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STABILITY INDICATING METHOD EVALUATION AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF GLIMEPIRIDE, METFORMIN AND VOGLIBOSE IN ORAL DOSAGE FORM USING LCMS

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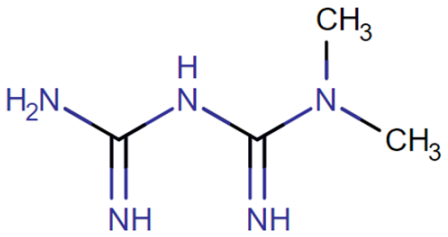
ABSTRACT
A specific, precise, accurate ultra pressure liquid chromatography (UPLC) method is developed for estimation of Glimepiride + Metformin + Voglibose in bulk drug and market dosage form. The method employed, with Hypersil C18 (100 mm x 2.1 mm, 1.7 µm) in a gradient mode, with mobile phase of Acetonitrile and Methanol in the ratio of 68:32%v/v. The flow rate was 1.0 ml/min and effluent was monitored at 260 nm. The method was validated in terms of linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) etc. in accordance with ICH guidelines. Linear regression analysis data for the calibration plot showed that there was good linear relationship between response and concentration in the range of 20- 100 µg/ml respectively. The LOD and LOQ values for were found to be 0.2099 (µg/ml) and 0.6362 (µg/ml) respectively. No chromatographic interference from excipients and degradants were found. The proposed method was successfully used for estimation of Glimepiride + Metformin + Voglibose in market dosage form.

KEYWORDS: Metformin, Glimepiride, Voglibose, oral dosage form, UPLC Stability indicating method.

INTRODUCTION
Glimepiride is indicated to treat type 2 diabetes mellitus; its mode of action is to increase insulin secretion by the pancreas. However, it requires adequate insulin synthesis as prerequisite to treat appropriately. It is not used for type 1 diabetes because in type 1 diabetes the pancreas is not able to produce insulin.



"Fig. 1: "Molecular Structure of Metformin, 1-carbamimidamido-N,N-dimethylmethanimidamide.

Therapeutic category	Antidiabetic drug
CAS Registry number	93479-97-1
Chemical name	*3-ethyl-4-methyl-2-oxo-N-(2-{4-[({[(1r,4r)-4-methylcyclohexyl]-C-hydroxycarbonimidoyl}amino)sulfonyl]phenyl}ethyl)-2,5-dihydro-1H-pyrrole-1-carboximidic acid*
Molecular formula	$C_{24}H_{34}N_4O_5S$
Molecular Weight	490.62
Solubility	Partly miscible in water
pka	2.23
λ_{max}	231 nm
Pharmacology	Glimepiride is indicated for the management of type 2 diabetes in adults as an adjunct to diet and exercise to improve glycemic control as monotherapy. It may also be indicated for use in combination with metformin or insulin to lower blood glucose in patients with type 2 diabetes whose high blood sugar levels cannot be controlled by diet and exercise in conjunction with an oral hypoglycemic (a drug used to lower blood sugar levels) agent alone

Metformin is used with a proper diet and exercise program and possibly with other medications to control high blood sugar. It is used in patients with type 2 diabetes. Controlling high blood sugar helps prevent kidney damage, blindness, nerve problems, loss of limbs, and sexual function problems.

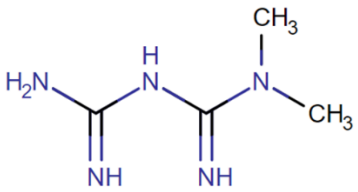


Fig. 2: Molecular Structure of Metformin, 1-carbamimidamido-N,N-dimethylmethanimidamide.

Therapeutic category	Antidiabetic drug
CAS Registry number	657-24-9
Chemical name	1-carbamimidamido-N,N-dimethylmethanimidamide
Molecular formula	$C_4H_{11}N_5$
Molecular Weight	129.163
Solubility	Soluble in 10mL of water
pka	12.4
λ_{max}	230 nm
Pharmacology	Metformin is indicated as an adjunct to diet and exercise to increase glycemic control in adults and pediatric patients 10 years of age and older diagnosed with type 2 diabetes mellitus

Voglibose is an alpha-glucosidase inhibitor used for lowering post-prandial blood glucose levels in people with diabetes mellitus. Voglibose delays the absorption of glucose thereby reducing the risk of macrovascular complications.

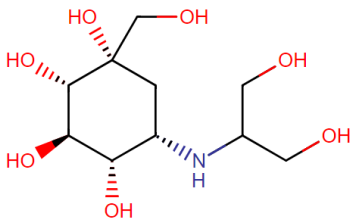


Fig. 3: Molecular Structure of Voglibose, (1S,2S,3R,4S,5S)-5-[(1,3-dihydroxypropan-2-yl)amino]-1-(hydroxymethyl) cyclohexane-1,2,3,4-tetrol.

Therapeutic category	Antidiabetic drug
CAS Registry number	83480-29-9
Chemical name	*(1S,2S,3R,4S,5S)-5-[(1,3-dihydroxypropan-2-yl)amino]-1-(hydroxymethyl)cyclohexane-1,2,3,4-tetrol*
Molecular formula	$C_{10}H_{21}NO_7$
Molecular Weight	267.276
Solubility	"190.0 mg/mL
pka	12.46
λ_{max}	242 nm
Pharmacology	For the treatment of diabetes. It is specifically used for lowering post-prandial blood glucose levels thereby reducing the risk of macrovascular complications.

Validation of Analytical Methods (USP/ICH)

Method validation, according to the United States Pharmacopeia (USP), is performed to ensure that an analytical methodology is accurate, specific, reproducible, and rugged over the specified range that an analyte will be analyzed. Regulated laboratories must perform method validation in order to be in compliance with FDA regulations. In a 1987 guideline (Guideline for Submitting Samples and Analytical Data for Methods Validation), the FDA designated the specifications in the current edition of the USP as those legally recognized when determining compliance with the Federal Food, Drug and Cosmetic Act can be referred to as the "eight steps of method validation".

