A Novel Dried Matrix Microsampling Device that Eliminates the Volume Based Hematocrit Bias Associated with DBS Sub-punch Workflows

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PO21860414_W_2

Introduction

Dried blood spots (DBS) have gained attention as a microsampling tool due to the small blood volumes required, simplicity, low cost, and ease of transport. In a typical DBS workflow a small disk of the spotted and dried blood spot is punched out. These “sub-punches” are assumed to contain a fixed volume of blood. However, the viscosity of blood has a positive correlation with the hematocrit (HCT) or packed cell volume (PCV) of the blood. Therefore, if a uniform volume of blood is placed onto a DBS card then the size of the spot formed will decrease as the hematocrit of the blood increases. This leads to a volumetric assay bias that is associated with the hematocrit of the blood sample.

In order to overcome this volumetric assay bias we have developed a novel microsampling technology that is designed to allow for precise volumetric absorptive microsampling (or VAMS™). We then illustrate the use of the tool in the collection and analysis of blood samples following the approach of drying the samples and extracting them in a fashion similar to current practice with DBS cards.

Materials and Methods

Gravimetric analysis was performed using a Sartorious microbalance with six digit readability. The densities of the matrices were determined for the conversion of mass to volume. Generally, the densities conformed to 1.025 mg/µL for plasma and 1.125 mg/µL for red blood cells, and an appropriate density/volume weighting was applicable when considering various hematocrit levels. The protocol for mass measurement was as follows: The microbalance was tared with the fluid to be sampled in place on a weighing dish. An autozero function was used to eliminate evaporative effects. The samples were collected using prototypes of the Mitra™ microsampling device based on the VAMS technology (**Illustration 1**), and absorbed mass was determined by loss. Analysis of drying of the device was also determined by loss using a fixture to hold the tips on the microbalance.

The general protocol for sampling is as follows: The tip of the Mitra microsampling device was touched to, but not completely immersed into the fluid to be collected. An additional two seconds of contact is required after the user visually determines that the tip is completely full in order to ensure uniform collection.

Blood with hematocrits above or below the normal range (43-48 %) was formulated through pulse centrifugation of whole blood at 7,000 RPM followed by the addition or removal of plasma as necessary to generate the appropriate hematocrit. Hematocrit determination was performed by centrifugation of a capillary tube containing ~60 µL of blood at 10,000 RPM for five minutes and then reported as a percentage of the length of the packed cell volume versus the total length.

A Multi-Matrix Extraction Study was performed by preparing matrices (Plasma, and whole blood at 20 %, 45 %, and 65 % Hematocrit) spiked with known concentrations of Acetaminophen. These matrices were prepared for analysis by collection with a Mitra microsampler (as above), or by spotting 10 µL onto a Whatman™ FTA™ DMPK-C card (lot# FE6847009). The Mitra microsamplers and DMPK-C Cards were allowed to dry for between 2-8 hours prior to extraction. The whole spot was collected from the DMPK-C card for extraction. Samples were placed into a 2 mL deep well collection plate and extracted by vortexing in the presence of 300 µL of methanol containing deuterated internal standard for Acetaminophen at 1200 rpm for 15 minutes. Extracts were centrifuged at 500g for 5 minutes, and 150 µL of supernatant was transferred to a new collection plate for evaporation followed by reconstitution in 150 µL of 90/10 Water/Methanol by vortexing at 1200 RPM for 10 minutes. These samples were directly injected for analysis.

An on tip stability study for naproxen was performed by generating a matrix matched calibration curve at 0.5, 1, 2.5, 5, 25, and 50 µg/mL of naproxen on each day of analysis for determination of the assay bias. Stability samples were prepared on the 1st day of the study at concentrations of 2, 8, and 40 µg/mL. QC samples were prepared on the 3rd, 5th, 9th, and 14th day of the study at concentrations of 2, 8, and 40 µg/mL. Stability samples and QC samples were analyzed (N=6) on days 3, 5, 9, and 14 while only stability samples were analyzed on the 1st day of the study. These results were compared against the matrix matched calibration curve generated on each day and presented as an assay bias for each day of the study.

The analytical method for the multi-matrix extraction and naproxen stability analysis was as follows:

Column: Kinetex 2.6 µm C18 100A
Dimensions: 50 x 2.1 mm
Part No.: 00B-4462-AN
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

Gradient:

Time(min)	B (%)
0	5
2	95
4	95
4.01	5
7.01	5

Flow Rate: 0.4 mL/min
Temperature: Ambient
Detection: AB Sciex API 3000™
Tandem Mass Spec (MS-MS) (ambient)
Sample: LLOQs of: Acetaminophen (25 ng/mL), Metoprolol (5 ng/mL), Naproxen(100 ng/mL), Amitriptyline (50 ng/mL), or multiples of LLOQ as shown in figure.

