Analytical Method Development and Validation by RP-HPLC for Simultaneous Estimation of Ketorolac and Febuxostat in Combined Tablet Dosage Form

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A B S T R A C T

A simple, Accurate, precise method was developed for the simultaneous estimation of the Ketorolac and Febuxostat in Tablet dosage form. Chromatogram was run through Inertsil C18 (4.6 x 150 mm, 5μm, Make: X Terra) or equivalent. Mobile phase containing methanol and phosphate buffer in the ratio of 60:40 was pumped through column at a flow rate of 1 ml/min. Buffer used at pH 3.5. Temperature was maintained at 30°C. Optimized wavelength for Ketorolac and Febuxostat was 292 nm. Retention time of Ketorolac and Febuxostat were found to be 4.981 min and 3.54 min. %RSD of the Ketorolac and Febuxostat were and found to be less than 2% respectively. %Recovery was found to be 97% and 103% for Ketorolac and Febuxostat respectively. LOD, LOQ values are obtained from regression equations of Ketorolac and Febuxostat was found to be within the limits respectively. The linearity range of Ketorolac and Febuxostat were found to be from 10-50 μg/ml of Ketorolac and 5-25μg/ml of Febuxostat. Linear regression coefficient was not more than 0.999.

Keywords: Ketorolac, Febuxostat, RP-HPLC

A R T I C L E  I N F O

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1. Introduction
Ketorolac is a chemically named as 5-benzoyl-2, 3-dihydro-1H-pyrrolizine-1-Carboxylic acid [1]. Ketorolac is a nonsteroidal anti-inflammatory drug (NSAID) chemically related to indomethacin and tolmetin. Ketorolac tromethamine is a racemic mixture of [-]S- and [+R]-enantiomeric forms, with the S-form having analgesic activity. Its anti inflammatory effects are believed to be due to inhibition of both cylooxygenase-1 (COX-1) and cylooxygenase-2 (COX-2) which leads to the inhibition of prostaglandin synthesis leading to decreased formation of precursors of prostaglandins and thromboxanes from arachidonic acid [2].

The resultant reduction in prostaglandin synthesis and activity may be at least partially responsible for many of the adverse, as well as the therapeutic, effects of these medications [3]. Analgesia is probably produced via a peripheral action in which blockade of pain impulse generation results from decreased prostaglandin activity. However, inhibition of the synthesis or actions of other substances that sensitize pain receptors to mechanical or chemical stimulation may also contribute to the analgesic effect [4]. In terms of the ophthalmic applications of ketorolac - ocular administration of ketorolac reduces prostaglandin E2 levels in aqueous humor, secondary to inhibition of prostaglandin biosynthesis [5]. Febuxostat is a chemically named as 2-[3-cyano-4-(2-methylpropoxy) phenyl]-4-methylthiazole-5-Carboxylic acid [6].

Febuxostat is a non-purine selective inhibitor of xanthine oxidizes. It works by non-competitively blocking the molybdenum pterin center, which is the active site on xanthine oxidase. Xanthine oxidase is needed to successively oxidize both hypoxanthine and xanthine to uric acid. Hence, febuxostat inhibits xanthine oxidase, therefore reducing production of uric acid [7]. Febuxostat inhibits both oxidized as well as reduced form of xanthine oxidase because of which febuxostat cannot be easily displaced from the molybdenum pterin site [8].

2. Materials and Methods

Instruments Used

<table>
<thead>
<tr>
<th>S.No</th>
<th>Instrument</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPLC</td>
<td>WATERS, software: Empower, 2695 separation module. 996 PDA detector</td>
</tr>
<tr>
<td>2</td>
<td>UV/VIS spectrophotometer</td>
<td>LABINDIA UV</td>
</tr>
<tr>
<td>3</td>
<td>pH meter</td>
<td>Lab India</td>
</tr>
<tr>
<td>4</td>
<td>Weighing machine</td>
<td>Sartorius</td>
</tr>
<tr>
<td>5</td>
<td>Pipettes and Burettes</td>
<td>Borosil</td>
</tr>
<tr>
<td>6</td>
<td>Beakers</td>
<td>Borosil</td>
</tr>
</tbody>
</table>

Chemicals Used

<table>
<thead>
<tr>
<th>S.No</th>
<th>Chemical</th>
<th>Brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Febuxostat</td>
<td>KP Labs</td>
</tr>
<tr>
<td>2</td>
<td>Ketorolac</td>
<td>KP Labs</td>
</tr>
<tr>
<td>3</td>
<td>KH2PO4</td>
<td>FINER chemical LTD</td>
</tr>
<tr>
<td>4</td>
<td>Water and Methanol for HPLC</td>
<td>LICHROSOLV (MERCK)</td>
</tr>
<tr>
<td>5</td>
<td>Acetonitrile for HPLC</td>
<td>Merck</td>
</tr>
</tbody>
</table>

HPLC Method Development

**Mobile Phase Optimization:**
Initially the mobile phase tried was methanol: Phosphate buffer pH 3.5 as well as varying proportions. Finally, the mobile phase was optimized to potassium dihydrogen phosphate with buffer (pH 3.5), Methanol 40: 60 v/v respectively [9].

