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Determination of voriconazole in human plasma by liquid chromatography–tandem mass spectrometry

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A rapid and sensitive method for the determination of voriconazole in human plasma was developed. Voriconazole and the internal standard were extracted from plasma samples by liquid–liquid extraction with 2 ml of diethyl ether: dichloromethane (60: 40, v/v). The chromatographic separation was accomplished isocratically on a 150×4.6 mm, 5 μm Zorbax extend C_{18} column at a flow rate of 0.7 mL/min. Detection by electrospray positive ionization mass spectrometry in the multiple-reaction monitoring mode was completed within 3.2 min. Linearity was over the concentration range 20-2000 ng/ml with a limit of detection of 5 ng/ml. Intra- and inter-day precision measured as relative standard deviation was <4.40% and <5.44%, respectively. The method was applied in a bioequivalence study of two tablet formulations of voriconazole.

Keywords: Voriconazole; LC-MS/MS; Human plasma

INTRODUCTION

Voriconazole (*2R, 3S*) –2– (2, 4-difluorophenyl) –3–(5-fluoro-4-pyrimidinyl)–1–(1*H*-1,2,4-triazol-1-yl)–2–butan–2–ol) is a novel triazole antifungal agent and shows a broader spectrum of activity against such common fungal pathogens as *Candida* and *Aspergillus*[1]. The pharmacokinetics of voriconazole in volunteers and patients has shown that voriconazole exhibits a nonlinear pharmacokinetic profile, secondary to saturable clearance [2, 3]. In addition, voriconazole is metabolized by the cytochrome P450 system, with less than 2% of the dose excreted unchanged [4-7].

Various analytical methods have been developed to determine voriconazole in plasma, such as high-performance liquid chromatography (HPLC) with ultraviolet [8-13] and mass spectrometric detection [14-17]. Limitations of some of these methods include lack of the requisite sensitivity and selectivity necessary for accurate assessment of the pharmacokinetics of the drug, large sample volumes and the extraction procedure of protein precipitation which is the dirtiest method existing. To overcome these problems, a new HPLC-UV method with a lower limit of quantification (LLOQ) of 200 ng/mL was published recently [13]. But the procedure of liquid–liquid extraction with 3 ml of hexane–methylene chloride (70:30, v/v) requested relative big amount of organic reagents and long run time (6 min), which were inappropriate in clinical studies with large numbers of samples.

This paper describes the development and validation of an improved method for the quantification of voriconazole in human plasma using LC-MS/MS. The assay is accurate and precise and requires only a small sample volume (0.10 ml), achieving a lower limit of quantification (LLOQ) of 20 ng/ml. The advantages of this method include the use of a small sample volume, liquid-liquid extraction (2 ml diethyl ether: dichloromethane, 60: 40, v/v) with high extraction efficiency and short chromatographic run times (3.2 min). This method was applied to a bioequivalence study of two oral tablet formulations of voriconazole in 20 healthy volunteers.

EXPERIMENTAL

Materials and reagents

Voriconazole (99.8%) and diazepam (99.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). Heparinized blank (drug-free) human plasma (different batches examined) was obtained from Changchun Blood Donor Service (Changchun, China). Acetonitrile and methanol were HPLC-grade. Distilled water, prepared from demineralized water, was used throughout the study.

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All other chemicals were of analytical grade and used without further purification.

Preparation of standard solutions

Stock solutions of voriconazole and diazepam (both 1 mg/ml) were separately prepared in 10 ml volumetric flasks with methanol. Voriconazole standard solutions with concentrations of 20, 50, 100, 200, 500, 1000 and 2000 ng/ml were prepared by dilution of aliquots of the stock solution with heparinized blank plasma. Low, medium and high concentration quality control (QC) solutions (50, 200, 1600 ng/ml) were prepared in a similar way. A working internal standard (I.S.) solution (diazepam, 250 ng/ml) was prepared in methanol: water (50: 50, v/v). All solutions were stored at 4°C.

Instrumentation and conditions

Chromatographic analysis was performed using an *Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA).* Separation of the analyte from potentially interfering material was achieved using a 150×4.6 mm, 5 μm Zorbax extend C_{18} column maintained at 40°C. The mobile phase used for the chromatographic separation was composed of acetonitrile–10 mM ammonium acetate (85:15, v/v) and delivered isocratically at a flow rate of 0.7 ml/min.

