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Translating Innovative Pharmacokinetic Sampling Techniques into Improved Antibiotic Dosing Regimens in Critically Ill Patients

Suzanne Louise Parker

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Abstract

Antibiotic resistance is a significant and immediate global health concern. An increasing prevalence of multi-drug resistant bacteria is steadily decreasing the number of antibiotics that can be used, and few new antibiotics are available for effective treatment of multi-drug resistant infections. Effective dosing of antibiotics can have a two-fold effect by firstly improving patient outcomes and secondly suppressing the emergence of antibiotic resistance. In critically ill patients there are significant pathophysiological changes that can complicate antibiotic dosing and knowledge of the pharmacokinetic (dose-concentration relationship) and pharmacodynamic (concentration-effect relationship) properties of antibiotics are essential to ensure effective treatment.

In an era of increasing antibiotic resistance, there is substantial interest in the optimal use of previously forgotten antibiotics like fosfomycin for the treatment of infections caused by multi-drug resistant bacteria. Given that resistance commonly arises in critically ill patients, a detailed understanding of antibiotic pharmacokinetics in these patients can lead to development of optimised dosing regimens that maximise bacterial killing and suppress the emergence of resistance of these antibiotics. However, pharmacokinetic studies are often expensive to perform and are resource-heavy. Innovative approaches to collecting, storing and transporting clinical samples, including microsampling techniques, could reduce some of these costs. The use of microsampling techniques in pharmacokinetic studies is likely to lead to simpler, less expensive, less invasive sample collection for more informative pharmacokinetic studies in critically ill patients that can then translate to more effective antibiotic dosing.

The principal aims of this Thesis are to investigate how innovative microsampling techniques can be translated into pharmacokinetic studies. Additionally, this Thesis aims to describe optimised dosing regimens for fosfomycin through the conduct of a pharmacokinetic study in critically ill patients.

This Thesis describes a quantitative bioanalytical validation performed using novel volumetric absorptive microsampling (VAMS) devices for sampling fosfomycin in whole blood. The use of the VAMS devices provided acceptable validation results for lower limit of quantification (LLOQ), linearity, and inter- and intra- day precision and accuracy, and matrix effects. However, the results from recovery and stability testing using VAMS devices for the quantitative bioanalysis of fosfomycin suggest challenges remain for the analysis of fosfomycin in whole blood.

The microsampling investigations also describe a validated process for quantitatively measuring fosfomycin in blood samples using a dried plasma spot (DPS) sampling technique. The results of the DPS samples from a clinical pharmacokinetic study were found to correlate with the 'gold-standard' of plasma sampling. The translation of this technique into pharmacokinetic studies can reduce the resource burden during the study and enhance opportunities for collaborative research between clinic-based and laboratory-based staff.

The results of the population pharmacokinetic study of fosfomycin in critically ill patients found a lower median clearance of fosfomycin (2.06 L/h) than that found in healthy patients (7.2 L/h). This demonstrates the need for careful monitoring of fosfomycin dosing in critically ill patients, particularly if there is evidence of renal dysfunction. Clearance was found to be proportional to calculated creatinine clearance (using the Cockcroft-Gault equation). A larger than normal apparent volume of distribution was found and is likely to reflect the degree of sickness severity in the patients. Application of the population pharmacokinetic model with Monte Carlo dosing simulations demonstrated that contemporary dosing of fosfomycin is inadequate for many pathogens in patients with augmented renal clearance.

This Thesis has provided an accurate description of the feasibility of innovative sampling approaches for use in pharmacokinetic studies. This sampling approach was shown to be appropriate for fosfomycin, which is being used to treat patients with multi-drug resistant infections. The characterisation of the pharmacokinetics of fosfomycin in critically ill patients in

this Thesis ensures evidence-based dosing is now available to maximise the likelihood of treatment success including reducing the emergence of resistance to fosfomycin. Future pharmacokinetic research with fosfomycin can be suitably undertaken using microsampling devices to increase the feasibility of these challenging studies.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Parker S., Lipman J., Koulenti D., Dimopoulos G., Roberts J.A. What is the relevance of fosfomycin pharmacokinetics in the treatment of serious infections in critically ill patients? a systematic review. International Journal of Antimicrobial Agents (42 (2013) 289-293)

Parker S., Lipman J., Roberts J.A., Wallis S.C. A simple LC-MS/MS method using HILIC chromatography for the determination of fosfomycin in plasma and urine: application to a pilot pharmacokinetic study in humans. Journal of Pharmaceutical and Biomedical Analysis (105 (2015) 39-45)

Parker S., Lipman J., Roberts J.A., Wallis S.C. Quantitative bioanalytical validation of fosfomycin in human whole blood with volumetric absorptive microsampling (VAMS). Bioanalysis (7: 19 (2015) 2585-2595)

Parker S., Lipman J., Dimopolous G., Roberts J.A., Wallis S.C. A validated method for the quantification of fosfomycin on dried plasma spots by HPLC-MS/MS: application to a pharmacokinetic study in humans. Journal of Pharmaceutical and Biomedical Analysis (115 (2015) 509-514).

Parker S., Frantzeskaki F., Wallis S.C., Diakaki C., Giamarellou H., Koulenti D., Karaiskos I., Lipman J., Dimopoulos G., Roberts J.A. Population pharmacokinetics and Monte Carlo dosing simulations for fosfomycin in critically ill patients. Antimicrobial Agents and Chemotherapy (59: 10 (2015) 6471-6476).

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Contributor	Statement of contribution
Parker S. (Candidate)	Performed review (80%) Wrote the paper (70%) Designed experiments (60%)
Lipman J.	Reviewing draft (30%)
Koulenti D.	Performed review (20%) Edited the paper (20%) Reviewed draft (20%)
Dimopoulos G.	Reviewing draft (20%)
Roberts J.A.	Designed experiments (40%) Wrote the paper (30%) Edited the paper (70%) Reviewed draft (30%)

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Contributor	Statement of contribution
Parker S. (Candidate)	Developed and validated method (100%) Analysed clinical samples (100%) Wrote the paper (100%)
Lipman J.	Reviewed draft (30%)

Roberts J.A.	Reviewed draft (30%) Edited the paper (40%)
Wallis S.C.	Edited the paper (60%) Reviewed draft (40%)

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Contributor	Statement of contribution
Parker S. (Candidate)	Experiment design (60%) Developed and validated method (100%) Analysed experimental samples (100%) Wrote the paper (100%)
Lipman J.	Reviewed draft (30%)
Roberts J.A.	Reviewed draft (30%) Edited the paper (40%)
Wallis S.C.	Experiment design (40%) Edited the paper (60%) Reviewed draft (40%)

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Contributor	Statement of contribution
Parker S. (Candidate)	Developed and validated method (100%) Analysed clinical samples (100%) Wrote the paper (100%)

	Experiment design (60%)
Lipman J.	Reviewed draft (30%)
Roberts J.A.	Reviewed draft (30%) Edited the paper (40%)
Wallis S.C.	Experiment design (40%) Edited the paper (60%) Reviewed draft (40%)

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Parker S. (Candidate)	Designed experiments (20%) Performed data analysis (80%) Interpreted results (60%) Wrote the paper (80%)
Frantzeskaki F.	Performed clinical trial (20%)
Wallis S.C.	Reviewing draft (20%)
Diakaki C.	Performed clinical trial (20%)
Giamarellou H.	Performed clinical trial (20%)
Koulenti D.	Performed clinical trial (40%) Coordinated clinical trial (20%) Reviewed draft (20%)
Karaiskos I.	Coordinated clinical trial (30%)
Lipman J.	Designed experiments (20%) Reviewing draft (20%)

Dimopoulos G.	Designed experiments (20%) Coordinated clinical trial (50%) Reviewing draft (20%)
Roberts J.A.	Designed experiments (40%) Wrote the paper (20%) Performed data analysis (20%) Interpreted results (40%) Reviewed draft (20%)

Contributions by others to the thesis

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Statement of parts of the thesis submitted to qualify for the award of another degree

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Keywords

Antibiotic, critically ill, fosfomycin, pharmacokinetics, pharmacodynamics, microsampling

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List of abbreviations used in the thesis

ANZICS-CTG	Australian and New Zealand Intensive Care Society Clinical Trials Group
APACHE	Acute physiology and chronic health evaluation
AUC	Area under the concentration–time curve
AUC_{0-4}	Area under the concentration–time curve from time 0 to 4 hours
AUC_{0-8}	Area under the concentration–time curve from time 0 to 8 hours
BSV	Between subject variability
CL	Clearance
C_{max}	Maximum concentration
CSF	Cerebrospinal fluid
CV	Coefficient of variation
CVC	Central venous catheter
DBS	Dried blood spots
DPS	Dried plasma spots
EDTA	Ethylenediaminetetraacetic acid
EPA	Ethylphosphonic acid
ESI	Electro-spray ionization
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ESBL	Extended spectrum beta-lactamase
FOM	Fosfomycin
FT	Freeze-thaw cycles
G-6-P	Glucose-6-phosphate
HILIC	Hydrophilic interaction liquid chromatography
HPLC-UV	High performance liquid chromatography – ultraviolet detection

ICU	Intensive care unit
IV	Intravenous
LC	Liquid chromatography
LC- MS/MS	High performance liquid chromatography tandem mass-spectrometry
LLOQ	Lower limit of quantification
LOD	Limit of detection
LTS	Long term storage
MIC	Minimum inhibitory concentration
MRM	Multiple reaction monitoring
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MWCO	Molecular weight cut-off
NONMEM	Non-linear mixed effects modelling
OFV	Objective function value
PD	Pharmacodynamics
PDR	Pan-drug resistant
PK	Pharmacokinetics
QC	Quality control sample
SOFA	Sepsis-related organ failure assessment
t1/2	Terminal elimination half-life
TID	Three times daily
t_{max}	Time to reach C_{max}
ULOQ	Upper limit of quantification
US FDA	United States Food and Drug Administration

UTI	Urinary tract infection
VAMS	Volumetric absorptive microsampling
Vd	Volume of distribution
VRE	Vancomycin-resistant enterococci

Part 1

Introduction

Chapter 1: Introduction and literature overview

Antibiotic resistance is a significant and immediate global health concern [1]. An increasing prevalence of multi-drug resistant bacteria is steadily decreasing the number of antibiotics available that can be used in the effective treatment of infection. During the past decade the incidence of multidrug-resistant organisms has reached unprecedented levels [2]. The potential for global spread is illustrated by the dissemination of organisms with transmissible resistance genes for either the enzyme New Delhi metallobetalactamase-1 or *Klebsiella pneumoniae*-producing carbapenemase (KPC) [3]. The presence of this bacterial resistance mechanism renders our normal treatment options prone to failure [4].

The burden of infection in an intensive care unit (ICU) is directly linked to the mortality and morbidity of critically ill patients [5, 6]. The incidence of infection is associated with the life-saving medical interventions routinely practiced by ICU clinicians [7, 8]. Changes to the pharmacokinetics in critically ill patients have been noted and may impact on the antibiotic concentrations at the site of infection [9]. Consideration of such changes are required to optimise dosing and increase the likelihood of a positive outcome for the patient

Current techniques for optimising the use of presently available antibiotics have also highlighted the possibility of reinventing old drugs [10, 11]. Where old antibiotics may have been discarded because of serious adverse effect profiles or unfavourable bacterial susceptibility patterns, some are now re-emerging as genuine potential therapies with our evolving understanding of pharmacokinetics/pharmacodynamics of these agents.

1.1 Mortality and Morbidity of Critically Ill Patients

Infection is an important determinant of the outcome of critically ill patients, with the incidence of infection directly linked to patient mortality [6]. Epidemiological studies show sepsis is a leading cause of death in Australia as reported by the Australian and New Zealand Intensive Care Society Clinical Trials Group (ANZICS-CTG), with a burden that is three times the annual Australian road toll [12]. In patients with septic shock, mortality rates are close to 60% [12]. The ANZICS-CTG also reported in 2004 that 11.8% of all admissions to Australian and New Zealand ICUs are associated with severe sepsis with an associated in-hospital mortality of 37.5%. The Extended Prevalence of Infection in Intensive Care (EPIC II) study, performed in 2007 in 1265 ICUs in 76 countries found that 71% of patients were being administered antibiotics with mortality rates more than two-fold higher in infected patients versus non-infected patients [5]. This same study found that patients who were admitted in the ICU for longer periods of time had an increased risk of acquiring an infection.

1.2 Burden of Infection in the Intensive Care Unit

The World Health Organisation identifies health-care associated infections as the most frequent adverse event associated with health-care delivery worldwide. The significant mortality and financial losses to health systems resulting from health-care associated infections are immense [1]. The World Health Organisation reported that for every 100 hospitalised patients at any given time, 7 in developed countries, and 10 in undeveloped countries, would acquire at least one healthcare-associated infection. Nosocomial infection is associated with common medical interventions in ICU such as use of central venous catheters, urinary catheters, endotracheal tubes and arterial catheters. More frequent use of medically invasive procedures is required after a nosocomial infection occurs [13].

Nosocomial infections are a significant burden to the health system in Australia with an estimated 175 000 cases each year – with the extra length of hospital stay accounting for over 850 000 bed days [14]. Cardiovascular, respiratory, neurological and hepatic failure were all found to significantly increase in both frequency and duration in patients who experienced a nosocomial infection, with renal and haematological organ failures also increasing, although not significantly (likely due to the study's low patient numbers) [13]. In a prospective study of sepsis patients from 35 French ICUs it was found that most patients experienced two organ dysfunctions with the three most frequent organs affected being the respiratory, circulatory and renal systems [15]. This study identified that, when comparing patients with severe sepsis and one organ dysfunction and patients with severe sepsis and at least two organ dysfunctions the latter group had a longer length of stay (20.4 vs 11.6 days), the highest requirement for organ support (respiratory 92 vs 57%; circulatory 83 vs 31%; renal 25 vs 3%, respectively) and the highest mortality rate: ICU mortality (43% vs 6%), and hospital mortality (49% vs 11%) [15].

Nosocomial infections are associated with creating additional patient suffering and come at a high cost for patients, their families and the healthcare system through prolonged hospital stays, long-term disability, increased resistance to antibiotics, as well as the additional financial burden to patients and their families [1].

Infections in the ICU have an associated cost for the use of resources, nutritional support, antibiotics, and interventions for the presence of organ dysfunction. In a study conducted in Mexico it was found that for each nosocomial infection there was an attributable increased length of ICU length of stay of 5 days and an additional 5 days in hospital costing approximately USD$12,000 per patient [13]. The World Health Organisation estimates the financial loss due to health care-associated infections at approximately €7 billion in Europe and US$6.5 billion in the USA [1]. In

Australia policy makers allocate resources with a mean valuation of a bed-day in hospital of $1005 – based on this the gross economic burden of hospital acquired infection in Australia is AUD $940 million annually [14].

1.3 Increased Prevalence of Multi-Drug Resistant Organisms

Resistance to antibiotics results in bacteria being able to survive exposure to an antibiotic. Resistance can be caused by spontaneous genetic mutation or can be transferred between bacteria by conjugation, transduction or transformation; if several genes of resistance are carried in bacteria it is termed multi-drug resistant [16]. An increasing prevalence of multi-drug resistant bacteria has been seen over the past decade and is steadily decreasing the number of effective antibiotics that can be used. This increasing prevalence is thought to be due, at least in part, to the misuse and overuse of antibiotics by both doctors and patients, with availability of antibiotics uncontrolled in some countries and the use of antibiotics in animals as growth promoters [16, 17].

Of greatest concern are the ESKAPE organisms (an acronym for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species). Furthermore, a transmissible resistance gene for producing the enzyme New Delhi metallobetalactamase-1, is increasingly described and has prompted global concern as this enzyme confers resistance to many antibiotic agents [18]. This enzyme was initially discovered in a strain of *K. pneumoniae*, which on initial testing was found to be resistant to all antibiotics tested except colistin and tigecycline [19]. Also of concern for the development of resistance are the pathogens: *Staphylococcus spp.*, *Escherichia coli*, *Stenotrophomonas maltophilia* and *Streptococcus pneumoniae* [16].

The EPIC II prevalence study, with data from 1265 participating ICUs, found 70% of infected patients had positive microbial isolates (some with more than one pathogen isolated): 47% were Gram-positive, 62% were Gram-negative, and 19% fungal. The most common Gram-positive organism was *S. aureus*, the most common Gram-negative organisms were *Pseudomonas* spp., and *E. coli* [5]. This study also found that patients who had longer ICU stays had higher rates of infection; the infection rate increased from 32% for patients with an ICU stay of 0-1 day to more than 70% for patients with an ICU stay of more than 7 days ($P < 0.001$). Infections due to multi-drug resistant-organisms such as methicillin-resistant *S. aureus* (MRSA), *A. baumannii* and *Pseudomonas spp.* especially became more common [5].

Antibiotic resistance means that we are more likely to observe clinical failures and death from untreated infection. Where no antibiotic is available to treat a serious infection, clinicians are faced with supportive care measures only. Where multi-drug resistant pathogens are present, the

likelihood of transmission to other patients appears considerable [20]. Reports of multi-drug resistant bacteria are common in the USA, Europe, Asia-Pacific and Middle East regions. As described by World Health Organisation director-general, Dr. Margaret Chan in a keynote address in 2012, 'a post-antibiotic era means, in effect, an end to modern medicine as we know it' [21].

1.4 Decreased Availability of Novel Antibiotics

Infections with resistant bacteria have been identified since the 1940s, very soon after the advent of the early antibiotics [22]. Bacteria that are intrinsically resistant or acquire resistance, survive and replace the antibiotic-susceptible bacteria, thus providing selective pressure that increases the presence of resistant bacteria [20]. When the existing antibiotic armamentarium is reduced, there is a requirement to develop novel antibiotics that are safe to use with low adverse-event profiles. Traditionally, antibiotics have been derived from natural compounds isolated from fungi and bacteria, although there are a small number that have been synthesised for a specific structure known to be effective against bacteria (for example, quinolones).

The development of novel antibiotics is increasingly difficult, and comprehensive searches for new sources of natural products have been on-going for more than 70 years [23] and synthetic molecules must be bio-available at the site of infection. The development of new drugs is a complex and expensive scientific process, with new drugs then requiring pre-clinical and clinical testing before becoming available for treatment – and finally, the resulting drug represents a poor return on investment for pharmaceutical industry relative to other classes of drugs [24]. Only two systemic antibacterial agents were approved for use in humans by the U.S. Food and Drug Administration (U.S. FDA) between 2008-2012, compared to sixteen that were approved from 1983-1987 [24].

1.5 Improving the Use of Existing Antibiotics

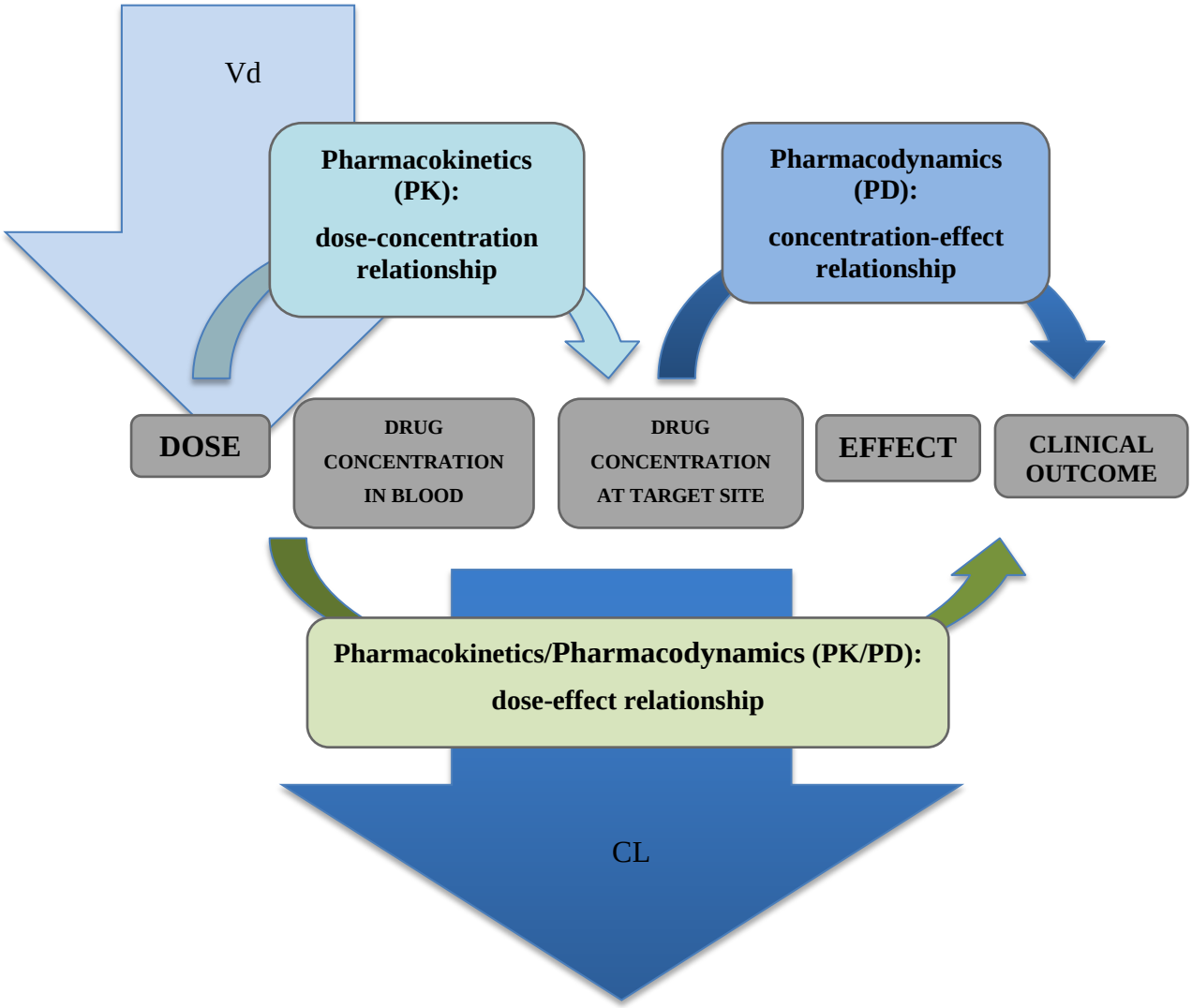
Effective use of antibiotics has a two-fold effect – it reduces the potential for bacteria to acquire resistance and improves patient outcomes [9, 25, 26]. Interestingly, these effects compete with a balance required between avoiding the unnecessary administration of antibiotics and providing essential treatment to patients [6].

In critically ill patients there are important pathophysiological changes that can affect antibiotic pharmacokinetics and complicate antibiotic dosing. Knowledge of the pharmacokinetic ('dose-concentration' relationship) and pharmacodynamic ('concentration-effect' relationship) properties of antibiotics is essential to ensure effective treatment.

Table 1.5-1 Important Pharmacokinetic/Pharmacodynamic definitions

Term	Abbreviation	Definition
Pharmacokinetics	PK	A description of the changes in concentrations of a drug in the body relative to the course of time
Pharmacodynamics	PD	A description of the relationship between drug concentrations and pharmacological effect
Volume of Distribution	Vd	The theoretical volume in which the total amount of drug would need to be uniformly distributed to produce the observed blood concentration of a drug
Clearance	CL	The volume of blood cleared of drug per unit time

Changes to important pharmacokinetic parameters, volume of distribution (Vd) and clearance (CL), of antibiotics in critically ill patients will affect the dosing requirements of the antibiotic [9]. In broad terms, the Vd determines the initial dose of the antibiotic, whereas the maintenance dosing is based on CL [27]. The importance of Vd, CL, pharmacokinetics and pharmacodynamics on antibiotic therapy are described in Figure 1.5-1.



Legend: Vd – volume of distribution; CL - clearance

Figure 1.5-1 The relationship between pharmacokinetics and pharmacodynamics

Adapted from Varghese *et al* [27, 28]

The hydrophilicity or lipophilicity of a molecule (i.e. the drug's affinity for water or lipids) can be used to characterise some of these pharmacokinetic principles – see Figure 1.5-2

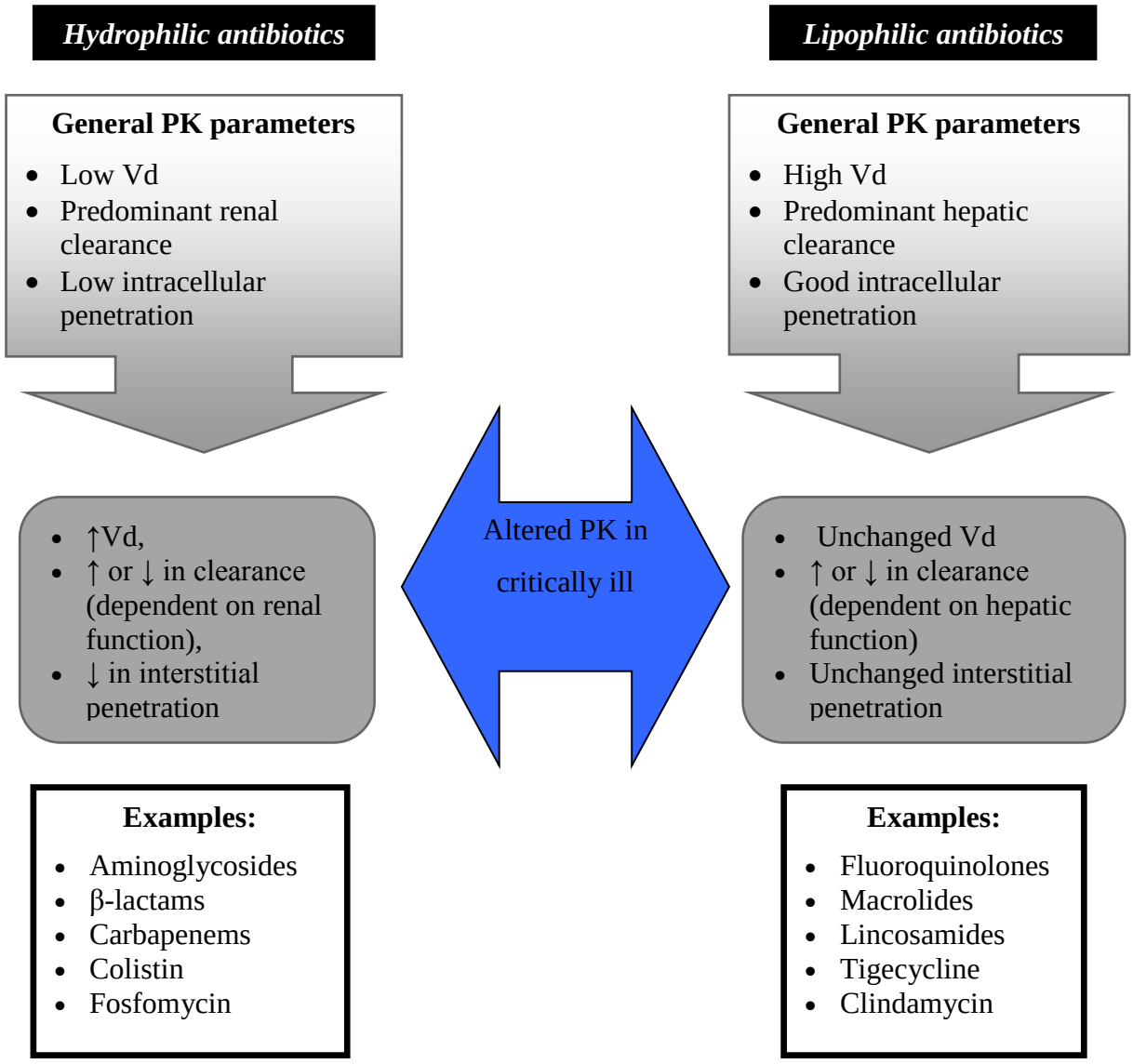
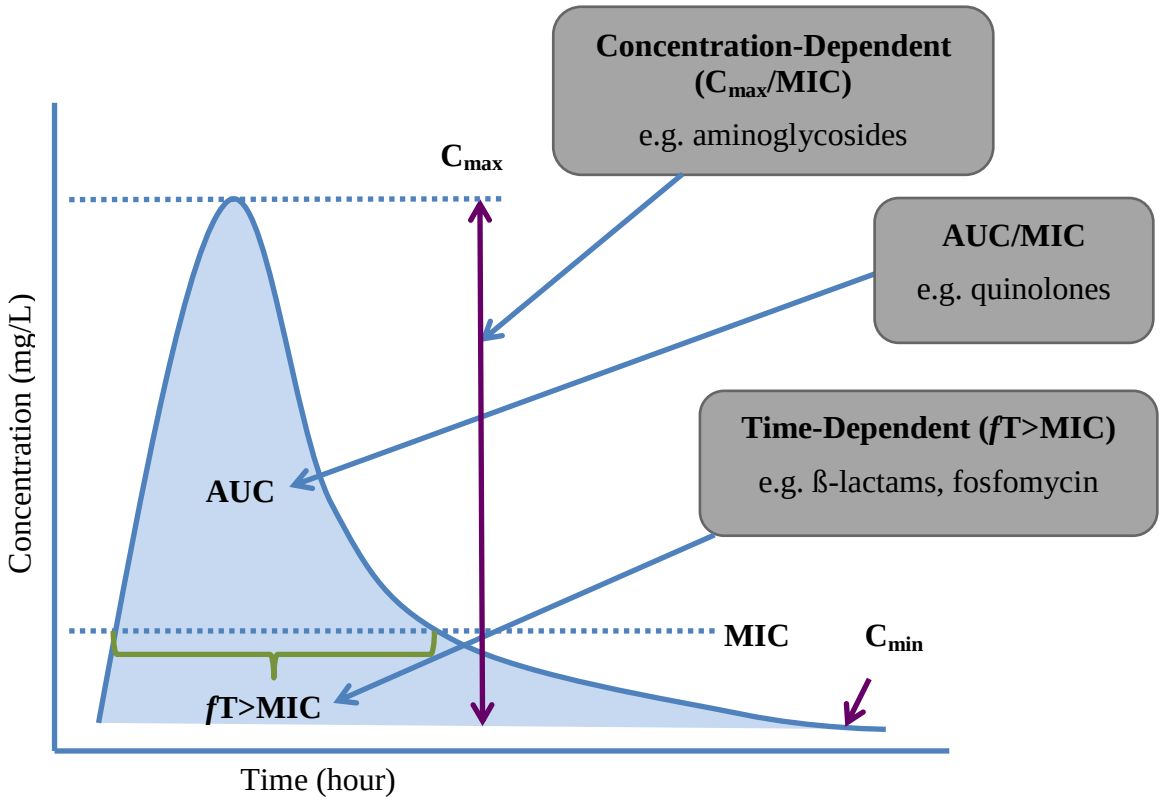


Figure 1.5-2 The association between antibiotic physicochemical characteristics and possible pharmacokinetic parameters alteration during critical illness

Adapted from Jamal *et al* [28]

The solubility of antibiotics is an important consideration alongside pharmacokinetic parameters as the solubility can help predict possible pharmacokinetic changes during critical illness. The bacterial kill-characteristics of antibiotics (the pharmacokinetic/pharmacodynamic relationship) also relate the ability of the antibiotic to eradicate or inhibit the growth of an infective organism [9]. Different antibiotic classes have been shown to have different kill-characteristics against bacteria (see Figure 1.5-3) and these must be considered to ensure any dosing adjustment achieves the optimal pharmacokinetic exposure given the susceptibility of the organism to the antibiotic - as described by the minimum inhibitory concentration (MIC)).



