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Bioanalytical applications

# Liquid chromatography tandem mass spectrometry method for simultaneous determination of antidiabetic drugs metformin and glyburide in human plasma

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**Abstract**

A simple and rapid liquid chromatography/tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the simultaneous quantitation of antidiabetic drugs metformin and glyburide in human plasma using glimepiride as internal standard (IS). After acidic acetonitrile-induced protein precipitation of the plasma samples, metformin, glyburide and IS were chromatographed on reverse phase C18 (50 mm × 4.6 mm i.d., 5 μm) analytical column. Quantitation was performed on a triple quadrupole mass spectrometer employing electrospray ionization technique and operating in multiple reaction monitoring (MRM) and positive ion mode. The total chromatographic run time was 3.5 min and calibration curves were linear over the concentration range of 20–2500 ng/ml for metformin and 5–500 ng/ml for glyburide. The method was validated for selectivity, sensitivity, recovery, linearity, accuracy and precision, dilution integrity and stability studies. The recoveries obtained for the analytes and IS (≥69%) were consistent and reproducible. Inter-batch and intra-batch coefficient of variation across four validation runs (LLOQ, LQC, MQC and HQC) was less than 8%. The accuracy determined at these levels was within ±8% in terms of relative error (RE). The method was applied to a bioequivalence study of 500 mg metformin and 5 mg of glyburide tablet after oral administration to 28 healthy human subjects under condition of fasting.


*Keywords:* Metformin; Glyburide; LC–MS/MS; Protein precipitation; Human plasma; Bioequivalence

## 1. Introduction

Diabetes and its abnormalities constitute a major health problem in the modern society [1]. It is characterized by disrupted insulin production, leading to high blood glucose concentration and other complications such as renal dysfunction, neuropathy and cardiopathy [2]. Many oral antidiabetic drugs with different mechanisms of action have been developed to lower blood sugar and delay the occurrence of serious complications in patients with type 2 diabetes [2]. For glycemic control in such cases, monotherapy with an oral antidiabetic agent is not adequate to achieve satisfactory blood glucose control [3]. Thus, combination regimens which include drugs with different and complementary mechanisms of action are recommended. The most commonly used combination therapy in clinical practice for type 2 diabetes includes metformin with a second generation sulfonylurea such as glyburide, gliclazide or glipizide [3].

Metformin is an orally administered antihyperglycemic drug that lowers glucose by reducing hepatic glucose production and gluconeogenesis and by enhancing peripheral glucose uptake [4]. Glyburide, a second generation sulphonylurea is one of the most widely used oral hypoglycemic drugs for noninsulin-dependant diabetic patients and is effective even at very low dosages [5]. Therapeutic drug monitoring necessitates the measurement of their plasma concentration for studying the pharmacokinetics of these drugs, assessment of bioequivalence of commercially available tablet formulation and for optimization of dosing in combination therapy [3,6]. Thus, reliable, sensitive and rapid bioanalytical methods are desired to simultaneously determine metformin and glyburide in human plasma.

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Several assay methodologies are reported for the determination of metformin and glyburide individually in different biological matrices using gas chromatography [7,8], high performance liquid chromatography [9–26] and capillary electrophoresis [27]. Some of these methods suffer from various disadvantages namely, sensitivity, long chromatographic run times or a cumbersome extraction procedure before analysis which prevent their use for routine sample analysis. To circumvent these problems, several methods are reported for the analysis of these drugs by LC–MS/MS with improved sensitivity, selectivity and efficiency [28–33]. The simultaneous determination of these analytes is important for the routine monitoring of diabetic patients who take combination medications and for studying the pharmacokinetics of the combined dosage forms. Zhong et al. [34] have developed a rapid and sensitive LC–MS/MS method to determine metformin and gliclazide after acetonitrile induced protein precipitation of plasma samples. The run time was very short (2 min) and the lower limits of quantitation of the method were 7.8 and 10.0 ng/ml for metformin and gliclazide, respectively.

However, there are very few reports on the simultaneous determination of metformin and glyburide in plasma. Abu-Ruz et al. [35] have developed an HPLC method employing SPE for the simultaneous determination of metformin and glipizide, gliclazide, glyburide or glimepiride in plasma. The limits of quantitation were between 5 and 22 ng/ml with a long chromatographic run time of 15 min, which may not be practical for high throughput analysis when large numbers of samples are involved. Using a nonaqueous solid phase extraction capillary electrophoresis technique, Lia and Feng [36] have determined metformin, phenformin and glyburide in human plasma. The separation of metformin from phenformin was achieved within 3 min using a non-aqueous buffer, acetonitrile + 5 mM ammonium acetate + 5% acetic acid, but the migration rate of glyburide was >6 min. When the electrokinetic injection time was increased to 36 s, the detection limits achieved for metformin and phenformin were 12 and 6 ng/ml, respectively.

In the present study, a rapid liquid chromatography tandem mass spectrometry method has been developed and validated for simultaneous determination of metformin and glyburide in human plasma. The use of mass spectrometry interfaced with HPLC helps to improve sensitivity and selectivity compared to traditional HPLC and GC methods. This rapid method (3.5 min run time) was successfully applied to a bioequivalence study of test and reference formulation (500 mg metformin and 5 mg of glyburide tablet) in 28 healthy human subjects under condition of fasting.

## 2. Experimental

### 2.1. Chemicals, reagents and materials

Standard reference materials of metformin hydrochloride, glyburide and glimepiride (internal standard) having purity of 99.30, 98.60 and 99.54%, respectively were provided by Themis Laboratories Pvt Ltd., (Mumbai, India). HPLC grade methanol and acetonitrile were purchased from J.T.Baker INC (Phillipsburg, NJ, USA). Ammonium acetate, formic acid and glacial acetic acid of AR grade were purchased from Qualigens Ltd., (Mumbai, India). Purified water was obtained from Milli Q A10 gradient water purification system (Millipore, Banglore, India). Blank human blood was collected with heparin from healthy and drug-free volunteers. After centrifugation at 4000 rpm at room temperature, plasma was collected and stored at −20 °C.

