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Short communication

Determination of cymipristone in human plasma by liquid chromatography–electrospray ionization-tandem mass spectrometry

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ABSTRACT

A rapid, specific and sensitive liquid chromatography–electrospray ionization-tandem mass spectrometry method was developed and validated for determination of cymipristone in human plasma. Mifepristone was used as the internal standard (IS). Plasma samples were deproteinized using methanol. The compounds were separated on a ZORBAX SB C_{18} column (50 mm × 2.1 mm i.d., dp 1.8 μm) with gradient elution at a flow-rate of 0.3 ml/min. The mobile phase consisted of 10 mM ammonium acetate and acetonitrile. The detection was performed on a triple-quadruple tandem mass spectrometer by selective reaction monitoring (SRM) mode via electrospray ionization. Target ions were monitored at $[M+H]^+$ m/z 498 → 416 and 430 → 372 in positive electrospray ionization (ESI) mode for cymipristone and IS, respectively. Linearity was established for the range of concentrations 0.5–100 ng/ml with a coefficient correlation (r) of 0.9996. The lower limit of quantification (LLOQ) was identifiable and reproducible at 0.5 ng/ml. The validated method was successfully applied to study the pharmacokinetics of cymipristone in healthy Chinese female subjects.

1. Introduction

Cymipristone (11β-[4-(N-methyl-N-cyclohexylmethylamino)-phenyl]-17α-(1-propinyl)-17β-hydroxyl-4,9-estradiene-3-one, Fig. 1) is a novel steroid compound developed by Shanghai Three-Alliance Biotechnologies Co. Ltd, which has been granted one United States Patent [1] and two China Patents [2,3]. The pharmacological effect of cymipristone is similar to mifepristone. Mifepristone has been used clinically as an abortifacient for many years and it also has been used to reduce glucocorticoid receptor activation as a potential therapy in the metabolic syndrome [4] and depression [5]. Although a large scale multi-center clinical trial is still ongoing, it was confirmed in a previous phase II clinical trial in China that compared with mifepristone, cymipristone on termination of early pregnancy is highly effective and causes less endometrial bleeding. Entrusted by Shanghai Three-Alliance Biotechnologies Co. Ltd, we carried out the study to determine the concentration of cymipristone in human plasma and investigate the pharmacokinetics of cymipristone after oral administration.

Cymipristone was synthesized based on the structure of mifepristone. As we can see, cymipristone is similar to mifepristone except for having a cyclohexylmethylamino group instead of a dimethylamino group in the side chain (Fig. 1). It was proposed that cymipristone had a different metabolic profile as opposed to mifepristone when used in humans [6]. However, methods of determination of cymipristone in biological fluids have never been reported. Earlier publications have described methods for the analysis of mifepristone by LC–MS/MS [6,7]. In this paper, a new liquid chromatography–electrospray ionization-tandem mass spectrometry method using mifepristone as the internal standard (IS) was established and validated, and it was successfully applied to a pharmacokinetic study of cymipristone in healthy Chinese female subjects.

2. Experimental

2.1. Materials and reagents

Cymipristone (100.5% purity, Lot060402-2RS) and mifepristone (IS, 99.9% purity, Lot 081215) were supplied by Shanghai Three-Alliance Biotechnologies Co. Ltd, (Shanghai, China). Acetonitrile and methanol were HPLC grade and purchased from Merck Company Inc. (Darmstadt, Germany). Ammonium acetate of HPLC grade was purchased from Fluck Company Inc. (Switzerland). Ultrapure water (Millipore, Bedford, MA, USA) was used from a Milli-Q system. Blank human plasma was obtained from healthy volunteers who had not taken any medications for 3 months, at the Blood Center of Hangzhou (Hangzhou, China).

