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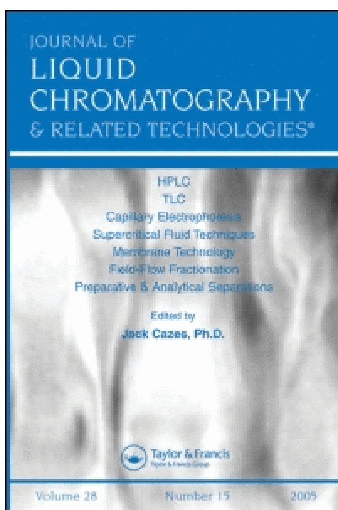

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Validated Ultra HPLC Method for the Simultaneous Determination of Atorvastatin, Aspirin, and their Degradation Products in Capsules

D. N. Vora[a]; A. A. Kadav[a]
[a] Department of Chemistry, Mithibai College of Arts, Chauhan Institute of Science & A.J. College of Commerce and Economics, Maharashtra, India

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Validated Ultra HPLC Method for the Simultaneous Determination of Atorvastatin, Aspirin, and their Degradation Products in Capsules

D. N. Vora and A. A. Kadav

Department of Chemistry, Mithibai College of Arts, Chauhan Institute of Science & A.J. College of Commerce and Economics, Maharashtra, India

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Abstract: An Ultra HPLC method was developed and validated for the simultaneous determination of atorvastatin, aspirin, and their major degradation products in capsules. Salicylic acid, acetylsalicylsalicylic acid, salicylsalicylic acid, and the lactone form of atorvastatin were separated along with six unknown degradation products within 4 min. The chromatographic separation was performed on acquity UPLC™ BEH C18 column (1.7 µm, 2.1 × 50 mm); using a gradient elution of acetonitrile and phosphate buffer (0.01 M, pH 2.0) at a flow rate of 0.6 mL/min. UV detection was performed at 247 nm. The method was validated for accuracy, repeatability, reproducibility, and robustness. Stability indicating capability was established by forced degradation experiments and separation of known degradation products. Linearity, LOD, and LOQ was established for atorvastatin, aspirin, and their known degradation products.

Keywords: Aspirin, Atorvastatin, Capsules, Degradation products, Stability indicating method, UHPLC

Correspondence: D. N. Vora, Department of Chemistry, Mithibai College of Arts, Chauhan Institute of Science & A.J. College of Commerce and Economics, Vile Parle (W), Mumbai 400 056, Maharashtra, India. E-mail: dn.vora@rediffmail.com

INTRODUCTION

Atorvastatin (ATO) calcium, chemically [R-(R*, R*)]-2-(4-fluorophenyl)-β, δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)-carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate, is a synthetic lipid lowering agent. ATO is an inhibitor of 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.[1] Atorvastatin calcium is an organic acid with a pKa of 4.46 and is a white to off white crystalline powder that is insoluble in aqueous solutions of pH 4 and below. It is very slightly soluble in distilled water, pH 7.4 phosphate buffer, and acetonitrile; slightly soluble in ethanol; and freely soluble in methanol.

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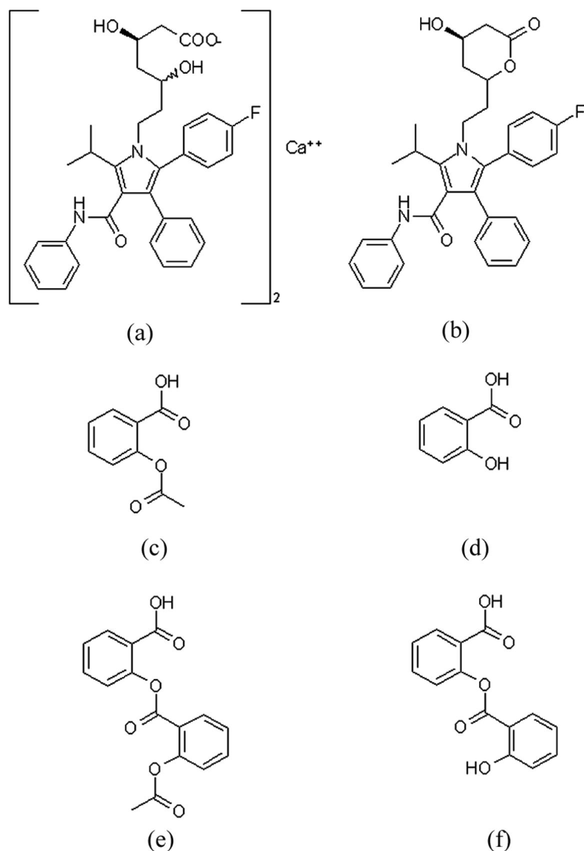

