Original Article

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A comparison of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and enzyme-multiplied immunoassay technique (EMIT) for the determination of the cyclosporin A concentration in whole blood from Chinese patients

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Summary **Cyclosporin A (CyA) is an immunosuppressive agent widely used in clinical therapy. In the therapeutic process, the blood concentration of CyA should be monitored to avoid or prevent rejection and toxicity. The objectives of this study were to compare the correlation of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and enzyme-multiplied immunoassay technique (EMIT) for the determination of the CyA concentration in human blood and to provide evidence for the rational usage of EMIT in clinical practice. Blood samples collected from 132 patients undergoing a liver or kidney transplant or patients with aplastic anemia at Qilu Hospital of Shandong University were tested using the two methods. The calibration curve was linear from 25-500 ng·mL^{-1} for LC-MS/MS and from 50-450 ng·mL^{-1} for EMIT. The inter- and intra-day RSDs were less than 15%. The CyA blood concentration according to EMIT was 3.5 ng·mL^{-1} more than that according to LC-MS/MS. The 95% confidence interval was –10.0~16.9 ng·mL^{-1}. The CyA blood concentration according to the two methods did not differ significantly ($p > 0.05$). LC-MS/MS and EMIT were suitable methods for determining the CyA blood concentration. The two methods were closely correlated ($r^2 = 0.969$), but the CyA blood concentration according to EMIT was slightly higher than that according to LC-MS/MS. The clinical significance of this finding needs to be further evaluated.**

Keywords: Cyclosporin A, LC-MS/MS, enzyme-multiplied immunoassay technique, comparison, therapeutic drug monitoring

1. Introduction

Cyclosporin A (CyA) is a highly lipophilic cyclic peptide consisting of 11 amino acids with selective and potent immunosuppressive activity, but it also causes a number of untoward adverse reactions. Therapeutic applications of CyA include kidney, liver, heart, lung, pancreas, and bone marrow transplants, treatment of autoimmune and rheumatoid diseases, and uses in dermatology and pulmonology (*1*). Immunosuppressive therapy with CyA follows a narrow path between the risk of rejection as a result of too little immunosuppression and toxic organ damage as a result of too much immunosuppression. Therapeutic drug monitoring (TDM) is recommended for monitoring blood/plasma CyA levels to avoid or prevent rejection and toxicity (*2,3*). Dose adjustment based on TDM leads to a better clinical outcome, especially in transplant recipients. During treatment with CyA, TDM and subsequent dosage adjustment for individual patients are required after liver transplantation to prevent rejection and over-immunosuppression. This leads to severe infection and adverse reactions including nephro-, hepato- and neurotoxicity (*4-*

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6). Many commercially available assays are used to monitor CyA levels; these assays involve techniques such as an enzyme-multiplied immunoassay (EMIT), an enzyme-linked immunosorbent assay (ELISA), and a fluorescence polarization immunoassay (FPIA) (*7*), that utilize the same monoclonal antibody against CyA. However, these methods are not specific enough as they cannot distinguish CyA from its metabolites, which may increase in concentration during CyA treatment or during liver dysfunction. Thus, more specific assays with better sensitivity are required to verify the CyA concentration.

High-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been used extensively in clinical laboratories over the last 10-15 years. This technique offers analytical specificity superior to that of immunoassays or conventional high-performance/pressure liquid chromatography (HPLC) for low molecular weight analytes. LC-MS/MS has superior analytical specificity and lower reagent cost, especially if one considers the ability to multiplex several different immunosuppressive drugs (8-10). LC-MS/MS is a sensitive and selective technique that could prove useful in TDM for CyA.

The current study created and validated a LC-MS/MS assay for CyA and the results of that assay were compared to those was obtained with an EMIT assay. This was done in order to determine whether the EMIT assays that are currently available are suitable for determining the concentration of CyA in blood as part of TDM and to provide an experimental basis for individualized CyA treatment.

