



Validation and Application of a Dried Blood Spot Amoxicillin Assay

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Abstract

Dried blood spot (DBS) antibiotic assays can facilitate pharmacokinetic (PK) investigations in situations where venous blood sampling is logistically and/or ethically challenging. The aim of this study was to establish, validate and demonstrate the application of a DBS amoxicillin assay for PK studies in vulnerable populations. The matrix effect, process efficiency (84–104%) and recovery (85–110%) of the liquid chromatography–mass spectrometry (LC–MS/MS) assay for amoxicillin in DBS was determined at 1, 10 and 100 µg/mL, and three different haematocrits. Thermal stability studies of amoxicillin in DBS were performed and a bridging study comprising 26 paired plasma and DBS samples was conducted in four healthy individuals. The limits of detection and quantification were 0.02 and 0.05 µg/mL for plasma and DBS amoxicillin assays, respectively. Accuracy and interday precision of amoxicillin in DBS (0.1–100 µg/mL) were 88–103% and 4.5–9.2%, respectively. At room temperature (22 °C) and 4 °C, amoxicillin was stable in DBS for ≈4 and 26 h, respectively. There was no degradation of amoxicillin in DBS at –20 °C for > 6 months. When comparing DBS and plasma collected from healthy volunteers, the slope of the Deming regression was 0.74. Amoxicillin CL/F estimates from DBS and plasma concentration data were 40.8 and 30.7 L/h/70 kg, respectively; V/F was 43.2 and 37.4 L/70 kg, respectively. In conclusion, amoxicillin can be reliably assayed from DBS in research studies but may have limited application in therapeutic drug monitoring. Due to poor stability at room temperature, amoxicillin DBS samples should be promptly dried and placed in frozen storage.

Keywords Amoxicillin · Dried blood spots · LC–MS/MS · Pharmacokinetics

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Introduction

Optimising antibiotic dosing through pharmacokinetic–pharmacodynamic (PK–PD) studies is an important strategy for improving clinical outcomes and mitigating the threat of antibiotic resistance [1, 2]. A lack of PK/PD data for older antibiotics such as amoxicillin, which is commonly used for infections in all age groups, has been identified as a research priority [3–6]. This is particularly relevant for oral preparations where intestinal bioavailability is dependent on a saturable ‘pump’ mechanism [3], resulting in highly variable concentrations in plasma as well as in the tissue compartments of interest. Potentially clinically relevant variability in amoxicillin PK has been observed in vulnerable populations including neonates [5, 6], and people living with diabetes [7] or obesity [8].

The measurement of drug concentrations in dried blood spots (DBS) collected on filter paper represents a promising way of characterising drug disposition in these patient groups, particularly where there are no laboratory facilities to process, store and transport blood or plasma samples [9]. An additional advantage of DBS sampling is that it can facilitate PK and PK–PD studies in ambulatory patients in community settings and/or remote and regional locations. In previous studies, we have developed and validated DBS assays in clinical ‘bridging studies’ involving samples of healthy adults and older children drawn from the populations of interest, such as children aged between 5 and 10 years who have a much larger circulating blood volume than very young infants and neonates [10–12]. Another important consideration of DBS assay development for use in ambulatory settings is thermal stability at ambient temperature, particularly given the need to air-dry blood collected onto DBS cards [13].

In light of these considerations, we conducted a suite of validation studies of an amoxicillin DBS assay. In addition to the necessary in-house laboratory validation, we performed a bridging study in healthy adults to compare DBS and plasma concentrations and the resulting PK exposure parameters of interest.

Materials and Methods

Analytical Materials and Reagents

Amoxicillin (MW = 365.4; pharmacopeial grade; 99% purity), trichloroacetic acid (TCA), and ammonium acetate were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA) and the deuterated internal standard amoxicillin- d_4 (anhydrous) from Toronto Research Chemicals Inc.

(Toronto, Ontario, Canada). Liquid chromatography–mass spectrometry (LC–MS) grade dimethylsulfoxide (DMSO), formic acid, methanol, and water were from Fisher Scientific (Fair Lawn, New Jersey, USA). All other chemicals were of analytical grade. Plasma was obtained from heparinised blood collected from five adult humans.

Instrumentation and Chromatographic Conditions

The triple-quadrupole mass spectrometer (LCMS-8060 Shimadzu, Kyoto, Japan) comprised a Nexera UHPLC pump (LC-30A), degasser (DGU-20A5), autosampler (SIL-30A), and column oven (CTO-30A). Interface sources and Electro-Spray Ionisation (ESI) were included in the system. Authentic standards of amoxicillin and the internal standard were scanned and optimised for parent and product ion. The specific precursor–product ion pair and collision energy for each compound of interest are provided in Supplementary Table S1.

Amoxicillin LC/MS–MS Parameters and Separation

Quantitation of amoxicillin was performed by multiple reaction monitoring in ESI positive mode. The optimised mass spectra were acquired with an interface voltage of 0.5 kV, a detector voltage of 1.0 kV, a heat block temperature of 400 °C, a desolvation temperature of 300 °C, and an interface temperature of 300 °C. Nitrogen was used as the nebulizer gas at a flow rate of 3 L/min and drying gas flow of 9 L/min. Argon was used as the collision gas at 230 kPa.

