Quantitation of Tacrolimus in Human Whole Blood Samples Using the MITRA Microsampling Device

Nasrullah Undre, PhD,* Imran Hussain, PhD,* John Meijer, MSc,† Johannes Stanta, PhD,‡
Gordon Swan, BSc,‡ Ian Dawson, MSc‡
*Astellas Pharma Europe, Ltd., Addlestone, UK
†Astellas Pharma Europe, B.V., Leiden, The Netherlands
‡Covance Laboratories, Harrogate, UK

Corresponding Author:
Dr Nasrullah Undre
Astellas Pharma Europe, Ltd, Bourne Business Park, 300 Dashwood Lang Rd, Addlestone,
KT15 2NX, UK
Telephone: +44 7437174856
Fax: n/a
Email: nundre0@gmail.com

Sources of Funding
This work was supported by Astellas Pharma Europe, Ltd. Editorial support was provided by Cello Health MedErgy, and was funded by Astellas Pharma, Inc.

This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.
Conflicts of Interest
N. Undre is a former employee of Astellas Pharma Europe, Ltd. I. Hussain was engaged as a consulting study manager during the course of the study by Astellas Pharma Europe, Ltd. J. Meijer is a former employee of Astellas Pharma Europe, B.V. J. Stanta, G. Swan, and I. Dawson are employees of Covance Laboratories Limited, a CRO working on behalf of Astellas.

Author Contributions
N. Undre, I. Hussain, and J. Meijer contributed to study planning and execution, data acquisition and analysis, and review of the validation data. J. Stanta, G. Swan, and I. Dawson contributed to method development, method validation, and data review. All authors were involved in data interpretation, critical review of the manuscript, and provided final approval of the submitted version.

Abstract
Background: The calcineurin inhibitor tacrolimus is a narrow therapeutic index medication, which requires therapeutic drug monitoring to optimize dose on the basis of systemic exposure. MITRA microsampling offers a minimally invasive approach for the collection of capillary blood samples from a fingerprick as an alternative to conventional venous blood sampling for quantitation of tacrolimus concentrations.

Methods: A bioanalytical method for the quantitation of tacrolimus in human whole blood samples collected on MITRA tips was developed, using liquid-liquid extraction followed by liquid chromatography with tandem mass spectrometry detection. Validation experiments were performed according to current Food and Drug Administration and European Medicines Agency guidelines on validation of bioanalytical methods. Validation
criteria included assay specificity and sensitivity, interference, carryover, accuracy, precision, dilution integrity, matrix effect, extraction recovery, effect of hematocrit and hyperlipidemia, and stability.

**Results:** All assay validation results were within the required acceptance criteria, indicating a precise and accurate tacrolimus quantitation method. The validated assay range was 1.00 to 50.0 ng/mL. No interference, carry-over or matrix effect was observed. Extraction recovery was acceptable across the assay range. Samples were stable for up to 96 days at -20°C and 20°C, and 28 days at 40°C. Hematocrit, hyperlipidemia and lot-to-lot differences in the nominal absorption volume of the 10 µL MITRA tips were shown not to influence tacrolimus quantitation by this assay method.

**Conclusions:** The bioanalytical method validated in this study is appropriate and practical for the quantitation of tacrolimus in human whole blood samples collected using the MITRA microsampling device.

**Keywords:** LC-MS/MS; MITRA; tacrolimus; quantitation; validation

**INTRODUCTION**

Tacrolimus, a macrolide lactone with a molecular weight of 822 Daltons (for the monohydrate form),¹ is the cornerstone of immunosuppressive therapy following solid organ transplantation. Tacrolimus has a narrow therapeutic index; thus, therapeutic drug monitoring is required to optimize dosing on the basis of systemic exposure.²⁻⁶ The pharmacokinetic variable associated with tacrolimus efficacy and safety is the area under the concentration–time curve profile over the dosage time interval (AUC₀⁻τ).⁴⁻⁶ Ideally, therapeutic drug monitoring of tacrolimus exposure should therefore be based on determination of the AUC.³⁻⁶ However, the routine determination of tacrolimus AUC in clinical practice is limited by the need to collect multiple blood samples over a 24-hour
period. Consequently, whole blood trough concentrations are generally used as a surrogate marker for tacrolimus AUC.\textsuperscript{3–6} More recently, limited blood sampling strategies for tacrolimus AUC estimation have been used.\textsuperscript{7}

