Rapid determination of immunosuppressive drug concentrations in whole blood by Coated Blade Spray-Tandem Mass Spectrometry (CBS-MS/MS)

Germán Augusto Gómez-Ríos, Marcos Tascon, Nathaly Reyes-Garcés, Ezel Boyacı, Justen James Poole, Janusz Pawliszyn*

Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

*Corresponding author:

Tel.: +1 519 888 4641; fax: +1 519 746 0435. E-mail address: Janusz@uwaterloo.ca (J. P.).

Abstract

Coated Blade Spray (CBS) is a technology that efficiently integrates sample preparation and direct coupling to mass spectrometry (MS) on a single device. In this article, we present CBS-tandem mass spectrometry (CBS-MS/MS) as a novel tool for the rapid and simultaneous determination of four commonly used immunosuppressive drugs (ISDs) in whole blood: tacrolimus (TAC) and cyclosporine-A (CycA), which are calcineurin inhibitors; and sirolimus (SIR) and everolimus (EVR), which are both mTOR (mechanistic target of rapamycin) inhibitors. Given that CBS extracts via free concentration, analytes that are largely bound to plasma proteins or red blood cells provide considerably lower extraction recovery rates. Therefore, we defy the solventless philosophy of SPME-based techniques, like CBS, by performing the analyte-enrichment step via direct immersion in a solvent-modified matrix. The assay was linear within the evaluated range of concentrations (between 1 and 100 ng/mL for EVR/SIR/TAC and 10-1000 ng/mL for CycA), and the limits of quantification were determined to be 10 ng/mL for CycA and 1 ng/mL for EVR/SIR/TAC. Good accuracy (87-119%) and linearity ($r^2 \geq 0.99$) were attained over the evaluated range for all ISDs. Interassay imprecision (CV) determined from incurred sample reanalysis was ≤ 10 % for all ISDs. Our method was validated using Liquichek™ whole blood immunosuppressant quality control (QC) standards purchased from Bio-Rad. Concentrations determined by CBS-MS/MS were inside the range specified by Bio-Rad and within 15% of the expected mean value for all ISDs at all QC levels. Furthermore, the effect of different hematocrit levels (20, 45, and 70%) in the entire calibration range was carefully studied. No statistical differences (RSD ≤ 15%) in the calibration curve slopes of ISDs in blood were observed. CBS offers a simpler workflow than that of traditional methods; it eliminates the need for chromatographic separation and provides a clean extract that allows for long-term MS instrumental operation with minimal maintenance. Additionally, because CBS integrates all analytical steps into one device, it eliminates the risk of instrumental carry-over and can be used as a low-cost disposable device for sample preparation and analysis. Fully-automated sample preparation simplifies the method and allows for total analysis times as short as 3 minutes with turn-around times of less than 90 minutes.

Keywords

Immunosuppressive drugs; Mass Spectrometry; Coated Blade Spray; SPME-MS

Abbreviations

CBS: Coated Blade Spray; ISDs: immunosuppressive drugs; TAC: tacrolimus; CycA: cyclosporine-A; SIR: sirolimus; EVR: everolimus; SPME: solid-phase microextraction; MS: mass spectrometry

Highlights

- This paper outlines a new SPME-MS method for the quantitation of four immunosuppressive drugs in whole blood.
- CBS-MS/MS offers a simpler workflow than that of traditional methods of immunosuppressive drug determination.
- The limits of quantitation for 1 $ng \cdot mL^{-1}$ for EVR/SIR/TAC and 10 $ng \cdot mL^{-1}$ for CycA were obtained.
- A total analysis time of less than 3 minutes can be attained by using a high-throughput sample preparation autosampler.
- This paper documents the first successful implementation of direct-immersion SPME (DI-SPME) on a solvent-modified matrix for the purpose of detecting ISDs in blood.

1. Introduction

Immunosuppressive drugs (ISDs) are a class of medication that control or reduce the immune system's activity[1]. ISDs are regularly used to prevent the rejection of transplanted organs and tissues, as well as to treat autoimmune diseases or illnesses, such as psoriasis, lupus, and rheumatoid arthritis. Traditionally, ISD therapy requires drug concentrations to be closely monitored due to their narrow therapeutic range[2]. Essentially, an under-dose of ISDs can lead to graft (*i.e.* a piece of living tissue that is transplanted surgically) rejection/impairment and an over-dose can cause severe nephrotoxicity and/or overimmunosuppresion, which can subsequently result in an excessive risk of infection and malignancies[3]. Therefore, frequent Therapeutic Drug Monitoring (TDM) of ISDs in whole blood concentrations is essential for patients who have recently experienced organ transplantation[4–6]. Historically, immunoassays have been used to assess ISD concentrations in a patient's blood[1,2]. However, a report recently published by Seger *et al.* shows that approximately 50% of tacrolimus (TAC) and cyclosporine (CycA) determinations are currently performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [3,7]. Furthermore, determinations for sirolimus (SIR) and everolimus (EVR), which are both mTOR (mechanistic target of rapamycin), are conducted using by LC-MS/MS in a range of 70 to 75%[5]. On average, approximately 60% of ISD determinations are performed via LC-MS/MS; this is largely due to immunoassay determinations' high level of risk for showing either matrix-induce method bias (*i.e.* susceptibility to false-positives caused by other matrix components)[3], or cross-reactivity-induced method bias (*e.g.* interaction of metabolites of the ISDs), which displays higher concentrations than those reported by LC-MS/MS[3,7]. State-of-the-art MS instruments have allowed limits of quantitation (LOQ) as low as 0.1 ng mL^{-1} for TAC [8,9], 0.2 ng mL^{-1} for SIR/EVR[10,11], and 2 ng mL^{-1} for CycA[12] to be reached. Despite the outstanding quantitative and selective capabilities provided by MS systems, these high-tech analytical instruments require clean sample extracts in order to generate reproducible and reliable results for long periods of time. Hence, there is a need for adequate sample preparation approaches that guarantee the following parameters: a.) the satisfactory release of ISDs from the erythrocytes; b.) acceptable enrichment

