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Method development and validation of simultaneous estimation of metformin and glibenclamide in combined tablet dosage form by RP-HPLC method

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ABSTRACT

A new simple, accurate, rapid and precise isocratic high performance liquid chromatographic (HPLC) method was developed and validated for the determination of Metformin and Glibenclamide in tablet formulation. The proposed HPLC method utilizes Develosil ODS HG-5 RP C18, 250 mm x 4.6 mm I.D; 5 µm with a flow rate of 1.0 mL/min, mobile phase consisting of Methanol : Acetate buffer (pH=3.0) = 75:25 (v/v) at a detection wavelength 256 nm. The method was validated in terms of accuracy, precision, linearity, limits of detection, limits of quantitation, and robustness. This optimized method has been successively applied to pharmaceutical formulation and no interference from the tablet excipients was found. Retention times of Metformin and Glibenclamide were found to be 2.24 min, and 3.28 min with a tailing factor 1.30, 1.29and 2603, 3534 as theoretical plates respectively which are within the limits. All the parameters were validated according to the ICH guidelines and found to be within limits. Limit of detection (LOD) and Limit of quantification (LOQ) were estimated from the signal-to-noise ratio. The LOD values of Metformin and Glibenclamide were found to be 0.062 and 0.018 µg/mL respectively. Metformin and Glibenclamide LOQ's were found to be 0.19, and 0.056µg/mL respectively. Linearity ranges for Metformin and Glibenclamide were 2-10 µg/mL, and 3-15 µg/mL respectively. Percent recovery study values of Metformin and Glibenclamide were found to be within 98-100 %. This new method was successfully developed and validated as per ICH guidelines, can be utilized for the quantitative estimation of Metformin and Glibenclamide in pharmaceutical dosage forms.

Keywords: Metformin, Glibenclamide, RP-HPLC, Validation, Simultaneous estimation.

INTRODUCTION

Metformin hydrochloride (MET) 1-Carbamimidamido-N-N-Dimethyl Methanimidamide is an antihyperglycemic agent agent. Metformin's (Metformin HCL) mechanisms of action differ from other classes of oral antihyperglycemic agents. Metformin decreases blood glucose levels by decreasing hepatic glucose

production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization. These effects are mediated by the initial activation by metformin of AMP-activated protein kinase (AMPK), a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats. Increased peripheral utilization of glucose may be due to improved insulin binding to insulin receptors. Metformin administration also increases AMPK activity in skeletal muscle. The rare side effect, lactic acidosis, is thought to be caused by decreased liver uptake of serum lactate, one of the substrates of gluconeogenesis.

Glibenclamide is the most extensively used sulphonylurea in many parts of the world for the management of noninsulin- dependent diabetes mellitus (NIDDM). It is practically insoluble in water; slightly soluble in alcohol and in methyl alcohol; sparingly soluble in dichloromethane. It is a second-generation sulfonylurea antidiabetic agent, appears to lower the blood glucose acutely by stimulating the release of insulin from the pancreas, an effect dependent upon functioning beta cells in the pancreatic islets. Glibenclamide bind to ATP-sensitive potassium channels on the pancreatic cell surface, reducing potassium conductance and causing depolarization of the membrane. Depolarization stimulates calcium ion influx through voltage-sensitive calcium channels, raising intracellular concentrations of calcium ions, which induces the secretion, or exocytosis of insulin [1-3].

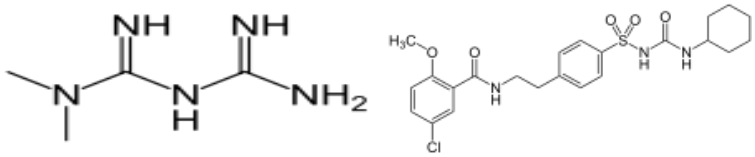


Fig 1.1: Structures of Metformin & Glibenclamide

MATERIALS AND METHODS

Instrument specifications: Waters HPLC 2965 system

Chemicals and reagents

Methanol obtained from local market, manufactured Pure METFORMIN and GLIBENCLAMIDE were obtained as gift sample from Aurabindo Pharma India Ltd; The tablet dosage form BEN Q MET 500mg (claim: 400.mg METFORMIN HCL and 2.5mg GLIBENCLAMIDE) was procured from local market.

Preparation of standard stock solutions

Working mixed standard solutions of concentrations at 50, 75, 100, 125 and 150% levels (i.e., 400mg/ml of Metformin HCl and 2.5mg/ml of Glibenclamide) were prepared by appropriate dilutions of the mixed standard stock solution with the diluent. The solutions thus prepared were filtered through 0.45µ membrane filter and the resulting filtrates were sonicated for 5min [4].

Preparation of sample solutions

BEN Q MET a commercial formulation containing a combination of Metformin HCl and Glibenclamide has been taken up for evaluating the proposed method for formulation. Twenty tablets were weighed and titurated to a fine powder, was weighed accurately weight equivalent to 10 mg (i.e., 9.525 mg) from the powdered sample was weighed and transferred into a 50ml volumetric flask and was dissolved in the diluent. The volume was made upto the mark with the same and the resulting solution was labeled as sample stock solution (contains 10mg of Metformin HCl and 0.1mg of Glibenclamide per ml). The solution was shaken well and allowed to stand for 15 min with intermittent sonication to ensure complete solubility of drug and filtered through a 0.45 µm membrane filter [5].

Preparation of Placebo solution

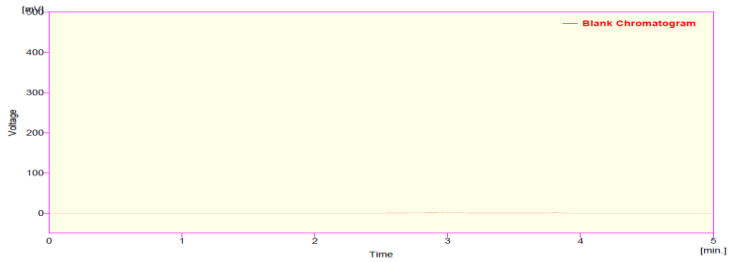
Placebo solution was prepared in the similar manner as that of the test solution. 500mg of placebo powder was accurately weighed and transferred into a 50ml volumetric flask and was dissolved in the diluent. The volume was made uptothe mark with the same and 0.5ml of the

resulting solution was transferred into 10ml volumetric flask and the volume was made upto the mark with the diluent. The solution thus prepared was filtered and the resulting filtrate was sonicated for 10 minutes [6].

Method Validation and Results

Procedure

The solutions of 100% level (i.e., solutions containing 500µg/ml of Metformin HCl and 2.5µg/ml of Glibenclamide) which were previously prepared in duplicate were injected at the optimized method conditions and the chromatograms were recorded and the percentage drug content was calculated.



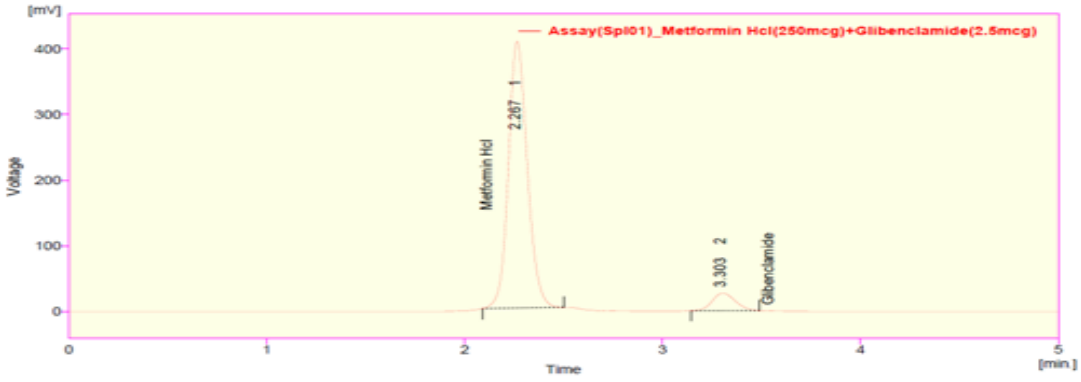
Result Table (Uncal - Blank Chromatogram)

Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
No peak to report			

Figure: 2.5.1 Chromatogram of Blank

Sample Info:

Sample ID	: Assay	Amount	: 0
Sample	: Metformin Hcl(250mcg)+Glibenclamide(2.5mcg)	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - Assay(Spl01)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.267	2696.018	406.682	92.54
2	3.303	217.364	26.882	7.46
	Total	2913.382	433.564	100.00

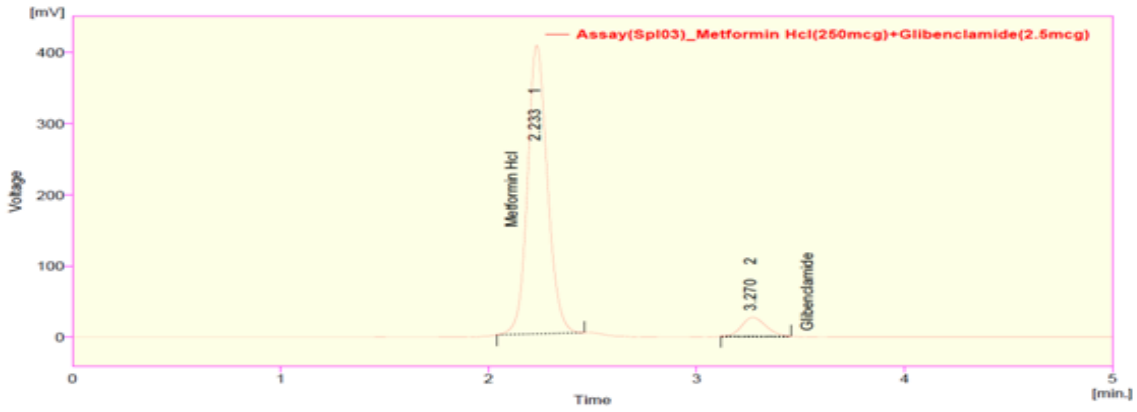
Column Performance Table (From 50% - Assay(Spl01)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Effi [t.p./m]	Resolution [-]
1	2.267	0.107	1.222	2502	25017	-
2	3.303	0.130	1.290	3577	35771	5.155

Figure: 2.5.2 Chromatogram of sample (01) Metformin HCl and Glibenclamide.

