Journal of Mass Spectrometry and Advances in the Clinical Lab 19 (2021) 7–19

Contents lists available at ScienceDirect

Journal of Mass Spectrometry and Advances in the Clinical Lab

journal homepage: www.elsevier.com/locate/jmsacl

Research Article

Validation of a simple liquid chromatography coupled to tandem mass spectrometry method for the simultaneous determination of tacrolimus, sirolimus, everolimus and cyclosporin A in dried matrix on paper discs

Ignacio Guillermo Bressán [a,b,*], María Isabel Giménez [a,c], Susana Francisca Llesuy [b]

[a] Laboratory of Mass Spectrometry, Hospital Italiano de Buenos Aires, Buenos Aires, Argentina
[b] Department of Chemistry, Instituto Universitario Escuela de Medicina del Hospital Italiano, Buenos Aires, Argentina
[c] Department of Clinical Biochemistry, Instituto Universitario Escuela de Medicina del Hospital Italiano, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 12 October 2020
Revised 20 January 2021
Accepted 21 January 2021
Available online 2 February 2021

Keywords:
Dried matrix on paper discs (DMPD)
Immunosuppressants
Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)
Hematocrit
Method comparison

ABSTRACT

Introduction: Due to its high specificity and sensitivity, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the gold standard method for immunosuppressant quantification in therapeutic drug monitoring. In this context, dried blood spots (DBS) have become a promising strategy as a sample collection procedure. Although the advantages of DBS over venipuncture are well known, this approach has limitations that strongly influence the acceptance of analytical results. Among them, the most important is hematocrit (Ht). The easiest way of overcoming this problem is by analyzing complete spots. In this strategy, called dried matrix on paper discs (DMPD), blood is volumetrically applied on pre-punched discs.
Objectives: To validate an LC-MS/MS method for the quantification of tacrolimus, sirolimus, everolimus and cyclosporin A using DMPD.
Methods: The procedure was validated according to international guidelines using a commercial kit. The following performance parameters were evaluated: selectivity, carryover, linearity, accuracy, precision, lower limit of quantitation, relative recovery, commutability and stability. In addition, a method comparison study was performed to evaluate the clinical influence of Ht on the results.
Results: All performance parameters were within acceptance criteria and, hence, it was determined that the validated method is fit for the intended purpose. Likewise, calculated bias values on medical decision levels showed that there was no clinical influence of Ht on the results.
Conclusion: Unlike other similar methodologies that have been published, here, a simple method has been fully validated. This is the first LC-MS/MS methodology adapting a commercial kit to use DMPD as a sampling strategy.

1. Introduction

Immunosuppression therapy is the most important and accepted pharmacological strategy for prevention and treatment of graft rejection in solid organ transplantation [1]. Despite the wide variety of immunosuppressive agents currently available in clinical practice, calcineurin inhibitors (tacrolimus and cyclosporin A) and mTOR inhibitors (sirolimus and everolimus) are some of the most used small molecules. These drugs can be administered in single or multiple regimens for induction, maintenance or rescue purposes [2].

Long-life therapeutic monitoring of immunosuppressants is a key tool in the treatment of transplant patients. It allows for improved and individualized therapies, which result in the best

Abbreviations: $[M+NH_4]^+$, Ammoniated adduct; mTOR, Mechanistic target of Rapamycin; C_0, Pre-dose trough concentration; C_2, 2-hour post-dose concentration; LC-MS/MS, Liquid chromatography coupled to tandem mass spectrometry; DBS, Dried blood spots; Ht, Hematocrit; PDBS, Perforated dried blood spots; PCDBS, Pre-cut dried blood spots; DMPS, Dried matrix on paper discs; CS, Calibration standard; QC, Quality control samples; LLOQ, Lower limit of quantitation; LSS, Limited sampling strategy; ESI+, Positive electrospray source ionization mode; PIs, Prediction intervals; CV%, Coefficient of variation; RE%, Percentage of the relative error; R %, Relative recovery; IC_m95%, 95% confidence interval for slopes; IC_b95%, 95% confidence interval for intercepts; $ZnSO_4{\cdot}7H_2O$, Zinc sulfate heptahydrate.

* Corresponding author at: Laboratory of Mass Spectrometry, Hospital Italiano de Buenos Aires, Tte. Juan Domingo Perón 4190 (C1181ACH), Buenos Aires, Argentina.
E-mail address: ignacio.bressan@hospitalitaliano.org.ar (I.G. Bressán).

https://doi.org/10.1016/j.jmsacl.2021.01.003
2667-145X/

clinical outcome. Measurement of calcineurin and mTOR inhibitors in whole blood (obtained via venipuncture) is especially recommended due to their high between-subject pharmacokinetic variability and narrow therapeutic ranges, which depend on several factors including the type of transplant, the type of therapy and the time of sample collection (pre-dose trough concentration (C_0) or 2-hour post-dose concentration (C_2)) (Table 1). Abbreviated area under the concentration time curve (AUC) obtained by limited sampling strategy (LSS) is the best in predicting response compared to C_0 and C_2 [3,4]. Nevertheless, this approach still faces logistical and financial disadvantages that make it difficult to implement in clinical practice [5]. Regarding the type of sample, whole blood is the gold standard matrix for patient monitoring because of the extensive binding of immunosuppressants to erythrocytes [2,6].

Currently, the available analytical procedures for monitoring immunosuppressive agents in patient specimens can be classified into two distinct categories: immunoassays and liquid chromatography-based methods. The first usually show significant positive bias because of metabolite cross-reactivity [8]. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is considered the gold standard methodology owing to its high sensitivity and specificity. In addition, the ability to develop multianalyte panels using a single method offers additional time, labor and expense savings [8,9].

At present, several companies offer commercial kits that include reagents, columns and recommendations to perform the quantification of these drugs in whole blood by LC-MS/MS [10]. The use of these kits has had a significant impact in clinical laboratories, including, improved sample throughput, and avoidance of several issues related to in-house developed methods such us weighing solid drugs standards, preparing stock and working solutions and spiking free-analyte matrices. Additionally, commercial kits reduce discrepancies in measurements between laboratories, and improve standardization [9].

With the introduction of LC-MS/MS into clinical laboratories, microsampling approaches have been increasingly considered as useful alternatives to conventional venous sampling. These strategies refer to procedures for collecting small volumes of blood (or other body fluids) in a minimally invasive manner [11]. In the field of therapeutic drug monitoring, dried blood spot (DBS) sampling seems to be particularly promising. DBS consists of applying capillary blood, obtained via a finger prick, to a sampling paper by either a medical professional or patients themselves. After drying and transportation, a sample disc is punched from the DBS and analytes are then extracted and analyzed [12].

Advantages of DBS over venous sampling are well known. Compared to venipuncture, DBS collection is minimally invasive and can be conducted in a nonclinical setting (e.g., patient's residence), with minimal training required for patients or caregivers. Low volumes required for analysis (usually less than 100 μL) make this sample collection method desirable for infants, young children, and critically ill patients [13,14]. Furthermore, most analytes are more stable in DBS than in frozen samples, allowing specimens to be shipped via standard post with no reasonable expectations of exposure to infectious material by handlers [11,13,15]. In the field of solid organ transplantation, these advantages are particularly useful to outpatients who frequently need to travel to the hospital for blood draws. Additionally, early transfer of DBS specimens to the laboratory provides clinicians with analysis results before patients visit the clinic for their routine checkup, allowing more efficient patient-physician contact time [15,16].

Despite the benefits of DBS sampling, there are three preanalytical variables that could impair result quality. These include (i) the blood spot volume, (ii) the chromatographic effect (homogeneity), and (iii) the hematocrit (Ht) [13,15,17]. Point (i) refers to the assumption that the blood volume contained within paper disc punches of the same size (e.g., 3, 5 or 8 mm) is constant. It must be considered that volume cannot be controlled when blood is directly spotted from the patients finger onto the DBS card. This could r0esult in different measured analyte concentrations from a fixed punch size for different patients when the concentrations may, in fact, be the same. For this reason, spot volume should be evaluated during method development or validation [18]. If the effect is known, it can be more easily managed.

To dispense accurate fixed blood volumes on the paper, capillary tubes or micro-collection pipettes can be used [13]. However, the ability to collect a sample of reasonable quality for clinical analysis depends on comprehensive training of both laboratory staff and patients, especially in the case of home-based self-sampling. Hence, the expected advantage of this technique for home sampling could be expected to be limited versus the simple process of sampling a single drop of blood; however, studies using this technique for DBS self-sampling in a hospital or clinical setting demonstrated that sample quality was comparable to that collected by trained personnel [12].

In addition to capillary tubes and micro-collection pipettes, other new commercial devices including volumetric absorptive microsampling (VAMS) and microneedles have recently been developed, showing promising results [11].

Point (ii), chromatographic effects, occur due to interactions between blood and/or analytes with the paper materials. This can result in significant differences in concentration between central and peripheral areas within a DBS [19].

Point (iii), Ht, is the most widely discussed DBS-related problem [19]. This hematological parameter is defined as the volume fraction of the blood that is occupied by erythrocytes. Ht is directly proportional to the viscosity of blood and affects the flux and diffusion properties of the matrix on the paper. In addition, a linear inverse relationship between DBS area and Ht has been well-established [20]. This means that at a high Ht the distribution of blood through the paper is poor, resulting in small blood spots. The opposite effect occurs when the Ht of the sample is low [13,19,20]. Considering that calcineurin and mTOR inhibitors are extensively bound to red blood cells, this phenomenon becomes critical.

Table 1
Therapeutic ranges for calcineurin and mTOR inhibitors, according to the type of transplant, type of therapy, and time of sample collection. Adapted from Sádaba (2016) [7].

