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

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The purpose of this study was to design a simple, sensitive and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for a febuxostat bioequivalence study in healthy Chinese male volunteers. In this method, febuxostat and etodolac (internal standard) were isolated from plasma samples by protein precipitation with acetonitrile. The supernatant was chromatographed on a Zorbax SB-C18 (150 × 3.0 mm, 3.5-μm particle size, Agilent) column with a SecurityGuard Inertsil Symmetry C18 column (12.5 × 4.6 mm, 5-μm particle size, Waters). The lower limit of quantification for febuxostat in 0.2 mL of human plasma was 13.40 ng•mL^{-1}, and the linearity was achieved over a concentration range from 13.40 to 21440 ng•mL^{-1}. Febuxostat tablets from Hengrui Medicine Co., Ltd (test, Jiangsu, China) and from Takeda pharmaceuticals america, Inc. (reference, Deerfield, IL) were evaluated following a single 80 mg oral dose to 18 healthy volunteers. Bioequivalence was determined by calculating 90% confidence intervals (90% CI) 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. The calculated 90% CIs for the ratio of C_{max} (88.7∼131.2%), $AUC_{0\text{-}t}$ (99.2∼122.7%) and $AUC_{0\text{-}\infty}$ (99.5∼123.1%) values for the test and reference products were all located within the bioequivalence criteria range (80∼125% 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 febuxostat formulations (test and reference) analyzed were bioequivalent in terms of rate and extent of absorption and the method met the principle of quick and easy clinical analysis.

1. Introduction

Febuxostat, 2-[3-cyano-4-(2-methylpropoxy)phenyl]-4-methylthiazole-5-carboxylic acid (CAS 144060-53-7), is a novel non-purine selective inhibitor of xanthine oxidase (NPSIXO, Okamoto et al. 2003; Takano et al. 2005; Osada et al. 1993; Komoriya et al. 1993, Horiuchi et al. 1999) and is currently under development for the management of hyperuricemia in patients with gout. Febuxostat is a potent xanthine oxidase inhibitor, has minimal effects on other enzymes involved in purine and pyrimidine metabolism, and is metabolized mainly by glucuronide formation and oxidation in the liver. In a study of subjects with renal impairment, the serum urate-lowering effect of febuxostat was unaltered (Mayer et al. 2005). The pharmacokinetic properties and clinical pharmacology of febuxostat have been studied in healthy American volunteers (Mayer et al. 2005; Khosvaran et al. 2006, 2008, 2007), but there is a lack of data for the Chinese population.

At present, several HPLC methods for the determination of febuxostat in human plasma were reported in the literature. Most of these methods employ fluorometric detection (Khosvararan et al. 2006, 2008, 2007), and show higher ranges in LLOQ and longer retention times (RT), which failed to meet the principle of quick and easy clinical analysis.

Thus in this study, a simple and rapid LC-MS/MS method using acetonitrile as precipitation solvent was developed to determine febuxostat in human plasma. The method described here required only small volumes of sample and mobile phase, short chromatographic run times and was sensitive, specific and fully validated. The method was developed successfully for a study of bioequivalence of two oral formulations of febuxostat (80 mg generic febuxostat table, Jiangsu Hengrui Medicine Co., Ltd, China; 80 mg ULORIC® tablet, Takeda Pharmaceuticals America, Inc.) in 18 healthy volunteers.

2. Investigations and results

2.1. Pharmacokinetic analysis

After oral administration, the following parameters were determined by non-compartmental analysis using Drug and Statistics (DAS) Software version 2.1.1 (University of Science and Technology, Hefei, People's Republic of 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 ($AUC_{0\text{–}\infty}$) 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

C_{max}, $AUC_{0\text{-}t}$, and $AUC_{0\text{-}\infty}$ were considered the primary variables to assess the bioequivalence of the two febuxostat formulations. Analysis of variance (ANOVA) using DAS 2.1.1 was performed on $AUC_{0\text{-}t}$, $AUC_{0\text{–}\infty}$, 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. The 90% CIs 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}, $AUC_{0\text{-}t}$ and $AUC_{0\text{–}\infty}$ located within the bioequivalence criteria range (80∼125% for AUC, and 70∼143% for C_{max}), the two formulations would be considered bioequivalent.

