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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 277–284
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Advantages of using tetrahydrofuran–water as mobile phases in the quantitation of cyclosporin A in monkey and rat plasma by liquid chromatography–tandem mass spectrometry

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Received 7 May 2006; received in revised form 22 June 2006; accepted 26 June 2006
Available online 2 August 2006

Abstract

A new analytical method is described here for the quantitation of anti-inflammatory drug cyclosporin A (CyA) in monkey and rat plasma. The method used tetrahydrofuran (THF)–water mobile phases to elute the analyte and internal standard, cyclosporin C (CyC). The gradient mobile phase program successfully eluted CyA into a sharp peak and therefore improved resolution between the analyte and possible interfering materials compared with previously reported analytical approaches, where CyA was eluted as a broad peak due to the rapid conversion between different conformers. The sharp peak resulted from this method facilitated the quantitative calculation as multiple smoothing and large number of bunching factors were not necessary. The chromatography in the new method was performed at 30 °C instead of 65–70 °C as reported previously. Other advantages of the method included simple and fast sample extraction—protein precipitation, direct injection of the extraction supernatant to column for analysis, and elimination of evaporation and reconstitution steps, which were needed in solid phase extraction or liquid–liquid extraction reported before. This method is amenable to high-throughput analysis with a total chromatographic run time of 3 min. This approach has been verified as sensitive, linear (0.977–4000 ng/mL), accurate and precise for the quantitation of CyA in monkey and rat plasma. However, compared with the usage of conventional mobile phases, the only drawback of this approach was the reduced detection response from the mass spectrometer that was possibly caused by poor desolvation in the ionization source. This is the first report to demonstrate the advantages of using THF–water mobile phases to elute CyA in liquid chromatography.

Keywords: Cyclosprin A; Quantitative determination; Reversed phase chromatography; LC–MS/MS

1. Introduction

The chemical structure (Fig. 1) of the well-known immunosuppressant cyclosporin A (CyA), a cyclic undecapeptide, is unique due to rapid conversion of its conformers. NMR studies of the molecule have showed a fairly rigid backbone and fixed combinations for the side chains except the amino acids in positions 1 and 2, where the (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine (MeBMT) unit is able to fold under the ply of the β-sheet or to protrude from the body of the molecule by rotation of 120° around the C_α- and C_β-bond [1,2]. This conformational conversion posed certain difficulties in liquid chromatography as broad peaks were reported [3–5]. The base widths of the peak in some literatures were as wide as 2 min [6,7]. Consequently, this peak broadening effect seriously reduced resolution of separation and sensitivity of LC detection. To solve this problem, researchers have applied high temperature (65–70 °C) to column in an attempt to facilitate the conformational conversion and narrow the eluted CyA peak [6–10]. But this approach has only had limited effects as CyA was eluted still as a fairly broad peak whereas high temperature was difficult to keep consistent and could result in accelerated column deterioration.

There have already been many analytical methods reported for the quantitative determination of CyA in biological fluids, including immunoassay and HPLC–UV methods. These methods suffer from different disadvantages and were briefly

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doi:10.1016/j.jpba.2006.06.040

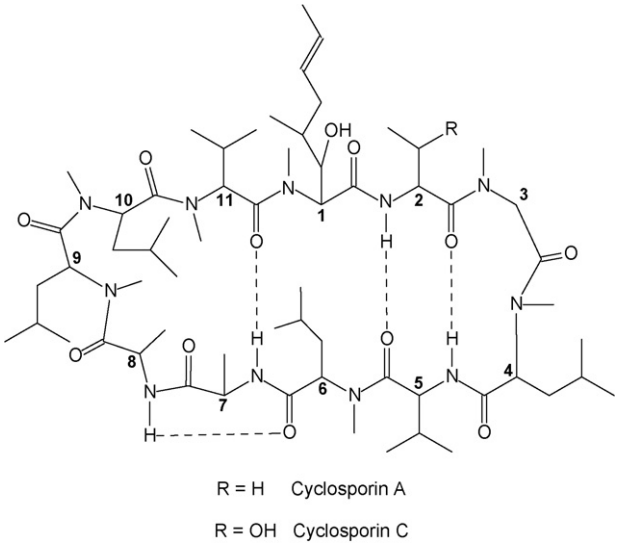

Fig. 1. Chemical structures of cyclosporin A and cyclosporin C.