2. Materials and Instruments

2.1. *Reagents and equipment*

CyA (Lot No. 30517, 95%) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Clarithromycin (Internal standard, IS, Lot No.130558-200501, 97.5%) of analytical grade was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Analytical ammonium formate (Lot No. F990215) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Zinc sulfate heptahydrate (Lot No. 20090210) of analytical grade was obtained from Tianjin Beifang Tianyi Chemical Reagent Factory (Tianjin, China). Acetonitrile (Lot No. 0000059829) and methanol (Lot No.0000118131) of chromatographic grade were obtained from J.T. Baker Company (Phillipsburg, New Jersey, USA), and pure water was obtained from the Hangzhou Wahaha Group Co., Ltd. (Hangzhou, Zhejiang Province, China). CyA-specific calibrators (Lot No. 6R119UL-H2), control CyA (Lot No. 6019), and a CyA sample preparation reagent (Lot No. J2) were obtained from Siemens Healthcare Diagnostic Ltd (Newark, New Jersey, USA).

The Agilent 1200 series HPLC system (Agilent Technology, Santa Clara, CA, USA), equipped with a G1367C auto-sampler connected to an Agilent 6410 Triple Quadruple mass spectrometer equipped with an electrospray ionization (ESI) source, was used in this study. The Siemens Viva-E automatic biochemical analyzer (Siemens Healthcare Diagnostics, Inc.) was also used. The PROIND centrifuge (Heraeus, Connaught, Germany), the XW-80A vortex mixer (Shanghai Jingke Industrial Co., Ltd., Shanghai, China), the PK514BP ultrasonic cleaner (BANDEL, Henstedt-Ulzburg, Germany), and the BHW-IV thermostatic water tank (Beijing Medical Equipment Factory, Beijing, China) were also used.

2.2. *Samples*

Blood samples used in this study were routinely collected from patients undergoing a liver or kidney transplant or patients with aplastic anemia receiving CyA treatment at Qilu Hospital of Shandong University. The study was conducted in accordance with the Declaration of Helsinki and patient consent was obtained. One hundred and thirty-two samples were collected from patients (males: 76, females: 56). Patient age ranged from 1 to 75 years, with a mean of 40.8 ± 18.6 years. All samples were collected between June and July 2016. Frozen blood samples were supplied in routine blood collection tubes and stored in the laboratory at -20°C prior to analysis.

2.3. *EMIT assay*

2.3.1. *Sample preparation*

A 300-μL extraction solution was added to 100 μL of whole blood. The mixture was then vortexed for 1 min and centrifuged at 10,800 rpm for 5 min. The supernatant was injected for analysis.

2.3.2. *Assay performance*

The CyA-EMIT kit consists of a CyA-specific assay, standard calibrators, controls (three levels), and a wash buffer. Samples were processed according to the kit's instructions.

2.4. *LC-MS/MS assay*

2.4.1. *Preparation of stock and working solutions*

A primary solution of CyA and the internal standard (IS) were separately prepared in acetonitrile at a concentration of 1 mg·mL^{-1}. Stock solutions of CyA were diluted from the primary solution with acetonitrile and then further diluted to working solutions of 250-

5,000 ng·mL^{-1}. A quality control (QC) working solution of CyA was diluted from the stock solution (1 mg·mL^{-1}) with acetonitrile to reach a concentration of 500, 2,000, or 4,000 ng.mL^{-1}. A working solution of IS (100 ng·mL^{-1}) was prepared by dilution of the stock solution (20 μg·mL^{-1}) with acetonitrile. All standard solutions were stored at 4°C. The stability of the standard solutions was verified throughout the study. A solution of zinc sulphate was prepared in water at a concentration of 100 mM to serve as a cell lysis buffer.

2.4.2. *Chromatography and mass spectrometry conditions*

CyA and clarithromycin (IS) in whole blood were separated on a Diamonsil C18 column (150 × 4.6 mm, 5 μm, Waters, Milford, MA, USA) and eluted with a mobile phase of 15 mM ammonium formate and 0.5% formic acid:acetonitrile (12:88, v/v) at a flow rate of 0.8 mL/min. The temperature of the auto-sampler was set at room temperature and the temperature of the column oven was set at 70°C.

The source parameters were as follows: sprayed gas (nitrogen) at a temperature of 350°C, a spray flow of 9 L/min, a nebulizer pressure of 40 psi, and a capillary voltage of 4,000 v. A full scan mass spectrum was obtained over a range of m/z 200 to 1,300. The fragmentor voltage was 100 V for CyA and 90 V for the IS, and the collision energy was 50 eV for CyA and 20 eV for the IS. The delta EMV was 300V. Multiple reaction monitoring (MRM) mode was used to detect CyA and the IS.

