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ORIGINAL RESEARCH

Simultaneous determination of metformin and glipizide in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT: A method has been developed for the simultaneous quantification of metformin (I) and glipizide (II) in human plasma. It is based on high-performance liquid chromatography with electrospray ionization tandem mass (LC-ESI-MS/MS) spectrometric detection in positive ionization mode. Phenformin (III) and gliclazide (IV) were used as internal standards for I and II, respectively. The MS/MS detection was performed in multiple reaction monitoring (MRM) mode. The precursor-product ion combinations of m/z 130 → 71, 446 → 321, 206 → 60 and 324 → 127 were used to quantify I, II, III and IV, respectively. This method was validated in the concentration ranges of 0.02–4 μg/mL for I and 0.004–0.8 μg/mL for II. It was utilized to support a clinical pharmacokinetic study after single dose oral administration of a combination of I and II. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: metformin; glipizide; LC-MS/MS; ESI; human plasma; determination

INTRODUCTION

Metformin (I, *N*,*N*-dimethyldiguanide) and glipizide (II, 1-cyclohexyl-3-[[p-[2-(5-methylpyrazinecarboxamindo) ethyl]phenyl]sulfonyl]urea) are used in the treatment of type 2 diabetes. Metformin improves hepatic and peripheral tissue sensitivity to insulin without the problem of serious lactic acidosis (Davis *et al.*, 2001; Setter *et al.*, 2003), whereas II glipizide can increase the secretion of insulin by stimulating islet β-cells (Rendell, 2004). Recently, I and II have been used in combination to achieve better glycolic control and patient compliance. To address the pharmacokinetics of the new combined formulation, a sensitive and specific method that allows simultaneous measurement of I and II in human plasma is needed.

Various analytical methods (Vasudevan *et al.*, 2001; Chen *et al.*, 2004; Ho *et al.*, 2004; Heinig and Bucheli, 2004; Lin *et al.*, 2004; Wang *et al.*, 2004; Koseki *et al.*, 2005) have been described for the quantification of I or II in biological samples separately. To our knowledge, only one article has been reported on the HPLC method (AbuRuz *et al.*, 2005) of simultaneous determination of several anti-diabetic drugs that include I and II in plasma with LLOQs of 0.100 and 0.05 μg/mL for I and II, respectively. However, complicated sample preparation by solid-phase extraction and lengthy analytical time (20 min) were involved. In this paper, a new method has been described for the simultaneous determination of I and II in human plasma. The method is based on the precipitation of plasma protein by organic solvent to afford sample for LC-MS/MS analysis. The two drugs can be quantified within 2 min, and a real high-throughput sample analysis is achieved due to the high efficiency of the liquid chromatography–tandem spectrometry method. This method has been successfully applied to the pharmacokinetic study of a combination of I and II with LLOQs of 0.02 and 0.004 μg/mL for I and II, respectively.

EXPERIMENTAL

Materials and reagents. Metformin (purity 99.3%), glipizide (purity 99.6%), phenformin (IS for metformin, purity 99.3%), and gliclazide (IS for glipizide, purity 99.0%) were obtained from Yung Shin Pharm. Ind. Co. Ltd (Kunsan, China). Acetonitrile and formic acid were of HPLC grade. Purified water was used throughout the study. Other reagents were of AR grade. The drug under clinical trial was compound tablets of I (250 mg) and II (2.5 mg; Yung Shin Pharm. Ind. Co. Ltd, Kunsan, China).

Preparation of stock and sample solutions. The standard stock solution was prepared with methanol to give final concentrations of 1 mg/mL for I and 200 μg/mL for II. The standard working solutions were prepared by dilution of stock

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Abbreviations used: MRM, multiple reaction monitoring.

solution. The internal standard stock solution was prepared with acetonitrile. The internal standard working solution was prepared from corresponding stock solution at the concentration of 1 μg/mL for III and 0.06 μg/mL for IV.

