A Rapid and Simple UPLC–MS-MS Method for Determination of Glipizide in Human Plasma and Its Application to Bioequivalence Study

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In this study, a simple, rapid and sensitive ultra performance liquid chromatography–tandem mass spectrometry method is described for the determination of glipizide in human plasma samples using carbamazepine as the internal standard (IS) from bioequivalence assays. Sample preparation was accomplished through protein precipitation with methanol, and chromatographic separation was performed on an Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm) with gradient profile at a flow rate of 0.4 mL/min. Mass spectrometric analysis was performed using an QTrap5500 mass spectrometer coupled with an electrospray ionization source in the positive ion mode. The multiple reaction monitoring transitions of m/z 446.1 → 321.0 and m/z 237.1 → 194.2 were used to quantify for glipizide and IS. The linearity of this method was found to be within the concentration range of 10–1,500 ng/mL for glipizide in human plasma. Only 1.0 min was needed for an analytical run. The method was applied to a bioequivalence study of two drug products containing glipizide in human plasma samples.

Introduction

Diabetes mellitus type 2 (Type 2 DM), also known as non-insulin-dependent DM, is a metabolic disorder characterized by high blood glucose resulting from insulin resistance and relative insulin deficiency. Drug treatment is the most effective therapeutic method for Type 2 DM, and a variety of antidiabetic agents have been approved for the treatment of this disease all over the world. Glipizide is a second-generation sulfonylurea that can stimulate insulin secretion of islet β-cells (1–3). It is a potent, rapid acting with short duration of action and well tolerated second-generation sulfonylurea effective in reducing post-prandial glucose levels (4).

During antidiabetic therapy using this preparation, it is of critical importance to monitor the plasma concentration of glipizide and investigate its pharmacokinetic in the human body (5, 6). To develop novel formulations, bioequivalence studies of glipizide are also required (7). Therefore, selective, sensitive and rapid methods are urgently required to determine glipizide in human plasma.

Several analytical methods have been reported to determine glipizide in biological samples, including radioimmunoassay (8), high-performance liquid chromatography (HPLC) (9, 10) and HPLC–MS-MS (6, 11, 12). These methods suffer from several disadvantages, such as complicated sample preparation by solid-phase extraction, low sensitivity, long running time, obvious matrix effect, large volume of plasma or injection volume. The negative factors may limit the application of these methods to high-throughput analysis in biological samples.

In this paper, a selective, sensitive and rapid ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS-MS) method was developed to measure glipizide in human plasma. The sample preparation of this method was simple one-step precipitation of plasma protein by methanol, which was time- and effort saving. The analysis time and the sensitivity could also meet the requirements of high-throughput bioanalysis. Once developed and validated, this method was successfully used for bioequivalence investigation of two different glipizide in 20 healthy Chinese volunteers.

Experimental

Chemicals and reagents

The standard reference material of glipizide was provided by Hainan Zambon Pharmaceutical Co., Ltd (Hainan, China). Test drug of glipizide was provided by Jiangxi Wanji Pharmaceutical Research Institute Co., Ltd (Jiangxi, China). Carbamazepine (purity >98.0%) was purchased from Sigma (St. Louis, MO, USA). LC-grade acetonitrile and methanol were from Amethyst Chemicals. Formic acid was an analytical grade and purchased from the Beijing Chemical Reagents Company (Beijing, China). Blank human plasma was obtained from The First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). Ultrapure water (resistance >18 mΩ) was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA).

Instrumentation and conditions

LC was performed on an Acquity UPLC unit (Waters Corp., Milford, MA, USA) with an Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm particle size) and an inline 0.2-μm stainless steel frit filter (Waters Corp.). The mobile phase consisted of acetonitrile and water containing 1% formic acid. A gradient elution was used, and the gradient profile can be seen in Table I. The flow rate was 0.4 mL/min. The overall run time was 1.0 min.

An AB Sciex QTRAP 5500 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Toronto, ON, Canada) was used for mass spectrometric detection. The detection was operated in the multiple reaction monitoring (MRM) mode under unit mass resolution (0.7 amu) in mass analyzers. The dwell time was set to 250 ms for each MRM transition. The MRM transitions were m/z 446.1 → 321.0 and m/z 237.1 → 194.2 for glipizide and internal standard (IS),
respectively. Figure 1 shows the product ion mass spectra of the analytes. After optimization, the source parameters were set as follows: curtain gas, 35 psig; nebulizer gas, 50 psig; turbo gas, 60 psig; ion spray voltage, 3.0 kV and temperature, 350 °C. Data acquiring and processing were performed using analyst software (version 1.5, AB Sciex).

