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Determination of four immunosuppressive drugs in whole blood using MEPS and LC–MS/MS allowing automated sample work-up and analysis

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ABSTRACT

In treatment with immunosuppressive drugs, monitoring of blood drug concentration is needed. The aim of this work was to explore micro extraction by packed sorbent (MEPS) as a possible on-line sample preparation method in combination with liquid chromatography–tandem mass spectrometry (LC–MS/MS) for quantification of cyclosporine, everolimus, sirolimus and tacrolimus in whole blood. An automated on-line MEPS system connected with a LC–MS/MS instrument was set up. A C_8 sorbent was used for the MEPS extraction. Subsequent analysis was performed with a gradient LC system. The adduct ions $[M + NH_4]^+$ of the analytes were monitored in SRM mode for quantification. Ascomycin and cyclosporine D were used as internal standards. The chromatographic run time 2.5 min and the quantification ranges were 3–1500 ng/mL ($r^2 \geq 0.999$, $n = 6$) for cyclosporine and 0.5–50 ng/mL for everolimus, sirolimus and tacrolimus ($r^2 \geq 0.998$, 0.994 and 0.993, respectively, $n = 6$). Precision and accuracy were documented at three levels. Accuracy results were between 102% and 109% with precision between 2% and 13% and carry over <0.02%. Matrix effects were characterized and found to be below 20%. The quantifications obtained were in agreement with a reference LC–MS/MS method based on protein precipitation, and results obtained from external proficiency test samples compared with the mean of all other LC–mass spectrometry methods showed good agreement. This method provides an accurate, precise and automated procedure that can be applied for therapeutic drug monitoring of immunosuppressive drugs in clinical laboratories equipped with LC–MS/MS.

1. Introduction

Cyclosporine (CYA), everolimus (EVE), sirolimus (SIR) and tacrolimus (TAC) are the major maintenance immunosuppressive drugs administered following organ transplantation. In current immunosuppressive therapycalcineurin inhibitors (CYA or TAC) can be combined with mTOR signal inhibitors, (SIR or EVE) and glucocorticoids [1–3]. This combination therapy has succeeded in significantly improving short and long term survival rates of transplant recipients and allografts [4]. Careful monitoring of immunosuppressive drug levels following transplantation is the key to the survival of both allograft and patient [2,4]. Due to this, therapeutic drug monitoring (TDM) of immunosuppressive drugs is established since it helps arriving at the optimal dose for therapeutic efficacy as well as minimizing toxicity. Therefore there is a need for robust and sensitive high-throughput methods, with short turn-around times [5,6].

Today, analytical methods for TDM of immunosuppressive drugs comprise immunoassays based on different methodologies and chromatographic methods mainly coupled to mass spectrometric detectors. Immunoassays methods suffer from inherent cross-reactivity between the drug and their metabolites and do have limited sensitivity. This results in overestimation of drug concentrations [7–13] and unacceptable uncertainty in measurement at low concentrations. The LC–MS/MS technique is able to overcome these problems by offering more selective and sensitive detection. Therefore, high performance liquid chromatography in combination with atmospheric pressure ionization tandem mass spectrometry is providing the best method of choice for the determination of immunosuppressive drugs in whole blood samples. Over the last ten years many LC–MS/MS methods for immunosuppressive drugs with different sample preparation procedures have been published [1,5,6,11,13–17]. The most commonly used sample preparation methods are protein precipitation and (on-line or off-line) solid phase extraction (SPE). Several publications for immunosuppressive measurements use two-dimensional chromatography switch columns as another approach for SPE automation [18–21]. Inspite of short sample preparation, these

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methods require two binary pumps and autosamplers with a programmed switching valve. Also pre-sample clean up is required, mainly protein precipitation, to remove proteins and lipids and cell residues. This will lead to a prolonged sample preparation process.

In this study a new sample preparation technology, micro extraction in packed sorbent (MEPS), was used in combination with LC–MS/MS to quantify immunosuppressive drugs in whole blood. MEPS is the miniaturisation of conventional SPE that use packed bed devices (bin) incorporated in the CTC-PAL injection syringe. It is a new development in the field of sample preparation and has the same functionality as SPE [22,23]. The MEPS technology allows removal of matrix components, isolation and concentration of targeted analytes, and also reduction of matrix effects comparing to protein precipitation method. In MEPS less sample volume and solvent is needed than in conventional SPE which decreases costs of solvents and labor. Studies with MEPS, the sample processing, extraction and injection steps are performed using the same syringe. One bin can be used for more than 100 samples which can significantly reduce the cost comparing with traditional solid phase extraction.

The syringes can be used manually or connected to LC or GC CTC PAL platforms for on-line or off-line analysis without any modification of the hardware [24–33].

