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Development of a simple LC-MS/MS method for determination of rebamipide in human plasma and its application to a bioequivalence study

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The purpose of this study was to design a simple and rapid liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for a rebamipide bioequivalence study in healthy Chinese male volunteers. In this method, sample pretreatment involved simple protein precipitation with venlafaxine as the internal standard. Analysis was achieved on a ZORBAX SB-C18 column with a concentration range of 6∼1200 ng/mL. Rebamipide tablets fromYuanlijian (test, Hangzhou, China) and from Otsuka (reference, Hangzhou, China) were evaluated following a single 300 mg oral dose to 20 healthy volunteers. Bioequivalence was determined by calculating 90 % confidence intervals (90 % CI) for the ratio of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ values for the test and reference products, using logarithmic transformed data. The 90 % confidence intervals for the ratio of C_{max} (83.7∼118.4 %), AUC_{0-t} (91.1∼113.4 %) and $AUC_{0-\infty}$ (90.6∼113.2 %) values for the test and reference products were within the interval (80.0∼125.0 % for AUC, and 70∼143 % for C_{max}), proposed by State of Food and Drug Administration [SFDA, 2005. China]. It was concluded that the two rebamipide tablets were bioequivalent in their rate and extent of absorption and the method met the principle of quick and easy clinical analysis.

1. Introduction

Rebamipide, (±)-2-(4-chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic acid (CAS 11911-87-6), is a novel quinolinone derivative with a molecular weight of 370.79 Da (Uchida et al. 1985, 1986). Rebamipide is a gastroprotective agent and has been prescribed for the treatment of gastric ulcer and gastritis to millions of patients in Asian countries and Egypt. The precise mechanism of rebamipide for the prevention of gastropathy and the acceleration of gastric ulcer healing is still unknown. However, rebamipide has been shown to increase gastric mucosal prostaglandin production (Yamasaki et al. 1987; Kleine et al. 1993; Suetsugu et al. 2000; Sun et al. 2000), increase gastric mucus production (Iijima et al. 2009; Ishihara et al. 1992), scavenge hydroxyl radicals (Yoshikawa et al. 1993; Naito et al. 1995; Sakurai et al. 2004), inhibit neutrophil activation (Yoshikawa et al. 1993; Ogino et al. 1992; Suzuki et al. 1994; Kim et al. 2001), suppress gastric mucosal inflammation (Yoshida et al. 1996; Murakami et al. 1997), regulate apoptosis-related genes (Naito et al. 2007), inhibit tyrosine nitration (Kinjo et al. 2008) and promote Sonic hedgehog restoration (Nishizawa et al. 2009).
Following a single oral administration at 100 mg, rebamipide is rapidly absorbed with a peak plasma level of 216 ng/ml in 2.4 h and eliminated with a half-life of 1.9 h (Hasegawa et al. 2003). Oral rebamipide mainly distributes to the gastrointestinal organs (Shioya et al. 1988) and its plasma concentration is much lower than the gastric mucosal concentration, which could be related to its safety profile and the presence of little or no drug-drug interactions. The majority of rebamipide is excreted in an unchanged form (Koyama et al. 2002). Approximately 10 % of the administered dose is excreted in urine and the remainder is found in the feces. The pharmacokinetic properties and clinical pharmacology of rebamipide have been studied in healthy Japanese and Korean volunteers (Hasegawa et al. 2003; Akamatsu et al. 2002; Cho et al. 2009), but there is a lack of data for the Chinese population.
Several high-performance liquid chromatographic (HPLC) methods with fluorescence detection for the determination of rebamipide in biological fluids have been published (Akamatsu et al. 2002; Cho et al. 2009; Naito et al. 1996). These methods used protocols with multiple steps for the extraction of rebamipide from biological samples. Hasegawa et al. (2003) published a LC/MS/MS method using solid-phase extraction (SPE). The method required a complicated and time-consuming sample preparation procedure, which failed to meet the principle of quick and easy clinical analysis.
Therefore, a simple and rapid LC-MS/MS method using methanol as precipitation solvent was developed to determine rebamipide in human plasma. Convenient sample preparation was the main advantage of the technique. The method was validated and applied successfully to a bioequivalence study of rebamipide in 20 healthy volunteers after an oral dose of 300 mg rebamipide tablets.

