An Investigation into the Use of Dried Blood Spot Analysis in Pharmacokinetic Studies

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Abstract

The ethical and practical issues of obtaining a blood sample pose a significant challenge to performing pharmacokinetic studies in children, infants and neonates. Dried blood spot analysis, based on the collection of a micro blood sample has potential to overcome these difficulties. There are at present a limited number of reports on the utility of dried blood spot analysis in clinical pharmacokinetic studies. The studies described in this thesis were undertaken to investigate the accuracy and precision of dried blood spot sampling coupled with mass spectrometry detection for drug quantification, and clinically validate the robustness and feasibility of this technique for pharmacokinetic studies in preterm neonates. Dried blood spot methods were developed for application to pharmacokinetic studies of test drugs dexamethasone and caffeine.

Investigations were focused on the blood collection system, analyte recovery and optimisation of the detection system. *In-vitro* validation results indicated developed methods were precise, accurate and selective in accordance with the Food and Drug Administration regulatory guidelines on the assessment of bioanalytical methods. Results were not significantly affected by small variations in the blood volume spotted or the presence of petroleum jelly, which is often used on the sampling site during capillary blood collection in neonates. Variability in haematocrit was determined to be the single most important factor affecting assay accuracy. Stability assessments by comparison with freshly prepared samples verified the suitability of sample drying, storage and post sample extraction conditions. An investigation of method transferability between different analytical instruments was undertaken with caffeine to provide an assessment of the robustness of dried blood spot analysis. Results generated from a single and triple quadrupole mass spectrometer were comparable with an expected lower limit of quantification with the latter technique most likely due to a greater ionisation and detection efficiency.

Intravenous dexamethasone pharmacokinetics was determined in 5 preterm neonates receiving treatment for chronic lung disease. Individual pharmacokinetic analyses were performed using a one compartment model to estimate primary pharmacokinetic parameters, clearance (mean, 0.18 l/h/kg) and volume of distribution (mean, 1.33 l/kg). The whole blood derived mean estimates were similar to previous plasma clearance and volume estimates of 0.14 l/h/kg and 1.91 l/kg, respectively reported in neonates (n=7). This highlights the potential for dried blood spot analysis as an alternative to conventional plasma based methods for dexamethasone dose optimisation studies in neonates. The population pharmacokinetics of oral / intravenous caffeine was determined in 67 preterm neonates. A one compartment model was used to describe the blood concentration-time data. Model evaluation using a bootstrapping technique confirmed the robustness and stability of the developed model. Pharmacokinetic parameters derived from dried blood spot drug measurements were estimated with precision (relative standard error < 10%) and were comparable to estimates of plasma clearance (mean, 7.3 *vs.* 7.0 ml/h/kg) and volume of distribution (mean, 593 *vs.* 851 ml/kg) from a previous population study in neonates (n=110). Weight and postnatal age were the most influential covariates in the clearance model which is in agreement with previous population studies.

These results demonstrate that dried blood spot analysis is a practical technique, with significant potential as a robust method for use in clinical pharmacokinetic studies in vulnerable populations such as preterms. Haematocrit related effects on paper will need to be accounted for if this potential is to be realised. Further investigations to determine the reproducibility of capillary blood sampling in neonates and the impact of using blood drug measurements on pharmacokinetic parameter estimation will be necessary before widespread use of the technique is possible.

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Publications and Presentations during Thesis

Papers

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- Patel, P., Mulla, H., Tanna, S. & Pandya, H. 2010. Facilitating pharmacokinetic studies in children: a new use of dried blood spots. *Archives of Disease in Childhood,* 95**,** 484-487.

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Posters

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- Patel, P. Patient clinical and demographic covariate data. University of Leicester, February 2011.
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- Patel, P. Pharmacokinetics of dexamethasone in preterm neonates using dried blood spot analysis. Neonatal Intensive Care Unit, Leicester Royal Infirmary, April 2008.

Abbreviations

AOP	Apnoea Of Prematurity
APCI	Atmospheric Pressure Chemical Ionisation
BLOQ	Concentration measured Below the Limit Of Quantification
CL	Clearance
Conc.	Concentration
CV%	Coefficient of Variation %
CYP1A2	Cytochrome P450 1A2
CYP3A4	Cytochrome P450 3A4
DBS	Dried Blood Spot
DPS	Dried Plasma Spot
ESI	Electrospray Ionisation
GA	Gestational Age
GC-MS	Gas Chromatography-Mass Spectrometry
IIV	Interindividual variability (the difference between the individual and the population mean parameter estimate)
IOV	Interoccasion variability (variability in parameter estimates between occasions)
IS	Internal Standard
IV	Intravenous
Ka	Absorption rate constant (h^{-1})
LC-MS	Liquid Chromatography-single quadrupole Mass Spectrometry
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LC-UV	Liquid Chromatography-Ultraviolet detection
LLOQ	Lower Limit Of Quantification
LOD	Limit Of Detection
Log P	Octanol-water partition coefficient
-2LL	Minus 2 Log Likelihood (objective function value)
MS	Mass Spectrometry
NICU	Neonatal Intensive Care Unit
NSQAP	Newborn Screening Quality Assurance Programme
OFV	Objective Function Value (i.e. maximum likelihood estimator) at the end of each iteration

PD	Pharmacodynamic
PK	Pharmacokinetic
PMA	Postmenstrual Age
PNA	Postnatal Age
RE%	Relative Error %
RIA	Radioimmunoassay
Residual	Observed minus predicted blood concentration
SD	Standard Deviation
SIM	Single Ion Monitoring
SRM	Selective Reaction Monitoring (precursor to product transition)
TDM	Therapeutic Drug Monitoring
TK	Toxicokinetic
$t_{1/2}$	Half life (time)
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet
V	Apparent volume of distribution

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Chapter 1

Introduction

1.1 Background

A significant limitation in performing neonatal and paediatric pharmacokinetic (PK) studies is the need for blood sampling. Traditional plasma based drug quantification methods most often require a relatively large blood volume (0.5 - 10 ml) at each time point to generate sufficient plasma for analysis. This poses ethical and practical challenges around obtaining PK data in these age groups. Dried blood spots (DBS) represent an alternative matrix for measurement of circulating drug concentrations. The DBS technique typically involves the collection of a few drops of blood onto filter paper. This sample format provides a method of handling liquid blood and is amenable to a range of quantitative and qualitative analyses. There is now an increased awareness of the potential advantages of DBS over conventional plasma drug measurements used in PK studies. Requiring only a micro blood volume (usually less than 50 μl), DBS analysis has the potential to overcome difficulties associated with blood collection in neonates and children and thereby facilitate much needed PK studies. The use of DBS was first widely introduced in the 1960's by Dr Guthrie, a neonatologist, to measure levels of phenylalanine in newborns for the diagnosis of phenylketonuria (Guthrie and Susi, 1963). DBS has subsequently been used as a sampling technique in the therapeutic drug monitoring (TDM) of some drugs in clinical practice (Coombesa *et al.*, 1984, AbuRuz *et al.*, 2006, la Marca *et al.*, 2008).

Plasma has been the preferred matrix for drug quantification studies largely due its relative ease of handling compared with liquid whole blood. As such it has become the gold standard for performing pre-clinical and clinical PK studies. Although there is some experience of DBS in drug quantification through TDM, its application to generate PK data is limited (Beaudette and Bateman, 2004, Filippi *et al.*, 2009,

Mohammed *et al.*, 2010, Spooner *et al.*, 2010, Kole *et al.*, 2011, Suyagh *et al.*, 2011). *In-vitro* validation reports clearly demonstrate the potential to accurately and precisely quantify pharmaceutical compounds using DBS (Liang *et al.*, 2009, Spooner *et al.*, 2009). There are however, relatively fewer reports on the clinical evaluation of DBS techniques applied to PK studies. All drug quantification methods must be demonstrably accurate and reliable so that the results from subsequent PK analysis of the data can be applied to optimise drug treatment schedules for patients. More extensive clinical validations of DBS analysis will therefore be essential before widespread use of the technique in PK studies is possible.

The *in-vitro* and clinical studies described in this thesis were designed to investigate the robustness and reliability of DBS quantification as an alternative to traditional plasma based methods in PK studies. Investigations were carried out in neonates and adults using the target drugs dexamethasone and caffeine.

1.2 Pharmacokinetic studies

Understanding the PK of a given compound in humans forms an essential part of the drug development process. PK studies typically involve the collection of repeat blood samples in order to determine the drug concentration-time profile. They are often combined with efficacy (pharmacodynamic, PD) studies to generate critical information for the safe and effective prescribing of medicines which include the dosage requirement, administration frequency and treatment length. The information gathered from these studies serve as the scientific foundation for dosing regimens used in clinical efficacy (and safety) trials of medicines (Howland, 2008). In turn, clinical efficacy trials form the basis for prescribing a medicine to the general population. Most medicines

used in adults have been formally tested in this manner and thus doses are prescribed on the basis of scientific evidence. In contrast, a significant proportion of medicines administered to children and neonates have not been subjected to such rigorous testing. In the absence of regulatory valid studies by pharmaceutical companies these drug treatments when clinically necessary are prescribed on an unlicensed and (or) off-label basis (Conroy *et al.*, 1999, Conroy and McIntyre, 2005, Lindell-Osuagwu *et al.*, 2009). Studies suggest the deficit in evidence based medicine is greatest in neonates with approximately 90% of neonates on the neonatal intensive care unit (NICU) and 70% of hospitalised children receiving at least one unlicensed or off-label drug treatment during their care (Conroy *et al*., 1999, Conroy *et al*., 2000). This raises serious concerns around short and long term safety for medicines currently used in children and neonates (Cuzzolin *et al*., 2006).

1.3 Barriers to performing neonatal and paediatric PK studies

1.3.1 Ethics, economics and logistics

A reluctance to perform PK and PD studies in children has been in part due to the perceived risks of involving vulnerable age groups in clinical studies (Saint Raymond and Brasseur, 2005). Economic reasons have also contributed to the inequality in drug data between different age groups. As children represent a small percentage of the market for medicines there is less financial incentive for the pharmaceutical industry to develop medicines specifically for children (Matsui *et al*., 2003). Furthermore, the conduct of large clinical studies can be logistically problematic due to limited sampling pools and poor recruitment.

1.3.2 Blood sampling

A major barrier to conducting pharmacokinetic trials in children is the relatively large volumes of blood (~ 0.5 - 10 ml) at a time required for analysis by most plasma based assays. The problem of blood sampling is most apparent in neonates due to their comparatively lower circulatory volume. Often numerous blood samples are collected from neonates during routine care on the NICU which already makes them at risk of iatrogenic blood loss (Weiss *et al*., 2002). Therefore any drug quantification method must enable the measurement of circulating drug concentrations from a micro volume of blood. The European Medicines Agency (EMA) guidelines on blood sampling in newborns state 'the trial-related blood loss (including any losses in manoeuvre) should not exceed 3% of the total blood volume during a period of four weeks and should not exceed 1% at any single time' (Guidelines on the Investigation of Medicinal Products in the Term and Preterm Neonate, 2007). Considering the total blood volume of a neonate is estimated at 80 ml/kg body weight, the maximum volume of blood that can be safely removed from a 500 g preterm for trial purposes is 1.2 ml over four weeks (Koren, 1997). PK studies should be designed and performed within these blood sampling limitations.

The problem of repeated blood sampling has been partly resolved by the use of population PK study designs that incorporate sparse sampling methodology. This means that 2 - 3 blood samples are required from each patient rather than in excess of 12 samples required by traditional PK modeling methods. The population PK approach is able to reliably estimate the values of PK parameters such as clearance (CL), volume of distribution (V) and half life ($t_{1/2}$) and their associated variability within the population. Indeed, population PK has been recommended by the regulatory agencies as the

preferred approach to studying pharmacokinetics in children (The Role of Pharmacokinetics in the Development of Medicinal Products in the Paediatric Population, 2007). This approach however, does not overcome the requirement for large blood volume samples.

In circumstances where the volume of blood does not prevent a PK study from taking place, blood sampling itself can make children, parents and ethics committees baulk at agreeing to a PK study. The degree of pain or discomfort associated with a blood sampling method is an important factor for parents when deciding whether to consent to their infant's participation in a study (Anderson *et al*, 2007). These ethical concerns can be reduced by coordinating blood sampling for research purposes with routine clinical sampling. Moreover, all blood sampling methods have a failure rate depending on the volume of blood needed and the skill of the phlebotomist. The collection of capillary blood following a heel-stick prick is a common method for obtaining blood samples on the NICU. It is technically less demanding to perform than venepuncture, but it is often not possible to collect large volumes of blood with this method. For these reasons, it is clear that PK data on drugs used in all age groups will only become readily available through the development of assays and methods that are compatible with micro volume blood samples.

1.4 Neonatal and paediatric medicines

1.4.1 Drug dose estimation in the absence of pharmacokinetic data

In the absence of age specific PK data, drug doses for administration to children and neonates have historically been derived from adult data adjusted according to body

weight or body surface area (Anderson *et al*., 2007). However, these approaches have a tendency to either over predict or under predict drug CL and are therefore often inappropriate for scaling neonates and children to adults (Holford, 1996). An additional problem of using adult PK data for dose selection is that it may have been derived for a different disease state to that which it is being intended for. On-going developmental changes in drug disposition are prevalent from birth to adolescence which adds to the complexity of studying medicines in children and neonates. Drug metabolic pathways are immature in the newborn which mean often a smaller dose per kilogram is required to produce an equivalent therapeutic drug level (Stevens *et al*., 2003, Hines, 2007). The maturation of specific hepatic enzymes are severely delayed and can extend over several years before reaching adult metabolic capacity. Renal function is also reduced in term and preterm neonates with adult glomerular filtration rates (GFR) not being reached until around one year of age (Rhodin *et al*., 2009). The International Conference on Harmonisation guidelines distinguishes at least four subgroups; newborns (birth to 28 days of life), infants (1 to 23 months of life), children (2 to 11 years of age) and young people (12 to 18 years of age) (Clinical Investigation of Medicinal Products in the Paediatric Population, 2001). Each subgroup warrants special consideration in relation to their developmental stage to ensure safe and effective prescribing practices and therefore the conduct of several clinical studies may be necessary.

Overdosing and toxicity are recognised risks associated with dosing on the basis of data generated in adults. Chloramphenicol and propofol are important examples where children and neonates have been prescribed inappropriately high and toxic doses of medicines because of unrecognised, age-specific differences in PK (Weiss, 1960, Knibbe *et al*., 2002). There are few instances of drugs prescribed at sub-therapeutic

doses based on adult PK data which could reflect the difficulty in detecting treatment failure due to age related differences in PK. Overall, potentially useful drugs may be perceived as either harmful or ineffective when the real issue may be one of a lack of PK data in the relevant age group to support effective treatment.

1.4.2 Better medicines

Recognition of the inequality in data between adults and children has led to the establishment of the paediatric EU legislation, the 'Best Pharmaceuticals for Children Act' and the 'Paediatric Research Equity Act' in the US and the foundation of the Medicines for Children Research Network (MCRN) in the UK (US Congress: Best Pharmaceuticals for Children Act, 2002, US Congress: Pediatric Research Equity Act of 2003, US Congress: Food and Drug Administration Amendments Act of 2007, Regulation (EC) No 1902/2006 of the European Parliament and of the Council on medicinal products for paediatric use, 2006, Medicines for Children Research Network, 2005). The aim of EU and US legislation is to improve the evidence base for drugs prescribed to children and neonates, whereas networks such as the MCRN aim to facilitate appropriate clinical studies of these drugs. According to legislative changes which came into force in Europe in 2007, marketing authorisation applications for new medicines must include data from paediatric and neonatal studies where there is potential for use. In return, pharmaceutical companies are given extended exclusivity on patent. The legislative changes also provide incentives to address existing problems with older medicines already on the market used on an unlicensed or off-label basis. This is a significant step forward to understanding drug pharmacology and improving the use of medicines in these age groups. Clinicians, academics and the pharmaceutical

industry are faced with the challenge of now performing PK and PK-PD studies to enable the licensing of age appropriate medicines.

1.5 Dried blood spots

1.5.1 Utility of the dried blood spot

DBS is a well established technique for collecting and storing blood (Guthrie and Susi, 1963). Since its use for the detection of phenylketonuria in newborns, advances in analytical capability, particularly with mass spectrometry (MS) based techniques have enabled the utilization of DBS in prospective newborn screening programmes for a range of compounds including acyl carnitines, amino acids, organic acids, thyroxine and steroids (Carpenter and Wiley, 2002, Keevil, 2011). With greater than 95% of newborns in the US included within the newborn screening programme DBS has become an important blood sampling method in the detection of treatable genetic and metabolic disorders (Mei *et al.*, 2001). DBS has been used for the detection of a variety of biomarkers in clinical and epidemiological studies including amino acids, hormones, cytokines, trace elements and vitamins (Nelson *et al.*, 1998, Butter *et al.*, 2001, Skogstrand *et al.*, 2005, Holub *et al.*, 2006, Chaudhuri *et al.*, 2009). It has also been used as a matrix for the measurement of human immunodeficiency virus, human immunodeficiency virus drug resistance genotyping, genotyping and metabolite profiling (Mwaba *et al.*, 2003, Ziemniak *et al.*, 2006, Wijnen *et al.*, 2008, Hollegaard *et al.*, 2009, Wang *et al.*, 2010, Yang *et al.*, 2010, Kong *et al.*, 2011). These applications highlight the breadth of DBS usage and the potential to study a range of compounds spanning a spectrum of low to high molecular weight.

1.5.2 Dried blood spots in drug quantification

Plasma or serum from venous blood sampling is the gold standard biological fluid for drug quantification and is reflected by the large volume of reported assays for these matrices in the literature. In comparison, there are fewer reports of the measurement of drug levels within DBS samples, although a significant increase in the use of the technique has been documented over the last few years (Li and Tse, 2010). Many of these reports stem from application to TDM through which a range of drugs including methylxanthines, anticonvulsants, antimalarials, antidiabetics, antivirals and immunosuppressants have been quantified (Coombesa *et al.*, 1984, AbuRuz *et al.*, 2006, la Marca *et al.*, 2008, Wilhelm *et al.*, 2009b, Blessborn *et al.*, 2010, Van Schooneveld *et al.*, 2010). These methods have been shown to perform to the accuracy and precision requirements of drug regulatory analytical guidelines on which plasma based assays are validated.

Understanding the application of DBS in PK studies is still in its infancy. As an emerging alternative method for performing drug quantification in PK studies, it is of particular interest to bioanalysts in the pharmaceutical industry because pre-clinical PK studies of drugs in small mammals face the same ethical and technical challenges as PK studies in neonates (Barfield *et al*., 2008). The need for large blood volumes per time point means that blood sampling must be performed using several animals. Composite sampling reduces blood loss per animal but introduces animal-to-animal variability and results in an increase in numbers of euthanized animals. A smaller sampling volume enables serial sampling from a single small animal whilst complying with the 3R's initiative which aims to reduce, refine and replace animal use in drug development research. This method also enables better quality PK data as all concentration

measurements are taken from a single animal. Development and application of DBS based methods for pre-clinical PK studies and toxicokinetic (TK) studies have been reported (Beaudette and Bateman, 2004, Barfield *et al.*, 2008, Liang *et al.*, 2009, González *et al.*, 2011, Kole *et al.*, 2011, Smith *et al.*, 2011). There are fewer applications to determine drug concentrations in clinical PK studies. Spooner *et al* reported on the development of an analytical method specifically for the measurement of paracetamol concentrations in 15 μl DBS samples (Spooner *et al.*, 2009). The developed method validated to regulatory standards was subsequently used in a PK study involving 11 healthy adult volunteers in a single dosing study with paracetamol (Spooner *et al*., 2010). This same test compound has been used to evaluate the potential of DBS in PK studies by other investigators (Mohammed *et al*., 2010). Youhnovski *et al* reported on the application of a naproxen DBS method in adult bioequivalence and PK studies (Youhnovski *et al*., 2010).

There are reports of the application of DBS methods for PK determination in paediatric and neonatal populations. The PK of topiramate has been investigated in 13 term newborns using a DBS assay requiring approximately 20 μl of blood (la Marca *et al.*, 2008, Filippi *et al.*, 2009). Suyagh *et al* developed a DBS based method for the investigation of metronidazole and reported on the subsequent application to determine the population PK of metronidazole in 32 preterm neonates (Suyagh *et al*., 2010a, Suyagh *et al*., 2011). The same investigators have also described the development and application of a DBS method for the estimation of canrenone levels in paediatric patients (Suyagh *et al.*, 2010b).

1.5.3 Dried blood spot technique

1.5.3.1 Blood sample collection, spotting and storage

In DBS sampling capillary blood is usually obtained from the heel or finger using a sterile disposable lancet. Due to the method of obtaining blood, the sample collected is a mixture of venuole, arteriole and capillary blood as well as intracellular and interstitial fluids (Blumenfeld *et al*., 1977). It has been recommended that the first blood drop containing a higher proportion of tissue fluid should be wiped away however, this practice is not standardised (Mei *et al.,* 2001). Droplets formed on the skin surface are allowed to saturate a specialised high quality cellulose based filter paper. This direct approach of blood collection has been widely adopted throughout newborn screening programmes. An alternative approach involves the collection of blood into a capillary tube containing anticoagulant. Blood is drawn up into the capillary via capillary action and then dispensed onto filter paper using a suction bulb (Spooner *et al.,* 2009). Blood may also be initially collected into a blood tube containing anticoagulant and then spotted using a pipette, but this method is associated with wastage of matrix. Whichever spotting method is selected it is important that it results in an even spreading of blood across the sample collection area. Samples must be spotted within the designated area and an even colour should be visible on both sides of the filter paper (which indicates complete saturation). Blood collected from venous and arterial sampling sites where available are equally suitable for producing DBS samples.

Collected samples must be thoroughly dried prior to storage as moisture may harm the sample by encouraging bacterial growth or altering its elution time (Mei *et al.*, 2001). A minimum drying time of 3 hours at 22°C over an open non-absorbent surface is

generally recommended, but this will vary according to the filter paper type used, temperature and humidity (Denniff and Spooner, 2010b). If necessary, DBS samples can be protected from the effects of moisture and humidity by storage in plastic sealable bags containing sachets of desiccant and humidity indicator cards.

Importantly, the DBS technique is compatible with blood collection procedures on the NICU. The simplicity of the DBS method also makes it a good candidate for collecting and storing blood for PK studies in other age groups.

1.5.3.2 Dried blood spot processing

In the first step of the analytical process a circular disc of blood of fixed diameter is punched from the centre of the DBS sample and transferred to a clean glass vial (Figure 1.1). A disc punched from a DBS sample this way provides a volumetric measurement, similar to the use of a pipette for liquid measurements. For this reason homogenous and reproducible distribution of analyte on the filter paper is of importance. It is therefore also important that a device which produces a reproducible disc diameter is used. Sample homogeneity may be affected by the type of filter paper, volume of blood spotted and analyte properties (Mei *et al.*, 2001, Clark *et al.*, 2010). The sub-sampled disc of blood is extracted with an organic solvent, most commonly acetonitrile or methanol or a mixture of organic and aqueous solvent containing an internal standard (IS) (Barfield *et al.*, 2008, la Marca *et al.*, 2008, Spooner *et al.*, 2009, Suyagh *et al.*, 2010b). Agitation, mixing or sonication may be necessary to aid analyte extraction. Sample clean-up procedures such as solid phase and liquid-liquid extraction have been used by some analysts (Liu *et al.*, 2010, Suyagh *et al.*, 2010b). Any residues arising from the filter paper can be removed via centrifugation and an aliquot of the resultant

extract is subsequently analysed using a selected detection method. A comparison of selected DBS based methods reported in the literature which have been specifically developed for application to PK or TK studies are presented in Table 1.1. A variety of filter papers and punch sizes have been employed, but the volume has been kept relatively low (15 to 50 µl) compared with earlier DBS methods used in TDM (20 to 200 µl) (Edelbroek *et al.*, 2009). Extraction procedures have commonly involved liquid extraction with minimal clean-up procedures and subsequent analysis using a MS based technique.

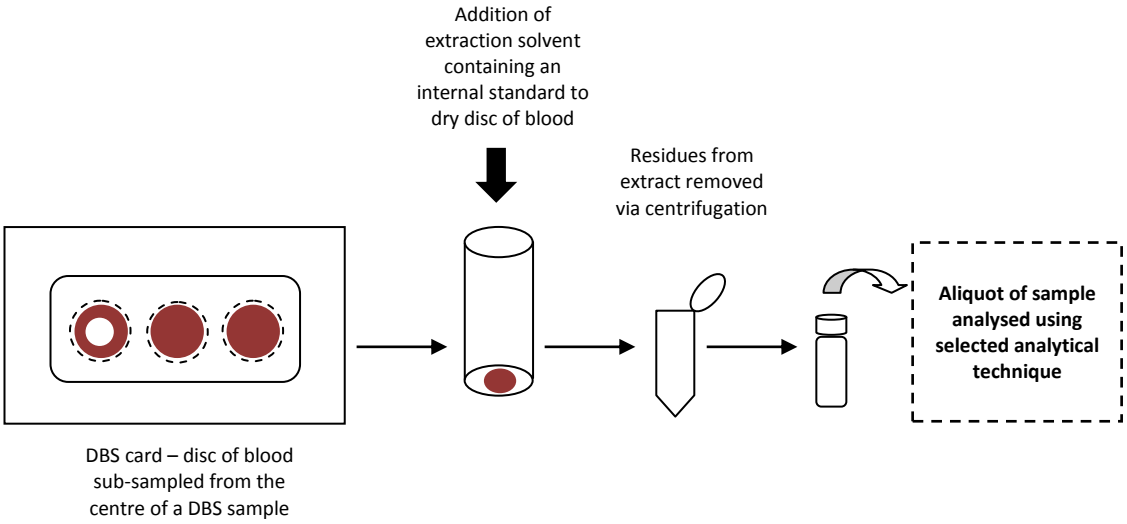


Figure 1.1 A schematic diagram of the typical DBS analytical process

The micro blood volume associated with DBS analysis is a clear advantage to PK studies in patient populations where blood sampling limitations apply. Another advantage is that many analytes are stable within DBS samples at room temperature for several weeks to months. Therefore storage is simplified and shipment of samples from clinical sites to analytical laboratories unlike plasma does not require extra-special

transport conditions (Ntale *et al*., 2008, Liang *et al*., 2009). This should make DBS a more economical sampling method. Samples in a DBS format also present less of a biohazard risk compared with liquid plasma or blood and can enhance the stability of some compounds (Bowen *et al.*, 2010, D'Arienzo *et al.*, 2010). Additionally, there is no need to centrifuge samples that is a requirement with plasma analysis thereby reducing the complexity of the blood collection process. DBS also provides a convenient way of handling small sample volumes that can be difficult with low plasma volume analysis. These factors make DBS quantification a potentially very useful method for performing multi-centre national and international PK studies as well as studies in resource limited settings (Johannessen *et al.*, 2009, Amsterdam and Waldrop, 2010). The ability to measure drug concentrations within alternative dried matrix spots such as plasma has been demonstrated (Barfield and Wheller, 2011). This enables the benefit of some of the advantages of DBS analysis and maybe useful in situations where plasma is preferred in PK studies.

Table 1.1 A comparison of selected DBS methods developed for application to pharmacokinetic and toxicokinetic studies

Author	Compound and application	Filter paper, DBS volume & punch diameter	Extraction procedure	Detection system
Liang *et al.*, 2009	Dextromethorphan Toxicokinetic	FTA Elute 50 µl, 3 mm	Solvent: 50 µl IS in acetonitrile:water (50:50, v/v) + 100 µl 2% ammonium hydroxide + 700 µl methyl tert-butyl ether. Samples vortexed (3 minutes) and centrifuged (5 minutes). 500 µl of organic layer concentrated. Residue reconstituted with 150 µl acetonitrile:water (20:80, v/v).	LC-MS/MS
Gonzalez *et al.*, 2011	Diphenylboroxazolidones Pharmacokinetic	S&S 903 40 µl, 3.2 mm	Solvent: 150 µl IS in acetonitrile:aqueous formic acid 0.1% (80:20, v/v). Samples placed in an ultra-sonic bath at 37°C for 15 minutes. Centrifugation at 12,000 x g for 5 minutes.	UPLC-MS/MS
Barfield *et al.*, 2008; Spooner *et al.*, 2009	Paracetamol Toxicokinetic / Pharmacokinetic	FTA Elute 15 µl, 3 mm	Solvent: 100 µl IS in methanol. Sample vortexed for 30 seconds and centrifuged at 3000 x g for 5 minutes.	LC-MS/MS

Table 1.1 Continued

Author	Compound and application	Filter paper, DBS volume & punch diameter	Extraction procedure	Detection system
Suyagh *et al*., 2010a	Metronidazole Pharmacokinetic	S&S 903 30 µl, 6 mm	Solvent: 25 µl IS in water + 975 µl water. Samples vortexed for 10 seconds at 10 minute interval over 30 minutes.	LC-UV
Suyagh *et al*., 2010b	Canrenone Pharmacokinetic	S&S 903 30 µl, 6 mm	Solvent: 2 ml IS in methanol. Sample shaken every 20 minutes over 60 minutes. Extract dried to a residue and reconstituted with 1 ml acetonitrile:water (10:90, v/v). Sample clean up using solid phase extraction, eluate dried to residue and reconstituted with 100 µl of mobile phase.	LC-MS
La Marca., *et al* 2008	Topiramate Pharmacokinetic	S&S 903 20 µl, two 3.2 mm discs	Solvent: 200 µl IS in acetonitrile: aqueous formic acid 0.05% (70:30, v/v). Samples shaken for 20 minutes.	LC-MS/MS

UPLC = ultra performance liquid chromatography; LC-UV = liquid chromatography ultraviolet; LC-MS = liquid chromatography mass spectrometry; LC-MS/MS = liquid chromatography tandem mass spectrometry.

1.5.3.3 Dried blood spot sampling paper

The sampling paper type Schleicher & Scheull 903 (S&S 903 or Whatman 903) is extensively used in newborn screening programmes and is classified by the US Food and Drug Administration (FDA) agency as a class II medical device. The paper is manufactured from high purity cotton linters to give an accurate and reproducible absorption of blood in accordance with the US National Committee on Clinical Laboratory Standards (Mei *et al.*, 2001). The quality of the filter paper is also tested independently by the Newborn Screening Quality Assurance Programme (NSQAP) at the Centre for Disease Control and Prevention. This provides an independent evaluation of the filter paper and importantly gives assurance that new filter paper batches perform to the standards of previous batches. The Ahlstrom grade 226 paper is an alternative FDA approved blood collection paper for newborn screening and also undergoes an independent assessment by the NSQAP. The two FDA approved filter papers have shown a comparable performance in analyte quantification (Mei *et al*., 2010).

Investigations conducted by the NSQAP to evaluate the uniformity and absorption characteristics of the filter paper involve the use of an isotopic method. Briefly, whole blood adjusted to 55% haematocrit is enriched with ^{125}I-L-thyroxine and spotted in 100 µl aliquots to the test filter paper. Once dried, a 3.2 mm disc is taken from the centre and four peripheral locations. The blood volume contained within the 3.2 mm disks is determined by measuring the total number of gamma counts in each disc and comparing them to the total number of gamma counts per unit volume of blood. The absorption time and spot size produced are also used to assess the absorption characteristics of the filter paper.

Whereas filter papers S&S 903 and Ahlstrom 226 are untreated, chemically treated cellulose papers (FTA & FTA Elute, Whatman, GE Healthcare) are also available. These papers upon contact lyse cells, deactivate enzymes and provide antimicrobial protection (Whatman FTA Elute). This paper was designed for nucleic acid and protein analysis but has also been utilised in drug quantification (Barfield *et al.*, 2008, Spooner *et al.*, 2009, Lad, 2010). Several other filter papers are available for analyte quantification of which S&S 903 is the most extensively investigated and used.

1.5.4 Analytical challenges in dried blood spot analysis

The advantages of DBS analysis over conventional plasma drug quantification methods are clear. There are however, many factors inherent to DBS analysis with potential to adversely affect drug quantification. The FDA guidelines on bioanalytical validation provide guidance on the range of tests to be carried out and the criteria which developed methods must meet to be considered suitable for drug bioanalysis. The outlined validation criteria apply equally to measurements in whole blood and plasma, but do not cover the additional factors such blood spot size and haematocrit that may need to be considered when validating the performance of DBS methods (O'Broin, 1993, Mei *et al.*, 2001). The blood collection and spotting procedure implemented are also potential sources of error. Although relatively simple to perform, experience from newborn screening programmes have highlighted that bed side errors can occur resulting in DBS samples that do not meet the minimum quality requirements for analysis. Reasons for sample rejection include multiple spotting, blood layering and insufficient sample for analysis (Guidelines for Newborn Blood Spot Sampling, UK Newborn Screening

Programme, 2008). Repeat sampling is not a viable option in PK studies where an inadequate sample would result in missing data.

The increased potential for problems in drug quantification with DBS mean that many more validation tests in addition to those already carried out for plasma are required. This significantly increases the amount of time that must be spent during the validation procedure. The time consuming process involved in DBS analysis has also been cited as a major disadvantage by some analysts. Many of these practical aspects are being overcome with better automation involving robotic punching of DBS samples and solvent addition (Wong *et al*., 2010). Investigations into the direct analysis of DBS samples with various techniques such as paper spray analysis, direct elution and direct desorption by desorption electrospray ionisation and direct analysis in real time have been reported (Abu-Rabie and Spooner, 2009, Kertesz and Van Berkel, 2010, Crawford *et al.*, 2011, Manicke *et al.*, 2011). These methods have the potential for high-throughput analysis with minimal sample handling.

The micro blood volume used in DBS analysis and the consequent need for higher detection sensitivities is likely to have contributed to its limited use outside the context of newborn screening programmes. Improvements in analytical instruments particularly with MS have increased the feasibility of drug analysis from low volume sampling (Zhang *et al*., 2008). Despite these advances, the sensitivity requirements with DBS sampling may mean that such an approach is less viable for some drugs at present.

1.5.5 Blood concentration measurements for pharmacokinetic evaluations

Conventionally, plasma measurements have been used to define the concentration-time profile of a compound, estimate PK parameters and provide an assessment of drug

exposure and safety. It is usually the unbound fraction of drug in plasma that is available to exert a pharmacological effect and this forms the basis for another important reason for its preference to whole blood drug concentration measurements (Rowland and Emmons, 2010). Regulatory guidelines stipulate drug exposure may be represented by concentration measurements in plasma, serum or whole blood matrices (ICH Harmonised Tripartite Guideline, 1994). Therefore the use of DBS is a valid matrix for determining drug PK. However, before it is possible for DBS to become a routine method in PK studies it will be necessary to ascertain the robustness of the technique through clinical validations. The problems which can occur in PK interpretation when using blood concentration measurements over plasma measurements have been highlighted (Rowland and Emmons, 2010). These are most likely to occur for drugs which show preferential distribution (into plasma or red blood cells) and where a large variability in factors which govern the partitioning process e.g. protein binding and haematocrit are expected in the study population. Therefore it will be important to consider the implications and appropriateness of DBS sampling within the context of planned use.

1.6 Bioanalysis of dried blood spots

The micro volume blood sample available for analyte quantification and complexity of the sample matrix pose several challenges for DBS analysis. For these reasons liquid chromatography with single quadrupole mass spectrometry (LC-MS) and more often tandem mass spectrometry (LC-MS/MS) quantification techniques have been preferred due to their inherent sensitivity and specificity. High resolution MS has the advantage of increased compound selectivity and has been applied to the quantification of drugs in

DBS samples (Tanna *et al.*, 2011). Other analytical techniques including LC with ultraviolet (UV) or fluorescence detection and radioimmunoassay (RIA) have also been used in DBS analysis with success (Neese and Sovka, 1977, Croes *et al.*, 1994, Mei *et al.*, 1998, AbuRuz *et al.*, 2006). The final choice of detection system will depend on the properties of the compound under study as well as the required level of detection sensitivity.

Detection methods used in the UK newborn screening programme have included immunoassay, LC-MS/MS and LC-UV depending on the condition screened (Pollitt *et al.*, 1997, Venditti *et al.*, 2003).

1.6.1 Liquid chromatography single quadrupole mass spectrometry

Mass spectrometry based techniques have been used in various fields including quantitative bioanalysis for PK studies, drug metabolite analysis, proteomics, and pharmaceutical degradation and stability testing (Lim and Lord, 2002). A variety of instruments are available with capabilities of performing qualitative and quantitative analyses to various levels. The simplest instrument is the LC-MS consisting of a liquid chromatography unit attached to a mass spectrometer via an interface which provides compatibility between the two techniques. In the first stage of analysis an aliquot of sample mixture is injected into a continuous flow of liquid mobile phase which is carried to a reversed phase column packed with bonded silica particles. The rate at which a given compound travels through the column is governed by its polarity and experimental conditions which include mobile phase composition and column properties (Snyder *et al.*, 1997). Compounds with little affinity for the stationary phase will travel at essentially the same rate as the mobile phase. Using these principles, LC is

used to achieve separation of a mixture of components. Compounds eluting from the column are carried by the mobile phase to the ionisation interface for analysis by MS.

1.6.1.1 Ionisation interfaces for mass spectrometry

Atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) are two of the most popular interfaces used in LC-MS and can be used to study a broad range of compounds (Kostiainen *et al.*, 2003). ESI techniques are useful for studying small to large molecules that become multiply charged $[M+nH]^{n+}$ including proteins to 100,000 daltons and above (Polettini, 2006). During ESI a strong electrical field is applied to the eluant from the LC column as it passes through a nebulizer needle. This creates an accumulation of charged liquid at the end of the needle which leads to the formation of highly charged droplets. The charged droplets are attracted toward the capillary sampling orifice through a counter flow of heated nitrogen gas, which shrinks the droplets and carries away uncharged material. The droplet size decreases until the surface tension cannot stabilize the charge at which point an explosion occurs resulting in the formation of charged molecular ions $[M+H]^{+}$ with little or no fragmentation (Agilent Technologies LC-MSD software operation, 2000).

In APCI a gas phase chemical ionisation process is used to ionise the sample. The process begins with gas mediated nebulisation into a hot (250 to 400°C) vaporizer chamber resulting in rapid evaporation of spray droplets (Agilent Technologies LC-MSD software operation, 2000). The gas phase (vaporized) solvent and analyte molecules produced are ionised by the discharge from a corona needle which serves to initiate the ionisation process. Here the solvent plays an important role in the ionisation process by transferring a proton to the analyte provided the proton affinity of the analyte

is greater than that of the solvent. This ionisation process usually produces a singly charged molecular ion $[M+H]^+$. Like electrospray, APCI is a soft ionisation technique but may result in a greater degree of fragmentation. APCI is only used for samples that can be vaporized and is particularly useful in the study of intermediate molecular weight and polarity compounds (Agilent Technologies LC-MSD software operation, 2000).

1.6.1.2 Ion transmission

All charged ions produced at the interface enter the mass spectrometer through the capillary sampling orifice (Figure 1.2) and are transmitted through a narrow glass capillary. An octopole is used to guide ions towards a quadrupole mass filter consisting of four parallel rods. Specific voltages are applied to these rods to filter out the ion of interest. The selected ions follow a trajectory towards the detector which creates a signal that is represented by a LC-MS chromatogram. The integrated peak area or height is proportional to the amount of analyte present and is used to quantify its concentration within a given sample. This mode of data acquisition is referred to as single ion monitoring (SIM). By varying the voltage applied it is also possible to monitor multiple ions from a sample and perform scans across a mass range for qualitative purposes.

1.6.2 Liquid chromatography triple quadrupole mass spectrometry

A triple quadrupole mass spectrometer consists of two quadrupole mass filters (Q1 and Q3) separated by a collision cell (Figure 1.3). Like LC-MS, an ESI or APCI interface is commonly used for sample ionisation. Here the first quadrupole is used to direct the molecular ion (precursor) of interest to the collision cell. Within this cell ions collide with an inert gas such as nitrogen or helium to induce fragmentation of the precursor ion (Barker, 1999). The degree of fragmentation which occurs is dependent on the

compound type and the fragmentor voltage applied (Takino *et al.*, 2003). A second quadrupole is used to separate and direct selected product (fragmentation) ions to the detector. This mode of operation is termed selected reaction monitoring, SRM. The product ions are pieces of the precursor and are therefore representative of the overall structure of the precursor ion. There are various types of instruments which perform MS/MS analysis, all however encompass an isolation and fragmentation process within the instrument. For drug quantification purposes, monitoring a product ion (SRM) compared to SIM in LC-MS confers additional assurance that the selected ion for monitoring represents the compound of interest. Additional specificity is gained through the ability to monitor a fragmentation pathway which is dependent on compound structure. This can lead to improved detection sensitivity for some compounds. The ability to fragment ions has also played a very important role in drug metabolic studies, an essential component of the drug development process (Want *et al.*, 2005).

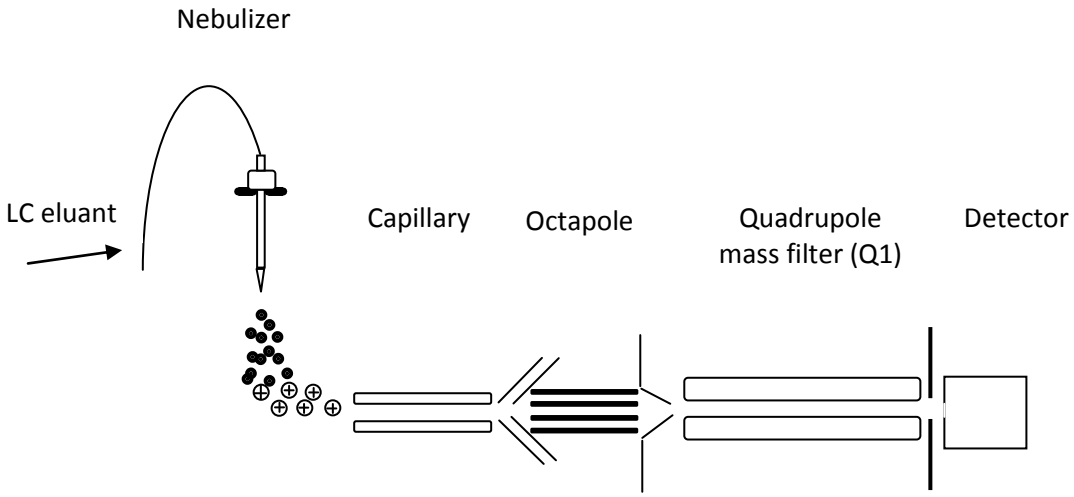


Figure 1.2 A schematic of a single quadrupole mass spectrometer

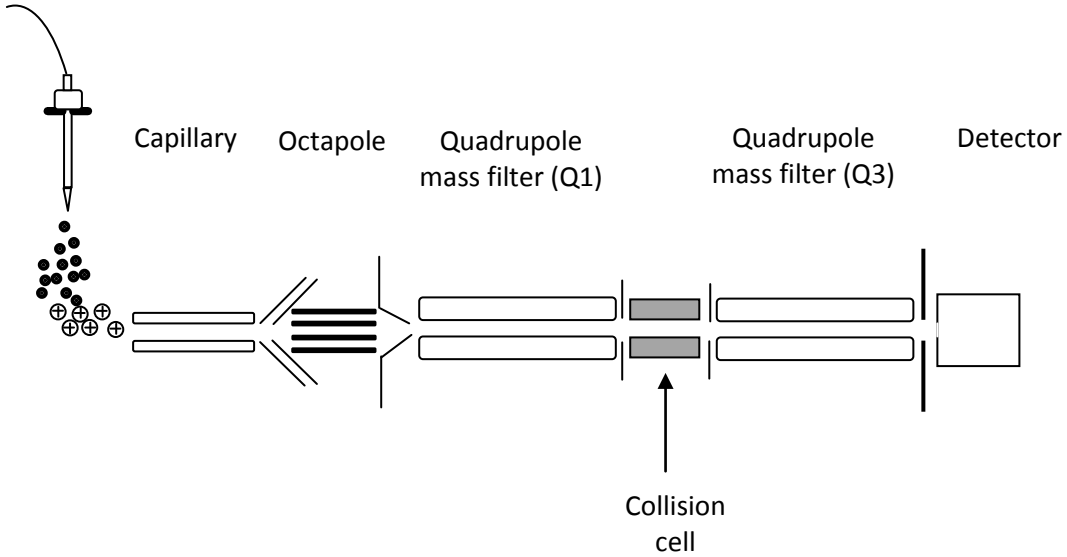


Figure 1.3 A schematic of a triple quadrupole mass spectrometer

1.7 Dexamethasone in the treatment of chronic lung disease of the newborn

Dexamethasone is amongst the most potent glucocorticosteroids used in clinical practice (British National Formulary, 2009). It is mainly used for the treatment of inflammatory conditions, allergic disorders, cerebral oedema and as an antiemetic with chemotherapy. The drug is also used to induce foetal lung maturation in women at risk of preterm labour and in the treatment of chronic lung disease in neonates (Brownfoot *et al.*, 2008, Halliday *et al.*, 2010).

1.7.1 Chronic lung disease of the newborn

Chronic lung disease (CLD) of the newborn predominantly affects premature neonates and is a significant and perenial contributor to the morbidity and mortality observed in this population (Jobe and Bancalari, 2001, Kinsella *et al.*, 2006). Though there are modern variations in the specific time frame, it has customarily been defined by the need for supplementational oxygen at 36 weeks postconceptual age, with persistant respiratory symptoms accompanied by an abnormal chest radiograph. The incidence of CLD amongst ventilated newborns is approximately 25% and the risk of developing CLD is strongly associated with the degree of prematurity with 75% of all cases occuring in newborns weighing less than 500 g compared with 5% in those above 1500 g (Kinsella *et al.*, 2006). The development of CLD has also been linked to mechanical ventilation and oxygen therapy for the treatment of respiratory distress syndrome and infection, which may have additive or synergistic roles in disease progression (Kinsella *et al.*, 2006). There is substantial evidence to suggest that an inflammatory response is central to the development of CLD (Kotecha *et al.*, 1996, Ramsay *et al.*, 1998, Kazzi *et al.*, 2001, Beresford and Shaw, 2002). Elevated levels of acute pro-inflammatory

cytokines IL-1β, IL-1α, IL-6 and IL-8 have been detected in lung lavage fluid during the first few postnatal days in infants who go on to develop CLD. These acute phase pro-inflammatory mediators are markedly increased during the first 2 to 3 postnatal weeks and remain elevated for several weeks thereafter. A combination of underdeveloped lungs, continuous injury and a reduced defence capacity is thought to explain the observed persistant inflammatory state (Jobe and Bancalari, 2001). Following the initial acute inflammtory response pro-inflammatory cytokines trigger a cascade of inflammatory events which lead to the release of enzymes responsible for lung remodeling (Sweet and Halliday, 2005). Profound structural lung changes have been observed in infants with CLD resulting in reduced respiratory function. Respiratory symptoms persist for two years after birth and may continue into adolescence (Kinsella *et al.*, 2006). Treament most often necessitates regular medication and frequent re-admissions to the hospital, which can be distressing for the young patient, parents and carers.

1.7.2 Treatment with dexamethasone

Dexamethasone has been widely used to oppose the pro-inflammatory state observed in premature neonates either developing or with CLD (Lemons *et al.*, 2001). Despite extensive usage it remains to be prescribed on an off-label basis on the NICU. Several studies have shown that dexamethasone treatment acutely improves lung mechanics and reduces the incidence of CLD, but is associated with short term complications (Halliday *et al.*, 2009a, Halliday *et al.*, 2009b, Halliday *et al.*, 2010). These include growth retardation, intestinal perforation, hypertension and hyperglycaemia requiring insulin therapy (Anderson *et al.*, 2001, Halliday *et al.*, 2010). Concerns over long term

neurodevelopmental impairment namely cerebral palsy led to joint guidelines from the European Association of Perinatal Medicine in 2001 and the American Academy of Pediatrics and the Canadian Paediatric Society in 2002 on the use of postnatal steroids for the treatment of CLD (Barrington, 2001, Halliday, 2001). The guidelines concluded that the routine use of systemic corticosteroids for the prevention or treatment of CLD in infants with very low birth weight is not recommended outside clinical trials 'except in exceptional circumstances'. Although use of dexamethasone has declined over the years, a substantial proportion of very low birth weight infants continue to be treated with postnatal steroids (Walsh *et al.*, 2006).

The doses used in the treatment of CLD have been empirically based. Initial investigative trials used high dexamethasone doses for prolonged periods with subsequent trials gradually using smaller doses for shorter periods. The dose in trials to date has ranged from 0.05 - 0.5 mg/kg per day (Cummings *et al.*, 1989, Yates and Newell, 2010). There is some evidence of a dose-response relationship of dexamethasone in CLD with higher doses more effectively reducing the incidence of CLD (Onland *et al.*, 2008). However, studies within which high versus low dosing regimens have been investigated are generally too small to draw any firm conclusions. Despite several trials, incorporating various dosing schedules, the optimum dexamethasone dosing regimen remains unknown. There is no consensus on the dose that should be used in clinical practice and at present dosing regimens vary significantly between NICUs. At the local Leicester NICU dexamethasone is initiated at 250 μg/kg 12 hourly and gradually reduced to 25 μg/kg 12 hourly over 10 days.

A major problem with the dose finding approach that has been used is that it does not take into account the disposition of dexamethasone in neonates. An initial study exploring the PK-PD relationship of dexamethasone would provide an efficacious dose range around which to design a much more efficient and possibly more conclusive trial. However, there are only two studies involving 16 patients reported on the PK of dexamethasone (Charles *et al.*, 1993, Lugo *et al.*, 1996). With limited PK data it is not possible to derive dosing information which take into account the variability which exist in infants between 24 and 36 weeks gestation.

The issue of postnatal steroid use is further complicated by a meta-analysis suggesting the beneficial effects of dexamethasone in premature infants at high risk of developing CLD (> 50%) may actually outweigh the risks of therapy (Doyle *et al.*, 2005). Therefore a proportion of premature neonates may be unfairly denied the beneficial effects of steroidal anti-inflammatory drugs based on disproportionate fears over adverse long term neurodevelopmental outcomes. This poses a dilemma for neonatologists whose primary objective is to arrest disease progression. The decision to treat CLD with dexamethasone may result in the development of neurodevelopment complications, whereas the decision to withdraw steroid treatment will lead to the progression of CLD and possibly death. Finding the optimum dose whilst minimising adverse events is now considered to be of great clinical importance.

1.7.3 Dexamethasone pharmacokinetics

The oral bioavailability of dexamethasone has not been studied in neonates, but the results from a healthy adult study suggest extensive absorption (~ 90%) with peak blood levels reached 1 hour after dose ingestion (English *et al.*, 1975). In both previous PK

studies reported in neonates a non compartment analysis was used to calculate estimates of PK parameters following intravenous (IV) dosing. The first study investigated the PK of dexamethasone after the administration of a single 400 μg/kg IV dose to 7 premature neonates with an average gestational age (GA) of 25.6 weeks (Charles *et al.*, 1993). Serial blood samples of 100 to 200 μl were collected at 0.5, 1, 2, 6, 12 and 24 hours after dosing and plasma concentrations were determined using LC-UV detection. The mean V and CL reported were 1.9 l/kg and 0.14 l/h/kg, respectively, and the half life was 9.3 h (range, 5.9 to 16.1 h). In the second study dexamethasone PK was investigated in 9 premature neonates with an average GA age of 27.3 weeks (Lugo *et al.*, 1996). Patients were recruited within 4 days of initiating treatment and received between 213 to 503 μg/kg of dexamethasone 12 hourly. Blood samples were collected at 0.5, 1, 2, 6, and 12 hour intervals after dosing and plasma concentrations were measured using a RIA method. Dexamethasone concentration measurements in plasma following a 250 μg/kg dose varied between 3.4 and 406.9 ng/ml and therefore significantly lower therapeutic levels would be expected in neonates during the course of treatment at the Leicester NICU. The mean estimates for V and CL were 1.7 l/kg and 0.30 l/h/kg, respectively. Similar to the findings of Charles *et al* the half life varied considerably between neonates (mean 6.8, range; 2.2 to 15.8 h). In comparison, mean half lives of 4.3 and 2.9 hours have been reported in children and adults, respectively (Tsuei *et al.*, 1979, Richter *et al.*, 1983). There is a limited understanding of the elimination pathway of dexamethasone in neonates, but a prolonged half life due to immature drug clearance pathways are likely to have contributed to the differences in half life reported. Further PK studies are urgently required to explore and characterise the variability in dexamethasone PK amongst the neonatal population.

1.8 Caffeine in the treatment of apnoea of prematurity

Caffeine is a methylxanthine drug primarily used as a respiratory stimulant in preterm neonates with idiopathic apnoea. It is also used to facilitate extubation in ventilated infants (British National Formulary for Children, 2009). Caffeine is associated with fewer adverse effects and has a wider therapeutic index compared with theophylline which belongs to the same class of drug. This has underpinned the preference of caffeine in the treatment of apnoea on many NICUs (Henderson-Smart and Steer, 2010).

1.8.1 Apnoea of prematurity

Apnoea of prematurity (AOP) is defined by the cessation of breathing for greater than 15 seconds with bradycardia and hypoxaemia (Schmidt *et al.*, 2006). The episodes of apnoea manifest due to immaturity of the centres of the brain which regulate breathing. It is a significant problem affecting approximately 85% of preterm neonates born at less than 34 weeks GA.

1.8.2 Treatment with caffeine

Caffeine is used as a first line treatment in AOP and has been shown to reduce the number of apnoaeic episodes and the need for mechanical ventilation during the first 7 days of treatment (Henderson-Smart and De Paoli, 2010). In infants with apnoea caffeine is believed to work through a combination of effects including stimulating the central respiratory centre, promoting fluid loss, decreasing the carbon dioxide threshold, increasing cardiac output and increasing the response to hypercapnia (Hascoet *et al.*, 2000, Mathew, 2011). AOP resolves as the breathing centres which control respirations mature, enabling discontinuation of caffeine treatment. Most neonates will receive

caffeine treatment until 34 to 35 weeks postmenstrual age (PMA) when the risk of apnoea is low.

