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Sensitive and Rapid Determination of Gliclazide in Human Plasma by UPLC-MS/MS and its Application to a Bioequivalence Study

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ABSTRACT

An ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method has been developed for the determination of gliclazide in human plasma using gliclazide-d4 as the internal standard (IS). The plasma samples were prepared by protein precipitation with acetonitrile employing 50 µL human plasma. Chromatography was performed on Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) analytical column under isocratic conditions using a mobile phase which consisted of 0.1% formic acid in water-acetonitrile (10:90, v/v). The MRM transitions for gliclazide (m/z 324.2 → 127.3) and gliclazide-d4 (m/z 328.2 → 127.4) were monitored on a triple quadrupole mass spectrometer, operating in the positive ionization mode. The method was validated over a dynamic concentration range of 1.0-2000 ng/mL for gliclazide. Matrix effect was assessed by post-column analyte infusion and the mean extraction recovery was 95.7 % across six quality control levels. Stability of gliclazide in plasma was evaluated under different conditions like bench top, auto sampler, dry and wet extract, freeze-thaw and long term stability. The method was applied to a bioequivalence study with 30 mg gliclazide tablet formulation in 28 healthy subjects under fasting. Further, the assay reproducibility was confirmed by reanalysis of 129 incurred samples.

Keywords: UPLC-MS/MS; protein precipitation; sensitive; bioequivalence study

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by disrupted insulin production, leading to increase in blood glucose level and other complications such as neuropathy, cardiopathy and renal dysfunction [1-3]. It is classified as type 1 or type 2 diabetes based on different stages of the disease. Type 1 diabetes is ascribed to an early-onset autoimmune disease marked by the destruction of β-cells of the pancreas, which results in a partial or complete lack of insulin production and the inability of the body to control glucose homeostasis [3]. Type 2, a non-insulin dependent diabetes is a complex metabolic disorder which occurs at a later stage and is most common in the overweight population [4]. It is caused by genetic and environmental factors, as evident from a study correlating the loss of function gene variants in *GPR120* with increased risk of type 2 complications [5, 6]. Gliclazide is a potent type 2 second generation antidiabetic drug used to enhance insulin secretion, and possesses beneficial extra pancreatic effects that makes it potentially useful in type 1 as well [7]. Gliclazide is completely absorbed from the gastro-intestinal tract with mean absolute bioavailability of 97 % and is highly protein bound. After oral administration the peak plasma concentration is achieved in about 6 h and is more than 90 % recovered unchanged in plasma. Although six main metabolites are identified in urine, no active metabolites are found in plasma [8].

Either monotherapy or combination therapy is required for maintaining long term glycemic control with oral antidiabetic agents. Further, it is essential to monitor their plasma concentration for effective control of blood glucose levels and for therapeutic drug monitoring to optimize dose strength and dosing regimen. Thus, development of adequately sensitive, selective and rapid method is required to determine these agents in plasma. Several methods are reported to determine gliclazide, either as a single analyte [9-14] or in combination with other antidiabetic agents [15-21] in human serum or plasma. These methods have employed high performance liquid chromatography with UV [9, 11, 14, 17, 19], electrochemical [12] or mass detection [10, 13, 15, 16, 18, 20, 21] for estimation of gliclazide. However, there are no reports on the use of UPLC-MS/MS for the quantification of gliclazide in human plasma. Thus, in the present study a selective, sensitive and rapid UPLC-MS/MS method has been developed and validated for accurate determination of gliclazide in human plasma for routine therapeutic drug monitoring. The method employs 50 µL plasma sample and a chromatographic analysis time of 1.2 min. The effect of endogenous matrix components on the quantification of gliclazide and its stability in plasma is extensively studied. Further, the utility of the method is demonstrated by a bioequivalence study in healthy subjects.

EXPERIMENTAL

Chemicals and materials

Reference standards of gliclazide (99.25 %) and gliclazide-d4 (99.58 %) were procured from Clearsynth Labs Pvt. Ltd. (Mumbai, India). HPLC grade acetonitrile and methanol were

procured from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). Formic acid (90%) was obtained from S.D. Fine Chemicals Ltd. (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at –20 °C until use.

