Bioequivalence study of cephradine capsules in Chinese volunteers

Yu-Hui Yan, Kun Wang, Rong Zhang, Guo-Zhu Han, Zhen-Shan Wang, Cheng-Ye Su, Hong Xu, Chun-Mei Yang, Shu Zhao, Xiu-Wen Xie

Aim To study the pharmacokinetics and relative bioavailability in humans of test and reference cephradine capsules and to evaluate the equivalence of the two preparations.

Methods A reversed-phase high performance liquid chromatography (RP-HPLC) method was developed to determine the plasma concentration of cephradine. 18 male healthy volunteers were given orally a single dose 500 mg of test (T) and reference (R) cephradine capsules in an open randomized two-preparation double-period cross-over design experiment. Pharmacokinetic parameters were obtained from the plasma concentration-time data.

Results The main pharmacokinetic parameters of T and R were as follows: AUC_0-5h was 1496.68 ± 272.38 µg·mL⁻¹·min⁻¹ and 1421.75 ± 268.09 µg·mL⁻¹·min⁻¹; C_max 14.41 ± 3.46 µg·mL⁻¹ and 14.39 ± 3.62 µg·mL⁻¹; t_max 59.17 ± 15.84 min and 63.33 ± 20.92 min; t_1/2 56.69 ± 9.68 min and 54.21 ± 7.53 min, respectively. The relative bioavailability of T to R was 106.3 ± 15.8%.

Conclusions The cephradine T and R capsules showed equivalence.

Key words Cephradine; Bioequivalence; reversed-phase high performance liquid chromatography; Pharmacokinetics; Healthy Volunteer

Introduction

Cephradine, a first – generation cephalosporin antibiotic, has broad spectrum antibacterial activity and is effective against gram positive and negative bacteria through inhibition of cell wall synthesis[1]. Although second and third generation cephalosporins have been introduced clinically, cephradine still is an often-used antibiotic, especially in treatment of infections of respiratory and urinary tracts, due to its superior antibacterial properties. The present study was designed to investigate the pharmacokinetics and relative bioavailability of test and reference cephradine capsules in healthy volunteers and to evaluate the bioequivalence of the two capsules.

Materials and Methods

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Drugs and reagents

Test preparation (T): cephradine capsule produced by Bolai Pharmaceutical Co., Ltd, each capsule containing 250mg cephradine, batch No.03072903. Reference preparation (R): cephradine capsule purchased from Zhejiang Yadong Pharmaceutical Co., Ltd, each capsule containing cephradine 250mg, batch No.04010503. Reference standard: cephradine purchased from China Drug & Biological Products Institute, batch No.30427-200306, purity 91.8%. Reagents: methanol (MeOH) and acetonitrile (CH₃CN) were of high-performance liquid chromatography (HPLC) grade; potassium phosphate monobasic dehydrate (KH₂PO₄), and trichloroacetic acid (TCA) were of AR grade.

Subjects

18 male healthy volunteers, aged 21.44 ± 1.25, with height 172.28 ± 4.69 cm and body weight 64.78 ± 4.92 kg, participated in this study. All subjects understood the study, signed a written-informed
consent, received a complete physical examination, and were found to be normal in renal, hepatic and cardiac function. No one had history of anaphylaxis to drugs including penicillins and cephalosporins. All subjects took no medicines within 2 weeks before and throughout the study. During the study a standard meal was served, and cigarette and alcohol were restrained.

**Study Protocol**

This study was carried out according to the two-preparation, double-period, cross-over experiment design. Subjects were assigned by random to 2 groups (group A and group B) of 9 subjects each. Each subject was orally given cephradine capsule as T (group A) or R (group B), then one week later as R (group A) or T (group B) respectively. Blood samples (3 mL each) were collected predose and at 15, 30, 45, 60, 75, 90, 120, 150, 180, 240 and 300 min postdose into heparinized glass centrifugal tubes. The plasma obtained after centrifugation (3000 r/min×10min) was transferred into EP tubes and kept frozen at -20°C until analysis.

The above experiment was approved by the Medical Ethics Committee of Dalian Medical University and supervised by physicians and nurses in a phase I clinical monitoring unit.

**HPLC Conditions**

Cephradine in plasma was chromatographed with a reverse phase Hypersil BDS C18 column (200×4.6mm ID, 5µm) equipped with a BDS C18 pre-column (20×4.6mmID, 10µm), and a mobile phase composed of 0.025mol·L⁻¹ KH₂PO₄: MeOH: CH₃CN (87.5:8 v/v) running at a flow rate of 1.2 ml/min. Detection wavelength was set at 261 nm.

**Pretreatment of plasma samples**

200µl of plasma sample was added with 100µl of 10% TCA solution and vortexed for 5min, then centrifuged (12000r-min⁻¹ for 10min). The resultant 50µl supernatant was injected onto the column.