2. Investigations and results

2.1. Pharmacokinetic analysis

After oral administration, the following parameters were determined by noncompartmental analysis using DAS Software-Version 2.0 (Chinese Pharmacological Society, China): half-life of drug elimination during the terminal phase ($t_{1/2}$), area under the plasma concentration–time curve from 0 to the last measurable concentration ($AUC_{0\text{-t}}$), area under the plasma concentration–time curve from 0 to infinity ($AUC_{0-\infty}$) and mean residence time (MRT). The maximum plasma concentration (C_{max}) and time point of maximum plasma concentration (T_{max}) were obtained directly from the measured data. The relative bioavailability (F%) of the tested formulation was calculated as follows:
F% = $AUC_{0\text{-t}}$ (test)/ $AUC_{0\text{-t}}$ (reference) * 100 %.

2.2. Statistical analysis

Bioequivalence of the two rebamipide formulations were assessed by calculating individual $AUC_{0\text{-t}}$, $AUC_{0-\infty}$, and C_{max} values. Analysis of variance (ANOVA) using Das 2.0 was performed on $AUC_{0\text{-t}}$, $AUC_{0-\infty}$, and C_{max} values evaluating for treatment, period, sequence, and subject within sequence effects. Intrasubject variability were estimated and period, subject, and sequence effects were determined on a significance level of $\alpha = 0.05$. Their ratios (test *versus* reference) of log-transformed data were analyzed for relative bioavailability. Ninety percent confidence intervals served as interval estimates and were determined by two one-sided t tests. If the parameters between the two formulations were not statistically different with each other ($\alpha = 0.05$), and the log-transformed ratios of C_{max} and AUC located within the predetermined equivalence range (70∼143 % for C_{max} and 80∼125 % for AUC), the two formulations would be considered bioequivalent.

2.3. Tolerability

Both the test and reference formulation of rebamipide appeared well tolerated in the population studied when administered orally. No adverse events occurred or were found on laboratory testing during the study, and no volunteers were withdrawn as a result of adverse events.

2.4. Analytical method validation

Figure 1 shows the product ion spectra of rebamipide and venlafaxine. The typical MRM chromatograms of a blank, a spiked plasma sample with rebamipide at 100 ng/mL and IS (1.1 ng/mL), and a plasma sample from a healthy volunteer 1.5 h after an oral administration are shown in Fig. 2. The retention times for rebamipide and IS were about 3.4 and 3.1 min, respectively. No interference peaks were detected for the analyte or IS from the six different sources of plasma.
The calibration curves of rebamipide in plasma were linear in the range from 6 to 1200 ng/mL. The regression equations of calibration curves were $Y = (0.0283 \pm 0.0060)X + (-0.0017 \pm 0.0291)$ ($n = 12$), $r^2 = (0.9983 \pm 0.0016)$. The lower limit of quantification (LLOQ) was 6 ng/mL with relative standard deviation (R.S.D.) lower than 5.0 %.
The recoveries of the protein precipitation for rebamipide were 98.98 ± 6.30 %, 91.80 ± 7.99 % and 98.09 ± 4.81 % at concentrations of 10, 100 and 1000 ng/mL, respectively. Mean recovery for the IS was 93.29 ± 2.35 %. By the analysis of six batches of samples, matrix effect values were calculated. Average matrix effect values obtained were 67.24 ± 2.23 %, 82.25 ± 3.71 %, 76.09 ± 2.88 %, and 79.52 ± 3.18 % for QC samples at