Legend: C_{max} – maximum concentration; MIC – minimum inhibitory concentration; AUC – area under the concentration-time curve; *f*T – time interval above the MIC; C_{min} – minimum concentration

Figure 1.5-3 Kill-characteristics of bacteria on a concentration vs. time curve

Adapted from *Roberts and Lipman* [9].

Concentration-dependent antibiotics kill bacteria maximally when the peak concentration (C_{max}) of the antibiotic is significantly greater than the MIC (C_{max}>>MIC) of the pathogen. Examples of concentration-dependent drugs are the hydrophilic aminoglycosides. Patients with a decreased C_{max} (resulting from an increased Vd which can result from aggressive fluid resuscitation) need larger than standard doses to ensure the desired C_{max} is achieved.

Time-dependent antibiotics have maximal bacterial killing when the free (unbound) concentration of the antibiotic is maintained above the MIC of the pathogen throughout the dosing interval (*f* $T_{>MIC}$). Examples of time-dependent antibiotics are the beta-lactams, including the carbapenems. Some pathophysiological changes, for example, hypoalbuminaemia, can result in altered pharmacokinetic which can result in normal doses of time-dependent antibiotics being suboptimal.

Antibiotics that are dependent on both concentration and time for efficacy have a pharmacokinetic/pharmacodynamic target described by the area under the concentration-time curve (AUC), a parameter which describes the cumulative systemic exposure to the drug. It is dependent on both the dose administered and the rate of elimination. The pharmacokinetic/pharmacodynamic target is the ratio of the AUC to the MIC (or AUC/MIC). Examples of antibiotics that are both concentration and time dependent are the quinolones and glycopeptides. As the kill-characteristics of these antibiotics are dependent on both time and concentration they are highly susceptible to variations in both Vd and CL in the critically ill because of the altered concentration-time profiles in these patients [27].

Dosing of antibiotics also requires consideration of toxicity. Some aminoglycosides commonly cause nephrotoxicity [29]. Some beta-lactams can cause neurotoxicities, like seizures [30]. Amoxicillin-clavulanate, flucloxicillin and nitrofurantoin can cause hepatotoxicity [31]. These toxicities are potential causes of morbidity and mortality, and can be confounded by both the infection being treated and medical interventions performed in an intensive care unit [31].

Based on the altered pharmacokinetics in critically ill patients suboptimal dosing or overdosing can result in therapeutic failures and poor clinical outcomes. The aim of effectively managing antibiotic dosing is to ensure the pharmacodynamic response to the treatment is maximised including minimising of the emergence of antibiotic resistance. New pharmacokinetic/pharmacodynamic techniques that optimise the use of current drugs have also provided approaches for reinventing old drugs. Where old antibiotics may have been discarded for serious adverse event profiles (for example, colistin was initially abandoned due to reports of serious and common neuro- and nephro-toxicity [32]) or unfavourable susceptibility patterns (for example, fosfomycin was abandoned due to difficulties in finding a formulation that was bioavailable and safe [33], as well as difficulties in

interpreting susceptibility to various bacteria until it was found the addition of endogenous glucose-6-phosphate (G-6-P) was required to achieve membrane lysis [34]) these and other older antibiotics are re-emerging as serious potential agents if considered alongside a pharmacokinetic/pharmacodynamic profile. This may provide options for ICU clinicians as they investigate the 'off-label' use of these drugs for critically ill patients for treating infections caused by multi-drug resistant pathogens.

1.6 Pharmacokinetic Data of Antibiotics for Treating Multi-Drug Resistant Infections in Critically Ill Patients

Despite multi-drug resistant infections being a serious, worldwide health threat there is a paucity of pharmacokinetic data on the use of antibiotics in critically ill patients. This information is crucial for determining optimal dosing strategies to maximise pharmacodynamic response in critically ill patients while minimising the potential for antibiotics to develop resistance.

1.7 Difficulties Associated with Pharmacokinetic Studies

Performing pharmacokinetic studies requires highly specialised scientific equipment and research expertise to ensure safe and minimally invasive procedures are practiced and robust data is acquired. There are difficulties in obtaining data for infections from multi-drug resistant bacteria, as hospitals capable of pharmacokinetic studies do not necessarily have the prevalence of multi-drug resistant infections in their hospital. The World Health Organisation has determined that the proportion of patients with ICU-acquired infections is almost three times higher in low- or middle-income countries compared to high-income countries. In some developing countries the occurrence of infection associated with invasive devices can be up to 19 times higher than those reported from higher income countries like Germany and the USA [1]. Given the prevalence of multi-drug resistant organisms and limited resources available to assess the suitability of dosing and optimise the therapeutic outcome to antibiotics there is a significant onus on the scientific community to offer collaborative research to what is emerging as a crisis without borders.

Chapter 2: The Effect of Altered pathophysiology of critically ill patients on pharmacokinetics

2.1 Synopsis

The aim of this chapter is to further describe the effect of ICU-related altered pathophysiology on the pharmacokinetic parameters of antibiotics in critically ill patients, using fosfomycin as an example antibiotic. This chapter will also describe how these changes can impact fosfomycin concentrations at the site of infection, which, in turn, may potentially reduce its bactericidal activity. Furthermore, this Chapter will examine the effectiveness of current fosfomycin dosing strategies in critically ill patients.

2.2 Published manuscript entitled: What is the relevance of fosfomycin pharmacokinetics in the treatment of serious infections in critically ill patients? A systematic review

The manuscript entitled, "What is the relevance of fosfomycin pharmacokinetics in the treatment of serious infections in critically ill patients? A systematic review" has been accepted for publication by the International Journal of Antimicrobial Agents (42 (2013) 289-293).

The co-authors contributed to the manuscript as follows: the PhD candidate, Suzanne Parker, under the supervision of Prof Jason Roberts, performed all literature reviews and analysis. The PhD candidate, Suzanne Parker, took the leading role in manuscript preparation and writing. Despoina Koulenti reviewed and edited the manuscript. Prof Jason Roberts, Prof George Dimopoulos and Prof Jeffrey Lipman oversaw all aspects of this paper.

The manuscript is presented as published; except figures and tables have been inserted into the text at slightly different positions. Also, the numbering of pages, figures and tables has been adjusted to fit the overall style of the Thesis. The references are found alongside the other references of the Thesis, in the section 'Bibliography'.

Google scholar citations: 22

Elsevier citations: 15

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What is the relevance of fosfomycin pharmacokinetics in the treatment of serious infections in critically ill patients? a systematic review

Suzanne Parker[1], *Jeffrey Lipman*[1,2], *Despoina Koulenti*[1,2,3], *George Dimopoulos*[3] , *Jason A. Roberts*[1,2]

[1]Burns, Trauma and Critical Care Research Centre, The University of Queensland, Brisbane, Australia, [2]Royal Brisbane and Women's Hospital, Brisbane, Australia,
[3] Attikon University Hospital, Athens, Greece

Address for correspondence:

Suzanne Parker
Burns, Trauma and Critical Care Research Centre
Level 7, Block 6,
Royal Brisbane and Women's Hospital
QLD, 4029
Phone no: + 61 (7) 3346 5104; Fax no: +617 3646 3542
Email address: suzanne.parker@uq.edu.au

Abstract

As treatment options for critically ill patients with multi-drug resistant bacteria diminish, older antibiotics, such as fosfomycin, are being investigated for use as last-resort drugs. Fosfomycin is a broad-spectrum antibiotic with antibiotic activity against both Gram-positive and Gram-negative bacteria. The aim of this review is to examine the effectiveness of current fosfomycin dosing strategies in critically ill patients. These patients can be subject to pathophysiology that can impact antibiotic pharmacokinetic profiles and potentially the effectiveness of their treatment. As a hydrophilic drug with negligible protein binding, fosfomycin is eliminated almost entirely by glomerular filtration and subject to patient renal function. If altered as is seen in augmented renal clearance, renal function in a critically ill patient may lead to low blood concentrations and predispose patients to a risk of treatment failure. If altered as seen in acute kidney injury, toxic blood concentrations may develop. Fosfomycin has volume of distribution comparable to beta-lactams and aminoglycosides and may therefore increase in critically ill patients. Altered dosing strategies may be required to optimise dosing given these pharmacokinetic changes, although the current paucity of data on fosfomycin in critically ill patients prevents accurate dosing guidance being recommended at this time.

2.2.1 Introduction

The escalation of antibiotic resistance is a significant and immediate global health concern. An increasing prevalence of multi-drug resistant Gram-negative bacteria is steadily reducing the number of antibiotics available that can be used as an effective treatment for many infections. Whilst serious Gram-negative infections are commonly treated with carbapenem antibiotics, resistance caused by bacteria, such as the increasingly described New Delhi Metallobetalactamase, means that our normal treatment options will result in an increased likelihood of treatment failure [4, 35].

Fosfomycin is a broad spectrum antibiotic with bactericidal activity against Gram-negative and Gram-positive bacteria. It was first discovered in Spain in 1969 and was used for the treatment of urinary tract infections [36]. Fosfomycin is structurally unrelated to other antibiotics: it is a small (138 Da), highly hydrophilic phosphonic acid and, with negligible protein binding [35], exhibits excellent penetration into tissue [37]. The bacterial killing mechanisms of fosfomycin is to inhibit the synthesis of peptidoglycan found in the inner cell wall of bacteria. Fosfomycin is very well tolerated with only minor adverse effects, with hypokalemia being the most frequent [38]. These characteristics support the effectiveness of fosfomycin for the treatment of multi-drug resistant pathogens, as a last-line antibiotic and as such, it has been used extensively in critically ill patients in some countries in these circumstances [39].

Despite its favorable chemical characteristics, there has been some debate over what appears to be a rapid development of *in vitro* resistance with monotherapy with fosfomycin [40]. However, in countries where fosfomycin has been used in clinical practice for many years, little change in the resistance patterns has been described [34]. Resistance may also be driven by low drug exposures as has been demonstrated for quinolones and beta-lactams [41] especially in association with what can be the relatively long duration of treatment with fosfomycin [39].

As a treatment reserved for the critically ill, pharmacokinetic changes of fosfomycin can significantly impact concentrations at the site of infection and as such dose alterations may be required to ensure optimal exposures are achieved. The aim of this paper is to examine the published data describing the pharmacokinetic of fosfomycin in critically ill patients and to review the effectiveness of current dosing strategies.

2.2.2 Methodology

2.2.2.1 Literature Search

We systematically searched Web of Knowledge and PubMed databases up until October 2012. The keyword searches were fosfomycin OR phosphomycin OR phosphonomycin AND critical care OR intensive care OR sepsis with a search limit of English language papers only. The bibliographies of relevant articles were also reviewed. Studies were selected if they included pharmacokinetic data on fosfomycin as well as a review of current microbiological or clinical data to report a summary of current clinical practice of fosfomycin and susceptibility patterns (Figure 2.2-1).

2.2.3 Pharmacokinetics in ICU patients vs. non-ICU patients

The effect of pathophysiological changes in critical illness on pharmacokinetic can be described via the changes that occur to pharmacokinetic parameters, specifically to CL and V_d. Such changes can impact fosfomycin concentrations at the site of infection, which, in turn may potentially reduce its bactericidal activity.

2.2.3.1 Clearance

CL of hydrophilic renally cleared antibiotics, such as fosfomycin, is subject to changes in patient renal function which may vary dynamically in critically ill patients. Being hydrophilic and with negligible protein binding (~0%, [42, 43]), elimination of fosfomycin is almost entirely via glomerular filtration [44] and total clearance of fosfomycin is highly correlated with glomerular filtration rate (as measured by creatinine clearance) [42].

An increase in CL is likely to lead to reduced blood concentrations of fosfomycin thereby exposing the patient to the risk of treatment failure. As seen in Figure 2.2-1, there is a scarcity of information on fosfomycin clearance in critically ill patients with impaired renal function. More research in this area is necessary to provide clinicians with evidence-based dosing strategies for rational adjustment of fosfomycin dosing.

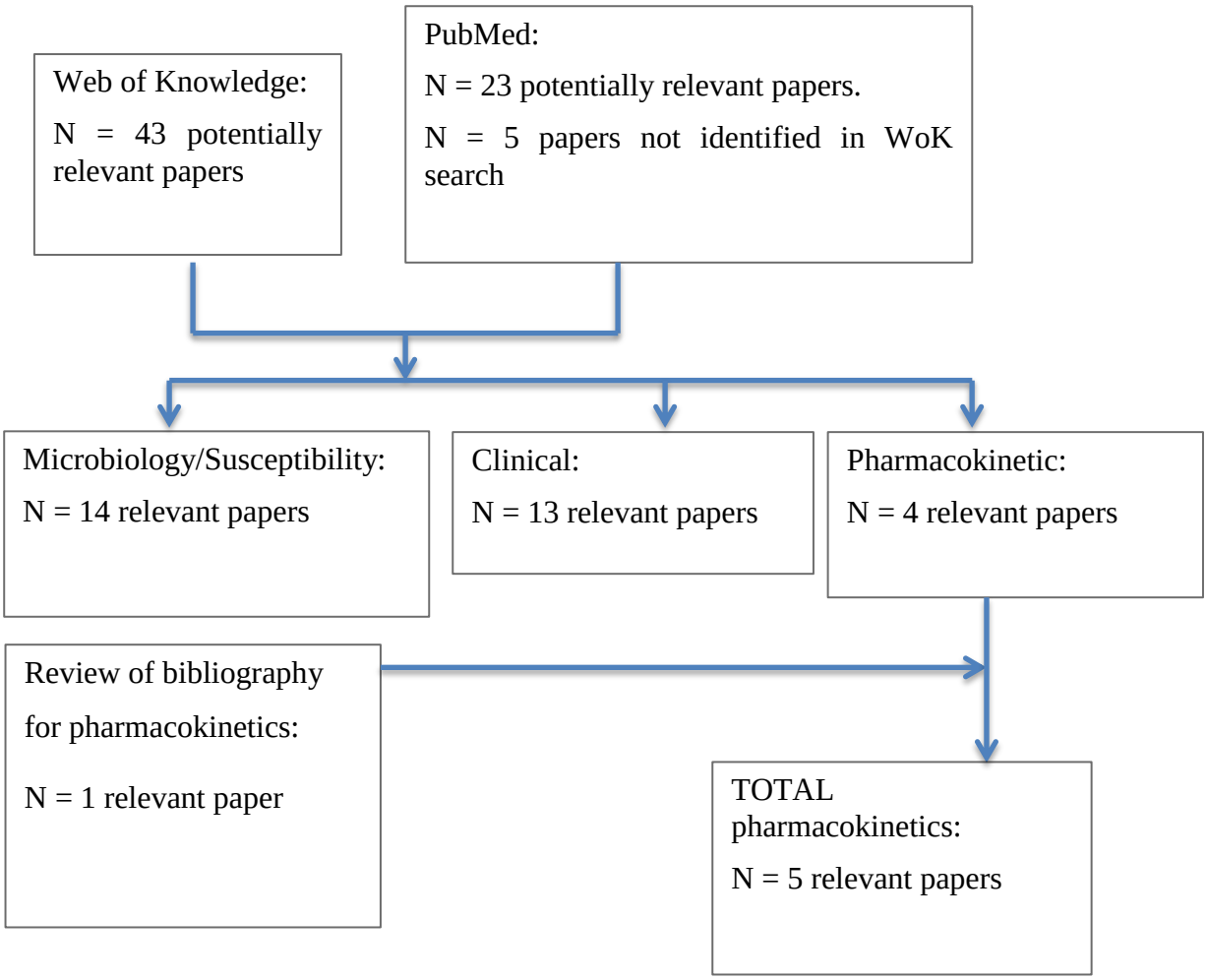

Figure 2.2-1 Summary of Literature Review

Table 2.2-1 Review of literature for pharmacokinetic data on ICU patients receiving fosfomycin

Reference	Study Population	Patient #	Dose	Pharmacokinetic Parameters					
				Vd (L)	$t_{1/2}$ (h)	CL (L/h)	C_{max} (mg/L)	t_{max} (h)	AUC (mg.h/L)
Bergan, et al. 1993 [45]	Healthy volunteers	12	3 g IV	20.6***	2.1 ± 0.1	6.8**	370.6 ± 92	0.02 ± 0	443.6 ± 48.9
Frossard, et al. 2000 [37]	Healthy volunteers	6	4 g IV	nd	nd	9.0** (serum)	97 ± 13 (muscle)		201.7 ± 56.7 (muscle)
							144 ± 1 (subcutis)		313.3 ± 43.3 (subcutis)
							202 ± 20 (serum)		$AUC_{(0-8)}$ 443.3 ± 41.7 (serum)
			8 g IV	nd	nd	9.0** (serum)	156 ± 16 (muscle)		460 ± 40 (muscle)
							209 ± 30 (subcutis)		596.7 ± 48.3 (subcutis)
							395 ± 46 (serum)		886.7 ± 70 (serum)
Gattringer, et al. 2006 [46]	Anuric ICU patients (CVVH)	12	8 g (cvc)	33.7 ± 12.7	12.1 ± 5.2	6.4 ± 7.6 (total)	442.8 ± 124.0	0.4 ± 0.1	2159.4 ± 609.8 $AUC_{(0-12)}$
						1.1 ± 0.2 (HF)*			

Joukhadar, et al. 2003 [47]	Sepsis	9	8 g IV	31.5 ± 4.5	3.9 ± 0.9	7.2 ± 1.3	357 ± 28 (plasma)	0.4 ± 0.1 (plasma)	$AUC_{(0-4)}$ 721 ± 66 (plasma)
					4.1± 0.6 (muscle)		247 ± 38 (muscle)	1.2 ± 0.2 (muscle)	$AUC_{(0-4)}$ 501 ± 69 (muscle)
Pfausler, et al. 2004 [48]	Bacterial ventriculitis	6	8 g IV	30.8 ± 10.2	3.0 ± 1.0	7.4 ± 2.3	260 ± 85 (plasma)	1.2 ± 0.4 (plasma)	929 ± 280 (plasma)
							43 ± 20 (CSF)	3.8 ± 1.8 (CSF)	225 ± 131 (CSF)
			8 g TID	26.3 ± 9.7	4.0 ± 0.5	5.0 ± 2.0	307 ± 101 (plasma)	1.5 ± 1.2 (plasma)	1035 ± 383 (plasma)
							62 ± 38 (CSF)	4.5 ± 2.7 (CSF)	295 ± 179 (CSF)
Pfeifer, et al. 1985 [49]	Perioperative neurosurgical patients	39	5 g IV	15.4	2	7.2	253 ± 108	0.25	
							9-10 (CSF)	3 - 6 h	
			10 g IV	nd	nd	nd	14-17 (CSF)	2 - 6 h	
			5 g IV TID	nd	nd	nd	32 (CSF)	2 to 5 days	
Matzi, et al. 2010 [50]	Sepsis	7	4 g IV	31.7***	2.5 ± 0.5 (plasma)	8.8**	243.3 ± 58.5 (plasma)	0.3 ± 0 (plasma)	AUC_{0-4} 453.0 ± 113.4 (plasma)

	Healthy lung	7		nd	2.2 ± 0.8[a]	nd	131.6 ± 110.6[a]	1.1 ± 0.4[a]	AUC_{0-4} 242.4 ± 101.6[a]
	Infected lung	5		nd	2.7 ± 1.5[b]	nd	107.5 ± 60.2[b]	1.4 ± 0.5[b]	AUC_{0-4} 203.5 ± 118.4[b]

Legend: nd: not described; ICU: intensive care unit; UTI: urinary tract infection; TID: dosing three times daily; AUC, area under the concentration–time curve; AUC_{0-4}, AUC_{0-8} area under the concentration–time curve from time 0 to 4 hours and 0 to 8 hours, respectively; Vd: volume of distribution; t1/2: terminal elimination half-life; CL: clearance; IV: intravenous infusion; C_{max}: maximum concentration; t_{max}: time to reach $C_{max;}$ CSF: cerebrospinal fluid; cvc: central venous catheter

[a] healthy lung

[b] infected lung

** calculated based on the approximation CL=dose/AUC

*** calculated based on the approximation that ke=0.693/t1/2=CL/Vd

* haemofiltration: CVVH clearance calculated with haemofiltration formula CLHF = (UFR · CUF)/CA, where UFR refers to the ultrafiltration rate and CUF and CA to ultrafiltrate and arterial (dialyser inlet) serum fosfomycin concentrations, respectively

2.2.3.2 Volume of Distribution

An increase in Vd of fosfomycin will prolong the half-life ($T_{1/2}$) and reduce the maximum concentrations during a dosing interval (C_{max}). Whilst a reduced C_{max} is not problematic, the time to reach therapeutic concentrations may be problematic.

Like most hydrophilic drugs, fosfomycin has a Vd consistent with extracellular body water (approx. 0.3 L/kg) in healthy volunteers [42]. Like aminoglycosides and beta-lactam antibiotics the Vd of fosfomycin is likely to increase in critically ill patients, which could result in a decreased C_{max}. As seen in Table 2.2-1 the C_{max} can be reduced by as much as 35% when compared to healthy patients [37, 48]. Increased Vd (by as much as 50%) is likely due to the pathogenesis of bacterial infections and resulting changes to the vascular endothelium with increased capillary permeability [51-53]. An increased Vd may suggest that the use of loading doses to ensure target concentrations are met are necessary where they may not traditionally be used [54].

2.2.3.3 Tissue Penetration

The apparent high permeability of fosfomycin manifests in favourable penetration of many tissue types including interstitial fluid of muscle, infected lung tissue, abscess fluid, heart valves, urinary bladder wall, prostate and cerebrospinal fluid [48, 50, 55-57].

Joukhadar et al. used the microdialysis technique to compare fosfomycin concentrations in the interstitial fluid of muscles with plasma concentrations in nine sepsis patients. The maximal concentrations of fosfomycin in plasma was similar to that found in the original study of healthy patients (357 and 395 mg/L, respectively). The concentrations found in muscle were higher in sepsis patients when compared to healthy volunteers (247 and 156 mg/L) which suggests sepsis may not affect penetration adversely with fosfomycin as has been found in other antibiotics (e.g. piperacillin, meropenem) [58-60], although the effect of microvascular failure on fosfomycin tissue penetration has not been fully investigated yet. The authors also found that muscle concentrations rapidly equilibrated with plasma in critically ill patients (within 80 minutes) which is important given that the interstitial fluid of tissues is typically the site of most infections [57].

Using microdialysis, Matzi et al. examined the ability of fosfomycin to penetrate both infected and healthy lung tissue in sepsis patients. The authors found that severe lung inflammation during bacterial pneumonia did not impair fosfomycin penetration. This supports the use of fosfomycin in achieving clinically relevant concentrations in severe pulmonary infection [50].

Another study by Pfeifer et al. in 39 patients examined fosfomycin pharmacokinetic in patients undergoing neurosurgical procedures resulting in cerebrospinal fluid drainage in the presence of an intact blood brain barrier. The authors concluded that there was adequate penetration of fosfomycin

in these patients for treatment of infections caused by susceptible organisms. Furthermore, the authors found increased cerebrospinal fluid penetration was observed in the presence of inflamed meninges [49]. Another study by Pfausler et al. also found the penetration of fosfomycin into the cerebrospinal fluid in six ventriculitis patients with extra-ventricular drainage was unaffected over 5 days, despite progressive healing and improvement of ventriculitis [48].

These studies support the favourable penetration of fosfomycin into tissues sites traditionally considered to be associated with low penetration thereby supporting its potential for use in many difficult to treat infection sites.

2.2.4 Pharmacodynamics

The bacterial killing of fosfomycin is categorised pharmacodynamically as time-dependent and as such maintaining concentrations above the MIC of the target pathogen for extended periods is required for optimal activity [44]. Static time-kill studies of fosfomycin tromethamine suggest it kills 99% of *Staphylococcus aureus*, *Escherichia Coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Citrobacter freundii* within 2.9 hours when fosfomycin concentrations are twice the MIC [61]. Increasing the concentrations to eight times the MIC slightly reduced this time to 2.2 hours [61], however this is not likely to be clinically significant. Bactericidal activity against *E.coli, Proteus mirabilis* or *K. pneumoniae* is seen at even 1 x MIC with no evidence of recovery by five hours of incubation [62].

