

A simple and accurate LC-MS/MS method for monitoring cyclosporin A that is suitable for high throughput analysis

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Abstract. With time, the number of samples in clinical laboratories from therapeutic drug monitoring has increased. Existing analytical methods for blood cyclosporin A (CSA) monitoring, such as high-performance liquid chromatography (HPLC) and immunoassays, have limitations including cross-reactivity, time consumption, and the complicated procedures involved. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has long been considered the reference standard owing to its high accuracy, specificity, and sensitivity. However, large numbers of blood samples, multi-step preparation procedures, and longer analytical times (2.5-20 min) are required as a consequence of the different technical strategies, to ensure good analytical performance and routine quality assurance. A stable, reliable, and high throughput detection method will save personnel time and reduce laboratory costs. Therefore, a high throughput and simple LC-MS/MS method was developed and validated for the detection of whole-blood CSA with CSA-d12 as the internal standard in the present study. Whole blood samples were prepared through a modified one-step protein precipitation method. A C18 column (50x2.1 mm, 2.7 μ m) with a mobile phase flow rate of 0.5 ml/min was used for chromatographic separation with a total running time of 4.3 min

to avoid the matrix effect. To protect the mass spectrometer, only part of the sample after LC separation was allowed to enter the mass spectrum, using two HPLC systems coupled to one mass spectrometry. In this way, throughput was improved with detection of two samples possible within 4.3 min using a shorter analytical time for each sample of 2.15 min. This modified LC-MS/MS method showed excellent analytical performance and demonstrated less matrix effect and a wide linear range. The design of multi-LC systems coupled with one mass spectrometry may play a notable role in the improvement of daily detection throughput, speeding up LC-MS/MS, and allowing it to be an integral part of continuous diagnostics in the near future.

Introduction

Cyclosporin A (CSA) is a potent immunosuppressant (ISD) that has been widely used in organ transplantation and autoimmune disorders. Long-term use of CSA may lead to several adverse effects, such as hypertension, hyperlipidemia, hepatotoxicity, and in particular, neurotoxicity (1,2). Furthermore, due to the narrow therapeutic window and large inter-individual variability, regular blood concentration monitoring of CSA with subsequent dosage adjustment to maximize treatment efficacy and reduce adverse effects is crucial.

Currently, CSA blood concentrations, including both trough and peak concentrations, which can be as high as 6,000 ng/ml in certain patients, are monitored primarily through detection methods including high-performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and immunoassays.

Among these methods, LC-MS/MS has long been regarded as the gold standard for the detection of ISD drug concentrations, as other methods have limitations. The greatest challenges with immunoassays are the non-specific matrix effects and cross-reactions between immunoassay antibodies and drug metabolites, which reduce the specificity, accuracy, and sensitivity of immunoassays (3). Although HPLC is less affected by cross-reacting metabolites, CSA lacks chromophores and functional groups that can be used to prepare derivatives and must be monitored at a short wavelength of

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210 nm (4), at which several other substances also absorb. Therefore, pretreatment is necessary for whole blood samples to enrich and purify CSA as much as possible.

LC-MS/MS combines the separation efficiency of HPLC with the high sensitivity of MS, although ease of use remains a challenge. Certain LC-MS/MS methods require cumbersome pretreatment steps, commonly using a multi-step protein precipitation procedure, liquid-liquid extraction methods, and solid-phase extraction (5-9). Both the time and cost of sample pretreatment and the long separation time together limit the throughput of LC-MS/MS, especially for those with a single liquid chromatography system with a long running time. Furthermore, the isotope abundance contributions of some internal standards with fewer isotopes, for example, CSA-d4 (3,10,11) also pose a challenge to accuracy (12). The aim of this study was to develop a high throughput LC-MS/MS method with a simple sample preparation method and high quantitative value for the detection of CSA in whole blood, using CSA A-d12 as the internal standard, to provide a method with high clinical application value to promote individualized precision drug monitoring.

Materials and methods

Reagents and specimen preparation. CSA was calibrated using a 6-point calibration curve with 6Plus1 Multilevel ISD calibrators in whole blood (Chromsystems) with concentrations of 23.40, 128.40, 294.00, 471.00, 745.00, and 1,890.00 ng/ml, which were also used for linearity validation. Internal quality controls (QCs) were evaluated using Bio-Rad Lyphochek Whole Blood ISD Controls levels 1, 2, 4, and level 5 were added when there was a high-concentration sample (Bio-Rad Laboratories, Inc.). CSA Standard (CAS 59865-13-3, $\geq 95\%$) was provided by MilliporeSigma, and [$^2\text{H}_{12}$]-Cyclosporin A (CSA-d12, AlsaChim) with a purity of 98% ^2H was used as an internal standard (IS). Formic acid and zinc sulfate were obtained from MilliporeSigma (analytical grade). Other solvents and reagents were HPLC grade. Ultrapure water of 18.2 M Ω cm resistivity was obtained from a Milli-Q (MilliporeSigma) water purification system.

Samples were pretreated by mixing 20 μl EDTA anti-coagulated whole blood with 400 μl sample pretreatment reagent consisting of 0.05 M zinc sulfate and 30.0 ng/ml CSA-d12 in 50% methanol/water. Samples were vortexed vigorously for more than 20 sec and mixed for 5 min in a 55-well oscillator. After centrifuging for 5 min at 10,000 \times g at 4°C, the obtained supernatant was used for analysis.

Instruments and parameters. LC-MS/MS analysis was performed on an LC-20AXR (Shimadzu Corporation) tandem AB SCIEX API4000 plus LC-MS/MS instrument (Applied Biosystems) at Guangzhou KingMed Center for Clinical Laboratory Co., Ltd. (Guangzhou, China). Two HPLC systems were paralleled via a six-port switching valve mounted on an MPX driver (Fig. 1). The system was controlled and data was processed using the Analyst software (AB SCIEX, version 1.6.2), followed by quantitative analysis using the MultiQuant software (AB SCIEX, version 3.0.1) with the default integration parameters except for a 0.05 min baseline

sub. window and a weighting factor of $1/x$ for area linear regression.

Chromatographic separation was achieved on a C18 column (MilliporeSigma, 2.1 \times 50 mm, 2.7 μm particles) with a C18 SecurityGuard column (Phenomenex, 2 \times 2.1 mm) at 60°C. A gradient elution program was set at a flow rate of 0.5 ml/min from 60% buffer B and changed to 100% buffer B (buffer A: 2 mM ammonia acetate and 0.1% formic acid in water; buffer B: methanol) at 1.5 min for 1.0 min. From 2.51 to 3.5 min the gradient was changed and kept at 60% buffer B to stabilize the column for the next injection (Table I). The injection volume was 10 μl .