Sample Info:

Sample ID	: Assay	Amount	: 0
Sample	: Metformin Hcl(250mcg)+Glibenclamide(2.5mcg)	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - Assay(Spl03)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.233	2702.791	405.438	92.62
2	3.270	215.416	26.671	7.38
	Total	2918.207	432.109	100.00

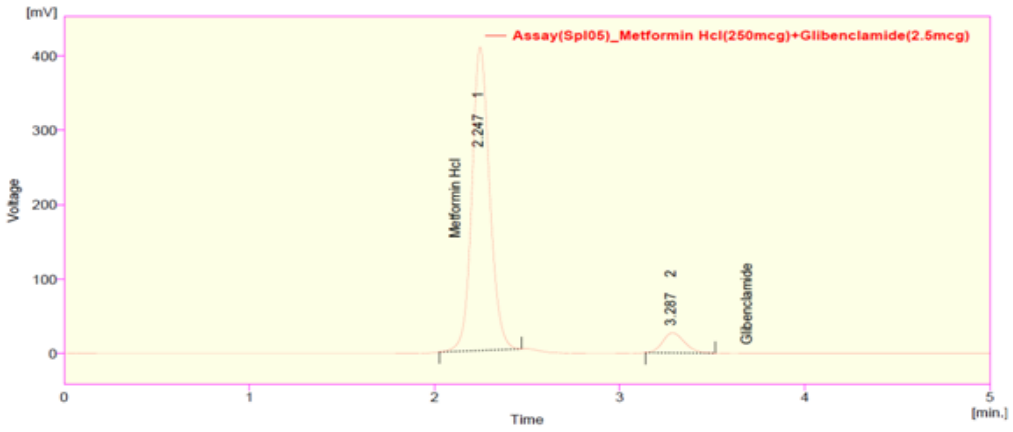
Column Performance Table (From 50% - Assay(Spl03)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.233	0.103	1.222	2588	25878	-
2	3.270	0.130	1.250	3505	35052	5.229

Figure: 2.5.3 Chromatogram of sample (02) Metformin HCl and Glibenclamide.

Sample Info:

Sample ID	: Assay	Amount	: 0
Sample	: Metformin Hcl(250mcg)+Glibenclamide(2.5mcg)	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



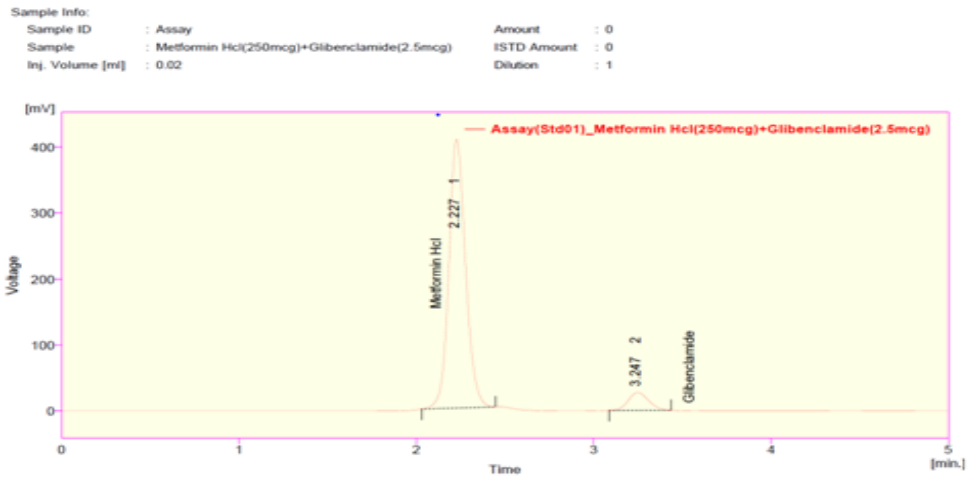
Result Table (Uncal - Assay(Spl05)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.247	2725.474	407.647	92.52
2	3.287	220.243	26.895	7.48
	Total	2945.717	434.542	100.00

Column Performance Table (From 50% - Assay(Spl05)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.247	0.103	1.222	2619	26188	-
2	3.287	0.130	1.281	3541	35411	5.245

Figure: 2.5.4 Chromatogram of sample (03) Metformin HCl and Glibenclamide.



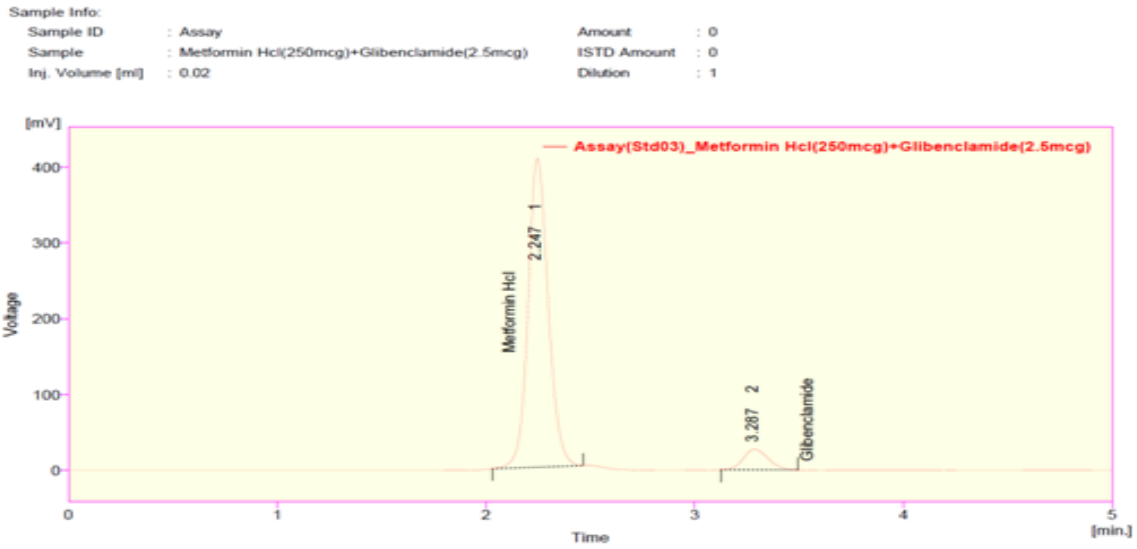
Result Table (Uncal - Assay(Std01)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.227	2671.765	407.455	92.48
2	3.247	217.271	27.131	7.52
	Total	2889.036	434.587	100.00

Column Performance Table (From 50% - Assay(Std01)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.227	0.103	1.269	2572	25724	-
2	3.247	0.127	1.290	3640	36397	5.219

Figure: 2.5.5 Chromatogram of standard (01) Metformin HCl and Glibenclamide.



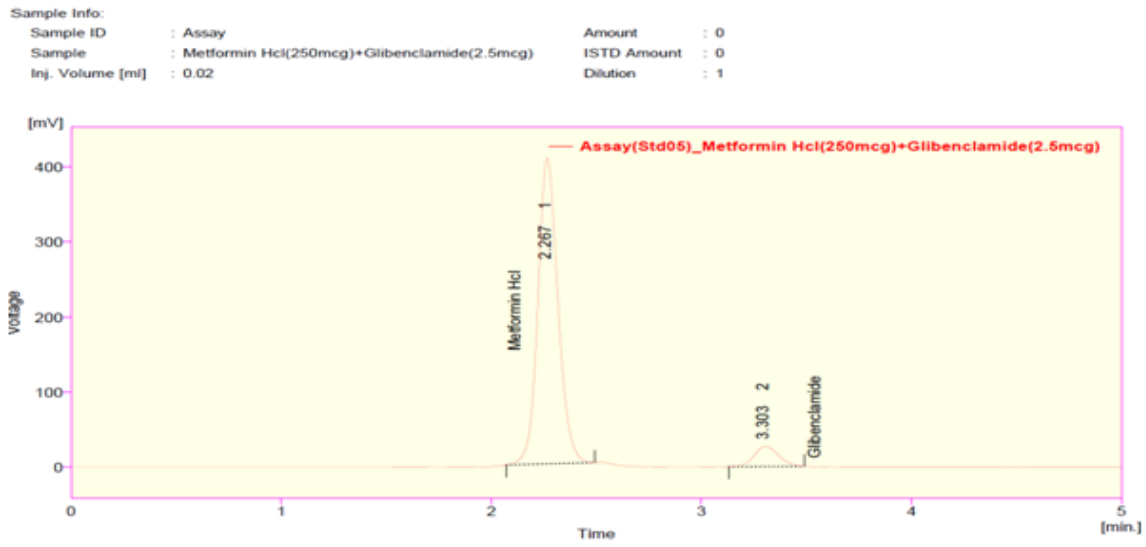
Result Table (Uncal - Assay(Std03)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.247	2725.489	407.475	92.44
2	3.287	222.936	27.056	7.56
	Total	2948.425	434.531	100.00

Column Performance Table (From 50% - Assay(Std03)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.247	0.107	1.222	2458	24577	-
2	3.287	0.130	1.281	3541	35411	5.172

Figure: 2.5.6 Chromatogram of standard (02) Metformin HCl and Glibenclamide.