Several investigations have been performed in neonates to evaluate caffeine safety and PK (Walther *et al.*, 1990, Thomson *et al.*, 1996, Ergenekon *et al.*, 2001). This has led to the development of a licensed caffeine citrate formulation (Peyona, Chiesi Farmaceutici SpA) approved by the Committee for Medicinal Products for Human Use (CHMP) in the treatment of AOP. The dosing regimen currently used in the UK consists of an initial loading dose of 10 to 20 mg/kg (caffeine base), followed by a once daily dose of 2.5 to 4 mg/kg (Natarajan *et al.*, 2007). Caffeine plasma concentrations between 5 and 20 mg/l are considered to achieve therapeutic effectiveness. However, in infants who continue to have apnoeas, it may be reasonable to target a much higher therapeutic range of around 30 to 40 mg/l (Lee *et al.*, 1997).

1.8.3 Caffeine pharmacokinetics

In neonates, caffeine is extensively and rapidly absorbed with bioavailability approaching 100% (Charles *et al.*, 2008). A significantly reduced CL capacity has been observed in this population (7.0 ml/h/kg) compared with non-smoking adults (124 ml/h/kg) resulting in a markedly prolonged half life (101 *vs.* 3.9 h) (Kaplan *et al.*, 1997, Lee *et al.*, 1997, Charles *et al.*, 2008). This is thought to be due to a maturational delay in the expression of cytochrome P450 enzymes responsible for the metabolism of caffeine (Aldridge *et al.*, 1979). The rate of caffeine CL increases progressively with postnatal age (PNA), with a transition to adult levels of elimination occurring at 3 to 5 months of age (Aranda *et al.*, 1979a). In contrast to dexamethasone, the PK of caffeine in neonates has been well characterised and several published population studies are

reported in the literature (Thomson *et al.*, 1996, Falcão *et al.*, 1997, Lee *et al.*, 1997, Lee *et al.*, 2002, Charles *et al.*, 2008).

1.9 Population pharmacokinetics

In traditional PK study designs healthy volunteers or specially selected patients are chosen for study to ensure a homogenous treatment group. This enables the study of drug disposition under controlled conditions and calculation of precise parameter estimates but ignores the biological variability that may exist in the treatment population for which the drug is intended (Ludden, 1988, Ette and Williams, 2004a). Population PK seeks to characterise and explain the variability in PK parameters within a group of individuals which are being clinically treated with the drug of interest. A range of factors can alter the dose-concentration relationship including body weight, renal and metabolic function and concomitant medication. Through population PK studies it is possible to quantify the extent of the influence of factors which alter the dose-concentration relationship. This information can then be used to develop a population model which predicts the dosage requirement or optimum dose for an individual. Such information provides guidance to the clinician for selecting an appropriate dosing regimen particularly where clinically significant changes in therapeutic outcome are observed (Guidance for Industry: Population Pharmacokinetics, 1999). PK models are an oversimplification of complex biological processes. Therefore although a population model may be useful, none are perfect and the estimated dosage requirement in an individual will vary from optimal by some extent. The study of the differences between model predicted and observed concentrations is thus a crucial component of the population PK model. The magnitude of the unexplained (random)

variability between model predicted and observed concentrations is important because the safety and efficacy of a drug may decrease as unexplainable variability increases. Sources of variability contributing to the goodness of fit between a PK model and the observed data can be categorised as interindividual and intraindividual. A PK parameter derived for an individual may vary from the population mean estimate and this difference is considered the interindividual variability (or between subject variability). Intraindividual variability (also known as residual unexplained variability or residual error) is the difference between the individual model predicted and observed (or measured) concentration values over a given time. Factors contributing to residual error include inexplicable changes in individual pharmacokinetics between dosing intervals (interoccasion or between occasion variability), errors in drug measurement, documentation errors and model misspecification. Population PK modeling aims to explain as much of the interindividual variability as possible in order to (i) differentiate information from the noise or random components in the system and (ii) improve the predictive properties of the model through a reduction in the residual unexplained variability (Guidance for Industry: Population Pharmacokinetics, 1999).

The major advantage of the population PK approach is that unlike traditional PK studies it is compatible with sparse sampling, unbalanced numbers of samples from patients and random sample collection. This approach therefore lends itself well to special populations such as neonates, the elderly and critically ill, where sample collection can be challenging and often opportunistic.

1.10 Approaches in population pharmacokinetic parameter estimation

There are a number of population PK methods that can be used to characterise the kinetics of a drug (Ette and Williams, 2004b). The approach selected will depend on the objectives of the PK analysis and the study design.

1.10.1 Naive average data approach

In this approach the dosing regimen and sample collection are identical for all subjects. The number of data points for each sampling interval is therefore equal to the number of subjects. The first step involves averaging the value of the data at each sampling time, which is possible since samples were collected under identical conditions. A model is then fitted to the averaged concentration data to determine the best-fit PK estimates which may be interpreted as mean parameter values. Using this approach it is not possible to measure the random intraindividual variability component and the observed interindividual variability is a result of all sources of error. Therefore it is not possible to determine the true interindividual variability and limits application of this method in PK data analysis.

1.10.2 Naive pooled data approach

All concentration data measured from all patients are treated as though they were collected from the same individual. A model is fit to the pooled data and population PK estimates are obtained. An imbalanced number of concentration data points from individual subjects can lead to the derivation of biased PK parameter estimates. This approach may be useful when the expected variations between subjects are small. However, as with the naive average data approach interindividual variability is

confounded and as a result the variance between individuals cannot be reliably estimated.

1.10.3 Standard two-stage approach

With this approach, dense concentration-time data from each subject is separately fitted using least squares to give individual estimates in the first stage. The individual estimates are combined in the second stage and standard statistical calculations are performed to determine population mean parameters and their variances. This approach offers a simple method of pooling individual parameter estimates but requires a large number of concentration data points for analysis and an equal contribution of data from each individual. A standard two-stage approach therefore does not lend itself to areas such as paediatrics where the collection of data is often limited by sparse sampling design. Although all individuals are allowed to contribute equally with this approach it is not possible to differentiate between interindividual and intraindividual variability. The random effects (variance) calculated for each parameter estimate represents all sources of error (between occasion variability, measurement error, and interindividual). Hence this method tends to overestimate the true variability of a given parameter in a population.

1.10.4 Non-linear mixed effects model approach

This is the standard population approach and can be used for the analysis of sparse, imbalanced, unstructured, and fragmented observational PK data (Sheiner *et al.*, 1977). It may also be used to analyse data following extensive sampling, alone or in combination with sparse data. Similar to the naive pooled method, concentration-time data from all patients is analysed in a single stage, but takes into account that different

data points came from different subjects. Therefore with this approach individuality of each subject is maintained and accounted for, even if the data are sparse.

A mixed effects population PK model provides estimates of population mean values (fixed effects) from the full set of individual concentration values and their variability within the population (random effects). The random effects encompass interindividual variability and intraindividual variability. Importantly, with this approach it is possible to model and estimate the magnitude of different sources of variability. In the final stage of the analysis, the individual parameters are regarded as random variables and the probability distribution of these (often the mean and variance, i.e., intersubject variance) is modelled as a function of individual-specific covariates (Guidance for Industry: Population Pharmacokinetics, 1999). The timing of sample collection is an important consideration in population PK study design, particularly when there are limitations in patients available for recruitment as well as the number of samples that can be collected from each individual. In this situation there are sampling strategies that can be implemented to ensure an informative study design. The collection of trough samples, obtained shortly before the next dose is useful for estimating CL. Peak concentration times may be targeted in situations where it is important to determine V. A full population sampling approach involves the collection of multiple blood samples (1-6) from each individual at various times following a dose (Sheiner and Beal, 1983). Provided samples are collected at a range of time points from a sufficient number of patients it is possible to accurately and precisely estimate a range of PK parameters including CL, V and half life and study there variability in the population. A sample size of 30 to 50 patients (approximately 180 samples) has been recommended for the precise estimation of primary PK parameters (Ette *et al*., 1998, Anderson *et al*., 2007).

The appropriate number of patients for a population study will vary depending on the objectives of the study, the age range of the study population and the number of covariates under investigation.

1.10.5 Computer software programme for analysis

A number of software programmes are available for performing population PK analyses but for the purposes of this thesis NONMEM (non-linear mixed effects modeling) version 7.0 was used to analyse all drug concentration-time data. Like most non-linear mixed effects modeling approaches, population PK parameter estimation in NONMEM is based on the maximum likelihood approach. NONMEM uses a maximum likelihood criterion to provide the 'best fit' of the data inputted. Estimates for population mean values of fixed effects parameters (CL, V and coefficients for covariates which influence either of these PK parameters) and the variances of random effects parameters (interindividual, intraindividual and interoccasion) are available in the NONMEM output. The correctness of the model cannot be determined from the NONMEM output. NONMEM however, does give the weighted residuals for each data point and generates standards errors associated with estimates which gives the modeller an indication of the precision with which population values were estimated (NONMEM Users Guide - Part I, 1989, Beal and Sheiner). It also calculates the objective function value (-2 log likelihood, -2LL) which is a metric of the goodness of fit of a model (Fisher/Shafer NONMEM Workshop, 2007). For two models based on the same data set the one that gives the lowest objective function value is the most statistically preferred. Constructing scatter plots of the data set prior to NONMEM analysis is an essential component of the PK analysis. The process enables the visual detection of imputation errors or outliers

with potential to bias parameter estimation at an early stage. They are also useful for identifying important covariate-parameter relationships.

1.10.6 Assessment of goodness of model fit

The difference in objective function value (OFV) is an important statistical test when choosing between two nested models, but it should be accompanied by other selection criteria during the model building process as the best model is not necessarily the one with the lowest OFV. The standard error and covariance matrix associated with the estimation of parameters gives an indication of the estimation process itself. Therefore a model with a higher OFV that more precisely estimates parameters may be preferred to a model which provides a statistically better fit with less precision. Precision of coefficients and exponents used to quantify covariate effects on PK parameters are also important to consider when deciding between models. The examination of weighted residuals forms an essential part of the model evaluation process. Residuals should be randomly distributed with no systematic deviations from predicted model concentrations. Graphical plots of residual values versus model predicted and time are a useful diagnostic for the detection of underlying problems within the structural model. This type of residual analysis also provides important information on the suitability of the chosen residual error variance model.

1.11 Study aims

The investigations described in this thesis were undertaken to clinically validate the robustness of DBS quantification methods for application to PK studies, with specific focus in neonates. Aims of the first stage were to develop and validate DBS methods for the test drugs dexamethasone and caffeine. In the proceeding stage *in-vitro* validated

methods were used to determine circulating drug concentrations in patients. Individual and population based PK studies were performed in preterm neonates for dexamethasone and caffeine, respectively to assess the reliability of the DBS technique for the estimation of PK parameters. The PK of caffeine was also determined in healthy adults. Clinical validations were facilitated through a comparison of DBS derived PK parameter estimates (CL, V and half life) with literature reported plasma estimates for agreement. The special ethical considerations around blood collection that apply to neonates underpinned the chosen method for performing the DBS and plasma matrix comparisons. Clinical investigations were also considered important for allowing an assessment of the feasibility of DBS sampling applied to PK studies in a clinical environment such as the NICU.

The decision to investigate dexamethasone was made following extensive discussions with study clinicians and on the assumption of sufficient patient numbers to perform a clinical validation. Investigations with dexamethasone were considered particularly important due to the current clinical need for PK data in neonates. A second test drug, caffeine, was selected to enable a more extensive investigation of DBS analysis in PK studies.

Chapter 2

Development of a Dried Blood Spot Method for the Quantification of Dexamethasone

2.1 Introduction

2.1.1 Dexamethasone quantification in biological fluids

A variety of analytical techniques have previously been used for the quantification of dexamethasone in biological fluids including LC with UV, fluorescence, chemiluminescence or MS detection, gas chromatography with mass spectrometry (GC-MS) and RIA (Ishida *et al.*, 1993, Katayama *et al.*, 1993, Schild and Charles, 1994, Lugo *et al.*, 1996, Hidalgo *et al.*, 2003, Qu *et al.*, 2007). A comparison of selected techniques reported in the literature is presented in Table 2.1. An LC-UV assay requiring a micro volume blood sample whilst maintaining a comparatively good detection sensitivity (15 ng/ml) has been reported (Schild and Charles, 1994). Most previously reported LC-UV assays have however, required a large blood volume sample (≥ 1 ml) to attain a higher degree of sensitivity or have relatively high quantification limits rendering the respective methodologies inappropriate for application to neonatal PK studies. Detection of levels as low as 0.6 pg/ml from a 100 µl plasma sample has been achieved by Katayama and co-workers using LC with fluorescence detection however, this method necessitates pre-column derivatisation of dexamethasone with a fluorescent compound that is not commercially available (Katayama *et al*., 1993). The need to synthesise a fluorescent tag via a complex series of reactions along with a resulting long column retention time makes the method practically less viable. Although GC-MS confers high sensitivity it is also associated with a lengthy pre-column derivatisation step (approximately 3 hours) and involves handling toxic reagents. LC-MS and LC-MS/MS have been used to quantify dexamethasone with the outcome of low detection limits without the need for time consuming derivatisation steps, however,

the majority of MS based methods have required a large blood volume sample (1 - 10 ml) for analysis. Both ESI and APCI techniques have been used to quantify dexamethasone in a variety of biological fluids. Recently an LC-MS/MS dried matrix spot method for the quantification of dexamethasone in bovine synovial fluid was described in the literature (Christianson *et al*., 2010). Requiring only a 15 µl sample a lower limit of quantification (LLOQ) of 5 ng/ml was achieved. RIA techniques have conferred good sensitivity from relatively small blood samples, but a significant degree of cross-sensitivity is observed with other corticosteroids and metabolites of dexamethasone which compromises its specificity, and thus accuracy (Hochhaus *et al.*, 1992, Lugo *et al.*, 1996).

The practice of removing serial large blood volumes for research purposes in neonatal and paediatric populations is now generally unacceptable. The EMA have provided guidance on the blood sampling volumes appropriate in research studies involving term and preterm neonates (Guidelines on the investigation of medicinal products in the term and preterm neonate, 2007). However, these guidelines are not evidenced based and therefore extra caution is necessary in blood sampling from vulnerable populations such as preterms where there is a recognised risk of sampling induced anaemia (Weiss *et al*., 2002). The blood volume requirement should be kept to a minimum during the development of drug assays. This will also increase the feasibility of opportunistic sample collection i.e. immediately after blood has been collected for clinical tests. Reported dexamethasone assays have either required large blood volumes or are associated with time consuming and complex work-up procedures. A simple micro analytical method tailored to the measurement of dexamethasone concentrations in neonates is therefore required in order to investigate its PK in this population.

Table 2.1 A comparison of selected analytical techniques reported for the quantification of dexamethasone in biological fluids with sensitivity expressed as either the lower limit of quantification (LLOQ) or limit of detection (LOD)

Reference	Analytical technique	LLOQ w/v	LOD w/v	Volume & type of biological fluid
(Lugo *et al*., 1996)	RIA	1 ng/ml		0.1 ml plasma
(Girault *et al*., 1990)	GC-MS		0.1 ng/ml	1 ml plasma
(Hidalgo *et al*., 2003)	GC-MS		0.2 ng/ml	Not specified urine
(Plezia and Berens, 1985)	LC-UV		5 ng/ml	2 ml plasma
(Schild and Charles, 1994)	LC-UV	15 ng/ml		0.1 ml plasma
(Grippa *et al*., 2000)	LC-UV	250 ng/ml		1 ml plasma
(Song *et al*., 2004)	LC-UV	10 ng/ml		1.5 ml plasma
(Kumar *et al*., 2006)	LC-UV	250 ng/ml		0.15 ml plasma
(Katayama *et al*., 1993)	LC-Fluorescence		0.6 pg/ml	0.1 ml plasma
(Wu *et al*., 1995)	LC-Fluorescence	1.57 ng/ml		0.2 ml plasma
(Ishida *et al*., 1993)	Chemiluminescence	15 ng/ml		0.2 ml plasma
(Chen *et al*., 2002)	LC-MS/MS	0.25 ng/ml		0.5 ml plasma
(Taylor *et al*., 2004)	LC-MS/MS	0.3 ng/ml		0.5 ml plasma
(Luo *et al*., 2005)	LC-MS/MS	0.1 ng/ml		1 ml plasma (equine)
(Qu *et al*., 2007)	μLC-MS/MS	5 pg/ml		1 ml plasma
(Christianson *et al*., 2010)	LC-MS/MS	5 ng/ml		15 μl synovial fluid (dried matrix spot)
(Zhang *et al*., 2011)	LC-MS/MS	0.5 ng/ml		50 μl plasma
(Damonte *et al*., 2007)	LC-MS	5.9 ng/ml		10 ml blood

2.1.2 Steroid quantification using dried blood spots

Several endogenous steroids have been successfully studied using DBS which supports the investigation of the technique for the measurement of synthetic steroidal compounds such as dexamethasone. The use of DBS analysis for the quantification of 17α-hydroxyprogesterone (17-OHP) was proposed in 1977 for the diagnosis of congenital adrenal hyperplasia (CAH) in neonates (Pang *et al.*, 1977). The test has now become an essential component in newborn screening programmes. Early 17-OHP quantification methods were based on immunological assays which resulted in cross-reactions of specific antigens with other steroids thereby producing a large number of false positive results (Wong *et al.*, 1992). To improve the selectivity of the DBS screening method and reduce the number of false positive results LC-MS/MS techniques were subsequently evaluated for the quantification of 17-OHP (Lai *et al.*, 2002). Preterm neonates may exhibit elevated levels of 17-OHP because of stress or delayed maturation of 11-hydroxylase which represents an additional contributory factor to the rate of false positive results. In order to improve the specificity of the screening process for CAH, second tier DBS techniques based on MS/MS detection were developed which enable the simultaneous measurement of multiple endogenous steroids important in the diagnosis of CAH including 17-OHP, cortisol, corticosterone, deoxycorticosterone, progesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, testosterone and dihydrotestosterone (Janzen *et al.*, 2008). Outside newborn screening programmes, DBS has been used for the measurement of testosterone and estradiol in steroid pharmacology and biobehavioural studies (Howe and Handelsman, 1997, Shirtcliff *et al.*, 2000). DBS coupled with LC-MS/MS has also been used to determine vitamin D

status during the perinatal period and study inter-seasonal fluctuations in concentration (Eyles *et al.*, 2010).

As with the majority of compounds measured using DBS, the blood collection method in steroid analysis has involved the use of the filter paper S&S 903. Relatively high steroid extraction efficiencies have been achieved with the use of either aqueous or organic solvents or a combination of both with the result of good assay accuracy and precision.

2.1.3 Aim

The aim of this part of the thesis was to develop a sensitive and robust DBS based method for the quantification of circulating dexamethasone concentrations in neonates, and to perform an *in-vitro* validation of the developed methodology.

2.2 Preliminary investigations for the selection of a detection system

2.2.1 Sensitivity

The detection sensitivity required to measure therapeutic dexamethasone concentrations in neonates was assessed by performing a simulation of the PK profile under the 10-day dose tapering regimen used on the Leicester NICU. This was done within NONMEM (version 7.0) using the First Order estimation method with a GNU Fortran Compiler 95. Simulations were based on the mean PK estimates (CL, 143 ml/h; V, 1845 ml) from two published reports for dexamethasone in neonates and the assumption of an underlying one compartment model (Charles *et al.*, 1993, Lugo *et al.*, 1996). Figure 2.1 shows the range of concentrations predicted in plasma during the treatment period for a neonate

weighing 1 kg. Based on the mean predicted concentration-time profile a LLOQ of at least 1 ng/ml was considered desirable for the measurement of therapeutic concentrations during the course of treatment.

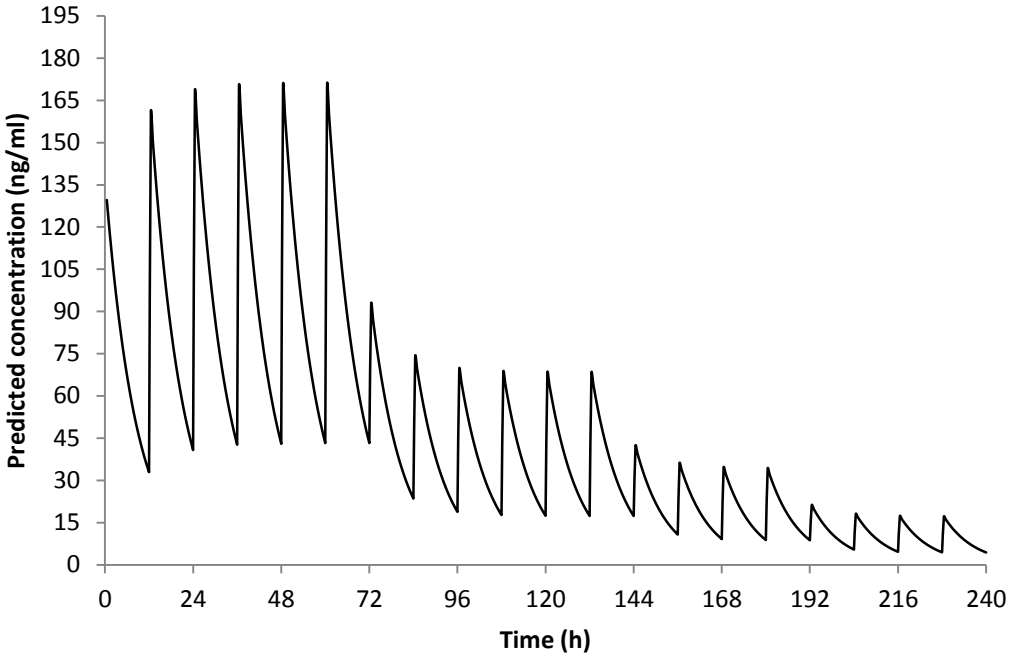


Figure 2.1 One compartment model used to predict plasma dexamethasone concentrations in a 1 kg neonate prescribed a 10-day tapering IV dosing regimen of 250 µg/kg 12 hourly for 72 hours, then 100 µg/kg 12 hourly for 72 hours, then 50 µg/kg 12 hourly for 48 hours followed by 25 µg/kg 12 hourly for 48 hours.

2.2.2 Selection of a detection system

Initial investigations into potential analytical techniques for the quantification of dexamethasone were carried out using LC with UV detection. The detection system was selected on the basis of the relatively good LLOQ (15 ng/ml) from a micro volume plasma sample (100 µl) previously reported (Schild and Charles, 1994). Analysis was performed on an Agilent 1100 LC system consisting of a quaternary solvent delivery pump, an automatic sample injection system, a micro vacuum degasser and a multiple

wavelength UV detector. The development of a working LC-UV method was undertaken using standard solutions of dexamethasone in acetonitrile:water (50:50, v/v). An initial UV scan was perfomed to determine the maximum absorption wavelength for dexamethasone. UV detection settings (band width, slit width and response time) and chromatographic parameters were considered during the method development stage in order to optimise the chromatographic peak shape and intensity, whilst achieving reasonably short run times. A substantial amount of work was undertaken to investigate the effects of mobile phase composition, buffers, pH, column temperature, injection volume and composition and flow rate on the resulting chromatographic signal. Validation of the final method was performed by analysing calibration standards prepared in solvent in replicate (n=3) over a dexamethasone concentration range of 1 to 1000 ng/ml. The developed analytical technique provided good reproducibility and accuracy within a relatively short analysis time (< 5 minutes). With an accuracy (relative error %, RE%) and precison (coefficient of variation %, CV%) of ≤ 15% and a signal-to-noise ratio of 10, the LLOQ achieved was 2.5 ng/ml from a 90 µl injection volume. This was considered too high with respect to the micro blood volume available for quantification with DBS sampling and the expected dilution during the extraction process.

Due to a requirement for better analytical sensitvity to measure expected therapeutic concentrations, an alternative approach using LC with fluorescence detection was considered. Dexamethasone is not a naturally fluorescent compound and therefore a derivatisation step must be performed using a fluorescent tag such as EDTN (1-ethoxy-4-(dichloro-s-triazinyl) naphthalene). EDTN reacts with phenolic hydroxyl groups to produce the respective derivatised compound and has previously been used to improve

the detection capability of dexamethasone in plasma. On the basis of the relatively low detection limit achieved and commercial availability, EDTN was selected for study (Wu *et al.*, 1995). The derivatisation procedure as described in the literature was carried out using a solution of 1 mM EDTN in acetontitrile and potassium carbonate. Analysis pre and post derivatisation was performed using an Agilent 1100 LC system with a fluorescence detector and the maximum wavelength of excitation and emission of labelled dexamethasone was determined using a PerkinElmer LS45 luminescence spectrometer. The chromatograms generated from the analysis of dexamethasone following the derivatisation procedure revealed a peak at a retention time of approximately 20 minutes which was assigned to the dexamethasone-EDTN derivative. However, due to the impurity of the purchased EDTN unknown reagent peaks severely interfered with the integration of the dexamethasone-EDTN peak. Attempts to improve the purity of EDTN via recrystallization with acetonitrile and methanol were not successful and hampered further progress with the fluorescent tag.

Given the difficulties encountered with fluorescence derivatisation and the need for better detection sensitivity LC-MS with ESI was subsequently investigated. Initial results with LC-MS indicated at least a 10 fold improvement in detection sensitivity compared with the LC-UV method. Therefore LC-MS was selected as the analytical detection system for dexamethasone quanitification work.

2.3 Experimental

2.3.1 Chemicals and materials

Dexamethasone (9-fluoro-11, 17, 21-trihydroxy-16-methylpregna-1, 4-diene-3, 20-dione) ≥ 98% and the internal standard triamcinolone acetonide (9-fluoro-16-hydroxyprednisolone 16, 17-acetonide) ≥ 99% (Figure 2.2) were purchased from Sigma–Aldrich (Poole, UK). Triamcinolone was selected as the internal standard (IS) due to its structural similarity to the analyte of interest and demonstrated suitability in dexamethasone quantification work (Taylor *et al.*, 2004, Kumar *et al.*, 2006). As triamcinolone is not used on the NICU there is no potential for alteration of the analyte to IS ratio and therefore to affect dexamethasone quantification. HPLC grade water, acetonitrile and methanol and formic acid ≥ 98% were obtained from Fisher Scientific (Loughborough, UK). Autosampler vials with 0.3 ml inserts, eppendorf cups and volumetric pipettes (Eppendorf Research) were obtained from Fisher Scientific (Loughborough, UK). The vortex (TopMix Wizard X), centrifuge (Eppendorf 5415R), shaker (IKA VXR Vibrax) and weighing scales (Mettler Toledo XS105) were obtained from Fisher Scientific (Loughborough, UK). Specimen collection filter paper S&S 903 was obtained from Fisher Scientific (Loughborough, UK). FTA Elute filter paper was obtained from Whatman, UK (part of GE Healthcare) and filter paper Ahlstrom 226 was supplied by ID Biological systems (South Carolina, USA). Sample glass tubes and polythene bags for storage of blood spot specimens were obtained from Richardson's of Leicester (Leicester, UK). The punching device was obtained from Maun Industries Ltd. (Nottingham, UK) and desiccant sachets were supplied by Sud-Chemie (Northwich, UK). Lithium heparin coated capillaries were obtained from Sangius Counting

(Numbrecht, Germany) and blood collection tubes (containing lithium heparin) were obtained from Sarstedt (Leicester, UK). Blank human venous blood for method development and patient sample analysis was obtained from healthy adult volunteers.

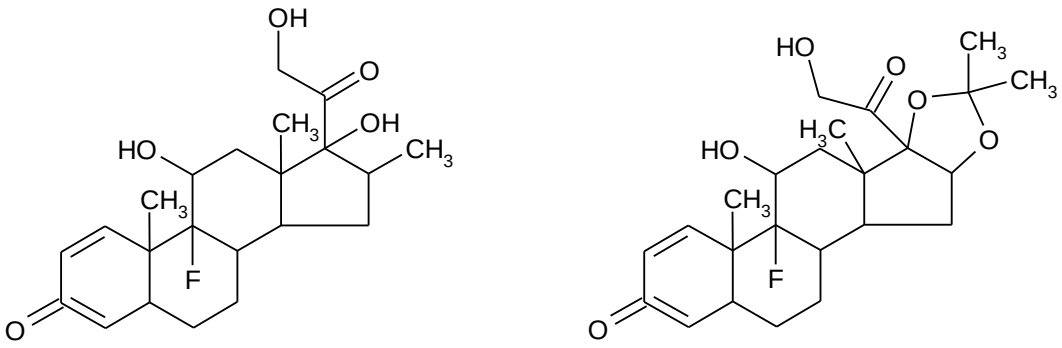


Figure 2.2 Chemical structures of dexamethasone (left) and triamcinolone acetonide (right)

2.3.2 Preparation of dexamethasone stock, working and spiking solutions

Stock solutions of dexamethasone were prepared by dissolving 5 mg in 5 ml of methanol:water (80:20, v/v) to produce a 1 mg/ml concentration. A relatively high concentration of methanol was used in the preparation of stock solutions to ensure complete dissolution of the corticosteroid. The stock solution was diluted to produce a concentration of 20 μg/ml in methanol:water (50:50, v/v), this was further diluted with methanol:water (50:50, v/v) to produce a series of working solutions of concentrations 315, 1050, 2100, 5250, 10500 and 16800 ng/ml. All solutions were freshly prepared on the day of analysis. Samples stored at 4°C were allowed to reach room temperature before use.

2.3.3 Preparation of internal standard, extraction solution and mobile phase

Stock solutions of IS were prepared by dissolving 2 mg in 10 ml of methanol:water (80:20, v/v) to produce a 200 µg/ml concentration. The stock solution was diluted to a concentration of 10 µg/ml in methanol:water (50:50, v/v), which was then further diluted with methanol:water (70:30, v/v) to produce an extraction solvent containing 5 ng/ml of IS.

Mobile phase A was made by adding 650 µl of formic acid to 500 ml of water to produce a 0.13% formic acid aqueous solution. Mobile phase B consisted of 100% acetonitrile.

2.3.4 Preparation of calibration and validation dried blood spots

Haematocrit levels are initially higher in the newborn and decrease with PNA. As dexamethasone is now reserved for use late in the neonatal period and the treatment population are preterms lower haematocrit values of approximately 30 to 40% are expected (Jopling *et al.*, 2009). A haematocrit level of 35% was selected for the preparation of DBS samples to represent the average value expected in the target population planned for study and limit any haematocrit related effects on filter paper. Blank human whole blood was centrifuged at 7,000 x g for 4 minutes and the plasma generated transferred to a clean eppendorf. The red blood cell (RBC) suspension and plasma were mixed in proportions (35:65, v/v) to give whole blood with an adjusted haematocrit of 35%. Calibration and validation DBS samples were prepared fresh on a daily basis by diluting 25 µl of each dexamethasone spiking solution with 500 µl of control human whole blood adjusted to a haematocrit of 35%. The final concentration of calibration standards were 15, 50, 100, 250, 500 and 800 ng/ml. These concentrations

were selected on the basis of plasma concentrations reported in neonates and performed simulations (Lugo *et al.*, 1996). 30 µl aliquots of spiked whole blood were spotted onto filter paper S&S 903 using a volumetric pipette and allowed to air dry overnight at room temperature prior to processing. A 30 µl volume applied onto filter paper gave a spot size of approximately 10 mm in diameter.

2.3.5 Single quadrupole mass spectrometry

2.3.5.1 Selection of ions for monitoring

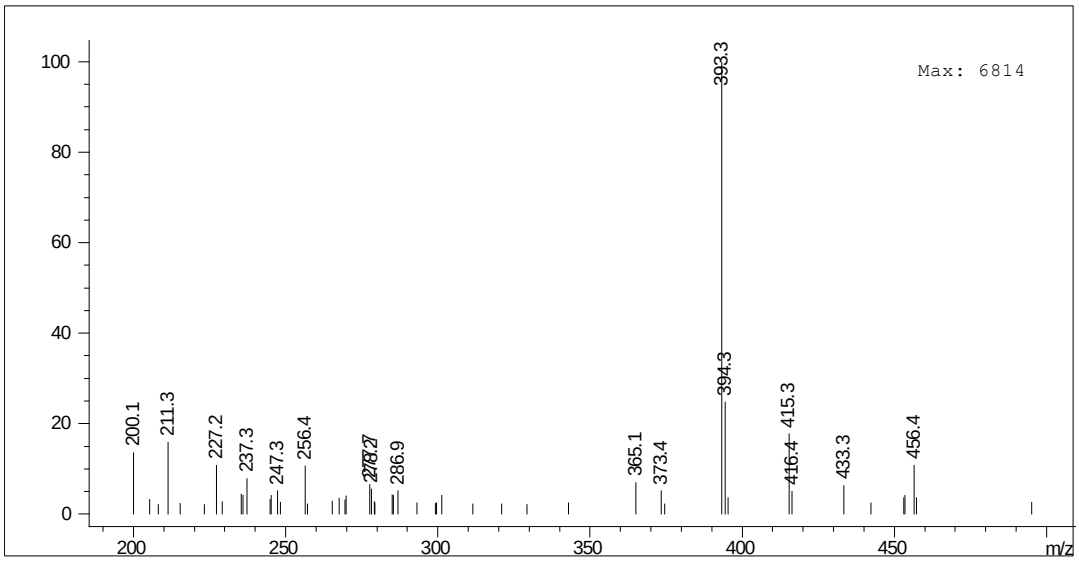
In the initial stages of method development experiments were focused on studying the behaviour of dexamethasone and IS in the ESI source and establishment of the respective base peak. A positive full scan was performed across a range of 200 to 500 *m/z* for each steroid at a concentration of 1000 ng/ml in solvent. The mass spectrum obtained for each steroid under optimised MS conditions is shown in Figure 2.3. For dexamethasone, a predominant peak was observed at *m/z* 393 corresponding to the molecular ion $[M+H]^+$. Characteristic of dexamethasone a fragment ion at *m/z* 373 $[M+H-HF]^+$ was observed in the spectra at 5% relative abundance. The formation of sodium adducts $[M+Na]^+$ was evidenced by the peak at *m/z* 415, however, this corresponded to less than 20% of the base peak. The mass spectra of the IS revealed a prominent base peak at *m/z* 435 which corresponds to the protonated molecular ion $[M+H]^+$.

The behaviour of dexamethasone in the ESI source was also investigated in negative mode. However, due to better signal intensity subsequent ion transmission optimisation was conducted in positive ESI mode.

2.3.5.2 Optimisation of ion transmission

Ion transmission optimisation was undertaken using the protonated molecular ion for dexamethasone (*m/z* 393) in SIM mode to optimise the detection sensitivity of analyte. The parameters known to have the greatest effect on the performance of ESI are capillary voltage, fragmentor voltage, nebuliser gas pressure, gas flow rate and drying gas temperature. The effect of each of these on analyte signal intensity was thus investigated using flow injection analysis. The optimum fragmentor voltage is known to be compound dependant and was therefore studied between 5 and 110 V in 15 V increments. An increase in signal intensity was observed with increasing fragmentor voltage up to a value of 65 V, thereafter the signal intensity dropped significantly (Figure 2.4). The trend observed can be explained by the effect of fragmentor voltage on ion transmission and fragmentation. Fragmentor voltage can improve ion transmission and thus sensitivity by facilitating the passage of ions through the high pressure region between the exit of the capillary and skimmer. When the compound specific threshold for this effect is reached (approximately 65 V for dexamethasone) fragmentation dominates and a decrease in signal intensity is observed. Full scans revealed the fragment ion at *m/z* 373 $[M+H-HF]^+$ dominated the mass spectrum at fragmentor settings above 65 V. Other studied operating parameters had little effect on the overall signal intensity of analyte.

A



B

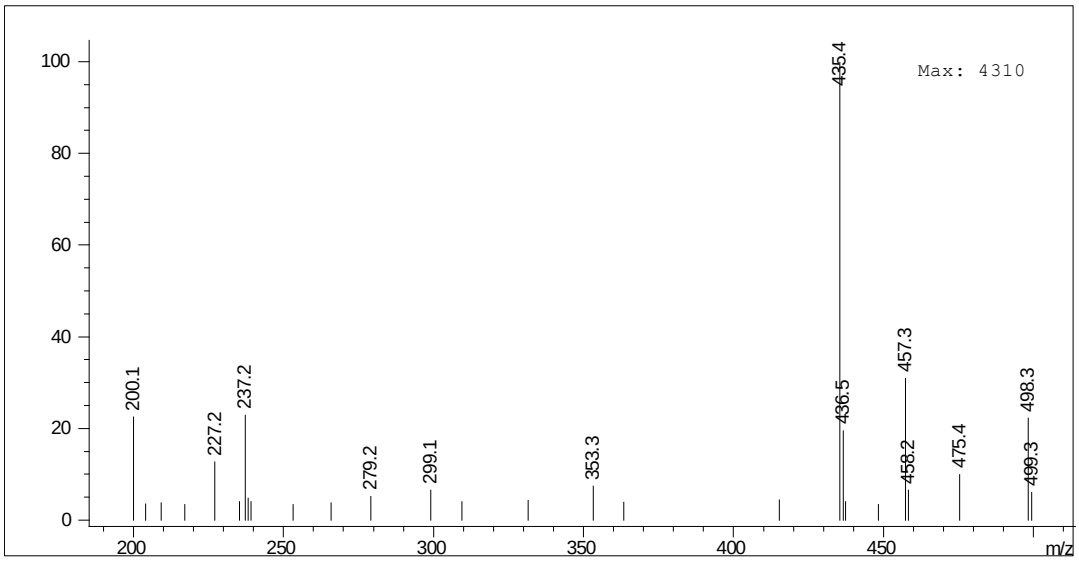


Figure 2.3 Full scan mass spectra under optimised MS conditions for dexamethasone (A) and internal standard triamcinolone acetonide (B) in solvent

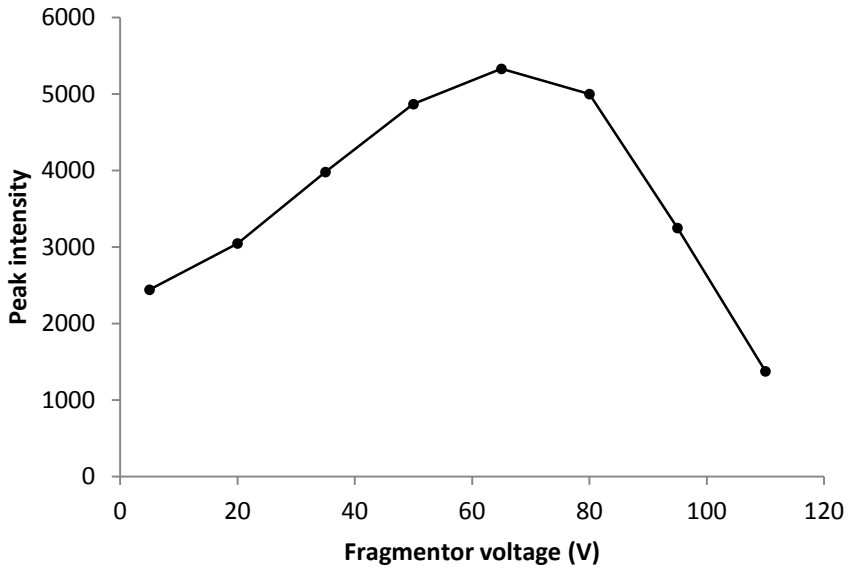


Figure 2.4 Effect of fragmentor voltage on dexamethasone signal intensity using a 100 ng/ml solution in SIM mode

2.3.5.3 Final conditions and instrumentation

Detection of samples was achieved using an Agilent 1200 mass spectrometer with a positive ESI source. The LC-MS system was calibrated daily using a tuning mixture supplied by Agilent Technologies. The ionisation source parameters optimised to give maximum analyte signal intensity were: fragmentor voltage, 65 V; drying temperature, 300°C; capillary voltage, 3500 V; nebuliser pressure, 25 psig; nitrogen gas flow, 7 l/min. The mass spectrometer was operated in SIM mode for the protonated molecular ion $[M+H]^+$ of dexamethasone at *m/z* 393 and IS at *m/z* 435. A dwell time of 289 msec was used for each ion. The analytical software programme Chemstation (series B.1.3, Agilent Technologies) was used to operate the system and aquire all data. Microsoft Excel 2007 (Microsoft Corporation, USA) was used to analyse acquired data.

2.3.6 Liquid chromatography

2.3.6.1 Final conditions and instrumentation

The chromatographic system consisted of an Agilent 1100 series quaternary solvent delivery pump, autosampler and vacuum degasser. Dexamethasone was analysed on a Zorbax Eclipse Plus C18 column (Agilent Technologies, Cheshire, UK; 150 mm x 2.1 mm i.d., 3.5 μm) attached with a C18 guard column (Phenomenex, Macclesfield UK; 3.0 x 4.0 mm). Under isocratic conditions dexamethasone and the IS eluted at overlapping retention times. Separation could not be achieved with changes in the mobile phase organic solvent, the organic to aqueous ratio or flow rate. Gradient elution was therefore implemented to improve the resolution between steroids. Variations in starting mobile phase composition and gradient steepness were studied to separate the steroids within a relatively short run time. Gradient elution was carried out using a combination of water with 0.13% formic acid (A) and acetonitrile (B). The mobile phase was initiated at 40% B and maintained for 0.15 minutes before increasing to 70% B by 3.0 minutes. A final increase to 80% B was achieved by 6.0 minutes before returning to 40% B. Thereafter, a 7.5 minute post-run at 40% B was maintained prior to the next injection. The flow rate was 200 μl/min and injection volume 25 μl. The column oven temperature was set to 22°C.

2.3.7 Dried blood spot extraction

2.3.7.1 Optimisation of extraction procedure

Initial extraction conditions using methanol:water (50:50, v/v) with gentle shaking for 50 minutes resulted in almost 100% recovery of dexamethasone from an entire 30 μl

250 ng/ml DBS sample. The effect of varying methanol concentration within a range of 30 - 100% in increments of 10% did not significantly affect analyte recovery. However, as the ratio of methanol to water increased the extract appearance changed significantly from reddish and cloudy to clear and straw like in appearance. The darker extracts arising with a lower methanol concentration were due to the extraction of water soluble components of whole blood and effectively resulted in the reconstitution of the blood spot. Due to better selectivity, 70% methanol was chosen for DBS extraction. Further investigations revealed that maximum recovery was achieved within 20 minutes using 70% methanol thereby enabling a reduction in the extraction time. Similar extraction efficiencies were noted on changing the organic component of the extraction solution to acetonitrile.

2.3.7.2 Punch size

To determine the suitability of using S&S 903 filter paper for dexamethasone analysis different sized disc diameter punches (3, 6, 8 and 10 mm) were investigated using a slightly larger 35 µl DBS sample at a concentration of 800 ng/ml. The analyte peak area increased linearly with increasing punch size explained by the larger volume of blood sampled from the DBS (Figure 2.5). This indicated the appropriateness of filter paper S&S 903 for dexamethasone quantification work and the validity of using a punch diameter between 3 and 10 mm. An 8 mm punch size was chosen as it enabled a disc of blood to be sampled from a 30 µl spot (approximately 10 mm in diameter) with relative ease whilst maximising detection sensitivity.

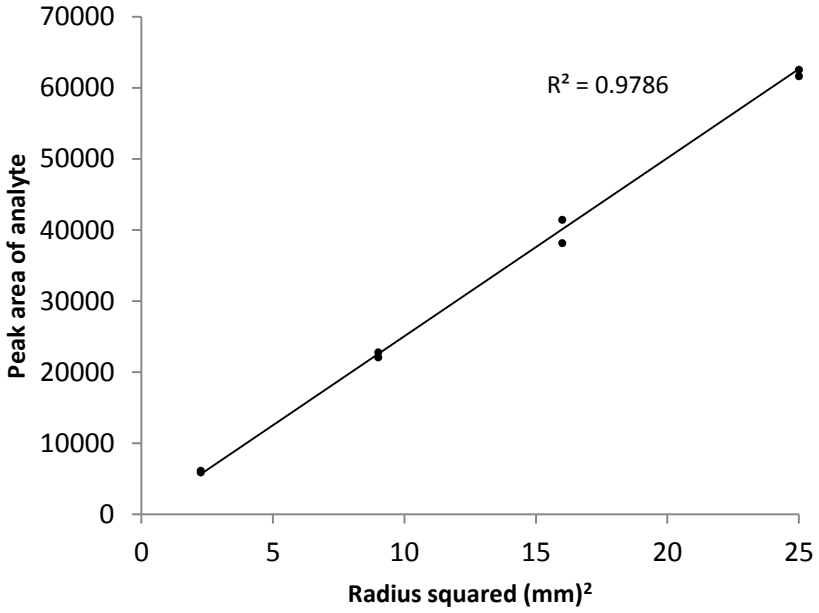


Figure 2.5 Effect of punch size (radius^2) on dexamethasone signal intensity

2.3.7.3 Final extraction method

An 8 mm diameter disc (equivalent to approximately 21µl of blood) was punched from the centre of a 30 µl DBS sample and transferred to a clean glass tube. A 250 µl volume of extraction solvent consisting of methanol:water (70:30, v/v) plus IS (5 ng/ml) was added and the tube was shaken at 300 rpm for 20 minutes. The extract was centrifuged at 13,500 x g for 10 minutes to remove any insoluble residues arising from the filter paper and the supernatant was transferred to an autosampler vial for analysis by LC-MS.

2.3.8 Filter paper selection

During method development two other filter papers FTA Elute and Ahlstrom 226 were investigated to determine their respective performances for dexamethasone quantification. DBS calibration standards within the range of 15 to 800 ng/ml (n=3) were prepared using S&S 903, FTA Elute and Ahlstrom 226 paper. The spots produced

were of similar size and shape, however, as previously noted a corona edge was observed with the FTA Elute paper. Extraction was carried out using the final method developed for recovery of dexamethasone from paper S&S 903 (section 2.3.7.3). High recoveries and acceptable precision (CV ≤ 15%) was observed for each filter paper type at the tested concentrations (Table 2.2). Furthermore, on comparison there was little difference in assay performance between the different specimen collection papers and therefore subsequent method development work was continued using S&S 903 filter paper.

Table 2.2 Assay precision results with filter paper S&S 903, FTA Elute and Ahlstrom 226 at low, medium and high dexamethasone concentrations n=3

	Precision (CV%)		
Nominal Conc. (ng/ml)	S&S 903	FTA Elute	226 Ahlstrom
50	5.0	5.8	9.7
250	3.4	9.9	3.6
800	6.1	10.3	3.8

2.4 Validation results and discussion

Following the development of an LC-MS method, a suitable blood collection method and optimisation of extraction conditions the microanalytical method was validated using FDA regulatory guidelines (Shah *et al.*, 2000, Guidance for Industry: bioanalytical method validation, 2001). Other tests specific to DBS analysis such as the influence of spot volume and haematocrit on analyte recovery were performed to

provide an assessment of the robustness of the developed method for dexamethasone quantification and its suitability for application to PK studies involving neonates.

2.4.1 Extraction efficiency

The overall recovery of dexamethasone was assessed at DBS concentrations of 50, 250 and 800 ng/ml in replicate (n=5). To determine recovery 30 µl spots were produced and allowed to dry. The entire spot on the filter paper was then extracted with methanol:water (70:30, v/v). The analytical results from extracted samples were compared to those obtained from the same amount of dexamethasone in solvent methanol:water (70:30, v/v). Recovery was calculated using peak area by the equation:

$$Recovery\ \% = \frac{Response\ in\ DBS\ extract}{Response\ in\ pure\ solvent} \times 100$$

The overall recovery for dexamethasone varied between 94 to 106% (Table 2.3, SD = standard deviation). Consistent and precise (CV% ≤ 15%) recovery values at low, medium and high concentrations indicate the extraction process is acceptable across the calibration range and reproducible. The high mean recovery (99%) observed indicate analyte stability under the extraction conditions applied.

Table 2.3 Recovery of dexamethasone from DBS samples n=5

	Nominal conc. (ng/ml)		
	50	250	800
Recovery (%)	94.3	105.7	97.8
SD	13.0	6.1	5.7
Precision (CV%)	13.8	5.8	5.8

2.4.2 Matrix effects

Matrix effects can alter the detector response of an analyte due to the presence of other compounds in the ionisation source and is particularly associated with ESI (Venn, 2008). Signal suppression rather than enhancement is often observed with MS based techniques which can have a detrimental effect on the detection sensitivity (Rogatsky and Stein, 2005). To evaluate suppression or enhancement of the detector response due to constituents within the DBS matrix replicate (n=5) samples of dexamethasone spiked in extracted blank DBS samples at concentrations of 50 and 800 ng/ml were produced. These samples were compared to standards of the same concentration spiked into pure methanol:water (70:30, v/v). Sample preparation involved the addition of 10 µl of spiking solution to 90 µl of either solvent or matrix solution. The matrix effect was calculated using dexamethasone peak area by the equation:

$$Matrix\ effect\ \% = \frac{Response\ in\ DBS\ extract}{Response\ in\ pure\ solvent} - 1\ \times\ 100$$

No significant (< 15%) ion suppression or enhancement of the analyte signal due to endogenous components of blood or the filter paper was observed at the two tested concentrations (Table 2.4). These results provide assurance on the selectivity of the extraction procedure and the ionisation method.

Table 2.4 Matrix effects arising from dried blood spots on dexamethasone signal intensity n=5

	Nominal conc. (ng/ml)	
	50	800
Matrix effect % (mean)	-0.4	3.4
Precision (CV%)	4.3	4.9

2.4.3 Linearity, selectivity and sensitivity

Dexamethasone calibration standards were prepared in replicate (n=5) and analysed on three separate days. A calibration plot of analyte/IS peak area ratio against nominal dexamethasone concentration was produced and an equally weighted linear regression applied. The assay showed linearity ($r^2 \geq 0.99$) within the tested concentration range of 15 to 800 ng/ml (Figure 2.6).

Chromatographic conditions used provided sufficient resolution with elution of dexamethasone and IS at 4.8 and 5.9 minutes, respectively. The selectivity of the method was determined by analysing DBS samples (n=1) collected from five individual human subjects. There were no significant interferences (≥ 20% of LLOQ peak area) at either of these retention times in any chromatograms. The selectivity of the method is demonstrated by the representative LC-MS chromatogram presented in Figure 2.7.

The LLOQ was defined by the lowest concentration that gave a signal-to-noise ratio equal to or greater than 10 whilst exhibiting a RE% and CV% of ≤ 15%. Signal-to-noise ratios were calculated by dividing peak height by the baseline noise (where noise is given as six times the standard deviation of the linear regression). The LLOQ was

determined to be 15 ng/ml in whole dried blood (Figure 2.7). This detection limit was not sufficient for the quantification of dexamethasone during the entire 10-day treatment course. The sensitivity of the assay was however, considered to be sufficient to capture the PK profile during the first 6 days when concentrations were predicted within the quantifiable range of the assay (section 2.2.1, Figure 2.1). The sensitivity of the assay was therefore considered to be acceptable for the purposes of the study objectives.

To improve the LLOQ the incorporation of a sample concentrator step during sample preparation was investigated. DBS calibration standards at concentrations of 7.5, 15, 50, 100, 250, 500, and 800 ng/ml were prepared in replicate (n=5). Samples were processed as described in section 2.3.7.3 except IS in 100% methanol was used as the extraction solvent. This was to facilitate evaporation of the extract to a residue. A 180 µl aliquot of the methanol extract was transferred to an eppendorf and evaporated to dryness under a stream of nitrogen. The residues were reconstituted with 72 µl of methanol:water (70:30, v/v) and vortexed for 2 minutes. Samples were then centrifuged at 13,500 x g for 5 minutes prior to LC-MS analysis. This resulted in an improvement in detection sensitivity of at least 2 fold but insufficient precision, particularly at the lower end of the calibration line (CV%, 23.2 for 7.5 ng/ml level) was observed. Further investigations to optimise the reproducibility of drug recovered were not undertaken.

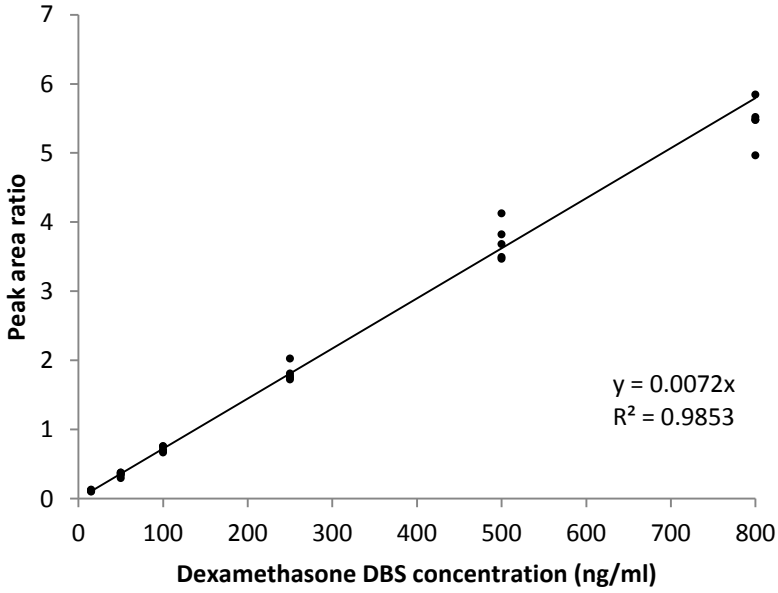


Figure 2.6 A calibration plot of nominal dexamethasone DBS concentration against analyte to IS peak area ratio (n=5)

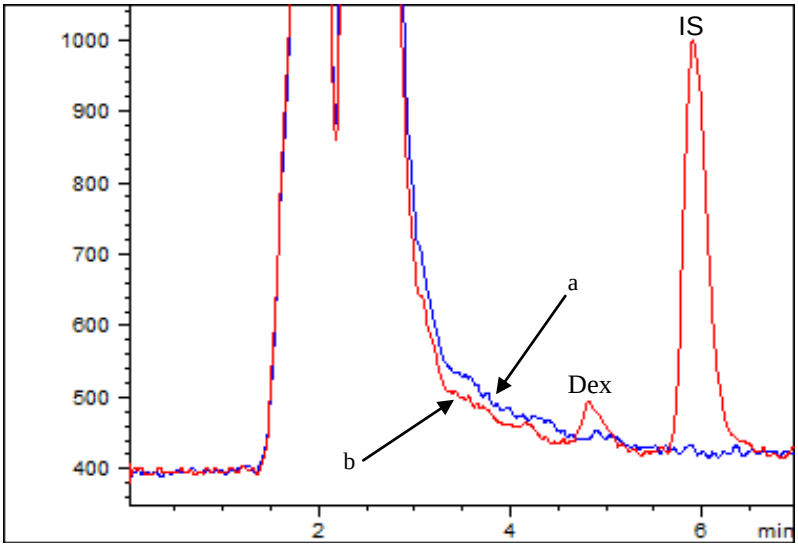


Figure 2.7 Representative LC-MS chromatogram of (a) a blank DBS sample extracted without IS and (b) a DBS sample spiked with dexamethasone (Dex) at 15 ng/ml (LLOQ) and extracted with IS

2.4.4 Accuracy and precision

2.4.4.1 Calibration

Intraday and interday assay accuracy and precision was determined from the analysis of replicate (n=5) calibration standards at six dexamethasone concentrations within the range of 15 to 800 ng/ml on three separate days. A RE and CV of ≤ 20% at the LLOQ and ≤ 15% at all other concentrations was considered acceptable. RE% and CV% values were within these pre-defined limits at each concentration for all runs (Table 2.5). The overall variation in assay performance between runs (interday) was within 15%.

