DEVELOPMENT AND VALIDATION OF NOVEL SPECTROPHOTOMETRIC METHODS FOR SIMULTANEOUS ESTIMATION OF PIOGLITAZONE AND METFORMIN IN BULK AND FIXED DOSAGE FORMS BY AREA UNDER CURVE AND DUAL WAVELENGTH MODE

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ABSTRACT

Objective: Two simple, accurate and reproducible spectrophotometric methods have been developed and validated for simultaneous estimation of metformin (MET) and pioglitazone (PIO) in bulk and tablet dosage forms.

Methods: (1) Area under curve method (Area calculation): The proposed area under the curve method involves measurement of area at selected wavelength ranges. Two wavelength ranges were selected 228-238 nm and 265-275 nm for estimation of MET and PIO respectively. (2) Dual wavelength method: In the dual-wavelength method, two wavelengths were selected for each drug in a way so that the difference in absorbance is zero for another drug. PIO shows equal absorbance at 235 and 266 nm, where the difference in absorbance was measured for determination of MET. Similarly, the difference in absorbance at 216 and 241.5 nm was measured for determination of MET.

Results: Linearity range for MET and PIO is 2-10 µg/ml and 10-50 µg/ml at respective selected wavelengths. Accuracy and precision studies were carried out and results were satisfactory. The proposed methods have been validated as per ICH guidelines and successfully applied to the estimation of MET and PIO in their combined tablet dosage form.

Conclusion: The developed methods are simple, precise, rugged and economical. The utility of the methods has been demonstrated by analysis of commercially available formulations.

Keywords: Metformin, Pioglitazone, Area under curve method, Dual wavelength method

INTRODUCTION

Diabetes mellitus, commonly referred to as diabetes, is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. Globally, diabetes is likely to be the fourth leading cause of death [1]. Approximately 90% of people with diabetes have type 2 diabetes. It usually begins as insulin resistance, a disorder in which the cells do not use insulin properly. As the need for insulin rises; the pancreas gradually loses its ability to produce insulin. Type 2 diabetes is associated with older age, obesity, family history of gestational diabetes, impaired glucose metabolism, physical inactivity and race/ethnicity [2]. If the glycemic target level is not achieved with one oral agent alone, combination oral and/or insulin therapy is recommended [3, 4]. Combination oral therapy becomes an obvious choice when glycemic control is not achieved with conventional monotherapy [5]. The advantages of oral dose combinations as compared to their components which are taken alone are lower cost and better patient compliance [6, 7]. Combination therapy has been shown to have achieved greater blood glucose lowering than non-combination therapy because different classes have different and complementary mechanisms of action. Therefore, it is more logical to add another drug than replace the existing drug. The rapid introduction of combination therapy involves two or three complementary oral antidiabetic agents helps in targeting the dual effect and also reduced adverse effects [8].

Pioglitazone (PIO) is chemically [(S)-5-[[4-[(5-ethyl-2-pyridinyl)ethoxy]phenyl]methyl]-2,4]-thiazolidinedione [9] [fig. 1 (a)]. It is an orally-active thiazolidinedione with antidiabetic properties and potential antineoplastic activity. PIO activates peroxisome proliferator-activated receptor gamma (PPAR-gamma), a ligand-activated transcription factor, thereby inducing cell differentiation and inhibiting cell growth and angiogenesis. This agent also modulates the transcription of insulin-responsive genes, inhibits macrophage and monocyte activation, and stimulates adipocyte differentiation [10]. Metformin (MET) is chemically (N, N-dimethyl imidodicarbonimidic diamide) [11] [fig. 1 (a)]. MET is a member of the biguanide class of oral antihyperglycemics improves glucose tolerance in patients with type 2 diabetes, lowering both basal and postprandial plasma glucose. MET decreases hepatic glucose production, decreases intestinal absorption of glucose and improves insulin sensitivity by increasing peripheral glucose uptake and utilization [12].

The review of literature stated that various analytical methods involving spectrophotometry [13], HPLC [14], and HPTLC [15] have been reported for PIO in a single form and in combination with other drugs. Several analytical methods have been reported for MET in a single form and in combination with other drugs, including spectrophotometry [16], HPLC [17-19], HPTLC [20], LC-MS [21] methods.

