Pharmacokinetic Study of Guanfu Base G in Rats by LC–ESI-MS

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A sensitive and simple liquid chromatography–electrospray ionization mass spectrometry method has been established and validated for the quantification of Guanfu base G in rats. Phenoprolamine hydrochloride was selected as the internal standard. Sample preparation involved simple liquid–liquid extraction by ethylacetate with high efficiency. The chromatographic separation was performed on a Shimadzu C18 column (150 × 2.0 mm, 5 μm) with a gradient elution of 0.2% acetic acid–acetonitrile (30:70, v/v). The method was sensitive with the lowest limit of detection at 1 ng/mL (S/N ≥ 3) in 100 μL of rat plasma. Good linearity (r = 0.9996) was obtained covering a concentration of 5–2000 ng/mL. The intra- and interday assay precision ranged from 4.3 to 6.1% and 5.4 to 8.3%, respectively. In addition, the stability, extraction recovery and matrix effect involved in the method were also validated. After intravenous dosing, rat plasma Guangfu base G (GFG) concentration declined in a biphasic manner with a terminal elimination half-life of 3.72 h. The total plasma clearance values were 1.15 L/h/kg. After oral dosing, the plasma GFG concentration reached a maximum within 0.5 h. The absolute bioavailability of GFG was 83.06%.

Introduction

Aconitum coreanum (Lév.) Rapaics (Guanaifu in Chinese) is one of the traditional Chinese herbs. It has been used to treat various kinds of disorders such as cardialgia, facial distortion, epilepsy, migraine headache, vertigo, tetanus, infantile convulsion and rheumatic arthralgia (1). Pharmacological studies and clinical practice demonstrated that its extract has anti-arrhythmia (2), analgesic and anti-inflammatory effects (3). Guangfu base G (GFG) and Guangfu base A (GFA) (chemical structures shown in Figure 1) are diterpenoid alkaloids isolated from the root of A. coreanum (Lév.) (4, 5). They were all esters of C20-diterpenoid, and only differed in the number of acetyl. GFA has been verified that it can prevent arrhythmias (6–9) and is currently undergoing Phase IV clinical study. Compared with GFA, GFG are also anti-arrhythmic and showed a much more powerful effect (10–12). However, up to now, the pharmacokinetics of GFG were not reported. Therefore, we developed a simple, sensitive high-performance liquid chromatography–mass spectrometry (LC–ESI-MS) method for the determination of GFG in rat plasma for the pharmacokinetic study.

Experimental

Chemicals and reagent

GFG (>98% purity) and phenoprolamine hydrochloride (DDPH, >98% purity, internal standard (IS)) were supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile was of HPLC grade (Fisher, Waltham, MA, USA). Ethyl acetate was product of J.T. Baker, Inc. (Philipsburg, NJ, USA). All other chemicals were of the highest quality or analytical reagent grade. Deionized water was purified using a Milli-Q system (Millipore, MA, USA).

Equipment and chromatographic conditions

The HPLC system consisted of two Shimadzu LC-10Advp pumps with a high-pressure mixer, a Shimadzu CTO-10Avp column oven and a Shimadzu SIL-HTC autosampler (Shimadzu, Kyoto, Japan). The Shimadzu 2010A mass spectrometer (Q-array-Octapole- Quadrupole mass analyzer) equipped with an ESI interface was used for MS detection. Nitrogen (99.99%), the carrier gas, was supplied by the Gas Supplier Center of Zhengzhou University (Zhengzhou, Henan, China). Chromatographic separation of analytes was carried out by a Shimadzu C18 column (150 × 2.0 mm, 5 μm), equipped with a C18 guard column (2.1 mm ID × 12.5 mm, Agilent Technologies, Santa Clara, USA), maintained at 40 °C. The mobile phase was 0.2% acetic acid–acetonitrile (30:70, v/v), at a flow rate of 0.2 mL/min.

Mass spectrometric conditions were optimized to obtain maximum sensitivity of the target. The final MS parameters were as follows: gas flow: 1.5 L/min; CDL (curved desolvation line) temperature: 250 °C; block temperature: 200 °C. Detector voltage: 1.65 kV, probe voltage: 4.5 kV; CDL voltage fixed as in Tuning. Mass vacuum was obtained by Turbo molecular pump. Peak areas for all components were automatically integrated using LC/MS solution Version 2.04 (Copyright © 1997–2002 Shimadzu Corp.). The injection volume was 10 μL. The ESI ion source was set in the positive ion polarity mode for acquiring all mass spectrometry data. The selective ion monitoring (SIM) was used, and protonated molecules [M+H]+ were detected at SIM at m/z 472.20 for GFG and m/z 344.25 for DDPH.

