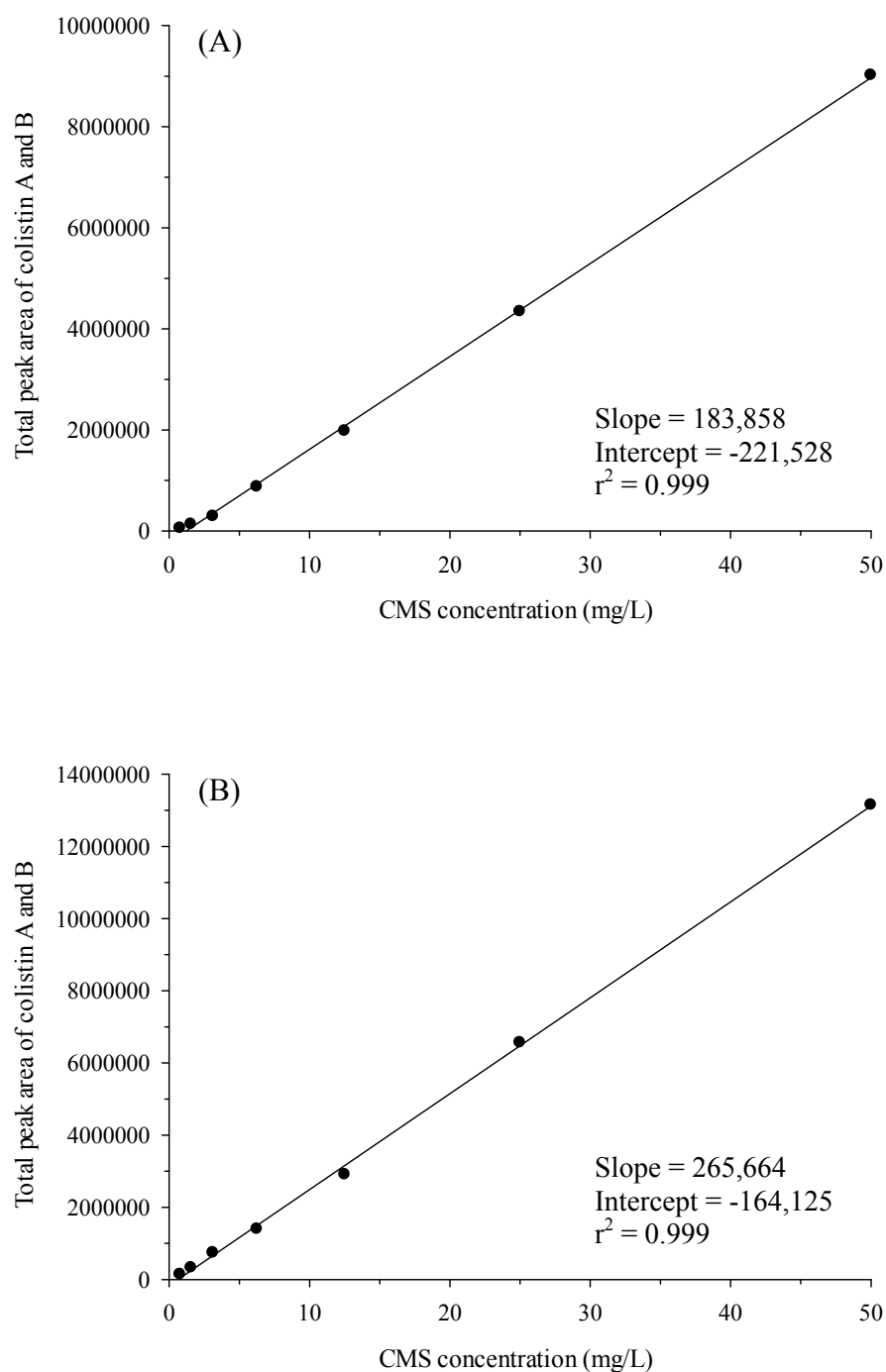
Figure 2-5: A typical calibration curve of CMS prepared in (A) drug-free BAL fluid and (B) BAL fluid-ACN (50:50, v/v). Note that CMS concentrations in the x-axis refer to sodium CMS concentrations.

CMS calibration standards ranging from 0.78 to 50 mg/L with the incorporation of QC samples of 1.25, 20, 40 and 250 mg/L were used for validation purposes. Above the calibration curve QC samples (250 mg/L) were diluted to within calibration curve range with

drug-free BAL fluid-ACN, prior to loading on to the SPE cartridges. Calibration standards and QC samples were prepared by serial dilution with appropriate volumes of drug-free BAL fluid-ACN. An aliquot of 20 µL of 1.0 M H_2SO_4 was added to 100 µL of calibration and QC sample, vortex mixed and allowed 10 min reaction time to enable accelerated conversion of CMS to colistin. The reaction was stopped by mixing 40 µL of 1.0 M NaOH and a 1:1 ratio of drug-free BAL fluid-ACN was added for protein precipitation. Following centrifugation at $10,621 \times g$ for 10 min, 110 µL of supernatant was transferred onto a SPE cartridge. Following sample pre-treatment as per Section 2.2.2.2.2, 25 µL of supernatant was injected onto the HPLC column. To ensure BAL fluid samples collected from the pharmacokinetic studies (Chapter 4) had the same composition of BAL fluid-ACN to that of calibration standards and QC samples, a 1:1 ratio of 100% ACN was added to the rat BAL fluid sample (100 µL aliquot), vortex mixed and processed in the same manner as described above. The composition of the mobile phase was MeOH-THF-water (35:39:24, v/v/v) and a run time of 10 min. Colistin A and B eluted at approximately 6.4 min and 5.5 min, respectively, with the generated HPLC chromatograph similar to that of the CMS plasma assay (Figure 2-3). No interfering peaks with colistin peak A and B were observed.

2.2.2.3.2 Intravenous administration

Following IV administration, concentrations of colistin and CMS in BAL fluid were below the LOQ of the colistin and CMS assays mentioned above. The observed low colistin/CMS concentrations in BAL fluid following IV administration can be explained by, 1) the anatomical location of the ELF when compared to the systemic site of drug administration, and 2) extensive dilution of colistin/CMS concentrations during the BAL procedure. Therefore, development of assays which involved concentrating up drug concentrations were carried out as discussed below.

Colistin and colistin methanesulphonate

To concentrate up drug concentrations, a two-step freeze-drying process was incorporated into the sample preparation stage. Colistin and CMS standards ranging from 0.015 to 0.25 mg/L and QC samples 0.040 mg/L and 0.20 mg/L in 5 mL of drug-free BAL fluid were prepared for the assay run. Due to the limited availability of drug-free BAL fluid only two QC samples were included in each HPLC run. For the same reason serial dilution was not utilised for preparation of calibration standards and QC samples, and instead calibration working solutions of colistin and CMS ranging from 0.38 to 6.3 µg/mL and QC working solutions of 1.0 µg/mL and 5.0 µg/mL were prepared in Milli-Q water by serial dilution. A 200 µL aliquot of working solution was spiked into 4.8 mL of drug-free BAL fluid and vortex mixed. Samples were stored at -80ºC for 1 h and placed in the freeze-dryer (VirTis AdVantage 2.0) which was maintained at 3-5 mTorr at a temperature of -40ºC. Samples remained in the freeze-dryer for 72 h. Samples were reconstituted with 1 mL of drug-free BAL fluid, vortex mixed and stored at -80°C for 1 h and reloaded onto the freeze-dryer for 24 h. Samples were reconstituted with 300 µL of drug-free BAL fluid. For the colistin assay, 100 µL of each calibration and QC sample was vortex mixed with 100 µL of ACN and centrifuged at 10,621 $\times$ *g* for 10 min. The volume of supernatant transferred onto a SPE cartridge was 170 µL. Following sample pre-treatment as per Section 2.2.2.2.2, 50 µL of supernatant was injected onto the HPLC column. The composition of the mobile phase was MeOH-THF-water (35:39:24, v/v/v) and a run time of 10 min. Colistin A and B eluted at approximately 6.7 min and 5.7 min, respectively, with the generated HPLC chromatograph similar to that of the colistin plasma assay (Figure 2-2). No interfering peaks with colistin peak A and B were observed.

For the CMS assay, 100 µL of each calibration and QC sample was vortex mixed with 20 µL of 1.0 M H_2SO_4. Following 10 min reaction time, 40 µL of 1.0 M NaOH was added and vortex mixed. An aliquot of 160 µL of ACN was added to the sample, vortex mixed and centrifuged at 10,621 × g for 10 min. The volume of supernatant transferred onto a SPE cartridge was 300 µL. Following sample pre-treatment as per Section 2.2.2.2.2, 50 µL of supernatant was injected onto the HPLC column. The composition of the mobile phase was MeOH-THF-water (35:39:24, v/v/v) and a run time of 10 min. Colistin A and B eluted at approximately 6.6 min and 5.7 min, respectively, with the generated HPLC chromatograph similar to that of the CMS plasma assay (Figure 2-3). No interfering peaks with colistin peak A and B were observed. To confirm that *in vitro* conversion of CMS to colistin was minimised during the freeze-drying process, three replicates of CMS 0.040 mg/L and 0.20 mg/L were prepared as described above. Samples were not subjected to treatment with 1.0 M H_2SO_4 and 1.0 M NaOH since *in vitro* conversion of CMS to colistin during the freeze-drying treatment process was being investigated. Colistin calibration standards and QC samples in BAL fluid were incorporated into the assay run to enable quantification of any *in vitro* formed colistin. No colistin A and B peaks were detectable in the three replicates of CMS 0.040 mg/L and 0.20 mg/L samples which provide confirmation that *in vitro* colistin formation during the freeze-drying process was minimal.

2.2.2.4 Validation

Validation for the rat plasma and BAL fluid assays were conducted as outlined in Section 2.2.1.3. Calibration curves were constructed by summing the peak area of colistin A and B with the nominal concentrations of colistin sulphate or sodium CMS. To determine the slope, intercept and r^2, linear least-squares regression analysis of the calibration curves with weighting of 1/response or no weighting of the data (as detailed below) was conducted. Colistin concentrations in QC samples were determined as outlined in Section 2.2.1.3.

Concentrations of CMS in QC samples were estimated by multiplying the sodium CMS concentrations obtained from the assay by the ratio of M_w of CMS base (1633 Da) and sodium CMS (1743 Da) [187]. The performance characteristics of colistin and CMS assays in rat plasma and BAL fluid are shown in Tables 2-2 and 2-3, respectively.

Table 2-2: Intra-day (n=6), inter-day (n=3) and LOQ (n=6) quality control samples for colistin and CMS in rat plasma HPLC methods.

	Intra-day				**Inter-day**				**LOQ**
Colistin (mg/L)[£]	**0.16**	**0.62**	**2.49**	**8.29**	**0.16**	**0.62**	**2.49**	**8.29**	**0.083**
Mean conc (mg/L)	0.17	0.65	2.69	8.48	0.17	0.62	2.55	7.89	0.10
Accuracy (%)	9.00	4.29	8.06	2.31	7.01	0.70	2.40	-4.83	20.1
Precision (%)	2.49	2.43	4.63	4.14	4.07	2.35	6.13	7.57	1.08
Colistin (mg/L)[Υ]	**0.21**	**0.83**	**3.32**	**-**	**0.21**	**0.83**	**3.32**	**-**	**0.104**
Mean conc (mg/L)	0.19	0.76	3.44	-	0.20	0.79	3.72	-	0.12
Accuracy (%)	-9.34	-8.06	3.48	-	-5.94	-4.26	11.9	-	15.7
Precision (%)	3.33	3.61	8.71	-	5.37	4.81	4.47	-	9.04
CMS (mg/L)	**1.17**	**18.7**	**37.5**	**281**	**1.17**	**18.7**	**37.5**	**281**	**0.73**
Mean conc (mg/L)	1.27	19.1	36.3	264	1.21	18.3	36.8	270	0.82
Accuracy (%)	8.35	2.05	-3.17	-6.17	3.29	-2.21	-1.93	-4.08	11.9
Precision (%)	3.63	3.90	3.53	5.44	7.09	4.64	5.76	5.94	5.85

[£] Colistin plasma assay used to quantify colistin concentrations in rat samples from the pharmacokinetic studies in Chapter 3.
[Υ] Colistin plasma assay used to quantify colistin concentrations in rat samples from the pharmacokinetic studies in Chapter 4.

Table 2-3: Intra-day (n=6), inter-day (n=3) and LOQ (n=6) quality control samples for colistin and CMS in rat BAL fluid HPLC methods.

	Intra-day				Inter-day				LOQ
Colistin (mg/L)[¥]	**0.62**	**2.49**	**4.97**	**-**	**0.62**	**2.49**	**4.97**	**-**	**0.21**
Mean conc (mg/L)	0.56	2.37	4.70	-	0.59	2.51	5.07	-	0.23
Accuracy (%)	-9.10	-4.87	-5.35	-	-5.36	0.68	2.06	-	10.3
Precision (%)	5.77	5.30	3.27	-	8.44	4.54	4.09	-	9.03
Colistin (mg/L)[δ,Υ]	**0.033**	**0.17**	**-**	**-**	**0.033**	**0.17**	**-**	**-**	**0.012**
Mean conc (mg/L)	0.035	0.18	-	-	0.033	0.16	-	-	0.011
Accuracy (%)	6.51	4.93	-	-	1.12	-3.34	-	-	-12.5
Precision (%)	2.98	4.72	-	-	7.90	5.06	-	-	15.4
CMS (mg/L)[¥]	**1.17**	**18.7**	**37.5**	**234**	**1.17**	**18.7**	**37.5**	**234**	**0.73**
Mean conc (mg/L)	1.13	18.6	38.9	231	1.19	18.4	37.8	234	0.74
Accuracy (%)	-3.65	-0.77	3.64	-1.41	1.80	-1.38	0.88	0.17	1.76
Precision (%)	13.9	11.3	8.30	3.31	9.06	9.53	11.7	4.60	2.57
CMS (mg/L)[δ,Υ]	**0.037**	**0.19**	**-**	**-**	**0.037**	**0.19**	**-**	**-**	**0.014**
Mean conc (mg/L)	0.042	0.22	-	-	0.037	0.22	-	-	0.014
Accuracy (%)	13.9	14.7	-	-	-0.86	15.3	-	-	2.64
Precision (%)	4.12	3.92	-	-	9.85	7.11	-	-	6.82

[¥]Colistin and CMS BAL fluid assays used to quantify drug concentrations in rat samples following pulmonary administration from the pharmacokinetic studies in Chapter 3 and 4.
[δ]Colistin and CMS BAL fluid assays used to quantify drug concentrations in rat samples following IV administration from the pharmacokinetic studies in Chapter 3 and 4.
[Υ]No weighting of the calibration curves.

2.2.2.5 Estimation of colistin and CMS concentrations in rat biological samples

Colistin concentrations in rat plasma and BAL fluid samples from the pharmacokinetic studies in Chapters 3 and 4 were estimated as outlined in Section 2.2.1.4. CMS concentrations in rat biological samples from the pharmacokinetic studies in Chapter 4 were determined as described for CMS QC samples in Section 2.2.2.4. The estimated CMS concentrations represent both fully and partially sulphomethylated derivatives of CMS and *in vitro* formed colistin. Therefore to determine the actual *in vivo* CMS concentrations (fully and partially sulphomethylated derivatives), the CMS concentration was subtracted from the colistin

concentration obtained from the same rat biological sample. This was carried out after correcting for differences in the M_w of colistin (1163) and sodium CMS (1743).

2.2.2.6 Summary

The HPLC assays for colistin and CMS in rat biological matrices were validated and used for the quantification of colistin and CMS in rat plasma and BAL fluid (Chapters 3 and 4).

2.2.3 Quantification of colistin and CMS in epithelial lining fluid

2.2.3.1 Method

Urea concentrations in rat BAL fluid and plasma were determined using a commercially available kit, QuantiChrom^{TM} Urea Assay Kit (BioAssay Systems, California, USA). A colorimetric reaction facilitated by mixing two reagents (*o*-phthaldialdhyde and primaquine phosphate) with urea in BAL fluid and plasma samples enabled quantification of urea concentrations in the biological matrices. Condensation of urea following reaction with *o*-phthaldialdhyde results in the formation of either 1,3-dihydroxy-isoindoline or 1-ureido-3-hydroxyphthalan [193]. The isoindoline and/or phthalan derivative reacts with primaquine phosphate to facilitate the formation of the characteristic yellow-orange colour in the biological matrices [193]. The QuantiChrom^{TM} Urea Assay Kit specifications for quantification of urea concentrations (mg/dL) in rat BAL fluid and plasma are detailed out in Table 2-4. Multiskan EX Microplate Photometer as per Section 2.1.2 was used to measure the optical density (OD) of rat BAL fluid and plasma samples.

Table 2-4: QuantiChrom^{TM} Urea Assay Kit specifications for quantification of urea concentrations (mg/dL) in rat BAL fluid and plasma.

	BAL fluid	**Plasma**
Sample volume (μL)	50	5
Blank (Milli-Q water) volume (μL)	50	5
Standard volume	50 μL of 5 mg/dL urea	5 μL of 50 mg/dL urea
Reagent volume (μL)$^{£}$	200	200
Incubation period (min)	50	20
Incubation temperature	Room temperature	Room temperature
Optical density (nm)	450	520

$^{£}$ Composed of reagent A:B (50:50, v/v).

Preparation of quality control samples

Urea stock solutions of 1.0 mg/mL and 35 mg/mL in Milli-Q water were used for preparation of QC samples in BAL fluid and plasma, respectively. Fresh stock solutions were prepared for each assay run.

Bronchoalveolar lavage fluid

Urea QC samples 0.20 and 1.0 mg/dL were prepared in blank rat BAL fluid. Urea working solutions of 0.10 mg/mL and 0.50 mg/mL were prepared by serial dilution with appropriate volumes of Milli-Q water. A 4 μL aliquot of working solution was spiked into 196 μL of blank BAL fluid and vortex mixed. Into a 96-well plate, three replicates of 50 μL QC sample, blank BAL fluid, Milli-Q water and urea 5 mg/dL standard were dispensed and 200 μL of reagent mixture (reagent A:B, 50:50, v/v) was added to each well. Following a 10 sec shaking period, samples were left at room temperature for 50 min before the OD of the samples were measured at 450 nm (Table 2-4).

Plasma

Urea QC samples of 30 and 70 mg/dL were prepared in blank rat plasma. Serial dilution was utilised to prepare urea working solution of 15 mg/mL in Milli-Q water. A 4 µL aliquot of working solution 15 and 35 mg/mL was spiked into 196 µL of blank plasma and vortex mixed. Three replicates of 5 µL QC sample, blank plasma, Milli-Q water and urea 50 mg/dL standard were dispensed into a 96-well plate followed by addition of 200 µL of reagent mixture (reagent A:B, 50:50, v/v). The samples underwent a 10 sec shaking period, and were left at room temperature for 20 min before the OD of the samples were measured at 520 nm (Table 2-4).

2.2.3.2 Estimation of urea concentrations in quality control and rat biological samples

Estimation of urea concentrations (mg/dL) in QC samples was calculated using Equation 2.3.

$$[Urea](mg/dL) = \frac{OD_{QC\ sample} - OD_{Blank\ BAL\ fluid/plasma}}{OD_{Standard} - OD_{Blank}} \times n \times [STD]$$

Equation 2.3

where $OD_{QC\ sample}$, $OD_{Blank\ BAL\ fluid/plasma}$, $OD_{Standard}$, OD_{Blank} are the optical density values of BAL fluid/plasma QC sample, blank BAL fluid/plasma sample, 5 mg/dL or 50 mg/dL urea standard, and Milli-Q water, respectively; *n* is the dilution factor and STD is the standard concentration. The equation used to estimate the urea concentrations in QC samples was slightly modified to that used for rat samples (below) due to the requirement for quantification of only 'exogenous' urea in the QC samples. This was undertaken by subtracting the 'exogenous and endogenous' urea OD value in the QC samples from the 'endogenous' urea OD value in the blank BAL fluid/plasma used to prepared the QC samples as shown in Equation 2.3. For the rat BAL fluid and plasma sample from the pharmacokinetic studies

(Chapters 3 and 4), urea concentration (mg/dL), referring to the endogenous concentrations, were calculated using Equation 2.4.

$$[Urea](mg/dL) = \frac{OD_{Sample} - OD_{Blank}}{OD_{Standard} - OD_{Blank}} \times n \times [STD]$$

Equation 2.4

where OD_{Sample}, $OD_{Standard}$ and OD_{Blank} are the optical density values of BAL fluid/plasma rat sample, 5 mg/dL or 50 mg/dL urea standard and Milli-Q water, respectively.

2.2.3.3 Estimation of the epithelial lining fluid volume and colistin and CMS concentrations in lining fluid

Estimation of the urea concentration in the recovered BAL fluid and plasma rat samples enabled the calculation of the dilution factor and thereby calculation of the ELF volume as shown in Equation 2.5.

$$V_{ELF} = \frac{[Urea]_{BAL\ fluid}}{[Urea]_{Plasma}} \times V_{BALF}$$

Equation 2.5

where *[Urea]$_{BAL\ fluid}$*, *[Urea]$_{Plasma}$* and *V$_{BALF}$* are the urea concentration in BAL fluid (mg/dL), plasma (mg/dL) and the volume of recovered BAL fluid, respectively. Subsequently, ELF colistin/CMS concentration (*[Colistin/CMS]$_{ELF}$*) was estimated by multiplying the colistin/CMS concentration in the BAL fluid (*[Colistin/CMS]$_{BALF}$*) by the ratio of recovered BAL fluid and ELF volumes as shown in Equation 2.6. Colistin/CMS concentration in BAL fluid was calculated as detailed in Section 2.2.2.3.

$$[Colistin/CMS]_{ELF} = [Colistin/CMS]_{BALF} \times \frac{V_{BALF}}{V_{ELF}}$$

Equation 2.6

2.2.3.4 Validation

To assess the reproducibility of the assay, intra-day and inter-day assays were carried out. Estimation of the accuracy and precision was carried out using Equations 2.1 and 2.2, respectively. The performance characteristics of urea assays in rat BAL fluid and plasma are shown in Table 2-5.

Table 2-5: Intra-day (n=6) and inter-day (n=3) quality control samples for urea in rat BAL fluid and plasma.

	Intra-day		Inter-day	
BAL fluid (mg/dL)	**0.20**	**1.0**	**0.20**	**1.0**
Mean conc (mg/dL)	0.22	1.16	0.20	1.06
Accuracy (%)	8.29	16.1	-2.34	6.01
Precision (%)	9.03	9.41	9.65	10.8
Plasma (mg/dL)	**30**	**70**	**30**	**70**
Mean conc (mg/dL)	32.5	67.9	30.6	66.2
Accuracy (%)	8.47	-3.02	1.86	-5.42
Precision (%)	6.97	3.72	10.8	4.83

2.2.3.5 Summary

The assays for urea in rat biological matrices were validated and used for the quantification of urea in rat BAL fluid and plasma (Chapters 3 and 4). This enabled estimation of the volume of ELF and the corresponding colistin and CMS concentrations in ELF (Chapters 3 and 4).

2.3 Quantification of colistin and CMS following pharmacokinetic studies in cystic fibrosis subjects

2.3.1 HPLC assay for quantification of colistin and CMS concentrations in human biological samples

Concentrations of colistin and CMS in human plasma, urine and sputum were determined using previously developed and validated HPLC assays for quantification of colistin [191] and CMS [192] in biological matrices, with minor modifications as detailed below.

2.3.1.1 Method

The HPLC specifications are as outlined in Section 2.2.2.1, except for the mobile phase composition of MeOH-THF-water (35:39:24, v/v/v) and a run time of 10 min for all the below mentioned assays. Preparation of colistin and CMS stock solutions and quantification of CMS in human biological matrices are as outlined in Section 2.2.2.1.

2.3.1.2 Human plasma samples

Preparation of calibration and quality control samples

Two independent stock solutions of colistin 1.0 mg/mL and CMS 1.0 mg/mL were prepared in Milli-Q water.

2.3.1.2.1 Colistin

Two colistin calibration standards in drug-free human plasma were prepared to encapsulate the colistin concentration range following IV and pulmonary CMS administration. Following IV CMS administration, colistin calibration standards ranged from 0.13 to 8.0 mg/L and QC samples at three concentrations levels were included in all HPLC runs (0.38, 3.0 and 6.0 mg/L). Calibration standards and QC samples were prepared by serial dilution with appropriate volumes of drug-free human plasma. A 100 µL aliquot of each calibration and QC

sample was mixed with 100 µL of ACN, vortex mixed and centrifuged at 10,621 × *g* for 10 min (Eppendorf centrifuge 5430®). The volume of supernatant transferred onto a SPE cartridge was 170 µL. Following pulmonary CMS administration, plasma colistin concentrations were at the lower concentration range of the above assay. Therefore colistin calibration standards ranging from 0.13 to 2.0 mg/L were constructed with an effort to increase the assay sensitivity by increasing the initial plasma volume (200 µL) and the volume of supernatant (300 µL) transferred onto the SPE cartridges. Colistin concentrations of 0.38, 0.75 and 1.5 mg/L were incorporated as QC samples. Calibration standards and QC samples were prepared by serial dilution with appropriate volumes of drug-free human plasma. An aliquot of each calibration and QC sample was mixed with 200 µL of ACN, vortex mixed and centrifuged at 10,621 × *g* for 10 min. The volume of supernatant transferred onto a SPE cartridge was 300 µL. For both assays, following sample pre-treatment as per Section 2.2.2.2.2, 50 µL of supernatant was injected onto the HPLC column. Colistin A and B were eluted at approximately 6.6 min and 5.7 min, respectively, with the generated HPLC chromatograph for the first colistin plasma assay shown in Figure 2-6. No interfering peaks with colistin peak A or B were observed for both assays.
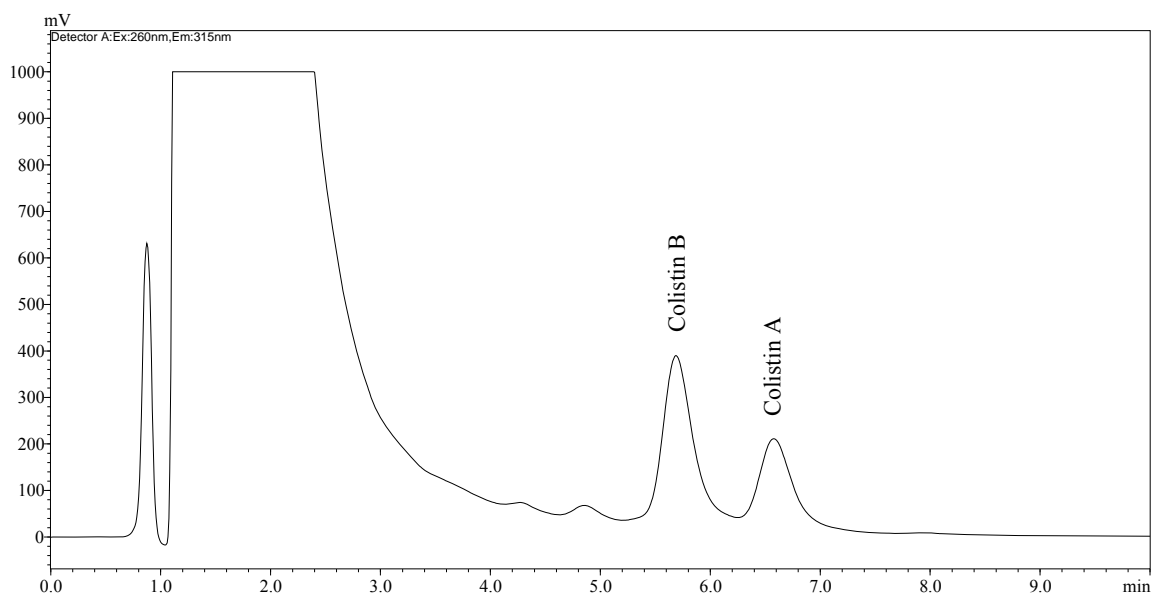
Figure 2-6: A typical HPLC chromatograph of fluorescent derivatives of colistin A and B for colistin 8.0 mg/L spiked in human plasma.

2.3.1.2.2 Colistin methanesulphonate

Two separate CMS calibration standards in drug-free human plasma were prepared to encompass the CMS concentration range following IV and pulmonary CMS administration. For IV CMS administration, CMS calibration standards ranged from 0.78 to 50 mg/L with concentrations of 1.3, 20 and 40 mg/L prepared for QC samples. Calibration standards and QC samples were prepared by serial dilution with appropriate volumes of drug-free human plasma. To facilitate accelerated conversion of CMS to colistin, 13 µL of 1.0 M H_2SO_4 was added to 100 µL of calibration standard and QC sample, vortex mixed and allowed 10 min reaction time. Following this, 27 µL 1.0 M NaOH was mixed to stop the conversion reaction and 140 µL of ACN vortex mixed for protein precipitation. Samples were centrifuged at $10,621 \times g$ for 10 min and the volume of supernatant transferred onto a SPE cartridge was 230 µL. Following sample pre-treatment as per Section 2.2.2.2.2, 20 µL supernatant was injected onto the HPLC column. Following pulmonary CMS administration, plasma CMS concentrations were below the LOQ of 0.78 mg/L of the above CMS assay. Therefore, CMS calibration standards which encapsulated a concentration range of 0.13 to 2.0 mg/L were

constructed in drug-free human plasma. To increase the sensitivity of the plasma assay, a higher initial plasma volume (200 µL), supernatant volume (300 µL) and HPLC injection volume (50 µL) were utilised. CMS concentrations of 0.38, 0.75 and 1.5 mg/L were incorporated as QC samples. Calibration standards and QC samples were independently prepared by serial dilution with drug-free human plasma. To 200 µL of calibration standard and QC sample 27 µL of 1.0 M H_2SO_4 was mixed and allowed a 10 min reaction time followed by addition of 53 µL of 1.0 M NaOH. Protein precipitant with 280 µL of ACN was followed by centrifugation at 10,621 × g for 10 min. Sample pre-treatment was as per Section 2.2.2.2.2 and the volume of supernatant transferred onto a SPE cartridge was 300 µL. Colistin A and B (corresponding to CMS A and B, respectively) eluted at approximately 6.9 min and 5.9 min, respectively, with the generated HPLC chromatograph for the second CMS plasma assay shown in Figure 2-7. No interfering peaks with colistin peak A or B were observed for both assays.
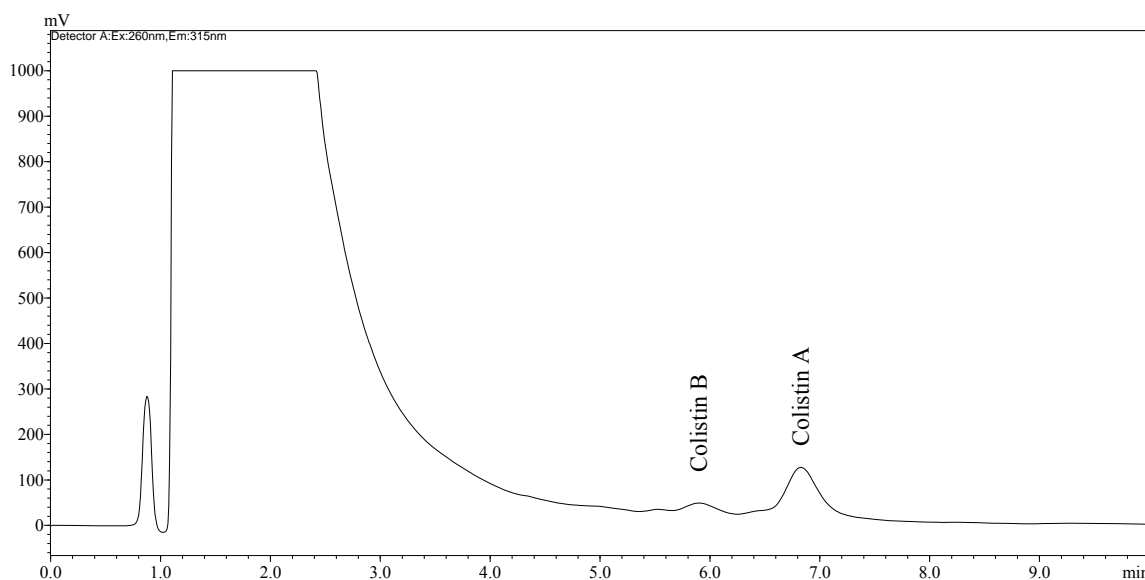
Figure 2-7: A typical HPLC chromatograph of fluorescent derivatives of colistin A and B for CMS 2.0 mg/L spiked in human plasma. Note that colistin A and B corresponds to CMS A and B, respectively.

2.3.1.3 Human urine samples

Preparation of calibration and quality control samples

Two independent stock solutions of colistin 1.0 mg/mL and CMS 1.0 mg/mL were prepared in Milli-Q water.

2.3.1.3.1 Colistin and colistin methanesulphonate

Due to low concentrations of proteins in human urine, similar to that in rat BAL fluid, preparation of colistin and CMS calibration standards in 100% human urine was expected to result in non-linearity. To confirm this hypothesis, calibration standards of colistin ranging from 0.13 to 8.0 mg/L and CMS ranging from 0.78 to 50 mg/L were prepared in 100% drug-free urine, with the concentration range selected based on the respective IV plasma assays. Samples were prepared by serial dilution with appropriate volumes of drug-free urine. As shown in Figures 2-8A (for colistin) and 2-9A (for CMS), non-linearity was evident at lower concentrations in both calibration curves, with 0.13 mg/L colistin not detectable in the colistin assay. Similar to the colistin and CMS BAL fluid assays (Section 2.2.2.3), to overcome the observed non-linearity, colistin and CMS calibration standards ranging from 0.13 to 16 mg/L were prepared in a matrix composed of drug-free urine-ACN (50:50, v/v). Modifications to the calibration standard concentration range compared to the previous urine assay were due to the anticipated concentrations of colistin and CMS in patient urine samples. Calibration standards prepared in drug-free urine-ACN (50:50, v/v) showed linearity (Figures 2-8B and 2-9B for colistin and CMS, respectively). Therefore, validation of the colistin and CMS assays for human urine samples was carried out in a matrix composed of drug-free urine-ACN (50:50, v/v), as described below.
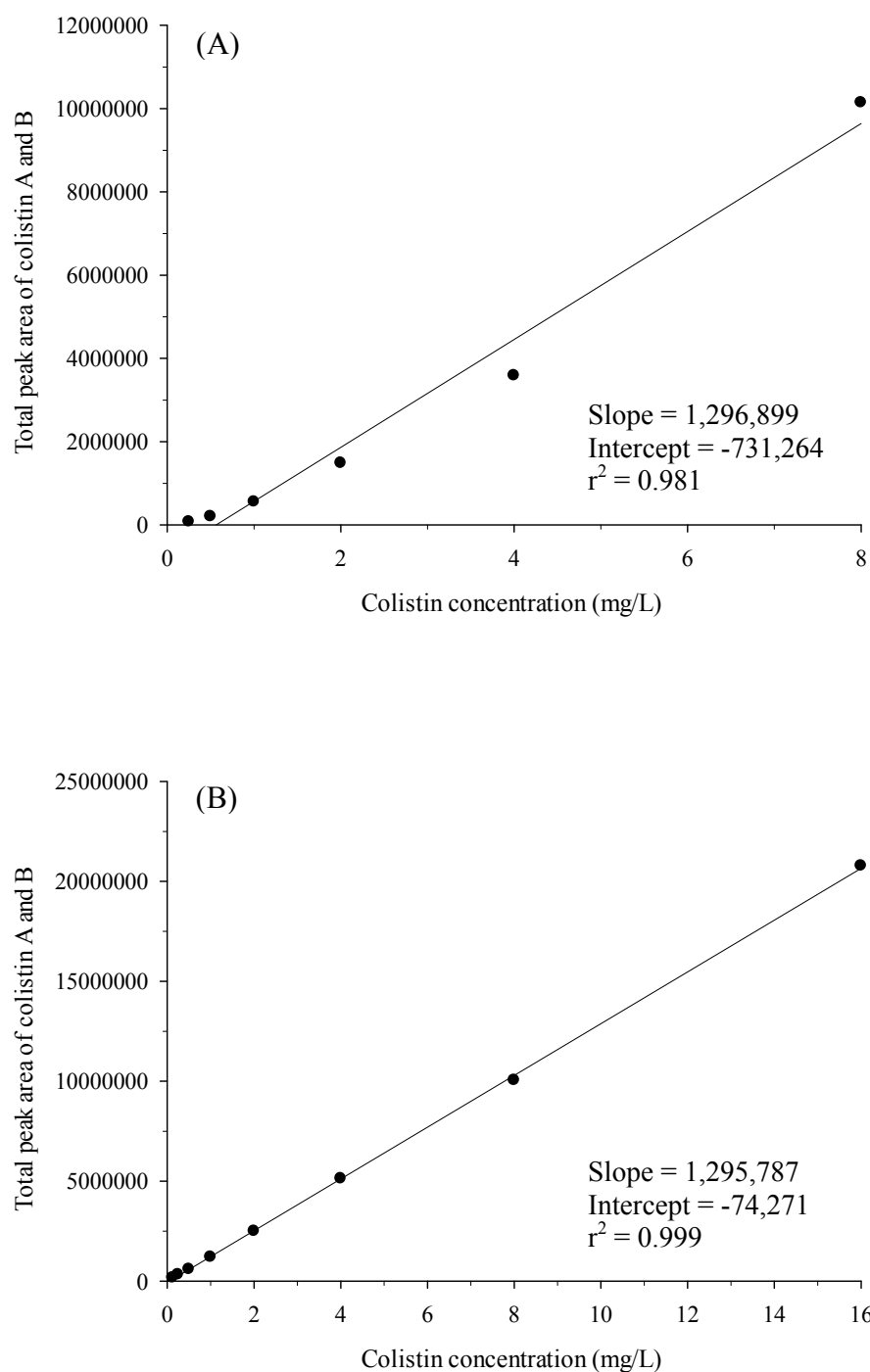
Figure 2-8: A typical calibration curve of colistin prepared in (A) drug-free human urine and (B) drug-free human urine-ACN (50:50, v/v). Colistin 0.13 mg/L was not detectable in panel A. Note that colistin concentrations in the x-axis refer to colistin sulphate concentrations and that panel B has a different concentration range.
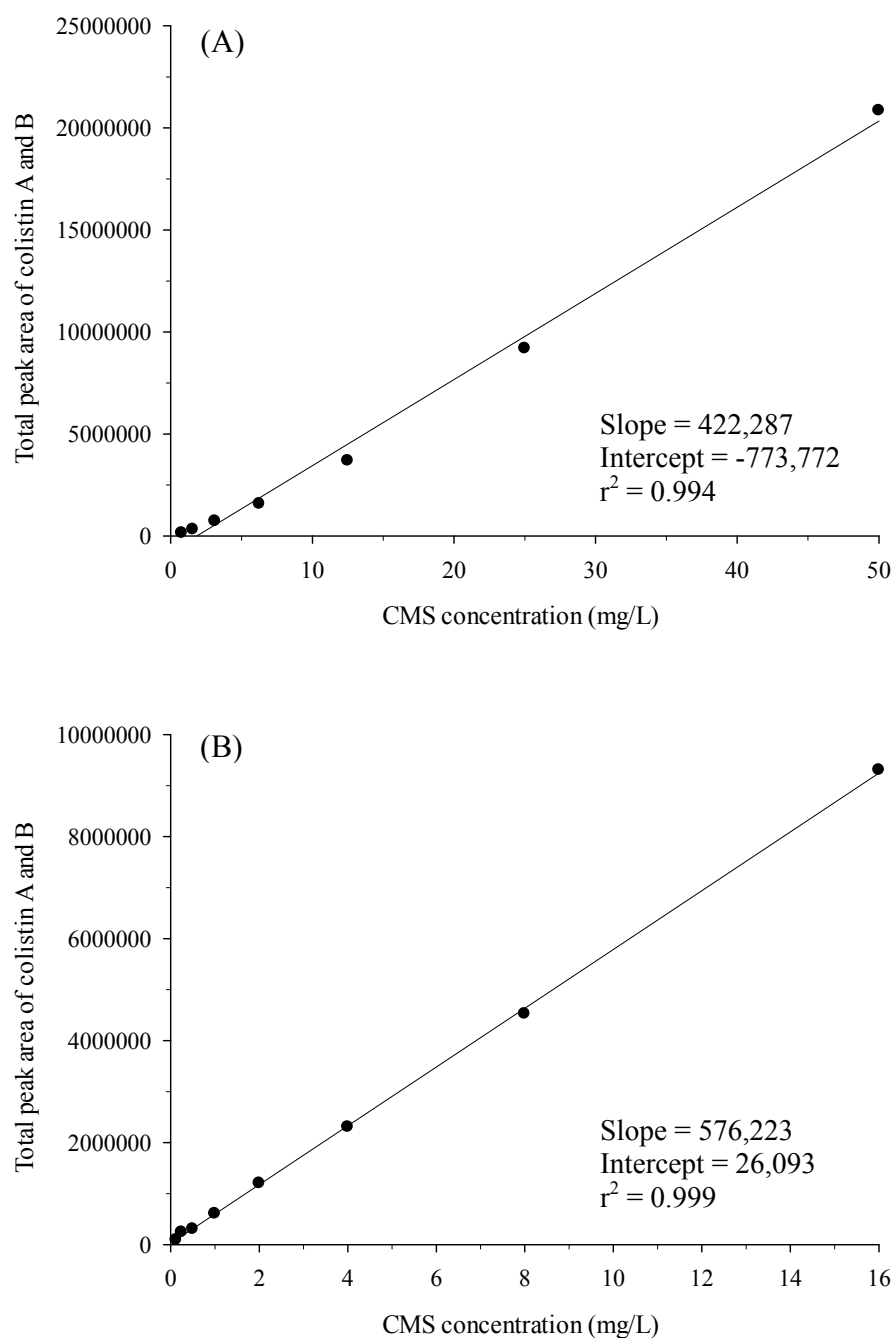
Figure 2-9: A typical calibration curve of CMS calibration prepared in (A) drug-free human urine and (B) drug-free human urine-ACN (50:50, v/v). Note that CMS concentrations in the x-axis refer to sodium CMS concentrations and that panel B has a different concentration range.

Colistin and CMS calibration standards ranged from 0.13 to 16 mg/L. Colistin concentrations of 0.35, 7.0, 14 and 40 mg/L were used as QC samples. For the QC samples for CMS, concentrations of 0.35, 7.0, 14 and 150 mg/L were utilised. High QC samples were diluted to

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within the calibration curve range with drug-free urine-ACN (50:50, v/v) prior to loading onto SPE cartridges. Calibration standards and QC samples were independently prepared by serial dilution with drug-free urine-ACN. For the colistin assay, 200 µL of sample was centrifuged at 10,621 × g for 10 min and 175 µL of supernatant was transferred onto a SPE cartridge. Following sample pre-treatment as per Section 2.2.2.2.2, 25 µL of supernatant was injected onto the HPLC column. For the CMS assay, 27 µL of 1.0 M H_2SO_4 was added to 200 µL of sample and following 10 min reaction time, 53 µL of 1.0 M NaOH was vortex mixed. After centrifugation at 10,621 × g for 10 min, 230 µL of supernatant was transferred onto a SPE cartridge. Following sample pre-treatment as per Section 2.2.2.2.2, 35 µL of supernatant was injected onto the HPLC column. Colistin A and B eluted at approximately 6.7 min and 5.7 min for the colistin and CMS assays, with the generated HPLC chromatograph similar to that of the colistin plasma assay (Figure 2-6) and CMS plasma assay (Figure 2-7). For both assays, no peaks interfering with colistin peak A or B were observed.

2.3.1.4 Human sputum samples

2.3.1.4.1 Colistin

Colistin calibration standards ranging from 0.63 to 20 mg/L were initially chosen due to the potential wide range of colistin concentrations in CF human sputum samples following IV and pulmonary CMS administration. Colistin/CMS-free CF sputum samples were collected from inpatients at the CF Service at the Alfred Hospital which meant that the sputum samples used to prepare the calibration standards and QC samples contained bacteria. To prepare calibration standards 10 µL colistin working solution (prepared in Milli-Q water) was spiked into 100 µL of homogenised sputum. Due to the relative viscous nature of the homogenised sputum, serial dilution was not utilised for preparation of samples as this may have led to inaccurate dilutions. Samples were treated with a 1:1 ratio of ACN and after centrifugation at 10,621 × g for 10 min a volume of supernatant (100 µL) was transferred onto a SPE cartridge.

As shown in Figure 2-10A the resultant calibration curve of nominal concentrations *versus* total peak area showed non-linearity especially at the lower concentration range of 0.63 to 10 mg/L. From previous observations in human urine and rat BAL fluid this was thought to be due to non-specific binding of colistin to microcentrifuge tubes during preparation of working solutions and therefore an alternate method of preparing working solutions in a matrix composed of Milli-Q water-ACN (50:50, v/v) was conducted. Calibration standards were prepared as described above except for transferring of a greater volume (190 μL) of supernatant onto a SPE cartridge to improve the assay sensitivity limit. The generated calibration curve is shown in Figure 2-10B, with evidence of non-linearity still present at the lower concentration range of 0.63 to 10 mg/L; this suggested that the observed non-linearity may in fact be arising from the sputum matrix.
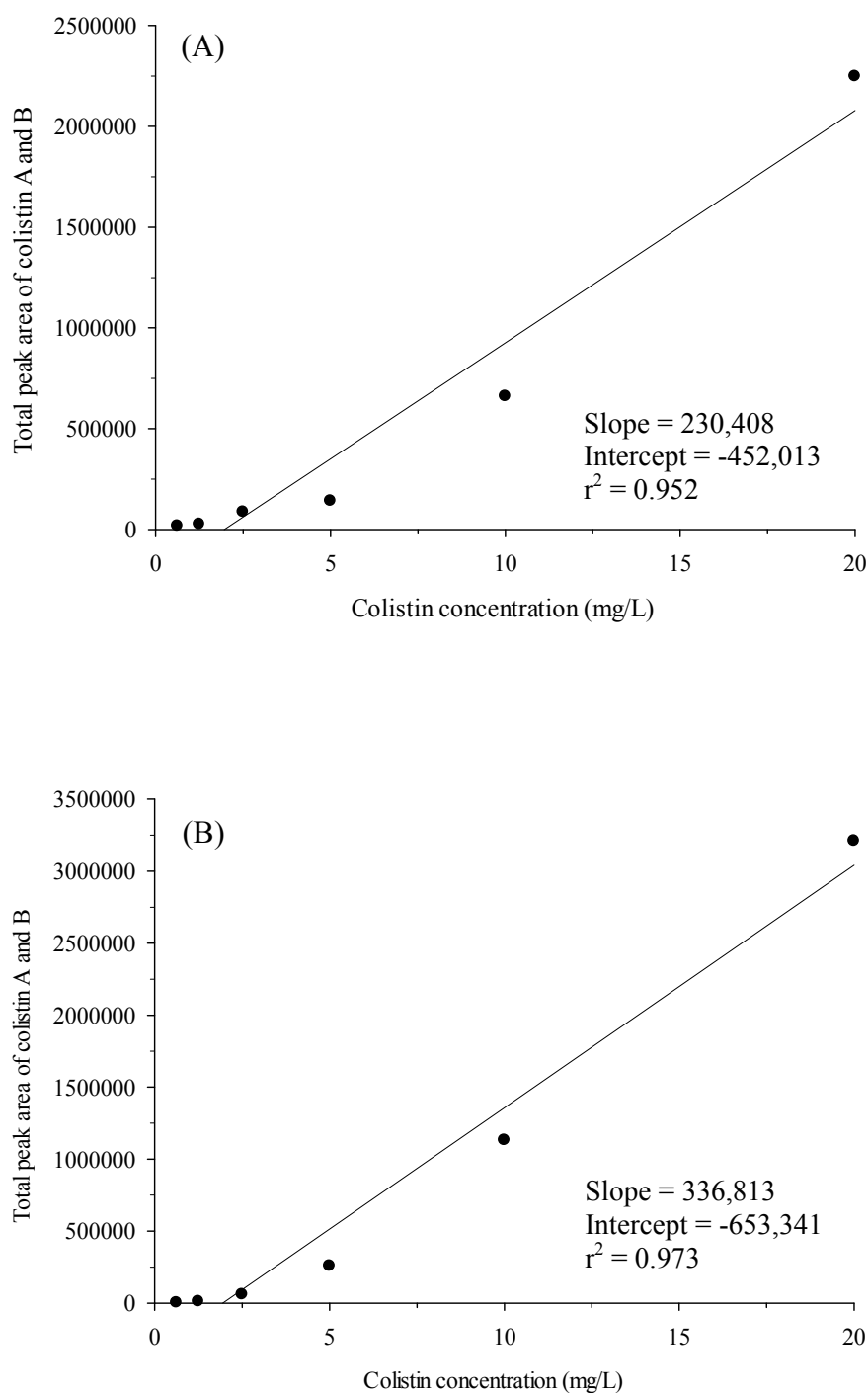
Figure 2-10: A typical calibration curve of colistin in human sputum prepared with working solution composed of (A) Milli-Q water and (B) Milli-Q water-ACN (50:50, v/v). Note that colistin concentrations in the x-axis refer to colistin sulphate concentrations.