2.3. Tolerability

Both febuxostat formulations appeared to be well tolerated in the population studied when administered orally. Safety was evaluated by monitoring adverse events during the whole bioequivalence study in the clinic. No adverse events occurred or were found and no volunteers were withdrawn as a result of adverse events.

2.4. Analytical method validation

Figure 1 shows product ion mass spectra of: (M_1) febuxostat (m/z 271.0) and (M_2) IS (m/z 242.1). The typical MRM chromatograms of a blank, a spiked plasma sample with febuxostat (101.8 ng•mL^{-1}) and IS, and a plasma sample from a healthy volunteer 1 h after an oral administration are shown in Fig. 2. The retention times for febuxostat and IS were 4.52 and 4.07 min, respectively. The total chromatographic run time was 5.5 min. No interference peaks were detected from the analyte or IS from the six different sources of plasma.

The calibration curves of febuxostat in plasma were linear in the range from 13.40 to 21440 ng•mL^{-1}, with a typical equation for the calibration curve being $Y = (-0.0527 \pm 0.0175)X^2 + (1.0545 \pm 0.3740)X + (0.0015 \pm 0.0015)$ (n = 6), $r^2 = (0.9990 \pm 0.0007)$. The LLOQ for febuxostat was 13.40 ng•mL^{-1}.

The mean extraction recoveries of febuxostat from human plasma were 80.59 ± 3.43%, 82.16 ± 3.40%, 78.19 ± 2.43% and 72.89 ± 1.37% for the final spiked concentrations of febuxostat at 26.8, 335.0, 3350.0 and 13400.0 ng•mL^{-1}, respectively. By the analysis of five batches of samples, matrix effect values were calculated. Average matrix effect values obtained were 80.59 ± 3.43%, 82.16 ± 3.40%, 78.19 ± 2.43%, 72.89 ± 1.37%, and 56.66 ± 1.37% for QC samples at 26.8, 335.0, 3350.0, 13400.0 ng•mL^{-1}, and IS.

The intra-assay accuracy ranged between 95.34 and 105.18% with a precision of 1.83∼5.07%. The inter-assay accuracy ranged between 98.09 and 102.83% with a precision of 4.05∼7.03%. Plasma samples were stable after three freeze-thaw cycles (R.S.D. from 6.16% to 12.24%). 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 3.73 to 7.09%). The precipitated plasma samples showed no significant degradation in the LC autosampler (25 °C) for at least 23 h (R.S.D. from 3.78 to 9.45%). 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 227 days at − 20 °C (R.S.D. from 5.70 to 12.05%). The good stability of febuxostat simplified the precautions needed for laboratory manipulations during the analytical procedures.

