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## Liquid chromatographic–electrospray mass spectrometric determination of cyclosporin A in human plasma

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**Abstract** A rapid, sensitive and selective liquid chromatography–mass spectrometry (LC–MS) assay has been developed for determination of cyclosporin A (CyA) in human plasma; cyclosporin B (CyB) was used as internal standard (IS). The method utilized a combination of a column-switching valve and a reversed-phase symmetry column. The mobile phase was a 25:75 (v/v) mixture of 10% aqueous glacial acetic acid and acetonitrile. Running time per single run was less than 10 min. Sample preparation included C<sub>8</sub> SPE of human plasma spiked with the analyte and internal standard, evaporation of the eluate to dryness at 50°C under N<sub>2</sub> gas, and finally reconstitution in the mobile phase. Detection of cyclosporin A and the IS was performed in selected ion-monitoring mode at *m/z* 601.3 and 594.4 Da for CyA and IS, respectively. Quantitation was achieved by use of the regression equation of relative peak area of cyclosporin to IS against concentration of cyclosporin. The method was validated according to FDA guideline requirements. The linearity of the assay in the range 5.0–400.0 ng mL<sup>-1</sup> was verified as characterized by the least-squares regression line  $Y = (0.00268 \pm 1.9 \times 10^{-4})X + (0.00078 \pm 1.8 \times 10^{-3})$ , correlation coefficient,  $r = 0.9986 \pm 1.1 \times 10^{-3}$  ( $n = 48$ ). Intra and inter-day quality-control measurements in the range 5.0–350.0 ng mL<sup>-1</sup> revealed almost 100% accuracy and  $\leq 9\%$  CV for precision. The mean absolute recovery of CyA was found to be  $84.01 \pm 9.9\%$  and the respective relative recovery was  $100.3 \pm 9.19$ . The limit of quantitation (LOQ) achieved was 5 ng mL<sup>-1</sup>. Eventually, stability testing of the analyte and IS in plasma or stock solution revealed that both chemicals

were very stable when stored for long or short periods of time at room temperature or  $-20^{\circ}\text{C}$ .

**Keywords** Cyclosporin A · Cyclosporin B · LC–MS · Human plasma

### Introduction

Cyclosporin A (CyA), Neoral, or Sandimmune is a very lipophilic, neutral undecapeptide immunosuppressive drug of fungal origin [1–6]. It has the formula C<sub>62</sub>H<sub>111</sub>N<sub>11</sub>O<sub>2</sub> and a cyclic polypeptide structure [1, 5, 6] (Fig. 1).

CyA is not soluble in water or *n*-alkanes; it is, however, readily soluble in alcohol and natural oils. It lacks any chromophore and thus has weak far-UV absorption (ca 205 nm) [1, 6–8].

Pharmacologically, CyA has specific and potent immunosuppressive activity. Since its use was approved by the FDA in the 1980s [5] it has been used in conjunction with organ and bone marrow transplantation to prevent allograft rejection [1–3]. It has also been found to be very beneficial in the treatment of autoimmune diseases such as psoriasis, rheumatoid arthritis, and bowel inflammation [6, 9].

The mode of action of CyA is believed to be inhibition of the production and/or release of lymphokines and thus it acts against proliferation of certain types of lymphocyte and  $\delta$ -interferon [2, 3, 5, 10–12].

In vivo, CyA is widely distributed throughout the body and is found to be mainly bound to the hydrophobic sites of erythrocyte cells and plasma lipoprotein [1, 2, 4]. In liver, CyA is prone to metabolic changes, producing more than 25 metabolites within a half-life period greater than 10 years [5, 13, 14]. The major routes of metabolism include hydroxylation, cyclic ether formation, *N*-demethylation, and intra-molecular rearrangement [5, 13]. It is worth mentioning reports that CyA has a narrow therapeutic index [3]; its evaluation in blood is thus very crucial

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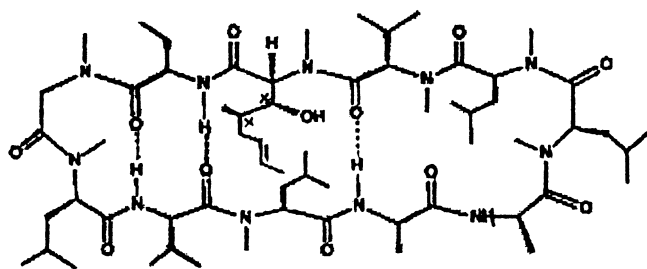


Fig. 1 Chemical structure of cyclosporin A

to guide the dose adjustments necessary for maximizing clinical benefits and avoiding incidence of organ rejection at lower dosage while minimizing overdose side-effects such as nephro, hepato, and neurotoxicity [3, 6]. Drug analysis is therefore very important and is a demanding analytical challenge, especially in quality control and pharmacokinetic studies.