**Optimization of Column:**

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. Symmetry C8 (4.6 x 150 mm, 5µm, Make: XTerra) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow [10].

**Optimized Chromatographic Conditions:**

<table>
<thead>
<tr>
<th>Instrument used</th>
<th>Waters HPLC with auto sampler and PDA Detector.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Column</td>
<td>Symmetry C8 (4.6 x 150nm, 5µm, Make: XTerra) or equivalent</td>
</tr>
<tr>
<td>Buffer</td>
<td>7.0 grams of potassium dihydrogen ortho phosphate in1000 ml water pH adjusted with ortho phosphoaric acid.</td>
</tr>
<tr>
<td>pH</td>
<td>3.5</td>
</tr>
</tbody>
</table>
Mobile phase : 40% buffer 60% Methanol
Flow rate : 1 ml per min
Wavelength : 292 nm
Injection volume : 20 µl
Run time : 8.0 min.

**Optimized chromatogram is obtained by following conditions**

**Trial 1:**
- Column : Inertsil C18 (4.6 x 150mm, 5µm, Make: or equivalent
- Buffer pH : 3.5
- Mobile phase : 20% buffer 80% methanol
- Flow rate : 1.0 ml per min
- Wavelength : 292 nm
- Temperature : ambient.
- Run time : 10 min.

**Figure 3:** Chromatogram for trial 1

From the above chromatogram, it was observed that the Febuxostat peak was splitted but Ketorolac peak was not separated properly.

**Trial 2:**
- Column : Symmetry C8 (4.6 x 150mm, 5µm, Make: XTerra) or equivalent
- Buffer pH : 3.5
- Mobile phase : 30% buffer 70% acetonitrile
- Flow rate : 1 ml per min
- Wavelength : 292 nm
- Temperature : ambient.
- Run time : 10 min.

**Figure 4:** Chromatogram for trial 2

From the above chromatogram, it was observed that the Febuxostat and Ketorolac peaks are splitted [11].

**Trial 3:**
- Column : Symmetry C8 (4.6 x 150mm, 5µm, Make: XTerra) or equivalent
- Buffer pH : 3.5
- Mobile phase : 40% buffer 60% acetonitrile
- Flow rate : 1.0 ml per min
- Wavelength : 292 nm
- Temperature : ambient.
- Run time : 8.0 min.

**Figure 5:** Chromatogram for trial 3

From the above chromatogram, it was observed that the Febuxostat and Ketorolac peaks are well separated but system suitability parameters are not obtained [12].

**Trial 4:**
- Column : Symmetry C18 (4.6 x 150 mm, 5µm, Make: X Terra) or equivalent
- Buffer pH : 3.5
- Mobile phase : 40% buffer 60% Methanol
- Flow rate : 1.0 ml per min
- Wavelength : 292 nm
- Temperature : ambient.
- Run time : 8.0 min.

**Figure 6:** Chromatogram for optimization

**Table 3:** Chemicals used

<table>
<thead>
<tr>
<th>Peak Results</th>
<th>Name</th>
<th>RT</th>
<th>Area</th>
<th>Height</th>
<th>USP Resolution</th>
<th>USP Tailing</th>
<th>USP Plate Count</th>
<th>Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Febuxostat</td>
<td>3.549</td>
<td>503691</td>
<td>35817</td>
<td>1.42</td>
<td>3.923</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Ketorolac</td>
<td>4.891</td>
<td>747698</td>
<td>55296</td>
<td>4.79</td>
<td>1.46</td>
<td>31492</td>
<td>2</td>
</tr>
</tbody>
</table>
From the above chromatogram, it was observed that the Febuxostat and Ketorolac peaks are well separated and system suitability parameters are also obtained.

**Preparation of buffer and mobile phase**

**Preparation of Phosphate buffer:**

Accurately weighed 7.0 grams of KH₂PO₄ was taken in a 1000 ml volumetric flask, dissolved and diluted to 1000 ml with HPLC water and the volume was adjusted to pH 3.5 with Orthophosphoric acid [13].