2. Experimental

2.1. Chemicals

Cyclosporine and everolimus were provided by Novartis, (Basel, Switzerland) and purchased from Fluka, sirolimus was provided by Wyeth and tacrolimus was provided by Astellas Pharma Inc. (Tokyo, Japan). The internal standards ascomycin (ASC) and cyclosporine D (CYD) were purchased from Alexis Biochemicals (San Diego, CA).

Acetonitrile, methanol, formic acid, isopropanol and ammonium formate (LC grade) were purchased from Merck (Darmstadt, Germany). The water used was from a Reagent Grade Milli-Q Plus water purification system (Millipore Co, Billerica, MA).

2.2. Blood samples

The blood used for preparing calibrators and QC samples was obtained from the Karolinska University Hospital Blood Bank. The patient's (transplant recipients) samples used for method comparison were decoded left-over samples from the TDM service at the Karolinska University Hospital In all instances, EDTA was used as anticoagulant. External proficiency samples were supplied by Analytical services international Ltd., London, UK.

2.3. Instrumentation

All work was conducted using a triple quadrupole mass spectrometer (TSQ Quantum, Thermo Scientific, Waltham, MA) equipped with an electrospray ionization source (ESI) and operated in positive ion mode. For data handling and quantification, Xcalibur software (version 2.07 sp1) was used. The MEPS syringe was 250 μL while the bin contained 1–2 mg, C_8 sorbent packing, particle size 45 μm and pore size 60 Å (SGE Analytical, Melbourne, Australia). The Accela liquid chromatography (LC) instrument was from Thermo Scientific and included pump and column oven. The CTC-Pal autoinjector was from CTC Analytics AG (Zwingen, Switzerland). The Kinetex C_{18} column (50 mm × 2.1 mm, 2.6 μm) was obtained from Phenomenex (Torrance, CA) and was used as analytical column. The Hypersil Gold C8 (10 mm × 2.1 mm) guard column was obtained from Thermo Scientific. The loop volume was 20 μL.

Table 1
Mass spectrometric parameters.

	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Cyclosporine, CYA	1219.8	1203.2	17
Everolimus, EVE	975.6	908.6	16
Sirolimus, SIR	931.5	864.7	17
Tacrolimus, TAC	821.5	768.6	18
Ascomycin, ASC	809.4	756.6	20
Cyclosporine D, CYD	1233.9	1217.4	16

2.4. HPLC conditions

A gradient mobile phase system was used with solvent A being aqueous 0.1% formic acid with 2 mM ammonium formate, and solvent B being methanol (MeOH) with 0.1% formic acid and 2 mM ammonium formate.

The gradient started with 57% of mobile phase B with a hold time of 0.15 min and then increased gradually to 95% over 0.3 min followed by increasing to 98% over 0.55 min with a hold of 0.21 min. Then the mobile phase B was set to 57% again. The flow rate was 400 μL/min and the injected sample volume was 20 μL, the injection was performed by over-filling mode. The column oven temperature was 60 °C and the CTC-Pal tray temperature was 8 °C. The automatic switch valve in the mass detector was set to divert the first 1.0 min and last 0.3 min of the gradient program. This was done to protect the mass spectrometer from early and late eluting compounds. The total analysis time between injections was 2.5 min.

2.5. MS conditions

The monitoring mode was selected reaction monitoring (SRM) using ammoniated precursor ions $[M+NH_4]^+$. The spray voltage was 3500 V; the sheath pressure was 60 and capillary temperature was 350 °C. Nitrogen was used both as drying and nebulizing gas and argon (ICP 5.0 grade, AGA gas AB, Sundbyberg, Sweden) was used as collision gas.

Compound specific mass spectrometric parameters settings were as shown in Table 1.

2.6. Calibrators and controls

Stock solutions were prepared in methanol. Calibrators and controls were prepared by mixing the methanol solutions with blank whole blood, with methanol content not exceeding 4%. The calibration curves covered 3.0–1500 ng/mL for CYA and 0.5–50 ng/mL for EVE, SIR and TAC. Quality control (QC) samples concentrations and the calibrators are as shown in Table 2. Blank samples and zero samples were prepared and analyzed in conjunction with calibrator analysis. The internal standard intermediate solution was prepared in methanol at concentrations of 350 ng/mL for ASC and 1500 ng/mL for CYD.

The internal standard working solution was prepared by diluting internal standard intermediate solution 160 times with aqueous 0.1% formic acid (final concentration ASC 2.18 ng/mL, CYD 9.37 ng/mL).

2.7. Sample preparation and MEPS conditions

A 50 μL aliquot of patient blood samples was diluted with 1.25 mL internal standard working solution thereafter, the samples were centrifuged for 2.0 min at speed of 2100 × *g*.