EXPERIMENTAL

MATERIALS

EQUIPMENTS	SOURCE
Ultra Pressure Liquid Chromatography (UPLC)	Acquity UPLC Systems, Waters Laboratories
Electrospray ionization and MS-MS	Mass Spectrometer PE Sciex Model: API 3000
Chromatographic data software	Empower
Column	C18 column (250 ×4.6 mm id)—ACE Generix
Detector	PDA
Injector	Automated
Electronic Balance	Eagle
Sonicator	Band Line Sonerex
p^H Meter	Lab India p^H meter

METHODOLOGY

Method Validation

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc. The described method extensively validated in terms of specificity, system suitability, linearity, accuracy, precision, limit of detection, limit of quantification and robustness.

- **Forced degradation studies of our selected pharmaceutical drugs**

In order to establish the analytical method for a stability indicating method, the drugs are subjected to various stress conditions to conduct forced degradation studies. Stress studies were carried out under the conditions of acid/base hydrolysis, oxidation, reduction, in accordance with ICH Q1A (R2). Several trials with different severity of each stressed condition are to be conducted, so that upto 10-30% degradation is to be achieved.

RESULTS

Preparation of Standard Stock Solution

Preparation of Diluent

In order to achieve the separation under the optimized conditions after experimental trials that can be summarized. Stationary phase like Hypersil C18 (100 mm x 2.1 mm, 1.7 µm) column was most suitable one, since it produced symmetrical peaks with high resolution and a very good sensitivity and with good resolution. The flow rate was maintained 0.5 mL min-1 shows good resolution. The PDA detector response of Glimepiride + Metformin + Voglibose was studied and the best wavelength was found to be 226 nm showing highest sensitivity.

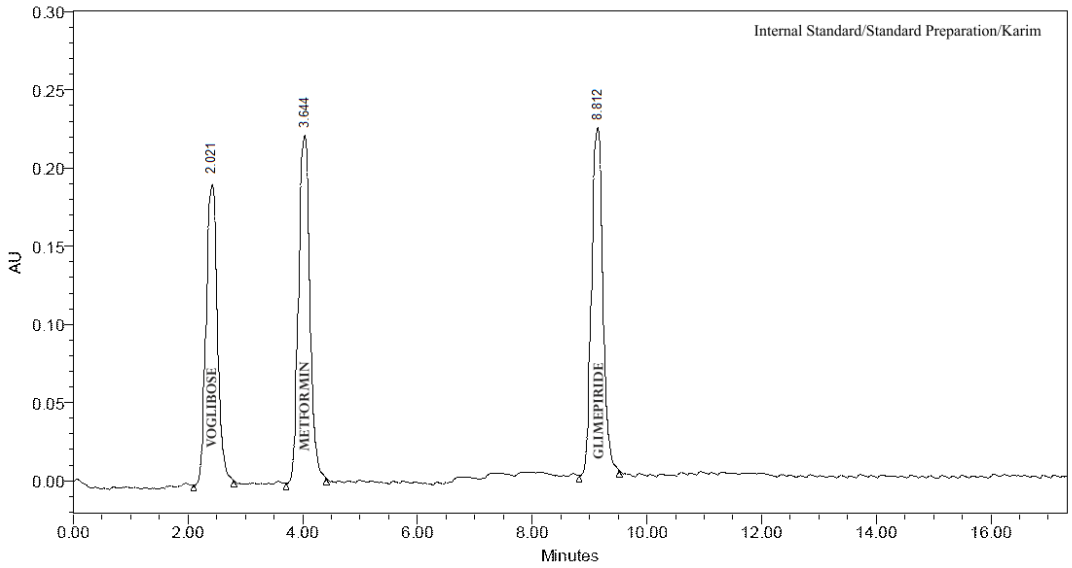
The mixture of two solutions Acetonitrile and Methanol in the ratio of 68:32%v/v with gradient programming was used as mobile phase at 0.5mL/min was found to be an appropriate mobile phase for separation of Glimepiride + Metformin + Voglibose. The column was maintained at ambient temperature.

Preparation of internal standard solution

Weighed accurately about 10 mg of Quinine sulphate working standard and transfer to 100 ml volumetric flask, add 50 ml of mobile phase and sonicate to dissolve it completely and then volume was made up to the mark with mobile phase to get 100 µg/ml of standard stock solution of working standard. Then it was ultrasonicated for 10 minutes and filtered through 0.20 µ membrane filter.

Preparation of Glimepiride + Metformin + Voglibose standard solution

Weighed accurately about 10 mg of Glimepiride + Metformin + Voglibose and transfer to 100 ml volumetric flask, add 50 ml of mobile phase and sonicate to dissolve it completely and then volume was made up to the mark with mobile phase to get 100 µg/ml of standard stock solution of working standard. Then it was ultrasonicated for 10 minutes and filtered through 0.20 µ membrane filter.



Chromatogram: Chromatogram of standard preparation of Glimepiride + Metformin + Voglibose

Accuracy

Glimepiride						
Level %	**Amount added (µg/ml)**	**Amount found (µg/ml)**	**% Recovery**	**Mean recovery (%)**	**Std.Dev**	**% RSD**
50	02.25	02.23	99.11	99.39%	0.24846	0.25%
100	04.50	04.48	99.53			
150	06.75	06.72	99.55			

Table No: 193. Results of Accuracy Study (Glimepiride + Metformin + Voglibose)

Metformin						
Level %	**Amount added (µg/ml)**	**Amount found (µg/ml)**	**% Recovery**	**Mean recovery (%)**	**Std.Dev**	**% RSD**
50	02.45	02.33	95.10	96.49%	0.3432	0.45%
100	04.75	04.54	95.57			
150	06.80	06.72	98.82			

Voglibose						
Level %	**Amount added (µg/ml)**	**Amount found (µg/ml)**	**% Recovery**	**Mean recovery (%)**	**Std.Dev**	**% RSD**
50	02.33	02.21	94.84	97.76%	0.3586	0.35%
100	04.30	04.28	99.53			
150	06.50	06.43	98.92			

System Precision

Procedure

The parameters, retention time (RT), theoretical plates (N), tailing factor (T), peak asymmetry (As) and repeatability were evaluated at a concentration of 4 µg/mL (Glimepiride + Metformin + Voglibose)."

