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Simultaneous for the estimation of Glimepiride and Pioglitazone in pharmaceutical dosage form by Reversed Phase-HPLC method

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***Abstract**: To develop accurate, fast, simple and precise reversed-phase high-pressure liquid chromatography method for simultaneous determination of the binary mixture of glimepiride and pioglitazone HCl in Glimepiride plus 4 mg/30 mg tablets. The chromatographic separation was conducted on Agilent 1200 with DA detector using Luna C18 (150mm x 4.6mm; 5-µm) as stationary phase. Isocratic mobile phase consisted of Phosphate Buffer pH 3.0 : Acetonitrile in ratio (45:55 v/v); The buffer solution is composed of 6.8 gm of potassium dihydrogen orthophosphate in 900 ml adjust pH to 3.0 by ortho-phosphoric acid then complete to 1000 ml by water and filtered through 0.45 µm nylon membrane filter. The flow rate of 1.0 ml min^{-1} was used. An injection volume of 20 µL was used for both glimepiride and pioglitazone HCl. The detection wavelength (λ_{max}) was 228 nm using a diode array detector. Linearity of the method was established over the concentration ranges of 4.0 – 12.0 µg ml^{-1} for glimepiride, with a retention time of 6.90 minutes and 30.0 – 90.0 µg ml^{-1} for Pioglitazone HCl, with a retention time of 2.36 minutes. Correlation coefficients were greater than 0.999. The relative standard deviation (RSD) was found to be < 2. The method was fast, accurate, precise, and sensitive hence it can be employed for routine quality control of tablets containing both drugs in quality control laboratories and pharmaceutical industries.*

***Keywords:** HPLC, Glimepiride, Pioglitazone HCl, Method validation.*

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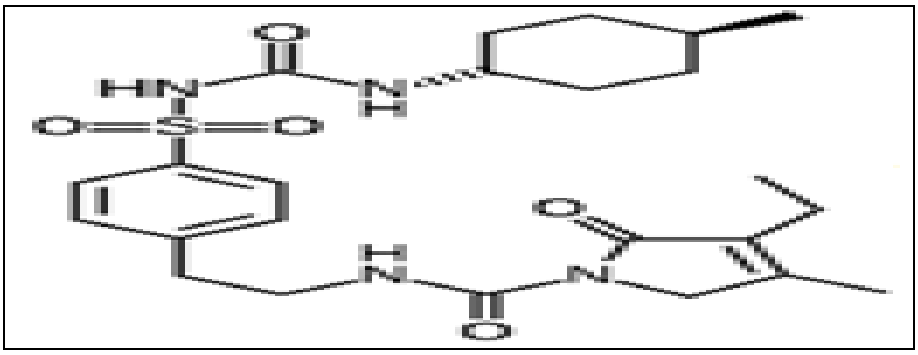
I. Introduction

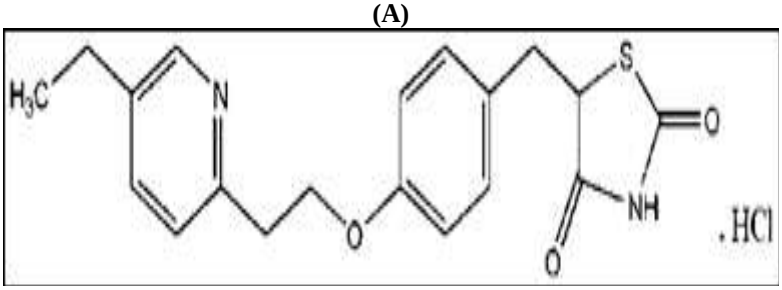
Diabetic patients are more prone to heart disease which is the leading cause of premature death in diabetic patients so the concurrent handling of both diseases is an essential issue [1]. Glimepiride is an oral sulfonylurea derivative which is indicated for the treatment of type 2 diabetes mellitus (DM). It is 1-[[p-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]phenyl]sulfonyl]-3-(trans-4-methylcyclohexyl) urea as shown in Fig. 1 (A). Glimepiride lowers blood glucose by stimulating the release of insulin from functioning pancreatic cells [2]. Various spectrophotometric methods [3-17] have been reported for the determination of glimepiride in pure as well as in dosage forms.

Pioglitazone, a thiazolidinedione derivative that is chemically named [(±)-5-[4-[2-(5-ethyl-2-pyridinyl) ethoxy]phenyl]-methyl]-2,4-thiaolidinedionemonohydro-chloride, is widely used in patients with type-2 diabetes (non-insulin dependent diabetes).[18] Pioglitazone HCl has been shown to affect abnormal glucose and lipid metabolism associated with insulin resistance by enhancing insulin action on peripheral tissues. Many patients suffering from type-2 diabetes require treatment with more than one antihyperglycemic drug in order to achieve optimal glycemic control.

