DEVELOPMENT AND VALIDATION RP-HPLC-PDA METHOD FOR THE DETERMINATION OF METFORMIN IN BULK AND DOSAGE FORM

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ABSTRACT
A simple, sensitive, precise and accurate reversed phase liquid chromatographic method has been developed for the estimation of metformin from bulk dosage form. The method was developed and validated using a Thermo Accucore Hillic (150 × 4.6 mm, 5 µm) column with a mobile phase consisting 20 mM sodium acetate buffer and acetonitrile (45:55, v/v with pH-4); at a flow rate of 0.7 mL min⁻¹. The PDA detection was achieved at 236 nm, over a wide dynamic range of 0.039 to 1.25 µg mL⁻¹. The retention time of metformin was 3.9 minutes. The method was successfully validated in accordance to ICH guidelines acceptance criteria for specificity, linearity, accuracy, precision, robustness, ruggedness and system suitability. Intra-day and inter-day assay accuracy and precision of the metformin were less than 2%, and the average recovery were in the range of 98–102%. Metformin was subjected to the stress conditions of oxidation, acid and base hydrolysis and thermolysis. The method was successfully applied for analysis of metformin in the presence of excipients in commercially available tablet and bulk dosage form.

Keywords: Metformin, HPLC, Hillic column, dosage form.

INTRODUCTION
Metformin (1,1-dimethylbiguanide) (Fig-1) is an oral anti-hyperglycemic agent not hypoglycemic, which improves glucose tolerance in patients with type II diabetes. That has been used in the treatment of diabetes mellitus since 1957. It decreases hepatic glucose
production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. It has no significant effects on the secretion of glucagon, cortisol, growth hormone, or somatostatin [1,2]. It is reported to be the most prescribed drug for treatment of type II diabetes [3] and also used for diabetes prevention, particularly in obese people and those with unimpaired renal function [4].

Method validation is the process of proving that an analytical method is accepted for its intended purpose. For pharmaceutical methods, guidelines from the Unites States of Pharmacopoeia (USP), International Conference on Harmonization (ICH) and the Food and Drug Administration (FDA) provides a framework for performing such validations [5,6]. Several analytical and bioanalytical methods have been reported for quantitative analysis of metformin in various biological fluids, these include ultraviolet (UV) spectrometry, and HPLC-UV [7-9]. More sensitive LC–MS–MS methods have also been used for quantification of metformin in plasma[10-12]. Some papers have been published applications using reversed-phase columns such as C 18 and C 8 [13-17].

Therefore aim of the present work was to develop simple, precise and accurate fast and rugged analytical RP- HPLC-PDA method for determination of Metformin in pharmaceutical and bulk dosage form. The established method was validated with respect to specificity, linearity, precision, accuracy and ruggedness. In addition, forced degradation studies were performed in order to prove the suitability of the method for the stability-indicating assay of Metformin.

**Experimental**

**Chemicals and Materials**

Metformin pure powder (Purity: 99.95) of pharmaceutical grade was gifted by Zydus Cadila Limited, (Sikkim, India). HPLC grade acetonitrile and methanol were from Sigma Aldrich Chemicals Pvt Ltd (Mumbai, India). Sodium acetate AR, glacial acetic acid AR, and ammonia solution (25%) were purchased from E Merck Pvt. Ltd (Mumbai, India). Ultrapure water was obtained from a Milli-Q PLUS PF water purification system. Metformin tabs were purchased from local pharmacy within their shelf-life period.

**Instrumentation and Chromatographic Conditions**
The method was developed using a Waters HPLC system (Milford USA) consisted of a binary pump (model 515), auto sampler (model 717) and photo diode array (PDA) detector. The separation was achieved on Thermo Accuacore Hillic (150 × 4.6 mm, 5 µm) column with a mobile phase consisting 20 mM sodium acetate buffer and acetonitrile (45:55, v/v with pH-4); at a flow rate of 0.7 mL min⁻¹. Detection was carried out with PDA at 236 nm. Total run time was 6 min and volume of injection was 50 µL, prior to injection of analyte, the column was equilibrated for 30-40 min with mobile phase. Analysis was performed at ambient temperature. The data collection and analysis were performed using breeze - version 3.1 software.

