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Stability Indicating Reverse Phase High-performance Liquid Chromatographic Determination of Pioglitazone Hydrochloride in Pharmaceuticals and Human Urine

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Abstract: A simple, sensitive, specific and stability-indicating high-performance liquid chromatographic (HPLC) method is presented for the determination of of pioglitazone hydrochloride (PGH) in its tablets. The assay was performed on an Inertsil ODS 3V (250 x 4.6 mm; 5 µm) column using phosphate buffer (pH 3.6)-methanol (60:40 v/v) as mobile phase. The flow rate was 1 mL min^{-1} and the analyte was monitored at 220 nm. The method provides a linear response over the concentration range 0.1 – 300 µg mL^{-1} (r = 0.9999). The limits of detection (LOD) and quantification (LOQ) were 0.03 and 0.1 µg mL^{-1}, respectively. The method showed intra-day and inter-day precision of <0.5% (RSD) and an accuracy of <2% (RE). Four variables: column temperature, mobile phase composition, flow rate and wavelength were slightly altered, and these were found to have no impact on the method performance indicating robustness of method. Person-to-person and column-to-column variations were also studied as a part of ruggedness study. The developed method was applied to the determination of PGH in its tablet dosage form with acceptable accuracy (%RE, ≤1.28) and precision (%RSD, ≤2.08). Accuracy was also checked by recovery study *via* standard addition procedure which yielded a mean recovery of 101.1% with a standard deviation of <0.5%. As a part of stress study, the drug was subjected to acid, base, peroxide, heat and light-induced stress conditions; the results showed slight vulnerability to base-induced stress condition and with no change under other stress conditions, thus revealing the stability-indicating ability of the developed method.

Keywords: Pioglitazone, determination, HPLC, pharmaceuticals, stability-indicating.

1 Introduction

Pioglitazone hydrochloride (PGH), chemically known as 5-[[4-[2-(5-ethyl-2-pyridinyl) ethoxy] phenyl] methyl]-2,4-thiazolidinedione monohydrochloride (Figure 1) [1], is an anti-hyperglycemic agent.

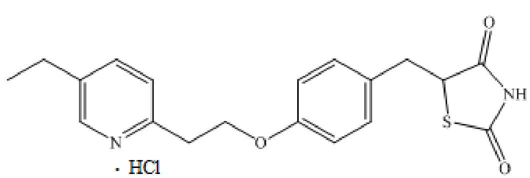


Figure 1: Structure of PGH

It is used to treat type-2 diabetes mellitus. PGH is a potent and highly selective agonist for the nuclear receptor, peroxisome proliferator-activator receptor gamma (PPAR-γ). PPARs are found in tissues like adipose tissue, skeletal muscle, and liver, which are critical to insulin action. Activation of PPAR-γ modulates the transcription of a number of insulin- responsive genes involved in the control of glucose and lipid metabolism [2, 3]. It acts by reducing peripheral and hepatic resistance to insulin, resulting in increased insulin-dependent glucose disposal and decreased hepatic glucose control [4, 5].

Due to its medicinal use, number analytical methods have been developed for its determination in pharmaceuticals and body fluids, and the same have been recently reviewed [6]. The drug is not official in any pharmacopeia. Methods based on several techniques like uv-spectrophotometry [7-25], visible spectrophotometry [11, 26], potentiometry [27-29], voltammetry [30, 31], capillary electrophoresis [32], ultra-performance liquid chromatography [33, 34] and high-performance thin layer chromatography [35-40] have been reported for the determination of PGH in pharmaceuticals.

In the realm of pharmaceutical analysis, HPLC offers enhanced detection sensitivity, improved accuracy, and reproducibility of drug analysis in the course of drug research, development and quality control testing of marketed drug products. Many wet analysis and classical test methods for existing drug products have also been replaced by HPLC methods for more accurate measurements, better precision and much faster analytical run time. This translates into lower cost per test in research and development and quality control laboratories.

Considering these advantages, several workers have reported the HPLC assay of PGH in pharmaceuticals. Jiladia *et al.* [41] have developed a method for PGH in its single-component tablets, where the chromatography was performed on a Waters spherisorb CNRP column with a mobile phase consisting of pH 6.0 phosphate buffer: acetonitrile (70:30 v/v) and uv detection at 268 nm. PGH in bulk and tablet forms was assayed by Srinivasulu *et al.* [42] by carrying out chromatography on a C_{18} column. A mixture of buffer and acetonitrile in the ratio 55:45 was used as the mobile phase at a flow rate of 1 mL min^{-1} with uv detection at 254 nm. By performing chromatography on a Nova- Pak® C_{18} column using a mixture of formate buffer of pH 3 and acetonitrile (75:25 v/v) as mobile phase at a flow rate of 1 mL min^{-1} and UV- detection at 225 nm, PGH was assayed in tablets [43].

