BIOANALYTICAL METHOD DEVELOPMENT FOR ESTIMATION OF DEFERASIROX IN HUMAN PLASMA

M. S. KALSHETTI, R. Y. PATIL, R. A. KARALE*, A. A. KULKARNI
Department of Quality Assurance, D. S. T. S. Mandal’s College of Pharmacy, Solapur University, Solapur, Maharashtra, India 413004
Email: rohitkpharmacist@gmail.com

Received: 08 May 2015 Revised and Accepted: 27 Jul 2015

ABSTRACT
Objective: A rapid and selective high performance liquid chromatography (HPLC) method for estimation of Deferasirox in Human plasma was developed and validated.

Methods: Analyte was recovered by protein precipitation technique and subsequently separated on C18 column (150x4.5 mm, 5µ) by using acetonitrile: phosphate buffer pH 3.0(60:40) as mobile phase at a flow rate of 1.0 ml/min at 248 nm.

Results: The calibration curve was linear over the concentration range 0.4-4.8 µg/ml in human plasma. Within-batch and between-batch precision were <15% (at LLOQ<20%) and accuracy was all within 15%. (at LLOQ<20%).

Conclusion: The developed and validated HPLC method was specific, sensitive and reproducible which can be used for routine drug analysis and bioanalysis.

Keywords: HPLC-UV, Bioanalytical, Deferasirox, Human plasma.

INTRODUCTION
Deferasirox belongs to the class Antidote. Chemical name is 1-[3, 5-bis (2-hydroxyphenyl)-1H 1, 2, 4-triazol-1-yl]-benzoic acid.

Deferasirox is an oral iron chelator. Its main use is to reduce chronic iron overload in patients who are receiving long term blood transfusions for conditions such as beta thalassemia and other chronic anemias. Deferasirox is an orally active chelator that is selective for iron (as Fe3+). It is a tridentate ligand that binds iron with high affinity in a 2:1 ratio. It is not official in any of the pharmacopoeia. It is listed in the Merck index 14th edition and Martindale the complete drug reference 35th edition [1-3].

Literature survey revealed that validated HPLC method for the quantification of Deferasirox in plasma is not reported [4-10]. For the estimation of the drugs present in the biological fluid, HPLC method is considered to be more suitable since this is a specific, linear, precise, accurate, sensitive and rapid method. In this study, we have developed a HPLC-UV method with a protein precipitation extraction and improved sensitivity for the determination of Deferasirox in plasma and the developed method is validated as per regulatory requirements [11].

MATERIALS AND METHODS
Chemicals
Deferasirox was gifted by Torrent Pharmaceuticals Pvt. Ltd. Gujarat. HPLC Grade Solvents (Acetonitrile, Methanol, Water) and Di potassium hydrogen phosphate were obtained from Merck Specialties Pvt. Ltd., Mumbai and ortho phosphoric acid were from S. D. Fine chem. Ltd., Mumbai.

Instrument
Younglins Acme 9000 LC system were equipped with a UV detector. Chromatographic separations were performed using the Phenomenex C18 (150x4.6 mm, 5µ) column and analyzed by LC software Autochro-3000.

Preparation of solutions
Phosphate buffer was prepared by dissolving approximately 0.34g of potassium dihydrogen phosphate in 250 ml of HPLC water and the pH was adjusted to 3.0 with acetic acid.

Preparation of standard
Deferasirox stock solutions were prepared at a concentration of 1 mg/ml by dissolving in methanol and the stock solutions were stored in the refrigerator. Spiking solutions of deferasirox for the preparation of calibration standards and quality control samples were prepared in methanol and spiked into the plasma. The calibration curve from 0.8μg/ml to 4.8μg/ml was generated using six calibration standards at the concentrations of 0.8μg/ml (STD 1), 1.6μg/ml (STD 2), 2.4μg/ml (STD 3), 3.2μg/ml (STD 4), 4.0μg/ml (STD 5), and 4.8μg/ml (STD 6). The quality control samples were prepared at concentrations of 2.4μg/ml (LQC), 3.2μg/ml (MQC) and 4.0μg/ml (HQC).

