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Development and Validation of Glimepiride and Metformin in Human Plasma by HPLC: An application study

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Abstract

A simple, accurate and cost effective RP-HPLC method is developed for simultaneous estimation of glimepiride (GLIM) and metformin (MET) at tablet dosage form using C-18 column (4.6 x 250mm, 5µ, 100 A°) with a mobile phase composed of methanol: water (90:10% v/v) buffered with ortho phosphoric acid at a flow rate of 1.0 mL/min (UV – detection at 231 nm). The retention time of both drugs (GLIM & MET) are observed as 4.286 & 2.262 respectively. Human plasma spiking studies of both the API and the formulation at the concentration of (0.2µg/mL - 1µg/mL) for glimepiride and metformin (1µg/mL - 5µg/mL) expressed the standard correlation coefficients of 0.9998 & 0.9999 respectively for API and 0.9917 & 0.99 respectively for the tablet dosage form. The mean (%) recoveries of glimepiride and metformin are 99.98 and 99.9% respectively. The % RSD below 0.5 shows the high precision of the proposed method. Assay studies revealed that 98.05% of purity is observed for glimepiride and 99.69 for metformin in a tablet dosage form. Human plasma spiking studies revealed that a minimal quantum of glimepiride had been bound with the plasma proteins compare to metformin in the tablet dosage form. The plain plasma chromatogram observed that there is absorption of the human plasma proteins, but there is no interference of drug in the plasma matrix. The method was validated as per the ICH guidelines.

Keywords: RP-HPLC; Glimepiride; Metformin; Human plasma

1. Introduction

Glimepiride is a medium to long acting sulfonyl urea antidiabetic drug. The primary mechanism of action of glimepiride in lowering blood glucose appears to be dependent on stimulating the release of insulin from functioning pancreatic beta cells [1]. Glimepiride is chemically known as 3-ethyl -4- methyl –N-(4[N-(1r, 4r)-4-methyl cyclohexylcarbamoyl) sulfamoyl] phenethyl)-2-oxo-2, 5dihydro-1H-pyrrole-1-carboxamide (Figure 1). Metformin is an oral antidiabetic drug belongs to the biguanide class. It is a first line drug for the treatment of type2 diabetes. Metformin mechanism of action differs from other classes of oral hypoglycemic 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 [2]. Metformin is chemically identified as1-carbamimidamido-N, N-dimethylmethanimidamide (Figure 2). Till date, all analytical methods described simultaneous determination of glimepiride and metformin in liquid chromatography [3], liquid chromatography-tandem mass spectrometry methods [4]. In the present investigation, we developed a simple, accurate, selective liquid chromatographic method for the simultaneous estimation of glimepiride and metformin in both API and pharmaceutical dosage form. This developed method is applied in the human plasma spiking studies.

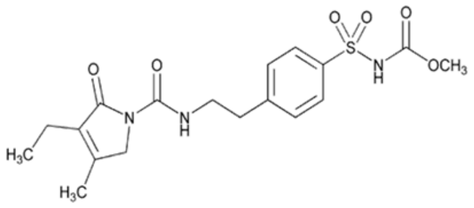

Figure 1: Chemical structure of Glimepiride

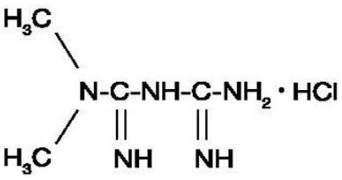

Figure 2: Chemical structure of Metformin hydrochloride

2. Experimental

2.1 Chemicals and Reagents

Glimepiride (GLIM) active pharmaceutical ingredient (API) is supplied by department of pharmaceutical analysis, JSS College of pharmacy, Ooty, India and Metformin (MET) API is obtained from yarrow chemicals, Mumbai, India. Compound Glimepiride and Metformin tablets (each tablet containing 0.2mg of GLIM and 500mg of MET) and the tablets are procured from Sanofi pharmaceuticals, India. The purity of glimepiride and metformin is determined by FT IR spectroscopy and thermogravimetric analysis.

HPLC grade methanol and water is obtained from SD fine chemicals (India). Ortho phosphoric acid AR is procured from Merck (Germany). All other related chemicals which are utilized for the investigation were of analytical grade.

2.2 HPLC instruments and analytical conditions

Chromatographic separation is achieved by utilizing a Shimadzu 20 A liquid chromatographic system (Tokyo, Japan) equipped with 20 AT pump, C-18 column (250mmx4.6mm I.D; particle size 5µm) and a PDA detector (SPD-20A). The system is controlled by LC solutions software in a personal computer. The column temperature is maintained at 25+/-2°C.

