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Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies

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ABSTRACT

A reversed phase HPLC-MS/MS method has been developed and validated for the quantitative bioanalysis of acetaminophen in dried blood spots (DBS) prepared from small volumes (15 μL) of dog blood. Samples were extracted for analysis with methanol. Detection was by positive ion TurboIonSpray™ ionisation combined with selected reaction monitoring MS. The analytical concentration range was 0.1–50 μg/mL. The intra-day precision and bias values were both less than 15%. Acetaminophen was stable in DBS stored at room temperature for at least 10 days. The methodology was applied in a toxicokinetic (TK) study where the data obtained from DBS samples was physiologically comparable with results from duplicate blood samples (diluted 1:1 (v/v) with water) analysed using identical HPLC-MS/MS conditions. This work demonstrates that quantitative analysis of a drug extracted from DBS can provide high quality TK data while minimising the volume of blood withdrawn from experimental animals, to an order of magnitude lower than is current practice in the pharmaceutical industry. This is the first reported application of DBS analysis to a TK study in support of a safety assessment study. The success of this and similar, related studies has led to the intent to apply DBS technology as the recommended analytical approach for the assessment of pharmacokinetics (PK)/TK for all new oral small molecule drug candidates, which have previously demonstrated a successful bioanalytical validation.

1. Introduction

Traditionally, the toxicokinetics (TK) of exposure to new chemical entities in preclinical safety studies is reported in plasma, due to the ease of sample collection and storage. In order to derive the appropriate volume of plasma required for quantitative bioanalysis, relatively large volumes of blood (between 100 and 500 μL) need to be taken from the animals. Due to the physiological and ethical limitations of obtaining multiple serial plasma samples from individual animals, especially small or juvenile rodents [1], composite sampling is often chosen. This leads to an increased number of animals being utilised and may result in lower quality TK data.

Regulatory authorities have long acknowledged that blood is an acceptable biological matrix for the measurement of drug exposures [2,3]. However, due to practical difficulties with shipping and storing blood samples, plasma is usually selected as the matrix of choice. Filter paper blood sampling is an established technique for the screening of in-born errors of metabolism [4,5]. There have also been a number of reports on its use in humans for therapeutic drug monitoring and pharmacokinetic (PK) studies [6–13]. Dried blood spots (DBS) offers the advantage of less invasive sampling, simpler matrix preparation and transfer (no centrifugation to produce plasma) and easy storage and shipment to laboratories (no requirement for freezers and dry ice). It is notable that there are very few reports of application of this technology to preclinical PK assessments [14–16]. For these studies, in addition to the advantages associated with the clinical use of DBS, the small blood volumes used for DBS facilitates increased PK data quality, through the ability to take serial bleeds from the same animal, particularly when coupled with sensitive and selective HPLC-MS/MS techniques. This benefit becomes even more significant as the pharmaceutical industry focuses on the FDA requirement to evaluate juvenile animals as part of the Safety Evaluation phase for drugs for paediatric use [17]. A further important consideration is that the use of DBS in pre-clinical rodent TK studies for the safety assessment of new chemical entities has the potential to significantly reduce the number of animals required, giving significant ethical and cost benefits.

This paper describes the development and validation of a method for the quantification of acetaminophen in dog blood, as a DBS sample. The results of these tests were evaluated against internationally used acceptance criteria described by Shah et al. [18].

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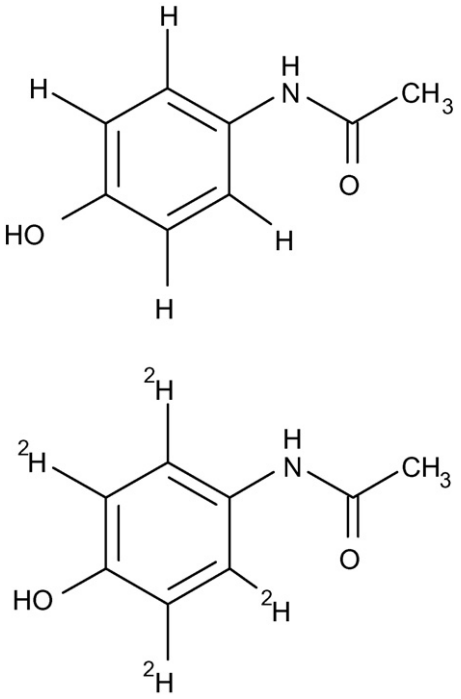

Fig. 1. Chemical structures of acetaminophen and [$^2H_4$]-acetaminophen.

