A validated RP-HPLC method for the determination of Eplerenone in human plasma

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ABSTRACT

A highly sensitive and accurate HPLC method was developed for the determination of Eplerenone in human plasma using Hydrochlorothiazide as internal standard. Diethyl ether was used as solvent in the liquid – liquid extraction process. A non-polar Phenomenex Prodigy ODS-2, C18 column (150 X 4.6 mm id) was chosen as the stationary phase and a binary mixture of 20 mM Sodium acetate buffer (pH 4.0 ± 0.05) and methanol in a ratio of 30:70 v/v was used as mobile phase. The drug and the internal standard were eluted under isocratic condition at a flow rate of 1.0 mL/min of the mobile phase and the wavelength of detection is 220 nm (UV – detector). The injection volume is 20 µL and the runtime of the method is 6 minutes. A good linearity was observed for the method over the range of 52.52 to 3089.48 ng/mL. The recovery of Eplerenone was 45.48 % with a standard deviation of 1.62 and recovery of internal standard was 75.32%. The LOD of Eplerenone was 52.52 ng/mL. Matrix effects were not observed.

Key words: Eplerenone, Anti-hypertensive, RP – HPLC, Isocratic elution etc.

INTRODUCTION

Eplerenone (Molecular formula is C24H30O6) (EPL), (Figure 1), (7α, 11 α, 17 α)-9, 11-Epoxy-17-hydroxy-3-oxopregn-4-ene-7, 21-dicarboxylic acid γ-lactone methyl ester), is the first selective aldosterone receptor antagonist (SARA) [1] widely used for the treatment of hypertension and left ventricular dysfunction after acute myocardial infarction [2-8]. A SPE-LC-MS/MS method [9] for the determination of EPL with a lower limit of quantification (LLOQ) of 10 ng/mL in human plasma has been reported. The hydrolyzed metabolite of EPL was an open lactone ring [10, 11]. A number of analytical and bio analytical methods were reported for the determination of Eplerenone alone and in combination with several other drugs [12-28]. The earlier methods on HPLC based bio analytical estimation of Eplerenone resulted in low sensitivity and high noise in the base line. The present investigation aims at developing a more efficient, rapid, sensitive and simple method with suitable chromatographic conditions for the determination of EPL using Hydrochlorothiazide ( Figure-2) as an internal standard(IS) in human plasma. The present method was developed as per FDA guidelines [29].
MATERIALS AND METHODS

Reagents and chemicals
The reference sample of EPLERENONE was gifted by M/s Laurus Pharma Ltd., Hyderabad. HYDROCHLOROTHIAZIDE was gifted by M/s Roorke Drugs Pvt Ltd. The chemicals like methyl-t-butyl ether, diethyl ether and methanol of HPLC Grade and sodium acetate and glacial acetic acid (GR grade) were used.

Chromatographic System
Chromatographic separation was performed on Shimadzu HPLC equipment comprising of binary LC 10AT vp pumps, CTO 10A vp column oven, SIL 10AD vp auto sampler, SPD 10Avp UV-Visible detector. The detector is set at a wavelength 255nm. Chromatographic separation were accomplished using a Phenomenex C18 column (150 X 4.6 mm id, 5 µm, ODS 2). The mobile phase consists of a mixture of 30 parts of 20mM Sodium acetate buffer and 70 parts of methanol operated using a binary HPLC. The mobile phase was pumped isocractically at a flow rate of 1.0ml/min during analysis at ambient temperature. The rinsing solution consists of mixture of 50:50 % v/v of Milli-Q –Water: acetonitrile.

Standard Solutions
The stock solutions of EPL and internal standard were made up in methanol to a concentration of 1.0mg/ml and 1000µg/ml respectively. The stock solutions were stored below 10°C in a refrigerator and these solutions were stable for at least two weeks. Aqueous stock dilution of EPL was prepared in diluent solution (mixture of 50:50%v/v of HPLC grade water: methanol).

Sample Preparation
Aqueous stock dilutions were prepared initially. 0.5 ml of each aqueous stock dilution is transferred into a 10 mL volumetric flask. The final volume is made up with screened drug-free K2EDTA human plasma and mixed gently for 15 minutes to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations are 0.0 (Blank; no EPL added), 52.52, 105.04, 400.49, 800.98, 1201.46, 2059.65, 2631.78 and 3089.48 ng/ml. Each of these standard solutions was distributed in disposable polypropylene micro centrifuge tubes (2.0 ml, eppendorf) in volume of 0.6 ml and stored at -70°C until analysis. Similarly quality control samples were prepared in plasma such that the final concentrations were 54.86, 163.40, 1544.74 and 2860.63 ng/ml and labeled as Lower limit of quantification (LLOQ), Low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively.

