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A Validated Reversed Phase HPLC Assay for the Determination of Gliclazide in Human Plasma

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Original Research Article

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Abstract: A simple and precise reversed-phase high performance liquid chromatography (HPLC) method for the determination of gliclazide in human plasma was developed and validated. Using glipizide as an internal standard (IS), separation was achieved on Atlantis dC18 column. The mobile phase consisted of acetonitrile and water (pH= 2.5±0.2 adjusted with phosphoric acid) (55:45, v:v), and delivered at a flow rate of 1 ml/min. 0.5 ml plasma samples were deproteinized with dichloromethane and centrifuged. 100 µl supernatant clear solutions were injected to HPLC system. The eluent was monitored spectrophotometricly at 230 nm. No interference in blank plasma or of commonly used drugs was observed. The relationship between the concentration of gliclazide in plasma and peak height ratio of gliclazide to the IS was linear over the range of 0.05-10.0 µg/ml. Intra-day and inter-day coefficient of variation (CV) and bias were 3.1% to 6.8% and 3.8% to 9.3% respectively. Mean extraction recovery of gliclazide and the IS from plasma samples was 94% and 87% respectively. The method was applied to assess the stability of gliclazide under various conditions generally encountered in the clinical laboratory. Stability for processed and unprocessed samples was ≥ 90% and ≥ 93% respectively.
Keywords: Gliclazide, Glipizide, Human plasma, HPLC

INTRODUCTION

Gliclazide (CAS: 21187-98-4), N- (Hexahydrocyclopenta [c] pyrrol-2 (1H)-yl carbamoyl-4-methyl benzene sulfonamide) is an oral hypoglycemic agent used widely in the treatment of type-II diabetes mellitus [1]. Gliclazide is absorbed rapidly with a peak plasma concentration 4.69±1.38 mg/L within 3.45±1.11 h after ingestion of single oral therapeutic dose of 80 mg [2].

Several analytical methods have been reported for the determination of gliclazide in pharmaceutical formulations and biological samples [3-18]. Gliclazide levels in human plasma have been mainly determined by high performance liquid chromatography (HPLC) with ultra violet detection. Some of the reported methods involved filtration [13-15], protein precipitation [16], liquid-liquid extraction [17], and solid phase extraction [18], as sample preparation procedures. Liquid chromatography- tandem mass spectrometry (LC-MS/MS) technique [19] was applied in bioequivalence and pharmacokinetic studies of gliclazide. LC-MS/MS method although it is simple and convenient, but limited by their high analysis cost.

In the present study, we describe a simple, precise, rapid, and low-cost HPLC assay that requires 0.5 ml human plasma, and is based on liquid- liquid extraction. The method was validated and successfully applied to assess the stability of gliclazide under various laboratory conditions.

MATERIAL AND METHODS

Apparatus

Chromatography was performed on a Waters Alliance HPLC 2695 (Waters Associates Inc., Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. A reversed-phase column Atlantis dC-18 (4.6 x 150 mm, 5-µm) protected by (3.9 x 20 mm, 5 µm guard column) symmetry C18. Data were collected with a Pentium IV computer using Empower Chromatography Software.

Chemical and reagents

All reagents were of analytical-reagent grade unless stated otherwise. Gliclazide and glipizide were purchased from Sigma-Aldrich Co, Steinheim, Germany. Acetonitrile, methanol, dichloromethane (all HPLC grade), hydrochloric acid, and phosphoric acid were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and was further purified by passing through a

Synergy Water Purification System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC) Riyadh, Saudi Arabia.

Chromatographic conditions

The mobile phase consisted of acetonitrile and water (pH= 2.5±0.2, adjusted with phosphoric acid) (55:45, v:v). The analysis was carried out under isocratic conditions using a flow rate of 1.0 ml/min at ambient temperature and a run time of 10 minutes. A photodiode array detector set at 230 nm was used.

Preparation of standard and quality control samples

Stock solutions of gliclazide and glipizide (1.0 mg/ml) were prepared in methanol. They were diluted with methanol to produce working solutions of 10 µg/ml for gliclazide and the IS. Ten calibration standards in the range of 0.05 – 10.0 µg/ml and four quality control (QC) samples (0.05, 0.15, 5.0, and 9.0 µg/ml) were prepared in human plasma. Calibration standards and QC samples were vortexed for one minute and 0.5 ml aliquots were transferred into microcentrifuge tubes and stored at -20 ºC until used.

