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ORIGINAL ARTICLE

Development and Validation of a U-HPLC-MS/MS Method for the Concurrent Measurement of four Immunosuppressants in Whole Blood

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SUMMARY

Background: **This study aimed to develop and validate a U-HPLC-MS/MS method for simultaneous determination of four immunosuppressants in human whole blood.**
Methods: **The method was based on the injection of 20 µL of calibrators and controls pretreated with the liquid phase extraction method for chromatography separation and mass spectrometry determination. LPE offline was performed by adding 0.1 mol/L $ZnSO_4$ and acetonitrile, while separation of target compounds was achieved within 2.5 minutes by a Zorbax Eclipse XDB-C8 column using ammonium acetate and ACN mixed with formic acid as solvents.**
Results: **The assay offers ng/mL detection limits (from 1.1 to 12.4 ng/mL), accuracy (% deviation from -4.4% to 5.6%), precision (CV less than 15% at all QC levels), and linearity (from 23.4 to 948 ng/mL for CsA, from 2.11 to 45.5 ng/mL for TAC, SIR and EVR). The recovery and matrix results were acceptable, and the carryover was less than 1%. The results of method comparison show that IA-based methods overestimated the concentration of drugs compared with the MS-based method. Comparing our MS-based method with external LC-MS/MS showed that the results were within 2 SDs.**
Conclusions: **We have developed a reliable assay for the analysis of CsA, TAC, SIR and EVR in whole blood using U-HPLC-MS/MS.**
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INTRODUCTION

Immunosuppressive drugs (ISDs) are used for various conditions, such as organ transplantation [1-3]. Since ISDs share complementary mechanisms of action with each other, treatment regimens in clinical practice are often composed of combinations of the four categories of ISDs: calcineurin inhibitors, mammalian target of rapamycin (mTOR) inhibitors, anti-metabolites and antibody-based agents [4].

Among ISDs, cyclosporine A (CsA), tacrolimus (TAC), sirolimus (SIR) and everolimus (EVR) are four important and commonly used agents. CsA is a metabolite of *Tolypocladium inflatum* and a calcineurin inhibitor [5]. TAC, another calcineurin inhibitor, is a macrolide antibiotic from *Streptomyces tsukubaensis* [6]. SIR is a macrocyclic fermentation product of *S. hygroscopicus* and a mTOR inhibitor [7]. EVR is a derivative of SIR [8].
ISDs have a narrow therapeutic range, and therefore, therapeutic drug monitoring (TDM) is crucial to prevent transplant rejection or side effects including the numerous renal, hepatic, immunological and neurological complications due to inadequate dosage [9,10]. In addition, due to the increasing number of transplant patients and the usage of combined drugs, simultaneous determination of ISDs is required for routine TDM.
For TDM of ISDs, immunoassays have been used traditionally. However, cross-reactions with some metabolites of these drugs and the resulting overestimation of the concentrations were major problems [11]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) using stable-isotope-labeled (SIL) internal standards (ISs) is being generally accepted as the technique of choice due to its improved specificity and sensitivity. In addition, a multiplexed approach in LC-MS/MS technique enabled simultaneous measurements of several ISDs, resulting in increased operational efficiency and cost reduction [12]. Ultra-high performance LC-MS/MS (U-HPLC-MS/MS) increased throughput while maintaining resolution and decreasing analysis time by operating at higher pressures and allows for lower particle sizes. Although it has existed in the laboratory for many years, this method is not standardized and is being used as a laboratory-developed test (LDT) [13]. In this study, we developed and validated a U-HPLC-MS/MS method for the simultaneous determination of four commonly prescribed ISDs, CsA, TAC, SIR, and EVR, in human whole blood for the purpose of TDM.

MATERIALS AND METHODS

Materials

LC-MS grade methanol and HPLC grade acetonitrile (ACN) were purchased from Merck KGaA (Darmstadt, Germany). Formic acid, analytical grade ammonium acetate and zinc sulfate heptahydrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Water was prepared using a Milli-Q ultrapure water system (> 18.2 megohm-cm resistivity) (Millipore, Milford, MA, USA). CsA-d4, TAC-d3, SIR-d3, and EVR-d4 standards were purchased from Chromsystems (Munich, Germany). Calibration standards were also purchased from Chromsystems (6PLUS1® Multilevel Calibrator Set, Chromsystems, Munich, Germany) at the following concentrations: 23.4, 128, 294, 471, 745, and 948 ng/mL for CsA; 2.25, 6.05, 12.1, 17.7, 24.3, and 42.9 ng/mL for TAC; 2.11, 5.97, 12.1, 18.4, 27.5, and 45.5 ng/mL for SIR; and 2.32, 6.16, 12.4, 18.4, 25.5, and 44.8 ng/mL for EVR. Five-level quality control (QC) samples were purchased from RECIPE (ClinChek® Controls, RECIPE, Munich, Germany). This study was approved by the Ethics Committee of Anam Hospital, Korea University, South Korea.