Type of transplant	Type of therapy	Therapeutic Range (ng/mL)				
		Cyclosporine A		Tacrolimus	Sirolimus	Everolimus
		C_0	C_2	C_0	C_0	C_0
Kidney	*Induction*	(200–400)	(1400–2000)	(10.0–15.0)	(5.0–15.0)	(5.0–15.0)
	Maintenance	(100–275)	(700–1800)	(5.0–10.0)	(5.0–15.0)	(3.0–8.0)
Liver	*Induction*	(250–350)	(800–1200)	(10.0–20.0)	–	(3.0–8.0)
	Maintenance	(100–200)	(600–1000)	(5.0–10.0)	–	(3.0–8.0)
Heart	*Induction*	(250–350)	–	(15.0–20.0)	–	(3.0–8.0)
	Maintenance	(100–200)	(300–600)	(5.0–10.0)	–	(3.0–8.0)

To avoid the variable effect of Ht on analytical results, several strategies have been proposed: these include application of a range of calibrators prepared in blood with varying Ht values covering the full range of the samples to be analyzed [21–23]; the mathematical correction of the obtained results for the Ht of the sample (determined from whole blood [19,24], estimated from another endogenous compound such as potassium or creatinine [25–28] or directly on the paper card [29]); and the analysis of whole-cut DBS discs instead of partial cuts [13]. The last one seems to be the most practical way of overcoming the Ht problem.

To analyze whole-spot concentrations, two strategies have been described in the literature. For the first approach, a complete DBS is punched after volumetric application of the blood [13,24,30,31]. The second approach consists of the volumetric application of blood on pre-punched discs [32–35]. Variants of this methodology include perforated dried blood spot (PDBS) and pre-cut dried blood spot (PCDBS) [33–35]. In the PDBS format, paper discs are punched out from the filter paper and placed back into it before DBS sampling. In the case of PCDBS, the perforated discs are attached on a support system instead of being placed back into the sheet of filter paper. A variant of the latter is called "dried matrix on paper discs" (DMPD), which applies a dedicated support system for the pre-cut paper discs. Advantages of DMPD approach include improved storage conditions and transportation of samples [35].

These microsampling methods have been successfully applied for the quantification of several drugs in human and animal samples [33–35]. However, neither of them has been used for the quantification of immunosuppressants. Due to its advantages, DMPD appears to be especially promising for clinical purposes. For that reason, the presented work describes the full validation of a simple LC-MS/MS method for the simultaneous quantification of tacrolimus, sirolimus, everolimus and cyclosporin A in DMPD, adapting a commercial kit (MassTrak Immunosuppressants XE Kit from Waters Corporation). In addition, a method comparison study using paired samples was performed to evaluate the clinical influence of Ht on the analytical results.

2. Materials and methods

2.1. Reagents and chemicals

Optima LC-MS grade acetonitrile and methanol were purchased from Fischer Scientific (Pittsburg, PA, USA) and ultra-pure water was obtained with an Arium Pro UV Water Purification System (Sartorius Stedim Biotech GmbH, Göttingen, Germany). Formic acid was purchased from Honeywell Fluka (Seelze, Germany). Ammonium acetate and zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$) were obtained from Merck (Darmstadt, Germany)

2.2. Kit components

The MassTrak Immunosuppressants XE Kit was purchased from Waters Corporation (Milford, MA, USA). The kit contains 7 lyophilized vials of primary assay calibrator standards (CS) (6 nonzero and 1 blank whole blood calibrator), 3 lyophilized vials of quality control samples (QC) (low, medium and high), an internal standard solution (ascomycin for tacrolimus and sirolimus, $[^{13}C_2^2H_4]$-everolimus for everolimus and $[^2H_{12}]$-cyclosporin for cyclosporin A), an instrument tuning mixture and a chromatographic column (MassTrak™ TDM C18 2, 1 × 10 mm, 3.5 μm).

All vials and solutions were prepared according to the manufacturer's directions. Briefly, CS and QC samples were reconstituted using ultra-pure water, while the tuning mixture and the internal standard solution were prepared using methanol, and methanol followed by acetonitrile, respectively. All CS and QC samples were used in both whole blood and DMPD quantification assays. Nominal concentrations are summarized in Table 2.

The MassTrak Immunosuppressants XE Kit is routinely used in this laboratory in order to measure immunosuppressants in whole blood samples obtained via venipuncture. This method was fully validated before performing the present study.

2.3. Sampling papers and support system

Whatman 903™ paper cards were purchased from Sigma Aldrich (St. Louis, MO, USA). DMPD cartridges (disk diameter; 5 mm) and 10 μL fixed-volume capillary micropipettes were provided by Rock Town Technologies & Services (Libertyville, IL, USA). 5 mm paper discs were obtained using a WALLAC DBS Puncher (Perkin Elmer, Waltham, MA, USA).

2.4. Whole blood samples

2.4.1. Collection and storage

Venous samples were collected from children and adult transplant patients, from in- and out-patients on single or multiple drug therapy, during their routine clinical check-ups. Specimens were taken from January to March of 2018. K_2EDTA was used as anticoagulant. To obtain trough concentrations, collection was coordinated prior to dose administration (30–60 min) and was performed by phlebotomists. Samples received from other health centers were also used, providing the measurement of immunosuppressive drugs in whole blood and Ht was documented in the medical analysis request; Ht was a mandatory inclusion criterion for all samples. Specimens were analyzed within a day as they were part of routine care and were stored at 2 °C for 7 days. The use of anonymous patient data and samples was approved by the Institutional Review Board (IRB00010193).

2.4.2. Preparation

According to the manufacturer's instructions, 50 μL of CS, QC or whole blood patient samples were transferred into 1.5 mL Eppendorf tubes. Then, 200 μL of 0.1 M $ZnSO_4{\cdot}7H_2O$ were added and the mixture was vortexed for 20 s to promote red blood cells lysis. After this step, 500 μL of the internal standard solution were added. The contents were vortexed for 20 s to facilitate protein precipitation, and then centrifuged at 12′500 rpm for 5 min at 4 °C. Finally, 25 μL of the supernatant were injected onto the LC-MS/MS system.

2.5. DMPD samples

2.5.1. Collection and storage

Pre-cut 5 mm Whatman 903™ paper discs were situated on empty DMPD cartridges. Then, a single 10 μL aliquot of CS, QC or

Table 2
Nominal concentrations of CS and QC levels provided in the MassTrak Immunosuppressants XE Kit. The kit does not provide a QC sample at the LLOQ level, for that reason a calibrator 1 from another lot, but with the same nominal concentration, was used.

Vial	Analyte (ng/mL)			
	Tacrolimus	Sirolimus	Everolimus	Cyclosporin A
CS 1	1.1	1.0	1.0	27.7
CS 2	3.1	2.9	3.4	52.4
CS 3	6.1	5.6	6.6	102
CS 4	12.0	11.1	13.0	201
CS 5	20.1	18.2	19.3	499
CS 6	30.8	27.5	33.4	1483
QC LLOQ	1.1	1.0	1.0	27.7
QC Low	2.1	2.0	2.2	159
QC Medium	8.4	7.5	8.9	411
QC High	22.5	20.3	24.6	922

whole blood patient samples (collected as described in 2.4) was spotted on each disc by trained laboratory staff members. Specimen spreading was performed using a fixed-volume capillary micropipette. DMPD samples were left to dry at least for 6 h at room temperature prior to analysis.

2.5.2. Preparation

DMPD samples were transferred into 2.0 mL Eppendorf tubes. Then, 40 μL of 0.1 M $ZnSO_4 \cdot 7H_2O$ were added and vortexed for 20 s. Subsequently, 100 μL of the internal standard solution were added and vortexed again for 20 s. The mixture was sonicated for 60 min at room temperature using a TB024 ultrasonic bath (Testlab®, Buenos Aires, Argentina). Finally, 25 μL of the supernatant were injected onto the LC-MS/MS system.

2.6. Ht measurement

Ht values from all whole blood samples were measured using a UniCel DxH-800 analyzer (Beckman Coulter Inc.; Brea, CA, USA). This measurement was made as part of each patient's routine checkups as requested by their physician and was not specifically performed for this study.

2.7. Instruments and analytical conditions

2.7.1. Chromatographic conditions

Liquid chromatographic analysis for whole blood and DMPD samples was performed using an Acquity UPLC system equipped with a binary solvent manager, a sample manager and a column heater. The chromatographic instrument was coupled to a Xevo TQ MS triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). Separation was performed on a MassTrak™ TDM C18 column (2.1x10 mm 3.5 μm), according to the directions for use. The mobile phases consisted of 2 mmol/L ammonium acetate in water, 0.1% formic acid (A) and 2 mmol/L ammonium acetate in methanol, 0.1% formic acid (B). The column temperature was maintained at 55 °C and a linear gradient elution was performed at 0.4 mL/min as follows: 0–0.6 min 50% A – 50% B; 0.6–1.2 min 0% A – 100% B; 1.2–2.0 min 50% A – 50% B. The auto-sampler temperature was kept at 10 °C.

2.7.2. Mass spectrometric conditions

The mass spectrometer was operated in positive electrospray source ionization mode (ESI +). Detection parameters applied to both whole blood and DMPD analyses were as follows: capillary voltage, 2.80 kV; cone voltage, 20 V; desolvation temperature, 550 °C; desolvation gas (N_2), 800 L/h; cone gas (N_2), 50 L/h; and collision gas (Ar), 0.15 mL/min. Multiple reaction monitoring (MRM) transitions for each compound are summarized in Table 3. The MassLynx™ software (Version 4.1, Waters Corporation, Milford, MA, USA) was used for instrument control, data acquisition and processing.

2.8. Analytical method validation on DMPD

The analytical method was validated according to international guidelines [36–40]. The following performance parameters were evaluated: selectivity, carryover, linearity, accuracy, precision, lower limit of quantitation (LLOQ), relative recovery, commutability (matrix effect) and stability under different conditions.

