Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 12, 2015

Original Article

DEVELOPMENT OF STABILITY INDICATING RP-HPLC METHOD FOR THE DETERMINATION OF RELATED SUBSTANCES IN ATORVASTATIN SOLID DOSAGE FORM AND BULK DRUGS

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Received: 08 Sep 2015 Revised and Accepted: 27 Oct 2015

ABSTRACT

Objective: The present paper reports the development of simple, rapid, accurate and stability indicating reversed phase high-performance liquid chromatography method (RP-HPLC) for estimation of related substances in Atorvastatin calcium (ATV) bulk drug as well as in solid dosage form. The method was validated using Agela, Unisol C18 (250 mmX 4.6 mm; 5µ) column.

Methods: A method was developed to separate clearly the drug peak from the synthetic/process impurities and degradation products formed under stress conditions is attained on (T/%B) were set at 0/43, 18/43, 20/60, 33/60, 35/43, and 40/43 of 0.02 mM ammonium acetate buffer of pH4.9 was used as mobile phase A and 90:10 v/v, ratio of acetonitrile and tetrahydrofuran was used as mobile phase B. A flow rate of 1.4 ml/min and column temperature of 25 °C was used. The wavelength selected was 246 nm. The developed method was validated as per ICH guidelines for the specificity, precision, linearity, accuracy, limit of detection, limit of quantitation and robustness.

Results: Linearity of the impurities was accomplished in the range 0.3-6.0µg/ml for impurity A (Imp A), B (Imp B), C (Imp C), H (Imp H) and 0.4-6.0µg/ml for impurity D (Imp-D), correlation coefficient was found to be more than 0.999 for all impurities. Recovery of impurities was found to be in the range 93%-111%.

Conclusion: The developed method was simple, precise, accurate, robust and also cost effective as it has shorter run time for quantification of impurities in drug substance and drug product as well.

Keywords: Atorvastatin calcium, Impurities, ICH guidelines, Method development, Analytical method validation, Degradation.

INTRODUCTION

ATV is a synthetic hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitor that has been demonstrated to be efficacious in reducing both cholesterol and triglycerides. It is administered as the calcium salt of the active hydroxy acid and is used between 10 and 80 mg per day to reduce the raised lipid levels in patients with primary hyperlipidemia (familial and non-familial) or combine hyperlipidemia [1]. As per the literature available, five impurities reported in drug substance (refer table 1) named as Imp A, B, C, D, and H [2] and for pharmaceuticals, there is no compendia method available either in the United States Pharmacopoeia (USP) and European Pharmacopoeia (EP). So far many methods are available for quantification of impurities, but the current developed method has a shorter runtime, which was obtained by using a gradient program ensuring that all the impurities elute out efficiently. The aim of the study is to develop a simple, reliable and accurate stability indicating a method for the determination of ATV and its process and also storage related impurities. This manuscript provides a detailed procedure for the determination of relative response factors (RRF) for the quantitative determination of impurities.

MATERIALS AND METHODS

Chemicals and reagents

Working standard of ATV and five impurities namely Imp A, Imp B, Imp C, Imp D, and Imp H were taken from the Ashland analytical lab, Hyderabad, Telangana, India. All reagents used were of analytical reagent grade. HPLC grade acetonitrile, tetrahydrofuran, and acetic acid used from Merck (Darmstadt, Germany). Ammonium acetate was from Merck (Darmstadt, Germany). Milli-Q water of high purity and filter through 0.22 µm from millipore water system was used.

Instrument

A Waters alliance HPLC System e2695 equipped with a PDA detector and a quaternary gradient pump was used for the study. All the data was acquired using empower 3 data acquisition and integration software. Agilent 8453 ultraviolet, visible spectrophotometer was used for recording the spectrum.

Methods

Method development and optimization

Four experiments were conducted using sample solution containing ATV and its impurities at the specified concentration (refer table 2) [3] and injected onto the HPLC in order to obtain a better separation in a shorter run time.