Samples for standard curve and quality control were prepared by spiking drug-free human plasma with appropriate standard working solutions. The calibration samples were prepared at 0.02, 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 μg/mL for I and at 0.004, 0.02, 0.04, 0.1, 0.2, 0.4, and 0.8 μg/mL for II. Samples for quality control were prepared at 0.02, 1.0 and 4.0 μg/mL for I and 0.004, 0.2 and 0.8 μg/mL for II. Samples for standard curve and quality control were prepared independently.

Instrumentation. The HPLC system consisted of a Shimadzu LC-10Advp pump, a Shimadzu SIL-HTC auto sampler, and a Shimadzu CTO-6A column oven. A triple-quadrupole mass spectrometer (API 3000, Applied Biosystem Sciex, Ontario, Canada) was used for mass analysis and detection. An electrospray ionization source was used as interference between the HPLC system and mass spectrometer.

Chromatographic conditions. Chromatographic separation was achieved on a Resteck C_{18} column (50 × 2.1 mm, 5 μm) with a guard column (C_{18}, 4 × 3 mm, Phenomenex Ltd). The column oven temperature was set at 30°C. The mobile phase consisted of acetonitrile, 20 mM ammonium acetate, and 96% formic acid (70:30:1, v/v/v). The flow rate was set at 0.2 mL/min. The injection volume was 5 μL.

MS/MS detection. The mass spectrometer operated on an electrospray atmosphere press ionization source in positive mode (ESI^+). Operation parameters for nebulizer, curtain gas and heated temperature were optimized by successive sample injections while introducing mobile phase into the ionization source at 0.2 mL/min. Nitrogen, used as nebulizer and curtain gas, was set at 11 and 7 L/min, respectively. The source temperature was set at 450°C and the spray voltage at 5.5 kV. The collision gas (nitrogen) pressure was set at 5 psi. The four compounds were detected using multiple reaction monitoring (MRM). The optimized parameters are shown in Table 1.

Sample preparation. To precipitate 150 μL of the plasma protein, 350 μL of internal standard working solution were added, then mixed briefly, and centrifuged at 10,000 rpm for 10 min. The supernatant liquid was for direct injection to the LC-MS/MS system.

Assay validation. The specificity of the method was evaluated using blank plasma sample from nine volunteers. The sensitivity of the method was evaluated by determining samples at LLOQ. As the assay parameter, plasma samples were quantified by the peak area ratio of the analyte and that of its corresponding IS. Peak area ratios plotted against concentrations was calculated using weighted ($1/c^2$) least squares linear regressions. The two analytes were calculated separately. Linearity was assessed by preparation of three independent calibration curves. Accuracy and precision were evaluated by determining QC samples at three concentration levels ($n = 5$), on three independent validation days.

Post-preparation stability and freeze–thaw stability were evaluated by determining QC samples at three concentration levels. Post-preparation stability samples were tested at 5°C. Freeze–thaw stability of plasma samples was determined after three freeze–thaw cycles.

Application of the method. This method was applied to a single oral dose study of a combination of I (500 mg) and II (5 mg). Nine healthy male volunteers aged 24–29 years and weighing 55–72 kg participated in this study. They were on fasted for 10 h before dosing and 4 h post-dosing. Standardized meals were served according to the schedule throughout the study. Two tablets (I, 250 mg; II, 2.5 mg) were given to each one of the volunteers with 250 mL water. Water was allowed only after the first 2 h post-dosing. Blood samples (3 mL) were collected by venipuncture prior to dosage and at 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 and 15 h thereafter. Following centrifugation (3000 rpm for 10 min), plasma samples were stored in polypropylene tubes at –20°C until they were taken out for analysis. The collected plasma samples were analyzed within 2 weeks. All the plasma samples were thawed at room temperature before preparation for analysis.

RESULTS AND DISCUSSION

Method development

Double internal standards were used in this assay, III for I and IV for II. The analytes and their corresponding internal standards had similar chemical structures; therefore, they showed similar ionization under the same conditions. This phenomenon contributed to better precision and accuracy.

