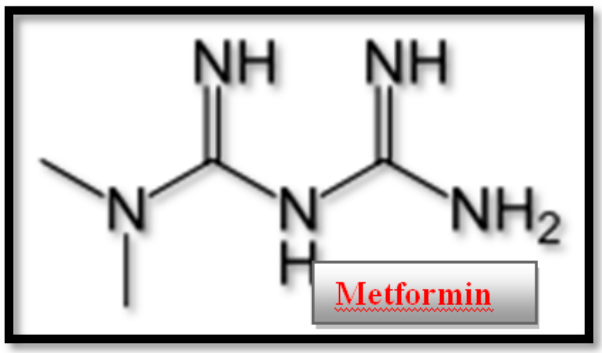
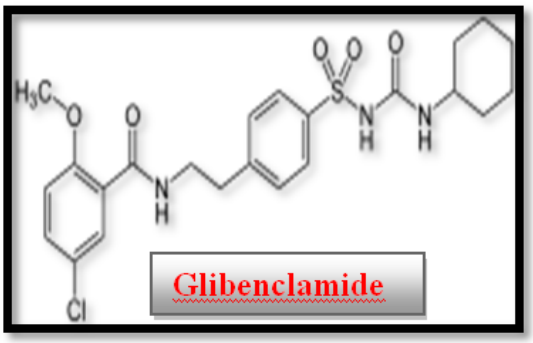
CHAPTER 7





RP - HPLC Method development and validation for simultaneous estimation of Metformin and Glibenclamide in formulation dosage forms

7.1: Introduction:

Detailed description of drugs Metformin and Glebenclimide were given in chapter 4.1

7.2 Literature review:

Detailed description of available literature for the estimation of Metformin and Glebenclimide using different analytical methods were given in chapter 4.2

7.3 Materials and Methods

7.3.1 Chemicals and solvents:

HPLC grade Methanol, Acetonitrile and water were purchased form Merck chemicals, Mumbai. Working standards of pharmaceutical grade Metformin and Glebenclimide were gifted from Cipla, Hyderabad. The market available brand Ben – Q- Met Fort containing Metformin -500mg and Glebenclimide -6.5mg was purchased from local pharmacy.

7.3.2 Instrumentation:

Chromatographic separation was performed on a PEAK chromatographic system with Oyster BDS RP- C18 Column (250mm×4.6mm; 5µm particle size) equipped with LC-P7000 isocratic pump; Rheodyne injector with 20µl fixed volume loop, variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Teccomp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Sonicator (1.5L) Ultrasonicator was used to sonicating the mobile phase and samples. Standard and sample drugs were weighed by using Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted by using Systronics digital pH meter.

7.3.3 Experimental conditions:

According to the solubility and chemical characteristics of both the drugs the mobile phase composition is specified, methanol: acetonitrile: water in 60:20:20 (v/v) at pH 4.3 was used. The isocratic mode of elution was employed for the analysis for the simultaneous determination of drugs. The flow rate was maintained as 1.0ml/min. The

column used was Oyster BDS RP- C18 Column and UV Detector wavelength of 228nm was used to carry total analysis.

7.3.4 Preparation of standard stock solution:

In order to prepare separate stock solutions accurately weigh 10mg of Metformin and Glibenclamide and transfer them into separate 10ml volumetric flask and made up to the volume with methanol. From this stock solution of concentration 1000µg/ml standard solution and different dilutions were prepared.

7.3.5 Preparation of tablet assay solutions:

The average weight of the tablet was calculated by weighing 20 tablets of Ben – Q-Met Fort. The tablet formulation was prepared by crushing and grinding 20tablets of Ben – Q- Met Fort containing 500mg of Metformin and 5mg of Glibenclamide and weigh approximately 10mg from the powdered drug sample. Then transfer it into 10ml volumetric flask and made up to the mark with methanol. The drug solution was sonicated for 2min for proper mixing and then filtered through nylon filter membrane of pore size 0.45µm. from this required sample concentration of 350µg/ml of Metformine was prepared by selected serial dilution method.