The mobile phase consisted of methanol and water (90:10v/v). The apparent pH is to be adjusted at 2.5 with orthophosphoric acid. The isocratic program is used. The flow rate of 1.0mL/min and the injection volume 20µL. Peak purity analysis is carried out over a wavelength range of 190nm – 400nm by the Shimadzu LC solution software. The detection wavelength is set at 215 nm because both the components have higher responses.

2.3 Sample preparation

For the system suitability conditions, 1:5 ratios have been adopted for the dilution of glimepiride and metformin. 10mg/mL of glimepiride and 50mg/mL of metformin is prepared by mobile phase in a 10mL volumetric flask. 5mL of diluent is added and sonicated to dissolve the contents. Finally the volume is made up to 10mL using diluent (mobile phase). These solutions are protected from light by wrapping with aluminum foil and stored at 5° C in the refrigerator.

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The test sample solution is prepared from pulverized compounds of glimepiride and metformin tablets. A 10- tablet equivalent mass corresponding to 0.2 mg of GLIM and 500 mg of MET is weighed and dissolved in mobile phase (methanol: water -90:10v/v) in a 250mL volumetric flask. The volume is made up with mobile phase. After ultra-sonicated for 15 min, the mixture is centrifuged at 6000 rpm for 10 min. The supernatant was separated and transferred in to the HPLC instrument to be for analyzation.

2.4 Validation of developed RP-HPLC method:

As per the International conference of Harmonization guide lines, the developed method should be validated by assessing the parameters such as system suitability, specificity, accuracy, precision, linearity, limit of detection/quantization and assay were optimized.

2.5 ***System suitability:***

2.5.1 Standard stock solution (Mixed stock) preparation: Glimepiride and Metformin standard solutions were prepared in the 1:3 ratios. Glimepiride (0.2mg) and Metformin (1500mg) were transferred in to 10mL volumetric flask. Diluent (5mL) was added and sonicated to dissolve the contents. Finally the volume is made up to 10mL using diluents. The mixed standard solution was injected for 3 times.

2.5.2 Acceptance criteria: Tailing factor for the peaks due to GLIM and MET should not be more than 2

Theoretical plates for the GLIM and MET peaks in standard solution should not be less than 2500

2.6 Specificity:

Glimepiride and Metformin standard solutions were prepared in the 1:3 ratios. Glimepiride (0.2mg) and Metformin (1500mg) were transferred in to 10mL volumetric flask. Diluent (5mL) was added and sonicated to dissolve the contents. Finally the volume is made up to 10mL using diluents.

2.6.1 Preparation of Blank solution: HPLC grade water was used as blank

2.6.2 Standard I solution preparation: Working samples were prepared at 2µg/mL & 6µg/mL of glimepiride and Metformin respectively.

2.6.3 Standard II solution preparation: Working samples were prepared at 4µg/mL & 12µg/mL of glimepiride and metformin respectively.

2.6.4 Standard III solution preparation: Working samples were prepared at 6µg/mL & 18µg/mL of glimepiride and metformin respectively.

2.6.5 Procedure: The blank, standard I, standard II and standard III were injected in replicates

2.7 Accuracy:

Glimepiride and Metformin standard solutions were prepared in the 1:3 ratios. Glimepiride (0.2mg) and Metformin (1500mg) were transferred in to 10mL volumetric flask. Diluent (5mL) was added and sonicated to dissolve the contents. Finally the volume is made up to 10mL using diluents.

2.7.1 Preparation of sample solutions:

2.7.1.1 Preparation of 80% solution: For 80% level, working samples were prepared at 3µg/mL and 9µg/mL of glimepiride and metformin respectively (1:3 ratio of GLIM: MET)

2.7.1.2 Preparation of 100% solution: For 100% level, working samples were prepared at 4µg/mL and 12µg/mL of glimepiride and metformin respectively (1:3 ratio of GLIM: MET)

2.7.1.3 Preparation of 120% solution: For 120% level, working samples were prepared at 5µg/mL and 15µg/mL of glimepiride and metformin respectively (1:3 ratio of GLIM: MET)

2.8 Precision:

Glimepiride and Metformin standard solutions were prepared in the 1:3 ratios. Glimepiride (0.2mg) and Metformin (1500mg) were transferred in to 10mL volumetric flask. Diluent (5mL) was added and sonicated to dissolve the contents. Finally the volume is made up to 10mL using diluents. Working samples were prepared at 6µg/mL and 18µg/mL of glimiperide and metformin respectively. The sample solution was injected for six times and measured the area for all six injections in HPLC.