Tacrolimus concentrations in whole blood samples can be determined using immunoassays or liquid chromatography tandem mass spectrometry (LC-MS/MS).\textsuperscript{4,6,8,9} Whole blood samples for quantitation of tacrolimus are usually obtained by venous sampling. However, for convenience, assays based on dried blood spot (DBS) sampling of capillary blood from a fingerprick have been developed.\textsuperscript{10–12} DBS assays have also been used to estimate tacrolimus pharmacokinetics.\textsuperscript{10,13}

The MITRA microsampling device (Neoteryx; Torrance, CA, USA) is a Food and Drug Administration (FDA) Class I, CE marked, blood sample collection device (Figure 1). The MITRA tip uses Volumetric Absorptive Microsampling (VAMS) technology to collect a specified absorption volume for quantitative bioanalysis. MITRA microsampling offers a minimally invasive approach to collect capillary blood samples from a fingerprick as an alternative to conventional venous blood sampling for quantitating tacrolimus concentrations.

The present study was undertaken to develop and validate a bioanalytical method for the quantitation of tacrolimus in human whole blood samples collected on MITRA tips, using liquid-liquid extraction with LC-MS/MS detection.
MATERIALS AND METHODS

Chemical Reagents and Equipment

The tacrolimus reference standard and the deuterated internal standard, \([^{13}\text{C}]\text{-FK-506-D}_2\), were purchased from Toronto Research Chemicals (North York, ON, Canada). All organic solvents and chemicals used were obtained from commercial suppliers and were of the highest commercially available grade.

Human whole blood samples, containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, were obtained from healthy donors and stored at ambient temperature for \(\leq 8\) hours or refrigerated for \(\leq 72\) hours (never frozen). MITRA tips with a nominal absorption volume of 10 \(\mu\text{L}\) were acquired from Neoteryx and stored at room temperature (i.e., nominal +20°C) until use. Five different lots of MITRA tips were used. Each lot was supplied with a certificate of conformance specifying a calculated average blood wicking volume (e.g., 9.90, 10.0, 10.6, and 10.9 \(\mu\text{L}\) for the lots used in this study). The data were not corrected for the calculated average wicking volume.

Stock Solutions

Tacrolimus and internal standard stock solutions were prepared in methanol at a concentration of 100 \(\mu\text{g/mL}\), and stored in amber glass vials at -20°C. The maximum storage periods for the tacrolimus and internal standard stock solutions were 36 days and 365 days, respectively. Tacrolimus spiking solutions were also prepared in methanol. Internal standard addition solutions (used during extraction) were prepared in acetonitrile:water (50:50).
Calibration Standards and Quality Control Samples

Calibration standards were prepared in EDTA whole blood at concentrations of 1.00, 2.00, 4.00, 6.00, 12.0, 25.0, 45.0, and 50.0 ng/mL. Quality control (QC) samples were prepared in EDTA whole blood at the following concentrations: 1.00 ng/mL (lower limit of quantification [LLOQ] QC), 3.00 ng/mL (low QC), 10.0 ng/mL (medium QC), and 40.0 ng/mL (high QC). Calibration standards and QC samples were freshly prepared prior to analysis and discarded after use (within 24 hours of preparation).

MITRA Sample Preparation and Extraction

Whole blood samples were mixed for ≥5 minutes on a roller mixer at room temperature prior to spiking with tacrolimus stock solution. The spiking volume was adjusted according to the blood volume, but did not exceed 1% of the blood volume (i.e. 10 µL added to 990 µL of blood). Once spiked, samples were gently inverted and mixed on a roller mixer for ≥15 minutes prior to portioning into aliquots for loading onto MITRA tips. To load, the MITRA tip was gently touched to the surface of the blood, ensuring that the tip was not fully submerged. Blood was allowed to permeate through the entire tip until visibly saturated. After a further 2 seconds, the tip was smoothly removed from the sample. Care was taken to avoid partitioning of blood and plasma during tip spotting by regularly inverting the blood tube and/or returning the sample to the roller mixer at 10-minute intervals. Tips were dried for a minimum of 3 hours under air flow and then stored in a sealed bag at room temperature until analysis.