When comparing results from studies reporting the oral formulation fosfomycin tromethamine with the intravenous formulation, fosfomycin sodium, it is important to be aware that the inactive salt component (tromethamine) of fosfomycin tromethamine contributes to almost half of dose reported (i.e. 1g fosfomycin tromethamine = 0.53g fosfomycin and 0.47g tromethamine). In intravenous fosfomycin (as a sodium salt) 76% of active material is fosfomycin. This is of relevance where interpreting what doses of drug should be used based on the formulation to be administered and the study used. For example, a 6g IV dose of fosfomycin sodium would contain 76% of 6g of fosfomycin, or 4.56g of fosfomycin.

2.2.4.1 Relevance of Microbiology data

The EUCAST MIC breakpoints for fosfomycin IV for *Enterobacteriaceae spp*. and *Staphylococcus sp*. are 32 mg/L (irrespective of site of infection) while there is insufficient evidence to nominate breakpoints for *Enterococcus spp*., *Streptococcus pneumoniae*, *Haemophilius influenza*e, and *Moraxella catarrhalis.* For *Pseudomonas spp*. anecdotally, these should be considered resistant to

fosfomycin and would require treatment with another agent or fosfomycin as part of combination therapy.

A considerable difference exists in the definition of breakpoints for fosfomycin by various breakpoint committees and professional societies and this may be due to the susceptibility testing procedures. *In-vitro* studies have found the activity of fosfomycin is potentiated by the presence of the physiologically available compound glucose-6-phosphate (G-6-P) and the addition of G-6-P produces results that more closely correlate to the *in vivo* activity of fosfomycin [44].

Fosfomycin also exhibits a high level of spontaneous bacterial mutation rates when tested *in vitro* [63]. However, little resistance to fosfomycin has been described in countries where fosfomycin has been used routinely [64, 65] suggesting that *in vitro* data may not correlate with clinical observations. There is, however, a recent report of three ICU patients developing resistance to fosfomycin during *K. pneumoniae* infections associated with Klebsiella producing Carbapenemase (KPC) when fosfomycin was used as adjunct therapy. Additional data is required to determine the role of fosfomycin in combination therapy for infections mediated by multi-drug resistant bacteria [66].

2.2.4.2 Interpretative Pharmacokinetics/Pharmacodynamics

Fosfomycin has been used in the past for the treatment of a variety of infections (urine, CNS, neurosurgical, etc.) due to multi-drug resistant pathogens where doses until 12 gr/daily were added. During the last years, because of the increasing incidence of multi-drug resistant /pan-drug resistant infections in critically ill patients a dose of 24 gr/daily was considered adequate in order to treat these infections.

The Joukhadar et al. study into tissue penetration of fosfomycin found the concentrations in the interstitium and plasma remained above 70 mg/L during a 4 hour observation period. However, with a half-life of 2.5+ hours (as has been found in critically-ill patients) and an MIC of 32 mg/L, twice daily dosing is unlikely to provide adequate concentrations of fosfomycin in interstitial fluid of some tissues [58]. This highlights the need for further investigations into dosing with fosfomycin, particularly where altered pathophysiological changes, are impacting on drug distribution.

Supporting the need for further investigations are the studies by Matzi et al. 2010 and Pfausler et al. 2004 [48, 50]. Matzi also found that while the single IV dose of 4 g administered was suitable for pathogens with an MIC of up to 32 mg/L there was considerable inter-patient variability in tissue and plasma pharmacokinetic profiles, and hypothesised that there was a potential risk of under-dosing individual patients [50]. Pfausler found that penetration of fosfomycin into the cerebrospinal

fluid at 8 g TID was potentially unsuitable for eradicating pathogens with an MIC_{90} of 32 mg/L with cerebrospinal fluid concentrations generally four-fold lower than observed in plasma [48]. In most of these studies the critically ill patients exhibited higher volumes of distribution and a high level of inter-patient variability than seen in non-critically ill patients.

From these studies and a review of the data available (see Table 2.2-1) a reasonable approach to dosing critically ill patients may be to provide frequent, higher doses of fosfomycin over the first 24-48 hours (to counter the increased Vd observed) and then to continue frequent but lower doses, based on estimates of creatinine clearance using urinary collection. Fosfomycin has a favourable safety profile and appears well tolerated [46] and this dosing schedule may be suitable. However, the data to categorically support this approach is not available and further investigations on dosing are required. There is also no strong data available in the literature on suggested duration of treatment for different sites of infection. An advanced description of the pharmacokinetic and pharmacodynamic parameters of fosfomycin in critically ill patients would ensure optimal dosing is achieved for successful therapeutic outcomes and to prevent the development of resistance.

2.2.5 Conclusion

This systematic review has found that there is limited information available on the pharmacokinetics of fosfomycin in the critically ill. The lack of data could be considered problematic given the serious nature of infections requiring use of this last line antibiotic. When comparing the pharmacokinetic data of healthy volunteers to critically ill patients, significant changes in the volume of distribution and clearance of fosfomycin are evident. This data emphasises that standard dosing, particularly in line with the earliest pharmacokinetic studies, is likely to be inappropriate for critically ill patients and that revised dosing strategies must be considered. A more advanced understanding of the pharmacokinetics and pharmacodynamics is clearly required to ensure optimal therapy for fosfomycin for critically ill patients.

2.2.6 Acknowledgements

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2.3 Conclusion

This Chapter has described the effect of ICU-related altered pathophysiology on the pharmacokinetic parameters of antibiotics in critically ill patients, using fosfomycin as an example antibiotic. Compared to healthy volunteers the pharmacokinetic data of critically ill patients, as evidenced by changes in the volume of distribution and clearance of fosfomycin, is significantly altered. These changes can impact on fosfomycin concentrations at the site of infection, which, in turn, may potentially reduce its bactericidal activity. These data emphasise that standard dosing is likely to be inappropriate for critically ill patients and a more advanced understanding of the pharmacokinetics and pharmacodynamics of fosfomycin in critically ill patients, combined with innovative dosing strategies, is required to ensure optimal therapy.

Chapter 3: Introduction to the Innovative Pharmacokinetic Sampling Techniques to Clinical Studies (from concept to application)

3.1 Innovative Pharmacokinetic Microsampling Techniques

A successful pharmacokinetic study requires the acquisition of a sample that is representative and, once acquired, has its integrity maintained. For a pharmacokinetic study, sampling requires information on the patient, as well as the time the sample was taken in relation to the dose of drug received. Sample acquisition requires careful record-keeping to ensure the data is representative of the patients' profile.

Samples are commonly taken in a variety of matrices, such as: whole blood, urine, cerebrospinal fluid and tissue. To create a plasma sample, by way of example, blood can be collected and transferred to a blood collection tube containing an anticoagulant. This is then mixed and, to prevent enzymatic degradation of the drug, may require being stored briefly on ice or combined with a stabilizing agent before being centrifuged with the plasma component then transferred and stored into a non-reactive tube suitable for storing at -80°C.

A scientific validation is performed to ensure the integrity of a sample is maintained during collection and storage, that the data derived from the sample represents an accurate result, and that the method is reliable and reproducible. The fundamental parameters of a bioanalytical validation investigation require adequate results of accuracy, precision, selectivity, sensitivity, reproducibility, and stability performed in matrix samples spiked with the analyte [67].

The collection, transport, processing, and storage of specimens pose unique challenges. The handling of samples prior to analysis should follow standardised procedures to ensure integrity [68]. An assessment of the impact of sample collection, transport and storage is required for a pharmacokinetic study. Any modification of these processes, that improves the assurance of the integrity of the sample, is highly sought by the scientific community.

Microsampling is a sampling technique in which a small volume (<50 μL) of a biological matrix is collected and then used in a quantitative bioanalytical analysis.

The microsampling techniques that are gaining prominence for use in clinical studies include:

1. Whole blood samples, often collected by thumb or heel prick, are spotted on absorbent filter paper and dried, to prepare dried blood spots (DBS, Section 3.2.1)
2. Whole blood samples can be collected on volumetric absorptive microsampling (VAMS) devices and dried (Section 3.2.2).

3. Whole blood can be collected onto Noviplex cards. Once applied the plasma is separated through a membrane from red blood cells (which are discarded), and dried onto a filter paper at a known volume.
4. Whole blood is collected, centrifuged to separate plasma, and the plasma is spotted on absorbent filter paper and dried, to prepare dried plasma spots (DPS, Section 3.2.3).
5. Urine is collected, spotted on absorbent filter paper and dried, to prepare dried urine spots.

There is considerable interest in applying these techniques to clinical applications such as pharmacokinetic studies [69, 70], newborn and infant screening [71, 72], microbiological screening [73], forensic and toxicology investigations [74-76] and therapeutic drug monitoring [77, 78].

Microsampling strategies can potentially allow less invasive sampling, small sample volumes, safer sample handling (viruses and bacteria are deactivated), simplified sample handling and laboratory analysis, and a potential cost saving for shipment and storage.

This Thesis includes a discussion of three of the microsampling techniques that have received considerable attention recently, DBS, VAMS and DPS, as well as a comparison of these techniques to the gold standard of sampling for clinical pharmacokinetic studies, liquid plasma sampling.

3.2 Microsampling vs. Traditional Pharmacokinetic Blood Sampling

Traditional pharmacokinetic studies require the frequent sampling of blood from a patient to create a concentration-time profile that reflects the distribution of the drug (see Figure 1.5-3). The process of preparing a plasma sample suitable for bioanalysis is described in Section 3.1 and it is a time-consuming, labour-intensive and expensive process. Minimally, it requires collection and preparation of the plasma sample. This is sometimes performed by two different persons and can consume a high level of human resources at a considerable cost when hundreds to thousands of samples are to be assayed. An overview of the challenges associated with microsampling, and how these compare to traditional plasma sampling, is presented in Table 3.2-1

Table 3.2-1 Overview of Microsampling Challenges

Collection and sample treatment	Plasma	DBS	VAMS	DPS
Gold standard for bio-analysis	✓			
Measure total drug concentrations	✓	✓	✓	✓
Measure unbound drug concentrations	✓			
Less-invasive sampling		✓	✓	✓
Low sampling volume (<100 µL)		✓	✓	✓
Simple sample handling		✓	✓	
Cost saving on transport and storage		✓	✓	✓
Simple extraction procedure		✓	✓	✓

3.2.1 Dried blood spots

One alternative to the traditional method of blood sampling is to use DBS. This technique allows sampling acquired from a finger-prick (or ear-lobe or heel -prick) of whole blood that is applied to an absorbent filter paper, known as sample collection paper, and either the entire sample or a sub-punch or 'spot' is removed from the centre of the sample for analysis. DBS is well-established globally for both screening metabolic disorders and HIV detection/monitoring in newborns [79], see Figure 3.2-1.

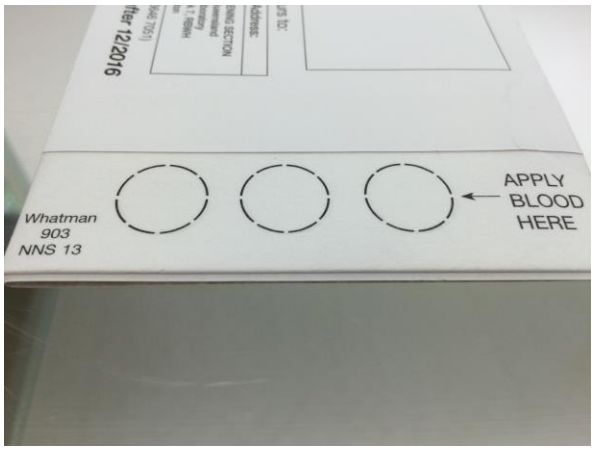

Figure 3.2-1 Dried blood spot sampling card

There is growing interest in the use of DBS in pharmacokinetic investigations as the sensitivity of analytical instrumentation (such as high performance liquid chromatography tandem mass spectrometry, HPLC-MS/MS) allows for assays with a limited volume of sample.

Some of the strengths of the DBS technique include less invasive sampling and a preparation that uses smaller sample volumes, a feature that has made it favourable for paediatric studies [79]. DBS can allow for safer sample handling (viruses and bacteria can be deactivated), simpler sample handling (no need for centrifugation, storage on ice, transfer of separated plasma or serum) and a potential cost saving for shipment and storage (no need for storage on dry ice during transport, no need to store in specialised freezers, all storage and transport is performed at ambient temperatures). These features are of particular interest for developing countries where suitable processing and storage facilities are not always available.

The primary weaknesses of DBS is that it has not been widely used for pharmacokinetic studies and therefore requires more validation testing than might be required for other sampling techniques. A major hurdle in the use of DBS is the impact of haematocrit on the sample. The variability of haematocrit levels has been identified to directly affect the spot size and homogeneity and can also cause variable recovery with differing matrix effects when used with mass spectrometry detection [80-84]. Haematocrit influences the viscosity and, thus, the distribution or 'spread' of whole blood on DBS cards [81, 82]. High haematocrit samples have high viscosity, resulting in a poor spread of blood and the formation of a relatively small spot [85]. Conversely, low haematocrit samples (as is often exhibited in critically-ill patients, as these patients can commonly experience anaemia and haemodilution [86] and is also relevant to developing countries) can result in increased spread and the formation of a relatively large spot.

The repeatability of the assay requires validation by assessing precision and accuracy; particularly at the level of the LLOQ. As part of the validation process, the precision and accuracy need to be correlated against haematocrit levels. The impact of haematocrit on the accuracy and precision of the assay has been identified as the most important parameter influencing DBS assay performance [87].

Another aspect requiring consideration is that with a limited volume of sample available, the sensitivity of the assay needs to be assessed to determine the appropriateness of the LLOQ and whether it is sufficiently low to accurately characterise the full pharmacokinetic profile of the drug from the patient. Initially it was thought that DBS required 'no need for accurate sample collection' (based on the assumption that the drug would distribute itself evenly on the DBS card and this would correlate with sample volume), however, factors such as blood composition (which change in different patient populations) can impact on the distribution of the sample on the paper through altered viscosity and recovery [79]. Spot volume and homogeneity of the spot (or sub-punch), across a range of haematocrit levels, requires careful assessment [85]. Sub-punched DBS samples

have been reported as having a 30% variation in blood volume across a haematocrit range of 20 and 80% haematocrits [88]. Non-homogeneity of a DBS can affect the distribution of the analyte, with some DBS cards exhibiting lower analyte concentrations at the centre of the spot, while other DBS cards exhibit higher analyte concentrations at the centre of the spot [89].

Haematocrit has also been identified as influencing matrix effects, and a validation would require an assessment to determine if variable haematocrit influences the consistency and reproducibility of mass spectrometric bioanalytical assays [85] and whether signal enhancement or suppression is observed [81]. And finally, haematocrit has been identified as having an influence on the extraction recovery [79, 81, 85, 88], with some compounds the recovery from high haematocrit blood is reduced relative to lower haematocrit levels [79].

Plasma has been the mainstay matrix for the measurement of systemic drug concentration to describe pharmacokinetics [90]. From a physiological perspective, once a drug is absorbed its distribution is influenced by how well each organ is perfused with whole blood, organ size, binding of drug within blood and tissue, and the permeability of tissue [91]. Drugs in the blood are in equilibrium between drug "bound" to plasma proteins and "unbound" to plasma proteins, as well as the equilibrium between drug bound to the red blood cell and in the plasma. It may be argued that we should therefore be directly measuring the unbound (the pharmacologically active component) rather than total drug concentrations, although this is traditionally not the case [90]. For antibiotics in particular, knowledge of the pharmacologically active unbound concentration is necessary for more accurate characterisation of pharmacokinetics. Therefore, a significant weakness of DBS is that the microsampling technique is only able to measure total drug concentrations. This is problematic for drugs with protein binding because protein binding often varies between patients and between samples from the same patient. Furthermore, knowledge of the distribution of the drug between red blood cells and plasma would be required if the data is to be compared to previous studies conducted in plasma. The suitability of dried blood spot sampling for use in a pharmacokinetic study requires an understanding of the binding of the drug of interest to both red blood cells and plasma proteins.

One strategy for overcoming some of the haematocrit problems is to avoid using a sub-punch of the dried blood spot, instead simply analyse the whole, volumetric44ally applied spot [83, 92]. This can eliminate the issues of non-homogeneity and sample volume, however, variable recovery and matrix effects remain challenges. Measuring or predicting haematocrit can be used for verification of whether haematocrit falls within a validated range and for the correction of a haematocrit-induced bias [83, 92].

Other weaknesses that require consideration include where any drug-specific characteristics exist, such as unstable in light, prone to oxidization/hydrolysis, chemical instability (some pro-drugs or acyl-glucuronide metabolites) [79]. In those cases, a feasibility assessment should be made and validation of the method, with reference to the specific issues, undertaken.

Finally, the collection of microsamples using peripheral sampling, for example, by finger- or heel-prick, provides a potential difference from a sample acquired from venous sampling. Study design using peripheral sampling requires consideration of the time required for equilibration from the initiation of treatment to the distribution of the drug to muscle or tissue concentrations from which the peripheral sample is to be taken.

Microsampling presents bioanalytical challenges for measuring drug concentrations in pharmacokinetic studies. The appropriateness of microsampling compared to the gold standard of plasma sampling should be evaluated where possible using quantitative bioanalytical validation. The principle underlying this is whether the microsampling technique maintains the integrity of the original sample and if it represents an accurate surrogate of the original sample (see Section 6.3).

3.2.2 Volumetric absorptive microsampling

VAMS devices are a novel dried sample collection technique for quantitative bioanalysis [93]. The device consists of a sample handler similar in design and size to a pipette tip, attached to a porous, hydrophilic tip. Whole blood is absorbed onto the tip as a fixed volume (ca. 10 μL) through wicking, ensuring the sample collection has a consistent blood volume regardless of haematocrit and the homogeneity of sample [93, 94]. The tip is then dried and the VAMS device is available for storage and shipment (see Figure 3.2-2).

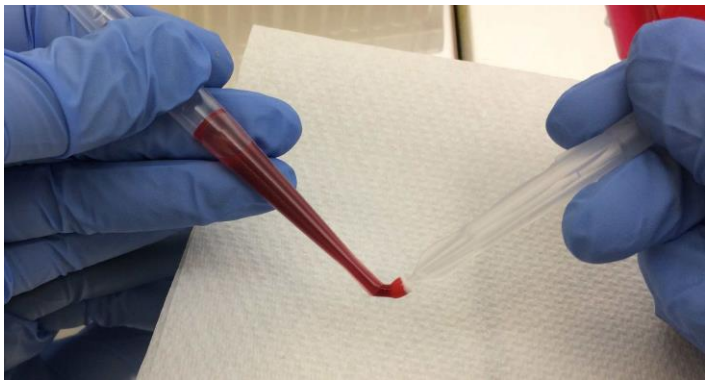

Figure 3.2-2 VAMS sampling technique

The VAMS sampling technique offers an alternative to DBS sampling, with both techniques providing advantages over traditional liquid plasma sampling, as described in Table 3.2-1. VAMS

devices offer an advantage over DBS sampling as a fixed volume of whole blood is applied to the tip of the VAMS device. The volume of blood absorbed by the VAMS tip has been assessed using radiolabelled ^{14}C caffeine, with an average volume of blood 10.5 ± 0.1 µL (precision 3.6%) across a range of haematocrit, 20% to 70% [93]. Precise and accurate sample volume obviates the haematocrit-associated non-homogeneity bias from sub-punching a DBS sample.

However, any haematocrit-related effects for matrix suppression or enhancement, or recovery must still be considered for influences on the consistency and reproducibility of mass spectrometric bioanalytical assays.

Like DBS, other weaknesses that require consideration for an assessment of the use of the VAMS devices include where any drug-specific characteristics exist, such as drugs that are: light sensitive, prone to oxidization/hydrolyzation, chemically unstabile (some pro-drugs or acyl-glucuronide metabolites) [79]. A feasibility assessment should be made and then testing for the impact of these characteristics in the validation of the method undertaken. Like DBS, the VAMS devices provide data on total drug concentrations only, and a determination of whether drug bound to plasma proteins is required.

3.2.3 Dried Plasma Spots

DPS samples are obtained by collecting plasma samples and applying these to sample collection paper (often the same paper as used in DBS) and drying it. The sample is then available for analysis or transport. This sampling strategy requires a longer clinical procedure where whole blood samples are centrifuged to obtain the plasma, however it removes any haematocrit-associated sampling bias. DPS microsampling provides the same dried sample advantages as DBS and VAMS devices over traditional liquid plasma sampling.

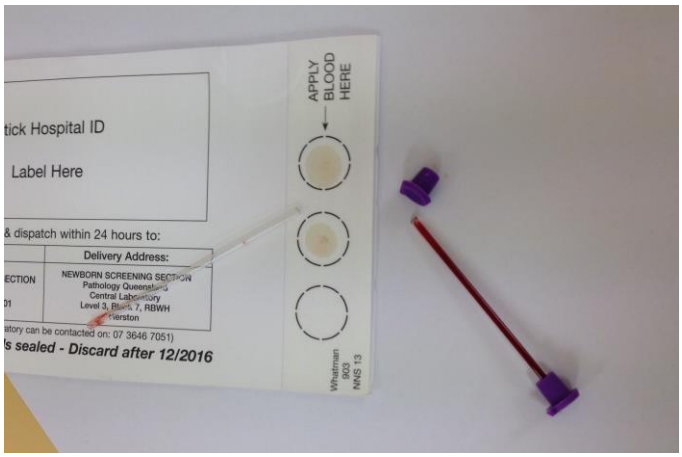

Figure 3.2-3 DPS sampling technique combined with capillary sampling

The removal of a haematocrit-associated bias from the dried plasma spot sample provides a distinct advantage over DBS and VAMS devices, particularly for clinical pharmacokinetic studies in critically-ill patients, where these patients can commonly exhibit low levels of haematocrit [86]. A study of 84 critically-ill patients to assess anaemia levels found an average peripheral haematocrit of 26.7% [95], compared to normal levels of haematocrit for men in the range of 38.8 to 50.0% and for women in the range of 34.9 to 44.5% [96]. Low haematocrit is associated with a lower blood viscosity which has been demonstrated to: increase spot size, decrease analyte response in a DBS sub-punch, affect recovery (with some compounds demonstrating reduced recovery at lower haematocrit, others increased recovery at lower haematocrit), and finally, affect the homogeneity of the spot (with more water soluble compounds and those with lower protein binding, following the migration of the water in the blood spot [81]).

DPS samples can be created as an adjunct sampling storage technique to traditional plasma sampling. Alternatively, the sample may be collected from a thumb or toe prick into a capillary tube (ca. 100 μL), centrifuged and then the capillary tube cut to separate the red blood cells from the plasma, with the plasma either analysed as a liquid sample or applied to sample collection paper [97-99]. Current investigations into the use of membrane filtration devices to directly form DPS samples from a spot of whole blood may also offer a simplification to dried plasma spot preparation [100, 101].

3.3 Application of Pharmacokinetic Microsampling Techniques

Microsampling techniques are an unproven and experimental sampling technique in the assessment of antibiotics in pharmacokinetic studies. As already discussed, if a microsampling technique is proven to be a robust and a reliable sampling technique, it may improve the convenience of pharmacokinetic studies.

A full assessment of a microsampling technique would require a holistic approach to assess the technique across a full validation protocol for an assessment of accuracy, precision, selectivity, sensitivity, reproducibility, and stability. This will be a requirement for each antibiotic, and include pivotal investigations on antibiotics that are known to have difficulties associated with their current analysis.

Validation protocols using microsampling are currently being discussed in the scientific literature as there are no specific regulatory guidelines for the quantitative determination of drugs in microsampling [85]. An overview of the validation requirements comparing microsampling techniques to the gold standard of plasma sampling is described in Table 3.3-1. This table has been compiled based on the regulatory requirements provided by the U.S. FDA draft guidelines, as

provided in the Guidance for Industry: Bioanalytical Method Validation document from September 2013, as well as current scientific literature on validation of microsampling techniques [79, 81, 85, 102-105]

Table 3.3-1 Summary of Bioanalytical Method Validation Requirements

Validation requirements	Plasma	DBS	VAMS	DPS
Intra-assay Accuracy (n=5) [b,*]	✓	✓	✓	✓
Intra-assay Precision (n=5) [b,*]	✓	✓	✓	✓
Inter-assay Precision (n=5) [b,≠]	✓	✓	✓	✓
Linearity[b,&] (n=6)	✓	✓	✓	✓
LLOQ[b] (n=5)	✓	✓	✓	✓
ULOQ[b] (n=5)	✓	✓	✓	✓
Matrix Effects[b]	✓	✓	✓	✓
Recovery[b,*]	✓	✓	✓	✓
Reproducibility (re-injection)[b]	✓	✓	✓	✓
Incurred sample reanalysis[b,%]	✓	✓	✓	✓
Sample dilution[c]	✓	✓	✓	✓
Stability[b] – benchtop (short term)	✓			
Stability[b] – frozen (long term)	✓			
Stability[b] – aqueous standard	✓	✓	✓	✓
Stability[b] – freeze/thaw	✓			
Stability[b] – autosampler	✓	✓	✓	✓
Stability[c] – room temp (long term)		✓	✓	✓
Stability[c] – dried, extreme (transport)		✓	✓	✓
Spot volume[c]		✓		✓
Spot homogeneity[c]		✓		
Spot-to-spot carry over[c]		✓		✓
Haematocrit[c] - recovery		✓	✓	✓
Haematocrit[c] – matrix effects		✓	✓	✓

[a] relative to the gold-standard of plasma sampling; [b] FDA Guidance for Industry, draft 2013; [c] Jager et al 2014; [*]performed at three concentration levels; [≠]performed over time and may include different

analysts, equipment, reagents; and at least four concentrations (LLOQ, low, medium, and high) performed in duplicate; [%]performed on 7% of study sample size.

As can be seen from Table 3.3-1 the traditional plasma validation requires stability testing for room temperature stability (short term), frozen storage (long term) and freeze-thaw stability, which are not requirements of dried micro-sampling techniques. Conversely, micro-sampling techniques require testing for stability at room temperature (long term) and at extreme temperatures to mimic the potential storage temperatures during transport. Current literature suggests extreme temperature stability considers high temperature and low temperature testing based on geographical location, as well as seasonal and transport conditions [85].

Both DBS and DPS require validation for spot volume if sub-punching is being performed. As haematocrit is removed from the DPS sample, the impact of a haematocrit-associated viscosity bias is greatly reduced. However, if the antibiotic is known to bind to red blood cells an assessment of the distribution in whole blood is required as changes to haematocrit may yield different plasma concentrations. Spot-to-spot carry-over during the sub-punch process must also be assessed. The impact of haematocrit on recovery and matrix effects for DBS and VAMS device is required with the challenges previously discussed in 3.2.1.

3.4 Conclusion

Investigations into the performance of microsampling techniques are required prior to their use in a clinical environment to determine their suitability for use – this is achieved by performing a quantitative bioanalytical validation. This chapter has provided an introduction to the concepts involved in the translation of innovative pharmacokinetic microsampling techniques into clinical studies. This chapter has described the potential and pitfalls of three microsampling techniques: DPS sampling, VAMS devices, and DPS sampling. Further to this has been an examination of the regulatory requirements provided by the U.S. FDA draft guidelines 2013 for quantitative bioanalytical validation, as well as current scientific literature on validation of microsampling techniques.

Part 2

Methods

Chapter 4: Improving Analytical Methodology for the Translation of Innovative Pharmacokinetic Sampling Techniques.

4.1 Synopsis

The aim of this chapter is to describe the methods used for the sample analyses performed in this Thesis. The development of analytical methodology for determination of antibiotic concentrations in plasma and urine, using LC-MS/MS allows the very small sample volumes because of the superior sensitivity of this instrumentation to traditional high performance liquid chromatography. Methodology using smaller sampling volumes facilitates translation of microsampling techniques from a clinical environment into laboratory methodology as part of a pharmacokinetic study. The development of analytical methodology using low sample volumes, and its subsequent validation, is an imperative for the successful translation of microsampling techniques into clinical practice.