Chromatographic separation was performed on an Acquity UHPLC® HSS PFP column (2.1 × 100 mm, 1.8 μm; Waters Corp, Wexford, Ireland). The mobile phase comprised solvent A (water plus 0.1% v/v formic acid) and solvent B (methanol plus 0.1% v/v formic acid). The flow rate was 0.5 mL/min and the mobile phase was run in a gradient mode of 0.5–5 min (solvent B = 5–95%) and 5.1–6 min (solvent B = 95%), with re-equilibration from 6.1 to 7.5 min (solvent B = 5%) for a total run time of 7.5 min. The sample injection volume was 2 μL and the retention time for amoxicillin was 2.2 min.

Plasma Sample Preparation and Extraction

Amoxicillin and amoxicillin- d_4 stock solutions were prepared separately at 1 mg/mL in DMSO and stored at –80 °C. Working standards were prepared by serial dilution from two independent primary stock solutions. Standard curve and quality control (QC) samples in plasma were prepared using 10 μL of relevant working standards, spiked into 1 mL of matrix. QC samples for amoxicillin were prepared in blank plasma at concentrations of 0.1, 0.5, 1, 5, 10, 20, 50, and

100 µg/mL, and stored at -80°C prior to use. The standard curve range was 0.05–100 µg/mL.

Plasma was extracted using a protein precipitation method. The plasma sample (10 µL) was added to 100 µL of 50 mM ammonium acetate (pH 6.8) containing 2 µg/mL of internal standard amoxicillin- d_4 . Precipitation was by addition of 100 µL of 10% TCA and 100 µL of methanol. The sample was vortex mixed for 1 min and centrifuged at $1500\times g$ for 10 min. A 100 µL aliquot of supernatant was then mixed with 100 µL of 50 mM ammonium acetate (pH 6.8). Subsequent sample preparations were adapted to a deep well plate method using 10 µL plasma with the same process and volumes as described above. The plate was shaken on a Thermomixer C (Eppendorf AG-22331, Hamburg, Germany) at 1000 rpm for 30 min. The plate was centrifuged at $2000\times g$ for 10 min using a plate bucket (model M-20; Heraeus Megafuge 16R Centrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA) and the supernatant was processed as also described above.

Plasma Method Validation

The plasma standard curves were linear ($r^2 \geq 0.998$). Plasma intraday and interday coefficients of variation (CV; %) were determined at 0.1, 0.5, 1, 5, 10, 20, 50, and 100 µg/mL amoxicillin. Chromatographic data were processed using LAB Solution (Version 5.56, Shimadzu, Japan). Matrix effects (ion suppression/enhancement), absolute recovery, and process efficiency were determined for three concentrations (1, 10 and 100 µg/mL). Three matrix sets were prepared [14]. Set 1 comprised blank plasma spiked prior to extraction, Set 2 were blank plasma spiked post-extraction, and Set 3 were pure solutions of analyte in methanol and 1% formic acid and water. The matrix effect (%) was determined from: $[\text{Set 2 response} \times 100]/[\text{Set 3 response}]$. The process efficiency (%) was determined from: $[\text{Set 1 response} \times 100]/[\text{Set 3 response}]$. The absolute recovery (%) was determined from: $[\text{Set 1 response} \times 100]/[\text{Set 2 response}]$. Accuracy was determined from the QC sample measurements during this validation process and found to be within 20% of the analytical validation range. The lower limit of quantification (LLOQ) was determined at a signal-to-noise ratio of 10:1 and confirmed by a CV < 20%. The lower limit of detection (LOD) was based on a signal-to-noise ratio of 3:1.

Preparation of Dried Blood Spot (DBS) Samples for Analysis

Venous blood was collected from healthy volunteers into 4 mL lithium heparin tubes. The haematocrit was measured using a haemocentrifuge (Kendro Laboratory Products GmbH, Hanau, Germany) at 5000 rpm for 5 min. DBS

standards were prepared from spiked whole blood. After 30 min of equilibration, the blood standards (50 µL) were spotted onto Whatman 903™ Protein Saver Cards (GE Healthcare Australia Pty Ltd, Parramatta, NSW, Australia) using a positive displacement pipette and air-dried at room temperature (22°C) for approximately 3 h. The DBS cards were then placed in a Whatman™ foil bag with desiccant and stored at -80°C until analysed.

A single 6 mm chad was punched manually and placed into a borosilicate tube with 100 µL of 50 mM ammonium acetate pH 6.8 containing 2 µg/mL of deuterated internal standard. The tube was vortexed briefly and added to 100 µL of 10% TCA and 100 µL methanol. The tube was sonicated in a water bath for 30 min and the sample was then centrifuged at $1500\times g$ for 10 min. Supernatant (100 µL) was separated and mixed with 100 µL of 50 mM ammonium acetate (pH 6.8), for LC–MS/MS assay. This method was also adapted for use with a deep well plate by extracting analytes from the 6 mm chads with 300 µL of extraction solution. The plate was shaken at 1000 rpm on a Thermomixer C for 1 h, centrifuged at $2000\times g$ for 10 min, and a 100 µL aliquot of supernatant was mixed with 100 µL of water, prior to LC–MS/MS assay.

Haematocrit Effect and Blood-to-Plasma Partition Ratio

Low and high haematocrits (0.26 and 0.59) were prepared by adding plasma or red cells to whole blood with normal haematocrit (0.40). The matrix effect, process efficiency, and recovery were assessed as described above for plasma.