Since non-linearity was observed at the lower concentration range, it was proposed that colistin may be binding to bacteria and/or sputum constituents present in the colistin/CMS-

free sputum matrix. A search of the literature showed that Ratjen *et al* had developed an assay for quantification of colistin concentrations in human sputum involving FMOC-Cl derivatisation of colistin followed by reversed-phase HPLC [170]. The assay involved addition of an aliquot of blank human serum (~ 6% of the total sputum volume) into sputum samples; however an explanation for this procedure was not given [170]. Therefore in the current assay, an investigation to whether the presence of blank human plasma in calibration standards would overcome the non-linearity was conducted. The presence of human plasma proteins in the colistin sputum sample may facilitate the binding of colistin predominantly to plasma proteins and thereby hinder colistin binding to bacteria and/or sputum constituents. The percentage of human plasma needed to achieve this was not known; therefore two calibration standards ranging from 0.63 to 10 mg/L were prepared with 10 µL (~ 8% of the total sputum volume) and 110 µL (50% of the total sputum volume) of blank human plasma. Colistin working solutions were prepared in a matrix composed of Milli-Q water-ACN (50:50, v/v). To colistin sputum samples, either 10 µL or 110 µL of blank human plasma was added and vortex mixed for 20 sec. Following addition and vortex mix of a 1:1 ratio of ACN, the samples were centrifuged at $10,621 \times g$ for 10 min and the supernatant transferred onto a SPE cartridge. As shown in Figure 2-11 both colistin calibration curves demonstrated non-linearity with colistin of 0.63 mg/L undetectable in Figure 2-11B. This suggested that addition of blank human plasma may not be a suitable solution to overcome the potential binding of colistin to bacteria and/or sputum constituents.
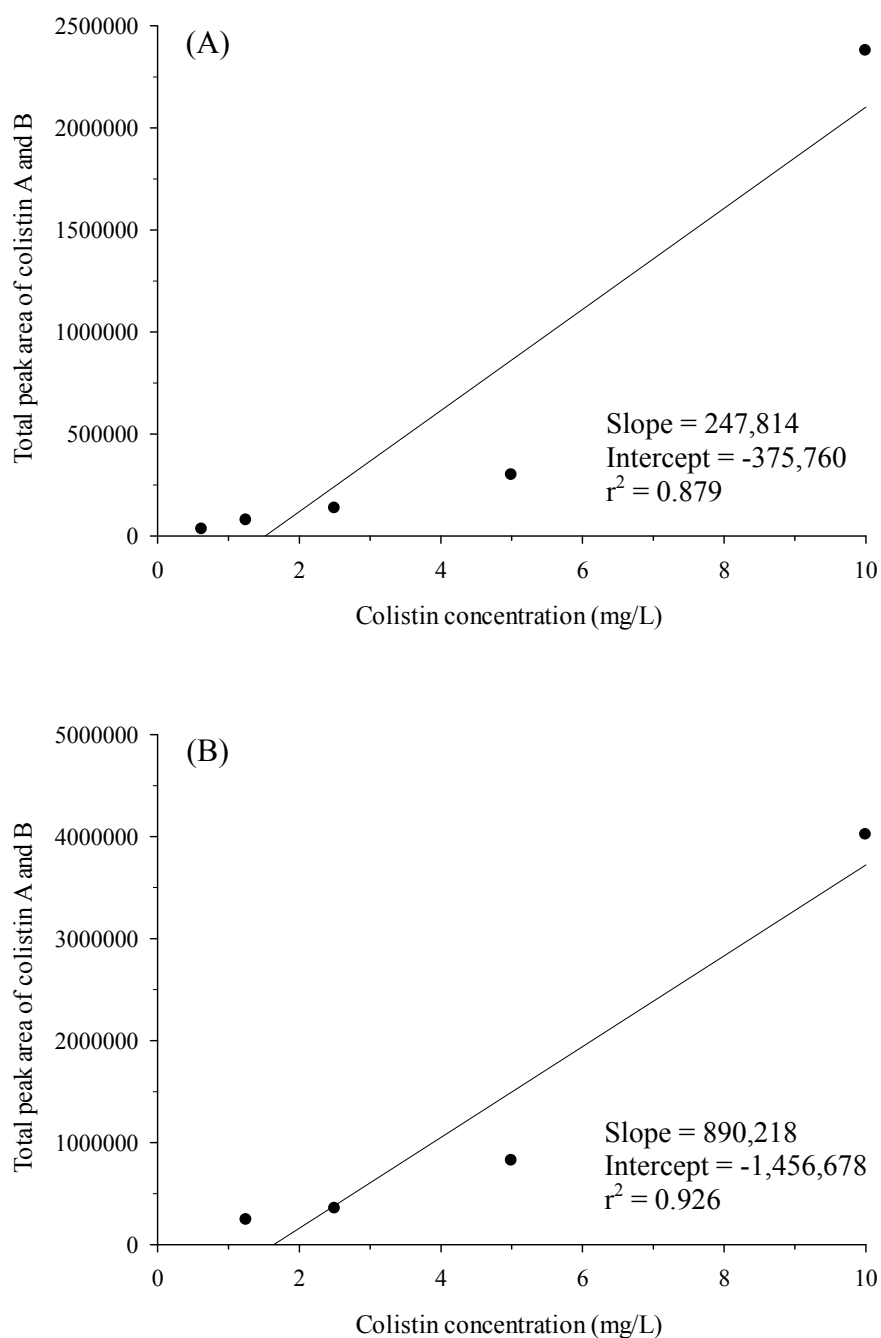
Figure 2-11: A typical calibration curve of colistin in human sputum spiked with blank human plasma that was (A) ~ 8% and (B) 50% of the total sputum volume. Colistin 0.63 mg/L was not detectable in panel B. Note that colistin concentrations in the x-axis refer to colistin sulphate concentrations.

If the non-linearity was arising from binding of colistin to bacteria and/or sputum constituents then knowing the mechanism of this binding would facilitate selection of a method to

overcome this non-linearity. The mechanism of colistin interaction with sputum constituents is not known; however the manner in which colistin exerts antibacterial activity is through an electrostatic interaction between colistin and lipopolysaccharide (LPS) on the outer layer of Gram-negative bacteria (Chapter 1, Section 1.5.4) [94]. Therefore, if disruption of colistin binding to bacteria could be achieved, this may improve linearity, and therefore the technique of 'cell lysis' was proposed. Cell lysis can be carried out by optical, mechanical, acoustic (sonication), electrical, and chemical (detergent and alkaline treatment) mechanisms [194]; however due to potential *in vitro* conversion of CMS to colistin some of mentioned cell lysis techniques could not be carried out. Chemical lysis, first proposed by Birnboim *et al*, involved preparation of a 'alkaline sodium dodecyl sulphate (SDS) solution' which was comprised of 200 mM NaOH and 1% SDS in Milli-Q water [195]. The mechanism by which the alkaline SDS solution aids in cell lysis is via cell wall breakdown and cell membrane solubilisation by NaOH and SDS [194].

Chemical lysis of colistin calibration standards was carried out by addition of alkaline SDS solution in a 1:1 ratio to 110 µL of colistin spiked sputum sample. Following vortex mixing for 30 sec, samples was left in ice water for 5 min to allow for cell lysis. For protein precipitation, an equal volume of ACN was added, vortex mixed for 30 sec and centrifuged at $20,817 \times g$ for 10 min. The supernatant was transferred onto a SPE cartridge. The resultant colistin calibration curve ranging from 0.13 to 10 mg/L is shown in Figure 2-12A with a repeat of the assay carried out with standard concentrations ranging from 0.63 to 20 mg/L (Figure 2-12B). Incorporation of alkaline SDS solution in the sample preparation stage enabled linear calibration curves to be constructed over the concentration range of 0.63 to 20 mg/L (Figure 2-12 A and B). This suggests that binding of colistin to bacteria present in the CMS/colistin-free sputum matrix may have led to the observed non-linearity.
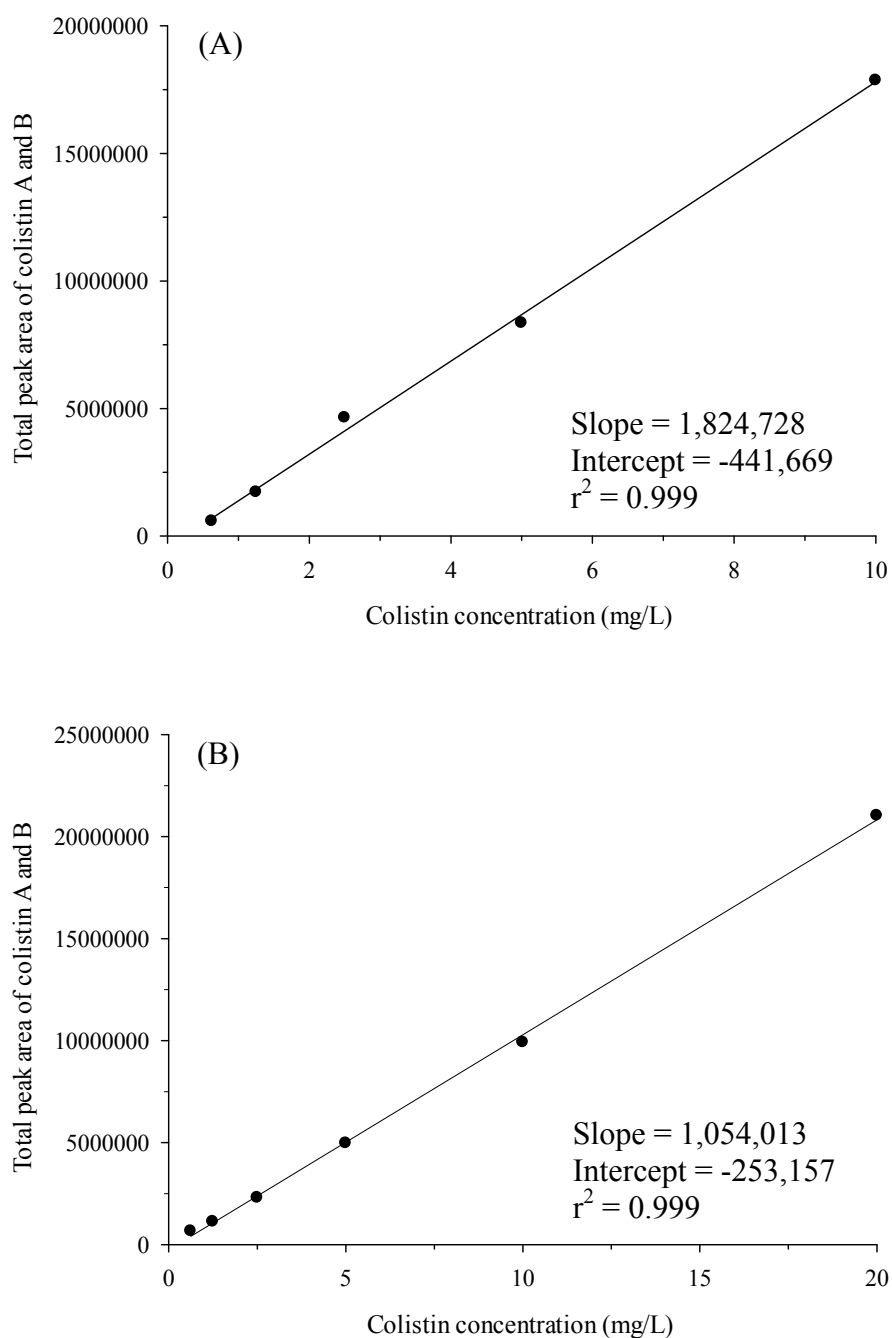
Figure 2-12: A typical calibration curve of colistin in human sputum prepared with alkaline SDS solution with concentrations ranging from (A) 0.63 to 10 mg/L and (B) 0.63 to 20 mg/L. Note that colistin concentrations in the x-axis refer to colistin sulphate concentrations and that panel B has a different concentration range.

To further confirm whether presence of bacteria in sputum were contributing to the observed non-linearity, a study which compare colistin calibration curves prepared in sputum

containing bacteria and sputum without bacteria was needed. However obtaining sputum with

and without bacteria was difficult and therefore a surrogate matrix containing bacteria, cation-

adjusted Mueller-Hinton broth (CaMHB), with an overnight culture of *Pseudomonas*

aeruginosa (10^8 colony forming units/mL, ATCC 27853) was chosen. *P. aeruginosa* was

selected as it is the predominant pathogen present in CF sputum [10, 196]. Colistin calibration

curve A was prepared in blank broth, calibration curve B and C in *P. aeruginosa* containing

broth. Calibration curves A and B were prepared by spiking in 10 µL of colistin working

solution (prepared in Milli-Q water-ACN, 50:50, v/v), addition of ACN and transferring of

supernatant onto a SPE cartridge. Calibration curve C standards had an additional 1:1 ratio of

alkaline SDS solution, left to stand in ice water, addition of ACN and supernatant transferred

onto a SPE cartridge. As shown in Figure 2-13A the absence of bacteria in the matrix resulted

in a linear calibration curve, while the presence of bacteria without alkaline SDS treatment

resulted in a non-linearity (Figure 2-13B). This suggests that the observed non-linearity for

calibration curve B when compared to A was due to presence of bacteria in the matrix. This

non-linearity observed in the *P. aeruginosa*-broth was overcome by alkaline SDS treatment as

shown in calibration curve C (Figure 2-13C). Therefore these findings indicate that the

presence of bacteria in the sputum matrix used to prepare colistin calibration standards was

likely to be contributing to the non-linearity observed. It is also possible that other

constituents in the sputum contribute to the observed non-linearity however at the present

time this cannot be investigated.
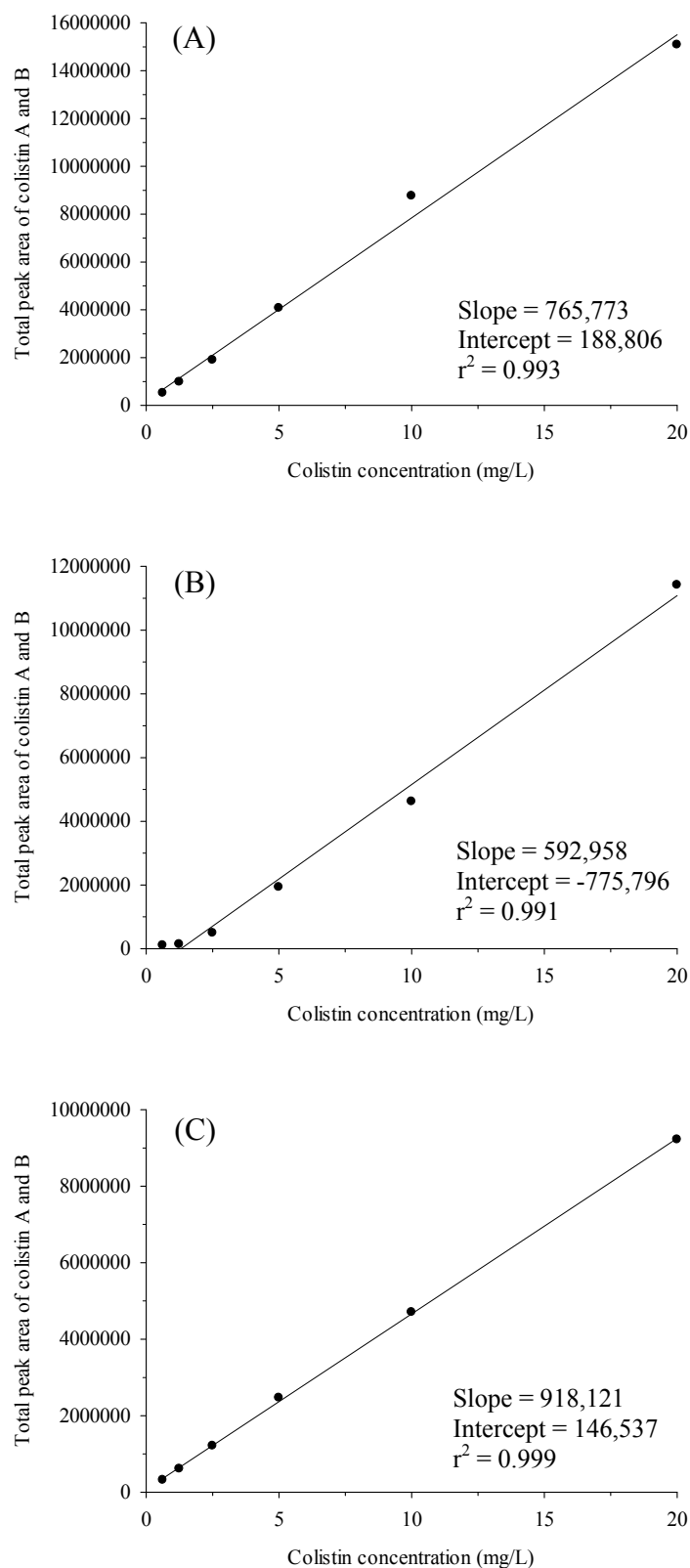
Figure 2-13: A typical calibration curve of colistin in prepared in (A) blank broth, (B) *P. aeruginosa*-broth with no alkaline SDS treatment and (C) *P. aeruginosa*-broth with alkaline SDS treatment. Note that colistin concentrations in the x-axis refer to colistin sulphate concentrations.

To allow for consistency in the colistin calibration standards for the sputum assay with that of the plasma and urine assay as per Sections 2.3.1.2 and 2.3.1.3, calibration standards ranging from 0.13 to 16 mg/L were prepared in sputum with incorporation of alkaline SDS solution. Colistin calibration curve demonstrated linearity as shown in Figure 2-14 and this assay with incorporation of QC samples were prepared and validated.
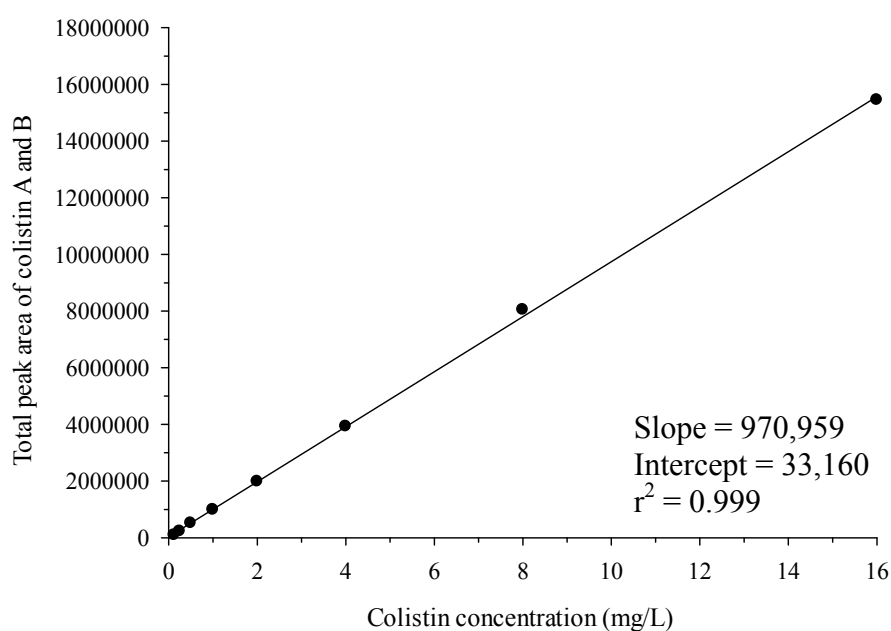
Figure 2-14: A typical calibration curve of colistin in human sputum prepared with alkaline SDS treatment. Note that colistin concentrations in the x-axis refer to colistin sulphate concentrations.

Preparation of calibration and quality control samples

Two independent stock solutions of colistin 2.0 mg/mL were prepared in Milli-Q water.

Colistin standards range from 0.13 to 16 mg/L with concentrations of 0.35, 7.0, 14 and 40 mg/L incorporated as QC samples. CMS/colistin-free sputum were homogenised and a positive-displacement pipette was used to aliquot 100 μL of sputum into 1.5 mL microcentrifuge tubes. Colistin working solutions ranging from 0.0014 to 0.18 mg/mL for calibration standards and 0.0039, 0.077, 0.15 and 0.44 mg/mL for the QC samples were

prepared in a matrix comprised of Milli-Q water-ACN (50:50, v/v). A 10 µL aliquot of

working solution was spiked into the sputum sample, vortex mixed for 10 sec and 110 µL of

alkaline SDS solution was added. Following vortex mixing for 10 sec, the sample was left to

stand in ice water for 5 min, 220 µL of ACN added, vortex mixed for 10 sec and centrifuged

at $20,817 \times g$ for 10 min. A supernatant volume of 320 µL was transferred onto a SPE

cartridge. Quality control samples above the calibration curve (40 mg/L) was diluted to within

the calibration curve range with eluting matrix of boric acid-ACN-acetone (30:25:45, v/v/v).

Following sample pre-treatment as per Section 2.2.2.2.2, 30 µL of supernatant was injected

onto the HPLC column. Colistin A and B eluted at approximately 6.6 min and 5.6 min,

respectively as shown in Figure 2-15. No interfering peaks with colistin peak A or B were

observed.
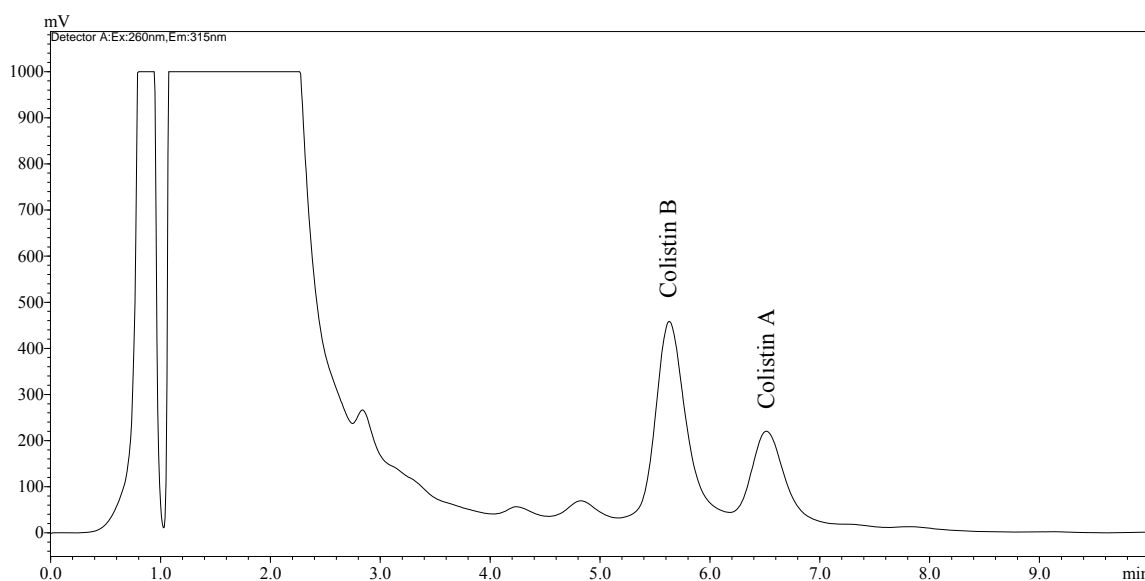
Figure 2-15: A typical HPLC chromatograph of fluorescent derivatives of colistin A and B
for colistin 16 mg/L in human sputum.

2.3.1.4.2 Colistin methanesulphonate

An assay for the quantification of CMS in human sputum has not been reported in the

literature. CMS calibration standards ranging from 6.25 to 200 mg/L was selected and

prepared by spiking in 10 µL of CMS working solution (prepared in Milli-Q water-ACN,

50:50, v/v) into 100 µL of homogenised CMS/colistin-free sputum. Following conversion of CMS to colistin, a 1:1 ratio of ACN was added, centrifuged and supernatant transferred onto a SPE cartridge. The generated CMS calibration curve is shown in Figure 2-16 which shows linearity throughout the concentration range. When compared to the colistin sputum assay, the absence of non-linearity was possibly due to higher CMS concentrations present in the calibration curve. In order to ensure consistency in the treatment of sputum samples for the quantification of CMS and colistin, alkaline SDS solution was included in the preparation of CMS calibration standards and QC samples.
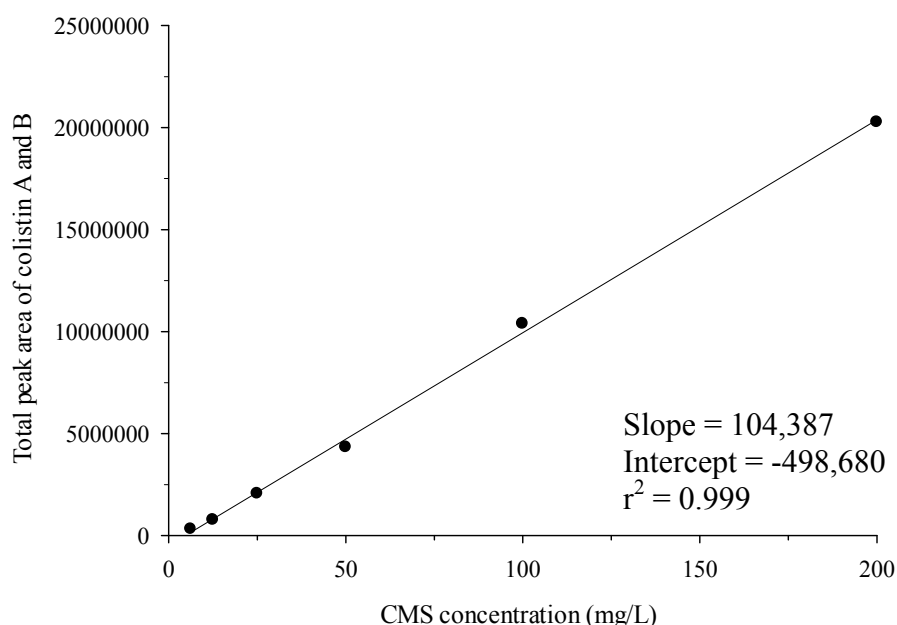
Figure 2-16: A typical calibration curve of CMS in human sputum. Note that CMS concentrations in the x-axis refer to sodium CMS concentrations.

Due to the limited availability of colistin/CMS-free sputum, initial CMS assay runs were prepared in *P. aeruginosa*-broth matrix. Following IV and pulmonary CMS administration, a wide CMS concentration range in sputum was expected and therefore CMS calibration standards ranging from 0.125 to 24 mg/L in *P. aeruginosa*-broth matrix were prepared with incorporation of QC sample of 0.38, 12, 20 and 200 mg/L. The high QC sample was diluted

to within the calibration curve range using eluting matrix of boric acid-ACN-acetone (30:25:45, v/v/v) (therefore dilution post-cartridge loading). Calibration standards and QC samples were prepared by spiking in 10 µL of CMS working solution (prepared in Milli-Q water-ACN, 50:50, v/v) into 100 µL of *P. aeruginosa*-broth sample, 1:1 ratio of alkaline SDS solution added, let to stand in in ice water for 5 min, CMS converted to colistin followed by protein precipitation with a 1:1 ratio of ACN. The generated CMS calibration curve was linear and the low, medium, and high QC samples were within the accuracy limit of ± 15%. However the estimated CMS concentration for the 200 mg/L QC sample which was diluted to 10 mg/L post-cartridge loading was 38% lower than the nominal concentration. From these findings we speculated that potential drug loss was occurring during the cartridge loading step, despite the total volume of supernatant for the 200 mg/L CMS sample loaded onto the cartridge in 4 aliquots (4 × 125 µL). The assay was repeated (calibration standards ranging from 0.125 to 16 mg/L; similar to the colistin sputum assay) and the estimated CMS concentration for the 200 mg/L QC sample was 20% lower than the nominal concentration. Therefore dilution of QC samples to within the calibration range with eluting matrix was not feasible. Thereby dilution of QC samples before loading onto SPE cartridges needed to be investigated.

In colistin/CMS-free sputum matrix, CMS calibration standards ranging from 0.125 to 16 mg/L with incorporation of 0.35, 7.0, 14 and 200 mg/L QC samples were prepared as described above. High QC samples (200 mg/L) were diluted to within calibration curve range with CMS/colistin-free sputum (therefore dilution pre-cartridge loading). The generated CMS calibration curve was linear and the low, medium and high QC samples were within the accuracy limit. However for CMS 200 mg/L the estimated concentration was 27% lower than the nominal concentration. To confirm these findings, the sputum assay was repeated a further two times (with incorporation of the 200 mg/L QC sample) with the estimated CMS

concentration being 28% and 15% lower than the nominal CMS 200 mg/L concentration. As initially proposed during the development of the colistin sputum assay (Section 2.3.1.4.1), dilution with homogenised sputum may not be accurate due to the viscous nature of the matrix and therefore a possible explanation for the 200 mg/L CMS samples not been within the accuracy limits. To avoid the need for dilution of sputum samples (pre- and post-cartridge loading), a decision was made to prepare two separate CMS calibration curves with concentrations ranging from 5.0 to 320 mg/L and 0.13 to 2.0 mg/L to analyse CMS concentrations following pulmonary and IV administration, respectively.

In *P. aeruginosa*-broth matrix, CMS calibration standards of 5.0 to 320 mg/L were linear and QC samples of 9.4, 125 and 300 mg/L were within the accuracy limit. However, on three separate occasions, preparation of CMS calibration standards ranging from 5.0 to 320 mg/L in sputum matrix showed a plateau at CMS concentrations above 100 mg/L as shown in Figure 2-17. The absence of such observations in CMS calibration prepared in *P. aeruginosa*-broth suggested that the sputum matrix may be contributing to the low recovery of CMS at the higher concentrations.
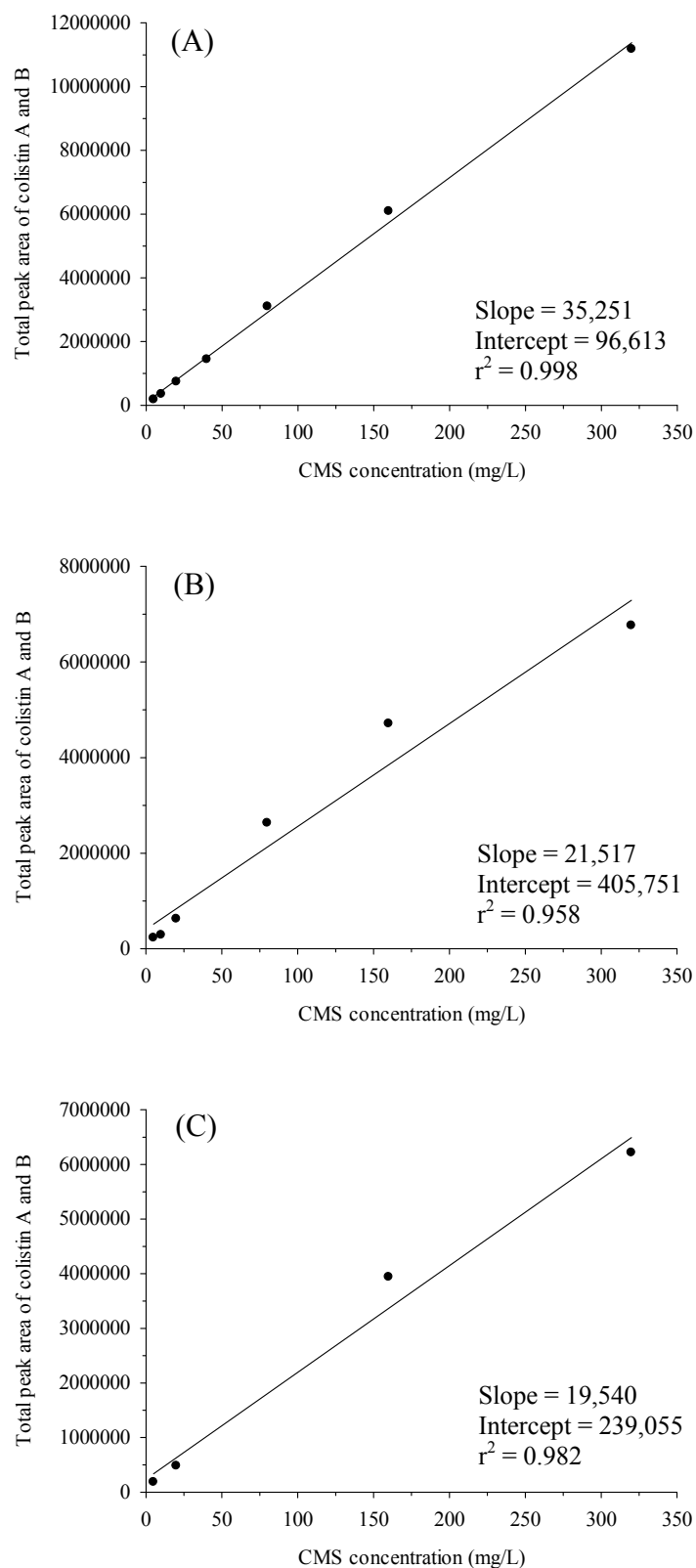
Figure 2-17: Calibration curves of CMS prepared in human sputum on three separate occasions (A, B and C). Note that CMS concentrations in the x-axis refer to sodium CMS concentrations.

To overcome the issues relating to the low recovery of CMS at high concentrations, an assay that involved dilution of CMS calibration standards back down to a concentration range below 100 mg/L was considered. CMS calibration standards ranging from 5.0 to 320 mg/L were prepared in sputum as described above and a five-fold dilution with blank broth prior to cartridge loading was conducted which resulted in a concentration range of 1 – 64 mg/L. CMS QC samples at concentrations of 9.4, 300 and 500 mg/L which underwent similar dilution were incorporated into the assay. Samples were treated with alkaline SDS solution and following conversion of CMS to colistin, a 1:1 ratio of ACN was added and following centrifugation the supernatant was transferred onto SPE cartridges. QC samples above the calibration curve (500 mg/L) were diluted to within the calibration curve range with eluting matrix of boric acid-ACN-acetone (30:25:45, v/v/v), following cartridge loading. For this assay a linear relationship between nominal CMS concentrations and total peak area was observed and the estimated concentrations of all QC samples were within the accuracy limit of ± 15%. Therefore this CMS sputum assay with incorporation of QC samples were prepared and validated.

In *P. aeruginosa*-broth and sputum matrix, CMS calibration standards ranging from 0.13 to 2.0 mg/L were linear with QC samples of 0.38, 0.75 and 1.5 mg/L falling within the accuracy limit. The following CMS calibration standards with incorporation of QC samples were prepared and validated.

Preparation of calibration and quality control samples

Two independent stock solutions of CMS 40 and 60 mg/mL (to prepare calibration standards ranging from 5 to 320 mg/L and QC samples) and two independent stock solutions of CMS 3.0 mg/mL (to prepare calibration standards ranging from 0.13 to 2.0 mg/L and QC samples) were prepared in Milli-Q water.

CMS calibration standards ranging from 5 to 320 mg/L with QC samples of 9.4, 125, 300 and 500 mg/L were prepared in colistin/CMS-free sputum. Working solutions (prepared in Milli-Q water-ACN, 50:50, v/v) ranging from 0.055 to 3.5 mg/mL were prepared for calibration standards and 0.10, 1.38, 3.3 and 5.5 mg/mL for QC samples. To 100 µL of homogenised CMS/colistin-free sputum 10 µL of working solution was added and vortex mixed for 10 sec. To enable a five-fold dilution of CMS concentrations, 440 µL blank broth was added and vortex mixed for 10 sec. A 1:1 ratio of alkaline SDS solution was added, vortex mixed and left to stand in ice water for 5 min. A 500 µL aliquot of the sample was transferred into a separate microcentrifuge tube and for CMS conversion to colistin 67 µL of 1.0 M H_2SO_4 was added, allowed 10 min reaction time and followed by addition of 133 µL of 1.0 M NaOH. Protein precipitation was carried out with a 1:1 ratio of ACN after vortex mix for 10 sec. Following centrifuge at $20,817 \times g$ for 10 min, a supernatant volume of 1 mL in two loading steps (2×500 µL) was transferred onto SPE cartridge. QC samples (500 mg/L) above the calibration curve was diluted to within the calibration curve range with eluting matrix of boric acid-ACN-acetone (30:25:45, v/v/v), following cartridge loading. Following sample pre-treatment as per Section 2.2.2.2.2, 10 µL of supernatant was injected onto the HPLC column. Colistin A and B eluted at approximately 6.7 min and 5.7 min, respectively as shown in Figure 2-18. No interfering peaks with colistin peak A and B were observed.
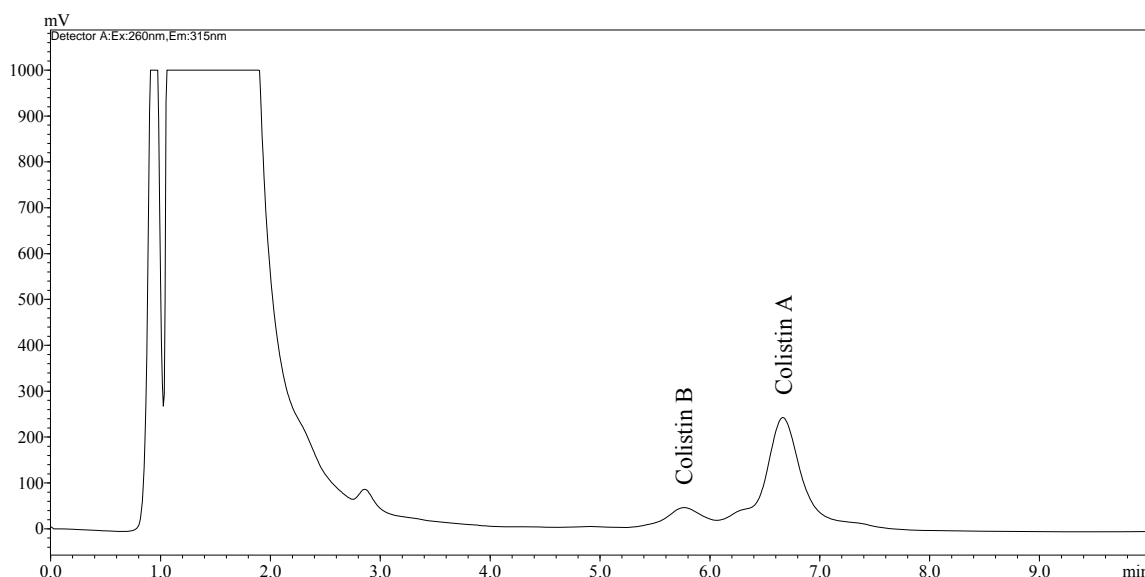
Figure 2-18: A typical HPLC chromatograph fluorescent derivatives of colistin A and B for CMS 32 mg/L in human sputum. Note colistin A and B corresponds to CMS A and B, respectively.

CMS calibration standards ranging from 0.13 to 2.0 mg/L with QC samples of 0.38, 0.75, 1.5 and 5 mg/L were prepared in colistin/CMS-free sputum. Independent working solutions of CMS (prepared in Milli-Q water-ACN, 50:50, v/v) ranging from 0.0014 to 0.022 mg/mL were used to prepare calibration standards and for the QC samples concentrations of 0.0041, 0.0083, 0.017 and 0.055 mg/mL were used. A 10 µL aliquot of working solution was added to 100 µL homogenised CMS/colistin-free sputum, vortex mixed for 10 sec and 110 µL of alkaline SDS solution was added. Following vortex mixing for 10 sec, samples were left to stand in ice water for 5 min. To convert CMS to colistin, 29 µL of 1.0 M H_2SO_4 was mixed and allowed 10 min reaction time and 59 µL of 1.0 M NaOH was added to stop the reaction. A 1:1 ratio of ACN was vortex mixed for 10 sec and centrifuged at 20,817 × *g* for 10 min. A supernatant volume of 500 µL (in 2 aliquots of 250 µL) was transferred onto a SPE cartridge. QC samples above the calibration curve (5 mg/L) was diluted to within the calibration curve range with eluting matrix of boric acid-ACN-acetone (30:25:45, v/v/v), following cartridge loading. Following sample pre-treatment as per Section 2.2.2.2.2, 50 µL of supernatant was

injected onto the HPLC column. Colistin A and B eluted at approximately 7.0 min and 6.0 min, respectively, with the generated HPLC chromatograph similar to that of the CMS sputum assay (Figure 2-18) developed to quantify CMS concentrations following pulmonary administration. No interfering peaks with colistin peak A or B were observed.

2.3.1.5 Validation

Validation for the human plasma, urine and sputum assays were conducted as outlined in Section 2.2.2.4. To determine the slope, intercept and r^2, linear least-squares regression analysis of the calibration curves with weighting of 1/response of the data was carried out. The performance characteristics of colistin and CMS assays in human plasma, urine and sputum are shown in Tables 2-6, 2-7 and 2-8, respectively.

Table 2-6: Intra-day (n=6), inter-day (n=3) and LOQ (n=6) quality control samples for colistin and CMS in human plasma HPLC methods.

	Intra-day			Inter-day			LOQ
Colistin (mg/L)[¥]	**0.31**	**2.49**	**4.97**	**0.31**	**2.49**	**4.97**	**0.104**
Mean conc (mg/L)	0.33	2.65	5.25	0.31	2.48	5.10	0.13
Accuracy (%)	5.23	6.51	5.67	-1.54	-0.60	2.65	20.0
Precision (%)	6.69	2.63	4.61	6.35	4.27	5.52	3.27
Colistin (mg/L)[§]	**0.31**	**0.62**	**1.24**	**0.31**	**0.62**	**1.24**	**0.104**
Mean conc (mg/L)	0.27	0.57	1.19	0.32	0.59	1.22	0.12
Accuracy (%)	-11.5	-7.36	-3.65	1.71	-4.30	-1.97	18.5
Precision (%)	3.72	3.34	5.08	4.92	6.68	2.72	8.43
CMS (mg/L)[¥]	**1.17**	**18.7**	**37.5**	**1.17**	**18.7**	**37.5**	**0.73**
Mean conc (mg/L)	1.10	19.0	35.7	1.21	20.0	39.4	0.76
Accuracy (%)	-6.05	1.34	-4.88	3.77	7.16	4.97	3.50
Precision (%)	6.40	1.45	4.57	7.03	6.04	6.79	2.26
CMS (mg/L)[§]	**0.35**	**0.70**	**1.41**	**0.35**	**0.70**	**1.41**	**0.12**
Mean conc (mg/L)	0.32	0.65	1.30	0.34	0.69	1.38	0.12
Accuracy (%)	-9.38	-7.15	-8.09	-2.36	-0.96	-1.84	0.52
Precision (%)	11.3	7.18	6.59	10.6	7.31	5.98	5.11

[¥] Colistin and CMS plasma assays used to quantify drug concentrations in human samples following IV CMS administration from the pharmacokinetic studies in Chapter 6.
[§]Colistin and CMS plasma assays to quantify drug concentrations in human samples following pulmonary CMS administration from the pharmacokinetic studies in Chapter 6.

Table 2-7: Intra-day (n=6), inter-day (n=3) and LOQ (n=6) quality control samples for colistin and CMS in human urine HPLC methods.

	Intra-day				Inter-day				LOQ
Colistin (mg/L)	**0.29**	**5.8**	**11.6**	**33.2**	**0.29**	**5.8**	**11.6**	**33.2**	**0.104**
Mean conc (mg/L)	0.29	6.11	12.1	34	0.29	5.61	11.4	34.3	0.12
Accuracy (%)	-1.18	5.35	4.67	2.39	0.77	-3.22	-1.68	3.28	11.6
Precision (%)	2.86	7.48	11.3	12.5	14.1	4.73	4.17	2.27	7.06
CMS (mg/L)	**0.33**	**6.56**	**13.1**	**141**	**0.33**	**6.56**	**13.1**	**141**	**0.12**
Mean conc (mg/L)	0.31	6.22	12.7	137	0.30	6.53	13.5	151	0.14
Accuracy (%)	-6.51	-5.24	-3.37	-2.88	-10.5	-0.46	2.75	6.91	14.4
Precision (%)	3.36	6.95	3.74	7.20	10.3	9.99	12.9	8.85	9.78

Table 2-8: Intra-day (n=6), inter-day (n=3) and LOQ (n=6) quality control samples for colistin and CMS in human sputum HPLC methods.

	Intra-day*				Inter-day				LOQ
Colistin (mg/L)	**0.29**	**5.80**	**11.6**	**33.2**	**0.29**	**5.80**	**11.6**	**33.2**	**0.104**
Mean conc (mg/L)	0.29	5.78	11.7	34.4	0.29	6.03	11.9	34.3	0.10
Accuracy (%)	-1.00	-0.30	0.77	3.55	-0.03	3.94	2.98	3.30	-3.37
Precision (%)	6.84	3.99	3.99	3.96	5.46	4.02	5.32	2.66	9.32
CMS (mg/L)[¥]	**8.78**	**117**	**281**	**468**	**8.78**	**117**	**281**	**468**	**4.68**
Mean conc (mg/L)	9.02	108	260	460	8.73	114	287	484	5.31
Accuracy (%)	2.74	-7.92	-7.45	-1.75	-0.60	-2.28	2.21	3.49	13.5
Precision (%)	7.40	6.40	2.91	5.37	7.77	9.28	9.92	13.6	7.21
CMS (mg/L)[§]	**-**	**-**	**-**	**-**	**0.35**	**0.70**	**1.41**	**4.68**	**0.12**
Mean conc (mg/L)	-	-	-	-	0.39	0.71	1.51	5.17	0.14
Accuracy (%)	-	-	-	-	11.7	1.97	7.00	10.4	17.3
Precision (%)	-	-	-	-	12.8	12.0	7.79	10.8	3.34

*Due to the limited availability of CMS/colistin-free sputum an intra-day run for CMS calibration standards ranging from 0.13 to 2.0 mg/L could not be carried out.
[¥]CMS sputum assay to quantify drug concentrations in human samples following pulmonary administration from the pharmacokinetic studies in Chapter 6.
[§]CMS sputum assay to quantify drug concentrations in human samples following IV administration from the pharmacokinetic studies in Chapter 6.

2.3.1.6 Estimation of colistin and CMS concentrations in human biological samples

Colistin and CMS concentrations in subject plasma, urine and sputum samples were estimated as detailed out in Section 2.2.2.5.

2.3.1.7 Summary

The HPLC analytical methods for assay of colistin and CMS in human biological matrices (plasma, urine and sputum) were validated and used for the quantification of colistin and CMS in plasma, urine and sputum samples from CF subjects (Chapter 6).

2.4 Conclusion

Methods for the quantification of colistin in dosing solutions and quantification of colistin and CMS in rat and CF subject biological samples of various matrices have been developed and validated. All assays demonstrated accuracy and precision over the specified concentration ranges. Quantification of colistin and CMS concentration for the pharmacokinetic studies in rats (Chapters 3 and 4) and CF subjects (Chapter 6) were conducted using these validated assays.

Chapter 3: Evaluation of pulmonary and systemic pharmacokinetics of colistin in rats following direct administration to the lungs *versus* intravenous administration

3.1 Introduction

Over the last 20 years there has been an increase in the delivery of colistin via inhalation for the treatment of multidrug-resistant (MDR) Gram-negative respiratory infections in cystic fibrosis (CF) patients and critically-ill patients with ventilator-associated pneumonia (VAP) [94, 97-99]. In clinical settings colistin methanesulphonate (CMS), the inactive prodrug of colistin [9] is typically administered, since it is associated with fewer pulmonary adverse effects than colistin, which is the active antibacterial moiety [159, 160, 166, 167]. Despite the increasing frequency of inhaled delivery of CMS there is a dearth of information on the pharmacokinetic characteristics of CMS and formed colistin following pulmonary administration. Such information is vital to optimise inhaled dosing regimens to ensure effective therapeutic outcomes and minimise the potential for emergence of resistance for an antibiotic that is reserved as a last line of defence against MDR Gram-negative infections [94, 97, 98].

Delivery of antibiotics directly to the site of respiratory infections has potential benefits when compared to systemically administered antibiotics. A relatively rapid onset of action with prolonged exposure to high concentrations of antibiotic whilst minimising systemic exposure can be achieved following pulmonary when compared to intravenous (IV) delivery [20, 21, 197]. Pulmonary delivery is more likely to achieve therapeutic concentrations at the respiratory infection site at relatively lower doses compared to IV administration [20, 21]. To achieve similar lung antibiotic exposure following IV administration higher doses and/or increase in frequency of dosing is required [20, 21] since systemically administered antibiotics must cross several biological barriers, such as endothelial and epithelial membranes, before reaching pulmonary target sites; and there are competing systemic dispositional events occurring. Higher systemic exposure following IV administration is likely to be associated with increased probability of systemic adverse effects.

Overall there is a lack of information on the relative concentrations of antibiotics in the lungs and plasma following either IV or pulmonary administration; this is certainly the case for colistin. Absence of this knowledge has the potential to lead to inadequate dosing regimens which promote therapeutic failure and facilitate bacterial resistance. Therefore, understanding the pharmacokinetics of CMS and formed colistin in the lungs and systemic circulation after pulmonary and IV administration is important to preserve the effectiveness of this important antimicrobial agent. Recognising that colistin (as its sulphate salt) is not routinely administered via inhalation or intravenously in the clinical setting a number of research groups have primarily focused on understanding the pharmacokinetics of CMS and formed colistin [126, 129, 170, 184, 185, 187, 189, 198-202]. A limited number of clinical studies have investigated the pulmonary tolerability [159, 160] and systemic exposure of colistin [160] following pulmonary administration of colistin. Pre-clinical studies which have investigated the pharmacokinetics of colistin following IV and subcutaneous (SC) administration in Sprague-Dawley rats were carried out by Li *et al* [203] and Marchand *et al* [184], respectively, with the main focus being on the systemic pharmacokinetics of colistin. However to date, no single study has been conducted to define the pharmacokinetics of colistin in plasma and in the lungs following both IV and pulmonary administration of colistin. Therefore the targeting advantage (i.e. maximising pulmonary exposure whilst minimising the systemic exposure) that can be achieved with pulmonary administration compared to IV delivery of colistin has not been investigated. This pharmacokinetic information for colistin will also be important to develop a comprehensive understanding of the disposition of formed colistin following administration of the prodrug, CMS (discussed in Chapter 4). Thus, there is need for a pre-clinical study that investigates the relative concentrations of colistin in the lungs and in plasma following pulmonary and IV administration of colistin.

This chapter describes, for the first time; 1) the pharmacokinetics of colistin in plasma following IV and pulmonary dose-ranging studies, and 2) the pharmacokinetics of colistin in the lung epithelial lining fluid (ELF) following a single pulmonary and IV administered dose of colistin, in rats.

3.2 Hypotheses and aims

It was hypothesised that:

1. Relative to IV delivery, pulmonary administration of colistin will result in higher ELF colistin concentrations and reduced systemic exposure to colistin.

To address this hypothesis, the study aims were to:

1. Determine the pulmonary and systemic pharmacokinetics of colistin in rats following direct administration to the lungs and IV delivery, over a range of doses; and

2. Undertake pharmacokinetic analysis to elucidate the targeting advantage (pulmonary exposure *versus* systemic exposure) achieved by direct administration of colistin to the lungs.

3.3 Materials

Colistin sulphate was purchased from Sigma-Aldrich (Missouri, USA) and Millex®-GV 0.22 μm filter units from Millipore (Country Cork, Ireland). Sodium chloride (NaCl) was purchased from Chem-Supply (South Australia, Australia), potassium chloride (KCl) from Ajax Laboratory Chemicals (New South Wales, Australia), disodium hydrogen phosphate (Na_2HPO_4) from Ajax Finechem Pty Ltd (New South Wales, Australia) and potassium dihydrogen phosphate (KH_2PO_4) from Merck Pty Ltd (Victoria, Australia). All chemicals were of analytical grade. Water was purified using a Milli-Q® water purification system from Millipore Corp. (Massachusetts, USA).

Isoflurane and acepromazine maleate was obtained from Delvet Pty Ltd (New South Wales, Australia) and medical-grade carbanox purchased from Coregas (New South Wales, Australia). Xylazine hydrochloride and ketamine hydrochloride were purchased from Ilium (New South Wales, Australia), sterile 0.9% sodium chloride from Baxter Healthcare Pty Ltd (New South Wales, Australia) and pentobarbitone sodium from Virbac Pty Ltd (New South Wales, Australia). Heparin sodium was purchased from Hospira Pty Ltd (Victoria, Australia), bupivacaine hydrochloride from AstraZeneca (New South Wales, Australia) and ethanol from Merck (Hesse, Germany).