In this context, a considerable body of literature exists describing a wide range of methods and techniques used for determination of CyA alone or with its metabolites in different matrices; among those reported are immunoassay (radio- and/or non-isotopic) and HPLC–UV [8, 12, 15–17]. The first is, however, limited by the expensive and commonly unavailable assay kits and by the non-specificity, as a consequence of drug metabolite cross-reactivity with the respective antibodies [18].

In turn, conventional HPLC–UV, despite its usefulness as a common reference method for quantitation of polar and thermally labile compounds, frequently suffers from difficulties [18] such as chromatographic interferences [11, 17], need for gradient elution [19], long analysis times [7, 20–23], peak broadening and column deterioration at high temperatures [2, 10, 17, 24], unavailability of a suitable internal standards [1, 25], lack of desired sensitivity [2, 3, 10, 14, 20, 24], and need for derivatization [27]. Other drawbacks include poor recovery [2, 3, 6, 20, 22, 28], low correlation coefficient [16], irreproducibility, confinement to matrices other than human plasma [9, 14–16, 22, 25, 26], large volume of blood used [1], and, finally, laborious sample preparation [31]. Such drawbacks have justified a substantial number of publications [17] and have emphasized the need to search for more reliable and efficient surrogates. Accordingly, more convenient methods in terms of sensitivity and rapidity have emerged utilizing more advanced technology, for example LC–MS [25] and LC–MS–MS [10, 17, 28]. However, these methods are not without disadvantages, because they are dedicated to measurement of CyA with other drugs and/or CyA metabolites in whole blood [10, 17, 28]. The LC–MS method reported used no internal standard [25] and the tandem-MS was performed at high temperature and recovery was poor [28] ( $\text{ca} \approx 72\%$ ). Also, many of the reported methods were conducted for comparison of HPLC and immunoassay [2, 3, 5, 8, 14, 24, 28].

Because of these difficulties and/or disadvantages coupled with the conclusion of various consensus panels of the need for CyA metabolite measurement [14, 30], the preference for using human plasma instead of whole blood in routine CyA analysis [29], and the importance of monitoring the narrow-index activity of CyA, it was deemed necessary to develop and validate a reliable, relatively simple, fast, sensitive and specific method, utilizing an available LC–MS technique, for determination of CyA in human plasma. Needless to say, many drug firms demand the use of LC–MS or tandem-MS for contract agreement. Therefore, the proposed method will be very useful in clinical monitoring and pharmacokinetic studies.

## Experimental

### Materials and reagents

Acetonitrile and methanol were of HPLC gradient grade (Merck, Germany), and glacial acetic acid was HPLC grade (Galena, France). Deionized water was laboratory prepared using EASYpure-RO and/or -UV system (USA). Solid CyA was B:15850 H, USP, CyB:12083 (Galena), SPE cartridges were (Oasis HLP, 1 cc, 30 mg/30  $\mu\text{m}$ ). The mobile phase was a 25:75 (v/v) mixture of 10% aqueous glacial acetic acid and acetonitrile.

### Equipment

Mass spectral analysis was performed with an AQA single-quadrupole LC–MS system (Thermoquest, Finnigan, UK). The system used consisted of a Symmetry C<sub>8</sub> (150 mm $\times$ 3.9 mm, 5  $\mu\text{m}$  particles) liquid chromatography column from Waters (Ireland), a column oven set at 60°C (COT-10A VP, Shimadzu, Japan) and a switching valve, PR-700-100-01, Lab Pro (Rheodyne, USA). The system included a P2000 LC pump, an SN4000 system controller, and an AS300 autosampler, all from Thermoquest (UK). The mass detector was equipped with (+)-ESI (Finnigan, UK) positive electrospray ionization and was operated in the SIM mode at  $m/z$  601.3 and 594.4 Da for CyA and CyB, respectively. LC–MS was controlled by Xcalibur software version 1.1 (Finnigan, UK).

