Dried blood spots and sparse sampling: a practical approach to estimating pharmacokinetic parameters of caffeine in preterm infants

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Drug measurements based on wet matrices cannot be easily applied to some patient populations, particularly the young. Here, 'dried blood spots' (DBS) are an alternative bioanalytical matrix. However, DBS-based drug studies in relevant clinical settings are needed to identify methodological issues.

WHAT THIS STUDY ADDS

- This study demonstrates that DBS methods can be applied to PK studies in a vulnerable population group and are a practical alternative to wet matrix sampling techniques. The study emphasizes that differences in haematocrit in the study population can significantly affect DBS drug measurement and their interpretation.

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AIMS

Dried blood spots (DBS) alongside micro-analytical techniques are a potential solution to the challenges of performing pharmacokinetic (PK) studies in children. However, DBS methods have received little formal evaluation in clinical settings relevant to children. The aim of the present study was to determine a PK model for caffeine using a 'DBS/microvolume platform' in preterm infants.

METHODS

DBS samples were collected prospectively from premature babies receiving caffeine for treatment of apnoea of prematurity. A non-linear mixed effects approach was used to develop a population PK model from measured DBS caffeine concentrations. Caffeine PK parameter estimates based on DBS data were then compared with plasma estimates for agreement.

RESULTS

Three hundred and thirty-eight DBS cards for caffeine measurement were collected from 67 preterm infants (birth weight 0.6–2.11 kg). 88% of cards obtained were of acceptable quality and no child had more than 10 DBS samples or more than 0.5 ml of blood taken over the study period. There was good agreement between PK parameters estimated using caffeine concentrations from DBS samples ($CL = 7.3 \text{ ml h}^{-1} \text{ kg}^{-1}$; $V = 593 \text{ ml kg}^{-1}$; $t_{1/2} = 57 \text{ h}$) and historical caffeine PK parameter estimates based on plasma samples ($CL = 4.9\text{--}7.9 \text{ ml h}^{-1} \text{ kg}^{-1}$; $V = 640\text{--}970 \text{ ml kg}^{-1}$; $t_{1/2} = 101\text{--}144 \text{ h}$). We also found that changes in blood haematocrit may significantly confound estimates of caffeine PK parameters based on DBS data.

CONCLUSIONS

This study demonstrates that DBS methods can be applied to PK studies in a vulnerable population group and are a practical alternative to wet matrix sampling techniques.

Introduction

Blood spotted and dried on paper cards (dried blood spots, DBS) is a versatile matrix that has been applied successfully in several bioanalytical settings including genetic studies, diagnosis of infectious diseases and, most commonly, in measuring levels of biomarkers that predict for inherited diseases [1–5].

More recently, the DBS platform is being re-applied to the problem of quantifying drugs. In conjunction with new micro volume (<20 µl) analytical techniques, DBS appears to overcome some of the drawbacks associated with more traditional 'wet' matrices. Specifically, DBS methods have the potential to facilitate pharmacokinetic (PK) studies in neonates and young children, a population in whom the large blood volume sampling requirements of traditional PK studies are unacceptable.

The 'mechanics' of drug quantification using DBS samples/microvolume analysis has been systematically investigated and defined through a combination of testing *in vitro* and studies involving animals and adult volunteer humans [6–11]. However, as a method for generating PK data for children, the DBS platform has had little evaluation in 'real-life' clinical settings [12–14]. Clearly, this is an important step in validating DBS as a platform for generating PK data in all age groups. If the techniques can be applied successfully in vulnerable populations such as infants and children then, by extension, the methods should be applicable to all other populations.

Caffeine is used to prevent and treat apnoea in preterm infants. The PK of caffeine in infancy has been defined using wet plasma samples [15–19]. Caffeine does not accumulate nor bind extensively to plasma proteins or red blood cell (RBC) constituents and has a blood:plasma partition ratio of 1 [20–21]. These characteristics indicate that differential drug partitioning is unlikely to influence interpretation of 'whole blood' PK data and importantly should allow direct comparison of DBS with historical plasma derived models of caffeine PK. Consequently, caffeine is a good candidate drug for evaluating DBS methods as a means of obtaining PK data in children.

