WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF impact Factor 8.084
Volume 9, Issue 1, 1291-1305. Research Article ISSN 2277– 7105

DEVELOPMENT OF NEW SIMULTANEOUS RP-HPLC METHOD FOR THE ESTIMATION OF PIOGLITAZONE AND GLIMEPIRIDE IN THE COMBINED TABLET DOSAGE FORM AND THEN VALIDATION OF THE METHOD

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Article Received on
26 Oct. 2019,

Revised on 16 Nov. 2019,
Accepted on 06 Dec. 2019,

DOI: 10.20959/wjpr20201-16439

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ABSTRACT

In the present work, a rapid, accurate and precise RP-HPLC method for the estimation of Pioglitazone and Glimepiride in tablets dosage form [500/15/2mg] by selecting the various chromatographic parameters. A new method was developed using 250mm x 4.6 mm, reverse phase C 18 column, 5µm (XBridge C18, 250 X 4.6 mm; 5µ) with mobile phase of 40 volumes of potassium dihydrogen phosphate (Phosphate buffer pH 6.8) and 60 volumes of Methanol as mobile phase and Methanol as diluent run as isocratic elution. Flow rate was 1.0 mL-1 with UV detection at 257nm and the injection volume was set at 20µL with 20 minutes of runtime. The method was validated by using various validation parameters like accuracy, precision, linearity, specificity and stability in analytical solution and robustness. All the validation parameters were found to be well within the acceptance criteria. Hence the method can be used for routine estimation of Pioglitazone and Glimepiride tablets [500/15/2 mg].

KEYWORDS: RP-HPLC, Pioglitazone, Glimepiride, Method Development, Analytical Method Validation.

INTRODUCTION

Reversed-Phase HPLC (RP HPLC or RPLC)

Reversed-phase chromatography employs mainly dispersive forces (hydrophobic or van der Waals interactions). The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP HPLC is hydrophobic and mobile phase is polar, where mainly water-based solutions are employed.

Reversed-phase HPLC is by far the most popular mode of chromatography. Almost 90% of all analyses of low-molecular-weight samples are carried out using RP HPLC. One of the main drivers for its enormous popularity is the ability to discriminate very closely related compounds and the ease of variation of retention and selectivity. Principal characteristic defining the identity of this technique is the dominant type of molecular interactions employed such as dispersive forces. As opposed to normal-phase HPLC, reversed-phase chromatography employs mainly dispersive forces (hydrophobic or van der Waals interactions). The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP HPLC is hydrophobic and mobile phase is polar, where mainly water-based solutions are employed.

Reversed-phase HPLC is by far the most popular mode of chromatography. Almost 90% of all analyses of low-molecular-weight samples are carried out using RP HPLC. One of the main drivers for its enormous popularity is the ability to discriminate very closely related compounds and the ease of variation of retention and selectivity. The origin of these advantages could be explained from an energetic point of view: Dispersive forces employed in this separation mode are the weakest intermolecular forces, thereby making the overall background interaction energy in the chromatographic system very low compared to other separation techniques. This low background energy allows for distinguishing very small differences in molecular interactions of closely related analytes. As an analogy, it is possible to compare two spectroscopic techniques: UV and fluorescence spectroscopy. In fluorescence spectroscopy, emission registers essentially against zero background light energy, which makes its sensitivity several orders of magnitude higher than in UV spectroscopy, where background energy is very high. A similar situation is in RP HPLC, where its sensitivity to the minor energetic differences in analyte–surface interactions is very high attributed to the low background interaction energy.

Experimental Study

MATERIALS AND METHODS

Materials and Reagents: Pioglitazone and Glimepiride were procured from Medley Pharmaceuticals Ltd.

Tablets of combined dosage form were purchased from the local drug store.

Potassium di hydrogen phosphate AR Grade, Acetonitrile, and Methanol HPLC grade from Merck chemicals, Mumbai.

Preparation of buffer solution: Buffer was prepared by dissolving 6.8g of Potassium dihydrogen orthophosphate in 1000mL of water and adjusts the pH 6.0 ± 0.02 with dilute KOH filtered followed by the degassing of the solution.

Preparation of mobile phase: 1000mL of mobile phase was prepared by mixing 600ml of buffer solution and 400ml of Acetonitrile.

Preparation of stock solutions: 165mg of Pioglitazone and 20mg of Glimepiride together was transferred to 100mL volumetric flask, dissolved and diluted to volume with mobile phase and kept in an ultrasonic bath until it dissolved completely. This yielded solution containing.