Using a 5 µm Symmetry C_{18} column and phosphate buffer: acetonitrile (50:50 v/v) as mobile phase at a flow rate 1 mL min^{-1} and uv detection at 225 nm, PGH was determined in bulk and tablet formulation [44]. The method was capable of detecting all process related compounds. Jedlicka *et al.* [45] have described a method for purity test and assay of PGH in tablets. To accomplish this, the authors used symmetry C_{18} column and a mixture of ammonium formate buffer (pH 4.1): acetonitrile (45:55 v/v) as mobile phase at a flow rate of 1 mL min^{-1} with UV- detection at 266 nm. Employing symmetry-extend- C_{18} column and 0.01M buffer: methanol (40:60 v/v) as mobile phase and UV-detection at 240 nm, PGH was determined by Madhukar *et al.* [46].

Other than the assays described above, several workers have reported stability-indicating assay methods (SIAM) for PGH. A study of stressed degradation behaviour of PGH in bulk and pharmaceutical formulation was reported by Sharma *et al.* [47]. The study was performed on a Phenomenex Luna C18 column using phosphate buffer (pH 3.5): methanol (55:45 v/v) as mobile phase at a flow rate of 1.5 mL min^{-1} and UV- detection at 241 nm. The method was specific to drug and selective to degradation products. An RP-HPLC method for PGH in the presence of its impurities and degradation products was developed by Rashmitha *et al.* [48]. The assay was achieved on an Inertsil ODS-3V column. The gradient method employed phosphate buffer of pH 3.1 as solution A and acetonitrile as solution B. The drug was found to undergo degradation under base and oxidative stress conditions and stable under other stress conditions studied. Reddy and Rao [49], in their method, employed a Prontosil C_8 column and phosphate buffer (pH 4): acetonitrile: methanol (55:30:15 v/v) as mobile phase at a flow rate 1.5 mL min^{-1} with UV-detection at 245 nm for the separation and assay of PGH in the presence of its degradation products. One more stability-indicating RP-HPLC method for PGH from its tablets has been described by Wanjari and Gaikwad [50]. The analysis was performed on an Hypersil C_8 column using acetonitrile: triethylamine (pH 4.6) (40:60 v/v) at a flow rate of 1.5 mL min^{-1} as the mobile phase with UV-detection at 220 nm. A stability-indicating assay method for the determination of impurities in PGH was developed and validated by Sriram *et al.* [51]. The method used Gemini C_{18} column and phosphate buffer (pH 3): acetonitrile (50:50 v/v) as mobile phase at a flow rate of 1 mL min^{-1} with UV-detection at 225 nm for the separation and analysis of PGH and its impurities.

In addition to the above, HPLC methods have also been reported for the determination of PGH in pharmaceuticals when it is present in combined dosage forms [52-71].

The above methods are not completely satisfactory with respect to linear range, sensitivity and optimum conditions.

Chemical stability of pharmaceutical molecules is a matter of concern as it affects the safety and efficacy of the drug product. The United States Food and Drugs Administration (USFDA) [72] and International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [73] guidance states the requirement of stability testing data to understand how the quality of drug substance and drug product change with time under the influence of various environmental factors. Knowledge of the stability of the molecule helps in selecting proper formulation and package as well as providing proper storage conditions and shelf life, which is essential for the regulatory documentation.

Realising the need for a stability-indicating HPLC method, this work describes the development and validation of a simple, sensitive and specific method for PGH. Forced degradation study revealed that the drug undergoes slight degradation under base-induced stress condition and stable to other stress conditions.

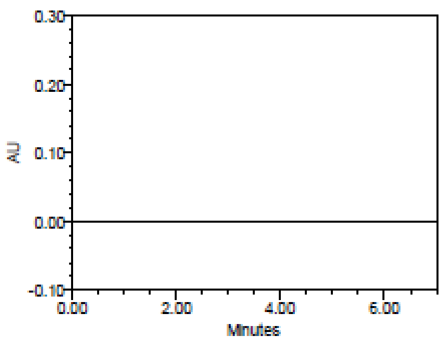
2 Results and Discussion

The aim of the study was to establish an HPLC method suitable for the determination of pioglitazone in its bulk and dosage form, and to investigate its behaviour under various stress-conditions.

