New Analytical Techniques for Determining Pharmacokinetics of Drugs in Neonates

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Declarations

Statement of Originality

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

Acknowledgments

They say it takes a village and that may be true of this thesis more than most. There are too many people who have contributed to this work in some way so a heartfelt Thank you to everyone who has provided wisdom, support and kindness over the years. I will never be able to convey how much it has meant and it would not have been finished with out you.

In the end, this is *FOR LUKE*, without whom nothing I achieve would be possible.

List of publications as a result of thesis

O'Hara K, Wright I.M.R., Schneider J.J, Jones A.L., Martin J.H. *Pharmacokinetics in neonatal prescribing: evidence base, paradigms and the future*. **British Journal of Clinical Pharmacology** 2015;80(6):1281-1288

O'Hara K, Schneider J.J, Jones A.L, Wright I.M.R, Martin J.H, Galettis P. *Development of a UHPLC-MS/MS method for remifentanil quantification in a small plasma volume*. **Journal of Liquid Chromatography & Related Technologies** 2019; 42(15-16):521-527

O'Hara K, Martin J.H, Schneider J.J *Barriers and Challenges in Performing Pharmacokinetic Studies to Inform Dosing in the Neonatal Population*. **Pharmacy** 2020; 8: 16

Under Review:

O'Hara K, Johnstone JM, Schneider JJ, Quail AW, Cottee DBF. *Determination of midazolam in rabbit plasma by high performance liquid chromatography with diode array detection.* **International Journal of Pharma Sciences and Scientific Research**

O'Hara K, Martin J.H., Schneider J.J**.** *In vitro degradation of remifentanil in neonatal blood and plasma* **Bioanalysis**

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O'Hara K. *Pharmacokinetic changes with growth and development between birth and adulthood.* **Journal of Pharmacy Practice and Research** 2017;47:313-318.

O'Hara K. *Paediatric pharmacokinetics and drug doses*. **Australian Prescriber** 2016;39(6):208-210.

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Abstract

Determining the appropriate dose of medication to use in a paediatric or neonatal patient is a clinical challenge. Many doses of medication currently used are extrapolated from adult dosing regimens due to a lack of pharmacokinetic studies in children and neonates. The lack of paediatric clinical trials and dosing information has been highlighted by many different international bodies, including the Food and Drug Administration (FDA) and European Medicines Agency (EMA) who acknowledged that this is an area of clinical need and there is now a requirement for more paediatric data in the licensing of new drugs. There is an urgent need for pharmacokinetic studies to be performed in paediatric and neonatal patients. Pharmacokinetic studies require the availability of a suitable assay to measure the drug concentration in blood or plasma. Without these analytical techniques, it is impossible to perform the required pharmacokinetic studies to develop dosing information in these patient groups. Neonates, in particular, have very small total blood volume and any samples taken must reflect this. The primary aim of this thesis is to describe the complex development of analytical techniques capable of measuring drug concentrations in small volume blood samples. Addressing this lack of suitable assays is a critical first step in pharmacokinetic research in this patient group.

As part of the research performed for this thesis, a clinical study in neonates was conducted. From the experience of designing and conducting this clinical study in neonates, combined with a review of the literature, several barriers to this type of research were identified. These barriers included gaining ethics approval, parental consent issues, sufficient number of patients and multicentre trials, minimising blood sampling requirements and availability of suitable analytical techniques. Reflecting on these identified barriers, potential solutions to overcome these barriers have been proposed to assist researchers in the future.

Similar to the adult population, the pharmacological therapies in neonatal patients span a huge range of medical conditions and illnesses. As a result of the review conducted for this thesis, priority areas requiring pharmacokinetic studies and further work were identified. This included analgesia, antibiotics and sedatives. In this thesis, small volume assays for drugs with clinical significance for neonatal patients in each of these areas were developed and validated as a first step towards determining pharmacokinetic data and evidence based dosing information.

Remifentanil has been identified as a potentially useful analgesic in neonates. In this research, an assay capable of measuring concentrations as low as 0.25ng/mL in 100µL of plasma was developed using HPLC-MS/MS. Applicability to use in pharmacokinetic studies was demonstrated by analysing small volume samples to determine pharmacokinetics in a rabbit model. Remifentanil undergoes metabolism via hydrolysis by esterases. Few data are available about extent of esterase activity in neonates. Work in this project explored esterase activity in neonatal red blood cells and plasma. This research provided data not previously reported in the literature on the extent of metabolism of remifentanil in neonatal blood. It was able to demonstrate that developmental changes are likely to occur and extrapolating dosing information from adults may present dangers to neonatal patients.

Neonates often require antimicrobial therapy and benzylpenicillin is a commonly used agent in this group. Detailed pharmacokinetic data would assist in optimising dosing, particularly with concerns about antibiotic resistance. An analytical technique capable of measuring concentrations as low as 10ng/mL in 50µL of plasma was developed and validated. This assay uses HPLC-MS/MS and would enable quick turnaround in analysis, making it potentially suitable for both clinical pharmacokinetic studies and therapeutic drug monitoring. As sample stability is a concern in previous benzylpenicillin studies this is also addressed. Contrary to some previously reported data in the literature,

benzylpenicillin was observed to be stable for up to 24 hours at room temperature, providing information to determine appropriate sample collection for clinical studies.

Providing adequate sedation is a challenge in neonates. The benzodiazepine, midazolam, has been used but little is known about its pharmacokinetics and the optimal dose. It is metabolised by CYP3A4, which is known to undergo developmental changes. In order for future clinical pharmacokinetic studies, as assay that would be suitable for use in a neonatal pharmacokinetic study using a limited sampling strategy was developed using HPLC-UV detection. The assay requires a 300μL plasma sample and is capable of measuring concentrations a slow as 10ng/mL. The applicability of this assay for use in neonates was demonstrated by performing pharmacokinetic analysis in a rabbit model.

This thesis outlines the experimental difficulties that must be overcome to develop practical and accurate analytical techniques, and develops methods to overcome some of the analytical barriers. Specifically, in the following chapters different strategies to overcome these difficulties are described including sample stability, accurate small volume detection and use of HPLC-MS/MS technology. Practical application of the analytical techniques is shown in analysis of midazolam in rabbit samples, benzylpenicillin in simulated patient samples to determine stability and remifentanil in a rabbit pharmacokinetic study and an in-vitro assay to determine differences in the rate of metabolism between adults and neonates.

Outline

Chapter 1 describes the unique pharmacokinetics of neonates along with the research tools and methods needed to conduct pharmacokinetic studies in neonates.

Chapter 2 outlines the difficulties in designing and developing neonatal pharmacokinetic studies and describes the solutions used throughout this work that will be of use to other researchers in the field.

The development of a low volume highly sensitive assay for remifentanil in blood samples is covered in Chapter 3. This assay was designed for use with neonatal patients and clinical applicability is demonstrated in rabbits.

Developing population pharmacokinetic models for remifentanil dosing in neonates is complicated by a lack of knowledge of the activity of remifentanil metabolising non-specific esterases in this age group and how they may differ from adults. Chapter 4 is a preliminary study of the differences in esterase metabolism in blood between the two groups to gather information for use in developing safer dosing information.

Benzylpenicillin is a commonly used antibiotic for treating infections in neonates. Chapter 5 describes the development of a low volume highly sensitive assay for determining benzylpenicillin concentrations in clinical samples. This chapter also includes a sample stability study to assist in collecting data from clinical samples. The work described in this chapter also highlights the need for appropriate analytical equipment for analysing neonatal samples by describing the failures during method development.

Development of a midazolam assay that is suitable for use in neonates is described in Chapter 6. This assay was applied clinically by completing a pharmacokinetic study in rabbits, which are able to provide similar blood sampling volumes to neonatal patients.

Chapter 7 discusses the relevance and future directions of this body of work.

List of Figures and Tables

List of abbreviations

CBP	Cardiopulmonary Bypass
CNS	Central Nervous System
CYP	cytochrome enzyme
CYP450	cytochrome P450
EMA	European Medicines Agency
FDA	Food and Drug Administration
GABA	gamma-Aminobutyric acid
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
IV	Intravenous
LLOQ	Lower limit of Quantitation
mL	millilitre
MS	Mass Spectrometry
ng	nanogram
NICU	Neonatal Intensive Care Unit
PICC	Peripherally Inserted Central Catheter
QC	Quality Control
SPE	Solid phase extraction
TDM	Therapeutic Drug Monitoring
TGA	Therapeutic Goods Administration
UGT	UDP-glucuronosyltransferase
UV	Ultraviolet
µg	microgram
µl	microliter

Chapter 1: Introduction

Use of Off-label Drugs in Children and Neonates

Many medications used in children, especially neonates, are used off-label which means that their use is without approval from the regulatory authority in Australia, the Therapeutic Goods Administration (TGA). Using a drug off-label requires the clinician to acknowledge that there is not enough evidence of safety or efficacy in the population being treated or the indication to register this drug. It does not imply that the drug is ineffective or necessarily unsafe but means information is lacking. This lack of information makes it difficult for the clinician to select appropriate doses and regimens and this does potentially increase the risk of adverse effects. Off-label use of drugs is associated with an increased risk of adverse effects including serious adverse effects, particularly in patients under the age of 2 [1].

Gaining approval and registration from the TGA requires clinical testing, including pharmacokinetic and pharmacodynamic studies, of the drug when it is used for specific conditions in a specified patient population. Given the increased risk of adverse effects and the clinical need to use drugs off-label to treat certain conditions, research investigating the pharmacokinetics and pharmacodynamics of these off-label drugs is urgently needed to improve the safety and efficacy of medications in these vulnerable patient groups [2].

Pharmacology and the Paediatric Population

Drugs used off-label in neonates are generally approved in adults, having undergone appropriate pharmacological and pharmacokinetic studies in the adult population. The paediatric population presents many different challenges in terms of determining the pharmacology of drugs administered. Neonates are particularly difficult to predict pharmacological effects in as development occurs

quickly and this results in rapid changes in pharmacokinetics and pharmacodynamics and increases difficulty determining doses and dosage regimens [3].

The factors influencing the pharmacology, pharmacokinetics and dosing of drugs in the paediatric population will be discussed in the following sections.

General Pharmacological Principles

The two main arms of clinical pharmacology involve pharmacokinetics and pharmacodynamics. Pharmacokinetics is the study of the time course of a drug in the body while pharmacodynamics involves the action of the drug on the body (Figure 1).

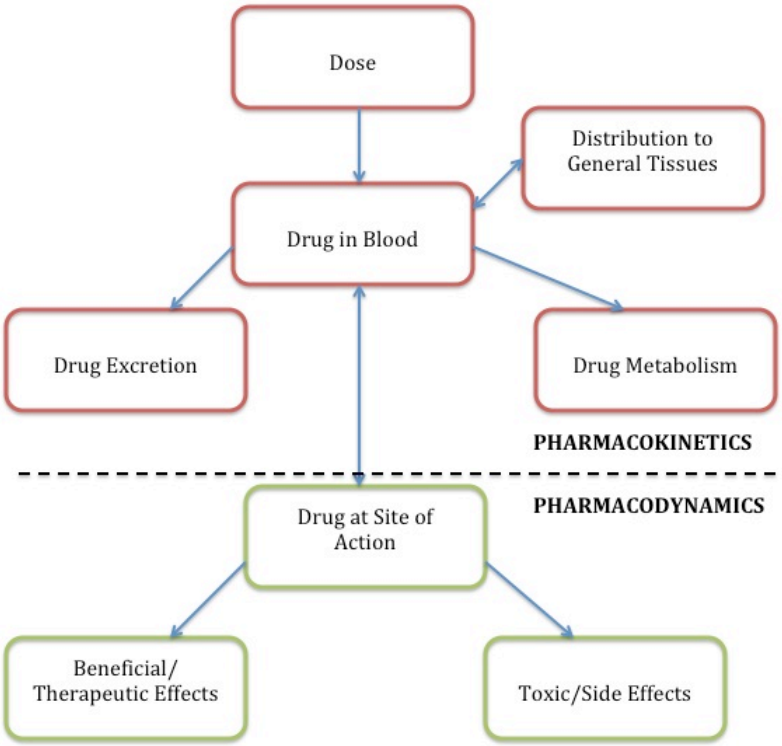


Figure 1: The main processes of pharmacokinetics and pharmacodynamics. Adapted from [4].

Pharmacokinetics in Neonates

The four major pharmacokinetic processes of absorption, distribution, metabolism and excretion present the most important differences between neonates and adults. These processes reach adult levels at different times during growth. The combined action of metabolism and excretion relate to the clearance of the drug from the body. Absorption, distribution, metabolism and excretion will each be addressed in order.

Absorption

Absorption is the ability of drugs administered by extravascular routes to overcome chemical, mechanical and physical barriers and be distributed to their site of action. The development and differences in these barriers between neonates and adults can change the rate and extent of drug absorption [2].

Absorption of enterally administered drugs is dependent on a number of factors. Acid content of the stomach and gastrointestinal transit time are two factors that are known to change between neonates and adults. Typically neonates have a higher gastric pH [5]. This can result in increased bioavailability of acid labile compounds and decreased bioavailability of weak acids.

The rate of gastric emptying and intestinal motility affect the rate at which drugs are presented to mucosal surfaces for absorption. In general, these processes are slower in neonates compared to older infants and children which results in longer times to peak serum concentrations. Villous formation is complete by 20 weeks gestation so decreases in absorption are not related to the surface area of the intestine [6]. The type of feeding can affect gastrointestinal transit time and it is proposed that the type of feed can influence the development of intestinal CYP enzymes with formula feed infants showing faster rates of CYP development [7].

High calories feeds containing long chain fatty acids slow gastric emptying while breast milk and low calorie feeds increase the rate of gastric emptying [8].

Biliary function, including bile composition and gall bladder function, can affect the absorption and transport of lipophilic compounds that require bile salt conjugation. Bile acids are present from 14 to 16 weeks gestation [9]. Pancreatic enzymes are low at birth and develop rapidly in the immediate postnatal period [10] assuming normal physiological processes are established.

The absorption of drug via other routes also changes throughout infancy. The thinner stratum corneum results in increased absorption via the skin along with increased water loss [11]. The larger body surface area to volume ratio increases the amount of absorption possible via the skin in a neonate as opposed to an older child or adult [12].

Rectal administration of drugs is problematic due to a higher frequency of pulsatile contractions of the rectum in children compared to adults often resulting in expulsion of solid dose forms reducing the practicality of this approach [13].

Pulmonary administration is increasing in popularity in this age group. Physical differences in the structure and surface of the lungs are expected to change the absorption characteristics compared to adults and older children [14]. The particle deposition rate has been shown to be higher for children aged between 3 and 14 than adults, with deposition decreasing as a function of height [15].

Buccal administration of sucrose for procedural pain is well established [16] as is the administration of glucose for hypoglycaemia. Research continues into the administration of other drugs via this route, including buprenorphine for

neonatal abstinence syndrome [17] and desmopressin for diabetes insipidus [18].

Distribution

The distribution of a drug is an important step in pharmacokinetics as it relates to time to achievement of steady state concentrations and therefore effectiveness. Body composition is an important determinant of the physiological spaces a drug can distribute into. Changes in body composition are particularly relevant to drug which require a peak or threshold concentration for pharmacodynamics effect [2]. The increased percentage of total body water and the ratio of intra to extracellular fluid will also influence the distribution of drugs into tissues [14]. The corresponding decrease in body fat results in a change to the distribution of both hydrophilic and lipophilic drugs. Both these factors are more pronounced in the premature neonate. The total body water decreases in the first few months of life and then remains stable throughout childhood, while fat percentage doubles within the first six months of life and varies throughout childhood [2]. Neonates have a higher percentage of total body water (Figure 2) along with a larger extracellular space changing the distribution pattern of medication from what is commonly seen in adults.

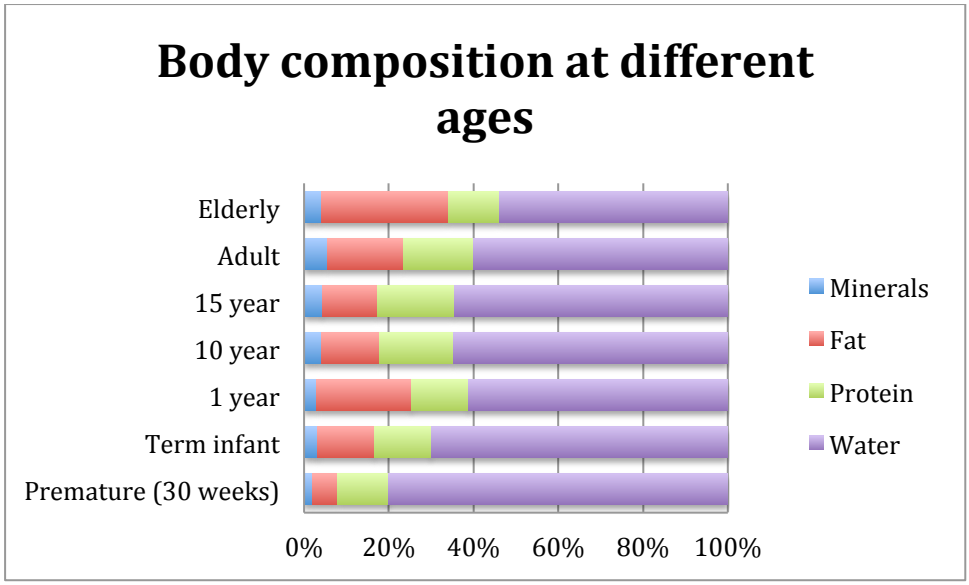


Figure 2: Changes in body composition with age. Adapted from [19].

Protein binding

Neonates and infants have less circulating plasma proteins which increases the amount of active drug available for highly protein bound drugs and increases the rate of clearance for drugs usually bound to plasma proteins. Along with a lower amount of plasma protein neonates also have higher circulating bilirubin and free fatty acid which can displace drugs from albumin binding sites [14]. In normal health albumin production begins at around 16 weeks gestation before which alpha-fetoprotein is the major serum protein. Albumin concentrations have usually reached adult levels by birth. Changes in the volume of distribution are also related to changes in blood flow, tissue perfusion, membrane permeability and cardiac output [10].

Blood-brain barrier

The diffusion of some drugs into the central nervous system presents a risk to both preterm and term neonate's developing brain. With the defensive mechanism of the blood brain barrier not fully formed the developing brain is at greater risk of damage from toxic levels of drugs. Studies in animals have linked

different blood flow and an increase pore size to increased permeability of the blood brain barrier but this has yet to be shown with humans [14]. Disease states such as hypoxia, sepsis and acidosis also increase the permeability of the blood-brain barrier [8]. Changes in expression of transporter proteins within the blood brain barrier have been demonstrated in neonates born between 23 and 42 weeks of gestational age in both pharmacological studies and samples taken during autopsy [14].

P-glycoprotein transporters

P-glycoprotein transporters are associated with drug efflux from cells and transport of drugs across cellular barriers. Lymphocytes have been shown to have high levels of P-glycoprotein at birth decreasing over six months before stabilising at adult levels around 2 years of age [20]. It is likely that P-glycoprotein activity at other sites is also affected by age.

Metabolism

The metabolism of drugs is an essential step in both drug activity and clearance. The ability to metabolise drugs is present in the foetus and newborn and changes throughout growth and development [21]. Variation in drug metabolism is dependent on a number of factors, including disease, environment and genes. The most important factor in the variation of neonatal drug metabolism is the development of the different biotransformation enzymes [2]. Determining the development of these enzymes is challenging. Functionality testing of the enzyme requires introduction of a substrate, in this case a drug, which requires ethical consideration. Studies thus far have been limited to drugs already used in practice, keeping the therapeutic pool for neonates small.

Drug metabolism enzymes are divided into Phase I and Phase II enzymes. Phase I enzymes are involved with primary oxidation, reduction and hydrolysis processes while Phase II enzymes conjugate drug molecules to allow excretion.

The most important group of enzymes involved in Phase I metabolism are CYP450 [2] but alcohol dehydrogenase and esterases also form part of Phase I drug metabolism [22]. A lack of activity of these enzymes can be responsible for the extreme toxicity syndromes that have been seen in premature infants [23-25].

The rate of hepatic blood flow can also affect the rate of drug metabolism by the liver. Hepatic blood flow increases as part of neonatal development. This is particularly important for drugs with a high hepatic extraction ratio [8]. Following birth blood flow through the liver changes with the ductus venous closing and the oxygen saturation increasing. Following the infants first feed post birth portal blood flow increases and bacterial colonisation of the gut begins. After six weeks these bacteria are able to produce sufficient vitamin K to induce hepatic production of clotting factors. The rapid rise in processing functions requires induction of a number of enzyme groups [26]. These changes make standard pharmacological models of hepatic extraction, metabolism and blood flow invalid in neonates. These models can be used once the ductus venous is closed [27] around day seven of life and hepatic blood flow matches older children and adults.

CYP enzymes

CYP enzymes are responsible for the majority of drug metabolism. When corrected for weight the content of CYP enzymes in foetal livers is 30-60% of adult values [2]. The family of CYP450 enzymes develop at different rates. Some are active in-utero while others do not demonstrate activity until some time after birth. Fully adult activity is usually achieved by 2 years of age [14]. Significant inter-individual differences in development and activity of CYP enzymes are seen [28] and the small number of samples available for enzyme activity studies may not always allow for a complete population picture to be elucidated.

The development of the CYP family of enzymes (Table 1) is difficult to generalise and enzyme specific development needs to be determined before an accurate estimate of drug metabolism is possible [2]. Development can be affected by many factors during early life [29]. Diet may also play a role in the development of CYP enzymes, with formula fed infants being shown to develop activity of CYP1A2 and CYP3A4 enzymes at a faster rate than breastfed infants [7] by inducing transcription of the CYP genes. A number of compounds are known to be either enzyme inhibitors or enzyme inducers [4]. Exposure to tobacco smoke during gestation can affect the activity of a number of CYP450 enzymes during gestation and after birth by inducing activity [30].

Table 1:CYP enzyme activity adapted from [14].

Enzyme	Becomes active .at	Substrates	Inhibitors	Inducers
CYP 1A2	1-3 months	Caffeine Paracetamol	Ciprofloxacin	Broccoli Tobacco Insulin Chargrilled Meat Omeprazole
CYP 2C9	First weeks	Ibuprofen Phenytoin	Fluconazole Sulfamethoxazole	Rifampicin
CYP 2C19	First weeks	Omeprazole Phenytoin Indomethacin	Omeprazole Indomethacin	Carbamazepine Prednisone
CYP 2D6	Hours-days	Amphetamines Codeine Flecainide Lignocaine Metoclopramide	Cocaine Methadone Ranitidine	Phenobarbitone Phenytoin
CYP 2E1	Hours	Ethanol Paracetamol		Ethanol Isoniazid
CYP 3A4	First weeks	Statins Amiodarone Amlodipine	Fluconazole Grapefruit Juice	Phenobarbitone Phenytoin

Phase II metabolism

Less is known about the development of Phase II enzymes [14]. Phase II reactions include glucuronidation, sulfation, methylation and acetylation. Phase II reaction are an important part of drug metabolism and the biotransformation of endogenous compounds, including steroids. The largest group of enzymes involved in these reactions are uridine diphosphate glucuronosyltransferase

isoenzymes [31]. The neonatal development of these enzymes appears to be less specific than the development of the CYP450 enzymes (Table 2). The development of UDP-glucuronosyltransferase (UGT) has both pharmacokinetic (as in chloramphenicol toxicity) and pharmacodynamic (as part of morphine glucuronidation) importance. [2]. While postmenstrual age is important in the development of the cytochrome pathways both postnatal age and postmenstrual age are relevant to the development of glucuronidation pathways [32].

Table 2: The development of phase II metabolism pathways. Taken from[33].

Key: + =activity shown;-=no activity; ?=unknown activity.

Gene	1st Trimester	2nd Trimester	3rd Trimester	Neonate	1 month to 1 year	1 to 10 years	Adult
GSTA1/A2	+	+	+	+	+	+	+
GSTM	+	+	+	+	+	+	+
GSTP1	+	+	+	+	-	-	-
NAT2	+	+	+	+	+	+	+
UGT1A1	-	-	-	+	+	+	+
UGT1A3	?	+	+	+	+	+	+
UGT1A6	-	-	-	+	+	+	+
UGT2B7	?	+	+	+	+	+	+
UGT2B17	?	+	+	+	+	+	+
EPHX1	+	+	+	+	+	+	+
EPHX2	?	+	+	+	+	+	+
SULT1A1	?	+	+	+	+	+	+
SULT1A3	?	+	+	+	+	+	+
SULT2A1	-	-	+	+	+	+	+

Differences have been seen between early and late neonatal life. Glucuronidation reactions are not thought to reach adult levels for at least three years [34]. Conjugation increases from minimal levels to almost adult levels within two weeks post birth in most cases. Conjugation is important for dextoxifying products of metabolism and drugs, particularly lipophilic compounds. Delays in achieving normal concentrations are seen in septic and preterm babies [26].

While other conjugation pathways are active, neonatal stores of sulphate and glycine are low, which can limit the capacity of those pathways [34].

Genetic influences on drug metabolism

Pharmacogenomics, or the effects of genetic polymorphisms on drug metabolising enzymes, is expected to have an effect on the development of drug metabolism pathways. Genetic polymorphisms are well documented to result in drug metabolism variability in adults it should be expected to have similar effects on neonates although observations of this are currently absent [2]. The ontogeny of drug metabolism pathways can make it difficult to determine the effects of genetic polymorphisms. The phenotype may not be immediately apparent in a neonate, as the gene may not be fully expressed. Genetic differences in antenatal life may also be responsible for changes in drug metabolism resulting in birth defects or malformations [35].

The genetic differences in Phase I and Phase II enzymes are documented in adults and children. The impact of individual polymorphisms on drug metabolism in neonates is dependent on the rate of maturation of the enzyme pathway [36] and the individual polymorphism. A number of genetic polymorphisms in CYP2D6 are relevant to opiate metabolism, particularly the conversion of codeine to morphine [37]. This particular polymorphism has resulted in toddler fatalities [38] highlighting the importance of genetic polymorphisms in drug metabolism from early life.

Excretion

Excretion is an important step as it is the final removal of the drug and/or its metabolites from the body. Excretion is usually via the renal or hepatic routes but it is possible from drugs to leave the body by many other routes. The development of elimination pathways may be the most important factor affecting pharmacological response in infants and children [3].

Excretion of drugs via the kidney is dependent on the development of the structure of the kidney and changes rapidly after birth. Nephrogenesis is complete at 34 weeks of gestation although growth retardation, nephrotoxic drugs administered during pregnancy and renal malformations can have a negative effect on kidney development [39]. Creatinine at birth is not a reliable marker of renal function and largely reflects maternal renal function. It becomes a more accurate marker of renal function during the first weeks of postnatal life [40]. The administration of other drugs, such as ibuprofen, can decrease renal function in preterm neonates regardless of gestational age at birth [41].

Glomerular filtration is an important determinant of the rate of excretion via the kidney as the secretion and absorption functions are immature at birth [2]. The glomerular filtration rate undergoes significant changes in early postnatal life [14]. Glomerular filtration rate increases to half adult value by three months of age and reaches adult levels by 2 years of age [8]. Premature neonates have a slower rate of kidney development [42]. Post-menstrual age is an important predictor of kidney function in the neonate, while size is also important. Babies who are born small for gestational age have been shown to have lower renal clearance than appropriate for gestational age counterparts [43].

Less effective tubular reabsorption and secretion in neonates also affect the rate of clearance of renally cleared drugs. Drug transporters are an important part of drug clearance by the kidneys. Organic anion transporters are responsible for the final elimination of compounds formed during stage II biotransformation. Organic anion transporters have low activity at birth then increase rapidly to high levels, higher than those seen in adults, over the first few weeks of life and then begin to decline to adult levels. This change in secretion happens independently of changes in renal mass. Exposure to hormones and organic acids also increases organic anion transporter activity [42].