**Preparation of mobile phase:**

Accurately measured 400 ml (40%) of above buffer and 600 ml of Methanol HPLC (60%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration [14].

**Diluent Preparation:** The Mobile phase was used as the diluent.

**Validation Parameters:**

**Method Precision:**

**Preparation of Standard Solution:**

Accurately weighed amount of 5 mg Febuxostat and 10 mg Ketorolac and Febuxostat were taken to a 10 ml cleaned and dried volumetric flask. This was then diluted with 7 ml of diluent and was sonicated [15]. The volume was made to 10 ml with the same solvent. This was marked and labeled as Stock solution. Further, an amount of 0.3 ml and Ketorolac and Febuxostat at each was pipette from the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluents to get 15 µg/ml for febuxostat and 30 µg/ml for Ketorolac [16].

**Preparation of Sample Solution:**

Accurately weighed amount Equivalent to 5 mg and 10 mg of Febuxostat and Ketorolac and Febuxostat were taken to a 10 ml cleaned and dried volumetric flask. This was then diluted with 7 ml of diluent and was sonicated. The volume was made to 10 ml with the same solvent. This was marked and labeled as Stock solution [17]. Further, an amount of 0.3 ml and Ketorolac and Febuxostat each was pipette from the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluents to get 15 µg/ml for febuxostat and 30 µg/ml for Ketorolac [16].

**Intermediate Precision/Ruggedness:**

30 µg/ml of and 15 µg/ml of Ketorolac and Febuxostat of the above sample solution were injected for five times in five different days and peak areas were recorded.

**Accuracy:**

For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same.

**Preparation Sample solutions:**

**Preparation of 50% solution (15 µg/ml of and 7.5 µg/ml of Ketorolac and Febuxostat):**

About 5 mg of 2.5 mg of Ketorolac and Febuxostat were weighed and transferred to 10 ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same solvent. Further 0.3 ml of above solution was diluted to 10 ml with the diluent to get 15 µg/ml of and 7.5 µg/ml of Ketorolac and febuxostat [19].

**Limit of Detection (for Ketorolac):**

**Preparation of 30 µg/ml solution:**

Pipette 0.3 ml of the stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

**Preparation of 0.58% solution At Specification level (0.017 µg/ml solution):**

pipette 1 ml of the stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent. Further Pipette 0.58 ml of 1 µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

**Limit of Quantification:**
Preparation of 30 µg/ml solution:
Pipette 0.3 ml of the stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent.

Preparation of 1.95% solutions At Specification level (0.058 µg/ml solution):
Pipette 1 ml of the stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent.
Further Pipette 1.95 ml of 1 µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluents

Limit of Detection: (for Febuxostat)
Preparation of 15 µg/ml solution: Pipette 0.3 ml of the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluents [23].
Preparation of 0.6% solution At Specification level (0.009 µg/ml solution):
Pipette 1 ml of the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent.
Further Pipette 0.6 ml of 1 µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent.

Limit of Quantification:
Preparation of 15µg/ml solution:
Pipette 0.3 ml of the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluents [24].

Preparation of 2.15% solution At Specification level (0.03 µg/ml solution):
Further pipetted 1ml of the above stock solution into a 10 ml volumetric flask and diluted up to the mark with diluent.
Pipe the 2.15 ml of 1 µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent.

Robustness:
The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

Preparation of sample solution (30 µg/ml of 15 µg/ml of Ketorolac)
About 10 mg of Ketorolac and 5 mg of Ketorolac and Febuxostat were weighed and transferred to 10 ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same solvent. Further 0.3 ml of above solution was diluted to 10 ml with the diluent to get 30 µg/ml of 15 µg/ml of Ketorolac and Febuxostat [25].

Effect of Variation of flow:
The sample was analyzed at 0.8 ml/min and 1.2 ml/min instead of 1.0 ml/min, remaining conditions are same. 10 µl of the above sample was injected twice and chromatograms were recorded.

Effect of Variation of mobile phase organic composition:
The sample was analyzed by variation of mobile phase i.e. phosphate buffer: Methanol was taken in the ratio 45: 55 and 70:30 instead of 40:60, remaining conditions are same. 10 µl of the above sample was injected twice and chromatograms were recorded.

3. Results and Discussion
Validation Parameters:
Precision:
Precision of the method was carried out for both sample and standard solutions as described under experimental work.

