Journal Pre-proof

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PII: S0003-2670(20)31186-7

DOI: https://doi.org/10.1016/j.aca.2020.11.056

Reference: ACA 238124

To appear in: *Analytica Chimica Acta*

Received Date: 31 May 2020

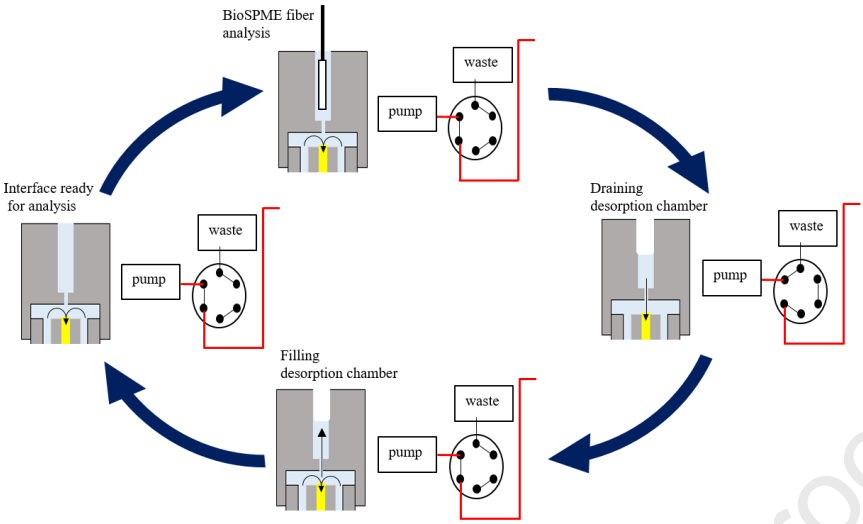
Revised Date: 23 November 2020

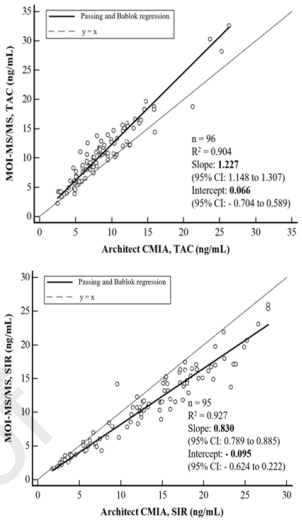
Accepted Date: 30 November 2020

Please cite this article as: E. Nazdrajić, M. Tascon, D.A. Rickert, G.A. Gómez-Ríos, V. Kulasingam, J.B. Pawliszyn, Rapid Determination of Tacrolimus and Sirolimus in Whole Human Blood by Direct Coupling of Solid-phase Microextraction to Mass Spectrometry via Microfluidic Open Interface, *Analytica Chimica Acta*, https://doi.org/10.1016/j.aca.2020.11.056.

AUTHOR CONTRIBUTION

E.N., M.T., D.R., G.A.G.R. V.K. and J.P. had contributed to design of the project. V.K. provided the residual whole blood samples of patients undergoing immunosuppression therapy. Experiments were performed by E.N., M.T., D.R. and G.A.G.R. under supervision of J.P. The manuscript and figures were prepared by E.N. and M.T. All authors reviewed the manuscript and supplementary information.





Rapid Determination of Tacrolimus and Sirolimus in Whole Human Blood by Direct Coupling of Solid-phase Microextraction to Mass Spectrometry via Microfluidic Open Interface

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List of abbreviations:

Tacrolimus (TAC), sirolimus (SIR), everolimus (EVE), cyclosporine A (CYC), matrix compatible solid-phase microextraction fibers (Bio-SPME), microfluidic open interface (MOI), chemical microparticle immunoassay (CMIA)

Abstract

Immunosuppressive drugs are administered to decrease immune system activity (e.g. of patients undergoing solid organ transplant). Concentrations of ISDs immunosuppressive drugs in circulating blood must be closely monitored during the period of immunosuppression therapy due to adverse effects that take place when concentration levels fall outside of the very narrow therapeutic concentration range of these drugs. This study presents the rapid determination of four relevant immunosuppressive drugs (tacrolimus, sirolimus, everolimus, and cyclosporine A) in whole human blood by directly coupling solid-phase microextraction to mass spectrometry via the microfluidic open interface (Bio-SPME-MOI-MS/MS). The BioSPME-MOI-MS/MS method offers ≤ 10 % imprecision of in-house prepared quality controls over a 10-day period, ≤ 10 % imprecision of ClinCal® Recipe calibrators over a three-day period, and single total turnaround time of ~60 min (4.5 min for high throughput). The limits of quantification were determined to be 0.8 ng mL^{-1} for tacrolimus, 0.7 ng mL^{-1} sirolimus, 1.0 ng mL^{-1} for everolimus, and 0.8 ng mL^{-1} for cyclosporine. The limits of detection were determined to be 0.3 ng mL^{-1} for tacrolimus, 0.2 ng mL^{-1} for sirolimus, 0.3 ng mL^{-1} for everolimus, and 0.3 ng mL^{-1} for cyclosporine A. The R^2 values for all analytes were above 0.9992 with linear dynamic range from 1.0 mL^{-1} to 50.0 ng mL^{-1} for tacrolimus, sirolimus, and everolimus while from 2.5 ng mL^{-1} to 500.0 ng mL^{-1} for cyclosporine A. To further evaluate the performance of the present method, 95 residual whole blood samples of tacrolimus and sirolimus from patients undergoing immunosuppression therapy were used to compare the Bio-SPME-MOI-MS/MS method against a clinically validated reference method based on chemiluminescent microparticle immunoassay, showing acceptable results. Our results demonstrated that Bio-SPME-MOI-MS/MS can be considered as a suitable alternative to existing methods for the determination of immunosuppressive drugs in whole

