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Short communication

High performance liquid chromatography – tandem mass spectrometric determination of cyclovirobuxine D in human plasma

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Abstract

A sensitive, specific and rapid high performance liquid chromatography – tandem mass spectrometric (LC/MS/MS) method for the determination of cyclovirobuxine D in human plasma was developed and validated. The triple–quadrupole tandem mass spectrometric detector with an electrospray interface (ESI) was operated under the selected reaction monitoring (SRM) mode. After the addition of citalopram as an internal standard (IS), plasma samples were extracted with ethyl acetate. Chromatographic separation of the analytes was performed on a Kromasil CN column with a mobile phase of methanol/water (88/12, v/v) containing 0.4% formic acid. Linearity was established for the range of concentration 0.2–40 ng/ml. Under optimized conditions, the mean recovery was 86.6%. The intra-day precision ranged from 4.56% to 7.81%, while the intra-day accuracy ranged from 2.75% to 11.0%. The inter-day precision was in the range 3.87–10.7%, and the inter-day accuracy was in the range −4.00% to 2.50%. The cyclovirobuxine D was stable in human plasma after three freeze–thaw cycles, under storage at room temperature for 12 h, in a freezer at −20 °C for 15 days and during processing (in autosampler) at 10 °C for 24 h. The validated method is suitable for quantitative determination of cyclovirobuxine D in human plasma in pharmacokinetics study and has been successfully applied to the analysis of clinical samples.
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Keywords: Cyclovirobuxine D; LC/MS/MS; Human plasma; Pharmacokinetic study

1. Introduction

Cyclovirobuxine D is a steroid alkaloid extracted from the plant *Buxux microphylla* [1]. Pharmacological investigations showed that cyclovirobuxine D caused primary effects of coronary vasodilatation through the endothelial release of nitric oxide [2], and that cyclovirobuxine D markedly affected intracellular Ca^{2+} homeostasis in ECV304 endothelial cells by promoting discharge of intracellular pools and by interfering with the operation of store-dependent channels via plasma membrane depolarization [3]. In China, cyclovirobuxine D has been widely used in clinical for treatment of cardiac insufficiency, angina pectoris and arrhythmias for many years, and recorded by *Pharmacopoeia of People's Republic of China* [4]. However, up to our best knowledge, the method of quantitative determination of cyclovirobuxine D plasma for clinical analysis has never been reported.

Despite the clinical use of cyclovirobuxine D for many years, few methods are available on the determination of cyclovirobuxine D in biological fluids. In 1982, a radiochemical method was developed to study the absorption, distribution, excretion and metabolism of ^{3}H-cyclovirobuxine D in rats [5], but it was hardly considered as a quantitative analysis. A method by LC–TOF (time of flight)–MS was used to determine the concentration of cyclovirobuxine D in rat plasma [6] and in dog plasma [7]. Although the method of LC–TOF–MS in some sense made a progress, its sensitivity (LOQ was 0.5 ng/ml when 1.0 ml volume of plasma was analyzed.) was not enough for the low dose clinical pharmacokinetics studies in which the peak plasma concentration (C_{max}) was estimated at 2–5 ng/ml. Actually, the initial experiments showed that it was hard to achieve the LOQ of 0.5 ng/ml while the method was applied to human plasma samples. Besides, a comparatively long run time (more than 10 min) limited the throughput of analysis.

We present here an improved bioanalytical methodology for quantifying cyclovirobuxine D in human plasma using high performance liquid chromatography/triple–quadrupole tandem mass spectrometry (LC/MS/MS) with an electrospray interface.

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This method offers a number of analytical advantages, including excellent selectivity, quantitation limits and improved sample throughput. And the validated method has been successfully applied to the analysis of clinical samples.

2. Experimental

2.1. Materials and reagents

Cyclovirobuxine D reference standard was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China), and citalopram (internal standard, IS) reference standard was purchased from Sigma (Sigma Chemical Company, USA). Chemical structures of cyclovirobuxine D and citalopram (IS) are presented in Fig. 1. Methanol (Merck, Germany) was HPLC grade. Purified water was obtained using a Purelab® classic water purification system (ELGA Labwater, France). Blank human plasma was obtained from Nanjing Blood Center (Jiangsu, PR China). All other chemicals were of analytical grade and were used as received.