### Standards

Stock standard solutions of CyA and IS were prepared separately by dissolving 100 mg of each in 100 mL methanol to give concentrations of 1 mg mL<sup>-1</sup>. Working standard solutions were then prepared by diluting 500  $\mu\text{L}$  of each stock solution to 100 mL with deionized water to afford concentrations of 5  $\mu\text{g}$  mL<sup>-1</sup>. Standards of CyA for calibration were prepared in 10-mL volumetric flasks by diluting appropriate volumes of the

working solution with drug-free human plasma to give 10 concentrations defined as blank (0.0), standard zero (0.0), 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, 300.0, and 400.0 ng mL<sup>-1</sup>.

Quality control standards of CyA in plasma for regular runs were also prepared in 25-mL volumetric flasks at concentrations of 5.0, 15.0, 175.0, and 350.0 mg mL<sup>-1</sup>.

### Sample preparation

Aliquots of 1,000  $\mu$ L from each calibration standard, including blank plasma, in separate Eppendorf microcentrifuge tubes were spiked with 100  $\mu$ L of the IS working solution (5.0  $\mu$ g mL<sup>-1</sup>). The samples were vortex mixed for 30 s and extracted using SPE cartridges. The cartridges were conditioned with methanol (1 mL) and equilibrated with deionized water (1 mL). The prepared samples were then loaded and the cartridges were washed with deionized water and finally eluted with methanol (1.5 mL). Eluates were evaporated to dryness at 50°C under N<sub>2</sub> gas. Residues were then reconstituted in 100  $\mu$ L mobile phase, vortex mixed for 1 min and finally transferred to glass microcentrifuge tubes and centrifuged for 5 min at a speed of 13,000 rpm.

### Measurements and calculations

Aliquots (50  $\mu$ L) of the prepared samples were injected and chromatographed using a combination of a switching valve and a Symmetry C<sub>8</sub> (150 mm $\times$ 3.9 mm, 5  $\mu$ m particle) HPLC column in an oven at 60°C. The analyte and the IS were separated by the mobile phase with a flow rate gradient of 0.7 mL min<sup>-1</sup> from 0 to 4.5 min and then 0.4 mL min<sup>-1</sup> from 4.5 to 11 min. MS detection was performed in SIM mode at *m/z* 601.3 and 594.4 Da for CyA and IS, respectively.

Calibration curves were constructed by plotting drug to IS peak-area ratio against nominal prepared concentration. The best fit least-squares linear equations were estimated and later used to back-calculate the concentration of standards and/or unknown sample concentrations.

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## Method validation

To check the reliability and overall performance of the assay and to meet the guidelines required by the FDA and/or ICH (International Conference on Harmonization) [32], the method was validated in terms as described below.

### Specificity and selectivity

The specificity of the assay was determined by screening six different batches of control blank human plasma to

ensure the absence of co-eluted peaks of endogenous compounds and/or drug metabolites at the retention times studied, as shown in Fig. 2.

The selectivity, in turn, was tested to check any potential interference peaks from six commonly used drugs, viz., acetaminophen, aspirin, ascorbic acid, ibuprofen, caffeine, and nicotine. The drugs were prepared and chromatographed in the mobile phase. It was observed there was no interfering peaks at the retention times studied.

### Linearity

Six individual internal standard addition calibration plots over the range 5.0–400.0 ng mL<sup>-1</sup> of the analyte were constructed. The respective best fit linear regression equations and correlation coefficients were deduced and averaged to give a representative general equation.

The mean, standard deviation, precision quoted in terms of CV%, and accuracy of the back-calculated concentrations are given in Table 1.

### Sensitivity

The lowest standard concentration in the calibration plot is regarded as the lower limit of quantitation (LLOQ) if its chromatographic response is  $\geq 5$  times that of blank response, with accuracy  $\geq 80\%$  and precision  $\leq 20\%$ . No attempts were made to obtain measurements below this limit.

### Accuracy and precision

The reliability of the method in terms of accuracy, repeatability, and/or reproducibility was evaluated from chromatograms obtained from pooled plasma samples spiked with a fixed concentration of the IS and different amounts of quality control standards at 5.0, 15.0, 175.0, and 350.0 ng mL<sup>-1</sup>. Peak areas of ten replicates of each concentration were measured and concentrations were back-calculated by employing the regression equation established on the same day.