To prepare the MITRA samples for analysis, the tips were removed by placing the tip over the edge of a well of a 2-mL 96-well plate. Then, they were gently pulled until the tip was released into the well, and 100 µL acetonitrile:water (50:50) containing 1 ng/mL [13C]-FK-
506-D₂ as the internal standard was added to each well (except for blank samples). The plate was sealed with a silicone plate lid and vortex-mixed for 2 minutes at ~1250 revolutions per minute (rpm) using a MixMate (Eppendorf, Hamburg, Germany), sonicated for 30 minutes at 30°C, and then centrifuged for 1 minute at ~1000 × g at 20°C. Subsequently, 100 µL acetonitrile was added to each well and the sealed plate was vortex-mixed for 5 minutes at ~1250 rpm and centrifuged for 5 minutes at ~3000 × g and 20°C. An 100-µL aliquot of the supernatant was transferred to a clean 1.2-mL 96-well plate, and 50 µL water added to each well. The plate was sealed, pulse centrifuged at a minimum ~250 × g to ensure all of the liquid was at the bottom of the well, vortex-mixed for 2 minutes at ~1250 rpm, and centrifuged for 5 minutes at ~3000 × g and 5°C. The plates were stored under refrigerated conditions (i.e., at nominal +5°C) for up to 119 hours prior to LC-MS/MS analysis.

**LC-MS/MS Analysis**

Analyses were performed using the Waters ACQUITY UPLC system (Waters, Milford, MA, USA). Chromatographic separation was achieved using a Kinetex 1.7 µm XB-C18 50 × 2.1 mm analytical column, with a KrudKatcher in-line 0.5 µm filter (both Phenomenex, Torrance, CA, USA). The column was maintained at 65°C, and the autosampler temperature was 5°C. Mobile phase A consisted of 0.01 mol/L aqueous ammonium formate:formic acid (100:0.2), and mobile phase B was acetonitrile. The gradient settings are shown in Table 1. The flow rate was 0.6 mL/min, the injector run time was 2.5 minutes, and the injection volume was 10 µL.

Detection was conducted using an AB Sciex 5500 mass spectrometer (AB Sciex, Framingham, MA, USA). Chromatographic integration and data collection were
performed using Analyst Software (version 1.6.3, AB Sciex). The mass spectrometer was operated in atmospheric-pressure chemical ionization (APCI) mode, generating positive ions at the following instrument settings: nebulizer current, 5 V; temperature, 350°C; acquisition time, 1.5 minutes; and cycle time, 3.5 minutes. The transitions monitored were 821.5→768.5 m/z for tacrolimus and 826.5→773.5 m/z for the internal standard. The dwell time was 100 milliseconds; the declustering potential was 60 V, the collision energy was 29 V, and the collision cell exit potential was 20 V. The typical mean retention time was 1.05 minutes for tacrolimus and the internal standard.

Bioanalytical Validation

The assay was developed and validated at Covance Laboratories (Harrogate, UK), funded by Astellas Pharma Europe. All work was performed according to applicable Covance and Astellas standard operating procedures and policies. Blood samples were obtained from healthy volunteers in accordance with Covance protocols concerning the collection and use of human tissue. The protocol and subsequent amendments were approved by the relevant local research ethics committees (NHS Health Research Authority REC reference: 05/Q1107/91). All volunteers provided written informed consent for blood collection and use.

