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Short communication

Rapid determination of metformin in human plasma by liquid chromatography−tandem mass spectrometry method

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Abstract

A rapid, sensitive and specific liquid chromatography−tandem mass spectrometry method is described for quantitation of metformin in human plasma. After a simple, one-step protein precipitation using acetonitrile, metformin and the internal standard diphenhydramine were chromatographed on a C_8 column and detected by tandem mass spectrometry. An atmospheric pressure chemical ionization interface was chosen to reduce ion suppression from sample matrix components and provide high sensitivity. The method has a chromatographic total run time of 3.4 min and was linear within the range 2–2000 ng/ml. Intra- and inter-day precision, expressed as the relative standard deviation (R.S.D.), ranged from 4.4 to 5.7% and from 1.3 to 2.8%, respectively. Assay accuracy was less than 1% in terms of %RE (relative error). The assay was used to evaluate the pharmacokinetics of metformin after an oral administration of multicomponent formulation containing 500 mg metformin and 2.5 mg glyburide to 20 healthy volunteers.

Keywords: Metformin

1. Introduction

Metformin is an oral antihyperglycaemic agent used in the management of non-insulin-dependent diabetes mellitus (type 2 diabetes). It reduces blood glucose levels, predominantly by improving hepatic and peripheral tissue sensitivity to insulin without affecting the secretion of insulin [1–3]. Results of the United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that intensive blood glucose control with metformin reduces the risk of diabetic complications and death in overweight patients with type 2 diabetes [4].

Gastrointestinal absorption of metformin is incomplete with an absolute bioavailability of 50–60% and approximately 90% of the absorbed drug is eliminated via renal route with a plasma elimination half-life of approximately 2–6 h [5,6]. To improve therapeutic effect and to reduce side-effect, a combination formulation of metformin and glyburide (a second-generation sulfonylurea), and a sustained-release formulation of metformin have been successfully used to treat patients with type 2 diabetes [7,8]. To address the pharmacokinetics of these new formulations, a sensitive method that allows an accurate measurement of low concentrations of metformin in plasma is required.

Various analytical methods have been described for the measurement of metformin in biological fluids, including GC with electron-capture [9,10] or mass spectrometry detection [11], capillary electrophoresis (CE) [12] and HPLC with UV detection [13–19]. Due to high polarity of metformin, the GC methods required a complex and time-consuming derivatization procedure [9–11], while CE method used an ion-pair extraction to remove ionic substances from plasma samples [12]. The reported HPLC methods also suffered from several disadvantages, such as lack of sensitivity [13–15], using complex extraction procedures which needed protein precipitation with evaporation of the supernatant or two-step extraction including protein precipitation and liquid-liquid extraction [16,17] or solid-phase extraction [18], requiring the use of ultrafiltration and column-switching system [14] and requiring long chromatographic times [19].

In this paper, we reported a relatively simple and rapid liquid chromatography−tandem mass spectrometry (LC−MS−MS) method to determine metformin in human plasma using one-step protein precipitation without evaporation.

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The method with a lower limit of quantification of 2 ng/ml offered increased sensitivity, compared with existing methods. It was successfully applied to a pharmacokinetic study after an oral administration of multicomponent formulation containing 500 mg metformin hydrochloride and 2.5 mg glyburide to 20 healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Metformin hydrochloride (99.6% purity) and diphenhydramine hydrochloride (internal standard, IS, 99.4% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and methanol were of HPLC-grade, and other chemicals used were of analytical grade. Blank (drug free) human plasma was obtained from Shenyang Blood Donor Service (China). Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrumentation

A Shimadzu LC-10ADvp pump (Kyoto, Japan) and a Agilent 1100 autosampler (Wilmington, DE, USA) were used for solvent and sample delivery. A Finnigan TSQ (API II) triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (San Jose, CA, USA) was used for mass analysis and detection. A six-port switching valve (Rheodyne, Cotati, CA, USA) was used to direct HPLC eluate to a waste container in the first 2.0 min of the chromatographic run and afterwards to the ionization source. Data acquisition was performed with Finnigan Xcalibur 1.1 software.

