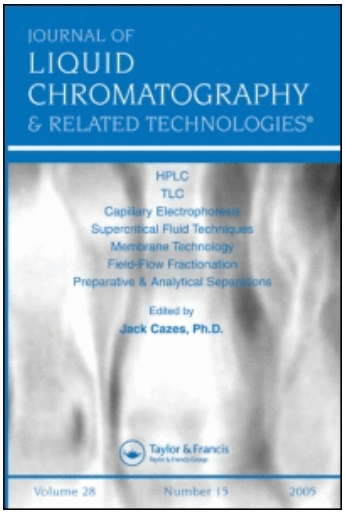

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Quantification of Cyclosporin A in Human Cerebrospinal Fluid by Liquid Chromatography-Mass Spectrometry using Atmospheric Pressure Chemical Ionization

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Abstract: Cyclosporin A (CsA) is widely used as an immunosuppressant in organ transplantation, and the treatment of autoimmune disorders. This is the first publication describing a rapid, sensitive, and selective liquid chromatography-mass spectrometry (LC-MS) method for analysis of CsA in cerebrospinal fluid (CSF). Chromatographic separation was achieved using a reversed-phase column with a linear gradient mobile phase. A column switching procedure was incorporated to minimize contamination in the ion source of the mass spectrometer. The sample volume requirement was 50 μL and no internal standard was employed. Quantification was carried out with selected ion reaction (SIR) monitoring of the protonated sodium ion adduct ($m/z = 1224.7$). Calibration curves were linear from 0.5 ng/mL to 20 ng/mL with a weighting factor of $1/x$. Precision and accuracy, and reverse predicted concentration residuals were within 15%. The method has been used successfully for the analysis of CSF samples from a clinical study.

Keywords: Cyclosporin A, Cerebrospinal fluid, LC-MS, APCI-SIR

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INTRODUCTION

Cyclosporin A (CsA), a cyclic polypeptide of fungal origin has been primarily used as a powerful immunosuppressive drug in solid organ and bone marrow transplantations, and in conditions such as psoriasis, eczema, and autoimmune disorders.[1] CsA is a neutral compound at physiological pH and has a relative molecular mass of 1202. It is abundant in hydrophobic amino acids such as methyl leucine. It is insoluble in water and n-hexane but very soluble in many organic solvents. CsA has the potential to cause substantial nephrotoxicity, hypertension, neurotoxocity, and seizures.[2–4] The pharmacokinetics of CsA is complex and unpredictable.[5] It is a 'critical dose drug' and several commentaries and reviews report issues with bioequivalence in development of generic cyclosporin formulations.[6–8] Also, CsA is subject to interactions with several drugs that may increase or decrease its bioavailability and metabolism.[8,9] A therapeutic drug monitoring strategy thus assumes significance post treatment.[10,11]

The analyses of CsA by immunochemical methods and high performance liquid chromatography (HPLC) have been reported.[12–16] Immunoassays for CsA are widely used in clinical practice. These suffer from a lack of specificity however. Carruthers et al. have attributed this problem to a non-specific antibody relative to the metabolites of CsA, when results of a radioimmunoassay were compared to a more selective HPLC assay.[17] In recent years, LC-MS-MS has become increasingly popular for the analysis of small volume samples. At therapeutic concentrations and at room temperature, however, CsA is partitioned between erythrocytes (40–50%), leukocytes (10–20%), and plasma (30–40%) in which it is primarily lipoprotein bound.[18–20] Therefore, for routine pharmacokinetic evaluations of CsA, whole blood has been used as the matrix of choice. Simpson et al. described a selective method for the measurement of Cyclosporin A in human whole blood by liquid chromatography-tandem mass spectrometry, using a reversed-phase (C_{18}) narrow bore column with a limit of quantification of 5 ng/mL.[21] Zhou et al. optimized an analytical method for cyclosporin A in whole blood, using electrospray ionization mass spectrometry without removal of salts prior to analysis.[22] The reported assay run times are still longer than immunoassay methods and required sample clean up procedures that utilize either solid phase extraction (SPE) or liquid-liquid extraction (LLE). The sample preparation procedure described in this paper employs a simple, single step, protein precipitation that enabled time and resource savings during routine analyses.