The prepared extract was subjected to on-line MEPS preparation. Sample loading was performed by taking six replicate 100 μL aliquots of the diluted blood sample. This was done

Table 2
Concentrations of calibrators and QC samples prepared in blood.

	CYA (ng/mL)	EVE (ng/mL)	SIR (ng/mL)	TAC (ng/mL)
Calibrator 1	3.0	0.50	0.50	0.50
Calibrator 2	20	1.0	1.0	1.0
Calibrator 3	50	4.0	4.0	4.0
Calibrator 4	100	10	10	10
Calibrator 5	500	25	25	25
Calibrator 6	1000	40	40	40
Calibrator 7	1500	50	50	50
QCL	10	2.0	2.0	2.0
QCM	360	16	16	16
QCH	960	38	38	38

by withdrawing and ejecting six times into the syringe by the autosampler (draw-eject in the same vial). Next, the MEPS sorbent was washed once with 100 μL of water/methanol (95:5, v/v). The analytes were eluted and injected by withdrawing 50 μL methanol/isopropanol/acetonitrile/water 50:30:10:10 (v/v/v/v) and injecting directly into the LC sample injector (20 μL). Washing of the MEPS sorbent was carried out using 4× 250 μL elution solution followed by 4× 250 μL of a washing solution (5% methanol in 0.1% formic acid) between every injection. The second cycle of washing also functioned as equilibration steps before the next injection. The same bin was used for about 120 extractions before it was discarded. Before using a new MEPS needle, the sorbent was manually activated and equilibrated with 50 μL methanol followed by 50 μL of water.

2.8. Reference methods

A modified LC–MS/MS method based on sample preparation was available in the lab [14,17,34]. It was based on precipitation with MeOH and zinc sulphate ($ZnSO_4$). In brief, 85 μL of blood was pipetted followed by 85 μL of water and 300 μL of precipitation solution containing internal standard (MeOH:aqueous 0.40 M $ZnSO_4$ 4:1) without intermediate mixing. This was followed by vortexing for five min and centrifugation for five min. A 20 μL aliquot of the supernatant was injected.

Immunoassay methods for CYA (CEDIA Cyclosporine PLUS) and TAC (EMIT 2000 Tacrolimus assay) were applied on Hitachi 917 instruments. Immunoassay methods for SIR (Abbott MEIA) and EVE (Seradyn FPIA) were applied on IMX and TD_X instruments.

2.9. Validation

Each calibration-curve consisted of 7 points in singlicate covering from 3 to 1500 ng/mL for CYA and 0.5–50 ng/mL for EVE, SIR and TAC. Blank samples were run simultaneously. The peak area ratios of solutes and the IS were measured and a calibrator curve without the zero concentration was constructed. The calibration curves were weighted ($1/x$). The QC samples were treated in the same way as the calibrators. The intra- and inter-assay of accuracy and precision were determined by using three levels of concentrations in human blood (QC: low, medium and high). Accuracy was defined as the degree of deviation of the determined value from the nominal value: ((measured value − nominal value)/nominal value) × 100).

Precision (CV %) was defined as the percentage of standard deviation of the observed values divided by their mean values: ((standard deviation/mean value) × 100).

Stability was tested for extracted and non extracted QC samples at 8 °C; Long-term stability in blood was assessed after QC samples were stored at −20 °C.

Carry-over was investigated by running a blank sample after running the highest calibrator.

Matrix effect assessment was studied quantitatively and qualitatively.

2.10. Method comparison

The method was applied for the analysis of frozen and stored left-over samples from the TDM service at the Karolinska University Hospital. The comparison method was based on protein precipitation as sample preparation. This method was validated and used in routine in our laboratory. Twenty-one patient samples for every immunosuppressive were analyzed in singlicate. The analysis took place in the same laboratory but sequentially in time.

3. Results

3.1. Method development

The chromatographic system was based on a 50 mm reversed-phase C_{18} column and used a gradient of from 57% to 98% mobile phase B in 1.21 min. A C_8 guard column was used to protect the chromatographic column and did not prolong retention, except for the additional void volume. In this system, which was developed to obtain a short time of analysis, the analytes and internal standards were not fully separated (Fig. 1). The first eluting substance was ASC (1.60 min), which was used as internal standard for EVE, SIR and TAC. These eluted within 0.1 min after ASC and overlapped chromatographically. CYA eluted slightly before its internal standard CYD and these also overlapped chromatographically.

More than 5 different MEPS-C8 bins were compared for their influence on analytes relative response. No statistically significant differences in recovery were observed between different bins. The bin was discarded after noticing loss of internal standard peak area. A 30% loss of the internal standard peak area, was used as indication of bin change. The bins could be used for 120 injections before being discarded.