Animals and blood sampling

Sprague–Dawley rats (three males, three females) weighing 201.3 ± 18.9 g (mean ± SD) were purchased from Shanghai Experimental Animal Center (Shanghai, China), Chinese Academy of Science, and subjected to the study after a 1-week acclimatization period. The animals were housed in stainless steel metabolic cages equipped with an automated watering valve. They were cared for at a constant temperature of 22 ± 1 °C, a humidity of 50 ± 20%, 12 h light/12 h dark cycle and 10–15 air changes per hour according to the regulations of the Animal Committee. Diet

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was prohibited for 12 h before the experiment, while water was taken freely. After starving overnight, Guanfu base G hydrochloride was administered intravenously (i.v.) via the intravenous route and oral at doses of 5 mg/kg to rats for the plasma concentration–time course study. Blood samples were collected in 1.5 mL Eppendorf tubes (containing sodium heparin) via the eye venous plexus at specified time intervals after dosing. All experimental procedures were carried out in accordance with the guidelines of the Animal Ethics Committee of Zhengzhou University (No. 40 of Daxue road, Zhengzhou Henan, The medical campus of Zhengzhou University. Permission number: 2012014). The animal experiment was carried out under the basic principles of Zhengzhou University experimental animal ethics review. The content includes the principle of the unity of science ethics, experimental animal protection principle, the experimental animal welfare principles and ethics review supervision principle. The expiration date of permission is 13 January 2014.

**Sample preparation**

**Standard solution, calibration curve and quality control samples**

The standard stock solution of 1 mg/mL of GFG was prepared in doubly distilled water. A series of standard working solutions with concentrations in the range 5–2000 ng/mL were obtained by further dilution of the standard stock solution with the mobile phase. The IS stock solution of 100 μg/mL of DDPH was prepared in doubly distilled water. Internal standard working solution (5 μg/mL) was prepared by diluting IS stock solution with the mobile phase. All solutions were stored at 4°C. A series of standard working solutions were evaporated to dryness in a Thermo Savant SPD 2010 SpeedVac system (Thermo Electron Corporation) set at 30°C. The residue was reconstituted in 0.1 mL of blank plasma to prepare the calibration standards containing 2000, 1000, 500, 200, 100, 50, 20, 10 and 5 ng/mL.

Quality control (QC) samples were prepared in the same way as calibration standards with blank plasma. QC sample low, medium and high concentrations were 5, 100 and 2000 ng/mL, respectively, and samples were stored at −20°C until analysis.

**Sample extraction**

Each collected blood sample was immediately centrifuged at 1500 × g for 10 min at 4°C (Himac CT 13R, Hitachi Co., Japan). The resulting plasma (0.1 mL) and the IS solution 20 μL (5 μg/mL) and 40 μL saturated Na₂CO₃ were added to an Eppendorf tube. The sample was mixed and then extracted with 1 mL of ethyl acetate using vortex for 3 min and centrifuged at 3000 × g for 5 min. The top organic layer 0.5 mL was transferred to a new Eppendorf tube and evaporated to dryness in a Thermo Savant SPD 2010 SpeedVac system (Thermo Electron Corporation) set at 30°C. The residue was reconstituted in 0.1 mL of mobile phase and centrifuged at 10 000 × g at 4°C for 10 min. The supernatant (10 μL) was transferred to an MS vial for LC–MS analysis.

**Validation of the method**

**Specificity**

The specificity of the method was investigated by analyzing drug-free rat plasma (without IS nor analytes) for the exclusion of any endogenous co-eluting interferences at the peak region of each analyte and IS.

**Linearity**

Linearity of calibration was tested by extraction and assayed (n = 5). Calibration curves in the concentration range 5–2000 ng/mL were constructed by plotting the peak-area ratios of analyte/IS versus GFG concentration in rat plasma. Weighted (1/conc²) least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. The concentration of GFG in plasma was determined from the peak-area ratios by using the equations of linear regression obtained from the calibration curves.

