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Short communication

A validated LC–MS/MS method for determination of sertaconazole nitrate in human plasma

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ABSTRACT

A specific and sensitive liquid chromatography electrospray ionization tandem mass spectrometric (LC–ESI-MS/MS) method for quantitative determination of sertaconazole in human plasma was developed. The analysis was performed and validated in positive ion multiple reactions monitoring mode using loratadine as an internal standard (IS). Sample preparation involved one-step liquid–liquid extraction using ether–dichloromethane (80/20, v/v). Sertaconazole and IS was separated on a C_{18} column using isocratic elution with a mobile phase of methanol: 0.2% formic acid aqueous solution (70:30, v/v,) at the flow rate of 0.2 mL/min. The transition monitored were m/z 439 $[M+H]^+ \rightarrow m/z$ 181 for sertaconazole and m/z 383$[M+H]^+ \rightarrow m/z$ 337 for IS. The lower limit of quantification was 0.1 ng/mL based on 500 μL of plasma, and no interferences were detected in chromatograms. Calibration curve was linear over the range of 0.1–10 ng/mL, and correlation coefficients were 0.999. Intra- and inter-day assay variations were <10%, and the accuracy values were between −0.4% and 9.0% relative error (RE). The extraction recoveries ranged from 60% to 70% across the calibration curve range. The described method provides a sensitive analytical tool to determine sertaconazole in plasma, and was successfully applied to a pharmacokinetic study in 10 healthy human subjects after administration of 300 mg vaginal suppository formulation of sertaconazole nitrate.

1. Introduction

Sertaconazole (RS)-1-[2-[(7-chloro-1-benzothiophen-3-yl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole nitrate (Fig. 1) is a broad spectrum antifungal agent with excellent activity against yeasts, dermatophytes and opportunistic fungi [1]. Up to now, the activities of sertaconazole have been widely studied. However, reports on the determination of sertaconazole nitrate especially in biological matrix were rare. Albet et al. [2] and Nemutlu et al. [3] determined sertaconazole nitrate in pharmaceutical preparations using HPLC and capillary zone electrophoresis, respectively. Besides, one review [4] mentioned that HPLC with UV detection was also utilized to quantify sertaconazole in plasma, urine and vaginal secretion. The LOQ of the method for sertaconazole were 20 ng/mL in plasma, 25 ng/mL in urine and 80 ng/mL in vaginal secretion, respectively, which were not enough for pharmacokinetic study of sertaconazole nitrate vaginal suppository. Thus a more sensitive analytical method is required to assay sertaconazole in biological matrix. So, the objective of the present study was to develop a more selective and sensitive method to determine sertaconazole in human plasma using liquid chromatography/tandem mass spectrometry (LC/MS/MS) for the first time. The assay was validated and applied to a clinical pharmacokinetic study in 10 healthy volunteers administered a 300 mg vaginal suppository formulation of sertaconazole nitrate.

2. Experimental

2.1. Chemicals and reagents

Sertaconazole nitrate (purity >99.5%) and sertaconazole nitrate vaginal suppository were supplied by Hainan Haishen pharmaceutical Group, China; Loratadine (internal standard, IS, purity >99.5%) was supplied by Hunan Warrant Pharmaceutical Co., Ltd., China. HPLC grade methanol and formic acid were purchased from Merck (Merck Company, Germany). Water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). All other chemicals and solvents were of highest analytical grade available.

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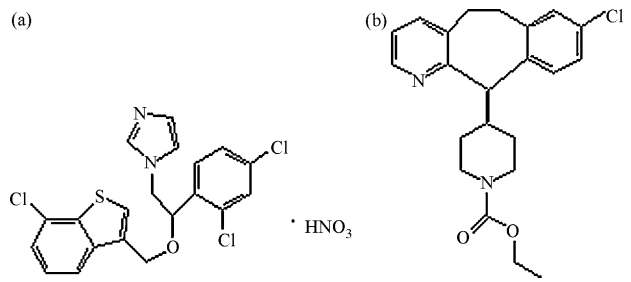

Fig. 1. Chemical structure of sertaconzaole nitrate (a) and loratadine (b).

