JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2005; **40**: 1462–1471
Published online 28 October 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jms.907

JMS

Simultaneous determination of metformin and gliclazide in human plasma by liquid chromatography–tandem mass spectrometry: application to a bioequivalence study of two formulations in healthy volunteers

Guo-ping Zhong,[1†] Hui-chang Bi,[1†] Shufeng Zhou,[2*] Xiao Chen[3] and Min Huang[1]**

[1] Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-sen University, 74 Zhongshan Road II, Guangzhou 510080, China
[2] Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore
[3] Department of Pharmacy, the First Affiliated Hospital, Sun Yat-sen University, 58 Zhongshan Road, Guangzhou 510080, China

Received 28 May 2005; Accepted 21 July 2005

A rapid and sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed and validated to simultaneously determine gliclazide and metformin in human plasma using huperzine A as the internal standard (IS). After acetonitrile-induced protein precipitation of the plasma samples, gliclazide, metformin and the IS were subjected to LC/MS/MS analysis using electro-spray ionization (ESI). Chromatographic separation was performed on a Hypersil BDS C_{18} column (50 mm × 2.1 mm, i.d., 3 μm). The method had a chromatographic running time of 2.0 min and linear calibration curves over the concentration ranges of 10–10 000 ng ml^{-1} for gliclazide and 7.8–4678.9 ng ml^{-1} for metformin. The recoveries of the method were found to be 71–104%. The lower limits of quantification (LOQ) of the method were 10.0 and 7.8 ng ml^{-1} for gliclazide and metformin, respectively. The intra- and interday precision was less than 15% for all quality control samples at concentrations of 100, 500, and 2000 ng ml^{-1}. The validated LC/MS/MS method has been used to study bioequivalence in healthy volunteers. These results indicate that the method was efficient with a very short running time (2.0 min) for metformin and gliclazide compared to the methods reported in the literature. The presented method had acceptable accuracy, precision and sensitivity and was used in clinical bioequivalence study. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: metformin; gliclazide; liquid chromatography/tandem mass spectrometry; pharmacokinetics; bioavailability; bioequivalence

INTRODUCTION

Diabetes is characterized by disrupted insulin production and sensitivity, leading to high blood glucose and a series of complications such as renal dysfunction, neuropathy and cardiopathy.[1–3] Type 2 (non-insulin-dependent) diabetes is a progressive and complex disease that is difficult to manage effectively in the long term. Many oral hypoglycemic drugs with different mechanisms of action have been developed to lower blood glucose and delay the occurrence of serious complications in patients with type 2 diabetes.[3–5] However, a moderate proportion of patients with type 2 diabetes will eventually require insulin therapy to maintain long term glycemic control, either as monotherapy or in combination with oral antidiabetic agents.[5,6] Metformin (1,1-dimethylbiguanide; Diabex, Fig. 1) is an orally administered biguanide that increases peripheral insulin sensitivity, inhibits hepatic gluconeogenesis and reduces hepatic glucose production in type 2 diabetic patients.[5,7,8] Gliclazide (Diamicron, Fig. 1) is a second generation sulfonylurea that stimulates insulin secretion and is used in the treatment of type 2 diabetes mellitus.[5] For many patients with type 2 diabetes, monotherapy with an oral antidiabetic agent is not sufficient to achieve satisfactory blood glucose control and thus combination regimens are always necessary to achieve better glycemic control.[6,9–11] In many cases, metformin has been coadministered with the second generation sulfonylureas such as glipizide, gliclazide, or glibenclamide.[5,6,12] During antidiabetic therapy using these drugs, it is important to monitor their plasma concentrations in diabetic patients.

*Correspondence to: Shufeng Zhou, Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore. E-mail: phazsf@nus.edu.sg
**Correspondence to: Min Huang, PhD, Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-sen University, 74 Zhongshan Road II, Guangzhou 510080, PR, China. E-mail: huangm@gzsums.edu.cn
†Zhong GP and Bi HC have equal contribution to this work. Both persons are the first authors.

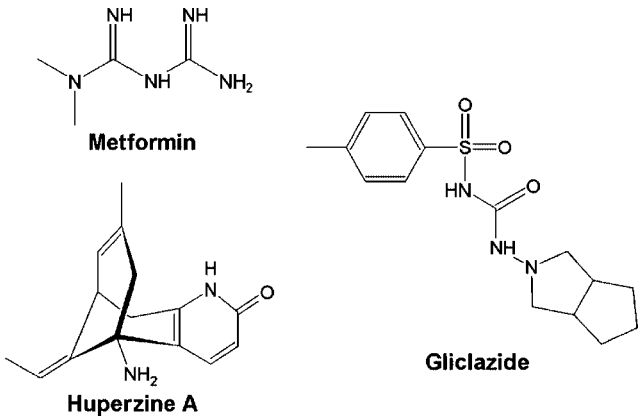

Figure 1. Chemical structure (drawn with mass frontier software, V.3.0) of metformin, gliclazide and huperzine A (I.S.).

Such therapeutic drug monitoring is always necessary for pharmacokinetic studies of these drugs, for optimization of dosing and dosing regimen and for diagnostic purpose in factitious hypoglycemia (e.g. suicidal patients).[5,6,13] Therefore, sensitive, reliable and rapid analytical methods are required to simultaneously determine metformin and the sulfonylurea (e.g. gliclazide) in human plasma.

Various analytical methods have been reported for the measurement of metformin and gliclazide individually, such as gas chromatography (GC), capillary chromatography and high performance liquid chromatography (HPLC).[14–18] However, these methods often suffered from several disadvantages including low sensitivity,[14,15,18] a complex and time-consuming derivatization and/or extraction procedure before analysis[16,17] and, in some cases, the need for a column-switching system.[15] To overcome these problems, several new methods for analysis of metformin in animal- or human plasma using liquid chromatography/mass spectrometry (LC/MS) with improved specificity, efficiency and sensitivity have been recently published.[19–22] On the other hand, gliclazide is usually analyzed in various biological matrices using HPLC methods, which often employ liquid or solid-phase extraction procedure and thus make the analysis tedious and time-consuming.[16] Recently, a sensitive LC/MS method for the determination of antidiabetic sulfonylurea drugs including gliclazide in equine- or human plasma has been reported by Ho *et al*.,[23] Maurer *et al*.[24] and Susanto and Reinauer.[25] These studies used LC/MS with atmospheric-pressure chemical-ionization (APCI) or electrospray ionization (ESI) to identify and quantitate various sulfonylureas including gliclazide, glipizide, glibornuride, glibenclamide and gliquidone in plasma. However, the LC/MS identification procedure of Susanto and Reinauer[25] is of minor specificity because they used only one ion per compound for selected ion mode (SIM) detection. Thus, full quantitation of the sulfonylureas is difficult.