2.4.3. *Preparation and disposition of samples*

Calibration and QC samples were prepared during the validation of the two methods and testing of whole blood samples. The concentration of the calibration samples was 25, 50, 75, 100, 200, 300, 400, and 500 ng·mL^{-1}. The concentration of the QC samples was 50, 200, and 400 ng·mL^{-1}.

Protein precipitation was used in sample preparation. A zinc sulfate heptahydrate solution (50 μL, 100 mM) was added to blood samples (150 μL) and mixed. The IS working solution (10 μL) was then added, and the mixture was vortexed for 1 min. Afterwards, 700 μL of methanol was also added and the mixture was vortexed again for 2 min and then centrifuged at 10,800 rpm for 5 min. The supernatant was injected into the LC-MS/MS system for analysis.

2.4.4. *Method validation*

The LC-MS/MS method was validated in terms of specificity, the matrix effect and extract recovery, linearity and lowest limit of quantitation (LLOQ), intra- and inter-day precision, and storage stability. Validation strictly conformed to the Chinese Food and Drug Administration guidance on the validation of methods of biomedical analysis.

Specificity. The specificity of method was evaluated by comparing chromatograms of blank blood, a standard solution of CyA and the IS, blank blood spiked with CyA and the IS, and blood from patient 88 after oral administration of CyA.

Matrix effect and extraction recovery. The matrix effect and extraction recovery of CyA and the IS were evaluated with five replicates of the QC samples at three concentrations (50, 200, and 400 ng·mL^{-1}) and five replicates of the IS (100 ng·mL^{-1}). Extraction recovery was evaluated by comparing peak areas of CyA or the IS in mixed blank blood samples to which CyA or IS had been added after extraction.

Calibration curve and LLOQ. A calibration curve was generated with concentrations of 25, 50, 75, 100, 200, 300, 400, and 500 ng·mL^{-1} in the blood. Samples of each concentration were analyzed in five replicates. A calibration curve was derived by plotting the peak area ratios of CyA to the IS as a function of concentration of CyA. The calibration curve was described by the equation $y = ax + b$, where y corresponds to the peak-area ratio and x to the ratio of the concentration of CyA to IS. The linearity of the calibration curve was assessed using linear regression with the reciprocal of the concentration squared ($1/x^2$) serving as a weighting factor. The LLOQ was evaluated by analyzing five replicates of mixed whole blood samples at the concentration of 25 ng·mL^{-1}.

Accuracy and precision. Accuracy and precision were determined using the same data set. Intra-day precision was determined based on 3 determinations: the LQC (25 ng·mL^{-1}), the MQC (200 ng·mL^{-1}), and the HQC (400 ng·mL^{-1}). The inter-day precision of each assay was analyzed based on the LQC, MQC, and HQC on three different days over a period of one week. Precision was expressed as the coefficient of variation (RSD).

Stability. The stability of CyA in the biological matrix was evaluated as follows and the results were expressed as the percentage recovery. The stability of CyA in blood samples was examined when samples were stored at -20°C for 60 days. Freeze-thaw stability was determined after two cycles of freezing (-20°C) and thawing (25°C) QC samples. The stability of samples on the bench after extraction and in the LC-MS/MS auto-sampler was also determined for 6 and 12 h at room temperature.

2.5. *Statistical analysis*

Data are presented as the mean ± SD. Statistical analysis was performed using regression analysis. A *p*-value of < 0.05 was considered statistically significant. The statistical software SPSS was used to

evaluate the correlation between the two assays and Med-Calc software was used to draw a Bland-Altman plot. The Bland-Altman plot is useful in revealing the relationship between the differences and the magnitude of measurements, indicating any systematic bias, and in identifying possible outliers.

3. Results

3.1. *EMIT assay*

Calibration curve. A calibration curve was automatically obtained from the Viva-E automatic biochemical analyzer. A four-point logarithmic curve was used to obtain the formula for the calibration curve, which was $A = Ro + K^* (1 / [1 + \exp(-a + b^*(\ln C)])$. The parameters were $Ro = 2.61010 \times 10^2$, $K = 8.19348 \times 10^1$, $a = -7.22146$, and $b = 1.40956$.