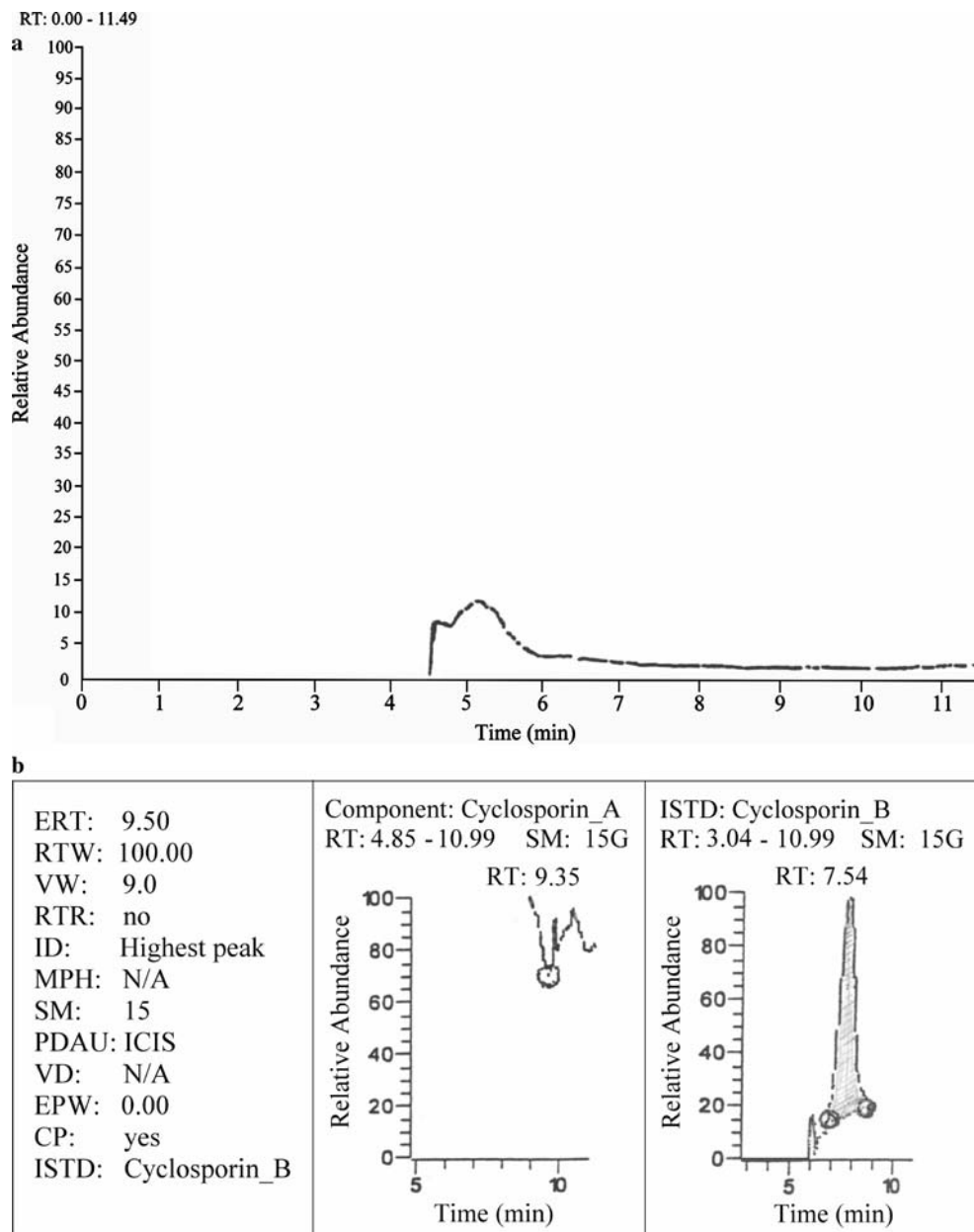
The relative accuracy was assessed as percentage accuracy or (100  $\pm$  relative error (bias%)). Precision, in turn, was quoted as percentage relative standard deviation or coefficient of variation (CV%).

Intra-day accuracy and precision were determined from measurements performed on the same day and inter-day reproducibility was established from quality control measurements made over three consecutive days. Results are presented in Table 2.