2.4.4.2 Carryover and injector precision

The effect of sample carryover on assay accuracy and precision was assessed by injecting two blank matrix extracts into the LC-MS system following the analysis of the highest (800 ng/ml) DBS calibration standard (Hughes *et al.*, 2007). The extent of carryover observed was acceptable with peak areas in the blank chromatograms at less than 20% of the peak area of the lowest DBS concentration (15 ng/ml). To determine if there was any carryover due to the punch, the device was used to cut out 10 discs from 800 ng/ml DBS samples followed by 2 discs from blank DBS samples. A peak attributable to dexamethasone was not observed in the resulting blank DBS extract chromatograms and therefore the punching device was excluded as a source of carryover during the extraction procedure.

Injector precision was determined by analysing 5 repeat injections of the lowest DBS concentration. The injection-to-injection precision (CV%) was 2.2% and the variability in retention time for dexamethasone and IS was 0.6% and 1%, respectively (Table 2.6).

Table 2.5 Intraday and interday accuracy and precision data for dexamethasone n=5

		Intraday					
	Nominal conc. (ng/ml)	15	50	100	250	500	800
run 1	Mean conc. (ng/ml)	15.8	45.7	91.3	225.4	498.9	777.1
	SD	1.8	1.1	4.2	16.7	17.2	33.1
	Accuracy (RE%)	5.2	-9.3	-9.6	-10.9	-0.2	-3.0
	Precision (CV%)	11.4	2.5	4.6	7.4	3.4	4.3
run 2	Mean conc. (ng/ml)	16.7	48.9	99.9	253.0	517.0	758.5
	SD	1.5	4.6	5.6	16.8	37.4	43.7
	Accuracy (RE%)	10.3	-2.2	-0.1	1.2	3.3	-5.5
	Precision (CV%)	9.1	9.2	5.6	6.6	7.2	5.8
run 3	Mean conc (ng/ml)	16.2	52.9	98.2	248.3	565.2	847.7
	SD	2.3	6.1	12.9	36.3	27.0	48.7
	Accuracy (RE%)	7.5	5.5	-1.9	-0.7	11.5	5.6
	Precision (CV%)	14.0	11.5	13.1	14.6	4.8	5.7
		Interday					
	Mean conc. (ng/ml)	16.3	49.2	96.5	242.2	527.1	794.4
	SD	1.8	5.1	8.8	25.3	38.4	54.3
	Mean accuracy (RE%)	7.7	-2.0	-3.8	-3.5	4.9	-0.9
	Overall precision (CV%)	11.0	10.4	9.1	10.5	7.3	6.9

Table 2.6 Injector precision and retention time variability results

Injection number	Peak area ratio	Dexamethasone retention time	IS retention time
1	0.417	4.873	5.944
2	0.433	4.825	5.839
3	0.408	4.833	5.902
4	0.426	4.895	5.969
5	0.424	4.859	5.979
Mean	0.422	4.857	5.923
SD	0.009	0.029	0.057
Precision (CV%)	2.2	0.6	1.0

2.4.5 Petroleum jelly application

Petroleum jelly is often used on the NICU to facilitate the collection of capillary blood. It is applied as a thin layer onto the heel of the infant before skin puncture with an automated lancet. The hydrophobicity of the petroleum jelly encourages the formation of a droplet of blood on the skin surface which can be neatly collected into a capillary tube for routine clinical blood tests. This helps to minimise blood loss due to overflow and reduces the blood collection time. Although blood is sampled from the droplet formed there is potential for chemical moieties to interfere with drug quantification.

The effects of petroleum jelly on dexamethasone quantification were investigated using blood spiked at 50 and 800 ng/ml (n=5). A thin layer of petroleum jelly was used to coat

a glass slide and 45 µl aliquots of spiked blood were applied. After 10 minutes 30 µl volumes of blood were collected using a pipette and spotted onto S&S 903 paper. This process was repeated without the application of petroleum. An 8 mm disc was punched from the centre of DBS samples and extracted with IS. The difference in peak area ratio between samples exposed to petroleum with those that were not was less than 15% at both concentrations (Table 2.7). In addition all precision values were within 15%. This would suggest that the use of petroleum jelly during capillary blood collection does not significantly affect dexamethasone quantification.

Table 2.7 Effect of petroleum jelly on dexamethasone assay performance n=5

Nominal conc. (ng/ml)	50		800	
	None	Petroleum jelly	None	Petroleum jelly
Peak area ratio (mean)	0.79	0.71	10.69	9.69
SD	0.07	0.05	0.44	0.68
Precision (CV%)	8.9	6.7	4.1	7.1
Difference (%)	-9.2		-9.4	

2.4.6 Blood collection and spotting device

Quantification of analyte in the DBS relies on the assumption of an even distribution of blood across the filter paper. The method of DBS sampling in clinical practice therefore becomes a critical factor in ensuring the collection of samples of an acceptable standard. Different DBS sampling approaches have been implemented one of which is skin puncture with direct application of blood and has been the method of choice in newborn screening programmes (Guidelines for Newborn Blood Spot Sampling, UK Newborn

Screening Programme, 2008). This method must be performed whilst avoiding skin contact with the collection paper and relies on the skill of the operator to ensure there is no double spotting i.e. blood is not applied to the same area twice. A potential problem with this approach is that the concentration of analyte in DBS samples is determined from a calibration line prepared using whole blood containing anticoagulant, whereas patient samples do not contain anticoagulant. This is important as anticoagulants can affect analyte quantification through matrix effects (Mei *et al.*, 2003, Holtkamp *et al.*, 2008, Bergeron *et al.*, 2009). Alternatively, blood may be collected into a capillary tube containing anticoagulant before spotting. The blood contained within the capillary tube is then dispensed in one smooth application using a suction bulb. Capillary tubes are already used in neonatal practice as they provide a convenient and practical way of collecting micro blood volumes. Due to better control offered by the latter technique capillary tube sampling was incorporated into the blood collection methodology for this study.

The influence of spotting device on the formation of blood spots was tested by producing 30 µl blood spots in replicate (n=5) using a capillary tube or a pipette (eppendorf) at two concentrations (50 and 800 ng/ml). The concentrations of dexamethasone were determined from a calibration line generated from DBS standards spotted using a pipette. A comparison of the accuracy and precision of dexamethasone quantification when samples were spotted using either a pipette or capillary is given in Table 2.8. An acceptable and comparable assay performance was observed suggesting the suitability of a capillary tube for blood collection and spotting.

2.4.7 Blood spot volume

Experiments conducted by the NSQAP using an isotopic method have demonstrated that the volume of blood applied onto filter paper S&S 903 can affect absorption reproducibility and is therefore an important variable to consider in DBS sampling (Mei *et al.*, 2001). They reported a 13% increase in serum held by a 6 mm disc when increasing blood spot size from 25 to 125 µl with constant haematocrit. This is corroborated by the results obtained by Liang *et al* who found varying the blood volume spotted on FTA Elute within a 10 to 50 µl range for dextromorphan quantification yielded a maximum assay inaccuracy of < 19%, however this difference was less than 10% when limiting the variability of blood volume collected to between 25 to 50 µl (Liang *et al.*, 2009). These results indicate that although a consistent volume is important for assay accuracy a degree of flexibility around the blood volume collected is acceptable.

To assess the effect of blood volume collected on dexamethasone quantification, 25, 30 and 35 µl DBS samples at 50 and 800 ng/ml were prepared in replicate (n=5). An 8 mm diameter disc was punched from the centre of each sample and extracted. The concentrations of extracts were determined using the linear regression equation generated from a calibration produced from 30 µl DBS samples. The accuracy and precision data as shown in Table 2.9 was within the 15% limit for 25 and 35 µl spot sizes at the two tested concentrations. Furthermore, the maximum variation in accuracy between spot sizes of 25 to 35 µl was 7%, indicating that the amount of blood spotted did not significantly affect the distribution of dexamethasone across the filter paper and therefore drug quantification. Others have also shown an acceptable assay performance

with small variations in sample volume spotted (ter Heine *et al.*, 2008, Spooner *et al.*, 2009).

Table 2.8 Effect of spotting device on accuracy and precision of dexamethasone assay n=5

Spotting device	Pipette		Capillary	
Nominal conc. (ng/ml)	50	800	50	800
Mean conc. (ng/ml)	45.6	838.5	50.6	822.8
SD	6.5	54.6	7.5	34.4
Accuracy (RE%)	-9.6	4.6	1.7	2.8
Precision (CV%)	14.2	6.5	14.7	4.2

Table 2.9 Effect of varying blood spot volume on accuracy and precision of dexamethasone assay n=5

Nominal conc. (ng/ml)	50			800		
Volume	25 µl	30 µl	35 µl	25 µl	30 µl	35 µl
Mean conc. (ng/ml)	52.0	48.9	51.1	807.3	758.5	804.0
SD	2.0	4.5	3.4	35.1	43.7	47.6
Accuracy (RE%)	3.8	-2.2	2.1	0.9	-5.5	0.5
Precision (CV%)	3.9	9.2	6.7	4.3	5.8	5.9

2.4.8 Haematocrit

Haematocrit has been identified as a factor with potential to affect drug quantification when using DBS. The effect is directly related to viscosity and the resulting

spreadability of blood on cellulose based paper. With high haematocrit values (due to a greater cellular composition) blood is more viscous and may lead to smaller blood spots being formed. For a fixed volume of blood, changes in haematocrit can therefore affect the absorption of blood per unit area of filter paper and consequently the final spot size. For methodologies based on taking a disc from within a DBS sample, the amount of analyte recovered can theoretically vary which has important implications for the accuracy of the concentration measurement. The haematocrit range varies according to age, for healthy adult males and females it is 42 to 52% and 37 to 47%, respectively (Hillman *et al.*, 2005). Values may deviate outside of these ranges in certain disease states and critically ill patients. Neonates exhibit considerable inter and intraindividual variation in haematocrit values during the first 28 days of life (Alur *et al.*, 2000, Christensen *et al.*, 2009, Jopling *et al.*, 2009). They are initially higher in the newborn (42 to 65%) and gradually decrease with increasing PNA. In addition, increased haematocrit values (greater than 65%) are observed in a small percentage of neonates due to polycythaemia. Changes in haematocrit during the neonatal period are also affected by GA and illness severity with lower values observed in more premature and critically ill infants (Alkalay *et al.*, 2003). Dexamethasone therapy is now reserved for use late in the neonatal period and since the majority of patients receiving treatment are critically ill preterms lower haematocrit levels of around 30 to 40% are expected.

Accordingly, the influence of haematocrit on the assay performance was determined at 50, 250 and 800 ng/ml in replicate (n=5) using 30 µl blood spots with an adjusted haematocrit of 30, 35 and 40%. The plasma and RBC components of whole blood were separated as previously described (section 2.3.4) and recombined in the correct proportions to give bloods of differing haematocrit %. An 8 mm disc was punched from

each spot and analysed. Concentrations of extracts were determined using the linear regression equation generated from a calibration produced from standards of 35% haematocrit.

A decrease in the size of the blood spot formed was noticeable with increasing haematocrit value. Other investigators have found similar relationships between haematocrit and spot size or volume held by a fixed diameter disc. Investigations on the performance of S&S 903 filter paper by the NSQAP found haematocrit to be the most important determinant of overall variation with results indicating 47% less serum volume for 6.0 mm punches taken from 100 µl DBS samples made with 30% haematocrit compared with those made with 70% haematocrit (Mei *et al.*, 2001). O'Broin *et al* using blood labelled with [125I]-thyroxine showed that the volume of blood in a 6.35 mm diameter punch taken from a 100 µl spot on 903 paper increased by 32% (from 8.2 to 10.8 µl) when haematocrit varied between 24 to 50% (O'Broin, 1993, O'Broin *et al.*, 1995).

The results from the haematocrit investigation gave RE% and CV% values within the pre-defined limit of ≤ 15% at all haematocrit levels for each tested dexamethasone concentration (Table 2.10). A haematocrit effect was however noticeable with higher haematocrit levels associated with an increase in the amount of drug recovered at all three concentrations. This suggests an altered distribution of analyte across the filter paper determined from an 8 mm disc. The positive correlation could be explained by blood viscosity, with blood of higher viscosity leading to smaller spots which results in a greater volume of blood being sampled and higher dexamethasone concentration

measurements. The effect of haematocrit changes on blood and analyte distribution, spot size and drug recovery from a fixed punch size have been reported by others.

Table 2.10 Influence of haematocrit on the accuracy (RE%) of dexamethasone measurements presented as the difference from the analyte/IS peak area ratio at the 35% haematocrit level. Precision (CV%) values for respective values are shown in brackets n=5.

	Nominal conc. (ng/ml)		
Haematocrit	50	250	800
40%	+11.6% (12.6%)	+13.8 (7.9%)	+4.1 (5.2%)
35%	Normalised (2.5%)	Normalised (7.4%)	Normalised (4.3%)
30%	-3.4% (7.0%)	+3.6 (3.4%)	-3.2 (4.3%)

Investigations by Adams *et al* using S&S 903 paper found a 3.1% increase in phenylalanine recovery from 3 mm discs taken from 100 µl DBS samples prepared using 53% haematocrit compared with samples of a lower haematocrit (50%) (Adam *et al.*, 2000). Holub *et al* investigated the effects of haematocrit on the measurement of numerous amino acids using S&S 2992 paper by taking 3 mm punches from the centre and periphery of 15 mm DBS samples prepared from blood adjusted to give a haematocrit value of 20, 30, 50 and 60% (Holub *et al.*, 2006). Significantly higher levels of amino acids were measured in discs with increasing haematocrit. However, for some amino acids the effect was either less pronounced or no significant correlation was observed. Wilhelm *et al* studied the effect of haematocrit on cyclosporin A measurements with 903 paper over a haematocrit range of 20 to 72% with normalisation at 35%. A haematocrit effect was noted, however, the overall variation in assay bias was

within 15% (-12 to 14%) across the range (Wilhelm *et al.*, 2009b). No influence on assay bias was detected when haematocrit varied from 29 to 59%. This is in contrast to the findings for dexamethasone where relatively small changes in haematocrit (± 5%) influenced drug quantification. Nevertheless, the maximum difference in the accuracy of the dexamethasone assay when changing haematocrit from 30 to 40% was less than 15%. An acceptable % bias has been reported for other analytes with relatively small variations in haematocrit (Butter *et al.*, 2001, Wilhelm *et al.*, 2009a, Al-Ghazawi and AbuRuz, 2010).

The importance of filter paper type on the observed haematocrit effect has been reported by Denniff and Spooner using test compounds paracetamol and sitamaquine (Denniff and Spooner, 2010a). In this study, the influence of varying haematocrit between 20 and 80% on spot appearance and drug recovery (determined against a 44% calibration line) was investigated using 15 µl DBS samples prepared on three different filter papers (903, FTA and FTA Elute). A clear inversely proportional relationship was observed between blood spot size (surface area) and haematocrit with all filter papers. Subsequent investigations highlighted a general trend towards an increased % bias with increasing haematocrit for both analytes on S&S 903 paper. Interestingly, for paracetamol the effect decreased between 60 and 80% indicating additional factors other than spot size (or blood viscosity) must account for the effect observed. It is noteworthy that the % bias observed for sitamaquine on 903 was within the acceptable limit (± 15%) across the haematocrit range studied and for the majority of haematocrit values for paracetamol. An initial increase in bias % followed by a decrease between 60 and 80% was also observed for sitamaquine on FTA paper. The difference in % bias, however, with FTA paper varied between approximately -30 and 10%. In contrast, the % bias was within ±

5% for paracetamol on FTA paper. A strong haematocrit effect was noted for sitamaquine using FTA Elute with a bias of ± 20%, however, there was no clear trend observed to enable a correlation between haematocrit and % bias. The results from this work highlight the complexity of the relationship between haematocrit and % bias and in turn the difficulties of predicting the amount of drug recovered. There appears to be a distinct relationship between the volume of blood held by a fixed diameter disc and haematocrit, however, this does not necessarily translate into a linear relationship that can be easily extrapolated for prediction of the amount of drug recovered.

This provides evidence that the nature and magnitude of a haematocrit effect is linked to the properties of the analyte under investigation as well as the characteristics of the selected specimen collection paper. O'Broin investigated the distribution of erythrocytes using an isotopic method by measuring counts of ^{51}Cr. Unlike serum, erythrocyte absorbency (or volume) appeared to remain constant with increasing haematocrit (O'Broin, 1993). Therefore the distribution of a drug between RBCs and plasma, and the extent of binding to components in each phase may be influential in the resulting haematocrit effect.

The current validation data and findings of other investigators highlight the importance of investigating haematocrit effects. This is of even greater importance in cases where the study population is expected to exhibit a large variability in haematocrit.

2.4.9 Red blood cell association

It is usually the free fraction or unbound drug in plasma that exerts a pharmacological effect and is available to be cleared from the body. The relationship between bound and unbound drug in plasma and RBCs is shown in Figure 2.9. The majority of drug assays

whether they are based on plasma or whole blood samples measure unbound and bound drug i.e. total concentration due to the difficulties associated with measurement of unbound concentrations. Therefore in most situations the total drug concentration is measured and used as a surrogate for unbound drug. The unbound concentration, plasma concentration and blood concentration at equilibrium are related by the equations below (Rowland and Emmons, 2010):

$$Total\ plasma\ concentration = \frac{Cu}{fu}$$

$$Total\ blood\ concentration = \left[1 - \frac{H}{fu} + H\ \times\ p\right] Cu$$

Where, *Cu* = unbound concentration

fu = fraction unbound in plasma

H = haematocrit

p = red blood cell to plasma concentration ratio

In plasma, the total drug concentration is proportional to the unbound concentration *(Cu)* provided the fraction unbound in plasma remains *(fu)* constant. Factors which may alter the extent of protein binding would therefore need to be considered when using plasma. In blood, the total drug concentration is proportional to the unbound concentration *(Cu)* provided the fraction unbound in plasma *(fu)* does not change and haematocrit (*H*) and partitioning (*p*) also remain constant. Based on these principles either blood or plasma could potentially be used to measure drug concentrations provided the ratio of total drug to unbound drug does not change. If there is a large degree of variability in these variables, using the total whole blood concentration as a

surrogate for unbound concentration has the potential to affect PK and PK-PD interpretation. When using DBS it therefore becomes important to understand the distribution kinetics of a drug in whole blood.

Knowledge of the blood-to-plasma ratio is useful for understanding the distribution of a given drug between RBCs and plasma. It can be used to make an assessment of the variable (*fu*, *H* or *p*) that is most likely to govern the ratio of total drug concentration to unbound drug concentration in plasma or whole blood and the suitability of each respective matrix. This value is also useful when a comparison or conversion between plasma and whole blood drug concentration data is required. To investigate the partitioning of dexamethasone into RBCs a conventional *in-vitro* method was performed using spiked human whole blood (Hinderling, 1997, Yu *et al.*, 2005).

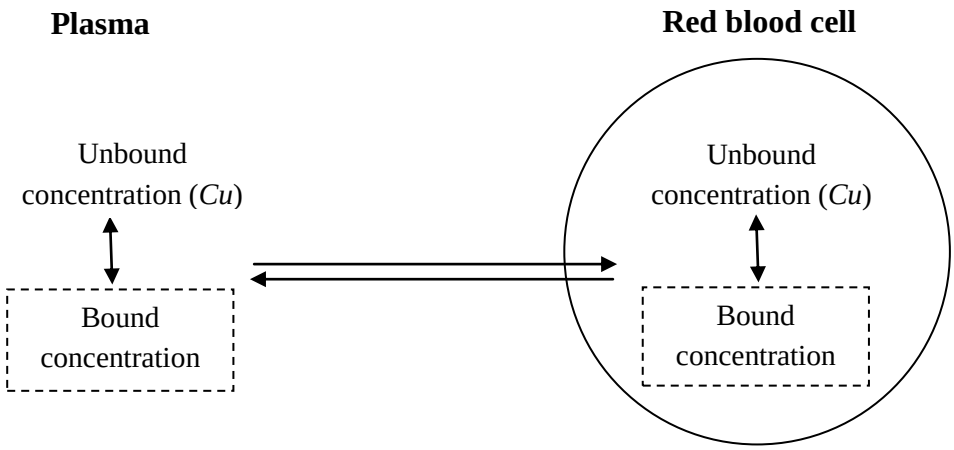


Figure 2.9 An illustration of the potential distribution of drug in whole blood

The rate of partitioning was studied to determine equilibration time between RBCs and plasma under controlled conditions. The determined time period was then used in a

subsequent experiment which focused on the extent of partitioning (*p*) with changes in dexamethasone concentration.

2.4.9.1 Time to reach equilibrium

Whole unadjusted blood of haematocrit 43% was spiked with dexamethasone to produce a single concentration of 250 ng/ml and incubated at 37°C with gentle agitation for 120 minutes. At 0, 15, 30, 60 and 120 minutes a 150 µl aliquot of blood was removed to determine the blood-to-plasma ratio. Whole blood was spotted onto filter paper in triplicate to produce 15 µl DBS samples and the remaining whole blood was centrifuged at 7,000 x g for 2 minutes. The plasma generated was applied onto filter paper in triplicate to produce 15 µl dried plasma spot (DPS) samples. Once dried, the entire DBS or DPS was punched from the filter paper and extracted using methanol:water (70:30, v/v). Concentrations of dexamethasone in the resulting extracts were analysed using LC-MS. The equilibration time was selected when no further increases in the blood-to-plasma ratio were observed. The blood-to-plasma ratio was calculated by the equation*:*

$$Blood\ to\ plasma\ ratio = \frac{PAb}{PAp}$$

Where *PAb* = peak area in blood

PAp = peak area in plasma

An increase in the blood-to-plasma ratio was observed between 0 and 30 minutes after which no further increases were observed (Table 2.11). Therefore equilibrium between RBCs and plasma appears to occur between 0 and 30 minutes but no further experimentation was conducted to determine a more accurate equilibration time. The

maximum incubation period was set to 120 minutes to avoid problems in interpreting results as with longer incubation times (of 180 minutes) the plasma generated was slightly pink in colour indicative of haemolysis. At 120 minutes, the ratio decreased by approximately 10% and although a change in the colour of plasma was not observed this could be due to haemolysis that cannot be detected visually. To ensure whole blood stability of dexamethasone under the experimental conditions a control blood sample spiked at 250 ng/ml was tested after incubation at 37°C for 120 minutes. In comparison to freshly prepared blood the difference in dexamethasone concentration was less than 5% indicating analyte stability under the experimental conditions used.

Table 2.11 Effect of incubation time on whole blood-to-plasma ratio of dexamethasone

	Time (minutes)				
	0	15	30	60	120
Blood-to-plasma ratio	0.75	0.96	1.00	0.99	0.91
SD	0.08	0.05	0.05	0.01	0.02
Precision (CV %)	10.5	5.2	4.7	1.1	1.8

An extraction efficiency experiment was conducted as described in section 2.4.1 using 15 µl spots to determine the % recovery from DBS and DPS samples at a single concentration of 250 ng/ml in replicate (n=5). Recoveries were 100.4% (CV%, 2.5%) and 104.7% (CV%, 3.8%) for dexamethasone from DPS and DBS, respectively. On investigation of matrix effects (using the method outlined in section 2.4.2) the DPS matrix was not found to significantly (≤ 15%) affect dexamethasone signal intensity

(Table 2.12). Comparisons between DPS and DBS measurements could therefore be based on the assumption of equal recoveries of dexamethasone from each dried matrix.

Table 2.12 Matrix effects arising from dried plasma spots on dexamethasone signal intensity n=5

	Nominal conc. (ng/ml)	
	50	800
Matrix effect % (mean)	14.7	0.5
Precision (CV%)	8.2	3.0

2.4.9.2 Extent of red blood cell partitioning

The extent of RBC partitioning was assessed using blood spiked to produce a single sample of dexamethasone at 50, 250 and 800 ng/ml. Liquid blood samples were incubated at 37°C with mild agitation. After a pre-determined incubation time of 30 minutes, replicate (n=3) 15 µl DBS and DPS were produced at each concentration and allowed to dry prior to extraction. The whole blood-to-plasma ratio was calculated as described in section 2.4.9.1. The following equation formed the basis for the RBC association calculation (Rowland and Tozer, 1995):

$$Cb\,.Vb = Cp\,.Vp \;+\; Cbc\,.Vbc$$

Amount in blood	Amount in plasma	Amount in blood cells

Where *Cb* = blood concentration of drug

Vb = blood volume

Cp = plasma concentration of drug

Vp = plasma volume

Cbc = blood cell concentration of drug

Vbc = volume occupied by blood cells

The volume occupied by blood and plasma is related to haematocrit (*H*) and therefore the above equation can be re-written as:

$$Cb.1 = Cp(1 - H) + Cbc\,.H$$

Rearrangement of the equation gives:

$$\left[1 - \left(Cp\frac{(1-H)}{Cb}\right)\right] = fraction\ of\ drug\ in\ blood\ cells$$

Using the haematocrit value of the control blood (packed cell volume, 0.43) and substitution of concentration with peak area the equation below was used to calculate the extent of association with RBCs. The fraction calculated was multiplied by 100 to give the percentage association value.

$$\left[1 - \left(PAp\frac{(1-H)}{PAb}\right)\right] \times 100$$

Factors which affect the extent of RBC partitioning include the chemical nature of the compound, pH, temperature and concentration (Hinderling, 1997). Drug lipohilicity has been identified as the most important factor determining the extent of partitioning. Dexamethasone is a lipophilic compound (log *P*, 1.83) and therefore would be expected to associate with RBCs (Núñez and Yalkowsky, 1997). The blood-to-plasma ratio determined for dexamethasone was close to 1 indicating a degree of association with RBCs. The determined value is comparable to the reported literature *in-vitro* values of

0.80, 0.81 and 0.93 in healthy adults (Araki *et al.*, 1966, Tsuei *et al.*, 1980, Petersen *et al.*, 1983). Dexamethasone has been reported to be 68% and 77% bound to proteins, mainly albumin in the plasma component of whole blood (Peets *et al.*, 1969, Tsuei *et al.*, 1980, Cummings *et al.*, 1990). Therefore protein binding may be restricting a more extensive RBC association. For dexamethasone, plasma protein binding is likely to be the most important factor in interpreting PK data when using either plasma or whole blood measurements. Previous studies have shown linear protein binding kinetics for dexamethasone in plasma at therapeutic drug concentrations between 10 and 1000 ng/ml (Peets *et al.*, 1969, Cummings *et al.*, 1990). However, a decrease in albumin concentration or displacement of bound drug can result in decreased protein binding and therefore an increase in unbound drug that is available to associate with RBCs (Cummings *et al*., 1990). Under these circumstances, changes in the extent of partitioning would depend on the binding affinity and capacity of RBCs for dexamethasone (Brinkmann *et al.*, 1972). Studies involving other steroids have shown differing extents of RBC association (20 - 60%) according to compound polarity (Holzbauer, 1972). In the current study, the percentage of dexamethasone associated with RBCs did not change significantly with increasing concentration indicating linear distribution kinetics over the calibration range of the assay (Table 2.13). Similar findings have been previously reported for dexamethasone under *in-vitro* conditions for concentrations between 100 and 300 ng/ml (Tsuei *et al.*, 1980).

A blood-to-plasma ratio of 1 suggests an equal distribution of drug between plasma and RBC components of whole blood. Furthermore, the partitioning of dexamethasone does not appear to be concentration dependent over the concentration range studied. Therefore either blood or plasma could potentially be used to measure dexamethasone

Table 2.13 Effect of concentration on the extent of dexamethasone partitioning

	Nominal conc. (ng/ml)		
	50	250	800
Blood-to-plasma ratio	1.05	1.11	1.06
SD	0.11	0.04	0.12
Precision (CV %)	10.1	3.9	11.3
Red blood cell association (%)	45.7	48.8	46.3

drug concentrations provided there are no significant changes in plasma protein binding. Additionally, DBS PK estimates can be compared with those in plasma without a prior need for conversion of concentration measurements.

The results obtained from the current experiment reflect the partitioning behaviour of dexamethasone in adult blood. RBC association affinities have however, been shown to vary between neonates and adults. For example in infants the blood-to-plasma ratio of digoxin is 3 fold higher compared to adults (Kelly *et al.*, 1983). Due to obvious ethical reasons all experiments were carried out using adult venous blood. A practical solution could be to use umbilical cord blood as a surrogate for neonatal blood. This approach was used by Tsuei *et al* to investigate *in-vitro* differences in dexamethasone RBC association between adults and neonates. A small but significant difference in blood-to-plasma ratio between adult venous blood (0.81) and umbilical venous blood (1.04) was reported (Tsuei *et al.*, 1980). The investigators went on to show differences in dexamethasone protein binding between adult venous plasma (67%) and umbilical venous plasma (61%). This could explain the greater blood-to-plasma ratio found in umbilical vein blood. The difference was not as large as that reported for digoxin but

highlights the importance of considering differences which may exist in blood cell association between adults and neonates.

2.4.10 Stability

2.4.10.1 Solvent

The stability of dexamethasone in stock solution was determined in replicate (n=5) by comparing the peak area of solutions analysed after storage to those which had been freshly prepared. Stock solutions were considered stable if differences in peak area were ≤ 15%. To test stability the lowest spiking concentration (315 ng/ml) was prepared using stock solutions as described in section 2.3.2. Dexamethasone was determined to be stable in solvent for at least 9 days stored at 4°C and 48 hours at room temperature with a difference of less than 11.7% (CV%, 6.5%) and 6.1% (CV%, 7.9), respectively.

2.4.10.2 Dried blood spot

The stability of dexamethasone in DBS samples was investigated following storage at 4°C and room temperature (22°C) with and without desiccant. Samples for stability assessment were prepared by applying 30 µl aliquots of blood at concentrations of 50 and 800 ng/ml onto filter paper. Following storage under various conditions the entire spot on the filter paper was extracted with a 250 µl volume of methanol:water (70:30, v/v). DBS samples were considered stable if differences in peak area between stored and fresh samples were ≤ 15%. Dexamethasone was found to be stable within a DBS sample for at least 7 days at room temperature and 28 days at 4°C. Differences in peak area between samples stored at 4°C for 28 days and fresh samples were -7.5 and -1.6% at 50 and 800 ng/ml, respectively, indicating analyte stability under these storage

conditions (Table 2.14). The stability of samples was not affected by storage with desiccant.

Table 2.14 Stability of dexamethasone in DBS samples stored at 4°C for 28 days n=5

Nominal conc. (ng/ml)	50		800	
	Fresh	Stored	Fresh	Stored
Peak area (mean)	3134.30	2899.59	44572.92	43838.61
SD	387.62	396.26	2299.82	2557.30
CV%	12.4	13.7	5.2	5.8
% Difference	-7.5		-1.6	

2.4.10.3 Drying temperature

In comparison to laboratory drying conditions the NICU can be warmer with temperatures ranging between 22 and 25°C (Thomas *et al.*, 2010). To determine the effect of drying temperature on dexamethasone stability 30 µl DBS samples at 50 and 800 ng/ml were prepared and immediately transferred to an oven set to 30°C (n=5). Following storage for a period of 48 hours under these conditions, 8 mm discs from DBS samples were extracted and the resulting peak area ratios compared with those obtained from the analysis of DBS samples left to dry at room temperature (22°C). The difference in peak area ratio between samples at 50 and 800 ng/ml was 1.8% and 4.6%, respectively (Table 2.15). This indicates that dexamethasone is stable in DBS samples at the temperatures expected in the environment where sample collection and drying will take place. There is an increased potential for shorter drying times and consequently smaller spot sizes at elevated temperatures. The results do not suggest

dexamethasone quantification was adversely affected and noticeable differences in spot size were not apparent.

Table 2.15 Stability of dexamethasone in DBS following drying and storage at 30°C for 48 hours n=5

Nominal conc. (ng/ml)	50		800	
	22°C	30°C	22°C	30°C
Peak area ratio (mean)	0.55	0.56	8.51	8.91
SD	0.03	0.08	0.56	0.83
CV%	5.2	14.4	6.6	9.3
% Difference	+1.8		+4.6	

2.4.10.4 In-process

The stability of extracted DBS samples were tested at concentrations of 50 and 800 ng/ml. Replicate (n=5) 8 mm discs taken from DBS samples at each concentration were extracted, transferred to an LC vial and stored at room temperature for 48 hours. In-process stability was determined by comparing the peak area ratios of fresh extracts with those stored for 48 hours. Differences at each concentration were within 7% giving assurance of stability post extraction and during LC-MS analysis (Table 2.16).

Table 2.16 Stability of dexamethasone in processed samples for 48 hours at 22°C n=5

Nominal conc. (ng/ml)	50		800	
	Fresh	Stored	Fresh	Stored
Peak area ratio (mean)	0.52	0.56	6.67	6.61
SD	0.02	0.08	0.84	0.45
CV%	4.1	13.7	12.5	6.8
% Difference	+6.7		+1.0	

2.5 Conclusion

A simple LC-MS method utilising DBS sampling has been developed for the quantification of dexamethasone in human whole blood based on the requirement of a 30 µl blood sample. The validated method was determined to be accurate and precise with a RE and CV ≤ 15% at all tested concentrations. High recovery of dexamethasone (99%) was observed from DBS samples using a simple liquid extraction method. This is similar to the reports of Christianson *et al* who reported a recovery of 83 to 94% for dexamethasone from dried synovial fluid spots on Ahlstrom paper 226 (Christianson *et al.*, 2010). The LLOQ of 15 ng/ml is not as low as some previously reported methods, however, the degree of sensitivity conferred was considered acceptable for application to the current study objective.

The degree of flexibility around blood volume collection and the spotting device used is particularly advantageous to test sites such as the clinical environment where accurate pipetting may be difficult to achieve. Exposure of blood to petroleum jelly did not affect the quantification of dexamethasone in DBS samples and therefore its use on the NICU

during capillary blood collection is not considered to be a problem. In agreement with the findings of others the most noticeable effect on assay performance was observed with changing haematocrit. However, the overall variation in % bias was within the accepted criteria of ≤ 15% for the tested haematocrit range and therefore the developed method was considered to be robust. Stability of dexamethasone within DBS samples has been shown following storage at room temperature for 7 days and up to 28 days for samples kept at 4°C in a refrigerator. In addition, an elevated drying temperature of 30°C with subsequent storage for 48 hours in this warmer environment does not affect the stability of dexamethasone.

The validated DBS method was used for the measurement of circulating dexamethasone concentrations in preterm neonates on the NICU. The study design and results from application of the DBS methodology are presented in Chapter 3.

Chapter 3

Pharmacokinetics of Dexamethasone in Preterm Neonates Using Dried Blood Spot Analysis

3.1 Introduction

Several studies have shown that prenatal corticosteroids in women at risk of preterm delivery reduce the incidence of respiratory distress syndrome and infant mortality (Roberts and Dalziel, 2010). The beneficial effects of exogenous surfactants in reducing the incidence of CLD and mortality are also clear (Stevens *et al.*, 2007). Despite the use of these treatments CLD of the newborn remains a major problem on the NICU. Inflammation has been identified as central to the pathogenesis of the disease and many of the prevention and treatment modalities have been focused on potential anti-inflammatory drugs (Watterberg, 2006). Postnatal dexamethasone is a proven effective anti-inflammatory treatment for the prevention and treatment of CLD (Halliday *et al.*, 2009b, Halliday *et al.*, 2009a, Halliday *et al.*, 2010). It is now apparent that modulation of the inflammatory response and protection of the lungs afforded by dexamethasone may occur at the expense of other organs including the brain. Routine use in neonatal practice is not recommended until there is sufficient scientific knowledge to support the selection of the optimal dose, frequency of administration and corticosteroid in CLD (Halliday, 2001). At present, our understanding of the PK of dexamethasone in neonates is limited to two small studies and therefore further investigations are necessary (Charles *et al*., 1993, Lugo *et al*., 1996).

3.2 Aim

The aim of this study was to clinically evaluate the potential of DBS analysis for the quantification of dexamethasone in PK studies involving preterm neonates. For this purpose DBS samples were collected from preterms receiving dexamethasone treatment for CLD. Dexamethasone levels determined from DBS samples using LC-MS

quantification (Chapter 2) were used to estimate individual PK parameter estimates (CL, V and half life). DBS derived estimates were then compared with plasma PK values reported in neonates.

3.3 Patients and study design

3.3.1 Patients

Patients were recruited from the NICU at University Hospitals of Leicester NHS Trust. A significantly smaller percentage of patients were treated with dexamethasone than expected based on the results of an audit of dexamethasone usage in CLD over 2007 to 2008. It therefore became necessary to include a second NICU centre located at Sheffield Teaching Hospitals NHS Trust to provide support with patient recruitment. Ethical approval for the conduct of the research and study protocol was granted by the Leicestershire, Northamptonshire and Rutland NHS Research Ethics Committee 2, the Research and Development departments at Leicester and Sheffield hospitals and De Montfort University Research Ethics Committee. All patients prescribed dexamethasone for the treatment of CLD were eligible for study participation on the provision of informed written consent from their parents or guardians (Appendix I). Exclusion criteria included infants whose parents or guardians refused consent for participation and any infant whom the neonatology consultant responsible for their care considered unsuitable for study.

3.3.2 Study protocol

Patients recruited received IV dexamethasone according to local NICU protocol. The dosing protocol at Leicester was 250 µg/kg twice a day for three days, then 100 µg/kg

twice a day for three days, then 50 µg/kg twice a day for two days, and finally 25 µg/kg twice a day for two days. Patients recruited at the Sheffield NICU received 150 µg/kg once a day for four days, then 100 µg/kg once a day for two days, then 50 µg/kg once a day for two days followed by 20 µg/kg once a day for two days. Blood spots (30 µl) for dexamethasone quantification were collected at random time intervals from each patient during the course of dexamethasone treatment. Where possible a single DBS sample was collected prior to dexamethasone treatment and after discontinuation. Samples were collected into lithium heparin coated capillary tubes and immediately spotted onto 903 paper with the aid of a suction bulb (Figure 3.1). All blood samples were collected opportunistically after bloods had been obtained for the clinical care of the patient. Therefore patients were not subjected to extra needles for the purposes of the study. In cases where blood flow ceased further attempts to obtain blood via a second skin puncture were not made. The number of samples collected per day was therefore governed by the number of sampling opportunities available. No more than three DBS samples were collected on any single day and the total number of DBS samples that could be collected during the entire study period was limited to twenty (equivalent to 0.6 ml of whole blood). A record of blood volumes collected was kept on a daily basis for each patient for the duration of the study. All dexamethasone dosing and DBS sampling information along with patient data collected during the study was recorded on case report forms (Appendix II).

3.3.3 Capillary blood collection procedure on the neonatal unit

In the first stage the infant's heel is cleaned, usually with an alcohol swab and allowed to dry. A thin layer of petroleum jelly may be applied onto the skin surface to facilitate

blood collection. An automated disposable lancet device is used to obtain blood from the lateral part of the heel. All blood including the first droplet is collected into a capillary tube or blood tube for clinical tests. In the process of blood collection gentle squeezing is used if necessary to encourage blood flow. After all clinical samples had been obtained capillary blood for the PK study was collected from the sampling site into a capillary tube.

3.3.4 Sample storage and analysis

Blood samples collected onto 903 paper were transferred to a dedicated research room on the NICU for drying. To avoid risk of contamination from the bench surface samples were dried on an elevated rack. Once dried, samples were sealed in plastic bags and transported to De Montfort University laboratories for storage at 4°C. Patient samples were analysed by LC-MS using the method outlined in Chapter 2 (section 2.3). Concentrations of dexamethasone within DBS samples were determined from the equation generated from a DBS calibration line analysed alongside the patient samples.

A

B

Figure 3.1 An example of a DBS sample collected from a patient (A) and a capillary tube with suction bulb attached (B)

3.4 Pharmacokinetic and statistical analysis

Non-linear regression analysis was performed using NONMEM (version 7.0) with a GNU Fortran Compiler 95. Post processing of NONMEM data was undertaken with PDx-POP (version 4.0, ICON Development Solutions, USA) and Microsoft Excel 2007 (Microsoft Corporation, USA). Initial parameter estimates for dexamethasone were obtained from previous PK studies conducted in neonates (Charles *et al.*, 1993, Lugo *et al.*, 1996). The IV administration of dexamethasone was duration modelled using a fixed value of 0.08 h. This value was suitable since the drug is routinely administered as a bolus dose over approximately 5 minutes. There are various methods that can be used to deal with concentration levels which fall below the LLOQ of an assay. In the current study, for data below the limit of quantification (BLOQ) a replacement approach with BLOQ/2 was implemented where the observed concentration is replaced with a value corresponding to half the LLOQ (i.e. 7.5 ng/ml) (Beal, 2001). For all patients, levels of dexamethasone were undetectable during the last few days of therapy when the lowest doses were administered. Due to the limited information gained from inclusion of all BLOQ values during this period the following criteria was applied. The first two levels BLOQ following a level above the LLOQ were incorporated in the analysis using a BLOQ/2 approach. Subsequent levels BLOQ were removed from the PK analysis. Despite efforts to include a second study centre a limited number of patients were recruited over the 18 month study duration which precluded the execution of the planned population based PK analysis or a formal investigation of covariate effect. Instead, an individual PK analysis was undertaken where DBS concentration data from each patient were separately fitted to a one and two compartment model in the first stage (Appendix III). The First Order estimation method was used as the model fitting

algorithm. Once individual estimates of CL and V were determined these were pooled to determine the mean and variability (SD, CV%) of PK estimates for the study group.

3.5 Results

3.5.1 Demographic and clinical characteristics of study patients

A total of six patients were enrolled onto the study over an 18 month period. Patient 6 was excluded from the PK analysis as dexamethasone concentrations were undetectable in all three DBS samples collected (on days 9 to 10 of treatment). The demographic and clinical characteristics of patients 1 to 5 are presented in Table 3.1. The majority of patients studied were extremely low birth weight (≤ 1 kg) infants. The GA varied between 23.7 and 28.7 weeks and the mean PNA at which patients received dexamethasone treatment was 20 days. No known inhibitors or inducers of dexamethasone metabolism were identified in co-medications prescribed to patients. Clinical data including haematocrit and albumin were recorded with each DBS sample if available from the results of tests ordered for routine care purposes. The range of haematocrit values measured in patients during DBS collection is shown in Figure 3.2. With the exception of a few, haematocrit values were centred around the assay calibration line (haematocrit 35%) and thus within the acceptable ± 5% validation limit.

3.5.2 Dried blood spot samples

A total of 69 DBS samples were collected from patients 1 to 5 (mean 14, range 3 - 20). Five of these samples were collected whilst patients were not on dexamethasone treatment and three samples were below the required standard of quality (due to insufficient sample or visual coagulation) and were therefore not included in the

analysis. The sampling times of the remaining 61 samples collected during dexamethasone treatment are shown in Figure 3.3.

Table 3.1 Characteristics of study patients

Patient	**Sex**	**GA (weeks)**	**BW (kg)**	**PNA (days)**	**PMA (weeks)**	**[a]WT (kg)**	**Co-medications**
1	F	23.7	0.57	23	27	0.59	CA, ER, CE, RA, VA, IN
2	M	24.3	0.75	12	26	0.88	CE, VA, IN, FZ, DO, MO
3	M	28.7	1.30	16	31	1.44	CA, GE, FL, JO, NY, CL
4	F	24.1	0.64	28	28.1	1.12	CA, NY, FU, ME, MI, SP
5	F	27.6	0.57	23	30.9	0.68	CA, RA, FZ
Mean		25.7	0.77	20.4	28.6	0.94	
SD		2.3	0.31	6.4	2.3	0.34	

a. Weight on day of study recruitment.

GA = gestational age; BW = birth weight; PNA = postnatal age; PMA = postmenstrual age.

Co-medications: CA = caffeine, ER = erythromycin, CE = cefotaxime, RA = ranitidine, VA = vancomycin, IN = insulin, FZ = fluconazole, DO = dopamine, MO = morphine, GE = gentamicin, FL = flucloxacillin, JO = Joulie's phosphate, NY = nystatin, CL = clotrimazole, FU = furosemide, MI = miconazole, ME = meropenem, SP = spirinolactone.

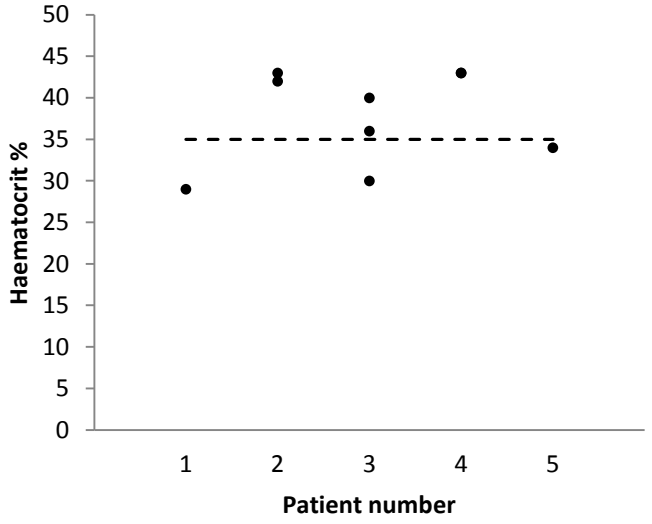


Figure 3.2 Haematocrit values (%) recorded for each patient during the course of dexamethasone treatment

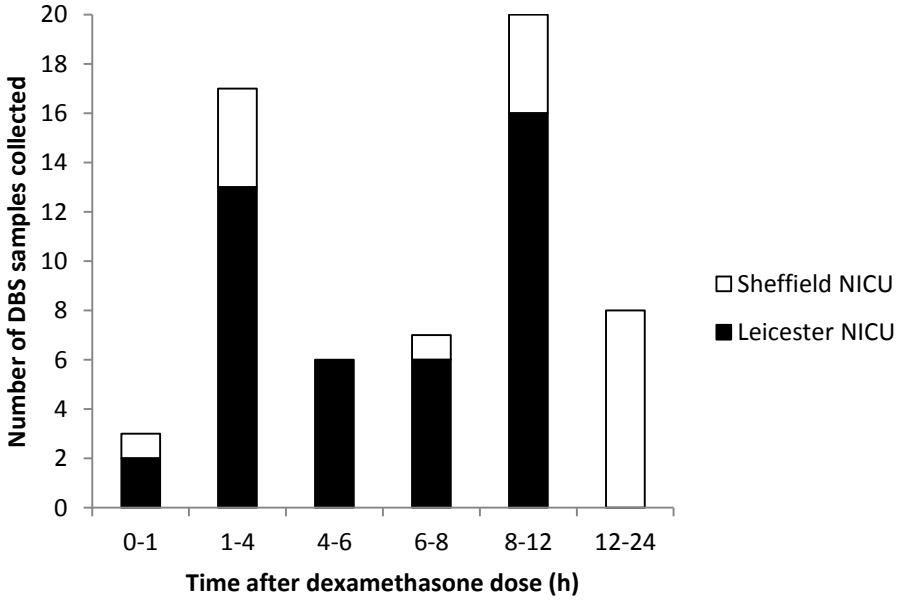


Figure 3.3 DBS sampling times post dexamethasone administration at the Leicester and Sheffield NICU

3.5.3 Pharmacokinetic analysis

A substantial number (32, 52%) of the (61) DBS samples collected during dexamethasone treatment were determined to be BLOQ. 17 of these were replaced with BLOQ/2 and kept in the PK data set and 12 were excluded. Thus the final number of samples used in the PK analysis was 49. The majority of DBS samples BLOQ were collected during the last few days of treatment when the lowest dexamethasone doses were administered or at the end of a dosing interval (Figure 3.4). The sampling times of the 49 DBS concentration measurements used in the PK analysis are shown in Figure 3.5 according to patient number. Samples were collected at a range of time points post dexamethasone administration necessary for the estimation of CL and V.

Three common approaches to handling data reported as BLOQ include omitting data, imputing zero and imputing BLOQ/2 (Beal, 2001). Each of these methods was investigated for their influence on PK parameter estimation. The residual error (difference between model predicted and observed concentrations) was used as an indication of the goodness of fit of the data. A complete omission of data BLOQ resulted in an increase in the estimation of V and a decrease in the estimated CL value for some patients. This type of effect can occur with this method due to an under prediction in the decline of the terminal phase of the slope resulting in a lower CL and a larger V (Duval and Karlsson, 2002). There was no significant difference between the replacement with zero method and BLOQ/2 method on either parameter estimation or residual error. A replacement with BLOQ/2 was applied to the data as previous investigations have shown the zero method can bias estimates. A one compartment model with duration modelled IV infusion and first order elimination was used to fit the data. The primary PK parameters (CL, V) and calculated half life for each patient is

shown in Table 3.2. A mean CL of 0.18 l/h/kg and an interindividual variability of ± 0.05 l/h/kg SD (CV%, 27.8) were estimated for the study group. The interindividual variability in V (CV%, 52.6) was larger in comparison and the half life varied significantly between individuals (1.5 to 7.6 h). The parameter estimates were used to

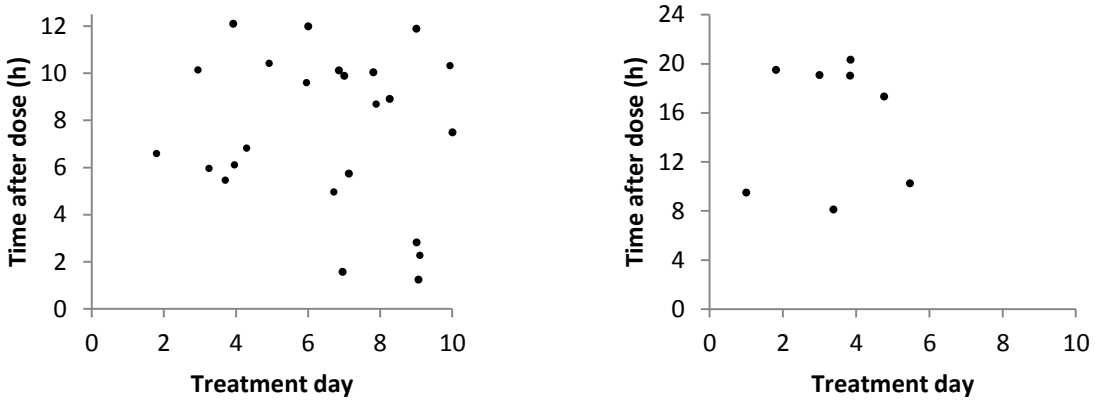


Figure 3.4 Day and time (post dexamethasone dose) of DBS samples for which concentrations determined were BLOQ at Leicester NICU (left) and Sheffield NICU (right)

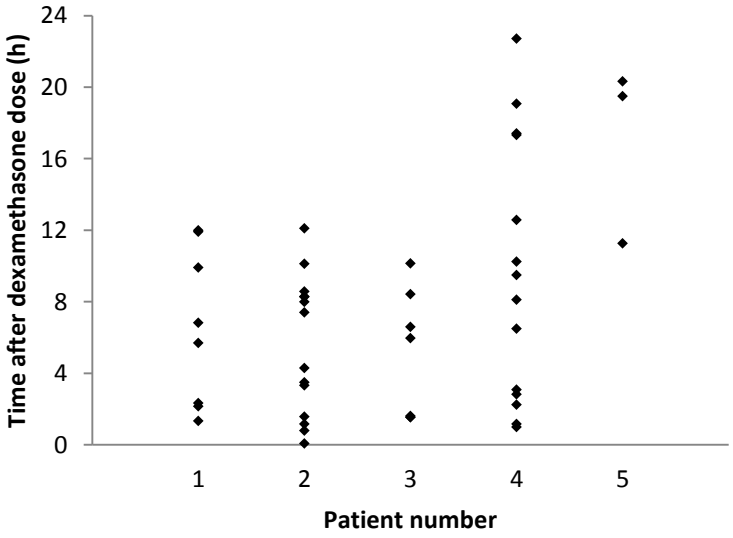


Figure 3.5 DBS sample collection times post dexamethasone dose for each patient

simulate a model predicted concentration-time profile under the weight individualised dosing regimen for each patient. An overlay of individual model predicted PK profiles with observed DBS concentration data show the goodness of fit of the concentration-time data to a one compartment model following non-linear regression (Figure 3.6). For patient 4, DBS samples were collected over two dosing periods (days 3 to 5 and 1 to 5, respectively) separated by a 2 week interval where no dexamethasone was administered. The PK profiles during the first and second dosing period are therefore presented separately in Figure 3.6. From the visual examination of these plots it would appear that a one compartment model provided a reasonably good fit of the DBS data. The under predictions of dexamethasone concentration observed during peak times may suggest distribution into a second compartment prior to a decline in concentration. Supporting this possibility, for patient 2 a two compartment model captured peak concentrations and provided a better fit of the data evidenced by a significant drop in the residual error (Figure 3.7). The sparse nature of the data and limited sampling during the early phase after dosing did not support a more complex model for all patients.