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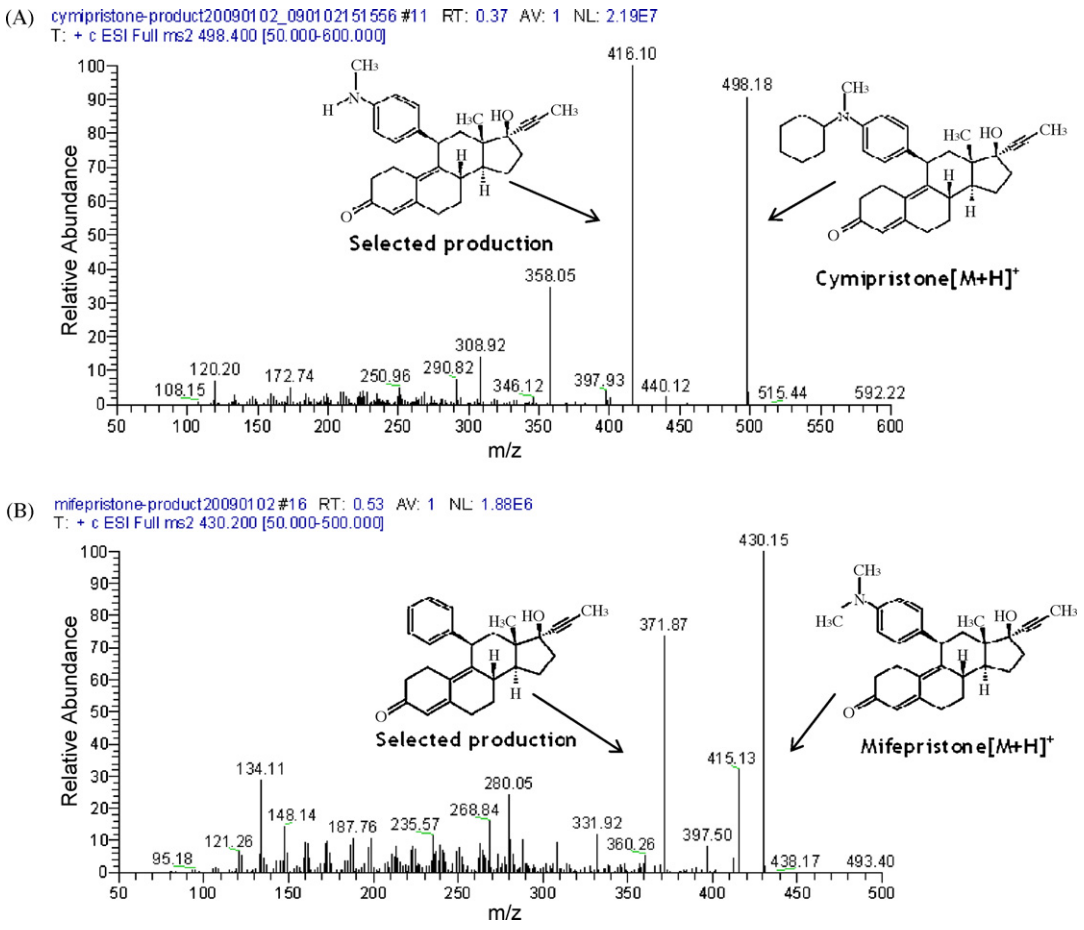

Fig. 1. Full-scan parent and product ion spectra of $[M+H]^+$ of (A) cymipristone, (B) mifepristone.

2.2. Preparations of calibration curve and quality control samples

Stock solutions of cymipristone (1 mg/ml) were accurately prepared in methanol and stored in −20 °C. Dilutions prepared in methanol at concentrations of 10 μg/ml, 5.0 μg/ml, 1.0 μg/ml, 500 ng/ml, 100 ng/ml, 50 ng/ml were used as working solutions to prepare the calibration standards. Appropriate amount of the working solutions were added into test tubes. After evaporation to dryness under a stream of nitrogen, the residues were dissolved in 1 ml blank plasma. Then the calibration standards in concentration levels of 0.5, 1, 5, 10, 50, 100 ng/ml were prepared. The quality control (QC) samples were prepared by the same way at concentrations of 0.8, 8, 80 ng/ml of cymipristone. All plasma samples were stored at −80 °C before being used.

Stock solution of IS was prepared by dissolving the accurately weighed reference compound in methanol to reach a final concentration of 1 mg/ml. A working solution of the IS at 20 ng/ml was prepared by diluting the stock solution with methanol and stored at 4 °C.

2.3. Sample preparation

Each collected blood sample was immediately centrifuged at 735 × *g* for 10 min and then transferred into a microcentrifuge tube (Eppendorf, 1.0 ml). The plasma samples were stored at −80 °C until analysis. A 50 μl volume of plasma was transferred to an Eppendorf tube, and 250 μl of IS working solution (20 ng/ml IS prepared in methanol) added and vortex-mixed again for 30 s. After centrifugation for 10 min at 20,664 × *g* at 6 °C, the clear supernatant was transferred to the autosampler and a 10 μl aliquot was injected into the LC–MS/MS system for analysis.

