Simultaneous Estimation of Atorvastatin Calcium and Aspirin in Combined Dosage Form by Liquid Chromatographic Method

D.A. SHAH*, K.K. BHATT, AND S.L. BALDANIA

Indukaka Ipcowala College of Pharmacy, P.B. No. 53, New Vallabh Vidyanagar - 388 121, Dist. Anand, Gujarat, India
E-mail: dimalgroup@yahoo.com

Summary. An isocratic reversed-phase liquid chromatographic assay method was developed for the quantitative determination of atorvastatin and aspirin (ASP) in combined dosage form. A Phenomenex Gemini C-18, 5-μm column with mobile phase containing 0.02 M potassium dihydrogen phosphate–acetonitrile–methanol (30:30:40, v/v/v) adjusted to pH 3 using o-phosphoric acid was used. The flow rate was 1.0 mL min\(^{-1}\) and effluents were monitored at 240 nm. The retention times (RTs) of atorvastatin calcium (ATV) and ASP were 10.5 and 3.8 min, respectively. ATV and ASP stock solutions were subjected to acid and alkali hydrolysis, chemical oxidation, and dry heat degradation. The degraded product peaks were well resolved from the pure drug peak with significant difference in their RT values. Stressed samples were assayed using developed LC method. The proposed method was validated with respect to linearity, accuracy, precision, and robustness. The method was successfully applied to the estimation of ATV and ASP in combined capsule dosage forms.

Key Words: aspirin, atorvastatin calcium, forced degradation, reversed-phase liquid chromatography, validation

Introduction

Atorvastatin calcium (ATV) is chemically \([\text{R-(R*, R*)}-2-(4-	ext{fluorophenyl})-\beta,\delta\text{-dihydroxy}-5-(1-	ext{methylethyl})-3-	ext{phenyl}-4-[(\text{phenyl amino) carbonyl}]-1\text{H-pyrrole-1-heptanoic acid}, calcium salt trihydrate. ATV is an inhibitor of 3-hydroxy-3 methyl glutaryl coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate limiting step in cholesterol biosynthesis [1, 2]. Aspirin (ASP) is chemically 2-acetoxy benzoic acid and used as an analgesic, antipyretic, anti-inflammatory, and antithrombic agent. Combination dosage form of ATV and ASP is used in the treatment of dyslipidemic patient with coronary heart diseases. A clinical study reported that the combination therapy proved beneficial for the patient after coronary artery bypass grafting compared to ASP monotherapy [3]. The study also reported that a combination therapy of ASP and ATV has an additive effect in reducing cardiovascular events in dyslipidemic patients with coronary heart disease [4].
A literature survey regarding quantitative analysis of these drugs revealed that attempts have been made to develop analytical methods for the estimation of ATV using extractive spectrophotometry [5], HPLC [6–10], GC–MS [11], LC–electrospray tandem mass spectrometry [12–14], and HPTLC [15] methods. ASP is official in USP and BP, and titrimetric [16] and HPLC [17] methods have been reported for its estimation. For the estimation of atorvastatin and ASP in combination, RP-HPLC [18] method has been reported, which has been developed using mobile phase 50 mM KH₂PO₄–acetonitrile–methanol (30:50:20, v/v/v). The reported method is linear in the concentration range of 11.2–56 μg mL⁻¹ and 75–375 μg mL⁻¹ for ATV and ASP, respectively.

The International Conference on Harmonization (ICH) guidelines [19] require the implementation of stress testing procedures for the identification of degradation products that potentially occur in drug substances, which can help to understand the possible degradation pathway for the drugs. No stability indicating method was reported for the estimation of ATV and ASP; so, attempt was made to develop a stability indicating LC method for the estimation of ATV and ASP in combined dosage form. This study reports a forced degradation study of atorvastatin and ASP under a variety of conditions such as acid and alkali hydrolysis, oxidative stress hydrolysis, and dry heat degradation.

