Method Development and Validation of Metformine, Pioglitazone and Glibenclamide in Tablet Dosage Form by using RP-HPLC

Anandkumar R. Tengli*, Gurupadayya BM, Neeraj Soni1 and Vishwanathan B
Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS University, S. S. Nagar, Mysore-570015, Karnataka, India

Abstract
Reverse phase high performance liquid chromatographic method has developed for the simultaneous estimation of metformine, pioglitazone and glibenclamide in tablet dosage form for using gliclazide as an internal standard. The separation was achieved at ambient temperature with low pressure gradient mode by using 5 µ size phenomenex luna CN (100R 250×4.60 (mm) column with mobile phase containing acetonitrile, water and buffer (0.5% potassium dihydrogen phosphate) pH 2.5 adjusted with orthophosphoric acid in the ratio of 60:20:20. The flow rate was 1 mL min⁻¹ and eluent was monitored at 230 nm by using UV detector. The selected chromatographic conditions effectively separated Metformine, pioglitazone and Glibenclamide with the retention time of 2.2, 2.8 and 5.6 min respectively. The linearity range for metformine, pioglitazone and glibenclamide is found in the range of 50-300 µg mL⁻¹, 1.5-9.0 µg mL⁻¹ and 0.5-3.0 µg mL⁻¹ respectively. The developed method was found to be accurate, simple, specific and reproducible. It can also be used for routine quality control analysis of these antidiabetic drugs in combinational dosage forms.

Keywords: HPLC; Metformine; Pioglitazone; Glibenclamide; Gliclazide; Simultaneous estimation

Introduction
Metformin HCl is an antidiabetic drug. Chemically, it is 1,1-dimethyl biguanide hydrochloride. Metformin decrease the gluconeogenesis and increases the glucose uptake by muscles and fat cells. It is indicated for the treatment of type II diabetes mellitus, used alone or in combination with sulfonylureas, alpha-glycosidase inhibitors, or insulin. It has fewer adverse effects and is generally tolerable and self limiting. Although its exact mechanism of action is not completely understood, it mainly has blood glucose-lowering activity which appears to be primarily through suppression of hepatic glucose output. Its therapeutic blood glucose-normalizing action is dependent on the presence of circulating insulin. Various methods have been reported for the analysis of metformine alone or its combination with other drugs in different dosage forms is capillary electrophoresis [1,2], HPLC [3,4], Liquid chromatography–mass spectrometry (LCMS), [5], spectrophotometric [6], High performance thin layer chromatography (HPTLC) [7] and spectrofluorometric method [8].

Pioglitazone is a thiazolidinedione antidiabetic agent, chemically it is (RS)-5-[(4-[2-(5-ethylpyridin-2- yl)ethoxy]benzyl)thiazoledine-2,4-dione. It selectively stimulates the peroxisome proliferator-activated receptor gamma (PPAR-γ) and to a lesser extent PPAR-α. Pioglitazone modulates the transcription of the insulin-sensitive genes involved in the control of glucose and lipid metabolism in the muscle, adipose tissue, and in the liver. It is used for the treatment of type II diabetes mellitus either alone or in combination with other oral antidiabetic drugs. Various methods reported for the assay of pioglitazone or its combination with other drugs is HPLC [9-12], HPTLC [13], spectrophotometry [14,15], LCMS/MS [16], chemometrics [17] and voltammetry [18].

Glibenclamide is a second generation sulphonyl urea oral hypoglycemic agent, chemically it is 1-[4-[2-(chloro-2-methoxybenzamido) ethyl]-benzenesulphonyl]-3-cyclohexylurea, 5-chloro-N-[2-[4-[[[cyclohexylamino]carbonyl]-amino]sulphonyl] phenyl] ethyl-2-methoxy benzamide. It is more potent than first generation sulfonyl ureas. It is used to assist to control mild to moderately severe type II diabetes mellitus that does not require insulin that can be adequately controlled by diet alone. It is drug of choice for initiating treatment in noninsulin-dependent diabetes mellitus when diet and weight control fails. It stimulates the secretion and enhances the utilization of insulin by appropriate tissues. Different methods have been reported for the analysis of Glibenclamide alone or its combination with other drugs is HPLC [19-24], voltametric [25], spectrophotometry [25] and LCMS/MS [26].