4.2 Published manuscript entitled: A simple LC-MS/MS method using HILIC chromatography for the determination of fosfomycin in plasma and urine: application to a pilot pharmacokinetic study in humans

The manuscript entitled, "A simple LC-MS/MS method using HILIC chromatography for the determination of fosfomycin in plasma and urine: application to a pilot pharmacokinetic study in humans" has been accepted for publication by the Journal of Pharmaceutical and Biomedical Analysis (105 (2015) 39-45).

The co-authors contributed to the manuscript as follows: the PhD candidate, Suzanne Parker, under the supervision of Dr. Steven Wallis developed and validated the LC-MS/MS method and subsequently applied the method to the analysis of samples from critically ill patients in a clinical pharmacokinetic study. The PhD candidate, Suzanne Parker, took the leading role in manuscript preparation and writing. Prof Jason Roberts, and Prof Jeffrey Lipman oversaw all aspects of this paper.

The manuscript is presented as published; except figures and tables have been inserted into the text at slightly different positions. Also, the numbering of pages, figures and tables has been adjusted to fit the overall style of the Thesis. The references are found alongside the other references of the Thesis, in the section 'Bibliography'.

Google scholar citations: 7

Elsevier citations: 3

Journal of Pharmaceutical and Biomedical Analysis, impact factor: 2.979

A simple LC-MS/MS method using HILIC chromatography for the determination of fosfomycin in plasma and urine: application to a pilot pharmacokinetic study in humans.

Suzanne L. Parker[1,a], *Jeffrey Lipman*[a,b], *Jason A. Roberts*[a,b] , *Steven C. Wallis*[a]

[a] Burns, Trauma and Critical Care Research Centre,

The University of Queensland, Brisbane, Australia

[b] Royal Brisbane and Women's Hospital, Brisbane, Australia

[1]Author for correspondence: Tel: +6173346 5104

E-mail: suzanne.parker@uq.edu.au

Present address: School of Medicine, The University of Queensland, Herston, Queensland, AUSTRALIA

Key terms:

Fosfomycin; Antibiotic; LC-MS/MS; Pharmacokinetic; HILIC

Abstract

A high performance liquid chromatography - tandem mass spectrometry method, using hydrophilic interaction liquid chromatography (HILIC) chromatography for the analysis of fosfomycin in human plasma and urine, has been developed and validated. The plasma method uses a simple protein precipitation using a low volume sample (10 μL) and is suitable for the concentration range of 1 to 2000 μg/mL. The urine method involves a simple dilution of 10 μL of sample and is suitable for a concentration range of 0.1 to 10 mg/mL. The plasma and urine results, reported respectively, are for recovery (68, 72%), inter-assay precision (≤9.1%, ≤8.1%) and accuracy (range -7.2 to 3.3%, -1.9 to 1.6%), LLOQ precision (4.7%, 3.1%) and accuracy (1.7% and 1.2%), and include investigations into the linearity, stability and matrix effects. The method was applied to samples from a pilot pharmacokinetic study with a critically ill patient receiving IV fosfomycin, which measured a maximum and minimum plasma concentration of 222 μg/mL and 172 μg/mL, respectively, after the initial dose, and a maximum and minimum plasma concentration of 868 μg/mL and 591 μg/mL, respectively, after the fifth dose. The urine concentration was 2.03 mg/mL after the initial dose and 0.29 mg/mL after the fifth dose.

HIGHLIGHTS

- A simple and robust LC-MS/MS method for the quantification of fosfomycin in human plasma and urine has been developed.
- The method uses HILIC chromatography that supports a simple treatment of fosfomycin from biological fluids.
- The developed LC–MS/MS method has been validated according to published U.S. FDA guidelines and shows excellent performance.
- Results of a critically ill patient from a pilot pharmacokinetic study with receiving IV fosfomycin are included.
- This is the first method published that is suitable for the quantification of fosfomycin in both plasma and urine.

4.2.1 Introduction

Fosfomycin is a broad-spectrum antibiotic that is generating substantial interest as an intravenous or enteral therapy for the treatment of multi-drug resistant infections [64, 106]. Antibiotic resistance is a significant and immediate global health concern and an increasing prevalence of multi-drug resistant bacteria is steadily decreasing the number of usable antibiotics available [107]. In this context, fosfomycin represents an important treatment option.

Fosfomycin was first discovered in Spain in 1969 and is used as treatment therapy for uncomplicated urinary tract infections [36] and often combined with beta-lactams or aminoglycosides for a synergistic effect against *Pseudomonas aeruginosa* [108, 109]. Fosfomycin is structurally unrelated to other antibiotics: it is a small (138 Da), highly hydrophilic phosphonic acid (see Figure 4.2-1) and, with negligible protein binding [35], exhibits excellent penetration into tissue [37]. Fosfomycin has a unique mechanism of action by way of its ability to inhibit the synthesis of peptidoglycan found in the inner cell wall of Gram-negative and Gram-positive bacteria [110]. These characteristics support the effectiveness of fosfomycin for the treatment of multi-drug resistant pathogens and it has been used extensively as a last-line antibiotic for treatment of critically ill patients [106].

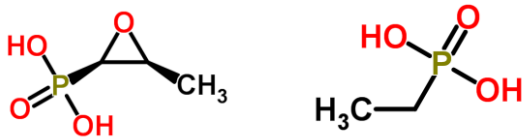

Figure 4.2-1 Structure of fosfomycin and the internal standard, ethylphosphonic acid [111, 112].

Investigating the pharmacokinetic/pharmacodynamic characteristics of fosfomycin may enable development of robust dosing strategies that maximise the pharmacodynamic response to treatment while minimizing the potential future development of antibiotic resistance. A reliable method of quantification of fosfomycin in plasma is needed to define the pharmacokinetic profile of the drug and urine data can provide valuable information on elimination rates. The information obtained can be used to characterise the pharmacokinetic/pharmacodynamic relationship.

There are several analytical techniques available for the determination of fosfomycin in biological fluids, using gas chromatography [113-117], liquid chromatography (LC) - spectrophotometric detection [118], LC - photometric detection [119], capillary zone electrophoresis [120, 121], and,

more recently, with derivatization and LC - atmospheric pressure chemical ionization mass spectrometry [122] and LC – tandem mass spectrometry (MS/MS) [123-125].

A method suitable for use in a pharmacokinetic study with patients receiving multiple intravenous doses, up to around 12-24 g/day (as are now being used clinically [106]) would require a LLOQ of around 1 μg/mL for plasma and 0.1 mg/mL for urine. However, patients with renal insufficiency or with altered pharmacokinetics – as is commonly seen in the critically ill – receiving multiple doses of antibiotics can exhibit very high concentrations in their plasma. This has, therefore, led to an unusually extended concentration range (from 1 to 2000 μg/mL for plasma) being described here, which is supported by the data from the pilot study. While there are many methods currently available for the analysis of fosfomycin in biological fluids, and the method by Li offers a rapid and sensitive alternative for plasma [123], we are unaware of any methods suitable for the analysis of fosfomycin in both plasma and urine that offer the concentration range to meet these clinical specifications at this time.

This paper describes an analytical technique using HILIC – tandem mass spectrometry that offers a simple and reliable determination of fosfomycin in plasma and urine, with a quick and reproducible sample preparation.

4.2.2 Experimental Section

4.2.2.1 Chemicals & materials

Fosfomycin (FOM), ethylphosphonic acid (EPA, internal standard) and acetonitrile (HPLC gradient-grade solvent) were purchased from Sigma-Aldrich and ammonium acetate was obtained from Ajax Univar. Ultra-pure water was obtained using a Permutit system. Drug-free human plasma was obtained from the Australian Red Cross Blood Service and drug-free urine was obtained from healthy volunteers.

4.2.2.2 Instrumentation & conditions

The LC-MS/MS used is a Shimadzu Nexera UHPLC equipped with a triple quadrupole 8030+ Shimadzu mass spectrometer (MS) detector. An electro-spray ionization (ESI) source interface operating in negative-ion mode was used for the multiple reaction monitoring (MRM) LC-MS/MS analysis. MS conditions for fosfomycin and the internal standard (IS) are reported in Table 4.2-1The interface settings consisted of the nebulizing gas flow of 3 L/min, a de-solvation line temperature of 250°C, heat block temperature of 400°C and a drying gas flow of 15 L/min.

The compounds were separated on a Merck SeQuant zic-HILIC, 2.1 x 50 mm, 5.0 μm analytical column (operated at 24° C) protected by a 20 mm SeQuant zic-HILIC guard cartridge using an

isocratic mobile phase containing acetonitrile with 2 mM ammonium acetate, pH 4.8 (85/15 v/v) at a flow rate of 0.3 mL/min. The injection volumes used were 0.1 µL for the plasma assay and 0.5 µL for the urine assay. The retention time for both fosfomycin and ethylphosphonic acid was 2.5 min.

Table 4.2-1 MS conditions for fosfomycin and ethylphosphonic acid

MS	FOM	EPA
Product Ion	137.1 (M.H-)	109.1 (M.H-)
Daughter Ion	78.9	78.9
Dwell Time (ms)	100	100
Q1 (V)	+14	+10
Q3 (V)	+28	+13
Collision Energy (V)	+25	+20

4.2.2.3 Stock and standard solution preparation

4.2.2.3.1 Standards for plasma analysis

Aqueous stock solutions for plasma standard preparation (at 1, 2 and 10 mg/mL) were stored at -80°C. On the day of assay these were diluted with drug free plasma to yield calibration standards from 1 to 2000 µg/mL that were processed alongside the clinical samples.

4.2.2.3.2 Standards for urine analysis

Aqueous stock solutions for urine standards of fosfomycin (2, 5, 10, and 50 mg/mL) were stored at -20°C. On the day of assay these were diluted with drug free urine to prepare calibration standards from 0.1 to 10 mg/mL that were processed alongside the clinical samples.

4.2.2.3.3 Internal standard solution

Ethylphosphonic acid in acetonitrile was used as internal standard for the plasma assay (10 µg/mL), and an aqueous ethylphosphonic acid solution was used as internal standard for the urine assay (1 mg/mL). Solutions were stored at -20°C.

4.2.2.3.4 Quality Controls

Quality controls were prepared by spiking drug free plasma with fosfomycin to concentrations of 3, 800 and 1600 µg/mL, and stored at -80°C until assay. On the day of assay an additional QC at 80

µg/mL was prepared by diluting with blank plasma the QC at 800 µg/mL. The four sets of QCs were assayed alongside clinical samples.

Quality controls for urine analysis were prepared at fosfomycin concentrations of 0.3, 2 and 8 mg/mL. The urine QCS were stored at -80°C until assayed alongside clinical samples.

4.2.2.4 Analytical procedure

4.2.2.4.1 Clinical plasma sample preparation

Clinical samples were prepared by combining 10 µL of clinical sample, 10 µL of water, and 90 µL of drug-free blank plasma. Internal standard (300 µL, 10 µg/mL of ethylphosphonic acid in acetonitrile) was then added and the sample vortexed and then centrifuged (for 5 min at 14,000 rpm) to remove precipitated proteins. A volume of 0.1 µL was injected onto the LC-MS/MS system.

4.2.2.4.2 Clinical urine sample preparation

All urine samples were filtered using a 0.22 µm filter prior to use. Clinical samples were prepared by combining 10 µL of sample with 10 µL water, followed by internal standard (20 µL, 1 mg/mL of ethylphosphonic acid). The sample was then diluted with 200 µL of mobile phase and 0.5 µL was injected into the LC-MS/MS system.

4.2.2.4.3 Data Analysis

For both plasma and urine the concentration of each clinical sample was obtained using the data from the calibration curve prepared (in either plasma or urine) within the batch using a quadratic regression with peak-area ratio (drug/internal standard area responses) against concentration (x), with a $1/x^2$ weighting as the mathematical basis of the quantification.

4.2.2.5 Method of Validation

The validation was performed in accordance with the guidelines provided by the U.S. FDA and met the criteria required to demonstrate the method is suitable for intended purpose [126]. The validation for both plasma and urine was assessed for matrix effects, LLOQ, linearity, inter-day precision and accuracy, freeze-thaw stability of quality control samples and the stability of standard solutions.

4.2.2.5.1 Limit of quantification

The lower limits of quantification for fosfomycin were evaluated by analysis of replicate standards, for both plasma and for urine samples.

4.2.2.5.2 Linearity

To investigate linearity, calibration curves were prepared using the corresponding concentration ranges suitable for each matrix.

4.2.2.5.3 Inter-day Precision and Accuracy

Precision and accuracy for fosfomycin throughout the calibration range of both plasma and urine was evaluated by the analysis of QC samples at four different concentrations for plasma and three different concentrations for urine with the QC concentrations determined against freshly prepared standard curves. In addition to precision and accuracy data obtained from QC samples, an incurred sample reanalysis was performed.

Acceptance criteria were applied according to the U.S. FDA guidelines [126]; with acceptance criteria on an incurred sample reanalysis applied according to the European Medicines Agency guidelines [127].

4.2.2.5.4 Matrix effects

Plasma matrix effects were evaluated to identify any suppression or enhancement of signal from an interfering substance around the retention time of fosfomycin and ethylphosphonic acid by using the matrix factor test. Five blank plasma samples were assayed at spiked low and high concentration levels and with internal standard, and the area results compared to those produced following the same extraction procedure using water instead of plasma. The precision of the matrix factor (normalised against the internal standard) was used to determine if any concentration level demonstrated a trend of variation from the expected result.

Five urine blanks were assayed at a spiked low and high concentration level and the precision and accuracy calculated, with respect to the nominal concentrations, to determine if any concentration level demonstrated a trend of variation from the expected result.

4.2.2.5.5 Recovery

The recovery of fosfomycin and ethylphosphonic acid was evaluated by comparing the peak area for plasma or urine samples spiked prior to protein precipitation (for plasma) or dilution (for urine) with samples spiked after protein precipitation or dilution. Care was taken to ensure the injection matrix was identical in comparable samples.

4.2.2.5.6 Stability

Stability of fosfomycin in plasma and urine was assessed across three freeze-thaw cycles (from -80°C to ambient temperature) using three replicates of the QC samples at low, medium and high

concentrations. Stability of stock solutions was assessed comparing aqueous solutions stored at both -20°C and -80°C to freshly prepared solutions.

4.2.2.6 Pharmacokinetic application

The method was developed for the analysis of plasma and urine samples from a pharmacokinetic study with critically ill patients receiving an intravenous dose of 6 g of fosfomycin every 6 hours with expected peak plasma concentrations of around 200 µg/mL, an expected plasma half-life of 2 h, and urinary concentrations of around 5 mg/mL [42].

One critically ill patient was administered an intravenous dose of 6 g fosfomycin disodium. Blood samples (3 mL) were taken prior to dosing (0 h) and 0.5, 0.75, 1, 1.5, 2, 4, and 6 h post administration using heparinised vacuum tubes (Greiner Bio-One, Vacuette® LiHep) on the first day of fosfomycin administration and after receiving the fifth fosfomycin dose. Blood samples were centrifuged at 3000 rpm (926 g) for 10 min to obtain plasma samples. Plasma samples were transferred into 2 mL polypropylene tubes, capped and stored at -80°C until analysis.

Similarly, a urine sample was collected 6 h post administration of the fosfomycin dose. The urine was transferred into a urine specimen vial, capped and stored at -80°C until analysis.

This procedure was conducted in accordance with the principles laid down by the ICH guidelines for Good Clinical Practice and approved by the University of Queensland Medical Research Review Committee (clearance # 2012000870) and the Epistimoniko Symvouleio (Scientific Committee) of Attikon University Hospital (approval ΜΕΘ-84/13-3-12).

4.2.3 Results & Discussion

4.2.3.1 Chromatography

This method has been established using HILIC technology which offers excellent selectivity for polar hydrophilic compounds like fosfomycin, and the use of a high organic-solvent content in the mobile phase leads to a rapid evaporation of the solvent during electrospray ionization [128], endowing the method with a simple compatibility with mass spectrometry. Additionally, the use of HILIC technology in this method obviates the requirement for further modification of the sample, after a solvent-based protein precipitation, to closely correspond with the organic content of the mobile phase for improved peak shape. This simplicity of extraction leads to low detection levels when using low sample volumes.

Retention of the analyte by the stationary phase is caused by hydrophilic partitioning within an aqueous-enriched liquid layer and/or with the positive or negative charges on the HILIC stationary

phase [129]. The balance of the partition is provided by the aqueous content and pH of the mobile phase. Therefore, the aqueous layer is critical to the efficiency of HILIC separation. Published HILIC methodology often recommends mobile phases consisting of high ionic strength (from 5 – 20 mM, with upper limits of 200-300 mM) [130] but this presented difficulties in development. The use of mobile phases with high ionic strength of buffers, low and variable aqueous content, combined with the high pressure applied in an HPLC system led to blocking of the column and the capillary in the ionization source on the mass spectrometer. This isocratic method uses a low ionic strength of 2 mM ammonium acetate buffer in 85% acetonitrile which has been reliable and provided consistent results with minimal loss to chromatographic shape and reproducibility. Regenerating the column after every 300 – 400 injections of samples, particularly the urine samples, and keeping buffer concentration to as low an ionic strength as possible, was advantageous to long term use. Using a guard column extended the column life but as the separation is highly dependent on the salt concentration and its impact on the stationary phases aqueous-rich layer, the regeneration of the column was critical to maintaining the quality of chromatography.

Another aspect of the method development that was found to affect both the chromatographic retention time and peak shape was the injection volume and the composition of the sample being injected. The retention time for plasma extracts was 2.4 min and for diluted urine was 1.3 min. Despite the low injection volumes being used, 0.1 µL for plasma and 0.5 µL for urine, we conclude that the changes in the retention times and quality of the chromatography observed may have been due to the sensitivity of the interaction of fosfomycin with the stationary phase to changes in ionic strength and pH of the buffer [128, 129], as well as the age of the analytical column. Considering each matrix separately, with each being validated separately several months apart, the robustness of the chromatography for each matrix is evidenced by very low variability in retention times (less than 1.0% relative standard deviation) as seen across the validation and clinical samples for each matrix. For the development of a bioanalytical assay using HILIC chromatography, consideration of the organic to aqueous ratio, concentration of salts, and finally, the presence of acids or base, is required. A dilution with plasma was used in the plasma method as the ratio of acetonitrile to aqueous concentration had an impact on the quality of the chromatographic peak shape and retention time; the concentrations found in clinical samples from the pilot study allowed this dilution. The urine method included a dilution of sample that improved the chromatographic separation by either reducing the presence of endogenous salts in the sample or controlling the pH.

Buszewski [128] and Alpert describe the mechanism of HILIC separation as being based on an interplay of a partitioning equilibrium in the aqueous layer (based on the hydrophilicity of the analyte), weak electrostatic mechanisms, and dipole-dipole interactions (including hydrogen-

bonding) [131] the impact of each parameter on the selectivity and reproducibility of chromatography requires a more sophisticated management than in general reversed-phase chromatography, but which once overcome can lead to a highly stable and robust method.

4.2.3.2 Validation

The LLOQ was determined as 1 μg/mL for plasma and 0.1 mg/mL urine with precision calculated as 4.7 and 3.1%, respectively, and accuracy calculated as 1.7 and 1.2%, respectively (Table 4.2-2).

Table 4.2-2 Lower limit of quantification

Matrix	Mean	Precision (%)	Accuracy (%)
Plasma (n = 13)	1.02 μg/mL	4.7	+1.7
Urine (n = 7)	0.10 mg/mL	3.1	+1.2

The signal to noise ratio of the lowest standard in the calibration curve was 23.2 for plasma and 178 for urine and this data, combined with the excellent precision and accuracy obtained at 1 μg/mL for plasma and 0.1 mg/mL for urine, suggests substantial scope for achieving a lower LLOQ for both matrices (see **Figure 4.2-2** and **Figure 4.2-3** for representative chromatograms of the LLOQ standard extracted from plasma and urine, respectively). The limit of detection (LOD) is defined as being reliably distinguished from the background noise and calculated as ≥ three-times the noise of the blank plasma sample. From the validation the LOD was determined as being approximately 0.01 μg/mL for plasma and <0.01 mg/mL for urine.

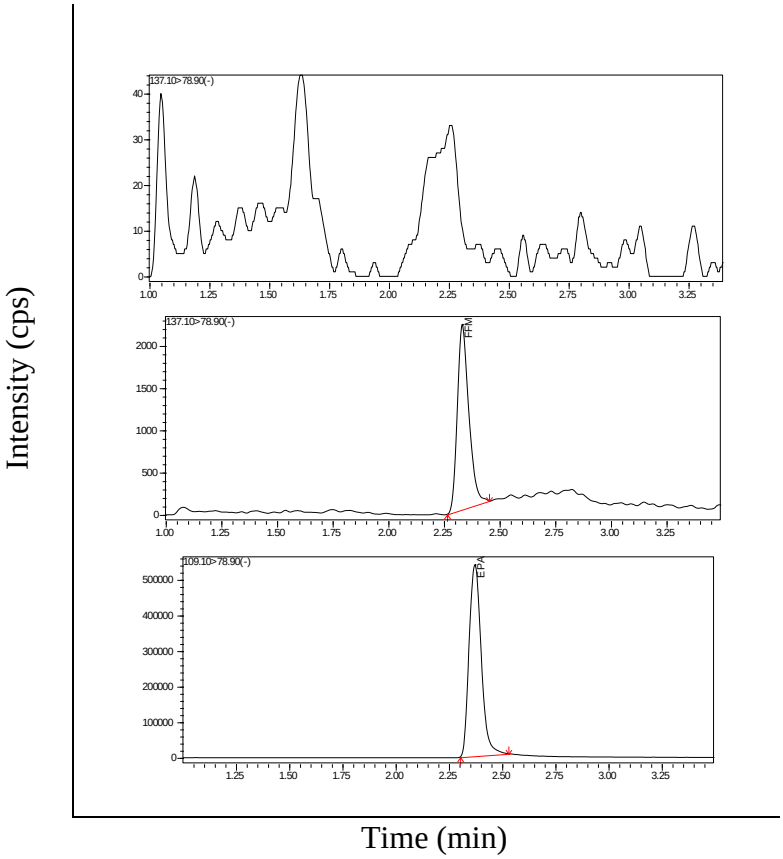

Figure 4.2-2 Chromatograms of a blank sample (top) and the LLOQ (1 µg/mL) plasma standard (FOM, centre; EPA, bottom).

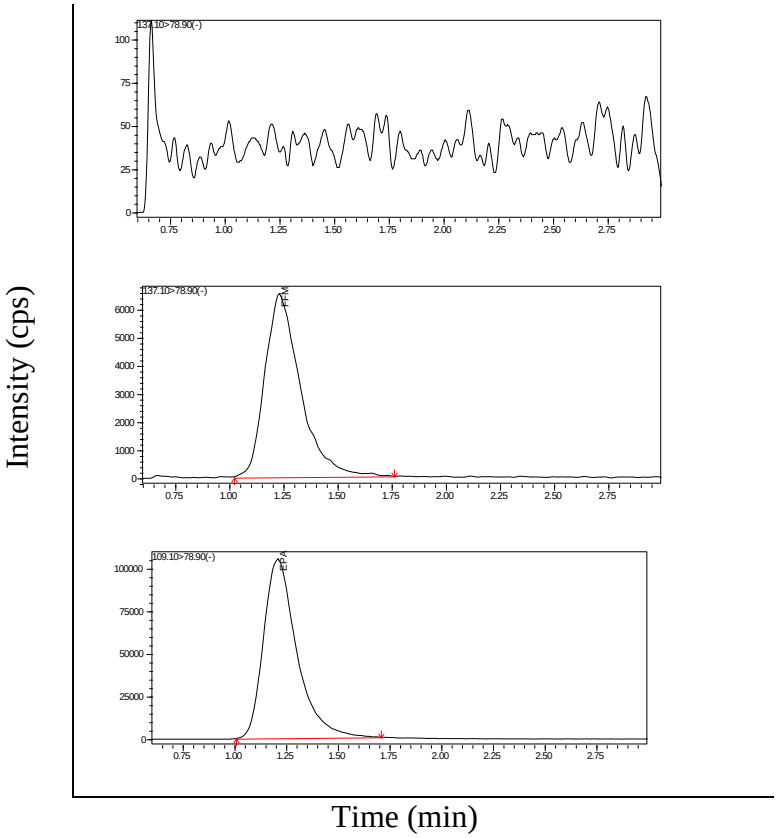

Figure 4.2-3 Chromatograms of blank sample (top) and the LLOQ (0.1 mg/mL) urine standard (FOM, centre; EPA, bottom)

A regression model with a weighted ($1/x^2$) quadratic curve provided the lowest distribution of error across the substantial concentration range (1 to 2000 µg/mL for plasma and 0.1 to 10 mg/mL for urine). The results of the quadratic regression study are described in Table 4.2-3.

Table 4.2-3 Regression analysis

Matrix	Calibration Range	Correlation Coefficient (Mean)	Maximum deviation* (%)
Plasma (n=12)	1 to 2000 µg/mL	0.9963	-14.0
Urine (n=5)	0.1 to 10 mg/mL	0.9959	+10.5

* Reported maximum deviation from nominal (%) across all standard curves and all concentration levels.

Precision and accuracy of the QC samples are shown in Table 4.2-4 for both plasma and urine. All results were within the acceptance criteria of ± 15% of the nominal concentration, indeed the results of all plasma QCs samples were within 9.1% and urine within 4.2%. An incurred sample reanalysis was performed on a subset of clinical samples and the results meet the current guidelines [126, 127] with >67% of repeated results being within 30% of the mean. Indeed, 100% of the repeated results were within 11%.

Table 4.2-4 Inter-assay Precision and Accuracy

Matrix	Concentration	Replicates	Mean	Accuracy (%)	Precision (%)
Plasma	3 µg /mL	22	2.93	-2.4	9.1
	80 µg /mL	27	78.0	-3.1	7.8
	800 µg/mL	22	826	3.3	4.3
	1600 µg/mL	23	1486	-7.2	5.9
Urine	0.3 mg /mL	9	0.30	0.0	4.2
	2 mg /mL	9	2.0	1.6	8.1
	8 mg/mL	9	7.9	-1.9	2.3

No signal suppression/enhancement was evident for either fosfomycin or the internal standard from the matrix study performed. The matrix effect evaluation is reported in Table 4.2-5.

Table 4.2-5 Matrix, recovery and freeze-thaw stability studies

Study	Matrix	Concentration	Mean	Accuracy (%)	Precision (%)
Matrix	Plasma	3 μg /mL	1.05[a]		8.8
		800 μg /mL	0.98[a]		3.9
	Urine	0.2 mg/mL	0.189 mg/mL	4.6	7.2
		5 mg/mL	5.36 mg/mL	-5.4	7.1
Recovery	Plasma	80 μg /mL	68 %		7.7
	Urine	10 μg/mL	98 %		4.2
Stability (freeze-thaw)	Plasma	0.3 μg /mL	0.31 μg /mL	3.2	4.0
		5 μg /mL	4.8 μg /mL	-3.1	5.4
		80 μg /mL	86 μg /mL	7.2	5.8
	Urine	0.3 mg /mL	0.30 mg /mL	0.9	2.7
		2.1 mg /mL	2.3 mg /mL	8.2	3.1
		7.8 mg /mL	8.2 mg /mL	4.4	1.0

[a] matrix factor: calculated as a ratio of peak area of fosfomycin in the presence of matrix to the peak area in the absence of matrix (normalised using the internal standard).