To date, there is no report on the use of LC/MS methods for the simultaneous determination of both metformin and gliclazide in human plasma. A method for determination of metformin and glipizide or gliclazide has been described previously[26] for studying pharmaceutical preparations but not for analysis in biological matrices. Recently, an HPLC method for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in human plasma was reported.[27] Unfortunately, this method required a relatively large sample volume (1.0 ml) and a complicated and time-consuming sample preparation procedure including a five-step solid-phase extraction. It also needed relatively long running time for each sample (about 20 min per sample), which is not suitable for high-throughput analysis when a large number of samples need to be quantitated. The coupling of LC with MS, being a more definitive technique with improved sensitivity, high specificity and selectivity compared to traditional HPLC and GC methods, can usually overcome these problems. Therefore, we developed a rapid and sensitive LC/MS/MS method to measure these two drugs simultaneously in human plasma using a one-step protein precipitation without evaporation. The method exhibited excellent performance in terms of high selectivity, robustness and excellent efficiency (2.0 min per sample) with simplicity of sample preparation.

EXPERIMENTAL

Chemical and reagents

Metformin hydrochloride and gliclazide were provided by Zhong-lian Pharmaceutical Co. (Shengzhen, China). Huperzine A (used as the Internal Standard, IS, Fig. 1) was provided by Xiang-xue Pharmaceutical Co. (Guangzhou, China). Both metformin hydrochloride and gliclazide had a relative purity of 100.1 and 99.8% for huperzine A as compared with the standards from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol, formic acid and acetonitrile of HPLC grade were all purchased from Tedia Company Inc (Guangzhou, China). All other reagents were of analytical grade. Human plasma was obtained from healthy volunteers who did not take any medications in one month, at Guangzhou Blood Center (Guangzhou, China). Ultrapure water was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA).

Preparation of standard and quality control samples

The stock standard solutions of metformin hydrochloride and gliclazide were prepared by dissolving the accurately weighted reference compounds in methanol–water (50 : 50, v/v) to give a final concentration of 500 μg ml^{-1}. The solutions were then serially diluted with methanol–water (50 : 50, v/v) to obtain working solutions at concentrations over 0.1–100.0 μg ml^{-1}. A standard stock solution of huperzine A (IS) at 500 μg ml^{-1} was also prepared in methanol–water (50 : 50, v/v) and then diluted to obtain a working solution at 1 μg ml^{-1}. All the solutions were stored at 4 °C and were brought to room temperature before use.

The analytical standard and quality control (QC) samples were prepared by spiking blank human plasma with standard working solutions in validation and during each experimental run for bioequivalence study. Calibration samples were made at concentrations of 10, 100, 200, 500, 1000, 2000, 3000, 6000 and 10 000 ng ml^{-1} for gliclazide and 10, 100, 200, 500, 1000, 2000, 3000 and 6000 ng ml^{-1} for

metformin hydrochloride (equivalent to 7.8, 78, 156, 389.9, 779.8, 1559.6, 2339.4 and 4678.9 ng ml^{-1}, respectively, for metformin base). Quality control samples were prepared at concentrations of 100, 500 and 2000 ng ml^{-1} for gliclazide and 78.0, 389.9 and 1559.6 ng ml^{-1} for metformin.

Sample preparation

To 200 µl human plasma in a 1.5-ml test tube, 100 µl of the internal standard solution (1 µg ml^{-1}) and 500 µl of acetonitrile were added. After vortex-mixing for 10 s and centrifugation at 15 000 rpm for 3 min, the supernatant was transferred to another clean test tube, with 500 µl of 10-mM KH_2PO_4 solution added. The mixture was vortexed strongly for 10 s, centrifuged at 15 000 rpm for 3 min, and 20 µl of the clear supernatant was directly injected onto the LC/MS/MS system for analysis.

Liquid chromatographic and mass spectrometric conditions

A Finnigan Surveyor MS pump (San Jose, CA, USA) and a Finnigan Surveyor autosampler were used for solvent and sample delivery. Chromatographic separation was achieved by using a C-18 column (Hypersil BDS C-18, I.D. 2.1 × 50 mm, 3 µm, Elite HPLC, China) at room temperature. The mobile phase consisted of methanol–water (containing 1% formic acid)–acetonitrile (30 : 31 : 39, v/v/v), pumped at a flow rate of 200 µl min^{-1}. Total running time was 2.0 min for each injection.

A Finnigan TSQ triple-quadrupole mass spectrometer equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive-ion mode (ESI+) and set up in the selected reaction monitoring (SRM) mode. Nitrogen was used as the sheath gas (35 psi) and the auxiliary gas (5 psi). The capillary temperature was at 350 °C. The spray voltage was set at 3500 V. Collision-induced dissociation (CID) studies were performed and argon was used as the collision gas with a collision cell gas pressure of 1.0 mtorr (1 torr = 133.3 Pa). The optimized source CID was 15 V for both metformin and gliclazide, and 10 V for IS. The optimized collision energy was 20 V for metformin, 38 V for gliclazide and 37 V for IS. On the basis of the full-scan mass spectra of each analyte, the most abundant ions were selected and the mass spectrometer was set to monitor the transitions of the precursors to the product ions as follows: m/z 130 → 71 for metformin, m/z 324 → 110 for gliclazide and m/z 243 → 210 for IS. The scanning time for each analyte was set to 0.1 s. Data acquisition was performed with the Finnigan Xcalibur 1.3 software, while peak integration and calibration were obtained with the Finnigan Lcquan software.

Method validation

The method was validated for selectivity, accuracy, precision, recovery, calibration curve and reproducibility according to the FDA guideline for validation of bioanalytical methods.[28] The selectivity was investigated by preparing and analyzing six individual human blank plasma samples at the lower limit of quantification (LOQ). The LOQ was defined as the lowest concentration on the calibration curve of the analytes measured with acceptable precision and accuracy (i.e. coefficient of variation (CV) and relative error <20%) and with at least five times the response compared to blank response. Linearity was assessed by preparing and analyzing metformin standard samples over 7.8–4678.9 ng ml^{-1} and gliclazide over 10–10 000 ng ml^{-1} in human plasma. The limit of detection was the amount that could be detected with a signal-to-noise ratio of 3. Calibration curves were analyzed by weighted linear regression ($1/x^2$) of the peak area of analyte over that of IS.