### Recovery

To check the performance of the method, three quality-control standards of CyA at low, medium, and high

**Fig. 2 a** LC-chromatogram of blank human plasma.  
**b** Xcalibur LCQuan chromatogram of blank plasma containing zero standard CyA and 500.0 ng mL<sup>-1</sup> internal standard (CyB)



**Table 1** Data from determination of standard calibration curves for CyA in human plasma

Conc. (ng mL <sup>-1</sup> )	Peak area ratio <sup>a</sup>	Back calculated conc. <sup>a</sup>	SD	CV%	Accuracy%
5	0.01322	4.63	0.59	12.74	92.60
10	0.02658	9.58	0.82	8.56	95.80
20	0.05332	19.48	1.84	9.45	97.40
50	0.1373	50.80	4.11	8.09	101.60
100	0.2840	105.77	5.46	5.16	105.77
200	0.5275	197.03	10.23	5.19	98.52
300	0.7798	290.65	10.74	3.70	96.88
400	1.0916	407.00	10.23	2.51	101.75
Mean				6.92 ± 3.38	98.79 ± 4.11

<sup>a</sup>Average from six replicates

**Table 2** Intra and inter-day accuracy and precision for determination of CyA in human plasma

Conc. (ng mL <sup>-1</sup> )	Intra-day				Inter-day			
	Found <sup>a</sup> (ng mL <sup>-1</sup> )	SD	CV%	Accuracy%	Found <sup>b</sup> (ng mL <sup>-1</sup> )	SD	CV%	Accuracy%
5	4.96	0.28	5.65	99.20	5.13	0.31	6.04	102.60
15	14.67	0.25	1.70	97.80	15.22	0.73	4.80	101.47
175	192.92	4.82	2.50	110.24	181.31	12.91	7.12	103.61
350	363.77	23.33	6.41	103.93	344.15	31.25	9.08	98.33
Mean			4.06	102.79			6.76	101.50

<sup>a</sup>Average from 10 replicates.

<sup>b</sup>Average from 20 replicates.

concentrations, viz. 15.0, 175.0, and 350 ng mL<sup>-1</sup>, were chosen. Five replicates of each concentration were extracted and measured.

The absolute recovery of CyA was determined by comparing peak areas of the extracted quality control samples with that of an equivalent unextracted aqueous standard. Results are tabulated in Table 3.

The relative analytical recovery of CyA from plasma was similarly established by measuring three quality control standards, peak areas were determined, and concentrations were back calculated. The relative recovery was estimated by comparing the amount of the analyte recovered with its nominal concentration. The mean recovery, standard deviation, and CV were deduced as presented in Table 4.

### Stability

The stability of CyA in human plasma was studied by using two quality-control standards at low and high concentrations, viz. 15.0 and 350.0 ng mL<sup>-1</sup>. Replicates of each concentration were stored at room temperature for short periods (2, 4, and 6 h). Other replicates were kept at -20°C for long periods (25 h, 1 month, and 4 months). Samples at the end of each study period were left to thaw and attain ambient temperature before analysis. Stability (%) was estimated by comparing the back-calculated concentrations with that of equivalent freshly prepared samples measured at zero time. Results of short-term and freeze/thaw cycle stabilities are shown in Tables 5 and 6.

**Table 3** Data from determination of absolute analytical recovery of CyA from human plasma

Sample no.	Peak area from aqueous standard solution			Peak area from QC sample		
	15.0 ng mL <sup>-1</sup>	175.0 ng mL <sup>-1</sup>	350.0 ng mL <sup>-1</sup>	15.0 ng mL <sup>-1</sup>	175.0 ng mL <sup>-1</sup>	350.0 ng mL <sup>-1</sup>
1	557991	6345754	14523340	475953	5439708	11304525
2	551722	5856726	14304319	484080	5607661	10307633
3	576424	5856556	13474604	474327	5457162	8661373
4	539079	5975754	13934619	509725	5316845	8782815
5	557349	5929205	12815813	476123	5774979	11321898
Mean	556513	5992799	13810539	484042	5519271	10075649
Recovery%				86.98	92.10	72.96
Mean recovery%					84.01	
SD					9.91	
CV%					11.80	

**Table 4** Data from determination of relative analytical recovery of CyA from human plasma

Sample no.	QC low (15.0 ng mL <sup>-1</sup> )		QC medium (175.0 ng mL <sup>-1</sup> )		QC High (350.0 ng mL <sup>-1</sup> )	
	Measured conc.	Relative recovery%	Measured conc.	Relative recovery%	Measured conc.	Relative recovery%
1	16.6	110.67	171.8	98.17	346.9	99.11
2	16.1	107.33	175.3	100.17	328.4	93.83
3	16.3	108.67	170.8	97.60	280.1	80.03
4	16.5	110.00	167.4	95.66	330.1	94.31
5	15.7	104.67	177.7	101.54	358.9	102.54
Mean	16.2	108.27	172.6	98.63	328.8	93.96
SD		2.39		2.29		8.58
CV%		2.21		2.32		9.13