Polyethylene tubing (single lumen, 0.80×0.50 mm/0.96×0.58 mm/1.70×1.20 mm (outer diameter (o.d) $\times$ inner diameter (i.d))) was purchased from Microtube Extrusions (New South Wales, Australia). Hypodermic needles (18 Gauge (G)/23 G/25 G $\times$ 40 mm, single use) were purchased from Terumo Corporation (Kantō, Japan). Surgical sutures were obtained from Johnson and Johnson (New South Wales, Australia). The small animal laryngoscope (rat) Model LS-1 and laryngoscope blade were from PennCentury Inc (Pennsylvania, USA).

3.4 Methods

3.4.1 Animals

Male Sprague-Dawley rats (300 to 320 g) were obtained from Monash Animal Services (Monash University). Animals were acclimated for a minimum of seven days in the animal house within a temperature range of 18-24°C, 40-70% relative humidity and 12 h light/dark cycles. Food and water were available *ad libitum* during the acclimation period and throughout the study. All pharmacokinetic studies were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the study protocols were reviewed and approved by the Monash Institute of Pharmaceutical

Sciences Animal Ethics Committee at Monash University (Monash University Ethics approval number VCPA2008.7).

3.4.2 Surgical procedures

Rats were lightly anaesthetised with gaseous isoflurane (2.5% v/v) prior to administration of a SC injection of an anaesthetic cocktail defined as 'cocktail I' in Table 3-1. During surgery if further anaesthesia was required a SC injection of a second cocktail, defined as 'cocktail II', was administered (Table 3-1). Depth of anaesthesia was monitored by visual observation of respiration rate, movement of whiskers/paws and response to the toe-pinch test.

Table 3-1: Composition, dose, route and typical duration of action of cocktail I and II anaesthetic.

	Cocktail I	Cocktail II
Composition:		
Ke	1.9 mL	1.0 mL
Xyl	0.5 mL	-
Ace	0.2 mL	0.2 mL
NaCl	2.5 mL	1.2 mL
Dose (mL/kg)	1.0	0.44
Dosing route	SC	SC
Typical duration of action (h)	1.5	1.0

Ketamine hydrochloride 100 mg/mL (Ke), xylazine hydrochloride 100 mg/mL (Xyl), acepromazine maleate 10 mg/mL (Ace), sodium chloride 0.9% (NaCl).

For animals receiving IV doses of colistin, the right jugular vein and carotid artery were cannulated in order to infuse dosing solutions and to collect blood samples, respectively. The right carotid artery was cannulated for collection of blood samples from the animals receiving pulmonary doses. The rat was placed in a supine position on a heated surgical board (37ºC) and the incision site was shaved and swabbed with ethanol (97% v/v). A longitudinal 2.5 cm incision was made anterior to the cervical vertebrae above the right sternum using surgical scissors. By means of blunt dissection, connective tissues were separated to expose the right

jugular vein and carotid artery. Using fine forceps the vein and artery were isolated from surrounding tissue and nerves, and blood supply to the vessel incision site was occluded to facilitate cannulation. Following a small incision on the artery and vein, the vessels were cannulated with 0.80 × 0.50 mm (o.d. × i.d.) polyethylene tubing which was pre-filled with heparinised saline (10 international units (IU)/mL) to prevent blood clot formation and occlusion of the cannula. The cannula was advanced approximately 2.5 cm from the site of cannulation. A surgical clamp temporarily held the cannula in position while four surgical sutures were positioned to permanently secure the cannula. The in-dwelling cannulae were exteriorised by tunnelling subcutaneously, surfacing from an incision posterior to the cervical vertebrae. The incision sites (anterior and posterior to the cervical vertebrae) were closed with four sutures and Marcain® (bupivacaine 0.5%) was applied topically for local analgesia. Subsequently the cannulae were connected to a harness and swivel system. The animals remained on the heated surgical board in a prone position until initial signs of recovery from anaesthesia were evident, such as movement of whiskers and an increase in breathing rate. The rats were placed in individual metabolism cages and the harness/swivel systems were attached to clamp-retort stands. The rats were closely monitored for full recovery from anaesthesia. The rats had free access to water and food and were given 24 h to recover from surgery prior to commencement of the study.

3.4.3 Pulmonary administration and sampling techniques

3.4.3.1 Intratracheal instillation

Intratracheal (IT) instillation was utilised as the pulmonary delivery method to assess the pharmacokinetics of colistin. Intratracheal instillation is widely used for pulmonary pharmacokinetic studies as it delivers accurate and reproducible dosing volumes into the lungs [204].

Prior to IT instillation each rat was lightly anaesthetised with gaseous isoflurane (2.5% v/v). The anaesthetised rat was rested in a supine position against a restraining board angled at approximately 60-70° from the horizontal [205] as shown in Figure 3-1A. The rat was held in the supine position by securing a wire (attached to the board) under the upper incisor teeth (Figure 3-1A) [205]. The longitudinal positioning was required for visualisation of the vocal cords. Using forceps, the tongue was gently pulled outwards and the blade of the laryngoscope was guided and positioned in the distal part of the mouth to enable visualisation of the vocal cords [205] as shown in Figure 3-1B. With the laryngoscope securing the tongue in place, the forceps were placed aside and a 2.5 cm polyethylene cannula (0.96 x 0.58 mm (o.d. x i.d.)) attached to a 23G needle and 1 mL syringe was manoeuvred past the vocal cords to the trachea-bronchus bifurcation and the dose delivered into the lungs (Figure 3-1C) [205]. The length of 2.5 cm cannula is representative of the distance from the vocal cords to the trachea-bronchus bifurcation in a Sprague-Dawley rat (300-320 g). A 100 μL aliquot of dosing solution was delivered into the rat lung followed by a 200 μL bolus of air. The air was delivered to ensure that the dosing solution was completely emptied from the syringe and cannula and ensured a consistent volume of dosing solution delivered to the rat [205]. Following IT instillation, rats were returned to the metabolic cage and monitored for recovery from anaesthesia, which typically occurred within 3 min.
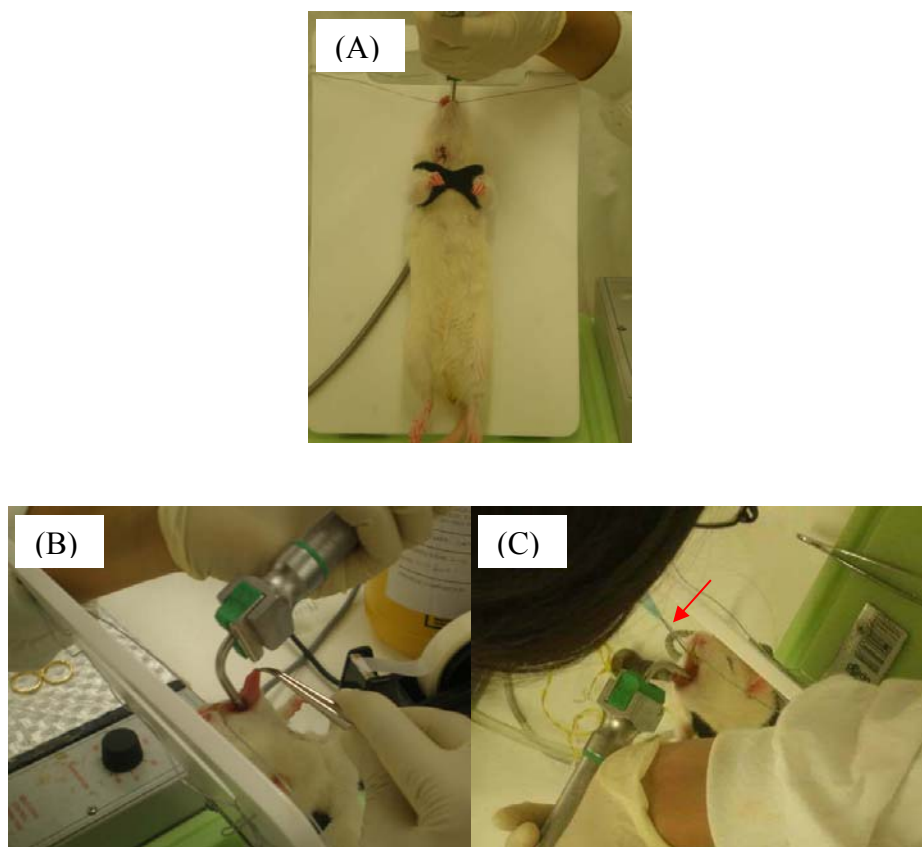
Figure 3-1: Intratracheal (IT) instillation, (A) supine position of the rat against a 60-70º restraining board, (B) insertion of the laryngoscope to visualise the vocal cords and (C) manoeuvring of a 2.5 cm cannula attached to a needle and syringe (shown by the red arrow) to the vocal cords.

3.4.3.2 Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was a technique carried out to sample the lung ELF. Rats were initially anaesthetised with gaseous isoflurane (5% v/v) and humanely sacrificed via exsanguination prior to the collection of BAL fluid sample. A 2.5 cm longitudinal incision was made anterior to the cervical vertebrae over the position of the trachea, and by means of blunt dissection, connective tissue was separated until the vocal cords and trachea were exposed. During the dissection process, rupture of surrounding blood vessels was avoided as the presence of blood can contaminate the BAL fluid sample and interfere with the urea analysis. The exposed trachea was isolated from surrounding tissues using fine forceps and maintained by placing a suture around the trachea. At a distance of 0.5 cm from the vocal

cords a small incision between two trachea cartridge rings was made allowing insertion of a polyethylene tube (1.70 × 1.20 mm (o.d × i.d)). The cannula was advanced (attached to a 18G needle) approximately 1 cm from the site of incision and was tightly secured to the trachea using a suture. A 5 mL syringe containing phosphate buffered saline (PBS, pH 7.4, 4°C) was attached to the 18G needle and the lungs were gently lavaged. This step was repeated a further two times using fresh PBS. The process of bronchoalveolar lavaging involves instillation and aspiration of PBS into the lungs, with an inflation of the rib cage evident with the instillation step; this process encompasses lavaging of the trachea, bronchioles and peripheral lungs. The composition and preparation of PBS used for lung lavage are summarised in Table 3-2.

Table 3-2: Composition and preparation of phosphate buffered saline (PBS) pH 7.4.

Salt	Amount* (g)
NaCl	8.0
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24

* Amount required for one litre. The appropriate amounts of salts were weighed and dissolved in 800 mL of Milli-Q water using sonication (~ 0.5 h). The volume was made up to 1 L with Milli-Q water and the pH checked. The solution of PBS was stored at 4°C until required for the BAL procedure.

3.4.4 Pharmacokinetic studies following intravenous and intratracheal administration of colistin

3.4.4.1 Preparation of colistin dosing solution

Colistin (sulphate) dosing solutions were prepared in normal saline on the day of dosing to deliver target doses outlined in Table 3-3. Dosing solutions were filtered using Millex®-GV

0.22 μm filter units and the concentration of the dosing solutions was confirmed using a ultraviolet high-performance liquid chromatography (UV-HPLC) assay as outlined in Chapter 2 (Section 2.2.1).

Table 3-3: Target doses and number of rats per treatment group for IV and IT pharmacokinetic studies.

Intravenous studies				
Target colistin sulphate dose (mg/kg)	0.25	0.50	0.75	
Volume administered (μL)	100	100	100	
Plasma pharmacokinetic study - number of rats (n)	3	3	3	
BAL pharmacokinetic study - number of rats (n)	-	14	-	
Intratracheal instillation studies				
Target colistin sulphate dose (mg/kg)	0.50	0.75	1.20	1.80
Volume administered (μL)	100	100	100	100
Plasma pharmacokinetic study - number of rats (n)	3	3	3	3
BAL pharmacokinetic study - number of rats (n)	-	21	-	-

3.4.4.2 Administration of intravenous colistin

Intravenous colistin sulphate doses of 0.25 mg/kg, 0.50 mg/kg and 0.75 mg/kg, hereafter expressed as colistin base doses 0.21 mg/kg, 0.41 mg/kg and 0.62 mg/kg in 100 μL were administered as a bolus injection in less than 5 sec via the jugular vein cannula. Immediately following administration, the cannula was flushed with 300 μL of heparinised saline (10 IU/mL) to ensure that the total dose was administered. The 25G needle attached to 1 mL syringe was weighed pre- and post-administration to determine the volume of the administered dosing solution for the calculation of the actual dose delivered. Following administration, animals were observed for signs of adverse effects.

In order to fully characterise the IV pharmacokinetics, two cohorts of animals were required. As described in more detail in the following paragraph, the rats in the first cohort were randomised into one of the three dosage treatment groups for serial blood sample collection

and animals in the second cohort received a single dose of colistin and animals were humanely sacrificed at predetermined time points for BAL fluid sample collection.

Animals in the first cohort were administered IV colistin at 0.21 mg/kg, 0.41 mg/kg or 0.62 mg/kg (n=3). Blood samples (200 µL) were collected via the carotid artery cannula prior to administration and at 0.02, 0.05, 0.08, 0.17, 0.33, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 h post-dose. Rats in the second cohort were administered IV colistin 0.41 mg/kg and BAL fluid and blood (200 µL) samples were collected at the following time points: 0.08, 0.25, 0.5, 1, 2, 3 and 4 h post-dose (n=2 rats per time point).

For blood sample collection, a 100 µL volume of heparinised saline/blood was initially drawn into a 1 mL syringe followed by 200 µL of blood drawn into a separate syringe. To minimise blood loss, the initial 100 µL of heparinised saline/blood was returned via the cannula followed by complete flushing with fresh heparinised saline (10 IU/mL). The 200 µL blood samples were transferred into pre-heparinised 1.5 mL microfuge tubes and immediately centrifuged ($6700 \times g$, 24°C, 10 min) in an Eppendorf Mini Spin® centrifuge. The plasma supernatant was removed and stored at -20ºC pending HPLC analysis for colistin concentration (described in Chapter 2, Section 2.2.2.2) and quantification of urea for the animals in the second cohort (described in Chapter 2, Section 2.2.3). At the conclusion of the pharmacokinetic study the rats were humanely sacrificed via an overdose of pentobarbitone administered through the carotid artery cannula. For the second cohort of animals, collection of BAL fluid samples was conducted as detailed in Section 3.4.3.2. The recovered lavage fluid was pooled, centrifuged ($6,700 \times g$, 4°C, 10 min) in an Eppendorf 5804 R centrifuge and the volume recorded. The BAL fluid supernatant was stored at -20°C pending analysis for colistin concentration (Chapter 2, Section 2.2.2.3) and urea (Chapter 2, Section 2.2.3).

3.4.4.3 Administration of intratracheal colistin

Colistin sulphate doses of 0.50 mg/kg, 0.75 mg/kg, 1.20 mg/kg and 1.80 mg/kg, hereafter expressed as colistin base doses 0.41 mg/kg, 0.62 mg/kg, 0.99 mg/kg and 1.49 mg/kg were administered into the lungs via IT instillation as described in Section 3.4.3.1. The needle (attached to the cannula) and syringe was weighed pre- and post-administration to determine the volume of dosing solution and calculation of the actual administered dose. Following colistin administration, animals were observed for signs of adverse effects.

Similar to the IV colistin studies, two cohorts of animals were required to fully characterise the pharmacokinetics. Rats in the first cohort were randomised into one of the four pulmonary dosage treatment groups for serial blood sample collection and whereas animals in the second cohort received a single dose of colistin and were humanely sacrificed at predetermined time points for BAL sample collection.

Animals in the first cohort were administered IT instillation of colistin at 0.41 mg/kg, 0.62 mg/kg, 0.99 mg/kg or 1.49 mg/kg (n=3). Blood samples (200 μL) were collected via the carotid artery cannula prior to colistin administration and at 0.02, 0.05, 0.08, 0.17, 0.33, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 h post-dose. Rats in the second cohort were administered IT instillation of colistin 0.62 mg/kg and BAL fluid and blood (200 μL) samples were collected at the following time points: 0.08, 0.5, 2, 4, 6, 8 and 12 h post-dose (n=3 rats per time point). The collection and processing of samples was the same as that outlined above. Plasma and BAL fluid samples were stored at -20ºC pending analysis for colistin concentration (Chapter 2, Section 2.2.2.2, 2.2.2.3 and 2.2.3) and urea (Chapter 2, Section 2.2.3).

3.5 Pharmacokinetic analysis

To adjust for any differences between target and actual administered doses (Section 3.4.4.2 and 3.4.4.3), dose-normalised concentrations were calculated as defined by Equation 3.1.

$$Dose-normalised\ colistin\ concentration$$

$$= measured\ colistin\ concentration\ (mg/L)$$

$$\times \frac{target\ dose\ (mg/kg)}{actual\ dose\ administered\ (mg/kg)}$$

Equation 3.1

Non-compartmental pharmacokinetic analysis (NCA) of the dose-normalised colistin concentration-*versus*-time profiles in individual rats was performed using WinNonlin (Version 5.3, Pharsight Corporation, Cary, North Carolina, USA). Peak plasma colistin concentration (C_{max}) and the time to reach peak concentration (T_{max}) were determined from the concentration-*versus*-time profiles following colistin pulmonary administration. The terminal rate constant (λ_z) and corresponding half-life $\left(t_{1/2}\right)$ were calculated by linear least-squares regression analysis using the last four log-transformed concentration-*versus*-time points. The area under the concentration-*versus*-time profile from time of dosing to the last sampling time ($AUC_{0-tlast}$) was calculated by the linear trapezoidal rule. The extrapolated area beyond the last quantifiable colistin concentration (C_{last}) of the concentration-*versus*-time profile was calculated from C_{last}/λ_z. Pharmacokinetic parameters $t_{1/2}$, CL and V_{ss} were calculated using the following equations:

$$t_{1/2} = 0.693/\lambda_z \qquad\qquad\qquad\qquad \textbf{Equation 3.2}$$

$$CL = D/AUC_{0-\infty} \qquad\qquad\qquad\qquad \textbf{Equation 3.3}$$

$$V_{ss} = D \times AUMC_{0-\infty}/AUC_{0-\infty}^2 \qquad\qquad\qquad \textbf{Equation 3.4}$$

where CL is the systemic clearance (determined after IV administration), D is the dose, $AUC_{0-\infty}$ is the area under the curve to time infinity, $AUMC_{0-\infty}$ is the area under the first moment curve to time infinity and V_{ss} is the volume of distribution at steady state (determined

after IV administration). Absorption half life $\left(t_{1/2,ab}\right)$ following pulmonary administration was calculated according to Equation 3.5, where k_a is the absorption rate constant. Absorption rate constant was estimated using a compartmental model (WinNonlin model 3: 1 compartment, 1st order absorption, no lag time, 1st order elimination).

$$t_{1/2,ab} = 0.693/k_a \qquad \qquad \textbf{Equation 3.5}$$

The mean systemic bioavailability (F%) following pulmonary administration was calculated from Equation 3.6. For the pulmonary doses, the systemic bioavailability was calculated using the mean $AUC_{0-\infty}$, the corresponding colistin IT dose and the mean systemic CL calculated from IV colistin doses of 0.21 mg/kg, 0.41 mg/kg and 0.62 mg/kg. Individual systemic bioavailability estimates were not calculated as the study was not a cross-over study where each rat received both an IV and pulmonary dose, instead each rat was administered either a single IV or pulmonary dose.

$$F = \frac{Mean\ AUC_{0-\infty,IT}}{D^{Colistin,IT}} \times CL_{Colistin,IV} \qquad \textbf{Equation 3.6}$$

The advantage of delivering colistin directly to the respiratory tract can be estimated by calculation of the therapeutic availability (TA) (Equation 3.7) [206] and drug targeting index (DTI) (Equation 3.8) [206, 207]. The TA for colistin was calculated using the ratio of the dose-normalised AUC (truncated to time *t*) in ELF following IT and IV colistin administration (Equation 3.7). The DTI for colistin was calculated using the ratio of the dose-normalised AUC (truncated to time *t*) in the ELF and plasma following IT colistin administration divided by the same ratio following IV colistin administration (Equation 3.8). Time *t* refers to 2 h or 12 h post-dose for estimation of DTI and TA, respectively, as discussed in Section 3.7.2.

$$TA_{Colistin} = \frac{\left(Mean\ ELF\ AUC_{0-t}/D^{Colistin}\right)_{IT}}{\left(Mean\ ELF\ AUC_{0-t}/D^{Colistin}\right)_{IV}}$$

Equation 3.7

$$DTI_{Colistin} = \frac{\left(\dfrac{Mean\ ELF\ AUC_{0-t}/D^{Colistin}}{Mean\ Plasma\ AUC_{0-t}/D^{Colistin}}\right)_{IT}}{\left(\dfrac{Mean\ ELF\ AUC_{0-t}/D^{Colistin}}{Mean\ Plasma\ AUC_{0-t}/D^{Colistin}}\right)_{IV}}$$

Equation 3.8

The volume of epithelial lining fluid, $V_{ELF,}$ and colistin concentrations in ELF were calculated as described in Chapter 2, Section 2.2.3.3. For the BAL studies, terminal BAL fluid sample collections were carried out to define the colistin concentrations in ELF. Thus at a single sample time the ELF concentration was the mean value from two individual rats following IV administration and three individual rats following pulmonary administration, and the pharmacokinetic parameters were calculated as described above from the mean values.

3.6 Statistical analysis

Dose-normalised plasma colistin concentrations were expressed as mean ± S.D. and dose-normalised ELF colistin concentrations were expressed as the mean value. Statistical analysis was performed using SPSS® Statistics (Version 20, IBM®, USA). Between dose comparisons were conducted on AUC, $t_{1/2}$, CL, V_{ss}, $t_{1/2,ab}$, V_{ELF} using one-way ANOVA and independent sample *t*-test analysis, as appropriate; $p < 0.05$ was regarded as a statistically significant difference.

3.7 Results

3.7.1 Dose-linearity

3.7.1.1 Intravenous colistin

Mean (± S.D.) plasma colistin concentrations as a function of time following IV bolus administration of colistin 0.21, 0.41 and 0.62 mg/kg are shown in Figure 3-2. Within the first 5 min following IV administration of the two higher doses the rats were noticeably less active within the metabolic cage and slight cyanosis was evident in the paws, ears and tail. These effects were transient with complete recovery observed 0.75-1 h after IV administration. Pharmacokinetic parameters of colistin in plasma following IV administration are summarised in Table 3-4. Linear pharmacokinetic behaviour was observed between $AUC_{0-\infty}$ and colistin doses as shown in Figure 3-3.

3.7.1.2 Intratracheal colistin

Mean (± S.D.) plasma colistin concentrations as a function of time following IT instillation of colistin 0.41, 0.62, 0.99 and 1.49 mg/kg are shown in Figure 3-4. Rapid appearance of colistin in plasma was observed with concentrations detected at 10 min for the 0.41 mg/kg dose, 3 min for the 0.62 mg/kg and 0.99 mg/kg doses and 1 min for the colistin dose of 1.49 mg/kg. Following IT administration of colistin 1.49 mg/kg, adverse effects similar to those observed after IV administration were evident at 20 – 30 min which corresponds to the maximal colistin concentrations (C_{max}) following pulmonary administration (Figure 3-4). These effects were transient with complete recovery observed at 1 h post-dose. Pharmacokinetic parameters of colistin in plasma following IT instillation are summarised in Table 3-5. Time to reach C_{max} ranged from 10 – 30 min as shown in Table 3-5. Linear pharmacokinetic behaviour was observed between $AUC_{0-\infty}$ and colistin doses as shown in Figure 3-5 and between C_{max} and colistin doses as shown in Figure 3-6. Systemic bioavailability across the dose range studied were 31%, 43%, 39% and 46% (Table 3-5).
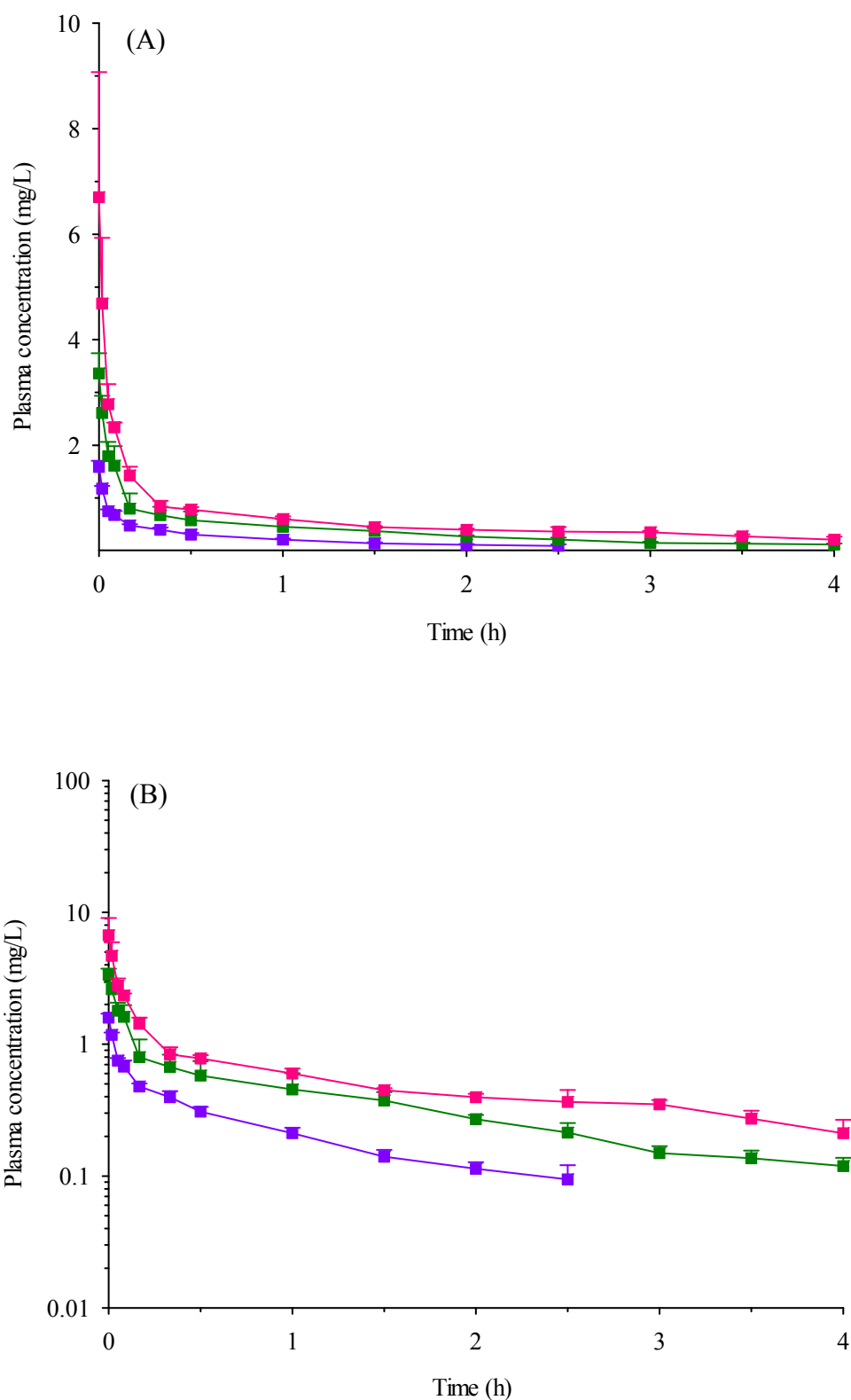
Figure 3-2: Plasma colistin concentration-*versus*-time profiles following IV administration of 0.21 (■), 0.41 (■) and 0.62 (■) mg/kg colistin (n=3). Colistin concentrations were below the limit of quantification (LOQ) at 3 h post-dose for colistin 0.21 mg/kg. (A): linear-linear coordinates; (B): semi-logarithmic coordinates. Concentrations are expressed as mean ± S.D.

Table 3-4: Pharmacokinetic properties of colistin in plasma following IV administration of colistin 0.21, 0.41 and 0.62 mg/kg (n=3). Data are expressed as mean ± S.D.

Parameters	0.21 mg/kg	0.41 mg/kg	0.62 mg/kg
CL (L/h/kg) [a]	0.27 ± 0.040	0.23 ± 0.033	0.22 ± 0.026
V_{ss} (L/kg) [a]	0.43 ± 0.032	0.51 ± 0.061	0.53 ± 0.075
$t_{1/2}$ (h) [a]	1.3 ± 0.25	1.9 ± 0.46	1.9 ± 0.51
$AUC_{0-\infty}$ (mg·h/L)	0.78 ± 0.12	1.8 ± 0.28	2.9 ± 0.33

[a] No statistically significant difference among doses after one-way ANOVA.
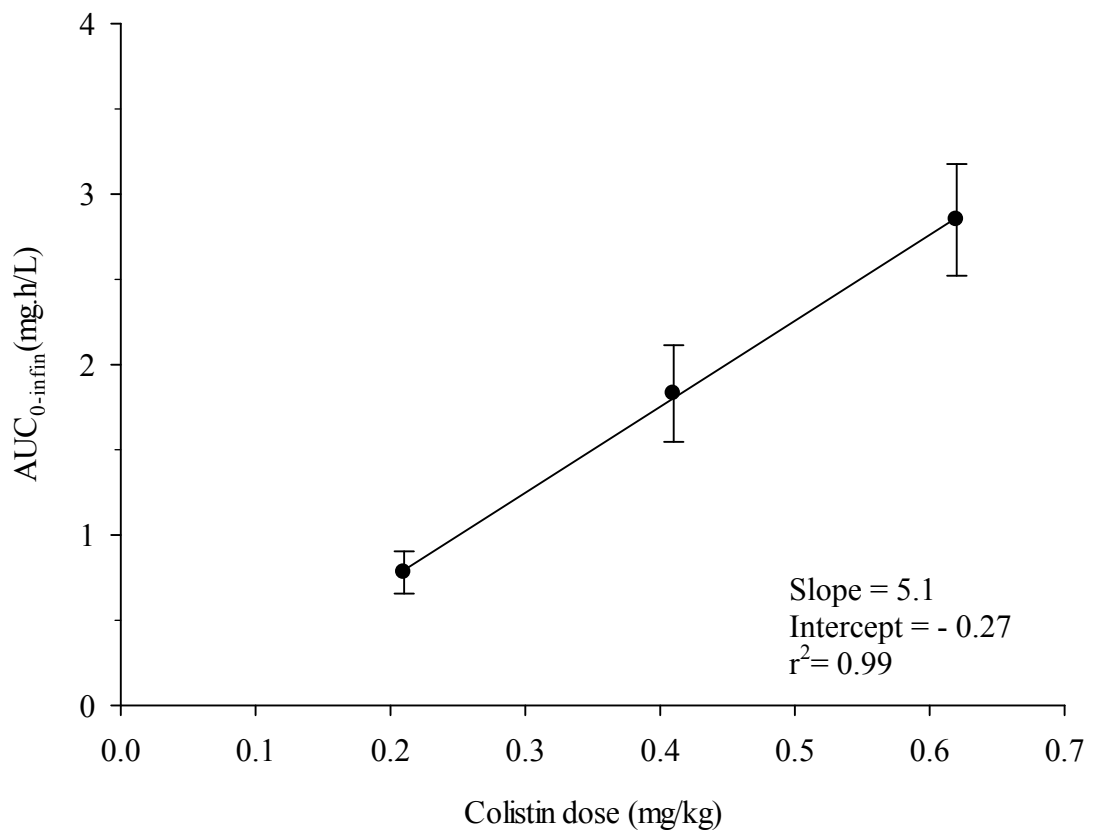
Figure 3-3: $AUC_{0-\infty}$ plotted against colistin dose following IV administration of 0.21, 0.41 and 0.62 mg/kg colistin (n=3). $AUC_{0-\infty}$ values are expressed as mean ± S.D.
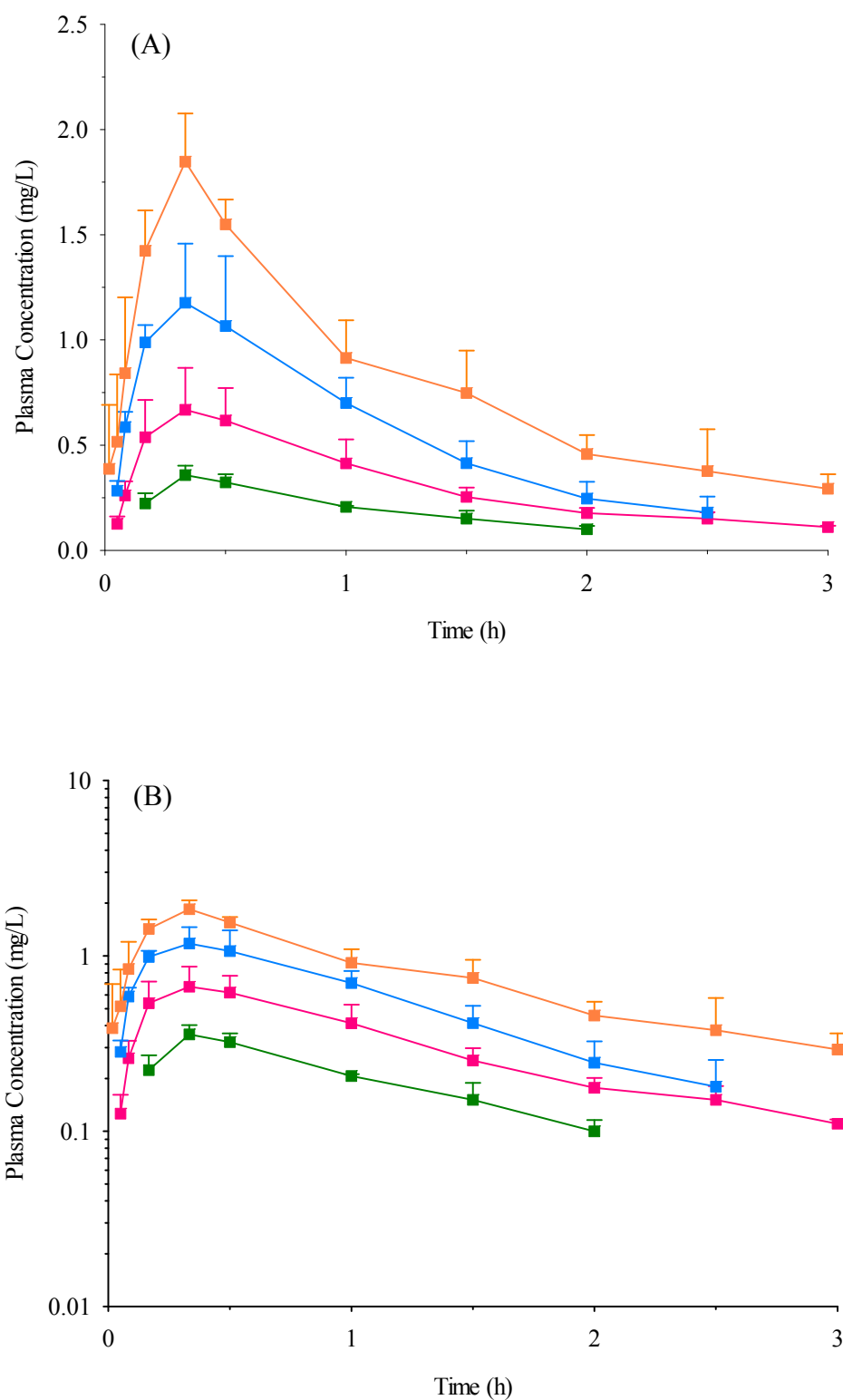
Figure 3-4: Plasma colistin concentration-*versus*-time profiles following IT instillation of 0.41 (■), 0.62 (■), 0.99 (■) and 1.49 (■) mg/kg colistin (n=3). Colistin concentrations were below the LOQ at 2.5 h post-dose for colistin 0.41 mg/kg, at 3 h for colistin 0.99 mg/kg and at 3.5 h for colistin 0.62 mg/kg and 1.49 mg/kg. (A): linear-linear coordinates; (B): semi-logarithmic coordinates. Concentrations are expressed as mean ± S.D.

Table 3-5: Pharmacokinetic properties of colistin in plasma following IT instillation of colistin 0.41, 0.62, 0.99 and 1.49 mg/kg (n=3). Data are expressed as mean ± S.D.

Parameters	0.41 mg/kg	0.62 mg/kg	0.99 mg/kg	1.49 mg/kg
Terminal $t_{1/2}$ (h) [a]	0.93 ± 0.25	1.3 ± 0.13	0.75 ± 0.17	1.2 ± 0.31
Absorption $t_{1/2}$ (h) [a]	0.16 ± 0.064	0.14 ± 0.036	0.13 ± 0.063	0.11 ± 0.052
T_{max} (h)	0.33	0.33-0.50 [ώ]	0.17-0.33 [ώ]	0.33
C_{max} (mg/L)	0.36 ± 0.049	0.67 ± 0.20	1.2 ± 0.24	1.8 ± 0.23
$AUC_{0-\infty}$ (mg·h/L)	0.55 ± 0.090	1.1 ± 0.20	1.6 ± 0.41	2.9 ± 0.59
F%	31	43	39	46

[a] No statistically significant difference among doses after one-way ANOVA.
[ώ] Represents T_{max} range.
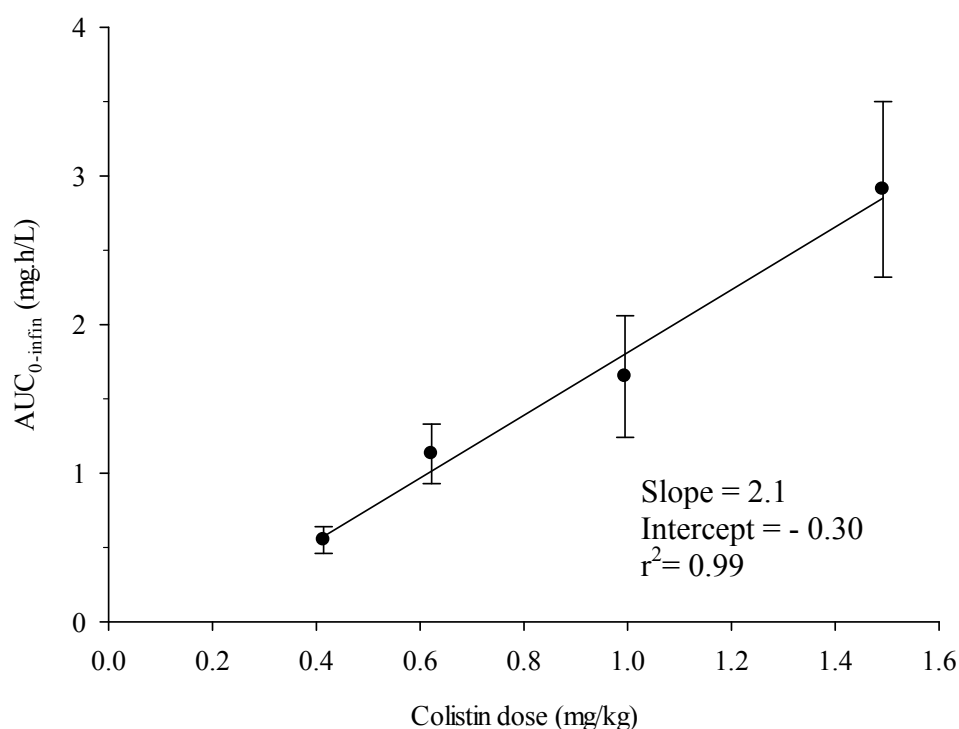
Figure 3-5: $AUC_{0-\infty}$ plotted against colistin dose following IT instillation of 0.41, 0.62, 0.99 and 1.49 mg/kg colistin (n=3). $AUC_{0-\infty}$ values are expressed as mean ± S.D.
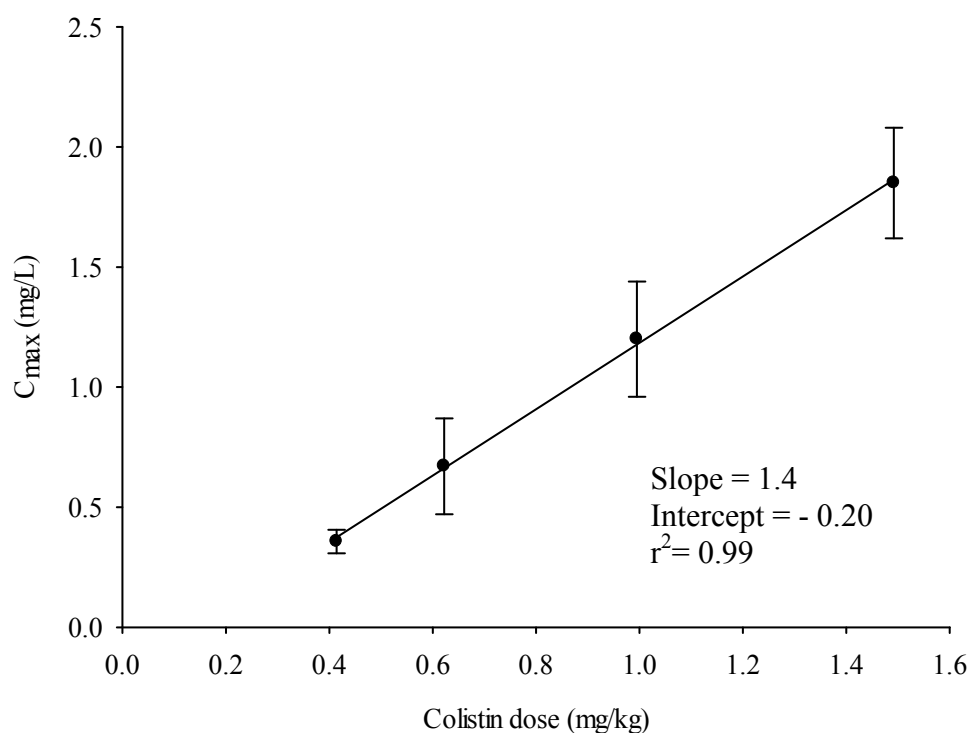
Figure 3-6: C_{max} plotted against colistin dose following IT instillation of 0.41, 0.62, 0.99 and 1.49 mg/kg colistin (n=3). C_{max} values are expressed as mean ± S.D.

3.7.2 Relative pulmonary and systemic exposures

Mean (± S.D.) urea concentration in BAL fluid and plasma, the percentage of recovered BAL fluid and the ELF dilution factor following IT and IV administration are summarised in Table 3-6. The V_{ELF} in rats (weighing between 300-320 g) was estimated to be 0.11 (± 0.023) mL and 0.088 (± 0.017) mL following IT and IV administration, respectively (Table 3-6).

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Table 3-6: Bronchoalveolar lavage parameter estimations following IT (n=21) and IV (n=14) administration of colistin. Parameters expressed as mean ± S.D.

Parameters	IT	IV
Recovered BAL fluid (%)	89 ± 7.1	89 ± 6.7
Urea conc - BAL fluid (mg/dL)	0.41 ± 0.14	0.42 ± 0.14
Urea conc - Plasma (mg/dL)	50 ± 9.2	63 ± 14
Dilution Factor	132 ± 36	156 ± 32
ELF volume (mL) [b]	0.11 ± 0.023	0.088 ± 0.017

[b] Statistically significant difference in ELF volume following IT and IV administration after independent samples *t*-test ($p < 0.05$).
dL denotes decilitre.

Mean colistin ELF concentrations and mean (± S.D.) colistin plasma concentrations as a function of time following IT instillation of colistin 0.62 mg/kg are shown in Figure 3-7A. Colistin concentrations in ELF remained at a steady level for 2 h post-dose and thereafter declined with a terminal half-life of 5.3 h. Relatively high colistin concentrations were present in ELF 12 h post-administration (46 mg/L) when compared to 3 h in plasma as shown in Figure 3-7A. Pharmacokinetic parameters of colistin in ELF and plasma following IT instillation of 0.62 mg/kg colistin are summarised in Table 3-7. Following pulmonary instillation of colistin 0.62 mg/kg, the $AUC_{0-\infty}$ of colistin in ELF was 1908 mg·h/L compared to 1.1 (± 0.20) mg·h/L in plasma (Table 3-7 and Figure 3-7B). Following absorption, a shorter terminal half-life of colistin in plasma (1.3 ± 0.13 h) was apparent compared to colistin in ELF (terminal half-life 5.3 h) (Table 3-7).
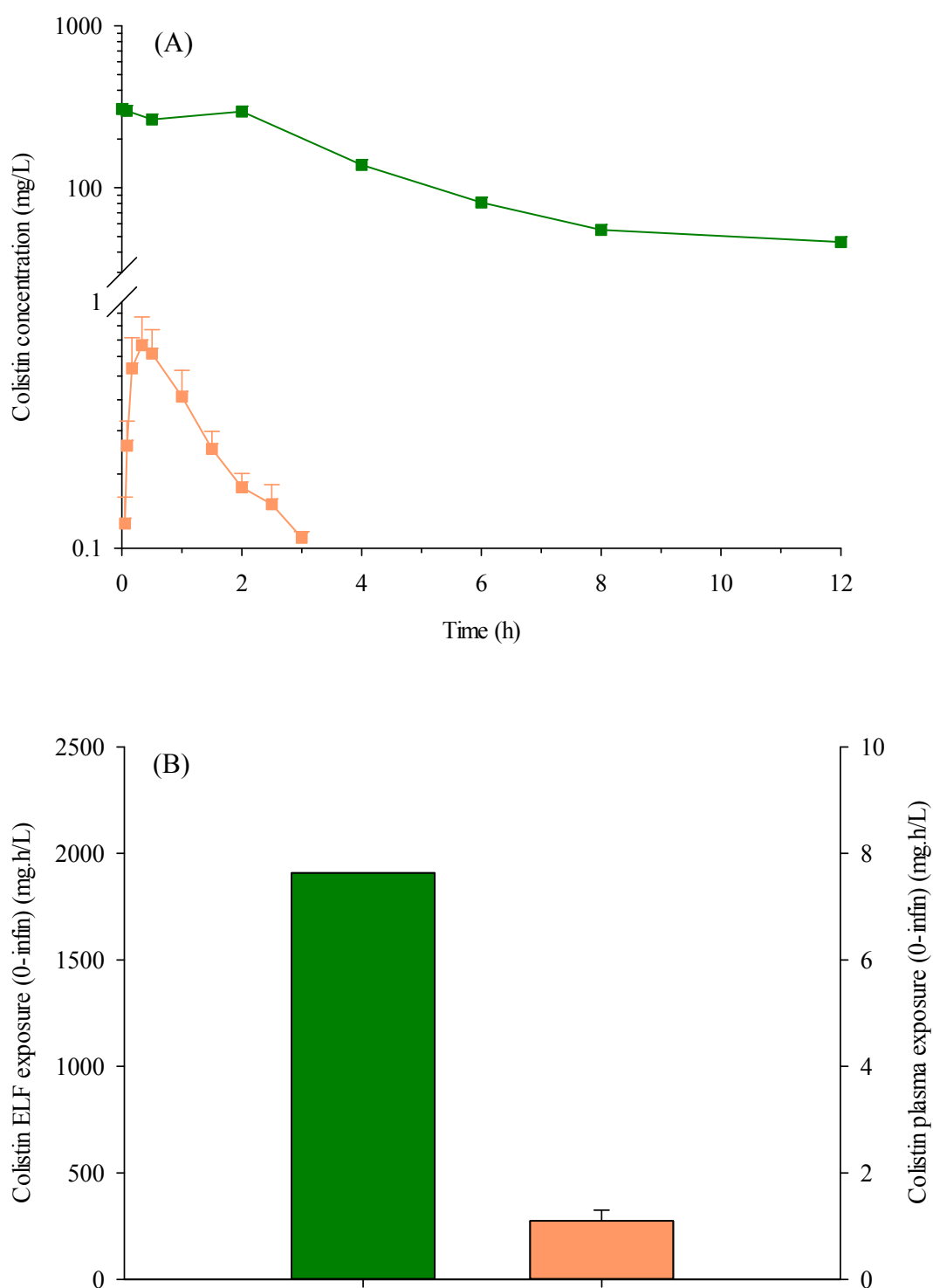
Figure 3-7: (A) ELF (■) (n=3 at each time point) and plasma (■) (n=3) colistin concentration-*versus*-time profiles following IT instillation of 0.62 mg/kg colistin (B) ELF and plasma $AUC_{0-\infty}$ following IT instillation of 0.62 mg/kg colistin. Colistin concentrations and exposure in ELF are represented as mean values and in plasma are represented as mean ± S.D. Note the broken scale for concentration in panel A, and the different scales for colistin exposure in ELF and plasma in panel B.

Table 3-7: Pharmacokinetic parameters of colistin in ELF and plasma following IT instillation of colistin 0.62 mg/kg. Colistin ELF estimates are expressed as mean values and colistin plasma estimates are expressed as mean ± S.D.

Parameters	ELF	Plasma
Terminal $t_{1/2}$ (h)	5.3	1.3 ± 0.13
Absorption $t_{1/2}$ (h)	-	0.14 ± 0.036
$AUC_{0-\infty}$ (mg·h/L)	1,908	1.1 ± 0.20
F%	-	43

Following IV administration of colistin 0.41 mg/kg, colistin concentrations were below the limit of quantification (LOQ) in BAL fluid of 0.012 mg/L at all sample times up to 4 h post-dose (n=2 rats per time point). It should be noted that three rats per time point was initially chosen to quantify colistin concentration in ELF after IV dosing, similar to the BAL studies following pulmonary administration. However given that concentrations were below the LOQ in BAL fluid a decision was made to reduce this number to two rats per time point. Plasma colistin concentrations were quantifiable for 4 h post-administration with concentrations ranging between 0.10 - 3.8 mg/L.

For the BAL studies, to enable comparison of colistin pharmacokinetics in ELF and plasma following IT and IV administration of the same colistin dose, a dose-normalisation of the pulmonary colistin dose of 0.62 mg/kg to 0.41 mg/kg was carried out. In the BAL studies a lower IV colistin dose (0.41 mg/kg) was administered to determine the ELF pharmacokinetic of colistin due to observation of transient adverse effects following IV colistin dose of 0.62 mg/kg in the IV dose-ranging studies (Section 3.7.1.1). Mean colistin ELF concentrations and mean (± S.D.) colistin plasma concentrations as a function of time following dose-normalised IT colistin 0.41 mg/kg and IV administration of colistin 0.41 mg/kg are shown in Figure 3-8. For all sample times, plasma colistin concentrations were higher following IV administration when compared to after IT administration, as shown in Figure 3-8. The pharmacokinetic

parameters of colistin in ELF and plasma after dose-normalised IT colistin 0.41 mg/kg and

following IV administration of colistin 0.41 mg/kg are summarised in Table 3-8. The $AUC_{0-\infty}$

of colistin in ELF was 1272 mg·h/L following dose-normalised IT colistin 0.41 mg/kg with

no quantifiable ELF concentrations following IV administration of the same dose as shown in

Table 3-8. The $AUC_{0-\infty}$ of colistin in plasma was 0.77 (± 0.17) mg·h/L and 1.8 (± 0.28)

mg·h/L for dose-normalised IT colistin dose and IV administration, respectively (Table 3-8).

