DEVELOPMENT OF SIMPLE HPLC METHOD TO ESTIMATE THE BLOOD PLASMA CONCENTRATION OF EFAVIRENZ IN RAT AFTER ORAL ADMINISTRATION

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ABSTRACT

Objective: The present study was designed with an objective of developing a simple and rapid high performance liquid chromatography (HPLC) method for the determination of Efavirenz (EFA) in rat plasma.

Methods: Chromatographic separation was achieved on C18 column using acetonitrile-50 mM potassium phosphate (55:45 v/v) as mobile phase at a flow rate of 1 ml/min and UV detection at 250 nm.

Results: The retention time of EFA was found to be 13.2 min. The developed method was validated for linearity, limit of quantification (LOQ), limit of detection (LOD), Stability and selectivity. Linearity studies were found to be acceptable over the range of 5-50 µg/ml.

Conclusion: The present analytical method was found to be specific, sensitive, accurate and precise for quantification of EFA in rat plasma. It can be successively applied for pharmacokinetics studies also.

Keywords: Efavirenz, Atorvastatin, HPLC, Pharmacokinetics.

INTRODUCTION

The development of a large number of new antiretroviral agents and the increasing resistance of HIV virus has led to changes in the treatment of this infection. Therapeutic monitoring of these drugs is recommended in order to avoid or to delay resistance from the virus, to avoid the usually underestimated non-adherence and to manage drug interactions. In view of this, analytical methods have already been described to quantify single [1-6] and combined [7-10] anti-HIV agents in biological media.

EFA (fig. 1) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) of HIV-1 is used in association with other antiretroviral agents in the treatment of HIV infection [11].

![Chemical structure of EFA](image)

Fig. 1: Chemical structure of EFA

Literature survey revealed that number of HPLC methods for estimation of plasma EFA alone [12-16] and in combination with other antiretroviral agents [17-26] have been reported. While some of these methods involve cumbersome and lengthy extraction procedures [15, 16, 19, 21], few other methods have utilized a complex mobile phase [17, 21] or gradient mobile phase [18,25]. Sample pretreatment by liquid-liquid extraction with diethyl-ether [12, 14, 18, 26] or protein precipitation with acetonitrile [13, 23] has also been carried out in some methods.

We have developed simple, sensitive and accurate HPLC method for determination and quantification of EFA following solid-phase extraction of plasma. In comparison to all above reported method, the validation result demonstrated a higher sensitivity and need for lower plasma volume. After validation, this assay was successfully applied to pharmacokinetics after administration EFA to rats and its results would be helpful for evaluating the clinical application of this drug.

MATERIALS AND METHODS

Chemicals

Pure EFA was kindly supplied as the gift sample from Lupin Research Park Pvt. Ltd. Pune, India. Atorvastatin (fig. 2) was a kind gift from Lupin Research Park Pvt. Ltd. Pune, India. Acetonitrile (HPLC grade) was purchased from Merck (India) and potassium dihydrogen orthophosphate was purchased from Qualigen (India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA).

![Chemical structure of Atorvastatin](image)

Fig. 2: Chemical structure of Atorvastatin

Preparation of standard solution

A standard stock solution was prepared by dissolving EFA in methanol with concentration 500 µg/ml. The standard solutions (n=6) were prepared by spiking drug free rat plasma with known amount of EFA ranging between 5-50 µg/ml. Six EFA concentrations were prepared (5, 10, 20, 30, 40, 50 µg/ml) as in house quality control and kept at -20 ºC until assay.
Sample preparation

The rat plasma samples of 250 μl were transferred to tubes in which 50 μl of atorvastatin (internal standard i.e. I. S.), 100 μl of EFA were added, followed by one milliliter of ethyl acetate. The samples were vortexed for 1 min. The samples were centrifuged at 7000 rpm and organic phase was evaporated to dryness. The dried residue was reconstituted with 100 μl of mobile phase and 20 μl was injected into the HPLC column.

