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Sensitive and selective LC-MS/MS assay for quantitation of flutrimazole in human plasma

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Abstract. – OBJECTIVE: A highly sensitive liquid chromatography-tandem mass spectrometry method was developed and validated for the determination of flutrimazole in human plasma. This study was to investigate the application of sensitive and selective LC-MS/MS method for quantitation of flutrimazole in human plasma.

MATERIALS AND METHODS: The analysis and internal standard were extracted with ether and hexane (*v:v*, 1:1) followed by a rapid isocratic elution with a 0.1% formic acid/methanol (*v:v*, 20:80) on a C18 column (50 mm × 2.1 mm I.D.) and subsequent analysis by mass spectrometry in the multi-reaction-monitoring mode. The precursor to production transitions of m/z 279.0 → 183.1 and m/z 441.0 → 295.1 were used to measure the analyte and the internal standard.

RESULTS: The assay was linear over the concentration range of 0.996-99.6 ng·mL^{-1} for flutrimazole in human plasma. The lower limit of quantification was 0.996 ng·mL^{-1} and the extraction recovery was larger than 78.83% for flutrimazole. The inter- and intra-day precision of the method at three concentrations was less than 9.26%.

CONCLUSIONS: The LC-MS/MS method was firstly applied to quantitation of flutrimazole in human plasma.

Key Words:
Flutrimazole, Cream, Human plasma, LC-MS/MS, Plasma concentration.

Introduction

The principal component of flutrimazole emulsifiable paste is flutrimazole (Figure 1) with chemical name 1-[(2-Fluorophenyl) - (4-fluorophenyl) phenylmethyl] - 1H – imidazole. Flutrimazole is a type of topical anti-fungal ointment[1]. As in other imidazole derivatives, flutrimazole achieves anti-fungal pharmacological functions by inhibiting the activity of lanosterol-14α-demethylase and interfering the synthesis of ergosterol to cause denaturation of fungal cell membrane[2,3]. Flutrimazole is applicable in topical treatment of the epidermis mildew infection such as tinea pedis, tinea corporis, facial tinea and tinea cruris, which are usually caused by trichophyton favosa, microsporum canis and epitermophyton floccosum[4-6].

The pharmacokinetics research of flutrimazole and clotrimazole with C_{14} marker on 6 Beagle dogs has been completed previously[7]. Both flutrimazole and clotrimazole showed significant first pass effect and only 10% of the administration dose can reach the systemic circulation after metabolism. Most of the radioactive substances were detected in the feces. Less than 1% of flutrimazole was excreted from the urine. Moreover, only about 0.5% of the drug dosage was collected in urine[7]. In the current study, a more sensitive and specific liquid chromatography-tandem mass spectrometry method (LC-MS/MS) has been developed for the determination of flutrimazole in human plasma for the first time.

Materials and Methods

Reagents and Materials

Flutrimazole (99% purity) was purchased from SihuanKebao pharmaceutical Co. Ltd. (Batch number: 20081030, Beijing, China). Simvastatin was obtained from National Institutes for Food and Drug Control (Batch number: 1006016-200502, Beijing, China). Methanol and acetonitrile were of HPLC grade (Merck, Kenilworth, NJ, USA). Others were of AR grade. Ultrapure water used for the LC/MS/MS was from Milli-Q water purification system (Millipore, Billerica, MA, USA).

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LC/MS/MS Instrumentation and Conditions

Analyses were performed by an Alliance 2695 LC system (Waters, Milford, MA, USA) coupled with a triple-quadrupole tandem Quattro Micro mass spectrometer (Waters, Milford, MA, USA). The Mass Lynx 4.1 software was used for instrumental control, acquisition and processing of the data. The LC separation was performed on a Waters XTerra C_{18} column (2.1×50 mm I.D., 3.5 µm, Waters, Milford, MA, USA) with a security guard column (12.5 mm × 2.1 mm I.D., 5 µm, Agilent Zorbax SB-C_{18}, Santa Clara, CA, USA). The mobile phase consisted of methanol and deionized water (*v:v*, 80:20) containing 0.1% formic acid at a flow rate of 0.2 mL/min. The auto sampler temperature was maintained at 15°C. The total LC run time was 4 min with the column temperature kept at 30°C.