Precision and accuracy. Intra-day precision was determined based on LQC, MQC, and HQC on 3 different days, and the inter-day precision of each assay was analyzed based on the LQC, MQC, and HQC. The precision of QC for CyA was expressed as the coefficient of variation (RSD). The inter- and intra-day RSDs were less than 15% (Table 1).

3.2. *LC-MS/MS assay*

Specificity. The full scan and product ion mass spectrum of CyA and the IS are shown in Figure 1, while typical

Table 1. The intra-day and inter-day precision of the CyA concentration according to EMIT ($n = 5$)

Concentration (ng mL^{-1})	Intra-day			Inter-day		
	Mean ± SD	Accuracy (%)	RSD (%)	Mean ± SD	Accuracy (%)	RSD (%)
76.6	67.7 ± 5.0	88.4	7.4	75.6 ± 8.0	98.8	10.6
190.4	201.6 ± 26.7	105.9	13.2	192.9 ± 17.7	101.3	9.2
350.2	307.9 ± 41.1	87.9	13.4	325.1 ± 31.3	92.8	9.6

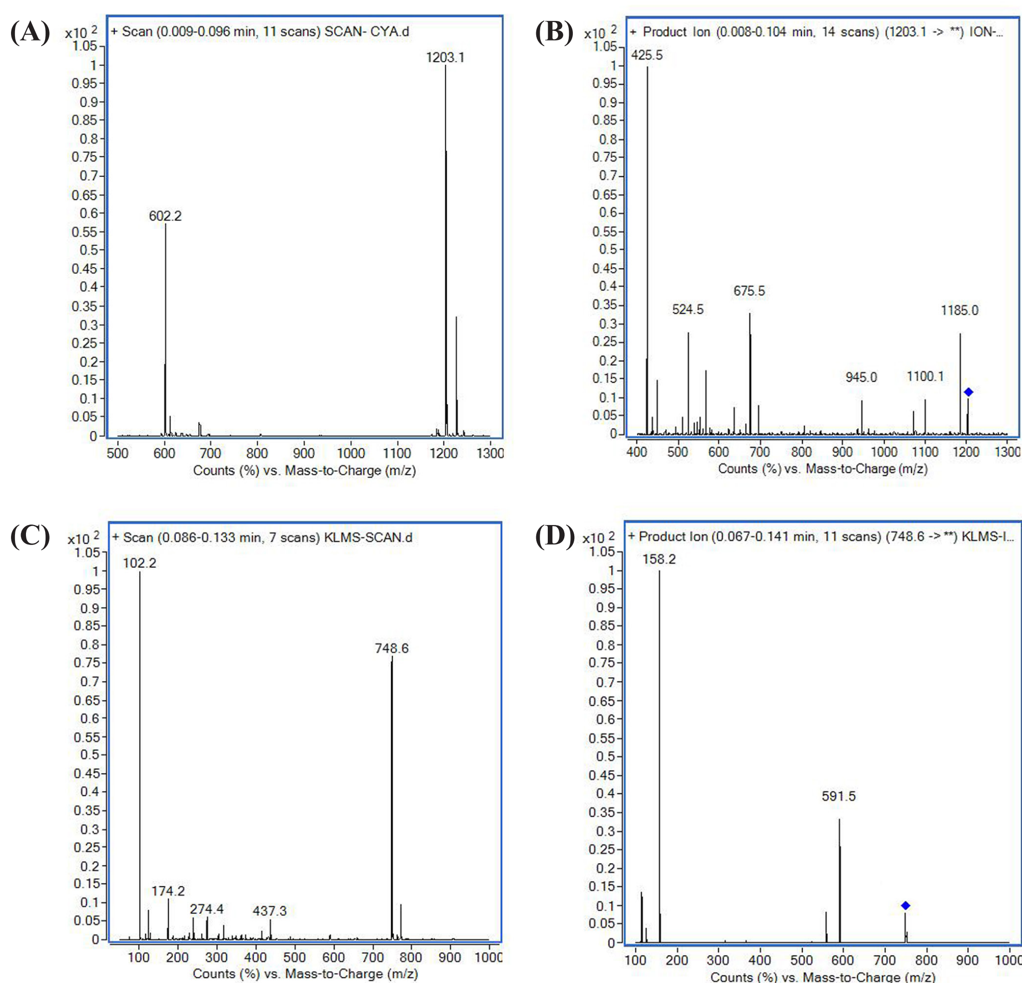

Figure 1. Full scan and product ion mass spectra for CyA (A, B) and an IS (C, D)

MRM chromatograms are shown in Figure 2. MRM mode was used to detect CyA and the IS, with an ion transition of m/z 1203.1→425.5 (CyA) and 748.6→158.2 (IS), respectively. The retention time was about 1.8 min for the IS and 5.5 min for CyA. There was no significant interference with endogenous blank human blood at the retention time for the analyte and the IS.

