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

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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 the 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 1.00–50.0 ng/mL. No interference, carryover 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.

Key Words: LC-MS/MS, MITRA, tacrolimus, quantitation, validation

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INTRODUCTION

Tacrolimus, a macrolide lactone with a molecular weight of 822 Da (for the monohydrate form),[1] is the cornerstone of immunosuppressive therapy after 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 ($AUC_{0–tau}$).[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.[3–6] More recently, limited blood sampling strategies for tacrolimus AUC estimation have been used.[7]

Tacrolimus concentrations in whole blood samples can be determined using immunoassays or liquid chromatography–tandem mass spectrometry (LC-MS/MS).[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.[10–12] DBS assays have also been used to estimate tacrolimus pharmacokinetics.[10,13]

The MITRA microsampling device (Neoteryx, Torrance, CA) is a Food and Drug Administration (FDA) Class I, CE marked, blood sample collection device (Fig. 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

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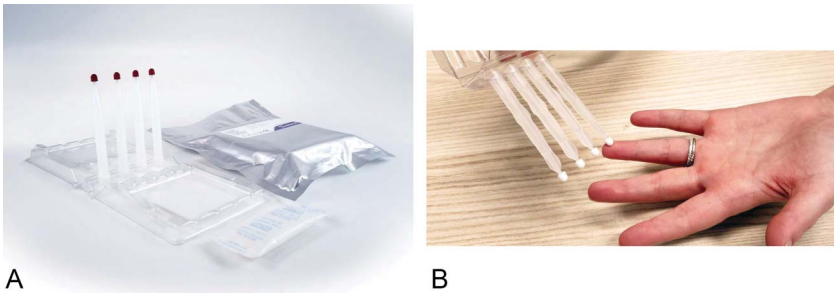

FIGURE 1. The MITRA microsampling device: (A) Example of the clamshell collection kit; (B) collection of capillary blood samples, that is, from a fingerprick. Images provided courtesy of Neoteryx, LCC (Torrance, CA).

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}C]-FK-506-D$_2$, were purchased from Toronto Research Chemicals (North York, Ontario, 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 ≤8 hours or refrigerated for ≤72 hours (never frozen). MITRA tips with a nominal absorption volume of 10 μL were acquired from Neoteryx and stored at room temperature (ie, 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 (eg, 9.90, 10.0, 10.6, and 10.9 μL 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 mcg/mL, and stored in amber glass vials at −20°C. The maximum storage periods for the tacrolimus and internal standard stock solutions were 36 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 before 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 before spiking with tacrolimus stock solution. The spiking volume was adjusted according to the blood volume, but did not exceed 1% of the blood volume (ie, 10 μL added to 990 μL of blood). Once spiked, samples were gently inverted and mixed on a roller mixer for ≥15 minutes before 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 of acetonitrile:water (50:50) containing 1 ng/mL of [^{13}C]-FK-506-D$_2$ 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 of 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 of 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 (ie, at nominal +5°C) for up to 119 hours before LC-MS/MS analysis.

LC-MS/MS Analysis

Analyses were performed using the Waters ACQUITY UPLC system (Waters, Milford, MA). 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). The column was maintained at 65°C, and the autosampler temperature was 5°C. Mobile phase A consisted of 0.01 mol/L of 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). 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, United Kingdom), 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.[14,15] 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 SD [%RSD]) at ≤15% (≤20% at the LLOQ) and for accuracy (expressed as mean % bias) at ±15% (±20% at the LLOQ).

TABLE 1. Chromatography Gradient Elution Profile

Time (min)	Mobile Phase A [Ammonium Formate 10 mM (aq):Formic Acid (100:0.2)]	Mobile Phase B [Acetonitrile]
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.

Specificity and Selectivity

Specificity and selectivity were determined by confirming the absence of interference and carryover. A range of representative chromatograms was obtained, including (1) a blank sample, (2) a blank sample spiked with the internal standard, (3) a sample spiked with tacrolimus and internal standard at the LLOQ; and (4) 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 4 independent runs of 6 samples at each of the 4 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, 6 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 (eg, all recovery values should fall within 75.0%–105.0%).

Matrix Effects and Factor

To determine the matrix effects, blank whole blood samples from 6 individual lots were analyzed without the internal standard; moreover, matrix samples from 6 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 6 individual lots and reagent blank samples (water), and spiked postextraction 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 4 hematocrit levels: 20%, 30%, 40%, and 50% (6 samples at each hematocrit level). To assess the effect of hyperlipidemia, 6 low QC and 6 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 (ie, 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 3 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 1.00–50.0 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-assay 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 1 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 (±SD) 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 ≤7.3% and %bias of ≤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 (ie, 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 ≤6.7% and %bias of ≤8.0%, as assessed between 3 different lots of MITRA tips with average blood wicking volumes of 10.0, 10.6, 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 that had been stored for several days. Matrix effects were noted in both the sample extraction, which