**Table 5** Data from determination of the short-term stability of cyclosporin A at room temperature

Sample no.	QC low (15.0 ng mL <sup>-1</sup> )			QC high (350.0 ng mL <sup>-1</sup> )		
	Initial anal.	After 2 h	After 4 h	Initial anal.	After 2 h	After 4 h
1	15.9	15.8	16.7	397.7	398.4	390.2
2	16.2	16.4	16.2	384.7	397.7	351.3
3	14.6	16.9	16.3	390.6	397.6	389.6
4	14.8	22.7 <sup>a</sup>	16.6	396.6	391.5	394.9
5	15.4	16.0	14.8	399.2	395.2	370.8
Mean	15.38	16.28	16.12	393.76	396.08	379.36
SD	0.69	0.49	0.77	6.03	2.83	18.20
CV%	4.49	3.01	4.78	1.53	0.71	4.80
Accuracy%	102.53	108.53	107.47	112.50	113.17	108.39
Stability%		105.85	104.81		100.59	96.34

**Table 6** Data from determination of freeze-thaw cycle stability for CyA

Sample no.	QC low (15.0 ng mL <sup>-1</sup> )				QC high (350.0 ng mL <sup>-1</sup> )			
	Initial anal.	After first cycle	After second cycle	After third cycle	Initial anal.	After first cycle	After second cycle	After third cycle
1	15.0	14.9	14.4	14.5	368.4	368.6	385.3	398.3
2	15.5	16.8	16.5	16.6	394.7	<sup>a</sup> 454.7	395.5	394.9
3	13.4	17.1	16.2	16.6	381.0	349.1	394.2	396.9
4	15.8	15.1	16.7	16.5	378.8	396.9	395.0	395.5
5	16.9	15.5	15.8	15.8	398.7	392.7	396.8	391.1
Mean	15.32	15.88	15.92	16.00	384.32	376.83	393.36	95.34
SD	1.28	1.01	0.91	0.90	12.34	22.30	4.60	2.71
CV%	8.36	6.36	5.72	5.63	3.21	5.92	1.17	0.69
Accuracy%	102.13	105.87	106.13	106.67	109.81	107.67	112.39	112.95
Stability%		103.66	103.92	104.44		98.05	102.35	102.87

Long-term stability, post-preparative stability and stability of CyA in stock solution were also examined and evaluated.

## Results and discussion

Most of the methods previously reported for CyA analysis are not without limitations or disadvantages. The method described in this study is a relatively simple, quick, and reliable procedure for the determination of CyA in human plasma using LC-MS. The use of SPE and the column-switching technique resulted in removal of potential interferences, giving a clean chromatogram in a reasonable time. Figures 2 and 3 show typical chromatograms obtained from drug-free plasma and plasma spiked with 5.0 and 15.0 ng mL<sup>-1</sup> CyA and 500.0 ng mL<sup>-1</sup> CyB.

To avoid various measurement uncertainties and enable constant day-to-day reproducibility, CyB, a demethylated analog of CyA, was chosen as internal standard because it behaves very similarly to the analyte in terms of both solubility and detectability. The drug and IS were well resolved and eluted at 9.25 and 7.21 min, respectively. Neither endogenous matter response nor any commonly used drugs injected with the mobile phase were observed to interfere with the peaks of the measurands, indicating to the specificity and

selectivity of the assay. The measurands were monitored by use of a mass detector in SIM mode at  $m/z$  601.3 Da for CyA and 594.4 Da for the IS.