In addition to other analytical challenges during method development, it was observed that cyclosporin A exhibited non-specific adsorption to plastic (PVC) and glassware that led to a loss of peak intensity with the mass spectrometer. To minimize this, a bile acid derivative, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), was employed during sample preparation. A few studies have been published on the efficiency of

CHAPS as a stationary phase modifier.[23–25] This compound has several polar functional groups, such as sulfonate, ammonium, and hydroxyl, and exists as an electrically charged zwitterion over a wide pH range (Figure 1). Its micelle forming properties have been employed in the fabrication of modified ODS chromatographic columns. This work reports the application of CHAPS as an additive added extraneously during sample preparation.

Current bioanalytical publications for CsA report methods for whole blood, serum, and plasma. Our objective, however, was to employ a simple and selective analytical procedure for quantification of cyclosporin A in cerebrospinal fluid of clinical subjects. The high performance liquid chromatography-mass spectrometric (LC-MS) method described in this paper employs minimal time for sample preparation and analysis, and the technique has been applied to clinical CSF samples.

EXPERIMENTAL

Chemicals

Cyclosporin A reference standard and CHAPS (3-[3-cholamidopropyl]-dimethylammino propanesulfonic acid) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol was purchased from Burdick & Jackson (Honeywell International, Inc. MI, USA). Ammonium acetate was purchased from Fisher Scientific (Fairlawn, NJ, USA). Normal saline was obtained from Baxter Healthcare Corp. (Deerfield, IL, USA). Water was obtained in-house using the Nanopure Diamond water system (Barnstead International, IO, USA).

Stock Solution and Stock Dilutions

Approximately 10 mg of cyclosporin A was accurately weighed and transferred into a 10 mL volumetric flask. It was dissolved in methanol to yield

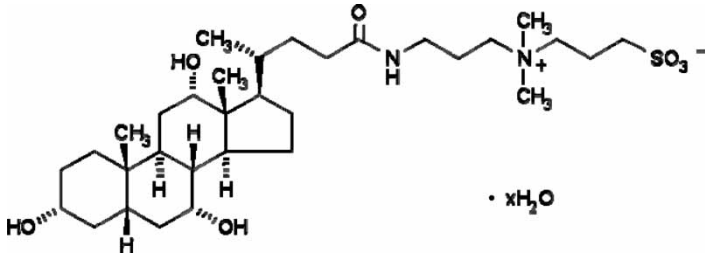

Figure 1. CHAPS, a zwitterionic surfactant.

a stock solution of 1 mg/mL. The stock solution was stored below 10°C in a refrigerator. A serial dilution in methanol yielded stock solutions in the concentration range of 2.5 to 100 ng/mL that were added to CSF immediately prior to analysis.

Biological Matrix Preparation and Evaluation

CSF from two batches was checked for potential interference at the retention time of the analyte. These were pooled to provide the matrix for the study. An evaluation of matrix effects was conducted by the post-column infusion method of Bonfiglio et al.[26] A 5 ng/mL solution of cyclosporin A was prepared in methanol and continuously infused at 5 μL/minute into the mass spectrometer using a 'tee', the third end of which was connected to the injector. Upon stabilization of the baseline response, a processed sample of blank CSF was injected and the resulting profile was evaluated for changes in ionization.

Calibration Curve Standards, Quality Control Samples, and Sample Preparation

Due to the relatively low volume of matrix available for the study, a bulk spiking of the calibration curve (CC) standards and quality control (QC) samples was not performed. CC standards were prepared as required at 0.5, 1, 2.5, 5, 10, and 20 ng/mL in the following manner: 10 μL of each stock dilution was added to stoppered glass tubes containing 30 μL of 20 mM CHAPS in normal saline to minimize non-specific adsorption of the drug to glass. CSF of 50 μL was then added and the tubes were vortex mixed. Similarly, quality control samples were prepared at 1.8, 9, and 18 ng/mL, representing low (LQC), middle (MQC), and high (HQC) controls, respectively. The final sample preparation step involved precipitation of 110 μL methanol, as described below for real-time samples.