For the simultaneous estimation of MET and PIO in tablets, vierodt’s simultaneous equation method by Rathod et al. [22] and another two methods; derivitive spectrophotomagry and Q-analysis by Goswami et al. have been reported [23].
However, no references have been found for simultaneous estimation of MET and PIO in their combined tablet dosage form by the area under the curve (AUC) and dual wavelength (DW) method. Both these methods are simple, accurate and precise for estimation of MET and PIO in combined tablet dosage form and do not require any complicated sample treatment like heating or organic solvent extraction and costly instrument like HPLC.

AUC method offers an efficient method which involves measurement of area under the curve at two sampling wavelength ranges for the estimation of MET and PIO. DW method is another easy and efficient method for analyzing a component in the presence of an interfering component. For elimination of interferences, dual analytical wavelengths were selected in a way to make the absorbance difference zero for one drug while it is directly proportional to the concentration of the other drug (a component of interest).

MATERIALS AND METHODS

Instrumentation

UV/Visible spectrophotometer: SICAN-2301, Inkarp instruments Pvt Ltd.
Analytical balance: Sartorius BSA223S-CW
Magnetic stirrer: REMI 1MLH, Remi laboratories limited.

Reagents and chemicals

MET: Gift sample from covalent laboratories Pvt. Ltd., Hyderabad.
PIO: Gift sample from Neuland laboratories Ltd., Hyderabad.
Formulation of MET and PIO: moxicip FC, Cipla limited and mahacef, Mankind Ltd.
Solvent: Methanol analytical grade, Merck.

Preparation of stock standard solutions

The stock solutions of MET and PIO were prepared separately by dissolving accurately 50 mg of the drug in methanol and the volume was made up to 50 ml with methanol to prepare standard stock solution (1 mg/ml).

Preparation of sample solutions

The stock standard solutions (1 mg/ml) of MET and PIO were further diluted to obtain the final concentration 2, 4, 6, 8, 10, 12 μg/ml and 10, 20, 30, 40 and 50 μg/ml respectively. Both the solutions were scanned in the spectrum mode from 200 nm to 400 nm.

Method 1: Area under curve method

For the selection of the analytical wavelength solutions of MET (6 μg/ml) and PIO (30 μg/ml) were prepared separately by appropriate dissolution from stock standard solutions and scanned between 200 to 400 nm using methanol as blank. From the overlain spectra (Fig.3) of both drugs the AUC was determined at both the selected analytical wavelength ranges.
Wavelength range selected were 228-238 nm for determination of AUC of MET and 265-275 nm for determination of AUC of PIO. The Calibration curve was prepared in the concentration range of 2-10 μg/ml for MET at 228 to 238 nm. The Calibration curve was prepared in the concentration range of 10-50 μg/ml for PIO at 265 to 275 nm. The 'X' value is the ratio of AUC at selected wavelength ranges (228-238 nm and 265-275 nm) with a concentration of a component in μg/ml. The concentration of each drug was calculated using following "Cramer's and Matrix rule" equation:

\[
\text{CMET} = \frac{A_2a_2y_2 - A_1a_1y_2}{a_2x_2y_1 - a_1x_1y_2} - (1)
\]

\[
\text{CPIO} = \frac{A_1a_2x_2 - A_2a_1x_1}{a_2x_2y_1 - a_1x_1y_2} - (2)
\]

Where,

\[
\text{CMET} = \text{Concentrations of MET,}
\]

\[
\text{CPIO} = \text{Concentrations of PIO,}
\]

\[
A_1 = \text{Area at 228-238 nm,}
\]

\[
A_2 = \text{Area at 265-275 nm,}
\]

\[
a_1 = \text{X value of MET at 228-238 nm,}
\]

\[
a_2 = \text{X value of MET at 265-275 nm,}
\]

\[
y_1 = \text{X value of PIO at 228-238 nm,}
\]

\[
y_2 = \text{X value of PIO at 265-275 nm.}
\]

**Method 2: Dual wavelength method**

The principle of DW method is "the absorbance difference between two points on the mixture spectra is directly proportional to the concentration of the component of interest".