The plasma terminal half-life values for colistin were 1.3 ± 0.24 h and 1.9 ± 0.46 h following

dose-normalised IT colistin dose and IV administration, respectively and were not

significantly different (Table 3-8).
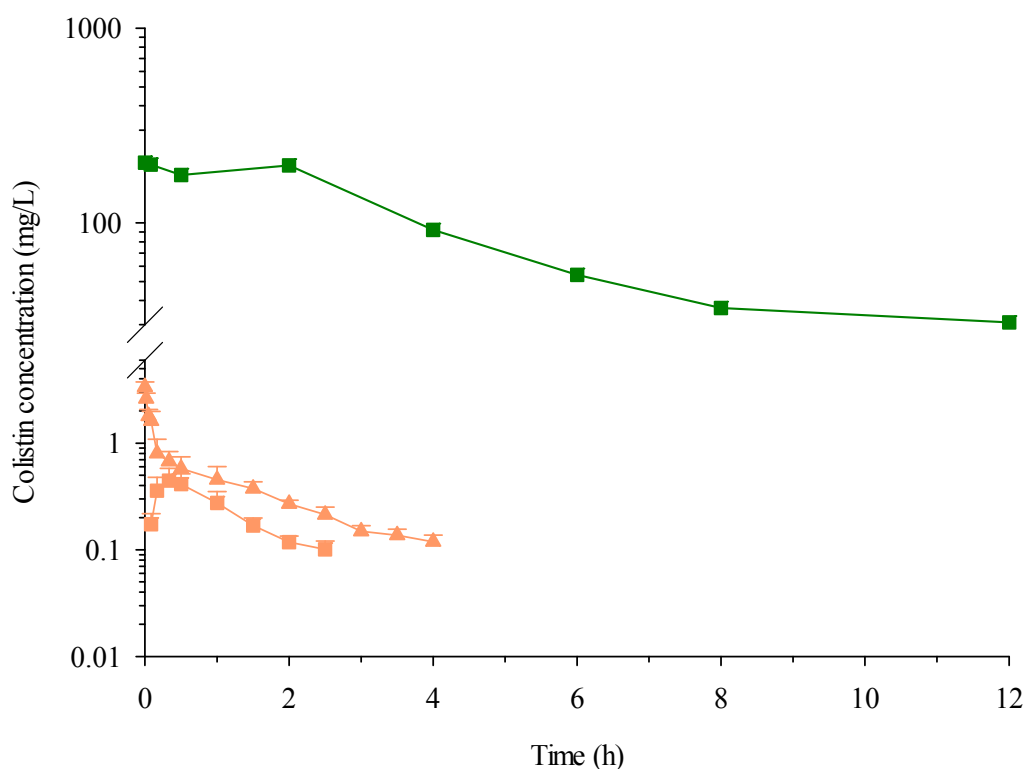
Figure 3-8: ELF (■) and plasma (■) colistin concentration-*versus*-time profiles following dose-normalised IT (■) colistin 0.41 mg/kg and IV (▲) administration of 0.41 mg/kg colistin. Colistin ELF concentrations following IV administration of 0.41 mg/kg colistin were below the LOQ concentration in BAL fluid (0.012 mg/L). Colistin concentrations in ELF are represented as mean values and in plasma are represented as mean ± S.D. Note the broken scale for concentration.

Table 3-8: Therapeutic availability (TA), drug targeting index (DTI) and pharmacokinetic parameters of colistin in ELF and plasma following dose-normalised IT colistin 0.41 mg/kg and following IV administration of colistin 0.41 mg/kg. Colistin ELF estimates are expressed as mean values and plasma estimates are expressed as mean ± S.D.

Parameters		
IT administration	**ELF**	**Plasma**
Terminal $t_{1/2}$ (h)	5.3	1.3 ± 0.24 [a]
AUC_{0-2} (mg·h/L)	375	0.52 ± 0.13
AUC_{0-12} (mg·h/L)	1,037	-
$AUC_{0-\infty}$ (mg·h/L)	1,272	0.77 ± 0.17 [b]
IV administration	**ELF**	**Plasma**
Terminal $t_{1/2}$ (h)	N.Q.	1.9 ± 0.46
AUC_{0-2} (mg·h/L)	3.9 [¥]	1.1 ± 0.18
AUC_{0-12} (mg·h/L)	23 [¥]	-
$AUC_{0-\infty}$ (mg·h/L)	N.Q.	1.8 ± 0.28
TA [§]	45	
DTI [ϒ]	210	

[a] No statistically significant difference in plasma terminal half-life following IT and IV administration after independent samples *t*-test.
[b] Statistically significant difference in plasma $AUC_{0-\infty}$ following IT and IV administration after independent samples *t*-test ($p < 0.05$).
N.Q. Not quantifiable as concentrations were less than the LOQ concentration in BAL fluid of the colistin assay.
[¥] ELF AUC following IV colistin administration was calculated using the LOQ concentration for colistin in ELF of 1.9 mg/L.
[§] TA estimated from AUC_{0-12} ELF values following IT and IV administration.
[ϒ] DTI estimated from AUC_{0-2} ELF and plasma values following IT and IV administration.

The targeting advantage of administering colistin via the IT rather than the IV route was demonstrated by a TA estimate of 45 and DTI estimate of 210 following dose-normalised IT colistin 0.41 mg/kg and following IV administration of colistin 0.41 mg/kg (Table 3-8). For estimation of TA and DTI, as colistin ELF concentrations were below the LOQ following IV administration the ELF exposure was calculated using the LOQ concentration in ELF for the colistin assay. The LOQ concentration in BAL fluid was 0.012 mg/L; with correction for the ELF-BAL fluid dilution factor following IV administration (Table 3-6) the LOQ in ELF was estimated to be 1.9 mg/L. Since the ELF exposure to infinity following IV administration

could not be calculated in the absence of the colistin terminal rate constant, the colistin ELF exposure to 2 h and 12 h was estimated as 1.9 mg/L × 2 h and 1.9 mg/L × 12 h, respectively. This method of estimating ELF colistin exposure following IV colistin administration results in an over-estimation of ELF exposure and hence an under-estimation of the targeting value. For estimation of TA, the ELF exposure following pulmonary and IV administration was calculated from time zero to 12 h post-dose. For estimation of DTI, the ELF and plasma exposure was calculated from time zero to 2 h post-dose.

3.8 Discussion

This chapter describes for the first time the pharmacokinetics of colistin in plasma and in the lungs following IV and pulmonary administration of colistin in rats. This enabled a better understanding of the disposition of colistin, the active antibacterial moiety of CMS, and the targeting advantage that can be achieved following direct delivery of colistin into the lung when compared to after IV administration. These findings facilitated a comprehensive understanding of the kinetics of formed colistin following CMS administration (Chapter 4).

Intravenous colistin dose-ranging studies were carried out to define the key pharmacokinetic parameters of colistin. Following IV administration, colistin followed linear pharmacokinetic behaviour for the dose-range studied (Table 3-4 and Figure 3-3). The negative y-intercept for the $AUC_{0-\infty}$ *versus* dose plot is likely to be due to the sensitivity limit of the analytical method and non-linear pharmacokinetic behaviour for IV doses below 0.21 mg/kg (Figure 3-3). The terminal half-life for colistin (Table 3-4) was comparable to that reported by Li *et al* (terminal half-life of 1.2 ± 0.22 h) following IV administration of 1.0 mg/kg colistin sulphate (corresponding to 0.83 mg/kg colistin base) to Sprague-Dawley rats [203]. Similarly, the systemic CL and V_{ss} estimated in the current study (Table 3-4) were consistent to that reported by Li *et al* (CL and V_{ss} of 0.31 ± 0.024 L/h/kg and 0.49 ± 0.060 L/kg, respectively) [203]. A

more recent pharmacokinetic study undertaken by Marchand *et al*, published after the current studies were conducted, reported the pharmacokinetics of colistin following SC administration of 1.5 mg/kg colistin sulphate (corresponding to 1.2 mg/kg colistin base) in Sprague-Dawley rats [184]. The estimated systemic CL (0.51 ± 0.060 L/h/kg) and V_{ss} (0.94 ± 0.25 L/kg) for colistin in the Marchand *et al* study [184] were higher than those observed in both the current study and the Li *et al* [203] study. The most likely reason for the inconsistency in the reported pharmacokinetic parameters is due to the assumption by the authors of 100% bioavailability of colistin in the systemic circulation following SC administration [184]. The authors may have made this assumption since administration of some drugs via the SC route can result in complete bioavailability; however in the case of colistin this has not been proven. Therefore the higher CL and V_{ss} is likely to be due to discrepancies in the amount of dose (D) available in plasma and the systemic exposure (AUC) following SC dosing, and as such these findings need to be interpreted with caution. The terminal half-life of colistin after IV dose-ranging studies was consistent to that of formed colistin following IV CMS administration as further discussed in Chapter 4 and consistent with the findings of Li *et al* [198, 203].

Following pulmonary administration, colistin followed linear pharmacokinetic behaviour for the dose-range studied (Table 3-5, Figure 3-5 and 3-6). Similar to the IV dose-ranging studies, the negative y-intercept observed for the $AUC_{0-\infty}$ *versus* dose plot (Figure 3-5) is likely to be due to the sensitivity limit of the analytical method and possible non-linearity at IT colistin doses below 0.41 mg/kg. The pulmonary dose-ranging studies demonstrated that colistin was rapidly absorbed into the systemic circulation with maximum colistin concentrations in plasma reached at 10 - 30 min post-administration (Table 3-5). During IT instillation an unknown fraction of the dose may have been delivered into the gastrointestinal tract however the contribution of oral absorption to the overall absorption profile would be expected to be

negligible [94, 208]. The systemic bioavailability across the IT colistin doses administered ranged from 31 – 46% and given the physicochemical properties of colistin (M_w 1163 Da, PSA 490 Å, Log P 3.42, pK_a ~ 10, Chapter 1, Section 1.5.2) the proposed mechanism of absorption through the pulmonary epithelium is likely to be via paracellular pathways such as passive diffusion through aqueous-filled pores (Chapter 1, Section 1.3.2.1). Studies to determine the exact mechanism of colistin absorption via lung epithelium have not been carried out, with a recent study by Marchand *et al* reporting that the apparent permeability (P_{app}) of colistin through a monolayer of Calu-3 cells was relatively low (0.042 ± 0.020 cm·s^{-1}) [184]. This suggests that colistin is slowly absorbed from the lungs which is in contrast to the rapid appearance of colistin in plasma in the current study. Following pulmonary administration, the plasma terminal half-life of colistin was significantly shorter than that after IV administration (Table 3-4 and Table 3-5), with such findings likely to be reflective of the study design (i.e. it was not a cross-over study). If the discrepancies in terminal half-life following IT and IV dose-ranging studies were due to the study design alone, this indicates that the systemic disposition of colistin following pulmonary administration was not rate-limited by absorption. This is an interesting observation given that the colistin ELF profiles demonstrated retention in the lining fluid with the colistin terminal half-life in ELF four-fold longer than the corresponding terminal half-life in plasma following IT instillation of colistin 0.62 mg/kg (Table 3-7) These findings suggest that following rapid absorption of a fraction of the IT colistin dose into plasma the remaining fraction resides in lung ELF with the major determinant for the persistence of colistin in ELF likely to be binding of colistin to lung tissue (discussed in more detail below).

Comparison of colistin disposition following pulmonary dosing with previously published data (both pre-clinical and clinical) was not possible as there are no studies which comprehensively analyse the pharmacokinetics of colistin following pulmonary

administration. Limited information in the literature is likely due to the fact that inhaled colistin is not used clinically as a result of colistin-induced lung adverse effects [159, 160]. Polymyxin B, belonging to the same class of antibiotics as colistin, differing only by a single amino acid in the chemical structure [91], is clinically administered via the pulmonary route and intravenously for the management of respiratory infections (VAP) in critically-ill patients [91, 92]. Therapeutic efficacy has been demonstrated when inhaled polymyxin B is used in combination with IV polymyxin B and other antibiotics [68, 89]. Unfortunately there is also limited information available about the pulmonary pharmacokinetics of polymyxin B [90-92]. Therefore findings from the current pre-clinical study will enable a better understanding of kinetics of polymyxin B in addition to that of formed colistin after CMS delivery.

A significant finding of the current study was that extensive exposure of colistin in ELF (45 – 307 mg/L) for an extended duration of time (12 h post-dose) was achieved following pulmonary instillation of 0.62 mg/kg colistin. Colistin concentrations in ELF were maintained well above the minimum inhibitory concentration required for 50 to 90% inhibition of bacteria growth based on clinical isolates of *P. aeruginosa* and *Acinetobacter* spp. (MIC_{50-90}) of 1.0 mg/L for colistin [172] for the 12 h sampling period. In rats the estimated ELF volume of 0.11 ± 0.023 mL (110 ± 23 μL) after IT instillation and 0.088 ± 0.017 mL (88 ± 17 μL) after IV delivery were within the range of previously published volumes of 7.6 ± 5.8 μL and 233 ± 59 μL by Marchand *et al* [184] and Zhang *et al* [209], respectively. Following IT instillation of 0.62 mg/kg colistin, rapid absorption of a fraction of the colistin dose into plasma was evident (as previously discussed) with a fraction of the dose retained in the lungs. In ELF the terminal half-life of colistin was four-fold longer (5.3 h compared to 1.3 ± 0.13 h) and the concentrations of colistin in ELF quantifiable for 12 h in comparison to 3 h, when compared to in plasma. The change in colistin concentrations in ELF can be accounted for by absorption processes and potentially by other pulmonary clearance mechanisms (Chapter 1,

Section 1.3.2.3, 1.3.2.4). The kinetics of colistin in ELF however does suggested a delay in the loss of colistin from ELF (similar to observations for formed colistin following IT CMS dosing in Chapter 4), which may be due to retention of colistin (binding) in the lungs following IT instillation. Basic amines ($pK_a > 8.5$) with lipophilic characteristics are known to be retained in the human, rabbit and rat lungs [48-50, 52, 53, 210] (Chapter 1, Section 1.3.2.2). The degree of protonation of the nitrogen atom has been proposed to play an important role in lung retention of these drugs [48-50]. Overall two accumulation sites in the lungs have been identified of which one is the alveolar macrophages [49-53, 210]; Wilson *et al* reported that in the isolated perfused rabbit lung, imipramine was slowly efflux from an accumulation site which was proposed to be the macrophages [51]. Bysani *et al* investigated the binding of polymyxin B to rat alveolar macrophages and reported that saturable cell membrane binding sites for polymyxin B exist and that this binding process was reversible [211]. Given the similarity in chemical structure between colistin and polymyxin B [91] it is reasonable to suggest that colistin may be binding to macrophages and over time being released due to the reversible nature of this binding. In addition, Kunin *et al* investigated the binding mechanism of polymyxin antibiotics to tissues and suggested that binding occurred via an electrostatic attraction between the positively charged amine groups to the negatively charged phospholipids of cell membranes [212]. Ziv *et al* reported that the bound concentration of colistin when compared to the unbound concentrations in the kidney, liver, lung, heart, skeletal muscle and brain was higher up to 48 h following IV administration of 5 mg/kg colistin sulphate (corresponding to 4.1 mg/kg colistin base) in calves [213]. Therefore the findings from these numerous studies suggest that binding may play a role in the disposition of colistin in the lungs. However at the present time, the exact mechanism/s of colistin binding to lung tissue is not known and more rigorous studies are needed to further

investigate the kinetics of binding as it may be a significant determinant for persistence of colistin in the lungs.

In contrast to the high ELF colistin concentrations observed following pulmonary administration, colistin pulmonary exposure was unquantifiable following IV administration of 0.41 mg/kg colistin. Following administration of colistin 0.41 mg/kg, the ELF exposure was 1272 mg·h/L after IT dosing (dose-normalised) whereas no ELF exposure was seen following IV dosing. Despite this, it is important to note that a significant difference in the systemic exposure was observed, with a two-fold greater exposure evident following IV compared to IT administration (Table 3-8). This was reflected in much lower IV colistin doses (0.21 mg/kg) required for animals to tolerate the dose when compared to pulmonary administration (0.41 mg/kg, 0.62 mg/kg and 0.99 mg/kg). Given that there are no pre-clinical studies in the literature reporting on the disposition of colistin in the lungs following IV administration, explanations for the unquantifiable exposure in the ELF was proposed to be due to binding of colistin to lung tissue (as previously discussed following IT colistin dosing), the BAL procedure diluting the relatively low concentrations of colistin in ELF and sensitivity limit of the analytical method. Interestingly, similar ELF exposure for formed colistin following IV CMS administration was observed as further explained in Chapter 4.

The targeting advantage achieved following pulmonary administration when compared to systemic delivery of colistin can be highlighted in a discussion about the TA and DTI [206, 207, 209]. The TA and DTI values give a quantitative assessment of the targeting benefit achieved following pulmonary administration of colistin. Since ELF colistin exposure following IV administration was below the LOQ concentration in BAL fluid, the ELF exposure was estimated using the LOQ concentration in ELF and as such it needs to be noted that the estimated TA and DTI values are an under-estimation of the targeting value. The TA provides an indication of the ELF availability of colistin. The estimated TA value was

magnitudes greater than unity which indicate that colistin exposure in ELF after IT administration was substantially higher than that achieved following IV administration (Table 3-8). The DTI gives an indication of the degree or effectiveness of targeting to the ELF that can be achieved following pulmonary when compared to IV administration [209]. The estimated value for DTI was far greater than unity which indicates that lung administration was an effective delivery route for targeted delivery to ELF as high lung exposure with lower systemic exposure can be achieved when compared to after the IV route (Table 3-8). Therefore for the first time this pharmacokinetic analysis has demonstrated that pulmonary administration of colistin was an effective delivery route to target colistin directly to the ELF, maximise lung exposure and minimise systemic exposure, when compared to IV administration. Whether similar targeting benefits are observed following administration of the clinically relevant form of colistin, CMS is further discussed in Chapter 4.

In conclusion this is the first study that has investigated the pharmacokinetics of colistin in plasma and in the lungs following IV and pulmonary administration of colistin in rats. Linear pharmacokinetics for colistin was observed following IV and IT dose-ranging studies. Pulmonary administration of colistin resulted in high ELF colistin concentrations that were maintained for an extended period of time and low systemic exposure when compared to IV administration. Pulmonary administration of colistin in rats has been demonstrated to be an effective delivery method for targeting colistin into the ELF while reducing the systemic exposure. The non-compartmental analysis in the present chapter has enabled a greater understanding of the pharmacokinetics of colistin in plasma and ELF, and such information will facilitate interpretation of the disposition of formed colistin following CMS administration in Chapter 4 and for the building of a population pharmacokinetic model for CMS and colistin as outlined in Chapter 5.

Chapter 4: Evaluation of pulmonary and systemic pharmacokinetics of colistin methanesulphonate and formed colistin in rats following direct administration to the lungs *versus* intravenous administration

4.1 Introduction

Colistin methanesulphonate (CMS) was introduced onto the market as a result of an increase in the prevalence of colistin induced toxicity, namely nephrotoxicity and neurotoxicity [93, 94, 97, 99]. Colistin methanesulphonate is an inactive prodrug of colistin [9]; therefore liberation of colistin is a prerequisite for antibacterial activity against multidrug-resistant (MDR) Gram-negative bacteria *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Klebsiella pneumoniae* [93, 94, 97, 99].

Colistin methanesulphonate has been administered intravenously for the treatment of MDR Gram-negative respiratory infections in cystic fibrosis (CF) patients experiencing acute pulmonary exacerbation with *P. aeruginosa* [13, 15, 187, 201] and in critically-ill patients with ventilator-associated pneumonia (VAP) caused by the above mentioned Gram-negative bacteria [18, 19, 123, 126, 188, 202, 214, 215]. Colistin-induced nephrotoxicity affects a significant proportion (30 – 55%) [154-156] of patients receiving intravenous (IV) CMS, and it is a dose-limiting adverse effect since it appears to be related to the duration of therapy and the cumulative amount of CMS delivered [97, 146, 154, 216]. For an antibacterial constrained by dose-limiting adverse effects, treatment of extravascular respiratory infections via the IV route may not be ideal since high doses administered more frequently would potentially be needed to achieve therapeutic CMS and formed colistin concentrations in the lungs.

Over the past two decades inhaled delivery of nebulised CMS for the treatment of MDR Gram-negative respiratory infections has increasingly been used in CF and critically-ill patients [3, 6, 129, 170, 184, 217, 218]. Pulmonary delivery of antibiotics directly to the site of respiratory infection has potential benefits such as achieving high concentrations, rapid onset of action, extensive duration of pulmonary exposure and minimising systemic exposure when compared to IV delivery of a similar dose [20, 21]. Therefore delivery of CMS directly

to the respiratory tract is likely to achieve high concentrations of CMS, and potentially formed colistin, at the infection site while minimising systemic exposure. In contrast, to achieve similar pulmonary exposure following IV administration greater systemic exposure would most likely be needed thereby increasing the potential for adverse effects, including nephrotoxicity.

Despite CMS being delivered via inhalation for over two decades only recently have studies investigating the concentrations of CMS and formed colistin in the lung following nebulised CMS in animals [184] and patients [129, 170] been conducted. The major limitations of these studies is the lack of data point/time course of CMS or formed colistin in the lungs which consequently means a thorough pharmacokinetic description was not possible [129, 170, 184]. As a consequence, there still is a lack of information on the relative concentrations of CMS and formed colistin in plasma and lung following either IV or pulmonary administration. Therefore, the advantage of local delivery of CMS directly to the lungs (i.e. maximise pulmonary exposure while minimising systemic exposure) has not been proven. As colistin is one of the last line of defence against MDR Gram-negative bacteria [94, 97, 98] there is a need for a pre-clinical study that investigates the pharmacokinetics of CMS and formed colistin in lung epithelial lining fluid (ELF) and plasma following IV and pulmonary administration of CMS.

This chapter describes, for the first time; 1) the pharmacokinetics of CMS and formed colistin in plasma following IV and pulmonary administration of CMS across a range of doses, and 2) the pharmacokinetics of CMS and formed colistin in lung ELF following pulmonary and IV administration a single dose of CMS in rats. Intravenous and pulmonary dose-ranging studies were conducted to determine the pharmacokinetic linearity of CMS and formed colistin.

Additionally, the pharmacokinetics of formed colistin following CMS administration was compared to the pharmacokinetic profile of colistin as described in Chapter 3.

4.2 Hypotheses and aims

It was hypothesised that:

1. Relative to IV delivery, pulmonary administration of CMS will result in higher ELF CMS and formed colistin concentrations;

2. Relative to IV delivery, pulmonary administration of CMS will result in a reduction in systemic exposure to CMS and formed colistin; and that

3. The pharmacokinetics of formed colistin in ELF following pulmonary administration of CMS will be similar to the pharmacokinetic observed after administration of colistin.

To address these hypotheses, the study aims were to:

1. Determine the pulmonary and systemic pharmacokinetics of CMS and formed colistin in rats following direct administration of CMS to the lungs and IV delivery, over a range of doses; and

2. Undertake pharmacokinetic analysis to elucidate the targeting advantage (pulmonary exposure *versus* systemic exposure) achieved by direct administration of CMS to the lungs.

4.3 Materials

Sodium colistin methanesulphonate was purchased from Link Pharmaceuticals Ltd (Auckland, New Zealand). All other materials are as that described in Chapter 3, Section 3.3.

4.4 Methods

4.4.1 Animals

Male Sprague-Dawley rats (300 to 320 g) were purchased and acclimatised as outlined in Chapter 3, Section 3.4.1. All pharmacokinetic studies were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the study protocols were reviewed and approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee at Monash University (Monash University Ethics approval number VCPA2008.7).

4.4.2 Surgical procedures

A day prior to the pharmacokinetic studies, the right carotid artery was cannulated in each rat to allow collection of blood samples and the jugular vein was cannulated in the cohort of animals receiving IV CMS. Details of the cannulation procedures are described in Chapter 3, Section 3.4.2.

4.4.3 Pharmacokinetic studies following intravenous and intratracheal administration of colistin methanesulphonate

4.4.3.1 Preparation of colistin methanesulphonate dosing solution

Colistin methanesulphonate (sodium) dosing solutions were prepared in normal saline on the day of dosing to deliver target doses outlined in Table 4-1. The dosing solutions were administered within 10 min following preparation to minimise potential *in vitro* CMS conversion to colistin. The dosing solutions were filtered using Millex®-GV 0.22 μm filter units.

Table 4-1: Target doses and number of rats per treatment group for the IV and IT pharmacokinetic studies.

Intravenous studies			
Target sodium CMS dose (mg/kg)	15	30	60
Volume administered (µL)	200	200	200
Plasma pharmacokinetic study - number of rats (n)	3	3	3
BAL pharmacokinetic study - number of rats (n)	22	-	-
Intratracheal instillation studies			
Target sodium CMS dose (mg/kg)	15	30	
Volume administered (µL)	100	100	
Plasma pharmacokinetic study - number of rats (n)	3	3	
BAL pharmacokinetic study - number of rats (n)	21	-	

BAL denotes bronchoalveolar lavage

4.4.3.2 Administration of intravenous colistin methanesulphonate

Intravenous sodium CMS doses of 15 mg/kg, 30 mg/kg and 60 mg/kg, hereafter expressed as CMS base doses 14 mg/kg, 28 mg/kg and 56 mg/kg in 200 µL were administered as a bolus injection in less than 5 sec via the jugular vein cannula. Immediately following administration, the cannula was flushed with 300 µL of heparinised saline (10 international units (IU)/mL) to ensure that the total CMS dose was administered. The 25 Gauge (G) needle attached to 1 mL syringe was weighed pre- and post-administration to confirm the volume of the dosing solution delivered and hence the actual dose administered. Following administration, animals were observed for signs of adverse effects.

In order to fully characterise the IV pharmacokinetics of CMS and formed colistin, two cohorts of animals were required. The rats in the first cohort were randomised into one of the three dosage treatment groups for serial blood sample collection and animals in the second cohort received a single dose of CMS and animals were humanely sacrificed at predetermined time points for bronchoalveolar lavage (BAL) fluid sample collection.

Animals in the first cohort were administered IV CMS at 14 mg/kg, 28 mg/kg or 56 mg/kg (n=3). Blood samples (320 µL) were collected via the carotid artery cannula prior to dosing and at 0.08, 0.25, 0.5, 1, 2, 3 and 4 h post-dose. Rats in the second cohort received IV CMS 14 mg/kg and BAL fluid and blood (320 µL) samples were collected at the following time points: 0.08, 0.25, 0.5, 1, 2, 3 and 4 h post-dose (n=3 rats per time point).

For blood sample collection, a 100 µL volume of heparinised saline/blood was initially removed into a 1 mL syringe followed by 320 µL of blood drawn into a separate syringe. To minimise blood loss, the initial 100 µL of heparinised saline/blood was returned via the cannula followed by complete flushing with fresh heparinised saline (10 IU/mL). The 320 µL of blood samples were transferred into pre-heparinised 1.5 mL microfuge tubes and immediately centrifuged (6,700 × *g*, 4°C, 10 min) in an Eppendorf 5804 R centrifuge. The plasma supernatant was removed and stored at -80°C pending high-performance liquid chromatography (HPLC) analysis for CMS and formed colistin concentration (Chapter 2, Section 2.2.2.2) and quantification of urea for the animals in the second cohort (Chapter 2, Section 2.2.3). At the conclusion of the pharmacokinetic study the rats were humanely sacrificed with an overdose of pentobarbitone administered via the carotid artery cannula. For the second cohort of animals, collection of BAL fluid samples was carried out as detailed in Chapter 3, Section 3.4.3.2. The recovered lavage fluid was pooled, centrifuged (6,700 × *g*, 4°C, 10 min) in an Eppendorf 5804 R centrifuge and the volume of BAL fluid recorded. The BAL fluid supernatant was stored at -80°C pending HPLC analysis for CMS and formed colistin concentration (Chapter 2, Section 2.2.2.3) and urea (Chapter 2, Section 2.2.3). To minimise potential *in vitro* CMS conversion to colistin during sample collection, blood and BAL fluid samples were placed on ice and immediately processed at 4°C proceeded by storage at -80°C. Furthermore, to minimise potential *in vitro* CMS conversion to colistin

during storage at -80°C, plasma and BAL fluid samples were analysed for CMS and formed colistin concentrations within 4 months of sample collection [219].

4.4.3.3 Administration of intratracheal colistin methanesulphonate

Pulmonary sodium CMS doses of 15 mg/kg and 30 mg/kg, hereafter expressed as CMS base doses 14 mg/kg and 28 mg/kg were administered into the lungs via pulmonary intratracheal (IT) instillation as described previously (Chapter 3, Section 3.4.3.1). Inclusion of a lower dose (7.0 mg/kg) was not feasible because plasma concentrations would likely be below the limit of quantification (LOQ) for the analytical method, nor was it appropriate to examine higher pulmonary doses where adverse effects were likely. The 23G needle (attached to a 2.5 cm polyethylene cannula (0.96×0.58 mm (o.d. $\times$ i.d.)) and 1 mL syringe was weighed pre- and post-administration to determine the volume of dosing solution and calculation of the actual administered dose. Following CMS administration, animals were observed for signs of adverse effects.

Similar to the IV studies, to fully characterise the pharmacokinetics of CMS and formed colistin following IT administration, two cohorts of animals were required. Rats in the first cohort were randomised into one of the two pulmonary dosage treatment groups for serial blood samples collection and animals in the second cohort received a single dose of CMS and animals were humanely sacrificed at predetermined time points for BAL fluid sample collection.

Animals in the first cohort were administered IT instillation of CMS at 14 mg/kg and 28 mg/kg (n=3). Blood samples (320 µL) were collected via the carotid artery cannula prior to CMS administration and at 0.08, 0.5, 1, 2, 3, 4, 5, 6 and 8 h post-dose. Rats in the second cohort received IT instillation of CMS 14 mg/kg and BAL fluid and blood (320 µL) samples were collected at the following time points: 0.08, 0.5, 2, 4, 6, 8 and 12 h post-dose (n=3 rats

per time point). The collection and processing of blood samples were as described in Section 4.4.3.2. The collection and processing of BAL fluid samples was as outlined in Chapter 3, Section 3.4.3.2 and Section 4.4.3.2, respectively. Plasma and BAL fluid samples were stored at -80ºC pending HPLC analysis (Chapter 2, Section 2.2.2.2, 2.2.2.3 and 2.2.3) and quantification of urea for the animals in the second cohort (Chapter 2, Section 2.2.3). To minimise potential *in vitro* CMS conversion to colistin during storage at -80°C, plasma and BAL fluid samples were analysed for CMS and formed colistin concentrations within 4 months of sample collection [219].

4.5 Pharmacokinetic analysis

To adjust for any difference between target and actual administered doses (Section 4.4.3.2 and 4.4.3.3), dose-normalised concentrations were calculated as defined by Equation 4.1.

$$Dose-normalised\ CMS\ and\ formed\ colistin\ concentration$$
$$= measured\ CMS\ and\ formed\ colistin\ concentration\ (mg/L)$$
$$\times \frac{target\ CMS\ dose\ (mg/kg)}{actual\ CMS\ dose\ administered\ (mg/kg)}$$

Equation 4.1

Non-compartmental pharmacokinetic analysis of the dose-normalised CMS and formed colistin concentration-*versus*-time profiles in individual rats was performed using WinNonlin (Version 5.3, Pharsight Corporation, Cary, North Carolina, USA). Peak CMS and formed colistin plasma concentrations (C_{max}) and the time to reach peak concentrations (T_{max}) were determined from the concentration-*versus*-time profiles. The terminal rate constant (λ_z) and corresponding half-life $\left(t_{1/2}\right)$ were determined by linear least-squares regression analysis using the last three or four log-transformed concentration-*versus*-time points. The exception to this was for the calculation of CMS terminal half-life in ELF following IV CMS

administration where limited terminal CMS concentrations meant that only the last two log-transformed concentration-*versus*-time points were utilised. Following pulmonary administration of CMS 14 mg/kg, weighting (1/response) was employed in the linear least-squares regression analysis of ELF CMS concentrations as the concentration range was large (100 – 3,000 mg/L). The area under the concentration-*versus*-time profile from time of dosing to the last sampling time ($AUC_{0-tlast}$) was calculated using the linear trapezoidal rule. The extrapolated area beyond the last quantifiable CMS and formed colistin concentration (C_{last}) of the concentration-*versus*-time profile was calculated from C_{last}/λ_z. Pharmacokinetic parameters $t_{1/2}$, CL and V_{ss} were calculated using the following equations:

$$t_{1/2} = 0.693/\lambda_z \qquad \qquad \textbf{Equation 4.2}$$

$$CL = D/AUC_{0-\infty} \qquad \qquad \textbf{Equation 4.3}$$

$$V_{ss} = D \times AUMC_{0-\infty}/AUC_{0-\infty}^2 \qquad \qquad \textbf{Equation 4.4}$$

where CL is the systemic clearance (determined for CMS after IV administration), D is the CMS dose, $AUC_{0-\infty}$ is the area under the curve to time infinity, $AUMC_{0-\infty}$ is the area under the first moment curve to time infinity and V_{ss} is the volume of distribution at steady state (determined for CMS after IV administration). The absorption half-life $\left(t_{1/2,ab}\right)$ for CMS following pulmonary administration was calculated according to Equation 4.5, where k_a is the absorption rate constant. Absorption rate constant was estimated using a compartmental model (WinNonlin, model 6: 1 compartment, 1st order absorption, lag time, 1st order elimination).

$$t_{1/2,ab} = 0.693/k_a \qquad \qquad \textbf{Equation 4.5}$$

The mean systemic bioavailability of CMS $\left(F_{systemic,CMS}\%\right)$ following pulmonary administration of CMS was calculated using Equation 4.6. For the pulmonary doses, the systemic bioavailability of CMS was calculated using the mean $AUC_{0-\infty}$, the corresponding CMS IT dose and the mean systemic CL for CMS calculated from IV CMS doses of 14 mg/kg, 28 mg/kg and 56 mg/kg. Individual systemic bioavailability estimates were not calculated as this study was not a cross-over study where each rat received both an IV and pulmonary dose; instead each rat was either administered a single IV or pulmonary dose.

$$F_{systemic,CMS} = \frac{Mean\ AUC_{0-\infty,CMS,IT}}{D^{CMS,IT}} \times CL_{CMS,IV}$$

Equation 4.6

The fraction of the IV CMS dose converted to colistin in the systemic circulation, $f_{m,systemic}$, was calculated using Equation 4.7 [198]. The calculation of $f_{m,systemic}$ relies on the inherent assumption that the disposition of formed colistin following administration of IV CMS is the same as the disposition of colistin following IV administration of colistin. For calculation of $f_{m,systemic}$ the mean area under the plasma concentration-*versus*-time profile of colistin and molar dose of IV colistin was obtained from Chapter 3, Section 3.7.1.1 following administration of 0.21 mg/kg colistin. This IV colistin dose was chosen as the plasma colistin concentrations were within a similar range (0.10 − 1.0 mg/L) to that of formed colistin concentrations following administration of IV CMS 14, 28 and 56 mg/kg. As mentioned above individual pharmacokinetic parameters were not determined.

$f_{m,systemic}$

$$= \frac{[(Mean\ plasma\ AUC_{0-\infty}\ of\ formed\ colistin\ after\ IV\ CMS) \times (Molar\ dose\ of\ IV\ colistin)]}{[(Mean\ plasma\ AUC_{0-\infty}\ of\ colistin\ after\ IV\ colistin) \times (Molar\ dose\ of\ IV\ CMS)]}$$

Equation 4.7

The apparent fraction of the IT CMS dose converted to colistin in ELF, $f_{m,ELF}$, was calculated using Equation 4.8 [198]. The calculation of $f_{m,ELF}$ relies on the assumption that the disposition of formed colistin in ELF following IT CMS administration is the same as the disposition of colistin in ELF following IT administration of colistin. This is contingent on the assumption that the same fraction of the dosing solution was administered into the ELF following IT CMS and colistin administration. For calculation of $f_{m,ELF}$ the mean area under the ELF concentration-*versus*-time profile of colistin and molar dose of IT colistin was obtained from Chapter 3, Section 3.7.2 following IT administration of colistin 0.62 mg/kg. The ELF colistin concentrations following IT administration of colistin were within a similar range (100 – 1000 mg/L) to that of formed colistin concentrations following administration of IT CMS 14 mg/kg. Individual estimates for $f_{m,ELF}$ were not calculated as this was not a cross-over study.

$$f_{m,ELF}$$

$$= \frac{[(Mean\ ELF\ AUC_{0-\infty} of\ formed\ colistin\ after\ IT\ CMS) \times (Molar\ dose\ of\ IT\ colistin)]}{[(Mean\ ELF\ AUC_{0-\infty} of\ colistin\ after\ IT\ colistin) \times (Molar\ dose\ of\ IT\ CMS)]}$$

Equation 4.8

Following pulmonary administration of CMS, the systemic exposure of formed colistin $\left(AUC_{colistin,plasma}^{CMS,IT}\right)$ is representative of systemically formed colistin following CMS absorption and absorption of pre-systemically formed colistin from the lungs, and can be calculated using Equation 4.9 [184].

$AUC_{colistin,plasma}^{CMS,IT}$

$$= \left[\frac{D \times \left(F_{systemic,CMS} \times f_{m,systemic} \right)}{CL} \right]$$

$$+ \left[\frac{D \times \left(f_{m,ELF} \times F_{systemic,colistin} \right)}{CL} \right]$$

Equation 4.9

where $F_{systemic,colistin}$ is the systemic bioavailability of pre-systemically formed colistin, CL is the systemic clearance of colistin following IV colistin administration (Chapter 3, Section 3.7.1.1) and D is the pulmonary CMS dose. Rearrangement of Equation 4.9 to Equation 4.10 enables calculation of the fraction of the pulmonary CMS dose that is bioavailable as colistin $\left(f_{m,ELF} \times F_{systemic,colsitin} \right)$ [184].

$$f_{m,ELF} \times F_{systemic,colistin} = \left(\frac{AUC_{colistin,plasma}^{CMS,IT} \times CL}{D} \right) - F_{systemic,CMS} \times f_{m,systemic}$$

Equation 4.10

As mentioned in Chapter 3 (Section 3.5) the advantage of pulmonary administration of antibiotics directly to the respiratory tract can be quantified by calculation of therapeutic availability (TA) (Equation 4.11) [206] and drug targeting index (DTI) (Equation 4.12) [206, 207]. The TA for CMS and formed colistin was calculated as the ratio of the dose-normalised AUC (to time *t*) in ELF following IT and IV CMS administration (Equation 4.11). The DTI for CMS and formed colistin was calculated as the ratio of the dose-normalised AUC (to time *t*) in the ELF and plasma following IT CMS administration divided by the same ratio following IV CMS administration (Equation 4.12). Time *t* refers to 4 h, 12 h post-dose for estimation of DTI and TA for colistin, respectively, and to infinity time for estimation of TA and DTI for CMS, as discussed in Section 4.7.2.

$$TA_{CMS/Formed\ colistin} = \frac{(Mean\ ELF\ AUC_{0-t}/D^{CMS})_{IT}}{(Mean\ ELF\ AUC_{0-t}/D^{CMS})_{IV}}$$

Equation 4.11

$$DTI_{CMS/Formed\ colistin} = \frac{\left(\dfrac{Mean\ ELF\ AUC_{0-t}/D^{CMS}}{Mean\ Plasma\ AUC_{0-t}/D^{CMS}}\right)_{IT}}{\left(\dfrac{Mean\ ELF\ AUC_{0-t}/D^{CMS}}{Mean\ Plasma\ AUC_{0-t}/D^{CMS}}\right)_{IV}}$$

Equation 4.12

The volume of epithelial lining fluid, $V_{ELF,}$ and CMS and formed colistin concentrations in ELF were calculated as detailed in Chapter 2, Section 2.2.3.3. For the BAL studies, terminal BAL fluid sample collections were carried out to define the CMS and formed colistin concentrations in ELF. Thus at a single sample time the ELF concentration was the mean value from three rats, and the pharmacokinetic parameters were calculated as described above from the mean values.

4.6 Statistical analysis

Dose-normalised plasma CMS and formed colistin concentrations were expressed as mean ± S.D. and dose-normalised ELF CMS and formed colistin concentrations were expressed as the mean value. Statistical analysis was performed using SPSS® Statistics (Version 20, IBM®, USA). Between dose comparisons were conducted on AUC, $t_{1/2}$, CL, V_{ss}, $t_{1/2,ab}$, V_{ELF} using one-way ANOVA and independent samples *t*-test analysis, as appropriate; $p < 0.05$ was regarded as a statistically significant difference.

4.7 Results

4.7.1 Dose-linearity

4.7.1.1 Intravenous CMS

Mean (± S.D.) plasma CMS and formed colistin concentrations as a function of time following IV bolus administration of CMS 14, 28 and 56 mg/kg are shown in Figure 4-1. Formed colistin concentrations were detected in plasma 5 min following administration of CMS. Subsequently, colistin concentrations increased with $C_{max,colistin}$ observed within 0.5-1 h of administration (Figure 4-1). No adverse effects were observed across the dose-range studied. Pharmacokinetic parameters of CMS and formed colistin in plasma following IV CMS administration are summarised in Table 4-2. Linear pharmacokinetic behaviour was observed between CMS and formed colistin $AUC_{0-\infty}$ and CMS doses as shown in Table 4-2, Figure 4-2A and Figure 4-2B. The estimated terminal half-life for formed colistin was longer than that of CMS (Table 4-2). A relatively low proportion of the CMS dose (1.9 – 3.3%) was converted to colistin following IV administration of CMS (Table 4-2).

4.7.1.2 Intratracheal CMS

Mean (± S.D.) plasma CMS and formed colistin concentrations as a function of time following IT instillation of CMS 14 mg/kg and 28 mg/kg are shown in Figure 4-3. Appearance of CMS in plasma was evident at the first sample time of 5 min for both CMS doses and $C_{max,CMS}$ was achieved at 2-3 h following IT instillation (Figure 4-3). Colistin was detectable in plasma 1-2 h post-dose, with $C_{max,colistin}$ occurring at 3 h following administration of either CMS 14 mg/kg or 28 mg/kg (Figure 4-3). At 2-2.5 h following instillation of CMS 28 mg/kg, rats were notably less active within the metabolism cage and light cyanosis was evident in the paws, ears and tail. These effects were transient with complete recovery observed 3 h post-administration. Pharmacokinetic parameters of CMS and formed colistin in plasma following pulmonary instillation are summarised in Table 4-3. Linear

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pharmacokinetic behaviour was observed between CMS and formed colistin $AUC_{0-\infty}$ and CMS dose as shown in Table 4-3. Following pulmonary instillation the terminal half-life of formed colistin was longer than that of CMS as shown in Table 4-3. The terminal half-life of CMS following pulmonary instillation (Table 4-3) was approximately double that following IV administration (Table 4-2). The systemic bioavailability across the dose-range studied for CMS was 80 - 89% (Table 4-3). Following IT instillation of CMS 14 mg/kg and 28 mg/kg the $AUC_{0-\infty}$ of formed colistin was 2.5- and 3.8-fold higher, respectively (Table 4-3), than that following IV administration (Table 4-2). The fraction of the pulmonary administered CMS dose that was bioavailable as colistin was 3.2% and 4.5% for CMS 14 mg/kg and 28 mg/kg, respectively (Table 4-3).
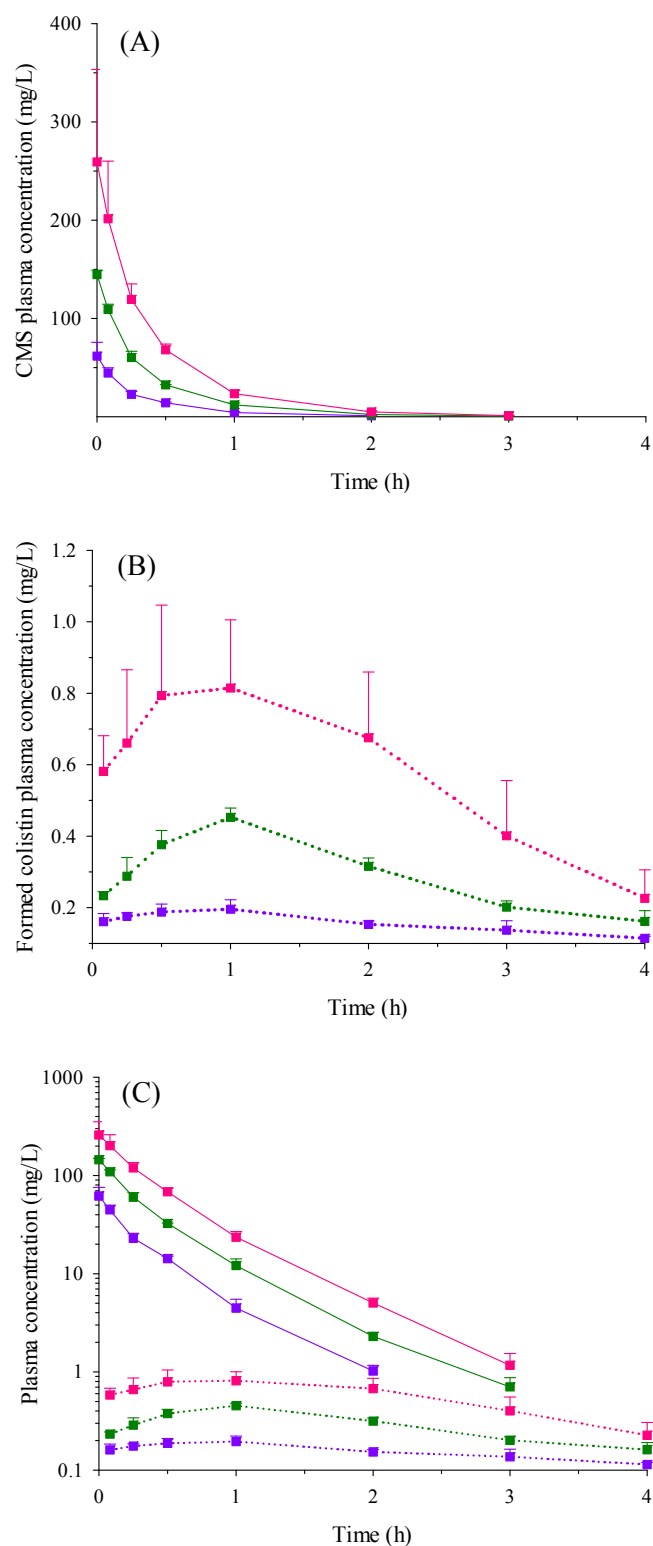
Figure 4-1: Plasma CMS (-■-) and formed colistin (··■··) concentration-*versus*-time profiles following IV administration of 14 (■), 28 (■), 56 (■) mg/kg CMS (n=3). CMS concentrations were below the limit of quantification (LOQ) at 3 h post-dose for CMS 14 mg/kg and at 4 h post-dose for CMS 28 mg/kg and 56 mg/kg. (A) and (B): linear-linear coordinates; (C) semi-logarithmic coordinates. Concentrations are expressed as mean ± S.D.

Table 4-2: Pharmacokinetic properties of CMS and formed colistin in plasma following IV administration of CMS 14, 28, 56 mg/kg (n=3). Data are expressed as mean ± S.D.