2.3. Chromatographic conditions

Chromatographic separation was achieved using isocratic conditions on a Zorbax SB C_8 column (150 mm × 4.6 mm i.d., 5 μm, Agilent, Wilmington, DE, USA) with a 4 mm × 3.0 mm i.d. SecurityGuard C_{18} (5 μm) guard column (Phenomenex, Torrance, CA, USA). The column temperature was maintained at 25 °C. The mobile phase consisted of acetonitrile−water−formic acid (70:30:1, v/v) at a flow-rate of 0.5 ml/min.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in the positive mode. The tuning parameters were optimized for metformin and IS by infusing a solution containing 4 μg/ml of both analytes in the mobile phase (0.5 ml/min) at a flow-rate of 10 μl/min using a post-column "T" connection. The sheath and auxiliary gases (nitrogen) were set at 80 psi and 3 l/min, respectively. The optimized temperatures of the vaporizer and heated capillary were 450 and 250 °C, respectively. For collision-induced dissociation (CID), argon was used as the collision gas at a back-pressure of approximately 0.19 Pa. Quantitation was performed using selected reaction monitoring (SRM) of the transitions *m/z* 130 → 60 for metformin and *m/z* 256 → 167 for IS, respectively, with a dwell time of 200 ms per transition. The optimized collision energy of 15 eV was used for the analyte and 25 eV for IS. The mass spectrometer was operated at unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3.

2.5. Preparation of stock and sample solutions

The solution concentrations of the analyte and IS were calculated as free base. A stock solution of metformin was prepared by dissolving the accurately weighed reference compound in methanol to give a final concentration of 400 μg/ml. The solution was then serially diluted with methanol to achieve standard working solutions at concentrations of 0.01, 0.02, 0.06, 0.2, 0.8, 2.0, 5.0 and 10.0 μg/ml for metformin. A 0.5 μg/ml internal standard working solution was prepared by diluting the 400 μg/ml stock solution of diphenhydramine with acetonitrile. Structural formulae of metformin and the internal standard are shown in Fig. 1.

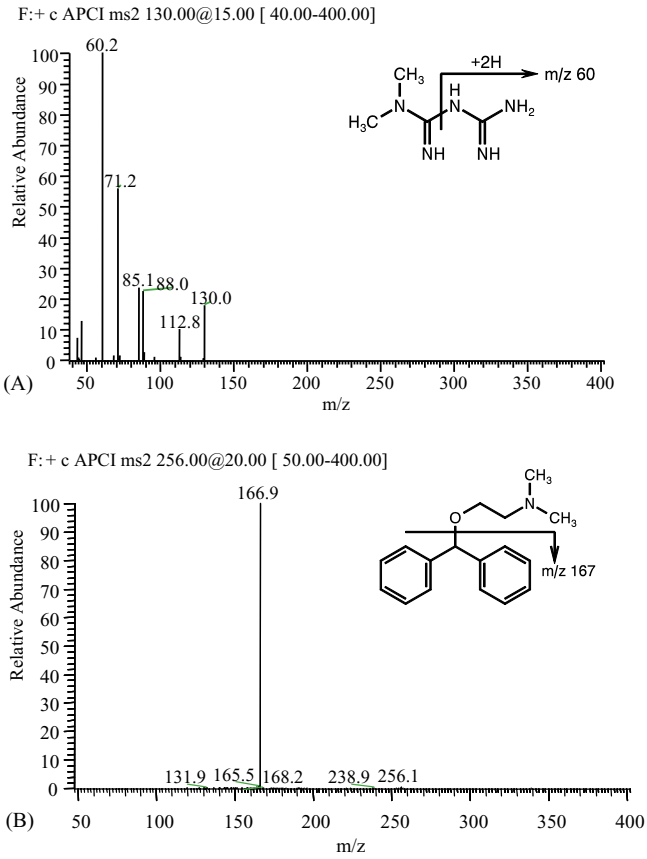

Fig. 1. Product ion mass spectra of $[M + H]^+$ of metformin (A) and diphenhydramine (B).

All the solutions were stored at 4 °C and were brought to room temperature before use.

For preparation of standard curves or quality control, the standard working solutions of metformin (50 μl) were used to spike blank plasma samples (250 μl), both in prestudy validation and during the pharmacokinetic study.