of the drugs on the extractive phase (*i.e.* solvent or particles); and c.) removal of potential matrix interferences. Most of the methods developed to date rely on protein precipitation (PP) followed by either liquid-liquid extraction (LLE) or solid-phase extraction (SPE). According to a survey conducted by Seger *et al.*[3], approximately 72% of the sample preparation work currently performed in clinical laboratories for ISD determinations is done manually by the analyst rather than by an automated liquid handling platform[2,3]; as such, this manual analytic procedure is both more tedious and prone to increased error in the determination[13]. Although the chromatographic step is not necessarily the bottleneck of the entire analytical process, the time required for each chromatographic run is between 2 and 4 minutes per sample[2]. Hence, the turnaround times can be anywhere from two hours up to multiple days, depending on the laboratory protocols[3]. Undeniably, there is a need for a method that can provide faster throughput—not only in the analysis stage, but also during the sample-preparation steps[14]. Methods that allow rapid interfacing with mass spectrometry[15]—for example, Paper Spray (PS)[16,17], Rapid-Fire (RF)[6], Turbo-Flow (TF)[18,19], and Laser Diode Thermal Desorption[20]—have been recently developed as alternatives for the rapid determination of immunosuppressants in whole blood[4]. Unfortunately, none of these technologies are capable of both sample preparation and the direct coupling with the instrument. Coated Blade Spray (CBS) is a technology that efficiently integrates sample preparation and direct coupling to MS on a single device [21]. Essentially, CBS consists of a stainless-steel sheet carved in a sword-like fashion which is coated with a biocompatible extractive composite polymer (Figure 1). As a solid-phase microextraction (SPME) device, CBS simultaneously isolates and enriches analytes of interest present in the matrix without collecting the matrix itself [22]; as an ambient ionization device, CBS acts as a solid-substrate electrospray ionization (ESI) source [14,23]. Owing to its simplicity, CBS-MS/MS results suitable for fast ($\leq$ 1 min per sample) and quantitative (low or sub-ng/mL) determinations of various substances have been previously demonstrated in urine, plasma, and whole blood samples[21,22,24]. In this study, we present CBS-MS/MS as a novel tool for the rapid and simultaneous determination of TAC, SIR, EVR, and CycA. By using a fully automated sample preparation procedure, we dramatically simplify the method and enable total analysis times as short as 3 minutes with turn-around times of less than 90 minutes.

2. Experimental section

2.1 Chemical, reagents and materials

LC-MS grade Methanol (MeOH), acetonitrile (ACN), isopropanol (IPA), and water were purchased from Fisher Scientific. Formic acid was purchased from Sigma–Aldrich (Saint Louis, USA). TAC, SIR, EVR, and CycA (see Figure S1) were obtained from Sigma–Aldrich (Milwaukee, USA). Deuterated analogs, namely tacrolimus-d2C1, sirolimus-d3, everolimus-d4, and cyclosporineA-d4 were purchased from TRC-Chemicals (Toronto, ON, Canada). Liquichek™ whole blood immunosuppressant quality control (4 levels QC) standards purchased from Bio-Rad (Mississauga, ON, Canada). Human whole blood (with K2-EDTA as the anticoagulant) from different patients was acquired from Bioreclamation IVT (Westbury, New York, USA). All blood samples were spiked and stored overnight at 4 °C prior to use in order to reach drug-protein binding equilibrium. HLB particles (~5 µm particle diameter) were kindly provided by Waters Corporation (Wilmslow, UK). Stainless steel blades (15mm), which were purchased from Shimifrez Inc. (Concord, Ontario, Canada), were coated with HLB-polyacrylonitrile (HLB-PAN) slurry according to a proprietary protocol developed in our laboratory.

2.2 Mass Spectrometry

All the experiments reported herein were performed using a TSQ Quantiva mass spectrometer (Thermo Scientific, San Jose, CA, USA), and the acquired data was processed using Trace Finder version 3.3 (Thermo Scientific, San Jose, CA, USA). A custom-made coated blade spray interface was built at the University of Waterloo; a thorough description of the operation of this system can be found elsewhere[21]. Each drug was detected as a single charged ammonium adduct[5], with one selected reaction monitoring transition recorded for each analyte (dwell time ~ 50ms): *m/z* 821→768 for TAC; *m/z* 931→864 for SIR; *m/z* 975→908 for EVR; and *m/z* 1219→1202 for CycA. Further details regarding collision energy and RF-lens values are presented in Table S1 in the supporting information.