2.2. Equipment and conditions

A Finnigan TSQ LC/MS/MS system (Thermo Electron Corporation, CA, USA) operating under Xcalibur® 1.1 software was used. The LC/MS/MS system consisted of a Finnigan Surveyor LC pump, a Finnigan Surveyor refrigerated autosampler with a 20 μl loop, and a Finnigan TSQ Quantum Ultra AM triple quadrupole tandem mass spectrometer with an electrospray interface (ESI). The LC/MS/MS system was operated at ambient temperature (25 ± 2 °C).

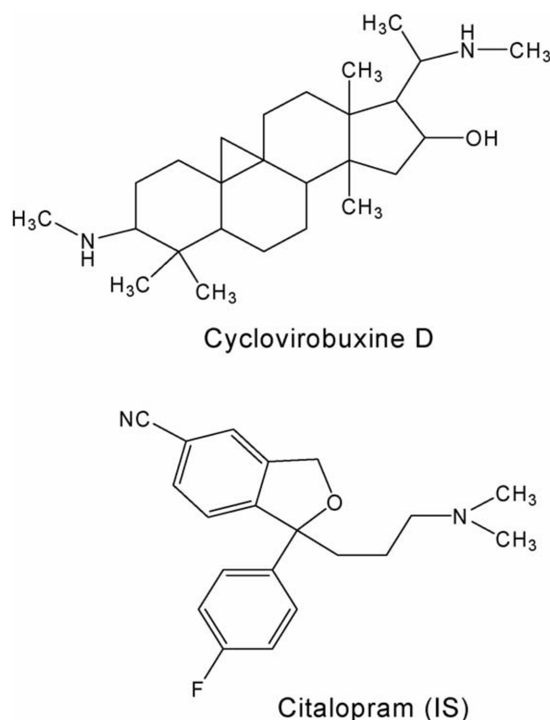

Fig. 1. The chemical structures of cyclovirobuxine D and citalopram (IS).

Chromatographic separation of the analytes from potential interfering material was performed on a Kromasil CN column (4.6 mm × 150 mm, 5 μm, Hanbon Sci. &. Tech. Co. Ltd., PR China) with the mobile phase of methanol/water (88/12, v/v) containing 0.4% formic acid. The analytical column was protected by a 4 mm × 2.0 mm i.d., Phenomenex Fusion-RP guard column (Phenomenex, USA). The mobile phase was set at a flow rate of 1.0 ml/min, and was split with the split ratio of 1/3 at the entrance of the mass spectrometer. A divert valve was utilized to limit interfering material in the HPLC eluate from entering the ESI source, and the eluate flow destination was switched alternatively between the detector and waste as follows: 0–3.6 min, waste; 3.6–6 min, detector.

Operation conditions for the ESI source were optimized by constantly adding cyclovirobuxine D stock solution (500 ng/ml) to the HPLC flow by a syringe pump via a T-connector in the fusion mode. The most sensitive detector response was achieved when ionization of analytes was carried out at the positive ionization mode using the following settings: spray voltage, 5000 V; sheath gas (N_2) pressure, 35 psi; auxiliary gas (N_2) pressure, 5 psi; capillary temperature, 350 °C; source CID, 10 V; collision energy, 25 V; unit resolution for Q1 and Q3, 0.7 u; and collision gas (Ar) pressure, 1.5 mTorr. Under these conditions, ions detected (selected reaction monitoring, SRM) were *m/z* 403.0 (parent) → 372.0 (product) for cyclovirobuxine D and *m/z* 325.0 (parent) → 234.0 (product) for citalopram (IS).

2.3. Preparation of stock solutions and standards

The cyclovirobuxine D stock solution (100 μg/ml) was prepared in HPLC mobile phase and stored at 4 °C. Different working solutions were prepared by diluting the stock solution with HPLC mobile phase, resulting in cyclovirobuxine D concentrations at eight levels of 5, 10, 20, 50, 100, 200, 500 and 1000 ng/ml. The internal standard stock solution was prepared by dissolving 10 mg of citalopram in 100 ml methanol. The stock solution of citalopram was further diluted with methanol to prepare the working internal standard solution containing 20 ng/ml of citalopram.

