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A high-throughput LC-MS/MS method for the quantification of four immunosu- ppressants drugs in whole blood

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ABSTRACT

Background: Immunoassays and liquid chromatography tandem mass spectrometry (LC-MS/MS) are two major methods for therapeutic drug monitoring (TDM) of immunosuppressant drugs. Compared to the relatively limited analytical performance and cross reactivities of immunoassays, the LC-MS/MS method is considered as a gold standard; however, the lack of systematic evaluation and standardization needs to be addressed.
Methods: A LC-MS/MS method for the determination of cyclosporine A, sirolimus, tacrolimus, and everolimus was developed. One-step protein precipitation was used to prepare blood samples. The newly developed method was systematically evaluated and validated according to the standard guidelines.
Results: The quantitative method for four immunosuppressant drugs in human whole blood was validated according to the guidelines. The lower limits of the measuring interval (LLMI) for cyclosporine A, sirolimus, tacrolimus, and everolimus were 5, 0.5, 0.5, and 0.5 ng/mL, respectively. Linear correlation coefficients were all > 0.999. Internal standard-normalized (IS-normalized) matrix correction factor was within the range 0.88–1.17. The average spiked recoveries of five replicates for the four immunosuppressant drugs were in the range 87.4–109.6%.
Conclusion: An LC-MS/MS method combined with one-step protein precipitation was developed, providing short sample preparation and chromatographic run time, thus allowing easy clinical diagnosis.

1. Introduction

Organ transplantations, such as heart, liver, kidney, lung, pancreas, intestinal tract and skin, have saved many lives each year [1]. However, available organs are not enough, and many people still die of rejection or other infections of the transplanted organ in the past. Maintaining organ function after transplantation is one of the most crucial procedures. Immunosuppressants are a class of drugs that suppress or reduce the strength of the body's immune system and play key roles in the prevention of organ rejection in transplant recipients and the treatment of diverse autoimmune disorders [2]. Because the range of drug exposure between lack of efficacy and increased toxicity is small, immunosuppressant drugs are often described as critical dose drugs or narrow therapeutic index (NTI) drugs, exhibiting a very close margin between the therapeutic and toxic blood concentrations [3–6]. Therefore, TDM is necessary to guide therapies and improve patient care. TDM is generally performed once or twice a day prior to the administration of a new dosage [7]. Cyclosporine A (CsA), sirolimus (SIR), tacrolimus (TAC), and everolimus (EVE) are four common immunosuppressant drugs. CsA discovered in 1976 is a cyclic peptide consisting of 11 amino acids isolated from the fungus hypocladium inflatum gams [8], followed by the discovery of SIR in 1977. SIR is a macrocyclic triene antibiotic and was originally isolated from streptomyces hygroscopicus. Its immunosuppressive properties were recognized soon afterwards the study by Martel et al. [9]. TAC discovered in 1987 [10] is a macrocyclic triene extracted from a culture filtrate of streptomyces tsukubaensis. TAC and CsA are both calcineurin inhibitors; however, the immunosuppressive activity of TAC is 100 times than that of CsA. The reason is the different bonding proteins for CsA and TAC. CsA binds to the immunophilin cyclophilin, whereas tacrolimus binds to the immunophilin FKBP12. EVE is a derivative of SIR; therefore, it is also known as 40-o-(2-hydroxyethyl)-sirolimus. EVE was initially approved for the prevention of transplanted organ rejection by the US Food and Drug Administration (FDA) in 2009 [2].

Currently, immunosuppressant drugs are monitored mainly by analytical methods such as immunoassays based on fluorescence or

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colorimetric detection and LC-MS/MS [11–15]. Immunoassays have the advantages of being fast, requiring less sample volumes, and lower costs for instrumentation and technical support compared to LC-MS/MS. Disadvantages are the limited analytical performance and cross reactivities [14,16]. For example, EVE and SIR only differ slightly in structure, and the similarity immunoassays for these two analytes are often plagued by high cross-reactivity [13]. The detection accuracy is also greatly affected by the matrix effect from autoantibodies and/or heterophile antibodies [17]. Most clinical laboratories use LC-MS/MS methods as a gold standard for the TDM of immunosuppressant drugs owing their selectivity, sensitivity, and flexibility [18,19]. Using a MS/MS as detector allows the achievement of very low limits of detection and quantification. LC–MS/MS also provides a high specificity [7].