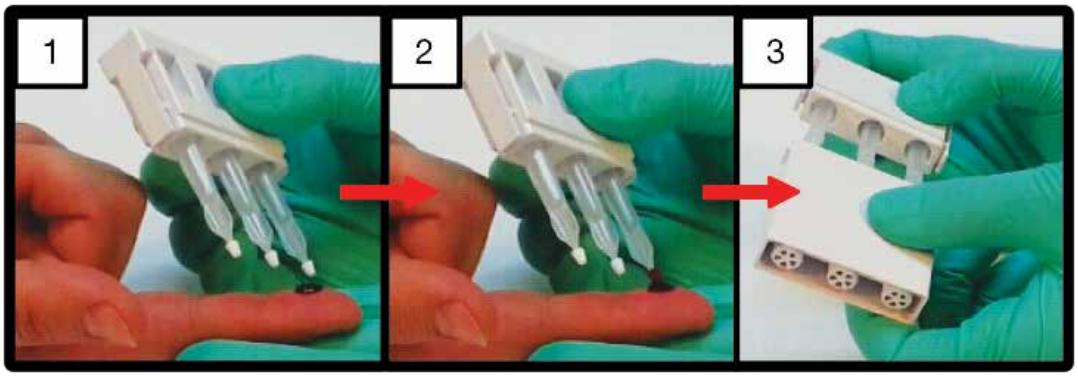
Transition Table:

Q1 Mass	Q3 Mass	Time	ID	DP	FP	EP	CE	CXP
Probes (ESI+)								
152	110	50	Acetaminophen	21	100	10	23	18
231	185	50	Naproxen	26	120	10	19	10

Results and Discussion

The Mitra microsampling device based on the Volumetric Absorptive Microsampling (VAMS) technology is a device for collecting fluid, and it is formatted to allow direct sampling from a host (**Illustration 1**). The process that is used for collection of the fluid is simple. The white tip of the device is placed in contact with the fluid to be collected and the fluid enters the tip. The user holds the tip in place for two seconds after the tip is completely full and the sampling is finished. In order to test the volumetric precision of this device we chose to use whole blood as a matrix for our analysis, and to evaluate a wide range of sampling conditions.

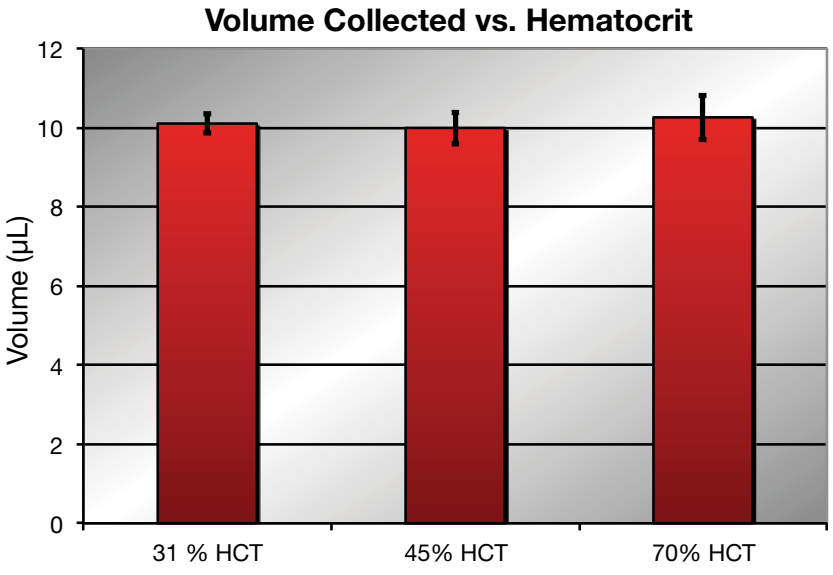
ILLUSTRATION 1.
VAMS sampling tool before (1) and after (2) sampling, and then placed into a cartridge (3).



Volumetric Accuracy and Precision

We performed an analysis of the volumetric precision of the Mitra microsamplers when sampling low, normal, and high hematocrit blood (**Figure 1**). We determined that across 30 probes and 3 different hematocrit levels the average volumes collected were 10.1, 10.0, 10.3 µL respectively for 31 %, 45 %, and 70 % HCT Blood. The RSDs for these measurements were between 2.4 % and 4.0 % and the volumes collected at all three hematocrit levels are indistinguishable statistically.