The pre-requisite for the DW method is the selection of two such wavelengths where the interfering component shows the same absorbance whereas the component of interest shows a significant difference in absorbance with concentration.

The overlain spectrum of PIO and MET suggested that a DW spectrophotometric method is a suitable method for simultaneous determination of MET and PIO.

The wavelengths selected for determination of MET were 235 nm and 266 nm, where the absorbance difference was zero for PIO. The wavelengths selected for determination of PIO were 216 nm and 241.5 nm where the absorbance difference was zero for MET.

The calibration curve was prepared in the concentration range of 2-10 μg/ml for MET at 235 nm to 266 nm. The calibration curve was prepared in the concentration range of 10-50 μg/ml for PIO at 216 nm to 241.5 nm.

![Graph of overlay of MET (6 μg/ml) and PIO (30 μg/ml)](image)

Fig. 5: Overlay of MET (6 μg/ml) and PIO (30 μg/ml)

**Assay of tablet formulation**

20 tablets each of MET and PIO were weighed and average weight noted. Then the tablets were crushed and powdered, and tablet powder equivalent to 500 mg of MET and 5 mg of PIO respectively was weighed and dissolved in 100 ml methanol. The solution was then filtered through Whatmann filter paper No.41 and diluted further to obtain a final concentration of 6 μg/ml of MET and 30 μg/ml of PIO. The sample solutions were analyzed as per the procedure for mixed standards. The concentrations of each drug in sample solutions were calculated using equations (i) and (ii) for the AUC method. In the DW method, the responses of the sample solution were measured at 216 nm, 235 nm, 241.5 nm and 266 nm for quantification of MET and PIO. The amounts of the MET and PIO present in the sample solution were calculated by fitting the responses into the regression equation for MET and PIO in the proposed method.

<table>
<thead>
<tr>
<th>Level of % recovery</th>
<th>Methods</th>
<th>%Recovery*</th>
<th>% RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MET</td>
<td>PIO</td>
<td></td>
</tr>
<tr>
<td>80%</td>
<td>AUC</td>
<td>101.49</td>
<td>0.953</td>
</tr>
<tr>
<td></td>
<td>DW</td>
<td>99.938</td>
<td>1.454</td>
</tr>
<tr>
<td>100%</td>
<td>AUC</td>
<td>100.93</td>
<td>1.677</td>
</tr>
<tr>
<td></td>
<td>DW</td>
<td>99.883</td>
<td>1.293</td>
</tr>
<tr>
<td>120%</td>
<td>AUC</td>
<td>100.76</td>
<td>0.894</td>
</tr>
<tr>
<td></td>
<td>DW</td>
<td>99.579</td>
<td>1.039</td>
</tr>
</tbody>
</table>

*Average of three estimations at each level of recovery

**Method validation**

The proposed methods were validated according to ICH Q2B guidelines for validation of analytical procedures in order to determine the linearity, sensitivity, precision and accuracy, etc. of the analytes [24].

**Accuracy**

Accuracy may often be expressed as % Recovery by the assay of known, added amount of analyte. It is a measure of the exactness of the analytical method. Recovery studies were carried out for both the methods by spiking standard drug in the powdered formulations 80%, 100%, 120% amount of each dosage content as per ICH guidelines. The recovery study was performed three times at each level for both methods.

![Table of statistical validation of recovery studies](image)

**Table 1: Statistical validation of recovery studies**

<table>
<thead>
<tr>
<th>Level of % recovery</th>
<th>Methods</th>
<th>%Recovery*</th>
<th>% RSD*</th>
</tr>
</thead>
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<tr>
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<td>0.894</td>
</tr>
<tr>
<td></td>
<td>DW</td>
<td>99.579</td>
<td>1.039</td>
</tr>
</tbody>
</table>

*Average of three estimations at each level of recovery
**International Conference on Harmonization (ICH) guidelines.**

The reproducibility of the proposed method was determined by performing tablet assay on the same day (Intra-day assay precision) and on three different days (Inter-day precision).

