A HPLC Method for the Determination of Tinidazole in Human Plasma and Application to a Bioequivalence Study

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SUMMARY. In this study, a simple, rapid and sensitive high performance liquid chromatography (HPLC) method is developed for determination of tinidazole (TNZ) in human plasma samples using metronidazole as the internal standard (IS). Sample preparation was accomplished through protein precipitation with 70% perchloric acid, and chromatographic separation was carried out on a ZORBAX Eclipse XDB-C18 (4.6 × 250 mm, 5 μm) at 40 °C. Mobile phase composed of a mixture of acetonitrile-2% glacial acetic acid-water (35:40:25) at a flow rate of 0.8 mL/min. Wavelength was set at 318 nm. The method was successfully applied to a bioequivalence study of oral TNZ drugs in Chinese healthy volunteers.

RESUMEN. En este estudio se ha desarrollado una cromatografía líquida de alta resolución (HPLC) sencilla, rápida y sensible para la determinación de tinidazol (TNZ) en muestras de plasma humano usando metronidazol como patrón interno (IS). La preparación de la muestra se logró por precipitación de las proteínas con ácido perchlorico al 70% y la separación cromatográfica se llevó a cabo en una columna Zorbax Eclipse XDB-C18 (4.6 x 250 mm, 5 μm) a 40 °C. La fase móvil estuvo compuesta de una mezcla de acetonitrilo-agua-ácido glacial (35:40:25) a un caudal de 0,8 mL/min. La longitud de onda se fijó en 318 nm. El método se aplicó con éxito a un estudio de bioequivalencia de medicamentos orales conteniendo TNZ en voluntarios sanos chinos.

INTRODUCTION

Tinidazole (TNZ) is a second generation member of the 5-nitroimidazole group with selective activity against anaerobic bacteria and protozoa, such as Trichomonas vaginalis, Entamoeba histolytica and Giardia lamblia. It can also be used effectively against metronidazole-resistant strains of T. vaginalis and recurrent periodontitis 1. TNZ has been widely used in Europe as well as developing countries for over two decades with an established efficacy and an acceptable tolerability 2. TNZ has also proven to be effective in the treatment of respiratory tract infections, intra-abdominal sepsis, obstetrical and gynecological infections, colonic and abdominal surgery, emergency appendectomy and amebic liver abscess 3. TNZ is metabolized by the CYP2C9 and CYP3A4 isozymes of the liver microsomal enzymes. The active hydroxymethyl metabolite of TNZ has insignificant activity because of very low serum concentrations 4. Bioavailability following oral administration approaches 100%. It is about 12% protein bound and diffuses into most tissues.

Since TNZ is widely used in the antibacterial therapy, it is important to develop and validate analytical methods for its determination in human plasma. Reviewing the literature revealed that numerous analytical methods have been developed for the assay of TNZ, either individually or in combination with other drugs; these methods include spectrophotometry 5, high performance liquid chromatography (HPLC) 6-9 and high performance liquid chromatography-mass spectrometry (HPLC-MS) 10. However, in bioequivalence studies, the proposed method should be simple and able to process hundreds of samples in a limited time. While some of these methods are successful in the determination of TNZ, these methods have several limitations. These include a long chromatographic running time, complicated, time-consuming sample pre-
treatment procedures and insufficient selectivity and stability.

For a very large number of samples are generated in bioequivalence assay, determination of TNZ at low concentrations, simple, rapid and efficient sample pre-treatment and reasonable elution time, are valid for TNZ quantification method for this kind of study. Thus, there were several requirements such as simple liquid extraction, small sample volume and shorter analytical run times, which are highly critical for routine sample analysis. The HPLC method has been highly used in the quality control of drugs because of its sensitivity, reproducibility and specificity. On the other hand, this method is very simple, rapid, economical, and allows the determination of drugs with sufficient reliability.

The purpose of this study was to develop a HPLC method for the estimation of TNZ in human plasma and to evaluate the bioequivalence of two brands of TNZ 500 mg tablets in Chinese fasting, healthy volunteers.

**MATERIALS AND METHODS**

**Chemicals**

Standard reference material of TNZ was provided by Guangdong Bidi Pharmaceutical Co. Ltd. (Guangdong, China). Test drug of TNZ was provided by Hubei Baikengdi Pharmaceutical Co. Ltd. (Hubei, China). Metronidazole (purity > 98.0 %) was purchased from Sigma (St.Louis, MO, USA). LC-grade acetonitrile, perchloric acid and glacial acetic acid were from Amethyst Chemicals. Blank human plasma was obtained from The First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). Ultra-pure water (resistance > 18 mΩ) was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA).

