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HPLC Method for Determination of Gliclazide in Human Serum

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A simple, rapid and specific method for analysis of gliclazide in serum by a sensitive high-performance liquid chromatographic method is described. Only 100 μL of serum and a little sample work-up is required. A simple procedure of extraction by toluene followed by evaporation to dryness under a gentle stream of air and dissolving the dried residue in mobile phase was used. The gliclazide peak was separated from endogenous peaks on a C_{18} column by a mobile phase of acetonitrile-methanol-water (50:30:20, v/v), pH 3. Gliclazide and internal standard (phenytoin) were eluted at 4.85 and 3.8 min, respectively. The limit of quantification (LOQ) for gliclazide in serum was 50 ng/mL at 230 nm. The method was linear over the range of 50-10,000 ng/mL with r^2 of 0.999. Mean recovery for gliclazide and internal standard was 85.5 and 86.0 %, respectively.

Key Words: Gliclazide, Phenytoin, Limit of quantification.

INTRODUCTION

Gliclazide, 1-(4-methylbenzenesulphonyl)-3-(3-azabicylco[3.3.0]octyl)urea (**I**), is an oral hypo-glycemic drug, belonging to second-generation sulphonyl ureas, which is used in type **II** diabetes, previously known as non-insulin-dependent diabetes mellitus [NIDDM]. It has been suggested that due to short-term acting, gliclazide may be suitable for a diabetic patients with renal impairment and also in elderly patients whose reduced renal function may increase the risk of hypoglycemia following some sulphonylureas[1].

Several methods have been reported for the determination of gliclazide, either *per se* or in pharmaceutical preparations and biological fluids. Different analytical methods including colorimetry[2], radioimmunassay[3], gas chromatography[4] and HPLC[5-12] have been reported for determination of gliclazide in biological fluids. Some reported analytical methods involve time-consuming and laborious extraction steps[7,8], complex derivatization techniques[8], lengthy retention time or large volumes of biological samples[7-9], solid-phase extraction[10] or use of mass spectrometry for

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detection and identification of the drug[12]. A simple, rapid and applicable HPLC method is developed, which requires a small sample volume and minimal sample work-up. This method is suitable for pharmacokinetic studies in terms of specificity and sensitivity.

Hong *et al.*[13] have assayed serum gliclazide by an HPLC method using glibenclamide as an internal standard. To 0.5 mL of serum sample, 20 mL of an aqueous methanol solution (50 %) of glibenclamide (50 mg/mL) was added. The analytical column was a CLC ODS-M column (C18, 5 mm, 25034.6 mm I.D.). The mobile phase was a mixture of 0.04 M potassium dihydrogenphosphate (pH 4.6)-acetonitrile-iso-propyl alcohol (4:5:1, v/v). The flow rate of the mobile phase was 1.0 mL/min. The eluate was monitored at 227 nm. The gliclazide concentration in the sample was determined using a calibration curve, prepared by the peak height ratio of gliclazide against the internal standard. The calibration curve was linear over the range of 0.05-10 mg/mL, with a correlation coefficient of 0.999.

Gayatri *et al.*[14] have developed a simple and accurate HPLC method for the simultaneous estimation of gliclazide and rosiglitazone from the pharmaceutical preparation. The determination was carried out on resolved C_{18} stainless steel ODS Hypersil column (5 µ, 250 × 4.6 mm) in isocratic mode using acetonitrile:methanol: ammonium dihydrogen phosphate buffer:trichloroacetic acid (30:30:40:0.05 %) as a mobile phase. Various dilutions were made to get a series of concentrations between 500-3000 µg/mL of gliclazide and 25-150 µg/mL of rosiglitazone, using methanol. 20 µL of the sample solution was injected in triplicate at an interval of 10 min. The flow rate was maintained at 1.75 mL/min and the effluent was monitered at 254 nm. The retention time of gliclazide was 5.14 min and rosiglitazone was 3.25 min.

Kuo and Wu[15] have developed a sensitive HPLC-electrochemical detection method for the analysis of gliclazide in human plasma. After deproteination of 100 µL of plasma by acetonitrile, evaporation and reconstitution, gliclazide was separated on a C_{18} column (150 mm × 4.6 mm) by the mobile phase (70 mM disodium tetraborate, pH 7.5, containing 26.5 % of acetonitrile). The regression equations were linear ($r > 0.9990$) over the range of 50 nM to 4.00 µM. The limit of detection for plasma was 10 nM for GL (S/N = 3, 10 µL injection). This newly developed method was applied for monitoring blood levels with one healthy volunteer dosing with a gliclazide tablet.