but very well reviewed by Santori et al. [11] and Zaater et al. [7]. More specific and sensitive LC–MS or LC–MS/MS methods have been reported for the measurement of CyA in plasma or whole blood, but still with disadvantages. These disadvantages include complicated extraction, such as protein precipitation followed with solid phase extraction (SPE) [6,8], manual liquid–liquid extraction [9], elevated column temperature [7–9] up to 80 °C [6], and broad chromatographic peak leading directly to reduced chromatography resolution and detection sensitivity [6,7,12,13]. For example, the base peak width of CyA was about 0.75 min reported by Brignol et al. [13] when the column was kept at 40 °C in a high-throughput setting. The reduced resolving power of the LC could very possibly lead to co-elution of interfering substances, matrix effects or metabolites [12] with CyA and consequently causing inaccurate quantitation. Also, as experienced in our previous work using an unpublished Covance method [14], integration of the broad peak during calculation required many times of smoothing and large numbers of bunching factors, but manual integration was still inevitable. Quantitation based on this type of integration was apparently vulnerable to noise level caused by large number of smoothing and bunching, interference caused by co-elution, and inconsistency caused by manual integration. Moreover, all previously reported chromatography settings for CyA were overwhelming reversed phase using acetonitrile–water or methanol–water mobile phases containing different modifiers. Here, based on our search for alternative LC condition to improve CyA peak shape and reduce column temperature, we describe our simplified analytical method that was used to quantitate CyA in monkey and rat plasma. The use of THF and water as mobile phases successfully eluted CyA as a sharp peak from the column under room temperature, and allowed an easy protein precipitation extraction procedure and elimination of the evaporation–reconstitution steps that were needed in liquid–liquid extraction or solid phase extraction methods reported before.

2. Experimental

2.1. Chemicals and reagents

CyA ($C_{62}H_{111}N_{11}O_{12}$, MW 1202) was purchased from Sigma (St. Louis, MO, USA). Internal standard (ISTD) CyC ($C_{62}H_{111}N_{11}O_{13}$, MW 1218) was synthesized by LKT Laboratories, Inc. (St. Paul, MN, USA). Control monkey and rat plasma were purchased from Biochemed Pharmacologicals (Winchester, VA, USA). Ammonium acetate was also purchased from Sigma. HPLC grade of tetrahydrofuran and acetonitrile were all from Fisher (St. Louis, MO, USA). The 96-well elution plates and sealing mats were from Axygen (Union City, CA, USA). Deionized water used for HPLC mobile phase A was prepared from an in-house Purelab Plus system (Elga LabWater, Buckinghamshire, UK).

2.2. Sample preparation

Calibration standards. A working standard solution at 40,000 ng/mL was prepared by diluting 80.0 μL of the standard stock solution at 1.00 mg/mL to 2.00 mL acetonitrile. The solution was then sequentially diluted by four folds (0.500–2.00 mL in acetonitrile) to generate working standard solutions at 10,000, 2500, 625, 156, 39.1, 9.77 ng/mL. Five microliters of the working standard solutions were respectively spiked to 45 μL of control blank plasma in a 96-well plate, generating calibration standards at the concentrations of 4000, 1000, 250, 62.5, 15.6, 3.91 and 0.977 ng/mL.

QC samples. QC-High (3200 ng/mL) was prepared by diluting 160 μL of the working standard at 40,000 ng/mL to 2.00 mL with control blank plasma. QC-Mid (1600 ng/mL) was prepared by diluting 80 μL of the working standard at 40,000 ng/mL to 2.00 mL with control blank plasma. Dilution of 60 μL of the working standard solution at 2500 ng/mL to 2.50 mL in acetonitrile generated working QC-Low solution at 60 ng/mL. A 100 μL of this solution was mixed with 1.90 mL control plasma and generated QC-Low (3.00 ng/mL). LLOQ was prepared by diluting 50 μL of the working standard at 39.1 ng/mL to 2.00 mL with control blank plasma.

Extraction. Aliquots (50 μL) of quality control (QC) samples and study samples were transferred to a 96-well plate. To each well and the well containing calibration standard, was then added 200 μL of internal standard working solution (50 ng/mL of CyC in acetonitrile) using an Eppendorf Repeater pipette. The samples were mixed on a flat-bed vortexer (VWR International, CA, USA) for 20 s and centrifuged at 3000 rpm for 10 min. The supernatant (250 μL) was then transferred to a 96-well elution plate using a Tomtec Quadra 96™ (Model 320) liquid handling workstation (Hamden, CT, USA) for direct injection of LC–MS/MS analysis.