### 2.2. Liquid chromatography and mass spectrometric condition

The liquid chromatography system (Shimadzu, Kyoto, Japan) consisted of a binary LC-20AD prominence pump, an autosampler (SIL–HTc), an online solvent degasser (DGU-20$A_3$ prominence) and a temperature-controlled compartment for column (CTO 10AVP). Chromatographic separation was performed on Hypersil, hypurity C18 (50 mm × 4.6 mm i.d., 5 μm) analytical column (Thermo Electron Corporation, Cheshire, UK) maintained at 25 °C temperature. The mobile phase was prepared by mixing 700 ml of acetonitrile with 300 ml of 5 mM ammonium acetate pH 3.0 adjusted with glacial acetic acid. The flow rate of the mobile phase under isocratic condition was kept at 0.4 ml/min. The auto sampler temperature was set at 10 °C and the injection volume was 5 μl. The total LC run time was 3.5 min. Detection of analytes and IS was performed on a triple quadrupole mass spectrometer, API-2000, (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray source in the positive ion mode. Analyst software version 1.4 was used to control all parameters of LC and MS. Quantitation was performed using multiple reaction monitoring (MRM) mode, based on parent → product ion transitions for metformin (130.1 → 60.1), glyburide (494.0 → 352.1) and IS (491.1 → 369.0). Source dependent parameters optimized were gas 1(Nebuliser gas): 40 psi; gas 2(heater gas): 60 psi; ion spray voltage (ISV): 5000 V; temperature (TEM): 400 °C. Compound dependent parameters declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were set at 21, 20 and 4 V, respectively for metformin; 22, 19 and 12 V, respectively for glyburide; 22, 20 and 11 V, respectively for glimepiride. Entrance potential (EP) and focusing potential (FP) were set at 10 and 400 V, respectively for both the analytes and IS. Nitrogen was used as collision activated dissociation (CAD) gas and was set at 4 psi. Quadrupole 1 and quadrupole 3 were maintained at unit resolution and dwell time was set at 200 ms.

### 2.3. Standard and quality control preparation

The standard stock solutions of metformin, glyburide and IS were prepared by dissolving their accurately weighted compounds in methanol to give a final concentration of 1000 μg/ml. The combined working solutions of analytes in the desired concentration range were prepared by appropriate dilution of standard stock solutions with methanol–water (60:40 v/v). All the solutions were stored at 2–8 °C and were brought to room temperature before use.

The calibration standards (CS) and quality control (QC) samples were prepared by spiking blank plasma (5% of total volume of blank plasma) with respective working solutions. Calibration standards were made at concentration of 20, 40, 100, 200, 500, 1000, 1500, 2000 and 2500 ng/ml for metformin; 5, 10, 25, 50, 100, 200, 300, 400 and 500 ng/ml for glyburide. Quality controls were prepared at 60 ng/ml (low quality control, LQC), 750 ng/ml (middle quality control, MQC) and 2250 ng/ml (high quality control, HQC) for metformin; 15 ng/ml (LQC), 150 ng/ml (MQC) and 450 ng/ml (HQC) for glyburide. Spiked plasma samples were aliquoted in microcentrifuge tubes and stored at −20 °C until use.

### 2.4. Sample preparation

All frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature prior to analysis. The samples were vortexed to mix and centrifuged at 3200 rpm at 10 °C for 5 min to settle down any clots present. To a 0.5 ml plasma sample, 50 μl of 10.0 μg/ml of internal standard was added and vortexed to mix for 10 s. The samples were precipitated using 1 ml, 0.1% (v/v) formic acid in acetonitrile and centrifuged at 10,000 rpm for 10 min. Clear supernatant was collected and transferred into 1.5 ml vials, capped and placed in auto sampler rack for injection in the chromatographic system.

### 2.5. Bioanalytical method validation

The method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, stability, matrix effect and dilution integrity following the USFDA guidelines [37].

Selectivity tests were performed with two sets, each having ten heparinised blank plasma in which eight were normal, one was haemolysed and the other was lipemic plasma. In the first set, blank plasma samples were extracted and directly injected for LC–MS/MS detection. The blank plasma samples of the other set were spiked with LLOQ working solution, extracted and analysed in the similar manner. The second set was also used for sensitivity (LLOQ) determination.

The linearity of the method was determined by analysis of standard plots associated with a nine-point standard calibration curve. Seven linearity curves containing nine non-zero concentrations were analysed. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of the analytes were calculated from calibration curve ($y = mx + c$; where $y$ is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor $1/x^2$ for metformin and $1/x$ for glyburide. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each QC level. The peak area ratio values of calibration standards were proportional to the concentration of the drug in plasma over the range tested.

Inter-batch and intra-batch accuracy and precision was evaluated at four different concentrations levels (LLOQ, LQC, MQC and HQC) in six replicates for both the analytes. Mean and standard deviation (S.D.) values were obtained for calculated drug concentration over these batches. The accuracy and precision was calculated and expressed in terms of relative error (% RE) and coefficient of variation (% CV), respectively.

Recovery of the extraction procedure was performed at LQC, MQC and HQC levels using the proposed extraction procedure. It was evaluated by comparing peak area of extracted samples (spiked before extraction) to the peak area of unextracted samples (quality control working solutions spiked in extracted plasma).

Stability experiments were performed to evaluate the analyte stability in stock solutions and in plasma samples under different conditions, simulating the same conditions, which occurred during study sample analysis. Stock solution stability was performed by comparing area response of stability sample of analytes and internal standard with the area response of sample prepared from fresh stock solutions. Bench top stability, extracted sample stability (process stability), freeze–thaw stability and long-term stability were performed at LQC and MQC levels using six replicates at each level.

Ion suppression/enhancement were checked by infusing fresh aqueous samples and unextracted sample (spiked externally in extracted blank plasma) at equivalent concentration (MQC level). Further, to study the effect of matrix on analyte quantitation with respect to consistency in signal suppression/enhancement, matrix effect was checked in six different lots of plasma. These lots of heparinised plasma comprised of: four lots of normal plasma, one lot of lipemic plasma and one lot of haemolysed plasma. Three replicates, each of LQC and HQC levels were prepared from these lots of plasma (total 36 QC samples) and checked for the accuracy in terms of relative error in all the QC samples.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. Dilution integrity experiment was carried out at 1.5× the ULOQ concentration (3750 and 750 ng/ml for metformin and glyburide, respectively). Six replicate samples each of ½ (1875 and 375 ng/ml) and 1/4 (937.5 and 187.5 ng/ml) concentration were prepared and their concentrations were calculated, by applying the dilution factor of 2 and 4 against the freshly prepared calibration curve for metformin and glyburide, respectively.

