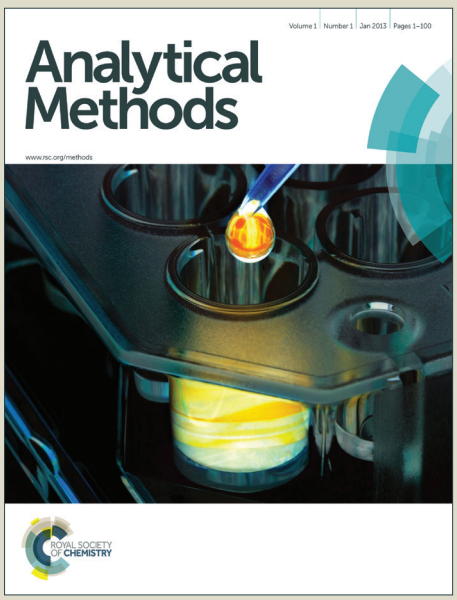
Analytical Methods

Accepted Manuscript



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A Sensitive LC-ESI-MS/MS method for the determination of clotrimazole in human plasma

Rui Zhou [1,#], Fang Tang [1,#], Sanwang Li [1], Xiang Xie [1], Jie Peng [1], Feifan Xie [1], Lingli Mu [2,*], Peng Yu [1,*]

([1] School of Pharmaceutical Sciences, Central South University, Changsha 410013, China; [2] Medical College, Hunan Normal University, Changsha 410006, China)

# These authors contributed equally.

* Corresponding author: Tel./fax: +86 731 82650446. E-mail: peng.yu@csu.edu.cn (Peng Yu); Tel./fax: +86 731 88912400. E-mail: mulingli@sina.com (Lingli Mu).

Abstract

A rapid, sensitive and specific liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the determination of clotrimazole in human plasma was developed and validated. The plasma samples were extracted with mixed solvent of methyltert butyl ether-dichloromethane (4:1, *v/v*), and estazolam was selected as the internal standard. Then the separation was carried out on a Phenomenex Luna CN column (2.0×150 mm, 5 μm), using a mobile phase of methanol-0.1% aqueous formic acid solution (85:15, *v/v*). A triple quadrupole mass spectrometer with the positive ionization mode was used for the determination of target analytes. The monitoredion transitions were *m/z* 276.9→165.1 for clotrimazole and *m/z* 294.9→266.9 for estazolam, respectively. The calibration

curve of clotrimazole was established over the range of 0.01563~1.000 ng·mL^{-1} (r^2=0.9935). The intra- and inter-day precisions were less than 10% and all the biases were not more than 9%. The mean extraction recovery of clotrimazole was greater than 68.4% and no significant matrix effect was detected. The LLOQ of 0.01563 ng·mL^{-1} is sensitive enough to perform pharmacokinetic studies after clotrimazole administration.

Keywords: Clotrimazole; LC-ESI-MS/MS; Human plasma; Clinical monitoring; Pharmacokinetic study

1 Introduction

Clotrimazole (CTZ; 1-[(2-chlorophenyl)diphenylmethyl]-1H-imidazole) is an antimycotic imidazole derivative which is commonly used for the treatment of human pathogenic non-invasive fungi infections such as candidiasis, dermatophytoses, tinea vesicolor and erythrasma[1]. It was first described in 1969, and has been in clinical use for more than 20 years. As a member of antimycotic imidazoles, clotrimazole can bind to phospholipids in the cell membrane and then inhibits the biosynthesis of sterols, such as ergosterol, which are required for cell membrane production. Thereafter the permeability of cell membrane is changed, which leading to the fungi cell death[2]. Besides the antimycotic activity, clotrimazole is also used to inhibit proliferation of human tumor cells and vascular endothelial cells, and the growth of chloroquine-resistant strains of the malaria parasite[3-8].