2.2. Instrumentation and conditions

The LC/MS/MS equipment consisted of a Surveyor LC pump, a Surveyor auto-sampler, a Finigan TSQ Ultra EMR tandem mass spectrometer (Thermo Electron Corporation, USA), equipped with an ESI ion source. Xcalibur software v.2.0 SUR 1 was used for data acquisition and analysis. LC separation was performed on the HYPERSIL GOLD C_{18} (150 mm × 2.1 mm, 5 μm; Thermo Electron Corporation, USA) column using isocratic elution with a mobile phase of methanol–0.2% formic acid (70:30, v/v) at a flow rate of 0.2 mL/min. The column temperature was maintained at 35 °C. The mass spectrometer was operated in the positive ionization mode. The electrospray voltage was set to 4000 V and capillary temperature was at 350 °C. The nitrogen sheath and auxiliary gasses were set at 35 and 5 arbitrary units, respectively. The source collision induced dissociation (Source CID) was set at −10 V. Detection of the ions was performed in the multiple reaction monitoring (MRM) mode, using precursor to product ion transition of m/z 439 $[M+H]^+$ to m/z 181 for sertaconazole and 383 $[M+H]^+$ to m/z 337 for IS. Argon was selected as collision gas at 1.5 mtorr (1 torr = 133.3 Pa). Collision energy (CE) were 33 V (m/z 439 → m/z 181) for sertaconazole and 35 V (m/z 383 → m/z 337) for IS, respectively.

2.3. Preparation of standard and quality control (QC) samples

Primary stock solution of sertaconazole nitrate was prepared by dissolving sertaconazole nitrate reference standard in methanol at concentration of 100 μg/mL. Working solutions of sertaconazole nitrate were prepared by serial dilutions with methanol, at final concentration of 1, 2.5, 5, 10, 25, 50 and 100 ng/mL from the primary stock solution. IS primary stock solution was also made up in methanol at 100 μg/mL and diluted with methanol to prepare of IS working solution (100 ng/mL). These were then stored at 4 °C and brought to room temperature before use.

Calibration standard samples were prepared by spiking addition of the working solutions to drug-free human plasma, resulting in calibration standards with concentrations of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 ng/mL. Quality control (QC) samples were prepared at concentrations of 0.25 (low), 1.0 (medium) and 5.0 ng/mL (high), by the same process.

2.4. Sample preparation

After thawing at room temperature, 500 μL plasma and 20 μL IS solution (100 ng/mL) was pipetted into glass tube. After vortexing briefly, 4 mL of ether–dichloromethane (80/20, v/v) was added to each sample and the mixture was vortex-mixed for 3 min and then centrifuged at 3500 × g for 10 min. The organic phase was transferred to another clean glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 200 μL mobile phase and the sample was transferred to an auto-sampler vial. Finally, a 5 μL aliquot was injected into the LC/MS/MS system.

2.5. Method validation

The selectivity of the assay was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank plasma sample was tested using the extraction procedure and chromatographic conditions described above to ensure no interference of sertaconazole and IS from plasma.

Calibration standards of seven sertaconazole concentration levels at 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 ng/mL were extracted and assayed. To evaluate the linearity, calibration curves were prepared and assayed on 5 days. The calibration curve was constructed by plotting the peak area ratios of sertaconazole to the IS versus the concentrations of sertaconazole, using weighted least-squares linear regression ($1/X$). The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20% [9], and it was established using five samples independent of standards.

To evaluate the accuracy and precision of the method, QC samples at three concentration levels (0.25, 1.0 and 5 ng/mL) were analyzed in five replicates on three validation days. Assay precision was calculated using the relative standard deviation (RSD%). The accuracy is the degree of closeness of the determined value to the nominal or known true value under prescribed conditions [9]. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated by using the formula RE% = $(E - T)/T \times 100$.

The extraction recovery was determined by comparing the peak areas of each compound after extraction with those obtained by direct injection of the same amount of analyte in the supernatant of the processed blank plasma. The matrix effect (ME) was examined by comparing the peak areas of the analyte resolved in the blank plasma sample's reconstituted solution and to those obtained for the standards in mobile phase at equivalent concentrations. Replicate analysis (n = 5) of QC samples at three concentration levels was used for determining extraction recovery and matrix effect of the assay.

