

Evaluation of the Mitra™ Microsampling Device for In Vivo Pharmacokinetic Studies

A novel technology that makes sample collection easy, saves on animals, and is simple to implement in the discovery setting

Dr. Daniel B. Kassel¹, Dr. Nurith Amitai², Dr. Stuart Kushon³, Dr. Hong Xin²

¹SciAnalytical Strategies, Inc. 505 Coast Blvd. South, La Jolla, CA 92037, ²Explora BioLabs, 3030 Bunker Hill St., Suite 300 San Diego, CA 92109,

³Neoteryx LLC, 421 Amapola Ave., Torrance, CA 90501

Abstract

A novel, patent-pending microsampling device (Mitra) has been evaluated in the drug discovery setting to support pharmacokinetic (PK) evaluation of drug candidates in rodents. The Mitra Microsampling Device has been designed to reproducibly collect a small volume (e.g. 10µL) of whole blood in a hematocrit-independent manner (Spooner et al., 2014). and, as a result, overcomes the major challenge and limitation of conventional dried blood spot cards that have historically been used for microsampling. For this current evaluation, three male Sprague Dawley rats were intravenously dosed with acetaminophen (2 mg/kg). Blood samples were collected over six time points spanning 4 hours using the Mitra microsampling device. The concentration of acetaminophen was determined against whole blood standard curves, also prepared using Mitra. The resulting whole blood IV PK curves are consistent with the published pharmacokinetics of acetaminophen in rats, and indicates that data obtained using the Mitra microsampling device can be used effectively to facilitate decision making in the discovery setting.

Introduction

Pharmacokinetic studies are routinely performed throughout the drug discovery process, initiating with screening PK during the lead generation phase and culminating in comprehensive PK studies to support candidate selection. During hit and lead generation, compounds are synthesized at the mg level, formulated for intravenous (IV) and oral (PO) dosing in a rat or mouse (e.g., 1 mg/kg for IV and 5 mg/kg for PO). Blood samples are typically taken over a defined time-course (e.g. 0-8 hours for IV dosing and 0-24 hours for PO dosing) and three animals are used to obtain an average drug exposure at each time point. A typical study design implemented in discovery PK is shown in **Table 1**. In general, for rodent PK studies, 250µL of whole blood is retrieved at each time point, processed to plasma (yielding approximately 100µL), and stored at -80°C prior to bioanalysis. LC/MS/MS bioanalysis is performed to assess the pharmacokinetic properties of the molecules and their potential as lead candidates. In general, compounds exhibiting >20% oral bioavailability, moderate to low clearance, and moderate to long terminal half-life are prioritized for further profiling in pharmacodynamic and efficacy studies.

Table 1: Standard PK Study Design

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 = 250µL, processed to plasma, stored at -80°C prior to bioanalysis

Microsampling, and more specifically, the dried blood spot (DBS) technique, has gained considerable attention as an alternative to the conventional whole blood plasma workup used for PK analysis. There are several compelling reasons for the interest in DBS. First, with improvements in LC/MS/MS technology, the blood volume requirements for bioanalysis make it unnecessary to collect such large volumes of blood prior to analysis. It is somewhat ironic that in the last 10 years, the sensitivity of triple quadrupole mass spectrometers (the “gold standard” for PK bioanalysis) has increased 100x-1000x relative to earlier generation instruments while blood sampling protocols have stayed the same for many in vivo DMPK and toxicology groups. Second, DBS, relative to plasma, offers a simplified sample collection, storage, and shipping process. Third, and perhaps most importantly, DBS offers the very real opportunity to reduce the number of animals required for pharmacokinetic, toxicokinetic (TK), pharmacodynamic (PD) and/or efficacy studies. For example, many groups still perform the conventional “one mouse, one time-point” mouse PK study design. As shown in **Table 1**, 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. Performing multiple bleeds from each animal, rather than using different animals for each time point, also eliminates inter-animal variability and is therefore likely to produce more consistent data. For PD and efficacy studies, PK data generally comes if satellite PK groups are part of the study protocol, as the traditional larger volume blood collections precludes the ability to acquire this information from the main study (efficacy) animals. The fact remains that large volumes of whole blood continue to be used for “traditional” reasons rather than out of necessity and this has translated into the use and sacrifice of more animals than necessary.

With the clear benefits of DBS/microsampling, the legitimate question to ask is why more groups in drug discovery have not fully embraced the technology. There are several reasons for this. One of the major reasons is the well-documented “hematocrit bias” of DBS cards. The blood hematocrit (HCT; the volume percentage of the whole blood that is comprised of red blood cells) influences the viscosity of blood, and therefore dramatically influences the extent to which a given volume of blood spreads out onto a DBS card. High hematocrit blood will be viscous and tend to not spread out across the card, whereas low HCT blood will be more fluid and spread out farther across the DBS card. Thus, a given volume of blood will generate different diameter spots on the DBS card as a function of hematocrit. In a typical DBS workflow a small circular punch is removed from the overall spot for analysis. The effective volume contained in this sub punch is not uniform from sample to sample because it is a function of the overall spot size (which is controlled by the hematocrit of the blood sample).