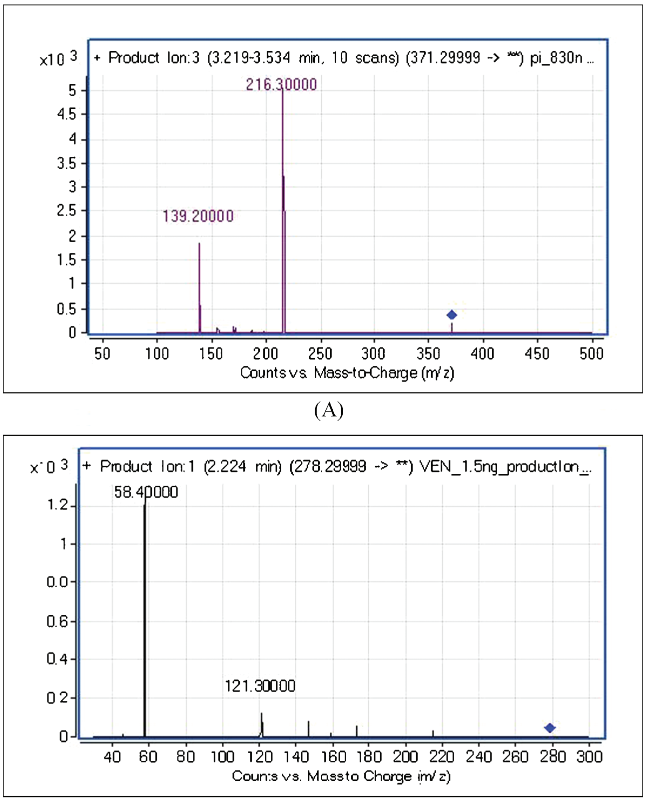

Fig. 1: Product ion spectra of rebamipide (A) and venlafaxine (B).

concentrations of 10, 100, 1000 ng/mL, and IS. The intra-assay accuracy ranged between 107.93 and 112.45 % with a precision of 1.45∼6.45 %. The inter-assay accuracy ranged between 97.37 and 104.11 % with a precision of 9.83∼11.60 %. Plasma samples were stable after three freeze-thaw cycles (R.S.D. from 4.55 % to 6.54 %). In the bench stability study, QC plasma samples were found to be stable for at least 6 h at ambient temperature (25 °C) (R.S.D. from 6.11 to 8.80 %). The precipitated plasma samples showed no significant degradation in the LC autosampler (10 °C) for at least 21 h (R.S.D. from 6.16 to 8.11 %). In the long-term stability study, the plasma samples spiked with the QC plasma samples also showed no loss of analytes when they were stored for 145 days at −70 °C (R.S.D. from 3.88 to 8.66 %). The good stability of rebamipide simplified the precautions needed for laboratory manipulations during the analytical procedures.

2.5. Bioequivalence evaluation

The mean plasma concentration versus time curves after administration of reference and test products to 20 healthy volunteers are shown in Fig. 3.
The corresponding pharmacokinetic parameters ($t_{1/2}$, C_{max}, T_{max}, $AUC_{0\text{-t}}$, $AUC_{0-\infty}$) after administration of reference and test products to 20 healthy volunteers are presented in Table 1.
The results of the analysis of variance (ANOVA) for assessment of product, group and period effects and 90 % confidence intervals for the ratio of C_{max}, $AUC_{0\text{-t}}$ and $AUC_{0-\infty}$ values for test and reference products, using logarithmic transformed data, are shown in Table 2. Power of statistical test was 99.6 % for C_{max}, 101.7 % for $AUC_{0\text{-t}}$ and 101.3 % for $AUC_{0-\infty}$.

3. Discussion

The analytical method developed for rebamipide quantification in plasma samples showed good specificity, sensitivity,

