DEVELOPMENT AND VALIDATION OF AN RP-HPLC METHOD FOR THE DETERMINATION OF FEBUXOSTAT IN HUMAN PLASMA

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ABSTRACT

A rapid, specific and accurate HPLC method was developed for the determination of Febuxostat in human plasma using Ornidazole as internal standard. The extraction process involved a liquid–liquid extraction using 70:30 % v/v of t-butyl methylether and dichloromethane. Both Febuxostat and the internal standard were eluted under isocratic mode using an YMC, C4 (4.6 X 150 mm, 3 µm) column. The mobile phase composed a mixture of 60:40 % v/v Methanol and 0.1% v/v ortho phosphoric acid at a flow rate of 1.0 mL/minute. The wavelength of detection is 310 nm. The injection volume is 20 µL. The runtime of the method is 6.0 minutes. The retention time of Febuxostat and the internal standard were 4.63 and 2.75 minutes respectively. The method showed good linearity in the range of 45.42 – 2559.64 ng/mL. The recovery of Febuxostat is 74.36 % with a standard deviation of 5.661 and recovery of internal standard was 73.77 % with a standard deviation of 5.649. The LOD of Febuxostat is 45.42 ng/mL. Matrix effects were not observed. This method is simple, specific and precise for determination of febuxostat in human plasma.

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INTRODUCTION

Febuxostat (molecular formula C_{16}H_{16}N_{2}O_{3}S, 2-(3-cyano-4-isobutoxyphenyl)-4-methyl-3-thiazole-5-carboxylic acid) [FEB] [Figure-1(A)] is a xanthine oxidase inhibitor used for treating gout caused by excessive levels of uric acid in the blood (hyperuricemia). A number of analytical and bioanalytical methods were reported for the determination of FEB alone and in combination with several other drugs. Febuxostat was estimated by spectrophotometric [4], LC–MS [5], HPLC–FLU [6], Spectroscopic [7-9], and HPLC methods [10-21]. The present investigation aims at developing a more efficient, rapid, sensitive and simple method with suitable chromatographic conditions for the determination of FEB using ornidazole as an internal standard in human plasma. The proposed method was simple, highly selective extraction method with good recovery, fully validated as per FDA guidelines [22].

A) Structure of Febuxostat

B) Structure of Ornidazole

Figure 1: Structure of Febuxostat and Ornidazole

MATERIALS AND METHODS

Solvents and Chemicals

Febuxostat (purity 96.50 % w/w) was used as received from M/s Enaltec Labs Ltd., Hyderabad. ORNIDAZOLE [ORZ],(used as an internal Standard [IS], purity) was gifted by M/s Roorkee Drugs Pvt Ltd. HPLC grade Methanol, Ortho phosphoric acid was purchased from Merck Ltd (Mumbai, India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). All other chemicals and reagents were of analytical grade.

Chromatographic System

The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system were controlled using SCL-10Avp System Controller. Data acquisition was done using LC Solutions software. The detector was set at a wavelength of 310 nm. Chromatographic separations were accomplished using an YMC C4, 3 μm, 150 mm×4.6 mm column. The mobile phase was a mixture of 40 parts of 0.1 % phosphoric acid solution and 60 parts of methanol. The mobile phase was pumped isocratically at a flow rate of 1.0 ml/min during analysis, at ambient temperature. The rinsing solution consists of a mixture of 50: 50 % v/v of acetonitrile: HPLC Grade Water.

Preparation of Standard Solutions

A stock solution of Febuxostat was prepared in methanol such that the final concentration is approximately 1.462 mg/mL. Stock solution of Ornidazole (approx 1 mg/mL) was prepared in HPLC Grade methanol. The solutions were stored at 10°C in a refrigerator and these solutions were stable for at least two weeks. Aqueous stock dilution of Febuxostat was prepared in diluent solution (mixture of 50: 50 % v/v of methanol: HPLC Grade water).
Sample Preparation

Aqueous stock dilutions were prepared initially. 0.5 ml of each aqueous stock dilution was transferred into a 10 mL volumetric flask. The final volume was made up with screened drug-free K$_2$EDTA human plasma and mixed gently for 15 minutes to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations were 0.0 (Blank; no Febuxostat added), 45.42, 90.83, 438.80, 1023.86, 1535.78, 1828.31, 2120.84 and 2559.64 ng/mL. Each of these standard solutions was distributed in disposable polystyrene micro centrifuge tubes (2.0 ml, eppendorf) in volume of 0.7 ml and stored at $-70^\circ$C until analysis. Similarly quality control samples were prepared in plasma such that the final concentrations were 47.43, 141.29, 1279.82 and 2340.24 ng/mL respectively and labeled as Lower limit of quantification (LLOQ), Low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively.