Validation experiments were performed according to the current FDA and European Medicines Agency guidelines on validation of bioanalytical methods. The validation criteria included assay specificity and sensitivity, interference, carryover, accuracy, precision, dilution integrity, matrix effect, extraction recovery, effects of hematocrit and hyperlipidemia, and stability. For all experiments, the acceptance criteria were set for
precision (expressed as % relative standard deviation [%RSD]) at \(\leq 15\%\) (\(\leq 20\%\) at the LLOQ) and for accuracy (expressed as mean % bias) at \(\pm 15\%\) (\(\pm 20\%\) at the LLOQ).

**Specificity and Selectivity**

Specificity and selectivity were determined by confirming the absence of interference and carryover. A range of representative chromatograms was obtained, including (i) a blank sample, (ii) a blank sample spiked with the internal standard, (iii) a sample spiked with tacrolimus and internal standard at the LLOQ; and (iv) a matrix blank spiked with tacrolimus at the upper limit of quantification (50.0 ng/mL, with no internal standard).

**Accuracy and Precision**

The accuracy and precision of the method were determined from four independent runs of six samples at each of the four QC concentrations.

**Dilution Integrity**

Dilution integrity was investigated by analyzing samples prepared at 100 ng/mL and then diluted 2.5-fold with blank matrix extract into the calibration range. In total, six samples were analyzed in a single run.

**Extraction Recovery**

For the analysis of extraction recovery, blank samples were taken through the full extraction procedure before being spiked with both analyte and internal standard at the low QC (3.00 ng/mL), medium QC (10.0 ng/mL), and high QC (40.0 ng/mL) levels (assuming 100% recovery). The peak areas of these samples were then compared with those of extracted low QC, medium QC, and high QC samples to generate a percentage recovery.
value that should be within 30.0% across the concentration range (e.g., all recovery values should fall within 75.0–105.0%).

Matrix Effects and Factor
To determine the matrix effects, blank whole blood samples from six individual lots were analyzed without the internal standard; moreover, matrix samples from six individual lots were spiked at the LLOQ QC and analyzed with the internal standard. To calculate the matrix factor, blank matrix samples were extracted from six individual lots as well as reagent blank samples (water), and spiked post-extraction at the low QC and high QC concentrations including the internal standard, assuming 100% recovery. The matrix factor was calculated as the ratio of the peak response in the presence of matrix ions (individual blanks) to the mean peak response in the absence of matrix ions (reagent blanks). The internal standard-normalized matrix factor was calculated by dividing the matrix factor of the analyte by the matrix factor of the internal standard.

Hematocrit and Hyperlipidemia
To assess the variation effects of hematocrit levels on tacrolimus quantitation, low QC and high QC samples were prepared at four hematocrit levels: 20%, 30%, 40%, and 50% (six samples at each hematocrit level). To assess the effect of hyperlipidemia, six low QC and six high QC samples were prepared in matrix with an intrinsic lipid content of ≥300 mg/dL triglyceride.

Stability
Stability was assessed using both low and high QC samples stored at -20°C, 20°C, and 40°C for 7, 14, 28, and 96 days. Processed sample stability was assessed for the low QC
and high QC samples refrigerated for up to 119 hours (i.e., stored at 5°C). Freshly extracted low, medium, and high QC samples were included for run acceptance. The samples were considered to be freshly extracted when extraction of the sample was commenced within 24 hours of sample preparation.

**MITRA Lot-to-Lot Comparison**

MITRA tips are supplied with a certificate of conformance, which includes a calculated average blood wicking volume, which may vary between lots. To compare the influence of the different wicking volumes, the calibration line and QC samples were prepared with three different lots of MITRA tips, including tips with 10.0 µL (calibration line, high QC, medium QC, and low QC), 10.6 µL (high QC and low QC), 10.9 µL (high QC and low QC) volumes.

**RESULTS**

**Specificity and Selectivity**

The validated assay range for tacrolimus quantification in whole blood was 1.00 to 50.0 ng/mL. Representative ion chromatograms are shown in Figure 2. There was no significant interference of the analyte on the internal standard, and no evidence of carryover within the chromatographic regions of the analyte and the internal standard.