To deal with this hematocrit bias, whole spot collections can be made from a DBS card to reduce the overall hematocrit bias. This approach provides a uniform volume; however, it is difficult to automate due to the

varied spot sizes. Another reason for the slow adoption of DBS 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. 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 effectively by discovery groups to generate PK in discovery.

Recently, a microsampling device (Mitra) has been designed to simplify the blood collection process and, most importantly, overcome the hematocrit bias that is associated with DBS cards. 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 hematocrit bias associated with the 10 µL volume capture on the Mitra tips. 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. Following blood collection, the tip is placed upright on a microtiter plate drying rack. After drying, the tips are immersed into a small volume of extraction solvent, such as 200 µL of acidified water, directly in the microtiter plate.

In the present study, the primary goal 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.

Materials and Methods

Sample Collection

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, 1 h, 2 h, 4 h, 8 h, and 24 h 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

Whole blood from naïve rats was collected into tubes containing anti-coagulant. K2-EDTA, K3-EDTA, and Li-Heparin tubes were all evaluated and deemed acceptable for temporary storage and transfer of whole blood. Initially, the collected whole blood was placed on either ice or dry ice and transferred to the bioanalytical lab for standard curve preparation. However, it was found that after warming the whole blood to room temperature, pipetting, aliquoting, and mixing of the whole blood with standards was challenging. Therefore, we adopted a procedure of collecting the whole blood and transporting 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. It should be noted that the Mitra tips show a bright, shiny red color after initial application of the whole blood to the tip and after complete drying the tip appears dull, reddish-brown in color.

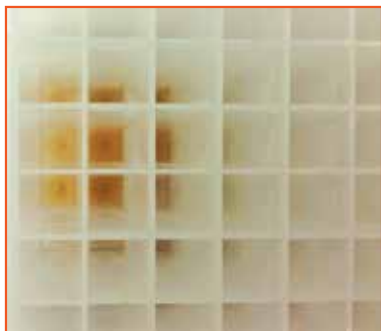
Extraction Method Development

One of the strengths of the Mitra technology is that the tips are well-suited to loading into microtiter plates. Extraction method development is greatly simplified because it is as simple as placing the dried Mitra tips into wells of a deep well microtiter plate containing 100 µL or 200 µL of extraction solvent. The following extractions solvents were evaluated: H₂O; 1% formic acid in H₂O; Methanol; Acetonitrile; 50/50 Methanol/H₂O; 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 microtiter plate shaker for 60 minutes at 1100 rpm. This allowed for good mixing of the extraction solvents with the Mitra tips. 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 or further cleaned-up by applying one of three protein precipitation methods. These different protein precipitation methods were: a) 2% ammonium sulfate, b) methanol (1:3 sample:methanol) and c) acetonitrile (1:3 sample:acetonitrile). To a 100 µL aliquot of extracted sample, 300 µL of cold protein precipitation solvent was added. Samples were placed on ice for 10 minutes prior to centrifugation at 10,000 rpm for 10 minutes. After centrifugation, the samples showed visible precipitate in the bottom of the well. Aliquots were taken, evaporated to dryness, and reconstituted in water containing 0.1% formic acid and D4-acetaminophen internal standard prior to LC/MS/MS analysis. 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.

Figure 1.

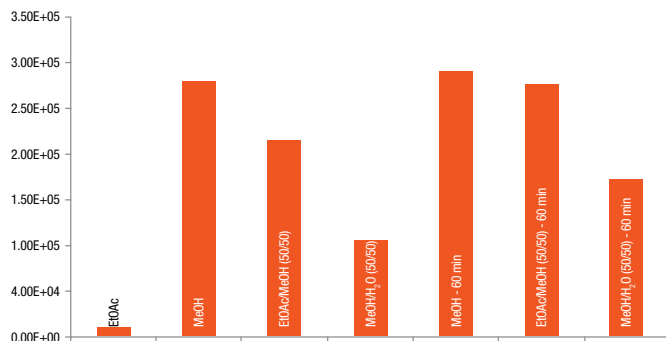
Photograph of the wells of the deep well plate show various colors, indicating selective extraction of whole blood matrix components depends on extraction solvent strength.



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 show 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.