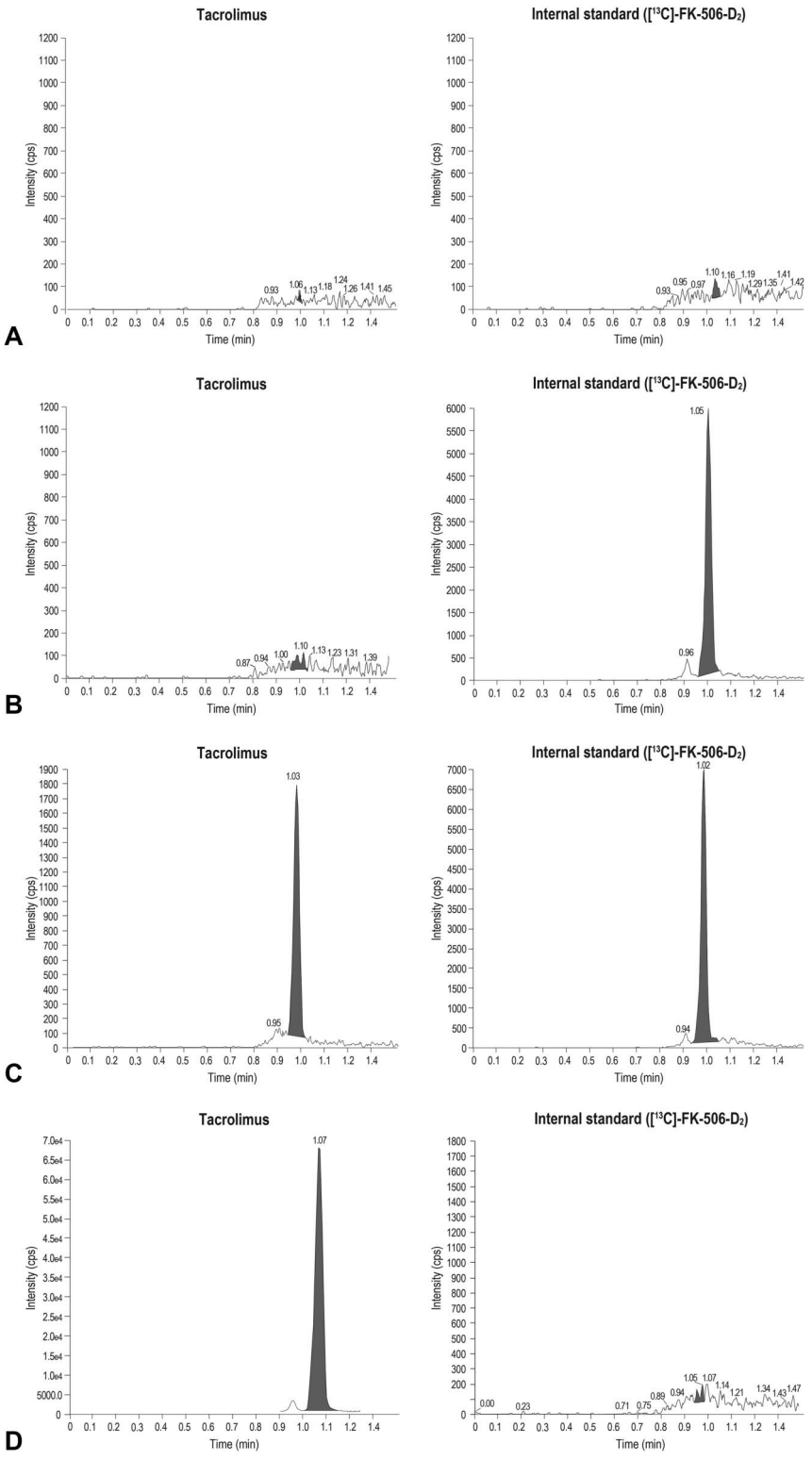

FIGURE 2. Representative ion chromatograms of (A) a blank sample (B) a blank sample spiked with the internal standard (C) a sample spiked with tacrolimus and internal standard at the LLOQ (1.00 ng/mL), and (D) a matrix blank spiked with tacrolimus at the ULOQ (50.0 ng/mL) and no internal standard. The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

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[4,16–18] and would typically be compensated for by an isotopically labelled internal standard. Although we had used electrospray ionization,

TABLE 2. Assay Accuracy and Precision

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

%bias, mean percentage bias; %RSD, percentage of relative standard deviation; HQC, high quality control; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; QC, quality control.

we found that moving to 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.

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 to 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 was similar to that of immunoassay methods used for therapeutic drug monitoring of tacrolimus.[4] Although a number of LC-MS/MS assay methods have reported an LLOQ in venous blood samples of 0.1 ng/mL,[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).[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 (ie, 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.[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% to 65%.[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.[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.[24–29] 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.[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

TABLE 3. Effect of Hematocrit on Assay Accuracy and Precision

	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

%bias, mean percentage bias; %RSD, percentage of relative standard deviation; HQC, high quality control (40.0 ng/mL); HT, hematocrit; LQC, low quality control (3.0 ng/mL).

monitoring of tacrolimus (eg, 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.

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Researchers may request access to anonymized participant level data, trial level data, and protocols from Astellas sponsored clinical trials at (www.clinicalstudydatarequest.com). For Astellas' criteria on data sharing see: (https://clinicalstudydatarequest.com/Study-Sponsors/Study-Sponsors-Astellas.aspx).

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