It is difficult to remove the interfering endogenous components by routine treatment, such as liquid–liquid extraction and solid-phase extraction. In this investigation, advantage was taken of dilution with the organic

Table 1. Summary of performing parameters for mass spectrometer setting

Compound	MRM (*m*/*z*)	Dwell time (ms)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
Metformin	130.0 → 71.0	200	10	400	10	28	5
Glipizide	446.4 → 321.1	200	60	400	10	25	15
Phenformin	206.1 → 60.0	200	10	400	10	32	5
Gliclazide	324.2 → 127.1	200	60	400	10	26	10

DP – Declustering Potential; FP – Focusing Potential; EP – Entrance Potential; CE – Collision Energy; CXP – Collision Cell Exit Potential.

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solvent. During sample preparation procedure, 350 μL acetonitrile was added to 150 μL plasma, both analytes and endogenous components were diluted, and the interference was decreased to a negligible quantity. Ionization of metformin decreased by about 40% due to matrix effects. However, the effects on low and high concentrations were also found to be almost equal. Therefore, the response of metformin still showed good linearity to its concentration at the range of 0.02–4 μg/mL.

Because I is a very strong basic compound, it is poorly retained on reverse-phase HPLC column, whereas II is a relatively less basic compound. Because of this, simultaneous detection of I and II by a HPLC-UV method (AbuRuz *et al.*, 2005) takes a long time. Taking the advantage of MS/MS detection, the analytes were not separated completely on chromatography. In this study, under optimized HPLC conditions, I and II were detected within 2 min. The retention times were 0.71, 0.74, 1.00 and 1.32 min for I, III, II and IV, respectively.

As I and II have different chemical properties, it is difficult and time-consuming to extract them from plasma simultaneously by a conventional liquid–liquid or solid-phase extraction method. In this study, 350 μL internal standard working solution was added to 150 μL plasma sample, and acetonitrile was utilized to precipitate protein followed by extraction of I and II from the mixture. The supernatant has almost the same constituent with the mobile phase. Only 5 μL supernatant liquid was required to minimize the potential spoilage to the column and the MS/MS detector.

Method validation

Full-scan positive ion mass spectra of I–IV yielded predominately the protonated molecules at *m*/*z* 130, 446, 206 and 324, respectively. The product ion mass spectra of these protonated molecules (Fig. 1) showed the presence of main product ions at *m*/*z* 71, 321, 60 and 127, respectively.