2.4. Instrumentation and LC–MS/MS condition

A Thermo Scientific TSQ Quantum tandem mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, CA, USA) and a Accela pump and an autosampler were used for LC–MS/MS analyses. The data processing was carried out using Finnigan Lcquan data analysis program.

Liquid chromatographic separations were achieved using a ZORBAX SB C_{18} column (50 mm × 2.1 mm i.d., dp 1.8 μm) and a Hypersil Gold C_{18} guard column (10 mm × 2.1 mm i.d., 3.0 μm; Thermo Scientific, San Jose, CA, USA). The mobile phase consisted of a mixture of solvent A (10 mM ammonium acetate) and solvent B (acetonitrile) and was delivered at a flow-rate of 0.3 ml/min. The gradient started at 70% solvent B and changed linearly to 90% B in the first 2.5 min, then changed to 95% B in the next 0.10 min, and maintained at 95% B for 1.5 min. A subsequent re-equilibration time (2 min) was allowed before the next injection. The total runtime of one sample was about 6.2 min. The sample injection volume was 10 μl, the autosampler tray temperature was kept constant at 4 °C, and the column temperature was maintained at 30 °C.

Samples were ionized by positive-ion electrospray ionization mode (ESI^+) and were monitored in the selective reaction monitoring (SRM) mode under the following source conditions: Sheath gas (nitrogen): 0.6 L/min; auxiliary gas: 1.08 L/min; capillary temperature: 350 °C; spray voltage: 4000 V; collision gas (argon) pressure: 1.5 mTorr. Analysis was carried out using the transitions of *m*/*z*

498 → 416 for cymipristone and m/z 430 → 372 for IS, respectively, with a scan time of 0.05 s per transition. The optimized collision energy of 20 eV and 22 eV were used for the analyte and IS compounds, respectively.

2.5. Assay validation

The method was validated for sensitivity, specificity, accuracy, precision, recovery, calibration curve and reproducibility according to the FDA guidelines [8].

The specificity of the assay for the analytes versus endogenous substances in the matrix was assessed comparing the lowest concentration in the calibration curves with reconstitutions prepared with drug-free plasma from six different individuals. The lower limit of quantification (LLOQ) is defined as the minimum concentration that could be accurately and precisely quantified (lowest data point of the calibration curve).

Linearity of calibration was tested and assayed in consecutive 5 days. Calibration curves in the concentration range of 0.5–100 ng/ml for cymipristone were constructed by plotting the peak-area rations of analyte/IS against the spiked concentrations. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ for which the maximum acceptable deviation was set at 20%.

The accuracy and precision of the assay were determined using QC samples at 0.8 ng/ml (low), 8 ng/ml (middle) and 80 ng/ml (high). The precision was calculated using the relative standard deviation (RSD) with RSD % = (standard deviation of the mean/mean) × 100. Accuracy was calculated as the relative error (RE) with RE % = (measured concentration − nominal concentration)/nominal concentration × 100. The acceptance criteria for precision and the accuracy were 20% for LLOQ and 15% for the other concentrations.

Absolute recovery of the analyte was evaluated by comparing peak area of QC samples with peak areas of direct injection of the pure authentic standard solutions dissolved in the mobile phase.

The stability of cymipristone in plasma was studied under a variety of conditions using three aliquots at each QC level. Freeze and thaw stability was determined after three freeze and thaw cycles at −20 °C. Short-term temperature stability was evaluated by keeping the QC samples at room temperature for 4 h. Long-term stability was tested by assaying frozen QC samples at −80 °C for 30 days. Stability of the extracted samples were evaluated by analysis of extracted QC samples stored at 4 °C in the autosampler for 24 h.

2.6. Application of the method

The method was applied to a pharmacokinetic study of 40 mg cymipristone tablet in Chinese female volunteers, which was approved by the Ethics Committee of the 2nd Affiliated Hospital (School of Medicine, Zhejiang University) and in accordance with the rules of Good Clinical Practice (GCP) promulgated by State Food and Drug Administration (China). A written informed consent was given before 20 volunteers participated in the study. After overnight fasting (12 h), the volunteers were orally administered a single dose of the assigned tablet in 250 ml water. No food was allowed until 4 h after dose administration. Blood samples (1.0 ml) were collected into tubes containing EDTA anticoagulant (BD, NJ, USA) before and 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0, 72.0, 96.0, 120.0 h after dosing. Then the samples were centrifuged at 735 × g (Allegra™ 6R centrifuge, Beckman Coulter, USA) for 10 min, and the plasma was separated. The plasma samples were stored at −80 °C until analysis.