Figure 1. Chemical structures of (a) ATO, (b) A-LCT, (c) ASP, (d) SA, (e) ASSA, and (f) SSA.

The lactone form of ATO (A-LCT) is the major degradation product of ATO. Chemical structures of ATO and A-LCT are shown in Figure 1 (a) and (b), respectively.

Aspirin (ASP), chemically 2-(acetyloxy) benzoic acid, is often used as an analgesic, antipyretic, and anti-inflammatory drug. It also has an antiplatelet effect and is used in long term low doses to prevent heart attack and cancer.[2] It has a pKa of 3.5 and is slightly soluble in water, freely soluble in alcohol, soluble in chloroform, and in ether. Literature indicates that Salicylic acid (SA), acetylsalicylsalicylic acid (ASSA), and salicylsalicylic acid (SSA) are major degradation products of ASP.[3] Chemical structures of ASP, SA, ASSA, and SSA are given in Figure 1 (c), (d), (e), and (f), respectively.

The combination of ATO and ASP is useful for cholesterol lowering and reducing the risk of a myocardial infarction. For this purpose, the capsule dosage form containing 10 mg of atorvastatin and 75 mg of aspirin are widely available on the market.

ASP is official in USP,[4] BP,[5] and IP.[6] Atorvastatin and its pharmaceutical formulation with aspirin is not yet official in any pharmacopoeia. Several analytical methods such as HPLC,[7–15] spectrophotometry,[16] and HPTLC[17] are reported for determination of ATO in drug substance, formulations, and biological matrices. HPLC methods[3,18–22] are available for determination of aspirin and its degradation products from formulations. A HPLC method[23] has been reported for simultaneous determination of ATO and ASP, but it is still only ATO and ASP determination without demonstrating its separation from degradation products. Nowadays, the determination of impurities is one of the main and difficult tasks during the development of separation methods for pharmaceutical analysts, especially if more and more impurities are required to be determined. It should be taken into consideration, and ASP + ATO should be monitored together with their degradation compounds in one analytical run.

Ultra High Performance Liquid Chromatography (UHPLC) is a recent technique in liquid chromatography, which enables significant reductions in separation time and solvent consumption. Literature indicates that the UPLC system allows about a nine fold decrease in analysis time, as compared to the conventional HPLC system using 5 μm particle size analytical columns, and about a three fold decrease in analysis time in comparison with 3 μm particle size analytical columns without compromising on overall separation.[24,25]

The purpose of this study was to utilize advantages of UHPLC and to develop a stability indicating method for the simultaneous determination of ATO and ASP along with their degradation compounds SA, SSA, ASSA, and A-LCT in capsules. The proposed method was able to separate these compounds as well as six other unknown degradation

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products within 4 minutes, which includes reequilibration time of 1 minute. Thereafter, this method was validated[26] and successfully applied for separation and quantification of all compounds of interest in the pharmaceutical formulation—atorvastatin and aspirin capsules.

EXPERIMENTAL

Chemicals and Reagents

Reference standards of ASP, ATO calcium, and A-LCT were kindly gifted by Indoco Remedies (Mumbai, India) with declared purity of 99.5%, 99.0%, and 80%, respectively. Salicyclic acid (99% pure) was obtained from Qualigens (Mumbai, India). Salicylsalicylic acid (99% pure) and acetylsalicylsalicylic acid (97% pure) was procured from ACROS (NJ, USA). All the standards were used as received.