Sample preparation

Upon receipt, internal standards were stored at -18°C until use, undergoing only one freeze-thaw cycle with 2.5 mL of reconstitution buffer and storing at room temperature for 5 minutes as recommended by the manufacturer.
Calibrators and controls (50 µL) were added into 100 µL of 0.1 mol/L $ZnSO_4$ solution with 25 µL internal standards in a 1.5 mL silanized conical test tube (Sigma-Aldrich, St. Louis, MO, USA) for erythrocyte hemolysis. The sample was vortexed for 15 seconds and then stored for 2 minutes at room temperature. Next, 250 µL of ACN was added to precipitate the proteins; the samples were vortexed for 15 seconds and then clarified by centrifugation (15,000 g) for 5 minutes. Next, 100 µL of the supernatant was placed into the autosampler and injected in 20 µL volumes per run for analyte quantification.

Analyte separation and quantification

The U-HPLC system consisted of a Shimadzu Nexera X2 Ultra Performance Liquid Chromatograph (Shimadzu, Kyoto, Japan) with a high-pressure binary pump, autosampler, column oven, and a flow-through needle for injections. Chromatography was performed using a Zorbax Eclipse XDB-C8 column (50 mm × 2.1 mm, 3.5 µm particle size) (Agilent, CA, USA), which was maintained at 60°C. The two mobile phases consisted of solvent A (2 mmol/L ammonium acetate in Milli-Q filtered water with 0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile). The flow rate was 0.65 mL/minute.
Solvent B gradient conditions were as follows: initially set to 50% for 0.20 minutes, linear gradient from 50% to 95% for 0.45 minutes, sustained at 95% for 0.80 minutes, lowered linearly to 50% over 0.1 minute, and continued at 50% for 1.0 minute, for a total run time of 2.5 minutes.
The syringe and flow through needle were cleaned between runs using a strong and a weak wash (1:1:1:1 Milli-Q filtered water/MeOH/IPA/ACN v/v/v/v and 9:1 Milli-Q filtered water:ACN v/v, respectively).
MS analysis was performed in the positive electrospray ionization mode (ESI) and collision-induced dissociation (CID) MS/MS on a SCIEX TRIPLE QUAD 4500 mass spectrometer (AB SCIEX, Toronto, Canada). Precursor-product ion transitions, retention time, cone voltage, and collision energy were optimized for each analyte and IS (Table 1).
The following flow-dependent parameters were used: desolvation temperature, 550°C; desolvation gas flow, 1,000 L/hour; capillary voltage, 3.5 kV; source tempera-

ture, 150 °C; cone gas flow, 0 L/hour; extractor, 3.0 V; and RF lens, 2.5 V. Drug levels were quantified from the most abundant and reliable product ion for each analyte using MultiQuant MD 3.0.2 (Ab Sciex, Framingham, MA, USA)

Method validation

The method was validated according to Bioanalytical Method Validation Guidance for Industry (FDA, Docket number FDA-2013-D-1020, 2018) and partially validated with method modification.

Lower Limit of Quantification (LLOQ)

The LLOQ was established by meeting the coefficient of variation (CV) ≤ 20% and an inaccuracy of ± 20% during the validation process.

Inaccuracy

Accuracy was investigated by running commercial calibration materials (6PLUS1® Multilevel Calibrator Set, Chromsystems, Munich, Germany) at six levels in pentaplicate measurements. Accuracies were defined as % deviation from the nominal value and the acceptance criteria for accuracy were ±15% (±20% for LLOQ-level).

Imprecision

Precision was investigated by running quality control samples (ClinChek® Controls, RECIPE, Munich, Germany) at five concentration levels for each analyte assessing intra-day (n = 4) and inter-day characteristics (five independent runs, total n = 20). Imprecision was expressed as CV%, and the within-run and between-run CV values should not exceed 15% (20% for the LLOQ samples).

Linearity

Six-level analyte calibrators were assayed at the beginning and end of each experimental run, and calibration curves were created using a $1/x^2$-weighted linear regression, without inclusion of origin. Back-calculated values were not allowed to deviate more than ± 15%, except for LLOQ where a ± 20% criterion was applied. In addition, 75% and a minimum of six non-zero calibrator levels met the above criteria in each validation run.

Ion suppression/enhancement

The matrix effects and recovery efficiency were assessed by post-extraction addition set-up, infusing analytes simultaneously as injection of a pooled blank matrix sample over the column. Four QC levels for CsA, TAC, and SIR and three QC levels for EVR were prepared by spiking each analyte into six specimens of drug-free pooled human serum before and after protein precipitation and comparing to neat specimens, in which drugs were spiked into 58.8% ACN/H_2O (v/v) prior to protein precipitation. The four levels of concentration were as follows: 20, 50, 100, and 500 ng/mL for CsA; 1, 2.5, 5, and 30 ng/mL for TAC; 2.5, 5, 10, and 20 ng/mL for SIR; 2.5, 5, and 10 ng/mL for EVR.