Mass spectrometric detection was performed on an Applied Biosystems Sciex Q-trap™ mass spectrometer (Concord, Ontario, Canada) equipped with an electrospray ionization (ESI) interface. The detector was operated at unit resolution in the multiple-reaction monitoring (MRM) mode using the transitions of the protonated molecular ions of voriconazole at *m/z* 350.3 → 127.0 and diazepam at *m/z* 285.2 → 193.1. The MS operating conditions were optimized as follows: curtain gas, gas 1 and gas 2 (nitrogen) 20, 40 and 60 units, respectively; dwell time 200 ms; source temperature 500°C; IonSpray voltage 5000 V. Declustering potential and collision energy were 30 V and 40 eV for voriconazole and 55 V and 33 eV for diazepam, respectively. Data acquisition and integration were controlled by Applied Biosystems Analyst version 1.3.2 Software.

Sample Preparation

An aliquot of plasma (100 μl) was placed in a 10 ml-glass tube followed by 100 μl I.S. solution, 100 μl 1M sodium carbonate solution and 2 ml diethyl ether: dichloromethane (60: 40, v/v). The mixture was vortex-mixed for 30 s and shaken for 10 min. After centrifugation at 3500 g for 5 min, the organic phase was transferred to another 10 ml-glass tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 150 μl mobile phase and a 10 μl aliquot of the mixture was injected into the LC-MS/MS system.

Assay validation

Three independent calibration curves and six replicates of QC samples (50, 200, 1600 ng/ml, respectively) were analyzed on three different days. Linearity was analyzed by weighed linear regression ($1/x^2$) of analyte-internal standard peak area ratios. Accuracy and precision were based on assay of six replicates of QC samples analyzed on three different days. The LLOQ was the concentration below which the inter-day coefficient of variation (CV) exceeded 20%. The limit of detection was determined as the concentration with signal-to-noise ratio of 3. The recovery rate was determined by comparing peak areas of QC samples with those of corresponding concentration QC solutions (prepared in methanol: water (50: 50, v/v)) dissolved in the supernatant of the processed blank plasma. The matrix effect of voriconazole was evaluated by comparing the peak areas of analyte in extracted blank plasma samples spiked with QC solutions (prepared in methanol : water (50 : 50, v/v)) with the peak areas of analyte in extracted water samples spiked with QC solutions (prepared in methanol : water (50 : 50, v/v)). The matrix effect of diazepam was investigated in a similar way except that I.S. solution was used instead of voriconazole solutions.

Stability tests including three freeze–thaw cycles, storage for one month at –20°C and at room temperature for 12 h were evaluated by QC samples.

Bioequivalence study

The method was applied to evaluate the bioequivalence of two tablet formulations of voriconazole in 20 healthy adult male volunteers who received a single dose (200 mg voriconazole) in a two-period randomized crossover design with a one-week washout period between doses.

Venous blood samples were collected into heparinized tubes at the following times: immediately before administration, 0.25, 0.50, 0.75, 1, 1.5，2.0，3.0，4.0，6.0，8.0，10，12 and 24 h after dosing. Plasma samples were obtained by centrifugation of the whole blood at 3000 g for 10 min and stored at –20 °C. Bioequivalence of the two formulations was assessed according to US-FDA methodology [18].

RESULTS AND DISCUSSION

Mass spectrometry

Tandem mass spectrometry with ESI source detection was used to provide a sensitive and selective assay for voriconazole and diazepam in human plasma. The structures and positive electrospray ionization mass spectra of voriconazole and diazepam are shown in Fig. 1. MRM was performed at unit resolution using the mass transition ion-pairs *m/z* 350.3 → 127.0 for voriconazole and *m/z* 285.2 → 193.1 for diazepam, respectively.