**Accuracy and Precision**

Accuracy and precision requirements were fulfilled (Table 2). All intra- and inter-assay accuracy (expressed as %bias) and precision (expressed as %RSD) values were within the acceptance criteria, with the exception of low QC samples in one run. The maximum run size validated was 192 injections. The precision value of the internal standard peak areas
from the extracted calibration standards and QC samples was consistent throughout the
validation runs, varying between 2.6% and 3.4%.

**Dilution Integrity**

Dilution integrity acceptance criteria were fulfilled. The mean (±standard deviation)
tacrolimus concentration was 96.2 (±4.14) ng/mL, with %RSD of 4.3% and %bias of -
3.8%.

**Extraction Recovery**

The extraction recovery was acceptable to obtain precise and accurate quantitation within
the assay range. The percentage recovery values were within 30% across the concentration
range (mean recovery was 95.3% at 3.0 ng/mL, 104.7% at 10 ng/mL, and 97.5% at 40
ng/mL), and the recovery of the internal standard mirrored that of the analyte (mean
recovery of 102.9%).

**Matrix Effect and Factor**

All matrix data met the acceptance criteria, indicating that the matrix had no impact on
assay performance. The internal standard-normalized matrix factor ranged from 0.94 to
1.03, with %RSD of ≤2.8%.

**Effect of Hematocrit and Hyperlipidemia**

Hematocrit was not found to affect the quantitation of tacrolimus in whole blood samples
obtained via MITRA sampling (Table 3). The accuracy and precision acceptance criteria
were met for all samples at all hematocrit levels tested (20–50%). Hyperlipidemia was also
shown not to influence tacrolimus quantitation using this method, with %RSD of $\leq 7.3\%$ and %bias of $\leq 3.7\%$.

**Stability**

The stability of tacrolimus on MITRA tips was confirmed for up to 96 days at -20°C and 20°C. The stability of tacrolimus on MITRA tips was also stress tested at 40°C for up to 96 days; however, it only showed stability at the interim stability time point at 28 days and failed the stability test at 96 days. Processed samples were stable for 119 hours when refrigerated (i.e. stored at 5°C).

**MITRA Lot-to-Lot Comparison**

The lot-to-lot differences and their calculated average blood wicking volumes were not found to influence tacrolimus quantitation by this assay method, with %RSD of $\leq 6.7\%$ and %bias of $\leq 8.0\%$, as assessed between three different lots of MITRA tips with average blood wicking volumes of 10.0 µL, 10.6 µL, and 10.9 µL.

**DISCUSSION**

In this study, we developed and validated an LC-MS/MS-based method for the quantitation of tacrolimus in human whole blood samples collected on MITRA tips.

**Method Development**

The initial assay development progressed well, displaying good accuracy and precision for calibration standards and QC samples. However, some issues were observed in samples with different hematocrit levels and samples which had been stored for several days. Matrix effects were noted in both the sample extraction, which were attributed to
differences in recovery, and on the MS instrumentation, which were attributed to
differences in ion suppression. The potential for ion suppression effects with LC-MS/MS
is well known,\textsuperscript{4,16–18} and would typically be compensated for by an isotopically labelled
internal standard. Although we had used electrospray ionization (ESI), we found that
moving to atmospheric pressure chemical ionization (APCI) completely removed this
effect and assisted with our investigations into extraction-based effects on recovery.

Extraction-based effects were observed by the poor recovery of tacrolimus in higher
hematocrit samples, with internal standard levels remaining consistent across hematocrit
levels. Various approaches to address this issue were assessed, with sonication in a heated
bath offering the best and most consistent results for both varying hematocrit levels and
storage periods (up to 96 days). In general, higher recovery was associated with an
increased recovery of red blood cell components from the MITRA tip, with a resulting
darker hue in the extract.