The subsequent use of the method for the analysis of samples taken from dogs in a routine TK dose range finding toxicology study following the oral administration of acetaminophen is described. This work was conducted in support of a current GSK drug development programme. The methodology was validated in dog as this species is to be used in the development of the molecule. Comparison of the DBS data obtained from analysis of replicate whole blood samples (diluted with water (1:1, v/v)) using the same HPLC-MS/MS conditions was used to validate the new technology. The outcome demonstrates the potential advantages of further application of DBS in pre-clinical and regulated drug development.

2. Experimental

2.1. Chemicals and reagents

Methanol and water were of HPLC gradient grade and were obtained from Fischer (Loughborough, UK). All other chemicals were of AnalaR grade, supplied by VWR International Limited (Poole, UK). Dog (Beagle) blood was supplied by Harlan (Hull, UK). Acetaminophen (Fig. 1) was obtained from Aldrich (Poole, UK). The stable isotopically labelled internal standard, [$^2H_4$]-acetaminophen (Fig. 1) was produced by Isotope Chemistry, GlaxoSmithKline (Stevenage, UK).

2.2. Equipment

FTA® Elute blood spot cards were supplied by Whatman (Sanford, USA). Sample tubes were obtained from Micronics (Platinastraat, The Netherlands). Centrifuge (model 5810R) was supplied by Eppendorf (Hamburg, Germany). Harris punch and cutting mat was supplied by Whatman (Sanford, USA). Plastic bags for the storage of blood spot cards were supplied by Fischer (Loughborough, UK). Sachets of desiccant were obtained from Sud-Chemie (Northwich, UK).

2.3. Preparation of standard stock and working solutions

Primary stock solutions of acetaminophen (in duplicate) and [$^2H_4$]-acetaminophen (internal standard) were prepared in dimethylformamide (1 mg/mL). Working standard solutions of acetaminophen in H_2O:acetonitrile (1:1, v/v) were prepared from the primary stocks at concentrations of 100 and 10 μg/mL. Internal standard working solutions (0.5 μg/mL) were prepared from the primary stock using methanol for blood spot analysis and acetonitrile for blood/water analysis. All the solutions were stored at 4 °C and brought to room temperature before use.

2.4. Preparation of calibration standards and quality control (QC) samples

Calibration standards were prepared fresh on the day of analysis (for both validation and TK study sample analysis) by diluting the appropriate working solutions with blank whole dog blood for DBS analysis and blank dog blood/water (1:1, v/v) for blood/water analysis. The concentrations for both DBS and blood/water calibrants were 0.1, 0.2, 0.5, 2, 5, 20, 40 and 50 μg/mL whole blood.

QC samples for all analyses were prepared from a separate stock solution to that used for the calibration standards. QC samples for the DBS validation were prepared by diluting the appropriate working solutions with blank dog matrix to give concentrations of 0.1, 0.3, 5, 40 and 50 μg/mL whole blood. For the analysis of TK study samples, QC's were prepared with blank whole dog blood (DBS) and blank dog blood/water (1:1, v/v) for blood/water analysis at concentrations of 0.3, 5, 40 μg/mL whole blood.

For DBS analyses 15 μL aliquots of calibration standards and QC samples were spotted onto FTA® Elute cards and allowed to dry at room temperature for at least 2 h prior to analysis. When required, the DBS QCs were stored at room temperature in a sealed plastic bag containing desiccant until analysis.

For blood/water analyses, 30 μL aliquots were transferred to a clean tube, prior to analysis. When required, the blood/water QCs were transferred to sample tubes for storage at −20 °C until analysis.

