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Analysis of cyclosporine A and its metabolites in rat urine and feces by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the quantification of cyclosporine A (CyA) and the identification of its metabolites in rat urine and feces. The analytes were extracted from waste samples via liquid–liquid extraction. A Turboionspray source was used as a detector. It was operated in a positive ion mode with transitions of m/z 1225 → m/z 1112 for CyA and in a selected multiple reactions monitoring (MRM) mode with transitions of m/z 1239 → m/z 1099 for the internal standard (cyclosporine D, CyD). Linear calibration curves were obtained for CyA concentration ranges of 12.5–250 ng mL^{-1} in urine and 2.5–375 ng mg^{-1} in feces. The intra- and inter-day precision values (relative standard deviation) obtained were less than 8%, and the accuracy was within ±15% for each of the analytes. Extraction recoveries of CyA and CyD were both over 80%. The identification of the metabolites and elucidation of their structure were performed on the basis of their retention times and mass spectrometry fragmentation behaviors. A total of seven metabolites in rat feces were identified as dimethyl CyA, hydroxy CyA, and dihydroxy CyA after the oral administration of cyclosporine A-Eudragit® S100 nanoparticles (CyA-NP). Six of these metabolites were also detected in rat urine. A possible metabolic pathway was also proposed. The newly developed method was proven to be sensitive, simple, reproducible, and suitable for the rapid determination of CyA. It was successfully employed to study the excretion of CyA in rats and could be used to better understand the *in vivo* metabolism of CyA-NP, a potentially effective nanoparticle system.

1. Introduction

Cyclosporine A (CyA), a lipophilic cyclic polypeptide, has been widely used as an immunosuppressant for the prevention of organ transplant rejection in humans and for the treatment of immune-mediated diseases [1]. Each patient has a unique narrow therapeutic index as well as significant intra- and inter-individual variability in drug absorption, distribution, metabolism, and elimination. Without monitoring of the blood CyA concentrations, adjustment of necessary dosages remains difficult. As such, therapeutic drug monitoring (TDM) plays a key role in post-transplant regimens. Variations in concentrations outside the narrow therapeutic ranges could result in adverse clinical consequences [2,3]. Several analytical methods are available for the TDM of CyA. Immunoassays, for example, have been largely used for CyA measurements. However, immunoassays often lack specificity, and the cross-reactivity of immunoassay antibodies with metabolites causes a very significant and unpredictable overestimation especially in the case of CyA concentrations [4]. Therefore, the method of high performance liquid chromatography with UV detection (HPLC–UV) appears to be more appropriate for such analyses than immunoassays [5–7]. Recently, analytical methods for CyA detection by liquid chromatography–mass spectrometry (LC–MS) or LC–MS/MS were developed [8–11]. Compared with HPLC–UV, LC–MS/MS assays were found to be over ninefold times more sensitive than HPLC–UV [12,13].

CyA is extensively metabolized by cytochrome P-450 3A enzymes (CYP 3A) into more than 30 metabolites that differ in their therapeutic activities and toxicity [14]. Identification of CyA metabolites in biological matrices is usually a very complicated process. HPLC–UV assays have been reported for the separation of CyA and its metabolites in biological samples [7,15,16]. Since the development of tandem mass spectrometry techniques, LC–MS/MS has been proven to be a more powerful analytical approach for the identification of CyA metabolites owing to its soft-ionization technique, high specificity for qualitative analysis at trace levels, and maximum amount of structural information [17]. This technique allies

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the discrimination power of mass spectrometry with the higher separation power of HPLC [18]. Thus, the LC–MS/MS technique is the primary choice for comprehensive structural characterization and quantification of the CyA metabolites.

Depending on the administration route, targeting drug delivery systems (TDDSs) have previously been proven to be an efficient approach to achieving better pharmacokinetic profiles and even to increasing the bioavailability of several drugs [19]. Previous studies of CyA-loaded nanoparticles (NP) *in vivo* had demonstrated that Eudragit® S100 NP could be used as a potential vehicle for drug administration as it showed significant increases in oral bioavailability of the drug. Prepared from the enteric polymers of Eudragit® S100, CyA-NP, with particle sizes less than 100 nm, exhibited perfect pH-sensitivity and protected CyA from degradation by gastric acids or enzymes [20,21]. With fast stomach emptying rates, absorption-site specificity, low degradation rates of luminal contents, high levels of bioadhesion of nanoparticles to intestinal mucosa, and the use of a P-glycoprotein inhibitor, CyA-NP increased the absorption of CyA [22]. This vehicle has demonstrated the potential for the development of new CyA-loaded NP formulations. The metabolic study of CyA-NP can be valuable in the development of new medical preparations and better clinical applications.

The aim of the present study is to develop and validate a method for the quantification of CyA and the identification of its metabolites in rat urine and feces based on LC–MS/MS. To the best of our knowledge, no previous study has reported the determination of CyA in rat feces using this method. This study also demonstrates the application of this assay method after a single oral dose of CyA-NP is administered to rats.

2. Experimental

2.1. Chemicals, reagents and test subjects

CyA and CyD were provided by Fujian Kerui Pharmaceutical Co. Ltd. (China). CyA-NP was prepared by the authors based on a previously described method [21]. Acetonitrile and methanol (HPLC-grade) were purchased from Tedia Company Inc. (USA). All other chemicals and solvents used were of analytical grade.

Male Sprague–Dawley (SD) rats, weighing 220–270 g, were obtained from the Laboratory Animals Center of the Medical College of Soochow University. All animal procedures were conducted in accordance with the guidelines from the National Institutes of Health for the care and use of animals and were carried out under the approval of the Laboratory Animals Center of the Medical College of Soochow University.

2.2. Liquid chromatography and mass spectrometry conditions

Chromatographic analysis was performed using an Agilent 1100 LC system (Agilent Technologies, Palo Alto, CA, USA), consisting of a quaternary pump and an autosampler. Separation of the analytes was achieved on a Hypersil C18 column (5 μm in 2.1 mm × 150 mm, Thermo Fisher Scientific, USA) maintained at 70 °C. The optimum wavelength was set at 210 nm. The mobile phase consisted of acetonitrile–methanol–water (22:9:9, v/v/v) passed at a flow rate of 0.2 mL min^{-1}. The injection volume was 5 μL for each sample.