blood providing faster analysis, better selectivity and sensitivity, and a wider dynamic range than current existing approaches.

Keywords

Solid-Phase Microextraction; Microfluidic Open Interface; Tacrolimus; Sirolimus; Everolimus; Cyclosporine; Mass Spectrometry

1. Introduction

Patients that require suppression of their immune system's activity are usually prescribed immunosuppressive drugs (ISD), especially in cases involving solid organ transplantation, where it is critical to reduce the possibility of organ rejection. Due to the toxicity of ISDs, and to decrease the risk of organ rejection, the concentration of the prescribed ISDs in the patients' blood must be closely monitored in order to maintain concentrations within their narrow therapeutic range (5 – 20 ng mL^{-1} for tacrolimus, 5 – 10 ng mL^{-1} for sirolimus, 3 – 8 ng mL^{-1} for everolimus, and 150 – 350 ng mL^{-1} for cyclosporine A) [1]. Over or under administration of ISDs can lead to severe adverse effects. For instance, over administration can cause infection, malignancy, cardiovascular diseases, and diabetes, whereas under administration can result in graft loss resulting from acute and/or chronic organ rejection [2]. Besides the narrow therapeutic range and the likelihood of ISD misadministration, some additional challenges regarding ISD analysis include highly variable dose/exposure relationships, as well as toxicodynamic effects that cannot be easily differentiated from clinical diseases' symptoms [3].

In this context, several methods have been developed to monitor the concentration of ISDs in whole blood. Among them, the most widespread and commonly used methods are immunoassay-based [1–3]. Some popular immunoassay methods are enzyme-linked

immunosorbent assay [4], quantitative microsphere system [5], electrochemiluminescence immunoassay [6,7], microparticle enzyme immunoassay [8], affinity column-mediated immunoassay [8], and chemiluminescent microparticle immunoassay [9]. These methods have some important advantages such as fast turnaround time (due to total automation of their work flow) and commercial availability [5,6,9,10]. However, immunoassay methods have majors limitations: the possibility of interference and lack of clinical accuracy stemming from cross-reactivity with other drugs or metabolites [1,3]. Liquid chromatography-mass spectrometry (LC-MS) has become the gold standard for analysis of ISDs since it addresses most of the limitations of immunoassay-based methods without compromising the time of analysis and workflow simplicity [11–14]. In general, MS-based methods are capable of monitoring multiple analytes simultaneously in a single instrument, a feat otherwise impossible in the case of immunoassays. This feature is especially advantageous when some immunosuppression therapies include coadministration of ISDs with different modes of action, which is generally carried out with the aim to reduce adverse effects of individual ISDs [1,15–19]. From an economical point of view, even though initial investment costs for an LC-MS system are higher; the lower cost of reagents and consumables offsets the initial investment over the time [14,20]. This fact is clearly reflected in the increased utilization of LC-MS assays in the market in recent years (~ 50 % for CYC and TAC and ~ 70 % for SIR and EVE) [3,11]. As a consequence of growing MS market, various direct-to-MS approaches targeting rapid determination have been developed, such as the Phytronix® Technologies laser diode thermal desorption module [21] and paper spray [22,23], among others. However, despite their practicality, these approaches have several drawbacks, the most detrimental being the higher susceptibility to matrix effects, and instrumental contamination due to the lack of separation and sample preparation, respectively [24].

It is crucial to emphasize that sample preparation is a critical step in any analytical workflow given that proper sample preparation can provide sufficient sample cleanup as well as suitable isolation and enrichment of target analyte(s) [25]. In this context, several sample preparation strategies have been employed to determine ISDs in whole blood, generally involving protein precipitation, solid-phase extraction, or liquid-liquid extraction [1,18]. Specifically for direct-to-MS methodologies with online sample preparation, Turboflow [26] and Rapidfire^{TM} [27] have been developed. A methodology worth highlighting for direct-to-MS analysis is the coupling of solid-phase microextraction (SPME), an open-bed extraction methodology that has been shown to be an efficient way of introducing a rapid sample preparation step into the analytical workflow [28,29]. SPME-MS provides rapid analyte enrichment onto the matrix-compatible extraction phase with minimal co-extraction of matrix components that cause matrix effects, thus enabling the introduction of clean extracts for quantitative and reproducible results [30–35]. Furthermore, it addresses the drawbacks of the aforementioned direct-to-MS approaches by offering a more streamlined process since it consolidates a lot of manual steps into a single step, while simultaneously eliminating the risk of cartridge/column clogging. For determination of ISDs in whole blood by SPME-MS, coated blade spray [25,36] and fibers [37] using biocompatible hydrophilic-lipophilic balanced coatings have been employed to date.