Acetonitrile for HPLC was obtained from J.T. Baker (NJ, USA), potassium dihydrogen orthophosphate and 85% phosphoric acid was from Merck (Mumbai, India). The 0.45 μ nylon filter used to filter sample preparation was mdi SY25NN, which was manufactured by Advanced Microdevices (P) Ltd. (Ambala, India). Capsules containing 10 mg of atorvastatin and 75 mg of aspirin were procured from a local market in Mumbai, India.

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Chromatographic System

Analyses were performed on Acquity UPLC™ system (Waters, Milford, USA), consisting of a binary solvent manager, sample manager, and PDA detector. The detector was set at sampling rate of 20 points/sec and filter time constant of 0.2 sec. System control, data collection, and data processing were accomplished using Waters Empower™ chromatography data software. The analytical column was 50 mm × 2.1 mm acquity UPLC™ BEH C18, 1.7 μm particle size (Waters, Milford, USA). The separation of ATO, ASP, and impurities was achieved by gradient elution using acetonitrile and phosphate buffer (0.01 M, pH 2.0). The finally selected and optimized conditions were as follows: injection volume 2 μL, gradient elution (Table 1), at a flow rate of 0.6 mL/min at ambient temperature, detection wavelength 247 nm, sample compartment temperature 10°C.

Buffer Preparation

The solution of potassium dihydrogen orthophosphate (0.01 M) was prepared by dissolving about 1.36 g of potassium dihydrogen orthophosphate

Table 1. Gradient program for elution of ASP, ATO and impurities

Time (min)	Flow rate (mL/min)	% A (0.01 M phosphate buffer pH 2.0)	% B (Acetonitrile)
Initial	0.600	72.0	28.0
1.50	0.600	48.0	52.0
2.50	0.600	39.0	61.0
3.00	0.600	72.0	28.0
4.00	0.600	72.0	28.0

in 1 L of water for HPLC. The pH of this solution was adjusted to 2.0 with 85% orthophosphoric acid.

Assay Standard Solution Preparation

The assay standard solution was prepared by dissolving standard substances in a diluent (mixture of acetonitrile and methanol, 85:15). The concentrations of the sample and reference standards were about 1500 μg/mL of ASP and 200 μg/mL of ATO.

Impurity Standard Solution Preparation

Impurity standard solutions were prepared by dissolving standard substances in diluent. The final concentrations were about 7.5 μg/mL of each of ASP, SA, SSA, and ASSA, and about 1 μg/mL of ATO and A-LCT.

Sample Preparation

The contents of twenty capsules were collected and crushed to a fine powder. An accurately weighed portion of the powder equivalent to 10 mg of ATO and 75 mg of ASP was taken in a 50 mL volumetric flask. About 30 mL of diluent was added to this flask and sonicated in an ultrasonic bath for 3 minutes. This solution was then diluted to the mark with diluent, filtered through a 0.45 μm nylon filter, and used for analysis after discarding the first few mL.

Method Validation

System Suitability

System suitability parameters were measured so as to verify the system performance. System precision for ASP and ATO peaks were determined

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on five replicate injections of assay standard preparation. All important characteristics including capacity factor, peak resolution, and theoretical plate number were measured and calculated by injecting the impurity standard solution in six replicates.

Specificity

Forced degradation studies were performed to demonstrate the selectivity and stability indicating capability of the proposed method. The powdered samples of capsules were exposed to acidic, alkaline, strong oxidizing, thermal, and photolytic degradation conditions. Also, standards of ATO and ASP were exposed to the above stress conditions, individually and in combination with each other to identify the source of the degradation peaks. All the exposed standards and capsule samples were then analyzed by the proposed method.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ of ATO, ASP, and known degradation products were determined by using signal to noise approach as defined in International Conference on Harmonization (ICH) guideline.[26] The increasingly dilute solution of each drug was injected into the chromatograph and signal to noise ratio was calculated for each compound at each concentration.

