INTRODUCTION

Pioglitazone is a thiazolidinedione oral antidiabetic drug. It is used in the management of Type-2 diabetes mellitus [1]. It is chemically designated as (±)-5-[4-(2-(5-Ethyl-2-Pyridinyl) ethoxy) benzyl]-2,4-thiazolidinedione hydrochloride. Its molecular weight is 392.90, and its empirical formula is C_{20}H_{22}N_{2}O_{4}S.HCl. It is soluble in N, N-dimethylformamide, slightly soluble in anhydrous ethanol, very slightly soluble in acetone and acetonitrile, practically insoluble in water, and insoluble in ether. Pioglitazone hydrochloride is an odorless white crystalline powder. Pioglitazone has one chiral centre; Active substance is produced as racemic mixture. Both enantiomers are having similar pharmacological properties.

Pioglitazone and its impurities chemical structure, as shown in Fig.1 [I-VII]. Pioglitazone undergoes hydrolysis to PIO-II, and undergoes oxidation to N-oxide as shown in Fig.1 [2]. Possible degradation pathway summarized in fig.1 [2].

So far to our present knowledge, no HPLC method was available in literature for estimation of Pioglitazone degradation product and known impurities in Pioglitzone Tablet. Methods are available for estimation of Pioglitazone in bulk and pharmaceutical Dosage [9]. An assay method for determination of Pioglitazone in pharmaceutical formulations and human plasma is also available [3-12]. Method is also available for impurity profiling in tablet formulation [8]. Attempts were made to develop a LC method for the estimation of degradants and known impurities in Pioglitzone tablet. This paper also deals with the validation of the developed method for the accurate quantification of degradants in dosage form.

In order to improve the sensitivity and selectivity of the chromatographic determination of Pioglitazone impurities, a simple reversed-phase HPLC method with UV detection at 225 nm, have been developed, where all six impurities have been separated in a single analytical column with a run time of 40 minutes. In our study, Water HPLC has been successfully used for the quantitative estimation of (PIO-II), (PGR-II), (PGS-II), Acid metabolite-(M-V), Keto metabolite-(M-III), and hydroxy metabolite-(M-IV). A reduction in separation time has been achieved, without compromising separation quality compared to other traditional Liquid Chromatography (LC) methods.

MATERIALS AND METHODS

Chemicals and reagents

Pioglitazone, impurities (PIO-II), (PGR-II), (PGS-II), Acid metabolite-(M-V), Keto metabolite-(M-III), and hydroxy metabolite-(M-IV) from Dr. Reddy's Laboratories Ltd., Hyderabad, India. Acetonitrile (HPLC-grade from J.T. Baker, USA), and triethylamine, Potassium dihydrogen phosphate monohydrate, Hydrochloric Acid, Hydrogen Peroxide were from Merck (Darmstadt, Germany). Water was purified by a Millipore (Bedford, MA, USA) Milli-Q water-purification system and passed through a 0.22 µm membrane filter (Durapore; Millipore, Dublin, Ireland) before use.

Standard and Test samples were prepared in Milli-Q water in the ratio of 900:200 v/v, and then adjust the pH 3.0 with dilute orthophosphoric acid (10%v/v).

Equipment

HPLC analysis was performed with a Waters (Milford, MA, USA) PDA 2996 system consists of a Quatemary solvent manager, a sample heating compartment, and Photodiode array detector. This system was controlled by Waters Empower software,Alltima C18, 250 x 4.6 mm, 5 µm employed as stationary phase for chromatographic separation. All samples were centrifuged for 10 minutes in order to eliminate insoluble excipients. The supernatant liquid was used for chromatographic analysis.

The impurity stock solution was prepared by dissolving an accurately weighed amount of impurity (PIO-II), 5 desethyl-N-Oxide, (PGS-II), Keto metabolite-(M-III), and hydroxy metabolite-(M-IV) in diluent, resulting in a concentration of 55 µg/mL of each impurity.

The identification solution was prepared by dissolving 83 mg of Pioglitazone working standard mixed with 3 mL of impurity stock solution and diluted to 100 mL in diluent.

The standard stock solution of Pioglitazone was prepared by dissolving an accurately weighed amount of Pioglitazone working standard in diluent, resulting in a concentration of 5.5 mg/mL. Then above solution was further diluted in diluent to get a final solution of 1.65 µg mL⁻¹.