The aim of this study was to develop an efficient and rapid LC-MS/MS method for the determination of CsA, SIR, TAC, and EVE. The blood samples were prepared by one-step protein precipitation. This sample preparation method is easy and has high throughput, making the clinical diagnosis possible. Systematic and multiple parameters evaluation were validated to access the method strictly according to the guidelines.

Guidelines mainly referred to C62-A [18], CLSI document EP32-R [19], CLSI document NBS04 [20] and CLSI document EP05 [21].

2. Experimental

2.1. Chemicals and material

All the solvents and reagents were at least LC/MS grade. Ultrapure water (18.2 MΩ CM resistivity) obtained from a Millipore water purification device (Millipore, USA). Methanol, acetonitrile and formic acid were obtained from Fisher Scientific (Waltham, MA, USA). Ammonium acetate and Zinc sulfate heptahydrate ($ZnSO_4●7H_2O$) were obtained from Sigma-Alorich (Shanghai, China). Cyclosporine A, sirolimus, tacrolimus, everolimus, ascomycin (Asc), and stable isotope-labeled internal standards (IS) were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Drug-free healthy whole blood was obtained from volunteers of Physical Examination Center. Quality control samples were home-made in our lab.

2.2. Preparation of stock and working solutions

Stock solutions of CsA, CsA-d_4, SIR, SIR-d_3, TAC, Asc, EVE, and EVE-d_4 were prepared in methanol at the concentrations of 500, 10, 10, 10, 1, 1, 1, and 1 μg/mL, respectively. The working solutions of CsA and SIR were mixed together at the concentrations of 50, 100, 500, 1000, 5000, 10,000, and 12,500 ng/mL for CsA and 5, 10, 50, 100, 500, 1000, and 1250 ng/mL for SIR. The working solutions were at the concentrations of 5, 10, 20, 40, 100, 200 and 400 ng/mL for TAC and 5, 10, 20, 50, 100, 200 and 500 ng/mL for EVE. CsA-d_4 and SIR-d_3 were mixed together and diluted with methanol to get the working solution of IS (500 ng/mL for CsA-d_4 and 500 ng/mL for SIR-d_3). Asc was diluted with methanol to get 100 ng/mL working solution of IS. EVE-d_4 was diluted with methanol to get 200 ng/mL working solution of IS. All the stock and working solutions were stored at −20 °C before use. Under this condition, the immunosuppressants in stock and working solutions were stable for at least one year.

2.3. Preparation of calibration and QC samples

10 μL of working solutions were added into 90 μL of EDTA-treated whole blood, followed by adding 100 μL of 100 mmol/L $ZnSO_4$. After 10 s vortex-mixing and 5 min storing at room temperature, erythrocyte hemolysis was obtained. 310 μL of acetonitrile (containing 10 μL IS working solutions) was added to precipitate the proteins. After 1 min, the sample was vortexed and centrifuged for 5 min at 15000 r/min. 10 μL of the supernatant was injected into LC-MS/MS for analysis. Series of calibration samples at 5, 10, 50, 100, 500, 1000, and 1250 ng/mL for CsA, 0.5, 1, 5, 10, 50, 100, and 125 ng/mL for SIR, 0.5, 1, 2, 4, 10, 20, and 40 ng/mL for TAC, and 0.5, 1, 2, 5, 10, 20 and 50 ng/mL for EVE were prepared.

QC samples were prepared in-house at low, medium, and high concentration levels, and defined as 110% of the LLMI, 90% of the upper limit of the measuring interval (ULMI), and (LLMI + ULMI)/2.

2.4. Instrumentation

LC-20AXR (Shimadzu, Japan) tandem AB SCIEX API 4000 (AB Applied Biosystems, USA) was used. The data was acquired and processed using Analyst software (AB SCIEX, version 1.5) and Origin 85.