Linearity

Linearity was demonstrated from the LOQ to 150% of standard concentration using minimum six calibration levels for both the compounds and their impurity standards. The method of linear regression was used for data evaluation. The peak area of standard compounds was plotted against respective concentrations. Linearity was described by an equation, and the correlation coefficient was determined as well.

Precision

Precision was investigated using the sample preparation procedure for six real samples of a commercial brand of capsules. Intermediate precision was studied using a different column, and performing the analysis on a different day.

Accuracy

To confirm the accuracy of the proposed method, recovery experiments were carried out by the standard addition technique. Three different

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levels of standards were added to pre-analyzed capsule samples in triplicate. The mean percentage recoveries of ATO, ASP, and impurities were calculated.

Robustness

The robustness, as a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions was studied by testing the influence of small changes in pH of buffer (±0.2 units), change in column temperature (30°C), and change in flow rate (±5%).

Stability of Sample Solution

Stability of the sample solution was established by storage of the sample solution at 10°C up to 12 hrs. The sample solution was injected after every 2 hours and assay/impurities were compared against a fresh sample.

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RESULTS AND DISCUSSION

Method Development and Optimization

The main criteria for development of a successful UHPLC method for determination of ATO, ASP, and impurities in capsules were: the method should be able to determine assay and impurities of both drugs in single run and should be accurate, reproducible, robust, stability indicating, free of interference from degradation products/impurities, and straightforward enough for routine use in the quality control laboratory.

In order to optimize the LC separation of ATO, ASP, and impurities, initially, the retention behavior of all components was studied in the pH range of 2.5–6.8, using mobile phases of buffer (pH 2.5–6.8) and acetonitrile. Being acidic in nature, all components except A-LCT were more retained in acidic pH. At higher pH, ASP and its impurities were found coeluting in the void volume. Hence, it was decided to work at pH of 2.5. The components eluted in the order of ASP, SA, ASSA, SSA, ATO, and A-LCT. To ensure that all possible degradation products are eluted and make the method as fast as possible, a gradient run was optimized using buffer pH 2.5 and acetonitrile.

The ratio of the content of active ingredients in capsule formulation was 1:7.5 (ATO: ASP). In order to determine the assay and impurities of both drugs in a single run, it was necessary to have sufficient concentration of ATO in the sample solution so that at least 0.1% of its concentration could be precisely quantifiable in order to determine its impurities. The concentration of 200 µg/mL of ATO was found suitable to give a

quantifiable response for 0.1% ATO (=0.2 μg/mL). Hence, it was decided to work at the concentration of 200 μg/mL of ATO and, consequently, ASP concentration became 1500 μg/mL. However, the separation between ASP and SA was found to be poor at the concentration of 1500 μg/mL of ASP in the gradient run of buffer pH 2.5 and acetonitrile. Any effort to separate ASP and SA by increasing initial aqueous content resulted in higher retention of ATO and A-LCT, resulting into a longer run time. To improve the separation between ASP and SA without significantly affecting the total run time, pH of buffer was further decreased to 2.0 and final gradient was optimized using this buffer.

The final gradient run was chosen with regards to the peak resolutions and analysis time as well. The gradient program is given in Table 1. The flow rate of 0.6 mL/min was optimized with regard to the column efficiency, back pressure, and analysis time as well.