2.8.1 Acceptance criteria: The percentage (%) RSD for the area of six standard injections results should not be more than 2%

2.9 Limit of detection (LOD) and Limit of Quantization (LOQ)

The LOD and LOQ of glimepiride and metformin were determined on the basis of response and slope of the regression equation.LOD and LOQ values were calculated using the formula: LOD = 3σ/S & LOQ = 10σ/S

Where σ = The standard deviation of the response; S = Slope of the calibration curve.

2.10 Linearity:

0.2mg of glimepiride and 1500mg of Metformin were weighed (1:3ratio) were weighed and transferred to 10mL volumetric flask. 5mL of diluents was added and sonicated to dissolve the contents. Finally, the volume was made up of 10mL using diluents. Linearity range for glimiperide (1µg/mL - 5µg/mL) & metformin (5µg/mL - 25µg/mL) respectively.

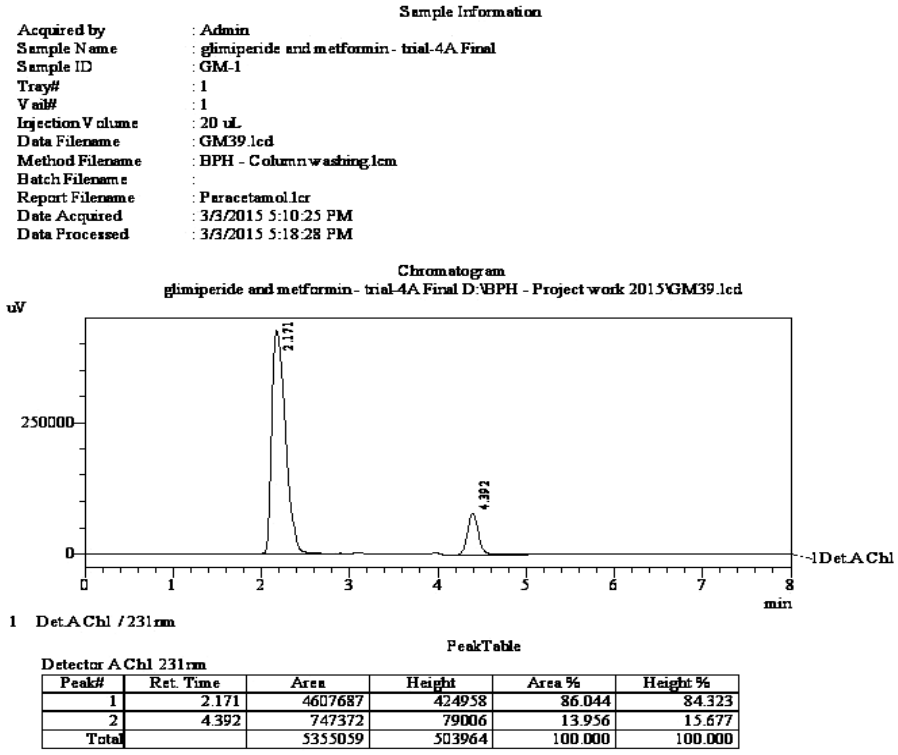
2.11 Human Plasma spiking studies

Interference of matrix effect on developed RP-HPLC method is determined by spiking known concentrations of glimepiride and metformin in human plasma. Healthy human volunteers have been selected and briefed about the research. After briefing, blood samples are collected in the vacutiner mixed with sodium citrate (anticoagulant). The plasma has been separated in a cooling centrifuge (6000rpm) and stored in a cooling refrigerator maintaining the temperature at 2° C +/- 1° C for 48 hr and filtered through 0.22µm filter. Known concentrations of GLIM and MET are spiked in 200µL of plasma and incubated for 6hours. Later, plasma proteins are precipitated using methanol followed by centrifugation at 6000 rpm in a cooling centrifuge. Methanol fractions are collected and purified by solid phase extraction cartridges (Oasis C18). Samples are concentrated and analyzed by RP-HPLC method. Linearity studies were carried out in the concentration range of GLIM (1-5µg/mL) and MET (5-25µg/mL) performed in both API and formulation [5].

3. Results and discussion

3.1 Method development

An isocratic HPLC method is adopted to obtain a shorter run time and high sensitivity based on the polarity differences among GLIM and MET. A stainless steel column (250x4.6mm) is used for chromatographic conditions. To optimize the mobile phase, various combinations of buffer, methanol and water are studied in C18 column. Initially, combination of methanol: water (50:50) is used on trial basis for the separation of GLIM and MET in combined dosage form. It is found that the resolution is not satisfactory. On similar lines, various ratios of combinations of methanol and water are trialed for the resolution of GLIM and MET. The resolution effects are not obtaining good shape. The combination of the solvents was modified by using methanol: water (80:20) found that the peaks with good shape and co-eluted at the RT2.393 & 4.427 for metformin and glimepiride respectively. A typical chromatogram for the method development is shown in Figure 3.