Preparation of Standard solution: Spiked accurately about 5ml of standard stock solution and transferred it into a 100mL volumetric flask. Made the volume up to the mark with mobile phase and mixed well. This yielded solution containing Pioglitazone 82.5ppm and Glimepiride 40ppm.

Table 1: List of Equipment used in the method.

S. No	Name	Make/ Model
1	Analytical balance	Aicoset
2	HPLC instrument Series Software	A HPLC system (WATERS) Alliance e2695 EMPOWER- 2
3	Columns	PHENOMENX LUNA C_{18}(250mm,4.6mm,5µ) SUNFIRE C_{18}(250mm,4.6mm,5µ) SYMMETRYC_{18}(250mm,4.6mm,5µ)
4	Detector	UV – Visible Model SPD 10 AV P
5	Sonicator	SONICA 2200MH
6	pH meter	ELICO
7	Vacuum filter	Model XI 5522050 of Millipore

METHOD DEVELOPMENT

Various chromatographic conditions were experimented to achieve better resolution and efficiency of the chromatographic system. Parameters such as mobile phase composition, wavelength of detection, column, column temperature, and pH of mobile phase were optimized.

According to literature, Pioglitazone and Glimepiride are freely soluble in methanol and acetonitrile. And it was checked for different dilutions of methanol and acetonitrile for solubility of Pioglitazone and Glimepiride. Finally acetonitrile was chosen as solvent for present work.

From the UV- Visible spectrophotometric results, the detection wavelength of 260nm (for pioglitazone) and 230nm (for Glimepiride) was selected because at this wavelength they shows maximum absorbance and then 230nm was selected as common wavelength for simultaneous estimation of both the drugs as these are eluting in the same mobile phase at maximum absorbance. Also at 230nm the chromatogram was observed in PDA detector which was having very less absorbance compare to 260nm. So the chromatographic condition was optimized at 230nm.

The different columns like SUNFIRE C_{18} (250mm, 4.6mm, 5µ), SYMMETRYC_{18} (250mm, 4.6mm, 5µ) did not show adequate resolution and column efficiency. The column PHENOMENX LUNA C_{18} (250 X 4.6mm, 5µ) had shown good resolution and efficiency.

Buffers like sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate did not yield desired results.

The composition of mobile phase Buffer: Acetonitrile (60:40%v/v) of pH 6 with flow rate of 1.5ml/min and detection at 230nm of runtime of 30 min obtained a peaks eluted at 4.26minute of Glimepiride and 6.26 minute of pioglitazone. Tailing and fronting observed with the analyte peaks. The composition of mobile phase Buffer: Acetonitrile (80:20%v/v)of pH 6 with flow rate of 1.3 ml/min and detection at 230nm of runtime of 30 min yielded peaks at 2.56 min for Glimepiride and 4.27minute for pioglitazone, which showed fronting. The peaks were very broad.

At Buffer: Acetonitrile (60:40%v/v) of pH 6 with flow rate of 1.5 ml/min and detection at 230nm of runtime of 30 min, a perfect chromatogram was eluted.

Table 2: Optimized Chromatographic conditions of Pioglitazone and Glimepiride.

Parameters	Method
Stationary phase (column)	PHENOMENX LUNA C_{18} (150 X
	4.6mm, 5 µ)
Mobile Phase	Buffer: Acetonitrile (60:40%v/v)
pH	6 ± 0.02
Flow rate (ml/min)	1.5
Run time (minutes)	30.0
Column temperature (°C)	Ambient
Volume of injection loop (□l)	20

The typical chromatogram obtained from final HPLC conditions are depicted in **Figure 3.**

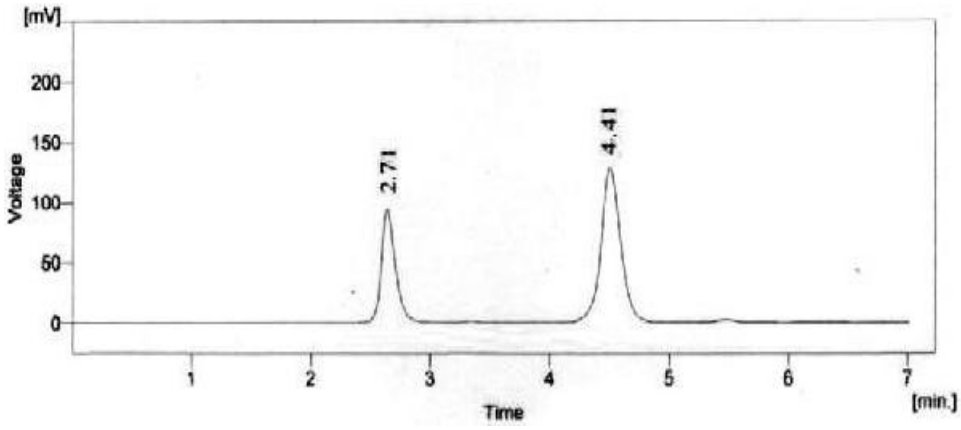

Figure 1: Typical chromatogram of Pioglitazone and Glimepiride by optimised method

Method validation.