Glomerular perfusion is determined by the vascular tone of the glomerular arterioli which further depends on the vasodilation mediated by prostaglandins. Dosing regimens of renally excreted drugs should be modified to reflect the renal function of the neonate [14]. Along with kidney development other factors than can affect drug clearance including drug interactions and severity of disease and should be taken into account when accounting for neonatal differences in drug clearance [2]. Administration of cyclo-oxygenase inhibitors has a negative effect on renal function [2]. The administration of non-steroidal anti-inflammatories in the first day of life has been shown to reduce the clearance of amikacin by 22% [44]. It is not yet known if co-administration with non-steroidal anti-inflammatories effect other medications commonly administered in the early neonatal period. The changes in renal clearance due to administration of non-steroidal anti-inflammatories, in infants between 24-34 weeks gestation, have been shown to be independent of gestational age [41].

Pharmacodynamics in Neonates

Pharmacodynamic differences are apparent in the neonate. These can be more difficult to define and are often unpredictable. These differences relate to the actions of the drug at its receptors. It is often unknown whether the receptor is present, inactive or has not yet developed in this difficult to manage population [14]. In addition the number of receptors, binding, affinity and the ability of these receptors to stimulate intracellular effects may be different from older and more mature neonates [45]. Tissue binding affinity and receptor capacity that changes with age can also affect the activity of drugs administered to neonates and children in ways that are not yet defined.

Determining the drug dose

Drug doses used in children have historically been extrapolated from adult pharmacokinetic data. However, human growth and development is not a linear

process and this approach is not adequate for determining doses across the range of developmental processes that occur throughout childhood [14].

The change from treating children and neonates as "small adults" has occurred gradually. Previously size or gestational age were viewed as the main determinants of drug metabolism but this has been replaced with the view that the individual organ capacity and function and the development of biochemical pathways are of greater importance [34]. A study conducted by the FDA in the United States examined different methods of predicting paediatric clearance of drugs based on adult values and concluded that no single method of prediction is suitable for all drugs or age groups [3].

Weight based and surface area based dosing regimens for children are simple and are used in most clinical situations. With the lack of paediatric specific pharmacokinetic data available these dosing equations are often based on adult information and scaled based on size and age. While this method may have some value in older children and adolescents who have similar values to adults in terms of body composition, it may be less accurate in toddlers and neonates [14].

As estimation cannot replace clinical studies there is an urgent clinical need to complete studies which will inform dosing in this population. Regulatory bodies internationally are providing incentives to drug companies to conduct studies in paediatric patients resulting in an increase in studies in recent years. This pattern is expected to continue improving the safety of medicines in children [46]. Previously, research into the use of drugs in children and neonates provided little financial incentive reducing progress.

Development of pharmacokinetic data to determine dosing information for clinical use

In order to guide safe dosing in paediatric patients, it is essential that pharmacokinetic studies be performed. There are two different approaches to pharmacokinetic studies; traditional or population pharmacokinetic approaches. Traditional pharmacokinetic models require a large number of consecutive concentration-time points from each participant in order to determine the pharmacokinetic parameters of the drug in individuals. This often precludes modelling drugs in children as the large number and volume of blood samples required are not possible or ethical to take from children [47], even less so in neonates.

Pharmacokinetic models

Different types of pharmacokinetic modelling are available. Population pharmacokinetic models use concentration time points from a number of subjects to determine the pharmacokinetics of drugs in that population [47] and are appealing in populations where traditional approaches are not ideal or not possible. Another approach is physiologically based pharmacokinetic models which require detailed information about the physiology of the study population. This includes organ maturation, body composition and maturation of elimination pathways. Including information about protein binding, genetic differences and clinical information further increases the accuracy of the model predictions [36].

The information needed to develop population pharmacokinetic models can be from paediatric studies or scaled from adult data [48]. The data required includes, but is not limited to, age (both postnatal and gestational), weight, height and renal function. These variables become the co-variates that contribute to the model and are tested to determine effects on the pharmacokinetics of a particular drug. By using what is known in adults the

optimal number of samples and sampling times can be set for neonatal studies [49].

Determining the appropriate number of patients for a population pharmacokinetic study can be difficult and is dependent on the number of co-variates being studied. Studies in children and neonates require a larger number of subjects than studies in adults in order to account for pharmacokinetic changes that occur as part of growth and development. Smaller numbers can be used as part of studies in specific age groups but this does limit the amount that can be learnt about fixed covariates such as weight [50, 51]. Small numbers of samples can be used to determine individual pharmacokinetic parameters, such as clearance. However, larger numbers are required for additional parameters or for increased accuracy [52]. As the interindividual variability in neonates is unknown and could be large developing a population pharmacokinetic model may need to be done in two stages. Samples can be used to develop an estimation model, which can then be used to refine sampling to the optimal time for detailed model development [49]. Determining sampling time is a significant problem for drugs, such as remifentanil, where little is known about the pharmacokinetics in neonates. Significant preliminary work is required before accurate models to determine drug dosing can be developed for these drugs.

Computer programs are available to undertake the statistical analysis required to develop the models. The most common is NONMEM; which is a nonlinear mixed effects model. Bayesian algorithms are used to develop the population information [48] with computer stimulations used to test the models applicability to real world situations, including dosing information and clinical trial design [47]. Modelling program can also be used to develop pharmacodynamic models [53]. A disadvantage of using computer models is applicability to real-world scenarios where variables may be unknown and uncontrolled. Information developed using computational models should always be tested by clinical studies to ensure accuracy.

The use of population pharmacokinetic models in preterm neonates is further limited by the knowledge of the development of drug metabolism pathways and physiological changes following birth. This can limit the ability of the model to predict inter-patient variability and pharmacodynamic effects [48] and remains a significant gap in developing evidence based dosing information. Models should be developed from credible data. Models should be designed using the optimisation techniques described below to avoid inaccurate and unreliable modelling which could result in erroneous conclusions [47]. Once a model is developed it should applied to clinical scenarios to ensure it accurately represents what occurs in real life situations. All models aim to produce clinically significant findings and to perform well when used to make predictions from new data sets. Assessment of covariate effects is required to produce clinically relevant individual dose predictions. The type of model and the amount of data will also influence the number of covariates that can be analysed [50]. Different model approaches are available which have different strengths and weaknesses.

Naïve Pooled Data Approach

The naïve pooled data approach combines all time- concentration data as if they have come from a single subject. This approach works best when there is expected to be little between-subject variability and each subject can contribute large amounts of data. Interpretation of information developed in this way can be difficult if there are few data points from some subjects or if the timing of sample collection is skewed towards one part of the curve. Models developed this way provide no information about inter-subject variability and its causes [50].

Two Stage Approach

In the standard two-stage approach to population pharmacokinetic modelling each individual data point are analysed to create a profile. These individual

profiles are then combined to create a population profile. If the number of data points differs between individuals in the population weighting can be used when creating the final model. With this approach, variability between subjects can be determined from the standard deviations of the individual estimates. Due to imprecision in the individual estimates this can overestimate the variability [50]. Both the naïve pooled data and standard two-stage approach method rely on a number of samples from a few subjects.

Mixed Effects Model

Mixed-effects models allow the study of variability in drug response in a small sample population that is representative of the population the drug will be used in clinically. This method uses the population as the unit of analysis rather than the individual. Analysing sparse data from many individuals produces a more representative sample to the target clinical population. Mixed effects models differ from other methods as they can use a small number of samples from a large number of subjects. This approach allows the data to be described using a mixture of fixed and random effects. Fixed effects predict the average effect of a covariate, such as weight or sex, as a possible explanation of part of the inter-subject variability in a pharmacokinetic parameter such as clearance. Random effects are used to describe the variability that remains and is not predictable from an average of the relevant fixed effects. The predictable parts of the inter-subject variability can be assigned explanatory covariates such as age, size, sex, temperature or renal function [50]. Mixed effects models in particular are useful for examining developmental aspects of drug metabolism [54] and are suitable for use in neonates.

D-Optimal Designs

D-optimal designs can be particularly useful in neonatal models as they can be used to determine the best time points for incorporation into a model, so that the optimal model for the limited points available can be achieved [51]. This

approach also allows for refining of the model as more becomes known about how it behaves with different data points from the set. D-optimal designed models replace data points in the set until the final estimate cannot be improved [55]. This determines the number of points required for a full model with all factors considered including determination of the number of samples required and the sampling times that will provide the optimal data set [56]. It is useful when designing studies when considering the ethics of blood sampling in this population and when using sparse samples from leftover clinical sampling collection [51]. Models designed in this way may be flawed by the production of replicates, particularly when the number of samples from an individual is higher than the variates being studied [47]. This is unlikely to be an issue in neonatal studies where the number of samples from an individual is limited.

The Fisher information matrix can be applied to d-optimised population pharmacokinetic models to improve modelling efficiency [57]. Computational time for the development of models can be lengthy. The timing can be reduced by using the most efficient estimates for the parameters tested and only testing for parameters that can be reliably estimated by the model with the data available. The application of the Fisher information matrix reduces time required to develop a population model by allowing rapid comparison of designs without simulated modelling that results in failed estimations. A theoretical comparison using the Fisher information matrix will identify model designs that will not work quickly [57] and has been shown to be comparable to traditional simulations [51, 57]. The use of D-optimal designs and the Fisher information matrix are especially useful in neonatal models designed for limited sampling [51].

Detection of drug concentrations in human samples

With the advances in pharmacokinetic analysis and determining optimal sampling times, the next requirement is ability to measure drug concentrations

in biological fluids such as blood or plasma. Measuring drug concentrations in samples is dependent on developing an assay that can quantify the drug in the particular fluid. Some fluids are more difficult to develop drug assays for due to high fat content (i.e. breastmilk [58]) or low concentrations requiring large sample volumes (i.e. ocular fluids). Clinically, blood samples are used most often due to the ease of sampling and volume available. Blood samples are more challenging to analyse in paediatric and neonatal populations as limits to sampling volumes are necessary, requiring assays with low limits of detection from small blood volumes. Paediatric and neonatal studies are not possible without microanalysis techniques [47].

Different techniques are available for analysis of human body fluids for pharmacokinetic studies. These include high performance liquid chromatography (HPLC) with different detection techniques and immunoassays.

Immunoassay

Immunoassays use an antibody mediated mechanism to identify drug in body fluids. Developing an immunoassay involves finding an appropriate antibody and a large number of steps. Automated systems are available for measuring blood or plasma concentrations for some drugs and are routinely used clinically for therapeutic monitoring of some drugs. While immunoassays have the ability to measure very low concentrations of drug in relatively small volumes of fluid, they suffer from cross-reactivity with metabolites, similar structured drugs or endogenous materials in the human sample resulting in false positives or falsely elevated concentrations. Interpretation of results from immunoassays can be difficult if cross-reactivity is suspected and sometimes immunoassays cannot be used to quantify drug in a sample when known interfering compounds are present.

High Performance Liquid Chromatography

High performance liquid chromatography assays are commonly used to measure drug concentrations in pharmacokinetic studies and clinical samples. Detection techniques used with HPLC include mass spectrometry, ultraviolet/visible (UV/Vis) where absorption of drug at particular wavelengths of light is measured, amperometric detection where drug is subject to a voltage potential to produce oxidation or reduction or fluorescence detection where the drug is excited to a higher level of energy at one wavelength of light and the emitted wavelength as it returns to lower energy state is measured. Detection techniques such as UV/Vis, fluorescence and amperometric detection do offer better selectivity than immunoassays since drugs will differ in the shape of their light absorption curve, emitted light wavelengths with fluorescence or ability to be oxidised and reduced at various voltage potentials. Since these techniques also involve chromatography prior to detection, the ability exists to separate interfering or compounds with similar chemical structure prior to measurement is feasible. However, sometimes resolution of interfering compounds is not achievable and this will confound results obtained. Detection techniques such as UV/Vis and fluorescence detection can, usually with extensive development work and sample clean up procedures, achieve concentrations in the low nanogram per mL range when plasma sample volumes of 500µL or more are used. For amperometric detection, drugs must have the ability to be oxidised or reduced, a feature not available for many drugs. These detectors are often also difficult to work with as frequent cleaning of electrodes is required to maintain sensitivity.

HPLC coupled with Mass Spectrometry (MS) offers the greatest sensitivity and specificity. The development of this technology has allowed analysis of very small samples containing very low drug concentrations, opening up the ability to conduct pharmacokinetic studies in children and neonates. HPLC/MS offers improvements over previous HPLC technologies with the addition of the mass spectrometer reducing cross reactivity and reducing the sample clean up required. MS detection following separation, as seen in Figure 3, via

chromatography by detecting compounds based on molecular weight reducing the risk of contamination by other compounds. MS also offers detection of smaller amounts of drug then can be detected by UV detection methods.

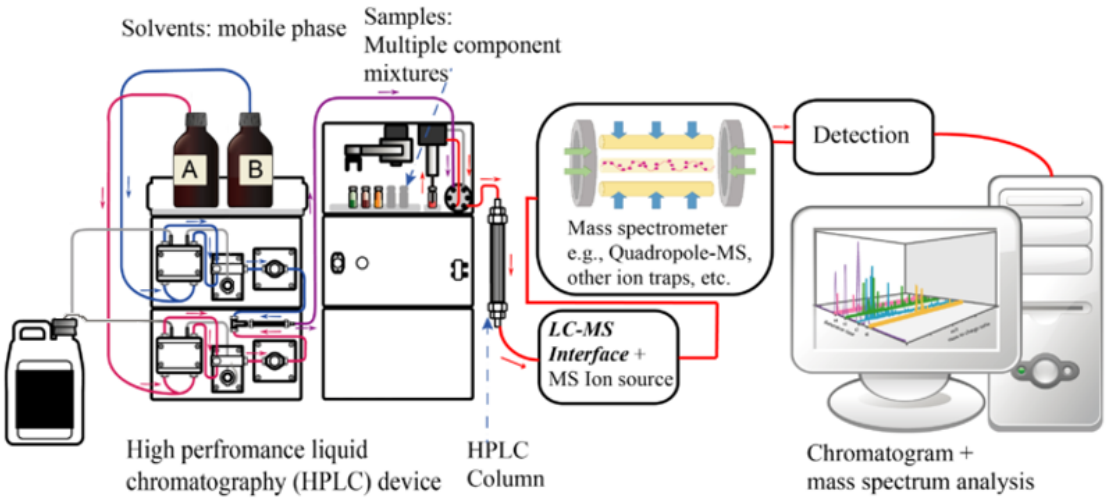


Figure 3: Diagram of HPLC-MS set up showing liquid chromatography followed by mass spectrometry with computerised detection setup [59].

The use of clinical samples for research purposes

Physiologically, it is important to limit the number of blood samples taken from premature neonates. A major factor which has limited the wider use of population pharmacokinetic models for neonatal research is access to and development of appropriate small volume drug analysis methodologies that have sufficient sensitivity to detect the expected concentrations in neonates. With the advent of HPLC-MS technology the volume of the sample required has decreased significantly, increasing the feasibility of pharmacokinetic studies in neonates.

In general, accessing blood samples usually requires the use of venepuncture or sampling via an existing indwelling line. There are clinical and ethical implications associated with using additional venepunctures for blood samples in pharmacokinetic studies. Using an existing line instead of venepuncture for blood sampling also has risks. An important ethical consideration of working

with neonates concerns the risk of infection associated with line access as opposed to the increased pain burden from extra venepunctures and the risks of each should be considered.

The use of blood samples taken for other clinical purposes is an option that can be employed once assay methodologies capable of measuring drug in very small volumes are developed. This use of leftover clinical samples reduces the burden on research participants improving the ethics of conducting research on vulnerable patient groups but can be subject to variable handling, affecting the stability of some drugs and the volume available is limited. For some drugs sampling time may also be an issue [49] and timing of clinical samples may be able to be adjusted to accommodate both clinical care and research. Sampling times are more flexible for population pharmacokinetic studies and samples taken as part of routine clinical care could be used. A number of studies have been taken from therapeutic drug monitoring data that has been collected as part of routine clinical care [60] for example with gentamicin.

In summary, developing small volume analytical techniques, along with low patient impact sampling, is an essential step in this work. When combined with small volume, highly sensitive assays these left over clinical samples become an important source of samples for population pharmacokinetic models with no effect on participants.

Clinical Use of off-label drugs in neonates

While there are many medications used off-label in neonates, there are three important clinical scenarios which urgently need addressing. These are the treatment of pain, sedation and the use of antimicrobial agents such as penicillin in neonates. These areas will be discussed in the following chapters.

Rationale of research project

While there are many different aspects requiring clinical studies and research in neonatal care, given the sources of variability in pharmacokinetics discussed in the literature, providing good analgesia and ensuring appropriate treatment of infections without adverse effects are high priority concerns. Since a major limitation in determining dosing guidelines for neonates is the lack of suitable assay techniques for measuring drug concentrations in small volumes of biological fluid to develop modern pharmacokinetic models, it is hypothesised that, with the advent of newer technologies in drug analysis, suitable methods for use in neonatal studies could be developed to enable research, including the study of esterase activity in neonates.

Pharmacokinetic studies to inform the use and dosing of neonates are urgently required. A major barrier to this work is the lack of suitable assays capable of measuring low drug concentrations in very small volumes of plasma. Three major clinical areas that urgently need addressing are pain management, antimicrobials to treat infections and sedation. To address these areas, the drugs remifentanil, benzylpenicillin and midazolam will be the focus of this research.

Hypothesis

Assays which use very small volumes of plasma and capable of measuring low concentrations and suitable for use in clinical pharmacokinetic studies in neonates are able to be developed using technology that is now available. The development of such assays will provide a platform for future pharmacokinetic studies in neonates.

Aims

The specific aims of this Thesis are:

- To develop a suitable analytical technique for use in measuring plasma concentrations of remifentanil in the neonate/paediatric population which can be used for future pharmacokinetic studies

- To design and perform a clinical study which investigates the activity of remifentanil metabolising esterases in blood and plasma obtained from neonates and compare this to adult esterase activity to determine if developmental changes are possible

- To develop a suitable analytical technique, including determining sample stability, for use in measuring serum concentrations of benzylpenicillin in the neonate/paediatric population for use in future pharmacokinetic studies

- To develop a suitable analytical technique for use in measuring plasma concentrations of midazolam in the neonate/paediatric population for use in future pharmacokinetic studies

CHAPTER 2: Difficulties with neonatal pharmacokinetic research

Introduction

Although neonatal pharmacokinetic research has been identified as a therapeutic imperative [61] for a number of years, progress has been slow. The majority of neonatal drug doses are still based on extrapolation rather than pharmacokinetic studies. As a result, a number of drugs have been introduced to the nursery without sufficient research with disastrous consequences, and many others causing unnecessary morbidity [23-25]. This would not be accepted as best practice in other patient populations, and yet research into neonatal drug dosing is uncommon and this practice is accepted. Extensive literature and practical analysis and understanding of why neonatal research is so difficult was a critical preparation step for this Thesis. The barriers identified were large and many, resulting in significant additional work and regulatory approvals before the research questions around specific drugs and methods could be developed. Acknowledgement of these issues and efforts to overcome them must be entertained to ensure neonatal drug dosing research is possible for future clinicians and researchers who are often limited by time.

The following description is of the key barriers I elicited when planning to undertake my clinical and research studies into optimal drug dosing in neonates.

Ethical considerations

The admission of a child to hospital is a stressful time for a family, particularly so with neonates. A parent or guardian is required to consent for neonatal participation for any research study [62]. Parents may struggle with the idea of volunteering their child for research studies during a period of illness. The thought of additional procedures will add to parental concerns regarding

research participation. The evidence that informed and voluntary consent can be obtained under these conditions is limited [63], although it has successfully occurred in the past with clinical trials for surfactant, where consent was gained antenatally [64]. There are some limits to parental consent, with parents not able to consent their children for as wide a range of studies as an autonomous adult [62]. Parents themselves have also noted concerns about consent, including that both parents should provide signatures [65].

Ethics committees may lack members with neonatal expertise and, therefore, may err towards caution when reviewing neonatal studies. As a result, the number of studies conducted may be limited or studies may be significantly delayed, even those with a minimal impact on patients and their families. The current "standard of care" may also influence the decision to approve a study, even if the current therapies are not based on evidence [63], due to concerns that the proposed interventions differ from standard practice. Health professionals with experience and training in neonatal care, including pharmacists, should be encouraged to pursue opportunities to be involved as members of local ethics committees to address this imbalance.

Ethical considerations are an important factor in designing any neonatal research project. Neonates are a vulnerable population with complex medical and developmental needs [66] and most standard trial interventions carry greater risk in neonates [67]. Any project should ensure these are considered. The use of clinical samples and interventions for research purposes results in no additional handling or sampling burden on the study participants and has been used successfully [68].

In all studies involving neonates the benefit of conducting the study must outweigh any risks to the participants [69]. For remifentanil, for example, the benefit of improved analgesia for painful procedures would outweigh the risks associated with a low impact study requiring small blood volumes for analysis.

While ethical considerations are an important part of designing these studies, it is also important to consider the scientific necessity of completing this research [63] to ensure improvements in safe and appropriate care for neonates in the future.

The ethical issues that were raised in the development of these studies centred around blood sampling and consent. In my clinical study, blood sampling volume concerns were overcome by the use of left over clinical samples. A system was developed where patients who had consented to take part in the study were identified to pathology and samples left over following processing for clinical need for collection by research staff. Working closely with the pathology team, combined with research assuring sample stability, allows collection of samples under appropriate conditions. While a system like this will not be suitable for all drugs it will expand the available samples for a large number of drugs in clinical use with no impact on patients.

The consent forms for the study were developed in conjunction with experienced neonatal research nurses who had experience with gaining consent from parents for research studies. The forms were developed to provide the information required by the ethics committee while still providing plain English information for parents without overwhelming them at a difficult time.

Sample size and appropriate study power

While population pharmacokinetic studies significantly reduce the ethical burden of neonatal pharmacokinetic research recruiting the appropriate number of participants remains a problem [69]. Only a small number of babies require neonatal intensive care resulting in studies taking a long time. Use of data collected clinically and data sharing between sites improves the rate and amount of data that can be collected to build pharmacokinetic models [70].

The small sample size is compounded by the developmental differences across the neonatal population. As information regarding the development of drug metabolising pathways is limited is difficult to determine how best to analyse any data. Whether weight or gestational age groupings are best is still unknown [61].

Multisite studies for population pharmacokinetics are challenging due to the variation of drug dosing used across sites [63]. Although a number of resources exist they are based on limited evidence and are often overruled by individual clinical experience. The first step in successfully completing this type of research is standardising drug dosing across a number of sites that will allow for participant recruitment at a higher rate than could be achieved in a single unit. The Netherlands have demonstrated that this standardisation works, enabling completion of a large number of neonatal pharmacokinetic studies. Work has recently begun on drug dosing standardisation in Australia with the NeoMed group and this will improve the ability to complete pharmacokinetic research, assuming appropriate analysis techniques are available.

Technical issues

Following the recruitment of an appropriate number of participants who have received the same drugs at the same dose the analysis of the samples becomes the next step.

Analysis of neonatal samples requires techniques which can detect low drug concentrations in small samples. Previous iterations of HPLC systems have not been able to do this. Newer HPLC-MS/MS systems with triple quadropole technology have greatly increased the sensitivity for drug assays while also requiring smaller samples for analysis. Throughout the duration of the study access to HPLC-MS/MS increased, improving the usefulness of HPLC-MS/MS methodologies. Work originally started on HPLC-UV however it quickly became

apparent that this equipment was not suitable for analysing low plasma concentrations in the small blood sampling volumes available from neonates. Switching to HPLC-MS/MS was an important step in achieving the aims of this study at was identified that access to suitable equipment in an important factor in completing neonatal pharmacokinetic research.

Samples of 50 to 100μl are now commonly used with this technology, compared to the 1mL samples required previously. Neonates are not able to provide a large number of samples or the large volumes previously required for analysis. These new systems also require low injection volumes allowing for re-analysis, if required, without re-sampling. Availability of a microassay, which require small amounts of blood, is an important consideration in neonatal research. It is recommended that studies limit samples to a blood volume of 3mL/kg [71]. Assays that can measure drug concentration in 200-300μl are required to achieve these goals [69] with the technology now available to exceed these requirements, further reducing the samples required for patients.

In addition to microassay methodologies new blood sampling techniques have also been developed which improve the ability of neonates to participate in pharmacokinetic research studies. Re-purposing older techniques like dried blood spots and newer devices such as Mitra® will further reduce the sample size taken from neonates. Full integration of these sampling options requires analysis techniques capable of detecting the low levels of drug in samples of less than 10μl along with the ability to analyse whole blood or plasma.

While this will increase the number of samples able to be taken, samples of this size will only be able to be used for drugs with sufficiently high plasma concentrations with the currently available HPLC-MS/MS equipment. These new techniques, however, provide hope for the future.

Access to equipment

HPLC-MS/MS offers other advantages for drug concentration analysis. Mass spectrometry allows for individual compounds to be detected reducing the risk of false positives while providing a cleaner baseline to ensure result integration of peaks is correct.

Access to the appropriate technology to analyse these samples is often limited to large tertiary referral hospitals and universities limiting the ability to conduct these kinds of studies. As the technology is new, methodologies to detect drugs are still being developed. Skilled personnel to conduct the assays are also required. This was an initial difficulty encountered in this work. When this project commenced, only HPLC with UV detection was available for development of analytical techniques. HPLC-MS/MS facilities only became available in recent years. Having to use HPLC-UV in the initial stages of this project clearly highlighted how lack of appropriate equipment could impede the conducting of neonatal research. When HPLC-MS/MS became available, it offered the opportunity to lead in the development of suitable analytical methodologies at a much faster rate.

Funding considerations

Like all research, neonatal pharmacokinetic studies require funding. The use of clinical sampling reduces costs. HPLC consumables are also reasonably inexpensive. However, HPLC-MS/MS equipment is very expensive which limits availability.

The drugs used in neonates have usually been in clinical use for long periods of time. Research into drug use in neonates offers no advantages to pharmaceutical companies, as it will not change usage or profits, reducing interest in conducting research. Funding sources for these studies are usually charities or competitive

grants. The challenges associated with this work can concern funding bodies, as they pose a risk to the completion of the work. Currently, practice does not allow for the completion of studies for most drugs in a reasonable time frame reducing the feasibility of these studies [69].

Licensing considerations and support from regulatory bodies

Currently, most drugs used in the NICU are unlicensed for use in the neonatal population [63, 69]. As there is no financial benefit to seeking licensing in Australia regulatory oversight will remain limiting. Without this oversight adverse effect reporting and other safety signalling systems are not present for neonates. The FDA [69] and EMA have encouraged pharmaceutical companies to seek this licensing by offering financial incentives, such as extended patents. Without such support for regulatory bodies this type of research is unlikely to occur on a wide scale and will remain dependent on individual groups of researchers.

The FDA convened a panel of experts in 2003 to prioritise the investigations of off-patent drugs in the neonatal population [69]. The conclusion of the panel was that judicious use of limited resources in this area remains an urgent public health need. The factors outlined [69] in prioritizing drugs for study in neonates were considered in the design of this work.

Solutions

The solutions to these complicated issues are multifactorial and dependent on wide-scale change and increased organizational support for neonatal research, particularly around the streamlining of ethics and governance procedures for multi-site studies. The increased representation of multidisciplinary neonatal clinicians to advocate on ethics committees, hospital leadership committees, funding organisations and regulatory bodies could help raise the profile of this

research area. However, it is well known that clinician time for additional service outside of clinical care is unfunded and burdensome. Support for clinical staff to have the dedicated time to conduct translational research, within their current workload, would improve the completion rate of this research.