**Experimental**

**Apparatus**

The LC system consisted of Shimadzu HPLC model (VP series) containing LC-10AT (VP series) pump, variable wavelength programmable UV/VIS detector SPD-10AVP and Rheodyne injector (7725i) with 20-μL fixed loop. The analytes were monitored at 240 nm. Chromatographic analysis was performed on Phenomenex Gemini C-18 column having 250 × 4.6 mm i.d. and 5-μm particle size. All the drugs and chemicals were weighed on Shimadzu electronic balance (AX 200, Shimadzu Corp., Japan).

**Chemicals and Reagents**

Analytically pure ATV and ASP were obtained as gift samples from Blue Cross Laboratory Limited, Mumbai, India and Mercury Laboratories Limited, Vadodara, India, respectively. HPLC grade acetonitrile, methanol, and water were obtained from E. Merck Ltd., Mumbai, India, while analytical reagent grade o-phosphoric acid and potassium dihydrogen phosphate
were obtained from S. D. Fine Chemicals, Mumbai, India. Capsule formulations A (Ato Plus, Triton Pharma. Ltd., Mumbai, India) and B (Atchol ASP, Aristo Pharma. Ltd., India) containing labeled amounts of 10 mg of atorvastatin and 75 mg of ASP, respectively, were procured from the local market.

**Chromatographic Conditions**

A Phenomenex Gemini C-18 (250 × 4.6 mm i.d) chromatographic column equilibrated with mobile phase 0.02 M potassium dihydrogen phosphate–acetonitrile–methanol (30:30:40, v/v/v) adjusted to pH 3 with o-phosphoric acid (1 M) was used. Mobile phase flow rate was maintained at 1 mL min⁻¹ and effluents were monitored at 240 nm. The sample was injected using a 20-μL fixed loop, and the total run time was 15 min.

**Preparation of Standard Stock Solutions**

ATV and ASP were weighed (25 mg each) and transferred to two separate 25-mL volumetric flasks, and dissolved in a few milliliters of the mobile phase. Volumes were made up to the mark with mobile phase to yield a solution containing 1000 μg mL⁻¹ of ATV and ASP, respectively. An aliquot portion of the stock solution of ATV was appropriately diluted with the mobile phase to obtain a working standard of 100 μg mL⁻¹ of ATV.

**Method Validation**

The method was validated for accuracy, precision, linearity, specificity, detection limit, quantification limit, and robustness.

**Linearity**

Appropriate aliquots of ATV and ASP working standard solutions were taken in different 10-mL volumetric flasks and diluted up to the mark with the mobile phase to obtain final concentrations of 0.1, 0.5, 1, 4, 8, 12, and 20 μg mL⁻¹ of ATV and 0.5, 1, 5, 10, 40, 80, and 120 μg mL⁻¹ of ASP, respectively. The solutions were injected using a 20-μL fixed loop system, and chromatograms were recorded. Calibration curves were constructed by plotting average peak area versus concentrations, and regression equations were computed for both drugs.
Precision

The intra-day and inter-day precision studies were carried out by estimating the corresponding responses three times on the same day and on three different days for three different concentrations of ATV (0.5, 4, and 20 μg mL\(^{-1}\)) and ASP (5, 40, and 120 μg mL\(^{-1}\)), and the results are reported in terms of relative standard deviation (RSD). The instrumental precision studies were carried out by estimating the response of three different concentrations of ATV (0.5, 4, and 20 μg mL\(^{-1}\)) and ASP (10, 80, and 120 μg mL\(^{-1}\)) six times, and the results are reported in terms of RSD.