According to ClinicalTrials.gov registered information, several clinical trials of

topical administration of clotrimazole (e.g. vaginal formulations) in healthy volunteers or patients are in progress, showing that there is a still great interest in this very old drug. Also, as an antifungal agent, clotrimazole exhibit marked variability in bloodstream concentrations that are difficult to predict on the basis of dosing alone, it has recommended therapeutic drug monitoring (TDM) for this drug in select patient populations[9]. Thus, development of reliable analytical methodologies for determination of clotrimazole in biological matrix is an essential step in optimizing and monitoring its therapy. However, most of analytical studies were focused on the quantification of clotrimazole in different pharmaceutical preparations and environmental water samples[10-13], and only few analytical methods for the determination of clotrimazole in biological matrix were reported[14-16]. To our knowledge, a previous study[15] employed capillary electrophoretic method to determine the concentrations of clotrimazole in mice plasma. Another study[14] used HPLC-UV assay for the quantification of clotrimazole in whole blood and plasma. The lower limit of quantification (LLOQ) for these two methods were 0.5 $\mu g \cdot mL^{-1}$ and 0.17 $\mu g \cdot mL^{-1}$ respectively. However, these LLOQ are still a little high for clotrimazole pharmacokinetics and clinical monitoring studies, since the low concentration ($ng \cdot mL^{-1}$) of clotrimazole in human plasma via both oral and topical administration were reported[17]. A method combining HPLC and mass spectrometry (MS) for the simultaneous quantification of 10 antifungal drugs in the liver and muscles of chickens has also been described[16]. However, this method was done in chicken matrices other than human plasma, and run over 8.1 min for each sample.

In the present study, a rapid, sensitive and specific liquid chromatography-electrospray ionization- tandem mass spectrometry (LC-ESI-MS/MS) method was developed and validated for determining clotrimazole in human plasma using estazolam as internal standard (IS). The advantages of this method over the reported method include a shorter run time and a much greater sensitivity. To our knowledge, this is the first report of the systematic validation of a HPLC-MS/MS assay for quantifying clotrimazole in human plasma.

2 Materials and methods

2.1 Chemicals and reagents

Clotrimazole and the internal standard (IS) estazolam (chemical structures are shown in Fig. 1) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade) was obtained from Merck (Merck, Germany). Sodium hydroxide, methyl tert-butyl ether and dichloromethane (analytical grade) were purchased from Sinopharm Chemcial Reagent Co., Ltd. (Shanghai, China). Formic acid (analytical grade) was purchased from Tianjin Kemiou Chemcial Reagent Co., Ltd. (Tianjin, China). Deionized water was purified in a Purelab classic system ELGA Labwater (Shanghai, China). Blank human plasma was obtained from the healthy volunteers. All other chemicals and reagents were of analytical grade from commercial sources.

2.2 Instrumentation and conditions

Analysis was performed on an Agilent 6460 triple quadrupole LC/MS system (Agilent, Germany) equipped with an electrospray ionization source (ESI). Data

acquisition was performed with Agilent Mass Hunter Workstation Software.

Separation was achieved by an Agilent 1200 HPLC system using a Phenomenex Luna CN column (2.0×150 mm, 5 μm, Phenomenex, Torrance, USA), and a Gemini C_{18} column (4×3.0 mm, 5 μm, Phenomenex, Torrance, USA) was employed as guard column. The mobile phase consisted of methanol-0.1% aqueous formic acid solution (85:15, *v/v*) at a flow rate of 0.2 mL·min^{-1}. The injection volume was 10 μL and the column temperature was maintained at 30 □.

The mass spectrometer was operated in positive ionization mode, and quantification was performed using multiple-reaction-monitoring (MRM) mode. The monitored ion transitions were *m/z* 276.9→165.1 for clotrimazole and *m/z* 294.9→266.9 for estazolam. The other optimized MS/MS parameters were as follows: fragmentor voltage 140 V for clotrimazole and 135 V for estazolam, collision energy (CE) 22 eV for both clotrimazole and estazolam, source temperature 300 □, drying gas (N_2) flow 11 L·min^{-1}, nebulizer pressure 15 psi, and capillary voltage 4 kV.