2.3 Sample preparation

All of the CBS devices were cleaned for 30 min using a 40:40:20 (MeOH/ACN/IPA, v/v/v) solution before being conditioned for an additional 30 min with a 50:50 (MeOH/water, v/v) solution prior to the extraction process. It is important to point out that, regardless of the application, CBS can be dry prior to the extraction step. The analytical workflow consisted of three stages (see Figure 1): a.) analyte enrichment; b.) coating cleaning, wherein nonspecific attachments of matrix components were quickly removed from the coated surface (10 s water-rinse); and c.) MS analysis, in which a minute droplet of elution/ionization solvent (10 µL of methanol:water 95:5, 0.1% FA, 10 mM AcNH4) was placed onto the coating[21,24]. Following analyte desorption (~20s), a high-voltage was applied (~5.5 kV) to the non-coated area of the blade in order to generate an electrospray from the CBS tip for 20s (see Figure 2)[21]. The extraction procedure was performed using a 1:9 mixture of 150 µL of EDTA-anticoagulated blood sample with a water-acetonitrile solution (Water:ACN, 85:15, 0.1M $ZnSO_4$); the objective of this procedure was to burst cells that were present in the matrix and to denature proteins that were bound to the target analytes[5], thereby increasing the free concentrations of ISDs[22]. Next, analyte enrichment was performed by immersing the CBS in the denatured sample for 90 minutes at 25 ºC. This process was done by working in batches (*i.e.* automated 96-samples; see Figure 3), producing an average analysis time of 90s, and an individual sample turnaround time of approximately 90 min. The MS analysis time was approximately 30s. Automated CBS extractions were carried out using a Concept-96 system (Professional Analytical Systems, PAS, Magdala, Germany). This robotic sample preparation unit has been described in detail elsewhere[25,26]. Matrix-match whole-blood calibration functions were constructed based on the signal ratio of the analyte and the isotope-labelled internal standard (A/Is) with seven calibrators in four independent experiments covering a range between 1 and 100 ng/mL for EVR/SIR/TAC and 10-1000 ng/mL for CycA (see Tables S2 and S3). Linear regressions were plotted using weighted linear least squares (1/x). The quantitation of the ISDs was validated in terms of LOD, LOQ, linearity, repeatability, reproducibility, accuracy, and relative matrix effect for whole blood containing different hematocrit levels.

3 Results and discussion

The quantitative determination at low concentration levels (*e.g.* low part-per-billion) of drugs heavily bound (≥80%) to plasma proteins is a challenge for any sample preparation technology that extracts via-free concentration, like CBS[27]. This challenge is particularly great if the targeted drugs are not only bound to proteins, but are also partitioned into the red blood cells, as is the case with ISDs. As a consequence, the most commonly used methods for ISD analysis involve protein precipitation using a solution consisting of methanol and zinc sulfate ($ZnSO_4$), in order to break the cell walls and denature the proteins that bind the drugs. Once this has been done, sample clean-up using online-SPE and analyte separation/detection via LC-MS/MS analysis is conducted. Unsurprisingly, early attempts at quantifying ISDs in intact blood samples using protocols that had previously been designed for plasma and urine[21,24] were not successful. Given that SPME has traditionally been a solvent-free sample preparation technology, our next step consisted in conducting extraction using a water-modified matrix (mixture 1:1 of 100 µL blood and 100 µL of a 0.1M $ZnSO_4$ solution). However, as can be seen in Figure S2, we only attained acceptable results for CycA, while the LOQs for TAC and EVR were significantly above the therapeutic range (~10-25 ng mL^{-1}). Moreover, SIR, the analyte with the largest partitioning with erythrocytes (~95%), was barely detected using this protocol. Therefore, we increased the sample volume (*i.e.* 200 µL of blood) and the volume of "denaturing" mixture (500 µL 0.1M $ZnSO_4$ solution and 500 µL of water) in an attempt to increase the free-concentration of SIR. Although these conditions allowed us to improve the LOQ for TAC and EVR (~5-10 ng mL^{-1})—and to slightly improve the detectability for SIR (see Figure S3)—the results fell far short of the required values. As an alternative, we also evaluated extraction using whole blood samples that had been frozen (-80 °C) and thawed. Nonetheless, as shown in Figure S4, the results were only satisfactory for CycA. In order to determine if the HLB-coated blades were effective for the extraction of ISDs, extractions were performed using non-modified human plasma samples that had been spiked with four ISDs. As can be seen in Figure S5, the results revealed that HLB-CBS was capable of extracting ISDs from plasma with LOQs in the order of 1 ng mL^{-1} for SIR/ TAC, 5 ng mL^{-1} for EVR (~74% protein binding in plasma[5]), and 10 ng

