a comparison of conventional vs. microsampling methodologies for *in vivo* pharmacokinetic studies in rodents

Dr. Daniel B. Kassel[1], Dr. Stuart Kushon[2], Dr. Nurith Amitai[3], Dr. Hong Xing[3]
[1]SciAnalytical Strategies, Inc. 505 Coast Blvd. South, La Jolla, CA 92037,
[2]Neoteryx, LLC, 421 Amapola Ave., Torrance, CA 90501,
[3]Explora BioLabs, 3030 Bunker Hill St., Suite 300 San Diego,CA 92109

Introduction

Microsampling has gained considerable attention as an alternative to the conventional whole blood plasma workup used for PK analysis. This is largely because improvements in LC/MS/MS technology have reduced the blood volume requirements for bioanalysis and make it unnecessary to collect large volumes of blood for analysis. Additionally, collection methods that result in dried blood, relative to plasma, offer a simplified sample collection, storage, and shipping process. Combining these factors, dried blood microsampling offers the very real opportunity to reduce the number of animals required for pharmacokinetic, (PK), toxicokinetic (TK), pharmacodynamic (PD) and/or efficacy studies.

Many groups still perform the conventional "one mouse, one time-point" mouse PK study design. For a standard mouse PK study design, this means a total of 27 mice are required for each dosing group. Microsampling blood collection enables far fewer animals to be used, since multiple bleeds can be done from each animal. This "serial sampling" provides the added benefit of eliminating inter-animal variability and is likely to produce more consistent data.

One of the major drawbacks of dried blood microsampling is the well-documented "hematocrit bias" of DBS cards.1–3 The blood hematocrit (HCT; the volume percentage of the whole blood that is comprised of red blood cells) influences the viscosity of blood, and controls the extent to which a given volume of blood spreads out onto a card. Another reason for the slow adoption of microsampling is that procedures and techniques for preparing animals, and bleeding them to only collect such a small volume of blood, must be developed and made to be routine through training.4 In addition, sample manipulation, including card punching, sample extraction and manipulation prior to bioanalysis is more labor intensive when using DBS cards than the simple plasma crash approach that is widely adopted and used by discovery groups to generate PK in discovery.

The patent pending Mitra® microsampler was designed to simplify the blood collection process and to overcome the hematocrit bias that is associated with DBS cards by achieving a volumetrically precise sample.[5,6] The Mitra device is an inert, porous, hydrophilic material that reproducibly samples and collects small volumes (e.g. 10 µL) of whole blood and other biological fluids. A small droplet of blood, derived from the tail or saphenous vein of the animal, for example, is simply placed in contact with the sampling device and rapidly "wicks" the whole blood onto the porous material, independent of hematocrit. Extensive work has been done and shows that, over a wide range of hematocrit values (20-70 %), there is no volumetric hematocrit bias associated with the 10 µL volume capture on the Mitra tips.[7] It was shown that Mitra has no volumetric hematocrit bias in a recent study comparing DBS and Mitra microsampling (a clinical evaluation of Caffeine and Paraxanthine in human blood).[8] Another important differentiating feature of the Mitra device is that it simplifies the post-sample collection handling and sample extraction protocol which is critical in the drug discovery environment.[4] Following blood collection, the tip is dried either in a cartridge or on a rack. After drying, the tips are immersed into the extraction solvent directly in a microtiter plate, shaken vigorously, processing by any necessary sample preparation steps and then switched to an initial mobile phase solvent and then transferred to an autosampler for bioanalysis. Extraction methods can be found in recent publications that illustrate approaches to achieve efficient analyte recovery from a Mitra microsampler and thereby reduce potential extraction biases.[8–11].

Our first goal, in study 1, was to assess the robustness and reproducibility of the Mitra technique as an alternative to conventional PK sampling and bioanalysis. We conducted rat PK studies using acetaminophen as the test article, collecting blood directly onto Mitra tips and then analyzing whole blood after Mitra extraction. Acetaminophen-D4 was used as the internal standard for all of the studies.

In subsequent studies (studies 2 and 3) we compare the pharmacokinetic profiles for acetaminophen following intravenous dosing in mice using a conventional design (standard plasma processing, n=21 animals) vs. a dried whole blood design (Mitra, n=3 animals for entire time-course).