**Cephradine Plasma Calibration Curve**

190 µL of blank plasma were spiked with 10 µL of different concentrations of cephradine standard working solutions, which were prepared by serial dilution of cephradine standard stock solution, to produce serial standard plasma samples containing cephradine 30, 20, 10, 5, 2, 1, and 0.5µg·mL⁻¹. The standards were then pretreated as described above prior to HPLC injection. Both the chromatograms and peak areas were recorded, then a calibration curve was constructed by plotting peak area against the concentration of cephradine added.

**Sample Assay**

The frozen plasma samples were thawed naturally at room temperature, then treated as described in the section pretreatment of plasma samples, and quantified using an external standard method with retention time as a qualification index and peak area as a quantification index.

**Validation of Analysis Methodology**

Method validation was carried out according to the guidance principle issued by China SFDA[2]. Recovery and precision of the method were calculated at low (1.0 µg·mL⁻¹), middle (5.0µg·mL⁻¹) and high (20.0µg·mL⁻¹) concentrations for 5 repeated analysis separately.

**Data Processing**

Concentration(C) – time (T) data were treated by the Practical Pharmacokinetic Program (3P97) edited by the Chinese Mathematical Pharmacological Society. The C-T curves and main Pharmacokinetic parameters were all obtained automatically by the 3P97 Program. The AUC was measured using the trapezoidal rule. Cmax and Tmax were obtained directly from determined values, the τ₁/₂ from the terminal C-T curve (τ₁/₂ = 0.693 / Kₑ), and the relative bioavailability (Fₑ) was calculated using the formula: Fₑ = AUCₑ / AUCₑ ×100%.

**Statistical Analysis**

All results were expressed as mean±SD, significance level as α=0.05. For evaluation of bioequivalence, an ANOVA and two one-sided t test, (1-α) confident interval were used on natural log transformed AUC and Cmax, and non-parameter test (wilcoxon method) was used on τ₁/₂.

**Results**

**Specificity and Linearity**
From Fig 1 it can be seen that the cephradine peak eluted at 7.2 min and resolved well from plasma substances, which eluted before 5.4 min. It follows that there was no interference with cephradine analysis in plasma. The calibration curve of cephradine in plasma was linear over the range of 0.5 – 30 µg·mL⁻¹ with linear regression equation of \( Y = 18.014X \) (concentration, µg·mL⁻¹) –3.9461 \((R^2=0.9990, n=3)\) and LOQ of 0.5 µg·mL⁻¹ as well as least detection limit of 0.125 µg·mL⁻¹ \((S/N = 3)\).

<table>
<thead>
<tr>
<th>Concentration (µg·mL⁻¹)</th>
<th>Within-day</th>
<th>Between-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean±SD</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>20</td>
<td>324.81±6.38</td>
<td>1.96</td>
</tr>
<tr>
<td>5</td>
<td>82.12±1.98</td>
<td>2.41</td>
</tr>
<tr>
<td>1</td>
<td>17.33±1.11</td>
<td>6.41</td>
</tr>
</tbody>
</table>

**Assay Precision and Recovery**

Table 1 shows that the within-day and between-day precisions (RSD, %) of plasma spiked with 20, 5 and 1 µg/ml of cephradine were 1.96, 2.41, 6.41, and 1.86, 5.47, 4.84, respectively. The mean recovery (%) at concentrations of 20, 5 and 1 µg·mL⁻¹ was 86.22±4.99, 85.64±2.52 and 90.80±4.12, respectively.

**Stability**

It had been demonstrated that cephradine plasma samples kept frozen at −20°C were stable for at least 1 month and at room temperature for at least 8h. Cephradine standard stock solution stored frozen at −20°C was also stable for at least 1 month, and the TCA-deproteinized plasma samples at 2-4°C for at least 8h.

**Pharmacokinetics**

Fig 2 depicts mean plasma drug concentration-time curves, and Table 2 lists a summary of the main pharmacokinetic parameters obtained from 18 volunteers following a single administration of 500 mg T / R. The relative bioavailability of T vs R was 106.3±15.8 (based on AUC₀–₅h data) and 106.8 ±15.3% (based on AUC₀–∞ data).
Evaluation of bioequivalence

The analysis of variance of ln AUC and ln C$_{\text{max}}$ indicated no significant difference between the two preparations and between the two periods, but showed significant difference between individuals (P< 0.05). The two-way one-side t-test and 1-2α confidence interval test of ln AUC and ln C$_{\text{max}}$ displayed that $t_{\alpha}$ and $t_{H}$ were all greater than 1.746 (critical value), while 90% confidence intervals were within 80 – 125% for AUC and 70-143% for C$_{\text{max}}$, indicating that the two preparations were bioequivalent (Table 3).

The non-parametric test for $t_{\text{max}}$ by the wilcoxon method exhibited $U_{0.05}$ and $U_{0.01}$ of 1.96 and 2.58, respectively, the values larger than $U_{\text{calculation}}$ of 1.20, implying no significant difference in $t_{\text{max}}$ between T and R.