Recovery (peak area) for different number of sample loading volume was investigated. Logically, sample response increased as applied sample volume increased, e.g. recovery increased 45% from 4× 100 μL to 6× 100 μL. Selective detection was achieved in the mass spectrometer by detecting products ions formed from the ammoniated molecules. No signs of interference due to cross-talk between the SRM channels were observed. Formic acid and ammonium formate were added to both mobile phase A and B and the concentrations were optimized for sensitivity and chromatographic performance.

The MEPS procedure was developed manually and was then transferred to the CTC-PAL platform. C18, C8, C4 and polymer sorbents have been tested. The lowest recovery was obtained with C18 sorbent (40% less than C8), this is the most non polar sorbent. Recovery using C8 was 15% higher than with C4.

Dilution of blood with water gave less recovery than using acidic water for dilution.

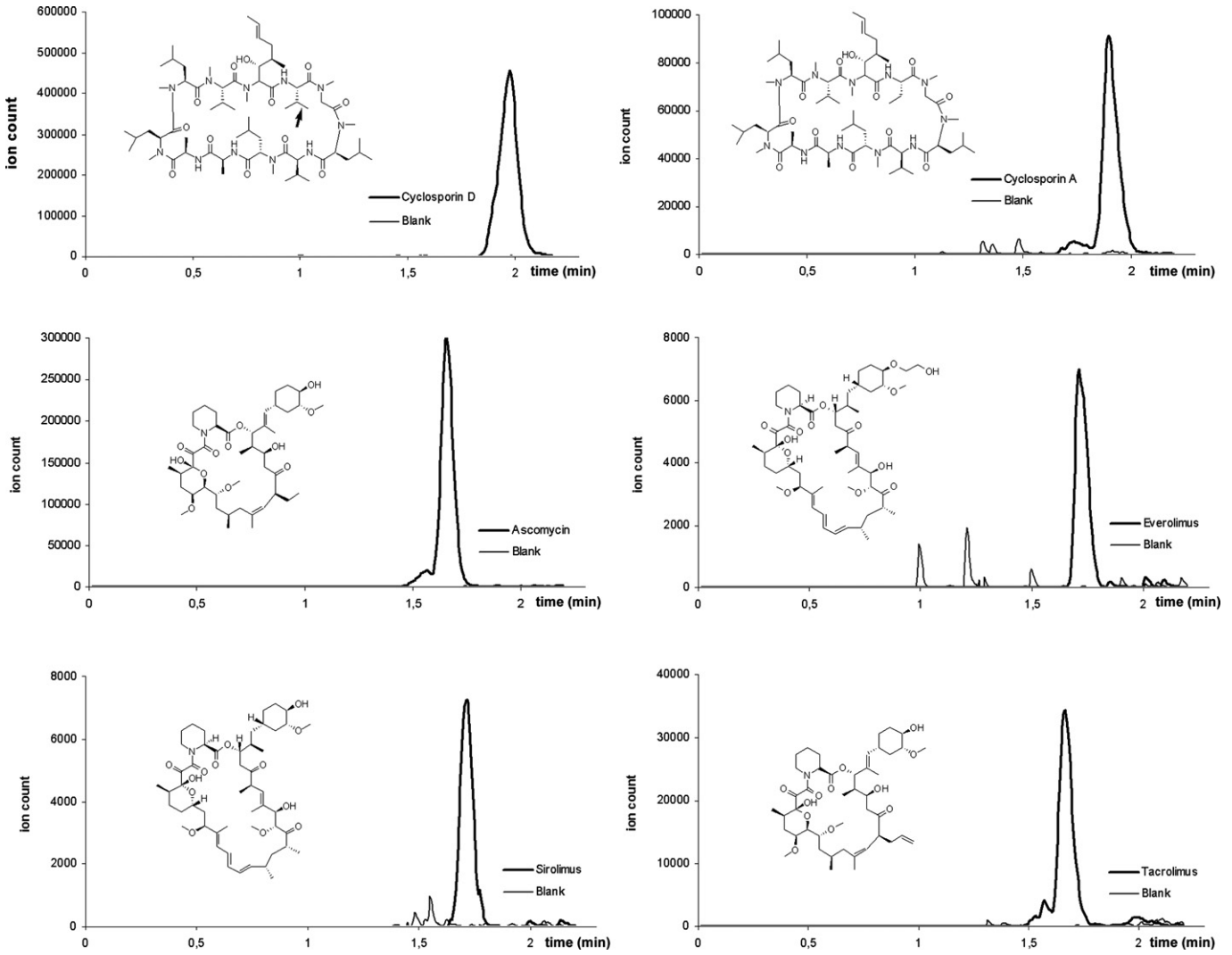

Fig. 1. SRM chromatograms obtained from the analytes of calibrator level 1; 3.0 ng/mL for CYA (B) and 0.5 ng/mL for EVE (D), TAC (E), and SIR (F) together with a blank sample shown for comparison. The concentrations of the internal standards, CYD (A) and ASC (C), corresponded to 150 and 35 ng/mL, respectively.