Parameters	14 mg/kg	28 mg/kg	56 mg/kg
CMS			
CL_{CMS} (L/h/kg) [a]	0.62 ± 0.044	0.50 ± 0.029	0.52 ± 0.076
$V_{ss,CMS}$ (L/kg) [a]	0.28 ± 0.011	0.24 ± 0.023	0.26 ± 0.070
$t_{1/2,CMS}$ (h) [a]	0.40 ± 0.024	0.49 ± 0.074	0.46 ± 0.057
$AUC_{0-\infty,CMS}$ (mg·h/L)	23 ± 1.6	57 ± 3.3	110 ± 15
$f_{m,systemic}$	0.033	0.022	0.019
Formed colistin			
$t_{1/2,colistin}$ (h) [b]	3.8 ± 0.66	2.0 ± 0.32	1.6 ± 0.18
$T_{max,colistin}$ (h) [ώ]	0.5-1.0	0.5-1.0	0.5-1.0
$C_{max,colistin}$ (mg/L)	0.20 ± 0.025	0.45 ± 0.027	0.83 ± 0.21
$AUC_{0-\infty,colistin}$ (mg·h/L)	1.2 ± 0.079	1.7 ± 0.19	2.8 ± 0.92

[a] No statistically significant difference among doses after one-way ANOVA.
[b] Statistically significant difference among doses after one-way ANOVA. A Tukey post-hoc test showed a statistically significant difference between dosage treatment groups of 14 mg/kg and 28/56 mg/kg, ($p < 0.05$).
[ώ] Represents T_{max} range.
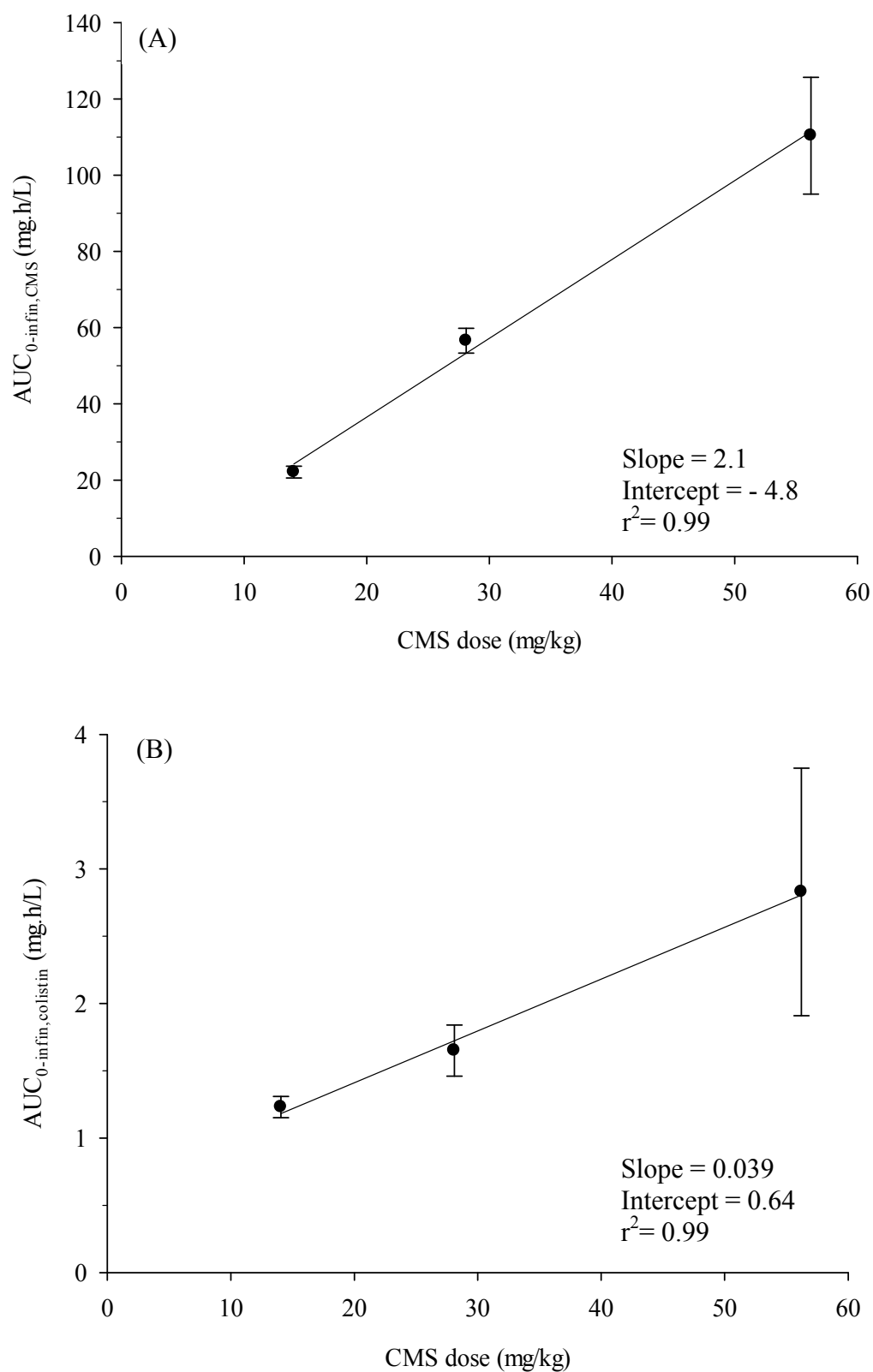
Figure 4-2: (A) $AUC_{0-\infty,CMS}$ and (B) $AUC_{0-\infty,colistin}$ plotted against CMS dose following IV administration of 14, 28 and 56 mg/kg CMS (n=3). $AUC_{0-\infty}$ values are expressed as mean ± S.D.
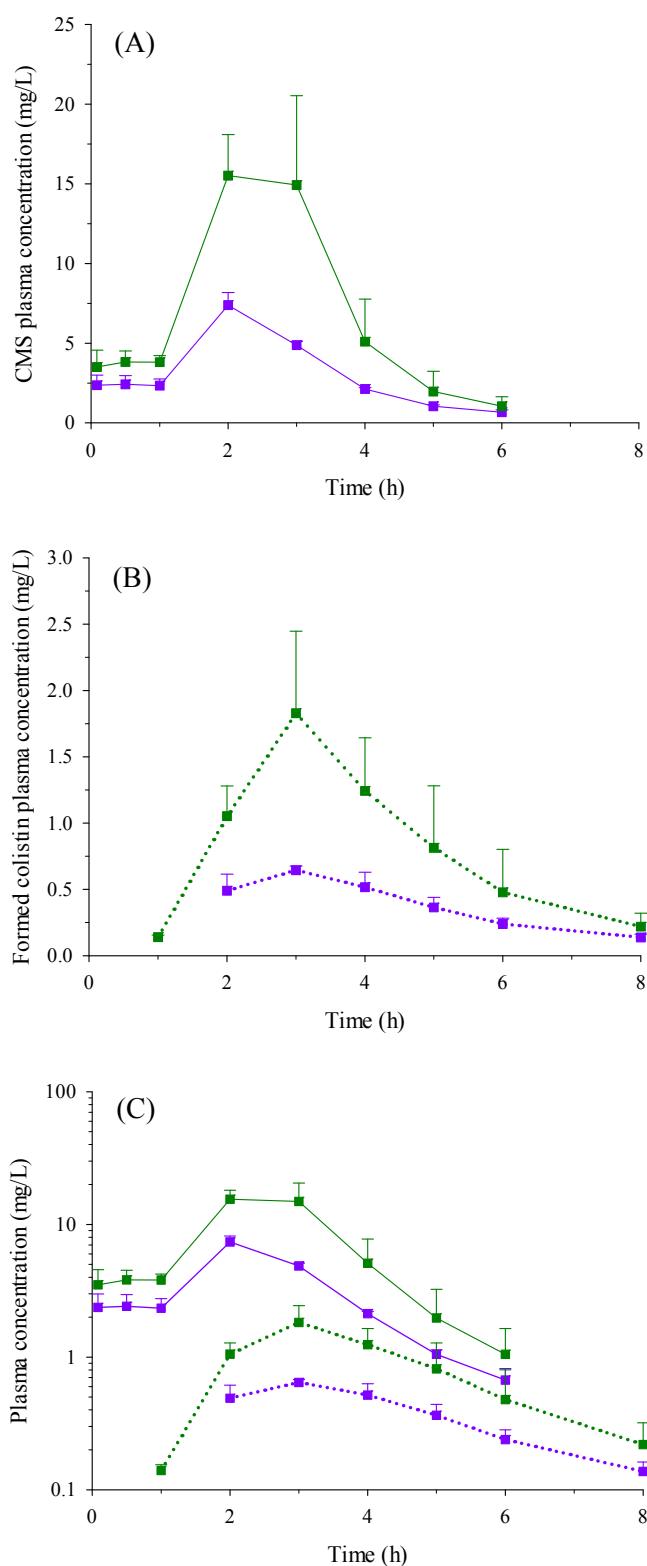
Figure 4-3: Plasma CMS (-■-) and formed colistin (··■··) concentration-*versus*-time profiles following IT administration of 14 (■) and 28 (■) mg/kg (n=3). CMS concentrations were below the LOQ at 8 h post-dose for CMS 14 mg/kg and 28 mg/kg. (A) and (B): linear-linear coordinates; (C): semi-lograithmic coordinates. Concentrations are expressed as mean ± S.D.

Table 4-3 Pharmacokinetic properties of CMS and formed colistin in plasma following IT instillation of CMS 14 and 28 mg/kg (n=3). Data are expressed as mean ± S.D.

Parameters	14 mg/kg	28 mg/kg
CMS		
Terminal $t_{1/2,CMS}$ (h) [a]	1.0 ± 0.12	0.79 ± 0.12
Absorption $t_{1/2,CMS}$ (h) [a]	0.86 ± 0.15	0.79 ± 0.22
$T_{max,CMS}$ (h)	2	2-3 [ώ]
$C_{max,CMS}$ (mg/L)	7.4 ± 0.79	18 ± 3.3
$AUC_{0-\infty,CMS}$ (mg·h/L)	20 ± 1.3	45 ± 9.5
$F_{systemic,CMS}$ (%)	89	80
Formed colistin		
Terminal $t_{1/2,colistin}$ (h) [b]	2.1 ± 0.20	1.6 ± 0.093
$T_{max,colistin}$ (h)	3	3
$C_{max,colistin}$ (mg/L)	0.64 ± 0.032	1.8 ± 0.62
$AUC_{0-\infty,colistin}$ (mg·h/L)	3.2 ± 0.53	6.5 ± 2.2
$f_{m,ELF} \times F_{systemic,colistin}$	0.032	0.045

[a] No statistically significant difference among doses after independent samples *t*-test.
[b] Statistically significant difference among doses after independent samples *t*-test ($p < 0.05$).
[ώ] Represents T_{max} range.

4.7.2 Relative pulmonary and systemic exposures

Mean (± S.D.) urea concentration in BAL fluid and plasma, the percentage of recovered BAL fluid and the ELF dilution factor following IT and IV administration are summarised in Table 4-4. The V_{ELF} in rats (weighing between 300-320 g) was estimated to be 0.084 (± 0.016) mL and 0.082 (± 0.027) mL following IT and IV administration, respectively as shown in Table 4-4.

Table 4-4: Bronchoalveolar lavage parameter estimations following IT (n=21) and IV (n=22) administration of CMS. Parameters expressed as mean ± S.D.

Parameters	IT	IV
Recovered BAL fluid (%)	90 ± 4.2	91 ± 1.6
Urea conc - BAL fluid (mg/dL)	0.35 ± 0.13	0.30 ± 0.11
Urea conc - Plasma (mg/dL)	56 ± 13	51 ± 11
Dilution Factor	167 ± 34	180 ± 42
ELF volume (mL) [a]	0.084 ± 0.016	0.082 ± 0.027

[a] No statistically significant difference in ELF volume following IT and IV administration after independent samples *t*-test ($p < 0.05$).
dL denotes decilitre.

Mean CMS and formed colistin ELF concentrations and mean (± S.D.) CMS and formed colistin plasma concentrations as a function of time following IT instillation of CMS 14 mg/kg are shown in Figure 4-4A. CMS ELF concentrations declined exponentially (terminal half-life 3.0 h), with relatively high concentrations 151 mg/L quantified in ELF 12 h post-administration (Figure 4-4A). CMS concentrations in ELF were detectable for 12 h compared to 6 h in plasma as shown in Figure 4-4A. Formed colistin concentrations of 60 mg/L were detected in ELF 5 min post-administration with $C_{max,colistin}$ of 803 mg/L achieved at 4 h (Figure 4-4A). Colistin concentrations of 271 mg/L were present in ELF 12 h post-dose (Figure 4-4A). Pharmacokinetic parameters of CMS and formed colistin in ELF and plasma following IT instillation of CMS 14 mg/kg are summarised in Table 4-5. Formed colistin had

a longer terminal half-life than CMS (Table 4-5) with the ELF terminal half-life for CMS and formed colistin longer than the corresponding plasma terminal half-life as shown in Table 4-5. Following IT instillation of 14 mg/kg CMS, the $AUC_{0-\infty}$ of CMS in ELF was 33,726 mg·h/L compared to 20 ($\pm$ 1.3) mg·h/L in plasma and the $AUC_{0-\infty}$ of formed colistin in ELF was 7,564 mg·h/L compared to 3.2 ($\pm$ 0.53) mg·h/L in plasma (Table 4-5 and Figure 4-4B). Following pulmonary administration of CMS a greater fraction of the CMS dose was converted to colistin in ELF ($f_{m,ELF}$ 25%) (Table 4-5) when compared to in plasma after IV CMS administration ($f_{m,systemic}$ 1.9 – 3.3%) (Table 4-2).
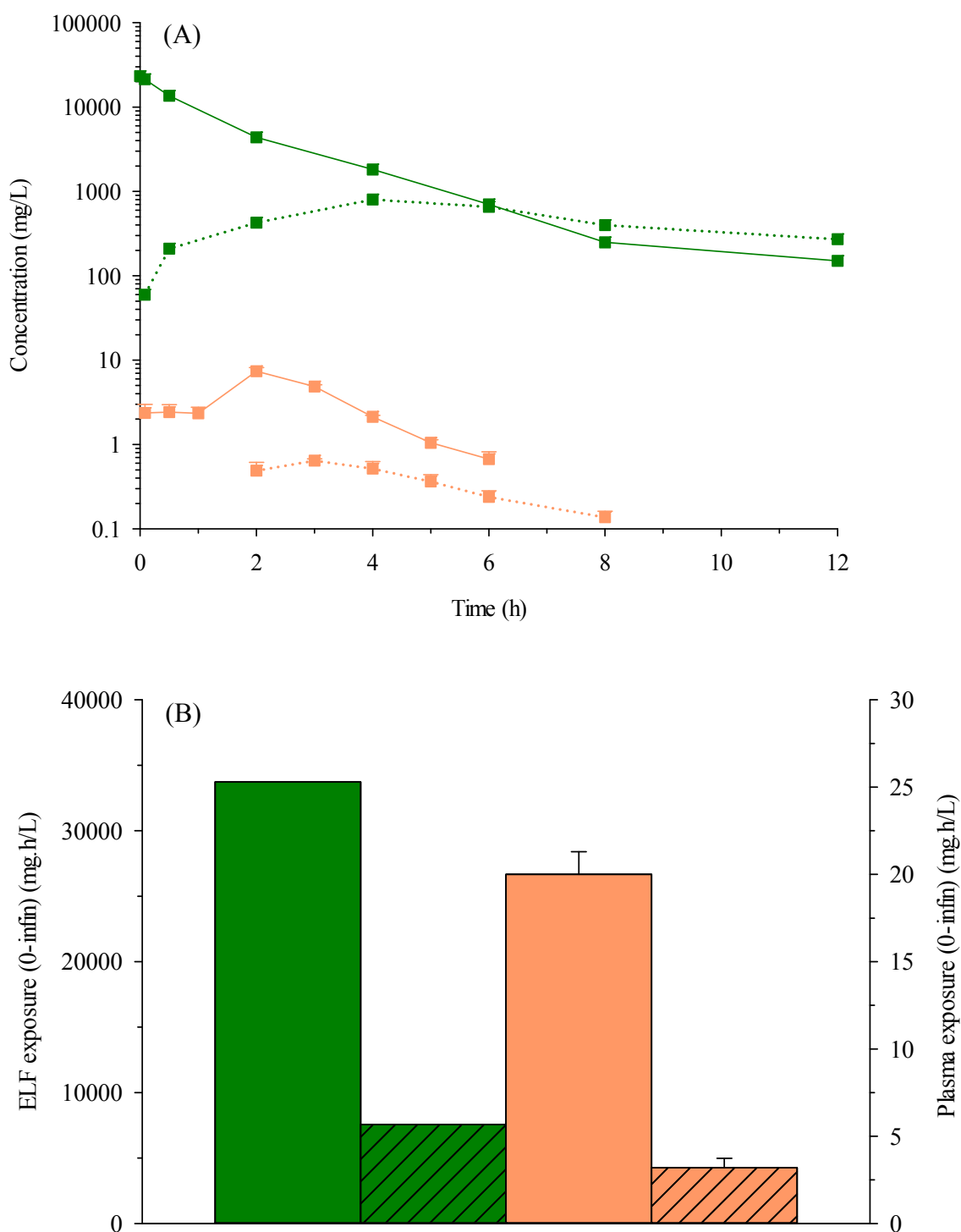
Figure 4-4: (A) ELF (■) (n=3 at each time point) and plasma (■) (n=3) CMS (-■-) and formed colistin (··■··) concentration-*versus*-time profile following IT instillation of 14 mg/kg CMS, (B) ELF and plasma AUC$_{0-\infty}$ of CMS (solid) and formed colistin (lines) following IT instillation of 14 mg/kg CMS. ELF concentrations and exposure are represented as mean values and plasma concentrations and exposure are represented as mean ± S.D. Note the different scales for CMS/colistin exposure in ELF and plasma in panel B.

Table 4-5: Pharmacokinetic properties of CMS and formed colistin in ELF and plasma following IT instillation of CMS 14 mg/kg. ELF estimates are expressed as mean values and plasma estimates are expressed as mean ± S.D.

Parameters	ELF	Plasma
CMS		
Terminal $t_{1/2,CMS}$ (h)	3.0	1.0 ± 0.12
Absorption $t_{1/2,CMS}$ (h)	-	0.86 ± 0.15
$AUC_{0-\infty,CMS}$ (mg·h/L)	33,726	20 ± 1.3
$f_{m,ELF}$ ^{Ɣ}	0.25	-
$F_{systemic,CMS}$ (%)	-	89
Formed colistin		
Terminal $t_{1/2,colistin}$ (h)	5.0	2.1 ± 0.20
$T_{max,colistin}$ (h)	4	3
$AUC_{0-\infty,colistin}$ (mg·h/L)	7,564	3.2 ± 0.53

^{Ɣ} Represents apparent $f_{m,ELF}$ ($f_{m,ELF}/F_{pulm}$) where F_{pulm} is the pulmonary bioavailability of the CMS dose following IT administration.

Mean CMS ELF concentrations and mean (± S.D.) CMS and formed colistin plasma concentrations as a function of time following IV administration of CMS 14 mg/kg are shown in Figure 4-5A. Formed colistin ELF concentrations were below the LOQ (0.012 mg/L) in BAL fluid at all post-dose sampling times. Maximum ELF CMS concentrations were achieved immediately following administration, with a secondary peak observed at 1 h post-IV dose (Figure 4-5A). CMS ELF concentrations declined exponentially (terminal half-life 2.3 h) with concentrations quantifiable up to 3 h post-dose (Figure 4-5A). CMS concentrations in ELF were detected for 3 h compared to 2 h in plasma as shown in Figure 4-5A. Pharmacokinetic parameters of CMS in ELF and CMS and formed colistin in plasma following IV CMS administration of 14 mg/kg are summarised in Table 4-6. The terminal half-life of CMS was longer in ELF compared to plasma (Table 4-6). Following IV administration of 14 mg/kg CMS, the $AUC_{0-\infty}$ of CMS in ELF was 28 mg·h/L compared to

23 ($\pm$ 1.6) mg·h/L in plasma and the $AUC_{0-\infty}$ of formed colistin in ELF was unquantifiable compared to 1.2 ($\pm$ 0.079) mg·h/L in plasma (Table 4-6 and Figure 4-5B).
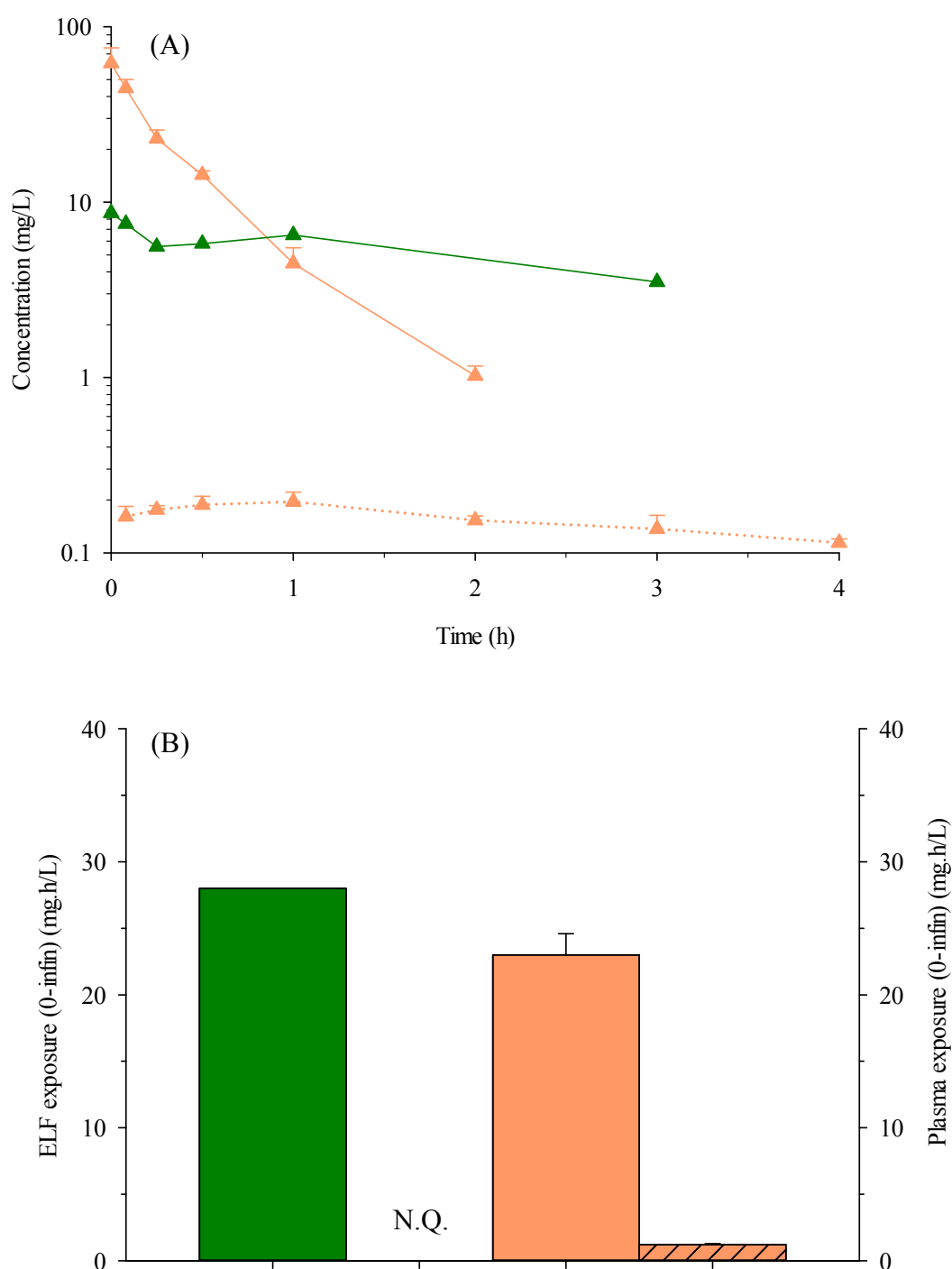
Figure 4-5: (A) ELF (■) (n=3 at each time point) and plasma (■) (n=3) CMS (-■-) and formed colistin (··■··) concentration-*versus*-time profile following IV administration of 14 mg/kg CMS, (B) ELF and plasma AUC$_{0-\infty}$ of CMS (solid) and formed colistin (lines) following IV administration of 14 mg/kg CMS. Formed colistin ELF concentrations following IV CMS 14 mg/kg were below the LOQ concentration in BAL fluid (0.012 mg/L), therefore ELF exposure is denoted as N.Q. (not quantifiable). ELF concentrations and exposure are represented as mean values and plasma concentrations and exposure are represented as mean ± S.D.

Table 4-6: Pharmacokinetic properties of CMS and formed colistin in ELF and plasma following IV administration of CMS 14 mg/kg. ELF estimates are expressed as mean values and plasma estimates are expressed as mean ± S.D.

Parameters	ELF	Plasma
CMS		
Terminal $t_{1/2,CMS}$ (h)	2.3	0.40 ± 0.024
$AUC_{0-\infty,CMS}$ (mg·h/L)	28	23 ± 1.6
Formed colistin		
Terminal $t_{1/2,colistin}$ (h)	N.Q.	3.8 ± 0.66
$AUC_{0-\infty,colistin}$ (mg·h/L)	N.Q.	1.2 ± 0.079

N.Q., Not quantifiable as concentrations were less than the LOQ concentration in BAL fluid of the colistin assay.

The ELF and plasma exposure of CMS and formed colistin following IT and IV CMS administration of 14 mg/kg are summarised in Table 4-7. Following pulmonary instillation of CMS 14 mg/kg, the $AUC_{0-\infty}$ of CMS in ELF was approximately 1,200-fold higher when compared to following IV administration (Table 4-7). No significant difference in the $AUC_{0-\infty}$ of CMS in plasma was evident following IT and IV administration (Table 4-7). The $AUC_{0-\infty}$ of formed colistin in ELF was 7,564 mg·h/L following IT administration with no quantifiable concentrations following IV administration (Table 4-7). The $AUC_{0-\infty}$ of formed colistin in plasma following IT administration was significantly higher when compared to after IV administration (Table 4-7). Comparable CMS terminal half-lives in ELF were observed following pulmonary (3.0 h) and IV (2.3 h) CMS administration (Table 4-5 and 4-6).

Table 4-7: Therapeutic availability (TA), drug targeting index (DTI) and ELF and plasma exposure of CMS and formed colistin following IT and IV administration of CMS 14 mg/kg. ELF estimates are expressed as mean values and plasma estimates are expressed as mean ± S.D.

Parameters	ELF	Plasma
IT administration		
$AUC_{0-\infty,CMS}$ (mg·h/L)	33,726	20 ± 1.3[a]
$AUC_{0-\infty,colistin}$ (mg·h/L)	7,564	3.2 ± 0.53[b]
$AUC_{0-4,colistin}$ (mg·h/L)	1,767	1.64 ± 0.27
$AUC_{0-12,colistin}$ (mg·h/L)	5,626	-
IV administration		
$AUC_{0-\infty,CMS}$ (mg·h/L)	28	23 ± 1.6
$AUC_{0-\infty,colistin}$ (mg·h/L)	N.Q.	1.2 ± 0.079
$AUC_{0-4,colistin}$ (mg·h/L)	8.9[¥]	0.61 ± 0.037
$AUC_{0-12,colistin}$ (mg·h/L)	27[¥]	-
TA_{CMS}[§]	1,218	
$TA_{colistin}$[ϒ]	209	
DTI_{CMS}[§]	1,366	
$DTI_{colistin}$[£]	73	

[a] No statistically significant difference among plasma CMS exposure to infinity following IT and IV CMS administration after independent samples *t*-test.
[b] Statistically significant difference among plasma formed colistin exposure to infinity following IT and IV CMS administration after independent samples *t*-test.
N.Q. Not quantifiable as concentrations were less than the LOQ concentration in BAL fluid of the colistin assay.
[¥] Formed colistin ELF AUC following IV CMS administration was calculated using the LOQ concentration for colistin in ELF of 2.2 mg/L.
[§] TA_{CMS} and DTI_{CMS} estimated from $AUC_{0-\infty,CMS}$ ELF and plasma values following IT and IV administration.
[ϒ] $TA_{colistin}$ estimated from $AUC_{0-12,colistin}$ ELF values following IT and IV administration.
[£] $DTI_{colistin}$ estimated from $AUC_{0-4,colistin}$ ELF and plasma values following IT and IV administration.

The targeting advantage of administering CMS via the lungs when compared to the IV route was demonstrated by a TA and DTI estimate for CMS of 1,218 and 1,366, respectively and a TA and DTI estimate for formed colistin of 209 and 73, respectively following IT and IV administration of CMS 14 mg/kg (Table 4-7). For the estimation of TA and DTI for formed colistin, since ELF concentrations were below the LOQ following IV administration the exposure was calculated using the LOQ concentration in ELF. The LOQ concentration in ELF (2.2 mg/L) was calculated using the LOQ concentration in BAL fluid (0.012 mg/L) after

correction for the ELF-BAL fluid dilution factor (Table 4-4). The ELF exposure to infinity following IV administration could not be calculated in the absence of the colistin terminal rate constant therefore the formed colistin ELF exposure to 4 h and 12 h was estimated as 2.2 mg/L × 4 h and 2.2 mg/L × 12 h, respectively. This method results in an over-estimation of the formed colistin exposure in ELF and hence an under-estimation of the targeting value. For estimation of TA for CMS and formed colistin, the ELF exposure was calculated from time zero to infinity and 12 h post-dose, respectively (Table 4-7). For estimation of DTI for CMS and formed colistin, the ELF/plasma exposure was calculated from time zero to infinity and 4 h post-dose, respectively (Table 4-7). The latter was estimated to 4 h since formed colistin concentrations in plasma following IV CMS were below the LOQ thereafter.

Pharmacokinetics parameters of formed colistin in ELF following administration of IT CMS 14 mg/kg and of colistin in ELF after IT colistin 0.62 mg/kg (Chapter 3, Section 3.7.2, Table 3-7) are summarised in Table 4-8. Comparison following IV administration was not possible as colistin ELF concentrations were undetectable following both IV CMS and colistin administration (Chapter 3, Section 3.7.2). Consistent terminal half-lives were observed for formed colistin (5.0 h) and colistin (5.3 h) in ELF (Table 4-8). Pulmonary administration of the prodrug resulted in a four-fold decrease in colistin ELF exposure when compared to IT administration of colistin (after dose-normalisation of formed colistin $AUC_{0-\infty}$ for CMS 14 mg/kg to colistin 0.62 mg/kg) (Table 4-8). Exposure of formed colistin and colistin in ELF were observed for 12 h post-dose (Table 4-8).

Table 4-8: Pharmacokinetic properties in ELF of formed colistin following IT CMS 14 mg/kg and colistin following IT administration of colistin 0.62 mg/kg. Data are expressed as mean values.

Parameters	Formed colistin	Colistin
Terminal $t_{1/2}$ (h)	5.0	5.3
$AUC_{0-\infty}$ (mg·h/L)	7,564	1,908
$AUC_{dose-normalised,0-\infty}$ (mg·h/L) [¥]	469	-
T_{last} (h)	12	12

[¥] Formed colistin $AUC_{0-\infty}$ estimate dose-normalised for a colistin 0.62 mg/kg dose.

4.8 Discussion

This chapter describes for the first time the pharmacokinetics of CMS and formed colistin in plasma and in the lungs following pulmonary and IV administration of CMS in rats. This enabled the quantification of the targeting advantage achieved following administration of CMS via the pulmonary route.

Intravenous CMS dose-ranging studies were carried out to define the key pharmacokinetic parameters of CMS and formed colistin. Linear pharmacokinetic behaviour was evident for CMS and formed colistin across the dose-range studied (Table 4-2 and Figure 4-2). The sensitivity limit of the analytical methods is likely to contribute to the negative and positive y-intercept for the $AUC_{0-\infty}$ *versus* dose plots for CMS and formed colistin, respectively (Figure 4-2). The pharmacokinetic estimates obtained in the following study are generally in agreement with previously publish data by Li *et al* following IV administration of sodium CMS 15 mg/kg (corresponding to 14 mg/kg CMS base) [198] and by Marchand *et al* who showed a linear pharmacokinetic relationship for CMS and formed colistin following IV sodium CMS doses of 5 – 120 mg/kg (corresponding to 4.7 – 112 mg/kg CMS base) [199]. The systemic CL, V_{ss} and terminal half-life of CMS were comparable to previously reported values by Li *et al* [198] and Marchand *et al* [199]. In the present study, a longer terminal half-

life for formed colistin (Table 4-2) was observed when compared to that reported by Li *et al* (0.93 ± 0.32 h) [198] and Marchand *et al* (mean value: 0.63 ± 0.077 h) [199]. Consistent with previous findings, a longer terminal half-life for formed colistin when compared to CMS indicated that the elimination of colistin was not rate-limited by formation from CMS (Table 4-2) [198, 199]. For colistin, similar clearance kinetics were evident following administration of the prodrug and active moiety as the terminal half-life of formed colistin following IV CMS (Table 4-2) was comparable to that of colistin following IV colistin (Chapter 3, Section 3.7.1.1, Table 3-4).

Following IV CMS dosing, a progressive rise in formed colistin concentrations in plasma was evident (T_{max} 0.5-1 h), similar to observations in humans [126, 200-202], and as would be expected for the conversion of a prodrug to the active moiety. However the studies by Li *et al* [198] and Marchand *et al* [184, 199] in rats, reported that peak formed colistin concentrations were achieved at the initial sampling times of 5 – 10 min. Li and colleagues confirmed that this rapid appearance of formed colistin in plasma was not due to *in vitro* colistin formation in CMS dosing formulations nor due to sample handling and storage procedures and therefore concluded that mechanisms other than the blood/plasma mediated conversion was leading to such observations [198]. The fraction of the CMS dose converted to colistin in the current study ranged from 1.9 – 3.3% in comparison to 6.2% and 13% by Li *et al* [198] and Marchand *et al* [184], respectively, following IV administration of sodium CMS 15 mg/kg (corresponding to 14 mg/kg CMS base). The variability in the findings may be due to the use of different brands of sodium CMS (Colistin Link[®] [Link Pharmaceuticals Ltd (present study)], CMS [Sigma [198]], Colymicine[®] [Sanofi-Aventis [184]]) where the fractional conversion of CMS to colistin may differ. Furthermore, the manner in which biological samples were handled, processed and stored may have contributed to the observed differences with Dudhani and colleagues reporting that to minimise *in vitro* conversion samples needed to

be stored at -80ºC and analysed within 4 months of collection [219]. The higher fractional conversion of CMS in Marchand *et al* study [184] in comparison to Li *et al* [198] and the present study is likely to be due to the manner in which $f_{m,\text{systemic}}$ was estimated. The authors used the systemic CL of colistin, and as discussed in Chapter 3 (Section 3.8), the assumption of 100% bioavailability of colistin in the systemic circulation following SC administration may have resulted in a larger estimation for systemic CL [184]. Therefore the higher fractional conversion of CMS may be reflective of the larger colistin systemic CL utilised [184]. Overall, an explanation for the low proportion of CMS converted to colistin in rats is unclear as there is limited information in the literature about the conversion kinetics of CMS. In *in vitro* studies, CMS conversion is reported to be concentration- and temperature-dependent [9, 110, 190, 219] however the exact mechanism/s of conversion is unknown. Therefore one of the primary objectives for building a population pharmacokinetic model described in Chapter 5 was to develop a better understanding of the conversion kinetics of CMS in the systemic circulation and in the lungs.

Pulmonary dose-ranging studies in rats demonstrated that CMS was slowly absorbed into systemic circulation with maximal plasma concentrations observed between 2-3 h following administration (Figure 4-3). The slow absorption of CMS from the lungs is consistent with the findings by Marchand *et al*, with the authors reporting that CMS has low apparent permeability through a monolayer of Calcu-3 cells (apical-to-basolateral direction) [184]. During IT instillation an unknown fraction of the CMS dose may have been delivered into the gastrointestinal tract however the contribution of oral absorption to the overall absorption profile is expected to be negligible [94, 208]. Linear pharmacokinetics was evident for CMS and formed colistin following pulmonary dose-ranging studies (Table 4-3). The systemic bioavailability of CMS across the pulmonary dose range studied (Table 4-3) was consistent with the 70% systemic bioavailability reported by Marchand *et al* following pulmonary

administration of 15 mg/kg of sodium CMS (corresponding to 14 mg/kg CMS base) [184]. The absorption profile for CMS was characterised by steady plasma concentrations for 1 h post-dose, consistent to findings by Marchand *et al* [184], after which a greater than two-fold increase in CMS concentrations was observed (Figure 4-3). Given the physicochemical properties of CMS (M_w 1633 Da, PSA 741 Å, Log P -12.09) (Chapter 1, Section 1.5.2) the proposed mechanism of absorption through the lung epithelium is likely to be via paracellular pathways such as passive diffusion through aqueous-filled pores (Chapter 1, Section 1.3.2.1). As further discussed in the later paragraphs, competitive displacement of CMS from the lungs by formed colistin may help explain the unusual absorption profile for CMS. Chapter 5 provides a more detailed discussion of the absorption kinetics based on the population pharmacokinetic model.

Following IT instillation of 14 mg/kg and 28 mg/kg of CMS, appearance of formed colistin in plasma occurred 1-2 h post-dose with peak concentrations observed at 3 h post-dose, consistent with the Marchand *et al* study [184]. Thereafter an exponential decline in formed colistin concentrations was evident which was characterised by terminal half-life that was approximately two-fold longer (Table 4-3) than the reported in Marchand *et al* study (terminal half-life of 1.1 ± 0.14 h) [184]. The delayed absorption of formed colistin in plasma was unlike the rapid absorption of colistin (T_{max} 10 − 30 min) following IT colistin dosing (Chapter 3, Section 3.7.1.2), with such variations reflective of a delay due to conversion to colistin in the lungs. The proposed absorption mechanism of formed colistin is the same as that for colistin in Chapter 3 (Section 3.8), via paracellular pathways by passive diffusion through aqueous-filled pores (Chapter 1, Section 1.3.2.1). Following IT CMS administration, clearance of formed colistin from plasma was not rate-limited by absorption (Table 4-3 and 4-2). However, absorption was the rate-limiting step in clearance of CMS as the terminal half-life following IT instillation (Table 4-3) was approximately doubled that after IV

administration (Table 4-2). Interestingly, Marchand *et al* reported the opposite findings with absorption being a rate-limiting step for clearance of formed colistin from plasma following pulmonary administration of 15 mg/kg of sodium CMS [184]. The inconsistency between the two studies may be attributed to the use of different pulmonary dosing techniques (IT instillation *versus* IT nebulisation [184]) which can influence which regions of the lung CMS/colistin are deposited in and thereby the metabolism and absorption kinetics.

The systemic exposure of formed colistin following pulmonary administration of CMS 14 mg/kg and 28 mg/kg were 2.5- to 3.8-fold higher when compared to after IV administration. These findings were different to the systemic exposure of colistin (bioavailability of 31 – 46%) following IT colistin dose-ranging studies as described in Chapter 3 (Section 3.7.1.2). Marchand *et al* reported similar results with a four-fold increase in colistin systemic exposure following pulmonary administration compared to IV administration of 15 mg/kg of sodium CMS [184]. The absorption of pre-systemically formed colistin is likely to result in the greater systemic exposure of colistin following lung dosing. As previously discussed, the fraction of the IV CMS dose converted to colistin in plasma was low ($f_{m,systemic}$ 1.9 – 3.3%), therefore if it is assumed that the fractional conversion of CMS in plasma is similar following IV and IT dosing, then of the 80 - 89% of CMS bioavailable in plasma the proportion of systemically formed colistin would be low. In comparison, a greater proportion of the IT CMS dose was estimated to be converted to colistin in the ELF ($f_{m,ELF}$ 25%) and of that 3.2 – 4.5% ($f_{m,ELF} \times F_{systemic,colistin}$) was absorbed into the systemic circulation and this likely to contribute to the 2.5 to 3.8-fold increase in formed colistin systemic exposure. The greater fractional conversion of CMS to colistin in the lungs indicates that there are differences in the conversion rates of CMS in the two biological fluids. As further discussed in Chapter 5, the population pharmacokinetic model provides details of the different conversion kinetics occurring in the lungs and plasma following IT and IV CMS dosing. When compared to the

<5% of the pulmonary CMS dose converted to colistin in ELF prior to absorption into plasma, Marchand *et al* reported a proportion of 39% and since the estimated CMS systemic bioavailability was ~70% concluded that the total inhaled CMS dose was available in the systemic circulation [184]. Despite the greater exposure of formed colistin in the systemic circulation in rats, these findings were not paralleled in humans as discussed in Chapter 6.

A significant finding of the current study was that following pulmonary administration of 14 mg/kg of CMS, there was an extensive, prolonged exposure of both CMS and formed colistin in ELF. Formed colistin concentrations in ELF were detectable at the first sampling time (5 min post-dose) followed by a gradual increase in colistin concentrations (T_{max} at 4 h), consistent to findings by Marchand *et al* [184], and similar to the formation kinetics in plasma. Following IT CMS dosing, the $AUC_{0-\infty}$ of CMS and formed colistin in ELF was greater than 1,500-fold higher when compared to the $AUC_{0-\infty}$ in plasma and the duration of ELF exposure was approximately double the exposure in plasma. The lung exposure to colistin was four-fold lower following IT administration of CMS when compared to an equimolar dose of colistin (Table 4-8), a consequence of incomplete conversion of CMS to colistin. The estimated ELF volume in rats following IT and IV administration were 0.084 ± 0.016 mL and 0.082 ± 0.027 mL, respectively, which is consistent with the ELF volumes reported in Chapter 3 (Section 3.7.2). The relatively small volume of lining fluid that CMS and formed colistin resides in is likely to result in the greater exposure in the lungs when compared to that in plasma. Additionally, the high concentrations of CMS in ELF (Figure 4-4A) may be facilitated by the slow absorption of CMS from the lungs and thereby may act as reservoir for formation of colistin in the lungs over time. Such findings of relatively high lung exposure when compared to systemic exposure for formed colistin are consistent with the outcomes in CF patients ([170] and Chapter 6) and critically-ill patients [129] following nebulisation of sodium CMS.

In ELF, the terminal half-life of formed colistin was approximately double that of CMS following IT instillation indicating that the elimination of colistin was not rate limited by formation from CMS (Table 4-5). Comparable terminal half-lives for colistin in ELF following IT instillation of CMS (5.0 h) and colistin (5.3 h) suggest similar clearance kinetics in the lungs. In ELF, a longer terminal half-life for CMS and formed colistin following pulmonary CMS administration (Table 4-5) when compared to in plasma following IV dosing (Table 4-2) suggests that different clearance kinetics are involved in ELF and plasma. For colistin an explanation for the slower clearance in ELF may reside in the binding characteristics to lung tissue with a study by *Ziv et al* showing that the bound concentration of colistin in the kidney, liver, lung, heart, skeletal muscle and brain is greater when compared to the unbound concentrations following IV colistin (sulphate) administration in calves [213]. However more rigorous studies are needed to confirm such findings. The estimation of CMS and formed colistin terminal half-life has not been reported in any of the studies investigating pulmonary delivery of CMS [129, 170, 184].

Following IT instillation of CMS 14 mg/kg, the absorption profile of CMS in plasma was not mirrored in the ELF concentration-*versus*-time profile (Figure 4-4A). This may be as a result of CMS concentrations in ELF being influenced by two kinetic processes; 1) conversion of CMS to colistin and 2) absorption of CMS from the lungs; the former is likely the predominant pathway as relatively high formed colistin ELF concentrations (427 mg/L) were observed at 2 h post-dose when compared to CMS plasma concentrations of 10 mg/L at the same time period (Figure 4-4A). The approximately two-fold increase in CMS concentrations in plasma at 1 h may be explained by the binding properties of CMS and formed colistin to tissue. Kunin *et al* have shown that the bound proportion of polymyxin B (similar in structure to colistin, with the chemical structures differing by a single amino acid on the peptide ring [91]) to tissues is greater when compared to CMS with such findings proposed to be due to

the presence of more uncapped amine groups [212]. The reversible binding nature of lipophilic basic amine drugs (pKa > 8.5) to lung tissue has been reported in the literature with potential displacement of the bound amine drug in the presence of a more basic amine compound [49, 52, 53, 210]. Therefore it is proposed that following CMS pulmonary administration, a fraction of the delivered CMS dose will be bound to lung tissue however with a gradual increase in formed colistin concentrations, displacement of CMS from the binding sites could potentially be occurring as colistin is a more basic amine compound. This displacement of CMS from the lung binding sites may result in the observed increase in plasma CMS concentration (T_{max} 2 h) as this occurs in parallel with an increase in formed colistin concentrations in ELF (T_{max} 4 h) (Figure 4-4A). However due to the limitations of the tissue binding studies undertaken by Kunin *et al* (the use of microbiological assays and sample pre-treatment methods potentially promoting *in vitro* conversion to colistin [212]) such proposed mechanisms need to be interpreted with caution and highlights the urgent need for more robust pharmacokinetic studies.

In contrast to the extensive ELF exposure of both CMS and formed colistin following IT instillation, IV administration of CMS achieved relatively low CMS concentrations and unquantifiable formed colistin concentrations in ELF (Figure 4-5A). Unquantifiable formed colistin concentrations in ELF can be explained by; 1) low formed colistin concentrations in plasma and thereby a smaller concentration gradient from plasma to ELF and 2) extensive binding of colistin to tissues (as previously discussed following pulmonary dosing). These results are consistent with the findings reported in Chapter 3 (Section 3.7.2) and with recently published data of unquantifiable formed colistin concentrations in BAL fluid sample under steady-state conditions following IV infusion of CMS in critically-ill patients [189] and in the lung tissues of infected piglets following multiple dosing of IV CMS [185]. On the contrary, Markou *et al* [220], Reed *et al* [201] and Aoki *et al* [144] have all observed formed colistin

concentrations in human ELF of critically-ill patients, sputum samples of CF patients and in mice homogenised lung tissue, respectively, following various IV CMS dosing regimens. The differences between studies is likely to be due to different species and variations in the IV CMS dosing regimens, sample preparation techniques and analytical methods [144, 185, 189, 201, 220]. Additionally, as proposed in Chapter 3 (Section 3.8) and reported by Imberti and colleagues, binding of colistin to lung tissue may contribute to the low or absent concentrations in the lung lining fluid [189]. Even though lung tissue CMS/colistin concentrations were not quantified in the present study, based on the findings of others these concentrations are expected to be higher than that in ELF [144, 212].

The targeting benefit of directly administering CMS into the lungs was demonstrated with extensive exposure of CMS and formed colistin in ELF when compared to IV administration of the same dose (Table 4-7). Despite this, a greater systemic exposure for formed colistin when compared to IV dosing suggests a significant increase in systemic exposure following lung administration and thereby contrary to the goals of targeted pulmonary drug delivery. However, the observed formed colistin ELF concentrations were orders of magnitudes higher than the minimum inhibitory concentration required for 50 to 90% inhibition of bacteria growth based on clinical isolates of *P.aeruginosa* and *Acinetobacter* spp. (MIC_{50-90}) of 1.0 mg/L for colistin [172], such that a reduction in total dose could be implemented to minimise the systemic exposure. In comparison, after IV administration of 14 mg/kg of CMS, formed colistin concentrations were below the MIC_{50-90} of 1.0 mg/L and therefore to achieve therapeutic relevant concentration an increase in IV CMS dose and/or increase in frequency of dosing interval would be needed which would increase the systemic exposure and potentially the systemic adverse effects.

A quantitative method of highlighting the targeting benefit of pulmonary administering of CMS into the ELF when compared to IV administration is by the estimation of TA and DTI values [206, 207]. Since formed colistin ELF exposure following IV CMS administration was below the LOQ in BAL fluid, the ELF exposure was estimated using the LOQ concentration in ELF and as such it needs to be noted that the estimated TA and DTI values are an under-estimation. As discussed in Chapter 3 (Section 3.8), the TA and DTI provide an indication of the ELF availability of CMS and formed colistin and the degree of targeting that can be achieved following IT when compared to IV administration, respectively. The TA for CMS and formed colistin were magnitudes greater than unity which provides confirmation that direct delivery to the lung results in a significant increase in exposure of CMS and colistin at the respiratory site when compared to delivery via the IV route of the same dose. Similarly the DTI was far greater than unity for both CMS and formed colistin and is indicative of effective targeted delivery to the ELF following pulmonary delivery with achievement of relatively higher lung exposure and lower systemic exposure when compared to following IV administration. The targeting benefit achieved for formed colistin is consistent with that of colistin following pulmonary when compared to IV administration of active antibacterial moiety (Chapter 3, Section 3.7.2). Such quantitative assessment of the targeting benefit following pulmonary administration of the therapeutically relevant form of colistin, CMS, has not been reported in the literature. Therefore these studies are the first to have demonstrated the extent and degree of targeting that can be achieved following pulmonary *versus* IV administration of the prodrug in Sprague-Dawley rats.

In conclusion, this is the first study that comprehensively investigated the pharmacokinetics of CMS and formed colistin in plasma in the ELF following both IV and pulmonary CMS administration in rats. Linear pharmacokinetic behaviour was observed for CMS and colistin following IV and pulmonary dose-ranging studies. Pulmonary administration of CMS in rats

has been demonstrated to be an effective method of directly targeting CMS and formed colistin into the ELF. Formed colistin concentrations in ELF were maintained well above the MIC_{50-90} following IT administration of CMS that a reduction of the dose can be implemented which in turn can significantly reduce the systemic exposure of formed colistin. Similar to conclusion drawn in Chapter 3, non-compartmental analysis has enabled a comprehensive understanding of the pharmacokinetics of CMS and formed colistin in plasma and ELF. Further analysis with incorporation of population pharmacokinetic modelling is carried out in Chapter 5 to facilitate a better understanding of the kinetics of absorption and conversion of CMS and formed colistin in rats.

Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Concept and design of studies, planning and execution of experimental work, data analysis and interpretations, formulation of conclusions and hypotheses resulting from the relevant studies. Drafting and revision of manuscript.	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%)
M.P. McIntosh[1]	Project supervisor, data analysis and manuscript review	N/A
R.L. Nation[1]	Project co-supervisor, manuscript review	N/A
Jian Li[1]	Project co-supervisor, manuscript review	N/A
C.J.H. Porter[1]	Project co-supervisor, manuscript review	N/A
K. Patel[2]	Data analysis and manuscript review	N/A

Candidate's Signature		Date

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	[1]Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Australia, [2]Centre for Medicine Use and Safety, Monash University, Australia,

Signature 1		**Date**
Signature 2		
Signature 3		
Signature 4		
Signature 5		

Chapter 5: Population pharmacokinetics of colistin methanesulfonate in rats: achieving sustained lung concentrations of colistin for targeting respiratory infections

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For a detailed description of the experimental designs refer to Chapter 3 and 4. For the analytical methods utilised for quantification of colistin and colistin methanesulphonate in the biological matrices refer to Chapter 2, Section 2.2.

Manuscript in submission.

5.1 Abstract

Colistin methanesulfonate (CMS), the inactive prodrug of colistin, is administered by inhalation for the management of respiratory infections. However, limited pharmacokinetic data is available for CMS and colistin following pulmonary delivery. This study investigates the pharmacokinetics of CMS and colistin following intravenous (IV) and intratracheal (IT) administration in rats and determines the targeting advantage after direct delivery into the lungs. In addition to plasma, bronchoalveolar lavage (BAL) fluid was collected to quantify drug concentrations in lung epithelial lining fluid (ELF). The resulting data was analyzed using a population modeling approach in S-ADAPT. A three-compartment model described the disposition of both compounds in plasma following IV administration. The estimated mean clearance from the central compartment was 0.122 L/h for CMS and 0.0657 L/h for colistin. Conversion of CMS to colistin from all three compartments was required to fit the plasma data. The fraction of the IV dose converted to colistin in the systemic circulation was 0.0255. Two BAL fluid compartments were required to reflect drug kinetics in the ELF after IT dosing. A slow conversion of CMS (mean conversion time, MCT_{CMS} = 3.48 h) in the lungs contributed to high and sustained concentrations of colistin in ELF. The fraction of the CMS dose converted to colistin in ELF ($f_{m,ELF}$ = 0.226) was higher than the corresponding fractional conversion in plasma after IV administration. In conclusion, pulmonary administration of CMS achieves high and sustained exposures of colistin in lungs for targeting respiratory infections.

5.2 Introduction

Pulmonary administration of antibiotics for the treatment of respiratory infections has gained significant interest over the last decade, due to the potential for achieving high local drug concentrations at the site of infection [20, 21, 197, 221]. Other favorable attributes for

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targeting antibiotics to the respiratory tract include a rapid onset, whilst minimizing systemic exposure and adverse effects [20, 21, 197, 221]. Several antibiotics are in clinical development for the treatment of lung infections, with tobramycin, aztreonam and colistin methanesulfonate (CMS) currently approved for inhalational administration in cystic fibrosis (CF) patients [12].

Colistin (also known as polymyxin E) was introduced into the market in the late 1950s for parenteral delivery but, due to the development of adverse effects including nephro- and neurotoxicity, was replaced by other antibiotics [93, 94, 99]. In the last two decades, however, there has been resurgence in the use of colistin as last line therapy against multidrug-resistant (MDR) Gram-negative bacteria, particularly *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* [93, 94, 98]. Clinically the inactive prodrug of colistin, CMS [9] is administered, with formation of colistin a pre-requisite for antibacterial activity [97]. CMS is administered via the pulmonary route in CF patients for the treatment of initial colonization of *P. aeruginosa* in the airways [10], and as maintenance therapy in chronic infections [3, 4]. Effective treatment with inhaled CMS is crucial for delaying lung function deterioration [3, 4, 10]. More recently, inhaled CMS has been introduced as adjunctive therapy in critically-ill patients with ventilator-associated pneumonia (VAP) caused by *P. aeruginosa* and *A. baumannii* [7, 17].

Despite the increase in inhalational administration of CMS, there are limited pharmacokinetic data available for CMS and formed colistin after pulmonary dosing. Recently, pharmacokinetic evaluation in rats [184], CF patients [170] and critically-ill patients [129] have reported on CMS and/or formed colistin exposure in sputum or epithelial lining fluid (ELF) and plasma following inhalation of a single dose of CMS. Relative to plasma exposure, high CMS and/or formed colistin exposure in sputum/ELF were evident in all studies [129, 170, 184]. However, none of these studies characterized the lung and systemic

pharmacokinetics following administration of both CMS and colistin via the pulmonary and intravenous (IV) route.

