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DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR ESTIMATION OF GLICLAZIDE IN RABBIT PLASMA

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ABSTRACT

This study presents rapid, sensitive and accurate HPLC method for determinations of gliclazide in Rabbit Plasma (RP). Rabbit plasma samples were subjected to protein precipitation by methanol and protein free plasma samples were injected to HPLC C 18 coloums. Glipizide was used as internal standard. Acetonitrile and pH 6.8 Phosphate buffer at 50:50 ratio, at a flow rate of 1mL /min and a pressure of 150-200 Kg/cm^2 were used to get the chromatogram. A good separation of gliclazide and glipizide was achieved by this method with retention times of 7.4 and 4.5 min respectively. The calibration curve of the gliclazide in a concentration range of 50-800 ng/mL resulted in the linear least square regression equation i.e. Y=0.032X+0.5187. Peak areas were reproducible as indicated by low coefficient of variation (<1.79%).

KEYWORDS

HPLC, Rabbit plasma, Gliclazide, Accuracy and Precision.

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INTRODUCTION

Gliclazide, 1-(hexahydrocyclopental [c]pyrrol-2(1H)-yl)-3-[(4methyl phenyl) sulphonyl) urea, is a second generation sulfonylurea[1,2] about 95% of gliclazide is bound to plasma proteins, mostly to albumin. Ethyl alcohol has been shown to reduce the binding of gliclazide to albumin[3].

Several HPLC methods were developed for estimation of gliclazide in plasma[4-14] (serum, plasma, blood or urine) and most of these methods require large volume of samples, multiple steps of sample preparation, laborious time consuming extraction processes. Hence studies were carried

out to develop a simple HPLC method for estimation of gliclazide in plasma samples. In present study, plasma samples were subjected to protein precipitation and protein free plasma samples were directly injected into HPLC column.

The rabbit is a standard laboratory animal in biomedical research can be used as animal models for a variety of human diseases Because of these criteria rabbits were widely used in pharmaceutical studies[15,16]. Hence in the present investigation it is proposed to estimate the gliclazide concentrations in rabbit plasma (RP) by using present HPLC method. The study was carried out in accordance with the guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) with Institutional Animal Ethics Committee number of Reg. No. 516/01/a/CPCSEA.

EXPERIMENTAL

MATERIAL

Gliclazide was provided by Dr Reddy's Laboratories Ltd, Hyderabad, India. HPLC grade methanol and acetonitrile were purchased from Qualigens Fine chemicals Ltd Mumbai, India. All other chemicals used were of HPLC grade.

Quantitative HPLC was performed on HPLC (SHIMADZU, Japan) with LC-10AT VP gradient solvent module, SPD-M10A VP model PDA detector and LC solutions software. Samples were chromatographed on C18 analytical column (250 mm X 4.6 mm packed with particles of 5 µm diameter). The composition of mobile phase was acetonitrile and pH 6.8 Phosphate buffer in 50:50 v/v ratio. The mobile phase was filtered through 0.45µ filter and pumped from the reservoir to the column at a flow rate of 1 mL/min and the pressure was 150-200 Kg/cm^2 and the coloum temperature was maintained at 35^0C. The volume of injection loop was 20 µL. Initially the column was equilibrated for at least 45 min with the mobile phase and then the prepared drug solutions were injected. The eluents were monitored at 226 nm by PDA detector and the data acquired was stored and analyzed with LC solutions software.

Preparation of stock solutions of Gliclazide

Gliclazide (100 mg) was dissolved in minimal quantity of methanol in a 100 mL volumetric flask and the final volume was adjusted to 100mL by using triple distilled water to get 1000 µg/mL solution. From this stock solution 1mL was diluted up to 100 mL with triple distilled water to get 10000 ng/mL solution (stock I). Then aliquots (0. 5, 1, 2, 4 and 8 mL) of stock I solutions were transferred to 10 mL volumetric flasks and the volume was made up to 10 mL with triple distilled water to get 500, 1000, 2000,4000 and 8000 ng/mL solutions. An aliquot of 0.1 mL of this solution represent 50. 100, 200, 400 and 800 ng/mL solutions.