### 2.6. Bioequivalence study

The validated method was successfully applied to the assay of metformin and glyburide in healthy human subjects who received reference and test formulation.The reference formulation used in the present bioequivalence study was Glucophage® 500 mg metformin tablet (Bristol Myers Squibb, New York, USA) and Daonil 5 mg glyburide tablet (Aventis, Mumbai, India). The active ingredients in the test formulation were 500 mg metformin and 5 mg glyburide.

The design of study comprised of a randomized, open label, single dose, two treatments, two sequence bio equivalence study of metformin (500 mg) and glyburide (5 mg) tablet in twenty-

eight normal healthy subjects under condition of fasting. All the subjects were informed of the aim and risk involved in the study and written consent were obtained. Ethics committee approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [38]. Health check-up for all subjects was done by general physical examination, ECG and laboratory tests like hematology, biochemistry and urine examination. All subjects were negative for HIV, HBSAg and HCV tests. They were orally administered a single dose of test and reference formulation after recommended wash out period with 240 ml of water. Drinking water was not allowed and supine position was restricted 2 h postdose. Standardized meals were provided as per schedule. Blood samples were collected in tubes containing heparin before (0 h) and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.5, 12, 24 and 30 h of administration of drug. Blood samples were centrifuged at 3200 rpm for 10 min and plasma was separated, stored at −20 °C until use.

## 3. Results and discussion

### 3.1. Method development

It was important to optimize chromatographic conditions, mass spectrometry parameters and extraction technique to develop and validate a selective and rapid assay method for simultaneously quantitation of metformin and glyburide in human plasma. MS parameters were optimized by infusing standard analyte solution of 500 ng/ml into the mass spectrometer having electrospray as the ionization source and operating in the multiple reaction monitoring (MRM) mode. The signal intensities obtained in positive mode were much higher than those in negative ion mode since the analytes and glimepiride (internal standard) have the ability to accept protons. Metformin, glyburide and glimepiride gave predominant protonated $(M+H)^+$ parent ions at m/z 130.1, 494.0 and 491.1 ions, respectively in Q1 MS full scan spectra. Fragmentation was initiated using sufficient nitrogen for collision-activated dissociation and by applying 20 V collision energy to break the parent ions. The most abundant ions found in the product ion mass spectrum were m/z 60.1, 352.1 and 369.0 for metformin, glyburide and glimepiride, respectively. It was observed that higher nebuliser gas pressure (40 psi) had a better impact on spectral response. The intensity was further enhanced after acidifying the solution, as it increases the ionization (protonation) resulting in high response in positive ion mode. Ion spray voltage and temperature did not have much impact on behavior of compounds and were maintained at 5000 V and 400 °C, respectively. Q1 and Q3 were set at unit resolution and dwell time kept was 200 ms. There was no cross talk between the MRMs of analytes and IS. Fine tuning of gas 1 (nebuliser gas), gas 2 (heater gas) and CAD gas was done to get a consistent and stable response with high signal to noise ratio. Figs. 1–3 show the mass spectra of parent and product ions for analytes and IS, respectively. Electrospray ionization (ESI) was selected as the ionization source as it gave high spectral response for both the analytes and the regression curves obtained were linear. Also, ESI source provided reliable

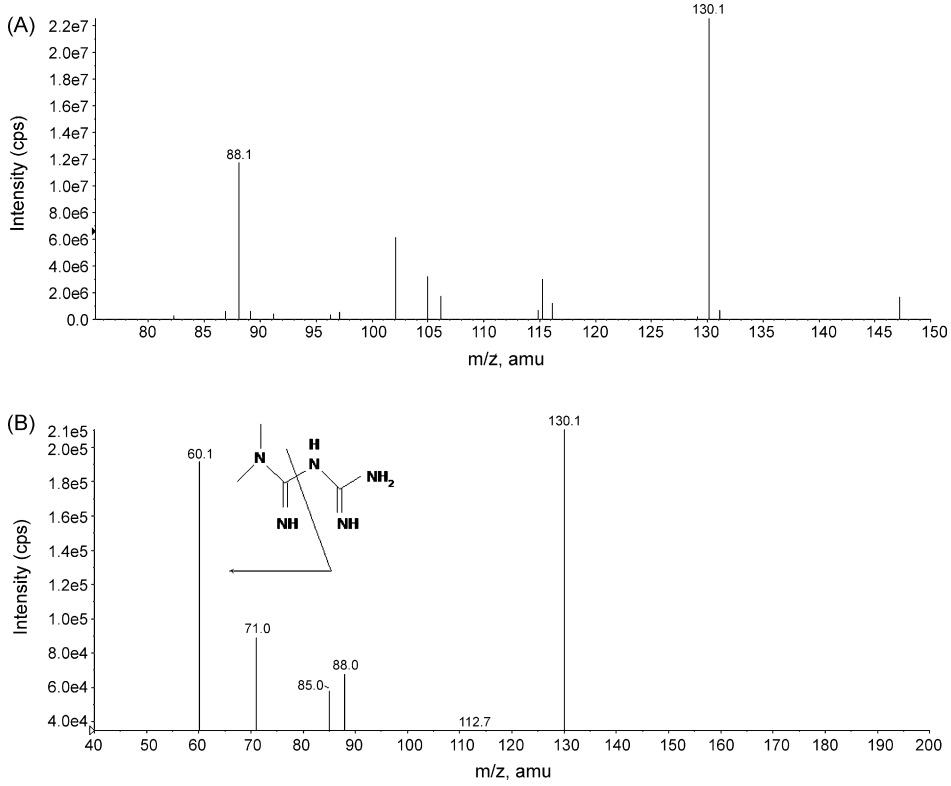


Fig. 1. Mass spectra of parent (A) and product ion (B) for metformin.