\textbf{Validation}

The results of this study show the described bioanalytical assay method to be suitable for
the determination of tacrolimus concentrations in human whole blood samples collected on
MITRA tips over a calibration range from 1.0–50.0 ng/mL. All assay validation criteria
were fulfilled, indicating a precise and accurate quantitation method. The LLOQ for this
assay (1.0 ng/mL) was in line with that recommended by the 2007 European Consensus
Conference on Tacrolimus Optimization, and similar to that of immunoassay methods used
for therapeutic drug monitoring of tacrolimus.\textsuperscript{4} Although a number of LC-MS/MS assay
methods have reported an LLOQ in venous blood samples of 0.1 ng/mL\textsuperscript{4,9}, the LLOQ of
the described assay was below the target range used for patients maintained on low
tacrolimus dose therapy (3 ng/mL).\textsuperscript{4} Samples were shown to be stable for up to 96 days at -20°C and 20°C, and 28 days at 40°C, which exceeds the range of temperatures likely to be encountered during shipping and storage with remote sampling.

Hematocrit was not found to have an effect on the quantitation of tacrolimus in whole blood samples obtained using MITRA sampling over the range of hematocrit levels likely to be seen in clinical settings (i.e., between anemic and normal adult reference levels). The MITRA microsampling device is designed to enable collection of a fixed volume of blood (10 µL) and the entire sample is extracted, which would be expected to reduce the hematocrit effect that has previously been observed with DBS sampling methods.\textsuperscript{19–21} Our findings agree with the results of a previous study, which found no notable difference in the volume of blood absorbed by MITRA tips for hematocrit levels ranging from 20–65%.\textsuperscript{22} Other studies using different bioanalytical assays have also shown hematocrit to have a minimal impact on the quantitation of tacrolimus blood concentrations using this microsampling device.\textsuperscript{23,24}

Our findings are in line with those of other recent studies undertaken to validate different bioanalytical methods for the quantitation of tacrolimus and other immunosuppressant drugs in human whole blood samples collected on MITRA tips.\textsuperscript{24–29} In order to assess the suitability of this method for determination of tacrolimus concentrations in clinical settings, a clinical validation study (NCT03465969) has been undertaken in kidney and liver transplant patients to compare tacrolimus concentrations determined in capillary whole blood concentrations obtained using the MITRA microsampler device with those determined using an established whole blood venipuncture method.\textsuperscript{30}
Capillary blood sampling using the MITRA microsampling device offers a number of potential benefits over venous blood sampling for determining tacrolimus blood concentrations. It is less invasive and more convenient than venous blood sampling, and phlebotomy services are not required. Moreover, the blood samples do not require refrigeration during shipping or storage, which offers the potential for remote collection of samples for therapeutic drug monitoring of tacrolimus (e.g., in the patient’s own home). This method would also be expected to facilitate collection of serial blood samples in clinical trial settings, with a reduced blood sample volume compared with venous sampling which is particularly advantageous in pharmacokinetic and pediatric studies.

CONCLUSIONS
In summary, the described bioanalytical method has been validated for the quantitation of tacrolimus in human whole blood samples collected using the MITRA microsampling device across the range of expected tacrolimus blood concentrations during therapeutic drug monitoring in transplant patients.

ACKNOWLEDGEMENTS
This work was supported by Astellas Pharma Europe, Ltd. Medical writing support was provided by Jennifer Coward for Cello Health MedErgy, under the direction of the authors. Editorial support was funded by Astellas Pharma, Inc.

DATA SHARING
Researchers may request access to anonymized participant level data, trial level data, and protocols from Astellas sponsored clinical trials at (www.clinicalstudydatarequest.com).

16
For Astellas’ criteria on data sharing see: (https://clinicalstudydatarequest.com/Study-Sponsors/Study-Sponsors-Astellas.aspx).

REFERENCES

1. National Center for Biotechnology Information. Prograf, CID=5282315. PubChem Database. https://pubchem.ncbi.nlm.nih.gov/compound/Prograf. Published 2005. Updated 2020. Accessed October 8, 2020.

2. Jusko W, Thomson A, Fung J, et al. Consensus document: therapeutic monitoring of tacrolimus (FK-506). Ther Drug Monit. 1995;17(6):606-614.

3. Venkataramanan R, Swaminathan A, Prasad T, et al. Clinical pharmacokinetics of tacrolimus. Clin Pharmacokinet. 1995;29(6):404-430.