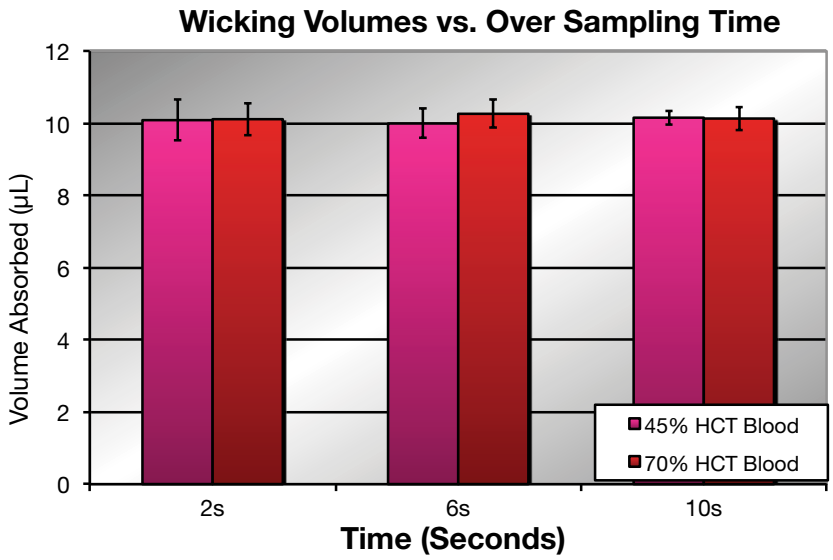
FIGURE 1.
Determination of absorbed volume at 31 %, 45 %, and 70 % Hematocrit.



Results and Discussion *(Cont'd)*

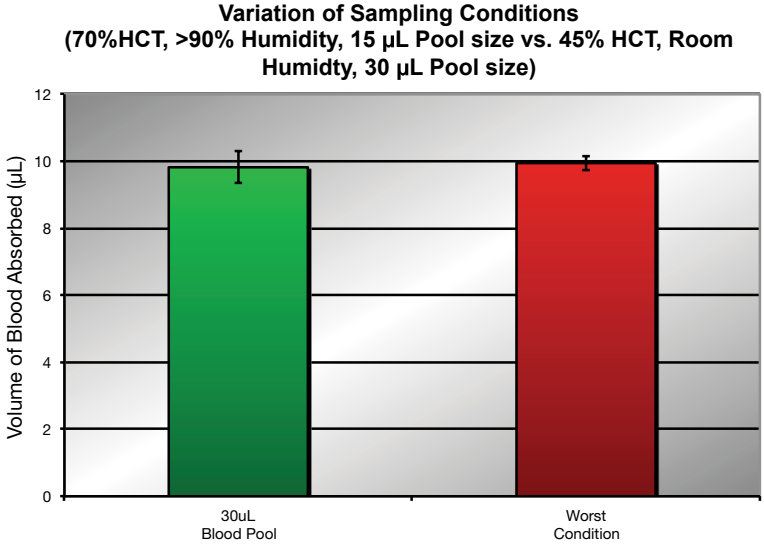
Next, we determined the effect of longer than prescribed exposure times to a pool of 45 % HCT blood (**Figure 2**). We sampled from 30 µL pools of either 45 % or 70 % HCT blood until the sampling tool was completely full, and then kept the tool in contact with the blood for an additional 2 seconds (as prescribed), 6 seconds, and 10 seconds. The sampling was again volumetrically precise at ~10 µL and there was no additional effect due to longer contact times with the pool of blood

FIGURE 2.
Absorbed volume at 45 %, and 70 % Hematocrit with long exposure times to sample.



As a final evaluation of the volumetric sampling characteristics of the Mitra microsampler we performed a collection of 70 % HCT blood from a limited sample of 15 µL after storing the part at > 90 % humidity for two days, and compared volume absorbed to 45 % HCT blood from a larger sample pool of 30 µL under ambient conditions. Once again, we were unable to distinguish between the volumes collected by the Mitra microsampler under these two distinct sets of conditions (**Figure 3**).