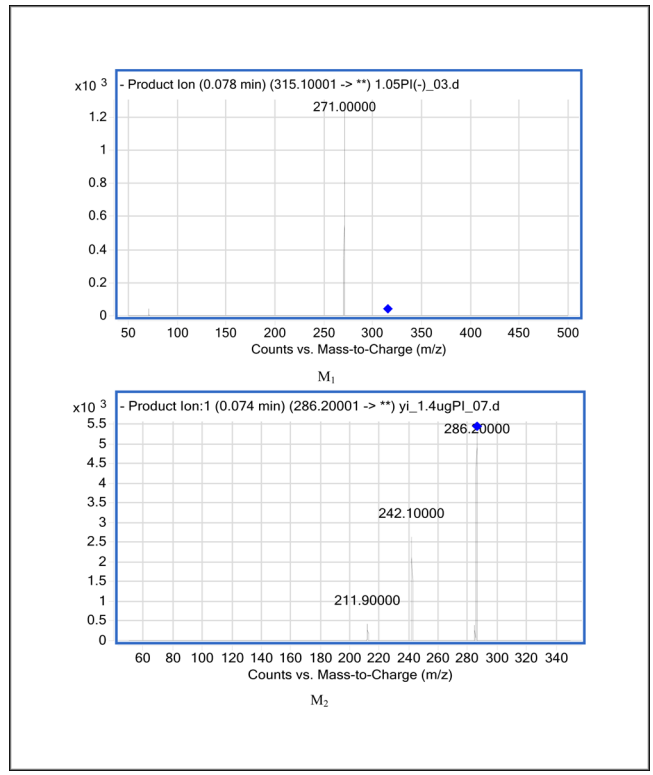

Fig. 1: Product ion mass spectra of: (M_1) febuxostat (m/z 271.0) and (M_2) IS (m/z 242.1)

2.5. Bioequivalence evaluation

The mean plasma concentration-time profiles of febuxostat after oral administration of a single 80-mg dose of test and reference formulations in 18 healthy Chinese male volunteers are shown in Fig. 3.

The pharmacokinetic parameters of febuxostat after an oral administration 80 mg test and reference formulations to 18 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 $AUC_{0\text{-}\infty}$ values for test and reference products,

Table 1: Pharmacokinetic parameters of febuxostat in 18 volunteers after an oral administration 80 mg test and reference formulations (Mean ± SD, n = 18)

Parameters	Test formulation	Reference formulation
$t_{1/2}$, h	5.11(2.26)	4.26(2.16)
T_{max}, h	2.00(1.03)	1.64(1.28)
C_{max}, ng•mL^{-1}	4354.98(1105.22)	4338.39(2077.82)
$AUC_{0\text{-}t}$, ng•mL^{-1}•h^{-1}	15570.28(4642.10)	14310.07(5130.15)
$AUC_{0\text{-}\infty}$, ng•mL^{-1}•h^{-1}	15803.19(4657.49)	14490.72(5163.22)
F, %	114.42 ± 33.48	