The aim of the present study was to evaluate DBS/microvolume platforms by determining estimates for caffeine PK parameters in preterm infants. For this purpose, DBS samples were collected prospectively from premature babies receiving caffeine for treatment of apnoea of prematurity (AOP) managed in a moderately large neonatal intensive care unit. A non-linear mixed effects approach was used to develop a population PK (Pop-PK) model from measured DBS caffeine concentrations. DBS derived PK parameter estimates were then compared with plasma estimates for agreement.

Methods

Subjects

All infants admitted to the neonatal intensive care unit at University Hospitals of Leicester NHS Trust who were treated with caffeine were eligible for inclusion into the study. Infants were excluded from the study if they were acutely unwell, e.g. due to sepsis or had evidence of cardiac, hepatic or renal impairment or had a congenital abnormality.

Study design

The study received ethics approval from the Trent NHS Research Ethics Committee. Parents provided prior informed written consent for all infants recruited into the study. As per clinical protocols, recruited infants were given 10 mg kg⁻¹ of caffeine base loading dose followed by a 2.5 mg kg⁻¹ once daily maintenance dose. The route of drug administration (i.v. and oral/nasogastric tube) and any drug dose adjustments were managed by the clinical team. No changes to drug treatment or care were affected by inclusion into the study. Blood samples (capillary, arterial or venous) for DBS caffeine measurement were only collected on occasions when blood was obtained for clinical reasons. The DBS sampling 'design' was therefore unstructured and samples were collected at random time intervals following either oral or i.v. doses. Blood samples were collected into EDTA coated capillary tubes and immediately applied onto FTA Elute paper (Whatman, part of GE Healthcare). Samples were required to be spotted within the designated area with the result of an even colour visible on both sides of the filter paper indicative of complete saturation. Between 1–10 DBS cards (each consisting of 3 × 15 µl spots) were planned for collection from each infant. The study was constrained to collecting a maximum of 500 µl of blood or 10 DBS card samples from any one infant. The sampling phase of the study was completed between January and November 2010.

Measurement of caffeine in DBS

As per clinical protocols, recruited infants were given 10 mg kg⁻¹ of caffeine base loading dose followed by a 2.5 mg kg⁻¹ once daily maintenance dose. Concentrations of caffeine within DBS samples were determined using liquid chromatography triple quadrupole mass spectrometry on a Sciex API-4000 (Applied Biosystems/MDS Sciex, Canada) with a TurbolonSpray™ ion source in positive ion mode (GSK Laboratories, Ware, Hertfordshire, UK) [11]. The assay range was between 250–25 000 ng ml⁻¹ with a lower limit of quantification of 250 ng ml⁻¹. For concentrations measured above 25 000 ng ml⁻¹, a validated matrix matched dilution was used to bring samples into the working assay range. The maximum within-run and between-run precision for the caffeine assay was 11.2 and 5.4% respectively with a bias range between –10.9

and 3.4%). From a total of 384, 338 DBS cards were deemed suitable for caffeine quantification.

Pharmacokinetic analyses

Structural model The population PK model was developed using NONMEM (version 7) 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) and R software (R Development Core Team, version 2.12.2, Austria) [1]. Caffeine bioavailability following enteral dosing was assumed to be 100% [19,20]. There were insufficient data to estimate the rate of caffeine absorption, therefore K_a was fixed to 4.0 h^{-1} [21]. Moreover, the goodness of fit of the concentration data at early time intervals or estimates of the disposition parameters were not altered using different values for K_a . Caffeine administered i.v. was modelled with zero order duration of input fixed to a value of 0.08 h. Initial PK estimates were obtained from reported plasma data.

Random effects model The inter-individual variation (IIV) in CL and V were estimated using an exponential error model which assumes a log normal distribution of differences between an individual and the population. Deviations of caffeine CL and V for the i th individual from the population mean values were estimated using the equations:

$$CL_i = TVCL \cdot e^{(\eta_{i,CL} + K_{j,CL})}$$

$$V_i = TVV \cdot e^{(\eta_{i,V} + K_{j,V})}$$

where CL_i and V_i are the estimated structural parameters in the i th individual on the j th occasion. TVCL and TVV are the true (but unknown) population mean values for CL and V predicted by the regression model, respectively. The differences between an individual and the population in CL and V are denoted by $\eta_{i,CL}$ and $\eta_{i,V}$, respectively.