Figure 7: Chromatograms for precision injection -1 to 5.
Table 4: Results of method precision for ketorolac

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>RT (min)</th>
<th>Area (µV ms)</th>
<th>Height (µV)</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
<th>USP Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>5.11</td>
<td>12960</td>
<td>3370</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>2.0</td>
<td>5.12</td>
<td>12980</td>
<td>3370</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>3.0</td>
<td>5.13</td>
<td>12990</td>
<td>3370</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>4.0</td>
<td>5.14</td>
<td>13000</td>
<td>3370</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>5.0</td>
<td>5.15</td>
<td>13010</td>
<td>3370</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Mean: 13000, SD: 0.3

Acceptance criteria:
- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate Precision (ruggedness)
There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation.

Table 5: Results of method precision for febuxostat

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>RT (min)</th>
<th>Area (µV ms)</th>
<th>Height (µV)</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
<th>USP Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>5.16</td>
<td>14000</td>
<td>3400</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>2.0</td>
<td>5.17</td>
<td>14020</td>
<td>3400</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>3.0</td>
<td>5.18</td>
<td>14040</td>
<td>3400</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>4.0</td>
<td>5.19</td>
<td>14060</td>
<td>3400</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>5.0</td>
<td>5.20</td>
<td>14080</td>
<td>3400</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Mean: 14060, SD: 0.3

Acceptance criteria:
- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Figure 8: Showing results for intermediate precision injections 1-3

Figure 9: Chromatograms showing accuracy 50%: 1 to 3.

Table 6: Results of Intermediate precision for Febuxostat

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>RT (min)</th>
<th>Area (µV ms)</th>
<th>Height (µV)</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
<th>USP Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>5.01</td>
<td>15000</td>
<td>3500</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>2.0</td>
<td>5.02</td>
<td>15020</td>
<td>3500</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>3.0</td>
<td>5.03</td>
<td>15040</td>
<td>3500</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Mean: 15040, SD: 0.3

Acceptance criteria:
- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

Accuracy:
Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.
Fig 10: Chromatograms showing accuracy 100%: 1 to 3.

Fig 11: Chromatograms showing accuracy 150%: 1 to 3.

<table>
<thead>
<tr>
<th>% Concentration</th>
<th>Area</th>
<th>Amount present (mg)</th>
<th>Amount Found (mg)</th>
<th>% Recovery</th>
<th>Mean Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>852858</td>
<td>2.5</td>
<td>2.52</td>
<td>101.2%</td>
<td>100.7%</td>
</tr>
<tr>
<td>100%</td>
<td>1119197</td>
<td>5</td>
<td>4.99</td>
<td>99.9%</td>
<td></td>
</tr>
<tr>
<td>150%</td>
<td>1038553</td>
<td>7.5</td>
<td>7.50</td>
<td>101.0%</td>
<td></td>
</tr>
</tbody>
</table>

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%.

Table 9: Accuracy (recovery) data for Ketorolac

<table>
<thead>
<tr>
<th>% Concentration</th>
<th>Area</th>
<th>Amount present (mg)</th>
<th>Amount Found (mg)</th>
<th>% Recovery</th>
<th>Mean Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>1424941</td>
<td>5</td>
<td>5.01</td>
<td>100.4%</td>
<td>100.8%</td>
</tr>
<tr>
<td>100%</td>
<td>1499296</td>
<td>10</td>
<td>10.02</td>
<td>100.5%</td>
<td></td>
</tr>
<tr>
<td>150%</td>
<td>2021862</td>
<td>15</td>
<td>15.86</td>
<td>101.4%</td>
<td></td>
</tr>
</tbody>
</table>

Acceptance Criteria: The percentage recovery was found to be within the limit (97-103%). The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence, method is accurate.

Linearity:
The linearity range was found to lie from 10 µg/ml to 50 µg/ml of Ketorolac, 5 µg/ml to 25 µg/ml of Febuxostat and chromatograms are shown below.
4. Conclusion

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of ketorolac and febuxostat was done by RP-HPLC. The Phosphate buffer was pH 3.5 and the mobile phase was optimized with consists of methanol: Phosphate buffer mixed in the ratio of 60:40 % v/v. A C8 column (4.6 x 150mm, 5µm, Make: XTerra) or equivalent chemically bonded to porous silica particles was used as stationary phase. The solutions were chromatographed at a constant flow rate of 1.0 ml/min. the linearity range of Ketorolac and febuxostat were found to be from 10-50 µg/ml of ketorolac and 5-25 µg/ml of febuxostat. Linear regression coefficient was not more than 0.999. The values of % RSD are less than 2% indicating accuracy and precision of the method. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

5. References


