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BIOANALYTICAL METHODS FOR ESTIMATION OF GLICLAZIDE IN HUMAN PLASMA

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ABSTRACT

The objective of this study is to estimate gliclazide in human plasma by both HPLC and HPTLC method. For HPLC method, SunQ C18 column was used as stationary phase and Methanol: 0.1% Formic acid in Water was used as mobile phase in ratio of 70:30 v/v. For HPTLC method, TLC plate precoated with silica gel 60 $F_{254}$was used as stationary phase and mobile phase selected was Toluene: Methanol: Ethyl acetate in ratio of 7:2:1 v/v/v. Simple protein precipitation method was employed as sample pretreatment in both HPLC and HPTLC methods. The detection wavelength set was 228 nm. Molnupiravir and Glipizide were used as internal standard for HPLC and HPTLC method respectively. With optimised HPLC method, Gliclazide eluted at 7.5±0.2 mins and the Rf value found was 0.49±0.06 for HPTLC. Both the methods were found to be linear over the range of 2-10 µg/ml. The r^2 value found for HPLC and HPTLC method was 0.9842 and 0.9835 respectively. Both of the methods were validated for as per USFDA guidelines for bioanalytical method. The developed methods were found to be simple, accurate, precise and economical for estimation of Gliclazide in human plasma.

Keywords: Gliclazide, HPLC, HPTLC, Internal Standard, Bioanalytical

INTRODUCTION

Gliclazide is an oral antihyperglycemic agent which belongs structurally to the group of first-generation sulfonylureas and based on pharmacological efficacy gliclazide considered as second-generation sulfonylurea **[1]**. It is used to treat noninsulin dependent diabetic mellitus **[2]**. It is chemically1-(3,3*a*,4,5,6,6*a*-hexahydro-1*H*-cyclopenta[c]pyrrol-2-yl)3-(4-methylphenyl) sulfonylurea **[3]**.

Gliclazide acts by binding to receptor on β cells of pancreas and causes blocking of ATP sensitive potassium channels. Closure of potassium channels leads to decrease in potassium efflux and polarization of β cells. This in turn cause opening of voltage gated calcium channels and activation of calmodulin. This cause exocytosis of insulin which leads to control in blood glucose level **[1]**.

As per the literature search, there are few LC-MS/MS, UHPLC and HPLC methods reported for estimation of Gliclazide in biological matrix **[4-11]**. There is no report of HPTLC method and comparison study of HPLC and HPTLC method for Gliclazide estimation in human plasma. The aim of this study is to develop and compare HPLC and HPTLC method for estimation of Gliclazide in human plasma.

MATERIALS AND METHODS

Chemicals and Reagents

Gliclazide was obtained as gift sample from Bal Pharma Ltd, Maharashtra, India and Glipizide was obtained as gift sample from Harind Pharmacal Pvt. Ltd, Maharashtra, India. Molnupiravir was obtained as gift sample from Natco Pharmaceuticals, Hyderabad, India. Glipizide and Molnupiravir were employed as internal standards for HPTLC and HPLC respectively. Methanol of HPLC grade was purchased from Thomas Baker Chemicals Pvt. Ltd., Mumbai, India. Ethyl acetate of HPLC grade was purchased from Merck Lifesciences, Pvt. Ltd., Mumbai, India. Other chemicals like Toluene and Formicacid were purchased from Loba Chemie, Pvt. Ltd., Mumbai, India. Pooled plasma was obtained as a gift sample from Sassoon hospital, blood bank, Pune, India.Maharashtra, India.

Instrumentation and Chromatographic conditions

HPLC method

The Jasco HPLC system coupled with UV detector UV-2075 plus, pump PU-2080 plus and operated with Borwin software version 1.50 was used. A SunQ C18 column (4.6mm id ×250 mm L) was used as stationary phase. The mobile phase consistsof methanol and water with 0.1 % formic acid in the ratio of 70:30 v/v. The isocratic elution with flow rate of 1 ml/min was set for analysis. The run time set was 10 mins and retention time of Gliclazide was found to be 7.5 mins. Molnupiravir was used as internal standard and its retention time was found to be 3.2 mins. The 20 µl of sample was analyzed. The detection wavelength set was 228 nm.