The extraction of the plasma samples involved Liquid-Liquid Extraction process. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 400 µL was then transferred to pre-labeled 2.0 mL eppendorf micro centrifuge tubes. 50 µL of internal standard dilution (approximately 100 µg/mL) was added and mixed. 1.2 mL of extraction solvent was added to extract the drug and internal standard. The samples were kept on a reciprocating shaker and allowed to mix for 20 minutes. Samples were centrifuged at 5000 rpm for 5 minutes at 4 °C. 1 mL of the supernatant was transferred into prelabelled polypropylene tubes and allowed to evaporate to dryness under nitrogen at constant temperature of 40 °C. The dried residue was dissolved in 300 µL of mobile phase and transferred into shell vials and containing vial inserts for analysis. 20 µL of the samples was injected into the system for analysis. The autosampler temperature is maintained at 4°C throughout the analysis. The column temperature oven is maintained at ambient temperature.

Validation of quantitative HPLC method

The quantitative HPLC-UV method was validated to determine selectivity, calibration range, accuracy and precision, limit of detection (LOD), limit of quantitation, % recovery, freeze–thaw, and auto sampler stability. The initial assay was fully validated for Febuxostat analysis in human plasma according to FDA guidelines.

Selectivity

The selectivity of the method was evaluated by analyzing six independent drug-free K$_2$EDTA human plasma samples with reference to potential interferences from endogenous and environmental constituents.

Calibration curve

Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of FEB in the standard samples. Fresh calibration standards were extracted and assayed as described above on three different days and in duplicate. Calibration curves for FEB were represented by the plots of the peak-area ratio (FEB/ORZ) versus the nominal concentration of the FEB in calibration standards. The regression line was generated using 1/concentration$^2$ factor as the mathematical model of best fit. FEB concentrations in QC samples, recovery, and stability samples were calculated from the resulting area ratio and the regression equation of the calibration curve.

Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC; n = 6 at each level) on the same day. Inter-day precision and the accuracy were determined by analyzing four QC levels on 3 separate days (n = 6 at each level) along with three separate standard curves done in duplicates.

The accuracy of an analytical method describes how close the mean test results obtained by the method are to the nominal concentration of the analyte. Accuracy was calculated by the following equation, expressed as a percentage:

$$\text{Accuracy (\%)} = \frac{\text{mean observed concentration}}{\text{nominal concentration}} \times 100$$

The precision was expressed by co-efficient of variation (CV). The CV % indicates the variability around the mean in relation to the size of the mean, and is defined as:

$$\text{CV (\%)} = \frac{\text{standard deviation}}{\text{mean observed concentration}} \times 100$$

Stability Studies

Auto sampler, and freeze–thaw stability of FEB was determined at low, medium and high QC concentrations. To determine the impact of freeze–thaw cycles on FEB concentration, samples were allowed to undergo 3 freeze ($-70^\circ$C) thaw (room temperature) cycles. Following sample treatment/storage conditions, the FEB concentrations were analyzed in triplicates and compared to the control sample that had been stored at $-70^\circ$C. Autosampler stability of extracted samples was determined by comparing FEB concentration in freshly prepared samples and samples kept in autosampler at 4 °C for 24 h.

Recovery

Recovery was determined by comparing the area under the curve of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of FEB as in the QC samples. This should highlight any loss in signal due to the extraction process. IS recovery was determined for a single concentration of 100 µg/mL.
Data analysis

HPLC data acquisition and processing was performed by Shimadzu LC Solutions Ver 1.23 SP 1 software. Standard curves for quantitation of FEB were constructed using a 1/concentration$^2$ weighted linear regression of the peak area ratio versus FEB concentration. Unknown and QC sample concentrations were back-calculated from the standard curves.