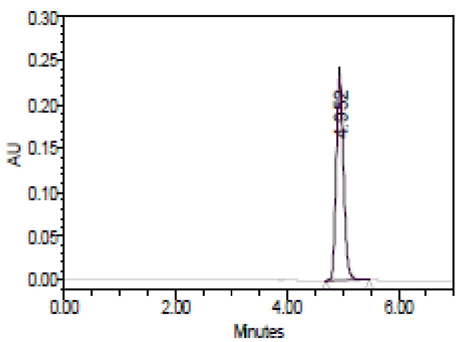
2.1 Method optimization

A well defined symmetrical peak (Figure 2) and good results were obtained upon measuring the response of eluent under the optimized conditions after thorough experimental trials that could be summarized as follows:

Five different columns were used for performance investigations, including hypersil BDS C_8 (250 mm × 4.0 mm, 5.0 µm particle size); chromatopack (250 mm × 4.6 mm, 5 µm particle size); Zorbax XDB (250 mm × 4.0 mm, 5.0 µm particle size); Luna C_{18} (250 mm × 4.0 mm, 5.0 µm particle size) and Inertsil ODS 3V (250 mm × 4.0 mm, 5.0 µm particle size). Inertsil ODS 3V column was found most suitable in terms of sensitivity. The UV detector response of PGH was studied and the best wavelength was found to be 220 nm showing the highest sensitivity. Several modifications in the mobile phase compositions were tried to achieve better performance characteristics. These modifications included, type and ratio of the organic modifier, pH, strength of the phosphate buffer, and flow rate. The results of this study are shown in **Table 1**.



(a)



(b)

Figure 2: Chromatograms for; **a)** Blank (mobile phase) **b)** Pure PGH solution (200 µg mL^{-1}).

Compared to acetonitrile, methanol was better suited as the organic modifier, giving elegant and highly sensitive peak.

The effect of proportion of organic modifier in the mobile phase on the peak shape and retention time of the test solute was investigated using mobile phases containing up to 30-60% methanol. A mobile phase consisting of 40% methanol gave well defined peak and the highest number of theoretical plates. The effect of pH of the mobile phase on the peak shape and retention time of the test solute was investigated using mobile phases of pH values ranging from 2.0- 6.0. The results (Table 1) revealed that pH 3.6 was the most appropriate, giving well defined peak and the highest number of theoretical plates. At lower and higher pH non-symmetrical peak and smaller number of theoretical plates were observed. The same trend was observed after making alteration in the ionic strength of the buffer and 10mM KH_2PO_4 solution containing 0.1% H_3PO_4 was used as working buffer throughout the investigation. The results of this investigation are also presented in Table 1. The effect of flow rate on the symmetry, sensitivity and retention time of the peak was studied, and a flow rate of 1 mL min^{-1} was optimal for better symmetry and reasonable retention time (**Table 1**).

Table 1. Effect of ratio of organic modifier, pH and ionic strength of buffer on the number of theoretical plates

Ratio (A/B)[a]	Number of theoretical plates (N)	pH of the medium	Number of theoretical plates (N)	%H_3PO_4	Number of theoretical plates (N)	Flow rate, mL min^{-1}	Number of theoretical plates (N)
40/60	4789	2.0	4090	0.050	4022	0.50	6902
50/50	5467	2.5	4560	0.075	4689	0.75	7089
55/45	6898	3.0	5783	0.090	5390	1.00	7737
60/40	7980	3.6	7922	0.100	7790	1.25	7590
70/30	6783	4.0	7910	0.150	7600	1.50	7390
-	-	5.0	6579	0.200	7019	1.75	7211
-	-	6.0	6490	0.250	6256	2.00	7489

[a]A- phosphate buffer and B- methanol

2.2 Method validation

2.2.1 Linearity, LOD and LOQ

The least squares method was used to calculate the slope, intercept and the correlation coefficient (r) of the regression line. The relationship between mean peak area, y (n=3) and concentration x, expressed by the equation y = 110177 x + 20656 (r^2 = 0.9999), was linear. A plot of peak area *versus* concentration was a straight line with the slope of 110177 and this coupled with a high value of the correlation coefficient (r- value > 0.9999) indicated excellent linearity between mean peak area and concentration in the range 0.1-300 µg mL^{-1} PGH (Figure 3).