Sample preparation and extraction
Deferasirox from the plasma was extracted using protein precipitation extraction technique. Aliquot of 490μl plasma was taken into micro centrifuge tubes and added 10 μl of spiking solution then vortexed to mix the contents. Deferasirox is extracted by using acetonitrile as a precipitating solvent. Vortexed for 30 sec then the solution was centrifuged at 4, 15000rpm for 10 min. The supernatant is taken and injected to HPLC.

HPLC method
The mobile phase used was acetonitrile and phosphate buffer (pH 3.0) (60:40). An isocratic method is developed table-1. Before analyses, the mobile phase was filtered through 0.45 μm filter and then degassed ultrasonically for 10 min. The analyses were conducted at a flow rate of 1.0 ml/min. The eluent was monitored at a wavelength of 248 nm for deferasirox. The total run time was 10 min and injection volume was 20μl.
Table 1: Optimized chromatographic conditions for analysis of deferasirox by RP-HPLC

<table>
<thead>
<tr>
<th>Mobile Phase</th>
<th>Rt (min)</th>
<th>Tailing Factor</th>
<th>Theoretical Plates (TP)</th>
<th>Resolution</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:50</td>
<td>10.3</td>
<td>0.7804</td>
<td>523.0</td>
<td>5.3</td>
<td>TP &lt; 2000</td>
</tr>
<tr>
<td>55:45</td>
<td>6.7</td>
<td>0.958</td>
<td>2371.3</td>
<td>6.3</td>
<td>Bad peak shape</td>
</tr>
<tr>
<td>60:40</td>
<td>4.3</td>
<td>1.1316</td>
<td>2633.3</td>
<td>3.8</td>
<td>Capacity factor, therapeutic plates, tailing factor within accepted range</td>
</tr>
</tbody>
</table>

(C18 Column, at 1 ml/min flow rate, detection wavelength is 248 nm, mobile phase ratio containing ACN: Buffer (pH-3) respectively)

Method validation

The method performance was evaluated for selectivity, accuracy, precision, linearity, and stability during various stress conditions including bench top stability, freeze thaw stability, stability of stock solutions etc. and recovery [11].

Linearity

Calibration curves were constructed using linear regression (with the weighting of $1/x^2$) within the range of 0.8-4.8μg/ml of deferasirox.

Recovery

Recovery of analyte was evaluated by comparing the response of deferasirox in three quality control samples (LQC, MQC and HQC) with the response of deferasirox in equivalent aqueous solutions.

Precision and accuracy

For precision and accuracy studies, samples of three concentration levels were prepared as low (LQC), medium (MQC) and high (HQC) quality controls, corresponding to 2.4, 3.2 and 4.0μg/ml respectively with three replicates each. Precision was evaluated within and between batches.

Stability studies

The stability of deferasirox in solutions and plasma samples was evaluated during method validation. Deferasirox stability was evaluated using two concentration levels (low and high quality control, corresponding to 2.4 and 4.0μg/ml respectively). The stability of deferasirox was evaluated in plasma samples kept at freezer and after being stressed to 3 freeze-thawing cycles (24 hours each cycle). All samples described above were compared to freshly prepared deferasirox samples at the same concentration level.

RESULTS AND DISCUSSION

Chromatographic Optimization

A RP-HPLC method was developed for deferasirox, which can be conveniently employed for routine analysis in biological fluids. The chromatographic conditions were optimized in order to provide a good performance of the assay. The mobile phase for drug was selected based on its polarity. Different trials were taken and the final optimized mobile phase is listed in table 1. The retention time of deferasirox was 4.3 min. The chromatogram of deferasirox in plasma has been shown in fig. 2. The method is validated as per regulatory guidelines.

Selectivity

The described method used reversed-phase HPLC for separation of deferasirox was shown to be selective. No interfering peaks were observed with the same retention time of the analyte when different plasma samples were analysed. Fig. 1, fig.2 and fig.3 represent the chromatograms of blank plasma sample, plasma sample spiked with drug and overlain chromatogram of blank plasma and spiked plasma respectively.

Linearity

Linearity was demonstrated from 0.8-4.8μg/ml. Fig. 4 shows calibration curve of deferasirox. The calibration curve includes 6 calibration standards which are distributed throughout the calibration range. Correlation coefficient was demonstrated for the evaluation of goodness fit. The average correlation coefficient was found to be 0.9914 with goodness of fit.

