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Rapid and sensitive liquid chromatography–tandem mass spectrometric method for the quantitation of metformin in human plasma

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Abstract

A rapid and sensitive liquid chromatography–tandem mass spectrometric (LC–MS–MS) method for the determination of metformin in human plasma using phenformin as internal standard has been developed and validated. Sample preparation of plasma involved acidification with acetic acid, deproteination with acetonitrile and washing with dichloromethane. Samples were then analyzed by HPLC on a short Nucleosil C_{18} column (5 μm, 50 mm × 4.6 mm i.d.) using a mobile phase consisting of acetonitrile:methanol:10 mM ammonium acetate pH 7.0 (20:20:60, v/v/v) delivered at 0.65 ml/min. Detection was performed using an Applied Biosystems Sciex API 4000 mass spectrometer set at unit resolution in the multiple reaction monitoring (MRM) mode. Atmospheric pressure chemical ionization (APCI) was used for ion production. The assay was linear over the range 1–2000 ng/ml with intra- and inter-day precision of <8.6% and accuracy in the range 91–110%. The limit of detection was 250 pg/ml in plasma. The method was successfully applied to a clinical pharmacokinetic study of an extended-release tablet of metformin hydrochloride (500 mg) administered as a single oral dose.
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Keywords: Metformin

1. Introduction

Metformin hydrochloride is an orally administered biguanide widely used in the treatment of type 2 (non-insulin dependent) diabetes mellitus [1,2]. It improves hepatic and peripheral tissue sensitivity to insulin without the problem of serious lactic acidosis commonly found with its analogue, phenformin. Metformin is a hydrophilic drug with an oral bioavailability of 50–60% and a relatively short half-life of 1.5–4.5 h [3]. Since its mode of action is antihyperglycemic rather than hypoglycemic, an extended-release formulation has the potential to improve control of blood glucose without the danger of the hypoglycemia that can occur with, for instance, sulfonylureas. As an essential part of our development of such a formulation, we required a rapid and sensitive assay for application in pharmacokinetic studies.

Various analytical methods have been developed for the determination of metformin in biological samples including gas chromatography (GC) [4–6] and high-performance liquid chromatography (HPLC) [7–11]. These methods suffer from a number of disadvantages including low sensitivity, the need for extensive sample preparation and/or derivatization before analysis and, in some cases, the need for a column-switching system [12].

Liquid chromatography with tandem mass spectrometric detection (LC–MS–MS) has been widely used for the analysis of drugs in biological fluids because of its excellent specificity, speed, and sensitivity [13,14]. However, small molecules like metformin pose particular difficulties due to the increased possibility of matrix effects arising when analyzing biological samples. To our knowledge, an LC–MS–MS method for the determination of metformin has not been reported. This paper describes the development and validation of a rapid, sensitive and specific LC–MS–MS method for the determination of metformin in plasma using phenformin as internal standard. The assay has been applied to a clinical pharmacokinetic study of an extended-release

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tablet of metformin hydrochloride (500 mg) in healthy volunteers.

2. Experimental

2.1. Materials and chemicals

Metformin hydrochloride and phenformin hydrochloride (Fig. 1) were kindly supplied by Beijing Juneng Life Scientific Research Center (Beijing, China). Acetonitrile and methanol were HPLC-grade, and all other chemicals were of analytical grade and used without further purification. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Preparation of solutions

All concentrations of metformin and phenformin refer to the free bases. Stock solutions (100 μg/ml) of metformin and phenformin were prepared in methanol using the hydrochloride salts. A series of metformin standard solutions with concentrations of 1.0, 4.0, 10, 40, 100, 400, 1000 and 2000 ng/ml were prepared by dilution of individual aliquots of the stock solution with methanol. A working internal standard solution (1600 ng/ml) was also prepared in methanol. High, medium and low QC solutions (1600, 200 and 2.5 ng/ml, respectively) were prepared in methanol, stored at 4 °C and used within 1 month of preparation.