Selectivity was investigated for potential interference of endogenous substances by using ten independent batches of venous blood samples from unexposed patients. The absence of interfering components was accepted if the area was<20.0% of the LLOQ for each analyte and 5.0% for the internal standards.

Table 3
Mass spectrometric conditions for immunosuppressants and their internal standards. All precursor ions create ammoniated adducts $[M + NH_4]^+$.

Analyte	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Cone voltage (V)	Collision energy (eV)
Tacrolimus	821.7	768.6	28	20
Sirolimus	931.8	864.6	20	16
Everolimus	975.7	908.6	20	16
Cyclosporin A	1220.0	1202.9	15	21
Ascomicin	809.5	756.5	28	20
$[^2H_{12}]$-cyclosporin	1232.0	1214.9	15	21
$[^{13}C_2^2H_4]$-everolimus	981.7	914.6	22	18

Carryover was examined by injecting a blank after the highest calibrator. Criteria for acceptability included that the blank sample area following the highest calibrator should not be>20.0% of the LLOQ area for each immunosuppressant and 5.0% for the internal standards.

Linearity was evaluated by linear regression. Each calibrator was injected five successive times on three consecutive days. Response was defined as the peak area ratio of the analytes to the internal standards (y) and was plotted against each drug's nominal concentration (x). Concentration of each immunosuppressive drug in unknown DMPD samples was calculated by the following equation: $x = (y$ - intercept)/slope.

The within-day precision and accuracy were assayed by replicate analysis (n = 5) of the four levels of QC samples during each analytical run. The between-day precision and accuracy were determined by evaluating the QC samples during three different analytical runs on three consecutive days. Furthermore, each analytical run was prepared by a different analyst. Precision was expressed as coefficient of variation (CV%), calculated as [CV% = (standard deviation/mean of measured values) × 100]. Accuracy was expressed as a percentage of the relative error (RE%), determined by the formula [RE% = [(mean measured concentration – nominal concentration)/nominal concentration] × 100]. Raw data were processed in Microsoft Excel version 16.0.6568.2036 (Microsoft Corporation, Redmond, WA, USA). Criteria for acceptability included accuracy within ±15.0% deviation from the nominal values and precision within ±15.0% of CV% for each QC level.

The LLOQ was defined as the lowest concentration of analyte that gave precision and accuracy values within the limits of ± 20.0%, and a signal-to-noise ratio (S/N) of at least 10.

The relative recovery (R%) between DMPD and whole blood for each analyte was determined according to Eurachem Guidelines [38]. At each QC level, ten independent preparations of both DMPD and whole blood were processed as described in 2.4.2 and 2.5.2 and analyzed in the LC-MS/MS system. Relative recovery for each analyte and internal standard was calculated using the formula [R% = (mean of the measured values of each analyte in DMPD/mean of the measured values of each analyte in whole blood) × 100]. In addition, reproducibility of the extraction process was evaluated as follow: [CV% = (standard deviation/mean of the measured values in DMPD) × 100].

Matrix effect was studied according to the Clinical Laboratory Standards Institute guideline, in terms of commutability [40]. Hence, 20 paired patient samples covering the linear range were prepared as described in 2.4.2 and 2.4.5. Randomly, 5 paired QC samples (1 LLOQ, 1 low, 2 medium and 1 high) were integrated throughout the specimen run. The procedure was performed in triplicate, with calibrations performed for each matrix. Subsequently, the mean values obtained for the 20 patient and 5 QC paired samples were plotted (whole blood results on the x axis and PCDBS values on the y axis). Once the linear relationship between the two variables was visually verified, Deming linear

regression was applied using the Analyse-it software version 5.01 (Leeds, United Kingdom). Where a processed QC result fell outside the 95% prediction intervals (PIs) for human samples, the material was considered as non-commutable.

Finally, the stability of the DMPD samples was evaluated at ambient conditions (24 °C),up to 10 days, packing the samples in zip lock plastic mini bags with a desiccant, as previously described in the literature [23,41]. In addition, the extracts were kept at 10 °C in the auto-sampler and the stability was determined after 24 h. In every case, stored QC samples (low, medium and high) were analyzed against a calibration curve obtained from freshly spiked CS. The measured levels were compared to the nominal values. To be accepted as stable, the mean concentration at each level had to be within ±15.0% of the nominal concentration with a CV% less than 15.0%.

2.9. Method interchangeability and study of the Ht effect

To test the interchangeability between DMPD and whole blood, a method comparison study was performed. In this step, it was assumed that the spot volume and the chromatographic effects were negligible because of the characteristics of the DMPD technique. Therefore, the potential non-interchangeability between methods could be attributed to the Ht effect.

The study was completed according to the Clinical Laboratory Standards Institute recommendations [42]. At least 100 whole blood samples for each analyte were collected from exposed transplanted patients as described in 2.4.1. Specimens were selected using the Laboratory Informatic System, considering drug and Ht values, which were previously measured as part of each patient's routine checkups. For that reason, samples received from other health centers were also included. The aim of this step was to cover not only the linear range of each immunosuppressant, but also a wide interval of Ht values.

To obtain paired specimens, 10 μL aliquots of each whole blood sample were collected directly from the $EDTAK_2$ tubes, as described in 2.4.1. Both types of samples were prepared as mentioned in 2.4.2 and 2.5.2, and then analyzed by LC-MS/MS.

Statistical analysis was performed on three main steps using the *Analyse-it* software version 5.01 (Leeds, United Kingdom). First, a non-parametric Passing-Bablok regression analysis was performed to estimate constant or proportional errors between methods. Statistical significance was set at $p < 0.05$ and results were presented with 95% confidence intervals for slopes and intercepts.

Second, a graphical concordance study for each analyte using difference plots was assembled to estimate the global bias of the DMPD methodology. To select the best bias estimator (average or median), verification of the normality of the differences between methodologies was performed. Then, the limits of agreement (LoA) were calculated. In addition, clinical relevance limits were established by a multidisciplinary team at a range of ±15.0% for all the studied immunosuppressants, as values outside this range would lead to different dosing advice.

Finally, the calculated regression line was used to estimate the bias values on medical decision levels (lower and higher limits values of the therapeutic ranges according to Table 1) for each analyte. The quality requirements to contrast the obtained bias values were established according to the recommendations of the Royal College of Pathologists of Australasia (43) at ±0.5 ng/mL if concentrations were less than 5.0 ng/mL for tacrolimus and sirolimus, and ±10.0 ng/mL for cyclosporin A if concentrations were less than 100 ng/mL. Likewise, bias values were set at ±10% if concentrations were higher than 5.0 ng/mL for tacrolimus and sirolimus and 100 ng/mL for cyclosporin A. At this time, no quality requirements for everolimus have been published. Nevertheless, in this study the quality requirements for sirolimus have been applied for everolimus, because of their similar structure and mechanism of action.

3. Results

3.1. Analytical method validation

None of the free immunosuppressive drug samples showed endogenous interferences at the retention times of analytes or internal standards. Therefore, the selectivity was considered acceptable.

Calibration curves were linear over the declared concentration range for each analyte. Weighting indexes (1/x for tacrolimus and cyclosporin A, and $1/x^2$ for sirolimus and everolimus) were selected and used to determine slopes, intercepts and determination coefficients (r^2) each day. The latter exceeded 0.995 for each drug on each analytical run. The LLOQ was determined to be 1.1, 1.0, 1.0 and 27.7 ng/mL for tacrolimus, sirolimus everolimus and cyclosporin A, respectively. All back-calculated standard concentrations were within 15.0% deviation from the nominal value (Table 4). The residuals showed no tendency of variation with concentration (data not shown). No significant carry over was observed for any immunosuppressant.

For all of the immunosuppressive drugs, the within-run and between-run precision and accuracy CV% and RE% values were within the acceptable limits stated for bioanalytical method validation. According to these results, the assay is accurate and precise enough for the studied concentration range. The relative recovery values for all the analytes were near 100% at all the studied concentration levels and the precision was acceptable (Table 5).

Results of the commutability study are shown in Fig. 1 and Table 6. Deming regression was performed on the individual sample data, and 95% PIs were calculated as recommended in the CLSI EP14-A3 document [40]. For any of the studied immunosuppressive drugs, neither of the QC results fell outside the 95% PIs, showing that matrix effect was absent.

Regarding the stability studies, there was no difference in the concentration of any of the drugs maintained at auto-sampler conditions 24 h after sample preparation. Stability of the drugs on DMPD was maintained at room temperature (24 °C), results showed that tacrolimus was stable for 10 days, sirolimus for 9 days, everolimus for 8 days and ciclosporin A for 7 days (Table 7).

3.2. Method interchangeability

3.2.1. Demographic characteristics

The validated method was applied to the analysis of 399 paired samples collected from 289 transplant patients (109 (37.7%) females; 180 (62.3%) males; median age: 44 years, range: 0.5 to 86 years; median of samples per patient: 1, range: 1 to 8). In 50 specimens (12.5%), more than one drug was quantified.

With regard to the type of patient, 59 (20.4%) were inpatients and 125 (43.3%) were outpatients. From the remaining 105 (36.3%), data was not available because their samples were obtained from another health center.

The study included 92 kidney (31.8%), 37 liver (12.8%), 15 bone marrow (5.2%), 12 reno-pancreas (4.2%), 11 heart (3.8%), 7 lung (2.4%), 3 liver-kidney (1.0%), 1 heart-kidney (0.3%), and 1 bowel transplant patients (0.3%). For the remaining individuals (38.2%), this information was not available.

The median Ht value for analyzed samples was 35.5% (range: 17.9 to 57.0%). These values were obtained via the Laboratory Informatic System and were distributed over the analytical measurement range for each immunosuppressant (Fig. 2).

Table 4
Results of the linearity assay.