**Preparation of Standard Stock Solution and Calibration Curves**

The stock solutions of 1.0 mg mL⁻¹ of metformin were prepared in mobile phase composition. Calibration samples and quality control samples (QC) were prepared by diluting working standard solution with mobile phase to give concentrations in the range of 0.039–1.25 µg mL⁻¹ for metformin. QC samples at four different concentrations (0.015, 0.39, 0.156 and 1.25 µg mL⁻¹ as LLOQ, low, medium and high, respectively) were prepared separately in five replicates, independent of the calibration standards. Test samples and quality control samples were then interpolated from the calibration curve to obtain the concentrations of the respective analyte. Calibration curves were plotted as concentration of drugs versus peak area response.

**Analysis of Marketed Formulation**

Three replicates of the required dilutions were prepared from suspension stock solution and sonicated for 10 min. For analysis of suspension dosage form of metformin was filtered through whatman filter paper.

**METHOD VALIDATION**

The method was validated in compliance with ICH guidelines (2006); in terms of specificity, Selectivity, linearity, precision, accuracy, limit of quantification, limit of detection, robustness and other aspects of analytical validation.

**Specificity and Selectivity**

The specificity of the method was checked by comparing chromatograms obtained from standard, sample and the corresponding placebo. The selectivity of the method was established from the resolution of the drug peak from the nearest and also among all other
peaks. The analyte with a resolution factor greater than 4.2 exhibited the selectivity of the method.

**Linearity**
The linearity of the method was determined at six concentration levels ranging from 0.039 to 1.25 µg mL⁻¹ for metformin. The calibration curves were established by plotting the peak area versus concentration. The regression parameters of slope, intercept and correlation coefficient were calculated by fitted to the y = mx + c using weighing factor (1/x²).

**Precision and Accuracy**
The precision (% RSD) and accuracy (% bias) mean relative error (RME, %) of this analytical method were determined using QC samples (n = 5) in five replicates of LLOQ, LOQ, MQC and HQC 0.015, 0.039, 0.156 and 1.25 µg mL⁻¹, respectively. The accuracy of the sample preparation was determined by injection of calibration samples and four QC samples in five replicate for 5 days. The criteria for acceptability of the data included accuracy within ± 2% standard deviation (SD) from nominal values and precision of within ± 2% relative standard deviation (RSD).

**Recovery**
The recovery of the method was assessed by adding known amounts of metformin to commercial available suspension containing a known amount of the drug (standard addition method). The average recovery was obtained in the range of 98–102 % for all with % RSD below 2%. The recovery of the added pure drug was calculated as:

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

where \(C_v\) is the total amount of drug measured after standard addition, \(C_u\) the amount of drug in the formulation, and \(C_a\) the amount of drug added to the formulation.

**Limit of Detection and Limit of Quantification**
The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the standard deviation of responses and slopes using signal-to-noise ratio as per ICH guidelines. This study was carried out to determinate the limit of detection (LOD, S/N = 3) and limit of quantitation (LLOQ, S/N = 10) in order to apply this method for the quantification.

**Robustness**
Robustness of the method was studied by changing the extraction time of metformin from dosage form by ± 5 min, composition of mobile phase by ± 2% of organic solvent, flow rate by ± 0.1 mL min⁻¹ and buffer pH by ± 0.2. The changes in the response of the analyte were noted.

**System-Suitability Test**
System suitability testing was done by CDER (Centre for drug and evaluation research) guideline [18]. It is used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were asymmetry of the chromatographic peak, peak resolution, tailing factor, theoretical plates and repeatability, as %RSD of peak area for replicate injections.