Increasing the neonatal expertise on ethics committees will reduce the number of concerns raised when neonatal research proposals are submitted. Comprehensive international guidelines addressing areas such as appropriate blood sampling and consent for neonates, are available and could be adopted by ethics committees worldwide. Increasing the use of antenatal consent from parents may help in improving participation rates by allowing more time for discussions about being involved in the study.

The development of low volume plasma/blood analysis techniques improves the feasibility of conducting this type of research. These techniques, combined with population pharmacokinetic modelling, allow researchers to use blood left over from clinical samples, resulting in negligible effects on the research participants.

Further research into drug concentration analysis techniques using low plasma volume or DBS technology will expand the number of drugs than can be studied in neonates. Once access to the equipment is established, this research will have low running costs beyond staff time. The fast throughput of the methodologies allows for large numbers of samples to be processed in a single session.

Once the research is completed, an efficient approach is required to quickly translate the findings to practice and update medicine formularies [72]. Research networks should work closely with local formulary committees and governance bodies to develop evidence-based dosing information that can be used for patients. Pharmacists, as part of their clinical practice, should consider opportunities for the publication of data collected during clinical care to help

inform the prioritization and design of clinical studies. Neonatal pharmacy should continue to be promoted as a specialist option to allow pharmacists to bring their unique skills to improving the use of medicines in this population.

Summary

In order to make neonatal drug dosing research feasible in Australia a number of factors have to come together. This work will provide a starting point for developing large-scale projects by sharing the experiences and solutions described in this Chapter. Beginning with sharing these experimental techniques, working with ethic committees, improving representation of clinicians working with neonates on research bodies and engaging clinicians involved in consensus dosing across Australia it will be possible to convert this preliminary work into the necessary large-scale pharmacokinetic studies of Australian neonates that are required to provide evidence based dosing.

Chapter 3: Development of an HPLC-MS/MS method for remifentanil quantification in a small plasma volume.

Introduction

Remifentanil (Figure 4) is an ultra short acting synthetic μ opioid receptor agonist used in anaesthesia and as an analgesic agent. In common with other opioid analgesics, remifentanil exhibits dose dependent analgesia across the therapeutic range and side effects of respiratory and central nervous system depression [73, 74]. Remifentanil differs from many other opioids in its routes of metabolism. In contrast to other opioids, which undergo metabolism via cytochrome enzymes and glucuronidation pathways, remifentanil is hydrolysed by nonspecific esterases to remifentanil acid. This is 800 to 2000 times less potent than the parent and not considered to have any relevant clinical effect [73, 75].. The relatively short half life of remifentanil reduces the clinical significance of respiratory depression [76]. As the metabolism is not dependent on liver or kidney function it is useful in populations where these organs are impaired or not yet fully developed, such as neonates [77].

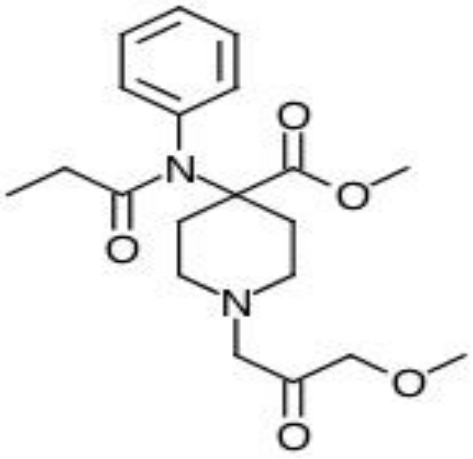


Figure 4: Chemical structure of remifentanil.

Use of remifentanil in neonates

Although described as an appropriate option for neonatal pain relief uptake has been slow due to a lack of clinical trial data. Small trials have been conducted but these have not included pharmacokinetic analysis that would assist with determining the appropriate dose for indication [78-80]. Studies in neonates have difficult ethical considerations and previous assays have not been able to achieve the levels of detection required [81, 82] from the available volume of blood. Current information regarding remifentanil metabolism in neonates is based on umbilical cord samples that are not limited by volume [81, 83] but do not provide a complete picture of expected metabolism in neonates post the transition phase. A major challenge in describing the pharmacokinetics of remifentanil is that dosing of this drug is in the microgram per millilitre range. Following distribution in the body, the resulting plasma drug concentrations will be extremely small. Access to sensitive, specific, reliable, small volume assay methodologies for determining remifentanil concentrations will improve the understanding of remifentanil pharmacokinetics and expand its use across diverse populations and clinical settings, not just neonatal care.

Remifentanil has been shown to reduce the time to extubation and awaking in intubated premature neonates when compared to morphine [84] and has been used safely in an infant with respiratory distress syndrome [85]. 48 neonates born between 25 and 33 weeks gestations were provided with remifentanil analgesia and sedation for mechanical ventilation without serious adverse events [86]. The use of remifentanil for pain relief for short procedures in the intensive care unit has been suggested for procedures including percutaneous central catheter placement and laser surgery for retinopathy of prematurity [87]. While remifentanil has been shown to be non inferior to suxamethonium and fentanyl for intubation, concerns were raised regarding muscle rigidity at higher doses and further research was suggested [78].

Pharmacokinetics of remifentanil in neonates

The only published pharmacokinetic study of remifentanil in preterm infants was conducted using umbilical blood by Welzing et al (2011). Umbilical cord blood was collected from infants born at 24 weeks gestation to 36 weeks gestation. The activity of the nonspecific esterases was comparable between the youngest group of infants at 24-27 weeks and term infants, however the half-life of remifentanil was increased in the most preterm infants. The half-life was increased in all groups of infants compared with adult values. This study was conducted using serum samples from a small number of neonates (40) which may not consider the activity of whole blood as non specific esterase is found in red blood cells [83]. A summary of the limited pharmacokinetic data for remifentanil in neonates and infants reported in the literature is seen in Table 3 below.

Table 3: Summary of reported remifentanil pharmacokinetics in neonates and infants

Age	Clearance	V_d	Author
114-360 days	6.03x(wt/70kg) L/min	19.6x(wt/70kg) L**	Chang et al [88]
<2 months	90.5mL/kg/min	452.8mL/kg **	Ross et al [89]
10 months- 15 years Undergoing cardiopulmonary bypass (CPB)	pre-CPB: 38.7+/- 9.6 mL/kg/min post CPB: 46.8 +/- 14 mL/kg/min	pre-CPB: 234.5 +/-105.5 mL/kg post-CPB: 235.3 +/- 110.2 mL/kg **	Davis et al [90]
6 months- 4 years Undergoing CPB	pre-CPB 0.244L/min	pre-CPB 1.4L *	Sam et al [91]

*One Compartment model

**Two Compartment model

Analgesia from opiates is generated by spinal or supraspinal activation of opioid receptors. This leads to inhibition of the nociceptive pathways altering perception and response to pain [92]. The time to a 50% decrease in serum drug concentrations is 3.2 (SD 0.9) minutes [93]. Remifentanil equilibrates between the blood and brain within 1 to 1.5 minutes and has a context-sensitive half time of between 3 and 5 minutes. Computer modelling has predicted that remifentanil concentration decreases by 80% in less than 15 minutes [73]. The context sensitive half time is the time it takes for the concentration of a drug to decrease by 50% in the blood and brain following an infusion [94]. This half-time is what makes remifentanil useful in a clinical setting as its quick onset makes it easily titratable while the short half-life reduces the risk of long lasting side effects.

Metabolism

Remifentanil differs structurally from other opiates as it contains an ester linkage making it susceptible to non-specific esterases. Metabolism occurs in blood and other tissues, most likely skeletal muscle. Red blood cells metabolise remifentanil at a much higher rate than circulating esterases in plasma [95]. A comparison of in-vitro and in-vivo metabolism showed a significantly longer half-life in in-vitro samples. This suggests that the majority of remifentanil metabolism occurs in other tissues rather than blood [96].

Remifentanil is metabolised to a compound, known as remifentanil acid (Figure 5), which is 800 to 2000 times less potent and is not considered to have any clinical effect [73, 75]. Remifentanil acid accounts for more than 98% of the metabolites of remifentanil and occurs by cleavage of the propionic acid methyl ester linkage. A minor metabolite is also produced by dealkylisation [95]. There is thought to be some binding competition between remifentanil and remifentanil acid as both bind the μ opioid receptor. The low binding affinity of remifentanil acid results in no pharmacological effects of this competition [97].

This metabolite is mostly excreted by the kidneys [75] but can also be excreted in the faeces [98]. Remifentanil is not a substrate for pseudocholinesterase, acetylcholinesterase or carbonic anhydrase [95].

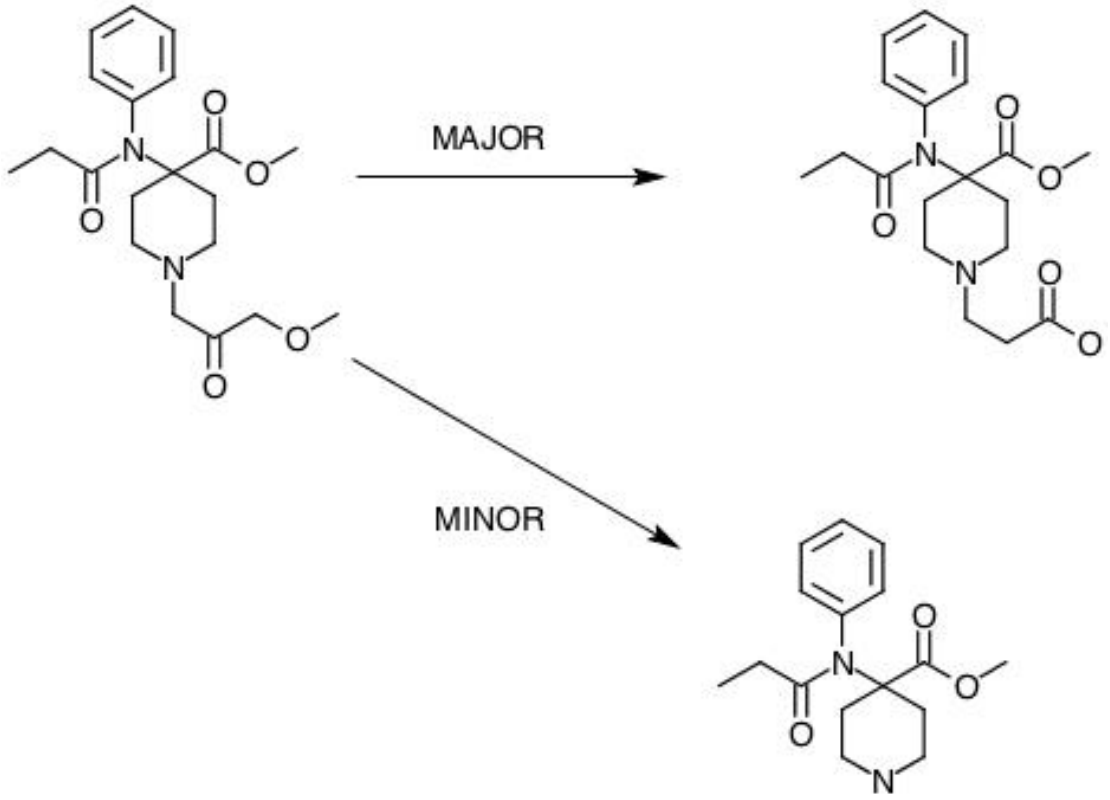


Figure 5: Pathways of Remifentanil metabolism. The major metabolite remifentanil acid shown top right and minor metabolite GI-94219 shown bottom right

Differences have been noted between arterial and venous concentrations of remifentanil. Venous concentrations are lower as the non-specific esterases present in blood and tissue have metabolised remifentanil [99]. Effect is more closely linked to arterial rather than venous concentrations [100].

Elimination

The elimination of remifentanil is thought to be independent of the rate of infusion due to the capacity of the tissue esterase system [101]. The rate of remifentanil hydrolysis in blood is limited but occurs rapidly in a number of other tissues with high levels of esterase activity [98]. Remifentanil is not metabolised in the lung [95].

Metabolism in renal and hepatic impairment

Renal and hepatic impairment are not associated with changes in remifentanil metabolism although patients with liver disease may be more sensitive to opiates [73]. Studies conducted during liver transplant surgery have shown that the presence or absence of the liver does not affect the rate of remifentanil metabolism [96]. Renal impairment does not significantly affect remifentanil clearance delivered by infusions lasting up to 72 hours although the clearance of metabolites was decreased. Remifentanil acid did not accumulate to a level that resulted in clinical effects [102]. Remifentanil acid is extracted during haemodialysis [103].

Other factors affecting metabolism

Increasing age has been shown to affect remifentanil pharmacodynamics as the incidence of adverse effects and sensitivity to opioids increases [95, 104]. Obese patients do not require an increase in dose [100]. It is recommended that remifentanil dose be calculated on lean or ideal body weight rather than total body weight. Studies comparing the two observed significantly higher effect site concentrations in obese patients whose dose was calculated on their total body weight [105]. There are no known pharmacogenetic differences in remifentanil metabolism [106].

Side effects

Remifentanil is associated with similar side effects to other opiates. These include nausea, vomiting, muscle rigidity, bradycardia, urinary retention, seizures, sedation and hypotension [73, 92]. Parasympathetic inhibition by atropine does not completely prevent negative chronotropic effects induced by remifentanil in children [107].

Placental Transfer

Remifentanil readily crosses the placenta [108]. The rate of placental transfer of remifentanil was assessed by comparing maternal arterial concentrations with umbilical arterial and venous concentrations in samples taken at birth. A significant amount of placental transfer is seen with an average ratio of 0.88 between the umbilical vein and maternal artery samples following infusion. Differences in remifentanil concentration between the umbilical artery and vein suggest metabolism is occurring in the term foetus [75]. A second study showed similar ratios between maternal and umbilical circulation with a ratio of 0.73 (SD 0.17) following bolus dosing [108]. Both studies had only a small number of participants, which may not allow for individual variability in esterase activity and placental transfer. No significant differences in the number of term neonates requiring resuscitation was seen in cases where remifentanil was used as part of general anaesthesia for caesarean section compared to standard anaesthesia and only mild respiratory depression was seen [108]. A study of 50 women using a remifentanil PCA in labour showed minimal effects on the neonates after birth with acceptable levels of adverse effects in the mother [109].

HPLC assays for remifentanil

Previously published assays for remifentanil have required over 200µl of plasma [82] and the use of complex extraction methods [81] to determine plasma remifentanil concentrations. The volume of blood or plasma required in these assays is a barrier to being able to perform the required pharmacokinetic studies

in neonates, while complex extraction techniques reduce the assay efficiency. For pharmacokinetic studies to be performed in neonates, microsampling techniques for blood sampling and assay methods capable of accurately measuring drug concentrations in small blood/plasma volumes are required (6). Methods of remifentanil analysis using HPLC-UV are not common in the English language literature and from review of abstracts do not reach the required level of sensitivity for neonatal studies. Based on these issues, HPLC-UV methodologies were not explored as part of this work. A summary of published assays for remifentanil using HPLC-MS/MS below in Table 4.

Table 4: Summary of published HPLC-MS/MS assays for remifentanil.

Study	Equipment	Plasma Volume	Limit of Detection	Extraction method	Run Time	Injection volume
Bossu et al [81]	HPLC-MS single quadrupole	250µl	0.5ng/mL	Solid phase	24 min	125µl
El Hamd et al [82]	HPLC-MS Triple quadrupole	200µl	0.17ng/mL	Liquid-Liquid	Not published	50µl
Alvarez et al [110]	HPLC-MS Triple quadrupole	500µl	0.1ng/mL	Liquid-Liquid	10 min	75µl
Said et al [111]	HPLC-MS Triple quadrupole	20µl	0.05ng/mL	Micro extraction in packed syringe	5 min	10µl

It is evident from the literature that further research is needed to investigate remifentanil pharmacokinetics when it is administered to neonates. While some assays have been published, there is still a need for an assay suitable for use in neonatal studies.

The aims of this study were:

1. To develop and validate a HPLC MS/MS assay suitable for use in conducting remifentanil pharmacokinetic studies in neonates
2. To demonstrate applicability in vivo, by measuring plasma remifentanil concentrations in small volume plasma samples obtained from rabbits administered a remifentanil infusion

Experimental

Chemicals and materials

The remifentanil standard was obtained from Sandoz (Sydney, Australia). Sufentanil, which has similar chemical properties to remifentanil, was obtained from Janssen-Cilag (High Wycombe, United Kingdom) and used as an internal standard. HPLC-grade acetonitrile, formic acid and methanol were obtained from Sigma-Aldrich (Sydney, Australia). Citric acid was obtained from PCCA (Sydney, Australia). Purified water was obtained from a Milli-Q® purifying system (Millipore Co. Bedford, MA, USA).

Instrumentation and chromatographic conditions

HPLC was performed using a Shimadzu Nexera system equipped with two pumps, a DGU-20A5 degassing unit, autosampler and CTO-20A column oven (Kyoto, Japan). Chromatographic separation was achieved using a Phenomenex Kinetex® C18 50x3mm 2.6µm column maintained at 40°C. The mobile phase consisted of acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). A

gradient was used with solvent A increasing from 5% at time 0 to 95% at five minutes with a flow rate of 0.5mL/min. Injection volume was 3µl.

The mass spectrometer was a 6500QTRAP (SCIEX, Framingham, MA, USA). The MS operated in positive electrospray mode with the following settings: Curtain gas-20, Collision Gas-Nitrogen, IonSpray Voltage-5500, Temperature-550°C Ion Source Gas 1-20 and Ion Source Gas 2-20. Scheduled MRM mode was used for compound detection with a detection window set to 60 seconds around the expected retention time. Data acquisition was controlled by Analyst 1.6.3 and processed with MultiQuant® 3.0 software. (SCIEX, Framingham, MA, USA).

Transitions of 377.05-317 (Collision energy 23 CXP 20), 377.05-113 (Collision energy 35 CXP12), 377.05-228.1 (collision energy 25, CXP 12) were used for remifentanil with 387.2-238.1 (collision energy 30 CXP12), 387.2-111.1 (collision energy 40 CXP12), 387.2-355.2 (collision energy 25 CXP12) for sufentanil. Product ion scan for remifentanil (Figure 6), remifentanil transitions (Figure 7) and sufentanil transitions (Figure 8) are shown below.

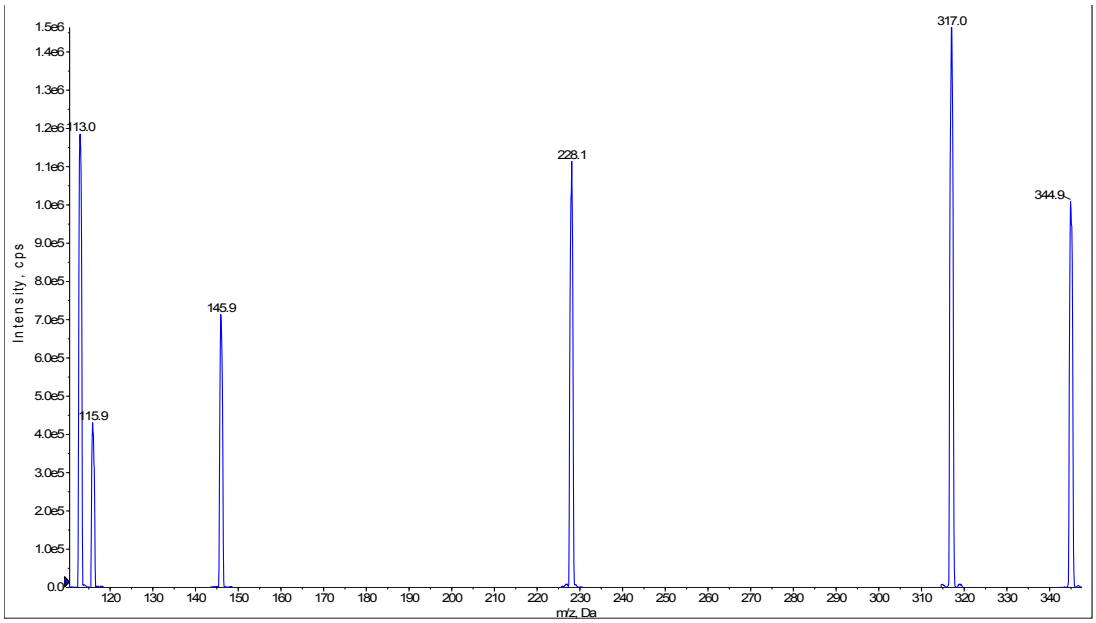


Figure 6: Product ion scan for remifentanil. Parent molecule at 377.

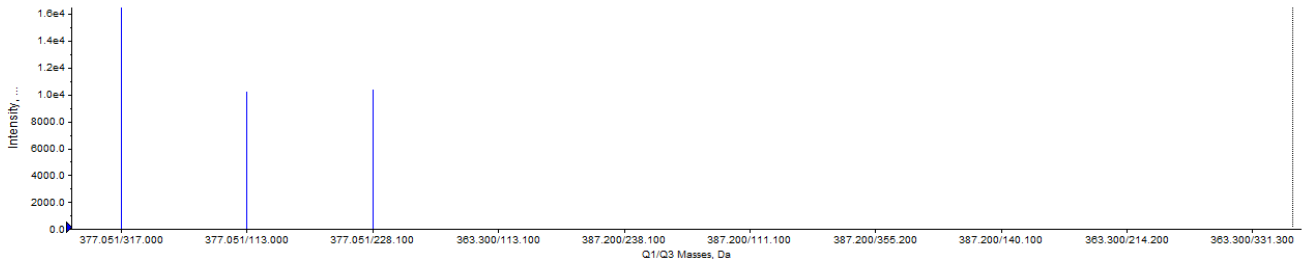


Figure 7: Remifentanil transitions.

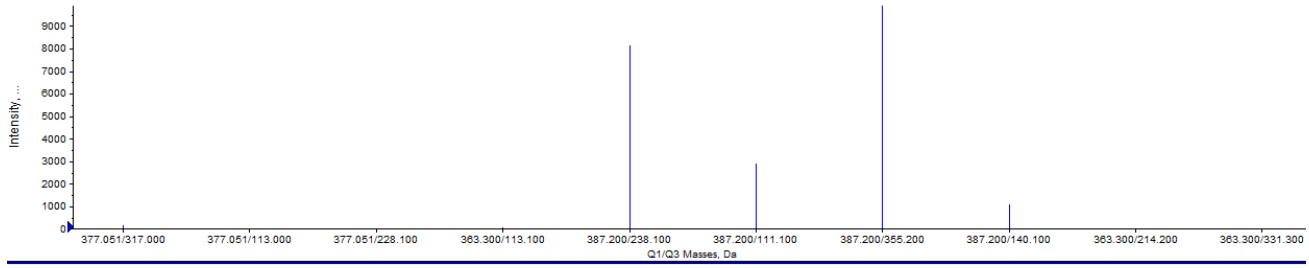


Figure 8: Sufentanil transitions.

Observed retention time of remifentanil is 1.93 minutes with sufentanil at 2.5 minutes.

Standard solutions

Stock solutions of remifentanil and sufentanil were obtained by diluting reconstituted solutions with HPLC-grade methanol. Calibration standards were prepared by diluting an aliquot of the stock solution in plasma. Calibration standards were prepared at the following concentrations 0.25ng/mL, 0.5ng/mL, 1ng/mL, 5ng/mL, 10ng/mL, 50ng/mL. Stock solutions, calibration standards and quality control (QC) standards were stored at -40°C.

Sample preparation

An acetonitrile precipitation method was used to extract and prepare samples. Sufentanil was added to acetonitrile for a final concentration of 0.5ng/mL. 300µl of this solution was added to 100µl of the plasma sample. 10µl/mL of 50%w/w

citric acid was added to all samples and standards to prevent in sample degradation of remifentanil by esterases in plasma. Samples were then vortexed for ten seconds and allowed to stand. After 5 minutes samples were centrifuged at 6800 *x g* for 10 minutes. 100µl of supernatant was transferred to polypropylene HLPC vials for injection into the HPLC system.

Bioanalytical method validation

The assay was validated by examining system suitability, carryover, selectivity, matrix effect, linearity, precision, accuracy and stability. System suitability was determined by injecting neat solutions of remifentanil and sufentanil (internal standard). The effect of carryover was evaluated by injecting blank plasma samples following spiked samples at both upper and lower limits of quantitation to ensure the retention time had passed before subsequent analytical runs.

The lowest limit of quantification (LLOQ) was used to define the sensitivity of the analytical method. The sensitivity of the method was determined by analysing 7 sets of spiked LLOQ samples. QC standards were included in each run. A high concentration of 15ng/mL and a low concentration of 0.75ng/mL was selected. Three QCs of each strength were included with each run.

Linearity was determined by generating a calibration curve from samples containing concentrations ranging from 0.25ng/mL to 50 ng/mL. Each calibration curve was analysed individually and a linear regression with $1/x^2$ weighting was used. Intra day precision and accuracy was assessed by determining the average and standard deviation calibration curves and QC's. The acceptable precision (CV) was set at 15% and no more than 20% at LLOQ. For accuracy, the accuracy % must be within +/- 15% of their nominal value and for the lowest concentration +/- 20%. Blank adult human plasma was screened using the method of detection for remifentanil. The freeze thaw stability was determined by analysis of the QC material that had been frozen at -80°C three

times. Stability was determined by storage of the standard and QC material at -40°C for 30 days. Both standards and QCs had 10µl/mL of 50%w/w citric acid added to denature the enzymes and improve stability [81].

Matrix effect was tested using pooled rabbit plasma and pooled adult human plasma samples. Rabbit was chosen as an animal model as the blood volumes available for testing are similar to neonates. Both water and pooled plasma was spiked with remifentanil and extracted.

In vivo performance of assay

In order to test the applicability of this small volume sample assay, plasma samples from six rabbits (University of Newcastle Ethics Approval A-2013-314) receiving an IV infusion of remifentanil were analysed. Samples were taken after 12 minutes of a remifentanil infusion of 0.1,0.2,0.3 or 0.4µg/kg/min. Samples were collected in tubes containing 10µl/mL of 50%w/w citric acid to denature drug metabolising enzymes. Blood samples were centrifuged for 10 minutes at 6800 x g to separate plasma and cell components. Samples were frozen before analysis. The acetonitrile precipitation method described above was used to prepare the samples for analysis.