2.3. Assay protocol

Sample preparation was carried out using a protocol based on that of Cheng and Chou [15]. To 100 μl plasma sample (or blank plasma) in a 10 ml glass tube, 100 μl of the internal standard solution, 100 μl methanol (or metformin standard solution or QC solution), 300 μl water and 20 μl 1 M acetic acid were added. After vortexing, 2.0 ml of acetonitrile were added and the contents of the tube vortexed for 30 s and centrifuged at 3000 × *g* for 5 min. The supernatant was then transferred to another tube, vortexed with 2.0 ml of dichloromethane for 30 s and centrifuged at 3000 × *g* for 5 min. Finally, a 20 μl aliquot of the aqueous layer was injected onto the column.

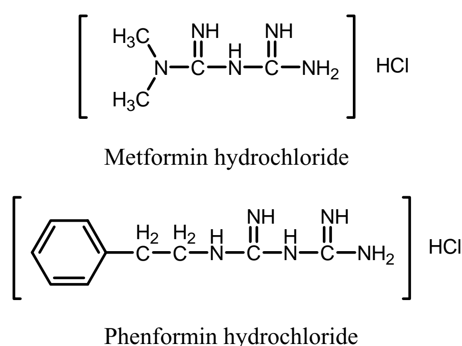

Fig. 1. Structures of metformin hydrochloride and phenformin hydrochloride (internal standard).

2.4. LC–MS–MS

The HPLC system (Agilent 1100 series) consisted of a binary pump, an autosampler and a Nucleosil C_{18} column (5 μm, 50 mm × 4.6 mm i.d. from Dalian Johnsson Separation Science and Technology Corp, Dalian, China). The mobile phase was prepared by mixing 10 mM ammonium acetate (adjusted to pH 7.0 with triethylamine) with acetonitrile and methanol in a ratio 60:20:20 (v/v/v). Chromatography was performed at ambient temperature at a flow-rate of 0.65 ml/min. Detection was performed on an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) using atmospheric pressure chemical ionization (APCI) for ion production.

APCI was performed in the positive ion mode with nitrogen as nebulizer, gas 1 and curtain gas. High-flow gas flow parameters were optimized by making successive flow injections while introducing mobile phase into the ionization source at 0.65 ml/min. Optimum values for nebulizer and curtain gas flow-rates were 55 and 10 units, respectively. The nebulizer current and temperature were 4.3 μA and 280 °C, respectively. Instrument response was optimized by syringe pump infusion (5 μl/min) of a solution of metformin and phenformin in mobile phase into the stream of mobile phase eluting from the column. The pause time was set at 5 ms and the dwell time at 200 ms.

The LC–MS–MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode using the transitions of the protonated molecular ions of metformin at m/z 130 → 60 and phenformin at m/z 206 → 60. The collision gas (N_2) was set at 4 units and collision energies of 35 and 20 eV were used for metformin and phenformin, respectively. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.3 software. Product ion mass spectra of metformin and phenformin are shown in Fig. 2.

2.5. Assay validation

Matrix effects were evaluated using aqueous supernatant from processed blank plasma from six different drug free volunteers. Linearity was assessed by preparation of three independent calibration curves based on eight metformin standards with concentrations in the range 1.0–2000 ng/ml in plasma. Calibration curves were analyzed by weighted linear regression ($1/x^2$) of drug-internal standard peak area ratios. Accuracy [relative error (RE)] and precision [relative standard deviation (R.S.D.)] were assessed by analyzing replicate ($n = 6$) QC samples on 3 different days and determined by one-way analysis of variance (ANOVA). Absolute