Table 3.2 Pharmacokinetic parameters of dexamethasone during multiple dose administration to 5 premature neonates

Patient	[a]Dosing regimen [b](day)	CL (l/h/kg)	V (l/kg)	[c]$t_{1/2}$ (h)
1	1 (6)	0.24	2.14	6.1
2	1 (1)	0.12	0.75	4.2
3	1 (1)	0.21	0.47	1.5
4	2 (1)	0.14	1.50	7.6
5	2 (3)	0.18	1.79	6.0
Mean		0.18	1.33	5.1
SD		0.05	0.70	2.3
CV%		27.8	52.6	

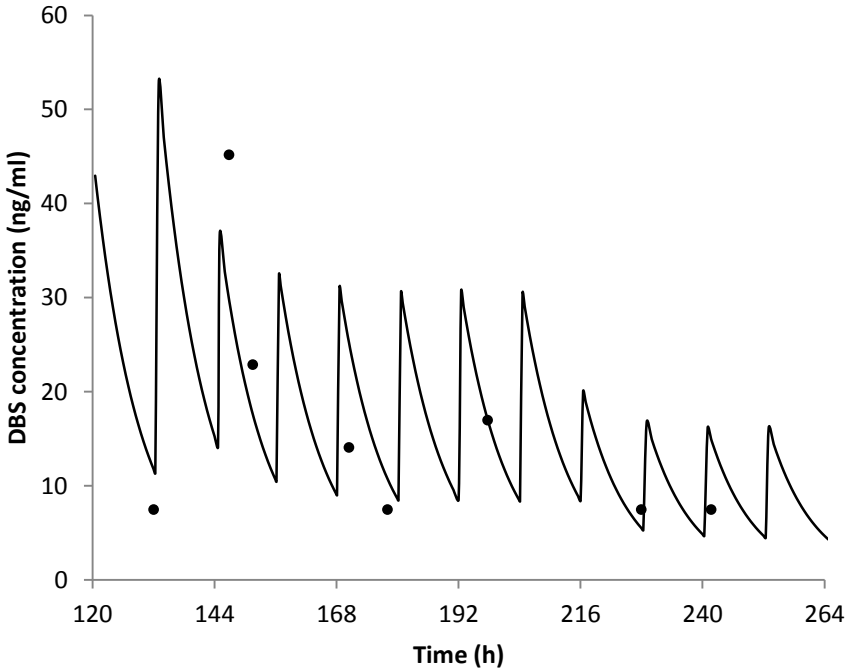
a. Dosing regimen 1 (Leicester NICU) : dexamethasone 250 µg/kg twice a day for three days, then 100 µg/kg twice a day for three days, then 50 µg/kg twice a day for two days, and finally 25 µg/kg twice a day for two days.

Dosing regimen 2 (Sheffield NICU): dexamethasone 150 µg/kg once a day for four days, then 100 µg/kg once a day for two days, then 50 µg/kg once a day for two days followed by 20 µg/kg once a day for two days.

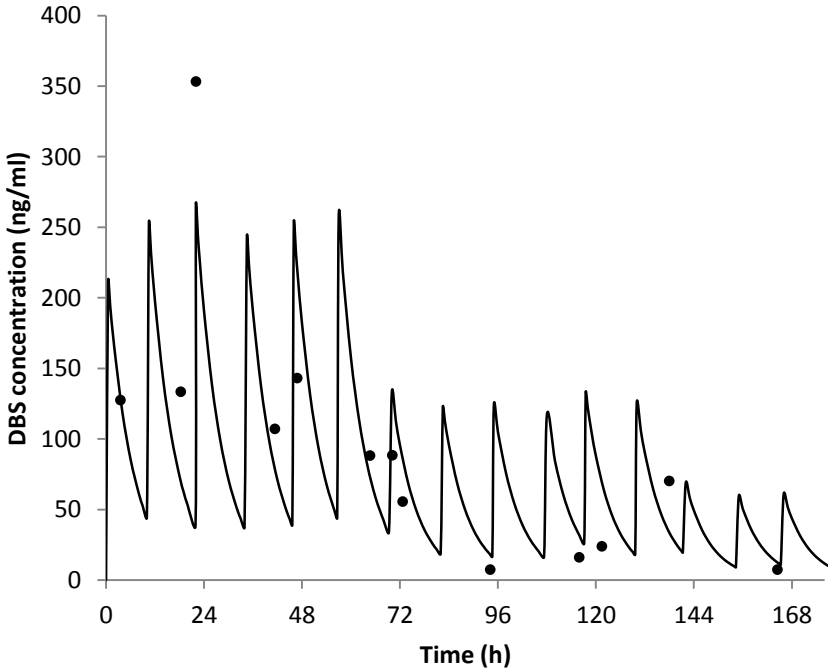
b. Day on which DBS sampling commenced.

c. $t_{1/2}$ calculated using CL and V estimates using the equation: $t_{1/2} = V \times 0.693 / CL$.

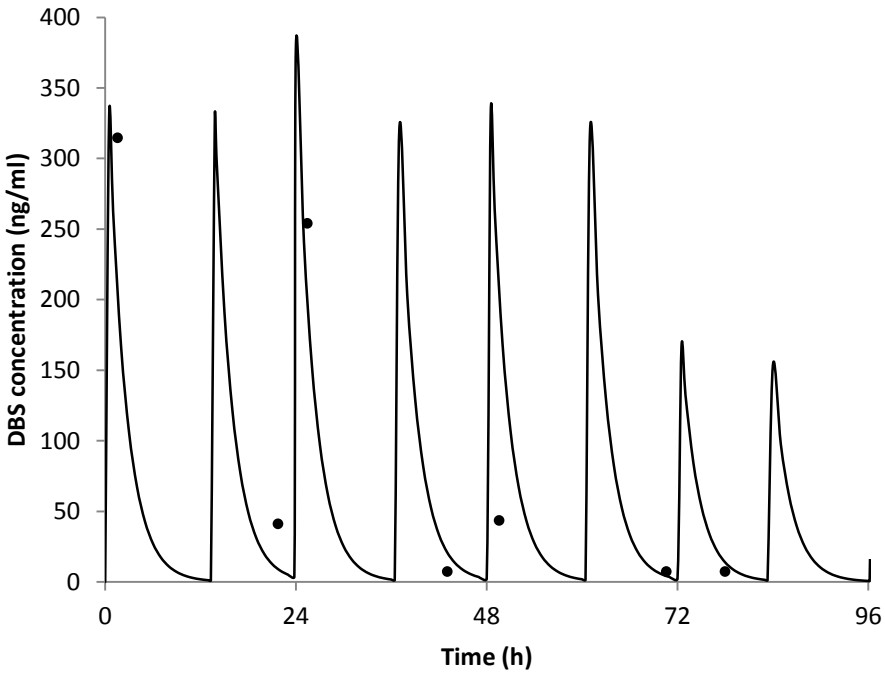
Patient 1



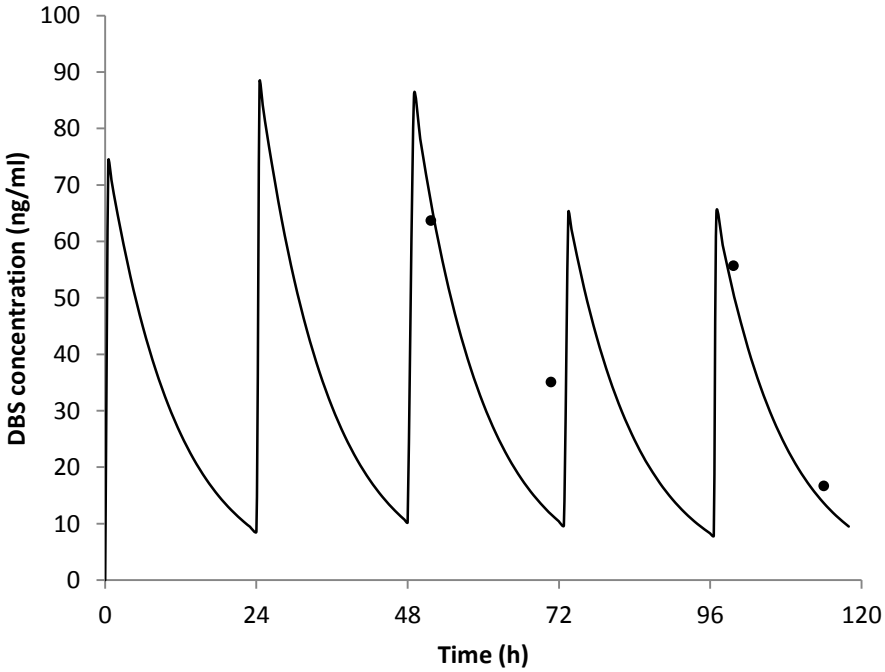
Patient 2



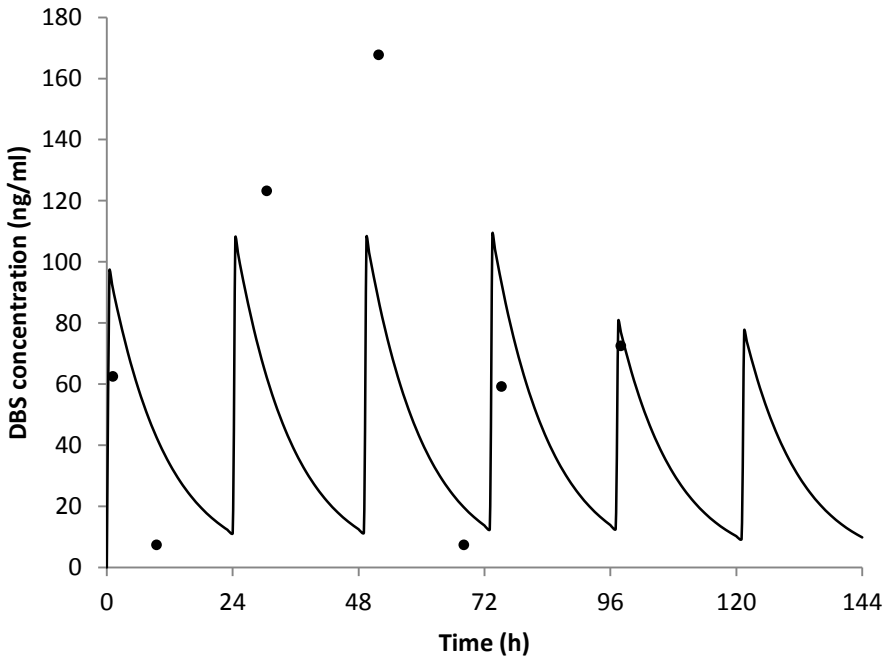
Patient 3



Patient 4: dosing period 1



Patient 4: dosing period 2



Patient 5

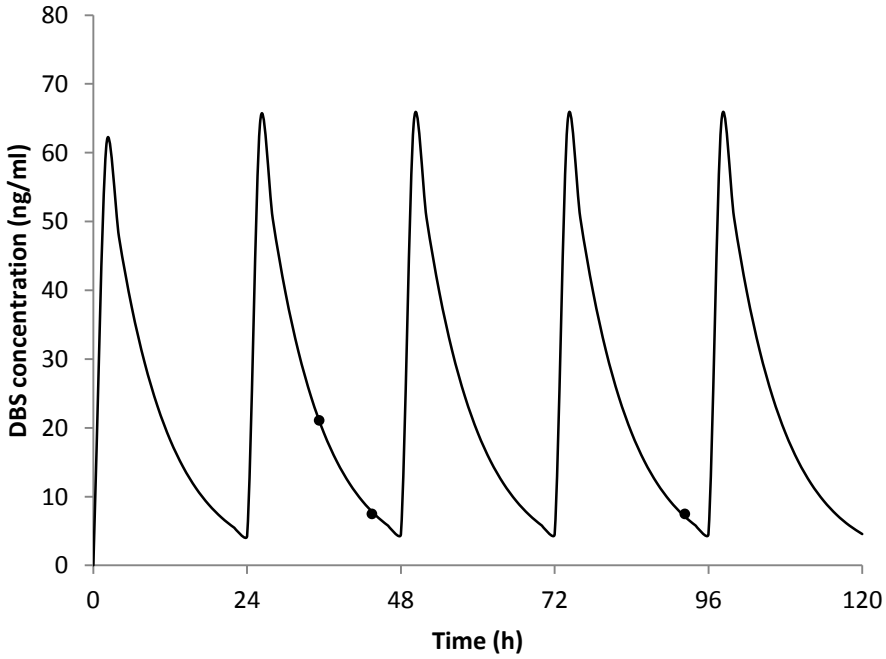


Figure 3.6 Predicted dexamethasone DBS-concentration time profiles (—) and observed DBS concentration data (•) for patients 1 to 5

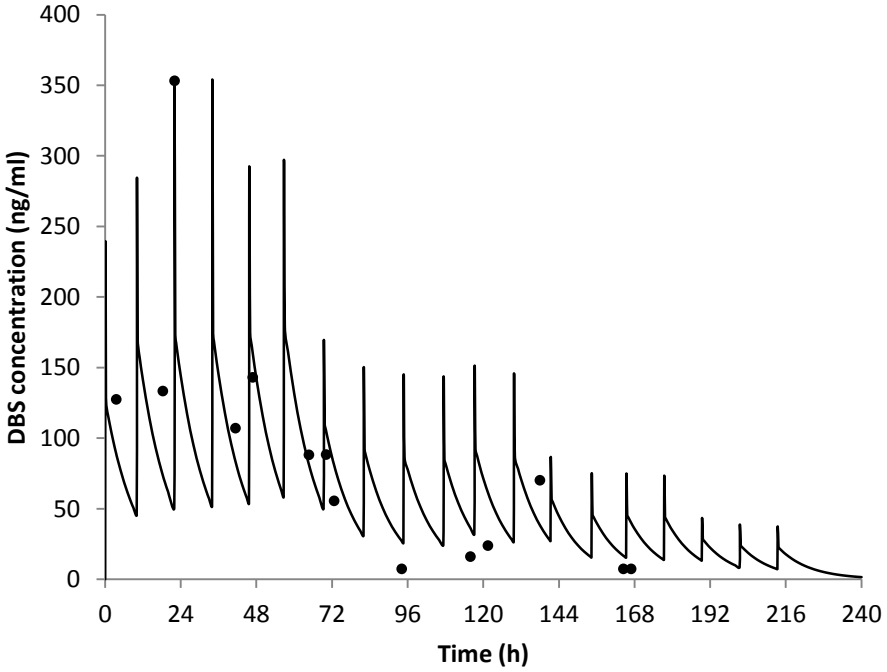


Figure 3.7 Predicted dexamethasone DBS concentration time profile (—) and observed concentration data (•) for patient 2 data fitted to a two compartment model

The limited number of patients studied precluded a formal covariate regression analysis to facilitate an understanding of the variability in parameter estimates. Instead, covariate-parameter relationships were explored by simple linear regression of individual estimates against age (GA, PNA and PMA) and weight. A positive correlation ($r^2 = 0.531$) between GA and CL was observed but this was no longer evident after the weight normalisation of CL estimates. Figure 3.8 shows the regression of weight on study enrolment against PK parameter estimates. A correlation ($r^2 = 0.644$) was observed between weight and CL highlighting the justification for normalising CL by incorporating weight into the dosage calculation. After weight normalisation of CL estimates, the correlation between CL and weight was no longer evident. An inverse correlation ($r^2 = 0.626$) between V and weight became apparent following the weight

normalisation of V. In addition, the elimination half life decreased with increasing weight. Based on the positive correlation observed between V and half life (Figure 3.9), it appears that a larger V significantly contributes to the extended half life in low weight infants in this study group.

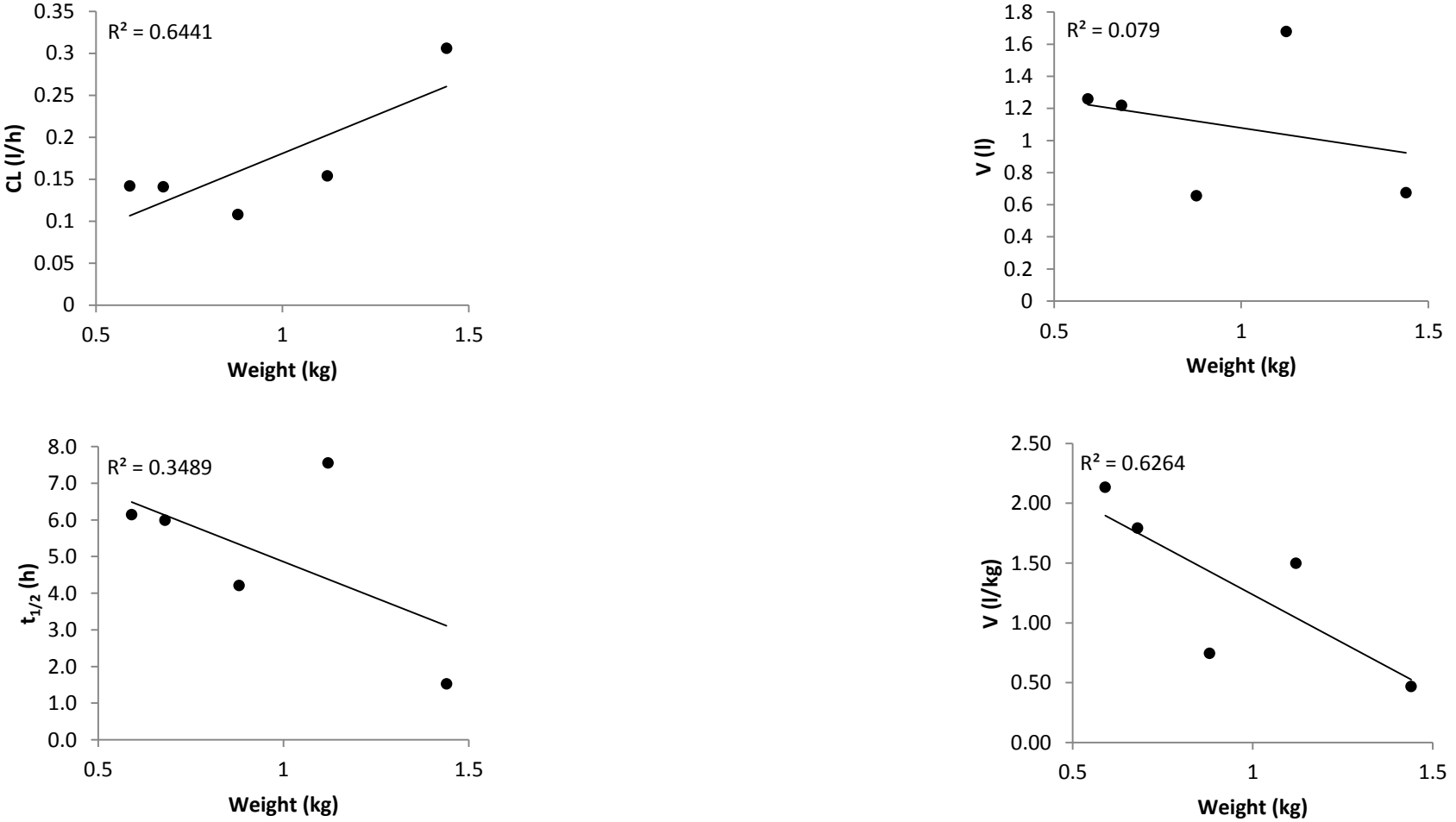


Figure 3.8 Relationship between dexamethasone PK parameters (CL, V, weight normalised V and $t_{1/2}$) and weight on study enrolment

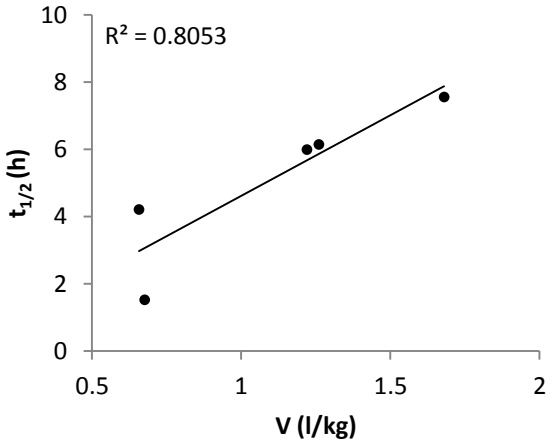


Figure 3.9 Relationship between V and $t_{1/2}$

3.6 Comparison with plasma pharmacokinetic estimates

The measured dexamethasone DBS concentrations (7.5 to 353.3 ng/ml) were comparable to values of 3.4 to 406.9 ng/ml previously reported in plasma for this age group after administration of the same weight normalised dose (250 μg/kg). A comparison of PK estimates determined using DBS analysis with those obtained in two previously reported plasma based non compartment studies in neonates is shown in Table 3.3 (Charles *et al.*, 1993, Lugo *et al.*, 1996). The mean CL estimated (0.18 l/h/kg (SD, 0.05)) was similar to the mean value of 0.14 l/h/kg (SD, 0.03) reported in the study by Charles *et al* (n=7). In a later study by Lugo *et al* (n=9) a mean CL value of 0.30 l/h/kg (SD, 0.25) was found which is almost twice the value obtained here. A large degree of variability in CL was observed amongst the study population reported by Lugo *et al* with estimates ranging between 0.06 and 0.73 l/h/kg. Following an analysis of CL stratified according to GA the authors noted a significant difference in CL between neonates ≤ 27 weeks (mean, 0.10 l/h/kg; SD, 0.05) and above 27 weeks (mean, 0.45 l/kg/h; SD, 0.22). This suggests that the greater mean GA of the patients in the

study by Lugo *et al* (27.3 weeks) compared with that of Charles *et al* (25.6 weeks) and the present study (25.7 weeks) may account for the differences in mean CL estimates. The mean CL was lower than the values of 0.23 l/h/kg and 0.34 l//h/kg in healthy adults (Tsuei *et al.*, 1979, Rose *et al.*, 1981) and 0.33 l/h/kg reported in children with croup or head injury (Richter *et al.*, 1983). The mean V (1.33 l/kg) in the current study was lower than that reported by Lugo *et al* (1.78 l/kg) and Charles *et al* (1.91 l/kg). However, when the V estimates in the study by Lugo *et al* were examined according to GA the authors found that for infants born at less than 27 weeks (mean, 25.8 weeks) the V was considerably smaller compared with infants born after 27 weeks (mean, 28.5 weeks) gestation (1.26 versus 2.19 l/kg). The distribution volume obtained in the current study was therefore very similar after consideration of GA.

The mean half life determined for the study group (5.1 h) was lower than previous reports of 6.8 and 9.3 h. The elimination half life is not an independent PK parameter and is determined by both CL and V. The reason for the shorter mean half life in the current study appears to be due to the smaller V determined in patients. In healthy adults, mean half lives of 2.9 h (SD, 0.8), 3.1 h (SD, 1.1) and 5.6 h (SD, 1.4) have been reported (Tsuei *et al.*, 1979, Rose *et al.*, 1981, O'Sullivan *et al.*, 1997). The difference in half life between neonates and adults determined by the current study is thus less marked compared with previous findings. However, if the range of half lives (1.5 to 7.6 h) is considered, the results of this study like previous ones highlight the large interindividual variability of PK parameters and the prolonged half life that can be expected in low weight neonates.

Table 3.3 A comparison of PK estimates obtained using DBS quantification with previous studies in plasma. PK estimates are reported as mean (SD) and range. GA = gestational age; BW = birth weight; PNA = postnatal age; PMA = postmenstrual age; WT = weight.

Study	Dexamethasone dose regimen	Patient demographics					Sampling design	CL (l/h/kg)	V (l/kg)	$t_{1/2}$ (h)
		GA (wk)	BW (kg)	PNA (days)	PMA (wk)	WT (kg)				
This study n=5	Tapering regimen 250 to 25 µg/kg twice daily or 150 to 20 µg/kg once daily (IV)	25.7 (2.3)	0.77 (0.31)	20.4 (6.4)	28.6 (2.3)	0.94 (0.31)	Random sampling 9 (mean) samples 30 µl capillary DBS LC-MS, LLOQ 15 ng/ml	0.18 (0.05) 0.12 - 0.24	1.33 (0.70) 0.47 - 2.14	5.1 (2.3) 1.5 - 7.6
(Charles *et al.*, 1993) n=7	Patients studied on day 1 after a 400 µg/kg once daily dose (IV)	25.6 (0.5)	0.74 (0.15)	16.9 (6.0)	27.9 (1.1)	0.84 (0.16)	5 time points (0.5, 1, 2, 6, 12, 24 h) ~0.2 ml arterial blood 0.1 ml plasma, LC-UV, LLOQ 10 ng/ml	0.14 (0.03) 0.10 - 0.17	1.91 (0.48) 1.18 - 2.52	9.3 (3.3) 5.9 - 16.1
(Lugo *et al.*, 1996) n=9	Patients studied on day 1, 2 or 3 of treatment. Dose range of 208 to 503 µg/kg twice daily (IV)	27.3 (1.7)	0.89 (0.27)	21.8 (10.5)	30.4 (2.2)	Not given	5 time points post single dose (0.5, 1, 3, 6, 12 h) Arterial/venous blood, plasma volume not given, RIA, LLOQ 1ng/ml	0.30 (0.25) 0.06 - 0.73	1.78 (0.52) 1.00 - 2.36	6.8 (4.9) 2.2 - 15.8

3.7 Discussion

Both compartment and non compartment PK analysis has been previously used to describe the disposition of dexamethasone in humans. In children and adults dexamethasone has been shown to display one and two compartment disposition (Tsuei *et al.*, 1979, Richter *et al.*, 1983). Whilst in neonates PK analysis has been performed using a non compartmental approach (Charles *et al.*, 1993, Lugo *et al.*, 1996). In the current study a one compartment model provided a reasonable fit of the DBS concentration-time data across the duration of a dexamethasone dose reducing regimen (Figure 3.5). With sparse data the model selected is often an oversimplification of the underlying true PK model (Aarons *et al.*, 1996). A more complex model may have been more suitable but was not supported by the data. Model misspecification is thus a potential contributory factor to the differences between predicted and observed concentration data. Other sources of error are inaccuracies in records of time of drug administration and sample collection and analytical measurement error. Random biological variability in CL and V parameters on different sampling occasions will also contribute to the difference between model predicted and observed concentrations (Karlsson and Sheiner, 1993).

In agreement with previous PK reports in neonates a significant degree of variability in dexamethasone disposition was observed amongst the patients in the present study. The results from the linear regression of covariates against individual parameter estimates indicated the higher V in the lowest weight neonates accounted for their relatively greater half life. It should be noted that the correlation found between V and half life is limited to 5 patients and therefore it is not possible to draw any conclusions on the

relationship observed. As dexamethasone exhibits low aqueous solubility, differences in V would most certainly be expected to arise from differences in tissue binding and not because of distribution into the extracellular fluid compartment. The large interindividual variability in dexamethasone PK in neonates and extended half life in the smallest neonates do however, demonstrate the sub-optimal dosing schedule currently adopted based on body weight alone.

There is only limited information available on the elimination pathway of dexamethasone during the neonatal period. Studies by Araki *et al* in adults have highlighted the difficulties of conjugating synthetic steroids to more water soluble derivatives within the human body (Araki *et al.*, 1966). Subsequent studies in adults have shown that a large percentage (~ 60%) of an administered dexamethasone dose is eliminated renally, mainly in the form of unconjugated polar steroids (Haque *et al.*, 1972). The major urinary metabolite has been identified as 6β-hydroxydexamethasone with dexamethasone or conjugated dexamethasone accounting for only 2.3% of the total dose (Minagawa *et al.*, 1986). Evidence suggests that cytochrome P450 3A4 (CYP3A4) is the key enzyme responsible for the 6β-hydroxylation of dexamethasone (Gentile *et al.*, 1996, Puisset *et al.*, 2007). Enzymes from the CYP3A family make up the largest percentage of enzymes in the adult and foetal liver of which CYP3A7 is the most abundant detected as early as 7 to 8.5 weeks gestation (Yang *et al.*, 1994). After birth levels of CYP3A7 begin to gradually decrease throughout the neonatal period into infancy (Lacroix *et al.*, 1997). In contrast, very low CYP3A4 amounts are detectable prior to birth. Expression of this enzyme steadily increases during the postnatal period reaching adult levels not until after 1 year of age (Stevens *et al.*, 2003). Although it is clear that a transition between CYP3A7 and CYP3A4 expression occurs after birth,

because of the variability in expression observed the exact time course of these changes are not yet fully understood (Hines, 2007). To assess CYP3A activity in neonates Nakamuru *et al* measured the 6β-hydroxycortisol to cortisol (6β-OHC/C) ratio in the urine of preterm and term neonates (Nakamura *et al.*, 1998). A positive correlation was found between GA and the 6β-OHC/C ratio with term neonates showing the highest 6β-OHC/C ratios on day one of life compared to preterm neonates and adults. By day 5 the ratio of 6β-OHC/C had dropped to that below adults in term neonates, perhaps due to changes in CYP3A expression whilst no change was observed during the first 14 days in neonates born prematurely. Although neonates may at least have some capacity these studies suggest that their metabolic capacity for drugs metabolised via CYP3A is likely to be significantly reduced and most pronounced in premature neonates. It is possible that alternative metabolic routes predominate in the neonate to compensate for immaturity of CYP3A enzyme activity and or renal excretion of dexamethasone in the unchanged form plays an important role. Investigations to identify covariates which reflect differences in dexamethasone disposition amongst neonates will be important for determining the most appropriate treatment dose. The findings of a previous study strongly indicate factors other than weight alone (such as GA) are likely to be of importance in accounting for interindividual differences in dexamethasone PK (Lugo *et al.*, 1996). In a recent study, Yang *et al* reported on the population PK of dexamethasone in children with acute lymphoblastic leukaemia (Yang *et al.*, 2008). The regression analysis results from this study indicated age, and to a greater extent albumin to be the most important covariates accounting for the large intraindividual and interindividual variability in CL. The positive correlation between CL and albumin concentration was assigned to differences in hepatic function rather than to changes in

protein binding. In this study, albumin was therefore suggested as a biomarker of hepatic function. The variability in albumin concentration observed was thought to be secondary to prior asparaginase in some children which is known to cause hypoalbuminaemia. Thus albumin may also be of importance in explaining the variability in dexamethasone PK that results from developmental processes and potentially protein binding. On the other hand, if renal mechanisms of drug CL predominate indicators of renal development such as GFR may provide a better understanding of the interindividual variability in PK parameters.

The primary aim of this study was to evaluate the potential of DBS analysis for the determination of dexamethasone PK parameters (CL, V and half life) in neonates. To avoid the practical and ethical difficulties of performing a direct comparison between plasma and blood, a method of validation by comparison with literature plasma PK estimates was chosen. Furthermore, opportunistic sampling provided a minimally invasive study design thereby helping to reduce patient discomfort and improve parental and clinical staff acceptability. The PK estimates determined in the current study were found to be comparable with values reported in studies that have utilised conventional plasma analysis. This indicates the usefulness of DBS in providing information on the PK of dexamethasone in a population that is extremely difficult to study. There is however, potential for the PK of dexamethasone to be complicated by a number of factors specific to normal developmental changes which occur in the neonatal age group. The presence of foetal plasma proteins, qualitatively different albumin and presence of endogenous compounds e.g. bilirubin able to compete for binding sites will invariably lead to unexpected changes in the fraction of unbound drug (Notarianni, 1990). The potential for misinterpreting dexamethasone PK and difficulties in studying

PK-PD (dose-effect) correlations thus hold true whether plasma or whole blood is used to measure drug concentration under these conditions. When the measured total concentration no longer reflects the unbound fraction of drug, it may be difficult to predict a pharmacological effect or make dosing recommendations. The additional variables to consider when using DBS measurements are blood cell partitioning and haematocrit. Changes in haematocrit would not be expected to alter the drug distribution ratio according to the results from the RBC association experiment (Chapter 2, section 2.4.9.2). In the presence of an increased free fraction of dexamethasone in plasma it is possible that an affinity for RBCs drives the movement of increased amounts of dexamethasone into RBCs thereby altering the blood-to-plasma ratio. There is at least some evidence of this possibility from the reported lower plasma protein binding of dexamethasone in conjunction with a higher blood-to-plasma ratio in umbilical venous blood compared with venous blood from adults (Tsuei *et al.*, 1980). Therefore with changes in protein binding, haematocrit may become an influential determinant in the percentage of dexamethasone that associates with RBCs. Partitioning in blood and plasma as a function of albumin concentration was not investigated, but would enable a better understanding of the distribution kinetics of dexamethasone.

Although current data would suggest DBS to be a suitable matrix for the investigation of dexamethasone PK, the study design used has some limitations. Firstly, a larger number of subjects are required to enable an informative conclusion to be made regarding the accuracy as well as precision of dexamethasone PK estimation using DBS. Secondly, a more informative study design would involve simultaneous plasma and DBS concentration measurements to determine the correlation between DBS and plasma concentration-time profiles in neonates themselves. A PK sampling design

involving the collection of a limited number of samples (~3 to 4) at pre-determined time intervals post dose would be suitable for this purpose. Extension of the current dexamethasone DBS method to include DPS analysis would be useful for the simultaneous determination of drug levels using a single methodology. Thirdly, an improvement in the current sensitivity of the DBS assay will be essential prior to future application. The doses received by patients in the current study are relatively high compared with some NICUs where doses as low as 50 μg/kg once a day are used (Yates and Newell, 2010). It is clear that a successful population PK study of dexamethasone in CLD will only be possible through a multi-centre study. Therefore DBS analysis will only be useful if it is adequately sensitive to measure dexamethasone levels in neonates following the range of doses used in clinical practice. A LLOQ of at least 1 ng/ml would be required to support a population PK study and subsequent PK-PD dose finding studies.

The contrasting accumulative doses administered to neonates at the Leicester and Sheffield NICU (2400 and 940 μg/kg, respectively) highlight the current clinical problem and the resulting variability in dexamethasone exposure in preterms (Figure 3.5). Interindividual differences in dexamethasone PK and variations in clinical practice mean that some infants may be at greater risk of adverse effects. A population based PK study to evaluate dexamethasone pharmacokinetics and explore potential covariate relationships in neonates treated for CLD is urgently needed. The simplicity and ethical advantages of DBS analysis to facilitate such studies are clear, but further investigations will be important to understand plasma to whole blood differences in dexamethasone concentration which may result in neonates and how these may affect PK estimation.

Chapter 4

Quantification by Mass Spectrometry of Caffeine in Dried Blood Spot Samples

4.1 Introduction

4.1.1 Caffeine quantification in biological fluids

Various analytical techniques have been applied to the quantification of caffeine concentrations in biological fluids including RIA, gas chromatography and LC with UV or MS detection (Table 4.1). A relatively large proportion of published methods have used LC-UV methodologies due to the simplicity of the technique and the relatively good quantification limits (80 ng/ml) achieved from micro volume samples (25 µl plasma) (Dorrbecker *et al.*, 1984). To overcome the possibilities of co-eluting endogenous interferences and improve the selectivity of caffeine quantification both LC-MS and LC-MS/MS based techniques have been utilised (Hieda *et al.*, 1995, Ptolemy *et al.,* 2010). Selectivity gained with MS/MS detection has enabled the separation of multiple caffeine metabolites in some cases without the need for full chromatographic separation (Ptolemy *et al.*, 2010). In addition to the selectivity afforded by MS, good detection limits have been achieved (Zhang *et al*., 2008, Li *et al*., 2010). Caffeine has been quantified in plasma, whole blood and urine. It has also successfully been measured in saliva where it is present at appreciable concentrations following dosing (Emory *et al*., 1988). A DBS technique for the simultaneous quantification of caffeine and theophylline was reported as early as 1981 by Brazier *et al* for the purposes of TDM in newborns with AOP (Brazier *et al*., 1981). The methodology was based on the collection of a 30 µl whole blood sample onto S&S 2992 paper using a direct approach. Once dried the entire blood spot was cut out using a 10 mm punching device and transferred to a clean glass tube for solvent extraction with chloroform:isopropanol (95:5, v/v). Methylxanthine derivatisation was subsequently

performed prior to GC analysis. The validation results indicated linearity ($r^2 = 0.998$) over a calibration range of 500 to 20000 ng/ml. Good accuracy (RE% < 5%) and precision (CV% < 9%) was reported at the concentration levels assessed (2000, 5000, 10000 and 15000 ng/ml). The use of a direct blood sampling approach with extraction of the entire spot does however, raise questions on the accuracy of the DBS method for concentration measurements in patient samples. The sensitivity of the method (LOD, 500 ng/ml) is not as high as other caffeine methods however, it was sufficient for its intended purpose. The investigators reported that the developed DBS method had been successfully used in PK studies and TDM in preterms in the clinical setting. Results were not published and therefore a comparison with plasma data was not possible. More recently a caffeine DBS method using LC-MS/MS detection has been established at GlaxoSmithKline (GSK) laboratories, UK (Lad, 2010). A LLOQ of 250 ng/ml was achieved from a 15 µl DBS sample on FTA Elute paper. Without the need for several work-up steps and compound derivatisation the developed method is simple, sensitive and practical.

4.1.2 Dried blood spot method for caffeine quantification

The bioanalysis arm of the caffeine PK study in neonates was supported through collaboration with the analytical team at GSK (Hertfordshire, Ware). All DBS samples collected from neonates were analysed at GSK laboratories using the caffeine DBS method developed by Lad, 2010. The methodology was validated in accordance to FDA regulatory criteria (Guidance for Industry; bioanalytical method development, 2001). A description of this methodology with validation results is presented in section 4.2.

Table 4.1 A comparison of selected analytical techniques previously used for the quantification of caffeine in biological fluids

Reference	Analytical technique	LLOQ	LOD	Volume & type of biological fluid
(Emory *et al*., 1988)	RIA		250 ng/ml	0.3 to 0.5 ml saliva
(Dorrbecker *et al*., 1984)	LC-UV	80 ng/ml		25 µl plasma
(Schreiber-Deturmeny and Bruguerolle, 1996)	LC-UV	100 ng/ml		50 µl plasma
(Ghosheh *et al*., 2000)	LC-UV	30 ng/ml		0.5 ml plasma
(Abu-Qare and Abou-Donia, 2001)	LC-UV	100 ng/ml		0.2 ml plasma
(Hieda *et al*., 1995)	LC-MS		10 ng/ml	0.2 to 1 ml plasma or urine
(Zhang *et al*., 2008)	LC-MS/MS	49 ng/ml		50 µl plasma
(Ptolemy *et al*., 2010)	LC-MS/MS	485 ng/ml		0.5 ml plasma, saliva or urine
(Li *et al*., 2010)	LC-MS/MS	10 ng/ml		0.1 ml plasma
(Lad, 2010)	LC-MS/MS	250 ng/ml		15 µl dried blood spot
(Jafari *et al*., 2011)	ES-ion mobility MS	200 ng/ml		1.0 ml serum
(Teeuwen *et al*., 1991)	GC		50 ng/ml	1.0 ml plasma
(Brazier *et al*., 1981)	GC		500 ng/ml	30 µl dried blood spot

4.1.3 Study aim

An assessment of method transferability is an important component of method validation and becomes necessary when methods are transferred to different analysts and different instruments. The aim of this part of the thesis was to determine the transferability of the caffeine DBS method developed at GSK to a simple LC-MS

instrument. For this purpose a working LC-MS method for the quantification of caffeine was initially established and validated. A series of validation tests were subsequently performed to determine the transferability and thus the robustness of DBS analysis. This included an assessment of accuracy, precision, selectivity, sensitivity and matrix effect (section 4.3). A clinical validation of the developed LC-MS DBS method was performed in two healthy adult volunteers following the administration of a single oral caffeine dose.

4.2 GSK Dried blood spot method and validation results

The caffeine DBS LC-MS/MS method used to support the PK study in preterm neonates (Chapter 5) is described here. All method development and validation work presented in this section was carried out by GSK analysts.

4.2.1 Chemicals and materials

Caffeine (1,3,7-trimethylxanthine) and caffeine-trimethyl-$^{13}C_3$ (IS) of analytical grade were obtained from Sigma-Aldrich (Poole, UK). The chemical structures of caffeine and IS are given in Figure 4.1. HPLC grade acetonitrile, methanol and water were obtained from Fisher Scientific (Loughborough, UK). Analytical grade formic acid, ammonium acetate, ammonia solution, dimethylformamide and isopropanol were supplied by VWR International Limited (Poole, UK). Sample tubes were obtained from Micronics (Platinastraat, The Netherlands). FTA Elute cards, drying racks and Harris punch and cutting matt were obtained from Whatman (part of GE Healthcare). Desiccant sachets were supplied by Sud-Chemie (Northwich, UK) and plastic bags for storage of samples were obtained from Fisher Scientific (Loughborough, UK). EDTA coated capillary tubes were obtained from Sarstedt (Leicester, UK). Blood (EDTA) for

method development and patient sample analysis was supplied by GSK volunteers in accordance with GSK ethical procedures (Lad, 2010). Blood donors were asked to adhere to caffeine free diets for at least two weeks beforehand.

4.2.2 Preparation of caffeine stock and spiking solutions

Stock solutions of caffeine were prepared by dissolving in dimethylformamide to produce a 10 mg/ml concentration. An aliquot of stock solution was used to produce a spiking solution of concentration 1000 µg/ml in acetonitrile:water (50:50, v/v). This was then used to make spiking solutions of concentration 100 and 10 µg/ml via serial dilution.

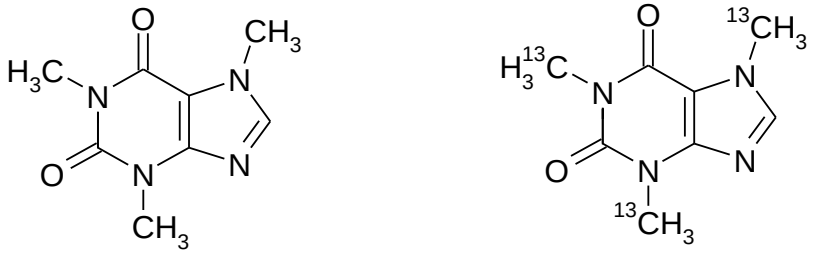


Figure 4.1 Chemical structures of caffeine (left) and caffeine-trimethyl-$^{13}C_3$ (right)

4.2.3 Preparation of extraction solution and mobile phase

Stock solutions of IS were prepared by dissolving in dimethylformamide to give a 100 µg/ml concentration. A 250 µl aliquot of this solution was added to 50 ml of methanol to give an extraction solution containing 500 ng/ml of IS.

Mobile phase A was made by diluting 10 ml of 1 M ammonium acetate solution with water to give a final volume of 1000 ml and adjusted to pH 8 with ammonia solution. Mobile phase B consisted of 100% acetonitrile.

4.2.4 Preparation of calibration and validation dried blood spots

Calibration and validation standards were prepared by diluting an aliquot of (10, 100 or 1000 µg/ml) working solution to 1000 µl with blank human blood. The volume of solvent used to produce calibration standards varied between 8 to 50 µl and therefore did not exceed 5% of the final volume. The final concentration of calibration standards were 250, 500, 1000, 5000, 8000, 15000, 20000, and 25000 ng/ml. Replicate 15 µl aliquots of standards were spotted onto FTA Elute paper using a volumetric pipette and allowed to dry at room temperature for at least 2 hours. Dried samples were stored at room temperature with desiccant.

4.2.5 Dried blood spot extraction

A 3 mm disc was punched out from the centre of DBS samples and transferred to a tube to which 100 µl of methanol containing 500 ng/ml of IS was added. The tubes were mixed using a vortex and extraction was facilitated by gentle shaking for 60 minutes. Extracts were transferred to a clean tube and vortexed briefly prior to LC-MS/MS analysis.

4.2.6 Liquid chromatography triple quadrupole mass spectrometry conditions

The LC system consisted of a HTS PAL autosampler (Presearch, Hitchin, UK) and an Agilent 1100 binary pump with a column oven. Separation was achieved on a Synergi Fusion C18 Column (50 x 3.0 mm i.d., 4 µm) (Phenomenex, Macclesfield, UK) within 1.5 minutes under isocratic conditions with 10 mM ammonium acetate (pH 8):acetonitrile (84:16, v/v). An oven column temperature of 60°C was maintained during the analysis. The flow rate was set to 0.6 ml/min and the injection volume to 3 µl.

MS/MS detection was performed with a Sciex API-4000 (Applied Biosystems/MDS Sciex, Canada) with a TurboIonSpray® ion source in positive ion mode. The ionisation temperature was set at 650°C. Nitrogen was used for gas 1 and 2 at a setting of 40 and 50 psi, respectively. The curtain gas (nitrogen) was set to 25 psi and collision gas (nitrogen) to 6 psi. The declustering potential and collision energy value was set to 30 and 27, respectively. Quantification was carried out under SRM using the precursor ion $[M+H]^+$ to product ion transition 195 to 138 *m/z* for caffeine and 198 to 140 *m/z* for the IS. The product ion monitored for caffeine and IS correspond to the ion $[M+H\text{-}OCNCH_3]^+$ and $[M+H\text{-}OCN^{13}CH_3]^+$, respectively. A dwell time of 200 msec and 150 msec was used for caffeine and IS, respectively. All data was processed using Analyst software (version 1.4.1 Applied Biosystems/MDS Sciex). Concentrations in DBS samples were determined from peak area ratios using a GSK laboratory information management system SMS2000 (version 2.0, GSK, UK).

4.2.7 Validation results

4.2.7.1 Linearity, selectivity and sensitivity

A weighted ($1/x_2$) linear regression was fitted to calibration plots of caffeine concentration against analyte/IS peak area ratio in triplicate. Linearity ($r^2 > 0.99$) was observed within the tested concentration range of 250 to 25000 ng/ml (Figure 4.2). Under the chromatographic conditions caffeine and IS co-eluted at a retention time of 1.0 minute. Method selectivity was assessed by analysing control DBS samples from six individual volunteers. No unacceptable (> 20% LLOQ) interferences were observed in any of the chromatograms at the retention time of caffeine or IS (Figure 4.3). A LLOQ

of 250 ng/ml was achieved whilst maintaining a precision (CV%) of less than 15% and an accuracy (RE%) within 15% of the nominal concentration (Figure 4.4).

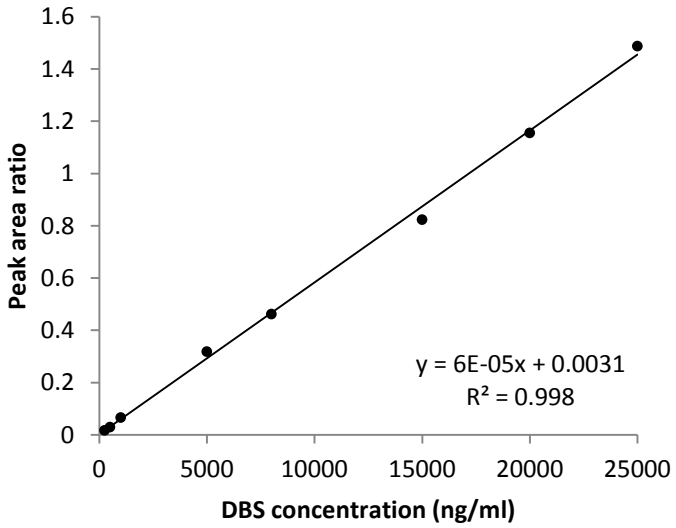
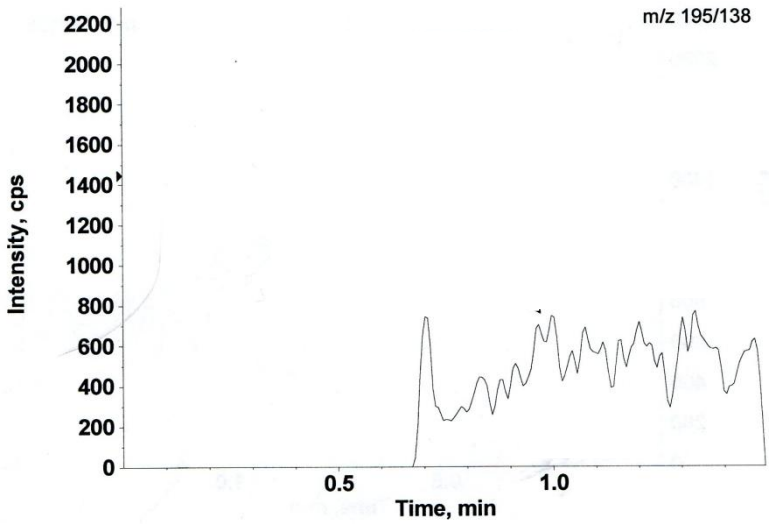


Figure 4.2 A representative calibration plot of nominal caffeine DBS concentration against mean caffeine to IS peak area ratio n=2

4.2.7.2 Accuracy and precision

Assay accuracy (RE%) and precision (CV%) was assessed using samples at five concentrations (250, 1000, 8000, 20000 and 25000 ng/ml, n=6) in triplicate. Concentrations of caffeine within DBS samples were determined from a calibration line (250 - 25000 ng/ml) prepared using a separate stock solution. A RE% and CV% value of less than 20% at the LLOQ and ≤ 15% at all other concentrations was considered acceptable. Values determined were all within these limits (Table 4.2).

A



B

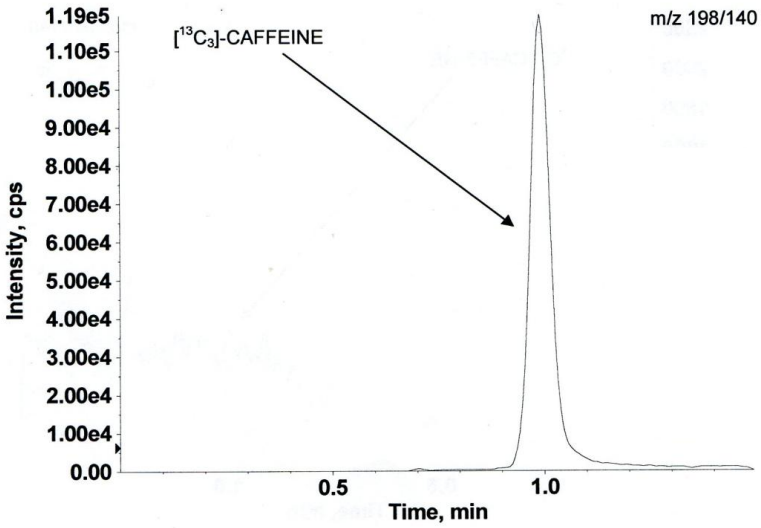


Figure 4.3 Representative LC-MS/MS chromatograms of a blank DBS sample extracted with IS where (A) is the SRM chromatogram for caffeine and (B) is the SRM chromatogram for IS

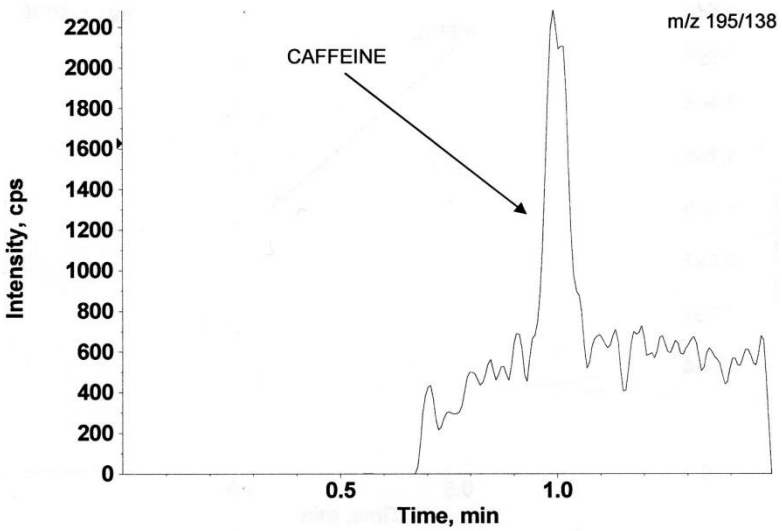


Figure 4.4 Representative LC-MS/MS chromatogram of a caffeine DBS extract at the LLOQ of 250 ng/ml

Table 4.2 Intraday and interday accuracy (RE%) and precision (CV%) results for the caffeine LC-MS/MS DBS method n=6

		Intraday				
	Nominal conc. (ng/ml)	250	1000	8000	20000	25000
run 1	Mean conc.					
	(ng/ml)	244	955	8083	18377	24838
	SD	22	99	179	819	2098
	RE%	-2.6	-4.5	0.5	-8.1	-0.6
	CV%	8.9	10.3	2.2	4.5	8.4
run 2	Mean conc.					
	(ng/ml)	223	910	7692	19535	23210
	SD	21	58	441	1042	1974
	RE%	-10.9	-9.0	-3.9	-2.3	-7.2
	CV%	9.4	6.4	5.7	5.3	8.5
run 3	Mean conc.					
	(ng/ml)	238	1034	8059	20054	24090
	SD	17	95	548	1057	2699
	RE%	-4.9	3.4	0.7	0.3	-3.6
	CV%	7.3	9.2	6.8	5.3	11.2
		Interday				
	Mean conc.					
	(ng/ml)	235	966	7930	19322	24046
	SD	21	96	430	1168	2248
	Average RE%	-6.1	-3.4	-0.9	-3.4	-3.8
	Overall CV%	3.0	5.4	1.5	3.9	Negligible

4.2.7.3 Spot size and haematocrit

The effect of varying the blood volume spotted was investigated between 10 and 20 µl at concentrations of 1000 and 20000 ng/ml in replicate (n=6). Spiked blood was used to produce 10, 15 and 20 µl spots on FTA Elute paper. The concentration of caffeine in 3mm samples was calculated from a calibration line produced with 15 µl DBS samples. Results indicated a trend towards an increased caffeine concentration with increasing spot volume (Table 4.3). However, differences from the 15 µl spot were less than 8% and all RE% and CV% values were < 15% indicating method robustness to small variations in blood volume spotted.

Haematocrit effects with FTA Elute paper was assessed at concentrations of 1000 and 20000 ng/ml at haematocrit levels of 20%, 25%, 30%, 35%, 40% and 45% in replicate (n=6) using 15 µl spots. Blood haematocrit was adjusted by either removing or adding plasma. Concentrations of caffeine in DBS samples were determined from the regression equation generated from a calibration performed using blood with a haematocrit value of 45%. In Table 4.4 the accuracy (RE%) observed in caffeine measurements is presented as the difference from the analyte/IS peak area ratio at a haematocrit value of 45%. At each tested concentration a trend towards an increasingly negative bias was observed with decreasing haematocrit. The most marked difference in RE% (-18.4 and -14.1) at both concentrations was observed at a haematocrit level of 40%. Thereafter changing from a haematocrit of 40% to 20% only gave further differences in RE% of -15% and -9.6% at 1000 ng/ml and 20000 ng/ml levels, respectively. Overall, the effect of haematocrit on assay accuracy (RE%) was found to significantly deviate from the acceptable ± 15% limit. The relationship between assay bias and haematocrit over the haematocrit range studied is shown in Figure 4.5.