Chromatographic conditions

The HPLC system was composed of UV/VIS detector (Jasco UV 975). Chromatographic separation was performed on a Hypersil ODS (250×4.6 mm i.d., 5 μm) column and protected by the guard column. The isocratic mobile phase composed of acetonitrile and 50 mM potassium phosphate (55:45 v/v) with 1.5% of triethylamine was used and pH was adjusted to 4.3 with orthophosphoric acid. The flow rate and the detection wavelength was 1 mL/min and 250 nm respectively. All determination was performed at ambient column temperature. The software used was Jasco BORWIN version 1.5 LC-net/B/ADC system.

Linearity

Calibration plots were constructed for EFA standard solutions by plotting the concentration of compounds versus peak area responses. Standard solutions containing 5 and 50 μg/ml of EFA in plasma were prepared and injected into the HPLC column. The calibration equation \( y = mx+b \) was calculated from the calibration curves. The regression equations were calculated from the calibration graphs with the standard deviations of the slope and y-interception.

Accuracy and precision

Accuracy of the assay method was determined for both within-day and between-day variations using the six times analysis of the samples. Precision of the assay was determined by repeatability (within-day) and intermediate precision (between-day). Intermediate precision was assessed by comparing the assays on different days (6 days). All data were expressed as % CV (coefficient of variation). The acceptance value for precision and accuracy for each concentration should not exceed 15% of percentage of coefficient of variation (% CV) from the theoretical value.

Detection and quantitation limits (sensitivity)

LOD and LOQ were estimated from the signal-to-noise ratio. The detection limit was defined as the lowest concentration level resulting in a peak height of three times the baseline noise. The quantitation limit was defined as the lowest concentration level that provided a peak height with a signal-to-noise ratio higher than 10, with precision (% RSD) and accuracy (% bias) within±10%.

Stability

Stability of EFA was measured by preparing three replicates (n=3) of EFA samples at three different concentrations of 10, 20 and 30 μg/ml, respectively. The stability of the samples was assessed by the percentage recovery based on criteria’s. The procedures were designed as follow; (a) 30 min at 50 °C in plasma and 18 hr at 25 °C in mobile phase after solid-phase extraction.

RESULTS AND DISCUSSION

Linearity

Calibration curves of EFA standard in rat plasma showed a linearity line over the range of 5-50 μg/ml. The data was consistent throughout the experiment which showed a consistency of coefficient, intercept and slope. The calibration curve gives R2 of 0.999 and y-interception was 0.0766 as shown in fig. 3. Peak height ratio of EFA and I. S. of calibration standards were proportional to the concentration of EFA in plasma over the range tested (5-50 μg/ml). The results of the linearity of the method are presented in table 1.

Table 1: Result of linearity of calibration of EFA

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Area of EFA</th>
<th>Area of I. S.</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>110104.2</td>
<td>400900.6</td>
<td>0.274642</td>
</tr>
<tr>
<td>10</td>
<td>336933.6</td>
<td>602078.6</td>
<td>0.559622</td>
</tr>
<tr>
<td>20</td>
<td>791335.5</td>
<td>755243.1</td>
<td>1.047789</td>
</tr>
<tr>
<td>30</td>
<td>1147214</td>
<td>770496.1</td>
<td>1.488929</td>
</tr>
<tr>
<td>40</td>
<td>1492710</td>
<td>760635.9</td>
<td>1.96245</td>
</tr>
<tr>
<td>50</td>
<td>2171868</td>
<td>903399.4</td>
<td>2.404107</td>
</tr>
</tbody>
</table>

Fig. 3: Calibration curve of EFA

Accuracy and precision

The accuracy of this method was assessed by the determination of the percentage recovery of EFA in rat plasma for both between-day and within-day variations. Six different standard solutions of concentration in the range of 0.5 to 50 μg/ml were analyzed.

The obtained result is summarized in table 2. The results obtained were summarized in table 2. The results described the accuracy of this method with a mean recovery of 97.03 % for within-day samples and 98.36% for between-day samples, respectively.