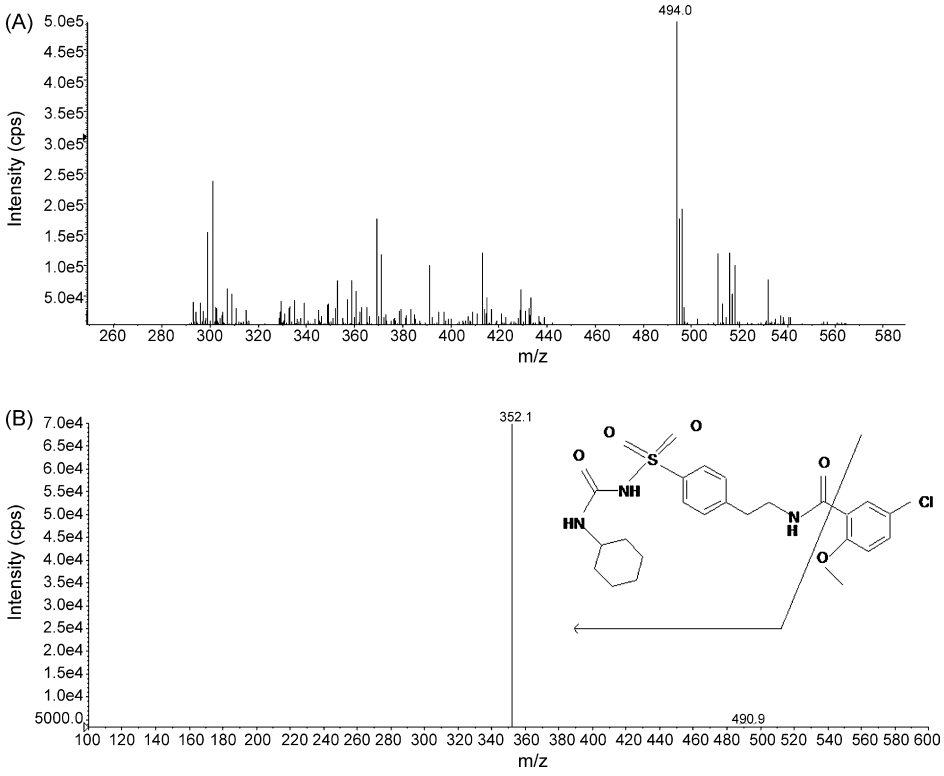


Fig. 2. Mass spectra of parent (A) and product ion (B) for glyburide.

data on method validation and for quantitation of samples from human volunteers.

Since metformin and glyburide have different physicochemical properties, it was difficult to set chromatographic conditions that produced sharp peak shape and adequate response. This included mobile phase selection, flow rate, column type and injection volume. Methanol, acetonitrile were tried in different ratio with buffers like ammonium acetate, ammonium formate as

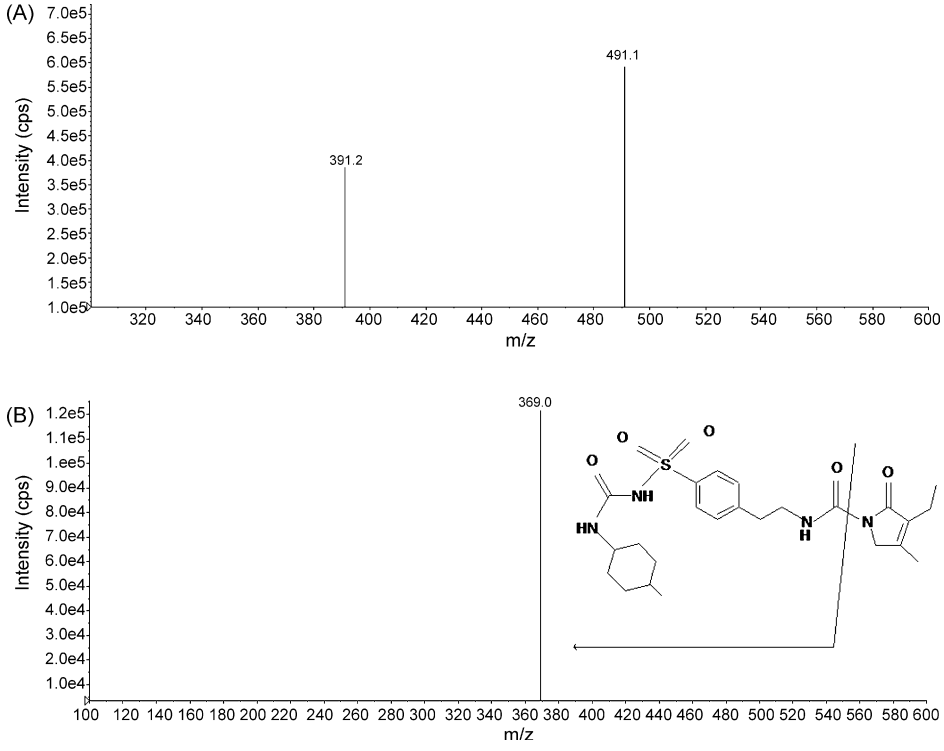


Fig. 3. Mass spectra of parent (A) and product ion (B) for glimepiride (IS).

well as acid additives like formic acid and acetic acid in varying strength. It was observed that 5 mM ammonium acetate (pH 3.0): acetonitrile (30:70 v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. Acidic buffer helped to improve the peak shape and spectral response. 30% aqueous part was adequate to retain the polar compound metformin. The use of a short chromatography column C18 Hypersil, hypurity (50 mm × 4.6 mm i.d., 5 μm) helped in the separation and elution of all three compounds in a very short time. The total chromatographic run time was 3.5 min for each run.

Simultaneous recovery of both the analytes from plasma was difficult as metformin is highly polar while glyburide is comparatively a less polar compound. Solid phase extraction was unsuccessful in giving consistent and adequate response. Liquid–liquid extraction in different solvents like diethyl ether, dichloromethane or methyl tert butyl ether gave consistent recovery for glyburide with negligible matrix effect but not for metformin. Moreover, the method required back extraction, drying and reconstitution steps, making it more tedious and time consuming. Thus, simple protein precipitation technique was tried with acetonitrile, methanol and acetone to recover both the analytes and IS. Precipitation with acetonitrile containing 0.1%(v/v) formic acid caused the lowest matrix with better peak shape compared to other organic solvents. The major advantage of this method was its efficiency in extracting both the analytes and IS. The extract (supernatant liquid) was directly used for injection without drying and reconstitution. Minor ion suppression due to biological matrix found at the retention time of metformin was rendered insignificant by modifying the mobile phase ratio that separated the undetected interference from metformin peak. Overall, this method is fast and simple in terms of chromatography and analyte extraction (sample preparation), respectively, which helped in giving a high turnaround for routine sample analysis. As per FDA guideline, an ideal internal standard should be a structurally similar analog, stable and a labeled compound. Glimepiride has structural similarity with glyburide but is markedly different from metformin. Since all three compounds had similar chromatographic behavior and were precipitated easily with the same protein precipitant, it was selected as the internal standard. Moreover, there was no significant matrix effect of IS on both the analytes. Also, the validation results obtained from this LC–MS/MS methodology encouraged its selection as an IS for the present study.