Parameters	**Glimepiride**	**Metformin**	**Voglibose**
Retention time (min) ± % RSD	8.846 ± 0.12	3.587 ± 0.12	2.176 ± 0.12
Theoretical plates ± % RSD	4673.63 ± 0.50	4455.75 ± 0.50	4055.55 ± 0.50
Asymmetry ± % RSD	1.08 ± 0.05	1.05± 0.05	1.10± 0.05
Repeatability (% RSD)	0.32	0.28	0.26

Table:. Results of System Precision (Glimepiride + Metformin + Voglibose)

Method Precision

"***Procedure:*** Precision was investigated using the sample preparation procedure for six consecutive replicates of sample of concentration 4 µg/mL for Glimepiride + Metformin + Voglibose."

Replicate		***Glimepiride + Metformin + Voglibose***			
S.No.	**Concentration Taken (µg/ml)**	**Area *Glimepiride***	**Area *Metformin***	**Area *Voglibose***	**%LC**
1	20.00	363431	358284	273221	99.98%
2		363486	354567	273266	99.97%
3		363393	355246	273213	99.99%
4		363434	357267	273323	99.98%
5		363452	356611	273443	99.98%
6		363391	357332	273656	99.99%
Average					99.98%
Std.Dev					0.00752
% RSD					0.01%
Standard weight					20 mcg
Standard potency					98.60%

Linearity

Glimepiride + Metformin + Voglibose				
Linearity level	**Concentration in µg/mL**	**Area *Glimepiride***	**Area *Metformin***	**Area *Voglibose***
1	20 µg/mL	162728	193814	173214
2	40 µg/mL	263389	294573	263389
3	60 µg/mL	374233	405466	374233
4	80 µg/mL	472884	493373	469884
5	100 µg/mL	562632	593345	562632
Correlation co-efficient		0.9968	0.9974	0.9985
Slope		25465.15	27214.13	24756.25
Intercept		260382.3	290145.5	265136.5