The stock solutions of cyclovirobuxine D were diluted further in blank human plasma on each day of analysis to prepare eight calibration samples containing cyclovirobuxine D at the following concentrations: 0.2, 0.4, 0.8, 2, 4 8, 20 and 40 ng/ml. Quality control (QC) samples were prepared daily by spiking 0.5 ml aliquots of blank plasma each with 20 μl of the corresponding standard solutions to produce final concentrations equivalent to low level (0.2 ng/ml), middle level (2 ng/ml) and high level (40 ng/ml) of cyclovirobuxine D.

2.4. Sample processing

Prior to extraction, frozen plasma samples were thawed in a water bath at 37 °C. A 0.5 ml aliquot of plasma sample was transferred to a 10 ml centrifuge tube, and spiked with 20 μl of working internal standard solution (20 ng/ml). The mixture was basified by addition of 100 μl of potassium carbonate solution (20%, w/v) and vortex-mixed for 30 s. Then 4 ml of ethyl acetate

was added, and the sample was vortex mixed for 3 min. After centrifugation of the sample at 3000 × *g* for 10 min, 3 ml of the upper organic phase was transferred to another 10 ml centrifuge tube and evaporated under a gentle stream of nitrogen gas in a water bath at 60 °C. The dried extract was reconstituted in 100 μl of mobile phase, and a 10 μl aliquot was injected into the HPLC.

2.5. Pharmacokinetic study

This was a single-dose, open-label pharmacokinetic study in healthy volunteers. The study was planned and performed according to the current GCP guidance. Sixteen healthy male Chinese volunteers (aged 18–24, body weight 60–75 kg) checked perfect for this study were selected as subjects. Each volunteer was administered a single dose of 2 mg cyclovirobuxine D intravenously in dextrose 5% in water over 2 h. Subjects were required to fast 12 h prior to drug administration, during administration (2 h), and 4 h after drug administration.

Venous blood samples about 4 ml were collected in heparinized tubes prior to the drug administration (0 h), 0.5, 1, 1.5 h after the start of the infusion, at the end of the infusion, and at 0.5, 1, 2, 4, 8, 12, 16, 24, 48 and 72 h after the end of the infusion. The plasma samples were separated by centrifugation at 3000 × *g* for 10 min and stored at −20 °C until analysis.

The maximum plasma concentration (C_{max}) and time of the maximum plasma concentration (T_{max}) were identified directly from the observed data. The area under the plasma concentration–time curve (AUC) from the time zero to the last measured concentration ($AUC_{0\rightarrow t}$) was calculated according to the linear trapezoidal rule. The terminal elimination rate constant (λ_Z) was calculated by least-square regression of the logarithm scale concentrations to time for the last four measurable points, the terminal half-life was calculated with $t_{1/2} = 0.693/\lambda_Z$ accordingly, and the $AUC_{0\rightarrow\infty}$was the corresponding area extrapolated to infinity by $AUC_{0\rightarrow t} + C_t/\lambda_Z$, where C_t is the last measurable drug concentration. And other major pharmacokinetic parameters calculated through the model fitting of the time–concentration curves of each subject, including the clearance (CL/*F*, l/h) and the volume of distribution (*V*/*F*, l).

3. Results and discussion

3.1. HPLC conditions

Cyclovirobuxine D is an alkalescent steroid alkaloid. Generally, in order to obtain a symmetric peak shape, the mobile phase should be acidic when an alkaloid is separated on a chromatographic column. The optimization experiments of the operating conditions for the ESI source also indicated that the most sensitive detector response of cyclovirobuxine D resulted from the positive ionization mode with a proper acidic mobile phase. However, under acidic mobile phases, cyclovirobuxine D was hardly retained on C18 (Luna C18, 5 μm, 4.6 mm × 150 mm, Phenomenex, USA), C8 (Zorbax SB-C8, 5 μm, 4.6 mm × 150 mm, Agilent, USA), and Phenyl (Luna Phenyl-Hexyl, 5 μm, 4.6 mm × 150 mm, Phenomenex, USA) columns. Finally, on a CN column (4.6 mm × 150 mm, 5 μm, Hanbon Sci. &. Tech. Co. Ltd., PR China) an acceptable retention time was achieved by adjusting the proportion of methanol and the concentration of formic acid in the mobile phase. The proportion of methanol, the concentration of formic acid and addition of ammonium acetate were evaluated through a number of trials. As a result, the mobile phase of methanol/water (88/12, v/v) containing 0.4% formic acid was chosen because an acceptable retention time, a good separation, a symmetric peak shape and a sensitive detect response were simultaneously obtained under this condition. Furthermore, plasma samples were prepared and analyzed using the optimized HPLC conditions with an ELSD (evaporation light scattering detector), and the chromatograms of six individual blank human plasma samples showed that main endogenous material was eluted before switching the divert valve from waste to detect at 3.6 min.