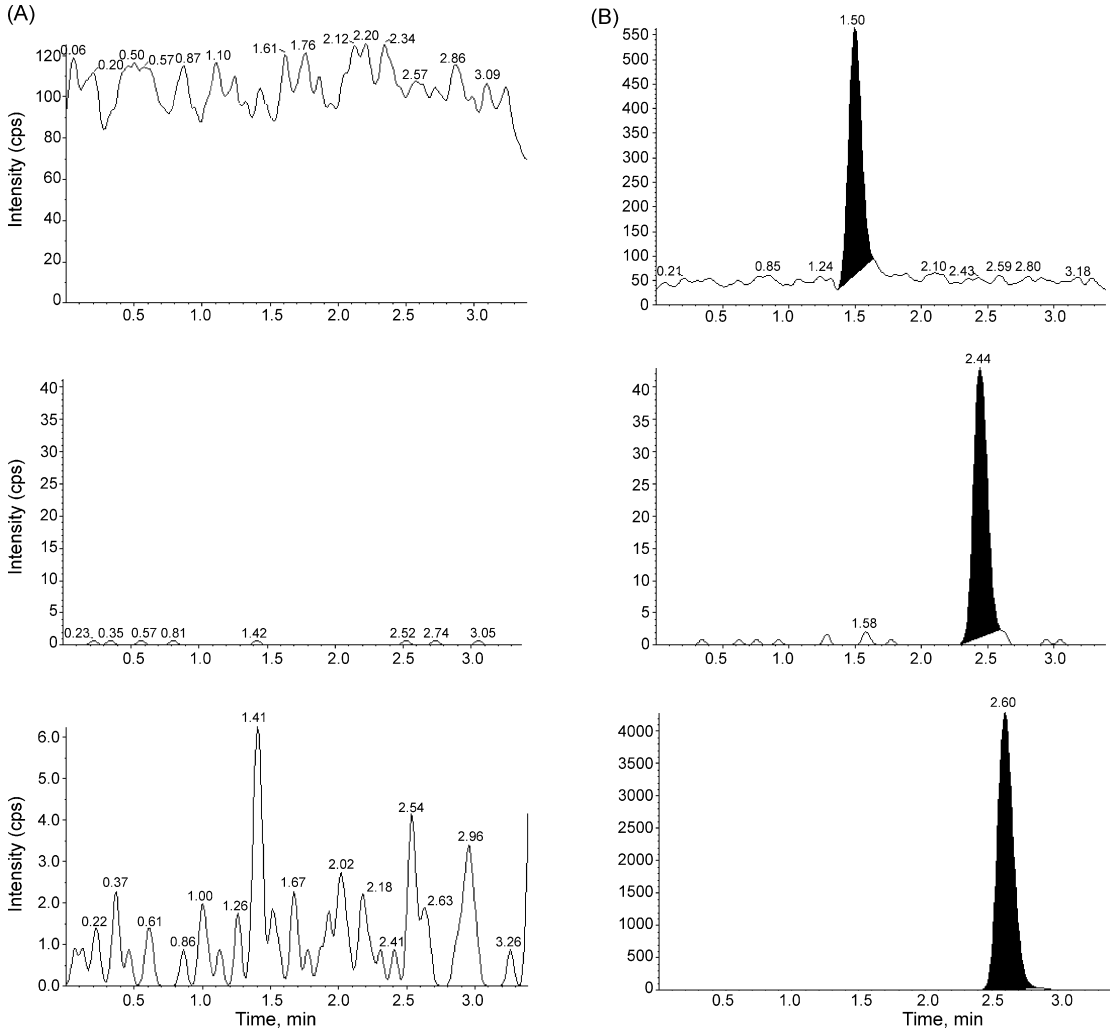


Fig. 4. Blank plasma (A) and LLOQ (B) chromatograms for metformin (130.1/60.1), glyburide (494.0/352.1) and IS (494.1/369.0), respectively.

Table 1
Summary of calibration curves for metformin (A) and glyburide (B) with back-calculated concentrations

| Linearity | CS-1 | CS-2 | CS-3 | CS-4 | CS-5 | CS-6 | CS-7 | CS-8 | CS-9 |
|---|---|---|---|---|---|---|---|---|---|
| added concentration | 20.00 | 40.00 | 100.000 | 200.00 | 500.00 | 1000.00 | 1500.00 | 2000.00 | 2500.00 |
| Metformin (A), concentration in ng/ml | | | | | | | | | |
| 1 | 19.15 | 42.94 | 103.75 | 214.25 | 524.19 | 1029.89 | 1474.00 | 1848.37 | 2301.27 |
| 2 | 20.10 | 39.70 | 100.00 | 214.38 | 518.96 | 997.27 | 1527.43 | 1930.67 | 2391.93 |
| 3 | 19.50 | 41.76 | 103.20 | 210.80 | 513.39 | 1037.04 | 1542.45 | 1831.99 | 2330.25 |
| 4 | 19.63 | 41.56 | 100.09 | 213.29 | 535.08 | 1005.01 | 1481.77 | 1912.65 | 2344.98 |
| 5 | 19.91 | 39.90 | 103.73 | 213.87 | 513.36 | 1032.88 | 1520.50 | 1933.10 | 2263.71 |
| 6 | 19.49 | 40.92 | 108.84 | 210.92 | 517.44 | 1024.14 | 1509.99 | 1874.48 | 2254.75 |
| 7 | 19.24 | 43.22 | 118.49 | 211.43 | 523.87 | 982.18 | 1526.92 | 1840.37 | 2431.57 |
| Mean | 19.57 | 41.43 | 105.44 | 212.71 | 520.90 | 1015.49 | 1511.86 | 1881.66 | 2331.21 |
| S.D. | 0.34 | 1.36 | 6.47 | 1.60 | 7.63 | 20.812 | 25.24 | 43.48 | 64.87 |
| % CV | 1.75 | 3.29 | 6.13 | 0.75 | 1.47 | 2.05 | 1.67 | 2.31 | 2.78 |
| Accuracy (%) | 97.86 | 103.57 | 105.44 | 106.35 | 104.18 | 101.55 | 100.79 | 94.08 | 93.25 |
| Linearity | CS-1 | CS-2 | CS-3 | CS-4 | CS-5 | CS-6 | CS-7 | CS-8 | CS-9 |
| added concentration | 5.00 | 10.00 | 25.00 | 50.00 | 100.00 | 200.00 | 300.00 | 400.00 | 500.00 |
| Glyburide (B), concentration in ng/ml | | | | | | | | | |
| 1 | 4.61 | 10.88 | 24.78 | 51.86 | 102.67 | 191.58 | 310.08 | 407.47 | 498.80 |
| 2 | 5.11 | 10.05 | 24.56 | 50.50 | 99.84 | 209.59 | 302.55 | 394.30 | 506.23 |
| 3 | 5.23 | 8.94 | 25.31 | 50.31 | 108.96 | 207.29 | 295.59 | 402.22 | 498.89 |
| 4 | 5.20 | 9.38 | 25.48 | 50.67 | 104.80 | 198.09 | 300.26 | 408.13 | 500.72 |
| 5 | 4.29 | 11.51 | 23.58 | 52.88 | 103.61 | 195.77 | 313.65 | 412.40 | 485.02 |
| 6 | 5.10 | 10.61 | 24.01 | 48.15 | 101.32 | 202.05 | 318.20 | 394.12 | 499.17 |
| 7 | 4.89 | 10.13 | 28.40 | 47.78 | 98.40 | 192.53 | 295.55 | 412.24 | 512.80 |
| Mean | 4.92 | 10.21 | 25.16 | 50.31 | 102.80 | 199.56 | 305.13 | 404.41 | 500.23 |
| S.D. | 0.35 | 0.88 | 1.58 | 1.84 | 3.49 | 7.02 | 8.95 | 7.76 | 8.47 |
| % CV | 7.11 | 8.61 | 6.27 | 3.65 | 3.39 | 3.52 | 2.93 | 1.92 | 1.69 |
| Accuracy (%) | 98.39 | 102.13 | 100.64 | 100.61 | 102.80 | 99.78 | 101.71 | 101.10 | 100.05 |