The blood-to-plasma partition ratio was determined for each drug at concentrations of 1, 10, and 100 µg/mL. Aliquots of blood were either (i) centrifuged and the plasma spiked with the appropriate concentration of amoxicillin or (ii) spiked and processed as whole blood. The samples were incubated at 37°C in a water bath for 1 h to optimise equilibration of drug within plasma and blood. The blood-to-plasma partition ratio was determined using the equation [15]

$$\text{Partition ratio} = \frac{\left(\frac{\text{Blood[Drug]}}{\text{Plasma[Drug]}} - (1 - \text{Haematocrit}) \right)}{\text{Haematocrit}}$$

Thermal Stability

Venous blood was collected from a healthy volunteer into 4 mL lithium heparin anticoagulant tubes. Amoxicillin standard (10 µg/mL) in whole blood was prepared and 50 µL spotted on to a Whatman 903™ protein saver card using a

positive displacement pipette and dried at 4 °C, room temperature (22 °C) and 35 °C for up to 8 h. The time zero sample was stored at 4 °C for 10 min, then placed inside the foil bag with silica gel, and stored at –80 °C until analysed. The long-term stability of the samples was assessed at 35 °C, 22 °C, 4 °C, and –20 °C, for pre-determined periods of 8, 10, 30, and 175 days, respectively.

Preliminary determination of the degradation reaction order was by visual inspection of the concentration–time plots and confirmed by goodness of fit, according to the Akaike Information Criterion (SigmaPlot v14.5; Systat Software, San Jose, CA). Where a first-order reaction was apparent, a single exponential equation was fitted to the concentration–time data to determine the degradation rate constant.

Healthy Volunteer (Bridging) Study

The healthy adult volunteer study was approved by the Curtin University Human Research Ethics Committee (HRE2021-0392). Four participants provided written informed consent and received a single oral dose of 500 mg amoxicillin with water. Finger prick capillary blood samples were collected onto DBS cards (Whatman 903™ Protein Saver Cards) at baseline, 10, 20, 30, 45, 60, 90, 120, 180, and 240 min following drug administration. Contemporaneous venous blood samples were collected into lithium heparin tubes. To enable comparisons between venous and capillary DBS concentrations, venous blood was spotted onto filter paper prior centrifugation. After centrifugation, plasma and red cell pellets were separated and kept on ice before storage at –80 °C. All DBS cards were air-dried at room temperature (22 °C) for 1 h, placed in an airtight foil envelope with a single desiccant sachet and stored at –80 °C.

Pharmacokinetic Modelling

A simple population PK model was developed to establish if the PK parameters derived from DBS values were comparable to plasma values. Each data set from the healthy volunteers, comprising one set from plasma and another from DBS amoxicillin samples, was individually analysed using a 1-compartment model (ADVAN2 TRANS2). This process employed the First-Order Conditional Estimation method with ETA–EPS interaction (FOCE-I) alongside Bayesian post hoc estimation, all executed using NONMEM 7.5.1 (Icon, Dublin, Ireland).

To examine differences in population PK parameter estimates between plasma and DBS samples, the same model was run separately for DBS and plasma matrices. Individual concentrations of DBS were converted to ‘predicted-plasma’ concentrations using the equation: $1.35 \times [\text{DBS}] = [\text{Plasma}]$ (based on the relationship between capillary DBS and plasma amoxicillin concentrations, whereby the slope of the

Deming regression was 0.74; i.e., $[\text{DBS}] = 0.74 \times [\text{Plasma}]$). This conversion allowed for a comparison between the actual and estimated plasma concentrations derived from measured DBS concentrations.

Data Analysis

Correlations between raw values for plasma, whole blood and DBS (from venous and capillary blood) amoxicillin concentrations were assessed using Spearman’s rank correlation co-efficient (r_s). Deming regression was used to determine the slope of the correlation and presented with 95% confidence intervals (CI_{95}). Analyses and graphs were prepared using GraphPad Prism v6.05 (GraphPad Software Inc, La Jolla, CA, USA), SigmaPlot v14.5 (Systat Software, San Jose, CA, USA) or NCSS 2022 Statistical Software (v22.0.4; Kaysville, UT, USA). Data are presented as mean \pm standard deviation, unless otherwise indicated.

Results

Assay Validation

Comprehensive assay validation data are shown in Table 1 and representative chromatograms are provided in Supplementary Figure S1. Plasma and DBS precision, accuracy, and other validation data were within acceptable ranges [16–19]. The LOD and LLOQ were 0.02 $\mu\text{g}/\text{mL}$ and 0.05 $\mu\text{g}/\text{mL}$ for plasma and DBS amoxicillin assays, respectively. The blood-to-plasma partition ratio for amoxicillin was close to unity.

The stability data for amoxicillin in DBS at 10 $\mu\text{g}/\text{mL}$ are shown in Fig. 1. A bi-exponential equation provided the most plausible model and superior goodness of fit at higher temperatures, suggesting a consecutive first-order degradation reaction. Equation parameters were used to calculate the time required to reach 95% of the starting concentration (t_{95}). At room temperature (22 °C) and 35 °C, the t_{95} values were 3.6 and 3.0 h, respectively. However, at 4 °C, the t_{95} was 26 h. No degradation was observed after 175 days at –20 °C and, although our stability data currently extend to only 6 months, the estimated t_{95} for amoxicillin in DBS at –20 °C is > 2 years. Further investigation of amoxicillin stability during drying of the DBS at 4 °C demonstrated $\approx 1\%$ degradation at 8 h and an extrapolated t_{95} of at least 40 h (Panel B). By comparison, at 22 °C degradation was negligible (< 1%) during the first 3 h and approximately 5% at 4 h, indicating minimal degradation occurred during drying of amoxicillin DBS and confirming the need for refrigerated or frozen storage of DBS within 2–3 h.

