bioanalytical methods for microsamples examined

immunosuppresants

neoteryx®
The blood microsampling innovator

Introduction

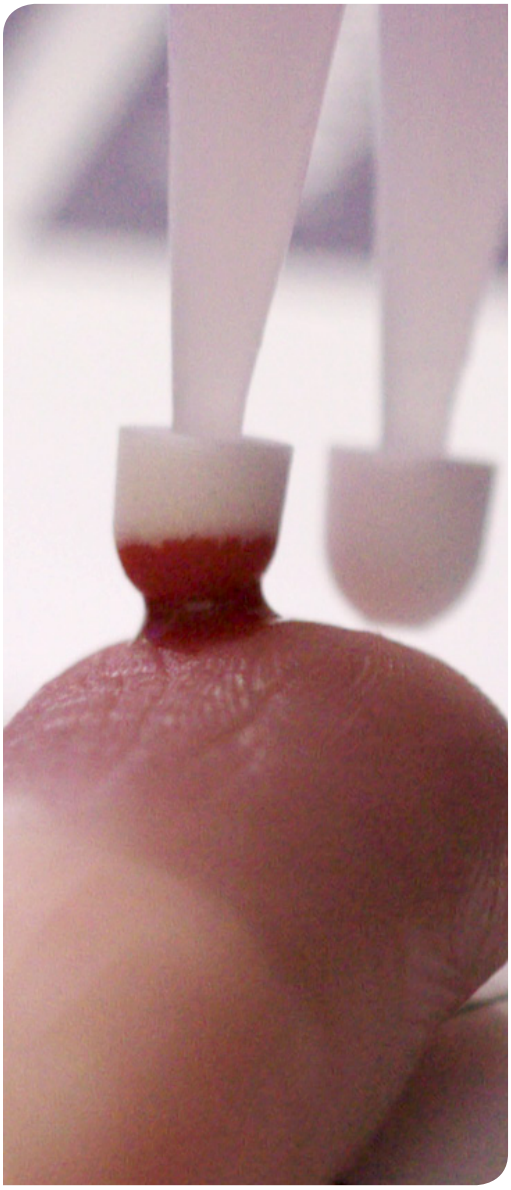
Recently, there has been significant and growing interest in the use of microsampling devices in the therapeutic drug monitoring community[1–12]. These devices have shown potential to streamline blood collection and transportation processes, drive innovation in precision medicine and patient-centered care and enable remote patient monitoring and virtual clinical trials.

When compared with traditional whole blood sampling methods, microsampling devices provide advantages in all aspects of the patient monitoring process. Patient experience is improved through what is considered a less painful and invasive sampling event than venipuncture; blood draws can be as low as 10 microliters (instead of up to 4 milliliters); and there is potential for an at home sampling event that would eliminate a trip to a blood draw center. Benefits to healthcare in general include: the potential for better testing adherence due to the improved patient experience, access to patients with low blood flow and access to patients in remote or underserved populations.

Cost benefits include: shipment by post instead of carrier; elimination of cold chain and biohazard shipping requirements; and more time to be productive without the need to travel to a blood draw center. Laboratory sample processing advantages include: the fact that freezing and thawing can often be eliminated, as well as numerous reports that indicate improved analyte stability in dried matrices.

In drug monitoring, therapeutic drugs often have narrow therapeutic ranges. With regards to immunosupressants, low blood concentration can lead to an increased risk of organ rejection, while high concentration can lead to: neurotoxicity, hypertension, photosensitivity, hyperkalemia, and organ based toxicity. By sampling patients remotely and having immunosuppressant concentrations on hand during consultations, physicians can optimize and individualize their clinical health decisions. With remote sampling, clinicians have access to a broader range of time points for sampling, and patients are given a greater sense of agency in their treatment.

Within the past several years, research groups have developed validated methods for the determination of a few common immunosuppressant drugs using the Mitra® microsampler [8,10,12–14]. This review summarizes the methods developed by six different labs. It aims to be a guide to the important workflow elements to consider when developing and validating bioanalytical methods for specimens collected with a Mitra microsampling device.