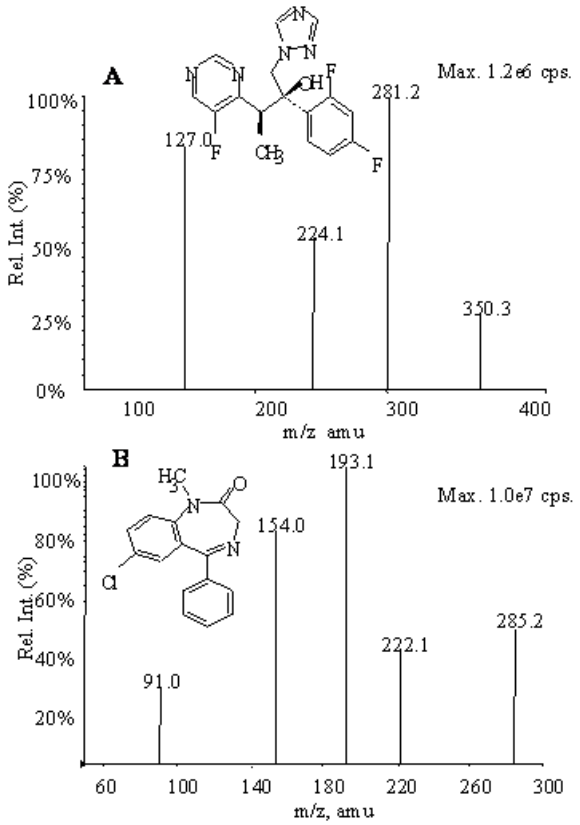

Fig. 1. Chemical structures and full-scan product ion mass spectra of $[M+H]^+$ ions of voriconazole (A) and diazepam (B) (internal standard).

Chromatography and specificity

The composition of the mobile phase was the critical factor for achieving good chromatographic peak shape and resolution. In the present study, acetonitrile–10 mM ammonium acetate (85:15, v/v) was selected as an isocratic mobile phase. The retention time of voriconazole and diazepam was <3 min. The selection of diazepam as the I.S. was based on its chromatographic and extraction behavior. Fig. 2 shows typical chromatograms. Voriconazole and diazepam were well separated from the biological background under the chromatographic conditions used with retention times of 2.27 and 2.74 min, respectively. The total analysis time for each run was 3.2 min, and no interference by the constituents from the blank human plasma samples at these retention times was registered.

Precision and accuracy

The calibration curves were linear in the plasma concentration range 20-2000 ng/ml ($r > 0.9991$) with a limit of detection of 5 ng/ml. Intra- and inter-day precision was 2.83-4.40% and 1.97-5.44% respectively and the relative error was below 3.76% (Table 1). All results were within the ranges of precision (%) and accuracy (%) specified by the FDA for bio-analytical applications.

Extraction recovery, matrix effect and stability

The recoveries of voriconazole at 50, 200 and 1600 ng/ml were 92.7%, 96.5% and 89.4%, respectively. The recovery of the internal standard was 95.9%. The matrix effect of the assay was evaluated at 50, 200 and 1600 ng/ml for voriconazole and three samples were analyzed at each level. The percent nominal concentrations determined were 92.7±4.2%, 94.2±4.8%, and 94.6±2.6% at each concentration level. The same evaluation was performed for the I.S. and the percent nominal concentration was 95.0±4.5%. The results indicate that ion suppression or enhancement from plasma matrix was negligible for this analytical method.

Table 2 summarizes the data from the short-term, freeze/thaw, and long-term stability tests for voriconazole. The results indicate that no stability-related problems are expected during the routine analyses for the study.

Table 1. Precision and accuracy for the determination of voriconazole in human plasma. (Data are based on the assay of 6 replicates on 3 different days)

Nominal conc. (ng/mL)	Mean found conc. (ng/mL)	Intra-day RSD(%)	Inter-day RSD(%)	Relative error (%)
50	48.13	2.83	5.44	-3.74
200	193.1	3.15	3.39	-3.43
1600	1540	4.40	1.97	-3.76

Table 2. Stability data of voriconazole in human plasma (three samples of each concentration).

Storage conditions	Nominal conc. (ng/mL)	Mean found conc. (ng/mL)	Relative error (%)
Freezing for 30 days at −20°C	50	48.27	–3.5
	200	195.0	–2.5
	1600	1557	–2.7
Three freeze/thaw cycles	50	48.47	–3.07
	200	194.3	–2.8
	1600	1570	–1.9
Autosampler stability for 12 h (after extracting and reconstitution)	50	47.4	–5.3
	200	187.6	–6.2
	1600	1511	–5.5

