SIMULTANEOUS DETERMINATION OF LOSARTAN AND ATORVASTATIN IN RAT PLASMA AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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ABSTRACT

A simple, rapid, economic and precise RP-HPLC method for simultaneous analysis of Losartan (LOS) and Atorvastatin (ATR) in rat plasma has been developed and validated. Valsartan (VAL) was used as an internal standard. Extraction of the drug from the plasma was carried out by precipitation method. Analysis was performed using Kromasil C18 column (250 × 4.6 mm; 5µ) with mobile phase consisting of acetonitrile and 0.02 M sodium dihydrogen phosphate (containing 0.1% heptanesulphonic acid, pH adjusted to 3.0 with ortho phosphoric acid) in the ratio of 55:45 (v/v) at a flow rate of 0.8 mL min⁻¹. Chromatographic separation was monitored at 235 nm. The method was linear over a range of 10-1000 ng mL⁻¹ for both the drugs. Limits of detection and Limits of quantification were 2.9 ng mL⁻¹ and 8.8 ng mL⁻¹ for LOS and 3.2 ng mL⁻¹ and 9.8 ng mL⁻¹ ATR respectively. The method was validated for accuracy, precision, specificity, recovery and stability. The applicability of this method in pharmacokinetic studies was demonstrated.

Keywords: Losartan, Atorvastatin, Valsartan, simultaneous determination, Pharmacokinetic studies

INTRODUCTION

Losartan (LOS), chemically described as 2-butyl-4-chloro-1-[(p-(1Htetrazol-5-ylphenyl) benzyl] imidazole-5-methanol, is an effective nonpeptide angiotensin II receptor antagonist [1]. Losartan and its principal active metabolite block the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor [2, 3]. The individual determination of losartan has been carried out in tablets by HPLC, capillary electrophoresis and super-critical fluid chromatography [4], in bulk and solid dosage forms by colorimetric method [5]. Losartan was estimated simultaneously with its degradants in stressed tablets by LC-MS/MS [6] and HPTLC [7]. The estimation of losartan in combination with amlodipine besylate, ramipril and atenolol by RP-HPLC in tablet dosage form were reported [8-10]. Losartan and its active metabolite were estimated in biological fluids by HPLC [11-15]. Pharmacokinetics and bioequivalence study in Chinese male volunteers was reported for two losartan potassium tablets [16]. Losartan in combination with hydrochlorothiazide in human plasma by LC-MS/MS was reported [17, 18].

Atorvastatin (ATR), chemically known as 1H-pyrorole-1-heptanoic acid, [R-(R*, R*)]-2-(4-fluorophenyl)-β, d-dihydroxy-5- (1-methylethyl)-3-phenyl-4- [(phenyl amino) carbonyl], is an antihyperlipidimic drug that inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis. Atorvastatin is administered as the calcium salt of the active hydroxyl acid and is used at a dose between 10 and 80 mg per day to reduce the raised lipid levels in patients with primary hyperlipidemia (familial and non familial) or combined hyperlipidemia [19-23]. Several procedures of chromatographic techniques such as LC/MS/MS, microbore LC/ESI-MS/MS, HPLC with electro spray tandem mass spectrometry and LC methods with UV detector have been tested for the determination of...
atorvastatin in biological fluids and pharmaceutical dosage forms. The estimation of atorvastatin in combination with amlopidine, ezetimibe, ramipril and aspirin simultaneously by RP-HPLC in tablets were reported. The simultaneous estimation of atorvastatin in combination with rosuvastatin and lercanidipine and also simultaneous estimation of atorvastatin with amlopidine, ramipril and benazepril in biological matrix were reported. The combination of losartan with atorvastatin is used in the treatment of coexisting hypertension and hyperlipidemia in adult patients. The methods that are reported for both losartan and atorvastatin in biological matrices involve complex procedures and are time consuming. The reported methods are not available for most laboratories because of their specialty requirement and financial reasons. However, so far, no single method was reported for the simultaneous estimation of LOS and ATR in rat plasma by RP-HPLC. Therefore, the aim of this study was to develop and validate a new simple, rapid and economic method for the simultaneous determination of these drugs. The developed bioanalytical method has been validated according to ICH guidelines and successfully applied to pharmacokinetic study.