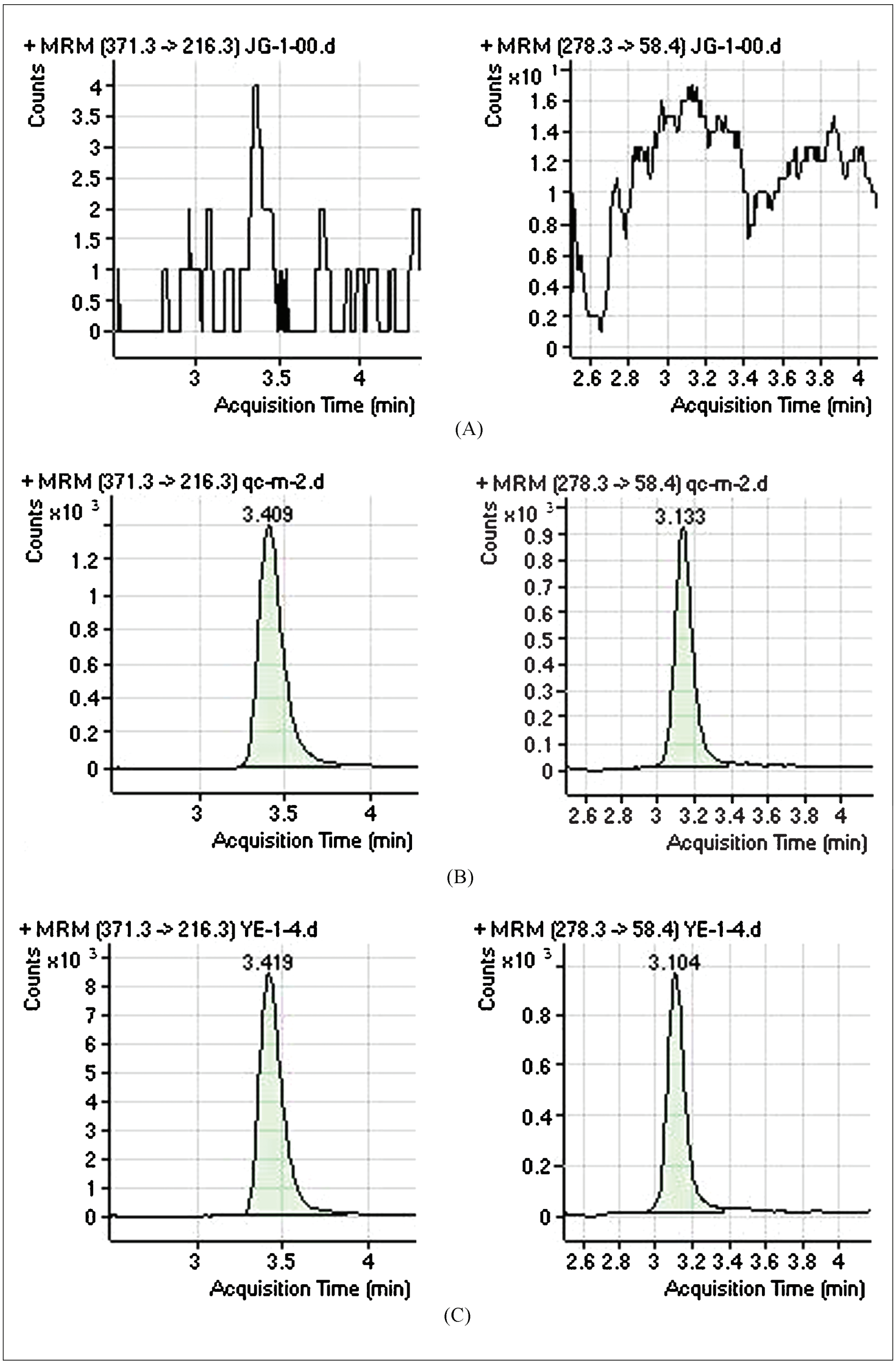

Fig. 2: Typical MRM chromatograms of rebamipide (left) and venlafaxine (IS, right) in human plasma samples, (A) blank plasma sample; (B) plasma sample spiked with rebamipide (100 ng/ml) and IS (1.1 ng/ml); (C) plasma sample 1.5 h after single oral dose of 300 mg rebamipide to a subject.

linearity, precision and accuracy, and was successfully applied in bioequivalence trials in Chinese volunteers.