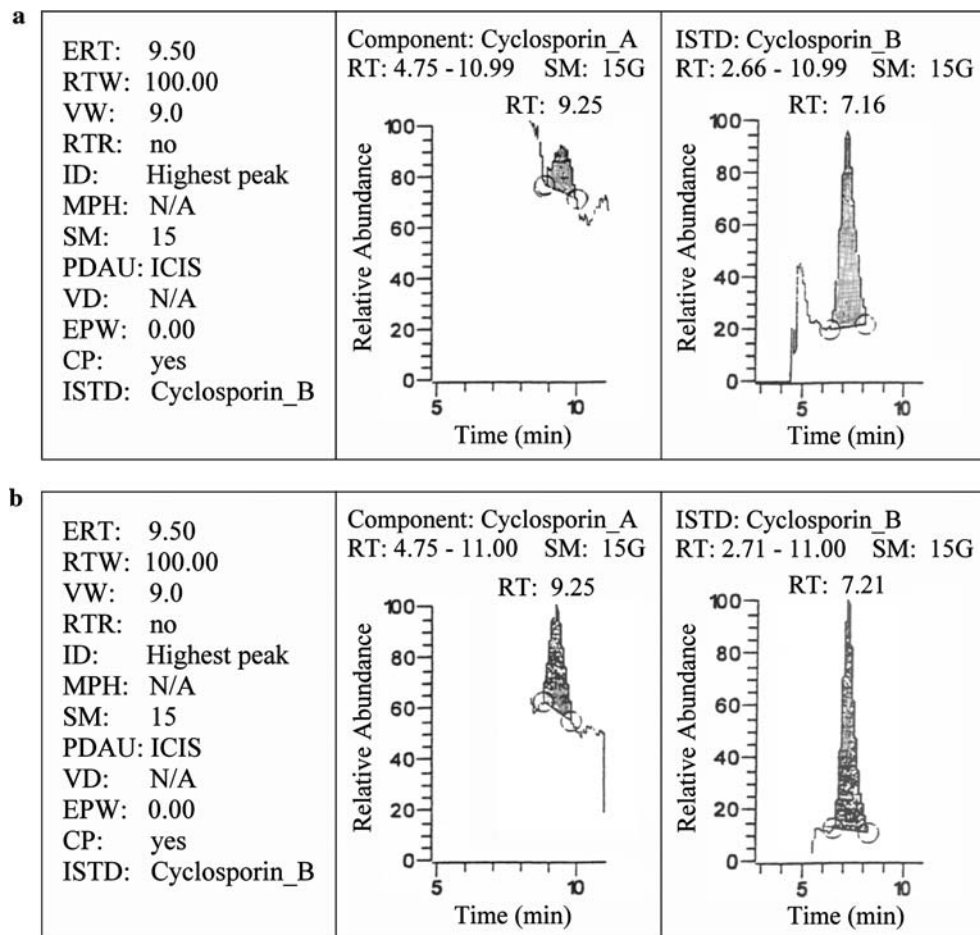
To check the linearity of the method, standard calibration plots of eight points (non-zero standards) over the range of 5.0–400.0 ng mL<sup>-1</sup>, enough to cover the therapeutic window of the drug, were individually constructed. The linearity of the plots were verified over the limits of quantitation and found to obey the least square regression equation  $Y = (0.00268 \pm 1.9 \times 10^{-4})X + (0.00078 \pm 1.8 \times 10^{-3})$  with a correlation coefficient,  $r = 0.9986 \pm 1.1 \times 10^{-3}$  ( $n = 48$ ), where  $Y$  represents peak-area ratio of the drug to that of the IS, and  $X$  represents CyA concentration in ng mL<sup>-1</sup>. The normalized equation was used for back-calculation of the concentrations of CyA in the calibration standards, quality control samples, and unknown concentrations in real samples.

The lowest LLOQ, defined as the lowest repeatable measured concentration with an accuracy  $\geq 80\%$  and precision  $\leq 20\%$ , was found to be 5.0 ng mL<sup>-1</sup>, which is sufficient for pharmacokinetic and clinical studies of CyA.

Statistical data, including the mean, standard deviation, precision quoted as CV, and percent accuracy of the method are presented in Table 1.

The intra- and inter-day variability of accuracy and precision for quality control samples at low, medium,

**Fig. 3** Xcalibur LCQuan chromatogram of plasma spiked with (a) 5.0 ng mL<sup>-1</sup> CyA and 500.0 ng mL<sup>-1</sup> IS and (b) 15.0 ng mL<sup>-1</sup> CyA and 500.0 ng mL<sup>-1</sup> IS