Extraction Procedure
Liquid-Liquid Extraction process was involved in the extraction of plasma samples. The stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature for processing. Into a pre-labelled 2.0 mL polypropylene centrifuge tubes an aliquot of 400 µL was then transferred. 50 µL of internal standard dilution (100.00 µg/mL) was then added and vortex-mixed for 30seconds followed by the addition of 1ml of the extraction solvent. The samples were subjected to flash –freeze using a mixture of acetone and dry ice. The resulting
supernatant liquid of 1 mL was then transferred into pre labelled polypropylene tubes and allowed to evaporate to
dryness under nitrogen at constant temperature of 40°C. The dried residue was reconstituted with mobile phase (300
μL) and 20 μL of the sample was then injected into the HPLC column for analysis. Throughout the analysis, the
auto sampler temperature was maintained at 4°C. The column temperature was maintained at ambient temperature.

Selectivity
The selectivity of the method was evaluated by analyzing six independent drug-free K2EDTA 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
EPL in the standard samples. Fresh calibration standards were extracted and assayed as described above on three
different days and in duplicate. Calibration curves for EPL were represented by the plots of the peak-area ratio
(EPL/IS) versus the nominal concentration of the EPL in calibration standards. The regression line was generated
using 1/concentration² factor as the mathematical model of best fit. EPL concentrations in QC samples, recovery,
and stability samples were calculated from the resulting area ratio and the regression equation of the calibration
curve (figure 5).

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. The precision was expressed by coefficient of variation (CV).

Stability Studies
Auto sampler, and freeze–thaw stability of EPL was determined at low, medium and high QC concentrations. To
determine the impact of freeze–thaw cycles on EPL concentration, samples were allowed to undergo 3 freeze
(−70°C) thaw (room temperature) cycles. Following sample treatment/storage conditions, the EPL concentrations
were analyzed in triplicates and compared to the control sample that had been stored at −70°C. Auto sampler
stability of extracted samples was determined by comparing EPL concentration in freshly prepared samples and
samples kept in auto sampler 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 EPL 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 100.00 µg/mL.

RESULTS AND DISCUSSION

Method Development
The HPLC procedure was optimized with a view to develop a sensitive and reproducible method for the
determination of EPL in Human Plasma. Since both EPL and IS are highly non-polar [30] we employed the usage of
liquid-liquid extraction process with diethyl ether. (Solvents such as Ethyl acetate, 100 % t-butyl methyl ether and
combinations of t-butyl methyl ether and Dichloromethane were used for extraction. The recovery of EPL and
internal standard was poor when Ethyl acetate or methyl tertiary butyl ether was used individually). The highest
recovery from the plasma samples is obtained with diethyl ether.

To get a better response the pH of the mobile phase is set to the acidic side. During our observation, a pH value
around 3 resulted in better peak shape for the internal standard while that of the drug is not acceptable. Due to
alkaline hydrolysis of end-capped silica [31, 33] alkaline mobile phase causes deterioration of the bonded phase in
the column. The hydrolysis of end-capped silica in alkaline conditions is usually rapid compared to acid catalyzed
hydrolysis. 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 4.0 ±
0.05 gave maximum response for the analyte at 220 nm. A similar response was observed with the usage of 20mM
sodium acetate buffer. Therefore the final mobile phase consisted of 70: 30 % v/v methanol and 20mM mixed
phosphate buffer.
The run time of analysis is higher when a longer normal phase column (250 X 4.6 mm id) is used. The resolution between the peaks was decreased and peaks shape was not acceptable when the experiment is carried out using shorter column (50 X 4.6 mm id). However better resolution, less tailing and high theoretical plates are obtained with a Phenomenex column C18 150 X 4.6 mm 5 µm column. The flow rate of the method is 1.0 ml/min. The column temperature is 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 70:30 v/v methanol and 20mM Sodium acetate buffer leading to better resolution of the drug and internal standard. Increasing the organic portion of the mobile phase caused IS to elute early. A mobile phase containing aqueous portion greater than 60 % led to very late elution and very poor peak shape for EPL. The peaks were also broad with unacceptable asymmetry factor.

Extraction methods were initially attempted using Protein precipitation technique. Precipitation technique was adopted using acetonitrile and or methanol. Initial experiments of protein precipitation were done using 1:3 ratio of plasma: Organic solvents. The recovery of the EPL is poor while that of the internal standard is relatively unchanged as compared with liquid-liquid extraction.

**Method validation**

**Selectivity**

We have demonstrated the absence of interfering endogenous compounds in blank plasma. Fig. 3 shows the typical chromatograms of blank human plasma sample, a zero blank sample with IS (figure 4) and with a sample containing ULOQ sample extracted using internal standard indicating the specificity of the method (figure 5). The retention times for EPL and IS were 4.43 and 2.38 minutes respectively.