Liquid Chromatographic and Mass Spectrometric Conditions

An Acquity UPLC system from Waters Corporation (Milford, MA, USA) consisting of binary solvent manager, sample manager and column manager and linked to a triple quadrupole Quattro Premier XE mass spectrometer with an electrospray ionization source (Milford, MA, USA) was used in the study. The analysis of gliclazide and IS was performed on a Waters Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) analytical column, maintained at 25 °C in a column oven. The mobile phase consisted of 0.1% formic acid in water - acetonitrile (10:90, *v/v*). The flow rate of the mobile phase was kept at 0.300 mL/min. The sample manager temperature was maintained at 5°C and the pressure of the system was 6200 psi. Ionization and detection of gliclazide and IS was carried out on a triple quadrupole mass spectrometer in the positive ionization mode. The source dependent parameters set for the analyte and IS were, cone gas flow: 120 L/h; desolvation gas flow: 640 L/h; capillary voltage: 1.4 kV, source temperature: 120°C; desolvation temperature: 400°C; extractor voltage: 4.0V. The pressure of argon used as collision activation dissociation gas was 0.120 Pa. The optimum value for cone voltage and collision energy was kept at 25 V and 31 eV for gliclazide and 24 V and 32 eV for IS respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 100 ms. Mass Lynx software version 4.1 was used to control all parameters of UPLC and MS.

Standard stock, calibration standards and quality control samples

The standard stock solution of gliclazide (500 µg/mL) was prepared by dissolving requisite amount in methanol. Further, an intermediate solution (100 µg/mL and 50.0 µg/mL) for spiking was prepared in methanol:water (50:50, *v/v*). Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with the intermediate solutions. Calibration curve standards for gliclazide were prepared at 1.00, 2.00, 5.00, 15.0, 45.0, 100, 250, 500, 1000, 2000 ng/mL concentrations, while quality control samples were prepared at six levels, 1600 ng/mL (HQC, high quality control), 1200/800 ng/mL (MQC-1/2, medium quality control), 400/3.00 ng/mL (LQC-1/2, low quality control) and 1.00 ng/mL (LLOQ QC, lower limit of quantification quality control). Stock solution (100 µg/mL) of the internal standard was prepared by dissolving 1.0 mg of gliclazide-d4 in 10.0 mL of methanol. Its working solution (2.50 µg/mL) was prepared by appropriate dilution of the stock solution in methanol:water (50:50, *v/v*). All standard stock and working solutions used for spiking were stored at 5 °C, while CSs and QC samples in plasma were kept at -70 °C until use.

Protocol for sample preparation

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 50 µL of spiked plasma sample/subject sample, 25 µL of internal standard was added and vortexed for 10s. Further, 600 µL of acetonitrile was added and vortexed for another 60 s. The samples were then centrifuged at 13148 × g for 10 min at 10°C. The supernatant layer was separated and evaporated to dryness in a thermostatically controlled water-bath maintained at 40 °C under a gentle stream of nitrogen for 10 min. After drying, the residue was reconstituted with 250 µL of mobile phase solution. The solution was briefly vortexed for 15s and 10 µL was used for injection in the chromatographic system.

Method validation procedures

Validation of the method for the estimation of gliclazide was carried out as per the USFDA guidelines [22]. System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of analyte (1200 ng/mL) and IS (2.50 µg/mL) at the start of each batch. System performance was studied by injecting one extracted blank (without analyte and IS) and one extracted LLOQ sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. The carryover effect of the auto-sampler was evaluated by sequentially injecting extracted blank plasma → ULOQ sample → extracted blank plasma → LLOQ sample→ extracted blank plasma at the beginning of each batch. The selectivity of the method towards endogenous plasma matrix components was assessed in eight batches (5 normal of K_3EDTA plasma, 1 heparinized, 1 haemolysed and 1 lipemic) of blank human plasma. The selectivity of the method towards commonly used medications in human volunteers was done for paracetamol, chlorpheniramine maleate, diclofenac, caffeine, acetylsalicylic acid and ibuprofen in six different batches of plasma having K_3-EDTA as anticoagulant. Their stock solutions (100 µg/mL) were prepared by dissolving requisite amount in methanol. Further, working solutions (1.0 µg/mL) were prepared in the mobile phase and 10 µL was injected to check for any possible interference at the retention time of analyte and IS. The cross talk of MRM for analyte and IS was checked using highest standard on calibration curve and working solution of IS.

The linearity of the method was ascertained through six calibration curves containing ten non-zero concentrations. The area ratio response (analyte/IS) obtained from multiple reaction monitoring was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression. A correlation coefficient (r^2) value >0.99 was desirable for all the calibration curves. Reinjection reproducibility for extracted samples was also checked by analyzing one entire analytical batch, stored at 5 °C.