Literature surveys revealed that sensitive LC-MS methods are available for analysis of antidiabetic drugs and its metabolites in human plasma and urine. [19-20] Even though various methods were reported in the literature for estimation of metformin, glimepiride and pioglitazone alone and in combination with other drugs [21-26]. Present study aimed for the simultaneous estimation of glimepiride and pioglitazone by reverse phase HPLC method. The method was validated according to the ICH guidelines [27].



(A)



(B)

Fig. 1. Chemical structures of Glimepiride (A) and Pioglitazone HCl (B).

II. Materials and Methods

Chemicals and reagents

Glimepiride and pioglitazone hydrochloride were obtained from Sinochem, China. Ortho-phosphoric 85% was HPLC grade from Fluka chemicals. Acetonitrile was HPLC grade from Fisher chemicals. Potassium dihydrogen orthophosphate was from EL Nasr Pharmaceuticals Chemicals. Hydrochloric acid 37% was HPLC grade from Merck. Distilled water. Mobile phase was filtered using 0.45 µm nylon membrane filter, ChromTech.

Pharmaceutical product containing glimepiride and Pioglitazone hydrochloride were purchased from the Egyptian pharmaceutical market. The pharmaceutical formulations were:-

1) Glimepiride plus 4 mg/30 mg tablets, Batch number 059018. Each tablet contains pioglitazone base 30 mg, glimepiride 4 mg, product by Biopharm for scientific research & Pharmaceutical manufacturing for Al Andalous for Pharmaceutical industries (October City, Egypt).

Equipment and chromatographic conditions

Agilent 1200 with DA detector was used. The HPLC separation and quantitation were achieved on a Luna C18 (150mm x 4.6mm; 5-µm). The mobile phase was prepared by Phosphate Buffer pH 3.0 : Acetonitrile in ratio (45:55 v/v); The buffer solution is composed of 6.8 gm of potassium dihydrogen orthophosphate in 900 ml adjust pH to 3.0 by ortho-phosphoric acid then complete to 1000 ml by water which was run isocratic. The mobile phase was delivered to the system at a flow rate of 1.0 ml/min. All determinations were performed at 25 ^{0}C temperature. The injected volume was 20 µl. The detector was set at 228 nm. The run time was set for 8.0 min. The optimized chromatographic condition is shown in Table 1.

Table 1. Optimized chromatographic conditions

Parameters	Conditions
Stationary phase	Luna C18, 150 x 4.6 mm, 5 µm
Mobile phase	Phosphate Buffer pH 3.0 : Acetonitrile (45:55 v/v)
Flow rate (mL min^{-1})	1.0
Run time (min)	8.0
Column temperature (^{0}C)	Ambient (25 ^{0}C)
Injection volume (µL)	20
Detection wavelength (nm)	228nm

Preparation of standard solution

Transfer accurately weighed 30 mg of Pioglitazone working standard into 100 ml volumetric flask, Add 75 ml diluent, sonicate for 5 min then cool and complete the volume with diluent (S1).

Weigh 40 mg of Glimepiride working standard into 100 ml volumetric flask, add 75 ml of diluent, sonicate for 5 min then cool and complete the volume with diluent (S2).

Dilute 10 ml from (S1 solution) and 1 ml from (S2 solution) to 50 volumetric flask then complete to volume with mobile phase. Filter through a 0.45µ syringe filter into HPLC vials rejecting the first portion of the filtrates and inject into HPLC.

Preparation of sample solution

Weigh and grind not less than 10 tablets. Transfer an accurately weighted portion of the powder equivalent to one tablet into 100 ml volumetric flask. Then add 75 ml diluent, sonicate for 20 min, cool at room temperature and then complete the volume with diluent. Dilute 5 ml to 25 volumetric flask then complete to volume with mobile phase. Filter through a 0.45µ syringe filter into HPLC vials rejecting the first portion of the filtrates and inject into HPLC.

Diluent: 0.1 N concentration hydrochloric acid : acetonitrile (1:9) .

Linearity

Linearity of an analytical procedure is its ability, within a given range, to obtain test results which are directly proportional to the concentration of analyte in the sample. Linearity suitable for single point standardization should extend to at least 20% beyond the specification range and include the target concentration. Linearity is defined by the correlation coefficient, which should be found to be ≥ 0.999, using peak area responses.

Linearity was performed by preparing a minimum five different concentrations (50, 80, 100, 120 and 150 %) of working standard solution and then making three replicates of each concentration.

Accuracy

Accuracy was evaluated by spiking standard solution. The measurements are made at a concentration of glimepiride and pioglitazone HCl in Glimepiride plus 4/30 mg tablets, which is found to be the target concentration, and at suitable intervals around this point.

Experimental procedure: Placebo except the active ingredient was spiked with known quantities of glimepiride and pioglitazone HCl working standard. Accuracy was assessed using 4 determinations over three concentrations level covering the specified range (i.e. three concentrations and three replicates). The measurements were made at a concentration, which is to be the (100%) specification, and at suitable concentration intervals around this concentration.