3.2. Method validation

3.2.1. Limit of detection and quantification

The limit of detection (LOD) was estimated based on a 3:1 signal to noise ratio. Calibrator 1 diluted with blank blood was used for this purpose. The LOD was found to be 0.9 ng/mL for CYA and 0.15 ng/mL for EVE SIR and TAC.

The LLOQ was estimated as a 10:1 signal to noise ratio. The LLOQ was then determined experimentally by analysing five replicates of spiked samples (3.0 ng/mL for CYA and 0.5 for EVE, SIR and TAC) in three batches during different days. The LLOQ was found to be 3.0 ng/mL for CYA (CV 10%), 0.5 ng/mL for EVE (CV 11%), 0.5 ng/mL for SIR (CV 8%) and 0.5 ng/mL for TAC (CV 7%).

3.2.2. Accuracy, precision and linearity

Accuracy and precision was studied by the analysis of six replicates of each of the three QC samples in five batches at different days. The peak area ratios of analytes to the internal standard were measured. Calibrator curves were calculated by linear regression with weighting factor of $1/x$ (Table 3). The accuracy varied from 102% to 109%, the intra-day precision from 2.0% to 11.7% (CV) and the total precision from 5.1% to 13.7% (CV) (Table 3).

The response was linear for all target compounds within the studied concentration range of the seven calibrators used. The results of the validation are presented in Table 3.

3.2.3. Selectivity

No interfering peaks were observed in chromatograms from calibrators, QC and patient samples. Three different blank blood specimens from the Karolinska University Hospital blood bank were analyzed. In addition, for each of the four immunosuppressive drugs, 20 blood samples from patients not receiving the respective drugs were analyzed for interfering peaks. No interference was observed.

3.2.4. Carry-over

The carry-over was investigated by analyzing a blank blood sample after injection of calibrator 7 ($n = 7$). The carry-over found to be 0.02% for CYA and less for EVE, SIR and TAC.

3.2.5. MEPS extraction efficiency

The extraction efficiency when using MEPS was examined by testing multiple loading cycles ($n = 1$–10) on a blood sample spiked at 1500 ng/mL for CYA and 50 ng/mL for EVE, SIR and TAC. The relationship between the MS response (peak area) and number of

Table 3
Summary of method validation results.

	Measuring range ng/mL	Linearity $n=6$	LOD ng/mL $n=6$	LLOQ ng/mL	Sample	Intra-day precision CV (%) $n=6$	Inter-day precision CV (%) $n=30$	Accuracy % $n=30$
Cyclosporine (CYA)	3–1500	$r^2 \geq 0.999$	0.9	3.0	QCL	4.2	5.1	102
					QCM	6.2	6.6	102
					QCH	5.8	7.1	103
Everolimus (EVE)	0.5–50	$r^2 \geq 0.998$	0.15	0.5	QCL	11.7	13.7	109
					QCM	3.5	6.3	103
					QCH	4.5	6.8	104
Sirolimus (SIR)	0.5–50	$r^2 \geq 0.994$	0.15	0.5	QCL	5.0	8.9	108
					QCM	4.6	7.0	102
					QCH	3.8	6.8	104
Tacrolimus (TAC)	0.5–50	$r^2 \geq 0.993$	0.15	0.5	QCL	8.4	9.1	106
					QCM	3.8	7.4	103
					QCL	2.0	7.6	104

extraction cycles was linear. Six sample loadings were found to be satisfactory for the needed sensitivity.

The extraction efficiency was further studied by analysing the waste volume after each sample loading cycle. The mean extraction yield for cycles 2–6 was calculated to be 97% for CYA, 92% for EVE, 90% for SIR and 96% for TAC.

3.2.6. Matrix effects

A post-column infusion experiment was performed for all analytes. Infusion of analytes in methanol (20 μg/mL for CYA and 1 μg/mL for the others) was made at a rate of 10 μL/min in conjunction with normal mobile phase flow. A prepared blank blood extract was injected and all the channels were monitored. A modest suppression at the retention time of the column void volume was followed by suppression just before the elution of analytes (Fig. 2).

For qualitative study of matrix effects an extracted blank blood sample was spiked with analytes (96 ng/mL for CYA, 6 ng/mL for the others). A reference sample prepared in mobile phase B was made in the same way. Triplicate samples were analyzed and the comparison of matrix to reference were +15% for CYA, +16% for EVE, +11% for SIR and −9% TAC.