Table 1 Laboratory validation data for amoxicillin in plasma and dried blood spots

Amoxicillin concentration ($\mu\text{g/mL}$)	0.1	0.5	1	5	10	20	50	100
Plasma								
Matrix effect [†]			103.0 \pm 10.6		92.3 \pm 10.8			95.8 \pm 10.2
Process efficiency [†]			95.4 \pm 12.2		95.5 \pm 8.6			92.9 \pm 11.5
Absolute recovery [†]			93.0 \pm 11.9		104.2 \pm 12.6			97.2 \pm 9.6
Intraday variation (RSD)	5.7	6.5	8.4	4.2	5.8	7.1	3.1	2.1
Interday variation (RSD, $n=25$)	9.9	7.6	8.1	8.0	6.3	8.5	7.8	6.3
Accuracy ($n=15$)	94.7 \pm 9.4	96.3 \pm 7.7	101.6 \pm 9.2	93.4 \pm 8.1	104.8 \pm 4.5	106.9 \pm 7.5	93.5 \pm 8.0	98.1 \pm 7.2
Blood:plasma partition ratio			1.01 \pm 0.07		1.06 \pm 0.08			0.98 \pm 0.05
Dried blood spots								
Matrix effect [‡]			93.1 \pm 2.5		87.2 \pm 4.3			90.9 \pm 6.1
Hct 0.26			98.7 \pm 4.9		97.1 \pm 5.9			95.1 \pm 2.6
Hct 0.40			93.9 \pm 7.9		95.2 \pm 1.5			96.9 \pm 3.7
Hct 0.59								
Process efficiency [‡]			103.9 \pm 8.4		84.6 \pm 3.6			88.5 \pm 3.4
Hct 0.26			84.1 \pm 2.4		83.6 \pm 7.7			85.3 \pm 2.5
Hct 0.40			89.2 \pm 7.2		97.3 \pm 9.4			92.0 \pm 4.2
Hct 0.59								
Absolute recovery [‡]			110.5 \pm 6.3		97.2 \pm 8.8			97.3 \pm 3.3
Hct 0.26			85.3 \pm 2.6		86.1 \pm 5.5			89.8 \pm 3.7
Hct 0.40			95.2 \pm 6.3		102.3 \pm 11.6			95.1 \pm 8.0
Hct 0.59								
Intraday variation (RSD)	5.2	4.0	5.3	6.2	4.7	3.2	4.0	5.2
Interday variation (RSD, $n=25$)	9.2	7.2	9.1	8.4	7.7	4.5	6.1	5.4
Accuracy ($n=50$)	98.9 \pm 10.9	98.6 \pm 12.8	102.8 \pm 9.8	88.4 \pm 9.0	94.6 \pm 7.6	95.2 \pm 6.9	102.2 \pm 8.2	94.5 \pm 6.5

Data are mean $\% \pm$ SD, unless otherwise indicated

Hct is haematocrit and RSD is relative standard deviation (co-efficient of variation)

[†] $n=5$ different sources of plasma; [‡] $n=5$ replicates of a single source of whole blood

Healthy Volunteer Study

Four adults aged between 35 and 55 (three males and one female; haematocrits 0.39–0.49) were recruited for the healthy volunteer study. A total of 44 paired plasma and capillary DBS samples were available for analysis, but 18 (those prior to 30–45 min) were below the LLOQ, leaving 26 paired samples with measurable concentrations. Deming regression and Bland–Altman plots for the relationship between capillary DBS and plasma amoxicillin concentrations are shown in Fig. 2. The Spearman correlation (r_s ; 95% confidence interval [CI_{95}]) was 0.93 (0.83–0.97) and the slope of the Deming regression was 0.74 (0.64–0.83). The relationship between DBS measurements from venous and capillary blood was not clinically significant (Deming regression = 0.92; $r_s = 0.93$; Supplementary Figure S2A). However, when comparing whole blood and plasma measurements, the r_s was 0.98, the slope of the Deming regression was 0.73 and the blood-to-plasma partition ratio was 0.34 (Supplementary Figure S2B). PK parameters for the DBS and plasma matrices based on the population PK model are shown in Table 2. Concentration–time profiles for each individual with measured plasma-predicted and

DBS-predicted plasma concentrations are shown in Supplementary Figure S3.

Discussion

The present study shows that highly sensitive measurement of amoxicillin in DBS samples is feasible and can be used as an alternative to plasma assays in a wide range of PK and PK–PD studies, including vulnerable or ambulatory patients where variability in bioavailability and clearance may have an impact on amoxicillin exposure and therapeutic efficacy. Our validated DBS assay has excellent characteristics in terms of LLOQ (0.05 $\mu\text{g/mL}$), recovery, process efficiency, precision, and accuracy (Table 1). The relationship between DBS and plasma concentration–time profiles in the healthy volunteer bridging study provides reassurance that DBS is a viable alternative to plasma samples. However, due to issues with thermal degradation, particularly at room- and ambient ‘tropical’ temperatures, it is imperative that future PK studies using this platform ensure the air drying of DBS cards is performed promptly before frozen storage at ≤ -20 °C. The present suite of laboratory and clinical experiments suggests