mL^{-1} for CycA. This results suggest that the poor extraction of ISDs in whole blood was undoubtedly linked to their partitioning into the erythrocytes (see Table 1). Thus, we were compelled to defy the traditional solventless philosophy of SPME[28] as a result of the complexity of the matrix-analyte interactions. Essentially, this class of compounds can only be quantified via SPME-based technologies under the following conditions: the matrix consists of blood cells that are fully denatured; the analytes have been released into the solution phase; and the CBS devices are used as open-bed SPE substrates[22]. Basically, this approach entails adding a mixture of water, 0.1M $ZnSO_4$ solution, and organic solvent (*e.g.* methanol or acetonitrile, MeOH or ACN) to the biofluid in order to modify the matrix viscosity and the analyte-protein binding properties, and to denature the red blood cell walls. This approach results in a marked increase in the free concentration of the ISDs, which promotes their extraction onto the coating particles. Nevertheless, the addition of solvent to a matrix creates extra challenges for analyte extraction. For instance, if the amount of organic solvent is too large, analyte partition may be driven onto the solvent layer rather than onto the extractive particles due to a dramatic decrease in the analyte partition coefficient (K_{fs}). This will result in a notable reduction in the amount that is extracted. Likewise, an insufficient amount of organic solvent will lead to an unsatisfactory release of the analytes, and, consequently, inadequate LOQs. Our initial assessments were performed using diverse ratios of MeOH. As shown in Figure S6, a mixture containing 50% MeOH allowed for the highest recoveries for all ISDs. Based on these observations, and knowing that more than 50% organic solvent will dramatically affect the extraction recovery, we also studied ACN as a substitute for MeOH. Our results indicated that, when using ACN only 25% of organic solvent was sufficient to get the best recovery (see Figure S7). Furthermore, and more importantly, ACN-denaturing mixtures displayed better performance than MeOH-based mixtures (see Figure S8). Hence, this ratio was selected as the ideal compromise for quantitating ISDs, and it was used to determine the best extraction time. Our results indicate that extraction times around of 100 minutes are necessary to collect a sufficient amount of ISDs to allow us to reaching the required LOQs (see Figure S9)[2]. A further comparison under optimum extraction conditions showed that HLB coatings performed approximately two times better than C18 coatings for the enrichment of ISDs (see Figure S10). Additionally, we found that using a larger amount

of blood is a viable alternative strategy for increasing the amount of analyte extracted from the matrix (see Figure S11). However, this option would be limited to the amount of sample available. Finally, we found that we were able to achieve a significant improvement in the extraction efficiency for all ISDs by increasing the extraction temperature (see Figure S12). Indeed, we observed that the amount of analyte extracted reached a maximum at 35°C. This behavior is mainly related to a compromise between the increasing of the diffusion coefficient of the target analytes and the reduction of the affinity of the coating for the ISDs as the temperature increases.

Based on all of the above-mentioned findings, we proceeded to evaluate the capabilities of CBS-MS/MS for the quantitation of ISDs in whole blood. Our final protocol consisted of performing extractions from 150 µL of blood mixed with 325 µL of ACN (25%) and 1025 µL of 0.1M $ZnSO_4$-solution. The extraction time used in this protocol was 90 min at 1500 rpm using a 96-well plate that had been heated to 35°C. Figure 4 presents the linear calibration curve attained for each of the studied compounds. The LOQs achieved using this methodology were 10 ng mL^{-1} for CycA, 1 ng mL^{-1} for EVR/ TAC, and 2.5 ng mL^{-1} for SIR (see Table 1). Good linearity ($R^2 \geq 0.996$) was attained over the evaluated range for all ISDs. As can be seen in Table 2, the coefficient of variation (CV) of the calibrators for all ISDs was in accordance with the assay precision levels prescribed by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology Immunosuppressive Drug Scientific Committee (IATDMCT; ≤ 10%)[3]. Furthermore, this method had a median accuracy of 100.5% (see Table 2), which was evaluated using third-party-prepared calibrators (*i.e.* Liquichek™ calibrators by Bio-Rad, see Table S4). Finally, as presented in Table 3, no statistical differences (RSD ≤ 7 %) were observed in the calibration curve slopes of ISDs in blood with different hct values (20, 45, and 70 hematocrit %)[29]. The above-mentioned result can be primarily attributed to the strongly denatured media employed during the extraction step. In other words, due to the extreme conditions of the solution, a quantitative and reproducible amount of analyte is released and extracted in its free form regardless of the hct level. Although this manuscript strictly presents a proof-of-

concept of CBS's suitability for ISD determination, a full validation of the technology using a certified LC-MS/MS method with real samples is presently being undertaken in our laboratory.

4 Conclusions

This study demonstrates that CBS-MS/MS is a suitable tool for routine ISD determinations in clinical laboratories. Moreover, CBS offers a workflow that is simpler than that of traditional methods by eliminating the need for chromatographic separation, while providing a clean extract that allows for long-term instrumental operation with minimal maintenance. Furthermore, as CBS integrates all analytical steps in one device, it eradicates the risk of instrumental carry-over. Fully automated sample preparation simplifies the method and allows for total analysis times as short as 3 minutes with turn-around times of less than 90 minutes. LOQs of 10 ng mL^{-1} for CycA, 1 ng mL^{-1} for EVR/ TAC and 2.5 ng mL^{-1} for SIR, were obtained. Moreover, excellent linearity and more than acceptable CV (≤ 10%) for all the validation points evaluated within the therapeutic range. Finally, it was demonstrated that no statistical differences (RSD ≤ 7 %) in sensitivity were obtained when blood with different hct values was analyzed. However, it is worth noting that, beyond the analytical validation herein reported and, in order to use CBS-MS/MS in routine clinical practice for TDM measurements, some clinical performance parameter must be considered; for example, long-term stability, performance of the instrument/assay combination under typical routine circumstances (*e.g.* several samples a day), different medical conditions (e.g. co-medication, lipemia, jaundice, impaired renal function) or different physical blood storage approaches (e.g. hemolysis).[3] Further validation is required using samples from real patients, as this will allow for more detailed examination of potential in-source fragmentation-related bias (e.g. inadvertent dissociation of labile conjugated metabolites in the API region). The aims of our current efforts have been threefold: to decrease the sample volume required to perform analysis; to provide a fully-automated CBS-MS platform; and to assess tacrolimus (ERM-DA110a), which is the only certified reference material available in the market for ISD quantitation[1,7].

Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council (NSERC) of Canada for their financial support through an Industrial Research Chair program. We would also like to thank Thermo Fisher Scientific for lending us the triple quadrupole mass spectrometer that was used in this work (TSQ Quantiva), which was part of the Industrial Research Chair (IRC) program. 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.

Author contributions

G.AG.R., M.T., N.R.G. and J.P. designed the project. Experiments were performed by G.AG.R., M.T. and N.R.G. with support from E.B. and J.J.P. The manuscript and figures were prepared by G.AG.R. All authors reviewed the manuscript and supplementary information.

5 References

1 D. M. Levine, G. T. Maine, D. A. Armbruster, C. Mussell, C. Buchholz, G. O'Connor, V. Tuck, A. Johnston and D. W. Holt, *Clin. Chem.*, 2011, **57**.

2 M. Shipkova and H. Valbuena, *TrAC Trends Anal. Chem.*, 2016, **84**, 23–33.

3 C. Seger, M. Shipkova, U. Christians, E. M. Billaud, P. Wang, D. W. Holt, M. Brunet, P. K. Kunicki, T. Pawiński, L. J. Langman, P. Marquet, M. Oellerich, E. Wieland and P. Wallemacq, *Ther. Drug Monit.*, 2016, **38**, 170–189.

4 M. Shipkova and D. Svinarov, *Clin. Biochem.*, 2016, **49**, 1009–1023.

5 C. Seger, K. Tentschert, W. Stöggl, A. Griesmacher and S. L. Ramsay, *Nat. Protoc.*, 2009, **4**, 526–534.

6 D. Grote-Koska, S. Czajkowski and K. Brand, *Ther. Drug Monit.*, 2015, **37**, 400–404.

7 T. M. Annesley, D. A. McKeown, D. W. Holt, C. Mussell, E. Champarnaud, L. Harter, L. J. Calton and D. S. Mason, *Clin. Chem.*, 2013, **59**.

8 I. R. Dubbelboer, A. Pohanka, R. Said, S. Rosenborg and O. Beck, *Ther. Drug Monit.*, 2012, **34**, 134–142.

9 I. Laverdière, P. Caron, F. Couture, É. Lévesque and C. Guillemette, *J. Chromatogr. B*, 2012, **885**, 131–137.

10 M. Marinova, C. Artusi, L. Brugnolo, G. Antonelli, M. Zaninotto and M. Plebani, *Clin. Biochem.*, 2013, **46**, 1723–1727.

11 M. Ivanova, C. Artusi, G. Polo, M. Zaninotto and M. Plebani, *Clin Chem Lab Med*, 2011, **49**, 1151–1158.

12 A. Buchwald, K. Winkler and T. Epting, *BMC Clin. Pharmacol.*, 2012, **12**, 2.

13 D. W. McClymont and P. S. Freemont, *Nat Biotech*, 2017, **35**, 312–314.

14 C. R. Ferreira, K. E. Yannell, A. K. Jarmusch, V. Pirro, Z. Ouyang and R. G. Cooks, *Clin. Chem.*, 2015, **62**, 99–110.

15 P. J. Jannetto and R. L. Fitzgerald, *Clin. Chem.*, 2015, **62**, 92.

16 R.-Z. Shi, E. T. M. El Gierari, N. E. Manicke and J. D. Faix, *Clin. Chim. Acta*, 2015, **441**, 99–104.

17 R.-Z. Shi, E. T. M. El Gierari, J. D. Faix and N. E. Manicke, *Clin. Chem.*, 2015, **62**.

18 R. W. A. Peake, C. R. Hartigan, C. L. Esposito, M. D. Kellogg, J. Gabler, S. Wang, A. Breaud, J. Di Bussolo, J. Moskowitz and W. Clarke, *Ther. Drug Monit.*, 2015, **37**, 161–171.

19 D. M. Mueller and K. M. Rentsch, *Sensitive quantification of sirolimus and everolimus by LC–MS/MS with online sample cleanup*, 2010, vol. 878.

20 J.-F. Jourdil, P. Picard, C. Meunier, S. Auger and F. Stanke-Labesque, *Anal. Chim. Acta*, 2013, **805**, 80–86.

21 G. A. Gómez-Ríos and J. Pawliszyn, *Angew. Chemie*, 2014, **53**, 14503–14507.

22 H. Piri-Moghadam, F. Ahmadi, G. A. Gómez-Ríos, E. Boyacı, N. Reyes-Garcés, A. Aghakhani, B. Bojko and J. Pawliszyn, *Angew. Chemie*, 2016, **55**, 7510–7514.