In most infants, DBS caffeine concentrations were measured over several dosing occasions. The potential for random variances in PK parameters was therefore investigated using an inter-occasion (IOV) error model. 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. IOV in PK parameters ($K_{j,CL}$ and $K_{j,V}$) was modelled exponentially and assumed to be sampled from the same sampling distribution as suggested by Karlsson & Sheiner [22].

The residual error was estimated using the following 'additive' error model:

$$C_{ij} = \hat{C}_{ij} + \varepsilon_{ij}$$

where C_{ij} is the j th observed concentration in the i th individual, $\hat{C}_{ij}$ is the j th predicted concentration in the i th individual and ε_{ij} is the residual error.

Covariate model Scatter plots of covariates vs. individual Bayesian parameter estimates were examined to detect biologically plausible patient factors that could influence estimation of PK parameters. Patient characteristics that improved the goodness of fit of the DBS data and/or explained variability in the individual parameter estimates were identified by a forward inclusion-backward elimination method within the base model. An objective function value (OFV) > 3.84 corresponding to a P value of 0.05 was used to test the significance of a covariate influence on CL and V. Covariates screened included weight, age in various forms (gestational age (GA), post-natal age (PNA), post-menstrual age (PMA)), gender, ethnicity, albumin, haematocrit, creatinine, feeding regimen and maternal smoking status. During backward elimination an OFV > 7.81 corresponding to a P value = 0.005 was used to test the significance of a covariate on CL and V. A higher value was chosen to compensate for multiple comparisons. Other important criteria used to discriminate between two models included the interindividual variance (ω^2), residual variance (σ^2), precision (CV%) in parameter estimation, precision in estimates of coefficients (95% confidence intervals (CI) for estimates do not contain 0) and improvement in goodness of fit plots.

Model evaluation

Graphical plots of CWRES values vs. model predicted, time and other fixed effects were used as a diagnostic to assess model suitability. An internal validation technique with nonparametric bootstrapping was used to evaluate the robustness of the final model. Validation was performed using 500 datasets within PDx-POP (version 4.0, ICON Development Solutions, USA).

Results

Demographics

A total of 72 parents were approached and 67 infants were enrolled into the study. The distribution of weights and gestational ages at birth are typical of a preterm population (Table 1). Most mothers were of Caucasian origin and just over 20% reported smoking during pregnancy. Between 1 and 10 (mean 5) DBS samples were collected from infants over a mean 'sampling-phase study period' of 21.6 days (Table 1). Of a total of 384 DBS cards collected, 338 (88%) cards were deemed suitable for caffeine quantification. Reasons for poor quality samples included visually apparent coagulation, decentred spots (which included 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. It was possible to collect three 15 μ l spots per time point on 77% of sampling occasions and the total blood volume collected over the entire study period from

Table 1

Demographics, caffeine dosing, sampling and blood concentration data in the study population ($n = 67$)

Patient characteristic	Maintenance dose
Gender	M/F = 33/34
Mean gestational age at birth (weeks)	29.0 (24.6–32.7)
Mean birth weight (kg)	1.25 (0.6–2.01)
Post-natal age at day of study entry (days)	9.7 (1–69)
Weight at day of study entry (kg)	1.26 (0.65–2.5)
Post-menstrual age at day of study entry (weeks)	30.4 (25.4–37.1)
Caucasian	50
Indian	10
Afro-Caribbean	2
Other	5
Maternal smoking	Yes = 15 No = 46 Unknown = 6
Total length of caffeine treatment (days)	28.2 (6–65)
Day of caffeine therapy on study enrolment	8.4 (1–47)
Loading dose (mg kg^{-1})	10.1 (9–12.5)
Maintenance dose (mg kg^{-1})	
Study participation (days)	21.6 (1–61)
Number of samples per infant	5 (1–10)
Blood caffeine concentration (ng ml^{-1})	16476 (2780–4304)

Measurements performed on caffeine therapy following either oral (245) or i.v. (93) doses. Blood sample type; capillary = 297; venous = 27; arterial = 13; () = range.

each infant did not exceed 500 μl (range 30–480 μl). Whilst only opportunistic sampling was available, the range of post caffeine-dose time intervals allowed estimation of all important PK parameters except drug absorption.