The test solution was prepared by dissolving an accurately weighed portion of the powder, equivalent to 75 mg of Pioglitazone in 70 mL diluent. After sonication for around 20 minutes, the volume was made up to 100 mL. The above solution was centrifuged at 4000 rpm for 10 minutes in order to eliminate insoluble excipients. The supernatant liquid was used for chromatographic analysis.
Chromatographic Conditions

The analytes were separated on an Alltima C18, 250 x 4.6 mm, 5 µm column at column oven temperature of 45°C with a gradient run program at a flow-rate of 1.0 mL min⁻¹. The separation was achieved by gradient elution and the beginning ratio of mobile phase was A-B 55:45 (V/V); constant at the same ratio for 4 minutes then the ratio was changed linearly 35:65 (V/V) within 9 minutes; 20:80 (V/V) within 18 minutes; 15:85 (V/V) within 26 minutes; constant at the same ratio for 31 minutes. The system came back to initial ratio at 32 minutes and continued at the same ratio for 8 minutes. The mobile phase was filtered through a 0.45 µm Millipore filter, before use. UV detection was performed at 225 nm. The sample injection volume was 20 µL.

Method Validation

The method was validated for specificity, precision, accuracy, sensitivity and linearity range as per the International Conference on Harmonization (ICH) guidelines [15-16].

Specificity

A study was conducted to demonstrate the effective separation of pioglitazone and its degradants. Also, study was intended to ensure the effective separation of degradation peaks of formulation ingredients at the retention time of pioglitazone and its impurities. Separate portions of drug product and ingredients were exposed to following stress conditions to induce degradation.

The drug product was subjected to base hydrolysis using 0.5 N Sodium hydroxide, acid hydrolysis with 5 N Hydrochloric acid and neutral hydrolysis with water at 70°C for duration of 15 hours. Oxidation study was performed with 5 % Hydrogen Peroxide solution at room temperature for 15 hours. On photostability study, drug product was sufficiently spread on petri plates (1 mm thick layer), exposed to sunlight and UV light (1.2 million lux hours) at ambient conditions for 7 days. Humidity study was performed separately by exposing the drug product to humidity at 25°C, 90% RH for 7 days. Thermal degradation study was performed by heating drug product at 70°C for 24 hours. Similarly placebo samples were prepared as like as drug product by exposing formulation matrices without drug substance.

Stressed samples were injected into the HPLC system with photo diode array detector by following test method conditions.

Precision

The precision of test method was evaluated by using six samples of pioglitazone tablet test preparation, spiking with impurities blend solution to get the concentration of 1.5 µg mL⁻¹ of each impurity and analyzed as per test method. Intermediate precision was also studied using different column and performing analysis on different day.

Accuracy

To confirm the accuracy of the proposed method, recovery studies were carried out by standard addition technique. Samples were prepared in triplicate by spiking impurities in test preparation at the level of Limit of Quantification, 50%, 100%, and 150% (A nominal concentration of about 0.05 µg mL⁻¹ to 5.1 µg mL⁻¹) of the standard concentration.

Sensitivity

Sensitivity of the method was established with respect to Limit of detection and limit of quantification for Pioglitazone impurities (i.e. PIO-II), (PGR-II), (PGS-II), Acid metabolite-(M-V), Keto metabolite-(M-III), and hydroxy metabolite-(M-IV)). Known concentration of drug solution and its impurities were injected; limit of detection (LOD) and limit of quantification (LOQ) was established by signal to noise ratio method as mentioned below.

LOD = s/n ratio should be between 2 to 3.4

LOQ = s/n ratio should be between 9 to 11.4

LOD and LOQ were experimentally verified by injecting six replicate injection of each impurity at the concentration obtained from above formula.

Linearity of Detector Response

A series of solutions of Pioglitazone impurities in the concentration ranging from limit of quantification (0.05 µg mL⁻¹) to 200% (6.7 µg mL⁻¹) of standard concentration were prepared and injected into the HPLC system.

Application of Developed Method

The method suitability was verified by analyzing three different strengths of finished product in-house formulated product. The content of 20 Tablets (each containing 45mg/30mg/15mg of Pioglitazone) were emptied and intimately mixed. Quantity equivalent to 75 mg of drug weighed accurately and dissolved in 100 mL of diluent by 20 minutes sonication. The solution was centrifuged and injected.

