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Liquid chromatography-mass spectrometry method for the quantification of posaconazole in human plasma: Application to pharmacokinetics following single-dose administration in the fasted state and with a high-fat meal

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A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method was developed to determine concentrations of posaconazole in human plasma precipitated by acetonitrile including internal standard. Rapid chromatographic separation was achieved in the mobile phase composition of acetonitrile, water and formic acid (v/v/v, 55:45:0.1) with a flow rate of 0.25 ml/min. Posaconazole-d4 was used as internal standard. Detection was undertaken with cation electrospray tandem mass spectrometry on a Sciex/API3000. The method was accurate, specific and sensitive for the analysis of posaconazole in human plasma in the concentration range of 2–1000 ng/ml. The inter- and intra-batch accuracy was within ± 10% and the lower limit of quantification was 2 ng/ml. The method facilitated a clinical pharmacokinetic study after oral administration of a single-dose of posaconazole suspension in the fasted state and with a high-fat meal in a two-period crossover design. C_{max} (maximum concentration) and AUC (area under serum drug concentration) were significantly increased, and T_{max} (time to maximum plasma concentration) was delayed under fed condition, which suggested that simultaneous administration of posaconazole with food may help to achieve higher plasma concentrations and result in better antifungal efficacy.

1. Introduction

Posaconazole (Fig. 1) is a novel triazole that is active against a wide variety of fungi, including fluconazole-resistant *Candida albicans* isolates. Its mechanism of action involves selective inhibition of the enzyme lanosterol 14-demethylase (CYP51), which catalyses an essential step in ergosterol biosynthesis in yeasts and moulds (Munayyer 2004). The *in vivo* activity of the drug, evaluated in several animal models, shows posaconazole was substantially more activity than amphotericin B in the treatment of aspergillosis (Graybill et al. 1998). It has also been shown to have significantly greater activity than fluconazole and itraconazole in a Coccidioides model (Lutz et al. 1997), and was of similar efficacy to amphotericin B in a Zygomycetes model (Sun et al. 2002).

Several assay methods have been developed for determining posaconazole in plasma. In each of these methods the limit of quantification was not lower than 5 ng/ml. The methods included high-performance liquid chromatography with ultraviolet detection (HPLC-UV) (Kim et al. 2003; Chhun et al. 2007; Gordien et al. 2009; Kim et al. 2002), or fluorescence detection (Neubauer 2009) and HPLC with mass spectrum (LC-MS/MS) (Shen et al. 2007; Cunliffe et al. 2009; Rochat et al. 2010). Compared with HPLC-UV and HPLC-fluorescence, LC-MS/MS displayed good sensitivity and high specificity. Based on these findings, a validated LC-MS/MS method was developed by Cunliffe et al. (2009), which adopted a mixed-mode cation exchange solid phase extraction from human plasma and a TurboIonSpray ionization source with the positive ion mode, and a calibration curve ranging from 5 to 5000 ng/ml. Ultra-performance LC-MS/MS using protein precipitation with a calibration curve ranging from 14 to 12000 ng/ml has been reported by Rochat et al. (2010). However, the quantitation limit 5 ng/ml was not sufficient to evaluate the pharmacokinetics of posaconazole after administration 200 mg posaconazole suspension to Chinese subjects in the fasted state.

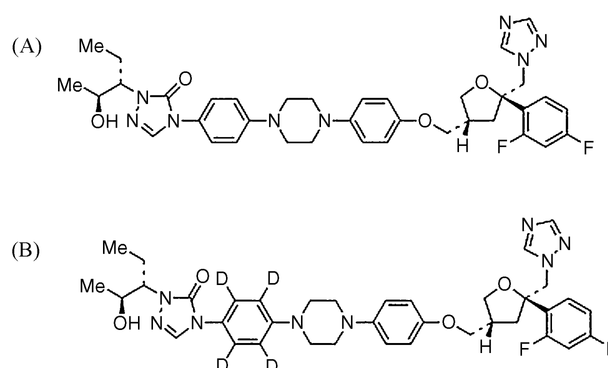

Fig. 1: Chemical structure of posaconazole (A) and the internal standard posaconazole-d4 (B)

In order to support a clinical pharmacokinetic investigation after oral administration of 200 mg posaconazole suspension in the fasted state and with a high-fat meal, a more sensitive LC-MS/MS method with a lower limit of quantitation of 2 ng/ml was established in this study to determine posaconazole in human plasma samples handed by 0.6 ml of acetonitrile precipitation solution.