In literature, several analytical methods for detecting plasma levels of rebamipide in biological matrix have been reported. These assays were mainly based on the use of high performance liquid chromatography (HPLC) coupled with fluorescence detection (Akamatsu et al. 2002; Cho et al. 2009; Naito et al. 1996). However, all these reported methods need complicated sample pretreatment procedures. Among the currently available bio-analytical techniques, liquid chromatography coupled with mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) has been emerged as the preeminent analytical tool for quantification of small molecular weight drugs in biological matrix. To our knowledge only one method has been developed and validated for quantification of rebamipide in human plasma using LC-MS/MS (Hasegawa et al. 2003). But the SPE method used in the plasma sample preparation is time-consuming and expensive. The LC-MS/MS we adopted has several advantages compared to the previously reported methods, as it used methanol as precipitation solvent. Convenient sample preparation was the main advantage of the technique. Rather, this method meets the principle of quick and easy clinical analysis without impairing high sensitivity.

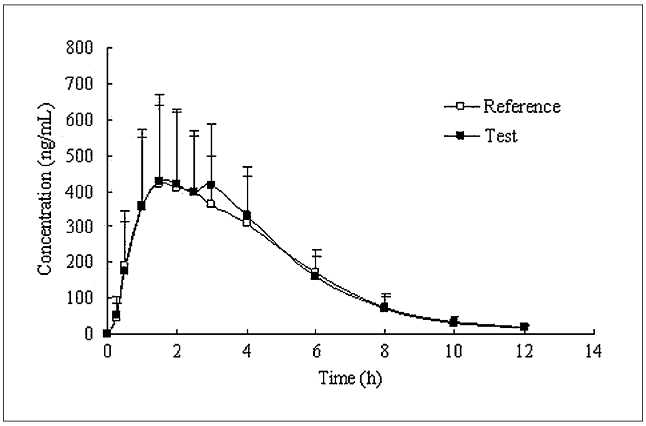

Fig. 3: Mean (+S.D.) plasma concentration–time profiles of rebamipide following a single oral dose of 300 mg rebamipide tablet from two companies to 20 healthy male subjects.

No significant differences in $AUC_{0\text{-t}}$ (reference, 2182.17 ng·h/mL; test, 2235.19 ng·h/mL), $AUC_{0\text{-}\infty}$ (reference, 2230.62 ng·h/mL; test, 2279.10 ng·h/mL), or C_{max} (reference, 547.05 ng/mL; test, 556.35 ng·mL^{-1}) were found between the test and reference formulations.
Previous pharmacokinetic data on rebamipide were few. Examination of rebamipide granules and tablets in humans, accomplished by Hasegawa et al. (2003), showed a mean $AUC_{0\text{-t}}$, $AUC_{0\text{-}\infty}$, C_{max}, T_{max}, and $t_{1/2}$ about 873.55 ng·h/mL, 936.99 ng·h/mL, 216.99 ng/mL, 2.4 h, and 1.93 h, repectively after a single administration of 100 mg rebamipide tablets to 28 healthy male Japanese volunteers during fasting conditions. Cho et al. (2009) reported that rebamipide tablets 100 mg orally administered to 30 Korean healthy male volunteers with produced mean reference (test) $AUC_{0\text{-t}}$, $AUC_{0\text{-}\infty}$, C_{max}, T_{max}, and $t_{1/2}$ of 903.46 (831.09) ng·h/mL, 923.58 (851.68) ng·h/mL, 220.57 (218.12) ng/mL, 2.1 (2.05) h, and 1.93(1.96) h, respectively. These data were close to those in the present study, considering the dose difference.
The multivariate analysis accomplished through analysis of variance revealed the absence of period, group and product effects for $AUC_{0\text{–t}}$, $AUC_{0\text{-}\infty}$ and C_{max}.
According to the present study, power of the statistical test was 99.6 % for C_{max}, 101.7 % for $AUC_{0\text{-t}}$ and 101.3 % for $AUC_{0\text{-}\infty}$, and the 90 % confidence intervals for the ratio of C_{max} (83.7∼118.4 %), $AUC_{0\text{-t}}$ (91.1∼113.4 %) and $AUC_{0\text{-}\infty}$ (90.6∼113.2 %) values for the test and reference products are within the interval (80.0∼125.0 % for *AUC*, and 70∼143 % for C_{max}), proposed by SFDA (2005).
It was concluded that the two formulations are bioequivalent in their rate and extent of absorption and, thus, may be used interchangeably, with no affection on therapeutic effect.