An API-2000 triple quadrupole mass spectrometer was operated with a Turboionspray source interface in positive ion mode. Instrument control and the acquisition and analysis of data were all processed by Analyst 1.4.1 software (Applied Biosystems, Foster City, CA, USA). The positive ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) conditions were as follows: 5500 V ion spray voltage, 380 °C ion source heater temperature, 30 psi ion source gas for nebulizing, 50 psi ion source gas for drying solvent, 25 psi curtain gas, and high collision gas. All gases used were nitrogen (N_2). Ramping in the multiple reactions monitoring (MRM) mode further improved the sensitivity for both CyA and CyD ion pairs. Accordingly, the ramped parameters in the MRM mode for CyA and CyD included: a 115 V declustering potential (DP), 11 V entrance potential (EP), 80.2 V collision energy, and a 3.0 V collision cell exit potential. Quantification was performed using the MRM of the transitions of m/z 1225 → m/z 1112 for CyA and m/z 1239 → m/z 1099 for CyD.

2.3. Standard and quality control (QC) solution preparation

A stock solution of CyA was prepared by dissolving 40 mg of CyA in 100 mL of HPLC-grade methanol. An internal standard stock solution (10 μg mL^{-1}) was obtained by dissolving CyD powder in methanol. A series of working solutions was prepared by serially diluting the stock solution with methanol. Quality control (QC) solutions (low, medium, high) were also prepared in the same manner using a separately weighed stock solution. All solutions were stored at 4 °C before use.

2.4. Method validation

Selectivity was determined by analyzing blank urine and feces samples from five different sources for interferences based on the retention times of the analytes and internal standard.

Calibration standards of CyA were prepared by adding appropriate amounts of working solutions to blank samples in order to obtain concentrations ranging from 12.5 to 250 ng mL^{-1} and 2.5 to 375 ng mg^{-1} for urine and feces, respectively. Calibration curves were constructed using a weighted least squares linear regression analysis of the drug concentrations (C) versus peak area ratios of CyA/CyD (Ai).

The precision and accuracy of the assay were determined by analysis of the QC samples obtained by spiking urine and feces with CyA in five replicates for three validation days. Precision was expressed as a percentage of relative standard deviation (% RSD), while the accuracy (%) was expressed as [(mean calculated concentration − nominal concentration)/(nominal concentration) × 100]. The accuracy was required to be within ±15%, and the intra- and inter-day precision values (% RSD) were not to exceed 15%. The QC samples were run at CyA concentrations of 25, 125, and 200 ng mL^{-1} for urine and concentration of 25, 100 and 250 ng mg^{-1} for feces.

The extraction recoveries of CyA and CyD were determined by comparing the peak areas obtained from urine and feces samples at low, medium, and high concentrations for the analytes and at a single concentration for the internal standard with the peaks found by the direct injection of standard solutions containing equivalent concentrations of CyA and CyD.

The stability of the biological standard samples of CyA was investigated by analyzing QC samples exposed to different time and temperature conditions. The stability of samples was evaluated after storing spiked rat urine and feces for 24 h at ambient temperature and after 7 days at 4 °C. The stability of extracted samples during storage in the autosampler was studied after storing for 24 h at ambient temperature.

2.5. Preparation of analytical samples from urine and feces

The preparation of analytical samples from urine and feces was adopted from a method by Yang et al. [21]. Urine samples (1 mL) were added to a centrifuge tube and spiked with 200 μL of internal standard (CyD) solution and 1 mL of hydrochloric acid (180 mmol L^{-1}). After vortexing for 1 min, 5 mL of ether was added to the solution. The extraction was carried out by shaking the tube