Ruz *et al.*[16] have developed solid phase extraction (SPE) and HPLC methods for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma. The separation was performed using a Discovery C_{18} Supelco analytical column (250 mm × 4.6 mm, 5 µ). The guard column was a Supelco Discovery (20 mm × 4 mm, 5 µ). The limits of quantification were between 5 and 22.5 ng/mL. The intraday and interday precision was always less than 9 %. The accuracy was always less than 12 %. Stability analysis showed that all analytes are stable for at least 3 months when stored at -70 ºC.

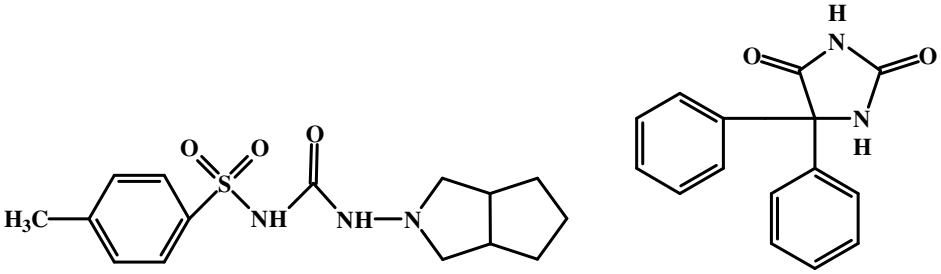
Park *et al.*[17] have developed a new HPLC method for the pharmacokinetic analysis of gliclazide by using a semi-micro column to quantify gliclazide in plasma samples. Chromatographic separation was achieved with a semi-micro C_{18} column and 40 mM KH_2PO_4 (pH 4.6)-acetonitrile-isopropyl alcohol (5:4:1, v/v/v) as the mobile phase and with UV detection at 229 nm. The method displayed good precision, was fast (total analysis time 8 min) and required only a small amount of mobile phase (0.22 mL/min), with a reasonable limit of quantification (0.1 μg/mL). The calibration curve was linear in the concentration range 0.1-10 μg/mL.

Gandhimathi *et al.*[18] have developed a simple, efficient and reproducible method for the simultaneous determination of metformin and gliclazide from tablets using reversed phase HPLC. The separation was done using a mobile phase consisting of 0.025 M disodium hydrogen phosphate and acetonitrile (25:75 % v/v) with pH adjusted to 3.2 with dilute orthophosphoric acid. Column used was Shimpack CLC C8 (250 × 4 mm ID) 5 μ with flow rate of 1 mL/min with detection at 240 nm. An elution order was metformin (2.8 min) and gliclazide (4.3 min). The linear dynamic range was 5-500 μg/mL and 10-100 μg/mL for metformin and gliclazide, respectively. Analytical parameters were calculated and a full statistical evaluation included.

Vasudevan *et al.*[19] have developed a simple, precise and accurate HPLC method for the simultaneous estimation of metformin with gliclazide and glipizide present in multicomponent dosage forms. The method was carried out on Inertsil C_{18} column. A mobile phase composed of acetonitrile-water containing camphor sulphonic acid (adjusted to pH 7 using 0.1 N sodium hydroxide; 75 mM) at a flow rate of 1 mL min^{-1} was used for the separation. Detection was carried out at 225 nm. Tolbutamide was used as internal standard.

EXPERIMENTAL

Gliclazide (USP reference standard) and phenytoin working standard (used as internal standard, IS) were kindly donated by Panachea Biotech Ltd., (Baddi, Himachal Pradesh, India) (Fig. 1). HPLC grade acetonitrile and methanol (S.D. Fine Chemicals, Mumbai, India) and double distilled water were used throughout the analysis. All other chemicals and reagents were of analytical grade.



(I) **(II)**

Fig. 1. Structure formula of gliclazide (**I**) and phenytoin (**II**)

The HPLC isocratic system consisted of a model 600 pump, a model 486 UV tunable absorbance detector, a 746 data module and a 600E system controller (all from Waters, Milford, USA).