Specificity

It provides an indication of the selectivity and specificity of the procedure. The method is to be selective, if the main peak is well resoluted from any other peak by resolution of minimum 2. This was done by injecting placebo and comparing it with that of standard and placebo; spiked with standard and sample. Then the peak purity was ascertained using of PDA.

System suitability

System suitability was performed by injecting six replicates of standard solution at 100% of the test condition at a 100% level to verify the precision of the chromatographic system. The purposed HPLC method permits the concurrent determination of glimepiride and pioglitazone HCl in sample drug; through having different retention times. System suitability data are given in Table 2.

Table 2. System suitability parameters for glimepiride and pioglitazone HCl

S. No.	Parameters	Glimepiride	Pioglitazone
1	Tailing factor	1.01	1.23
2	Retention time	6.90	2.36

Ruggedness

It's defined the degree of reproducibility of test results obtained by the analysis of the same samples under variety of conditions such as different analysts, different columns, different days etc.

- **Day to day**: Five replicates of a single sample of powder material (100%) were used for each determination. On the first day; five replicates was analyzed. Then, on the second day, another five replicates of freshly prepared test from the same sample were analyzed by same analyst.
- **Analyst to analyst**: It determines ruggedness between different analysts. Five replicates of a single sample were analyzed. Then, a second person analyzed five replicates from the same sample, prepared by him.
- **Column to column**: The same analytical method was performed on columns of the same packing material and length but of different batch number.

Robustness

Robustness is determined by observing how a method stands up to slight variations in normal operating parameters. For instance, for HPLC, this could change if slight variation in sonication time or in aliquot stability.

Limit of detection (LOD) and limit of quantitation (LOQ)

Detection and quantitation limits were determined by the signal-to-noise (S/N) approach. In order to examine the limit of quantitation and limit of detection, solutions of different concentrations were prepared by spiking known amounts of glimepiride and pioglitazone HCl. Each solution was prepared according to the defined protocol and analyzed repeatedly to determine the S/N ratio. The average S/N ratio from all the analyses at each concentration level was used to calculate the limit of quantitation and limit of detection. The concentration level that gives an S/N ratio of 10:1 at which analytes can be readily quantified with accuracy and precision was reported as the limit of quantitation. The concentration level that gives an S/N ratio of 3:1 at which analytes can be readily detected was reported as the limit of detection.

III. Results and Discussion

The proposed HPLC method required fewer reagents and materials, and it is simple and less time consuming. This method could be used in quality control test in pharmaceutical industries. The chromatogram of glimepiride and pioglitazone HCl was shown in Fig. 2, 3. There was clear resolution between glimepiride and pioglitazone HCl with retention time of 6.90 and 2.36 minutes; respectively. The developed chromatographic method was validated using ICH guidelines [27]. Validation parameters include linearity, accuracy, precision, robustness, specificity, limit of detection and quantitation.

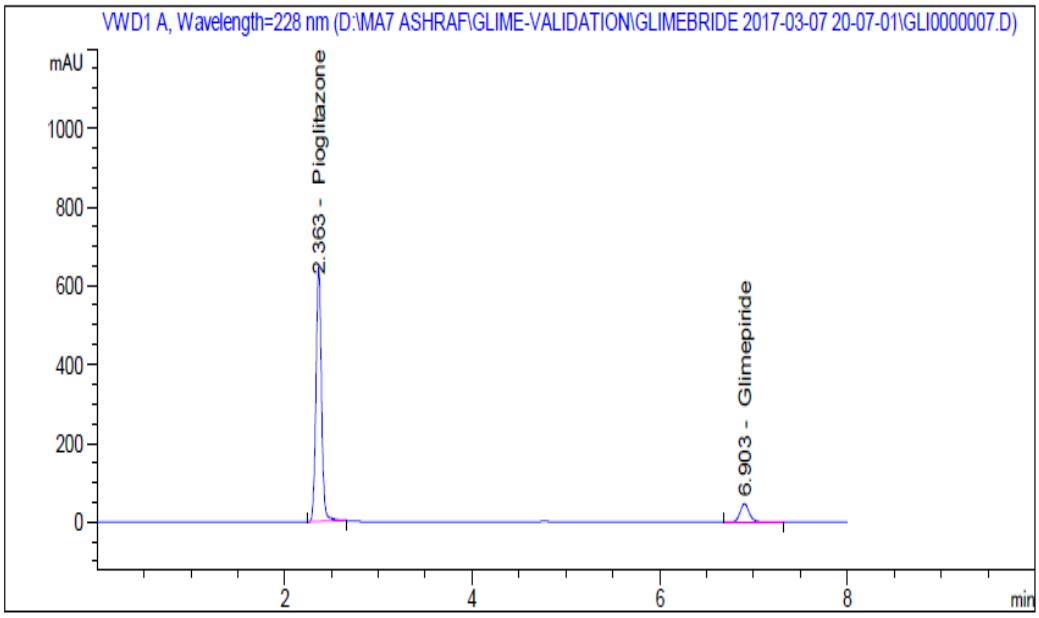


Fig. 2. HPLC chromatogram for glimepiride and pioglitazone HCl (standard drug).