**Forced Degradation Stability Studies**
The forced degradation of the drug molecule can help to identify the likely degradation pathway and the intrinsic stability of the analyte. Specificity is the ability of the method to measure the analyte response in presence of its potential impurities [19]. All forced degradation studies were performed at an initial drug concentration of 20 µg mL⁻¹. Acid hydrolysis was performed in 1.0 N HCl at 60 °C for 1 hr. The study in basic solution was carried out in 1.0 N NaOH at 60 °C for 1 hr. The oxidation studies were carried out at ambient temperature in 3% and 30% hydrogen peroxide (H₂O₂) for 1 hr. For study in neutral solution, the drug dissolved in water and kept at 60 °C temperature for 1 hr. Thermal degradation was performed at 60 °C for 7 days. Assessment of mass balance in the degraded samples was carried out to confirm the amount of impurities detected in stressed samples matches with the amount present before the stress was applied. Quantitative determination of metformin was carried out in all the stressed samples against qualified working standard and the mass balance (% assay + % sum of all degradation products) was tabulated in Table 5.

**Method Development**
Analysis of metformin is a challenge owing to its high polarity and small molecular size, which lead to poor retention on reversed-phase liquid chromatographic columns. The chromatographic conditions were optimized with respect to specificity, resolution, and time of analysis. Hence we started the development activity with C₁₈ stationary phase of various manufacturers such as Zorbax, ODS (250 × 4.6 mm, 5 µm), Spherisorb ODS (250 × 4.6 mm, 5 µm) Symmetry shield C₁₈ (250 mm × 4.6 mm, 5.0 µ), Phenomenex Luna C₁₈ (250 ×4.6 mm, 5 µm), Spheri-5, CYANO column (30 × 4.6 mm, 5 µm) and Thermo Accuaacore Hillic
(150 × 4.6 mm, 5 µm) column. Metformin was retained in C18 Column with high aqueous condition. Peak broadening with tailing problem was created. Metformin was also retained in CYANO column (30 × 4.6 mm, 5 µm) with high organic condition (>90%). Same problem was created. Problems was tried to solve by adding 1-5 % triethylamine but could not find better resolution. However, Thermo Accuacore Hillic (150 × 4.6 mm, 5 µm) column was used as a good resolution and optimum elution time were obtained. The stationary phase was not only the parameter which could give better resolution. Mobile phase, pH and organic modifies also played very important role which leads the best separation.

Different mobile phases containing acetonitrile, methanol, water and buffer were examined. Initially the methanol was used as an organic modifier which gives the poor baseline with baseline drift. Hence the response for the metformin was reduced. To improve the resolution and response, acetonitrile was tried as an organic modifier. The base line was found good and response metformin was improved. Effects of pH (3–7) and ionic strength (5–50 mM) were investigated using phosphate and acetate buffer. It was found that at higher and lower pH the tailing of the metformin peak was more and also resolution was poor of the analyte. The effect of buffer concentration on the retention of metformin was also studied. The mobile phase containing acetonitrile: sodium acetate buffer 20 mM (55: 45 v/v, pH 4), was selected as optimal for obtaining well-resolved peaks with acceptable system suitability parameters. Flow rates from 0.5 to 1 mL min⁻¹ were tested. Flow rates less than 0.5 ml/min led to an increase in retention times peak broadening and the time of analysis. High flow rates led to a remarkable increase in column pressure and decrease in resolution. It was found that 0.7 mL min⁻¹ was optimal as it compromised between resolution and run time.

Effect of the wavelength on the response factor was observed over the wavelength range 200–300 nm. The detection wavelength, 236 nm was found optimal due to the high absorbativity at this wavelength for metformin (fig-2). Complete separation was achieved in <6.0 min at ambient temperature (Fig. 3). The average retention times ± RSD % for metformin was found to be 3.9 ± 0.10 (n = 10).

**METHOD VALIDATION**

Specificity
There was no cross interference from impurity, excipients or additives at the retention time of Metformin was found. Representative chromatograms of blank and metformin are shown in Fig. 3. The retention time of the analyte represented less variability with a relative standard deviation (R.S.D.) well within the acceptable limit of 5%.