The short-term stability of sertaconazole was assessed by determining QC samples kept at room temperature for 6 h, which exceeded the routine preparation time of samples. The long-term stability was evaluated by determining QC samples kept at low temperature (−70 °C) for 15 days. The post-preparative stability was measured by determining QC samples kept under the auto-sampler conditions (4 °C) for 24 h. The freeze and thaw stability was tested by analyzing QC samples undergoing two freeze (−70 °C) and thaw (room temperature) cycles on consecutive days. The stock solution stabilities of sertaconazole and the IS were evaluated by analyzing their working solutions kept at 4 °C, respectively.

2.6. Pharmacokinetic study

The proposed analytical method was applied to a pharmacokinetic study. This trial was carried out in accordance with the Declaration of Helsinki, as well as other local guidelines defining the protection of human beings and approved by the Human Ethics Committee of the hospital. A single dose of 300 mg vaginal suppository was given to 10 healthy female volunteers. Blood samples were collected in tubes containing heparin before and after 1, 2.5, 4, 5.5, 7, 9, 12, 16, 24, 36, 48 and 72 h of administration of drug. These were centrifuged at 3500 × g for 10 min to obtain plasma, and the supernatant plasma was put into microtube and then frozen at −70 °C.

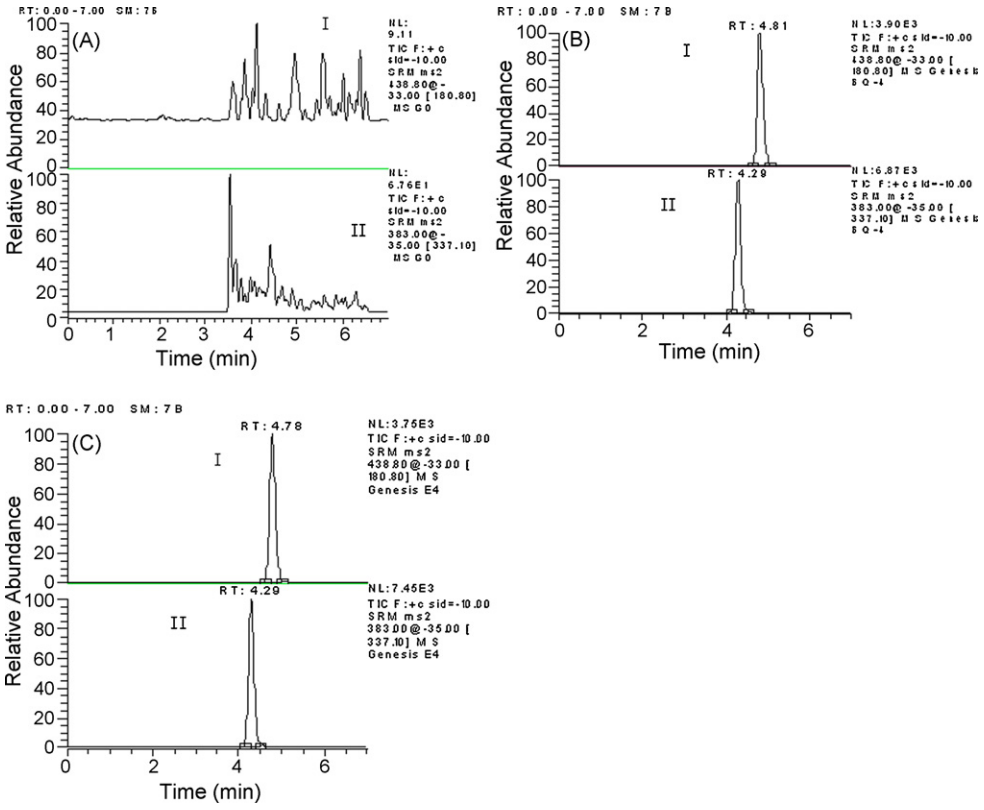

Fig. 2. Multiple reaction monitoring (MRM) chromatograms of (A) double blank plasma. (B) Plasma spiked with sertaconazole (I, final plasma concentration: 1.0 ng/mL) and 20 μL of IS (II, 100 ng/mL), and (C) a volunteer plasma sample (5.5 h after the administration of a 300 mg vaginal suppository of sertaconazole nitrate).