ASP is rapidly hydrolyzed to SA in the presence of hydroxylic solvents and, thus, makes it difficult to accurately determine the content of SA as the impurity. Hence, diluting solvent needed to be carefully chosen so as to retard the rate of degradation of ASP to SA, and at the same time ATO should be soluble and stable in the chosen diluent. Therefore, after optimizing the separation, optimization of diluent for sample preparation was worked upon. USP indicates the use of 1% formic acid in acetonitrile as diluent for determination of SA in ASP tablets.[4] Also, Fogel et al. had demonstrated a slow rate of decomposition of ASP to SA in the extraction solvent, acetonitrile-methanol-phosphoric acid (92:8:0.5).[18] These diluents used acetonitrile as the major portion to limit degradation of ASP to SA. Both these solvents were tried for the sample preparation of ATO and ASP capsules. However, ATO was found to be poorly soluble in these solvents. Since it is readily soluble in methanol, it was decided to increase methanol in the diluent to the extent which would be just sufficient to dissolve ATO. A mixture of acetonitrile-methanol-phosphoric acid (85:15:0.5) was found to be suitable for dissolution of both the drugs at the required concentration. Later, it was observed that ATO was rapidly degrading to A-LCT in this diluent system and, thus, makes it difficult to accurately determine A-LCT as the impurity. It was further investigated that, in the presence of acid, ATO rapidly degrades to A-LCT. Hence, phosphoric acid was removed from the diluent. The final diluting solvent was a mixture of acetonitrile and methanol (85:15), which was most appropriate with respect to solubility and stability of both the drugs. To further enhance the stability of both the drugs in solution, the sample compartment of the system was maintained at 10°C.

ATO, ASP, and their impurities were well resolved in the reasonable time of about 3 minutes and then the column was re-equilibrated for 1 minute. A typical chromatogram of assay standard preparation and impurity standard preparation is shown in Figures 2 and 3, respectively.

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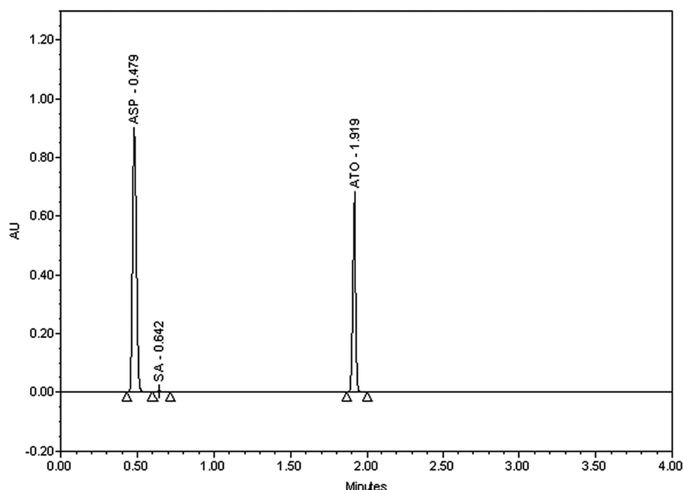

Figure 2. Typical chromatogram of assay standard solution containing 1500 μg/mL of ASP and 200 μg/mL ATO, chromatographed on acquity UPLC™ BEH C18 column (1.7 μm, 2.1×50 mm) using gradient elution of 0.01 M phosphate buffer pH 2.0 and acetonitrile as mobile phase and measured at 247 nm.

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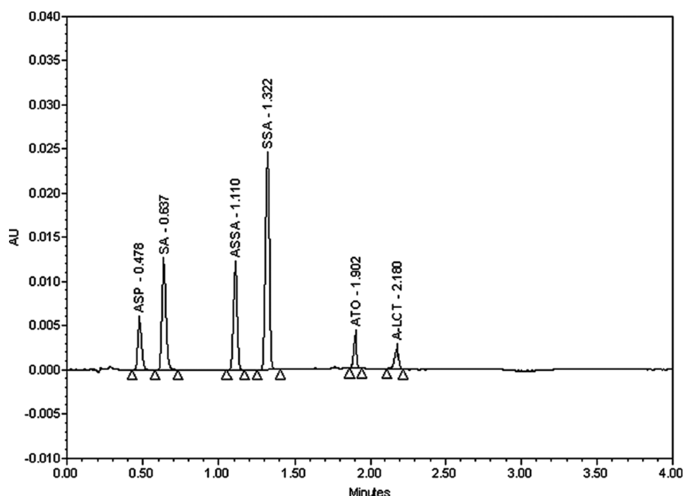

Figure 3. Typical chromatogram of impurity standard solution containing 7.5 μg/mL each of ASP, SA, SSA, ASSA, and 1 μg/mL of ATO and A-LCT, chromatographed on acquity UPLC™ BEH C18 column (1.7 μm, 2.1×50 mm) using gradient elution of 0.01 M phosphate buffer pH 2.0 and acetonitrile as mobile phase and measured at 247 nm.