Sample preparation

Aliquots of 0.5 ml of calibration standards or QC samples were allowed to equilibrate to room temperature. To each tube, 100 µl of the IS working solution and100 µl of 1M hydrochloric acid were added and the mixture was vortexed for 10 seconds. After the addition of 2 ml of dichloromethane, the mixture was vortexed again for 10 min and then centrifuged for 15 min at 4200 rpm at room temperature. The organic layer was carefully collected into a clean tube and dried under a gentle stream of nitrogen, and the residue was reconstituted in 200 µl water and centrifuged at 13000 rpm for 5 min at room temperature. The supernatant was transferred into an auto-sampler vial and 100 µl were injected into the HPLC system.

Stability studies

A total of 40 aliquots of each QC samples (0.05, 0.15, and 9.0 µg/ml) were used for stability studies. Five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed (counter stability, 24 hours at room temperature), five aliquots were stored at –20 °C for eight weeks before being processed and analyzed (long term freezer storage stability), and five aliquots were processed, reconstituted, and stored at room temperature for 24 hours or 48 hours at –20 °C before analysis (autosampler stability). Finally, fifteen aliquots of each QC sample were stored at –20 °C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to –20 °C for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [20]. The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Under the optimal experimental conditions consisting of mobile phase of acetonitrile and water (55:45, v:v) (pH= 2.5±0.2, adjusted with phosphoric acid). The mobile phase was delivered at a flow rate of 1 ml/min, gliclazide, glipizide, and plasma components exhibited a well-defined chromatographic separation within 10 minutes run. The retention times of gliclazide and glipizide (IS) were around 6.1 and 3.8 respectively.

Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. No endogenous component co-eluted with gliclazide or the IS. **Figure 1** depicts a representative chromatogram of drug free human plasma used in preparation of standards and QC samples.

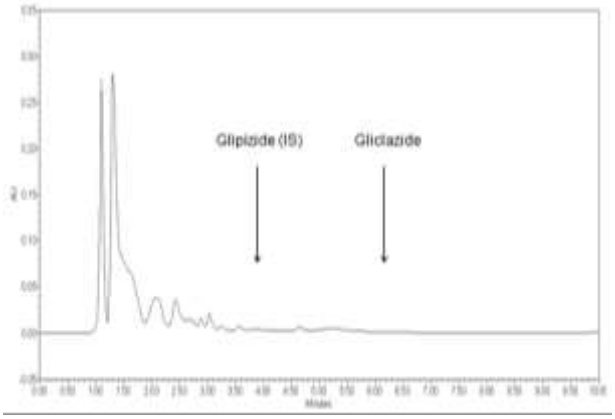

Fig-1: Representative chromatogram of gliclazide and glipizide-free human plasma. The arrows indicate the retention times of and glipizide (internal standard, IS; 3.8 min) and gliclazide (6.1 min)

Linearity, Accuracy and Precision

Linearity of gliclazide was evaluated by analyzing ten curves of ten standard concentrations over the range (0.05-10.0 µg/ml) prepared in human plasma. Figure 2 represents an overlay of chromatograms of extracts of 0.5 ml human plasma spiked with the IS and one of ten concentrations of gliclazide. The peak height ratios were subjected to regression analysis (regression

equation, Y = 0.102 X + 0.006). The suitability of the calibration curves was confirmed by back-calculating the concentration of gliclazide in human plasma from the calibration curves (Table 1). All calculated concentrations were well within the acceptable limits. Precision and bias were also determined for four QC concentrations (0.05, 0.15, 5.0, and 9.0 µg/ml). The intra-day (n=10) and inter-day (n=20, over 3 days) precision was ≤ 6.8% and ≤ 9.3%, respectively. The intra-day and inter-day bias was in the range of -0.1-14.0% and 1.2-11%, respectively. The results are summarized in Table 2.

Table 1: Back-calculated gliclazide concentrations from ten calibration curves

Nominal Level (µg/ml)	Calculated Level (µg/ml)		*CV (%)	**Accuracy (%)
	Mean	SD		
0.05	0.049	0.006	11.7	98
0.10	0.097	0.008	8.0	97
0.25	0.244	0.016	6.7	98
0.50	0.472	0.043	9.2	94
1.0	0.936	0.074	7.9	94
2.0	2.001	0.122	6.1	100
4.0	4.219	0.193	4.6	105
6.0	5.715	0.511	8.9	95
8.0	8.060	0.332	4.1	101
10.0	10.013	0.296	3.0	100

*Coefficient of variation (CV) = standard deviation (SD) divided by mean measured concentration x 100.
** Accuracy = measured level divided by nominal level x 100