Preparation of standard and quality control samples

The stock solution of glipizide that was used to make the calibration standards and quality control (QC) samples was prepared by dissolving 10 mg in 10 mL of methanol to obtain a concentration of 1.00 mg/mL. The working solutions for calibration and QCs were made from the stock solution by diluting with methanol. Calibration curve standards were prepared by spiking blank human plasma with appropriate amounts of the working solutions at final drug concentrations of 10, 25, 50, 100, 250, 500, 1,000 and 1,500 ng/mL for glipizide. The preparation of QC samples was the same, with the three levels of plasma concentrations (20, 400 and 1,200 ng/mL). IS stock solution was made at an initial concentration of 1 mg/mL. The IS working solution (200 ng/mL) was made from the stock solution using methanol for dilution. All the solutions were stored in a refrigerator at 4 °C.

Sample preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5-mL centrifuge tube, an aliquot of 400 μL of the IS working solution (100 ng/mL in methanol) was added to 200 μL of collected plasma samples. The tubes were vortex mixed for 60 s and centrifuged at 13,000 rpm for 10 min to precipitate the plasma protein. The supernatant (10 μL) was injected into the UPLC–MS-MS system for analysis.

Method validation

Specificity was determined by the analysis of blank human plasma samples from six different volunteers, and every blank sample was handled by the procedure described in “Sample Preparation” and confirmed that endogenous substances did not have the possible interference with the analyte and the IS.

Plasma samples were quantified using the calibration curve. Calibration curves were validated by analyzing spiked calibration samples on 3 days in a row. The peak area ratio of glipizide to IS was plotted against analyte concentrations, and standard curves were fitted by weighted (1/x²) least-squares linear regression in the concentration of 10–1,500 ng/mL for glipizide. A correlation of >0.99 was desirable for all the calibration curves. The sensitivity of the method was determined by quantifying the lower limit of quantification (LLOQ). An LLOQ was defined as the lowest acceptable point in the calibration curve that had an acceptable precision and accuracy.

To determine the matrix effect, six different blank plasma samples were utilized to prepare QC samples and used for assessing the lot-to-lot matrix effect. Matrix effect was evaluated at three QC levels by comparing the peak areas of analytes obtained from plasma samples spiked with analytes after extraction to those of the pure standard solutions at the same concentrations. The matrix effect of IS was evaluated at the working concentration (200 ng/mL) in the same manner.

The extraction recoveries of glipizide at three QC levels (n = 6) were determined by comparing the 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 extraction recovery of the IS at the working concentration (200 ng/mL) was determined in a similar way as a reference.

The intraday precision and accuracy of glipizide were evaluated by analyzing QC samples (20, 400 and 1,200 ng/mL) with six replicates for each concentration. The interday precision and accuracy were evaluated by analyzing QC samples with six replicates for each concentration over 6 days. The precision was expressed by coefficient of variation (CV) and the accuracy by relative error (RE).

The stabilities of glipizide in human plasma were tested by analyzing five replicates of plasma samples at three concentration levels (20, 400 and 1,200 ng/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 autosampler at room temperature for 24 h. The freeze–thaw stability was evaluated after three complete freeze–thaw cycles (−20 to 25 °C) on consecutive days. The long-term stability was assessed after storage of the standard-spiked plasma samples at −20 °C for 14 days.

Bioequivalence study

The validated method was successfully applied to the assay of glipizide in healthy human subjects who received reference and test formulation. The design of study comprised of a randomized, open-label, single-dose, two treatment, two sequence bioequivalence study of glipizide (10 mg) tablet in 20 normal
healthy subjects under condition of fasting. The study was conducted at the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) in accordance with the principles of the Declaration of Helsinki after receiving approval from the independent ethics committee in the hospital. All the subjects were informed of the aim and risk involved in the study, and written consent was obtained. The study was conducted strictly in accordance with guidelines laid down by the International Conference on Harmonization and USFDA. Health checkup for all subjects was done by general physical examination, ECG and laboratory tests like hematology, biochemistry and urine examination. All subjects were negative for HIV, HBsAg and HCV tests. They were orally administered a single dose of test and reference formulation after recommended wash out period with 200 mL of water. Drinking water was not allowed, and supine position was restricted 2 h postdose. Standardized meals were provided as per schedule. Blood samples (5 mL) were collected in tubes containing heparin at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14 and 24 h of administration of drug. Blood samples were centrifuged at 3,000 rpm for 10 min, and plasma was separated and stored at −20°C until use. The pharmacokinetic parameters of glipizide were calculated by non-compartmental analysis using the computer program DAS (DAS V3.0, Medical University of Wenzhou, China).

Results

Specificity

UPLC chromatograms of human plasma showed that the retention times for glipizide and IS were ∼0.62 and 0.37 min, respectively. Figure 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with glipizide 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 and sensitivity

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range of 10−1,500 ng/mL for glipizide in human plasma. The typical equations of the calibration curve were as follows: $y = 571.973x + 4.747$, $r = 0.9997$, where $y$ represents the ratio of peak area to that of IS, and $x$ represents the plasma concentration. The LLOQ was determined to be 10 ng/mL for glipizide.