Intra-batch accuracy and precision was checked through replicate analysis of plasma samples on a single batch. The run consisted of a calibration curve and six replicates of LLOQ, LQC-2, LQC-1, MQC-2, MQC-1 and HQC samples. The inter-batch accuracy and precision were assessed by analysis of six batches at six QC levels on three consecutive validation days.

Ion suppression/enhancement effects on the MRM LC-MS/MS sensitivity were evaluated by post column analyte infusion experiment. A standard solution containing gliclazide (1200 ng/mL) and IS was infused post column into the mobile phase at 10 µL/min employing infusion pump. Aliquots of 10 µL of extracted control plasma were then injected into the column and MRM chromatograms were acquired for the analyte and IS.

The extraction recovery and matrix effect were assessed as recommended by Matuszewski *et al.* [23]. Both the parameters were evaluated at HQC, MQC-1/2 and LQC-1/2 and LLOQ levels in six replicates. Extraction recovery (RE) was calculated by comparing the mean area response of extracted samples (spiked

before extraction) with unextracted samples (spiked after extraction) at each QC level. The recovery of IS was similarly estimated. Matrix effect, expressed as matrix factors (MFs), was assessed by comparing the mean area response of post-extraction fortified samples with mean area of solutions prepared in mobile phase solutions (neat standards). IS-normalized MFs (gliclazide/IS) were calculated to access the variability of the assay due to matrix effects. Relative matrix effect was assessed from the precision (% CV) values of the slopes of the calibration curves prepared from eight different plasma lots/sources.

All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against freshly prepared comparison standards. Stock solutions of gliclazide and IS were checked for short term stability at room temperature and long term stability at 5 °C. The solutions were considered stable if the deviation from nominal value was within ±10.0 %. Bench top (at room temperature), dry extract, wet extract and freeze-thaw stability (at -20 °C and -70 °C) were performed at LQC-2 and HQC levels using six replicates. Long term stability of spiked plasma samples stored at -20 °C and -70 °C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0 %.

Method ruggedness was studied with two precision and accuracy batches. The first batch was analyzed on two different columns (same make but different batch no.), while the second batch was analyzed by two different analysts. Dilution integrity experiment was conducted by diluting the stock solution prepared as spiked standard at concentration of 4000 (2 times of ULOQ) ng/mL for gliclazide. The precision and accuracy for dilution integrity standards at 1/5 (800.0 ng/mL) and 1/10th (400.0 ng/mL) dilutions for the analyte were determined by analyzing the samples against freshly prepared calibration curve standards.

Bioequivalence study and incurred sample results

The bioequivalence study was conducted with a single dose of a modified release tablets (30 mg gliclazide, Valpharma International S.p.A., Italy) and DIAMICRON® modified release tablet (30 mg of gliclazide, Les Laboratories Servier, France) formulations to 28 normal, healthy, adult subjects under fasting. Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. The study was conducted as per the International Conference on Harmonization, E6 Good Clinical Practice guidelines [24]. Blood samples were collected at 0.00 (pre-dose), 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 9.00, 10.0, 11.0, 12.0, 14.0, 16.0, 24.0, 48.0, 72.0, 96.0 and 120 h after oral administration of the dose for test and reference formulation. Plasma was separated by centrifugation and kept frozen at -20°C till the completion of period and then at -70°C until analysis. During study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters of gliclazide were estimated by non-compartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA).

An incurred sample reanalysis was also carried out by reanalysis of 129 subject samples. The selection criterion was based on samples which were near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The results obtained were compared with the initial results. The percent change in the value should not be more than ±20% [25].

RESULTS AND DISCUSSION

I. UPLC-MS/MS method optimization

In the present work electrospray ionization (ESI) source was used to maximize sensitivity and obtain good linearity in the regression curves. During ionization in the positive ionization mode, gliclazide and IS formed predominantly protonated precursor ions in the full scan Q1 mass spectra at *m/z* 324.2 and 328.2 respectively. The most abundant product ions in Q3 mass spectra were found at *m/z* 127.3 and 127.4 for gliclazide and its deuterated analog as IS but applying optimum collision energy of 31 and 32 eV respectively (Figure 1). This product ion can be attributed to the substructure containing hexahydro-cyclopenta[c]pyrrole moiety. In addition to the quantification transition, a qualifying transition was also monitored for the identification of gliclazide (*m/z* 324.2 → 110.2) and IS (*m/z* 328.2 → 110.4). A dwell time of 100 ms was adequate to have sufficient no. of data points for quantification.