Accuracy and precision were assessed by determining QC samples at three concentration levels (five samples for each concentration) on three different validation days. The precision was determined as the RSD (%) and the accuracy was expressed as a percentage of the measured concentration over the nominal (theoretical) concentration. The criteria used to assess the suitability of precision and accuracy were as follows: the RSD did not exceed 15% and the accuracy was within 15% of the actual value. The recovery (extraction efficiency) of analytes from human plasma after the extraction procedure was determined by comparing the areas of extracted analytes with those of the nonextracted pure standards that represent 100% recovery.

During routine analysis, each analytical run included a blank plasma, a blank plasma spiked with IS, a set of calibration samples, a set of QC samples and unknowns. The stability of analytes was assessed by determining QC samples at three concentrations (100, 500 and 2000 ng ml^{-1} for gliclazide; 78.0, 389.9 and 1559.6 ng ml^{-1} for metformin) with five samples for each concentration, exposed to different time and temperature conditions. The stability studies included: (a) stability at room temperature for 4 h; (b) stability after three freeze–thaw cycles; (c) stability of the extracted samples at room temperature for 12 h and (d) the long-term stability after storage at −30 °C for 39 days.

Bioequivalence study

The method was applied to a bioequivalence study of a multicomponent formulation containing 250 mg metformin hydrochloride and 40 mg gliclazide. Twenty healthy adult male volunteers participated in this study. The volunteers were selected after a thorough assessment of medical history, physical examination and laboratory biochemical examination. Informed consent was obtained from all the subjects after explaining to them the aim and risks of the study. The study protocol was approved by the Human Investigation Ethical Committee of School of Pharmaceutical Sciences at the Sun Yat-sen University, Guangzhou, China.

The study was of a single-dose, randomized, two-period crossover design. Dosing periods were separated by a one-week washout period. After overnight fasting (10 h), the volunteers were orally administered a single dose of the assigned tablet with 200 ml of water. Regular standardized low-fat meals were provided 4 h after dose administration; and water intake was allowed at 2 h following drug administration. Following drug administration, venous blood samples (3.0 ml) were collected into heparinized tubes at the following times: immediately before administration, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 14, 24, 36, 48 and 60 h after dosing.

Blood samples were centrifuged at 4000 rpm for 10 min, and the plasma was separated and obtained. The plasma samples were labeled and kept frozen at −30 °C until analysis.

Calculation of pharmacokinetic parameters was done using the Nonmem program (Version 1.1). The elimination rate constant (λ_z) was obtained as the slope of the linear regression of the log-transformed concentration values *versus* time data in the terminal phase. The elimination half-life ($t_{1/2\beta}$) was calculated as $0.693/\lambda_z$. Time-to-peak plasma concentration (T_{max}) and peak plasma concentration (C_{max}) were read directly from the observed concentration *versus* time profiles. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve to infinity ($AUC_{0\to\infty}$) was calculated as $AUC_{0\to\infty} = AUC_{0-t} + C_t/\lambda_z$, where C_t is the last measurable concentration.

For the bioequivalence analysis, the C_{max}, AUC_{0-t} and $AUC_{0\to\infty}$ were considered as primary variables. Statistical significance of variations in the different formulations was tested according to a one-way analysis of variance (ANOVA) followed by Dunnett's test using the Excel 2000 program. The products were considered bioequivalent if the difference between the two compared parameters was statistically insignificant ($P \geq 0.05$) and the 90%-confidence interval (CI) for these parameters fell within 80–125%.

RESULTS AND DISCUSSION

Method development

In this study, ESI was chosen as the ionization source. It was found that the signal intensity of the analytes and IS in human plasma was high using ESI source and the regression curves were linear. By using ESI, the analytes and IS formed predominantly protonated quasi-molecular ion $[M + H]^+$ in full scan spectra, with m/z 130 for metformin, m/z 324 for gliclazide, and m/z 243 for the IS. To determine metformin and gliclazide using SRM mode, full scan product-ion spectra of the analytes and IS were investigated. The most abundant ion in the product-ion mass spectrum was at 71 for metformin, 110 for gliclazide and 210 for IS. It was found that the capillary temperature and the spray voltage did not significantly influence the MS behavior of these compounds and remained unchanged at the recommended value of 350 °C and 3.5 kV, respectively. Other MS conditions, including source CID and collision pressure, were maintained at the auto-tuned values, since they did not significantly affect the collision behavior of the analytes. But the collision behavior of the $[M + H]^+$ of these compounds was strongly dependent on the collision energy. An increase in the collision energy caused a marked increase of the fragmentation processes. After optimization of the collision energy, the collision behavior was carried out using 20, 38 and 37 V collision energy for metformin, gliclazide and IS, respectively, to obtain the maximum intensity of product ions. Therefore, the SRM transition of m/z 130 → 71 for metformin, m/z 324 → 110 for gliclazide and m/z 243 → 210 for IS were selected to obtain maximum sensitivity. Positive-ion ESI mass spectrum for these target compounds and MS/MS product-ion spectrum of these compounds are shown in Figs 2, 3 and 4.

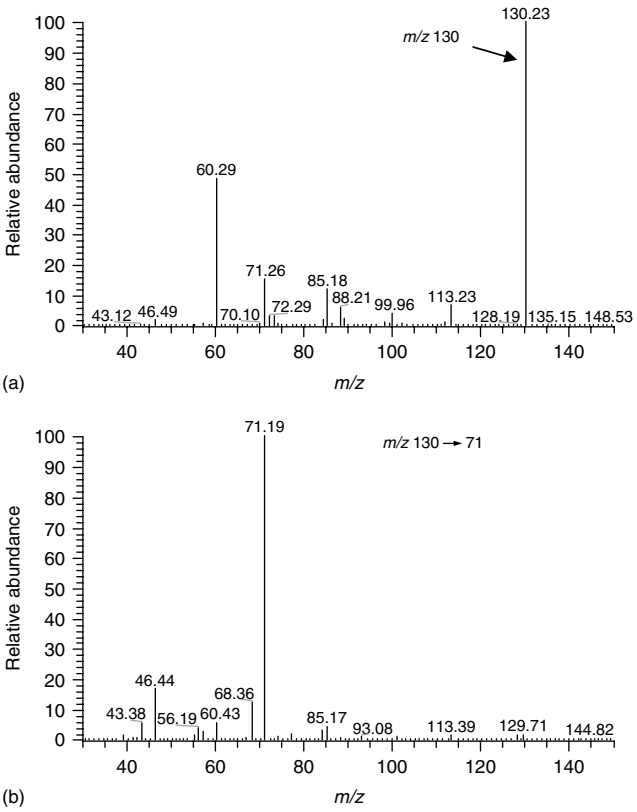

Figure 2. (a) Positive-ion ESI mass spectrum for metformin; (b) MS/MS product-ion spectrum of metformin with $[M + H]^+$ at m/z 130 as the precursor ion.