Experimental Methods

Sample Collection (Study 1, rats)
Three Sprague Dawley rats, approximately 250 gram by body weight and implanted with jugular vein catheters (JVC), were administered 2 mg/kg acetaminophen as a single dose at time 0 via intravenous injection. Prior to dosing and at 5 min, 15 min, 30 min, 1h, 2h, 4h, 8h, and 24h after dosing, blood was collected for control plasma and Mitra microsampling. Approximately 250 µL of blood was drawn from the JVC into a syringe. The syringe needle was removed and a drop of blood was expressed at the tip of the syringe. 10 µL of blood was sampled from this drop into a Mitra device by immersing the Mitra tip just below the surface of the blood for 7-8 seconds. Two more Mitra samples were collected in the same manner. One of the three Mitra samples was collected into Mitra devices pre-treated with K2-EDTA. The tip was pretreated by dipping it for 6 seconds into a stock solution of K2-EDTA, that was at the target blood concentration, and drying it in the open air for 2 hours. The other two Mitra samples were collected into standard untreated Mitra devices. The remaining blood was transferred to a K2-EDTA tube and processed to plasma by centrifugation at 2,300 g for 5 min at 4 °C. Plasma was collected, snap frozen on dry ice, and stored at -80 °C until bioanalysis. Mitra tips were placed into a drying rack and allowed to dry at ambient temperatures for at least 3 hours before bioanalysis.

Preparation of Mitra Tips for Standard Curve Generation in Whole Blood (Study 1)
Whole blood from naïve rats was collected into tubes containing the anticoagulants K2-EDTA, K3-EDTA, and Li-Heparin.The whole blood was transported to the bioanalytical lab at room temperature for immediate processing. Standard curves were generated in the following manner: First, stock solutions of acetaminophen were made in methanol at concentrations of 2500 ng/mL, 1250 ng/mL, 625 ng/mL, 312 ng/mL, 156 ng/mL, 78 ng/mL, 39 ng/mL, 19.5 ng/mL, 9.8 ng/mL and 4.9 ng/mL. 5 µL of each of the standards was transferred to separate 1.5 mL Eppendorf® tubes containing 95 µL of whole blood. Samples were pipetted up and down repeatedly to mix. Next, the blood was transferred to the Mitra device by briefly touching the tip to the surface of the blood in the appropriate tube. Approximately 5 to 6 seconds after the whole blood completely wicked onto the tips, the Mitra devices were removed and set aside to dry in an upright position at room temperature for 3 hours.

Extraction Method Development (Study 1)
200 µL of the following extractions solvents were evaluated: H_2O; 1 % formic acid in H_2O; Methanol; Acetonitrile; 50/50 Methanol/H_2O; 50/50 Methanol/EtOAc. For extraction method development, acetaminophen was added to fresh whole blood at either 10 ng/mL, 100 ng/mL or 1 µg/mL. Mitra tips for extraction method development were prepared in a manner identical to the calibration standards described previously. Samples were shaken vigorously on a micotiter plate shaker for 60 minutes at 1100 rpm. Shown in the photo (**Figure 1**) are examples of solution colors following extraction of the 1 µg/mL acetaminophen spiked whole blood solution. Following extraction, aliquots were removed from the deep well microtiter plate, transferred to an Eppendorf Tube and diluted 5-fold with water containing 0.1 % formic acid and D4-acetaminophen internal standard and analyzed directly by LC/MS/MS. The LC/MS/MS response for acetaminophen was compared under various extraction procedures. In this particular study, there was no attempt to measure absolute recovery. Rather, the goal was to identify an extraction solvent that provided high reproducible signal to support quantification of acetaminophen in whole blood. As an example, shown in **Figure 2**, is the signal of extracted acetaminophen using four different extraction solvents and vigorous shaking for either 30 minutes or 60 minutes. The data shows that 60 minutes provides slightly improved signal relative to 30 minutes. EtOAC showed the poorest signal of the 4 extraction solvents evaluated in this example. Additionally, solvents containing base and acid were evaluated as well as other organic solvents, such as acetonitrile (data not shown). For this particular study, it was found that the 60 min extraction in 100 % methanol provided the highest signal for acetaminophen extracted from the Mitra tips.

Study Design, Dosing and Blood Sampling (Study 2 and 3, mice)
The mouse pharmacokinetic profile of acetaminophen was evaluated using the following protocol. In study 2, 3 animals were sacrificed at each time point. A total of 21 mice were therefore required for the entire time-course (pre-dose, 0.08hr, 0.25h, 0.5h, 1h, 2h, 4h). Time points past 4h were not required due to the short half-life of acetaminophen in mice. Study 3 animals received acetaminophen through the same route of administration. However, just 3 mice were used in this study, with each animal bled at every time point across the entire time-course.