2.5. LC and MS conditions

An Inertsil C18 column (Shimadzu, 4.6 × 100 mm, 5 μm particles) was used for separation by using water phase (2 mmol/L ammonium acetate and 0.1% fromic acid, solvent A) and methanol phase (2 mmol/L ammonium acetate and 0.1% fromic acid, solvent B) as the mobile phase under gradient elution as follows: initial, 60% A; 0.01–0.4 min, 60% A; 0.4–2.0 min, 60%–0%A; 2.0–5.0 min, 0% A; 5–5.5 min, 0–60% A; 5.5–6.0 min, 60% A; 6.0–7.0 (1.0 min for equilibration), 60% A. The flow rate was 1 mL/min with an injection volume of 10 μL. The column oven was set to 60 °C.

The electrospray positive electrospray ionization mode was used for ionization. The temperature and ion spray voltage were 300 °C and 5500 V, respectively. The curtain gas, ion source gas 1, and ion source gas 2 were at 37 psi, 60 psi, and 15 psi, respectively. Amonium adducts ions $[M + NH_4]^+$ ions were detected for quantification. For each analyte, two pairs of selected-reaction monitoring (SRM) were selected, one for qualifier and the other for quantifier. For each internal standard, only a quantifier SRM is needed. High purity nitrogen was used as the sheath gas, auxiliary gas, and collision gas. Parameters dwell time, declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were optimized. The optimized results are listed in Table 1.

2.6. Quantification algorithm

The quantification module of Analyst 1.5.2 was used to generate the quantification method, including peak detection, peak integration, and analyte quantification. Within the Analyst Classic Integration Algorithm, the smoothing factor and the bunching factor were set to 3 and 1, respectively. Calibration function generation: plot the peak area ratio of an analyte and its respective internal standard against the corresponding analyte standard concentration. A weighted least-squares method was used with a weighting factor of $1/x^2$.

Table 1
SRM conditions and optimized mass parameters for the monitored immunosuppressant drugs.

Immunosuppressant	Parent ion (*m/z*)	Product ion (m/z)	SRM type	Dwell Time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
CsA	1219.9	1202.8	Quantifier	100	53	11	30	19
	1219.9	1184.6	Qualifier	100	53	11	45	18
CsA -d_4	1223.9	1206.8		100	46	11	31	20
SIR	931.6	864.5	Quantifier	100	50	11	25	13
	931.6	882.3	Qualifier	100	50	11	18	15
SIR -d_3	934.6	864.5		100	50	11	25	13
TAC	821.5	768.6	Quantifier	100	76	9	30	11
	821.5	576.1	Qualifier	100	78	9	34	17
Asc	824.5	771.6		100	75	11	30	24
EVE	975.6	908.5	Quantifier	100	78	8	25	14
	975.6	926.4	Qualifier	100	80	8	20	32
EVE-d_4	979.6	912.5		100	64	10	25	35

2.7. Method validation

LLMI, selectivity, linearity, carry-over effect, matrix effect, precision and recovery were validated in this study.

2.7.1. LLMI, selectivity and linearity

The LLMI is the lowest actual amount of an analyte that can be reliably detected and meets the laboratory's requirements for accuracy and precision. LLMI of each analyte was determined by analyzing 11 replicates from four different concentration level samples close to the predetermined limit of detection (LoD). The LLMI should meet an acceptable inaccuracy within ± 15% and imprecision within ± 20% according to the guidelines.

To evaluate the selectivity, LLMI, and double blank (DB) blood samples were used. Quantitation of a DB sample reflects the background in the whole testing system including sample processing. According to the guidelines, the selectivity was acceptable when the area of the background peaks was < 20% of the peak area for the analyte at the LLMI.

As for a quantitative measuring procedure, linearity experiment is an essential component of testing and confirming the detection range of the measurement procedure. The calibration standards should be prepared in the same biological matrix as the samples. In this experiment, nine points were analyzed with three replicates each, and the linear range was evaluated by the polynomial regression method. Linear correlation coefficient should be larger than 0.995 for accurate quantification.