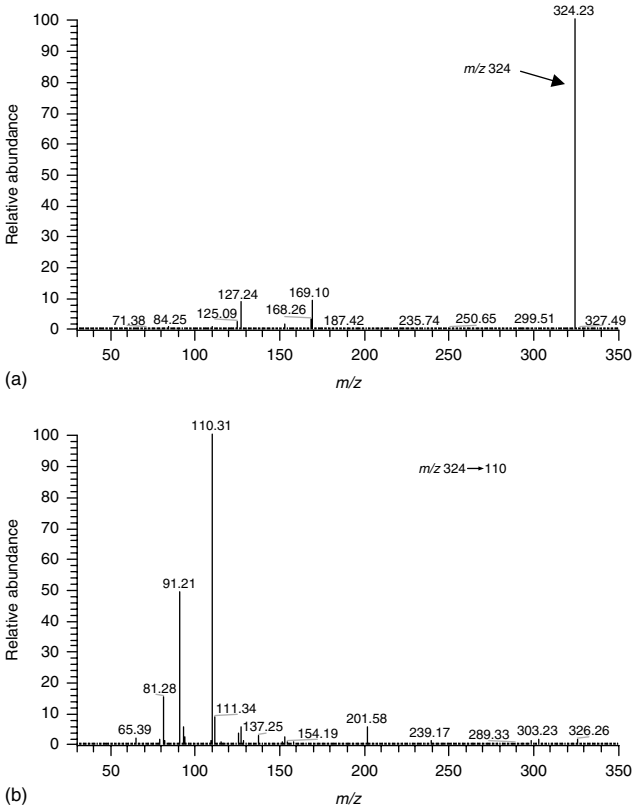

Figure 3. (a) Positive-ion ESI mass spectrum for gliclazide; (b) MS/MS product-ion spectrum of gliclazide with $[M + H]^+$ at m/z 324 as the precursor ion.

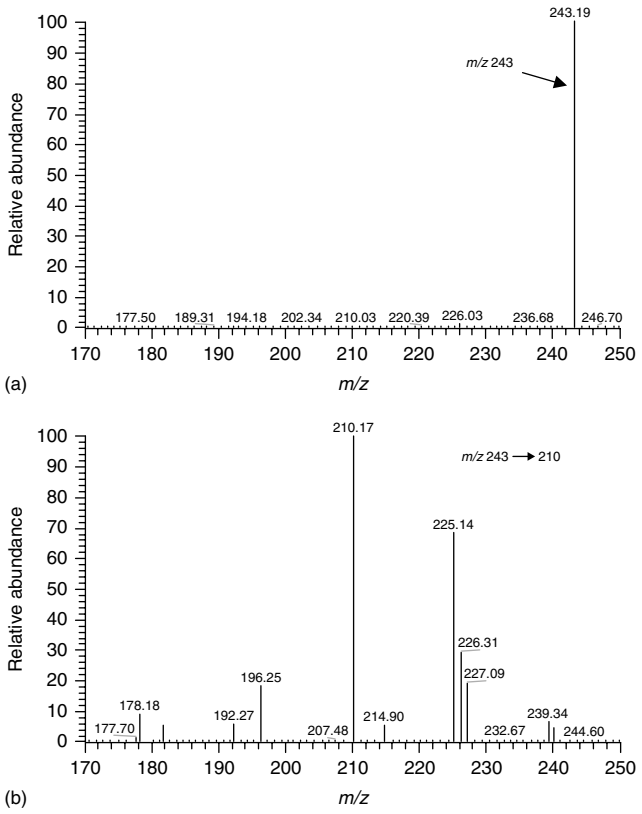

Figure 4. (a) Positive-ion ESI mass spectrum for IS; (b) MS/MS product-ion spectrum of IS with $[M + H]^+$ at m/z 243 as the precursor ion.

In this study, we chose ESI as the ionization source. Since the ESI source provided satisfactory data on method validation and subsequent quantitation for plasma samples from healthy volunteers, we did not try electron impact (EI) or APCI ionization source. Some studies indicate that APCI is much less susceptible to analyte-ion suppression compared with ESI and EI techniques.[29,30] However, APCI is often used only after ESI has not provided the required sensitivity, as APCI needs strict operating conditions such as high flow-rate, high temperature and high voltage, and thus result in incompatibility with thermally labile compounds.[29]

In the present study, a simple single-step liquid–liquid protein precipitation procedure was used. The extraction efficiency and matrix effect of different protein precipitants including acetonitrile, acetone, alcohol and methanol were compared during our method development. All solvents gave a >70 extraction of efficiency for the two analytes. It was found that methanol as a precipitant increased the matrix effect and caused a marked decrease in mass spectral response to the two analytes. Acetone and alcohol also showed a matrix effect and decreased the mass spectral response to the analytes, but to a lesser extent compared to methanol. However, acetonitrile caused the lowest matrix effect and insignificant effect on spectral response to the analytes with a better peak shape compared to other organic solvents. Thus, acetonitrile was finally used as the protein precipitant throughout the study.

Various combinations of acetonitrile, methanol and water with changed content of each component were investigated and compared to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. The acidic modifier, formic acid, in the mobile phase and an increase in the acetonitrile content could improve peak shape, whereas an increase in the water content broadened the peak. A mobile phase consisting of methanol–water (containing 1% formic acid)–acetonitrile was finally used and the ratio 30 : 31 : 39 (v/v/v) was optimal. Each chromatographic run was completed within 2.0 min.

An ideal IS should be a structurally similar analog, stable, labeled compound according to the FDA guideline.[28] However, it was difficult to find a suitable IS that has similar chemical and physical behavior to both metformin and gliclazide when we started the method development. In the present study, huperzine A was chosen as the IS. Huperzine A is an alkaloid isolated from the Chinese herb *Huperzia serrata*. It is a reversible and selective acetylcholinesterase inhibitor.[31] Although there is little structural similarity between huperzine A and the two analytes (metformin and gliclazide), there is a tremendous similarity in the solubility behavior of these agents. On the basis of the solubility behavior of the drugs and the IS, all of them could be readily extracted using acetonitrile as a protein precipitant. Also, these agents had similar chromatographic behavior under the present LC/MS conditions. Additionally, huperzine A did not show any suppressing effect on the analyte ions throughout the LC/MS/MS study. Bonfiglio *et al*.[32] reported that the analyte ions might be suppressed by IS as determined by the postcolumn infusion technique. Importantly, the results of method validation using huperzine A as the IS were acceptable in this study based on the FDA guideline.[28] Moreover, there are numerous reports in the literature where a structurally different compound was used as the IS but acceptable validation data and chromatographic or mass spectral behaviors have been obtained, and these developed HPLC and LC/MS methods to be successfully applied to the determination of samples in biological matrices.[30,33–35] Taking all these together, huperzine A was a suitable IS in this study, though it is structurally different from the two analytes.