Therefore, the principal objective of the current study was to investigate the pharmacokinetics of CMS and colistin following IV and pulmonary administration of both compounds in rats. The development of a population pharmacokinetic model provided a better understanding of the CMS to colistin conversion kinetics and the disposition in plasma and ELF. Overall, the study identified the targeting advantage achieved following direct dosing of CMS into the lungs compared to IV administration.

5.3 Materials and methods

5.3.1 Chemicals

Colistin sulfate and sodium colistin methanesulfonate were from Sigma-Aldrich (Missouri, USA) and Link Pharmaceuticals Ltd (Auckland, New Zealand), respectively. All other chemicals were of analytical reagent grade and solvents were of at least high-performance liquid chromatography (HPLC) grade.

5.3.2 Animals

All experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The study protocols were reviewed and approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. Male Sprague-Dawley rats (300-320 g) were acclimated for a minimum of seven days in the faculty animal house within a temperature range of 18-24°C, 40-70% relative humidity and 12 h light/dark cycles. Food and water were available *ad libitum* during the acclimation period and throughout the study. The day prior to the pharmacokinetic study, the right carotid artery of each rat was cannulated for collection of blood samples; the jugular vein was cannulated

for IV dosing. Following surgery, rats were individually housed in metabolic cages and allowed 24 h for recovery.

5.3.3 Drug formulations and administration

Immediately prior to IV and pulmonary administration, CMS (sodium) and colistin (sulfate) dosing solutions were freshly prepared in sterile 0.9% sodium chloride (Baxter Healthcare Pty Ltd, New South Wales, Australia). For the IV studies, CMS or colistin solutions were administered by a bolus injection via the jugular vein cannula. Intratracheal (IT) instillation was utilized as the technique for pulmonary administration, as it delivers an accurate and reproducible volume of dosing solution into a localized region of the lungs [204]. For IT instillation, rats were lightly anesthetized with gaseous isoflurane (Delvert Pty Ltd, New South Wales, Australia) and rested in a supine position against a restraining board angled at approximately 60-70° from the horizontal. The tongue of the rat was gently pulled outwards using forceps, and the blade of a small animal laryngoscope (PennCentury Inc, Pennsylvania, USA) positioned in the distal part of the mouth to enable visualization of the vocal cords. A 2.5 cm polyethylene tube (PE) (0.96 × 0.58 mm (o.d. × i.d.)) attached to a 23 gauge (G) needle and 1 mL syringe was then maneuvered past the vocal cords to the trachea-bronchus bifurcation. A 100 μL aliquot of dosing solution followed by a 200 μL bolus of air was then delivered into the rat lungs. The air was administered to ensure complete delivery of the dosing solution from the syringe and cannula. Following IT instillation, rats were returned to metabolism cages where they rapidly recovered from the isoflurane anesthesia.

5.3.4 Pharmacokinetic studies

Animals were administered IV CMS at doses of 14 mg/kg, 28 mg/kg or 56 mg/kg (n=3 per dose). Blood samples (320 μL) were collected via the carotid artery prior to dosing and at 0.08, 0.25, 0.5, 1, 2, 3 and 4 h post-dosing. In an independent study, rats were administered

IV colistin at doses of 0.21 mg/kg, 0.41 mg/kg or 0.62 mg/kg (n=3 per dose); blood samples (200 µL) were collected prior to dosing and at 0.02, 0.05, 0.08, 0.17, 0.33, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 h post-dose. In the dose-ranging CMS study above, blood samples were immediately placed on ice prior to centrifugation (6,700 × *g*, 4°C, 10 min), to minimize any potential *in vitro* conversion of CMS to colistin. Additionally, any potential *in vitro* conversion was minimized by storage of plasma samples at -80ºC, and subsequent HPLC analysis within 4 months of collection [219].

For the pulmonary dosing studies, two cohorts of animals were required to fully characterize systemic and lung exposure. Rats in the first cohort were administered IT CMS at doses of 14 mg/kg or 28 mg/kg (n=3 per dose). Blood samples (320 µL) were collected pre-dose and at 0.08, 0.5, 1, 2, 3, 4, 5, 6 and 8 h post-dose. In the second cohort, rats received a pulmonary dose of 14 mg/kg CMS, to allow for the collection of terminal bronchoalveolar lavage (BAL) fluid and a corresponding blood sample. Samples were collected at 0.08, 0.5, 2, 4, 6, 8 and 12 h post-dose (n=3 rats per time point). In a separate study, rats in the first cohort were administered colistin at IT doses of 0.41 mg/kg, 0.62 mg/kg, 0.99 mg/kg or 1.49 mg/kg (n=3 per dose). Blood samples were collected as described above following IV colistin dosing. A second cohort of rats received an IT dose of 0.62 mg/kg colistin; terminal BAL fluid and blood samples were collected as described for IT dosing of CMS. Blood samples were processed as above and the plasma collected to quantify drug and urea concentrations (for the second cohort of rats). Bronchoalveolar lavage and processing of BAL fluid samples were undertaken as detailed below.

5.3.5 Bronchoalveolar lavage

Rats were anesthetized with gaseous isoflurane and sacrificed via exsanguination. For BAL, the trachea was exposed and a small incision made to allow the insertion of a PE tube (1.70 ×

1.20 mm (o.d. × i.d.)) attached to an 18G needle. The lungs were gently lavaged with 5 mL of phosphate buffered saline (PBS, pH 7.4, 4°C) for three cycles, using fresh PBS each time. The recovered lavage fluid was pooled and centrifuged (6,700 × *g*, 4°C, 10 min), and the resulting supernatant removed and stored at -80ºC prior to HPLC analysis.

5.3.6 Plasma and bronchoalveolar fluid analysis

The concentrations of colistin and CMS in plasma and BAL fluid were determined using previously validated HPLC assays [191, 192] with minor modifications. Briefly, the analytical methods involved quantification of colistin before and after forced *in vitro* conversion from CMS; the concentration of CMS was calculated as the difference between the two assay results and adjusted for molecular weight [191, 192]. For each of colistin and CMS assays, calibration standards and quality control (QC) samples were from independently prepared stock solutions. For the plasma assay, colistin and CMS calibration standards were prepared in drug-free rat plasma, and ranged from 0.071 − 2.85 μmoL/L and 0.45 − 29 μmoL/L, respectively. For the analysis in BAL fluid, calibration standards were prepared in drug-free rat BAL fluid:acetonitrile (50:50, v/v), and ranged from 0.18 − 5.70 μmoL/L (colistin) and 0.45 − 29 μmoL/L (CMS). Rat plasma and BAL fluid samples with colistin and CMS concentrations above the upper end of the respective plasma and BAL fluid calibration curve were diluted to within the linear range with drug-free matrix; QC samples of colistin and CMS were treated similarly to confirm satisfactory assay performance. The limit of quantification (LOQ) was defined as the lowest concentration in the calibration curve, at which the accuracy and precision were within ± 20%; at all other concentrations, the accuracy and precision were within ± 15%.

Urea concentrations in plasma and BAL fluid were determined using a commercially available kit, QuantiChrom[TM] Urea Assay Kit (BioAssay Systems, California, USA). Urea is

an endogenous marker of dilution, and was used to estimate the apparent volume of the ELF (V_{ELF}) [31]. The apparent V_{ELF} was calculated as shown in Equation 1:

$$V_{ELF} = \frac{[Urea]_{BAL\,fluid}}{[Urea]_{Plasma}} \times V_{BALF} \qquad (1)$$

where *[Urea]$_{BAL\,fluid}$*, *[Urea]$_{Plasma}$* and *V$_{BALF}$* are the urea concentration in BAL fluid (mg/dL), plasma (mg/dL) and the volume of recovered BAL fluid, respectively. The V_{ELF} was used to calculate the concentration of CMS or colistin in ELF (*[CMS/Colistin]$_{ELF}$*) using Equation 2:

$$[CMS/Colistin]_{ELF} = [CMS/Colistin]_{BALF} \times \frac{V_{BALF}}{V_{ELF}} \qquad (2)$$

where *[CMS/Colistin]$_{BALF}$* is the concentration of CMS or colistin in the recovered BAL fluid.

5.3.7 Pharmacokinetic modeling

Initially, a non-compartmental analysis (NCA) of dose-normalized concentration-*versus*-time profiles was conducted using WinNonlin (version 5.3, Pharsight Corporation, USA) to guide model development. Population pharmacokinetic analysis of the observed (not dose-normalized) concentrations was then performed using nonlinear mixed-effects modeling in S-ADAPT (version 1.57) facilitated by SADAPT-TRAN [222]. Initially, models were independently developed for CMS and colistin following their IV administration. One-, two-, three- and four-compartment models were tested to obtain key disposition parameters of CMS or colistin in plasma. The concentration-*versus*-time data of CMS and colistin in plasma, BAL fluid and ELF were then combined to simultaneously analyze the pharmacokinetic profiles after IV and IT administration. Two compartments (BAL fluid$_1$ and BAL fluid$_2$) were required to reflect drug kinetics in the BAL fluid. The total amount of drug in the BAL fluid

was therefore represented by the sum of the predicted amounts in BAL fluid$_1$ and BAL fluid$_2$. These total drug amounts were divided by V$_{ELF}$, for the prediction of CMS or colistin concentrations in the ELF.

First-order and mixed-order kinetics were tested to describe the conversion of CMS to colistin, and absorption of both compounds from the lungs into the systemic circulation. These models also explored whether a delay (represented by a series of transit compartments) in conversion or absorption was required to adequately fit the data. Figure 1 illustrates the structure of the final composite model.

The between-subject variability (BSV) in parameter estimates was assumed to follow a log-normal distribution, with the magnitude reported as a coefficient of variation (CV). Residual unexplained variability (RUV) in the plasma model was evaluated using a combined exponential and additive random error. For the BAL fluid and ELF model, the exponential and additive components were fixed to assay precision (15%) and LOQ (0.18 μmoL/L for colistin and 0.45 μmoL/L for CMS), respectively. This allowed for the estimation of BSV, since only a single observation was obtained from each rat due to terminal BAL sampling. Data below the LOQ were handled using a likelihood-based (M3) method [223].

Visual inspection of diagnostic scatter plots, the objective function value (OBJ, reported as -1 × log likelihood in S-ADAPT) and the biological plausibility of the parameter estimates were used for model selection. Statistical comparison of the models was performed using a χ^2 test; a decrease in the OBJ of 1.92 units ($\alpha = 0.05$) was considered significant.

The final model was evaluated by performing a visual predictive check (VPC). For this, 1000 data sets were simulated from the final parameter estimates using the original data. The median and the 10th and 90th percentiles (25th and 75th percentiles for the ELF models) of the simulated predictions were computed and plotted against the observed values. All reported

parameter estimates and VPCs were from the simultaneous modeling of CMS and colistin pharmacokinetics after IV and IT dosing.

5.3.8 Histopathology

Histopathology examination was undertaken to determine whether cellular damage to the lung epithelium occurred following pulmonary dosing of CMS or colistin. Two control groups (n=3 rats per group), were dosed with 100 μL of blank saline via IT instillation; the treatment groups (n=3 rats per group) received either 14 mg/kg CMS or 0.62 mg/kg colistin (doses corresponding to the studies where BAL fluid was collected) in a 100 μL aliquot. Following dosing, rats were anesthetized and sacrificed via exsanguination at 4 h (for CMS) or 0.75 h (for colistin); sample collection times reflect maximal concentrations in plasma. The trachea was exposed and an incision made to allow for the insertion of an 18G needle. The trachea/lungs were then immediately fixed with 5 mL of formalin solution (neutral buffered, 10%, Sigma-Aldrich), and stored in 10 mL of formalin solution. Histopathology examination (Cerberus Sciences, South Australia, Australia) was performed on the following lung tissues, 1) trachea in the lumen, mucosa, submucosa, submucosal glands, connective tissue and cartilage regions, and 2) lung lobes in the external/internal bronchi, terminal bronchioles/alveolar ducts, tracheal bifurcation (lumen, mucosa, submucosa, connective tissues), pleura, blood vessels and the bronchus-associated lymphoid tissues regions. BAL was not performed prior to tissue harvesting to ensure that the histopathology was not affected by excess fluid in the alveoli.

5.4 Results

5.4.1 Pharmacokinetics following intravenous administration

Following administration of IV CMS, average maximal plasma concentrations of CMS were 27 μmoL/L (lowest dose) and 123 μmoL/L (highest dose), and declined by approximately

three-orders of magnitude over the study period (Figure 2A). The corresponding concentrations of formed colistin in plasma were quantifiable at the first sampling time (5 min), with mean peak concentrations of 0.17 μmoL/L (lowest dose) and 0.70 μmoL/L (highest dose) (Figure 2B). These maximal concentrations were achieved between 0.5 – 1 h after CMS administration, and declined relatively slowly during the sampling period (Figure 2B). Administration of the active antibacterial moiety, colistin, was undertaken to characterize the disposition in plasma, with average maximal plasma concentrations ranging from 1.0 to 4.0 μmoL/L, and concentrations declining exponentially over the 4 h sampling period (Figure 2C).

5.4.2 Pharmacokinetics following intratracheal instillation

Pulmonary administration of CMS resulted in average ELF concentrations of 13,099 μmoL/L at 5 min post-dose, and thereafter concentrations declined over the 12 h sampling period (Figure 3A). Maximal concentrations of formed colistin in ELF were achieved at 4 h after CMS dosing, with relatively high concentrations maintained throughout the 12 h sampling period (Figure 3B). Measured concentrations of urea after IT CMS were 56 ± 13 mg/dL in plasma and 0.35 ± 0.13 mg/dL in BAL fluid, with an estimated apparent V_{ELF} of 0.084 ± 0.016 mL. Similar urea concentrations (50 ± 9.2 mg/dL and 0.41 ± 0.14 mg/dL in plasma and BAL fluid, respectively) and apparent V_{ELF} estimates (0.11 ± 0.023 mL) were obtained after IT administration of colistin.

5.4.3 Population pharmacokinetic model

In the full composite model, the disposition of both CMS and colistin in plasma was best described by a three-compartment model. Table 1 presents the parameter estimates from the final model. The estimated mean clearance from the central compartment was 0.122 L/h (BSV 10.7%) for CMS and 0.0657 L/h (BSV 24.7%) for colistin. Steady-state volumes of

distribution were 0.292 L and 0.170 L for CMS and colistin, respectively. Conversion of CMS to colistin in the central compartment was described by a first-order process (CL_{pm} = 0.0016 L/h; corresponding rate constant of 0.102 h^{-1}). Similar conversion clearances were incorporated from the shallow and deep peripheral compartments to fit the high concentrations of formed colistin at 2 h after IV administration of CMS. In the absence of these peripheral conversion terms, the model was statistically inferior (Δ OBJ + 20.1). The rate constant for central conversion was more than three-fold faster than that estimated in shallow (0.0303 h^{-1}) and deep peripheral (0.00121 h^{-1}) compartments. The estimated fraction of the IV CMS dose converted to colistin ($f_{m,systemic}$) was 0.0255. Figure 2 presents the VPCs for both compounds after their IV administration.

Following IT instillation, the fractional dose (F_{pul}) available for exposure to the lungs was 40.9% for CMS and 48.5% for colistin. Two compartments (BAL fluid$_1$ and BAL fluid$_2$) described drug movement from the IT dosing site to the remaining regions of the respiratory airways (see Figure 1). For CMS the estimated mean time ($1/k_{BALF}$) for movement from BAL fluid$_1$ to BAL fluid$_2$ was 0.315 h, which was faster than that for colistin (2.52 h). Additional first-order terms were included to describe the distribution of both compounds from the lung lining fluid to the deep peripheral compartment in the plasma model.

The model required a slow rate of CMS conversion (mean conversion time, MCT$_{CMS}$ 3.48 h in BAL fluid$_1$) to describe the high concentrations of colistin maintained in the ELF (Figure 3B, Figure 3 provides the VPCs for both compounds in ELF and in plasma after IT dosing). This slow conversion adequately explained the subsequent delay in absorption of colistin into plasma (Figure 3E). In contrast, only a single first-order term (kpm = 0.0154 h^{-1}) was required to describe the kinetics of CMS to colistin conversion in BAL fluid$_2$. The estimated fraction of the CMS dose converted to colistin in ELF ($f_{m,ELF}$) was 0.226, and was almost nine-fold

higher than the corresponding fractional conversion in plasma (0.0255) after IV administration (Figure 4A).

First-order kinetics best described the absorption of both CMS (ka_{cms} = 0.936 h^{-1}) and colistin (ka_{col} = 2.15 h^{-1}) from the IT dosing compartment (BAL fluid$_1$) to plasma. For CMS, an additional pathway incorporating zero-order with sequential delayed first-order absorption, was required to fit the high concentrations observed at 2-3 h in plasma (Figure 3D). This route was a minor component of the model, and only contributed to 0.3% of total CMS absorption.
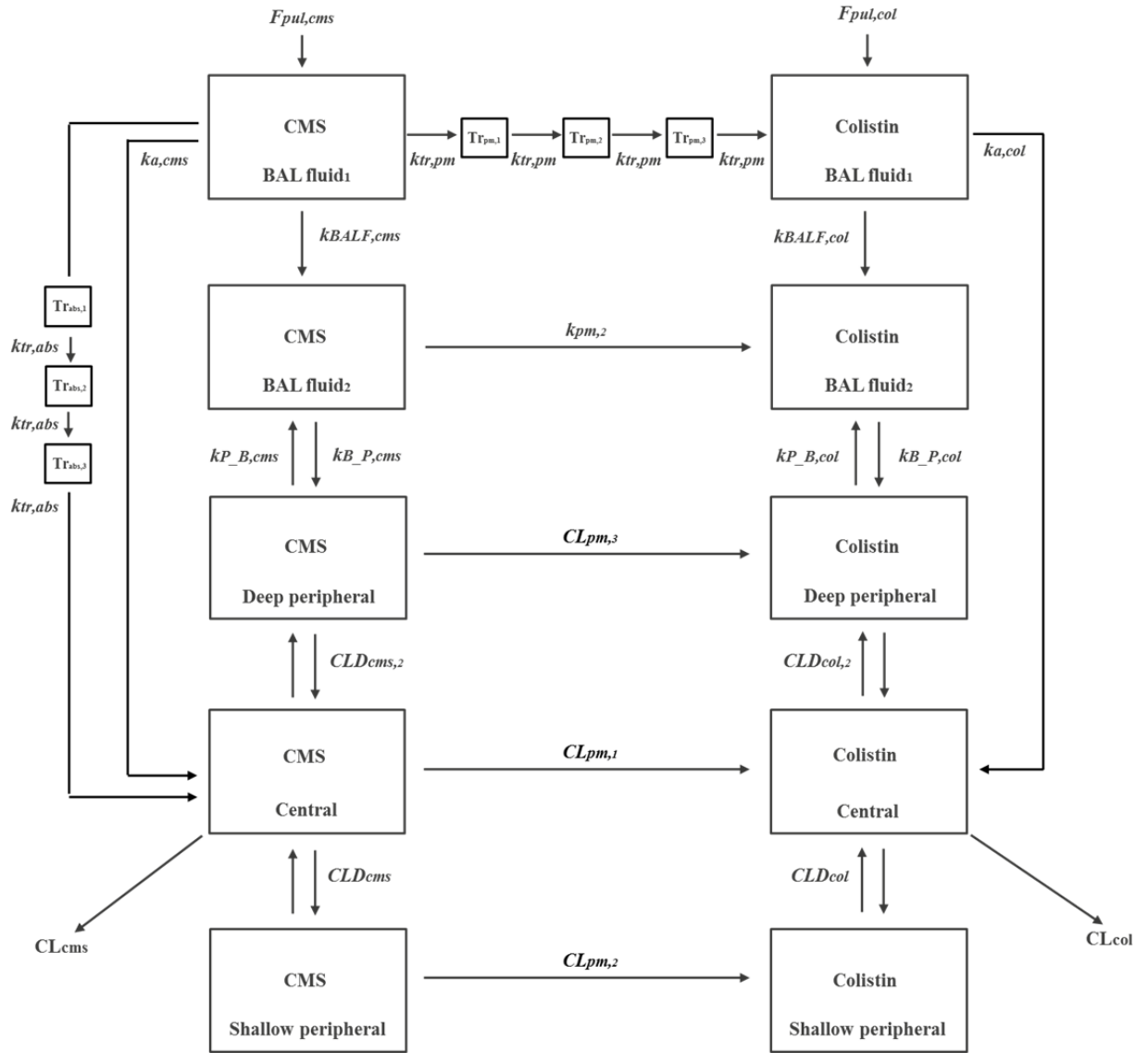
Figure 1: Structure of the population pharmacokinetic model for CMS and colistin following intravenous and intratracheal dosing in rats. Abbreviations are defined in Table 1.

Table 1: Population pharmacokinetic parameter estimates for CMS and colistin after intravenous and pulmonary dosing.

	Parameter	Description	Unit	Estimate	BSV (CV%)
Plasma					
Colistin	VC_{col}	Central volume of distribution	L	0.0464	20.9
	CL_{col}	First-order central compartment clearance	L/h	0.0657	24.7
	$VP_{col,1}$	Shallow peripheral volume of distribution	L	0.0294	128
	CLD_{col}	Shallow peripheral inter-compartmental clearance	L/h	0.366	23.5
	$VP_{col,2}$	Deep peripheral volume of distribution	L	0.0942	23.6
	$CLD_{col,2}$	Deep peripheral inter-compartmental clearance	L/h	0.0402	32.0
CMS	VC_{cms}	Central volume of distribution	L	0.0157	17.9
	CL_{cms}	First-order central compartment clearance	L/h	0.122	10.7
	$VP_{cms,1}$	Shallow peripheral volume of distribution	L	0.0432	6.65
	CLD_{cms}	Shallow peripheral inter-compartmental clearance	L/h	0.356	16.1
	$VP_{cms,2}$	Deep peripheral volume of distribution	L	0.233	31.8
	$CLD_{cms,2}$	Deep peripheral inter-compartmental clearance	L/h	0.0178	35.2
	$CL_{pm,1}$ [a]	Central compartment clearance of CMS to colistin	L/h	0.00160	16.4
	$CL_{pm,2}$ [a]	Shallow peripheral clearance of CMS to colistin	L/h	0.00131	20.4
	$CL_{pm,3}$ [a]	Deep peripheral clearance of CMS to colistin	L/h	0.000282	71.8
Lung					
Colistin	$F_{pul,col}$	Fractional dose available for pulmonary disposition	-	0.485	69.6
	$k_{a,col}$	First-order rate constant for lung absorption	1/h	2.15	20.6
	$k_{BALF,col}$	First-order transfer from BAL fluid$_1$ to BAL fluid$_2$	1/h	0.397	29.1
	$k_{B\ P,col}$	Distribution from BAL fluid$_2$ to deep peripheral	1/h	0.0843	24.9
	$k_{P\ B,col}$	Distribution from deep peripheral to BAL fluid$_2$	1/h	0.00416	69.5
CMS	$F_{pul,cms}$	Fractional dose available for pulmonary disposition	-	0.409	54.5
	$k_{a,cms}$	First-order rate constant for lung absorption	1/h	0.936	45.1
	$Dur_{abs,cms}$	Duration for zero-order absorption	h	2.21	13.3
	$MTT_{cms,abs}$	Mean transit time for pulmonary absorption	h	1.50	10.4
	$k_{BALF,cms}$	First-order transfer from BAL fluid$_1$ to BAL fluid$_2$	1/h	3.17	27.8
	$k_{B\ P,cms}$	Distribution from BAL fluid$_2$ to deep peripheral	1/h	0.608	9.45
	$k_{P\ B,cms}$	Distribution from deep peripheral to BAL fluid$_2$	1/h	0.0121	35.6
	$MCT_{cms,conv}$	Mean conversion time for CMS in BAL fluid$_1$	h	3.48	16.5
	$k_{pm,2}$	Rate constant for CMS conversion in BAL fluid$_2$	1/h	0.0154	162
Residual Error [b]					
	$SDsl_{col,plasma}$	Exponential error for colistin in plasma	%	18.1	-
	$SDsl_{CMS,plasma}$	Exponential error for CMS in plasma	%	16.7	-

[a] Plasma $k_{pm,1}$, $k_{pm,2}$ and $k_{pm,3}$ were 0.102, 0.0303 and 0.00121 h^{-1}, respectively.
[b] Residual error for the BAL fluid and ELF model was fixed to the assay precision and limit of quantification, since only a single observation was obtained from each rat due to terminal BAL sampling.
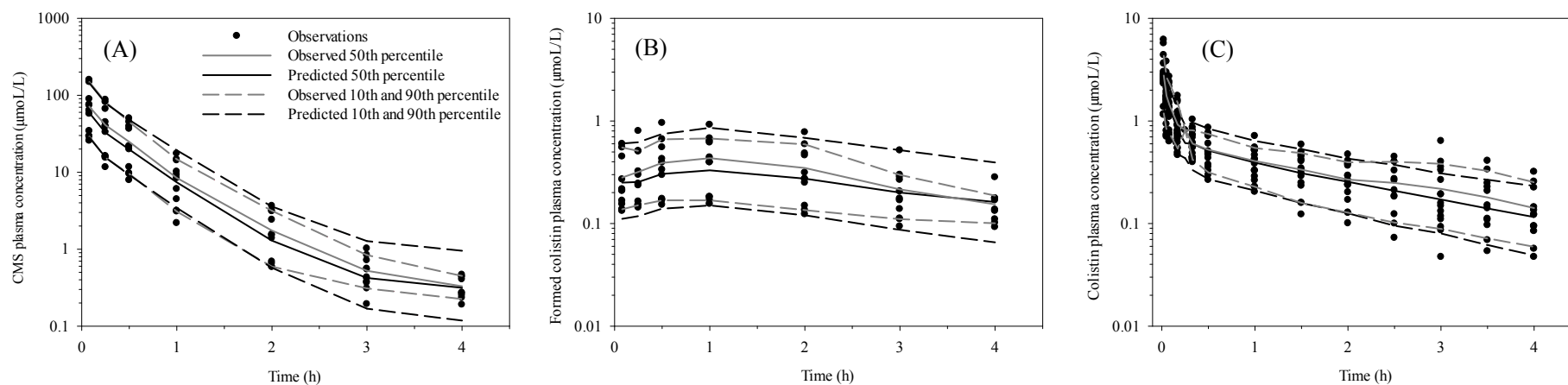
Figure 2: Visual predictive check for (A) CMS and (B) formed colistin plasma concentration following intravenous (IV) CMS dosing, and (C) colistin plasma concentration following IV colistin dosing in Sprague-Dawley rats. The model-predicted median and 10th and 90th percentiles closely match corresponding observed percentiles, indicating the suitability of the pharmacokinetic model. Closed circles represent actual observed data in rats.
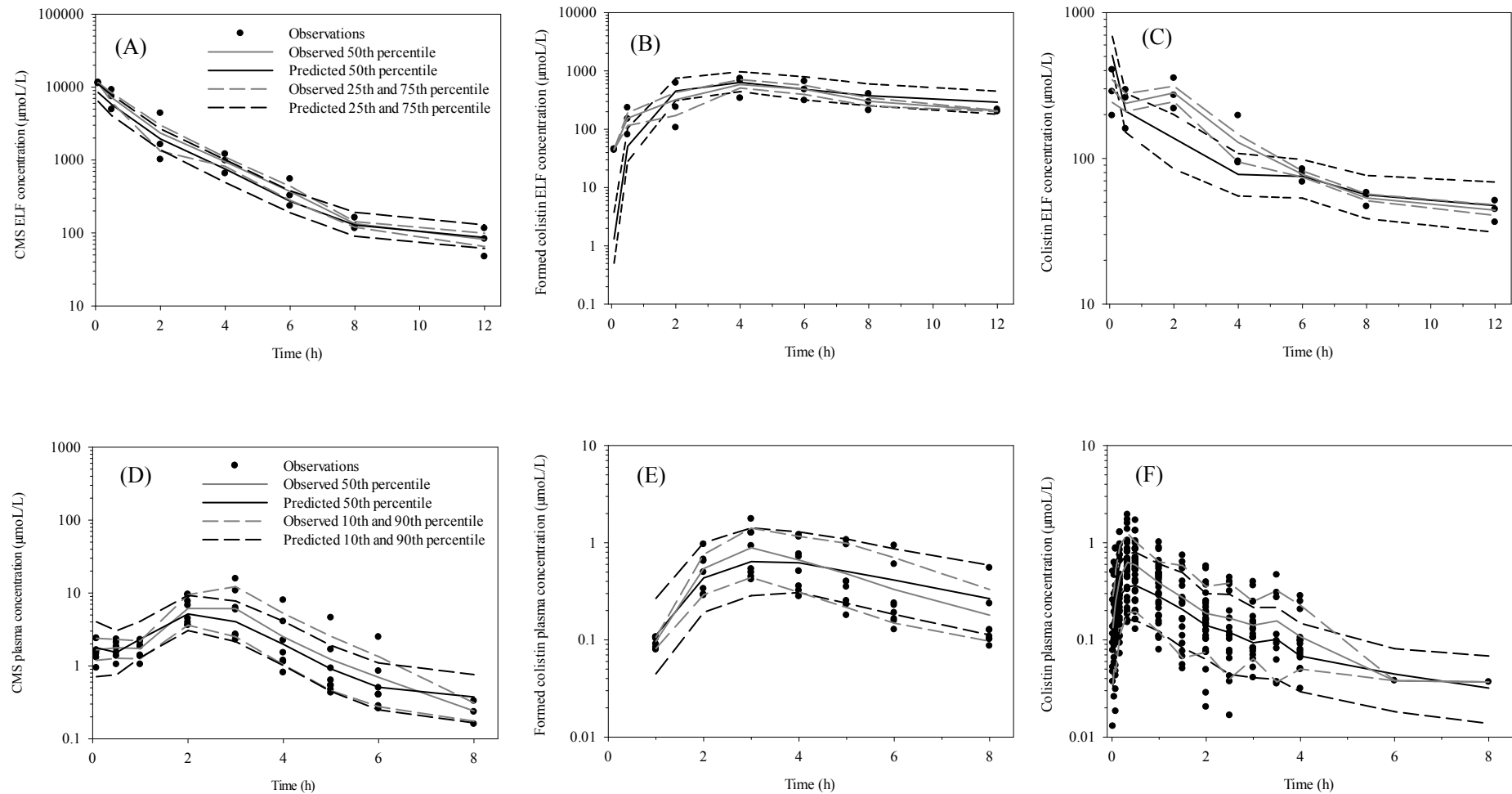
Figure 3: Visual predictive check for (A) CMS, (B) formed colistin in ELF following intratracheal (IT) CMS dosing and (C) colistin in ELF following IT colistin dosing; (D) CMS, (E) formed colistin in plasma after IT CMS dosing and (F) colistin in plasma following IT colistin dosing in Sprague-Dawley rats. The model-predicted median and 25th/75th and 10th/90th percentiles broadly match corresponding observed percentiles, suggesting reasonably good predictive performance. Closed circles represent actual observed data in rats. The 25th and 75th percentiles are presented for the ELF profiles, due to the small number of observations obtained via this sampling route
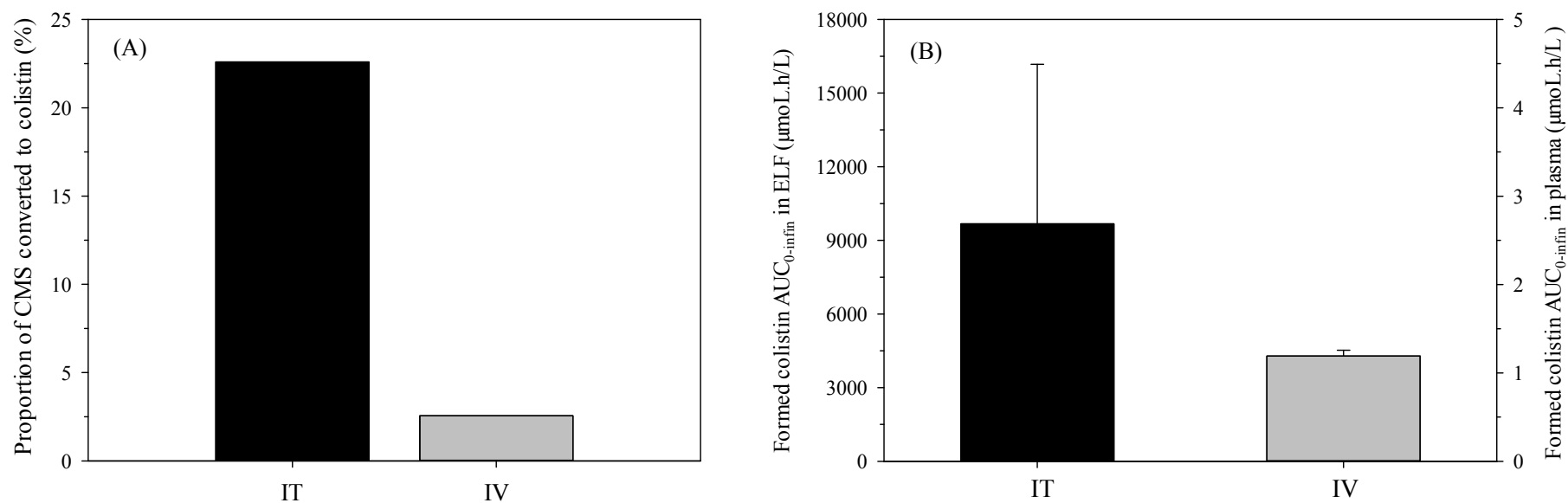
Figure 4: (A) Percentage of IT (14 mg/kg) and IV (dose-ranging) CMS dose converted to colistin in ELF (black) and plasma (grey) and (B) formed colistin exposure ($AUC_{0-\infty}$, μmoL·h/L) in ELF and plasma following IT and IV administration of CMS 14 mg/kg, respectively. Formed colistin exposure was approximately 8000-fold higher in ELF after IT dosing when compared to in plasma after IV dosing. For panel B, note the different scale for the right y-axis.

5.4.4 Exposure of formed colistin following intratracheal instillation

Following IT instillation of CMS (14 mg/kg), the AUC of formed colistin in ELF was 9,669 ± 6,507 μmoL·h/L. Of this, only 0.03% of formed colistin from ELF was absorbed into plasma (AUC = 2.59 ± 0.409 μmoL·h/L). After IV dosing of CMS, the AUC of formed colistin in plasma was 1.19 ± 0.0666 μmoL·h/L. Thus, IT dosing gave a formed colistin exposure in ELF that was 8000-fold higher than that in plasma after IV dosing (Figure 4B).

5.4.5 Histopathology examination

Histopathology examination of the trachea and lung lobes following IT administration of either CMS or colistin showed no significant difference between the control and treatment groups (*data not shown*).

5.5 Discussion

The current study evaluates the pharmacokinetics of CMS and colistin following IV and IT administration of both compounds in rats. The data showed that direct administration of CMS into the lungs achieved high ELF exposure to formed colistin, thus demonstrating the potential for local targeting of antibiotics for the treatment of respiratory infection. Population pharmacokinetic analysis was advantageous in this study, because it allowed for the simultaneous modeling of plasma sampled via serial bleeds in one cohort of animals and ELF data from a second cohort where terminal BAL samples were obtained. The pharmacokinetic model provided a better understanding of the conversion kinetics for CMS to colistin in ELF and in plasma. The population pharmacokinetic analysis identified that high colistin concentrations in the ELF were obtained due to slow and sustained conversion kinetics of CMS in ELF after IT administration.

Intravenous dose-ranging studies demonstrated linear pharmacokinetics for CMS and colistin, consistent with the findings by Marchand *et al* in Sprague-Dawley rats [199]. The pharmacokinetic parameter estimates were broadly consistent with previously reported values arising from NCA after IV administration of CMS [184, 198, 199] and colistin [203] in rats.

There is limited knowledge on the mechanism by which colistin is formed from CMS *in vitro* and *in vivo* [110, 190, 219]. Conversion of CMS generates a complex mixture of partially sulfomethylated derivatives and colistin, formation of the latter being a pre-requisite for antibacterial activity [9, 110]. While previous studies in rats have reported on the fraction of an IV CMS dose that is converted to colistin systemically [184, 198], the kinetics of this conversion is currently unknown. Li *et al* [198] and Marchand *et al* [184], estimated a fractional systemic conversion of ~6% and ~13%, respectively, which is higher than that estimated in the present study (3%). The population pharmacokinetic model required conversion in all three disposition compartments (central, shallow and deep peripheral), to characterize the gradual appearance of formed colistin in plasma following IV administration of CMS. Maximal plasma concentrations of formed colistin were achieved at $0.5 - 1$ h post-CMS administration, which was in contrast to studies by Li *et al* [198] and Marchand *et al* [184, 199] where peak concentrations of formed colistin were observed at the initial sampling time of 5 min. The inter-study variation in the conversion kinetics is potentially the result of the use of different brands of sodium CMS which can have varying ratios of fully and partially sulfomethylated CMS derivatives and yield different plasma concentration-*versus*-time courses for formed colistin [224]. Importantly, in all of the above pre-clinical evaluations, a low proportion (~3 - 13%) of the IV dose of CMS was converted to colistin.

This study demonstrates that direct pulmonary administration of CMS achieves high ELF exposure to formed colistin compared to the exposure in plasma. The concentrations of colistin in ELF were maintained, over the 12 h sampling period, above the minimum

inhibitory concentration required for 50 to 90% inhibition of bacterial growth based on clinical isolates of *P. aeruginosa* and *Acinetobacter* spp. (MIC_{50-90}) of 1.0 mg/L [172]. The high concentrations of CMS and formed colistin in ELF described here are consistent with the findings by Marchand *et al*, following IT nebulization to rats at a dose equivalent to 14 mg/kg CMS [184].

Population pharmacokinetic analysis identified slower conversion kinetics in the lung following IT instillation, with a greater fraction of the CMS dose (23%) converted to colistin in ELF compared to conversion in plasma after IV administration (3%) (Figure 4A). This slow and sustained conversion (in BAL fluid$_1$) contributed to the high colistin concentrations in ELF and the corresponding delay in its plasma appearance after IT administration of CMS. With limited information available about the conversion mechanism of CMS to colistin, one potential explanation for the slower, but ultimately more extensive conversion observed in the lungs is that while CMS resides within the lung it is not available for renal clearance, which is the major systemic clearance mechanism [97]. A further contributing factor to the increased ELF exposure is that CMS will be dissolved within a relatively small volume of lung lining fluid (ELF volume estimated to be 0.084 ± 0.016 mL) and thereby creating a reservoir of CMS available for ongoing conversion to colistin, which will be reflected in the high exposure of formed colistin in ELF and a greater fractional conversion of CMS in the lungs. Marchand *et al* proposed similar explanations for observations of high CMS concentrations in ELF (slow conversion and absorption), with the authors reporting a slightly higher fraction of the IT CMS dose converted to colistin (39%) in the lungs [184].

Two BAL fluid compartments were included in the model to reflect the site of IT dosing and the movement of drug over time to other regions of the lungs. Thus, BAL fluid$_1$ potentially represents localized antibiotic concentrations in the trachea, upper airways and to some extent the peripheral airways, followed by mono-directional transfer into the remaining peripheral

lung regions (represented as BAL fluid$_2$). Relative to CMS, a slower movement of colistin into the peripheral airways may have occurred due to colistin binding to lung tissues [212, 213, 225]. Several sites have been proposed for the binding of lipophilic basic amines (pKa > 8.5) in the lungs [49-51, 53]. Further antibiotic distribution from the ELF to the deep peripheral compartment suggests that this compartment could equate to the lung tissue, although additional studies are required to confirm this hypothesis.

In the current model, the absorption of CMS and colistin from the lungs into the systemic circulation was best described by first-order kinetics, with an additional minor (<1%) delayed pathway for the former compound. Appearance of colistin in plasma following IT administration of colistin was more rapid (plasma concentrations quantifiable at 1 – 10 min post-dose, Figure 3F) than for formed colistin (1 h post-dose) after IT dosing of CMS (Figure 3E). For formed colistin this is likely to be due to the ongoing conversion from CMS in the lungs. Given the physicochemical properties of CMS (average M_w 1633 Da, polar surface area (PSA) 741 Å, Log P -12.09) and colistin (average M_w 1163 Da, PSA 490 Å, Log P 3.42), the proposed mechanism of absorption is passive diffusion via paracellular transport mechanisms. This is supported by Schanker and colleagues, who reported that the rat lung epithelium is characteristic of the classic lipoid-pore of biological membrane [226-228]. Tronde *et al* have also illustrated passive diffusion as the dominant transport mechanism for drug absorption from the rat lungs [229, 230]. In the present study, the slow absorption of CMS from the lungs was consistent with the low apparent permeability for CMS through Calu-3 cells (apical-to-basolateral direction) [184].

Notwithstanding the extensive lung exposure to formed colistin, its relative systemic exposure following IT administration of CMS (14 mg/kg) was two-fold higher than the systemic exposure after IV administration of the same dose of CMS. The greater fraction of CMS dose converted to colistin in the ELF (23%) and absorption of a proportion of this pre-systemically

formed colistin is the most likely cause for these findings. These results are, in-part, consistent with findings by Marchand *et al* who reported that 39% of the IT CMS dose was converted to colistin in the lungs prior to absorption into the systemic circulation which the authors speculated led to the increased exposure to formed colistin in plasma [184]. The potential for lung epithelium damage and resultant increased drug permeability was considered as an alternative explanation for the increased plasma exposure to formed colistin in this study, however histopathological analysis of lung tissue following IT administration confirmed that this was not the case. This study in rats suggests that a reduction in the pulmonary CMS dose could achieve colistin concentrations in ELF above the MIC_{50-90} of 1.0 mg/L [172] and still reduce total systemic exposure and hence reduce the likelihood of adverse effects.

Population pharmacokinetic analysis demonstrated good predictive performance of a model that simultaneously fits six dependent variables. While minor model misspecification is apparent in some profiles (e.g. colistin in ELF following IT administration of colistin, Figure 3C), these would not alter the pharmacological implications of the exposure achieved after direct (targeted) administration to the lungs. Future applications of the current model could test whether similar CMS to colistin conversion occurs with the use of different inhalational formulations (e.g. dry powder) in the pre-clinical setting. Furthermore, the model serves as a basis for the translation of CMS and colistin pharmacokinetics from rodents to larger animal models and humans. Ultimately, these models could be usefully employed to develop inhalational dosing recommendations for CMS in CF patients that simultaneously consider target endpoints in respiratory infection whilst avoiding potential adverse effects.

In conclusion, we have for the first time developed a detailed model that describes the pharmacokinetics of CMS and colistin after both IV and IT administration. We demonstrate that high concentrations of colistin in rat ELF are achieved as a result of slow and sustained

CMS conversion following IT instillation. Slow absorption of CMS into the systemic circulation and less competing clearance pathways for CMS in the lungs contribute to the greater fractional conversion in the lungs. The higher colistin systemic exposure after IT administration compared to exposure after IV dosing of CMS is a result of absorption of formed colistin from the lungs. This has the potential to increases systemic toxicity, but may be modulated by a dose reduction, since colistin exposures in the current study were well above the MICs of *P. aeruginosa*. The current population pharmacokinetic model can be utilized to further investigate the conversion kinetics of CMS and to characterize the disposition of CMS and formed colistin following inhalational administration in larger animals and in humans.

5.6 Acknowledgements

We thank Professor Carl Kirkpatrick, Drs Jürgen Bulitta and Cornelia Landersdorfer for advice and assistance with development of the population pharmacokinetic model.

5.7 Funding

SWSY was supported by a Monash Postgraduate Research Scholarship.

5.8 Transparency declaration

None to declare.

Monash University

Declaration for Thesis Chapter 6

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Concept and design of studies, planning and execution of experimental work, data analysis and interpretations, formulation of conclusions and hypotheses resulting from the relevant studies. Drafting and revision of manuscript.	85%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%)
M.P. McIntosh[1]	Project supervisor, data analysis and manuscript review	N/A
R.L. Nation[1]	Project co-supervisor, data analysis and manuscript review	N/A
Jian Li[1]	Project co-supervisor, manuscript review	N/A
C.J.H. Porter[1]	Project co-supervisor, manuscript review	N/A
K. Patel[2]	Data analysis and manuscript review	N/A
J.W. Wilson[3]	Manuscript review	N/A
M. J. Dooley[2,4]	Manuscript review	N/A
J. George[2]	Manuscript review	N/A
D. Clark[3]	Manuscript review	N/A
S. Poole[2,4]	Manuscript review	N/A
E. Williams[3]	Sample collection, patient recruitment, manuscript review	N/A

Candidate's Signature		Date

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	[1]Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Australia, [2]Centre for Medicine Use and Safety, Monash University, Australia, [3]Cystic Fibrosis Service, Department of Allergy, Immunology and Respiratory Medicine, Alfred Hospital, Australia, [4]Pharmacy Department, Alfred Health, Australia.

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Signature 11		

Chapter 6: Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulphonate in cystic fibrosis patients: targeting advantage of inhalational administration

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For a detailed description of the analytical methods utilised for quantification of colistin and colistin methanesulphonate in plasma, urine and sputum refer to Chapter 2, Section 2.3. Participant information and consent form (A2-1), case report forms (A2-2, A2-3 and A2-4) and adverse effect report forms (A2-5, A2-6 and A2-7) used for the current study are presented in Appendix II.

Manuscript in submission.

6.1 Abstract

Objective: The purpose of this study was to define the pulmonary and systemic pharmacokinetics of colistin methanesulphonate (CMS) and formed colistin following intravenous (IV) and inhalation administration in cystic fibrosis (CF) patients.

Methods: Six CF subjects were administered nebulised CMS doses of 2 and 4 million international units (IU) and IV CMS infusion of 150 mg of colistin base activity. Plasma, sputum and urine samples were collected for 12 to 24 h post-dose. To assess tolerability, lung function tests, serum creatinine concentrations and adverse effect reports were undertaken.

Results: The pharmacokinetic parameters for CMS following IV delivery were consistent with previously reported values. Sputum concentrations of formed colistin were maintained below 1.0 mg/L for 12 h post-dose. Nebulisation of CMS resulted in relatively high sputum concentrations of CMS and formed colistin when compared to IV administration. The systemic availability of CMS was low following nebulisation of 2 and 4 million IU (7.93 ± 4.26% and 5.37 ± 1.36%, respectively) and plasma colistin concentrations were below the limit of quantification. Less than 2 - 3% of the nebulised CMS dose was recovered in urine in 24 h. The therapeutic availability and drug targeting index ratios for CMS and colistin following inhalation when compared to IV delivery were significantly greater than one. All doses were well tolerated in subjects.

Conclusions: Inhalation of CMS is an effective means of targeting CMS and formed colistin into the lungs as high lung exposure and minimal systemic exposure were achieved in CF subjects.

6.2 Introduction

Cystic fibrosis (CF) is characterised by persistent airway colonisation and subsequent chronic airway infections, most commonly caused by *Pseudomonas aeruginosa* [5, 10, 196]. Establishment of chronic airway infections results in a vicious cycle of infection and inflammation that leads to permanent lung damage, pulmonary insufficiency and eventual mortality [3-5]. Therefore, effective management of CF lung infections with the use of antibiotic therapy plays a crucial role in reducing the progression of lung function deterioration [1, 3, 4, 10, 196].

As a consequence of rising rates of resistance to multiple antibiotics among *P. aeruginosa* isolates and the lack of new antibiotics available, colistin (also known as polymyxin E) which entered clinical use in late 1950s is now used increasingly in CF patients [94, 98]. Colistin methanesulphonate (CMS), an inactive prodrug that converts to the antibacterial form colistin [9], is administered intravenously (IV) or *via* inhalation to manage various stages of pulmonary colonisation and infection with *P. aeruginosa* [3, 4, 10, 11, 13, 15]. Intravenous administration of CMS relies on achievement in the airways of sufficiently high exposure of formed colistin to elicit the required antibacterial effect, ideally without precipitating nephrotoxicity which is the major dose-limiting adverse effect of systemically administered CMS [154]. Delivery of antibiotic *via* inhalation when compared to IV administration has the potential to achieve higher concentrations within the respiratory tract whilst minimising systemic exposure. Although CMS has been administered IV or by inhalation in CF patients for more than two decades, there is a paucity of comparative information on the pharmacokinetics of CMS and formed colistin following these modes of administration. Whilst there are data in CF patients on the concentrations of colistin in plasma and/or sputum following dry powder inhalation and nebulised CMS [160, 167, 170, 186, 231] and of CMS/colistin in plasma and/or sputum following IV CMS [187, 201], no studies have

reported the relative concentrations of CMS, and in particular formed colistin, in plasma and sputum following IV *versus* pulmonary administration of CMS in the same patient. Thus, the targeting advantage that may be achieved by delivering CMS directly to the airway to maximise bacterial killing (and potentially reduce bacterial resistance) while minimising systemic exposure (and the possibility of nephrotoxicity) has not been explored systematically in CF patients.

The principal aim of the current study was to examine the pulmonary and systemic pharmacokinetics of CMS and formed colistin following administration of CMS by the IV and pulmonary routes in CF patients. On three separate occasions, each patient received two single doses of nebulised CMS (two escalating dose levels) and a single dose of IV CMS. The tolerability of the respective doses of nebulised and IV CMS was also monitored. Our study enabled an assessment of the targeting advantage that may be achieved by administration of CMS directly to the airways for the treatment of pulmonary infections which may be used to facilitate optimisation of dosing regimens in CF patients.

6.3 Methods

6.3.1 Setting and subjects

The study population consisted of six inpatients at the CF Service at the Alfred Hospital (Melbourne, Australia). The study was approved by the Human Research Ethics Committees of the Alfred Hospital and Monash University (Melbourne, Australia), and subjects provided informed written consent before participating in the study. Subjects were eligible to participate in the study if they (1) were aged 18 years or older, (2) had a documented diagnosis of CF, (3) had a positive respiratory culture of *P. aeruginosa* in the absence of other Gram-negative and/or Gram-positive organisms, (4) were willing and able to use nebulised antibiotics, (5) had adequate birth control measures if sexually active while participating in

the study, and (6) had baseline forced expiratory volume in 1 second ($FEV_{(1)}$), that was 25 - 75% of predicted. Subjects were excluded if they had a history of allergy to colistin or polymyxin B or had recovery of *Burkholderia cepacia* in respiratory secretions over the previous two years. Inhaled or systemic CMS were not used by subjects 1 - 2 weeks prior to commencement of the study. Prior to commencement of this pharmacokinetic study, subjects were stabilised for their lung infection which was demonstrated by improvement in clinical symptoms and lung function tests.