4. Wallemacq P, Armstrong VW, Brunet M, et al. Opportunities to optimize tacrolimus therapy in solid organ transplantation: report of the European consensus conference. Ther Drug Monit. 2009;31(2):139-152.

5. Tanzi MG, Undre N, Keirns J, et al. Pharmacokinetics of prolonged-release tacrolimus and implications for use in solid organ transplant recipients. Clin Transplant. 2016;30(8):901-911.

6. Brunet M, Van Gelder T, Åsberg A, et al. Therapeutic drug monitoring of tacrolimus – Personalized therapy: Second consensus report. Ther Drug Monit. 2019(3):41:261-307.

7. Woillard JB, de Winter BCM, Kamar N, et al. Population pharmacokinetic model and Bayesian estimator for two tacrolimus formulations - twice daily Prograf and once daily Advagraf. Br J Clin Pharmacol. 2011;71(3):391-402.

8. Alak AM, Moy S, Cook M, et al. An HPLC/MS/MS assay for tacrolimus in patient blood samples. Correlation with results of an ELISA assay. J Pharm Biomed Anal.
9. Kalt DA. Tacrolimus: a review of laboratory detection methods and indications for use. Lab Med. 2017;48(4):e62-e65.

10. 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.

11. Shokati T, Bodenberger N, Gdapaille H, et al. Quantification of the immunosuppressant tacrolimus on dried blood spots using LC–MS/MS. J Vis Exp. 2015;105:e52424.

12. 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.

13. Stifft F, Stolk LML, Undre N, et al. Lower variability in 24-hour exposure during once-daily compared to twice-daily tacrolimus formulation in kidney transplantation. Transplantation. 2014;97(7):775-780.

14. US Food and Drug Administration. Bioanalytical method validation. Guidance for Industry. https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf. Published 2018. Accessed October 8, 2020.

15. European Medicines Agency. Guideline on bioanalytical method validation. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf. Published 2012. Accessed October 8, 2020.

16. King R, Bonfiglio R, Fernandez-Metzler C, et al. Mechanistic investigation of ionization suppression in electrospray ionization. J Am Soc Mass Spectrom. 2000;11(11):942-950.

17. Taylor P. Matrix effects: the Achilles heel of quantitative high-performance liquid
chromatography-electrospray-tandem mass spectrometry. *Clin Biochem.* 2005;38(4):328-324.

18. George R, Haywood A, Khan S, et al. Enhancement and suppression of ionization in drug analysis using HPLC-MS/MS in support of therapeutic drug monitoring: A review of current knowledge of its minimization and assessment. *Ther Drug Monit.* 2018;40(1):1-8.

19. De Kesel PM, Sadones N, Capiau S, et al. Hematocritical issues in quantitative analysis of dried blood spots: challenges and solutions. *Bioanalysis.* 2013;5(16):2023-2041.

20. Wilhelm AJ, den Burger JC, Swart EL. Therapeutic drug monitoring by dried blood spot: progress to date and future directions. *Clin Pharmacokinet.* 2014;53(11):961-973.

21. Koster RA, Botma R, Greijdanus B, et al. The performance of five different dried blood spot cards for the analysis of six immunosuppressants. *Bioanalysis.* 2015;7(10):1225-1235.

22. Spooner N, Dennif P, Michielsen L, et al. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated with blood hematocrit. *Bioanalysis.* 2015;7(6):653-659.

23. 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;143:168-175.

24. 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.

25. Vethe N, Gustavsen M, Midtvedt K, et al. Tacrolimus can be reliably measured with
volumetric absorptive capillary microsampling throughout the dose interval in renal transplant recipients. *Ther Drug Monit.* 2019;41(5):607-614.

26. Gruzdys V, Merrigan SD, Johnson-Davis KL. Feasibility of immunosuppressant drug monitoring by a microsampling device. *J Appl Lab Med.* 2019;4(2):241-246.

27. Paniagua-González L, Díaz-Louzao C, Lendoiro E, et al. Volumetric Absorptive Microsampling (VAMS) for assaying immunosuppressants from venous whole blood by LC–MS/MS using a novel atmospheric pressure ionization probe (UniSpray™). *J Pharm Biomed Anal.* 2020;189:113422.