Result Table (Uncal - Assay(Std05)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.267	2709.080	407.269	92.48
2	3.303	220.328	27.033	7.52
	Total	2929.408	434.302	100.00

Column Performance Table (From 50% - Assay(Std05)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.267	0.107	1.222	2502	25017	-
2	3.303	0.130	1.250	3577	35771	5.155

Figure: 2.5.7 Chromatogram of standard (03) Metformin HCl and Glibenclamide.

The % assays of Metformin HCl and Glibenclamide were found to be 100.02% and 99.36% respectively and were within the acceptance limits. Hence the developed method can be routinely used for the simultaneous estimation of Metformin HCl and Glibenclamide in the marketed formulations.

Table: 2.5.1 Assay Results Of Metformin HCl&Glibenclamide

Metformin HCl			**Glibenclamide**	
Standard Area	1	2671.765		217.271
	2	2725.489		222.936
	3	2709.08		220.328
	Average	2702.111	Average	220.178
Sample area	1	2696.018		217.364
	2	2702.791		215.416
	3	2725.474		220.243
	Average	2708.094	Average	221.007
Tablet average weight		500	Mg	5mg
Standard weight		250	Mg	2.5mg
Sample weight		250	Mg	2.5mg
Label amount		500	Mg	5mg
std.purity		99.6%		99.7%
Cal.:		**502.70**	Mg	**5.01mg**
	%Assay	**100.54**	%	**100.16%**

METHOD VALIDATION

Specificity

Procedure

The Placebo solution was injected at the optimized conditions and the chromatogram was recorded. As there were no peaks were found at the retention times of Metformin HCl and Glibenclamide, the proposed method was specific for the detection of the same [7].

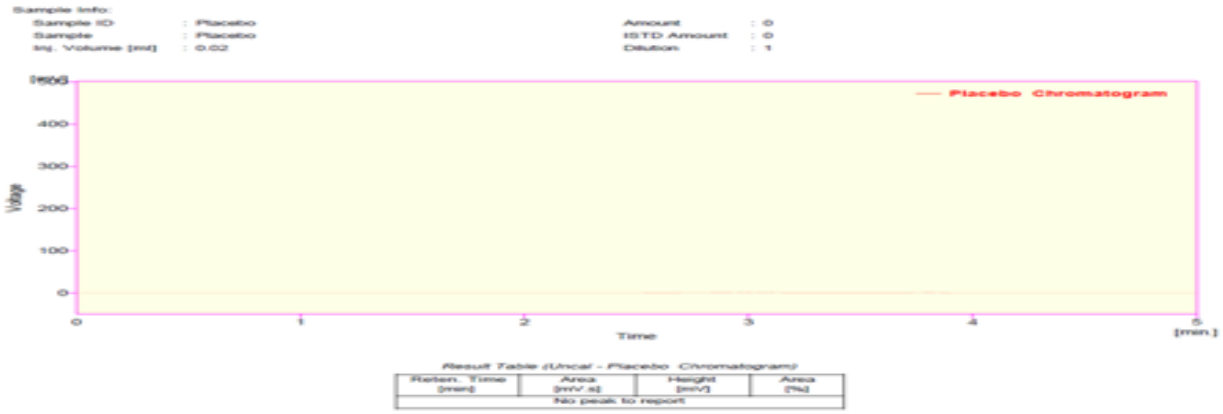
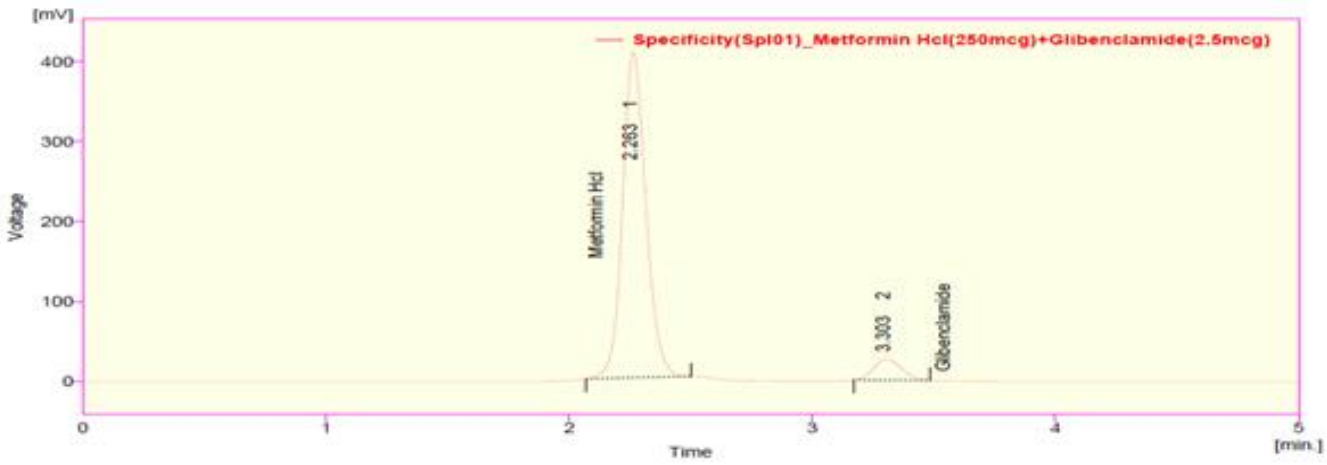


Figure: 3.1.1 Chromatogram of placebo



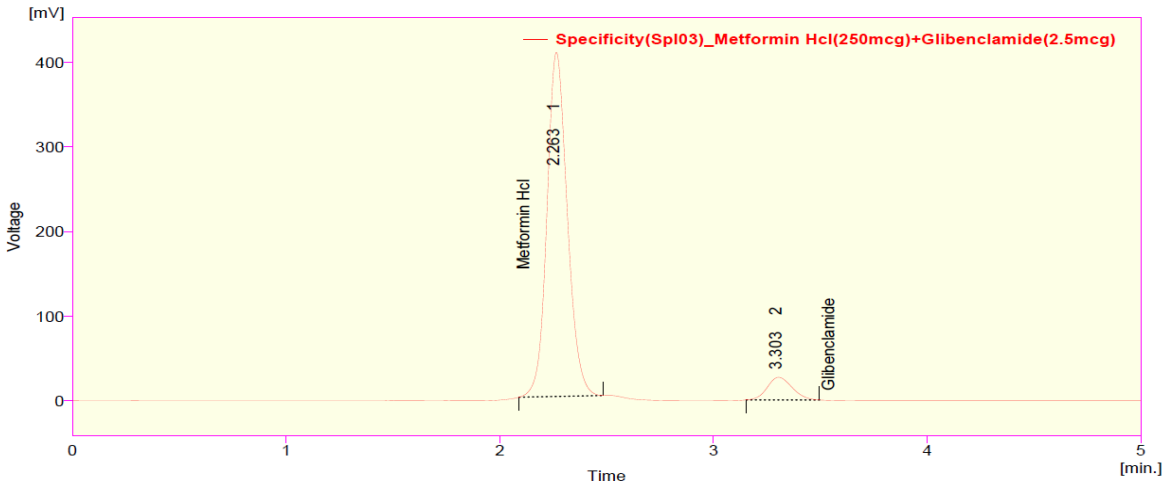
Result Table (Uncal - Specificity(Spl01)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.263	2711.618	407.276	92.83
2	3.303	209.359	26.376	7.17
	Total	2920.977	433.653	100.00

Column Performance Table (From 50% - Specificity(Spl01)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.263	0.103	1.308	2658	26578	-
2	3.303	0.127	1.333	3768	37678	5.321

Figure: 3.1.2 Specificity sample (01) Metformin HCl & Glibenclamide



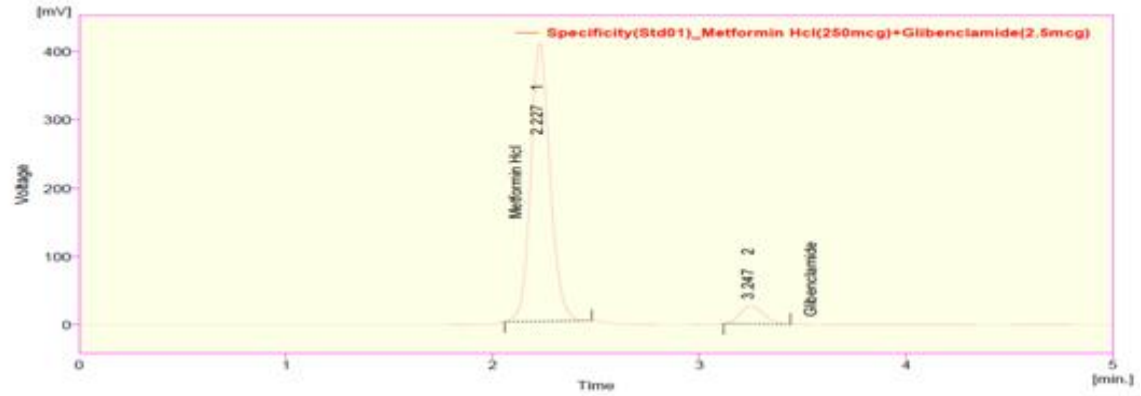
Result Table (Uncal - Specificity(Spl03)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.263	2697.080	406.624	92.55
2	3.303	217.057	26.795	7.45
	Total	2914.137	433.419	100.00

Column Performance Table (From 50% - Specificity(Spl03)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.263	0.103	1.308	2658	26578	-
2	3.303	0.130	1.290	3577	35771	5.245

Figure: 3.1.3 specificity sample (02) Metformin HCl & Glibenclamide



Result Table (Uncal - Specificity(Std01)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.227	2655.240	406.687	92.72
2	3.247	208.522	26.618	7.28
	Total	2863.762	433.305	100.00

Column Performance Table (From 50% - Specificity(Std01)_Metformin Hcl(250mcg)+Glibenclamide(2.5mcg))

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.227	0.103	1.269	2572	25724	-
2	3.247	0.127	1.300	3640	36397	5.219

Figure: 3.1.4 specificity standard (01) Metformin HCl & Glibenclamide

System suitability

Five replicate injections of standard solution were injected and the chromatograms were recorded. The system was suitable for analysis if the % relative standard deviation (%RSD) of area counts in five replicate injections should be not more than 2.0%.USP tailing factor for Metformin HCl and Glibenclamide peak should be not more than 2.0.USP resolution factor between the peaks corresponding to Metformin HCl and Glibenclamide should be more than 2.0 [8].