Through the paralleled HPLC systems, during the 1.5 min interval from 0.8 to 2.3 min, the LC was set to the mass spectrometer, and another sample could be detected during the remaining time (Fig. 1). The autosampler needed an additional 0.4 min per sample for injection. In this way, within 4.3 min, two samples were detected, thus allowing for a shorter analysis time for each sample of 2.15 min, thus improving the instrument throughput.

An electrospray ionization source was used in positive ion mode (Applied Biosystems). Precursor/production pairs were used (1,219.9/1203.0 m/z for CSA and 1,232.0/1,215.2 m/z for CSA-d12) in multiple reaction monitoring (MRM) mode with a dwell time of 120 msec. Qualitative ion pairs were also monitored (1,221.0/1,204.0 m/z for CSA and 1,233.0/1,216.1 m/z for CSA-d12) with a dwell time of 60 msec. MRM pairs were processed at 60, 6, 30, and 20 V for declustering potential, entrance potential, collision energy, and collision cell exit potential, respectively. The curtain gas, collision gas, nebulizer gas, and auxiliary gas were set at 25, 5, 50, and 60 psi, respectively. The ion spray voltage was 5,500 V, and the source temperature was 400°C.

Method validation. The modified LC-MS/MS assay was evaluated according to the guidelines in the CLSI LC-MS C62-A documentation and the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) expert consensus group (12,13). HPLC-MS, affinity chrome-mediated immunoassay (ACMIA), chemiluminescent microparticle immunoassay (CMIA), electrochemiluminescence immunoassay (ECLIA), and cloned enzyme donor immunoassay (CEDIA) were performed as described previously (6,14-16).

Inter-day and intra-day precision. Whole blood samples at three concentration levels were used to assess the intra-day precision and inter-day precision ($n=20$ for each concentration). Intra-day precision was processed on the same day and day-to-day variability was assessed by analysis of two sets of samples on 10 different days. The acceptance criterion for total imprecision was based on the recommendation of the IATDMCT expert consensus group of $\leq 10\%$ (13).

Linearity and sensitivity. A total of six samples of each concentration were measured in duplicate on 3 different days. Whilst 23.40, 128.40, 294.00, 471.00, 745.00, and 1,890.00 ng/ml were obtained from the 6-point calibration curve (Chromsystems), 5.85 and 7.80 ng/ml were obtained by dilution from 23.40 ng/ml by the blank point. The acceptance

Table I. The gradient elution used for LC-MS/MS detection of CSA

Step	Analysis time (min)	Flow rate (ml/min)	Mobile phase A %	Mobile phase B %
1	0	0.5	40	60
2	1.5	0.5	0	100
3	2.5	0.5	0	100
4	2.51	0.5	40	60
5	3.5	0.5	40	60

Buffer A: 2 mM ammonia acetate and 0.1% formic acid in water; buffer B: methanol.

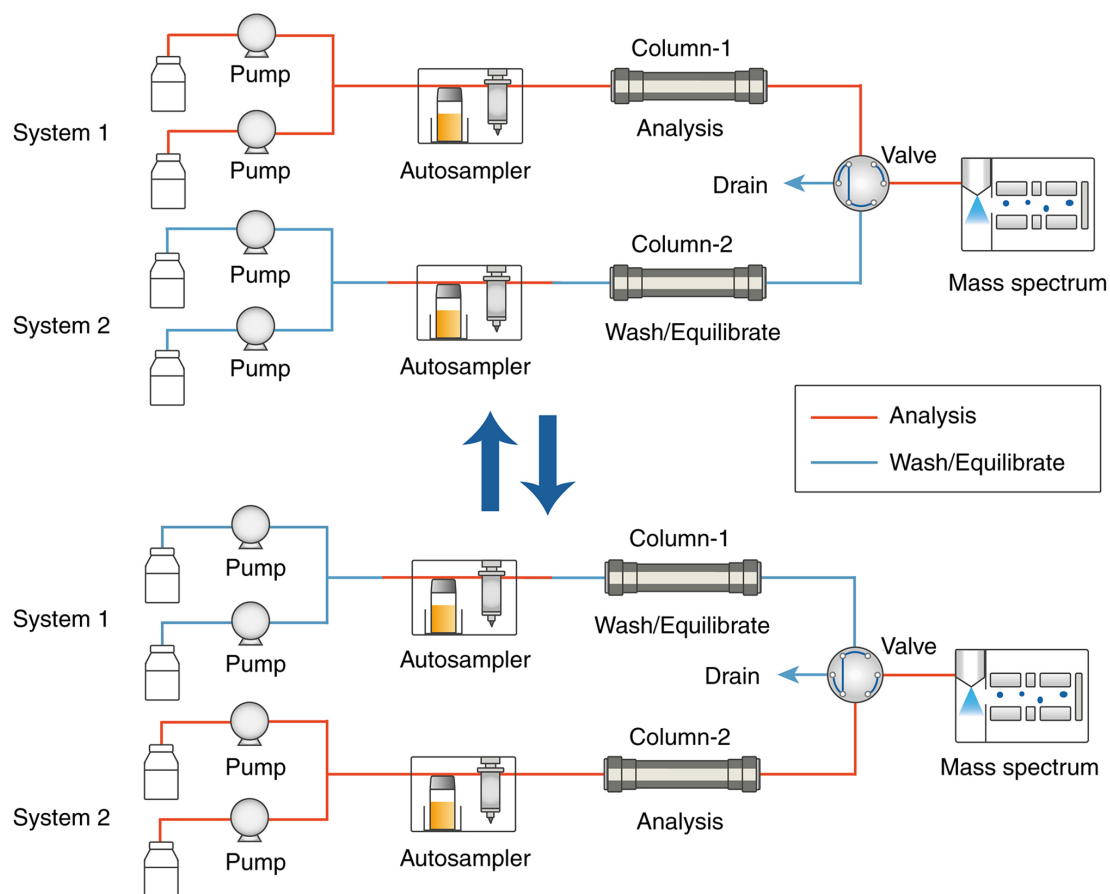


Figure 1. Schematic diagram of the modified liquid chromatography-tandem mass spectrometry method.

criterion was defined as a regression deviation <10% and the calibration curve had to have a correlation coefficient (r) of 0.99 or better. The lowest concentration that met the above criteria and had a signal-to-noise ratio >10 was accepted as the lower limit of quantitation (LLOQ).