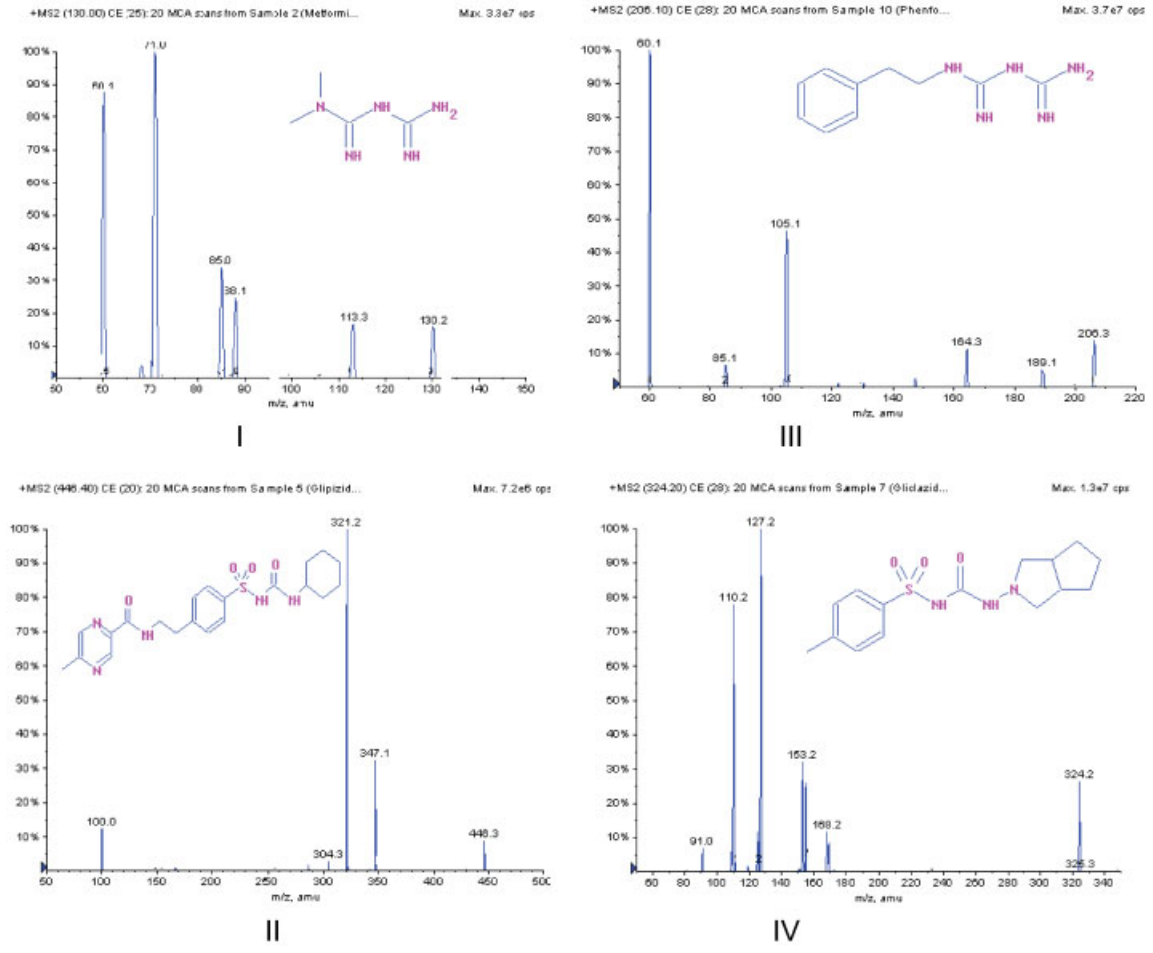

Figure 1. Product ion mass spectra of four protonated molecules. I, metformin; II, glipizide; III, phenformin; IV gliclazide. This figure is available in colour online at www.interscience.wiley.com/journal/bmc

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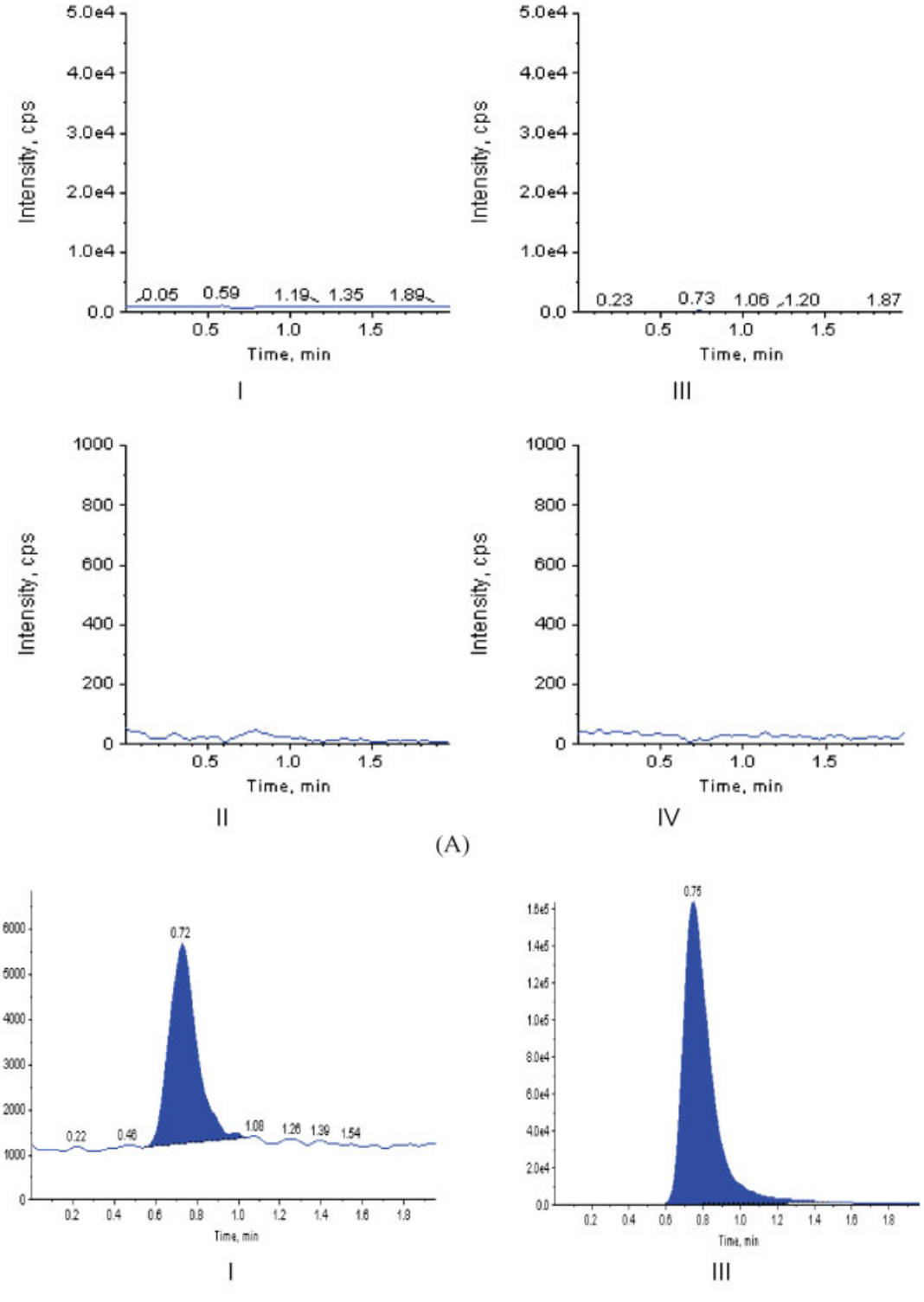