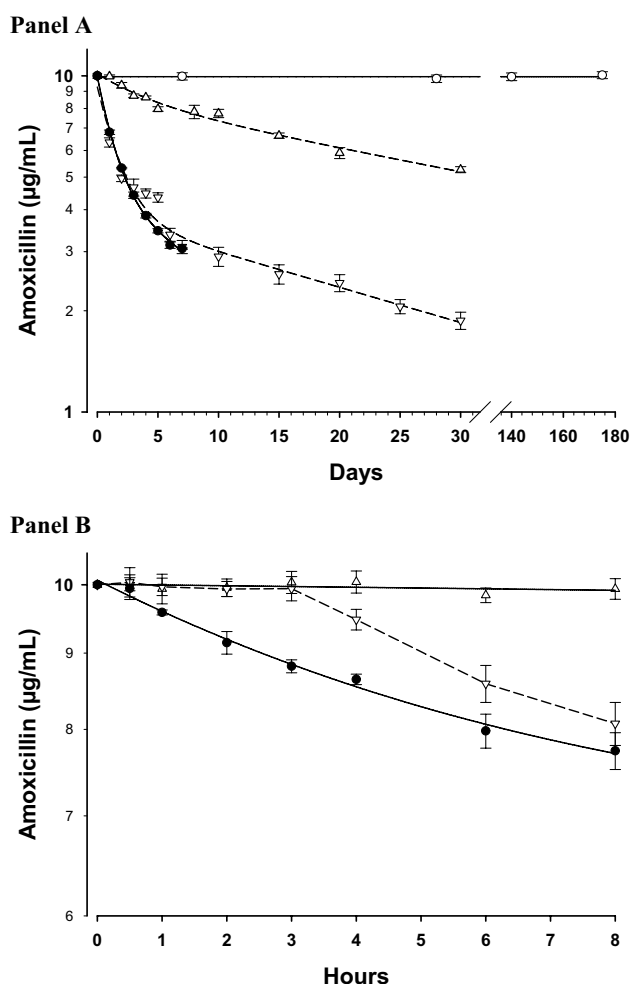


Fig. 1 Concentration–time data for amoxicillin stability in dried blood spots at an initial concentration of 10 µg/mL when stored at 35 °C (filled circle), 22 °C (unfilled delta), 4 °C (unfilled triangle) and –20 °C (unfilled circle). Data are mean ± standard deviation. **Panel A.** A mono-exponential (first-order) equation was fitted to the data at –20 °C and a bi-exponential equation was fitted to the data at 35 °C, 22 °C and 4 °C. At 4 °C, 22 °C and 35 °C, the t_{95} was 26, 3.6 and 3.0 h, respectively. No degradation was observed after 175 days at –20 °C. **Panel B.** Concentration–time data for amoxicillin in dried blood spots during drying at 35 °C (filled circle), 22 °C (∇) and 4 °C (Δ). A mono-exponential (first-order) equation was fitted to the data at 4 °C, demonstrating approximately 1% degradation at 8 h. Degradation at 35 °C was rapid, with a t_{95} of one hour. At 22 °C, degradation was <1% at 3 h and approximately 5% at 4 h, indicating minimal degradation during drying of amoxicillin dried blood spots (2–3 h)

that amoxicillin DBS assays should only be used for therapeutic drug monitoring under strict sampling and handling conditions.

Our data complement and extend recent reports of analytical methods to measure aminopenicillins (amoxicillin and ampicillin) in DBS, plasma, and other microsampling devices in vulnerable populations [5, 20–23]. For example, measuring amoxicillin from heel- or finger prick sampling

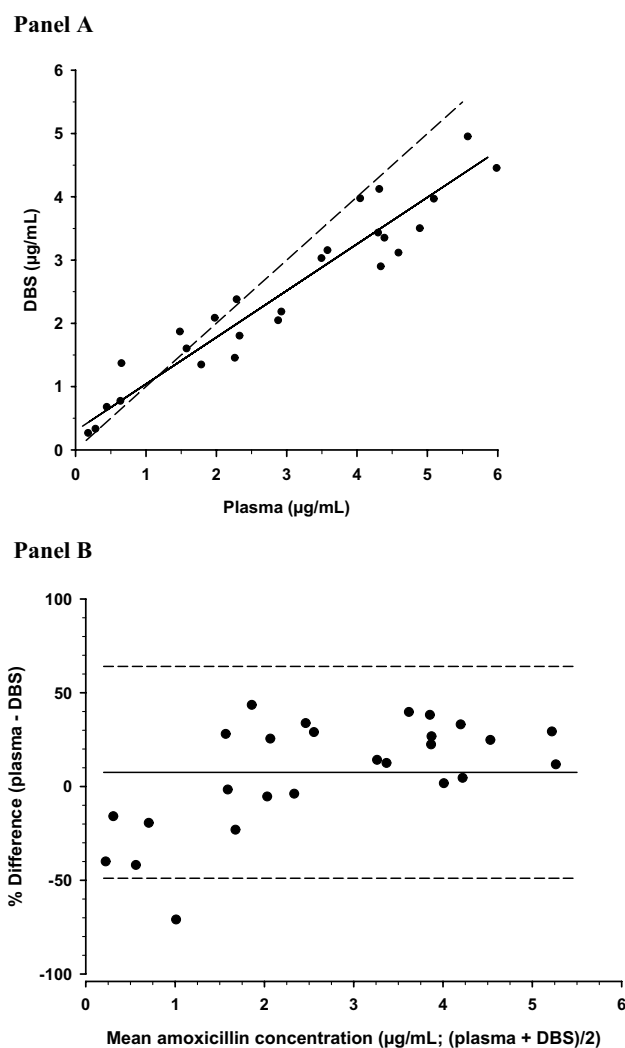


Fig. 2 Relationship between capillary dried blood spot (DBS) and plasma amoxicillin concentrations from healthy adult volunteers following a single oral dose of amoxicillin. **Panel A:** Deming regression (—; slope=0.74; CI_{95} =0.64–0.83; n =26 paired samples) and (dashed) line of unity. **Panel B:** Bland–Altman plot of percent difference versus mean amoxicillin concentration (µg/mL). The solid horizontal line is mean percent difference (8%; SD =29%); the dashed lines are the upper and lower 95% confidence limits ($\pm 1.96 \times SD$).