Analyte	CS (n = 3)	Nominal concentration (ng/mL)	Day 1		Day 2		Day3	
			RE%	r^2	RE%	r^2	RE%	r^2
Tacrolimus								
	1	1.1	1.7	0.9981	−2.7	0.9968	−1.1	0.9990
	2	3.1	−3.9		2.5		−0.8	
	3	6.1	−5.3		−0.3		−1.0	
	4	12.0	1.1		3.5		−2.2	
	5	20.1	4.1		−3.7		−0.5	
	6	30.8	−1.9		1.8		0.5	
Sirolimus				0.9998		0.9963		0.9962
	1	1.0	2.4		1.9		0.5	
	2	2.9	−1.9		−6.1		−0.8	
	3	5.6	−8.1		3.3		3.5	
	4	11.1	3.8		3.8		1.7	
	5	18.2	9.0		−4.4		2.7	
	6	27.5	−2.4		−3.0		3.1	
Everolimus				0.9961		0.9967		0.9974
	1	1.0	2.3		1.5		3.1	
	2	3.4	−7.5		−7.3		−9.8	
	3	6.6	−1.3		5.7		−1.0	
	4	13.0	2.8		3.5		−0.2	
	5	19.3	6.6		−4.6		6.0	
	6	33.4	−2.9		0.9		3.1	
Cyclosporin A				0.9983		0.9978		0.9981
	1	27.7	−0.4		−3.2		5.7	
	2	52.4	−6.5		−2.4		−7.3	
	3	102	−2.2		3.1		3.7	
	4	201	6.2		0.0		−1.5	
	5	499	5.1		3.6		−1.0	
	6	1483	−2.2		5.0		0.5	

Table 5
Results of the accuracy, precision and recovery assays.

Analyte	QC (n = 5)	Nominal concentration (ng/mL)	Day 1		Day 2		Day 3		Inter-run		Recovery	
			RE%	CV%	RE%	CV%	RE%	CV%	RE%	CV%	R%	CV%
Tacrolimus	LLOQ	1.1	2.9	11.6	−4.6	8.6	−12.9	5.7	−4.9	10.8	–	–
	Low	2.1	2.1	9.7	5.8	6.8	1.0	5.3	3.0	7.3	98.6	7.9
	Medium	8.4	−0.4	4.0	−1.9	7.9	2.6	3.7	0.1	5.4	99.0	4.8
	High	22.5	−3.5	6.1	−7.3	6.6	2.5	2.4	−2,8	6.5	99.6	5.1
Sirolimus	LLOQ	1.0	−7.4	9.1	−1.5	11.2	−1.4	10.0	−3.4	10.1	–	–
	Low	2.0	−2.4	11.7	−2.1	9.7	−3.2	10.0	−2.6	9.8	103.6	12.7
	Medium	7.5	−3.4	1.6	−2.0	5.5	3.2	5.4	−0.8	4.5	100.4	11.9
	High	20.3	−5.1	6.6	−8.7	5.1	4.3	5.6	−3.2	8.0	100.3	4.9
Everolimus	LLOQ	1.0	0.7	14.6	−3.3	12.1	1.7	11.6	−0.3	12.7	–	–
	Low	2.2	−0.1	11.1	−3.3	10.2	1.2	9.8	−0.7	9.8	102.0	11.5
	Medium	8.9	4.4	6.0	0.4	7.8	2.5	2.8	2.4	5.7	99.6	11.7
	High	24.6	−4.8	8.2	−10.2	5.9	7.7	4.4	−2.5	9.8	99.2	5.8
Cyclosporin A	LLOQ	27.7	3.7	5.1	−2.8	9.8	1.7	3.4	0.3	6.7	–	–
	Low	159	−1.0	9.0	−3.4	8.5	0.5	5.3	−1.3	7.4	100.1	9.2
	Medium	411	−2.1	6.5	−2.2	7.1	1.8	3.0	−0.8	5.7	99.3	5.2
	High	922	−1.5	7.5	−9.5	3.0	4.5	3.9	−2.2	7.8	103.1	6.9

Demographic characteristics for each immunosuppressive drug are summarized in Table 8.

3.2.2. Statistical analysis

Passing-Bablok regression analysis showed a significant relationship between DMPD and venous whole-blood levels for all studied drugs. In all cases, correlation coefficient values were greater than 0.990 and the 95% CI for the intercepts and slopes were 0 and 1, respectively. Therefore, no constant or proportional bias was detected (Fig. 3 and Table 9).

Difference plots displayed the results of whole blood concentrations on the *x*-axis, and the percent difference between the DMPD and whole blood measurement procedures on the *y*-axis. That choice was made because, for all drugs, the variability of the differences between the two measurement procedures changed with increasing concentrations (data not shown).

Due to the lack of normality found for the measurement differences between analytical methods (p values obtained via Shapiro Wilks test were <0.001 for tacrolimus, 0.011 for sirolimus, 0.054 for everolimus and 0.028 for cyclosporin A), the median value was selected as the bias estimator. The limits of agreement were calculated using a non-parametric method considering both 2.5 and 97.5 percentiles. In addition, clinical relevance limits were also established by a multidisciplinary team at a range of ±15.0% for all of the studied immunosuppressants. In all cases, less than 5.0% of the results were excluded based on the agreement limits (4.3, 4.6, 4.8 and 4.9% for tacrolimus, sirolimus, everolimus, and cyclosporin A respectively).

Tacrolimus and everolimus difference plots showed no significant difference in bias between methodologies; 95% confidence intervals for the medians contained the value 0. The 95% confidence intervals for the median of the remaining drugs did

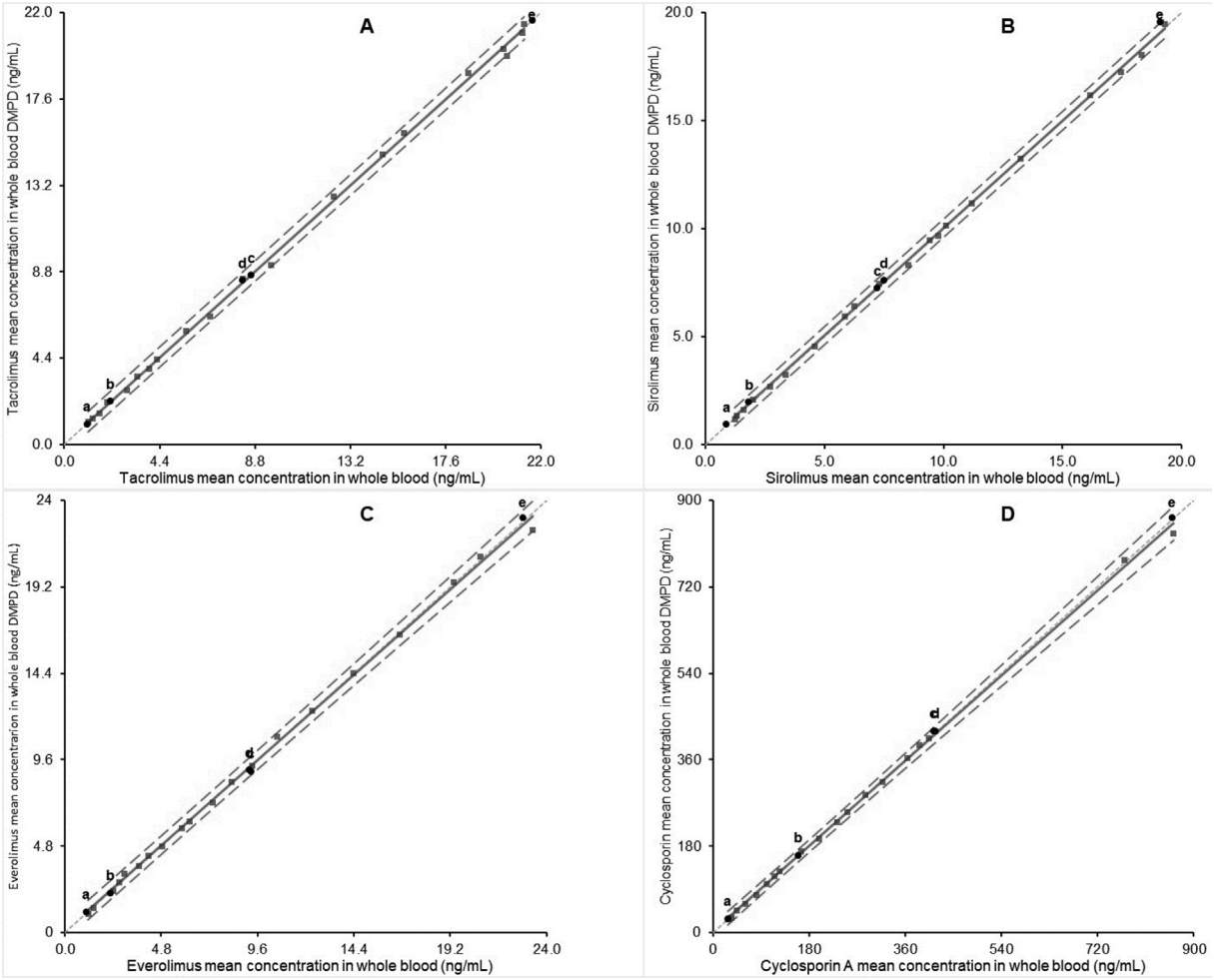

Fig. 1. Deming regression lines for tacrolimus (A), sirolimus (B), everolimus (C) and cyclosporin A (D). Dotted lines are the 95% prediction intervals, and the continuous lines are the Deming regression lines. Patient samples are presented as grey dots. Processed LLOQ (a), low (b), medium (c,d) and high (e) QCs are showed as black dots.

Table 6
Results of the commutability assay (matrix effect).