Method validation

Selectivity and matrix effects

The LC/MS/MS method demonstrated high specificity because only ions derived from the analytes of interest were monitored. The selectivity toward endogenous plasma matrix was tested in six different batches of human plasma samples by analyzing blanks and samples at LOQ levels. The ratio of response of visible interferences from blank plasma over that of the analytes at LOQ level was determined. It was found that the area of interference observed at the expected retention time for gliclazide was <7.5% of the area of gliclazide at the LOQ level, <5.7% for metformin and <0.2% for the IS. This indicated no significant interference at the expected retention times of the analytes and the IS. Chromatograms of blank human plasma and the plasma at LOQ levels are shown in Fig. 5. The retention times for gliclazide, metformin and IS were 1.5, 0.9 and 0.95 min, respectively. The method had a short

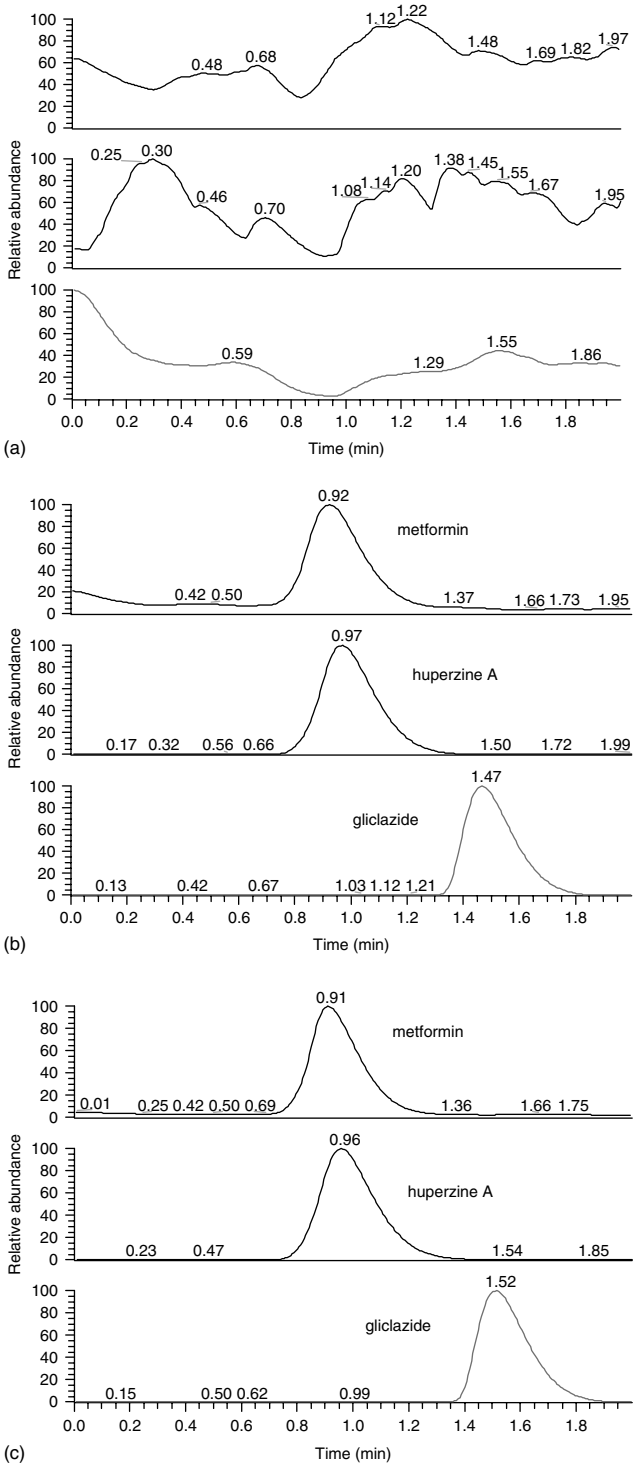

Figure 5. Representative SRM chromatograms of metformin, IS and gliclazide in human plasma. (a) a blank plasma sample; (b) plasma sample at LOQ; (c) plasma sample from a volunteer 24 h after an oral administration of 250 mg metformin hydrochloride in combination with 40 mg gliclazide.

total running time (2.0 min) for simultaneous determination of metformin and gliclazide compared with those reported in the literature.[7,10,11,13,15]

Since potential matrix effect is a concern with the fast isocratic system, the co-elution effect and potential ion suppression were evaluated. To assess the co-elution effect, pooled blank plasma was spiked with each analyte or IS, and the corresponding peak area was compared to that from the spiked sample of combined IS and analytes. Triplicate of the QC sample at medium concentration were tested. Peak area ratios (individual/combined) ranging from 0.98–1.02 showed no co-elution effect and these three co-eluted compounds in the plasma did not cause significant mutual enhancement or suppression of the response. To assess the 'absolute' matrix effect, i.e. the potential ion suppression due to the matrix components, six different batches of blank plasma were extracted by acetonitrile and then spiked with each analyte at medium QC concentration and IS. The corresponding peak areas were then compared to those of the aqueous standards at equivalent concentrations. Peak-area comparison showed that there was a decrease of the area of these compounds in the post-extraction spiked plasma samples, suggesting a matrix effect on the ionization of these compounds. Although ionization suppression was indeed observed for these compounds, a significant change in the ionization response of each analyte was not found, and the ratio of analyte over IS was consistent over the analytical period. Thus, despite the matrix effects that were observed, the present analytical method was considered reliable.

Linearity and lower limit of quantification

The slope, the intercept and the correlation coefficient (r) for each standard curve from each analytical run were determined automatically by the Finnigan Lcquan software program. Table 1 shows the mean slope, intercept and correlation coefficient values for both metformin and gliclazide. The mean squared correlation coefficients (r^2) for the daily calibration curves were all $\geq$0.998 ($n = 5$) for both metformin and gliclazide and the within- and between-run CVs of the response factors for each concentration assayed were <10%. The mean y intercepts were 0.068 and 0.3045 ($n = 5$) for metformin and gliclazide, respectively. For each point on the calibration curves for the two analytes, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for accuracy and precision of ±10%. A linear regression of the back-calculated concentrations *versus* the nominal values provided a unit slope and an intercept not significantly different from zero. The distribution of the residuals showed random variation, was normally distributed and was centered on zero. The bias was not statistically different from zero, and the 95% confidence intervals included zero (data not shown). Overall, both metformin and gliclazide gave linear response as a function of the concentration ranges studied.

