Evaluation and Optimization of Blood Micro-Sampling Methods: Serial Sampling in a Cross-Over Design from an Individual Mouse

Nita J Patel[1], Enaksha Wickremsinhe[1], Yu-hua Hui[1], Alexander Barr[2], Nicholas Masterson[2], Kenneth Ruterbories [1], Jennifer Weller[3], Jennifer Hanes[2], Tom Kern[2], Everett Perkins[1]

[1] Drug Disposition Eli Lilly and Company, Indianapolis, USA. [2] Covance Laboratories, Greenfield, USA. [3] Project Management Eli Lilly and Company, Indianapolis, USA

Received, August 29, 2016; Revised, October 6, 2016; Accepted, November 3, 2016; Published, November 29, 2016.

ABSTRACT - Purpose: Current practices applied to mouse pharmacokinetic (PK) studies often use large numbers of animals with sporadic or composite sampling that inadequately describe PK profiles. The purpose of this work was to evaluate and optimize blood microsampling techniques coupled with dried blood spot (DBS) and LC-MS/MS analysis to generate reliable PK data in mice. In addition, the feasibility of cross-over designs was assessed and recommendations are presented. **Methods**: The work describes a comprehensive evaluation of five blood microsampling techniques (tail clip, tail vein with needle hub, submandibular, retro-orbital, and saphenous bleeding) in CD-1 mice. The feasibility of blood sampling was evaluated based on animal observations, ease of bleeding, and ability to collect serial samples. Methotrexate, gemfibrozil and glipizide were used as test compounds and were dosed either orally or intravenously, followed by DBS collection and LC-MS/MS analysis to compare PK with various bleeding methods. **Results**: Submandibular and retro-orbital methods that required non-serial blood collections did not allow for inter-animal variability assessments and resulted in poorly described absorption and distribution kinetics. The submandibular and tail vein with needle-hub methods were the least favorable from a technical feasibility perspective. Serial bleeding was possible with cannulated animals or saphenous bleeding in non-cannulated animals. **Conclusions:** Of the methods that allowed serial sampling, the saphenous method when executed as described in this report, was most practical, reproducible and provided for assessment of inter-animal variability. It enabled the collection of complete exposure profiles from a single mouse and the conduct of an intravenous/oral cross-over study design. This methodology can be used routinely, it promotes the 3Rs principles by achieving reductions in the number of animals used, decreased restraints and animal stress, and improved the quality of data obtained in mouse PK studies.

INTRODUCTION

The conduct of rodent PK and TK studies designed to understand the circulating concentrations of the drug moiety (and metabolites) are fundamental for drug discovery and development (1). Conventional methods require periodic collection of blood samples from the test animal, which are usually immediately centrifuged to separate the red blood cells in order to yield sufficient quantities of plasma samples to enable the quantification of the analyte(s) of interest. Overall, the sampling volumes and the collection techniques have undergone very little change over the past few decades. The volume of blood collected at each sampling time-point has been dictated by the volume of sample needed for the analysis, typically about 150 to 200 µL of blood from a mouse, which limits the number of sampling time-points to two or three bleeds per mouse (one or two surviving bleeds followed by a terminal bleed) (2-5). Therefore, generating a complete PK or TK profile in a single mouse with conventional plasma collection methods is not possible due to the limitations in blood volumes that can be collected without impacting the animal's health and investigators resort to the generation of composite PK/TK profiles with non-serial bleeds (6). Recently, significant advancements have been made with regard to the bioanalytical techniques and associated instrumentation (predominantly LC- MS/MS) that has enabled the

Corresponding Author: Nita J. Patel, PhD, Drug Disposition, DC0710, Lilly Research Laboratories, Indianapolis, USA; E-mail: patel_nita@lilly.com

use of smaller volumes for analysis (7). In addition, the adoption of various bleeding techniques to enable micro-sampling of blood or plasma (generally defined as the collection of blood volumes of less than 50 µL) has enabled the collection of multiple PK time points and the generation of a complete PK profile from a single mouse. These techniques include micro-sampling of plasma either as liquid plasma (8-10), or as dried plasma spots (11), and micro-sampling of blood either as liquid blood (12-15), or as dried blood spots (DBS) (2-4, 16-18). Overall, micro-sampling also benefits the adoption and compliance with the 3Rs principles (Reduction, Refinement, and Replacement in animal usage) (19).

Dried blood spot (DBS) sampling has recently gained broader implementation, likely due to a combination of its simplicity in sampling, shipping, and storage, as well as the overall cost savings (20, 21). One of the challenges in employing DBS analysis is related to the sensitivity of the LC-MS/MS system, since the actual volume of blood used for sample extraction and analysis is equivalent to approximately 2 to 4 µL of blood (from a 3-mm diameter DBS punch), in contrast to 50 to 100 µL of plasma used for a standard analysis. Another bioanalytical challenge includes the bias caused as a result of extremes of hematocrit. However, the effect/impact of hematocrit on assay bias is minimized in nonclinical studies using purpose-bred animals, for which inter-animal variability is expected to be low relative to clinical populations (2, 22-24), but may be important when conducting studies in diseased animals where blood counts could be altered. Finally, in rare instances the stability of the analyte(s) in blood may interfere with the assay results, in which case the stability of suspect structures would need to be evaluated up front.

Multiple blood sampling techniques have been evaluated and suggested for sampling from small laboratory animals such as rats and mice (25, 26). Blood can be sampled from multiple sites using various methods and sampling sites that target both arteries and veins. The most commonly used techniques in rats include tail vein, jugular vein, saphenous vein, retro-orbital, and cardiac puncture (26). In mice, tail clip, tail vein with a needle hub, saphenous vein, retro-orbital, and cardiac puncture are the most widely used (26, 27). The practical aspects and ease of execution of these techniques vary, since some require the use of anesthesia and additional training and personnel. Vascular cannulation in rodents is also an option however; this requires surgery, additional care, longer timelines, and increased cost.

Although multiple sampling techniques have been established for sampling in mice (25, 27-29); a comprehensive evaluation of the respective PK profiles with cross-over designs using DBS methods has not been reported. Tail vein sampling (tail clip and tail vein with needle hub) seems to be the most commonly used micro-sampling technique in mice (2, 12, 30, 31) while saphenous bleeds and retro-orbital bleeds have also been used (3, 4, 12). The use of tail clips and tail vein with a hub needle should be avoided to reduce contamination of samples with administered dose, especially in small rodents such as mice when drug/test compounds are administered IV via the tail vein. Although saphenous vein puncture bleeding has been demonstrated to be a useful and practical method for repeated sampling from mice and other rodents (25, 26, 29) its implementation has been very limited as it may require further optimization to enable the conduct of cross-over studies (29).

This work combines an optimized microsampling technique with dried blood spot (DBS) collection and LC-MS/MS analysis as a most efficient and practical way of performing mouse PK studies in drug discovery and development.

METHODS

Animals: Male CD1 mice were purchased from Harlan Laboratories (Indianapolis, IN) and were housed in groups of four per cage in standard open-topped polycarbonate shoebox-style cages with corncob bedding. The animal rooms were maintained on a 12:12hr light:dark cycle at a room temperature that ranged from 68 to 79 °F with a relative humidity between 30% and 70%. Enrichment was provided in the form of small cotton pads, which the mice treat as nesting material and tear apart to sleep on. Cages were changed every 7 days. A commercial diet (Harlan Teklad 2010, Harlan Labs, Indianapolis, IN) was provided and animal weights ranged from 18-35 g at study start. All procedures were in compliance with the U.S. Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1, 2, and 3); the Guide for the Care and Use of Laboratory Animals: Eighth Edition, (Institute for Laboratory Animal Research, The National Academies Press, Washington, D.C.); and the National Institutes of Health, Office of Laboratory Animal Welfare. Whenever possible,

procedures in this study were designed to avoid or minimize discomfort, distress, and pain to animals.