2.3 Subjects

Four healthy Chinese volunteers were included in this study. Prior to study, the protocol of this study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University (Changsha, China) and written informed consent was obtained from all participants after explanation of the potential risks and benefits, as well as the investigational nature of the study. No other prescribed or OTC drugs were taken from three months before this trial to the end of the study. Four female healthy volunteers, aged 18 to 40 years with a body mass index of 18.5 to 24.9 kg·m^{-2},

received a single dose of 0.150 g clotrimazole suppository via vaginal administration. The blood samples (approximately 5 mL) were collected at 0 (pre-dose), 1, 2, 3, 4, 6, 8, 10, 12, 24, 48 and 72 h in labeled Na-heparin vacuettes (5mL). Plasma was separated by centrifugation (3000 ×g, 4 ℃, 10 min) and stored at -20 ℃ until analysis. During the trial, volunteers have standard diet while water intake was unmonitored.

2.4 Preparation of stock and standard solutions

The stock solution of clotrimazole (1 mg·mL^{-1}) was prepared in methanol and stored in 4 □. The stock solution of IS estazolam (1 mg·mL^{-1}) was freshly prepared in 50% aqueous methanol solution and stored in 4 □. The IS working solution was prepared by diluting the IS stock solution to a concentration of 50 ng·mL^{-1} with 50% aqueous methanol solution before use.

A series of standard solutions of 0.1563, 0.3125, 0.6250, 1.250, 2.500, 5.000, 10.00 ng·mL^{-1} for clotrimazole was prepared by serially diluting the stock solution with 50% aqueous methanol solution. A 50 μL of standard working solution was transferred into 450 μL of blank plasma to yield the final plasma concentrations of 1.000, 0.5000, 0.2500, 0.1250, 0.06250, 0.03125, and 0.01563 ng·mL^{-1}. Quality control (QC) samples were prepared independently in the same way at three concentration levels (0.03125, 0.1250 and 0.8000 ng·mL^{-1}). All calibration standard samples and QC samples were freshly prepared daily.

2.5 Sample preparation

To a 500 μL standard plasma sample, 50 μL of internal standard working solution (50 ng·mL^{-1}) was added andvortex-mixed for 30s, then 100 μL of sodium hydroxide solution (1 mol·L^{-1}) and 4 mL mixed solvent of methyl tert-butyl ether and dichloromethane (4:1, *v/v*) were added and vortexed for 3 min. After the sample centrifugated at 2500 rpm for 10 min, 3 mL of the organic phase was transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen at 40□. Residue was reconstituted with 200 μL 50% aqueous methanol solution and 10 μL was injected into the LC-MS/MS system.

2.6 Method validation

Validation of the method was based on the Food and Drug Administration Guidance for Industry: Bioanalytical Method Validation[18]. The specificity of the method was assessed in six different sources of plasma, of which, four were normal plasma and one each of lipemic and heamolyzed plasma. By comparing the chromatograms of extracted blank plasma from six different sources with the corresponding plasma samples spiked with IS and clotrimazole. The chromatogram of typical plasma samples from different subjects after using clotrimazole suppository 3 hours was also analyzed to check for the endogenous interference. The linearity of the calibration curve for clotrimazole was assessed by analyzing seven standard solutions in the range of 0.01563~1.000 ng·mL^{-1} in plasma. Calibration curves were generated using the analyte to internal standard peak area ratios by weighted ($1/X^2$) least-squares linear regression. The acceptance criterion for each back-calculated standard concentration above the LLOQ was ± 15% deviation from the nominal value, except

