Introduction

There is significant and growing interest in the use of microsampling devices to streamline blood collection and transportation processes; to drive innovation in precision medicine and patient-centered care; and to enable remote patient monitoring, virtual clinical trials, and more. Microsampling devices provide several advantages over older methods, including the elimination of trained venipuncture staff and the travel required for venipunctures, along with the ability to generate better-quality data from smaller specimens than ever before.

In drug monitoring, microsampling applications have gained special attention. Therapeutic drugs often have narrow therapeutic ranges – by sampling patients remotely and having the concentration of the drug on hand during consultation, physicians can optimize and individualize their clinical health decisions. With remote sampling, clinicians also have access to a broader potential range of time points for sampling, and patients are given a greater sense of agency in their treatment.

Between 2017 and 2018, four unrelated research groups developed validated methods for the determination of antiepileptic drugs (AEDs) using the Mitra® microsampler. This review summarizes the methods developed by the four labs and aims to be a guide to the important workflow elements to consider when developing and validating a bioanalytical method for specimens collected with a Mitra microsampling device.

Case Studies: Group 1 | Group 2 | Group 3 | Group 4

Analysis of Work

The four groups evaluated their extractions of AEDs independently. The drugs evaluated are shown in **Table 1** along with their associated logP data. The general extraction approaches that were optimized are shown in **Table 2.**

COLLECTION	Abbreviation	Research Group	Generation	LogP
10-hydroxycarbamazepine	10-OH-OXC	1	newer	2.23
Carbamazepine	CBZ	1, 3, 4	1st	2.77
Carbamazepine-epoxide	CBZ-EP	1, 3	newer	2.58
Clobazam	COA2	4	newer	2.55; >pH 9 (2.55 --> -0.6)
Clonazepam	COA	4	newer	3.15
Ethosuximide	ETX	1	1st	0.55
Felbamate	FBM	1, 4	newer	0.68
Lacosamide	LCM	1	newer	-0.02
Lamotrigine	LTG	1, 4	newer	1.93; <pH 6 (1.93 --> -1.0)
Levetiracetam	LEV	1, 4	newer	-0.59
N-Desmethylclobazam	DSA	4	newer	2.69; >pH 4.5 (2.69 -->-0.5)
Nitrazepam	NTA	4	newer	2.55
Oxycarbazepine	OXC	1	newer	1.82
Perampanel	PMP	1	newer	
Phenobarbital	PHB	1, 3, 4	1st	1.4; >pH 6 (1.4 --> -2.5)
Phenytoin	PHT	1, 3, 4	1st	2.15; <pH 7 (2.15 --> -0.2)
Primidone	PRM	1	1st	1.12
Rufinamide	RUF	1, 4	newer	1.93
S-Licarbazepine	ELN	4	newer	1.73
Stiripentol	SIO	4	newer	3.12
Topiramate	TPM	1, 4	newer	0.13
Valproic Acid	VPA	3, 4	1st	2.8; <pH 4 (2.8--> -0.7)
Zonisamide	ZNS	1, 4	newer	0.11; pH<8 (.011 --> -0.8)

Table 1: Analyte List by Group - LogPs are approximated, and if variation occurs across pH range it is reported between pH 3-10, deviations outside that range are not reported.

	Research Group 1	Research Group 2	Research Group 3	Research Group 4
Analytes	16 AEDs (see table 1)	Novel AED	CBZ, VPA, PHT, PB, CBZ-E	16 AEDs (see table 1)
Pre-extraction Step	Presoak in Aqueous - 10 seconds	None	None	None
Initial Extraction	"Organic"	Aqueous w/0.1% formic acid	Acetonitrile/Water (80/20 v/v) at 60°C	Methanol/Water (80/20)
Sample Clean up	None	Precipitation w/ Acetonitrile	None	None
Agitation Methods	Shake 60 minutes at 600 RPM	Sonication followed by 60 minutes of Shaking	Shaking for 10 minutes at 1000 RPM	Stainless Steel Bead Grinding, 1750 RPM 10 minutes
Extraction Efficiencies	86%-112%	~100%	85.2%-93.7%	84%-99%
Hematocrit Bias Evaluated	Yes	Yes	Yes	Yes
Stability Evaluated	Yes	Yes	Yes	Yes
Address Whole Blood/ Plasma Ratios	Yes	Novel AED	Yes	Yes

Table 2: Extraction Methods

Review of Extraction Methods

Pre-extraction

Group 1 (de Grazia et al.) began their extraction study with a presoak under aqueous conditions.[1] The primary function of this step is to soften and solubilize the dried matrix. Group 1 used an organic extraction solvent. The main benefit of this type of extraction is the cleanliness of the extract due to the low solubility of many of the components of dried whole blood in these solvents (i.e. Methanol or Acetonitrile). The partial reconstitution of the dried blood prior to exposure to the organic extraction was shown to improve recoveries for their analytes.