2. Investigations and results

2.1. LC/MS/MS optimization

The mobile phase was optimized for sensitivity, speed and peak shape. Various proportions of acetonitrile in water were initially used but gave poor and unstable chromatography. The addition of formic acid to the mobile phase was subsequently found to improve peak symmetry and reduce the tailing factor without loss of sensitivity. Posaconazole-d4, an isotope of posaconazole, was used as an internal standard and showed retention and ionization identical to those of posaconazole.
In the process of sample pretreatment, Fully automatable solid phase extraction (Gordien et al. 2009; Cunliffe et al. 2009; Vogeser et al. 2009) and liquid-liquid exaction (Chhun et al. 2007) were often used for sample pretreatment, however, these processes were complicated, and not all laboratories have access to automatable solid phase equipment. We, therefore, adopted the protein precipitation method using acetonitrile. The result of the matrix effect (described below) did not compromise the performance of posaconazole and IS.
We also discovered that a high ionspray voltage (4900 V) and MRM improved single-to-noise ratios better than low ionspray voltages and selected ion monitoring. Protonated posaconazole and posaconazole-d4 were the major ions produced when an *m/z* range of 450–800 amu was scanned during Q1 scan, and these were selected as precursor ions. Under the optimized collision energy, posaconazole gave a major product ion at 683.3 amu and posaconazole-d4 gave a major product ion at 687.3 amu (Fig. 2).

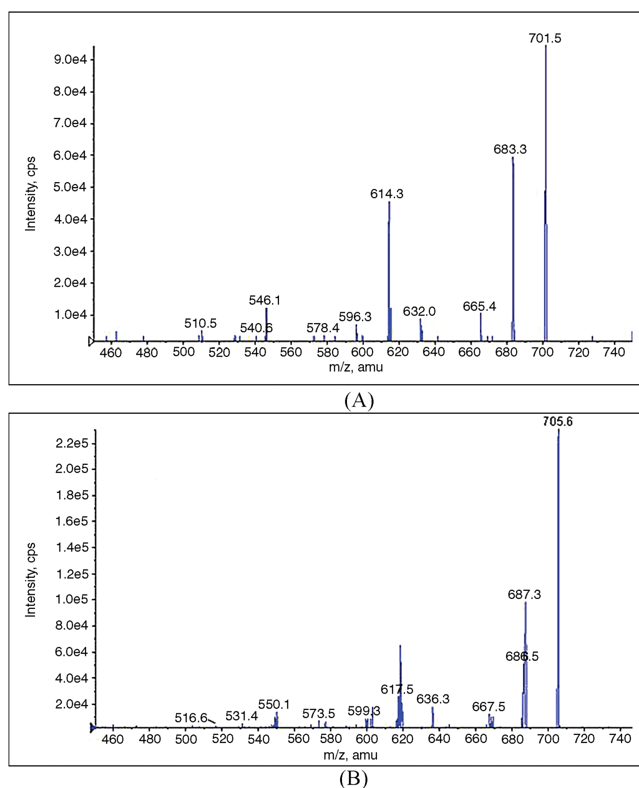

Fig. 2: Products ion spectra of protonated (A) posaconazole and (B) posaconazole-d4 (internal standard, IS)

2.2. Method validation

2.2.1. Calibration curve and lower limit of quantitation (LLOQ)

Calibration curves were constructed using duplicated of eight calibration standards with concentration in the range of 2–1000 ng/ml. Peak area ratios of posaconazole-to-IS were used for weighted (1/x) linear least-squares linear regression analysis. One of the regression equations and its correlation coefficient (r) were: $y = 0.0104x + 0.0108$ ($r = 0.9995$), where y was the peak area ratio and x was the concentration of posaconazole. The equation showed a good linear relationship between the peak area and the concentration. The LLOQ was defined as the lowest concentration in the calibration curve, based on visual evaluation, at which the analysis could be reliably detected with acceptable precision and accuracy. Posaconazole concentration (2 ng/ml) producing a signal that was 10-times higher than the noise peaks was regarded as the LLOQ.