Model development (base and covariate model)

A one compartment model best described the Pop-PK of caffeine in this group of babies. A first order oral absorption model with zero order input of i.v. doses into the central compartment was used to enable the simultaneous analysis of DBS concentration data collected following oral and i.v. doses. The absorption rate constant (K_a) and duration of zero order input (D1) were fixed with clearance (CL) and volume of distribution (V) parameters to be estimated. In the selected base model residual variability was modelled using an additive residual error model and IIV and IOV were modelled using an exponential error model. 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^{-1} (CV%, 4.6) and 767 ml (CV%, 7.9) for CL and V, respectively.

During the initial covariate screening process body weight, PMA and albumin were found to be statistically significant determinants of V (OFV > 3.84, P value 0.05),

Table 2

The effect of covariates on caffeine V and CL ($n = 67$)

PK parameter-covariate relationship	OFV	$\Delta\text{OFV}^\dagger$	Interpretation
CL	6020		Base model
V			
CL = f(WT, PNA, HCT, ALB, PN) V = f(WT, ALB)	5904		Full model
CL = f(WT, PNA, HCT, ALB) V = f(WT, ALB)	5905	$\uparrow 0.9$	PN does not affect CL
CL = f(WT, PNA, HAEM) V = f(WT, ALB)	5905	$\uparrow 0.8$	ALB does not affect CL
CL = f(WT, PNA) V = f(WT, ALB)	5908	$\uparrow 3.1$	HCT does not affect CL
CL = f(WT) V = f(WT, ALB)	5923	$\uparrow 14.7$	PNA does affect CL
CL = f(PNA) V = f(WT, ALB)	5946	$\uparrow 37.4$	WT does affect CL
CL = f(WT, PNA) V = f(WT)	5909	$\uparrow 5.8$	ALB does not affect V
CL = f(WT, PNA) V	5934	$\uparrow 25$	No covariate influence on V WT does affect V

† Change in objective function ($-2 \times \log$ likelihood) resulting from the removal of individual covariates from the model. Assuming a Chi squared distribution, a change in OFV of 7.84 units was accepted as statistically significant ($P < 0.005$). ALB, albumin; HCT, haematocrit; PN, parenteral nutrition; PNA, post-natal age; WT, weight.

whereas in the CL model weight and age (PNA, PMA), haematocrit, parenteral nutrition and albumin were identified as significant covariates. After the inclusion of weight on CL and V, PMA was no longer statistically significant for either PK parameter. However, PNA was still a significant covariate for CL. At this stage GA was formally tested as a dichotomized 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 GA. During the backward elimination process (Table 2) weight and PNA were found to be the only significant covariates in the CL model, whereas weight alone was the only influential covariate in the V model which was demonstrated by an increase in OFV of >7.84 units (P value 0.005) when either of these covariates was removed from the model.

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 IIV and therefore did not explain any of the population variability in CL but was kept in the final model as it improved the fit of the data. A reduction in the IOV from 41.1% to 34.1% and 31.4% was observed following the inclusion of weight and PNA on CL, respectively.

Table 3

Final model estimates

Parameter	Final model estimate (CV %)	Bootstrap estimate (CV %)	Difference (Δ bias%)
Fixed effects			
CL (ml h ⁻¹)	8.6 (4.0)	8.6 (4.1)	0.07
V (ml)	774 (7.9)	781 (9.2)	-0.94
K _a (h ⁻¹)	4.0 (fixed)		
Duration (i.v. bolus) (h)	0.08 (fixed)		
Exponent for WT on CL	0.786 (17.7)	0.784 (18.9)	0.17
Exponent for WT on V	1.030 (24.9)	1.031 (29.0)	-0.15
Coefficient for PNA on CL	0.013 (32.9)	0.014 (36.3)	-5.29
Random effects			
IIV CL	24.7% (25)	24.5% (30.9)	0.58
IIV V	45.7% (44.2)	43.9% (45.5)	3.88
IOV CL	31.2 (22.5)	30.1% (28.4)	3.30
Residual additive error (SD) (mg l ⁻¹)	1.8 (23.7)	1.9 (39.8)	-5.11

Δ bias% calculated using following equation: Bias = (final model estimate/ bootstrap estimate)/final model estimate $\times$ 100.