at LLOQ. The LLOQ was defined as the concentration of the sample that could be quantified with less than 20% variation in precision (n = 5) and provided a signal-to-noise ratio ≥ 10. Precision and accuracy of the method were evaluated by analyzing QC samples at four concentration levels (0.01563, 0.03125, 0.1250 and 0.8000 $ng \cdot mL^{-1}$) on five replicates of each level in three validation days. Precision was expressed using the relative standard deviation (RSD %). Accuracy was defined as the relative deviation in the determined concentration of a standard from that of its nominal concentration expressed as a percentage. Extraction recoveries of clotrimazole were determined at three QC concentrations (0.03125, 0.1250 and 0.8000 $ng \cdot mL^{-1}$) by comparing the peak areas that were extracted from plasma samples with standard solutions without extraction procedure at the same nominal concentrations. To evaluate the matrix effect, blank human plasma samples were processed according to the sample preparation procedure described above and then spiked with clotrimazole and IS at the final concentration after extraction. The matrix effect of the plasma were expressed as the ratio of the mean peak area of analyte spiked post-extraction to that of the neat standard solution with 50% aqueous methanol solution at corresponding concentrations. Stability tests were performed for analyte-spiked plasma samples under various conditions: post-preparative stability (at 4 □ for 24 h), short-term storage stability (at ambient temperature for 4 h), through 3 freeze/thaw cycles (from -40 □ to room temperature) and long-term storage stability (at -40 □ for 3 month) by analyzing three replicates at low, medium and high QC concentrations. Dilution integrity experiment was performed with an aim to validate

the dilution test to be carried out on higher analyte concentration (above upper limit of quantification), which may encountered during real subject samples analysis. Dilution integrity experiments were carried out by a 10-fold dilution of the plasma samples with blank plasma for five replicates. The acceptable precision and accuracy were required to be within ±15%.

2.7 Incurred sample reanalysis

An incurred sample reanalysis (ISR) was performed by selecting four subjects (2 samples from each subject) near C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The ISR values were compared with the earlier value for the same sample using the same procedure. The percent change in the value should not be more than ±20%.

Change % = (repeat value – initial value)/(mean of repeat and initial values)×100

3. Results

3.1 Specificity

The typical chromatogram profiles of blank plasma, blank plasma spiked with clotrimazole and IS, and plasma obtained after using clotrimazole suppository 3 hours are shown in Fig.2. The retention time of clotrimazole and IS were approximately 4.4 min and 2.9 min, respectively. No obvious interferences from endogenous substances were observed. These results showed that the method exhibited good specificity and selectivity.

3.2 Linearity and LLOQ

An excellent linear relationship was observed between peak area ratios of

clotrimazole and IS versus plasma concentrations over a range of 0.01563 ~ 1.000 ng·mL^{-1}. Both the regression models (1/x and 1/x^2) were compared and the best fit for the concentration response relationship was obtained with a weighting factor of 1/x^2. The mean regression equation of the calibration curve for clotrimazole was Y=0.5353X+0.0139 (r^2=0.9935). The back calculated concentrations at all point on the plasma calibration curves were within the ±15% of the nominal concentration (Table 1). The LLOQ for clotrimazole in plasma was shown to be 0.01563 ng·mL^{-1} on the basis that the accuracy of LLOQ was 98.8% and precision (RSD %) was 1.1%.

3.3 Precision and accuracy

The intra- and inter-day precision and accuracy values are presented in Table 2. As shown, all the intra- and inter-day precisions were less than 10% and all the biases were not more than 8%. The data demonstrated that the precision and accuracy of this assay were within the acceptable range and the method was satisfied.

3.4 Extraction recovery and matrix effects

Then liquid–liquid extraction technique was chosen as extracting method, and the extracting effects of tert-butyl ether-dichloromethane (4:1, *v/v*), cyclohexane–methylene dichloride (2:1, *v/v*) and ethylether solvents were assessed. It was found that tert-butyl ether-dichloromethane (4:1, *v/v*) gave the highest recovery (about 70%) and no significant interference was observed. Mean recoveries of clotrimazole from human plasma were 68.7 ± 1.2%, 68.4 ± 3.2% and 72.3 ± 4.5% at concentrations of 0.03125, 0.1250 and 0.8000 ng·mL^{-1}, respectively, and the mean recovery of IS was 87.6 ± 1.9%. Matrix effects (%) of clotrimazole at 0.03125, 0.1250

and 0.8000 ng·mL^{-1} were found to be 100.6 ± 6.5%, 98.9 ± 3.3%, and 100.3 ± 3.0% of the nominal concentrations, respectively, and this value was 99.1 ± 2.7% in terms of IS. This indicates that matrix effects are not an issue using the current method.