Experiment 1

Initially, solutions containing 0.8 mg/ml of ATV, 0.004 mg/ml of all impurities, placebo equivalent to one tablet was prepared and injected onto RP-HPLC equipped with Agilent Zorbax Rx-C8 column of dimension 250 mm X 4.6 mm i. d, 5µ particle size. A mixture of acetonitrile, tetrahydrofuran, and buffer (3.9 g/l of ammonium acetate in water, adjusted to pH 5.0 with glacial acetic acid) in the ratio of 21:12:67 and 61:12:27 v/v/v were used as mobile phase A and B respectively. The column temperature was maintained at 35 °C and the flow rate was 1.5 ml/min. A gradient program (T/%B) was set at 0/0, 40/0, 70/80, 85/100, 100/100, 105/0, and 115/0. The detector wavelength was 246 nm.

Experiment 2

In order to overcome the difficulties in the experiment 1, the column was replaced with Phenominex Luna C18, 250 mm x 4.6 mm i. d, 5µ particle size. And different combinations of buffer (1.5 g/l of ammonium acetate in water, adjusted with glacial acetic acid to a pH of 4.45) acetonitrile and tetrahydrofuran were used. The flow rate was 1.0 ml/min, column oven temperature was 35 °C and the gradient program (T/%B) was set at 0/20, 15/26, 45/35, 75/65, 80/20, and 90/20.

Experiment 3

Here, Waters X-Bridge column having dimensions 50 mm x 4.6 mm i. d, 3.5 µ particle size was used to decrease the analysis time, considering the same mobile phase A and B as in experiment 2. Different combination of buffer, acetonitrile, and tetrahydrofuran were used to optimize the method. The flow rate was 1.4 ml/min and the column temperature was at 25 °C. The gradient program (T/%B) was set at 0/20, 15/20, 20/35, 22/20, and 30/20.

Experiment 4

Here, Agela, Unisol C18 column having dimensions of 250 mm x 4.6 mm i. d, 5 µ particle size was used. 0.02 M ammonium acetate buffer adjusted to pH 4.9 was used as mobile phase A, different combination mixtures of acetonitrile, tetrahydrofuran were used and finally the ratio of 90:10 v/v, was set as mobile phase B. The gradient program (T/%B) was set at 0/43, 18/43, 20/60, 33/60, 35/43, and 40/43. The flow rate was 1.4 ml/min and the column temperature was at 25 °C. The detector wavelength was 246 nm.

Table 1: ATV and its related impurities

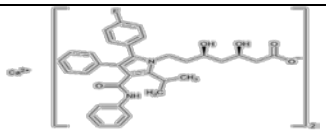
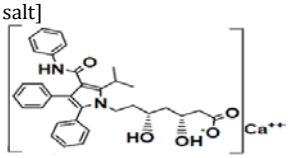
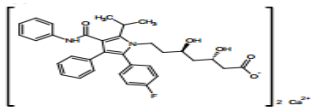
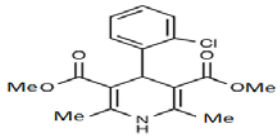
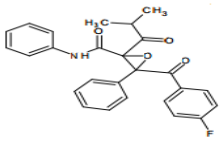
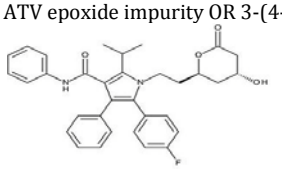
Compound	Structure
ATV	 [(3R,5R)-7-[3-(Phenylcarbamoyl)-5-(4-fluorophenyl)-2-isopropyl-4-phenyl-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid, calcium salt]
Imp A	 (3R, 5R)-7-[3-(phenylcarbamoyl)-2-isopropyl-4,5-diphenyl-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid calcium salt)
Imp B	 (3S,5R)-7-[3-(phenylcarbomoyl)-5-(4-fluorophenyl)-2-isopropyl-4-phenyl-1Hpyrrol-1-yl]-3,5-dihyroxyheptanoicacid, calcium salt.
Imp C	 (3R,5R)-7 [3-(phenylcarbomoyl)-4,5-bis(4-fluorophenyl)-2-isopropyl-1H-pyrrol-1-yl]-3,5-dihyroxyheptanoicacid, sodium salt.
Imp D	 ATV epoxide impurity OR 3-(4-fluorophenyl)-2-isobutyryl-3-phenyloxirane-2-carboxylic acid phenylamide
Imp H	 5-(4-Fluorophenyl)-2-(1-methylethyl)-N,4-diphenyl-1-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1H-pyrrole-3-carboxamide