Research Studies Referenced:

Group 1a	Group 2
Group 1b	Group 3
Group 4	Group 5

Table 1: Analyte List by Group

LogPs are approximated

Immunosuppressant	Abbreviation	Research Group	LogP
Cyclosporin-A	CsA	**1a, 2, 1b, 5**	3.64
Tacrolimus	FK-506	**2, 4, 5**	5.59
Everolimus	RAD001	**3, 4**	7.4
Sirolimus	RAPA SLM	**4**	7.45
Mycophenolic acid	L04AA06	**4**	3.53

Extraction Methods

The research methods summarized here typically used water, methanol, or a mixture of both in their initial extractions from the Mitra microsampling device.

Most groups utilized some methanol during their extraction workflows, which is common for high LogP analytes (the immunosuppressants of interest). One of the main advantages of a primarily methanolic extraction solvent is sample cleanliness. Most of the biological matrix of blood is not soluble in methanol, and thus a relatively clean sample is produced.

However, as more water is introduced into the initial extraction solvent, more matrix is solubilized into the extract. Thus, the introduction of water will generally introduce the need for an additional clean-up step to remove these unwanted matrix components.

The use of a primarily methanolic extract was pursued by groups **1a**, **1b**, and **3**. **Group 3** showed that a purely methanolic extraction of everolimus yielded low extraction efficiencies and a significant hematocrit bias. **Group 1a** was able to make a direct comparison between a methanolic workflow and a more aqueous workflow paired with an additional protein precipitation step for sample clean up.

They reported considerably worse correlation to traditional wet methods when utilizing the more methanolic extraction solvent[14].

Research groups in this review that utilized a more aqueous initial extraction step typically used a protein precipitation step to clean their samples and rid them of the complex matrix of proteins solubilized in an aqueous extraction solvent [8,13,14]

This allows for better correlation to standard wet methods when compared to an organic extraction method (see section on correlation to standard methods below). Zinc sulfate (ZnSO4) was the precipitation agent most commonly used in the literature reviewed here, however pure methanol[13] has also been used successfully.

Table 2: Extraction Method Summary

Research Group	1a (Ref Site A)	2	1b (Ref Site B)	3	4	5
Reference	13	14	13	12	10	9
Volume of Mitra® Microsampler Used	10 µL	20 µL	10 µL	10 µL	20 µL	10 µL
Initial Extraction	100 µL of Water: Methanol (95:5)	150µL Ultra-pure water (containing IS)	200 µL Methanol	500 µL of Methanol w/ 10 µL of IS solution (1ng/mL in methanol)	100µL of Water:Methanol (60:40) containing IS	Compared three different extraction solutions; 100uL methanol-water (1:1), 0.2 mol/L zinc sulfate in methanol water (1:1), and 0.5 mol/L zinc sulfate in methanol-water (4:1)
Agitation Method 1	Plate shake for 20 minutes	Vortex 20 minutes at 2500 rpm	Shake collection plate on orbital shaker for 1 hour at 1100 rpm	Vortex, ultrasonicate for 5 minutes, then shake at 500 rpm for 5 minutes	Ultrasonicate for 30 minutes at 47 kHz	Vortexed for 10 s followed by sonication for 15 minutes (in water bath)
Sample Clean up	Protein precipitation w/ ZnSO4 (2.88 g in 100 mL) then 100uL Acetonitrile	Protein precipitation w/ 150 µL 0.1 M ZnSO4, followed by agitation and further precipitation with Methanol	None	None	Protein precipitation w/ 200 uL Methanol	Solution was centrifuged at 13000 rpm (15700g) for 5–15 minutes at 4°C, aliquot of supernatant was taken for LC/MS analysis
Agitation Method 2	Plate shake for 5 min	Vortex 5 minutes at 2500 RPM after each addition of precipitation solvent (ZnSO4 then methanol, total of two agitations; 10 minutes)	None	Vortex, then centrifuge for 3 min at 15,000 rpm.	Vortex 15 minutes at medium speed, then 1 minute at maximum speed, followed by 15 minutes of sonification at 47 kHz, 15 minute vortexing at medium speed and 1 minute at maximum speed	None

Table 2: Extraction Method Summary (continued)

Research Group	1a (Ref Site A)	2	1b (Ref Site B)	3	4	5
Extraction efficiencies	Not Available	Not Available	Not Available	20.2–23.1%	77.5–94.5%	30%–120%
Hematocrit Bias Evaluated	No	No	No	Yes	Yes	Yes
Stability Evaluated	No	No	No	Yes	Yes	Yes
Address Whole Blood/ Plasma Ratios	No	No	No	No	No	Yes
Correlation to Wet Methods	Yes - R^2=0.9838	Yes- R^2=0.979	Yes	Yes - R^2 not reported, however data is within 95% confidence interval	No	No

Agitation Methods

Choice of agitation is critical to the extraction methodology. The most traditional method is shaking via a platform shaker or vortex, with the main variables being RPM and time of shaking. The optimal speed for deep well plates is 1000-1100 RPM, as this is the maximum speed that will maintain a vortex.