RESULTS AND DISCUSSION

A reversed-phase chromatographic technique was developed to quantitate Pioglitazone and its impurities at 225 nm. The presence of non-aqueous solvents in the mobile phase, such as methanol and Acetonitrile, was studied. A mixture of acetonitrile and methanol was chosen as organic modifier. Satisfactory separation was achieved when the acetonitrile concentration was 50% in mobile phase B with 30% methanol.

The effect of triethyl amine concentration on analyte retention was studied. Triethyl amine is known as ion pair showing effect on peak resolution by decreasing the analyte interaction with silane groups at the chromatographic surface and decreased peak tailing and peak broadening. At low pH (less than 2) with high operating column temperature, hydrolysis of the siloxane bond can occur, stripping the bonded phase from the silica support. To avoid this, pH 3.6 buffer used in mobile phase A. Satisfactory resolution was achieved with use of a mixture of water, methanol and acetonitrile in mobile phase B as demonstrated in Fig. 2B.

C8 and C18 column were first evaluated as stationary phase for the separation of Pioglitazone and its degradants. C18 column was adopted for the analysis because it provided a similar separation of the analytes. Selectivity, sensitivity, resolution, and speed of chromatographic separation were optimized for the HPLC method. Comparing the signal to noise ratio of Pioglitazone shows that proposed method has better sensitivity. Present HPLC method offers well resolution within 40 minutes. The retention times of Pioglitazone at 17.8, impurity PIO-II at 5.8, PGS-II at 22.0, 5-Desethyl at 8.1, N-Oxide at 12.6, Keto metabolite-(M-III) at 13.8, and hydroxy metabolite-(M-IV) at 10.3 minutes respectively, under the chromatographic conditions described. Chromatograms obtained from placebo, drug spiked with impurities mixture solution are shown in Fig. 2A, Fig. 2B respectively. The retention times were much more reproducible on a C18 column and mixture of A and B mobile phase.

HPLC system has been proved to be a promising tool for separation of Pioglitazone and its impurities. Pioglitazone its degradants were well separated with good peak shape and resolution. No interfering peaks were observed in blank & placebo, indicating that signal suppression or enhancement by the product matrices was negligible. Use of HPLC resulted in a reduction in run-time to 40 min, without compromising the efficiency, compared with a run-time.

After satisfactory method development it was subjected to method validation as per ICH guideline [15-16]. The method was validated to demonstrate that it is suitable for its intended purpose by standard procedure to evaluate adequate validation characteristics. The result of system suitability parameter was found to be complying with acceptance criteria: relative standard deviation of replicate injection is not more than 5.0% and resolution between N-Oxide and Keto metabolite-(M-III) is not less than 2.0 as shown in Table 1. The result of specificity study ascertained the separation of degradation peaks from Pioglitazone peak and the spectral purity of all exposed samples were found spectrally pure and data of degradation studies are shown in Table 2. Fig.2C-G. The % RSD of replicate determination was found to be <5% in both precision and intermediate precision, which indicates that the method is precise and the data of precision studies
are shown in Table 3. The result obtained from the recovery study were found within the range of 90% to 110% (LOQ to 150%), which indicates that method is accurate and data for the same are shown in Table 4. Sensitivity of the method was verified and the method was found to be linear, accurate and precise at limit of quantification and the data of LOD & LOQ studies are given in Table 4 and 5. The calibration curve of all impurities were obtained by plotting the peak area of individual impurity versus concentration over the range of about 0.05-6.7 μg/mL and were found to be linear (r = 0.999). The data of regression analysis of the calibration curves are shown in Table 3. The impurity content in in-house formulations was found to be satisfactory.

### Table 1: System Suitability

<table>
<thead>
<tr>
<th>Compound name</th>
<th>RT</th>
<th>RRT*</th>
<th>Tailing factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIO-H Imp.</td>
<td>5.9</td>
<td>0.33</td>
<td>1.1</td>
</tr>
<tr>
<td>5-Desethyl imp</td>
<td>8.2</td>
<td>0.46</td>
<td>1.0</td>
</tr>
<tr>
<td>Hydroxy Imp.</td>
<td>10.4</td>
<td>0.58</td>
<td>1.0</td>
</tr>
<tr>
<td>N-oxide</td>
<td>12.7</td>
<td>0.71</td>
<td>1.0</td>
</tr>
<tr>
<td>Keto imp</td>
<td>13.9</td>
<td>0.78</td>
<td>1.0</td>
</tr>
<tr>
<td>PGS-2 imp</td>
<td>22.0</td>
<td>1.24</td>
<td>1.0</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>17.8</td>
<td>1.00</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Relative retention times (RRT) were calculated against the retention time (RT) of Pioglitazone.