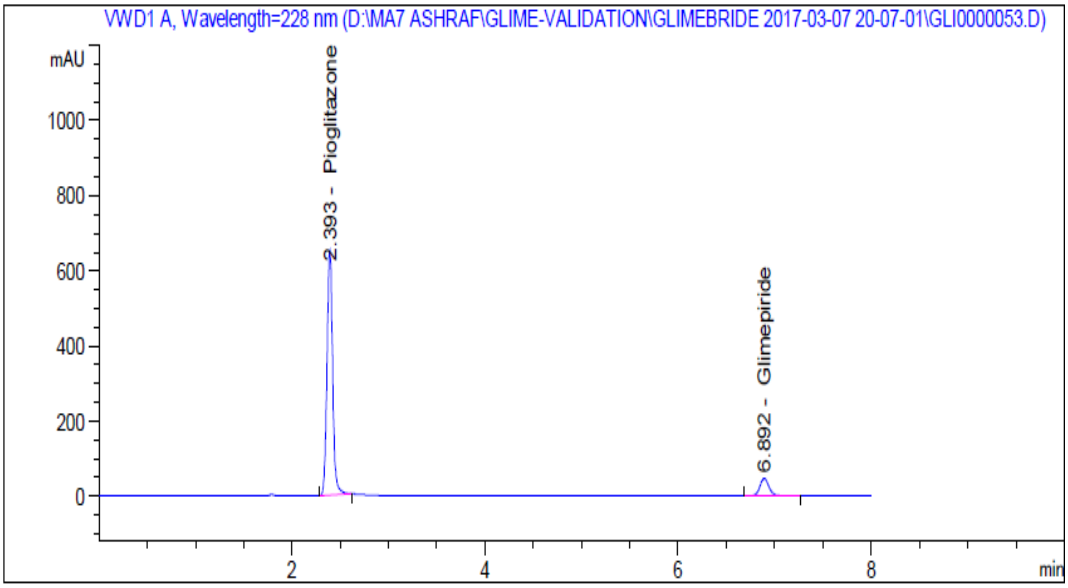


Fig. 3. HPLC chromatogram for glimepiride and pioglitazone HCl (sample drug).

Linear calibration plots for the proposed method were obtained in concentration ranges of 4.00-12.00 µg mL^{-1} (4.00, 6.40, 8.00, 9.60 and 12.00 µg mL^{-1}) for glimepiride and as shown in Fig. 5 and data are shown in Table 3 and 30.00-90.00 µg mL^{-1} pioglitazone HCl (30.00, 48.00, 60.00, 72.00 and 90.00 µg mL^{-1}) as shown in Fig. 6 and data are shown in Table 4.

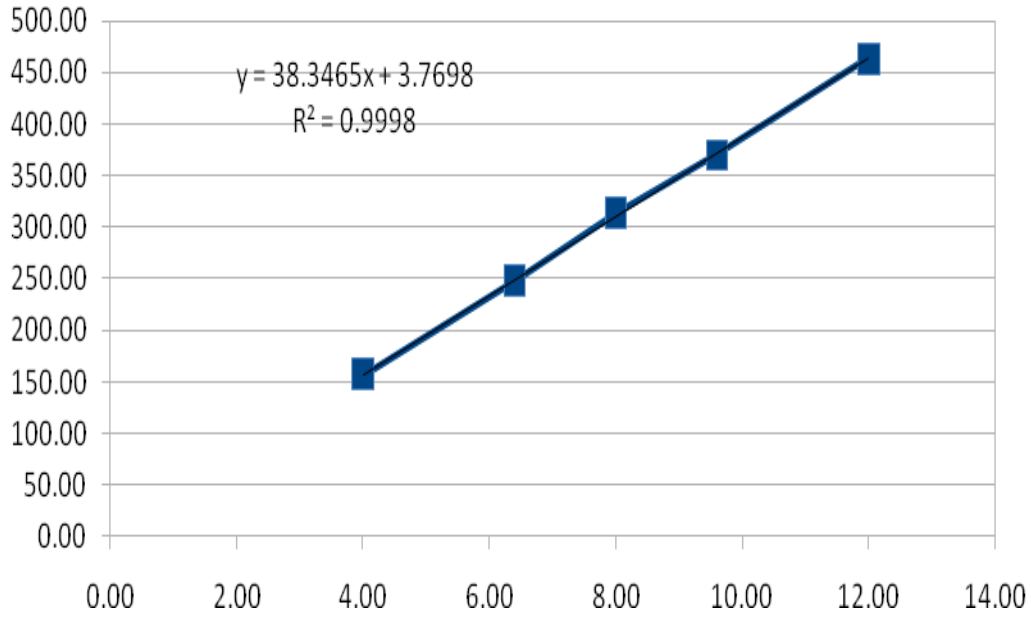


Fig. 4. Calibration curve of glimepiride.