2.2.2. Specificity and matrix effect

Typical MRM chromatograms of human plasma with and without standards are showed in Fig. 3. Retention times for posaconazole and posaconazole-d4 were 1.5 and 1.5 min, respectively. The overall chromatographic run time was within 3.0 min. No significant interfering peaks were present at the retention times of the analysis or the IS.

The matrix effects of the analysis and the IS were assessed by comparing the peak areas of the analysis and the IS in post-extraction blank samples from six different individuals with those in neat solution at the corresponding concentrations. The matrix effects at concentrations of 2, 100 and 1000 ng/ml for the analysis were 95.93 ± 8.47 %, 118.13 ± 8.45 % and 122.13 ± 13.44 %, respectively. The matrix effect for the IS at 100 ng/ml was 114.83 ± 5.76 %. It was concluded that the ionization effect slightly increased the performance of posaconazole and IS.

2.2.3. Accuracy, precision and recovery

Accuracy was defined as (detected concentration - nominal concentration)/ (nominal concentration) × 100% (denoted as RE). Precision was determined by calculating the coefficient of variation (denoted as CV(%)). As shown in Table, the posaconazole

Table: Intra- and inter-batch accuracy and precision of posaconazole in human plasma

	LOQ 2 ng/ml	Middle 100 ng/ml	High 1000 ng/ml
Intra-batch accuracy and precision			
Mean	2.18	107.08	1024.58
SD	0.16	3.54	21.48
CV(%)	7.42	3.31	2.10
RE	9.17	7.08	2.46
n	6	6	6
Inter-batch accuracy and precision			
Mean	2.09	108.91	1019.87
SD	0.23	2.48	22.15
CV(%)	11.01	2.28	2.17
RE	4.46	8.91	1.99
n	18	18	18