Analyte	Samples' concentration range (ng/mL) (n = 20)	Slope	95% confidence Interval	Intercept	95% confidence Interval	QC level	Predicted DMPD concentration (ng/mL)	Prediction interval (ng/mL)	Commutability
Tacrolimus	(1.1–21.4)	0.996	(0.977–1.013)	0.080	(−0.038 to 0.199)	LLOQ	1.1	(0.6–1.7)	Yes
						Low	2.2	(1.7–2.7)	Yes
						Medium	8.7	(8.2–9.2)	Yes
						Medium	8.3	(7.8–8.8)	Yes
						High	21.6	(21.0–22.2)	Yes
Sirolimus	(1.0–19.5)	0.997	(0.982–1.011)	0.046	(−0.054 to 0.147)	LLOQ	1.0	(0.5–1.4)	Yes
						Low	1.9	(1.5–2.3)	Yes
						Medium	7.3	(6.8–7.7)	Yes
						Medium	7.5	(7.1–7.9)	Yes
						High	19.1	(18.6–19.5)	Yes
Everolimus	(1.0–24.0)	0.988	(0.950–1.027)	0.116	(−0.122 to 0.355)	LLOQ	1.2	(0.6–1.7)	Yes
						Low	2.4	(1.8–2.9)	Yes
						Medium	9.3	(8.8–9.8)	Yes
						Medium	9.2	(8.7–9.7)	Yes
						High	22.7	(21.8–23.5)	Yes
Cyclosporin A	(27.7–890)	0.987	(0.939–1.034)	3.554	(−4.024 to 11.130)	LLOQ	30.6	(16.4–44.6)	Yes
						Low	161	(148–174)	Yes
						Medium	411	(393–428)	Yes
						Medium	414	(397–432)	Yes
						High	852	(816–887)	Yes

not contain 0 (Fig. 4). For that reason, the calculated regression line for each immunosuppressant was used to estimate the bias values on medical decision levels.

Neither of the studied drugs presented bias values out of the defined acceptable error range, at each clinical decision level (Table 9). For that reason, the Ht effect was considered as non-

Table 7
Results of the autosampler and ambient conditions stability assays.

Analyte	QC level (n = 5)	Stability condition									
				DMPD maintained at room temperature (24 °C)							
		Autosampler (24 h)		Day 8		Day 7		Day 9		Day 10	
		RE%	CV%	RE%	CV%	RE%	CV%	RE%	CV%	RE%	CV%
Tacrolimus	Low	0.9	1.1	1.2	3.9	−1.9	11.1	−2.5	3.6	−7.7	4.6
	Medium	1.8	5.3	4.9	7.1	−4.3	10.7	−3.4	5.7	−2.5	1.9
	High	2.3	4.2	11.5	8.2	−2.9	4.9	−4.9	11.2	−3.0	3.5
Sirolimus	Low	−0.5	9.4	5.4	1.2	−2.7	0.9	−3.0	4.4	–	–
	Medium	5.4	6.2	−1.2	8.7	−8.2	11.9	−7.6	1.6	–	–
	High	3.9	1.9	5.0	4.6	−2.4	5.9	−10.2	4.7	–	–
Everolimus	Low	1.8	1.2	−9.1	6.9	−2.8	8.5	–	–	–	–
	Medium	−4.7	6.3	−11.2	12.1	−11.7	1.7	–	–	–	–
	High	−2.2	5.7	−8.7	1.8	−1.9	5.4	–	–	–	–
Cyclosporin A	Low	4.0	6.8	−14.8	5.2	–	–	–	–	–	–
	Medium	−1.2	4.4	−10.5	4.2	–	–	–	–	–	–
	High	−6.3	9.7	−10.2	4.6	–	–	–	–	–	–

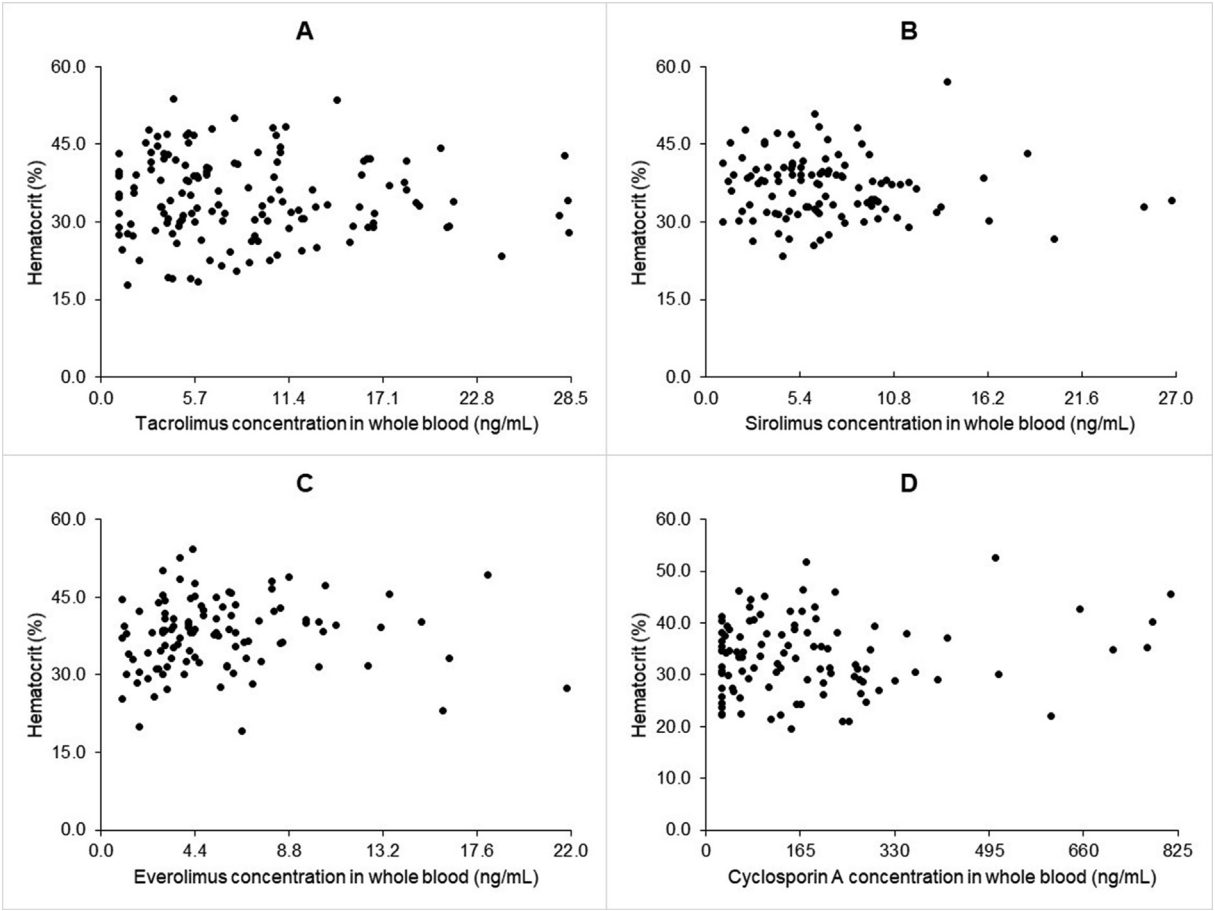

Fig. 2. Scatter plots of hematocrit (Ht) values according to whole blood concentration for tacrolimus (A), sirolimus (B), everolimus (C) and cyclosporin A (D).

clinically significant and methods were accepted as interchangeable.

4. Discussion

In this work, a simple and fast analytical method to simultaneously measure tacrolimus, sirolimus, everolimus and cyclosporin A in whole blood on DMPD by LC-MS/MS was validated. Capillary blood obtained via fingerpick was not used in this study. Although this modality of sample collection appears promising, clinical validation incorporating this collection step would be necessary to assess and implement the procedure into routine clinical practice.

Advantages associated with the validated method include: (a) removing the need for in-house prepared CS and QCs; (b) the use of easy-to-prepare internal standards; (c) a facile sample preparation step; (d) the reduction of CS, QCs, blood samples and reagents volumes required for immunosuppressants analysis (five-times less than the routine method); (e) a high process throughput; and (f) time and expense saving.

Considering the different therapeutic ranges for each immunosuppressive drug (Table 1), the analytical method showed

Table 8
Demographic characteristics for each immunosuppressive drug.

Variable	Immunosuppressant			
	Tacrolimus	Sirolimus	Everolimus	Cyclosporine A
Number of samples	141	109	105	103
Number of patients	105	70	93	69
Sex				
n (%)				
Female	39 (37.1)	29 (41.4)	36 (38.7)	30 (43.5)
Male	66 (32.9)	41 (58.6)	57 (61.3)	39 (56.5)
Age	44 (3–86)	44 (0.5–74)	49 (3–81)	47.5 (2–79)
(years)				
(median; range)				
Type of patient				
n (%)				
Outpatients	59 (56.2)	43 (61.4)	19 (20.4)	37 (53.6)
Inpatients	42 (40.0)	8 (11.4)	3 (3.2)	13 (18.8)
Data not available	4 (3.8)	19 (27.1)	71 (76.3)	19 (27.5)
Hematocrit (%)* (median; range)	33.9 (17.9–53.7)	37.3 (23.4–57.0)	38.2 (19.1–54.3)	33.6 (19.5–52.7)
Whole blood concentration range (ng/mL)	(1.1–28.4)	(1.0–26.8)	(1.0–21.8)	(27.7–813)
Type of transplant				
n (%)				
Heart	9 (8.6)	1 (1.4)	3 (3.2)	2 (2.9)
Heart-kidney	1 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)
Liver	20 (19.0)	5 (7.1)	10 (10.8)	13 (18.8)
Liver-kidney	2 (1.9)	1 (1.4)	1 (1.1)	0 (0.0)
Bowel	1 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)
Bone marrow	8 (7.6)	3 (4.3)	0 (0.0)	4 (5.8)
Lung	6 (5.7)	3 (4.3)	0 (0.0)	0 (0.0)
Kidney	46 (43.8)	34 (48.6)	8 (8.6)	25 (36.2)
Reno-pancreas	8 (7.6)	5 (6.1)	0 (0.0)	3 (4.3)
Data not available	4 (3.8)	18 (25.7)	71 (76.3)	19 (27.5)

* For inpatients, median Ht value was 30.4% (range: 17,9–46.1). For outpatients, median Ht value was 38.1% (range: 18.4–57.0). Statistical differences were found by the Wilcoxon-Mann-Whitney test ($p < 0.001$).

adequate linearities if trough concentrations need to be measured. Furthermore, the assay was accurate and precise enough, and lower limits of quantitation were adequate, for assessment of C_0 levels. Nevertheless, numerous pharmacokinetic studies have demonstrated that cyclosporine A levels at 2 h post-dose (C_2) are the best single time point predictor of AUC for several types of transplant recipients [44]; in this case, the validated linear range for cyclosporine A would not be acceptable. The same criteria must be considered if an abbreviated AUC needs to be measured. In the Hospital Italiano de Buenos Aires, C_2 and abbreviated AUC monitoring are feasible for clinical practice and C_0 levels are routinely requested by physicians.