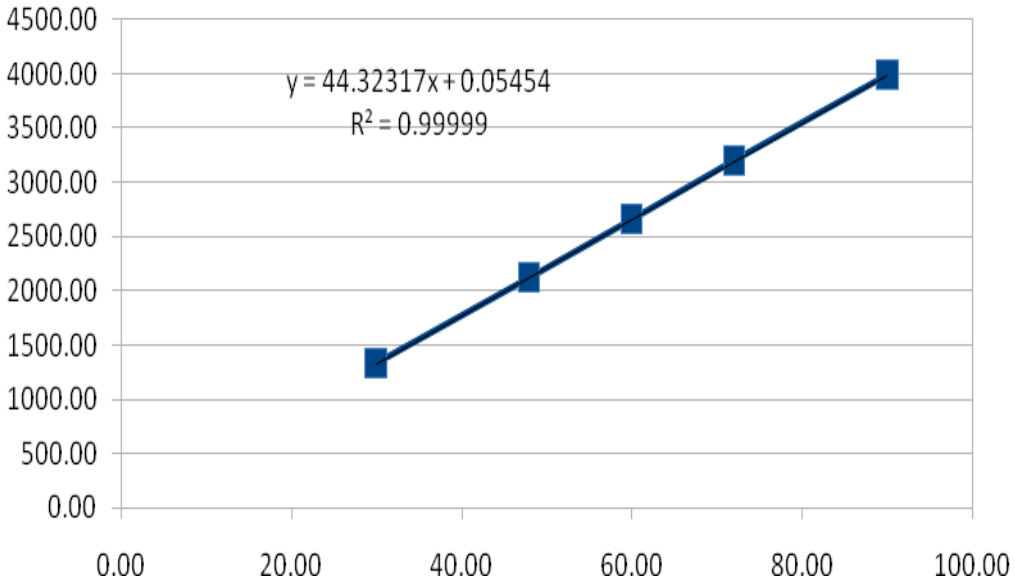


Fig. 5. Calibration curve of pioglitazone HCl.

Table 3. Statistical data of calibration curves of glimepiride

Working Conc. (%)	.Working Conc. (µg/ml)	Observed peak areas		
		Mean	SD	RSD%
50%	**4.00**	156.82	0.87	0.55
80%	**6.40**	248.31	0.54	0.22
100%	**8.00**	313.42	2.49	0.79
120%	**9.6**	370.26	1.85	0.50
150%	**12.00**	463.90	0.50	0.11
Slope				**38.3465**
Intercept				**3.7698**
R 2				**0.9998**

Table 4. Statistical data of calibration curves of pioglitazone HCl

Working Conc. (%)	.Working Conc. (µg/ml)	Observed peak areas		
		Mean	SD	RSD%
50%	**30.00**	1331.62	1.67	0.13
80%	**48.00**	2122.89	4.12	0.19
100%	**60.00**	2660.29	4.34	0.16
120%	**72.00**	3194.60	44.76	1.40
150%	**90.00**	3987.83	4.24	0.11
Slope				**44.32317**
Intercept				**0.05454**
R 2				**0.9999**

Each of the concentrations was injected in triplicate to get reproducible response. Calibration curves were constructed by plotting peak area versus concentration. Each reading was average of three determinations. They were represented by the linear regression equation.

$Y_{glimepiride} = 38.3465x + 3.7698$, $r^2 = 0.9998$
$Y_{pioglitazone} = 44.32317x + 0.05454$, $r^2 = 0.9999$

Slopes and intercepts were obtained by using regression equation (Y = mx + c) and least square treatment of the results used to confirm linearity of the method developed.

The limit of detection (LOD) and quantitation (LOQ) were determined by making serial dilutions. LOD was found to be 0.171 µg.ml^{-1} and 0.267 µg.ml^{-1} for glimepiride and pioglitazone HCl; respectively (signal to noise ratio of 3:1). LOQ was found to be 0.519 µg.ml^{-1} and 0.808 µg.ml^{-1} for glimepiride and pioglitazone HCl; respectively (signal to noise ratio of 10:1).

Accuracy was calculated by addition of standard drugs to reanalyzed sample at three different concentration levels and computing percentage recoveries. Standard limit of % recovery study is 98 - 102 % as per ICH guideline. From the studies it was concluded that % recovery study of glimepiride and pioglitazone HCl complies with standard limit of ICH guideline. Results of accuracy were proven by the Table 5,6.

Table 5. Accuracy and recovery results for determinations of glimepiride

Level (%)	Actual µg/ml	Theoretical µg/ml	(%) Recovery (n=3)
80	6.50	6.436	100.99
100	8.01	8.045	99.56
120	9.71	9.654	99.42
		Average Recovery	**99.99**
		SD	**0.87**
		% RSD	**0.87**

Table 6. Accuracy and recovery results for determinations of pioglitazone HCl

Level (%)	Actual µg/ml	Theoretical µg/ml	(%) Recovery (n=3)
80	47.51	48.269	98.43
100	59.37	60.336	98.40
120	71.59	72.403	98.88
		Average Recovery	**98.57**
		SD	**0.27**
		% RSD	**0.27**

Selectivity & Specificity: Forced degradation studies were performed to provide an indication of the stability indicating properties, selectivity and specificity of the procedure. Accelerated degradation was attempted using acid and base hydrolysis, effect of heat and oxidation; in addition to injection of well known degradation products (resolution solution).