### 3.2. Selectivity and sensitivity

Selectivity was evaluated by calculating signal (LLOQ) to noise (blank) ratio for all the samples. Mean ratio found was 29.37 and 20.63 for metformin and glyburide, respectively. Back-calculated concentrations were obtained from calibration curve and the mean accuracy for both analytes was within $95 \pm 1\%$ and precision (% CV) was less than ten. Endogenous peaks at the retention time of the analytes were not observed for any of the plasma lots. Chromatograms (Fig. 4) for blank plasma, LLOQ concentration of analytes and internal standard (IS) demonstrate the selectivity of this method for routine sample analysis. The retention times for metformin, glyburide and IS were 1.5, 2.44 and 2.6 min, respectively.

### 3.3. Linearity, accuracy and precision

Calibration curves were linear from 20–2500 ng/ml with correlation coefficient $r \geq 0.9977$ for metformin and 5–500 ng/ml with $r \geq 0.9992$ for glyburide. The $r$ values, slopes and intercepts were calculated using linear regression ($1/x^2$) and ($1/x$) weighing analysis, respectively. The observed mean back-calculated concentration of calibration standards with accuracy (%) and precision (% CV) of seven linearities are given in Table 1A and B.

Intra and inter batch accuracy and precision were performed by analyzing one and three batches, respectively. Each batch had six replicates at four concentration levels (LLOQ, LQC, MQC and HQC). Concentrations were calculated from calibration curve and the intra-batch and inter-batch precision was less than 7.9% for metformin and glyburide. Accuracy expressed in terms of relative error was within $\pm 8\%$ for both the analytes of their nominal concentration as given in Table 2.

### 3.4. Recovery and matrix effect

Six replicates at LQC, MQC and HQC levels were prepared for recovery determination. Mean recovery found was 69.3 and 71.2% and the precision (% CV) was 4.1 and 2.2 for metformin and glyburide, respectively. Recovery of IS was 70.9% with a % CV of 4.2.

Ion suppression was checked by comparing the area response of unextracted samples to that of aqueous sample. Unextracted samples were prepared in three different lots of blank plasma. Ion suppression was minor in all three lots and it was consistent as CV of area ratio (unextracted/aqueous) was < 5%. Assessment of matrix effect was performed with the aim to see the effect of different lots of plasma on the back-calculated value of QC's nominal concentration. The results found for normal, haemolysed and lipemic plasma samples were well

Table 2
Intra-batch and inter-batch precision and accuracy

| | | Metformin | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Intra-batch | | | | Inter-batch | | | |
| Level | Concentration added (ng/ml) | *n* | Mean concentration found (ng/ml)[a] | % RE | % CV | *n* | Mean concentration found (ng/ml)[b] | % RE | % CV |
| LLOQ | 20.00 | 6 | 19.31 | −3.47 | 7.8 | 18 | 20.34 | 1.17 | 6.7 |
| LQC | 60.00 | 6 | 59.14 | −1.44 | 3.9 | 18 | 60.75 | 0.74 | 3.3 |
| MQC | 750.00 | 6 | 750.38 | 0.05 | 2.1 | 18 | 745.00 | −1.16 | 3.8 |
| HQC | 2250.00 | 6 | 2082.36 | −7.45 | 2.4 | 18 | 2140.38 | −5.35 | 4.0 |
| | | Glyburide | | | | | | | |
| | | Intra-batch | | | | Inter-batch | | | |
| Level | Concentration added (ng/ml) | *n* | Mean concentration found (ng/ml)[a] | % RE | % CV | *n* | Mean concentration found (ng/ml)[b] | % RE | % CV |
| LLOQ | 5.00 | 6 | 4.70 | −6.05 | 7.1 | 18 | 4.71 | −6.41 | 7.1 |
| LQC | 15.00 | 6 | 15.20 | 1.30 | 7.9 | 18 | 14.90 | −1.45 | 7.9 |
| MQC | 150.00 | 6 | 150.74 | 0.50 | 2.7 | 18 | 149.37 | −1.21 | 2.5 |
| HQC | 450.00 | 6 | 462.55 | 2.79 | 1.4 | 18 | 463.19 | 2.11 | 2.4 |

RE, relative error; CV, coefficient of variation; *n*, total number of observation.
[a] Mean of six replicate observations at each concentration.
[b] Mean of eighteen replicate observations over three different analytical runs.

within the acceptable limits as shown in Table 3. Moreover, the minor suppression of analyte signal due to endogenous matrix interferences does not affect the quantitation of analytes and IS peak. Thus, the extraction method was rugged and gave accurate and consistent results when applied to real patient samples.

