

Validation of an UPLC method for the determination of Pioglitazone Hydrochloride active substance in Pharmaceutical Dosage Forms

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ABSTRACT: The present work reports an ultra-performance liquid chromatography (RP-UPLC) method for the quantitative determination of pioglitazone HCl (PGZ) in pharmaceutical dosage form. The chromatographic separation was performed on C₁₈, BEH (50 mm x 2.1 mm, 1.7 µm) column using isocratic elution. The optimized mobile phase consists of phosphate buffer (pH:6) as a solvent-A and 55:45 v/v mixture of acetonitrile as solvent-B. Flow rate was 0.4 ml/min with UV detection at (λ_{max}) 225 nm and the injection volume was set at 2 µL with retention time 1 min. The developed RP-UPLC method was validated as per International Conference on Harmonization (ICH) guidelines with respect to system suitability, specificity, precision, accuracy, linearity.

KEYWORDS Diabetes; Pioglitazone hydrochloride; UPLC; Validation; Pharmaceutical Dosage Form.

1. INTRODUCTION

The regulation of the ratio of blood sugar in the body is caused by the interaction of many chemicals and hormones. The most important hormones that contribute to the regulation of sugar ratio is insulin hormone, which is secreted from the pancreas. Diabetes Mellitus is used to identify a number of groups of conditions caused by insufficient insulin secretion or an increase in blood sugar as a result of a disorder caused by insulin [1].

There are two reasons why diabetes occurs. The first is due to reduced insulin production (Type 1 diabetes). The latter consists of the body developing resistance to the effect of insulin (Type 2 diabetes and gestational diabetes). The result of both conditions is an increase in blood sugar (hyperglycemia).

PGZ is an antidiabetic used in Type 2 diabetes to keep blood sugar levels under control. It acts by increasing the insulin effect and stimulating nonoxid glucose metabolism in the muscle so inhibiting gluconeogenesis in the liver. It has no effect on insulin secretion. Drugs reduce glucose concentration, accompanied by a decrease in plasma insulin concentration [2].

PGZ belongs to the thiazolidinedione class of drugs, which are characterized by a five-membered ring containing a nitrogen and sulfur atom. The pyridine ring in PGZ is an aromatic heterocyclic compound consisting of a six-membered ring containing five carbon atoms and one nitrogen atom. The ethoxy group is an organic functional group consisting of an oxygen atom bonded to an ethyl group. The phenylmethyl group is a common substituent in organic chemistry, consisting of a phenyl ring attached to a methyl group.

PGZ is a white to off-white crystalline powder that is insoluble in water and soluble in organic solvents such as ethanol and methanol. It is synthesized chemically and undergoes various purification and quality control measures to ensure its purity, potency, and safety. The chemical name of PGZ is 5-[[4-[2-(5-ethyl-2-pyridinyl)ethoxy]phenyl]methyl]-2,4- thiazolidinedione. Its molecular formula is C₁₉H₂₀N₂O₃S, and its molecular weight is 356.44 g/mol (3).

Literature indicates several analytical methods for the determination of PGZ in pharmaceutical formulations, drug substances and biological matrices [4-21].

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High performance liquid chromatography (HPLC) is a proven technique that has been used in laboratories worldwide over the past 30-plus years and also, the researchers have been reported determination of PGZ, mainly using reverse phase HPLC with UV and PDA detection [7-22]. One of them is analysis for PGZ tablets has been developed by Adukondalu et al. This method consists of phosphate buffer: acetonitrile in the ratio of (50:50, v/v) as a mobile phase. C18 (4.6×250 mm, 5µm) column was used. Analysis was performed at a flow rate of 1ml/min and the eluents are monitored at 245 nm (retention time was recorded as 6.3 min) [8].

Sayed was used a C-8 (mm) column and a mobile phase consisting acetonitrile-water (60:40 %v/v) at a flow rate of 1 ml/min and detection at 266 nm. The retention time of PGZ have been found to be 6.40 min and recoveries were between 98.82- 101.21 % (9).

Saber used ammonium phosphate buffer and acetonitrile (75:25, v/v) adjusted to pH 3 with formic acid as mobile phases of Nova-Pak C18 column (3.9 mm × 150 mm, 5 µm) on HPLC with UV detector of the PGZ drug substance in tablet form and analyzed at a wavelength of 225 nm on UV detector with flow rate of 1.0. mL/s and validated the drug substance assay method. Linearity was ≥ 0.5 µg/mL, precision was ≥ 99.14 %, and precision was ≤ 0.6 % [10].