As per ICH guidelines, the method validation parameters checked were system suitability, specificity, precision, accuracy, linearity, and robustness, limit of detection and limit of quantification.

System Suitability

Standard solutions of Pioglitazone and Glimepiride were prepared as per procedure and were injected six times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from six replicate injections.

1. The % RSD for the retention times of principal peak from 6 replicate injections of each standard solution should be not more than 2.0%.

2. The number of theoretical plates (N) for the Pioglitazone and Glimepiride peaks should be NLT 2000.
3. The Tailing factor (T) for the Pioglitazone and Glimepiride peaks should be NMT 2.0.

Assay

$$\text{Assay\%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AVGWt}{\text{Label Claim}} \times 100$$

Where, AT = Peak Area of obtained with test preparation.

AS = Peak Area of obtained with standard preparation.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Precision: The system precision of the test method was performed by injecting 6 replicate determination of test sample against a qualified reference standard and the % RSD was calculated (% RSD should not be more than 2%).

Accuracy: The accuracy was carried out using samples prepared for assay. Accuracy studies were conducted using triplicate determination as per the test method by injecting the sample thrice into HPLC system and the average peak area was calculated from which Percentage recoveries were calculated. (% Recovery should be between 98.0 to 102.0%).

Linearity: The linearity of detector response was established by plotting a graph to concentration versus area of Pioglitazone and Glimepiride standard and determining the correlation coefficient. A series of solution of Pioglitazone and Glimepiride standard solution in the concentration ranging from about 240-350µg/ml of Pioglitazone and 32-50µg/ml of Glimepiride respective levels of the target concentration were prepared and injected into the HPLC system.(Correlation coefficient should be not less than 0.999.)

Limit of Detection (LOD) and Limit of Quantification (LOQ): LOD and LOQ for the were determined at signal to noise ratios of 3:1 and 10:1, respectively by injecting series of dilute solutions with known concentrations.

Robustness

For the evaluation of robustness, in the concentration range 240-350µg/ml of Pioglitazone and 32-50µg/ml of Glimepiride were injected thrice in to the system in all deliberately varied conditions such as flow rate from 1.3ml/min to 1.7ml/min (□0.2ml/min) and the variation in wavelength from 228 to 232 (□ 0.2ml/min) do not affect the method significantly.

Specificity

In case of simultaneous assay of Pioglitazone and Glimepiride, demonstration of Specificity requires that the procedure is unaffected by the presence of impurities or excipients. For the evaluation of specificity one blank and standard were injected to HPLC and checked for the additional peaks in the chromatogram.

RESULTS AND DISC USSION

System suitability: The system suitability parameter tailing factor for the proposed HPLC method from the standard injection of Pioglitazone and Glimepiride are 1.122 and 1.273 respectively. Theoretical Plates obtained from the standard injection of Pioglitazone and Glimepiride are 3888 and 2577 respectively. The resolution obtained with the proposed method was 7.395. The results proved that the optimized HPLC method fulfils these requirements within the U SP accepted limits.

Table 3: The summary of System Suitability for Pioglitazone and Glimepiride.

Parameter	Pioglitazone	Glimepiride
Resolution	7.395	
Tailing Factor	1.122	1.273
Number of theoretical plate	3888	2577