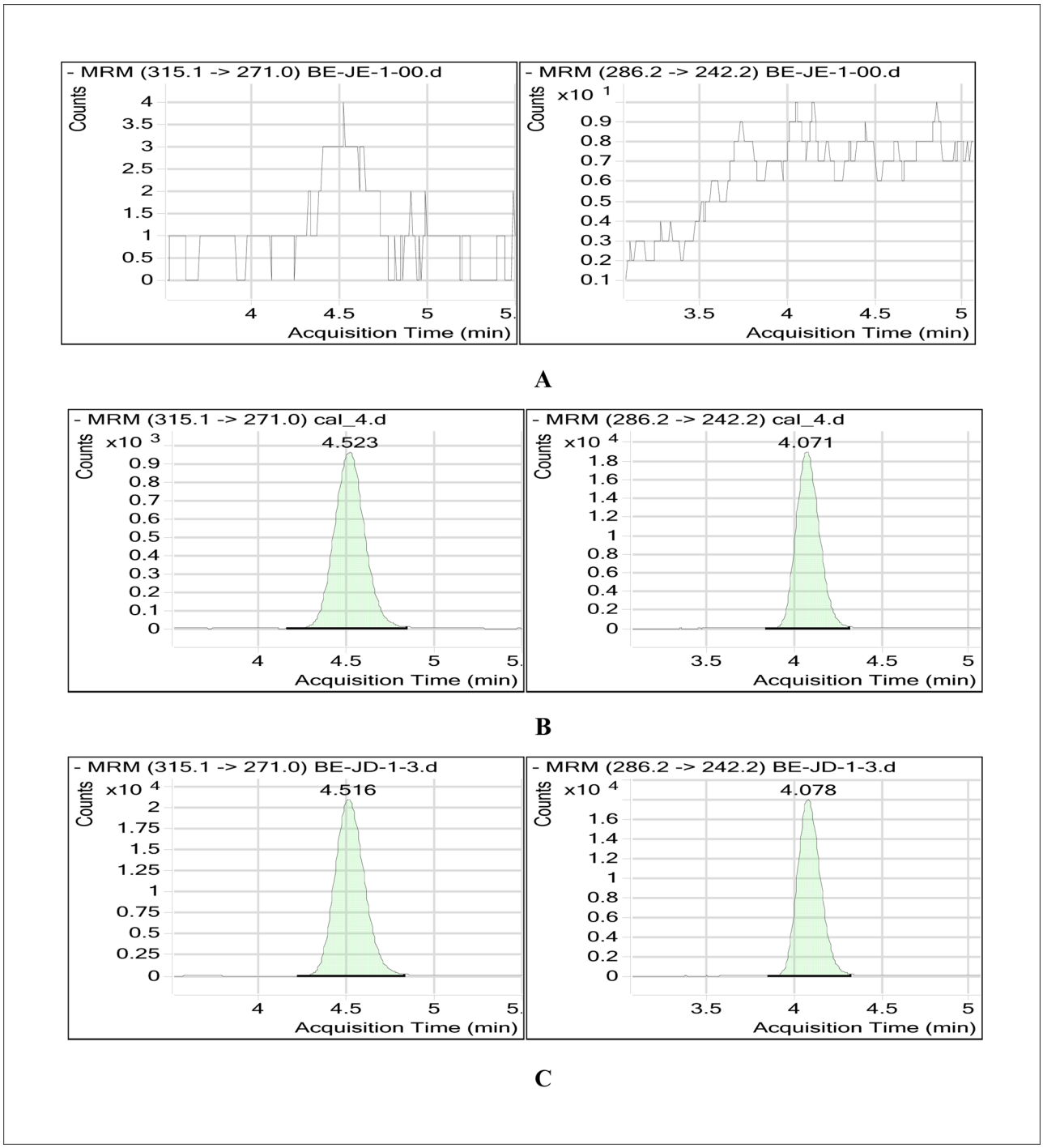

Fig. 2: Representative MRM chromatograms of: (A) blank human plasma of five volunteers, (B) blank plasma spiked with febuxostat(101.8 ng•mL^{-1}) and IS, (C) a study plasma sample of volunteer 1.0 h after an oral administration of 80 mg febuxostat

using logarithmic transformed data, are shown in Table 2. Power of statistical test was 107.8% for C_{max}, 110.3% for $AUC_{0\text{-}t}$ and 110.6% for $AUC_{0\text{-}\infty}$.

3. Discussion

The LC-MS/MS method developed for febuxostat quantification in plasma samples has proved to have good specificity, sensitivity, linearity, precision and accuracy, and was successfully applied to the pharmacokinetic study in human plasma.

Several analytical methods for the determination of febuxostat in human plasma were reported in the literature. Most of these methods were mainly based on the use of high performance liquid chromatography (HPLC) coupled with fluorescence detection (Khosvararan et al. 2006, 2008, 2007). However, all these reported methods show higher ranges in LLOQ and longer retention times (RT), and 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 matrices. The LC-MS/MS we adopted has several advantages compared to the previously reported methods, as it used acetonitrile 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.

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 ($\alpha = 0.05$)

Pharmacokineticparameters	*ANOVA*($p-value$), *variationsource*			*Statisticalpower*(%)	90%*C*.I.
	Product	Period	Group		
C_{max}	0.510	0.556	0.232	107.8	88.7–131.2
$AUC_{0\text{-}t}$	0.128	0.125	0.088	110.3	99.2–122.7
$AUC_{0\text{-}\infty}$	0.117	0.144	0.098	110.6	99.5–123.1

No significant differences in $AUC_{0\text{-}t}$, or C_{max} were found between the test and reference formulations.

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}.