Drug material and administration: Methotrexate and gemfibrozil were purchased from Sigma-Aldrich (St. Louis, MO) and glipizide was obtained from Research Biochemical Inc. (Natick, MA). The compounds were administered at 10 mg/kg via oral dose (PO) and at 1mg/kg intravenous (IV) dose and the serial collection time-points were as follows; for IV dosing, 0.08, 0.25, 0.75, 2, 4, 8 and 24 hours, and for PO dosing, 0.25, 0.5, 1, 2, 4, 8 and 24 hours, (N=4/dose). For non-serial collections, 8 mice were dosed IV and a separate group of 8 mice were dosed PO with a distribution of 4 mice per time point. Animals requiring non-serial collections were bled at alternate timepoints to allow for recovery and terminal samples were obtained by cardiac puncture (3-4 timepoints/animal). The dose formulations for both PO and IV groups were 10% hydroxypropyl-beta-cyclodextrin in purified water and doses were administered at 10 mL/kg, PO and 1 mL/kg, IV.

Blood sampling: Five bleeding methods were compared: (1) tail clip and (2) tail vein with needle hub, using cannulated mice, (3) submandibular, (4) retro-orbital, and (5) saphenous bleeding, and the latter three using non-cannulated mice approved by the Animal Care and Use Committee at Covance Laboratories, Greenfield, IN. Blood samples were obtained at specified time points, where approximately 20 µL of blood was filled to capacity into EDTA-coated capillary tubes (Fisher Scientific, Pittsburgh, PA) and spotted on to Whatman® DMPK-C DBS collection cards obtained from GE Healthcare Bio-Sciences (Piscataway, NJ). Once spotted, the cards were allowed to dry for 2 hours, placed in Ziploc® bags (S.C. Johnson & Son, Inc.) and shipped to the bioanalytical facility at ambient temperature.

Tail clip and tail vein with needle hub sampling was performed using cannulated mice where IV dosing was performed through the cannula and serial sampling was performed from the tail in a cross-over design with a two day washout. Each mouse was placed in a whole body restrainer and its tail was swabbed with alcohol and a warm moist pad. A sharp surgical blade was then used to cut 1 mm from the tip of the tail and blood flow was initiated by gentle milking of the tail. Following the initial blood-collection procedure, direct pressure was applied to the incision site with a clean cotton swab for about 10 seconds to stop the bleeding and allow for the blood to clot. At subsequent collection time points, the clot was removed and the tail was milked again from the initial incision site. A fresh cut was needed for the 24-hour collection.

In the case of the tail vein with needle hub method, each mouse was placed in a whole body restrainer and its tail was swabbed with alcohol followed by a warm water moistened gauze pad. A needle with a Luer lock hub was inserted into the distal tail artery or vein and blood was allowed to collect in the hub. A capillary tube inserted into a bulb pipette was then used to collect blood from the hub for spotting onto DBS cards. Bruising and/or needle stick marks were noted for some animals that indicated damage to tail vessels and added to the difficultly of later collections.

Submandibular bleeding was conducted in conscious mice. The mice were placed into a cotton glove for restraint. The body and head were restrained to present the mandibular region and visualize the submandibular vein. Using a blood collection lancet, the submandibular vein was opened to produce a drop of blood. Blood was then collected using a capillary tube inserted into bulb pipette. Bruising and/or prior lancet marks complicated later collections.

Studies with retro-orbital bleeding, conducted in a parallel design with intravenous and oral dose groups, were designed to derive composite plasma concentration versus time profiles with four animals per time-point. Isoflurane was used to anesthetize animals prior to each blood-collection time point. A glass capillary tube was inserted into the retro-orbital sinus venous plexus and blood was allowed to flow via capillary action into a collection tube. To ensure that less than 10% of blood volume was removed, retro-orbital blood collections were performed no more than twice from each eye of a single animal. A terminal blood sample was collected from animals under deep anesthesia via cardiac puncture.

A previously published saphenous bleeding technique (25) was used initially for non-serial blood collections, followed by an additional modification (29) which was further optimized for adoption to serial collections. Application of petroleum jelly (Vaseline®, Conopco, Inc.) following the shaving of the bleeding site was an added step that helped visualize the vein and facilitated serial micro-sampling. Additionally, when the vessel was punctured with a needle, the blood welled up and formed a drop instead of dissipating across the surface of the leg (**Figure 1**). After blood collection with a micro-capillary tube, the restraint was

released and the puncture site was compressed for approximately 10 seconds with light pressure. Using the same procedure, subsequent blood collections from the same leg were executed with ease. The feasibility of serial collections with cross-over designs was evaluated.

The tail clip, tail vein with needle hub and the saphenous method allowed for individual animal and variability assessments with the resulting PK parameter calculations. Sampling using submandibular, saphenous, and retro-orbital methods was limited to non-serial blood collections with IV and PO arms run in parallel, basically due to the limitations in the number of blood samples that could be obtained from each of these sampling options. Observations regarding feasibility of the different techniques were noted during each study with respect to both ease of collection/timing and limitations due to animal comfort and wellbeing.

Test analytes and bioanalyses: Methotrexate, glipizide and gemfibrozil were selected as the test materials for this evaluation since they had been previously used to evaluate blood sampling techniques and have had well-behaved PK and bioanalytical properties (32). Bioanalysis was performed using a fit-for-purpose/discovery methodology where a large standard curve range (1-10000 ng/mL) was employed to ensure that the standard curve points bracketed all the concentrations in the sample without the need for reanalysis with dilutions. This approach enabled the generation of PK concentration data for each sample/time-point collected in a single DBS containing approximately 20 µL of blood.

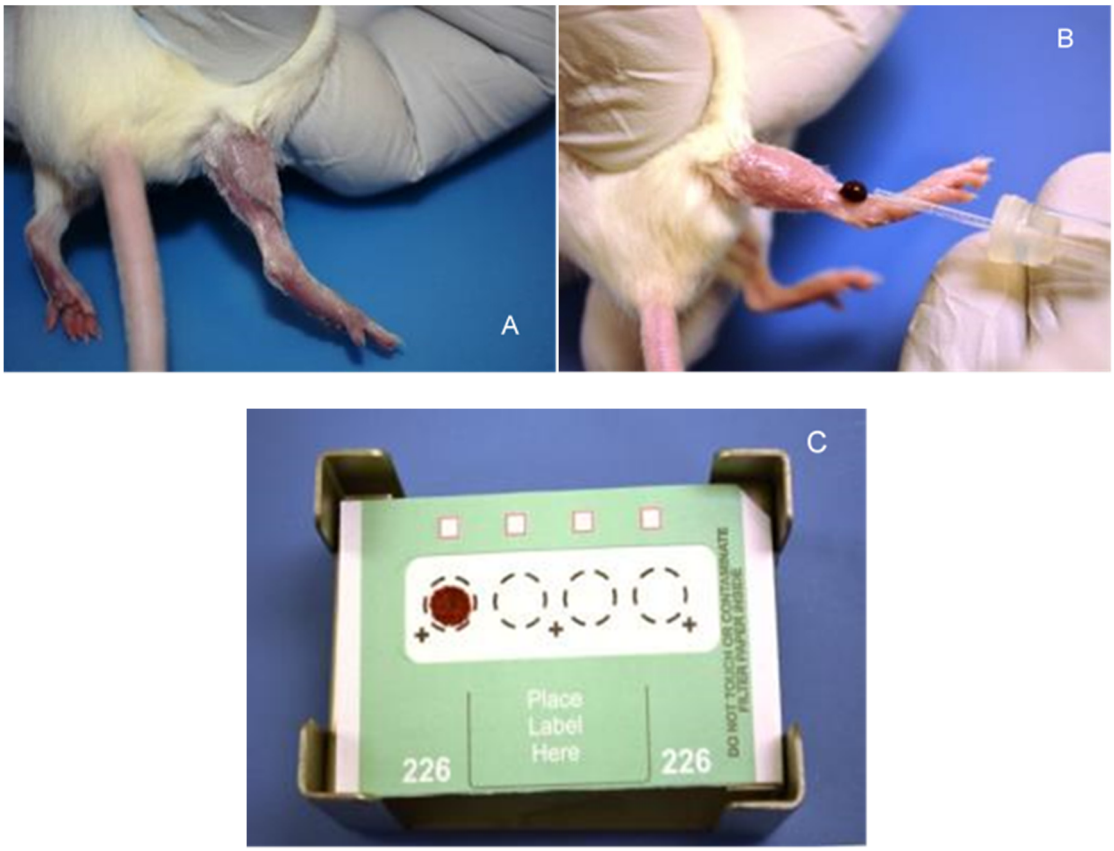


Figure 1. Optimization of the saphenous micro-bleeding technique: (A) Visualization of the vein and application of petroleum jelly; (B) use of capillary tube to collect drop of blood produced by needle puncture; (C) placement of blood (20 µL) onto DBS cards.