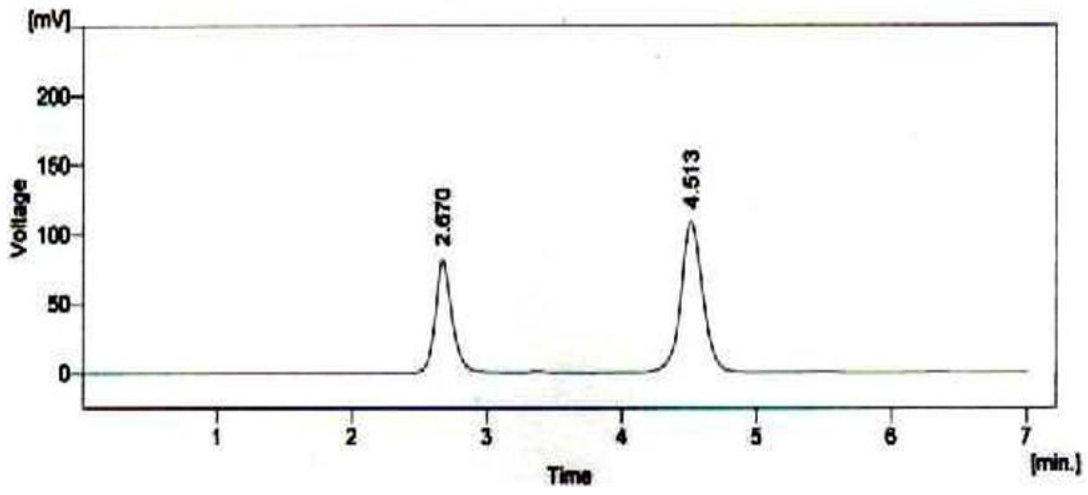

Figure 2: Chromatogram of System suitability.

Precision: The % R.S.D. of Pioglitazone and Glimepiride assay during the method precision was found to be 0.052948% and 0.089858% respectively, indicating excellent precision of the method.

Table 4: Summary of results of Method Precision parameter for Pioglitazone and Glimepiride.

Injection	Pioglitazone			Glimepiride		
	Area			Area		
S.No.	Inj-1	Inj-2	Avg.	Inj-1	Inj-2	Avg.
MP-1	1243.459	1235.157	1239.308	644.166	653.352	648.759
MP-2	1247.629	1241.357	1244.493	650.229	657.245	653.737
MP-3	1245.456	1233.257	1239.356	648.241	656.984	652.612
MP-4	1239.452	1245.214	1242.333	646.321	654.325	650.323
MP-5	1238.258	1246.321	1242.289	644.256	654.021	649.138
MP-6	1244.245	1236.425	1240.335	653.254	648.221	650.737
MEAN	1241.352			650.884		
SD	6.349054			9.807		
% RSD	0.511162			0.5028		

Table 5: Summary of results of Injection Precision parameter for Pioglitazone and Glimepiride.

S.No.	Pioglitazone	Glimepiride
SP-1	1237977	643296
SP-2	1243238	650187
SP-3	1244499	664745
SP-4	1242860	649951
SP-5	1243500	650067
SP-6	1239251	641870
Mean	1241887.5	650019.33
SD	2624.23	8105.49
% RSD	0.21	1.25

Accuracy: Percent recovery of Pioglitazone samples ranged from 98.6% to 101.3%, and the Percent recovery of Glimepiride samples ranged from 98.9% to 101.5% showing the good accuracy of the method.

Table 6: Summary of results of Accuracy parameter for Pioglitazone and Glimepiride Pioglitazone.

Recovery Level	Standard Injections				% Recovery	% RSD
	Inj-1	Inj-2	Inj-3	Average		
80%	1029743	1029744	1029742	1029743	101.3	
100%	1243605	1243606	1243607	1243606	99.8	1.35
120%	1474804	1474802	1474803	1474803	98.6	
Glimepiride						
80%	527151	527153	527152	527152	101.5	
100%	646426	646427	646425	646426	100.5	1.31
120%	763119	763118	763117	763118	98.9	

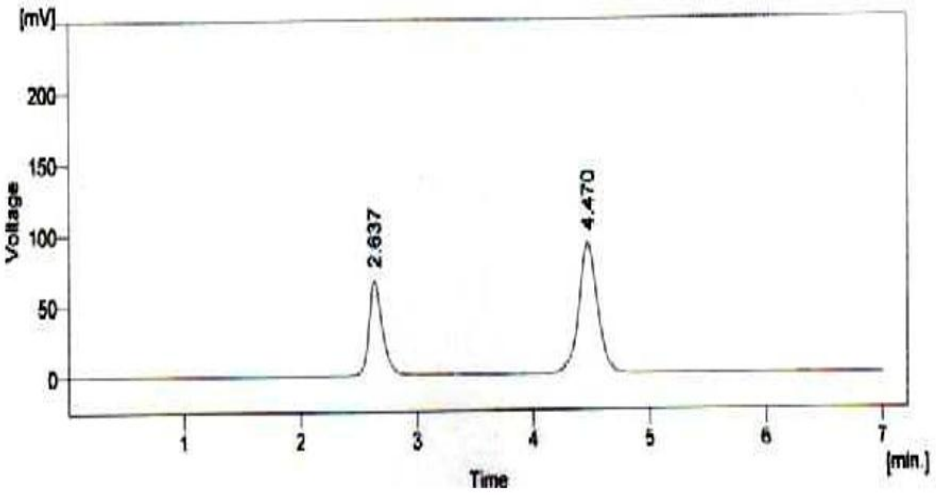

Figure 3: Chromatogram for recovery of 80%.