Final model and bootstrap results

The final PK model is shown below where CL is modelled as a function of weight and PNA and V is modelled simply as a function of weight alone. Covariate effects centred on population median weight and PNA resulted in the most numerically stable covariate models in NONMEM.

The final PK model:

$$CL \text{ (ml h}^{-1}\text{)} = \theta_1 \times (WT/1.28)^{\theta_5} \times (1 + \theta_7 \times (PNA - 15))$$

$$V \text{ (ml)} = \theta_2 \times (WT/1.28)^{\theta_6}$$

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

$$\text{i.v. bolus duration (h)} = \theta_3 = 0.08 \text{ (fixed)}$$

The population mean (%CV) PK estimates for CL and V were 8.6 ml h⁻¹ (4.0) and 774 ml (7.9), respectively. The final structural and random error model parameter estimates along with the bootstrap results are presented in Table 3. For all parameters, precision was comparable between the bootstrap and final model results indicating good model stability.

The relationship between the model-predicted and observed DBS concentrations is shown in Figure 1. Scatter plots of conditional weighted residuals (CWRES) against model predicted concentrations (A) and time after last dose (B) along with individual weighted residuals vs. individual predicted concentrations (C) are shown in Figure 2. CWRES were randomly and evenly distributed around the

Figure 1

Goodness of fit plots for observed vs. population model predicted concentration (A) and observed vs. individual model predicted concentration (B)

mean (zero ordinate, line of unity) with no systematic deviations in predicted concentration indicating the suitability of the error model chosen. Similar random residual scatters 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. A slight positive bias was observed on examination of the absolute individual weighted residuals at low caffeine concentrations which may have resulted from the presence of baseline caffeine in some infants (Table 3).

Discussion

To our knowledge, this is the first 'in-field' study using DBS methods to generate a Pop-PK model of caffeine drug

Figure 2

Diagnostic goodness of fit plots of (A) weighted residual vs. model predicted concentration, (B) weighted residual vs. time (IWRES) after dose and (C) individual predictions against absolute individual weighted residuals. In plots (A) and (B) the dotted lines represent perfect agreement between observed and predicted concentrations. In plot (C) the line is a locally weighted polynomial regression (LOESS) smoothing line

disposition in preterm infants. The primary aim of this study was to scope out potential problems associated with developing such a model based on DBS samples in a 'vulnerable' paediatric population. The success or otherwise of this study centred on whether a 'baby-, parent- and

staff-friendly' study design would facilitate recruitment of sufficient numbers of infants into the study and whether a busy clinical environment could cope with collecting large numbers of good quality DBS samples within the 12 month study schedule.

It is well established that PK studies in humans depend on recruitment of individuals, accurate gathering of demographic and dosing data and methods of collecting and storing blood samples that minimize analyte degradation. However, it is also recognized that these tasks are more difficult in studies involving paediatric populations and in clinical settings where interventions need to be kept to a minimum such as a neonatal intensive care unit. Hence, we were pleasantly surprised by the above expectation rate of patient recruitment (67 infants in 11 months), the relatively small numbers of parents ($n = 5$) electing not to participate in the study and by collection of large numbers of DBS samples. Several factors may have contributed to the success of this study. It is clear that clinical staff not directly involved in research were particularly enthused by the study design. Specifically, nurses were very receptive to the opportunistic nature of blood sampling, the very small volumes of blood taken for study purposes and the use of a research method (DBS) that is practised by them routinely. The positive light in which the clinical staff viewed the study may have been helpful in a number of ways including indirectly re-assuring parents that participation would not be harmful to their child and facilitating accrual of DBS samples by alerting doctors to blood taking opportunities.

For newborn screening purposes, it is common practice to draw blood from a heel prick and spot the card by allowing a droplet to 'kiss' the paper. For this study, staff members were trained to collect blood samples into a 15 μ l capillary tube attached to a vented plastic bulb at one end. By covering the vent, blood then flows in droplets allowing accurate spotting of blood onto the card, i.e. only within the confines of the target area and minimizing the risk of damaging the cards. This technique resulted in at least one analyzable spot from approximately 88% of cards. The high success rate suggests that this technique is relatively simple and can be performed with minimal training in the smallest of small babies managed in busy clinical areas.