Acetaminophen was formulated in saline to a concentration of 5 mg/mL and a 2 mg/kg dose was administered into the tail vein of the mice. Blood collection was achieved in the following manner: For study 2, at the specific time point, mice were anesthetized with 2 % isoflurane and blood harvested by cardiac puncture; animals were euthanized by cervical dislocation after exsanguination. The Mitra tips were dipped into the collected blood and set aside to dry. The remaining blood was processed to plasma and stored at -80 °C until analysis. For study 3, blood was retrieved at the specific time point by saphenous vein sampling.

Plasma Standard Curve Generation (Study 2 and 3)
Plasma standard curves were generated using control plasma and standard procedures. A 5 µL aliquot of a 50 µg/mL solution of acetaminophen in acetonitrile was added to 95 µL of blank plasma to make the top plasma calibrator (2.5 µg/mL). Subsequent calibration standards were prepared by serial dilution of the preceding plasma calibrator into blank plasma. 10 µL aliquots were then precipitated with 90 µL methanol containing 50 ng/mL D4-acetaminophen. Mouse whole blood Mitra standards were prepared within 1 hour after collecting whole blood into tubes containing K2-EDTA. A 5 µL aliquot of 50 µg/mL stock solution of acetaminophen in acetonitrile was spiked into an Eppendorf tube containing 95 µL of fresh whole blood, resulting in a top whole blood calibrator of 2.5 µg/mL. Remaining standard calibrators were prepared by serial dilution of the preceding calibrator into an equal volume of blank whole blood.

Experimental Methods *(cont'd)*

Mitra Extractions (Study 2 and 3)
Mitra tips were dipped into each of the Eppendorf tubes containing whole blood calibrations standards, and set aside to dry for 3 hours. After drying, the tips were then placed into a deep-well microtiter plate containing 100 µL of water containing 1 % formic acid and shaken on a plate shaker for 30 minutes at 1100 rpm. Following this initial aqueous extraction, the tips were temporarily removed and a 100 µL aliquot of methanol containing 50 ng/mL D4-acetaminophen internal standard was added to each well, and the tips re-immersed and shaken on a plate shaker for an additional 30 minutes at 1100 rpm. After extraction, tips were removed, extract was removed from the plate, transferred to new Eppendorf tubes, mixed with 600 µL of cold acetonitrile and centrifuged for 10 minutes at 10,000 rpm. Supernate was removed, evaporated to dryness, and reconstituted in 100 µL water containing 0.1 % formic acid prior to LC/MS/MS analysis.

Collected plasma and blood were processed in a manner identical to the calibration standards. For plasma, a 10 µL aliquot of plasma was transferred to a tube containing 90 µL of cold methanol containing 50 ng/ mL D4-acetaminophen internal standard. The sample was vortexed, placed on a microfuge and centrifuged at 10,000 rpm to pellet the protein precipitate. Supernate was removed, evaporated to dryness and then reconstituted in Mobile Phase A prior to analysis. Dried Mitra tips from the study samples were processed in an identical manner to the whole blood Mitra calibration standards.

LC/MS/MS
A Spark Holland Symbiosis HPLC coupled to an API 4000™ QTRAP® triple quadrupole ion trap mass spectrometer (SCIEX) was used for LC/MS/MS analysis. Chromatographic separations were performed using a Kinetex® C18 5 µm 2 mm ID x 50 mm column (Phenomenex). D4-acetaminophen was used as the internal standard. Mobile Phase A was water containing 0.1 % formic acid. Mobile Phase B was acetonitrile containing 0.1 % formic acid. The gradient was: 1 % B to 70 % B in 2.5 minutes following an initial hold at 1 % B for 0.5 minutes.