The same experiment was done for the reference method and the results were as the following:

+40% for CYA, +35% for EVE, +50% for SIR and +65% TAC

Matrix effects for ASC and CYD for the MEPS method was found to be −11% and +19%, respectively.

Matrix effects for ASC and CYD for the reference method was found to be +37% and +30%, respectively.

3.2.7. Stability of samples and extracts

Extracted QC samples at three levels ($n=3$) were stable for 24 h at 8 °C while there was 15% loss at 48 h at the same temperature.

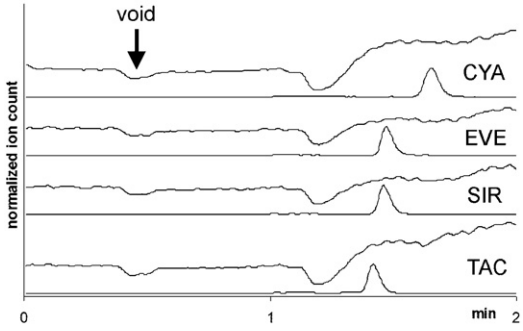

Fig. 2. Matrix effect after injecting a blank blood extract. The analytes were infused post column at a flow rate of 10 μL/min. For comparison the retention time of the analytes are shown in the lower traces.

Non-extracted samples (diluted blood samples) were stable for 24 h at 8 °C (autosampler temperature). Blood samples were stable for at least 60 days at −20 °C.

3.2.8. External proficiency testing

The method was used to run stored external proficiency samples from Analytical Services International Ltd. (UK). Results of the proficiency testing program are presented in Table 4.

3.2.9. Reference method (protein precipitation) validation results

The working range was 3–1500 ng/mL for CYA and 0.5–50 ng/mL for EVE, SIR and TAC. For CYA accuracy was between 92.5% and 100.3% and the total imprecision was 2.5–3.4 (CV%). For EVE, the accuracy results were 94.8–105.1% and the total precision results were between 3.7 and 6.9 (CV%). SIR accuracy results were 90.5–108.9% and the total precision 4.4–11.7 (CV%). TAC accuracy results were 95.0–101.9%, the total precision results were between 4.3 and 6.9 (CV%).

3.2.10. Method comparison

The performance of the method was further assessed by the comparison with the reference LC–MS/MS method and immunoassay methods, using authentic patient samples. The comparison showed that the MEPS method was in agreement with the reference LC–MS/MS method (Fig. 3). Representative chromatograms obtained from the analysis of patient samples are shown in Fig. 4. The linear regression analysis between MEPS method and immunoassay methods was as the following:

CYA: 1.24 ± 0.41 ($r^2 = 0.97$) ($n = 20$), EVE: 1.48 ± 0.23 ($r^2 = 0.84$) ($n = 20$), SIR: 0.97 ± 0.11 ($r^2 = 0.98$) ($n = 20$) and TAC: 1.36 ± 0.41 ($r^2 = 0.95$) ($n = 20$).

4. Discussion

An LC–MS/MS method for quantitative determination of four immunosuppressive drugs in patient whole blood samples using MEPS for automated online sample preparation was successfully developed and validated guided by the FDA guidelines [35,36]. Sufficient sensitivity was obtained in agreement with the clinical needs [3], with LLOQ of 0.5 ng/mL for TAC, EVE and SIR and 3.0 ng/mL

Table 4
Results f the proficiency testing program.

Drug	Samples (n)	Concentration range (ng/mL)	Min–Max agreement (%)
CYA	10	74–309	87–101
SIR	10	5–10	88–93
EVE	10	1.7–6.5	95–108
TAC	10	3.9–12	84–107