Preparation of stock solution of internal standard

Glipizide was used as internal standard for estimation of gliclazide. Glipizide 100 mg was dissolved in minimal quantity of methanol in 100 mL volumetric flask and then the volume was made up to 100 mL by using tripled distilled water to get 1000 µg/mL solution. From this stock solution 1 mL was diluted to 100 mL with triple distilled water to get 10000 ng/mL solution (stock I). 1 mL of this stock was diluted up to 10 mL in volumetric flask with triple distilled water to get 1000 ng/mL solution. An aliquot of 0.1 mL of this solution represent 100 ng/mL.

Preparation of Mobile Phase[17]

Transfer 250 ml of 0.2 M monobasic potassium phosphate solution, 112 ml of 0.2 M NaOH into 1000 mL volumetric flask and make up the volume with triple distilled wanted to get 6.8pH phosphate buffer.

Procedure

Blood samples (1mL) were collected from marginal ear vein of healthy rabbits by standard procedure[18], transferred into K_3-EDTA tubes, centrifuged at 7500 rpm for 15 min and plasma was separated. 0.1mL of these plasma samples and 0.1mL of standard gliclazide solution were transferred into an Eppendorf micro centrifuge tubes. To this 0.1mL of internal standard (glipizide equivalent to 100ng/mL) and 0.7mL of methanol were added to get concentrations of 50, 100, 200, 400 and 800 ng/mL

of gliclazide. These samples Were vortex-mixed for 3 min using Remi Cylo mixture followed by centrifugation at 7500 rpm for 20 min. 20 µL of supernatant was collected by micro syringe and directly injected into HPLC column.

Precision, Accuracy[19]

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of sample. The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true concentration of an analyte. The intra-day (within run) and inter-day (between run) Precision and Accuracy of the present HPLC method were estimated by subjecting 100, 200 and 400ng/mL of gliclazide solutions for analysis. Intraday accuracy and precision were determined by assaying samples of five each for each concentration within one day whereas inter day precision and accuracy were determining the five samples for each concentration for five consecutive days. In each case the coefficient of variation (%C.V.) and %relative error (%RE) were calculated to find out the precision and accuracy of the present HPLC method.

Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method. Recovery pertains to extraction efficiency of an analytical method within limits of variability.

Recovery of the procedure was conducted by adding 100, 200 and 400 ng/mL of gliclazide to the pre analyzed plasma drug samples containing 100 ng/mL of gliclazide and 100 ng/mL of internal standard. Then these samples were subjected to the present HPLC method. Five spiked plasma samples at these different concentration levels were subjected to analysis to calculate mean recovery.

RESULTS AND DISCUSSION

Method development

No of methods are available for estimation of gliclazide in plasma samples but most of the involves large volume of samples, multiple steps of sample preparation, laborious time consuming extraction processes. To get good separation between gliclazide and internal standard (glipizide) no of coloums and mobile phases were examined. Glipizide due to its structural similarity to gliclazide was selected as internal standard to normalize erratic recoveries and to improve precision of analysis.

Among plasma extraction methods, protein precipitation with methanol was found to be optimal and produced clean chromatogram for a blank sample and yielded highest and stable recovery for gliclazide from rabbit plasma. This may be due to the reason that alcohol has been shown to reduce the binding of gliclazide to albumin[3]. Composition of mobile phase was set up by several trails to get good resolution and symmetric peak shapes of analyte as well as short run time. Finally mixture of acetonitrile and pH 6.8 Phosphate buffer (50:50) as mobile phase at a flow rate of 1 mL /min and pressure of 150-200 Kg/cm^2 was optimized as chromatographic conditions.