This work describes the development of a Bio-SPME-MS/MS method for simultaneous determination of four immunosuppressive drugs (tacrolimus, sirolimus, everolimus, and cyclosporine A) from whole human blood via the microfluidic open interface (MOI). The present work serves as an evaluation and further development of the proof-of-concept method that was reported by Tascon et al. in 2018 [37]. Briefly, the MOI system allows analytes on Bio-SPME

devices to be rapidly desorbed in the flow-isolated chamber, while at the same time, the desorption solution is continuously supplied to the electrospray source of the instrument. This setup offers great sensitivity due to the small volume (< 4 µL) of the flow-isolated region (desorption chamber) and rapid transfer of the concentrated analyte plug towards the electrospray source (**Supplementary material, Figure S1**) [30,37,38]. In addition, general method development criteria were followed to evaluate the inter-day stability, carryover, precision, accuracy, and sensitivity of the method [3,39]. Finally, cross-validation against the routinely used method for ISDs analysis, chemiluminescent microparticle immunoassay (CMIA), was performed, obtaining acceptable values [9].

2. Materials and Methods

2.1 Materials and Supplies

LC-MS grade methanol, acetonitrile, isopropanol, and dimethylformamide were purchased from Fisher Scientific (Bartlesville, OK, USA). LC-MS formic acid, polyacrylonitrile, and ammonium acetate were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Tacrolimus (TAC), Sirolimus (SIR), Everolimus (EVE), Cyclosporine A (CYC), TAC ^{13}C d_2, EVE d_4, and CYC $^{15}N_{11}$ were purchased from Cerilliant Corporation (Round Rock, TX, USA). CYC d_4 was purchased from Toronto Research Chemicals (Toronto, ON, CA). All the physicochemical parameters can be found in **Supplementary Material Table S1.** Analyte stock solutions were prepared in acetonitrile at 100 µg mL^{-1} and stored at -80 °C. Recipe ClinCal® whole blood immunosuppressant quality control (QC) levels (Blank, Level 1- 6; LOT#: 1366) were purchased from Recipe (Munich, Germany). Pooled whole human blood containing K_2-EDTA was purchased from BioIVT (Westbury, NY, USA). Whole blood samples were collected

from patients receiving immunosuppressant therapy (TAC and SIR) at the University Health Network (UHN; Toronto, ON; Canada). Informed consent was obtained prior to sample collection, and ethics approval was waived by the Research Ethics Board at UHN for use of collected samples for evaluation of method performance. Five micrometer Oasis® hydrophilic-lipophilic balance (HLB) particles used to coat the BioSPME fibers were graciously provided by Waters Corporation (Wilmslow, United Kingdom). Bio-SPME fibers were prepared by dipping ~~coating~~ nitinol wire (200 μm diameter) in HLB particles suspended in a polyacrylonitrile-dimethylformamide mixture where procedure is described in the **Supplementary material**. All Bio-SPME fibers used in this study had a coating length of 10 mm and 25 μm thickness. All fibers were made for single use, and as such, were discarded in a biohazard bin following analysis.

2.2 Sample preparation

In-house QCs and calibration levels were prepared by spiking pooled whole blood with the ISDs mixture, keeping the organic solvent content in the sample below 1 %. Spiked whole blood samples were incubated overnight at 4°C so as to allow analytes to bind with the whole blood matrix. Following overnight incubation, spiked samples were pipetted into 200 μL aliquots, and subjected to the following mechanical lysis process: three freeze-thaw cycles (a cycle consisted out of placing the sample for 1 min in liquid nitrogen and then for 1 min in an ice bath). Finally, samples were stored at -80 °C until the moment of the experiment.

Prior to analysis, the samples were subjected to an additional chemical lysis process where 1.3 mL of a lysis solution containing the internal standards 0.1M zinc sulfate:acetonitrile:water (6:3:1, v/v) with 1.1 ng mL^{-1} of TAC ^{13}C d_2, EVE d_4, and 11.2 ng mL^{-1} of CYC d_4; was added to thawed 200 μL aliquot of whole blood. Immediately after the Bio-

SPME fiber was directly immersed in the solution to initiate the extraction. Extractions were performed using a VWR® Thermal Shake Touch set at 2200 rpm and at 55 °C for 60 min.

2.3 Analysis via MOI-MS/MS

The analytical workflow consisted of four steps: 1) Extraction of analytes with a Bio-SPME fiber (60 min); 2) Rinsing of fiber in water for 5 seconds so as to remove salt residuals and non-specific matrix components from blood that could be loosely attached to the coating surface; 3) Placement of the Bio-SPME fiber into the pre-filled MOI chamber; and 4) Introduction of the desorbed analytes into the API 4000 (SCIEX, Concord, ON, Canada) by the self-aspiration of the TurboIon™ electrospray source [37]. More information regarding instrumental parameters for analysis of ISD can be found in the **Supplementary Material Table S2**. Furthermore, detailed information on the analytical workflow is shown in **Figure 1**.