The clinical reportable range was determined according to the linear range and the maximum dilution multiple. A high-concentration sample was diluted 2, 4, or 8 times with homogeneous drug-free whole blood (EDTA anticoagulant). Sample concentrations after dilution should be within the linear range and not threefold below the LLOQ. Taking the concentrations before dilution as references, the dilution multiple was determined by analyzing five replicates of patient samples before and after dilution separately. The recovery of

the diluted samples should range from 85 to 115%, and the deviation of five duplicates should be <15%.

Accuracy and recovery. A total of six external quality assessment (EQA) samples [Laboratory of the Government Chemist (LGC) Institution] under the ISD program (CICTAC-Cyclosporin) were detected to assess the method's accuracy. A bias within $\pm 25\%$ and a Z score under ± 3 was considered acceptable.

In recovery experiments, three concentration levels of whole blood samples were spiked with low, moderate, and high concentrations of standard solutions separately to calculate recoveries. All the spiked and unspiked samples were analyzed with three replicates. The spiked recovery, which refers to

(spiked sample concentration - unspiked sample concentration)/standard spiked value, should be within 85-115%.

Matrix specificity and matrix effect. Matrix specificity was validated by evaluation of the presence of any peaks in the corresponding position for the CSA and CSA-d12 when the blank matrix (the point of STD-0 in the standard curve) and the blank patient samples were detected.

The matrix effect is independent of the presence of the analyte and influences the accurate quantification for CSA (10). In this matrix admixing experiment, the matrix effect between whole blood patient sample and QC or calibration was evaluated. Standard solutions (a and b: commercial QC at low and high concentrations respectively, Bio-Rad Laboratories, Inc; c-f: matrices at blank, low, moderate, and high concentrations of the calibration respectively, Chromsystems Instruments & Chemicals GmbH), patient samples, and mixed samples (standard solutions mixed with six samples A-F from different patients separately in different proportions) were prepared. The matrix effect was evaluated through the analysis of three replicates of the three different matrix samples above separately. The deviation between the response of the mixed samples and the response average of the patient samples and standard solutions should be <20%.

A post-column infusion experiment was processed to evaluate the matrix, in which the analytes dissolved in solvent (1.0 mg/l CSA or CSA-d12 in 50% methanol/water) was directly infused into the mass spectrometer using a syringe pump (30 μ l/min) to gain a stable total ion count (TIC). Then three different extracted matrix samples were separately injected concurrently by the HPLC system. There should be no decrease or increase in TIC of the direct infusion observed at the time point when the matrix components are eluted from the column.

Carryover. Carryover was evaluated through repeated (n=3) injections of samples with low concentration (C1, LLoQ), immediately followed by high concentration [C2, upper (U) LoQ] and low concentration (C3, LLoQ) injections. The acceptance criterion was $\leq 20\%$ carryover, which was calculated using the following formula: $\text{Bias} = (\text{C3 mean} - \text{C1 mean}) / \text{C1 mean} \times 100\%$.

Clinical application of the LC-MS/MS method

Subjects. This modified method was used for the detection of CSA concentrations in 41 nephrotic patients (33 with nephrotic syndrome and 8 with kidney transplants) who were treated with CSA between April 2016 and July 2021 in the Guangdong Provincial Hospital of Chinese Medicine (Guangdong, China). Among these patients, 10 were women and 31 were men, with an age range of 17-78 years, a median age of 44 years and an mean age of 43.46 ± 16.54 years. EDTA anticoagulated whole blood was collected from patients before the morning CSA dose using the aforementioned LC-MS/MS method to obtain the CSA trough concentration. Patient information, such as clinical diagnosis, CSA trough concentration, laboratory indicators, and drug combinations were recorded. This study was approved by the Ethics Committee of Guangdong Provincial Hospital of Chinese Medicine (approval no. ZE2020-240-01). Written informed consent was obtained from all participants.

Definitions. Chronic kidney disease (CKD) was graded on the basis of the estimated glomerular filtration rate (eGFR) according to the KDIGO guidelines (17): CKD1, eGFR ≥ 90 ml/min/1.73 m²; CKD2, eGFR 60-90 ml/min/1.73 m²; CKD3, eGFR 30-59 ml/min/1.73 m²; CKD4, eGFR 15-29 ml/min/1.73 m²; CKD5, eGFR <15 ml/min/1.73 m².

Statistical analysis. Statistical analyses were performed using MedCalc version 20.0.22 (MedCalc Software bvba), SPSS Statistics version 20.0 (IBM Corp.), and GraphPad Prism version 8.3 (GraphPad Software, Inc.). The results of proficiency testing were evaluated using Passing-Bablok regression and Bland-Altman plots. Continuous data are presented as the mean \pm SD for the normally distributed data or otherwise as the median (range). For intergroup comparisons, normally distributed data were analyzed using a Welch one-way ANOVA test with a Games-Howell post hoc test. Non-normally distributed data were assessed using a Mann-Whitney U test or a Kruskal-Wallis test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Method validation. The retention times for CSA and CSA-d12 were 1.74 and 1.71 min (0.94 and 0.91 min for the MS monitoring period), respectively. For the blank matrix and blank patient sample, no peak was detectable in the corresponding position. A high-concentration sample (1,692.87 ng/ml) and a low-concentration sample (10.10 ng/ml) were used to assess carryover. And cross-contamination between samples was negligible.

A total of six whole blood samples were analyzed to assess precision, three of these were tested as 20 parallels in 1 day and the remaining three samples were analyzed in duplicate, one run per day over 10 days (Table II). For the two HPLC systems, the total imprecision was <3.64%, which met the goal of $\leq 10\%$ for all concentrations.

The method showed good correlation in the linear range of 5.85-1,890.00 ng/ml (weighting 1/x, Fig. 2) with an $r > 0.99$, and the LLoQ was set to 5.85 ng/ml (Fig 3). The linear regression equation was $y = 0.0016x + 0.00144$ ($r = 0.99948$) for HPLC system 1, and $y = 0.0016x + 0.00284$ ($r = 0.99954$) for HPLC system 2. The dilution performance was assessed by diluting a high-concentration sample with homogeneous drug-free whole blood (EDTA anticoagulant) 2, 4, or 8 times. Dilution recoveries were within the pre-defined acceptance limits and the deviation of five duplicates was <4.15%. Hence, the sample could be quantified by dilution when the concentration of the analyte exceeded the ULoQ (1,890.00 ng/ml), which meant that this method could obtain quantitative results for samples with concentrations between 5.85 and 15,120 ng/ml.