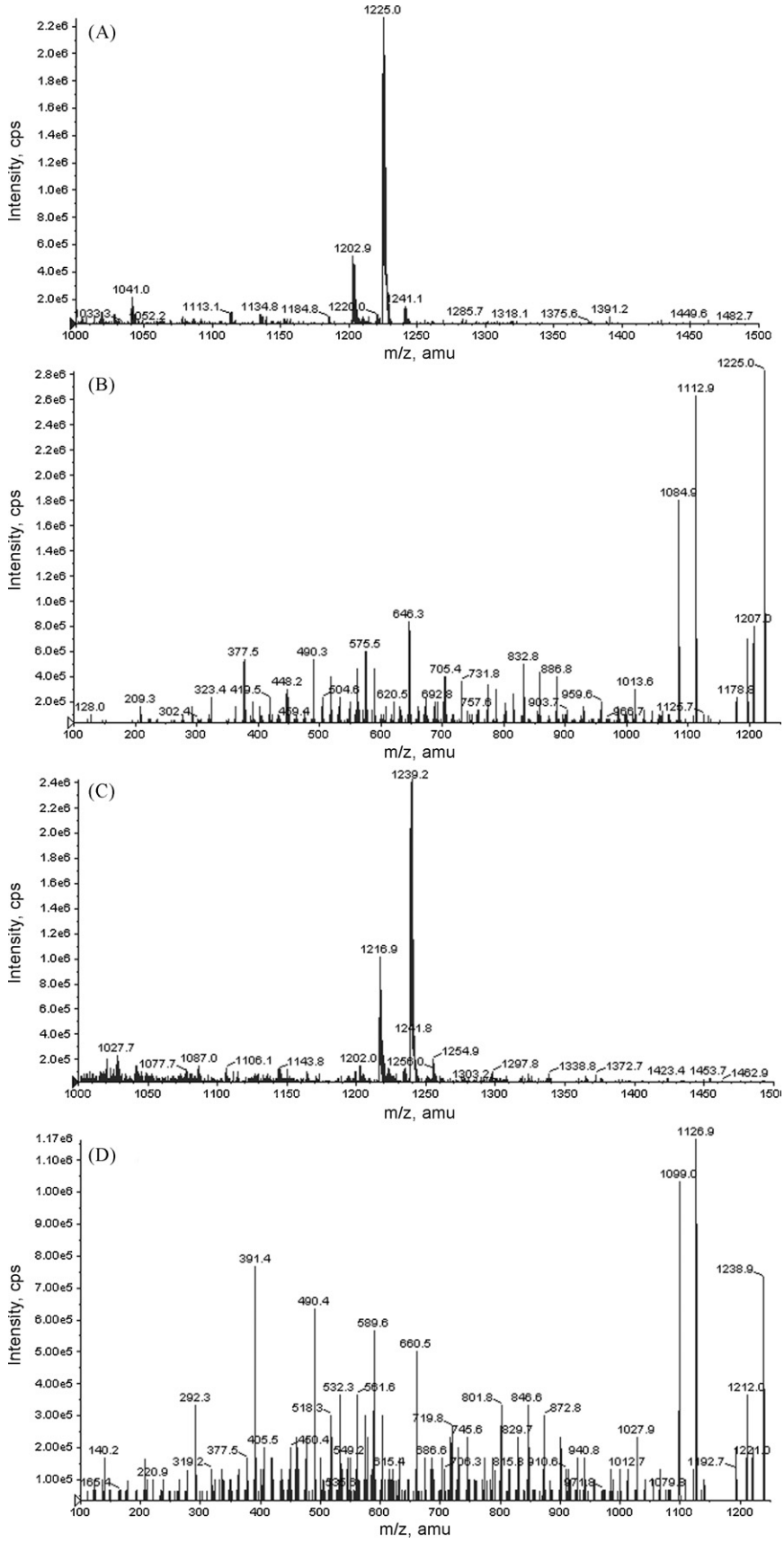

Fig. 1. Mass spectra of CyA and CyD. (A) Enhanced mass spectrum of CyA; (B) enhanced product ion spectrum of $[M+Na]^+$ of CyA at m/z 1225.0; (C) enhanced mass spectrum of CyD; and (D) enhanced product ion spectrum of $[M+Na]^+$ of CyD at m/z 1239.2.

horizontally for 15 min at 100 rpm, centrifuging it for 15 min at 4000 rpm, and separating the ether phase into another centrifuge tube. Afterwards, 1 mL of sodium hydroxide (95 mmol L^{-1}) and 2.5 mL of 1% sodium pyrosulfite were added to this new centrifuge tube, ether extraction was repeated. The ether layer was transferred to a clean tube and evaporated to dryness under nitrogen at 40 °C. The residue was reconstituted with 250 μL of acetonitrile/water (70/30, v/v). The reconstituted samples were washed three times with 1 mL of n-hexane and then transferred to HPLC autosampler vials. About 5 μL of residual solution was injected into the LC–MS/MS system.

Feces samples were crushed, weighed, and homogenized in water at a rate of 10 mL g^{-1}. A 1 mL aliquot was added to a centrifuge tube and spiked with 400 μL of CyD solution. The following procedure was similar to that of urine sample preparation.

2.6. Collection and analysis of samples

Five rats were housed in stainless steel metabolic cages provided with urine–feces separators. The rats were fasted overnight but had free access to water. The rats were orally administered with CyA-NP at a dosage of 15 mg kg^{-1}. Urine and feces samples were collected at pre-dose (0 h) and at different time intervals (i.e., 0–3, 3–6, 6–12, 12–36, and 36–84 h post-dose. After the volumes of urine obtained were measured, the samples were stored at −70 °C until analysis.

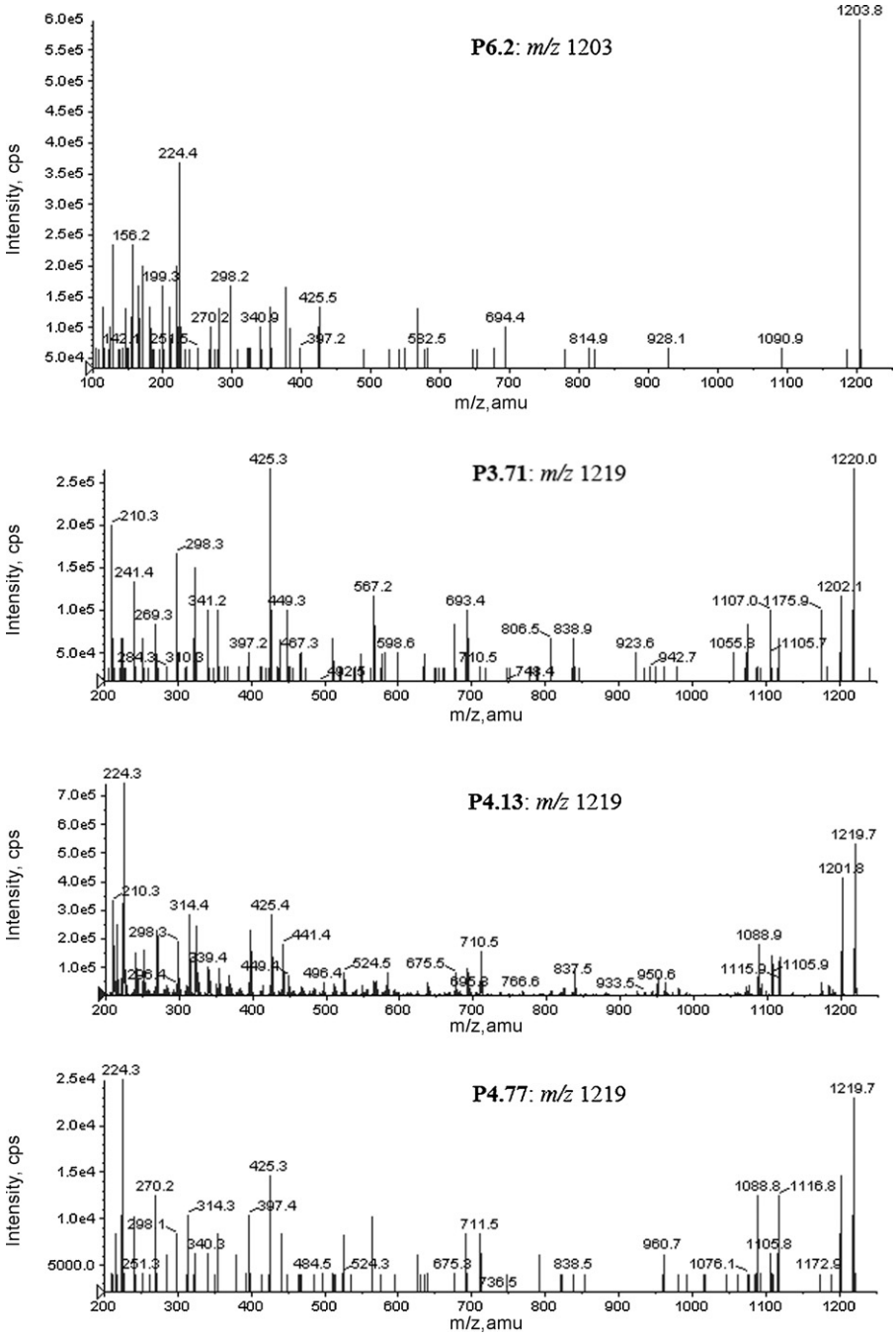

Fig. 2. MS/MS product ion spectra of CyA and its metabolites in rat feces.