2.3. LC–MS/MS methods

The LC–MS/MS system consisted of a Shimadzu series 10 ADVP HPLC system (Kyoto, Japan) and an API 3000 Spectrometer (Applied Biosystems, Concord, Canada) with

TurboIonspray interface. The chromatography was performed on a Zorbax C18 (50 mm × 2.1 mm, 5 μm particle size, Agilent Technologies, Palo Alto, CA, USA), the analyte and ISTD were eluted with a typical high-throughput gradient program. The gradient started from 75% mobile phase A (5 mM ammonium acetate in water) and 25% mobile phase B (100% THF) and held for 0.5 min, followed with a gradual increase of mobile phase B to 95% within 1 min. This condition was held for 0.8 min before going back to initial condition (5 mM NH_4OAc in water–THF, 25:75) within 0.1 min. The column was equilibrated for 0.6 min for next injection. The total run time was 3 min including column equilibration. The total mobile phase flow rate was set at 0.5 mL/min, and the column temperature was controlled at 30 °C. To avoid contamination to the ion source, the LC flow of the first 1 min was diverted to waste.

The mass spectrometer was operated under MRM positive mode with collision energy of 28 eV for the ion transitions *m/z* 1220 $[M+NH_4]^+ \rightarrow$ 1203 $[M+H]^+$ (CyA) and 22 eV for *m/z* 1236 $[M+NH_4]^+ \rightarrow$ 1219 $[M+H]^+$ (CyC), respectively.

All parameters for the mass spectrometer were optimized by standard infusion experiments. Both quadupoles were maintained at unit resolution. Chromatograms were integrated using Analyst 1.4 software. A weighed 1/concetration^2 linear regression was used to generate calibration curves.

2.4. Pharmacokinetic study design and plasma sample collection

Monkey. Four groups of randomized cynomolgus monkeys (*Macaca fascicularis*) were respectively dosed with control material, 25, 50 and 100 mg/kg of CyA once daily by oral gavage for at least 28 consecutive days. Doses were based on the most recently recorded body weights. Blood samples were collected via a femoral vein from all animals. On Day 28, all animals were bled predose and at approximately 1, 2, 4, 8, and 24 h postdose. Blood samples (approximately 1 mL) were collected in tubes containing sodium heparin as the anticoagulant. The plasma was separated from blood cells by centrifugation, harvested and stored at −70 °C for quantitative analysis.

Rat. CyA was administered once daily via oral gavage to male SD rats for 28 days. CyA was administered at 3, 10, and 30 mg/kg/day to 10 male rats/group. Blood samples for toxicokinetic analysis were collected on day 24 as follows: the first set of three animals/group was bled predose and approximately 4 h postdose. The second set of three animals/group was bled approximately 1 and 8 h postdose. The remaining animals/group were bled approximately 2 and 24 h (prior to day 25 dose administration) postdose. Blood samples (approximately 0.75 mL) were collected in tubes containing sodium heparin as the anticoagulant. The plasma was separated from blood cells by centrifugation and then harvested and stored at −70 °C for quantitative analysis.

Non-compartmental analysis [18] was applied to the mean CyA plasma concentration data in male rats on day 24 and individual CyA plasma concentration data in male monkeys on day 28. The following parameters were estimated, maximum concentration in plasma (C_{max}), time to maximum concentration (T_{max}) and area under the plasma concentration–time curve from hour 0 to time of last measurable concentration in plasma, estimated by linear trapezoidal rule (AUC_{0-24}). All analysis was performed using WinNonlin Professional Edition (Pharsight Corporation, Version 4.1).