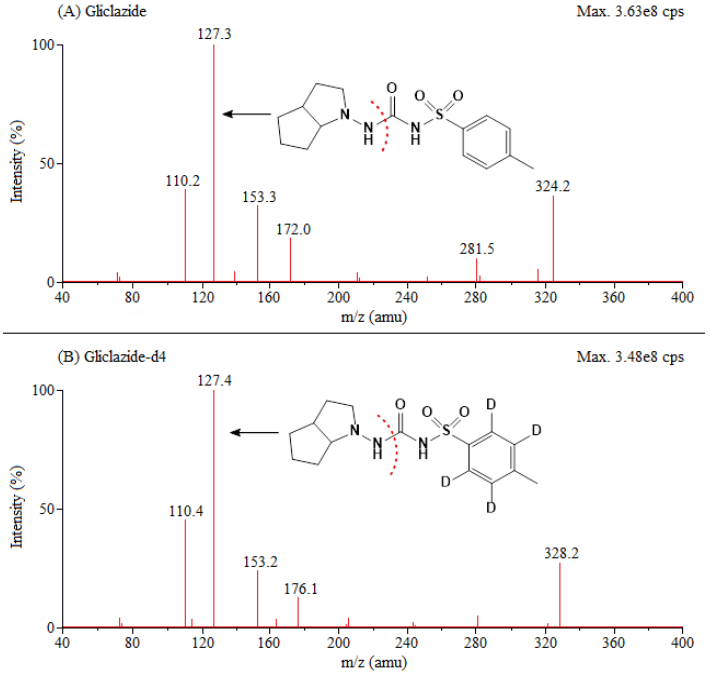

Figure 1 Product ion mass spectra of (A) gliclazide (m/z 324.2 → 127.3, scan range 40-400 amu) and (B) internal standard, gliclazide-d4 (m/z 328.2 → 127.4, scan range 40-400 amu) in the positive ionization mode.

Reported methods have used several reversed-phase columns like Techsphere C8 [9], Hypersil BDS C18 [10, 15], Cepcell Pak C18 [11], Apollo C18 [12], Diamonsil C18 [13] with different dimensions for chromatographic separation of gliclazide. Foroutan et al. [14] have used a monolithic column for the analysis of gliclazide from human plasma. In the present work, the chromatographic analysis of gliclazide was suitably optimized on UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) column using various combinations of acetonitrile/methanol with acidic buffers (formic acid-ammonium formate and acetic acid-ammonium acetate) and acidic modifiers like formic acid and acetic acid in different concentrations. The aim was to have adequate retention, response and peak shape with a short run time. Although adequate retention was obtained under all mobile phase conditions, the peak shape was not acceptable when the aqueous part was greater than 25 %

with longer retention time. Further, the response was much higher in formic acid compared to both the acidic buffers. Thus, a mobile phase consisting of 0.1% formic acid in water - acetonitrile (10:90, v/v) was found best in terms of sensitivity, peak shape and short analysis time.