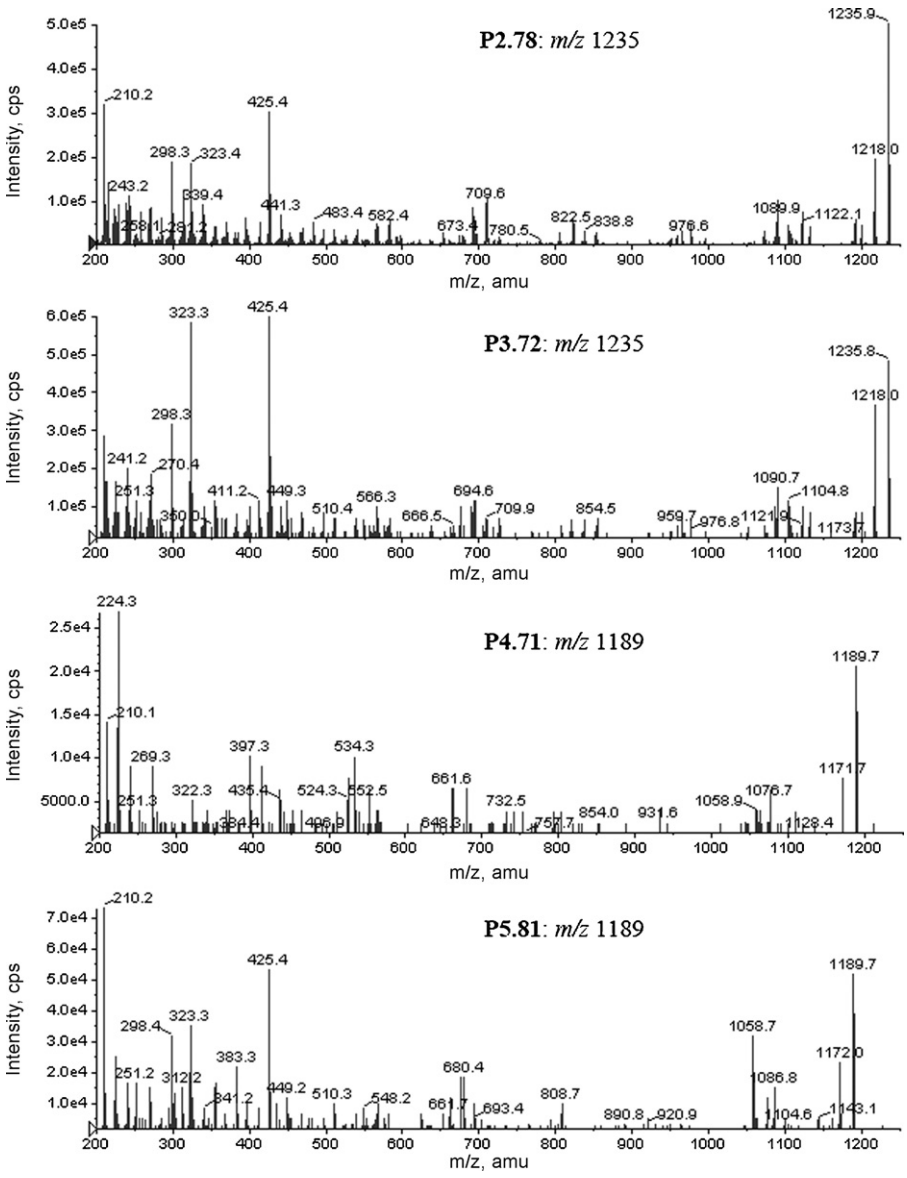

Fig. 2. (*Continued*).

The CyA concentration in the samples was analyzed by HPLC–MS/MS. The excretion ratio was calculated as follows:

$$\text{Excretion ratio (\%)} = \frac{\text{Drug concentration in sample} \times \text{sample volume or weight}}{\text{Amount of drug administered}} \times 100$$

3. Results and discussion

3.1. Mass spectrometry and in vivo identification of rat metabolites

The responses of CyA and CyD to ESI were evaluated by recording the mass spectra scanned from *m*/*z* 200 to 1500 in positive ionization modes. As shown in Fig. 1(A) and (B), the positive mode yielded stronger signals of the sodium adduct ion $[M+Na]^+$ for CyA (*m*/*z* 1225.0) and CyD (1239.2) than those of the protonated molecule $[M+H]^+$ and potassium adduct $[M+K]^+$ ions. The $[M+Na]^+$ of CyA and CyD was selected as the precursor ion. In the product ion spectra, several fragment ions were obtained. For CyA, the most abundant product ion was *m*/*z* 1112 and that for CyD was *m*/*z* 1099 (Fig. 1C and D). These product ions were extracted for quantification.

3.1.1. LC–MS/MS analysis of CyA

Since metabolites could retain the base substructure of the parent drug, the first step of this work involved the characterization of the chromatographic and mass spectral properties of the parent drug, which provide the substructural template for interpreting the structures of the metabolites.

The chromatographic and mass spectrometric conditions were optimized using the CyA standard in positive ion detection mode. A full scan mass spectral analysis of CyA showed protonated molecular ions at *m*/*z* 1203. The MS/MS product ion spectrum of the protonated molecular ion (*m*/*z* 1203) and the fragmentation patterns are shown in Fig. 2. The principle fragment ions are listed in Table 1. The product ions at *m*/*z* 1185 were formed by the loss of H_2O from the parent ion at *m*/*z* 1203.

Table 1
The principle fragment ions (m/z) observed in the mass spectra of $[M+H]^+$ ions of CyA metabolites from rat urine and feces.