Shaik et al. performed analysis on an isocratic system at a flow rate of 0.7 mL/min at a wavelength of 269 nm using Intersil ODS C18 (150 mm x4.6 mm, 5µm), ammonium acetate buffer solution as mobile phase, acetonitrile and acetic acid (50:50:1, v/v) [13].

Otherwise, combination therapy is a common approach for clinical treatment of type 2 diabetes mellitus. Jain et al. developed a method for drug substance analysis on a single HPLC system of 3 different samples using a UV-PDA detector for forms of metformin HCl, PGZ and glimepiride tablets. Inertsil-ODS-3 C18 column (250 × 4.60 mm, 5 µm) and methanol-phosphate buffer solution as mobile phase (pH 4.3) (75:25, v/v) at a flow rate of 1 mL/min and validated the method [17].

Lakshmi et al. also developed a drug substance analysis method for metformin and PGZ tablet forms. Gemini C18 column (150 × 4.6 mm, 5 µm) was used and selected as mobile phase in a ratio of acetonitrile and ammonium acetate buffer solution (pH 3) (42:58, v/v). The flow rate was 0.3 mL/min and the metformin peak at 5.17 min and PGZ peak at 8.1 min in the chromatogram in the device studied at a wavelength of 255 nm. The calibration curve is labeled 0.5-50 µg/mL for metformin and 0.3-30 µg/mL for PGZ [18].

Validated method was used in the form of diltiazem, metformin, pioglitazone and rosiglitazone tablets in human blood serum by Sultana N et al. The Hiber, 250×4.6 mm RP-C18 column was used. Acetonitrile-methanol-water (30:20:50, v/v ratio and pH 2.59 ± 0.02) was used as the mobile phase and analysis was performed at room temperature with a flow rate of 1.0 mL/min and at a wavelength of 230 nm in the UV-VIS detector. With good resolution, all antidiabetics were separated from each other [19]. Likewise, Lakshmi and Rajesh developed the HPLC method for the simultaneous determination of glipizide, rosiglitazone, pioglitazone, glibenclamide and glimepyridine, analysis from drug forms and human plasma. Phenomenex used as mobile phase with a triethylamine, acetonitrile and methanol (55:15:30; pH 3.5) at a flow rate of 1 mL/minute with a C18 (150 x4.6 mm, 5 µm) column. The drugs were separated in less than 20 minutes at a wavelength of 248 nm [20].

Another study developed an HPLC-DAD method for the simultaneous determination of metformin, glyclazide, PGZ, dapagliflozin, empagliflozin, saxagliptin, linagliptin and teneligliptin, Kant et al. They used a mixture of acetonitrile and 0.01% v/v formic acid as mobile phase. They performed the analysis with the Waters C18 column (250 mm × 4.6 mm, 5 µm), at 25 °C, with 20 µL injection volume and at a wavelength of 230 nm and determined the retention time to be 4.9 minutes [21]. Wang et al, validated method was applied to pharmacokinetic study of PGZ combined with omarigliptin in rats a by UHPLC-MS/MS [22].

In recent studies conducted with UPLC, Fachi and et al., proposed a UPLC-QToF-MS method for the simultaneous quantification of chlorpropamide, glibenclamide, gliclazide, glimepiride, metformin, nateglinide, PGZ, rosiglitazone, and vildagliptin in human plasma was developed and validated, using isoniazid and sulfaquinoxaline as internal standards [23]. Cijo and et al were described the experimental design an UPLC method components including column, pH, and mobile phase effects [24]. Abdel-Ghany et al. have developed UPLC-MS/MS method for the simultaneous determination of alogliptin and pioglitazone. 0.1% formic acid: acetonitrile (40:60, v/v) were used as mobile phase. They performed analysis with BEH C18 column (50 mm ×2.1 mm, 1.7 µm), at 25 °C, with 10 µL injection volume and flow rate of 0.3 mL/min. They determined the retention time for PGZ to be 1.33 minutes [25].