Extraction

Group 2 (York Bioanalytical) performed an aqueous extraction followed by a protein precipitation step.[2] This approach is most effective with a low logP analyte. Their initial extraction method used a 20 minute vortex with water. However, a stability bias was observed as a function of increased drying and/or storage times of the samples. This resulted in an improved method that added 0.1% formic acid to the extraction solvent, a sonication step, and increased the shaking time to 60 minutes. The improved method was shown to eliminate the stability bias that was initially observed.

It is common for a stability bias to be observed with dried whole blood samples on the Mitra microsampler. The first situation where this is observed is when extractions are optimized with samples that have not completely dried. In this circumstance, samples for testing are normally very well dried, and extractions are less effective against a well dried matrix resulting in a negative stability bias.

The second situation where stability bias is observed is when extractions are optimized against samples that have only been dried for a short period of time (i.e. 1 day) and samples being received are much older (i.e. 5-30 days old). As the blood matrix ages, extractions become more difficult unless extractions are optimized.[5]

The other groups used a mixed aqueous organic extraction approach. Following the extraction Groups 1, 3, and 4 optioned for a dilute and shoot methodology to achieve higher throughputs. Group 1, as mentioned above, exposed their samples to a pre-wetting step with water, while Group 3 (Altasciences)[3] and Group 4 (Stove et al.)[4] used a mixture of aqueous and organic (ratio 1:4) in their extractions. The LogPs of the analytes extracted range from -0.59 to 3.15 (Table 2).

The extraction efficiencies achieved by all the groups was at least 84% illustrating the suitability of this solvent system. The use of water improves recovery as a function of *solubilization* of the matrix, however, this will also increase matrix effects. Therefore, minimal amounts of water are generally used in favor of reducing matrix effects.

Additional compensation for matrix effects is achieved using internal standards (as is common for LC-MS applications), and in these workflows the internal standards are present in the extraction solvent.

Agitation

The choice of agitation is critical to the extraction methodology. The most traditional method is shaking via a platform shaker or vortex, and the main variable is the RPM of shaking. The optimal RPM when using deep well plates is 1000-1100 because this is the maximum speed that will create a vortex. Sonication is also a strategy for improving extraction efficiencies as shown by Group 2 in their improved method. **Group 4 extracted using a bead beating apparatus.**

This tool utilizes stainless steel balls to create a mechanical disruption of the matrix through bead beating (an approach traditionally used for tissue homogenization). This mode of extraction is extremely efficient, only taking 10 minutes for the complete extraction.

Notably the tip of the Mitra microsampler is not masticated or destroyed during this high energy process allowing the sample to be easily removed from the wells for analysis. Finally, Group 3 elevated the temperature of their extraction solvent to 60 °C. While not an agitation approach, heating the solvent used for extraction is *expected to improve the kinetics of extraction*.

QCs and Standards

An approach used by Group 2 prepared all standards and QCs en-masse by aliquoting 80 µL of blood into all wells of a 96-well plate and dipping a full rack (96 samplers at a time) for efficient preparation of validation samples. They ensured that the standards and QCs were dried for a minimum of overnight to appropriately match the dried state of their samples.

Data Evaluation and Quality

Each of the groups performed an analysis of hematocrit (HCT) bias in their method development efforts. The normal approach is to evaluate three to five different HCT levels (from 0.20 to 0.65). Each group compared at least a high and low HCT sample to a median HCT reference sample (0.40-0.45 HCT), and in each case the results of the hematocrit bias studies yielded acceptable results for method validation.

For example, the evaluation of HCT bias by Group 4 indicated that at 4 HCT levels (0.21, 0.42, 0.52, and 0.62) the maximum observed bias was 18% for valproic acid at an HCT level of 0.62, all other analytes were within 15% at all HCT levels.

Each group also evaluated stability, and in each case acceptable results were obtained. **Group 3** illustrates a thorough stability study that explores multiple parameters: Time (4 days, 1 week, and 1 month), temperature (-20°C, 4°C, and 60°C), and concentration. Only at 1 month, did any analyte show a bias greater than 15%.

Whole blood to plasma correlations are also discussed in the works of Group 1 and Group 3. Slide 20 of the presentation of Group 1 provides references for the partitioning of analytes between blood cells and plasma, while Group 3 discusses the importance of the development of references ranges for whole blood.

Conclusion

These works provide a useful guide for those who are interested in the extraction of AEDs and other small molecules from Mitra microsamplers. In each case, the groups achieved a validated method with high extraction efficiency that was free of hematocrit bias. Stability biases were explored and found to be acceptable. **Group 2** **stated of their method**, "*The method performed with a 100% success rate and generated high quality pharmacokinetic data*".

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