Figure 2. MRM chromatograms of metformin (I), glipizide (II), phenformin (III) and gliclazide (IV). (A) Blank plasma sample; (B) blank plasma sample spiked with I at 0.02 μg/mL and II AT 0.004 μg/mL; (C) plasma sample from a volunteer 1.5 h after dosing. This figure is available in colour online at www.interscience.wiley.com/journal/bmc

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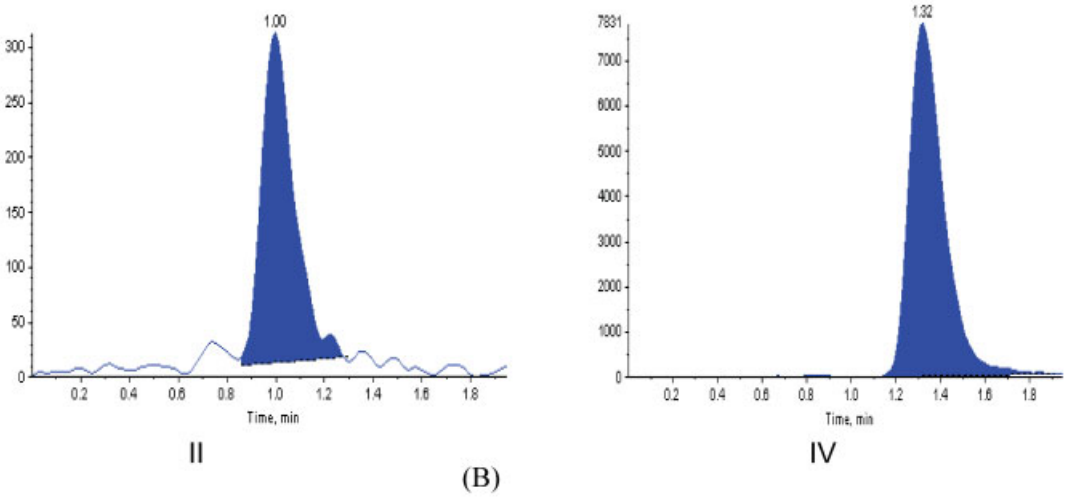