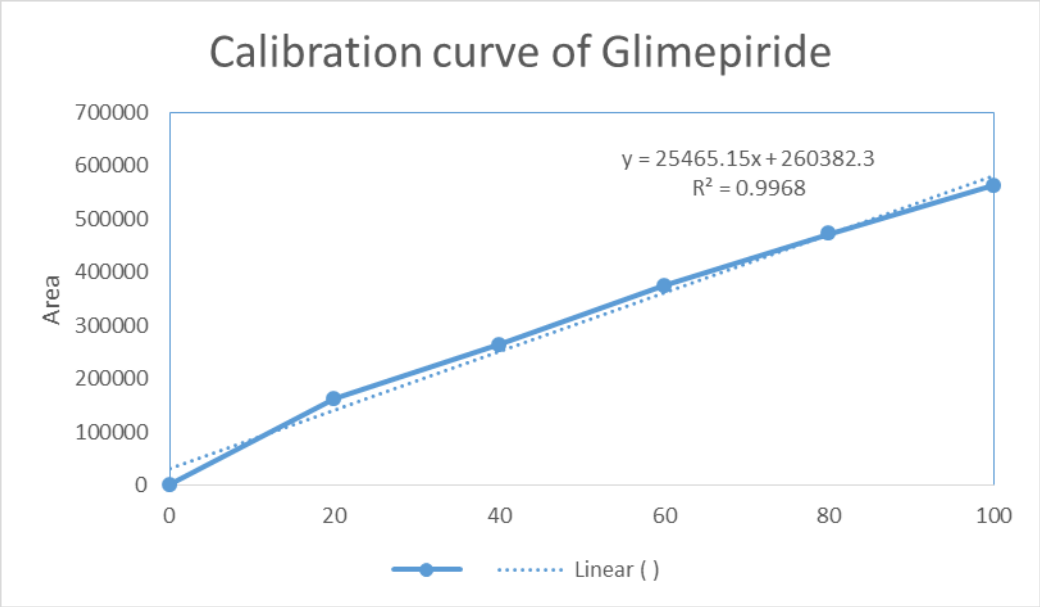


Fig.: Calibration curve of Glimepiride

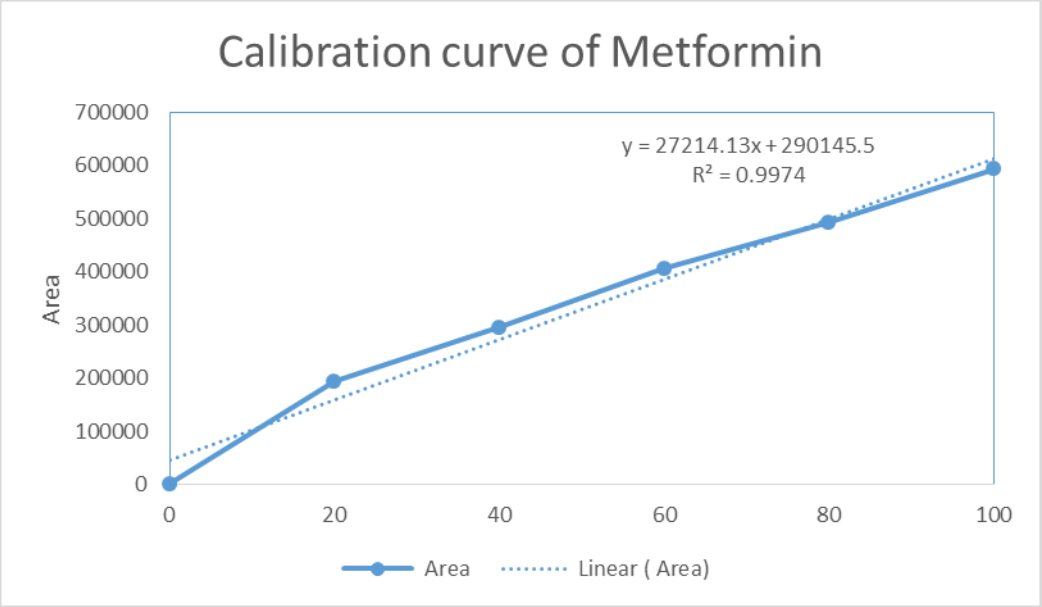


Fig.: Calibration curve of Metformin

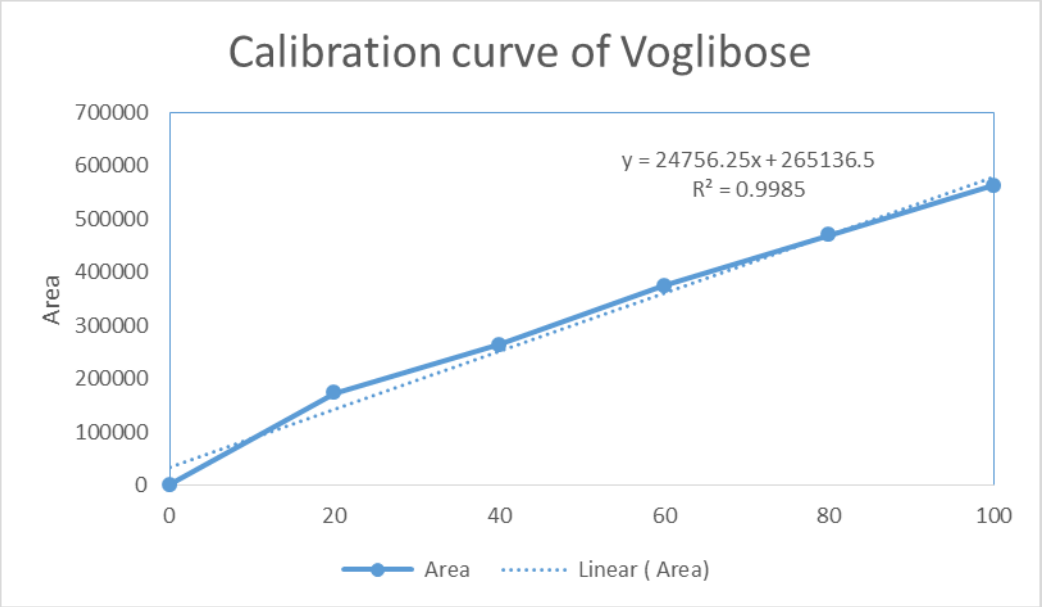


Fig.: Calibration curve of Voglibose

Robustness

Robustness Studies					
Parameter	**Value**	**Peak Area Glimepiride**	**Peak Area Metformin**	**Peak Area Voglibose**	**% RSD**
Flow Rate	**Low**	364739	358286	273337	0.12%
	Actual	365312	358313	273215	
	Plus	365589	358456	273443	
Temperature	**Low**	364864	358357	273329	0.05%
	Actual	365039	358589	273246	
	Plus	365245	358633	273452	
Wavelength	**Low**	364934	358344	273315	0.14%
	Actual	365477	358441	273182	
	Plus	365973	358538	273461	

Ruggedness

Glimepiride + Metformin + Voglibose					
Ruggedness					
Parameter	**Peak Area Glimepiride**	**Peak Area Metformin**	**Peak Area Voglibose**	**% RSD**	**%LC**
Intraday precision	363489	354286	271337	0.32%	99.36%
	364953	355313	274218		99.76%
	365768	356245	273325		99.99%
Inter day precision	363491	355322	276214	0.30%	99.37%
	364949	356266	274315		99.76%
	365761	356325	275237		99.98%
Instrument:1 Acquity UPLC Waters, 2695H	365288	356241	276338	0.25%	99.86%
	365434	357372	273229		99.90%
	363767	356271	272336		99.44%
Instrument:2 Agilent Technologies, 1290	365289	356315	271224	0.24%	99.86%
	365437	355256	276327		99.90%
	363764	357324	274246		99.44%
Average					99.71%
Std.Dev					0.24431
%RSD					0.25%

LOD and LOQ

Procedure

"The limit of detection and limit of quantification were evaluated by serial dilutions of Glimepiride + Metformin + Voglibose stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ as per ICH guidelines."

Calculations of LOD and LOQ

Slope = a; Intercept = b; The number of tests = N
Standard Error (SE) of Intercept = EXCEL function data analysis → Regression → Table
SD of Intercept = SE of Intercept / Square root of N

LOD

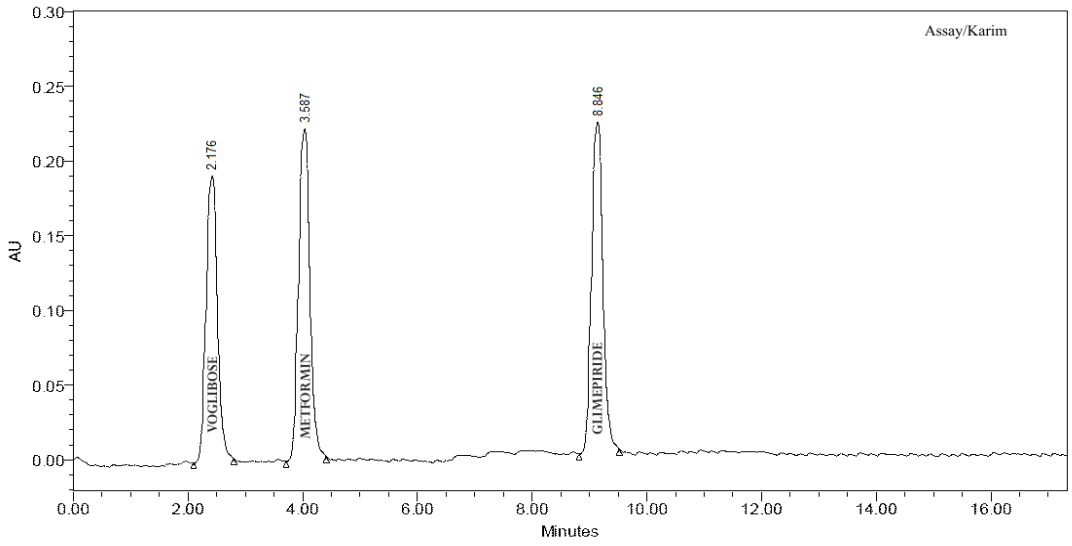
LOD=3.3(SD of intercept/Slope)
Total numbers: 5
SE of Intercept: 3612.82
SD of Intercept: 1620.09
LOD= 3.3*(1620.09/ 25465.15)
LOD= 3.3*(0.06362)
LOD= 0.2099(µg/ml)

