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Discovery stage pharmacokinetics using dried blood spots

Patrick Beaudette, Kevin P. Bateman*

Merck Frosst Canada Inc., 16711 Trans Canada Hwy., Kirkland, Que., Canada H9H 3L1

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Abstract

Early in the discovery stage, the measurement of drug candidates in biological fluids as a function time provides important information used in decision making for lead optimization. The detection methodology primarily used is liquid chromatography coupled to triple quadrupole mass spectrometry (LC–MS). Sample preparation is an important aspect of these experiments and robotic-based automation is commonly used. The often overlooked aspect of these experiments is the sample collection itself. Typically, several hundred microliters of whole blood is collected and the plasma fraction separated for each time-point. The plasma is then transferred to an appropriate vessel for subsequent aliquoting and processing. We describe a method for performing discovery stage pharmacokinetic analysis using whole blood dried onto filter paper. The use of dried blood spots is a well established technique for neo-natal screening, and its application to early screening of drug candidates proves to be robust, reliable and reproducible.

Keywords: Discovery stage pharmacokinetic analysis; Dried blood spots

1. Introduction

Pharmacokinetic (PK) analysis is vital to drug discovery as it provides insight into how new drug candidates may be absorbed and excreted by the human body. Discovery stage PK studies involve dosing an animal with the compound of interest and measuring the drug concentration in biological fluids as a function of time. This yields valuable information such as the time needed to reach peak concentration and the half-life of the drug, other parameters such as volume of distribution, clearance and bioavailability can also be determined from these experiments. Such information helps to define the "disposition" of a compound, allowing it to be assigned a priority ranking relative to a lead compound and giving medicinal chemists targets for optimization. With many candidates to screen, PK studies are routine but demand a significant amount of time and resources. Hence improvements to current protocols are always welcome.

Current methodology for PK determinations in larger mammals (i.e. rats, rabbits, dogs, etc.) uses plasma (separated from whole blood) collected from the dosed animal(s) at designated times. The plasma is then analyzed directly after on-line [1] or off-line [2] solid phase extraction [3], liquid–liquid extraction [4] or protein precipitation [5]; the latter method has been adopted at this particular site. However, these methods suffer the drawback of involving time-consuming sample preparation, which creates a bottle-neck in the analysis process. This puts a limitation on throughput, which means that fewer compounds can be advanced to pharmacokinetic analysis.

The use of dried blood spots (DBSs) has been around for nearly 40 years and has been widely adopted in newborn screening applications [6]. In the United States, >95% of newborns are screened for inherited metabolic disorders in this fashion [7]. An impressive range of compounds have been analyzed from dried blood spots, spanning a molecular weight range from amino acids [8] to hormones [9] and RNA [10]. Therapeutics agents have also been analyzed from dried blood spots. These studies have focused on anti-malarial drugs, with the goal of developing methodology for the collection of samples in remote areas [11–14]. These papers showed that the compounds are stable at room temperature for 2 months, and that analysis of metabolites is also possible. The filter paper blood collection device itself has become a precision analytical tool with well-characterized

* Corresponding author. Present address: Merck Frosst Canada Inc., P.O. Box 1005, Pointe Claire-Dorval, Que., Canada H9R 4P8.
Tel.: +1-514-428-8689; fax: +1-514-428-8615.
E-mail address: kevin_bateman@merck.com (K.P. Bateman).

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properties in terms of blood volume per unit area, chromatography and lot-to-lot variability [7,15,16].

This paper describes a method for obtaining small animal pharmacokinetic data from whole blood dried onto collection cards. The method is suitable for obtaining a fast "snapshot" of the disposition of discovery stage compounds; especially when a full set of PK parameters is not required to make the quick kill decisions used early in discovery programs. Compared to the plasma precipitation method currently used, DBSs offer the advantage of being faster in terms of sample collection and sample preparation for LC–MS analysis. As a result, more compounds can be screened in a shorter period of time and ranked relative to a lead compound with respect to their bioavailability and half-life. When the rate-determining step in the PK assay procedure, sample preparation, is more efficient, the entire process of drug discovery and development is accelerated.

2. Experimental

2.1. Reagents and materials

All compounds under study (A, B, C and D) were synthesized at Merck Frosst and Co. All solvents were HPLC-grade. Formic acid was from Acrôs, acetonitrile was from Fisher and water was Milli-Q (Millipore). Xterra columns were purchased from Waters. Blood was collected onto Schleicher and Schuell #903 specimen collection paper.