2.7.2. Carry-over effect and matrix effect

The carry-over effect was evaluated in this experiment and operated as follows: The blank sample was injected immediately following a high concentration of the calibration sample. At the elution time of analytes and IS, the peak area of the blank sample should be < 20% of the peak area of the LLMI sample for analytes.

Matrix effect, which is independent of the presence of the analyte, influences the accurate quantification for analyte. Matrix correction factor (MF) is defined as the ratio of the intensity of the extracted blank matrix added the analytes to the intensity of the analytes in pure solution (methanol in this experiment). The IS-normalized MF was used to evaluate the matrix effect and is defined the ratio of $\mathrm{MF}_{\mathrm{analyte}}$ to $\mathrm{MF}_{\mathrm{IS}}$. The formula can be expressed as follows:

$$\mathrm{IS-normalized\ MF} = \mathrm{MF}_{\mathrm{analyte}}/\mathrm{MF}_{\mathrm{IS}}$$

where $\mathrm{MF}_{\mathrm{analyte}}$ and $\mathrm{MF}_{\mathrm{IS}}$ are the MF of the analyte and internal standard, respectively. IS-normalized MF should be within the range 0.8–1.2, and the CV of the IS-normalized MF should not exceed 15%.

2.7.3. Recovery

The reliability of measurements was assessed by the recovery of the known standards into the blank human whole EDTA blood. Accuracy was determined by the means of average recoveries for five replicates of drug-free whole blood by adding three levels of standard concentration (low, medium, and high). The inaccuracy should not exceed 15% CV except for the low concentration samples, where ≤20% CV was acceptable.

2.7.4. Intra-day and inter-day precision

The evaluation of precision should include the entire testing process, including sample collection and storage, pretreatment, extraction, and analysis. To evaluate the intra-day and inter-day precision, five replicates of the in-house prepared QC samples at three levels of concentration (110% LLMI, 90% ULMI, and (LLMI + ULMI)/2) were analyzed on the same day and three consecutive days. For the QC samples, the precision of each concentration should not exceed 15% CV except for the LLMI, where ≤20% CV is acceptable.

Table 2
The concentration and peak areas of LLMI, peak areas of DB and DB/LLMI for CsA, SIR, TAC and EVE.

Immunosuppressant	LLMI		Peak areas of DB	DB/LLMI
	Concentration (ng/mL)	Peak areas (counts)		
CsA	5	6082.9	69.4	1.14%
SIR	0.5	463.3	78.8	17.0%
TAC	0.5	115.7	3.31	2.86%
EVE	0.5	882.5	37.8	4.28%

3. Results and discussion

3.1. LLMI, selectivity and linearity

The LLMI of CsA, SIR, TAC, and EVE measured were 5, 0.5, 0.5, and 0.5 ng/mL, respectively. The inaccuracy was from −9.0% to +11.0%, and the imprecision was < 3.95%, 4.31%, 14.4%, and 10.5% for CsA, SIR, TAC, and EVE, respectively and were all acceptable according to the guidelines that the inaccuracy should be within ± 15% and the imprecision should be < 20%. The ratio of the background peak area to the analyte peak area at the LLMI (DB/LLMI) was 1.14%, 17.0%, 2.86%, and 4.28%, respectively. The results are listed in Table 2.

The ion chromatograms and calibration curves of CsA, SIR, TAC, and EVE are shown in Fig. 1. The typical linear regression equation is $y = 0.05057\,x + 0.00468$ (x, CsA concentration; y, peak area ratio of CsA to CsA-d_4, $r^2 = 0.9993$) for CsA, $y = 0.05604\,x + 0.03571$ (x, SIR concentration; y, peak area ratio of SIR to SIR-d_3, $r^2 = 0.9998$) for SIR, $y = 0.01784\times + 0.00701$ (x, TAC concentration; y, peak area ratio of TAC to Asc, $r^2 = 0.9992$) for TAC, and $y = 0.02788\times + 0.00465$ (x, EVE concentration; y, peak area ratio of EVE to EVE-d_4, $r^2 = 0.9996$) for EVE.