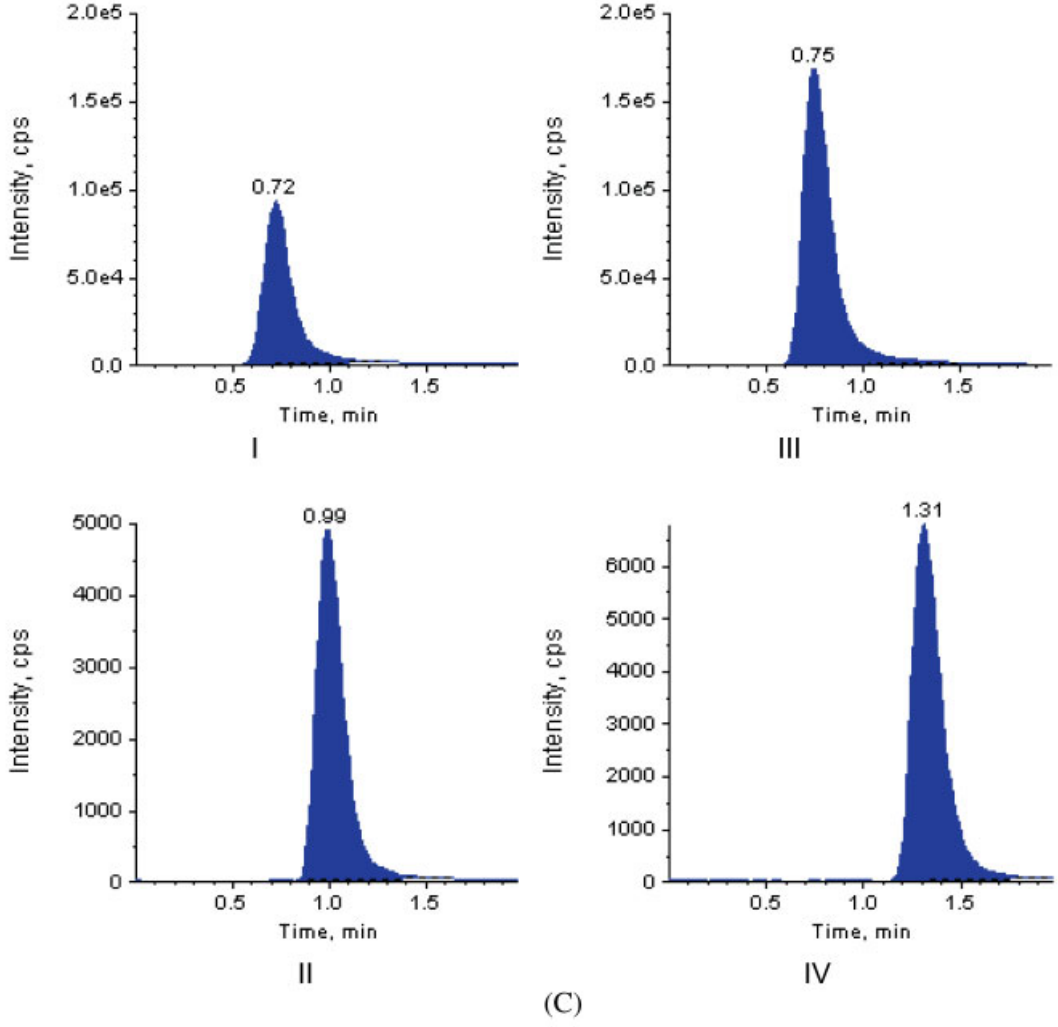

Figure 2. (*Continued*)

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Table 2. Precision and accuracy for the analysis of metformin and glipizide in human plasma (n = 3 days, five replicates per day)

Compound	Added (μg/mL)	Found (μg/mL)	Intra-run RSD (%)	Inter-run RSD (%)	Relative error (%)
Metformin	4.00	4.20 ± 0.06	0.96	2.8	5.2
	1.00	1.01 ± 0.03	0.79	4.2	0.6
	0.020	0.021 ± 0.001	5.02	8.6	6.2
Glipizide	0.800	0.801 ± 0.032	3.94	2.7	0.1
	0.200	0.202 ± 0.003	1.54	0.9	1.1
	0.004	0.0042 ± 0.0006	6.09	6.9	4.1

Table 3. Summary of post-preparation and freeze–thaw stability for metformin and glipizide (n = 3)