The method to be selective and stability indicating, the peak of glimepiride and pioglitazone HCl standard should be resolute from any other peak that may appear due to degradation.

Placebo preparation: Weigh and grind not less than 10 tablets. Transfer an accurately weighted portion of the powder equivalent to the one tablet placebo into 100 ml volumetric flask. Then add 75 ml diluent, sonicate for 20 min, then cool and complete the volume with diluent. Dilute 5 ml of filtrate in 25 ml volumetric flask and complete volume by mobile phase was shown in Fig. 6. Filter through a 0.45µ syringe filter into HPLC vials rejecting the first portion of the filtrates and inject into HPLC.

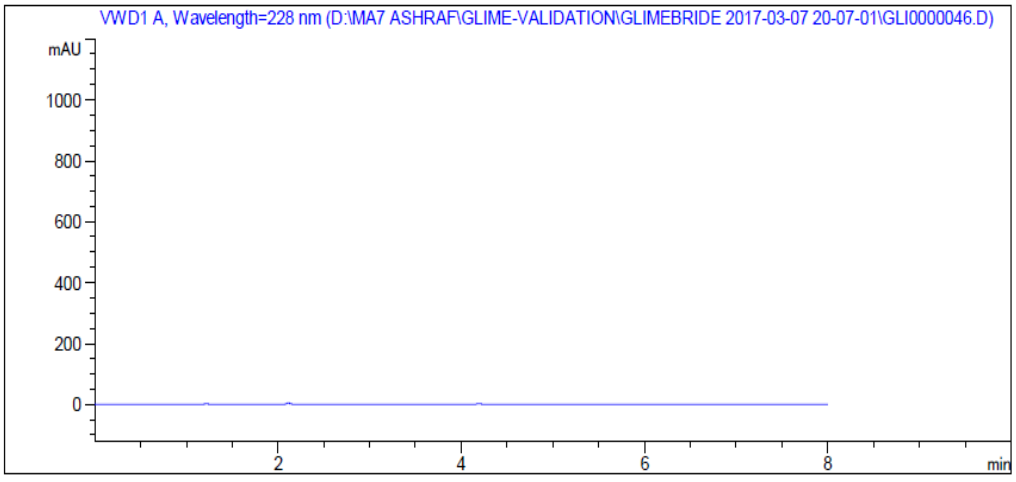


Fig. 6. HPLC chromatogram of placebo.

Basic hydrolysis: Weigh and grind not less than 10 tablets, transfer accurately equivalent one tablet in 100 ml volumetric flask, add 10 ml of 0.1 N NaOH then put in water bath for 3 hrs. Cool to ambient temperature then neutralize with 10 ml of 0.1 N HCl, then add 75 ml diluent, sonicate for 20 min .Cool and complete the volume with diluent. Dilute 5 ml of filtrate in 25 ml volumetric flask and complete volume by mobile phase was shown in Fig. 7. Filter through a 0.45µ syringe filter into HPLC vials rejecting the first portion of the filtrates and inject into HPLC.

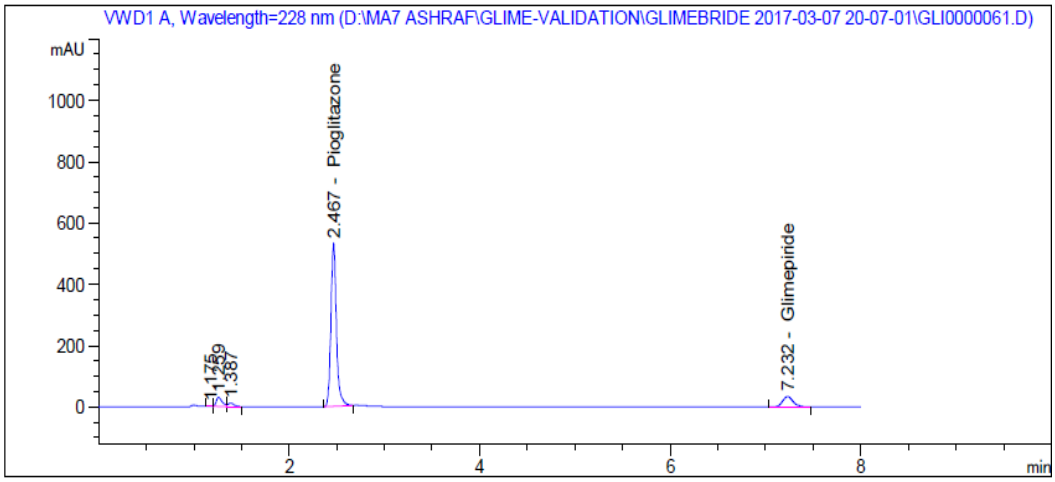


Fig. 7. HPLC chromatogram of basic hydrolysis.