28. Mbughuni MM, Stevens MA, Langman LJ, et al. Volumetric microsampling of capillary blood spot vs whole blood sampling for therapeutic drug monitoring of tacrolimus and cyclosporin A: Accuracy and patient satisfaction. *J Appl Lab Med.* 2020;5(3):516-530.

29. Marshall DJ, Kim JJ, Brand S, et al. Assessment of tacrolimus and creatinine concentration collected using Mitra microsampling devices. *Ann Clin Biochem.* 2020;57(5):389-396.

30. Undre N, Dawson I, Aluvihare V, et al. Validation of a capillary dry blood sample MITRA-based assay for the quantitative determination of systemic tacrolimus concentrations in transplant recipients. Submitted for publication.
FIGURE LEGENDS

FIGURE 1. The MITRA microsampling device: (A) example of the clamshell collection kit; (B) collection of capillary blood samples, i.e., from a fingerprick. Images provided courtesy of Neoteryx, LCC (Torrance, CA, USA).

FIGURE 2. Representative ion chromatograms of (A) a blank sample, (B) a blank sample spiked with the internal standard, (C) a sample spiked with tacrolimus and internal standard at the LLOQ (1.00 ng/mL), and (D) a matrix blank spiked with tacrolimus at the ULOQ (50.0 ng/mL) and no internal standard. The gray shading shows the integrated signal peak. cps, counts per second; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.
TABLE 1. Chromatography Gradient Elution Profile

| Time (min) | Mobile Phase A          | Mobile Phase B   |
|------------|-------------------------|-----------------|
|            | [ammonium formate 10 mM | [acetonitrile]  |
|            | (aq):formic acid (100:0.2)] |                |
| Initial    | 50                      | 50              |
| 0.50       | 50                      | 50              |
| 0.51       | 15                      | 85              |
| 1.50       | 15                      | 85              |
| 1.51       | 5                       | 95              |
| 2.00       | 5                       | 95              |
| 2.01       | 50                      | 50              |
| 2.50       | 50                      | 50              |

aq, aqueous
| Parameter                          | LLOQ QC (1.0 ng/mL) | LQC (3.0 ng/mL) | MQC (10.0 ng/mL) | HQC (40.0 ng/mL) |
|-----------------------------------|---------------------|-----------------|------------------|------------------|
| n                                 | 24                  | 24              | 24               | 24               |
| Mean tacrolimus concentration found (ng/mL) | 1.05                | 3.24            | 10.2             | 40.1             |
| Inter-assay %RSD                  | 6.8                 | 11.8            | 7.0              | 6.1              |
| Inter-assay mean %bias            | 5.0                 | 8.0             | 2.0              | 0.3              |

QC, quality control; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control; %RSD, percentage of relative standard deviation; %bias, mean percentage bias
|                | HT 20% LQC | HT 20% HQC | HT 30% LQC | HT 30% HQC | HT 40% LQC | HT 40% HQC | HT 50% LQC | HT 50% HQC |
|----------------|------------|------------|------------|------------|------------|------------|------------|------------|
| n              | 6          | 6          | 6          | 6          | 6          | 6          | 6          | 6          |
| Mean tacrolimus concentration (ng/mL) | 2.94       | 40.6       | 3.16       | 42.3       | 3.06       | 40.9       | 2.93       | 45.5       |
| %RSD           | 2.8        | 4.4        | 3.7        | 2.6        | 4.1        | 3.5        | 9.0        | 4.7        |
| %bias          | -2.0       | 1.5        | 5.3        | 5.8        | 2.0        | 2.3        | -2.3       | 13.8       |

HQC, high quality control (40.0 ng/mL); HT, hematocrit; LQC, low quality control (3.0 ng/mL); %RSD, percentage of relative standard deviation; %bias, mean percentage bias
© 2020. The Author(s). Published by Wolters Kluwer Health, Inc. on behalf of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology.
Tacrolimus

Internal standard ([13C]-FK-506-D2)