3. Results and discussion

3.1. LC–MS/MS optimization

MS/MS optimization was performed by directly infusing solutions of sertaconazole nitrate and loratadine (IS) into the electrospray injection (ESI) unit of a mass spectrometer at a constant flow rate of 10 μL/min. Quadrupole full scans (Q1 scans) were carried out in positive ion detection mode in order to optimize ESI conditions. The mass spectra of sertaconazole and loratadine revealed base peaks at m/z 439 $[M+H]^+$ and 383 $[M+H]^+$, respectively. The single 37Cl isotope is the most abundant in the cluster for sertaconazole, so m/z 439 was selected as parent ions. MRM detection was adopted for assay, which condition parameters including collision gas, collision energy were set to maximize the amounts of product ions. The product ions of sertaconazole and loratadine were at m/z 181 and m/z 337, respectively. The instrumental parameters used are mentioned in Section 2.2.

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve proper retention time, sensitivity and symmetric peak shapes for sertaconazole and IS. Different percentages of methanol–0.2% formic acid were attempted. Finally, methanol–0.2% formic acid (70:30, v/v) was adopted as the mobile phase, due to the proper retention time, higher sensitivity, symmetric peak shapes and stable MS signal.

3.2. Sample preparation and the selection of IS

Liquid–liquid extraction was necessary and important because this technique cannot only purify but also concentrate the sample. Several solvent combinations were tested for liquid–liquid extraction of the analyte and ether–dichloromethane (80/20, v/v) mixture was selected for its high recovery and time-saving during drying.

It is necessary to use an IS to get high accuracy when HPLC is equipped with a tandem mass spectrometer. Loratadine was adopted in the end because of the similarity of its retention time, ionization and extraction efficiency with the analyte. The structure of loratadine is shown in Fig. 1.

3.3. Method validation

The specificity of the method was evaluated by analyzing individual blank plasma samples from six different sources. All samples were found to have no interferences from endogenous substances at the retention time of either the analyte or the IS. The retention times of sertaconazole and IS were about 4.8 and 4.3 min, respectively, Typical chromatograms of a blank plasma, a spiked plasma sample with sertaconazole and IS, and a plasma sample from a subject are shown in Fig. 2.

The method exhibited excellent linear response over the selected concentration range of 0.1–10 ng/mL by weighted least-squares linear regression analysis. The representative standard curve was described by the equation: $Y = 0.6235X - 0.0212$, $r = 0.9998$. The LLOQ for sertaconazole was proved to be 0.1 ng/mL. The mean inter-assay accuracy and precision at 0.1 ng/mL were −2.8% relative error (RE) and 10.4% R.S.D., respectively ($n = 5$). The mean inter-assay accuracy and precision for the rest of the calibration standards were between −4.1% and 7.4% RE and between 3.2% and 6.8% R.S.D., respectively. The assay range was confirmed as appropriate for the measurement of sertaconazole in human plasma samples.

Data for intra- and inter-batch precision and accuracy of the method for sertaconazole as determined from the QC samples runs at the concentrations of 0.25, 1.0 and 5.0 ng/mL are presented in

Table 1
The precision and accuracy of the method for determining sertaconazole in human plasma.

Concentration added (ng/mL)	Intra-batch ($n=5$)			Inter-batch (3 days, five replicates per day)		
	Concentration found (mean ± S.D., ng/mL)	Accuracy (%)	Precision (%)	Concentration found (mean ± S.D., ng/mL)	Accuracy (%)	Precision (%)
0.25	0.257 ± 0.01	2.8	5.3	0.249 ± 0.01	−0.4	4.4
1.0	1.09 ± 0.04	9.0	3.8	1.05 ± 0.07	5.0	6.9
5.0	5.35 ± 0.34	7.0	6.4	5.12 ± 0.39	2.4	7.7