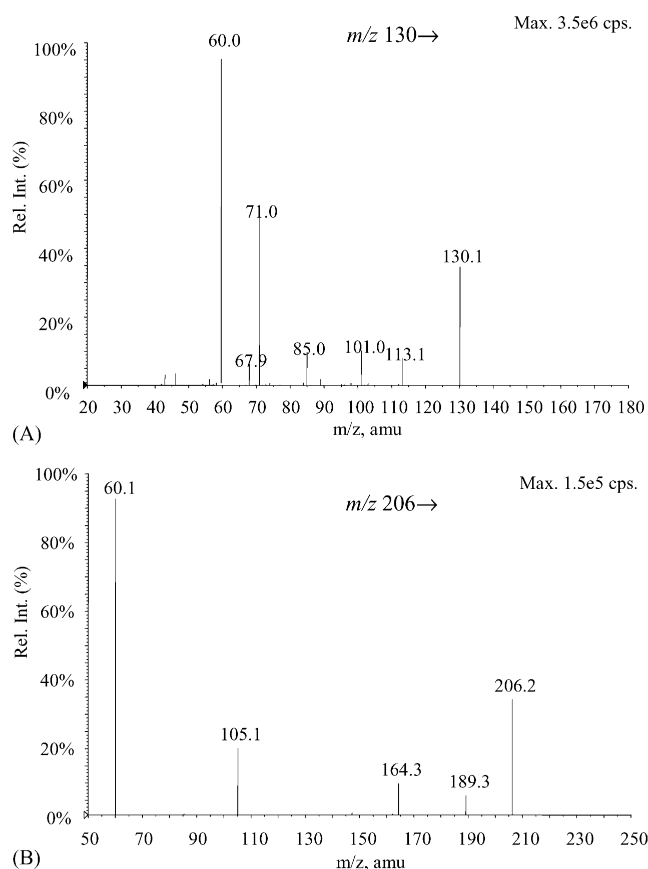

Fig. 2. Full-scan product ion spectra of $[M + H]^+$ for (A) metformin and (B) phenformin.

recoveries were determined in triplicate by comparing peak area ratios with those obtained by direct injection of the compounds dissolved in aqueous supernatant from processed blank plasma. The lower limit of detection (LOD) of the method was determined as the concentration with a signal-to-noise ratio of 3. Stability of the analyte and internal standard were examined in methanol stock solutions, spiked plasma (prepared by adding 100 μl QC solutions to 100 μl blank plasma) and aqueous supernatant from processed blank plasma.

2.6. Application of the method

The method was applied to a single oral dose study of an extended-release tablet of metformin hydrochloride (500 mg) in 24 healthy male volunteers. Blood samples (3 ml) were collected by venepuncture prior to dosage and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 14 and 24 h thereafter. Following centrifugation (3000 × *g* for 10 min), plasma samples were stored in polypropylene tubes at −20 °C and analyzed within 1 month. Each analytical run included a plasma blank, a zero-level standard (blank plasma plus internal standard), a set of calibration standards and QC samples in duplicate. Pharmacokinetic parameters were calculated using Topfit 2.0.

3. Results and discussion

3.1. Mass spectrometry

Analyte and internal standard responded best to positive ionization and, under electrospray ionization (ESI) conditions, protonated molecular ions $[M + H]^+$ were present as major peaks. In fact, direct injection of a 10 ng/ml standard solution of metformin gave a signal-to-noise ratio of >200. However, ESI was found to be very sensitive to ion suppression effects consequent to the protein precipitation procedure such that no signal was obtained for metformin using ESI. Given that liquid–liquid extraction is not an option for preparation of samples containing the highly water-soluble metformin and that APCI is less prone to matrix effects [16], it was decided to use APCI for ion production.

3.2. Chromatography

In order to minimize the run time of the assay, a short C_{18} column was used. A number of different commercial columns (Nucleosil, Nova-Pak, Hypersil and Zorbax) were evaluated and Nucleosil was found to give the best chromatography with minimal matrix effects. The pH of the mobile phase was found to markedly affect the retention of the analyte. At pH <5.0 metformin was not retained and was subject to considerable matrix effects. At pH >7 the analyte gave reasonable retention but the life of the column was reduced. The best compromise was to adjust the pH to 7.0 with triethylamine. Although this decreased the response of metformin, it gave adequate sensitivity with minimal matrix effects. Under these optimum conditions, analyte and internal standard were free of interference from endogenous substances and gave retention times of 1.20 and 2.04 min, respectively. In order to avoid shifts in retention time due to variations in mobile phase pH, a volume of mobile phase sufficient for the anticipated number of samples to be analyzed was prepared.