in neonates may overcome the disadvantages of traditional plasma PK protocols [5, 6, 21, 22], particularly in very young children from resource limited settings. There are, however, challenges to address, so that valid, clinically applicable results can be obtained [19, 24]. Contemporary analytical validation typically comprises the use of spiked blood/plasma to demonstrate the assay performance characteristics, such as LLOQ, which has varied from 0.01–0.25 µg/mL for amoxicillin in 30–200 µL plasma samples [23, 25, 26] to 0.15 µg/mL in DBS [22] and 0.05 µg/mL in the present study. In addition to the volume of matrix required,

Table 2 Pharmacokinetic parameters of healthy volunteers following a single dose of amoxicillin according to measurement from dried blood spot or plasma matrices

Parameter	Dried blood spot		Measured plasma concentration	
	Point estimate	%RSE	Point estimate	%RSE
A_{lag} (h)	0.626	10.1	0.632	10.3
k_a (h^{-1})	0.74	8.9	0.69	11
CL/F (L/h/70 kg)	40.8	8.4	30.7	10.3
V/F (L/70 kg)	43.2	13.8	37.4	10.5
A_{lag} IIV	19.7	26.2	21	28.8
CL/F IIV	14.5	27.5	19.1	15.5
Proportional residual variability	38.9	13.6	42.1	12.7

A_{lag} , absorption lag time; k_a , absorption rate constant; CL/F, clearance relative to bioavailability; V/F, volume of distribution relative to bioavailability; IIV, interindividual variability; RSE, relative standard error; variability parameters (%) were calculated using the equation: $100 \times \sqrt{\text{(estimated variance)}}$

processing, storage and transport of samples to the analytical laboratory are important factors in the study design. In the present study, amoxicillin was stable in DBS for 26 h (Fig. 1), which is comparable to the stability in plasma [26, 27], and consistent with the report by Mensitieri and colleagues [22] who found that DBS was more efficient than volumetric absorptive microsampling devices at preventing drug degradation. Importantly, the present study has shown that amoxicillin DBS should be promptly dried and frozen at ≤ -20 °C within 3–4 h (Fig. 1).

Due to the scientific and clinical interest in microsampling, industry and regulatory guidance is emerging to inform the approaches to robust validation of DBS bioanalytical assays in therapeutic drug monitoring and clinical PK studies [9, 19, 24, 28, 29]. These guidelines and consensus documents specify that storage and handling, thermal stability, homogeneity of spotting, haematocrit, carryover and reproducibility should be considered as minimum requirements. However, there is less guidance on appropriate participant characteristics, sample size and reportable outcome requirements for clinical bridging studies [19, 30]. Nevertheless, recent guidelines have provided additional recommendations for the number of paired plasma and DBS samples ($n=40$), the ideal number of participants ($n>20$) and statistical approaches for development of an assay intended for regular therapeutic drug monitoring [19, 30].

Consequently, for DBS assays in research settings, our approach is that, in addition to demonstration of in vitro feasibility and suitability of a microsampling assay, population-specific PK bridging studies can be performed in healthy volunteers [10, 12] and accessible patients with

the infection and/or clinical condition of interest [11]. This should confirm that, in addition to adequate correlations between plasma and DBS concentrations, PK profiles have acceptable accuracy across a broad clinical spectrum. Where this is not possible, a hybrid approach to validating DBS assays for clinical/field studies could be adopted, whereby paired DBS and plasma samples are collected at one or more time points for each individual (preferably across a broad concentration/time range), therefore minimising logistic challenges and concerns regarding blood volume loss with frequent plasma sampling [30]. Furthermore, we have previously demonstrated that bridging studies for DBS assays in healthy adults may not be directly translatable to paediatric patients due to significant haematocrit bias [31]. Therefore, DBS assay validation in specific patient groups is recommended.

The potential value of determining the relationship between paired DBS and plasma samples in clinical/field studies is evident from the finding from our bridging study that the blood-to-plasma partition ratio was 0.34 (Figure S2B), whereas the blood-to-plasma partition ratio in the initial LC–MS/MS assay validation was close to unity. Although detailed investigation of this discrepancy was beyond the scope of the present study, a notable difference was that the bridging study comprised plasma, blood and capillary DBS samples from volunteers who had received an oral dose of amoxicillin, whereas the initial validation comprised (single) volunteer blood spiked with amoxicillin. Hence, as there may be significant limitations if the in vitro blood-to-plasma partition ratio is used to predict a clinical relationship between the DBS and predicted-plasma concentrations in patients, the most important comparative data in the context of the present study are DBS versus plasma concentrations (Fig. 2).

The present study had some limitations. First, the sample size of the bridging study was small, with only four participants. Although the total number of samples collected was in the range typically recommended for clinical validation studies (>40), there were only 26 measurable paired samples. A further limitation relevant to DBS assays is that free amoxicillin concentrations were not measured. However, due to low protein binding, total plasma amoxicillin concentrations are thought to closely parallel free concentrations [31].

In conclusion, our data show that a reliable DBS amoxicillin assay can serve as an alternative to conventional plasma drug measurement in an appropriate clinical setting. For therapeutic drug monitoring, DBS storage ≤ 4 °C and assay within the first 8 h after sampling would be mandatory. For research studies, freezer storage at or below -20 °C (preferably ≤ -80 °C) would be essential for valid results.