A MS detector with an electrospray ionization (ESI) interface in positive ion mode was used for quantitative analysis. Quantitation was performed using the multi-reaction-monitoring (MRM) mode of transitions of m/z 279.0→183.1 for flutrimazole, m/z 441.0→295.1 for simvastatin. The optimized conditions used for the ESI^+ source were as follows: capillary voltage: 3.5 kV; cone voltage: 50 V; source temperature: 110°C; desolvation temperature: 350°C; desolvation gas flow (nitrogen): 500 l/h; collision energy 28 V for flutrimazole and 25 V for simvastatin; Argon was used as the collision gas with the gas pressure of 3.0×10^{-3} mbar.

Preparation of Standard and Quality Control (QC) Samples

The standard stock solutions were prepared by dissolving flutrimazole (996 µg/ml) and simvastatin as IS (746 µg/ml) in methanol. The stock solution with methanol to make flutrimazole standard solution of 0.996, 2.49, 4.98, 9.96, 24.9, 49.8, 99.6 $ng \cdot ml^{-1}$ was diluted. Quality control (QC) samples were prepared at concentrations of 1.992, 7.968, 79.68 $ng \bullet ml^{-1}$, in the same way as the plasma samples for calibration. All the standard calibration samples and QC samples were stored at -20°C.

Sample Preparation

200 µl of plasma samples and 20 µl of internal simvastatin standard solution (1.492 $\mu g \cdot ml^{-1}$) were added to glass centrifuge tubes. After vortex-mixing for 30 s, 3 ml organic solvent solution (ether: hexane = 1:1) was added, then vortex-mixed for 3 min. The solution was centrifuged for 10 min at 3000 $r \cdot min^{-1}$. 2.8 ml of supernatant was transferred into another tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted with 100 µl mobile phase and vortex for 1 min, then centrifuged for 5 min at 12000 rpm. 10 µl supernatant was injected onto the LC-MS/MS system for analysis.

Method Validation

The method was validated for the selectivity, sensitivity, matrix effect (ME), linearity, precision and accuracy, recovery and stability. Specificity was assessed by comparing the total ion chromatogram (TIC) of 6 blank plasma samples with TIC of plasma samples, which spiked with flutrimazole and IS. Further, 10 µl working solution was prepared in the mobile phase and was injected to check the interference. ME was defined as the ion suppression/enhancement on the ionization of analytes. It was evaluated by comparing the area response of post-extraction blank plasma samples. The samples had two analytes at 3 QC levels (A) to those of the equivalent concentration standard solutions dried directly and reconstituted with the same mobile phase (B). The ratio (A/B × 100)% was used to evaluate the effect of the matrix. The same procedure was performed for the IS.

The lowest limit of quantification (LLQ), defined as the amount that could be detected with a signal-to-noise ratio of 10, was determined in five replicates with the precision of less than 20% using the relative standard deviation (RSD) and 80% to 120% accuracy of the spiked concentration. The linearity for each analyte was assessed by analyzing the calibration curves from seven non-zero concentrations of calibration samples in duplicate in 5 separate runs. Blank plasma samples were analyzed to discard the presence of interferences. The linearity was confirmed through the comparison of the ratio of the peak area of the analytes to that of the IS solution with the analyte concentrations through least squares linear regression analysis, which is described in the form of $Y = aX + b$ (weighting factor = $1/x^2$).

Recoveries of flutrimazole were determined at 3 different concentration levels by comparing the peak areas of the extracted samples (spiked before extraction) with those from the standard solutions at the same concentration. The intra-batch precision and accuracy were evaluated in 5 replicate analyses for flutrimazole at 3 QC levels on the same analytical run. Inter-batch precision and ac-

curacy were calculated after repeated analysis in 3 different analytical runs. Concentrations were calculated from the calibration curve. The accuracy and precision was calculated and expressed in terms of % bias and relative standard deviation (% RSD), respectively.

The stabilities of flutrimazole in human plasma were determined in triplicate through different storage conditions in the following ways: (1) Stability of flutrimazole in human plasma during sample preparation was assessed by detecting samples after storage for 4 h at room temperature. (2) For freeze-thaw stability, the plasma samples were determined through 3 freeze (-20°C)-thaw (room temperature) cycles. (3) To evaluate the stability of the treated plasma samples in the auto-sampler, QC samples were prepared and placed in the auto-sampler at 4°C for 24 h, and then injected for analysis. (4) The long-term stability was performed by assaying the plasma at -20°C.

Application for Pharmacokinetic Study

Test Persons

10 volunteers were enrolled (5 females and 5 males). All volunteers were 20-30 years old and did not smoke or consume alcohol. The average age was 24.6 years (s = 2.5) with an average body mass index of 22.3 (s = 1.7). The study was approved by the Medical Ethics Committee of the Affiliated Hospital of Nanjing University of TCM (Nanjing, Jiangsu, China). All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki.