EXPERIMENTAL

Materials and Reagents: Losartan, atorvastatin and valsartan reference standards were kind gift of Aurobindo Pharma Ltd., Hyderabad, India. The purity of all chemicals was above 98 %. The structures are shown in Fig. I. HPLC grade acetonitrile and methanol was purchased from Merck, India. Sodium dihydrogen phosphate (AR grade) and ortho-Phosphoric acid were purchased from S.D.Fine-Chem Ltd., India. Heptane-1- sulfonyc acid sodium salt was purchased from Merck, India. Deionized water was purified using Millipore water purification system (Barnstead, USA).

Instrumentation and chromatographic conditions: The Shimadzu HPLC system consisted of two pumps (LC-10 Ai, Japan), a system controller (SCL-10 AVP), an auto injector (SIL-10 ADVP) and a diode array detector (SPD-M10 AVP). The data was analyzed and processed by using class LC-10 software (Version 1.6). Chromatographic separation was achieved using a Kromasil ODS analytical column (250mm × 4.6mm, 5 µm) with a mobile phase consisting of acetonitrile and 0.02 M Sodium dihydrogen phosphate containing 0.1% heptanesulphonic acid (HSA), pH adjusted to 3.0 with ortho phosphoric acid (OPA) in the ratio of 55:45 (v/v). The mobile phase was filtered, degassed and pumped at a flow rate of 0.8 ml/min. The chromatographic separation was monitored at 235 nm.

Preparation of standard stock and working solutions: Primary standard stock solutions of LOS and ATR were prepared separately by dissolving accurately weighing 10mg of drug in 10ml of methanol to produce a concentration of 1.0 mg/ml. Further concentrations of working stock solutions were prepared as a mixture in methanol from standard stock solutions of LOS and ATR for calibration curve and quality control (QC) samples. Valsartan (VAL) was used as an internal standard (IS) at a working concentration of 20 µg mL⁻¹.

Extraction Procedure: Extraction of the drug from the plasma was carried out by precipitation method. An aliquot of 100µl of drug-free plasma spiked with 10µl of different working standards of LOS and ATR mixture and IS, and were vortexed for 30 s. A volume 200 µL of methanol was added as a precipitating agent, vortexed for 30 s and then centrifuged for 5 min at 5000 rpm. The supernatant solution was separated and filtered through 0.45 µ membrane filter and 50µl of the solution was injected.

Preparation of calibration standard and quality control (QC) samples: Working standards of 1, 2, 5,10,20,50 and 100 µg mL⁻¹ were prepared from the working stock standard solution. A volume of 10µl of each working standard was spiked to 100µl of plasma separately to get the concentrations of 10, 20, 50,100, 200, 500 and 1000 ng mL⁻¹ for each drug. A volume of 10 µL of IS was then added from 20 µg mL⁻¹ working standard concentration. The sample extraction was followed as mentioned above. Linearity of the two drugs was evaluated over the concentration range of 10 to 1000 ng mL⁻¹ using least square linear regression analysis and regression equations were used for determination of concentrations. The QC samples were prepared in same way as standard solutions to yield low, medium and high concentrations (30, 450 and 750 ng mL⁻¹).
concentrations and QC concentrations were calculated by using linear regression equation, \( y = mx + c \), where \( x \) is concentration of drug, \( y \) is ratio of peak area of drug and IS, \( m \) is slope of calibration curve and \( c \) is intercept of calibration curve. Accuracy was determined as the absolute value of the ratio of the back-calculated mean values to their respective nominal values. Precision was measured by the percentage relative standard deviation (\%RSD) over the linearity and QC concentrations. The system precision was determined by injecting six replicates of standard preparation of 750 ng mL\(^{-1}\). The autosampler stability was evaluated by keeping plasma samples of binary mixture (LOS and ATR) in autosampler for 12 h and injecting to HPLC. The short-term stability was determined by storing QC samples at \(-20^\circ C\) for 24 h. Freeze thaw stability of QC samples was analyzed after three freeze-thaw cycles by freezing at \(-20^\circ C\) for 24 h and then thawing at room temperature for 24 hr.