Compound	Added (μg/mL)	Post-preparation for 20 h (μg/mL)	Freeze–thaw for three cycles (μg/mL)
Metformin	4.00	3.84	4.07
	1.00	0.92	0.96
	0.020	0.019	0.019
Glipizide	0.800	0.801	0.740
	0.200	0.204	0.194
	0.004	0.0041	0.0041

Table 4. Pharmacokinetic parameters obtained from nine male healthy volunteers

Compound	C_{max} (μg/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-t} (μg/mL/h)	AUC_{0-t} (μg/mL/h)	MRT (h)
Metformin	1.491 ± 0.525	1.94 ± 0.85	3.10 ± 0.39	7.496 ± 2.142	7.761 ± 2.170	4.96 ± 0.19
Glipizide	0.552 ± 0.087	2.06 ± 0.77	3.40 ± 0.38	2.690 ± 0.6450	2.837 ± 0.7416	5.26 ± 0.64

MRM chromatograms are shown in Fig. 2. The excellent selectivity shown proves that there was no interference from endogenous substances with the analytes or IS. The calibration curves of I and II were typically described by $R = -0.001733 + 0.4750C$ (r = 0.9993, n = 3) and $R = -0.0004975 + 3.246C$ (r = 0.9995, n = 3), at the concentration ranges 0.02–4.0 and 0.004–0.8 μg/mL, respectively. Chromatograms of the sample at LLOQ (0.02 μg/mL for I and 0.004 μg/mL for II) are shown in Fig. 2. The signal-to-noise (S/N) ratio was about 30. The result indicated that LLOQ was sufficient for clinical pharmacokinetic studies of I and II as compared with the general case of S/N 10. Accuracy and precision data proving that the method is satisfactory are shown in Table 2.

The results of post-preparation and freeze–thaw stability test are shown in Table 3. I and II were found stable under the above-mentioned conditions. All the measured concentrations of tested samples were in the range 85–115% of added concentrations.

Pharmacokinetic study

Mean plasma concentration–time profiles of I and II after a single oral dose of 500 mg metformin and 5 mg glipizide given in combination to nine male healthy volunteers are shown in Fig. 3. The corresponding pharmacokinetic parameters are shown in Table 4.

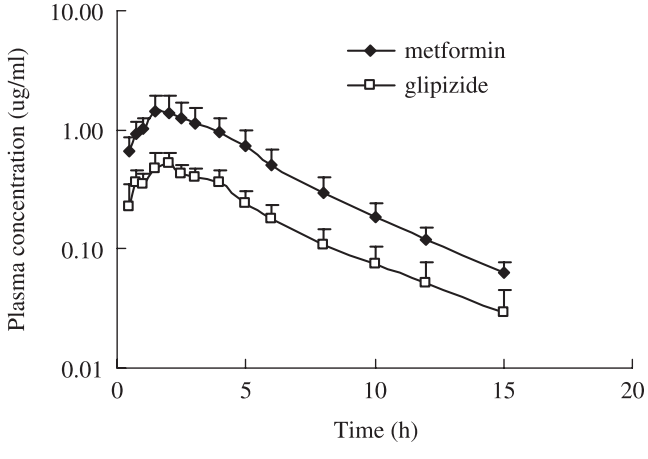

Figure 3. Mean plasma concentration-time profiles of metformin and glipizide after a single oral dose of 500 mg metformin and 5 mg glipizide in combination to nine male healthy volunteers.

The T_{max} and $T_{1/2}$ obtained in this study were approximately consistent with the reported values

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(Davis *et al.*, 2001; Setter *et al.*, 2003; Kobylinska *et al.*, 2004) obtained in experiments in which I and II were administered separately. It may be assumed that the absorption and elimination character of the two drugs did not interfere with each other in the compound tablets.

CONCLUSION

A rapid and sensitive LC-MS/MS method was developed for the simultaneous quantification of I and II in human plasma. It has been successfully applied to determine concentration–time profiles of the two drugs in combined formulation in a clinical pharmacokinetic study. The method shows high efficiency and convenience due to simple sample preparation procedure and optimized LC-MS/MS conditions.

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