A Shimadzu 10AD LC pump was used to supply the desorption solution, which was comprised of 12 mM ammonium acetate and 0.1 % of formic acid (v/v) in methanol, to the MOI setup. The MOI set-up consisted of a Shimadzu LC pump providing the desorption solution through a switch valve to the ESI source and the desorption chamber. Once the desorption chamber was filled and equilibrium was reached between the LC pump supply flow rate and aspiration flow rate (generated by the ESI source), the Bio-SPME fiber was introduced into the desorption chamber. After 5 seconds of fiber immersion for desorption, the Bio-SPME fiber was withdrawn, and the valve was switched in order to rapidly move the desorbed plug of analytes towards the ESI source [30,37,38]. Once the desorption chamber was emptied, the switch valve was returned to the initial position. At this stage, a higher desorption flow rate was used to fill the desorption chamber. Finally, the chamber was refilled and drained three consecutive times to prevent any carryover from previous desorption stages. Electrospray ionization parameters for

optimum performance of the MOI and for ionization in positive mode were auxiliary gases GS1 = 90 psi, GS2 = 70 psi and, curtain gas = 25 psi, voltage = 5000 V, and temperature = 300 °C. A schematic of the MOI setup and operational features are shown in **Figure S3** of the **Supplementary Material**.

2.4 Inter-day stability and imprecision evaluation of in-house prepared whole blood QC samples

The inter-day stability was evaluated by performing measurements for ten consecutive days, using four independent replicates per day for three in-house prepared QC levels (i.e. 2.5, 7.5, and 15 ng mL^{-1} for TAC, SIR, and EVE; and 50, 150, and 300 ng mL^{-1} for CYC).

2.5 Calibration plots

To evaluate the ability of internal standards to correct for experimental and instrumental variances following their addition to lysis solutions, three calibration plots were prepared with three different hematocrit levels in whole blood (20%, 40% and, 70% hematocrits) and analyzed in triplicate, covering the following concentration range: 1.0 – 50.0 ng mL^{-1} for TAC, SIR, and EVE and 10 – 500 ng mL^{-1} for CYC.

The sensitivity of the MOI-MS/MS method was assessed by analyzing seven calibration levels, one blank level, and three in-house prepared QC levels, all in quadruplicate. The calibration solutions were spiked between 1.0 – 50 ng mL^{-1} for TAC, SIR, EVE and 10 – 500 ng mL^{-1} for CYC. In-house QC levels were prepared with different lots of whole blood to meet general method development criteria [3,39]. The limit of quantitation (LOQ) was estimated using the lowest calibration point that met the following requirements: 1) lowest calibration point with

the signal to noise ratio ≥ 10, 2) 80 – 120 % back-calculated accuracy using linear regression line, and 3) imprecision of < 20 %.

2.6 Bio-SPME-MOI-MS/MS cross-validation against CMIA

Experimental information regarding CMIA method is described elsewhere [9]. Concentrations calculated using the Bio-SPME-MOI-MS/MS assay were compared to those obtained by the Abbot ARCHITECT i2000 CMIA. Two sets of patient samples (n = 95) containing TAC and SIR were analyzed separately for this purpose, using respective immunoassay kits. Each set was analyzed in a single replicate together with a single replicate of calibration levels, in-house QC, and Recipe ClinCal®. Additionally, inter-day imprecision analysis (n = 3) was performed using Recipe ClinCal® QC levels. Previously determined concentrations with the CMIA assay ranged between 2.5 – 26.3 ng mL^{-1} for TAC and 1.8 – 27.8 ng mL^{-1} for SIR. Statistical analysis comprised slope, intercept, and correlation coefficient determination using Passing-Bablok regression with a 95 % confidence interval. Relative and absolute method bias was determined and visualized using a Bland-Altman plot. The acceptance criteria for method comparison were defined as the slope of 1.00 ± 0.25 and $R^2 \geq 0.9$.

All statistical analysis was performed on MedCalc® (v. 14.8.1) and Microsoft® Excel® 2013 (v. 15.0.5059.1000).

3. Results and Discussions

3.1 Bio-SPME-MOI-MS/MS and general method development

The imprecision of the calibration plot slopes prepared with three different lots of blood containing different hematocrit levels was evaluated. Total imprecision was < 6 % for TAC, < 4 % for SIR, < 6 % for EVE, and < 2 % for CYC **(Supplementary Material, Figures S4-S7)**. These results succinctly proved that the spiking of internal standards in the lysis solution was able to accurately correct for hematocrit level variability in whole blood [40].