Acid hydrolysis: Weigh and grind not less than 10 tablets, transfer accurately equivalent one tablet in 100 ml volumetric flask, add 10 ml of 0.1N HCL then put in water bath for 3 hrs. Cool to ambient temperature then neutralize with 10 ml of 0.1 N NaOH, then add 75 ml diluent, sonicate for 20 min .Cool and complete the volume with diluent. Dilute 5 ml of filtrate in 25 ml volumetric flask and complete volume by mobile phase was shown in Fig. 8. Filter through a 0.45µ syringe filter into HPLC vials rejecting the first portion of the filtrates and inject into HPLC.

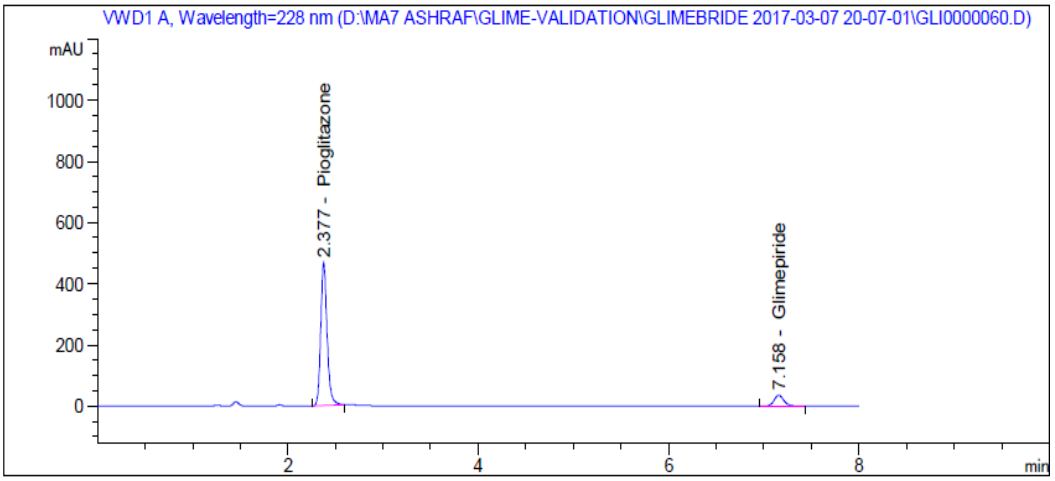


Fig. 8. HPLC chromatogram of acid hydrolysis.

Oxidation by H2O2: Weigh and grind not less than 10 tablets, transfer accurately equivalent one tablet in 100 ml volumetric flask, add 10 ml of 10% H2O2 then put in water bath for 3 hrs, then add 75 ml diluent, sonicate for 20 min .Cool and complete the volume with diluent. Dilute 5 ml of filtrate in 25 ml volumetric flask and complete volume by mobile phase was shown in Fig. 9. Filter through a 0.45µ syringe filter into HPLC vials rejecting the first portion of the filtrates and inject into HPLC.

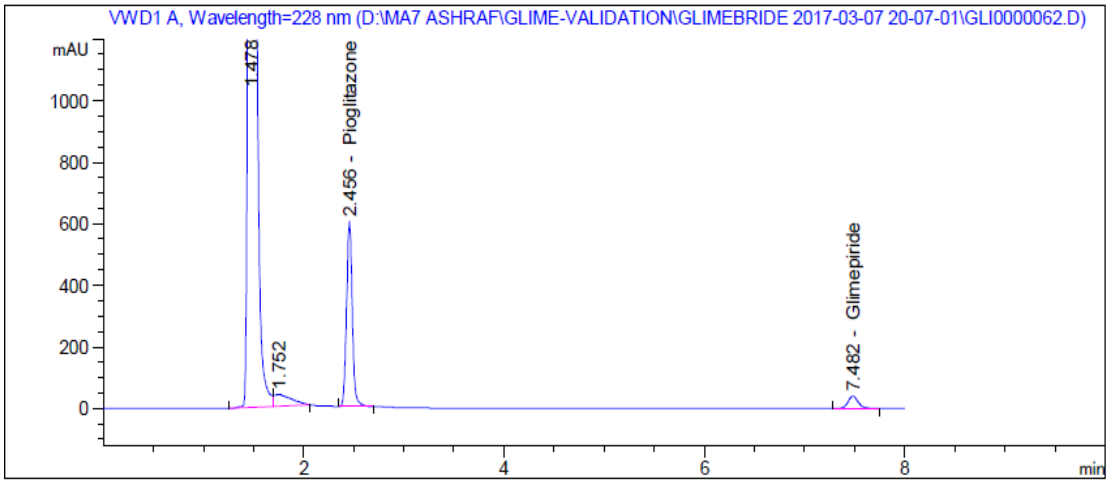


Fig. 9. HPLC chromatogram of oxidation hydrolysis.