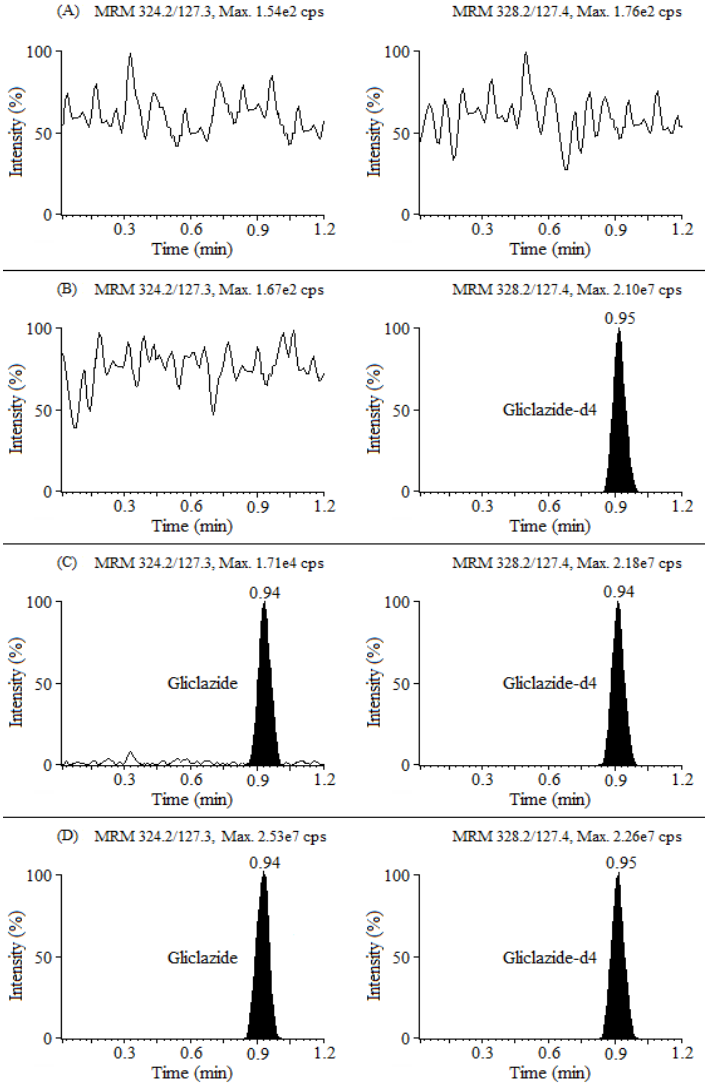

Figure 2 Representative MRM ion-chromatograms of (A) double blank plasma without gliclazide and gliclazide-d4, (B) blank plasma with working solution of gliclazide-d4 (m/z 328.2 → 127.4), (C) gliclazide (m/z 324.2 → 127.3) at 1.0 ng/mL concentration of gliclazide and gliclazide-d4 (D) real subject sample at Cmax and gliclazide-d4 after administration of 30 mg gliclazide.

The entire chromatographic run was completed within 1.2 min. All previous methods [9-16] have used general internal standards (ISs) for the analysis of gliclazide. However, according to the FDA guidelines [22] a stable and labeled compound should be preferred which has more structural similarity compared to general standards. Gliclazide-d4, used in this work gave excellent results with acceptable accuracy and precision at each QC level. The MRM chromatograms illustrated in Figure 2 of extracted blank plasma (double blank), blank plasma fortified with IS, gliclazide (at 1.0 ng/mL) and IS and a subject sample at Cmax demonstrates the overall performance and selectivity of the method. Results of post-column infusion experiment in Figure 3 indicate no ion suppression or enhancement at the retention time of gliclazide and IS.

Reported methods have used either protein precipitation (PP) [12, 14-16] or liquid-liquid extraction (LLE) [9, 11, 13] for sample preparation of gliclazide from plasma or serum samples. However, to keep the extraction protocol simple and quick with minimum steps, PP was tried with different protein precipitants like acetonitrile, methanol, ethanol and acetone. The extraction efficiency obtained in these solvents was quantitative (≥ 81 %), but minimum interference of endogenous components was found in presence of acetonitrile. At the same time the peak shape and analyte response was much higher and consistent at all QC levels with acetonitrile and hence was selected in the present work. The salient features of the present work in comparison with reported methods are presented in Table 1.

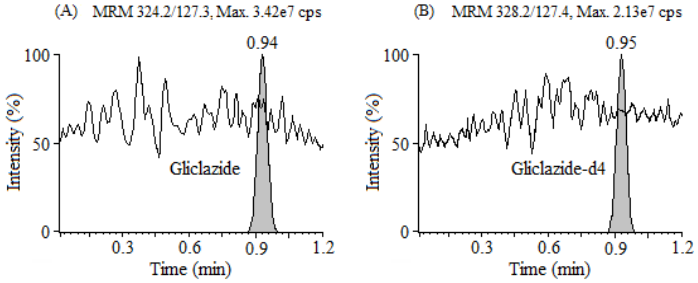

Figure 3 Injection of extracted blank human plasma during post column infusion of (A) gliclazide at 2000 ng/mL and (B) gliclazide-d4.