LOQ

LOQ=10*(SD/S)
LOQ= 10*(1620.09/ 25465.15)
LOQ= 0.6362(µg/ml)

Forced Degradation Studies

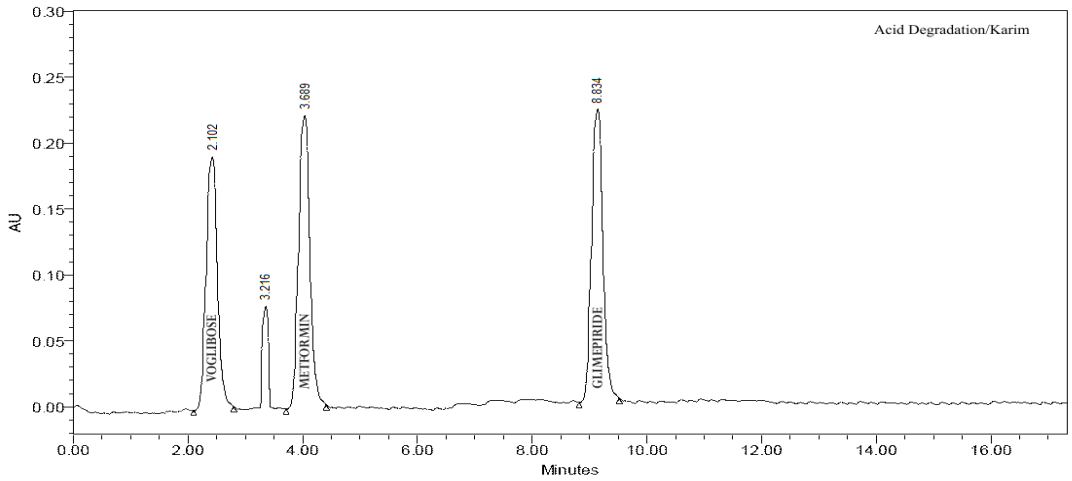
Sample Control: An accurate 10 ml of the prepared pure drug stock solution of working standard was transferred to a clean and dry RBF. The volume of the sample was 100 µg/ml. It was injected into the UPLC system against a blank of Acetonitrile and Methanol in the ratio of 68:32%v/v after optimizing the mobile phase composition, chromatogram was recorded.



Chromatogram: Assay of Glimepiride + Metformin + Voglibose (Sample Control)

a. Acidic Degradation

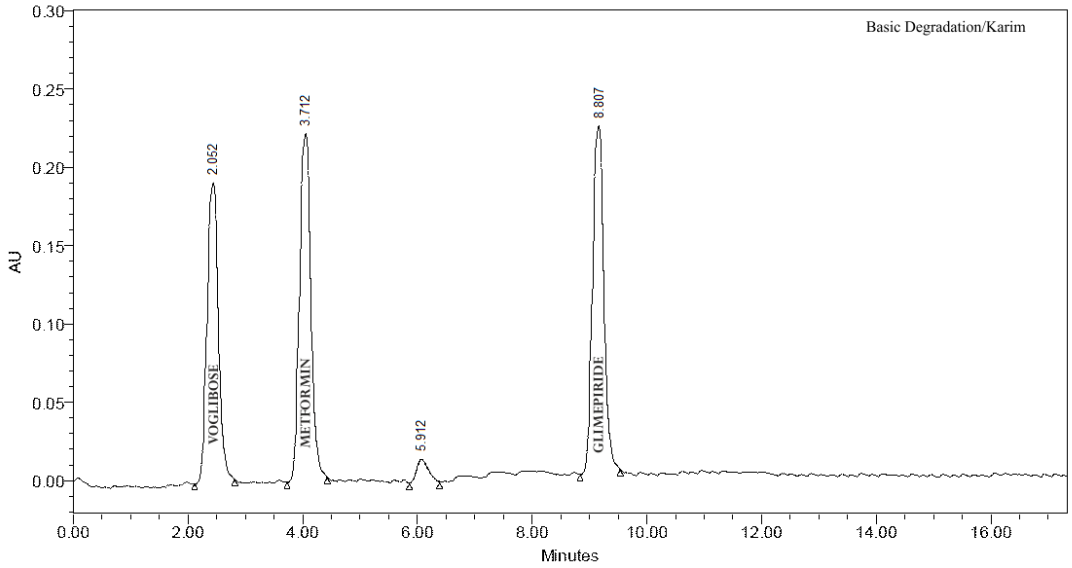
An accurate 10 ml of pure drug sample solution was transferred to a clean and dry round bottom flask (RBF). 30 ml of 0.1 N HCl was added to it. It was refluxed in a water bath at 60°C for 4 hours. Drug became soluble after reflux which was insoluble initially. Allowed to cool at room temperature. The sample was then neutralized using 2N NaOH solution and final volume of the sample was made up to 100ml with water to prepare 100ppm solution. It was injected into the UPLC system against a blank of Acetonitrile and Methanol in the ratio of 68:32%v/v after optimizing the mobile phase composition, chromatogram was recorded.



