Eliminating Volumetric Haematocrit Bias Associated with DBS Sub-Punch Workflows Using a Novel Microsampling Device which Absorbs a Fixed Volume of Blood

James Rudge[1], Stuart Kushon[3]; Allen Bischofberger[1]; Anna Carpenter[1]; Philip Denniff[2]; Yibo Guo[1]; Peter Rahn[1]; Neil Spooner[2]; Sally Osborne[1], Emmet Welch[1] Cathy Cordova[1], Jeff Layne[1]

[1]Phenomenex, Torrance, CA, USA; [2]GSK, Ware, UK, [3]Neoteryx, Torrance, CA

Dried blood spot (DBS) cards are routinely used as simple, low cost microsampling tools for clinical and preclinical applications. In general, when using a typical DBS workflow, the dried blood sample is sub-punched to generate a disk which is assumed to contain a fixed volume of blood. This sub-punch is then extracted for analysis. The drawback of this approach is that viscosity of the collected blood will influence the extent to which the blood spot spreads across the DBS card. Thus, fixed volumes of blood which have different haematocrit (HCT) levels will generate different sized spots on the DBS card, and the resulting sub-punches will therefore contain different volumes of blood. This can lead to assay bias as a function of blood haematocrit levels. Here we describe a novel microsampling device that eliminates the volumetric assay bias seen with conventional DBS sub-punch sampling.

Key words

DBS, Haematocrit, Assay bias, microsampling, Dried Blood Spot, Mitra™, VAMS™

Introduction

Microsampling of blood and other biological fluids is an attractive means to collect, transport and extract low volumes (e.g. <100 µL) of samples. Processing blood samples through standard blood collection techniques raises concerns relating to total volume of blood taken and the quality of the resulting data. Currently, sampling for preclinical and clinical LC/MS assays typically requires plasma volumes of around 100 µL or more. For healthy individuals, plasma accounts for approximately 50% of the total blood volume. Thus for every 100 µL of plasma, 200 µL of blood is needed. For an adult human, 200 µL is a comparatively small volume of blood, but the situation becomes more of a challenge for subjects with limited blood volumes.

Microsampling can also be a very attractive technique when applied in preclinical toxicokinetics (TK) studies. Historically, pooled data from a number of rodents is often employed to generate the blood volumes necessary for data spanning several time points. However, it has been noted that better TK profiles are often generated when blood is taken from one individual, as pooling data from several animals causes more data scatter and increased uncertainty in the TK data [1,2]. The reduced sample volumes required by effective microsampling techniques, as compared to traditional sampling methods, enables the use of serial sampling in rodent or other small animal TK studies. Moreover, there is a general desire to reduce the overall use of animals in laboratories, and in the UK both pharmaceutical companies and animal testing facilities are tasked with aligning themselves with the recommendations from NC3Rs (National Centre for the replacement, refinement & reduction of animals in research) [3]. Microsampling provides solutions to these issues and one such approach is Dried Blood Spot (DBS) analysis.

Dried blood spotting (DBS) is a very popular microsampling technique. To process a sample through the DBS technique, blood is first spotted onto a specialised absorbent card. The technique for collection of the initial blood spot is depending upon the application. In the case of neonatal screening, the heel of a baby is pricked with a lancet and the paper is then directly applied to the blood bolus and a suitable diameter spot is allowed to absorb onto the card [4]. The card can then be dried and sent to the laboratory via the post. In the laboratory, the dried blood spot is 'sub-punched' to create a smaller disk (typically 3mm in diameter) containing an undefined volume of blood. The disk is then added to an extraction solution often containing an internal standard. If the extraction solvent is an organic solvent, then the extraction is very similar to protein precipitation where the cellular contents including the protein, precipitate and then are immobilised on the disk. However, small molecules, including some peptides which are soluble in organic solvent, can also be extracted into the medium. The presence of these co-extracted molecules can sometimes interfere with subsequent downstream analysis of the target analytes. Thus, once extracted, the samples are sometimes further processed using techniques such as solid-phase extraction (SPE) prior to use in an analytical method.