3.5 Stability

In terms of stability, the results are shown in Table 3 indicated that CTZ and IS were stable in human plasma and in processed samples under the conditions described above.

3.6 Dilution integrity

Dilution integrity experiments were carried out in five replicates by a 10-fold dilution of the plasma samples with blank plasma, and assay precision and accuracy were tested using the same sample pretreatment method. The precision (RSD %) for dilution integrity of 1/10 dilution was found to be 11.2%, while the accuracy results were found to be 97.9%. The results suggested that samples whose concentrations exceeded the upper limit of the calibration curve could be reanalyzed by an appropriate dilution.

3.7 Incurred samples reanalysis

The reproducibility of the present method was established by reanalysis of incurred samples. Two plasma samples from each subject were selected and re-assayed in a single bioanalytical run. The changes in concentrations between ISR and the initial values for the samples were less than 20% (Table 4), indicating good reproducibility of the present method.

4. Discussion

Over the past decade or so, modern liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has greatly multiplied the sample analysis throughput. Moreover, technological advances in column technology and mass spectrometers have also driven ever lower the limit of quantitation of bioanalysis required to fully understand the pharmacokinetics of the low dose/high potency drug candidates and have made LC–MS/MS the choice for the antifungal agents. In this work, we obtained excellent sensitivity for the quantifiation of clotrimazole in human plasma by LC-MS/MS and the LLOQ up to 0.01563 $ng \cdot mL^{-1}$, which was much greater sensitive than the reported method while using only 500 μL human plasma.

Due to the complexity of the sample matrices and the low concentration of clotrimazole in biological samples, a sample preparation step was necessary to reduce the endogenous interferences before LC-MS/MS analysis. In the present study, in order to simultaneously obtain suitable recoveries and minimum interference for the determination of clotrimazole, different sample preparation procedures were investigated. Initially, the extraction method was performed by protein precipitation (PPT) using methanol and acetonitrile respectively, the PPT method is simplest, but the sensitivity was relatively low. Solid phase extraction (SPE) has high recovery but is much time and money consuming, which may restrict its application in determination of analytes in human plasma. Then liquid–liquid extraction technique was chosen as extracting method, and the extracting effects of tert-butyl ether-dichloromethane (4:1, *v/v*), cyclohexane–methylene dichloride (2:1, *v/v*) and ethylether solvents were assessed. It was found that tert-butyl ether-dichloromethane

(4:1, *v/v*) gave the highest recovery (about 70%) and no significant interference was observed. Clotrimazole, the chemical structure of which shown in Fig.1.A, is a kind of alkaline compound with the imidazole ring. The alkaline medium was helpful to release more clotrimazole in a free state. So, the addition of 100 μL of sodium hydroxide solution (1 $mol \cdot L^{-1}$) in sample preparation could increase the liquid–liquid extraction rate and decrease the interference. The recovery results of the sample preparation with and without the addition of sodium hydroxide solution were also compared. The recovery was higher with the addition of sodium hydroxide solution.

The choice of the internal standard (IS) is of crucial importance since it affects precision and accuracy of the analytical method. Physicochemical properties may vary to some degree and cause high variability during sample pretreatment; therefore, differences in detector response would usually appear. Estazolam was used as internal standard here for its similar structure and polarity to clotrimazole, which made its retention time close to that of clotrimazole. Estazolam is relatively stable with few fragments, which could reduce the possibility of interference in the quantification of clotrimazole. Further results showed that the internal standard also had similar recovery and suitable retention time to the target analyte.

The bioanalytical methodology for clotrimazole described above is rapid, sensitive and specific for analysis of routine samples in pharmacokinetic studies and therapeutic drug monitoring, and it was successfully applied to a pharmacokinetic study of clotrimazole suppository enrolling four Chinese healthy volunteers (the results are not shown). This is the first report of the systematic validation of a

LC-MS/MS assay for quantifying clotrimazole in human plasma.