**Lower limit of quantification**

The lower limit of quantification (LLOQ) was defined as the lowest concentration at which the signal-to-noise (S/N) ratio was ≥10 and both the precision and accuracy were ≤20% by analyzing the six replicates of samples spiked with each analyte.

**Recovery**

The extraction recovery (absolute recovery) was determined by measuring an extracted sample against a postextraction spiked sample and expressed as the ratio of the peak-area responses. The recovery experiments were performed with three QC concentrations (low, medium and high QC concentrations), with triplicate determinations at each concentration.

**Precision and accuracy**

Intraday accuracy and precision (each n = 5) were evaluated by analysis of QC samples at different times during the same day. Interday accuracy and precision were determined by repeated analysis of QC samples over five consecutive days (n = 1 series per day). The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy of the method was determined by the equation: (mean of determined concentration − actual concentration)/actual concentration × 100%. Precision was determined by the coefficients of variation (CVs). The intra- and interday precision and bias were set at ≤15%, except that at LLOQ, they were set at ≤20%.

**Matrix effect**

The matrix effect on the ionization efficiency of each analyte was evaluated by comparing the peak response of analytes dissolved in blank sample extract (i.e. the final solution obtained from blank plasma after extraction and reconstitution) with...
those for analytes dissolved to the same concentrations in deionized water. The experiment was performed in triplicate for each QC concentration. If the peak-area ratios for the plasma extracts versus deionized water were \(< 85\%\) or \(> 115\%\), a matrix effect was implied.

**Stability**

The three freeze-thaw cycles stability for the samples going through three cycles of storage at \(-20\,^\circ\text{C}\) for 24 h and thawing at room temperature, the compound stability for 8 h at room temperature and placed in the autosampler at \(4\,^\circ\text{C}\) for 24 h were evaluated by repeated analysis \((n = 3)\) of QC samples. Long-term stability in plasma was also tested by assaying frozen QC samples after storage at \(-20\,^\circ\text{C}\) for 2 weeks. The amount of them in the plasma samples was determined using a newly prepared calibration curve. Stability was expressed as a percentage of nominal concentration.

**Pharmacokinetic study design**

The present method was used to determine concentration time profiles of GFG in rat plasma after i.v. and oral administration of 5 mg/kg GFG. The blood samples (~0.25 mL) were collected into heparinized tubes at 5, 15, 30, 45, 60, 120, 240, 360, 480, 600, 720 and 960 min. Following centrifugation at 1500 \(\times g\) for 10 min, resultant plasma was separated and stored at \(-20\,^\circ\text{C}\) until analysis. Plasma (100 µL) was then extracted and analyzed by the same procedure as that of calibration samples.

**Results**

Figure 2 shows the mass spectra of GFG and IS, respectively. SIM chromatograms for standard solutions, blank plasma, extracts of spiked blank plasma and rat plasma samples after i.v. administration of GFG are shown in Figure 3. The retention times of GFG and the IS DDPH were 2.57 and 4.21 min, respectively.

The analytical method for rat plasma sample concentration of GFG showed a non-significant matrix effect in rats.

Tables I, Table II and Table III show the confidence limits of the analytical methods for the determination of plasma concentration of GFG. The validated method was successfully applied to pharmacokinetic study in rats after a single intravenous and oral administration of GFG. The mean plasma concentration vs time profile is illustrated in Figure 4. The main pharmacokinetic parameters were obtained: Maximum concentration \((C_{\text{max}})\) and time to maximum concentration \((T_{\text{max}})\) were the experimentally observed values following oral administration of GFG. The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule. AUC\(_t\) was calculated from time 0 to 8 h and AUC\(_\infty\) from time 0 to \(\infty\). The terminal elimination half-life \((T_{1/2})\) was obtained by dividing \(\ln 2\) by \(b\) (\(b\) is the absolute value of the slope of the least-square regression line for the terminal datum point). The absolute bioavailability (F%) was estimated from the dose-normalized ratios of [AUC\(_\infty\)] oral administration to [AUC\(_\infty\)] i.v. Plasma clearance (CL) was calculated as CL = dose/AUC\(_\infty\). The estimated pharmacokinetic parameters are shown in Table IV.