3.2. Specificity

Six individual human blank plasma samples were prepared and analyzed, and there were no interfering peaks at the retention time of either analyte or internal standard. The specificity of the assay was further verified by comparing the chromatograms of the six blank plasma samples before and after spiking with cyclovirobuxine D and citalopram (IS). Representative chromatograms of human plasma samples are shown in Fig. 2. The retention times for cyclovirobuxine D and citalopram (IS) were 4.7 ± 0.2 and 4.3 ± 0.2 min.

3.3. Linearity and LOQ

The peak area ratios of cyclovirobuxine D to citalopram (IS) in human plasma were linear with respect to the analyte concentrations over the range 0.2–40 ng/ml. The calibration curve was fit by a least-square $1/x^2$-weighted linear regression method. The average slope and intercept of regression equations were 0.2776 and 0.005843, and the correlation coefficients were consistently greater than 0.99.

The limit of quantification (LOQ), defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy and precision, was established at 0.2 ng/ml (signal-to-noise ration, $S/N \geq 10$). The chromatogram of an extracted sample that contained 0.2 ng/ml (LOQ) of cyclovirobuxine D is shown in Fig. 2B.

3.4. Precision and accuracy

The intra-day precision and accuracy of the assay was investigated by analyzing quintuplicate spiked samples of cyclovirobuxine D at each QC level (0.2, 2 and 40 ng/ml). The inter-day precision and accuracy was determined over three separate batches by analyzing 45 QCs. Precision and accuracy of the method, presented as relative standard deviation (R.S.D.%) and relative error (RE%), are demonstrated in Table 1. The intra-day precision ranged from 4.56% to 7.81%, while the intra-day accuracy ranged from 2.75% to 11.0%. The inter-day precision was in the range 3.87–10.7%, and the inter-day accuracy was in the range −4.00% to 2.50%.