A sample of 50 μL was added to tubes containing 30 μL of 20 mM CHAPS in normal saline to process real-time samples, vortex mixed, and added to 120 μL of methanol The tubes were again vortex mixed, centrifuged at 4000 rpm for 15 minutes, and the supernatant transferred into 200 μL silanized HPLC vials for injection. The sample (50 μL) was injected into the LC-MS-MS system.

HPLC Conditions with Column-Switching

Three high pressure pumps (Shimadzu DGU-14A; designated Pumps A, B, and C), a system controller (Shimadzu SCL10ADVP, and an HTS-PAL

Table 1. Gradient conditions for mobile phase

Time (minutes)	%A	%B	Curve
0.0	50	50	1
3.0	0	100	6
3.5	0	100	1
3.51	50	50	1

autosampler (Carrboro, NC, USA) were used. A Beta Basic C_8 (5 μm; 2.1 × 50 mm) column (Thermo-Electron, Bellafonte, PA, USA) maintained at 50°C was also used. The mobile phases in Pumps A and B consisted of 10 mM ammonium acetate in water (pH 5.5) and methanol, respectively. The flow rate was fixed at 0.3 mL/minute for a linear gradient described in Table 1.

A "heart-cut" column switching procedure was adopted, it was, therefore, necessary to allow a continuous flow of solvent into the heated ion source. Pump C employed 10 mM ammonium acetate in water (pH 5.5): methanol (50:50, v/v) at 0.2 mL/minute flowing directly into the mass spectrometer through an 8-port selector valve. The column switching technique is described in Figure 2 and Table 2. Based upon the retention time of the drug (2.9 minutes) that was well resolved from the region of ion suppression, a front-cut time of 2.0 minutes was fixed. The acquisition time was optimized at 1.7 minutes. The total run time for each injection was 4.5 minutes.

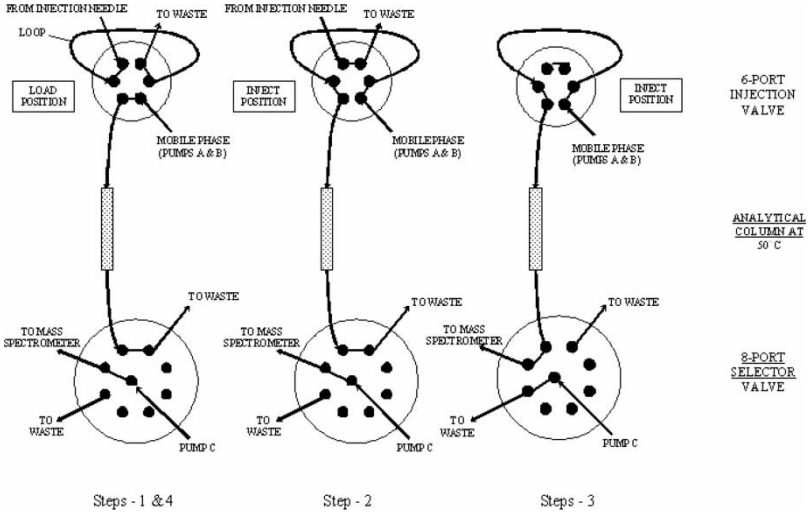

Figure 2. Representation of the column switching technique.

Table 2. The timetable used for column switching

Step	Time (min:sec)	Switching positions	Flow stream event	Comment
1	0:00–0:30	−, +	Pump A & B → Column → Waste Pump C → MS	Sample injection and retention in loop
2	0:30–2:00	+, +	Pump A & B → Loop → Column → Waste Pump C → MS	Sample retention on column for clean-up
3	2:00–3:36	+, −	Pump A & B → Loop → Column → MS Pump C → Waste	Sample injected into mass spectrometer
4	3:36– End	−, +	Pump A & B → Column → Waste Pump C → MS	Switch back to initial condition

Note: The '+' or '−' sign on either side of comma indicates a clockwise or counter-clockwise position for the 6-port and 8-port valve, respectively.