3.3. Sample preparation

Using a protein precipitation method based on that of Cheng and Chou [15], we found acidification with acetic acid led to better chromatography than hydrochloric acid and addition of water was necessary to obtain good separation of the dichloromethane and aqueous phases. Initially all reagents including the dichloromethane were added to a single tube but this procedure led to metformin peak broadening after analysis of about 150 samples. Separation of the supernatant before washing with dichloromethane gave good chromatography and extended column life. The adopted procedure allowed analysis of 150–200 samples per day on a single instrument and a column life of at least 800 samples.

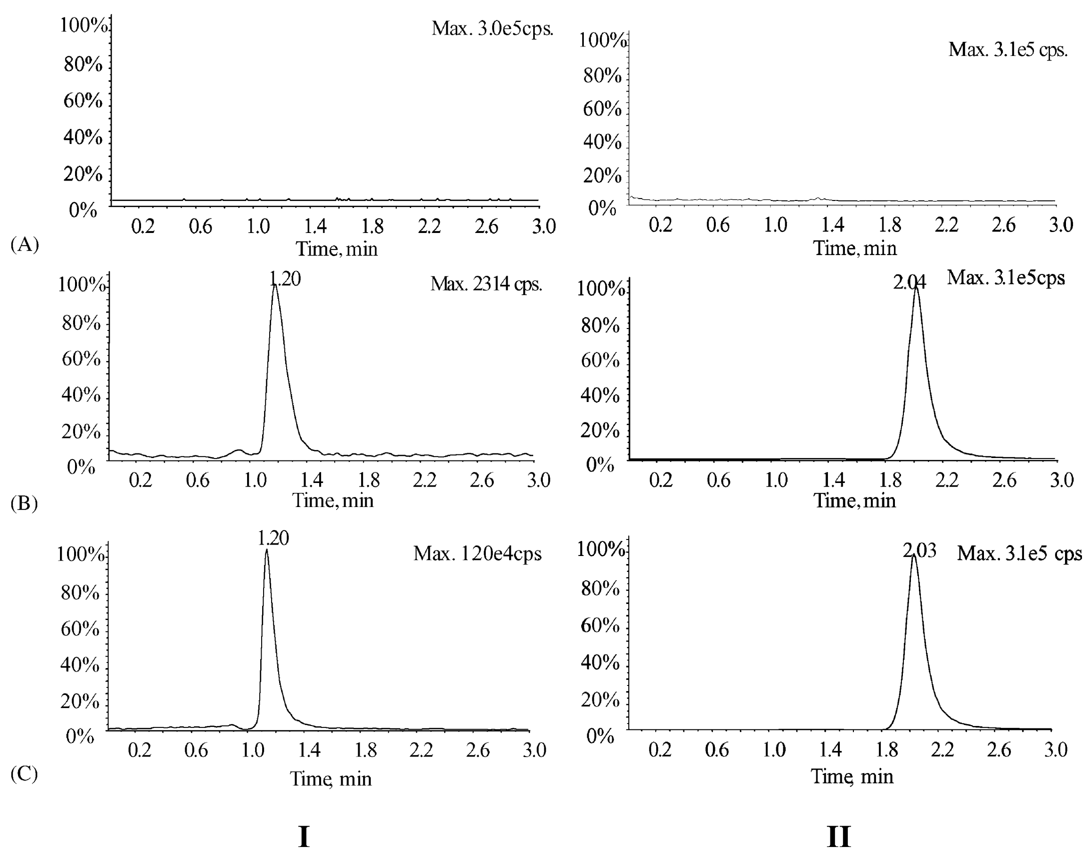

Fig. 3. Representative single reaction monitoring chromatograms of (A) blank plasma, (B) plasma spiked with metformin and phenformin at the limit of quantitation (1.0 ng/ml) and (C) a plasma sample 24 h after a single oral dose of an extended-release tablet of metformin hydrochloride (500 mg) to a healthy male volunteer. Peak I, metformin; Peak II, phenformin.