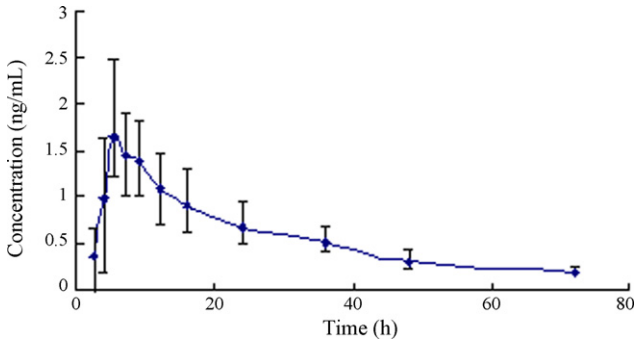

Fig. 3. Mean (±SD) plasma concentration–time profile of sertaconazole after the vaginal administration of a 300 mg sertaconazole nitrate vaginal suppository to each of 10 volunteers.

Table 1. Intra-batch R.S.D. was below 6.4% and inter-batch R.S.D. was below 7.7%. The accuracy was within −0.4 to 9.0% RE. The results revealed good precision and accuracy.

The mean extraction recoveries were measured at three different concentration levels for sertaconazole (0.25, 1.0 and 5.0 ng/mL) by comparing the peak areas of sertaconazole prepared in plasma with those obtained from direct injection of standards dissolved in the supernatant of the processed blank plasma. The mean extraction recoveries at each QC level (0.25, 1.0 and 5.0 ng/mL) were 66.4 ± 7.6%, 60.3 ± 3.3% and 70.3 ± 6.1%, respectively, and the extraction recovery of IS was 74.4 ± 10.8%.

Co-elution compounds of endogenous may affect the ionization efficiency. Therefore, matrix effects were also evaluated by comparing the peak areas of sertaconazole from the spike-after-extraction samples (the blank plasma samples were from six different sources) to those obtained for the standards in mobile phase at equivalent concentrations. The ratios were 106.3 ± 5.8%, 98.1 ± 3.1% and 90.5 ± 1.7% at the concentration of 0.25, 1.0 and 5.0 ng/mL. The same assay was preformed for the IS and the ratio was 105.6 ± 3.1%. These observations indicated that the matrix effect should not have a significant impact on assay performance in our study.

Stability tests were performed by analyzing five replicates at low, medium and high QC concentrations. The measured concentrations for sertaconazole at each QC concentrations deviated within 13.8% of nominal concentrations. The results demonstrated that sertaconazole was stable in human plasma after two freeze/thaw cycles, at room temperature for 6 h, at 4 °C in the autosampler for 24 h and in a freezer set to −70 °C for 15 days. Stock solutions of sertaconazole nitrate and IS were found to be stable for 4 weeks at 4 °C.

3.4. Pharmacokinetic study

The validated method was successfully used to quantify sertaconazole nitrate concentration in a pharmacokinetic study. The mean plasma concentration–time profiles are shown in Fig. 3. The pharmacokinetic parameters for sertaconazole nitrate are as follows: C_{max} 1.79 ± 0.70 ng/mL; T_{max} 7.0 ± 1.5 h; $t_{1/2}$ 20.25 ± 9.09 h; AUC_{0-t} 37.11 ± 13.73 ng h ml^{-1}; $AUC_{0-\infty}$ 43.31 ± 15.05 ng h ml^{-1}.

Previous studies [4–8] reported that there was no systemic absorption for sertaconzole nitrate when applied to the skin in human. Another, it was mentioned that no sertaconzole was detected in human plasma after the application of vaginal dosage forms using the reported method with a LOQ of 20 ng/mL [4,6,10]. However, our current study revealed that sertaconazole concentrations in human plasma were determinable after the application of 300 mg vaginal suppository when the limit of quantification of the assay was downed to 0.1 ng/mL, which also indicated the existing of systemic absorption for sertaconazole.

4. Conclusions

In this study, a LC–MS/MS method was developed and validated to quantify sertaconazole in human plasma using a simple liquid–liquid extraction procedure. The proposed LC–MS/MS method provided greater sensitivity than previously published method. The devised method was satisfactory in terms of its selectivity, sensitivity, reproducibility and matrix effects. The method was successfully applied to a pharmacokinetic study in 10 healthy volunteers administered 300 mg sertaconazole nitrate vaginal suppository, which revealed the existing of systemic absorption of sertaconazole nitrate when administration vaginally.

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