Accuracy and precision

Accuracy and precision were evaluated by analyzing 3 batches. Each batch consists of three replicates of LQC, MQC and HQC. Precision was evaluated both within and between batches. The within and between batch precision and accuracy of the method for each deferasirox concentration levels (2.4, 3.2 and 4.0μg/ml) are represented in table 2. The mean accuracy for each concentration level ranged from 100.6 to 102.3 and the mean precision for each concentration level ranged from 0.835 to 2.0%.

Recovery

The recovery was evaluated by comparing response of extracted and unextracted samples. Extracted samples include three replicates of extracted LQC, MQC and HQC samples. Unextracted samples included the aqueous solutions equivalent to extracted samples. The mean recovery for deferasirox in plasma was ranged from 90.3 to 92.2% for the low, medium and high quality control samples represented in table 3.

Stability studies

Stability studies were performed to evaluate the stability of deferasirox both in aqueous solution and in plasma after exposing to...
various stress conditions. The stability studies performed include stock solution stability, bench top stability in plasma and freeze thaw stability in plasma. All Stability evaluations were performed as per regulatory guidelines.

Deferasirox stock solution (1 mg/ml) remained stable when stored at room temperature for 6 hr. Deferasirox was stable in plasma samples when stored at room temperature for 6 hours. Deferasirox was found to be stable for three freeze and thaw cycles.

**Table 2: Within batch and between batch precision and accuracy of deferasirox**

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Accuracy (% Mean)</th>
<th>Precision (%CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Within batch</td>
</tr>
<tr>
<td>2.4</td>
<td>102.3</td>
<td>2.0</td>
</tr>
<tr>
<td>3.2</td>
<td>101.2</td>
<td>0.8</td>
</tr>
<tr>
<td>4.8</td>
<td>100.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>

(The % mean accuracy for all QC samples should be in the range of 85.00-115.00%. The % CV for all QC samples should be within 15.00 %.)

**Table 3: Recovery result of deferasirox from plasma**

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>% Mean recovery (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>91.6</td>
</tr>
<tr>
<td>3.2</td>
<td>90.3</td>
</tr>
<tr>
<td>4.0</td>
<td>92.2</td>
</tr>
</tbody>
</table>

**Table 4: Validation parameters of deferasirox by HPLC method**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Selectivity</td>
<td>Pass</td>
</tr>
<tr>
<td>2.</td>
<td>System suitability</td>
<td>Pass</td>
</tr>
<tr>
<td>3.</td>
<td>Accuracy and precision</td>
<td>Pass</td>
</tr>
<tr>
<td>4.</td>
<td>Linearity</td>
<td>R²=0.991</td>
</tr>
<tr>
<td>5.</td>
<td>Range</td>
<td>0.8-4.8μg/ml</td>
</tr>
<tr>
<td>6.</td>
<td>Recovery</td>
<td>Pass</td>
</tr>
<tr>
<td>7.</td>
<td>Short term stock stability</td>
<td>6 Hr</td>
</tr>
<tr>
<td>8.</td>
<td>Bench top stability</td>
<td>6 Hr</td>
</tr>
<tr>
<td>9.</td>
<td>Freeze and thaw stability</td>
<td>Pass (3 cycles)</td>
</tr>
</tbody>
</table>

**CONCLUSION**

In the present work, a rapid, sensitive, specific, precise and accurate bioanalytical method for deferasirox in human plasma has been developed and validated with a larger calibration curve range (i.e. 0.8 to 4.8μg/ml) using high performance liquid chromatography which can be used for routine drug analysis and bioanalysis.

**ACKNOWLEDGEMENT**

I take this golden opportunity to express my humble gratitude and respect to our honourable principal, Professor R. Y, Patil and my research guide Dr. M. S. Kalshetti M. Pharm. PhD. Professor, for their inspiring guidance, constant encouragement and intellectual suggestions throughout the course of the dissertation.

Last but not least I am highly grateful to my parents for their moral support.

**CONFLICT OF INTERESTS**

Declared None

**REFERENCES**

5. Chauzit, Emmanuelle, Bouchet. A method to measure deferasirox in plasma using HPLC coupled with ms/ms
detection and its potential application. Ther Drug Monit 2010;32:476-81.