3. Results and discussion

As mentioned in Section 1, the broad peak of CyA eluted with previously reported methods [6,7] was primarily because of its molecular characteristics. This peak broadening effect reduces the column separation power. Even though the high degree of specificity offered by the tandem mass spectrometry has greatly reduced the requirements of sample preparation and chromatography, and has enabled high-throughput analysis as routine practices, this specificity should not be assumed as absolute and unlimited. Vogeser and Spohrer [12] pointed out that fast run on a short column caused co-elution of possible metabolites or endogenous materials with CyA that had the same MRM transition, resulting significantly elevated CyA concentrations compared with those measured with an extended run on a longer column. By examination of the chromatograms presented in the article, we found that the peaks of both CyA and the interference were broad, with base widths of at least 0.75 and 1.5 min, respectively, from the long run. The base width of the merged peak in the short run was slightly narrower but still spread for about 1 min. Apparently, the co-elution in the short run was caused by peak broadening effect. The chromatographic method described here effectively resulted a narrow peak of CyA, of which the base width ranged from only about 12 s (0.2 min) at the LLOQ (0.977 ng/mL) to 20 s (0.3 min) at ULOQ (4000 ng/mL) (Fig. 2). The narrowed peak in turn effectively improved the resolution power of the column as shown in Fig. 2 that CyA was baseline separated from the endogenous component eluted at 2.0–2.3 min.

On the other hand, endogenous components that could potentially enhance or suppress the ionization of CyA could also co-elute with CyA, given the wide peak span from previous methods. We have tested the matrix effects for both our and previous chromatography methods. Fig. 3 shows a chromatogram of CyA eluted from the same column but with acetonitrile–water mobile phases under a column temperature of 75 °C. The peak was five times broader than the one eluted with THF–water mobile phases, and most of the peak was in the ionization-suppressing region. This in turn required more sophisticated extraction methodologies, such as SPE and LLE, to remove the ionization-suppressing materials. Fig. 4 demonstrates that our THF–water mobile phases effectively resolved CyA from the ionization-suppressing substances.

The THF–water mobile phases used here to elute CyA into a sharp peak offered different selectivity than the widely used acetonitrile–water or methanol–water mobile phases. In terms of mobile phase selectivity, according to Snyder and Kikland [15], THF was categorized as a Type II solvent on reversed phase chromatography, but both acetonitrile and methanol were Type I solvents. Even though different selectivity can be achieved by different combinations of mobile phases and columns, the

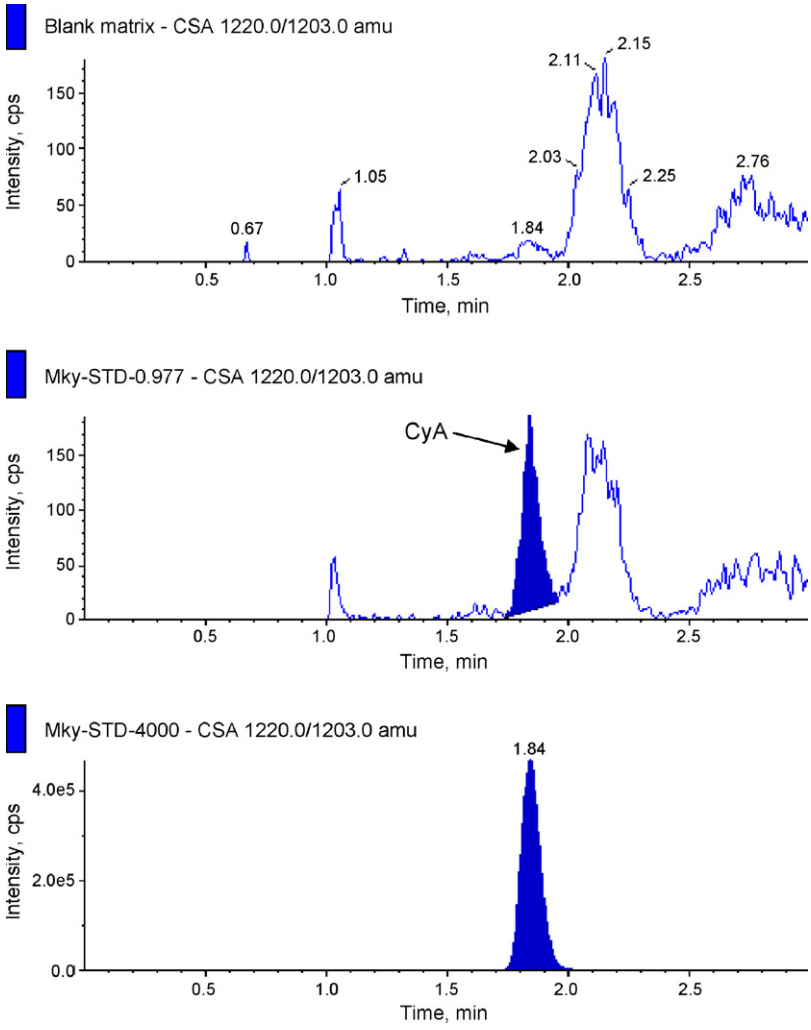

Fig. 2. Chromatograms of cyclosporin A at blank matrix, 0.977 (LLOQ) and 4000 (ULOQ) ng/mL in monkey plasma.