Optimized Mass Spectrometer Conditions

A Micromass Quattro triple quadrupole mass spectrometer (Manchester, UK) was used with Mass Lynx ver. 3.4 software for data acquisition and analysis. Atmospheric pressure chemical ionization (APCI) in the positive ion mode was employed, although an electrospray ionization (ESI) source was also investigated for detector response, as described in the following section. During method development using single ion reaction monitoring, the intensity for the sodium adduct (m/z: 1224.7) was found to be consistently greater than that of the parent ion (m/z: 1203) and the potassium adduct (m/z 1241); the intensity of this mass ion peak was, therefore, optimized. This was performed by infusion of a 1 μg/mL solution of the compound in methanol and carrying out an iterative, manual optimization of other instrument parameters. The following values were obtained and set for analysis - Corona needle 2 kV, Cone 100V, Extractor 5V, Source temperature 150°C, and APCI probe temperature 600°C. Nitrogen was used as the desolvation gas at 500 L/hr.

RESULTS AND DISCUSSION

Method Optimization

Cyclosporin A readily forms adducts with sodium and potassium that are ubiquitous in glassware. This led to problems in quantitative analysis, especially

when employing an electrospray ionization (ESI) source. Different techniques have been reported to solve the problem, such as calculation of the sum of all adducts for quantification, or through use of an internal standard. Also, electrospray ionization has been reported to be susceptible to ion suppression effects and generates large number of multiple charged species. During initial method development using an ESI source, this was indeed found to be the case. A multiple charged $[CsA + 2Na]^{++}$ ion was observed in addition to singly charged species. This phenomenon usually compromises the sensitivity of LC-MS methods used for quantification due to loss of intensity of the peak of interest. Atmospheric pressure chemical ionization (APCI) is known to be less susceptible to suppression of ionization than the ESI and, therefore, it was decided to employ this source. Molecular ions were observed at m/z 1203, 1225, and 1241 that corresponded to $[CsA + H]^+$, $[CsA + Na]^+$, and $[CsA + K]^+$, respectively. An APCI mass spectrum of CsA is shown in Figure 3. The m/z peak of 1224.7 was optimized for maximum intensity. This resulted in an excellent chromatographic peak as represented in Figure 4.

LC-MS-MS methods may be prone to complications such as matrix effects, suppression of ionization, etc. that impair their reliability. To overcome these problems, extensive and lengthy sample pretreatment and chromatographic separations are often required, which lower the throughput efficiency of the method and make these methods difficult for routine laboratory use. The method described in this paper overcomes many of these potential problems. The gradient change from 50% to 100% methanol in 3 minutes ensured that the region of ion suppression (Figure 5) eluted much earlier than the elution of the analyte ($\sim$2.7 minutes). Also, since ion suppression could be attributed primarily to salts in the matrix, the column switching technique ensured that contamination of the ion source of the mass spectrometer was minimal.

The blank cerebrospinal fluid sample was devoid of interference at the retention time of cyclosporin. Figure 6 shows a representative chromatogram, each of blank and LOQ samples.

Linearity, Precision, Accuracy, and Recovery Studies

The peak areas of m/z 1224.7 were employed for quantification throughout. The calibration curves were linear in the range of 0.5–20 pg/mL ($r^2 > 0.98$) using a weighting factor of 1/concentration. The concentration residuals were between 94.7% and 107.3% (RSD = 1.4%−6.4%). The data are summarized in Table 3.

The LLOQ of the method was tested at 0.5 ng/mL by injection of CSF samples prepared and processed independent of the calibration curve. The deviation was found to be −1.7% of the nominal concentration (RSD = 7.1%; n = 6).