**Linearity**

The calibration plot for the method was linear over the concentration in the range of 0.039 to 1.25 µg mL⁻¹ for metformin. The correlation coefficient ($r^2$) of the regression was 1 (fig-4). The best-fit linear equation obtained was $y = 43878x + 98.26$. This result demonstrates linearity of this method over the specified range.

**Accuracy and Precision**

Accuracy and precision (intra- and inter-day) were calculated at four different concentration levels of LLOQ, LOQ, MQC and HQC ($n = 5$), for analyte on five days are presented in Table 1. The results showed that the analytical method is accurate, as the % bias is within the acceptance limits of ± 2.0% of the theoretical value. The precision around the mean value was never greater than ± 2.0 % at any of the concentrations studied.

**Recovery**

The Value of recovery (%), standard deviation and % coefficient of variance (% COV), indicating method accuracy, is listed in Table 2.

**Limit of Detection and Limit of Quantification**

The LOD for metformin was found to 0.0.01 µg/ml at a signal to noise ratio of 3:1, while the limit of quantification on was 0.039 ng/ml.

**Robustness & System-Suitability Test**

The method is found to be robust as the results were not significantly affected by slight variation in composition of mobile phase, extraction time, flow rate and Buffer pH (data not shown).

The SST measured from six replicate injections of metformin was capacity factor, theoretical plates, column efficiency and tailing factor and the results are tabulated in Table 3. For all six injections the tailing factor was less than 1.5.

**Stability**
The stability of metformin was investigated thoroughly under auto-sampler storage and bench-top storage (Table 4). The results obtained were well within the acceptable limits. At 4 °C, metformin showed no degradation for a 24 hr period.

Analysis of metformin Dosage Form.
The proposed validated RP-HPLC method was successfully applied to determine metformin in marketed tablet form. Three replicates of the required dilutions were prepared from suspension stock solution and sonicated for 10 min. These solutions (50 µL) were injected for quantitative analysis. The amounts of metformin were calculated by extrapolating the peak area from the calibration plot. The mean percentage of metformin were found to be 100.12 ± 0.025, which were comparable to the corresponding labeled amounts.

RESULTS OF FORCED DEGRADATION STUDIES
Degradation in acidic solution: The metformin was exposed to 0.1 N HCl at 60 °C temperature for 1 h. The metformin rapidly underwent degradation with time in acidic condition.

Degradation in basic solution: The metformin was exposed to 0.1 N NaOH at 60 °C temperature for 1 h, prominent degradation was observed.

Oxidative conditions: The metformin was exposed to 30% and 3% fresh hydrogen peroxide preparation. metformin has shown significant sensitivity towards the treatment of 30% hydrogen peroxide. The metformin rapidly underwent degradation with time in 30% hydrogen peroxide. The major degradation product was eluted with solvent front.

Thermal degradation: The metformin powder was exposed to dry heat at 60 °C for 7 days and the metformin solution was kept at room temp for 7 days. No significant degradation (~4 %) was observed.

Degradation in neutral (water) solution: No degradation was observed after 1 h at 60 °C temperature.

Tables
Table 1 .The Accuracy (% bias) and precision (% R.S.D.) of Metformin
Table 2. The recovery of Metformin

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>%Recovery ± S.D</th>
<th>% COV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.015 µg/mL</td>
<td>99.87 ± 0.034</td>
<td>0.87</td>
</tr>
<tr>
<td>0.039 µg/mL</td>
<td>101.88 ± 0.001</td>
<td>0.379</td>
</tr>
<tr>
<td>0.156 µg/mL</td>
<td>99.68 ± 0.026</td>
<td>1.02</td>
</tr>
<tr>
<td>1.25 µg/mL</td>
<td>99.35 ± 0.216</td>
<td>1.08</td>
</tr>
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</table>