Table 2: Concentration of ATV and its impurities

Compound	Concentration	
	mg/ml	µg/ml
ATV	0.8	800
ATV impurities	0.004	4
Unknown concentration	0.0016	1.6

RESULTS AND DISCUSSION

Method development

Experiment 1

Here, separation of all the peaks was observed, but the baseline fluctuations were more and also total run time was kept long for elution of all the impurities. Representative chromatogram is shown in fig. 1.

Experiment 2

Here, the baseline fluctuation was observed to be less as compared to experiment 1.

However, HPLC run time was still longer and could not be shortened due to elution of all the peaks. Representative chromatogram is shown in fig. 2.

Experiment 3

Here, one of the impurity named as Imp B was not seen, as it probably merged with the main peak, since peak purity was out of the range for ATV peak and resolution between ATV and Imp C was found to be lesser than 1.5, this experiment did not work, since it was not meeting the system suitability criteria. Representative chromatogram is shown in fig. 3.

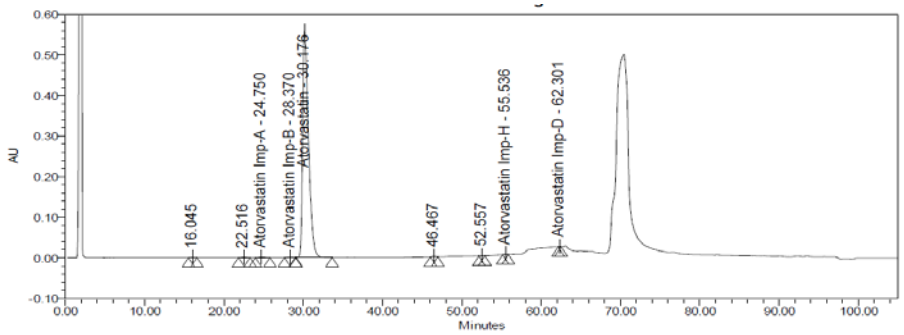


Fig. 1: Typical chromatogram of ATV and its impurities from experiment 1

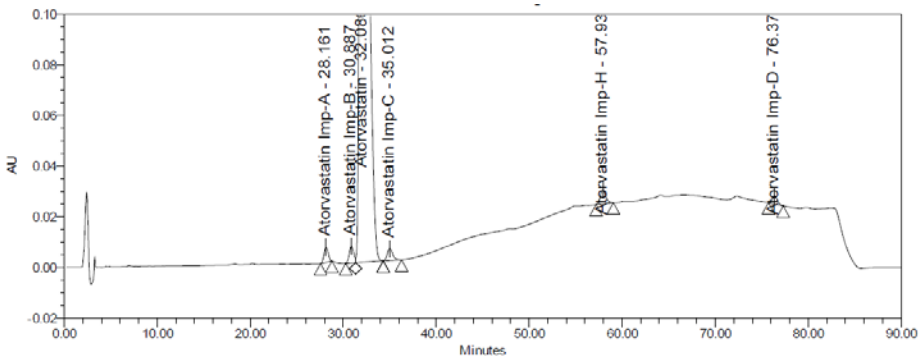


Fig. 2: Typical chromatogram of ATV and its impurities from experiment 2

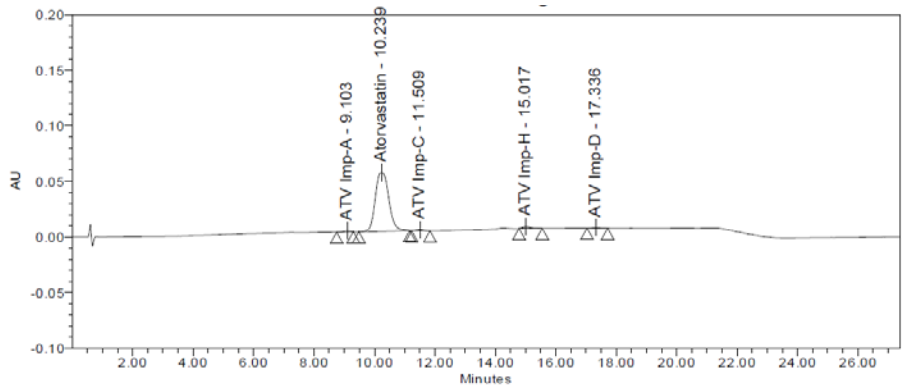


Fig. 3: Typical chromatogram of ATV and its impurities from experiment 3

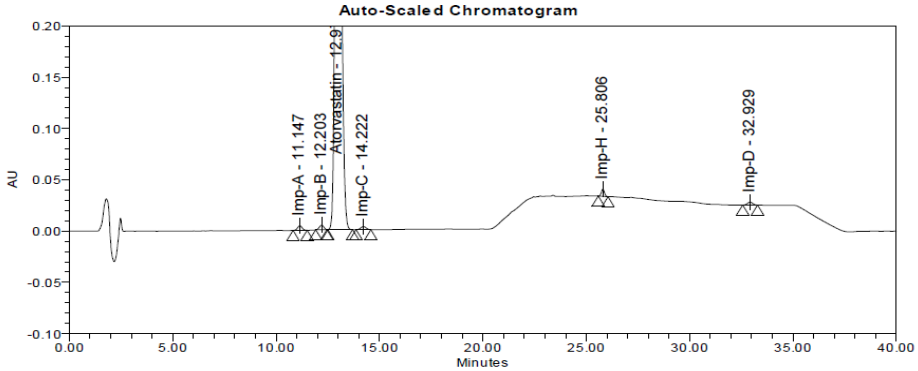


Fig. 4: Typical chromatogram of ATV and its impurities from experiment 1

Experiment 4

Imp-B is one of the isomers of ATV, and its peak separation was not seen even on a shorter column used in RP-HPLC.

Here in this experiment Agela, Unisol C18 column was considered, due to its properties of high carbon loading (18%). Assuming that all the impurities can retain well on this column as compared with the previous columns, this was chosen. A representative chromatogram is shown in fig. 4.