**Mixed standard solutions containing 4, 6, 8 μg/ml MET and 20, 30, 40 μg/ml of PIO were analyzed on three consecutive days. The results were reported in terms of %RSD by both the methods.**

### Table 2: Statistical validation of Inter-day precision

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Methods</th>
<th>%Amount found±SD*</th>
<th>%RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET</td>
<td>AUC</td>
<td>101.14±0.08</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>DW</td>
<td>100.91±0.09</td>
<td>1.55</td>
</tr>
<tr>
<td>PIO</td>
<td>AUC</td>
<td>100.45±0.10</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>DW</td>
<td>99.96±0.21</td>
<td>0.74</td>
</tr>
</tbody>
</table>

*Average of six estimations

### Table 3: Statistical validation of intra-day precision

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Methods</th>
<th>% Amount found±SD*</th>
<th>% RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET</td>
<td>AUC</td>
<td>101.74±0.06</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>DW</td>
<td>101.18±0.08</td>
<td>1.37</td>
</tr>
<tr>
<td>PIO</td>
<td>AUC</td>
<td>100.38±0.08</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>DW</td>
<td>100.33±0.08</td>
<td>0.33</td>
</tr>
</tbody>
</table>

*Average of six estimations

### Table 4: Optical characteristics of MET and PIO

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AUC method</th>
<th>DW method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>MET</td>
<td>PIO</td>
</tr>
<tr>
<td>Wavelength Range</td>
<td>228-238 nm</td>
<td>265-275 nm</td>
</tr>
<tr>
<td>Concentration Range</td>
<td>2-12 μg/ml</td>
<td>10-50 μg/ml</td>
</tr>
<tr>
<td>Regression Equation</td>
<td>y = 0.7514x-0.6636</td>
<td>y = 0.1541x-0.2398</td>
</tr>
<tr>
<td>Slope</td>
<td>0.7514</td>
<td>0.1541</td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.6636</td>
<td>-0.2398</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>0.9986</td>
<td>0.9969</td>
</tr>
<tr>
<td>LOD (μg/ml)</td>
<td>0.252</td>
<td>0.266</td>
</tr>
<tr>
<td>LOQ(μg/ml)</td>
<td>0.763</td>
<td>0.806</td>
</tr>
</tbody>
</table>

LOD=Limit of Detection, LOQ=Limit of Quantification

**Intra-day precision**

Mixed standard solutions containing 4, 6, 8 μg/ml MET and 20, 30, 40 μg/ml of PIO were analyzed at different time intervals (morning, afternoon and evening) on the same day. The results were reported in terms of %RSD by both the methods.

**Linearity**

The measurement of linearity was evaluated by analyzing different concentrations of the standard solution of MET and PIO. For both the methods, the Beer law was obeyed in the concentration range 2-10 μg/ml and 10-50 μg/ml for MET and PIO respectively. The absorbance was plotted against the corresponding concentrations to obtain the calibration plots.

**Limit of detection and limit of quantitation**

Limit of detection is the lowest amount of analyte in a sample which can be detected, but not necessarily quantitated as an exact value and limit of quantitation is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The limit of detection (LOD) and the limit of quantification (LOQ) of the drug was derived by calculating the signal-to-noise ratio (S/N) using the following equations designated by International Conference on Harmonization (ICH) guidelines.

\[
\text{LOD} = \frac{3\sigma}{S} \\
\text{LOQ} = \frac{10\sigma}{S}
\]

Where, \(\sigma\) = the standard deviation of the response and \(S\) = slope of the calibration curve. The LOD and LOQ were separately determined based on the calibration curve.

**RESULTS AND DISCUSSION**

In the AUC method solutions of PIO and MET were scanned separately between 200 to 400 nm using methanol as blank. Maximum absorbance is obtained at 266 nm for PIO and 232 nm for MET. Two wavelength ranges were selected 265-275 nm and 228-238 nm for estimation of PIO and MET respectively (fig. 3 and fig. 4). Linear regression equations for PIO and MET were found to be \(y = 0.1541x-0.2398\) and \(y = 0.7514x-0.6636\) respectively. Linearity data as summarized in table 4 proves that the method is linear and is within specified criteria of ICH guidelines.