Despite using a very simple protein-precipitation for the extraction of fosfomycin from plasma the recovery was somewhat low at 68%. However, this extraction recovery is not atypical for a drug with a highly hydrophilic nature due to preferential aqueous partitioning. As was seen from the LLOQ testing, the variability was reliable (precision 6.1%) and sensitivity (LLOD ca. 0.01 μg/mL) easily achievable. The recovery of the internal standard, ethylphosphonic acid, was good at 72% when tested at the undiluted concentration of 10 μg/mL. The urine preparation was a simple dilution with internal standard and as such provided recoveries of 98% when tested at 0.4 mg/mL. The recovery results are reported in Table 4.2-5.

Stock solution stability for fosfomycin was tested for aqueous solutions stored for over 16 months at -80°C and for over 11 months at -20°C and it was found to be stable. Fosfomycin was also found to be stable in plasma and urine across three freeze-thaw cycles when stored at -80°C and thawed at ambient temperature in water (see Table 4.2-5).

Overall, the validation of this method was highly successful for both plasma and urine with the method showing an excellent degree of reproducibility and accuracy, and is suitable for use in the analysis of patient samples in a pharmacokinetic study.

This technique offers a simple and robust method for the analysis of fosfomycin in both plasma and urine in patient samples. Other quantitative methods have been described for the determination of fosfomycin in serum or plasma [113, 115, 117, 118, 123, 125] and urine [114]. However, these methods often require a significant amount of time in sample preparation or technique, and none offers a chromatographic system suitable for a pharmacokinetic study of fosfomycin in both plasma and urine.

4.2.3.3 Pharmacokinetic analysis

The plasma concentration-time profile obtained in the pilot pharmacokinetic study is shown in Figure 4.2-4. The peak plasma concentration in this patient after receiving the initial dose was 222 µg/mL, and the trough concentration was 141 µg/mL. Increased plasma concentrations were observed after receiving the fifth dose of fosfomycin, with the peak plasma concentration recorded as 868 µg/mL, and the trough concentration was 592 µg/mL. The urinary concentration determined from a 6 h sample taken post-dose and was 2.03 mg/mL after the initial dose and 0.29 mg/mL after the fifth dose of fosfomycin.

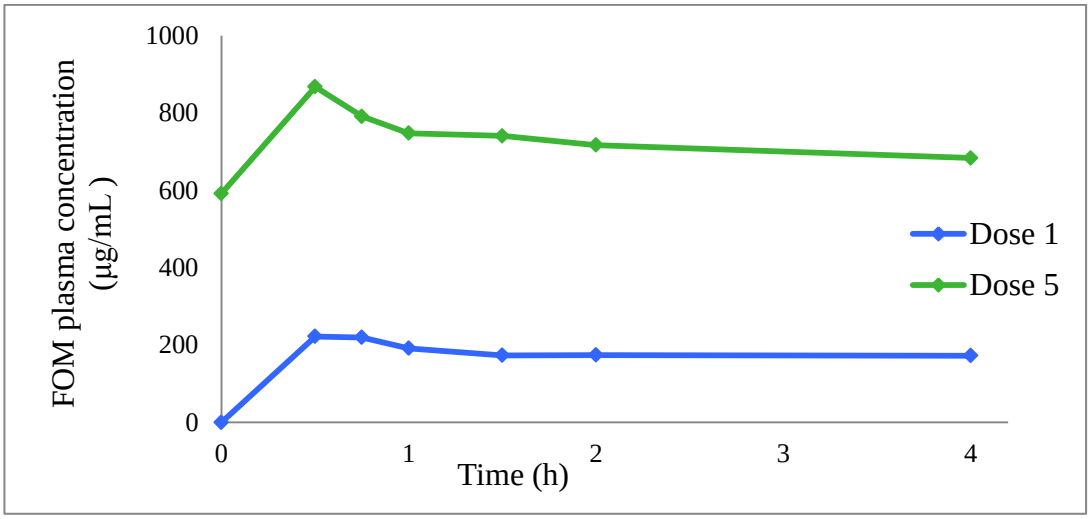

Figure 4.2-4 Plasma concentration – time profiles of fosfomycin in a critically ill patient receiving a 6 g fosfomycin IV dose every 6 hours, for the first and fifth doses.

4.2.4 Conclusion

The developed analytical method is a sensitive, simple and robust tool to analyse fosfomycin in plasma and urine of patients. With the increasing prevalence of multi-drug resistant organisms and the reduced effectiveness of currently available antibiotics this method allows the opportunity to study the disposition of fosfomycin, particularly in at-risk patient groups. This research may improve dosing strategies which could minimise the risk of increasing resistance and bring an effective antibiotic back into the hands of treating physicians.

4.3 Conclusion

This Chapter has described a sensitive, simple and robust LC-MS/MS method using HILIC technology for the quantitative bioanalysis of fosfomycin in plasma and urine. The method allows analysis of very small sample volumes because of the superior sensitivity of the instrumentation combined with the compatibility of HILIC chromatography. The small clinical volumes (10 µL) required for this analysis provide a platform for the translation of innovative microsampling techniques for use in pharmacokinetic studies which can then be used to define robust antibiotic doses that may improve patient outcomes, including suppression of the emergence of antibiotic resistance.

Part 3

Results

Chapter 5: Investigations into Translating Innovative Pharmacokinetic Microsampling Techniques for Clinical Studies

5.1 Synopsis

The aim of this chapter is to describe the suitability of microsampling techniques in clinical studies. This is achieved by assessing their performance through a quantitative bioanalytical validation. Specifically, given that haematocrit has been identified as a confounding factor for the use of whole blood sampling techniques including DBS, this preliminary investigation sought to understand the impact of haematocrit on fosfomycin measurements in whole blood samples using VAMS devices. Further to this the performance of VAMS devices in a quantitative bioanalytical validation for use in a clinical pharmacokinetic study of fosfomycin in critically ill patients is assessed. Finally, a complete validation of DPS sampling for fosfomycin as applied to a clinical pharmacokinetic study in critically ill patients is described.

5.2 Submitted manuscript entitled: Quantitative bioanalytical validation of fosfomycin in human whole blood with volumetric absorptive microsampling (VAMS)

The manuscript entitled, "Quantitative bioanalytical validation of fosfomycin in human whole blood with volumetric absorptive microsampling (VAMS)" has been accepted for publication by Bioanalysis (7: 19 (2015) 2585-2595).

The co-authors contributed to the manuscript as follows: the PhD candidate, Suzanne Parker, under the supervision of Dr. Steven Wallis, developed and validated the microsampling technique for application to a LC-MS/MS method. The PhD candidate, Suzanne Parker, took the leading role in manuscript preparation and writing. Prof Jason Roberts and Prof Jeffrey Lipman oversaw all aspects of this paper.

The manuscript is presented as submitted; except figures and tables have been inserted into the text at slightly different positions. Also, the numbering of pages, figures and tables has been adjusted to fit the overall style of the Thesis. The references are found alongside the other references of the Thesis, in the section 'Bibliography'.

Bioanalysis, impact factor: 3.003

Quantitative bioanalytical validation of fosfomycin in human whole blood with volumetric absorptive microsampling (VAMS)

Suzanne L. Parker[*, a], *Jason A. Roberts*[a,c], *Jeffrey Lipman*[a,b,d], *Steven C. Wallis*[a]

[a]Burns, Trauma and Critical Care Research Centre,

The University of Queensland, Brisbane, Australia

[b]Department of Intensive Care Medicine,

Royal Brisbane & Women's Hospital, Brisbane, Australia

[c]Department of Pharmacy,

Royal Brisbane & Women's Hospital, Brisbane, Australia

[d]Faculty of Health,

Queensland University of Technology

[*]Author for correspondence: Tel: +617 3346 5104

E-mail: suzanne.parker@uq.edu.au

Present address:

F Floor, Block 6,

School of Medicine,

The University of Queensland,

Royal Brisbane and Women's Hospital, Herston,

Queensland, AUSTRALIA

Abstract

Background: Fosfomycin is an antibiotic of considerable interest for the treatment of infection by multi-drug resistant bacteria. Translating microsampling techniques such as volumetric absorptive microsampling (VAMS) devices into clinical pharmacokinetic studies can lead to defining effective dosing information to improve patient outcomes. *Results:* Intra- and inter- assay results were all within 15%; the method was validated across the range of 5 to 2000 µg/mL of fosfomycin using VAMS devices. *Conclusion:* The VAMS technique provides acceptable validation data as assessed for lower limit of quantification (LLOQ), linearity, inter-day precision and accuracy, selectivity, and matrix effects. Results from the recovery and stability studies suggest challenges remain for the analysis of fosfomycin in whole blood using VAMS.

Key Terms

Volumetric absorptive microsampler: A device used for the collection of a fixed volume (10 µL) of a biological sample that can be dried for ease of transport and storage.

Fosfomycin: A broad-spectrum (MurA inhibitor) antibiotic used to treat some resistant bacteria.

Pharmacokinetic study: A study to characterize the disposition of a drug in the body, from administration to elimination. It can be used to improve the effectiveness of dosing.

5.2.1 Background

Fosfomycin is a small, highly polar phosphonic acid (see Figure 4.2-1) that has gained considerable interest recently as an antibiotic that is effective in the treatment of serious infection by multi-drug resistant bacteria [107], including extended spectrum beta-lactamase and carbapenemase–producing bacteria. There is little information available on the pharmacokinetics of fosfomycin in critically ill patients. Significant changes in the clinical pharmacokinetic of antimicrobial agents are common in critically ill patients and traditional strategies for dosing with antibiotics in patients who are critically ill are unlikely to consistently achieve the pharmacokinetic/pharmacodynamic targets associated with maximum antibiotic activity. This situation raises the risk of clinical failure, or development of resistance, or both, for a patient who is critically ill [132]. Data from a pharmacokinetic study can be used to define new dosing regimens that can lead to improved clinical effectiveness of the antibiotic and minimise the potential development of bacterial resistance.

Volumetric absorptive microsampling (VAMS) is a novel sample collection technique in which small volumes of whole blood can be collected, dried, stored and shipped for use in quantitative bioanalysis [93]. The translation of this microsampling technique using dried, whole blood samples for quantitative bioanalysis requires a validation that meets published US FDA guidelines and current scientific standards on microsampling.

The VAMS device consists of a sample handler similar in design and size to a pipette tip, attached to a porous, hydrophilic tip. Whole blood is absorbed onto the tip as a fixed volume (~10 μL) through wicking, ensuring the sample collection has a consistent blood volume regardless of haematocrit and the homogeneity of sample [93, 94]. The tip is then dried and the device available for storage and shipment.

The VAMS device offers an alternative to dried blood spots (DBS) sampling, with both providing advantages over traditional blood sampling. These advantages include: reduced blood volume requirements; simplification of obtaining sample from finger or heel prick rather than cannulae; simplification of sample processing as centrifuges are not required to make plasma; and, reduced costs of sample shipping and storage as samples often do not require freezing and may not be classed as biohazards during shipment [88, 94, 104]. The DBS technique is being used across a wide range of applications, including pre-clinical pharmacokinetic studies [69, 70], new-born and infant screening [71, 72], microbiological screening [73], forensic and toxicology investigations [74-76] and therapeutic drug monitoring [77, 78].

The substantial interest in the use of the DBS technique in quantitative bioanalysis has generated significant discussion on the problem of haematocrit impacting on the viscosity and thereby volume

of blood obtained from a fixed-diameter, sub-punch sample taken from DBS paper [76, 77, 83, 88, 92, 104, 133]. Deviating haematocrit values may cause significant assay bias for DBS [104] and, additionally, different volumes applied to the DBS paper may cause bias because of the non-homogeneity of the analyte across the site of punching [83, 93]. De Kesel *et al* has summarised the strategies that have been employed to minimise haematocrit problems [83] including whole-spot analysis [92], dried plasma spots [98], and estimation of haematocrit using a correlation to endogenous potassium [133].

Early investigations into the VAMS devices have demonstrated that the VAMS device is capable of collecting reproducible volumes of blood: with variation of 3.6%, across a wide range of haematocrit (20 to 70%) when using VAMS spiked whole blood with ^{14}C caffeine to measuring radioactivity [93] compared to variation of 30% observed from a DBS sample [88]; and intra-laboratory variability (n=6) based on weight of blood absorbed by the VAMS tip across a range of haematocrit (20 to 65%) was 8.7% [134]. Issues of non-homogeneity experienced by sub-punching DBS samples are negated with the VAMS device by the use of the whole sampling tip in extraction. De Kesel *et al* found no haematocrit bias in measuring caffeine and paraxanthine, although there was a consistent overestimation in the VAMS device measurement [105]; this positive bias has also been described by Denniff *et al* [103].

The first aim of this study is to perform an investigation into the extraction performance of the VAMS technique using whole blood samples, with varying hematocrit, containing fosfomycin. The second aim is to develop a validated method for extraction and quantification of fosfomycin in whole blood using the VAMS technique that would be suitable for use in a clinical pharmacokinetic study. The final aim is to describe practical issues associated with use of VAMS. We can report that critically ill patients experiencing renal insufficiency while receiving multiple doses of fosfomycin can exhibit very high concentrations (>1000 µg/mL) in their plasma [135] and this has led to the unusually high concentration range described here.

5.2.2 Experimental

5.2.2.1 Materials, Reagents and Equipment

Fosfomycin, ethylphosphonic acid (internal standard), methanol and acetonitrile (HPLC gradient-grade solvents) were purchased from Sigma-Aldrich (Sydney, Australia) and ammonium acetate was obtained from Ajax Univar (Sydney, Australia). Ultra-pure water was obtained using a hi-Pure Permutit system (Bayswater, Australia). Drug-free, screened whole blood was obtained from healthy volunteers from the Australian Red Cross Blood Service. Whole blood samples with altered haematocrit levels were prepared by centrifuging whole blood and removing or diluting with the

plasma produced, as required. Values for haematocrit in the modified and unmodified whole blood samples were processed on a Roche Sysmex XE5000 (Boston, USA) using the red blood cell pulse height detection method. All whole blood was stored at 4°C. VAMS devices were developed by Neoteryx™ (supplied by Phenomenex®, Sydney, Australia; marketed as Mitra™).

The liquid chromatograph – tandem mass spectrometer (LC-MS/MS) used was a Shimadzu Nexera high performance liquid chromatograph equipped with a Shimadzu 8030+ triple quadrupole mass spectrometer (MS) detector (Shimadzu Scientific Instruments, Rydalmere, Australia). An electro-spray ionization (ESI) source interface operating in negative-ion mode was used for the multiple reaction monitoring (MRM) MS/MS analysis. The compounds were separated on a Merck SeQuant zic-HILIC obtained from Merck Australia (Frenchs Forest, Australia), 2.1 x 50 mm, 5.0 µm analytical column protected by a 20 mm SeQuant zic-HILIC guard cartridge using an isocratic mobile phase containing acetonitrile with 2 mM ammonium acetate, pH 4.8 (85/15 v/v). The LC-MS/MS conditions for fosfomycin and the internal standard (ethylphosphonic acid) have been previously described [136]. The injection volume used was 0.5 µL. The retention time for both fosfomycin and ethylphosphonic acid was 2.3 min.

5.2.2.2 Standard and stock solution preparation

Aqueous stock solutions for whole blood standard preparation (at 1, 2 and 50 mg/mL of fosfomycin) were stored at -80°C. These aqueous solutions were serially diluted with drug-free whole blood to yield calibration standards in a range from 5 to 2000 µg/mL of fosfomycin (with the aqueous dilution of whole blood maintained at less than 4%) and applied to VAMS devices (see below). Ethylphosphonic acid in methanol was used as internal standard for the assay (at 10 µg/mL) and was stored at -20°C.

5.2.2.3 Quality control preparation

An aqueous stock solution for the quality control samples (at 50 mg/mL of fosfomycin) was stored at -80°C. This solution was diluted with drug-free whole blood to yield quality control samples containing fosfomycin at 15, 80 and 500 µg/mL for total whole blood concentrations (with an aqueous dilution of whole blood of 3%) and applied to VAMS devices (see below).

5.2.2.4 Preparation of VAMS dried blood samples

Calibration standards or quality control samples prepared with fosfomycin in whole blood were applied to VAMS devices by touching the tip into a bead of blood sitting on a small plastic tray (mimicking a needle prick), allowing the blood to wick up into the tip until it changed to red. The

tips were dried for 2 hours at room temperature in a Level 2 biosafety cabinet with filtered, circulating air and subsequently stored at room temperature in a sealed container.

5.2.2.5 Extraction of VAMS samples for LC-MS/MS analysis

Fosfomycin sampled using the VAMS device was extracted by inserting the VAMS device into a 96-deep well plate containing 200 µL of internal standard solution (10 µg/mL ethylphosphonic acid in methanol). The 96-deep well plate was mixed for 30 minutes at 1200 r.p.m. (800 g) on a lateral shaker. The VAMS device was removed, the 96-deep well plate sealed and mixed briefly, followed by LC-MS/MS analysis.

5.2.2.6 Data Analysis

Calibration curves were prepared within the batch using a linear regression with peak-area ratio (drug/internal standard area responses) against concentration (x), with a $1/x^2$ weighting as the mathematical basis of the quantification.

5.2.2.7 Assessment of the effect of haematocrit variability

An assessment of the effect of variable haematocrit in whole-blood matrices was performed prior to commencing validation. In this assessment three different patients' whole blood were adjusted (using the patient's own plasma for volumetric dilutions) to create a range of three haematocrit levels, with subsequent haematocrit results ranging from 24.6 to 42.9%. These nine whole blood vehicles were then spiked at low and high concentrations (see: quality control preparation) and applied to the VAMS devices, to prepare samples in triplicate (thus creating a total of 54 VAMS samples). A calibration curve containing 5 to 2000 µg/mL of fosfomycin was prepared alongside these samples using one subject's whole blood (haematocrit 35.2%) and applied using the VAMS device.

5.2.3 Method of Validation

The validation was performed with reference to the guidelines provided by the U.S. Food and Drug Administration (FDA) [137]. The use of the VAMS devices in a quantitative analysis of fosfomycin from whole blood includes a validation study with an assessment for lower limit of quantification (LLOQ), linearity, intra- and inter- day precision and accuracy, matrix effects, recovery, selectivity, and stability of dried matrix.

5.2.3.1 Limit of quantification

The LLOQ for fosfomycin was evaluated by analysis of replicate standards (n=5) on a single day, using the VAMS devices and defined as the lowest calibration standard that could be measured with precision and accuracy below 20%.

5.2.3.2 Linearity

To investigate linearity, calibration curves were prepared using the VAMS devices for concentrations of 5, 10, 20, 50, 100, 200, 500, 1000, 1500, and 2000 µg/mL of fosfomycin in whole blood. On three occasions, 10-point calibration curves were analysed in duplicate, alongside a blank whole blood sample and a blank whole blood sample containing internal standard only, to evaluate linearity. Regression models were evaluated using a linear and quadratic regression analysis, with unweighted and weighted parameters evaluated, including weighting factors of $1/x$, $1/x^2$, $1/y$, $1/y^2$.

5.2.3.3 Intra-assay Precision and Accuracy

Intra-assay precision and accuracy for fosfomycin was evaluated by the analysis of quality control samples (n=5) at three different concentrations and at the LLOQ concentration, on a single day, with concentrations determined against a freshly prepared standard curve.

5.2.3.4 Inter-assay Precision and Accuracy

Inter-assay precision and accuracy for fosfomycin was evaluated by the analysis of quality control samples at three different concentrations and at the LLOQ, in duplicate on three occasions, with concentrations determined against freshly prepared standard curves.

5.2.3.5 Matrix effects

Matrix effects were evaluated to identify any suppression or enhancement of signal from an interfering substance around the retention time of fosfomycin by using the matrix factor test. Five drug-free whole blood samples from healthy volunteers were prepared using the VAMS devices, extracted and then spiked at 15, 80 and 500 µg/mL fosfomycin concentrations and with internal standard, and the area results compared to water spiked with fosfomycin and internal standard at the same concentrations. The precision of the matrix factor (normalised against internal standard) was used to determine if any concentration demonstrated unacceptable variability from the expected result.

An additional study was performed to evaluate the impact of variable haematocrit on matrix effects. For this study drug-free whole blood was obtained from a healthy volunteer and adjusted (using the patient's own plasma for volumetric dilutions) to create a range of three haematocrit levels, with subsequent whole blood samples containing haematocrit levels of 27, 41, and 52%. These three variable-haematocrit whole blood samples were extracted and then spiked at 15, 50 and 500 µg/mL fosfomycin concentrations (in duplicate) and with internal standard. The area results of the samples containing whole blood matrix were compared to water spiked with fosfomycin and internal standard at the same concentrations. The precision of the matrix factor (normalised against internal

standard) was used to determine if any concentration demonstrated unacceptable variability from the expected result.

5.2.3.6 Recovery

Whole blood samples were spiked at three concentrations of fosfomycin (15, 50, and 500 µg/mL) and using three levels of hematocrit (27, 41, and 52%) and applied to the VAMS tip (identified as: extracted samples). Blank whole blood, using three levels of hematocrit (27, 41, and 52%), were also applied to the VAMS tip (identified as: non-extracted samples). All samples were prepared in duplicate and by the volumetric application of 10 µL to the VAMS tip. The extracted and non-extracted samples were then dried for 2 hours at room temperature in a Level 2 biosafety cabinet with filtered, circulating air and then extracted. The non-extracted samples were then spiked with 10 µL of stock fosfomycin solutions to produce the three concentrations of fosfomycin (15, 50, and 500 µg/mL). The extracted samples were diluted with 10 µL of water.

The recovery of fosfomycin from extracted samples prepared using the VAMS technique was then evaluated by comparing the peak area for extracted samples to the non-extracted samples.

5.2.3.7 Selectivity and Carry-Over

The selectivity of the method was evaluated to identify any interfering peaks around the retention time of fosfomycin or the internal standard. Six blank samples from healthy volunteers, and a blank sample containing internal standard only, were analysed. Six blank samples spiked with fosfomycin at the LLOQ were also analysed and assessed for precision. Furthermore, during every analytical run a blank sample was injected after the highest calibration standard and inspected for carry-over.

5.2.3.8 Stability

Stability of fosfomycin in whole blood as a dried sample on a VAMS device was assessed by comparing the peak area ratio of fosfomycin to internal standard for freshly prepared samples at 500 µg/mL to samples stored (i) at room temperature for 10 days, 2.5 months and again at 4 months, (ii) at -20°C for 4 months, and (iii) at +50°C for 4 days. Freshly prepared samples were generated using the original whole blood that had been stored at 4°C for 10 days for comparison of the samples stored for 10 days at room temperature, while samples prepared using fresh whole blood (a separate donor) were used for all remaining stability tests. The stability of samples extracted from VAMS devices (n = 5) was also tested by comparison of the peak area ratios obtained on initial injection, to the results after the extracts were stored on an autosampler at 4°C for 48 hours.

5.2.3.9 Practical issues (observations from the bench)

Samples in small and large batches (>30 samples) were extracted using the VAMS devices to facilitate a direct qualitative investigation of the practical advantages of the sampling method for use in a bio-analytical research laboratory.

5.2.4 Results & Discussion

5.2.4.1 Development of extraction

Initial extraction solutions tested included mobile phase (2 mM ammonium acetate buffer in 85% acetonitrile), 50% methanol in water (v/v), and methanol; all included the internal standard at 10 μg/mL ethylphosphonic acid. The results from the extraction with methanol gave the highest recovery of fosfomycin for the VAMS devices. The mixing of the VAMS devices on a lateral shaker was tested for 10, 20, 30 and 60 minutes, with less than 3% difference between the results observed between 30 and 60 minutes. The VAMS devices were also extracted with sonication, without an improved recovery. Furthermore, the extraction was tested with the tip removed from the VAMS handler and while remaining attached, without evidence of an improved recovery from either method. The development of the extraction was simple, quick and the final extracted sample was compatible with HILIC chromatography and tandem mass spectrometry.

5.2.4.2 The effect of haematocrit variability

The use of the VAMS devices was assessed across a range of laboratory prepared haematocrit-adjusted (24.6 to 42.9%) whole blood samples (n=54 samples). Using the VAMS devices resulted in 9% of samples failing to meet acceptance criteria. The accuracy of all patient samples at 75.5 ± 6.9 μg/mL of fosfomycin was -5.6% and at 506 ± 44.8 μg/mL of fosfomycin was +1.3% using VAMS devices.

The mean accuracy of the triplicate analyses at 80 μg/mL and 500 μg/mL of fosfomycin plotted against haematocrit levels is reported in Figure 5.2-1. The VAMS device produced evidence of bias for the analysis of fosfomycin (r^2 0.33), where it produced an inverse correlation, with a positive bias at lower haematocrit levels and a negative bias at higher haematocrit levels (Figure 5.2-1). As the VAMS device reduces bias from applied volume, the bias observed when using the VAMS device for fosfomycin may be due to a change in recovery of the analyte or a matrix effect across haematocrit levels. While the mean of the results falls within the 15% acceptance criteria, the results warranted further investigation, and this was performed as part of the recovery and matrix studies.

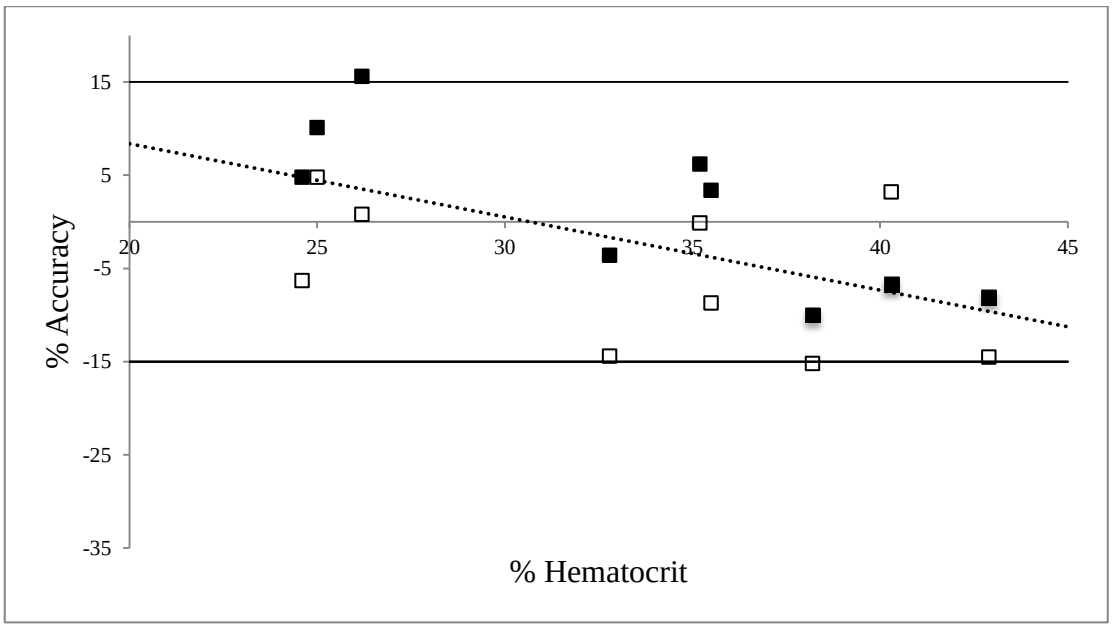

Figure 5.2-1 Variation in the accuracy of concentration of fosfomycin with changes in haematocrit for the volumetric absorptive microsampling (VAMS) device (squares open 80 µg/mL, squares closed 500 µg/mL) with each square representing the mean result of a triplicate analysis. Solid lines represent the acceptance criteria of +/- 15%. The dotted lines represent the linear regression line.

5.2.4.3 Validation

A linear regression provided the simplest calibration model across the concentration range, and the 1/concentration^2 weighting improved the correlation coefficient considerably. This model met the acceptance criteria with the back-calculated concentration results being within 20% deviation from the LLOQ and within 15% deviation of standards other than the LLOQ, from nominal concentrations, for all of the non—zero standards. The calibration range of 5 to 2000 µg/mL for the six standard curves produced a mean correlation coefficient of 0.9955 and the percentage of maximum deviation (inaccuracy) of the standards of the three calibration curves was 14.7%.