Procedure

The standard solution was prepared as per the proposed assay method and was injected into the HPLC system. The tailing factor and theoretical plate count of Metformin Hcl and Glibenclamide peak from fifth injection and % RSD on replicate injections were recorded.

Table 3.2.1: System Suitability parameters of Metformin HCl

Injection	R_t(min)	Area	USP Plate Count	USP Tailing
1	2.240	2706.929	2443	1.22
2	2.240	2727.230	2603	1.308
Mean	-	2717.079	-	
%RSD	-	1.7	-	-

Table 3.2.2: System Suitability parameters of Glibenclamide

Injection	R_t (min)	Area	USP Plate Count	USP Tailing
1	3.283	218.399	3359	1.281
2	3.283	215.848	3534	1.290
Mean		217.123		
%RSD	-	1.6%	-	-

Linearity and Range

The linearity of an analytical method is its ability to elic it test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

Procedure

Standards equivalentto50%, 75%, 100%, 125% & 150% of the stated amount of standard were weighed individually and the solutions were prepared according to the assay method. A graph of weight taken (%) versus chromate graphic area was plotted. There gressionline obtained was linear. From the data obtained, co-relation coefficient, slopeandy-intercept were calculated. Ideally co-relation coefficient should be not less than 0.999 [9].

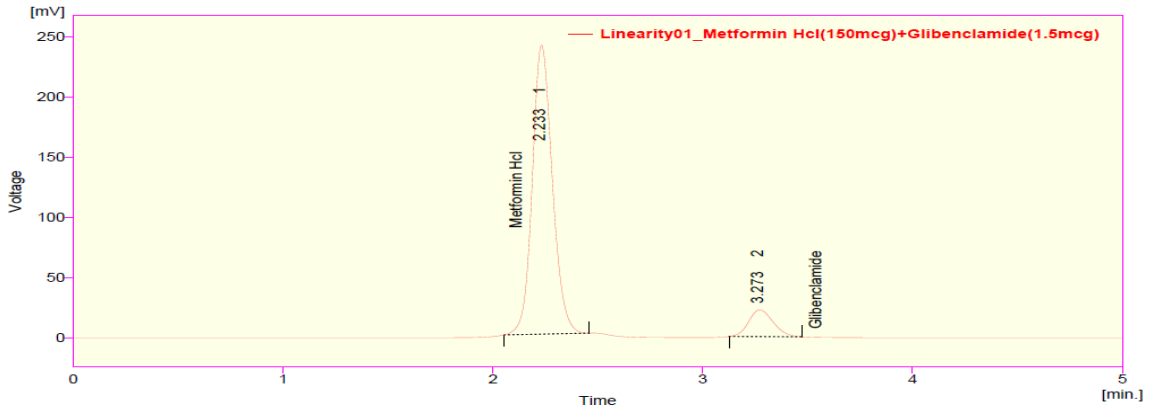


Figure 3.3.1 Chromatogram of Metformin HCl & Glibenclamide at 50%

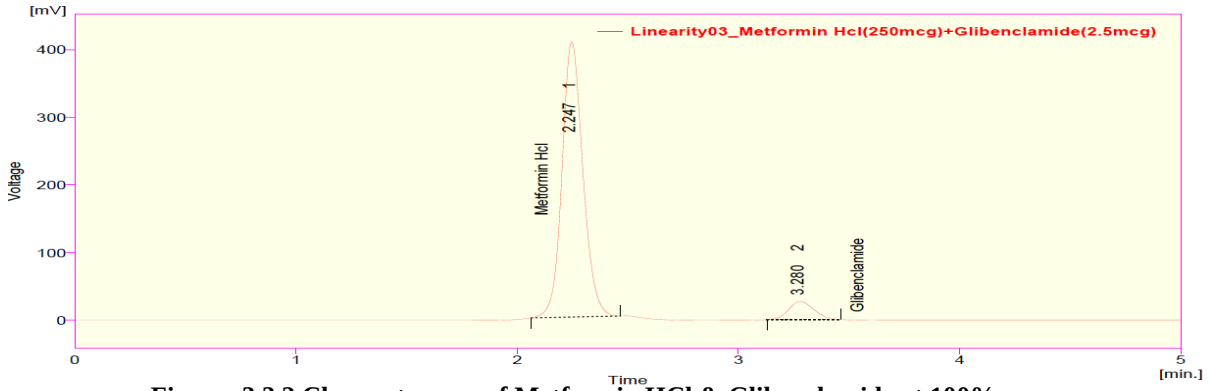


Figure: 3.3.2 Chromatogram of Metformin HCl & Glibenclamide at 100%

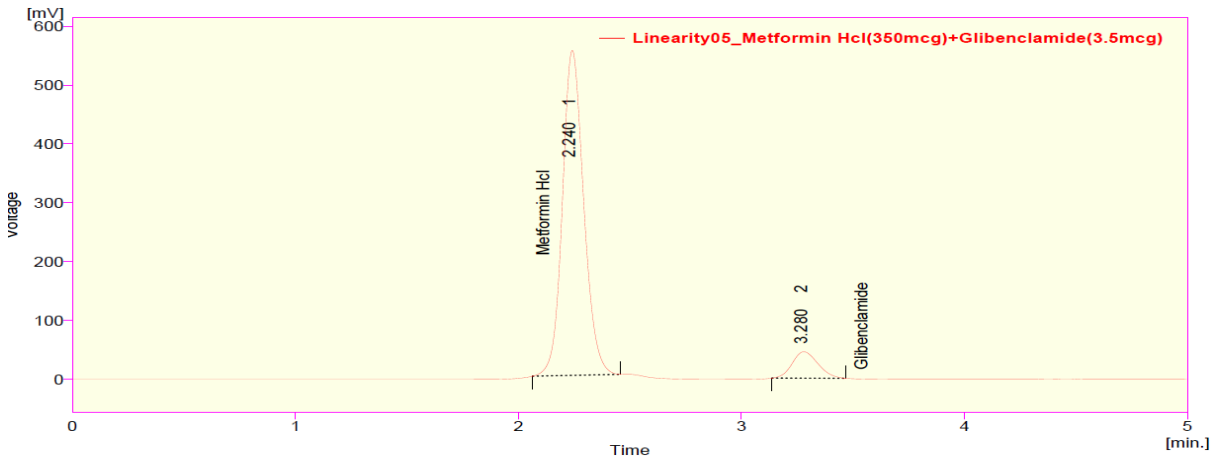


Figure: 3.3.3 Chromatogram of Metformin HCl & Glibenclamide at150%

Table 3.3.1 linearity data of Metformin HCl and linearity data of Glibenclamide

Linearity Levels	Metformin HCl	Peak areas
	Mcg	Area
L1-50%	150	1598.165
L2-75%	200	2141.807
L3-100%	250	2777.099
L4-125%	300	3218.275
L5-150%	350	3680.663

Linearity Levels	Glibenclamide	
	mcg	Area
L1-50%	1.5	109.89
L2-75%	2	167.979
L3-100%	2.5	233.116
L4-125%	3	308.562
L5-150%	3.5	361.164

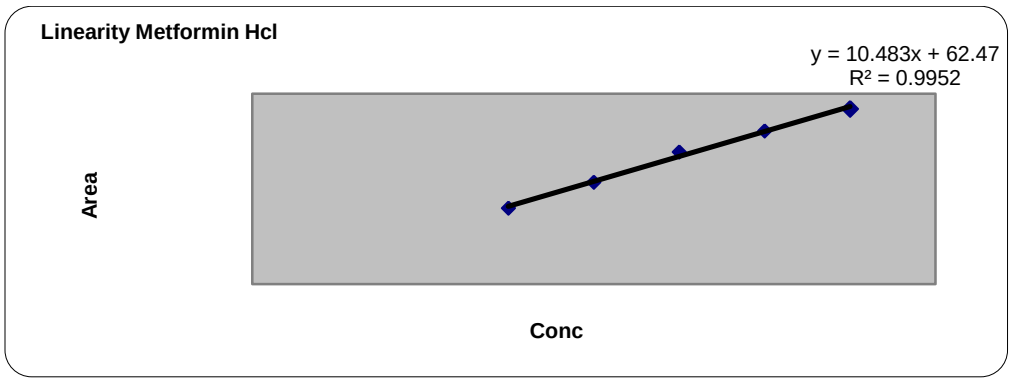


Figure 3.3.4 Graphical Representation of Linearity range of Metformin HCl.