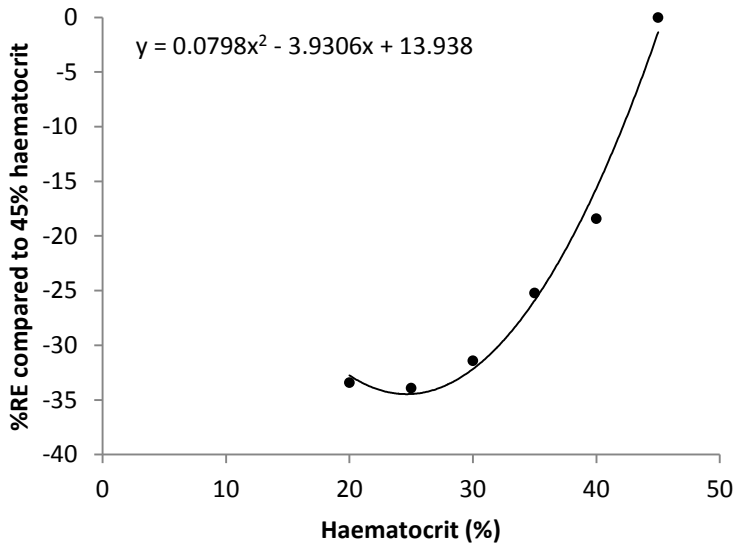
Table 4.3 Effect of spot volume on accuracy and precision of caffeine assay n=6

Nominal conc. (ng/ml)	1000			20000		
Volume	10 µl	15 µl	20 µl	10 µl	15 µl	20 µl
Mean conc. (ng/ml)	888	955	990	17348	18377	19828
SD	109	99	57	950	819	1112
Accuracy (RE%)	-11.2	-4.5	-1.1	-13.3	-8.1	-0.9
Precision (CV%)	12.3	10.3	5.7	4.3	5.8	5.9

Table 4.4 Influence of haematocrit on the accuracy (RE%) of caffeine measurements presented as the difference from the analyte/IS peak area ratio at the 45% haematocrit level. Precision (CV%) values for respective values are shown in brackets n=6.

	Nominal conc. (ng/ml)	
Haematocrit	1000	20000
20%	-33.4 (9.6%)	-23.7 (3.4%)
25%	-33.9 (12.7%)	-31.6 (3.8%)
30%	-31.4 (9.5%)	-20.5 (3.1%)
35%	-25.2 (8.8%)	-17.2 (4.7%)
40%	-18.4 (12.2%)	-14.1 (6.8%)
45%	Normalised (11.1%)	Normalised (5.6%)

A



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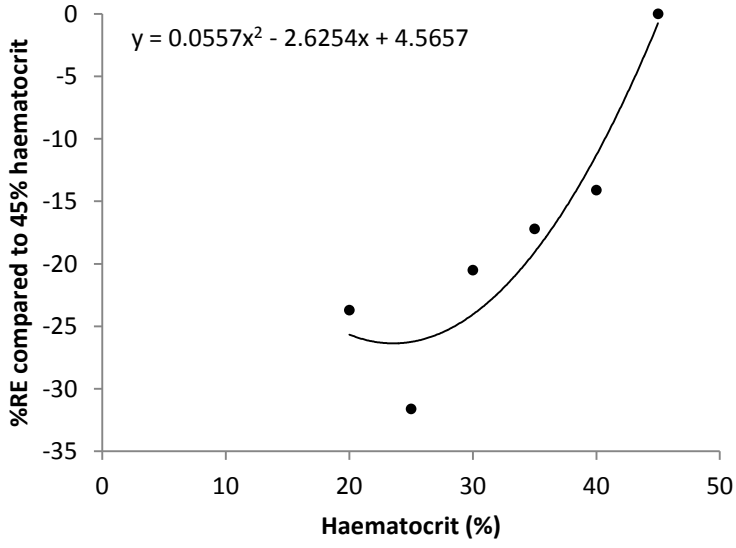


Figure 4.5 Relationship between accuracy (RE%) of caffeine DBS measurement and haematocrit (%) at concentrations of (A) 1000 ng/ml and (B) 20000 ng/ml

4.2.7.4 Petroleum jelly

The effect of petroleum jelly on caffeine DBS quantification was assessed using blood spiked at concentrations of 1000 and 20000 ng/ml (n=6). 30 µl spots were applied onto a glass slide covered with a thin layer of petroleum jelly. Using a pipette 15 µl aliquots were spotted onto FTA Elute paper after a contact time of 1 minute. This was then repeated without petroleum jelly and for a longer exposure time of 10 minutes. Differences in analyte to IS peak area ratio between samples exposed to petroleum with those that had not were less than 15%. Precision values were also within 15% (CV%) and were comparable. Therefore the use of petroleum jelly was not considered to significantly affect caffeine quantification.

4.2.7.5 Matrix dilution

Caffeine plasma concentrations greater than 25000 ng/ml can be expected in some neonates during treatment for AOP (Lee *et al*., 1997). To accommodate any concentration measurements falling above the higher limit of quantification the use of a 10 fold matrix dilution was investigated during method validation using 50000 ng/ml DBS samples. A 3 mm punch was taken and extracted as described in section 4.2.5. 5 µls of the resulting extract was diluted with a 45 µl aliquot of matrix solution (prepared by extracting blank DBS samples with IS). Concentrations in diluted extract samples were determined from a calibration line and corrected for using the dilution factor. Accuracy and precision values were within 15% indicating a 10 fold dilution using blank DBS samples extracted with IS is suitable (Table 4.5).

4.2.7.6 Stability

Stability of caffeine stock solutions was tested in replicate (n=6) by comparing the

Table 4.5 Matrix dilution data for caffeine DBS extracts (10 fold dilution) n=6

Nominal conc. (ng/ml)	50000
Mean conc. (ng/ml)	53552
SD	3298
Accuracy (RE%)	7.1
Precision (CV%)	6.2

analyte to IS peak area ratio obtained from the analysis of stored solutions with those which had been freshly prepared. Concentrations of caffeine at 500 ng/ml were prepared from stock solutions for stability testing. Caffeine was stable for at least 31 days at 4°C with differences between fresh and stored stocks of less than 5%.

The stability of caffeine in EDTA whole blood was determined by comparing DBS samples prepared using blood stored at 37°C for 4 hours with those that had been immediately spotted, at concentrations of 1000 and 20000 ng/ml. Differences in the analyte to IS peak area ratio resulting from the analysis of 3 mm discs were less than 2% indicating stability during blood sample collection and handling.

Caffeine stability within DBS samples was determined by analysing 3 mm discs taken from 15 µl DBS samples at 1000 and 20000 ng/ml after storage at room temperature for 6 days in sealed plastic bags containing desiccant. Compared with freshly prepared DBS samples, differences were less than 15% indicating analyte stability within DBS on FTA Elute paper (Table 4.6). Long term stability data has shown caffeine is stable within DBS samples for at least 5 months stored with desiccant at room temperature.

In-process stability was assessed at five concentrations after storage of extracts for 120 hours at room temperature by comparison against a freshly prepared calibration line. Accuracy and precision values were all within 15% indicating sample extract stability (Table 4.7).

Table 4.6 Stability data for caffeine in DBS samples collected on FTA Elute paper stored at room temperature with desiccant for 6 days n=6

Nominal conc. (ng/ml)	1000		20000	
	Fresh	Stored	Fresh	Stored
Mean conc. (ng/ml)	955	1034	18377	20054
SD	99	95	819	1057
CV%	10.3	9.2	4.5	5.3
% Difference	+8.3		+9.1	

Table 4.7 In-process caffeine stability at room temperature for 120 hours n=6

	Nominal conc. (ng/ml)				
	250	1000	8000	20000	25000
Mean conc. (ng/ml)	251	1057	8498	20644	24734
SD	15	38	535	1022	2146
CV%	6.0	3.6	6.3	5.0	8.7
RE%	0.3	5.7	6.2	3.2	-1.1

4.3 Method development and validation on a liquid chromatography single quadrupole mass spectrometer

The methodology and validation results from investigations of caffeine DBS quantification by LC-MS are presented in this section.

4.3.1 Chemicals and materials

Caffeine (1,3,7-trimethylxanthine) ≥ 99.0% (HPLC) and caffeine-trimethyl-$^{13}C_3$ (IS) 1 mg/ml 99% were obtained from Sigma-Aldrich (Poole, UK). FTA Elute paper and Harris punch and cutting mat were purchased from Whatman, UK (Part of GE Healthcare). Blood collection tubes (containing EDTA) and EDTA coated capillary tubes were obtained from Sarstedt (Leicester, UK). All other chemicals and materials used are given in Chapter 2 (section 2.3.1). Blank blood (EDTA) for method validation and patient sample analysis was obtained from healthy adult volunteers asked to abstain from dietary sources of caffeine for at least two weeks.

4.3.2 Preparation of caffeine stock, working and spiking solutions

A stock solution of caffeine was prepared by dissolving 0.05 g in 5 ml of methanol:water (80:20, v/v) to produce a 10 mg/ml concentration. This was diluted with methanol:water (50:50, v/v) to produce a 1000 µg/ml working solution which was then used to make a spiking solution for each calibration level. The final concentrations of spiking solutions were 5.25, 10.5, 21, 105, 168, 315, 420 and 525 µg/ml.

4.3.3 Preparation of extraction solution and mobile phases

An aliquot of the 1 mg/ml IS solution was diluted with methanol to produce a final concentration of 500 ng/ml. Mobile phase A was made by adding 650 µl of formic acid

to 500 ml of water to produce a 0.13% formic acid aqueous solution. Mobile phase B consisted of 100% acetonitrile.

4.3.4 Preparation and extraction of dried blood spots

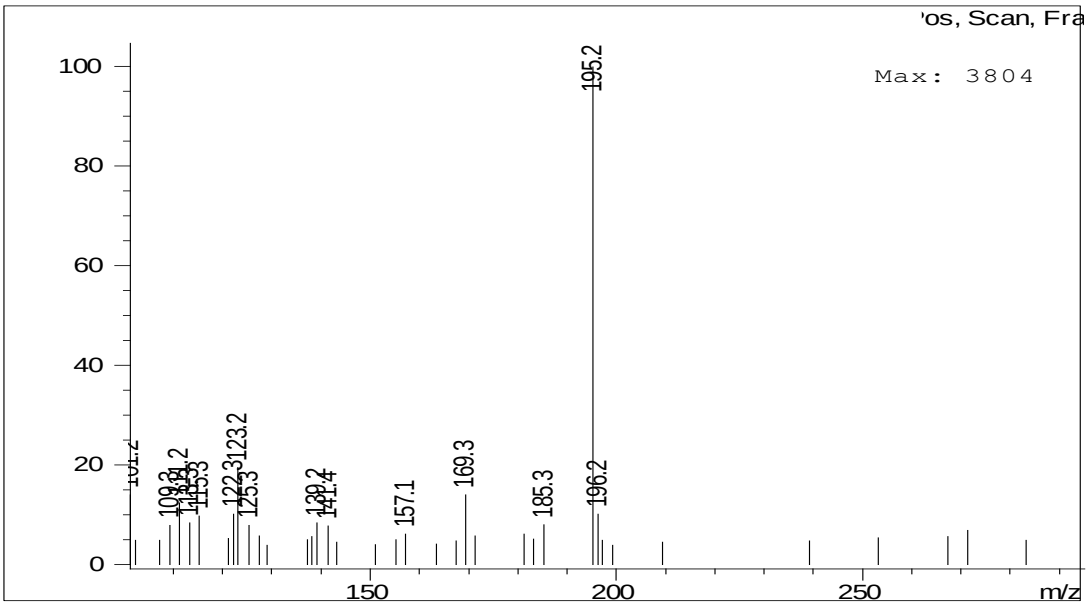
Calibration and validation standards were prepared by diluting 25 µl of each spiking solution with 500 µl of blank human whole blood. 15 µl volumes of spiked blood were applied onto FTA Elute paper and left to dry at room temperature overnight. The final concentration of calibration standards were 250, 500, 1000, 5000, 8000, 15000, 20000 and 25000 ng/ml. Once dried, samples were stored at room temperature in plastic bags with desiccant. The extraction procedure used to recover caffeine is outlined in section 4.2.5. Punched 3 mm discs were extracted in eppendorfs with methanol containing 500 ng/ml of IS and analysed via LC-MS.

4.3.5 Single quadrupole mass spectrometry

4.3.5.1 Selection of ions for monitoring

The behaviour of caffeine and IS in the ESI source was investigated in positive polarity mode. Figure 4.6 represents the mass spectrum obtained following the qualitative analysis of each compound using a 1000 ng/ml solution over a scan range of 100 to 300 *m/z*. Major ions at *m/z* 195 $[M+H]^+$ and *m/z* 198 $[M+H]^+$ were identified corresponding to the protonated molecular ions of caffeine and IS, respectively. These ions were therefore selected for caffeine quantification work.

A



B

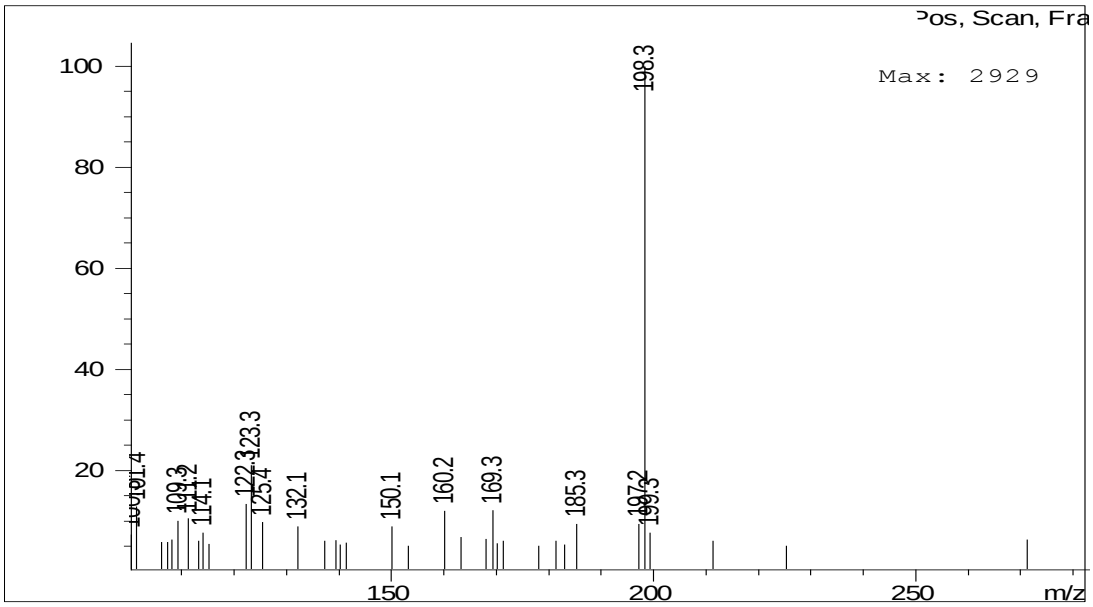


Figure 4.6 Full scan mass spectra under optimised MS conditions for (A) caffeine and (B) internal standard caffeine-trimethyl-$^{13}C_3$

4.3.5.2 Optimisation of ion transmission

ESI parameter optimisation was based on transmission of the analyte ion at *m/z* 195 in SIM mode. The settings investigated were capillary voltage, fragmentor voltage, nebuliser gas pressure, gas flow rate and drying gas temperature. Fragmentor voltage had the greatest effect on the resulting detection sensitivity of caffeine. The effect of fragmentor voltage on analyte peak intensity between 5 V and 125 V in 15 V increments is shown in Figure 4.7. A maximum response was achieved at 95 V and therefore selected for optimal ion transmission.

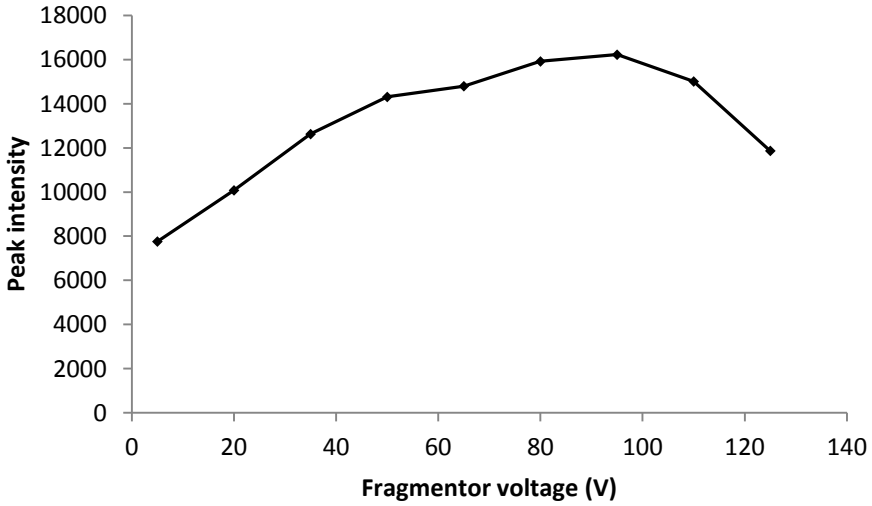


Figure 4.7 Effects of fragmentor voltage on caffeine signal intensity using a 100 ng/ml solution in SIM mode

4.3.5.3 Final conditions and instrumentation

MS detection was performed with an Agilent 1200 mass spectrometer with a positive ESI source. The system was calibrated on a daily basis using a tuning mixture supplied by Agilent Technologies. The ionisation source settings optimised to give maximum caffeine signal intensity were: fragmentor voltage, 95 V; drying temperature, 300°C;

capillary voltage, 3000 V; nebuliser pressure, 25 psig; nitrogen gas flow, 7 L/min. The protonated molecular ion $[M+H]^+$ of caffeine at *m/z* 195 and IS at *m/z* 198 were monitored using SIM mode. A dwell time of 289 msec was used for each ion. The software programme Chemstation (series B.1.3, Agilent Technologies) was used to operate the system and aquire all data. Microsoft Excel 2007 (Microsoft Corporation, USA) and Chemstation were used to analyse the data.

4.3.6 Liquid chromatography

The chromatographic system consisted of an Agilent 1100 series quaternary solvent delivery pump, autosampler and vacuum degasser. Caffeine was analysed on a Zorbax Eclipse Plus C18 column (150 mm x 2.1 mm i.d., 3.5 μm) attached with a C18 guard column (Phenomenex, Macclesfield UK, 3.0 x 4.0 mm) under isocratic conditions. Mobile phase A (water with 0.13% formic acid) and B (100% acetonitrile) were held at 18% and 82%, respectively over a run time of 5 minutes. The column oven temperature was set to 40°C and the flow rate was 200 μl/min. The injection volume was 10 μl.

4.3.7 Validation results and discussion

4.3.7.1 Recovery of caffeine from DBS samples

The overall efficiency of the extraction method utilising 100% methanol was investigated at DBS concentrations of 500, 8000 and 25000 ng/ml. At each concentration level replicate (n=5) 15 μl spots were made and allowed to dry. The entire DBS was subsequently cut out and extracted with 100% methanol. Recovery was determined by comparing the peak area obtained with DBS extracts to those obtained from caffeine in solvent containing an amount which assumes 100% recovery.

Recoveries were calculated from the equation:

$$Recovery\ \% = \frac{Response\ in\ DBS\ extract}{Response\ in\ pure\ solvent} \times 100$$

The overall recovery was relatively low (44 to 47%) but consistent and reproducible across the calibration range of the assay (Table 4.8).

Table 4.8 Recovery of caffeine from DBS at three concentrations n=5

	Nominal conc. (ng/ml)		
	500	8000	25000
Recovery (%)	47.0	44.2	44.9
SD	7.4	2.3	3.0
Precision (CV%)	15.6	5.2	6.6

4.3.7.2 Matrix effects

Matrix effects following plasma protein precipitation with organic solvents have been documented and therefore such concerns are not limited to DBS analysis. Phospholipids have been identified as an important endogenous source of matrix effects and are present in different amounts in various biological fluids including blood, plasma and urine (Lahaie *et al*., 2010, Guo and Lankmayr, 2011). Other factors which may affect ionisation efficiency of a compound are sub-optimal chromatography, the ionisation method and exogenous compounds e.g. anticoagulant.

The effect of matrix on the resulting caffeine LC-MS signal intensity was investigated at concentrations of 500 and 25000 ng/ml in replicate (n=5). An assessment was made by comparing the peak area of caffeine spiked into blank DBS extracts with that of

caffeine in solvent. A 90 µl volume of blank DBS extract or 100% pure methanol was added to 10 µl of caffeine spiking solution. The matrix effect was calculated using peak area by the equation:

$$Matrix\ effect\ \% = \frac{Response\ in\ extract}{Response\ in\ pure\ solvent} \times 100$$

Initial results indicated substantial ion suppression (≥ 15%) at both concentrations tested. To identify which components of the DBS sample where giving rise to the observed matrix effect the influence of FTA Elute paper was studied. FTA Elute paper extracts spiked with caffeine were prepared and compared to standards of the same concentration spiked into 100% methanol. The magnitude of the matrix effect was also studied using a different filter paper (S&S 903) and following the inclusion of IS. For the experiment including an IS the matrix effect % was calculated using the analyte/IS peak area ratio. Figure 4.8 shows the extent of the matrix effect observed under each of the experimental conditions. Components within extracts of blank DBS samples on FTA Elute paper resulted in 32 and 40% ionisation suppression of the caffeine signal under ESI at 500 and 25000 ng/ml, respectively. Results from the FTA Elute paper showed a similar matrix effect on the caffeine signal suggesting that chemical components within FTA Elute paper rather than endogenous components in blood are responsible for the ion suppression. Under identical chromatographic conditions using paper S&S 903 no significant matrix effects were observed. This provided further evidence of matrix effects arising from the impregnated chemicals in FTA Elute paper. These results are in agreement with the findings of a study where three different paper types (FTA Elute, FTA and Ahlstrom 226) were studied for potential matrix effects (Clark *et al*., 2010). Using 10 different compounds the investigators showed that FTA

Elute paper produced the most marked matrix effects. Signal suppression varied considerably from not significant to greater than 80% depending on the compound tested. Paper 226 which is not chemically treated was not associated with any significant matrix effects. Despite the severity of the matrix effect observed for some compounds, FTA Elute was found to be the only paper that enabled an acceptable degree of accuracy and precision for greater than 80% of test compounds (which was one of three core assessment criteria during the investigations). On that basis the authors concluded that FTA Elute was the preferred choice of paper substrate for method development.

Several approaches have been used to remove matrix effects including improved sample clean-up (Guo *et al.*, 2005, Van Hoof *et al.*, 2005) and liquid chromatography parameter changes to separate the co-eluting interference from analyte (Chambers *et al.*, 2007). Alternatively, labelled IS can be very useful in this situation by compensating for the matrix effect. As it co-elutes with analyte its response is expected to be affected to the same extent (Van Eeckhaut *et al.*, 2009). After the inclusion of IS the matrix effect observed was within the accepted limit of 15% at both concentrations and therefore IS corrected for the effect observed. Although a correction was possible with the use of a labelled IS there is still the problem of a loss in sensitivity. This will be of particular importance in DBS analysis where detection is already limited by small sample volumes and for drugs which are less well ionised. The size of the matrix effect would account for the relatively low recoveries of caffeine calculated using methanol extraction (section 4.3.7.1). A matrix effect was not apparent at this stage since comparisons of DBS extracts were undertaken with standards made in 100% methanol. For the purposes of the current research objective the loss in sensitivity was not considered to be a

problem and therefore efforts were not made to remove the matrix effect. The benefits of sample clean-up procedures on matrix effects and sensitivity when using FTA Elute paper have been reported (Liu *et al.*, 2010).

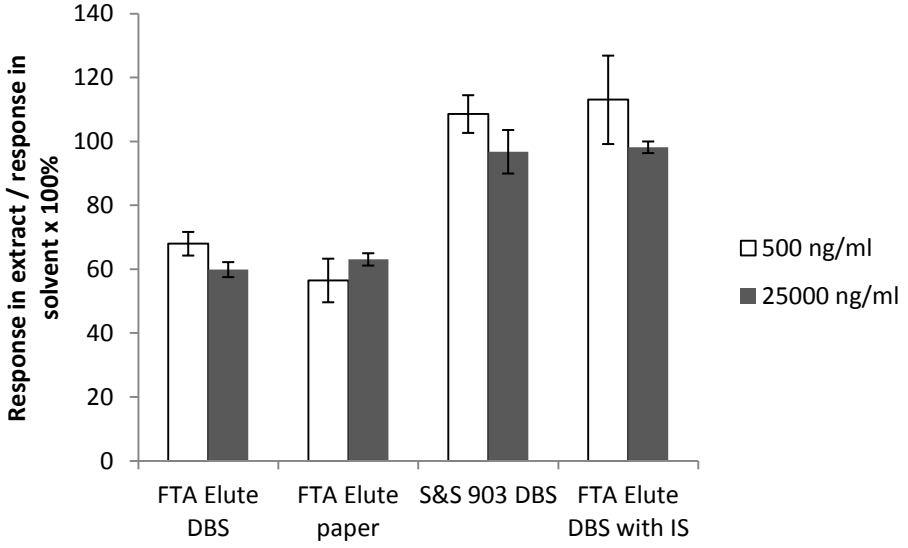


Figure 4.8 Matrix effect observed for caffeine at two concentrations in FTA Elute DBS extract, FTA Elute paper extract, 903 paper DBS extract and FTA Elute DBS extract containing IS. Results are presented as a percentage of the peak area observed in pure solvent n=5.

4.3.7.3 Linearity, selectivity and sensitivity

A calibration plot of caffeine concentration against analyte/IS peak area ratio was constructed and a weighted $1/x^2$ linear regression was applied. The response was linear ($r^2 \geq 0.99$) over the calibration concentration range of 500 to 25000 ng/ml (Figure 4.9). Under the chromatographic conditions a run time of 5 minutes was achieved with co-elution of caffeine and IS at a retention time of 3.3 minutes. Method selectivity was demonstrated following the analysis of DBS samples (n=1) collected from five individual human subjects. There were no significant (≥ 20% of LLOQ peak area) interferences observed in any of these chromatograms at the retention time of analyte

and IS. The selectivity of the method is shown in the representative chromatogram in Figure 4.10. Signal-to-noise ratios were calculated by dividing peak height by the baseline noise (where noise is given as six times the standard deviation of the linear regression). The LLOQ with a signal-to-noise ratio of ≥ 10 and CV% and RE% less than 20% was determined to be 500 ng/ml (Figure 4.11).

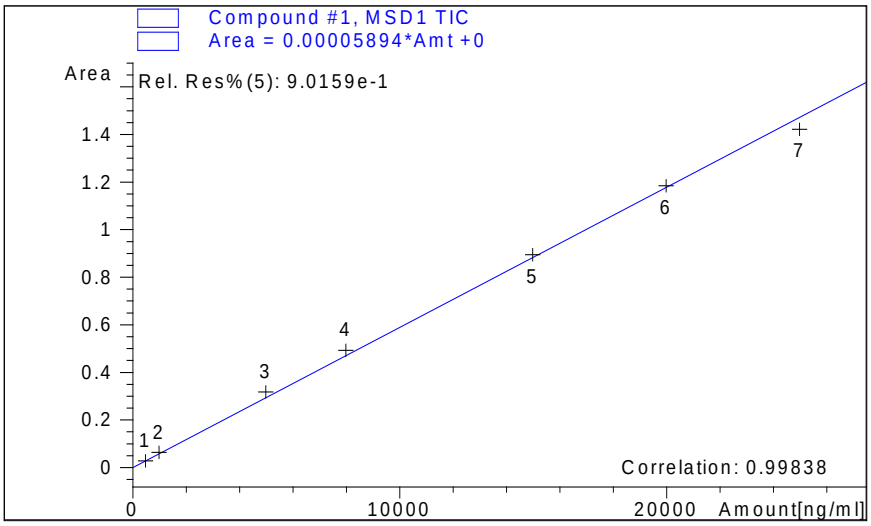


Figure 4.9 Representative calibration plot of nominal caffeine concentration against mean analyte to IS peak area ratio (n=5)

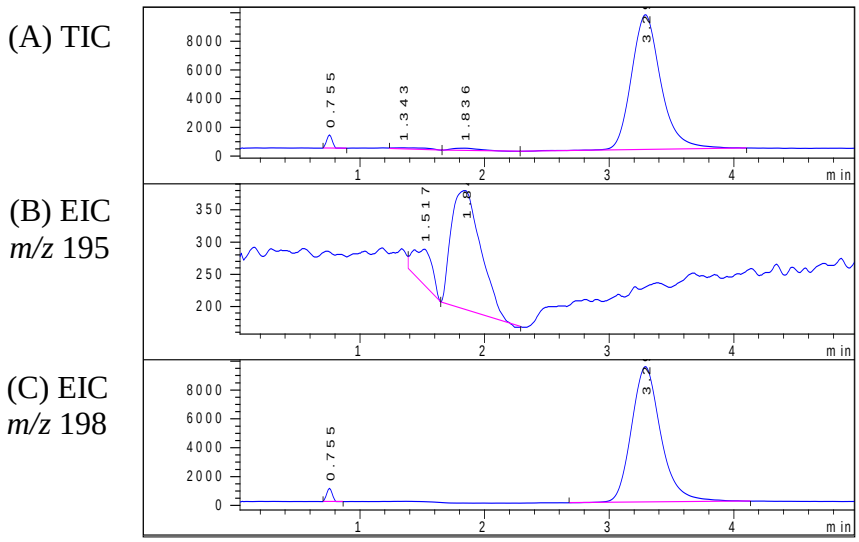


Figure 4.10 A representative LC-MS chromatogram of a blank DBS sample extracted with IS where (A) is the total ion chromatogram (TIC), (B) is the extracted ion chromatogram (EIC) for m/z 195 and (C) is the EIC for m/z 198

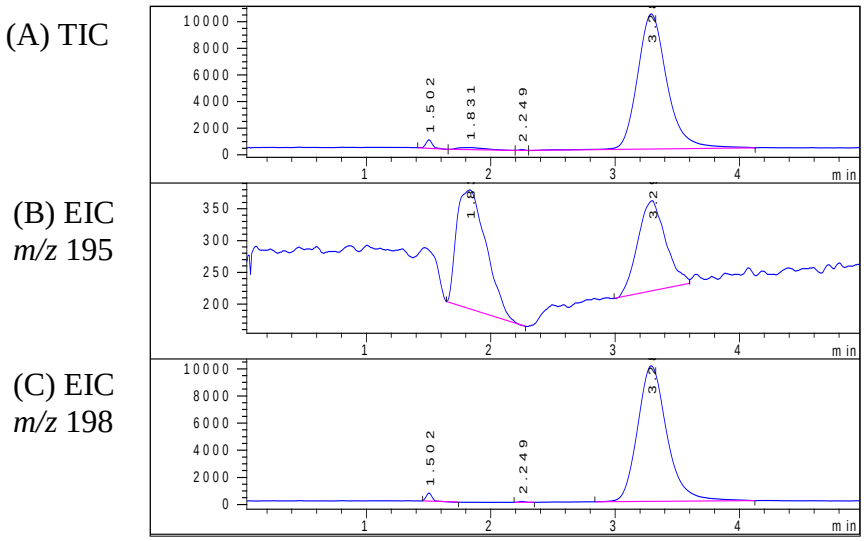


Figure 4.11 A representative LC-MS chromatogram of a spiked DBS at 500 ng/ml extracted with IS where (A) is the TIC, (B) is the EIC for m/z 195 and (C) is the EIC for m/z 198

4.3.7.4 Accuracy and precision

The assay accuracy and precision was assessed by analysing calibration standards at 7 concentration levels (n=5) within the calibration range of 500 - 25000 ng/ml on three separate days. Intraday CV% and RE% was within the acceptable 20% limit at the LLOQ and within 15% for all other concentrations. In addition, the interday CV% and RE% was within 15% (Table 4.9).

4.3.7.5 Carryover and injector precision

Sample carryover was assessed by analysing blank DBS extracts following the analysis of the highest DBS concentration (25000 ng/ml) in the calibration range. No significant peak (> 20% of LLOQ peak area) was observed in blank DBS chromatograms. Similarly, potential contamination resulting from the punch was assessed by analysing two blank DBS discs cut from the same punching device previously used to cut out 10 discs of blood from 25000 ng/ml DBS samples. In the absence of a significant peak attributable to caffeine in resulting chromatograms the punching device was also excluded as a major factor affecting assay accuracy and precision. Injector precision was determined by analysing 5 repeat injections using a 500 ng/ml DBS extract (Table 4.10).

Table 4.9 Intraday and interday accuracy (RE%) and precision (CV%) results for the LC-MS caffeine DBS method n=5

		Intraday						
	Nominal conc. (ng/ml)	500	1000	5000	8000	15000	20000	25000
run 1	Mean conc.							
	(ng/ml)	441.8	1034.2	5346.7	8325.0	15138.9	20061.8	24087.7
	SD	75.0	20.2	698.2	339.6	668.4	1668.9	140.1
	RE%	-11.8	3.2	6.8	3.9	0.8	0.1	-3.8
	CV%	17.0	2.0	13.1	4.1	4.4	8.3	4.7
run 2	Mean conc.							
	(ng/ml)	562.0	1113.4	5237.2	7820.4	15204.0	17404.8	21902.6
	SD	38.3	92.9	182.4	202.2	2112.4	604.3	1519.3
	RE%	12.4	11.3	4.7	-2.2	1.4	-13.0	-12.4
	CV%	6.8	8.3	3.5	2.6	13.9	3.5	6.9
run 3	Mean conc.							
	(ng/ml)	503.6	971.5	4970.3	7387.8	15702.9	21355.2	24908.2
	SD	60.1	38.9	341.1	318.0	1466.4	1766.7	2160.4
	RE%	0.7	-2.8	-0.6	-7.7	4.7	6.8	-0.4
	CV%	12.4	4.0	6.9	4.3	9.3	8.3	8.7
		Interday						
	Mean conc.							
	(ng/ml)	502.4	1039.7	5184.7	7844.4	15348.6	19607.3	23632.8
	SD	75.1	77.6	456.9	469.6	1485.7	2165.5	2070.2
	Average RE%	0.4	3.9	3.6	-2.0	2.3	-2.0	-5.5
	Overall CV%	14.9	7.5	8.8	6.0	9.7	11.0	8.8

Table 4.10 Injector precision and retention time variability results for caffeine and IS

Injection number	Peak area ratio	Caffeine retention time (min)	IS retention time (min)
1	0.028	3.327	3.321
2	0.029	3.312	3.315
3	0.025	3.287	3.307
4	0.026	3.292	3.315
5	0.028	3.287	3.311
Mean	0.027	3.301	3.314
SD	0.002	0.018	0.005
Precision (CV%)	6.4	0.5	0.2

4.3.7.6 Stability

Bench top stability of caffeine solutions was tested using the lowest spiking standard concentration in replicate (n=5). The peak area obtained from the analysis of freshly prepared spiking standard was compared to those stored at room temperature for a period of 48 hours. The difference in peak area between stored and fresh solutions was 2.8% (CV, 1.3%) indicating caffeine stability at 22°C for at least 48 hours.

The stability of caffeine within DBS samples and extracts (in-process stability) has been demonstrated at room temperature (section 4.2.7.6). An important, additional consideration is the effect of drying and storage temperature on the stability of caffeine in DBS samples. To investigate the effect of temperature 15 µl blood spots were applied in replicate (n=5) to FTA Elute paper using blood spiked with caffeine at concentrations

of 500 and 25000 ng/ml. Samples were immediately transferred to an oven set to 30°C or left to dry at 22°C. Following a period of 48 hours under these storage conditions, 3 mm discs from DBS samples were extracted and resulting peaks area ratios were compared. The difference in peak area ratio at concentrations of 500 and 25000 ng/ml was -1.1% and 9.5%, respectively (Table 4.11). This provides assurance of analyte stability at warmer temperatures likely to be experienced where sample collection and drying will take place.

Table 4.11 Stability of caffeine in DBS following drying and storage at 30°C for 48 hours n=5

Nominal conc. (ng/ml)	500		20000	
	22°C	30°C	22°C	30°C
Peak area ratio (mean)	0.04	0.04	1.17	1.28
SD	0.007	0.002	0.151	0.068
CV%	18.9	6.1	12.9	5.3
% Difference	-1.1		+9.5	

4.3.7.7 Caffeine red blood cell association

Caffeine exhibits relatively low plasma protein binding, mainly to albumin (Blanchard, 1982). *In-vitro* investigations have shown a constant unbound concentration with increasing caffeine concentration indicating the suitability of plasma for PK studies (Blanchard, 1982). The drug enters RBCs but does not bind to cellular constituents there, freely moves between RBCs and plasma and has a blood-to-plasma ratio equal to 1 (Poulin and Theil, 2009, Rowland and Emmons, 2010). The factors that can affect PK interpretation when using whole blood (e.g. RBC partitioning) would therefore not be

expected to affect caffeine. Based on these drug distribution kinetics either plasma or blood should be a suitable matrix for performing PK studies. As the blood-to-plasma ratio is 1 a comparison between plasma and DBS caffeine PK data is possible without a prior need to convert drug concentration values.

4.3.8 Clinical validation

4.3.8.1 Patients and method

The validated caffeine DBS LC-MS method was applied to samples collected from two healthy non-smoking (NS) adult volunteers (1 male, 1 female) following the ingestion of a single 100 mg oral caffeine dose. Prior to caffeine administration individuals were asked to abstain from dietary sources of caffeine for at least two weeks. Replicate (n=2) 15 µl capillary blood samples were collected following a finger-prick before caffeine administration and thereafter at intervals of 0.5, 1, 2, 4, 6, 12 and 24 hours post dose. Each 15 µl blood sample was collected into separate EDTA coated capillary tubes and immediately spotted onto FTA Elute paper. Samples were left to dry at room temperature for at least 2 hours prior to storage.

Patient samples were analysed alongside a freshly prepared caffeine DBS calibration line. Measured concentrations for each patient were fitted individually to a one compartment PK model within NONMEM (version 7.0) with a GNU Fortran Compiler 95. Post processing of NONMEM data was undertaken with PDx-POP (version 4.0, ICON Development Solutions, USA) and Microsoft Excel 2007 (Microsoft Corporation, USA).

4.3.8.2 Pharmacokinetic profile in adults

The DBS concentration-time profiles for patient 1 and 2 along with the model predictions are shown in Figures 4.12 and 4.13, respectively. A one compartment model with first order oral absorption and elimination fit the data well. First order absorption with lag time was required to capture the absorption phase for patient 2. The absorption rate constant (Ka) was fixed to 12 h^{-1} (Shi *et al*., 1993). No detectable caffeine levels were present in DBS samples collected prior to caffeine administration. Results from previous studies demonstrate caffeine exhibits rapid and complete absorption when administered orally with peak plasma levels achieved within 30 to 90 minutes for most individuals (Newton *et al*., 1981). Similarly, peak DBS levels were reached within 30 to 60 minutes in both patients. The PK parameter estimates for CL and V for patients 1 (male) and 2 (female) are presented in Table 4.12. They are in agreement with Joeres *et al* who reported a mean plasma CL and V of 1.43 ml/min/kg and 0.51 l/kg, respectively, in adults (Joeres *et al*., 1988). The CL and V estimates obtained were also comparable to other previous PK reports for caffeine in plasma (Table 4.13). A higher CL estimate was obtained for patient 1 compared to patient 2 (2.02 versus 1.31 ml/min/kg) which may in part be explained by gender related differences since there is evidence to suggest increased activity of the key enzyme responsible for caffeine metabolism, cytochrome P450 1A2 (CYP1A2), in males (Ou-Yang *et al.*, 2000). A relatively small caffeine dose of 100 mg was used in the current study, however, a comparison of PK parameters with reported studies was possible as caffeine is known to obey first order kinetics, at least up to a dose of 750 mg (Newton *et al.*, 1981). The similarity of the DBS concentration-time profiles and PK estimates to those obtained in plasma demonstrate the suitability of the DBS method for application to PK studies.

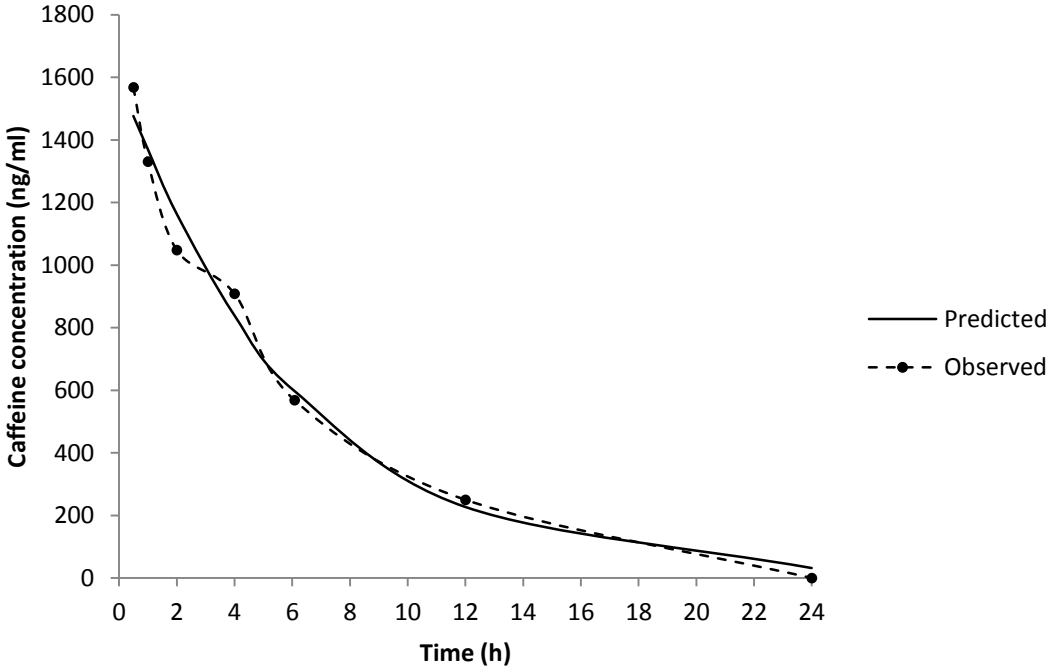


Figure 4.12 DBS concentration-time profile for patient 1 following the ingestion of 100mg of caffeine. The solid line represents the model predicted PK profile.

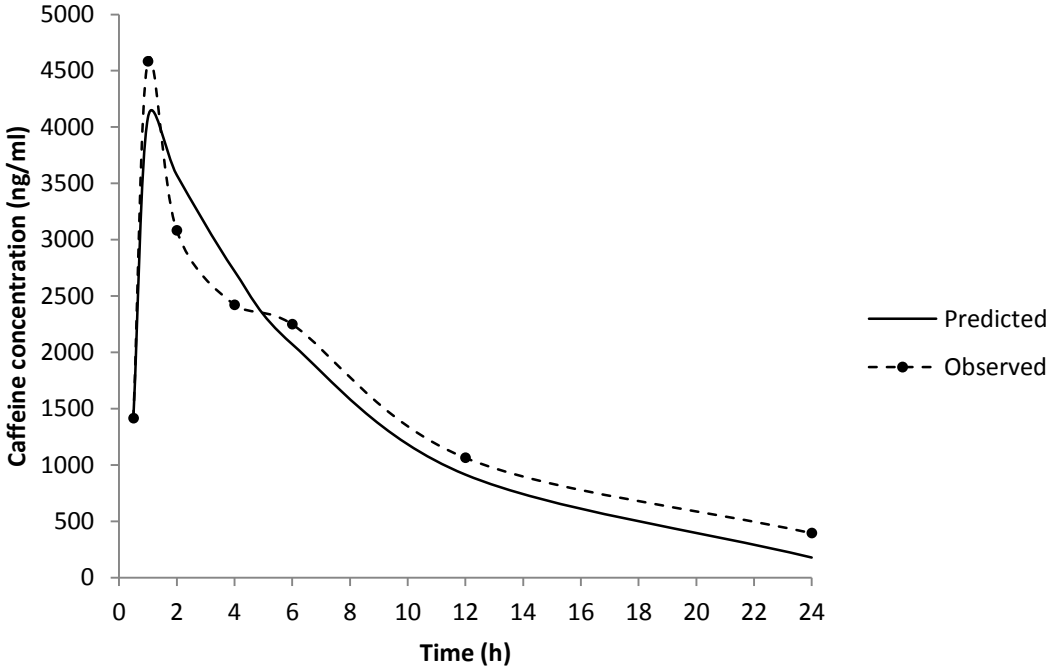


Figure 4.13 DBS concentration-time profile for patient 2 following the ingestion of 100mg of caffeine. The solid line represents the model predicted PK profile.

Table 4.12 DBS estimates of caffeine clearance, volume of distribution and half life following a single 100 mg oral dose

Parameter	Patient 1	Patient 2
CL (ml/min/kg)	2.02	1.31
V (l/kg)	0.74	0.58
[a]$t_{1/2}$ (h)	4.2	5.1

a. $t_{1/2} = V \times 0.693 / CL$.

Table 4.13 A comparison of plasma caffeine PK estimates reported in the literature for adults. CL and V are presented as mean (SD).

Reference	Study design	CL (ml/min/kg)	V (l/kg)	$t_{1/2}$ (h)
(Newton *et al*., 1981)	6 [a]NS adults Dose 50 mg	1.12 ± 0.30	0.35 ± 0.1	5.7
(Joeres *et al*., 1988)	7 NS adults Dose 366 mg	1.43 ± 0.54	0.51 ± 0.13	4.8
(Kaplan *et al*., 1997)	12 adults (10 NS) Dose 250 mg	2.07 ± 0.40	0.58 ± 0.03	3.9
(Seng *et al*., 2009)	14 NS adults Dose 3mg/kg	1.81 ± 0.67	0.85 ± 0.48	4.8

a. NS = non smoker

4.3.8.3 Incurred sample reanalysis

There are differences between liquid plasma and DBS samples which may be of importance for incurred sample reanalysis (ISR). Plasma represents a homogenous sample from which serial analyses are possible. In DBS analysis, the sample is usually only used once and therefore necessitates the collection of replicate spots at each time point. These could be viewed as separate samples and raises questions on the reproducibility and quality of capillary blood sampling for PK determinations.

To assess the accuracy of caffeine measurements in DBS samples collected from patients 1 and 2 a repeat analysis was performed. This involved the analysis of a second spot alongside a fresh calibration line (for 9 out of 14 samples) 3 weeks after the original analysis. Differences from the original results were within the ± 20% limit of the original concentration measurement providing confidence in the reliability and reproducibility of the developed method (Fast *et al.*, 2009). However, higher concentrations were measured in all repeat DBS samples giving a positive bias (Figure 4.14). Investigations into analytical errors due to incorrect preparation of stock solution and calibration standards were made but could not account for the positive bias observed. A possible explanation may be the blood collection method used to obtain samples from patients. The initial droplet of blood was not wiped away prior to the collection of DBS samples. Therefore it is possible that the first spot collected was diluted by the presence of tissue fluid resulting in lower caffeine concentration measurements. The reanalysis of a larger number of samples would enable a more conclusive assessment of systematic bias on assay accuracy.

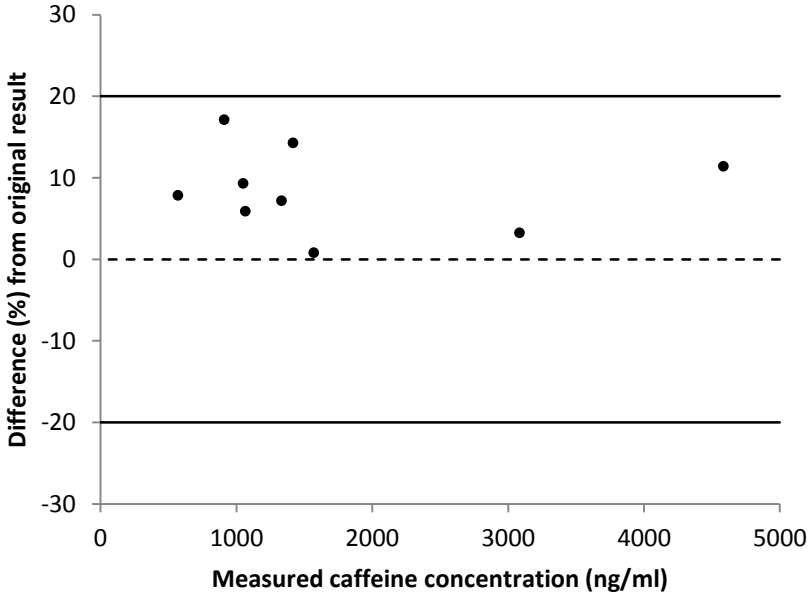


Figure 4.14 Results from the reanalysis of DBS samples from patient 1 and 2. Differences calculated as: (repeat – original / mean) × 100.

4.4 Comparison of analytical techniques for caffeine DBS quantification

The accuracy (RE%) and precision (CV%) results for LC-MS and LC-MS/MS methods (Table 4.14) were within the accepted validation criteria of less than 20% at the LLOQ and below 15% at all other tested concentrations. Linearity was observed with a weighted $1/x^2$ regression up to a DBS concentration of 25000 ng/ml for both methods (mean $r^2 = 0.9960$ and 0.9970 with method 1 and 2, respectively). A better detection sensitivity was however achieved with LC-MS/MS (250 ng/ml *vs.* 500 ng/ml) despite the comparatively smaller amount of caffeine analysed on column (injection volume 3 µl *vs.* 10 µl). TurboIonSpray® (a form of electrospray) enables the use of higher flow rates whilst maintaining detection sensitivity. In addition, depending on the compound under study, improved sensitivity due to more efficient ionisation has been noted with the use of this technique (Contin *et al.*, 2001). The two fold difference in sensitivity

observed could therefore be attributable to better ionisation of caffeine in the TurboIonSpray® source. A more efficient detection system in the newer GSK instrument is also likely to have contributed to this difference. Both formic acid and ammonium acetate are recommended for the improvement of ionisation when using MS based techniques (Maurer, 1998). A simple isocratic elution method with water containing formic acid (mobile phase A) and acetonitrile (mobile phase B) was chosen for the LC-MS analysis of caffeine. An alternative mobile phase additive such as ammonium acetate may have improved the detection sensitivity by improving molecular ion formation but was not investigated.

The dosing protocol at the Leicester NICU for AOP is 10 mg/kg of caffeine base as the loading dose followed by a 2.5 mg/kg once daily maintenance dose. Previously reported population PK studies in neonates which incorporated a similar dosing strategy would suggest that the LLOQ achieved with LC-MS/MS and LC-MS detection are approximately 10 and 5 fold, respectively greater than the lowest caffeine concentration (~ 2500 ng/ml) expected during the treatment period (Lee *et al.*, 1997, Lee *et al.*, 2002). Therefore a sufficient degree of sensitivity is afforded by either quantification method for application to caffeine PK studies or TDM in neonates.

There were no significant interferences at the retention time of caffeine and IS in any of the control DBS chromatograms for either method indicating the selectivity of the developed analytical techniques. LC-MS/MS techniques are often preferred due to their inherent selectivity. The results obtained here indicate the potential of SIM and SRM in providing a selective method for the quantification of caffeine within DBS samples under the sample preparation conditions used. Results from the matrix effect experiment

suggested significant ion suppression from the FTA Elute paper itself and demonstrate the importance of understanding the source and extent of matrix effects. Matrix effects have been shown to be dependent on the ionisation source design (Mei *et al.*, 2003) but a comparison between LC-MS (Agilent) and LC-MS/MS (Sciex) instruments was not possible as a matrix effect evaluation was not carried out during LC-MS/MS method development.

Table 4.14 Chromatographic and mass spectrometer settings for in-house (method 1) and GSK laboratories (method 2) caffeine DBS methods

	Method 1 (LC-MS)	**Method 2 (LC-MS/MS)**
Chromatographic conditions		
Column	Zorbax Eclipse Plus C18 150 mm x 2.1 mm i.d., 3.5 µm	Synergi Fusion C18 50 x 3.0 mm i.d., 4 µm
Column oven temperature	40°C	60°C
Mobile phase A	0.13% formic acid in water	10 mM ammonium acetate (pH 8) in water
Mobile phase B	Acetonitrile	Acetonitrile
Injection volume	10 µl	3 µl
Flow rate	0.2 ml/min	0.6 ml/min
Elution and run time	Isocratic (82% A: 18% B)	Isocratic (84% A: 16% B)
Run time	5 minutes	1.5 minutes
Mass spectrometer conditions		
Instrument	Agilent LC-MS	Sciex API-4000 LC-MS/MS
Ionisation	Electrospray	TurboIonSpray®
Polarity	Positive	Positive
Acquisition mode	SIM $[M+H]^+$ 195 *m/z* (caffeine) 198 *m/z* (IS)	SRM precursor ion $[M+H]^+$ to product ion transitions*:* 195 to 138 *m/z* (caffeine) 198 to 140 *m/z* (IS)

4.5 Conclusion

Method transferability is an important issue when transferring methods across laboratories and to other analysts. Although it is expected that a cross-validation should be performed in these circumstances there is currently no specific criteria on how this should be performed or which experiments should be included (Viswanathan *et al.*, 2007). The extent of validation required will depend on the differences likely to be encountered as well as whether there will be a change in the analytical technique altogether. At least in situations where a method is transferred to a different analytical instrument (or mode of detection) it is clear a complete validation would be necessary. To determine the transferability of the caffeine DBS method developed at GSK to an LC-MS instrument validation experiments were focused on linearity, selectivity, sensitivity, accuracy, precision and matrix effects. The validation results suggest assay performance to regulatory validation criteria, highlighting the robustness of DBS analysis and transferability of the technique to simpler MS detection methods and different laboratories. Further assessment of method transferability would involve the simultaneous analysis of patient samples on the LC-MS and LC-MS/MS instrument. Although this was not done, application of the developed LC-MS method to samples collected from two adult volunteers demonstrated the successful application of the DBS technique.

A simple and sensitive DBS LC-MS/MS method for caffeine quantification has been developed at GSK laboratories. Requiring only a 15 µl blood sample for quantification the method is compatible with the blood sampling restrictions which apply to neonates. The flexibility in blood volume spotted (10 to 20 µl) helps to maintain simplicity in the blood collection procedure. Long term stability under simplified room temperature

conditions has been demonstrated. In addition, caffeine is stable within DBS samples at elevated temperatures likely to be encountered during the drying process. Results from the validation of the DBS method, however, suggest variability in haematocrit to be a significant contributory factor to assay bias.