A total of six EQA samples were in good agreement with the results from the LGC institution (44.05-2,129.50 ng/ml) and met the acceptable range for the LC-MS/MS method (bias $< \pm 4.71\%$, Z scores $< \pm 0.57\%$, Fig. 4).

A total of three concentration levels of whole blood samples (59.73 ± 0.63 , 124.87 ± 1.79 , and 365.21 ± 0.78 ng/ml) were spiked with low, moderate, and high concentrations of standard solutions (54.04, 104.49, and 511.73 ng/ml) separately, and the spiked recoveries ranged from 89.4-104.7%.

Table II. Validation results.

Performance metrics	HPLC system 1		HPLC system 2	
Intra-day (CV%, n=20)	84.34±1.36 ng/ml 541.51±8.51 ng/ml 1,279.68±16.83 ng/ml	1.62% 1.57% 1.32%	83.26±1.07 ng/ml 530.14±8.68 ng/ml 1,249.94±17.89 ng/ml	1.29% 1.64% 1.43%
Inter-day (CV%, n=20)	83.72±2.58 ng/ml 467.41±17.03 ng/ml 1,774.72±47.63 ng/ml	3.08% 3.64% 2.68%	83.41±2.20 ng/ml 464.04±13.86 ng/ml 1,770.31±39.66 ng/ml	2.64% 2.99% 2.24%
Linearity (n=6) Dilution (n=5)	5.85-1,890.00 ng/ml		5.85-1,890.00 ng/ml	
High concentration sample	1,841.90±18.56 ng/ml		1,831.10±21.87 ng/ml	
2 times (Recoveries):	881.29±16.30 ng/ml	95.69±1.77%	881.71±7.56 ng/ml	96.30±0.83%
4 times (Recoveries):	459.28±19.08 ng/ml	99.74±4.14%	448.42±15.26 ng/ml	97.96±3.33%
8 times (Recoveries):	232.07±3.22 ng/ml	100.79±1.40%	229.90±8.76 ng/ml	100.44±3.83%
Carryover (Bias, n=3)		0.8%		1.7%
Accuracy (Recoveries, n=9)	54.04 ng/ml 104.49 ng/ml 511.73 ng/ml		95.53±4.25% 95.64±5.65% 95.11±1.92%	

CV, coefficient of variation.

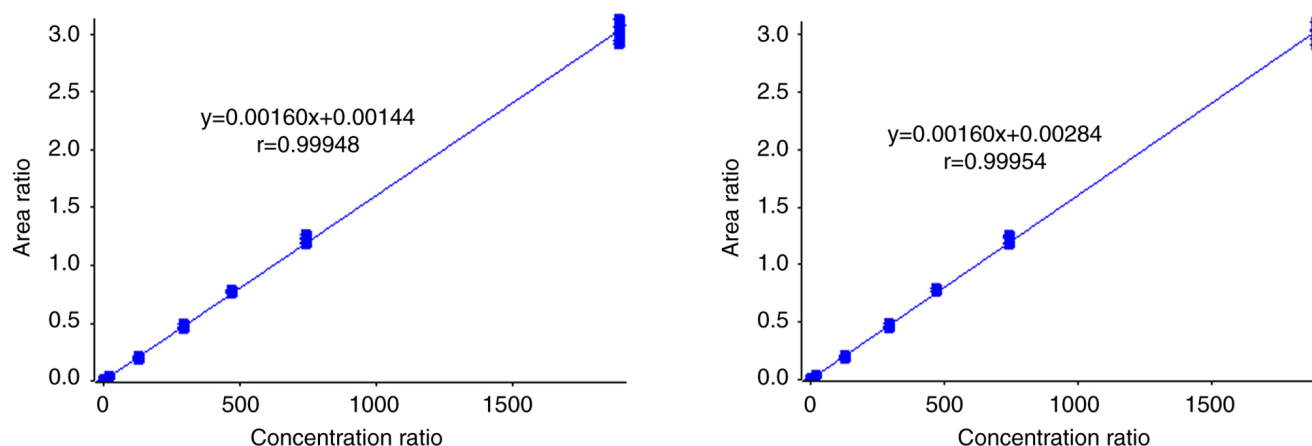


Figure 2. Linear ranges for system 1 and 2 (1/x weighting).

Post-column infusion experiments showed no observable decrease or increase in the TIC of the direct infusion when the matrix components were eluted from the column (Fig. 5). For the matrix admixing experiment, standard solutions and patient samples were mixed in a 1:4 ratio. The deviations of the mean responses ranged from -1.9-5.5%, thus meeting the acceptance criterion of <20% and confirming no relative matrix effect.

A total of 12 standard samples with a concentration range of 121.74-1,439 ng/ml were applied to compare the modified LC-MS/MS with five other CSA detection methods, including HPLC-MS, ACMIA, CMIA, ECLIA, and CEDIA. The Z score represents the deviation between the measured value and the target value. In the modified LC-MS/MS, the Z scores of the 12 samples were between -0.5 and 1, which were statistically different

from ACMIA and CMIA (Fig. 6). However, the Z score of the LC-MS/MS method (median=0.1,100; 25th-75th percentile=-0.0925-0.4375) was less discrete when compared with HPLC-MS (median=-0.425; 25th-75th percentile=-0.830-0.410), ECLIA (median=0.395; 25th-75th percentile=-0.1425-0.7025) and CEDIA (median=0.405; 25th-75th percentile = -0.245-0.700). Both ECLIA and CEDIA are a type of immunoassay based on the specific reaction of antigen and antibody. The obvious disadvantage was cross-reactivity with metabolites, particularly in liver transplant recipients where hepatic dysfunction can lead to alterations in CSA metabolism and elimination (18). CEDIA had cross-reactivity for CSA metabolites AM1, AM4n, and AM9, resulting in a positive bias of the results (16). Likewise, Shigematsu *et al* (19) evaluated the analytical performances of immunoassays in monitoring tacrolimus,