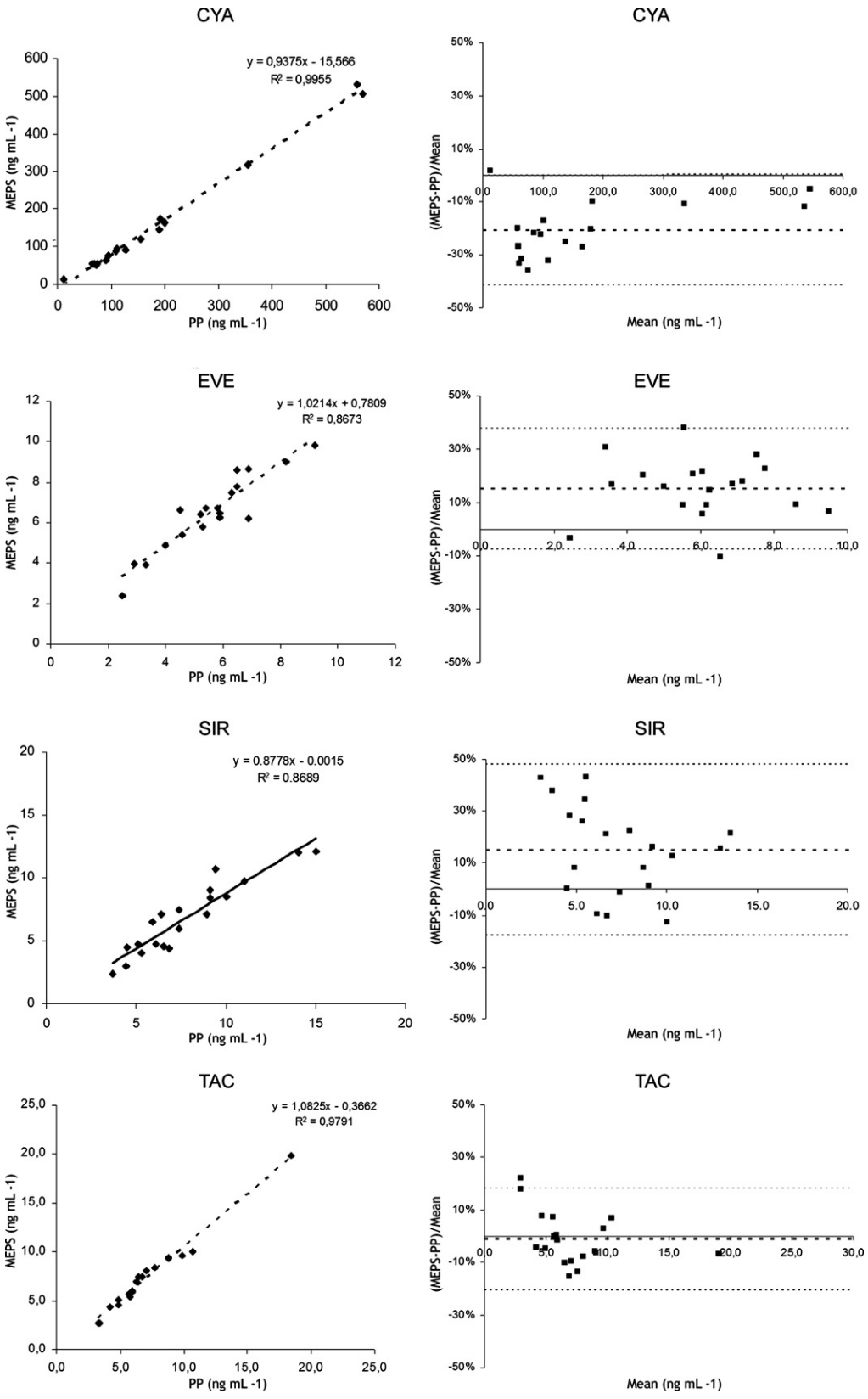

Fig. 3. (A). Method comparison between MEPS and PP; using linear regression method (left-hand side) and Bland–Altman plot (right-hand side).

for CYA. The sensitivity of the method can easily be enhanced by reducing the dilution and also by increasing the number of sample loading cycles, although bin lifetime would be expected to decrease correspondingly. The suitability of the method for its intended use was confirmed by application to authentic patient samples and by the agreement of the results with a reference LC–MS/MS method. The slope of the linear regression analysis of the investigated immunosuppressive show good agreement for all analyte

