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An liquid chromatography–tandem mass spectrometry assay for determination of trace amount of new antifungal drug iodiconazole in human plasma

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ABSTRACT

A simple, rapid and sensitive LC–MS/MS method for determination of trace amount of new antifungal drug iodiconazole in human plasma was developed (1-(1H-1,2,4-triazole)-2-(2,4-diflurophenyl)-3-[*N*-methyl-*N*-(3-chlor-benzyl)amino]-2-propanol), was used as internal standard (IS). The analytes were extracted by liquid–liquid extraction with *n*-hexane after internal standard spiked. The separation was performed by a ZORBAX SB-C_{18} column (3.5 μm, 2.1 mm × 100 mm) with an isocratic mobile phase consisting of methanol–water–formic acid (50:50:0.05, v/v/v) at a flow rate of 0.3 mL/min. The lower limit of quantification (LLOQ) was 0.10 ng/mL. This method was successfully used to determine the concentration of iodiconazole in human plasma following dermal administration.

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1. Introduction

Currently, the incidence of serious fungal infection is becoming very prominent in patients undergoing chemotherapy for cancer, organ transplants and patients with AIDS [1]. Triazoles, as an important class of antifungal agents, inhibit the biosynthesis of ergosterol in fungi which is the main constituent of fungal cell membrane. Triazole antifungal agents such as fluconazole, itraconazole, and voriconazole have improved tolerability profiles relative to amphotericin B, but their extensive use has led to the emergence of resistance among susceptible strains. To meet the challenge of the rising incidence of invasive fungal infections caused by opportunistic filamentous molds and the emergence of fungal resistance, there is an increasing need for new antifungal agents [2]. Aiming to obtain new compounds with more potent activity, less toxicity and broader spectrum, some compounds were synthesized on the basis of computer-aided drug designing, according to the crystal structure of cytochrome P450 14α-steroldemethylase (CYP51) and the docking results of inhibitors to the active site of the enzyme [3]. One of these compounds is iodiconazole [4] 1-(1H-1,2,4-triazole)-2-(2,4-diflurophenyl)-3-[*N*-methyl-*N*-(4-iodo-benzyl)amino]-2-propanol), whose structure is shown in Fig. 1.

In order to avoid the irritation of gastrointestinal tract, minimize systemic toxicity and achieve a better therapeutic effect, iodiconazole was developed as a topical transdermal formulation. Topical application concentrates a drug in the tissues to which it is applied and reduces the concentration of the drug in blood and other tissues. It can also minimize the side-effects of gastrointestinal damage and the first-pass effect observed after oral administration. Besides the pharmacodynamic evaluation, it is also important to determine the pharmacokinetic properties of iodiconazole. Some published papers were concerned with the determination of similar azole drugs such as itraconazole, involving liquid chromatography (LC) with ultraviolet (UV) detection [5] or fluorescence detection [6] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [7]. The low plasma concentration and long resident time after topical application of the drug justify the necessity of developing a sensitive LC–MS/MS method which could present a more accurate description for the pharmacokinetic profile of iodiconazole after the application of a transdermal formulation. This method was simple, sensitive and can be rapidly assayed simultaneously.

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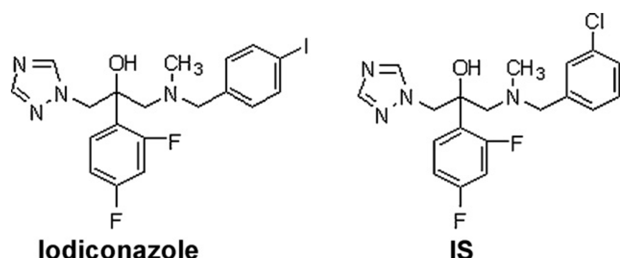

Fig. 1. Chemical structures of iodiconazole and the internal standard (IS).