**Pharmacokinetic Study:** Five healthy male wistar rats were taken with average weight of 190 g \( \pm \) 10 g were randomly selected in the pharmacokinetic study. After overnight fasting of animals, LOS and ATR mixture was prepared as a suspension and administered orally at a dose of 5 mg kg\(^{-1}\) and 1 mg kg\(^{-1}\) respectively. A volume of 0.3 mL of blood was collected in EDTA coated glass tubes at time intervals of 0.083, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 24 h after drug administration. Blood samples were centrifuged at 5000 rpm for 10 min and the plasma was separated and stored at \(-80^\circ C\) until analysis. Pharmacokinetic parameters were estimated using non-compartmental analysis with PK-ANALYST software.

**RESULTS AND DISCUSSION**

The reverse phase LC method was developed to provide a specific procedure for the rapid separation of binary mixture containing LOS and ATR. During the preliminary investigations the chromatographic separation of LOS and ATR were done using different columns, different mobile phases, varying contents of organic modifier and changing flow rate. Separation of these two drugs was attempted using deionised water, sodium periodate and phosphate buffer (0.02M NaH\(_2\)PO\(_4\)), all at pH 3.0, with different organic solvents such as methanol and acetonitrile. With deionised water and sodium periodate at pH 3.0, there was interference between the peaks of LOS and IS. The sensitivity was less when methanol was used in the composition of mobile phase. When phosphate buffer (pH 3.0) was used individually with acetonitrile, the tailing was more. To reduce the tailing an ion-pairing agent, heptane sulfonic acid (HSA) was used. An optimized concentration of 0.1% HSA was used to reduce tailing. Different columns such as RP-C\(_{18}\), RP-C\(_{16}\) and RP-NH\(_2\) were used for the selection of appropriate column. Amino column was not eluting the drugs and RP-C\(_{18}\) column shows less sensitivity and resolution compared to RP-C\(_{16}\). The composition of mobile phase mixture was altered to optimize the ratios of two mobile phase components using RP-C\(_{18}\) column. With the change in the acetonitrile composition, there was interference between LOS, ATR and IS a peak and with the change in buffer composition, a broad peak of ATR was obtained. At a composition of 55% acetonitrile and 45% buffer ratio has shown a good resolution with sharp peaks. Decrease in flow rate to 0.7 mL min\(^{-1}\) increased the tailing where as increase in flow rate to 0.9 mL min\(^{-1}\) merged the peaks of IS and ATR. Effect of pH on chromatographic conditions was studied to determine the optimum pH for the separation of drugs with good resolution and high sensitivity. The increase in pH from 3.0 resulted in decreased peak area and lower resolution. The optimum wavelength for the estimation of drugs was selected based upon the maximum area using a shimadzu SPD-M10 AVP diode array detector. On the basis of the above results, appropriate LC conditions for separation of these drugs were reverse phase Kromasil RP-C\(_{18}\) (250 mm \( \times \) 4.6 mm, 5 \( \mu \)m) with mobile phase of acetonitrile and phosphate buffer (0.02M NaH\(_2\)PO\(_4\)) containing 0.1% HSA, pH adjusted to 3.0 with ortho-phosphoric acid in the ratio of 55:45 v/v. The optimum wavelength for detection of LOS, ATR and IS was 235 nm at which better detector responses were obtained.

To reduce the endogenous-related substances in plasma the method of precipitation of protein is usually performed. Acetonitrile and methanol were used for the selection of deproteinizing agent. When the drugs in plasma were extracted with acetonitrile the selectivity was less with more peak width. Extraction with methanol has increased sensitivity with sharp peaks. The results showed that the best protein precipitation was achieved at a ratio of 1:2 (plasma: methanol).

The developed LC method showed a linearity form 10 to 1000 ng mL\(^{-1}\) with desirable correlation coefficient of more than 0.999 for both LOS and ATR. Regression equations for LOS and ATR were \( y = 0.0058x + 0.0234 \) and \( y = 0.0036x + 0.031 \) respectively.