Since the ATO concentration in sample preparation was 7.5 times lower than ASP, the wavelength of ATO maximum (247 nm) was used to have good detection of ATO and impurities. At this wavelength, ASP and impurities also have good responses and, thus, simultaneous determination of assay and impurities of both the drugs was possible in a single run.

Analytical Parameters and Validation

After satisfactory development of the method it was subjected to method validation as per ICH[26] guidelines The method was validated to demonstrate that it is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics (accuracy, precision, linearity, robustness, and stability indicating capability).

System Suitability

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The results of system precision are presented in Tables 2 and 3. Results of other system suitability parameters are presented in Table 5.

Table 2. Method validation results for assay of individual compound

Parameter	ASP	ATO
System precision[a] (% R.S.D.)	0.40	0.55
Tailing factor	1.21	1.17
Repeatability[b] (% assay)	99.2	99.8
Repeatability[c] (% R.S.D.)	0.3	0.1
Intermediate precision[b] (% assay)	99.0	99.4
Intermediate precision[c] (% R.S.D.)	0.1	0.2
Linearity[d] (correlation coefficient)	0.9996	0.9996
Linearity[d] (equation)	$y = 984.8 \times +7098.3$	$y = 5306.3 \times +3773.9$
LOQ (μg/mL)	1.8	0.2
Accuracy[e] (% R.S.D.)	0.2	1.1
Accuracy[e] (% recovery)	99.6	99.9
Selectivity[f]	No interference	No interference
Stability—12 hr @ 10°C (%)[g]	100.4%	99.3%

[a]Determined on five replicate injections.
[b]Average of six determinations.
[c]Determined on six values.
[d]Six levels, from LOQ–150% of standard concentration.
[e]Determined at three levels with triplicate determination at each level.
[f]Demonstrated by forced degradation and separation of known degradation products.
[g](%) correlation with freshly prepared sample.

Table 3. Precision, accuracy, selectivity and stability results for impurities

Parameter	SA	ASSA	SSA	A-LCT
System precision[a] (% R.S.D.)	2.3	2.6	2.6	2.6
Repeatability[b] (% impurity)	0.98	0.49		0.30
Repeatability[c] (% R.S.D.)	2.6	1.6	N.A.[g]	1.7
Intermediate precision[b] (% impurity)	0.98	0.52	N.D.[f]	0.34
Intermediate precision[c] (% R.S.D.)	2.6	0.96	N.A.[g]	1.5
Accuracy[d] (% recovery)	103.6	103.7	102.8	100.9
Accuracy[d] (% R.S.D.)	0.7	2.4	2.3	1.2
Selectivity[e]	No interference	No interference	No interference	No interference
Stability—10°C [%]				
% in fresh sample	0.98	0.49	N.D.[f]	0.30
% after 12 hrs	1.08	0.49	N.D.[f]	0.31

[a]Determined on six replicate injections.
[b]Average of six determinations.
[c]Determined on six values.
[d]Determined at three levels with triplicate determination at each level.
[e]Demonstrated by separation of known degradation products.
[f]Not Detected.
[g]Not Applicable.