2.5. Sample preparation

For DBS analyses, a 3 mm diameter disk was punched from the centre of the DBS into a clean tube. Methanol (100 μL) containing internal standard ([$^2H_4$]-acetaminophen) was added and the tube vortex mixed for approximately 30 s. The tube was centrifuged for 5 min at 3000 × *g* and the supernatant transferred to a clean tube and a portion injected onto the HPLC-MS/MS system.

For blood/water analyses, a 30 μL aliquot was extracted with 300 μL acetonitrile containing internal standard ([$^2H_4$]-acetaminophen). This was vortex mixed for approximately 30 s and then centrifuged for 5 min at 3000 × *g* and a portion of the supernatant injected onto the HPLC-MS/MS system.

2.6. HPLC-MS/MS analysis

The HPLC-MS/MS system consisted of a CTC HTS PAL autosampler (Presearch, Hitchin, UK) with fast wash, an Agilent 1100 binary pump (Palo Alto, CA, USA) with integrated column oven and divert valve, and a Polaris Amide C18, 3 μm, 50 mm × 3.2 mm i.d. HPLC column (Varian Limited, Oxford, UK). The post column flow was diverted to waste for first 0.5 min of each chromatographic run. During this time, flow (0.25 mL/min, methanol:water (1:1, v/v)) was provided to the MS by a Knauer pump (Presearch, Hitchin, UK).

The chromatographic separation was achieved using a solvent gradient employing the mobile phases 10 mM ammonium formate

containing 0.3% ammonia (A) and methanol (B). Following sample injection (3 μL) the mobile phase was held at 98% A for 0.2 min. A ballistic gradient to 5% A at 0.3 min, was followed by an isocratic period at 5% to 0.8 min. The mobile phase was then returned to 98% A by 0.9 min and was held at this composition until 1.5 min, before the injection of the next sample. The flow rate was 1 mL/min, the column was maintained at 40 °C. The HPLC effluent was split approximately 1:3 before entering the ion source.

MS detection was by a Sciex API-3000 (Applied Biosystems/MDS Sciex, Canada) equipped with a TurboIonspray™ ion source. The source temperature was 450 °C with a turbo gas flow of 7 L/min (N_2) and a nebuliser gas setting of 10 (N_2). The curtain gas and collision gas settings were 10 and 6, respectively (both N_2). The characteristic precursor $[M+H]^+$ to product ions transitions, m/z 152–110 and 156–114 were consistent with the structures of acetaminophen and the internal standard (loss of CH_2CO), respectively, and were used as selected reaction monitoring transitions to ensure high selectivity. A dwell time of 200 ms was used for both transitions. The pause time was 5 ms.

HPLC-MS/MS data were acquired and processed (integrated) using Analyst software (v1.4.1 Applied Biosystems/MDS Sciex, Canada). Concentrations were determined from the peak area ratios of analyte to internal standard using the in-house laboratory information management system, SMS2000 (v1.6, GlaxoSmithKline, UK).

2.7. Assessment of matrix suppression effects

To assess the suppression of HPLC-MS/MS detector response due to matrix components associated with DBS, the peak areas of the internal standard in extracts of replicate DBS QC samples at 0.3, 5 and 40 μg/mL whole blood, were compared to those of the same concentration of internal standard spiked directly into methanol.

2.8. Application of the DBS assay to a toxicokinetic study

One male and one female dog received a single daily oral gavage administration of a suspension of acetaminophen at a target dose of 50 mg/kg for 7 days. Blood samples (1 mL) were collected by jugular venepuncture into EDTA tubes on days 1 and 7 at 0.5, 1, 2, 4, 8, 12 and 24 h after administration.

For DBS analyses, replicate ($n=3$ for each time point) 15 μL aliquots from each blood sample were spotted onto FTA® Elute paper and allowed to dry at room temperature for 2 h. These were then stored and shipped at room temperature in a sealed plastic bag containing desiccant before analysis.

For blood/water analyses, a further 15 μL of blood from each sample was aliquoted into a tube containing 15 μL of HPLC grade water. The tubes were mixed by inversion and frozen immediately over carbon dioxide. The diluted blood samples were stored and shipped at −20 °C before analysis.