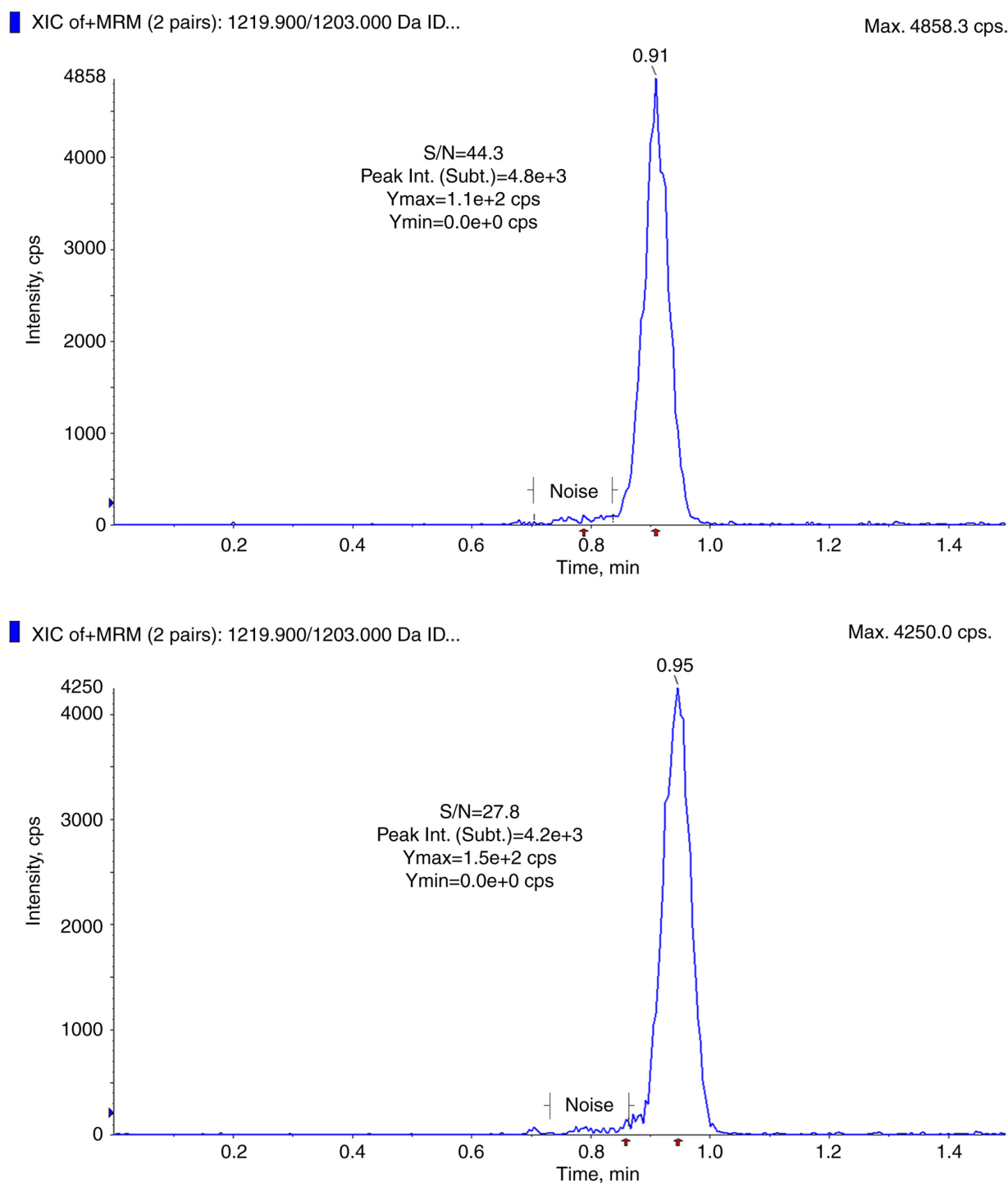


Figure 3. Defined lower limit of quantitation (5.85 ng/ml) for system 1 (S/N=44.3) and 2 (S/N=27.8). S/N, signal-to-noise ratio; cps, counts per second; XIC, extracted ion chromatogram; MRM, multiple reaction monitoring; Ymax, highest signal response; Ymin, lowest signal response.

using LC-MS/MS as a standard, and observed a bias of 7.46% in ECLIA. Thus, the accuracy and stability of the modified LC-MS/MS showed improvement to some extent.

Clinical application

Overall monitoring. Patients with kidney transplants received more frequent monitoring than those with nephrotic syndrome (NS). The CSA concentration was significantly higher for patients with kidney transplants than for those with NS ($P<0.05$). Of the 33 patients with NS,

13 underwent renal biopsies, and nine were diagnosed with membranous nephropathy, whereas four showed minimal changes in the disease. The results of the monitoring are shown in Table III.

Concomitant medications. Concomitant medications were more common in patients with NS. The CSA treatment was most frequently combined with antihypertensive agents. The results of the monitoring of patients with different drug combinations are shown in Table IV.

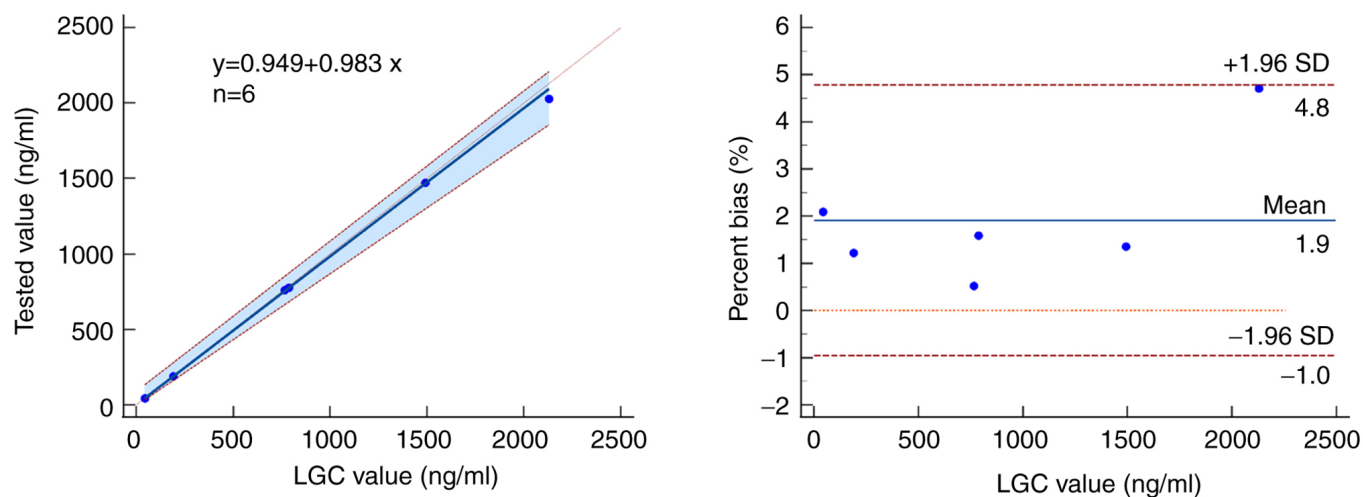


Figure 4. Passing and Bablok Regression and Bland-Altman plots of LGC samples.