Sample extraction: Punches (3 mm) from DBS cards that represented samples, standards, or QCs were extracted with 180 µL of a 20 ng/mL analog IS (proprietary Lilly compound, D6-gemfibrozil, or D11-glipizide) in methanol/acetonitrile (1:1, v/v) solution in 96-well plates. Extraction was conducted overnight in a refrigerator and supernatants were analyzed for each compound as described below.

Analysis of methotrexate: A volume of each sample supernatant was transferred to a new 96-well plate and diluted 2-fold with water then analyzed by LC-MS/MS using a Betasil C18 (Thermo Scientific, Waltham, MA) 20 mm x 2.1 mm 5-µm Javelin column and a gradient mobile phase system with A consisting of 1000:4:1 water/trifluoracetic acid/1M ammonium bicarbonate and B consisting of 1000:4:1 acetonitrile/trifluoroacetic acid/1M ammonium bicarbonate delivered at 1.5 mL/min.

Analysis of gemfibrozil and glipizide: A volume of each sample's supernatant was transferred to a new 96-well plate and diluted 2-fold with water/methanol (1:1, v/v) then analyzed by LC-MS/MS using a Betasil® C18 (Thermo Scientific, Waltham, MA) 20 mm x 2.1 mm 5 µm Javelin column. For gemfibrozil, a gradient mobile phase system with A consisting of 1000:1 water/formic acid and B consisting of methanol delivered at 1.5 mL/min was used. For glipizide, a gradient mobile phase system was applied with A consisting of 1000:5 water/1M ammonium bicarbonate and B consisting of 1000:5 methanol/1M ammonium bicarbonate delivered at 1.5 mL/min.

HPLC and LCMS conditions: The HPLC column was held at ambient temperature, and a flow rate of 1.5 mL/min was used with an injection volume of 10 µL. Mass spectrometric data were generated by using an AB Sciex® API 4000 triple-quadrupole mass spectrometer (Applied BioSystems, Foster City, CA) and acquired using Analyst software, version 1.4 (Applied BioSystems, Foster City, CA). Selected Reaction Monitoring (SRM) transitions (positive ion mode) with precursor and product ions for the analyte and internal standard were acquired with an AB Sciex® API 4000 tuned to achieve unit resolution (0.7 DA at 50% FWHM) using Analyst software (version 1.4.2). Acquisitions for methotrexate and glipizide were performed in positive-ion mode and gemfibrozil data were acquired in negative ion mode using a TurboIonSpray® (Applied BioSystems, Foster City, CA) source set at approximately 700 °C and an ion spray voltage of approximately 1500 V (positive ion) and -3000 V (negative ion).

Pharmacokinetic analysis: PK parameters were calculated for concentration time profiles with non-compartmental analysis using Watson LIMS (version 7.4) from Thermo Scientific (Billerica, MA). For non-serial sampling, concentration data were represented as mean values and the PK parameters were calculated using composite curves and with serial collections, individual animal data were used for PK analysis. Differences in PK parameters that were less than 2-fold were considered to be within experimental variability based on in-house experience.

RESULTS

Technical feasibility of various micro-sampling methods: Animal observations during the conduct of PK studies using various micro-sampling methods are summarized in **Table 1**.

The tail vein with needle hub collection method using cannulated animals for IV dosing allowed for relatively smooth blood collections at all time-points, but the tail seemed to have quite a few puncture marks and made bleeding more difficult due to reduced blood flow as each time-point progressed in the cross-over arm of the study. Additionally, bleeding was complicated by the fact that animals needed approximately two minutes of warm-up time to enhance tail blood flow, as well as by the fact that some animals did not bleed well and resulted in delayed collection times. The use of a heat lamp for pre-warming the animals was attempted, but was found to be ineffective since the cannulated mice needed to be individually housed. Overall, a maximum of approximately ten time-points could be collected from the tail bleeding method in a cross-over design.

Bleeding from the submandibular vein was evaluated in non-cannulated conscious mice. The submandibular region appeared swollen with some sites exhibiting slight hematomas. Additionally, decreased activity that lasted more than one minute was observed. Overall, the appearance of the animals was rough and disheveled. These observations limited the number of blood collections and did not allow for serial collections or application of a cross-over design.

Table 1: Comparison of standard blood sampling techniques employed in mice to obtain blood or plasma samples for computing pharmacokinetic profiles**.**

Micro-bleeding method	Collections per mouse	# animals per two arm study	Crossover (Y/N)	Anesthesia (Y/N)	Cannulation	Observations
Sub-mandibular	4 plus terminal	12-18	N	N	N	Most technically challenging Animal distress due to swollen submandibular region Multiple collections led to decreased activity Difficult to obtain multiple samples from same site
Tail vein w/ needle hub	4-8	12-18	N	N	Y	Tissue trauma in tail. Bleeding time errors; Bleeding difficult with progression of collections Long warm-up times (~2min) for adequate blood flow Use of heat lamp inconvenient with individual housing
Retro-orbital	4 plus terminal	12-18	N	Y	N	Commonly used for composite curves, not serial bleeding
Tail clip	8-10	3-4	Y	N	Y	Some animals lose patency during study
Saphenous	6-8	3-4	Y	N	N	Petroleum jelly helps vein stand out; blood wells up instead of dissipating across skin surface Multiple blood collections from the same leg possible Minimal to no bruising and vein continues to be visible. Animals bled very quickly and efficiently. Slight bruising after 6 bleeds; recovery between arms ≥ 3 days Animals appear to be bright and alert at all times.

Table 2. Comparison of mean (±SD) pharmacokinetics generated using different blood sampling methods, following the administration of a single intravenous (IV) and oral dose (PO) dose of methotrexate to male mice (n=4/timepoint).

Design Parameter		Non Cannulated/Non-cross-over[a]						Cannulated/Cross-over			
		Sub-mandibular		Initial Saphenous		Retro-Orbital		Tail Clip		Tail vein with Hub Needle	
Route	Units	IV	PO	IV	PO	IV	PO	IV	PO	IV	PO
Dose	mg/kg	1	10	1	10	1	10	1	10	1	10
$AUC_{0\text{-}24h}$	µg*Hours/mL	0.9	1.5	1.0	1.1	0.7	1.3	1.5±0.4	1.9±0.4	1.2±0.3	1.3±0.4
C_{max} or C_0	µg/ml	0.6	0.3	0.8	0.3	0.5	0.3	0.7±0.3	0.3±0.1	0.6±0.2	0.2±0.1
$T_{1/2}$	Hours	12.7	6.8	8.3	8.8	10.4	6.8	14.2±5.3	13.7±0.9	10.2±0.8	13.9±7.7
CL_p	mL/min/kg	14.5		15.0		19.7		9.2±2.6		13.1±3.7	
V_{dss}	mL/kg	10700		6900		10300		8300±3200		7400±3100	
% F	Dose * AUC	15.6		10.9		17.6		13.0 ± 2.2		12.8 ± 8.5	

[a] Submandibular, initial saphenous and retro-orbital bleeds were composite bleeds, and therefore did not allow SD calculations.

Retro-orbital and tail-clip methods were relatively straightforward with no atypical live-phase observations. However, the retro-orbital method required anesthesia and with a limitation of less than four time-point collections per animal, this method could only be used for non-serial blood collections in a non-cross-over design. The tail clip method allowed for a cross-over design without the use of anesthesia, but required the use of cannulated animals for IV dosing.