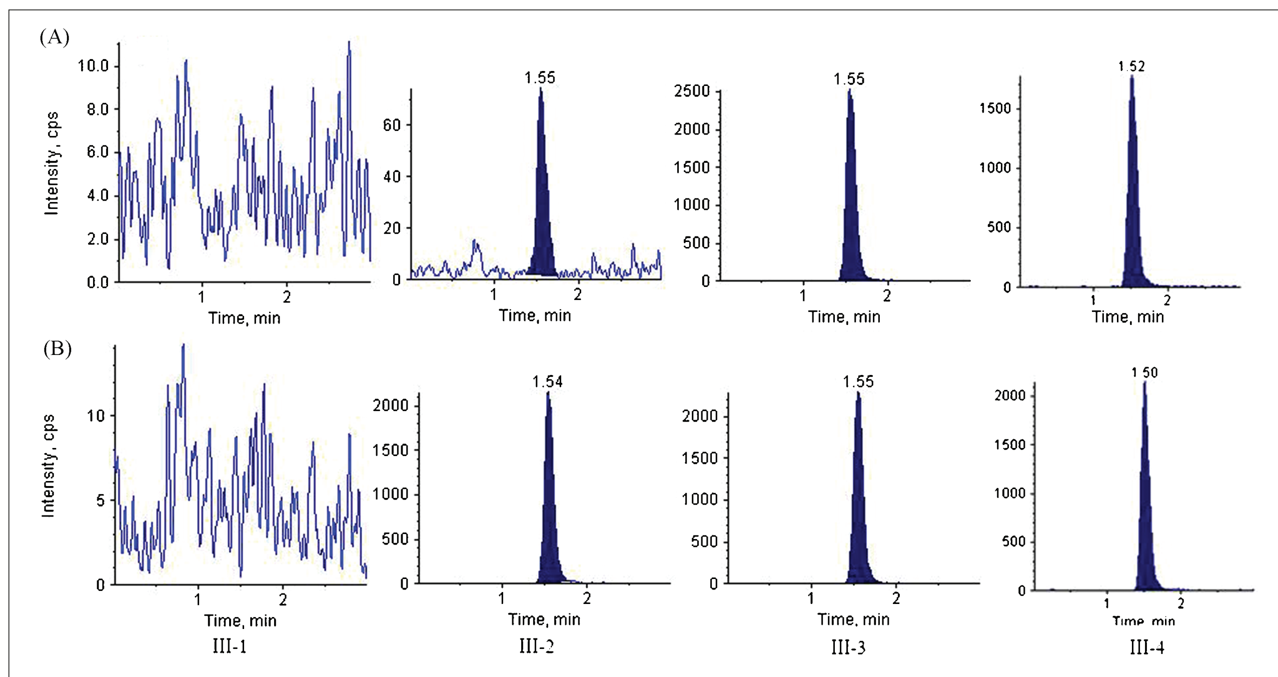

Fig. 3: Chromatography of (A) posaconazole and (B) posaconazole-d4 (internal standard, IS) in human plasma. III-1: blank plasma, III-2: 2 ng/ml posaconazole added into blank plasma (LLOQ), III-3: 100 ng/ml posaconazole and IS added into blank plasma, and III-4: a human plasma collected 2.0 h after an oral single dose of posaconazole suspension 200 mg with high-fat food

assay in plasma was accurate and precise for each QC level. All QC levels for posaconazole had intra- and inter-batch RE within ± 10%. The largest CV(%) for the LOQ, which appeared in the inter-batch precision of posaconazole, was 11.01%.

The extraction recoveries of the analysis and the IS were evaluated by comparing the peak areas of the analysis and the IS in pre- and post-exaction plasma samples from six different individuals. The exaction recoveries at concentrations of 2, 100, 2000 ng/ml of analysis were 84.54 ± 4.41 %, 88.73 ± 5.86 % and 88.41 ± 3.72 %, respectively. The corresponding result for 100 ng/ml of the IS was 90.86 ± 5.54 %.

2.2.4. *Stability*

The stability of posaconazole in human plasma was investigated by analyzing duplicates of three QC levels. The test conditions included three freeze-thaw cycles, at room temperature for 4 h, at 15 °C for 48 h in the autosampler and at -20 °C for 41 days. Posaconazole was found to be stable under these conditions. Changes in the concentrations of posaconazole at any QC level were in the range of -14.92% to 14.87%.

2.3. Clinical application

The current assay facilitated assessment of the concentration-time profiles of posaconazole in human plasma after oral administration of 200 mg posaconazole suspension in the fasted state and with a high-fat meal (Fig. 4). The C_{max} of posaconazole in 12 subjects ranged from 62.46 to 528.39 ng/ml. The mean C_{max} in the fasted state was 128.02 ± 92.21 ng/mL compared with a C_{max} of 354.29 ± 82.13 ng/mL when administered with a high fat meal ($P < 0.05$). The corresponding values for T_{max} were 3.88 ± 1.11 and 7.38 ± 2.87 h, respectively ($P < 0.05$). The mean $AUC_{0\text{-}t}$ was 3075.27 ± 1917.00 ng.h/mL in the fasted state and 12229.08 ± 2923.37 ng h/ml when administered with a high fat meal ($P < 0.05$). The corresponding values for $AUC_{0\text{-}\infty}$ were 3196.18 ± 1933.04 and 12612.35 ± 3191.94 ng·h/ml, respectively. The half life ($t_{1/2}$) of posaconazole was 22.11 ± 3.02 h when administered in the fasted sate and 21.39 ± 5.34 h when administered with a high-fat meal ($P > 0.05$). The relative bioavailability of the drug administered with a high-fat meal was 509.58 ± 257.92%.