Chapter 5

Pharmacokinetics of Caffeine in Preterm Neonates Using Dried Blood Spot Analysis

5.1 Introduction

Caffeine is a highly water soluble compound (Log *P* = -0.091), displays limited plasma protein binding and does not accumulate or bind to constituents of RBCs (blood-to-plasma ratio = 1) (Blanchard, 1982, Poulin and Theil, 2009, Rowland and Emmons, 2010). As previously discussed, additional factors such as haematocrit and blood cell partitioning that need to be considered when interpreting whole blood derived PK data therefore should not be a concern here. In the absence of PK related issues, caffeine is a suitable test drug for performing a clinical validation of DBS analysis for drug quantification in PK studies involving neonates. Furthermore, caffeine has been extensively studied in neonates thereby enabling a DBS versus plasma comparison to be made (Aldridge *et al.*, 1979, Aranda *et al.*, 1979b, Gorodischer and Karplus, 1982, Thomson *et al.*, 1996, Falcão *et al.*, 1997, Lee *et al.*, 1997, Lee *et al.*, 2002, Charles *et al.*, 2008).

5.2 Study aim

The aim of this study was to clinically evaluate the reliability of DBS analysis as a method for generating PK data in neonates, and to identify any practical issues associated with developing a population model based on DBS samples in a busy clinical unit. For this purpose DBS samples were prospectively collected from preterm neonates receiving caffeine therapy for AOP. Concentrations of caffeine within samples were determined using the LC-MS/MS quantification method in Chapter 4 (section 4.2). A non-linear mixed effects approach was used to develop a population PK model based on DBS caffeine measurements and estimate PK parameters and their variability. Model evaluation techniques were used to determine the robustness of the final DBS model

and the accuracy and precision of population mean parameter estimates. At this stage a comparison was made between DBS derived PK parameter estimates and reported plasma PK data in neonates.

5.3 Patients and study protocol

5.3.1 Patients

Patients were recruited from within the University Hospitals of Leicester NHS Trust NICUs. All infants on the NICUs treated with caffeine for AOP were eligible for study inclusion on the provision of informed written consent from their parents or guardians (Appendix IV). Exclusion criteria included congenital or chromosomal abnormalities, cardiac arrhythmia, hypoxic ischaemic encephalopathy, sepsis, grade 3 or 4 cerebral haemorrhage, evidence of renal or hepatic damage or if the clinical team for any reason considered inclusion unsuitable. The study was approved by the Trent NHS Research Ethics Committee, the Research and Development Department at University Hospitals of Leicester NHS Trust and De Montfort University Research Ethics Committee.

5.3.2 Study design

Infants recruited onto the study received oral or IV caffeine base as directed by the clinical team and unit protocol. A caffeine loading dose of 10 mg/kg followed by a 2.5 mg/kg once daily maintenance dose was administered to patients. The maintenance dose was increased at the discretion of the clinical team in the event of increased apnoeic episodes. The length of caffeine therapy is variable and in some infants can continue for several weeks. The sampling design was unstructured and DBS samples were collected opportunistically at random time intervals post caffeine administration. Between 1 and

10 (replicate 15 µl, n=3) DBS samples were planned for collection from each neonate during caffeine treatment. In order to define the terminal phase of caffeine elimination DBS samples where possible were also collected after the discontinuation of treatment. The total volume of blood removed from each infant for the entire study period did not exceed 0.45 ml (10 x 45 µl) and records of the volume of blood removed were kept on a daily basis for each patient for the duration of the study. The sampling process involved the collection of blood (either capillary, venous or arterial) into an EDTA coated capillary tube with a suction bulb attached at one end. A separate capillary tube was used to collect each 15 µl blood sample. Capillary blood collection was performed as outlined in Chapter 3 (section 3.3.3). On occasions of blood collection from arterial or venous sampling sites, blood was initially drawn into a syringe without anticoagulant by clinical staff. Blood samples for the study were collected into capillary tubes directly from the syringe. Samples were immediately spotted within the designated area onto FTA Elute paper. An example of a typical DBS card collected from study patients is shown in Figure 5.1. It was estimated that approximately 250 DBS samples from 50 patients (average of 5 samples per patient) would be required to develop a robust population model of caffeine PK. Accurate caffeine administration and DBS sampling information was recorded for each patient on a case report form (Appendix V). Demographic data was collected for each infant from medical notes. Clinical data (if available from tests ordered by the clinician) were recorded for days on which DBS samples were collected.

5.3.3 Sample storage and analysis

Samples collected were left to dry on an elevated drying rack for a minimum of 2 hours in a dedicated room on the NICU. Once dried samples were placed in sealable plastic bags containing desiccant and transferred to the Child Health Laboratories at the University of Leicester for storage at room temperature until analysis. All DBS samples were transported via courier delivery to GSK laboratories (Ware, UK) where caffeine bioanalysis was performed as described in Chapter 4 (section 4.2). Concentrations of caffeine were determined from a single spot per card using the equation generated from a DBS calibration line analysed alongside the patient samples.

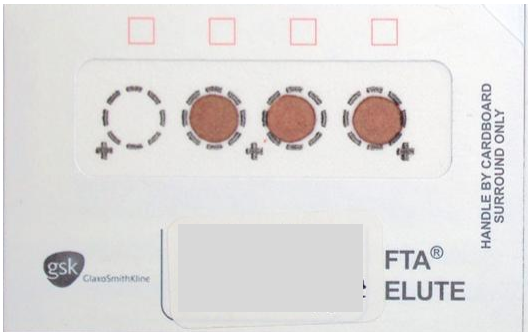


Figure 5.1 An example of a DBS card collected from a patient during the study. Replicate (n=3) 15 µl blood samples collected for a single time point.

5.4 Pharmacokinetic and statistical analysis

5.4.1 Non-linear mixed effects modeling

Non-linear mixed effects modeling was performed within NONMEM (version 7.0) using the First Order Conditional Estimation method with a GNU Fortran Compiler 95. Data generated by NONMEM was processed using PDx-POP (version 4.0, ICON Development Solutions, USA), R software (R Development Core Team, version 2.12.2,

Austria) and Microsoft Excel 2007 (Microsoft Corporation, USA). Using this approach it is possible to estimate population mean PK parameters, the interindividual variability (IIV) in these parameters as well as the interoccasion variability (IOV). NONMEM also enables the estimation of the residual error which is the difference between the observed and model predicted concentrations. In the initial stages of the model building process one and two compartment models were fitted to all data. The most suitable structural model was identified using statistical criteria (OFV, i.e. -2 log likelihood difference) and examination of graphical goodness of fit and residual error plots. Initial estimates of PK parameters were obtained from a reported population PK model in neonates (Charles *et al.*, 2008). Caffeine is known to be rapidly and completely absorbed when given orally and thus for oral doses bioavailability was assumed to be 100% (Giacoia *et al.*, 1989, Charles *et al.*, 2008). Sufficient post dose samples were not available to support the estimation of the rate of caffeine absorption and therefore Ka was fixed to 4.0 h^{-1} on the basis of previous PK reports (Falcão *et al.*, 1997). Varying the value of Ka did not affect the goodness of fit of the concentration data at early time intervals following oral dosing and thus the chosen value was considered to be suitable. IV doses of caffeine (administered over 5 minutes) were duration modelled using a fixed value of 0.08 h. An initial analysis was conducted by permitting NONMEM to estimate the base model parameters. Clinical and demographic covariates were subsequently tested within the base model to identify patient characteristics which improved the goodness of fit of the DBS data and explained the population variability in the parameters (CL and V) estimated.

5.4.2 Interindividual and interoccasion variability

The IIV in CL and V was modelled using an exponential error model in which differences between an individual and the population (ηi) are assumed to be log normally distributed. Exponential error models are advantageous as they avoid negative parameter estimation which can occur with additive error models. Deviations of caffeine CL and V for the ith individual from the population mean values were estimated as follows:

$$CL_i = TVCL.e^{(\eta i,CL + Kj,CL)}$$

$$V_i = TVV.e^{(\eta i,V + Kj,V)}$$

CL_i and V_i = Estimated parameters in the i^{th} individual on the j^{th} occasion.

TVCL and TVV = Population mean values for CL and V predicted by the regression model, respectively.

ηi,CL and ηi,V = Random variables, which are normally distributed with variance, ω^2, mean 0 and represent the difference between an individual and the population in CL and V, respectively.

Ignoring IOV in population models can result in biased parameter estimates (Karlsson and Sheiner, 1993). As samples were collected over several dosing occasions the inclusion of an error model to account for variability in PK parameters on different occasions (i.e. from one day to the next) was investigated. IOV in PK parameters was modelled exponentially as suggested by Karlsson and Sheiner, where Kj,CL and Kj,V are random variables which represent the variability in CL_i and V_i estimates on j occasions for an individual, respectively (Karlsson and Sheiner, 1993). An occasion was

defined as a dose or sequential doses followed by at least one observation. There were a median of 4 (range 1 to 10) occasions. Kj,CL and Kj,V were assumed to be normally distributed with means of 0 and a variance of π^2_{CL} and π^2_{V}, respectively. The IOV variability for each parameter (π^2_{CL} and π^2_{V}) was assumed to be sampled from the same sampling distribution.

5.4.3 Intraindividual variability

The residual variability was evaluated using additive, proportional and combined error models.

Additive model: $C_{ij} = \hat{C}_{ij} + \varepsilon_{1,ij}$

Proportional model: $C_{ij} = \hat{C}_{ij} . (1 + \varepsilon_{1,ij})$

Proportional and additive model: $C_{ij} = \hat{C}_{ij} . (1+ \varepsilon_{1,ij}) + \varepsilon_{2,ij}$

C_{ij} = The jth observed concentration in the ith individual

$\hat{C}_{ij}$ = The jth model predicted concentration in the ith individual.

$\varepsilon_{1,ij}$ and $\varepsilon_{2,ij}$ = Residual error terms, normally distributed, mean of 0 and variance of σ^2_1 and σ^2_2, respectively.

5.4.4 Covariate analysis

The regression model was developed within NONMEM using a forward inclusion backward elimination method (Wählby *et al.*, 2002). The OFV (-2LL) was used to test for statistical significance. The difference in OFV value between two models (one nested in another) approximates a chi squared distribution, with the degrees in freedom being the difference in the number of parameters. During the forward inclusion step for

models which differed by one parameter a drop in the OFV of greater than 3.84 which corresponds to a *p* value 0.05 was considered as statistically significant. All biologically plausible continuous and categorical covariates were tested one at a time in the base model and the change in OFV noted. Other criteria used to discriminate between two models included the interindividual variance (ω^2), residual variance (σ^2), precision in parameter estimation, precision in estimates of coefficients (95% confidence intervals for estimates do not contain 0) and improvement in goodness of fit plots. All significant covariates identified were incorporated into the base model to produce a full model. The backward elimination step was performed by removing each covariate one at a time to determine its influence within the model. Covariates which failed to show statistical significance were deleted from the model. A change in the OFV of 7.81 corresponding to a *p* value of 0.005 was required at this stage for covariates to be kept in the final model.

5.4.5 Model validation

Model validation is an important component of the model development process. Since there is no right or wrong model the subjectivity associated with model development also extends to the validation process. As such there is no single test or series of tests for determining the validity of a model and in practice a range of methods have been used (Guidance for Industry: Population Pharmacokinetics 1999). Although there is currently no consensus on the statistical approach that constitutes a validated model the method and extent of validation should be based on the purpose for which the model was developed and its intended use (Guidance for Industry: Population Pharmacokinetics 1999). Internal validation techniques involve resampling from the

original data set used to build the model and are particularly useful for paediatric studies where patient numbers are limited and there is no test data set available. External validation techniques require an additional data set but provide the most stringent method for evaluating the predictive performance of a model (Sun *et al.*, 1999). The purpose of the current model was to estimate PK parameters and describe the variability in caffeine disposition. The model was not intended for predictive applications or dose adjustment recommendations and thus an internal validation technique with bootstrapping was used to test the developed final model. With bootstrapping it is possible to calculate the precision, bias and confidence intervals of parameter estimates (Parke *et al.*, 1999). The method entails the generation of a series of new datasets of equal size to the original dataset by sampling individuals with replacement from the original dataset. The final model is then fitted to each dataset and the distribution of parameter estimates examined for bias and precision. Validation was performed using 500 datasets within PDx-POP (version 4.0, ICON Development Solutions, USA).

5.5 Results

5.5.1 Demographics of study patients

A total of 67 patients over a 10 month period were recruited onto the study from which 384 DBS samples were collected. The demographic characteristics of the study population are presented in Table 5.1. The study group consisted of preterm infants all born at less than 33 weeks gestation (24.6 - 32.7 weeks) with equal numbers of males and females. The PNA on the day of study enrolment varied significantly (range, 1 - 69), but the majority of patients were recruited within the neonatal period (Figure 5.2). Patient weight was recorded at each sampling time point and whenever infants were

weighed on the unit between sample collection times. Figure 5.3 shows the distribution of weights recorded for patients on the day of study enrolment. Except for a single patient, all preterms were of low weight (< 2 kg), approximately 20% of which were very low weight (< 1 kg). The only maternal covariate collected was smoking status during pregnancy and was obtained from medical notes. Clinical covariate data is presented in section 5.5.3.

Table 5.1 Characteristics of 67 preterm neonates

Patient characteristics	**Mean (± SD, CV%) and range**
Sex	33 Males 34 Females
Gestational age (weeks)	29.0 (± 1.9, 6.7%) 24.6 - 32.7
Postnatal age on day of study inclusion (days)	9.7 (± 12.2, 125.1%) 1 - 69
Postmenstrual age on day of study inclusion (weeks)	30.4 (± 2.1, 7.1%) 25.4 - 37.1
Postnatal age at end of study (days)	31.3 (± 18.2, 58.1%) 7 – 79
Birth weight (kg)	1.25 (± 0.34, 27.2%) 0.6 - 2.01
Weight on day of study inclusion (kg)	1.26 (± 0.34, 27.3%) 0.65 - 2.5
	Number of patients
Ethnicity	
White	50
Indian	10
African-Caribbean	2
Other	5
Maternal smoking	Yes 15 No 46 Unknown 6

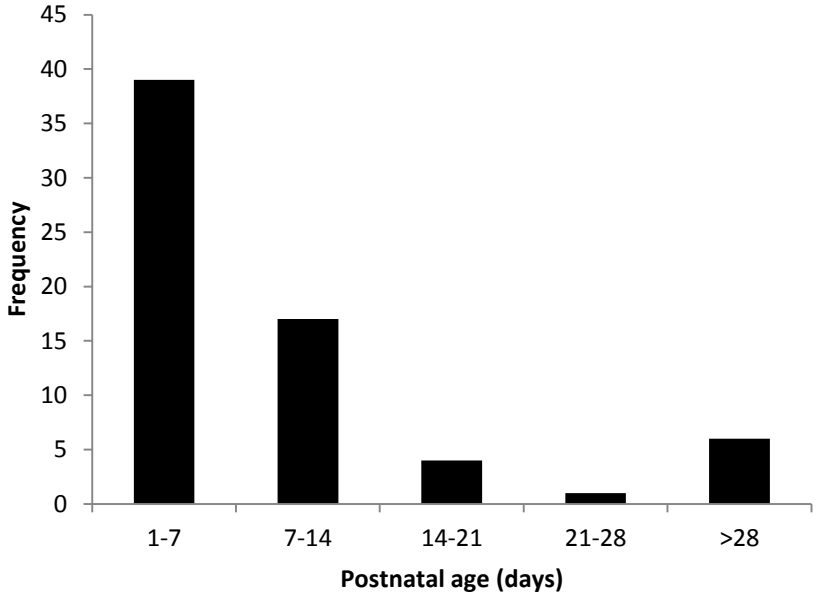


Figure 5.2 Postnatal age of study population on day of recruitment n=67

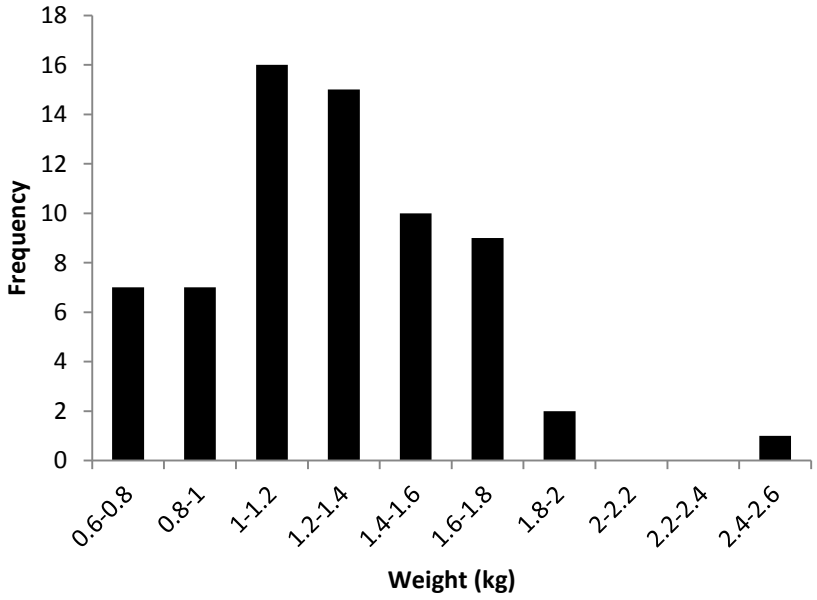


Figure 5.3 Distribution of weights recorded on day of study recruitment n=67

5.5.2 Dried blood spot sampling and caffeine measurements

Recruited patients received a mean caffeine loading dose of 10.1 mg/kg (range 9 - 12.5 mg/kg) followed by a once daily mean maintenance dose of 2.8 mg/kg (range 1.4 - 7 mg/kg) orally or intravenously (Table 5.2). Of the 384 DBS samples collected by researchers and clinical staff trained in the DBS technique, approximately 10% (40 DBS samples) did not meet the minimum standard of quality required for drug quantification and were not analysed. A further 6 DBS samples all collected several days after caffeine had been discontinued were determined to be BLOQ and were omitted from the data set. Therefore a total of 338 DBS caffeine concentration measurements from 67 patients were available for the PK analysis. Between 1 and 10 (mean, 5.6; mode, 5) DBS samples were collected from each patient over a mean study period of 21.6 days (Figure 5.4). DBS samples were collected following 245 enteral and 93 IV doses of caffeine.

The times of DBS collection post caffeine administration are shown in Figure 5.5. Samples were collected at a range of post dose intervals which is necessary for the estimation of PK parameters, CL and V. For most patients a DBS sample was collected after the discontinuation of caffeine, but none were collected prior to the start of treatment. Most blood samples (297) were collected following a capillary heelstick procedure. A small number of blood samples were collected from venous (27) and arterial (13) sites for patients with catheters *in situ*. Caffeine DBS concentrations determined via LC-MS/MS analysis (Chapter 4, section 4.2) ranged between 2780 to 43045 ng/ml in patients during treatment (Figure 5.6) (Appendix VI).

Table 5.2 Caffeine dosing and DBS sampling information

Dosing and sampling information	Mean (range)
Caffeine dose	Loading: 10.1 mg/kg (9 - 12.5 mg/kg) Maintenance: 2.8 mg/kg (1.4 - 7 mg/kg)
Total length of caffeine treatment (days)	28.2 (6 - 65)
Day of caffeine therapy on study enrolment	8.4 (1 - 47)
Study participation (days)	21.6 (1 - 61)
Number of samples per infant	5 (1 - 10)
[a]Concentrations measured in DBS samples (ng/ml)	16124 (665 - 43045)
Concentrations measured in DBS samples during treatment (ng/ml)	16475 (2780 - 43045)

a. Includes levels measured after discontinuation of caffeine treatment.

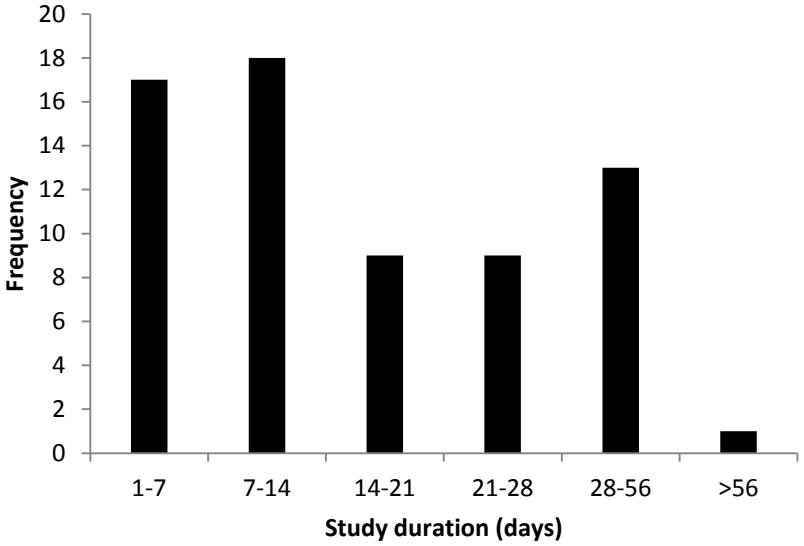


Figure 5.4 Number of days that patients were in the study

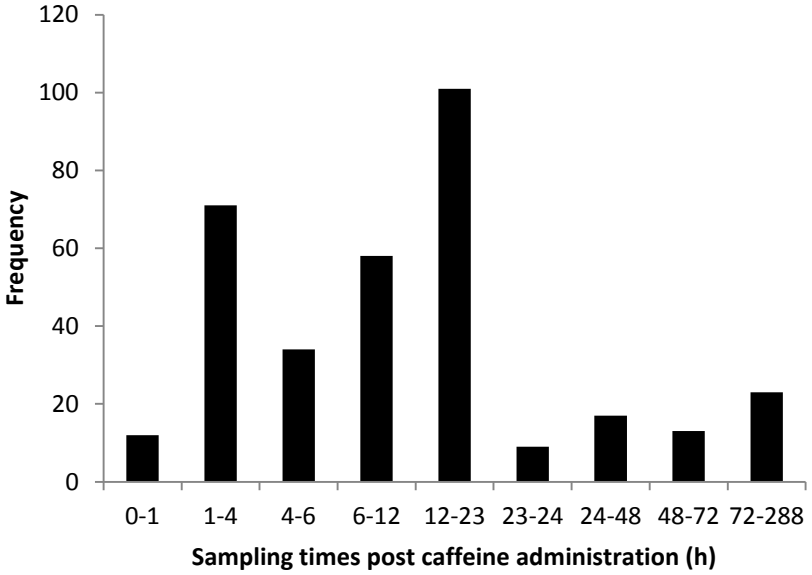


Figure 5.5 DBS sampling times post caffeine administration n=338

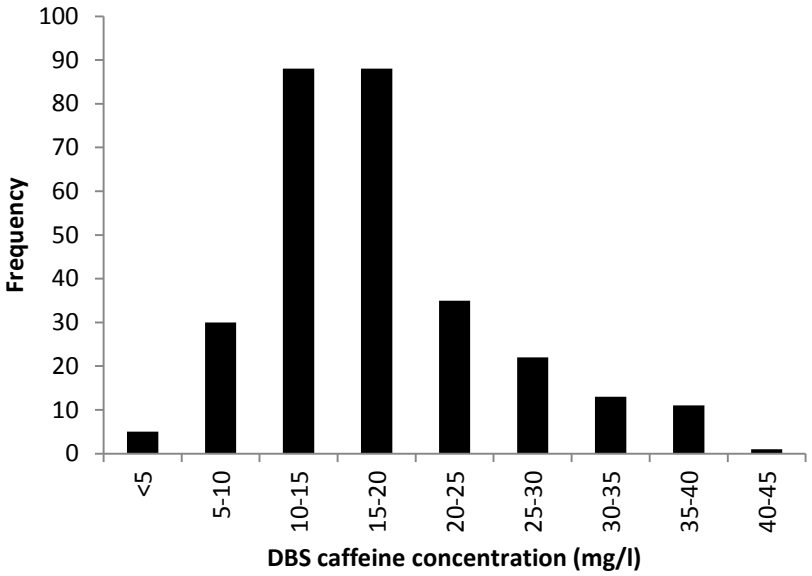


Figure 5.6 Caffeine concentrations measured in DBS samples collected from patients whilst receiving treatment

5.5.3 Clinical covariate data

Clinical covariate data recorded with each DBS sample included haematocrit, albumin, creatinine, feeding regimen and co-medications. Caffeine disposition has been shown to be affected by the feeding method used and therefore information was collected on the type of enteral feeding received (formula, breast milk or a combination) and parenteral nutrition status (Falcão *et al.*, 1997, Blake *et al.*, 2006). During the study 106 (33%) samples were collected from patients receiving parenteral nutrition. The types of enteral feeds received by patients are shown in Figure 5.7. The majority of samples (n=195, 58%) were collected from infants who were receiving breast milk. A smaller number were collected from patients on formula feeds or a combination of formula and breast milk. Significant variability in haematocrit (mean, 34%; range, 21 - 58%) was observed in the study population (Figure 5.8). As expected, the highest values were observed in the youngest neonates and a general decrease in haematocrit was observed with increasing PNA. A wide range of albumin concentrations were recorded for patients (mean, 30.9 g/l, range, 20 - 41 g/l) but correlations with age (GA, PNA or PMA) could not be detected (Figure 5.9). Patients received between 1 and 12 (mean 4) co-medications. A single patient was prescribed phenobarbitone, but no other known inhibitors or inducers of caffeine metabolism were identified. Medications most frequently prescribed to the study population (> 15%) were benzylpenicillin, flucloxacillin, gentamicin, sytron, dalavit and Joulie's phosphate.

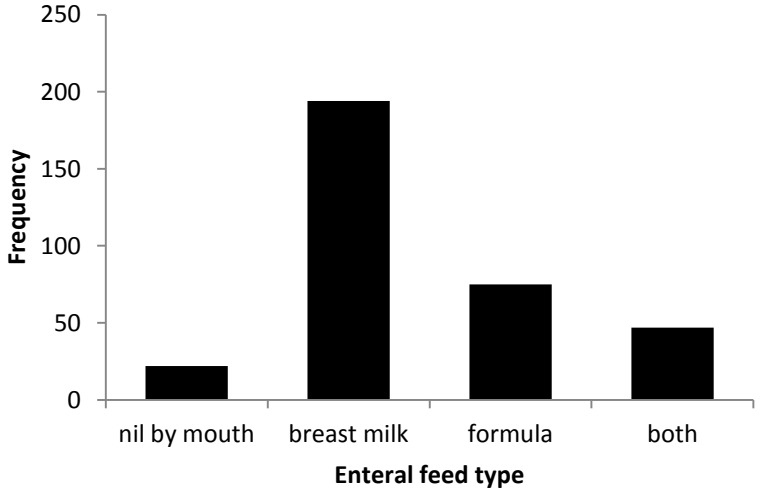


Figure 5.7 Information on the type of enteral feed study patients received during DBS sample collection

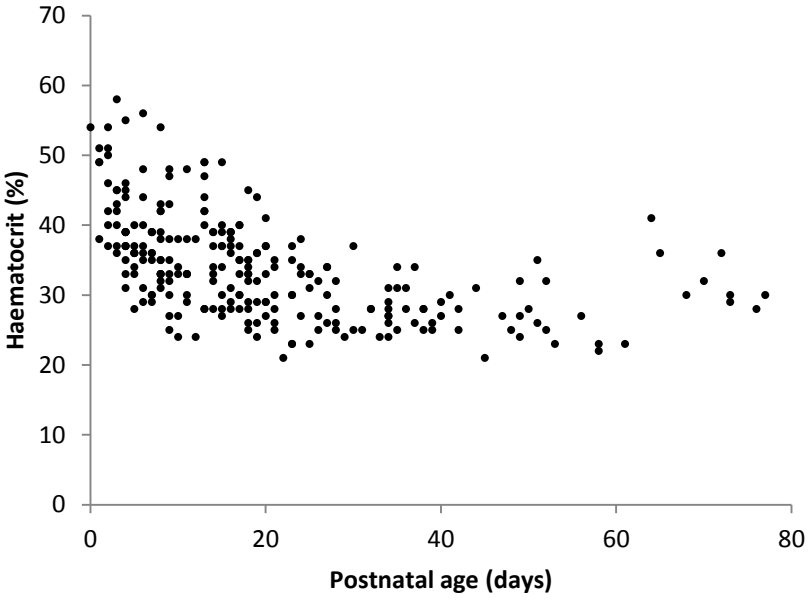


Figure 5.8 A plot of patient haematocrit (%) against postnatal age

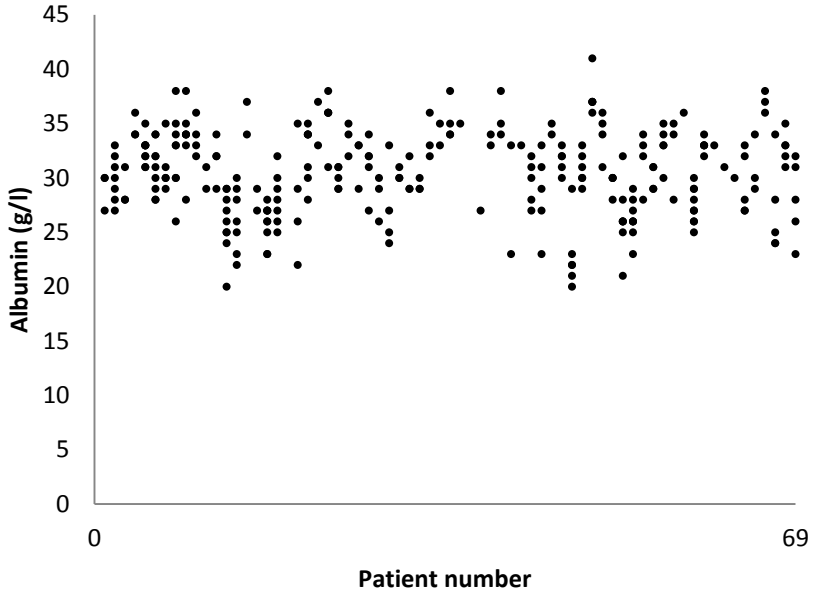


Figure 5.9 Albumin concentrations (g/l) measured in each patient

5.5.4 Pharmacokinetic analysis

5.5.4.1 Base model

All caffeine DBS concentration measurements (n=338) were allocated to the development of the PK model. Residual analysis and the OFV were used to assess goodness of fit in the structural model selection process and discriminate between different error models. A one compartment model resulted in a better fit of the data (OFV, 6135 units) compared with a two compartment model (OFV, 8822 units) and was therefore chosen to fit the DBS concentration-time data. An oral absorption model (Figure 5.10) with direct input of IV doses into the central compartment was used to enable the simultaneous analysis of DBS concentration data collected following oral and IV dosing.

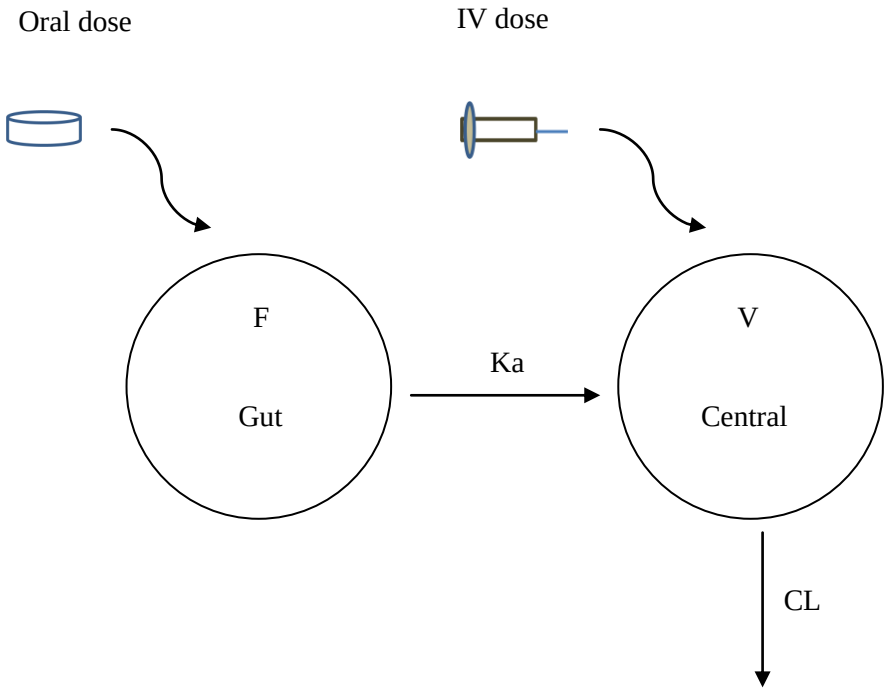


Figure 5.10 Structural model of the PK of caffeine: an oral absorption model with direct dose input into central compartment for IV doses.

The model assumes first order oral absorption for oral doses, zero order infusion for IV doses and first order elimination from the central compartment. Bioavailability (F) and Ka were fixed with CL and V parameters to be estimated. Both additive and combined error models were found to be suitable for modeling the residual error. In the selected base model residual variability was modelled using an additive residual error model and IIV and IOV were modelled using exponential error models. The inclusion of IOV in CL significantly reduced the OFV (from 6135 to 6020 units) and reduced the residual variance, but an IOV on V could not be estimated. The base model converged successfully to give initial population mean estimates of 9.1 ml/h (CV%, 4.6) and 767 ml (CV%, 7.9) for CL and V, respectively.

5.5.4.2 Covariate model

Scatter plots of covariates versus individual Bayesian parameter estimates were examined to aid in the identification of factors which may have an influence in the model. These plots were also used to identify the likely nature of the covariate-parameter relationship (linear or non-linear) and derive initial numerical estimates of coefficients and exponents. A correlation between CL and weight ($r^2 = 0.28$) as well as PNA ($r^2 = 0.21$) was evident (Figure 5.11). A positive correlation between weight and V was also observed.

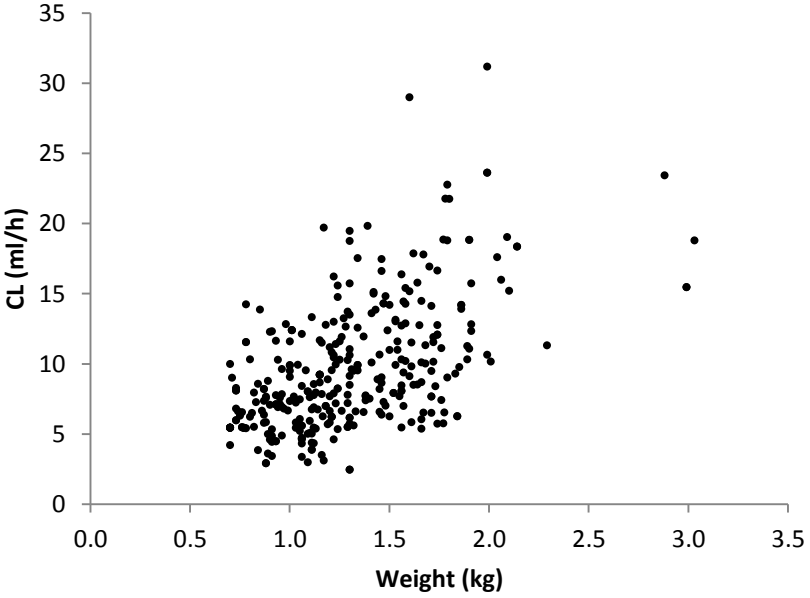
Covariate-PK parameter correlations are not always evident from the visual examination of scatter plots and thus all biologically plausible covariates were tested in the base model. During the forward selection process the effect of the continuous covariates weight, age in various forms (GA, PNA and PMA) and albumin were tested in the V model. Continuous and categorical covariates screened for influence on CL estimation included weight, age, albumin, creatinine, urea, haematocrit, feeding regimen, sex, ethnicity and maternal smoking status. Current weight resulted in the largest drop in OFV (14 units) in the V model followed by PMA and albumin. In the CL model, weight (58 units) and age (PNA, 74 units; PMA, 56 units) resulted in the largest drop in OFV. Haematocrit, parenteral nutrition and albumin were also identified as significant covariates for CL. After the inclusion of weight on CL and V, PMA was no longer statistically significant on either PK parameter. However, PNA was still a significant covariate for CL. At this stage GA was formally tested as a dichotomised variable in the CL model with patients divided into two groups; those born at less than 28 weeks (very premature infants) and greater than 28 weeks gestation. No improvement in the fit of the

data resulted from the inclusion of GA. The full model which consists of the base model with all statistically significant covariates incorporated is shown in Table 5.3.

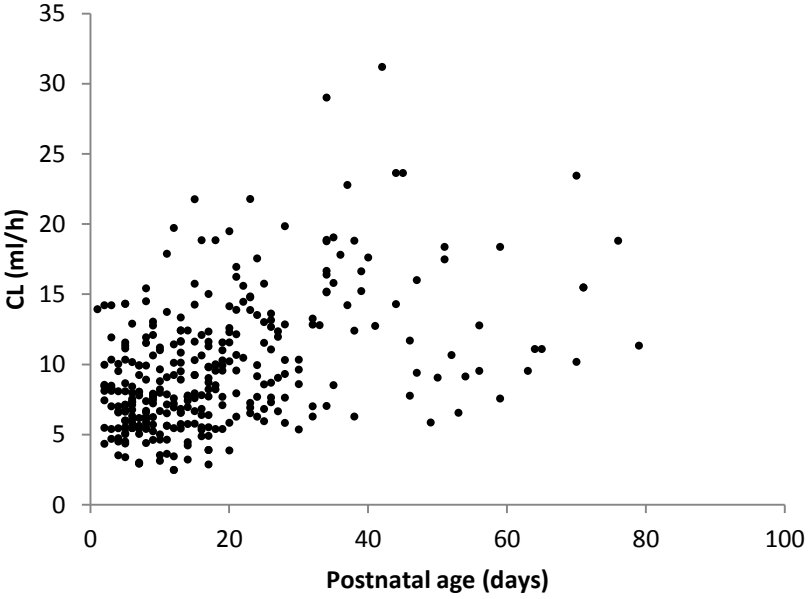
During the backward elimination process only weight and PNA were found to be significant covariates in the CL model, whereas weight alone was the only covariate influential in the V model which was demonstrated by an increase in OFV of > 7.84 units when either of these covariates was removed from the model. Figure 5.12 shows the distribution of weighted residuals before and after the inclusion of weight as a covariate in the CL model. Before the inclusion of weight a systematic deviation in the residuals could be seen with an under prediction at low weights and over prediction at high weights. Following the inclusion of weight an improvement was observed with residuals more evenly distributed around the zero ordinate. Similarly the systematic deviation in the differences between the population mean and individual CL estimates (ETAs) were no longer apparent after modeling the effect of weight on CL (Figure 5.13).

Other covariates which were significant in the base model during the initial (forward selection) screening process did not show significance even at a *p* value of 0.05 (change in OFV of 3.84) after the inclusion of weight and PNA. As a final check each covariate in the final model was removed one at a time and the reduced model was tested for statistical significance against the final model.

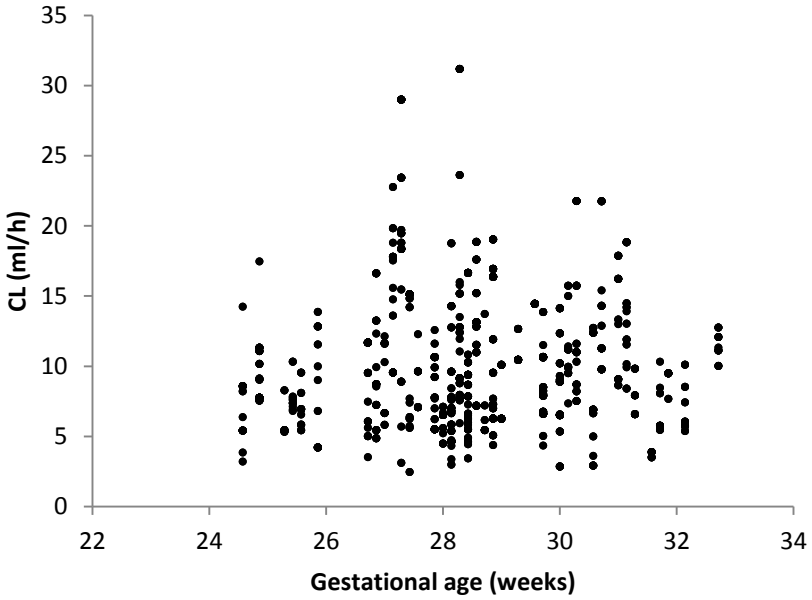
A


CL (ml/h)
Weight (kg)
0
5
10
15
20
25
30
35
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

B


CL (ml/h)
Postnatal age (days)
0
5
10
15
20
25
30
35
0
20
40
60
80
100

C



D

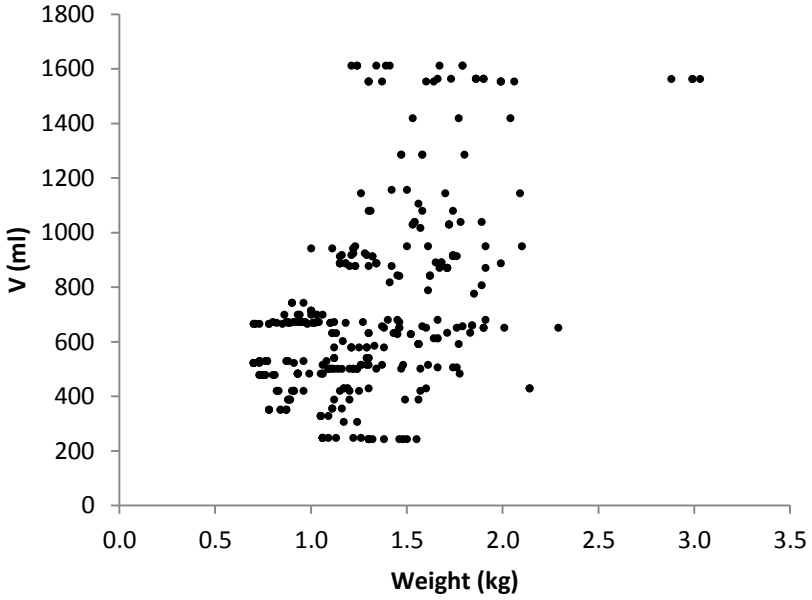


Figure 5.11 Scatter plots of individual Bayesian estimates of CL versus weight (A), postnatal age (B), gestational age (C) and V versus weight (D).

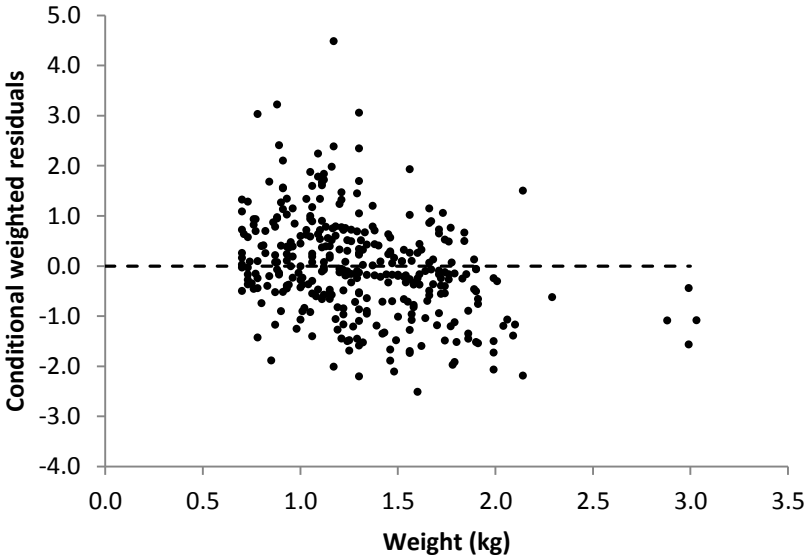
Table 5.3 Covariate model development; the effect of covariates in the determination of V and CL on the objective function value

Model	**OFV**	**[a]Δ OFV**	***p* value**	**Interpretation**
CL V	6020.391			Base model (no covariate influence)
CL = f(WT, PNA, HAEM, ALB, PN) V = f(WT, ALB)	5904.203		$p < 0.005$	Full model
CL = f(WT, PNA, HAEM, ALB) V = f(WT, ALB)	5905.093	↑ 0.890	$p < 0.005$	PN does not affect CL
CL = f(WT, PNA, HAEM) V = f(WT, ALB)	5905.874	↑ 0.781	$p < 0.005$	ALB does not affect CL
CL = f(WT, PNA) V = f(WT, ALB)	5908.920	↑ 3.046	$p < 0.005$	HAEM does not affect CL
CL = f(WT) V = f(WT, ALB)	5923.642	↑ 14.722	$p < 0.005$	PNA does affect CL
CL = f(PNA) V = f(WT, ALB)	5946.290	↑ 37.371	$p < 0.005$	WT does affect CL
CL = f(WT, PNA) V = f(WT)	5909.877	↑ 0.957	$p < 0.005$	ALB does not affect V
CL = f(WT, PNA) V	5934.938	↑ 25.061	$p < 0.005$	No covariate influence on V WT does affect V

a. Change in objective function (-2 log likelihood) resulting from the removal of individual covariates from the model. Assuming a chi squared distribution, a change in OFV of 7.84 units ($p < 0.005$) was accepted as statistically significant.

WT = weight; PNA = postnatal age; HAEM = haematocrit; ALB = albumin; PN = parenteral nutrition.

A



B

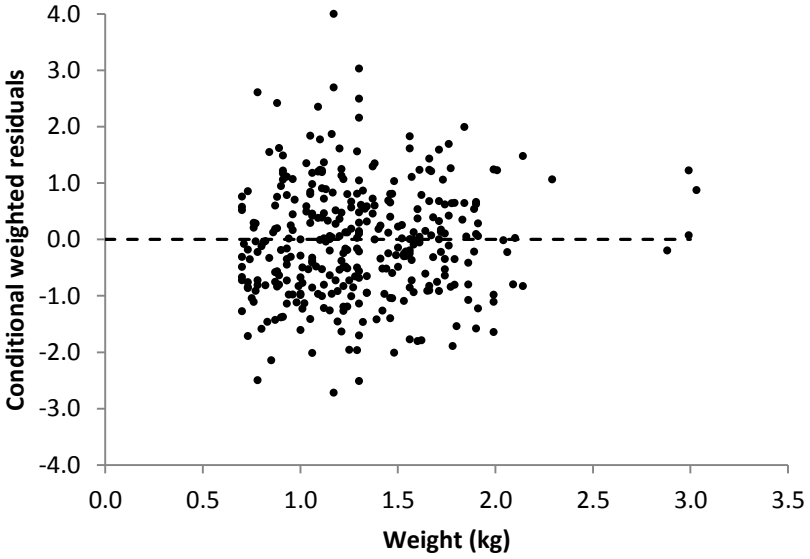
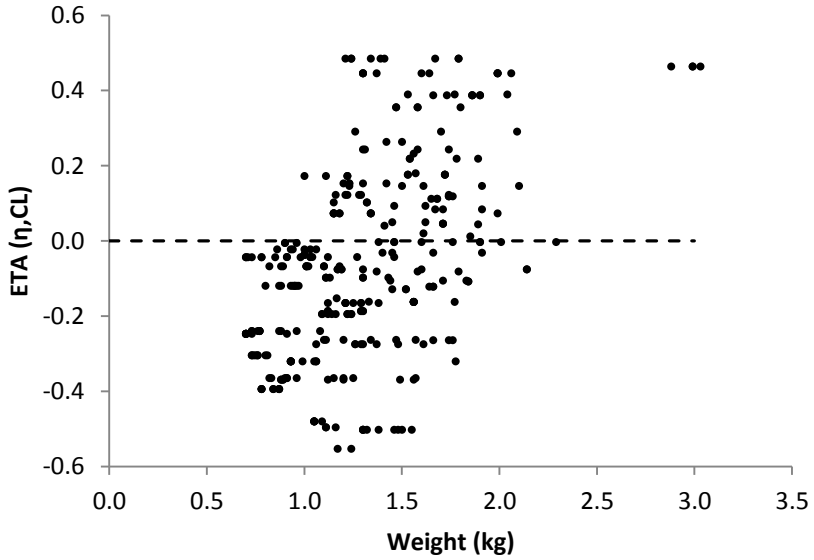


Figure 5.12 Scatter plot of weight versus conditional weighted residuals before (A) and after the inclusion of weight (B) as a significant covariate in the CL model

A



B

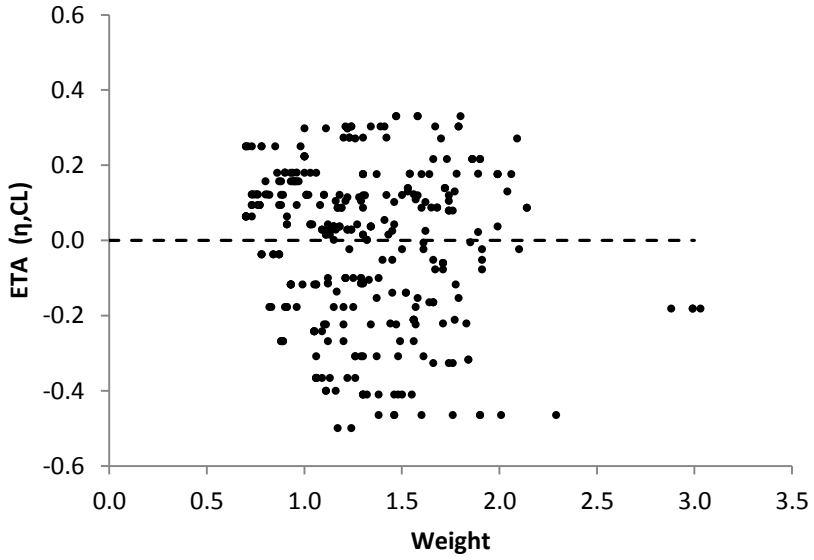


Figure 5.13 Scatter plots of weight versus ETA (η,CL), the difference between an individual and the population mean in CL before including weight as a covariate (A) and after the inclusion of weight (B).

5.5.4.3 Final model

The final PK model is shown below where CL (theta 1, θ_1) is modelled as a function of weight and PNA, and V (θ_2) is modelled simply as a function of weight alone. θ_5 to θ_7 are exponents and coefficients for the covariate influences on PK parameters. Covariate effects centred on median weight and age resulted in the most numerically stable covariate models in NONMEM. It was possible to model the influence of weight on parameters linearly and non-linearly using a power equation with little difference in the resulting drop in OFV. A power model was chosen due to their purported biological basis for relating physiological processes such as CL and V to size (weight) (Mordenti *et al.*, 1991). The relationship between PNA on CL could be modelled linearly and exponentially. A larger drop in the OFV with better precision in estimating covariate influence was possible using a linear equation and was therefore used in the final model.

The final PK model:

$$CL\ (\text{ml/h}) = \theta_1 \times \left(\frac{\text{WT}}{1.28}\right)^{\theta 5} \times (1 + \theta_7 \times (\text{PNA} - 15))$$

$$V\ (\text{ml}) = \theta_2 \times \left(\frac{\text{WT}}{1.28}\right)^{\theta 6}$$

$$Ka\ (\text{h}^{-1}) = \theta_3 = 4.0$$

$$IV\ bolus\ duration\ (\text{h}) = \theta_4 = 0.08$$

The population variability in CL and V was calculated by taking the square root of the individual variances (ω^2_{CL} and ω^2_V) in the OMEGA matrix of the NONMEM output and expressing the value as a percentage:

$$Population\ estimate\ variability\ (\%) = 100 \times (\text{variance})^{0.5}$$

The IOV in CL was similarly calculated by taking the square root of the variance component (π^2_{CL}) in the OMEGA matrix. Residual variability was calculated by taking the square root of the residual variance estimate (σ^2) in the SIGMA matrix of the NONMEM output to give the SD. Table 5.4 summarises the influence of each covariate addition on the population variability in CL and V. The addition of weight as a covariate into the base model resulted in a decrease in the IIV in CL and V from 30.1% to 26.1% and 50.5% to 43.4%, respectively. The incorporation of PNA in the CL model did not reduce the interindividual variance and therefore explain any of the population variability in CL but was kept in the final model as it provided an improvement in the OFV. Although CL was influenced by body weight the covariate only described 25% of the interpatient variability. Weight as a covariate accounted for 20% of the IIV in V. A reduction in the IOV from 41.1% to 34.1% and 31.4% was observed following the inclusion of weight and PNA, respectively on CL.

The final structural and random error model parameter estimates along with their associated precision (CV%) are presented in Table 5.5. The precision (CV%) of parameter estimates was calculated by dividing the standard error of the parameter estimate by its value and expressing the ratio as a percentage as shown below (Grasela and Sheiner, 1991):

$$Estimate\ precision\ (CV)\% = \frac{Standard\ error\ of\ the\ estimate}{Parameter\ estimate} \times 100$$

Table 5.4 Effect of covariates on objective function value and random effect variables (IIV, IOV and residual error).

Model	**IIV in CL (ω_{CL}) %**	**IIV in V (ω_V) %**	**IOV in CL (K_{CL}) %**	**Residual error (σ) (ng/ml)**	**[a]OFV**
Base model					
$CL = \theta_1$	30.1	50.5	41.1	1749	6020.391
$V = \theta_2$					
Clearance model					
$CL\ (ml/h) = \theta_1 \times (WT/1.28)^{\theta 5}$	26.1	_	34.1	1863	5962.291
$V = \theta_2$					
$CL\ (ml/h) = (1+\theta_7 \times (PNA-15))$	31.5	_	31.4	1900	5946.799
$V = \theta_2$					
Volume model					
$V\ (ml) = \theta_2 \times (WT/1.28)^{\theta 6}$	_	43.4	_	1712	6006.329
$CL = \theta_1$					
Final model					
$CL\ (ml/h) = \theta_1 \times (WT/1.28)^{\theta 5} \times (1+\theta_7 \times (PNA-15))$	24.7	45.7	31.2	1764	5909.877
$V\ (ml) = \theta_2 \times (WT/1.28)^{\theta 6}$					

a. Objective function value (-2 log likelihood).