2. Experimental

2.1. Chemicals and reagents

Iodiconazole and internal standard (IS) (purity of both ≥99.0%) were confirmed by IR, MS and NMR spectroscopy, and it was synthesized and refined at the laboratory in School of Pharmacy, School of Second Military University (Shanghai, China). The iodiconazole cream was provided by Anhui Jiren Pharmaceutical Co., Ltd. (Anhui, China). Methanol and *n*-hexane of HPLC (high-performance liquid chromatography) grade were purchased from Merck Company (Darmstadt, Germany). HPLC grade formic acid was purchased from Tedia Company Inc. (Tedia Fairfield, OH, USA). Ultrapure water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). All other reagents were of analytical grade. Human blank plasma was obtained from Shanghai Red Cross Blood Center (Shanghai, China).

2.2. Instrumentation

All experiments were carried out on an Agilent 1200 series high-performance liquid chromatography and interfaced to an Agilent 6410 triple-quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Corporation, MA, USA). All data were acquired and analyzed using Agilent 6410 Quantitative Analysis version B.01.02 analyst data processing software (Agilent Corporation, MA, USA).

2.3. Chromatographic conditions

The separation was performed by a ZORBAX SB-C_{18} column (3.5 μm, 2.1 mm × 100 mm i.d., Agilent Corporation, MA, USA) and a C_{18} guard column (5 μm, 4.0 mm × 2.0 mm, Agilent Corporation, MA, USA) with an isocratic mobile phase consisting of methanol–water–formic acid (50:50:0.05, v/v/v) at a flow rate of 0.3 mL/min. The column temperature was maintained at 35 °C. The injection volume was 10 μL and the analysis time was 3 min per sample.

2.4. Mass spectrometric condition

Ionization was achieved using electrospray in the positive mode with the spray voltage set at 4000 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set at 40 psi with a source temperature of 105 °C. Desolvation gas (nitrogen) was heated to 325 °C and delivered at a flow rate of 10 L/min. For collision-induced dissociation (CID), high purity nitrogen was used as collision gas at a pressure of about 0.1 MPa. Quantitation was performed using multiple reaction monitoring (MRM) mode of the transitions of m/z 485 → 217 for iodiconazole, m/z 393 → 125 for IS, respectively. The peak widths of precursor and product ions were maintained at 0.7 amu at half-height in the MRM mode.

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

A stock solution of iodiconazole was prepared by dissolving the accurately weighed iodiconazole in methanol to yield a final concentration of 1 mg/mL. The solution was sonicated for 5 min to ensure complete dissolution. Calibration standards of iodiconazole at concentrations of 0.10, 0.20, 0.50, 1.00, 2.00, 5.00, 10.0 and 20.0 ng/mL were prepared by spiking appropriate amounts of the standard solutions in blank plasma. A 100 μg/mL stock solution of IS was prepared by dissolving the drug in methanol. This solution was diluted with water to a final concentration of 100 ng/mL. All the solutions were stored at 4 °C and brought to room temperature before use.

Quality control samples for iodiconazole were made up in plasma by an independent analyst using a new stock solution, at the concentrations of 0.20, 2.00 and 10.0 ng/mL, representing low, medium and high concentration of QC samples, respectively. Samples were aliquoted into cryovials, and stored frozen at −20 °C for use with each analytical run. The results of the QC samples provided the basis of accepting or rejecting the run. A total of 12 QC samples (4 for each concentration) were analyzed during each run. At least four of the six QC samples had to be within ±20% of their respective nominal values. Two of the six QC samples could be outside the ±20% of their respective nominal value, but not at the same concentration.

2.6. Sample preparation

To a 400 μL aliquot of plasma sample, 50 μL of 2 mol/L sodium hydroxide solution and 20 μL of the IS working solution (100 ng/mL) were added. The mixture was then vortex-mixed for 30 s and extracted with 4.0 mL *n*-hexane by vortex-mixing for 3.0 min. After centrifugation at 2000 × *g* for 10 min, the upper organic layer was transferred to another tube and evaporated to dryness at 45 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL mobile phase followed by vortex-mixing for 1.0 min and centrifugation at 2000 × *g* for 10 min. Then, a 10 μL aliquot of supernatant was injected into the LC–MS/MS system.