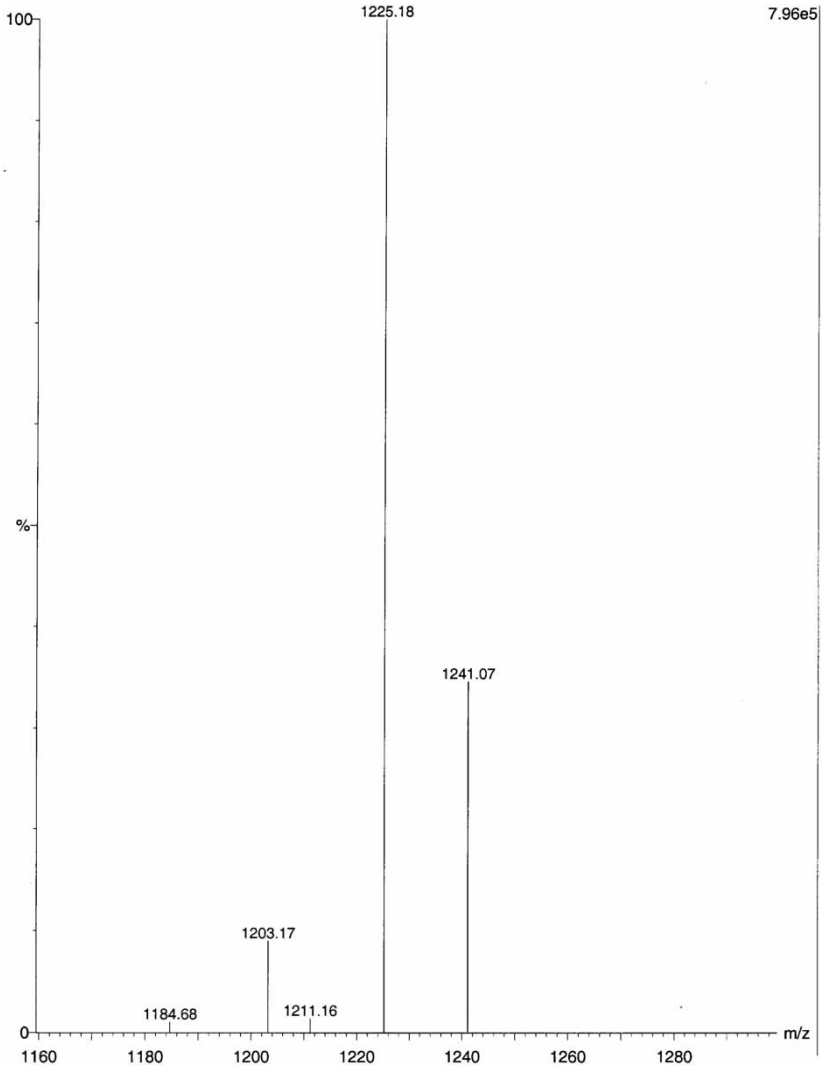

Figure 3. The APCI mass spectrum for cyclosporin A.

To evaluate the precision and accuracy of the assay, three batches were processed and analyzed. Each batch consisted of 3 sets each of quality control samples (low, middle, and high concentrations) that were quantified using calibration curves. The precision and accuracy of the method were reported as the relative standard deviation (%RSD) and percent of nominal value, respectively. The global inter-batch precision and accuracy ranged between 4.5%–11.9%, and 94.0%–99.4%, respectively [refer to Table 4]. The intra-batch precision and accuracy ranged between 2.1%–14.5%, and