The inter-day imprecision of the proposed methodology was evaluated by analyzing the in-house prepared QC levels in quadruplicate. The effect of the mechanical lysing step has been studied and shown in **Supplementary Material Figures, S1-S2**. It can be seen from **Supplementary Material Figure S1** that for first several time points across all in-house QC Levels for TAC there is an increase in the analyte/internal standard ratio. This suggests that chemical lysing step alone is not capable of fully lysing the red blood cells. For SIR, EVE, and CYC, this effect is not observed. Obtained imprecision over the interday period is shown in **Supplementary Material Table S3,** and is between 6 – 20 % for all analytes across all in-house QC levels. After introducing a mechanical lysing step as an aid to the aforementioned chemical lysing, the results have improved. For TAC, the increasing trend over the first several time points is no longer observed, and across all in-house QC levels for all analytes, the deviation of the analyte/internal standard ratios has been decreased in comparison chemical lysing step alone. This is evident from imprecision values in Table 2, which ranged from 4 – 10 % for all analytes across all in-house QC levels. Additionally, an inter-day evaluation of Recipe® ClinCal QCs in a single replicate was carried out in three days as an alternative imprecision evaluation of the MOI-MS/MS method. Imprecision values are summarized in **Table 2**. Recipe® ClinCal QC imprecision values were ≤ 9 % for TAC, ≤ 10 % for SIR, ≤ 7 % for EVE, and ≤ 9 % for CYC.

Since the desorption solution does not continuously flow through the desorption chamber during analysis, there is a chance that carryover may occur between introduction of the isolated volume for the analysis. The desorbed plug of analytes is carried to the detector surrounded by a pure desorption solution [41]. To further prevent carryover from affecting results, three additional 'dummy' desorption steps (fill the desorption chamber, hold for 5 seconds, drain the desorption chamber to the MS) were carried out prior to the introduction of each Bio-SPME fiber. This set of measures was able to clean the desorption chamber prior to subsequent analysis and prevent carryover (**Supplementary Material Figures S8-S11**).

3.2 Limit of quantitation, limit of detection, and linearity evaluation

The method´s LOQ achieved for a given ISD should be at least one-third to one-half of the lower limit of its target concentration window [3]. LOQs and linearity of the MOI-MS/MS method were determined for each ISD analyte using matrix-matched calibration plots (**Table 1** and **Figure 2**). The LOQs were determined to be 0.8 ng mL^{-1} for TAC, 0.7 ng mL^{-1} for SIR, 1.0 ng mL^{-1} for EVE, and 0.8 ng mL^{-1} for CYC. These were determined using lowest calibration point, and limits of detection we taken as one-third of limit of quantitation. In this manner, the therapeutic range of all ISD is completely covered with a determined linear dynamic range [3,25].

3.3 Bio-SPME-MOI-MS/MS vs CMIA for TAC and SIR

Anonymized residual whole blood samples from patients undergoing immunosuppressive therapy were quantified with the developed MOI-MS/MS method and statistically compared to

an already validated CMIA assay (**Figure 3; Supplementary Material Figures S16-17**). The acceptance criteria were defined as a slope of 1.00 ± 0.25 and $R^2 \geq 0.9$ for Passing and Bablok regression analysis.

For the set of TAC samples, MOI-MS/MS showed acceptable agreement with CMIA with a slope of 1.227 (95 % confidence interval: 1.148 to 1.307), an intercept of 0.066 (95 % confidence interval: - 0.704 to 0.589), and R^2 of 0.904. The lack of a slope value of 1 in the 95 % confidence interval explains the proportional differences between the two methods. Additionally, no systematic differences could be studied given that the 95 % confidence interval contains an intercept value of 0. A Bland-Altman plot identified 93.75 % (90/95) of samples within a 95 % confidence interval. The average bias was determined to be + 1.9 ng mL^{-1} or 19.3 %.

Regarding the set of samples containing SIR, the method showed good agreement with CMIA, providing a slope of 0.830 (95 % confidence interval: 0.789 to 0.885) and an intercept of - 0.095 (95 % confidence interval: - 0.624 to 0.222). Similarly to TAC samples, SIR samples showed some proportional bias and no systematic bias. Additionally, the Bland-Altman plot identified 95.8 % (91/95) of samples within a 95 % confidence interval. The average bias was determined to be – 2.3 ng mL^{-1} or approximately – 19.3 %.

Several studies have shown the positive bias of immunoassay-based methods compared to LC-MS/MS-based methods, and these differences have been mostly attributed to the cross-reactivity of ISD metabolites. Proportional differences are especially observed for SIR, but it also must be kept in mind that proportional and systematic differences depend on the type of commercial immunoassay kit and external calibrator reference materials used [8,9,42,43]. Moreover, similar results have been achieved herein as compared to another recent publication in the based on the SPME [36].

3.4. Comparison of Bio-SPME-MOI-MS/MS vs other technologies

Reports in the literature suggest that a total turnaround time of 3 – 6 hours is desirable for clinical applications so as to allow for daily dose adjustments [3]. Therefore, in this case, aiming to achieve total turnaround times that meet the requirements demanded in clinical environments, the IS's are added to the lysis solution instead of being spiked to the whole blood. Using this approach, a single sample can be completely analyzed in less than 62 minutes, an appropriate timeframe for clinical environments. Additionally, taking into account the high throughput capabilities of the method (reduction by a factor of 24, number of vials VWR® Thermal Shake Touch can accept at once), a total time of 4.5 minutes per single sample can be achieved. By doing this, the IS mixture can still accurately correct for subsequent experimental and/or instrumental variations. This is evidenced from **Supplementary Material Figures S4-S7** where an imprecision $\leq$ 10 % was achieved for method evaluation by using different whole blood lots with varying hematocrit levels.