Li et al. conducted a co-assay with simvastatin, fenofibrate, gemfibrozil, metformin, glimepiride, nateglinide, PGZ and sitagliptin to determine the drug interaction potential of berberine hydrochloride. For

this, they developed a gradient method based on the new UPLC-MS/MS method. As mobile phase, they used water with 3 mM ammonium and 0.1% formic acid and acetonitrile with 3 mM ammonium and 0.1% formic acid. They performed the analysis with Luna Omega C18 column below 30 °C (20.0 mm × 2.0 mm, 1.6 µm), with 2 µL injection volume and flow rate of 0.4 mL/min. They determined the retention time for PGZ to be 0.79 minutes [26].

Based on the literature survey, a suitable method was developed, validated and compared with previous research studies. The results of the study reveal some unique or different findings compared to the existing literature. One possible way to explain the unique or different findings is to highlight the specific advantages or improvements of the newly developed and validated method. For example, the method may provide higher accuracy, sensitivity, specificity, reproducibility, or speed compared to the previously reported methods. The method may also use a simpler, more cost-effective or environmentally friendly approach, or it may be more suitable for specific sample types or matrices. Another way to explain the unique or different findings is to focus on the novel or unexpected results obtained from the study. These results may challenge or complement the existing knowledge and understanding of the studied system or phenomenon. For instance, the study may have identified new compounds, pathways, mechanisms, interactions, or effects that were not previously reported or explored. The study may also have revealed unexpected trends, correlations, or patterns that were not predicted or explained by the existing models or hypotheses.

Overall, the unique or different findings of the study should be clearly and objectively presented, supported by relevant data and analysis, and interpreted in the context of the existing literature and research gaps. It is also important to acknowledge the limitations and uncertainties of the study, and to suggest future directions for further research and applications.

2. RESULTS

2.1. Method Validation

The method was validated by the analysis of specificity, linearity, accuracy, precision to demonstrate reproducibility and reliability [16].

2.1.1. Specificity and Selectivity

The specificity parameter is a reliable analysis of the substance to be analyzed in the chromatogram formed by its interaction with excipients and solutions. The solvent and placebo solution should not interfere at the retention time of the main peak of the active ingredient in the chromatogram. The solvent, placebo solution, standard solution and test solution were analyzed in the method developed to determine the peaks from their chromatograms. The results are shown in solvent chromatogram, placebo solution chromatogram, standard PGZ solution chromatogram, sample solution chromatogram Figure 1 and Figure 2.