23 X. Ma and Z. Ouyang, *TrAC Trends Anal. Chem.*, 2016, **85**, 10–19.

24 M. Tascon, G. A. Gómez-Ríos, N. Reyes-Garcés, J. Poole, E. Boyacı and J. Pawliszyn, *J. Pharm. Biomed. Anal.*, 2017, 10.1016/j.jpba.2017.03.009.

25 N. Reyes-Garcés, B. Bojko and J. Pawliszyn, *J. Chromatogr. A*, 2014, **1374**, 40–9.

26 N. Reyes-Garcés, B. Bojko, D. Hein and J. Pawliszyn, *Anal. Chem.*, 2015, **87**, 9722–9730.

27 M. N. Alam, L. Ricardez-Sandoval and J. Pawliszyn, *Ind. Eng. Chem. Res.*, 2017, **56**, 3679–3686.

28 É. A. Souza-Silva, N. Reyes-Garcés, G. A. Gómez-Ríos, E. Boyaci, B. Bojko and J. Pawliszyn, *TrAC Trends Anal. Chem.*, 2015.

29 B. K. Matuszewski, M. L. Constanzer and C. M. Chavez-Eng, *Anal. Chem.*, 2003, **75**, 3019–3030.

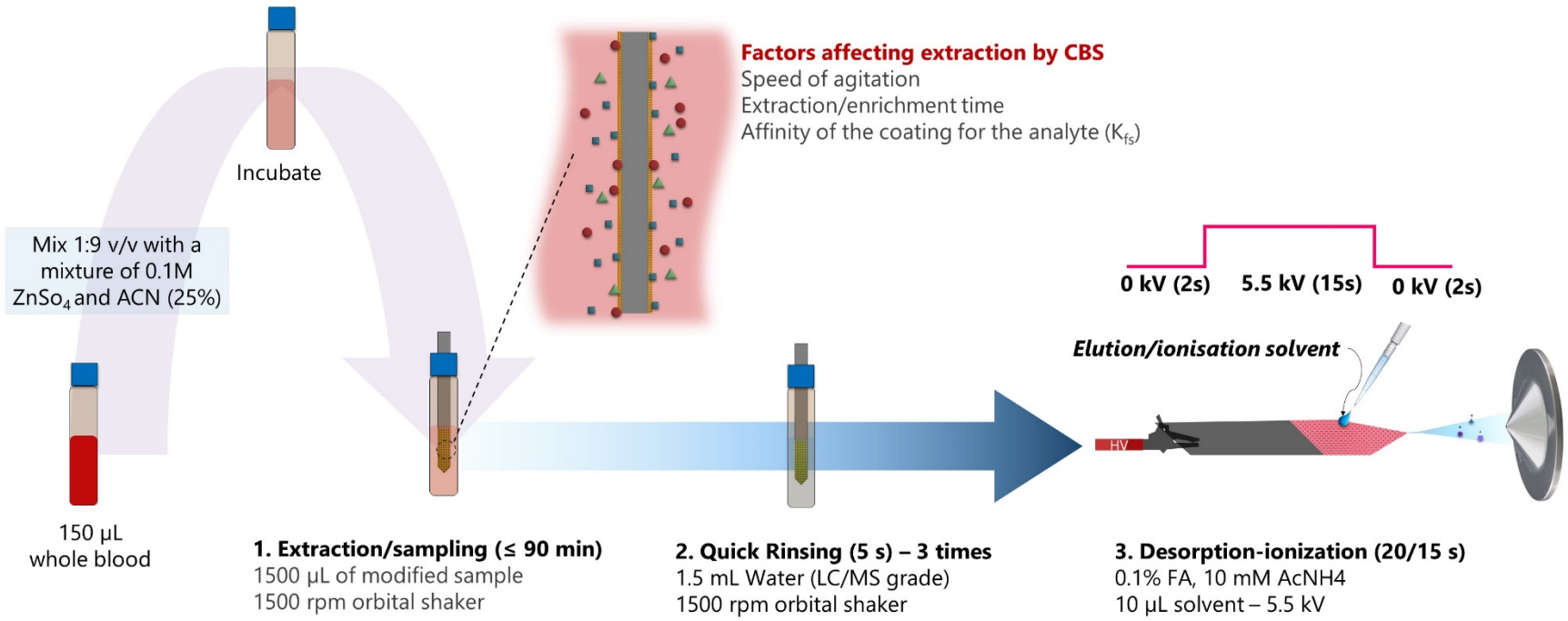

Fig. 1 Experimental workflow for the determination of ISDs in whole blood via CBS-MS/MS