Even though the method herein proposed met the desired turnaround time for daily dose adjustments in clinical environments [3], the Bio-SPME-MOI-MS/MS method is at disadvantage compared to other methods in terms of single sample turnaround time [23,26,27,44]. However, the method turnaround time is comparable to other reported assays when samples are run in a high-throughput fashion. Most traditional sample methodologies involve manual steps that reduce their high throughput capability (e.g. centrifugation of samples [9,21,26,27,44] or drying of samples [22,23]), whereas Bio-SPME fibers can be arranged to be operated on liquid handlers previously used for other SPME devices in high-throughput applications [32,45,46]. Thus, simplifying the overall workflow to minimize manual work. Additionally, BioSPME-MOI-MS/MS does not require a pressurized system for analysis, an advantage that reduces overall

system cost and contributes to design simplicity in comparison to existing methodologies. [14,26,27]

Additionally, the developed method uses a combination of mechanical and chemical lysing of the matrix (freeze-thaw cycles and addition of zinc sulfate:acetonitrile composition) to ensure that all analytes are released from the matrix prior to the extraction process, which is desirable for a non-exhaustive technique like SPME, which extracts exclusively via the free concentration of analytes. This allows our method to obtain comparable LOQs with other methodologies [26,27,36,44,47] for determination of ISDs in whole human blood, with other methods capable of reaching the recommended requirement of $\leq$ 1.0 ng mL^{-1} for tested analytes (with the exception of paper spray [23]) [3].

4. Conclusions

This manuscript presents a method for the simultaneous analysis of four ISDs from whole human blood using Bio-SPME-MOI-MS/MS technology. The method contributed to a single total turnaround time of 62 min, which surpasses the total turnaround time desirable in clinical environments (3 – 6 hours) without compromising the sensitivity, precision and/or the accuracy of the assay. Additionally, it is important to mention that when experiments are carried out in a high-throughput fashion, the total turnaround time decreases by a factor of 24, yielding a total turnaround time of 4.5 min per single sample. The imprecision of the assay was determined to be $\leq$ 10 % for in-house prepared QCs of each ISD over a 10-day period, while commercial QCs ((Recipe ClinCal®). also yielded an imprecision of $\leq$10 %. LOQs of less than one-third of the lowest concentration level for TAC, SIR, EVER, and CYC were achieved. Results of a cross-validation against a well-established CMIA assay for TAC and SIR did not show any systematic

differences (TAC: Passing and Bablok intercept of 0.066 with the interval of -0.704 – 0.589 and SIR: -0.905 with the interval of -0.624 – 0.222), but it has evidenced some proportional differences for both TAC and SIR (TAC: Passing and Bablok slope of 1.227 with an interval of 1.148 – 1.307 and SIR: Passing and Bablok slope of 0.830 with an interval of 0.789 – 0.885). The latter being inherent of comparisons between any MS-based method and immunoassay based methods. In order to make a more appropriate method comparison, the future work will involve the comparison of the SPME-MOI-MS/MS to validated LC-MS methods instead of knowingly biased immunoassay-based methods. In conclusion, this method offers a fast option for ISDs determination from whole blood matrixes while meeting general method development criteria.

Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council of Canada (NSERC) of Canada for their financial support through an Industrial Research Chair program. We would also like to thank SCIEX for the funding which was part of the Industrial Research Chair (IRC) program. We would also like to thank Waters Corporation for providing us with HLB particles. Finally, we would like to express our sincere gratitude to the University of Waterloo's Science Technical Services staff for their outstanding technical support and collaboration during the construction and improvement of the high-throughput system.

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Table 1. Figures of merit for determination of tacrolimus (TAC), sirolimus (SIR), everolimus (EVE), and cyclosporine A (CYC) in whole human blood. TAC, SIR, and EVE in-house quality control (QC) concentrations were 2.5 ng mL^{-1}, 7.5 ng mL^{-1}, and 15 ng mL^{-1} for QC Level 1, QC Level 2, and QC Level 3, respectively; while CYC concentrations were 50 ng mL^{-1}, 150 ng mL^{-1}, and 300 ng mL^{-1} for QC Level 1, QC Level 2, and QC Level 3, respectively.

				Accuracy, % (n=4)		
Analyte	LDR (ng mL^{-1})	LOQ (ng mL^{-1})	LOD (ng mL^{-1})	QC Level 1	QC Level 2	QC Level 3
TAC	1.0 – 50.0	0.8	0.3	86	115	101
SIR	1.0 – 50.0	0.7	0.2	112	107	93
EVE	1.0 – 50.0	1.0	0.3	81	87	83
CYC	2.5 – 500.0	0.8	0.3	96	91	89

LDR: linear dynamic range; LOQ: limit of quantitation; LOD: limit of detection, QC: quality control

Table 2. Total inter-day imprecision of method for TAC, SIR, EVE, and CYC from Recipe ClinCal® and in-house prepared QCs.