3. Results and discussion

3.1. Optimization of LC–MS/MS condition and IS selection

In this study, ESI was chosen as the ionization source. It was found that the signal intensity of the analytes and IS in human plasma was high using ESI source and the regression curves were linear over 0.5–100 ng/ml. By using ESI, the analytes and IS formed predominantly protonated molecular ions $[M+H]^+$ in full-scan spectra, with m/z 498 for cymipristone and m/z 430 for the IS. To determine these compounds using SRM mode, full-scan product ion spectra of the analytes and IS were investigated. The most abundant ion in the product ion mass spectrum was at m/z 416 for cymipristone and m/z 372 for IS. Capillary and cone voltages and collision energies were optimized to obtain the greatest intensity of the most abundant product ion for further MS/MS experiments. The collision behavior of the $[M+H]^+$ of these compounds was strongly dependent on the collision energy. An increase in the collision energy caused a marked increase of the fragmentation processes. After optimization of the collision energy, the collision induced dissociation was carried out using 20 eV and 22 eV collision energy for the analyte and IS compounds, respectively, to obtain the maximum intensity of product ions. Therefore, the SRM transition of m/z 498 → 416 for cymipristone, m/z 430 → 372 for IS were selected to obtain maximum sensitivity. Full-scan parent and product ion spectra of $[M+H]^+$ of these compounds are shown in Fig. 1.

It is critical to optimize the chromatographic conditions to achieve symmetrical peak shapes with high selectivity and sensitivity. Ammonium acetate was commonly employed to supply the ionic strength and eliminate tailing peak. However, mobile phase with high concentration of ammonium acetate is not good for instrument maintenance. It was found that a mixture of 10 mM ammonium acetate buffer–acetonitrile could achieve the required purpose and was finally adopted as the mobile phase. Gradient elution is usually used in order to elute the analyte rapidly, which allowed HPLC to push the limits of both peak capacity (due to higher efficiency) and speed of analysis (due to higher linear velocities) without compromising resolution. Therefore, gradient elution was commonly used to provide a better peak shape. Meanwhile, 95% acetonitrile maintained for 1.5 min in the gradient elution benefits the elution of the impurity, which is good for extending the column life.

An ideal IS should be a structurally similar analog or stable isotope-labelled compound according to the FDA guideline [8]. However, stable isotope-labelled compound is too expensive for most research institutes. There's great similarity in the structure and chemical–physical properties between cymipristone and mifepristone. Also, they had similar chromatographic behavior under the present LC–MS/MS conditions. So, mifepristone was chosen as the IS in the present study.

3.2. Method validation

3.2.1. Specificity

The specificity of the method was determined by analyzing six different lots of blank control both with and without the IS. The chromatograms of protein precipitation produced clean extracts with no interference from endogenous compounds at the retention times for cymipristone and the IS.

Fig. 2 shows representative chromatograms of blank human plasma, blank human plasma with IS, the LLOQ (0.5 ng/ml) of cymipristone with IS in plasma and volunteer's plasma sample. Typical retention time for cymipristone and the IS were 3.5 min and 1.6 min. And the peak shapes were sharp and symmetrical. The total run time was about 6.2 min. In Fig. 2, the divert valve was used