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Specificity

The results of forced degradation study are given in Table 6. ATO was found highly sensitive to heat and acid hydrolysis as seen from the significant drop in assay value and appearance of degradation peaks in the chromatogram. The major degradation product of acid hydrolysis or heat degradation was A-LCT. Similarly, ASP was found sensitive to all conditions except light. SA was the major degradation product of ASP. ASSA and SSA were also observed as degradation products in heat degradation. A few unknown degradation products were also observed in the chromatogram of heat degraded sample. The chromatogram is presented in Figure 4. The peaks labeled as DP1 to DP5 are degradation products of ASP, whereas DP6 is the degradation product of ATO. The source of these degradation products in the capsule sample was ascertained from chromatogram of standards of ATO and ASP, which were simultaneously degraded under the same

Table 4. LOD, LOQ and Linearity results for impurities

Parameter	SA	ASSA	SSA	A-LCT
LOD (μg.mL)[a]	0.18	0.18	0.09	0.2
Signal to noise ratio at LOD[b]	4.8	4.8	6.1	6.8
LOQ (μg/mL)[a]	0.6	0.6	0.3	0.4
Signal to noise ratio at LOQ[c]	15.7	17.4	16.6	13.6
Linearity range (μg/mL)	0.3–66.9	0.3–66.6	0.3–64.6	0.1–2.0
Linearity[d] (correlation coefficient)	0.9998	0.9998	0.9999	0.9998
Linearity[d] (equation)	$y = 2772.2 \times + 769.5$	$y = 2486.0 \times + 124.6$	$y = 5612.7 \times - 67.6$	$y = 4952.7 \times - 177.6$

[a]Based on signal to noise (S/N) ratio.
[b]Acceptance criteria–$S/N > 3$.
[c]Acceptance criteria–$S/N > 10$.
[d]Determined on seven levels.

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conditions. Further, PDA spectra of degradation products in the capsule sample were similar to that of degradation products of individual standards eluting at the respective retention time. Peaks due to ASP and ATO in the chromatogram of all exposed samples and standards were investigated for spectral purity using a PDA detector and found spectrally pure.

LOD and LOQ

The LOD and LOQ results of ATO, ASP, and impurities are presented in Tables 2 and 4. The signal to noise ratio was greater than 3.0 at the level of LOD and greater than 10.0 at the level of LOQ, as required by ICH guidelines.

Linearity

The response was found linear from limit of quantitation to 150% of assay concentration. For all compounds, the correlation coefficient was greater than 0.999. Results are presented in Tables 2 and 4.

Precision

The % assay of ATO, ASP, and impurities analyzed by the proposed method is shown in Tables 2 and 3. Low values of RSD, indicates that the method is precise.

Table 5. System suitability parameters and robustness

System suitability Parameter	Robustness parameter	ASP	SA	ASSA	SSA	ATO	A-LCT
Capacity Factor (k')	No change (repeatability)	1.37	2.17	4.55	5.59	8.49	9.89
	pH of buffer (+0.2 units)	1.39	2.16	4.61	5.68	8.54	9.95
	pH of buffer (−0.2 units)	1.40	2.24	4.55	5.64	8.51	9.92
	Column temperature 30°C	1.34	2.10	4.46	5.50	8.48	9.84
	Flow (+5%)	1.28	2.05	4.39	5.44	8.32	9.69
	Flow (−5%)	1.52	2.37	4.70	5.87	8.74	10.17
Resolution factor (R)	No change (repeatability)	–	3.22	9.51	4.28	12.19	5.67
	pH of buffer (+0.2 units)	–	3.06	9.77	4.29	11.95	5.81
	pH of buffer (−0.2 units)	–	3.43	9.23	4.28	11.78	5.73
	Column temperature 30°C	–	3.10	9.99	4.46	13.22	5.84
	Flow (+ 5%)	–	3.19	9.50	4.26	12.16	5.66
	Flow (−5%)	–	3.34	9.64	4.40	12.02	5.86
Column efficiency (N)	No change (repeatability)	1615	2604	8550	10860	29768	27129
	pH of buffer (+ 0.2 units)	1597	2476	8665	10823	30192	27798
	pH of buffer (−0.2 units)	1633	2700	8157	10116	29239	26941
	Column temperature 30°C	1538	2673	9271	12093	31759	31200
	Flow (+ 5%)	1560	2463	8095	10209	28715	27873
	Flow (− 5%)	1676	2794	9309	11895	30885	28471

Accuracy

The results of recoveries for the assay are shown in Table 2 and for impurities are shown in Table 3, which indicates that the method is accurate.