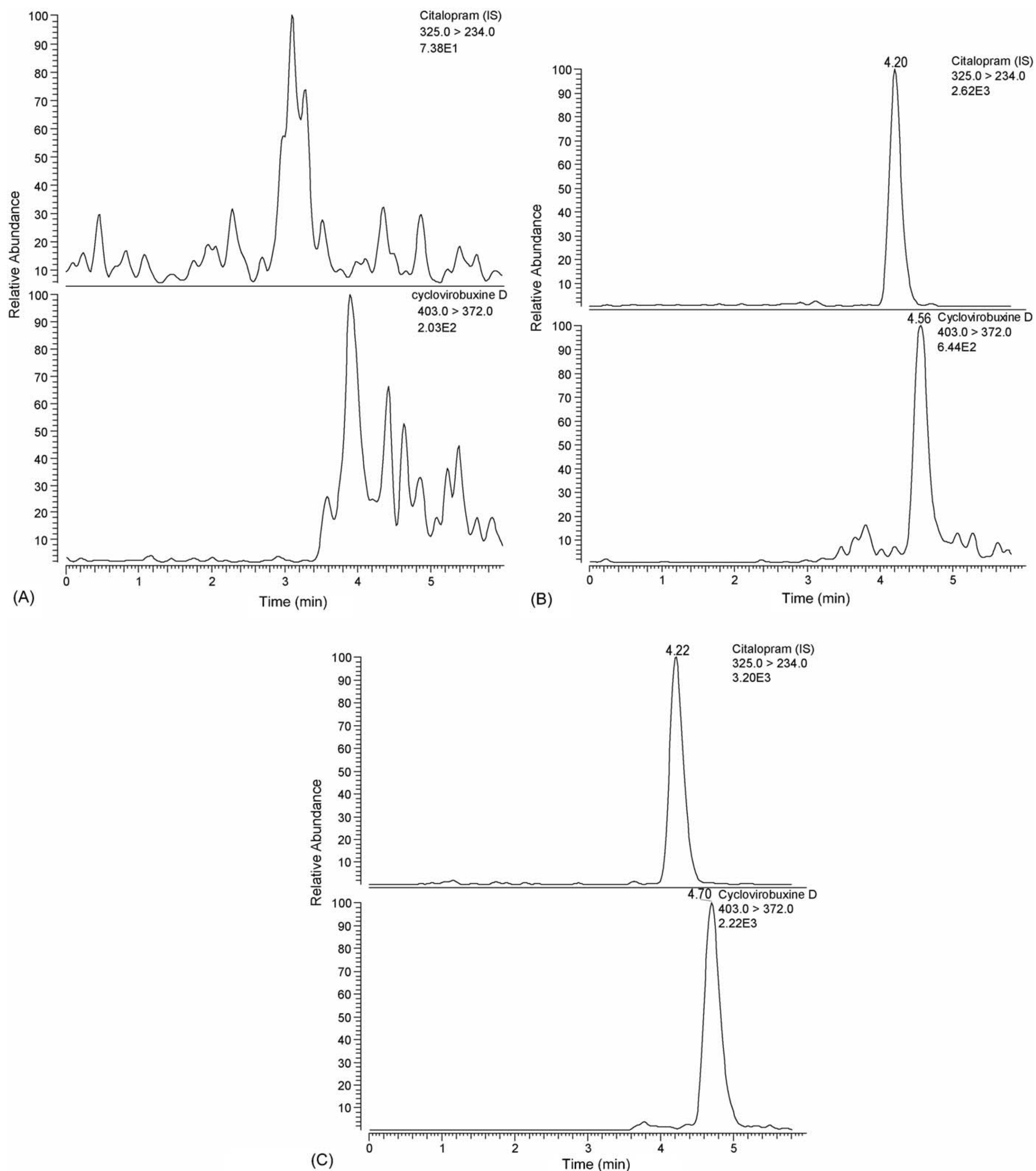

Fig. 2. Representative chromatograms obtained from human plasma samples: (A) a chromatogram obtained from blank human plasma; (B) a chromatogram of plasma sample spiked with cyclovirobuxine D at 0.2 ng/ml and citalopram (IS) at 0.8 ng/ml; (C) a typical chromatogram of clinical sample.

3.5. Extraction

Liquid–liquid extraction (LLE) was used for the sample preparation to produce a condensed spectroscopically clean sample. Organic solvents including ethyl acetate, *n*-hexane, cyclohexane, methyl *t*-butyl ether, diethyl ether, methylene chloride and their mixtures in different combinations, and pH modifiers including sodium hydroxide, sodium carbonate, sodium bicarbonate, ammonia and potassium carbonate were evaluated. Finally, the highest extraction recovery was achieved when

Table 1
Data for the intra- and inter-day accuracy and precision ($n = 5$)

Nominal (ng/ml)	Intra-day			Inter-day		
	Mean (ng/ml)	R.S.D. (%)	RE (%)	Mean (ng/ml)	R.S.D. (%)	RE (%)
0.2	0.222	4.56	11.0	0.205	10.7	2.50
2	2.060	5.98	3.00	1.920	6.33	−4.00
40	41.101	7.81	2.75	39.725	3.87	−0.69

plasma samples were spiked with 100 μl 20% (w/v) potassium carbonate solution and then extracted with ethyl acetate. Under this condition, the recovery of cyclovirobuxine D was 88.2 ± 11.0%, 84.2 ± 6.4% and 87.6 ± 8.2% at the concentrations of 0.2, 2 and 40 ng/ml. The mean recovery was 86.6% while the recovery of internal standard, citalopram was 79.6% at the concentration used in the assay (0.8 ng/ml).