Percent results were determined by comparing the ratio of analyte to IS raw peak areas between the following groups: ME (matrix factor), post-precipitated spiked serum to spiked ACN/H_2O; RE (recovery), pre-precipitated spiked serum to post-precipitated spiked serum; and PE, pre-precipitated spiked serum to spiked ACN/ H_2O. Extraction recovery with CV% for six matrix lots should be below 15%.

For CsA and TAC, the qualitative matrix effect was evaluated additionally from the post-column infusion experiment to see if there was any ion enhancement or suppression from endogenous substances for the analytes. At the retention times of the analytes, the analyte signals should not be exceeded or decreased more than ± 25%.

Carryover

Carryover was evaluated by injecting high and low analyte samples in the following order: H1, H2, H3, H4, L1, L2, L3, L4. The potential increase in the low sample values were calculated using equation: carryover (%) = [L1- (L3+L4)/2] × 100/[(H2+H3)/2-(L3+L4)/2].

Method Comparison

We obtained 298 patient samples for CsA, 884 patient samples for TAC, 40 patient samples for SIR, and 22 patient samples for EVR from Korea University Anam Hospital. CsA and TAC samples were analyzed by a cloned enzyme donor immunoassay (CEDIA) Cyclosporine Immunoassay (Boehringer Mannheim Ltd., CA, USA) and a chemiluminescent microparticle immunoassay (CMIA) Tacrolimus Immunoassay Test (Abbott, IL, USA), respectively. Since the LLOQ of the CsA CEDIA assay was 40 ng/mL, samples below this limit by immunoassay were excluded. SIR and EVR were measured by LC-MS/MS at Green Cross Laboratories (Yongin, South Korea).

Comparison between methodologies was accomplished using Passing-Bablock model and Pearson's test using Analyse-it (Analyse-it Software, Ltd., Leeds, UK) by plotting results from U-HPLC/MS/MS versus results from the immunoassay or other U-HPLC/MS/MS. The differences between methods were assessed using the Bland-Altman regression, which plots the relative difference between two methods versus the mean concentration. The mean values of the two measurements were evaluated by Wilcoxon test considering data distribution.

Statistical analysis

Analyse-it and Medcalc (MedCalc Software, Mariakerke, Belgium) software were used for statistical analysis. A p-value < 0.05 was considered statistically significant.

Table 1. Monitored transitions, collision energies and retention times of the analyzed compounds.

	MRM transition	Collision energy (volts)	Retention time (minutes)
CsA	1,219.600 → 1,202.800	29.000	1.43
TAC	821.300 → 768.300	30.000	1.25
SIR	931.400 → 864.400	25.000	1.28
EVR	975.500 → 908.400	25.000	1.28
CsA-d4	1,223.900 → 1,206.800	29.000	1.43
TAC-d2	824.449 → 771.400	27.000	1.24
SIR-d3	934.500 → 864.500	25.000	1.28
EVR-d4	979.600 → 912.400	27.000	1.28

Table 2. LLOQ and accuracy results.

Analyte	LLOQ (ng/mL)	Assigned conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (%)	Percent error (%)	CV (%)
CsA	12.4	23.4	23.5	100.4	0.4	0.2
		128	126.1	98.5	1.5	1.2
		294	287.4	97.8	2.2	1.7
		471	497.5	105.6	5.6	1.9
		745	760.5	102.1	2.1	1.6
		948	907.9	95.8	4.2	1.9
TAC	1.0	2.25	2.3	100.4	2.2	0.5
		6.05	6.0	99.0	0.8	2.5
		12.1	11.8	97.4	2.5	1.9
		17.7	17.8	100.3	0.6	1.8
		24.3	24.9	102.4	2.5	2
		42.9	43.0	100.3	0.2	2.2
SIR	1.2	2.11	2.1	99.5	0.5	1.4
		5.97	6.1	102.2	2.2	5.7
		12.1	11.6	95.5	4.1	3.5
		18.4	19.0	103.4	3.3	3.4
		27.5	27.3	99.2	0.7	1.9
		45.5	46.6	102.3	2.4	2.3
EVR	1.1	2.32	2.3	100.4	0.9	1.5
		6.16	6.1	99.5	1.0	4.6
		12.4	12.2	98.1	1.6	1.7
		18.4	18.7	101.5	1.6	3.9
		25.5	25.1	98.2	1.6	2.3
		44.8	45.8	102.3	2.2	2.3

LLOQ - Lower limit of quantification, CV - Coefficient of variation, CsA - Cyclosporine, TAC - Tacrolimus, SIR - Sirolimus, EVR - Everolimus.

Table 3. Precision (n = 20).