The modelling analysis showed that a one compartment model adequately described the DBS concentration–time course of caffeine and enabled relatively precise estimation of primary PK parameters ($CL_{CV} = 4.0\%$; $V_{CV} = 7.9\%$), population parameter and residual variability. The only PK parameter that could not be estimated in this study was the oral caffeine absorption rate because there were insufficient DBS samples obtained at early time points. However, it is likely that even this parameter could have been estimated with precision had we early time points during its course. Although we investigated the influence of several covariates on caffeine CL, only weight

and PNA appeared as significant factors. In contrast only weight significantly influenced V . The estimated exponents for weight on CL (0.79) and V (1.03) in our DBS model of caffeine were close to the values of 0.75 and 1 commonly used to relate weight to CL and V , respectively in theory-based allometric equations [23, 24]. Thus, our data suggest that the relationship between weight and caffeine CL is non-linear whereas for caffeine V it is essentially linear. The estimated coefficient for PNA on CL was associated with the highest imprecision ($CL_{PNA CV} = 32.9\%$), and reflects the relatively weak influence of PNA in this population.

Several caffeine PK studies have been conducted in neonates using plasma samples [15–19]. The DBS-based PK model and parameters presented here are in good agreement with PK models and parameters of caffeine based on 'wet' plasma samples (Table 4). 'Wet' plasma sample based models also found that the two most important covariates accounting for the variability in caffeine disposition in a preterm population are weight and PNA [18, 19]. We estimated significant IOV in CL, similar to that determined in the study by Charles *et al.* (31.2 and 35.1% respectively) confirming that therapeutic drug monitoring of caffeine is not indicated in this population. However, the additive residual variability (SD, 1.8 mg l^{-1}) is slightly higher than reported by Charles *et al.* using a combined additive and proportional error model ($\sigma_1 = 0.9 \text{ mg l}^{-1}$, $\sigma_2 = 6.0\%$). The absence of an error model to account for between occasion variability in structural parameters as well as differences in bioanalytical methods may have contributed to the larger residual error reported by Lee *et al.* [18] and Thomson *et al.* [17] (3.9 mg l^{-1} and 2.5 mg l^{-1} respectively). However, a more robust comparison of the PK parameters

estimated from the two different sampling approaches would require paired DBS and plasma samples from patients.

It is well recognized that interpretation of PK data needs to account for the matrix used to measure drug concentrations. Conveniently, the RBC : plasma partition ratio for caffeine is 1:1, and therefore interpretation of PK data from DBS and plasma samples for caffeine are interchangeable. However this is not always the case. Rowland and others have discussed the general principles associated with using whole blood (DBS) drug concentration data sets in PK studies [25]. Nevertheless, common to all DBS studies in term and preterm infants, the variability in haematocrit observed after birth could influence the accuracy and precision of drug concentrations. In the present study, we noted a significant drop in haematocrit (range, 21–58%) with PNA. In general, the size of a blood spot produced on a DBS card will vary in direct proportion to the blood volume applied and in inverse proportion to blood viscosity. haematocrit is the single most important contributor to blood viscosity. Thus, for bioanalysis based on a 'fixed' disc of blood sub-sampled from a DBS, variations in haematocrit may reduce the accuracy of analyte quantification and thereby affect PK parameter estimation. For the caffeine DBS assay, results from experiments *in vitro* demonstrated a +30% bias in concentration measurements when changing haematocrit from 45% to 20%. We performed a sensitivity analysis to assess the potential impact of this systematic assay bias on the final PK model. Unfortunately, haematocrit data were available for only 138 DBS samples. For purposes of the sensitivity analysis, only these 138 DBS caffeine concentration measurements

Table 4

Comparison of a DBS-based population-PK model for caffeine in preterm neonates with models based on plasma samples