5. Conclusion

A rapid, sensitive and specific LC-ESI-MS/MS method was established and fully validated for the quantification of CTZ in human plasma, which was the first time according to previous reports about CTZ. A simple LLE method was employed for sample preparation which provided stable recoveries with no significant endogenous interference. The optimized HPLC-MS/MS performance demonstrated a much greater sensitivity and a shorter run time than previous methods. The LLOQ of 0.01563 $ng \cdot mL^{-1}$ is sensitive enough to perform pharmacokinetic studies of clotrimazole.

Acknowledgements

This work was supported by the Hunan Science and Technology Project (No. 2011SK3261) and the Fundamental Research Funds for the Central Universities of Central South University (No. 2015zzts286).

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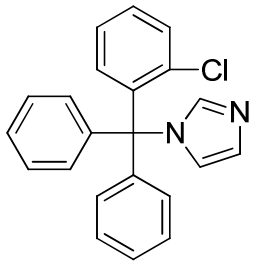
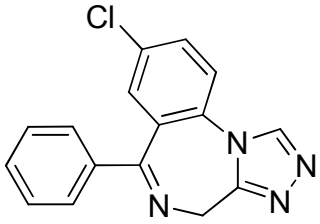
Figure captions

Fig.1. Chemical structure of clotrimazole (A) and IS estazolam (B).

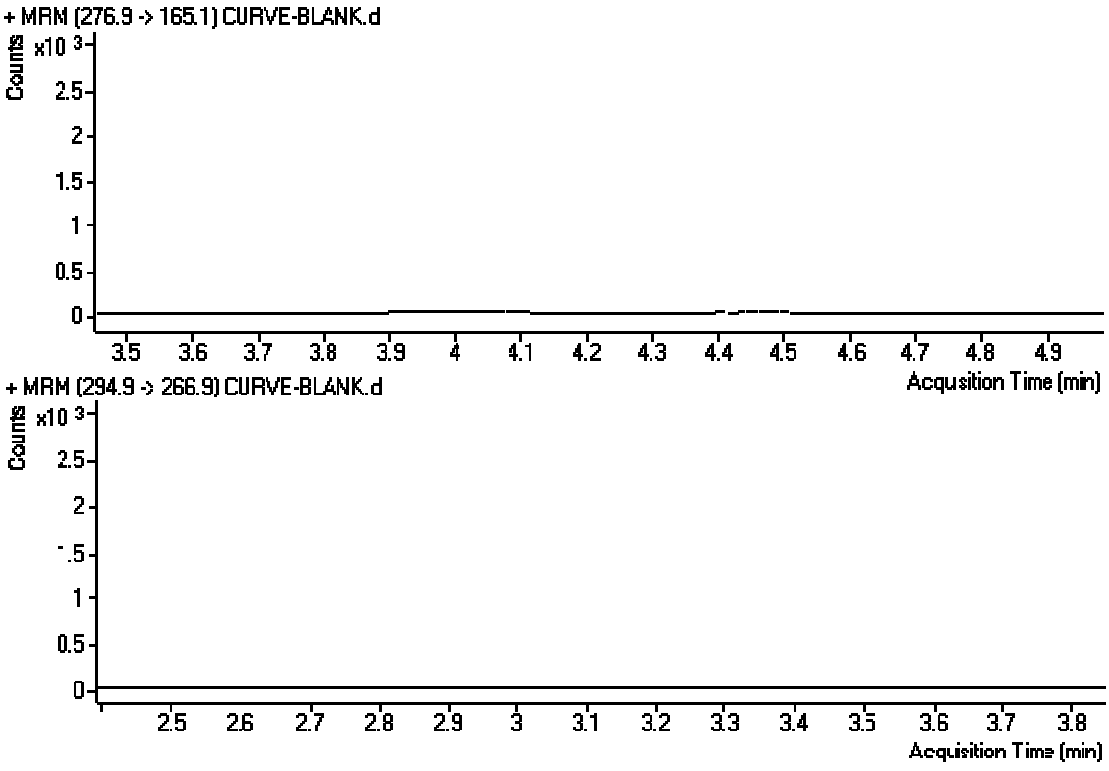


A



B

Fig.2. Typical chromatograms of clotrimazole in human plasma: (A) blank human plasma from healthy volunteers; (B) blank plasma spiked clotrimazole (0.01563 ng·mL^{-1}) and IS; (C) Plasma sample from a healthy volunteer 3 h after a single-dose administration of 150 mg clotrimazole suppository. The sample concentration was determined to be 0.8990 ng·mL^{-1}.



