VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF FEBUXOSTAT AND DICLOFENAC POTASSIUM IN BULK DRUG AND IN BI LAYER TABLET FORMULATION

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ABSTRACT
A simple, rapid, accurate and precise isocratic reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for simultaneous estimation of febuxostat and diclofenac potassium in combined dosage form by using phenomenex C18 column (stationary phase) having dimension of 4.6 × 250 mm and particle size of 5 µm and mobile phase containing a mixture of 0.02 M potassium dihydrogen orthophosphate buffer (adjusted to pH 7 with sodium hydroxide): acetonitrile: methanol (35:9:56 v/v/v) at a flow rate of 1 ml/min and detection was carried out at 290 nm. The retention times of febuxostat and diclofenac potassium were 6.01 ± 0.02 minutes and 7.10 ± 0.02 minutes respectively. The developed method was validated as per ICH guideline for specificity, linearity, accuracy and precision, limit of detection and limit of quantification. Linearity studies for the developed method were found in the range of 5 to 30 µg/ml for febuxostat and 12.5 to 75 µg/ml for diclofenac potassium respectively. The accuracy of the method was studied by recovery study and found to be in the range of 98 % - 102 %. The % RSD for intraday and interday precision was found less than 2. The new RP-HPLC method was successfully applied to marketed formulation of Febuxostat and Diclofenac potassium without any interference from excipients.

Keywords: RP-HPLC, Febuxostat, Diclofenac potassium, Validation

INTRODUCTION
Febuxostat (FEBU) chemically is 2- [3- cyano-4- (2- methyl propoxy) phenyl] - 4- methyl thiazole- 5 -carboxylic acid. Febuxostat is novel non- purine selective inhibitor of xanthine oxidases that is use in the treatment of hyperuricemia and gout. Clinical studies revealed that febuxostat lowers the serum urate levels below 6 mg/dL compared with conventionally used doses of allopurinol. Febuxostat was well tolerated in long term treatment in patients with hyperuricemia including those experiencing hypersensitivity/intolerance to allopurinol. Febuxostat inhibits both oxidized and reduced forms of xanthine oxidase and has minimal effects on other enzymes of purine and pyrimidine metabolism.1 Diclofenac potassium is chemically potassium (o-(2, 6-dichloroanilino) phenyl) acetate, a non-steroidal anti-inflammatory drug (NSAID) exhibiting anti-inflammatory and analgesic properties.2 Diclofenac potassium is a non-steroidal anti-inflammatory drug (NSAID) that shows preferential inhibition of the cyclooxygenase-2 (COX-2) enzyme.3 (Figure 1)

Literature survey revealed that FEBU is not official in any pharmacopoeia, whereas DP is official in I.P., B.P. and U.S.P. For FEBU estimation UV4, HPLC5 and stability indicating analytical method6 have been reported. For DP estimation UV7, HPLC8-12, RP-Plasma in human plasma13,14 have been already reported in literature but not a single method was reported for the estimation of FEBU and DP in combined dosage form. So, in the present work RP-HPLC method was developed and validated for simultaneous estimation of febuxostat and diclofenac potassium.

MATERIALS AND METHOD
Standard Febuxostat was obtained from Lupin pharmaceuticals Pune and Diclofenac potassium was obtained from Lincoln pharmaceuticals Ahmedabad as gift samples. HPLC grade Methanol and acetonitrile were procured from Spectrochem Pvt Ltd. Mumbai, India. Potassium dihydrogen orthophosphate AR grade was procured from Loba Chemie. 0.22 µm nylon filter paper was procured from Pall life sciences Mumbai. The bilayer tablet formulation (XANFEB DSR, INDOCO REMEDIES) containing 40 mg FEBU and 100 mg DP (sustained release form) was procured from the local market.

Instruments
Liquid chromatographic system (shimadzu LC-20 AT) was fitted with double reciprocating plunger pump, PDA 20A detector and manual injection loop of 20 µl. Chromatographic data was analyzed by spinchorm software.

Preparation of Mobile Phase
Potassium dihydrogen orthophosphate was dissolved in water and pH adjusted to 7 with Sodium hydroxide. Phosphate buffer, acetonitrile and methanol were mixed in ratio of 35:9:56 v/v/v. The mixture was passed through 0.22 µm nylon filter paper. The mixture was sonicated for 10 minutes and used as mobile phase. The analysis was carried out in isocratic mode using reverse phase C18 phenomenex column equilibrated with mobile phase. The separation was achieved with of 1 ml/min to elute FEBU and DP. Effluents were detected at 290 nm and runtime was 9 minutes.