current practice in the field of LC–MS/MS bioanalysis is largely dependent upon the change of columns, not mobile phases. This practice is confirmed by a brief survey [16] the authors conducted for the chromatographic methods reported recently. The authors here would propose the consideration of diverse mobile phases, instead of only acetonitrile–water or methanol–water, to achieve needed selectivity for different compounds with diverse nature of physico-chemical properties.

One of the advantages of the method described here was the "normal" column temperature. It was only 30 °C instead of 65 °C

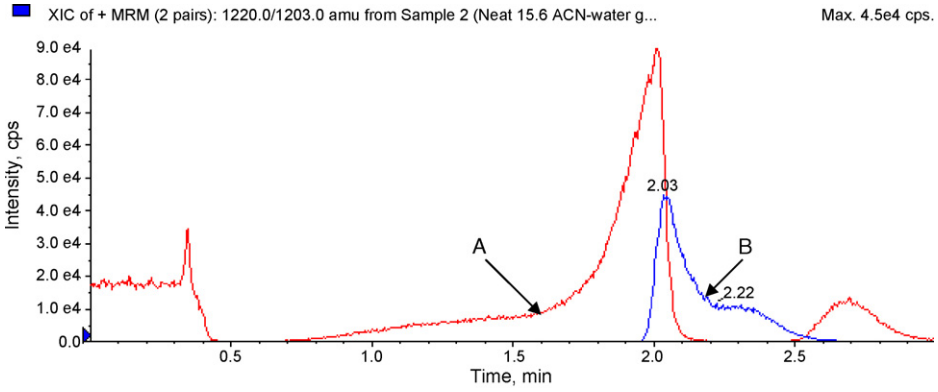

Fig. 3. Chromatograms of post-column infusion experiment eluted with gradient 5 mM ammonium acetate in water–acetonitrile mobile phases. *Note*. A constant flow of 10 μL/min of 100 ng/mL CyA neat solution was combined with the LC flow post-column through a Tee and delivered to the MS. Trace A was from the injection of extracted control monkey plasma. Trace B indicated the broad CyA peak. The gradient elution program was the same as the THF–water elution except, mobile phase B was 100% acetonitrile; the starting mobile phase was acetonitrile–5 mM NH_4OAc in water (55:45, v/v); and the column temperature was set at 75 °C.

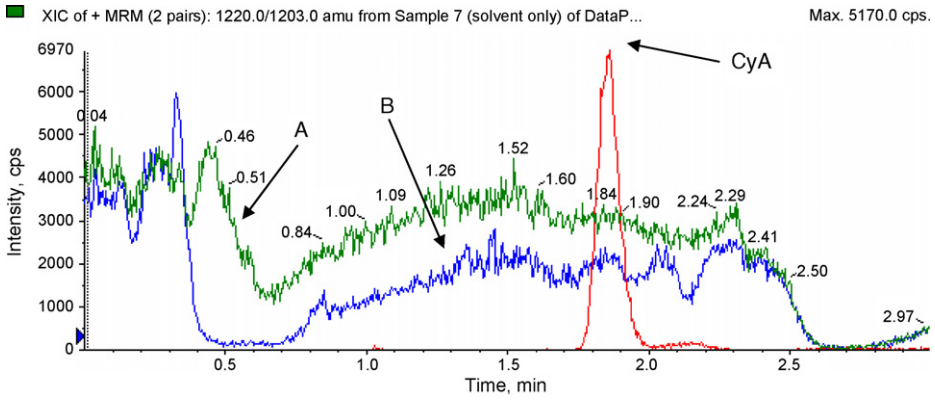

Fig. 4. Chromatograms of post-column infusion experiment eluted with gradient THF–5 mM ammonium acetate in water mobile phases. *Note*. A constant flow of 10 μL/min of 100 ng/mL CyA neat solution was delivered to combine with the LC flow post-column using a Tee and introduced to the MS. Trace A was from injection of neat solvent (acetonitrile–water, 70:30, v/v) only. Trace B was from the injection of extracted control monkey plasma. "CyA" indicated retention time and sharp peak of CyA eluted with the gradient mobile phases.

or higher reported in previous methods. High temperature could potentially accelerate deterioration of packed stationary phase and shorten column life. It was also difficult to keep the column oven at the set temperature, and slight fluctuation of column temperature could cause inconsistent chromatography results. This phenomenon was observed in our laboratory when a previous Covance method was used [14].