Sample Information

Acquired by : Admin
Sample Name : glimiperide and metformin - trial-4A Final
Sample ID : GM-1
Tray# : 1
V ail# : 1
Injection V olume : 20 uL
Data Filename : GM39.lcd
Method Filename : BPH - Column washing.lcm
Batch Filename :
Report Filename : Paracetamol.lcr
Date Acquired : 3/3/2015 5:10:25 PM
Data Processed : 3/3/2015 5:18:28 PM

Chromatogram
glimiperide and metformin - trial-4A Final D:\BPH - Project work 2015\GM39.lcd

1 Det.A Ch1 / 231nm

PeakTable

Detector A Ch1 231nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.171	4607687	424958	86.044	84.323
2	4.392	747372	79006	13.956	15.677
Total		5355059	503964	100.000	100.000

Glimepiride RT: 4.392 & Metformin RT: 2.171
Figure: 3 Chromatogram of Glimepiride and Metformin in methanol: water (80:20)

3.2 Method Validation

The proposed method is validated as per the ICH (International Conference of Harmonization) guidelines with the aspect of system suitability, specificity, linearity, accuracy, precision, LOD, LOQ and assay according to the ICH guidelines[6].

3.2.1 System suitability test: System suitability is determined by six replicate injections of the system suitability solution. The acceptance criteria is less than 2% relative standard deviation for glimepiride and metformin for peak areas, greater than 5000 column plates, less than 1.5 of the USP tailing factor and greater than 1.5 of the resolution. The results obtained were all within the acceptable limits.

3.2.2 Specificity: The selectivity of the method is confirmed by observing the potential interferences caused by the excipients of tablet formulation under stress conditions prescribed by the ICH [7]. The chromatograms of the excipients are confirmed in Figure 4.

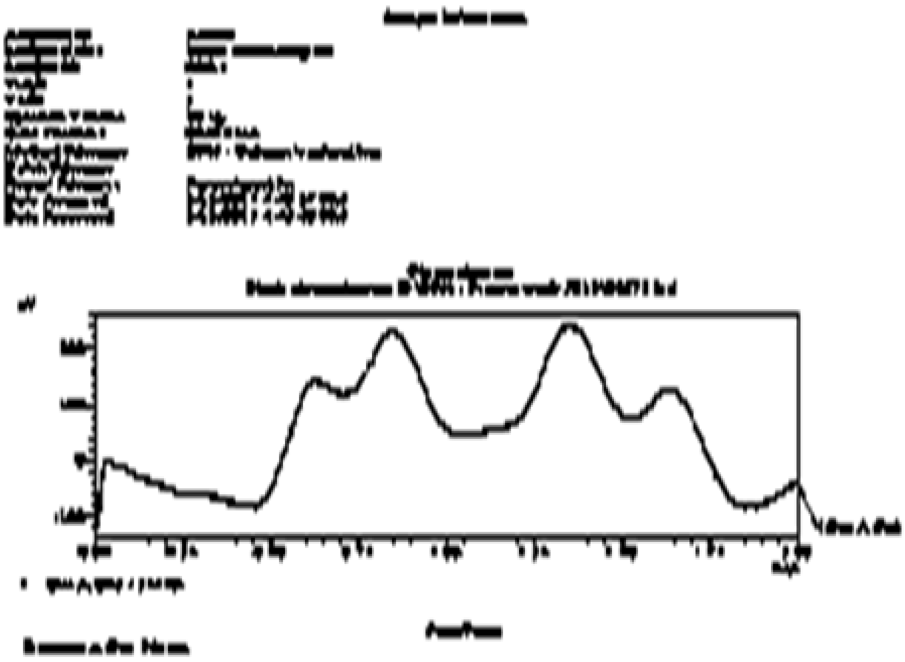
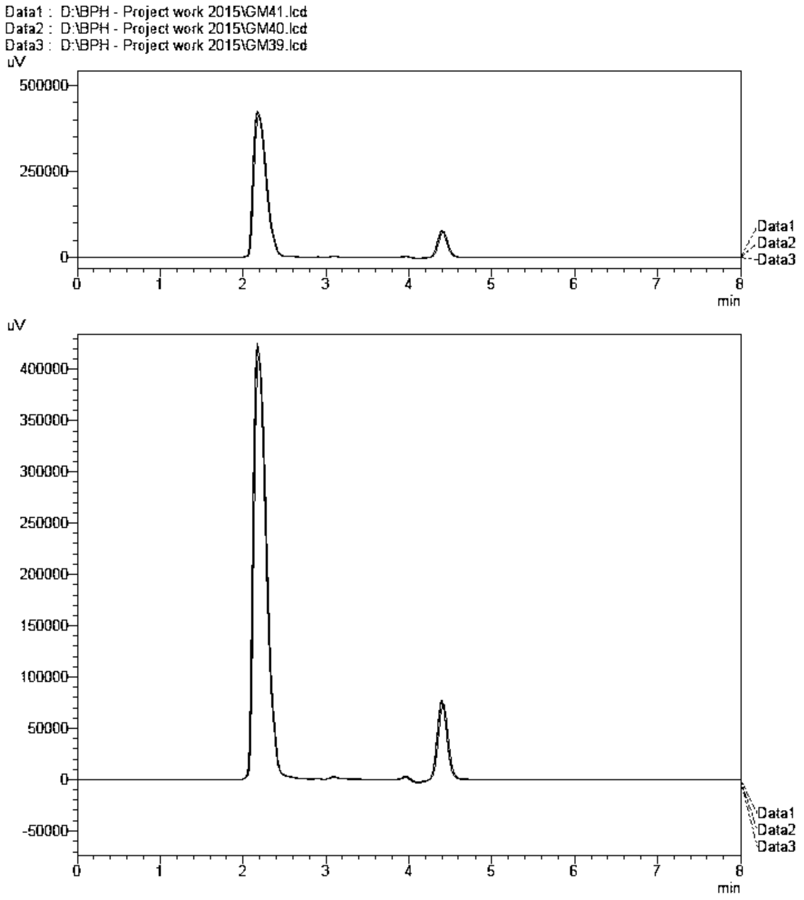