3.6. Sample matrix effects

It is widely assumed that the highly specific nature of LC/MS/MS permits short chromatographic analysis times. However, when cyclovirobuxine D was eluted before 2.5 min on a C18 column, the detector response for a cyclovirobuxine D was weakened due to the ionization suppression caused by coeluting endogenous components. The response was improved with the improvement of the retention action of cyclovirobuxine D while a CN column was used, which suggested that the matrix effects should be investigated. The sample matrix effects were assessed by comparing A_i (peak area obtained from analysis of post-extraction blank human plasma samples spiked with the analyte) with A_s (peak area obtained from analysis of the neat reference standard solutions). The respective ratio of A_i to A_s was 97.4%, 87.2% and 92.7% for cyclovirobuxine D at three concentration levels (0.2, 2 and 40 ng/ml), while the value of A_i/A_s was 85.8% for citalopram at 0.8 ng/ml. The results indicated that there were no significant sample matrix effects under the optimal conditions.

3.7. Stability

The stock solutions were stable for at least one month when stored at 4 °C. The stability of cyclovirobuxine D in human plasma was assessed at the QC levels (0.2, 2 and 40 ng/ml) after storage at room temperature (RT) for 12 h, after three freeze–thaw cycles and after storage at −20 °C for 12 days. The autosampler stability was conducted reanalyzing extracted QC samples kept under the autosampler conditions (10 °C) for 24 h. Evaluation of the stability of QC samples was based on the comparison of the calculated mean concentration of the respective sample with its nominal value. No degradation of cyclovirobuxine D was observed after stability tests (Table 2).

3.8. Pharmacokinetic study results

This validated method was successfully applied to the analysis for plasma samples in a pharmacokinetics study. About 150 samples were extracted and analyzed within a day. The mean plasma concentration–time curve of cyclovirobuxine D in sixteen healthy Chinese volunteers tested was shown in Fig. 3. The major pharmacokinetic parameters found were listed in Table 3.

Table 2
Assessment of stability ($n = 3$)

	Detected concentration (ng/ml)		
	0.2	2	40
Short-term stability at room temperature	0.188	1.996	40.728
Three freeze and thaw cycles	0.210	1.816	37.765
Long-term stability at −20 °C	0.202	2.015	41.536
Autosampler stability for 24 h	0.228	2.144	43.043
Mean	0.206	1.994	40.614
R.S.D. (%)	7.19	5.86	4.81
RE (%)	3.00	−0.30	1.54

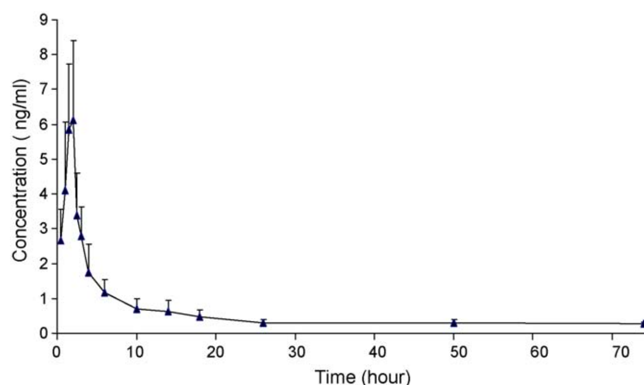

Fig. 3. Mean plasma concentration vs. time profiles of sixteen healthy Chinese volunteers. The error bars represent + standard deviation (S.D.).

Table 3
Pharmacokinetic parameter of cyclovirobuxine D in sixteen healthy Chinese volunteers

Parameters	Mean ± S.D.
C_{max} (ng/ml)	6.10 ± 2.39
T_{max} (h)	2.00 ± 0.32
$t_{1/2}$ (h)	27.51 ± 8.92
$AUC_{0\to t}$ (ng h/ml)	42.81 ± 12.54
$AUC_{0\to\infty}$ (ng h/ml)	47.22 ± 15.05
CL/F (L/h)	37.54 ± 65.01
V/F (L)	1489.6 ± 714.3

4. Conclusion

For the first time, a method of quantitative determination for cyclovirobuxine D in human plasma was developed and validated. The LC/MS/MS method presented here has major advantages: a high degree of specificity, an improved sensitivity and a short run time. The validation data demonstrates acceptable precision and accuracy. The validated method allows quantification of cyclovirobuxine D over the concentration range 0.2–40 ng/ml. The specific, sensitive, rapid and cost-effective method is suitable for the determination of cyclovirobuxine D

in human plasma in pharmacokinetics studies and clinical monitoring.

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