Analyte	Ion	$[M+H]^+$	Amino acid position												
			5 b_3^{2-3}	6 b_4^{2-3}	8 b_6^{2-3}	9 b_7^{2-3}	10 b_8^{2-3}	11 b_9^{2-3}	2 b_3^{1-11}	3 b_3^{1-11}	4 b_4^{1-11}	5 b_5^{1-11}	6 b_6^{1-11}	7 b_7^{1-11}	8 b_8^{1-11}	9 b_9^{1-11}	10 b_{10}^{1-11}	9 b_4^{5-6}	10 b_5^{5-6}	11 b_6^{5-6}
CyA	Calc.	1202.8	298.2	425.3	567.4	694.5	821.6	934.7	269.2	340.2	467.3	566.4	693.5	764.5	835.6	962.7	1089.8	397.3	524.4	637.5
P6.3	Meas.	1203.8	298.2	425.5	567.5	694.4	821.5	n.f.	269.2	340.9	n.f.	n.f.	694.4	n.f.	n.f.	n.f.	1090.9	397.2	524.0	n.f.
AM4N	Calc.	1188.8	284.2	411.3	553.4	680.5	807.6	920.6	269.2	340.2	453.3	552.4	679.5	750.5	821.6	948.7	1075.8	397.3	524.4	637.5
P4.71	Meas.	1189.7	n.f.	411.2	n.f.	680.6	807.8	n.f.	269.3	340.2	453.2	552.5	n.f.	750.6	822.5	948.7	1076.7	397.3	524.3	638.5
AM1N	Calc.	1188.8	298.2	425.3	567.4	694.5	821.6	934.7	255.2	326.2	453.3	552.4	679.5	750.5	821.6	948.7	1075.8	397.3	524.4	637.5
P5.81	Meas.	1189.7	298.4	425.4	567.3	693.4	n.f.	n.f.	255.4	326.1	n.f.	552.2	679.6	n.f.	n.f.	n.f.	1076.7	397.4	n.f.	637.6
AM1	Calc.	1218.8	298.2	425.3	567.4	694.5	821.6	934.7	285.2	356.2	483.3	582.4	709.5	780.5	851.6	978.7	1105.8	397.3	524.4	637.5
P3.71	Meas.	1220.0	298.3	425.3	567.2	693.4	n.f.	934.5	284.3	356.3	n.f.	582.2	710.5	n.f.	n.f.	979.5	1105.7	397.2	n.f.	637.6
AM1c	Calc.	1218.8	298.2	425.3	567.4	694.5	821.6	934.7	285.2	356.2	483.3	582.4	709.5	780.5	851.6	978.7	1105.8	397.3	524.4	637.5
P4.77	Meas.	1219.7	298.1	425.3	567.5	693.9	821.7	n.f.	285.1	356.1	484.5	582.2	711.5	n.f.	851.7	979.5	1105.8	397.4	524.3	637.4
AM9	Calc.	1218.8	298.2	425.3	567.4	710.5	837.6	950.7	269.2	340.2	467.3	566.4	693.5	764.5	835.6	978.7	1105.8	413.3	540.4	653.5
P4.13	Meas.	1219.7	298.3	425.4	n.f.	710.5	837.5	950.6	270.2	340.1	467.5	n.f.	695.8	766.6	837.5	979.5	1105.9	413.4	n.f.	654.5
AM19	Calc.	1234.8	298.2	425.3	567.4	710.5	837.6	950.7	285.2	356.2	483.3	582.4	709.5	780.5	851.6	994.7	1121.8	413.3	540.4	653.5
P2.78	Meas.	1235.9	298.3	425.4	567.5	710.8	838.8	951.7	285.1	356.3	483.4	582.4	709.6	780.5	853.4	n.f.	1122.1	413.5	n.f.	653.6
AM1c9	Calc.	1234.8	298.2	425.3	567.4	710.5	837.6	950.7	285.2	356.2	483.3	582.4	709.5	780.5	851.6	994.7	1121.8	413.3	540.4	653.5
P3.72	Meas.	1235.8	298.3	425.4	566.3	710.4	837.7	950.6	n.f.	356.3	n.f.	n.f.	709.9	n.f.	850.6	994.9	1121.9	411.2	540.6	653.3

n.f., not found.

3.1.2. LC–MS/MS analysis of metabolites

In order to identify the metabolites *in vivo*, the possible structures of metabolites were first speculated according to the metabolism rule of drugs [23]. Due to a lack of authentic standards, the full scan mass spectra of the biosamples after the administration of CyA-NP were compared with those of blank rat urine and feces samples to determine the possible metabolites. These compounds were then analyzed by HPLC–MS/MS to elucidate their structures through a comparison of their retention time, changes in observed mass (Δm), and mass spectral patterns of product ions with those of CyA.

Based on the method above, the parent drug and its metabolites were found in rat urine and feces after the administration of CyA-NP. Their molecular ions $[M+H]^+$ were found at m/z 1189, 1203, 1219, and 1235. The MS/MS product ion spectra (Fig. 2), obtained via fragmentation of these protonated molecular ions, were used for more precise structural identification of metabolites. Among them, the retention time and the MS and MS/MS spectra of the molecular ion at m/z 1203 were found to be the same as those of the CyA standard. Thus, this compound could be confirmed as the unchanged parent drug.

CyA is metabolized by cytochrome P-450 3A enzymes. This includes hydroxylation of amino acid residues 1, 4, 6, and 9, demethylation of amino acid residue 4, and intramolecular cyclization as well as oxidation of amino acid residue 1 [24]. The main fragmentation pathways start with the primary splitting between amino acids 2–3, 1–11, and 5–6. The corresponding N-terminal b-type ions are common fragment types in the mass spectra. The 1–11 splitting can be enhanced by the introduction of a lactone group into the peptide ring through the conversion of cyclosporins into isocyclosporins [25]. The principle fragment ions (m/z) observed in the mass spectra of $[M+H]^+$ ions of CyA metabolites from rat urine and feces are shown in Table 3.

The mass spectra of P3.71, P4.13, and P4.77 were detected at a retention time of 3.71, 4.13, and 4.77 min, respectively, and all showed the protonated molecular ion at m/z 1219. The molecular ion $[M+H]^+$ at m/z 1219 increased by 16 Da compared to that of the unchanged parent drug. There also appeared to be a hydroxylation (16 Da) reaction for CyA. The product ion at m/z 1202 was generated via the loss of a neutral H_2O fragment from the molecular protonated ion at m/z 1219. The product ions at m/z 284.3, 356.3, 582.2, 710.5, 979.5, and 1105.7 (P3.71) were all 16 Da more than those of CyA. The localization of the hydroxyl group should be at ^{1}MeBmt. Based on the appearances of these characteristic fragment ions in their spectra and according to past investigations and references [26], P3.71 was identified as AM1. The characteristic product ions of P4.77 were similar to those of P3.71. All known cyclized metabolites have longer retention times than uncyclized compounds [27]. P4.77 was identified as AM1c. The product ions at m/z 710.5, 837.5, 950.6, 413.4 and 654.5 (P4.13) were also all 16 Da more than those of CyA. These results indicate that the localization of the hydroxyl group should be at ^{9}MeLeu. Thus, P4.13 was identified as AM9.