3.2. Carry-over effect and matrix effect

The carry-over effect for the present method was < 0.52%, 17.2%, 3.0%, and 4.28% for CsA, SIR, TAC, and EVE, respectively (the raw data are not listed in the article), which was acceptable in accordance with the requirements of the guidelines (< 20%).

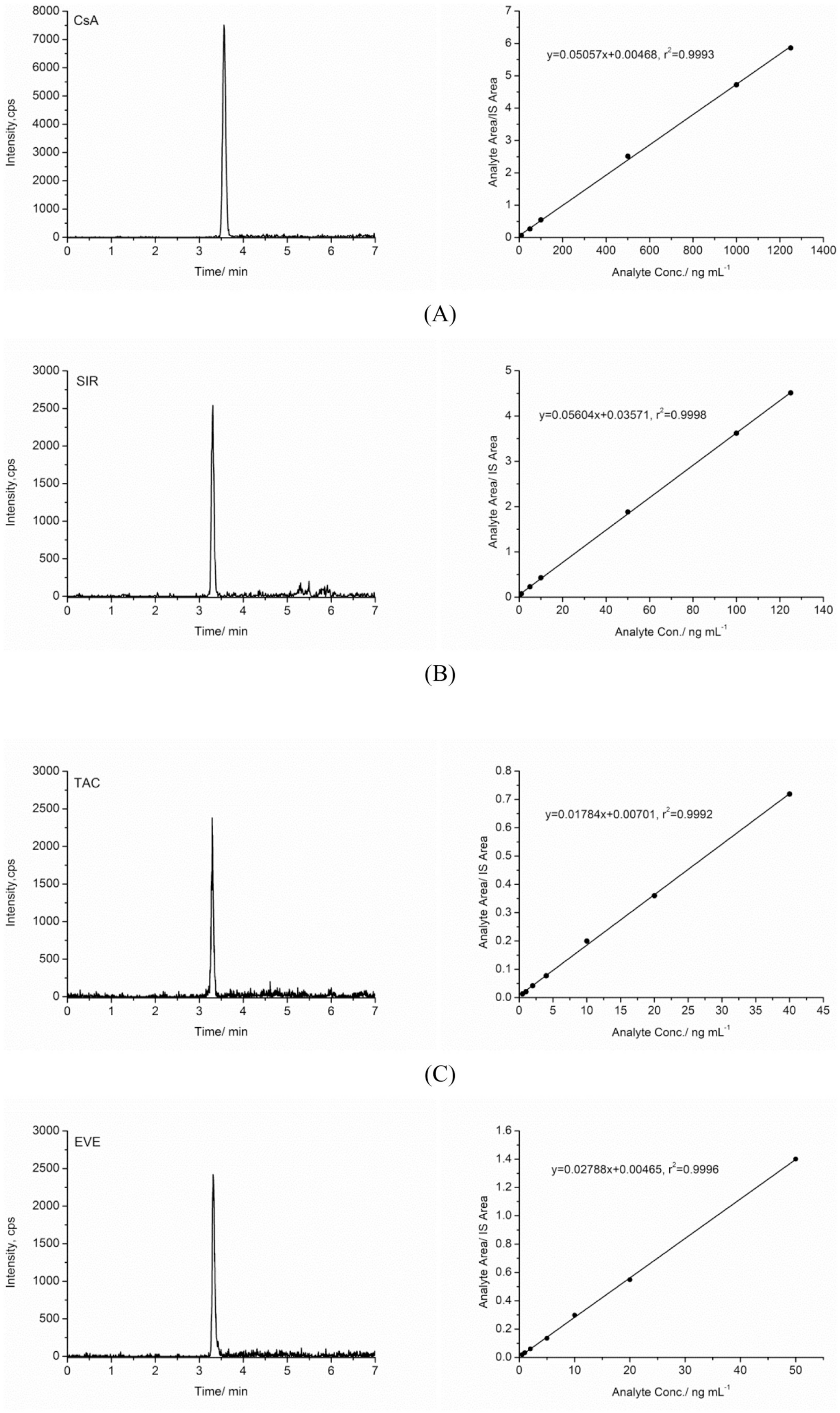
Three concentration levels were analyzed to evaluate the IS-normalized MF. Each level was determined by analyzing five replicates. The results are listed in Table 3. Both IS-normalized MF and CV were acceptable.