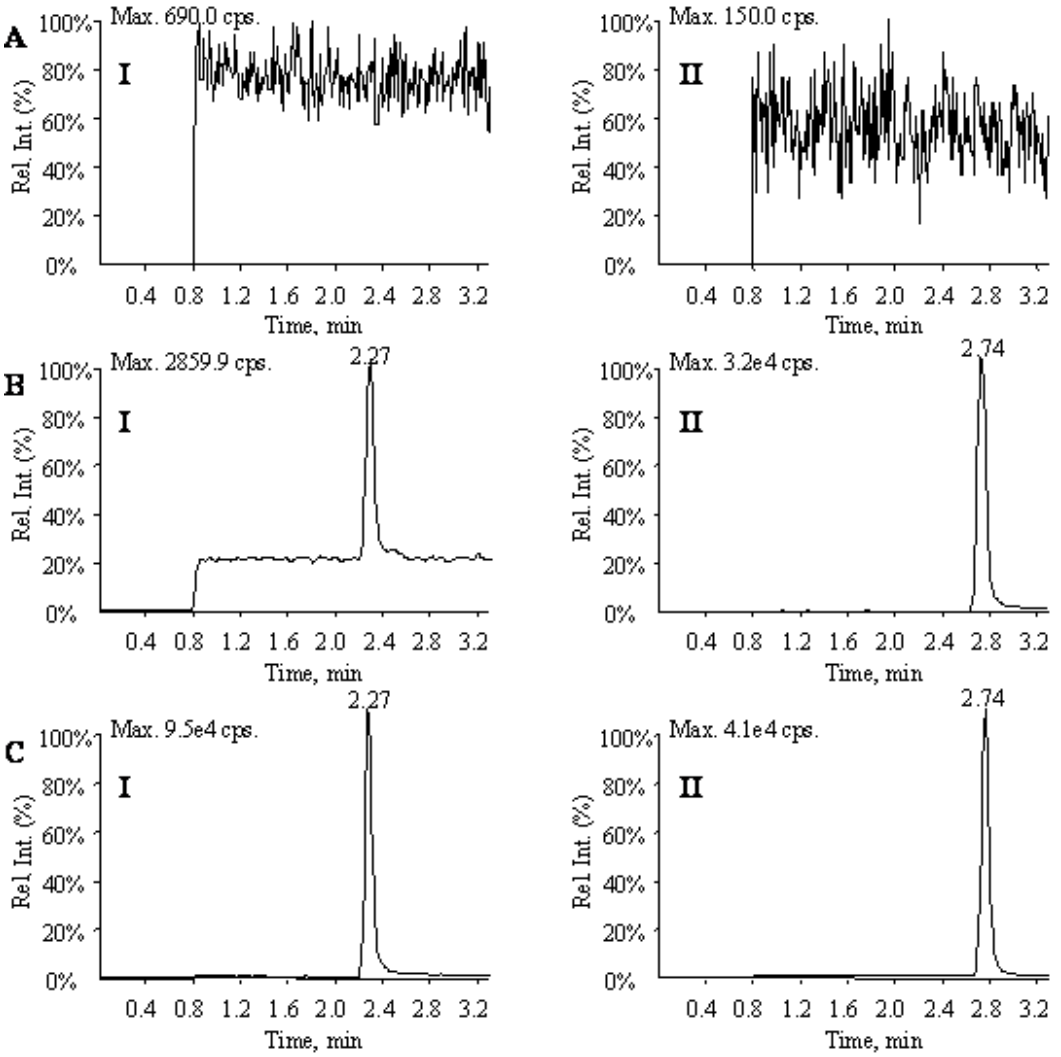

Fig. 2. Representative MRM chromatograms of (**A**) blank plasma, (**B**) standard sample at LLOQ (20 ng/ml) and (**C**) plasma sample from a volunteer 2 h after oral administration of 200 mg voriconazole. Peak **I**, voriconazole; Peak **II**, diazepam.

Application to the clinical test

The proposed method was applied to the determination of voriconazole in plasma samples for bioequivalence study in 20 healthy Chinese male volunteers orally administered 200 mg of voriconazole in tablet form. High-throughput sample analysis is of particular importance for studies that require the analysis of a large number of samples. The devised method of sample preparation using liquid–liquid extraction could resolve this problem. In the present study, the 560 clinical samples were divided into four batches, each batch consisting of a calibration curve and QC samples in triplicate.

Fig. 3 shows the mean plasma concentration–time curves for the two voriconazole formulations. The pharmacokinetic parameters derived from these curves are presented in Table 3.