Separation was performed on a C_{18} column (3 μm, 150 mm × 3.9 mm ID; HPLC) at ambient temperature (23-27 ºC). The pH of the acetonitrile-methanol-water mobile phase (50:30:20, v/v) was adjusted to 3 with phosphoric acid. Mobile phase was passed through column by a flow rate of 1.0 mL/min and the eluate was monitored at 230 nm. Prepared samples were injected to HPLC column through a 7725i Rheodyne injector fitted with a 50-μL loop.

Standards: The stock solution of gliclazide (100 μg/mL) and the IS (160 μg/mL) were prepared in acetonitrile, methanol and were stable for at least 1 month at 4 ºC. The working IS solution (12 μg/mL) was prepared in acetonitrile, methanol and water every day.

TABLE-1
DATA FOR STANDARD CURVE OF GLICLAZIDE IN ACETONITRILE-METHANOL-WATER BY HPLC

Concentration (ng/mL)	Retention time (min)	Height (cm)	Area (cm^2)	Area (%)
50	4.85	1.1805	8.1389	100
100	4.85	2.4327	17.1931	100
250	4.85	3.6561	24.7148	100
500	4.86	4.5919	32.4247	100
1000	4.85	7.7126	53.0255	100
1500	4.85	9.6787	70.8117	100
2000	4.85	12.8828	89.5360	100
4000	4.85	22.4589	155.7202	100
5500	4.85	41.2762	172.9484	100
10000	4.85	79.1146	209.7787	100

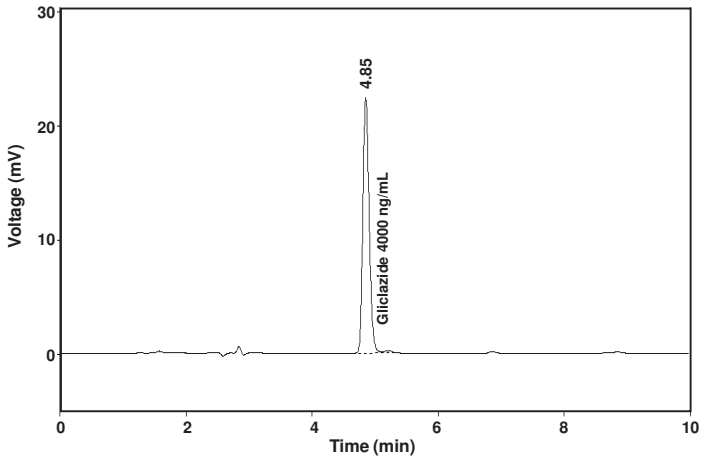


Fig. 2. Chromatograms of gliclazide of a 4000 ng/mL concentration

Serum extraction: To 100 µL of serum were added 50 µL of the IS working solution and 100 µL of 0.07 M phosphate buffer (pH 4.5). After vortex mixing for 10 s, 1 mL of toluene was added and the mixture was shaken vigorously for 1 min. The mixture was then centrifuged for 5 min at 10000 rpm. A 800 µL aliquot of the upper organic layer containing gliclazide and IS was transferred to a clean glass tube and evaporated under air stream to dryness at 50 ºC. The residue was redissolved in 100 µL of mobile phase and a 50 µL aliquot was injected onto the HPLC column.

Human serum study: After administration of 80 mg tablet of gliclazide [formulated by Panachea Biotech Ltd., Baddi, India] blood samples were taken up to 24 h. Blood sugar was also determined every hour up to 12 h.

RESULTS AND DISCUSSION

Fig. 3 shows representative chromatograms of extracted serum samples. The retention times of gliclazide and the IS were 4.85 and 3.8 min, respectively. No endogenous interference was observed with both gliclazide and the internal standard. The calibration curve was obtained by plotting the peak height ratios (gliclazide/IS) *versus* concentrations, which was linear over the range of 50-10 000 ng/mL with the regression equation of $y = 0.0355+0.354x$ and $r^2 = 0.999$. Estimates of within-day and between-day precisions of the assay were evaluated by analyzing 4 different known concentrations of gliclazide in serum. The results of 6 determinations (Table-2) showed a good reproducibility of the proposed method with the mean CV of 3.8 and 6.5 % for within-day and between-day precision, respectively.