The pharmacokinetic results in 12 healthy subjects showed that a high-fat meal significantly increased the extent of absorption of posaconazole and increased its bioavailability by about five-fold. The presence of a high-fat meal delayed the rate of absorption of posaconazole but did not affect its terminal elimination. These findings suggest that administration of posaconazole simultaneously with food may help to achieve higher plasma concentrations and result in better antifungal efficacy. These findings are consistent with previous studies which have also demonstrated that food substantially enhances the extent of posaconazole absorption in healthy subjects (Courtney et al. 2004; Krishna et al. 2009).

3. Experimental

3.1. Materials and reagents

Posaconazole (99.4% pure, Lot: 12467) was obtained from Schering-Plough Ltd. (Shanghai, China). Posaconazole-d4 (internal standard, 98.0% pure, Lot: 3-GJF-28-2) (Fig. 1) was purchased from Toronto Research Chemical

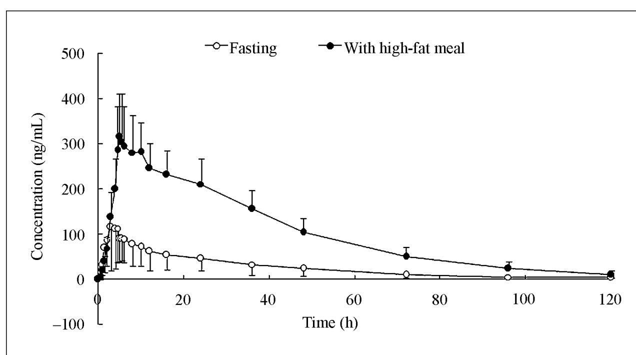

Fig. 4: Arithmetric mean (SD) plasma concentration-time profiles for posaconazole after administration of 200 mg posaconazole suspension in the fasted state and with a high-fat meal

Inc. (USA). Methanol and acetonitrile with HPLC grade were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Formic acid was reagent grade and deionized/distilled (DI) water was prepared from tap water in our own department.

3.2. LC/MS/MS analysis

The liquid chromatography separation system comprised LC-10AD VP (pump), DGU-14AM (degasser) and SIL-HTc (autosampler) (Shimadzu, Kyoto, Japan). The separation column was a CAPCELL PAK C18 column (50 mm × 2.0 mm, 5 μm) (Shiseido, Tokyo, Japan). An XW-80 vortex was purchased from Shanghai Medical University Apparatus (Shanghai, China). The Biofuge 28 RS centrifuge was purchased from Heraeus Sepatech (Osterode, Germany). The isocratic mobile phase consisted of 55% acetonitrile, 45% water and 0.1% formic acid. The flow rate was set to 0.25 ml/min. The injection volume was 10 μl and the run time was 3.0 min. Retention times for posaconazole and posaconazole-d4 (IS) were 1.50 and 1.49 min, respectively.
A Sciex API 3000 LC-MS/MS system (Foster City, CA, USA) was operated under Analyst 1.4 software. The electrospray ion source was run in a positive-ionization mode. The typical ion source parameters were: declustering potential, 80 V; collision energy, 46 eV for posaconzole and 24 eV for posaconazole-d4; focusing potential, 300 V; collision cell exit potential, 8 V; ionspray voltage, 4900 V; ion temperature, 350 °C. Nebulizer gas, curtain gas and collision gas using nitrogen were set to 75, 50, 90 psi, respectively. Samples were analyzed via multiple-reaction monitoring (MRM) with monitoring ion pairs at *m/z* 701.5→683.3 for posaconazole and *m/z* 705.5→687.3 for internal standard. The scan dwell time was set at 0.25 s for every channel. Sciex Analyst version 1.4 software was used for data collection and peak integration.