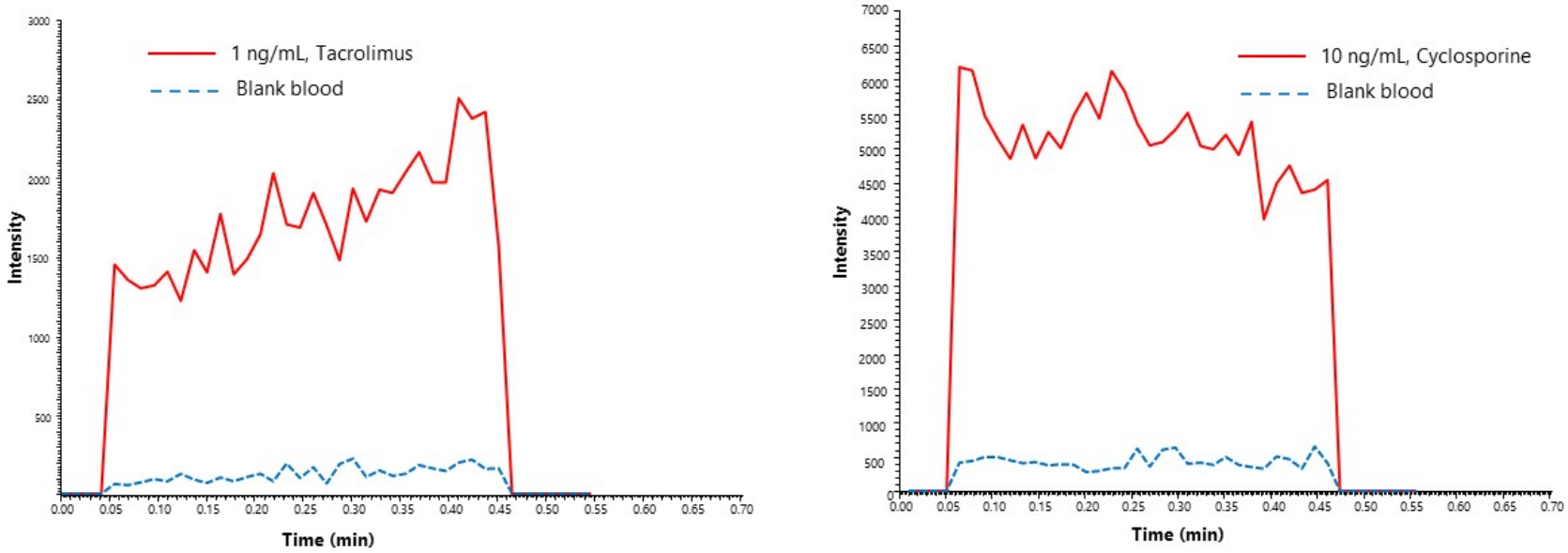

Fig. 2 Ion chronograms for TAC and CycA in whole blood spiked at 1 and 10 ng mL^{-1} (red line), respectively, overlay with representative chronograms from blank blood (blue line).