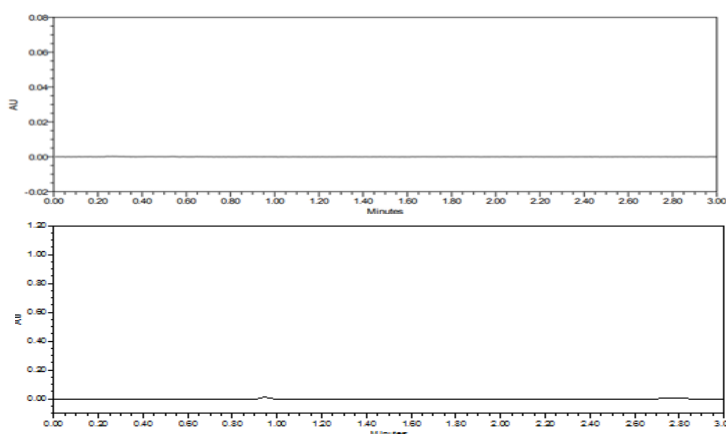


Figure 1. (a) Blank injection chromatogram, (b) Placebo injection chromatogram

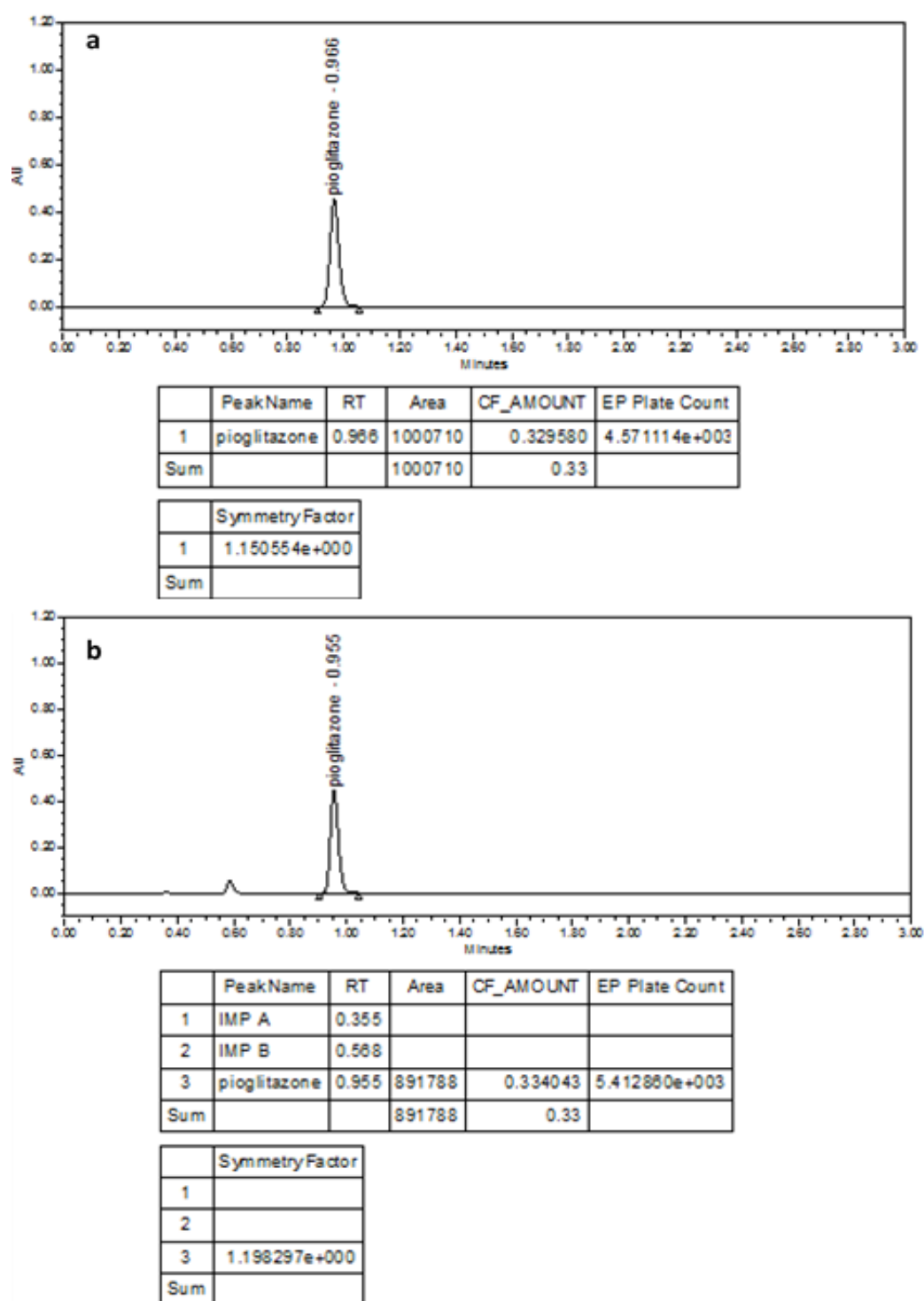


Figure 2. (a) Standard injection chromatogram of PGZ, (b) PGZ tablet injection chromatogram

2.1.2. Linearity

0.0015 (LOQ)– 0.384mg/mL concentration range. Solutions at a concentration of 80% - 90% - 100% - 110% - 120% with PGZ were given to the system in 3 replicates each. The calibration curve was generated by plotting the peak area against analyte concentration (Figure 3). Linear regression equations were calculated by the least squares method. Accordingly, the correct equation was $y = 3E+06x + 1455.8$ and the correlation coefficient was 0.9997. PGZ showed good linearity over the range tested.