Accuracy

The accuracy of the method was determined by calculating recoveries of ATV and ASP by the method of standard additions. Known amounts of ATV (0, 0.5, 4, and 12 μg mL\(^{-1}\)) and ASP (0, 1, 40, and 80 μg mL\(^{-1}\)) were added to a pre-quantified sample solution, and the amounts of ATV and ASP were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

Detection Limit and Quantification Limit

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using the following equations as per ICH guidelines.

\[
LOD = 3.3 \times \frac{\sigma}{S} \quad \text{and} \quad LOQ = 10 \times \frac{\sigma}{S},
\]

where \(\sigma\) is the standard deviation of \(y\)-intercepts of regression lines and \(S\) is the slope of the calibration curve.

Solution Stability

Stabilities of sample solutions were studied at 25 ± 2 °C for 24 h.

Robustness

Robustness of the method was studied by deliberately changing the experimental conditions such as flow rate and percentage of organic phase.
Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and degradation products. Commonly used excipients (starch, microcrystalline cellulose, and magnesium stearate) were spiked into a pre-weighed quantity of drugs. The chromatogram was taken by appropriate dilutions, and the quantities of drugs were determined.

Specificity was also studied by performing a forced degradation study using acid and alkali hydrolysis, chemical oxidation, and dry heat degradation studies, and the interference of the degradation products was investigated. ASP and ATV were weighed (25 mg each) and transferred to two separate 25-mL volumetric flasks, dissolved in a few milliliters of methanol, and diluted up to the mark with methanol. These stock solutions were used for forced degradation studies.

Alkali hydrolysis. In two different 25-mL volumetric flasks, 1 mL stock solutions of ATV and ASP, respectively, were taken, and 5 mL of 0.1 N NaOH were added. In another volumetric flask, 1 mL stock solutions of both drugs were taken to obtain a mixture, and 5 mL of 0.1 N NaOH were added to perform base hydrolysis. All flasks were heated at 80 °C for 1 h and allowed to cool to room temperature. Solutions were neutralized with 0.1 N HCl and diluted up to the mark with the mobile phase. Appropriate aliquots were taken from the above solutions and diluted with the mobile phase to obtain a final concentration of 6 μg mL⁻¹ of ATV and ASP separately and in the mixture.

Acid hydrolysis. In two different 25-mL volumetric flasks, 1 mL stock solutions of ATV and ASP were taken and 5 mL of 0.1 N HCl were added. In another volumetric flask, 1 mL stock solutions of both drugs were taken to obtain a mixture, and 5 mL of 0.1 N HCl were added to perform acid hydrolysis. All flasks were heated at 80 °C for 1 h and allowed to cool to room temperature. Solutions were neutralized with 0.1 N NaOH and diluted up to the mark with the mobile phase. Appropriate aliquots were taken from the above solutions and diluted with the mobile phase to obtain a final concentration of 6 μg mL⁻¹ of ATV and ASP separately and in the mixture.

Oxidative stress degradation. To perform oxidative stress degradation, appropriate aliquots of stock solutions of ATV and ASP were taken in two different 25 mL volumetric flasks and 5 mL of 3% hydrogen peroxide were added. Similarly, appropriate aliquots of stock solutions of ATV and ASP were taken in the same 25 mL volumetric flask and 5 mL of 3% hydrogen peroxide were added. All the mixtures were heated in a water bath at 80 °C for 1 h, allowed to cool to room temperature, and diluted up to the mark with the mobile phase. Appropriate aliquots were taken from above solu-
tions and diluted with the mobile phase to obtain a final concentration of 6 μg mL\(^{-1}\) of ATV and ASP separately and in mixture.

Dry heat degradation. Analytically pure samples of ATV and ASP were exposed in oven at 80 °C for 1 h. The solids were allowed to cool and 25 mg each of ATV and ASP were weighed, transferred to two separate volumetric flasks (25 mL), and dissolved in a few milliliters of methanol. Volumes were made up to the mark with the methanol. Solutions were further diluted with the mobile phase and appropriate aliquots were taken in a 10-mL volumetric flask to obtain a final concentration of 4 μg mL\(^{-1}\) of ATV and 40 μg mL\(^{-1}\) of ASP.