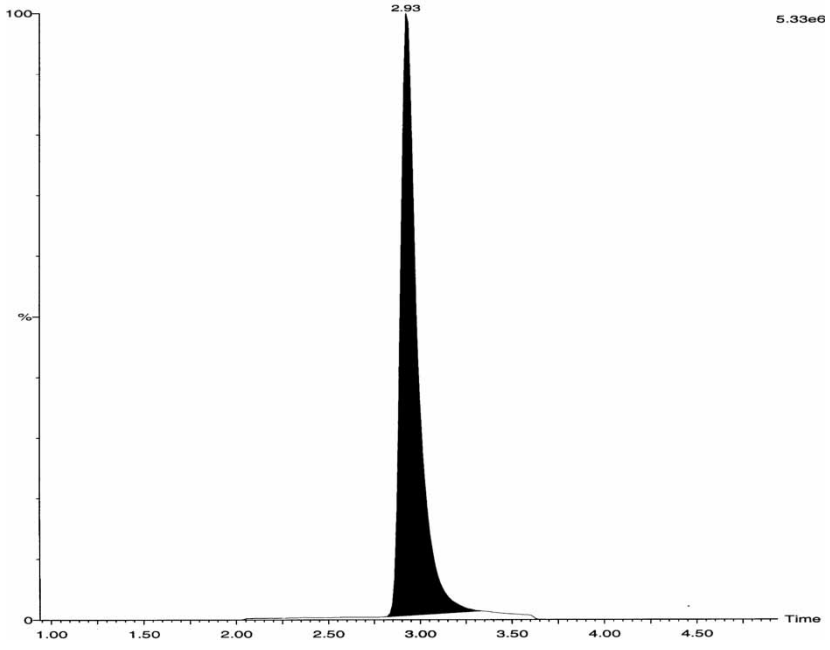

Figure 4. Chromatogram of a dilution of cyclosporin A in methanol containing 20 mM of CHAPS.

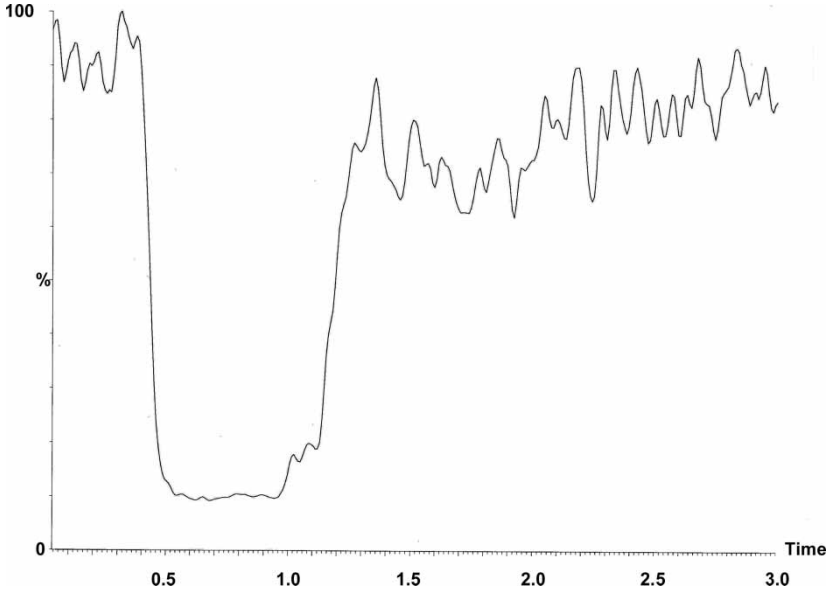

Figure 5. Ion suppression profile following post-column in fusion of cyclosporin A.

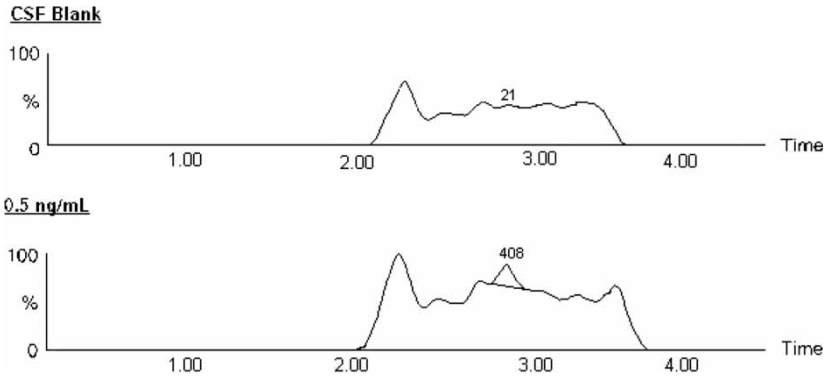

Figure 6. Representative chromatograms of blank and LOQ samples.