Heat hydrolysis: Weigh and grind not less than 10 tablets. Transfer an accurately equivalent to one tablet into 100 ml volumetric flask. Then add 75 ml diluent, sonicate for 20 min then put in water bath for 24 hrs then cool and complete the volume with diluent. Dilute 5 ml of filtrate in 25 ml volumetric flask and complete volume by mobile phase was shown in Fig. 10. Filter through a 0.45µ syringe filter into HPLC vials rejecting the first portion of the filtrates and inject into HPLC.

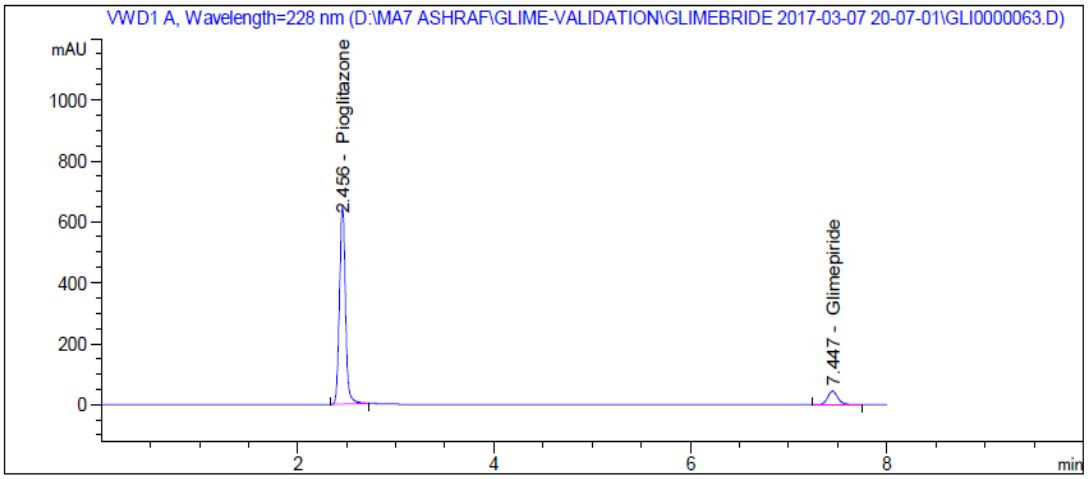


Fig. 10. HPLC chromatogram of heat hydrolysis.

Light hydrolysis: Weigh and grind not less than 10 tablets. Transfer an accurately equivalent to one tablet into 100 ml volumetric flask. Then add 75 ml diluent, sonicate for 20 min, then leave it in light for 24 hrs then complete the volume with diluent. Dilute 5 ml of filtrate in 25 ml volumetric flask and complete volume by mobile phase was shown in Fig. 11. Filter through a 0.45µ syringe filter into HPLC vials rejecting the first portion of the filtrates and inject into HPLC.

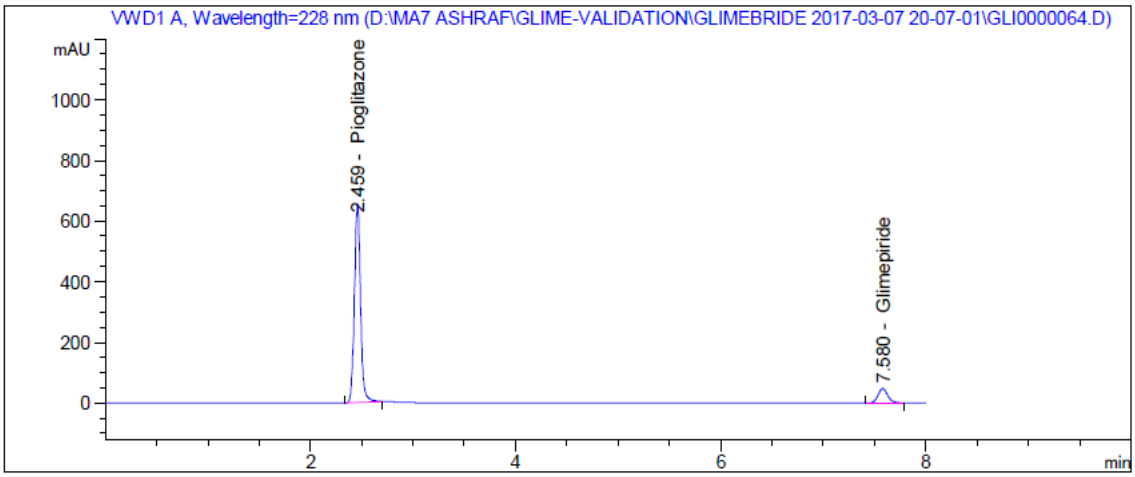


Fig. 11. HPLC chromatogram of light hydrolysis.

IV. Conclusion

The proposed analytical method of Glimepiride and Pioglitazone HCl in Glimepiride 4/30 mg Tablets was found to be precise, repeatable, linear, accurate, rugged, robust, specific and sensitive. Results demonstrate that the method is suitable for its intended use.

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