Due to the use of a commercial kit, recovery and matrix effect assays were challenging. Regarding the first performance parameter, most studies performed on DBS compare the areas of each analyte at different processed QC levels and spiked blank extracts at the same concentrations (normally low and high) [23,45]. According to this approach, all solid drug standards must be available for weighing and preparation of the spiked solutions. Due to their unavailability, recovery was evaluated by comparing the area of each immunosuppressant in processed DMPD and whole blood (reference matrix) at different QC levels. A similar approach has been previously reported [46]. Obtained recovery values were not only close to 100%, but also reproducible enough for all drugs at each QC level (Table 5), and were similar to other similar published DBS studies [21,23].

The analysis of the matrix effect is essential in the practice of LC-MS/MS. Typical approaches to study this parameter in bioanalytical method validation include post-column infusion, standard additions to pre-extractions and matrix factor estimation using post-extraction spiking [47]. Nevertheless, this parameter can also be determined by comparing an evaluated reference method and a comparative test procedure using patient samples [40,47]. In such cases, differences between measurements can be attributed to a matrix effect.

According to the Clinical Laboratory Standards Institute, once the linear relationship between measurements was visually evaluated, the magnitude of the matrix effect was evaluated by Deming regression analysis and the prediction interval for human samples of each analyte were estimated. The acceptance criteria set as the absence of QC results outside the prediction intervals was accomplished for all analytes (Table 6). Predicted values for each processed QC at each concentration level were within the estimated PI. These results showed that the influence of the matrix on the analytical results was not significant and neither ion suppression, nor enhancement, were present.

Regarding the stability studies, results showed that processed samples were stable enough to assure the chemical integrity of analytes if any operative issue (i.e., instrument failure) were to occur. DMPD sample stability at room temperature was adequate for transport and shipment purposes and was similar to other previously published studies using DBS (Table 8) [22,23,46]. Further storage conditions (i.e., −80 °C, 4 °C and higher temperatures that could be encountered during transportation) should be studied in future investigations.

In this work, the Ht effect was largely evaluated by performing a method comparison study to evaluate procedural interchangeability. The difference between methods was quantified in terms of bias. This phase of the study was carefully designed and planned according to the Clinical Laboratory Standards Institute guidelines [42]. In addition, the present work was not limited to a single age group, type of transplant or patient type; and a wide range of Ht values were selected (Table 8 and Fig. 3).

The first step of the data analysis was focused on a search for constant and proportional errors using a Passing-Bablok regression analysis. Results for all drugs revealed that confidence intervals for the intercepts and slopes were 0 and 1, respectively. Therefore, neither showed statistically significant constant or proportional errors. In addition, correlation coefficients (r) showed a strong linear relationship between concentrations obtained by both

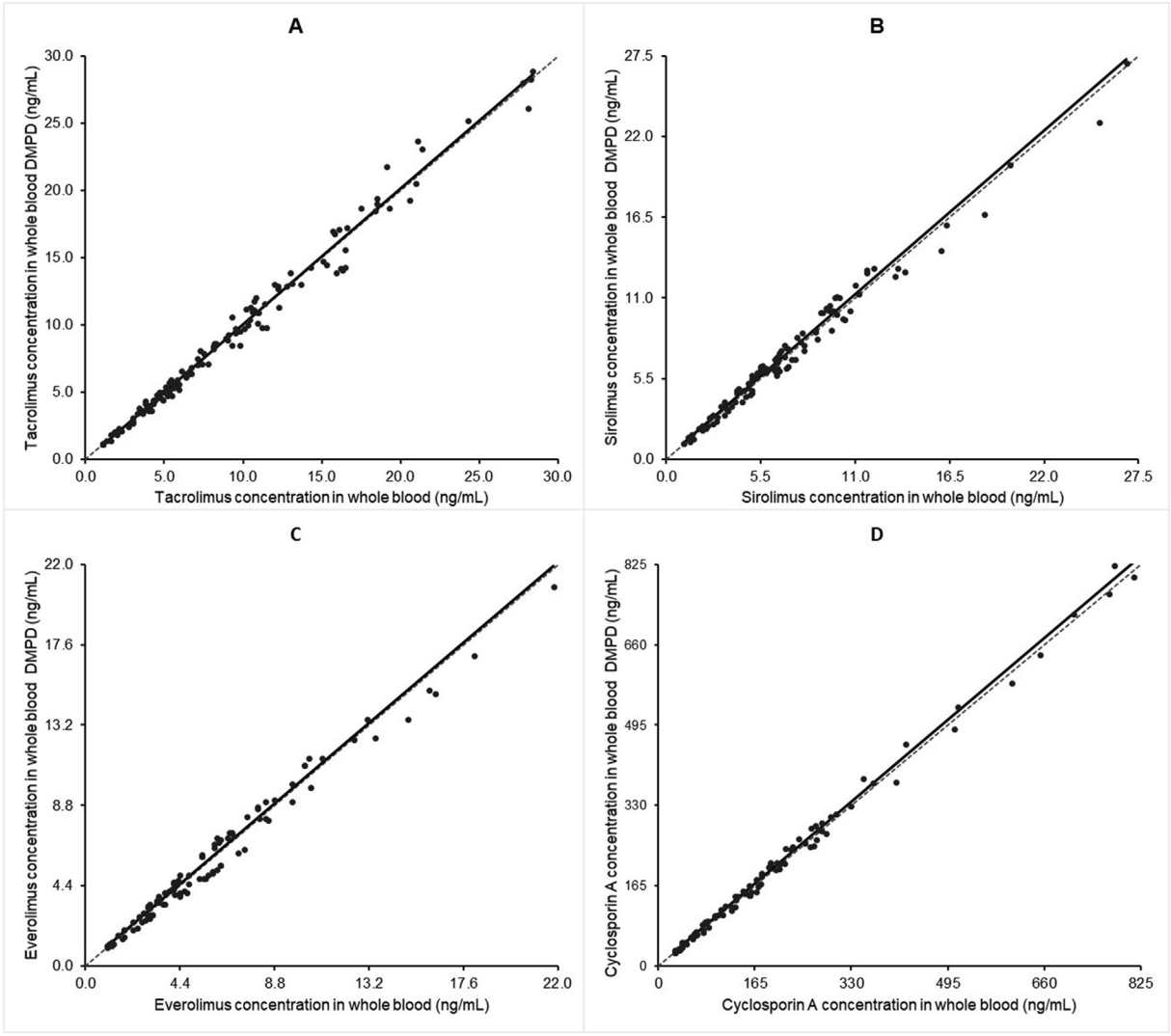

Fig. 3. Passing-Bablok regression lines for tacrolimus (A), sirolimus (B), everolimus (C) and cyclosporin A (D). Dotted lines are the identity lines and continuous lines are the Passing-Bablok regression lines.

Table 9
Results of the Passing-Bablok regression analysis.

Analyte	r^2	Slope	IC_m 95%	Intercept	IC_b 95%	Medical decision level (ng/mL)	Calculated BIAS (ng/mL, (%))	Acceptance criteria
Tacrolimus	0.993	1.011	(1.000–1.039)	−0.053	(−0.206 to 0.000)	Lower: 5.0	0.00 (0.03)	±0.5 ng/mL; <5.0 ng/mL
						Upper: 20.0	0.17 (0.83)	±10%; >5.0 ng/mL
Sirolimus	0.991	1.015	(0.9748–1.071)	0.085	(−0.190 to 0.244)	Lower: 5.0	0.16 (3.21)	±0.5 ng/L; <5.0 ng/mL
						Upper: 15.0	0.31 (2.07)	±10%; >5.0 ng/mL
Everolimus	0.992	1.000	(0.9624–1.057)	0.100	(−0.100 to 0.200)	Lower: 3.0	0.10 (3.33)	±0.5 ng/L; <5.0 ng/mL
						Upper: 15.0	0.10 (0.67)	±10%; >5.0 ng/mL
Cyclosporine A	0.998	1.019	(0.993–1.048)	0.771	(−0.669 to 1.952)	Lower: 100	2.67 (2.67)	±10 ng/mL; <100 ng/mL
						Uppler: 400	8.37 (2.09)	±10%; >100 ng/mL

IC_m 95%: 95% confidence interval for slopes; IC_b 95%: 95% confidence interval for intercepts.

analytical methods. These results were similar to other previously published works on DBS [22,23,46].