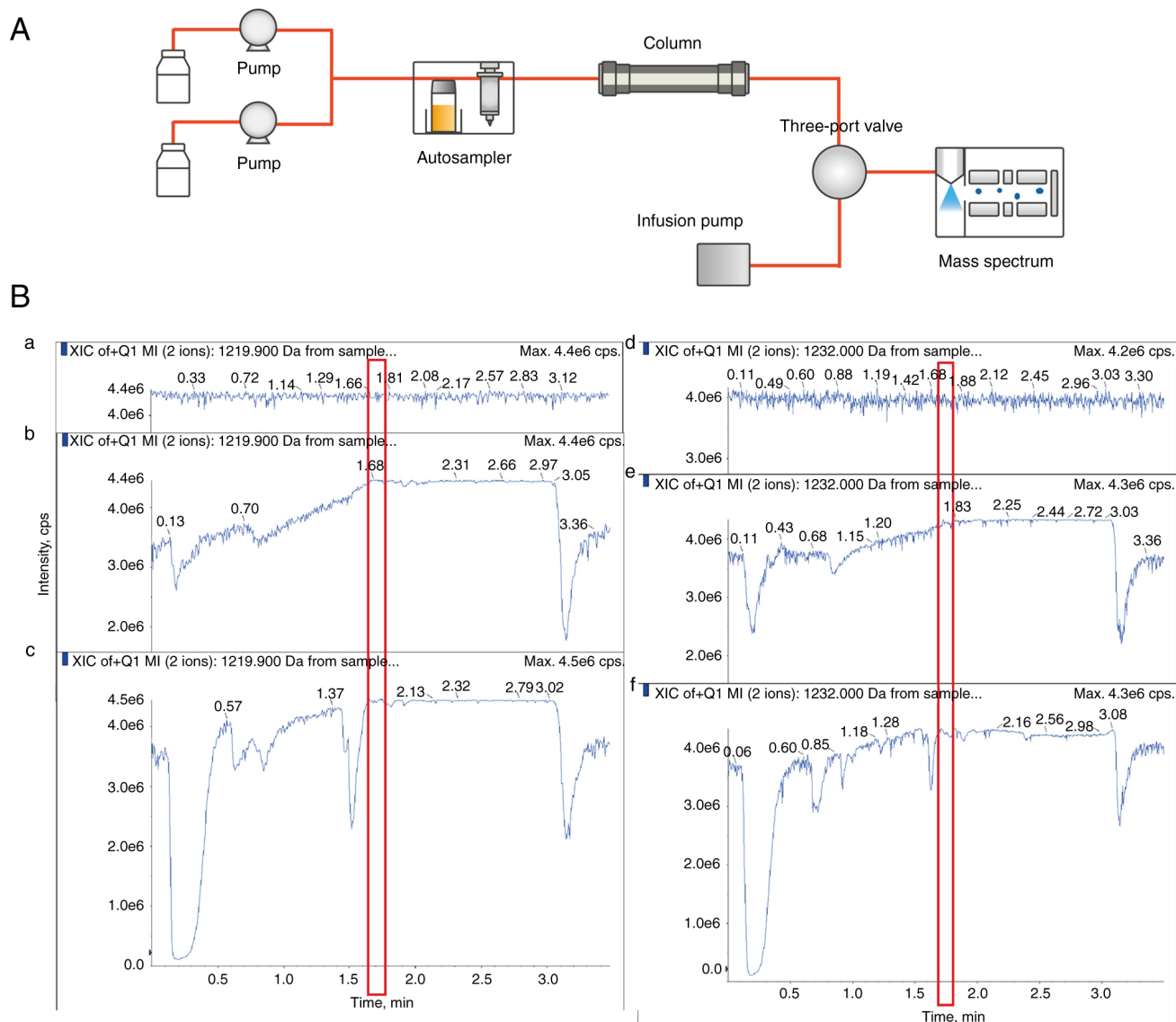


Figure 5. The post-column infusion system and ion chromatograms of cyclosporin A and cyclosporin A-d12. (A) Schematic of the post-column infusion system. (B) Comparison of (a, d) analytes directly infused into the mass spectrometer using a syringe pump (30 μ l/min) after reaching a stable total ion count, (b, e) 50% methanol/water injection whole blood sample prepared by protein precipitation, and (c, f) extracted whole blood sample injection using the post-column infusion method. The time points (around 1.7 min) when the matrix components are eluted from the columns are marked in the red box. a, b, and c for cyclosporin A; d, e, and f for cyclosporin A-d12. XIC, extracted ion chromatogram.

Table III. Results of cyclosporin A blood concentrations monitoring in patients with nephropathies.

Diseases	Monitor frequency	C ₀ , ng/ml (range)
Nephrotic syndrome, n=33	141	81.95 (<10.4-872.18)
Membranous nephropathy, n=9		72.02 (13.35-296.13)
Minimal change disease, n=4		76.57 (19.69-281.42)
Kidney transplant, n=8	103	149.95 (38.43-1079.25)

C₀, CSA trough concentration. Data presented as median (full range).

Table IV. CSA blood concentration in patients with nephropathy with different drug combinations.

Drugs	C ₀ in nephrotic syndrome, ng/ml	C ₀ in kidney transplant, ng/ml (range)
Antihypertensives	72.02 (<10.4-385.58), n=22	149.95 (48.63-1,079.25), n=6
Lipid-modifying drugs	60.75 (<10.4-296.13), n=13	n=0
Corticosteroids	55.00 (<10.4-296.13), n=12	153.00 (48.63-1,079.25), n=3
Calcitriol	48.13 (<10.4-296.13), n=9	151.76 (48.63-1,079.25), n=2
Diuretics	81.22 (15.50-360.11), n=8	n=0
Gastric mucosal protective agents	69.93 (<10.4-281.42), n=8	151.76 (48.63-1,079.25)
Liver-protection drugs	40.79 (<10.4-239.43), n=6	n=0
Mycophenolic acid	93.80 (40.9-167.81), n=2	153.00 (48.63-1,079.25), n=3

C₀, CSA trough concentration. Data presented as median (full range).