2.7. Assay validation

Comparison of the chromatograms of the blank and the spiked human plasma was used to assay the selectivity of the method. In order to develop a reliable and reproducible method, the matrix effect was also investigated. The matrix effect was evaluated by the following experiment. Triplicates of QC samples at three levels of all the five analytes and IS were added into 0.4 mL mixed blank human plasma and water separately, and then the spiked samples were pretreated with exactly the same procedure as described in Section 2.6.

Calibration curves ranging from 0.10 to 20.0 ng/mL of each analytes were run on three separate days. Calibration curves were constructed from the peak area ratios of each analyte to IS versus plasma concentrations using a $1/x^2$ weighted linear least-squares regression model.

The intra-day and inter-day assays of the method were evaluated by quintuplicate analyses of three quality control samples. The calibration standards and quality controls were analyzed on five different days in order to determine intra-day and inter-day precision and accuracy. The accepted criteria for each quality control were that the R.S.D. value and accuracy should not exceed 15%. The lower limit of quantification (LLOQ) is defined as the lowest concentration of standard that can be measured with an acceptable accuracy and precision (≤20% for both parameters).

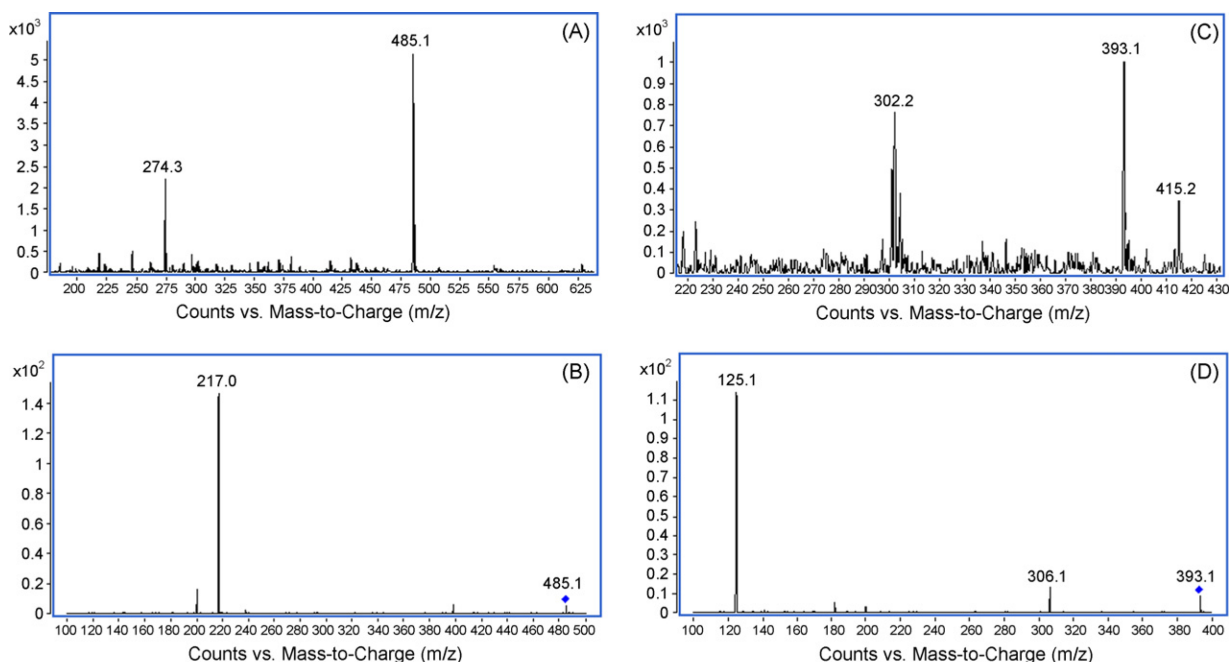

Fig. 2. ESI-MS spectrum of iodiconazole and IS. (A) Full-scan of iodiconazole, (B) product ion of iodiconazole, (C) full-scan of IS, and (D) product ion of IS.

The recoveries of iodiconazole from plasma were determined by spiked samples at three concentrations, 0.20, 2.00 and 10.0 ng/mL. The extraction recoveries were calculated by comparing mean peak areas of five low, medium and high spiked samples with mean peak areas of the five same amounts of unextracted iodiconazole solutions.