The 90% CIs for the ratio of C_{max} (88.7∼131.2%), $AUC_{0\text{-}t}$ (99.2∼122.7%) and $AUC_{0\text{-}\infty}$ (99.5∼123.1%) values for the test and reference products were all located within the bioequivalence criteria range (80∼125% 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 febuxostat formulations (test and reference) analyzed were bioequivalent in terms of rate and extent of absorption and, thus, may be used interchangeably, with no affection on therapeutic effect.

4. Experimental

4.1. Materials

Febuxostat (100%) was obtained from Hengrui Medicine Co., Ltd. (Jiangsu, China). Etodolac (>99.5%) used as internal standard (IS) was supplied by the Institute for Food and Drug Control (Zhejiang, China). HPLC grade reagents (methanol, acetonitrile) were obtained from Merck (Darmstadt, Germany). Formic acid (>98%) (HPLC grade) was purchased from Aladdin Reagent Ltd. (Shanghai, China). Water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA).The blank human plasma was obtained from the Blood Center of Zhejiang (Hangzhou, China).

The test generic formulation of febuxostat tables (batch no. 1003062) has been supplied by Hengrui Medicine Co., Ltd, Jiangsu, China. The reference formulation was ULORIC® tablet (batch no. 02009AF), which was commercially obtained from Takeda Pharmaceuticals America, Inc., Both formulations were labeled to contain 80 mg febuxostat.

4.2. Instrumentation

Analyses were performed on an Agilent liquid chromatographic system (Agilent RRLC1200, California, USA) consisting of a Gl312B Binary Pump, a Gl379B vacuum degasser, a G1367C autosampler, and a G1316B 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. Chromatographic separation was performed using a ZORBAX SB-C18 column (3.5-μm particle size, 150 × 3.0 mm internal diameter; Agilent Technologies, USA) with a SecurityGuard Inertsil Symmetry C18 column (5-μm particle size, 12.5 × 4.6 mm internal diameter; Waters Sciences, USA).

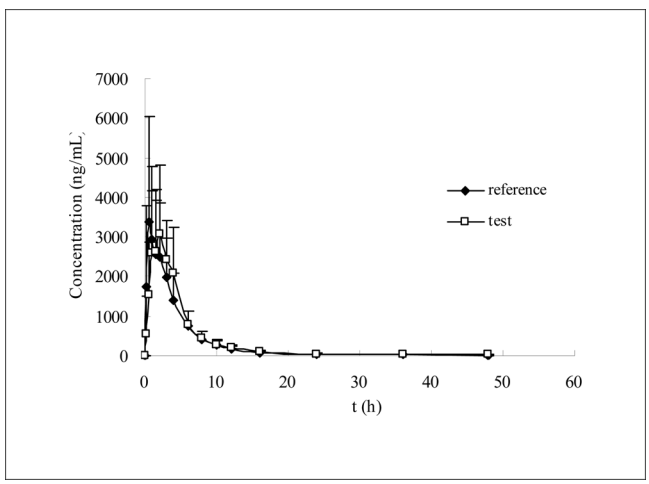

Fig. 3: Mean plasma concentration–time profiles of febuxostat after oral administration of a single 80-mg dose of test and reference formulations in healthy Chinese male volunteers. Data are expressed as mean ± SD (n = 18)

4.3. Chromatographic and mass spectrometric conditions

The isocratic mobile phase consisted of methanol/1% formic acid (80:20; v/v). The flow rate of the mobile phase, the column oven temperature and the UV detective wavelength were set at 0.5 mL• min^{-1}, 25 °C and 210 nm, respectively. The total chromatographic run time was not more than 5.5 min. The HPLC system was connected to the mass spectrometer through an ESI interface and was operated in the negative ion detection mode. The spray voltage was set at 4000 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set at 35 psi (1 psi = 6.895 kPa). Desolvation gas (nitrogen) temperature was set at 350 °C with a flow-rate of 11 L• min^{-1}. High purity nitrogen was used as collision gas with a pressure of 0.1 MPa for collision-induced dissociation (CID). Using multiple reaction monitoring (MRM)-mode for quantification at mass-to-charge ratio (m/z) 315.2→271.0 (fragmentation energy = 90 V, collision energy = 1 V) for febuxostat, and m/z 286.2→242.1 (fragmentation energy = 80 V, collision energy = 10 V) for etodolac.