We anticipated increased signal to noise ratio for our method due to the sharp peaks and the resulting increased peak concentration. However, our method failed to achieve this theoretical advantage as demonstrated in Fig. 5. The peak height from our method was only 1/6 of the one from the conventional acetonitrile–water method. This finding indicated the ionization efficiency of CyA in THF–water mobile phase was much lower. This could be due to difficult desolvation or difficult formation of ammonium adduct ion ($[M + NH_4]^+$) that was monitored in the first quadrupole (Q1). Nevertheless, adequate sensitivity was still achieved with ESI-MS/MS detection and the use of THF–water mobile phases greatly improved the ruggedness and reproducibility of the chromatographic separations.

The previously reported sample extraction methods used either SPE or LLE approaches to separate CyA and internal standard from matrices such as proteins, peptides and inorganic salts. Even though SPE methods can be automated by utilizing 96-well format sample aliquote and collection, it typically needs time-consuming steps such as conditioning the extraction plate, loading samples, washing, elution, evaporation of eluent and reconstitution. LLE, on the other hand, is more labor intensive and typically needs the steps of twice tube-labeling, freezing the aqueous layer in dry-ice–acetone bath and transfer (pouring) of the organic layer, evaporation and reconstitution. All these time-consuming and labor-intensive sample extraction steps seriously reduced the throughput of the overall sample analysis. Although the semi-automated LLE approach reported by Brignol et al. [13] significantly improved the analysis throughput, it still used multiple partition and organic layer transfer, the evaporation and reconstitution steps. It has been estimated that the evaporation and reconstitution steps in 96-well format SPE generally accounted for 50% of the total time for sample extraction [17]. We have found that by using THF–water mobile phases, the extraction method could be simplified as protein precipitation on the basis of merely 50-μL of plasma, and the supernatant could be directly injected to the column for final analysis.

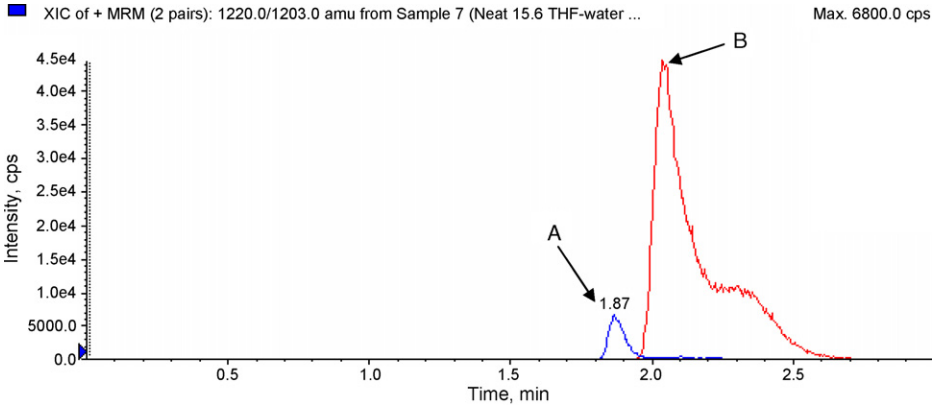

Fig. 5. Response comparison of cyclosporin A eluted with different mobile phases. *Note*. Same amount of CyA (25 μL of 15.6 ng/mL neat solution) was injected to column using: (A) THF–water mobile phase, peak height 7000 cps and (B) acetonitrile–water mobile phase, peak height 45,000 cps.