The stability of analytes in plasma was assessed by analyzing triplicate QC samples stored for 24 h at ambient temperatures, three cycles of freezing at −20 °C and thawing and stored for 1 month at −20 °C, respectively. The stability of analytes as well as IS in reconstituted extract at room temperature for 24 h was also assessed. Concentrations following storage were compared with freshly prepared samples of the same concentrations.

2.8. Application of the analytical method

Six healthy, male volunteers with a median age of 23.5 years (range 22–26) and a mean body weight of 59.5 kg (range 52–75 kg) participated in a controlled experiment. The experimental method was reviewed and approved by the Ethical Committee of Changzheng Hospital, and performed in Changzheng Hospital, Shanghai, China. The antifungal drugs were evenly applied to the forearm of the volunteers on areas (5 cm × 8 cm) each of 0.1 g/cm^2. Iodiconazole was continuous transdermal administered for 4 days in a single dose of 2%× 4 g, and blood samples were collected on 5th, 6th, and 7th day, respectively. Specifically, blood samples were drawn into disebrin tubes at different times (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 h) after transdermal administration on 7th day. Then plasma samples were immediately separated by centrifugation at 2000 × *g* for 10 min. All samples were stored at −20 °C until analysis.

3. Results and discussion

3.1. LC–MS/MS optimization

To determine iodiconazole using the MRM mode, full scan and product ion spectra of iodiconazole and IS were investigated under the present HPLC conditions. The possibility of using positive or negative ion detection was first evaluated. It was found that positive mode could offer higher sensitivities than negative mode. In the positive ESI mode, the analyte and IS formed predominately protonated molecular ions $[M+H]^+$ in full scan mass spectra. Fig. 2 displays product ion spectra of $[M+H]^+$ ions from two compounds. Two fragment ions were observed in the product ion spectra. The major fragment ions at m/z 485 → 217 and m/z 393 → 125 were chosen in the MRM acquisition for iodiconazole and IS, respectively.

Formic acid was added to the mobile phase for suppressing the production of $[M+Na]^+$ ions and enhancing the abundance of $[M+H]^+$ ions. After optimization, a proper concentration of formic acid was chosen. Further addition of formic acid would do little help for the production of precursor ions and would do more harm to the column.

3.2. Selection of internal standard

It is necessary to use an IS to get high accuracy when a mass spectrometer is equipped with HPLC as the detector. In this method

Table 1
Intra-day and inter-day precision and accuracy of iodiconazole plasma.

Concentration added (ng/mL)	Intra-day (n = 5)			Inter-day (n = 5)		
	Measured concentration (mean ± S.D., ng/mL)	Precision (R.S.D.%)	Accuracy (RE%[a])	Measured concentration (mean ± S.D., ng/mL)	Precision (R.S.D.%)	Accuracy (RE%[a])
0.20	0.20 ± 0.01	3.6	−2.0	0.21 ± 0.01	3.5	4.1
1.00	1.02 ± 0.05	5.2	1.9	1.05 ± 0.02	2.3	4.6
5.00	4.78 ± 0.24	4.9	−4.5	5.12 ± 0.55	10.7	2.3

[a] RE is expressed as [(mean measured concentration)/(spiked concentration) − 1] × 100%.