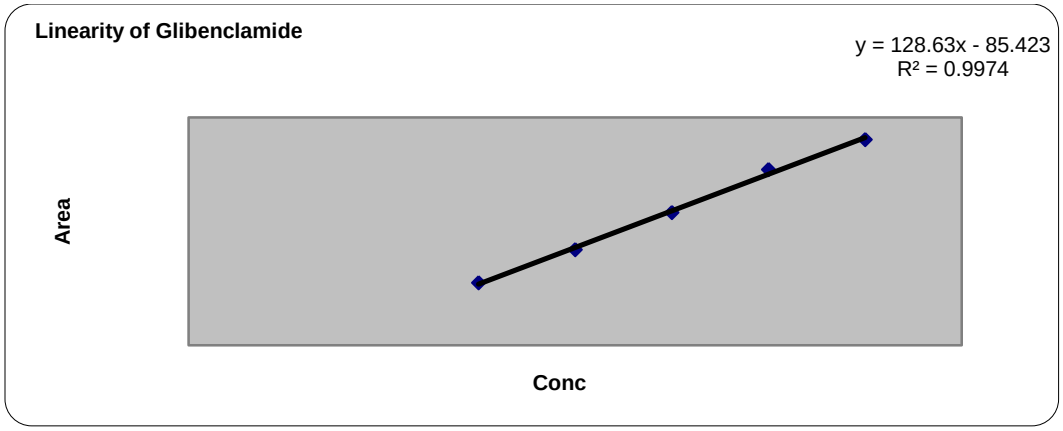


Figure 3.3.5 Graphical Representation of Linearity range of Glibenclamide.

Precision

The precision of an analytical method was the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurement [9].

System Precision

The system precision was checked by using standard Metformin HCl, Glibenclamide to ensure that the analytical system was precise. The retention time and area of five determinations was measured and RSD was calculated. % RSD of the assay value for five determinations should not be more than 2.0%.

Procedure

The standard solution was prepared as per the proposed assay method in five determinations and was injected into HPLC system. The retention time and peak area of five determinations was measured and RSD was calculated [9].

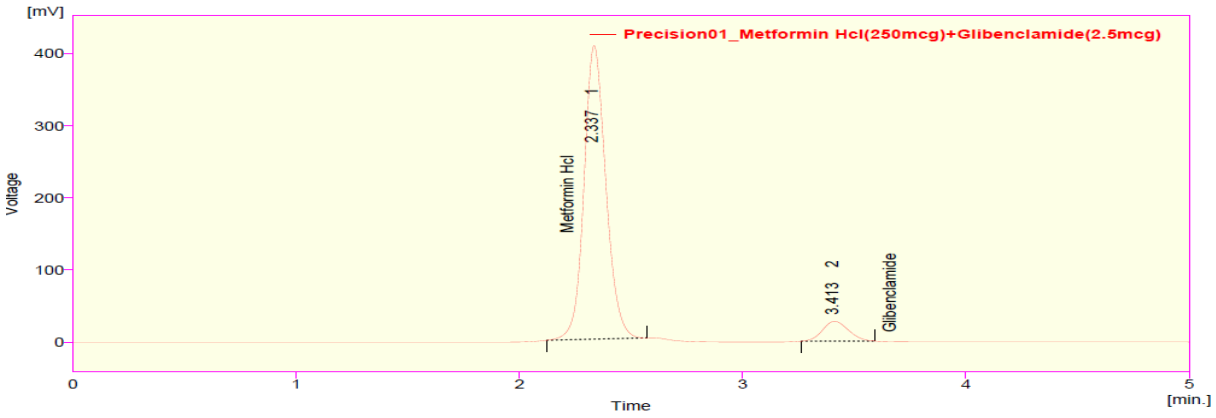


Figure: 3.5.1 precision 01 Metformin HCl & Glibenclamide

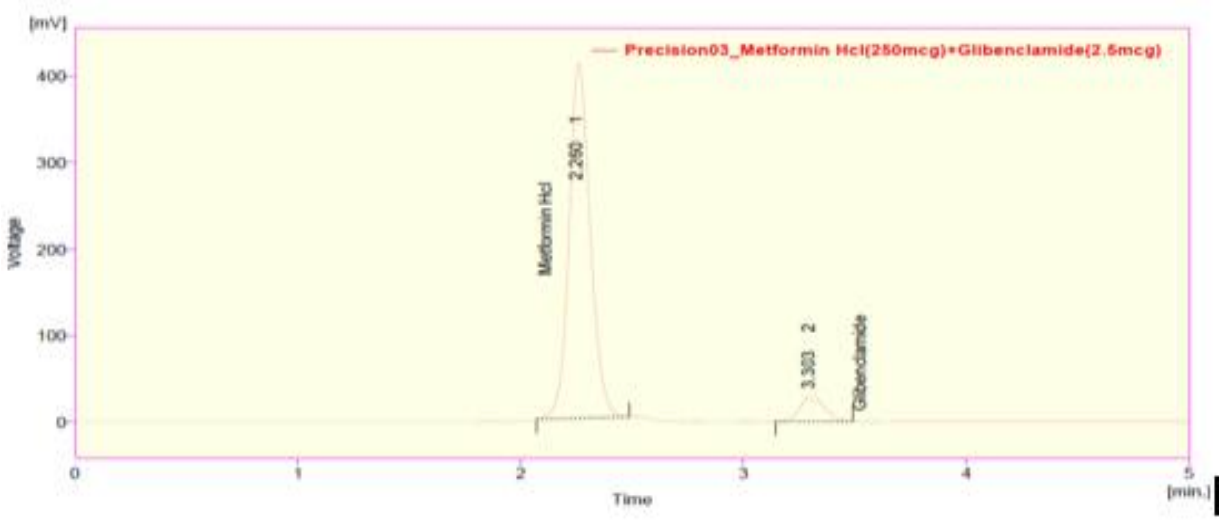


Figure: 3.5.2 precision 02Metformin HCl & Glibenclamide

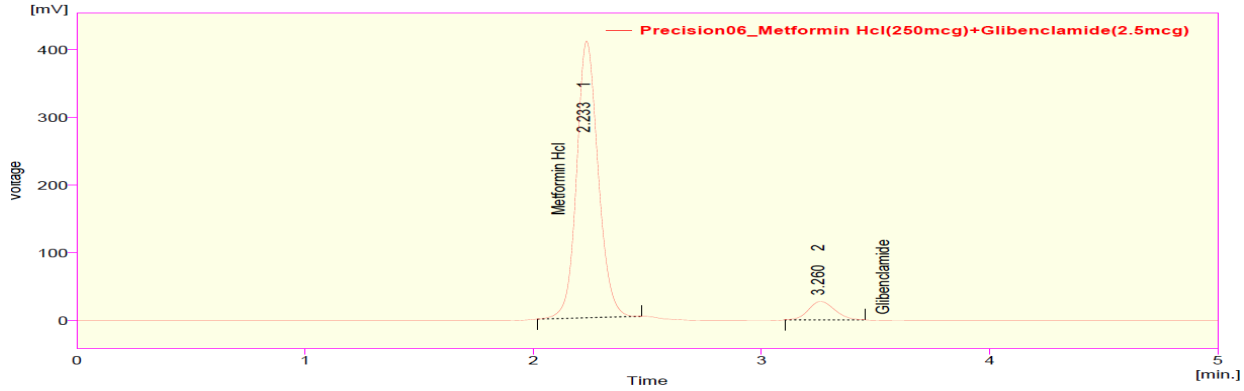


Figure: 3.5.3 precision 03 Metformin HCl & Glibenclamide

Data Interpretation

It was observed from the data tables above, that the retention time and area responses are consistent as evidenced by the values of relative standard deviation. Hence, it can be concluded that the system precision parameter meets the requirement of method validation.

Method Precision

In method precision, a homogenous sample of a single batch was analyzed six times. This indicates whether a method was giving consistent results for a single batch. The method precision was performed on Metformin HCl, Glibenclamide formulation. The % RSD of the assay value for six determinations should not be more than 2.0%.

Procedure

Method precision indicates whether a method is giving consistent results for a single material. The sample solution was prepared as per the proposed assay method in five determinations and was injected into HPLC system. The retention time and peak area of five determinations was measured and RSD was calculated [10].

Table 3.7.1 Method Precision of Metformin HCl and Method Precision of Glibenclamide

S. No	Retention time(Rt)	Area
1	2.337	2767.579
2	2.26	2710.928
3	2.233	2689.908
AVG	2.276	2722.495
STDEV	0.0381	29.538
%RSD	1.4	1.2

S. No	Retention time(Rt)	Area
1	3.413	222.354
2	3.303	221.339
3	3.26	218.775
AVG	3.325	220.822
STDEV	0.056	2.599
%RSD	1.7	1.8

Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. Accuracy is performed in three different levels for Metformin HCl and Glibenclamide at 50%, 100% and 150%. Samples analysed at each level in triplicate. From the results, % recovery was calculated. Average % recovery at each spike level not less than 98.0 and not more than 102.0 [10].

Preparation of Standard and Test Solutions

Mixed standard solutions containing 150µg/ml, 400µg/ml and 650µg/ml of Metformin HCl (60µg/ml, 160µg/ml and 260µg/ml of Glibenclamide respectively) were prepared in triplicate, from the mixed standard stock solution by appropriate dilutions. A test solution containing 100µg/ml of Mteformin Hcl and (40µg/ml of Glibenclamide) was prepared by appropriate dilution of the sample stock solution [11].