7.4 Method development

The HPLC method developed in this study was aimed at developing chromatographic system capable of eluting, resolving the simultaneous determination and separation of Metformin and Glibenclamide in pharmaceutical formulations with suitability conditions. Preliminary studies in method development involved analysis carried out on Chromosil (250 mm x 4.6 mm, 5µ) embedded with isocratic and testing mobile phase compositions with different proportions of methanol: Acetonitrile: water for the separation of Metformin and Glibenclamide but it was accompanied with poor resolution of peaks. Various trails were carried out changing the wavelength, mobile phase composition, pH and flow rate. Further trails were carried out on Oyster BDS RP- C18 Column (250 mm x 4.6 mm, 5µ) with isocratic elution maintaining the moderate flow rate of 0.5-1.0ml/min with mobile phase composition methanol: Acetonitrile: water in the ratio (80:20:20 v/v/) which resulted chromatograms with better resolution compared to previous trails.

Table 7.1: Table showing optimized condition for determination of Metformin and Glibenclamide.

S.NO	Parameter	Results
1	MP	Methanol: Acetonitrile: Water in 60:20:20 (v/v)
2	Wavelength	228 nm
3	Stationary Phase	Oyster BDS RP- C18 Column
4	pH of MP	4.3
5	Flow Rate	1.0 ml/min
6	Run Time	12 min
7	Pump Mode	Isocratic
8	Pump Pressure	15.5 ± 5MPa
9	Api Concentration	Metformin – 350µg/ml Glibenclamide- 3.5µg/ml
10	RT	Metformin – 3.17min Glibenclamide- 8.10min
11	Resolution	Metformin -------- Glibenclamide- 39.20
12	Area	Metformin – 438543 Glibenclamide- 9497
13	Theoretical Plates	Metformin – 7412 Glibenclamide- 96983
14	Tailing Factor	Metformin – 1.28 Glibenclamide- 1.03

The retention time was reported as 3.17min for Metformin and 8.10min for min Glebenclamide. However for improving the tailing factor and retention time was further improved by studying the separation of drug samples of different concentrations at different pH values ranging from 7.0 to 3.0, it was found that good resolution of the peaks were achieved at acidic pH of 4.3.