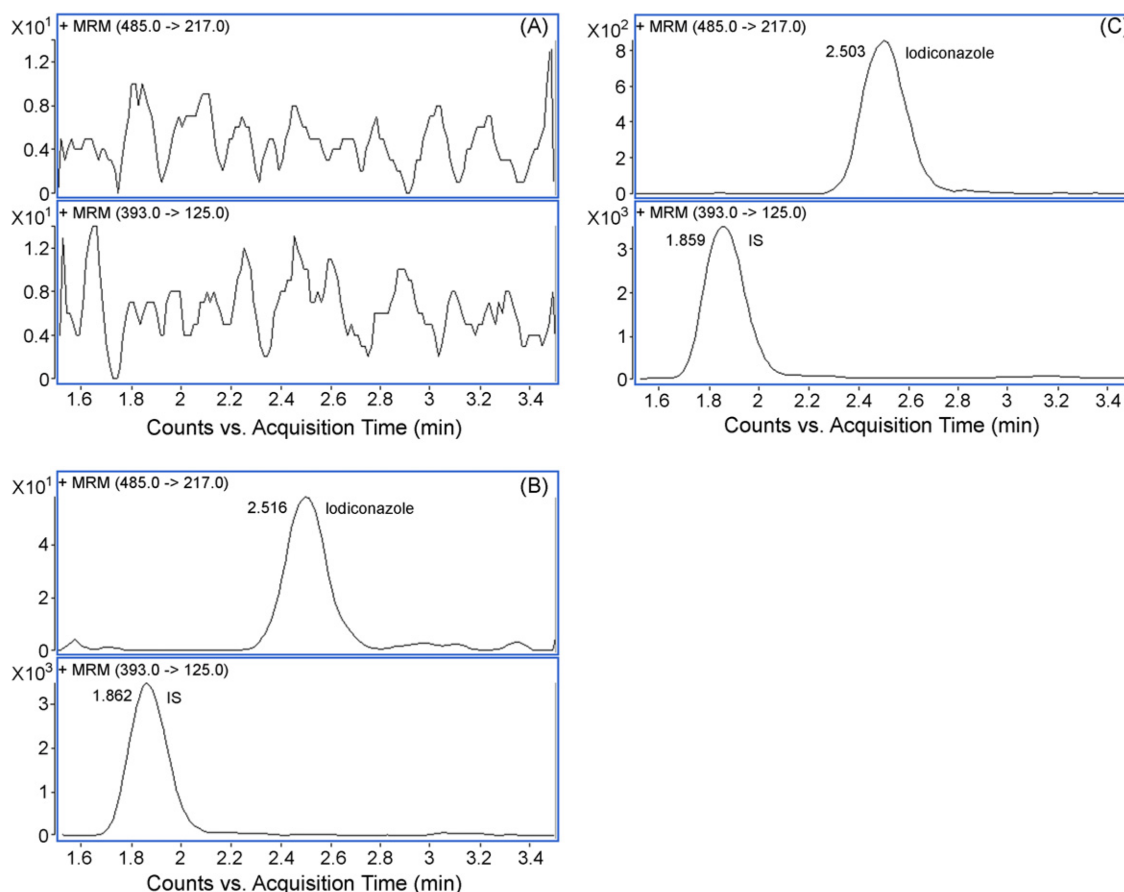

Fig. 3. Representative MRM chromatograms of iodiconazole plasma samples. (A) Blank human plasma, (B) blank plasma sample spiked with iodiconazole at the lower limit of quantification, and (C) test plasma sample.

the intermediate product was chosen as internal standard for its similar chemical structure, extraction recovery, ionization response in ESI mass spectrometry and chromatographic retention time. It was also stable during the period of pretreatment and assaying of the plasma samples.

3.3. Method validation

3.3.1. Assay selectivity and matrix effect

The LC–MS/MS method has high selectivity because only selected ions produced from selected precursor ions are monitored. Comparison of the chromatograms of the blank and the spiked human plasma (shown in Fig. 3) indicated no significant interference at the retention times of the analytes and the IS.

The results of matrix effect experiments showed that there was no significant difference between the peak areas of samples prepared from human plasma and from water which indicated that no co-eluting unseen compounds significantly influenced the ionization of analytes and IS.

3.3.2. Linearity of calibration curves and lower limit of quantification

The calibration curves were performed by the IS technique following linear regression analysis by plotting plasma concentrations of iodiconazole (0.10–20.0 ng/mL) against peak area ratio of iodiconazole to IS. The linear regression equation was $A = 0.2754C + 0.0161$ ($r = 0.9993$). The present LC–MS/MS method offered an LLOQ 0.10 ng/mL with an accuracy of −6.7% in terms of RE and a precision of 9.1% in terms of R.S.D. ($n = 6$).