In order to demonstrate the validity and suitability of the proposed method, intra-day and inter-day
accuracy and precision were performed for QC samples. Intra-day and inter-day accuracy of the method was found to be 93.82-106.77 % and 92.71-109.45 % for LOS and ATR respectively. Intra-day and inter-day precision of the method was found to be 0.119-2.271 % and 0.46-2.41% for LOS and ATR respectively. The results indicated that the method is reproducible with acceptable accuracy and precision. The results of accuracy and precision are enumerated in Table I.

The LLOQ of the assay for both LOS and ATR was 10 ng mL⁻¹. The reproducibility of LLOQ was determined by examining five LLOQ samples of both the drugs independent from calibration curve. The accuracy and precision were 98.38 ± 7.47 % and 96.56 ± 9.32 % for LOS and ATR respectively. The typical chromatograms of blank plasma and LLOQ sample are shown in Fig. II. The LOD of LOS and ATR were 2.9 and 3.2 ng mL⁻¹ respectively. The mean extraction efficiencies were all over 70 % with RSD’s less than 10 %.

System suitability parameters such as retention time, tailing factor, resolution and theoretical plates were taken into consideration. The approximate retention times of LOS, IS and ATR were found to be 5.5, 8.0 and 11.7 respectively. The tailing factor of LOS and ATR were 1.30 and 1.12 respectively. The theoretical plate counts of LOS and ATR were more than 5000, which was desirable. The resolution factor between LOS and IS was 4.54 and between IS and ATR was 8.23. All the values for the system suitability parameters were within acceptable range.

Stability of both the drugs in plasma was performed for three QC concentrations. After storage of plasma samples at −20°C for 24 h and three freeze-thaw cycles, the samples were found to be stable after analysis. The drugs present in plasma were remained unchanged when kept in auto sampler for 24 h. The recovery of both LOS and ATR in all stability studies were found to be more than 90 % with less than ± 15 % RSD which are shown in Table II.

The established method was applied to analysis of plasma samples after an oral administration of 5 mg kg⁻¹ and 1 mg kg⁻¹ of LOS and ATR simultaneously. The mean plasma concentration - time profile of LOS and ATR are shown in Fig. III. A non-compartmental model was used to estimate the pharmacokinetic parameters of both LOS and ATR in rat plasma, which are shown in Table III. After administration of both the drugs, peak plasma concentrations (Cₘₙ₅) were reached at 0.60 ± 0.22 h and 1.20 ± 0.27 h (Tₘ₅₅) with an elimination half-life (t₁/₂) of 2.16 ± 0.18 h and 4.83 ± 0.79 h for LOS and ATR respectively. Thus the developed method was successfully applied for pharmacokinetic study in rats after administration of LOS and ATR in combination.

**CONCLUSION**

A method for simple, rapid, specific, economic and simultaneous determination of LOS and ATR in rat plasma has been developed using HPLC with UV detection. The developed method involves simple sample preparation procedure and one-step precipitation extraction procedure which reduces time consumption. The sensitivity of this method is high with low quantitation limits. The method shows good recovery, accuracy and precision, indicating that it is valid enough to meet the requirement for pharmacokinetic study of LOS and ATR in combination. Finally, the developed method is not only suitable for assessing the pharmacokinetics in rats but also applicable for clinical pharmacokinetics.

**ACKNOWLEDGEMENT**

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**Table I: Intra-day and inter-day precision and accuracy of LOS and ATR in rat plasma** (n=3)

<table>
<thead>
<tr>
<th>Spiked (ng/ml)</th>
<th>Intra-day</th>
<th>Inter-day</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOS</td>
<td>ATR</td>
<td>LOS</td>
<td>ATR</td>
</tr>
<tr>
<td></td>
<td>Accuracy (%)</td>
<td>%RSD</td>
<td>Accuracy (%)</td>
<td>%RSD</td>
</tr>
<tr>
<td>30</td>
<td>98.65</td>
<td>7.82</td>
<td>97.55</td>
<td>8.27</td>
</tr>
<tr>
<td>450</td>
<td>97.71</td>
<td>4.61</td>
<td>95.22</td>
<td>6.12</td>
</tr>
<tr>
<td>750</td>
<td>99.75</td>
<td>2.86</td>
<td>96.77</td>
<td>3.99</td>
</tr>
</tbody>
</table>

*a. Results of the five replicates of LOS and ATR binary mixture obtained on four occasions.