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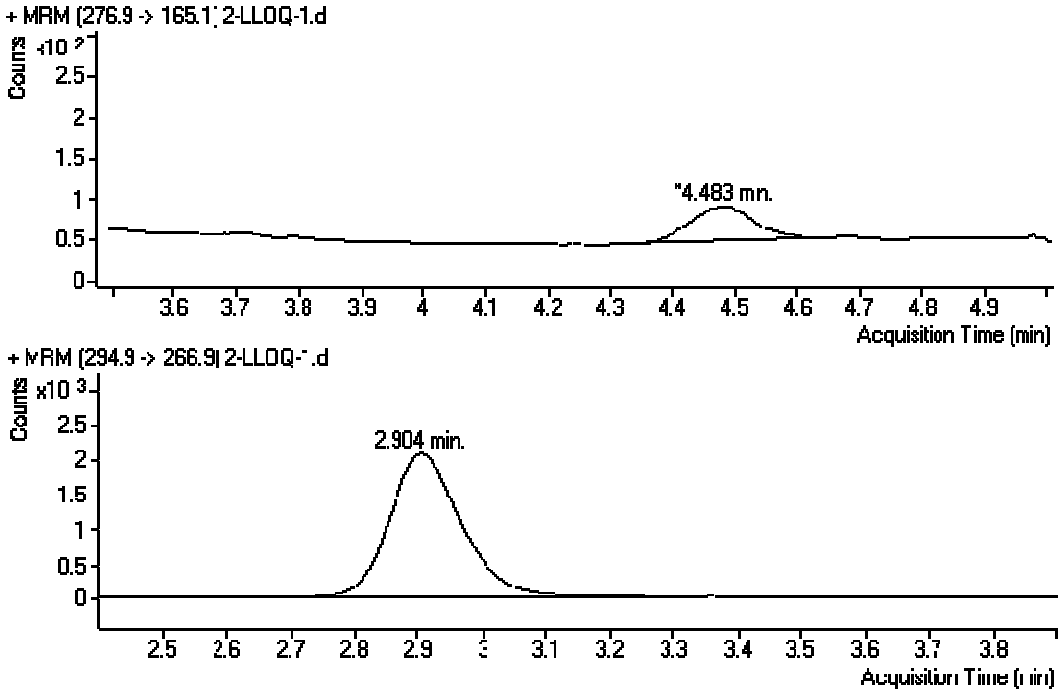