The elimination rate constant (K) for remifentanil for each of the tested samples was calculated using the first order elimination equation where slope

$$(-k) = \frac{\ln C2 - \ln C1}{t2 - t1}$$

C2 was the concentration during the infusion and C1 was the concentration post infusion as determined by the peak size detected. The half-life determined using the equation:

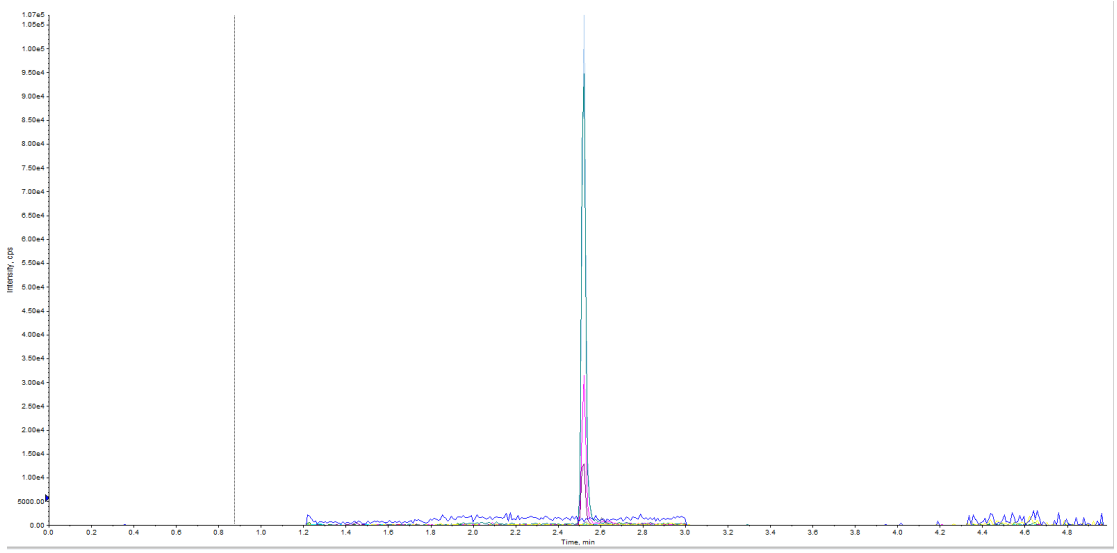
$$t\frac{1}{2} = 0.693/k$$

Results

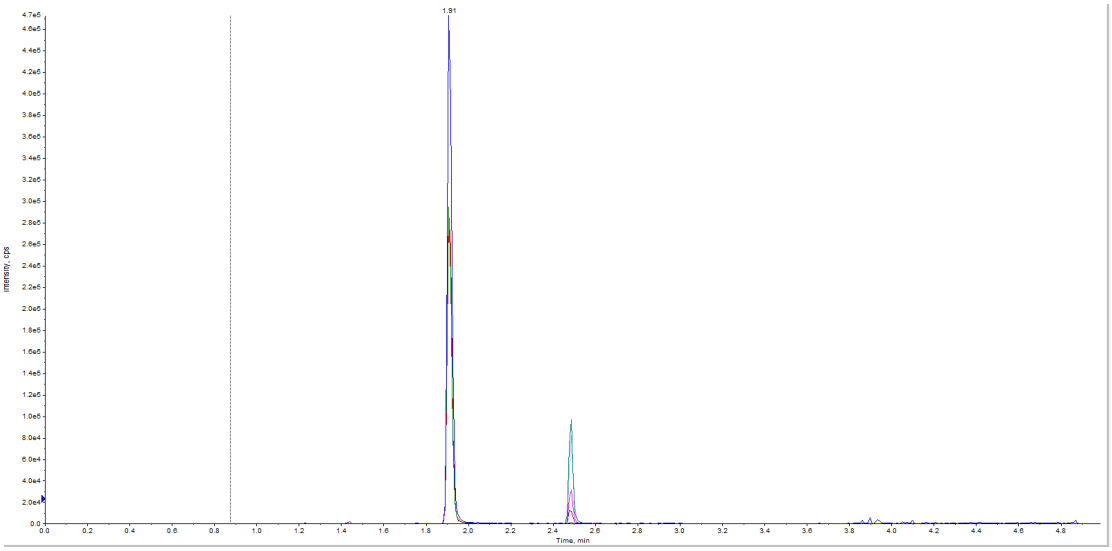
Liquid chromatography

In determining the optimal conditions for liquid chromatography several factors were assessed. The gradient of acetonitrile-water was adjusted from an acetonitrile starting position of 10 to 5 % to reduce peak width. Using acetonitrile instead of methanol in the mobile phase resulted in reduced noise and improved the lower limit of quantitation from 0.5ng/mL to 0.25ng/mL. A biphenyl column was tested but did not retain the compounds effectively. None of these factors either individually or in combination improved the chromatography of the samples.

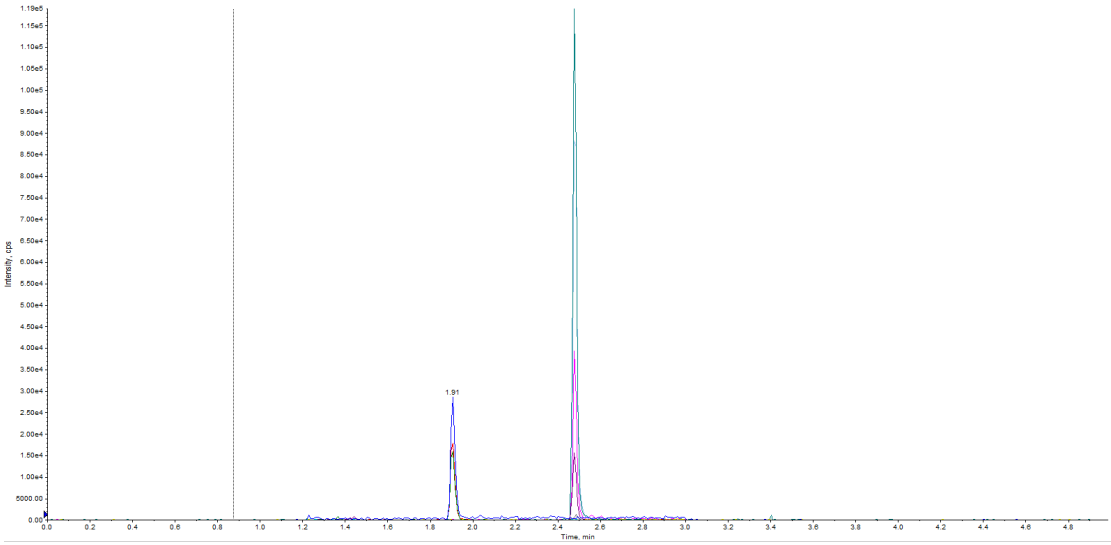
Flow rates of between 0.3mL/min and 1mL/min were tested with 0.5mL/min providing the optimal results with regard to peak shape and retention time as shown in Figure 9.



a)



b)



c)

Figure 9: Remifentanil chromatograms

Chromatograms showing:

a) Remifentanil blank

b) Remifentanil high concentration QC standard 15ng/mL

c) Remifentanil low concentration QC standard 0.75ng/mL .All extracted from human plasma with acetonitrile containing 0.5ng/mL internal standard. The chromatogram shows the three transitions overlaid for both remifentanil and internal standard. Retention time of remifentanil is 1.93 minutes with internal standard at 2.5 minutes.

Mass spectrometry

In order to determine the most sensitive ionization mode for remifentanil and sufentanil full scans were conducted in both positive and negative mode. Positive ion detection provided higher signal strength. The 377-317 MRM transition for remifentanil was used for quantification as it provided the most consistent peak for analysis.

Optimization of sample preparation and recovery

Precipitation of remifentanil was tested using methanol and acetonitrile, with acetonitrile extractions providing better chromatography and sensitivity. Plasma volumes for extraction between 50µl and 300µl were tested (at 50µl intervals) to determine the minimum plasma volume that could be used for extraction without reducing the sensitivity of the assay. Recovery using the precipitation method with acetonitrile was determined to be 90%. The addition of citric acid prevented degradation in the calibration and quality control standards.

Calibration curve

A calibration curve ranging from 0.25 to 50ng/mL was prepared by adding dilutions of remifentanil 100µl of blank plasma and centrifuging to mix. 300µl of acetonitrile containing 0.5ng/mL sufentanil as an internal standard was added, mixed via vortex mixer and allowed to precipitate for 5 minutes. Samples were then centrifuged and the supernatant transferred into HPLC vials for analysis. Calibration curves were accepted with a correlation coefficient (r^2) of >0.99. Calibration curves used analyte to internal standard peak area ratios with a weighted ($1/x^2$) least-squares linear regression.

As shown in Figure 10, a linear relationship between the remifentanil:IS ratio and drug concentration was observed in the range from 0.25 to 50ng/mL (r^2>0.99).

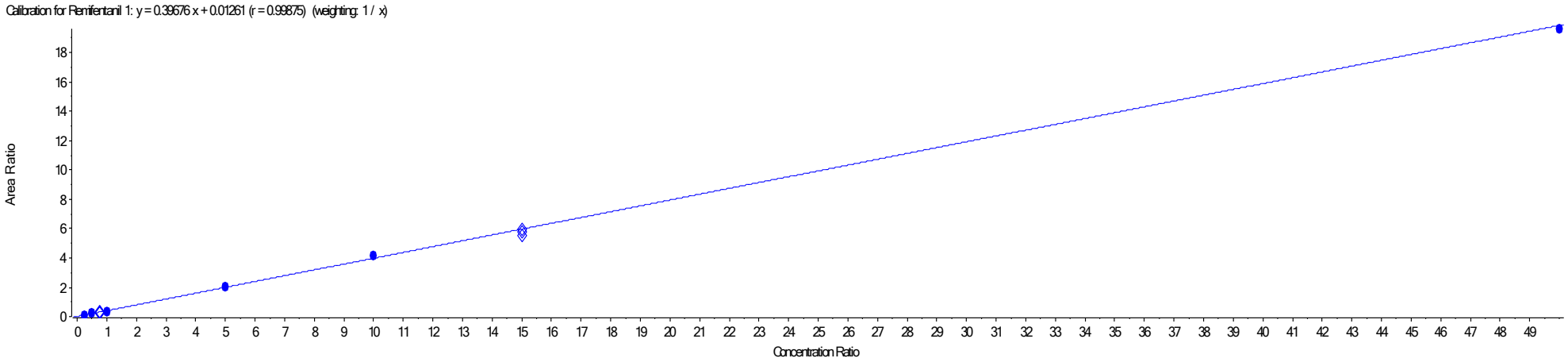


Figure 10: Calibration curve generated by plotting the ratio of remifentanil:sufentanil peak area versus plasma remifentanil concentration after extracted from plasma (ng/mL)

Accuracy and Precision

QC samples were prepared at 0.75ng/mL and 15ng/mL. To determine the between and within day coefficients of variation for the assay, standard curves and QC samples were run on seven occasions with three replicates of QC samples being analysed on each occasion plus an additional run containing seven replicates of the QC samples.

Seven replicate standard curves (Table 5) and 18 QC samples were run (Table 6). Plasma remifentanil concentrations obtained for each run were then analysed using Multiquant® software. The inter-day and intra-day variability was <10% at QC concentrations of 0.75ng/mL and 15ng/mL.

Table 5: Inter-day variability (expressed as coefficient of variation (CV) in accuracy) of standards.

	Inter day variability		**N=7**	
	Mean measured concentration ng/mL	**SD**	**Percent CV (accuracy)**	**Accuracy**
0.25ng/mL	0.23	0.056	25.6	92%
0.5ng/mL	0.53	0.04	8.2	106%
1ng/mL	0.98	0.165	18.5	98%
5ng/mL	4.8	0.551	11.4	96%
10ng/mL	10.4	1.951	16.5	104%
50ng/mL	49.6	4.961	10.1	99%

Table 6: Inter-day variability of QCs (CV=coefficient of variation)

	Inter-day variability		N=18	
	Mean measured concentration (ng/mL)	**SD**	**Percent CV (accuracy)**	**Accuracy**
0.75ng/mL	0.79	0.07	9.24	105%
15ng/mL	13.8	0.61	4.37	92%

The assay has been shown to be accurate over the range of 0.25ng/mL to 50 ng/mL. The LLOQ is 0.25ng/mL.

Stability

The freeze thaw stability was determined by analysis of the QC material that had been frozen at -80°C and thawed three times. Long-term stability was determined by storage of the standard and QC material at -40°C for 30 days. Following analysis of the freeze thaw samples, measured concentrations were within 10% of the original concentration. The samples stored for 30 days at -40 C were also within 10% of the original concentration. Based on this data, remifentanil was observed to be stable in extracted samples for at least one month when stored at -40 C.

Matrix effect

No identifiable matrix effect was determined with either pooled human or rabbit plasma. Peaks were similar size following extraction from water or plasma.

Analysis of in vivo samples

In order to provide proof of applicability of the assay to pharmacokinetic studies, the assay was used to determine the plasma concentration of remifentanil over time in a hypoxic rabbit study [112] (Figure 11).

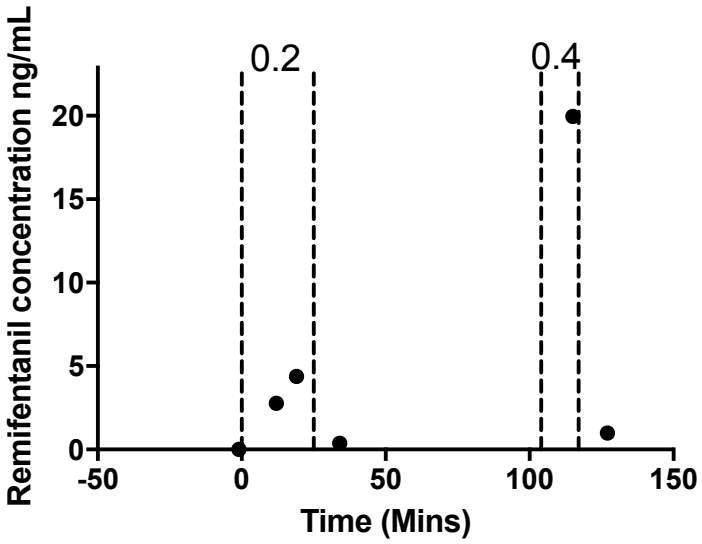


Figure 11: Concentration plot of remifentanil over time in a hypoxic rabbit. The infusion was run at 0.2mcg/kg/min for 25 minutes, then 60 minutes break was given for the rabbit to recover then an infusion of 0.4mcg/kg/min was commenced for 25 minutes.

When rabbits were administered increasing doses of remifentanil, the plasma remifentanil concentration increased. A plot of plasma remifentanil concentrations after each increasing. dose of infusion is shown in Figure 12.

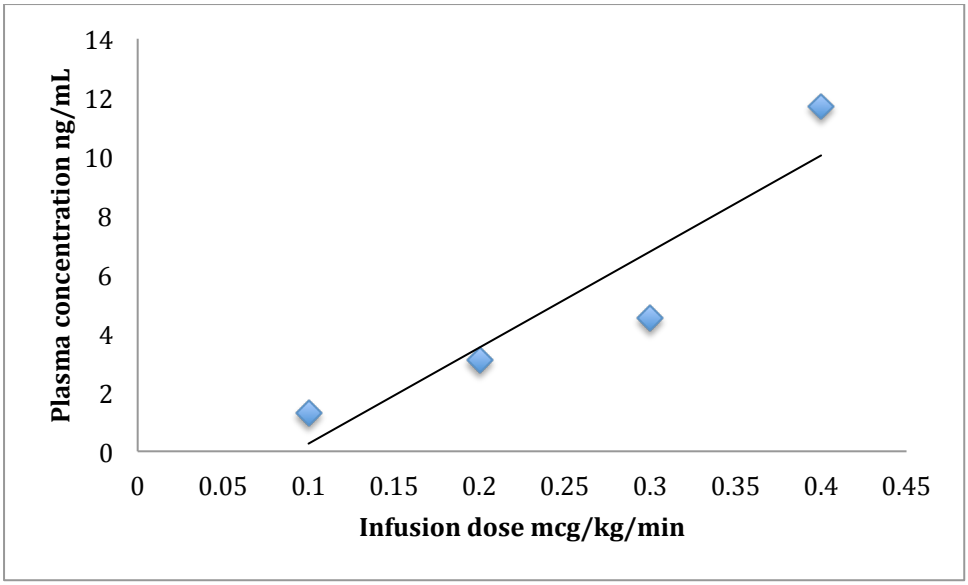


Figure 12: Plasma concentration of remifentanil in NZ white rabbits at doses of 0.1,0.2,0.3 and 0.4 mcg/kg/min

The elimination rate constants and elimination half-lives calculated in 6 rabbits after different doses are shown in table 7.

Table 7: Calculated values of elimination rate constant (k) and elimination half-life (t1/2) in rabbits

	0.1mcg/kg/min		0.2mcg/kg/min		0.3mcg/kg/min		0.4mcg/kg/min	
Rabbit	K	t ½ (min)	k	t ½ (min)	k	t ½ (min)	K	t ½ (min)
10 (a)			0.2	3.47			0.15	4.62
10 (b)	0.16	4.33			0.15	4.62		
11 (b)			0.36	1.93			0.45	1.54
12 (a)			0.39	1.78			0.17	4.08
12 (b)	0.28	2.5			0.16	4.33		
7 (a)			0.15	4.6			0.17	4.08
8 (a)			0.16	4.33			0.25	2.77
8 (b)	0.11	6.3			0.04	17.3		
9 (b)	0.4	1.73			0.12	5.78		

Discussion

The development of this assay demonstrates a significant improvement in the ability to analyse plasma samples for remifentanil concentrations. This assay is highly sensitive and can be used for small sample volumes.

Previously reported methods for detection of remifentanil in plasma by LC-MS used more complex methods for extraction in order to concentrate samples. One method required 500µl sample volumes and used ethyl acetate/hexane for extraction which carries risk for personnel [110]. This approach also required an evaporation step, which significantly increased the time required to perform the assay. Cartridge based extraction techniques [81, 111] have been used. However, this type of extraction requires additional equipment and carries significantly increased consumable cost limiting applicability to small-scale research projects. Cartridge extraction also is a more time consuming process than the precipitation method developed in this assay. This is relevant in the clinical setting where rapid turn-around times and cost minimisation are key factors for test availability for clinicians. Other methods require the addition of sodium carbonate buffer to the precipitation step [82] and have an additional drying and resuspending step which increases the time taken to prepare samples for analysis. Both these methods require larger plasma volume for analysis, needing 200-250µl compared with the 100µl in this assay. Only one method using cartridge based extraction uses a smaller sample volume [111]. None of the previously reported assays have achieved the sensitivity of this method with limits of detection of 1ng/mL [82] and 0.5ng/mL [81, 111] although this will also be related to the equipment used. The MS system used for this assay does represent improved technology compared with the single quadrupole [81] and triple quadrupole [82] used in the previous assays. The additional sensitivity of the UHPLC-MS used in this project allowed detection of much lower concentrations using a faster, simpler protein precipitation method of extraction. This fast and simple methodology without significant consumable cost is applicable for both research and clinical use. Its applicability for in vivo measurement of remifentanil in very small volumes has been demonstrated in an animal model of anaesthetised rabbits [112]. The data collected from the rabbit study allowed for the determination of the elimination constant (k) and half-life following intravenous infusion. The calculated half-life in the rabbit model falls between 1.73-5.78 minutes which is similar to the results expected in human patients, demonstrating this methods applicability for human research studies.

Clinical application

As a result of this study, a sensitive and specific low volume assay for measurement of plasma remifentanil concentration has been developed. This low volume, highly sensitive remifentanil assay represents a significant advance in the ability to analyse remifentanil and is suitable for clinical application. It has applicability in a wide range of settings and provides the capacity to perform pharmacokinetic studies in neonates and enable description of pharmacokinetic-pharmacodynamic relationship data to inform safe and effective dosing guidance.

The range, sensitivity and speed of the assay makes it suitable for both research and clinical use across a number of different populations, particularly neonates.

In order to ensure an assay is suitable for analysing neonatal standards a high degree of sensitivity and specificity from small amounts of blood is required. This assay uses only 100µl of plasma, which can be obtained from left over blood taken for testing as part of standard clinical care. This will improve the ability to recruit neonates to pharmacokinetic studies as there will be no impact to the patient.

Increasing use of remifentanil in neonates has been suggested [77] but is currently limited due to a lack of dosing information and quantitative studies of the pharmacokinetics in neonates, particularly those born preterm. To date a HPLC methodology has not been available to analyse the low concentrations in small volume blood samples that are required for neonatal research. The final sample volume with the low injection volume in this assay also allows for re-analysis if required reducing the risk of wastage. As well as in neonates, the ability to conduct further pharmacokinetic studies will be useful in the critically unwell or those with genetic conditions causing altered pharmacokinetics. Further development of this assay for use with microsampling techniques will

also increase its usefulness. However, current microsampling techniques will not provide a suitable volume of blood or plasma to be able to measure remifentanil concentrations in neonatal plasma at this time. The extremely low concentrations due to microgram doses administered presents a challenge to the currently available MS technology and future advances in this technology will be required to be able to consider this type of sampling.

Conclusion

This novel method of remifentanil detection using HPLC-MS/MS uses safe and simple extraction and analysis techniques on small samples with significantly improved sensitivity than previously reported. This work has resulted in development of an assay which can now feasibly be employed in studying the pharmacokinetics of remifentanil in neonates and could also be used for Therapeutic Drug Monitoring (TDM) to guide more accurate and individualised dosing.

Chapter 4. A Comparison of In-Vitro Remifentanil Degradation in Blood and Plasma from Neonates and Adults.

Introduction

Neonates in an intensive care setting frequently undergo painful and stressful procedures. One way to prevent pain is to reduce the number of procedures the neonate undergoes. Invasive procedures should also be conducted in the most effective way as this will reduce the pain and stress associated with the procedure [113]. Failing this, the use of both non-pharmacological and pharmacological interventions for pain relief should be considered.

Providing appropriate analgesia for neonates undergoing painful procedures is a major clinical challenge. In terms of pharmacological agents, the ideal analgesic for use in paediatric patients is not yet determined. Ideal properties of an analgesic in the paediatric population include rapid onset of action, predictable pharmacokinetics along with potency, inactive metabolites and a small number of adverse effects and hemodynamic changes [77].

A range of analgesics, including paracetamol, morphine and fentanyl are used in neonates. Remifentanil has been suggested as a suitable opioid for short-term analgesia in neonates [113] due its short half-life and ability to be metabolised independently of renal and hepatic function both of which are still developing in this group.

The chemical structure of remifentanil differs from other opiates as it contains an ester linkage making it susceptible to non-specific esterases. As a result, remifentanil does not undergo metabolism via CYP450 enzymes or glucuronidation seen with other opioids, instead it undergoes widespread extra-hepatic metabolism by blood and tissue non-specific esterases resulting in rapid

clearance of the drug. The identified metabolites are reported to have very little activity compared to remifentanil. The duration of therapeutic effect of remifentanil mainly depends on metabolic clearance and no redistribution within the body [100].

Identified sites of metabolism of remifentanil are the blood and skeletal muscle although other tissues are also likely to be involved. Red blood cells have been observed to metabolise remifentanil at a much higher rate than circulating esterases in plasma in adults [94]

Currently, few data are available on differences in the pattern of esterase enzyme expression in neonates. Any differences will affect the rate and extent of metabolism. Clinical observations have suggested that remifentanil is metabolised more slowly increasing the risk of adverse effects, particularly respiratory depression in premature neonates [79]. It is possible that neonates have less esterase activity than adults and more information is needed about the location, activity and amount of these important detoxification enzymes before drugs metabolised by this route can be used safely in this vulnerable population.

Esterases

Esterases are responsible for the metabolism of a number of drugs and endogenous compounds that have ester subgroups as part of their structure. The esterases found in red blood cells have different drug metabolising activity than those found in plasma [114]. While animal models are sometimes used to gain a preliminary insight where clinical studies are difficult to perform, this is not feasible for modelling neonatal esterase development. The range of esterases present in the serum of different species make extrapolation of metabolism studies of both drugs and endogenous compounds between species very difficult [114]. There are different types of esterases found in the human body. These are discussed in the following section.

Carboxylesterases

Carboxylesterase enzymes catalyse the hydrolysis of esters, amides and thioesters [115] and are responsible for cleaving carboxylic esters into the corresponding carboxylic acid and alcohol [116]. Although found throughout the body the highest concentration of carboxylesterases are found in liver microsomes [115]. Other locations are the small intestine and lung [117] testes, kidney, heart and monocytes/macrophages [118]. Carboxylesterases have been noted in the capillary cells of the brain and spinal cord suggesting they form part of the blood brain barrier [119].

Some carboxylesterases are found extracellularly in the plasma whilst others are associated with cell membranes both externally and on organelles such as the endoplasmic reticulum [120]. Animal studies have shown that expression of carboxylesterases can be increased or decreased based on exposure to drugs, in a similar fashion to CYP enzymes [120]. The active site of the enzyme is large allowing access to a variety of substrates [116]. Carboxylesterase content increases with age [121].

Non-specific Esterases

Non-specific esterases are a sub group of cellular carboxylesterases. They are designated as non-specific as no endogenous substrates have been identified [122]. They are best known as histochemical markers for macrophages [123].

The non-specific esterase responsible for converting beclomethasone from di to monopropionate has been shown to be expressed in the liver, colon, stomach breast, brain, monocytes and T-lymphocytes [122] and skin [124] suggesting that drug metabolising esterases occur in multiple sites around the body.

Esterase metabolism of remifentanil in neonates

A search of the literature for data on esterase metabolism of remifentanil in the blood of neonates yielded only one publication addressing this topic. A pharmacokinetic study of remifentanil in preterm infants was conducted on umbilical blood serum by Welzing et al (2011) [83]. Umbilical cord blood was collected from infants born at 24 weeks gestation to 36 weeks gestation. Degradation of remifentanil was observed in the serum samples collected. The activity of the nonspecific esterases in the serum from the umbilical cord blood was comparable between the youngest group of infants at 24-27 weeks and term infants. While no significant difference in the half-life of remifentanil was observed between different age groups, there was a tendency towards a longer half-life in the most immature preterm infants compared with the more mature preterm infants. This study was conducted using serum samples from a small number of neonates (40) and did not measure the activity in whole blood or red blood cells. While providing useful information that esterases are present in serum of neonates, it does not provide information on the location of esterases or a comparison between adult and neonate enzyme expression.

From the paucity of information available in the literature, it is evident that very little is known about the development of non-specific esterase at any stage of life and in particular the developmental changes of this drug metabolism pathways in preterm neonates. Given the utility of remifentanil in the preterm neonatal population, it is important to gain an understanding of where metabolism may be occurring and factors that may influence the rate of degradation of this drug. Having identified this lack of information as a crucial area where further data are required, as part of this research project, a study to provide information about esterase activity in neonatal blood was developed.

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The aims of this study were to

1. Demonstrate the feasibility of an in-vitro assay of remifentanil metabolism

2. Study the rate of degradation in neonatal samples compared with adult samples to determine if developmental differences exist
3. Investigate the in vitro metabolism of remifentanil in plasma and red blood cells obtained from preterm neonates to determine whether metabolism of remifentanil occurs in red blood cells

Methods

Sample collection

This study received ethical approval from the Hunter New England Human Research Ethics Committee (12/02/15/4.03). Following consent from parents left over blood from clinical samples was collected and used for analysis. Only samples collected in plain 1mL neonatal tubes were used, with samples collected in other tubes or in different volumes excluded from analysis. Red cell components obtained by centrifugation at 2000 *x g* for 10 minutes and plasma were stored separately at -80°C until analysis. Samples were collected from 16 patients. Ages ranged from 25 to 40 weeks gestation with a median age of 30 weeks. Male patients made up 44% of those recruited. Some participants provided multiple samples across their admission.

Adult samples (total = 30) were collected from a single female volunteer throughout the recruitment period and stored alongside the baby samples. Red blood cells and plasma was obtained using same technique as described for neonate samples. Samples obtained throughout in the study were stored at -80°C. Samples were tested from early and late recruitment to check for effects of freezing on esterase activity. A final adult sample was collected on the day of analysis. This sample was processed immediately and not frozen to determine effects of freezing on esterase activity.

Experimental method

Neonatal red blood cell components were resuspended with 0.9% sodium chloride 250µl upon thawing and vortexed for 3 minutes. Samples from neonates of 250µl plasma and 250µl of resuspended red cells were aliquoted into microfuge tubes for processing. For the adult samples 500µl was used. Samples were placed in a water bath at 37°C and allowed to stabilise for one hour to ensure consistent temperature. 10µl of a 1ng/mL solution of remifentanil was added at time point zero. 100µl was taken at time points 15 minutes, 30 minutes, 60 minutes and 120 minutes for adult samples. Due to volume constraints time points were only taken from neonate samples at 0 and 120 minutes. At each sampling time, the remifentanil metabolising non-specific esterase was denatured to stop drug degradation by adding 300µl of acetonitrile that also contained 500picogram/mL of sufentanil (the internal standard for the assay). The solution was vortexed, allowed to stand for 5 minutes and centrifuged for 10 minutes at 6500 x *g*. 100µl of supernatant was transferred to polypropylene HPLC vials for analysis.