Matrix effect and recovery

To avoid interference from exogenous compounds co-eluted with the target compound, MS-MS detection was performed which offers unique selectivity against matrix background and requires very limited sample preparation. Ionization of analytes was carried out using the ESI technique with positive polarity and MRM mode. In order to further reduce the matrix effect, we took only 100 μL of the plasma sample was precipitated with methanol, injecting 10 μL. The matrix effect for glipizide at concentrations of 20, 400 and 1,200 ng/mL and IS (200 ng/mL) were shown in Table II. As a result, matrix effect from plasma was negligible in this method. The recovery was calculated by comparing the mean peak areas of analytes spiked before extraction divided by the areas

Figure 2. Representative UPLC–MS/MS chromatograms for glipizide and carbamazepine (IS) in human plasma samples: (A) blank plasma sample; (B) blank plasma sample spiked with glipizide and IS; (C) human plasma sample 1.5 h after oral administration of single dosage 10 mg glipizide.
of analytes samples spiked after extraction and multiplied by 100%. Results are shown in Table II. The recovery in plasma ranged from 86.9 to 88.2% for glipizide. The recovery of IS (200 ng/mL) in plasma was 84.8%.

### Precision and accuracy

The precision of the method was evaluated by calculating CV for QCs at three concentration levels (20, 400 and 1,200 ng/mL) over 3 validation days. The intraday precision for glipizide was 6.36% or less, and the interday precision was 8.75% or less at each QC level.

The accuracy of the method ranged from −8.78% to 10.4% for glipizide at three QC levels. Assay performance data are presented in Table III. The above results demonstrated that the values were within the acceptable range and the method was accurate and precise.

### Stability

All the stability studies of glipizide in plasma were conducted at three concentration levels (20, 400 and 1,200 ng/mL) with five determinations for each under different storage conditions. The CV of the mean test responses was within 15% in all stability tests of glipizide in plasma.

No effect on the quantitation was observed for plasma samples kept at room temperature for 4 and 24 h in an autosampler. There was also no significant degradation when samples of glipizide in plasma were taken through three freeze (−20°C)–thaw (25°C) cycles. It was also stable at −20°C for 14 days.

### 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 glipizide under condition of fasting. Mean plasma concentration–time curve of glipizide, after oral administration of 10 mg to 20 healthy volunteers, is shown in Figure 3. Bioequivalence studies protocols generally recommend plasma sample collection for a time period corresponding to three to four times the drug plasma elimination half-life, which brings terminal concentration values of ~6% of the peak concentration value; for glipizide, mean peak plasma concentration of ~1,000 ng/mL (Figure 3) will produce plasma concentrations after a time period corresponding to four glipizide half-lives of ~60 ng/mL. Since the method LLOQ was 10 ng/mL, its sensitivity is adequate for bioavailability studies. The 90% confidence intervals for geometric mean ratio glipizide test/reference drug ranged from 94.3% to 100.4% (point estimate 96.0%) for $C_{max}$ from 98.3 to 107.4% (point estimate 102.8%) for $AUC_{0-24}$ and from 98.9 to 108.6% (point estimate 103.6%) for $AUC_{0-\infty}$. The 90% confidence interval of the individual ratio geometric mean for test/reference was within 80–125% for $AUC_{0-24}$, $AUC_{0-\infty}$ and $C_{max}$. These findings suggest that the drugs are bioequivalent. Main pharmacokinetic parameters of glipizide in 20 healthy volunteers after single-dose administration of 10 mg test and reference tablets are presented in Table IV.

### Discussion

This method was intended for rapid quantification of the plasma concentrations after glipizide exposure. A simple one-step protein precipitation procedure was chosen to shorten the sample preparation time. The optimization of the procedure was obtained after testing several precipitating solvents and different solvent compositions. The results with the best recoveries and...
lowest matrix effects for the analytes were achieved with the solvent of methanol. High-speed centrifugation at 13,000 rpm helped to remove tiny particles and to increase the service life of the column.

A number of commercially available UPLC columns and various mobile phases were evaluated for its chromatographic behavior and the ionization response of glipizide. The best response was obtained from acetonitrile and water (containing 0.1% formic acid). The addition of 0.1% formic acid to the mobile phase increased the sensitivity of glipizide. The gradient elution mode was applied in chromatographic separation and showed a better peak shape and appropriate retention time. A Waters Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm) with isocratic delivery provided satisfactory chromatographic results with minimal matrix effects. In this assay, no significant signal suppression or enhancement was found using the current conditions. The whole separation of the analyte and IS was completed within only 1.0 min per sample. Glipizide and IS were eluted at ~0.62 and 0.37 min, respectively.

Conclusions

In this study, a selective, sensitive and rapid UPLC–MS-MS method was developed for the determination of glipizide in human plasma. The sample pretreatment was a single-step protein precipitation with methanol, which was simple and effort saving. The method offered satisfactory selectivity, good sensitivity and a short-run time of 1.0 min per sample. The method has been successfully applied to a bioequivalence study of glipizide administered in tablet form to Chinese healthy volunteers.

References

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