Procedure of Spiking

Spiking at 50% level was accomplished in triplicate, by adding 2.5ml of sample stock solution to 3.75ml of mixed standard stock solution (containing 1mg/ml of Metformin HCl and 0.4mg/ml of Glibenclamide) in a test tube. The contents of test tube were then cautiously filtered through Whatmann filter paper. In order to collect the remnants of the solution, the test tube and filter paper were washed with small quantities of diluent, and the washings were added to the filtrate through the same filter paper. Then the volume of filtrate was made up to 25ml with the diluent and the resultant solution was filtered through 0.45µ membrane filter. In the similar manner, spiking at 100% and 150% levels was carried out by adding 2.5ml of sample stock solution separately to 10ml and 16.25ml of mixed standard stock solution respectively [12].

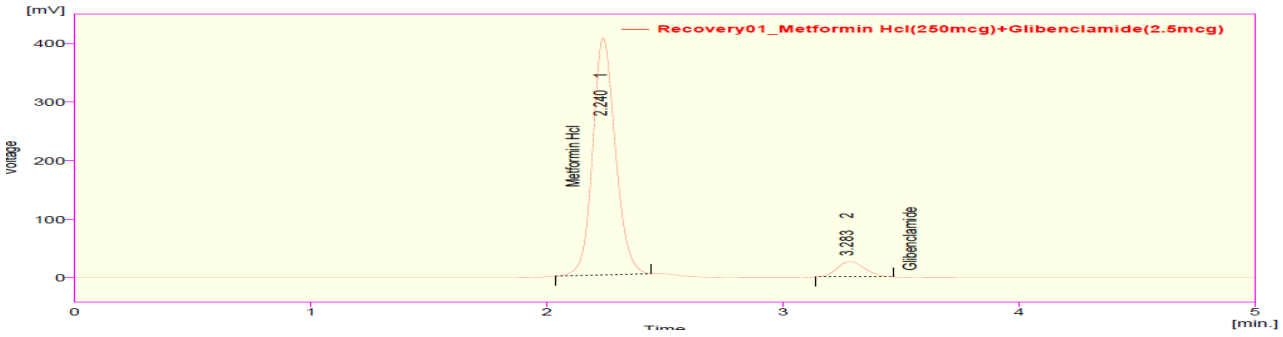


Figure 3.10.1 Chromatogram for Accuracy level

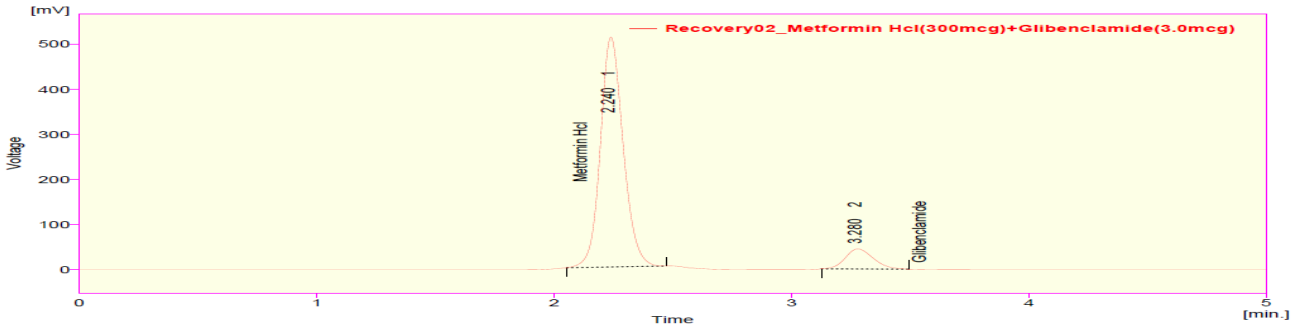


Figure 3.10.2 Chromatogram for Accuracy level -100%

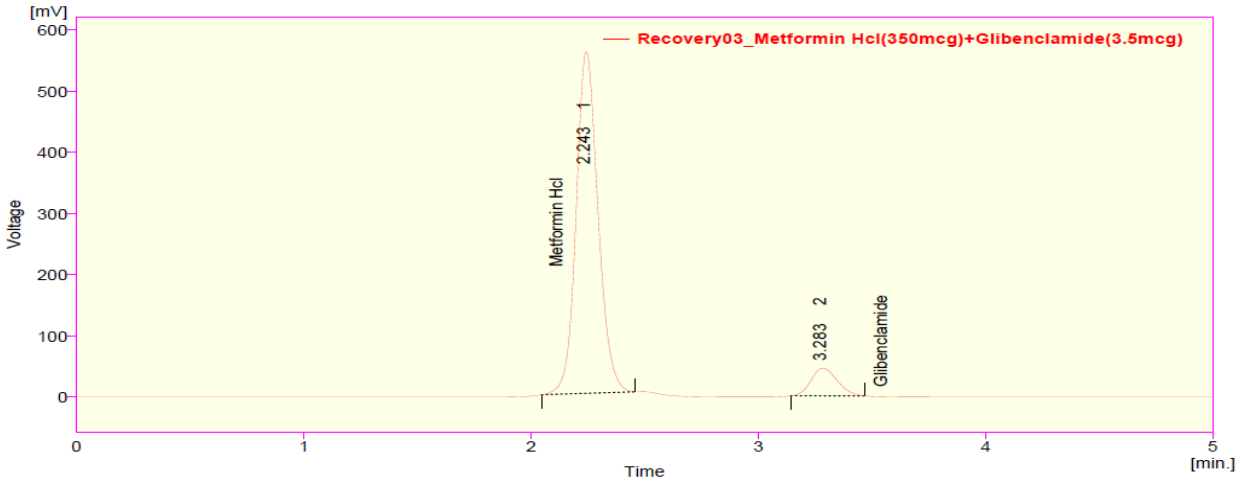


Figure 3.10.3 Chromatograms for Accuracy level -150%

Table 3.10.1 Standard area of Metformin HCl

Conc(µg/ml)	Peak Area
150	1598.165
200	2141.807
250	2777.099
300	3218.275
350	3680.663

Table 3.10.2 accuracy study of Metformin HCl

Conc (µg/ml)	Peak Area
1.5	109.89
2	167.979
2.5	233.116
3	308.562
3.5	361.164

Table 3.10.3 standard area of Glibenclamide

	Pure Drug Conc (µg/ml)	Formulation Conc(µg/ml)	%Recovery of pure drug	area	Average of area	results
50%	250	200	100.49%	2708.906	2690.426	251.23
50%	250	200		2683.091		
50%	250	200		2679.282		
100%	300	250		3323.625		
100%	300	250	100.83%	3401.711		
100%	300	250		3355.017	3360.118	302.48
150%	350	300		3692.432		
150%	350	300	98.75%	3685.647		
150%	350	300		3744.607	3707.607	345.41

Table 3.10.4 Accuracy of Glibenclamide

	PureDrug Conc (µg/ml)	Formulation Conc(µg/ml)	% Recovery of pure drug	Area	Average of area	Results mcg
50%	2.7	2.5			211.861	2.52
			100.90%	214.43		
50%	2.7	2.5				
				21.312		
50%	2.7	2.5		208.861		
100%	3.2	3.0		278.133		
100%	3.2	3,0	98.63%	273.698	275.917	2.96
100%	3.2	3.0		275.921		
150%	3.7	3.5		379.477		
150%	3,7	3.5	98.57%	361.001		
150%	3.7	3.5		362.629	367.629	3.57
	Pure Drug Conc (µg/ml)	**Formulation Conc(µg/ml)**	**% Recovery of pure drug**	**Area**	**Average of area**	**Results mcg**
50%	2.7	2.5				
			100.90%	214.43	211.861	
50%	2.7	2.5				2.52
				21.312		
50%	2.7	2.5		208.861		
100%	3.2	3.0		278.133		
100%	3.2	3,0	98.63%	273.698	275.917	
100%	3.2	3.0		275.921		2.96
150%	3.7	3.5		379.477		
150%	3,7	3.5	98.57%	361.001		
150%	3.7	3.5		362.629	367.629	3.57

Limit of Detection and Limit of Quantitation

The limit of detection and limit of quantitation of the present method were established based on the standard deviation of the response and slope. The slopes were calculated from the respective calibration.

Table 3.11.1 LOD and LOQ Data of Metformin Hcl and Glibenclamide

METFORMIN HCL			GLIBENCLAMIDE		
Conc.(x) (µg/ml)	**Peak Areas (y)**	**Statistical Analysis**	**Conc.(x) (µg/ml)**	**Peak Areas (y)**	**Statistical Analysis**
150	1598.165	S = 10.5	1.5	109.89	S = 128.6
200	2141.807	c = 79.1	2	167.979	c = 0.7906
250	2777.099	σ = 831	2.5	233.116	σ = 102
300	3218.663	LOD OF CON	3	308.562	LOD of con
350	3680.663	24.89µg/ml	3.5	361.164	0.02µg/ml
		Lod of area			Lod of area: 2.61µg/m
		261.59µg/ml			LOQ of con:
		LOQ: 75.44µg/ml			0.061µg/ml
		LOQ:792.68µg/ml			Loa of area7.92v

The lowest possible concentrations of Metformin HCl and Glibenclamide that can be detected by the present method were found to be 62.834µg/ml and 0.18.638µg/ml respectively and that can be Quantitated were found to be 190.409µg/ml and 56.4813µg/ml respectively.

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was done by changing the mobile phase (±1ml), flow rate (±1%), changing the wavelength (±5nm). All the system suitability parameters must be met as per the method.