HPTLC method

Camag HPTLC system with win CATS software version 1.4.2, was used. The TLC plate precoated with silica gel 60 $F_{254}$was used as stationary phase and mobile phase selected was Toluene: Methanol: Ethyl acetate in ratio of 7:2:1 v/v/v. The plate was allowed to develop upto the distance of 80 mm. The saturation time set was 20 min. The Sample volume of 20µl was applied on TLC plate in the form compact band of 6mm with help of Camag semiautomatic applicator using sample syringe of capacity 100µl.Densitometric scanning was performed at the228 nm. Glipizide was used as internal standard. The Rf values for Gliclazide and Glipizide were found to be 0.49±0.06 and 0.39±0.01 respectively.

Other instruments used were UV- Visible spectrophotometer (Shimadzu UV 1780), Analytical balance (Shimadzu AY 120), Cyclo-mixer (Remi) and centrifuge (Remi R-302), Sonicator (Prama SM15).

The statistical test applied for stability study comparing HPLC and HPTLC was paired t test.

Method development

Selection of analytical wavelength

To a clean calibrated volumetric flask, 10 mg of drug was transferred and it was made to dissolve in methanol and volume was made upto the mark to achieve concentration of 1000µg/ml. From this stock solution further dilution was made to get concentration of 10µg/ml and this solution was scanned over the range of 200-400 nm in a quartz cell. The spectrum indicates 228 nm as λmax.

Selection of Internal standard

Based on the λmax of drug, various internal standards like Eluxadoline, Molnupiravir, Torsemide, Linagliptin, Ramipril, Mesalamine, Glipizide were tried as internal standard. Molnupiravir was selected as internal standard based on resolution and extraction efficiency from plasma for HPLC and Glipizide for HPTLC.

Preparation of stock solution of Gliclazide

To clean and calibrated volumetric flask accurately weighed 10 mg of Gliclazide was transferred and dissolved in methanol. The volume made with same solvent to produce concentration of 1000 µg/ml. Then further dilutions were made from to get final concentration of 20-100µg/ml.

Preparation of Spiked plasma

To a clean and dry test tube 0.8 ml of plasma was taken, to it 0.1 ml of stock solution of Gliclazide (20 to 100µg/ml) and 0.1 ml of Internal standard (500µg/ml) was spiked to get required quality control samples, vortexed to mix well. To the quality control samples 1 ml of methanol was added as protein precipitating agent, mixed by vortexing, centrifuged and supernatant was injected to system.

HPLC method

Optimization of mobile phase

The mobile phase first tried was ACN: water in ratio of 70:30 v/v but retention time was found to be very low. To improve it, various trials were done to the ratio of mobile phase but retention time obtained was very low, less than 3 mins. Then methanol:water (0.1% formic acid) was tried as mobile phase in ratio of 80:20 v/v but still retention time was not acceptable hence ratio was changed to 70:30 v/v to get acceptable retention time.

Preparation of stock solution of Internal standard

The accurately weighed 10 mg of Molnupiravir was transferred to calibrated volumetric flask. This was dissolved in methanol and volume was made upto the mark with methanol to produce the concentration of 1000 µg/ml. It was appropriately diluted to get final dilution of concentration 500µg/ml.

HPTLC method

Selection of Mobile phase

Various mobile phases were tried e.g., Methanol: Chloroform 5:5v/v, Toluene: Ethyl acetate 5:5v/v, Toluene: Methanol 7:3v/v, Toluene: Ethyl acetate: Methanol 7.5: 0.5: 2 v/v/v. Peaks were not shifted from initial position in Methanol: Chloroform 5:5v/v, Toluene: Ethyl acetate 5:5v/v and Double peaks were seen in Toluene: Methanol 7:3 v/v. Toluene: Ethyl acetate: Methanol 7.5: 0.5: 2 v/v/v showed good peaks but no proper resolution was observed between drug and internal standard. Then the ratio of this mobile phase was modified to Toluene: Ethyl acetate: Methanol 7:1:2v/v/v to get well resolved peaks of internal standard and Gliclazide.

Preparation of stock solution of Internal standard

The accurately weighed 10 mg of Glipizide was transferred to calibrated volumetric flask. This was dissolved in methanol and volume was made upto the mark with methanol to produce the concentration of 1000 µg/ml. Further dilution was made from it to get final dilution of concentration 100µg/ml.

Method validation

The method is validated as per USFDA guidelines for bioanalytical methods **[12, 13]**.

Calibration curve

Linearity of method is checked to demonstrate direct proportionality between detector response and concentration of analyte of interest. Different replicates of seven spiked quality control sample were analysed and the peak areas were recorded. The response factor was determined by taking ratio of area of Gliclazide and area of internal standard. Calibration curve was plotted by taking response factor vs concentration (in HPLC) and vs amount spotted (in HPTLC).

Selectivity

The ability of bioanalytical method to give signal only for analyte of interest and not for other interfering components. Specificity of method was demonstrated by analysing blank plasma, Zero plasma and spiked plasma. There should not be any extra or interfering peak at the same Rt and Rf value of Gliclazide for HPLC and HPTLC respectively.

Accuracy

The accuracy of any bioanalytical method is closeness between observed value and true value of concentration. It was determined by analysing quality control samples of different range that is LQC, MQC and HQC. Their peak areas were recorded and % mean accuracy was determined.

Precision

The precision of bioanalytical method expresses its ability to achieve reproducible results between series of measurement. The precision was determined as intermediate precision and repeatability by analysing independent runs of different concentration and the % CV was calculated.

Recovery

The recovery of bioanalytical method expresses the efficiency at which it can recover the analyte from spiked quality control sample using efficient sample pre-treatment process. It is performed by comparing the results obtained from extracted samples at different concentrations with standard solutions.

Stability

The purpose of determining stability is to detect any degradation of analyte occurred during entire process of sample collection, storage, extraction and analysis. It is recommended to determine stability during short term storage, long term storage as well as during freeze thaw cycles. Stability samples are compared with freshly prepared QC samples.

RESULTS

Calibration curve

Considering the pharmacokinetic profile of Gliclazide (Cmax = 2-8 μg/ml) **[14]** the linearity range set was plasma concentration 2-10μg/ml for both HPLC and HPTLC. When concentration (for HPLC) and amount spotted (for HPTLC) was plotted vs response factor the method was found to be linear over the given range for both the HPLC and HPTLC methods. The regression coefficient was found to be 0.9842 for HPLC and 0.9835 for HPTLC **(Table 1)**.

Selectivity

Both the methods were found to be specific as no interfering peaks were observed at the same Rt or Rf of Gliclazide **(Figure 1-6)**.

Accuracy

The methods were found to be accurate as the% mean accuracy at each concentration was found to be in acceptable range as shown in **Table 2**.

Precision

After analysing the samples at different concentration like LQC, MQC and HQC the % CV obtained for each concentration level was found to be with in limit hence method was found to be precise based on result given in **Table 3**.

Recovery

The recovery was determined after comparing the standard samples with spiked plasma and the overall % CV was calculated which was found within limit for both the HPLC and HPTLC methods **(Table 4)**.

Stability

By comparing the peak areas of spiked stability quality control samples with peak areas of freshly prepared quality control samples the % mean stability was calculated and results are summarised in **Table 5**.

Statistical Analysis

Paired t test was used to compare stability results by HPLC and HPTLC. H_o = There is no statistically significant difference between stability results by HPLC and HPTLC. The calculated ‘t value’ is 7.84 and the table‘t value’is 2.262.

Inference - Since, calculated ‘t value’ is greater than tabulated ‘t value’, null hypothesis is rejected. Thus, difference in stability study results by both methods is statistically significant.

Remark - The significant difference between calculated ‘t value’ and table ‘t value’ may be due to variation in pooled plasma samples obtained from blood bank.

Table 1: Calibration curve

Concentration (µg/ml) n=5	HPLC	HPTLC
	Mean response factor ± SD	Mean response factor ± SD
2	0.0468±0.0004	0.269±0.0051
4	0.0956±0.0004	0.506±0.0458
5	0.1237±0.0004	0.584±0.0063
6	0.1522±0.0003	0.614±0.0585
7	0.2096±0.0004	0.723±0.0073
8	0.2264±0.0004	0.816±0.0028
10	0.3104±0.0005	0.939±0.004

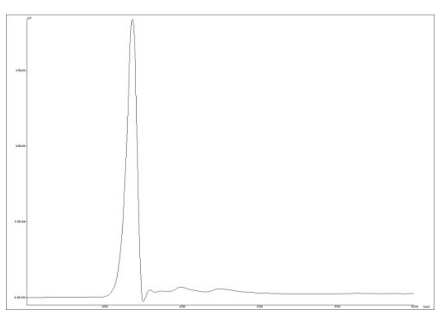

Figure 1: Representative chromatogram of Blank plasma

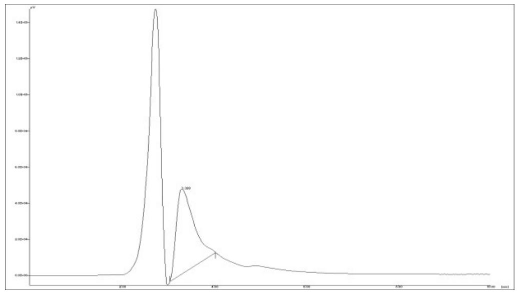

Figure 2: Representative chromatogram of Zero plasma (plasma spiked using IS)

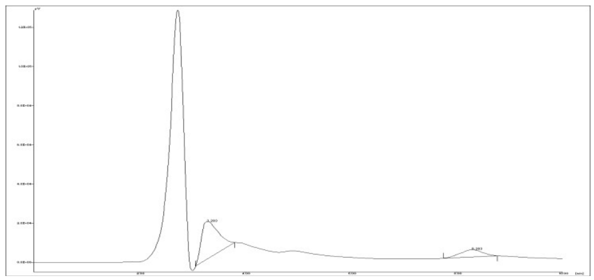

Figure 3: Representative chromatogram of Spiked plasma (Gliclazide Rt= 7.5±0.2 mins + Internal standard Rt=3.2 ±0.3mins)

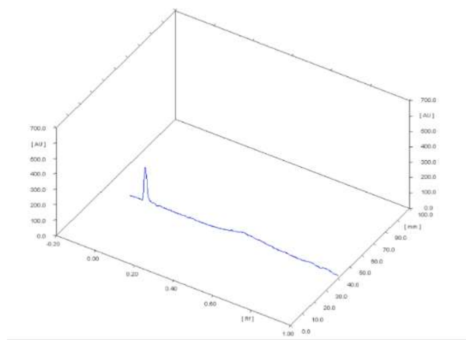

Figure 4: Representative densitogram of Blank plasma

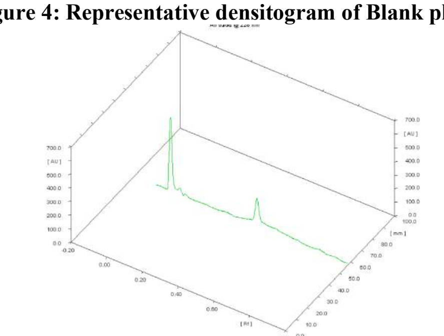

Figure 5: Representative densitogram of Zero plasma (plasma spiked using IS)

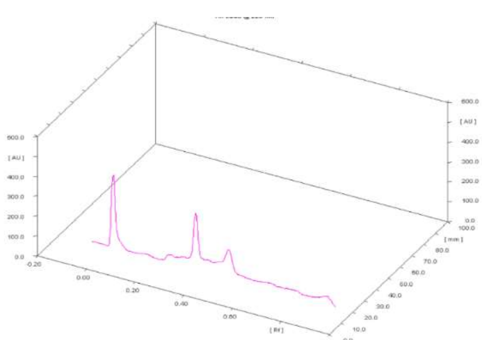

Figure 6: Representative densitogram of Spiked plasma (Gliclazide Rf= 0.49±0.06 + IS Rf= 0.39±0.01)