FIGURE 1.
Photograph of the wells of the deep well plate show various colors, indicating selective extraction of whole blood matrix components depends on extraction solvent strength

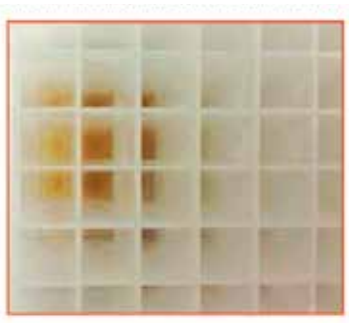


FIGURE 2.
Area counts for acetaminophen after extraction in various extraction solvents. From Left to Right: EtOAc for 30 minutes, MeOH for 30 minutes, EtOAc/MeOH (50/50) for 30 minutes, MeOH/H_2O for 30 minutes, MeOH for 60 minutes, EtOAc/MeOH (50/50) for 60 minutes, MeOH/H_2O for 60 minutes

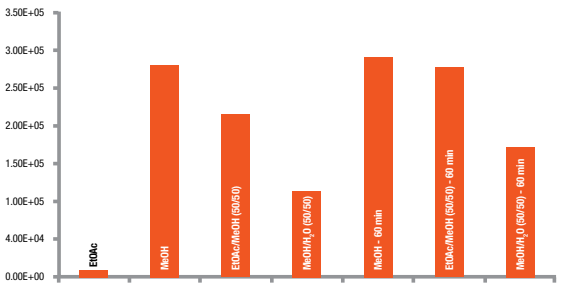
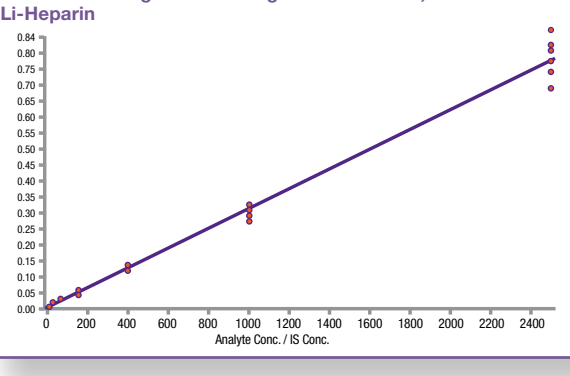


FIGURE 3.
Overlay of 6 Mitra calibration curves with tips either untreated or pre-treated with K2-EDTA with whole blood collected into tubes containing the anti-coagulants K2-EDTA, K3-EDTA or Li-Heparin



neoteryx™
The promise of microsampling, delivered

Continued on back

Continued from front

Results and Discussion

Shown in Figure 3 are the overlays of 6 acetaminophen standard curves over the concentration range of 5 ng/mL – 2500 ng/mL. The whole blood standard curves from study 1 were highly reproducible regardless of the blood anti-coagulant used in the collection device (tubes or Mitra Tips). There was also no significant difference between samples collected into Mitra devices pre-treated with K2-EDTA versus untreated ones. Representative LC/MS/MS chromatograms for the Mitra standard curves are shown in **Figure 4**. The LC/MS/MS chromatograms show excellent signal-to-noise, reinforcing the fact that only very small volumes of whole blood are needed to measure drug concentrations reproducibly and accurately from a 10 µL blood sample.

The whole blood IV PK curve (**Figure 5**) is consistent with published pharmacokinetics of acetaminophen in rats.[12] In the example shown, the whole blood bioanalysis properly confirmed that one of the animals (blue trace) received a slightly lower dose than the other two animals (this is confirmed by comparing directly to the plasma concentration data, which is not shown). Finally, based upon our experience, it is recommended that duplicate Mitra samples are collected at each time point, as it allows for more than one measurement of the Mitra whole blood concentrations, should it be necessary.

For studies 2 and 3, plasma and dried whole blood standard curves were generated for acetaminophen over the concentration range of 5 ng/mL – 5000 ng/mL, as shown in **Figure 6**. The r value for the mouse plasma and mouse whole blood (from Mitra) were 0.9975 and 0.9986, respectively. These standard curves were then used to quantify the plasma and whole blood exposures, respectively for acetaminophen following 2 mg/kg intravenous dosing. Shown in **Figure 7** are the plasma concentration vs. time profile and dried whole blood concentration (Mitra) vs. time profile for the study 2 animals.