Sonication is another common strategy for improving extraction efficiencies, with many groups using sonication in tandem with vortexing [8,10,12]. This generally resulted in significantly shorter required vortex time (5-30 minutes).

In general, shorter vortexing times were also used when a protein precipitation step was part of the experimental procedure (20-30 minute vortexing times).

As a method development strategy, one option is to start with longer agitation times and then evaluate the effect of reducing the agitation times to determine when a negative effect is observed.

Care should also be taken to not agitate for longer than necessary as this may lead to unwanted matrix components in the extract.

While not yet used for the extraction of immunosuppressants, a novel method of extraction has been published in the literature recently, referred to as “impact-assisted extraction”[16]. This method has been shown to provide extremely high extraction efficiencies as well as robust assays free of hematocrit biases when used with the Mitra microsampling device.

The method employs the use of mechanical disruption by “bead beating” the microsamplers, in a process that is normally used for tissue homogenization. The Mitra microsamplers will survive this process intact, and the analyte will be completely extracted in the process.

Other successful approaches (not explored in these reviewed manuscipts) include, higher extraction temperatures and even the use of microwaves to help improve extraction efficiencies and lessen vortexing time[16].

QCs and Standards

All research groups reported here managed to generate calibration curves with excellent linearity and R^2 values using Mitra tips under varying conditions. When creating a calibration curve using Mitra, it is extremely important to ensure all experimental conditions are kept the same between calibrators and experimental samples.

A particularly important parameter to consider is the drying time of QC and standard samples on the Mitra tips prior to extraction, as this can influence extraction efficiency. Drying times commonly employed in the literature range from 3 hours to 24 hours. For application to immunosuppressant testing, a drying time of 24 hours is most suitable[11].

The relationship between drying time, extraction efficiency, and stability bias should not be undervalued during the method development process. If drying times between QC and experimental samples vary, then extraction efficiencies of the QC samples may differ from your experimental samples. Other important factors in the generation of QC standards are the amount of analyte to spike, as well as the period of equilibration that is used to allow the QCs and standards to reach an equilibrium post spike. The volume of analyte spiked into blank whole blood should be limited to 5% or less[10,12], and the spiked blood should be mixed for at least 30 minutes prior to use, although some authors would recommend a few hours of equilibration[18]. Additionally, when using blood for preparation of standards and QCs, it is important to measure and control for the hematocrit of blood being spiked.

While there are many factors to keep in mind with regards to the consistency of your experimental samples and calibrators, the groups reviewed here illustrate that the Mitra microsampler does not add significant variation to their methods, even when inter operator variability is considered[12]. Thus, Mitra devices should provide linear calibration curves even when common experimental variables are introduced.

One useful approach to preparation of QCs and standards en-masse involves aliquoting 80 µL of blood into all wells of a 96-well plate and then dipping a full rack (96 samplers at a time) for efficient preparation of validation samples either manually or via automation.

Assay Precision

All groups here that evaluated accuracy and precision followed traditional assay acceptance criterion. Both accuracy and precision were required to be within ±15% at each concentration level studied. For the LLOQ, accuracy and precision were required to be within ±20%. All these groups reported acceptable criterion using their extraction methodologies. Most of the groups illustrated robust methods with hematocrit biases that were less pronounced than those previously illustrated with DBS cards, except for **Group 3**, who did not see an improvement relative to DBS cards in their analysis of everolimus.

Sample Stability

Research **Group 3** evaluated two types of stability in their analysis. The first was the stability of the extracted sample prior to injection. They show that for everolimus, the sample is stable for up to 48 hours when stored at 2-8 °C (with a maximum bias reported of 9.8% for their Low QC).