The population mean PK estimates for CL and V were 8.6 ml/h (range 2.4 - 30.1 ml/h) and 774 ml (range 214 - 1654 ml), respectively. Both CL (CV%, 4.0) and V (CV%, 7.9) were estimated within the acceptable precision limit of less than 20 - 25% which has been suggested for fixed effect parameters (Ette *et al.*, 1993, Ette *et al.*, 1998). The

estimated exponents for weight on CL (0.79) and V (1.03) were very close to the physiologically based values of 0.75 and 1 which have been used to relate weight to PK parameters (Mordenti *et al.*, 1991, Anderson *et al.*, 2000). Fixing the exponents in the final model to these theoretical values did not affect parameter estimation or improve the precision of estimates or the fit of the data. As the estimate of the exponent for weight on V was essentially 1, the correlation between weight and V is effectively reduced to a simple linear relationship ($V = \theta_2 \times (WT/1.28)$). The exponents for weight on CL (CV%, 17.7) and V (CV%, 24.9) were estimated with reasonable precision. Estimation of the coefficient for PNA on CL was associated with the highest imprecision (CV%, 32.9). This may have been due to the relatively small number of patients studied in relation to the large variability in PNA (1 - 69 days) amongst study participants. The population IIV in CL and V and IOV on CL was estimated with a precision (CV%) of 25.0%, 44.2% and 22.5%, respectively. The additive residual error was estimated with a precision (CV%) of 23.7%. The error (SD, 1.76 mg/l) associated with prediction of caffeine concentrations expressed as a percentage of the mean caffeine concentration (16.5 mg/l) measured during treatment was 10.7%. Despite the relatively high imprecision in estimating IIV in V all random effects parameters were estimated within the accepted 50% limit (Ette *et al*., 1993).

Scatter plots of conditional weighted residuals (CWR) against final model predicted concentration (A), time after last dose (B) and patient ID (C) are shown in Figure 5.14. CWR were randomly and symmetrically distributed around the mean (zero ordinate, line of unity) with no systematic deviations in predicted concentration indicating the suitability of the error model chosen. Similar CWR were observed according to time of sampling indicating suitability of the structural model. Most residuals were within ± 2

SD of the mean and thus the model enabled an adequate description of caffeine PK. The histogram presented in Figure 5.15 also shows weighted residuals were randomly and evenly distributed with a mean of 0 in the final model. The relationship between the population and individual model predicted and observed DBS concentrations are shown in Figure 5.16. Simulations using individual parameter estimates for selected patients presented in Figure 5.17 demonstrate the goodness of fit between model predicted and observed concentrations. Goodness of fit plots between model predicted and observed concentrations for all patients are presented in Appendix VII.

Table 5.5 Final caffeine population model parameter estimates

Parameter		[a]Final model estimate (units)	[b]Precision of estimate (CV%)
Fixed effect parameters (structural model & covariate model)			
θ_1	CL	8.6 ml/h	4.0
θ_2	V	774 ml	7.9
θ_3	Ka	4.0 h^{-1}	Not estimated
θ_4	Duration (IV bolus)	0.08 h	Not estimated
θ_5	Exponent for WT on CL	0.786	17.7
θ_6	Exponent for WT on V	1.030	24.9
θ_7	Coefficient for PNA on CL	0.013	32.9
[c]**Random effects parameters** (Variance model)			
	IIV ω_{CL}	24.7 %	25.0
	IIV ω_V	45.7 %	44.2
	IOV K_{CL}	31.2 %	22.5
Random effects parameter (Residual error model)			
	σ, additive (SD)	1.76 mg/l	23.7

a. Final model: CL (ml/h) = $8.6 \times (WT/1.28)^{0.786} \times (1 + 0.013 \times (PNA-15))$

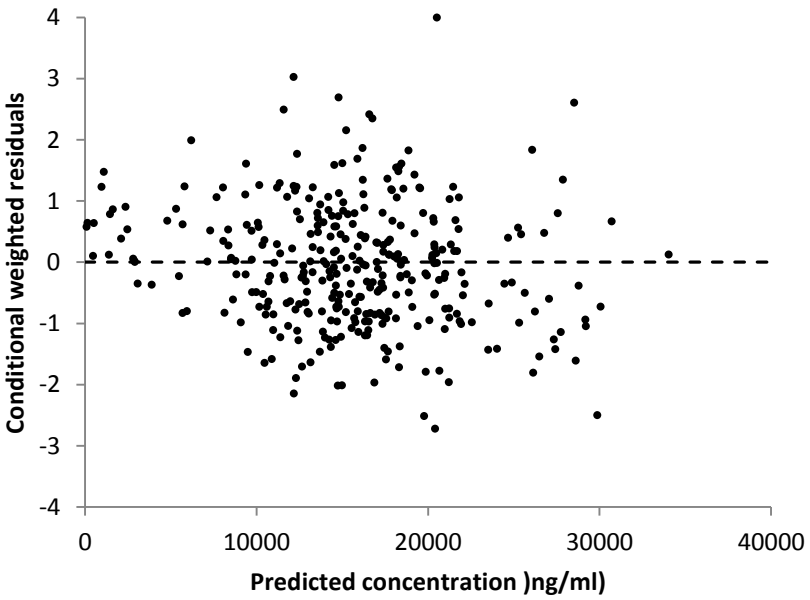
V (ml) = $774 \times (WT/1.28)^{1.03}$.

b. Standard error / parameter estimate × 100 = CV%.

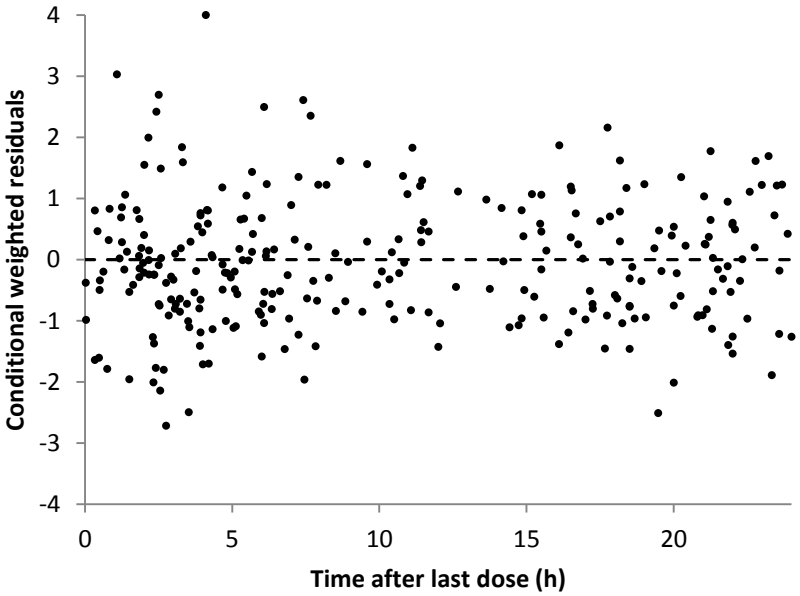
c. $(Variance)^{0.5} \times 100$ = % variability in population.

ETA Shrinkage (%): ω_{CL} = 18.8; ω_V = 21.2; EPS Shrinkage: σ = 38.5.

A


Conditional weighted residuals
4
3
2
1
0
-1
-2
-3
-4
0
10000
20000
30000
40000
Predicted concentration)ng/ml)

B


Conditional weighted residuals
4
3
2
1
0
-1
-2
-3
-4
0
5
10
15
20
Time after last dose (h)

C

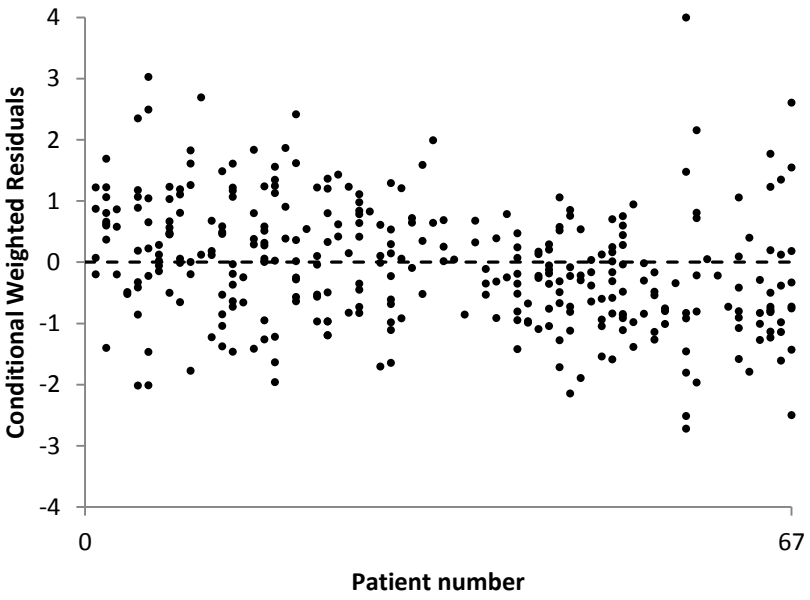


Figure 5.14 Diagnostic goodness of fit plots of conditional weighted residuals versus (A) model predicted concentration, (B) time after dose and (C) patient number. For each plot the dotted line represents perfect agreement (line of unity).

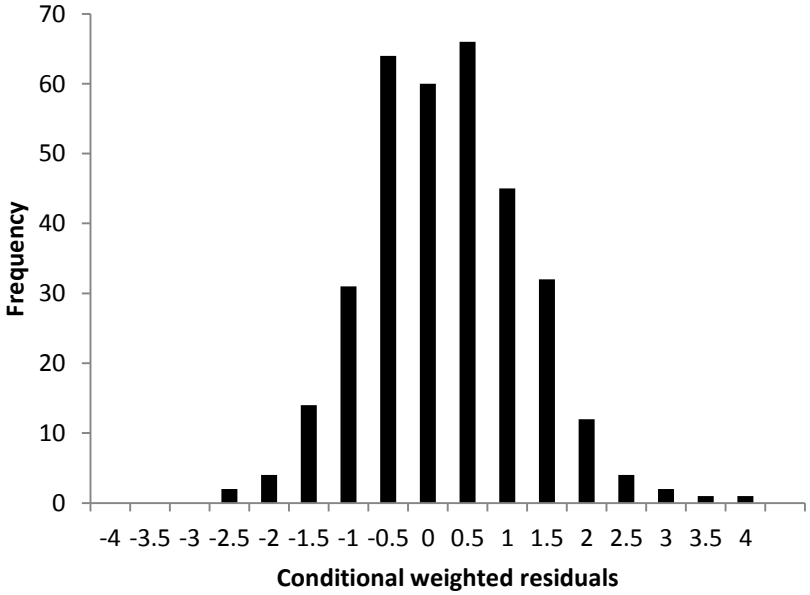
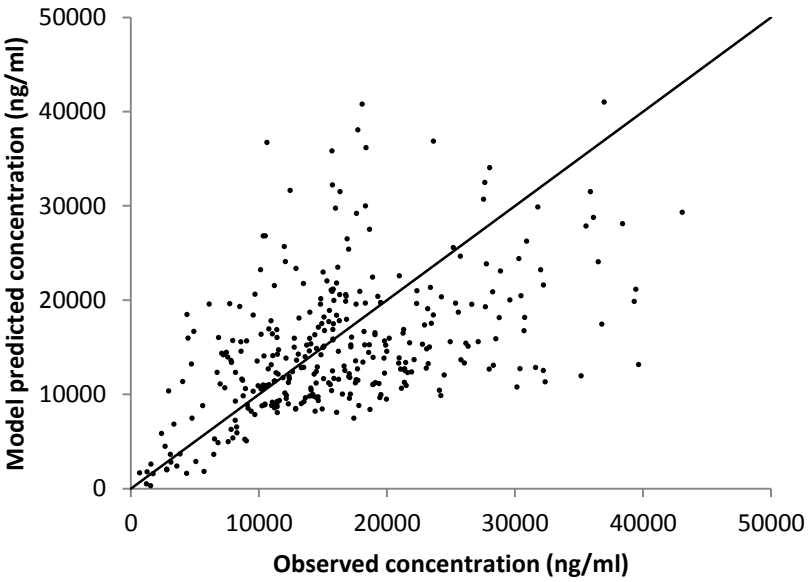


Figure 5.15 Distribution of conditional weighted residuals for final PK model

A



B

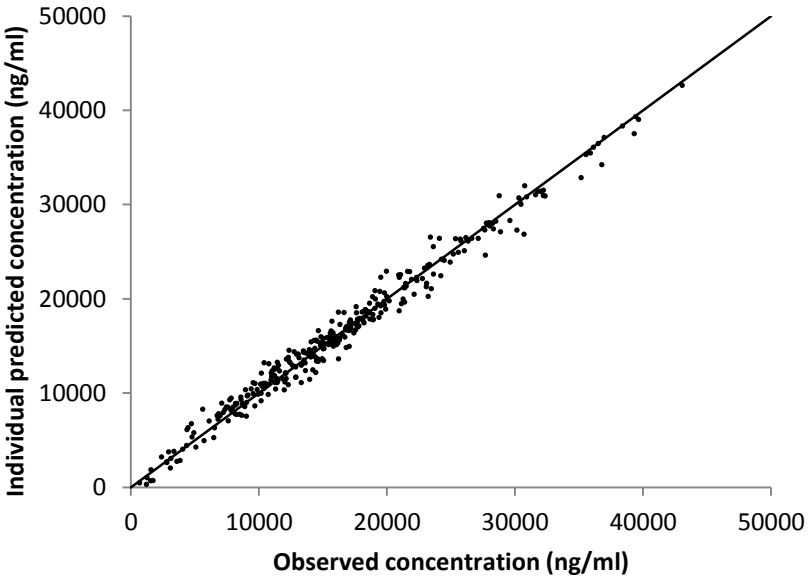
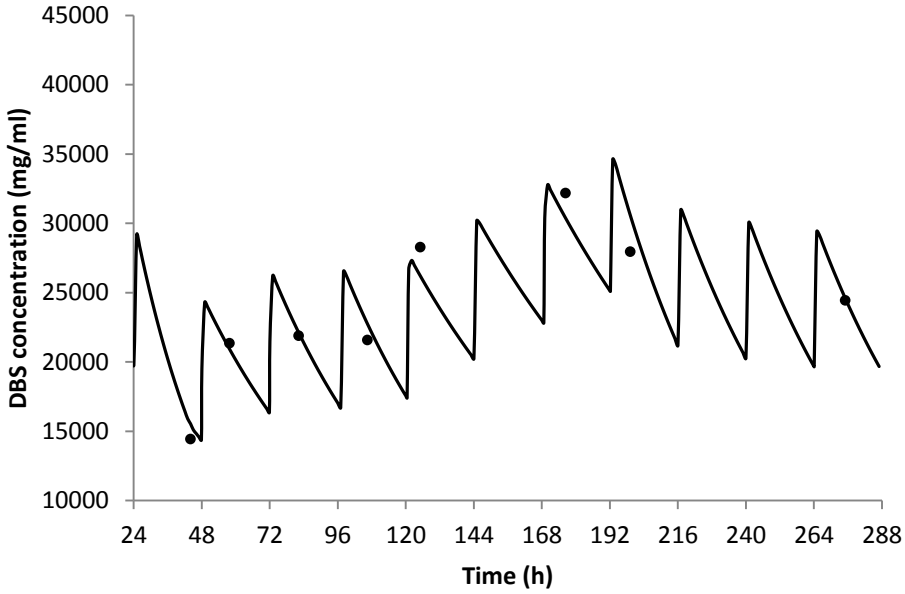


Figure 5.16 Goodness of fit plots for observed versus population model predicted concentration (A) and observed versus individual model predicted concentration (B)

A



B

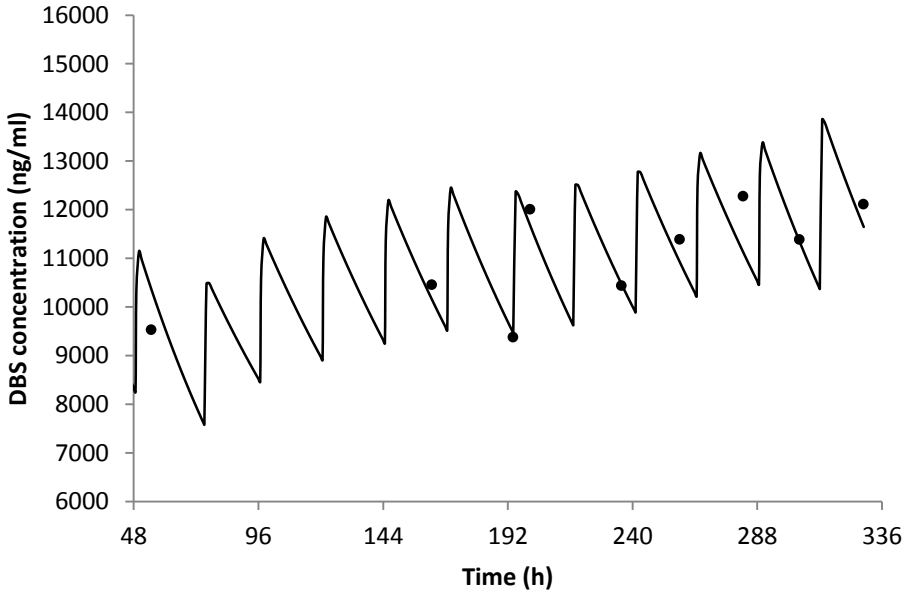


Figure 5.17 Predicted DBS caffeine concentration-time profiles (—) and observed DBS concentration data (•) for patient A and B.

5.5.4.4 Model validation

During the bootstrap validation 94% of (500 replicate) NONMEM runs minimised successfully indicating stability of the final population model. The bootstrap parameter estimates and 95% confidence intervals generated by fitting the final model to 500 datasets randomly constructed with replacement are shown alongside the final model parameters estimated from the original dataset in Table 5.6. The bootstrapped structural model parameters (CL, V and coefficients) and random effects parameters closely agreed with respective values from the final model (differences < 6%) indicating the robustness of the final model. Furthermore all parameter estimates were within the bootstrap generated 95% confidence interval. The precision associated with estimation of the residual error was higher in the bootstrap results (23.7% *vs.* 39.8%) but still acceptable. For all other parameters the precision was comparable between the bootstrap and final model results. These results indicate that the developed model is robust and has good stability.

Table 5.6 A comparison of nonparametric bootstrap estimates with original estimates

Parameter	NONMEM (original)		Bootstrap (500 replicates)		Difference [a](bias %)	Confidence Interval 95%
	Estimate	CV%	Estimate	CV%		
Fixed effects						
CL ml/h	8.6	4.0	8.6	4.1	0.07	7.9 - 9.3
V ml	774	7.9	781	9.2	-0.94	640 - 939
Ka h^{-1}	4.0	Ne	4.0	Ne	-	-
Duration (IV bolus) h	0.08	Ne	0.08	Ne	-	-
Exponent for WT on CL	0.786	17.7	0.784	18.9	0.17	0.504 - 1.090
Exponent for WT on V	1.030	24.9	1.031	29.0	-0.15	0.524 - 1.700
Coefficient for PNA on CL	0.013	32.9	0.014	36.3	-5.29	0.005 - 0.025
[b]Random effects						
IIV ω_{CL}	24.7 %	25.0	24.5 %	30.9	0.58	15.7 - 31.6
IIV ω_{V}	45.7 %	44.2	43.9 %	45.5	3.88	22.0 - 62.1
IOV K_{CL}	31.2 %	22.5	30.1 %	28.4	3.30	18.5 - 37.7
Random effects						
Residual error σ, additive (SD) mg/l	1.76	23.7	1.85	39.8	-5.11	1.36 - 2.78

Ne = Not estimated

a. % bias = (final model estimate - bootstrap estimate) / final model estimate × 100.

b. Estimates given as CV%.

5.6 Comparison of pharmacokinetic model with plasma data

Several traditional (Aranda *et al.*, 1979b, Gorodischer and Karplus, 1982, Le Guennec *et al.*, 1985) and population studies (Thomson *et al.*, 1996, Falcão *et al.*, 1997, Lee *et al.*, 1997, Lee *et al.*, 2002, Charles *et al.*, 2008) have been conducted to investigate the plasma PK of caffeine in neonates. As such caffeine PK has been well characterised in this age group and a selection of reported studies are shown in Table 5.7. For comparative purposes three large prospective population studies were chosen (Table 5.8) (Thomson *et al.*, 1996, Lee *et al.*, 1997, Charles *et al.*, 2008). There were differences in the maturity (GA and PNA) of patients in each study, the treatment dose and methods of sample collection and bioanalysis. The comparison was possible as linear disposition kinetics have been observed in neonates for caffeine doses up to at least 30 mg/kg (Lee *et al*., 1997). In each of these reported studies a one compartment model was also found to be the most suitable for modeling caffeine disposition in neonates. The plasma PK estimates are compared alongside estimates obtained in the current study in Table 5.9. The population mean CL value of 7.3 ml/h/kg determined in the current study is very close to the average estimates of 7.9 ml/h/kg and 7.0 ml/h/kg reported by Thomson *et al* and Charles *et al*, respectively. The lower CL (4.9 ml/h/kg) reported by Lee *et al* may be due to the lower PNA (4 days, range 2 - 15) of the patients studied since age has been shown to be positively correlated with caffeine CL both in the current and previous studies. The mean V determined in the current study of 593 ml/kg was lower than the values of 970 ml/kg, 640 ml/kg and 851 ml/kg previously reported. The lower V may be due to the changes which occur in the extracellular fluid (ECF) compartment with age. Neonates have an increased volume of ECF relative to total body water compared with infants and adults which can lead to higher distribution

volumes for drugs such as caffeine which readily distribute throughout the body. The ECF gradually decreases with PNA and can fluctuate with the relative humidity of the environment (Kearns *et al.*, 2003). Although the mean PNA of the study group was only 9.7 days on study entry, by study exit this had increased to 31.3 days. It is noteworthy that the lowest literature V estimates (of 640 ml/kg and 781 ml/kg) were reported in studies involving relatively more mature neonates (Gorodischer and Karplus, 1982, Thomson *et al.*, 1996). Thus, differences in population demographics as well as patient care may account for the lower V in this study compared to published data. A smaller V would also account for the comparatively lower mean caffeine half life (57 h) estimated in the current study.

On comparison of the random effects variables, the IIV in CL (24.5%) was similar to that reported by Lee *et al* (25.0%), Thomson *et al* (20.0%) and Charles *et al* (18.8%). The IIV in V (45.7%) however, was significantly higher than previous reports of 11%, 24.3% and 18.8%. This may have been a consequence of the limited (< 1%) DBS samples collected following the loading dose (and hence the distribution phase) in the present study. This contrasts to comparative studies where samples were collected at random time intervals following the loading dose for each patient. The precision associated with the estimation of IIV in V (44.2%) in the current study was similar to that reported by Lee *et al* (47.4%). The precision associated with parameter estimates were not reported in the other two studies. The IOV in CL was similar to that determined in the study by Charles *et al* (31.2 *vs.* 35.1%). Although the IOV was greater than the IIV in CL in the present study, this was not as pronounced as previously observed where an almost two fold difference was noted. The additive residual variability (SD, 1.8 mg/l) was slightly higher than that reported in the study by Charles

et al where the residual variance was modelled using a combined additive and proportional error model (σ_1, 0.9 mg/l; σ_2, 6.0 CV%). The absence of an error model to account for IOV in structural parameters as well as differences in bioanalytical methods may have contributed to the larger residual error reported by Lee *et al* and Thomson *et al* (3.9 mg/l and 2.5 mg/l, respectively).

Table 5.7 Reported plasma PK studies for caffeine in neonates

Reference	Sample size	Sampling site	PNA (days)	GA (weeks)	CL (ml/h/kg)	V (ml/kg)	$t_{1/2}$ (h)
(Aranda *et al*., 1979b)	n=12	C	11.5	28.5	8.9	916	103
(Charles *et al*., 2008)	n=110	V	12	27.5	[a]7.0	[a]851	[a]101
(Gorodischer and Karplus, 1982)	n=13	C	6.5	30.6	8.5	781	65
(Lee *et al*., 1997)	n=89	C	4.0	28.2	4.9	970	144
(Lee *et al*., 2002)	n=18	C	—	28.9	[b]5.6	[b]862	106
(Thomson *et al*., 1996)	n=60	—	[a]23	31.0	7.9	640	—

CL, V and $t_{1/2}$ estimates are expressed as mean values unless otherwise indicated; **—** = not reported.

a. Median value reported. b. Estimates were normalised to weight using the mean study group weight.

C = capillary; V = venous; PNA = postnatal age; GA = gestational age.

Table 5.8 A comparison of the current study design with three selected population PK studies

Study information	**Current study**	**Study I** **Lee *et al*., 1997**	**Study II** **Thomson *et al*., 1996**	**Study III** **Charles *et al*., 2008**
PNA (days)	9.7 (1 - 69)	4.0 (2 - 15)	[a]23 (1 - 100)	12 (1 - 45)
GA (wk)	29.0 (24.6 - 32.7)	28.2 (24 - 31)	—	27.5 (24 - 29)
Weight (kg)	1.26 (0.65 - 2.50)	1.17 (0.57 - 2.31)	[a]1.3 (0.6 - 2.9)	0.99 (0.66 - 1.86)
Study duration	1 - 61 days	≤ 7 days	—	≤ 7 days
Dose & sampling	10 mg/kg (L), 2.5 mg/kg (M) Opportunistic, random sampling design 338 DBS samples Mean 5 (range 1 - 10) time points per patient	30, 15 or 3 mg/kg (L), 15, 7.5 or 1.5 mg/kg (M) Pre-randomized sampling times 430 capillary blood samples Mean 5 (range 3 - 6) time points per patient	10 mg/kg (L), 2.5 mg/kg (M) Once weekly sampling 186 blood samples [a]3 (range 1 - 13) time points per patient	40 or 10 mg/kg (L), 10 or 2.5 mg/kg (M) Pre-randomized sampling times 431 venous blood samples Mean 4 (range 1 - 8) time points per patient
Bioanalysis	15 µl DBS sample LC-MS/MS LLOQ, 0.25 mg/l	Serum (250 µl blood) LC-UV LLOQ 0.2 mg/l	Serum (—) Enzyme immunoassay LLOQ, 1 mg/l	Plasma (2 ml blood) Enzyme immunoassay LLOQ, 0.1 mg/l

Values given as mean and range unless otherwise indicated; — = Not reported; L = loading dose; M = maintenance dose.

a. Median value reported. b. Postmenstrual age of study group was 31 weeks (median), range 25 - 41 weeks.

Table 5.9 A comparison of the final model parameter estimates with reported 1 compartment PK models for caffeine in plasma

Parameter	**Current study, n=67**	**Study I, n=89** **Lee *et al*., 1997**	**Study II, n=60** **Thomson *et al*., 1996**	**Study III, n=110** **Charles *et al*., 2008**
Structural parameters				
CL (ml/h/kg)	7.3 (2.3 - 16.9)	4.9 (2.3 - 9.0)	7.9 [b](± 1.9)	[a]7.0 (1.6 - 22.56)
V (ml/kg)	593 (174 - 1005)	970 (740 - 1400)	640 (—)	[a]851 (365 - 1761)
$t_{1/2}$ (h)	57 (13 - 112)	144 (86 - 277)	—	[a]101 (24.5 - 371)
Interindividual variability (IIV)				
CL (CV%) ωCL	24.7	25.0	20.0	18.8
V (CV%) ωv	45.7	11.0	24.0	22.3
Interoccasion variability (IOV)				
CL (CV%) K_{CL}	31.2	Not estimated	Not estimated	35.1
V (CV%) K_V	Not estimated	Not estimated	Not estimated	11.1
Residual error				
σ_1, additive (mg/l)	1.8	3.9	2.5	0.9
σ_2, proportional (CV%)				6.0

Estimates of CL, V and $t_{1/2}$ are given as the mean and range unless otherwise indicated; — = Not reported.

a. Median value reported.

b. Standard deviation reported.

5.7 Influence of haematocrit on final model

Patient haematocrit varied significantly amongst the study group (n=253, 21 - 58%). The majority of these values (92%) were within the experimentally investigated haematocrit range of 20 to 45% for which a negative bias of approximately 30% was determined for caffeine measurements using DBS analysis (Chapter 4, section 4.2.7.3). In order to determine the potential impact of this systematic assay bias on PK estimation a sensitivity analysis was conducted by normalising caffeine measurements according to haematocrit. On the assumption patient samples were analysed alongside a calibration line of haematocrit 45%, using the equation generated from the regression of assay bias (at the 1000 ng/ml level) against haematocrit the likely bias incurred and thus the correction factor required was calculated (Chapter 4, section 4.2.7.3). Of the 253 haematocrit measurements available only 138 were recorded on days for which a DBS sample was collected. Therefore it was only possible to adjust 138 out of 338 DBS caffeine concentrations according to haematocrit level. It was assumed that the remaining 200 caffeine concentration measurements were unaffected by assay bias. The final model with haematocrit normalised caffeine concentrations was re-run within NONMEM. The PK parameter estimates obtained are presented in Table 5.10 along with the original estimates from the final model. The results from this analysis indicated a decrease in CL (8.6 *vs.* 7.9 ml/h) and V (774 *vs.* 722 ml). A decrease in the unexplained interindividual variability in V was observed and the residual error increased from 1.76 mg/l to 2.82 mg/l. All other PK parameters were comparable and a similar level of precision was observed to that of the final model.

Table 5.10 Influence of patient haematocrit on final population PK model

Parameter		**Final model**		**Model incorporating normalised caffeine concentrations (n=138/338)**	
Fixed effects		Estimate	CV%	Estimate	CV%
θ_1	CL	8.6 ml/h	4.0	7.9 ml/h	4.1
θ_2	V	774 ml	7.9	722 ml	6.0
θ_3	Ka	4.0 h^{-1}	Ne	4.0 h^{-1}	Ne
θ_4	Duration (IV bolus)	0.08 h	Ne	0.08 h	Ne
θ_5	Exponent for WT on CL	0.786	17.7	0.745	18.4
θ_6	Exponent for WT on V	1.030	24.9	0.917	29.1
θ_7	Coefficient for PNA on CL	0.013	32.9	0.010	60.0
[a]**Random effects parameters** (Variance model)					
	IIV ω_{CL}	24.7 %	25.0	25.4 %	24.7
	IIV ω_V	45.7 %	44.2	34.8 %	44.1
	IOV K_{CL}	31.2 %	22.5	26.6 %	30.6
Random effects parameter (Residual error model)					
	σ, additive (SD)	1.76 mg/l	23.7	2.82 mg/l	24.5

Ne = Not estimated

a. Estimate given as CV%

5.8 Discussion

The primary aim of this study was to clinically evaluate the use of DBS analysis for PK parameter estimation and to identify potential problems associated with developing a population model based on DBS samples in a neonatal population. A total of 338 DBS samples were available from 67 preterms neonates for the development of the population model. The whole blood caffeine concentrations measured varied widely (2780 to 43045 ng/ml) despite study infants being on relatively similar maintenance doses of caffeine (1.4 - 7 mg/kg). A wide range in plasma caffeine concentration (2500 to 49900 ng/ml) has also been reported by Lee *et al* following the administration of maintenance doses of 1.5 and 7.5 mg/kg (Lee *et al.,* 1997). The DBS concentration data is therefore comparable to caffeine plasma levels which have been measured in patients which received similar maintenance doses to those in the current study. The modeling analysis showed a one compartment model adequately described the DBS concentration-time course of caffeine and enables the estimation of primary PK parameters CL (CV%, 4.0) and V (CV%, 7.9) with relatively good precision. The DBS dataset supported the precise estimation of the population variability in PK parameters and the residual variability. It was also possible to model the interoccasion variance in CL for the population.

A relatively large amount of clinical and demographic data was collected during the study to enable a thorough investigation into potential covariate effects which may be important in explaining the variability in caffeine CL that exists in neonates. Smoking status and ethnicity are significant covariates in adults, but were not found to be determinants of caffeine CL in neonates (Parsons and Neims, 1978, Landi *et al.,* 1999). Genetic factors alone determine around 72.5% of the variability in activity of CYP1A2,

the key enzyme involved in caffeine metabolism in adults (Rasmussen *et al.*, 2002). Bimodal and trimodal polymorphic distributions in CYP1A2 activity have been identified in adult populations (Butler et al., 1992). These types of covariate-parameter relationships may be difficult to detect in neonates due to the slow maturation of the CYP1A2 enzyme and the presence of developmental changes in renal function which predominate. Very low to absent CYP1A2 expression has been found in the foetal and neonatal liver (Cazeneuve *et al.*, 1994). Postnatally, around 4 - 5% of adult levels of CYP1A2 activity have been observed in neonates with progressive increases to 10 - 15% of adult levels by the age of 1 to 3 months (Sonnier and Cresteil, 1998). Adult levels of enzyme activity are not reached until beyond one year of age. Three major metabolites theobromine, paraxanthine and theophylline have been measured in the urine of adults following a caffeine dose with less than 2% of the dose recovered unchanged (Burg, 1975). In contrast, as much 85% of an administered caffeine dose has been measured in the urine of neonates in its unchanged form (Aldridge *et al.*, 1979). The predominance of a renal clearance pathway in neonates, which is less efficient compared with hepatic metabolism, would provide some explanation of their markedly increased caffeine half life compared to adults (Blanchard and Sawers, 1983). Significant changes in renal development occur during the postnatal period in term and preterm neonates. The GFR in the full term neonate is 35% of the adult value (Rhodin *et al.*, 2009). A large increase in the GFR occurs in the first two postnatal weeks in term and preterm neonates, thereafter it continues to increase until about one year of age to adult capacity (Rhodin *et al.*, 2009). In preterms, the maturation of renal function also depends on GA and therefore PMA (GA and PNA) is considered to be the best parameter of renal immaturity in neonates and infants (Rhodin *et al.*, 2009). In the

current study PMA provided no improvement in the fit of the data or a reduction in the interindividual variance term after the inclusion of weight. This is surprising given the range in GA (24.6 to 32.9 weeks) and PNA (1 to 69 days) of the study group. The addition of PNA age however, significantly improved the fit of the data. This is in agreement with other population models where weight and PNA were also found to be the two most influential covariates for estimating CL (Lee *et al.*, 1997, Charles *et al.*, 2008). Although a large variability in haematocrit and albumin was noted amongst study patients, these covariates were not determined to have an influential effect on the final model. However, this may not be true for other drugs.

The form of nutrition received by infants has previously been linked to the rate of CYP1A2 maturation. Blake *et al* reported accelerated CYP1A2 expression in infants receiving formula feeds compared with breast milk evidenced by higher levels of urinary CYP1A2 caffeine metabolites (Blake *et al.*, 2006). Significantly higher caffeine CL values have also been reported in neonates receiving parenteral feeds in a previous population PK study (Falcão *et al.*, 1997). A structural parameter–covariate relationship has similarly been demonstrated for theophylline indicating that parenteral nutrition may interfere with the elimination or metabolism of methylxanthine drugs (Moore *et al.*, 1989). Other PK studies which have looked for an influence of feeding regimen on CL including the present could not identify any correlation. Caffeine is known to be present in appreciable amounts in breast milk, but unfortunately DBS samples were not collected prior to starting caffeine treatment and therefore it was not possible to assess pre-dose caffeine concentrations. In a previous large population study 80% of neonates had detectable pre-dose caffeine concentrations which ranged between 0.2 to 5.1 mg/l (mean, 1.2 mg/l) (Lee *et al.*, 1997). No maternal covariate data on timing and amount of

caffeine consumption were collected in the present study but given the large number of DBS samples obtained from infants receiving breast milk it is likely that this will have contributed to the residual error of 1.8 mg/l.

On comparison with literature data, the PK parameter estimates derived from DBS measurements were found to be in good agreement with plasma values reported in previous population studies in neonates. These findings would suggest that the blood-to-plasma ratio for caffeine is 1, which is indeed the case, and, importantly, implies that interpretation of PK data from DBS and plasma samples is interchangeable. To assess the potential impact of the observed systematic assay bias from haematocrit effects on the final PK model, a sensitivity analysis was performed. Given the negative assay bias observed when changing from 45 to 20% haematocrit a decrease in primary PK estimates is predictable, and this was observed with an 8.9% and 7.2% decrease in estimates of CL and V, respectively (Table 5.10). A decrease in the IIV in V of 23.9% was also observed suggesting differences in haematocrit between patients also has the potential to significantly inflate the estimated between subject variability. The poorer predictive capability of the adjusted model (residual error, 1.76 vs. 2.82 mg/l) is likely to have arisen due to the partial normalisation of the dataset. A disadvantage of the modeling approach used is that only a fraction of the data set was normalised (138 of 338 data points) and therefore differences in PK estimates from the final model are likely to be an under prediction of the true impact of bias arising from variability in haematocrit. Despite this limitation, these results demonstrate the potential for haematocrit to significantly affect PK parameter estimation and will be important for analysts to consider during method development and certainly before reporting DBS concentration measurements for PK analyses.

During the bioanalysis of patient samples, whole blood for the preparation of calibration standards was ethically obtained from healthy adult female volunteers. Although a haematocrit measurement of the donated blood was not taken at the time, considering the normal haematocrit range for females, it would have been reasonable to assume an average level of 40%. Under these circumstances, based on the relationship observed between haematocrit and assay bias, a less pronounced negative systematic bias on PK parameter estimates would be expected. The sensitivity analysis was however, performed on the assumption of a 45% haematocrit level. This was done to test the robustness of the final model to the effects of haematocrit and to account for the possibility of a higher adult blood haematocrit. Based on the assumption patient samples were analysed alongside a 45% haematocrit calibration line, and taking into account the results of the sensitivity analysis as well as the average haematocrit for the study group (34%), it is likely for systematic assay bias to have affected the estimation of CL and V parameters by 10 to 20%. Factoring this bias into the DBS derived caffeine PK estimates, the results from this study are still comparable to reported plasma data.

The data generated here has little impact on clinical practice as the caffeine dose has been well established and clinicians in the UK generally treat infants according to therapeutic response and not therapeutic drug levels. In the treatment of AOP, caffeine levels between 5000 and 20000 ng/ml are generally considered therapeutic with some infants requiring higher levels of up to 40000 ng/ml to effectively treat apnoeic episodes (Natarajan *et al.*, 2007). Therefore for the majority of patients, concentrations of caffeine were attained within therapeutic levels (Figure 5.6). Considering the IOV in CL determined in this study and elsewhere, dose adjustment recommendations used on the

basis of plasma caffeine levels by some units may be of limited value (Charles *et al.*, 2008).

The results of this study provide important data on the practicality of DBS analysis and the problems likely to occur during the sample collection process in a clinical environment such as the NICU. The feasibility of the method was reflected by the number of DBS samples collected, the high sample accrual rate and the large percentage of these which met the standard of quality required for drug quantification (90%). The high success rate would suggest that this technique is relatively simple and can be performed with minimal training in a busy clinical unit. A 10% loss of data may however, be significant in situations where there are limited patient and sample numbers. Reasons for poor quality samples included visually apparent coagulation, decentred spots (which includes the printed circle on FTA Elute paper), application of spots too close to each other resulting in merged samples, insufficient sample and capillary tube indentation marks on the sample surface from an incorrect blood spotting technique. The provision of adequate practical training in the DBS technique will thus be crucial in minimising the percentage of samples collected below the standard of quality required. There is a greater risk of blood coagulation as the duration of capillary blood collection increases. Therefore it is possible for the use of an opportunistic blood sampling method to have contributed to the collection of coagulated DBS samples.

DBS analysis provides a potential solution to the blood sampling difficulties encountered in PK studies. However, the methodology will only be useful if it is well accepted by parents and clinical staff. A qualitative study aimed at determining parental and healthcare professional acceptability of the DBS method was carried out alongside

the caffeine PK study. Although this was not the focus of this thesis the outcomes from the study will provide important information on the design and conduct of how best to perform PK or PK-PD studies in neonates in the future. Preliminary data based on the percentage of parents approached who consented to the study (~93%) and sample accrual rate during the PK study would suggest DBS to be an acceptable method of blood collection in neonates. Similarly, a high percentage (6 out of 7, ~85%) of parents approached during the dexamethasone PK study consented to study participation.

5.9 Conclusion

The results from this study indicate that DBS analysis is a technique with significant potential as a robust method for drug quantification in neonatal PK studies. The PK interpretation of DBS data and comparison with reported plasma estimates were simplified by the distribution kinetics of caffeine in whole blood. Therefore the use of DBS would need to be considered in the context of the behaviour of a candidate drug with respect to RBC association and protein binding. The results from this study also demonstrate that DBS sampling within the context of an opportunistic sampling design is an accepted and feasible approach for performing PK studies in neonates, and potentially older age groups. There is clearly a greater potential for problems with assay bias when using DBS analysis. To minimise any adverse effects on PK parameter estimation, analytical issues such as filter paper related haematocrit effects will need to be understood and accounted for early in the method development stage.

Chapter 6

General Discussion

6.1 General discussion

The ability to accurately and precisely measure drug levels from micro blood volumes is an important stepping stone to understanding drug pharmacology and deriving scientifically based dosing regimens in neonates and children. However, there are a number of factors associated with DBS that have potential to affect the accuracy of drug measurements. For the developed dexamethasone and caffeine DBS quantification methods results from *in-vitro* validations demonstrate an accuracy, precision and selectivity in accordance with the requirements of current regulatory FDA guidelines. On testing for the robustness of the DBS assays to haematocrit effects, both drugs were found to be sensitive to changes in haematocrit. This was most noticeable for caffeine where a relatively small change in haematocrit (5%) resulted in a significant bias (RE%, 18.4% at 1000 ng/ml level). This suggests that for some drugs, even small fluctuations in blood haematocrit of ± 5% which are generally considered to be normal in healthy adults would need to be considered when developing DBS based methods (Hillman *et al.*, 2005). A regression of the relationship between haematocrit and bias (RE%) to a polynomial function provided a good fit indicating potential for modeling the effect of haematocrit on caffeine assay bias. The equation generated formed the basis of investigations undertaken to assess the effect of systematic measurement noise on PK parameter estimation. This may be a useful method of normalising caffeine concentration data prior to PK data analysis. A disadvantage is that it introduces an additional variable and a validation would be necessary to determine whether a correction within acceptable accuracy is possible across the calibration range of the assay. The accuracy of blood haematocrit readings can vary significantly between different instruments and therefore this would need to be considered during

investigations (Al-Odeh *et al.*, 1994). Furthermore, correction for the influence of haematocrit this way would be difficult to achieve in neonates since haematocrit tests are not carried out on a daily basis on the NICU and would thus necessitate additional blood collection. The difficulty in correlating haematocrit effect to assay bias has previously been highlighted and therefore the use of a correction factor to account for varying haematocrit amongst study patients will not be a universal approach (Denniff and Spooner, 2010a).

For compounds which show an unacceptable bias within the haematocrit range expected in a study population, a more practical solution may be through modification of the sampling method so that an accurate blood volume is spotted onto filter paper with a subsequent extraction of the entire spot. The approach has been reported in TDM for drugs including chloroquine and lumefantrine with adequate precision and accuracy (Blessborn *et al.*, 2007, Lejeune *et al.*, 2007). Drug quantification now becomes independent of changes in distribution of blood or drug on filter paper. However, the flexibility around the blood volume collection afforded by the current approach is no longer possible and the degree of skill with which samples must be collected and spotted is increased. This may be less feasible in a clinically orientated environment. An additional concern is that it may not be possible to collect the full volume required on each sampling occasion which would impact on the sampling success rate. An alternative approach may be to investigate different filter paper types as haematocrit effects have been shown to be compound and paper dependent (Denniff and Spooner, 2010a). Recently, a non-cellulose based blood collection paper has been reported with consistent spot homogeneity and minimal changes in spot size (11%) with haematocrit changes from 20 to 80% (Ritu *et al.*, 2011). This may provide a practical way of

overcoming haematocrit effects for some drugs whilst maintaining flexibility in the blood collection process.

The degree to which the DBS calibration line reflects concentrations measured in the patient is also important, particularly in patient groups where the average haematocrit level may be significantly different to the haematocrit of healthy adult blood used to produce the calibration line. For dexamethasone, an adjustment of the calibration line to 35% haematocrit was incorporated into the methodology to reflect the average haematocrit value expected in patients as well as to limit assay bias arising from interindividual variability in haematocrit. For caffeine an adjustment of the calibration line to the average haematocrit (34%) measured for the patient group would not have been more suitable due to the large interindividual variability in haematocrit (Chapter 5, section 5.5.3). When the assay bias is outside the recommended ± 15% limit there is an increased potential to affect PK parameter estimation as demonstrated with caffeine (Wieling and Tump, 2004). Analytical issues related to DBS such as haematocrit can be overcome, but will need to be carefully considered.

A limiting factor in the use of DBS analysis for some compounds such as dexamethasone may be sensitivity. For drugs such as caffeine that are dosed on a mg/kg basis and have a relatively long half life, quantifying therapeutic drug levels will evidently be easier. For dexamethasone, alternate methods of sample preparation and detection will be necessary to adequately capture the PK profile in neonates. A problem with liquid extraction techniques are the large solvent volumes used compared with the volume of sample available. Some investigators have minimised losses in sensitivity through sample concentrator techniques which involve drying extracts to a residue

followed by reconstitution in a smaller volume of solvent before analysis (Christianson *et al.*, 2010). Results from the investigation of sample drying and reconstitution methods for dexamethasone gave unacceptable precision values (CV% > 20) and further work is necessary to identify the reason for inconsistent drug recovery. Solid phase micro extraction has been suggested as a sample preparation method with potential to facilitate the quantification of drugs in micro volume fluids such as those handled in DBS (Millership, 2011). The technique involves the adsorption of analyte from either an aqueous or gaseous phase onto a fibre coating immobilised onto silica (Pawliszyn, 2001). For thermally labile or non-volatile compounds, analyte concentrated on the fibred coating can be detected using LC-UV following desorption with a solvent volume which is very small compared with the sample volume (Kumar *et al.*, 2008). The feasibility of this sample preparation methodology for the types of volumes in DBS sampling which are significantly lower compared with previous applications (1.5 ml blood) would need to be investigated (Szultka *et al.*, 2010). Direct DBS surface sampling techniques such as the thin-layer chromatography mass spectrometer interface (TLC-MS) have been reported with the benefit of significantly higher detection capability (Abu-Rabie and Spooner, 2009, Loppacher *et al.*, 2011). The TLC-MS interface is designed to elute DBS samples on-line into the LC-MS system with or without LC separation. Direct sampling techniques are associated with issues such as carryover, matrix effects and process efficiency which mean that they may not be a practical solution at present. However, considerable effort is being made on the development and improvement of automated methods which should facilitate the analysis of micro samples and encourage future widespread usage of DBS analysis. The use of an alternate MS detection system is a potential option for improving the current

dexamethasone sensitivity. An LC-MS/MS method has been reported in the literature which has enabled dexamethasone to be quantified in plasma to levels as low as 0.5 ng/ml from a 50 µl plasma sample (Zhang *et al.*, 2011). In this report separation was achieved using a shorter column (50 mm, flow rate 0.3 ml/min) and detection performed in SRM mode using an API 4000 triple quadrupole mass spectrometer with a TurboIonSpray® source. A TurboIonSpray® source due to a better dexamethasone ionisation efficiency has also been preferred by other analysts and combined with further investigations on a pre-column sample concentrator method may be very useful for improving detection capability (Samtani and Jusko, 2007).

There are other important analytical aspects of DBS analysis that need to be addressed such as the stage of incorporation of the IS. The IS is used to account for variability during the extraction, chromatography and ionisation of an analyte. Like many previous reported DBS methods for practical reasons the IS in the present study was added to the extraction solvent used to reconstitute the DBS sample. Therefore the variability in the extraction method is not accounted for. A two stage approach has been suggested to overcome this problem (Liu *et al.*, 2010). The first stage involves a reconstitution of the DBS sample in an aqueous liquid containing IS. In the second stage a liquid-liquid extraction is performed to recover the drug and IS using a water immiscible organic solvent, similar to the procedure used in plasma analysis. Alternate ways of introducing the IS at an earlier stage of the extraction process have been suggested and include impregnating the filter paper with IS and spraying the DBS specimen with IS (Christianson *et al.*, 2010, Manicke *et al.*, 2011, Zimmer *et al.*, 2011). These methods would be very useful provided they are validated to show reproducibility and are sufficiently standardised to avoid operator dependent variability. Drying conditions, in

particular the effects of humidity have been identified as a factor with potential to affect the integrity of the DBS sample (Denniff and Spooner, 2010b). Denniff and Spooner reported on the effects of high humidity (81%) on FTA Elute, FTA and S&S 903 paper. On visual examination only the FTA Elute paper was found to be sensitive to the effects of high humidity with noticeable deterioration of both the DBS and paper. Although such an extreme in humidity would not have occurred on the NICU in the current study this may be an important variable in neonatal studies. The NICU environment is generally well controlled, but temperatures can be warmer (23 to 25°C) due to incubators, and fluctuations in room humidity (20 to 50%) have been reported with seasonal changes (Thomas *et al.*, 2010). Both caffeine and dexamethasone were found to be stable under an elevated drying and storage temperature (30°C), but the stability of these compounds were not tested with respect to fluctuations in humidity. All FTA paper DBS samples were however, stored with desiccant after drying to protect samples from the effects of humidity. On visual examination, DBS samples collected during the study did not appear to be affected by humidity. Given the susceptibility of FTA Elute to humidity, this blood specimen collection paper may be the least preferred for use in future neonatal PK studies. Further work will be necessary to determine the effects of drying and storage humidity on analyte quantification within clinical environments such as the NICU.

A great deal of work has been undertaken to evaluate and improve the DBS method for drug quantification but a major concern is the current variability in extent of validation performed in reported DBS methods and lack of consensus on what constitutes a validated DBS method. The development of guidelines specifically on the validation of DBS assays will be important so that analysts, pharmacokineticists and regulatory

agencies have confidence in DBS derived PK data. This has been acknowledged and progress to a more standardised DBS validation practice is now of interest to many analysts developing DBS methods (Abbott *et al.*, 2010, Evans *et al.*, 2010, Timmerman *et al.*, 2011).

During the caffeine study replicate (n=3)15 µl blood spots were requested for collection at each sampling opportunity to ensure sample availability in the event of bioanalytical problems which may necessitate a repeat sample analysis. This was possible on 77% of sampling occasions which indicates the feasibility of repeat sampling during opportunistic blood collection. The low blood volume (15 µl) required per spot was an important factor governing the success of replicate sample collection and therefore this volume should be kept as low as possible. A larger blood volume of 30 µl was collected for dexamethasone analysis to maximise detection sensitivity. This sampling methodology will need to be modified to increase the feasibility of replicate sampling. Using an accurate spotting method with a smaller blood volume of 15 µl with subsequent extraction of the entire spot will be helpful in minimising the loss in dexamethasone sensitivity. With changes in regulatory guidelines on the bioanalysis of drugs, ISR is now mandatory (Savoie *et al.*, 2010). This involves the reanalysis of a percentage (5 to 10%) of the original study samples and therefore the collection of replicate DBS samples will need to be a standard practice. It is an important component of bioanalysis as it provides assurance on the precision and accuracy of the developed assay and can be used to test the robustness of repeat capillary DBS sample collection. A PK model can only be as good as the quality of drug concentration information provided and therefore it will be important to assess the reproducibility or quality of data obtained from capillary blood sampling. The experiences of ISR at GSK from 42

analyses would suggest DBS sampling and analysis is a robust method for drug quantification in PK studies (Barfield *et al.*, 2011). ISR may be a concern where excessive squeezing is used to obtain a capillary blood sample or when the initial droplet of blood formed is not wiped away. On the repeat analysis of caffeine concentrations in DBS samples collected from adult volunteers, whilst all measurement were within ± 20% of the origin result a positive bias was observed which may have been due to contamination from interstitial fluids. This is less of a concern where an opportunistic blood sampling approach is used as the initial drop of blood thought to contain high levels of tissue fluids has been removed. Poor capillary blood flow can necessitate squeezing and in this situation there is therefore potential for contamination of the blood sample collected. There is at present a lack of data on the reproducibility of capillary blood sampling in neonates. The blood sampling method is as important as the method of bioanalysis and therefore further work including the reanalysis neonatal patient samples from the caffeine PK study will be important for providing assurance on the robustness of capillary blood collection in this age group. A potential problem that is specific to capillary DBS sampling in neonates is the use of petroleum jelly which could contaminate the sample collected. However, *in-vitro* experiments suggest that exposure to petroleum jelly did not affect dexamethasone or caffeine quantification by DBS analysis.

There are alternative methods of blood collection and spotting that may be adopted to ensure a homogenous sample. For example, capillary blood may initially be collected into a blood collection tube containing anticoagulant, mixed and subsequently spotted onto filter paper using either a capillary tube or pipette. Alternatively a single large blood spot may be collected which allows ISR through repeat sub-sampling from

different areas of the DBS surface. This method is based on the assumption of an even distribution of drug across the entire DBS sample. However, differences in analyte distribution have been observed between peripheral and central locations and therefore sample homogeneity would need to be evaluated beforehand (Ren *et al.*, 2010). A summary of analytical issues associated with DBS analysis is presented in Table 6.1.

Table 6.1 Analytical issues in DBS analysis

Issue	Potential solutions
Haematocrit effect on filter paper	• Normalise measured concentration using patient haematocrit • Alternative cellulose filter paper • Investigate non-cellulose paper • Accurate blood spotting with extraction of entire DBS sample
Suitability of matrix used to prepare calibration standards	• Adjustment of blood haematocrit (through addition or removal of plasma) to reflect average haematocrit of study population • Limit blood matrix dilution through addition of minimal drug spiking solution (<5%)
Sensitivity	• Optimise analyte recovery • Optimisation of sample clean-up procedure to remove matrix effects • Pre-column sample concentration • Investigate micro sample preparation techniques e.g. solid-phase micro extraction • Use an alternative instrument or detection system for quantitative analysis • Direct DBS surface sampling techniques e.g. TLC-MS
Stage of IS incorporation	• Impregnate filter paper with IS prior to blood spot collection • Spray IS on the surface of DBS sample prior to extraction • Two stage approach incorporating liquid-liquid extraction with an immiscible organic solvent
Humidity and drying temperature	• Perform *in-vitro* investigations under expected study conditions to assess appearance of sample and recovery of analyte • Storage of DBS samples in sealed plastic bags with desiccant • Drying and storage of samples in a well controlled environment
Incurred sample reanalysis	• Collect replicate DBS sample per time point • Use a single large DBS sample to allow replicate sub-sampling • Collect blood into a blood collection tube prior to blood spotting
Use of creams or petroleum jelly during blood collection	• Perform *in-vitro* validation to ensure chemical moieties do not interfere with assay

Considerations also need to be given to the implications of using DBS as an alternative matrix to plasma in PK studies (Table 6.2). Problems in PK interpretation could arise when the total blood (DBS) concentration does not reflect the unbound drug concentration due to changes in either haematocrit, blood-to-plasma ratio or protein binding (Emmons and Rowland, 2010). An example of a drug affected by haematocrit is tacrolimus, a hepatically cleared immunosuppressant. Tacrolimus exhibits a high affinity for RBC binding resulting in a whole blood-to-plasma ratio of greater than 50 in humans (Jusko *et al.*, 1995). Alterations in albumin concentrations have negligible effects on the unbound to bound drug ratio, whereas high haematocrit levels are strongly correlated to low plasma concentrations due to increased RBC partitioning (Jusko *et al.*, 1995, Chow *et al.*, 1997). The total blood concentration does not change but the amount of (unbound) drug which is able to exert a pharmacological effect has changed. Under these circumstances a mechanistic approach to modeling PK data can result in assigning high blood concentrations to a low hepatic CL of tacrolimus when in fact the drug has accumulated in RBCs due to a high haematocrit and the intrinsic metabolic capacity of the liver has not changed. It may be argued that plasma levels should provide a better indicator of pharmacological events, but despite the problems with using whole blood it has been the preferred matrix for the TDM of tacrolimus in clinical practice (Hoogtanders *et al.*, 2007b). This is largely due to the difficulty in measuring tacrolimus concentrations in plasma which are much lower than in whole blood and the temperature dependence of RBC binding which needs to be considered during the separation (centrifugation) process (Machida *et al.*, 1991). Another reason whole blood is preferred is that sample haemolysis can significantly increase plasma concentrations thereby introducing assay bias.