Preparation of Stock Solutions
Accurately weighed 10 mg FEBU and 25 mg DP and transferred it in to 100 ml volumetric flask. Add 40 ml methanol in volumetric flask and shaken for 5 minutes and sonicated to dissolve drug. The final volume was made up with methanol up to 100 ml to produce 100 µg/ml FEBU and 250 µg/ml DP. Serial dilutions were prepared by appropriate dilution of stock solution with mobile phase.
Calibration curve of FEBU and DP
Suitable aliquots of FEBU and DP were taken accurately in 10 ml volumetric flask and diluted it with mobile phase up to the mark to get final concentration in the range of 5 – 30 µg/ml for FEBU and 12.5 – 75 µg/ml for DP. A 20 µl fixed Rheodyne injection loop was utilized for injection and chromatograms were recorded in the instrument. Calibration curve was prepared by plotting peak area of chromatogram Vs. Concentration. Regression equations were generated for both the drugs.

Selection of Analytical Wavelength
Analytical Wavelength was selected by scanning of 10 µg/ml of FEBU and 10 µg/ml of DP in the range of 200 – 400 nm. (Figure 2)

HPLC Method Optimization
HPLC method optimization was optimized for efficient separation and simultaneous quantification of the Febuxostat and Diclofenac potassium. Standard solution prepare from stock solution. The mixture of standard solution (20 µg/ml FEBU and 50 µg/ml DP) was injected in HPLC system. Different ratios of water and methanol were used but either the peak shape was broad or unresolved peak was obtained. 0.20 mM Phosphate buffer of pH 3 along with methanol and acetonitrile were also tried but the peak shape was very broad. Finally a combination of 0.20 mM Phosphate buffer pH 7: methanol: acetonitrile (35:56:9) was used at a flow rate of 1 ml/min which gave sharp and well resolved peak.

Method Validation
The method was validated according to ICH Q2R1 guidelines with respect to linearity, Specificity, Precision, Accuracy, limit of detection and limit of Quantification.

Specificity
Specificity is the ability to assess the analyte in the presence of components which are expected to be present. Qualitative information required for the specificity was the peak purity (Peak start, apex, peak end) of the standard sample and formulation sample. It was determined that excipients should not affect the assay of analyte drugs.

Linearity
Linearity studies were done by plotting concentration of the drugs against the peak area and evaluated by the calculation of regression line. Minimum 6 concentrations recommended for linearity study. A linear response was obtained in the concentration range from 5 – 30 µg/ml and 12.5 -75 µg/ml for FEBU and DP respectively.

Accuracy
Accuracy study of the method was done by applying the standard addition method. The addition of 80 %, 100 % and 120 % standard solution was made to previously analyzed test sample mixture or in the formulation mixture and chromatograms were obtained.

Precision
Precision of the developed method was studied by the repeatability and intermediate precision. Repeatability studies were done by the taking 5, 10 and 15 µg/ml for FEBU and12.5, 25 and 37.5 µg/ml for DP on the same day for three times and peak areas were obtained. For intermediate precision the above same concentration of both the drugs were applied for three days under same chromatographic conditions and peak areas were obtained. Calculate the % RSD.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)
LOD of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOQ of an analytical procedure is the lowest amount of the analyte which can be quantitatively determined by precision and accuracy. LOD and LOQ of FEBU and DP were calculated by calibration curve method. The equations of LOD and LOQ were as follow:

\[
\text{LOD} = 3.3 \times \sigma / S;
\]

\[
\text{LOQ} = 10 \times \sigma / S
\]

Where, \(\sigma\) = Standard deviation of y intercept of regression line; \(S\) = Slope of the calibration curve

Robustness
Robustness of the method was studied by deliberate variations in the method parameters. Robustness of the method was studied by using the parameters like change in flow rate and change in mobile phase concentration. The studied was carried out at three concentrations 5, 10 and 15 µg/ml for FEBU and 12.5, 25 and 37.5 µg/ml for DP.

RESULTS AND DISCUSSIONS
Linearity
For linearity six calibration standards of FEBU 5 – 30 µg/ml (5, 10, 15, 20, 25 and 30) and DP 12.5 – 75 µg/ml (12.5, 25, 37.5, 50, 62.5 and 75) were taken. Both FEBU and DP showed good correlation coefficient 0.9997 and 0.9995 respectively in the given concentration range. (Table 1)

Precision
The results of intraday precision and inter day precision showed % RSD value less than 2 which indicates that the method was precise. (Table 2)

Accuracy
The accuracy of the proposed method was confirmed by recovery studies. The results of the diclofenac potassium and febuxostat were found in the range of 99 -101 %. (Table 3)

LOD and LOQ
The LOD and LOQ for febuxostat were found to be 0.78 µg/ml and 2.3 µg/ml. Whereas LOD and LOQ for diclofenac potassium were 0.53 µg/ml and 1.60 µg/ml.

Robustness
Robustness of the method is the efficiency of the method to analyze the analytes with slight change in chromatographic conditions. The results of (Table 4) show that there was no effect of the change in flow rate and mobile phase on the result and the method was found to be robust.