The intra- and inter- precision and accuracy of the quality control samples are shown in Table 5.2-1 (see Figure 5.2-2 for representative chromatograms of the LLOQ standard extracted from whole blood using the VAMS devices) with the results meeting US FDA acceptance criteria [137]. Variation in retention time is observed between samples extracted using VAMS and that seen previously in plasma extracts (2.4 min) and diluted urine (1.3 min). We conclude that the changes in the retention times and quality of the chromatography observed may have been due to the

sensitivity of the interaction of fosfomycin with the stationary phase to changes in ionic strength and pH of the buffer [128, 129], as well as the age of the analytical column. The VAMS extractions were performed many months after those performed in plasma and urine [136], on different columns, and after a substantial reconfiguration of instrumentation. The robustness of the chromatography for samples extracted using VAMS is evidenced by very low variability in retention times (less than 1.1% relative standard deviation) as seen across the validation.

Table 5.2-1 Intra- and Inter- assay Precision and Accuracy

Study	Concentration (µg /mL)	Mean	Accuracy (%)	Precision (%)
Intra-assay Precision & Accuracy	5	5.15	103	± 5.6
	15	14.4	96.3	± 4.1
	200	182	91.1	± 2.9
	1500	1591	106	± 5.6
Inter-assay Precision & Accuracy	5	5.05	101	± 2.3
	15	14.6	97.4	± 5.2
	200	188	94.0	± 4.5
	1500	1606	107	± 5.8

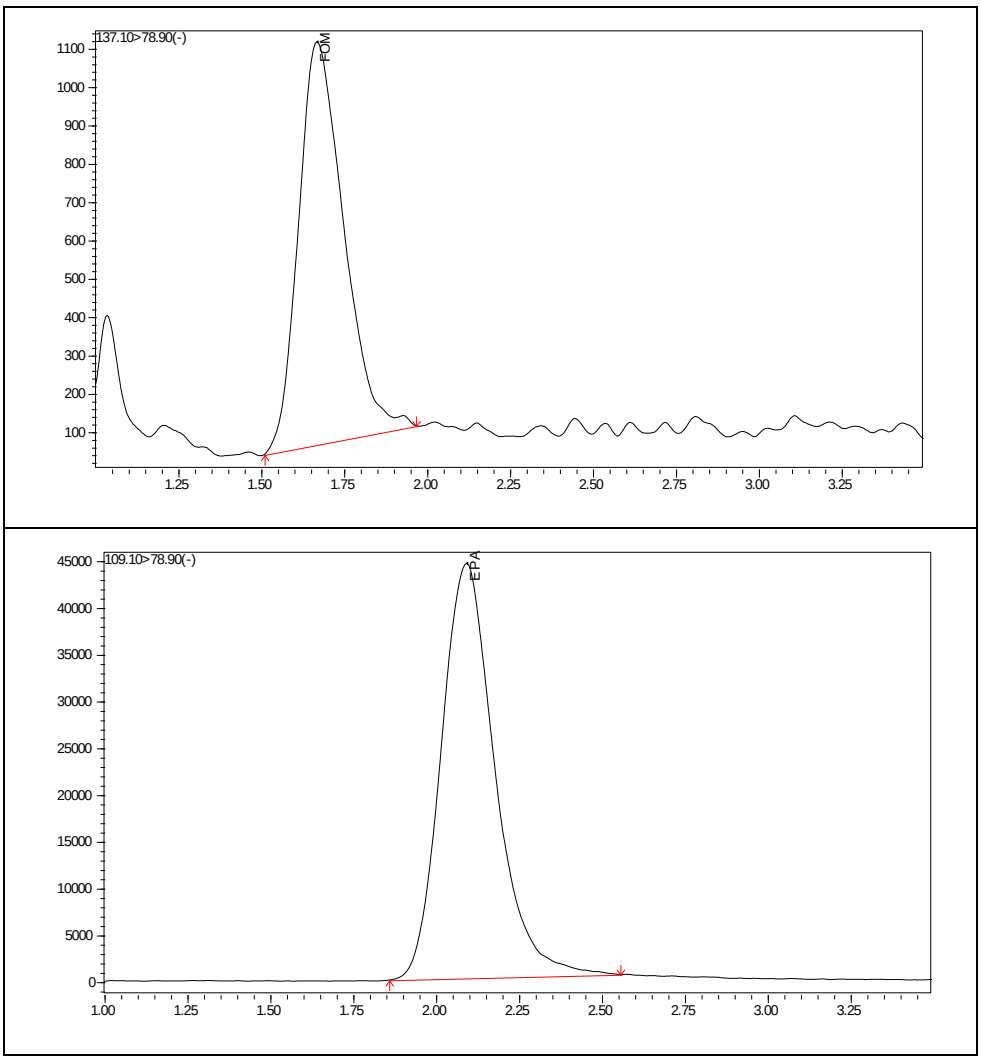

Figure 5.2-2 Representative chromatograms of the volumetric absorptive microsampling (VAMS) devices for (A) the mass transitions of fosfomycin (FOM) and the internal standard, ethylphosphonic acid (EPA) at the lower limit of quantification.

The extraction of fosfomycin or the internal standard (ethylphosphonic acid) using the VAMS devices found no evidence of signal suppression/enhancement. This was concluded from both of the matrix effect studies performed as (i) whole blood samples from five healthy volunteers, and (ii) whole blood of varying haematocrit, and as evidenced by a lack of significant bias or variability. The matrix effect evaluations are described in Table 5.2-2.

Table 5.2-2 Matrix Study

Study	Concentration (μg /mL)	Mean Matrix Factor	Precision (%)
Matrix [a]	15	1.06	± 2.8
	80	0.90[a]	± 1.9
	500	1.02[a]	± 1.3
Matrix [b]	15	1.01	± 3.9
	50	1.05	± 3.3
	500	1.03	± 5.0

[a] Prepared in whole blood samples from healthy volunteers

[b] Prepared in whole blood samples with varying haematocrit values (range 27 to 52%)

No interfering peaks were observed in blank samples obtained from five healthy volunteers, at the retention time of fosfomycin or the internal standard. No interfering peaks or unacceptable variability were observed in the whole blood samples from six healthy volunteers containing fosfomycin at the concentration of the LLOQ (precision 5.3%). There was no evidence of carry-over in any analytical runs performed.

The extraction recovery for fosfomycin from the VAMS device was low at 52.0, 50.5, and 52.5% across three concentrations of 15, 50 and 1000 μg/mL of fosfomycin, when tested in whole blood with a haematocrit level of 41%. Despite the low extraction recovery, as seen from the LLOQ testing, the variability of extraction was reliable (precision 5.6%) and sensitivity easily achievable. However, when tested across whole blood prepared for a varying range of haematocrit (at 27, 41 and 52%) an inverse correlation for recovery is identifiable (Table 5.2-3), at all three concentrations tested, where samples containing low levels of haematocrit have higher extraction recoveries. The recovery test is performed by the direct spotting of 10 μL of blood onto the VAMS device, as was performed by De Kesel *et al* who observed a similar result. This excludes the likelihood that the result reflects a low absorption of blood onto the tip, and is more likely that as haematocrit increases the higher relative amount of erythrocytes trapped in the VAMS tip may render it more difficult for compounds to be desorbed from the tip [105].

Table 5.2-3 Recovery Study

Haematocrit (%)	Concentration (μg /mL)	Recovery %	Precision (%)
27	15	61.8	± 1.8
	50	60.4	± 4.8
	500	63.9	± 15.9
41	15	52.0	± 6.2
	50	50.5	± 4.8
	500	52.5	± 13.3
52	15	45.6	± 5.9
	50	48.9	± 8.5
	500	47.1	± 7.9

Stability of fosfomycin in whole blood when applied to VAMS devices was demonstrated after 10 days when compared to whole blood stored for 10 days, and when compared to samples prepared in fresh whole blood (i.e. the whole blood was from two different subjects). After 2.5 months, samples applied to VAMS devices and stored at room temperature showed a decrease in peak area of 31.4% (precision 10.7%) and which was further decreased to 53.1% (precision 6.6%) after 4 months. Extreme temperature storage, at +50°C for four days, also showed a decrease in peak area of 78.4% (precision 2.4%). Degradation of antibiotics when applied to DBS and storage at room temperature has been previously observed [138]. Further to this, the decreased concentrations may also reflect a reduced recovery over time, as has been observed with some analytes in dried blood spots samples [139]. In-house testing of fosfomycin in whole blood when applied to sample collection paper (Whatman 903) and stored at room temperature for 2.5 months found a decrease in peak area of 14.0% at 500 μg/mL of fosfomycin (precision 3.9%; haematocrit 35.2%), however, fosfomycin in plasma, when applied to the same type of paper (Whatman 903) and stored at room temperature for 3 months produced a decrease in peak area of just 4.9% at 500 μg/mL of fosfomycin (precision 11.3%). These results (see Table 5.2-4) suggest the stability of fosfomycin in whole blood using the VAMS device may reflect a reduced recovery over time (or with temperature) or possible degradation by components removed during the preparation of plasma samples. Stability of fosfomycin in whole blood when applied to VAMS devices and stored at -20°C demonstrated a

difference in peak area ratios of +15.2% at 500 μg/mL for fosfomycin after 4 months. This final result suggests an increased recovery during frozen storage.

Table 5.2-4 Stability Studies

Study	% Difference	Precision (%)
Room temperature (10 day)	+ 0.7[a]	± 7.5
	+ 1.6[b]	± 3.6
Room Temperature (2.5 months)	-31.4	± 10.7
Room Temperature (4 month)	-53.1	± 6.6
Frozen Storage	+15.2	± 7.2
Extreme Temperature	-78.4	± 2.4
Extracts stored in autosampler	-3.2	± 4.5

[a] calculated against a freshly prepared standard from whole blood stored at 4°C for 10 days

[b] calculated against a freshly prepared standard from fresh whole blood.

Further investigations into long term stability and the possible impact on recovery of fosfomycin when using VAMS devices for whole blood sampling, at room temperature and at extreme temperatures (both high and low), may be required to ensure the samples will be stable during transport and storage prior to analysis. The results from the stability testing may reflect that optimisation of extraction may need to be performed on older samples, as well as those freshly prepared.

There was no instability detected from extracted samples stored on the autosampler at 4°C for 48 hours.

Overall, the validation of this method was successful for the extraction of fosfomycin from whole blood when sampled using the VAMS devices, with the results showing acceptable reproducibility and accuracy. However, a bias in recovery at varying haematocrit may complicate a quantitative analysis particularly if applied to critically ill patients, who commonly experience anaemia or haemodilution due to medical interventions [86]. Furthermore, the practicalities of analysing samples, often transported internationally, within a short time would need to be addressed.

5.2.4.4 Practical issues (observations from the bench)

The first advantage for the VAMS technique compared to a DBS sampling technique was labelling. That is, because the tip on the VAMS device was not removed during the analysis, sample identification remained intact during the sample processing. This advantage was best observed when performing a batch analysis of a large number of clinical samples, where being able to confirm the sample identity can eliminate potential dispensing and sample processing errors. Once a DBS sample has been sub-punched it is unidentifiable.

Care was taken to avoid the abuse scenarios as described by Denniff and Spooner in their investigation into the use of VAMS tips during sampling and handling [93], including: immersing the sampler tip past the shoulder, double dipping, handling the tip, dropping the sampler, touching the tip against a surface after collection. Despite avoiding these scenarios we observed a failure rate of approximately 3% in which the VAMS device simply failed to draw whole blood into the tip. After allowing up to 8-10 seconds for the sample to absorb onto the tip incomplete absorption was observed (it should be noted that within 3-4 seconds it was observed that absorption was very slow); the tip was subsequently discarded.

5.2.5 Limitations

The whole blood used for the preparation of quality control and standard samples in the laboratory contained either citrate buffer or lithium heparin as anti-coagulants. The matrix testing was performed without any anticoagulant present. Clinical samples may not contain anticoagulant if the sample is obtained using thumb or heel prick, however ICU patients commonly receive heparin. A more complete matrix study would include whole blood containing citrate buffer, lithium heparin, or other possible anticoagulants to investigate the possibility of interferences. Ion suppression or enhancement caused by citrate or EDTA anticoagulants has been identified as causing interferences and serious matrix effects for polar molecules [140]. Further to this the matrix study does not include an assessment of co-administered medications, including other antibiotics.

The stability testing does not meet the FDA validation requirements as it has been performed at only one concentration.

While fosfomycin is an unusual molecule, in that it is not metabolised, a clinical study could provide a direct comparison of the results from VAMS devices to the gold standard of liquid plasma sampling, and this is required to determine whether the VAMS devices are a suitable tool in the quantitative bioanalysis of fosfomycin.

5.2.6 Conclusion

A simple and quick extraction of fosfomycin from whole blood using the VAMS devices, with compatibility with HILIC chromatography using an LC-MS/MS, was developed. The use of the VAMS devices for whole blood sampling provides acceptable validation data as assessed for matrix effects, lower limit of quantification (LLOQ), linearity, and inter- and intra- day precision and accuracy.

Results from recovery testing show the method is suitable for the analysis of samples with normal or high haematocrit. Testing of the stability of dried whole blood samples on VAMS devices for use with the analysis of fosfomycin show the method is suitable for samples stored at room temperature for 10 days. The results from these tests suggest the impact of hematocrit or other components in whole blood may present challenges to the analysis of fosfomycin using VAMS devices, and if samples were outside the criteria established here for recovery and stability, further testing would be required.

Finally, we observed a practical advantage to the use of VAMS devices as they allow sample identification during analysis.

5.2.7 Future Perspective

The application of the use of VAMS devices for the sampling of fosfomycin in whole blood into a clinical study is required to assess its suitability as a tool in a pharmacokinetic study in critically ill patients. Using multimodal sampling would allow a comparison of the results obtained using the VAMS devices to both the gold standard of liquid plasma sampling and whole blood sampling. Furthermore, the comparison of liquid plasma sampling to whole blood sampling could be made. Clinical staff could perform a qualitative investigation into the ease of use of VAMS devices in the preparation and storage of patient samples.

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5.3 Published manuscript entitled: A validated method for the quantification of fosfomycin on dried plasma spots by HPLC-MS/MS: application to a pilot pharmacokinetic study in humans

The manuscript entitled, "A validated method for the quantification of fosfomycin on dried plasma spots by HPLC-MS/MS: application to a pilot pharmacokinetic study in humans" has been accepted for publication by the Journal of Pharmaceutical and Biomedical Analysis, (115 (2015) 509-514).

The co-authors contributed to the manuscript as follows: the PhD candidate, Suzanne Parker, under the supervision of Dr. Steven Wallis, developed and validated the microsampling technique for application to a LC-MS/MS method. The PhD candidate, Suzanne Parker, took the leading role in manuscript preparation and writing. Prof Jason Roberts, Prof George Dimopoulos, and Prof Jeffrey Lipman oversaw all aspects of this paper.

The manuscript is presented as submitted; except figures and tables have been inserted into the text at slightly different positions. Also, the numbering of pages, figures and tables has been adjusted to fit the overall style of the Thesis. The references are found alongside the other references of the Thesis, in the section 'Bibliography'.

Journal of Pharmaceutical and Biomedical Analysis, impact factor: 2.979

A validated method for the quantification of fosfomycin on dried plasma spots by HPLC-MS/MS: application to a pilot pharmacokinetic study in humans.

Suzanne L. Parker[*a], *Jeffrey Lipman*[a,b,c], *George Dimopoulos*[d,e], *Jason A. Roberts*[a,f], *Steven C. Wallis*[a]

[a]Burns, Trauma and Critical Care Research Centre,
The University of Queensland, Brisbane, Australia

[b]Department of Intensive Care Medicine,
Royal Brisbane Hospital, Brisbane, Australia

[c]Faculty of Health, Queensland University of Technology

[d]Medical School, University of Athens, Athens, Greece

[e]Internal Medicine Department, Hygeia Hospital, Athens, Greece

[f]Department of Pharmacy, Royal Brisbane Hospital, Brisbane, Australia

*Corresponding author

Mailing Address: Burns, Trauma and Critical Care Research Centre (BTCCRC),
Level 7, Block 6, Royal Brisbane Hospital QLD 4029

Phone: +61 7 3346 5104

Fax: +61 7 3636 7202

Email: suzanne.parker@uq.edu.au

Key terms:

Fosfomycin; dried plasma spots; LC-MS/MS; pharmacokinetic; antibiotic

Abstract

Quantification of fosfomycin in the plasma samples of patients is the basis of clinical pharmacokinetic studies from which evidence based dosing regimens can be devised to maximise antibiotic effectiveness against a pathogen. We have developed and validated a LC-MS/MS method to quantify fosfomycin using dried plasma spot sampling. Following HILIC chromatography, fosfomycin and ethylphosphonic acid, used as internal standard, were measured using negative-ion multiple reaction monitoring.

The method was linear over the calibration range of 5 to 2000 µg/mL of fosfomycin. Intra-day assay results for dried plasma spot quality control samples at 15.6, 79.9 and 1581 µg/mL of fosfomycin had precision of ± 4.2, 8.2, and 2.0%, respectively, and accuracy of +3.9, -0.1, and -1.2%, respectively. Recovery of fosfomycin from dried plasma spots was calculated as 83.6% and the dried plasma spot samples were found to be stable stored at room temperature for three months, and when stored for four hours at 50°C. A Bland-Altman plot comparing DPS to plasma sampling found a negative bias of 16.6%, with the mean limits of agreement ranging from -2.6 to 30.6%. Dried plasma spot sampling provides a useful tool for pharmacokinetic research of fosfomycin.

HIGHLIGHTS

- A simple and robust LC-MS/MS method for the quantification of fosfomycin in human plasma using dried plasma spot sampling has been developed.
- The developed LC–MS/MS method has been validated according to published U.S. FDA guidelines and current scientific standards on microsampling.
- Results from a pilot pharmacokinetic study with a critically ill patient receiving IV fosfomycin, with samples prepared using dried plasma spot sampling, are included.

5.3.1 Background

Fosfomycin is a broad-spectrum bactericidal antibiotic with a unique mechanism of action, inhibition of phosphoenolpyruvate transferase, the enzyme involved in the synthesis of peptidoglycan that is found in the cell wall of Gram-negative and Gram-positive bacteria [11, 110].

Fosfomycin is generating substantial interest as an intravenous or enteral therapy for multi-drug resistant pathogens [64, 106]. With an increasing prevalence of multi-drug resistant bacteria, combined with a steadily decreasing number of usable antibiotics available, fosfomycin offers a potential infection treatment option for critically-ill patients.

Critical illness is associated with a significant distortion of pharmacokinetics for many antibiotics [141]. Changes in fosfomycin pharmacokinetics can significantly impact concentrations at the site of infection, and as such, dose alterations may be required to ensure that optimal exposures are achieved [107]. Pharmacokinetic studies can provide evidence based dosing regimens and maximise antibiotic effectiveness against a pathogen.

Dry micro-sampling techniques offer several advantages over the gold-standard of traditional liquid plasma sampling for use in clinical pharmacokinetic studies including: low sample volumes, simplified collection, as well as a reduction in shipment and storage costs (traditional liquid plasma samples require frozen storage and transport).

However, dry whole-blood micro-sampling techniques, such as dried blood spots, are known to exhibit a bias due to haematocrit in quantitative analysis for sub-punched dried blood spots due to a non-homogeneous distribution of blood across the spot, viscosity-related diffusion properties of blood on dried blood spots, and may influence recovery and matrix effects in LC-MS applications [102, 142]. Dried plasma spots may therefore be better suited for clinical pharmacokinetic studies in critically-ill patients, as these patients can commonly experience anaemia and consequently exhibit low levels of haematocrit [86].

Quantification of drugs using the dried plasma spot sampling technique has been described for daptomycin [143], valproic acid and gabapentin [144, 145], linezolid [146], paroxetine[147], acetaminophen [76], guanfacine [101], iothalamate [148], vigabatrin [149], triazoles [150], and anti-epileptic drugs [151], as well as anti-HIV drugs [152].

There are several analytical techniques available for the determination of fosfomycin in human plasma: using gas chromatography [113, 117], LC - spectrophotometric detection [118], LC - photometric detection, capillary zone electrophoresis [120, 121], and, more recently, with derivatisation and LC - atmospheric pressure chemical ionization mass spectrometry [122] and LC

– MS/MS [123, 136]. However, no methods have been published describing the quantitation of fosfomycin in dried plasma spots.

The aim of this work was to develop a reliable, quick and sensitive method for the quantitation of fosfomycin (structure in Figure 4.2-1) using a DPS sampling technique, compare it to the gold standard of plasma sampling, and assess its suitability for a clinical pharmacokinetic study.

5.3.2 Experimental

5.3.2.1 Materials and reagents

Fosfomycin, ethylphosphonic acid (internal standard), methanol and acetonitrile (HPLC gradient-grade solvent) were purchased from Sigma-Aldrich and ammonium acetate was obtained from Ajax Univar. Ultra-pure water was obtained using a four-module Hi-Pure Permutit system manufactured by Permutit, Bayswater, Australia. Drug-free human plasma was obtained from the Australian Red Cross Blood Service. Whatman (GE Healthcare, Maidstone, U.K.) supplied the 903 Sample Collection Paper used for dried plasma spots.

5.3.2.2 Instruments and Conditions

The LC-MS/MS used is a Shimadzu Nexera UHPLC equipped with a Shimadzu 8030+ triple quadrupole mass spectrometer (MS) detector. An ESI source interface operating in negative-ion mode was used for the MRM LC-MS/MS analysis with the compounds were separated on a Merck SeQuant zic-HILIC, 2.1 x 50 mm, 5.0 µm analytical column. LC and MS conditions for fosfomycin and the internal standard have been previously published [136]. The injection volume used was 1.0 µL. The retention time for both fosfomycin and ethylphosphonic acid was 2.4 min.

5.3.2.3 Standards for dried plasma spot analysis

Aqueous stock solutions for plasma standard preparation (at 10 000, 20 000 and 50 000 mg/L of fosfomycin) were stored at -80°C. These were diluted with drug free plasma to yield ten calibration standards from 5 to 2000 µg/mL of fosfomycin, ensuring the aqueous dilution volume in each standard was below 5% v/v. The calibration standards were then dispensed (20 µL) onto sample collection paper and dried for two hours at room temperature in a Class 2 Biosafety Cabinet with filtered, circulating air. The calibration standards were then stored in sealed plastic bags and processed alongside the clinical samples.

5.3.2.4 Internal standard solution

Ethylphosphonic acid in methanol was used as internal standard for the assay (at 10 µg/mL) and stored at 4°C.

5.3.2.5 Quality control sample preparation

Quality control samples were prepared by spiking drug free plasma with fosfomycin stock solutions, prepared independently of standard solutions to concentrations of 15, 80 and 1600 µg/mL (ensuring the aqueous dilution volume in each standard was below 5% v/v), dispensed (20 µL) onto sample collection paper and dried for two hours at room temperature in a Class 2 Biosafety Cabinet with filtered, circulating air. The quality control samples were then stored at room temperature in sealed plastic bags.

5.3.2.6 Extraction of dried plasma spot samples for LC-MS/MS analysis

Dried plasma spot samples were extracted by manually punching a 3 mm disc from the centre of the DPS and inserting it into a 96-deep well plate containing 200 µL of internal standard solution (10 µg/mL ethylphosphonic acid in methanol). The 96-deep well plate was capped and mixed for 30 minutes at 1200 r.p.m. on a lateral shaker. The dried plasma spot disc was removed, the 96-deep well plate re-sealed, vortex mixed for 3 seconds, followed by LC-MS/MS analysis.

5.3.2.7 Extraction of liquid plasma samples for LC-MS/MS analysis

The analysis of plasma samples for LC-MS/MS analysis was performed by protein precipitation with acetonitrile and has been published elsewhere [136] and is used here for comparative purposes with dried plasma spot clinical samples only.

5.3.2.8 Data Analysis

The concentration of each clinical sample was back-calculated using least squares regression analysis based on the peak-area ratio (drug/internal standard area responses) against concentration (x) from the calibration curve prepared within the batch.

5.3.3 Method of Validation

The validation was performed in accordance with the guidelines provided by the U.S. FDA with pre-established acceptance criteria required to demonstrate the method is suitable for the intended purpose [126] and current scientific standards on microsampling [85, 153, 154]. The validation for extraction of fosfomycin from dried plasma spots was assessed for lower limit of quantification (LLOQ), linearity, inter-day precision and accuracy, sample spot volume, matrix effects, recovery, storage and transport stability.

5.3.3.1 Limit of quantification and detection limits

The LLOQ for fosfomycin was evaluated by analysis of replicate standards (n=5) prepared as dried plasma spot samples and subsequently extracted in a single batch. The acceptance criteria were established as that the back-calculated concentration results should have precision that does not

exceed 20% of the CV and accuracy within 20% of the nominal concentration. The LOD for fosfomycin was calculated based on its definition as being the lowest peak reliably distinguished from the background noise and calculated as ≥ three-times the noise of the blank dried plasma spot sample.

5.3.3.2 Linearity

To investigate linearity, three calibration curves consisting of ten non-zero standards, using the concentration range of 5 to 2000 mg/L of fosfomycin in plasma and prepared in different runs (n=3) as dried plasma spots. The linearity selected was the simplest calibration model and weighting that satisfied the criteria of results being within 20% deviation from the LLOQ and within 15% deviation of standards other than the LLOQ, from nominal concentrations. Regression models were evaluated using a linear and quadratic regression analysis, with unweighted and weighted parameters evaluated, including weighting factors of $1/x$, $1/x^2$, $1/y$, $1/y^2$.

5.3.3.3 Precision and Accuracy

Intra-assay (n=5) precision and accuracy for fosfomycin throughout the calibration range for dried plasma spot sampling was evaluated by the analysis of quality control samples at three different concentrations, determined against a standard calibration curve in a single batch. Inter-assay precision and accuracy was evaluated similarly, for the duplicate analysis of quality control samples in two separate batches (n=4). The acceptance criteria were established as that the mean value at each concentration should have precision that does not exceed 15% of the CV and accuracy within 15% of the nominal concentration.

5.3.3.4 Sample spot volume

The effect of sample spot volume was evaluated for a range of dried plasma spot sampling volumes by identifying a trend in fosfomycin concentration across a range of sample spot volumes (from 5 to 30 μL), and determining if there was unacceptable variability (>5%) in peak area obtained from samples prepared from low volumes (5 μL) to high volumes (30 μL).

5.3.3.5 Matrix effects

Matrix effects were evaluated to identify any suppression or enhancement of signal from an interfering substance around the retention time of fosfomycin by using the matrix factor test. Dried plasma spot samples were prepared with five different blank matrices and extracted in duplicate. The blank matrix extracts and non-matrix (water) samples were spiked with internal standard and low and high concentration levels of fosfomycin and the peak areas measured. The precision of the

matrix factor (normalised against internal standard, where applicable) was used to determine if any concentration level demonstrated unacceptable variability from the expected result.

5.3.3.6 Recovery

The recovery of fosfomycin was evaluated by comparing the peak area for samples spiked with fosfomycin in matrix prior to sample preparation, with samples spiked after sample preparation. Plasma spots, of known volume, were prepared and the whole spot used for analysis. Care was taken to ensure the injection matrix was identical in comparable samples.

5.3.3.7 Storage and Transport Stability

Stability of fosfomycin stored as dried plasma spot samples at room temperature was assessed by comparing the peak area ratio of the dried plasma spot samples stored at room temperature for three months to the peak area ratio of freshly prepared dried plasma spot samples, with each sample prepared at one concentration and analysis in triplicate. Stability of fosfomycin during transport was assessed by comparing the peak area of dried plasma spot samples, at two concentrations, stored at 50°C for four hours to the peak area of dried plasma spot samples stored at room temperature for four hours. Results for stability were considered acceptable when the % difference of the stored sample from the original result was within 15%.