Calibration curve and LLOQ. The calibration curve for CyA was linear over the concentrations governed by the regression equation (weight = $1/x^2$), which was y = 0.016 x + 0.0408. The correlation coefficient was 0.9950. The LLOQ of CyA was 25 ng·mL^{-1} (n = 5) with a precision of 3.1% and an accuracy of 106%.

Recovery and matrix effect. A high percentage of CyA and the IS were recovered and results of both methods were highly reproducible. No significant matrix effect was observed when the CyA concentration was determined at three different QC levels and when the IS was tested at a concentration of 100 ng·mL^{-1}. The mean recovery was 75.73% and the matrix effect was 93.75% for CyA, and the mean recovery was 94.33% and the matrix effect was 97.19% for the IS. The RSD was less than 15%, indicating that the analytical methods were free of endogenous substances in human blood.

Precision and accuracy. The intra- and inter-day precision and accuracy of the CyA concentration were acceptable for analysis. Results are shown in Table 2.

Stability. The stability of CyA in whole human

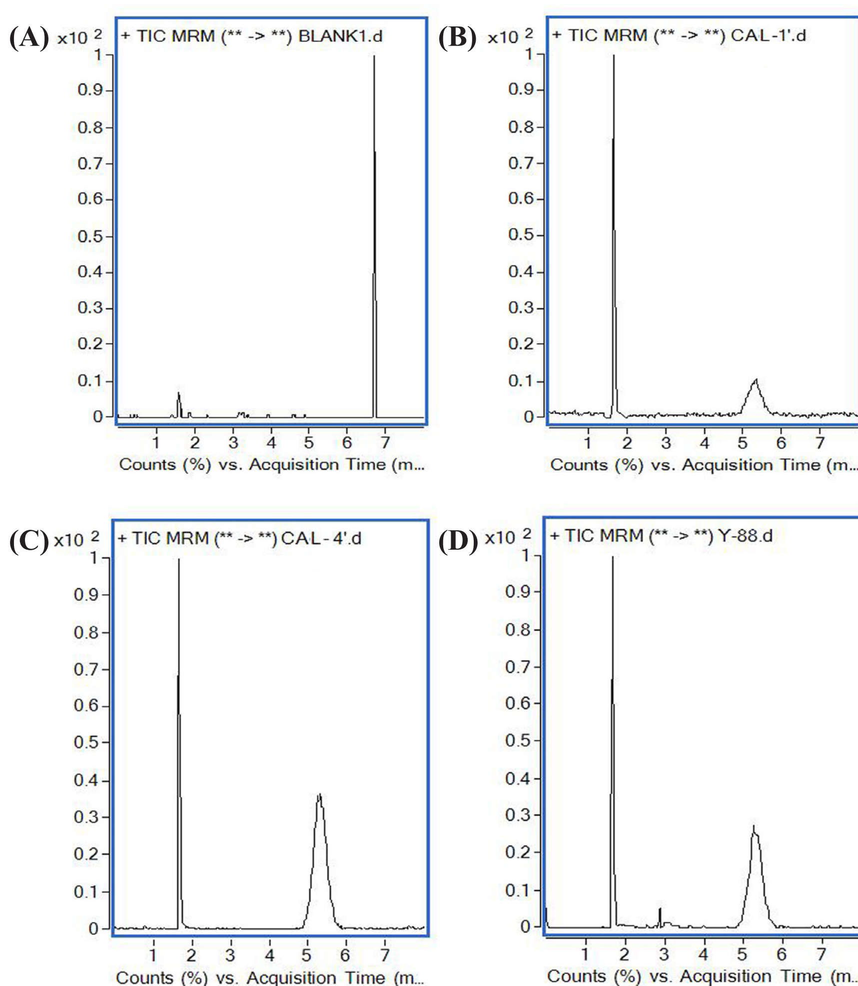

Figure 2. Typical MRM chromatograms for CyA and an IS. (A) blank blood; **(B)** LLOQ-blank blood spiked with CyA (25 ng/mL) and the IS (100 ng/mL); **(C)** blank blood spiked with CyA (200 ng/mL) and the IS (100 ng/mL); **(D)** blood spiked with the IS from patient 88 after oral administration of CyA.