Table 3. System suitability study (SST)

<table>
<thead>
<tr>
<th>SST limits</th>
<th>CDER guidelines</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability of peak response</td>
<td>≤1.0% for 5 replicates</td>
<td>0.787</td>
</tr>
<tr>
<td>Resolution</td>
<td>&gt;2.0 general</td>
<td>5.44</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>≤2.0</td>
<td>1.65</td>
</tr>
<tr>
<td>Column efficiency</td>
<td>&gt;2000 (plate count)</td>
<td>2867.74</td>
</tr>
<tr>
<td>Capacity factor</td>
<td>&gt;2</td>
<td>5.37</td>
</tr>
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</table>

Table 4. Bench top and auto sampler stability of Metformin.

<table>
<thead>
<tr>
<th>Storage Conditions</th>
<th>Nominal Conc. (µg/mL)</th>
<th>Initial amount (µg/mL)</th>
<th>Amount after 20 hrs (µg/mL)</th>
<th>% deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auto-sampler</td>
<td>0.039</td>
<td>0.0318</td>
<td>0.0322</td>
<td>-1.351</td>
</tr>
<tr>
<td>stability (4°C,</td>
<td>0.156</td>
<td>0.158</td>
<td>0.160</td>
<td>-0.21</td>
</tr>
<tr>
<td>24 h)</td>
<td>1.25</td>
<td>1.284</td>
<td>1.223</td>
<td>3.15</td>
</tr>
<tr>
<td>Bench-top</td>
<td>0.039</td>
<td>0.317</td>
<td>0.315</td>
<td>0.630915</td>
</tr>
<tr>
<td>stability for 20</td>
<td>0.156</td>
<td>0.15475</td>
<td>0.149</td>
<td>1.71</td>
</tr>
<tr>
<td>hrs at ambient</td>
<td>1.25</td>
<td>1.164</td>
<td>1.283</td>
<td>-1.334</td>
</tr>
<tr>
<td>tem</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Forced degradation stability studies of Metformin
<table>
<thead>
<tr>
<th>Stress condition</th>
<th>Time</th>
<th>% Assay</th>
<th>% degradation product</th>
<th>Mass balance (% assay + % degradation product)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid/0.1 N HCl/60°C</td>
<td>10 min</td>
<td>43.91</td>
<td>56.11</td>
<td>99.96</td>
</tr>
<tr>
<td>Base/0.1 N NaOH/60°C</td>
<td>10 min</td>
<td>4.39</td>
<td>95.61</td>
<td>99.64</td>
</tr>
<tr>
<td>Oxidation/3% H2O2/RT</td>
<td>60 min</td>
<td>97.074</td>
<td>2.72</td>
<td>99.32</td>
</tr>
<tr>
<td>Oxidation/30% H2O2/RT</td>
<td>60 min</td>
<td>9.89</td>
<td>90.11</td>
<td>99.59</td>
</tr>
<tr>
<td>Thermal degradation</td>
<td>Powder</td>
<td>95.942</td>
<td>4.061</td>
<td>99.99</td>
</tr>
<tr>
<td></td>
<td>Solution</td>
<td>97.325</td>
<td>3.786</td>
<td>100.11</td>
</tr>
<tr>
<td>Water hydrolysis</td>
<td>60 min</td>
<td>98.691</td>
<td>1.27</td>
<td>99.98</td>
</tr>
</tbody>
</table>

Fig. 1 Structure of Metformin

Fig. 2 PDA spectra of mobile phase base line and metformin peak
CONCLUSION
The validated RP-HPLC method employed here proved to be simple, specific, accurate, precise, sensitive and robust. The validated method showed satisfactory data for all the validation parameters tested. The short retention time of 3.9 min allows the analysis of a large number of samples in a short period of time and is therefore more cost effective. The information presented here could be very useful for quality monitoring of bulk samples and
as well as employed to check the quality of drug during stability studies in pharmaceutical formulations.

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REFERENCE