Table 1 Comparison of salient features of chromatographic methods developed for gliclazide in biological matrices

aAlongwith metformin; bAlong with benazepril and valsartan; LLE: liquid-liquid extraction; PP: protein precipitation; ACN: acetonitrile; DCM: dichloromethane

Sr. No.	Technique	Sample volume (µL); extraction procedure; internal standard	Linear range (ng/mL); retention time /run time (min)	Application	Ref.
1	HPLC-UV (230 nm)	100 (serum); LLE with toluene; phenytoin	75-10,000; 6.8/8.0	Pharmacokinetic study with 80 mg gliclazide in 12 healthy volunteers	9
2	LC-MS/MS	1000 (plasma); Non-porous membrane probe; tolbutamide	100-9850; 2.71/3.00	Pharmacokinetic study with 80 mg gliclazide in 1 healthy volunteer	10

3	Semi-micro HPLC-UV (229 nm)	100 (plasma); LLE with chloroform; glyburide	100-10,000; 4.1/8.0	Bioequivalence study with two formulations in 20 healthy subjects	11
4	HPLC-electrochemical detection	100 (plasma); PP with ACN; methyl-4-hydroxy benzoate	16-1300; 5.0/20.0	Pharmacokinetic study with 80 mg gliclazide in 1 healthy volunteer	12
5	LC-MS/MS	200 (plasma); LLE with *n*-hexane and DCM; tolbutamide	2.5-2000; 3.55/5.0	Pharmacokinetic study with 30 mg gliclazide in 20 patients	13
6	HPLC-UV (230 nm)	450 (plasma); PP with ACN; glibenclamide	10-5000; 3.2/6.0	Pharmacokinetic study with 80 mg gliclazide in 12 healthy volunteer	14
7[a]	LC-MS/MS	200 (plasma); PP with ACN; huperzine	10-10000; 1.47/2.0	Bioequivalence study with 40 mg gliclazide in 20 healthy subjects	15
8[b]	LC-MS/MS	100 (plasma); PP with methanol; ketaconazole	20-2000; 6.5/8.0	Protein binding interaction with Rhein	16
9	UPLC-MS/MS	50 (plasma); PP with ACN; gliclazide-d4	1.0-2000; 0.94/1.20	Bioequivalence study with 30 mg gliclazide in 28 healthy subjects	PW

Results for method validation

The calibration curves were linear over the concentration range of 1.0-2000 ng/mL with correlation coefficient (r^2) ≥ 0.9995 for gliclazide. The mean equation for five calibration curves was y = (0.00081 ± 0.00001) x - (0.000041 ± 0.000034). The standard deviation value for slope, intercept and correlation coefficient observed were 0.00001, 0.000034 and 0.0001 respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 97.6 to 101.6 % and 0.88 to 2.92 % respectively. The lowest concentration (1.00 ng/mL) in the standard curve was measured at a signal-to-noise ratio (S/N) of ≥ 10.0.

The intra-batch and inter-batch precision and accuracy results obtained at each QC level are shown in Table 2. The intra-batch precision (%CV) ranged from 1.04 to 2.89 % and the accuracy was within 99.6 to 100.7 %. For the inter-batch experiments, the precision varied from 1.09 to 2.95 % and the accuracy ranged from 98.6 to 100.4 %.

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

Nominal concentration (ng/mL)	Intra-batch (n = 6; single batch)			Inter-batch (n = 30; 6 from each batch)		
	Mean conc. found (ng/mL)	% CV	% Accuracy	Mean conc. found (ng/mL)	% CV	% Accuracy
HQC (1600)	1604.2	2.07	100.2	1591.0	2.23	99.4
MQC-1 (1200)	1195.0	1.04	100.3	1204.6	1.09	99.6
MQC-2 (800.0)	802.44	2.41	99.6	797.24	2.95	100.4
LQC-1 (400.0)	402.48	2.37	100.6	398.14	1.92	99.5
LQC-2 (3.00)	3.018	2.89	100.6	2.987	2.62	99.5
LLOQ QC (1.000)	1.007	2.81	100.7	0.986	2.91	98.6

CV: coefficient of variation; HQC: high quality control; MQC: medium quality control; LQC: low quality control; LLOQ QC: lower limit of quantitation quality control

The extraction recovery and matrix effect results for gliclazide and IS at different QC levels are presented in Table 3. The mean recovery across QC levels for gliclazide and IS ranged from 94.6 to 96.7 %, while the matrix effect expressed matrix factors varied from 0.983 to 1.014.

Table 3 Extraction recovery and matrix factor for gliclazide at different QC levels.