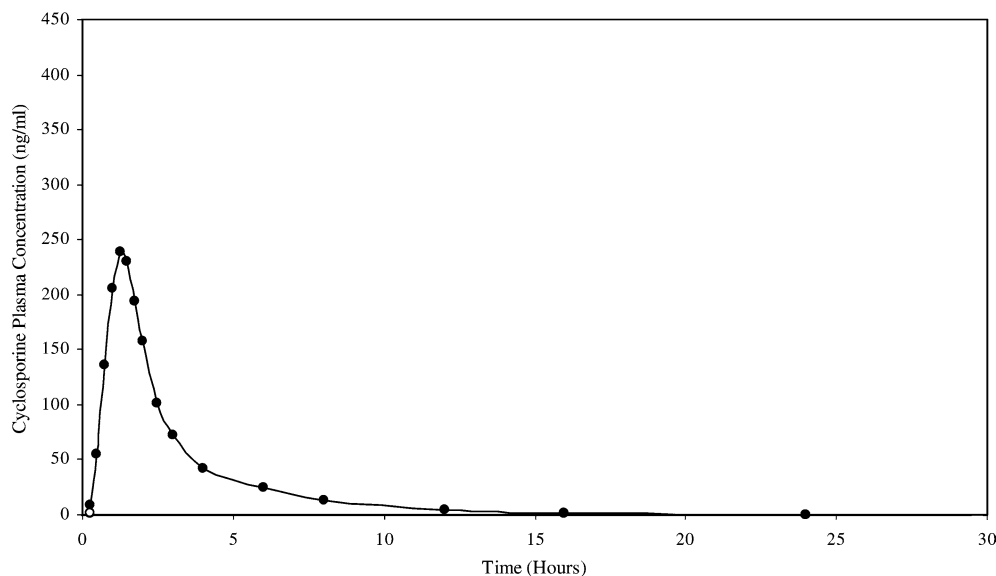


and high CyA concentrations were conducted in 1 day for intra-runs and in three consecutive days for inter-runs. The variation in CV ranged between 1.7 and 6.41 and between 4.8 and 9.08 for intra- and inter-day measurements, respectively. The reliability of the method is

apparent from the small variation in the CV and high percentage accuracy as shown in Table 2.

To ensure the performance and to account for losses and potential interferences, recovery measurements were conducted on fortified plasma samples containing CyA

**Fig. 4** Plasma concentration-time profile for cyclosporin after administration of a single dose of 100 mg mL<sup>-1</sup> Sandimmune solution



at three different concentrations in the range 15.0–350.0 ng mL<sup>-1</sup>.

The absolute recovery of the analyte was monitored by comparing the peak area from spiked plasma replicates with that from both equivalent aqueous and unextracted samples. However, relative recoveries were determined by comparing the measured concentrations of spiked plasma replicates with the actual concentrations added.

The mean absolute and relative recoveries as calculated were 84.01 ± 9.91 and 100.29 ± 9.19, respectively, indicating high performance of the assay.

Stability testing for CyA in biological plasma and stock solution under different conditions of time and temperature were studied at low and high concentration levels. Various types of stability testing were performed viz., short-term, freeze–thaw (unassisted cycles), post preparative, long-term, and stock solution stability. The short-term stability for CyA in plasma at room temperature ranged from 96.34 to 105.85 with an average of 101.90 and the freeze–thaw cycles stability for CyA stored at –20°C ranged from 98.05 to 104.44 with an average of 102.55. The respective data are shown in Tables 5 and 6.

Stability results of long-term, post-preparative and stock solution at room temperature and/or –20°C gave mean stabilities 99.18 ± 5.68, 98.0 ± 1.02, and 98.44 ± 4.97, respectively (raw data not included).

On the basis of these findings, it is inferred that CyA is entirely stable, in either human plasma or stock solution even when kept under various conditions of time and temperature.

Eventually, the applicability of the assay to real samples has been proven because hundreds of plasma samples collected from healthy volunteers after administration of a single dose of 100 mg cyclosporin solution were successfully analyzed and computed. A typical plasma concentration–time profile is presented in Fig. 4.

## Conclusion

A new method of SPE coupled with LC–MS was developed and validated to ensure quick and reliable determination of CyA in human plasma. The combination of SPE cleanup with a column-switching technique and Xcalibur LCQuan liquid chromatography guaranteed a clean analysis in a reasonable time, i.e. 10 min.

Compared with other reported methods mentioned in the introduction, the method presented has improved accuracy, repeatability and/or reproducibility, high recovery, low volume of plasma used, low volume of injection, high selectivity, and stability, among other advantages. All of these features confirm the superiority

of the method and ensure its suitability for assay of CyA in human plasma, particularly in clinical and pharmacokinetic studies.

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