3.3. Recovery

Blank human whole blood was spiked with the immunosuppressant stock or standard solutions to yield three concentration levels (low, medium, and high) for CsA (10, 100, and 500 ng/mL), SIR (2, 20, and 100 ng/mL), TAC (0.5, 4, and 20 mg/mL) and EVE (0.5, 5, and 20 ng/mL), respectively. The inaccuracy of each concentration was < 15% CV, which was acceptable according to the guidelines. The average spiked recovery of five replicates was in the range of 87.4–109.6%. The experimental results are listed in Table 4, exhibiting good accuracy of the method.

3.4. Intra-day and inter-day precision

For the four immunosuppressant drugs, the precision of Intra-day and inter-day were in the range of 4.92%–18.1% for the 110% LLMI, 0.61%–5.78% for the 90% ULMI and (LLMI + ULMI)/2. The validation results for precision were all well within the maximum tolerated CV (20% for the LLMI, 15% for the other concentrations) according to the guidelines. The results are listed in Table 5.



(A)

(B)

(C)

(D)

Fig. 1. Representative ion chromatograms (A-D) of whole blood samples containing CsA, SIR, TAC and EVE (left) and the calibration curves for each immunosuppressant (right) are presented.

Table 3
MF and IS- normalized MF of CsA, SIR, TAC and EVE.

Analyte		$MF_{analyte}$	MF_{IS}	IS-normalized MF	CV
CsA	Level I	0.82	0.77	1.07	2.83%
		0.78	0.78	1.01	
		0.89	0.85	1.05	
		0.78	0.80	0.98	
		0.81	0.89	0.92	
	Level II	0.86	0.74	1.16	3.15%
		0.89	0.85	1.05	
		0.82	0.78	1.06	
		0.88	0.90	0.98	
		0.91	0.83	1.10	
	Level III	0.82	0.76	1.08	4.55%
		0.79	0.78	1.01	
		0.88	0.88	1.00	
		0.87	0.89	0.99	
		0.88	0.82	1.07	
SIR	Level I	0.82	0.77	1.07	5.91%
		0.78	0.78	1.01	
		0.89	0.85	1.05	
		0.78	0.80	0.98	
		0.81	0.89	0.92	
	Level II	0.86	0.74	1.16	6.20%
		0.89	0.85	1.05	
		0.82	0.78	1.06	
		0.88	0.90	0.98	
		0.91	0.83	1.10	
	Level III	0.82	0.76	1.08	4.06%
		0.79	0.78	1.01	
		0.88	0.88	1.00	
		0.87	0.89	0.99	
		0.88	0.82	1.07	
TAC	Level I	1.77	1.78	1.00	
		1.89	1.95	0.97	1.32%
		1.77	1.76	1.00	
		1.62	1.64	0.99	
		1.64	1.67	0.98	
	Level II	2.18	2.23	0.98	7.50%
		2.44	2.45	1.00	
		2.42	2.42	1.00	
		2.01	1.87	1.07	
		2.60	2.23	1.17	
	Level III	2.30	2.23	1.03	2.79%
		1.95	1.99	0.98	
		2.41	2.29	1.05	
		2.43	2.30	1.05	
		2.20	2.12	1.03	
EVE	Level I	1.17	1.20	0.98	3.98%
		1.10	1.11	0.99	
		1.21	1.17	1.03	
		1.21	1.18	1.02	
		1.13	1.22	0.93	
	Level II	0.96	1.07	0.90	4.17%
		1.10	1.16	0.95	
		1.10	1.13	0.97	
		1.07	1.21	0.88	
		1.09	1.17	0.93	
	Level III	1.16	1.21	0.96	3.02%
		1.14	1.24	0.92	
		1.11	1.23	0.90	
		1.13	1.25	0.90	
		1.09	1.15	0.95	

4. Conclusion

A straightforward LC-MS/MS method combined with one-step protein precipitation is described herein to detect four immunosuppressant drugs. The method provided the short sample preparation and chromatographic run time, making the clinical diagnosis possible.

