Advantages and Limitations of Microsampling, Hematocrit and MicroLC in DBS: Quantitative Bioanalytical Assays for Immunosuppressants, Warfarin and Methylphenidate in Whole Blood

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WORLDWIDE CLINICAL TRIALS
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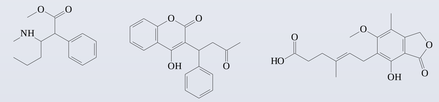
INTRODUCTION

Analysis of dried blood spots (DBS) in therapeutic drug monitoring, rodent pharmacokinetic and toxicokinetic studies, and newborn screening is popular due to its reduced volume requirements and ease of sample collection, handling and transportation. We present our latest findings on quantitative bioanalysis of a variety of immunosuppressants, warfarin and methylphenidate utilizing various microsampling techniques. Volumetric assay bias due to hematocrit has been a center of attention and the focus of our experiments. Fully automated direct on-line analysis of DBS cards by Camag DBS-MS 500, perforated DBS card samples and Mitra Volumetric Absorptive Microsampling were evaluated for hematocrit variability, recovery and quantitation. Both normal and low flow (50 µL/min) analyses were performed.

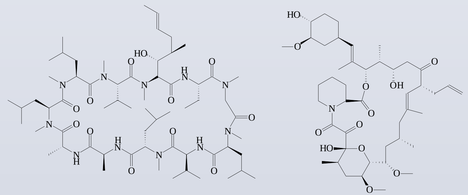
STRUCTURES

Monoisotopic mass in parenthesis

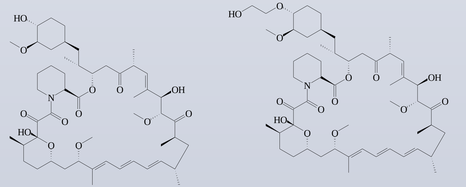
Methylphenidate (233.1) **Warfarin** (308.1) **Mycophenolic Acid** (320.1)



Cyclosporin A (1201.8) **Tacrolimus (803.5)**



Sirolimus (913.6) **Everolimus** (957.6)



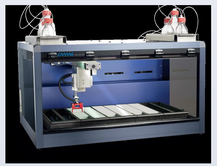
RESULTS and DISCUSSION

Sample volumes were 15 µL for Camag, 7 uL for perforated disc DBS cards, and 10 µL for Mitra. Linear calibration curve ranges were:

- 5 to 2500 ng/mL (cyclosporin A, sirolimus, mycophenolic acid and warfarin)
- 10 to 500 ng/mL (everolimus)
- 2 to 1000 ng/mL (tacrolimus)
- 0.10 to 100 ng/mL (methylphenidate)

For immunosuppresive drugs and warfarin, our initial evaluation of hematocrit values, between 0.20 and 0.70, by different microsampling techniques indicated variability ranged from 8% to 50%CV using acetonitrile/water (50:50) as extraction solvent. Extraction with various solvent mixtures indicated that 90% methanol in water was the best solvent mixture for punched DBS card samples; recovery ranged from 55% to 82% for all six analytes. For Mitra tips, zinc sulfate precipitation with microflow LC and QTRAP 6500 LC-MS/MS gave 90% or better recovery for all seven analytes. Micro LC and QTRAP mass spectrometer offer reduced solvent consumption and low limits of quantification with microsampling.

Camag DBS-MS 500



The Camag DBS-MS 500, capable of analyzing 500 DBS cards, contains an integrated camera for spot analysis and centering, an automated internal standard application and direct online extraction module interfaced with LC-MS/MS. We had previously presented the analysis of topiramate, warfarin, acetaminophen, and HIV protease inhibitors (ritonavir, lopinavir and saquinavir) in human blood using the Camag system [1]. We also presented the first proof of principle of direct elution [2]. In this study, we tested methylphenidate, immunosuppressive drugs and warfarin in human blood (15 uL) using the DBS-MS 500 integrated with a Sciex API 4000 and QTRAP 6500. Using a low extraction volume of 20 uL delivers enhanced sensitivity. Internal standard (20 µL) was applied by automated online spraying module and was dried for at least one hour before samples were eluted directly onto LC-MS/MS. Elution solvent was 90% methanol in water. For all analytes, bias for hematocrit (Hct) values of 0.30, 0.40, 0.50, and 0.60 was within ± 20%. Methylphenidate data for LLOQ and QC samples (15 µL) in whole blood are presented in **Table 1**. **Figure 1** presents a representative chromatogram of the LLOQ sample for methylphenidate in whole blood.