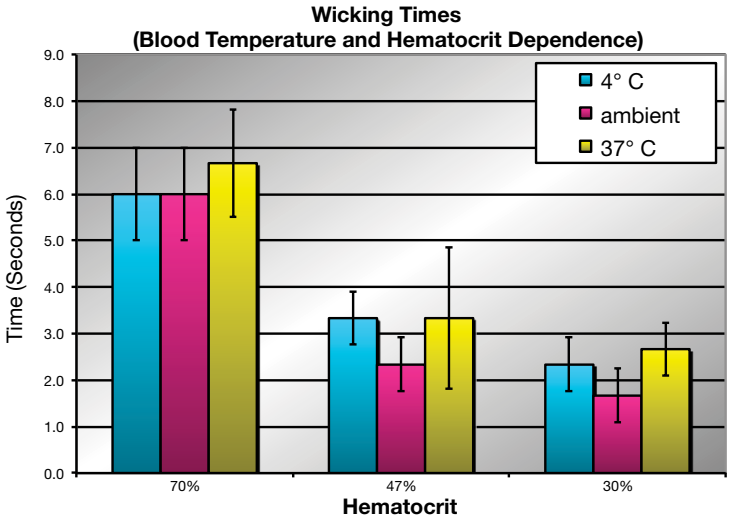
FIGURE 3.
Combined effect of humidity, small liquid pool size, and hematocrit on volume absorbed.



Results and Discussion *(Cont'd)*

Throughout the previous set of studies it was observed that the collection of high hematocrit blood (either 65 % or 70 %) was slower than with lower hematocrit levels of blood. We postulated that either the viscosity of the blood or the additional solids within the blood could be playing a role in the slower collection speeds. To understand this effect, we measured the amount of time required for the sampling tool to become completely filled with blood of various hematocrits across a range of temperatures (4 °C, ~25°, and 37 °C). Blood with an appropriate hematocrit (**Figure 4**) was prepared and stored at the temperature to be tested. When ready for testing, the blood was removed from its controlled temperature environment and immediately tested to determine the amount of time required for collection to be completed. There was very little observable effect from the storage of blood at different temperatures. However, the results clearly indicate that while 30 % and 47 % HCT blood are collected at approximately the same speed, the high hematocrit blood (70 %) took twice as long to be collected into the device (~6 seconds).

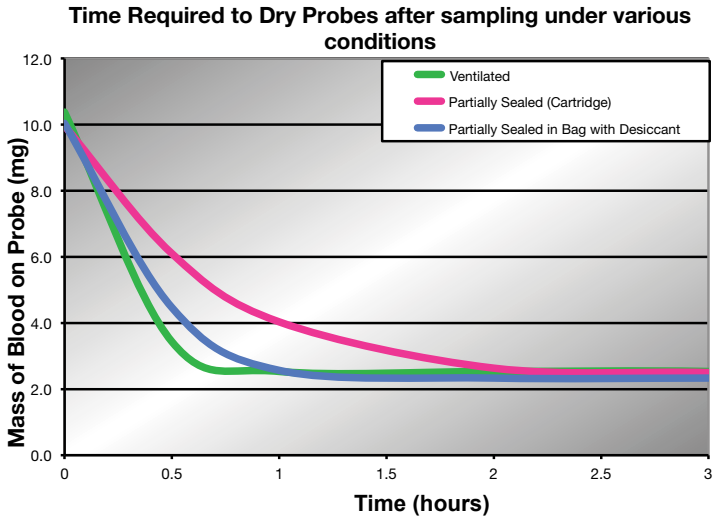
FIGURE 4.
Effect of blood temperature and Hematocrit on sampling times.



Drying Time Analysis

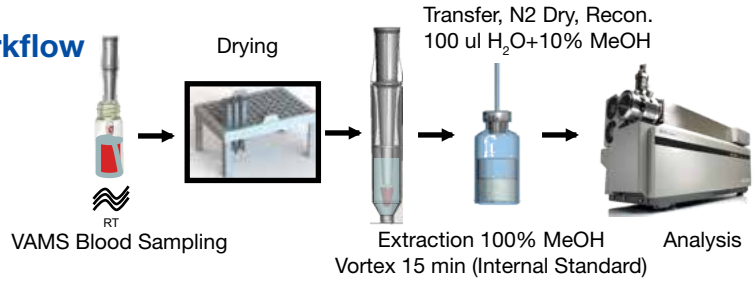
To determine the workflow for the Mitra microsampler in the context of dried matrix sampling we performed a gravimetric analysis of the time required to dry blood that is collected with the tool (**Figure 5**). We studied three different conditions 1) Complete ventilation 2) Sealed in a cartridge (shown as (**3**) in **Illustration 1**). Sealed in a cartridge placed into a bag with desiccant. The samples are dry with complete ventilation or packed with desiccant within 1 hour. When sealed in a cartridge the sample is dried at the two hour time point.

FIGURE 5.
Gravimetric analysis of drying times under various conditions.