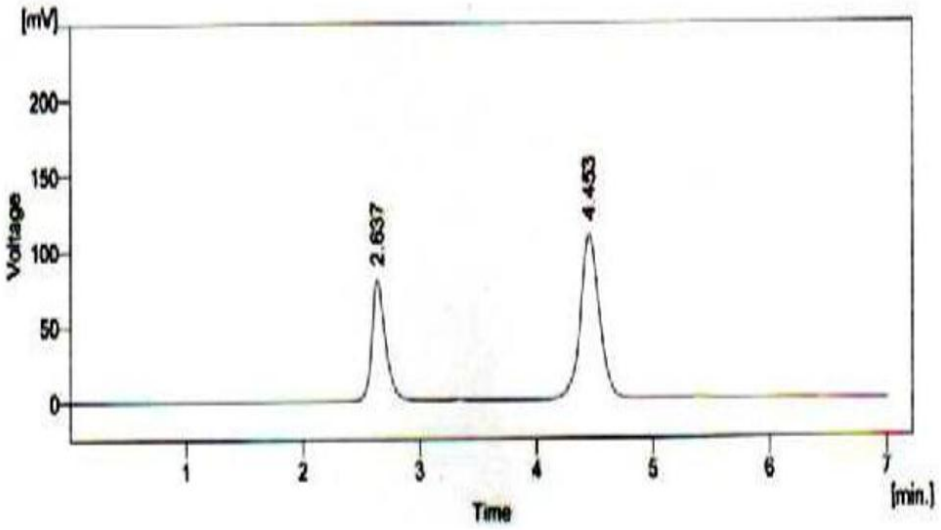

Figure 4: Chromatogram for recovery of 100%.

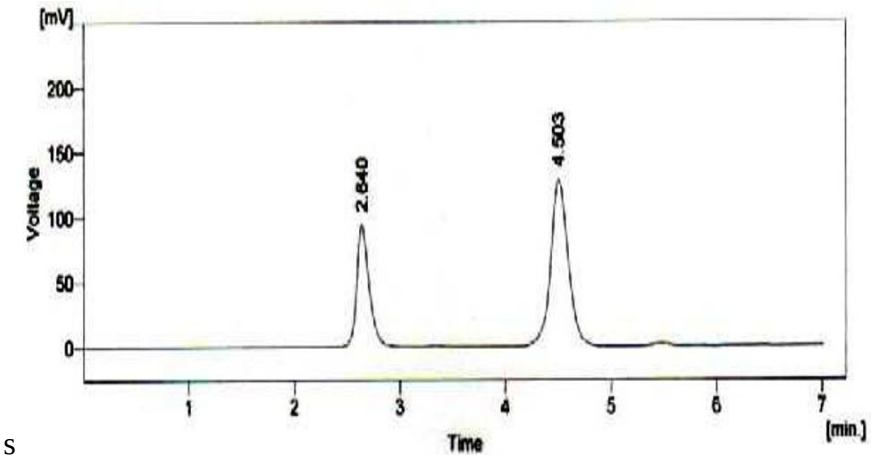

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Figure 5: Chromatogram for recovery of 120%.

Linearity: The linearity of the calibration plot for the method was obtained over the calibration ranges tested, i.e., 240-350µg/ml for Pioglitazone and32 - 48 µg/ml for Glimepiride three times, and the correlation coefficient obtained was 0.997 and 0.998 for Pioglitazone and Glimepiride respectively, thus indicating excellent correlation between peak areas and concentrations of the analytes.