A major drawback of microsampling via DBS cards, is the volumetric haematocrit bias which results from the differential behaviour of blood as a function of blood viscosity. Blood viscosity is governed by the percent haematocrit of the blood, which can range from 25-70% of the total blood volume. The higher the blood haematocrit and the more viscous the blood, the less the resulting blood spot spreads out on the DBS card (*Figure 1*). Thus, a sub-punch taken from a high haematocrit bloodspot will contain more blood than the same sized sub-punch obtained from blood spot of equivalent

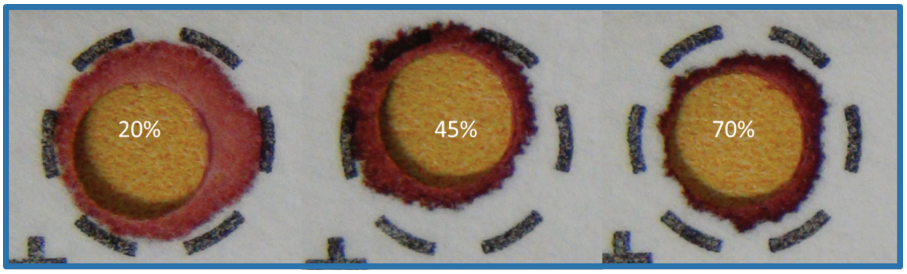

Figure 1. DBS spots obtained from an identical volume of blood at three different haematocrit levels (20%, 45%, and 70%). Identical sub-punches from each spot will contain differing volumes of blood.

volume but lower haematocrit level. The discrepancy in the actual volume of blood collected due to the differing haematocrit leads to an analytical bias which can be as high as 40% across the mean [1]. This bias makes accurate quantification across individuals with differing haematocrit levels extremely difficult, if not impossible.

In this paper, we present data obtained using a novel microsampling tool that was designed to overcome the deficiencies of the standard DBS technique. The Mitra™ Microsampler, using patent-pending VAMS™ (Volumetric Absorptive MicroSampling) technology, can simply, rapidly, and accurately (<4% RSD) collect a fixed volume (10 µL) of blood (or other biological fluid) and enable the generation of quantitative analytical results that are independent of blood haematocrit. The sampler is based on the design of a pipette tip and therefore has the potential to be compatible with automated liquid handlers.

Experimental

Sampling using the Microsampler

The white tips of the device were placed in contact with the top of the blood pool at a positive angle and the blood was allowed to enter the tip through capillary action until the tip was saturated (typically 2-6 seconds dependent on %HCT) and then held for a further two seconds (*Figure 2*). Upon saturation, the tips were measured for volumetric accuracy and precision before measuring drying times.

Gravimetric Analysis of Volumetric Accuracy and Precision

A Sartorious (Goettingen, Germany) Micro Balance Pro 11 was calibrated and tested using Mettler Toldeo (Greifensee, Switzerland) calibration weights prior to analysis (ensuring a result with a deviation of less than 1% from the expected mass of the weight). A weighing boat was loaded with 200 µL of the matrix for testing (of various HCT). The scale was then tared using the autozero function to account for any evaporation that might occur before the sampling event. Then a sample was collected using the standard collection method. Care was taken to touch only the tip to the surface of the blood, and to not submerge the entirety of the tip into the blood sample in order to allow capillary action to fill the tip. The loss of mass was recorded once the microbalance achieved weight stability. The result was converted to volume using the density of the blood sample that was used.

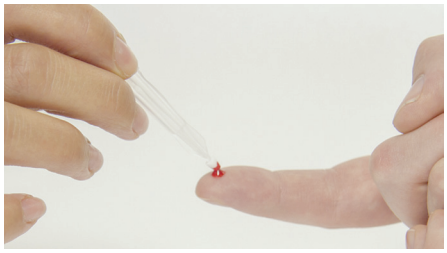

Figure 2. Blood being sampled using the microsampler.

Determination and Modification of Haematocrit

Pooled whole human blood (multiple lots) for this study was purchased from BioreclamationIVT (New York, USA). The HCT of the blood was determined by using a haematocrit capillary to collect a sample of blood and then using a haematocrit centrifuge to separate the packed cells from the plasma. The percent volume of the packed cells is reported as the haematocrit. The haematocrit of larger quantities of blood was adjusted by centrifugation of the blood sample at 2000 g for 3 minutes, and then either adding or removing plasma to achieve the desired haematocrit level.

Results and Discussion

Volumetric Accuracy and Precision

Analysis of the volumetric precision of the microsamplers was conducted by sampling a wide HCT range across the excepted normal level (~45%). This was determined across 30 samplers and 3 different hematocrit levels (31%, 45% and 70%). The average volumes collected were 10.11, 10.00, 10.26 µL. The