6.3.2 Study protocol

6.3.2.1 Administration of nebulised and intravenous colistin methanesulphonate

Subjects received each of the following single-dose treatments in the order indicated: (1) nebulised CMS dose of 2 million international units (IU) (equivalent to 160 mg sodium CMS or 60 mg of colistin base activity (CBA)), (2) nebulised CMS dose of 4 million IU (320 mg sodium CMS or 120 mg of CBA) and (3) IV CMS dose of 150 mg of CBA (400 mg sodium CMS or 5 million IU). The dose equivalence of CMS in regard to millions of IU, and milligrams of sodium CMS and CBA have been reported previously [97]. Dosing solutions for nebulisation were prepared by reconstituting CMS (Tadim®, Phebra Pty Ltd, NSW, Australia) in 4 mL of sterile 0.9% sodium chloride. The IV dose of 150 mg of CBA (Colistin Link Parenteral®, Link Pharmaceuticals Ltd, Auckland, New Zealand) was reconstituted with 2 mL of sterile water for injection and added to 100 mL of sterile 0.9% sodium chloride solution. There was a minimum 3 day wash-out period between each single-dose treatment and the subsequent treatment was administered only if the preceding treatment was well tolerated.

All subjects received chest physiotherapy and salbutamol (2.5 - 5 mg *via* nebuliser) 1 h prior to administration of inhaled CMS. For nebulisation of CMS, a Salter Ultra-Mist nebuliser

(No. 8960) attached to an Exhalation Filter (No. 8980) connected to an outlet supplying air at a rate of 8 L/min was utilised and the mean nebulisation time was 14 ± 4.0 min. Nebulisation was carried out immediately following preparation of the dosing solution to minimise any potential *in vitro* CMS conversion to colistin [190]. Subjects sat upright for the duration of the nebulisation period to promote lung inflation. Immediately after preparation, the IV dose of CMS was infused over 45 min *via* a single lumen, peripherally inserted, central catheter line. Following dose administration the line was flushed with 30 mL of 0.9% sodium chloride. During the study period, subjects were administered all routine medications for the management of their CF including other antibiotics.

6.3.2.2 Sampling of blood, sputum and urine

Blood samples (5 mL) were collected pre-dose and following nebulisation of CMS at 0.5, 1, 2, 4, 8 and 12 h for subjects 1, 2 and 3. An amendment to the blood sampling times to 0.5, 1, 2, 4, 6 and 8 h post-dose was requested and approved by the ethics committee for subjects 4, 5 and 6 as CMS/colistin plasma concentrations were below the limit of quantification (LOQ) at 12 h post-dose following nebulised CMS in subjects 1 - 3. Blood samples (5 mL) were collected pre-dose and following completion of IV infusion at 0.25, 0.5, 0.75, 1, 2, 4, 8 and 12 h. In all subjects, an additional 15 mL of blood was collected at pre-dose and 12 h post-dose for serum creatinine quantification and full blood examination. Sputum samples were obtained in subjects capable of spontaneously expectorating adequate amounts of sputum, pre-dose and at 1, 4 and 12 h following nebulised and IV administration. Urine voided over the 12 h pre-dose period and 24 h post-dose period was collected. To minimise any potential *in vitro* conversion of CMS to colistin [219], samples for CMS and colistin concentration analysis were immediately stored on ice and processed at 4°C prior to storage at -80°C pending high-performance liquid chromatography (HPLC) analysis. Samples were analysed for CMS and colistin concentrations within 4 months of completion of the study [219].

6.3.2.3 Assessment of tolerability

Following CMS administration, subjects were monitored for signs and symptoms of adverse effects. Lung function tests were carried out pre-dose and 1 - 2 h following completion of inhaled and IV CMS administration. The lung function parameters monitored were $FEV_{(1)}$, forced vital capacity (FVC), minimum inspiratory pressure (MIP) and maximal expiratory pressure (MEP). Measurement of serum creatinine concentrations pre-dose and 12 h following completion of CMS administration was carried out to enable estimation of the glomerular filtration rate (eGFR) for the assessment of nephrotoxicity [232, 233]. Monitoring of blood pressure, heart rate and oxygen saturation was conducted at baseline and throughout the 24 h post-dose period. Subjects completed an adverse effect questionnaire 0.5 h following completion of CMS administration to report any adverse effects and to rate the severity on a scale of 0 (no adverse effect) to 10. Subjects were asked specifically if they experienced any of the following adverse effects: cough, chest tightness, irritation of the throat and wheeziness following inhaled CMS delivery and burning/tingling/numbness, problems with co-ordination/balance/clumsiness following IV delivery.

6.4 Bioanalytical methods

6.4.1 Determination of colistin methanesulphonate and colistin in plasma and urine

Concentrations of CMS and formed colistin in plasma and urine were measured using validated HPLC assays [191, 192], with minor modifications. Briefly, the analytical methods involved quantification of colistin before and after forced *in vitro* conversion from CMS; the concentration of CMS was calculated as the difference between the two assay results and adjusted for molecular weight [191, 192]. Calibration standards and quality control (QC) samples were from independently prepared stock solutions of the analytes (colistin sulphate and sodium CMS); QC samples were used to assess the accuracy (% deviation from nominal

QC concentration) and precision (coefficient of variation) of the assays. For the plasma assay, two colistin calibration curves (0.125 – 2 mg/L and 0.125 to 8 mg/L) were prepared in drug-free plasma to cover the range of colistin concentrations encountered after pulmonary and IV delivery, respectively. CMS calibration standards ranged from 0.125 – 2 mg/L and 0.78 – 50 mg/L for pulmonary and IV delivery, respectively. For the analysis in urine, calibration standards containing colistin and CMS concentrations ranging from 0.125 – 16 mg/L were prepared. Subject urine samples with estimated CMS or colistin concentrations above the upper end of the respective calibration curve were diluted prior to re-analysis; QC samples of colistin 40 mg/L and CMS 150 mg/L were similarly diluted to ensure satisfactory assay performance. The lowest concentration on the calibration curve was the LOQ. In all cases the accuracy and precision were within ± 15% for non-LOQ QCs and within ± 20% for LOQ samples.

6.4.2 Determination of colistin methanesulphonate and colistin in sputum

For the determination of CMS and formed colistin concentrations in sputum, the assay was similar to that described above, with minor modifications. For the colistin assay, colistin calibration standard or independently prepared QC working solutions were spiked into pre-determined volumes of homogenised CMS/colistin-free sputum. Following vortex mixing, alkaline sodium dodecyl sulphate (SDS) solution (1% SDS, 200 mM sodium hydroxide, Sigma-Aldrich, MO, USA,[195]) was added in a 1:1 ratio. The tube contents were vortex mixed and samples left to stand in ice water for 5 min. Subsequently, protein was precipitated with a 1:1 ratio of acetonitrile and the supernatant was transferred onto solid-phase extraction (SPE) C_{18} cartridges (Sep-Pak[®], Waters, MA, USA). Calibration standards containing colistin concentrations in sputum ranging from 0.125 – 16 mg/L were prepared. As described above, a QC sample of colistin 40 mg/L was diluted to ensure satisfactory assay performance for subject samples that required similar dilution. For the CMS assay, two calibration curves

(0.125 – 2 mg/L and 5 – 320 mg/L) were required to encompass the CMS sputum concentrations following IV and pulmonary delivery, respectively. Both CMS calibration curves were prepared in a similar manner to that of the colistin sputum assay except for a 5-fold dilution of sputum calibration standards ranging from 5 – 320 mg/L with blank cation-adjusted Mueller-Hinton broth. A QC sample of 500 mg/L was diluted to assess assay performance for subject samples above the calibration range that required the same dilution step. The accuracy and precision were within ± 15% for non-LOQ QCs and for the LOQ were within ± 20%.

6.5 Data analysis

Colistin concentrations in subject samples were determined by multiplying the colistin sulphate concentrations obtained from the assay by the ratio of the molecular weights of colistin base and colistin sulphate [187]. CMS concentrations in subject samples were calculated from the difference between the measured colistin concentration with and without the forced within-assay conversion of CMS to colistin and adjusting for the molecular weights of colistin, sodium CMS and CMS base, as described previously [187].

Non-compartmental analysis of the pharmacokinetic properties of CMS and formed colistin was performed using WinNonlin (Version 5.3, Pharsight Corporation, NC, USA). Maximum concentration of colistin (C_{max}) and time to reach maximum concentration (T_{max}) were determined from the concentration-*versus*-time profiles following CMS delivery. The terminal rate constant (λ_z) was calculated by linear least-squares regression analysis using the last three log-transformed concentration-*versus*-time points, while the corresponding half-life ($t_{1/2}$) was calculated using $0.693/\lambda_z$. The area under the concentration-*versus*-time profile from time of initiation of IV infusion or nebulisation to the last sampling time ($AUC_{0-tlast}$) was calculated by the linear trapezoidal rule. The area under the concentration-*versus*-time profile

to infinity ($AUC_{0-\infty}$) was calculated as the sum of $AUC_{0\text{-tlast}}$ and C_{last}/λ_z where C_{last} is the concentration in the last sample. Clearance (CL) and volume of distribution at steady state (V_{ss}) of CMS following IV infusion was calculated using $Dose_{IV}/AUC_{0-\infty}$ and $Dose_{IV} \times [(AUMC_{0-\infty}/(AUC_{0-\infty})^2]$, respectively, where $AUMC_{0-\infty}$ is area under the first moment curve to infinite time. The percentage of CMS dose recovered in urine in 24 h was calculated from the cumulative molar amount of CMS and colistin recovered in urine in 24 h divided by the CMS dose expressed in molar terms; the percentage recovered as colistin was determined in a corresponding manner. Systemic CMS availability (F) following inhaled CMS delivery was calculated using plasma data as $[(AUC_{0-\infty})_{pulm} \times Dose_{IV}]/[(AUC_{0-\infty})_{IV} \times Dose_{pulm}]$, where $Dose_{pulm}$ is expressed as the nominal CMS dose that was nebulised. The systemic availability of CMS following inhaled CMS delivery was also calculated using urinary recovery data as $[(A_{e,0-24})_{pulm} \times Dose_{IV}]/[(A_{e,0-24})_{IV} \times Dose_{pulm}]$, where $A_{e,0-24}$ is the cumulative amount of CMS and formed colistin, expressed in molar terms, recovered in urine in 24 h. The targeting advantage in terms of pulmonary (sputum) exposure to CMS or formed colistin by direct administration of CMS to the respiratory tract *via* nebulisation, *versus* that achieved with IV administration of CMS, was estimated by the calculation of therapeutic availability (TA) [206] and drug targeting index (DTI) [206, 207]. The TA was calculated by the ratio of the dose-normalised AUC in sputum to time *t* following nebulised and IV CMS administration (Equation 1). The DTI was calculated by the ratio of the dose-normalised AUC in sputum and plasma to time *t* following nebulised CMS administration divided by the same ratio following IV CMS administration (Equation 2). Time *t* refers to either 12 h or up to the scheduled 4 h post-sampling time, as discussed in the Results section.

$$TA = \frac{(Mean\ sputum\ AUC_{0-t}/D^{CMS})_{Nebulised}}{(Mean\ sputum\ AUC_{0-t}/D^{CMS})_{IV}}$$

Equation 1

$$DTI = \frac{\left(\dfrac{Mean\ sputum\ AUC_{0-t}/D^{CMS}}{Mean\ plasma\ AUC_{0-t}/D^{CMS}}\right)_{Nebulised}}{\left(\dfrac{Mean\ sputum\ AUC_{0-t}/D^{CMS}}{Mean\ plasma\ AUC_{0-t}/D^{CMS}}\right)_{IV}}$$

Equation 2

Statistical analysis was performed using SPSS® Statistics (Version 20, IBM®, USA). Differences between the values of C_{max}, T_{max} and AUC following the two nebulised CMS doses and difference between the pre- and post-dose values of lung function parameters and eGFR were evaluated using paired-sample *t*-tests. A $p < 0.05$ was regarded as a statistically significant difference.

6.6 Results

Six subjects were included in the study and the subject demographic characteristics are summarised in Table 1. All subjects received all three study doses.

Table 1. Demographic characteristics of enrolled subjects (n = 6).

Subject	Age (years)	Sex	Body weight (kg)	Race	eGFR (mL/min/1.73 m^2)
1	29	Male	70	Caucasian	127
2	20	Male	56	Caucasian	130
3	35	Male	71	Caucasian	103
4	31	Male	80	Caucasian	148
5	30	Male	85	Caucasian	116
6	27	Male	65	Caucasian	143

6.6.1 Pharmacokinetics following intravenous administration

Notwithstanding that IV dosing was the third treatment for each subject, the pharmacokinetic data for this treatment is presented first because of the pivotal nature of IV administered drug

in defining key parameters. Plasma concentration-*versus*-time profiles for CMS and formed colistin following IV CMS infusion are shown in Figure 1a (left panel). Plasma pharmacokinetic parameters of CMS and formed colistin are presented in Table 2. Maximum plasma concentrations of formed colistin were reached within approximately 5 h following initiation of IV infusion (Table 2 and Figure 1a, left panel). The terminal half-life of CMS was shorter than that of colistin in each subject for whom the calculation was possible (Table 2). Of the IV dose of CMS, $40.0 \pm 18.7\%$ was recovered as CMS and colistin in urine collected over 24 h, with approximately half of the recovered CMS dose ($19.5 \pm 8.79\%$) in the form of colistin (Table 3).

Quantifiable concentrations of CMS (two subjects; 0.66 and 1.07 mg/L) and formed colistin (five subjects; 0.47 ± 0.28 mg/L) were present in pre-dose sputum samples prior to IV CMS administration, due to the persistence of CMS and formed colistin in sputum following nebulised delivery of CMS in the first two treatment arms. When only limited quantities of sputum could be collected, colistin concentrations were preferentially determined since colistin is the antibacterial moiety [9]. Sputum CMS concentrations following the IV administration of CMS ranged between 0.14 – 0.70 mg/L which were similar to the CMS concentrations in the pre-dose sputum samples (Figure 1a, right panel). Colistin sputum concentration-*versus*-time profiles following IV CMS infusion showed relatively little variability over time within individual subjects, and for all six subjects were within a narrow range of 0.12 – 0.72 mg/L over the post-dose sampling period again being similar to the colistin concentrations present in pre-dose sputum (Figure 1a, right panel).
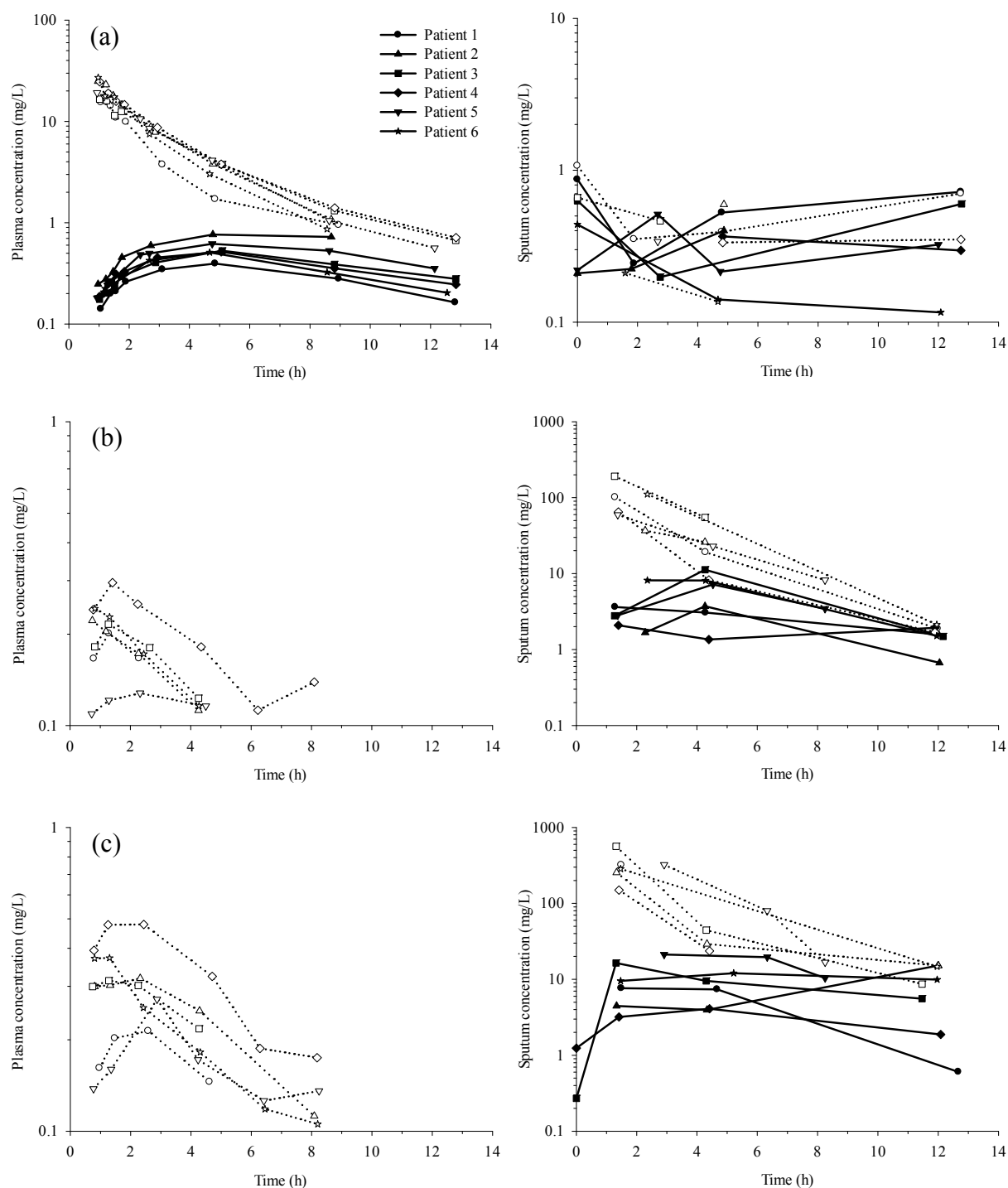
Figure 1: Plasma (left-side panels) and sputum (right-side panels) concentration-*versus*-time profiles of CMS (··hollow··) and formed colistin (-solid-) following (a) IV CMS dose of 150 mg CBA and nebulised CMS dose of (b) 2 million IU and (c) 4 million IU. Note the different range of concentrations on the y axis across the panels. The concentrations of CMS and colistin present in pre-dose sputum for the nebulised 4 million IU dose (second treatment arm) and the IV dose (third treatment arm) are shown at zero time in the respective panels.

Table 2. Pharmacokinetic parameters of CMS and formed colistin in plasma following a single IV infusion of CMS (150 mg of CBA).

	Colistin methanesulphonate				Colistin			
Subject	**$AUC_{0-\infty}$ (mg·h/L)**	**CL (L/h)**	**V_{ss} (L)**	**$t_{1/2}$ (h)**	**$AUC_{0-\infty}$ (mg·h/L)**	**$t_{1/2}$ (h)**	**C_{max} (mg/L)**	**T_{max} (h)**
1	46.9	8.00	23.5	3.17	4.91	6.04	0.40	4.9
2	71.7	5.23	12.2	1.99	N.C.[*]	N.C.[*]	0.77	4.8
3	62.3	6.02	21.4	3.12	8.14	8.42	0.53	5.1
4	75.1	4.99	16.1	3.23	7.28	7.38	0.52	5.1
5	65.2	5.75	16.6	2.55	10.2	9.03	0.62	4.8
6	65.1	5.76	12.0	1.92	5.87	5.84	0.51	4.7
Mean		5.96	16.9	2.66		7.34		
S.D.		1.07	4.68	0.60		1.41		

[*] N.C., not calculated, the t_{last} for subject 2 was 8.71 h as the 12 h sample could not be collected; thus, not able to estimate $t_{1/2}$ or $AUC_{0-\infty}$.

Table 3. Percentage of CMS and colistin recovered in urine following administration of CMS by nebulisation or IV infusion.

	% CMS dose recovered in urine as CMS plus colistin in 24 h	**% CMS dose recovered in urine as colistin in 24 h**
IV infusion of 150 mg of CBA[*]	40.0 ± 18.7	19.5 ± 8.79
Nebulised 2 million IU of CMS[¥]	1.30 ± 0.65	0.62 ± 0.26
Nebulised 4 million IU of CMS[λ]	0.68 ± 0.26	0.29 ± 0.13

[*]Subject 1 urine data excluded from analysis as urine was collected until 12 h post-IV CMS delivery.
[¥]Subject 4 urine data excluded from analysis as colistin concentrations were below LOQ of the assay.
[λ]Subject 5 urine data excluded from analysis as colistin concentrations were below LOQ of the assay.

6.6.2 Pharmacokinetics following nebulisation

Sputum concentration-*versus*-time profiles for CMS and formed colistin following nebulisation of 2 and 4 million IU of CMS in subjects are shown in Figure 1b and 1c (right panel), respectively. Mean sputum CMS concentrations of 94 ± 55 mg/L and 315 ± 137 mg/L were observed at the first sampling time following nebulised CMS doses of 2 and 4 million IU, respectively, with concentrations declining thereafter (Figure 1b and 1c, right panel). In two subjects, quantifiable colistin concentrations (0.27 and 1.24 mg/L) were present in pre-dose sputum samples prior to nebulisation of 4 million IU of CMS (the second treatment arm), again likely a result of a prolonged lung residence time after the earlier nebulisation delivery of 2 million IU of CMS. Pharmacokinetic parameters of colistin in sputum following pulmonary CMS delivery are presented in Table 4. Peak sputum colistin concentrations were reached within $1.3 - 5.2$ h following initiation of both nebulised CMS doses, with the exception of subject 2 where T_{max} was observed at 12 h after nebulisation of 4 million IU of CMS (Table 4 and Figure 1b and 1c, right panel). Limited sputum samples and the assay sensitivity precluded characterisation of the CMS and colistin terminal phase in sputum. Estimation of CMS dose-linearity in sputum was not possible due to unavailability of data for CMS at later time points in some subjects (Figure 1b and 1c, right panel) because of limited amounts of sputum, as mentioned above. For colistin, no significant difference was evident for the dose-normalised areas under the sputum concentration-*versus*-time profiles to t_{last} with doubling of the CMS dose (Table 4).

Plasma CMS concentrations following pulmonary delivery of both doses were relatively low $(0.11 - 0.48$ mg/L) and there were no quantifiable concentrations of formed colistin in plasma as shown in Figure 1b and 1c (left panel). The pharmacokinetic parameters of CMS in plasma following pulmonary CMS delivery are presented in Table 5. The percentage of the CMS dose recovered in urine as CMS plus colistin following pulmonary delivery of 2 and 4 million

IU of CMS was 1.30 ± 0.65% and 0.68 ± 0.26%, respectively, with approximately half of the recovered amount in the form of colistin (Table 3). The systemic availability of CMS following nebulisation of 2 and 4 million IU of CMS, calculated from areas under plasma concentration-*versus*-time curves to infinity, was 7.93 ± 4.26% and 5.37 ± 1.36%, respectively. Using urinary recovery data, the estimated systemic availability for CMS was 3.01 ± 0.50% and 2.69 ± 2.20% following nebulised CMS doses of 2 and 4 million IU, respectively.

Given the long residence time of CMS and colistin in sputum and the fact that following the nebulised CMS dose of 4 million IU the pre-dose sputum samples had quantifiable colistin concentrations, the calculation of the TA and DTI was carried out using the lower of the two nebulised CMS doses, which was the first treatment arm. Following IV administration, the sputum exposure was calculated including CMS and colistin pre-dose concentrations. The targeting advantage of delivering CMS *via* the pulmonary when compared to the IV route was demonstrated by TA estimations of 387 and 24 for CMS and formed colistin, respectively. The corresponding respective DTI values for CMS and formed colistin were 15,952 and 35, respectively. For estimation of TA for CMS and colistin, the sputum exposure following pulmonary and IV delivery was calculated from time zero to 12 h post-dose. For the estimation of DTI for colistin, as plasma colistin concentrations were below the LOQ following pulmonary delivery the systemic exposure was calculated using colistin LOQ concentration (0.125 mg/L) from time zero to 12 h post-dose. The DTI for CMS was estimated up to the schedule 4 h post-dose sampling time as plasma CMS concentrations were below the LOQ thereafter.

Table 4. Pharmacokinetic parameters of formed colistin in sputum following nebulisation of 2 and 4 million IU of CMS.

Subject	Nebulised 2 million IU of CMS			Nebulised 4 million IU of CMS		
	$AUC_{0-tlast}$ (mg·h/L)[a]	C_{max} (mg/L)[a]	T_{max} (h)[a]	$AUC_{0-tlast}$ (mg·h/L)	C_{max} (mg/L)	T_{max} (h)
1	30.3	3.63	1.3	61.5	7.63	1.5
2	24.3	3.72	4.3	88.9	15.2	12
3	73.1	11.3	4.3	104	16.4	1.3
4	18.9	2.09	1.4	37.0	4.10	4.4
5	37.5*	7.18	4.5	129*	21.2	2.9
6	62.2	8.15	2.4	122	12.1	5.2
Mean	41.8	6.00		82.6	12.8	
S.D.	24.3	3.45		33.7	6.19	

[a] No statistically significant difference for dose-normalised $AUC_{0-tlast}$, dose-normalised C_{max} and T_{max} between the two nebulised CMS doses.
* Subject 5 t_{last} following nebulised CMS doses was 8.3 h.

Table 5. Pharmacokinetic parameters of CMS in plasma following nebulisation of 2 and 4 million IU of CMS.

Subject	Nebulised 2 million IU of CMS			Nebulised 4 million IU of CMS		
	$AUC_{0-\infty}$ (mg·h/L)[a]	C_{max} (mg/L)[b]	T_{max} (h)[a]	$AUC_{0-\infty}$ (mg·h/L)	C_{max} (mg/L)	T_{max} (h)
1	1.39	0.20	1.3	1.67	0.21	2.6
2	1.16	0.22	0.74	2.41	0.32	2.3
3	1.27	0.22	1.3	2.53	0.31	1.3
4	3.53	0.30	1.4	3.53	0.48	1.3
5	3.77	0.13	2.3	4.10	0.27	2.9
6	1.26	0.24	0.82	2.46	0.37	0.8
Mean	2.06	0.22		2.78	0.33	
S.D.	1.24	0.055		0.88	0.092	

[a] No statistically significant difference for dose-normalised $AUC_{0-\infty}$ and T_{max} between the two nebulised CMS doses.
[b] Statistically significant difference for dose-normalised C_{max} between the two nebulised CMS doses ($p < 0.05$).

6.6.3 Tolerability following nebulised and intravenous administration

Inhaled CMS was generally well tolerated by subjects, as demonstrated by a lack of change in lung function parameters and eGFR as shown in Table 6. Following inhalation of 2 million IU of CMS, subject 5 reported chest tightness, while subject 6 complained of cough and chest tightness after the higher CMS nebulised dose. Subject 2 reported the sensation of a lump in the throat following inhalation of 2 million IU of CMS and on the morning of the third treatment arm (IV CMS administration) presented with haemoptysis with uncertain relation to the earlier inhalational CMS treatments. The above-mentioned adverse effects were minor in severity and transient. There were no remarkable changes in lung function parameters in subjects 2, 5 and 6 following each respective treatment. Following IV CMS, subjects did not report any pulmonary adverse effects and no significant changes in lung function parameters were evident (Table 6). Subject 2 reported transient dizziness 4 h after administration of IV CMS. A small but statistically significant difference ($p < 0.05$) in the pre- and 12 h post-dose eGFR was evident following IV CMS administration (Table 6).

Table 6. Lung function parameters and glomerular filtration rates following administration of CMS by nebulisation or IV infusion. Data represented as mean ± S.D. (n = 6).

Parameters	Nebulised 2 million IU of CMS		Nebulised 4 million IU of CMS		IV infusion of 150 mg of CBA [Y]	
	Pre-dose	Post-dose[£,α]	Pre-dose	Post-dose[£,α]	Pre-dose	Post-dose[£,α]
$FEV_{(1)}$ [a]	2.0 ± 0.65	1.9 ± 0.65	2.0 ± 0.61	$2.1 \pm 0.64^{\S}$	2.1 ± 0.62	2.1 ± 0.62
FVC [a]	3.6 ± 0.85	3.5 ± 0.94	3.9 ± 0.87	$3.8 \pm 0.93^{\S}$	3.9 ± 0.78	3.9 ± 0.88
MIP [a]	155 ± 64	151 ± 44	160 ± 40	161 ± 33	148 ± 39	153 ± 36
MEP [a]	157 ± 56	157 ± 55	158 ± 30	166 ± 38	152 ± 29	164 ± 30
eGFR [a*,b]	127 ± 18	$121 \pm 20^{\in}$	133 ± 22	121 ± 37	145 ± 22	$127 \pm 31^{\in}$

$FEV_{(1)}$, forced expiratory volume in 1 second.
FVC, forced vital capacity.
MIP, minimum inspiratory pressure.
MEP, maximum expiratory pressure.
[£] 1-2 h post-administration for lung function test.
[α] 12 h post-administration for eGFR.
[a] No statistically significant difference between pre- and post-dose measurements for $FEV_{(1)}$, FVC, MIP and MEP.
[a*] No statistically significant difference between pre- and post-dose measurements for eGFR following nebulised CMS delivery.
[b] Statistically significant difference between pre- and post-dose measurements for eGFR following IV CMS delivery.
[∈] n=4 for nebulised 2 million IU CMS and n=5 for IV 150 mg of CBA.
[§] n=5, due to time constraints in the lung function laboratory $FEV_{(1)}$ and FVC could not be carried out for subject 2.
[Υ] n=5, for lung function parameters as subject 2 did not undergo lung function tests as he presented with haemoptysis.

6.7 Discussion

This study was designed to evaluate the systemic and pulmonary pharmacokinetics of CMS and formed colistin following administration of inhaled and IV CMS in CF subjects. The results provide the first quantitative description of the targeting advantage that may be achieved by administering CMS by inhalation for treatment of a pulmonary infection.

Following IV CMS infusion (150 mg of colistin base activity, CBA), the CL, V_{ss} and $t_{1/2}$ for CMS were consistent with previous reported values in CF patients following IV infusion of 30 – 60 mg CBA every 8 h [187] and in healthy volunteers following IV CMS infusion of 30 mg of CBA [200]. The estimated terminal $t_{1/2}$ of formed colistin of 7.34 ± 1.41 h in this study

was slightly longer than in previous reports [187, 200] and peak colistin plasma concentrations were observed at approximately 5 h following initiation of IV infusion compared to ~1.3 − 2 h and 1 − 4 h in the reports by Li *et al* [187] and Couet *et al* [200], respectively. One possibility for variation in the time for achievement of peak plasma concentrations of formed colistin is brand-to-brand differences in the ratio of fully and partially sulphomethylated CMS entities which may impact on the time-course for formation of colistin. Consistent with the findings by Li *et al* [187] and Couet *et al* [200] colistin terminal half-life was ~2.5-fold longer than that of CMS which indicates that the elimination of colistin is not rate limited by formation from CMS. Following IV CMS infusion, 40.0 ± 18.7% of the CMS dose was recovered in urine in 24 h with colistin representing approximately half of the recovered CMS dose (Table 3). As described previously, most of the colistin recovered in urine is expected to result from conversion of CMS to colistin in the kidney and bladder [198, 200].

There is a paucity of information available on the disposition of CMS and formed colistin in sputum following IV administration of CMS. In this study, the sputum concentrations of CMS and colistin after IV administration of CMS were relatively low and similar to those observed in the pre-dose sputum samples; there was no convincing evidence for a time-course that one would expect if there was substantial ingress from the systemic circulation followed by egress (Figure 1a, right panel). Thus, it is apparent that the CMS and colistin concentrations observed were largely the result of carryover from the previously administered inhalational doses of CMS. Even so, colistin sputum concentrations were below the minimum inhibitory concentration (MIC$_{90}$) of 1.0 mg/L for *P. aeruginosa* [172] across the 12 h post-dose sampling period. In CF patients, intravenous CMS is used to treat acute exacerbation of lung infections where it is typically administered two to three times per day for several days [4, 11, 13, 15]. It remains to be determined whether higher concentrations of formed colistin in

sputum would be observed with such multiple-dose regimens. In critically-ill patients, Imberti *et al* reported that under steady-state conditions, formed colistin concentrations were undetectable in bronchoalveolar lavage (BAL) fluid following IV CMS infusion of 60 mg CBA every 8 hours [189]. The authors reported a limit of detection (LOD) concentration of 0.05 mg/L in BAL fluid, with no corresponding LOD concentration for lung epithelial lining fluid (ELF) [189]. Referring to the original study that Imberti and colleagues referenced for their BAL procedure [234] and utilising the percentage of recovered BAL fluid for a standard BAL procedure [31], we approximated that the ELF LOD concentration for the assay was ~5 mg/L (i.e. BAL fluid concentration ~100-fold dilution of ELF concentration). Therefore undetectable BAL fluid concentrations of formed colistin in the study of Imberti *et al* [189] must be interpreted with caution. Generation of higher concentrations of formed colistin in lung fluids may be achieved by increasing the IV dose of CMS, but this would be associated with potential for increased nephrotoxicity which is the major dose-limiting adverse effect for the IV route [154].

One of the major findings of this study was that pulmonary administration of 2 and 4 million IU of CMS resulted in much higher sputum concentrations of CMS and, more importantly, of the active antibacterial entity, colistin than were observed after IV administration of a higher dose of CMS (Figure 1b and 1c, right panels). Across the subjects, the higher nebulised dose led to a proportional increase in the sputum concentration exposure profile for formed colistin. In the majority of subjects, nebulisation of 2 million IU of CMS resulted in colistin sputum concentrations above the MIC_{90} of 1.0 mg/L for *P. aeruginosa* [172] for up to 12 h. Doubling of the nebulised CMS dose resulted in sputum colistin concentrations above 3.0 mg/L up to 12 h post-administration. A multiple dosing regimen of inhaled CMS would lead to higher sputum colistin concentrations due to accumulation. Ratjen *et al* reported significantly higher colistin concentrations (~5 – 45 mg/L) in sputum following nebulisation

of a single dose of 2 million IU of CMS in CF patients [170]. It should be noted that their analytical method for 'colistin' involved treatment of sputum with relatively high concentration of trifluoroacetic acid (~7%) [170]. This may promote conversion of CMS to colistin during the sample preparation procedures [170]. More recently Athanassa *et al* [129] reported that following nebulisation of 1 million IU of CMS every 8 hours in critically-ill patients, ELF concentrations of colistin were within a similar range (~1.0 – 15 mg/L) to the concentrations observed in sputum in the current single-dose study. The variability in the reported colistin concentrations in sputum and ELF across the studies will likely be a function of the efficiency of nebulised delivery, whether the study involved single or multiple dosing, the different biological matrices (sputum *versus* ELF), analytical issues as discussed above and variation in the pathophysiological status of the respective patient populations.

Administration of CMS *via* the pulmonary route resulted in achievement of very high sputum concentrations of CMS (~50 – 500 mg/L) approximately 1 h after nebulisation (Figure 1b and 1c, right panels). Over the ensuing hours, CMS in the lungs is expected to have acted as a reservoir for ongoing conversion of CMS to colistin and in doing so to have supported the observed persistence of formed colistin in the lungs (Figure 1b and 1c, right panels). For the treatment of respiratory infections, the prolonged residence of CMS/colistin in the lung has significant implications for therapeutic efficacy and therefore warrants further detailed investigation.

Despite the high and persistent concentrations of CMS and colistin in sputum following pulmonary delivery of CMS, the systemic exposure to CMS and colistin was minimal (Figure 1b and 1c, left panels). Following inhalation of 2 and 4 million IU of CMS, plasma concentrations of CMS were <0.5 mg/L and only quantifiable for up to 8 h post-administration (Figure 1b and 1c, left panels); the systemic availability of inhaled CMS was only ~5 – 8%. Importantly, colistin concentrations in plasma were below the LOQ of the

assay (0.125 mg/L) for the entire 12 h sampling period following nebulisation of 2 and 4 million IU CMS. Following nebulisation, the relatively low systemic exposure of CMS and formed colistin when compared to IV delivery is likely to result in a reduction in systemic adverse effects such as nephrotoxicity.

Overall, these findings highlight the major advantage of pulmonary when compared to IV delivery of CMS, as sputum concentrations of the active antibacterial entity, colistin, were maintained above the MIC_{90} of 1.0 mg/L for *P. aeruginosa* [172] whilst minimising systemic exposure to CMS and colistin. Following nebulisation of CMS (2 or 4 million IU), the colistin sputum exposure was >10-fold higher in comparison to that achieved with IV administration, while colistin concentrations in plasma following pulmonary administration were below the LOQ of the assay. Calculation of the therapeutic availability (TA) and drug targeting index (DTI) provides an opportunity for quantitative assessment of the benefits of local (inhalational) delivery over systemic (IV) administration. The TA (Equation 1) provides an indication of the sputum availability of CMS and colistin. The TA values for CMS (387) and colistin (24) were far greater than unity which indicated that the sputum exposures for CMS and colistin following pulmonary administration were substantially higher than after IV administration of the same CMS dose. The effectiveness or degree of targeting achieved following administration *via* the pulmonary route when compared to the IV route can be estimated by the DTI ratio (Equation 2). For CMS and colistin the respective DTI were 15,952 and 35, again values substantially higher than unity, indicating that a high degree of targeting to the lungs (greater sputum exposure and minimal plasma exposure) was achieved after inhalational delivery of CMS when compared to IV administration. The targeting values calculated for TA and DTI under-estimate the true targeting benefit for CMS and colistin following pulmonary delivery. In order to calculate DTI it is necessary to have a value for mean plasma AUC for colistin following lung dosing, however all samples were below the

LOQ (0.125 mg/L), therefore a minimum value for AUC was calculated assuming samples at all times contained 0.125 mg/L of colistin. Additionally, for the IV treatment the pre-dose sputum samples contained residual CMS and colistin from the prior nebulisation treatment arms and these values were included in the calculation of mean sputum AUC, which further contributes to the under-estimation of both TA and DTI. For an antibiotic such as colistin, which is a last-line of defence against *P. aeruginosa* infections, the ability to target the lungs to achieve high local concentrations and to simultaneously minimise systemic exposure has major advantage in terms of maximising efficacy and minimising the potential for the development of resistance and nephrotoxicity. We recognise that both the pulmonary exposure and the systemic availability will be influenced by the efficiency of the nebulisation equipment and process; nevertheless the colistin targeting advantage of inhalational *versus* IV administration of CMS is readily apparent.

In contrast to the low systemic availability of CMS and formed colistin (<2-3% of the nebulised CMS dose recovered in urine) observed in CF subjects in the current study, Marchand *et al* [184] reported that the total dose of CMS administered by intratracheal nebulisation to rats was absorbed into the systemic circulation either as CMS (systemic availability of ~70%) or following CMS conversion to colistin in the lungs (39% of the nebulised CMS dose). Following nebulisation of sodium CMS (15 mg/kg) in rats, a ~4-fold higher plasma concentration-*versus*-time exposure was observed for formed colistin when compared to IV administration of the same CMS dose [184]. This is in contrast to CF subjects where colistin concentrations in plasma were unquantifiable after CMS inhalation. The fraction of the CMS dose that was converted to colistin following IV delivery in rats was ~13%, which suggests that following nebulisation the absorption of pre-systemically formed colistin contributed significantly to the systemic exposure of colistin [184]. Such species differences can be attributed to the efficiency of the different nebuliser devices in delivering

the total dose into the lungs, the physiological size of the rat *versus* human lung, and the healthy *versus* infected status of the lungs which can influence the barriers for absorption and impact on clearance mechanisms (i.e. mucocilliary clearance, uptake by macrophages and coughing).

Cystic fibrosis centres worldwide have adopted different inhaled CMS dosing regimens (dose and dosing interval) with current therapies ranging from 1 million IU of CMS twice daily to 2 million IU of CMS three times daily [3, 4, 10, 11]. One reason for the variability in inhaled dosage regimens is the lack of robust pharmacokinetic data to inform dosage selection. Inclusion of two inhaled CMS doses (2 and 4 million IU) in the present study has enabled a better understanding of the kinetics of CMS and colistin in sputum and in plasma and has additionally shown that CMS inhalation at both dose levels was well tolerated by CF subjects.

In conclusion, we have for the first time demonstrated, in quantitative terms, the targeting advantage that may be achieved by nebulised delivery of CMS to the airways. Following pulmonary CMS administration, a greater lung exposure and minimal systemic exposure were observed for CMS and, importantly, colistin compared to IV administration, where systemic exposure was very substantially higher than the lung exposure. Thus, inhalation of CMS is an effective means of targeting colistin to the lungs to maximise antibacterial effect while minimising systemic exposure and the potential for nephrotoxicity. The present and future studies will inform the design of inhalational CMS dosing regimens for CF patients.

6.8 Acknowledgements

6.9 Funding

6.10 Transparency declaration

None to declare.

Chapter 7: Summary and perspectives

Over the last two decades, colistin, an old polymyxin antibiotic, has undergone a resurgence in clinical use due to emergence of multidrug-resistant (MDR) Gram-negative bacteria and the limited number of new antibiotics in the drug development pipeline. Since reintroduction into the clinical practice, colistin methanesulphonate (CMS), the inactive prodrug of colistin, has predominantly been used for treatment of Gram-negative respiratory infections in cystic fibrosis (CF) patients and more recently in critically-ill patients with ventilator-associated pneumonia (VAP) via the pulmonary and intravenous (IV) route. Despite an increase in CMS usage, there is non-uniformity in the inhaled CMS dosing regimens prescribed to patients. Furthermore, following IV CMS administration little is known about the concentrations of formed colistin achieved at the infection site. In the existing literature, the limited information on the pharmacokinetics of CMS and formed colistin is as a result of CMS undergoing less rigorous drug registration procedures prior to release into the market. Since the resurgence in CMS use over the last 20 years there has only been a few pre-clinical and clinical studies conducted to characterise the pharmacokinetics of CMS and formed colistin following inhalational and IV administration. Therefore studies to determine the targeting advantage (i.e. maximise lung exposure while minimsing systemic exposure) following pulmonary delivery when compared to systemic delivery of CMS had not been conducted prior to this thesis. Worryingly, if non-optimised dosing regimens continue to be used into the future, therapeutic failure and development of resistance to an antibiotic that is already a last line of defense against MDR Gram-negative bacterial infections will be inevitable.

The studies described in this thesis have investigated the pharmacokinetics of CMS and formed colistin following IV and pulmonary administration of CMS in Sprague-Dawley rats and CF subjects. The pharmacokinetics of the active antibacterial moiety, colistin, was determined in rats to enable a more thorough understanding of the kinetics of formed colistin following CMS administration. Specifically, the targeting advantage of administering CMS

via the pulmonary route when compared to systemic route has been addressed in this thesis, both in the pre-clinical and clinical setting.

7.1 Pharmacokinetic assessment of colistin in Sprague-Dawley rats

Understanding the disposition of colistin following IV and pulmonary administration of colistin was an essential first step since this information is required for a more comprehensive analysis of the kinetics of formed colistin following administration of the prodrug (Chapter 3). To the author's knowledge, these are the first studies that have defined the pharmacokinetics of colistin in both plasma and lung epithelial lining fluid (ELF) following IV and intratracheal (IT) instillation in rats. Linear pharmacokinetics was evident for colistin following IV and IT dose-ranging studies. A major finding of the current studies was that following pulmonary instillation of colistin 0.62 mg/kg, extensive exposure of colistin in ELF for an extended duration of time (12 h) was evident when compared to the exposure in plasma. In contrast, colistin ELF concentrations were unquantifiable following IV administration of colistin 0.41 mg/kg, with such observations and the persistence nature of colistin in ELF following IT instillation proposed to be due to binding of colistin to lung tissue.

7.2 Pharmacokinetic assessment of CMS and formed colistin in Sprague-Dawley rats

Studies described in Chapter 4 investigated the pharmacokinetics of CMS and formed colistin in plasma and lung ELF following IV and IT administration in rats. Linear pharmacokinetics was evident for CMS and formed colistin following IV and IT across the dose-range studied. A significant finding was that following pulmonary administration of CMS (14 mg/kg) the ELF exposure of CMS and formed colistin was 1,500-fold higher when compared to exposure in plasma. In contrast, IV administration of the same CMS dose resulted in significantly lower CMS concentrations and unquantifiable concentration of the antibacterial active moiety,

colistin, in ELF. The targeting advantage achieved following pulmonary delivery when compared to IV administration of CMS was demonstrated with the therapeutic availability (TA) and drug targeting index (DTI) values for CMS and formed colistin sufficiently greater than unity. These pre-clinical studies are the first to confirm that direct delivery of CMS into the lungs results in an increase in availability of CMS and formed colistin in ELF and confirms that pulmonary delivery is an effective method to target CMS and formed colistin into the lungs while minimise systemic exposure.

The high exposure of CMS in ELF following IT instillation was proposed to be due to slow absorption of CMS from the lungs and CMS not available for renal clearance and thereby creating a reservoir for ongoing conversion to colistin in the ELF. The population pharmacokinetic model for CMS and colistin indicated that the absorption kinetics for CMS was mostly first-order kinetics with a small contribution from zero-order processes. Non-compartmental pharmacokinetic analysis and population pharmacokinetic modelling provided a greater insight into the conversion kinetics of CMS in lung ELF and plasma. These studies indicated that in ELF and plasma the conversion kinetics of CMS to colistin were different with a higher fractional conversion of the IT CMS dose in ELF ($f_{m,ELF}$ 0.226) compared to the IV dose in plasma ($f_{m,systemic}$ 0.0255). The population model demonstrated that, both in ELF and plasma, first-order kinetics best described the conversion of CMS to colistin, however more delayed and slower conversion kinetics was occurring in the lungs when compared to in plasma, in particular at the lung dosing site. This slow and sustained conversion kinetics in the lungs contributed to the extensive exposure of formed colistin in lung lining fluid and to the delayed appearance of formed colistin in plasma (1 h post-dose) following IT CMS dose-ranging studies. The greater fractional conversion of the IT CMS dose in the lungs and absorption of a fraction of the pre-systemically formed colistin was likely to contribute to the greater systemic exposure of colistin following IT CMS dose-ranging studies.

This research has shown, for the first time that the disposition characteristics of formed colistin following CMS administration are consistent with that of colistin following administration of colistin. The terminal half-life of formed colistin and colistin in plasma following IV administration and in ELF following IT administration were not significantly different. Unquantifiable formed colistin and colistin concentrations in ELF following IV administration of either CMS or colistin, respectively, suggests similar distribution kinetics from the systemic circulation into lung ELF. The different time course of appearance of formed colistin (T_{max} of 3 h) and colistin (T_{max} of 10 – 30 min) in plasma following IT dose-ranging studies of CMS and colistin, respectively, is likely to be due to conversion kinetics of CMS to colistin in the lungs.

7.3 Pharmacokinetic assessment of CMS and formed colistin in cystic fibrosis subjects

The first quantitative description of the targeting advantage achieved following administration of CMS by the pulmonary route when compared to the IV for the treatment of respiratory infections in CF subjects was conducted as part of this thesis. Nebulisation of 2 and 4 million international units (IU) of CMS resulted in high CMS sputum concentrations and formed colistin sputum concentrations maintained above the minimum inhibitory concentration (MIC) for *Pseudomonas aeruginosa* of 1.0 mg/L for the 12 h sampling period. Similar to observations in rats, the high CMS concentrations in sputum may act as a reservoir for ongoing conversion of CMS to colistin and thereby may provide an explanation for the persistence of formed colistin in sputum. In the majority of subjects a dose proportional increase in formed colistin sputum exposure was evident. Despite the extensive exposure of CMS and formed colistin in the lungs, formed colistin concentrations in plasma were unquantifiable and the systemic bioavailability for CMS was low (~5 – 8%). Following IV CMS administration (150 mg of colistin base activity), CMS and formed colistin

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concentrations in sputum were low with formed colistin concentrations below the MIC for the duration of the sampling period (12 h). The fraction of the IV CMS dose recovered in urine in 24 h was ~40% compared to < 2% following delivery of both nebulised CMS doses, which further confirms low systemic exposure following inhaled CMS administration. These findings are significant since low systemic exposure to CMS and formed colistin will minimise systemic adverse effects such as nephrotoxicity, which is a dose-limiting adverse effect following IV CMS dosing. For the first time in CF subjects, the targeting benefit of administration of CMS into the respiratory site of action when compared to after IV administration was demonstrated with the TA and DTI values greater than unity. Consistent with the pre-clinical studies, inhalational administration to CF subjects resulted in a greater availability of CMS and formed colistin in sputum and was effective in targeting CMS and formed colistin into the lungs while minimising systemic exposure.

Following pulmonary administration of CMS, there was evidence of inter-species differences in the pharmacokinetics of CMS and formed colistin. The systemic exposure for both CMS and formed colistin was significantly lower in CF subjects when compared to rats. However in both clinically and pre-clinically, a greater pulmonary exposure relative to plasma exposure was observed following pulmonary administration of CMS.

7.4 Future studies

Further pre-clinical and clinical studies are needed to build on the outcomes generated from this thesis. *In vitro* and *in vivo* studies to investigate the conversion kinetics of CMS, the mechanism of CMS and colistin absorption from the lungs (cell culture models) and the distribution or binding kinetics of CMS and colistin in the lungs (*in vivo* studies - collection of lung tissue) are needed. Additionally, pharmacokinetic studies in larger animal models and in

human patients following pulmonary administration of multiple CMS dosing regimens are warranted.

7.5 Concluding comments

The pharmacokinetics of CMS and formed colistin following IV and pulmonary administration to Sprague-Dawley rats and CF subjects has been evaluated in this thesis. The findings from the pre-clinical and clinical studies have demonstrated that pulmonary administration of CMS for treatment of Gram-negative respiratory infections is an effective route to achieve targeted delivery and thereby increasing lung exposure of the antibiotic whilst simultaneously reducing systemic exposure compared to after IV administration. The knowledge generated from this thesis has provided a more comprehensive understanding of the pharmacokinetics of CMS and formed colistin in both plasma and the lungs; information that has been lacking in the literature. Further clinical studies are needed to allow for evidence based decisions of inhaled dosing regimens to preserve the therapeutic efficacy of an antibiotic that is a last line of defence against MDR Gram-negative infections.

Appendix I

Pharmacokinetics of colistin and colistin methanesulphonate following intravenous administration

Pre-clinical and clinical studies that have define the pharmacokinetics of colistin and colistin methanesulphonate (CMS) following intravenous (IV) administration are discussed below. This review focuses on the findings from recent studies which have employed validated high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS) methods to quantify colistin and CMS concentrations in biological matrices.