2.2. Animals

All procedures were approved by the Animal Care Committee at the Merck Frosst Center for Therapeutic Research according to the guidelines established by the Canadian Council on Animal Care. Sprague Dawley rats with an average mass of 300 g were used in each of the studies. Two animals per dosing route were used for each compound and results are presented as average values.

2.3. Sample collection and preparation

2.3.1. Dried blood spot method

Following the initial dosage, animals were tail bled directly onto the blood collection cards at the specified time-points: 0, 0.25, 0.5, 1, 2, 4 and 6 h for compounds A, B and C. Compound D had additional time-points at 8 and 24 h post-dosing. When the study was completed, the cards were air dried overnight, then stored in a resealable plastic bag containing ∼300 g of Drierite desiccant. Extraction of the compound into solvent was performed as follows: 3.2 mm discs were punched out of the dried blood spots and placed in a 500 μL Costar 96-well plate, one disc per well. For each time-point, two discs were assayed (if bloodspot size allowed) to ensure adequate precision. An extraction solvent of 1:1 acetonitrile/water containing internal standard (1000 ng/mL) was prepared, and 150 μL of this was pipetted into each well with the aid of a multichannel pipettor. The plate was sealed with a Beckman Capmat and vortexed briefly to ensure all discs were submerged in solvent. The plate was then incubated for 2 h in an Eppendorf Thermomixer at 37 °C with 500 rpm shaking. Following incubation, the plates were centrifuged to remove particulate matter and a portion of the extraction solvent was transferred to a clean 96-well plate, ready for LC–MS analysis.

Calibration standards were prepared by spiking the compound into whole blood to concentrations which spanned the expected range. This blood was then spotted onto the collection cards (40 μL per circle) and dried thoroughly before being processed as described. For the long-term stability study, these cards were stored at room temperature in a resealable plastic bag containing Drierite desiccant. Calibration curves were generated using $1/x$ weighting of standards.

2.4. Plasma precipitation method

Following initial dosage, blood was collected from animals at specified time-points. Blood was centrifuged to separate the plasma, 50 μL of which was then aliquotted into Eppendorf tubes. Acetonitrile (75 μL, containing internal standard), 1.5 times the volume of the plasma, was added to each Eppendorf and vortexed briefly to precipitate the plasma proteins. The tubes were centrifuged for 10 min at 14,000 × g to pellet the proteins, and the supernatant was transferred to a clean 300 μL HPLC vial insert prior to analysis. Calibration standards were prepared by spiking concentrated standards into blank plasma to achieve the desired concentration. After spiking, the plasma was processed as described above.

2.5. LC–MS analysis

Separations were performed on a Waters Alliance 2790 LC system (Milford, MA, USA) controlled with MassLynx V3.5 software. Samples (25 μL) were injected onto a Waters Xterra MS C18 column (4.6 mm × 50 mm, 5 μm particles) equipped with a Phenomenex SecurityGuard C18 guard column. A moderate gradient (10–90% acetonitrile in 3 min, versus 0.1% formic acid) at a flow rate of 1.0 mL/min was applied to elute both the compound and the internal standard. The flow was split 1:5 prior to entering the electrospray source such that ∼200 μL entered the source with the rest diverted to waste. Mass spectral data were generated using a Micromass Quattro LC (Manchester, UK) triple quadrupole mass spectrometer using multiple reaction monitoring for the detection of the parent and the internal standard.

Typical operating conditions for the mass spectrometer were as follows: ion source: electrospray in the positive-ion mode; scan mode: MS–MS; scan type: multiple reaction monitoring (MRM), dwell time 0.3 s per channel, nebulizing gas: 130 L/h, desolvation gas: 860 L/h; source block temperature: 120 °C, desolvation temperature: 375 °C. Collision

energies and cone voltages were optimized on a compound to compound basis.

3. Results and discussion

With hundreds of compounds in queue for pharmacokinetic screening, changes to the current assay which allow for increased throughput are welcome. Simpler sample collection would allow a lab animal technician to administrate more tests simultaneously. Utilizing blood collection cards eliminates the need for bleeding the animals into tubes, and subsequent centrifugation and liquid transfer of the separated plasma is no longer necessary. No anti-coagulation agent is required, and the dried blood spots are stable enough to allow mailing to a central laboratory for processing.