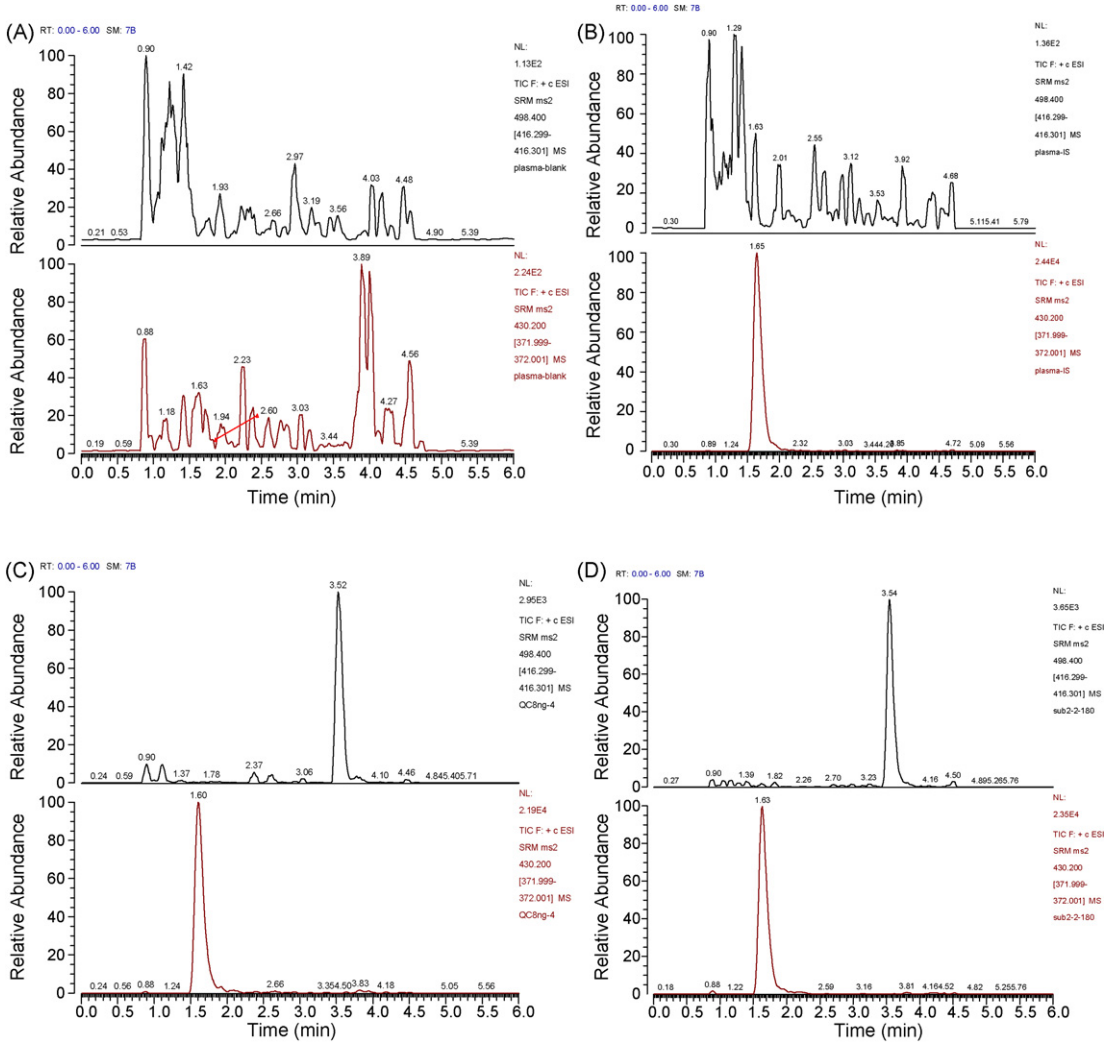

Fig. 2. Typical SRM chromatograms of blank plasma (A), blank plasma with IS (B), LLOQ for cymipristone in plasma (0.5 ng/ml) and IS (C), plasma sample obtained from a healthy subject 1 h after oral administration of 40 mg cymipristone tablet (D).

at 0–1.0 min and 4.5–6.2 min, so the eluant does not flow into the mass detector during those times.

3.2.2. Linearity and sensitivity

Over the concentration range of 0.5–100 ng/ml, the linear regression equation for the analyte was $y = 0.1601x + 0.0005$ ($n = 5$, $r = 0.9996$), where y was the peak area ratio of the analyte to IS and x was the concentration of the analyte.

The LLOQ of the method was 0.5 ng/ml of cymipristone in human plasma, the precision (RSD %) was 16.8% ($n = 6$), and the accuracy (RE %) was −10.68% ($n = 6$).

3.2.3. Accuracy and precision

As shown in Table 1, the intra-batch precision and accuracy ranged between 2.30 and 7.62%, −0.20 and −7.11% over the three QC levels. The inter-batch precision ranged from 5.91 to 8.81%, and the corresponding accuracy varied from −3.45 to 2.11%, respectively.

3.2.4. Matrix effect and absolute recovery

The matrix effect was evaluated by analyzing at three concentration levels. The first level set consists of the analyte and the IS in mobile phase (standard solution), the second level set consists of post-extraction spiked plasma samples (matrix matched standard), and the third set of pre-extraction spiked plasma samples (fortified real sample). From the peak areas acquired for these calibration plots, the percentage matrix effect (ME %) and recovery (RE %) can be calculated from:

$$\text{ME}(\%) = \frac{\text{Area of post-extraction spike}}{\text{Area of standard}} \times 100$$

$$\text{RE}(\%) = \frac{\text{Area of pre-extraction spike}}{\text{Area of standard}} \times 100$$

Data from our experiment show the matrix effect of cymipristone was 103.3, 102.8, 115.2% (with the RSD % of 13.5, 5.9, 12.7%, respectively) at the three QC concentration levels. This result suggested that endogenous substances had no effect on the ionization of the analyte. In addition, the mean peak area of I.S. was 90.5% (with the RSD % of 9.4%) of that from the reference solution.