Table 2  Main pharmacokinetic parameters of T/R in 18 subjects after oral administration of cephradine 500mg

<table>
<thead>
<tr>
<th>parameters</th>
<th>T</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>56.69±9.68</td>
<td>54.21±7.53</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (min)</td>
<td>59.17±15.84</td>
<td>63.33±20.09</td>
</tr>
<tr>
<td>C$_{\text{max}}$ (µg·mL$^{-1}$)</td>
<td>14.41±3.46</td>
<td>14.39±3.63</td>
</tr>
<tr>
<td>$\text{AUC}_{0-5h}$ (µg·mL$^{-1}$·min$^{-1}$)</td>
<td>1496.68±272.38</td>
<td>1421.75±268.09</td>
</tr>
<tr>
<td>$\text{AUC}_{0-\infty}$ (µg·mL$^{-1}$·min$^{-1}$)</td>
<td>1570.90±287.15</td>
<td>1485.25±284.80</td>
</tr>
<tr>
<td>MRT$_{0-5h}$ (min)</td>
<td>102.03±12.71</td>
<td>100.97±15.04</td>
</tr>
<tr>
<td>MRT$_{0-\infty}$ (min)</td>
<td>115.00±16.37</td>
<td>112.33±17.89</td>
</tr>
</tbody>
</table>

Table 3  Two-away one-side t-test and (1-2α) confidence interval

<table>
<thead>
<tr>
<th>parameter</th>
<th>$t_{\alpha}$</th>
<th>$t_{H}$</th>
<th>$t_{0.05/15}$</th>
<th>90% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ln (AUC$_{0-5h}$)</td>
<td>7.878</td>
<td>4.956</td>
<td>1.746</td>
<td>99.0%-111.8%</td>
</tr>
<tr>
<td>ln (AUC$_{0-\infty}$)</td>
<td>8.238</td>
<td>4.942</td>
<td>1.746</td>
<td>99.7%-112.2%</td>
</tr>
<tr>
<td>ln (C$_{\text{max}}$)</td>
<td>4.176</td>
<td>4.16</td>
<td>1.746</td>
<td>86.2%-116.3%</td>
</tr>
</tbody>
</table>

Discussion

Cephradine, like most cephalosporins, is a polar compound and almost insoluble in organic solvents. This property results in the inability of the antibiotic to be extracted with organic solvents. Therefore, we deproteinized plasma samples with TCA for cleanup and utilized RP-HPLC method for determination of cephradine. The method validation showed that the analytical procedure presented in this paper has high specificity and good precision, recovery and sensitivity, thus completely meeting bioanalytical requirements. The blood collection time ranged from 15 min to 300min, covering 5 half-lives of the drug. The $\text{AUC}_{0-\infty}$ accounted for > 95% of $\text{AUC}_{0-\infty}$.

Several previous papers have described RP-HPLC methods for the determination of cephradine in plasma$^{[3-5]}$. Unlike the previous papers in which ODS C$_{18}$ columns were used, we used a Hypersil BDS C$_{18}$ column, which is packed with base-deactivated silica to minimize residual silica hydroxyl groups, producing better peak shape and diminishing peak tailing.

To protect the analytical column, we employed a pre-column, which was replaced by a new one after every 100-sample batch was completed. Throughout the study we performed strict quality control analysis. Each batch of samples was accompanied by a freshly prepared calibration curve and QC samples of 3 different concentrations. In 6 batches of analysis QC samples of high and middle concentrations gave a relative error of ≤ 6.2%, and low concentration QC samples ≤ 11.00 %. We also investigated the stability during storage of samples and found this antibiotic was stable under the conditions mentioned above. These results are similar to those obtained by Dr. Johnson VM et.al$^{[3]}$. Even so, the sample pretreatment was conducted with a small number of samples (4-6 samples) each time to fully ensure that TCA-deproteinization and their HPLC analysis were finished within 1 h. These practices ensured the reliability of the analytical results.

In the present study, we took advantage of an external standard method instead of using the internal standard method reported by Dr. Mao Guoguang et.al$^{[3]}$ and Dr. Johnson VM et.al$^{[5]}$. This approach yielded good results, while also shortening the run time. The mobile phase composed of phosphate buffer plus MeOH / CH$_3$CN as modifier was chosen and found to be superior for eliminating endogenous interference, relative to using an acetate buffer plus MeOH$^{[3]}$. TCA was selected to deproteinize plasma samples and found to be as efficient as the perchloric acid (HClO$_4$) utilized by Dr. Liu Hongmei et.al$^{[4]}$ and Dr. Johnson VM et.al$^{[5]}$. 
The analysis of variance showed a significant inter–individual difference in AUC, suggesting that the extent of oral absorption of the drug had a great difference. Therefore, individualization of clinical oral medication of cephradine should be exercised.

References
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Papers published in this Acta not only have originality and frontier, but also have characteristics of the Chinese traditional medicine with high cultural value. According to uncompleted statistics about 10% of papers published in this Acta received prizes awarded by the nation, province, or ministry. The central government, province or ministry (Natural Science Foundation, National 973 and 863 Foundation, the 10th Five-year plan Foundation and Provincial and Ministry Foundations) funded about 60% of the papers. It would appear that papers published in this journal can represent the aims and development of the pharmaceutical domain in China and may play important role in international exchange.

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