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application note

A Comparison of Conventional vs. Microsampling Methodologies for In Vivo Pharmacokinetic Studies in Mice

Generate comparable PK data while simplifying sample collection and saving on animals

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Abstract

A novel microsampling device, the Mitra™ Microsampler, has been applied to pharmacokinetic (PK) evaluation of acetaminophen in mice. The microsampling device reproducibly collects 10 µL whole blood volumes, thereby allowing for serial bleeds over the pharmacokinetic time course, reducing animal usage and associated labor requirements to achieve full pharmacokinetic profiles. The Mitra Microsampler is an incredibly simple sampling device and has the critical benefit of being hematocrit-independent. This means that the whole blood sample collected is precisely 10 µL each time, independent of the hematocrit. No punching of blood spots from dried blood spot cards is needed, offering a superior dried microsampling method for discovery pharmacokinetic studies.

We have compared 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). The results suggest that Mitra will be a very useful microsampling collection technique for pharmacokinetic studies involving mice, greatly reducing the animal number requirement and opening the door to possibilities for combining PK and PD and /or efficacy assessments in the same study animals.

Introduction

Pharmacokinetic studies are routinely performed throughout drug discovery, initiating with screening PK during the lead generation phase and culminating in comprehensive PK, PK-PD and efficacy studies to support candidate selection and IND-enabling studies. Typically, for mouse PK studies, each animal is only bled once and n=3 animals are used at each time point to obtain an average drug exposure across a defined time course. For a 9-point pharmacokinetic time course, a total of 27 mice are required for each dosing route. Such a large animal "n" size is employed into these studies because collecting small, reproducible volumes of blood (multiple bleeds from a single animal) has proved challenging for in vivo and bioanalytical groups

With a plethora of high sensitivity LC-MS-MS systems available on the market today, blood volume is no longer a critical determinant of bioanalytical success, especially for discovery pharmacokinetic studies. Despite these analytical advances, the "one mouse, one time point" mouse PK study design remains common practice.

The Mitra Microsampler simplifies the blood collection process and overcomes the volumetric hematocrit (HCT) bias, well known to be problematic for dried blood spot (DBS) cards. Extensive work has been done and shows that over a wide range of HCT values (30-70 %), there is no HCT bias associated with the 10 µL volume capture on the Mitra tips (Spooner et al., Bioanalysis 2014 Epub ahead of print). 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, shaken vigorously and then transferred to an autosampler plate for bioanalysis.

Materials and Methods

Study Design, Dosing and Blood Sampling

The mouse pharmacokinetic profile of acetaminophen was evaluated using a traditional study design, as shown for group 1 in **Table 1** below. In group 1, 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. Group 2 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.

Table 1: Mouse PK Study Design

Group Number	Route	Time Points	Sample Number
1	IV	pre-dose, 0.08h, 0.25h, 0.5h, 1h, 2h, 4h	N=21
2	IV	pre-dose, 0.08h, 0.25h, 0.5h, 1h, 2h, 4h	N=3

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 group 1, 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 group 2, blood was retrieved at the specific time point by saphenous vein sampling.

Standard Curve Generation
Plasma standard curves were generated using control plasma and standard procedures. A 5 µL aliquot of an 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.

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.

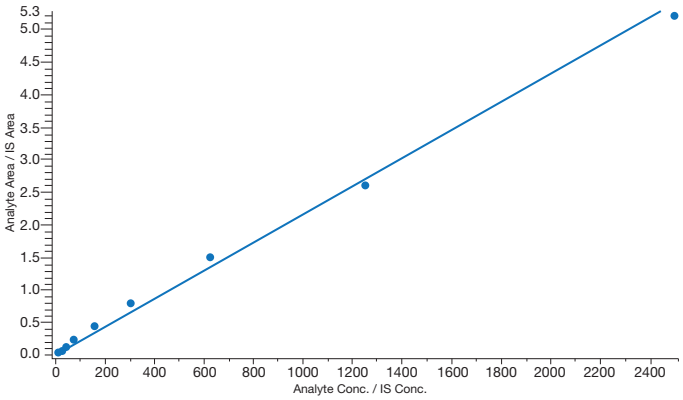
LC/MS/MS
A Spark Holland Symbiosis HPLC coupled to an API4000 QTRAP® triple quadrupole ion trap mass spectrometer (AB SCIEX) was used for LC/MS/MS analysis. Chromatographic separations were performed using a Kinetex C18 5 µm 2 mm ID x 50 mm column (Phenomenex). D4-acetaminophen was used as the internal 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.