CV (%)	CsA					TAC					SIR					EVR				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Within-run	2.4	1.0	2.3	4.6	3.3	2.0	1.9	3.6	4.1	2.8	3.4	5.2	4.3	4.5	2.0	1.8	4.1	2.9	3.8	3.2
Between-run	3.8	5.2	3.8	5.5	9.1	3.9	3.9	3.6	5.1	6.1	5.3	6.2	5.7	5.3	6.4	4.1	5.3	6.1	6.3	4.2
Total	4.5	6.0	4.4	7.2	9.7	5.3	5.2	5.1	7.8	7.2	6.3	8.1	7.1	7.6	6.7	4.7	6.7	6.7	7.3	5.4

Table 4. Matrix effect.

Analyte	Nominal concentration (ng/mL)	Recovery (%)	Recovery-IS (%)	IS-normalized recovery (%)	Matrix factor (%)	Matrix factor-IS (%)	IS-normalized matrix factor (%)
CsA	20	51.43 ± 1.67	61.35 ± 0.57	83.83 ± 2.37	181.94 ± 5.38	178.89 ± 3.57	101.72 ± 2.78
	50	50.24 ± 1.11	70.56 ± 1.09	71.23 ± 2.31	144.55 ± 2.53	143.29 ± 1.42	100.89 ± 2.26
	100	76.25 ± 1.67	67.92 ± 1.14	112.33 ± 4.20	151.34 ± 3.60	155.90 ± 3.57	97.13 ± 3.82
	500	68.73 ± 1.34	60.28 ± 0.99	114.06 ± 3.63	191.05 ± 5.29	166.04 ± 5.57	115.11 ± 2.87
TAC	1	82.34 ± 6.76	77.6 ± 2.66	106.11 ± 7.95	117.82 ± 4.73	115.11 ± 2.94	102.44 ± 5.57
	2.5	87.06 ± 11.71	90.15 ± 11.53	96.55 ± 3.13	98.73 ± 3.25	96.74 ± 2.35	102.07 ± 3.01
	5	68.0 ± 7.28	69.53 ± 11.46	98.55 ± 7.13	133.03 ± 13.17	128.97 ± 11.97	103.21 ± 5.68
	30	71.42 ± 2.36	72.24 ± 3.05	98.94 ± 3.30	123.32 ± 3.58	119.01 ± 2.98	103.68 ± 4.00
SIR	2.5	86.17 ± 8.92	83.18 ± 3.74	103.58 ± 9.60	126.01 ± 10.10	123.31 ± 5.84	102.37 ± 9.65
	5	90.36 ± 8.98	83.79 ± 4.45	107.89 ± 9.62	127.57 ± 13.35	133.98 ± 7.80	95.16 ± 7.85
	10	82.29 ± 7.65	86.02 ± 7.00	98.78 ± 6.69	129.31 ± 3.57	123.71 ± 3.79	104.61 ± 4.33
	20	78.33 ± 7.62	84.02 ± 8.80	93.43 ± 5.45	123.26 ± 3.55	124.01 ± 6.55	99.63 ± 6.35
EVR	2.5	72.19 ± 5.31	78.65 ± 4.22	91.93 ± 7.09	123.94 ± 11.84	122.26 ± 2.11	101.32 ± 8.67
	5	73.51 ± 5.79	87.14 ± 4.86	84.41 ± 5.76	122.02 ± 7.61	120.11 ± 4.86	101.72 ± 7.39
	10	68.22 ± 2.87	89.43 ± 2.87	76.33 ± 3.24	118.59 ± 5.04	117.96 ± 4.74	100.62 ± 4.64