Plasma Samples Collection

0.6 g of flutrimazole cream was smeared within 25 × 10 cm^2 area on the back of the subjects. Diet was prohibited for 12 h before the experiment while water was taken freely. 3 ml of the plasma were collected at 1, 2, 4, 8, 12, 24, 36, 48, 72, 96 h after administration. All the plasma was stored at -75°C.

Results

Specificity

The liquid-liquid extraction methodology in combination with mass spectrometry detection provided selectivity for the analytes and IS. Figure 2 showed the total ion chromatogram (TIC) of spiked plasma with flutrimazole (0.7968 ng•ml^{-1}) and IS, as well as TIC of plasma after an administration of flutrimazole at intervals of 2 h. The retention time was 1.88 min for flutrimazole, and 2.78 min for IS, respectively. There was not endogenous interference detected in blank plasma samples at the retention time of analytes and IS.

Linearity and Lower Limits of Quantification

The calibration curve for flutrimazole was linear well within the range of 0.996–99.6 ng·ml^{-1}. The mean value of regression equation was Y = (0.6312 ± 0.0467) X + (0.0539 ± 0.0153) (n = 5) with a correlation coefficient over 0.9992, where Y was the peak-area ratio of flutrimazole to IS and X was the plasma concentration of flutrimazole. The LLQ of flutrimazole was 0.996 ng•ml^{-1}.

Recovery and Matrix Effect

The recovery and matrix effect of flutrimazole were shown in Tables I and II. The recoveries of flutrimazole determined at 0.1992, 0.7968, 7.968 ng·ml^{-1} were 84.3180.54, and 78.83 , respectively, while the recovery of the IS determined at 149.2 ng·ml^{-1} was 70.59%. The mean matrix effect values obtained for flutrimazole were 92.84, 96.17 and 90.45 at low, medium and high QC level, respectively.

Accuracy and Precision

The results of intra and inter-day were as shown in Table III. The intra-day accuracy for flutrimazole ranged from 100.39 to 102.16 within the testing concentrations with the precision (R.S.D.) between 3.64 and 7.46 , and the inter-day accuracy for flutrimazole ranged from 100.63 to 101.56 with the precision (R.S.D.) be-

Table I. Recoveries of flutrimazole and IS from human plasma using ethyl acetate as extracting solvent.

	Concentration (ng▪ml^{-1})	Recoveries (n = 5)
Flutrimazole	0.1992	84.31 ± 6.84
	0.7968	80.54 ± 7.82
	7.968	78.83 ± 8.47
Simvastatin	149.2	70.59 ± 4.86

Table II. Matrix effect of flutrimazole and simvastatin (IS).

Matrix	Concentration (ng▪ml^{-1})	ME (%)
Flutrimazole (n=3)	1.992	92.84 ± 5.51
	7.968	96.17 ± 4.77
	79.68	90.45 ± 5.82
Simvastatin (n=9)	149.2	86.92 ± 6.35

Table III. Intra- and inter-day accuracy and precision of flutrimazole assay in human plasma.

		Intra-day (overall mean, n=5)			Inter-day (overall mean, n=15)		
	Concentration (ng▪ml^{-1})	Conc.found (ng▪ml^{-1})	Accuracy (%)	RSD (%)	Conc.found (ng▪ml^{-1})	Accuracy (%)	RSD (%)
Flutrimazole	0.1992	0.2008	100.39	4.05	0.2013	100.63	5.67
	0.7968	0.8079	100.98	7.46	0.8057	100.71	9.26
	7.968	8.1728	102.16	3.64	8.1251	101.56	5.20

tween 5.20% and 9.26%. These results indicated that the present method was accurate, reliable and reproducible.

Stability

Results of the stability (n =3) were summarized in Table IV. Flutrimazole was stable in human plasma for at least 3 freeze/thaw stability and at ambient temperatures up to 24 h. Besides, flutrimazole was stable in human plasma for up to 20 days at -20°C. This demonstrated that flutrimazole has a good stability under these conditions.

Application

The Table IV showed that most plasma samples of all 10 healthy subjects after single drug administration was lower than the LLQ level. Flutrimazole was detected only in a few healthy subjects while the concentration was only 1.18 ng/ml in plasma (Table V). Only a few of the drug were absorbed into the human circulation system. The result was consistent with the reported reference[8].