Table 1
Accuracy and precision of calibration standards

	Calibration standards (ng/mL)						
	0.977	3.91	15.6	62.5	250	1000	4000
Monkey plasma ($n=6$)							
Mean (ng/mL)	0.982	3.82	15.7	64.0	261	1019	3684
Accuracy (RE, %)	0.51	−2.3	0.53	2.5	4.5	1.9	−7.9
Precision (R.S.D., %)	15.3	4.9	8.4	3.8	5.0	4.9	4.0
Rat plasma ($n=2$)							
Mean (ng/mL)	0.975	3.87	16.7	62.9	268	985	3528
Accuracy (RE, %)	−0.22	−0.93	6.7	0.63	7.0	−1.50	−11.8
Precision (R.S.D., %)	13.7	7.2	0.73	4.8	4.0	2.5	5.7

As mentioned before, the sensitivity drop due to the use of THF as mobile phase made it necessary to inject 50 μL of the supernatant to the column to achieve the sensitivity of maintaining the LLOQ at 0.977 ng/mL. It proved that the starting mobile phase which contained only 25% THF was compatible with the high organic content (70% ACN) of the protein precipitation supernatant since both CyA and CyC were focused and chromatographed well in the run. Nevertheless, the use of THF–water mobile phases enabled the direct injection of the protein precipitation supernatant and eliminated the bottle-neck limiting step of evaporation and reconstitution.

The method performance has been extensively verified for CyA in monkey plasma and partially evaluated in rat plasma as well. Tables 1–3 list its performance parameters regarding linearity, accuracy and precision for both matrices. Compared with the most recently reported analytical approach [13], which used a sample volume of 300 μL plasma, our method only used 50 μL plasma and hence represented ease of use and improved sensitivity over previous methods.

The mean CyA concentration–time curves in monkeys (Fig. 6) and rats (Fig. 7) show that exposure to CyA increased as the dose level increased. Following oral administration, CyA was readily absorbed, with mean T_{max} values ranging from 4.80 to 6.40 h postdose on day 28 for monkeys and from 4:00 to 8:00 h postdose on day 24 for rats. In general, increases in C_{max} and AUC_{0-t} were greater than dose proportional in both monkeys (Table 4) and rats (Table 5).

Table 2
Accuracy and precision of monkey plasma quality control samples

	LLOQ (0.977 ng/mL)	LQC (3.0 ng/mL)	MQC (1600 ng/mL)	HQC (3200 ng/mL)
Day 1 ($n=6$)				
Mean	1.04	3.01	1507	2865
RE (%)	6.6	−0.43	−5.8	−10.5
R.S.D. (%)	6.6	2.1	2.4	1.6
Day 2 ($n=6$)				
Mean	0.956	3.02	1479	2940
RE (%)	−2.1	0.79	−7.6	−8.1
R.S.D. (%)	9.0	6.2	1.8	1.6
Day 3 ($n=6$)				
Mean	1.05	3.24	1589	2963
RE (%)	7.1	8.0	−0.68	−7.4
R.S.D. (%)	7.4	5.2	−2.0	2.2
Inter-day ($n=18$)				
Mean	1.02	3.09	1526	2923
RE (%)	3.9	3.0	−4.7	−8.6
R.S.D. (%)	8.4	5.8	3.7	2.2

Table 3
Accuracy and precision of rat plasma quality control samples

	LLOQ (0.977 ng/mL)	LQC (3.0 ng/mL)	MQC (1600 ng/mL)	HQC (3200 ng/mL)
Day 1 ($n=6$)				
Mean	0.965	2.81	1542	2805
RE (%)	−1.2	−6.4	−3.6	−12.4
R.S.D. (%)	13.2	3.9	2.9	2.1

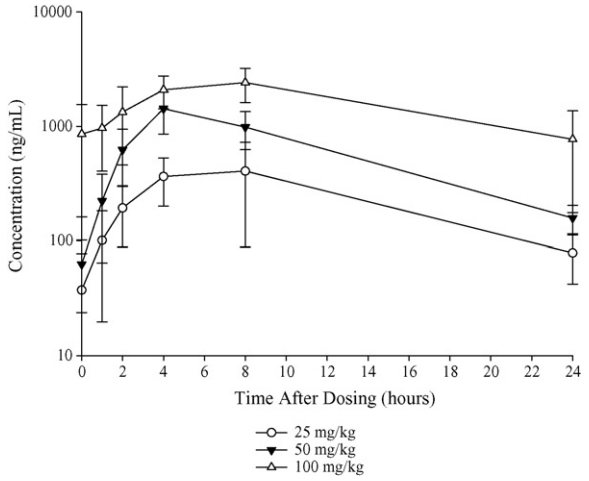

Fig. 6. Mean (S.D.) concentration (ng/mL) of cyclosporin A in male monkeys as a function of dose.