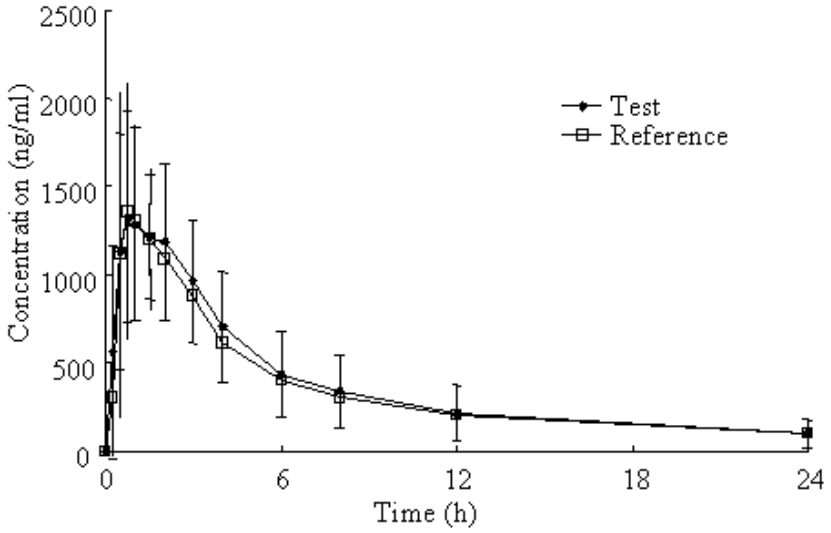

Fig. 3. Plasma concentration versus time curve for two voriconazole tablet formulations in healthy volunteers (n=20). Data are mean±S.D.

Table 3. Pharmacokinetic parameters of voriconazole in test and reference formulations (mean ± S.D., n=20)

Parameter	Voriconazole	
	Test formulation	Reference formulation
C_{max} (ng/ml)	1640.05±564.77	1772.08±676.26
T_{max} (h)	1.3±0.8	1.1±0.7
$T_{1/2}$ (h)	6.99±2.47	7.40±2.98
AUC_{0-t} (ng.h/ml)	8827.98±4243.32	8122.76±3740.73
$AUC_{0-\infty}$ (ng.h/ml)	9971.75±5409.05	9412.17±5006.66

According to the present study, the relative bioavailability of the test formulation was 109.28% (mean AUC_{0-t}) and 107.53% (mean $AUC_{0-\infty}$). There were no significant differences between the two formulations on the basis of assessment by a two one-sided t-test. The 90% confidence intervals of test to reference ratio (after log-transformation) of the AUC_{0-t} (101.20-115.07%) and $AUC_{0-\infty}$ (97.43-113.75%) were within the bioequivalence criteria range of 80–125%, and that of C_{max} (after log-transformation) was within 70–143%. Based on these, the two tablet formulations were found to be bioequivalent.

CONCLUSIONS

A simple, rapid and sensitive LC-MS/MS method is reported for the determination of voriconazole in human plasma. Moreover, the devised method fully meets FDA guidelines, and has high sensitivity and specificity. The method allows high sample throughput (more than 150 samples per day), making it suitable for PK or bioequivalence studies of voriconazole.

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ОПРЕДЕЛЯНЕ НА ВОРИКОНАЗОЛ В ЧОВЕШКА ПЛАЗМА ЧРЕЗ ТЕЧНА ХРОМАТОГРАФИЯ И МАС-СПЕКТРОМЕТРИЯ

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(Резюме)

Разработен е бърз и чувствителен метод за определяне на вориконазол в човешка плазма. Вориконазолът и вътрещният стандарт се екстрахират от плазмени проби с 2 мл смес от диетилов етер и дихлорметан (60: 40, v/v). Хроматографското разделяне се извършва изократично на колона C_{18} (150×4.6 mm, 5 μm Zorbax) при дебит 0.7 mL/min. Определянето става мас-спектрометрично при положителна йонизация с електроспрей в режим на мониторинг на множество реакции. Определянето завършва след 3.2 min. Зависимостта е линейна в интервала 20-2000 ng/ml с граница на откриване 5 ng/ml. Точността в рамките на един и повече дни е с стандартно отклонение съответно <4.40% и <5.44%. Методът е приложен в изследване за биоеквивалентност на две таблетни форми на вориконазол.