3. Results and discussion

3.1. Method validation

3.1.1. Linearity, selectivity and sensitivity

A calibration plot of analyte/internal standard peak area ratio versus the nominal concentration of acetaminophen was constructed and a weighted $1/x^2$ linear regression applied to the data. Linear responses were observed for DBS over the range 0.1–50 μg/mL dog blood. This is represented by the following linear regression equation: $y = 1.56 \times 10^{-5}x + 7.68 \times 10^{-4}$, $r^2 = 0.9947$, where y represents the peak area ratio of acetaminophen to that of IS and x represents the concentration of acetaminophen in μg/mL.

The selectivity of the blood spot method was established by the analysis of samples of control dog blood from six individual beagle dogs extracted from FTA® Elute paper. No unacceptable interferences, i.e. those with peak areas of >20% of that observed for the LLQ, at the retention times of acetaminophen or its internal standard were observed in any of these lots. A representative chromatogram of a control DBS double blank is shown in Fig. 2.

The lower limit of quantification (LLQ) for the analysis of DBS sample extracts was 0.1 μg/mL. This was defined as the lowest concentration that gave accuracy within 15% of nominal and precision not exceeding 15% for $n=6$ replicates. Representative mass chromatograms of blank and LLQ samples are shown in Figs. 3 and 4, respectively. The suppression of the response for the stable isotope labelled internal standard when analysing DBS extracts by HPLC-MS/MS was 76%.

3.1.2. Accuracy and precision

The accuracy (%bias) and precision (%CV) of the DBS method was evaluated using QC samples at five concentrations analysed against calibration standards prepared from a separate stock solution. Each QC was analysed in replicate ($n=6$) in a single validation batch. Accuracy was assessed by calculating the percent deviation from the theoretical concentration. Precision was determined by calculating the coefficient of variation for intra-day replicates. Table 1 shows a summary of the individual QC concentration data obtained during the validation. The maximum accuracy and intra-day preci-

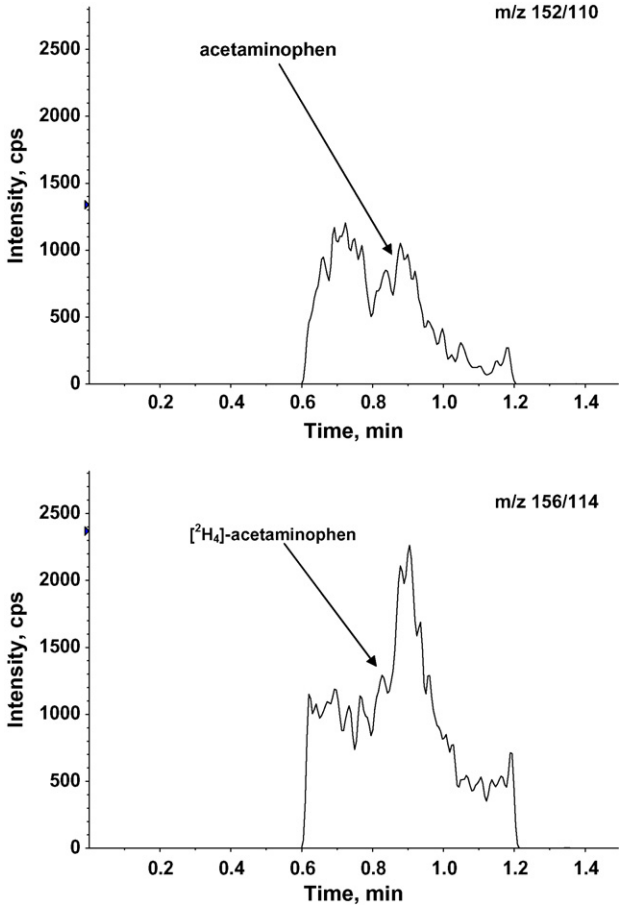

Fig. 2. Representative HPLC-MS/MS chromatograms of a double blank dried dog blood spot sample.