The extraction method described here is flexible in terms of the time required for incubation, the type and volume of solvent used (assuming it is HPLC-compatible), and the number of blood-discs incubated. Hence the assay can be tailored for optimization to a variety of compounds and applications. No modifications to the LC–MS protocols typically used for plasma analysis were made, and the data is processed in the same manner. The increased throughput the DBS method affords can provide medicinal chemists feedback on more candidates in a shorter period of time. Compounds can be ranked in terms of their absorption/excretion characteristics (bioavailability and half-life), providing the chemists insight into the structural motifs/elements which work for reference in lead compound development.

Four compounds (A, B, C and D), each from a different structural class, were investigated with rats being the animal chosen for dosing. In general, compound A is a coumarin, compound B is a carboxylic acid, compound C is an imidazole and compound D is a secondary amine. The calculated log *P* for each compound is 7.14 ± 1.01, 1.61 ± 0.75, 7.4 ± 0.88 and 3.43 ± 0.82, respectively. For each of the compounds, the equivalent of 50 ng/mL was easily detected with both methods (Fig. 1). The absolute sensitivity is approximately 10–20*x* greater using the plasma method. This is expected as the dilution factor for the DBS method is greater (∼35-fold versus 2-fold) than for the plasma method. Increased sensitivity could be obtained by doing the extraction in a smaller volume, or by drying down the extract and reconstituting in a smaller volume. Although, for discovery stage PK, sensitivity is usually not an issue. Side-by-side comparisons of the pharmacokinetic data obtained using both methods (plasma precipitation and DBS) are shown in Fig. 2. Dosage size and vehicle information is contained in Table 1. Table 2 summarizes the bioavailability and half-life measurements determined for each compound in the parallel studies. Depending on the partitioning of the compound into red blood cells, it would be expected that the peak concentrations in plasma to be higher in plasma vs. whole blood. The absolute value of drug compound concentration has no effect on the measurement of half-life and bioavailability. It is evident for each compound that the values obtained for the plasma precipitation and the DBS methods of analysis are in agreement within acceptable limits of error.

Table 1
Dosing parameters for compounds used in this study

Compound	Dose P.O. (mg/kg) ($n = 2$)	Vehicle	Dose I.V. (mg/kg) ($n = 2$)	Vehicle
A	20	0.5% Methocel	1	DMSO
B	10	60% PEG 200	2	60% PEG 200
C	20	0.5% Methocel	5	60% PEG 200
D	10	1% Methocel	2	60% PEG 200

Table 2
Comparison of $t_{1/2}$ and %*F* for the standard method and the dried blood spot method

Compound	Plasma		Blood	
	Half-life (h)	Bioavailability (%)	Half-life (h)	Bioavailability (%)
A	3.7	6	1.6	4
B	1.2	46	1	49
C	2.6	22	3.2	18
D	2.1	62	2.3	58

The intra-assay precision and accuracy were assessed by extracting and analyzing seven replicates of the DBS standards at three different concentrations. The intra-assay precision (expressed as percent relative standard deviation, %R.S.D.) ranged from 2.6 to 6.4% and the intra-assay accuracy (expressed as percent of nominal values) ranged from 97.2 to 104.1% (Table 3). The inter-assay precision and accuracy were determined by analyzing three replicates at three concentrations through eight assay runs. The method showed excellent reproducibility with an inter-assay precision ranging from 4.9 to 14.4% R.S.D. The inter-assay accuracy ranged from 99.1 to 109.4% (Table 4).

The stability of compounds in DBS was assessed over a one month period. A series of standards in whole blood were spotted onto collection cards and analyzed on days 1, 7, 14 and 28. A fresh standard curve was prepared on each day to quantify the compound in the stored cards. Three replicates were prepared and analyzed for each concentration on each day and the results are shown in Fig. 3. The data demonstrates that, for these compounds, the storage at room tem-

Table 3
Intra-assay precision and accuracy

Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL)[a]	Accuracy (%)	Precision (%)
50	48.6	97.2	6.40
250	260.3	104.1	3.20
1000	991	99.1	2.6

[a] $n = 7$ for each concentration.