**Instrumentation and Conditions**

The analytes were performed using an Agilent 1100 liquid chromatographic system equipped with a G1379A vacuum degasser, a G1311A quaternary pump, a G1316A column oven, a G1313A autosampler and G1315B DAD detector.

Samples were separated on a ZORBAX Eclipse XDB-C18 (4.6 × 250 mm, 5 µm) and Agilent XDB-C18 protection column (4.6 × 12.5mm, 5 µm) at 40 °C. The mobile phase consisted of acetonitrile-2% glacial acetic acid-water (35:40:25). All compounds were detected at an optimum wavelength of 318 nm, and the flow rate of mobile phase was 0.8 mL/min.

**Preparation of standard and quality control (QC) samples**

Individual stock solutions of TNZ (100 µg/mL) and metronidazole (internal standard, IS; 10 µg/mL) were dissolved in methanol. The working solutions solutions for calibration and quality controls were made from the stock solution by diluting with methanol. All of the solutions were stored in a refrigerator at 4 °C.

Calibration curve standards were prepared by spiking blank human plasma with appropriate amounts of the working solutions at final drug concentrations of 0.2, 0.5, 1, 2.0, 5.0, 10.0, and 25.0 µg/mL for TNZ. The preparation of QC samples were the same, with the three levels of plasma concentrations (0.4, 5.0, and 20.0 µg/mL).

**Sample Preparation**

Plasma samples were thawed to room temperature and vortexed thoroughly before use. To 500 µL plasma sample, 10 µL IS (10.0 µg/mL) and 30 µL 70% perchloric acid were added in a 1.5 mL test tube. Then, the test tubes were vortex-mixed thoroughly for 1 min and centrifuged at 15000 rpm for 10 min. Clear supernatant (300 µL) was collected and only 5 µL injected into the HPLC system for analysis.

**Method validation**

To evaluate the selectivity of the method, blank human plasma and blank plasma spiked TNZ and IS were analyzed. Calibration curves were constructed validated by analyzing spiked calibration samples on three days in a row. Peak area ratio of TNZ to IS was plotted against analyte concentrations, and standard curves were fitted by weighted (1/χ^2) least squares linear regression in the concentration of 0.2-25.0 μg/mL for TNZ. The lower limit of quantification (LLOQ) of TNZ in human plasma was selected as the lowest concentration used in the calibration curve.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels in six replicates (0.4, 5.0, and 20.0 μg/mL). The precision was expressed by coefficient of variation (RSD) and the accuracy by relative error (RE).

The extraction recoveries of TNZ at three QC levels (n = 6) were determined by comparing peak area of the analytes in samples that were spiked with the analytes prior to extraction with those of samples to which the corresponding solution was added after extraction. The extrac-
tion recovery of the IS (10.0 µg/mL) was determined in a similar way as a reference.

The stabilities of TNZ in human plasma were tested by analyzing five replicates of plasma samples at three concentration levels (0.4, 5.0, and 20.0 µg/mL) in different conditions. The short-term stability was determined after the exposure of the spiked samples at room temperature for 4 h, and the ready-to-inject samples (after extraction) in the HPLC autosampler at room temperature for 24 h. The freeze-thaw stability was evaluated after three complete freeze-thaw cycles (-80 °C) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at -80 °C for 4 weeks.

Bioequivalence study

The bioequivalence study for oral formulation of TNZ (500 mg capsules) was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice and Good Laboratory Practice requirements. The protocol and associated informed consent forms were reviewed and approved by the Medical Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University, and the informed consent forms were signed by the volunteers. Twenty healthy volunteers (aged 21-24) were enrolled in this study. The volunteers met the requirements of the inclusion/exclusion criteria. They had no history of cardiovascular, hepatic, renal, gastrointestinal, hematologic or nervous disease, or any acute or chronic diseases or drug allergy, and had stopped using any drugs 2 weeks prior to study enrollment. Physical examination and laboratory tests showed no abnormal findings. The volunteers were randomized into two groups of 10. The crossover design was applied to this study when one subgroup was administered the study drug followed by administration of the reference drug; another subgroup was administered the reference drug followed by administration of the study drug. All subjects were administered a single dose of 1 g TNZ after overnight fasting. The subjects were required to refrain from smoking, alcohol and caffeine, and were under direct medical supervision at the study site.