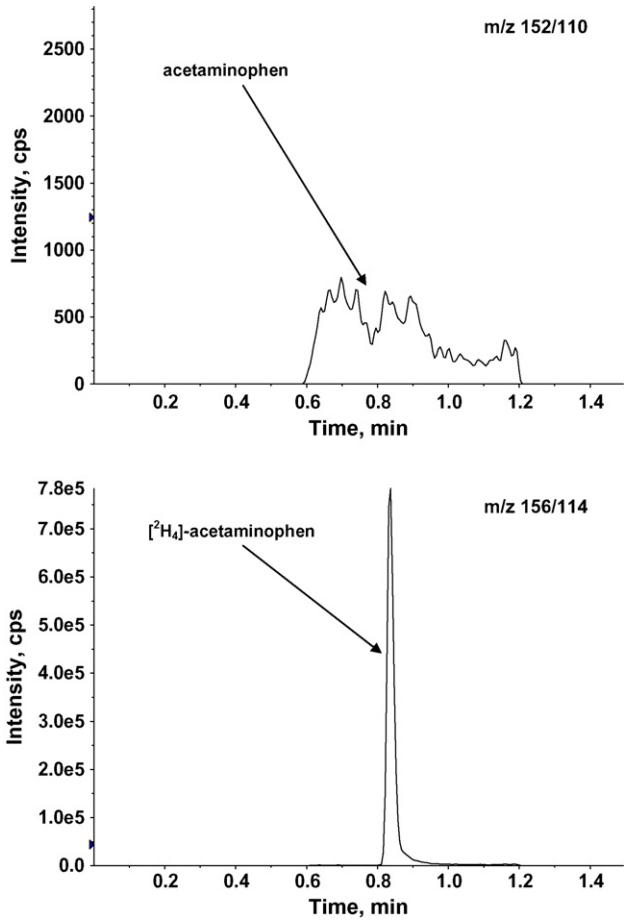

Fig. 3. Representative HPLC-MS/MS chromatograms of a blank dried dog blood spot sample.

sion values observed were 14.5% and 10.4%, respectively, which are well within internationally recognised acceptance criteria for assay validations [11].

3.1.3. Stability

The stability of acetaminophen in matrix during the process of collecting the blood sample, and spotting and drying on the paper, was assessed by comparing peak area ratios of replicate ($n = 6$) dog blood samples spiked at 40 μg/mL and stored for 4 h at 37 °C, with those of the fresh samples. Samples (30 μL) were extracted by the addition of acetonitrile (300 μL) containing IS. The difference was 2.2% (Table 2), indicating no acetaminophen instability under the conditions of storage.

The stability of acetaminophen in blood dried on the FTA® Elute paper for the maximum period a sample was likely to be stored before being analysed, was assessed by comparing concentrations of replicate ($n = 6$) dog blood samples spiked at 0.3 and 40 μg/mL after storage of the DBS at room temperature for 7 days, with those of the same samples extracted immediately after initial spotting and drying. The difference was less than 15% (Table 3), indicating no acetaminophen instability under the conditions of storage.

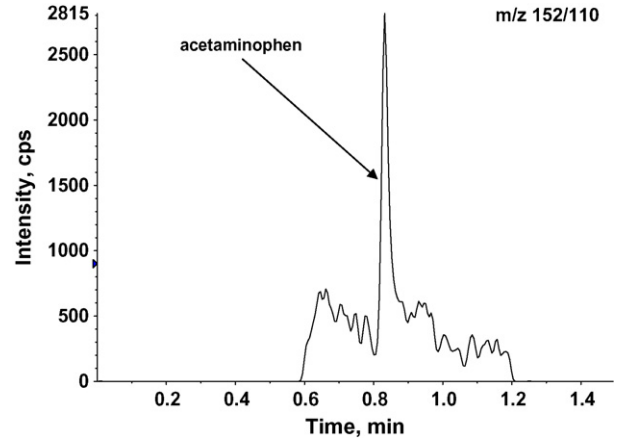

Fig. 4. Representative HPLC-MS/MS chromatogram of a dried dog blood spot sample at the LLQ (0.1 μg/mL acetaminophen).