		Imprecision (n=10) %				Concentration (ng mL^{-1})			
		TAC	SIR	EVE	CYC	TAC	SIR	EVE	CYC
In-house QC	Level 1	4	8	6	2	2.5	2.5	2.5	50.0
	Level 2	3	4	5	6	7.5	7.5	7.5	150.0
	Level 3	3	10	6	3	15.0	15.0	15.0	300.0
Recipe ClinCal®	Level 1	9	10	7	1	1.3	1.5	1.5	26.3
	Level 2	3	3	4	9	2.5	2.9	2.9	48.9
	Level 3	1	9	3	7	5.3	5.7	5.3	93.0
	Level 4	4	3	5	4	10.9	11.9	11.7	180.0
	Level 5	5	4	3	9	21.6	23.5	23.2	440.0
	Level 6	1	1	6	N/A	44.1	49.8	48	1287.0

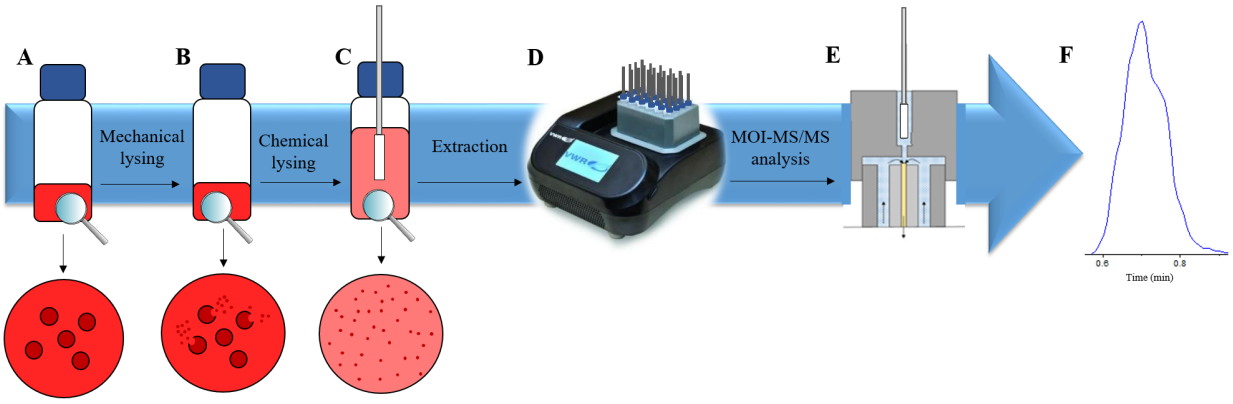
N/A: Concentration of this calibrator level exceeds the linear dynamic range of the method