A previously published saphenous bleeding technique was initially used (25) and then compared to a method published by Peng et.al (29) and optimized for application in mouse PK studies. With saphenous bleeding, bruising and puncture marks were absent in nearly all of the animals and the vein continued to be visible. Where bruising was present, it was very minimal compared to previous attempts where petroleum jelly was not applied. Overall, all animals bled very quickly, reproducibly, and efficiently via the saphenous vein. Using the modified procedure allowed for four additional collections amounting to a total of eight collections (four/leg) within a standard 24-hour PK collection period. While slight bruising was observed when sampling beyond a 24-hour time-point, the veins were still visible enough to support these additional bleeding points. All animals that were bled using the modified saphenous bleeding method appeared to be bright and alert at all times, did not show any signs of lack of movement or distress while walking around in their cages, and did not appear to be in pain when handled.

Overall, the tail vein with needle hub and submandibular methods were the most challenging techniques due to apparent animal discomfort and difficulty in obtaining multiple samples from the same site. With respect to feasibility and generation of PK data, the tail clip and saphenous methods were most preferable of all the methods examined, particularly as they required only three to four animals for a two-arm, cross-over study and were consistent with the 3Rs principles, whereas the other methods utilized up to 18 animals. The tail clip method with serial sampling provided the possibility of a cross-over design and was considered preferable in all aspects including assessment of inter-animal variability; however, this method required the use of cannulated animals for IV dosing, which was costly and reduced study turnaround time. In contrast, the saphenous bleeding method facilitated serial bleeding in a cross-over design, the ability to assess inter-animal variability, and was more advantageous and cost-effective since it used non-cannulated mice. Additionally, the saphenous method as described in this work did not result in hematomas or bruising if at least a three-day recovery period was allowed between cross-over arms or blood sampling periods.

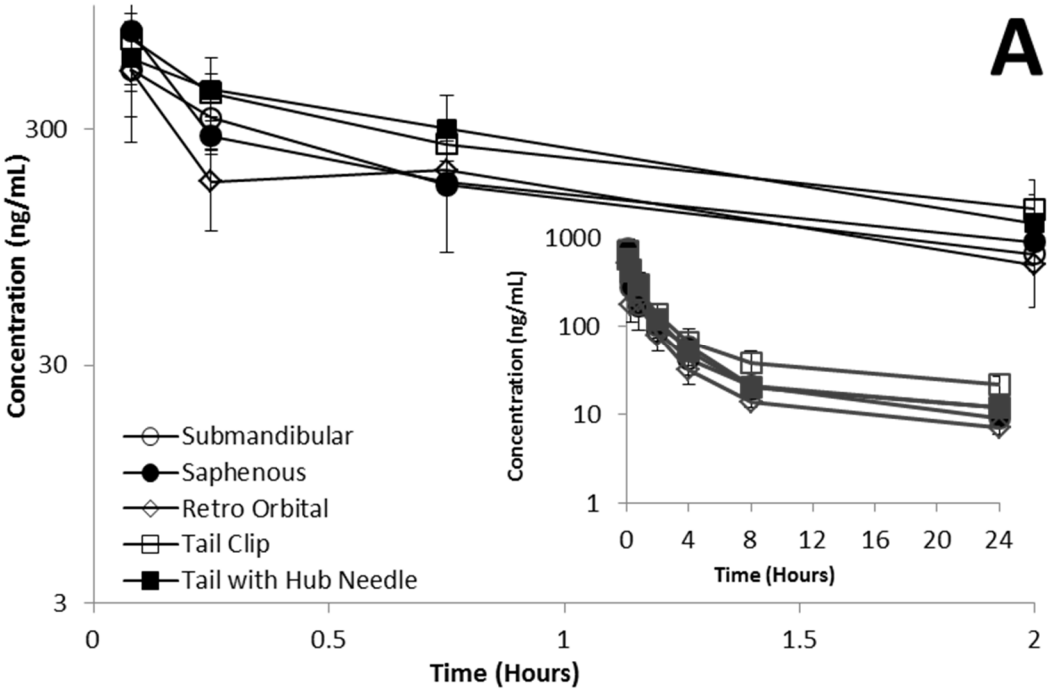
Pharmacokinetics with various micro-sampling methods: A key advantage of serial collections is the ability to assess inter-animal variability, which the non-serial collection does not provide. Since sampling via submandibular, initial saphenous, and retro-orbital methods, were limited to non-serial blood collections with PO and IV arms run in parallel, a statistical comparison of PK parameters could not be performed as they lacked individual animal concentration-time profiles (**Table 2**).

The inter-animal variability of bioavailability using tail vein with needle hub method (%CV = 66) was much higher than that using tail-clip method (%CV = 17). However, the bioavailability of methotrexate was comparable between all methods (within 2-fold) and ranged from 11 to 18% (**Table 2**). In examining the initial distribution phase within 2 hours after IV administration it was observed that retro-orbital bleeding led to an erratic distribution profile, while other methods were consistent with a typical rapid decline that allowed for a better definition of distribution kinetics (**Figure 2A**). This may be due to the non-serial collection method that may cause variability in the timing of collections during the initial distribution phase and/or the possibility of blood-flow differences resulting from various degrees of anesthesia. The PO arm using submandibular bleeding resulted in an atypical flat absorption profile compared to the other methods (**Figure 2B**). Using the initial saphenous method, most PK parameters for methotrexate were comparable between serial and non-serial collections, except that the volume of distribution with serial bleeding was 3.3 to 8.6-fold higher than the mean values obtained with non-serial bleeding (**Table 3**). The importance of accurately describing distribution and elimination kinetics with IV dosing and absorption kinetics with PO dosing is realized when these data are used for modeling and predictions that require a precise estimation of these parameters. Therefore, non-serial sampling as utilized in submandibular, initial saphenous or retro-orbital bleeding techniques would be less acceptable for PK modeling and prediction calculations since the resulting data did not adequately describe absorption and distribution kinetics essential for modeling and predictions.

The PK of gemfibrozil was evaluated using all the bleeding methods (including saphenous bleeds with serial collections). The exposure of gemfibrozil was higher with retro-orbital bleeding compared to other methods as shown in **Figure 3**. With the retro-orbital method performed in a non-crossover design generating composite animal profiles, the bioavailability was greater than 100% and higher than other methods (which ranged from 53 to 85%). The volume of distribution and clearance values were generally similar between all methods (statistical analysis was not feasible for composite profiles). The inter-animal variability of bioavailability using tail vein with needle hub method (% CV = 22) was similar to that using tail clip method (% CV = 17).

The PK of glipizide was also evaluated using all the bleeding methods including the saphenous bleeding method with serial collections. The PK profiles of glipizide were not influenced to any great extent by the bleeding methods utilized in this study as shown in **Figure 4**. Both the volume of distribution and clearance values were similar across all groups. The IV and PO profiles were similar between the submandibular, saphenous, retro-orbital and tail vein with needle hub methods. The bioavailability was greater than 100% with submandibular, tail clip and tail vein with needle hub, while retro-orbital and saphenous methods resulted in bioavailability values of 71% and 65%, respectively. The inter-animal variability for oral AUC was low with the tail clip (% CV = 17), tail vein with needle hub (% CV = 5), and saphenous (% CV = 9) serial bleeding methods.

Based on the previous evaluations and the preference for methods that afforded serial blood collections, the feasibility of using the saphenous bleeding and tail-clip methods with a seven time-point blood collection scheme and a cross-over design was evaluated for glipizide. The key difference between the two methods was that the saphenous method did not require cannulation, while the tail-clip method required placement of a cannula for IV dosing to avoid contamination of tail-clip collection samples. All seven time-points were successfully collected for both oral and IV arms using both methods.