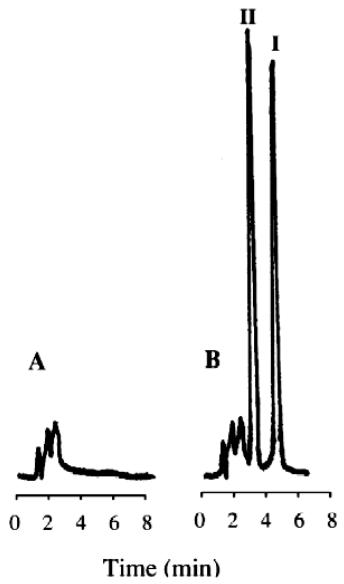


Fig. 3. Chromatograms of (A) blank serum and (B) serum sample 2 h after administration of an 80 mg tablet. (I: gliclazide, II: phenytoin)

TABLE-2
REPRODUCIBILITY AND ACCURACY OF THE ANALYSIS METHOD (n = 6)

Concentration (μg/mL)	Within-day C.V	Within-day accuracy	Between-day C.V	Between-day accuracy
2000	3.7	6.5	8.6	9.7
4000	3.8	-2.2	5.4	3.4
5500	4.5	-1.7	6.2	-4.3
10000	3.2	4.1	5.8	5.9

The limit of quantification (LOQ) of gliclazide was 50 ng/mL and the minimum detectable limit (LOD) was 30 ng/mL. The recovery was obtained by comparing the peak height of known serum samples spiked with gliclazide and IS to those of their respective aqueous solutions, correcting for volume. The results showed absolute recoveries of 85.5 ± 2.9 and 86.0 ± 3.70 % for gliclazide and IS, respectively. Fig. 4 shows a concentration-time profile of a human volunteer after taking an 80 mg single oral dose of gliclazide.

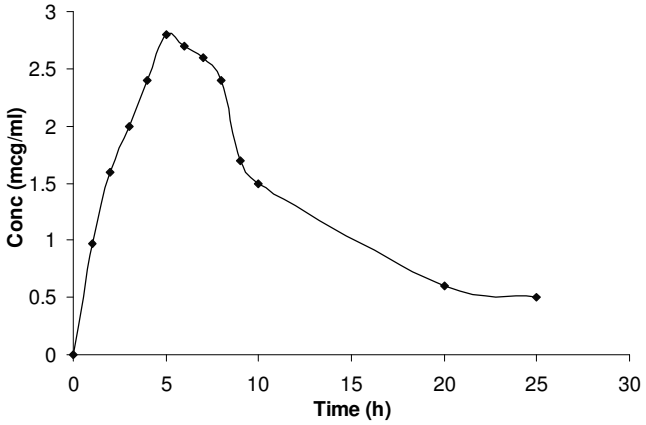


Fig. 4. Serum concentration time profile of gliclazide 80 mg tablet in a blood sample

The solid phase extraction procedure reported by Noguchi *et al.*[10] for sample preparation is an expensive sample clean-up method. The temperature should be kept at 37 ºC during their reported analysis and each run lasts *ca.* 15 min. Present method works at ambient temperature and uses liquid-liquid extraction and has a shorter run time. Igaki *et al.*[8] have developed an HPLC method using fluorescence detection and chemical derivatization, but nevertheless their method is less sensitive than present method (LOQ of 100 *vs.* 50 ng/mL, respectively). In another study Charles and Ravenseroft[6] used 3-chlorogliclazide as internal standard, which is not commercially available. The LOQ of their method is *ca.* 500 ng/mL, which is far below the therapeutic serum concentrations and sensitivity needed in pharmacokinetic studies.

The methods published by Kimura *et al.*[7] and Sener *et al.*[9] and Poirier *et al.*[11] need 0.5 and 0.25 mL of serum samples, respectively, compared to 0.1 mL in present method. Poirier *et al.*[11] have also used nadoxolol as IS which is not easily available. The perchloric acid used in their mobile phase reduces the column life remarkably. We also did not find any difference between the citrate-phosphate buffer used by Poirier *et al.*[11] compared to phosphate buffer alone in sample preparation. Present method has also the shortest run time in comparison to this and all previously mentioned methods.

The pharmacokinetic parameters obtained from present study using this method of analysis showed a maximum serum concentration of 3 ± 0.58 μg/mL, a t_{max} of 3.5 ± 0.7 h and a $t_{½}$ of 9.5 ± 2.4 h. The 24 h serum concentration was *ca.* 300 ng/mL after administration of 80 mg tablet.

In conclusion, present method is simple, sensitive and specific HPLC method and could easily be used in pharmacokinetic and bioequivalency studies when numerous daily samples have to be assayed relatively quickly; at least 40 samples can be analyzed during an 7 h working day.

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