### 3.5. Stability and dilution integrity

Stock solution of both analytes and IS were stable at room temperature for 24 h and at 2–8 °C for 32 days. Both analytes in control human plasma at room temperature were stable at least for 24 h and for minimum of five freeze and thaw cycles. Process stability was of 30 h at 10 °C. Spiked plasma samples, stored at −20 °C for long term stability experiment, were stable for minimum of 105 days. Different stability experiments in plasma with values for precision and percent change are shown in Table 4.

The mean back-calculated concentrations for ½ and 1/4 dilution samples were within 85–115% of their nominal values. The precision (% CV) for ½ and 1/4 dilution samples was < 3.3 for both the analytes.

Table 3
Matrix effect in human plasma

| | Metformin | | | |
|---|---|---|---|---|
| | LQC (60.00 ng/ml) | | HQC (2250.00 ng/ml) | |
| | Mean calculated concentration | % relative error | Mean calculated concentration | % relative error |
| Lot-1 | 55.95 | −7.22 | 1971.09 | −12.83 |
| Lot-2 | 61.39 | 1.81 | 2000.28 | −11.54 |
| Lot-3 | 56.44 | −6.40 | 2073.07 | −8.32 |
| Lot-4 | 56.44 | −6.40 | 2119.73 | −6.26 |
| Lot-5 | 56.78 | −5.83 | 2022.67 | −10.55 |
| Lot-6 | 59.19 | −1.84 | 2035.80 | −9.97 |
| | Glyburide | | | |
| | LQC (15.00 ng/ml) | | HQC (450.00 ng/ml) | |
| | Mean calculated concentration | % relative error | Mean calculated concentration | % relative error |
| Lot-1 | 14.83 | −1.13 | 437.40 | −3.44 |
| Lot-2 | 14.63 | −2.49 | 445.10 | −1.74 |
| Lot-3 | 14.94 | −0.38 | 459.36 | 1.40 |
| Lot-4 | 14.60 | −2.64 | 461.74 | 1.93 |
| Lot-5 | 14.50 | −3.31 | 454.24 | 0.27 |
| Lot-6 | 14.01 | −6.58 | 466.78 | 3.04 |

Table 4
Stability tests for metformin and glyburide

| Stability | Storage condition | Level | Metformin | | | | Glyburide | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | A (ng/ml) | % CV | B (ng/ml) | % CV | % mean change | A (ng/ml) | % CV | B (ng/ml) | % CV | % mean change |
| Bench top | Room temperature (24 h) | LQC | 56.46 | 2.6 | 55.78 | 7.3 | −1.2 | 15.40 | 4.6 | 15.58 | 6.5 | 1.2 |
| | | HQC | 2171.47 | 7.3 | 2090.64 | 3.6 | −3.7 | 483.80 | 6.1 | 465.65 | 3.3 | −3.75 |
| Process | Autosampler (10 °C, 30 h) | LQC | 59.57 | 3.4 | 63.43 | 3.8 | 6.5 | 115.00 | 4.9 | 14.73 | 8.8 | −1.8 |
| | | HQC | 2058.34 | 1.9 | 2153.14 | 3.3 | 4.6 | 473.93 | 1.2 | 441.39 | 1.4 | 6.9 |
| Freeze and thaw | After 5th cycle at −20 °C | LQC | 61.83 | 3.1 | 63.66 | 4.1 | 2.9 | 14.64 | 9.1 | 14.27 | 3.3 | −2.8 |
| | | HQC | 2226.08 | 3.3 | 2254.30 | 3.0 | 1.3 | 457.90 | 2.3 | 462.81 | 2.0 | 1.1 |
| Long term stability | 105 days at −20 °C | LQC | 59.14 | 3.9 | 56.82 | 2.7 | −3.9 | 15.20 | 7.9 | 16.66 | 5.2 | 9.6 |
| | | HQC | 2082.36 | 2.4 | 2216.77 | 2.0 | 6.5 | 462.55 | 1.4 | 454.47 | 2.8 | 1.8 |

A: mean comparison concentration; B: mean stability concentration; CV: coefficient of variance.

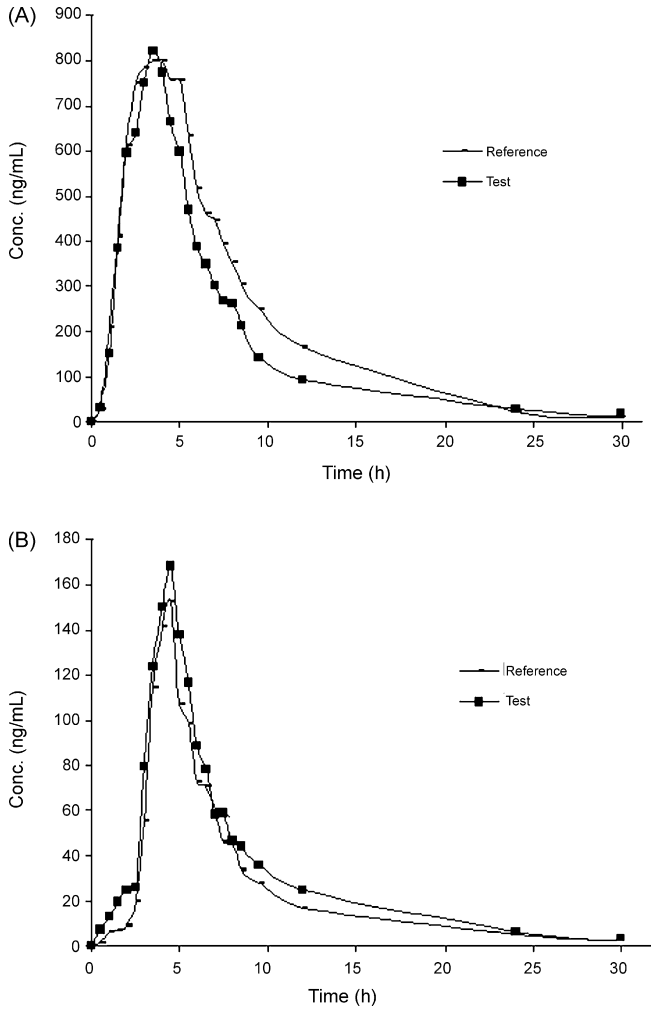


Fig. 5. Mean plasma concentration of metformin (A) and glyburide (B) after oral administration of single dose of 500 mg of metformin and 5 mg of glyburide tablet to 28 healthy human subjects under fasting condition.