The absolute recoveries of cymipristone and IS were 116.9 ± 11.5% (0.8 ng/ml), 99.1 ± 5.9% (8 ng/ml), 100.7 ± 7.9% (80 ng/ml), and 56.8 ± 5.4% (IS), respectively.

3.2.5. Stability

Stability results of cymipristone in QC samples are shown in Table 2, which indicate that cymipristone is stable in plasma samples under these storage conditions: after 4 h at ambient temperature; after sample processing and being retained at

Table 1
Inter- and intra-accuracy and precision data for assays of cymipristone ($n = 6$).

Spiked conc. (ng/ml)	Intra-assay			Inter-assay		
	Mean ± SD	Precision (RSD %)	Accuracy (RE %)	Mean ± SD	Precision (RSD %)	Accuracy (RE %)
0.8	0.809 ± 0.062	7.62	1.15	0.801 ± 0.071	8.81	0.16
8	7.984 ± 0.184	2.30	−0.20	8.169 ± 0.571	6.99	2.11
80	74.31 ± 2.54	3.42	−7.11	77.24 ± 4.57	5.91	−3.45

Table 2
Stability of cymipristone in human plasma under various storage conditions ($n = 3$).

Storage conditions	Concentrations (ng/ml)		RSD (%)	RE %
	Added	Found		
Short-term temperature stability (4 h at 25 °C)	0.8	0.758 ± 0.046	6.1	−5.3
	8	7.835 ± 0.275	3.5	−2.1
	80	78.72 ± 1.29	1.6	−1.6
Three freeze–thaw cycles	0.8	0.774 ± 0.088	11.4	−3.3
	8	8.011 ± 0.458	5.7	0.1
	80	74.76 ± 4.38	5.9	−6.5
Short-term temperature stability (30 days at −80 °C)	0.8	0.825 ± 0.072	8.7	3.1
	8	8.260 ± 0.548	6.6	3.3
	80	80.56 ± 1.10	1.4	0.7
Post-preparative stability (autosampler, 24 h at 4 °C)	0.8	0.768 ± 0.047	6.1	−4.0
	8	7.806 ± 0.212	2.7	−2.4
	80	73.43 ± 1.13	1.5	−8.2

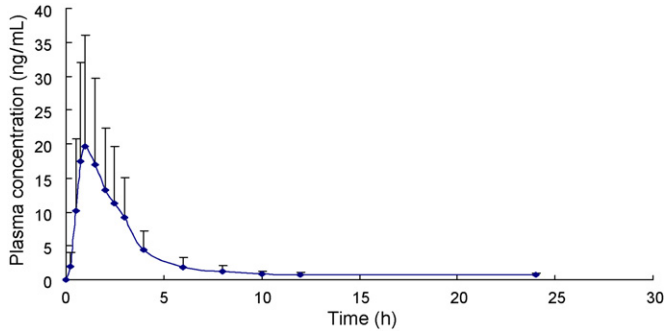

Fig. 3. Mean plasma concentration–time profiles after a single oral administration of 40 mg cymipristone tablets in 20 Chinese healthy female subjects.

autosampler for 24 h; after three freeze–thaw cycles; after being stored at −80 °C for 30 days.

3.3. Application of the method

The method was applied to a pharmacokinetic study of cymipristone in Chinese female subjects. The mean plasma concentration of cymipristone versus the time profile is shown in Fig. 3. The maximum concentration of cymipristone in plasma was 22.34 ng/ml (C_{max}) and the time to C_{max} was 1.45 h (t_{max}).

4. Conclusions

A rapid, specific and sensitive liquid chromatography–electrospray ionization-tandem mass spectrometry method was firstly developed and validated for the determination of cymipristone in human plasma. The assay used mifepristone as IS. This LC–MS/MS method required only 50 μl of plasma with a LLOQ of 0.5 ng/ml and the determination of one plasma sample needed only 6.2 min. These results indicated that it was suitable for routine analysis of large number of biological samples. It was successfully applied to characterize the pharmacokinetics of cymipristone in Chinese healthy female subjects.

Acknowledgement

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