Tacrolimus is an extreme example of the complications associated with using whole blood concentrations over plasma. It does however, highlight the potential for misinterpreting data if a drugs distribution kinetics are not adequately understood. This type of concentration dependent partitioning is most likely to be observed for compounds with a blood-to-plasma ratio > 2 (Rowland and Emmons, 2010, Phadnis *et al.*, 2011). At the other end of the spectrum, for hydrophilic drugs (blood-to-plasma ratio = 0.55) that do not associate with RBCs, variability in partitioning is not a concern and the only additional variable to consider when using DBS is haematocrit.

Neonates are amongst patient groups which display significant variability in haematocrit and albumin. Albumin concentrations are initially lower in neonates and show lower drug binding affinities compared with adults (Notarianni, 1990). Increases in albumin concentration from 20 g/l in 28 weeks gestation babies to approximately 30 g/l in term babies have been shown. During the first 8 postnatal weeks the increase in albumin concentration continues at a similar rate (Cartlidge and Rutter, 1986, Reading *et al.*, 1990). In addition, high levels of the foetal protein alpha-fetoprotein are observed in the neonates which is almost entirely replaced with albumin by the late neonatal period (Notarianni, 1990, Bader *et al.*, 2004). Studies suggest the binding affinity of alpha-fetoprotein to drugs is lower than for albumin (Hirano *et al.*, 1985). The changes which occur in protein composition and drug binding affinity continue in infants until about 1 year of age when levels of albumin and binding capacity are similar to adults (McNamara and Alcorn, 2002). Hyperbilirubinemia during the neonatal period adds to the variability in drug protein binding. The changes in haematocrit which occur during the first few months of life are also well known (Christensen *et al.*, 2009, Jopling *et al.*, 2009).

Therefore the effect of protein binding and haematocrit on PK parameter estimation may need to be investigated; just as it has been important to show linear protein binding kinetics as a function of drug concentration for the measurement of drug in plasma. This is not necessarily an inhibitory factor for the use of DBS concentration measurements in neonates. Drug distribution kinetics in blood such as the blood-to-plasma ratio, red blood cell partitioning concentration dependency and protein binding at therapeutic concentrations can be studied *in-vitro* prior to the conduct of PK studies. Once the important variables have been identified and the magnitude of the effect characterised it may be possible to account for their influence on PK estimation by incorporation into the PK model. The use of patient haematocrit for example has been used to guide the therapeutic dosing of tacrolimus in paediatric renal transplant patients (Zhao *et al.*, 2009).

To determine the factor most likely to influence changes in the total unbound to bound drug ratio of dexamethasone, investigations were focused on RBC association and the underlying concentration dependency of the blood-to-plasma ratio. Results indicated a constant ratio at all tested dexamethasone concentrations. On the basis of a blood-to-plasma ratio of 1 for dexamethasone and caffeine, a comparison between DBS derived and literature plasma PK data was made. This method of comparison may be easier to perform but a clinical validation involving direct DBS to plasma comparisons would be more informative and may be necessary in some cases. Despite the limitations of the study design used it was possible to show comparable parameter estimates for both test drugs.

In a recent publication, Suyagh *et al* reported on the application of an LC-UV DBS quantification method for the determination of the population PK of metronidazole in preterm neonates (Suyagh *et al.*, 2010a, Suyagh *et al.*, 2011). Blood samples (30 µl) were collected directly onto S&S 903 paper at times on most occasions when blood was collected for clinical reasons. A total of 203 DBS samples from 32 preterm neonates were collected and dedicated to the model building process. Structural and random effects parameters were estimated with precision and bootstrapping confirmed the accuracy of the estimates with differences between the original data set and bootstrap estimates of less than 5%. These results strengthen the evidence for DBS quantification as a robust methodology for PK studies. Furthermore, the population model was used to generate dose recommendations in the treatment of necrotizing enterocolitis for which age specific guidelines have been lacking. Metronidazole displays limited protein binding and has a high affinity for RBCs, therefore there is potential for haematocrit to affect PK estimation (Ralph *et al.*, 1974). However, the investigators could not identify a significant relationship between haematocrit and PK parameters. Direct DBS versus plasma data comparisons were not carried out, but DBS PK estimates of CL and V were found to be similar to estimates reported in plasma studies.

The ethical constraints associated with neonatal PK studies warrant careful consideration of the blood volume collected at each sampling point as well as the total volume collected over a given time period. With these in mind there is a degree of flexibility to allow for direct comparisons between plasma and DBS measurements. This could be achieved through the incorporation of a sparse sampling methodology based on the requirement of micro DBS and DPS samples. This may be particularly important to consider in light of the variability in physiological parameters expected in

neonates and the underlying disease mechanisms with potential to further complicate PK interpretation. Further clinical work involving the study of a limited number of drug candidates, encompassing a range of chemical properties, may be useful in facilitating a better understanding of the impact of using DBS measurements on PK estimation in neonates.

In adults, DBS to plasma concentration correlations have been investigated but for a limited number of drug candidates. Youhnovski *et al* reported on the plasma and DBS naproxen PK profiles following the administration of a single 440 mg dose to four subjects (Youhnovski *et al.*, 2010). Plasma concentrations were approximately twice that measured in DBS samples in all subjects indicating limited RBC association, but the data showed a strong correlation between plasma and DBS at all time points of the PK profile. For compounds like naproxen which largely reside in the plasma compartment RBCs can effectively be considered as a diluent. As there was very little difference in the haematocrit of studied patients and data from only 4 patients were available it was difficult to evaluate the influence of haematocrit on the plasma-DBS correlation. Differences in drug concentration between DBS and plasma have also been reported for theophylline, etravirine and darunavir, but similar to the findings of Youhnovski *et al*, a correlation was observed between the two matrices at all time points (Hibberd *et al.*, 1986, ter Heine *et al.*, 2009, ter Heine *et al.*, 2011). Comparisons between plasma and DBS will be necessary to provide assurance that whole blood data can be used to determine drug exposure. Ideally, as has been observed for some reported compounds the concentration ratio between the two matrices should be constant at all sampling time points. It is likely that drug regulatory agencies will initially want to see

comparisons between plasma and DBS in clinical trial and marketing authorisation applications (Beharry, 2010).

A combination of DBS samples from capillary, arterial and venous sampling sites were used in the PK analysis of caffeine. However, the interchangeability of DBS sampling this way may not be appropriate as sampling site dependencies in concentration measurements have been noted for a number of drugs in animals and humans (Chiou, 1989). In a clinical study which involved the administration of paracetamol to healthy adult volunteers significant differences in drug concentration were observed between capillary and venous DBS samples at 15, 30 and 60 minutes post dose (Mohammed *et al.*, 2010). The highest difference was observed at 15 minutes when the DBS finger-prick paracetamol median concentration (22.9 μg/ml) was approximately 4 times greater than the venous DBS concentration (5.1 μg/ml). By 60 minutes the concentration difference between the two sites was 9.3%. These results suggest a site concentration dependency during the early distribution phase for paracetamol. Spooner *et al* reported similar sampling site differences for the same drug with the greatest differences in paracetamol concentration at the earliest time points post dose (Spooner *et al.*, 2010). Differences in drug measurement during the early phase have been assigned to the diffusion of drug into the extravascular space through the capillary membrane giving higher arterial concentrations (Chiou, 1989). Since capillary blood is actually a combination of arterial, venous and capillary blood this would help to explain the higher concentrations at early sampling times. A constant concentration ratio between sampling sites at all time points post dose has been reported for AZD2019 (an AstraZeneca Pharmaceuticals compound) (Smith *et al.*, 2011). For this compound assuming an instant equilibrium would be suitable. Similarly tacrolimus and everolimus

capillary DBS concentrations have not been found to significantly vary from venous blood concentrations (Hoogtanders *et al.*, 2007a, van der Heijden *et al.*, 2009). Since differences in peripheral (capillary) DBS and venous DBS concentration appear to be compound dependent, to ensure correct use of DBS sampling clinical validation will be necessary where different blood sampling sites are to be incorporated into the study protocol. During the caffeine PK study only 3 venous and 0 arterial blood samples were collected within the first 2 hours post dose. Therefore potential differences in sampling site caffeine concentrations are unlikely to have had a significant effect on the final caffeine PK parameter estimates. A number of studies have reported higher haematocrit levels in capillary blood compared with venous blood and this difference is most marked in neonates (Linderkamp *et al.*, 1977, Rivera and Rudolph, 1982, Daae *et al.*, 1988). For drugs that are sensitive to haematocrit related effects on filter paper a bias may be introduced when making DBS capillary to DBS venous comparisons. To avoid confounding bias associated with the filter paper a DBS technique which involves the extraction of an accurate blood volume may be most appropriate for investigating sampling site drug concentration dependencies.

Table 6.2 Factors considered influential in pharmacokinetic interpretation when using DBS concentration measurements

- Red blood cell association (blood-to-plasma ratio)
- Concentration dependency in red blood cell partitioning
- Plasma protein binding
- Sampling site (capillary, venous, arterial)
- Capillary blood sampling method (dilution through contamination with interstitial fluid)

The ability to measure therapeutic drug concentrations well within the blood sampling limits considered to be acceptable in preterm and term neonates is a major advantage. The micro samples collected in DBS analysis also means that the feasibility of opportunistic blood sampling in population PK studies is increased. The results from this thesis have shown that DBS analysis with MS detection has significant potential as a robust method for use in clinical PK studies in neonates. The investigations described in this thesis are limited to two test drugs and therefore further *in-vitro* and *in-vivo* studies will be important in understanding the impact of using DBS as an alternative matrix to plasma for drug quantification in PK studies.

6.2 Final conclusion

- In-vitro investigations have shown developed DBS based drug quantification methods to be accurate and precise, in accordance with regulatory acceptance criteria on the validation of bioanalytical methods.

- The transferability of the DBS technique to different analytical instruments and laboratories was demonstrated thereby highlighting the robustness of the methodology for bioanalysis.

- Assay performance was not significantly affected by factors specific to the DBS process including small variations in blood volume spotted, spotting device, presence of petroleum jelly or an elevated drying and storage temperature (30°C). Variability in haematocrit level was the most influential factor affecting the accuracy of drug concentration measurements during *in-vitro* investigations.

- DBS sampling coupled with LC-MS/MS analysis enabled the precise and accurate estimation of caffeine population PK parameters in preterm neonates. Furthermore, DBS generated PK estimates were in agreement with reported plasma values in this age group.

- A sensitivity analysis performed using the population caffeine model highlighted the potential for systematic assay bias arising from paper related haematocrit effects to significantly affect PK parameter estimation.

- Dexamethasone DBS PK estimates were comparable to reported estimates of plasma CL and V, however, further investigations will be necessary to understand the impact of protein drug binding changes on the PK of dexamethasone.

- The results from this study indicate that DBS analysis is a technique with significant potential as a robust method for drug quantification, and within the context of an opportunistic sampling design is an accepted and feasible approach for performing PK studies in neonates.

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Appendix I

Dexamethasone pharmacokinetic study consent form

CONSENT FORM

Version 2 Dated 11th February 2009

Study Number: UHLPFL001

Patient Identification Number for this trial:

Title of Project: New microanalytical method to assess the effect of varying doses of dexamethasone on Chronic Lung Disease in premature newborns:

A Single-Centre Cohort Study

Name of Researchers: **Professor Field, Dr Kairamkonda,**

Dr Pandya, Ms Patel

Please read this form and sign it once the above named or their designated representative, has explained fully the aims and procedures of the study to you

- I confirm that I have read and understand the information sheet dated 11th February, 2009 Version 2 for the above study. ☐
- I have been given the opportunity to ask questions and discuss the study with one of the above investigators or their deputies on all aspects of the study and have understood the advice and information given as a result. ☐
- I understand that I can ask for further explanations at any time. ☐

- I understand that I am free to withdraw my baby from the study at any time, without having to give a reason for withdrawing. ☐

- I voluntarily agree to enable my baby to take part in this study ☐

_____	_____	_____
Name of Parent/Guardian	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for parent; 1 for researcher; 1 to be kept with hospital notes

Appendix II <u>Dexamethasone pharmacokinetic study case report form</u>

University Hospitals of Leicester NHS
NHS Trust

New microanalytical method to assess the effect of varying doses of dexamethasone on Chronic Lung Disease in premature newborns

CASE REPORT FORM

Complete form using a black ballpoint ink pen.
Record time using ITU clock (hh:mm)
Record date as dd/MMM/yyyy.

List of Abbreviations	
IV = intravenous	ND = Not done
PO= Oral	NA = not applicable
CAP = Capillary	
V = Venous	
A = Arterial	

1. Patient details

Patient Identification number .....

Date recruited onto study.....

Birth weight (kg) .....

Date of birth .....

Gestational age at birth.....

Gender .....

Ethnicity.....

Researcher Initials:

2. Record of Dexamethasone doses previously administered (i.e.Pre-recruitment)

Date (start and end)			
Dose (mcg)			
Weight (kg)			
Frequency			
Route (PO/IV)			
Initials			

3. Notes/Comments

Date & Time	Comment

4. Other medication prescribed

Drug name	Dose	Frequency	Route (PO/IV)	Start date	End date

5. Study completion

Date	
Completed or reason for withdrawal	

Sample number										
6. Dexamethasone administration										
Date of last dose										
Time drug given (if IV record start time of infusion)										
Total dose given (mcg)										
Weight (kg)										
PO/IV										
Initials										
7. Blood spot sample collection										
Date of blood collection										
Time of collection										
Number of 30µL capillary tubes collected										
Site of collection (CAP / V / A)										
Initials										
8. Laboratory Biochemistry Data (please input latest data related to sample)										
Date										
Haematocrit (PCV)										
Creatinine (µmol/l)										
Albumin (g/l)										
Bilirubin (µmol/l)										
Urea (mmol/l))										
Sodium (mmol/l)										
Potassium (mmol/l)										
9. Bloods (please input latest data related to sample)										
Date										
WCC ($x10^9$/L)										
RBC ($x10^9$/L)										
Haemaglobin (g/dL)										
Neutrophil ($x10^9$/L)										
Total lymphocyte ($x10^9$/L)										
Monocyte ($x10^9$/L)										
Eosinophil ($x10^9$/L)										
Basophil ($x10^9$/L)										

10. DAILY record of dexamethasone dose given during treatment

Date	Dose	Time	Route

11. Bloods (starting 48 hours before start of dexamethasone i.e. pre-recruitment)

Date					
WCC ($x10^9$/L)					
RBC ($x10^9$/L)					
Haemaglobin (g/dL)					
Haematocrit (PCV)					
Neutrophil ($x10^9$/L)					
Total lymphocyte ($x10^9$/L)					
Monocyte ($x10^9$/L)					
Eosinophil ($x10^9$/L)					
Basophil ($x10^9$/L)					

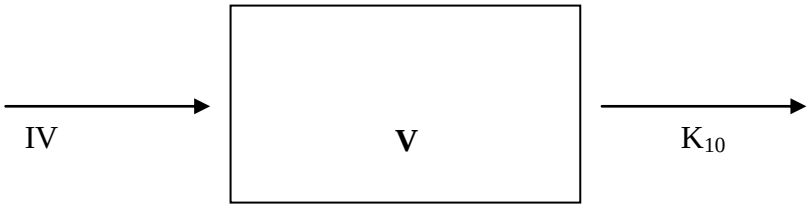
12. Bloods (48 hours after stopping dexamethasone)

Date					
WCC ($x10^9$/L)					
RBC ($x10^9$/L)					
Haemaglobin (g/dL)					
Haematocrit (PCV)					
Neutrophil ($x10^9$/L)					
Total lymphocyte ($x10^9$/L)					
Monocyte ($x10^9$/L)					
Eosinophil ($x10^9$/L)					
Basophil ($x10^9$/L)					

Appendix III

Compartmental models used in NONMEM to fit DBS concentration data

1a. One compartment PK model, IV bolus, first order elimination



$C(t) = Dose \times 1/V\ e^{-K10*t}$

Where C(t) = concentration in blood at time, t
V = distribution volume
K_{10} = elimination rate constant

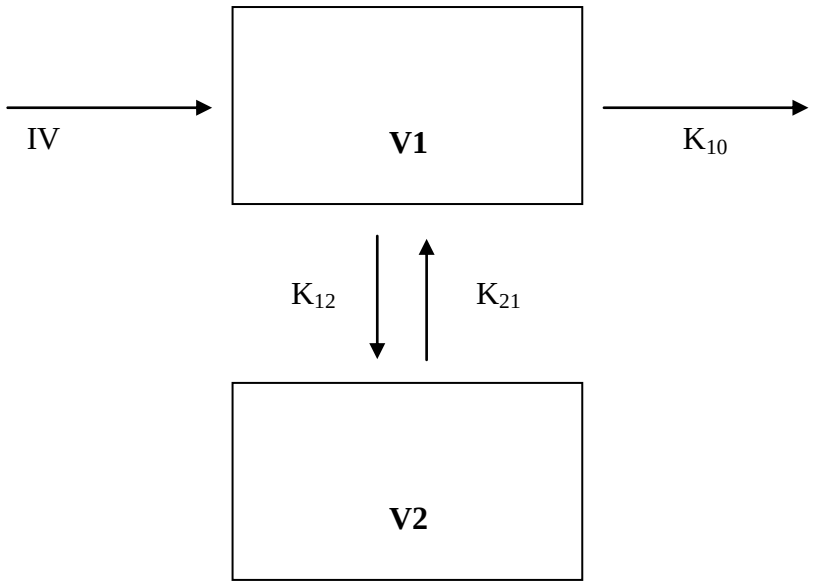
Estimated parameters*:*
V
$CL = V \times K_{10}$

1b. One compartment PK model, first order oral absorption, first order elimination

$C(t) = Dose \times 1/V\ (k_a /(k_a – k_{10}))\ (e^{-K10*t} – e^{-ka*t})$

Where K_a = absorption rate constant

2a. Two compartment model, IV bolus, first order elimination



$C(t) = Dose \times 1/V \ (A.e^{-\alpha*t} + B.e^{-\beta*t})$

Where $A = (k_{21} - \alpha) / (\beta - \alpha)$

$B = (k_{21} - \beta) / (\alpha - \beta)$

$\alpha = ½ [(k_{10} + k_{12} + k_{21}) + ((k_{10} + k_{12} + k_{21})^2 - 4*k_{10}*k_{21}))^{0.5}]$

$\beta = ½ [(k_{10} + k_{12} + k_{21}) + ((k_{10} + k_{12} + k_{21})^2 - 4*k_{10}*k_{21}))^{0.5}]$

Estimated parameters*:*

$V1 = 1 / (A + B)$

$V2 = V1 \times k_{12} / k_{21}$

$CL1 = V1 \times K_{10}$

$CL2 = V2 \times K_{21}$

2b. Two compartment model, first order oral absorption, first order elimination

$C(t) = Dose \times 1/V \times (k_a /(k_a - \alpha)) \ (A.e^{-\alpha*t} + B.e^{-\beta*t})$

Appendix IV

Caffeine pharmacokinetic study consent form

CONSENT FORM FOR PARTICIPATION OF CHILD

Version 5 dated 10th December 2009

Scientific Title: *An 'in field' assessment of 'Dried Blood Spot Methodology': Determination of caffeine pharmacokinetics in infants - A Single-Centre Cohort Study*

Lay title: *Measuring levels of the drug caffeine in preterm infants using micro-volumes of blood*

Participant Identification Number: **Centre Number:** **Study Number:**

Name of Researchers: Professor David Field, Dr Venkatesh Kairamkonda & Dr Hitesh Pandya

Please initial box

I confirm that I have read and understand the information sheet dated 20th November 2009 version 3 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
I understand that my baby's participation is voluntary and that I am free to withdraw at any time without giving any reason, without my baby's medical care or my legal rights being affected.	☐
I understand that relevant sections of my baby's care record, and data collected during the study, may be looked at by responsible individuals from the research team, individuals from regulatory authorities or from the NHS Trust where it is relevant to my baby taking part in this research.	☐

I agree for my baby to take part in this study ☐

_____	_____	_____
Name of Parent	Date	
Signature		

_____	_____	_____
Name of person taking consent	Date	
Signature		

Original to be kept in baby's medical notes; 1 copy to parent; 1 copy for research file

Appendix V Caffeine pharmacokinetic study case report form

University Hospitals of Leicester NHS
NHS Trust

An 'in field' assessment of 'Dried Blood Spot Methodology': Determination of caffeine pharmacokinetics in infants

CASE REPORT FORM

Complete form using a black ballpoint ink pen.
Record time using ITU clock (hh:mm)
Record date as dd/MMM/yyyy.

List of Abbreviations	
OD= once a day	CAP = capillary
BM = breast milk	V = venous
F = formula	A = arterial
C = combination	ND = not done
NBM = nil by mouth	NA = not applicable
PO= oral	
IV = intravenous	

1. Patient details

Patient Identification number .....

Date recruited onto study.....

Birth weight (kg) .....

Date of birth .....

Gestational age at birth.....

Gender .....

Ethnicity..... Researcher Initials:

Maternal smoking (Y/N).....

2. Record of Caffeine Base doses previously administered (i.e.Pre-recruitment)

Date (start and end)						
Dose (mg)						
Weight (kg)						
Frequency						
Route (PO/IV)						
Initials						

3. Notes/Comments

Date & Time	Comment

4. Other medication prescribed

Drug name	Dose	Frequency	Route (PO/IV)	Start date	End date

5. Study completion

Date	
Completed or reason for withdrawal	
Requirement of oxygen at 36 weeks gestation? (Y/N)	

Sample number	1	2	3	4	5	6	7	8	9	10
6. Caffeine base administration										
Date of last dose										
Time drug given (if IV record start time of infusion)										
Total dose given (mg)										
Weight (kg)										
PO/IV										
Initials										
7. Blood spot sample collection										
Date of blood collection										
Time of collection										
Number of 15µL capillary tubes collected										
Site of collection (CAP / V / A)										
Initials										
8. Laboratory Biochemistry Data (please input latest data related to sample)										
Haematocrit (PCV)										
Creatinine (µmol/l)										
Albumin (g/l)										
Bilirubin (µmol/l)										
Urea (mmol/l)										
Sodium (mmol/l)										
Potassium (mmol/l)										
9. Feeding Regimen Prescribed										
Enteral feed type (B /F /C /NBM)										
Parenteral feeds? YES/NO										
Date of last enteral feed before caffeine dose										
Time of last enteral feed before caffeine dose										
10. Level of care received										
Please indicate level i.e. ITU / HDU / SCBU										

11. DAILY record of Caffeine base dose given

Date	Weight	Caffeine Dose (mg)	Initials

Date	Weight	Caffeine Dose (mg)	Initials

Date	Weight	Caffeine Dose (mg)	Initials

Appendix VI

Caffeine drug concentrations measured in neonatal DBS samples n=338

TAD = Time after last caffeine dose
Caffeine concentrations given as ng/ml

Patient	TAD	Level	Patient	TAD	Level	Patient	TAD	Level
1	5.08	10320	6	6.78	19946	11	167.67	1249
1	7.92	11966	6	5.48	30375	11	2.50	39638
1	4.30	10481	6	20.40	17537			
1	30.61	8484	6	5.28	26325	12	27.58	6831
			6	1.08	35150	12	10.42	22302
2	21.85	18625	6	6.08	32339	12	3.25	24239
2	14.83	31767				12	6.00	22322
2	17.50	28009	7	2.58	9021			
2	1.84	36948	7	1.97	12750	13	6.08	8571
2	21.19	27527	7	1.85	13949	13	2.34	16635
2	22.00	30892	7	1.25	11396	13	5.90	8158
2	46.75	16994	7	5.67	18610	13	1.50	14548
2	23.68	27627				13	0.42	14461
2	23.22	35873	8	14.92	30287	13	4.17	16696
			8	11.67	36113	13	2.57	23056
3	0.62	8710	8	11.41	38385	13	22.08	16505
3	263.67	1198	8	213.97	5056			
3	98.78	3826	8	21.03	35532	14	55.00	3344
			8	15.50	21488	14	10.34	11178
4	6.62	7841	8	21.99	36489	14	7.54	12361
4	5.09	7807	8	5.40	43045	14	14.21	14374
						14	18.50	11420
5	20.00	14453	9	2.92	15771	14	22.78	13948
5	1.91	21444	9	6.14	23305	14	15.18	17791
5	7.00	27968	9	22.58	15731	14	3.77	17051
5	10.34	21907	9	1.75	20159	14	18.39	18659
5	9.42	21597	9	5.35	26146	14	22.99	17397
5	9.92	21367	9	16.50	26030			
5	10.95	24457				15	3.92	9845
5	4.66	28296	10	22.33	21288	15	2.17	12098
5	7.66	32197	10	11.12	27674			
			10	2.40	23378	16	3.90	28753
6	2.32	14628	10	10.08	25342	16	3.58	28486
6	6.00	25763	10	59.25	18882	16	4.17	39422
6	10.67	19968	10	8.67	29588	16	14.89	21346

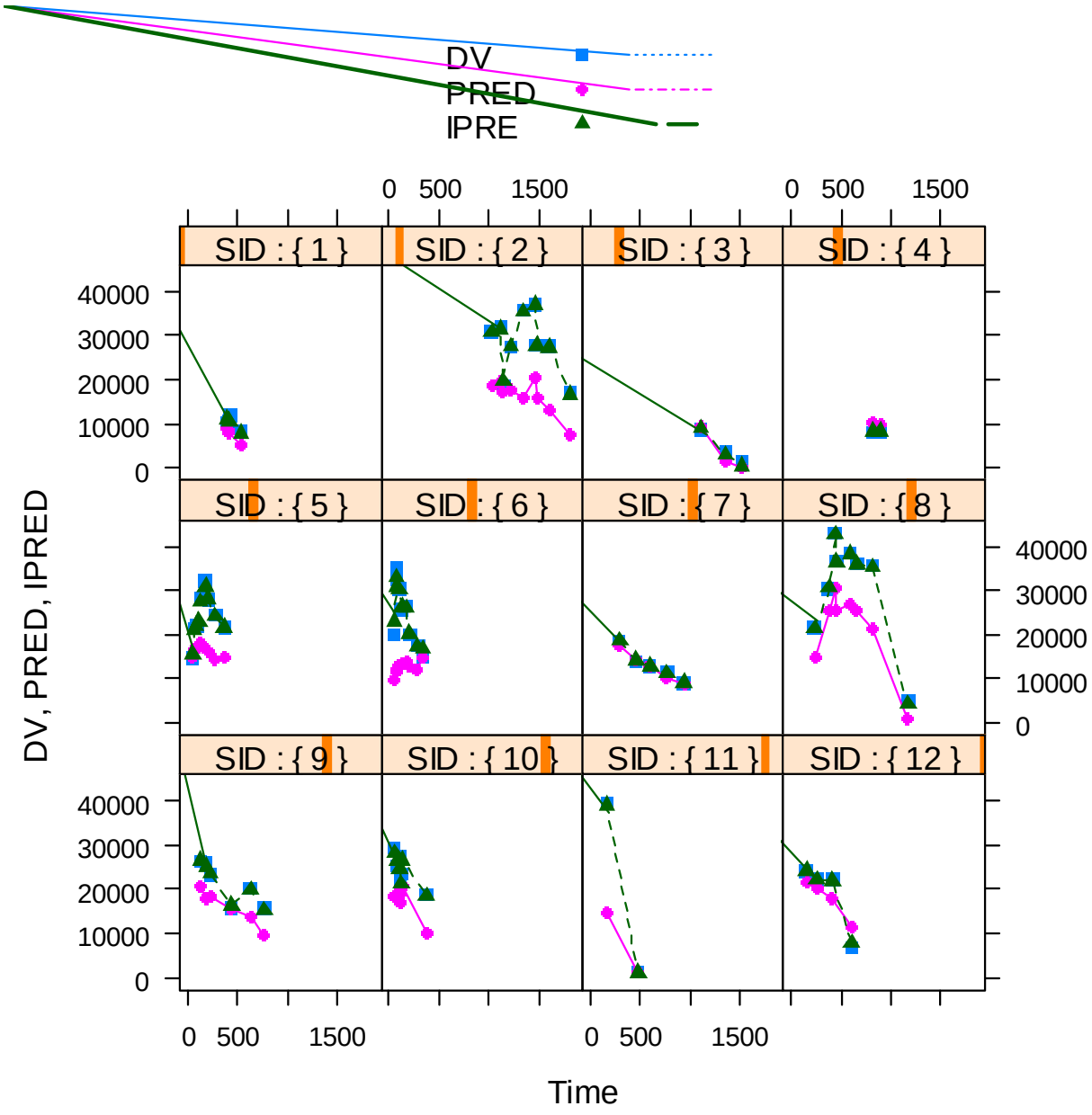
16	3.29	39296	23	18.67	13478	29	15.67	11482
			23	11.38	20940	29	18.17	11866
17	15.58	11518	23	22.50	14297			
17	225.83	2788	23	16.42	12926	30	2.84	15297
17	222.92	2780	23	4.67	17222	30	1.83	16051
17	144.25	6517	23	10.65	19753	30	23.50	30453
17	22.00	20949	23	0.33	18902			
17	51.17	10711	23	3.92	16190	31	2.50	16829
17	15.46	14484	23	10.80	22107	31	21.25	13593
17	0.80	15800				31	23.42	18106
17	73.50	8247	24	123.37	8881			
17	58.12	9666	24	23.87	22917	32	21.33	14991
			24	5.67	32205	32	44.83	11240
18	1.50	19502				32	3.32	25561
18	23.58	12283	25	99.70	4758			
18	20.25	21244	25	2.17	27750	33	288.07	1526
18	16.53	17040	25	6.17	31989	33	2.15	13618
18	35.71	14427						
18	28.11	10390	26	174.53	1716	34	168.82	3108
18	21.33	18245	26	22.25	17127	34	21.05	19048
18	9.58	23449	26	11.07	17576	34	1.22	28239
			26	2.50	21049			
19	179.09	5699	26	12.60	19082	35	46.80	9924
19	188.84	4337	26	14.15	18922			
19	16.11	31593	26	18.17	19519	36	3.22	7198
			26	12.67	21119			
20	2.92	14039	26	5.70	19793	37	2.67	19270
20	2.35	15441	26	13.63	19362	37	122.63	7586
20	1.17	17681				37	7.12	19486
20	16.50	14036	27	0.83	17085			
20	18.08	19430				38	191.36	1553
20	2.42	30135	28	4.20	7080	38	21.93	14790
20	5.17	24082	28	193.50	665	38	21.83	15491
20	18.17	24191	28	2.16	10155			
			28	11.50	10155	39	20.98	13114
21	3.83	26608				39	21.67	14895
			29	0.33	4363	39	19.93	16265
22	6.93	11031	29	17.00	4901			
22	6.36	13716	29	3.54	6096	40	2.33	16757
22	8.93	18554	29	51.47	4031	40	169.00	3574
22	8.50	15070	29	15.26	6717			
22	6.09	19789	29	20.00	8647	41	7.83	16317
22	8.20	23083	29	8.84	10947	41	3.05	18048
			29	11.45	13290	41	7.75	17711

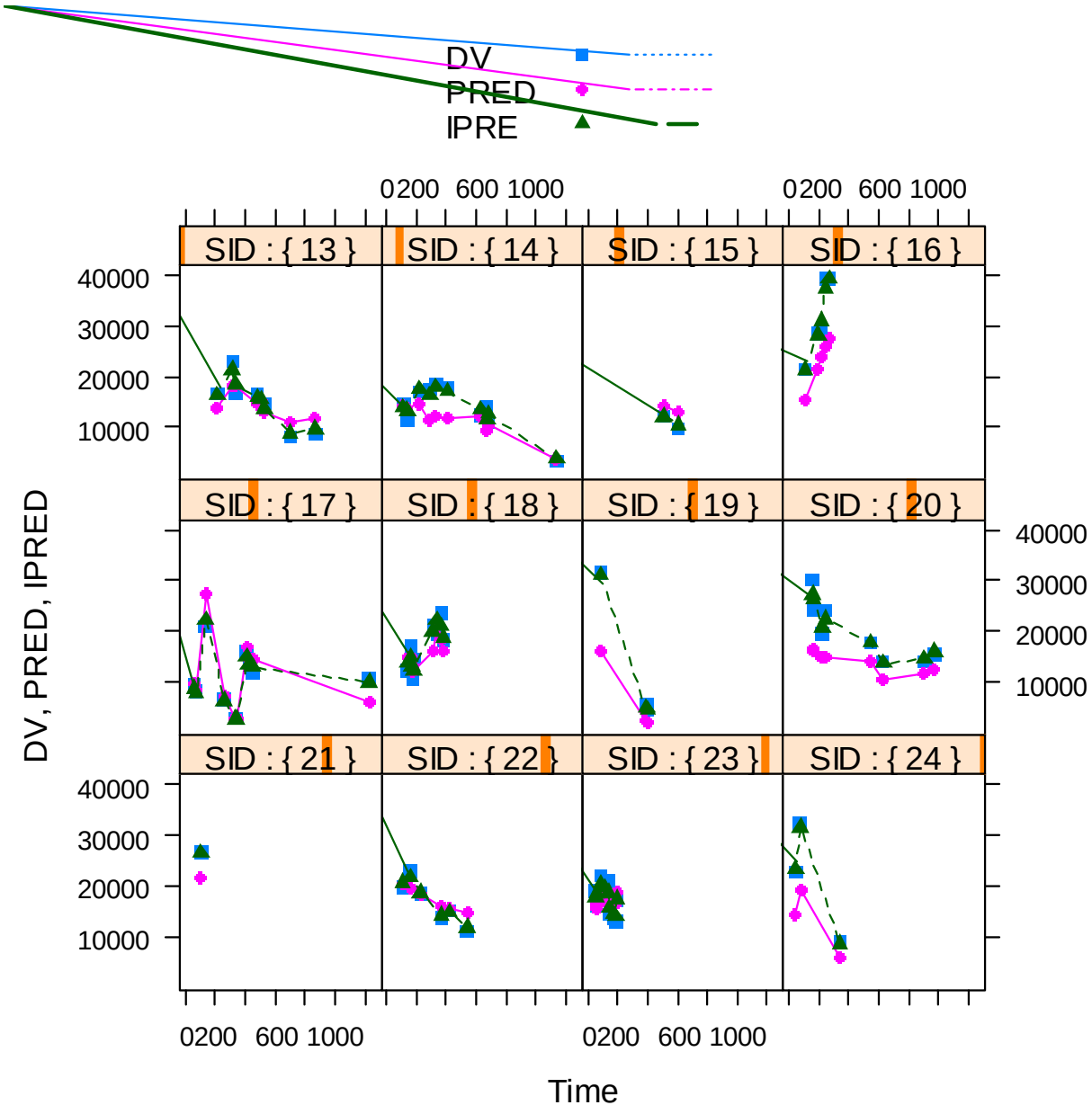
41	19.05	13462	47	23.33	4448	54	25.73	8588
41	17.15	14816	47	147.58	3067	54	24.00	7443
41	19.58	15699	47	4.95	15839	54	3.71	15066
41	24.08	15966	47	2.00	16762	54	25.42	11429
41	21.08	16145				54	1.33	17577
41	19.50	16047	48	3.22	7940			
			48	21.50	11322	55	50.83	2929
42	14.83	13923	48	0.02	12604	55	2.53	9537
42	7.88	15765	48	4.33	17164	55	3.50	13961
42	0.03	15758						
42	13.22	18191	49	22.00	12423	56	0.50	15110
			49	12.06	17606			
43	5.12	15626	49	20.23	16872	57	2.67	15686
43	6.88	17866	49	20.80	18306	57	2.75	10759
43	4.83	15783	49	1.42	23617	57	19.47	7702
43	6.16	16293				57	204.13	2656
43	5.24	15750	50	6.00	9534	57	27.43	10609
			50	15.50	11390	57	17.66	13060
44	18.90	16999	50	16.75	12116	57	17.73	14830
44	23.59	15662	50	18.00	10460	57	4.10	30691
44	18.50	15558	50	25.28	9381			
44	10.84	14207	50	6.42	12011	58	7.45	12741
44	18.59	13504	50	16.91	11392	58	4.14	22757
44	18.25	14063	50	17.83	12280	58	17.25	20899
44	7.58	17714	50	18.50	10441	58	3.92	27113
44	9.58	17521				58	17.75	23205
			51	13.75	10490	58	4.75	21756
45	4.00	12128	51	16.58	10975			
45	89.25	6458	51	11.67	11586	59	71.47	8756
45	61.25	9005	51	14.42	11031			
45	56.42	7951	51	3.98	15842	60	51.49	10378
45	64.58	6790	51	17.83	13296			
45	48.25	8265	51	20.83	11412	61	3.08	10928
45	43.25	7815	51	11.42	14604			
45	1.36	24925	51	16.67	14619	62	50.90	4713
			51	24.21	12851	62	3.06	12880
46	2.55	5592				62	5.97	14999
46	5.04	7321	52	16.10	10175	62	14.73	9677
46	20.10	8133	52	10.50	10306	62	1.63	12049
46	6.34	9135	52	21.83	14190	62	3.88	10113
46	6.05	11203				62	15.50	16801
46	4.67	12857	53	8.52	15729			
46	1.25	14928	53	8.28	11376	63	0.75	11192
46	3.92	16055	53	5.55	18347	63	2.00	25168

64	2.30	7546
64	75.22	2377
64	4.78	11070
64	1.84	15011
65	17.24	6958
65	18.50	8145
65	0.49	10768
65	7.25	8935
65	21.12	10226
65	24.75	9070
65	22.75	11289
65	19.00	15034
65	21.30	12337
65	21.25	16204
66	4.33	18298
66	0.47	18860
66	24.23	14352
66	2.75	25718
66	24.28	15433
66	7.25	28852
67	3.52	23608
67	3.00	23167
67	20.00	17167
67	12.00	19050
67	3.46	30743
67	19.34	19886
67	2.01	21365
67	7.41	36765

Appendix VII

The population predicted (PRED), individual predicted (IPRED) and observed caffeine concentration-time profile (DV) for each patient

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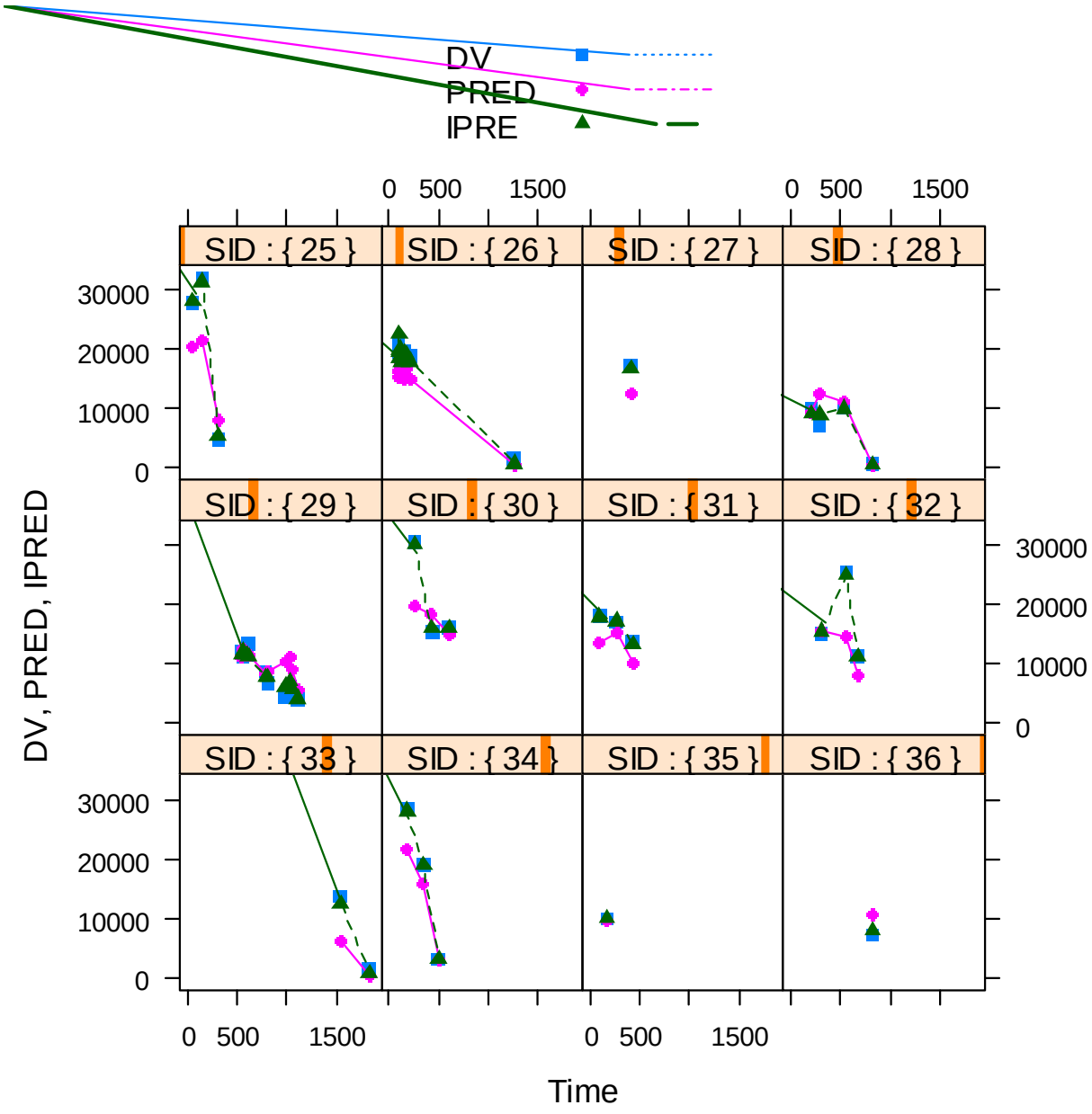
20000
10000
0200 600 1000
DV, PRED, IPRED
Time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SID : { 33 }
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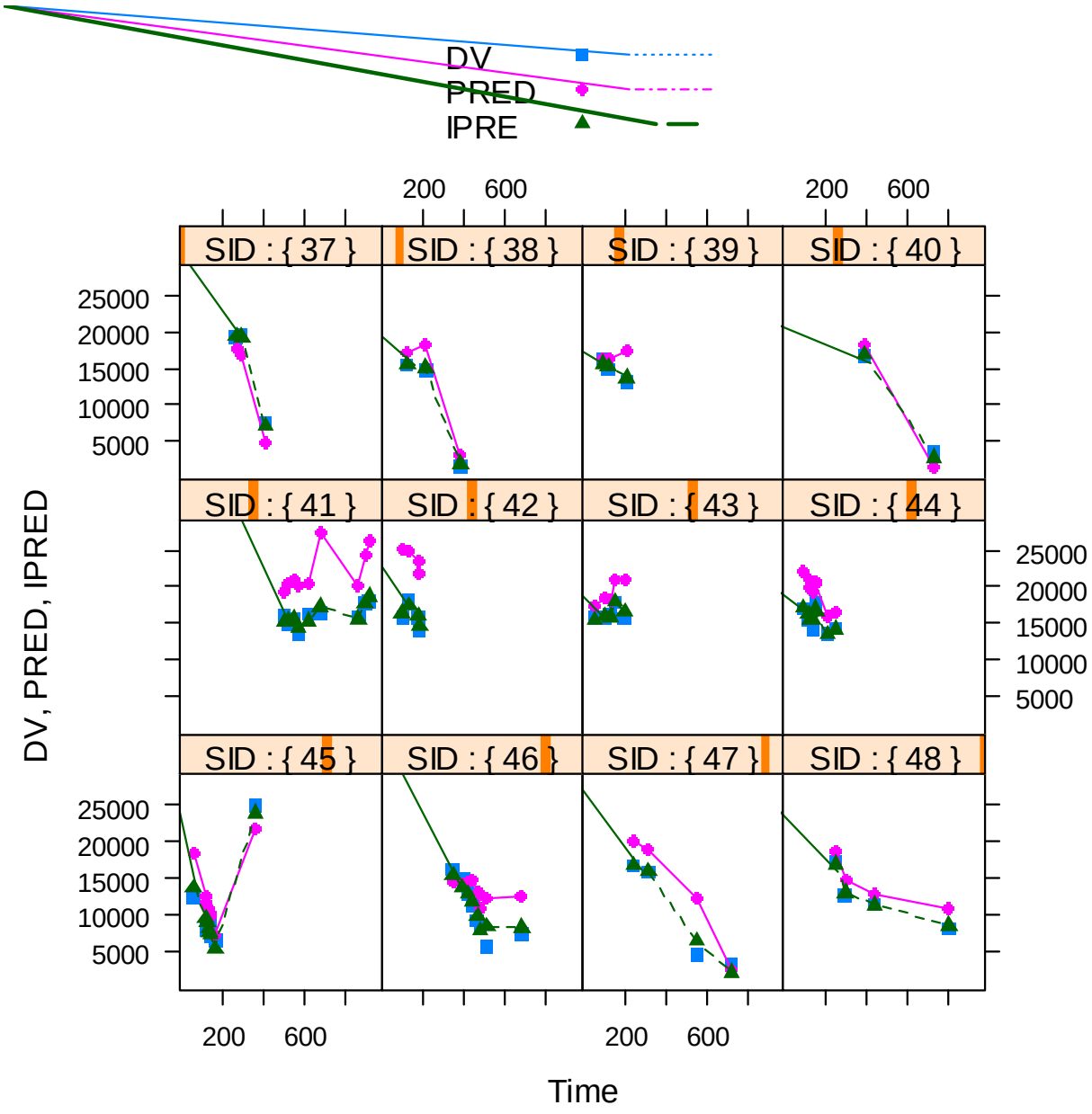
SID : { 36 }
0
500
1500
10000
20000
30000
DV, PRED, IPRED
Time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SID : { 48 }
DV, PRED, IPRED
Time
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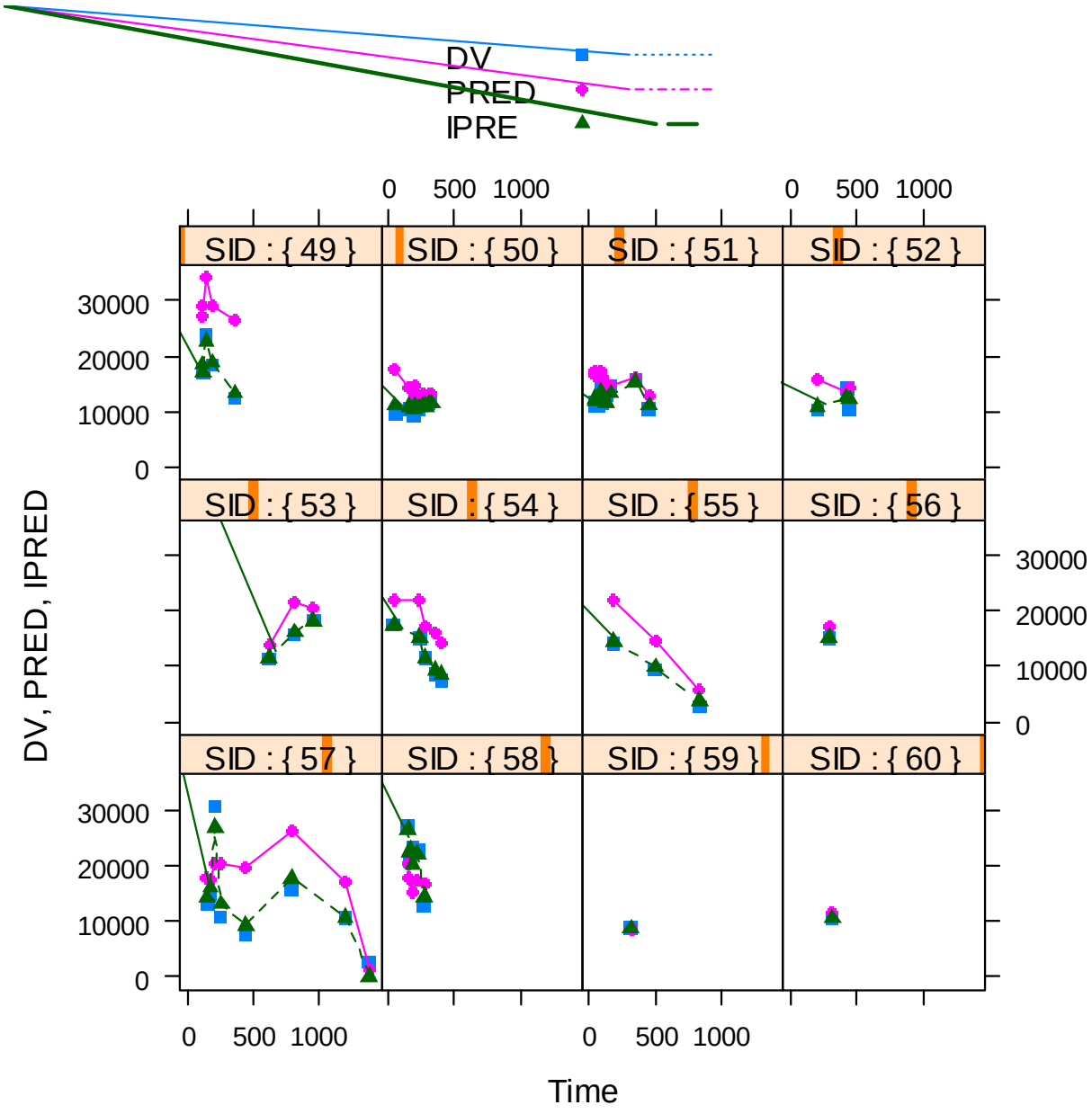
600
5000
10000
15000
20000
25000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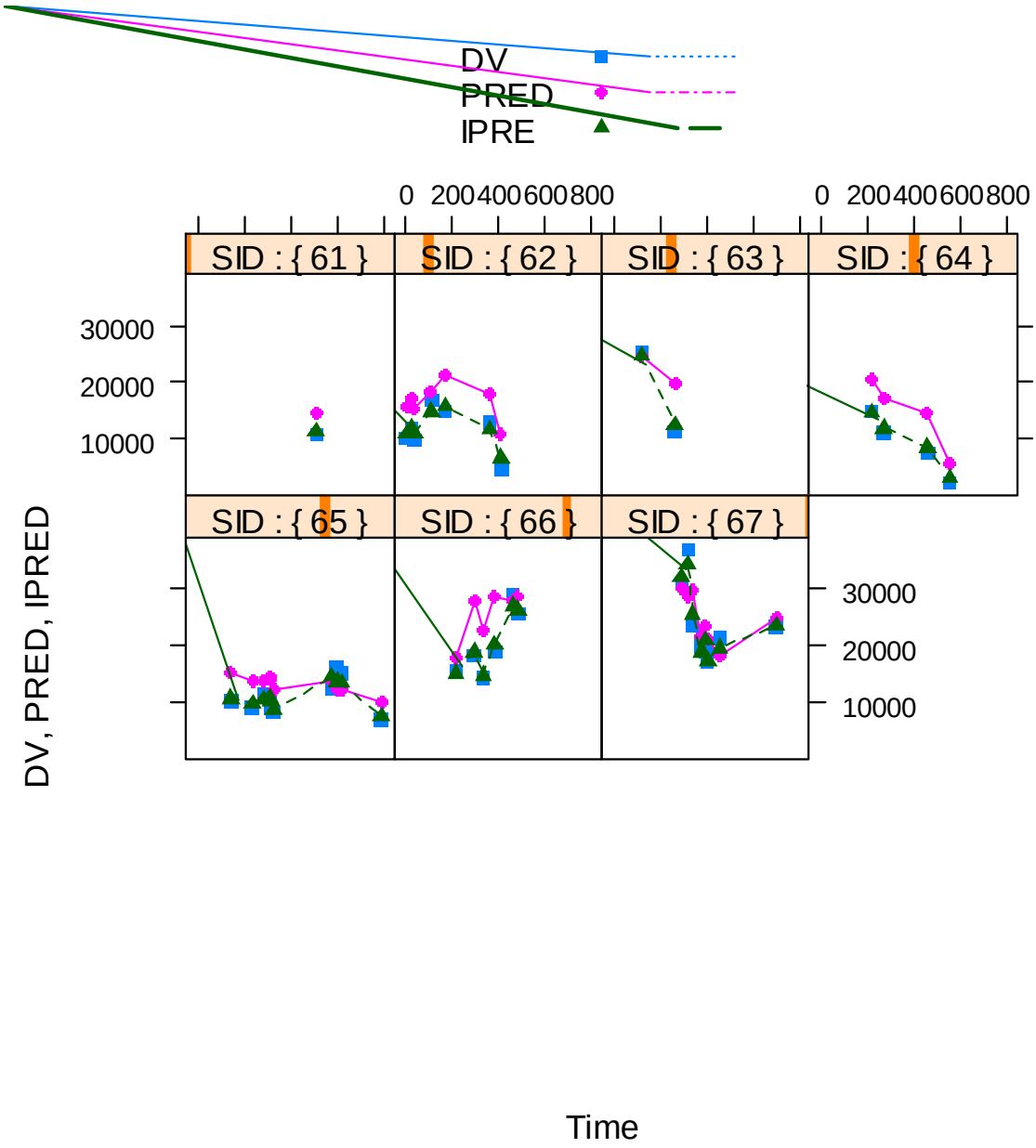
500
1000
10000
20000
30000
DV, PRED, IPRED
Time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IPRE
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10000
DV, PRED, IPRED
Time