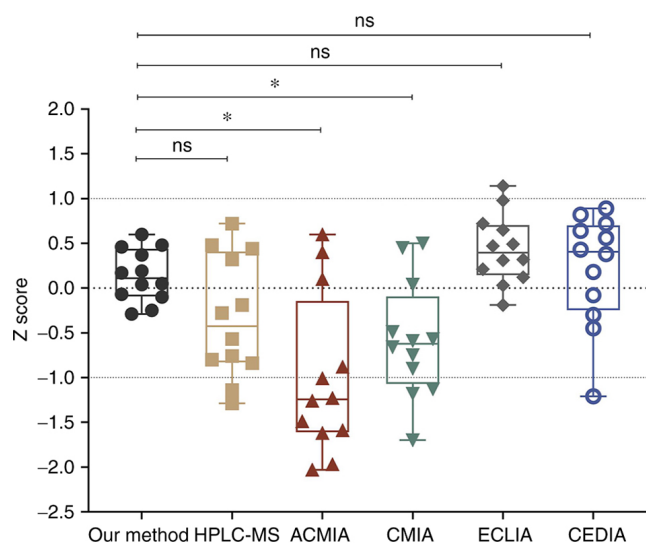


Figure 6. Comparison of the modified liquid chromatography-tandem mass spectrometry method with five other common methods using 12 samples with a concentration range from 121.74 to 1,439 ng/ml. The Z score represents the deviation between the measured value and the target value. * $P < 0.05$. ns, not significant; HPLC, high-performance liquid chromatography; ACMIA, affinity chrome-mediated immunoassay; CMIA, chemiluminescent microparticle immunoassay; ECLIA, electrochemiluminescence immunoassay; CEDIA, cloned enzyme donor immunoassay.

CKD stages. Patients with NS in the CKD3 stage had higher blood concentrations than those in the CKD1 stage ($P < 0.05$). However, no statistically significant differences between other

grades in patients with either NS or kidney transplants were observed ($P > 0.05$, Table V).

Discussion

In the monitoring of ISDs, sample preparation is typically regarded as the bottleneck of the analytical method (20). In the blood, CSA is often bound to plasma proteins and erythrocytes, in proportions influenced by multiple factors such as temperature, hematocrit, and drug concentration. Therefore, whole blood samples were used for CSA concentration detection to ensure accurate and stable results (20). When detected directly without any purification pretreatment, the complex matrix in whole blood blocks the chromatographic channels and contaminates the mass spectrometer, thereby increasing the maintenance costs and decreasing the lifetime of the instrument. Even after sample pretreatment, considerable daily maintenance is required for LC-MS/MS.

Notably, the complicated purification process is time-consuming and technically challenging (because of personnel requirements, quality control for the overall process, and auxiliary equipment demands). Protein precipitation (PPT), liquid-liquid extraction (LLE), and solid-phase extraction (SPE) are most frequently used for blood CSA preparation (5-9,21). Compared to existing protein precipitation methods, the sample preparation procedure often contains multiple liquid-adding and mixing steps (Table VI). In this study, zinc sulfate was pre-mixed with CSA-d12 in methanol/water and added together. This one-step PPT for sample preparation was rapid and easier to perform (Table SI).

Table V. Distribution of CSA blood concentrations in different CKD stages.

Stage	C ₀ in nephrotic syndrome, ng/ml	C ₀ in kidney transplant, ng/ml
CKD1	73.05 (<10.4-872.18)	-
CKD2	76.55 (<10.4-360.11)	154.79 (47.05-247.29)
CKD3	120.39 (<10.4-310.97)	149.94 (38.43-1079.25)
P-value	NS CKD1 vs. NS CKD2, 0.783; NS CKD1 vs. NS CKD3, 0.062; NS CKD2 vs. NS CKD3, 0.129.	Kidney transplant CKD2 vs. CKD3, 0.302; NS CKD2 vs. kidney transplant CKD2, <0.001; NS CKD3 vs. kidney transplant CKD3, 0.457.

CKD, chronic kidney disease. C₀, CSA trough concentration. NS, nephrotic syndrome. Data presented as median (full range).

Recently, increased efforts have been made to improve the throughput of CSA detection. Several of these reduced the LC running time using smaller diameter separation media (14,22,23), which may be accompanied by a high column pressure and a higher risk of column blockage. Additionally, a RapidFire high-throughput solid-phase extraction system with running times of <15 sec per sample has been reported in recent years (8). From a clinical point of view, the injection volume of 400 μ l makes the method less suitable for those patients whose sample collection is more difficult. This novel method is not yet widespread in domestic laboratories, thus normalization and standardization are required. In this study, a C18 column 50x2.1 mm, with 2.7 μ m pore size was used for separation, and the mass spectrometer was coupled to two HPLC systems. Furthermore, only part of the sample after LC separation was allowed to enter the mass spectrum, through this double HPLC system coupled with one mass spectrometry design. Consequently, the instrument throughput was improved allowing for a routine detection time of 4.3 min for two samples, that is, 2.15 min per sample. This study suggested that the two coupled LC systems can be considered an available alternative for improving the throughput in laboratories with a large number of samples. As CSA has a narrow therapeutic window, the rapid and high throughput LC-MS/MS method for drug-level monitoring may contribute to informing clinical decision-making better.

Importantly, this easily operable method has not only high throughput but also good analytical performance. A potential advantage is the use of CSA-d12 for the internal standard. Consistent with the view of Yang *et al* (24), internal standards should be carefully selected when using HPLC-MS/MS to measure immunosuppressants, as they are critical for the performance of detection. The findings of Taylor *et al* (25) revealed the superior performance of CSA-d12 when compared with CSD and ascomycin. They described the presence of interference in the CSD mass transition leading to negative skewing for high CSA concentrations. Regarding the deuterium-labeled CSA, the isotope abundance contributions of CSA-d4 with fewer isotopes also challenge the accuracy. Furthermore, the inter-day precision is <3.64%, and the intra-day precision is <1.64%. One possible reason is that the chromatographic separation time used in the present study was not long enough, which avoids the influence of the sample matrix. Meanwhile, there is a stable room temperature controlled within $\pm 2^{\circ}\text{C}$ in our laboratory, which is essential for reproducible results (26).