Results and Discussion *(Cont'd)*

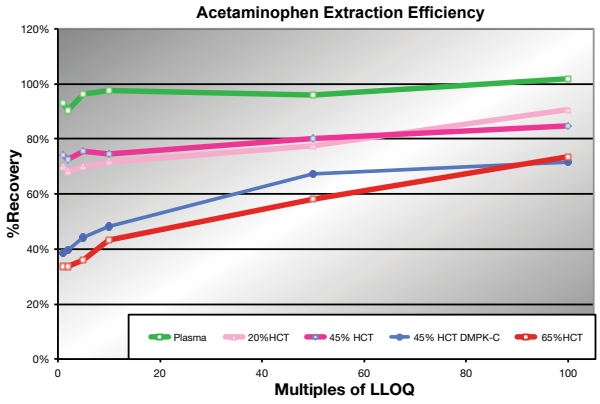
ILLUSTRATION 2.
Summary of VAMS workflow



Multi-Matrix Recovery Analysis

We studied the performance of the Mitra microsampler using a number of different matrices, analytes, and analyte concentrations. The workflow used follows the process described in the materials and methods section. Acetaminophen was evaluated using plasma, 20 % HCT, 45 % and 65 % HCT blood. These results were compared to a Whatman DMPK-C card using a whole blood spot extraction and 45 % HCT blood (**Figure 6**). We observed increasing absolute recovery with concentration, and a loss of recovery with increasing red blood cell content. The results using the Whatman DMPK-C card provided slightly lower recoveries than with the Mitra microsampler at 45 % HCT. In this study the collection of the blood was volumetric in nature, and it is clear that the hematocrit of the blood had an effect on the outcome of the assay. Our experience is that this effect is dependent on the analyte and the concentration of the analyte. Extraction optimization studies (not presented here) indicate that optimization of extraction methods can reduce or eliminate the effect of hematocrit on an assay, and that a suitable method of optimization is to identify the conditions with the poorest recovery (i.e. low concentration/high hematocrit) and to identify an extraction method that yields >85 % absolute recovery.

FIGURE 6.
Recovery of acetaminophen with variation of Hematocrit and concentration. Comparison to DMPK-C card at 45 % Hematocrit.

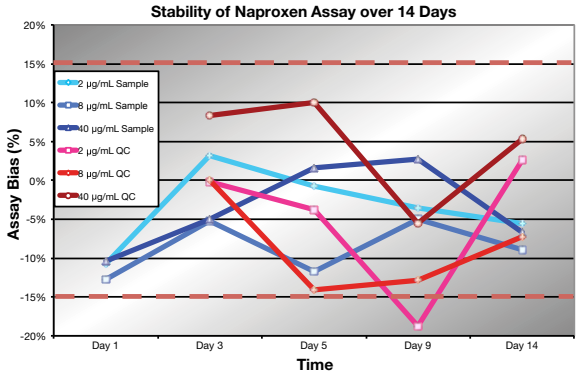


Results and Discussion *(Cont'd)*

Naproxen Stability Study

Finally, we performed an analysis of the stability of an assay using Mitra microsamplers that had been used for collection and then aged for up to 14 days using the methodology described above. Only one data point across the series fell outside of common acceptance criterion (± 15 % assay bias; indicated by the red dotted lines in **Figure 7**). The sample that "failed" was a QC sample at the lowest concentration used in the study (2 µg/mL) that was prepared on the 9th day of analysis. The sample that had been collected and stored for 9 days showed no such loss as compared to the calibration curve generated on day 9. Outliers such as this are not unusual is this type of study, but it does not escape our attention that a negative assay bias was common to the QC samples prepared on the day of analysis. Overall the results of this study suggest that an assay for naproxen will generate signals with acceptable responses for samples that are up to 14 days old.

FIGURE 7.
Naproxen assay bias for samples and QC's over a period of 14 days.



Conclusions

- The Mitra microsampling device based on the VAMS technology is volumetric in nature, and can collect a consistent amount of blood across a range of hematocrits and sampling conditions.
- The Mitra microsampler has a simple workflow that allows for direct sampling from a host, and then requires drying, followed by extraction. No physical manipulation of the sample is required before extraction.
- The elimination of the volumetric bias caused by sampling blood of different hematocrits does not completely eliminate the variation of signal/recovery that can occur in the extraction and analysis of blood samples with different hematocrits. Optimization of extraction methodology may be required to eliminate hematocrit effects.
- The extraction of naproxen yields consistent recoveries across a range of concentrations over a period of 14 days when collected and dried onto a Mitra microsampler.