Figure 4: Chromatogram of the tablet excipients

3.2.3 Accuracy and precision

The accuracy of the method is assessed by recovery test. A known amount of each standard powder is added to the blank samples at a concentrations of each drug (0.8, 1.0 &1.2µg/mL for GLIM and 4, 5 &6µg/mL for MET) respectively. The samples are analyzed in triplicate using optimal conditions of RP-HPLC method and chromatograms are recorded in Figure 5. The corresponding percentage recovery data is summarized in Table 1.



Glimepiride – RT: 4.324 & Metformin –RT: 2.156

Figure 5: Overlay representative chromatogram – Accuracy (80%, 100% and 120%)

Table 1: Results of accuracy studies of glimepiride and metformin

S. No	Level	Met	Glim	Glimepiride	Recovery	% of recovery	Metformin	Recovery	% of recovery
		Area	**Area**	**Actual Amt added**			**Actual Amt added**		
1	80	9732111	1985620	0.8	0.7959	99.48	4	4.002	100.05
2	80	9624280	1980842	0.8	0.8048	100.6	4	4.011	100.27
3	80	9691539	1993555	0.8	0.7992	99.9	4	3.986	99.65
	Avg	9682643.3	1986672.33		0.7999	99.99		3.99	99.99
	Std. Devation				**0.0037**	**0.4619**		**0.01141**	**0.2566**
4	100	15510649	3179012	1	1.001	100.1	5	4.994	99.88
5	100	15539415	3174113	1	0.999	99.9	5	5.002	100.04
6	100	15539415	3174113	1	0.999	99.9	5	5.002	100.04
	Avg	15529826.3	3175746		0.999	99.9		4.999	99.98
	Std. Devation				**0.001**	**0.1**		**0.0037**	**0.07549**
7	120	10688589	1903120	1.2	1.22	102	6	5.99	100
8	120	15507955	3172643	1.2	1.25	104	6	6.25	104
9	120	10647405	1929255	1.2	1.21	101	6	6.2	103
	Avg	12281316.33	2335006		1.22	102		6.14	102
	Std. Devation				**0.01732**	**1.4207**		**0.1126**	**1.8905**

Repeatability or intra-day precision is investigated by injecting six replicate of sample solution on the same day of the concentration of 5µg/mL and 25µg/mL of GLIM and MET respectively. Inter day precision is assessed by analyzing newly prepared sample solutions in triplicate over three consecutive days. % RSD values obtained at the peak areas of GLIM and MET on a single day (day1, n=6) are 0.35% and 0.47% respectively. RSD values on triplicate injections on three successive days (days 1-3, n=9) are 1.8% and 2.0% respectively. The results implied that the method developed was accurate for the determination. The results were summarized in Table 2.