	DBS (Current study)	Plasma Lee <i>et al.</i> [18]	Plasma Thomson <i>et al.</i> [17]	Plasma Charles <i>et al.</i> [19]
PNA (days)	9.7 (1–69)	4.0 (2–15)	^a 23 (1–100)	12 (1–45)
GA (weeks)	29.0 (24.6–32.7)	28.2 (24–31)	NR	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	21.6 days (mean)	≤7 days	NR	≤7 days
(i) Structural parameter				
CL ($\text{ml h}^{-1} \text{ kg}^{-1}$)	7.3 (2.3–16.9)	4.9 (2.3–9.0)	7.9 (± 1.9)	^a 7.0 (1.6–22.6)
V (ml kg^{-1})	593 (174–1005)	970 (740–1400)	640	^a 851 (365–1761)
$t_{1/2}$ (h)	57 (13–112)	144 (86–277)	NR	^a 101 (24.5–371)
(ii) Inter-individual variability				
CL	24.7	25.0	20.0	18.8
V	45.7	11.0	24.0	22.3
(iii) Inter-occasion variability				
CL	31.2	NE	NE	35.1
V	NE	NE	NE	11.1
(iv) Residual error				
Additive (mg l^{-1})	1.8	3.9	2.5	0.9
Proportional (CV%)				6.0

CL, V and $t_{1/2}$ are given as the mean and range unless otherwise indicated. ^a, Median value; GA, gestational age; NE, Not estimated; NR, Not reported; PNA, post-natal age.

were normalized according to haematocrit. For the remaining 200 DBS samples, we assumed that caffeine concentration measurements were unaffected by haematocrit. Predictably, there was a decrease in estimates of CL and V of 8.9% and 7.2%, respectively (Table 5). Considering 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 with reported plasma data. More importantly, these results demonstrate the potential for haematocrit to significantly affect PK parameter estimation and will be an important factor for bioanalysts to consider during DBS method development and certainly before reporting DBS concentration measurements for PK analyses.

One possible solution to eliminating haematocrit effects in DBS analysis is to collect a 'fixed' volume DBS sample and quantify the analyte using the whole sample rather than collecting a variable blood volume and using a 'fixed' punch size as is the case in the present study. However, the difficulty of collecting a precise microvolume (e.g. 15 µl) sample of blood from patients on every occasion should not be underestimated as it is very easy to entrap air bubbles into capillary tubes/pipette tips. Adopting such a technique would also require significant amounts of operator training and validation. An alternative approach is to use DBS cards that reduce or eliminate haematocrit effects [26]. 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% [27]. This may provide

a practical way of overcoming haematocrit effects for some drugs whilst maintaining flexibility in the blood collection process.

Although the caffeine PK model and parameters presented here should not be used to alter clinical practice, the results of this study demonstrate that as part of baby, parent and staff friendly study design, the DBS/microvolume platform is an excellent alternative to traditional 'wet' matrix based PK studies in small children. Confidence in the platform will come with experience of real-life data sets and we recommend that the DBS/microvolume platform should be trialled in a variety of scenarios to test its robustness. However, it is clear that this platform has the potential to generate PK data for all age groups.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare no support from any organization for the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years and no other relationships or activities that could appear to have influenced the submitted work.

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Table 5

Influence of haematocrit on caffeine pop-PK parameter estimates

Parameter	Final model Estimate (CV%)	Haematocrit-adjusted plasma caffeine concentrations Estimate (CV%)
CL (ml h ⁻¹)	8.6 (4.0)	7.9 (4.1) ↓
V (ml)	774 (7.9)	722 (6.0) ↓
K _a (h ⁻¹)	4.0 (NE)	4.0 (NE)
Duration (i.v.bolus) (h)	0.08 (NE)	0.08 (NE)
Exponent for WT on CL	0.786 (17.7)	0.745 (18.4)
Exponent for WT on V	1.030 (24.9)	0.917 (29.1)
Coefficient for PNA on CL	0.013 (32.9)	0.010 (60.0)
Random effects parameters		
IIV CL	24.7 % (25)	25.4 % (24.7)
IIV V	45.7 % (44.2)	34.8 % (44.1)
IOV CL	31.2 % (22.5)	26.6 % (30.6)
Random effects parameter (Residual error model)		
Additive (SD)	1.8 mg l ⁻¹ (23.7)	2.8 mg l ⁻¹ (24.5)

α, Estimate given as %; NE, Not estimated; PNA, post-natal age.

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