The protonated molecular ion $[M+H]^+$ at m/z 1235 increased by 32 Da compared with that of the parent drug. Two chromatographic peaks appeared in the LC–MS/MS chromatogram at m/z 1235 with retention times of 2.78 min (P2.78) and 3.72 min (P3.72). Dihydroxylation (16 + 16 Da) reactions at CyA appeared to have occurred. P2.78 and P3.72 eluted earlier than the mono-hydroxylated metabolites AM1, AM9, and AM1c, which is consistent with the fact that di-hydroxylated metabolites are more hydrophilic. The product ion at m/z 1219 was formed by the loss of a neutral H_2O fragment from the molecular protonated ion at m/z 1235. The product ions at m/z 710.8, 838.8, 951.7, 285.1, 356.3, 483.4, 582.4, 709.6, 780.5, 1122.1, 413.5, and 653.6 were all 16 Da more than those of CyA. The dehydroxylating position should be located at ^{9}MeLeu and

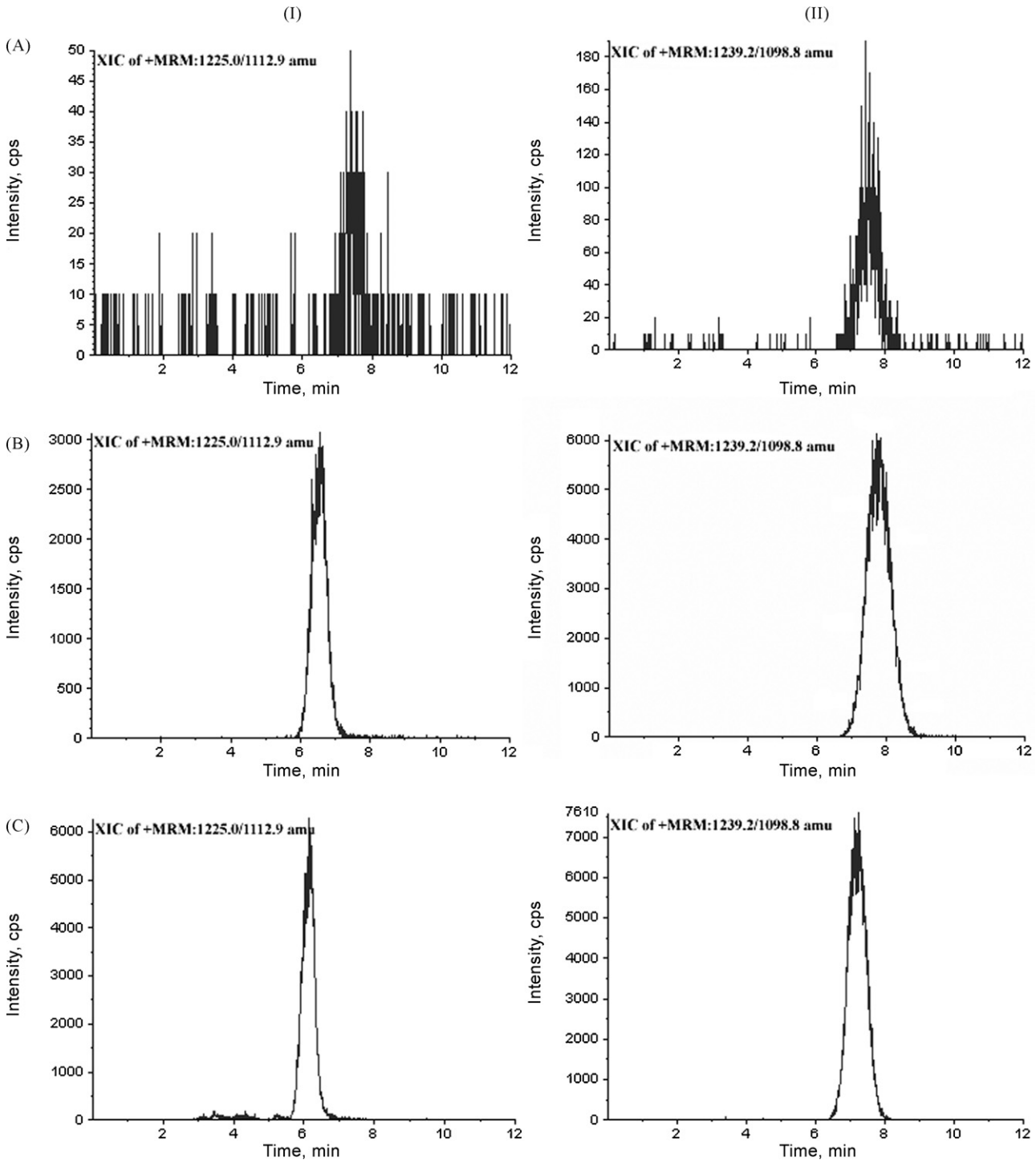

Fig. 3. Representative MRM chromatograms of CyA and CyD in rat urine (I: m/z 1225.0 → 1112.0 amu; II: m/z 1239.2 → 1099.0): (A) blank urine sample; (B) urine sample spiked with CyA and CyD; and (C) urine sample.

^{1}MeBmt. As mentioned above, cyclized metabolites have longer retention times than uncyclized compounds. As such, P2.78 and P3.72 were identified as AM19 and AM1c9, respectively.

Two peaks with retention times of 4.71 min (P4.71) and 5.81 min (P5.81) were identified for m/z 1189. The molecular ion $[M+H]^+$ at m/z 1189 was 14 Da less than that of the parent drug, and there appeared to be an N-demethylation (14 Da) reaction at CyA. The product ion at m/z 1171 was produced by the loss of a neutral H_2O fragment from the molecular protonated ion at m/z 1189. The product ions at m/z 411.2, 680.6, 807.8, 453.2, 552.4, 679.5, 750.5, 822.5, 948.7, and 1076.7 (P4.71) were all 14 Da less than CyA. The above results indicate that demethylation reactions occurred in the N group of ^{4}MeLeu. P4.71 could thus be confirmed as AM4N.

The product ions at m/z 255.4, 326.1, 552.2, 679.6, and 1076.7 (P5.81) were all 14 Da less than CyA. The demethylation position should be located at ^{1}MeBmt. As such, P5.81 was identified as AM1N.

By comparing the profiles of urine and feces samples after dosing with CyA-NP, it was shown that seven metabolites (AM1, AM1c, AM9, AM19, AM1c9, AM1N, and AM4N) were detected in rat feces samples *in vivo*, while six metabolites (all those mentioned above except for AM1c) were identified in rat urine.