Although LC-MS/MS is described as a golden method for the TDM of immunosuppressant drugs, there are still many problems to be solved before really going into clinical practice. First, there is a need for a high throughput method that could meet the needs of clinical standard. Second, sample pretreatment needs to be improved. Nowadays, liquid–liquid extraction after protein precipitation and solid-phase extraction are two common methods. Liquid–liquid extraction consumes a large amount of reagents and has an impact on the environment, whereas the extraction column of solid-phase extraction is relatively expensive. Third, the storage and stability of the sample is a challenge. Dried blood spot (DBS) is a good method, providing more convenient and frequent monitoring; however, DBS puts forward higher requirements for sample pretreatment and lower LLMI for the instrument. Fourth, from a clinical point of view, because of the lack of internationally recognized reference materials and methods, current LC–MS/MS method used to monitor ISD concentrations in whole blood is still not standardized.

Table 4
The added amount and recovery of CsA, SIR, TAC and EVE.

Analyte	Added ammount (ng/mL)	Determination (average ± SD) (ng/mL)	Average spiked recovery
CsA	10	10.96 ± 0.24	109.6%
	100	108.4 ± 3.36	108.4%
	500	525.6 ± 15.2	105.1%
SIR	2	1.92 ± 0.30	96.0%
	20	21.1 ± 1.32	105.5%
	100	109.2 ± 3.77	109.2%
TAC	0.5	0.46 ± 0.05	92.1%
	4	3.62 ± 0.32	90.5%
	20	17.5 ± 1.37	87.4%
EVE	0.5	0.46 ± 0.04	92.8%
	5	4.91 ± 0.25	98.2%
	20	19.56 ± 0.53	98.0%

Table 5
Intra-day and inter-day precision for CsA, SIR, TAC and EVE.

Analyte	Precision	CV% (110% LLMI)	CV% (LLMI + ULMI)/2)	CV% (90%ULMI)
CsA	Intra-day	4.92%	4.94%	2.23%
	Inter-day	15.3%	4.60%	3.93%
SIR	Intra-day	10.9%	5.78%	5.21%
	Inter-day	18.1%	5.41%	4.00%
TAC	Intra-day	9.34%	1.95%	4.83%
	Inter-day	7.10%	0.61%	3.57%
EVE	Intra-day	12.8%	2.91%	1.35%
	Inter-day	15.8%	1.48%	3.66%

Declaration of interest

None.

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References

[1] R.A. Koster, E.C. Dijkers, D.R. Uges, Robust, high-throughput LC-MS/MS method for therapeutic Drug Monitoring of cyclosporin A, tacrolimus, everolimus and sirolimus in whole blood, Ther. Drug Monit. 31 (2009) 116–125.

[2] Shery Jacob, A.B. Nair, A review on therapeutic drug monitoring of the mTOR class of immunosuppressants: everolimus and sirolimus, Drugs & Ther. Perspectiv. 33 (6) (2017) 290–301.

[3] A. Johnston, Equivalence and interchangeability of narrow therapeutic index drugs in organ transplantation, Eur. J. of Hospital Pharm. Sci. and Pract. 20 (5) (2013) 302–307.

[4] J.E. Ericson, K.O. Zimmerman, D. Gonzalez, C. Melloni, J.T. Guptill, K.D. Hill, H. Wu, M. Cohen-Wolkowiez, A systematic literature review approach to estimate the therapeutic index of selected immunosuppressant drugs after renal transplantation, Ther. Drug Monit. 39 (1) (2017) 13–20.

[5] David H. Adams, Alberto Sanchez-Fueyo, D. Samuel, From immunosuppression to tolerance, J. Hepatol. 62 (1) (2015) S170–S185 Suppl.

[6] M. Gharagozloo, S. Majewski, M. Foldvari, Therapeutic applications of nanomedicine in autoimmune diseases: from immunosuppression to tolerance induction, Nanomedicine 11 (4) (2015) 1003–1018.