3.3. Posaconazole standard, quality control (QC) and internal standard preparation

Primary stock solutions of posaconazole (100 μg/ml) and posaconazole-d4 (98 μg/ml) were prepared in methanol. Working standard solutions of posaconazole and posaconazole-d4 for internal standard (1 μg/ml) were prepared by diluting aliquots of corresponding primary stock solution with methanol. All solutions were stored at 4 °C in darkness when not in use. Calibration standards of posaconazole (2, 5, 10, 50, 100, 200, 500, 1000 ng/ml) were prepared by spiking the working standard solutions into a pool of blank human plasma.
QC stock solution of posaconazole (100 μg/ml) was prepared from a separate weighing and was also dissolved in methanol. The QC sample were prepared at high (1000 ng/ml), medium (100 ng/ml) and low (2 ng/ml) concentrations in the same way as the calibration standards. QCs were stored at -20 °C.

3.4. Plasma sample preparation

A 200 μl aliquot of the appropriate test sample was pipette into 1.5 ml polypropylene tube, then 0.6 ml of the precipitation solution of acetonitrile including internal standard posaconazole-d4 (60 ng/ml) and 20 μl methanol were added. The tubes were capped, vortexed and supersonicated for 30 s reprectively. The samples were then centrifuged in a Heraeus Biofuge 28RS at 26000 × g and 4 °C for 15 min. The supernatant layers were filtered through 0.45 μm diaphragm and an aliquot of 10 μl was directly injected into the high-performance liquid chromatography system equipped with an MS/MS detector.

3.5. Pharmacokinetic study protocol and parameters

The pharmacokinetic investigation was an open-label, randomized and 2 × 2 crossover study with a washout of 14 days. The protocol was approved by the Ethnic Committee of Zhongshan Hospital and conformed to the principles of the Declaration of Helsinki. Subjects signed informed consent before any screening item was performed.
Each of the 12 subjects enrolled in the study received one dose of 5 ml posaconazole suspension (200 mg) administrated in the fasted state and with a high-fat meal. The drug was administrated in different days with at least 13 days washout period between two doses. The subjects were served a light snack more than 10 h prior to each treatment administration on day -1. In the fasted state, food was withheld for approximately 4 h following treatment administration. For the fed state a standardized high-fat breakfast was served at 0.5 h before administration. This included two pork dumplings (50 g flour, 30 g meat), 20 g fried peanuts, one boiled egg and 10 g pickle. The calorie was 820 kJ, which comprised 41.12 g fat, 24.31 g protein, 88.28 g carbohydrate, 2.05 g dietary fiber 20 g salad oil.
Blood samples were collected into heparinized vacutainers at pre-dose and serially at 0.5, 1, 1.5, 2, 3, 4, 4.5, 5, 5.5, 6, 8, 10, 12, 16, 24, 36, 48, 72, 96 and 120 h post-dose, and immediately centrifuged at 1500 × g and 4 °C for 10 min. The plasma was pipette into screw-cap labeled polypropylene tubes and stored at the study center at -20 °C until assayed.
Pharmacokinetic parameters were estimated by non-compartmental model using WinNonlin version 6.1 (Pharsight Corporation, Mountain View, California). C_{max} and T_{max} were measured values. AUC_{0-t} was calculated by trapezoidal rule and $AUC_{0-\infty}$ was estimated as $AUC_{0-t} + C_{tn}/\lambda_z$ (where t_n was sampling time of last detectable concentration; C_{tn} was plasma concentration of the last sampling; λ_z was terminal elimination constant). Mean retention time MRT was calculated as the area under the moment curve (AUMC) divided by AUC, total body clearance CL/f was calculated as dose divided by $AUC_{0-\infty}$ (where f was absolute bioavailability, dose was for medication administration dosage).

3.6. Statistical analysis

The pharmacokinetic parameters were analyzed using a crossover analysis of variance model (ANOVA, SPSS 16.0). The effects due to subject, period and treatment were extracted. Relative bioavailability was expressed as the ratio of the AUC estimates based on log-transformed data for the suspension in the fasted state compared to the suspension with a high-fat meal. Ninety percent confidence interval (90% CI) between treatment means of AUC and C_{max} were also computed. Two-one side t test was used to compare logarithmically transformed of AUC and C_{max} obtained after a high-fat meal or under fasting conditions. The non-parameter rank sum test is used for t_{max} with $P < 0.05$ as statistical significance to compare PK parameters of different administrated ways.

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