88.9%–107.6%, respectively. A representative chromatogram each of low, middle, and high QC samples is shown in Figure 7.

The recovery of the method was determined by a comparison of mean peak area of processed samples at the high quality control level to the mean peak area of samples spiked at the same concentration level in methanol containing 20 mM CHAPS in normal saline (15%, v/v). The mean recovery was 40.4% (RSD = 1.3%).

Application to a Clinical Study

The analytical method was employed for analyses of CSF samples of an institutional review board (IRB) approved clinical study. This study was conducted in severe traumatic brain injury patients to assess penetration of cyclosporin A through the blood brain barrier. Thirty-six patients were enrolled for the double blind, placebo controlled study, and a 24-hour continuous intravenous infusion at 5 mg/kg dose of cyclosporin A was administered. The predose samples showed no interference at the retention time of CsA.

As part of the clinical study, this method was also extrapolated to the analyses of microdialysate and brain tissue samples. For microdialysate analysis, the method modification involved replacement of cerebrospinal fluid with normal saline for spiking calibration standards and quality control samples. The calibration curve was linear from 0.025–5 ng/mL, and precision and accuracy of quality control (0.09, 0.9, and 4.5 ng/mL) samples were within the 15% acceptance criteria. The results of the study with pertinent pharmacokinetic interpretation has been published elsewhere.[27]

Brain tissue samples were stored at −80°C until analysis. The samples were weighed and chopped into small pieces using a scalpel. The pieces were homogenized in KH_2PO_4 buffer (2 mL; pH 7.4) using a Polytron instrument. Blank homogenized matrix was used for spiking calibration standards

Table 3. Reverse predicted concentration residuals of cyclosporin A

	Nominal Concentration (ng/mL)								
Set #	0.50	1.00	2.50	5.00	10.00	20.00	r^2	Slope	Intercept
1	0.47	1.08	2.56	5.28	10.57	19.10	0.985	1206.12	691.22
2	0.51	1.04	2.49	5.27	9.71	20.52	0.994	1107.64	538.50
3	0.45	1.10	2.54	5.15	10.15	19.60	0.987	1014.06	945.80
Mean	0.47	1.07	2.53	5.24	10.14	19.74			
SD	0.03	0.03	0.03	0.07	0.43	0.72			
%RSD	6.4	2.9	1.4	1.4	4.2	3.6			
%nominal	94.7	107.3	101.2	104.7	101.4	98.7			

Table 4. Precision and accuracy data for cyclosporin A

	Nominal Concentration (ng/mL)		
Set #	1.80	9.00	18.00
1	1.71	8.38	15.24
	1.62	7.87	14.58
	1.63	7.74	18.61
Mean	1.65	8.00	16.14
SD	0.05	0.34	2.16
%RSD	3.0	4.2	13.4
%Nominal	91.9	88.9	89.7
2	1.65	10.27	19.14
	1.75	9.02	19.71
	1.58	9.75	18.91
Mean	1.66	9.68	19.25
SD	0.09	0.63	0.41
%RSD	5.1	6.5	2.1
%Nominal	92.2	107.6	107.0
3	1.82	10.69	17.53
	1.71	8.38	15.24
	1.75	8.39	16.76
Mean	1.76	9.15	16.51
SD	0.06	1.33	1.17
%RSD	3.2	14.5	7.1
%Nominal	97.8	101.7	91.7
	Global calculation		
Mean	1.69	8.94	17.30
SD	0.08	1.06	1.93
%RSD	4.5	11.9	11.1
%Nominal	94.0	99.4	96.1

and quality control samples in the range of 0.5–20 ng/mL. Samples (50 μL) were processed using the method described previously for CSF. The results were reported as nanograms of cyclosporin A per unit weight of tissue.