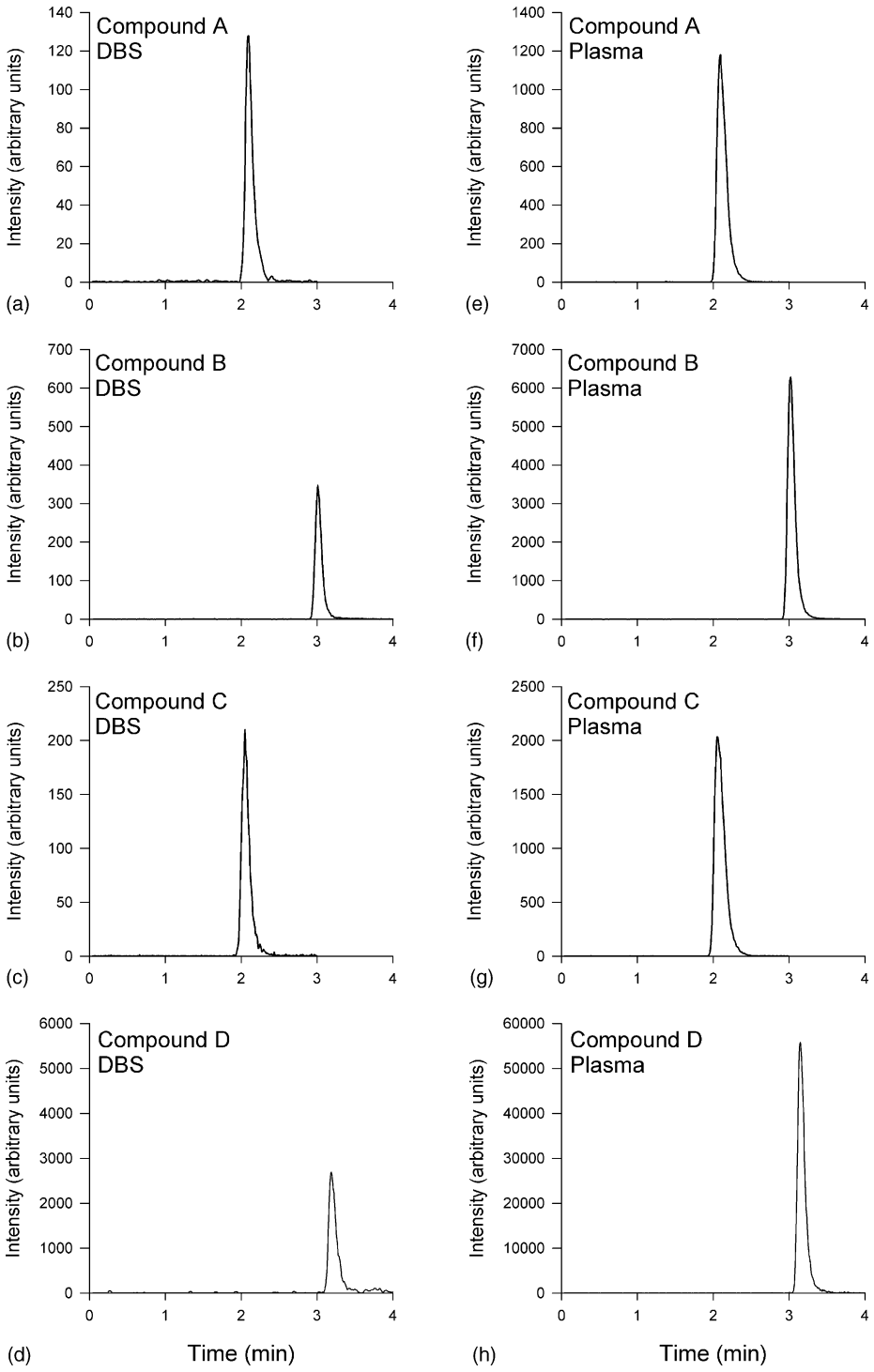

Fig. 1. Total ion chromatograms for each compound at 50 ng/mL analyzed from dried blood spots (a–d) or from plasma extracts (e–h).

perature had no affect on the ability to quantify the samples. This may provide a means for initial collection and analysis, followed by subsequent reanalysis if necessary to look for circulating metabolites.