Table 2. The intra-day and inter-day precision of the CyA concentration according to LC-MS/MS (n = 5)

Concentration (ng mL^{-1})	Intra-day			Inter-day		
	Mean ± SD	Accuracy (%)	RSD (%)	Mean ± SD	Accuracy (%)	RSD (%)
50	49.22 ± 2.08	98.4	4.24	49.45 ± 3.41	98.9	6.89
200	192.17 ± 1.61	96.1	0.84	202.65 ± 9.06	101.3	4.47
400	410.85 ± 19.15	102.7	4.66	390.48 ± 28.35	97.6	7.26

Table 3. Stability of CyA in human blood under different conditions (n = 5)

Conditions	50 ng·mL^{-1}		200 ng·mL^{-1}		400 ng·mL^{-1}	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
-20°C/60 d	48.77 ± 2.18	4.47	189.43 ± 7.75	4.09	384.89 ± 8.70	2.26
-20°C/1 freeze-thaw cycle	50.63 ± 3.50	6.91	199.38 ± 8.21	4.11	394.90 ± 46.90	11.88
-20°C/2 freeze-thaw cycles	49.28 ± 2.00	4.06	201.27 ± 13.42	6.67	368.40 ± 6.64	1.80
25°C/6 h (extracted samples)	50.43 ± 1.25	2.48	191.86 ± 17.27	9.00	383.69 ± 25.43	6.63
25°C/12 h (extracted samples)	51.18 ± 2.75	5.37	184.68 ± 4.50	2.44	358.48 ± 8.69	2.42

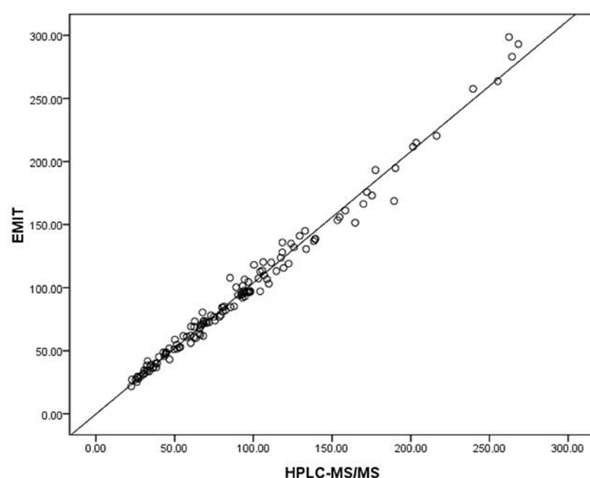

Figure 3. Correlation of the regression curve for LC-MS/MS and EMIT (n = 132).

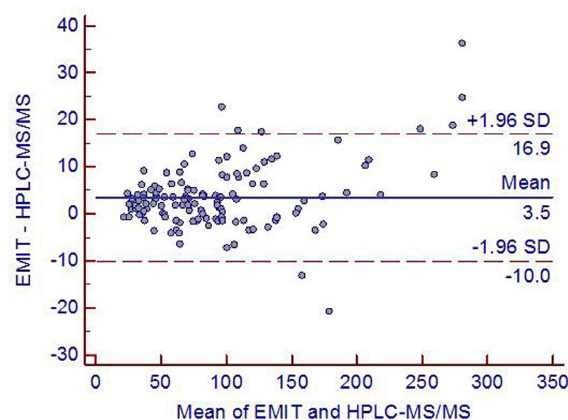

Figure 4. The Bland-Altman deviation graph of the CyA concentration according to LC-MS/MS and EMIT (n = 132).

blood was investigated under different storage conditions and processing. CyA was stable after 6 h and 12 h on the bench or in the auto sampler, it was stable after two freeze-thaw cycles, and it was stable after freezing at –20°C for 60 days. These results are shown in Table 3.