Robustness

No significant effect was observed on the system suitability parameters of respective components when small, but deliberate changes were made to chromatographic conditions. The results are presented in Table 5 along

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Table 6. Forced degradation data

Degradation condition	ASP		ATO	
	Assay(%)	Major degradation products	Assay(%)	Major degradation products
No degradation (Control)	99.2	–	99.8	–
Acid hydrolysis (0.1 N HCl, 40°C, 30 min)	94.1	SA	76.7	A-LCT
Alkali hydrolysis (0.1 N NaOH, 40°C, 30 min)	77.0	SA	99.0	–
Oxidation (5% H_2O_2, 40°C, 30 min)	93.6	SA	98.9	–
Thermal (80°C, 4 hrs)	69.0	SA, ASSA, SSA	34.6	A-LCT
Photolytic (UV@254 nm, 1 day)	99.2	–	99.5	–

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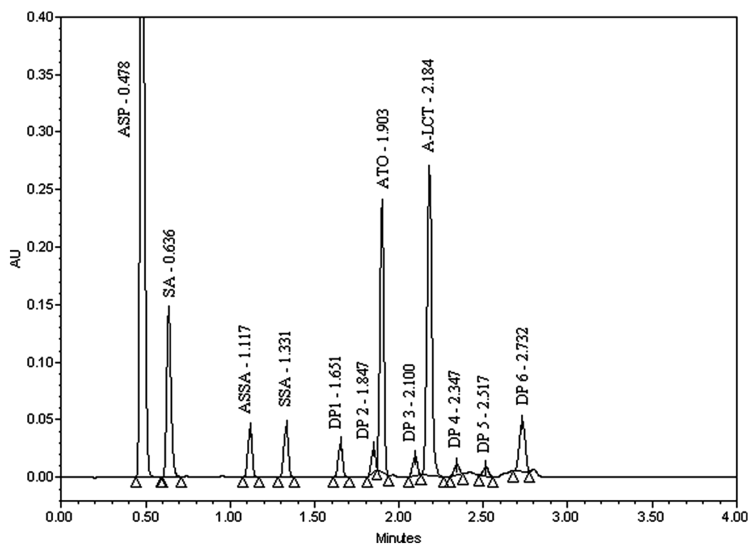

Figure 4. Chromatogram of heat degraded capsules sample, chromatographed on acquity UPLC^{TM} BEH C18 column (1.7 μm, 2.1 × 50 mm) using gradient elution of 0.01 M phosphate buffer pH 2.0 and acetonitrile as mobile phase and measured at 247 nm. DP1-DP5 are degradation products of ASP and DP 6 is degradation product of ATO.

with system suitability parameters of normal methodology. Thus, the method was found to be robust with respect to variability in above conditions.

Stability of Sample Solution

The sample solution did not show any appreciable change in assay and impurities value up to 12 hrs. Assay results are presented in Table 2 and impurities results are presented in Table 3.

CONCLUSION

A novel UHPLC method was successfully developed and validated for simultaneous determination of ATO, ASP, and impurities in capsules. The method was optimized with respect to chromatographic separation as well as sample preparation. The total run time was 4 min, within which both the drugs and their degradation products were separated. Method validation results have proven the method to be selective, precise, accurate, robust, and stability indicating. Sample solution stability was established for determination of the assay as well as impurities. This method can be successfully applied for the routine analysis as well as stability study. Also it can be utilized for determination of content uniformity and dissolution profiling of this product, where sample load is higher and high throughput is essential for faster delivery of results. Overall, the method provides a high throughput solution for determination of atorvastatin, aspirin, and their degradation products in capsules with excellent selectivity, precision, and accuracy.

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