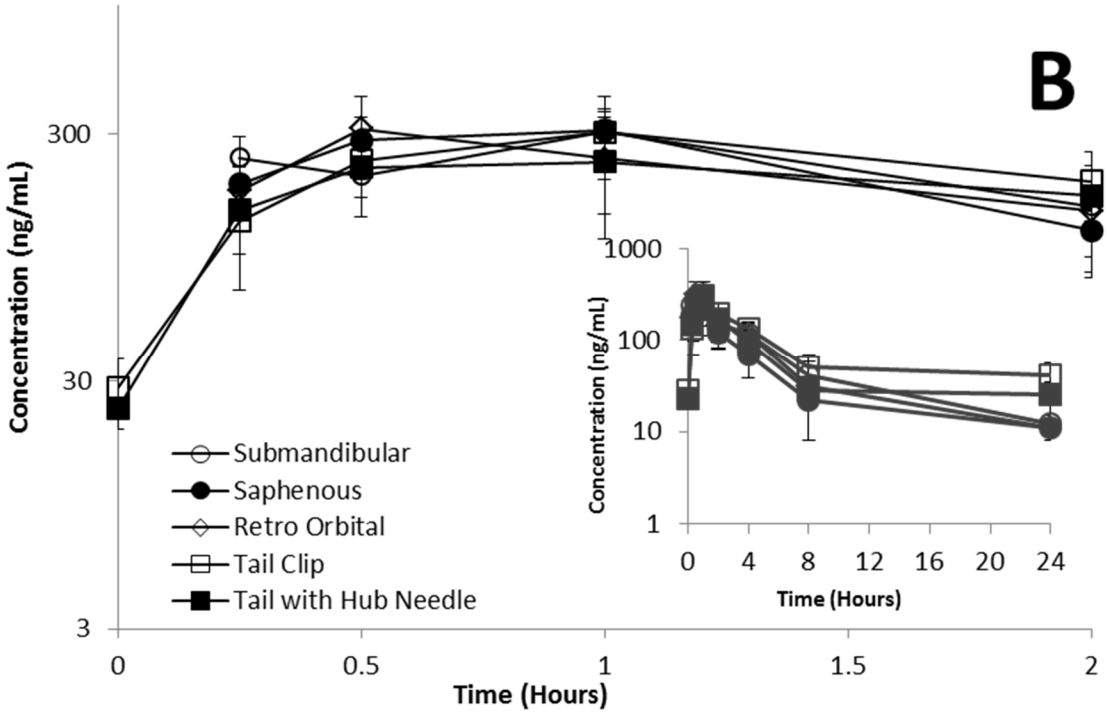
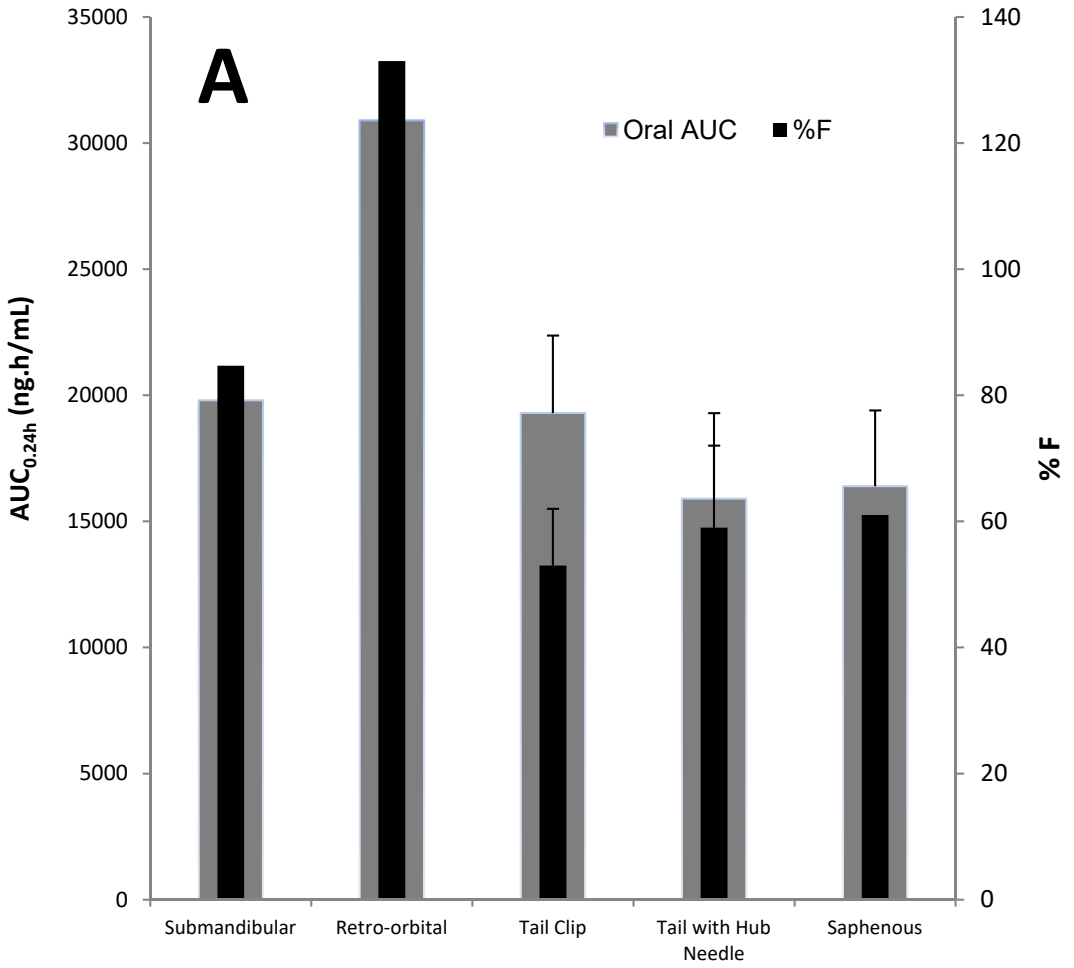


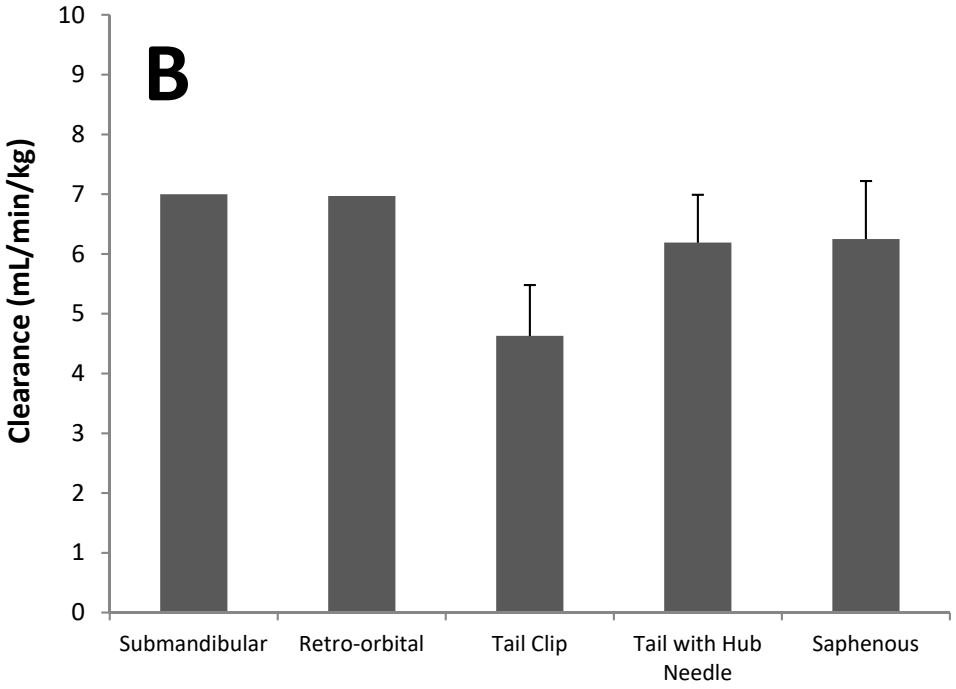
Figure 2. Mean (±SD) blood-versus-time concentration profiles generated following (A) a single 1 mg/kg IV dose or (B) a single 10 mg/kg PO dose administration of methotrexate using various micro-bleeding sampling methods in male mice (n=4/time-point). Saphenous bleeds were using the initial method; therefore, data from saphenous, submandibular, and retro-orbital bleeds were composite bleeds, and did not allow calculations of SD (no error bars).