All the reaction solutions were injected in the liquid chromatographic system and chromatograms were recorded.

**Analysis of Marketed Formulations**

The contents of 20 capsules were taken and weighed. An amount of powder equivalent to atorvastatin 10 mg (and 75 mg ASP) was accurately weighed and transferred to a 50-mL volumetric flask, and 20 mL of the mobile phase were added to the same; the flask was sonicated for 5 min. The solution was filtered using Whatman filter paper (no. 1) in another 50-mL volumetric flask and the volume was made up to the mark with the mobile phase. Appropriate volume of the aliquot was transferred to a 10-mL volumetric flask, and the volume was made up to the mark with the mobile phase to obtain a concentration of 10 μg mL\(^{-1}\) of atorvastatin and 75 μg mL\(^{-1}\) of ASP. The solution was sonicated for 10 min. The solution was injected at the above-mentioned chromatographic conditions and peak areas were measured. The quantification was carried out by keeping these values to the straight-line equation of calibration curve.

**Results and Discussion**

**Optimization of Mobile Phase**

The objective of method development was to resolve chromatographic peaks for active drug ingredients and degradation products produced under stressed conditions with less asymmetric factor.

Various mixtures containing aqueous buffer, methanol, and acetonitrile were tried as mobile phases in the initial stage of method development. Several mixtures, 0.02 M KH\(_2\)PO\(_4\)–acetonitrile–methanol (30:10:60, \(v/v/v\)), 0.02 M KH\(_2\)PO\(_4\)–acetonitrile–methanol (20:40:40, \(v/v/v\)), and 0.02 M KH\(_2\)PO\(_4\)–
methanol (20:80, v/v), were tried as mobile phases, but satisfactory resolutions of the drug and degradation peaks were not achieved.

The mobile phase 0.02 M KH$_2$PO$_4$–acetonitrile–methanol (30:30:40, v/v/v), with total pH adjusted to 3 using o-phosphoric acid, was found to be satisfactory and gave two symmetric and well-resolved peaks for ATV and ASP. The retention times (RTs) for ATV and ASP were 10.5 min and 3.8 min, respectively (Fig. 1). The resolution between ATV and ASP was found to be 23, which indicates good separation of both compounds. The asymmetric factors for ATV and ASP were 1.2 and 1.5, respectively. The mobile-phase flow rate was maintained at 1 mL min$^{-1}$. Overlaid UV spectra of both drugs showed that ATV and ASP absorbed appreciably at 240 nm; so, detection was carried out at 240 nm.

![Fig. 1. Liquid chromatogram showing well-resolved peaks of atorvastatin (ATV) and aspirin (ASP)](image)

**Method Validation**

**Linearity**

The calibration curve for ATV was found to be linear in the range of 0.1–20 μg mL$^{-1}$ with a correlation coefficient of 0.9998. The calibration curve for ASP was found to be linear in the range of 0.5–120 μg mL$^{-1}$ with a correlation coefficient of 0.9992. The standard deviation values of slopes for ATV and ASP were 0.41 and 0.26, respectively, which indicated a strong correlation between peak area and concentration. The regression analysis of calibration curves is reported in Table I.
Table I. Regression analysis of calibration curves

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ATV</th>
<th>ASP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>0.1–20 μg mL(^{-1})</td>
<td>0.5–120 μg mL(^{-1})</td>
</tr>
<tr>
<td>Slope</td>
<td>28.69</td>
<td>14.23</td>
</tr>
<tr>
<td>SD of slope</td>
<td>0.41</td>
<td>0.26</td>
</tr>
<tr>
<td>Intercept</td>
<td>−1.60</td>
<td>3.01</td>
</tr>
<tr>
<td>SD of intercept</td>
<td>0.34</td>
<td>0.52</td>
</tr>
<tr>
<td>Corr. Coefficient</td>
<td>0.9998</td>
<td>0.9992</td>
</tr>
</tbody>
</table>

**Precision**

Instrument precision was determined by performing injection repeatability test and the RSD values for ASP and ATV were found to be 0.18–0.63% and 0.61–0.79%, respectively. The intra-day and inter-day precision studies were carried out and the results are reported in Table II. The low RSD values indicate that the method is precise.