Assay of remifentanil

Assay was conducted using the remifentanil HPLC-MS/MS method described in Chapter 3. Assays were performed in triplicate and the average of the results used for comparison. The concentrations measured at different time points were used to calculate a percentage of the original concentration of remifentanil in the sample at time 0.

The elimination rate constant (k) for remifentanil for each of the tested samples was calculated using the first order elimination equation where slope

$$(-k) = \frac{\ln C2 - \ln C1}{t2 - t1}$$

C2 was the concentration at 120 min and C1 was the concentration at time zero as determined by the peak size detected. The half-life determined using the equation:

$$t\frac{1}{2} = 0.693/k$$

The total elimination rate constant for blood was estimated by summing the elimination rate constants observed in plasma and that observed in red blood cell component.

Results

Results obtained when the esterase activity in fresh adult red blood cells and fresh plasma and frozen red blood cells and frozen plasma were measured are shown in Table 8. The average remaining remifentanil across all samples in plasma was 69% (SD 4.6) and red blood cells was 33% (SD 12.5). Degradation of remifentanil in frozen samples was less than 15% different to that observed in fresh samples

Table 8: Activity of remifentanil metabolising non-specific esterases in fresh and frozen blood measured using remifentanil as the substrate

	Percentage at time 0 min	Percentage at time 120 min
Fresh plasma	100	90
Fresh red cells	100	37
Frozen plasma	100	79
Frozen red cells	100	35

The median elimination half-life in red blood cells and plasma was 75 min and 243 min respectively in the adult samples. Summing the two elimination rate constants in adult results in a total half-life in blood of 58 minutes.

The median half-life observed in neonatal plasma in this study was 138 min (range 69-1115 minutes). For red blood cells, the median elimination half-life was also 138 minutes (range 69.3 to 693 minutes). Table 9 shows detailed neonatal red blood cells analysis and Table 10 shows detailed neonatal plasma analysis.

Table 9: Degradation of remifentanil in neonatal red blood cell components expressed as percentage of time zero concentration and calculated values for k and the elimination half-life

Sample	0 min	120 min	k (min^{-1})	t 1/2 (min)
1	100	41	0.0075	92.4
2	100	40	0.0075	92.4
3	100	55	0.005	138.6
4	100	63	0.0038	182.3
5	100	47	0.006	115.5
6	100	28	0.01	69.3
7	100	34	0.009	77
8	100	54	0.005	138.6
9	100	35	0.008	86.6
10	100	73	0.003	231
11	100	30	0.01	69.3
12	100	93	0.0006	1155
13	100	59	0.0044	157.5
14	100	55	0.0049	141.4
15	100	54	0.005	138.6
16	100	60	0.004	173.2
17	100	43	0.007	99
Median Value+/-SD	100	54 +/- 17	0.005 +/- 0.002	138.6 +/- 253.7

Table 10: Degradation of remifentanil in neonatal plasma components expressed as percentage of time zero concentration and calculated values for k and elimination half-life.

Sample	0 min	120 min	k (min-1)	t 1/2 (min)
1	100	26	0.01	69.3
2	100	52	0.005	138.6
3	100	38	0.008	86.6
4	100	89	0.001	693
5	100	46	0.006	115.5
6	100	78	0.002	346.5
7	100	51	0.005	138.6
8	100	57	0.005	138.6
9	100	67	0.003	231
10	100	57	0.005	138.6
11	100	42	0.007	99
12	100	43	0.007	99
13	100	53	0.005	138.6
14	100	59	0.004	173.2
Median +/- SD	100	52.5 +/- 16.8	0.005 +/- 0.002	138.6 +/- 168.2

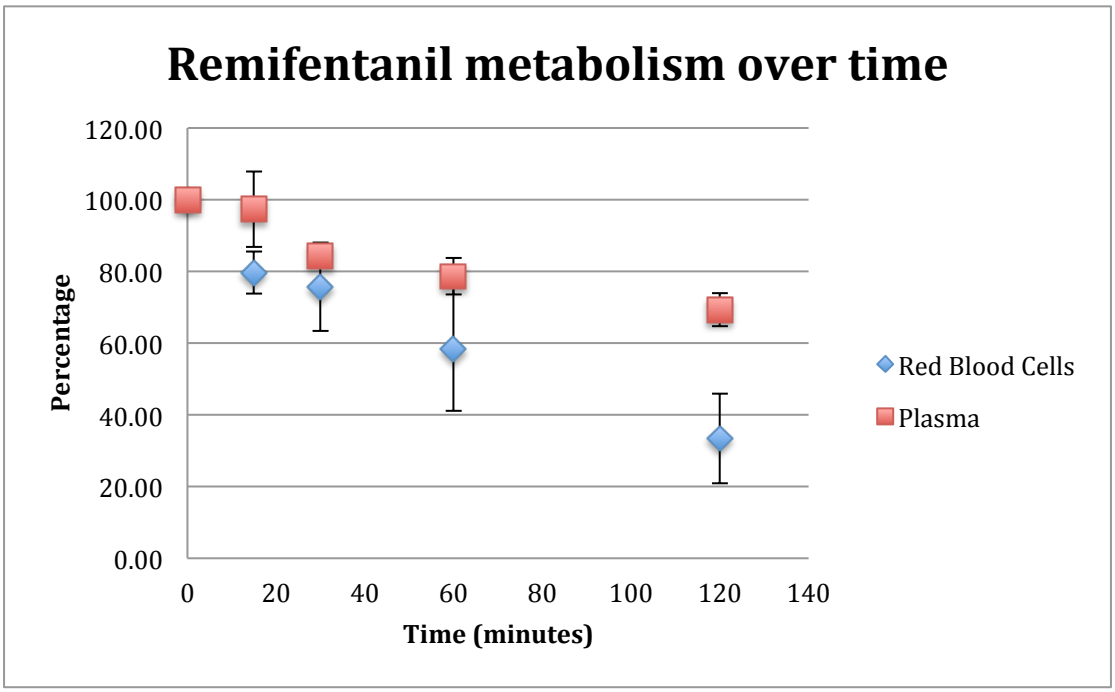


Figure 13: Plot showing percentage remifentanil remaining over time in adult blood

Figure 13 shows the percentage remaining over time in combined adult blood samples while figure 14 shows the same information collected from neonatal samples which shows red blood cells and plasma demonstrating similar rates of remifentanil metabolism.

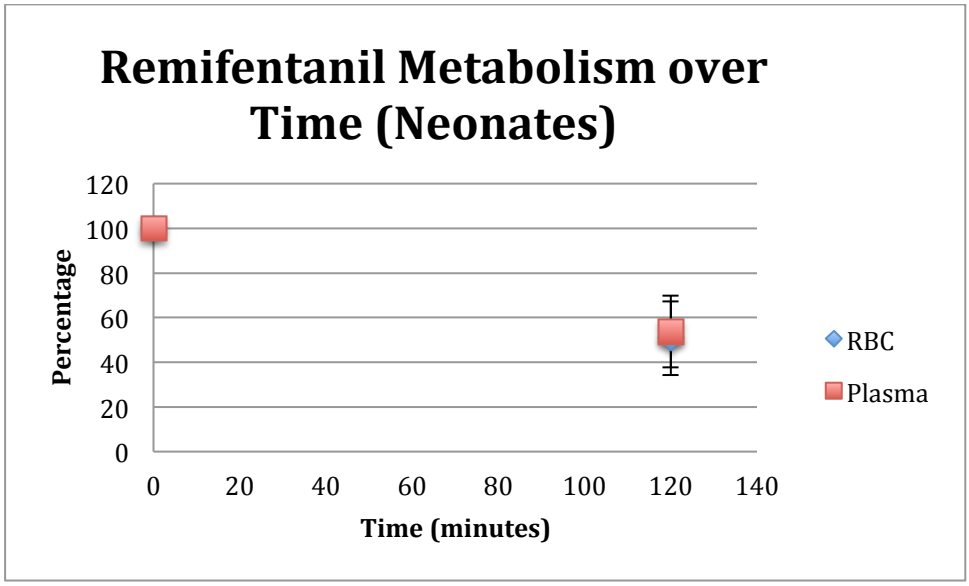


Figure 14: Chart showing remifentanil remaining over time in combined neonatal samples.

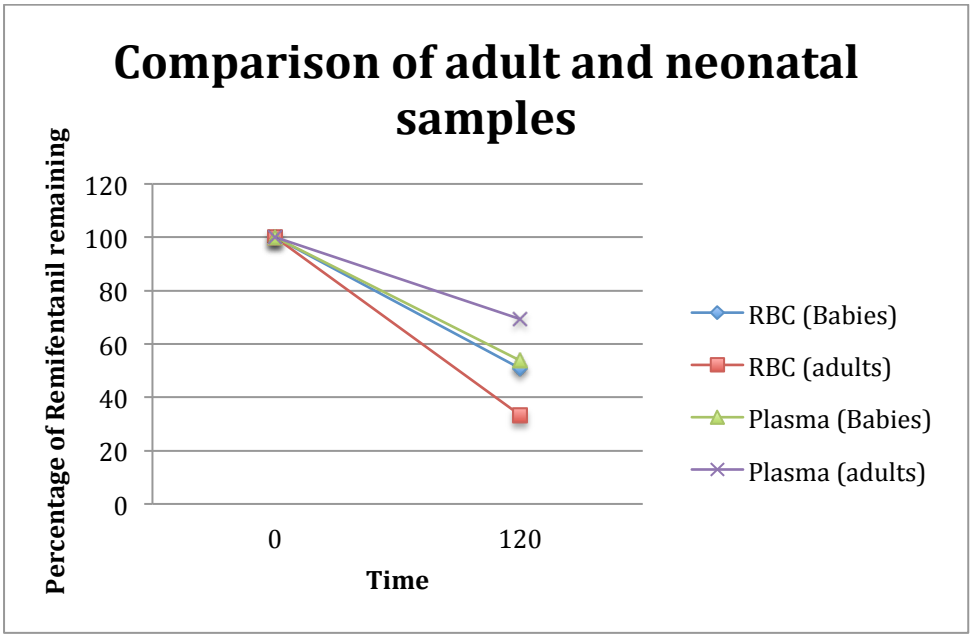


Figure 15: Comparison of adult and neonatal remifentanil metabolism over 120 minutes.

Adult and neonatal samples were compared directly to confirm that different rates and patterns of remifentanil metabolism (Figure 15). Adults were shown to have different rates of metabolism between red blood cells and plasma while neonates had the same rate in both red blood cells and plasma.

Discussion

The results of this study clearly show that extrapolation of remifentanil metabolism and pharmacokinetics from adult data for use in neonates should never be done. The study demonstrated that distinct differences in metabolism of remifentanil in neonate and adult blood exist. The rapid degradation of remifentanil by esterases is a key factor when considering selection of this opioid for use in neonates. Previous studies in adults have shown very rapid metabolism in whole blood with an elimination half-life ranging from 37-66 minutes [125] which is in agreement with the half-life of 58 minutes observed in this study. The median half-life in neonate plasma in this study of 138 minutes

was also similar to the median value of 143 minutes previously observed [83] although these observations were in umbilical cord serum. The elimination half-lives of remifentanil in neonatal red blood cells observed in this study were longer than in the adult sample suggesting that immature red blood cells may contain less esterase or if esterase is present it may not function as effectively as that present in adult samples. The elimination half-lives of remifentanil in plasma were also longer than in the adult sample and this suggests differences in development of esterases in plasma also occurs. Differences in elimination half-lives of elimination for both red blood cells and plasma are important observations as it indicates that different doses and dosing schedules will be required in neonates. If in-vitro elimination observed here is translated to in-vivo situation, then dosing by assuming similar half-life to adults could lead to higher drug levels and longer time to drug elimination in neonates, which could potentially lead to adverse effects. Comparison of the results in figure 12 and 13 show that for adult samples the rate of metabolism is higher in red blood cells than plasma. The same information collected from neonatal samples shows red blood cells and plasma demonstrating similar rates of remifentanil metabolism, suggesting either a developmental of distributional difference.

An important finding from this study is that esterase activity could be studied using frozen red blood cells as degradation observed in frozen red blood cells was similar to those seen in fresh red blood cells from the same subject. The results for the fresh and long term frozen samples fell within 15% of each other suggesting no significant difference between fresh and frozen samples. This observation enables future studies to be designed where a large number of samples could be collected across different ages and analysed as a batch. This overcomes the difficulties where the assay would have to be run every time a new sample was obtained if the sample had to be analysed fresh and this would not be practical to perform.

This is the first study the compares adult and neonatal blood in the same analysis, removing concerns that differences seen are due to analytical differences.

This study demonstrated that degradation of remifentanil does occur in both the plasma and red blood cells obtained from neonates and the rate of degradation in these samples is similar. Welzing et al [83] acknowledged that in their study it was only possible to study serum from umbilical cord samples. This study is the first to investigate remifentanil degradation in both red blood cells and plasma taken from the same neonate. Demonstrating the presence of esterase activity in neonatal red blood cells is an important observation. It shows that there is esterase present in red blood cells as well as plasma and this can contribute metabolism of remifentanil. Another significant observation is the large variability in rate of degradation for both red blood cells and plasma in the neonates studied. Elimination half-lives in red blood cells ranged from 69.3 to 693 minutes while half-lives in plasma ranged from 69 to 1115. This suggests that in this neonate population where ages ranged from 25 to 40 weeks, there may be significant variation in the rate and extent of esterase development. A limitation of this work is insufficient samples to undertake analysis by age group.

Interestingly, the median rate of degradation for both red blood cells and plasma were the same, suggesting that there is a developmental difference in the distribution, if not the activity or amount of remifentanil metabolising non-specific esterase. In neonatal plasma, the median elimination half-lives was 138.6 minutes while in the adult the elimination half-life was 243 minutes. The adult samples showed a much faster rate of degradation in red blood cells than plasma. The rates in samples of babies born between 25 and 40 weeks remain the same between red blood cells and plasma. This suggests there may be a difference in distribution of enzyme activity between red blood cells and plasma in neonates.

This study also demonstrated that left over clinical samples could be used to perform pharmacokinetic and related studies in neonates. Using left over clinical samples meant that participants were not subjected to additional blood samples and involvement in the study had no patient impact.

This study provides evidence that developmental changes in remifentanil metabolising non-specific esterases occur throughout life. This supports the observation made by other studies that the rate of remifentanil metabolism is slowed in the most preterm infants [83]. It also highlights the need for ongoing research into the development of drug metabolising non-specific esterases, particularly with regard to the effects of age.

The increased half-life suggested in preterm infants is an important clinical consideration. The major benefit of using remifentanil in this population is its short half-life which reduces the risk of respiratory depression and decreases the need for respiratory support. If this is not the case the benefits of using this untested therapy decrease. While the exact pharmacokinetics remain unknown, unexpected respiratory support requirements can arise.

Conclusion

This study provides preliminary information on esterase development in preterm neonates when compared with adults. This information, along with the validated remifentanil assay, can be used in the development of population pharmacokinetic models to determine the ideal dose for preterm neonates. This will allow for safe and effective pain management for neonates. These results demonstrate that remifentanil metabolising non-specific esterases undergo developmental changes and further dose determination studies are needed to properly inform the clinical use of remifentanil in the neonatal population.

Chapter 5. Development of a highly sensitive benzylpenicillin assay from low plasma volumes.

Introduction

Antimicrobials in Neonates

Prevention and treatment of infections is a common need for medications in neonatal intensive care. Limited dosing information is available for antimicrobials in neonates and there is limited incentive to study treatments that have been used for several decades in this population and have wide therapeutic indices. With increasing antimicrobial resistance optimising the use of antimicrobials in a clinical imperative. Detailed information on dosing and pharmacokinetics is required to achieve this.

Benzylpenicillin is commonly used in neonatal intensive care, and has a narrow spectrum of activity, which reduces the risk of resistance when used judiciously. For these reasons, benzylpenicillin was chosen as a drug for which a suitable assay would be developed for use in neonate pharmacokinetic studies.

Use of Benzylpenicillin in neonatal medicine

Benzylpenicillin is a beta-lactam antibiotic that is used worldwide to treat many different infectious conditions in paediatric and neonatal care. Current dosing in this setting is based on data from adults and older children scaled down and adapted based on clinical experience. While this dosing information has been in use for many years it cannot compare with a pharmacokinetic study to provide optimal information about safe and effective dosing.

Recently, the use of therapeutic cooling for hypoxic ischemic encephalopathy which can affect drug disposition in neonates, and the growing speciality of neonatal and paediatric intensive care where multiple drugs are used concurrently, has elevated concerns regarding the appropriate dosing of medications in this group. Benzylpenicillin is one of many drugs that could be affected. However the ability to provide detailed dosing information for benzylpenicillin is limited by access to appropriate methodologies to analyse samples in order to develop population pharmacokinetic models. As discussed previously, population pharmacokinetic approaches overcome the requirement of traditional pharmacokinetic studies for multiple blood samples from the one patient, which usually precludes their use in neonates due to their small total blood volume. However, even with limited sampling strategies and sampling times optimised using D-optimal design, analytical techniques still need to be able to measure drug concentrations in very small volume blood samples for population pharmacokinetic studies to be feasible.

Benzylpenicillin also forms the basis of regimens for treating and preventing early onset sepsis, particularly where infection with group B streptococcus is suspected in neonates. In this clinical setting doses used are based on experience and expert opinion only. High serum concentrations of penicillin have been associated with side effects, such as seizures, the risk of which could be reduced by conducting pharmacokinetic studies to provide dosing information. With growing concerns regarding antibiotic resistance, the ability to target specific penicillin serum concentrations is also of value. Therefore having a rapid assay suitable for use in neonates would be useful for therapeutic drug monitoring in neonates and patients in general.

Pharmacokinetics of Benzylpenicillin in neonates

Few pharmacokinetic studies have been conducted in this population. One study conducted in very low birth weight (<1200g) infants suggests that a 15mg/kg dose, as opposed to the 60mg/kg dose commonly used, reaches therapeutic

serum concentrations [126]. A population pharmacokinetic study in cooled infants also identified a risk of toxicity with doses at the higher end of the current range [127]. This preliminary research suggests much lower doses could be used, reducing the risk of side effects, such as seizures, that are associated with high serum concentrations. A summary of the reported pharmacokinetics in neonates is seen in Table 11 below.

Table 11: Summary of reported pharmacokinetic parameters of benzylpenicillin in neonates.

Age	Clearance	Volume of Distribution	Author
<28 weeks and 1200g	1.2mL/min/kg	0.41L/kg	Metsvaht et al [126]
> 32 weeks	0.21L/hr/kg	0.48L/kg	Padari et al [128]
26-32 weeks	0.1L/hr	0.36L/kg	Muller et al [129]

A recent study [127] used a modelling approach to describe the pharmacokinetics of benzylpenicillin in term neonates undergoing moderate hypothermia. Therapeutic whole-body hypothermia is a common treatment in patients with hypoxic-ischaemic encephalopathy and these patients often receive benzylpenicillin. In this study, it was noted that 22 samples could not be included in the study as the samples were below the limit of quantitation, which was 500ng/mL. This illustrates the importance of sensitive analytical techniques to ensure collection of as much data as possible. This study represents one of the few that have investigated benzylpenicillin dosing in neonates.

Importance of TDM with benzylpenicillin

With increased rates of antibiotic resistance, individualised dosing to ensure that effective concentrations are achieved in each person is critical. Whilst beta-lactam antibiotics are generally recognised to have a large therapeutic index, the variability in their pharmacokinetics in critically ill adults has recently emphasised the importance of therapeutic drug monitoring with these drugs [130, 131]. With even less dosing data in the paediatric and neonatal setting, TDM is therefore likely to be a useful tool to improve dosing.

Pharmacology of Benzylpenicillin

Mechanism of action

Benzylpenicillin, (Figure 16) or penicillin-G, is a beta-lactam antibiotic. It works to inhibit cell wall synthesis in a wide range of gram positive and some gram-negative bacteria. Following incorporation of benzylpenicillin into bacterial cell walls cell lysis occurs.

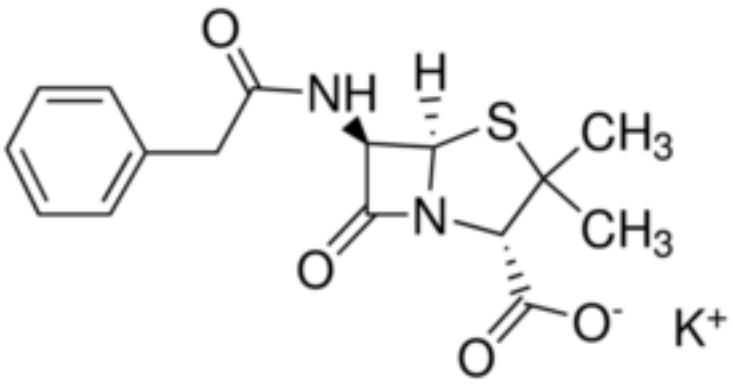


Figure 16: Chemical structure of benzylpenicillin.

Clearance

Based on adult studies, up to 30% of benzylpenicillin is metabolised to an inactive metabolite, penicilloic acid. Benzylpenicillin is also eliminated by the

kidneys in its original form while small amounts are also excreted via the bile and metabolised via the liver [132].

Side effects

Common side effects include gastrointestinal upset, particularly diarrhoea. High concentrations in the cerebrospinal fluid can result in seizures. Secondary infections such as pseudomembranous colitis can also occur [132].

HPLC assays for benzylpenicillin

A number of assays designed for use in TDM have been reported in the literature and are described in Table 1. Many of these methods use HPLC with UV [133, 134] detection and some involve either solid phase extraction or solvent extraction prior to analysis [135, 136]. However, the volumes required for these assays are at least 0.2mL [137] which is larger than ideal for neonatal studies. Currently only one published method [138] is suitable for neonate TDM and there is a need to develop other simple rapid method for clinical studies and TDM in children and neonates. A summary of published assays is presented in Table 7.

Table 12: Previously published HPLC methodologies for benzylpenicillin.

Study	Equipment	Plasma Volume	Limit of detection	Extraction method	Run time	Injection volume
Van Gulpen et al [139]	HPLC-UV	500µl	0.5µg/mL	Acetonitrile precipitation followed by liquid-liquid extraction	15 min	20µl
Verdier et al [140]	HPLC-UV	100µl	5µg/mL	Acetonitrile precipitation	22 min	20µl
Briscoe et al [133]	HPLC-UV	200µl	0.1µg/mL	Ultrafiltration	10 min	25µl
McWhinney et al [141]	HPLC-UV	200µl	5µg/mL	Acetonitrile precipitation followed by liquid-liquid extraction	30 min	10µl
Bruck Sime et al [137]	HPLC Triple quadrupole MS API 3000	300µl	0.1µg/mL	Acetonitrile precipitation	7 min	20µl
El-Najjar et al [138]	HPLC Triple quadrupole MS API 4000	40µl	0.05µg/mL	Methanol precipitation	4 min	15µl

Stability of Benzylpenicillin in plasma

There have been limited reports in the literature regarding stability of plasma samples with frozen sample stability for 14 days [142] and bench top stability for 4 hours [137] reported. Other methodologies have only reported stability for

extracted samples [140], which has limited usefulness when collecting samples from patients.

Rationale for assay development

Since benzylpenicillin is widely used in neonates for many different clinical scenarios, there is an urgent need for pharmacokinetic studies to inform dosing in all of these different scenarios. It is evident from the literature that few, if any, assays are available which meet the necessary criteria for use in neonatal pharmacokinetic studies.

A lack of information regarding the stability of benzylpenicillin samples in plasma at room temperature, refrigerated and frozen samples as well as extracted samples is required for a methodology to be used for collecting leftover clinical samples from neonates.

The necessity for TDM to personalise doses is also highlighted in the literature. For TDM and drug dosing studies, there must be access to appropriate equipment to perform assays and methods developed must have a quick analysis time and turnaround time for reporting to ensure sufficient throughput of samples. While the availability of HPLC-MS/MS is increasing, many laboratories may still mainly use HPLC-UV [134]. With this in mind and based on the current literature, the following aims were proposed:

1. Explore the feasibility of using HPLC-UV detection to measure benzylpenicillin concentrations in very small volumes of plasma
2. Develop a HPLC-MS/MS assay capable of measuring low concentrations of benzylpenicillin in as small a volume of plasma as possible

3. Investigate the stability of benzylpenicillin samples in plasma at room temperature (22°C), under refrigeration (4°C) and frozen at -80°C and at room temperature (22°C) after extraction

Development of HPLC-UV methodology

Method

Reagents and Chemicals

Benzylpenicillin and phenoxymethylpenicillin standards, HPLC-grade acetonitrile, formic acid, orthophosphoric acid, heptane, octanol and methanol were purchased from Sigma-Aldrich (Sydney, Australia). Purified water was obtained from a Milli-Q® purifying system (Millipore Co. Bedford, MA, USA).

Solutions

Stock solutions of benzylpenicillin and phenoxymethylpenicillin (the internal standard) were dissolved in HPLC-grade methanol. Calibration standards were prepared by diluting an aliquot of the stock solution in plasma. Calibration standards of 20, 100, 200ng/mL were used. The standards were stored at -40°C for up to one month.

Chromatographic system

The HPLC-UV system was an Agilent 1200 system consisting of a binary pump, UV detector, column oven and autoinjector (Agilent, Australia). Chromatographic separation was achieved using a Phenomenex Kinetex® C18 50x3mm 2.6u column maintained at 40°C. A pre-column Krudcatcher® (Phenomenex, Australia) was used to maximise column life. A 205nm wavelength was used for detection. Samples were stored in the autosampler at 4°C. A 1mL/min flow rate was used.

Mobile Phase preparation

Mobile phase was manufactured by taking 120mL acetonitrile and making up to 1000mL with 0.03M potassium dihydrogen phosphate solution. This combined solution was filtered through a 0.2μm filter and adjusted to pH 5 with 10M sodium hydroxide solution.

Sample preparation

Two approaches for sample preparation were evaluated. One method involved use of solid phase extraction cartridges while the other employed a solvent extraction technique.

Solid Phase Extraction method

Solid phase extraction was investigated using Prevail® C18 cartridges (Alltech, Germany). This method requires solid phase extraction (SPE) cartridges to be pre-conditioned with solvents such as methanol and water followed by application sample. A vacuum system was used to draw the sample through the cartridges. Various wash steps are employed to remove interfering compounds and the drug is eluted with a determined solvent blend that efficiently extracts drug from the cartridge. Different plasma volumes, cartridge washes and elution solution combinations were trialled and these will be discussed in the results section.

Solvent extraction method

A double extraction procedure using heptane:octanol mix was used. Different blends of heptane:octanol were studied to determine the optimal ratio. 100μl of 10% orthophosphoric acid and 100μl of 100ng/mL phenoxymethylpenicillin solution (internal standard) was added to the plasma sample (250-350ul) and

made up to 1mL with water. 900µl of heptane:octanol mix was added and the sample vortexed for 60 sec then centrifuged at 10 000 *x g* for 60 seconds. The upper layer was transferred to a vial and 600µl of heptane:octanol mix was added to the sample that was re-vortexed and centrifuged. The upper layer was removed and combined with the initial organic layer and the drug and IS were back extracted into an aqueous solution by adding 160µl of pH 8 0.1M potassium dihydrogen phosphate added and re-vortexing and centrifuging the sample. The aqueous layer was transferred to polypropylene HLPC vials for analysis.