Both analytes showed excellent linearity over 10–10 000 ng ml^{-1} for gliclazide and 7.8–4678.9 ng ml^{-1} for metformin (Table 1). Notably, the concentrations for 'metformin' instead of 'metformin hydrochloride' were used for all standard and QC samples, samples for recovery and stability studies and plasma samples from healthy volunteers (Tables 1–5).

The lowest concentration on the calibration curve of gliclazide and metformin was 10 ng ml^{-1} and 7.8 ng ml^{-1}, respectively. The analytes' response at these concentration

Table 1. Slope, intercept and correlation coefficient (r) for the calibration curves for metformin and gliclazide ($n = 5$)

Compound	Conc. Range (ng ml^{-1})	Slope[a]		Intercept[a]		r^2
		Mean ± SD	CV[b] (%)	Mean ± SD	CV[b] (%)	
Metformin	7.8–4678.9	0.0372 ± 0.0008	2.2	0.0680 ± 0.0032	4.7	≥0.998
Gliclazide	10–10 000	0.0294 ± 0.0020	6.8	0.3045 ± 0.0288	9.5	≥0.999

[a] Slope and intercept were determined automatically by Finnigan Lcquan software.
[b] CV = Coefficient of variation = $\frac{\text{SD}}{\text{Mean}} \times 100$.

Table 2. Intra- and interday precision and accuracy data for assays of metformin and gliclazide in human plasma ($n = 5$)

Compound	Nominal Conc. (ng ml^{-1})	Precision		Accuracy
		Mean ± SD	RSD[a] (%)	Mean relative error (%)
Intraday				
Metformin	78.0	82.6 ± 6.8	8.2	5.9
	389.9	420.4 ± 25.6	6.1	7.8
	1559.6	1577.8 ± 91.1	5.8	1.2
Gliclazide	100.0	91.5 ± 9.8	10.7	−8.5
	500.0	518.6 ± 38.8	7.5	3.7
	2000.0	2062.6 ± 203.9	9.9	3.1
Interday				
Metformin	78.0	83.8 ± 4.5	5.3	7.4
	389.9	425.7 ± 31.2	7.3	9.2
	1559.6	1598.8 ± 82.0	5.1	2.5
Gliclazide	100.0	101.2 ± 12.7	12.5	1.2
	500.0	555.1 ± 63.2	11.4	11.0
	2000.0	2168.6 ± 176.6	8.1	8.4

[a] RSD = Relative standard deviation.

levels were >5 times the baseline noise. The precision and accuracy at these concentration levels were acceptable, with <14.5% of the CVs and <12.0% of the relative errors. Thus, the lowest concentration on the calibration curve was accepted as the LOQ. However, the LOQ could be lowered by injecting a more concentrated solution into the LC/MS/MS system. But the current LOQ was already sufficient for the determination of the bioequivalence of gliclazide and metformin following a single-dose administration of the test or reference formulation in healthy human volunteers.

Precision and accuracy

The intra- and interday precision and accuracy data for metformin and gliclazide are summarized in Table 2. All values of accuracy and precision were within recommended limits. Intraday precision ranged between 5.8 and 10.7%, and the interday precision was between 5.1 and 12.5%. The mean intraday error was between −8.5 and 7.8%, and the mean interday error was between 1.2 and 11.0%.

Recovery

Table 3 shows the recovery (extraction efficiency) of metformin and gliclazide from human plasma following acetonitrile extraction. The recovery of metformin and gliclazide from human plasma ranged over 71.0–83.6% and 88.1–104.0%, respectively. Lower extraction of metformin compared with gliclazide was probably due to the differences in pK_a and hydrophobicity. There was a marked variability in the recovery of gliclazide at different concentrations (low concentration level at 104% and ∼88% for other two concentrations), though the concentration dependence was not significant. This might be caused by the matrix effect from plasma components. If the recovery was assessed by comparing the response of spiked plasma samples before and after liquid extraction, the variability could be minimized and the recovery could be consistent at all concentrations.

Table 3. The recovery (extraction efficiency) for metformin and gliclazide in human plasma ($n = 5$)

Compound	Nominal Conc. (ng ml^{-1})	Recovery[a] (Mean ± SD, %)	RSD[b] (%)
Metformin	78.0	77.5 ± 7.0	9.0
	389.9	71.0 ± 2.9	4.0
	1559.6	83.6 ± 1.0	1.2
Gliclazide	100.0	104.0 ± 4.1	4.0
	500.0	89.8 ± 3.1	3.5
	2000.0	88.1 ± 3.6	4.0

[a] The recovery (extraction efficiency) of analytes from human plasma after the extraction procedure was determined by comparing the areas of extracted analytes with that of the nonextracted pure standards that represent 100% recovery.
[b] RSD = Relative standard deviation.

Table 4. Stability of metformin and gliclazide in human plasma under various storage conditions ($n = 5$)

Storage condition	Compound	Nominal Conc. (ng ml^{-1})	Calculated conc. (ng ml^{-1}) Mean ± SD	E_r[a] (%)
−30 °C/39 days				
	Metformin	78.0	66.9 ± 4.6	−14.2
		389.9	418.9 ± 21.2	7.9
		1559.6	1421.7 ± 80.5	−8.8
	Gliclazide	100.0	111.8 ± 2.4	11.8
		500.0	534.2 ± 20.5	6.8
		2000.0	1967.3 ± 76.3	−1.6
−30 °C/3 freeze–thaw cycles				
	Metformin	78.0	76.4 ± 2.6	−2.1
		389.9	400.4 ± 25.8	2.7
		1559.6	1526.9 ± 52.2	−2.1
	Gliclazide	100.0	111.3 ± 2.6	11.3
		500.0	556.1 ± 14.1	11.2
		2000.0	2200.5 ± 84.8	10.0
Room temperature/4 h				
	Metformin	78.0	69.4 ± 1.9	−11.0
		389.9	364.4 ± 17.1	−6.5
		1559.6	1414.0 ± 60.1	−9.3
	Gliclazide	100.0	110.6 ± 4.4	10.6
		500.0	541.1 ± 2.8	8.2
		2000.0	2157.2 ± 82.1	7.9
Room temperature/12 h (Extracted sample)				
	Metformin	78.0	80.3 ± 1.7	3.0
		389.9	411.6 ± 18.4	5.6
		1559.6	1571.9 ± 53.1	0.8
	Gliclazide	100.0	101.8 ± 2.5	1.8
		500.0	489.4 ± 11.1	−2.1
		2000.0	1929.8 ± 85.0	−3.5

[a] Relative error $= \dfrac{\text{Overall mean assayed concentration} - \text{added concentration}}{\text{Added concentration}} \times 100$

Stability

The analytes are considered stable in biological matrix when 85–115% of the initial concentration can be detected.[28] The stability of both metformin and gliclazide in human plasma under different storage conditions is presented in Table 4. There was no significant degradation under these conditions described in this study, since their concentrations deviated by no more than 14.2% relative to the reference nominal concentrations. No degradation products were detected under the selected MS conditions. Both metformin and gliclazide in human plasma can therefore be stored at room temperature for 4 h, 39 days at −30 °C and after three freeze–thaw cycles. Analysis of the QC samples following protein precipitation procedure showed no significant degradation after 12 h at room temperature. These results indicate that both metformin and gliclazide are stable under routine laboratory conditions and no specific procedure (e.g. acidification, or addition or organic solvents) is needed to stabilize the compounds for daily clinical drug monitoring.