Table 1
Accuracy, precision and individual validation sample concentrations for acetaminophen in dried dog blood spot samples

Nominal concentrations (μg/mL)	QC 1	QC 2	QC 3	QC 4	QC 5
	0.1	0.3	5	40	50
	0.099	0.350	5.828	34.886	44.264
	0.096	0.391	5.298	42.138	45.588
	0.107	0.301	6.145	38.045	44.223
	0.105	0.345	5.432	40.821	47.384
	0.126	0.348	5.103	40.954	42.852
	0.117	0.326	4.903	37.009	45.682
Mean	0.108	0.344	5.452	38.976	44.999
S.D.	0.011	0.030	0.463	2.785	1.565
Precision (%CV)	10.4	8.6	8.5	7.1	3.5
Accuracy (%bias)	8.3	14.5	9.0	−2.6	−10.0

Table 2
Stability of acetaminophen in whole dog blood stored at 37 °C for 4 h

	Peak area ratio	
	Fresh	4 h at 37 °C
	0.973	0.962
	0.995	0.988
	0.903	0.905
	0.943	0.928
	0.879	0.990
	0.915	0.959
Mean	0.935	0.955
S.D.	0.044	0.033
Precision (%CV)	4.7	3.5
Difference (%)		2.1

Table 3
Stability of acetaminophen in dried dog blood spots on Whatman FTA® Elute paper stored at room temperature for 7 days

	Nominal concentrations			
	0.300 μg/mL		40 μg/mL	
	Fresh	Stored	Fresh	Stored
	0.293	0.299	38.947	36.503
	0.314	0.289	40.548	35.584
	0.295	0.272	37.431	35.241
	0.267	0.228	38.772	37.062
	0.307	0.232	40.625	33.492
	0.328	0.225	39.247	35.280
Mean	0.301	0.258	39.262	35.527
S.D.	0.021	0.033	1.200	1.233
%CV	7.0	12.9	3.1	3.5
%Bias	0.2	−14.2	−1.8	−11.2
%Difference	−14.4		−9.5	

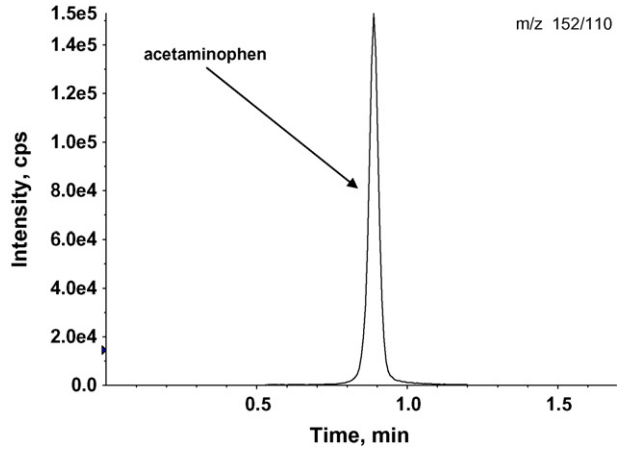

Fig. 5. Representative HPLC-MS/MS chromatogram of a dried dog blood spot sample obtained 0.5 h following daily oral administration of 50 mg/kg acetaminophen to a male dogs for 7 days (corresponding to the maximum observed peak concentration (C_{max})).

3.2. Application of the DBS assay to a toxicokinetic study

A representative mass chromatogram of a DBS sample extract, corresponding to a C_{max} sample is shown in Fig. 5. TK analysis of the DBS and blood/water concentration data obtained was performed by non-compartmental pharmacokinetic analysis using WinNonlin™, Enterprise Edition Version 4.1. The systemic exposure of acetaminophen was determined by calculating the area under the concentration–time curve (AUC) from the start of dosing to the last quantifiable time point (AUC_{0-t}) using the linear-logarithmic trapezoidal rule. The maximum observed peak concentration (C_{max}) was determined by inspection of the observed data. The AUC and C_{max} results obtained are presented in Table 4. As only one male and one female dog were used in the TK study, it is inappropriate to employ statistical methods to compare the data obtained from DBS and blood/water samples. However, data generated from such small group sizes over many years in our laboratories for multiple new chemical entities suggest that TK data can vary by up to two-fold and still be considered comparable. Hence, the <1.4-fold variability in data obtained from DBS and blood/water samples in this study demonstrates that TK exposure parameters for acetaminophen by DBS analysis are physiologically comparable to those obtained from direct analysis of blood/water. This is also illustrated graphically in the blood concentration–time plots shown in Fig. 6.