5.3.4 Pharmacokinetic Application

The method was developed and validated to determine the suitability of the analysis of fosfomycin samples stored as dried plasma spots from a pharmacokinetic clinical trial with critically ill patients receiving intravenous fosfomycin for the treatment of an infection in an Intensive Care Unit.

One critically ill patient was administered an intravenous dose of 6 g fosfomycin disodium, every eight hours. Blood samples (3 mL) were taken prior to dosing (0 h) and 0.5, 0.75, 1, 1.5, 2, 4, and 6 h post administration using heparinised vacuum tubes (Greiner Bio-One, Vacuette® LiHep) on the second day of fosfomycin administration and on the fifth day of fosfomycin dosing. Blood samples were centrifuged at 3000 rpm for 10 min to obtain plasma samples. Plasma samples were transferred into 2 mL polypropylene tubes, capped and stored at -80°C. On receipt in to the laboratory, and immediately prior to plasma analysis, samples were thawed and 20 µL dispensed onto sample collection paper. The dried plasma spot clinical samples were then dried for 2 hours at room temperature in a Class 2 Biosafety Cabinet with filtered, circulating air. The dried plasma spot clinical samples were then stored in sealed plastic bags at room temperature until analysis.

This clinical procedure was conducted in accordance with the principles laid down by the ICH guidelines for Good Clinical Practice and approved by the University of Queensland Medical

Research Review Committee (clearance # 2012000870) and the Epistimoniko Symvouleio (Scientific Committee) of Attikon University Hospital (approval ΜΕΘ-84/13-3-12).

The samples from the pharmacokinetic profile acquired are used to evaluate the correlation between the use of dried plasma spot sampling to the gold-standard sampling technique of frozen plasma. Correlation between the two methods was assessed with a Bland Altman plot (Figure 2b) using Microsoft® Excel® for Mac 2011, version 14.2.2, with 95% confidence intervals reported for the mean and limits of agreement.

5.3.5 Results and Discussion

5.3.5.1 Use of Dried Plasma Spot Extraction

The use of dried plasma spot allows a very simple extraction of drug from the proteinaceous matrix. The extraction performed in this method was clean and did not require centrifugation prior to LC-MS/MS analysis. The LOD and repeatability of LLOQ demonstrate the ability of the method to use very small samples - with a sub-punch from a 20 μL sample being used in the extraction – and sample volume testing demonstrating it is not necessary to apply precise volumes of plasma in order to obtain accurate results.

The preparation of dried plasma spots, with venous sampling and subsequent centrifugation, is more onerous than the preparation of dried blood spots, the advantages of transportation, storage and simplicity of extraction are still realised relative to traditional liquid plasma samples. The use of membrane filtration devices to directly form dried plasma spot samples may offer a simplification to preparation [100, 101] and with this the dried plasma spot sampling techniques may offer more of the benefits currently available with dried blood spots – smaller sample volumes from thumb or needle-prick, no pre-treatment prior to shipment – without the inherent haematocrit challenges of samples containing red blood cells.

5.3.5.2 Validation

A linear regression provided the simplest calibration model across the concentration range, and the $1/concentration^2$ weighting improved the correlation coefficient considerably. This model met the acceptance criteria with the back-calculated concentration results being within 20% deviation from the LLOQ and within 15% deviation of standards other than the LLOQ, from nominal concentrations, for all ten of the non—zero standards. The calibration range, mean correlation coefficient (r^2) and the percentage of maximum deviation (inaccuracy) of the standards of the three calibration curves are presented in Table 5.3-1.

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Table 5.3-1 Linearity Analysis

Sample	Calibration range	Correlation coefficient *	Maximum deviation (%)**
Dried plasma spot	5 to 2000 µg/mL	0.9983	7.6

*Mean (n = 3)

** Reported maximum deviation from nominal (%) across all standard curves and concentration levels

The precision and accuracy (n=5) of the dried plasma spot sample extraction at the LLOQ are reported in Table 5.3-2, with all five individual accuracy results within 10%. The limit of detection (LOD) is defined as being reliably distinguished from the background noise and calculated as ≥ three-times the noise of the blank plasma sample. From the validation the LLOQ ratio of signal to noise was calculated as 35, and therefore the LOD was estimated as being approximately 0.5 µg/mL for the dried plasma spot sample extraction.

Table 5.3-2 Intra- and inter-assay precision and accuracy, matrix, recovery and stability

Study	Concentration (mg /L)	Sample #	Mean	Accuracy (%)	Precision (%)
LLOQ	5	5	4.96	-0.8	±6.3
Intra-assay Precision & Accuracy	15	5	15.6	+3.9	±4.2
	80	5	79.9	-0.1	±8.2
	1600	5	1581	-1.2	±2.0
Inter-assay Precision & Accuracy	15	4	15.7	+4.3	±9.0
	80	4	77.8	-2.8	±5.2
	1600	4	1632	+2.0	±2.3
Matrix	50	5	1.00[a]		±0.8
	500	5	1.02[a]		±2.7
Recovery	500	3	83.6%		±4.7
Stability RT[b]	500	3		-4.9 loss	
Stability Trans[c]	500	3		0.2 increase	

[a] matrix factor: calculated as a ratio of peak area of analyte in the presence of matrix to the peak area in the absence of matrix (normalised using the internal standard).
[b] stability at room temperature - compared over three months of storage as a dried plasma spot sample.
[c] stability during transport at 50°C - compared to 4 hours of storage as a dried plasma spot sample

The precision and accuracy of the dried plasma spot sample assay at the prepared QC levels are presented in Table 5.3-2; the acceptance criteria were met in all cases.

Sample volume testing demonstrated no trends across a range of sample spot volumes and a +1.6% difference in peak area obtained between a sub-punch from a 5 μL sample spot and a 30 μL sample spot.

No signal suppression/enhancement was evident for fosfomycin or the internal standard (ethylphosphonic acid) for extraction using dried plasma spot samples, from the matrix studies performed as evidenced by significant bias or variability. The results of the matrix effect evaluations are described in Table 5.3-2.

Validation revealed adequate recoveries for both fosfomycin (83.6%) and ethylphosphonic acid (87.1%) from dried plasma spots. The performance of the method was not adversely affected by the less than complete recovery as demonstrated by there being sufficient signal at the LLOQ, and acceptable precision and accuracy validation.

Stability of fosfomycin in plasma when applied to sample collection paper and stored at room temperature for 3 months was acceptable with a difference in peak area ratio observed of -4.9% at 500 μg/mL of fosfomycin. Stability of fosfomycin during transport at 50°C for four hours was acceptable with a mean difference observed of 0.2%.

Stock solution stability for fosfomycin has been previously reported as being stable for aqueous solutions stored for over 16 months at -80°C and for over 11 months at -20°C; freeze-thaw stability in liquid plasma samples has also been previously reported as being stable across three freeze-thaw cycles [136]. In-house data on storage of frozen plasma samples containing fosfomycin was acceptable with a difference in concentration of 11.6% at -20°C for 20 months.

5.3.5.3 Application

This method has been successfully applied to samples of a clinical pharmacokinetic study. The plasma concentration-time patient profile for one patient is presented in Figure 5.3-1a. A 58 year old male provided samples after receiving his fourth and thirteenth dose of 6 g intravenous fosfomycin disodium, every 8 hours. The results of the Bland-Altman plot (Figure 5.3-1b) demonstrate that the DPS concentrations were subject to a consistent negative bias (16.6%) compared to the plasma samples, with the precision from the mean result relatively limited (7%). The resulting negative bias may reflect variability between the patient plasma and the plasma used to generate the calibration line. Substantial changes in protein content may influence the spreading on the filter paper and this has the potential to produce this result. The 95% confidence interval for

the mean is -12.9 to -20.4%. The mean limits of agreement ranged from -2.6 to 30.6%. The 95% confidence interval for the upper limit of agreement was -9.1 to 3.8%, and for the lower limit of agreement was -37.1 to -24.2%. Of the 16 samples analysed the difference between the DPS and plasma concentrations were within 20% of the mean result for 12 of the samples (75%). The FDA acceptance criteria of an incurred sample reanalysis is that 67% of the repeated samples should be within 20% for small molecules[67]. Therefore, the results fulfil the criteria of an incurred sample reanalysis. This criteria is intended for replicate analysis whereas the results here are from different matrix preparations, different extraction procedures, analysed in small and single analytical runs, and separate analytical runs (with whole plasma performed separately to the DPS analysis), and prepared from different standard solution preparations.

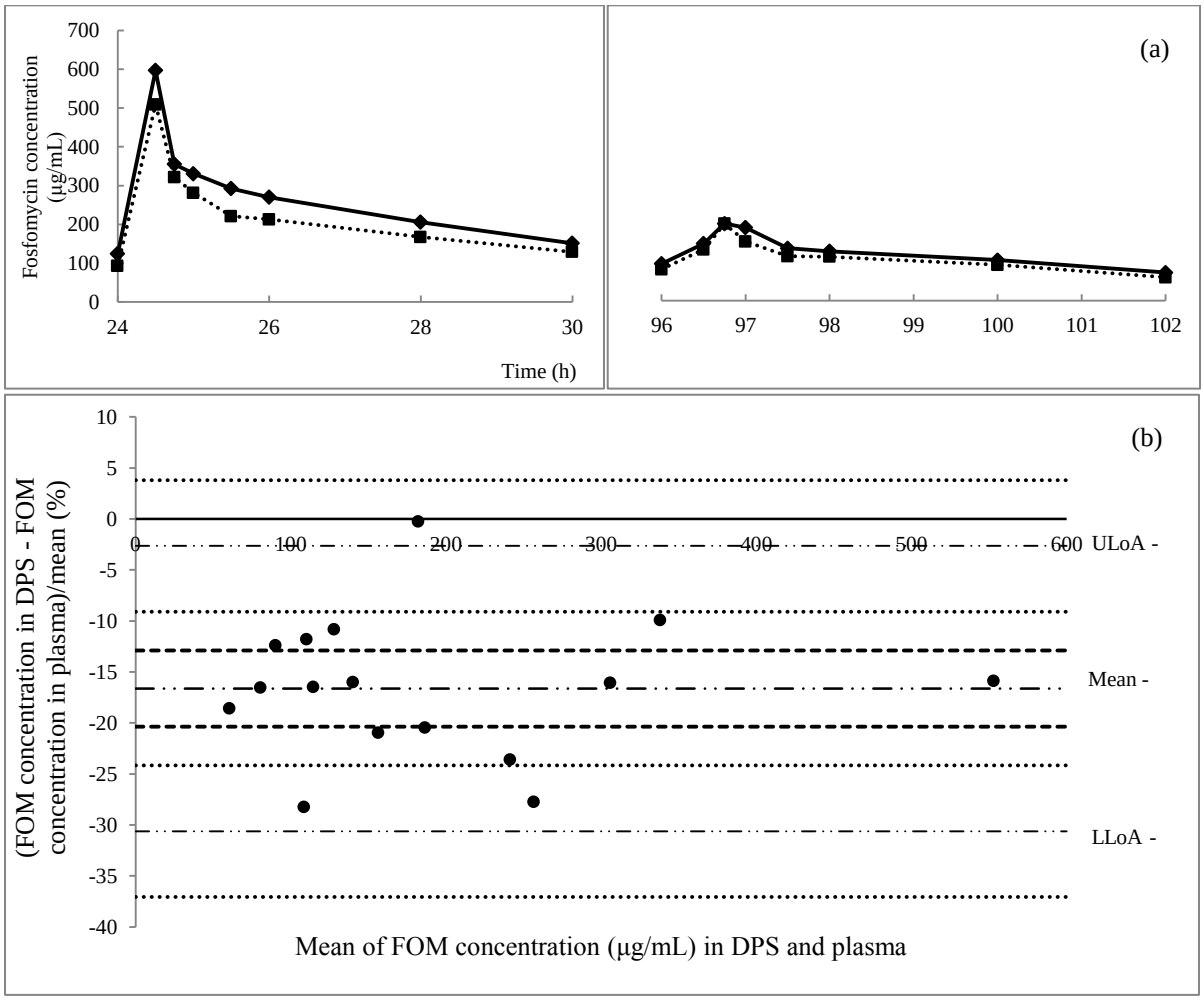

Figure 5.3-1(a) Plasma concentration – time profiles of fosfomycin in a critically ill patient receiving a 6 g fosfomycin IV dose every 8 hours, for the fourth and thirteenth doses, prepared using an extraction from plasma (diamond, full-line) and dried plasma spots (DPS, squares, dotted line); 2(b): Bland-Altman plot between plasma and DPS samples obtained for fosfomycin (FOM) concentrations from a critically-ill patient. The mean difference

demonstrated a negative bias of 16.6%, with the mean limits of agreement ranging from -2.6 to 30.6%.

5.3.6 Conclusion

The method presented here offers a validated quantitative analysis of fosfomycin using a dried plasma spot sampling technique. The assay performance is accurate and precise, with sufficient sensitivity and range of calibration for a clinical pharmacokinetic study. The dried plasma spot sampling technique has advantages over traditional plasma sampling in terms of storage temperature, transport and simplicity of sample preparation. The results of the dried plasma spot samples from a clinical pharmacokinetic study have been found to strongly correlate with the gold standard of plasma sampling. The results of the Bland-Altman plot demonstrate that the DPS concentrations were subject to a consistent negative bias (16.6%) compared to the plasma samples, with the precision from the mean result relatively limited (7%). The resulting negative bias may reflect variability between the patient plasma and the plasma used to generate the calibration line. Substantial changes in protein content may influence the spreading on the filter paper and this has the potential to produce this result. This method has been demonstrated to be suitable for clinical pharmacokinetic study applications for fosfomycin and may provide greater opportunities for collaborative research in critically ill populations, including, burns or obese patients.

5.4 Conclusion

This Chapter has described investigations into the suitability of translating pharmacokinetic microsampling techniques into clinical studies. As haematocrit has been identified as a confounding factor for the use of whole blood sampling, a preliminary investigation sought to measure the impact of haematocrit on fosfomycin measurements in whole blood samples using the VAMS devices. The VAMS devices produced evidence of bias for the analysis of fosfomycin, where an inverse correlation is observed, with a positive bias at lower haematocrit levels and a negative bias at higher haematocrit levels. As the VAMS devices reduce bias by sampling an applied volume (10 μL), the bias observed when using the VAMS device for fosfomycin may be due to a change in recovery of the analyte or a matrix effect across haematocrit levels. While the mean values of the results were within the 15% acceptance criteria, the results warranted further investigation that was subsequently conducted as part of the quantitative bioanalytical validation into recovery and matrix effects.

The quantitative bioanalytical validation investigating the VAMS devices for whole blood sampling of fosfomycin provided acceptable validation data as assessed for matrix effects, lower limit of quantification (LLOQ), linearity, and inter- and intra- day precision and accuracy.

Results from recovery testing and testing of the stability of dried whole blood samples on VAMS devices for use with the analysis of fosfomycin, suggest the impact of haematocrit or other components in whole blood may present challenges and require optimisation of the extraction when samples are stored. The inclusion of testing of recovery on these stored samples is warranted.

Additionally, a successful validation of DPS sampling for fosfomycin for application in a clinical pharmacokinetic study in critically ill patients was described in this Chapter. The results of this study support the translation of DPS sampling of fosfomycin into clinical pharmacokinetic studies, with the data provided by DPS sampling being comparable to the gold standard of liquid plasma sampling.

The antibiotic fosfomycin was selected for this analysis as it is of considerable interest due to its effectiveness as a treatment for multi-drug resistant pathogens [107], it has negligible protein binding [43] and has demonstrated considerable aqueous long-term stability (16 months at -80°C) [136].

Part 4

Results – clinical pharmacokinetic study

Chapter 6: The Potential Use of Fosfomycin in Treating Infections in Critically Ill Patients

6.1 Synopsis

The aim of this chapter is to describe the role of intravenous fosfomycin in the treatment of critically ill patients suffering from infections caused by multi-drug resistant pathogens. The population pharmacokinetics of fosfomycin in critically ill patients is described. This model is then used for Monte Carlo simulations to provide robust dosing regimens that can ensure the achievement of effective intravenous fosfomycin concentrations in critically ill patients.

6.2 Published manuscript entitled: Population pharmacokinetics of fosfomycin in critically ill patients.

The manuscript entitled, "Population pharmacokinetics of fosfomycin in critically ill patients" has been accepted for publication by Antimicrobial Agents and Chemotherapy (59: 10 (2015) 6471-6476).

The authors contributed to the manuscript as follows: the PhD candidate, Suzanne Parker, under the supervision of Dr. Jason Roberts, developed and validated the pharmacokinetic model. The PhD candidate, Suzanne Parker, took the leading role in manuscript preparation and writing. Dr Steven Wallis assisted with bio-analysis of the pharmacokinetic samples. Frantzeska Frantzeskaki, Chryssa Diakaki, Helen Giamarellou, Despoina Koulenti, and Elias Karaiskos performed and coordinated the clinical trial. Prof Jason Roberts and Prof George Dimopoulos and Prof Jeffrey Lipman oversaw all aspects of this paper.

The manuscript is presented as submitted; except figures and tables have been inserted into the text at slightly different positions. Also, the numbering of pages, figures and tables has been adjusted to fit the overall style of the Thesis. The references are found alongside the other references of the Thesis, in the section 'Bibliography'.

Antimicrobial Agents and Chemotherapy, impact factor: 4.476

Population pharmacokinetics of fosfomycin in critically ill patients

Suzanne L. Parker[a], *Frantzeska Frantzeskaki*[c,d], *Steven C. Wallis*[a], *Chryssa Diakaki*[c,d], *Helen Giamarellou*[e], *Despoina Koulenti*[a,e], *Ilias Karaiskos*[e], *Jeffrey Lipman*[a,b], *George Dimopoulos*[d,e*], *Jason A. Roberts*[a,b*#]

Burns, Trauma and Critical Care Research Centre,

The University of Queensland, Brisbane, Australia[a];

Royal Brisbane and Women's Hospital, Brisbane, Australia[b];

Critical Care Department, Attikon University Hospital, Athens, Greece[c];

Medical School, University of Athens, Athens, Greece[d];

Sixth Department of Internal Medicine, Hygeia Hospital, Athens, Greece[e]

Running head: Population pharmacokinetics of fosfomycin.

Address correspondence to:

Prof Jason A. Roberts j.roberts2@uq.edu.au

* Joint senior authors

Abstract

This study describes the population pharmacokinetics of fosfomycin in critically ill patients. In this observational study, serial blood samples were taken over several dosing intervals of intravenous fosfomycin. Blood samples were analysed using a validated liquid chromatography tandem mass spectrometry technique. A population pharmacokinetic analysis was performed using non-linear mixed effects modelling. Five hundred and fifteen blood samples were collected over one to six dosing intervals from 12 patients. The mean (SD) age was 62 (17) years, 67% were male and creatinine clearance (CL_{CR}) ranged from 30 to 300 mL/min. A two-compartment model with between-subject variability on clearance and volume of distribution of the central compartment (Vc) described the data adequately. Measured CL_{CR} was supported as a covariate on fosfomycin clearance, as was patient weight. The median parameter estimates for clearance on the first day was 2.06 L/h, Vc was 27.2 L, intercompartmental clearance was 19.8 L/h and volume of the peripheral compartment was 22.3 L. This study found significant pharmacokinetic variability of fosfomycin in this heterogeneous sample, which may be explained somewhat by variations in renal function.

6.2.1 Introduction

Inadequate treatment of infections among patients requiring intensive care unit (ICU) admission is an important determinant of hospital mortality [26]. Drug dosing should be considered an essential part of optimizing antibiotic use. However, critically ill patients have been shown to have significant pharmacokinetic variability for some antibiotics due to the physiological changes associated with this pathology [155]. This variability has been shown to impact on the achievement of therapeutic exposures of antibiotics [156-160]. If not considered in dosing regimens this pharmacokinetic variability can lead to clinical failure or toxicity [161].

Fosfomycin is a broad-spectrum antibiotic with bactericidal activity against Gram-negative and Gram-positive bacteria, and has been gaining considerable attention recently due to its effectiveness as a treatment for multi-drug resistant pathogens [107], including extended spectrum beta-lactamase and carbapenemase producing bacteria. Fosfomycin exhibits extensive penetration into many tissue types [37, 47, 49, 162] and is well tolerated, with only minor adverse events reported [163],[164]. However, there is little data on the pharmacokinetics of this potentially valuable antibiotic in critically ill patients and as such, significant uncertainty relating to appropriate dosing exists.

Dosing guidelines from the European Committee on Antimicrobial Susceptibility and Testing (EUCAST) recommend dosing schedules of 3-4 g fosfomycin three times daily up to maximum dose as 5-8 g fosfomycin three times daily [165]. However, the data supporting these recommendations are unclear given we are unaware of any studies investigating the population pharmacokinetics of intravenous fosfomycin in critically ill patients.

The aim of this study was to describe the population pharmacokinetics of fosfomycin in critically ill patients.

6.2.2 Materials and Methods

6.2.2.1 Patients

This study was performed in the ICU of two hospitals (Attikon University Hospital and Hygeia Hospital, Greece). Ethical approval to conduct the study was obtained from the local institutional ethics committee (approval ΜΕΘ-84/13-3-12). Consent to participate was obtained from the patient or the patient's legally authorised representative.

Critically ill patients who were prescribed fosfomycin by the treating physician were eligible for inclusion. In accordance with usual practice, all patients had an indwelling arterial cannula. Patients meeting any of the following criteria were excluded: (i) age less than 18 years; (ii) recent use of fosfomycin within the previous month; (iii) pregnancy or lactation; and (iv) consent not obtained.

The severity of illness of each patient was described using the Acute Physiology and Chronic Health Evaluation (APACHE) II [166] and sequential organ failure assessment (SOFA) [167] scores determined on two days of fosfomycin treatment, day 1 and 4. Serum creatinine concentrations were collected as a routine procedure in all patients, with creatinine clearance (CL_{CR}) calculated daily using the Cockcroft-Gault equation [168]. A measured urinary CL_{CR} over a 24 h time period was collected on the first day of sampling. Serum biochemistry, including albumin concentrations, white cell count, and bilirubin concentrations, were recorded. Concomitant antibiotic treatment was also recorded, as was length of stay in the ICU and hospital stay, overall mortality and outcome of treatment.

6.2.2.2 Fosfomycin Treatment

In accordance with the study protocol treatment with fosfomycin was added to therapy only after the culture results became available. The fosfomycin MIC was determined by Etest (Biomerieux, Marcy-I' Etoile, France). Results were interpreted according to EUCAST 2013 criteria [169]. Administration of fosfomycin was by IV infusion over 30 to 60 minutes in accordance with local guidelines. The choice of antibiotic dose was at the discretion of the treating physician; with doses of 4 or 6 g of fosfomycin used at a frequency of three or four times daily, common in both ICUs.

6.2.2.3 Sample Collection

Blood samples were collected from an indwelling arterial cannula before the drug administration and at 30 min, 45 min, 1 h, 1.5 h, 2 h, 4 h, and 6 h after administration of fosfomycin. Where possible sampling occurred during the first dosing interval and/or on days 2, 4, 5, 6 and 7.

6.2.2.4 Drug Assay

Plasma fosfomycin concentrations were determined using a high performance liquid chromatography tandem mass spectrometry method previously described [136] by the Burns Trauma and Critical Care Research Centre, The University of Queensland, Australia. The assay inter-day coefficients of variation for fosfomycin in plasma were ≤9.1 %, with an accuracy range of -7.2 to 3.3 %. The assay limit for plasma was 1 mg/L, with precision at 4.7 %, and accuracy of 1.7 %. The linearity of the assay (r^2) was 0.9963 (n = 12).

6.2.3 Pharmacokinetic and Statistical Analysis

The concentration-time data was analysed using non-linear mixed-effects modelling (NONMEM version 7.3, Globomax LLC, Hanover, MD, USA). A digital Fortran compiler was used and the runs were executed using Wings for NONMEM (http://wfn.sourceforge.net). First-order conditional estimation method with interaction was used throughout the model building.

6.2.3.1 Model Development

For the population pharmacokinetic analysis, the one- and two-compartment linear models were fitted into plasma fosfomycin concentration data, using subroutines from the NONMEM library. Between-subject variability (BSV) was best described using an exponential variability model. Residual unexplained variability was tested using various model iterations.

6.2.3.2 Model Diagnostics

The goodness of fit of the model was evaluated using visual inspection of diagnostic scatter plots and the NONMEM objective function (OFV). A statistical comparison of nested models was undertaken with a decrease in OFV of 3.84 units ($P<0.05$) considered statistically significant.

6.2.3.3 Covariate Screening

Covariate model building was performed using a sequential of assessment of biologically plausible clinical parameters. Forward inclusion was based upon the aforementioned model selection criteria and significant correlation with one of the pharmacokinetic parameters. The covariates evaluated were: calculated and measured urinary CL_{CR}, age, sex, weight and serum albumin concentration.

6.2.3.4 Bootstrap

A NONMEM non-parametric bootstrap method (n=1000) was used to study the uncertainty of the pharmacokinetic parameter estimates in the final model. Using the bootstrap empirical posterior distribution we obtained the 95% confidence interval (CI, 2.5 to 97.5 percentiles) for the model parameters, using methods previously described [170].

6.2.4 Results

6.2.4.1 Patient Characteristics

A total of 515 plasma samples were collected over one to six dosing intervals from 12 enrolled patients. The demographic and clinical characteristics of the patients are shown in Table 6.2-1. All patients received a dose of 6 g of fosfomycin every 6 hours, except for patients 7 and 9. Patient 7 received a dose of 4 g of fosfomycin every 6 hours and patient 9 received a dose of 6 g of fosfomycin every 8 hours. The microbiology for these infections, concomitant antibiotics and patient outcomes are described (next page) in Table 6.2-2. All patients were diagnosed as having septic shock and respiratory failure and all patients were intubated.

Table 6.2-1 Patient characteristics

Age (years), median (IQR*)	62.5 (17.3)
Weight (kg), median (IQR)	71.5 (10.5)
Body mass index (kg/m^2), median (IQR)	26.4 (3.6)
Male(%)/ Female (%)	8 (67)/4 (33)
APACHE II score on ICU admission, median (IQR)	11.5 (7.8)
SOFA score on ICU admission, median (IQR)	7 (5)
CL_{CR} on admission (mL/min), median (IQR)	59 (47)
Albumin concentration (g/dL), median (IQR)	2.7 (0.6)

*Interquartile range; APACHE, Acute Physiology and Chronic Health Evaluation; ICU, Intensive Care Unit; SOFA, Sequential Organ Failure Assessment; CL_{CR}, Creatinine Clearance

Table 6.2-2 Specimen, isolated organism and their susceptibility, concomitant antibiotics.