Procedure

The standard solution was prepared as per the proposed assay method and was injected into HPLC system by changing chromatographic conditions. The actual mobile phase ratio (75:25) and the standard solution was injected and also injected at 74:26 and 76:24. The retention time and peak area of chromatograms was measured and %RSD was calculated. The actual flow rate was 1ml/min and the standard solution was injected and also injected at flow rate 0.9ml/min and 1.1ml/min. The retention time and peak area of chromatograms was measured and %RSD was calculated. The actual wavelength was 256nm and the standard solution was injected and also injected at wavelength 249nm and 259nm.The retention time and peak area of chromatograms was measured and %RSD was calculated [13].

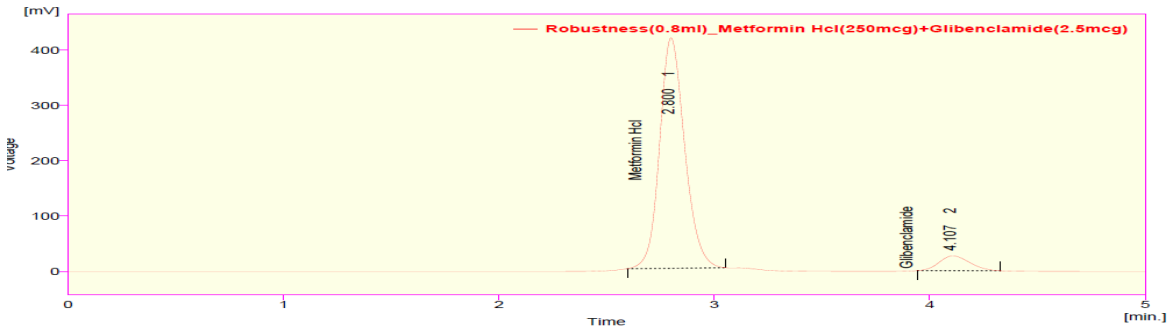


Figure 3.12.1 Robustness (0.8ml) Metformin HCl & Glibenclamide

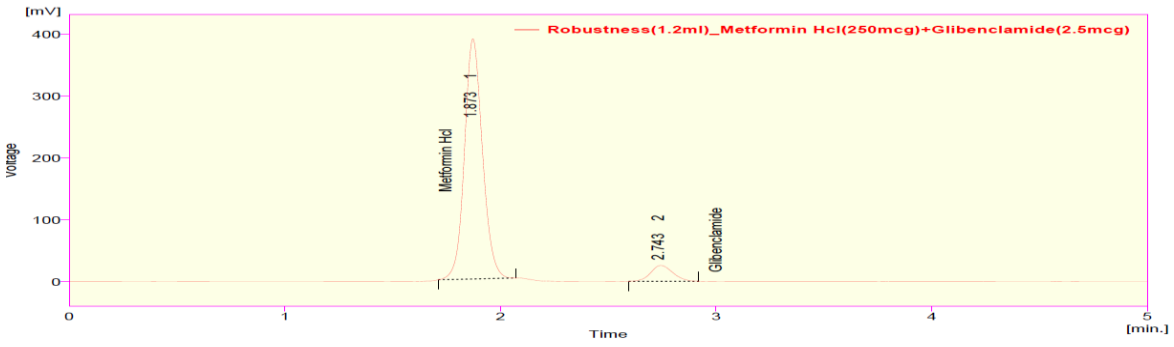


Figure 3.12.2 Robustness (1.2ml) Metformin HCl & Glibenclamide

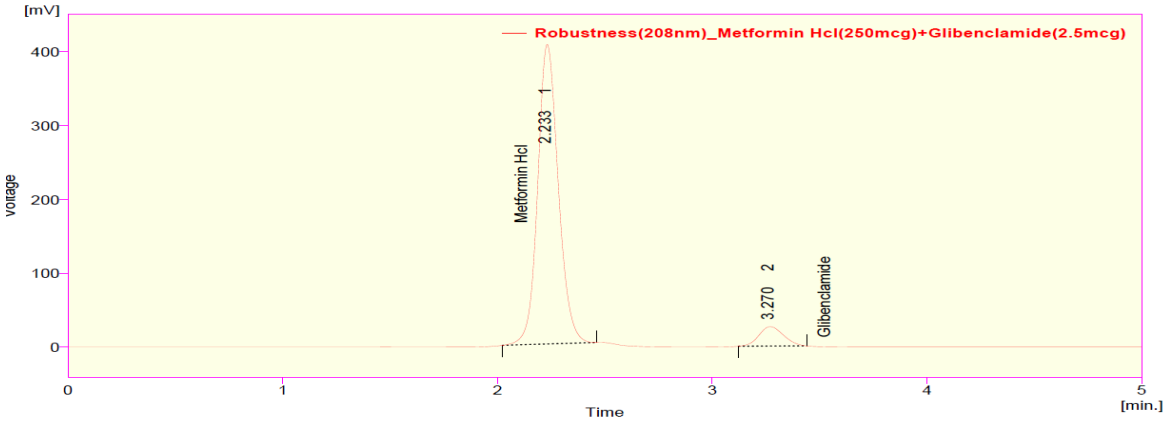


Figure 3.12.3 Robustness (208nm) Metformin HCl & Glibenclamide

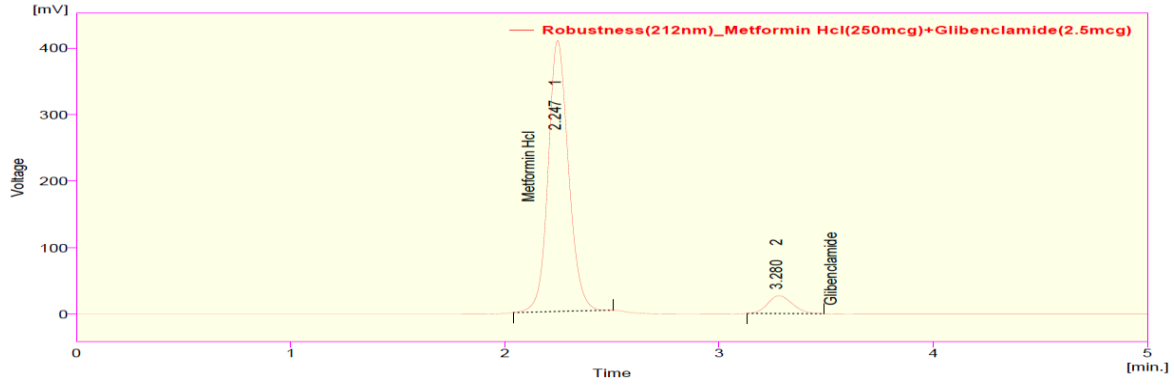


Figure 3.12.4 Robustness (212nm) Metformin HCl & Glibenclamide

Table 3.12.1 Robustness Peak results for Metformin HCl

S.No.	Parameter	Condition	Rt	System suitability results		
				Peak Area	USP tailing	USP Plate Count
1	Flow rate by ± 1%	0.8ml	2.80	3367.409	1.281	28554
		1.2ml	1.87	2256.523	1.167	24002
2	Wavelength of analysis ± 5nm	208nm	2.233	2711.187	1.227	25878
		212nm	.247	2693.483	1.269	27963

Table 3.12.2 Robustness Peak results for Glibenclamide

S. No.	Parameter	Condition	Rt	System suitability results		
				Peak Area	USP tailing	USP Plate Count
1	Flow rate by ± 1%	0.8 ml	4.107	261.38	1.324	38066
		1.2 ml	2.743	186.11	1.241	32466
2	Wavelength of analysis ± 5nm	208nm	3.270	209.94	1.258	36922
		212nm	3.280	216.09	1.290	35267

Ruggedness

Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory, from column to column and from analyst to analyst. All the system suitability parameters should be met as per the method.

Procedure

The sample solution was prepared as per the proposed assay method and injected into HPLC system. The same solution was injected into same HPLC system using another column to check column variability. The same solution was injected into another system to check system variability. The sample solution was prepared by another analyst as per assay method and injected into first HPLC system to check analyst variability. The retention time and peak area of all chromatograms was measured, %assay and RSD was calculated [13].