[7] Y. Zhang, R. Zhang, Recent advances in analytical methods for the therapeutic drug monitoring of immunosuppressive drugs, Drug testing and analysis 10 (1) (2018) 81–94.

[8] C.F.J.F. Borel, H.U. Gubler, H. Stähelin, Biological effects of cyclosporin A-a new anti-lymphocytic agent, Agents Actions 6 (4) (1976) 468–475.

[9] J.K.R.R. Martel, S. Galet, Inhibition of the immune response by rapamycin, new antifungal antibiotic, Can. J. Physiol. Pharmacol. 55 (1977) 48–51.

[10] T. Kino, H. Hatanaka, M. Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, H. Imanaka, FK-506, a novel immunosuppressant isolated from a Streptomyces. II, J. Antibiot. 40 (1987) 1256–1265.

[11] M. Andreas Meinitzer, Gabriele Gartner, M.D. Stefan Pilz, M.D. Mariana Stettin, Ultra fast liquid chromatography–tandem mass spectrometry routine method for simultaneous determination of Cyclosporin a, tacrolimus, Sirolimus, and Everolimus in whole blood using deuterated internal standards for Cyclosporin a and Everolimus, Ther. Drug Monit. 32 (2010) 61–66.

[12] A. Veringa, M.G.G. Sturkenboom, B.G.J. Dekkers, R.A. Koster, J.A. Roberts, C.A. Peloquin, D.J. Touw, J.-W.C. Alffenaar, LC-MS/MS for therapeutic drug monitoring of anti-infective drugs, TrAC Trends Anal. Chem. 84 (2016) 34–40.

[13] A.J. McShane, D.R. Bunch, S. Wang, Therapeutic drug monitoring of immunosuppressants by liquid chromatography-mass spectrometry, Clinica chimica acta; international journal of clinical chemistry 454 (2016) 1–5.

[14] K. Freudenberger, U. Hilbig, G. Gauglitz, Recent advances in therapeutic drug monitoring of immunosuppressive drugs, TrAC Trends Anal. Chem. 79 (2016) 257–268.

[15] R. Rigo-Bonnin, A. Arbiol-Roca, J.M.G. de Aledo-Castillo, P. Alía, Simultaneous measurement of cyclosporine a, Everolimus, Sirolimus and tacrolimus concentrations in human blood by UPLC–MS/MS, Chromatographia 78 (23–24) (2015) 1459–1474.

[16] F. Saint-Marcoux, F.L. Sauvage, P. Marquet, Current role of LC-MS in therapeutic drug monitoring, Anal. Bioanal. Chem. 388 (7) (2007) 1327–1349.

[17] E.K. Leung, X. Yi, C. Gloria, K.T. Yeo, Clinical evaluation of the QMS(R) tacrolimus immunoassay, Clinica Chimica Acta; Int. J. Clin. Chem. 431 (2014) 270–275.

[18] S. Mei, J. Wang, D. Chen, L. Zhu, M. Zhao, X. Tian, X. Hu, Z. Zhao, Simultaneous determination of cyclosporine and tacrolimus in human whole blood by ultra-high performance liquid chromatography tandem mass spectrometry and comparison with a chemiluminescence microparticle immunoassay, J. Chromatogr. B 1087-1088 (2018) 36–42.

[19] C. Seger, K. Tentschert, W. Stoggl, A. Griesmacher, S.L. Ramsay, A rapid HPLC-MS/MS method for the simultaneous quantification of cyclosporine a, tacrolimus, sirolimus and everolimus in human blood samples, Nat. Protoc. 4 (4) (2009) 526–534.

[20] CLSI, Liquid Chromatography-Mass Spectrometry Methods; Approved Guideline. CLSI Document C62-A, Clinical and Laboratory Standards Institute, Wayne, PA, 2014.

[21] CLSI, Metrological Traceability and its Implementation; a Report. CLSI Document EP32-R, Clinial and Laboratory Standards Institute, Wayne, PA, 2006.