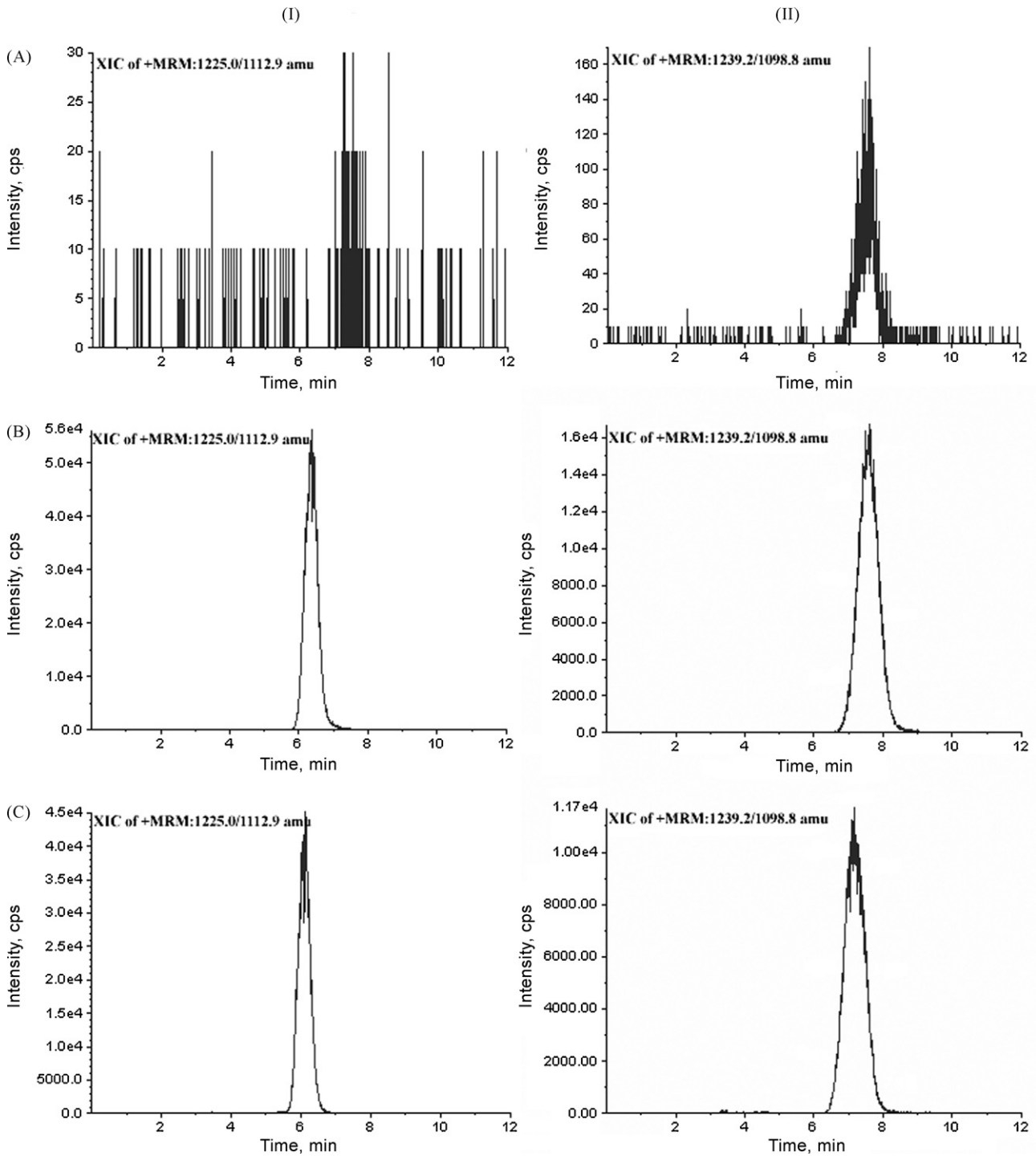

Fig. 4. Representative MRM chromatograms of CyA and CyD in rat feces (I: m/z 1225.0 → 1112.0 amu; II: m/z 1239.2 → 1099.0): (A) blank feces sample; (B) feces sample spiked with CyA and CyD; and (C) feces sample.

3.1.3. Elucidation of the possible metabolic pathway

Drugs are metabolized by oxidative processes, such as hydroxylation, carboxylation, demethylation, and cyclization via epoxide formation as well as conjugation reactions with sulphuric or glucuronic acid. CyA was extensively metabolized by enzymes different from the cytochrome P-450 3A subfamily, resulting in hydroxylation, N-demethylation, cyclization, and oxidation. All of its metabolites, the structures of which have been elucidated, retained their cyclic undecapeptide structure and were more hydrophilic than the original compound [24].

The metabolic pathway of CyA was similar between humans and rodents. The primary metabolites, mono-hydroxylated AM1, AM9, N-demethylated AM4N, and AM1N, were produced by metabolic alteration of single functional groups. Subsequently, various combinations of locations of primary attack generated a complex metabolic profile. Further oxidation of AM1 and AM9 resulted in di-hydroxylated AM19 [28]. AM1 and AM19 were subjected to further biotransformation, yielding AM1c and AM1c9 as the quantitatively most important secondary metabolites [14]. In addition, AM1c9 could be generated by the oxidation of AM1c. In humans and rabbits, the metabolite AM1 was the major metabolite observed,

Table 2
Intra- and inter-day precision and accuracy of the assay for urine and feces samples (3 days, 5 replicates per day).

Samples	Nominal concentration ($ng\,mL^{-1}$ or $ng\,mg^{-1}$)	Calculated concentration ($ng\,mL^{-1}$ or $ng\,mg^{-1}$)	RSD (%)	Accuracy (%)
Urine	Intra-day			
	25	25.8 ± 0.9	3.3	3.1
	125	140.6 ± 6.3	4.5	12.4
	200	185.4 ± 6.8	3.7	−7.3
	Inter-day			
	25	26.4 ± 1.4	5.4	5.4
	125	143.4 ± 5.4	3.7	14.6
	200	186.3 ± 5.7	3.1	−6.9
Feces	Intra-day			
	25	26.2 ± 0.6	2.5	4.7
	100	103.0 ± 2.5	2.4	2.9
	250	248.0 ± 8.2	3.3	−0.8
	Inter-day			
	25	27.1 ± 2.0	7.3	8.3
	100	105.2 ± 3.7	3.5	5.2
	250	255.1 ± 12.1	4.7	2.0

whereas AM9 predominated in rats and dogs. AM4N is mainly generated in humans and rats [29,30].