Chromatogram: Chromatogram showing the degraded products in Acidic degradation.

b. Basic Degradation

An accurate 10 ml of pure drug sample solution was transferred to a clean and dry RBF. 30 ml of 0.1N NaOH was added to it. It was refluxed in a water bath at 60°C for 4 hours. Drug became soluble after reflux which was insoluble initially. It was allowed to cool at room temperature. The sample was then neutralized using 2N HCl solution and final volume of the sample was made up to 100ml with water to prepare 100ppm solution. It was injected into the UPLC system against a blank of Acetonitrile and Methanol in the ratio of 68:32%v/v after optimizing the mobile phase composition, chromatogram was recorded.

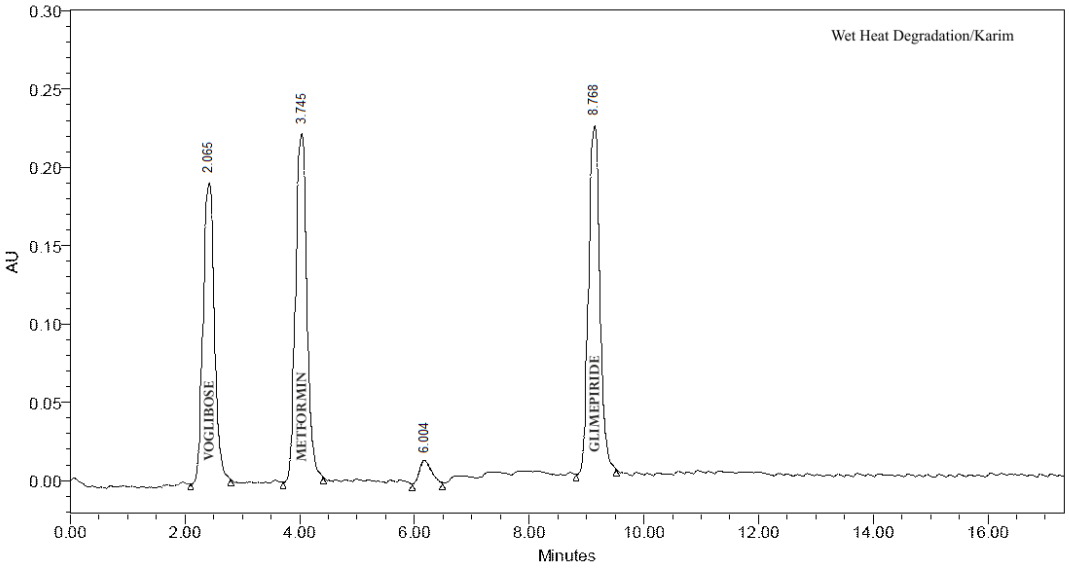


Chromatogram: Chromatogram showing the degraded products in Basic degradation

c. Wet heat degradation

Accurate 10 ml of pure drug sample was transferred to a clean and dry RBF. 30 ml of HPLC grade water was added to it. Then, it was refluxed in a water bath at 60°C for 6 hours uninterruptedly. After the completion of reflux, the drug became soluble and the mixture of drug and water was allowed to cool at room temperature. Final volume was made up to 100 ml with HPLC grade water to prepare 100 ppm solution. It was injected into the UPLC system against a blank of Acetonitrile and

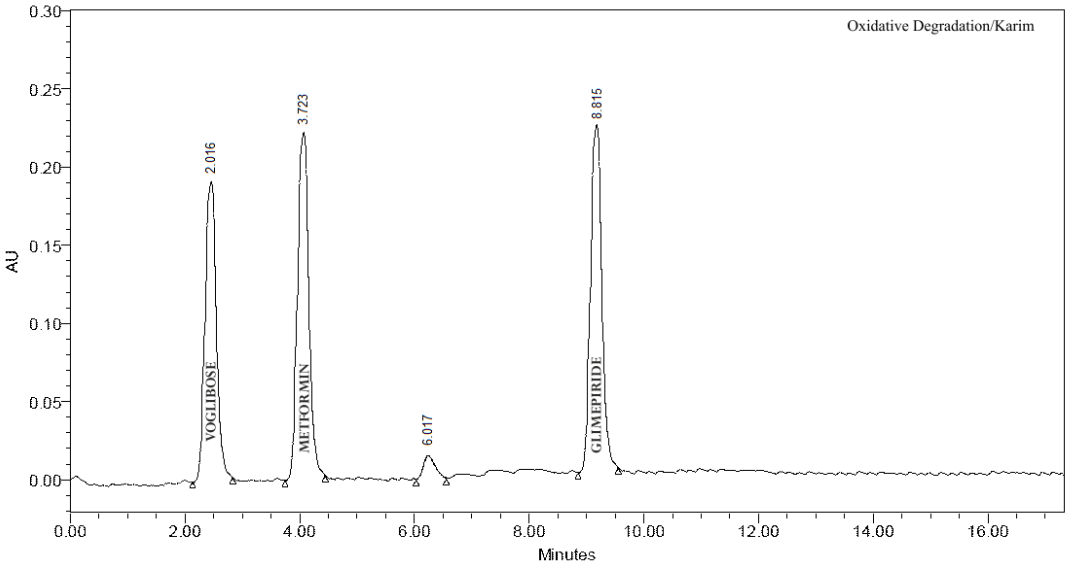
Methanol in the ratio of 68:32%v/v after optimizing the mobile phase composition, chromatogram was recorded.



Chromatogram: Chromatogram showing the degraded products in Wet heat degradation

d. Oxidation with (3%) H_2O_2

Approximately 10 ml of pure drug sample was transferred in a clean and dry 100 ml volumetric flask. 30 ml of 3% H_2O_2 and a little methanol was added to it to make it soluble and then kept as such in dark for 24 hours. Final volume was made up to 100 ml using water to prepare 100 ppm solution. The above sample was injected into the UPLC system. The chromatogram was recorded.