Chromatograms obtained with blank plasma and plasma spiked with different concentrations of gliclazide are shown in Figure No.1. A good separation of gliclazide and internal standard (glipizide) were achieved with the retention times of 7.4 and 4.5 min respectively without interference of endogenous compounds in rabbit plasma. Each sample was subjected three times and the same retention times were observed in all the cases. In addition, the total run time for each injection per sample was only 10 min. The concentrations of gliclazide and the corresponding peak area ratios of gliclazide to internal standard are given in Table No.1 and the calibration curve is shown in Figure No.2. The calibration curve of the gliclazide in a concentration range of 50-800 ng/mL resulted in the linear least square regression equation i.e. $Y=0.032X+0.5187$ where X is the concentration of

gliclazide in ng/mL and Y is the peak ratio of gliclazide to internal standard. A good linear relation was observed as indicated by r=0.9971. The peak areas were reproducible as indicated by low coefficient of variation (<1.79%).

Accuracy and Precision

The results of accuracy and precision were shown in Table No.2. Low coefficient of variation (<0.7%) was observed with intra-day and inter-day estimation of gliclazide by the present HPLC method indicated that this HPLC method is highly precise. RE% values were found to be within the limits of ±0.235, indicating the high accuracy of preset HPLC method.

Recovery

For different concentration (ng/mL), recovery of gliclazide (n=5) from pre analyzed drug solutions are shown in Table No.3 which indicated a good efficiency of the method confirmed by detection of more than 99% of gliclazide of precipitation procedure employed in this study.

Table No.1: Concentration vs. peak area ratio of gliclazide in rabbit plasma

S.No	Concentration of gliclazide (ng/mL)	Mean peak area ratio of gliclazide to internal standard	%C.V.
1	50	2.698±0.047	1.79
2	100	4.598±0.051	1.11
3	200	6.483±0.095	1.47
4	400	12.399±0.11	0.88
5	800	26.56±0.32	1.22

Table No.2: Accuracy and Precision of the HPLC method (n=5)

S.No	Concentration (ng/mL)		%C.V.		%RE	
	Actual	Measured				
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1	100	99.88	99.96	0.374	0.624	-0.114	-0.036
2	200	199.87	199.48	0.139	0.700	-0.064	-0.256
3	400	399.4	400.14	0.204	0.286	-0.150	0.235

Table No.3: Recovery of gliclazide form pre analyzed samples containing 100ng/mL (n=5)

S.No	Concentration (ng/mL)	Mean % recovery (± s.d.) (n=5)
1	100	99.92±0.91
2	200	99.87±1.05
3	400	99.97±1.34