A relatively large volume (>100 μL) of whole blood is required in order to produce enough plasma for analysis via LC–MS. For smaller mammals (i.e. mice) this volume is large enough such that one animal would need to be sacrificed per time-point. This is unacceptable due to the animal-to-animal variation intrinsic in this type of approach, as well as the practical and ethical considerations associated with the euthanasia of a large number of animals. When valuable gene knockout or other specialty mice strains are to be employed, the routine PK study can become prohibitively costly.

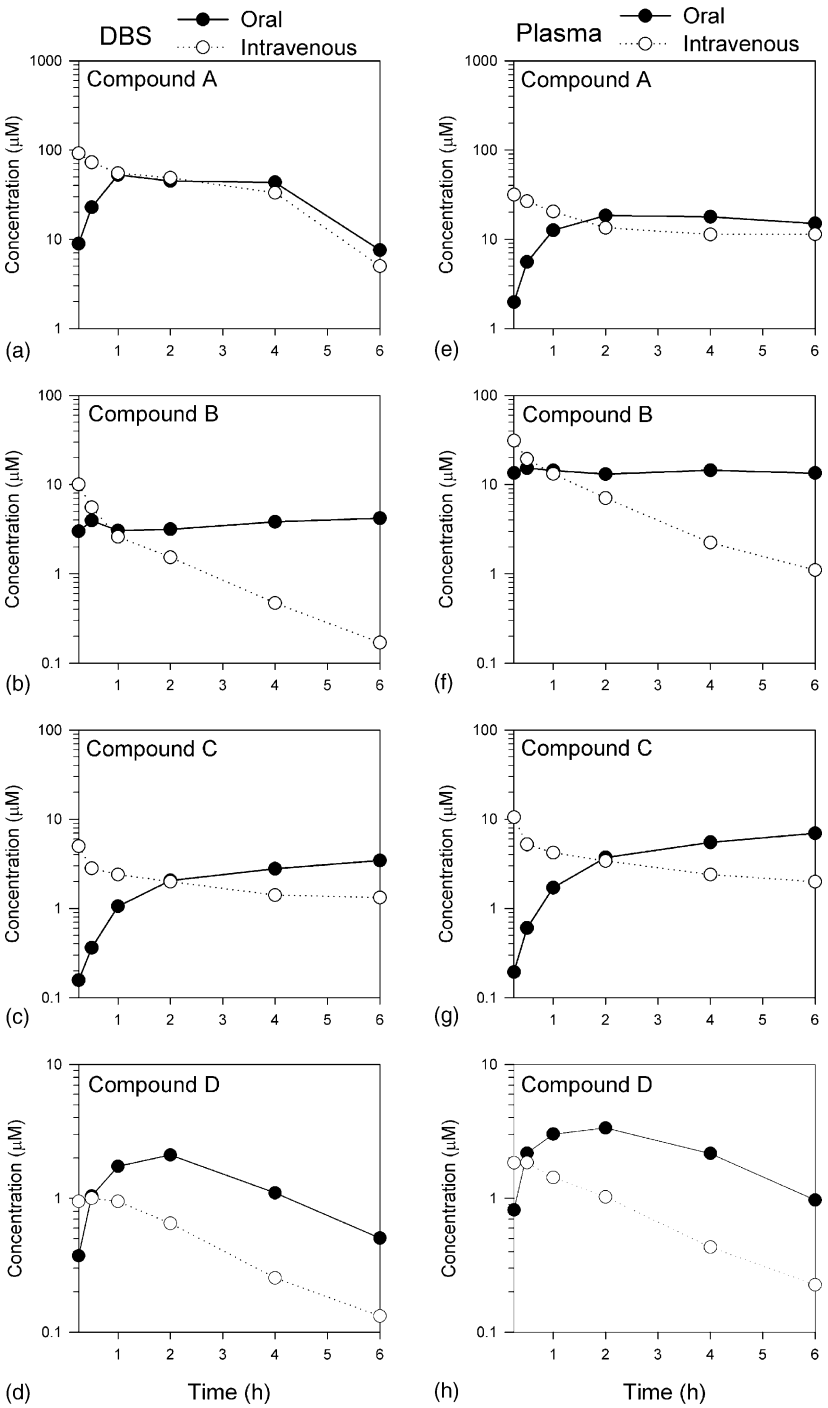

Fig. 2. Concentration vs. time profiles for each of the compounds A, B, C and D dosed orally and intravenously, analyzed from dried blood spots (a–d) or from plasma extracts (e–h).