Here, it was observed that all the peaks got separated with good peak shapes. And also the run time was very short, it has reduced almost three times as compared to the run times available in the existing literature for ATV molecule. In order to achieve separation of five impurities of ATV in a shorter run time and to save time chemicals and other sources, different HPLC parameters like buffer pH, the ratio of buffer and organic modifier and also the flow rate were optimized. The summary of method development is shown in table 3.

Table 3: Summary of method optimization

Experiment	Column	Observation
1	Agilent, Zorbax Rx-C8, 250 mm X 4.6 mm i. d, 5µ particle size	Longer run time and baseline fluctuations.
2	Phenominex Luna, C18, 250 mm X 4.6 mm i. d, 5µ particle size	Still longer run time and baseline fluctuations.
3	Waters X Bridge, 50 mm X 4.6 mmi. d, 3.5µ particle size	Complete merging of Imp B with ATV main peak.
4	Agela, Unisol C18, 250 mm X 4.6 mm i. d, 5µ particle size	Good separation, baseline and run time are reduced to 40 min.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness is a measure of the capacity of a method to remain unaffected by small but deliberate changes by varying the parameters like column temperature (±2 °C), flow rate (±0.1 ml/min), column (different lot number), pH (±0.1), mobile phase B (±10%) of least contribution component (tetrahydrofuran), and wavelength (±2 nm). ATV test preparation was prepared by spiking with blend impurity solution at release specification level of 0.5% of ATV test concentration and injected in to the HPLC system. The system suitability parameters were evaluated and were found to be within the acceptance criteria. The results are tabulated in the table 4.