2.6. Sample preparation

The plasma samples of metformin were treated as published [19]. To a 250 μl aliquot of plasma were added 100 μl of internal standard (0.5 μg/ml in acetonitrile) and 50 μl of methanol. The sample mixture was vortexed briefly and 400 μl of acetonitrile was added to precipitate the protein. The mixture was vortexed for 30 s, centrifuged at 2000 × *g* for 10 min, and 20 μl of clear supernatant was directly injected onto the LC−MS−MS system.

2.7. Method validation

Plasma samples were quantified using the ratio of the peak area of metformin to that of IS as the assay parameter. Peak area ratios were plotted against concentrations and metformin concentrations were calculated using a weighted ($1/x^2$) least squares linear regression.

During prestudy validation, plasma standard curves were prepared and assayed in triplicate on three separate days. The linearity of the standard curves was evaluated through least-squares linear regression analysis of peak area ratios of metformin/IS versus metformin concentrations in spiked plasma samples. Accuracy and precision were also assessed by determining QC samples at three concentration levels (see Table 1, six samples each) on three different validation days. The accuracy was expressed by (mean observed concentration—spiked concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (R.S.D.). During routine analysis, each analytical run included a set of calibration samples, a set of QC samples in duplicate and unknowns.

Absolute recoveries of metformin at three QC levels were determined by assaying the samples as described above and comparing the peak areas of both metformin and IS with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank plasma.

The stability of metformin in the reconstituted solution obtained by protein precipitation procedure was assessed by placing QC samples at three concentrations under ambient conditions for 24 h.

Table 1
Summary of precision and accuracy from QC samples of human plasma extracts ($n = 3$ day, six replicates per day)

Added C (ng/ml)	Found C (ng/ml)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Relative error (%)
4.00	3.97 ± 0.17	4.4	2.3	−0.75
160.0	160.9 ± 8.7	5.7	2.8	0.56
1800	1784 ± 92	5.5	1.3	−0.89

3. Results and discussion

3.1. Method development

To determine metformin using SRM mode, full scan and product ion spectra of the analyte and IS were investigated under the present HPLC conditions. Metformin and IS are strong basic compounds, which contain alkylamine groups in their structures. Therefore, they could be detected under positive APCI or electrospray ionization (ESI) condition. Compared with that of the standard solution at the same concentration, the signal intensity of metformin in plasma significantly decreased using ESI source. And it was also found that the regression curves were nonlinear. These results showed ion suppression from endogenous components in plasma samples occurred within ESI source. But the phenomenon was not observed using APCI source. Therefore, APCI was chosen as ionization source in the experiment. By APCI, the analyte and IS formed predominantly protonated molecules $[M + H]^+$ in full scan spectra. No sodium or other solvent adducts or dimers were observed. Fig. 1 displays the product ion spectra of metformin and the internal standard. The collision behavior of the $[M + H]^+$ ion of metformin is strongly dependent on the collision energy. A major fragment ion at *m/z* 60 was formed using 15–20 eV collision energy (Fig. 1A). When higher collision energy (25 eV) was used, the major fragment ion at *m/z* 71 was observed. Peak area obtained by the *m/z* 130 → 71 transition at 25 eV is lower about two times than that obtained by the *m/z* 130 → 60 transition at 15 eV. In the selected conditions, the maximum intensity for product ion at *m/z* 167 could be obtained for IS using 25 eV collision energy. Therefore, the SRM transitions of *m/z* 130 → 60 and *m/z* 256 → 167 were selected for metformin and IS, respectively, to obtain the maximum sensitivity.

Metformin is biguanide compound that is highly polar in nature, making its extraction from biological samples using organic solvents difficult. In the present experiment, a simple protein precipitation procedure was developed to reduce sample preparation time. The selected protein precipitant was acetonitrile, resulting from its high efficiency of precipitating [19]. Solvents such as perchloric acid, trichloroacetic acid and some heavy metal cations, which have been used in the literature, were not chosen in the present study to reduce ion suppression. To avoid chromatographic peak distortion, acetonitrile instead of methanol was used as the mobile phase, which ensured that the organic content of the mobile phase was approximately equal of the sample. In addition, the acidic modifier (formic acid) in the mobile could improve peak shape and improve the sensitivity of the assay. In the experiment, a mobile phase consisting of acetonitrile−water−formic acid (70:30:1, v/v/v) was

therefore used. Each chromatographic run time was completed within 3.4 min.