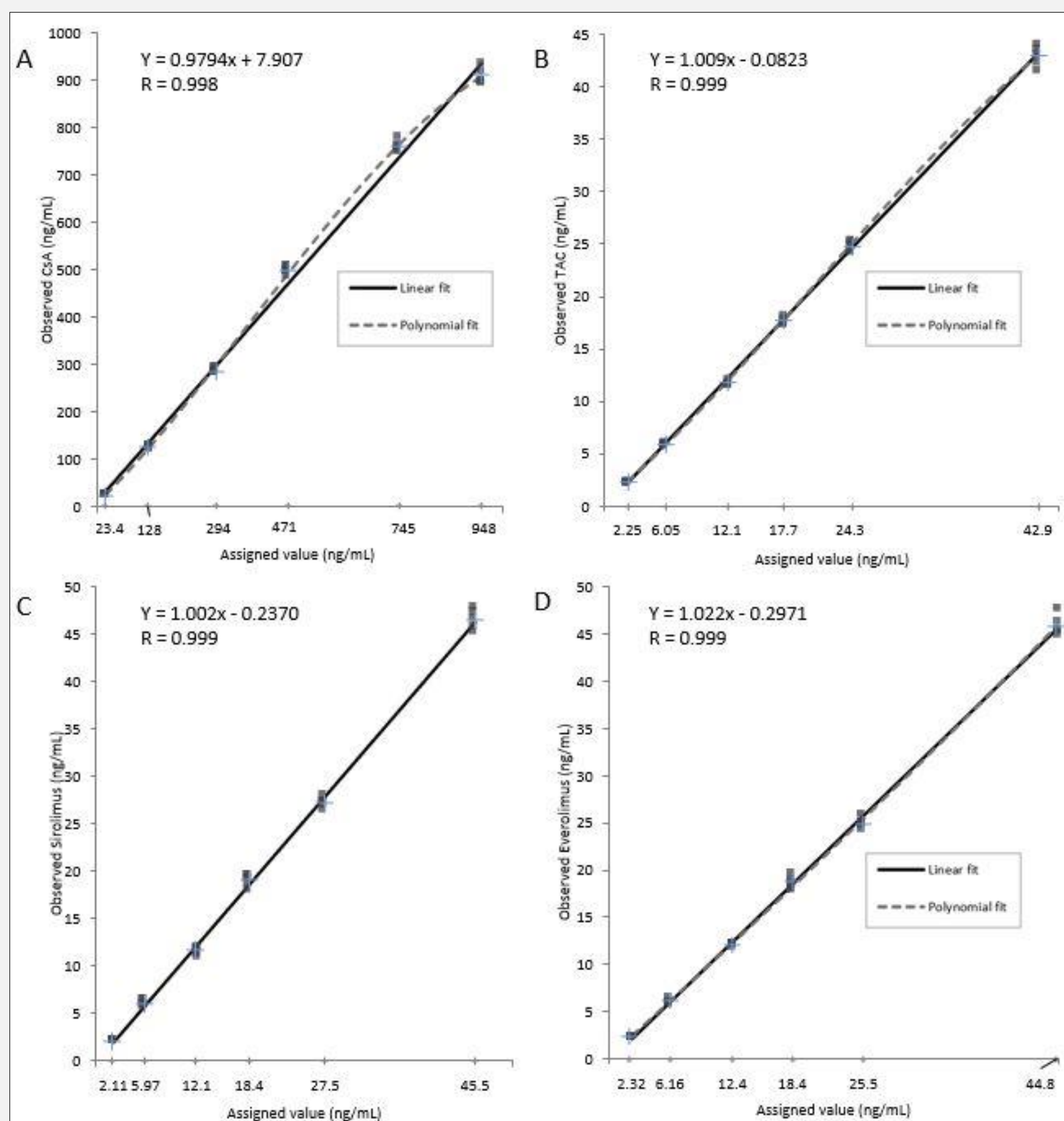

Figure 1. The calibration curve results and their linearity.

A - The result of CsA, B - The result of TAC, C - The result of SIR, D - The results of EVR.
CsA - Cyclosporine, TAC - Tacrolimus, SIR – Sirolimus, EVR - Everolimus.

RESULTS

LLOQ
LLOQ was determined to be 12.4 ng/mL, 1.0 ng/mL, 1.2 ng/mL and 1.1 ng/mL, for CsA, TAC, SIR, and EVR, respectively (Table 2).

Inaccuracy
The accuracy results are listed in Table 2. The accuracy was 95.8 to 105.6, 97.4 to 102.4, 95.5 to 103.4, and 98.1 to 102.3 for CsA, TAC, SIR, and EVR, respectively. At all levels, % deviation from the nominal value were less than < ± 15%, fulfilling the acceptance criteria.

Imprecision
The precision results are listed in Table 3. At all QC levels for the four analytes, the within-run and between-run imprecision CV values did not exceed 15%.

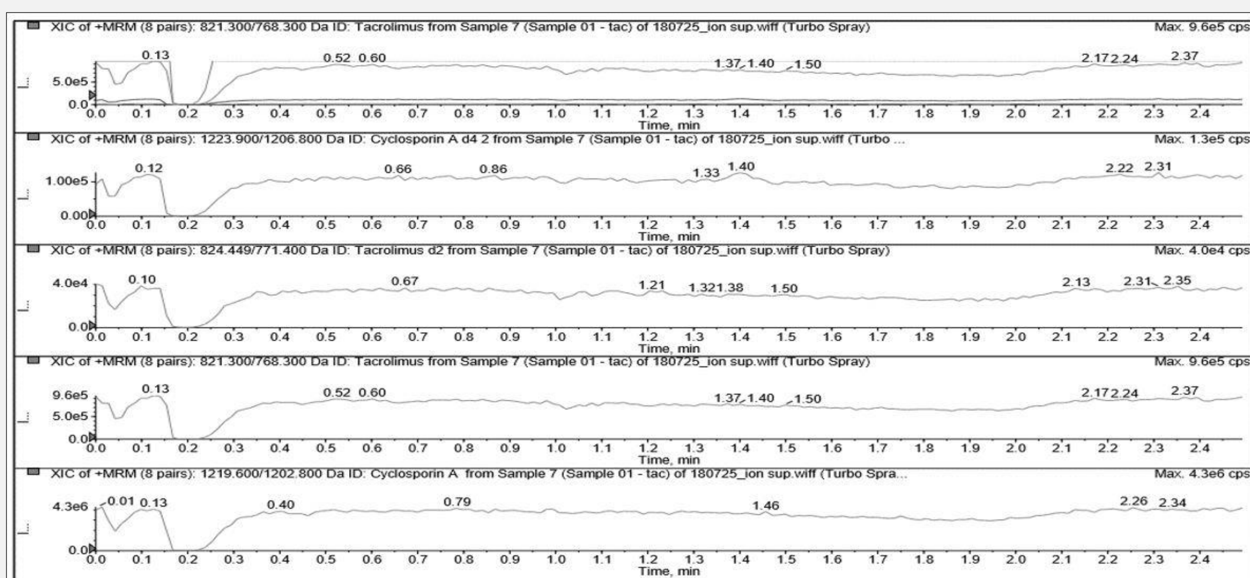

Figure 2. Ion suppression and enhancement of CsA and TAC.