Discussion

Flutrimazole is a new imidazole derivative with antifungal activity, which has been studied *in vivo* and *in vitro*. According to the comparisons, flutrimazole was considered to have similar antifungal activity as clotrimazole and higher antifungal activity than bifonazole[9]. Generally, flutrimazole is used for topical treatment of su-

Table IV. Stability of flutrimazole in human plasma under different storage conditions (mean ± SD, n = 3).

Storage conditions	Flutrimazole		
	Added (ng▪ml^{-1})	Measured (ng▪ml^{-1})	Deviation (%)
Short-term stability (at room temperature for 4 h)	0.1992	0.1951 ± 0.0112	5.73
	0.7968	0.8379 ± 0.0960	11.46
	7.968	8.2820 ± 0.7717	9.32
Three freeze-thaw cycles	0.1992	0.2019 ± 0.0189	9.38
	0.7968	0.7562 ± 0.0476	6.29
	7.968	7.9056 ± 0.2472	3.13
At 4°C in the auto-sampler for 24 h	0.1992	0.2039 ± 0.0108	5.31
	0.7968	0.8060 ± 0.0665	8.26
	7.968	8.2245 ± 0.2608	3.17
Long-term stability (at -20°C for 20 days)	0.1992	0.2143 ± 0.0010	0.46
	0.7968	0.7541 ± 0.0030	0.39
	7.968	7.0004 ± 0.1157	1.65

Table V. The concentration of flutrimazole in 10 volunteers with in vitro administration of flutrimazole (n = 10)

Time (h)	A	B	C	D	E	F	G	H	I	J
0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	1.18	ND	ND	ND
4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
36	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
72	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
96	ND	ND	ND	0.26	ND	ND	ND	ND	ND	ND

perficial mycoses of the skin by interfering with the synthesis of ergosterol by inhibiting the activity of the enzyme lanosterol 14 α-demethylase[10,11]. Fluconazole, a drug from the azole family, is considered as a safe and effective oral and intravenous systemic therapy treatment. However, due to the characteristics of the large molecular weight, it is difficult to be used as topical application. Moreover, strong hydrophilic property is another characteristic of flutrimazole[12,13].

In this work, we developed specific LC-MS/MS assay for the determination of flutrimazole in human plasma for the first time. LC-MS/MS has become an indispensable part of modern analytical methods[14]. The application of LC/MS was begun in 70s. Since the successful application of atmospheric pressure ionization and the development of mass spectrometry, the combination of liquid chromatography and mass spectrometry has been used since 90s. In particular, the use of tandem mass spectrometry (MS/MS) has received great attention and development. The advantage of LC-MS.MS combination is very significant[15,16]. The results of this study suggested that after a single and multiple applications of the treatment, the most of the flutrimazole concentrations in plasma of the healthy samples were lower than those detected by the LC-MS/MS method. The flutrimazole was detected only in a small number of healthy patients, and the concentration was lower than 1.18 ng/ml. The results also suggested that after a single use of the cream, the concentration of flutrimazole in the circulatory system of the involved patients were lower. After 10 weeks of continuous administration of a single dose of flutrimazole, the concentrations of flutrimazole from all the concentrations in the plasma samples were significantly lower than those of the present method. 6 healthy patients were discovered to have flutrimazole in the plasma. However, the concentration was lower than 0.25 ng/mL, indicating that, in multiple applications of the flutrimazole treatment, the concentrations of flutrimazole absorbed by the skin in the circulation systems of patients were low. There were 6 cases of adverse events during the whole experiment. Among them, 1 cases of high renal function examination of creatinine and urea, 1 case of upper respiratory tract infection, 1 case of high red blood cells, high white blood cells and high neutrophils in the urine routine examination. However, at the next day all patients with adverse events were turned normal. It not only filled up the blank of the study of flutrimazole on the circulation system, but also provided the basis for the safety evaluation. The concentration of flutrimazole in the circulatory system by skin absorption was lower, indicating that most of the external drugs used in the human body have not been absorbed into the blood through the skin. It was proved that the external preparation was safe, and the purpose of this experiment was achieved.

Conclusions

Our study was the first to demonstrate a novel and validated LC-MS/MS method for the analysis of flutrimazole in human plasma. The advantages of the method presented in this paper were the simple sample preparation and the determination of both analytes within a short run time. In addition, this LC-MS/MS with a simple liquid extraction can ultimately be applied to other preclinical and clinical bio-samples (plasma, urine, tissue, etc.) for future pharmacokinetic studies.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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