The second step of the statistical assessment was to estimate the method's global bias; this was achieved using difference plots and concordance analysis. Acceptance criteria require that the 95% confidence interval of the bias estimator should contain the value 0 (proving statistical concordance), and the limits of agreement (LoA) should be tighter than the acceptable bias width [48]. Currently, tolerable bias values are rarely published in the literature and are not available for all immunosuppressive drugs. The International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) suggests that a 0.28 ng/mL (5.6%) bias value for tacrolimus would not impact clinical decision making, but no values for sirolimus, everolimus or cyclosporin A are reported

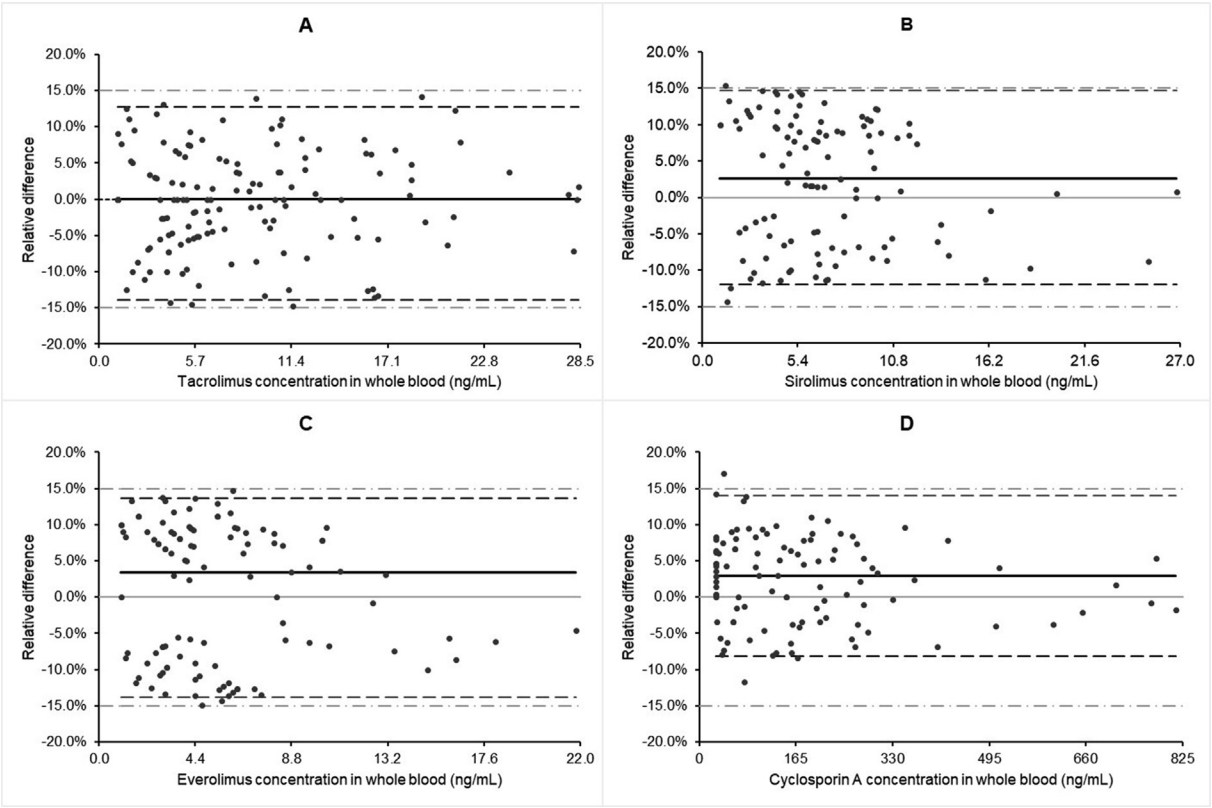

Fig. 4. Difference plots for tacrolimus (A), sirolimus (B), everolimus (C) and cyclosporin A (D). Median bias values for each analyte; relative difference is presented as a continuous black line. Dotted black lines represents lower and upper limits of agreement for each drug. Dotted grey lines are lower and upper limits of clinical relevance, set at ±15.0%. Obtained values were as follow: (A): median bias: 0.0%, 95% CI: (−1.1 to 0.8)%, LoA (−13.9 to 12.8)%; (B): median bias: 2.7%, 95% CI: (0.5–7.7)%, LoA (−12.0–14.7)%; (C): median bias: 3.4%, 95% CI: (−5.6 to 7.1)%, LoA (−13.8 to 13.7)%; (D): median bias: 2.9%, 95% CI: (0.4–4.7)%, LoA (−8.2 to 14.1)%.

[18]. On the other hand, the Royal College of Pathologists of Australasia (RCPA) recommends analytical quality requirements for calcineurin inhibitors and sirolimus [43].

Multiple studies have been published regarding the therapeutic monitoring of immunosuppressive agents in DBS. However, only three of them used the application of a drop of venous blood on paper cards as a paired sample [22,23,46], and just one estimated the method's global bias for tacrolimus and cyclosporin A via difference plot analysis [46]. Regardless of the selection of IATDMCT or RCPA quality requirements, neither this work, nor any other, completely fulfills the acceptance criteria (Fig. 4). Although statistical concordance was proven for some analytes (the 95% confidence interval of the bias estimator included the value 0), in all cases the limits of agreement were wider than the acceptable bias width (in both concentration and percentage units). Consequently, results must be carefully interpreted. Though the absence of statistical concordance denotes non-interchangeability between analytical methods, poor or non-agreement should not lead to premature rejection of a new measurement technique [49]. Quite often a candidate procedure may not fulfill the acceptability criteria for bias against the existing method, but it is still implemented in the laboratory [42]. Therefore, evaluation of clinical utility is needed.

To achieve this, two approaches can be considered. The first is the use of clinical relevance limits established by multidisciplinary teams in each health center. This strategy has been previously applied, considering maximum bias levels that could alter the defined dosing management. In this work, these values were set at ±15.0% for all immunosuppressive drugs and were similar to other published works [27,50].

The second strategy is the estimation of bias at medical decision points (lower and upper limits of the therapeutic range), using the regression line equation in both concentration and percentage units [48]. In this case, results were compared against the quality requirements of the IATDMCT and the RCPA. In the present study, bias values for all the immunosuppressants proved to be acceptable, demonstrating that the Ht has no clinical influence on the results. This result is especially interesting regarding the statistical significative differences between the Ht values of in- and outpatients (Table 8). This strategy was a distinctive feature of this work. None of the previously published studies using DBS have reported on this approach, although, the calculation of bias values at medical decision points using their reported regression lines showed that many were suitable for patient monitoring purposes [22,23,46].

Finally, it should be noted that, in this work, the influence of the sample volume on the analytical results could be minimized as the laboratory staff were previously trained, and blood spots were directly collected from anticoagulated whole blood tubes. Previous studies using a similar methodological approach suggest that results should not be extrapolated to capillary collected blood specimens, since the matrices are different [22,23,46]. Although results may not differ significantly from blood spots made by patients and trained personnel, this needs to be validated [12,23]. For that reason, in a future study, this procedure will be applied to fingerprick DMPD samples in order to assess its use for routine analysis.

5. Conclusion

A simple and fast analytical method for calcineurin and mTOR inhibitors was fully validated. The adaption of a commercial kit sig-