Table 1: Mean pharmacokinetic parameters of rebamipide following a single oral dose of 300 mg rebamipide tablets (reference and test products) to 20 healthy male subjects (mean ± SD)

Parameters	Reference	Test
$t_{1/2}$ (h)	1.92 ± 0.64	1.79 ± 0.56
C_{max} (ng/mL)	547.05 ± 194.05	556.35 ± 213.64
T_{max} (h)	2.15 ± .01	2.48 ± 1.04
$AUC_{0\text{-t}}$ (ng·h /mL)	2182.17 ± 609.45	2235.19 ± 667.72
$AUC_{0\text{-}\infty}$ (ng·h /mL)	2230.62 ± 616.33	2279.10 ± 697.26

4. Experimental

4.1. Materials

Rebamipide reference standard (purity: > 99 %) was provided by Zhejiang provincial laboratory for the control of drugs (Zhejiang, China). Venlafaxine (Batch No. 100543-200401), employed as the internal standard (IS), was purchased from the national institute for the control of pharmaceutical and biological products (Beijing, China). Methanol and acetonitrile HPLC grade were obtained from Merck (Darmstadt, Germany). Ammonium formate (purity: 99.995 %) was bought from Sigma (St. Louis, MO, USA) and formic acid from Tedia (Fairfield, OH, USA).Water was distilled using a Milli-Q plot water purification system (Millipore Corporation, Bedford, Massachusetts).
Pharmaceutical products used in the bioequivalence study were rebamipide tablet (conventional tablet containing 100 mg of rebamipide, lot no. 20100812, produced by Zhejiang Yuan Li Jian Pharmaceutical co. Ltd, China), used as the test product, and Mucosta (conventional tablet containing 100 mg of rebamipide, lot no. 100205R, produced by Otsuka, Zhejiang, China), as the reference product.

4.2. Instrumentation

Analyses were performed on an Agilent liquid chromatographic system (Agilent 1100, California, USA) consisted of a Gl311A quaternary-dimension infusion pump, a Gl379A vacuum degasser, a G1367A autosampler, and a G1316A column thermostat. This HPLC system was coupled with an Agilent 6410 mass spectrometric detection equipped with an electrospray ion source and operating under Agilent Mass Hunter Workstation B.01.04. The analytical column was a ZORBAX SB-C18 (150 mm × 3.0 mm i.d, 3.5 μm particle size) protected with a Waters symmetry C18 guard cartridge (20 mm × 3.9 mm i.d., 5 μm particle size) (Ireland).

4.3. Chromatographic and mass spectrometric conditions

The isocratic HPLC mobile phase consisting of methanol (containing 5 mM ammonium formate)- water (80:20, v/v) was used at a flow rate 0.3 mL/min. The column temperature was maintained at 30 °C, and the injection volume was set at 10 μL. The total running time was not more than 3.5 min. The HPLC system was connected to the mass spectrometer through an ESI interface and was operated in the positive ion detection mode. The spray voltage was set at 3500 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set at 35 psi. Desolvation gas (nitrogen) was heated to 350 °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 0.1 MPa. Quantification was performed using multiple reaction monitoring (MRM) mode at m/z 371.3→216.3 (fragmentation energy = 140 v, collision energy = 20 v) for rebamipide, and m/z 278.3→58.4 (fragmentation energy = 120 v, collision energy = 30 v) for venlafaxine (IS). The 2 pairs of ions were monitored simultaneously within the analytic procedure.

4.4. Calibration standards and quality control plasma samples

The stock solutions of rebamipide (600 μg/mL) and internal standard (venlafaxine, 1.1 mg/mL) were prepared in methanol. Both solutions were stored at −20 °C. Solutions of rebamipide with concentrations of 60, 120, 600, 1200, 6000, 12000 ng/mL were prepared by serial dilution of stock solutions with a mixture of water:methanol (20:80, v/v). The concentration of the internal standard working solution was 11 ng/mL. Calibration standard samples were freshly prepared in 200 μL of blank plasma by adding 20 μL of the rebamipide calibration solutions and 20 μL of the internal standard working solution to yield a series of standards for the calibration curve with concentration corresponding to 6,12,60,120,600,1200 ng/mL of rebamipide and 1.1 ng/mL of internal standard, respectively. Quality control (QC) samples were prepared at low (10 ng/ml), medium (100 ng/ml) and high (1000 ng/ml) concentrations in the same way as the plasma samples for calibration.