The plasma and whole blood concentration vs. time profiles are quite similar, with some modest differences. The plasma exhibited drug exposure out to 2 hours, whereas for dried whole blood using Mitra, the exposure was below the quantitation limit after 1 hour for this study. The 15 minute time point was also slightly higher for the plasma samples, which could simply be explained by the bioanalytical measurement (research-grade assay). Shown in **Table 1** are the pharmacokinetic parameters for the study 2 plasma and dried whole blood (Mitra) samples. The area under the curve (AUC), systemic clearance (CLs), and volume of distribution (Vd) were comparable. Only the terminal (elimination) half-life was significantly different ($T_{½}$ = 0.6h for plasma vs. 0.2h for dried whole blood). Importantly, in the context of drug discovery setting, the interpretation of data would be the same – that is, the compound exhibits high clearance, high volume of distribution and short half-life in mouse. Given the concordance between the mouse plasma and mouse whole blood pharmacokinetics incorporating a conventional 21 mouse, "one mouse – one time point" paradigm, we next assessed the pharmacokinetics of acetaminophen using only 3 mice (study 3), performing serial bleeding via saphenous vein sampling directly onto Mitra tips. Shown in **Figure 8** is the Mitra whole blood vs. time profile for acetaminophen incorporating an n=3 study design (i.e. the full time-course was taken from each animal via saphenous vein sampling) as compared to the study 2 results.

The mouse study 3 dried whole blood concentration vs. time profile was similar to the study 2 result, the primary difference being the measured concentration at the 5 minute and 15 minute time points. The acetaminophen dried whole blood concentration at t=0.08h was 1830 ng/mL, and at t=0.25h, 1000 ng/mL. In the case of the study 2 animals, the concentration of acetaminophen at the t=0.08h time-point was 1320 ng/mL. The 0.5h, 1h and 2 hr time-points showed whole blood concentrations similar between the two groups. The differences are unlikely attributable to the bioanalytical assay; rather, they may more likely be explained by the source of whole blood retrieval (saphenous vein vs. cardiac puncture) and/or the fact that the study 3 was conducted on a different day with a different (and fresh) preparation of test article. Further investigations will be needed to confirm the reason for the higher concentrations observed at these early time-points. The pharmacokinetic parameters for study 3 are shown in **Table 1**. Consistent with the higher exposure at the early time points, the AUC was higher relative to study 2 and the clearance lower. Importantly, the conclusions from study 3 are consistent with study 2 – that is, the compound exhibits moderate to high clearance, high volume of distribution, short half-life and moderate exposure after intravenous dosing of acetaminophen at 2 mg/kg.

FIGURE 4.
LC/MS/MS chromatogram for acetaminophen at the IV PK 15 minute time-point (top) following extraction. D4-Acetaminophen internal standard signal (bottom)

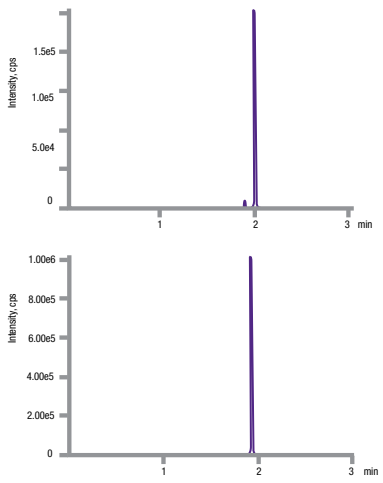


FIGURE 5.
Mitra whole blood concentration vs. time profiling following IV dosing

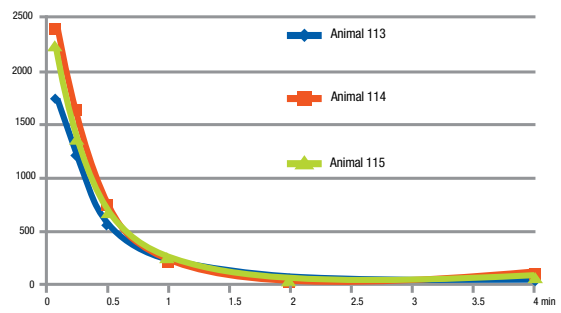


TABLE 1.
Pharmacokinetic parameters for the study 2 plasma and dried whole blood (Mitra) samples

Group #	Route	Time Points	# of Rats	# of Mice
1	IV	pre-dose, 0.08h, 0.25h, 0.5h, 1h, 1.5h, 2h, 4h, 8h	N=3	N=27
2	PO	pre-dose, 0.25h, 0.5h, 1h, 1.5h, 2h, 4h, 8h, 24h	N=3	N=27

Volume of Blood drawn at each time-point = 250 µL, processed to plasma, stored at -80 °C prior to bioanalysis