Table 3. Mean (±SD) blood pharmacokinetic parameters following a single 1 mg/kg IV bolus or a 10 mg/kg oral dose administration of methotrexate with blood collected via the saphenous vein in male mice.

Parameter		**Serial Bleeding / Non cross-over (Modified method)**		**Non-serial Bleeding / Non cross-over (Initial method)**	
Dose	mg/kg	1	10	1	10
Route		IV	PO	IV	PO
$AUC_{0\text{-}24h}$	µg*Hours/mL	0.6 ± 0.1	0.6 ± 0.1	1.0	1.1
AUC_{Extrap}	µg*Hours/mL	1.5 ± 0.6	0.9 ± 0.1	1.1	1.2
C_{max} or C_o	µg/mL	0.5 ± 0.2	0.17 ± 0.05	0.8	0.3
T_{max}	Hours	NA	0.88 ± 0.25	NA	1.00
$T_{1/2}$	Hours	52.0 ± 27.5	17.4 ± 8.8	8.3	8.8
CL_p	mL/min/kg	12.5 ± 4.2	NA	15.0	NA
V_{dss}	mL/kg	39800 ± 15100	NA	6900	NA
% F	Dose * AUC	10		10.9	

NA: Not Applicable


A
Oral AUC
%F
AUC0.24h (ng.h/mL)
% F
35000
30000
25000
20000
15000
10000
5000
0
140
120
100
80
60
40
20
0
Submandibular
Retro-orbital
Tail Clip
Tail with Hub Needle
Saphenous


B
Clearance (mL/min/kg)
10
9
8
7
6
5
4
3
2
1
0
Submandibular
Retro-orbital
Tail Clip
Tail with Hub Needle
Saphenous

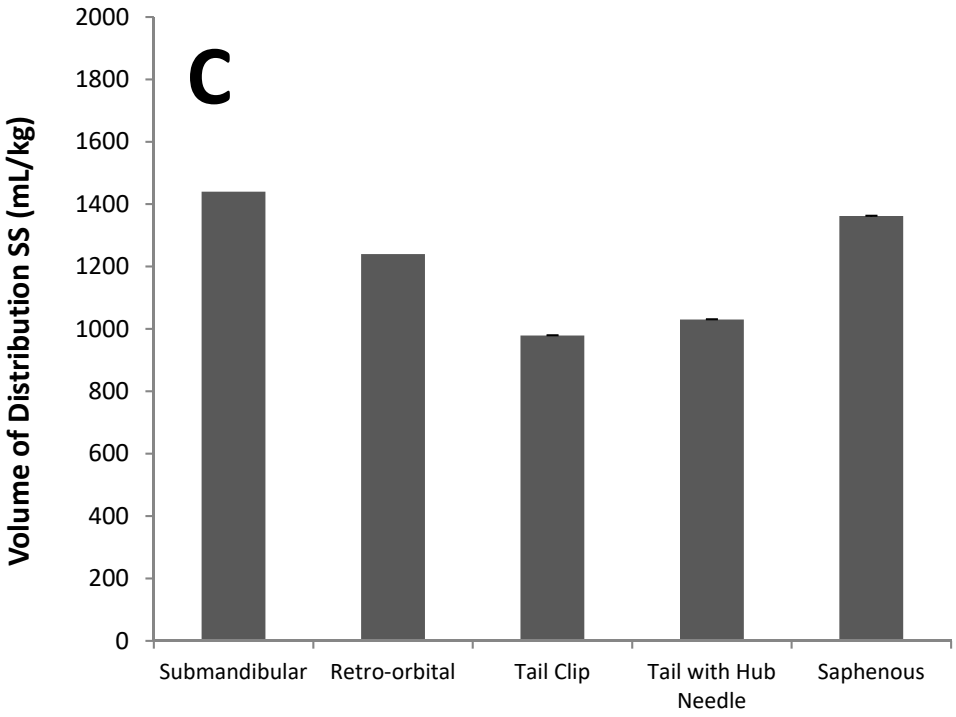


Figure 3: Comparison of pharmacokinetic properties resulting from the use of different micro-bleeding techniques following the administration of a single 10 mg/kg, PO or 1 mg/kg, IV dose of Gemfibrozil to male rats. (A) Exposure and bioavailability, (B) Clearance, (C) Volume of distribution. Submandibular and retro-orbital bleeds were composite bleeds, and therefore did not allow calculation of SD (no error bars).

The PK profiles shown in **Figure 4** and the data presented in **Table 4** showed good agreement between the two methods. This evaluation showed that the PO and IV PK profiles of glipizide were not influenced by either of the two bleeding methods, further confirming that serial sampling maintained the ability to determine variability in secondary PK parameters such as clearance, volume of distribution, and half-life. The inter-animal variability for oral AUC was very low; 5.5% CV for tail clip and 13% CV for the saphenous bleeding method. The overall bioavailability was greater than 100% with the saphenous method, while the tail clip method resulted in a bioavailability of 83%. The reproducibility of the PK parameters generated by the two bleeding methods was subsequently compared over two separate studies. While there were some differences in the half-lives, these were resolved upon careful optimization and execution of the saphenous method in the repeat study (**Figure 4)**. No significant differences were observed between either of the methods or between the two studies.

DISCUSSION

The generation of circulating drug (and metabolite) concentrations in experimental animals and evaluation of PK/TK during drug discovery and development is paramount to establishing activity of analyte(s) against the target (or targeted disease) as well as toxicity and safety. Additionally these data are used to develop *in-silico* models, understand *in vitro-in vivo* extrapolations, and ultimately in human PK predictions.

The intent of our work was to perform within and inter-study comparisons of methotrexate, gemfibrozil, or glipizide mouse PK that may be impacted by various micro-sampling techniques with identical dose levels, dosing methods, route of administration and formulations.