#	Specimen	Organism	Susceptibility	Concomitant antibiotic	Length of stay in ICU (day)	Length of stay in hospital (day)	Outcome of treatment
1	Tracheal aspirate	KPC- *Klebsiella pneumoniae*	I: Gentamicin S; Fosfomycin MIC 24 mg/L	Gentamicin Tigecycline	42	60	Improvement
2	Peritoneal fluid	KPC- *Klebsiella pneumoniae*	S: Gentamicin	Gentamicin Colistin Linezolid	28	90	Improvement
	Bronchial secretion	*Pseudomonas aeruginosa*	S: Colistin				
		Acinetobacter baumannii	S: Colistin				
		KPC- *Klebsiella pneumoniae*	S: Gentamicin S. Fosfomycin MIC 18 mg/L				
	Stool	KPC- *Klebsiella pneumoniae*	I: Amikacin S: Fosfomycin MIC 24 mg/L				
3	Tracheal aspirate	*Klebsiella pneumoniae*	PDR R : Fosfomycin 128 mg/L	Ampilcillin/SulbactamColistin Cefepime	28	48	Death on 8th day of treatment
		Acinetobacter baumannii	PDR				
		Stenotrophonomas maltophilia	PDR				
4	Bronchial aspirate	KPC-*Klebsiella pneumoniae*	I: Cefepime, I: Gentamicin I: Meropenem S: Fosfomycin MIC 32 mg/L	Cefepime	46	66	Death on 9th day of treatment
		Acinetobacter baumannii	I: Ampicillin/Sulbactam				

		Candida parapsilosis					
	Urine	*Candida parapsilosis*					
5	Pleural effusion	*Stenotrophonomas maltophilia*	S: Minocycline S: Trimethoprim/sulfamethoxazole S: Levofloxacin	Meropenem Linezolid	42	82	Death 11 days after end of treatment
		KPC-*Klebsiella pneumoniae*	S: Gentamicin S: Tobramycin S: Amoxicillin S: Colistin S: Fosfomycin : 32 mg/L				
6	Tracheal aspirate	*Acinetobacter baumannii*	PDR	Gentamicin, Meropenem, Colistin	40	55	Death on 2nd day of treatment
	Stool	KPC- *Klebsiella pneumoniae*	S: Gentamicin S; Fosfomycin MIC 42 mg/L				
7	Surgical wound of septic arthritis	*Acinetobacter baumannii*	S: Colistin	Tigecycline Meropenem Colistin	53	76	Death on 15th day of treatment
		Pseudomonas aeruginosa	S: Colistin S: Aztreonam				
	Bone from septic arthritis	*Klebsiella pneumoniae*	S: Gentamicin S: Trimethoprim/sulfamethoxazole S: Fosfomycin MIC 32 mg/L				
	Urine	KPC- *Klebsiella pneumoniae*	PDR R: Fosfomycin MIC 128 mg/L				

	Rectal	KPC- *Klebsiella pneumoniae* PDR S: Fosfomycin MIC 32 mg/L *Acinetobacter baumannii* S: Colistin					
8	Sputum	KPC- *Klebsiella pneumoniae* S: Amikacin S: Minocycline S: Tetracycline S: Tigecycline S: Colistin S: Fosfomycin MIC 32 mg/L	Colistin Tigecycline	12	35	Death on 4th day of treatment	
		Pseudomonas aeruginosa S: Colistin R: Fosfomycin					
9	Bronchial secretion	KPC- *Klebsiella pneumoniae* S: Fosfomycin MIC 64 mg/L		25	25	Death on 4th day of treatment	
10	Blood	*Klebsiella pneumoniae* S: Fosfomycin MIC 32 mg/L	Colistin	36	36	Improvement	
11	Bronchial secretion	*Klebsiella pneumoniae* S: Fosfomycin MIC 18 mg/L	Meropenem	14	14	Improvement*	
12	Blood	*Klebsiella pneumoniae* S: Colistin S; Fosfomycin 64 mg/L	Colistin	6	210	Improvement**	

* Fosfomycin discontinued after one day, due to infection identified as ESBL--*Klebsiella pneumoniae;* ** Fosfomycin discontinued after one day, due to allergic rash;KPC, Klebsiella pneumoniae Carbapenemase; I, Intermediate; S, Susceptible; PDR, Pan Drug Resistant; ICU, Intensive Care Unit; ESBL, Extended Spectrum beta-lactamases

6.2.4.2 Patient Plasma-Concentration Data

The median trough fosfomycin plasma concentration (C_{min}) for patients during the first sampling interval was 84.3 mg/L, with the range of concentrations observed from 41 to 172 mg/L. On Day 2 the C_{min} was 250 mg/L (range 76 to 684 mg/L). On Day 7 two patients had a C_{min} >1000 mg/L with two others have a C_{min} <100 mg/L.

6.2.4.3 Pharmacokinetic Analysis

The best base model consisted of a two-compartment linear model with zero-order input (ADVAN3 TRANS4) incorporating an exponential residual unknown variability. BSV was supported on clearance (CL) and volume (V) of the central compartment.

Calculated urinary CL_{CR} using Cockcroft-Gault equation and patient weight were supported as covariates in the final model. Inclusion of patient weight normalised to the population mean value improved the goodness of fit plots and was therefore included in the model.

The final model is represented is follows:

(1) $TVCL = (\theta_{1\text{-}6} \times CL_{CR}/90)$

(2) $TVV1 = \theta_2 \times (WT/70)^{0.75}$

Where TVCL is the typical value of CL and $\theta_{1\text{-}6}$ is the typical value of fosfomycin CL in the population, with each sampling day defined as an individual θ, from Day 1 (θ_1), 2 (θ_2), 4 (θ_3), 5 (θ_4), 6 (θ_5), and 7 (θ_6). CL_{CR} was normalised to the mean value for the patients and WT is patient weight.

The inclusion of between subject variability to CL and V1 was associated with a decrease in the objective function of 659 and 64.4, respectively. The inclusion of calculated CL_{CR} was associated with a decrease in the objective function of 79.7. Allometric scaling was applied *a priori* to V1 and was standardised to a bodyweight of 70 kg. All were therefore statistically significant and included in the final model. Figure 6.2-1(a) displays the diagnostic goodness-of-fit plot for the final population pharmacokinetic covariate model. Figure 6.2-1(b) displays a visual predictive check for the final covariate model from the first day of sampling through to the sixth day of sampling. As diagnostic checks of the model Figure 6.2-1 demonstrates that the final pharmacokinetic model provides an adequate description of the measured concentrations of fosfomycin from this highly heterogenous sample of patients of widely varied age and renal function. The fit of the model was acceptable in terms of visual or statistical biases for the prediction. Having met the criteria for inclusion the model was accepted

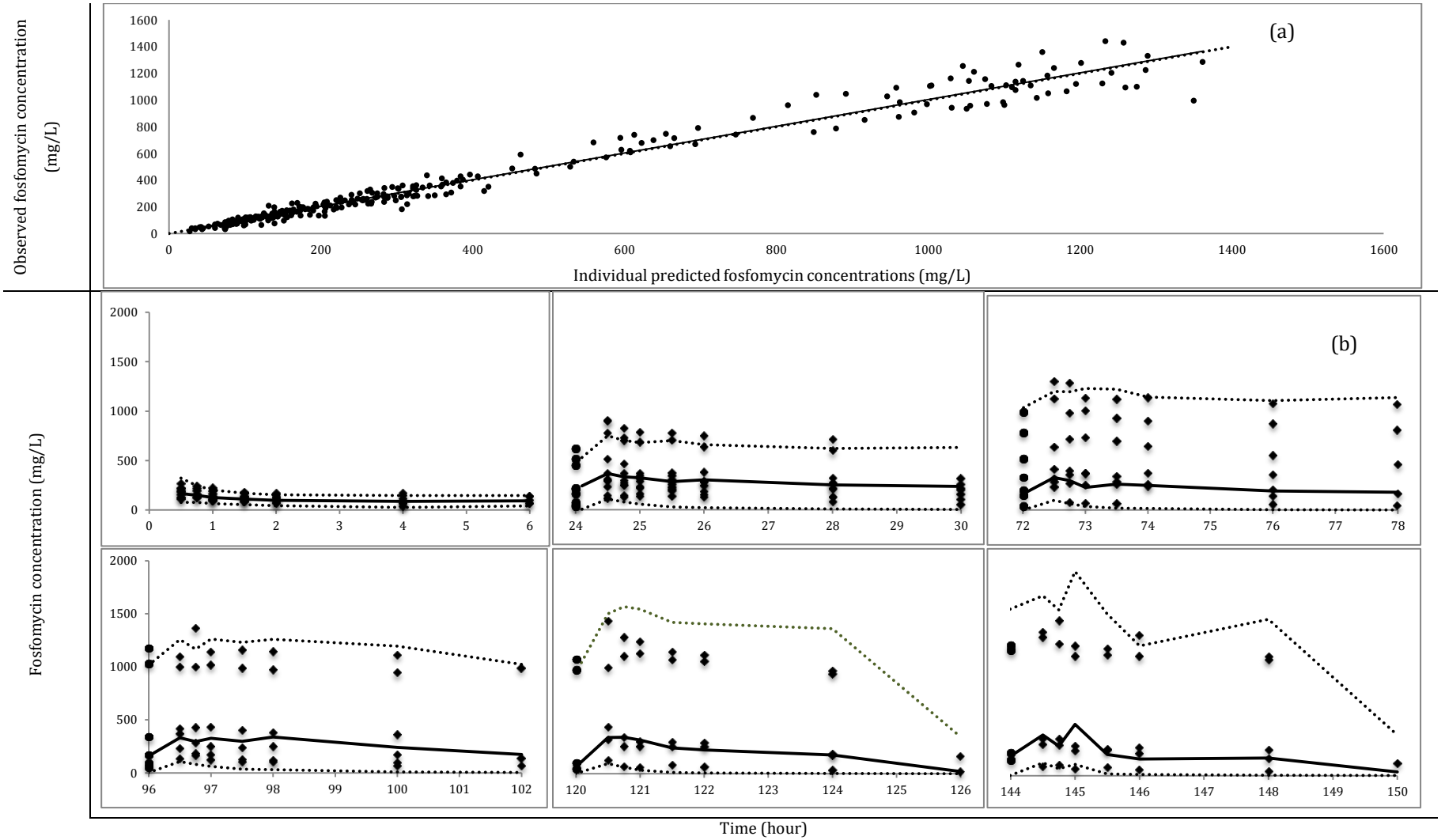

Figure 6.2-1 Diagnostic plots for the final population pharmacokinetic covariate model. (a) Individual predicted fosfomycin concentrations versus observed concentrations (r^2 = 0.97), non-linear regression line of best fit is shown by the black continuous line and the line of identity *xy* is shown by the black broken line. (b) Visual predictive check generated from a Monte Carlo simulation (n=1000) showing that the estimated population pharmacokinetic model has adequate performance (from 0 to 150 hours of dosing shown only), the raw data are shown as black dots.

The parameter estimates for the final model are given in Table 6.2-3 and include the 95% values from all bootstrap runs.

Table 6.2-3 Bootstrap parameter estimates of the final covariate model

	Model	Bootstrap		
			95% CI	
	median	median	2.5%	97.5%
Fixed Effects				
Clearance – occasion 1 (L/h)	2.06	2.19	1.11	5.11
Clearance – occasion 2 (L/h)	5.57	5.06	2.76	9.37
Clearance – occasion 4 (L/h)	4.67	4.05	2.46	7.42
Clearance – occasion 5 (L/h)	4.13	4.13	2.29	9.37
Clearance – occasion 6 (L/h)	5.42	4.93	2.59	9.96
Clearance – occasion 7 (L/h)	5.19	4.74	2.31	9.09
Volume of the central compartment (L)	26.5	26.4	19.0	34.2
Volume of the peripheral compartment (L)	22.3	21.6	12.7	34.9
Inter-compartmental CL (L/h)	19.8	19.8	8.01	46.0
Random effects, BSV (%CV)				
Clearance (L/h)	91.9	81.9	48.1	128
Volume of the central compartment (L)	39.0	36.7	18.0	57.2
Random error				
Exponential (% CV)	16.4	15.5	10.5	19.2

Legend: BSV – between subject variability; CV – coefficient of variation

6.2.5 Discussion

Fosfomycin is currently being used as a last-line treatment for critically ill patients for the treatment of serious infections, yet there is limited information available describing the pharmacokinetics of fosfomycin in this sub-population [107]. We found that the CL was proportional to calculated $CL_{CR,}$ with increasing CL_{CR} affecting the likelihood that fosfomycin doses would achieve target concentrations for relevant MICs. Standard dosing of 3 or 4 g of fosfomycin, as a 30 minute IV

infusion, every 8 h appears inadequate for achieving concentration targets for patients with $CL_{CR} \geq$ 130 mL/min for pathogens with a MIC of 16 mg/L [165].

A previous study from Kirby et al, tested intravenous doses of fosfomycin in healthy patients of 4 g every 6 h and showed accumulation with peak serum concentrations increasing from 195 mg/L after the first dose up to 253 mg/L after the second dose [42]. CL was reported as 7.2 L/h. This mean CL is substantially higher than our median CL 2.06 L/h on the first day of patient sampling and demonstrates the need for careful monitoring of fosfomycin dosing in critically ill patients, particularly if there is evidence of renal dysfunction. While fosfomycin is well tolerated and has a low adverse event profile [163], in this study peak plasma concentrations were observed in some patients with low CL_{CR} up to 1440 mg/L after multiple days of treatment.

The mean apparent volume of distribution in this study was 48.8 L, which is higher than that observed by Kirby (22 L) [42] in healthy volunteers, and is likely to due to presence of greater sickness severity. A larger than normal volume of distribution is one of the typical pathophysiological changes observed in critically ill patients and has been reported in multiple pharmacokinetic studies for different antibiotics [59, 160].

In this study of twelve patients, two patients consistently had CL_{CR} >200 mL/min. Augmented renal clearance – defined as a sustained elevation of CL_{CR} (>130 mL/min/1.73m^2) – manifests in over 65% of critically ill patients in the first week of admission into an ICU [171] and, as seen in this study, is likely to impact on the probability of attainment of therapeutic exposures of renally cleared antibiotics. Renal insufficiency is associated with dramatically higher fosfomycin concentrations, and two patients consistently had CL_{CR} <50mL/min/1.73m^2. The pharmacokinetic variability observed in this study is explained somewhat by the wide ranges of renal function.

This study has some limitations we would like to declare. We have not investigated the free concentrations of fosfomycin in plasma, or the concentrations at the site of infection. Instead we have measured the total drug concentration. However, the antimicrobial activity of fosfomycin has been shown to not be affected by the presence of albumin [43], and is considered to have negligible protein binding. Also, given this study is of a relatively small cohort of twelve patients, and given the high extremes of renal function in patients and sample collection over up to 6 dosing intervals, this could also be considered a limitation. Indeed the small heterogeneous sample may also impact on the model being able to identify other relevant covariates, although this number of patients provides useful data from which clinicians can use to procure more relevant doses [172]. The visual predictive check (Fig 1b) suggests there is a small overestimation of higher concentrations from the model, compared to the individual data points in the later days of sampling. However Fig 1a shows

that the line of x=y overlaps with the regression line confirming that it is not a systematic deviation which supports the adequacy of the final model. Finally, fosfomycin was co-administered as part of a more extensive antibiotic treatment in eight of the twelve patients and this model was not intended to provide any description of pharmacodynamic synergistic effects.

6.2.6 Conclusion

This population pharmacokinetic study found a substantially lower CL and larger than normal volume of distribution of fosfomycin compared to that reported in healthy subjects. The mean parameter estimates for clearance on the first day were 2.06 L/h, Vc 27.2 L, intercompartmental clearance 19.8 L/h and volume of the peripheral compartment 22.3 L. We found significant pharmacokinetic variability of fosfomycin in this heterogeneous patient sample, which may be explained in part by the variations observed in renal function.

6.3 Monte-Carlo Dosing Simulations

Monte Carlo simulations (n=1000) of the final covariate model were used to test the ability of various dosing regimens to achieve target exposures. We simulated various CL_{CR}, 30, 130 and 200 mL/min per dosing regimen. The simulated IV dosages (administered as 30 minute infusions) were of 4 g of fosfomycin every 6 and 8 hours daily; 6 g of fosfomycin every 6 hours daily; and 3 g of fosfomycin every 8 hours daily. We also simulated a 4 h infusion of both 4 g and 8g of fosfomycin every 8 hours. The probability of target attainment was then obtained by determining the proportion of patients with concentrations above MICs of 1, 2, 4, 8, 16, 32, 64, 128 and 256 mg/L for the entire dosing interval.

6.3.1 Results

The probability of MIC target attainment results for the simulated dosing regimens for the different CL_{CR} are shown in Figure 6.3-1. We observed that a standard dose of 3 or 4 g IV fosfomycin, administered as a 30 minute infusion, 8-hourly did not achieve target concentrations for patients with elevated $CL_{CR} \geq 130$ mL/min.

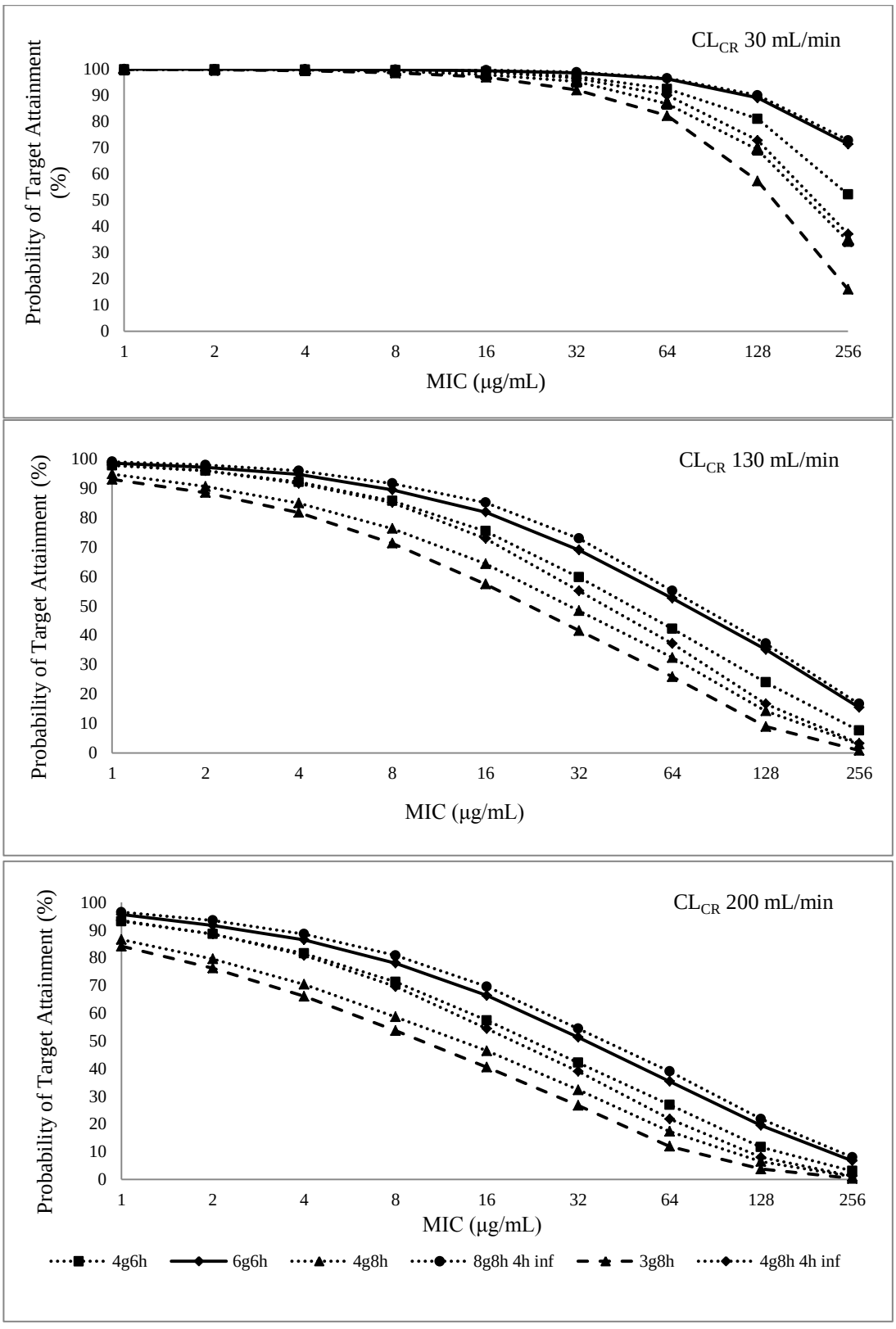

Figure 6.3-1 Probability of target attainment for different dosing strategies for creatinine clearances of (a) 30 mL/min, (b) 130 mL/min, and (c) 200 mL/min for doses (with a 0.5 h infusion) of 4 and 6 g every 6 h3 and 4 g every 8 h; and 4 g and 8g every 8 h with a 4 h infusion.

6.3.2 Discussion

The dosing simulations performed here provide useful guidance of how to dose IV fosfomycin in critically ill patients. EUCAST [165] refers to common dosing of IV fosfomycin as from 3 to 8 g fosfomycin administered three times daily. Simulations of doses of 3 g of fosfomycin, as a 30 minute IV infusion, administered three times daily for patients with $CL_{CR} \geq 130$ mL/min will not ensure 90% probability of target attainment for MIC of 16 mg/L. This standard dose leads to a 57.4% probability of target attainment for patients with $CL_{CR} = 130$ mL/min, for an organism with an MIC of 16 mg/L, and 41.6% probability of target attainment for an organism with an MIC of 32 mg/L. Even prolonged infusions (over 4 hours) of 4 g of fosfomycin administered three times daily did not ensure 90% probability of target attainment for MICs of 8 or 16 mg/L for patients with normal or augmented renal clearance.

6.3.3 Conclusion

The dosing simulations show that contemporary dosing of fosfomycin is inadequate for patients with augmented renal clearance ($CL_{CR} \geq 130$ mL/min) to achieve the target concentrations for most pathogens. Indeed, higher dosing of 4 or 6 g of fosfomycin administered four times a day is more likely to achieve the pharmacokinetic/pharmacodynamic targets in these critically ill patients with elevated CL_{CR}.

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6.4 Conclusion

This Chapter has described the population pharmacokinetics of fosfomycin in critically ill patients and found significantly altered pharmacokinetics in this patient group as compared to healthy subjects. A substantially lower clearance and larger than normal volume of distribution of fosfomycin was observed. When the model was used for Monte Carlo dosing simulations, it was demonstrated that common dosing regimens of fosfomycin remains inadequate for patients with normal or augmented renal clearance ($CL_{CR} \geq 130$ mL/min) to achieve the target concentrations for the eradication of most pathogens. Indeed, higher dosing of 4 or 6 g of fosfomycin administered four times a day was more likely to achieve the pharmacokinetic/pharmacodynamic targets in these critically ill patients with elevated CL_{CR}. Further studies should now be performed in subgroups of critically ill patients including patients receiving renal replacement therapy and extracorporeal membrane oxygenation and use of microsampling techniques would improve the feasibility of these studies.

Given that Chapter 5 confirms the appropriateness of VAMS and DPS microsampling techniques for use in pharmacokinetic studies and the present Chapter demonstrates the pharmacokinetic analyses possible in critically ill patients for antibiotics, it follows that microsampling can now be considered for use in pharmacokinetic studies.

Part 5

Conclusion

Chapter 7: Summary and direction for future research

An increasing prevalence of multi-drug resistant bacteria is steadily decreasing the number of antibiotics that can be used, and few new antibiotics are available for effective treatment of multi-drug resistant infections. In critically ill patients there are significant pathophysiological changes that can complicate antibiotic dosing and using knowledge of the pharmacokinetic and pharmacodynamic properties of antibiotics is essential to ensure effective treatment that will improve patient outcomes and reduce the potential for an organism to acquire resistance.

Improving sampling and bioanalysis methodology for antibiotic pharmacokinetic studies is an important advance in this area. The microsampling techniques offer a new approach to collecting, storing and transporting smaller, but sufficient, volumes of clinical samples. The potential significance of these sampling techniques is very high. This Thesis characterises the capabilities and suitability of microsampling so that these techniques can now be translated into improved collaborative clinical research.

7.1 Summary of Findings

Investigations using the novel VAMS devices for whole blood analysis of fosfomycin provided acceptable validation data for limit of quantification (LLOQ), linearity, and inter- and intra- day precision and accuracy, and matrix effects. However, results from recovery testing and testing of the stability of dried whole blood samples on VAMS devices for use with the analysis of fosfomycin, suggest the impact of haematocrit or other compounds found in whole blood is problematic and requires special attention to be given to extraction optimization.

A complete and successful validation of a quantitative analysis of fosfomycin using a DPS sampling technique was achieved. The results of the DPS samples from a clinical pharmacokinetic study were found to strongly correlate with the gold standard of plasma sampling. The results of the Bland-Altman plot demonstrate that the DPS concentrations were subject to a consistent negative bias (16.6%) compared to the plasma samples, with the precision from the mean result relatively limited (7%). The resulting negative bias may reflect variability between the patient plasma and the plasma used to generate the calibration line. Substantial changes in protein content may influence the spreading on the filter paper and this has the potential to produce this result. The results of this study support the translation of DPS microsampling of fosfomycin for use in clinical pharmacokinetic studies

Fosfomycin is being used as a last-line antibiotic to treat critically ill patients for severe infections against various Gram-negative and Gram-positive bacteria, and has been found to be effective as a

treatment for multi-drug resistant pathogens, including extended spectrum beta-lactamase and carbapenemase-producing bacteria. Yet prior to this research little information was available on the pharmacokinetics of fosfomycin in this patient population, despite the knowledge that there are significant changes in the pathophysiology of critically ill patients compared to that of healthy volunteers.

The results of the population pharmacokinetic study of fosfomycin in critically ill patients found a lower clearance of fosfomycin (CL = 2.06 L/h) than that found in healthy patients (CL = 7.2 L/h). The model described significant changes in clearance on different dosing occasions for individuals, as well as random unexplained differences between individuals on clearance and volume of the central compartment. This demonstrates the need for careful monitoring of fosfomycin dosing in critically ill patients, particularly if there is evidence of renal dysfunction. Clearance was found to be proportional to calculated creatinine clearance (using the Cockcroft-Gault equation) thus providing a measure for physicians to base dosing upon. While fosfomycin is well tolerated and has a low adverse event profile [163], we observed peak plasma concentrations in some patients with low CL_{CR} after multiple days of treatment up to 1440 mg/L. A larger than normal apparent volume of distribution was found. This is likely to reflect the degree of sickness severity in the patients and is a typical pathophysiological alteration observed in critically ill patients.

EUCAST [165] refers to common dosing of IV fosfomycin as from 3 to 8 g fosfomycin administered three times daily. Analysis of the pharmacokinetic data using population pharmacokinetic modelling and Monte Carlo dosing simulations demonstrated that contemporary dosing recommendations of fosfomycin are inadequate for patients with augmented renal clearance to achieve target concentrations for most pathogens. Higher dosing of 4 or 6 g of fosfomycin administered four times a day is more likely to achieve the pharmacokinetic/pharmacodynamic targets in these critically ill patients with elevated CL_{CR}.

7.2 Suggested Direction for Future Research

Based on the findings of this Thesis the importance of understanding the pharmacokinetics of critically ill patients and the impact of this on effective antibiotic treatment is again realised. Evidence based dosing is essential to improve the outcomes for critically ill patients and reduce the opportunity for bacterial resistance to evolve.

- The potential for microsampling to be used in clinical pharmacokinetic studies may reduce the current resource load and improve opportunities for collaboration. Further validation into the use of DPS and VAMS devices with other antibiotics can improve the opportunities for research, including in paediatrics and neonates.

- The VAMS devices require an assessment on the impact of haematocrit on recovery, particularly at lower levels of haematocrit as experienced by critically ill patients, and the impact of extraction recovery over time. The variability of recovery of fosfomycin using whole blood VAMS devices with variable haematocrit requires testing to ensure the integrity of the samples is maintained during storage and transport.
- The full advantages of dried microsampling are yet to be realised for DPS, as sampling still requires whole blood collection from a cannula or needle, and subsequently centrifuged to obtain the plasma sample, or the use of expensive and specialised sampling devices. Further research into micro-capillary sampling of whole blood from heel or thumb-prick, with subsequent processing in a bedside bench-top centrifuge may be a simple and inexpensive way to produce DPS samples in a clinical environment.
- Further investigations into the pharmacokinetic/pharmacodynamic of fosfomycin are warranted. The pharmacokinetic model described here may be used with laboratory-based hollow fibre infection models to describe the pharmacodynamics of fosfomycin, for associated bacterial killing and resistance emergence over clinically relevant durations of therapy
- Fosfomycin uses two uptake systems to enter bacteria – one of those, the hexose phosphate transport system, is induced by extra-cellular glucose-6-phosphate. An *in-vivo* investigation supplementing critically ill patients with glucose-6-phosphate while dosing with intravenous fosfomycin may increase penetration of the antibiotic.

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