Table 4: Robustness

Actual condition	Changed condition	Theoretical Plates (N)	Tailing factor (T)	USP resolution between ATV and	
				Imp B	Imp C
1.4 ml/min	1.3 ml/min	ATV: 10125 Imp B: 13977 Imp C: 11754	ATV: 1.4 Imp B: 1.0 Imp C: 1.1	2.1	2.5
	1.5 ml/min	ATV: 12015 Imp B: 15218 Imp C: 14125	ATV: 1.2 Imp B: 1.0 Imp C: 1.0	1.9	2.3
246 nm	244 nm	ATV: 11075 Imp B: 15087 Imp C: 12841	ATV: 1.3 Imp B: 1.0 Imp C: 1.1	2.0	2.5
	248 nm	ATV: 11084 Imp B: 15147 Imp C: 12321	ATV: 1.3 Imp B: 1.0 Imp C: 1.1	2.0	2.5
25 °C	23 °C	ATV: 989 Imp B: 12315 Imp C: 11254	ATV: 1.3 Imp B: 1.0 Imp C: 1.1	2.0	2.5
	27 °C	ATV: 13154 Imp B: 15214 Imp C: 13215	ATV: 1.2 Imp B: 1.0 Imp C: 1.0	1.9	2.3
4.9 pH	4.8 pH	ATV: 11055 Imp B: 14987 Imp C: 12744	ATV: 1.4 Imp B: 1.0 Imp C: 1.1	1.7	2.1
	5.0 pH	ATV: 11055 Imp B: 14987 Imp C: 12744	ATV: 1.3 Imp B: 1.0 Imp C: 1.1	1.9	2.1
100 ml THF in 1000 ml of mobile phase B	90 ml	ATV: 12015 Imp B: 15287 Imp C: 13748	ATV: 1.2 Imp B: 1.0 Imp C: 1.1	1.8	2.1
	110 ml	ATV: 10125 Imp B: 12587 Imp C: 12144	ATV: 1.3 Imp B: 1.0 Imp C: 1.1	2.0	2.3
Column change	Column 1 Lot# AQ-110523	ATV: 11055 Imp B: 14987 Imp C: 12744	ATV: 1.3 Imp B: 1.0 Imp C: 1.1	2.0	2.5
	Column 2 Lot#110523	ATV: 13127 Imp B: 15014 Imp C: 13015	ATV: 1.3 Imp B: 1.0 Imp C: 1.1	2.0	2.5

Here it was observed that by varying the above parameters, much impact was not shown on the resolution, tailing factor, theoretical plates, and relative retention times of ATV impurities. Hence, the method was found to be robust.

System suitability solution

The system suitability test is a critical parameter for an analysis as it evaluates both the instrument and method performance. The sample solution containing ATV spiked with Imp B and Imp C at the specification level with respect to the sample concentration were injected onto the HPLC system.

The representative chromatogram is shown in fig. 5 and the system suitability test results are tabulated in table 5.