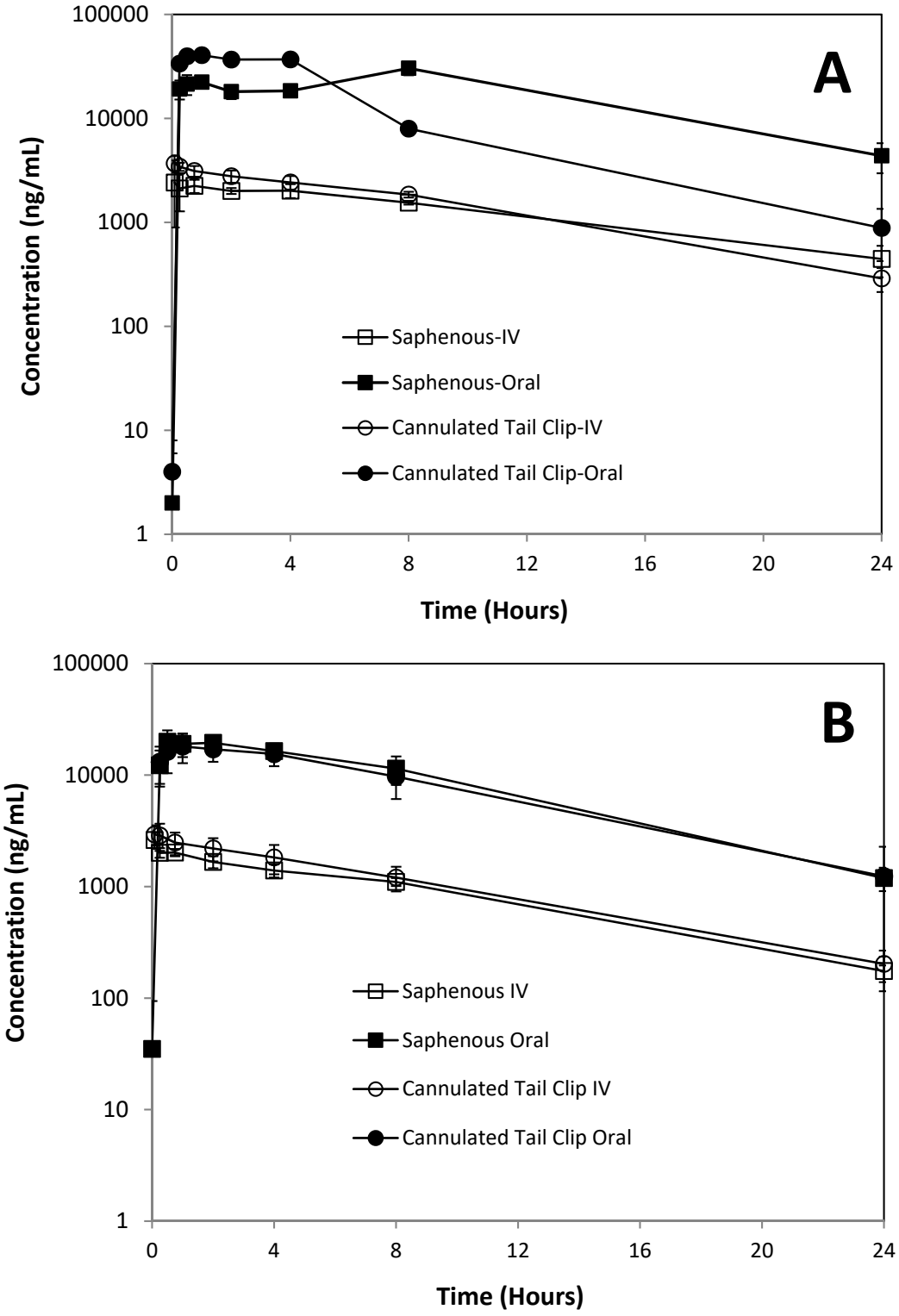


Figure 4. Time-vs-concentration profiles based on the saphenous bleeds (non-cannulated) and cannulated tail-clip method following the administration of a single 10 mg/kg dose of glipizide via oral dose (PO) and a single 1mg/kg dose via intravenous (IV) (N=4/time-point). Graph A shows data from initial study and graph B represents the repeat study for confirmation.

Table 4. Pharmacokinetic parameters based on a cross-over design comparing saphenous and cannulated tail-clip blood micro sampling methods following the administration of a single 10 mg/kg dose of glipizide via oral dose (PO) and a single 1mg/kg dose via intravenous (IV) (N=4/time-point).

Collection/Design	Units	Saphenous (non-cannulated) Serial/Cross-over		Tail Clip (cannulated) Serial/Cross-over	
Dose	mg/kg	1 mg/kg IV	10 mg/kg PO	1 mg/kg IV	10 mg/kg PO
Parameters[a]	**STUDY #1**				
$AUC_{0\text{-}24h}$	µg*Hours/mL	32.9 ± 2.2	450. ± 59	37.1 ± 1.9	307 ± 17
C_{max} (PO) or C_o (IV)	µg/mL	3.6 ± 0.4	30.4 ± 4.3	4.1 ± 0.8	41.5 ± 4.0
$T_{1/2}$	Hours	9.4 ± 1.6	8.0 ± 1.2	6.3 ± 0.6	4.0 ± 0.7
CL_p	mL/min/kg	0.42 ± 0.05	NA	0.42 ± 0.03	NA
V_{dss}	mL/kg	320 ± 20	NA	210 ± 7	NA
AUC Ratio Saph/Tail Clip	ng*Hours/mL	0.89	1.47	NA	NA
Parameters[a]	**STUDY #2**				
$AUC_{0\text{-}24h}$	µg*Hours/mL	22.2 ± 1.8	227 ± 29	26.4 ± 6.3	202 ± 53
C_{max} (PO) or C_o (IV)	µg/mL	3.0 ± 0.6	21.0 ± 4.0	3.3 ± 0.7	18.7 ± 5.2
$T_{1/2}$	Hours	6.5 ± 1.3	5.0 ± 0.3	6.4 ±1.1	5.4 ± 2.3
CL_p	mL/min/kg	0.70 ± 0.06	NA	0.61 ± 0.16	NA
V_{dss}	mL/kg	358 ± 65	NA	307 ± 99	NA
AUC Ratio Saph/Tail Clip	ng*Hours/mL	0.84	1.12	NA	NA

[a]No significant differences were observed between methods or between studies; $T_{1/2}$ differences in Study #1 were resolved upon optimization of the saphenous method in Study #2.

Overall, the comparison of PK for these three compounds with previous reports in the literature were challenging due to significant differences in the designs of reported studies including the species/strain, disease models, completeness of PK profiles, dose and route of administration, and formulations used (33-35). However, where comparisons were possible, the mouse PK for the compounds used in our studies were comparable to the literature. For example, gemfibrozil PK study in mouse reported a bioavailability of 73% which was similar to the 60% that we report in our studies (36) .

Using various micro-sampling techniques with identical dose levels, dosing methods, route of administration and formulations in this report we found that while various bleeding methods did not generally influence PK, considerations with respect to technical feasibility indicated that the submandibular and tail vein with needle hub methods were the least favorable. Methods that require non-serial collections (submandibular and retro-orbital) are not preferred as they tend not to adequately describe distribution and absorption kinetics that are necessary for applications to PK modeling and prediction work. Of the three methods that allowed serial sampling (tail-clip, saphenous and tail vein with a needle hub), the saphenous method was the most practical and appealing as it was reproducible and provided for assessment of variability. Additionally, the saphenous bleeding method did not require the use of cannulated mice (for IV dosing) or anesthesia (during bleeding) and was cost-effective. It was also consistent with the 3Rs principles by achieving significant reductions in the number of animals used, decreased restraints and animal stress, and improved data quality. On the basis of this work, we have successfully established the saphenous bleeding technique as a routine practice for mouse PK studies delivering consistent and reproducible results. This has resulted in a 66% reduction in the overall number of mice used per single arm studies (compared to traditional plasma studies) and 83% reduction when conducting mouse IV/PO crossover PK studies. Using the saphenous micro-sampling technique with DBS analysis as described in this paper is practical and highly recommended for the conduct of mouse PK/TK studies in drug discovery and development.

CONCLUSION

We have presented a comprehensive evaluation of five different micro-sampling techniques used for mouse pharmacokinetic evaluations demonstrating that existing methods have been limited to non-serial sampling that generate less acceptable PK calculations since the resulting data do not adequately describe absorption and distribution kinetics essential for modeling and predictions. Of the methods that allow serial sampling, the saphenous method when executed as described in this report, was most practical and appealing as it was reproducible, provided for assessment of variability and has the advantage of conducting these studies in a cross-over study between oral and intravenous dosing in a single mouse. Finally, this method did not require the use of cannulated mice or anesthesia was most cost-effective and promotes the 3Rs principles by achieving reductions in the number of animals used, decreased restraints and animal stress.

ACKNOWLEDGEMENTS

The authors thank Mr. Jason Manro (Eli Lilly and Co) for performing the appropriate statistical analysis of the PK data, and Ms. Amy Wakefield (Covance Labs) for supporting this collaboration. Bioanalysis was performed at Q^2 Solutions (Indianapolis, IN).