3.2. Method validation

3.2.1. Selectivity

Potential interference from endogenous substances was investigated by the analysis of six different sources of human plasma. Representative chromatograms of a blank plasma sample, a blank plasma sample spiked with metformin at the LLOQ and IS, and a volunteer sample are shown in Fig. 2. No interferences from endogenous substances with analyte or IS were detected.

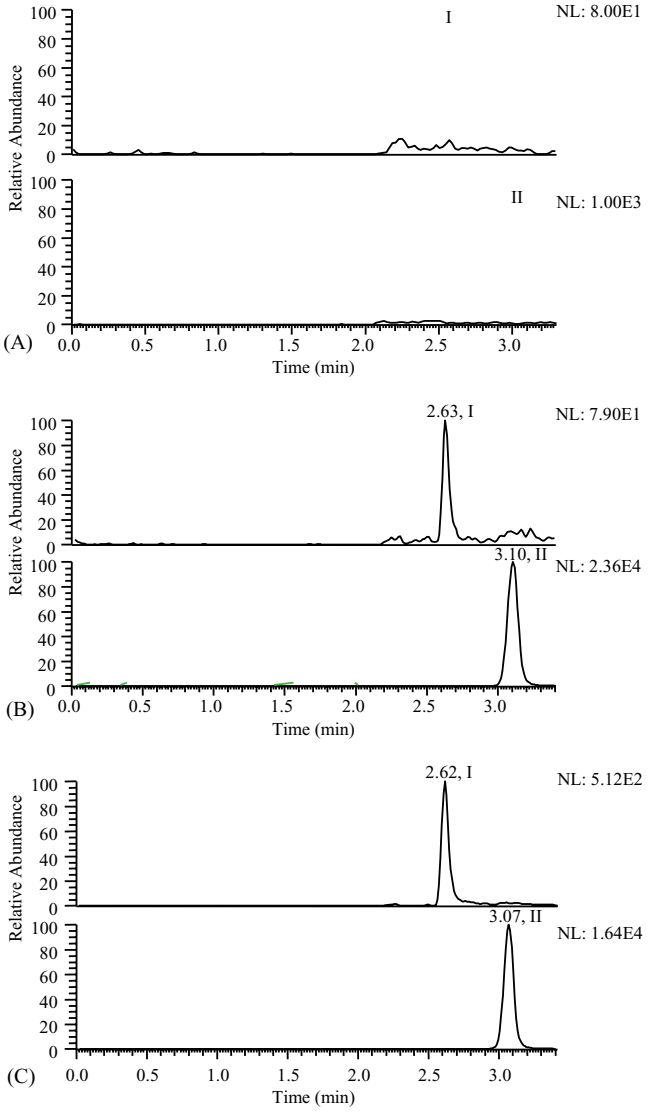

Fig. 2. Representative SRM chromatograms of metformin (I) and IS (II) in human plasma. (A) A blank plasma sample; (B) a blank plasma sample spiked with metformin at the LLOQ of 2 ng/ml and IS (200 ng/ml); (C) plasma sample from a volunteer 12 h after an oral administration of 500 mg metformin hydrochloride in combination with 2.5 mg glyburide (metformin, 36.8 ng/ml).

The LC−MS−MS method has high specificity because only the precursor and product ions derived from the analytes of interest are monitored. Therefore, compounds with different precursor or product ions, e.g. glyburide co-administered with metformin (SRM transition of *m/z* 494 → 370), could not be detected under the present MS conditions. Compared with the analytical methods reported previously, the present method proved to be superior with respect to selectivity and speed of analysis. Each sample required less than 3.4 min of chromatographic run time.

3.2.2. Linearity and lower limit of quantitation

Visual inspection of the plotted triplicate calibration curves and correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration ranges 2.0–2000 ng/ml. The slope and intercept of three regression lines obtained from the spiked plasma were $(2.753 \pm 0.273) \times 10^{-4}$ (mean ± S.D., $n = 3$) and $(1.377 \pm 0.464) \times 10^{-4}$ (mean ± S.D., $n = 3$), respectively.

The lower limit of quantitation (LLOQ), defined as the lowest concentration analyzed with accuracy within ±15% and a precision ≤15%, was 2 ng/ml for determination of metformin in plasma. The limit is already sufficient for pharmacokinetic studies of metformin.