Table 7: Summary of results of Linearity parameter for Pioglitazone and Glimepiride

Injection	Pioglitazone		Glimepiride	
S. No.	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	240	1002974	32	533916
2	270	1132050	36	587608
3	300	1244040	40	649797
4	330	1368367	44	710856
5	360	1481264	48	768578

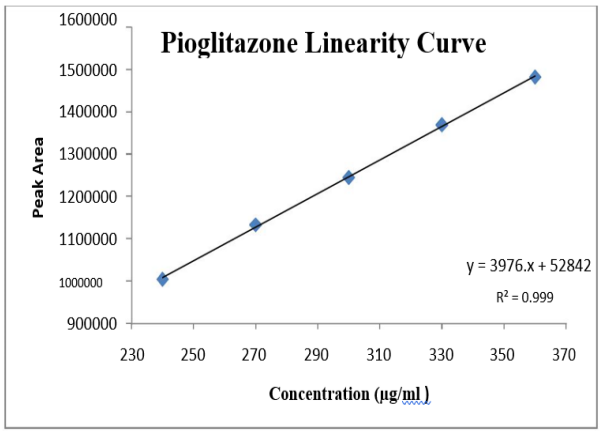


Figure 6: Linearity Curve of Pioglitazone

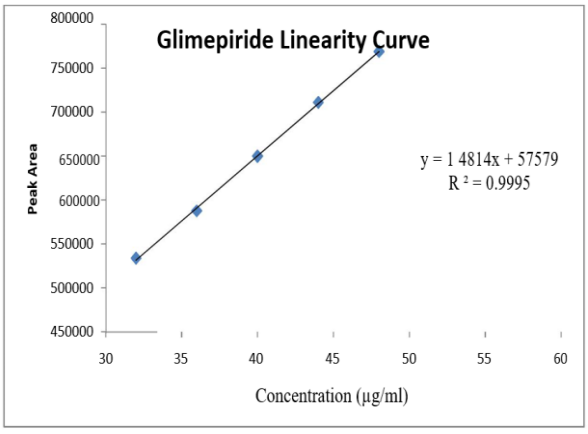


Figure 7: Linearity Curve of Glimepiride.

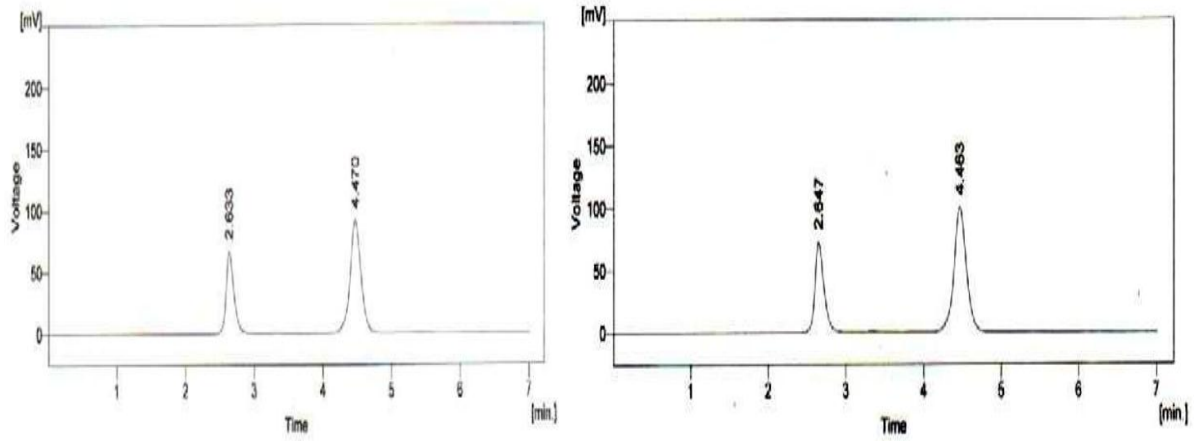


Figure 8: Chromatogram for Linearity of Pioglitazone and Glimepiride of Injection-1 and 2.