Table 4
Toxicokinetic parameters obtained from the analysis of dried blood spot and blood/water (1:1, v/v) samples obtained following daily oral administration of 50 mg/kg acetaminophen to dogs for 7 days

Period	Sex	AUC (μg h/mL)		C_{max} (μg/mL)	
		Blood/water	Blood spot	Blood/water	Blood spot
Day 1	Male	58.8	71.3	29.9	37.9
	Female	55.9	60.4	31.4	41.5
Day 7	Male	65.7	81.6	33.8	44.0
	Female	57.6	64.7	37.1	43.5

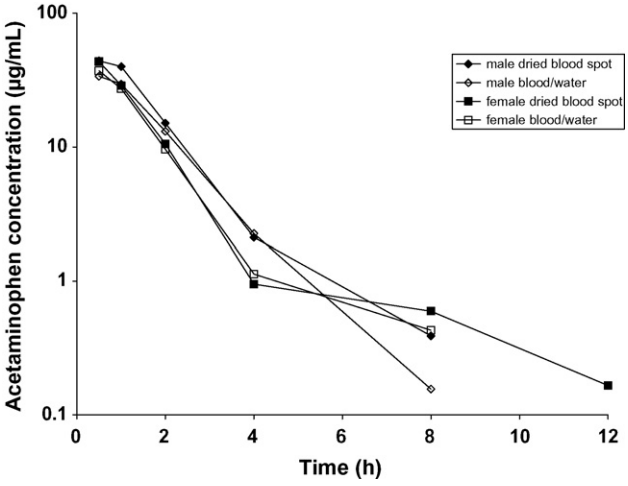

Fig. 6. Blood concentrations of acetaminophen obtained from the analysis of dried blood spot and blood/water (1:1, v/v) samples obtained following daily oral administration of 50 mg/kg acetaminophen to dogs for 7 days.

4. Conclusion

DBS has been reported to be a convenient matrix, readily amenable to measurement of biomarkers and drugs like the anti-malarials, in blood for both clinical and pre-clinical studies. However, this is the first reported application of DBS analysis for the assessment of TK in a pre-clinical safety assessment study with all the potential advantages this technology allows. This paper describes the validation of an acetaminophen method in dog blood by combining extraction of the drug from DBS and HPLC-MS/MS quantification. The method was selective for acetaminophen and linear over the concentration range 0.1–50 μg/mL using only 15 μL of blood, with intra-day precision and bias of less than 12%. The stability of acetaminophen in dog blood spotted onto the FTA Elute paper and stored at room temperature was demonstrated for at least 10 days. The methodology has been successfully employed in a TK study following daily oral administration of 50 mg/kg acetaminophen to dogs for 7 days. The resultant TK data from DBS samples are physiologically comparable with that obtained from duplicate blood/water samples analysed using identical analytical conditions. This work demonstrates the capability of DBS analysis to provide high quality TK information using significantly smaller volumes of blood than are conventionally required. Further, the reduction in blood volume leads to a decrease in animal numbers used, through serial rather than composite sampling regimes, giving ethical benefits and an increase in data quality. In addition, if continued through the life-cycle of a new drug, the technology offers the advantage of simpler sample collection, storage and shipment for both preclinical and clinical study samples, leading to notable ethical and financial benefits. The success of this and similar, related studies has led to the intent top apply DBS technology as the recommended analytical approach for the assessment of PK/TK data for all new oral small molecule drug candidates, which have previously demonstrated a successful bioanalytical validation.

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