3.2.3. Precision and accuracy

The method showed good precision and accuracy. Table 1 summarizes the intra- and inter-assay precision and accuracy for metformin from QC samples. The results were calculated using one-way ANOVA. The intra- and inter-assay precisions were measured to be below 5.7 and 2.8%, respectively. The inter-assay accuracy ranged from −0.9 to 0.6%.

3.2.4. Recovery and stability

The absolute recoveries of metformin at concentrations of 4.0, 160.0 and 1800 ng/ml ($n = 6$) were determined to be 97.7 ± 4.3, 96.5 ± 3.4, and 98.0 ± 3.8%, respectively; and the recoveries of IS were 99.2 ± 5.4% at concentrations of 200 ng/ml.

QC sample solutions of metformin obtained by protein precipitation procedure showed no significant degradation after at least 24 h at room temperature (7.8% deviation of the spiked values). Standard stock solution of metformin was shown to remain stable for at least 1 month at 4 °C.

3.3. Pharmacokinetic study

To investigate the potential of the validated method for clinical studies, it was applied to determine metformin concentrations in plasma samples after an oral administration of 500 mg metformin hydrochloride in combination with 2.5 mg glyburide to 20 healthy volunteers. Blood samples (4 ml) were collected by venepuncture prior to dosage and at 0.5, 1, 2, 2.5, 3, 4, 5, 6, 8, 12, 24 and 36 h thereafter, and were immediately centrifuged (2000 × *g* for 10 min) to separate the plasma fractions. Fig. 3 shows the mean plasma

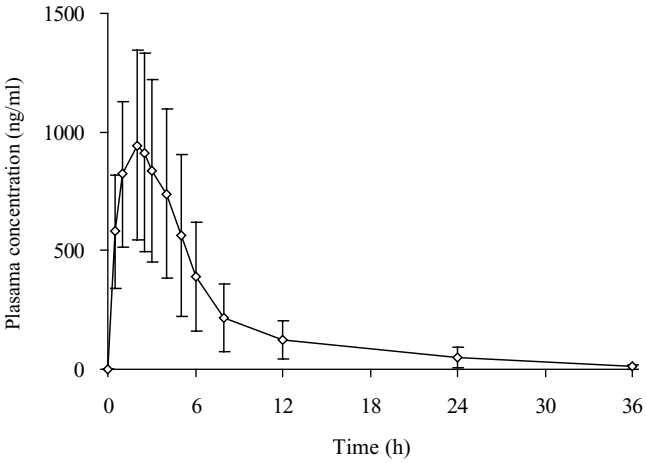

Fig. 3. Mean plasma concentration−time profile of metformin after an oral dose of 500 mg metformin hydrochloride in combination with 2.5 mg glyburide to 20 healthy volunteers. Each point represents the mean ± S.D.

concentration-time curve of metformin after the oral administration. The mean C_{max} values of 975.6 ± 346.9 ng/ml (range 441.9–1824) for metformin were obtained; corresponding mean t_{max} value was 2.5 ± 0.6 h (range 1.0–4.0). The mean plasma elimination hale-life was 5.20 ± 0.75 h (range 4.03–6.66). These calculated pharmacokinetic parameters in this study are similar to those reported in previous studies when metformin was administrated alone [5,6,20,21] or with glyburide [22]. The drug co-administered with metformin did not interfere with the determination of metformin. The present method could be applied to pharmacokinetic studies after a lower dose administration of metformin (250 mg). It could provide a reference to the quantitative determination of other basic drugs with high hydrophilicity.

4. Conclusions

A LC−APCI/MS−MS assay for metformin in human plasma has been developed and validated with respect to linearity, precision and accuracy, and analysis of real samples was demonstrated. The method only needed a simple protein precipitation procedure, which reduced the preparation time and allowed quantitation of metformin in plasma for concentrations ranging from 2 to 2000 ng/ml using 0.25 ml of plasma. It allows the determination of metformin up to 36 h after an oral administration. The short chromatographic cycle time (3.4 min) allowed rapid analysis with minimal matrix interference. The method proved to be superior with respect to sensitivity, selectivity and speed of analysis, compared with the analytical methods reported previously.

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