The limit of quantification (LOQ) was determined by establishing the lowest concentration that can be measured according to *ICH* recommendations [73], below which the calibration graph is non linear, and was found to be 0.1 µg mL^{-1}. The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected and the value was found to be 0.03 µg mL^{-1}**.** These values are collected in **Table 2**.

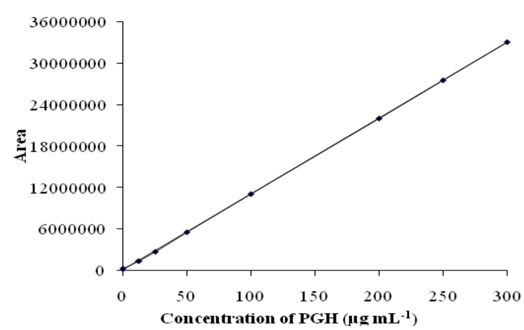


Figure 3: Calibration plot

2.3 Precision and accuracy

Method precision was evaluated from the results of seven independent determinations of PGH at three different concentrations, 100, 200 and 300 µg mL^{-1}, on the same day and on five successive days. The intra-day and inter-day relative standard deviation (%RSD) values for peak area and retention time for the selected concentrations of PGH were less than 0.42 and 0.45%, respectively. The method accuracy, expressed as relative error (%), was determined by calculating the percent deviation found between concentrations of PGH found and concentrations injected. This study was performed by taking the same three concentrations of PGH used for precision estimation. The intra-day and inter-day accuracy (expressed as RE) was less than 2% and the values are compiled in **Table 3**.

Table 2. Regression and sensitivity parameter

Parameter	Value
Linear range, μg mL^{-1}	0.1 -300
Limits of detection, (LOD), μg mL^{-1}	0.03
Limits of quantification, (LOQ), μg mL^{-1}	0.1
Regression equation, y*	
Slope (m)	110177
Intercept (b)	20656
Standard deviation of intercept (Sb)	897.9
Standard deviation of slope (Sm)	1681.2
Correlation coefficient (r)	0.9999

*y=mx+b, where y is the mean peak area, x is concentration in μg mL^{-1}, b intercept, m slope.

Table 3. Results of accuracy and precision study (n=5)

PGH injected, μg mL^{-1}	Intra-day				Inter-day			
	PGH found, μg mL^{-1}	% RE[a]	% RSD[b]	% RSD[c]	PGH found, μg mL^{-1}	% RE[a]	% RSD[b]	% RSD[c]
100	98.7	1.30	0.38	0.21	98.1	1.90	0.45	0.15
200	202.4	1.20	0.27	0.31	198.3	0.85	0.25	0.38
300	301.9	0.63	0.36	0.42	302.7	0.93	0.32	0.23

[a]Relative error
[b]Relative standard deviation based on peak area;
[c]Relative standard deviation based on retention time.

2.4 Method robustness

Table 4. Results of method robustness

Condition altered	Modification	Mean peak area ± SD*	%RSD	Mean R_t ± SD*	%RSD	Mean theoretical plates ± SD*	%RSD	Mean tailing factor ±SD*	%RSD
Actual	-	22059958 ± 28389	0.13	4.935 ± 0.002	0.04	7924± 48.86	0.62	1.252 ± 0.016	1.28
Column temperature	30 ±2 °C	22094431 ± 50454	0.23	4.936 ± 0.003	0.06	7946± 60.10	0.76	1.217 ± 0.008	0.66
Mobile phase composition	(Buffer: methanol) 65:35 60:40 55:45	22143861 ± 31058	0.14	4.939 ± 0.003	0.06	7954± 49.81	0.62	1.217 ± 0.012	0.99
Flow rate	1.0 ±0.1 mL min^{-1}	22097543 ± 49790	0.22	4.933 ± 0.010	0.20	7949± 46.66	0.59	1.210 ± 0.009	0.74
Wavelength	220±2 nm	22151963 ± 52937	0.24	4.934 ± 0.006	0.12	7949 ± 46.66	0.59	1.215 ± 0.014	1.15

*Mean value of three determinations at PGH concentration of 200 μg mL^{-1}.