FIGURE 6.
Acetaminophen standard curves

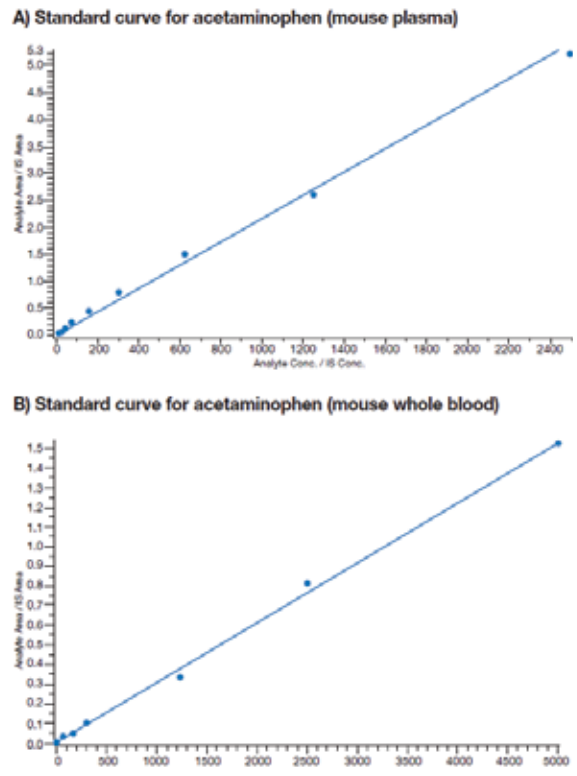


FIGURE 7.
Overlay of concentration vs. time profile for Mitra study 2 (cardiac puncture, whole blood wicked onto Mitra tips) and Plasma study 2 (cardiac puncture, whole blood processed to plasma)

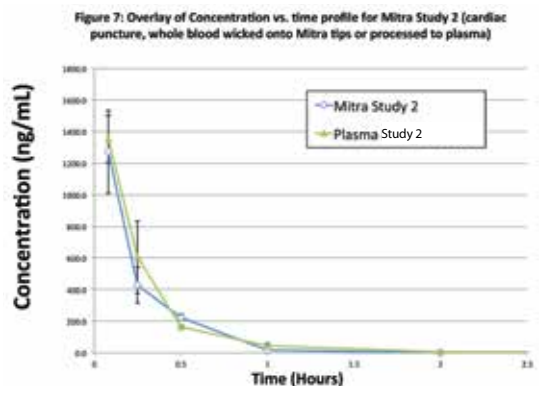
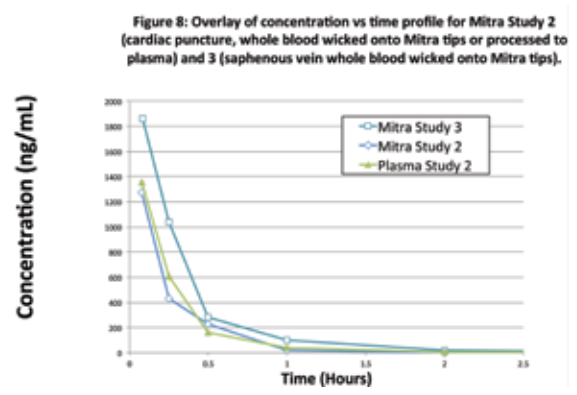


FIGURE 8.
Overlay of concentration vs. time profile for Mitra (study 2 - cardiac puncture, whole blood wicked onto Mitra tips and study 3 - saphenous vein whole blood wicked onto Mitra tips) and Plasma (study 2 - cardiac puncture, whole blood processed to plasma). The 5 minute (0.08h) Mitra study 3 time point shows slightly higher concentration than plasma and whole blood for study 2 animals.



Conclusion

In the discovery setting, compounds are screened in a wide range of in vitro and in vivo assays and the data is used to "rank order" and prioritize chemotypes and individual compounds for more extensive optimization and evaluation in animal models. The Mitra microsampling methodology provides a simple, reproducible method for whole blood sampling, extraction and bioanalysis. It also facilitates serial sampling which reduces the number of animals needed per study and eliminates inter-animal variability which may lead to more consistent data. These data support the use of the Mitra Microsampler for dried whole blood microsampling in the discovery setting for pharmacokinetic studies. The precise and hematocrit-independent transfer of 10 µL whole blood from the blood retrieval sites evaluated (cardiac puncture and saphenous vein) make it possible to conduct full pharmacokinetic time-course profiles in a small number of animals, supporting the ethical use of rodents in research and development.