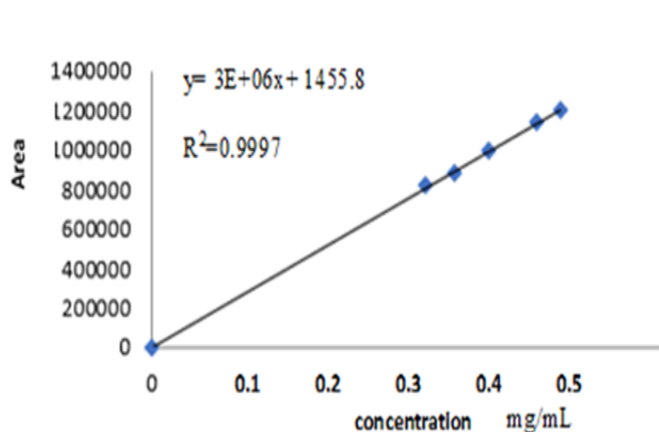


Figure 3. Calibration curve for PGZ

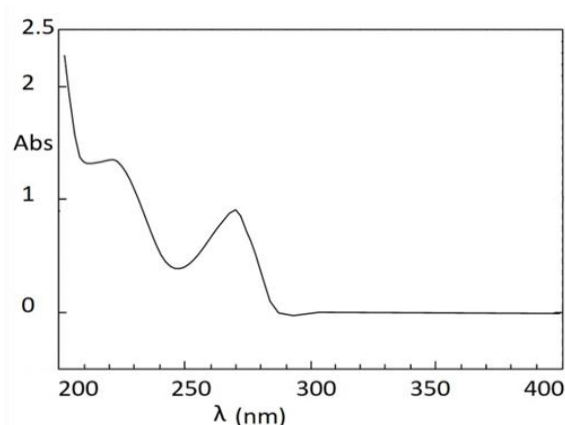


Figure 4. UV spectrum of standard PGZ

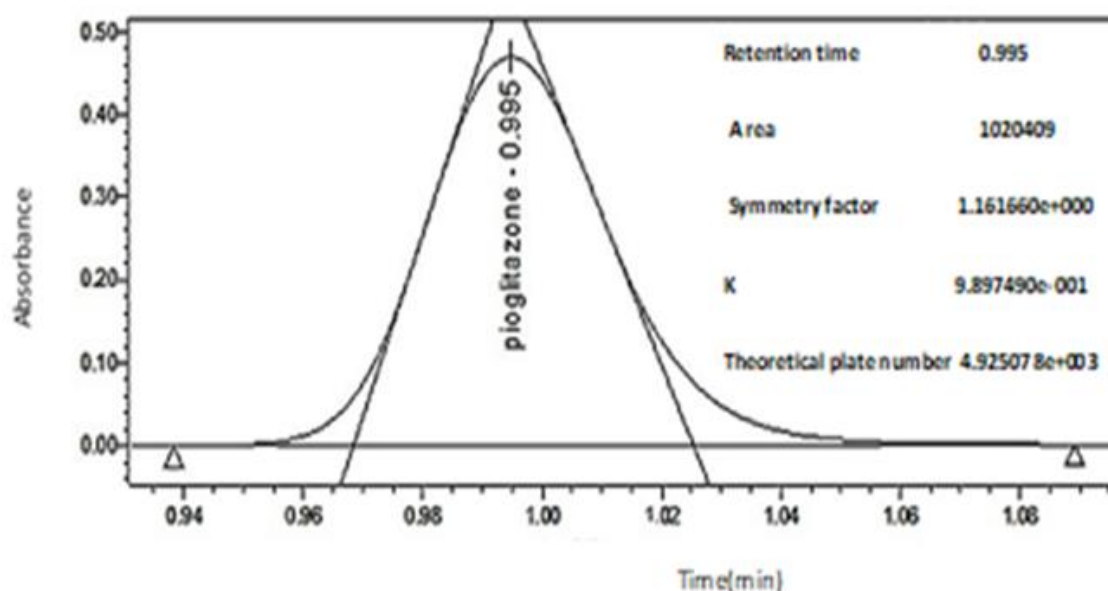


Figure 5. System Suitability Chromatogram of Standard PGZ Peak

2.1.3. Accuracy

Samples at concentrations of 80%, 100% and 120% were prepared to be a control sample containing PGZ drug substance and placebo and their accuracy (recoveries) were calculated based on their mean %. This process was repeated for 3 different samples. The results are given in Table 1.

Table 1. Accuracy study results (% recovery)

Level	Theoretical Concentration	Found Concentration	Accuracy RSD %	Mean RSD %
80%	0.26536	0.26721	100.70	99.97± 0.72
	0.26574	0.26556	99.90	
	0.26654	0.26457	99.30	

100%	0.33508	0.33024	98.60	100.00± 1.25
	0.33012	0.33176	100.50	
	0.32962	0.33258	100.90	
120%	0.39778	0.39826	100.10	100.50± 0.82
	0.39608	0.40157	101.40	
	0.39996	0.39936	99.80	
			AVG	100.10
			FAQ	0.28
			RSD%	0.28

2.1.4. Precision

The precision of the analytical method is expressed as 6 consecutive injections of a prepared homogeneous sample into the system and the relationship between these injections. The precision parameter is examined in 3 separate headings. The first is the precision of the system, the second is the precision of the method, and the third is the precision of the analysis conducted at the same laboratory on different days.