RESULTS and DISCUSSION

Method Development

The HPLC procedure was optimized with a view to develop a sensitive and reproducible method for the determination of FEB in Human Plasma. Since both Febuxostat and internal standard are highly non-polar, we employed the usage of liquid-liquid extraction process with a mixture of 70 parts of t-butyl methyl ether and 30 parts of dichloromethane. To get a better response the pH of the mobile phase was set to the acidic side. During our observation, a pH value around 7.4 resulted in better peak shape for the internal standard while that of the drug was not acceptable. Also, alkaline mobile phase characteristics causes deterioration of the bonded phase in the column due to alkaline hydrolysis of end-capped silica [23, 24]. Compared to acid catalyzed hydrolysis, the hydrolysis of end-capped silica in alkaline conditions is usually very rapid. Therefore experiments were performed using Potassium Dihydrogen phosphate in a limited pH range of 3.0 to pH 5.5. The response was checked at the detector using a connector (without the column). A pH value of 3.0 ± 0.1 gave maximum response for the analyte at 310 nm. A similar response was observed with the usage of 0.1% orthophosphoric acid. Therefore the final mobile phase consisted of 60: 40 % v/v methanol and 0.1 % orthophosphoric acid.

The run time of analysis is higher when a longer reverse phase column (250 X 4.6 mm id) is used. The resolution between the peaks was decreased and peaks were not acceptable peak shape when the experiment is performed using a shorter column (50 X 4.6 mm id). However better resolution, less tailing and high theoretical plates were obtained with a YMC C4 150 X 4.6 mm id, 3 µm column.

The flow rate of the method was 1.0 ml/min. The column temperature was maintained at ambient. At the reported flow rate, peak shape was acceptable, however increasing or decreasing the flow rate increased the tailing factor and resulting in poor peak shape and decreased resolution between the drug and internal standard. There was no interference in the drug and internal standard, from the extracted blank. The peak symmetry were found to be good when the mobile phase composition of 60:40 v/v methanol and 0.1% ortho phosphoric acid leading to better resolution of the drug and internal standard.

Detection and chromatography

The typical chromatogram in figure 2 was obtained when human plasma was not spiked with sample and IS. The chromatogram in figure 3 represents a peak corresponding to IS alone when human plasma was spiked with IS and not with sample. Figure 4 was the chromatogram of human plasma spiked with IS and FEB in its ULOQ. The retention times for FEB and IS were 4.63 and 2.75 min, respectively.

Method validation

Selectivity

The method was found to have high selectivity for the analytes; since no interfering peaks from endogenous compounds were observed at the retention time for FEB in any of the six independent blank plasma extracts evaluated (Fig. 2).
Figure 2: Chromatograms of (A) Extracted Blank Sample

Figure 3: Zero Blank Containing ORNIDAZOLE as Internal Standard

Figure 4: FEBUXOSTAT (drug) and ORNIDAZOLE (IS) extracted from human plasma

Calibration curves

A system suitability exercise was performed before the initiation of the validation. A system was assumed to be suitable for analysis if and only if the % CV for the retention times of FEB and internal standards was less than 2%. The results are tabulated in Table 1. Calibration curves for FEB in human plasma were fitted by weighted 1/concentration^2 quadratic regression, with the r^2 values of >0.99 for all curves generated during the validation. The calibration curve accuracy for plasma was presented in Table 2 demonstrating that measured concentration was within ± 15% of the actual concentration point (20% for the lowest point on the
standard curve, the LLOQ). Results were calculated using peak area ratios. A representative calibration curve showing the regression equation and $r^2$ value was depicted in Figure – 5.

Table 1: System Suitability Study

<table>
<thead>
<tr>
<th></th>
<th>Ornidazole Internal Standard (100 µg/mL)</th>
<th>Febuxostat (2340.24ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention Time (min)</td>
<td>Peak Area</td>
<td>Retention Time (min)</td>
</tr>
<tr>
<td>Mean (n = 6)</td>
<td>2.75</td>
<td>309627.45</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.01</td>
<td>8525.149</td>
</tr>
<tr>
<td>% CV</td>
<td>0.36</td>
<td>2.75</td>
</tr>
</tbody>
</table>

Figure 5: Calibration Curve of for FEBUXOSTAT (Curve – 1)

Table 2: Results of regression analysis of the linearity data

<table>
<thead>
<tr>
<th>Linearity parameters</th>
<th>Mean ± SD (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.00056</td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.00091</td>
</tr>
<tr>
<td>Correlation coefficient ($r^2$)</td>
<td>0.99970</td>
</tr>
</tbody>
</table>

Accuracy and precision

A detailed summary of the intra-day and inter-day precision and accuracy data generated for the assay validation was presented in Table 3. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations (LLOQ, LQC, MQC, and HQC) of three separate assays. Intra-assay variability was determined as the accuracy and precision of the six individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision was <5% for all QC concentrations, which was within the general assay acceptability criteria for QC samples according to FDA guidelines [22].