Experimental
Materials, reagents and chemicals
Working standards, Metformine, Glibenclamide, Pioglitazone and Gliclazide was from Ranbaxy laboratories, New Delhi, India. Potassium dihydrogen phosphate, orthophosphoric acid AR grade, HPLC grade Acetonitrile, methanol and water used were from Rankem, Mumbai. The pharmaceutical dosage form containing 500 mg MET, 15 mg PIOG and 5 mg GLIB, Triglycomet 520 mg, 20 tablets (USV Pharmaceuticals Ltd.) purchased from a local drug store. Gliclazide, which was employed as an internal standard (IS) was obtained from Ranbaxy Laboratories, New Delhi.

Equipment
The development and validation of the assay was performed on a Shimadzu LC2010 integrator a 4-liquid gradient HPLC system (Kyoto Japan), provided with high speed auto sampler, column oven, degasser and UV detector. LC solution software was used for data acquisition.

*Corresponding author: Anandkumar R. Tengli, Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS University, S. S. Nagar, Mysore-570015, Karnataka, India, E-mail: anandrtengli@gmail.com
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Chromatographic condition

The chromatographic column, phenomenex luna 5 μ CN 100R, 250×4.60 mm (5 micron size) column was used as stationary phase. The mobile phase was prepared with acetonitrile, water and buffer (60:20:20). Buffer solution was prepared by dissolving 0.5% potassium dihydrogen orthophosphate in 100 mL of water. The pH was adjusted to 2.5 with orthophosphoric acid; flow rate was 1 mL min⁻¹ and injection volume was 20 µl. The detection was performed at 230 nm using UV detector at 25°C.

Preparation of standard solution

Separate stock solutions of 1000 µg mL⁻¹ of metformine, pioglitazone and glibenclamide were prepared by dissolving in 25 mL HPLC grade methanol and diluted to 1000 mL with acetonitrile, then 1 mL of stock solution into a 10 mL of standard volumetric flask and diluted to 1000 mL with acetonitrile, then pioglitazone and glibenclamide were prepared by dissolving in 25 mL acetonitrile to furnish the concentration range listed in table 1.

Preparation of linearity solution

Linearity solutions were freshly prepared from the stock solution by diluting with acetonitrile as 50, 100, 150, 200, 250 and 300 µg mL⁻¹ of metformine, 1.5, 3, 4.5, 6, 7.5 and 9 µg mL⁻¹ of pioglitazone and 0.5, 1, 1.5, 2, 2.5 and 3 µg mL⁻¹ glibenclamide and IS (internal standard), respectively. Each solution was injected in triplicate and chromatographed under the same chromatographic conditions as specified above. Gliclazide (10 µg mL⁻¹) was used as internal standard for determination of mixtures of metformine, pioglitazone and glibenclamide with Gliclazide. Linear relationships were obtained when drug-to-internal standard peak-area ratios were plotted against the corresponding concentrations for each drug.

Preparation of sample solution for batch analysis

Average weight was calculated by weighing 20 tablets. The tablets were crushed into homogenous powder. A quantity of powder equivalent to one tablet containing 500 mg of metformine, 15 mg of pioglitazone and 5 mg of glibenclamide was transferred into a 100 mL volumetric flask. To this flask, 50 mL of methanol were added, and the solution was sonicated for 25 min with intermittent shaking. The solution was cooled to ambient temperature. Then the volume was made up with acetonitrile and centrifuged at 10,000 rpm for 10 min. The centrifuged solution filtered through 0.45 μm nylon filters (Millipore, Milford, MA, USA). From the filtered solution, aliquots of appropriate volume were transferred to 10 mL volumetric flasks and diluted to volume with acetonitrile to furnish the concentration range listed in table 1.

Analytical method validation

Specificity of the method: The terms selectivity and specificity are often used interchangeably. Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. This parameter was performed to know the retention time of each drug in a mixture and in the sample to understand if any drug-drug interaction or drug-excipients interaction is present.

System suitability: System suitability test is used to verify that the resolution and reproducibility of the chromatographic systems are adequate for the analysis to be done. The tests are based on the facts that the equipments, electronics, samples to be analyzed constitute an integral system that can be evaluated as such. The limits for system suitability were set for theoretical plates, resolution and asymmetry.