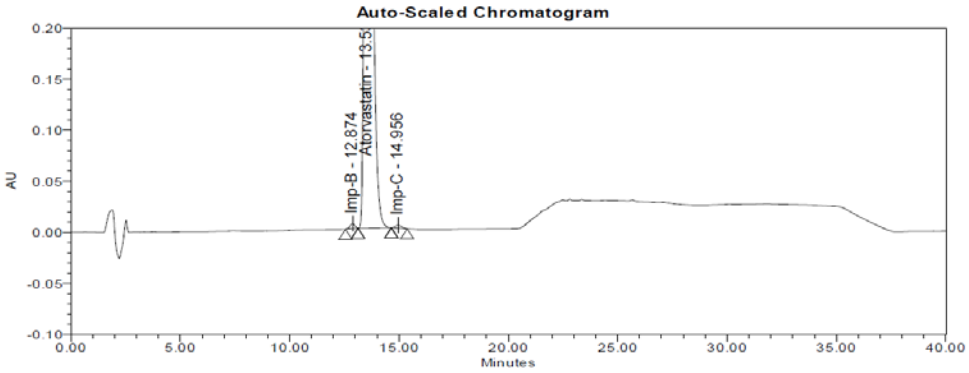


Fig. 5: System suitability solution chromatogram

Table 5: System suitability parameters

Parameter	Specification	Results
Theoretical plates (N)	≥ 2000	ATV: 11055 Imp B: 14987 Imp C: 12744
Tailing factor (T)	≤ 1.5	ATV: 1.3 Imp B: 1.0 Imp C: 1.1
Resolution between Imp B and ATV (R)	≥ 1.5	2.0
Resolution between ATV and Imp C (R)	≥ 1.5	2.5

Analytical method validation

The objective of validation of an analytical procedure is to prove that it is suited for its designated use. The proposed method has been validated for the related substances as per ICH guidelines [6].

Relative response factors determination

Relative response factors were calculated by injecting all the impurities and ATV as an unknown from 0.5 to 6 ppm concentration onto the HPLC system. The relative response factor (RRF) of each impurity was determined by dividing the slope of each impurity by the slope of ATV calcium from the linearity curve. The relative response factors and relative retention times for individual impurities are tabulated in table 6.

Validation parameters

- Specificity
- Precision
- Repeatability
- Intermediate Precision
- Reproducibility
- Detection and Quantization Limit
- Linearity
- Accuracy
- Range

Specificity

Specificity is the ability to assess the analyte unequivocally in the presence of components, which might include impurities, degradants and matrix. Specificity was performed in terms of forced degradation.

Degradation study

Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and also validate the stability indicating the power of the analytical procedures used [4, 5].

Table 6: Relative response factors and relative retention times

Compound name	Relative retention time	Relative response factor
ATV	1.00	1.00
Imp A	0.86	1.01
Imp B	0.94	0.89
Imp C	1.10	0.87
Imp D	2.53	0.63
Imp H	1.98	0.96

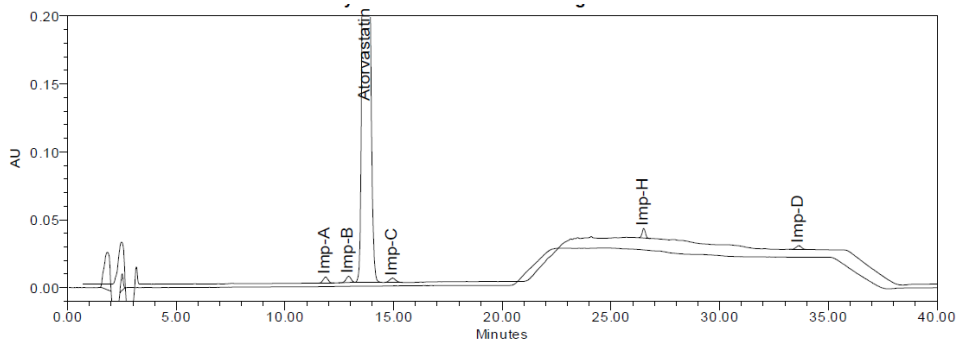


Fig. 6: Overlaid chromatograms of placebo and ATV with Imp A, B, C, D, and H

Conditions for degradation

Hydrolytic condition

Hydrolysis is one of the most common degradation chemical reactions over a wide range of pH. Hydrolytic study under acidic, basic and neutral condition involves catalyzation of ionizable functional groups present in the molecule. Here the sample was refluxed with 2N HCL/2N NaOH/Water at 60 °C for 5 h. The representative chromatograms are shown in fig. 7-9.

Oxidative condition

Generally, most of the samples undergo auto-oxidation under atmospheric oxygen conditions. Auto-oxidation is a free radical reaction that involves a free radical initiator to start out the chain reaction and here hydrogen peroxide act as an initiator for the same. Here the sample was oxidized using 3% hydrogen peroxide at a temperature not exceeding 60 for 2 h. The representative chromatogram is shown in fig. 10.