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/H₂O for 30 minutes, MeOH for 60 minutes, EtOAc/MeOH (50/50) for 60 minutes, MeOH/H₂O for 60 minutes

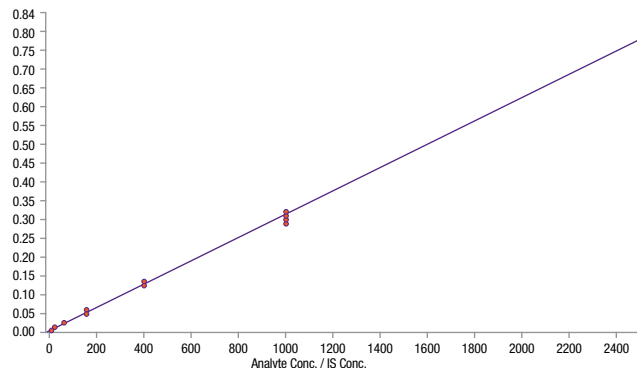


Results and Discussion

In the case of acetaminophen the whole blood standard curves 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. Additionally, the standard curves were reproducible for the majority of extraction solvents evaluated. Shown in **Figure 3** are the overlays of 6 acetaminophen standard curves over the concentration range of 5 ng/ml – 2500 ng/ml.

Figure 3.

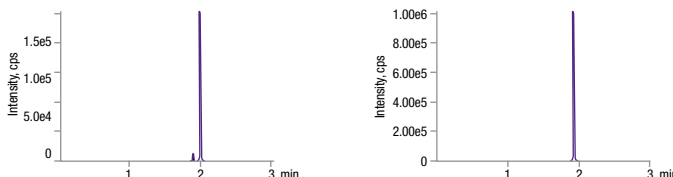
Overlay of 6 Mitra calibration curves with tips either untreated or pre-treated with K2-EDTA with whole blood collected into tubes containing the anti-coagulants K2-EDTA, K3-EDTA or Li-Heparin



Representative LC/MS/MS chromatograms for the Mitra standard curves are shown in **Figure 4**. A 4000 QTRAP® was used for the analysis. 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.

Figure 4.

LC/MS/MS chromatogram for acetaminophen at the IV PK 15 minute time-point (left panel) following extraction. D4-Acetaminophen internal standard signal (right panel)



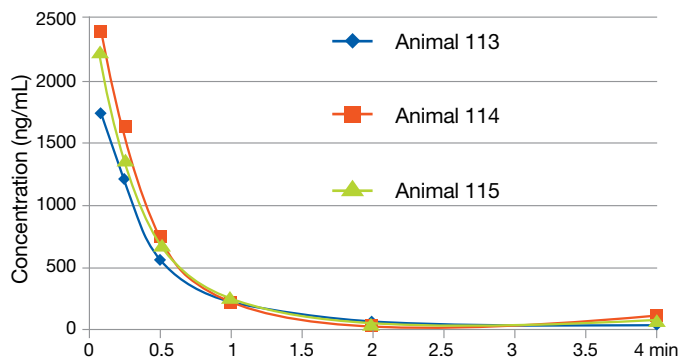
Shown in **Table 2** are the Mitra whole blood concentrations for acetaminophen following IV dosing at a dose of 2 mg/kg. The whole blood IV PK curve (**Figure 5**) is consistent with published pharmacokinetics of acetaminophen in rats (Jang et al., 1994). In the example shown, the whole blood bioanalysis 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).

Table 2: Whole Blood Concentrations for Acetaminophen Following IV Dosing at 2 mg/kg

Time (hours)	Animal 113	Animal 114	Animal 115	Average Concentration
4	62.2	111	84	85.7
2	72.3	46.3	53	57.2
1	246	227	267	246.7
0.5	565	750	701	672.0
0.25	1220	1640	1380	1413.3
0.08	1740	2390	2230	2120.0

Figure 5.

Mitra whole blood concentration vs time profiling following IV dosing



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.

Conclusions

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 to therefore likely produce more consistent data. The results of this study indicate that the data can be used with confidence to facilitate decision making in the discovery setting for the selection and advancement of compounds from lead generation through lead optimization.

Literature Cited

1. 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., Rudge, J.B. Journal of Bioanalysis. (2014). A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated blood hematocrit. Advance online publication. doi:10.4155/bio.14.310
2. Jang, S.H., Lee, M. H., and Lee, M. G. J Pharm Sci. 1994; 83(6):810-4.

Ordering Information

Part No.	Description	Unit
9R-K002-CA	Mitra 10 µL Microsampling Device 96-Well Plate Assembly	1 ea
9R-K002-CD	Mitra 10 µL Microsampling Device 96-Well Plate Assembly	12/pk
9R-K002-BF	Mitra 10 µL Microsampling Device 4-Pack Clamshell	60/pk
AK-9268-A	Mitra Drying Rack 96-Well Plate	1 ea
AK-9269-A	Mitra SBS Deck Adapter 96-Well Plate	1 ea
AK-9270-A	Mitra Sampling Tool	1 ea
AH0-8636	96-Well Collection Plate 2 mL round well with round bottom	50/pk

Trademarks

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Disclaimer

Mitra is patent pending. The Mitra Microsampling Device is for research-use-only and should serve no medical purpose. It should not be used with the intent or in the course of clinical diagnosis, treatment, or care of humans or animals.

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