Figure 7.A: Standard chromatogram of Metformin and Glibenclamide

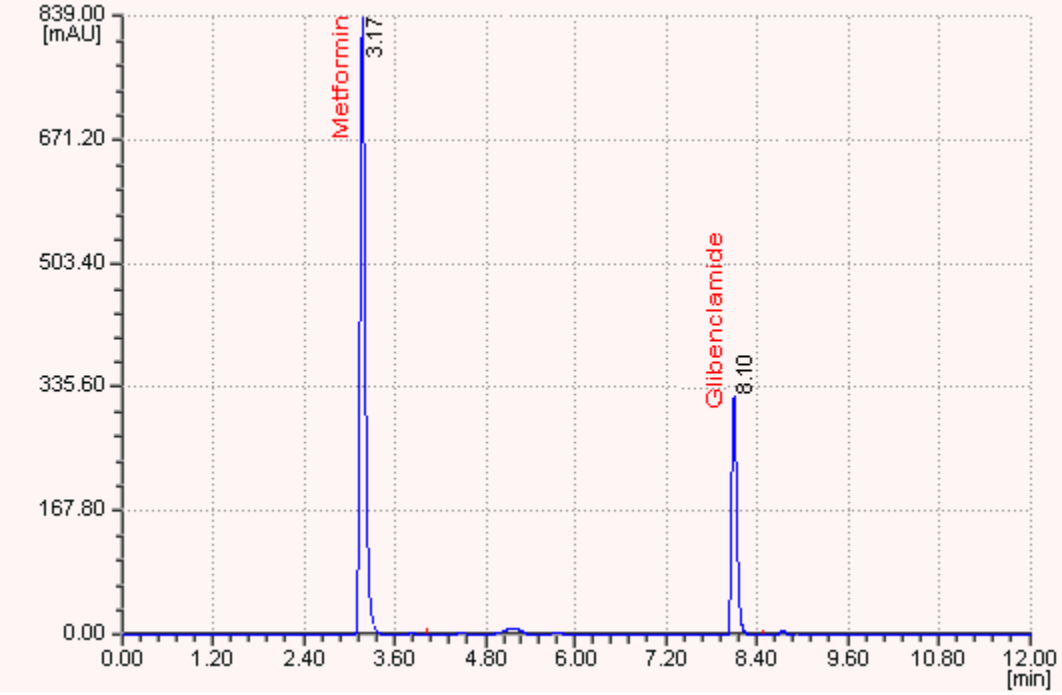
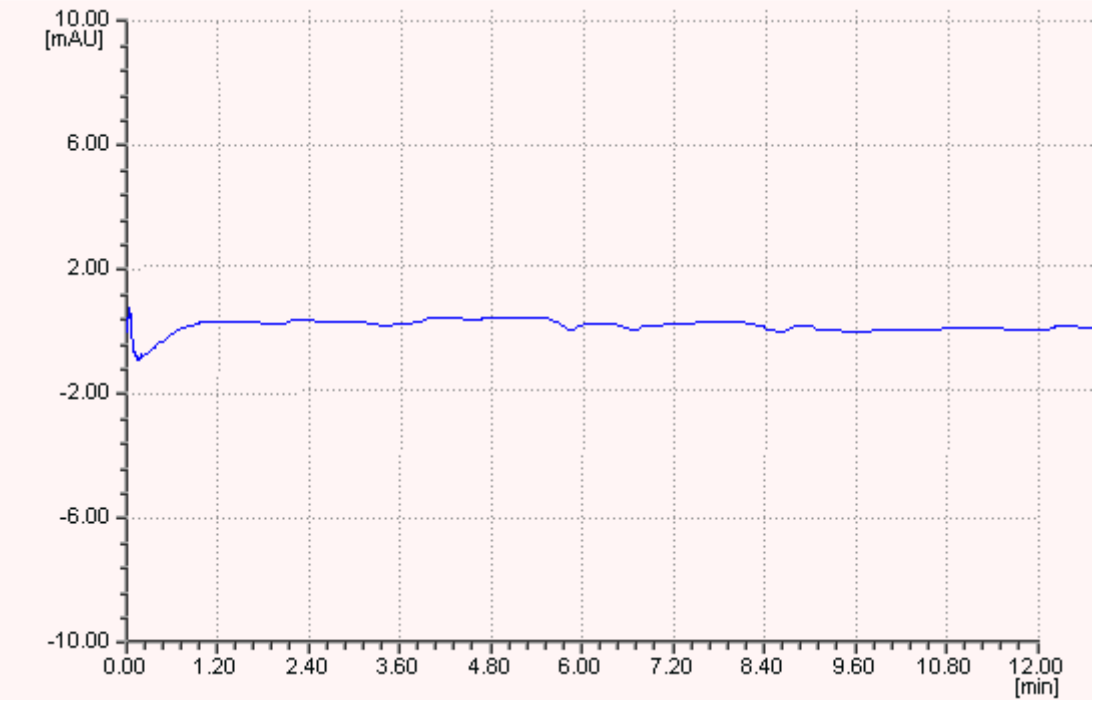


Figure 7.B: Blank chromatogram of Metformin and Glibenclamide



7.5 Method validation

Method validation was performed following ICH specifications for specificity, range of linearity, accuracy, precision, LOD, LOQ, robustness and ruggedness. Validation of the analytical method is to demonstrate that whether it is suitable for intended purpose. Validation of analytical methods is the process by which it is established by laboratory studies that the performance characteristics of the method meet the established by requirements of the analytical applications.

7.5.1 System Suitability

Having optimized the efficiency of a chromatographic separation, the quality of the chromatograph was monitored by applying the following system suitability tests: capacity factor, tailing factor and theoretical plates. The system suitability method acceptance criteria set in each validation run were: capacity factor >2.0, tailing factor ≤2.0 and theoretical plates >2500. In all cases, the relative standard deviation (R.S.D) for the analytic peak area for two consecutive injections was < 2.0%. A chromatogram obtained from reference substance solution is presented. System suitability parameters were shown in Table 7.1. Standard chromatogram was given in Figure 7.A.

7.5.2 Specificity:

The method specificity was assessed by comparing the chromatograms obtained from the drug and the most commonly used excipient mixture with those obtained from blank (excipient solution in water without drug). The excipients chosen are the ones used commonly in tablet formulation, which included di-calcium phosphate (DCP), lactose, starch, micro- crystalline cellulose (MCC), polyvinyl pyrrolidone (PVP), sodium starch glycolate (SSG) and magnesium stearate. The drug to excipient ratio used was similar to that in the commercial formulations. The methods were specific as none of the excipients interfered with the analytes of interest. Hence, the methods were suitably employed for assaying both the drugs in commercial marketed formulation.