The utility of dual wavelength data processing program is its ability to calculate the unknown concentration of the component of interest in a mixture containing an interfering component. For elimination of the effects of interfering components, two specific wavelengths were chosen 235 nm and 266 nm for the determination of MET, where the absorbance difference was zero for PIO and 216 and 241.5 nm for the determination of PIO where absorbance difference was zero for MET. Absorbance difference was determined between wavelengths 266-235 nm and 241.5-216 nm and calibration curves were plotted between absorbance difference values and concentration of the drug.

Linearity range for MET and PIO is 2-10 μg/ml and 10-50 μg/ml at respective selected wavelengths. The coefficient of correlation for
MET at the range 228-238 nm and PIO at the range 265-275 nm is 0.9996 and 0.9999 respectively, for AUC method and 0.9997 and 0.9999 for MET at 235-266 nm and PIO at 216-241.5 nm respectively for DW method (table 4). MET and PIO shows limit of detection 0.252 μg/ml and 0.266 μg/ml for AUC method and 0.251 μg/ml and 0.234 μg/ml for DW method and limit of quantification 0.763 μg/ml and 0.806 μg/ml for AUC method and 0.761 μg/ml and 0.710 μg/ml for DW method respectively (table 4). In both Intra and inter-day precision study for both shows % RSD are not more than 2.0%, which indicates good repeatability and intermediate precision (table 2 and 3). Mean % recovery studies resulted from 100.76% to 101.49% for MET and 100.63% to 102.28% for PIO in AUC method and 99.58% to 99.94% for MET and 100.58% to 100.89% for PIO in DW method with % RSD values, not more than 2.0%, which indicates that any small change in the drug concentration in the solution could be accurately determined by the proposed methods (table 1). The % assay was found to be 99.66% to 100.41 % for MET and 99.53 to 99.73 for PIO in AUC method and 99.48 to 99.64 for MET and 99.07 to 99.53 for PIO in DW method with % RSD values, not more than 2.0% indicating the precision of this method (table 5). No interference was observed from the pharmaceutical adjuvants/excipients.

### Table 5: Statistical validation of tablet formulation

<table>
<thead>
<tr>
<th>Brand</th>
<th>Drug</th>
<th>Method</th>
<th>Amount present (mg)</th>
<th>% Amount found</th>
<th>SD*</th>
<th>% RSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Davista-M (Dr. Reddy’s)</td>
<td>MET</td>
<td>AUC</td>
<td>500</td>
<td>100.41</td>
<td>0.51</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DW</td>
<td>500</td>
<td>99.64</td>
<td>0.11</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>PIO</td>
<td>AUC</td>
<td>15</td>
<td>99.73</td>
<td>0.06</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DW</td>
<td>15</td>
<td>99.53</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Exermet-P (Cipla)</td>
<td>MET</td>
<td>AUC</td>
<td>500</td>
<td>99.66</td>
<td>1.03</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DW</td>
<td>500</td>
<td>99.48</td>
<td>0.55</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>PIO</td>
<td>AUC</td>
<td>15</td>
<td>99.53</td>
<td>0.19</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DW</td>
<td>15</td>
<td>99.07</td>
<td>0.20</td>
<td>1.35</td>
</tr>
</tbody>
</table>

### CONCLUSION
Both of the proposed Spectrophotometric methods are simple, rapid, accurate, precise, and economical and validated in terms of linearity, accuracy, precision and reproducibility as per ICH guidelines. These two methods can be successfully used for simultaneous estimation of MET and PIO in pure and marketed tablet dosage form. These proposed two methods can also be used for routine quality-control analysis of these drugs in pure and its pharmaceutical dosage forms.

### ABBREVIATION
AUC-Area under the curve, DW-Dual Wavelength, MET-Metformin, PIO-Pioglitazone. ICH-International Conference on Harmonization, HPLC-High performance liquid chromatography, HPTLC-High performance thin layer chromatography, LC-MS-Liquid chromatography-mass spectrophotometer, μg-microgram (s), mg-milligram (s), nm-nanometer (s), %R. SD-Percentage Relative Standard Deviation, SD-Standard deviation, LOD-Limit of detection, LOQ-Limit of Quantification.

### ACKNOWLEDGEMENT
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### CONFLICTS OF INTERESTS
Declare none

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24. ICH, Q2 (R1), harmonized tripartite guideline, Validation of analytical procedures: text and methodology International Conference on Harmonization ICH, Geneva; 2005.

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