This modified LC-MS/MS method was applied for the detection of CSA concentrations in clinical practice. To the best

of our knowledge, multiple factors such as gene polymorphisms, concomitant medications, and other conditions play important roles in the pharmacokinetic variability of CSA and influence blood concentrations (27,28). For managing the patient's condition and preventing complications, ISD treatment among patients with nephropathy is frequently combined with corticosteroids, anti-hypertensives, or lipid-modifying drugs. From a metabolic perspective, the CSA concentrations in patients with different drug combinations might yield different results, owing to drug-drug interactions (5,29). In this study, the CSA concentrations detected by using the LC-MS/MS method were compared with those detected by other methods presented in previous studies in patients with NS or kidney transplants receiving the same drug combinations (30-33) (Table VII). However, from a detection perspective, tandem mass spectrometers provide highly specific information for drug monitoring. The mass pairs of precursor and product ions are unique to the specific compound (34). Therefore, the analyte concentrations detected by LC-MS/MS were not affected by PK-drug drug interactions. Furthermore, nephropathies directly affect drug pharmacokinetics (for example absorption, distribution, and metabolism) in parallel with the decrease in GFR and tubular secretion, thus resulting in a decline in kidney drug clearance, and molecular accumulation (35). The CSA concentrations may differ among patients with nephropathy at different stages of kidney function. In this study, the CSA concentrations detected by our LC-MS/MS method were compared with other methods presented in previous studies in patients with nephropathy and similar eGFR (31,36) (Table VIII). The LC-MS/MS method presented in this study may provide a much wider detection range and a lower limit of detection in actual clinical applications, which appeared to outperform other methods described in previous studies.

However, the limited sample size was a drawback of the study. In follow-up studies, the sample size will be expanded further to perform more in-depth and comprehensive research.

In conclusion, the design of multi-LC systems plays a significant role in the improvement of daily detection throughput, to allow for the application of LC-MS/MS as an integral part of 24/7 diagnostics in the near future. The high throughput LC-MS/MS method with a simple sample preparation procedure, a much wider detection range, and a lower limit of detection than those of prior methods is suitable for mass specimen detection and thus provides high clinical application value.

Table VI. Comparison of the modified LC-MS-MS with previous LC-MS-MS methods.

A, Analytical performance									
Performance parameters	Present study	31156436 ^a	33780006 ^a	29206806 ^a	30350284 ^a	19065123 ^a	20010460 ^a	18520600 ^a	33068377 ^a
Intra-precision	<1.64%	<7.43%	<4.93%	<6.48%	-	<6.30%	<3.30%	≤4.70%	<6.00%
Inter-precision	<3.64%	<7.43%	<5.88%	<8.57%	-	<6.00%	<7.00%	≤5.10%	<6.00%
Accuracy-recovery	89.4%-104.7%	±7.0%	>79.8%	100.4-110.5%	-	-2.5-2.7%	97.5-107.7%	89.0%	<1.3%
EQA Bias	1.90%	-	-	-	-	-	-	3.00%	-
Linear range, ng/ml	5.85-1,890.00	10-3,000	5-2,500	5-2,000	25-1,000	10-2,000	10-1,500	25-2,000	16-450
Quantitation range (ng/ml)	5.85-15,120.00	-	-	5-20,000	-	-	-	-	-
Dilution times	8	-	-	10	-	5	-	-	-
B, Workflow procedure									
Operating parameters	Present study	31156436 ^a	33780006 ^a	29206806 ^a	30350284 ^a	19065123 ^a	20010460 ^a	18520600 ^a	33068377 ^a
Sample volume, μ l	30	50	50	50	200	200	100	100	-
Sample preparation	One-step preparation	Two-step preparation	Solid phase extraction	Four-step preparation	-	Two-step preparation	One-step preparation and online clean up	Two-step PPT	Protein precipitation
Internal standard	CSA-d12	CSD	CSA-d4	Ticlopidine	CSA-13C2, d4	Ascomycin	CSA-d4	Ascomycin and cyclosporin D	-
Analysis time per sample	2.15 min	5.0 min	4.0 min	2.2 min	15 sec (RapidFire 365)	2.6 min	1.5 min	1.5 min	4.0 min

^aPMID. EQA, external quality assessment; CSA, cyclosporin A; CSD, cyclosporin D.

Table VII. Comparison of CSA concentrations between our method and others in patients with nephropathy treated with the same drug combinations.

First author <i>et al</i> , year	Drug Combinations	Methods	CSA daily dose, mg/day	CSA concentration, ng/ml (range)	(Refs.)
Inoue <i>et al</i> , 2013	CSA + corticosteroids	Enzyme immunoassay, n=11	100-150	70.00 (36.00-101.00)	(30)
Sommerer <i>et al</i> , 2008	CSA + corticosteroids	LC-MS/MS, n=20		45.46 (<10.4-98.11)	
		Enzyme multiplied immunoassay technique, n=20	175 (100-300)	105.00 (67.00-206.00)	(31)
		LC-MS/MS, n=30	200 (100-300)	152.88 (48.63-1079.25)	
Noreikaite <i>et al</i> , 2017	CSA + mycophenolic acid + corticosteroids	LC-MS/MS, n=83	204.80±46.26 120-300	59.00-254.00	(32)
		LC-MS/MS, n=30	208.33±43.71 100-300	48.63-1,079.25	
Wasilewska <i>et al</i> , 2007	CSA + antihypertensives + corticosteroids	Immunofluorescence, n=40	≤150	36.70-215.30	(33)
		LC-MS/MS, n=24	≤150	13.35-296.13	

LC-MS/MS, liquid chromatography-tandem mass spectrometry; CSA, cyclosporin A. Data presented as median (full range).

Table VIII. Comparison of CSA concentrations between the method used in the present study and previous methods.

First author <i>et al</i> , year	Methods	Estimated glomerular filtration rate, ml/min/1.73m ²	CSA daily dose, mg/day	CSA concentration, ng/ml	(Refs.)
Sommerer <i>et al</i> , 2008	Enzyme multiplied immunoassay technique, n=20	55 (28.00-112.00)	175 (100-400)	109 (69.00-176.00)	(31)
Trkulja <i>et al</i> , 2014	Affinity column-mediated immunoassay. n=43	37.8 (16.60-102.10)	-	70.00-341.00	(36)
The present study	Liquid chromatography-tandem mass spectrometry, n=76	58.47 (26.24-99.33)	200 (100-300)	109.74 (<10.40-1,079.25)	-

CSA, cyclosporin A. Data presented as median (full range).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BBZ and XZH designed the study. BBZ, BLL, and HD performed the method validation. KKY was responsible for recording clinical data. YSY, JML, XRL, and CMK performed the data analysis. YSY and JML wrote the manuscript. PFK, XRL, YX, and LC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Committee for Ethical Review of Research Involving Human Subjects of Guangdong Provincial Hospital of Chinese Medicine (Guangdong, China).

Patient consent for publication

Written informed consent was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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