The possible immunosuppressive activity or renal side effects of CyA metabolites are still controversial topics. Experiments to determine the immunosuppressive or toxic activity of metabolites have been restricted to *in vitro* and animal tests [31], most of which focus on their effects on lymphocyte proliferation in concanavalin A activation, mixed lymphocyte cultures, and primed lymphocyte test assays. The possible contribution of CyA metabolites to the immunosuppressive activity of CyA was first discussed by Fidelus and Ferguson [32]. Some metabolites exhibited higher levels of immunosuppressive activity than CyA [33,34], whereas other studies indicated that some of them retained a level of immunosuppressive activity that was lower or similar to CyA [34–36]. Furthermore, the structural–functional relationship of CyA with its metabolites has been reported. Changes to amino acids at positions 4, 6, and 11 led to a complete loss of immunosuppressive activity, while metabolites retained immunosuppressive activity with changes to amino acids at position 1.

3.2. Selectivity assay

The specificity of the method was tested by comparing the chromatograms of blank and spiked samples. In the MRM mode of positive ions, Figs. 3 and 4 show that no endogenous substances in rat urine and feces interfered with CyA or CyD. A typical chromatogram of an extract from a dosed rat's urine and feces containing CyA and CyD displayed peaks that were absent in the chromatogram of a blank sample. The retention times of CyA and CyD were about 6.2 and 7.4 min, respectively.

3.3. Linearity

The CyA determined in urine and feces samples at concentration ranges of 12.5–250 $ng\,mg^{-1}$ and 2.5–375 $ng\,mg^{-1}$, respectively, displayed good linear relationships. The mean values of the regression equation of CyA in rat urine and feces were $C = 316.56Ai - 32.316$ ($r = 0.9991$) and $C = 18.834Ai - 11.034$ ($r = 0.9990$), respectively. The assay proved to be linear and acceptable.

The lower limit of quantification (LLOQ) for the determination of CyA was 12.5 $ng\,mL^{-1}$ in urine and 2.5 $ng\,mg^{-1}$ in feces. At this LLOQ level, the intra-day precision values were 7.8 and 5.5%, respectively, while the accuracy was 12.4 and 13.1% for urine and feces.

Table 3
Extraction recovery of CyA and CyD from spiked rat urine and feces.

Compound	Added concentration ($ng\,mL^{-1}$ or $ng\,mg^{-1}$)	Recovery (%)	RSD (%)
CyA	Urine		
	25	86.8 ± 6.0	6.9
	125	89.9 ± 7.6	8.5
	200	80.3 ± 6.1	7.6
	Feces		
	25	93.3 ± 6.4	6.9
	100	89.0 ± 3.6	4.0
	250	81.4 ± 6.6	9.0
CyD	Urine		
	2000	86.6 ± 6.2	7.2
	Feces		
	40	89.1 ± 6.0	6.7

3.4. Precision and accuracy

The accuracy and precision values of the method for rat urine and feces are presented in Table 2. The intra-day precision ranged from 2 to 5% and that of inter-day precision ranged from 3 to 8%. The procedure was accurate for all the concentrations tested with RSD ranging from 2 to 15%. The data shows that the precision and accuracy of the method were acceptable.

3.5. Extraction efficiency and stability

The extraction recovery of CyA was measured using three levels of concentration in rat urine and feces, while the recovery of the internal standard was determined at only one concentration level. The results are listed in Table 3. The mean recoveries of CyA for urine and feces samples were both over 80% and the RSD were all below 10%. The mean recovery of CyD was 86% (RSD 7.2%) and 89% (RSD 6.7%) for urine and feces, respectively.

In the present study, liquid–liquid extraction (LLE) was explored as a technique for recovering CyA from the samples. Compared with solid-phase extraction (SPE), LLE exhibited high recoveries of the spiked standard compounds. Moreover, LLE was easily obtained and economical. During the extraction procedure, deproteinization was an important feature because impurities in the sample liquid resulting from incomplete deproteinization may block the LC–MS/MS. A two-step LLE with ether was selected because it could ensure the simultaneous extraction of all target compounds. In

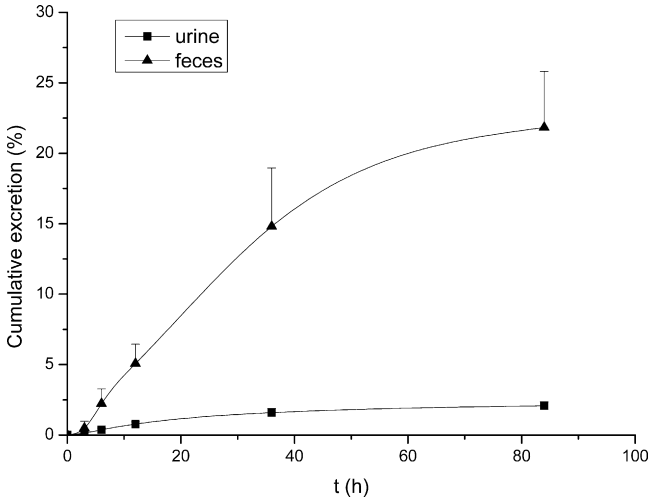

Fig. 5. Cumulative amounts of excreted in urine and feces–time curves of CyA after the oral administration of CyA-NP to rats ($n = 5$).

addition, n-hexane cleaning was successfully used to remove any interfering substances.

CyA concentrations were unchanged after the week-long storage of spiked urine and feces at 4 °C. During storage at room temperature, concentrations were stable for at least 24 h. Liquid-containing CyA (after extraction) were stable in the autosampler at room temperature over a period of 24 h.

3.6. Excretion study

The amount of CyA detected in urine during the first 12 h was only 2.78 ± 0.38 μg, indicating an extremely minor urinary excretion of CyA. About 84 h after the oral administration of CyA-NP, the cumulative amounts of CyA were found to be 625.8 ± 289.1 μg in feces and 6.48 ± 1.29 μg in urine, representing about 23.9 ± 2.76% of the dosage given. Cumulative excretion–time curves are depicted in Fig. 5.

4. Conclusion

An LC–MS based method for the qualitative and quantitative assay of CyA and its major metabolites *in vivo* in rat urine and feces was developed. Urine and feces samples were obtained in rats that had been orally administered with CyA-NP. Seven metabolites of CyA were identified in rat urine and feces. The results indicate that the parent drug was mainly excreted in the feces. The proposed method was found to be specific, sensitive, and accurate. It is particularly valuable for investigating the excretion profile of CyA-NP, and it provides better understanding of *in vivo* metabolism.

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