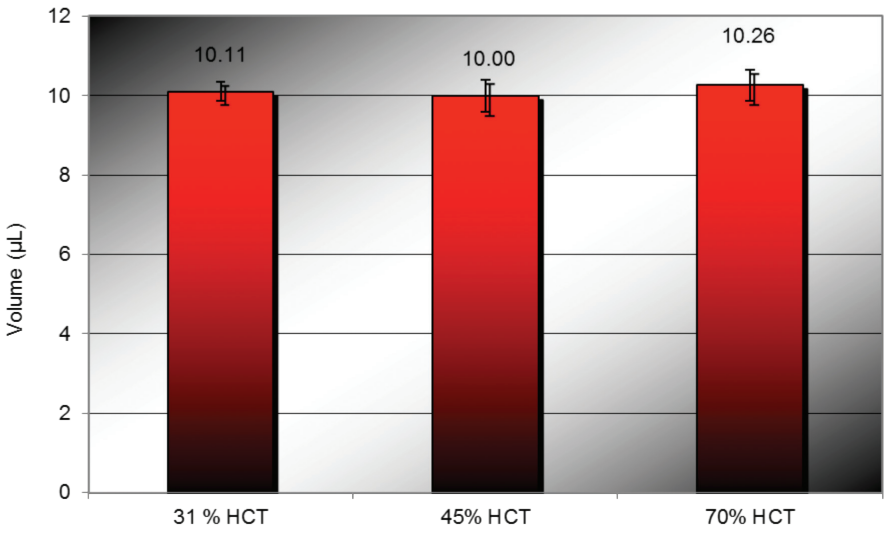

Figure 3. Haematocrit independent absorption of a fixed volume blood on the samplers

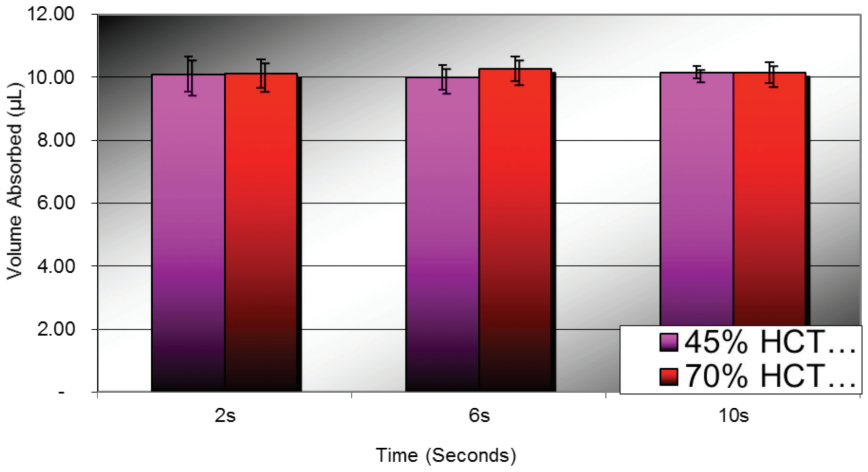

Figure 4. Haematocrit independent absorption of a fixed volume blood on the samplers over a range of sampling over sampling times.

RSDs for these measurements were between 2.4% and 4.0% (*Figure 3*). Confirmation of this data has been recently reported where Denniff and Spooner measured mass on tip with radioactive caffeine [5]. Further confirmation of this data has been reported in a joint publication (in press) [6].

In order to ensure that the samplers could not easily be oversaturated through timed exposure of the blood, the sampler was exposed to the 30 µl pools of blood (45% or 70% HCT) for an additional 2, 6 and 10 seconds after saturation. It was found that once the tips had saturated, no further increase in weight was observed, indicating that further absorption of blood had not taken place (*Figure 4*).

The samplers were then tested for their absorptive properties when exposed to extreme sampling conditions, such as humidity and temperature. Thus samplers from two days storage in high humidity (> 90%) were exposed to 15 µl pools of 70% haematocrit blood. These were compared to samplers stored in ambient conditions and sampled in 30 µl pools of 45% haematocrit blood. Once again there was no difference between the samplers under the two conditions tested (*Figure 5*).

The samplers were then tested for speed of absorption with respect to temperature as it had been noticed that high HCT blood (60% or 70%) absorbed more slowly into the sampler (6 seconds). It was postulated that this could be a viscosity related effect and was presumed that the cooler the blood, the more viscous it would be so the slower the ingress into the sampler. For this test, three different HCT blood samples taken at increasing HCT concentrations (31%, 47% and 70%). Speed of absorption was measured at three temperatures (4°C, ambient (~25°C), and 37°C). It was noted that the speed of absorption was independent of the temperatures tested and that wicking times were only effected by % HCT (*Figure 6*).

Drying Time Analysis

The CDC considers blood in its dry form as safe to send in the post with certain conditions applied and many other countries adopt this policy too [7]. It was, therefore, important to measure the drying time of the samplers in different conditions. The first set of samplers were totally exposed to the air, the second set were partially sealed and the final set were sealed with desiccant. *Figure 7* shows the results for different drying times which shows that the samplers will dry in the closed cartridge but it takes longer than when they are fully open to the air.