The second type is a standard stability bias, with the metric for stability being that the reported concentrations remain within ±15% of the nominal concentration. The manuscript does not mention QC and standard drying times, nor analyte equilibration times, however they do report that the sample was stable for 362 days while dried on the Mitra device. **Group 4** allowed their analytes to equilibrate in blood for 30 minutes with mixing prior to addition to the Mitra tips, and all samples were dried for between 24 and 48 priors prior to analysis. They established 7 days of stability post extraction at 10°C and stability of dried samples on the tip for 30 days at 37°C and 50 days at -20°C. Group 6 allowed 1 hour of spiked analyte equilibration time in whole blood prior to addition to Mitra tips, and at least 2 hours of drying. Under ambient conditions tacrolimus was only shown to be stable for 2 days.

The primary difference between the methodologies of groups **4** and 5 relate to their extraction methods. **Group 4** and **5** used similar initial extraction solvents containing both water and methanol. However, **Group 4** added even more methanol to facilitate protein precipitation. Additionally, **Group 4** allowed for longer drying times for their QCs and standards. One or both choices dramatically affected the reported stability for the immunosuppressants studied.

It should be noted that both groups **4** and **5** report significantly improved stability at lower temperatures (≤ -20°C). Therefore, it is recommended to store samples at lower temperatures once received if they need to be stored on-site for prolonged periods of time prior to analysis (as might be the case with batch testing).

Correlation to Wet Methods

The validated methods were used to correlate data between blood that was extracted from a Mitra device to previously established wet blood reference methods (referred to as the "wet method"). Without a reliable way to map data obtained using Mitra devices back to this standard method, you cannot reliably interpret concentration values. Thus, we have included this section to discuss the best methods to use when attempting to validate any analyte in reference to whole blood sampling.

Groups **1a**, **2**, **1b**, and **3** all included this correlation to a wet methods in their studied. Groups **1a** and **1b** evaluated a methanolic extraction, with **Group 1a** also evaluating a protein precipitation type extraction in their investigation. It is notable that most wet methods for immunosuppressant monitoring follow a protein precipitation based extraction.

The R^2 values for the correlation of the dried blood extraction from the Mitra microsampler to their respective wet methods for Cyclosporine A, ranged from 0.86-.90 when the methanolic extraction was used. When **Group 1a** reevaluated the correlation using a protein precipitation method, the R^2 value increased significantly to 0.9838. A possible reason for this improvement is that when the extraction methodologies are chemically similar the intrinsic biases of the methods are also more likely to be similar. The protein precipitation approach was followed by most of the other groups, except for **Group 3** which focused on a primarily methanolic extraction methodology.

Group 2 also evaluated a protein precipitation methodology, and the R2 value for their evaluation of tacrolimus was 0.947 when correlating the dried test method to their wet reference method with 45 patients. Likewise, **Group 2** illustrated an R2 value of 0.979 for the extraction of Cyclosporine when correlating to the wet reference method.

Instrument Carry-Over

With hydrophobic analytes such as these immunosuppressants, the analysis of carry over from analytical run to analytical run is crucial. The process used by Groups **2** and **3** are aligned in that they injected blank samples after the injection of a high concentration sample and show that the response is a fraction of the response expected by a sample that is at the LLOQ.

Conclusion

This review covers a body of work performed by multiple groups all aimed at optimizing analytical methods for the detection of immunosuppressants using the Mitra microsampling device.

Their results are largely positive, and most groups were able to develop robust methods and some effectively correlated their Mitra microsampling methods to standard wet methods. However, success was varied. The method development approaches that proved most successful were as follows:

First, choosing an overall extraction method most similar to the method used in the wet method i.e. if the wet method uses a protein precipitation, then a protein precipitation step is recommended for your Mitra extraction method.

Second, allowing suitable analyte equilibration. In the case of the immunosuppressants studied here, one hour appears acceptable.

Third, allowing suitable drying time for your analytes may be crucial, and a minimum of 24 hours will provide optimal results according to the groups reviewed here.

Lastly, choose the most powerful agitation approaches available, and evaluate your extraction efficiencies at the LLOQ of the assay. High extraction efficiencies have been shown to minimize or completely rid of any hematocrit and stability biases.

Based on research reviewed here, these appear to be the best practices for the development of analytical methods that will correlate well to traditional wet methods when using the Mitra microsampler for immuosuppressant analysis.