Linearity

The linearity results are shown in Figure 1. The method was found to be linear over the measuring ranges 23.4 - 948 ng/mL for CsA, 2.11 - 45.5 ng/mL for TAC, SIR, and EVR, with back-calculated values for calibrators all within ± 15%.

The typical linear regression equation is y = 0.9794x + 7.907 (x, nominal CsA concentration; y, observed CsA concentration, r = 0.998) for CsA, y = 1.009x - 0.0823 (x, nominal TAC concentration; y, observed TAC concentration, r = 0.999) for TAC, y = 1.002x - 0.2370 (x, nominal SIR concentration; y, observed SIR concentration, r = 0.999) for SIR and y = 1.022x - 0.2971 (x, nominal EVR concentration; y, observed EVR concentration, r = 0.999).

Ion suppression and enhancement

At four levels, the recovery and matrix factor were 50.24% to 76.25% and 144.55% to 191.05% for CsA, 68.0% to 87.06% and 98.73% to 133.03% for TAC, and 78.33% to 90.36% and 123.26% to 129.31% for SIR, respectively. At three levels, the recovery and matrix factor for EVR were 68.22% to 73.51% and 118.59% to 123.94%, respectively.

The IS-normalized recovery and matrix factor were 71.23% to 114.06% and 97.13% to 115.11% for CsA, 96.55% to 106.11% and 102.07% to 103.68% for TAC, 93.43% to 107.89% and 95.16% to 104.61% for SIR, and 76.33% to 91.93% and 100.62% to 101.72% for EVR, respectively; the CV of IS-normalized recovery and matrix factor were < 10% for all analytes. The recovery and matrix results were acceptable. Further validation results are described in Table 4.

The post-column infusion experiment result showed that no interfering peaks were found at the retention times of CsA and TAC (Figure 2).

Carryover

The carryover of the first low calibrator (L1) after the highest calibrators was < 1%, which was acceptable in accordance with the requirements of the guidelines.

Method comparison

All of the measurements were abnormally distributed ($p < 0.001$ for Shapiro-Wilk test). Upon evaluation of paired samples Wilcoxon test, the mean values of the measurements were different for both CsA and TAC ($p < 0.05$). The measurements of immunoassays and U-HPLC-MS/MS were 152.6587 ± 6.6361 (SE) (25.800 to 733.300) ng/mL and 149.2335 ± 7.2543 (SE) (4.300 to 872.300) ng/mL for CsA and 6.8441 ± 0.1430 (2.000 to 55.800) ng/mL and 6.2890 ± 0.1302 (1.555 to 45.384) ng/mL for TAC, respectively. The regression equation of the measurements by the two methods was CEDIA = 0.947 × U-HPLC-MS/MS + 12.667, r = 0.9689 for CsA and CMIA = 1.135 × U-HPLC-MS/MS - 0.116, r = 0.9529 for TAC (Figure 3).

By Bland-Altman plot, the mean bias and the 95% limits of agreement of measurements were -8.6468 and -60.7217 to 43.4282 for CsA and -8.2146 and -43.2475