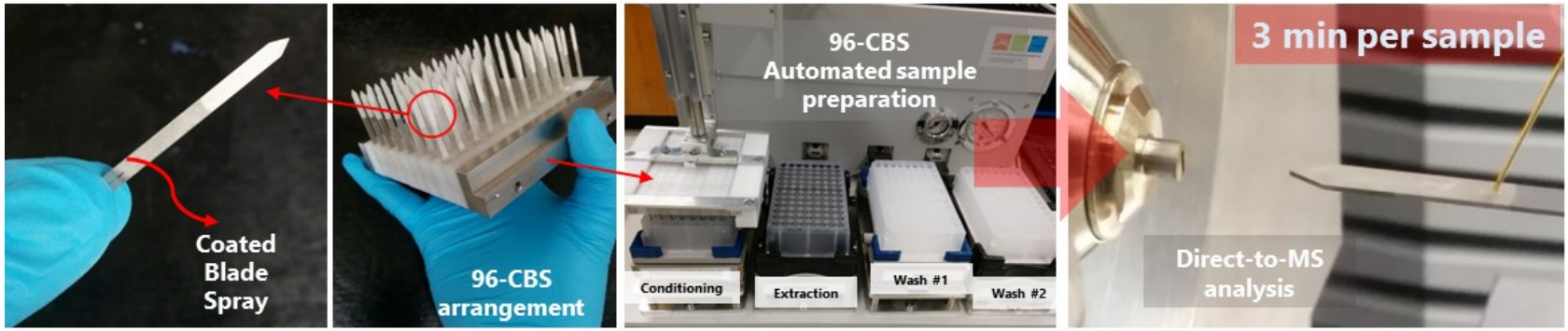

Fig. 3 Automated CBS sample preparation set-up for ISDs analysis

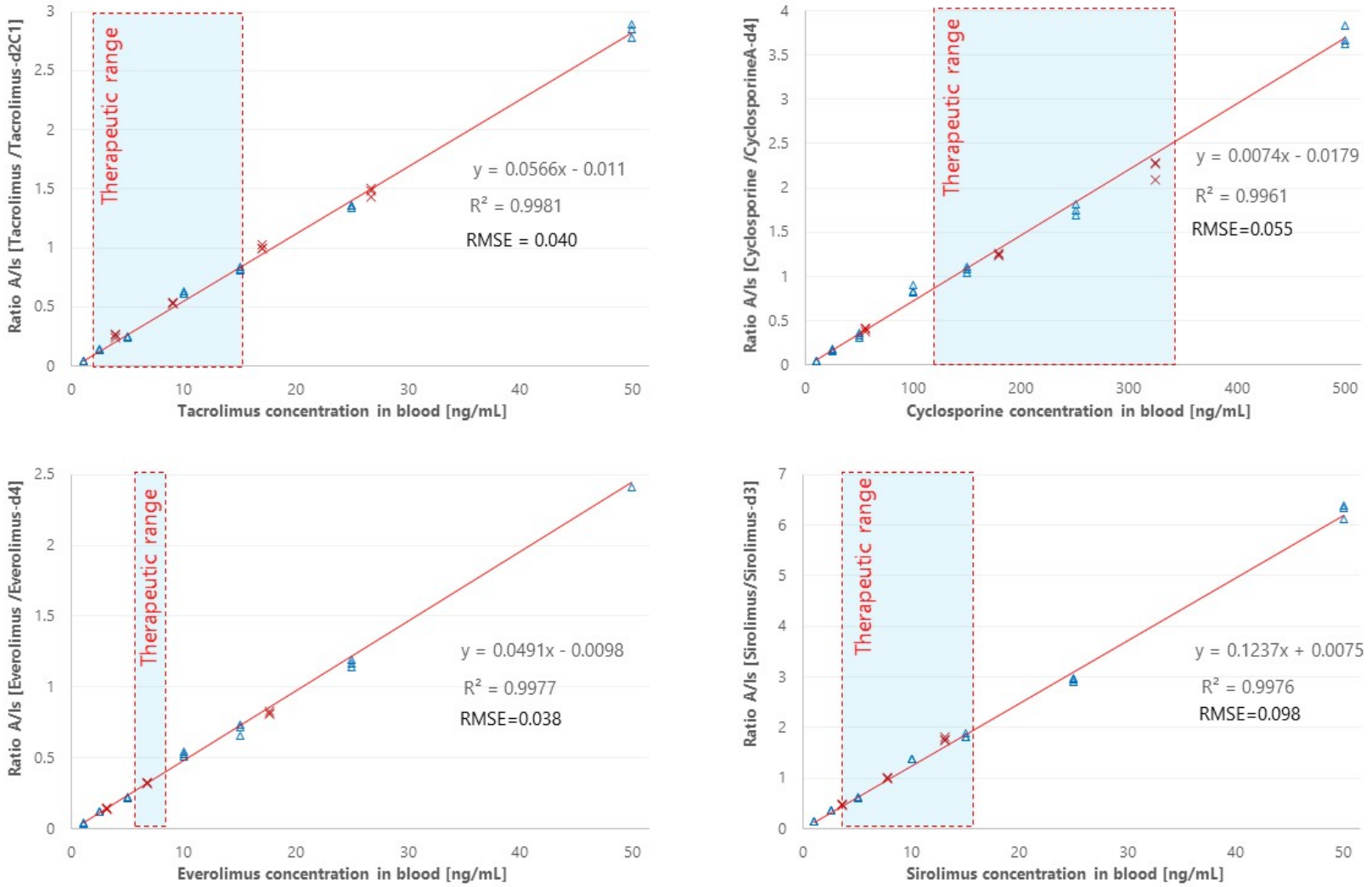

Fig. 4 Linear regression curves for TAC, CycA, EVR and SIR in whole blood. Analyses were performed using CBS-MS/MS from modified blood matrix with ACN and zinc sulfate. Quantification was performed with the entire area under the curve for each analyte, normalized by IS, to obtain calibrations curves as shown (blue triangles). Red marks represent the Liquichek™ QC standards.

Table 1 Figures of merit for the determination of ISDs in whole blood. Extractions were performed from 150 µL of blood mixed with 325 µL of ACN (25%) and 1025 µL of 0.1M $ZnSO_4$-solution. Extraction time was 90 min at 1500 rpm using a 96-well plate heated at 35°C. RMS: root-mean-square error; LDR: linear dynamic range

Compound	Distribution in Erythrocytes (%)	Typical Concentrations in ISD-TDM Samples [ng/mL][2,3]	LOD [ng/mL]	LOQ [ng/mL]	LDR [ng/mL]	RMSE
Cyclosporine	41-58	50−350	3	10	10-1000	0.055
Tacrolimus	~85	3−15	0.3	1	1-100	0.040
Sirolimus	~95	3−20	1	2.5	2.5-100	0.064
Everolimus	≥75	3−15	0.3	1	1-100	0.038

Table 2 Validation of protocol herein proposed using Liquichek® Bio-Rad standards (n=4)

Compound	QC-1	QC-2	QC-3	QC-4
Cyclosporine	101 ± 4.3	95 ± 1.1	93 ± 5.0	87 ± 2.1
Tacrolimus	119 ± 4.9	106 ± 1.3	106 ± 1.8	98 ± 2.8
Sirolimus	101 ± 2.8	103 ± 1.0	109 ± 1.9	-
Everolimus	-	96 ± 1.9	100 ± 0.4	96 ± 1.5

Table 3 Comparison of ISDs calibration curves obtained using blood with different hematocrit levels (n=4)

Hematocrit (%)	Linear least squares slope values			
	TAC	SIR	EVR	CycA
70	0.0226	0.0459	0.0228	0.0101
45	0.0219	0.0441	0.0210	0.0100
20	0.0205	0.0499	0.0220	0.0090
Average	0.0216	0.0466	0.0219	0.0097
RSD (%)	5	6	4	6