The system precision parameter is a measure of the reproducibility and stability of a chromatographic system over a given number of consecutive injections under the same analytical conditions. It is typically determined by analyzing a standard solution of a known compound multiple times, usually five or more, and calculating the relative standard deviation (RSD) of the peak area or retention time for each injection. The precision of the system is the parameter that determines the reliability of the sample-independent system. The reference solution prepared according to the method of analysis was administered to the system 6 times and based on our results, the %RSD was determined to be 0.74%, the theoretical number of plates was 4925 and the tailing factor was 1.16. The relative standard deviation values of the result of the analyses performed during the day were calculated as 0.60%. The relative standard deviation values of the samples given to the system on a different day were found to be between 0.99 and the results of the analysis (Table 2). The relative standard deviation determined for each concentration in the within day and between day precision runs was less than 2%.

Table 2. Intraday and between day precision results for the developed method

	Min Max	Average	%RSS
PGZ Intraday (n=6)	98.02-99.65	98.947± 0.589	0.60
PGZ Between days (n=6)	98.92- 101.12	99.620± 0.980	0.99
Acceptance Criterion: 2.00%			

2.1.5. Limit of Quantitation and Limit of Detection

LOD and LOQ are measures of the sensitivity of a test. LOD is a value defined as the lowest amount of an analyte that can be detected by the method but does not need to be accurately and precisely quantified. Conversely, the LOQ is a value that is the lowest amount of an analyte that can be measured with a reasonable level of accuracy and precision. The LOQ limit from the 100% concentration sample was 0.0015mg/mL, then the LOD limit was determined to be 0.0025mg/mL. The assay was used to quantify atorvastatin in a tablet containing 16 mg PGZ using the assay method developed and validated. The relative standard deviation calculated as a result of the analysis of a tablet containing.

3. DISCUSSION

In this study chromatographic conditions were optimized as the composition of the mobile phase, the flow rate and column temperature. Proposed method has been validated for accuracy, precision, linearity, specificity, and sensitivity. The method has proved to be specific, rapid, accurate, precise and reproducible according to ICH standards [27]. It is a high-pressure liquid chromatography technique that uses smaller particle sizes in the stationary phase to provide faster and more efficient separations. First of all, it was tried to determine the organic solvent ratio in the mobile phase. It is important to minimize the system volume, as this reduces the time required for the sample to travel through the system. So it aims to shorten the analysis time and obtain high yields. For this, it was determined that the best organic solvent for the separation process in the mobile phase was acetonitrile. One approach to achieving this is to make use of the loop volume change of the devices UPLC systems use a valve that can change the loop volume. This allows the loop to be filled with a larger volume of sample, which can then be injected into the system without reducing the concentration of the sample. Once the sample has been injected, the valve can be switched to the low-volume loop position, which reduces the system volume and improves the efficiency of the separation. In UPLC, the sample is injected into a small volume loop, typically less than 10 μ L. The loop is then connected to the UPLC system, which consists of a pump, a sample injector, a column, and a detector. The sample is then pushed through the column by the pump, and the components of the sample are separated based on their physicochemical properties. Overall, the use of loop volume change in UPLC can improve the speed and efficiency of separations, while still maintaining the concentration of the sample in the system. Reproducibility was ensured by using an injection volume of 0.5 μ L. System suitability parameters were applied to the developed analysis method and values that met the acceptance criteria were obtained as a result of this test. The calibration curve was selected for the PGZ in the concentration range of 0.0015 (LOQ) – 0.384 mg/mL. The correlation coefficient was 0.9997. In the precision studies performed on the same day and between different days, it was detected that the tablets were within the limit range of $\pm 5\%$. In the recovery studies, the average is 100.1% at the concentration limits of 80% - 100% - 120%. The retention time was determined to be 1 min with this method and less chemical usage was achieved with a flow of 0.4 ml/min in the UPLC system. In the UPLC method we performed, due to the advantage of using a short column even the total analysis time which was calculated for PGZ was shorter than the designated retention time. Compared with literature studies, Abdel-Ghany et al. determined the retention time of PGZ to be 1.33 minutes for simultaneous determination of alogliptin and PGZ using the UPLC-MS/MS method. 0.1% formic acid: acetonitrile (40:60, v/v) were used as mobile phase. In our study, phosphate buffer (pH:6) and acetonitrile (55:45 v/v) were used as mobile phase. The results show that, retention time is shorter than Abdel-Ghany's study also it is advantageous in terms of less solvent consumption [24]. As a characteristic of UPLC studies, less chemicals were used in our study and our flow rate was determined as 0.4 ml/min. Otherwise Cjioa et al. in a study conducted by PGZ, mobile phase condition was more suitable but LOQ and LOD were higher.