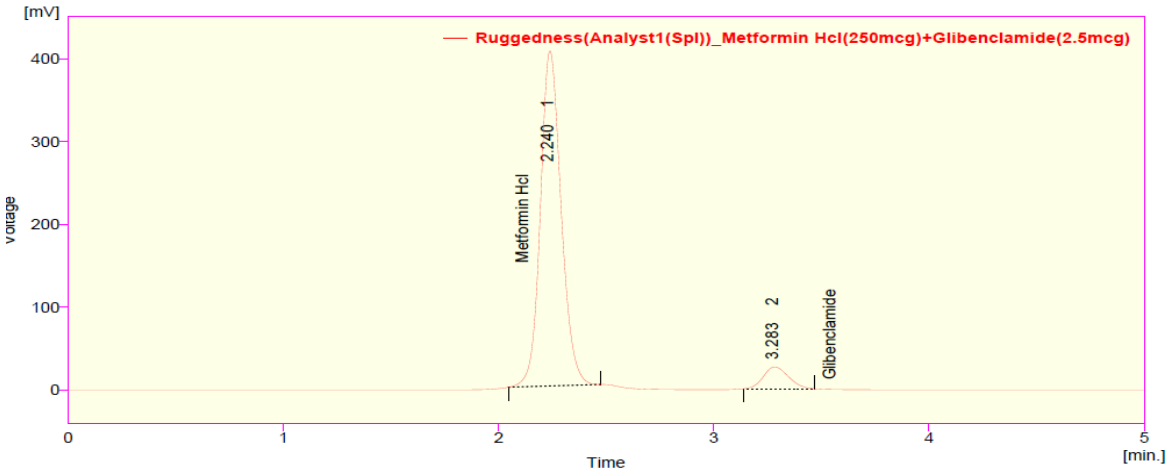


Figure: 3.13.1 Sample (01) Metformin HCl and Glibenclamide for Ruggedness

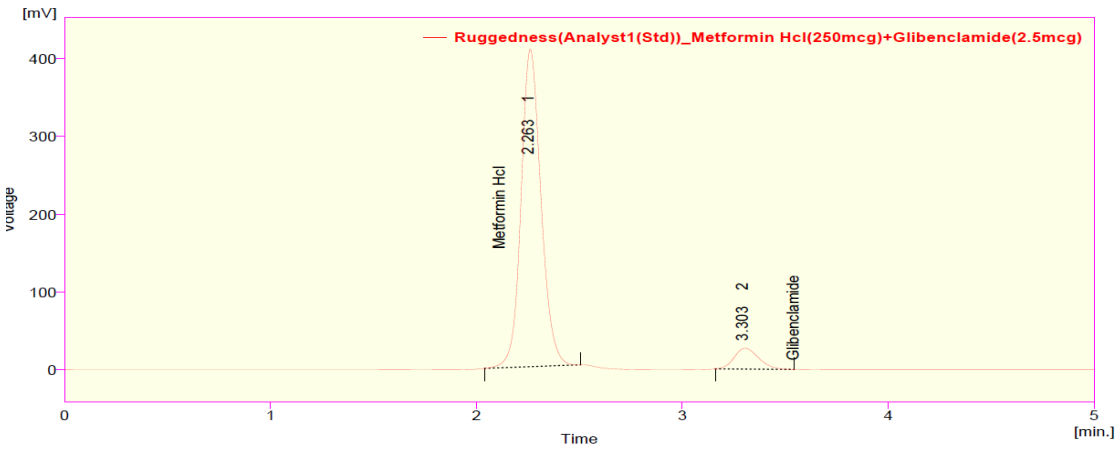


Figure: 3.13.2 Standard 01 Metformin HCl & Glibenclamide for Ruggedness

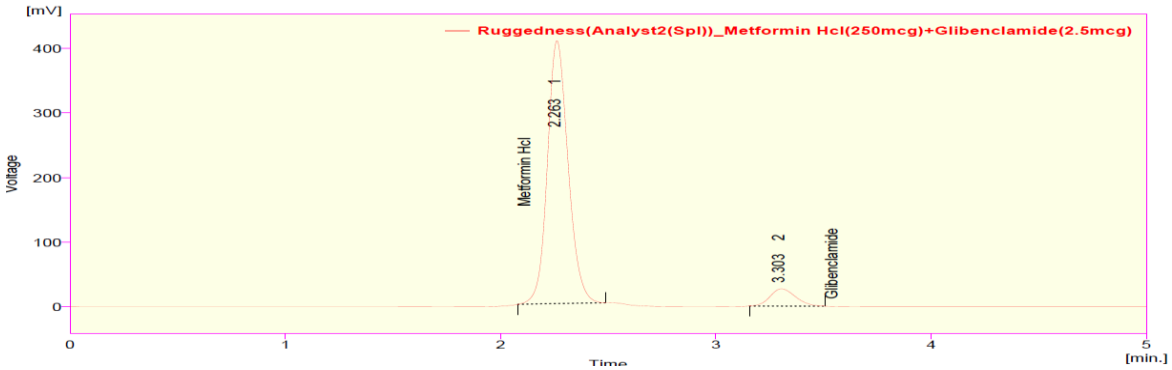


Figure: 3.13.3 Sample 02 Metformin HCl & Glibenclamide for Ruggedness

Table: 3.13.1 Sample & Standard Chromatogram values of Metformin HCl for Ruggedness

	Analyst-1		Analyst-2	
S.No.	**R_t**	**Area**	**Rt**	**Area**
1	2.240	2713.213	2.240	2723.213
2	2.247	2725.243	2.247	2728.829
AVG	2.244	2719.228	2.244	2726.021
STD	_	14.367	_	14.525
%RSD	_	0.526	_	0.532

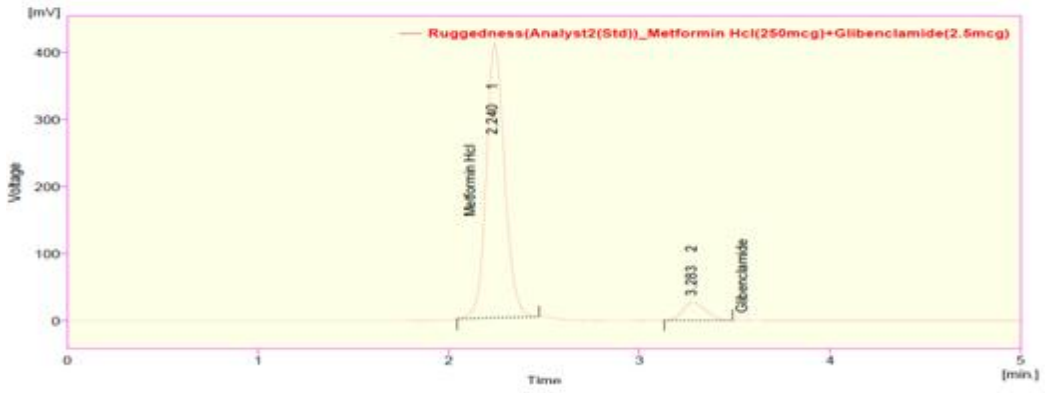


Figure: 3.13.4 Standard 02Metformin HCl and Glibenclamide for Ruggedness

Table. 3.13.2 Standard & Sample Chromatogram values of Glibenclamide for Ruggedness

	Analyst-1		Analyst-2	
S.No.	**Rt**	**Area**	**Rt**	**Area**
1	3.283	214.437	3.303	215.430
2	3.289	217.491	3.301	218.496
AVG	3.286	215.964	3.203	216.963
STD	_	3.307	_	3.321
%RSD	_	1.51	_	1.50

% RSD of peak areas of the solutions evaluated by different analysts was found to be within limits i.e., not more than 2.0%

Estimation of Stability of Drug Solutions

Stability was estimated with standard (at 100% level) and sample solutions. The standard and sample solutions were injected after their preparation and the peak area values were recorded. After 24 hours, the solutions were prepared in the similar way and were injected thrice (in order to minimize errors) along with the solutions of the initial day and the peak areas were recorded. The same procedure was repeated at an interval of 24 hours until there was a significant change (due to degradation) in the peak area values. The fresh solutions were prepared in order to eliminate the effect of the environmental conditions on the stability study [13].

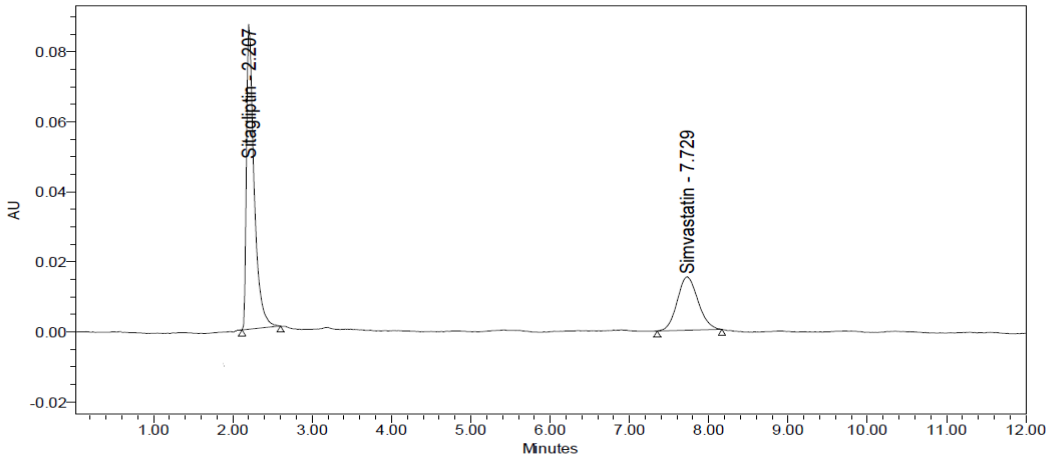


Figure 3.14.1 Chromatogram Recorded at 48th hour

SUMMARY& CONCLUSION

The solubility of the drugs was determined. The scanning of drugs for wavelength in UV region was carried out and wavelength was selected by using UV-Visible detector for the measurement of active ingredients in the proposed method. In HPLC method, the conditions were optimized to obtain an adequate elution of compounds. Initially, various mobile phase compositions were tried to separate the titled ingredients. Mobile phase, column selection, wavelength selection was based on peak parameters (height, tailing factor, theoretical plates, capacity or symmetry factor) and run time. The mobile phase with pH 3.0 buffer, Methanol in isocratic program and a flow rate of 0.1 ml /min was used. The optimum wavelength for detection was 256nm and a run time of 20min.

The HPLC method used for the estimation of Metformin HCl and Glibenclamide from tablets was validated in terms of system suitability, accuracy, precision, linearity, filter validation, solution stability, robustness and ruggedness.

Hence the proposed method was found to be rapid, accurate, precise, specific, robust and economical. The mobile phase is simple to prepare and economical. The method shows non-interference of formulation excipients in the estimation. This method is also having an advantage that the retention time of both the drugs is below 4 min and both the drugs can be assayed with the short time. Thus the method is not time consuming and can be used in laboratories for the routine analysis of combination drugs.

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