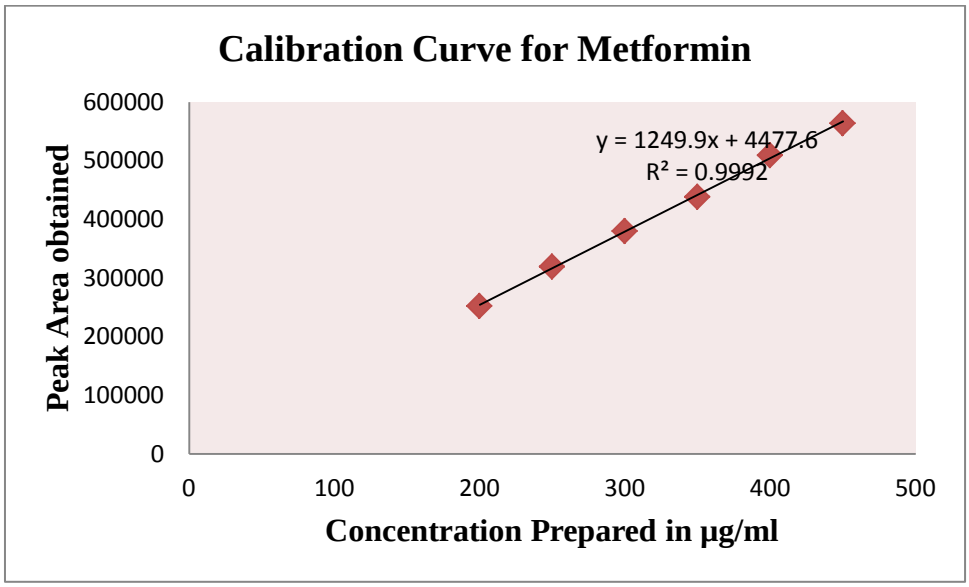
7.5.3 Linearity:

Linearity was determined by series of three to six injections of five of more standards. Different levels of solution were prepared for both the drugs and injected to the chromatographic system and the peak areas were measured. Plotted a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and correlation coefficient was calculated. The linearity of the method was determined at six concentration levels having concentration range 200 µg/ml to 450 µg/ml for Metformin HCl and 2.0 µg/ml to 4.5 µg/ml Glibenclamide. The calibration curve was constructed by plotting Area against Concentration of drugs. The slope and intercept value for calibration curve for Metformin Hydrochloride and Glibenclamide are shown in the Figure 7.C and 7.E for Metformin Hydrochloride and Glibenclamide respectively. Regression equation was found to be y = 1249.x + 4477 (r^2= 0.999) for Metformin and y = 27180x + 1220 (r^2 = 0.998) for Glibenclamide. Results of the linearity confirm that accurate calibration curve range was observed for both the drugs.

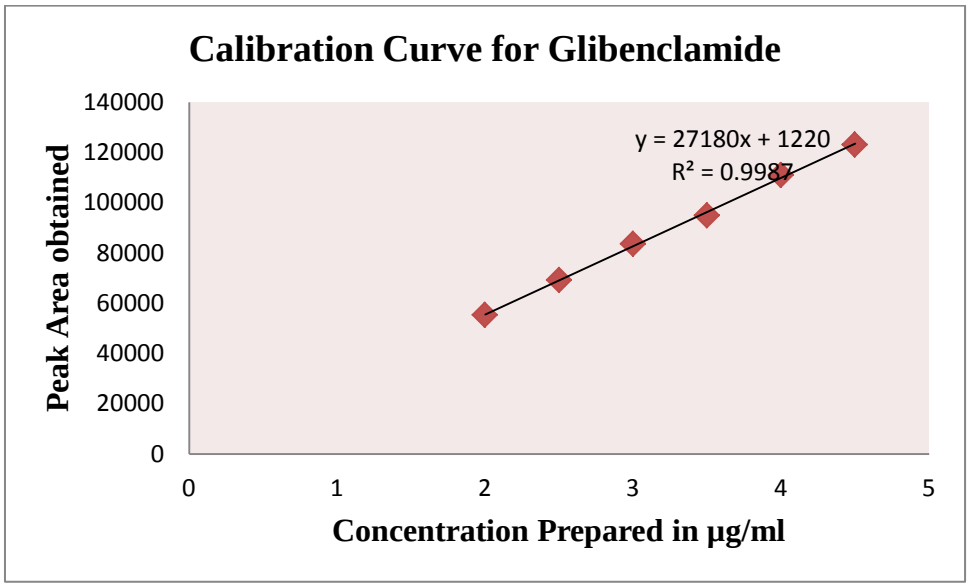
Table 7.2: Table showing linearity results