Samples (n=6)	%Nominal	%CV
LLOQ 0.100 ng/mL	113	5.35
QC Low 0.200 ng/mL	93.0	3.36
QC Medium 5.00 ng/mL	91.4	10.6
QC High 90.0 ng/mL	94.6	4.47

Table 1: Accuracy and Precision

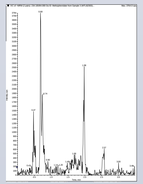
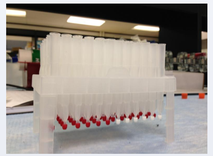


Figure 1: Extracted LLOQ Sample

RESULTS and DISCUSSION

Mitra VAMS



Mitra microsampling device is a simple dried matrix microsampling device, based on volumetric absorptive microsampling (VAMS™) technology, which collects a fixed volume of blood and other biological fluids. It is a novel approach for DBS quantitative bioanalysis that overcomes the Hct bias and homogeneity issues associated with conventional punched DBS samples [3]. Dried samples on Mitra tips in 96-well plate were vortexed with 100 µL of water containing internal standard for 30 minutes. Mitra tips were removed and 100 µL of zinc sulfate solution (28.8 mg/mL) was added and vortexed for 5 minutes. Lastly, 100 µL of acetonitrile was added and 5 minutes of vortexing was carried out. After centrifugation, 200 µL of supernatant was transferred to another 96-well plate by automation and injected onto the QTRAP 6500 using microflow LC. For methylphenidate, the quantitation range was linear from 0.10 to 100 ng/mL based on a 10 uL blood sample collected on Mitra tips. **Table 2** exhibits accuracy and precision data for methylphenidate LLOQ and QC samples. We found that bias between 0.20 and 0.70 Hct was within ± 20% at two concentration levels (1.0 and 45 ng/mL). Microflow LC-MS/MS (QTRAP 6500) was utilized to compensate for the low sample volume. Recovery of methylphenidate in dried blood from Mitra tips was 95% and bench top stability at room temperature was established for 72 hours. **Figure 2** presents extracted LLOQ sample chromatogram for methylphenidate. A chromatogram of extracted QC Low sample for immunosuppressants and warfarin is displayed in **Figure 3**.

Samples (n=12)	%Nominal	%CV
LLOQ 0.100 ng/mL	89.3	15.1
QC Low 0.200 ng/mL	84.2	10.4
QC Medium 5.00 ng/mL	86.8	5.58
QC High 90.0 ng/mL	97.7	2.97

Table 2: Accuracy and Precision

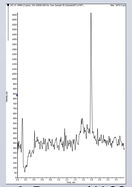


Figure 2: Extracted LLOQ Sample

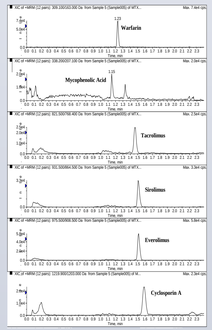
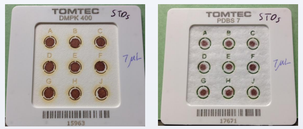


Figure 3: Extracted QC Low Sample

RESULTS and DISCUSSION

Tomtec Perforated DBS Discs



Tomtec perforated discs overcome Hct variability by sampling the entire spot contained within the perforation. Two types of Tomtec perforated discs, DMPK400 (cellulose) and PDBS7 (polyester) were evaluated for our analysis of Hct bias on the bioanalysis of immunosuppressive drugs and warfarin. The spotted sample volume was 7 uL. The entire sample spot was punched out into 96-well plate and vortexed with acetonitrile/water (1:1), containing internal standard, for 30 minutes. After centrifugation, an aliquot was transferred to another 96-well plate by automation and injected onto the LC-MS/MS. Bias between 0.20 and 0.70 Hct was ± 20% at both low and high concentration levels.