Thermal condition

In general, the rate of a reaction increases with an increase in temperature. Hence, the drugs are susceptible to degradation at a higher temperature. Here the sample was kept at 105°C for about 48 h. The representative chromatogram is shown in fig. 11.

Photolytic condition

Exposure of drug molecules to light may produce degraded photolytic products. The rate of photo degradation depends upon the intensity of incident light and quantity of light absorbed by the drug molecule. Photolytic degradation is carried out by exposing the drug product with a combination of visible and UV light. Here the sample tablet powder was exposed to ultraviolet radiation up to a lower limit of 200-watt h/m² and a lower limit of 1.2 million lux hours of visible light in the photostability chamber. The representative chromatogram is shown in fig. 12.

Humidity condition

Humidity is the key factor in establishing the potential degradants in the finished product and active pharmaceutical ingredient. Here the sample was exposed at 90% humidity for one week. The representative chromatogram was shown in fig. 13.

The mass balance of all the conditions was found to be more than 99.2%. The Purity angle was found to be less than purity threshold and also no purity flag is present (refer table 7).

Table 7: Degradation results

Condition	% net degradation	Purity angle	Purity threshold	Purity flag (yes/no)
Unstressed	0.52	0.033	0.263	No
Acid stressed	9.32	0.023	0.256	No
Water	0.90	0.036	0.263	No
Base stressed	0.52	0.029	0.261	No
Peroxide stressed	19.1	0.034	0.260	No
Photolytic stressed	0.54	0.029	0.263	No
Sunlight stressed	0.55	0.032	0.261	No
Thermal stressed	2.22	0.027	0.261	No
Humidity	0.58	0.028	0.259	No