### Table 2: Specificity

<table>
<thead>
<tr>
<th>Stress Condition</th>
<th>Drug Product</th>
<th>PIO-II % degradation</th>
<th>5-Desethyl % degradation</th>
<th>Hydroxy % degradation</th>
<th>N-oxide % degradation</th>
<th>Keto % degradation</th>
<th>PGS-2 % degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>kept the flask at 70°C for 1.5Hrs by adding the 5 mL of 5N HCL on shaking water bath</td>
<td>PIO-II</td>
<td>0.23</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.014</td>
</tr>
<tr>
<td>kept the flask at 70°C for 1.5Hrs by adding the 5 mL of 0.5N NaOH on shaking water bath</td>
<td>PIO-II</td>
<td>0.92</td>
<td>0.051</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.010</td>
</tr>
<tr>
<td>kept the flask at 70°C for 1.5Hrs by adding the 5 mL of 5% H2O2 on shaking water bath</td>
<td>PIO-II</td>
<td>2.17</td>
<td>0.283</td>
<td>ND</td>
<td>0.698</td>
<td>ND</td>
<td>0.013</td>
</tr>
<tr>
<td>kept the flask at 70°C for 1.5Hrs by adding the 5 mL of Water on shaking water bath</td>
<td>PIO-II</td>
<td>0.22</td>
<td>0.085</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.013</td>
</tr>
<tr>
<td>Exposed to sunlight for about 1.2 Million Lux hours in photostability chamber</td>
<td>PIO-II</td>
<td>0.08</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.021</td>
</tr>
<tr>
<td>Exposed to UV light both for about 200 watt hours / square meter in photostability chamber</td>
<td>PIO-II</td>
<td>0.07</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.019</td>
</tr>
<tr>
<td>Exposed to thermal degradation at 70°C for about 24 hours</td>
<td>PIO-II</td>
<td>0.08</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.015</td>
</tr>
<tr>
<td>Exposed to humidity at 25°C, 90% RH for about 7 days</td>
<td>PIO-II</td>
<td>0.08</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.017</td>
</tr>
</tbody>
</table>

### Table 3: Regression and Precision Data

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>PIO-II</th>
<th>5-Desethyl</th>
<th>Hydroxy</th>
<th>N-oxide</th>
<th>Keto</th>
<th>PGS-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD (μg/mL)</td>
<td>0.001</td>
<td>0.002</td>
<td>0.003</td>
<td>0.001</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>LOQ (μg/mL)</td>
<td>0.004</td>
<td>0.008</td>
<td>0.008</td>
<td>0.003</td>
<td>0.009</td>
<td>0.011</td>
</tr>
<tr>
<td>Slope (B)</td>
<td>583.48152</td>
<td>358.19159</td>
<td>300.91853</td>
<td>67229.355</td>
<td>53968.737</td>
<td>30047.911</td>
</tr>
<tr>
<td>Intercept [a]</td>
<td>84.3922636</td>
<td>722.67353</td>
<td>1166.3638</td>
<td>1466.36027</td>
<td>652.716221</td>
<td>-1407.441268</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>0.999</td>
<td>1.000</td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td>0.8</td>
<td>0.6</td>
<td>1.0</td>
<td>0.5</td>
<td>0.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Intermediate precision (%RSD)</td>
<td>0.3</td>
<td>0.7</td>
<td>0.7</td>
<td>0.2</td>
<td>0.3</td>
<td>1.8</td>
</tr>
</tbody>
</table>