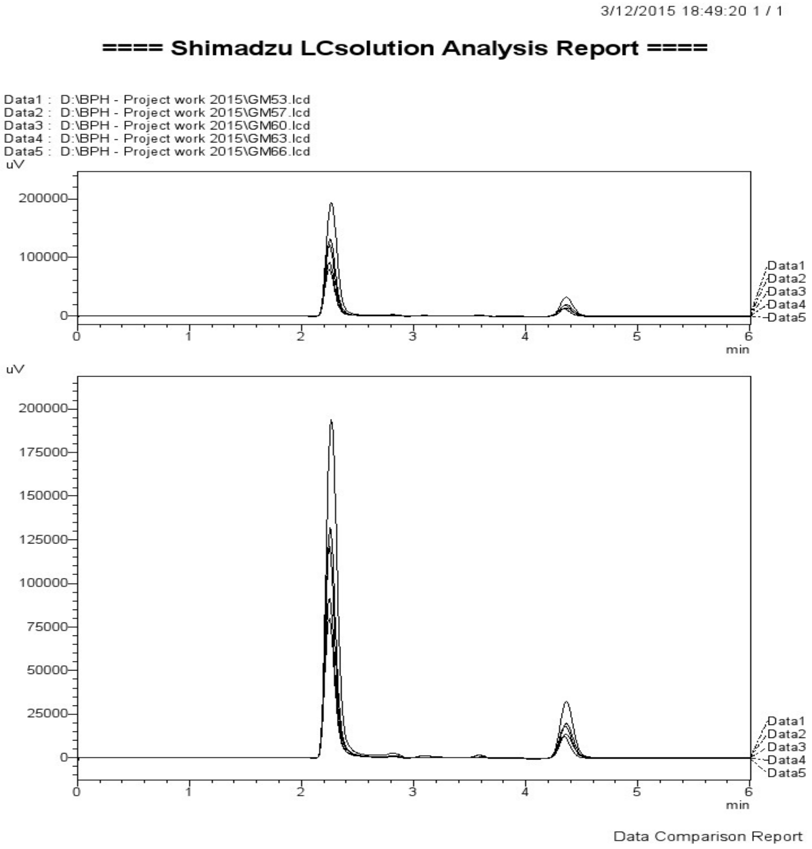
Table 2: Results of Inter & Intraday Precision
Intra day

S. No	Concentration	Glimepiride		Metformin	
		RT	AREA	RT	AREA
1	5µg/mL; 25µg/mL	4.873	325069	2.368	1584325
2		4.858	323544	2.369	1581402
3		4.831	323671	2.36	1583274
4		4.808	326604	2.358	1582188
5		4.789	326504	2.356	1582320
6		4.757	331270	2.342	1586010
	Average	4.8193333	326110.33	2.358833	1583253.16
	Std. Deviation	0	0	0	0
	%CV	0	0	0	0

Inter day

S. No	Concentration	Glimepiride		Metformin	
		RT	AREA	RT	AREA
1	5µg/mL; 25µg/mL	4.431	647647	2.283	3267747
2		4.429	655783	2.28	3293761
3		4.427	659859	2.281	3313726
4		4.436	661417	2.29	3319759
5		4.444	660954	2.295	3322349
6		4.44	663085	2.289	3336555
	Average	4.4345	658214.67	2.28633	3308982.83
	Std. Deviation	0	0	0	0
	%CV	0	0	0	0

3.2.4 Linearity: The linearity is checked by analyzing five working solutions of GLIM over the concentrations range 1-5µg/mL and 5-25µg/mL for MET. The following results were obtained: y = 48594x+60058 (r^2 =0.9999) for GLIM and y=22040x+332389 (r^2 =0.9979) for MET, where y= peak area, x= concentration of solution; r^2 = the square of determined correlation coefficient. The results indicate that the method is linear over the concentration range studied. The typical chromatogram and calibration curve are recorded in Figure.6.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==== Shimadzu LCsolution Analysis Report ====

Data1 : D:\BPH - Project work 2015\GM53.lcd
Data2 : D:\BPH - Project work 2015\GM57.lcd
Data3 : D:\BPH - Project work 2015\GM60.lcd
Data4 : D:\BPH - Project work 2015\GM63.lcd
Data5 : D:\BPH - Project work 2015\GM66.lcd