Pre-clinical studies

Intravenous administration of colistin (sulphate)

The pharmacokinetics of colistin following IV [203] and subcutaneous (SC) [184] administration of colistin in Sprague-Dawley rats are presented in Table A1-1. The findings from the Marchand *et al* study [184] are presented in this section since the authors made an assumption of 100% bioavailability following SC delivery and therefore this is similar to if colistin was administration via the IV route. The pharmacokinetic estimates from the two studies are generally in agreement as shown in Table A1-1. The volume of distribution at steady-state (V_{ss}) for colistin (Table A1-1) indicates limited extravascular distribution and is consistent with the physicochemical properties of colistin (Chapter 1, Section 1.5.2). Colistin is cleared predominantly via non-renal pathways with the exact mechanism of this clearance unknown [203]. A low percentage of the colistin dose is recovered in urine following IV administration to rats, with colistin undergoing extensive reabsorption back into the blood stream [203]. Ma *et al* reported that colistin is reabsorbed from the renal tubules in part by carrier mediated transport via organic cation transporter and polypeptide transport pathways

in a study conducted in isolated perfused rat kidney [235]. Hepatic clearance is not a major contributing pathway for colistin clearance with a very low hepatic extraction ratio reported for colistin [203]. In addition, Couet *et al* found no significant difference in the pharmacokinetics of colistin between control and acute hepatic failure-induced rats after SC administration of 1.5 mg/kg colistin [236]. In rats, colistin is approximately 55 – 57% bound to plasma proteins [203]. In neutropenic infected mice, Dudhani *et al*, reported that the unbound fraction of colistin in plasma is highly drug concentration-dependent, with a greater unbound fraction of colistin observed at higher plasma concentrations [237]. Binding of colistin to acute-phase plasma protein (AAG) indicates that in infected animals and patients the unbound fraction is dependent on both colistin and plasma protein concentrations [237].

Table A1-1: Pharmacokinetics properties of colistin in plasma following IV and SC administration of colistin (sulphate) to Sprague-Dawley rats. Expressed as mean ± S.D.

Colistin dose	CL (mL/min/kg)	V_{ss} (mL/kg)	$t_{1/2}$ (min)	Ref
IV 1.0 mg/kg	5.2 ± 0.4	496 ± 60	74.6 ± 13.2	[203]
SC 1.5 mg/kg	8.5 ± 1.0	939 ± 253	75.4 ± 14.1	[184]

CL, systemic clearance.
$t_{1/2}$, terminal half-life.

Intravenous administration of colistin methanesulphonate (sodium)

The pharmacokinetics of CMS and formed colistin following IV administration of CMS in Sprague-Dawley rats have been reported by Li *et al* [198] and Marchand *et al* [184, 199] with the pharmacokinetic estimates consistent between the studies as presented in Table A1-2. A longer terminal half-life for formed colistin when compared to CMS indicates that the elimination of colistin is not rate limited by formation from CMS (Table A1-2) [184, 198, 199]. The V_{ss} for CMS indicates limited extravascular distribution (Table A1-2) and was reported to be approximately 60% of that of colistin (following IV colistin [203]) in the Li *et*

al study with the difference likely to be due to the greater hydrophilic characteristics of CMS (Chapter 1, Section 1.5.2). Linear pharmacokinetics for CMS and formed colistin is evident following IV dose-ranging studies of CMS 5 – 120 mg/kg [199]. In rats, the fraction of CMS converted to colistin in the systemic circulation ($f_{m,systemic}$) is low with values of 0.068 and 0.125 reported by Li *et al* [198] and Marchand *et al* [184], respectively (Table A1-2). In all three studies maximal formed colistin concentrations is observed at the initial sampling time of 5 min [184, 198, 199]. These findings are in contrast to the kinetics of colistin formation in the systemic circulation in humans, as discussed below. The unbound fraction of CMS in rat plasma is not known [198].

Table A1-2: Pharmacokinetics properties of CMS and formed colistin in plasma following IV administration of CMS (sodium) to Sprague-Dawley rats. Expressed as mean ± S.D.

CMS dose	CL (mL/min/kg)	V_{ss} (mL/kg)	$t_{1/2}$ (min)	$f_{m,systemic}$	Ref
15 mg/kg					
CMS	11.7 ± 1.8	299 ± 55	23.6 ± 3.9	0.068	[198]
Formed colistin	N.R	N.R	55.7 ± 19.3	-	
15 mg/kg					
CMS	14.6 ± 3.6	330 ± 75	22.0 ± 3.4	0.125	[184]
Formed colistin	N.R	N.R	35.5 ± 5.6	-	
5 – 120 mg/kg					
CMS[¥]	9.5 – 14.9	224 – 380	20.6 – 25.5	N.R	[199]
Formed colistin[¥]	N.R	N.R	32.4 – 45.2	-	

N.R, not reported.
[¥]estimates expressed as a range (of the mean values) for the dose-escalating studies (5, 15, 30, 60 and 120 mg/kg).

Clearance via the renal route is the major elimination pathway for CMS with approximately 61.1 ± 14.4% of the IV CMS dose recovered in urine in the Li *et al* study [198]. Approximately half of the CMS dose recovered in urine is present as colistin, with the higher urinary recovery of colistin when compared to after IV colistin delivery likely to be due to

intra-renal formation of colistin from CMS within the tubular cells of the kidney, bladder and to a lesser extent in the collection vessel [198, 203]. In comparison to colistin, CMS undergoes extensive renal tubular secretion with the difference in renal disposition of CMS likely to be due to the presence of the sulphomethyl moieties [198]. The mechanism of clearance of the remaining ~40% of the CMS dose is unknown [198]. Studies investigating elimination of CMS via the liver is limited with a low hepatic extraction ratio reported for CMS [198]. In 1976, Abe *et al* identified tentatively a metabolite of colistin, colistin-*N*-glucuronide in the urine (1.7% of the dose) and bile (6.7% of the dose) following administration of IV CMS 100 mg/kg to rabbits [198]. Couet *et al* demonstrated that no significant difference in the pharmacokinetic of CMS and formed colistin is observed in control and acute hepatic failure-induced rats following IV administration of CMS 15 mg/kg [236].

Clinical studies

Intravenous administration of colistin (sulphate)

In the recent literature there has not been any clinical studies investigating the pharmacokinetics of colistin after IV colistin delivery.

Intravenous administration of colistin methanesulphonate (sodium)

Several studies have investigated the pharmacokinetics of CMS and formed colistin in plasma following IV CMS administration in healthy volunteers, cystic fibrosis (CF) patients and in critically-ill patients as presented in Table A1-3. One other study in the literature is that by Reed *et al* who reported on the pharmacokinetic of formed colistin in CF patients, however these results are not reported in the following discussion as the biological samples were subjected to conditions (54ºC for 1 h) which can facilitate *in vitro* formation of colistin [201]. The reported pharmacokinetic estimates presented in Table A1-3 are derived from a

combination of non-compartmental pharmacokinetic analysis and population pharmacokinetic analysis. A two- and one-compartment model with first order conversion best described the disposition of CMS and formed colistin, respectively, in healthy volunteers [200] and critically-ill patients [126, 202]. Contrary to findings in rats, following IV infusion of CMS a gradual formation of colistin in plasma is evident with peak concentrations achieved at 1 - 4 h after completion of infusion [200], or 45 min [188], 1 h [189] and 7 h [126] after the start of IV infusion. However consistent with observations in rats [184, 198, 199], the elimination of formed colistin following CMS administration is not rate limited by formation from CMS, as the terminal half-life for colistin is longer than that of CMS (Table A1-3) [126, 187, 200]. In healthy volunteers when compared to rats [198, 199], relatively similar volumes of distribution (V_D, per L/kg) were observed for both CMS (V_{ss} = 0.19 L/kg) and colistin (apparent V_D, V/f_m = 0.17 L/kg) (Table A1-3) [200], but in both species distribution into extravascular space is limited. The volume of distribution of CMS and formed colistin is significantly larger in critically-ill patients [126, 188, 189, 202] when compared to the other patient populations (Table A1-3), with such variations likely to be due to changes to the distribution kinetics in disease state. Inter-species variability in the fractional conversion of CMS in plasma is evident with Couet *et al* reporting a significantly higher value of 0.30 in human volunteers [200] in comparison to less than 0.13 in rats [184, 198].

In healthy volunteers following IV CMS infusion, approximately 50% of the dose was recovered in urine as CMS and ~15% as colistin in the 24 h sampling period [200]. These findings are consistent with the ratio of CMS and colistin recovered in urine following IV CMS administration in rats [198]. Intra-renal formation of colistin is confirmed as a reason for the higher recovery of colistin in urine after IV CMS delivery, with Couet *et al* reporting that after correcting for post-excretion conversion, ~64% of the dose is actually excreted in urine as CMS with minimal recovery of colistin [200]. In critically-ill patients, Markou *et al*

[188] and Plachouras *et al* [126] reported no significant correlation is evident between creatinine clearance and colistin kinetics with such observations likely to be due to colistin predominantly cleared by non-renal pathways. However Plachouras *et al* demonstrated a similar correlation for CMS which is unexpected as the prodrug is predominately cleared by the kidneys [126]. The authors proposed that these findings may be due to the small sample size (n=18) and the use of a narrow range of creatinine clearance values (41 – 126 mL/min) in the study [126]. Garonzik *et al* carried out a more comprehensive study with inclusion of 105 subjects and reported that creatinine clearance is an important covariant for both CMS and formed colistin clearance [202]. The significance of creatinine clearance on formed colistin clearance is explained due to the complex relationship of the overall disposition of CMS and formed colistin [202]. A reduction in renal clearance will result in a decrease in CMS clearance (and to a minor extent colistin renal clearance) which in turn can lead to a large fraction of CMS converting to colistin systemically and thereby affecting the overall colistin disposition and clearance [202].

Despite administration of IV CMS in CF patients and in majority of critically-ill patients for the treatment of respiratory infections (pneumonia), the pharmacokinetic of CMS and formed colistin in the lungs has not been thoroughly defined. As mentioned above, rigorous and robust pharmacokinetic studies with incorporation of non-compartmental and compartmental pharmacokinetic analysis have characterised the disposition of CMS and formed colistin in the systemic circulation after IV administration but none have defined the kinetics in the lungs. The study by Reed *et al* reported that relatively high, variable formed colistin sputum concentrations is evident when compared to plasma for all sampling times up to 8 h post-dose following IV infusion of 10 - 90 mg of colistin base activity (given every 8 h) in CF patients [201]. However the reliability of these findings are questionable due to analytical issues (discussed above) and no qualitative/quantitative sputum data reported in the study [201].

More recently Imberti *et al* reported undetectable formed colistin concentration in bronchoalveolar lavage (BAL) fluid sample collected under steady-state conditions following IV CMS administration in critically-ill patients [189]. Quantification of formed colistin concentrations in only the BAL fluid matrix (approximately 100-fold dilution of epithelial lining fluid concentrations) may have led to such findings [189]. Additionally the authors reported that colistin binding to lung tissue rather than low tissue penetration following IV delivery is likely to be the reason for low exposure in the BAL fluid [189]. The findings from these two studies have highlighted the need for more pharmacokinetic studies to define the kinetics of CMS and formed colistin in the lungs following IV CMS delivery in order to preserve the therapeutic value of one of the last line of defence against Gram-negative bacterial infections.

Table A1-3: Pharmacokinetics properties of CMS and formed colistin in plasma following IV administration of CMS (sodium) to healthy volunteers, CF patients and critically-ill patients.

Parameters	Healthy volunteers		CF patients	Critically-ill patients					
	Couet *et al* [Y,Ψ] [200]		Li *et al* [§,£,ℓ] [187]	Markou *et al* [£,ℓ] [188]	Imberti et al [φ,£,ℓ] [189]	Plachouras *et al* [Ψ] [126]		Garonzik *et al* [Ψ] [202]	
	Typical value	IIV (CV%)				Typical value	IIV (CV%)	Typical value	IIV (CV%)
CMS									
CL (mL/min)	148 (5)	15 (47)	113 ± 25.8	N.R	N.R	228 (10)	37 (15)	154.3$^{\upsilon}$ (-)	-
V_{ss} (L)	14.0 (7)	-	19.0 ± 5.32	N.R	N.R	42.4$^{\beta}$ (-)	-	30.2$^{\beta}$ (-)	-
$t_{1/2}$ (h)	2.0	6 (-)	2.07 ± 0.87	N.R	N.R	2.3	N.R	N.S	-
Formed colistin									
CL/f_m (mL/min)	48.7 (15)	-	N.R	227 ± 96.7	352 ± 244	151.5 (19)	59 (36)	45.3 (-)	23
V/f_m (L)	12.4 (15)	19 (53)	N.R	139.9 ± 60.3	123 ± 86.3	189 (12)	-	45.1 (6)	48
$t_{1/2}$ (h)	3.0	19 (-)	4.18 ± 1.32	7.4 ± 1.7	5.9 ± 2.6	14.4	N.R	N.S	-

N.R, not reported.

N.S, not shown as the authors have reported few $t_{1/2}$ based on different creatinine clearance values. Overall formed colistin $t_{1/2}$ was longer than that for CMS.

[Y] mean body weight 72.7 ± 9.1 kg.

[§] estimates reported for a mean body weight of 56 ± 9 kg.

[£] under steady-state conditions.

[φ] estimates reported for a mean body weight of 81.4 kg.

[β] summed values for V_D in central and peripheral compartment.

[υ] CL for CMS calculated from the author's formula for a creatinine clearance of 120 mL/min/1.73m^2.

CL/f_m and V/f_m are apparent CL and V_D estimates.

IIV, inter-individual variability.

CV, coefficient of variation.

[Ψ] population pharmacokinetic analysis and [ℓ] non-compartmental pharmacokinetic analysis.

Appendix II

Participant information and consent form, case report forms and adverse effects report forms for the pharmacokinetic studies described in Chapter 6.

Form A2-1: Participant Information and Consent Form

Participant Information and Consent Form
Version 1: Dated 17th June 2009
Site: The Alfred Hospital

Full Project Title: Pharmacokinetics and tolerability of inhaled and intravenous colistin methanesulphonate (CMS) in cystic fibrosis patients: a pilot study

Principal Researcher: Professor John Wilson

Associate Researcher(s): Professor Michael Dooley, Professor Roger Nation, Dr Michelle McIntosh, Professor Christopher Porter, Dr Jian Li, Dr Johnson George, Denise Clark, Susan Poole, Elyssa Williams, Shalini W.S.Yapa

This Participant Information and Consent Form is 9 pages long. Please make sure you have all the pages.

1. Your Consent

You are invited to take part in this research project.

This Participant Information contains detailed information about the research project. It's purpose is to explain to you as openly and clearly as possible all the procedures involved in this project before you decide whether or not to take part in it.

Please read this Participant Information carefully. Feel free to ask questions about any information in the document. You may also wish to discuss the project with a relative or friend or your local health worker. Feel free to do this.

Once you understand what the project is about and if you agree to take part in it, you will be asked to sign the Consent Form. By signing the Consent Form, you indicate that you understand the information and that you give your consent to participate in the research project.

You will be given a copy of the Participant Information and Consent Form to keep as a record.

2. Purpose and Background

The purpose of this project is to determine the activity and tolerability of an intravenous (IV) and inhaled antibiotic in adult cystic fibrosis (CF) patients.

An antibiotic called colistin methanesulphonate (CMS) has been used in the IV and inhalation form for the treatment of CF. This antibiotic has been approved by the Alfred Pharmacy for the treatment of CF patients with chronic lung infections.

Your eligibility to take part in this study will be determined by your doctor. If you agree to participate the treatment will be in addition to standard routine care. You can refuse or change your mind at any time your routine care will not be affected in any way.

A total of approximately 12 people will participate in this project.

You are invited to participate in this research project because:

1. You have cystic fibrosis and are over 18 years old.

2. You have grown *Pseudomonas aeruginosa* in your sputum and have no other bacteria in your sputum.

3. Your FEV_1 is 25-75% of predicted normal.

4. You have used antibiotics in hospital or at home for treatment of *Pseudomonas aeruginosa* infections.

5. You have no history of hypersensitivity and/or allergy to colistin, polymyxin B or other polymyxins.

6. You are willing and able to use nebulised antibiotics.

7. You have in place adequate birth control measures if you are going to be sexually active during the study.

8. You receive inpatient treatment and attend outpatient clinics at the Alfred hospital.

This study has been initiated by the investigator, Professor John Wilson.

3. Procedures

Participation in this project will involve:

- Participants receiving two doses of nebulised CMS and one dose of intravenous CMS:

  1. 160 mg of CMS via a Salter™ nebuliser,

  2. 320 mg of CMS via a Salter ™ nebuliser,

  3. 400 mg of CMS intravenously

     - Only participants who tolerated the lower nebulised dose will receive the higher nebulised CMS dose at an interval of 2 days.

- Under medical/nursing supervision, receiving chest physiotherapy and scheduled dose of inhaled bronchodilator(s) for example Ventolin prior to CMS being nebulised.

- Being closely monitored by medical and nursing staff for 12 hours following administration of the CMS doses.

- Taking your routine medications for the management of CF during the study period including the use of other antibiotics.

- Allowing research staff to carry out the following tests:

  - Airway function tests – spirometry

- Completing an adverse effect self-report form.

- Allowing research/nursing staff to collect the following samples:

  - 5 mL of blood being taken prior to dose administration and at 0.5, 1, 2, 4, 8 and 12 hours following nebulised CMS administration[§] and an additional 15 mL of blood taken prior to dose administration and at 12 hours following nebulised CMS administration.

- 5 mL of blood being taken prior to dose administration and at 0.25, 0.5, 0.75, 1, 2, 4, 8 and 12 hours following IV CMS administration and an additional 15 mL of blood taken prior to dose administration and at 12 hours following IV CMS administration.

- Less than 2 gram or 2 teaspoon of sputum prior to dose administration and at 1, 4 and 12 hours following dose administration.

- Exhaled breath condensate (approximately 1 mL) prior to dose administration and at 1, 4 and 12 hours following dose administration[¥].

- Urine collection for 24 hours after dose administration[£].

4.   Collection of Tissue Samples for Research Purposes

By consenting to take part in this study, you also consent to the collection, storage and use of tissue samples as specified below.

- Blood Samples:

  - 5 mL of blood being taken prior to dose administration and at 0.5, 1, 2, 4, 8 and 12 hours following nebulised dose administration[§] and prior to dose administration and at 0.25, 0.5, 0.75, 1, 2, 4, 8 and 12 hours following IV dose administration. An additional 15 mL of blood taken prior to dose administration and at 12 hours following nebulised and IV administration.

    The blood will be taken from a peripherally inserted central catheter (PICC line) which you would already have inserted for your standard inpatient antibiotic administration.

    The CF research nurse or Coordinator will take the blood as per the study protocol. One batch of blood will then be taken to Pathology for testing as per usual routine and the other batch of blood will be transported to Monash University (Parkville) to calculate the amount of antibiotic in the blood following administration of the three doses of CMS.

  - The blood will be destroyed after testing has taken place. It will not be used for further research or for genetic testing.

- Sputum Samples:

  - Sputum samples (less than 2 gram or 2 teaspoon) will be taken on admission, prior to dose administration and at 1, 4 and 12 hours following dose administration.

  - The sputum will be sent to Pathology lab at the Alfred to check for the presence of *Pseudomonas aeruginosa* and the density/amount of the bacteria in your lungs.

  - A small sample of the sputum (less than 1 gram or 1 teaspoon) will be sent to Monash University (Parkville) to calculate the amount of antibiotic in the lungs following administration of the three doses of CMS.

  - The sputum will be destroyed after testing has taken place. It will not be used for further research or for genetic testing.

- Exhaled Breath Condensate (EBC) Samples[¥]:

  - EBC samples (1 mL) will be taken prior to dose administration and at 1, 4 and 12 hours following dose administration.

  - The EBC samples will be sent to Monash University (Parkville) to calculate the amount of antibiotic in the lungs following administration of the three doses of CMS.

- The EBC samples will be destroyed after testing has taken place. It will not be used for further research or for genetic testing.

- Urine Samples£:

  - Urine will be collected for 24 hours after each dose of the study drug.

  - The urine will be sent to Monash University (Parkville) to calculate the amount of antibiotic in the body following administration of the three doses of CMS.

  - The urine will be destroyed after testing has taken place. It will not be used for further research or for genetic testing

5. Possible Benefits

We cannot guarantee or promise that you will receive any benefits from this research; however, possible benefits related to the study drug may include an improvement in lung function and a reduction in the number of acute exacerbations of CF lung disease. It is possible that the medication will not produce any benefits or that the benefits may only be temporary.

6. Possible Risks

As with any antibiotic there have been reported side effects which are mild to moderate in severity and are reversed once the drug is stopped. These side effects are mostly related to stomach upsets (nausea and vomiting, diarrhoea, abdominal pain), generalised rash, itching and skin problems and following inhalation bronchospasms (narrowing of the airways), cough, wheeze and chest pain. Administration of bronchodilator(s) prior to CMS dose inhalation will minimise these lung side effects. The potentially serious side effects that can occur especially following IV administration are nephrotoxicity (toxic build up of the drug in the kidneys) and neurotoxicity (toxic build up of the drug causing hearing problems and hearing loss). These side effects occur when the drug is used in higher then recommended doses and in patients who have impaired renal functions. Additionally patients will be closely monitored for 12 hours following dose administration to observe for early treatment response and side effects.

This list is not a complete list of side effects for this drug and like many other medicines, can on rare occasions cause extremely severe side effects such as severe swelling of the face or lips or other parts of the body resulting in an allergic reaction that has the potential to be life threatening. Such events cannot be predicted for any individual.

There may be side effects that the researchers do not expect or do not know about and that may be serious. Tell your doctor immediately about any new or unusual symptoms that you get.

This project does not include exposure to radiation (in the form of x-rays) at any time.

Safe use of colistin methanesulphonate during pregnancy and breast-feeding has not been established. Because of this, it is important that study participants are not pregnant or breast-feeding and do not become pregnant during the course of the study. You must not participate in the study if you are pregnant or trying to become pregnant, or breast-feeding. If you are male, you should not father a child. If you are female and child bearing is a possibility, you will be required to undergo a pregnancy test prior to commencing the study. Both male and female participants are strongly advised to use effective contraception during the course of the study. You should discuss methods of effective contraception with your doctor. If you do become pregnant whilst participating in the study you should advise your treating doctor immediately. He/she will withdraw you from the study and advise on further medical attention should this be necessary. You must not continue in the study if you become pregnant. There may be additional unforeseen or unknown risks.

7. Other Treatments Whilst on Study

It is important to tell your doctor and the research staff about any treatments or medications you may be taking, including non-prescription medications, vitamins or herbal remedies and any changes to these during your participation in the study.

8. Alternatives to Participation

Alternative procedures/alternative treatments include continuing with the treatment plan which is your routine care, as clinically indicated in consultation with your CF doctor.

9. Privacy, Confidentiality and Disclosure of Information

Any information obtained during the course of this study is potentially identifiable. Your details will therefore be coded and the code list stored separately from the details in a locked cabinet, within a locked office. Only the study investigator, researchers and trial coordinator will have access to the information.

At the end of the project the de-identified information will be used in a report which may be published in medical journals. As a participant you will be able to view the overall group results, but individual information will remain private and confidential. In accordance with the *Freedom of Information Act* 1982 (Vic), you have the right to access and to request correction of information held about you by The Alfred Hospital and Bayside Health. To do this you will need to contact the Freedom of Information Coordinator at the Alfred hospital on (03)90763002.

At the end of the study all data is securely stored indefinitely in accordance with hospital policy. The Hospital Research and Ethics Committee (HREC) will determine who is able to access the stored data.

10. New Information Arising During the Project

During the research project, new information about the risks and benefits of the project may become known to the researchers. If this occurs, you will be told about this new information. This new information may mean that you can no longer participate in this research. If this occurs, the person(s) supervising the research will stop your participation. In all cases, you will be offered all available care to suit your needs and medical condition.

11. Results of Project

Results of this study will appear in summary form in the Cystic Fibrosis Newsletter. You will be informed of this prior to publication date.

12. Further Information or Any Problems

If you require further information or if you have any problems concerning this project (for example, any side effects), you can contact the principal researcher Professor John Wilson, Professor Michael Dooley or Denise Clark. The researchers responsible for this project are:

Professor John Wilson

5th Floor, Alfred Hospital

(03) 9076 2315 business hours

(03) 9076 2000 page through hospital switchboard out of hours.

Denise Clark CF Research nurse/Trial Coordinator

(03) 9076 8590 business hours

Professor Michael Dooley – Director of Pharmacy

(03) 9076 2408 business hours

13.   Other Issues

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about your rights as a research participant, then you may contact

Name: **Ms. Rowan Frew**

Position: Ethics Manager, Alfred Research & Ethics Unit

Telephone: (03) 9076 3848

You will need to tell Rowan Frew the name of one of the researchers given in section 12 above.

14.   Participation is Voluntary

Participation in any research project is voluntary. If you do not wish to take part you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with The Alfred Hospital.

Before you make your decision, a member of the research team will be available so that you can ask any questions you have about the research project. You can ask for any information you want. Sign the Consent Form only after you have had a chance to ask your questions and have received satisfactory answers.

If you decide to withdraw from this project, please notify a member of the research team before you withdraw. This notice will allow that person or the research supervisor to inform you if there are any health risks or special requirements linked to withdrawing.

15.   Reimbursement for your costs

You will not be paid for your participation in this trial.

16.   Ethical Guidelines

This project will be carried out according to the *National Statement on Ethical Conduct in Human Research* March 2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.

The ethical aspects of this research project have been approved by the Human Research Ethics Committee of The Alfred Hospital.

17.   Injury

In the event that you suffer an injury as a result of participating in this research project, hospital care and treatment will be provided by the public health service at no extra cost to you.

18.   Termination of the Study

This research project may be stopped for a variety of reasons. These may include reasons such as: unacceptable side effects, the drug being shown not to be effective, the drug being shown to work and not need further investigation. At the end of the study period the treatment will be available as determined by your treating doctor.

Consent Form
Version 1: Dated 17th June 2009
Site: Alfred Hospital

Full Project Title: Pharmacokinetics and tolerability of inhaled and intravenous colistin methanesulphonate (CMS) in cystic fibrosis patients: a pilot study

I have read and I understand the Participant Information version 1 dated 17th June 2009

I have had an opportunity to ask questions and I am satisfied with the answers I have received.

I freely agree to participate in this project according to the conditions in the Participant Information.

I will be given a copy of the Participant Information and Consent Form to keep.

I understand that the researcher has agreed not to reveal my identity and personal details if information about this project is published or presented in any public form.

Participant's Name (printed) …………………………………………………

Signature   Date

Name of Witness to Participant's Signature (printed) ……………………………………

Signature   Date

Declaration by researcher*: I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Researcher's Name (printed) …………………………………………………

Signature   Date

* A senior member of the research team must provide the explanation and provision of information concerning the research project.

Note: All parties signing the Consent Form must date their own signature.

Revocation of Consent Form

Full Project Title: Pharmacokinetics and tolerability of inhaled and intravenous colistin methanesulphonate (CMS) in cystic fibrosis patients: a pilot study

I hereby wish to WITHDRAW my consent to participate in the research proposal named above and understand that such withdrawal WILL NOT jeopardise any treatment or my relationship with *Name of Institution*.

Participant's Name (printed) …………………………………………………….

Signature   Date

Minor amendments to the Participant Information and Consent Form:

§ Amendment to the blood sampling times (pre-dose, 0.5, 1, 2, 4, 6 and 8 post-dose) following nebulisation of both CMS doses in patient 4, 5, and 6 was implemented with approval by the ethics committee. Patients were notified of this change prior to written consent been given.

¥ Exhaled breath condensate was not collected in any patients as an analytical method for quantification of drug concentrations could not be developed.

£ Urine was collected 12 h prior to administration of all three CMS doses, with approval given by the ethics committee. Patients were notified of this change prior to written consent been given.

Form A2-2: Case report form A

CASE REPORT FORM A:
Version 1: Dated 28th January 2010
Project 221/09

**Pharmacokinetics and tolerability of inhaled and intravenous colistin
methanesulphonate (CMS) in cystic fibrosis patients: a pilot study**

Patient Eligibility Form

Date of recruitment: _ _ _ / _ _ _ / _ _ _

Patient Identification/Code: Pt…= #00…

Inclusion Criteria (Tick for Yes and Cross for No)

Have a documented diagnosis of CF, ☐

Have a respiratory tract culture of *Pseudomonas aeruginosa* without the presence of any
other Gram-negative or Gram-positive organisms, ☐

FEV$_1$ 25-75% of predicted, ☐

Have a history of using antibiotics at the hospital or home for treatment of Gram-negative
infections, ☐

Willing and able to use nebulised antibiotics, ☐

Have in place adequate birth control measures if subject will be sexually
active while participating in this study. ☐

Exclusion Criteria

Unable to provide written informed consent, ☐

<18 years and no guardian or carer to give consent on behalf of the patient, ☐

History of hypersensitivity and/or allergy to colistin, polymyxin B or other polymyxins, ☐

Recovery of *Burkholderia cepacia* from the respiratory tract within the previous two years. ☐

Eligible for inclusion to study?   Yes / No

250

Form A2-3: Case report form B

CASE REPORT FORM B:
Version 1: Dated 28th January 2010
Project 221/09

Pharmacokinetics and tolerability of inhaled and intravenous colistin methanesulphonate (CMS) in cystic fibrosis patients: a pilot study

DAY 1:

➢ **Patient Information**

- Patient Identification/Code: Pt… = #00…

- Date of recruitment: ……./……./…….

➢ **Participant Information and Consent Form (PI&CF)**

Date PI&CF given to patient: ……./……./…….

Date of Informed Consent: ……./……./…….
(If no Informed Consent was signed, patient cannot be included in the study).

Copy of Informed Consent given to participant: Yes / No

Copy of Informed Consent kept in locked cabinet: Yes / No

Copy of Informed Consent filed in Medical Record: Yes / No

➢ **Demographics**

Date of Birth: ……./……./…….

Postcode: ………………….

Race:
    Caucasian / white
    Black
    Asian
    Aboriginal / Torres strait islander
    Hispanic
    Other please specify ……….

Gender: Male: …….; Female: …….

Height: ……. cm   Weight: ……. kg

➤ **Baseline Data** (from patient / patient history / Cerner Power chart)

- Medical diagnosis: ……….

- Concurrent illness:

Conditions		Tick
Bronchiectasis		
Pancreatic insufficiency		
Renal disease		
Liver disease including:	Portal hypertension	
	Varices	
	Abnormal liver function	
CF related diabetes		
Gastroesophageal reflux:	Medicated	
	Non medicated	
Neutropenia		
CF arthropathy		
Transplant waiting list		
Asthma		
Other: please specify		

- Current medications:

Drug name *i.e. salbutamol*	Dosage form *i.e. tablet*	Frequency *i.e. BD*	Special directions *i.e. with food, break or crush*

Was patient on CMS/colistin nebulised or IV at home or at hospital prior to commencement of clinical trial? Yes / No

If Yes please provide details such as dose, dosing regimen and when the therapy was ceased prior to commencement of the trail:
………………………………………………………………………………………………………
………………………………………………………………………………………………………
………………………………………………………………………………………………………
………………………………………………………………………………………………………
………………………………………………………………………………………………………

Form A2-4: Case report form C

CASE REPORT FORM C:
Version 1: Dated 28th January 2010

CASE REPORT FORM C:
Version 1: Dated 28th January 2010
Project 221/09

Pharmacokinetics and tolerability of inhaled and intravenous colistin methanesulphonate (CMS) in cystic fibrosis patients: a pilot study

DAY 1: Nebulisation of 160 mg of sodium CMS = Pt…A

➢ **Patient Information**

-   Patient Identification/Code: Pt… = #00…

-   Date of recruitment: ……./……./…….

-   Patient Weight: ……. Patient Height: …….

➢ **Drug**

-   Sodium Colistin Methanesulphonate

➢ **Product Information**

-   Brand Name: …….
-   Sodium CMS quantity: …….
-   Drug Company: …….
-   Batch Number: …….
-   Expiry Date: …….

➢ **Delivery Route**

-   Pulmonary delivery

➢ **Delivery Device**

-   Salter Ultra-Mist nebuliser with Exhalation Filter

➢ **Nebulised CMS Dose**

-   Low dose of CMS: *160 mg sodium CMS*
-   Number of Tadim® vials:……………………………………………….......
-   Nebulisation Duration: Start Time: ……. End Time: ……. Duration: …….

➢ **Preparation of Nebulised CMS Dosing Solution**

- The required nebulised dose will be reconstituted with sodium chloride 0.9% up to a final volume of 4 mL.

➢ **Chest Physiotherapy and Salbutamol Dose**

- Yes/No; Time of salbutamol dose (2.5 – 5 mg): …….

➢ **CMS Low Dose**

- Date of Dosing: ……./……./…….
- Time of Dosing: …….

➢ **Collection of Biological Samples**

1) Blood Samples

Sample no.	Sample time (h)	Actual sample time (h)	Blood volume (mL)	Sample Centrifuged¥	Sample storage (-80ºC)
Pt..A P1	PTD		5 mL, 15 mL*		3 × 0.8 mL
Pt..A P2	0.5§		5 mL		3 × 0.8 mL
Pt..A P3	1		5 mL		3 × 0.8 mL
Pt..A P4	2		5 mL		3 × 0.8 mL
Pt..A P5	4		5 mL		3 × 0.8 mL
Pt..A P6	6		5 mL		3 × 0.8 mL
Pt..A P7	8		5 mL, 15 mL*		3 × 0.8 mL

PTD, denotes prior to dosing; § Following completion of nebulisation; * 15 mL of blood collected at PTD and 12 h post-dose for quantification of serum creatinine and full blood examination; ¥ Sample centrifugation conditions (10,640 × g, 4°C, 10 min); 3 × 0.8 mL, denotes three aliquots of 0.8 mL plasma.

NOTE: For patient 1, 2 and 3 blood samples were collected at PTD, 0.5, 1, 2, 4, 8 and 12 h post-dose, however CMS concentrations were below limit of quantification at 12 h following both nebulised CMS doses. Therefore amendments were made to ethics application and case report form to exclude the 12 h sample time but include a sample collection at 6 h. However a 15 mL blood sample was still collected at 12 h for serum creatinine and full blood examination.

2) Sputum Samples

Sample no.	Sample time (h)	Actual sample time (h)	Weight of sputum (g)	Sputum	Sample storage (-80ºC)	Notes
Pt..A S1	PTD			2 g or 2 teaspoon*	2 × 0.5 g/0.5 tsp	
Pt..A S2	1§			2 g or 2 teaspoon	2 × 0.5 g/0.5 tsp	
Pt..A S3	4			2 g or 2 teaspoon	2 × 0.5 g/0.5 tsp	
Pt..A S4	12			2 g or 2 teaspoon	2 × 0.5 g/0.5 tsp	

§ Following completion of nebulisation; * 1 g or 1 teaspoon (tsp) sent to pathology for analysis for presence and density of *P. aeruginosa*; 2 × 0.5 g/ 0.5 tsp, denotes two aliquots of 0.5 g or 0.5 tsp of sputum.

NOTE: Analysis for the presence and density of bacteria was not conducted as the Alfred Hospital laboratories did not have the facilities to carry out such procedures.

3) Urine Sample

Sample no.	Sample time (h)	Actual sample time (h)	Urine volume (mL)	Sample storage (-80ºC)
Pt..A U1	12 h pre-dose			2 × 5 mL 2 × 1 mL
Pt..A U2	24 h post-dose§			2 × 5 mL 2 × 1 mL

§ Following completion of nebulisation; 2 × 5 mL and 2 × 1 mL, denotes two aliquots of 5 mL and 1 mL urine, respectively.

Did the patient have day leave during the study? Yes / No

If Yes; did the patient discard all urine when away from the hospital in a container and return the container to the nurse? Yes / No

➢ Airway Function Test (Spirometry)

- Prior to dose administration: Time …….
- 1 h post-dose administration: Time …….

1) Airway Function Results (Spirometry)

Parameters*	Normal range	Pre-dose results (Time = …….)	Post-dose results (Time = …….)
$FEV_{(1)}$			
FVC			

*$FEV_{(1)}$ and FVC denotes forced expiratory volume in 1 sec and forced vital capacity, respectively.

2) Maximal Respiratory Pressures

Parameters*	Normal range	Pre-dose results (Time = …….)	Post-dose results (Time = …….)
MIP			
MEP			

* MIP and MEP denotes minimum inspiratory pressure and maximal expiratory pressure, respectively.

➢ Serum Creatinine Measurements

Sample Time	Serum Creatinine Levels (µmoL/L)
Pre-dose	
Post-dose (12 h)	

➢ CMS Dose Tolerability

Did the patient tolerate the 160 mg sodium CMS dose?

- Yes: ……. If Yes, patient can receive high CMS dose (320 mg sodium CMS dose).
- No: ……. If No, patient will NOT receive the high CMS dose.

Note down any adverse effects reported by the patient from the adverse effect report form:

………………………………………………………………………………………………………
………………………………………………………………………………………………………
………………………………………………………………………………………………………

➢ Washout Period between Doses

- 48/72 h washout period ……., Date/time of next dose:…………………………

Pharmacokinetics and tolerability of inhaled and intravenous colistin methanesulphonate (CMS) in cystic fibrosis patients: a pilot study

DAY 4: Nebulisation of 320 mg of sodium CMS = Pt…B

➢ **Patient Information**

- Patient Identification/Code: Pt… = #00…

- Date of recruitment: ……./……./…….

- Patient Weight: ……. Patient Height: …….

➢ **Drug**

- Sodium Colistin Methanesulphonate

➢ **Product Information**

- Brand Name: …….
- Sodium CMS quantity: …….
- Drug Company: …….
- Batch Number: …….
- Expiry Date: …….

➢ **Delivery Route**

- Pulmonary delivery

➢ **Delivery Device**

- Salter Ultra-Mist nebuliser with Exhalation Filter

➢ **Nebulised CMS Dose**

- Low dose of CMS: ***320 mg sodium CMS***
- Number of Tadim® vials:……………………………………………….......
- Nebulisation Duration: Start Time: ……. End Time: ……. Duration: …….

➢ **Preparation of Nebulised CMS Dosing Solution**

- The required nebulised dose will be reconstituted with sodium chloride 0.9% up to a final volume of 4 mL.

➢ **Chest Physiotherapy and Salbutamol Dose**

- Yes/No; Time of salbutamol dose (2.5 – 5 mg): …….

➢ **CMS High Dose**

- Date of Dosing: ……./……./…….
- Time of Dosing: …….

➢ **Collection of Biological Samples**

1) Blood Samples

Sample no.	Sample time (h)	Actual sample time (h)	Blood volume (mL)	Sample Centrifuged¥	Sample storage (-80ºC)
Pt..B P1	PTD		5 mL, 15 mL*		3 × 0.8 mL
Pt..B P2	0.5§		5 mL		3 × 0.8 mL
Pt..B P3	1		5 mL		3 × 0.8 mL
Pt..B P4	2		5 mL		3 × 0.8 mL
Pt..B P5	4		5 mL		3 × 0.8 mL
Pt..B P6	6		5 mL		3 × 0.8 mL
Pt..B P7	8		5 mL, 15 mL*		3 × 0.8 mL

PTD, denotes prior to dosing; § Following completion of nebulisation; * 15 mL of blood collected at PTD and 12 h post-dose for quantification of serum creatinine and full blood examination; ¥ Sample centrifugation conditions (10,640 × *g*, 4°C, 10 min); 3 × 0.8 mL, denotes three aliquots of 0.8 mL plasma.

NOTE: For patient 1, 2 and 3 blood samples were collected at PTD, 0.5, 1, 2, 4, 8 and 12 h post-dose, however CMS concentrations were below limit of quantification at 12 h following both nebulised CMS doses. Therefore amendments were made to ethics application and case report form to exclude the 12 h sample time but include a sample collection at 6 h. However a 15 mL blood sample was still collected at 12 h for serum creatinine and full blood examination.

2) Sputum Samples

Sample no.	Sample time (h)	Actual sample time (h)	Weight of sputum (g)	Sputum	Sample storage (-80°C)	Notes
Pt..B S1	PTD			2 g or 2 teaspoon*	2 × 0.5 g/0.5 tsp	
Pt..B S2	1§			2 g or 2 teaspoon	2 × 0.5 g/0.5 tsp	
Pt..B S3	4			2 g or 2 teaspoon	2 × 0.5 g/0.5 tsp	
Pt..B S4	12			2 g or 2 teaspoon	2 × 0.5 g/0.5 tsp	

§ Following completion of nebulisation; * 1 g or 1 teaspoon (tsp) sent to pathology for analysis for presence and density of *P. aeruginosa*; 2 × 0.5 g/ 0.5 tsp, denotes two aliquots of 0.5 g or 0.5 tsp of sputum.

NOTE: Analysis for the presence and density of bacteria was not conducted as the Alfred Hospital laboratories did not have the facilities to carry out such procedures.

4) Urine Sample

Sample no.	Sample time (h)	Actual sample time (h)	Urine volume (mL)	Sample storage (-80°C)
Pt..B U1	12 h pre-dose			2 × 5 mL 2 × 1 mL
Pt..B U2	24 h post-dose§			2 × 5 mL 2 × 1 mL

§ Following completion of nebulisation; 2 × 5 mL and 2 × 1 mL, denotes two aliquots of 5 mL and 1 mL urine, respectively.

Did the patient have day leave during the study? Yes / No

If Yes; did the patient discard all urine when away from the hospital in a container and return the container to the nurse? Yes / No

➤ **Airway Function Test (Spirometry)**

- Prior to dose administration: Time …….
- 1 h post-dose administration: Time …….

3) Airway Function Results (Spirometry)

Parameters*	Normal range	Pre-dose results (Time = …….)	Post-dose results (Time = …….)
$FEV_{(1)}$			
FVC			

*$FEV_{(1)}$ and FVC denotes forced expiratory volume in 1 sec and forced vital capacity, respectively.

4) Maximal Respiratory Pressures

Parameters*	Normal range	Pre-dose results (Time = …….)	Post-dose results (Time = …….)
MIP			
MEP			

* MIP and MEP denotes minimum inspiratory pressure and maximal expiratory pressure, respectively.

➢ **Serum Creatinine Measurements**

Sample Time	Serum Creatinine Levels (µmoL/L)
Pre-dose	
Post-dose (12 h)	

➢ **CMS Dose Tolerability**

Did the patient tolerate the 320 mg sodium CMS dose?

- Yes: ……. If Yes, patient can receive IV CMS dose (400 mg sodium CMS dose).
- No: ……. If No, patient will NOT receive the IV sodium CMS dose.

Note down any adverse effects reported by the patient from the adverse effect report form:

…………………………………………………………………………………………………
…………………………………………………………………………………………………
…………………………………………………………………………………………………

➢ **Washout Period between Doses**

- 48/72 h washout period ……., Date/time of next dose:…………………………

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DAY 7: IV infusion of 400 mg of sodium CMS = Pt…C

➢ **Patient Information**

- Patient Identification/Code: Pt… = #00…

- Date of recruitment: ……./……./…….

- Patient Weight: ……. Patient Height: …….

➢ **Drug**

- Sodium Colistin Methanesulphonate

➢ **Product Information**

- Brand Name: …….
- Sodium CMS quantity: …….
- Drug Company: …….
- Batch Number: …….
- Expiry Date: …….

➢ **Delivery Route**

- IV infusion
- Single or double lumen PICC line: …….

➢ **IV CMS Dose**

- IV CMS dose: ***400 mg sodium CMS*** or ***150 mg of colistin base activity (CBA)***
- One vial of Colistin Link Parenteral contains 150 mg of CBA which is equivalent to 400 mg of sodium CMS.
- IV infusion duration: Start Time: ……. End Time: ……. Duration: …….
- Flush volume: …….

➢ **Preparation of IV CMS Dosing Solution**

- Each vial is reconstituted with 2 mL of sterile water for injection (final volume of 75 mg CBA per mL). The reconstituted solution (2 mL) is then added to 100 mL of either sodium chloride 0.9% or glucose 5%.

➢ CMS IV Dose

- Date of Dosing: ……./……./…….
- Time of Dosing: …….

➢ Collection of Biological Samples

1) Blood Samples

Sample no.	Sample time (h)	Actual sample time (h)	Blood volume (mL)	Sample Centrifuged¥	Sample storage (-80ºC)
Pt..C P1	PTD		5 mL, 15 mL*		3 × 0.8 mL
Pt..C P2	0.25§		5 mL		3 × 0.8 mL
Pt..C P3	0.5		5 mL		3 × 0.8 mL
Pt..C P4	0.75		5 mL		3 × 0.8 mL
Pt..C P5	1		5 mL		3 × 0.8 mL
Pt..C P6	2		5 mL		3 × 0.8 mL
Pt..C P7	4		5 mL		3 × 0.8 mL
Pt..C P8	8		5 mL		3 × 0.8 mL
Pt..C P9	12		5 mL, 15 mL*		3 × 0.8 mL

PTD, denotes prior to dosing; § Following completion of IV infusion; * 15mL of blood collected at PTD and 12 h post-dose for quantification of serum creatinine and full blood examination; ¥ Sample centrifugation conditions (10,640 × *g*, 4°C, 10 min); 3 × 0.8 mL, denotes three aliquots of 0.8 mL plasma.

2) Sputum Samples

Sample no.	Sample time (h)	Actual sample time (h)	Weight of sputum (g)	Sputum	Sample storage (-80ºC)	Notes
Pt..C S1	PTD			2 g or 2 teaspoon*	2 × 0.5 g/0.5 tsp	
Pt..C S2	1§			2 g or 2 teaspoon	2 × 0.5 g/0.5 tsp	
Pt..C S3	4			2 g or 2 teaspoon	2 × 0.5 g/0.5 tsp	
Pt..C S4	12			2 g or 2 teaspoon	2 × 0.5 g/0.5 tsp	

§ Following completion of IV infusion; * 1 g or 1 teaspoon (tsp) sent to pathology for analysis for presence and density of *P. aeruginosa*; 2 × 0.5 g/ 0.5 tsp, denotes two aliquots of 0.5 g or 0.5 tsp of sputum.

NOTE: Analysis for the presence and density of bacteria was not conducted as the Alfred Hospital laboratories did not have the facilities to carry out such procedures.

3) Urine Sample

Sample no.	Sample time (h)	Actual sample time (h)	Urine volume (mL)	Sample storage (-80ºC)
Pt..C U1	12 h pre-dose			2 × 5 mL 2 × 1 mL
Pt..C U2	24 h post-dose§			2 × 5 mL 2 × 1 mL

§ Following completion of IV infusion; 2 × 5 mL and 2 × 1 mL, denotes two aliquots of 5 mL and 1 mL urine,

respectively.

Did the patient have day leave during the study? Yes / No

If Yes; did the patient discard all urine when away from the hospital in a container and return the container to the nurse? Yes / No

➤ **Airway Function Test (Spirometry)**

- Prior to dose administration: Time …….
- 1 h post-dose administration: Time …….

1) Airway Function Results (Spirometry)

Parameters*	Normal range	Pre-dose results (Time = …….)	Post-dose results (Time = …….)
$FEV_{(1)}$			
FVC			

*$FEV_{(1)}$ and FVC denotes forced expiratory volume in 1 sec and forced vital capacity, respectively.

2) Maximal Respiratory Pressures

Parameters*	Normal range	Pre-dose results (Time = …….)	Post-dose results (Time = …….)
MIP			
MEP			

* MIP and MEP denotes minimum inspiratory pressure and maximal expiratory pressure, respectively.

➤ Serum Creatinine Measurements

Sample Time	Serum Creatinine Levels (µmoL/L)
Pre-dose	
Post-dose (12 h)	

➤ CMS Dose Tolerability

Did the patient tolerate the 400 mg sodium CMS dose?

- Yes: …….
- No: …….

Note down any adverse effects reported by the patient from the adverse effect report form:

……
……
……

Form A2-5: Adverse effects report form for low nebulised sodium CMS dose of 160 mg

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Name: ………………..   Date: ………………..

1) Following the first inhaled dose of Colistin do you feel that your symptoms of cough have changed?

(*On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no change to your cough and 10 being cough as bad as it gets*).

      0   1   2   3   4   5   6   7   8   9   10

2) Following the first inhaled dose of Colistin do you feel that you have experienced any chest tightness?

(*On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no chest tightness and 10 being worst chest tightness you have experienced*).

      0   1   2   3   4   5   6   7   8   9   10

3) Following the first inhaled dose of Colistin do you feel that you have experienced any irritation of your throat for example a feeling that there is something stuck in your throat?

(*On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no throat irritation and 10 being worst throat irritation you have experienced*).

      0   1   2   3   4   5   6   7   8   9   10

4) Following the first inhaled dose of Colistin do you feel that you have experienced any wheeziness in your chest?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no chest wheeziness and 10 being worst chest wheeziness you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

5) Following the first inhaled dose of Colistin do you feel that you have experienced any chest pain other than tightness?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no chest pains and 10 being worst chest pains you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

6) Following the first inhaled dose of Colistin do you feel that you have experienced any burning, tingling or numbness in any part of your body?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no problems and 10 being worst problems you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

7) Following the first inhaled dose of Colistin do you feel that you have experienced any problems with co-ordination, balance or clumsiness?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no problems and 10 being worst problems you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

Form A2-6: Adverse effects report form for high nebulised sodium CMS dose of 320 mg

Project 221/09: Pharmacokinetics and tolerability of inhaled and intravenous colistin methanesulphonate (CMS) in cystic fibrosis patients: a pilot study

Name: ………………..   Date: ………………..

1) Following the second inhaled dose of Colistin do you feel that your symptoms of cough have changed?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no change to your cough and 10 being cough as bad as it gets).

      0   1   2   3   4   5   6   7   8   9   10

2) Following the second inhaled dose of Colistin do you feel that you have experienced any chest tightness?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no chest tightness and 10 being worst chest tightness you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

3) Following the second inhaled dose of Colistin do you feel that you have experienced any irritation of your throat for example a feeling that there is something stuck in your throat?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no throat irritation and 10 being worst throat irritation you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

4) Following the second inhaled dose of Colistin do you feel that you have experienced any wheeziness in your chest?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no chest wheeziness and 10 being worst chest wheeziness you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

5) Following the second inhaled dose of Colistin do you feel that you have experienced any chest pain other than tightness?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no chest pains and 10 being worst chest pains you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

6) Following the second inhaled dose of Colistin do you feel that you have experienced any burning, tingling or numbness in any part of your body?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no problems and 10 being worst problems you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

7) Following the second inhaled dose of Colistin do you feel that you have experienced any problems with co-ordination, balance or clumsiness?

(On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no problems and 10 being worst problems you have experienced).

      0   1   2   3   4   5   6   7   8   9   10

Form A2-7: Adverse effects report form for intravenous sodium CMS dose of 400 mg

Project 221/09: Pharmacokinetics and tolerability of inhaled and intravenous colistin methanesulphonate (CMS) in cystic fibrosis patients: a pilot study

Name: ………………..   Date: ………………..

1) Following the IV dose of Colistin do you feel that you have experienced any burning, tingling or numbness in any part of your body?

(*On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no problems and 10 being worst problems you have experienced*).

    0   1   2   3   4   5   6   7   8   9   10

2) Following the IV dose of Colistin do you feel that you have experienced any problems with co-ordination, balance or clumsiness?

(*On the following scale of 0 to 10 please put a mark on the line where you think any changes would lie: 0 being no problems and 10 being worst problems you have experienced*).

    0   1   2   3   4   5   6   7   8   9   10

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