S.NO	Metformin		Glibenclamide	
	Concentration [µg/ml]	Peak Area	Concentration [µg/ml]	Peak Area
1	200	252235	2.0	55334.5
2	250	319817	2.5	69207
3	300	380004	3.0	83667.2
4	350	438543	3.5	94976.1
5	400	509275	4.0	111033
6	450	564322	4.5	123106

7. C Calibration Curve for Metformin



7.D Calibration Curve for Glibenclamide



7.5.4 Precision

It is a measure of degree of repeatability of an analytical method under normal operation and it is normally expressed as % of relative standard deviation (% RSD). The standard solutions of both the drugs were injected for six times and measured the area for all six injections in HPLC. %RSD was calculated. The % RSD was found to be 0.62, 0.78 for Metformin and 0.59, 0.72 for Glibenclamide in Intraday and Inter day precision respectively. Table 7.3 shows the results of precision experiment for Metformin and Glibenclamide in the developed method.

Table 7.3: Precision results of Metformin and Glibenclamide

S.NO	Metformin at 350 µg/ml		Glibenclamide at 3.5 µg/ml	
	Intraday	Inter day	Intraday	Inter day
1	439593	438761	94282	93491.7
2	438994	431739	94579.9	94625.7
3	433319	433712	95016.8	94706.9
4	435044	438734	94789.7	94322
5	437105	432115	94690.3	95605.8
6	440114	438425	93438.5	94587.7
RSD	0.62	0.78	0.59	0.72

7.5.5 Recovery

To study the accuracy of the developed method, recovery study was carried out by external addition of standard of Metformin and Glibenclamide to the pre-analyzed sample at three different levels 50%, 100% and 150%. The % recoveries of Metformin were obtained in the range 98.87 to 100.008% and in case of Glibenclamide 98.64 to 100.71% were obtained. The recoveries of both the drugs lie in the acceptable range as 90-110% was most accurate for the analysis and estimation of both the drugs in pharmaceutical dosage form when present as combination in a tablet. The recovery results of Metformin and Glibenclamide were shown in Table 7 .4 and 7.5 respectively.

Table 7.4: Recovery results of Metformin

S.NO	%Recovery	Concentration in µg/ml			Amount Found	% recovery
		Target	Spiked	Total		
1	50%	200	100	300	296.65	98.88
2		200	100	300	298.84	99.61
3		200	100	300	297.05	99.02
4	100%	200	200	400	395.48	98.87
5		200	200	400	395.48	98.87
6		200	200	400	396.24	99.06
7	150%	200	300	500	500.35	100.08
8		200	300	500	498.47	99.69
9		200	300	500	498.08	99.61

Table 7.5: Recovery results of Glibenclamide

S.NO	%Recovery	Concentration in µg/ml			Amount Found	% recovery
		Target	Spiked	Total		
1	50%	2	1	3	2.97	99.09
2		2	1	3	2.98	99.35
3		2	1	3	2.98	99.21
4	100%	2	2	4	3.99	99.69
5		2	2	4	3.99	99.73
6		2	2	4	3.99	99.80
7	150%	2	3	5	5.01	100.20
8		2	3	5	4.93	98.64
9		2	3	5	5.03	100.71

7.5.6 Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, pH Variation was made to evaluate the impact on the method. The % change in retention time and all other system suitability conditions decide the robustness of the method. The robustness of the proposed method was evaluated by varying three of the essential chromatographic parameters mobile phase composition, pH and wavelength of the detector. It was reported that very slight changes were observed by changing the chromatographic conditions slightly. Table 7.6 shows the robustness results of Metformin and Glibenclamiode.

Table 7.6: Robustness results of Metformin and Glibenclamide

S.NO	Condition	Change	Metformin at 350 µg/ml		Glibenclamide at 3.5 µg/ml	
			Area	% Change	Area	% Change
1	Standard	……..	295655	……..	171735	…….
2	MP 1	35:55:10 (v/v)	440487	0.44	95506	0.56
3	MP 2	25:65:10 (v/v)	433112	1.24	94061	0.96
4	WL 1	233nm	430440	1.85	93959	1.07
5	WL 2	223nm	431553	1.59	95306	0.35
6	pH 1	4.2	436515	0.46	94952	0.02
7	pH 2	4.4	437077	0.33	95269	0.31

7.5.7 Ruggedness:

Ruggedness was performed by using six replicate injections of standard and sample solutions of concentrations which were prepared and analyzed by different analyst on three different. Ruggedness also expressed in terms of percentage relative standard deviation. Ruggedness of the method was evaluated by injecting the drug samples in six replicates 350 and 3.5µg/ml of Metformin and Glibenclamide respectively. The % RSD of both the drugs was obtained within the acceptable criteria as below 2, results were shown in Table 7.7.