### *3.6. Application of the method on human volunteers*

The validated method was successfully applied to a bioequivalence study in twenty eight healthy human male subject samples for reference and test formulations of metformin (500 mg) and glyburide (5 mg) tablet formulation under condition of fasting. All 1740 samples including the calibration, QC and volunteer samples were run and analysed in only 16 days and precision and accuracy for calibration and QC samples were within acceptable limits. The 90% confidence interval of the individual ratio geometric mean for test/reference was within 80–125% for AUC $_{(0-t)}$, AUC$_{(0-\infty)}$ and $C_{max}$ (AUC: area under curve, $C_{max}$: peak plasma concentration). Mean plasma concentration versus time profile for the treatment, under condition of fasting is presented in Fig. 5.

## 4. Conclusions

The developed LC–MS/MS assay for metformin and glyburide is selective, rapid and rugged, suitable for routine measurement of subject samples. This method has significant

advantages in terms of simple precipitation procedure and a shorter chromatographic run time (3.5 min). The method gave consistent and reproducible recoveries for analytes and IS from plasma, with minimum interference and ion suppression. The extract (5 μl) can be directly submitted for LC–MS analysis without drying and reconstitution to give high throughput. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with test formulation of metformin and glyburide.

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## References

[1] J.T. Cheng, C.C. Huang, I.M. Liu, T.F. Tzeng, C.J. Chang, Diabetes 55 (2006) 819–825.
[2] H.E. Lebovitz, Med. Clin. North Am. 88 (2004) 847–863.
[3] D.S. Bell, Am. J. Med. 116 (2004) 23S–29S.
[4] C.J. Bailey, R.C. Turner, N. Eng. J. Med. 334 (1996) 574–579.
[5] R.A. De Fronzo, Ann. Intern. Med. 131 (1999) 281–303.
[6] M.M. Funnell, D.F. Kruger, Nurse Pract. 29 (2004) 11–23.
[7] J. Brohon, M. Neol, J. Chromatogr. 146 (1978) 148–151.
[8] D. Castoldi, O. Tofanetti, Clin. Chem. Acta 93 (1979) 195–198.
[9] B.G. Charles, N.W. Jacobsen, P.J. Ravenscroft, Clin. Chem. 27 (1981) 434–436.
[10] J. Keal, A. Somogvi, J. Chromatogr. 378 (1986) 503–508.
[11] R. Huupponen, P. Ojala-Karlson, J. Rouru, M. Koulu, J. Chromatogr. 583 (1992) 270–273.
[12] K.H. Yuen, K.K. Peh, J. Chromatogr. B 710 (1998) 243–246.
[13] O. Vesterqvist, F. Nabbie, B. Swanson, J. Chromatogr. B 716 (1998) 299–304.
[14] A.R. Bonfigli, S. Manfrini, F. Gregorio, R. Testa, I. Testa, G. De Sio, G. Coppa, Ther. Drug. Monit. 21 (1999) 330–334.
[15] C.L. Cheng, C.H. Chou, J. Chromatogr. B 762 (2001) 51–58.
[16] M. Zhang, G.A. Moore, M. Lever, S.J. Gardiner, C.M. Kirkpatrick, E.J. Begg, J. Chromatogr. B 766 (2002) 175–179.
[17] A. Zarghi, S.M. Foroutan, A. Shafaati, A. Khoddam, J. Pharm. Biomed. Anal. 31 (2003) 197–200.
[18] S. AbuRuz, J. Millership, J. McElnay, J. Chromatogr. B 798 (2003) 203–209.
[19] H. Amini, A. Ahmadiani, P. Gazerani, J. Chromatogr. B 824 (2005) 319–322.
[20] L. Zecca, S. Trivulzio, A. Pinelli, R. Colombo, O. Tofanetti, J. Chromatogr. 339 (1985) 203–209.
[21] H. Emilsson, S. Sjoberg, M. Svedner, I. Christenson, J. Chromatogr. 383 (1986) 93–102.
[22] S. Othman, O. Shaheen, I. Jalal, A. Awidi, W. Al-Turk, J. Assoc. Off. Anal. Chem. 77 (1988) 942–944.
[23] J.R. Valdes Santurio, E. Gonzalez Porto, J. Chromatogr. B 682 (1996) 364–370.
[24] J. Khatri, S. Qassim, O. Abed, B. Abraham, A. Al-Lami, S. Masood, J. Pharm. Pharm. Sci. 4 (2001) 201–206.
[25] I. Niopas, A.C. Daftsios, J. Pharm. Biomed. Anal. 28 (2002) 653–657.
[26] P. Venkatesh, T. Harisudhan, H. Choudhary, R. Mullangi, N.R. Srinivas, Biomed. Chromatogr. 20 (2006) 1043–1048.
[27] J.Z. Song, H.F. Chen, S.J. Tian, Z.P. Sun, J. Chromatogr. B 708 (1998) 277–283.
[28] X. Chen, Q. Gu, F. Qui, D. Zhong, J. Chromatogr. B 802 (2004) 377–381.
[29] K. Heinig, F. Bucheli, J. Pharm. Biomed. Anal. 34 (2004) 1005–1011.
[30] Y. Wang, Y. Tang, J. Gu, J.P. Fawcett, X. Bai, J. Chromatogr. B 808 (2004) 215–219.
[31] L. Ramos, R. Bakhtiar, F. Tse, Rapid Commun. Mass Spectrom. 13 (1999) 2439–2443.
[32] M.R. Moura, G. De Nucci, S. Rath, F.G. Reyes, Anal. Bioanal. Chem. 378 (2004) 499–503.
[33] F. Albu, C. Georgita, V. David, A. Medvedovici, J. Chromatogr. B 846 (2007) 222–229.
[34] G.P. Zhong, H.C. Bi, S. Zhou, X. Chen, M. Huang, J. Mass Spectrom. 40 (2005) 1462–1471.
[35] S. AbuRuz, J. Millership, J. McElnay, J. Chromatogr. B 817 (2005) 277–286.
[36] E.P. Lia, S.Y. Feng, J. Chromatogr. B 843 (2006) 94–99.
[37] Guidance for industry: bioanalytical method validation, U.S. Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM). May 2001.
[38] FDA guidance for industry: bioavailability studies for orally administered drug- products-General Considerations, U.S. Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), 2000.