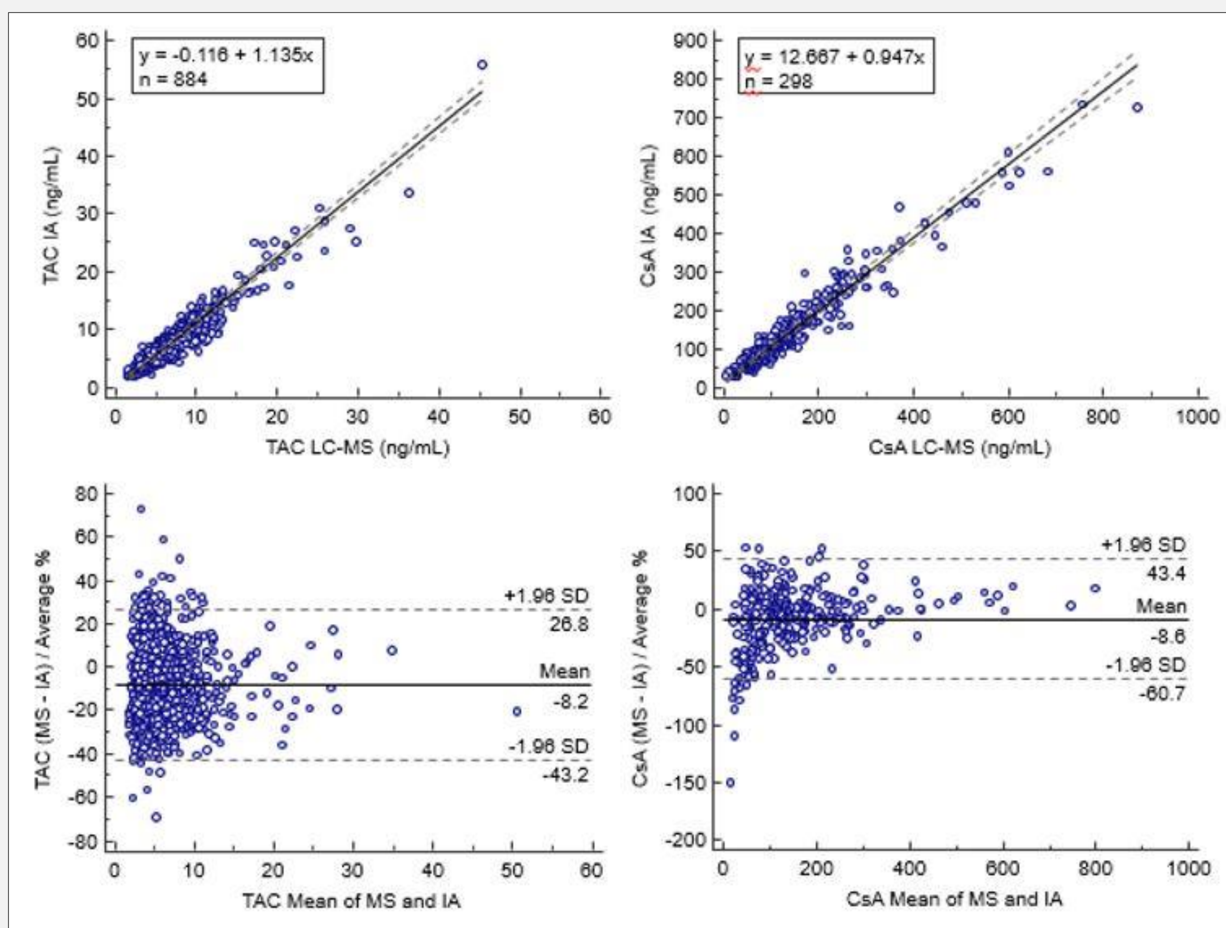

Figure 3. Passing-Bablok correlation and Bland-Altman plot for CsA and TAC.

(In Bland-Altman plot) Black solid line: Mean; Grey dashed line: Limits of Agreement (LOA).

to 26.8183 for TAC, respectively (Figure 3). Back-transformation was performed to obtain the ratio of the two measurements. The mean bias of the immunoassay was as follows: the U-HPLC-MS/MS ratio was 114.8% for CsA and 110.3% for TAC. In other words, the mean overestimation of immunoassay compared with LC-MS/MS was 14.8% for CsA and 10.3% for TAC.

On evaluation by paired samples Wilcoxon test, the mean measurements were different for both SIR and EVR ($p < 0.05$). External LC-MS/MS and U-HPLC-MS/MS measurements were 5.8365 ± 0.2588 (3.0950 to 9.7540) ng/mL and 3.8525 ± 0.2069 (1.7000 to 7.6000) ng/mL for SIR and 5.7630 ± 0.3756 (2.8050 to 8.7470) ng/mL and 4.6364 ± 0.3936 (1.1000 to 8.3000) ng/mL for EVR, respectively. The regression equation of the measurements by the two methods was U-HPLC-MS/MS = 0.717 × external LC-MS/MS - 0.342, r = 0.4982 for SIR, and U-HPLC-MS/MS = 0.995 × external LC-MS/MS - 1.250, r = 0.7565 for EVR.

DISCUSSION

In this study, we aimed to validate the quantification method of CsA, TAC, SIR, and EVR using U-HPLC-MS/MS. CsA and TAC were verified by comparing immunoassay and LC-MS/MS, and SIR and EVR were verified by comparing them with external LC-MS/MS equipment.

The primary disadvantage of immunoassay is that cross-reactions between drugs and metabolites can cause overestimation of the drug concentration [14]. However, evolved immunoassays produced results that were as high as 60% higher than LC-MS/MS down to as low as