Limit of detection and limit of quantification

LOD was defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 30ng/mL. The LLOQ has been accepted as the lowest points on the standard curve with a relative standard deviation of less than 20% and signal to noise ratio of 5:1. Results at lowest concentration studies (50ng/mL) met the criteria for the LLOQ (Table 3). The method was found to be sensitive for the determination of RIF in human plasma samples. The ULOQ has been accepted as the highest points on the standard curve with a relative standard deviation of less than 15%.
Table 3: Intra and Inter day accuracy and precision of HPLC assay

<table>
<thead>
<tr>
<th>Nominal Concentration in ng/mL (QC ID)</th>
<th>DAY 1</th>
<th></th>
<th>DAY 2</th>
<th></th>
<th>DAY 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>% CV</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>47.43 (LLOQ QC)</td>
<td>47.91</td>
<td>1.586</td>
<td>3.31</td>
<td>48.01</td>
<td>2.20</td>
</tr>
<tr>
<td>141.29 (LQC)</td>
<td>146.70</td>
<td>2.507</td>
<td>1.71</td>
<td>147.49</td>
<td>4.94</td>
</tr>
<tr>
<td>1279.82 (MQC)</td>
<td>1289.04</td>
<td>34.395</td>
<td>2.67</td>
<td>1295.93</td>
<td>49.60</td>
</tr>
<tr>
<td>2340.24 (HQC)</td>
<td>2399.88</td>
<td>36.490</td>
<td>1.52</td>
<td>2412.37</td>
<td>60.401</td>
</tr>
</tbody>
</table>

Each mean value is the result of triplicate analysis.

Carryover test

A critical issue with the analysis of many drugs was their tendency to get absorbed by reversed phase octa-decyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

Stability studies

The results of short-term, long term and freeze–thaw stability were presented in Table 4. Determination of FEB stability following three freeze–thaw cycles showed that for all QC samples there was a minor change in the FEB concentration.

Table 4: Short Term, long term and Freeze Thaw stability of FEBUXOSTAT

<table>
<thead>
<tr>
<th>Nominal Concentration (ng/mL)</th>
<th>Short-term stability (4 Days)</th>
<th>Long-term stability (12 Days)</th>
<th>Freeze – Thaw stability (3 Cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Accuracy (%)</td>
<td>S.D.</td>
<td>% CV</td>
</tr>
<tr>
<td>141.29 (LQC)</td>
<td>99.41</td>
<td>4.95</td>
<td>3.49</td>
</tr>
<tr>
<td>2340.24 (HQC)</td>
<td>94.71</td>
<td>40.30</td>
<td>1.63</td>
</tr>
</tbody>
</table>

Each mean value is the result of triplicate analysis.

Recovery

Percentage recovery of FEB was measured by dividing the peak area values of extracted QC samples with direct injection of solution containing the same nominal concentration of compounds as the QC samples in extracted blank plasma. The mean recovery of FEB from plasma spiked samples at LQC, MQC and HQC levels was 80.827 %, 72.130 % and 70.20 % respectively. The overall recovery is 74.39 % with a % Coefficient of variation of 7.61 %, respectively. IS recovery at 100 µg/mL of ORZ was 73.77 % with a % Coefficient of variation of 7.66 %.

CONCLUSION

The method described in this manuscript has been developed and validated over a concentration range of 45.42 to 2560 ng/ml in human plasma. The extraction process was a single-step liquid–liquid extraction procedure employing the use of 70:30 % v/v of t-butyl methyl ether and dichloromethane. LLE method was usually devoid of polar interferences thus rendering the sample clean for final analysis. The noise was usually absent or at minimum as compared to precipitation or SPE techniques. This assay requires only a small volume of plasma (500 µL). There was no carryover effect. Due to the LLE method of extraction, baseline noise is minimal.
Matrix effects are not observed. In conclusion, method validation following FDA guideline indicated that the developed method had high sensitivity with an LLOQ of 45.42ng/mL, acceptable recovery, reliability, specificity and excellent efficiency with a total running time of 6.0 min per sample, which was important for large batches of samples. Thus this method can be suitable for pharmacokinetic, bioavailability or bioequivalence studies of FEB in human subjects. This method has been successfully applied to analyze Febuxostat concentrations in human plasma. In future we are planning to carry similar research aimed at quantitative determination of same and several other classes of drugs in human plasma. 

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