4.5. Sample preparation

Before analysis, the plasma samples were thawed at ambient temperature. A 200 μL aliquot plasma sample was mixed with 20 μL IS (11 ng/mL) solution and 20 μL of water: methanol (20:80, v/v). After a thorough vortex mixing for 30 s, mixtures were precipitated with 600 μL methanol, vortex-mixed for 1 min, and centrifuged at 13000 r/min for 5 min. Its supernatants (10 μL) were injected into the LC-MS/MS system.

4.6. Experimental design

This study was performed according to the revised Declaration of Helsinki for biomedical research involving human subjects and the rules of Good Clinical Practice. The protocol of this study was approved by the ethics

Table 2: Analysis of variance (ANOVA) for the assessment of the product, period and group effects, statistical power and 90 % confidence intervals (90 % C.I.) for the ratio of C_{max}, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ values for the test and reference products, using logarithmic transformed data, after administration of reference and test products to 20 healthy volunteers ($\alpha = 0.05$)

Pharmacokinetic parameters	ANOVA (*p*-value), variation source			Statistical power (%)	90% C.I.
	Product	Period	Group		
C_{max}	0.966	0.764	0.607	99.6	83.7–118.4 %
$AUC_{0\text{-}t}$	0.797	0.301	0.470	101.7	91.1–113.4 %
$AUC_{0\text{-}\infty}$	0.848	0.328	0.505	101.3	90.6–113.2 %

committee of the First Affiliated Hospital of Zhejiang University (Hangzhou, Zhejiang, China). All participants signed a written informed consent after they had been informed of the nature and details of the study prior to any screening procedures.

A total of 20 healthy male Chinese volunteers participated in the study. Subjects had a mean age of 22.7 years, a mean bodyweight of 62 kg, and a mean height of 1.71 m. Eligible subjects were selected after passing a clinical screening procedure including a physical examination and laboratory tests, which included hematology, blood biochemistry, urine analysis, and hepatitis B and HIV antibodies. In addition, the medical history, body weight, height, vital signs, and a 12-lead electrocardiogram were recorded. No volunteers had a history or evidence of a renal, gastrointestinal, hepatic, or hematologic abnormality or any acute or chronic disease, or an allergy to any drugs. This was done to ensure that the existing degree of variation would not be due to an influence of illness or other medications. All volunteers avoided using other drugs for at least 4 weeks prior to the study and until after its completion. Screening procedures were repeated at the end of the trial.

A monocentric, single-dose, randomized-sequence, open-label, 2-period crossover study design was adopted, in which 20 healthy volunteers according to a computer-generated randomization schedule were assigned to one of two treatment sequences (test-reference or reference-test). Volunteers were hospitalized at 8:00 p.m. one day before this study and fasted 10 h before each drug administration. On the morning of the administration day, the subjects received a single 300-mg dose (administered with 250 mL of water) of the test (rebamipide tablet 100 mg, manufactured by Zhejiang Yuan Li Jian Medicine Co. Ltd, China, Lot: 20100812, manufactured by Otsuka Pharmaceutical Co. Ltd., Zhejiang, China, Lot: KG8823, expiration: 2012.8) or the reference (rebamipide tablet 100 mg, trade mark: Mucosta®, expiration: 2012.3). Drinking water and food intake was allowed 2 and 4 h after treatment, respectively. A one-week washout phase was between the two treatment periods.

During both treatment periods, free flowing blood samples (∼5 mL) were collected from a suitable ulnar vein using an indwelling catheter into heparin anticoagulated tubes before (0 h) and 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 h after dosing. The blood samples were centrifuged at 3500 r/min and 4 °C for 10 min, and plasma samples were separated and stored at −70 °C until required for analysis.

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