**Discussion**

**Chromatography and mass spectrum**

It is clear that the analytes and IS formed predominantly protonated molecules \([M+H]^+\) in full scan spectra and protonated...
administration of GFG are shown in Figure 3A–D, respectively. No sodium or other solvent adducts or dimmers were observed. The retention times of GFG and the IS DDPH were 2.57 and 4.21 min, respectively. Under the chromatographic conditions, there were no endogenous plasma components interfering with them. The overall chromatographic run time was finished within 5 min. Ion suppression was investigated and was not detected in the assay.

**Calibration curves**

GFG was dissolved in mobile phase and diluted to give a series of standard solutions for the calibration curves of the drug in rat plasma. The linear regression analysis of GFG was constructed by plotting the peak-area ratio of GFG to the IS (y) versus analyte concentration (ng/mL) in spiked plasma samples (x). The calibration curves were constructed in the range 5–2000 ng/mL. The regression equation of these curves and their correlation coefficients (r) were calculated as follows: \( y = 0.00398x - 0.0146 \) (r = 0.9993); it showed good linear relationships between the peak areas and the concentrations. Signals 3 and 10 times higher than the peak-noise height were regarded as the detection and quantification limits. The detection limit of GFG was 1 ng/mL (S/N ≥ 3), and the lower quantification limit was 5 ng/mL (S/N ≥ 10).

**Recovery**

The extraction recoveries from the rat plasma were determined at concentrations of 5, 100 and 2000 ng/mL in triplicate. The recoveries of GFG from rat plasma were shown in Table I. The mean recoveries of GFG were >80%. The %CV for recoveries was all <10%.

**Accuracy and reproducibility**

Analytical accuracy and precision data were shown in Table II. The reproducibility of the method was defined by examining both intra- and interday variance. The intraday precision (CV) of the assay was <6.1% for each of the three concentrations of the QC samples; assay accuracy was in the range 95–101%. The interday precision (CV) of the assay was <8.3% for all the QC samples, and assay accuracy was in the range 96–102%. At these concentrations, the intra- and interday CVs were determined to be <10%, and the accuracy was 95–102%. These results suggest that the present method is accurate, precise and reproducible for detecting GFG over the tested concentration ranges.

**Matrix effect**

The possibility of matrix effect caused by ionization competition between the analyte and the endogenous co-eluents was evaluated at three QC concentrations in triplicate. The results of matrix effect were acquired from comparing the peak responses of the postextraction spiked samples with those of the standard solution and suggested negligible matrix effect under the developed sample preparation and chromatographic conditions.
Stability

All stability results are shown in Table III. GFG was stable for at least 8 h at room temperature, in the autosampler at 4°C for 24 h, at −20°C for a 2-week period and following three freeze–thaw cycles. No significant degradation of GFG was observed during all of the sample storage, preparation and analysis periods.

The method described above is also outstanding with respect to simplicity in the samples pretreatment and short run time. These results indicated that the assay was simple, accurate, sensitive and reproducible.

Pharmacokinetic study

GFG and GFA have the same molecular structure and differ only in the number of acetyl. Therefore, the pharmacokinetic behavior of GFG and GFA in rats is probably similar. However, the pharmacokinetics of GFA in rats have not been reported. But the main pharmacokinetic parameters of GFA after i.v. injection in dogs and humans were similar; however, T1/2 was different, with the T1/2 in dogs (14 h) being longer than in humans (8 h) (13, 14). One reason probably is different race.

The pharmacokinetic behavior of a drug has to be considered during the drug discovery process in order to find compounds that will reach their site of action and maintain their concentration long enough to be able to produce the desired effect. According to the test results, the pharmacokinetic behavior of GFG is better in rats, the absolute bioavailability of GFG was 83.06% and T1/2 was 3.87 h. These results indicate that GFG has a relatively higher bioavailability and suitable elimination half-life and prompted that GFG has good absorption after orally administered and has lower first pass metabolism. As a promising anti-arrhythmias agent, GFG probably can be orally administered in clinic therapy. Further research in other animals and humans is under way.

Conclusion

A specific, sensitive, rapid, precise and accurate LC–ESI-MS assay for the determination of GFG in rat plasma was established. The simple liquid–liquid extraction procedure and short run time can curtail the cost and time of the test, which enables the analysis of large numbers of plasma samples. The assay was validated to meet the requirements of pharmacokinetic studies.

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