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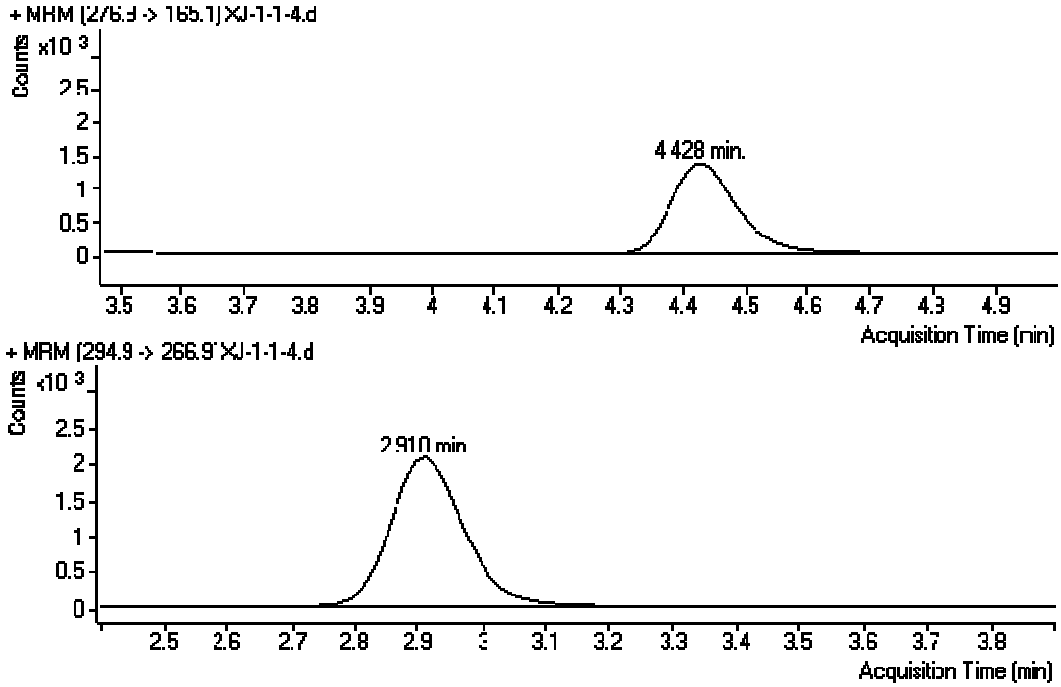

C

Table 1 Intra-day reproducibility of the standard plasma calibration curve of clotrimazole obtained by LC-MS/MS assay

Nominal conc. ($ng \cdot mL^{-1}$)	Conc. found ($ng \cdot mL^{-1}$)	Precision (RSD %)	Accuracy (%)
0.01563	0.01546	1.6	98.9
0.03125	0.02976	6.0	95.2
0.06250	0.06985	8.5	111.8
0.1250	0.1295	5.5	103.6
0.2500	0.2434	3.9	97.3
0.5000	0.5045	1.5	100.9
1.000	1.034	5.9	103.4

Table 2 Inter- and intra-day accuracy and precision of clotrimazole in human plasma.

	Concentration	Intra-day (n=5)			Inter-day (n=15)		
QC ID	added ($ng \cdot mL^{-1}$)	Concentration found ($ng \cdot mL^{-1}$)	Accuracy (%)	Precision (RSD %)	Concentration found ($ng \cdot mL^{-1}$)	Accuracy (%)	Precision (RSD %)
LLOQ QC	0.01563	0.01544	98.8	1.1	0.01521	97.3	2.4
LQC	0.03125	0.03012	96.4	9.0	0.03045	97.4	7.5
MQC	0.1250	0.1289	103.1	8.6	0.1296	103.7	7.2
HQC	0.8000	0.7433	92.9	4.0	0.7592	94.9	5.7

Table 3 Stability of clotrimazole in human plasma under various conditions (n＝3).

Condition	0.03125ng·mL^{-1}			0.1250 ng·mL^{-1}			0.8000 ng·mL^{-1}		
	Mean (ng·mL^{-1})	Accuracy (%)	RSD (%)	Mean (ng·mL^{-1})	Accuracy (%)	RSD (%)	Mean (ng·mL^{-1})	Accuracy (%)	RSD (%)
Post-preparative stability	0.0299	95.5	5.5	0.1168	94.5	3.3	0.7608	95.1	3.0
Short-term stability	0.0300	95.8	4.7	0.1154	92.3	4.0	0.7801	97.5	4.7
Freeze-thaw stability	0.0307	98.1	6.1	0.1171	93.7	8.6	0.7496	93.7	3.9
Long-term stability	0.0308	98.4	3.9	0.1303	104.2	2.9	0.8079	101.0	6.7

Table 4 Incurred samples re-analysis data of clotrimazole

Sample	Initial conc. (ng·mL^{-1})	Re-assay conc. (ng·mL^{-1})	Change (%)
1	2.154	2.302	6.6
2	0.6431	0.6191	-3.8
3	1.325	1.452	9.1
4	0.4131	0.4601	10.7
5	2.000	1.899	-5.1
6	0.6361	0.6142	-3.5
7	1.329	1.503	12.3
8	0.4343	0.4632	6.4