Analysis of Marketed Formulations
Ten tablets were accurately weighed and finely powdered. Powder equivalent to 25 mg FEBU and 62.5 mg DP was taken in 25 ml volumetric flask. To this 12 ml methanol was added in to the flask. The flask was shaken for 10 minutes, sonicated for 10 minutes and finally prepared volume up to the mark with methanol.
Table 1: Linearity Studies of Febuxostat and Diclofenac potassium

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Febuxostat</th>
<th>Diclofenac potassium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>5 - 30 µg/ml</td>
<td>12.5 - 75 µg/ml</td>
</tr>
<tr>
<td>Slope</td>
<td>43.973</td>
<td>21.132</td>
</tr>
<tr>
<td>Intercept</td>
<td>39.95</td>
<td>144.74</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9997</td>
<td>0.9995</td>
</tr>
</tbody>
</table>

Table 2: Precision Studies

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Repeatability (intraday precision)</th>
<th>Intermediate precision (interday precision)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured concentration ± S.D.</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>Febuxostat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.96 ± 0.032</td>
<td>0.64</td>
</tr>
<tr>
<td>10</td>
<td>9.97 ± 0.104</td>
<td>1.07</td>
</tr>
<tr>
<td>15</td>
<td>14.97 ± 0.16</td>
<td>1.09</td>
</tr>
<tr>
<td>Diclofenac potassium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>12.45 ± 0.092</td>
<td>0.74</td>
</tr>
<tr>
<td>25</td>
<td>25.00 ± 0.16</td>
<td>0.64</td>
</tr>
<tr>
<td>37.5</td>
<td>37.42 ± 0.25</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Table 3: Recovery Studies

<table>
<thead>
<tr>
<th>Amount of drug taken (mg)</th>
<th>Amount of drug added (mg)</th>
<th>Total amount (mg)</th>
<th>Amount recovered (mg) ± % RSD</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Febuxostat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>18</td>
<td>17.90 ± 0.23</td>
<td>99.44</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>20</td>
<td>20.04 ± 0.54</td>
<td>100.2</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>22</td>
<td>21.86 ± 0.87</td>
<td>99.36</td>
</tr>
<tr>
<td>Diclofenac Potassium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>45</td>
<td>45.07 ± 0.45</td>
<td>100.15</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>50</td>
<td>49.58 ± 0.58</td>
<td>99.16</td>
</tr>
<tr>
<td>25</td>
<td>30</td>
<td>55</td>
<td>54.5 ± 0.63</td>
<td>99.09</td>
</tr>
</tbody>
</table>

Table 4: Robustness Studies

<table>
<thead>
<tr>
<th>Method parameter</th>
<th>Deliberate changes</th>
<th>Retention time</th>
<th>Retention Factor</th>
<th>Asymmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9 ml/min</td>
<td>6.09</td>
<td>7.16</td>
<td>0.05</td>
<td>1.20</td>
</tr>
<tr>
<td>1.0 ml/min</td>
<td>6.01</td>
<td>7.10</td>
<td>0.03</td>
<td>1.18</td>
</tr>
<tr>
<td>1.1 ml/min</td>
<td>5.996</td>
<td>7.00</td>
<td>0.01</td>
<td>1.10</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>6.003 ± 0.041</td>
<td>7.00 ± 0.065</td>
<td>0.03 ± 0.016</td>
<td>1.16 ± 0.052</td>
</tr>
<tr>
<td>Mobile phase ratio (0.02 M KH2PO4 pH 7: Methanol:acetonitrile)</td>
<td>33:56:11 (v/v/v)</td>
<td>5.93</td>
<td>6.95</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>35:56:9 (v/v/v)</td>
<td>6.01</td>
<td>7.10</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>37:56:7 (v/v/v)</td>
<td>6.12</td>
<td>7.19</td>
<td>0.09</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>6.02 ± 0.095</td>
<td>7.08 ± 0.012</td>
<td>0.043 ± 0.041</td>
<td>1.19 ± 0.056</td>
</tr>
</tbody>
</table>

Figure 1: Structure of Diclofenac potassium (A) and Febuxostat (B)

Figure 2: Analytical Wavelength Selection
The solution was filtered by 0.45μm Whatman filter paper and makes the volume was made up to the mark. Aliquot of 0.2 ml solution from the above flask was taken in 10 ml volumetric flask and diluted to10 ml with mobile phase to obtain 20 μg/ml FEBU and 50 μg/ml DP. Sample was injected in to chromatographic system to obtain chromatogram which was quantified by using the regression equation. (Figure 4)

CONCLUSION
The developed isocratic RP-HPLC method is specific, accurate, precise and robust. The stastical analysis proves that method is suitable for analysis of Febuxostat and Diclofenac potassium in bilayer tablet dosage form.

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