nificantly improved sample throughput. Method validation showed that the procedure is suitable for the C_0 measurement of immunosuppressive drugs. In addition, the method comparison study showed that the Ht had no clinical influence on the analytical results and that the method could potentially be used for therapeutic monitoring of immunosuppressive agents.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] A.M. Krensky, W.M. Bennett, F. Vicenti, Immunosuppressants, tolerogens, and immunostimulants, in: L. Brunton, B. Chabner, B. Knollman (Eds.), Goodman & Gilman's The Pharmacological Basis of THERAPEUTICS, 12th ed., McGraw-Hill, New York, 2011, pp. 1005–1031.
[2] M. Oellerich, V.W. Armstrong, The role of therapeutic drug monitoring in individualizing immunosuppressive drug therapy: recent developments, Ther. Drug Monit. 28 (6) (2006) 720–725.
[3] L.S.L. Ting, E. Villeneuve, M.H.H. Ensom, Beyond cyclosporine: a systematic review of limited sampling strategies for other immunosuppressants, Ther. Drug Monit. 28 (3) (2006) 419–430.
[4] I. Robertsen, J. Debord, A. Åsberg, P. Marquet, J.B. Woillard, A limited sampling strategy to estimate exposure of everolimus in whole blood and peripheral blood mononuclear cells in renal transplant recipients using population pharmacokinetic modeling and bayesian estimators, Clin. Pharmacokinet. 57 (11) (2018) 1459–1469.
[5] G.A.J. Van Boekel, A.R.T. Donders, K.E.J. Hoogtanders, T.R.A. Havenith, L.B. Hilbrands, R.E. Aarnoutse, Limited sampling strategy for prolonged-release tacrolimus in renal transplant patients by use of the dried blood spot technique, Eur. J. Clin. Pharmacol. 71 (7) (2015) 811–816.
[6] N. Mohammadpour, S. Elyasi, N. Vahdati, A.H. Mohammadpour, J. Shamsara, A review on therapeutic drug monitoring of immunosuppressant drugs, Iran J. Basic Med. Sci. 14 (6) (2011) 485–498.
[7] B. Sádaba, Monitorización y efectos secundarios de los inmunosupresores en el trasplante Monitoring and secondary effects of immunosuppressants in the transplant. An Sist Navar. 2006 29(2):207–18.
[8] Y. Zhang, R. Zhang, Recent advances in analytical methods for the therapeutic drug monitoring of immunosuppressive drugs, Drug Test. Anal. 10 (1) (2018) 81–94.
[9] P.J. Jannetto, R.L. Fitzgerald, Effective use of mass spectrometry in the clinical laboratory, Clin. Chem. 62 (1) (2016) 92–98.
[10] A.J. McShane, D.R. Bunch, S. Wang, Therapeutic drug monitoring of immunosuppressants by liquid chromatography-mass spectrometry, Clin. Chim. Acta 454 (2016) 1–5.
[11] B.U.W. Lei, T.W. Prow, A review of microsampling techniques and their social impact, Biomed. Microdevices 21 (4) (2019) 21–81.
[12] A. Klak, S. Pauwels, P. Vermeersch, Preanalytical considerations in therapeutic drug monitoring of immunosuppressants with dried blood spots, Diagnosis (Berlin, Ger. 6 (1) (2019) 57–68.
[13] W. Li, F.L.S. Tse, Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules, Biomed. Chromatogr. 24 (1) (2010) 49–65.
[14] K. Kulmatycki, W. Li, X. Xu, V. Jarugula, Application of Dried Blood Spot Sampling in Clinical Pharmacology Trials and Therapeutic Drug Monitoring, in: W. Li, M.S. Lee, editors. DRIED BLOOD SPOTS Applications and Techniques. 1st ed. New Jersey: Wiley; 2014. p. 216–228.
[15] A.J. Wilhelm, J.C.G. den Burger, E.L. Swart, Therapeutic drug monitoring by dried blood spot: progress to date and future directions, Clin. Pharmacokinet. 53 (11) (2014) 961–973.
[16] K.L. Johnson-Davis, G.A. McMillin, Application of Liquid Chromatography Combined with Mass Spectrometry or Tandem Mass Spectrometry for Therapeutic Drug Monitoring of Immunosuppressants. In: Dasgupta A, Oellerich Mi, editors. Personalized Immunosuppression in Transplantation: Role of Biomarker Monitoring and Therapeutic Drug Monitoring. 1st ed. San Diego: Elsevier Inc.; 20p. 57–81. Available from: http://dx.doi.org/10.1016/B978-0-12-800885-0.00003-5.
[17] P.M.M. De Kesel, N. Sadones, S. Capiau, W.E. Lambert, C.P. Stove, Hematocritical issues in quantitative analysis of dried blood spots: challenges and solutions, Bioanalysis. 5 (16) (2013) 2023–2041.
[18] S. Capiau, H. Veenhof, R.A. Koster, Y. Bergqvist, M. Boettcher, O. Halmingh, et al., Official international association for therapeutic drug monitoring and clinical toxicology guideline: development and validation of dried blood spot-based methods for therapeutic drug monitoring, Ther. Drug Monit. 41 (4) (2019) 409–430.
[19] W. Li, Considerations in development and validation of LC-MS/MS method for quantitative analysis of small molecules in dried blood spot samples, in: W. Li, editor. DRIED BLOOD SPOTS Applications and Techniques. 1st ed. New Jersey; 2014. p. 168–178.
[20] P. Denniff, N. Spooner, The effect of hematocrit on assay bias when using DBS samples for the quantitative bioanalysis of drugs, Bioanalysis 2 (8) (2010) 1385–1395.
[21] J.C.G. Den Burger, A.J. Wilhelm, A. Chahbouni, R.M. Vos, A. Sinjewel, E.L. Swart, Analysis of cyclosporin A, tacrolimus, sirolimus, and everolimus in dried blood spot samples using liquid chromatography tandem mass spectrometry, Anal. Bioanal. Chem. 404 (6–7) (2012) 1803–1811.
[22] K. Sadilkova, B. Busby, J.A. Dickerson, J.C. Rutledge, R.M. Jack, Clinical validation and implementation of a multiplexed immunosuppressant assay in dried blood spots by LC-MS/MS, Clin. Chim. Acta. 421 (2013) 152–156.
[23] R.A. Koster, J.W.C. Alffenaar, B. Greijdanus, D.R.A. Uges, Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery, Talanta 115 (2013) 47–54.
[24] L. Fan, J.A. Lee, Managing the effect of hematocrit on DBS analysis in a regulated environment, Bioanalysis 4 (4) (2012) 345–347.
[25] R.A. Koster, B. Greijdanus, J.W.C. Alffenaar, D.J. Touw, Dried blood spot analysis of creatinine with LC-MS/MS in addition to immunosuppressants analysis, Anal. Bioanal. Chem. 407 (6) (2014) 1585–1594.
[26] R.A. Koster, H. Veenhof, R. Botma, A.T. Hoekstra, S.P. Berger, S.J. Bakker, et al., Dried blood spot validation of five immunosuppressants, without hematocrit correction, on two LC-MS/MS systems, Bioanalysis. 9 (7) (2017) 553–563.
[27] H. Veenhof, R.A. Koster, J.W.C. Alffenaar, S.P. Berger, S.J.L. Bakker, D.J. Touw, Clinical validation of simultaneous analysis of tacrolimus, cyclosporine A, and creatinine in dried blood spots in kidney transplant patients, Transplantation 101 (7) (2017) 1727–1733.
[28] S. Capiau, V.V. Stove, W.E. Lambert, C.P. Stove, Prediction of the hematocrit of dried blood spots via potassium measurement on a routine clinical chemistry analyzer, Anal. Chem. 85 (1) (2013) 404–410.
[29] S. Capiau, L.S. Wilk, M.C.G. Aalders, C.P. Stove, A novel, nondestructive, dried blood spot-based hematocrit prediction method using noncontact diffuse reflectance spectroscopy, Anal. Chem. 88 (12) (2016) 6538–6546.
[30] P. Abu-Rabie, P. Denniff, N. Spooner, J. Brynjolffssen, P. Galluzzo, G. Sanders, Method of applying internal standard to dried matrix spot samples for use in quantitative bioanalysis, Anal. Chem. 83 (22) (2011) 8779–8786.
[31] R. Garcia Boy, J. Henseler, R. Mattern, G. Skopp, Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots, Ther. Drug Monit. 30 (6) (2008) 733–739.
[32] P. Lc, D.B.S. Lc, Perforated dried blood spots: a novel format for accurate microsampling, Bioanalyisis. 3 (20) (2011) 2321–2333.
[33] F. Li, S. Ploch, D. Fast, S. Michael, Perforated dried blood spot accurate microsampling: the concept and its applications in toxicokinetic sample collection, J. Mass Spectrom. 47 (5) (2012) 655–667.
[34] N. Youhnovski, A. Bergeron, M. Furtado, F. Garofolo, Pre-cut Dried Blood Spot (PCDBS): An alternative to Dried Blood Spot (DBS) technique to overcome hematocrit impact, Rapid Commun. Mass Spectrom. 25 (19) (2011) 2951–2958.
[35] R.J. Meesters, J. Zhang, N.A. Van Huizen, G.P. Hooff, R.A. Gruters, T.M. Luider, Dried matrix on paper disks: The next generation DBS microsampling technique for managing the hematocrit effect in DBS analysis, Bioanalysis. 4 (16) (2012) 2027–2035.
[36] European Medicines Agency (EMA), Guideline on Bioanalytical Method Validation. London, U.K., 2011.
[37] Food and Drug Administration (FDA), Guidance for Industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services. Washington, DC, U.S.A., 2018.
[38] Eurachem, Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, 2014.
[39] Clinical and Laboratory Standards Institute (CLSI) document, Liquid Chromatography-Mass Spectrometry Methods; approved guidline, C62-A. 2014.
[40] Clinical and Laboratory Standards Institute (CLSI) document, Evaluation of Commutability of Processed Samples; approved guidline, EP14-A3, 2014.
[41] R.A. Koster, R. Botma, B. Greijdanus, D.R.A. Uges, J.G.W. Kosterink, J.-W.-C. Alffenaar, et al., The influence of the dried blood spot drying time on the recoveries of six immunosuppressants, J. Appl. Bioanal. 1 (4) (2015) 116–122.
[42] Clinical and Laboratory Standards Institute (CLSI) document, Measurement Procedure Comparison and Bias Estimation Using Patient Samples; approved guidline, EP09c, 2018.
[43] Royal College of Pathologists of Australasia (RCPA) [Internet]. 2014. Available from: http://www.rcpaqap.com.au/docs/2014/chempath/ALP.pdf.
[44] S.R. Knight, P.J. Morris, The clinical benefits of cyclosporine C2-level monitoring: a systematic review, Transplantation 83 (12) (2007) 1525–1535.
[45] K. Hoogtanders, J. van der Heijden, M. Christiaans, P. Edelbroek, J.P. van Hooff, L.M.L. Stolk, Therapeutic drug monitoring of tacrolimus with the dried blood spot method, J. Pharm. Biomed. Anal. 44 (3 SPEC. ISS.) (2007) 658–664.
[46] E. Hinchliffe, J.E. Adaway, B.G. Keevil, Simultaneous measurement of cyclosporin A and tacrolimus from dried blood spots by ultra high

performance liquid chromatography tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 883–884 (2012) 102–107.
[47] Y. Huang, R. Shi, W. Gee, R. Bonderud, Matrix effect and recovery terminology issues in regulated drug bioanalysis, Bioanalysis. 4 (3) (2012) 271–279.
[48] J. Pum, A practical guide to validation and verification of analytical methods in the clinical laboratory. In: Advances in Clinical Chemistry. 1st ed. Elsevier Inc.; 2019. p. 215–81. Available from: http://dx.doi.org/10.1016/bs.acc.2019.01.006.
[49] R. Kwiecien, A. Kopp-Schneider, M. Blettner, Concordance analysis—part 16 of a series on evaluation of scientific publications, Dtsch Arztebl. 108 (30) (2011) 515–521.
[50] A.E.C.A.B. Willemsen, L.M. Knapen, Y.M. de Beer, R.J.M. Brüggemann, S. Croes, C.M.L. van Herpen, et al., Clinical validation study of dried blood spot for determining everolimus concentration in patients with cancer, Eur. J. Clin. Pharmacol. 74 (4) (2018) 465–471.