LC-MS/MS Conditions

	Immunosuppressants & Warfarin	Methylphenidate
Instrumentation	CTC PAL autosampler, Shimadzu LC system, QTRAP 6500 and API 4000 LC-MS/MS	
Mobile Phase A	5mM ammonium formate 0.1% formic acid in water	2mM ammonium trifluoroacetate 0.2% acetic acid in water
Mobile Phase B	5mM ammonium formate - 5% 0.1% formic acid in acetonitrile - 95%	Mobile Phase A - 5% Acetonitrile - 95%
Column	Phenomenex Luna 5µ Phenyl-Hexyl 50 x 0.50 mm @ 50 µL/min. Phenomenex Luna 3µ Phenyl-Hexyl 50 x 4.6 mm @ 0.800 mL/min. 60ºC	Phenomenex Luna 3µ C18, 50 x 0.50 mm @ 80 µL/min. Room temp
Gradient	0.50 min. 15%B 1.50 min. 90%B 2.00 min. 90%B 2.10 min. 100%B 2.90 min. 100%B 3.00 min. 15%B 3.80 min. Stop	0.1 min. 5%B 1.50 min. 25%B 2.00 min. 25%B 3.10 min. 85%B 4.10 min. 85%B 4.20 min. 5%B 5.00 min. Stop
MRM Transition Ions DP, CE, CXP	1219.9→1203.0 Cyclosporin A [DP 130, CE 25, CXP 15] 975.6→908.5 Everolimus [DP 90, CE 27, CXP 15] 931.6→871.5 Sirolimus [DP 80, CE 27, CXP 15] 821.5→768.4 Tacrolimus [DP 80, CE 31, CXP 15] 338.2→207.1 Mycophenolic Acid [DP 70, CE 32, CXP 7] 309.1→163.0 Warfarin [DP 70, CE 16, CXP 7]	234.1→84.0 DP 60 CE 25 CXP 7
IS voltage Temp.	5000 550	3900 520
Note	Ammoniated molecules for Immunosuppressants gave the best sensitivity	

Microflow LC

Flow rate limitations in the past required the use of capillary or microbore LC to reduce demands on vacuum systems. Much progress has been made to operate instruments at higher flow rates, conditions well suited for larger samples commonly available in human studies. However, when dealing with limited volume samples, scaling to smaller extraction volumes and lower flow rates affords significant improvements in sensitivity. These gains are due to mass concentration of the eluted peak as well as more efficient ionization at lower flow rates. At the same linear flow velocity, mass concentration improves as the inverse square of the column inner diameter. This assumes that the same injection volume can be loaded. Only under pure electrospray conditions (< 3 uL/min) can a more focused spray with greatest efficiency be achieved. The gains here are the result of the chromatographic focusing when moving from a 4.6 mm to a 0.5 mm column.

CONCLUSIONS

Preliminary quantitative analysis and hematocrit evaluation for different types of microsampling have provided valuable insights. Matrix effects, column stability, injection volume and batch size need to be further explored before fully validating the methods. The combination of microflow LC and microsampling aids studies in small animals (mice, rats, marmosets), pediatric or limited volume (tears, intracellular) studies. These new and evolving techniques have spurred renewed interest in regulated bioanalysis as a viable alternative or addition to a traditional plasma or blood assays. Advances in microsampling and microflow LC also reduce the cost and improve the quality of pharmacokinetic data obtained from single animals. Robustness of the methods and the ability to meet stringent GLP guidelines is top priority. Most, if not all, of the challenges observed in DBS and microsampling have assignable causes and are scientifically manageable [4].

REFERENCES

1] Lloyd T, Nunez S, Johnson R, Tan M. Full automation of dried blood spot/LC/MS/MS methods for topiramate, warfarin, acetaminophen, ritonavir, lopinavir and saquinavir in human whole blood. Presented at: 2013 AAPS Annual Meeting, San Antonio, TX, USA, November 12, 2013.

2] T Lloyd, J Short, P Paul. Exploration of Automated Sonication and Direct Elution Approaches for the Analysis of Drugs in Dried Blood Spots and Other Matrices. Presented at 2010 APA Annual Meeting, Baltimore, MD, USA, September 2010.

3] Denniff P, Spooner N. Volumetric absorptive microsampling: A dried sample collection technique for quantitative bioanalysis. *Anal. Chem.* 86, 8489-8495 (2014).

4] Timmerman P, White S, Cobb Z et al. Update of the EBF recommendation for the use of DBS in regulated bioanalysis integrating the conclusions from the EBF DBS-microsampling consortium. *Bioanalysis* 5(17), 2129-2136 (2013).

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