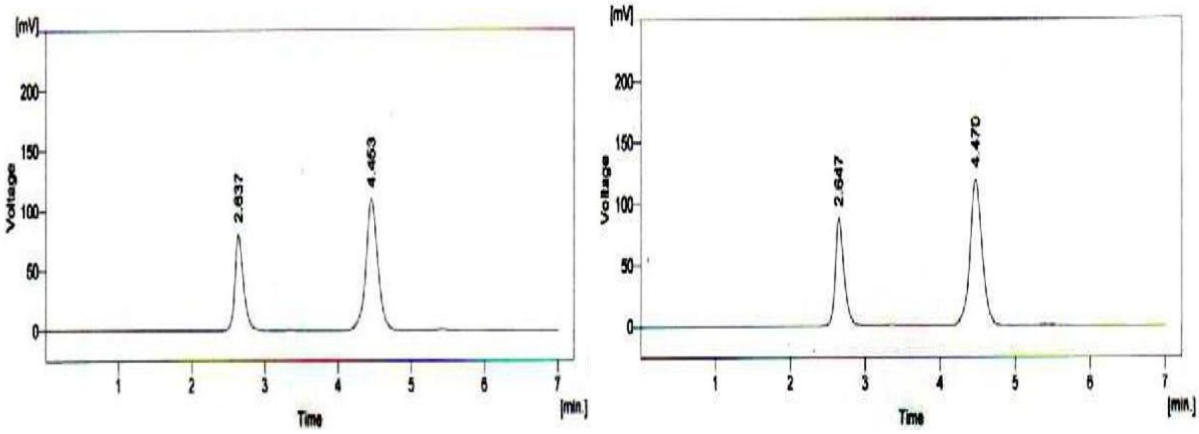


Figure 9: Chromatogram for Linearity of Pioglitazone and Glimepiride of Injection-3 and 4.

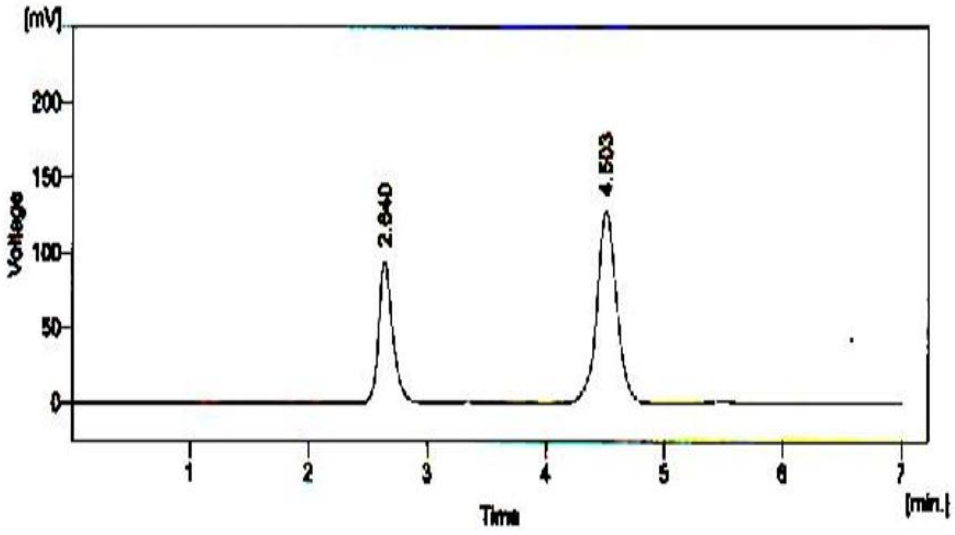


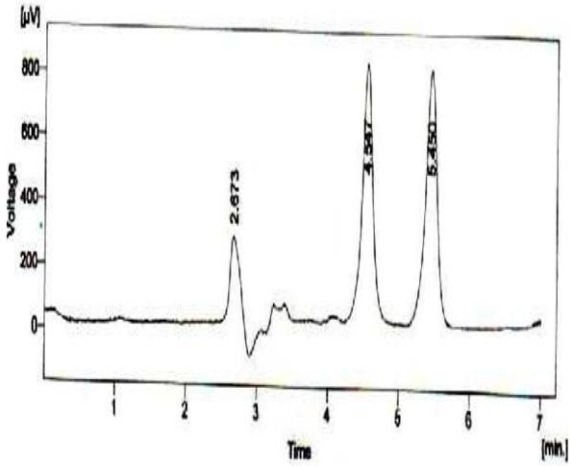
Figure 10: Chromatogram for Linearity of Pioglitazone and Glimepiride of Injection-5.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD of Pioglitazone and Glimepiride were found to be 7.5 µg/ml and 0.96µg/ml respectively. The LOQ w as 22.74µg/ml and 2.93µg/ml for Pioglitazone and Glimepiride respectively.

Table 8: LOD&LOQ for Pioglitazone and Glimepiride.

Parameter	LOD	LOQ
Pioglitazone	7.5 µg/ml	22.74 µg/ml
Glimepiride	0.96 µg/ml	2.93 µg/ml



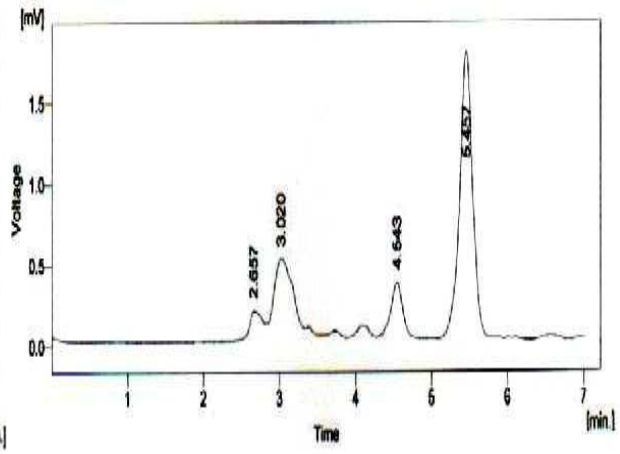


Figure 11: LOD Chromatogram (7.5&0.96µg/ml) Figure 12: LOQ Chromatogram (22.74&2.93µg/ml).