Table 7.7: Ruggedness results of Metformin and Glibenclamide

S.NO	Metformin at 350 µg/ml	Glibenclamide at 3.5 µg/ml
1	440137	95407.6
2	437358	94093.5
3	433495	95506.5
4	439857	94401
5	433968	95717
6	436863	94582
RSD	**0.64**	**0.71**

7.5.8 LOD and LOQ

For both the drug samples LOD were calculated using the formula of S/N where S is the signal obtained from LOD solution (least concentrated solutions of the drug) and N is the Average baseline noise obtained from blank solution. For both the drug samples LOQ were calculated using the formula of S/N where S is the signal obtained from LOQ solution (least concentrated solutions of the drug) and N is the Average baseline noise obtained from blank solution. Sensitivity results of Metformin and Glibenclamide were shown in table 7.8.

Table 7.8: Results of LOD and LOQ:

Parameter	Metformin	Glibenclamide
LOD	1.5µg/ml	0.05µg/ml
LOQ	5.0µg/ml	0.16µg/ml

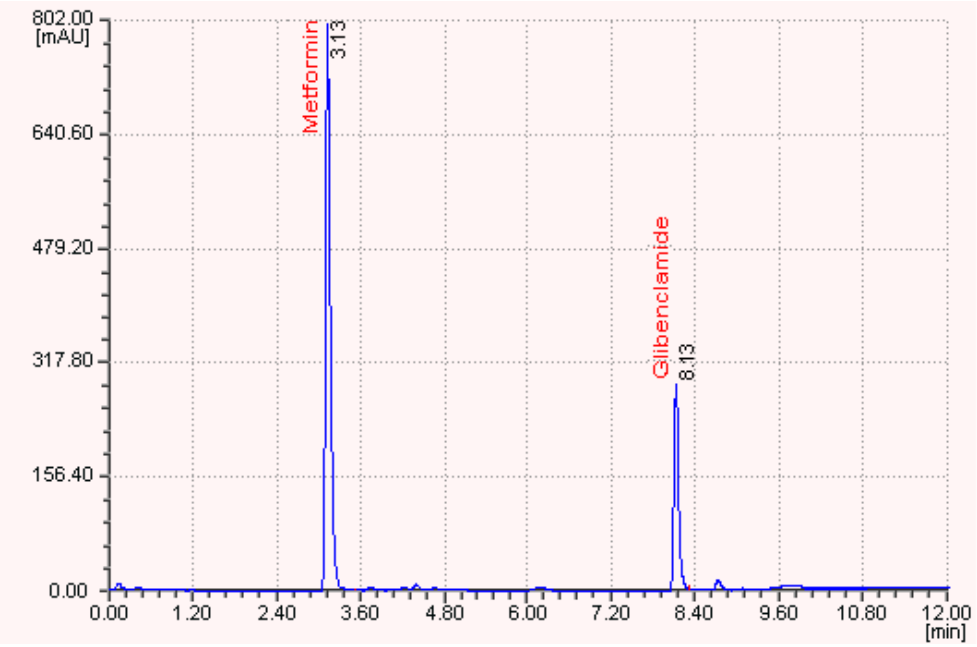
7.5.9 Assay of tablet formulation:

To determine the content of Metformin and Glibenclamide in conventional tablet (Brand name: Ben – Q- Met Fort, containing 500mg of Metformin and 5mg of Glibenclamide twenty tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 500mg of Metformin and 5mg of Glibenclamide into a 50 ml volumetric flask containing 50ml methanol, sonicated for 30min and diluted upto 50ml with methanol. The resulting solution was determined with the concentration (1000µg/ml). Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45-micron filter Nylon membrane. The above stock solutions were further diluted to get sample solutions of 350g/ml and 5g/ml of Metformin and Glibenclamide respectively. A 20 µl volume of sample solution was injected into HPLC, six times, under the conditions described above. The peak areas were recorded at 228nm and concentrations in the samples were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation. Formulation chromatogram was shown in figure 7.E.