Chromatogram: Chromatogram showing the degraded products in Oxidative degradation

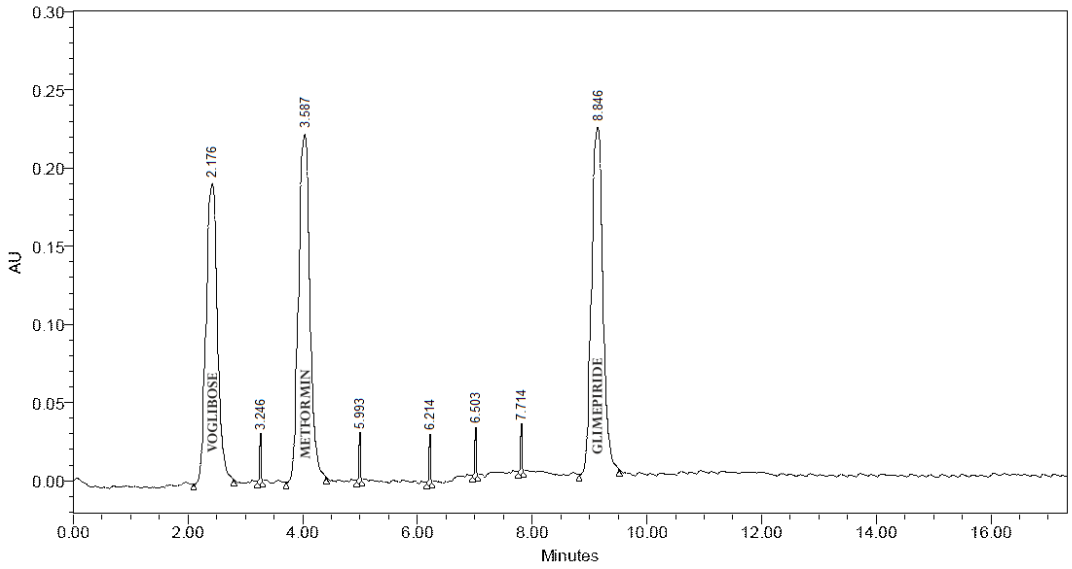
Nature of Stress	Degradation condition	Time(h)	Number of degradation products (Rt)
Acidic	60°C	3	2 (4.712, 7.206)
Basic	60°C	9	1 (8.994)
Oxidative	RT	48	1 (1.129)
Wet Heat	105°C	24	1 (9.247)

Table : Summary of Forced Degradation Studies (Glimepiride + Metformin + Voglibose)

Structure and Separation of the Known/ Unknown Impurities by LCMS/MS

An unknown impurity with a relative retention time (RRT) of 1.396 with respect to Glimepiride + Metformin + Voglibose was observed during the stability study of the drug product and we tried to enhance the impurity by using the forced degradations to separate it. But the impurity was not increased in any trial. So the impurity was separated by preparative UPLC from stability samples with a purity of > 98% and used for its characterisation by LC-MS -MS^n studies."

The positive ESI-MS spectrum of the unknown impurity showed a peak at m/z 612.24 amu [M+H]+ which was 26.22 amu higher than that of Glimepiride + Metformin + Voglibose (m/z 586.02). The comparison of MS/MS studies of the unknown impurity and Glimepiride + Metformin + Voglibose showed common fragment ions at m/z 590.58. The common fragment ion peak suggests that 7-chloroquinolin-2-yl was intact and changes were at the sulfanylmethyl- cyclopropyl - acetic atom."



Chromatogram: Chromatogram showing the impurities

Impurity Profile

S.NO	IMPURITY NAME	ACTIVE PHARMACEUTICAL INGREDIENT	RELATIVE RETENTION TIME
1	Impurity-A1	Glimepiride + Metformin + Voglibose	3.246
2	Impurity-A2		5.993
3	Impurity-B		6.214
4	Impurity-C		6.503
5	Any individual unknown impurity		7.714

Table : Summary of Impurity Profile (Glimepiride + Metformin + Voglibose)

Mass Spectrometry Conditions for MS/MS

The samples (5 µL) is injected directly into the source by the flow injection method using Acetonitrile and Methanol in the ratio of 68:32%v/v as mobile phase at a flow rate of 0.5 mL/min. The mass spectra were recorded in ESI negative mode. Ultra-high purity nitrogen and helium were used as curtain and collision gas, respectively. The typical ion source conditions were: nebulizer gas, 60 psi; dry temperature, 325˚C; dry gas, 5.0 mL/min; capillary voltage, 5kV; capillary current, 80.243 nA; vapourizer temperature, 400°C; dwell time, 200 ms. For the collision-induced dissociation (CID) experiments, the precursor ion was selected using the quadrapole analyzer and product ions were analyzed by the time-of-flight analyzer. HRMS data acquisition was performed by the following source conditions: capillary voltage, 5 kV; declustering potential (DP) and collision energy (CE) were −60 V and −10 V, respectively; focusing potential, 220 V; resolution 40,000 (FWHM)."