Table II. Summary of validation and system suitability parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ATV</th>
<th>ASP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>10.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Asymmetry</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Resolution</td>
<td>23</td>
<td>–</td>
</tr>
<tr>
<td>Theoretical Plates</td>
<td>11,585</td>
<td>8326</td>
</tr>
<tr>
<td>Detection limit (μg mL(^{-1}))</td>
<td>0.05</td>
<td>0.2</td>
</tr>
<tr>
<td>Quantitation limit (μg mL(^{-1}))</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>98.10–100.17</td>
<td>97.06–100.98</td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-day (n = 3)</td>
<td>0.53–1.74</td>
<td>0.57–1.40</td>
</tr>
<tr>
<td>Inter-day (n = 3)</td>
<td>0.90–1.96</td>
<td>0.45–1.88</td>
</tr>
<tr>
<td>Instrument precision (%RSD)</td>
<td>0.61–0.79</td>
<td>0.18–0.63</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>98.22–100.42</td>
<td>97.45–99.56</td>
</tr>
</tbody>
</table>
Accuracy

The accuracy of the method was determined by calculating recoveries of ATV and ASP by the method of standard addition. The recoveries were found to be 98.10–100.17% and 97.06–100.98% for ATV and ASP, respectively (Table III). The high values indicate that the method is accurate.

Table III. Accuracy study of the proposed method

<table>
<thead>
<tr>
<th>Amount of sample taken (μg mL⁻¹)</th>
<th>Amount of standard drug added (μg mL⁻¹)</th>
<th>Amount of drug recovered (μg mL⁻¹)</th>
<th>%Recovery ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATV</td>
<td>ASP</td>
<td>ATV</td>
<td></td>
</tr>
<tr>
<td>4.332</td>
<td>30</td>
<td>0</td>
<td>4.28 ± 1.24</td>
</tr>
<tr>
<td>4.332</td>
<td>30</td>
<td>0.5</td>
<td>4.74 ± 0.95</td>
</tr>
<tr>
<td>4.332</td>
<td>30</td>
<td>4</td>
<td>8.22 ± 1.20</td>
</tr>
<tr>
<td>4.332</td>
<td>30</td>
<td>12</td>
<td>16.36 ± 0.89</td>
</tr>
<tr>
<td>ASP</td>
<td>ATV</td>
<td>0</td>
<td>29.23 ± 1.90</td>
</tr>
<tr>
<td>4.332</td>
<td>30</td>
<td>0</td>
<td>30.09 ± 1.04</td>
</tr>
<tr>
<td>4.332</td>
<td>30</td>
<td>40</td>
<td>68.74 ± 1.40</td>
</tr>
<tr>
<td>4.332</td>
<td>30</td>
<td>80</td>
<td>111.07 ± 2.80</td>
</tr>
</tbody>
</table>

Limit of Detection and Limit of Quantification

The detection limits for ATV and ASP were 0.05 μg mL⁻¹ and 0.2 μg mL⁻¹, respectively, while quantitation limits were 0.1 μg mL⁻¹ and 0.5 μg mL⁻¹, respectively. The above data show that a nanogram quantity of both drugs can be accurately and precisely determined.

Specificity

The specificity study was carried out to check the interference from the excipients used in the formulations by preparing a synthetic mixture containing both drugs and excipients. The chromatogram showed peaks for both drugs without any interfering peak, and the recoveries of both drugs were above 97%.