Table 2: Intra - and inter-day bias and precision of gliclazide assay

Nominal Level (µg/ml)	Measured Level (µg/ml)		CV (%)	Bias (%)
	Mean	SD		
Intra-day (n=10)				
0.05	0.057	0.003	5.3	14.0
0.15	0.154	0.005	3.1	2.5
5.0	4.993	0.342	6.8	-0.1
9.0	9.026	0.573	6.3	0.3
Inter-day (n=20)				
0.05	0.056	0.003	6.0	11.0
0.15	0.154	0.006	3.8	2.6
5.0	5.062	0.275	5.4	1.2
9.0	9.293	0.867	9.3	3.3

SD, standard deviation, CV, standard deviation divided by mean measured concentration x100
Bias, measured level - nominal level divided by nominal level x 100

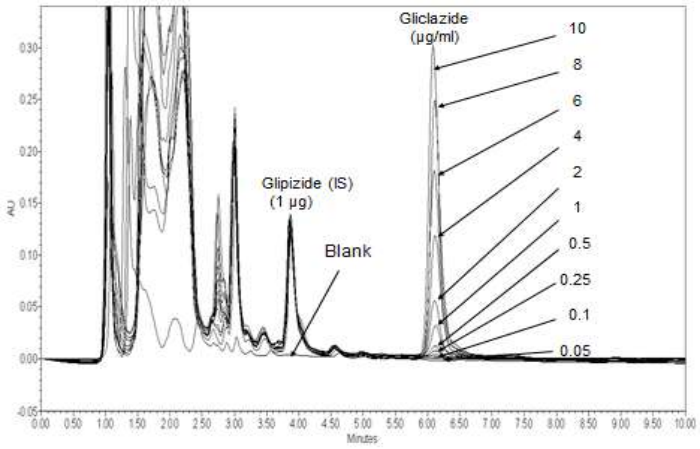

Fig-2: Overlay of chromatograms of extracts of 0.5 ml human plasma spiked with the internal standard (IS) and one of ten concentrations of gliclazide, zero, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 µg/ml

Recovery

The absolute recovery of gliclazide was assessed by direct comparison of peak height from plasma and water samples, using five replicates for each of four QC samples (0.05, 0.15, 5.0, and 9.0 µg/ml). Similarly, the recovery of the IS was determined by comparing the peak height of the IS in 5 aliquots of 0.5 ml human plasma spiked with 100 µl of IS (10 µg/ml) with the peak height of equivalent samples prepared in water. The results are presented in **Table 3**. The mean recovery of gliclazide and the IS were 94% and 87%, respectively.

Table 3: Recovery of gliclazide and the internal standard from 0.5 ml of human plasma

Concentration (µg/ml)	Mean peak height (SD)*		Recovery** (%)
	Human Plasma	Water	
Gliclazide 0.05	36388 (43)	36570 (370)	100
0.15	22231(690)	22317 (107)	100
5.0	31760 (18262)	33577 (14831)	95
9.0	35702 (7278)	42790 (3308)	83
Internal standard 10	149653 (9680)	171292 (6192)	87

* Mean peak height (SD), n = 5. * Recovery is equal to mean peak height in human plasma divided by mean peak height in water x 100

Table 4: Stability of gliclazide under various clinical laboratory conditions

Nominal	Unprocessed		Processed		Freeze-Thaw (Cycle)		
Level(µg/ml)	24 hrs (RT)	8 hrs (-20 °C)	24 hrs (RT)	48 hrs (-20 °C)	1	2	3
0.05	112	96	96	93	109	107	102
0.15	93	98	92	94	98	89	110
9.0	99	92	98	100	108	103	102

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after 24 hours at room temperature (24 hrs RT), after freezing at –20 ºC for 8 weeks (8 wks, –20 ºC), or processed and then analyzed after storing for 24 hours at room temperature (24 hrs, RT) or 48 hours at –20 ºC (48 hrs, -20 ºC).

Stability

Stability of analyte in biological matrices is an important pre-analytical variable. It is necessary to perform stability studies of the analyte and IS to determine the range of appropriate conditions and time of storage. Gliclazide and IS stability in processed and unprocessed plasma samples (0.05, 0.15 and 9.0 µg/ml) was investigated. Gliclazide was stable in processed samples for at least 24 hours at room temperature (≥ 90%) or 48 hours at -20 °C (≥ 92%). Gliclazide in unprocessed plasma samples was stable for at least eight weeks at -20 °C (≥ 93%), and after three freeze- and thaw cycles (≥ 81%).

CONCLUSION

The described HPLC assay is accurate, precise, and rapid. It requires only 0.5 ml plasma and utilizes a simple and convenient method for sample preparation. The assay was applied to monitor stability of gliclazide under various conditions generally encountered in the clinical laboratories.

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