QC conc. (ng/mL)	Mean area response (n = 6)			Extraction recovery, % (B/A)	Matrix factor		
	A	B	C		Analyte (A/C)	IS	IS-normalized

3.00	7292	6935	6718	95.1 (96.3)[a]	1.085	1.070	1.014
400.0	974865	935241	904587	95.9 (95.9)[a]	1.077	1.083	0.994
800.0	1985678	1878746	1848514	94.6 (96.7)[a]	1.074	1.062	1.011
1200	2928627	2816242	2737784	96.2 (95.1)[a]	1.069	1.079	0.990
1600	3895563	3762278	3688412	96.6 (94.7)[a]	1.056	1.074	0.983

[a] values for gliclazide-d4;

A: mean area response of six replicates prepared by spiking in extracted blank plasma;

B: mean area response of six replicates prepared by spiking before extraction;

C: mean area response of six replicates prepared by spiking in mobile phase (neat samples)

IS: Internal standard; LQC: low quality control; MQC: medium quality control; HQC: high quality control.

Further, another essential criteria to evaluate matrix effect, expressed as relative matrix in different plasma lots/batches showed % CV values in the measurement of slope of standard curves was 2.84 (Table 4).

Table 4 Relative matrix effect in eight different lots of human plasma for gliclazide

Plasma lot	Slope
Lot-1	0.000803
Lot-2	0.000792
Lot-3	0.000821
Lot-4	0.000845
Lot-5	0.000788
Lot-6 (heparinized)	0.000817
Lot-7 (haemolysed)	0.000832
Lot-8 (lipemic)	0.000778
Mean	0.000810
±SD	0.000023
%CV	2.84

SD: standard deviation; CV: coefficient of variation

The stock solutions for short term stability of gliclazide and IS were stable at room temperature up to 27 h and between 2-8 °C for a minimum period of 24 days. Gliclazide in control human plasma (bench top) at room temperature was stable for at least 20 h at 25°C and for minimum of five freeze and thaw cycles. Dry and wet extract stability of the spiked quality control samples was determined up to 24 h and 48 h respectively. Long term stability of the spiked quality control samples was remained unaffected up to 186 days. The detailed stability results at LQC and HQC levels in plasma are presented in Table 5.

For method ruggedness, the precision (%CV) and accuracy values for two different columns ranged from 2.13 to 4.45 % and 97.6 to 104.6 % respectively across five quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 1.29 to 2.05 % and 95.1 to 102.4 % respectively at these levels. The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. The precision and accuracy values for 1/2 and 1/10th dilution ranged from 2.95-3.46 % and 97.8-105.4 % for gliclazide.

Table 5 Stability results of gliclazide in plasma under various conditions (n = 6)

Storage conditions	Nominal concentration (ng/mL)	Mean stability sample (ng/mL) ± SD	Change (%)
Bench top stability at 25 °C, 20 h	1600	1604.4 ± 39.8	0.28
	3.00	2.99 ± 0.07	-0.19
Freeze & thaw stability at -20 °C	1600	1606.0 ± 35.8	0.38
	3.00	3.019 ± 0.06	0.65
Freeze & thaw stability at -70 °C	1600	1594.0 ± 36.0	-0.38
	3.00	2.99 ± 0.07	-0.22
Dry extract stability at 2-8°C, 24 h	1600	1598.6 ± 35.7	-0.09
	3.00	3.02 ± 0.07	0.51
Wet extract stability at 2-8°C, 48 h	1600	1608.2 ± 31.5	0.51
	3.00	2.98 ± 0.06	-0.63
Long term stability at -20 °C, 186 days	1600	1609.4 ± 30.5	0.59
	3.00	3.01 ± 0.08	0.33
Long term stability at -70 °C, 186 days	1600	1607.4 ± 19.8	0.46
	3.00	2.98 ± 0.06	-0.34

SD: standard deviation; n: number of replicates

$$*\text{Change } (\%) = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100$$

Application of the method in healthy human subjects and incurred sample results

The validated method was successfully applied for the assay of gliclazide in healthy Indian subjects. Figure 4 shows the plasma concentration vs. time profile of gliclazide in healthy subjects under fasting.

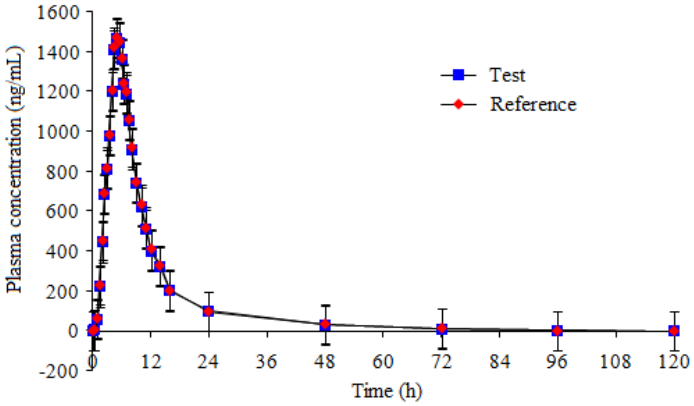

Figure 4 Mean plasma concentration-time profile of gliclazide after oral administration of 30 mg of test (modified release tablet, Valpharma International, Italy) and reference (DIAMICRON® tablet, Les Laboratories Servier, France) formulation to 28 healthy subjects.