Linear range: The linearity of the method was evaluated by analyzing different concentration of the drugs. According to ICH recommendations [25], at least five concentrations must be used. In this study, six concentrations were chosen in the ranges of 50-300 µg mL⁻¹ metformine, 1.5-9.0 µg mL⁻¹ pioglitazone and 0.5-3 µg mL⁻¹ glibenclamide respectively.

Accuracy and precision: The accuracy of the method was determined by recovery experiments using the standard addition method. Each solution was injected in triplicate and percentage recovery was calculated. The precision of the method was assessed by studying intra-day and inter-day variation. In the intra-day studies, standard and sample solutions were analyzed in triplicate on the same day and percentage RSD was calculated. In case inter-day studies, standard and sample solutions were analyzed in triplicate on three consecutive days and percentage RSD was calculated.

Limits of detection (LOD) and limit quantitation (LOQ): In accordance with ICH recommendations [27], the approach based on the standard deviation of the response and the slope of the calibration plots was used to determine detection and quantification limits. LOD and LOQ values were estimated as [(standard deviation of repeatability)/(Slope of the regression equation)] by multiplying with 3.3 and 10 respectively. The values obtained are given in table 1.

Selectivity: The selectivity of the method was evaluated by assessing whether excipients present in the pharmaceutical formulations interfered with the analysis. A placebo for each tablet was prepared by mixing the respective excipients, and solutions were prepared by following the procedure described in the section on sample preparation. The commonly used tablet excipients did not interfere with the method.

Robustness: Robustness is a measure of capacity of analytical methods to remain unaffected by small but deliberate variation of the operating conditions. It was tested by studying the effect of changing mobile phase pH by ± 0.2, the amount of buffer in the mobile phase by ± 2%, and detector wavelength by ± 2 nm

Ruggedness: To test the ruggedness of the method, the analysis was done on different days and different chemists to check for any changes in the chromatograph. Percentage RSD for the retention time and area was calculated.

Results and Discussion

To establish and validate an efficient method for analysis of
these drugs in pharmaceutical formulations, preliminary tests were performed with the objective of selecting optimum chromatographic conditions. The separation was tried using either column described previously in the literature or alternative stationary phases. The main problems encountered during these investigations were lack of resolution between metformine, pioglitazone, glibenclamide and IS. To solve these problems, three columns, C18, C8, and CN, were used for simultaneous determination of the drugs. The best resolution and peak shape, without excessive tailing, were obtained by use of the CN column. The effect of mobile phase composition, flow rate and pH were also studied. The best resolution with reasonable retention time was obtained with mobile phase containing acetonitrile, water and 0.5% of potassium dihydrogen phosphate buffer pH 2.5 adjusted with orthophosphoric acid (60:20:20) with 1.0 mL min⁻¹ flow rate. To avoid multiple peaks of peptides on reversed phase columns, the pH must be controlled with buffers, for example potassium dihydrogen phosphate. A major reason for using a concentration of 0.5 mM was to achieve maximum sensitivity of UV detection at low wavelengths. Members of this class of antidiabetic drugs contain weak chromophores and are characterized by low molar absorptive, so the detector was set at 230 nm to increase the sensitivity of the method. The specificity of the method is illustrated in figure 1 which indicates that separation of the compounds was complete. Average retention times ± standard deviation for IS, MET, PIOG and GLIB were 2.23785 ± 0.07850, 2.8217 ± 0.08578 and 5.8691 ± 0.017347 min, respectively, for six replicate analyses. In determination of accuracy and precision, recovery was 100 ± 3%, which indicates the method is accurate, and intra-day and inter-day variation, as RSD, were no more than 5.16%, indicating the method is precise. In determination of the robustness of the method, slight variation of mobile phase pH, amount of buffer, in the mobile phase, and detector wavelength had no significant effect on chromatographic resolution.

**Method validation**

**System suitability:** The RSD values of peak area and retention time for drugs and IS are within 2% indicating the suitability of the system (Table 2).

**Linearity:** The calibration curves were prepared by plotting the peak areas of the drug to IS which were linear in the range of 50-300, and other concentrations in the range of 0.5-1.5 µg mL⁻¹.