3.3. *Comparison of EMIT and LC-MS/MS*

One hundred and thirty-two patient samples were tested with EMIT and LC-MS/MS. Both methods can be used to determine the CyA concentration in blood. The LLOQ was 25 ng·mL^{-1} for LC-MS/MS and 50 ng·mL^{-1} for EMIT. EMIT had less reproducibility than LC-MS/MS did, but the two assays were closely correlated over a range of concentrations from 26.24-293.06 ng.mL^{-1} (R^2 = 0.969, Figure 3).

In addition, a plot of the differences between the two methods in terms of the mean concentrations they yielded indicated a close relationship between the two methods, albeit with slight differences. The Bland-Altman deviation graph of CyA is shown in Figure 4. As shown in the plot, the 95% confidence interval was –10.0~16.9 ng·mL^{-1}, and most of the data were within the 95% confidence interval. Thus, there was no systematic bias in terms of differences in CyA concentrations and the magnitude of those measurements.

The CyA blood concentration according to the EMIT assay was slightly higher than that according to the LC-MS/MS assay. One potential explanation for this would be non-specific binding to antibodies in the EMIT assay. The LC-MS/MS assay had excellent reproducibility, suggesting that sample handing was an unlikely source of error.

4. Discussion

The LC-MS/MS technique devised here is a reliable and sensitive assay of the CyA concentration in human blood with an LLOQ of 25 ng·mL^{-1}, which is 2-fold lower than that of EMIT (50 ng.mL^{-1}) assays that are currently available.

Simple protein precipitation with methanol was a robust way to prepare samples for LC-MS/MS and provided clean samples in this study. Previous studies have used liquid-liquid extraction with ethyl ester (*11*) or tert-butyl-methyl-ether (*12*) as extraction agents. Solid-phase extraction has been used to extract CyA from biological samples in other studies (*13,14*). Procedures for liquid-liquid extraction and solid-phase extraction are quite complicated, time-consuming, and potentially dangerous both to the environment and the experimenter. In contrast, protein precipitation is rapid,

environmentally friendly, and convenient for continuous batch analysis, making this technique suitable for analysis of the CyA concentration in human blood.

LC-MS/MS has proven to be useful in determining the CyA concentration as part of TDM since it is more reliable, sensitive, and rational than EMIT. In the current study, the CyA blood concentration according to the EMIT assay was slightly higher than that according to the LC-MS/MS assay. LC-MS/MS had excellent reproducibility, suggesting that sample handing is an unlikely source of error. The most likely explanation for these results would be non-specific binding to antibodies in the EMIT assay. EMIT depends on the reaction between an analyte and a biological antibody, so it may involve more inherent imprecision than other methods of pharmaceutical analysis (*e.g.* chromatography). The specificity of immunoassays depends mainly on the antibody targeting the analyte, but some immunoassays are not highly selective and they may respond to a group of compounds (*e.g.* metabolites) rather than individual compounds. Due to the cross-reaction between a drug and metabolites, EMIT may overestimate the CyA concentration, so monitoring the concentration of CyA with EMIT might lead to too low a dosage of CyA. This could affect the effective administration of CyA, and especially in patients with a low concentration of CyA in the blood since CyA is already a narrowly defined therapeutic index.

Techniques such as a chemiluminescent enzyme immunoassay (CLIA) and an electro-chemiluminescence immunoassay (ECLIA) have recently been used in TDM and have become key methods of measuring the CyA concentration in blood. CLIA systematically yielded a higher CyA concentration than UPLC-TMS did (*15*). Compared to EMIT, CLIA and ECLIA have a higher sensitivity and a higher precision when measuring the tacrolimus concentration in blood (*16*). However, no study has compared EMIT and CLIA or ECLIA when measuring the CyA concentration as part of TDM, so this topic should be studied further.

The current study found a close correlation between the LC-MS/MS assay and previous immunoassays. This finding is reassuring since clinical estimates of the boundaries of CyA therapy, which were identified using immunoassays, remain intact. In addition, the increased sensitivity of the LC-MS/MS assay will allow an accurate determination of the CyA concentration in other patients treated with lower doses.

5. Conclusion

In conclusion, a comparison of LC-MS/MS and EMIT to determine the concentration of CyA in human blood indicated that EMIT slightly overestimated the CyA concentration. This finding is consistent with cross-reactivity of the EMIT antibodies with one or more CyA metabolites. However, the two methods were closely correlated. Therefore, the EMIT assay is suitable for TDM of CyA.

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