It was possible to analyze about 2500 samples (subject samples, calibration and QC samples) during a period of 8 days and the results obtained were well within the acceptable limits. The main pharmacokinetic parameters of gliclazide, C_{max}, AUC_{0-t}, AUC_{0-inf}, T_{max}, K_{el} and $t_{1/2}$ for both the formulations are shown in Table 6. The C_{max}, T_{max}, K_{el} and $t_{1/2}$ values obtained were in good agreement with a previous report with 30 mg gliclazide modified release tablets in Chinese subjects [13]. Further, there was no apparent statistical difference between the two formulations in any parameter. The ratios of mean log-transformed parameters (C_{max}, AUC_{0-120}, and AUC_{0-inf}) and their 90% CIs were all within the defined bioequivalence range of 80-125 %. These observations confirm the bioequivalence of the test formulation with the reference product in terms of rate and extent of absorption.

Table 6 Mean pharmacokinetic parameters, comparison of treatment ratios of 90% CIs of natural log (Ln)-transformed parameters following oral administration of 30 mg gliclazide tablet formulation in 28 healthy Indian subjects under fasting.

Parameter	Test (Mean ±SD)	Reference (Mean ±SD)	Ratio (test/ reference), %	90% CI (Lower – Upper)	Power	Intra subject variation, % CV
C_{max} (ng/mL)	1465.52 ± 190.25	1472.31 ± 205.36	99.5	95.3-104.1	0.9995	4.65
$AUC_{0-120\ h}$ (h. ng/mL)	11245.2 ± 2045.5	12045.6 ± 2060.9	99.3	93.1-105.7	0.9997	5.25
AUC_{0-inf} (h. ng/mL)	11965.4 ± 2102.6	12445.0 ± 2132.6	96.1	92.7-99.5	0.9998	4.92
T_{max} (h)	5.05 ± 1.25	5.26 ± 1.35	--	--	--	--
$t_{1/2}$ (h)	9.19 ± 1.24	9.46 ± 1.02	--	--	--	--
Kel (1/h)	0.075 ± 0.006	0.073 ± 0.005	--	--	--	--

The incurred sample reanalysis (ISR) results are represented in Fig. 5. The % change for assay reproducibility in 129 incurred samples was within ±16 % for gliclazide. This authenticates the reproducibility of the proposed method.

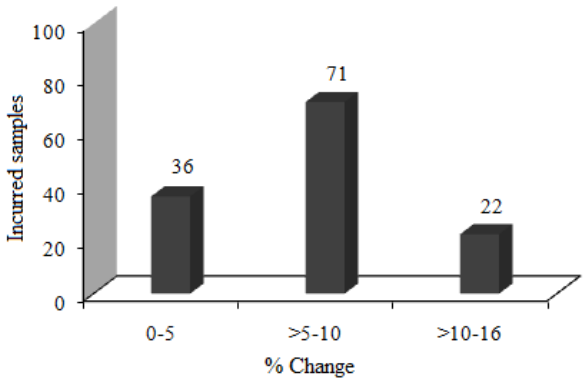

Figure 5 Graphical representation of % change in concentration during reanalysis of 129 incurred samples for gliclazide.

CONCLUSIONS

The validated UPLC-MS/MS method offers several advantages over reported procedures, in terms of lower sample requirements, simplicity of extraction procedure and overall analysis time. The proposed method is more sensitive and rapid (chromatographically) compared to all other procedures for determination of gliclazide in biological fluids. The present method employs small plasma volume (50 µL) for processing, which is lower compared to all existing methods for gliclazide. With dilution integrity up to two folds, it is possible to extend the

upper limit of quantification to 4000 ng/mL. Matrix effect is assessed through post-column analyte infusion and precision values for calculated slopes of calibration curves. The validated method showed acceptable study data for the quantification of gliclazide in a clinical setting. Further, incurred sample reanalysis of 129 samples authenticates the reproducibility of the proposed method and is reported in any previous method.

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