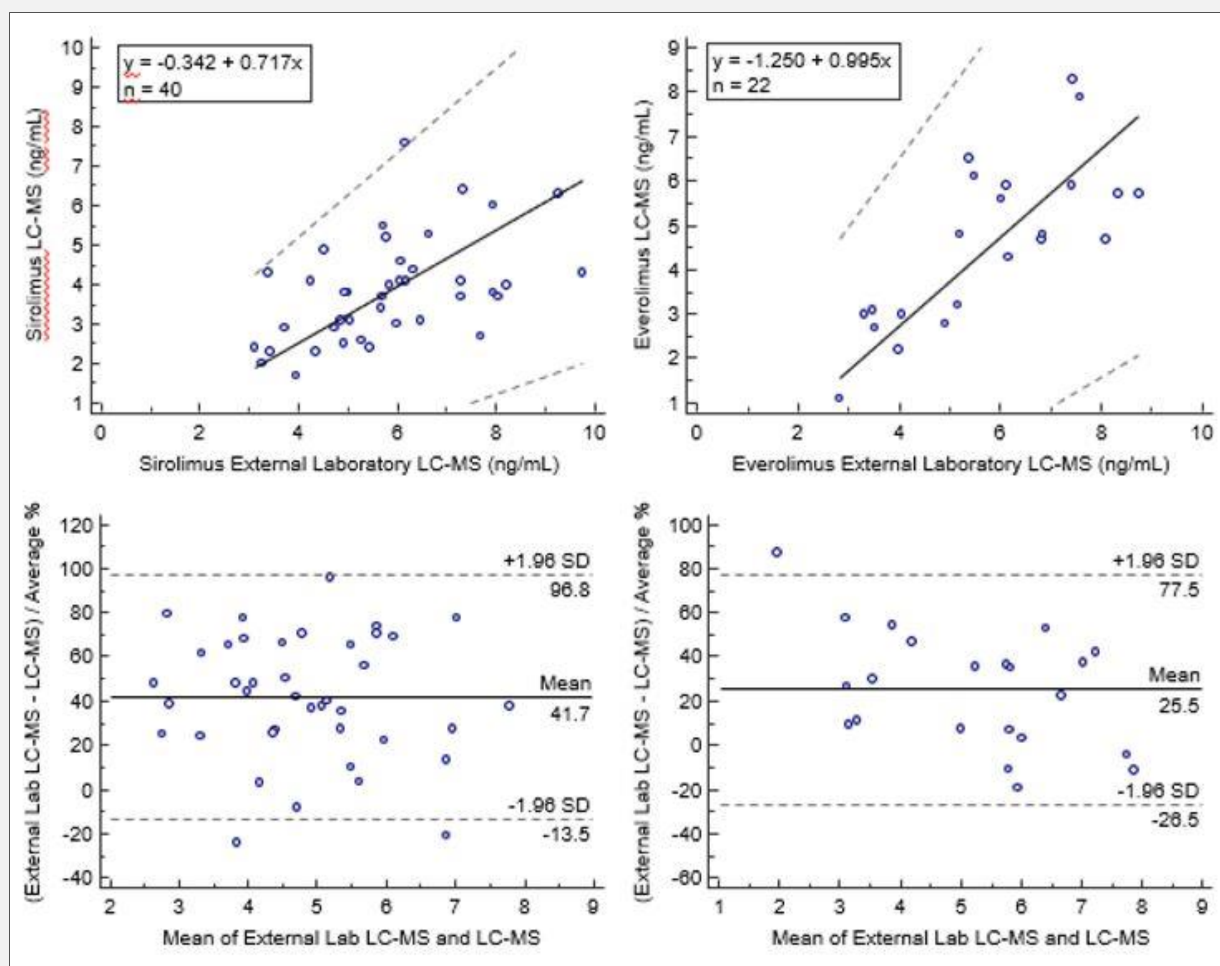

Figure 4. Passing-Bablok correlation and Bland-Altman plot for SIR and EVR.

15% [15]. Furthermore, Wei et al. showed that their highly specific sandwich assay has a similar dynamic range of LC-MS/MS methods [16]. The results of comparing two methods to verify TAC and CsA showed that IA methods were 14.8% higher than LC-MS/MS in CsA and 10.3% in TAC. These results suggest that an overestimation of IA methods was observed in this experiment.

The r values of the comparison of SIR and EVR on external LC-MS/MS were 0.4982 and 0.7565, respectively. In addition, the slopes of the regressions were over 0. This result can be explained by the lack of standardization [13]. The sample preparation methods vary from laboratory to laboratory. Another reason is the different combinations of equipment. The mass spectrometer and the external spectrometer we used were produced by the same company but were different series. The manufacturer of the combined LC in the external equipment is unknown. Kim et al. compared immunosuppressive drugs using interlaboratory LC-MS/MS [17]. This research showed that the different combinations of equipment, from the same or different companies, resulted in a variation of results. However, to compensate for these problems, we participated in the external quality assessment project with the Korea Association of External Quality Assessment Service twice a year. In comparison between peer groups, the results have been consistently confirmed within 2SD, guaranteeing the accuracy of this method.

This research aim was to establish a laboratory developed test for immunosuppressants. Despite the difficulty of comparing methods, we have developed a reliable assay for the analysis of CsA, TAC, SIR, and EVR in whole blood using U-HPLC-MS/MS. The assay offers an acceptable measurable range with excellent accuracy and precision. Further studies should be performed with

more samples and other equipment to develop standardized methods.

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Declaration of Interest:
None declared.

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