CONCLUSION

This paper describes a rapid and sensitive LC-MS method for the determination of cyclosporin A in human cerebrospinal fluid. The column switching technique has proven to facilitate a quick and simple sample

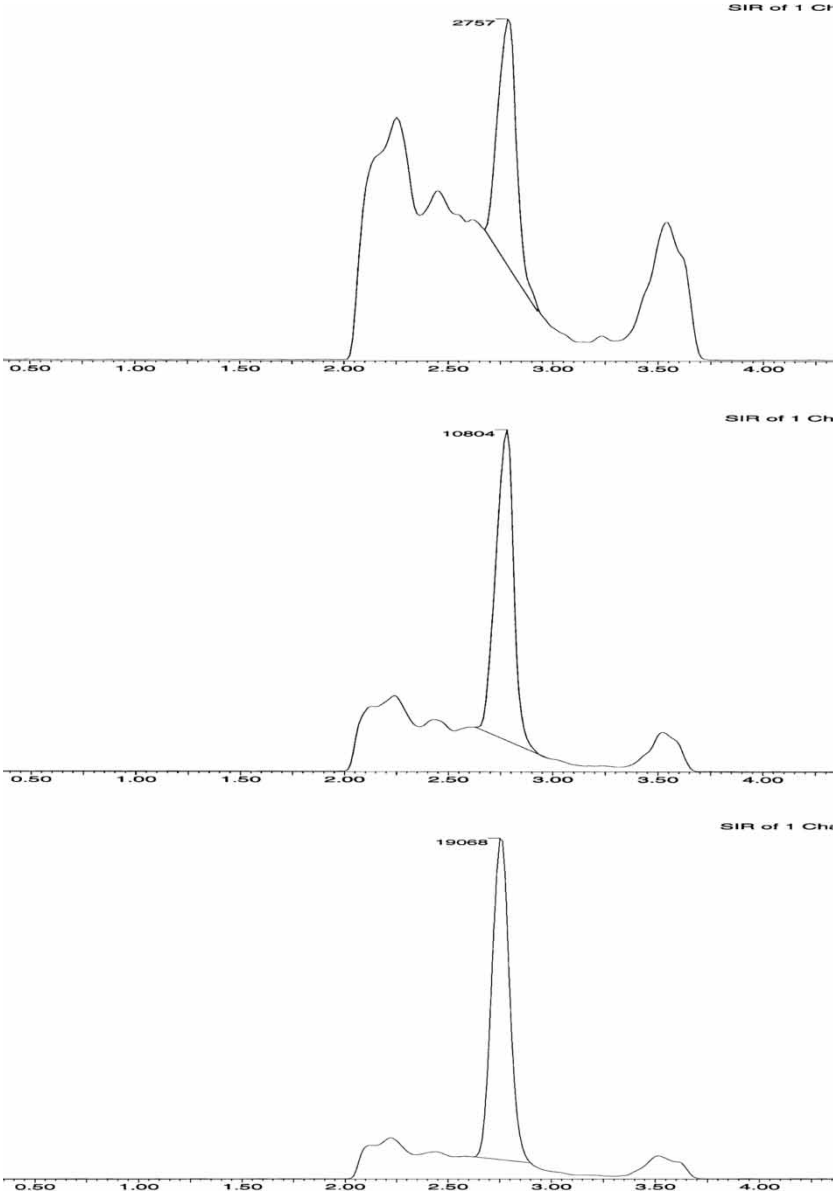

Figure 7. Representative chromatograms of 1.80, 9.00, and 18.00 μg/mL samples.

preparation. The calibration curves showed excellent linearity from 0.5–20.0 ng/mL for cyclosporin A, with precision and accuracy reported within 15% of the nominal values. The LC-MS method has been successfully applied for the quantification of cyclosporin A in cerebrospinal fluid

samples from a clinical study. An extrapolation of the method to microdialysate and brain tissue samples was conducted, and applied to analysis of real-time samples.

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