To determine the robustness of the method small deliberate changes in the chromatographic conditions like detection wavelength (220±2 nm), mobile phase composition (actual±5%), flow rate (1.0±0.1 mL) and column temperature (35±2 ºC) were made, and the results obtained under altered were compared with those of the optimized chromatographic conditions. The results indicated that changes had no significant effect. The results of this study expressed as RSD are summarized in **Table 4.**

2.5 Method ruggedness

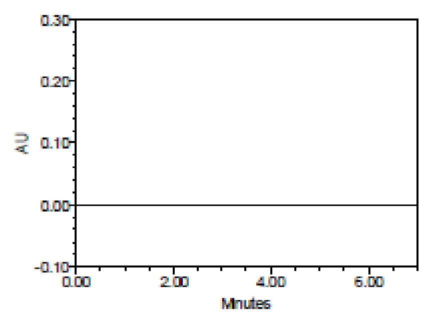
In method ruggedness, analyses using three columns (different lot with the same manufacturer), and analysts (n=3) were performed. The low %RSD values summarized in **Table 5** speak of acceptable ruggedness of the method.

Table 5. Results of method ruggedness (n=3)

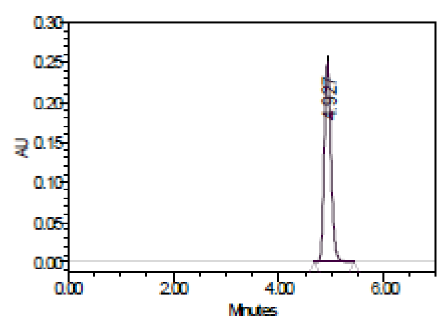
Variable	Mean Peak area ± SD*	%RSD	Mean Rt ± SD*	%RSD	Mean theoretical plates ±SD	%RSD	Mean tailing factor± SD*	%RSD
Analysts (n=3)	22121380 ± 47561	0.22	4.933± 0.010	0.20	7931± 49.54	0.62	1.220± 0.017	1.39
Columns (n=3)	22064716 ± 50454	0.23	4.931± 0.010	0.20	7945± 45.72	0.58	1.222± 0.015	1.23

*Mean value of three determinations at PGH concentration of 200 μg mL^{-1}.

2.6 Selectivity



(a)



(b)

Figure 4: Chromatograms obtained for: a) placebo blank and b) tablet extract (200 μg mL^{-1} PGH).

The chromatogram obtained for placebo solution (**Figure 4a)** was the same obtained for mobile phase. The peak area for the synthetic mixture solution (200 μg mL^{-1}) was nearly the same as that obtained for pure PGH solution of identical concentration. This unequivocally demonstrated the non-interference of the inactive ingredients in the assay of PGH. Further, the slope of the calibration plot prepared from the synthetic mixture solution was about the same as that obtained from pure drug solution. Method selectivity was checked by comparing the chromatograms obtained for placebo blank, pure PGH solution, synthetic mixture and tablet solution (**Figure 4b**). An examination of the chromatograms of the above solutions revealed the absence of peaks due to additives present in tablet extract.

2.7 Solution stability and mobile phase stability

Stability of PGH solution was evaluated by injecting the standard solution stored at room temperature at time intervals of 0, 12 and 24 h, and recording the retention and peak area. RSD values for retention time and peak areas calculated. The mobile phase stability was studied by preparing the drug solution afresh with the mobile phase stored at room temperature at the same intervals of time. The solution prepared at 0, 12 and 24 hours was injected and RSD values for retention time and peak areas were calculated (Table 7). The R_t and peak remained almost unchanged and no significant degradation was observed within the studied period, indicating that the sample solution and mobile phase were stable for at least 24 h as shown by small values of RSD. The results of this study are compiled in Table 6 and 7.

2.8 Application to tablets

The developed and validated method was applied to the assay of PGH commercial tablets. The results shown in Table 8 are in good agreement with those of the label claim and also with those obtained by the reference method [7], wherein absorbance of tablet extract in 0.1M HCl was measured at 269 nm.

2.9 Accuracy by recovery study

Table 6. *Results of solution stability*

Time, hour	Mean Peak area ± SD*	Pooled %RSD	Mean Rt ± SD*	Pooled %RSD	Mean theoretical plates ±SD	Pooled %RSD	Mean tailing factor± SD*	Pooled %RSD
0	22143861 ± 31058		4.939± 0.003		7895± 47.81		1.217± 0.012	
12	22121380 ± 47561	0.24	4.933± 0.010	0.20	7931± 49.54	0.74	1.220± 0.017	1.31
24	22097543 ± 49790		4.933± 0.010		7949± 46.66		1.210± 0.009	

*Mean value of three determinations for PGH concentration of 200 μg mL^{-1} at each time interval.