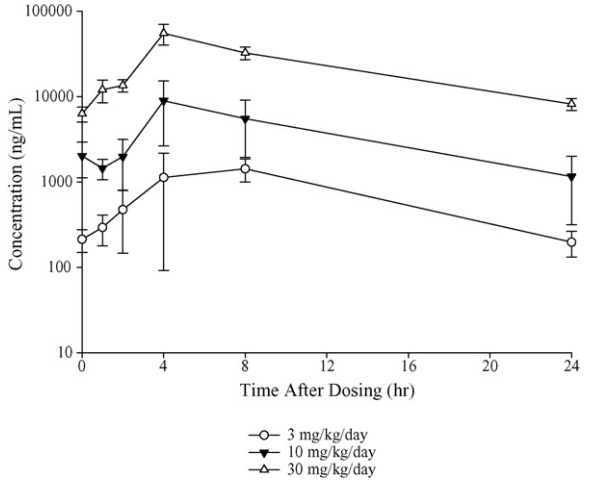

Fig. 7. Mean (S.D.) concentration (ng/mL) of cyclosporin A in male rats as a function of dose.

Table 4
Mean (S.D.) toxicokinetic parameters for cyclosporin A in monkey plasma

CyA dose level (mg/kg)	C_{max} (ng/mL)	T_{max} (h)	$AUC_{0-24\,h}$ (ng h/mL)
25	492 (226)	6.4 (2.19)	6205 (3680)
50	1615 (357)	4.8 (1.79)	16578 (2610)
100	2583 (807)	6.4 (2.19)	39947 (12155)

Table 5
Mean toxicokinetic parameters for cyclosporin A in rat plasma

CyA dose level (mg/kg)	C_{max} (ng/mL)	T_{max} (h)	$AUC_{0-24\,h}$ (ng h/mL)
3	1425	8	20324
10	8921	4	96294
30	54902	4	589553

4. Conclusions

The broad peak of CyA has been troubling chromatographers who used either methanol–water or acetonitrile–water as mobile phases. With the different selectivity offered by THF–water mobile phase, this peak broadening effect has been eliminated. The narrow peak improved the resolving power and hence avoided co-elution of possible interfering materials and ionization-suppressing agents from biological matrix. Other advantages included the use of normal column temperature for the chromatography, ballistic fast gradient elution as well as high-throughput analysis of biological samples. The later was realized due to the direct injection of the protein precipitation supernatant to the column and the elimination of labor-intensive liquid–liquid extraction or solid phase extraction. The only drawback of the new method was the reduced sensitivity from mass spectrometer that was probably due to difficult desolvation process in the ionization source. However, this reduced sensitivity could be well compensated by more injection volume, and the improved sensitivity offered by today's greatly improved mass spectrometers. CyA pharmacokinetics and observed concentrations were comparable to literature values [19,20]. In all, the use of THF–water mobile phase offered some unique advantages, and this approach could be an additional tool in the field of xenobiotic bioanalysis, which currently largely relies on the use of acetonitrile–water and methanol–water mobile phases.

Acknowledgement

The authors would like to thank Mr. Ryan Hurd of Covance Bioanalytical for the preparation of CyC solution.

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[14] Unpublished Covance method: determination of cyclosporin A in human whole blood by HPLC with tandem MS detection, Covance Laboratories, Inc., 2004. Brief description follows: column—Genesis C18, 50 mm × 4.6 mm, 3 μm particle size; mobile phase—isocratic acetonitrile–water–formic acid (85:15:0.1, v/v/v) at 1.0 mL/min for 3.5 min; column temperature: 60 °C confirmed with a temperature probe; sample extraction—manual liquid–liquid extraction of 100 μL human blood using methyl-*tert*-butyl ether (MTBE). The extraction comprised of twice tube-labeling, solvent partition, freezing aqueous layer in

acetone/dry-ice bath, pouring MTBE extract to second tube, evaporation of MTBE, reconstitution and final transfer of the extracts to 96-well plate for analysis. MS detection: MRM 1203 → 224 for CyA and 1219 → 224 for CyC (ISTD).

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