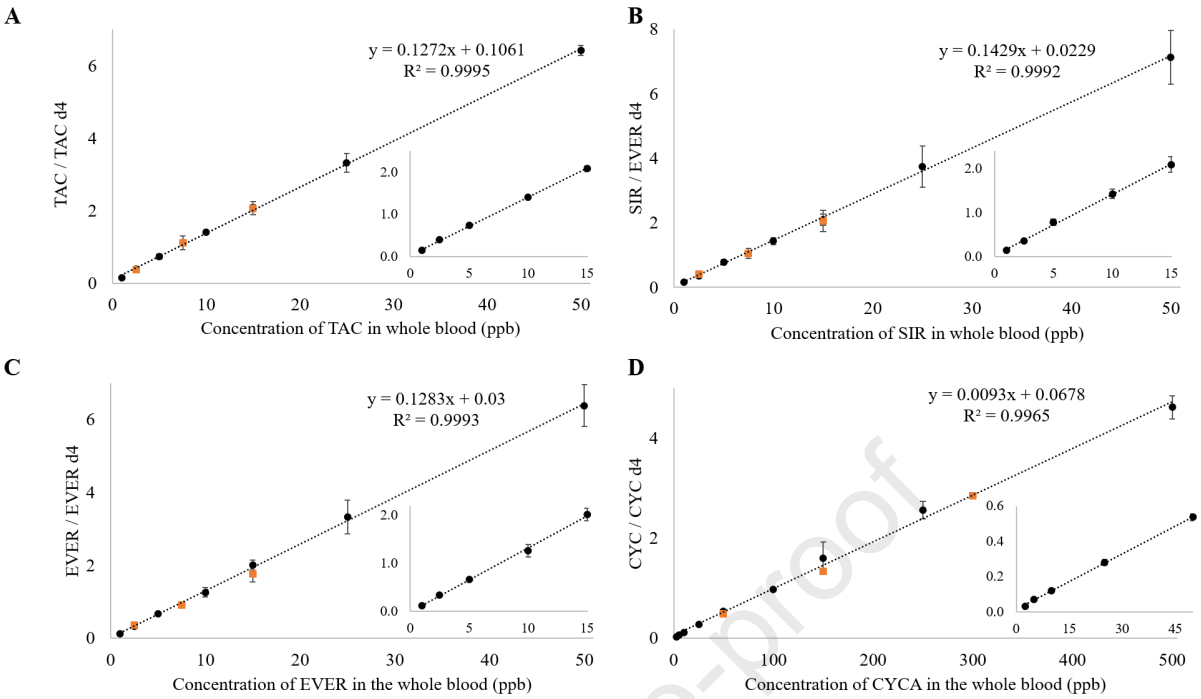
Figure Captions

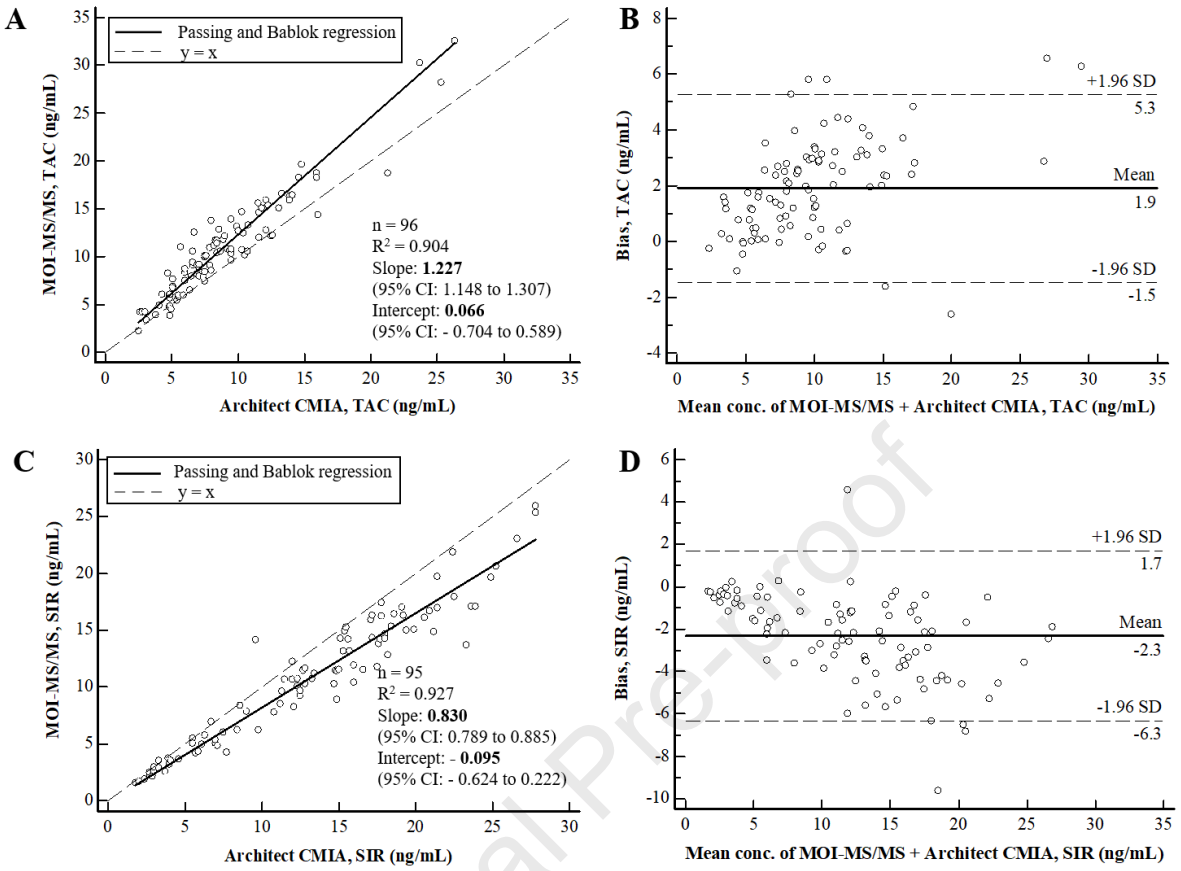
Figure 1. General workflow of the proposed method. **A.** Aliquot whole blood spiked with ISDs, **B.** Perform mechanical lysing (freeze-thaw cycle, three times), **C.** Perform chemical lysing (addition of 6:3:1, V:V, 0.1M Zinc Sulfate:Acetonitrile:Water mixture containing ISDs' internal standards), **D.** Extraction using VWR® Thermal Shake Touch at 2200 RPM and 55°C, **E.** Desorption of 5 seconds in the MOI, **F.** Instrumental analysis

Figure 2. Linear regression analysis of tacrolimus (TAC), sirolimus (SIR), everolimus (EVE), and cyclosporine A (CYC) in whole human blood. Black circles correspond to the calibration points, orange squares correspond to the internal quality control levels, and small insert on the right-bottom side of each graph is enlarged calibration plot at the limit of quantification levels for each analyte.

Figure 3. Passing and Bablok regression comparisons and Bland-Altman plots for TAC (**A** and **B**) and SIR (**C** and **D**) between MOI-MS/MS and Architect CMIA







Highlights

1. Determination of immunosuppressive drugs from whole blood
2. The limits of quantitation in sub 1 ng mL^{-1} range
3. Single turnaround time was ~60 min (4.5 min for high throughput) per single sample
4. Microfluidic open interface - mass spectrometry as an alternative in bioanalysis
5. Cross-validation against chemiluminescent microparticle immunoassay method

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: