STUDIES IN DEVELOPMENT, VALIDATION AND APPLICATION OF ANALYTICAL RP-HPLC METHOD FOR QUANTITATIVE ESTIMATION OF GLIPIZIDE FROM ITS BULK AND FORMULATION

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ABSTRACT

A RP-HPLC method has been developed for the estimation of glipizide (GLP). The proposed method is based on the separation of the drug in reversed-phase mode using BDS HYPERSIL C18 (4.6 mmø×250 mm) analytical column, mobile phase methanol:water 70:30 V/V, at the flow rate of 1.0 mL min^{-1} and detection wavelength 222 nm. GLP was well resolved and retained at t = 3.86 minutes. This RP-HPLC method was validated as per the recommendations of ICH Revised Q2(R1) guidelines of analytical method validation, in order to prove that the new analytical method meets the reliability characteristics. The method characteristics showed the capacity of an analytical method to keep, all over the time, the basic standards for validation: selectivity, linearity, precision, accuracy and sensitivity. The method was found linear over the range 1-7 µg mL^{-1}. The LOD and LOQ were 0.5281 and 1.761 µg mL^{-1} for GLP. The validated method was successfully used for quantitative estimation(assay) of GLP from in-house formulation and marketed formulations.

INTRODUCTION

Analytical method development and validation is an important aspect for the discovery, development and manufacture of pharmaceuticals so as to ensure the identity, purity, potency and performance of the drug products. Study of physicochemical properties of drug, selection of method parameters, sample preparation and optimization are the steps involved in development of RP-HPLC method. The main goal in developing a RP-HPLC method is to separate and to quantify the analyte of interest. RP-HPLC method has the ability to handle compounds with diverse polarity and molecular mass.

Glipizide (GLP) is an oral anti-diabetic drug. Diabetes is a chronic and potentially fatal disease which affects the whole human body. Billions of individuals have diabetes, and also the worrying fact is that a high percentage of affected patient aren't even aware that they are diabetic. GLP belongs to the sulfonyl urea class that controls blood glucose levels by helping pancreas to secrete insulin which is used together with diet and exercise to improve blood glucose control in adults with type II diabetes mellitus[1].

The basic purpose behind proposed method development was to have a robust analytical RP-HPLC method for estimation of GLP from our inhouse nanofiber formulation and single - component formulations, so that it can be used effectively for its routine quality control analysis, stability assay and content uniformity assay[2]. A new, rapid, economical RP-HPLC method described here for the quantification of the glipizide (GLP) in bulk, inhouse formulation and marketed formulation. The objective of the proposed work was to develop reliable analytical method and validate the same as per the recommendations of ICH Revised Q2 (R1) guidelines of analytical validation.

MATERIALS AND METHODS

Materials

Working standard of pharmaceutical grade glipizide was obtained as a generous gift sample from local API manufacturing unit. Marketed formulation of tablets containing 5 mg GLP were procured from local pharmacy shop. Methanol and acetonitrile were purchased from S.D. Fine Chemicals Ltd., Mumbai, India.

Method

Reversed-Phase High Performance Liquid Chromatographic method (RP- HPLC)

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Instrument used

The HPLC system; Make & Model: Shimadzu UFLC series employed with LAB SOLUTION software (Version 6.72 SP1).

Experimental

Analytical method development

Study of physicochemical properties of analyte (GLP)

Glipizide (GLP)

Chemically known as 1-cyclohexyl-3-[[4-[2-[[(5-methylpyrazin-2-yl) carbonyl] amino] phenyl]sulphomyl] urea (Fig.1). glipizide stimulates the beta cells of pancreatic islets of Langerhans insulin response, which cause more insulin to be released in response to glucose than would be without glipizide ingestion. Glipizide partially blocks potassium channels among beta cells of pancreatic islets which will cause depolarization of the cells. This results in the opening of voltage-gated calcium channels and the resulting calcium influx encourages insulin release from beta cells[3,4]. Physicochemical properties of glipizide are given in Table I[35,36].

Preparation of standard stock and working solutions for RP-HPLC method development studies

10 mg quantity of GLP was weighed accurately and transferred into 10 mL volumetric flask labelled as 'Standard/Stock solution A' and volume was made up with methanol.

Working solution (Solution B) was prepared from standard solution. 1 mL from stock solution was pipetted out and transfer to 10 mL capacity volumetric flask and volume was made up to the mark with mobile phase. Solution B was diluted 10 times with mobile phase and used as sample solution (10 µg mL^{-1}) during experimental trials of method development studies.

Selection of detection wavelength

A UV spectrum of 10 ppm solution of GLP was generated by scanning over the range of 200-400 nm using double beam UV-visible spectrophotometer (Shimadzu UV1801). The wavelength corresponding to higher absorbance analysed was selected as detection wavelength for estimation of GLP by UV detector in HPLC system (Shimadzu UFLC series).

Optimisation of chromatographic conditions

Many preliminary trials were carried out for selection and optimisation of stationary phase, mobile phase,

Table I: Physicochemical properties of glipizide (GLP) BP

Property	Glipizide (GLP)
Chemical name	1-cyclohexyl-3-[[4-[2-[[(5-methylpyrazin-2-yl)carbonyl]amino] phenyl]sulphomyl]urea
Molecular formula	$C_{21}H_{27}N_5O_4S$
Molecular weight	445.5 g mol^{-1}
Therapeutic category	Inhibition of ATP-dependent potassium channels (sulfonylurea); treatment of diabetes mellitus
State	White or almost white, crystalline powder
Melting point	208-209 ºC
Log P	1.91
Solubility	Practically insoluble in water, very slightly soluble in methylene chloride and acetone, practically insoluble in ethanol (96 %). It dissolves in dilute solutions of alkali hydroxides
pKa	5.9
λmax	At 226 nm and 274 nm
Bioavailability	100 % (regular formulation) 90 % (extended release)
Half life	2-5 h

flow rate, injection volume and column temperature. Experimental trials for mobile phase composition are tabulated in Table II.

Analytical method validation

Performance characteristics of analytical HPLC method were statistically validated as per ICH revised Q2 (R1) guideline for analytical method validation. Method validation protocol is given in Table III[34].

Studies for establishment of analytical method validation parameters

Accuracy

Accuracy experiments were performed as per method validation protocol (Table III) by conducting recovery studies of known added amount of GLP over three concentration levels viz. 80 %, 100 % and 120 %. Accuracy studies were also performed using marketed formulations.

Table II: Trials for optimization of mobile phase composition for estimation of GLP

Mobile phase components	Compositions (V/V)
DDW (pH 3.5- 4.0): MeOH: ACN	80:10:10
MeOH: DDW (pH 3.0- 3.5): ACN	85:10:05
MeOH: ACN: DDW (pH 3.0- 4.0)	80:20:10
DDW: CAN	80:20
DDW: CAN	70:30
ACN: DDW	70:30
ACN: DDW	80:20
DDW: MeOH	70:30

DDW= Double distilled water; CAN= Cerium ammonium nitrate

Precision

Experimental determinations for establishment of repeatability and reproducibility of analytical method were carried out as per method validation protocol (Table III).

Intra-day precision

Three replicate analyses were performed at three different concentration levels including low, mid and high concentrations. The concentration levels were coded as LQC, MQC and HQC for GLP respectively within the same day at three different times (1, 2, 3).

Specificity

To determine specificity, chromatograms were obtained for blank (mobile phase), GLP, placebo, marketed formulation and in-house formulation. All chromatograms were analysed and evaluated for any interference of mobile phase components, sample mixture and formulation additive with analyte of interest.

Linearity and range

Experimental determinations were carried out on seven serial dilutions of working solution (Solution B)

Table III: Protocol for analytical RP-HPLC method validation[34]

Parameter	Purpose	Recommendation as per ICH revised Q2(R1) guidelines		Acceptable criteria
Accuracy	Assay (Content/potency): Recovery studies	Accuracy was established across the specified range of analytical procedure by adding known quantities of analyte to the synthetic mixture of drug product components and to the combined dosage form. As per ICH, accuracy should be assessed using a minimum of 9 determinations over a minimum of three concentration levels covering the specified range i.e., 3 concentration levels in triplicate. (e.g., 3 Concentrations/3 replicates each). Accuracy of the method is reported as percent recovery of known added amount of analyte in sample.		98-102 % Recovery of known added amount of analyte
Precision	Assay (Content/potency): Repeatability and Reproducibility	**Repeatability**	**Intermediate Precision**	
		Repeatability was assessed by using minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentration/3 replicates each)	Intermediate precision was established to study the effects of random events i.e., days, on the precision of the analytical procedure. Intraday and interday precision studies were performed by taking 9 determinations of 3 concentration/3 replicates each, at 3 times in a same day and on 3 different days, respectively.	RSD≤ 2%

		Precision is reported as relative standard deviation (coefficient of variation) for each type of precision investigation.	
Specificity	Identification, testing for impurities and assay (content/potency)	As per ICH revised Q2 (R1), Specificity should be carried out to ensure identification tests, the determination impurity and assay.	No interference in analyte determination
Linearity	Testing for impurities and assay (content/potency): To check linear relationship of performed concentration	As per ICH, for the establishment of linearity, a minimum of 5 concentrations are approved. Linearity is reported by the value of the correlation coefficient, y-intercept, and slope of the regression line along with a plot of the data.	$R^2 \geq 0.99$
Detection limit and quantification limit	Testing for impurities sensitivity of analytical method and assay: Determination of minimum detectable and quantifiable concentration of analyte solution.	Detection limit and quantification limit is determined based on the standard deviation of the response and the slope. **LOD**$=3.3 \times \sigma/S$ **LOQ**$=10 \times \sigma/S$ σ = Standard deviation of response estimated based on the calibration curve. S = Slope of the calibration curve.	NA
Robustness	To establish reliability of analytical method.	Robustness was evaluated for proving the reliability of an analytical method with respect to deliberate variations in method parameters. To establish robustness of analytical method following factors were studied: ➢ Influence of variations in pH of a mobile phase. Or Influence of variations in mobile phase composition ➢ Flow rate ➢ Detection wavelength ➢ Injection volume	Pooled RSD$\leq$3% in every change item

prepared using mobile phase as diluting solvent. It was evaluated across the range of 1- 7 ppm for GLP. Experiments were performed in triplicate as per method validation protocol (Table III), linear relationship was checked by plotting average peak areas against sample concentrations.

Limit of detection and limit of quantification (LOD & LOQ)

Values for detection limit and quantification limit were determined based on the standard deviation of the response and the slop of regression line.

Robustness

To evaluate and check robustness of the newly developed analytical RP-HPLC method, deliberate
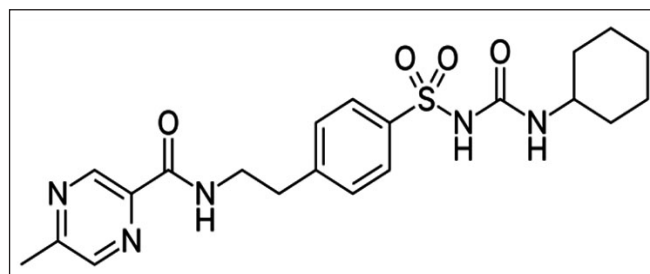

Fig. 1: Chemical structure of glipizide (GLP)

changes were made in critical method parameters as listed in Table IV.

Experimental studies for application of newly developed and validated RP-HPLC method for routine sample analysis of marketed and in-house formulations

Content uniformity assay

Content uniformity assay was performed on market tablet formulation and in-house formulation (MDF) containing GLP. 20 tablets of GLP were accurately weighed and powdered for assay of analyte and for estimation from in-house formulation. The weight of powder equivalent to label claim of GLP was transferred into individual 100 mL volumetric flask and dissolved completely in methanol with the aid of sonication for 10 mins (solution A). The solution A was filtered through 0.45 µm filter paper and dilutions were made up using mobile phase. Then these solutions were filtered through 0.45 µ syringe filter and 20 µL of this filtered solution was injected into HPLC column and corresponding chromatograms were recorded. The data was statistically processed for calculation of percent drug content of the stated amount. In-house formulation (MDF of GLP) was also analysed using same method.

RESULTS AND DISCUSSION

Analytical method development

Detection of wavelength

The wavelength of 222 nm was selected as the detection wavelength for chromatographic determination of GLP, refer UV absorbance a spectrum for 10 ppm solution of GLP Fig. 2.

Optimization of chromatographic condition

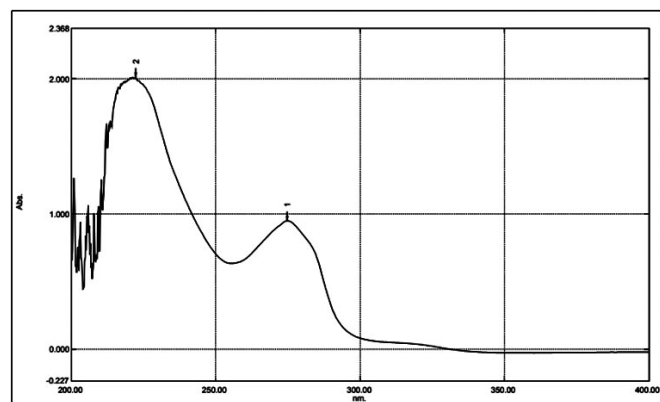According to the referred scientific analytical literature, it is found that when GLP is estimated or

Table IV: Robustness studies- variations in method parameters and levels of variation

Method parameter and variation	Level of variation	Actual value of method parameters after changes
Injection volume (20 ± 10 µL)	-10 µL	10 µL
	+10 µL	30 µL
	+30 µL	50 µL
	+70 µL	100 µL
Flow Rate (1.0 ± 0.5 mL min^{-1})	-0.5 mL min^{-1}	1.5 mL min^{-1}
	+0.5 mL min^{-1}	2.0 mL min^{-1}
Wavelength (222 ± 10 nm)	-10 nm	212 nm
	+10 nm	232 nm
Mobile Phase (70:30 V/V)	-	90:10 (V/V)
	-	50:50 (V/V)

analysed by HPLC from single component formulation or bulk this gets separated and retained on Octadecyl silane (ODS) C-18 HPLC columns. Thus, in order to get better separation optimum resolution and sharp peak in chromatogram, C18 column [BDS HYPERSIL C18 (4.6 mmø×250 mm) analytical column] was selected.
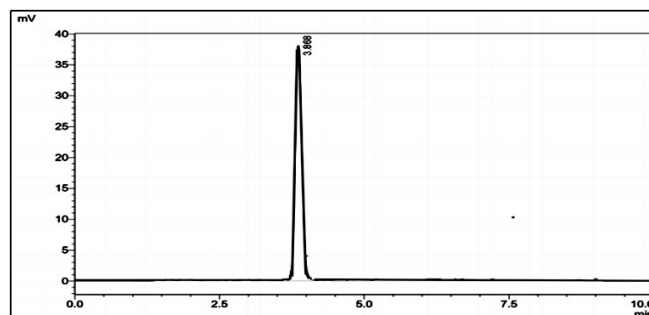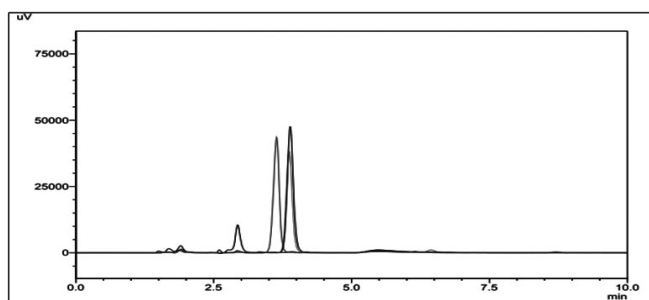

Fig. 3: Representative RP-HPLC chromatogram of GLP

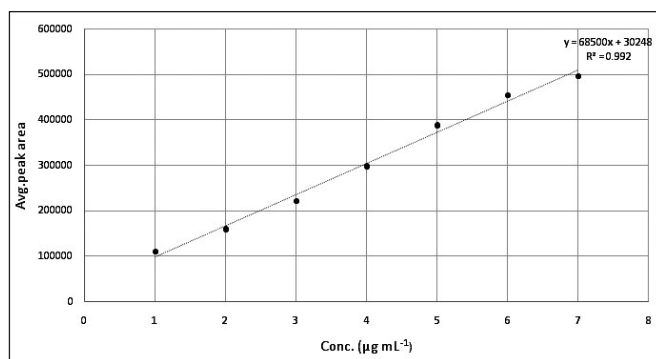**Fig. 4: Overlain chromatogram of blank (mobile phase), GLP, placebo and formulation**

Fig. 2: UV spectrum of GLP

Fig. 5: Linearity- calibration plot for GLP

Plot annotation: $y = 68500x + 30248$, $R^2 = 0.992$

Table V: Optimized chromatographic condition

Mobile phase	MeOH: DDW (70:30)
Stationary phase	BDS HYPERSIL C18 (4.6mm ø×250mm) analytical column
Flow rate	1.0 mL min^{-1}
Detection wavelength	222 nm
Injection volume	20 µL

Experiments were designed and experimental trials were carried out for selection of mobile phase; some of these are tabulated in Table II.

During experimental trials of RP-HPLC method development, different flow rates varying from 0.5 to 1.5 mL min^{-1} as well as variable injection volumes in the range of 10 µL to 50 µL were tried. At the end of all experimental trials, on the basis of results and experimental observations with respect to response, resolution, peak sharpness, and peak symmetry chromatographic conditions were

Table VI: Accuracy - recovery studies on bulk drug

Drug	% Level	Concentration before spiking (µg mL^{-1})	Total concentration after spiking (µg mL^{-1})	Amount Recovered (µg mL^{-1})	% Recovery	Inference
			Observation			
GLP	80	3	5.4	5.15	95.51	Acceptable recovery hence accurate
	100	3	6	6.01	100.01	
	120	3	6.6	6.79	102.91	

Table VII: Accuracy - Recovery studies on tablet formulation

Drug	% Level	Concentration before spiking (µg mL^{-1})	Total concentration after spiking (µg mL^{-1})	Amount recovered (µg mL^{-1})	% Recovery	Inference
			Observation			
GLP	80	3	5.4	5.13	95.01	Acceptable recovery hence accurate
	100	3	6	5.90	98.45	
	120	3	6.6	6.62	100.31	

Table VIII: Intra-day precision results

		Observation			Inference
		GLP			
Level		LQC	MQC	HQC	
Amount (µg mL^{-1})		1	3	5	
Peak Area	1	93993	218608	654797	Acceptable %RSD, hence precise
	2	94495	217622	652839	
	3	93267	220707	655666	
Average Peak Area		93918.33	218979	654434	
S.D.		617.3956	1575.607	1448.036	
% RSD		0.657375	0.719524	0.221265	

Table IX: Inter-day precision results

		Observation			Inference
		GLP			
Level		LQC	MQC	HQC	
Amount (µg mL⁻¹)		1	3	5	
Peak Area	1	91012	217136	333496	Acceptable %RSD, hence precise
	2	88166	215746	334531	
	3	88059	218996	327948	
Average Peak Area		89079	217292.7	331991.7	
S.D.		1674.882	1630.654	3539.949	
% RSD		1.880221	0.750441	1.066276	

Table X: Linear regression data of calibration plot

Drug	Range	R²	y- intercept	Slope
GLP	1- 7 µg mL⁻¹	0.9923	30248	68500

Table XI: Calculated value of LOD and LOQ

	GLP
LOD	0.58126 µg mL⁻¹
LOQ	1.761395 µg mL⁻¹

Table XII: Robustness - effect on retention time and response by variation in mobile phase composition, flow rate and injection volume

Method parameter and variation	Level of variation	Actual values of method parameters after changes	GLP	
			%RSD of recorded response (Area)	Retention time (Min)
Injection volume (20 µL ± 10 µL)	-10	10 µL	0.057098	4.05
	+10	30 µL	0.384793	4.12
	+30	50 µL	1.23801	4.15
	+70	100 µL	0.378997	4.10
Flow Rate (1± 0.5 mL min⁻¹)	-0.5	0.5 mL min⁻¹	0.500492	7.79
	+0.5	1.5 mL min⁻¹	0.242571	3.25
Wavelength (222± 10 nm)	-10	212 nm	1.825352	3.15
	+10	232 nm	0.047716	3.62
Mobile phase (70:30 V/V)	-	90:10	1.43561	4.03
		50:50	1.604282	4.01

Table XIII: Calculated values of drug content uniformity assay for marketed and in-house formulation of GLP

Drug	Formulation	Label claim (mg)	Amount of drug (mg)	% Drug content of the label claim
GLP	Marketed	5 mg tab^{-1}	4.33 mg tab^{-1}	86.60 %
Nanofiber film	In-house formulation	5 mg film^{-1}	4.38 mg film^{-1}	87.78 %

finalised. Table V gives optimal chromatographic conditions.

Chromatograms obtained using this optimised chromatographic condition showed that drug GLP was well resolved and retained at 3.86 minutes. Representative chromatogram of GLP is shown in Fig. 3.

Analytical method validation

Studies for establishment of analytical method validation parameters

Accuracy

Accuracy of the method is reported as percent recovery of known added amount of analyte in sample. Experimental observations and results are tabulated in Table VI.

Recovery studies were also performed using tablets containing GLP. The marketed tablets containing GLP were triturated and sample solution was prepared with drug concentration of 3 µg mL^{-1}. To each of these solutions of GLP, a known amount of drug was added. The 80 %, 100 %, and 120 % of 3 µg mL^{-1} concentrations were added, respectively, in each flask and dilutions were carried out with mobile phase and injected for RP-HPLC analysis. Experimental observations and results are tabulated in Table VII.

Precision

Percent RSD values for both intraday and inter-day precision were found within acceptable limit as mentioned in validation protocol. Experimental observations and results are tabulated in Tables VIII and IX.

Specificity

Separate chromatograms were obtained for blank (mobile phase), GLP, placebo and formulation. Overlain chromatogram of mobile phase (blank), analyte (GLP), in- house formulation (MDF) and placebo are shown in Fig. 4. Overlain chromatogram ensures the method is selective and specific for analyte of interest.

Linearity

The results of linearity studies reflected that the RP-HPLC method for estimation of GLP was found linear across the range of 1- 7 µg mL^{-1}. The linearity plot of GLP is given in Fig. 5. The values of correlation coefficient, y intercept and slope of regression line are shown in Table X.

Limit of detection and limit of quantification (LOD & LOQ)

The calculated values of limit of detection (LOD) and limit of quantitation (LOQ) for GLP are shown in Table XI.

Robustness

To determine robustness of analytical HPLC method, changes in retention time and response were recorded. Method was found to be reliable and robust, since method performance (retention time and response) is not much affected by deliberate variations in mobile phase composition, injection volume, flow rate and detection wavelength. The results obtained are tabulated in Table XII.

Application of newly developed and validated RP-HPLC method for routine sample analysis of marketed and in-house formulations

Content uniformity assay

The method was conveniently adopted for stability analysis, content uniformity assay for formulation containing mouth dissolving film of GLP. So, we propose that the method will be useful for quality control (QC) analysis of single component formulation of the drug. The results are tabulated in Table XIII.

CONCLUSION

An analytical RP-HPLC method for quantitative estimation and content uniformity assay of glipizide (GLP) from its bulk, and formulations was successfully developed and statistically validated.

Validation studies were performed as per the in-house validation protocol developed as per ICH revised Q2 (R1) guidelines in order to prove that the new analytical method, meets the reliability characteristics. Validation studies proved that newly developed RP-HPLC method is specific, accurate, precise and robust.

The newly developed and validated RP-HPLC method was successfully applied for quantitative estimation of GLP from marketed formulation and content uniformity assay of in-house formulation containing glipizide mouth dissolving formulation (Nanofiber).

Results and corresponding data obtained from all experimental studies indicated that the proposed method is suitable for the estimation of GLP in bulk and in pharmaceutical formulations[5-33].

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