REFERENCES

1. Lesko LJ, Rowland M, Peck CC, Blaschke TF. Optimizing the science of drug development: opportunities for better candidate selection and accelerated evaluation in humans. Pharmaceutical research. 2000;17(11):1335-44. Epub 2001/02/24.
2. Wickremsinhe ER, Perkins EJ. Using dried blood spot sampling to improve data quality and reduce animal use in mouse pharmacokinetic studies. Journal of the American Association for Laboratory Animal Science : JAALAS. 2015;54(2):139-44. Epub 2015/04/04.
3. Clark GT, Haynes JJ, Bayliss MA, Burrows L. Utilization of DBS within drug discovery: development of a serial microsampling pharmacokinetic study in mice. Bioanalysis. 2010;2(8):1477-88. Epub 2010/11/19.
4. Rahavendran SV, Vekich S, Skor H, Batugo M, Nguyen L, Shetty B, et al. Discovery pharmacokinetic studies in mice using serial microsampling, dried blood spots and microbore LC-MS/MS. Bioanalysis. 2012;4(9):1077-95. Epub 2012/05/23.
5. Joyce AP, Wang M, Lawrence-Henderson R, Filliettaz C, Leung SS, Xu X, et al. One mouse, one pharmacokinetic profile: quantitative whole blood serial sampling for biotherapeutics. Pharmaceutical research. 2014;31(7):1823-33. Epub 2014/01/28.
6. Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, et al. A good practice guide to the administration of substances and removal of blood, including routes and volumes. Journal of applied toxicology : JAT. 2001;21(1):15-23. Epub 2001/02/17.
7. Xu RN, Fan L, Rieser MJ, El-Shourbagy TA. Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS. Journal of pharmaceutical and biomedical analysis. 2007;44(2):342-55. Epub 2007/03/16.
8. Jonsson O, Villar RP, Nilsson LB, Eriksson M, Konigsson K. Validation of a bioanalytical method using capillary microsampling of 8 microl plasma samples: application to a toxicokinetic study in mice. Bioanalysis. 2012;4(16):1989-98. Epub 2012/09/06.
9. Nilsson LB, Ahnoff M, Jonsson O. Capillary microsampling in the regulatory environment: validation and use of bioanalytical capillary microsampling methods. Bioanalysis. 2013;5(6):731-8. Epub 2013/03/15.
10. Bowen CL, Licea-Perez H, Karlinsey MZ, Jurusik K, Pierre E, Siple J, et al. A novel approach to capillary plasma microsampling for quantitative bioanalysis. Bioanalysis. 2013;5(9):1131-5. Epub 2013/05/07.
11. Barfield M, Wheller R. Use of dried plasma spots in the determination of pharmacokinetics in clinical studies: validation of a quantitative bioanalytical method. Analytical chemistry. 2011;83(1):118-24. Epub 2010/12/09.
12. Bateman KP, Castonguay G, Xu L, Rowland S, Nicoll-Griffith DA, Kelly N, et al. Reduction of animal usage by serial bleeding of mice for pharmacokinetic studies: application of robotic sample preparation and fast liquid chromatography-mass spectrometry. Journal of chromatography B, Biomedical sciences and applications. 2001;754(1):245-51. Epub 2001/04/25.
13. Jonsson O, Palma Villar R, Nilsson LB, Norsten-Hoog C, Brogren J, Eriksson M, et al. Capillary microsampling of 25 microl blood for the determination of toxicokinetic parameters in regulatory studies in animals. Bioanalysis. 2012;4(6):661-74. Epub 2012/03/29.
14. Dillen L, Loomans T, Van de Perre G, Versweyveld D, Wuyts K, de Zwart L. Blood microsampling using capillaries for drug-exposure determination in early preclinical studies: a beneficial strategy to reduce blood sample volumes. Bioanalysis. 2014;6(3):293-306. Epub 2014/01/30.

15. Liederer BM, Berezhkovskiy LM, Ubhayakar SS, Deng Y. An alternative approach for quantitative bioanalysis using diluted blood to profile oral exposure of small molecule anticancer drugs in mice. Journal of pharmaceutical sciences. 2013;102(2):750-60. Epub 2012/12/12.
16. Beaudette P, Bateman KP. Discovery stage pharmacokinetics using dried blood spots. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2004;809(1):153-8. Epub 2004/07/30.
17. Spooner N, Lad R, Barfield M. Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method. Analytical chemistry. 2009;81(4):1557-63. Epub 2009/01/22.
18. Dainty TC, Richmond ES, Davies I, Blackwell MP. Dried blood spot bioanalysis: an evaluation of techniques and opportunities for reduction and refinement in mouse and juvenile rat toxicokinetic studies. International journal of toxicology. 2012;31(1):4-13. Epub 2012/01/10.
19. Guhad F. Introduction to the 3Rs (Refinement, Reduction and Replacement). Journal of the American Association for Laboratory Animal Science. 2005;44(2):58-9.
20. Demirev PA. Dried blood spots: analysis and applications. Analytical chemistry. 2013;85(2):779-89. Epub 2012/11/23.
21. Henion J, Oliveira RV, Chace DH. Microsample analyses via DBS: challenges and opportunities. Bioanalysis. 2013;5(20):2547-65. Epub 2013/10/22.
22. Viswanathan CT. Perspectives on microsampling: DBS. Bioanalysis. 2012;4(12):1417-9. Epub 2012/07/17.
23. Wickremsinhe E. Dried blood spot analysis for rat and dog studies: validation, hematocrit, toxicokinetics and incurred sample reanalysis. Bioanalysis. 2015;7(7):869-83. Epub 2015/05/02.
24. Pratt SE, Durland-Busbice S, Shepard RL, Donoho GP, Starling JJ, Wickremsinhe ER, et al. Efficacy of low-dose oral metronomic dosing of the prodrug of gemcitabine, LY2334737, in human tumor xenografts. Molecular cancer therapeutics. 2013;12(4):481-90. Epub 2013/02/02.
25. Hem A, Smith AJ, Solberg P. Saphenous vein puncture for blood sampling of the mouse, rat, hamster, gerbil, guinea pig, ferret and mink. Laboratory animals. 1998;32(4):364-8. Epub 1998/11/10.
26. Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. Journal of pharmacology & pharmacotherapeutics. 2010;1(2):87-93. Epub 2011/02/26.
27. Bazare J, Jr., Leamons ML, Young JF. Sampling methods for pharmacokinetic studies in the mouse. Journal of pharmacological methods. 1981;5(2):99-120. Epub 1981/03/01.
28. Kassel R, Levitan S. A jugular technique for the repeated bleeding of small animals. Science. 1953;118(3071):563-4. Epub 1953/11/06.
29. Peng SX, Rockafellow BA, Skedzielewski TM, Huebert ND, Hageman W. Improved pharmacokinetic and bioavailability support of drug discovery using serial blood sampling in mice. Journal of pharmaceutical sciences. 2009;98(5):1877-84. Epub 2008/09/23.
30. Watanabe A, Watari R, Ogawa K, Shimizu R, Tanaka Y, Takai N, et al. Using improved serial blood sampling method of mice to study pharmacokinetics and drug-drug interaction. Journal of pharmaceutical sciences. 2015;104(3):955-61. Epub 2014/12/03.
31. Kurawattimath V, Pocha K, Mariappan TT, Trivedi RK, Mandlekar S. A modified serial blood sampling technique and utility of dried-blood spot technique in estimation of blood concentration: application in mouse pharmacokinetics. European journal of drug metabolism and pharmacokinetics. 2012;37(1):23-30. Epub 2011/09/29.
32. Hui YH, Huang NH, Ebbert L, Bina H, Chiang A, Maples C, et al. Pharmacokinetic comparisons of tail-bleeding with cannula- or retro-orbital bleeding techniques in rats using six marketed drugs. Journal of pharmacological and toxicological methods. 2007;56(2):256-64. Epub 2007/07/10.
33. Hendel J, Nyfors A. Nonlinear renal elimination kinetics of methotrexate due to saturation of renal tubular reabsorption. European journal of clinical pharmacology. 1984;26(1):121-4. Epub 1984/01/01.
34. Ganti V, Walker EA, Nagar S. Pharmacokinetic application of a bio-analytical LC-MS method developed for 5-fluorouracil and methotrexate in mouse plasma, brain and urine. Biomedical chromatography : BMC. 2013;27(8):994-1002. Epub 2013/03/16.
35. Abolmaali S, Tamaddon A, Kamali-Sarvestani E, Ashraf M, Dinarvand R. Stealth Nanogels of Histinylated Poly Ethyleneimine for Sustained Delivery of Methotrexate in Collagen-Induced Arthritis Model. Pharmaceutical research. 2015;32(10):3309-23. Epub 2015/05/13.
36. Deguchi T, Watanabe N, Kurihara A, Igeta K, Ikenaga H, Fusegawa K, et al. Human pharmacokinetic prediction of UDP-glucuronosyltransferase substrates with an animal scale-up approach. Drug metabolism and disposition: the biological fate of chemicals. 2011;39(5):820-9. Epub 2011/02/02.