Results and Discussion

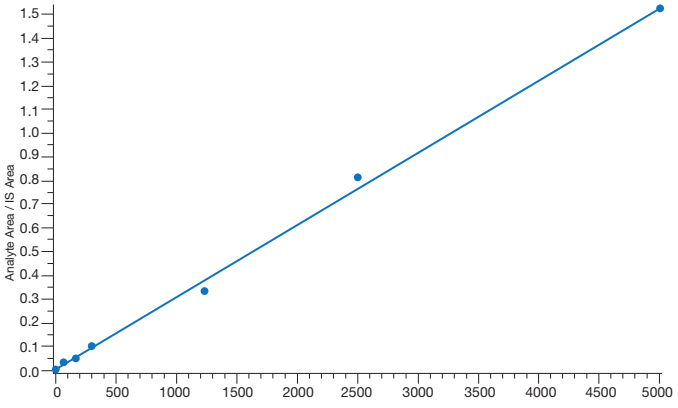
Plasma and dried whole blood standard curves were generated for acetaminophen over the concentration range of 5 ng/ml – 5000 ng/ml, as shown in **Figure 1**. The r value for the mouse plasma and mouse whole blood (from Mitra) were 0.9975 and 0.9986, respectively. These standard curves were then used to quantify the plasma and whole blood exposures, respectively for acetaminophen following 2 mg/kg intravenous dosing

Figure 1.
Standards met the criteria of the analytical assay, all measured to within +/- 30 % of their target values. R values for plasma and whole blood were 0.9975 and 0.9986, respectively. Standard curves were generated at the front-end and back-end of the study samples.

A) Standard curve for acetaminophen (mouse plasma)

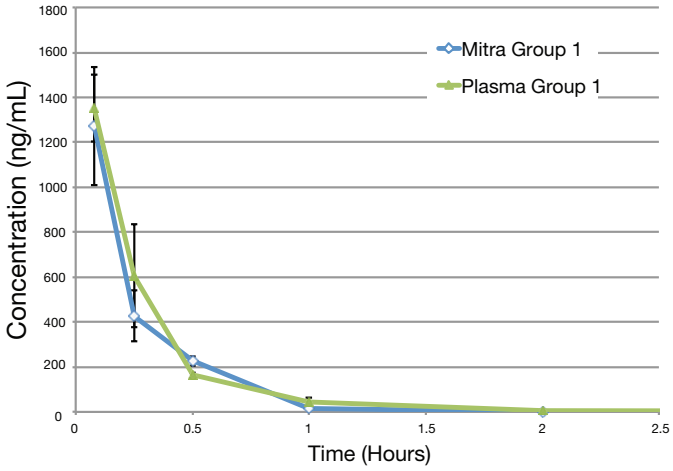


B) Standard curve for acetaminophen (mouse whole blood)



Shown in **Figure 2** below are the plasma concentration vs. time profile and dried whole blood concentration (Mitra) vs. time profile for group 1 animals.

Figure 2.
Overlay of concentration vs time profile for Mitra Group 1 (cardiac puncture, whole blood wicked onto Mitra tips) and Plasma Group 1 (cardiac puncture, whole blood processed to plasma)



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).