Table 7.9: Table showing Formulation results

S.NO	Drug	Brand	Dosage	Amount Prepared	Amount Found	%Assay
1	Metformin	Ben – Q-Met Fort	500mg	350µg/ml	393.44µg/ml	98.36
2	Glibenclamide		5mg	3.5µg/ml	3.48µg/ml	99.40

Figure 7.E: Formulation chromatogram of Metformin and Glibenclamide



7.6 Discussion of the results

The HPLC as optimized with a view to develop precise and effective assay method. Both the pure drugs Metformin and Glibenclamide were run in same composition of mobile phase and same column Oyster BDS RP- C18 Column. The chromatographic conditions was found as optimal for obtaining well defined and resolve peaks at a flow rate of 1.0 ml/min at ambient temperature. The optimum wavelength for detection was used at 228 nm, at which best detector response gave sharp and symmetrical peaks with 3.17 and 8.10min for Metformin and Glibenclamide respectively. All the system suitability parameters were verified lie in the acceptable range. The typical chromatogram of sample solution is shown in Figure.7.A.The percentage of individual drugs was calculated. The results of analysis shows that the amounts of drugs were in good agreement with the label claim of the formulations.

The linearity of the method was determined at six concentration levels having concentration range 200 µg/ml to 450 µg/ml for Metformin HCl and 2.0 µg/ml to 4.5 µg/ml Glibenclamide. The calibration curve was constructed by plotting Area against Concentration of drugs. The slope and intercept value for calibration curve for Metformin Hydrochloride and Glibenclamide are shown in the Figure 7.C and 7.E for Metformin Hydrochloride and Glibenclamide respectively. Regression equation was found to be y = 1249.x + 4477 (r^2= 0.999) for Metformin and y = 27180x + 1220 (r^2 = 0.998) for Glibenclamide. Results of the linearity confirm that accurate calibration curve range was observed for both the drugs.

The % RSD in precision experiment was found to be 0.62, 0.78 for Metformin and 0.59, 0.72 for Glibenclamide in Intraday and Inter day precision respectively. Table 7.3 shows the results of precision experiment for Metformin and Glibenclamide in the developed method.

The method obeys all the validation parameters like accuracy, ruggedness and robustness. LOD was found to be 1.5µg/ml, 0.05µg/ml and LOQ was found to be 5.0µg/ml, 0.16µg/ml for Metformin and Glibenclamide respectively. The % assay was found to be 98.36 for Metformin and 99.40 for Glibenclamide. Hence the method was successfully applied for the routine analysis of Metformin and Glibenclamide in pharmaceutical formulations. The summery results were given in table 7.10

Table 7.10: summery results for Metformin and Glibenclamide

	Parameter	Metformin	Glibenclamide
Method Developed	Elution	Isocratic	
	Mobile Phase	Methanol: Acetonitrile: Water in 60:20:20 (v/v)	
	pH	4.3	
	Column	Oyster BDS RP- C18 Column	
	Wave Length	228 nm	
	Flow	1.0 ml/min	
	Runtime	12 min	
	Temperature	Ambient	
Method validation	Retention Time	3.17 min	8.10 min
	Tailing factor	1.28	1.03
	Theoretical plate	7412	96983
	Resolution	…….	6.20
	Linearity range	200-450 µg/ml	20-4.5 µg/ml
	Slope	1249	27180
	Intercept:	4477	1220
	r^2	0.999	0.998
	Intraday Precision	0.62	0.59
	Interday Precision	0.78	0.72
	Ruggedness	0.67	0.71
	Recovery	98.87-100.08	98.64-100.71
	Robustness difference	0.33-1.85	0.02-1.07
	Limit of Detection	1.5µg/ml	0.05µg/ml
	LOQ	5.0µg/ml	0.16µg/ml
	Formulation assay	98.36	99.40

7.7 Conclusion

The developed RP-HPLC method was proved to be simple, fast and reliable. The method was validated for its performance parameters e.g. Linearity, Repeatability, Accuracy, Precision, Ruggedness, Robustness etc. The developed method offers several advantages in terms of simplicity in mobile phase, isocratic mode of elution and sample preparation steps and comparative short run time makes the method specific, repeatable and reliable for its intended use in simultaneous determination of Metformin and Glibenclamide in tablet dosage form as well as in other formulations.

7.8 References

The references available for the drugs Metronidazole and Diloxanide Fumarate were given in chapter 4.8