Recently we reported a method for performing PK analysis on serially bled mice that eliminated the animal-to-animal variation as well as the need for euthanasia [17]. Whole blood (10 μL) collected at each time-point was treated with a fixed volume of anti-coagulant agent, precipitated in acetonitrile containing internal standard and centrifuged prior to analysis. While this is an effective analytical method, it suffers the drawback of being time-consuming, despite the automation of some of the liquid transfer steps. HPLC vials and inserts still must be assembled and labeled, and a technician must perform the task of accurately aliquoting 10 μL of whole blood into each vial. The DBS method offers an attractive alternative to this approach, making the sample preparation less labor-intensive while still allowing small volumes (∼10 μL) to be serially sampled from a small mammal such as a mouse. A comparison of historical data from

Table 4
Inter-assay precision and accuracy

Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL)[a]	Accuracy (%)	Precision (%)
50	51.4	102.8	14.4
250	273.6	109.4	6.6
1000	991.3	99.13	4.9

[a] $n = 8$ days with three replicates per day.

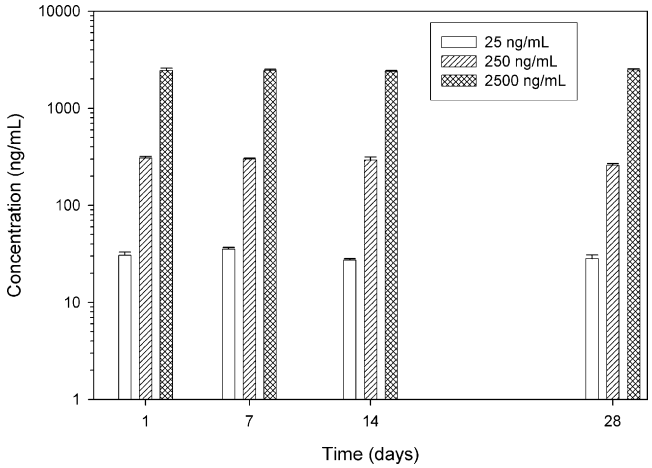

Fig. 3. Stability of compound D at 25, 250 and 2500 ng/mL for 1, 7, 14 and 28 days at room temperature in dried blood spots.

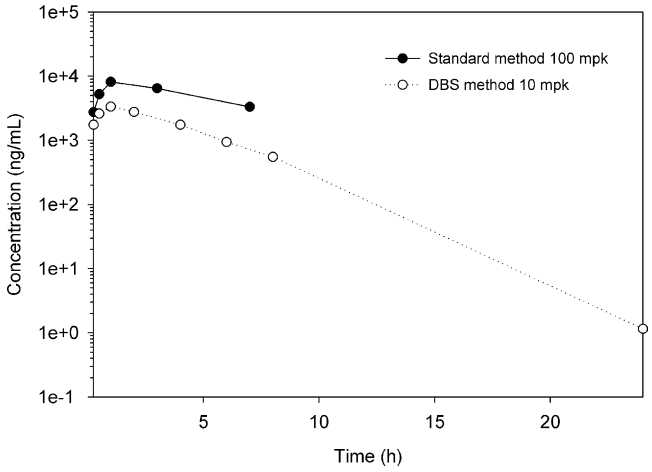

Fig. 4. Comparison of the concentration vs. time profile for compound D in serially bled mice using a published method and the method described in this work.

our previous method for mouse PK with the use of DBS is shown in Fig. 4. Further investigation of this method for mouse PK is ongoing.

4. Conclusions

We have developed a method for discovery stage pharmacokinetics using whole blood dried onto filter paper cards. The collection of samples from the dosed animal is easier, and sample preparation prior to analysis is less time-consuming and requires fewer resources, as it involves the simple extraction of compound from a disc punched from a dried blood spot into LC mobile phase solvent. The method is both precise and accurate for a variety of compounds from different structural classes, with acceptable inter and intra-assay variability. The compounds tested are stable for up to one month at room temperature. This technique is a viable alternative to sample collection procedures currently in place for PK analysis from both large and small laboratory mammals. It should be noted that in the absence of red blood cell partitioning information the method is most useful for ranking compounds based on $t_{1/2}$ and bioavailability. The dried blood spot technique described gives results comparable to the standard methods, but with significant savings in time and resources.

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