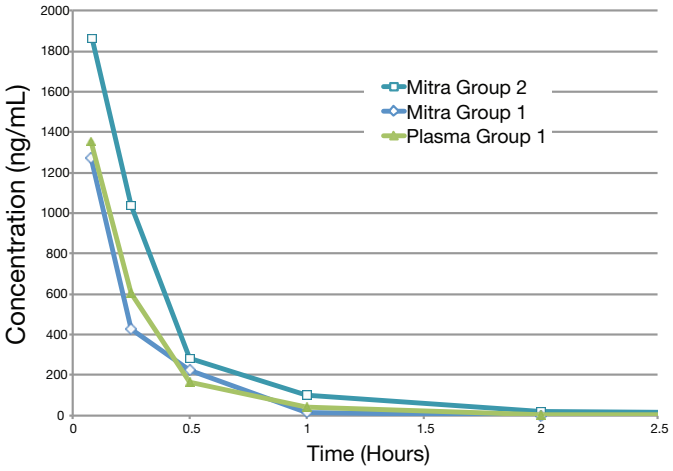
Shown in **Table 3** are the pharmacokinetic parameters for group 1 plasma and dried whole blood (Mitra). The area under the curve (AUC), systemic clearance (CLs), and volume of distribution (Vd) were comparable. Only the terminal (elimination) half-life was significantly different (T½ = 0.6h for plasma 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.

Table 3: Pharmacokinetic Parameters

Group Number	AUC (0-t) (ng*h/ml)	CLs (ml/min/kg)	T ½ (h)	Vd (ml)
Plasma Group 1	481	70	0.62	1270
Mitra Group 1	432	77	0.2	983
Mitra Group 2	739	45	0.4	835

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 (group 2), performing serial bleeding via saphenous vein sampling directly onto Mitra tips, leveraging the true Mitra advantage – reproducible, precise whole blood microsampling. Shown in **Figure 3** 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 group 1 results.

Figure 3.
Overlay of concentration vs time profile for Mitra Group 1 (cardiac puncture, whole blood wicked onto Mitra tips) and 2 (saphenous vein whole blood wicked onto Mitra tips) and Plasma Group 1 (cardiac puncture, whole blood processed to plasma). The 5 minute (0.08h) Mitra Group 2 time point shows slightly higher concentration than plasma and whole blood for study 1 animals.



The mouse group 2 dried whole blood concentration vs. time profile was similar to the group 1 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 group 1 animals, the concentration of acetaminophen at the t=0.08h time-point was 1320 ng/ml. The 0.5h, 1h and 2hr 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 group 2 study 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 group 2 are shown in **Table 3**. Consistent with the higher exposure at the early time points, the AUC was higher relative to group 1 and the clearance lower. Importantly, the conclusions from this group 2 are consistent with group 1 – 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.

Conclusions

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. **Literature Cited**

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. Advanceonline publication. doi:10.4155/bio.14.310

Ordering Information

Part No.	Description	Unit
10005	Mitra 10 µL Microsampler 96-Rack	1 ea
10006	Mitra 10 µL Microsampler 96-Rack	6/pk
10004	Mitra 10 µL Microsampler 4-Sampler Clamshell	52/pk
10002	Mitra 10 µL Microsampler 3-Sampler Clamshell	52/pk
10001	Mitra 10 µL Microsampler 2-Sampler Clamshell	52/pk
100	Mitra Drying Rack	1 ea
102	Mitra Sampling Tool	1 ea
104	Protein Precipitation Plates	2/pk
103	96-Well Collection Plates, 2 mL Round Well with Round Bottom, 8mm	50/pk
105	Sealing Mats, Pierceable, 96-round well, 8 mm, silicone	50/pk
106	Sealing Mats, Pre-slit, 96-round well, 8 mm, silicone	50/pk
107	Sealing Tape Pad	10/pk

CE IVD

The Mitra Microsampling Device is a FDA listed Class 1 device (D254956). Neoteryx complies with FDA good manufacturing practices, CFR 820 regulations, and ISO 13485

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