Bioequivalence study

Both test and reference formulations were readily absorbed from the gastrointestinal tract. Metformin and gliclazide were measurable at the first sampling time (0.5 h) in plasma samples from all 20 healthy volunteers. The mean plasma concentration *versus* time curves obtained after a single oral dose of each formulation for metformin and gliclazide are shown in Figs 6 and 7, respectively.

The pharmacokinetic parameters of metformin and gliclazide for the reference and test formulations are presented

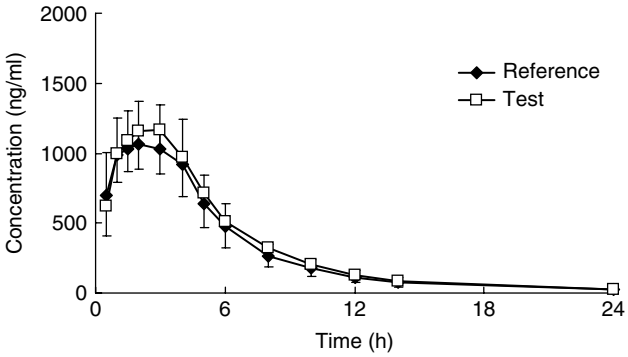

Figure 6. Mean plasma concentration of metformin after oral administration of single dose of two formulations to 20 healthy male volunteers.

Table 5. Pharmacokinetic parameters of metformin and gliclazide in the test and reference formulations (mean ± SD, $n = 20$)

	Metformin		Gliclazide	
Parameter	Test formulation	Reference formulation	Test formulation	Reference formulation
C_{max} (ng ml^{-1})	1264.4 ± 275.0	1161.7 ± 307.9	5266.7 ± 880.2	4958.8 ± 771
T_{max} (h)	2.0 ± 0.8	1.9 ± 1.0	3.5 ± 1.0	3.9 ± 0.8
$t_{1/2\beta}$ (h)	4.1 ± 0.6	4.6 ± 0.9	12.4 ± 4.4	12.6 ± 4.8
AUC_{0-t} (ng ml^{-1} h)	7758.5 ± 2096.4	7132.9 ± 1956.1	76752.9 ± 33971.9	75804.7 ± 34103.4
$AUC_{0-\infty}$ (ng ml^{-1} h)	8063.6 ± 2096.0	7287.3 ± 1998.9	81567.9 ± 44203.9	82114.4 ± 43993.4

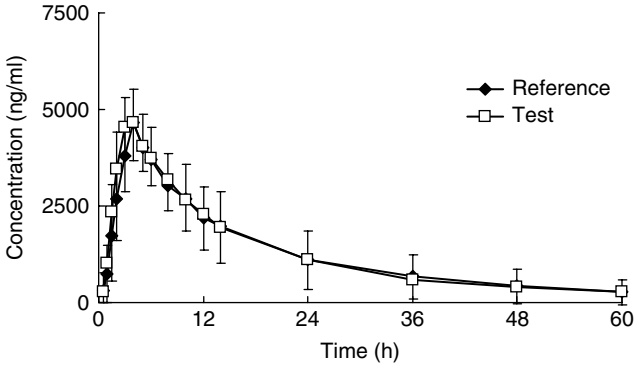

Figure 7. Mean plasma concentration of gliclazide after oral administration of single dose of two formulations to 20 healthy male volunteers.

in Table 5. There was no significant difference between the two formulations with regard to the pharmacokinetic parameters including C_{max}, AUC_{0-t} and $AUC_{0\to\infty}$. The pharmacokinetic parameters in this study are similar to those reported in previous studies when metformin or gliclazide was administrated alone in humans.[16,20,32,36]

From the plasma concentration *versus* time profiles of the 20 subjects, the relative bioavailability of metformin was 111.2 ± 23.6% based on mean AUC_{0-last} and $AUC_{0\to\infty}$ and that of gliclazide was 103.4 ± 16.6%. The 90%-CIs of metformin for C_{max}, AUC_{0-t} and $AUC_{0\to\infty}$ were 101.7–119.4%, 99.8–118.5% and 99.9–123.3%, respectively. The 90%-CIs of gliclazide for C_{max}, AUC_{0-t} and $AUC_{0\to\infty}$ were 100.5–111.8%, 95.5–109.2% and 92.4–107.4%, respectively.

CONCLUSIONS

In this study, we reported on a newly developed LC/MS/MS method for the simultaneous determination of gliclazide and metformin in human plasma. The sample pretreatment was a single-step liquid–liquid protein precipitation and extraction of the analytes using acetonitrile. The analytes, gliclazide, metformin and the IS were subject to LC/MS/MS analysis using ESI technique with satisfactory mass spectral response. Detailed validation following FDA guideline indicated that the developed method had high sensitivity, reliability, specificity and excellent efficiency with a total running time of 2.0 min per sample. The method was successfully applied to determine gliclazide and metformin plasma concentrations in a bioequivalence study in human volunteers. As the 90%-CIs for C_{max}, AUC_{0-t} and $AUC_{0\to\infty}$ for the two drugs analyzed were all within 80–125%, it was concluded that the test gliclazide/metformin formulation was bioequivalent to the reference formulation with regard to both the rate and the extent of absorption in humans.