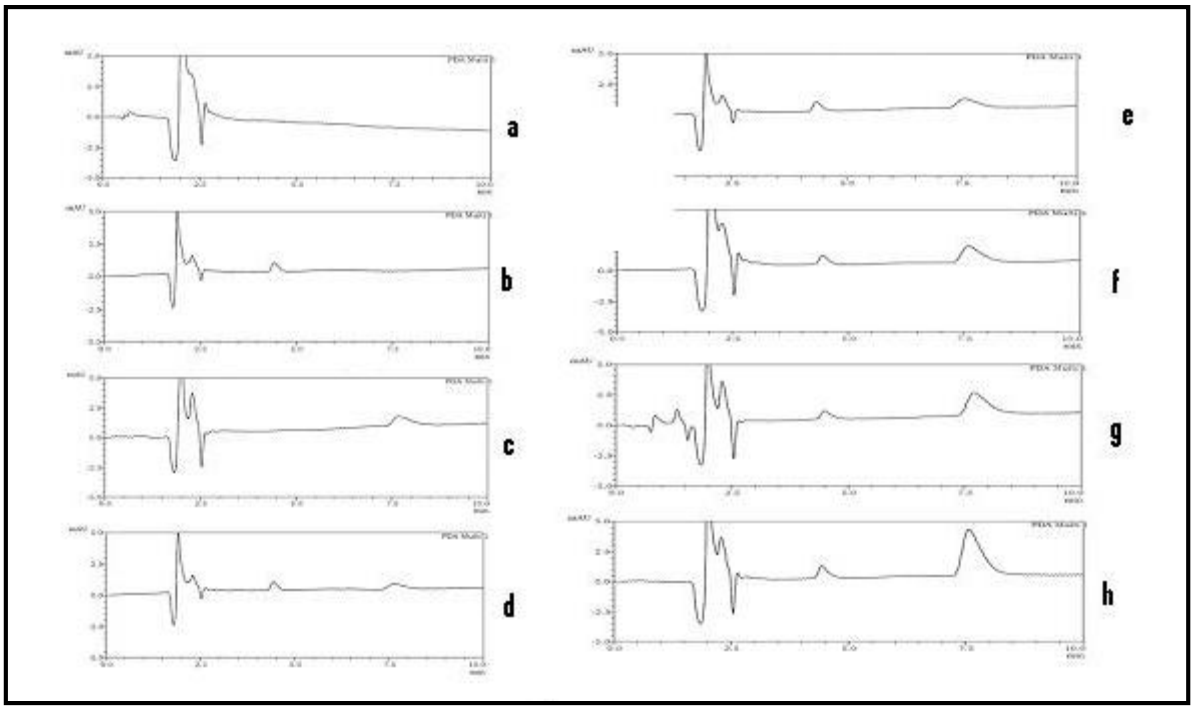

Figure No.1: HPLC Chromatogram of rabbit plasma spiked with (a) Blank plasma (b) IS (100 ng/mL) (c) pure gliclazide 100 ng/mL and IS (d) gliclazide 50 ng/mL and IS (e) gliclazide 100 ng/mL and IS (f) gliclazide 200 ng/mL and IS (g) gliclazide 400 ng/mL and IS (h) gliclazide 800 ng/mL and IS

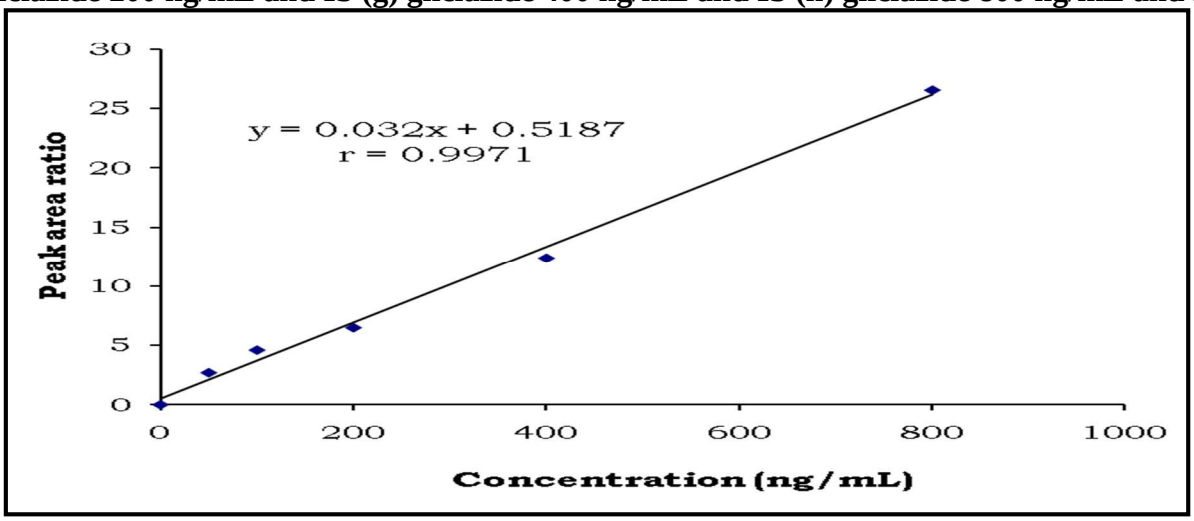

Figure No.2: Calibration curve of gliclazide in rabbit plasma

CONCLUSION

Present method has good sensitivity and specificy for determination of gliclazide in rabbit plasma. A good separation of gliclazdie and internal standard was achieved without interference of any endogenous compounds in RP. Standard curve of gliclazide demonstrated good linearity over a range of 50-800 ng/mL. The low intraday and inter day coefficients of variation at different concentrations demonstrated that the assay is accurate and reproducible. Hence this present HPLC method can be used for routine clinical monitoring of plasma levels of rabbits in pharmacokinetic research studies using small laboratory animals.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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