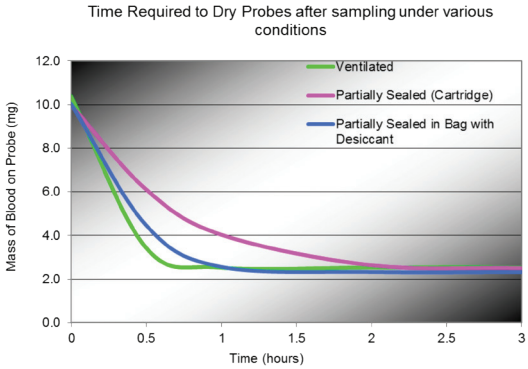

Figure 7. Differences in drying times of samplers based on their drying conditions.

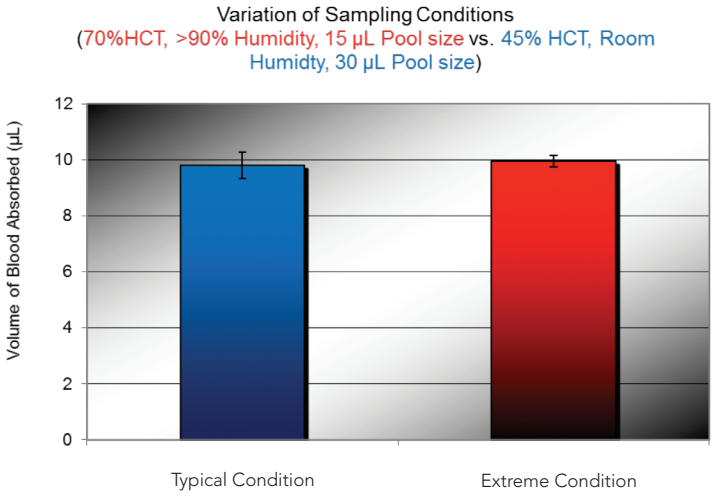

Figure 5. No reduction of blood absorption is seen when samplers have been stored in high humidity and used to sample a restricted volume of high haematocrit blood..

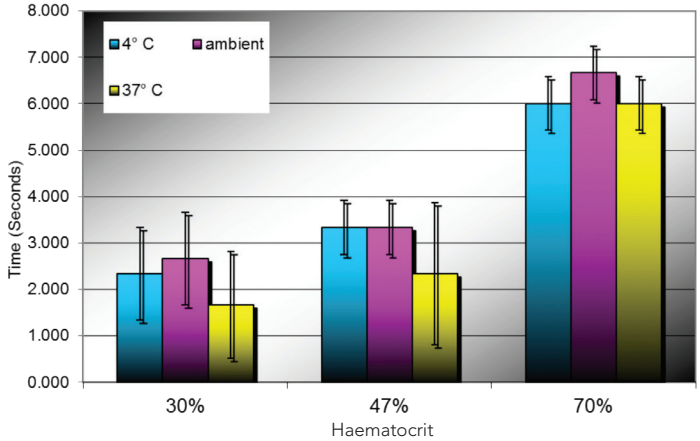

Figure 6. Temperature does not influence wicking times of a wide range of Haematocrit blood samples.

Conclusions

Interest in the use of microsampling techniques is increasing in a wide variety of applications and industries. While DBS cards are well-established for certain niche applications, such as newborn infant screening, the well-documented problem that DBS cards have with haematocrit bias has slowed its adoption for use in quantitative analyses. In this work, we have presented data generated using the new microsampler which can simply and accurately collect a fixed volume of blood independent of percent HCT. In addition, the device absorbs blood consistently at a range of temperatures, sample pools sizes, and time spent in the pool. Theses unique properties allows the microsampler to provide all of the benefits associated with DBS sampling (low sample volumes, easy shipment and storage, sample stability) without the drawback of volumetric haematocrit bias, enabling the microsampler to be used for both semi-quantitative and quantitative assays. Lastly, the simplified blood collection workflow of the microsampler reduces the amount of sample manipulation associated with the traditional DBS sub-punch work profile.

References

[1] P. Denniff, N. Spooner, Bioanalysis, 2(8), (2010) 1385

[2] N. Powles-Glover, S. Kirk, C. Wilkinson, S. Robinson, J. Stewart., Regul Toxicol Pharmacol., 68(3), (2014), 325

[3] http://www.nc3rs.org.uk/page.asp?id=7

[4] http://www.medhelp.org/lib/pku.htm

[5] P. Denniff, N. Spooner, Anal. Chem., 86 (16), (2014), 8489

[6] N. Spooner, P. Denniff, L. Michielsen, R. De Vries, Q.C. Ji, M.E. Arnold, K. Woods, E.J. Woolf, Y. Xu, V, Boutet, P. Zane, S. Kushon, J.B. Rudge, A device for dried blood microsampling in quantitative bioanalysis - Overcoming the issues associated blood hematocrit, Bioanalysis, (2014), In Press.

[7] http://www.cdc.gov/labstandards/pdf/nsqap/Blood-spot_Transportation_Guidelines.pdf