Data Comparison Report

Glimepiride – RT :4.365 & Metformin –RT :2.257

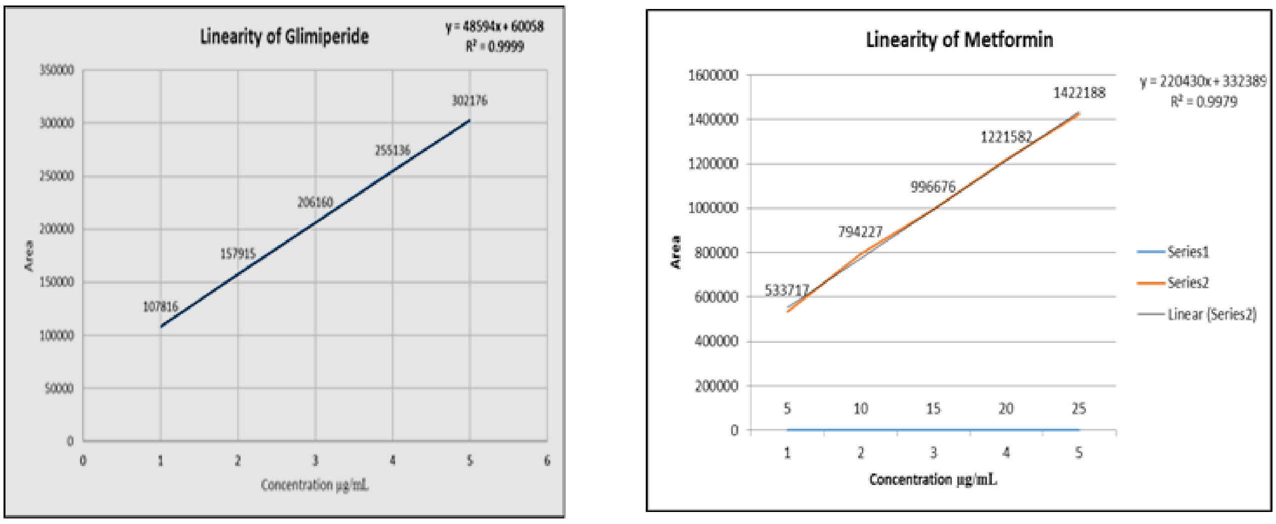


Figure 6: Linearity graph of glimepiride and metformin

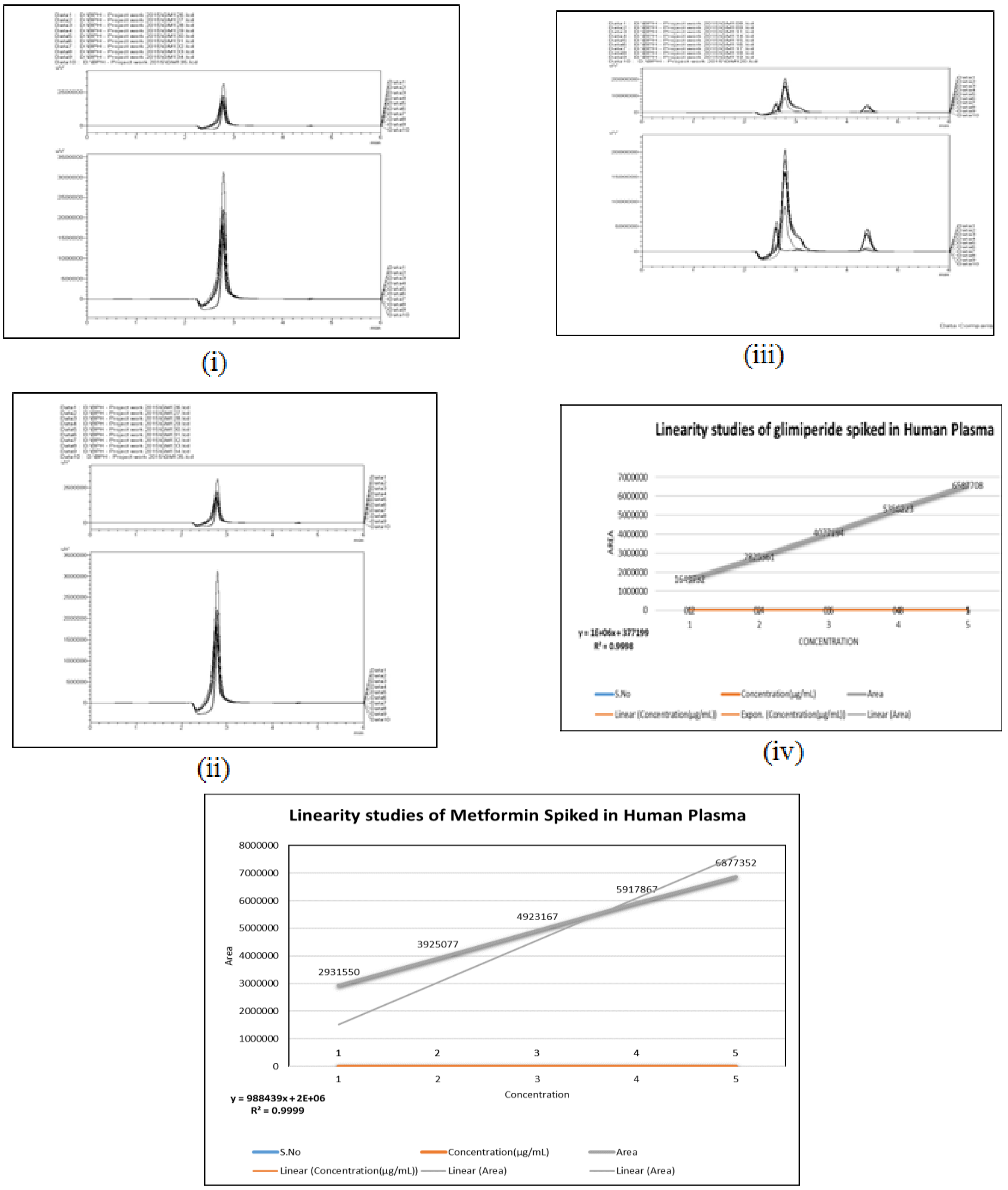
3.2.5 LOQ and LOD: The LOQs for GLIM and MET corresponding to a signal –to –noise ratio of 10 are 0.837 µg/mL and 0.188µg/mL for GLIM and MET respectively. On similar lines, LODs corresponding to a signal – noise ratio of 3 are 0.27µg/mL and 0.01µg/mL respectively.