3.3.3. Assay precision and accuracy

The method showed very good precision and accuracy. Intra-day and inter-day precision and accuracy for iodiconazole from plasma samples data are shown in Table 1. The intra-day and inter-day precisions were ranging 3.6–5.2% and 2.3–10.7% for plasma, respectively. These results suggested that the procedures described as above were satisfactory with respect to both accuracy and precision. All intra-day and inter-day precision and accuracy were acceptable. Analytical values of all QC samples were all within the 10% of their respective nominal values.

3.3.4. Extraction and absolute recovery

The extraction recoveries of the iodiconazole and IS under the liquid–liquid extraction conditions were summarized in Table 2.

Table 2
Extraction recoveries of iodiconazole and IS ($n = 5$).

Drug	Concentration added (ng/mL)	Recovery (mean ± S.D., %)	R.S.D.%
Iodiconazole	0.20	79.6 ± 4.2	5.2
	2.00	76.3 ± 3.9	5.2
	10.0	79.4 ± 2.8	3.5
IS	0.20	76.7 ± 5.2	7.4
	2.00	78.3 ± 2.9	4.1
	10.0	77.3 ± 3.2	4.7

Table 3
Absolute recoveries of iodiconazole plasma ($n = 5$).

Concentration added (ng/mL)	Recovery (mean ± S.D., %)	R.S.D.%
0.20	97.6 ± 3.5	3.6
1.00	101.6 ± 5.3	5.2
5.00	95.1 ± 4.7	5.0

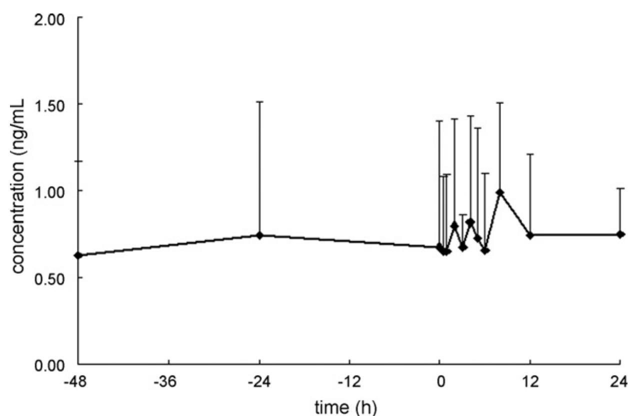

Fig. 4. Mean plasma concentration–time profile of iodiconazole in healthy volunteer after transdermal administration.

The absolute recoveries of iodiconazole at the three concentration levels for 5 days in plasma ranged from 95.1 to 101.6% (shown in Table 3).

3.3.5. Analyte stability

The stability of iodiconazole in human plasma and mobile phase was investigated. The analyte was found to be stable in human plasma stored for 1 month at −20 °C and in reconstituted mobile phase at room temperature for 24 h (<5% reduction). After a storage at −4 °C for 2 months, no obvious reduction was found in the stock and working solutions. The analyte was stable after three freeze–thaw cycles with a reduction of less than 15%. The analytes were also stable in human plasma at room temperature for at least 24 h with a reduction of less than 15%.

3.4. Application of the analytical method to concentration determination

The LC–MS/MS method developed was used for determination of trace amount of iodiconazole after transdermal administration. Six healthy volunteers' iodiconazole plasma concentration below the minimum detection limits most of the time showed that little iodiconazole was absorbed into the blood. Fig. 4 shows the mean plasma concentration–time curve of the iodiconazole after transdermal administration cannot be used by non-compartment model estimates pharmacokinetic parameters.

4. Conclusion

The present method for the determination of iodiconazole in human plasma has been proved to be rapid, sensitive, and selective, and it requires relatively small volumes of samples. It was suitable for the trace amount concentration study of iodiconazole in human plasma. To date, no application of LC–MS/MS for analysis of iodiconazole in human plasma has been reported. Sample pretreatment procedures for plasma were characterized by easy-to-use methods and speed. They provided a sufficient clean up of the biological samples prior to LC–MS/MS analysis and showed no significant loss of the analytes during sample handling. In conclusion, this paper describes a sensitive and accurate LC–MS/MS method for the quantification of iodiconazole suitable to monitor plasma concentrations.

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