REFERENCES

1. Andersson DKG, Svardsudd K. Long term glycemic control related to mortality in type II diabetes. *Diabetes Care* 1995; **18**: 1534.
2. Yale JF. Oral antihyperglycemic agents and renal disease: new agents, new concepts. *J. Am. Soc. Nephrol.* 2005; **16**: S7.
3. Lebovitz HE. Oral antidiabetic agents: 2004. *Med. Clin. North Am.* 2004; **88**: 847.
4. Chipkin SR. How to select and combine oral agents for patients with type 2 diabetes mellitus. *Am. J. Med.* 2005; **118**: 4S.
5. Krentz AJ, Bailey CJ. Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs* 2005; **65**: 385.
6. Bell DS. Type 2 diabetes mellitus: what is the optimal treatment regimen? *Am. J. Med.* 2004; **116**: 23S.
7. DeFronzo RA, Barzilai N, Simonson DC. Mechanism of metformin action in obese and lean noninsulin-dependent diabetic subjects. *J. Clin. Endocrinol. Metab.* 1991; **73**: 1294.
8. Bailey CJ, Turner RC. Metformin. *N. Engl. J. Med.* 1996; **334**: 574.
9. Marathe PH, Arnold ME, Meeker J, Greene DS, Barbhaiya RH. Pharmacokinetics and bioavailability of a metformin/glyburide tablet administered alone and with food. *J. Clin. Pharmacol.* 2000; **40**: 1494.
10. Riddle MC. Glycemic management of type 2 diabetes: an emerging strategy with oral agents, insulins, and combinations. *Endocrinol. Metab. Clin. North Am.* 2005; **34**: 77.
11. Padwal R, Majumdar SR, Johnson JA, Varney J, McAlister FA. A systematic review of drug therapy to delay or prevent type 2 diabetes. *Diabetes Care* 2005; **28**: 736.
12. Tack CJ, Smits P. New drugs for diabetes. *Neth. J. Med.* 1999; **55**: 209.
13. Funnell MM, Kruger DF. Type 2 diabetes: treat to target. *Nurse Pract.* 2004; **29**: 11–15, 19–23; quiz 23–5.
14. Yuen KH, Peh KK. Simple high-performance liquid chromatographic method for the determination of metformin in human plasma. *J. Chromatogr. B Biomed. Sci. Appl.* 1998; **710**: 243.
15. Vesterqvist O, Nabbie F, Swanson B. Determination of metformin in plasma by high-performance liquid chromatography after ultrafiltration. *J. Chromatogr. B Biomed. Sci. Appl.* 1998; **716**: 299.
16. Park JY, Kim KA, Kim SL, Park PW. Quantification of gliclazide by semi-micro high-performance liquid chromatography: application to a bioequivalence study of two formulations in healthy subjects. *J. Pharm. Biomed. Anal.* 2004; **35**: 943.
17. Cheng CL, Chou CH. Determination of metformin in human plasma by high-performance liquid chromatography with spectrophotometric detection. *J. Chromatogr. B Biomed. Sci. Appl.* 2001; **762**: 51.
18. Nunez M, Ferguson JE, Machacek D, Jacob G, Oda RP, Lawson GM, Landers JP. Detection of hypoglycemic drugs in human urine using micellar electrokinetic chromatography. *Anal. Chem.* 1995; **67**: 3668.

19. Wang Y, Tang Y, Gu J, Fawcett JP, Bai X. Rapid and sensitive liquid chromatography-tandem mass spectrometric method for the quantitation of metformin in human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2004; **808**: 215.
20. Chen X, Gu Q, Qiu F, Zhong D. Rapid determination of metformin in human plasma by liquid chromatography-tandem mass spectrometry method. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2004; **802**: 377.
21. Heinig K, Bucheli F. Fast liquid chromatographic-tandem mass spectrometric (LC-MS-MS) determination of metformin in plasma samples. *J. Pharm. Biomed. Anal.* 2004; **34**: 1005.
22. Koseki N, Kawashita H, Niina M, Nagae Y, Masuda N. Development and validation for high selective quantitative determination of metformin in human plasma by cation exchanging with normal-phase LC/MS/MS. *J. Pharm. Biomed. Anal.* 2005; **36**: 1063.
23. Ho EN, Yiu KC, Wan TS, Stewart BD, Watkins KL. Detection of anti-diabetics in equine plasma and urine by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2004; **811**: 65.
24. Maurer HH, Kratzsch C, Kraemer T, Peters FT, Weber AA. Screening, library-assisted identification and validated quantification of oral antidiabetics of the sulfonylurea-type in plasma by atmospheric pressure chemical ionization liquid chromatography-mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2002; **773**: 63.
25. Susanto F, Reinauer H. Screening and simultaneous quantitative measurement of six sulfonylureas in serum by liquid chromatography/mass spectrometry with atmospheric-pressure chemical-ionization (APCI LC/MS). *Fresenius' J. Anal. Chem.* 1997; **357**: 1202.
26. Vasudevan M, Ravi J, Ravisankar S, Suresh B. ION-pair liquid chromatography technique for the estimation of metformin in its multicomponent dosage forms. *J. Pharm. Biomed. Anal.* 2001; **25**: 77.
27. Aburuz S, Millership J, McElnay J. The development and validation of liquid chromatography method for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2005; **817**: 277.
28. FDA. Guidance for industry bioanalytical method validation, May 2001.
29. Mallet CR, Lu Z, Mazzeo JR. A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. *Rapid Commun. Mass Spectrom.* 2004; **18**: 49.
30. Jemal M, Schuster A, Whigan DB. Liquid chromatography/tandem mass spectrometry methods for quantitation of mevalonic acid in human plasma and urine: method validation, demonstration of using a surrogate analyte, and demonstration of unacceptable matrix effect in spite of use of a stable isotope analog internal standard. *Rapid Commun. Mass Spectrom.* 2003; **17**: 1723.
31. Ye JW, Cai JX, Wang LM, Tang XC. Improving effects of huperzine A on spatial working memory in aged monkeys and young adult monkeys with experimental cognitive impairment. *J. Pharmacol. Exp. Ther.* 1999; **288**: 814.
32. Gusler G, Gorsline J, Levy G, Zhang SZ, Weston IE, Naret D, Berner B. Pharmacokinetics of metformin gastric-retentive tablets in healthy volunteers. *J. Clin. Pharmacol.* 2001; **41**: 655.
33. Zaghloul AA, Hussain A, Khan MA, Ahsan F. Development of a HPLC method for the determination of cyclosporin-A in rat blood and plasma using naproxen as an internal standard. *J. Pharm. Biomed. Anal.* 2003; **31**: 1101.
34. Yin OQ, Lam SS, Lo CM, Chow MS. Rapid determination of five probe drugs and their metabolites in human plasma and urine by liquid chromatography/tandem mass spectrometry: application to cytochrome P450 phenotyping studies. *Rapid Commun. Mass Spectrom.* 2004; **18**: 2921.
35. Zhou S, Li Y, Kestell P, Paxton JW. Determination of thalidomide in transport buffer for Caco-2 cell monolayers by high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2003; **785**: 165.
36. Najib N, Idkaidek N, Beshtawi M, Bader M, Admour I, Alam SM, Zaman Q, Dham R. Bioequivalence evaluation of two brands of metformin 500 mg tablets (Dialon & Glucophage)–in healthy human volunteers. *Biopharm. Drug Dispos.* 2002; **23**: 301.