![Chemical structures of drugs.](image)

<table>
<thead>
<tr>
<th>Name of the drug</th>
<th>Actual concentration (µg mL⁻¹)</th>
<th>Intra-day precision</th>
<th>Inter-day precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found concentration (µg mL⁻¹) ± SD</td>
<td>RSD (%)</td>
<td>RME (%)</td>
</tr>
<tr>
<td>MET</td>
<td>50</td>
<td>49.28 ± 0.4124</td>
<td>0.837</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>99.88 ± 0.0525</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>149.90 ± 0.0746</td>
<td>0.049</td>
</tr>
<tr>
<td>PIOG</td>
<td>1.5</td>
<td>1.474 ± 0.018</td>
<td>1.232</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.920 ± 0.052</td>
<td>1.796</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
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</tr>
<tr>
<td>GLIB</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>1.5</td>
<td>1.480 ± 0.012</td>
<td>0.828</td>
</tr>
</tbody>
</table>

Table 2: Intra-day and inter-day precision and accuracy of MET, PIOG and GLIB.
1.5-9 and 0.5-3 µg mL⁻¹ MET, PIOG and GLIB, respectively. Peak area ratios and concentrations were subjected to least square linear regression analysis to calculate the calibration equations and correlation coefficients. The mean regression equations were found as Y=0.300x (R²=0.971, n=6), Y=0.300x (R²=0.971, n=6) and Y=354x (R²=0.97, n=6) for MET, PIOG and GLIB, respectively. y=ax+b where "y" is the peak area ratio of drugs, "a" is the slope, "b" is the intercept and "x" is the concentration of the measured solution in µg mL⁻¹. The result shows that there is an excellent correlation between the peak area ratios and the concentrations of drugs in the range tested.

**LOD and LOQ:** The LOD was 0.003 µg mL⁻¹ for MET, 0.02 µg mL⁻¹ for PIOG and 0.015 µg mL⁻¹ for GLIB at a signal to noise ratio of 3.1. The limit of quantification was determined as 0.05 MET, 0.2 µg mL⁻¹ for PIOG and 0.05 µg mL⁻¹ for GLIB at a signal to noise ratio of 10.1.

**Precision:** Intra-day precision was performed by relative standard deviation of five repeated assays of samples at the three concentration levels. Inter-day precision was determined by analyzing the same set of samples of five different days. The RSD values were found to be 0.021-0.837% for MET, 0.535-2.597% for PIOG and 0.741-5.168% for GLIB respectively, indicating good precision (Table 2).

**Recovery:** To examine the accuracy of the method, recovery studies were carried out by standard addition method [27,28]. The percent recovery of the added standard to the assay samples was calculated from:

\[
100\text{Recovery}\% = \left(\frac{C_1 - C_u}{C_a}\right) \times 100
\]

where \(C_1\) is the total concentration of analyte found; \(C_u\) is the concentration analyte present in the formulation; and \(C_a\) is the concentration added to the formulation. The average percent recoveries obtained as 98.0-100.04% indicate good accuracy of the method (Table 3).

**Specificity:** The specificity of the RP-HPLC method was determined by the complete separation of MET, PIO, GLIB and IS as shown in figure 2 with parameters like retention time (t<sub>R</sub>), resolution (R<sub>S</sub>) and tailing factor (T). The peaks obtained for MET, PIOG, GLIB and IS were sharp and have a clear baseline separation.

**Robustness:** To ensure the insensitivity of the HPLC method to minor changes in the experimental conditions, it is important to demonstrate robustness of the method. None of the modifications caused a significant change in the resolution between the drugs and IS, peak area RSD, USP tailing factor, peak width or theoretical plates.

**Conclusion**

A simple, rapid, and reliable LC method has been established for simultaneous determination of MET, PIOG and GLIB either alone or in their ternary formulations. The method has several advantages, including rapid analysis, a simple mobile phase, simple sample preparation and high accuracy and precision.
preparation, and improved sensitivity. It is suitable for analysis of these anti-diabetic agents in their ternary formulations in a single isocratic run, in contrast with previous methods. This makes the method suitable for routine analysis in quality-control laboratories.

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References