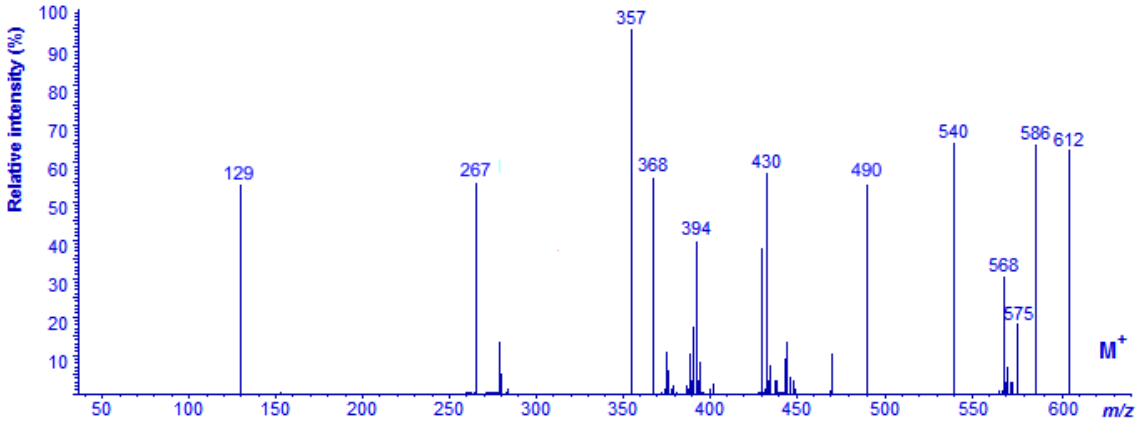


Fig. MS/MS^n characterisation of impurities.

Elemental compositions of Glimepiride + Metformin + Voglibose in MS/MS spectra

Analyte	Observed ion mass (Da)	Proposed formula	Calculated mass (Da)	Error (ppm)
Metformin	129.19	$C_4H_{11}N_5$	129.16	
Voglibose	267.27	$C_{10}H_{21}NO_7$	267.28	
Glimepiride	490.62	$C_{24}H_{34}N_4O_5S$	490.61	
Glimepiride + Metformin + Voglibose	586.02	$C_{35}H_{40}NO_3S_2$	586.83	1.34
	575.46	$C_{35}H_{47}N_2O_3S$	575.82	2.49
	568.38	$C_{35}H_{38}NO_2S_2$	568.81	-2.73
	540.63	$C_{35}H_{42}NO_2S$	540.78	-1.17
	430.71	$C_{25}H_{20}NO_4S$	430.49	-2.12
	612.24	$C_{35}H_{35}NOS_4$	613.92	1.26

Table:. Compositions in MS/MS spectra (Glimepiride + Metformin + Voglibose)

EVALUATION OF METHODS

Related Substance Studies

Analysis of Glimepiride + Metformin + Voglibose

Conditions	Sample Amount (µg/ml)	Peak Area	% claim	% Degradation
Sample Control	04.05	363393	97.89%	-
Acidic Degradation	04.03	357338	*96.26%*	1.63%
Basic Degradation	04.02	362849	*97.75%*	0.14%
Oxidative Degradation	04.04	359276	*96.78%*	1.11%
Wet Heat	04.01	362765	*97.72%*	0.17%

Results of Impurity Assays (Glimepiride + Metformin + Voglibose)

Calculation formula for Glimepiride + Metformin + Voglibose

$$\%\ Assay = \frac{AT}{AS} \times \frac{W1}{100} \times \frac{1}{25} \times \frac{100}{W2} \times \frac{25}{1} \times \frac{AW}{LC} \times P$$

Whereas,"

AT = Average area of test preparation, 363393"

AS = Average area of standard preparation, 365461"

W1 = Weight taken of reference standard (µg), 04.05"

W2 = Weight taken of test sample (µg), 04.06"

AW = Average weight of sample (µg), 1000.60"

LC = Label claim (µg), 1000.50"

P = Potency of reference standard (%), 98.60%"

Sample Control (Glimepiride + Metformin + Voglibose)

$$\%\ Assay = \frac{363393}{365461} \times \frac{04.05}{100} \times \frac{1}{25} \times \frac{100}{04.06} \times \frac{25}{1} \times \textbf{Error!} \times$$

98.60 = 97.89%

Acidic Degradation (Glimepiride + Metformin + Voglibose)

$$\%\ Assay = \frac{357338}{365461} \times \frac{04.05}{100} \times \frac{1}{25} \times \frac{100}{04.06} \times \frac{25}{1} \times \textbf{Error!}$$

$\times\ 98.60 = 96.26\%$

Basic Degradation (Glimepiride + Metformin + Voglibose)

$$\% \ Assay = \frac{362849}{365461} \times \frac{04.05}{100} \times \frac{1}{25} \times \frac{100}{04.06} \times \frac{25}{1} \times \textbf{Error!} \times 98.60 = 97.75\%$$

Oxidative Degradation (Glimepiride + Metformin + Voglibose)

$$\% \ Assay = \frac{359276}{365461} \times \frac{04.05}{100} \times \frac{1}{25} \times \frac{100}{04.06} \times \frac{25}{1} \times \textbf{Error!} \times 98.60 = 96.78\%$$

Wet Heat (Glimepiride + Metformin + Voglibose):

$$\% \ Assay = \frac{362765}{365461} \times \frac{04.05}{100} \times \frac{1}{25} \times \frac{100}{04.06} \times \frac{25}{1} \times \textbf{Error!} \times 98.60 = 97.72\%$$

Related Substance Stress (Glimepiride + Metformin + Voglibose)

$$\% \ Assay = \frac{363895}{365461} \times \frac{04.05}{100} \times \frac{1}{25} \times \frac{100}{04.06} \times \frac{25}{1} \times \textbf{Error!} \times 98.60 = 98.03\%$$

CONCLUSION

A short selective, precise, accurate and sensitive stability-indicating gradient LC-MS/MSn method was developed for the quantitative determination of process-related impurities and degradation products of Glimepiride + Metformin + Voglibose in pharmaceutical oral dosage formulations. During the stress study, the degradation products of Glimepiride + Metformin + Voglibose were well-resolved from Glimepiride + Metformin + Voglibose and its impurities and the mass balances were found to be satisfactory in all the stress conditions, thus proving the stability-indicating capability of the method. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection and quantification, accuracy, precision, ruggedness, and robustness. During the stability analysis of the drug product, one unknown impurity was detected by the above stability-indicating method. The flow rate was 0.5 ml/min and effluent was monitored at 226nm. The LOD and LOQ values were found to be 0.2099 (µg/ml) and 0.6362 (µg/ml) respectively.

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