The present developed reversed-phase ultra-performance liquid chromatography (RP-UPLC) method was accurate, precise, and robust for estimation of pioglitazone in Active Pharmaceutical Ingredient and tablet dosage form. The developed RP-UPLC method showed better resolution and low retention time, very good separation efficiency, and faster elution and tiny amount of sample consumed when compared to the reported RP-HPLC methods (25).

Dhani et al., were estimated alogliptin and PGZ in tablet and retention time was found 0.529 for PGZ. The retention time is lower, but the LOQ and LOD and organic solvent volume are higher than in our study [28]. Li et al. determined the retention time of PGZ in to be 0.79 minutes in the simultaneous determination of simvastatin, fenofibrate, gemfibrozil, metformin, glimepiride, nateglinide, PGZ and sitagliptin using the UPLC-MS/MS method. They used Luna Omega C₁₈ (20.0 mm $\times$ 2.0 mm, 1.6 μ m) as a column. The retention times are better than our study because the column they use is shorter in size. But in the current RP-UPLC method, little amount of sample was consumed because 0.5 μ L of injection volume and flow rate was maintained at 0.4 ml/minute. There are no large differences in other parameters. Besides that, the method we use in our study may be preferred as it is more economical than MS/MS methods [25].

4. CONCLUSION

In conclusion, the method was described and compared with other studies by using various validation parameters like system suitability, precision, specificity, linearity, accuracy. The results obtained had advantages and disadvantages compared to the presented literature, however all the validation parameters

were found to be suitable within the acceptance criteria. It is shown that the method was accurate, reproducible, repeatable, linear, precise, and selective, proving the reliability of the method. The retention time is 1 min which enables proper separation and resolution between the peaks and quality control analysis of tablet formulations. Therefore, this method was demonstrated to be appropriate for determination of antidiabetic agent.

5. MATERIALS AND METHODS

The study used PGZ reference standard (Zhejiang Huahai), acetonitrile (J.T. Baker), methanol (J.T. Baker), sodium dihydrogen phosphate (Merck), sodium hydroxide (Merck). Piogtan Film-Coated Tablet (Zentiva) was procured from the company during manufacture. All chemicals and solvents were purchased from Merck (Darmstadt, Germany) and J.T Baker (China). The analysis used UPLC (Waters Alliance ACQUITY UV detector), Precision Scale (Metts Toledo), pH Meter (Metts Toledo), Magnetic Agitator, Ultrasonic Bath (Isolab), Waters Acquity UPLC BEH C18 2.1×50mm, 1.7 µm UPLC column, Waters vial, Filter (PET 0.45 µm porous). Ultrapure water used in the validation study was provided by the Merck Milipore water system. The Empower 3 software Biostat stars computer pogram was used.

5.1. Preparation of Solutions

Standard Solution: Keep 16.00 mg PGZ reference standard in an ultrasonic bath using acetonitrile as a solvent in a 50.00 mL volumetric flask for 15 minutes and filter it through a PET filter.

5.1.1. Test Solution: 10 tablet samples were ground in air and weighed 1 tablet weight of sample to contain 16.00 mg of PGZ drug substance and completed to volume with solvent in a 50 mL volumetric flask. It was kept in ultrasonic bath for 15 minutes and filtered with a 0.45 µm porous PET filter.

5.1.2. Placebo solution: 43.00 mg of the active-free placebo solution was weighed into a 50.00 mL volumetric flask and filtered by means of a 0.45 µm porous PET filter by completing it with solvent and allowing it to stand in an ultrasonic bath for 15 minutes.