References

1. Mee A V, Wong PY, Sun C, et al. Monitoring of cyclosporine concentrations by using dry blood-spot samples. J Clin Lab Anal. 1991;5(1):74-77. doi:10.1002/jcla.1860050114

2. Cheung CY, van der Heijden J, Hoogtanders K, et al. Dried blood spot measurement of tacrolimus is promising for patient monitoring. J Pharm Biomed Anal. 2007;21(2):140-145. doi:10.1111/j.1432-2277.2007.00584.x

3. Hoogtanders K, van der Heijden J, Christiaans M, van de Plas A, van Hooff J, Stolk L. Dried blood spot measurement of tacrolimus is promising for patient monitoring. Transplantation. 2007;83(2):237-238. doi:10.1097/01.tp.0000250730.30715.63

4. Hoogtanders K, van der Heijden J, Christiaans M, Edelbroek P, van Hooff JP, Stolk LML. Therapeutic drug monitoring of tacrolimus with the dried blood spot method. J Pharm Biomed Anal. 2007;44(3):658-664. doi:10.1016/j.jpba.2006.11.023

5. Cheung CY, van der Heijden J, Hoogtanders K, et al. Dried blood spot measurement: application in tacrolimus monitoring using limited sampling strategy and abbreviated AUC estimation. Transpl Int. 2008;21(2):140-145. doi:10.1111/j.1432-2277.2007.00584.x

6. Van der Heijden J, de Beer Y, Hoogtanders K, et al. Therapeutic drug monitoring of everolimus using the dried blood spot method in combination with liquid chromatography-mass spectrometry. J Pharm Biomed Anal. 2009;50(4):664-670. doi:10.1016/j.jpba.2008.11.021

7. Martial LC, Hoogtanders KEJ, Schreuder MF, et al. Dried Blood Spot Sampling for Tacrolimus and Mycophenolic Acid in Children: Analytical and Clinical Validation. Ther Drug Monit. 2017;39(4):412-421. doi:10.1097/FTD.0000000000000422

8. Kita K, Noritake K, Mano Y. Application of a Volumetric Absorptive Microsampling Device to a Pharmacokinetic Study of Tacrolimus in Rats: Comparison with Wet Blood and Plasma. Eur J Drug Metab Pharmacokinet. 2019;44(1):91-102. doi:10.1007/s13318-018-0493-7

9. Kita K, Mano Y. Application of volumetric absorptive microsampling device for quantification of tacrolimus in human blood as a model drug of high blood cell partition. J Pharm Biomed Anal. 2017;143(5):168-175. doi:https://doi.org/10.1016/j.jpba.2017.05.050

10. Koster RA, Niemeijer P, Veenhof H, et al. A volumetric absorptive microsampling LC-MS/MS method for five immunosuppressants and their hematocrit effects. Bioanalysis. 2019;11(6):495-508. doi:10.4155/bio-2018-0312

11. Veenhof H, Koster RA, Alffenaar J-WC, et al. Clinical application of a dried blood spot assay for sirolimus and everolimus in transplant patients. Clin Chem Lab Med. April 2019. doi:10.1515/cclm-2019-0053

12. Verheijen RB, Thijssen B, Atrafi F, et al. Validation and clinical application of an LC-MS/MS method for the quantification of everolimus using volumetric absorptive microsampling. J Chromatogr B, Anal Technol Biomed life Sci. 2019;1104:234-239. doi:10.1016/j.jchromb.2018.11.030

13. Stephenson S, Dutton J, Davison A, Kushon S, Rudge J. Evaluation of the Mitra® Microsampling Device for Immunosuppressants Monitoring. 2016:Poster Presentation.

14. Janetto PJ. Immunosuppressant Monitoring by LC-MS/MS Using MitraTM Microsampling Devices. 2016:MSACL Poster.

15. Youhnovski N, Mayrand-Provencher L, Berube E-R, et al. Volumetric absorptive microsampling combined with impact-assisted extraction for hematocrit effect free assays. Bioanalysis. 2017;9(22):1761-1769. doi:10.4155/bio-2017-0167

16. Velghe S, Delahaye L, Stove CP. Is the hematocrit still an issue in quantitative dried blood spot analysis? J Pharm Biomed Anal. 2019;163:188-196. doi:10.1016/j.jpba.2018.10.010

17. Rowland M, Emmons GT. Use of dried blood spots in drug development: pharmacokinetic considerations. AAPS J. 2010;12(3):290-293. doi:10.1208/s12248-010-9188-y