![Representative Chromatograms](image-url)

**Figure 3** A chromatogram of the extracted blank plasma sample
Linearity
A system suitability exercise is performed before the initiation of the validation and the % CV for the retention times of EPL and IS was less than 2%. The results are tabulated in Table 1. The calibration curve accuracy for plasma is presented in Table 2 demonstrating that measured concentration is 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 r² value is depicted in Figure – 6.
Figure 6 Calibration curve for EPLERENONE

Table 1 - System Suitability Study

<table>
<thead>
<tr>
<th></th>
<th>Hydrochlorothiazide (100.00 µg/mL)</th>
<th>Eplerenone (3089.48 ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention Time (min)</td>
<td>Peak Area</td>
</tr>
<tr>
<td>Mean (n = 6)</td>
<td>2.38</td>
<td>78337</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.01</td>
<td>20552.77</td>
</tr>
<tr>
<td>% CV</td>
<td>0.42</td>
<td>2.62</td>
</tr>
</tbody>
</table>

Table 2 - Results of regression analysis of the linearity data

<table>
<thead>
<tr>
<th>Linearity parameters</th>
<th>Mean ± SD (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.00019</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0012</td>
</tr>
<tr>
<td>Correlation coefficient (r²)</td>
<td>0.99617</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 is 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.

Table 3 - Intra and Inter day accuracy and precision of HPLC assay

<table>
<thead>
<tr>
<th>Nominal Concentration (ng/mL)</th>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>54.86</td>
<td>163.40</td>
<td>1544.74</td>
</tr>
<tr>
<td>Mean</td>
<td>56.94</td>
<td>166.44</td>
<td>1565.36</td>
</tr>
<tr>
<td>S.D.</td>
<td>2.02</td>
<td>5.89</td>
<td>65.58</td>
</tr>
<tr>
<td>% CV</td>
<td>3.55</td>
<td>3.54</td>
<td>4.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CV</td>
<td>5.03</td>
<td>8.33</td>
<td>84.21</td>
</tr>
<tr>
<td>S.D.</td>
<td>2.88</td>
<td>7.44</td>
<td>77.61</td>
</tr>
<tr>
<td>% CV</td>
<td>3.33</td>
<td>4.88</td>
<td>3.02</td>
</tr>
</tbody>
</table>

Each mean value is the result of triplicate analysis

Limit of detection and limit of quantification

LOD is defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 52.52 ng/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 (54.86 ng/mL) met the criteria for the LLOQ (Table 3). The method was found to be sensitive for the determination of EPL 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%.

**Carryover test**

Many drugs during analysis have a tendency to get absorbed by reversed phase octa-decyl-based chromatographic packing materials, resulting in the carryover effect. 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 are presented in Table 4. Determination of EPL stability following three freeze–thaw cycles showed that for all QC samples there was a minor change in the EPL concentration.

<table>
<thead>
<tr>
<th>Table 4 - Short Term, long term and Freeze Thaw stability of Eplerenone</th>
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</thead>
<tbody>
<tr>
<td>**Nominal Concentration (ng/mL) **</td>
</tr>
<tr>
<td><strong>Bench Top Stability</strong></td>
</tr>
<tr>
<td>Mean Accuracy (%)</td>
</tr>
<tr>
<td>S.D.</td>
</tr>
<tr>
<td>% CV</td>
</tr>
<tr>
<td><strong>Long-term stability</strong></td>
</tr>
<tr>
<td>Mean Accuracy (%)</td>
</tr>
<tr>
<td>S.D.</td>
</tr>
<tr>
<td>% CV</td>
</tr>
<tr>
<td><strong>Freeze – Thaw stability (3 Cycles)</strong></td>
</tr>
<tr>
<td>Mean Accuracy (%)</td>
</tr>
<tr>
<td>S.D.</td>
</tr>
<tr>
<td>% CV</td>
</tr>
</tbody>
</table>

Each mean value is the result of triplicate analysis

**Recovery**

Recovery was determined by dividing the peak area obtained from analysis of the drug added to the plasma by that observed for the same amount of the drug injected directly into the chromatograph. The mean recovery of EPL from plasma spiked samples at LQC, MQC and HQC levels was 44.2%, 44.9 % and 47.3 % respectively. The overall recovery is 45.48% with a coefficient of variation of 3.57 %. IS recovery at 100.00 µg/mL was 75.32 % with a coefficient of variation of 4.60 %.

**CONCLUSION**

LLE method is devoid of polar interferences thus rendering the sample clean for final analysis. The noise is usually absent or at minimum as compared to precipitation or SPE techniques. This assay requires only a small volume of plasma (600 µL). There is 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 54.86 ng/mL, acceptable recovery, reliability, specificity and excellent efficiency with a total running time of 6.0 min per sample, which is important for large batches of samples. Thus this method can be suitable for pharmacokinetic, bioavailability or bioequivalence studies of Eplerenone in human subjects. This method has been successfully applied to analyze Eplerenone concentrations in human plasma.

**Acknowledgements**

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