5.1.3. Blank Solution: The solvent, acetonitrile, was administered to the system as a blank solution.

5.1.4. Mobile Phase solution: Dissolve 3.90 g of sodium dihydrogen phosphate in 1L of water and adjust pH = 6 with diluted NaOH solution. A 45:55 (v/v) mixture of this prepared buffer solution with acetonitrile was prepared.

5.2. Analysis of PGZ in Tablets

5.2.1. Preparation of Tablet Stock Solution

For this purpose, it was dissolved in a 50.00 mL volumetric flask containing 16.00 mg PGZ with acetonitrile and completed to 50.00 mL. It was sonicated for 15 minutes. At the end of this time, samples at a concentration of 0.32 mg/mL were filtered through the PET filter and injected into the UPLC system. This process was repeated for 6 different samples. The quantities of PGZ in the tablets were calculated using the equation of the previously prepared measurement curve.

5.2.2. Chromatographic Conditions Applied in UPLC system

Parameters; such as mobile phase difference, flow rate difference, injection volume difference were tried in order to determine the appropriate conditions in the development of the UPLC method. Chromatographic separation was performed using an ultra-performance high pressure liquid chromatography Waters Acquity UPLC BEH C18 (2.1×50 mm, 1.7µm) column was used for separation. The column temperature was set at 25°C. The flow rate was 0.4 mL/min and the volume of the injection was 0.5 µL and the wavelength was set at 225 nm. An isocratic separation was performed using phosphate buffer (pH 6) and acetonitrile (55:45) (v/v) as mobile phase. Under all these conditions, the total analysis time was 3 minutes and the retention time of the analyte was determined to be 1 minute.

5.2.3. Results of Analysis by PGZ Ultra High Performance Liquid Chromatography Determination of Chromatographic Conditions

The best available results of the trial studies for the method under development were obtained with phosphate buffer (pH 6): acetonitrile (55:45 v/v) mobile phase, at 0.5 µl injection volume and at 225 nm wavelength using a BEH C18 column with a length of 50 mm, an inner diameter of 2.1 mm, a particle diameter

of 1.7 μm . The retention time in the chromatographic system of this method is 1.0 minutes. The point at which the drug substance PGZ absorbs max. at 225 nm was used (Figure 4) Mobile phase ratios were first determined in the gradient system to give the drug substance PGZ the best retention time and peak shape in the UPLC system. pH adjustment was performed considering the pH sensitivity of the system. First of all, buffer solution was added to line A of the UPLC system and acetonitrile, which is an organic solution, was added to line B, and standard solution was injected into the system by trying gradient parameters. Mobile phase rates in the isocratic system were determined with the best injection. BEH C18 (2.1 $\times$ 50 mm, 1.7 μm) columns for Piog in the UPLC system gave the best results. To examine the contribution of the flow rate separation of the mobile phase used in the analysis, mobile phase containing buffer: acetonitrile (50:50), buffer: acetonitrile (60:40) and buffer: acetonitrile (55:45) was analyzed in the UPLC system at different flow rates at 25°C column temperature. The flow rate was given to the system at 0.3 and 0.4 ml/min, with the best result at a flow rate of 0.4 ml/min.. The optimal operating temperature was determined to be 30 °C.

5.2.4. System Suitability Test

The selectivity of a method is an indication that the substance to be analyzed can be accurately measured in the sample mix (example matrix). To verify the system suitability parameter, the solvent, placebo, and standard solution prepared at 100% test solution concentration and test solution were analyzed and the following parameters were examined. The wavelength is set at 225 nm. No apparent peak was observed at the retention time of the PGZ peak in the chromatograms obtained from solvent and placebo solution injections. To demonstrate injection repeatability, the RSD% was calculated by performing 6 standard injections into the UPLC device. For the suitability of the chromatographic conditions, the tailing, capacity factor, and theoretical number of plates were examined. The tailing was expected to be between 0.8-1.5 with a theoretical plate count of greater than 3000 and a %RSD of less than 2.0% (Figure 5).

5.2.5. Statistical analysis

The results were depicted graphically with means and standard deviations (SD) represented by vertical bars. Statistical significance was determined using Microsoft Office Excel (2016) and assessed through Student's t-test ($P < 0.05$) for differences in recovery values. All results are reported as mean $\pm$ SD, with statistical significance defined as $p < 0.05$.

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