### Table 4: Evaluation of Accuracy

<table>
<thead>
<tr>
<th>Amount Spiked</th>
<th>% Accuracy</th>
<th>5-Desethyl</th>
<th>Hydroxy</th>
<th>N-oxide</th>
<th>Keto</th>
<th>PGS-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOQ</td>
<td>93.9</td>
<td>104.3</td>
<td>101.1</td>
<td>100.1</td>
<td>110.7</td>
<td>94.6</td>
</tr>
<tr>
<td>% RSD</td>
<td>4.8</td>
<td>0.9</td>
<td>6.0</td>
<td>4.0</td>
<td>8.0</td>
<td>2.6</td>
</tr>
<tr>
<td>50 %</td>
<td>102.5</td>
<td>90.2</td>
<td>101.2</td>
<td>90.0</td>
<td>88.7</td>
<td>94.1</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.2</td>
<td>0.8</td>
<td>1.6</td>
<td>1.1</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>75 %</td>
<td>101.7</td>
<td>90.3</td>
<td>90.8</td>
<td>90.2</td>
<td>97.9</td>
<td>94.1</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.1</td>
<td>0.6</td>
<td>0.9</td>
<td>0.8</td>
<td>0.3</td>
<td>1.1</td>
</tr>
<tr>
<td>100 %</td>
<td>101.0</td>
<td>95.6</td>
<td>98.7</td>
<td>91.2</td>
<td>92.6</td>
<td>93.9</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.6</td>
<td>1.9</td>
<td>2.8</td>
<td>3.6</td>
<td>2.1</td>
<td>0.6</td>
</tr>
<tr>
<td>125 %</td>
<td>101.2</td>
<td>92.6</td>
<td>94.6</td>
<td>90.6</td>
<td>96.8</td>
<td>94.7</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.1</td>
<td>0.9</td>
<td>0.6</td>
<td>0.3</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>150 %</td>
<td>102.2</td>
<td>89.6</td>
<td>97.6</td>
<td>91.7</td>
<td>96.5</td>
<td>95.1</td>
</tr>
<tr>
<td>% RSD</td>
<td>1.1</td>
<td>0.2</td>
<td>0.6</td>
<td>5.2</td>
<td>2.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

% RSD values calculated with three sample recovery at each level.
(A) Placebo

(B) Impurity and Active Spiked Drug Product

(c) Base Stress Drug Product

(D) Acid Stress Drug Product
**CONCLUSION**

Although Liquid chromatography (LC) is a versatile technique for the analysis of drug in complex matrices, such as biological or pharmaceuticals, a number of analytical approaches have been previously described to determine Pioglitazone in biological materials and pharmaceutical preparation; however, this is the first study reporting a validated reversed phase method for quantification of impurity in Pioglitazone formulation. The simple HPLC method developed in this study makes it suitable for separation and estimation of impurities without interference from excipients and other related substances present in the pharmaceutical matrices. The analytical performance and the result obtained from analysis of two different formulations demonstrated that the method is reliable and sufficiently robust. In conclusion, the high sensitivity, good selectivity, accuracy and reproducibility of the HPLC method developed in this study makes it suitable for quality control analysis of complex pharmaceutical preparation containing Pioglitazone and its impurities.

**ACKNOWLEDGEMENT**

We wish to express our sincere thanks to the Management of Dr. Reddys Laboratories, Hyderabad, India for their support and encouragement.
(I) Pioglitazone Hydrochloride

![Chemical Structure of Pioglitazone Hydrochloride]

(II) PIO-II Impurity

![Chemical Structure of PIO-II Impurity]

(III) 5-Desethyl 5-Carboxy Pioglitazone Impurity

![Chemical Structure of 5-Desethyl 5-Carboxy Pioglitazone Impurity]

(IV) Hydroxy Pioglitazone Impurity

![Chemical Structure of Hydroxy Pioglitazone Impurity]

(V) N-Oxide Impurity

![Chemical Structure of N-Oxide Impurity]

(VI) Keto Pioglitazone Impurity

![Chemical Structure of Keto Pioglitazone Impurity]
(VII) PGS-2 Impurity

\[
\text{5-[[4-[2-(5-Ethyl-2-pyridinyl)-ethoxy]-benzylidene]-2,4-] thiazolidinedione monohydrochloride}
\]

[2] Possible Degradation Pathway:

I) Oxidation:

\[
\text{5-[[4-[2-(5-Ethyl-2-yl)ethoxy]benzyl]thiazolidine-2,4-dione}
\]

Pioglitazone

Oxidation

II) Hydrolysis:

\[
\text{5-[[4-[2-(5-Ethyl-2-yl)ethoxy]benzyl]thiazolidine-2,4-dione}
\]

Pioglitazone

Hydrolysis

\[
\text{2-Carbamoylmethyl-3-[4-[2-(5-ethyl-2-yl)-ethoxy]-phenyl]-propionic acid}
\]
III) Thermal

Fig. 1: Chemical structures and names of Pioglitazone Hydrochloride and its impurities
REFERENCES