To evaluate the accuracy and reliability of the method, recovery experiment *via* standard addition procedure was performed. To the pre-analyzed tablet powder, pure PGH was added at three levels and the total was determined by the proposed method in triplicates. When the test was performed on 15 and 30 mg tablets, the percent recovery of pure PGH was in the range of 98.97–102.6 with standard deviation values of 0.31-0.59. The results compiled in **Table 9,** reflect that the method is reliable and free from interference from co-formulated substances in tablets.

Table 7. Results of mobile phase stability

Time, hour	Mean Peak area ± SD*	Pooled %RSD	Mean Rt ± SD*	Pooled %RSD	Mean theoretical plates ±SD	Pooled %RSD	Mean tailing factor± SD*	Pooled %RSD
0	22143814 ± 31036		4.939± 0.009		7950± 47.72		1.217± 0.011	
12	22121327 ± 47563	0.24	4.933± 0.010	0.25	7941± 49.51	0.74	1.220± 0.014	1.19
24	22097503 ± 49792		4.933± 0.010		7949± 47.66		1.210± 0.010	

*Mean value of three determinations for PGH concentration of 200 μg mL^{-1} at each time interval.

Table 8. Results of determination of PGH in tablets and statistical comparison with the reference method

Tablet brand name	Nominal amount, mg	PGH found* (Percent of label claim± SD)	
		Reference method	Proposed method
Oglo-15	15	99.27±1.28	100.3±2.08 t = 0.91 F = 2.64
Neoglit-30	30	99.58±0.98	98.72±1.09 t = 1.31 F = 1.24

*Mean value of five determinations. Tabulated t-value at 95% confidence level is 2.77; Tabulated F-value at 95% confidence level is 6.39.

Table 9. Results of recovery study via standard addition procedure

Tablet studied	PGH in tablet, μg mL^{-1}	Pure PGH added, μg mL^{-1}	Total found, μg mL^{-1}	Pure PGH recovered* (%NTG ±SD)
Oglo-15	100.27	50	153.47	102.1±0.54
	100.27	100	203.63	101.7±0.36
	100.27	150	248.09	99.13±0.45
Neoglit-30	98.72	50	152.54	102.6±0.31
	98.72	100	196.67	98.97±0.52
	98.72	150	253.27	101.8±0.59

*Mean value of three determinations

2.10 Application to spiked human urine sample

The developed method was applied to determine PGH in spiked urine sample. Figure 5 shows the PGH peak obtained from spiked human urine, with no additional peaks. The recovery for PGH from spiked human urine was calculated at three concentrations (Table 10) indicates that endogenous substances present in urine did not elute under the stated chromatographic conditions.

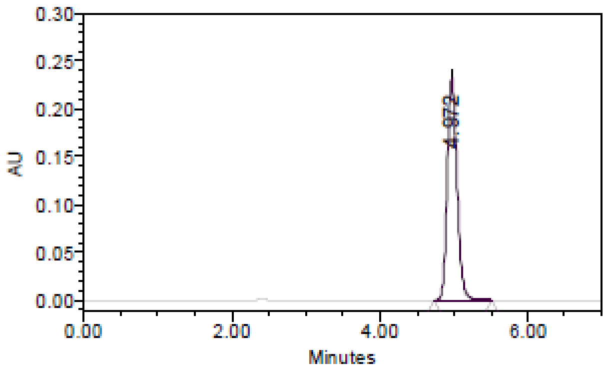


Figure 5: Chromatogram obtained for spiked human urine (200 µg mL^{-1} PGH)

Table 10. Results of PGH determination in spiked urine sample

Spiked concentration (µg mL^{-1})	Found[a]±SD	% Recovery±RSD
100	95.76±0.35	95.76±1.01
200	196.4±0.57	98.47±1.24
300	293.8±0.49	97.92±0.99

[a]Mean value of five determinations; RSD- relative standard deviation

2.11 Results of forced degradation study

All analyses of sample post-degradation were carried out at an initial concentration of 200 µg mL^{-1} PGH with the described HPLC conditions using a TUV detector to monitor the homogeneity and purity of the PGH peak. The purity angle was within the purity threshold limit obtained in all stressed samples except alkaline condition and demonstrates the analyte peak homogeneity. Marginal degradation was observed when the drug was subjected to base-induced stress condition. The purity and assay of PGH were unaffected by the presence of its degradation products resulting under other conditions except alkaline medium, which confirms the stability-indicating power of the developed method (Table 11). The chromatograms that obtained for PGH after subjecting to degradation are presented in Figure 6.