*b. Accuracy (%) = (mean of measured concentration/spiked concentration) x100.
Fig. I. Structures of losartan, atorvastatin and internal standard valsartan (IS).
Fig. II. Chromatograms of LOS, VAL and ATR from the study

a. Blank Plasma

b. Chromatogram for LOS and ATR (750 ng/ml) with IS
c. LLOQ concentration of LOS and ATR with IS in rat plasma

![Chromatogram of LOS and ATR with IS from pharmacokinetic study in rats (1 h)](Image)

**Data:** ALB01.K01  **Chrom:** ALB01.R01  **Method:** ALB01.H01  **Lib:** TEST.SLB

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d. Chromatogram of LOS and ATR with IS from pharmacokinetic study in rats (1 h)

![Chromatogram of LOS and ATR with IS from pharmacokinetic study in rats (1 h)](Image)

**Data:** ALB02.K01  **Chrom:** ALB02.R01  **Method:** ALB02.H01  **Lib:** TEST.SLB

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Fig. III. Mean plasma-time profile of a. Losartan and b. Atorvastatin in rat plasma after oral administration. Each point represents the mean ± SD (n = 5).
Fig. IV. Calibration curve of a. Losartan and b. Atorvastatin (n=3)

a

\[ y = 0.0036x + 0.031 \]
\[ R^2 = 0.9999 \]

b

\[ y = 0.0036x + 0.031 \]
\[ R^2 = 0.9999 \]
**Table II: Stability of LOS and ATR in rat plasma (n=3)**

<table>
<thead>
<tr>
<th>Spk Conc (ng/mL)</th>
<th>LOS</th>
<th></th>
<th></th>
<th>ATR</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obt Conc&lt;sup&gt;a&lt;/sup&gt; (ng/mL)</td>
<td>% RSD</td>
<td>Obt Conc&lt;sup&gt;a&lt;/sup&gt; (ng/mL)</td>
<td>% RSD</td>
<td>Obt Conc&lt;sup&gt;a&lt;/sup&gt; (ng/mL)</td>
<td>% RSD</td>
</tr>
<tr>
<td>30</td>
<td>30.31±3.33</td>
<td>1.09</td>
<td>32.57±1.11</td>
<td>1.34</td>
<td>29.63±2.16</td>
<td>1.54</td>
</tr>
<tr>
<td>450</td>
<td>451.77±5.69</td>
<td>2.51</td>
<td>443.41±3.30</td>
<td>1.74</td>
<td>452.99±7.03</td>
<td>1.49</td>
</tr>
<tr>
<td>750</td>
<td>745.59±8.61</td>
<td>1.17</td>
<td>748.97±2.33</td>
<td>2.82</td>
<td>746.71±1.39</td>
<td>1.19</td>
</tr>
</tbody>
</table>

<sup>a</sup> Obtained concentration expressed as Mean ± SD of the five replicates of LOS and ATR binary mixture

**Table III. Pharmacokinetic parameters of LOS and ATR in rat plasma (n = 5) after oral administration (mean ± SD)**

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>AUC&lt;sub&gt;0-4&lt;/sub&gt; (ng mL&lt;sup&gt;-1&lt;/sup&gt; h&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (ng mL&lt;sup&gt;-1&lt;/sup&gt; h&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOS</td>
<td>0.6 ± 0.22</td>
<td>825.88 ± 60.32</td>
<td>1800.77 ± 145.76</td>
<td>1888.95 ± 155.24</td>
<td>2.16 ± 0.18</td>
</tr>
<tr>
<td>ATR</td>
<td>1.2 ± 0.27</td>
<td>364.50 ± 24.52</td>
<td>1067.26 ± 146.16</td>
<td>1177.20 ± 235.48</td>
<td>4.83 ± 0.79</td>
</tr>
</tbody>
</table>

C<sub>max</sub>, the maximum plasma concentration; T<sub>max</sub>, the time to reach C<sub>max</sub>; t<sub>1/2</sub>, elimination half-life; AUC<sub>0-4</sub>, the area under the plasma concentration-time curve from time zero to the last sampling time; AUC<sub>0-∞</sub>, the area under the plasma concentration-time curve from time zero to infinity.

**REFERENCES**