Thirteen blood samples were drawn from each subject at each study time point. The blood sampling (each 5 mL) was conducted according to the following schedule: 0 h (pre-dose), 0.25, 0.5, 1, 1.5, 2, 4, 8, 12, 24, 36, 48, and 60 h post-dose. The samples were transferred into sodium heparinized tubes (6 mL volume) and centrifuged within 1 min after collection. The centrifugation was conducted in a temperature-stabilized rotary chamber at 4 °C for 10 min at 3000 rpm. The plasma was divided into two aliquots (the first one was for the analysis, the second for back-up), but the volume was not less than 1.3 mL for each aliquot, and transferred into the pre-labeled cryotubes. Plasma was kept at -80 °C at the study site and at the bioanalytical laboratory as well. The time period between the blood collection and the plasma transfer into a -80 °C freezer did not exceed 15 min. In total, 480 frozen samples were submitted to the bioanalytical laboratory for assessment. The pharmacokinetic parameters of TNZ were calculated by non-compartmental analysis using the computer program DAS (DAS V3.0, Medical University of Wenzhou, China).

RESULTS AND DISCUSSION

Method Development

A full validation of the developed method was carried out. The method was validated for selectivity, LLOQ, calibration curve, intra- and inter-day precision and accuracy, recovery, and stability. Method development was focused on the optimization of column detection, sample preparation and chromatographic separation. Majority of the ionizable pharmaceutical compounds can be well separated on reverse phase C18 column. Thus, in the present study, several C18 columns (Agilent ZORBAX SB C18, Agilent Extend C18, Agilent ZORBAX Eclipse XDB-C18) were compared on the basis of the peak shape and retention times. Finally, the Agilent ZORBAX Eclipse XDB-C18 (4.6 × 250 mm, 5 µm) column was chosen to determine TNZ in human plasma for its good peak shape and acceptable retention times. As for the sample preparation, a protein precipitation method with 70% perchloric acid was chosen in this study for its little interfering endogenous substances and the high recovery.

In order to achieve better chromatographic peak shape, acceptable retention times and resolution, several solvent mixtures were tested for optimizing the components of mobile phase. As a result, good separation of target compounds and short run time were obtained using a mobile phase system of acetonitrile-2% glacial acetic acid-water (35:40:25). The mobile phase in the proposed method instead of buffered systems was used in this HPLC method. Therefore, flushing of the column after analysis was not re-
quired. During the IS method, we preferred to employ metronidazole as an internal standard other than indapamide or phentolamine in this assay, because this allowed us to shorten the total run time of the complete separation. Under these elution conditions, all the peaks of interest were well separated and there were no others interfering peaks from endogenous compounds.

Sensitivity
HPLC chromatograms of human plasma showed that the retention times for TNZ and IS were approximately 5.74 and 3.32 min, respectively. Fig. 1 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with TNZ and IS, and a plasma sample from a healthy volunteer after an oral administration. No endogenous interferences were observed in the blank plasma samples for the analytes.

Linearity of Calibration Curve
The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range of 0.2-25.0 µg/mL for TNZ in human plasma. The typical equations of the calibration curve was as follow: \( y = 0.0866x - 0.102, \) \( r = 0.9998, \) where \( y \) represents the ratio of peak area to that of IS, and \( x \) represents the plasma concentration. The LLOQ for the determination of TNZ in plasma was 0.2 µg/mL. The precision and accuracy at LLOQ were 8.6 and 9.6 %, respectively.

Precision and Accuracy
The precision of the method was evaluated by calculating RSD for QC samples at three concentration levels (0.4, 5.0, and 20.0 µg/mL) during three validation days. The intra-day precision for TNZ was 3.7% or less, and the inter-day precision was 4.9% or less at each QC level.

The accuracy of the method ranged from -9.9 to 8.6% for TNZ at three QC levels. Assay performance data is presented in Table 1. The above results demonstrated that the values were within the acceptable range and the method was accurate and precise.

Recovery
Mean extraction recoveries of TNZ were 85.3 ± 2.3, 81.3 ± 1.1, and 82.0 ± 2.0% (n = 6) at the concentrations of 0.4, 5.0, and 20.0 µg/mL, respectively. The extraction recovery of the IS was 88.5 ± 3.4% (Table 2).

Stability
Three quality control plasma samples (0.4, 5.0, and 20.0 µg/mL) spiked TNZ have shown good stability in plasma for 4 h at room temperature, during three freeze-thaw cycles, and for 28 days at -80 °C. The samples after extraction were also analyzed after at least 24 h of storage at room temperature. The results of stability experiments are summarized in Table 3.