Table 11. Results of degradation study

Degradation condition	% Degradation
Acid hydrolysis	No degradation
Base hydrolysis	28
Oxidation	No degradation
Thermal (80 °C, 3 hours)	No degradation
Photolytic (1.2 million lux hours)	No degradation

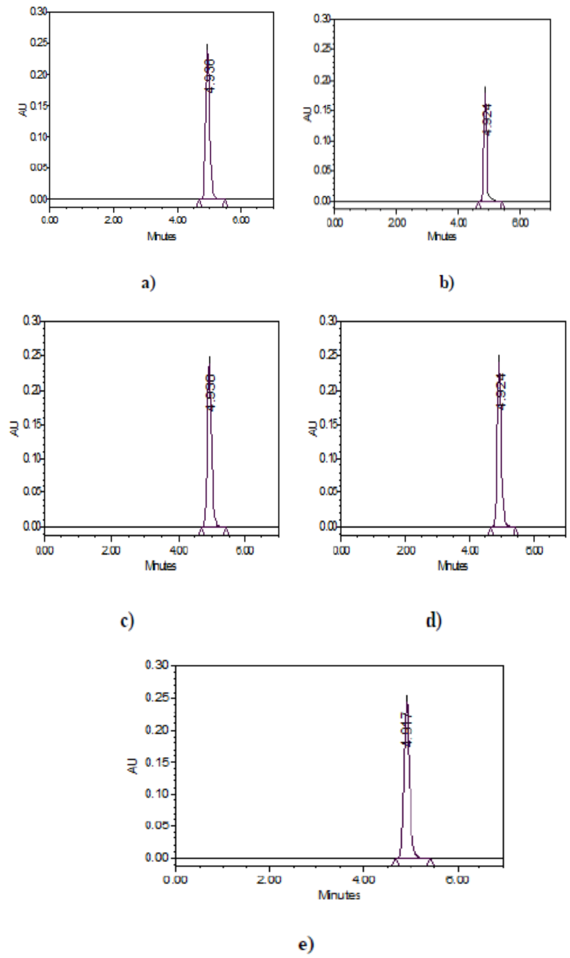


Figure 6: Chromatograms of PGH (200 µg mL^{-1}) after forced degradation: **a)** acid degradation; **b)** base degradation; **c)** peroxide degradation; **d)** photolytic degradation and **e)** thermal degradation

3 Materials and Methods

3.1 Apparatus and software

Chromatography was performed using Alliance Waters HPLC system (Waters Corporation, Milford, USA) equipped with Alliances 2657 series low pressure quaternary pump, a programmable variable wavelength UV detector, Waters 2996 photodiode array detector, and auto sampler. Data were collected and processed using Waters Empower 2.0 software.

3.2 Materials and reagents

All solvents used were HPLC grade. Pure sample of PGH was kindly supplied by Glenmark Pharmaceuticals, Mumbai, India, as gift. PGH-containing tablets; Neoglit-30 (30 mg) (Novus Life Sciences Private Limited, Mumbai, India), Oglo-15 (15 mg) (Panacea Biotech., Mumbai, India) were procured from the local market. Orthophosphoric acid, methanol, sodium hydroxide and hydrogen peroxide (all from Rankem, Hyderabhad, India) and de-ionized water (Millipore, Billerica, USA) were used in the investigation. Urine sample was collected from a 35 years old healthy male. Phosphate buffer (pH 3.6) was prepared by dissolving 1.3 g potassium dihydrogenorthophosphate in 1000 mL water, and pH with dilute H_3PO_4 adjusted using a pH meter (Metrohm AG, Herisau, Switzerland). Hydrochloric acid and sodium hydroxide solutions, 0.1M each were prepared by either diluting concentrated HCl (Sp. gr. 1.18) with water or dissolving the required quantity of NaOH in water. A 5% solution of H_2O_2 was obtained by dilution of commercial 30% chemical.