4.4. Calibration standards and quality control plasma samples

The stock solution of febuxostat was prepared by dissolving the accurately weighed standard compound in acetonitrile to give a final concentration of 1 mg•mL^{-1} and was further diluted into 13.40 to 21440 ng•mL^{-1} in 80% (v/v) methanol aqueous solution for the preparation of standard samples. The concentration of etodolac (IS) working solution was 20 μg•mL^{-1}. All the working standard solutions were stored in polypropylene vials at 4 °C in a freezer and brought to room temperature before use. An aliquot of 20 μL of each febuxostat standard solutions were spiked with 200 μL human control plasma and vortex-mixed for 30 s to yield calibration curve samples at concentrations of 13.4, 53.6, 134, 268, 536, 1340, 2680, 5360, 10720 and 21440 ng•mL^{-1}. Quality control solutions (QCs) of febuxostat were prepared in the same way as the plasma samples for calibration; the concentrations were 26.8, 335.0, 3350.0 and 13400.0 ng•mL^{-1}, respectively. The IS solution was added to each standard sample just prior to sample processing.

4.5. Sample preparation

Frozen human plasma samples (stored at -20 °C) were thawed at ambient temperature and vortexed for 20 s. In a 5.0-mL polypropylene centrifuge tube, an aliquot of 200-μL plasma, 20 μL of IS working solution (20 ng•mL^{-1}), 20 μL of methanol-water solution (80: 20, v:v), and 600 μL of acetonitrile were blended and vortexed for 1 min and then centrifuged at 13000 rpm for 5 min at 4 °C. The supernatant was transferred to autosampler vials and injected into the LC-MS/MS system (volume 10 μL).

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 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.

Eighteen Chinese male volunteers (mean [SD] age, 25.17 [2.62] years; height, 170.17 [4.96] cm; weight, 63.03 [8.64] kg; body mass index (BMI),

21.70 [2.14] kg• m^{-2}) were enrolled in and completed the study. 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. Exclusion criteria included a history of cardiovascular, hepatic, renal, psychiatric, neurologic, hematologic, or metabolic disease; allergic constitution; drug or alcohol abuse within 2 years before the start of the study; smoking; Syphilis, HIV, hepatitis B, or hepatitis C infection; consumption of any prescribed or over-the-counter drugs within 2 weeks before the study; or participation in a similar study within the past 3 months. This was done to ensure that the existing degree of variation would not be due to an influence of illness or other medications. Screening procedures were repeated at the end of the trial.

This study was a single-dose, randomized-sequence, open-label, 2-period crossover study with a 7-day washout period between doses. Eighteen healthy volunteers according to a computer-generated randomization schedule were assigned to one of two treatment sequences (test-reference or reference-test). After an overnight fast of at least 10 h, subjects received a single oral dose of either the test (manufactured by Jiangsu Hengrui Medicine Co., Ltd, China, batch no. 1003062) or reference (manufactured by Takeda pharmaceuticals america, Inc., USA, batch no. 02009AF) formulation of febuxostat 80-mg tablets with 250 mL of water. Within two hours after drug administration, the subjects were not allowed to drink water. Four hours after drug administration, the subjects were allowed to eat a low-fat standard breakfast.

During both treatment periods, blood samples (5 mL) were obtained at administration before and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 24, 36 and 48 h after drug administration, in heparinized tubes. Plasma was directly separated by centrifugation at 3500 rpm for 15 min, removed out and stored in the polypropylene test tube at -70 °C until assayed.

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