Validation of the method

Samples of water and plasma spiked to produce concentrations ranging from 20ng benzylpenicillin/mL to 200ng/mL and the different extractions methods were employed. The peak height ratio of benzylpenicillin to internal standard was plotted versus concentration to produce calibration curves. These curves were used to determine limit of quantitation.

System suitability was determined by injecting neat solutions of benzylpenicillin and phenoxymethylpenicillin (IS). The effect of carryover was evaluated by injecting blank plasma samples following spiked samples at both upper and lower limits of quantitation to ensure the retention time had passed before subsequent analytical runs.

Results

Chromatographic conditions

Selection of wavelength

The optimal UV wavelength for analysis of benzylpenicillin was determined by comparing the chromatography and intensity of response for a set concentration of benzylpenicillin measured at 190, 195, 200, 205, 210, 215 and 220nm. A

wavelength of 205nm was observed to produce the best peak shape, peak size and signal to noise ratio.

Chromatography column and mobile phase composition

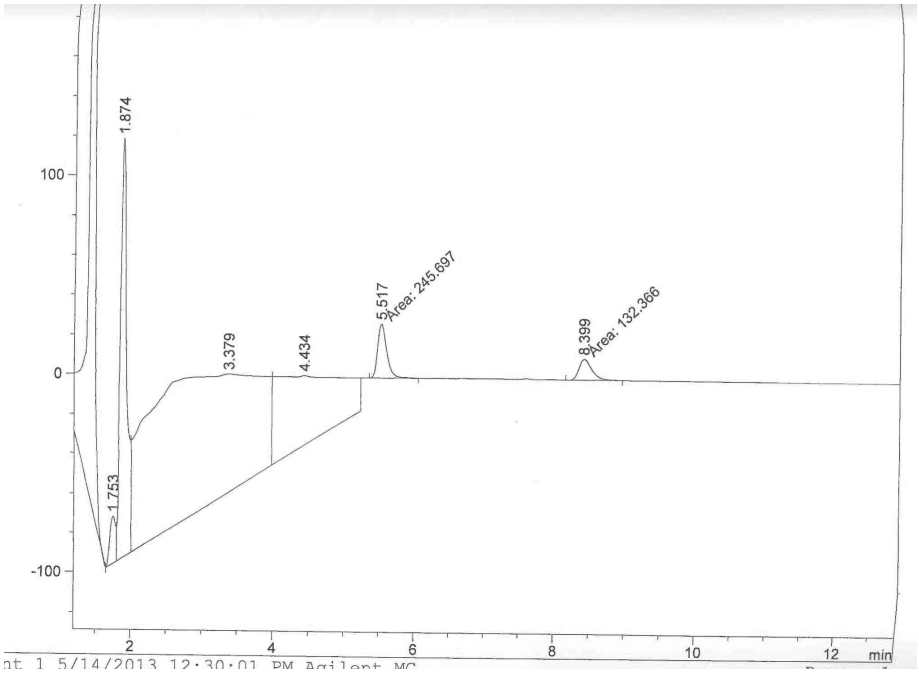
Various combinations of columns, mobile phase blends of organic solvent and buffer and flow rates were trialled to produce the best resolution of endogenous peaks from benzylpenicillin and internal standard. Chromatography using a C18 column was compared with that obtained with a C8 column. The C8 column did not produce adequate retention of analytes and a C18 column was observed to produce optimal separation. Flow rates ranging from 0.8 to 1mL/min were also investigated, with 1mL/min observed to produce the best compromise between peak shape and total run time.

Several mobile phases were trialled in method development. Higher concentrations of acetonitrile (18-36%) decreased the run time but did not improve the chromatography. Isopropyl alcohol and methanol were trialled as the organic phase, both resulted in poorer chromatography.

Reduction of pH to 3.5 from 6.45 reduced run time but increased baseline activity increasing he limit of detection due to interference. A mobile phase pH of 5 provided the best balance between peak shape and baseline.

Injection volumes of 60µl, 70µl and 100µl were trialled. 100µl provided the most consistent peak at 20ng/mL.

Using the final conditions determined from the variations described above, the retention time of benzylpenicillin was 9 minutes and phenoxymethylpenicillin was 15 minutes with a total run time of 25 minutes as shown in Figure 17.



Run Time (minutes)

Figure 17: Chromatogram obtained when benzylpenicillin was analysed using HPLC-UV.

Retention time of benzylpenicillin was 5.5 minutes and phenoxymethylpenicillin at 8.4 minutes

Sample preparation

Sample volumes of 200-350µl were trialled. Despite the trialling of several variations of the two extraction methods it was not possible to reduce the sample volume below 200µl and be able to detect the penicillin at 20ng/mL.

Solvent extraction method

Using the heptane:octanol method both a single and double extraction method were trialled with the double extraction producing a 15% larger peak. The double extraction method was determined to have an average extraction efficiency of 63%. The ratio of heptane:octanol was varied to determine the optimal ratio to produce maximal extraction of drug and minimum extraction of other endogenous material. The optimal ratio was determined to be heptane:octanol 60:40.

A heptane:octanol ratio of 70:30 was also tested but offered no improvement in extraction efficiency. The back extraction into potassium dihydrogen phosphate was tested at 0.1M, 0.075M, 0.05M, 0.025M (all at pH 8) with 0.1M demonstrating a 20% increase in peak size.

Solid phase cartridge method

Prevail® C18 columns were also trialled with various wash steps to improved extraction efficiency. 50µl sample volume followed by 100µl methanol, 100µl acetonitrile then 100µl water washes demonstrated 50% extraction efficiency. Increasing the volume of acetonitrile did not improve the extraction efficiency and reduced the sensitivity by diluting the sample. A test of a water wash followed by a mobile phase wash with increasing pH from 4.5 to 7.2 reduced the extraction efficiency to less than 10%.

Other steps trialled to improve extraction efficiency from the cartridges included pre-conditioning with methanol, pre-conditioning with 50:50 methanol:water pH10, elution with 50:50 methanol:water pH10, acidifying samples with 10µl 10% orthophosphoric acid before extraction. None improved extraction efficiency with plasma extractions or allowed for smaller plasma volumes to be used.

Due to concerns regarding contaminants from the polypropylene HPLC tubes washing the tubes with polypropylene before transferring the samples for analysis was trialled to see if a smoother baseline could be achieved. No difference was seen.

Calibration curves

Solid phase extraction method

The calibration curve produced following extraction of water samples spiked with benzylpenicillin and internal standard using Prevail® C18 cartridges is shown in Figure 18. While linearity was observed between 10 and 100ng/mL, these results could not be replicated when the same extraction procedure was performed using plasma spiked with benzylpenicillin.

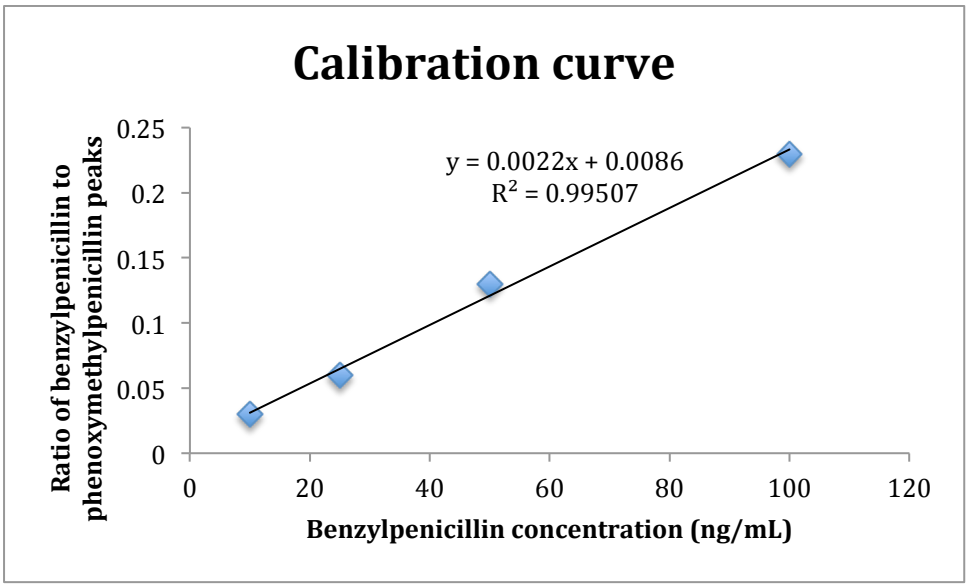


Figure 18: Calibration curve obtained when peak height ratio of benzylpenicillin:phenoxymethylpenicillin (IS) was plotted versus water containing different concentrations of benzylpenicillin.

A linear curve was identified between 10ng/mL to 200ng/mL (Figure 19) when extracted from plasma using the heptane:octanol method. The method was successful at higher concentration and the focus of the development was on the lower limit of quantification. The 10ng/mL peak was not consistently detected and was often affected by the baseline quality. The limit of detection was set at 10ng/mL but due to inconsistent detection it was not able to be set as the LLOQ.

Despite numerous attempts to lower the limit of quantification to less than 25ng/mL it was not possible using HPLC-UV.

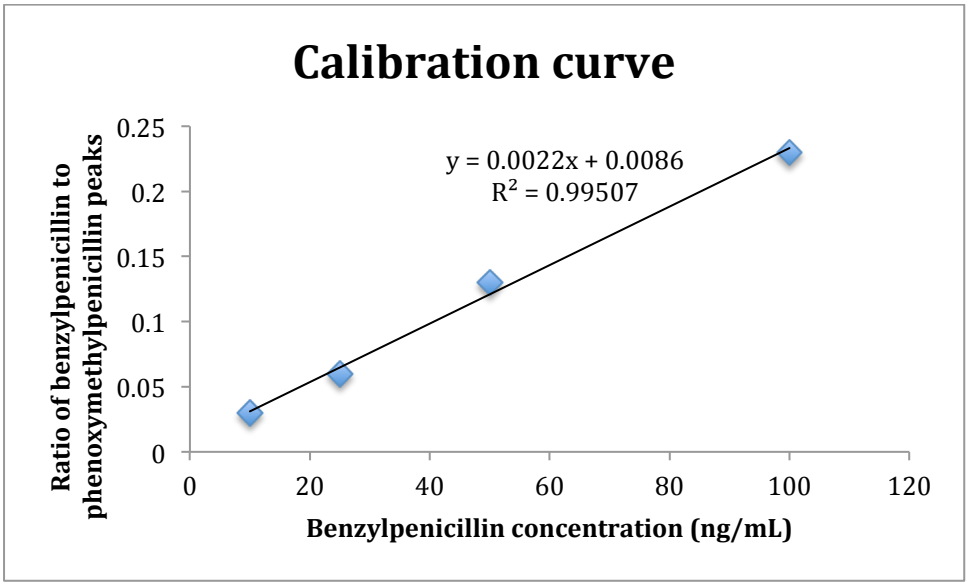


Figure 19: Calibration curve obtained by plotting the peak height ratio of benzylpenicillin:phenoxymethylpenicillin versus Benzylpenicillin concentration after extraction using heptane:octanol method

Discussion

Previous methods using HPLC-UV mainly used protein precipitation and injection of a small amount of supernatant. The work performed here explored the feasibility of using techniques which enable concentration of drug in extracted material by either elution from SPE cartridges using a small volume of eluting solvent or by extraction into organic solvent and then back extraction into very small volume of buffer.

Despite testing numerous techniques during development the HPLC-UV methodology was unable to reach the required sensitivity for analysing neonatal samples. The plasma volumes required for the assay are too high for sampling of neonates regardless of the extraction method used. HPLC-UV also has long run

times, which reduce the usefulness of the assay. The detection of penicillin using this method was also affected by contaminants in blank plasma and it was not possible to get clear baselines. Although a linear calibration curve across a small range on concentrations was achieved further validation was abandoned as the method was deemed to be not fit for purpose.

Development of HPLC-MS/MS methodology

Method

Reagents and Chemicals

Benzylpenicillin, HPLC-grade acetonitrile, formic acid and methanol were purchased from Sigma-Aldrich (Sydney, Australia). Purified water was obtained from a Milli-Q® purifying system (Millipore Co. Bedford, MA, USA).

Solutions

Stock solutions of benzylpenicillin and phenoxymethylpenicillin (IS) were dissolved in HPLC-grade methanol. Calibration standards were prepared by diluting an aliquot of the stock solution in plasma. Calibration standards of 10, 50, 100, 250, 500, 1000 ng/mL and quality control standards of 20, 150 and 750 ng/mL were prepared. The standards and quality control samples were stored at -40°C for up to one month. Quality control samples were prepared in plasma independently of standards and these were at three concentration levels 25, 150 and 750ng/mL.

Chromatographic system

HPLC was performed using a Shimadzu, Nexera with two pumps, autosampler with a DGU-20A5 degassing unit and CTO-20A column oven (Kyoto, Japan). Autosampler temperature was set at 4°C. Chromatographic separation was achieved using a Phenomenex Kinetex® C18 50x3mm 2.6u column maintained at

40°C. Mobile phase consisted of acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). A gradient was used with solvent A increasing from 10% at time 0 to 80% at 3 minutes with a flow rate of 0.5mL/min. Injection volume was 1µl. Expected retention time of benzylpenicillin is 2.06 minutes and phenoxymethylpenicillin at 2.21 minutes.

Mass spectrometry

The mass spectrometer was a 6500QTRAP (SCIEX, Framingham, MA, USA). The MS operated in electrospray positive mode with the following settings: Curtain gas-20, Collision Gas-nitrogen, Temperature-5500°C Ion Source Gas 1-15 and Ion Source Gas 2-15. Scheduled MRM mode was used for compound detection with a detection window set to 60sec around the expected retention time. Data acquisition was controlled by Analyst 1.6.3 and processed with MultiQuant® 3.0. (SCIEX, Framingham, MA, USA).

Transitions of 335-160 (Collision energy 15), 335-175.9 (Collision energy 17), 335-113.9 (Collision energy 45) were used for benzylpenicillin. For phenoxymethylpenicillin transitions of 350.9-160 (Collision energy 17), 350.9-114 (Collision energy 43) were selected.

Sample preparation

An acetonitrile precipitation method was used to extract and prepare samples. Phenoxymethylpenicillin internal standard was added to acetonitrile for a final concentration on 500ng/mL. 150µl of this solution was added to 50µl of the plasma sample. Samples were then vortexed for ten seconds. After 5 minutes samples were centrifuged at 6800 *x g* for 10 minutes. Supernatant was transferred to polypropylene HLPC vials for analysis.

Validation of the method

To ensure that the method was suitable for intended use for TDM, validation was conducted with reference to FDA guidelines for bioanalytical method validation [143].

System suitability was determined by injecting neat solutions of benzylpenicillin and phenoxymethylpenicillin (IS). The effect of carryover was evaluated by injecting blank plasma samples following spiked samples at both upper and lower limits of quantitation to ensure the retention time had passed before subsequent analytical runs.

Linearity of response was tested by extracting plasma samples spiked at nominal concentrations of 10, 50, 100, 250, 500, 1000 ng/mL. The calibration curve was generated by least squares regression of the peak area of benzylpenicillin/internal standard with a weighting of 1/x. The percent deviation from nominal value was back calculated at each standard concentration. The acceptance criterion was <= 15% for standards and <= 20% at LLOQ for inclusion in the calibration curve. Calibration curves were accepted with a correlation coefficient (r^2) of >0.99.

Precision and accuracy were assessed for both within day and between day by replicate analysis of plasma quality control samples at low, medium and high concentrations levels. Within day data was collected within a single run, while between day data was collected over several days. Specificity of the assay was demonstrated by testing donated adult human plasma samples.

The stability of prepared samples in the autosampler was tested by comparing the results of a set of QCs injected after 24 hours, compared with those obtained on immediate injection, calculated from the original standard curve.

Quality control samples were prepared at 20, 150 and 750ng/mL. The freeze thaw stability was determined by analysis of the QC material that had been frozen at -80°C three times. Stability was determined by storage of the standard and QC material at -40°C for 30 days.

Stability study, Samples and storage

Samples matching the calibration curve were prepared in triplicate and placed in varying conditions to determine stability. All samples were tested a time 0 then at 1, 2, 4, 6, 24, 48, and 168 hours after preparation for samples at room temperature. Samples were tested at 24, 48, 168 hours post preparation and storage at 4°C. Samples frozen at -40°C were analysed at hour 168, 672 post preparation. Processed samples were stored at room temperature and reanalysed after 14 days.

Results

Liquid chromatography

Acetonitrile and methanol mobile phases were tested with acetonitrile showing improved peak shape and sensitivity. Reducing the column length reduced retention time resulting in shorter run time. Flow rates between 0.5mL/minute and 1mL/minute were tested with 0.5mL/minute providing optimal peak shape. Injection volumes between 1 and 10µl were tested with 1µl producing the best peak size for benzylpenicillin and phenoxymethylpenicillin.

Mass spectrometry

In order to determine the most sensitive ionization mode for benzylpenicillin and phenoxymethylpenicillin full scans were conducted in both positive and

negative mode. Positive ion detection provided higher signal strength as shown in Figure 20 for benzylpenicillin and Figure 21 for phenoxymethylpenicillin.

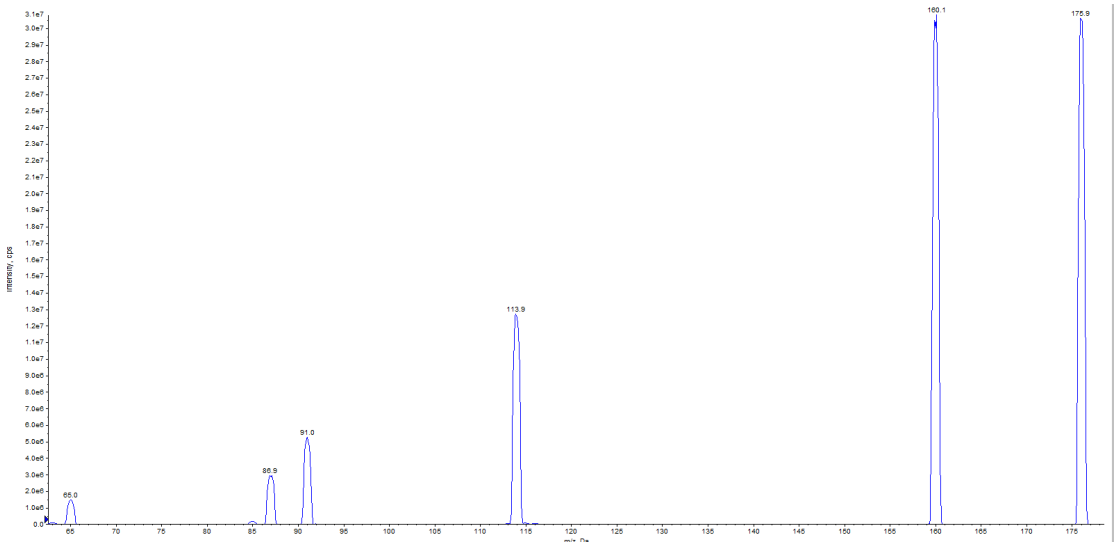


Figure 20: Final benzylpenicillin transitions.

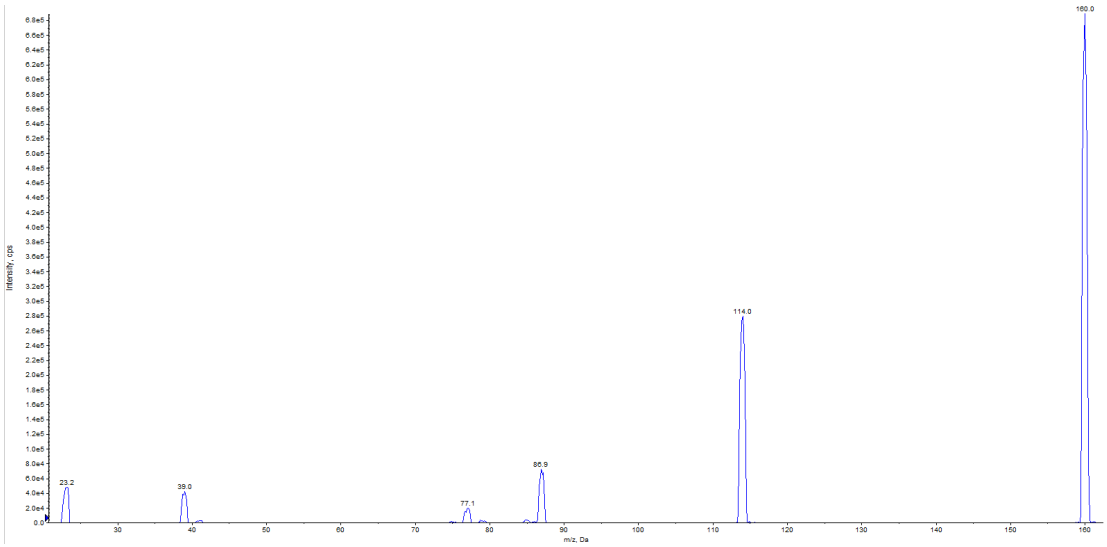


Figure 21: Final phenoxymethylpenicillin transitions.

Optimization of sample preparation and recovery

Prevail® C18 extraction cartridges were tested but did not offer any advantages over precipitation methods. Extraction using smaller volumes of plasma (20µl) was tested but did not provide adequate sensitivity.

Validation

Calibration curve (Figure 22) was linear between 10-1000ng/mL with an r^2 value of 0.99.

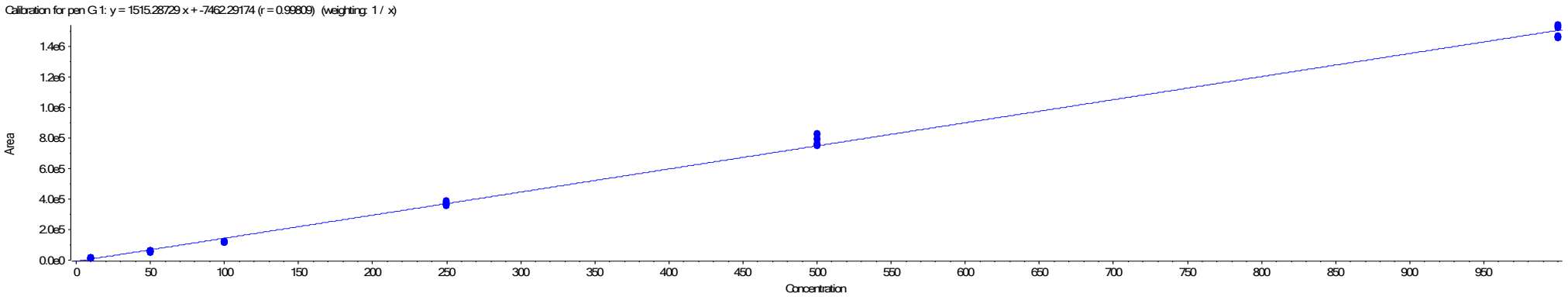


Figure 22: Calibration curve obtained when the peak area ratio of benzylpenicillin:phenoxymethylpenicillin was plotted versus concentration of benzylpenicillin in plasma (ng/mL)

Validation data for penicillin standards (Table 13) and QC samples (Table 14) are shown below. Standards and QCs met the standard for assay validation.

Table 13: Accuracy data obtained when plasma standards containing different concentrations of benzylpenicillin (pen G) were analysed between different runs and different days.

Component Name	Actual Concentration ng/mL	Mean measured concentration ng/mL	Standard Deviation	Accuracy Percent CV
pen G	10	12.41	0.59	4.79
pen G	50	43.3	1.45	3.35
pen G	100	84.8	0.88	1.04
pen G	250	251.04	8.29	3.3
pen G	500	522.33	21.55	4.13
pen G	1000	996.12	26.46	2.66

Table 14: Accuracy data obtained when quality control standards containing different concentrations of benzylpenicillin (pen G) were analysed between different runs and different days.

Component Name	Actual Concentration ng/mL	Mean measured concentration ng/mL	Standard Deviation	Accuracy Percent CV
pen G	25	25.32	3.02	11.92
pen G	150	148.55	10.47	7.05
pen G	750	758.21	29.06	3.83

Sample stability

The average of the triplicate measured concentrations for each temperature was determined. Samples were shown to be stable for 6 hours at room temperature (22°C) (Figure 23). Sample stability was demonstrated for 24 hours at room temperature (22°C) with rapid degradation over one week (Figure 24), one week at 4°C (Figure 25) and 1 month at -40°C (Figure 26). Processed samples were determined to be stable for two weeks at room temperature following reanalysis.

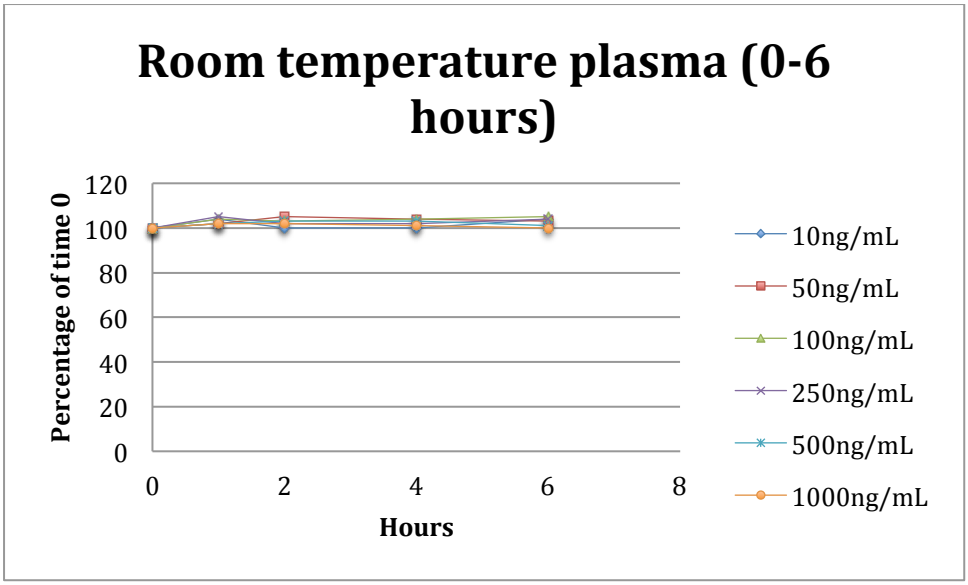


Figure 23: Stability of benzylpenicillin in plasma over 6 hours when stored at room temperature 22°C.

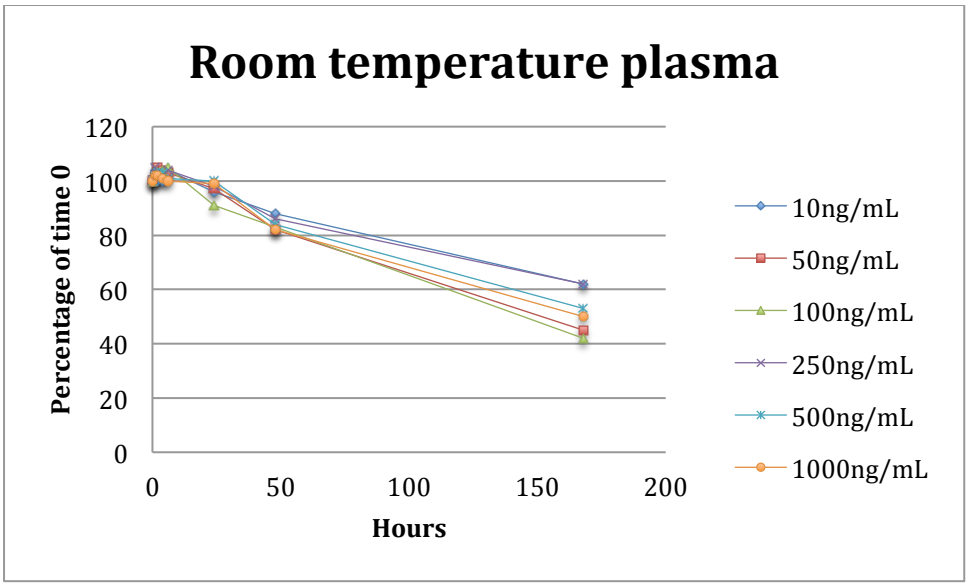


Figure 24: Stability of plasma containing benzylpenicillin when stored at room temperature 22°C for one week.