Forced Degradation Study

Chromatogram of base hydrolysis performed at 80 °C for 1 h showed complete degradation of ASP with degradation product peak at an RT of 4.73 min. ATV was found to be stable to base hydrolysis (Fig. 2).
The chromatogram of acid-degraded samples showed degradation product peaks at RT 4.587 min for ASP and 11.72 and 14.13 min for ATV. The degradation peaks of the ATV and ASP were well resolved from the pure drug peaks with a resolution of more than 2 (Fig. 3). The chromatogram of hydrogen peroxide-degraded samples showed degradation product peaks at RT 4.743 min for ASP and 12.583 min for ATV (Fig. 4). The degradation product peaks were well resolved with the drug peaks. The chromatogram of dry heat degradation study showed degradation product peaks at 4.563 min for ASP, and ATV was found to be stable (Fig. 5).
The degradation study thereby indicated that ATV was stable to base hydrolysis and dry heat degradation study, while it was susceptible to acid hydrolysis and oxidative stress degradation. ASP was indicated to be susceptible to acid and alkali hydrolysis, oxidative stress degradation, and dry heat degradation (Table IV). No degradation products from different stress conditions affected the determination of ATV and ASP.
Table IV. Forced degradation study of ATV and ASP for the proposed LC method

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Time (h)</th>
<th>Recovery (%)</th>
<th>Retention time of degradation products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATV</td>
<td>ASP</td>
</tr>
<tr>
<td>Base 0.1 N NaOH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>98.56</td>
<td>1.67</td>
</tr>
<tr>
<td>Acid 0.1 N HCl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>41.25</td>
<td>35.67</td>
</tr>
<tr>
<td>3% Hydrogen peroxide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>74.47</td>
<td>6.04</td>
</tr>
<tr>
<td>Dry heat&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>99.32</td>
<td>97.23</td>
</tr>
</tbody>
</table>

<sup>a</sup>Solutions were heated at 80 °C for a specified period of time

Solution Stability

The solution stability study showed that ASP underwent hydrolysis with percentage recovery of 96.43%, while ATV was found to be stable.

Robustness

Robustness of the method was studied by changing the flow rate of the mobile phase from 1 mL min\(^{-1}\) to 0.8 mL min\(^{-1}\) and 1.2 mL min\(^{-1}\). Using 1.2 mL min\(^{-1}\) flow rate, RTs for ATV and ASP were observed to be 9.2 and 3.1 min, respectively, and with 0.8 mL min\(^{-1}\) flow rate, RTs for ATV and ASP were found to be 11.7 and 4.5 min, respectively, without affecting the resolution of the drug. When the mobile phase composition was changed to 0.02 M KH\(_2\)PO\(_4\)–acetonitrile–methanol (25:35:40, v/v/v; pH 3) by increasing the percentage of acetonitrile, the RTs for ATV and ASP were observed to be 10.1 and 3.6 min, respectively. The assay result of both drugs was found to be more than 97%.

Analysis of Marketed Formulations

The proposed method was successfully applied to the determination of ATV and ASP in their combined dosage form (capsules A and B). The %recoveries for ATV and ASP are reported in Table V, which are comparable to the corresponding labeled amounts.
Conclusion

The proposed study describes a stability-indicating LC method for the estimation of ATV and ASP combination in a mixture. The method was validated and found to be simple, sensitive, accurate, and precise. Compared to the reported method [18], the developed method is sensitive, specific, and stability indicating containing a simple mobile phase. Statistical analysis proved that the method was repeatable and selective for the analysis of ATV and ASP in combination, without any interference from the excipients. The method was successfully used for the determination of drugs in their pharmaceutical formulations. Also, the above results indicate the suitability of the method for acid, base, dry heat, and wet heat degradation studies. As the method separates the drugs from their degradation products, it can be used for the analysis of stable samples.

Acknowledgments

Authors are grateful to Mercury Laboratory Limited, Vadodara, India for providing gift sample of aspirin and Blue Cross Labs. Ltd., Mumbai, India for providing gift sample of atorvastatin.

References


Accepted by DA