![Figure 1. Representative HPLC chromatograms for TNZ and metronidazole (IS) in human plasma samples. (A) blank plasma sample; (B) blank plasma sample spiked with TNZ (5.0 µg/mL) and IS (10 µg/mL); (C) human plasma sample 0.5 h after oral administration of single dosage 1 g TNZ. 1) metronidazole (IS); 2) TNZ.](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µg/mL)</th>
<th>RSD% intra-day</th>
<th>RSD% inter-day</th>
<th>RE% intra-day</th>
<th>RE% inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNZ</td>
<td>0.4</td>
<td>2.8</td>
<td>2.1</td>
<td>8.6</td>
<td>-9.9</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>3.7</td>
<td>4.9</td>
<td>4.5</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>2.5</td>
<td>0.8</td>
<td>2.3</td>
<td>-1.2</td>
</tr>
</tbody>
</table>

Table 1. Precision and accuracy for TNZ of QC samples in human plasma (n = 6).
Application of the Method

The validated method was successfully applied to a bioequivalence study in 20 healthy human male subject samples for reference and test formulation of TNZ under condition of fasting. Mean plasma concentration-time curve of TNZ, after oral administration of 1 g to 20 healthy volunteers is shown in Fig. 2. Bioequivalence studies protocols generally recommend plasma sample collection for a time period corresponding to three to five times the drug plasma elimination half-life, which brings terminal concentrations values of about 3% of the peak concentration value; for TNZ, mean peak plasma concentration of about 20 µg/mL (Fig. 2).

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Concentration added (µg/mL)</th>
<th>Concentration Measured (µg/mL)</th>
<th>RE%</th>
<th>RSD%</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>room temperature/4 h</td>
<td>0.4</td>
<td>0.42 ± 0.01</td>
<td>3.6</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.98 ± 0.16</td>
<td>3.1</td>
<td>-3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>22.00 ± 1.25</td>
<td>5.7</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>in an autosampler/24 h</td>
<td>0.4</td>
<td>0.41 ± 0.03</td>
<td>7.7</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.96 ± 0.19</td>
<td>3.9</td>
<td>-1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>19.79 ± 1.80</td>
<td>9.1</td>
<td>-0.8</td>
<td></td>
</tr>
<tr>
<td>-80 °C/3 freeze-thaw cycles</td>
<td>0.4</td>
<td>0.38 ± 0.02</td>
<td>5.2</td>
<td>-0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.96 ± 0.20</td>
<td>4.1</td>
<td>-2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>20.78 ± 1.72</td>
<td>8.3</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>-80 °C/28 days</td>
<td>0.4</td>
<td>0.39 ± 0.03</td>
<td>7.2</td>
<td>-0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.90 ± 0.16</td>
<td>3.4</td>
<td>-2.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>20.64 ± 1.55</td>
<td>7.5</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Stability of TNZ under various storage conditions (n = 5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Test</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{max} (µg/mL)</td>
<td>21.382 ± 3.641</td>
<td>20.217 ± 3.131</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>1.825 ± 1.162</td>
<td>1.825 ± 1.067</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>15.166 ± 1.602</td>
<td>15.769 ± 1.737</td>
</tr>
<tr>
<td>AUC_{0→60} (µg/mL.h)</td>
<td>423.772 ± 46.490</td>
<td>405.359 ± 39.734</td>
</tr>
<tr>
<td>AUC_{0→∞} (µg/mL.h)</td>
<td>454.726 ± 50.893</td>
<td>438.005 ± 49.666</td>
</tr>
<tr>
<td>AUC_{0→60}/AUC_{0→∞}</td>
<td>0.932 ± 0.017</td>
<td>0.927 ± 0.020</td>
</tr>
<tr>
<td>F (%)</td>
<td>104.73 ± 7.91</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Main pharmacokinetic parameters of TNZ in 20 healthy volunteers after oral administration of 1 g test and reference tablets.
will produce plasma concentrations after a time period corresponding to five TNZ half-lives of approximately 0.6 µg/mL. Since the method LLOQ was 0.2 µg/mL, its sensitivity is adequate for bioavailability studies. The 90% confidence intervals for geometric mean ratio TNZ test/reference drug ranged from 100.9 to 110.0% (point estimate 105.4%) for \( C_{\text{max}} \), from 101.3 to 107.7% (point estimate 104.4%) for \( \text{AUC}_{0-60} \), and from 100.5 to 107.3% (point estimate 103.8%) for \( \text{AUC}_{0-\infty} \). The 90% confidence interval of the individual ratio geometric mean for test/reference was within 80-125% for \( \text{AUC}_{0-60} \), \( \text{AUC}_{0-\infty} \), and \( C_{\text{max}} \). These findings suggest that the drugs are bioequivalent.

Main pharmacokinetic parameters of TNZ in 20 healthy volunteers after single dose administration of 1 g test and reference tablets are presented in Table 4.

CONCLUSIONS

We have developed and validated a selective and sensitive HPLC method for quantification of TNZ in human plasma. The method was successfully applied to a bioequivalence study of oral TNZ drugs in Chinese healthy volunteers.

REFERENCES