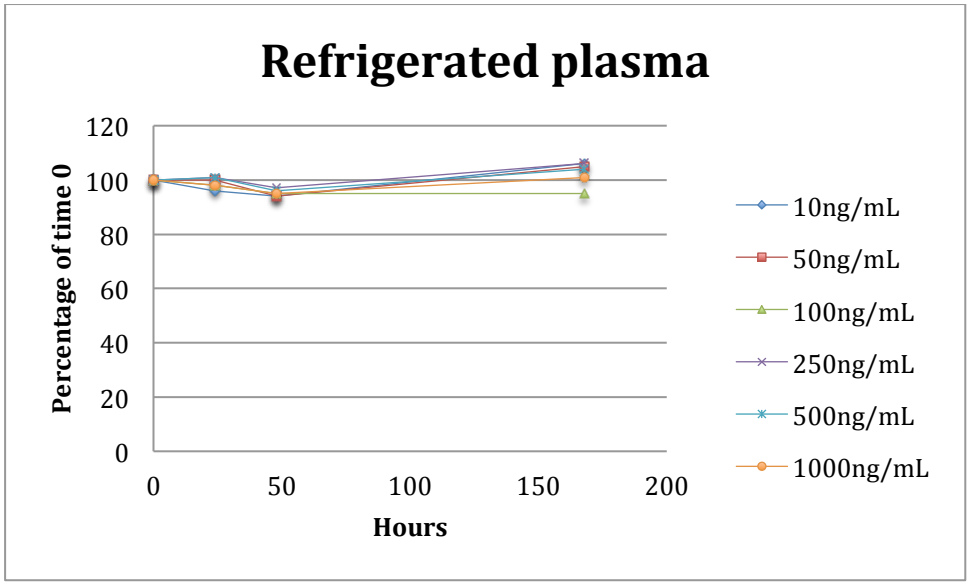


Figure 25: Stability of benzylpenicillin in plasma at refrigerated temperature 4°C for one week

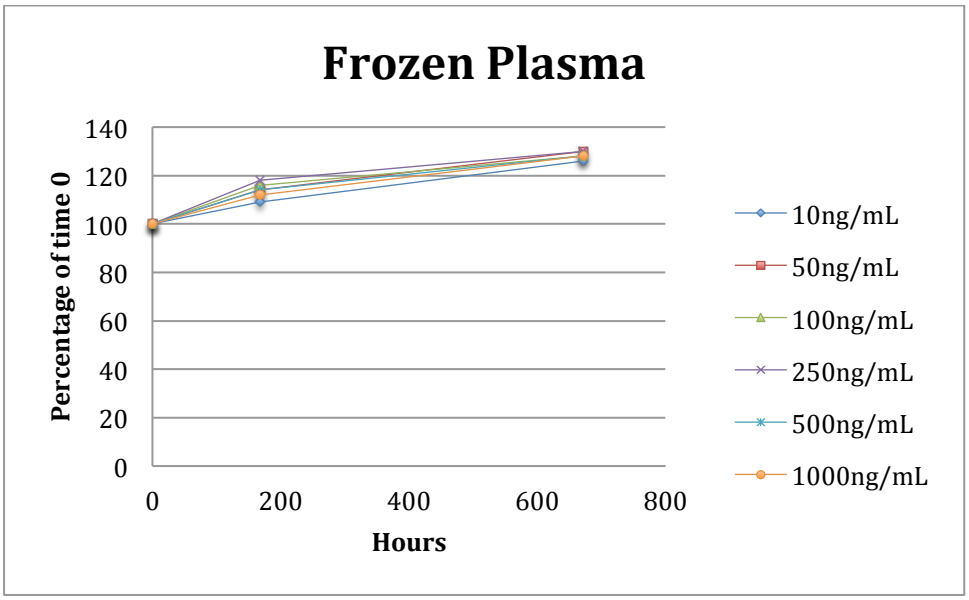


Figure 26: Stability of benzylpenicillin in a plasma when stored at -40°C.for one month

Discussion

The HPLC-MS/MS assay developed proved to be robust, reproducible and fast. It is able to reliably reach the low levels of quantitation required to analyse small volumes of blood taken from neonates and children. This assay also has the ability to measure benzylpenicillin concentration for dose adjustments, in situations where low concentrations are expected. This is the case in rheumatic

heart disease where patients are treated with long acting benzylpenicillin injections to provide bacterial prophylaxis. Currently no available assays are able to reach the low limits of detection to ensure sufficient antibacterial coverage. The speed of the assay makes it suitable for TDM as results can be conveyed to clinicians rapidly, allowing dose adjustments to be made as required, improving patient outcomes.

In this study, the stability of samples over different temperature ranges was studied. It is important to know about the stability of a drug in blood when a sample is collected for use in a pharmacokinetic study or for TDM. In previous work, benzylpenicillin stability has also been shown to be temperature dependent [144], with temperatures under 25 degrees showing better stability. In this study, benzylpenicillin was shown to be stable for 24 hours at room temperature in plasma and this indicates that, after collection in a study, there is no need to immediately rush to freeze samples. This compares to other assays that demonstrated 24 hour stability in extracted samples [141] while improving the utility of the assay for both clinical and research use.

The use of acetonitrile precipitation in preparing the sample for analysis also improved penicillin stability. The use of acetonitrile precipitation and by doing this changing the solvent composition is likely to be the mechanism that improves penicillin stability as benzylpenicillin degrades in acidic conditions [139]. Therefore, once samples are prepared for analysis, the samples were very stable and this allows for re-injection of samples if reanalysing is required. The demonstrated sample stability in this work confirms the suitability of this methodology for both research and clinical use across a range of indications while not having restrictive constraints on sample handling.

The speed of the extraction and analysis, along with the demonstrated room temperature stability of samples shows clearly that this assay would be appropriate for use in a clinical setting by assisting in determining optimal

dosing for individual patients. The growing interest in TDM of penicillin in adult critical care will soon be reflected in paediatric and neonatal populations as concerns regarding antibiotic resistance increase. Assays, such as the one described in this study will be important to improve the care provided to these patient groups. In addition to critical care patients and improving dosing strategies, the low limit of detection of this assay will be important in improving treatment of conditions requiring ongoing penicillin prophylaxis, such as rheumatic heart disease and asplenia.

Conclusion

HPLC-UV methods are not suitable for use in neonatal research studies as they are unable to consistently reach the required level of sensitivity with small volumes of plasma. Further, HPLC-UV techniques are also not able to achieve the short run times seen with HPLC-MS/MS, deeming them unsuitable for TDM assays in clinical use. The HPLC-MS/MS method described in this study has many advantages over previously published methods including small sample volume, simple extraction and fast detection time. It also presents a significant improvement on previously published assay limits of quantitation and detection along with confirmed stability criteria. This offers many opportunities for population pharmacokinetic studies in populations requiring small blood volume sampling, particularly neonates, and where relatively low concentrations of penicillin are expected. Along with research applicability this assay is suitable for TDM in clinical use.

Chapter 6: Determination of midazolam in rabbit plasma by high performance liquid chromatography with diode array detection

Introduction

In earlier stages of this research work, the opportunity to develop a HPLC assay for measurement of midazolam in small volume animal samples arose. Given the frequency of midazolam use in neonates, the development of this assay also provided the opportunity to develop an assay technique, which could form the basis for future work exploring pharmacokinetics in neonates. At time of development of this assay, only HPLC-UV analytical equipment was available.

Use of Midazolam in neonatal medicine

Along with appropriate analgesia, sedation is an important part of intensive care. Sedation can be used to keep babies still for procedures, during ventilation and to assist in managing post surgery wounds. Midazolam is used most commonly as it is easily reversible and there is experience with its use. Midazolam is a short acting benzodiazepine used as premedication and sedation during procedural and intensive care settings. It is generally administered as an intravenous bolus or a short-term infusion.

This drug is used in conjunction with opioids such as fentanyl and morphine to provide analgesia and sedation in neonates undergoing mechanical ventilation. Midazolam, at higher doses, has also been used as an anticonvulsant in this population.

Population pharmacokinetic studies that have been conducted in neonates have demonstrated a difference in required dosing between preterm and term

neonates [145, 146]. Recent models have particularly noted problems with predicting clearance [146]. Concerns have been raised regarding the use of midazolam in neonates due to the lack of evidence of effectiveness and safety [147] setting a requirement for additional research to support safe use in the NICU environment.

Pharmacology

Midazolam is a short acting benzodiazepine (Figure 27) with a rapid onset of effect. It produces its effects by interacting with inhibitory neurotransmitter receptors that are directly activated by gamma-Aminobutyric acid (GABA). Benzodiazepines act at $GABA_A$ receptors by binding directly to a specific site that is distinct from that of GABA binding. It is weakly basic (pKa=6), water soluble at pH4 but highly lipophilic at physiological pH [148].

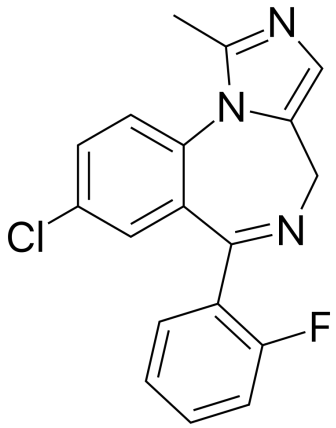


Figure 27: Midazolam

Metabolism and Elimination

Midazolam is mainly eliminated by the CYP450 enzymes CYP3A5 and CYP3A4, both of which undergo developmental changes in activity over the first few months of life. The glucuronidation of midazolam metabolites has been observed to be immature in preterm infants less than 2 weeks of age and the

proportion of midazolam excreted as glucuronide increased significantly with post-conceptional age [149].

While other benzodiazepines, such as diazepam, are metabolised to active metabolites often with a relatively long half-life, midazolam is converted to the main metabolite 1-OH-midazolam which has little or no activity and to a minor extent to 4-OH-midazolam. These metabolites undergo glucuronidation. The apparent lack of pharmacological effect of the main metabolite makes it an attractive alternative to other benzodiazepines. IV bolus of midazolam in neonates has been reported to produce hypotension in some neonates, especially if administered with fentanyl [8]. A summary of the known pharmacokinetics of midazolam in neonates is seen in Table 15 below.

Table 15: Summary of reported midazolam pharmacokinetics in neonates.

Age	Clearance	Volume of Distribution	Author
24-33 weeks	73.7 mL/min in 1.1kg neonate	1.03L in 1.1kg neonate	Voller et al [150]
26-34 weeks	1.8mL/kg/min	1.1L/kg	De Wildt et al [151]
26-42 weeks	0.07L/kg/hr	0.591L/kg	Burtin et al [145]
24-31 weeks >1000g	1.24mL/min	823mL	Lee et al [152]
24-31 weeks <1000g	0.783mL/min	473mL	Lee et al [152]

Side effects

The most common side effects associated with midazolam are drowsiness and respiratory depression, both of which worsen with increasing plasma concentrations.

HPLC assays for midazolam

Midazolam pharmacokinetic studies reported in the literature have commonly employed analytical techniques that require 1mL blood samples and samples being taken from arterial lines. This approach has provided some information

but for other clinical scenarios this volume of blood would not be suitable and there would not be access to arterial blood. There remains a gap in data required to inform midazolam dosing in early age brackets and in various clinical conditions.

Different methods for analysing midazolam in serum or plasma have been reported in the literature. These include gas chromatography methods requiring derivatisation prior to analysis, HPLC with UV/diode array detection (HPLC-UV/DAD) [153-164] and HPLC with mass spectrometry (HPLC-MS)[165].

Most methods employing HPLC-UV/DAD detection use liquid-liquid extraction procedures. Organic solvents such as diethyl ether [154, 155, 157, 161, 162], toluene [153], chloroform [163] or cyclohexane [159, 160] are used to extract midazolam from the sample matrix. The organic solvent is then evaporated under nitrogen to dryness or a back extraction step is used.

Few assay techniques for midazolam have reported the use of SPE cartridges. These have the advantage of avoiding the use of potentially dangerous organic solvents. Methods reported using SPE cartridges, used either C1 [148] or C18 [156] cartridges. Strong cation exchange cartridges present benefits of being able to target and concentrate analytes without evaporation. These have not previously been used for midazolam. Cartridges can be selected on the basis of individual drug characteristics to improve analyte retention increasing assay sensitivity.

Methods employing HPLC-UV/DAD reported in the literature usually require at least 1mL of serum or plasma [148, 155-157, 159, 161-164]. To obtain 1mL of serum or plasma, a volume of about 2mL of blood is often required. In some clinical situations, such as neonatal use and where midazolam is being studied in small animals it is not possible to withdraw multiple samples of this size due to

their small total blood volume. While HPLC/MS methodologies can use smaller blood volumes [165] access to this expensive equipment can be limited. A summary of published methodologies is below in Table 16.

Table 16: Summary of previously published HPLC UV/DAD assays for midazolam.

Study	Equipment	Plasma Volume	Limit of Detection	Extraction method	Run Time	Injection volume
Elbarbry et al [153]	HPLC/UV	n/a	5ng/mL	Liquid-Liquid	10 min	20µl
Ma & Lau [154]	HPLC/UV	50µl	10ng/mL	Complex liquid-liquid	8 min	50µl
Hamdy & Brocks [155]	HPLC/UV	500µl	5ng/mL	Liquid-liquid	10 min	75-125µl
Sautou et al [156]	HPLC/UV	1mL	50ng/mL	C18 cartridges	12 min	100µl
Jurica et al [157]	HPLC/UV	450µl	13.3ng/mL	Liquid-Liquid	10 min	50µl
Lee & Charles [158]	HPLC/UV	100µl	12.5ng/mL	Liquid-liquid	10 min	80µl
Iwasaki et al [160]	HPLC/UV	100µl	10ng/mL	Liquid-Liquid	20 min	100µl
Lehmann & Boulieu [161]	HPLC/UV	1mL	2ng/mL	Liquid-Liquid	7 min	100µl
Puglisi et al [162]	HPLC/UV	1mL	50ng/mL	Liquid-Liquid	15 min	10µl

Yasui-Furukori et al [163]	HPLC/UV	2mL	0.5ng/mL	Liquid-Liquid	15 min	100µl
El Mahjoub & Staub [164]	HPLC/DAD	1mL (whole blood)	30ng/mL	Liquid-Liquid	25 min	20µl

Here a new, simple, rapid and sensitive HPLC-UV method for analysis of midazolam using strong cation exchange cartridges is described. The method requires only 300µL of plasma and uses a solid phase extraction clean up procedure reducing the need for potentially dangerous organic solvents.

Experimental Section

Materials

Midazolam ampoules (1mg/mL) were obtained from Roche Products (Dee Why, NSW Australia). The internal standard, clonazepam, was obtained from PCCA (Houston, Texas). Drug free plasma was obtained from rabbits that had not received midazolam.

Methanol and Acetonitrile (HPLC grade) were obtained from VWR International (EC). Deionised water was generated with a Synergy Millipore water purification system with a 0.22µm Simpak filter (France). Formic acid was obtained from Fluka (Sigma Aldrich, Australia). Glacial Acetic Acid, Sodium Acetate, ammonium hydroxide and orthophosphoric acid were obtained from (Ajax Finechem Pty Ltd, Australia).

Preparation of Stock Solutions

Working stock solutions of midazolam were prepared by diluting 1mg/mL midazolam in deionised water on each day of analysis. For each analytical run, calibration standards were prepared in the drug free rabbit plasma at final plasma concentrations of 1000, 500, 100 and 50ng/mL. Quality control samples were prepared separately with final plasma concentration of 1000ng/mL and 100ng/mL. The internal standard solution was prepared by weighing 10mg of clonazepam and dissolving in isopropanol (Ajax Finechem Pty Ltd, Australia). This solution was stored in the refrigerator at 4°C. An aliquot of this solution was taken and diluted 1 in 10 with deionized water for each analytical run.

Sample Preparation

Oasis MCX® SPE cartridges (1mL, 30mg) (Waters Australia) were conditioned with 1mL of methanol and 1mL of water. To 300µL of plasma sample was added 25µL of internal standard solution and 300µL of 4% orthophosphoric acid solution. The sample was vortexed for 10 seconds and transferred to the SPE cartridge. The sample was then aspirated slowly through the sorbent. Following washing of the cartridge with 1mL deionized water, 1mL of 2% formic acid in water and 1mL of methanol, the analytes were then eluted with 1mL of 5% ammonium hydroxide in methanol and collected in glass tubes. The eluents were evaporated under air at room temperature using a Techne Dri-Block® sample evaporator (Cambridge, United Kingdom). Prior to HPLC analysis, the residues were dissolved in 140µL of mobile phase and 40µL of sample injected into the HPLC system.

Chromatography

The chromatography system consisted of a Shimadzu SIL-20A HT auto-injector, Shimadzu LC-10AT VP pump and a Shimadzu SPD-M10A VP Diode Array UV detector. The UV wavelength was set at 254nm. The chromatographic separation of midazolam and clonazepam was accomplished using a Synergi® Polar-RP

4um 150x4.6mm column (Phenomenex, Australia). Chromatographic data were collected and compiled by Shimadzu Class VP7.4 software. Mobile phase used was a 200:245:555 (v/v) mixture of acetonitrile:methanol:sodium acetate buffer. The buffer was adjusted to pH 3.4 after mixing with the organic phase. The mobile phase was pumped isocratically at 1mL/min.

Linearity

The ratio of the peak area of midazolam to the peak area of clonazepam was plotted against the midazolam concentration to generate a calibration curve for each run. Calibration curves were generated by least-squares regression.

Accuracy and precision

Accuracy and precision were evaluated by determining midazolam at two different concentrations of QC samples in 4 replicates on 4 different days. Analysis of Variance was performed on the data and used to determine the between and within day coefficient of variation. Accuracy was defined as the percentage difference between the mean observed concentration and the nominal concentration.

Recovery

The recovery of midazolam from plasma was determined using 4 samples at two concentrations (1000ng/mL and 100ng/mL). The plasma samples were extracted and the peak areas for midazolam were compared to the peak area of a direct injection of midazolam.

Stability

The stability of midazolam in plasma was determined by preparing plasma samples containing 1000ng/mL and 100ng/mL of midazolam. Samples were

assayed on day of preparation and then stored at -4°C for 14 days. The samples were then re-assayed.

Clinical Application

The applicability of this assay for measuring a wide range of midazolam concentrations in plasma was demonstrated by analyzing plasma samples drawn from 24 adult female New Zealand White Rabbits. Rabbits were randomised to receive either high (1.56mg/kg bolus followed by 1.32mg/kg/h infusion) or low (0.78mg/kg bolus followed by 0.66mg/kg/h infusion) for approximately 110 minutes on day One. On day two the alternate dose was given. Blood samples (2mL) were collected from an ear artery catheter at known periods and placed into heparin sodium tubes (Vacuette® 2mL LH Lithium Heparin tubes). Ten samples were collected from each rabbit to determine the pharmacokinetic profile. The samples were stored in a container of ice in the refrigerator till the completion of the experiment then centrifuged at 3000 rpm for 15mins in a single batch. Plasma samples were collected from the tubes then stored at -80°C until assayed. Experimental procedures were approved by the Animal Care and Ethics Committee of the University of Newcastle (A-2009-113).

The elimination rate constant (k) for remifentanil for each of the tested samples was calculated using the first order elimination equation where slope

$$(-k) = \frac{\ln C2 - \ln C1}{t2 - t1}$$

C2 was the concentration at 200 min and C1 was the concentration at time 100 min as determined by the peak size detected. The half-life determined using the equation:

$$t\frac{1}{2} = 0.693/k$$

Results and Discussion

The retention times for midazolam and clonazepam (internal standard) were 16.25min and 19.5min respectively (Figure 28). No interfering peaks were observed at the retention time of midazolam or the internal standard. The lower limit of quantitation observed for this assay was 10ng/mL when an aliquot of 100µL of reconstituted sample was injected.

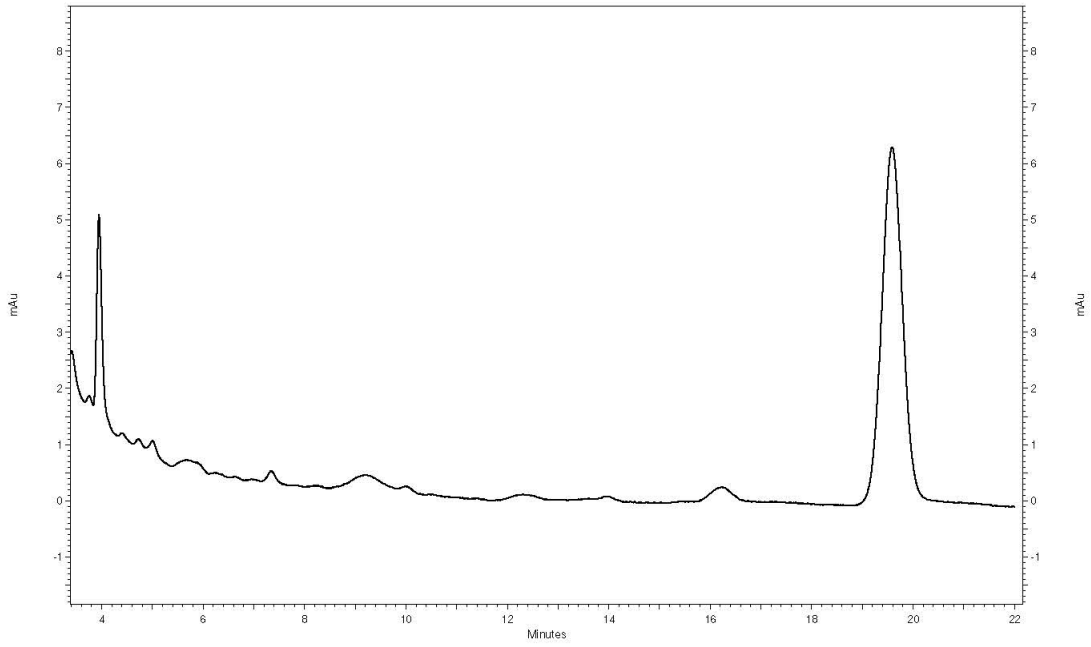


Figure 28: Chromatogram of plasma sample containing 50ng/mL midazolam

Calibration curve for midazolam was linear over the range of 50 to 2000ng/mL with a correlation coefficient (R^2) of 0.9998. The between-day assay coefficient of variation was 2.7% at 100ng/mL and 4.5% at 1000ng/mL. The within-day assay coefficient of variation was 3.3% at 100ng/mL and 4.4% at 1000ng/mL. The assay was demonstrated to be accurate with observed values being within +/- 5 % of the nominal concentration. The mean recovery of midazolam was observed to be 88.5% +/- 3.8% at 1000ng/mL and 90% +/- 1.2% at 100ng/mL.

Stability study demonstrated that plasma midazolam samples are stable for at least 14 days when stored at 4°C. Values obtained were within 5% of the original concentration.

When developing the assay it was noted that the retention time of midazolam and clonazepam was highly dependent on the pH of the mobile phase. Greater separation and faster run times were achieved by adjusting the pH (Figure 29). Midazolam direct injection were trialled at final pH 3.2, 3.4 and 3.8 and showed that faster run times were achieved by reducing the pH.

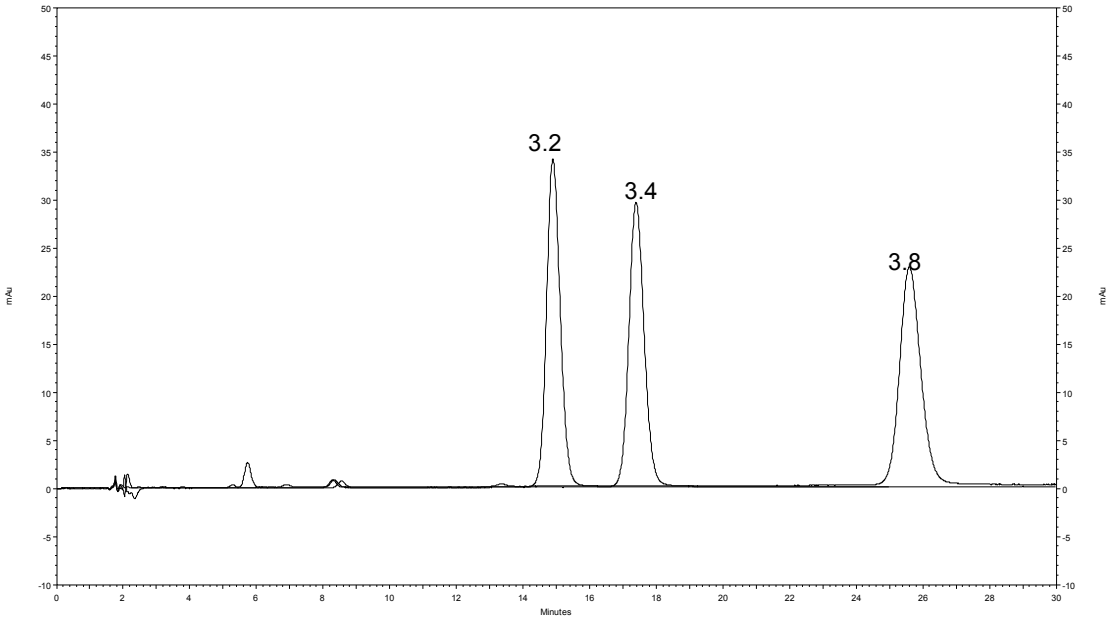
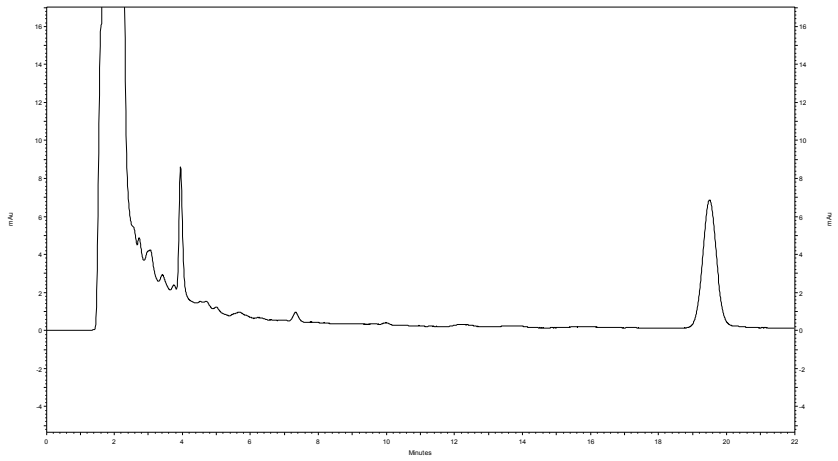


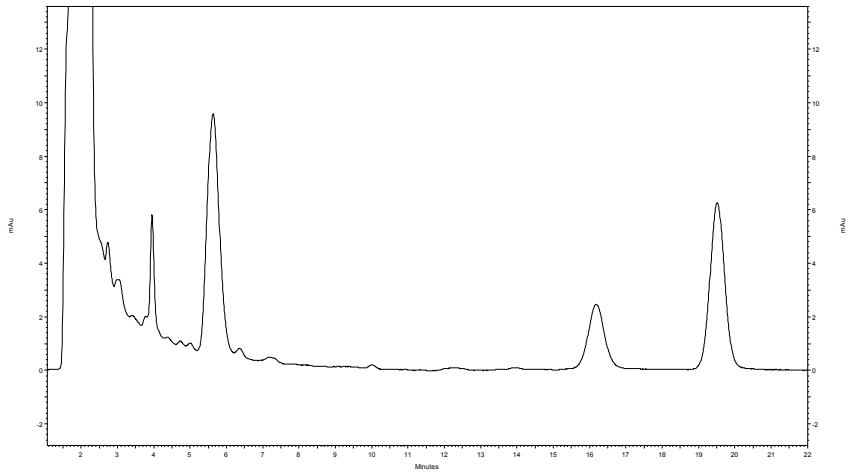
Figure 29: Effect of pH on midazolam retention time, with lower pH lowering retention times. A pH of 3.2 was found to be optimal

This assay was demonstrated to work effectively in biological samples. The assay was able to determine the concentration of midazolam in serum samples from NZ White rabbits with no interfering peaks (Figure 30). The assay was used to

determine the pharmacokinetics of midazolam in this animal model (Figure 31). The pharmacokinetic parameters calculated are described in Table 17.