Robustness: In all the deliberately varied chromatographic conditions such as variations in flow rate and wavelength, the robustness of the method was evaluated. It can b e concluded that the variation in flow rate and the variation in wavelength do not affect the method significantly. Hence it indicates that the method is robust even by change in the flow rate and change in the wavelength.

Tabl 9: Summary of results for Robustness parameters for Pioglitazone and Glimepiride.

S.No	Flow rates	Pioglitazone		Glimepiride	
		Rt	Peak Area	Rt	Peak Area
1	1.3	5.57	1559.416	3.31	879.518
2	1.5	4.49	1474.803	2.63	763.115
3	1.7	3.74	998.801	2.21	533.256
S.No	**wave length**	**Pioglitazone**		**Glimepiride**	
		Rt	**Peak Area**	**Rt**	**Peak area**
1	228	4.5	1106.866	2.66	574.869
2	230	4.49	1474.803	2.63	763.115
3	232	4.5	1247.096	2.66	693.371

Assay: The assay results for commercial tablets given in **Table 10.**

Table 10: Results of commercial formulation analysis.

Label claim (mg/Tab)	%Label claim estimated (Mean±S.D)(n=6)	%RSD
Pioglitazone	99.78±1.20	1.20
Glimepiride	100.1±1.28	1.28

SUMMARY

After several trials with various solvents, mobile phase system composed of phosphate Buffer: Acetonitrile (40:60v/v) was chosen for the estimation of Pioglitazone and Glimepiride by RP-HPLC. This mobile phase composition offered maximum resolution for drugs at the detection wavelength of 230 nm.

Mobile phase with the flow rate of 1.3ml/min gave optimum separation with good resolution between the peaks. A reverse phase PHENOMENX LUNA C_{18}(250 X 4.6mm, 5 µ) column was used as stationary phase.

From the results shown in precision **Table 5:** The % RSD was found that 0.511162 for Pioglitazone and 0.5028 for Glimepiride which is less than 2%; that indicates the proposed method has good reproducibility. From the results shown in accuracy **Table 7**, it was found that the percentage recovery values were 99.8 for Pioglitazone and 100.5 for Glimepiride, which indicates that the method was accurate and also reveals that the commonly used excipients and additives present in the pharmaceutical formulations were not interfering in the proposed method.

From the linearity **Table no 8**, it was found that the drug obeys linearity within the concentration range of 240- 360µg/ml for Pioglitazone and 32-48µg/ml for Glimepiride.

The system suitability parameters also reveal that the values (no of theoretical plates above 2000, tailing factor below 2) were within the specified limits for the proposed method.

The Robustness of the method was proved by varying the flow rate and wavelength from the optimized chromatographic conditions and the tailing factors were found to be within the limits (below 2).

The LOD of Pioglitazone and Glimepiride were found to be 7.5µg/ml and 0.96µg/ml respectively.

The LOQ was 22.74µg/ml and 2.93µg/ml for Pioglitazone and Glimepiride respectively.

CONCLUSION

The new, simultaneous RP-HPLC method proved to be economical, simple, linear, precise, accurate and robust. The developed method was capable maintaining good separation more than that achieved with other available HPLC methods. The above method does not suffer from any interference due to common excipients. Though the linearity range of this method is slightly more as compared to the reported RP-HPLC method, the newly developed RP-HPLC method leads to better resolution and peak symmetry. Hence the developed RP-HPLC method for the simultaneous determination of Pioglitazone and Glimepiride can be used for routine analysis of both these components in combined dosage form.

The system suitability parameter tailing factor for the proposed HPLC method from the standard injection of Pioglitazone and Glimepiride are 1.122 and 1.273 respectively. Theoretical Plates obtained from the standard injection of Pioglitazone and Glimepiride are 3888 and 2577 respectively. The resolution obtained with the proposed method was 7.395. The results proved that the optimized HPLC method fulfils these requirements within the U SP accepted limits.

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