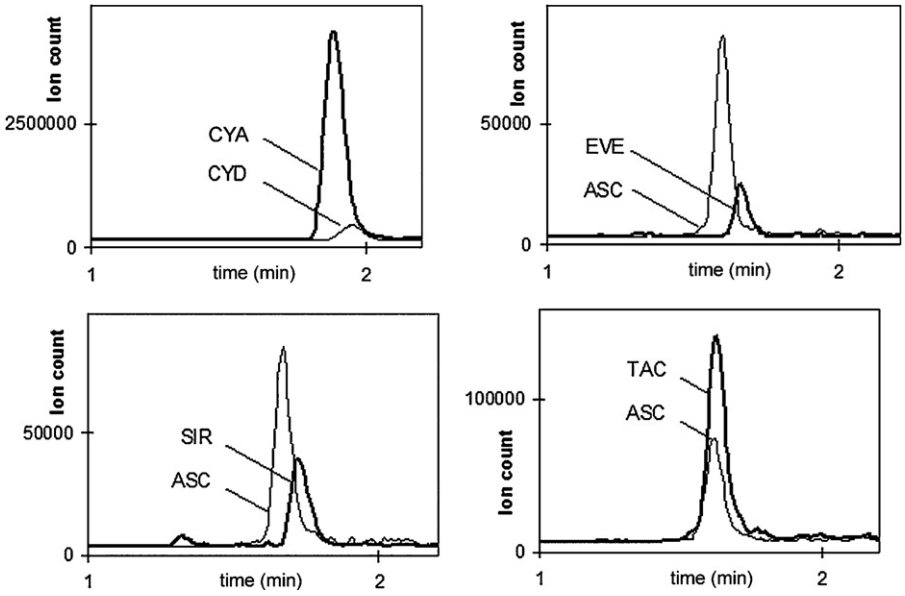

Fig. 4. SRM chromatograms obtained from four different patient samples. Quantifications were 20 ng/mL for CYA, 3.3 ng/mL for EVE, 4.2 ng/mL for SIR and 8 ng/mL for TAC.

but SIR where differences were around 13%, This discrepancy could depend on several different factors such as, the internal standard concentration since it was shown before that SIR quantification precision is affected by the internal standard concentration [6], matrix effect which is compound dependant and the run analysis time. However, the SIR discrepancy was acceptable. CYA and TAC seems to be influenced by the high concentration samples. However, excluding the highest points will not affect the slope and the regression coefficient. For the low concentration samples there was a noticeable deviation between the two methods but it was within acceptable range.

Furthermore the MEPS results correlated as expected with the immunoassay results with a noticed positive bias for the immunoassay results. This positive bias in the immunoassay results could be due to the overestimation of the drugs concentrations because of the non specific cross-reaction from their metabolites and this is in agreement with earlier findings [5].

The new MEPS technique is promising in term of extracting drugs and their metabolites from biological samples which makes it of interest in different fields of bioanalysis, e.g. environmental analysis, forensic toxicology and clinical pharmacology. At present, the approach has been used for a number of applications applicable to therapeutic drug monitoring (TDM), covering the clinically relevant matrices blood, urine and plasma. Demonstrated applications of MEPS for determination of therapeutic drugs comprise a wide range of analytes such as local anaesthetics, anticancer drugs, β-blockers and anti-depressants [24,25,28–32,37]. These applications have been reviewed recently [31]. MEPS method development can be performed starting from standard SPE protocols but every application need optimization of sorbent type, as well as of volumes and composition of activation, application, washing and elution solutions [27].

For this study, several types of sorbent material were considered and the best results were obtained with C_8 sorbent as compared to C_{18} and C_4. Also the composition of the elution solution was critical, since the elution solution should be able to displace the targeted analyte from the sorbent with a minimum volume. For the compounds in the present study it was found that methanol/isopropanol/acetonitrile/water 50:30:10:10 (v/v) was an optimal elution solution. Consecutive elutions with the solution yielded no significant analyte peaks, confirming effective desorbtion of the analytes with one elution cycle. Still four washing cycles were needed with the same solution to eliminate carry-over effects.

Carry-over is one of the expected problems when using MEPS. This problem may affect the results by effecting accuracy and precision [27,31]. Many factors may contribute to this problem such as; adsorption properties of the analytes, sensitivity of the method and the performance of the CTC-PAL hardware. This problem can usually be eliminated by choosing suitable washing solutions and optimal number of washing cycles. When using the CTC-PAL autosampler the washing can be done while the previous sample is running. In this method, elimination (<0.02%) of carry-over was achieved with a protocol of four washing cycles.

Using MEPS as sample preparation method may offer some advantages over other published methods for immunosuppressive determination [1,4,6,11,13,14,16,17,34,38]. In comparison with protein precipitation procedure, MEPS offer enrichment of analytes and depletion of non-polar matrix constituents. This may be important for increasing sensitivity and for robustness of the chromatography and mass spectrometry system. A stronger negative peak (ion suppression) at the void (0.45 min) was observed in our reference method than with MEPS and this was also reported for similar PP methods [17,34]. This suggests that cleaner extracted samples can be obtained with MEPS and that matrix related problems are reduced.

In comparison with conventional SPE, the MEPS method can handle a smaller blood volume, which is mainly of value for TDM in small children. Also smaller amount of sorbent is used in MEPS and thus less quantity of solvent needed for elution and washing. Moreover, MEPS is more suitable for on line automation than traditional SPE since it can be easily integrated to a CTC autosampler with only dilution as sample pretreatment. Moreover, one bin can be used for more than 100 times equalling 100 disposable traditional SPE cartridge.

5. Conclusions

The developed method using MEPS in combination with LC–MS/MS provides precise and accurate quantification of CYA, TAC, EVE and SIR in a sample volume of 50 μL of whole blood. The sample preparation procedure is automated, simple and fast. The use of MEPS in an online application can offer a possibility of an

automated analytical solution suitable for routine TDM laboratories with short turn- around-times.

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