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Data Availability Data not provided in the manuscript or supplementary information file are available on reasonable request to the authors.

Declarations

Conflict of Interest The authors declare no conflicts of interest.

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References

- Mi K, Zhou K, Sun L, Hou Y, Ma W, Xu X, Huo M, Liu Z, Huang L (2022) Application of semi-mechanistic pharmacokinetic and pharmacodynamic model in antimicrobial resistance. *Pharmaceutics*. <https://doi.org/10.3390/pharmaceutics14020246>
- Barreto EF, Webb AJ, Pais GM, Rule AD, Jannetto PJ, Scheetz MH (2021) Setting the beta-lactam therapeutic range for critically ill patients: is there a floor or even a ceiling? *Crit Care Explor* 3(6):e0446. <https://doi.org/10.1097/CCE.0000000000000446>
- de Velde F, de Winter BC, Koch BC, van Gelder T, Mouton JW (2016) Non-linear absorption pharmacokinetics of amoxicillin: consequences for dosing regimens and clinical breakpoints. *J Antimicrob Chemother* 71(10):2909–2917. <https://doi.org/10.1093/jac/dkw226>
- de Velde F, Mouton JW, de Winter BCM, van Gelder T, Koch BCP (2018) Clinical applications of population pharmacokinetic models of antibiotics: Challenges and perspectives. *Pharmacol Res* 134:280–288. <https://doi.org/10.1016/j.phrs.2018.07.005>
- Tang BH, Wu YE, Kou C, Qi YJ, Qi H, Xu HY, Leroux S, Huang X, Zhou Y, Zheng Y, Jacqz-Aigrain E, Shen AD, Zhao W (2019) Population pharmacokinetics and dosing optimization of amoxicillin in neonates and young infants. *Antimicrob Agents Chemother* 63(2):e02336–e2418. <https://doi.org/10.1128/AAC.02336-18>
- Keij FM, Schouwenburg S, Kornelisse RF, Preijers T, Mir F, Degraeuwe P, Stolk LM, van Driel A, Kenter S, van der Sluijs J, Heidema J, den Butter PCP, Reiss IKM, Allegaert K, Tramper-Stranders GA, Koch BCP, Flint RB (2023) Oral and intravenous amoxicillin dosing recommendations in neonates: a pooled population pharmacokinetic study. *Clin Infect Dis* 77(11):1595–1603. <https://doi.org/10.1093/cid/ciad432>
- Adithan C, Sriram G, Swaminathan RP, Shashindran CH, Bapna JS, Krishnan M, Chandrasekar S (1989) Differential effect of type I and type II diabetes mellitus on serum ampicillin levels. *Int J Clin Pharmacol Ther Toxicol* 27(10):493–498
- Soares AL, Montanha MC, Alcantara CDS, Silva SRB, Kuroda CM, Yamada SS, Nicacio AE, Maldaner L, Visentainer JV, Simoes CF, Locatelli JC, Lopes WA, Mazucheli J, Diniz A, Paixao PJ, Kimura E (2021) Pharmacokinetics of amoxicillin in obese and nonobese subjects. *Br J Clin Pharmacol* 87(8):3227–3233. <https://doi.org/10.1111/bcp.14739>
- Parker SL, Dorofaeff T, Lipman J, Ballot DE, Bandini RM, Wallis SC, Roberts JA (2016) Is there a role for microsampling in antibiotic pharmacokinetic studies? *Expert Opin Drug Metab Toxicol* 12(6):601–614. <https://doi.org/10.1080/17425255.2016.1178238>
- Knippenberg B, Page-Sharp M, Salman S, Clark B, Dyer J, Batty KT, Davis TM, Manning L (2016) Validation and application of a dried blood spot assay for biofilm-active antibiotics commonly used for treatment of prosthetic implant infections. *Antimicrob Agents Chemother* 60(8):4940–4955. <https://doi.org/10.1128/AAC.00756-16>
- Page-Sharp M, Coward J, Moore BR, Salman S, Marshall L, Davis TME, Batty KT, Manning L (2017) Penicillin dried blood spot assay for use in patients receiving intramuscular benzathine penicillin G and other penicillin preparations to prevent rheumatic fever. *Antimicrob Agents Chemother* 61(8):e00252–e317. <https://doi.org/10.1128/aac.00252-17>
- Page-Sharp M, Nunn T, Salman S, Moore BR, Batty KT, Davis TM, Manning L (2015) Validation and application of a dried blood spot ceftriaxone assay. *Antimicrob Agents Chemother* 60(1):14–23. <https://doi.org/10.1128/AAC.01740-15>
- Hand RM, Salman S, Newall N, Vine J, Page-Sharp M, Bowen AC, Gray K, Baker A, Kado J, Joseph J, Marsh J, Ramsay J, Sika-Paotonu D, Batty KT, Manning L, Carapetis J (2019) A population pharmacokinetic study of benzathine benzylpenicillin G administration in children and adolescents with rheumatic heart disease: new insights for improved secondary prophylaxis strategies. *J Antimicrob Chemother* 74(7):1984–1991. <https://doi.org/10.1093/jac/dkz076>
- Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem* 75:3019–3030. <https://doi.org/10.1021/ac020361s>
- Hung TY, Davis TM, Ilett KF (2003) Measurement of piperazine in plasma by liquid chromatography with ultraviolet absorbance detection. *J Chromatogr B Biomed Appl* 791:93–101. [https://doi.org/10.1016/S1570-0232\(03\)00209-5](https://doi.org/10.1016/S1570-0232(03)00209-5)
- Van Eeckhaut A, Lanckmans K, Sarre S, Smolders I, Michotte Y (2009) Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects. *J Chromatogr B Analyt Technol Biomed Life Sci* 877(23):2198–2207. <https://doi.org/10.1016/j.jchromb.2009.01.003>
- Li W, Tse FL (2010) Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules.