Table 2: Accuracy

Concentration n=5	HPLC			Concentration n=5	HPTLC		
	Mean±SD	% CV	% Mean accuracy		Mean±SD	% CV	% Mean accuracy
LQC (4µg/ml)	3.90±0.028	0.73	97.5	LQC (80ng/band)	78.03±4.036	5.17	97.53
MQC(6µg/ml)	5.61±0.003	0.06	93.59	MQC(120ng/band)	110.82±4.131	3.72	92.35
HQC (8µg/ml)	7.86±0.002	0.03	98.26	HQC(160ng/band)	170.40±5.212	3.05	106.5

Table 3: Precision

HPLC				
	Concentration	LQC (4µg/ml)	MQC (6µg/ml)	HQC (8 µg/ml)
Intermediate precision	Mean Conc.±SD	3.88±0.0189	5.60±0.0067	7.85±0.0062
	% CV	0.48	0.12	0.11
Repeatability	Mean Conc.±SD	3.89±0.0372	5.60±0.0064	7.86±0.0084
	% CV	0.95	0.11	0.10
HPTLC				
	Concentration	LQC (80ng/band)	MQC (120ng/band)	HQC (160ng/band)
Intermediate precision	Mean Conc.±SD	81.48±5.87	111.09±4.47	173.68±5.33
	% CV	7.21	4.91	2.92
Repeatability	Mean Conc.±SD	82.90±6.55	113.24±4.94	170.40±5.21
	% CV	7.90	4.36	3.05

Table 4: Recovery

Conc. (µg/ml)	% Mean Recovery (HPLC)	% Overall mean recovery± %CV (HPLC)	% Mean Recovery (HPTLC)	% Overall mean recovery± %CV (HPTLC)
2	63.41	63.81±0.75	65.15	65.72±2.93
4	65.31		66.78	
5	62.31		66.30	
6	61.40		66.51	
7	63.70		62.09	
8	65.80		64.52	
10	64.80		68.72	

Table 5: Stability

Sr. No.	Stability		HPLC		HPTLC	
			% Mean Stability	% CV	% Mean Stability	% CV
1	Freeze thaw stability (Three cycles)	LQC	97.4 %	0.06	87.14	2.45
		HQC	98.18 %	0.02	93.77	1.81
2	Short term stability (for 4hrs. at RT)	LQC	97.3 %	0.06	86.83	3.98
		HQC	98.16 %	0.03	92.19	0.82
3	Long term stability (for 14 days at 4°C)	LQC	93.46 %	0.02	86.83	3.98
		HQC	96.59%	0.21	92.54	2.29
4	Stock solution stability (for 5 days)	MQC	96.45 %	0.06	92.38	1.61
		IS	98.7%	0.03	93.68	3.56
5	Post preparative(for 5hrs. at RT)	MQC	98.2%	0.52	92.41	1.59
		IS	99.32%	0.05	89.83	4.17

DISCUSSION

Various methods reported for estimation of Gliclazide in biological matrix are based on HPLC, LC-MS, UPLC. The methods reported involve use of complex pre-treatment technique involving multiple steps with use of various chemicals example 1M hydrochloric acid, dichloromethane, etc. **[4-11]**. In this method very simple protein precipitation with methanol was used as pre-treatment technique, which is economic and time saving. The reported methods utilize complex mobile phase which require adjustment of pH and gradient mode of elution **[4-11]**, also column temperature was required to be maintained for some methods[9-11]whereas, this method involve use of very simple mobile phase with isocratic elution.

CONCLUSION

The developed HPLC and HPTLC methods were validated for various parameters like selectivity, calibration curve, accuracy, precision, recovery and stability. Both the methods found to be simple, economic, precise and accurate. For both methods simple protein precipitation method was adopted for sample pre-treatment.

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