Table 2: Within day and between day results expressed in mean±standard deviation (SD) and precision in coefficient of variation (% CV). Each data represents a set of triplicates

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Within-day</th>
<th>Between day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean±SD (μg/ml)</td>
<td>Accuracy (Recovery %)</td>
</tr>
<tr>
<td>10</td>
<td>5.2±0.8</td>
<td>96.02</td>
</tr>
<tr>
<td>20</td>
<td>6.3±1.5</td>
<td>97.05</td>
</tr>
<tr>
<td>30</td>
<td>7.1±1.9</td>
<td>98.02</td>
</tr>
<tr>
<td>10</td>
<td>5.4±0.9</td>
<td>97.01</td>
</tr>
<tr>
<td>20</td>
<td>6.2±1.3</td>
<td>99.03</td>
</tr>
<tr>
<td>30</td>
<td>8.1±2.4</td>
<td>99.04</td>
</tr>
</tbody>
</table>
We then assessed the precision of this method from the repeatability of three different standard solutions for six times in the same day (within-day). The intermediate precision used the same six standard solutions six times on different days (between-day).

The precision is described by the percentage relative standard deviation (RSD). In table, the relative standard deviation for within-day samples ranged from 1.7 to 9.2 % and between-day samples ranged from 2.1 to 9.0 % as stated in table 2.

**Selectivity**

Representative chromatograms of blank and spiked plasma samples showed no interfering endogenous substances neither with EFA nor with the I. S. Potentially co-administered drugs had retention times different from EFA or the I. S. and were not detected with the described bio-analytical method. The retention times for the drugs tested for interference are shown in fig. 4. The retention times for EFA, I. S., Atovaquone, Isoniazide and Saquinavir are given in table 2.

**Table 2: The retention time of drug and I. S**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atovaquone</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Isoniazide</td>
<td>2.3</td>
</tr>
<tr>
<td>Atorvastatin (I.S.)</td>
<td>8.3</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>10.9</td>
</tr>
<tr>
<td>EFA</td>
<td>13.2</td>
</tr>
</tbody>
</table>

**Fig. 4: Retention time of both EFA and I. S**

In this study, attempts were made to extract EFA from plasma using ethyl acetate using atorvastatin as I. S. and this has been reported so far. The present method has the advantages of being simple and a run time of 15 min, without any loss of analyte, and uses a small sample volume. The use of I. S. helped in monitoring the recovery of EFA from plasma. Under the chromatographic conditions described above, EFA was well separated as seen in the representative chromatograms. The retention times of the I. S. and EFA were 8.3 and 13.2 min, respectively. Blank plasma samples did not give any peak at the retention time of EFA.

**Sensitivity**

Six different concentration of EFA were assayed to calculate the LOD and LOQ. LOD represent the lowest concentration that can be detected by the method, while LOQ is the lowest concentration that can be determined at an acceptable precision and accuracy. In this study, the LOD and LOQ for EFA analysis were found to be 0.10 μg/ml and 0.30 μg/ml, respectively.

**Stability**

The stability of EFA under various conditions were determined. The recovery data was obtained by comparing the samples to the freshly prepared sample. The results of stability studies were within the acceptable range as shown in table 4.

**Pharmacokinetic study**

The method described was applied for the determination of EFA concentrations from rat plasma after receiving a single oral dose of EFA (30 mg). Fig. 6 presents the mean plasma concentrations of EFA at various time points (30 min, 1, 2, 3, 4, 5, 6, 12, 24 and 48 h) EFA was detectable up to 48 h after the dose. The plasma concentration of EFA shows maximum concentration (Cmax, fig. 7) of EFA at 2 hr to be 200 μg/ml.

**Table 4: Stability of EFA (n=3)**

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Concentration (μg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min at 50 °C in plasma</td>
<td>10</td>
<td>91.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>97.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>94.6</td>
</tr>
<tr>
<td>18 h at 25 °C in mobile phase after solid-phase extraction</td>
<td>10</td>
<td>94.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>93.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>95.4</td>
</tr>
</tbody>
</table>

**Fig. 6: Plasma EFA concentration in rats administered a single oral dose of 30 mg EFA**

**Fig. 7: Maximum concentration (Cmax) of EFA in plasma at 2 hr**
CONCLUSION
Current HPLC method is a simple, sensitive, reliable and easily applicable for determination and quantification of EFA in rat plasma. This method is suitable for studying the pharmacokinetic parameters of EFA in rat model. The analysis time was 15 min per sample. It was applied to a pharmacokinetic study of EFA. The applicability of method suggests its further application for bioequivalence, bioavailability, drug-drug interaction and clinical studies.

CONFLICT OF INTERESTS
Declared None

REFERENCES