Further advantages not discussed in detail include reduced test article requirement as well as reduced labor requirement associated with dosing and blood retrieval from a much smaller animal group. Current and future efforts are focused on applying Mitra to pharmacokinetic-pharmacodynamic (PK-PD) and early efficacy studies. For the vast majority of PK-PD and early efficacy studies, a "satellite" group of animals is required to obtain the necessary quantitative data to make PK-PD and efficacy correlations. Mitra microsampling, because of the small volume blood sampling requirement, opens the door to combining these critical measurements in the same animal (disease model animal). Combining PK-PD and efficacy assessment in the same animal will undoubtedly lead to additional insights into the relationship between drug exposure, drug duration and drug effect and will aid in the translation of such results from animal to human. A related paper by Denniff et. al., demonstrated that TK curves generated from acetaminophen dosed in rats showed similar profiles when comparing Mitra samples to traditional blood water work-up. In that study, the authors note that the assay using Mitra passed all international assay guidelines, and that the VAMS technique has the ability to supplant DBS for quantitative bioanalysis, since it retains all the recognized advantages of DBS as well as making the sample collection process simpler. Furthermore, it reduces the work flow within the bioanalytical laboratory and minimizes the effect of HCT related assay bias.[4]

References

1. Denniff, P., and Spooner, N. (2010) The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs. *Bioanalysis 2*, 1385–1395.
2. De Vries, R., Barfield, M., van de Merbel, N., Schmid, B., Siethoff, C., Ortiz, J., Verheij, E., van Baar, B., Cobb, Z., White, S., and Timmerman, P. (2013) The effect of hematocrit on bioanalysis of DBS: results from the EBF DBS-microsampling consortium. *Bioanalysis 5*, 2147–60.
3. De Kesel, P.M.M., Capiau, S., Lambert, W. E., Stove, C. P. (2014) Current strategies for coping with the hematocrit problem in dried blood spot analysis. *Bioanalysis 6*, 1871–874.
4. Denniff, P., Parry, S., Dopson, W., and Spooner, N. (2015) Quantitative bioanalysis of paracetamol in rats using volumetric absorptive microsampling (VAMS). *J. Pharm. Biomed. Anal.* 108, 61–69.
5. Denniff, P., and Spooner, N. (2014) Volumetric Absorptive Microsampling: A Dried Sample Collection Technique for Quantitative Bioanalysis. *Anal. Chem. 86*, 8489–8495.
6. Kushon, S., Bischofberger, A., Carpenter, A., Denniff, P., and Guo, Y. (2014) A Novel Dried Matrix Microsampling Device that Eliminates the Volume Based Hematocrit Bias Associated with DBS Sub-punch Workflows. 62nd ASMS Conference on Mass Spectrometry and Allied Topics, Baltimore, Maryland, June 15-19, 2014. WP 640
7. Spooner, N., Denniff, P., Michielsen, L., De Vries, R., Ji, Q. C., Arnold, M. E., Woods, K., Woolf, E. J., Xu, Y., Boutet, V., Zane, P., Kushon, S., and Rudge, J. B. (2015) A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. *Bioanalysis 7*, 653–65
8. De Kesel, P.M.M., Lambert, W. E., Stove, C. P. (2015) Does volumetric absorptive microsampling eliminate the hematocrit bias for caffeine and paraxanthine in dried blood samples? A comparative study. *Anal. Chim. Acta*. 881, 65–75.
9. Luo, Y., Korfmacher, W., Ho, S., Shen, L., Wang, J., Wu, Z., Guo, Y., Snow, G., O'Shea, T. (2015) Evaluation of two blood microsampling approaches for drug discovery PK studies in rats. *Bioanalysis*.
10. Mano, Y.; Kita, K.; Kusano, K. (2015) Hematocrit-independent recovery is a key for bioanalysis using volumetric absorptive microsampling devices, MitraTMNo Title. *Bioanalysis 7*, 1821–1829.
11. Miao, Z., Farnham, J. G., Hanson, G., Podoll, T., Reid, M. J. (2015) Bioanalysis of emixustat (ACU-4429) in whole blood collected with volumetric absorptive microsampling by LC – MS / MS. *Bioanalysis 7,* 2071–2083.
12. Jang, S.H.; Lee, M. H.; Lee, M. G. (1994) Pharmacokinetics of acetaminophen after intravenous and oral administration to spontaneously hypertensive rats and normotensive Wistar rats. *J Pharm Sci. 83,* 810–4.

neoteryx™

The promise of microsampling, delivered

PO43230616