3.3 Chromatographic conditions

The analysis was carried out on an Inertsil ODS 3V (250 mm × 4.6 mm, 5 µm particle size) column. The column oven temperature was maintained at 35 °C and the auto sampler maintained at ambient temperature.

Phosphate buffer of pH 3.6 and methanol (60:40 v/v) was used as a mobile phase after filtering through 0.22 µm membrane filter. The flow rate was 1 mL min^{-1}, the detector wavelength was set at 220 nm and injection volume was 20 µL. The retention time was 4.952 minutes and run time was <10 minutes.

3.4 Standard PGH solution

A 500 µg mL^{-1} stock standard solution was prepared by dissolving the required quantity of pure PGH in the mobile phase and filtered through 0.22 µm membrane filter.

4 General procedures

4.1 Procedure for bulk drug

4.1.1 Procedure for preparation of calibration curve

Working standard solutions equivalent to 0.1-300 µg mL^{-1} PGH were prepared by serial dilutions of stock solution with the mobile phase. Aliquots of 20 µL were injected (in triplicate) and eluted with the mobile phase under the stated chromatographic conditions. Average peak area *versus* the concentration plot was prepared. Alternatively, the regression equation was derived using mean peakarea-concentration data, and the concentration of the unknown was computed from the regression equation.

4.1.2 Procedure for tablets

An amount of tablet powder equivalent to 20 mg PGH was weighed into a 100 mL volumetric flask, 60 mL of mobile phase was added and sonicated for 20 min in an ultrasonic bath to complete dissolution of the PGH. Then, the mixture was diluted to the mark with the mobile phase, mixed well and filtered using a 0.45 µm nylon membrane filter. The tablet extract (200 µg mL^{-1} in PGH) was injected in five replicates and chromatographed.

4.1.3 Procedure for placebo blank and synthetic mixture

A placebo blank containing starch (10 mg), acacia (15 mg), hydroxyl cellulose (10 mg), sodium citrate (10 mg), talc (20 mg), magnesium stearate (15 mg) and sodium alginate (10 mg) was prepared by homogeneous mixing. A 20 mg of the placebo blank was accurately weighed and its solution prepared as described under 'procedure for tablets', and then subjected to analysis by performing chromatography.

A synthetic mixture was prepared by adding an accurately weighed 20 mg of pure PGH to 20 mg of placebo and thorough mixing. Synthetic mixture solution equivalent to 200 µg mL^{-1} PGH was prepared as described under "procedure for tablets". The resulting solution was assayed (n= 5) by the proposed method.

4.1.4 Procedure for analysis of spiked human urine

Twenty five mg of pure PGH was taken in a 50 mL volumetric flask containing 5 mL of drug free urine; 5 mL of mobile phase and 25 mL of methanol were added. The content was mixed well and the volume was brought up to mark with mobile phase. The solution was filtered through 0.45 µm nylon membrane filter and 20 µL aliquots were injected in five replicates and eluted with the mobile phase under the stated chromatographic conditions.

4.1.5 Procedure for stress study

An accurately weighed 5 mg of pure PGH was taken in three separate 25 ml volumetric flasks; 5 ml 0.1M HCl, 5 ml 0.1 M NaOH or 5 ml 5% H_2O_2 were added to the flasks. The flasks were stoppered and placed in a water bath maintained at 80±2 ^{O}C for 2 h. After cooling, the acid or base was neutralised with 5 mL of 0.1M NaOH or 0.1M HCl depending on the case, and the contents of the flasks diluted to the mark with the mobile phase, and chromatographed by injecting 20 µL in triplicate. For thermal and photolytic degradation, solid sample placed in an oven at 80 °C for 3 h, and separate portion was exposed to 1.2 million lux hours in a photo stability chamber for 3 h, respectively. Post-degradation, 200 µg mL^{-1} PGH solution in mobile phase was prepared and chromatographed.

5 Conclusions

A rapid, sensitive, and specific isocratic HPLC UV-method was developed for the determination of pioglitazone without an internal standard. The method was validated for linearity, LOD, LOQ, accuracy, precision, and stability. The method uses a simple mobile phase, which is easy to prepare. The rapid run-time of <10 min and relatively low flow-rate (1 mL min^{-1}) allow the analysis of a large number of samples with less mobile phase, which proves to be cost-effective. The method is stability-indicating as required by the current ICH-guidelines; and hence, can be used for routine analysis.

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