- Biomed Chromatogr 24(1):49–65. <https://doi.org/10.1002/bmc.1367>
18. Bansal S, DeStefano A (2007) Key elements of bioanalytical method validation for small molecules. *AAPS Journal* 9(1):E109–E114. <https://doi.org/10.1208/aapsj0901011>
 19. Enderle Y, Foerster K, Burhenne J (2016) Clinical feasibility of dried blood spots: Analytics, validation, and applications. *J Pharm Biomedical Anal* 130:231–243. <https://doi.org/10.1016/j.jpba.2016.06.026>
 20. Le J, Poindexter B, Sullivan JE, Laughon M, Delmore P, Blackford M, Yogev R, James LP, Melloni C, Harper B, Mitchell J, Benjamin DK Jr, Boakye-Agyeman F, Cohen-Wolkowicz M (2018) Comparative analysis of ampicillin plasma and dried blood spot pharmacokinetics in neonates. *Ther Drug Monit* 40(1):103–108. <https://doi.org/10.1097/FTD.0000000000000466>
 21. Liu Q, Liu L, Yuan Y, Xie F (2023) A validated UHPLC-MS/MS method to quantify eight antibiotics in quantitative dried blood spots in support of pharmacokinetic studies in neonates. *Antibiotics* 12(2):199. <https://doi.org/10.3390/antibiotics12020199>
 22. Mensitieri F, Coglianese A, Giudice V, Charlier B, De Rosa F, Filippelli A, Dal Piaz F, Izzo V (2022) Effects of selected pre-analytical variables on dried blood spot (DBS) and volumetric adsorptive microsampling (VAMS) based bioanalytical methods for the determination of four β -lactam antibiotics. *Biochimica Clinica* 46(2):141–153
 23. Gaikwad A, Gavali S, Narendiran KD, Bonde S, Bhadane RP (2013) An LC–MS–MS method for the simultaneous quantification of amoxicillin and clavulanic acid in human plasma and its pharmacokinetic application. *J Pharm Res* 6(8):804–812. <https://doi.org/10.1016/j.jopr.2013.07.019>
 24. Evans C, Arnold M, Bryan P, Duggan J, James CA, Li W, Lowes S, Matassa L, Olah T, Timmerman P, Wang X, Wickremsinhe E, Williams J, Woolf E, Zane P (2015) Implementing dried blood spot sampling for clinical pharmacokinetic determinations: considerations from the IQ consortium microsampling working group. *AAPS J* 17(2):292–300. <https://doi.org/10.1208/s12248-014-9695-3>
 25. Armoudjian Y, Lin Q, Lammens B, Van Daele J, Annaert P (2023) Sensitive and rapid method for the quantitation of amoxicillin in minipig plasma and milk by LC-MS/MS: a contribution from the IMI ConcePTION project. *J Pharmacol Toxicol Methods* 123:107264. <https://doi.org/10.1016/j.vascn.2023.107264>
 26. Van Vooren S, Verstraete AG (2021) A sensitive and high-throughput quantitative liquid chromatography high-resolution mass spectrometry method for therapeutic drug monitoring of 10 beta-lactam antibiotics, linezolid and two beta-lactamase inhibitors in human plasma. *Biomed Chromatogr* 35(7):e5092. <https://doi.org/10.1002/bmc.5092>
 27. Bahmany S, Ewoldt TMJ, Abdulla A, Koch BCP (2023) Stability of 10 beta-lactam antibiotics in human plasma at different storage conditions. *Ther Drug Monit* 45(5):606–615. <https://doi.org/10.1097/FTD.0000000000001100>
 28. Kothare PA, Bateman KP, Dockendorf M, Stone J, Xu Y, Woolf E, Shipley LA (2016) An integrated strategy for implementation of dried blood spots in clinical development programs. *AAPS J* 18(2):519–527. <https://doi.org/10.1208/s12248-015-9860-3>
 29. Moorthy GS, Vedar C, Downes KJ, Fitzgerald JC, Scheetz MH, Zuppa AF (2021) Microsampling assays for pharmacokinetic analysis and therapeutic drug monitoring of antimicrobial drugs in children: a critical review. *Ther Drug Monit* 43(3):335–345. <https://doi.org/10.1097/FTD.0000000000000845>
 30. Capiou S, Veenhof H, Koster RA, Bergqvist Y, Boettcher M, Halmingh O, Keevil BG, Koch BCP, Linden R, Pistos C, Stolk LM, Touw DJ, Stove CP, Alfenaar JC (2019) official international association for therapeutic drug monitoring and clinical toxicology guideline: development and validation of dried blood spot-based methods for therapeutic drug monitoring. *Ther Drug Monit* 41(4):409–430. <https://doi.org/10.1097/FTD.0000000000000643>
 31. Barker CIS, Kipper K, Lonsdale DO, Wright K, Thompson G, Kim M, Turner MA, Johnston A, Sharland M, Standing JF (2023) The neonatal and paediatric pharmacokinetics of antimicrobials study (NAPPA): investigating amoxicillin, benzylpenicillin, flucloxacillin and piperacillin pharmacokinetics from birth to adolescence. *J Antimicrob Chemother* 78(9):2148–2161. <https://doi.org/10.1093/jac/dkad196>

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