3.2.6 Assay of GLIM and MET in tablets: Three batches of mixture tablets are analyzed using the developed method. Results obtained were satisfactory that the mean percentage found for GLIM and MET are in good agreement with the label claimed. The mean percentage found and the RSD values are mentioned in the Table.3. It is indicated that the proposed method could be adopted for the determination of GLIM and MET in compound tablets.

Table 3: Assay of Glimepiride and Metformin Tablets

S. No	Sample Area (Glimepiride)	% of Assay of Glimepiride	Sample Area (Metformin)	% of Assay of Metformin
1	577358	98.23	73967191	100.22
2	566705	97.42	73919410	100.16
3	576356	99.08	72661541	98.45
4	561491	96.53	73720671	99.89
5	573398	98.58	73845300	100.06
6	572997	98.51	73328776	99.36
Average	571384.166	98.05	73573814.83	99.69
Standard area	1178435.5		7475927.66	
Std. Deviation		0		0
% RSD		0		0

3.2.7 Human Plasma spiking studies: Linearity of GLIM and MET (both API and Formulation) is performed by the interference matrix effect by spiking the known concentration range of 0.2 -1µg/mL for glimepiride and 1-5µg/mL for metformin in both API and formulation respectively. It is observed that quantitative linearity was obeyed in the above concentrations. The regression equations of concentration at peak levels were found to be y = 37719x (r^2 = 0.9998) and y= 988439x (r^2 = 0.999) for API of glimepiride and metformin respectively. Similarly, compound tablets shows a regression of concentration at peak levels y= 32451x (r^2 = 0.9917) and y= 40006x (r^2 = 0.9953) for glimepiride and metformin respectively. The quantitative linearity graphs and chromatograms are been expressed in Figure 7.



(i) (iii) (ii) (iv)

Figure 7: (i) HPLC overlay chromatogram of blank plasma extract (ii) HPLC overlay chromatogram of Glimepiride and Metformin (API) (iii) HPLC overlay chromatogram of Glimepiride and Metformin (formulation) (iv) Linearity studies of glimepiride spiked in human plasma (v) Linearity studies of metformin spiked in human plasma

4. Conclusion

An isocratic LC method is developed and validated for the analysis of glimepiride and metformin in tablet dosage forms. The results are highly specific and selective. It is observed in the interference of external matrix in human plasma studies revealed that there is absorption of plasma proteins in the glimepiride in the tablet dosage form. On the contrary, there is no interference of drugs (glimepiride and metformin) in plasma matrix in a pure active ingredient form. Hence the validated RP-HPLC method is simple, sensitive, precise and accurate for the simultaneous estimation studies in bioanalytical arena.

Acknowledgement

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