3.4. Assay validation

Representative chromatograms of blank plasma, blank plasma spiked with metformin at the limit of quantitation (1.0 ng/ml) and a study sample containing a low concentration of metformin are shown in Fig. 3. Matrix effects were minimal and no co-eluting "unseen" endogenous species interfered with the ionization of the analyte and internal standard.

The assay was found to be linear in the concentration range 1–2000 ng/ml ($r > 0.999$). Precision and accuracy were satisfactory at the three concentrations studied (Table 1). Absolute recoveries of metformin at concentrations of 2.5, 200 and 1600 ng/ml were 103 ± 3.9%, 98.7 ± 4.5% and 97.9 ± 3.8%, respectively. Stability of the analyte and internal standard in methanol stock solutions was verified on storage for 1 month at 4 °C. Stability of the analyte in spiked plasma was verified on storage for 2 months at −20 °C. Stability of the analyte in aqueous supernatant from processed blank plasma was verified on storage in plastic autosampler vials at room temperature for 24 h. The limit of detection of the assay was 250 pg/ml which is lower than for any previously reported method.

3.5. Application of the method

The concentration–time profile after a single oral dose of a metformin extended-release tablet is shown in Fig. 4. The C_{max} was 794 ± 257 ng/ml occurring at 2.4 ± 0.8 h. The plasma elimination half-life was 5.6 ± 2.2 h and the area under the plasma concentration–time curve was 5870 ± 900 ng h/ml. As expected for an extended-release dosage

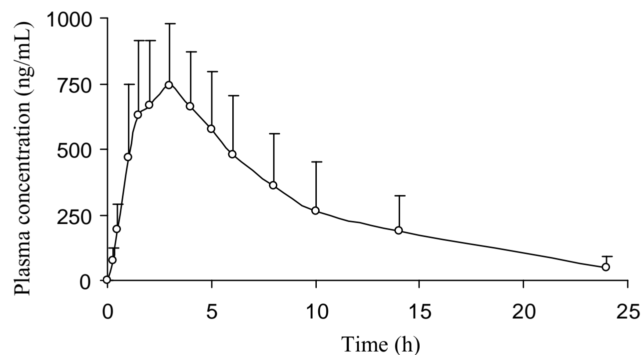

Fig. 4. Mean plasma concentration–time profile of metformin after a single oral dose of an extended-release tablet of metformin hydrochloride (500 mg) to healthy male volunteers. Data are mean ± S.D. ($n = 24$).

Table 1
Summary of precision and accuracy for determination of metformin in human plasma (data are based on analysis of six replicate QC samples on 3 different days)

Day	Added concentration (ng/ml)		
	2.50	200	1600
1	2.66	206	1570
	2.65	214	1480
	2.61	210	1490
	2.58	216	1570
	2.60	214	1530
	2.63	212	1500
2	2.67	194	1580
	2.66	193	1580
	2.51	194	1570
	2.77	224	1580
	2.78	220	1550
	2.81	224	1500
3	2.23	180	1601
	2.58	178	1620
	2.52	214	1590
	2.78	206	1650
	2.40	214	1680
	2.83	199	1650
Mean (ng/ml)	2.63	206	1572
Relative error (%)	5.04	3.12	−1.77
Intra-day R.S.D. (%)	6.69	8.30	8.60
Inter-day R.S.D. (%)	5.09	6.00	2.07

form, the half-life is somewhat longer and the C_{max} lower than previously observed with standard 500 mg tablets of metformin hydrochloride [3,17].

4. Conclusion

A rapid and sensitive LC–MS–MS method is reported for the determination of metformin in human plasma. The assay was successfully applied to determine concentration-time profiles of the drug in a clinical pharmacokinetic study of a metformin hydrochloride extended-release tablet. The method allows high sample throughput due to the short run time and relatively simple sample preparation procedure.

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