(a)



(b)

Figure 30: Chromatograms

(**a**) Typical chromatogram of drug free plasma (NZ White Rabbit 3.27kg); (**b**) Actual sample (453.1ng/mL) obtained from NZ White Rabbit receiving a 0.78mg/kg bolus midazolam injection immediately followed by an IV midazolam infusion at 0.011mg/kg/min.

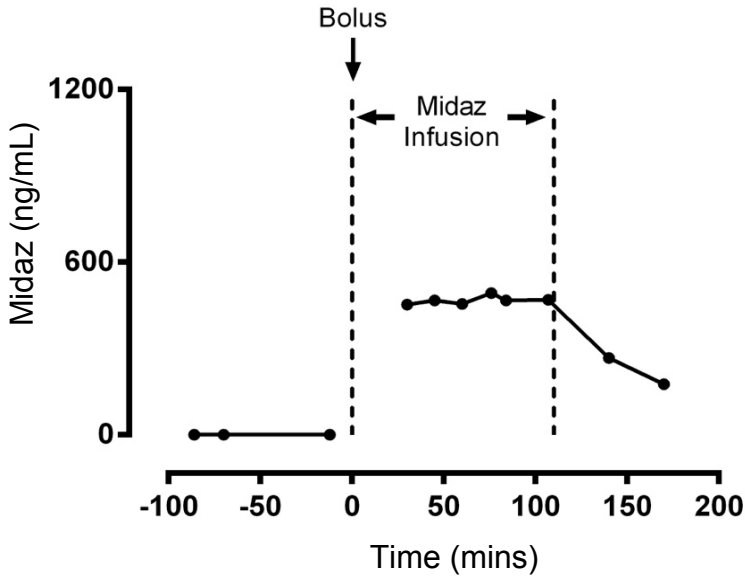


Figure 31: Concentration over time in rabbit plasma- Rabbit (3.27kg) was given a 0.78mg/kg i.v. bolus of midazolam at 0 mins followed immediately by an i.v. infusion of 0.011mg/kg/min. Infusion was stopped at 110 mins. Ordinate = measured plasma levels of midazolam.

Table 17: Summary of the pharmacokinetic parameters for midazolam including elimination rate constant (k) and elimination half-life (t1/2) calculated in five different rabbits.

Rabbit	k (min^{-1})	t1/2 (min)
1 (a)	0.010	69.3
1 (b)	0.011	63
2 (a)	0.014	49.5
2 (b)	0.014	49.5
17 (a)	0.009	77
17 (b)	0.015	46.2
18 (a)	0.017	40.7
19 (a)	0.013	53.3
19 (b)	0.014	49.5
Median	0.0135	49.5
Standard Deviation	+/- 0.003	+/- 11.87
Range	0.009-0.017	40.7-77

The calculated pharmacokinetics of the rabbits in this study demonstrate some interindividual variability between subjects with elimination half-life ranging from 40.7 to 77 minutes.

Conclusions

This method offers a number of advantages over previously published assays. The assay requires only a small volume of plasma (300µL) compared to the 1mL often required with other solid phase extraction techniques. It also avoids the use of organic solvents such as diethyl ether, chloroform and toluene. The extraction method is unique in that it uses a cation exchange SPE column for sample clean up. Previous methods have used cartridges with different packing material. The samples can be quickly prepared and the method has the ability to determine levels as low as 10ng/mL. While HPLC-MS methods are able to achieve lower limit of quantitation, many researchers do not have access to this expensive equipment. This method offers another approach that may be useful when studying the pharmacokinetics of midazolam- especially in small animal models or neonates.

Acknowledgements

Funding for this study was provided by Hunter Medical Research Institute Cardiovascular Health Program and the Australian and New Zealand College of Anaesthetists.

Chapter 7: Discussion

The research conducted for this thesis was designed to address the hypothesis that assays suitable for use in future pharmacokinetic studies in neonates could be developed. The drugs, benzylpenicillin, midazolam and remifentanil, were chosen as they represent important drugs used in this population, all previously without a suitable assay. Previously published assays required blood volumes for analysis that are too large for neonates or were unable to reach the level of sensitivity required for analysing neonatal samples. While the major focus of the research was on developing assay techniques for these drugs, the next step of using a developed assay in determining pharmacokinetic information was also achieved. The remifentanil assay, developed as part of this research project, was used in a clinical study where blood samples from neonates were collected and used to investigate esterase activity. As a result of my experience in designing and conducting this clinical study, insights into the barriers encountered in such studies were gained. Barriers identified included difficulties in gaining ethics approval and informed consent from parents, collecting sufficient samples and difficulties due to lack of dedicated research time for health staff. However, the study did demonstrate that it was feasible to use leftover clinical samples providing an assay with small volume of sample requirements and appropriate sensitivity was available.

The solutions to many of the identified barriers to neonatal clinical research are multifactorial and dependent on wide-scale change and increased organisational support for neonatal research, particularly around the streamlining of ethics and governance procedures for multi-site studies. Increasing representation of multidisciplinary neonatal clinicians on ethics committees, hospital leadership committees, funding organisations and regulatory bodies could help raise the profile of this research area. However, it is well known that clinician time for

additional service outside of clinical care is unfunded and burdensome. Support for clinical staff to have the dedicated time to conduct translational research, within their current workload, would improve the completion rate of this research.

Increasing the neonatal expertise on ethics committees will reduce the number of concerns raised when neonatal research proposals are submitted. Comprehensive international guidelines [63, 69, 71], addressing areas such as appropriate blood sampling and consent for neonates, are available and could be adopted by ethics committees worldwide. Increasing the use of antenatal consent from parents may help in improving participation rates by allowing more time for discussions about being involved in the study.

The development of low volume plasma/blood analysis techniques, like those developed in this project, is an important step in improving the feasibility of conducting pharmacokinetic studies in neonates. These techniques, combined with population pharmacokinetic modelling, will enable researchers to use blood left over from clinical samples to develop pharmacokinetic models, resulting in negligible effects on the research participants, as demonstrated in this work. I have published the insights gained from conducting a clinical study in neonates in a peer reviewed journal, so that these insights and proposed solutions will assist future researchers as they design clinical studies in the neonatal population.

As a result of this project, suitable assays for three important drugs are now available. It is anticipated that this work will provide a platform for further research into drug concentration analysis techniques using low plasma volume or dried blood spot technology for other drugs currently being used in neonates. Once access to the equipment required for suitable assays is established, this research will have low running costs beyond staff time. The fast throughput of

the methodologies allows for large numbers of samples to be processed in a single session.

Providing analgesia for neonates is clinically challenging. The opioid remifentanil has been proposed to be a good alternative to other opioids such as morphine because of its unique metabolism and short elimination half-life. Remifentanil is thought to be useful as clearance is via metabolism by esterases. Other opioids such as morphine and fentanyl undergo hepatic metabolism involving CYP450 enzymes or glucuronidation and, in the case of morphine, the glucuronide metabolites, one of which is more potent than the parent compound, undergo renal elimination. As seen in the data reported in Chapter 1, the development of CYP enzyme activity is variable and renal function develops over the first year of life. Remifentanil clearance by esterases means its elimination from the body is not dependent on metabolism by CYP450 enzymes or renal excretion that are still developing or where development is unknown.

When reviewing the literature, it was evident that few data are available on the pharmacokinetics of remifentanil in pre-term neonates. In the few studies that have reported pharmacokinetics from adults and children, assays required sample volumes of at least 500μL and were arterial blood. In a neonate this is a significant volume and limits using these assays for future pharmacokinetic studies and access to arterial blood may not be available. While the few data currently in the literature provides a starting point to developing population pharmacokinetic models for and producing evidence-based dosing information, assay techniques capable of measuring low concentrations in very small blood volume are required. These assays, combined with sampling strategies developed using approaches such as D-optimal design, which was discussed in Chapter 1 offer the opportunity to produce the data needed to inform dosing in neonates. Development of a small volume assay also reduces patient impact from research participation. The ideal method of collecting neonatal samples for developing pharmacokinetic models is to use leftover blood from samples taken

for other clinical use. This results in no additional blood samples and further reduces the ethical considerations of this work. The volumes left over from these samples are typically 100μL to 300μL.

The analytical technique for remifentanil developed in this project meets all of the criteria for a suitable assay described above. This assay is able to measure concentrations as low as 0.25ng/mL in only 100μL of plasma. This is ideal for leftover blood samples. The low limit of quantitation in this assay was achieved by using sophisticated analytical technology. The HPLC-MS/MS used to develop this assay was a 6500 triple quadrupole which offers greater ability to detect low concentrations in samples than other detection techniques such as HPLC with UV or fluorescence detection. The extraction technique used in this assay allows for rapid processing time. The acetonitrile extraction techniques used does not require complex steps or the use of potentially dangerous chemicals. This extraction method is not suitable for use with all HPLC equipment using other detection techniques. Equipment with less sensitive detection systems usually require more complex extraction from samples to remove impurities that could affect results and often require drying and resuspending steps to concentrate the analyte of interest in order to improve sensitivity. The combination of this advanced equipment with the extraction technique used is an important factor in the sensitivity of this assay.

The assay method developed was validated using the HPLC-MS/MS 6500 as described in Chapter 3. Different types and brands of tandem mass spectrometers are available. The opportunity to test this method on a different system was not available. While the general aspects of this assay should be transferrable, slight refinements of the method may be required if the assay were to be performed on different tandem mass spectrometry systems. This assay provides a significant advance in the volume required for pharmacokinetic studies, reducing the required volume to one-fifth of that required in previously reported studies. Work could also be done to reduce the sample size even further

to be able to work with the volumes collected with new microsampling devices (usually in the range of 10-20μL). To achieve sufficient sensitivity in the volume range of 10-20μL of blood or plasma, the extraction technique could be modified to include an evaporation and re-suspension step to concentrate the sample to provide greater sensitivity. In summary, the remifentanil assay developed as part of this work will enable many future research studies. As more laboratories become equipped with HPLC-tandem mass spectrometry, this method should simplify the process of setting up the analytical arm of pharmacokinetic studies.

As mentioned earlier, while it is relatively straightforward to access venous blood samples of a few millilitres in adults and gaining informed consent, these volumes and gaining consent of parents to draw blood samples from neonates is problematic. This is where left over blood samples from other clinical uses provides a potential source of blood, overcoming these problems. In this project, the feasibility of using left over blood samples was demonstrated when these samples and the remifentanil assay were used to investigate esterase activity in neonatal samples. Little is known about the development of esterase activity with age and the location of esterase activity within blood. The only study previously conducted on esterase activity in blood in newborns only analysed plasma taken from umbilical cord samples [83]. In adult patients remifentanil metabolising activity is strongly associated with red blood cells rather than plasma, which was reflected by the observations in this study.

The results of this study provided information previously not available on the existence and extent of esterase activity in neonates by comparing activity in both neonatal and adult red blood cell components and plasma. These results have shown that esterase activity is present in both plasma and also red blood cells with wide variability in extent being observed in the neonates studied. The results also suggest that there is difference between extent of activity or the distribution of esterases between plasma and red blood cells between adults and neonates. Showing that enzyme activity is still retained when samples are frozen

will also facilitate future work. Currently, very little is known about drug metabolising esterases. The demonstration that frozen samples can be used for in-vitro testing of esterase based metabolism has applicability to both developmental studies and studies of esterase metabolism of other drugs, for example ciclesonide.

From a clinical perspective, this study provides further understanding on the metabolism of remifentanil in neonates. Remifentanil is currently recommended as a short-term pain reliever for procedures for neonates. There is no evidence-based dosing for this drug based on pharmacokinetic studies and dosing information is extrapolated from adult and paediatric information. Information regarding the development of all drug metabolising pathways in neonates is limited, even more so for esterases. One of the reasons that remifentanil has been suggested as a suitable pain reliever, especially for short procedures, as its metabolism and clearance is not dependant on kidney or liver function [166], both of which are immature in neonates. An assumption has been made that esterase based metabolism does not undergo developmental changes, however studies have shown differences in half-life associated with age [83, 166]. Overdose with remifentanil can result in respiratory depression of the neonate, which is a considerable risk. This work provides information regarding the development of remifentanil metabolising non-specific esterases and the possible differences in neonates and adults in this drug metabolising pathway by clearly demonstrating that the rate of metabolism in blood is different between neonates and an adult. The difference seen between neonatal and adult metabolism in this study demonstrates that developmental differences in esterase occurrence occur. The data from this experiment is limited by the small number of patients recruited, which did not allow for age group sub-analysis. The adult samples only came from a single patient that may not account for genetic differences in adult remifentanil metabolism, although currently no genetic or inter-individual differences in remifentanil metabolism are known. This preliminary work suggests that further research should be conducted regarding metabolism and dosing before remifentanil use becomes widespread

in the neonatal intensive care environment and adds further weight to the overall understanding that extrapolation of adult data to neonates and children can result in dosing errors. This data provides a validated starting point for further research with no risk to patients, with the ongoing focus on determining specific age range differences in remifentanil metabolism between birth and adulthood.

Treatment of infections in neonates is another challenging area in neonatal care. Since benzylpenicillin is commonly used in this population, this drug was selected for my project. Benzylpenicillin is used as treatment and prophylaxis of Group B streptococcal infections that are common concerns in the first few days of neonatal life. It is often used with gentamicin in this setting and currently detailed, evidence based dosing information to optimise the use of benzylpenicillin is not available.

The current era of antimicrobial resistance requires antimicrobials to be adjusted using TDM to produce optimal effectiveness. Currently, in Australia, the ability to analyse neonatal samples quickly for clinical use is not readily available. Assays that are available are aimed at the adult population where volumes of plasma of about a millilitre are easy to obtain. The assay developed as part of my project offers the opportunity to make this important clinical tool available to all patients- adults and neonates. The volume of sample required, fast throughput, demonstrated stability and sensitivity of this assay make it suitable for use across all patient groups. There is of particular utility to intensive care patients who require TDM. Although penicillins are considered to have a wide therapeutic margin, they are not without side effects. The ability to adjust doses to the individual patient and infection, as already seen in this population with gentamicin, can only improve patient outcomes.

Previously published assays and clinical assays currently available generally do not have the ability to measure benzylpenicillin concentrations in the range of

20ng/mL. This is the concentration above which plasma concentration should be maintained in patients receiving long-term prophylaxis depot injections of benzylpenicillin for rheumatic heart disease. The assay I developed meets these requirements, which if the assay was established in routine pathology laboratories, could support achieving better outcomes in this group of patients.

This assay, along with the stability work, allows for the design of a pharmacokinetic study and also presents opportunities for clinical therapeutic drug monitoring [167], which is important in managing complex infections in paediatric and adult patients [168]. Previous dosing for benzylpenicillin was based on data extrapolated from studies in older children and adapted to the clinical setting. As described previously, in Chapter 1, this is not optimal and does not lead to the best outcomes for patients. The ability to conduct pharmacokinetic trials to produce evidence based dosing, as expected by modern standards of evidence-based practice, is an important safety improvement in neonatology.

Conducting stability studies was an important aspect of this work. The experiments done as part of this work were designed to simulate "real-world" studies, in which samples are not always kept at their optimal temperature and delays may occur in processing. Previous assays for benzylpenicillin have shown concerns regarding sample stability [137]. By demonstrating stability with this assay it can be immediately translated into clinical work with optimal outcomes. It also allows flexibility in analysing samples as the proven 24 hour room temperature stability and 4 weeks of freezer stability allows for samples to be stored so that multiple samples can be run at the same time.

Developing the benzylpenicillin assay required comparison of different analytical techniques – specifically HPLC-UV and HPLC-MS/MS. Although a large number of assay conditions and extraction techniques were trialled it was not possible to reach the required levels of sensitivity and specificity with HPLC-UV

and this equipment was determined to be unsuitable for use in neonatal studies for benzylpenicillin. The use of HPLC-MS/MS allowed for small volumes to be used and reach the required lower limits of sensitivity and specificity required. HPLC-MS/MS also provides faster run times allowing more samples to be processed improving the clinical applicability of this assay.

A further advantage of HPLC-MS/MS is less toxic extraction techniques are required. As mass spectrometry works on individual molecular weights less complex extraction procedures and sample clean up is required. The simple acetonitrile precipitation to remove protein from the sample, as seen with this method, further decreases the time taken to conduct the assay and reduces exposure to toxic chemicals.

The advantages of the assay I developed in this project are all features that would make this assay attractive for use in a routine TDM laboratory. For clinical use, rapid turnaround time is essential as clinicians need results prior to the next dose administration time. Simple assays reduce workload in routine laboratories. The observation that plasma samples are more stable than previously reported also reduces pressure on sample handling procedures in the clinic while supporting the use of clinical sample left overs in research.

Midazolam is used for sedation, anaesthesia and treatment of seizures in neonates. Although used commonly, there are concerns regarding the effects on neurodevelopment. Dose optimisation will help determine the lowest effective dose, possibly reducing adverse effects. The work in this thesis aimed to develop a low sample volume assay suitable for use in neonates. The assay developed represents an improvement over other published assays by using a sample volume of only 300μL and reaching a limit of detection of 10ng/mL. A complex extraction method, using solid phase extraction, was required. Significant improvement of the assay could be made by transferring from HPLC-UV to HPLC-

MS/MS equipment, which would increase sensitivity and specificity. This would also allow for smaller sample volumes with simpler extraction techniques that would speed up the assay.

This body of work demonstrates that once the difficulties associated with this type of research are overcome it is possible to complete pharmacokinetic research in neonates. The increased number of assays suitable for use in the neonatal population described in this thesis offers multiple opportunities for translation into clinical research. The small body of work conducted so far in determining evidence based dosing for neonates has focused on drugs where clinical therapeutic drug monitoring has been used clinically for some time, for example gentamicin [169-171].

The need for greater information on neonatal pharmacokinetics and pharmacodynamics is well established [172]. While this need is well described in Chapter 1 the methods required to conduct this work are currently limited and regulatory barriers exist. In order to overcome ethical barriers to the recommended pharmacokinetic studies in neonates, different methodologies to traditional studies are required. Developing the appropriate analytical techniques to detect low drug concentrations in small volume samples is an important step in establishing neonatal pharmacokinetic research programs in Australia. The work in this thesis is also significant because it enables the start of a library of analytical methods that can be used in small and large clinical pharmacokinetic studies. This enables researchers and clinicians to expand the evidence base around neonatal drug dosing. Regulatory bodies, including the FDA and EMA have already encouraged an increase in paediatric drug dosing studies [46]. An increase in neonatal drug dosing information for clinical use represents the next step in this work once research is established.

This work has demonstrated with three separate assays that it is possible to conduct neonatal research in this way, opening up greater opportunities for

future work. Chapters 3, 5 and 6 give specific examples of the work required to develop appropriate small volume assay work of commonly used drugs. It was noted that neonates are not able to provide the number and volume of blood samples required for traditional pharmacokinetic studies. In order to conduct this work ethically low blood volume drug assays coupled with population pharmacokinetic models must be developed. The methods developed in this Thesis presents a starting point for larger studies; specifically by describing low volume assays with in use testing, stability information and in-vitro assays to determine how drug metabolism pathways develop over the neonatal period.

These new methodologies reduce the ethical issues related to conducting this clinically required work. The work described in Chapter 4 also highlights the difficulties associated with developing these methodologies. This work also shows that appropriate HPLC-MS/MS equipment is required and access to this equipment is a limiting factor in completing neonatal pharmacokinetic research. Large portions of time during development of these assays was spent working with equipment and methodologies that are unsuitable for use in neonatal research, presenting a barrier to the completion of this work. This work demonstrates that collaboration between researchers is essential to complete neonatal pharmacokinetic studies and this collaboration is one of the next steps in expanding this research.

Future Directions

This work sets up a number of future studies. Currently study in neonatal pharmacokinetics is slowed by a lack of appropriate methodologies. The work in this Thesis offers three proven new drug assay methodologies which, when combined with population pharmacokinetic modelling, will allow for evidence based drug dosing regimens for neonates to be delivered.

Immediate ongoing plans from this work are to use the benzylpenicillin assay and stability information to conduct a clinical study to develop a population model of benzylpenicillin pharmacokinetics in preterm neonates. The midazolam assay offers similar opportunities to conduct dose-finding studies. Further improvements in sample volume required and reduced analysis time by using HPLC-MS/MS could be achieved which would make pharmacokinetic studies easier to perform.

Ongoing work using remifentanil assays in conjunction with pain scoring to look at the appropriate dosing of remifentanil for central line insertion in preterm neonates.

Current advances in microsampling technology present the next experimental steps in this work. Microsampling techniques, such as dried blood spots, along with new devices, such as Mitra® sampling devices have recently been identified as suitable for drug concentration analysis. In addition, these samples do not require refrigeration or freezing. Further development of these HPLC methodologies to work in conjunction with these technologies will increase the ability to conduct large-scale pharmacokinetic studies.

Overall this work provides opportunities to start contributing to this important, growing field. Specifically it has shown how to develop small blood volume analysis techniques that are suitable for neonatal studies. Providing safe and effective drug dosing information for neonates will continue to be a clear priority in both pharmacology and neonatology in the future.

Glossary

Co-variate	Independent variable added to a model
Gestational age	The length of pregnancy calculated from the first day of the womens menstrual cycle
Infant	Children under 1 year of age
Neonate	Children under 4 weeks of age
Pharmacodynamics	Biochemical and physiological effects of drugs on an organism
Pharmacokinetics	Effects of an organism on a drug
Post-menstrual age	Gestational age plus age post birth

Appendix

Appendix 1. Medicines in Babies – Esterases Participant Information Sheet

NEONATAL INTENSIVE CARE UNIT

JOHN HUNTER CHILDREN'S HOSPITAL

LOCKED BAG 1

HUNTER REGION MAIL CENTRE NSW 2310

TELEPHONE: (02) 4921 4362/4365/4410

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Dr Susan M Lord

Staff Specialist in Pain Medicine

Associate Professor Ian M R Wright

Senior Staff Specialist in Neonatal Medicine

Participant Information Sheet

Medicines In Babies

The development of remifentanil metabolising non-specific esterases

Principal Researcher: Dr Susan Lord, Assoc/Prof Ian Wright, Prof Alison Jones

PhD Student: Kate O'Hara, Clinical Pharmacist

Associate Researchers: Ros Black, Jane Buchan

This information package contains a double-sided Participant Information Form and a single-sided Consent Form. Please make sure you have received all pages.

Who are the researchers?

This research project is being conducted by Kate O'Hara, a Clinical Pharmacist, as part of her PhD program at the University of Newcastle, NSW. Her PhD supervisors are Dr Susan Lord, a Pain Medicine Specialist, Associate Professor Ian Wright, a Senior Specialist and Researcher in Neonatal Medicine, and Professor Alison Jones, a Clinical Pharmacologist and Physician.

Your Consent

This form contains detailed information about the study. Its purpose is to explain to you as openly and clearly as possible, the background and all the procedures involved in the study before you decide whether or not to take part. Please read all pages carefully, and feel free to ask questions about any of the information. You may also wish to discuss the study with a friend, relative or your local health worker. Once you understand what the study is about and if you agree to take part, you will be asked to sign a consent form. We will then provide you with a copy of the consent form to keep for your records.

Purpose of the study

After babies are given medications that they need for their care, the medications wear off because they are broken down in the body and the body gets rid of them. One group of medications is broken down by a natural enzyme (chemical) in the babys blood called 'esterase'. Currently, we do not know exactly when esterases develop or when they reach full activity during development.

This study does not involve any extra tests, treatments or risks for your baby. We are seeking permission to collect the small amount of blood that is left over in the test-tube after your baby's routine blood tests have been done. We will add the drug remifentanil to the leftover blood samples (in a test-tube) and measure how much the blood breaks down the drug. Your baby will not be given any extra medications. All information will be stored anonymously on secure computers and information will be analysed in groups.

What is involved for your baby?

During the course of your babys treatment here in the NICU blood samples will be taken regularly to check on your babys health. Once these samples are processed by pathology there may be a small amount of blood left over. We wish to use that blood to find out how much activity the esterase in the blood has. Your baby will not need to have any extra needles.

Possible Benefits

There are no direct benefits to your baby from being a part of this study.

Possible risks, side effects and discomforts

There are no risks to your baby from being a part of the study.

Participation is voluntary

- If you do not enrol for the study your baby will still receive the best care the nursery can give
- Only give your consent once you have had all of your questions answered to your satisfaction.
- If you later wish to withdraw from the study, you may do so at any time without giving a reason. Withdrawal will in no way affect the care that you or your baby receives.
- There is no financial payment for being in the study.

Privacy, Confidentiality and Disclosure of Information

All research data about your baby will be confidential. After enrolment, your baby's data is given a code. Any paper records will be kept in a locked cupboard. Your baby's data will be held in its coded form (so your baby cannot be identified) on the Study research computers at the Hunter Medical Research Institute, protected with secure passwords. Only the members of the Study team and the Trial Monitoring Committee are allowed access to the data.

At the end of the study, publication of the results will not contain any personal details that could identify you or your baby. Information from this study will be used to help neonatologists from all over the world make decisions about the doses of remifentanil used to treat babies at different stages of their development.

You have a right of access to, and to request correction of, information held about yourself or your baby by the John Hunter Children's Hospital in accordance with the NSW Health Records and Information Privacy Act 2002.

Further Information

If you require further information regarding any part of the study, please do not hesitate to contact Kate O'Hara at John Hunter Children's Hospital Neonatal Intensive Care Unit, on 49214362,

Other Issues

If you have any complaints about any aspect of the study, the way it is being conducted or any questions about your rights as a research participant then you may contact any of the research staff or, if an independent person is preferred, to Dr Nicole Gerrand, Manager, Research Ethics and Governance, Hunter New England Human Research Ethics Committee, Hunter New England Health, Locked Bag 1, New Lambton NSW 2305, telephone (02) 49214950, email Nicole.Gerrand@hnehealth.nsw.gov.au.

Ethical Guidelines

This study is carried out according to the *National Statement on Ethical Conduct in Research Involving Humans (2007)* produced by the National Health and Medical Research Council of Australia. This information sheet has been developed to protect the interests of people who participate in research studies.

This research has been approved by the Hunter New England Human Research Ethic Committee to be conducted in the Neonatal Intensive Care Unit.

The conduct of this study at the John Hunter Hospital has been authorised by Hunter New England Health. Any person with concerns or complaints about the conduct of this study may also contact the Research Governance Manager on 4921 4950 and quote reference number 12/02/15/4.03.

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