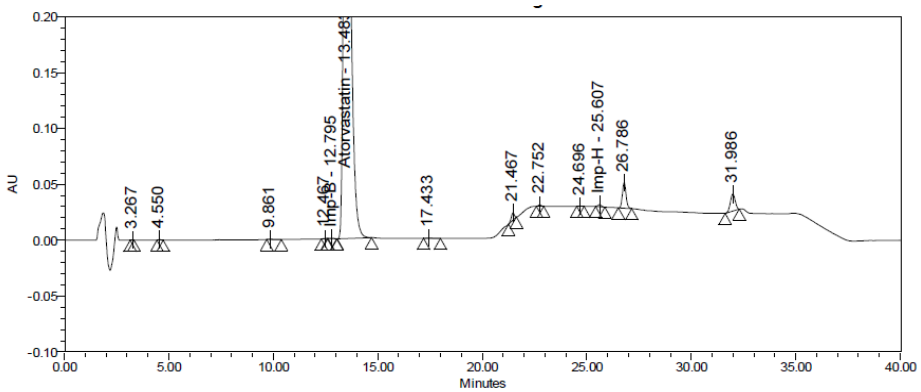


Fig. 7: Chromatogram represents acidic condition

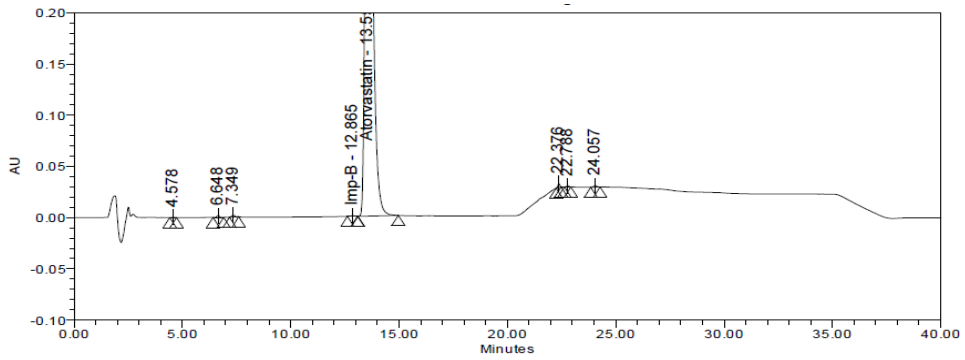


Fig. 8: Chromatogram represents basic condition

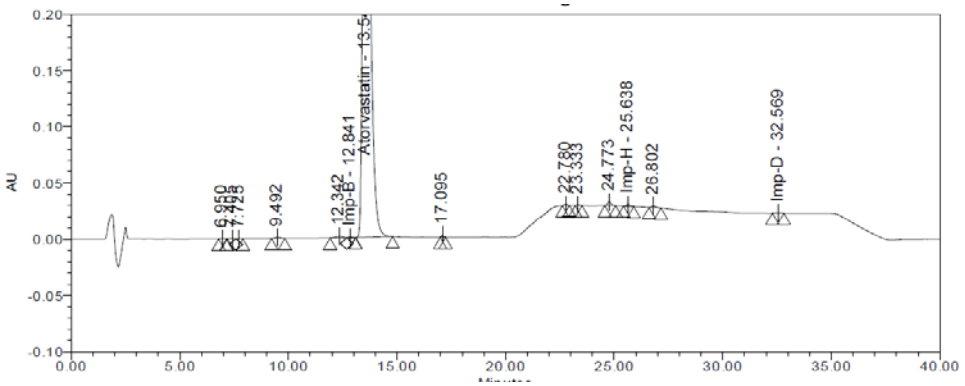


Fig. 9: Chromatogram represents water condition

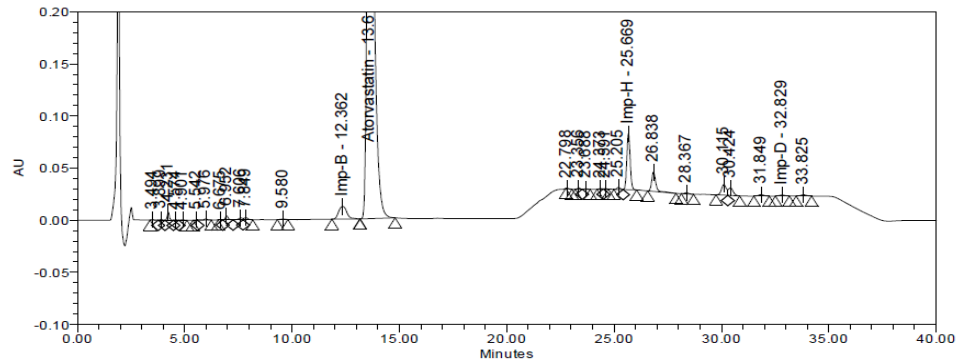


Fig. 10: Chromatogram represents oxidative condition

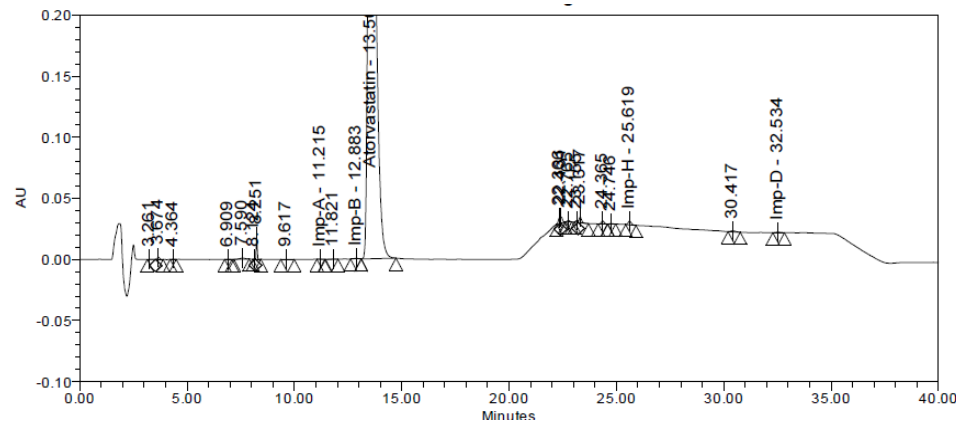


Fig. 11: Chromatogram represents thermal condition

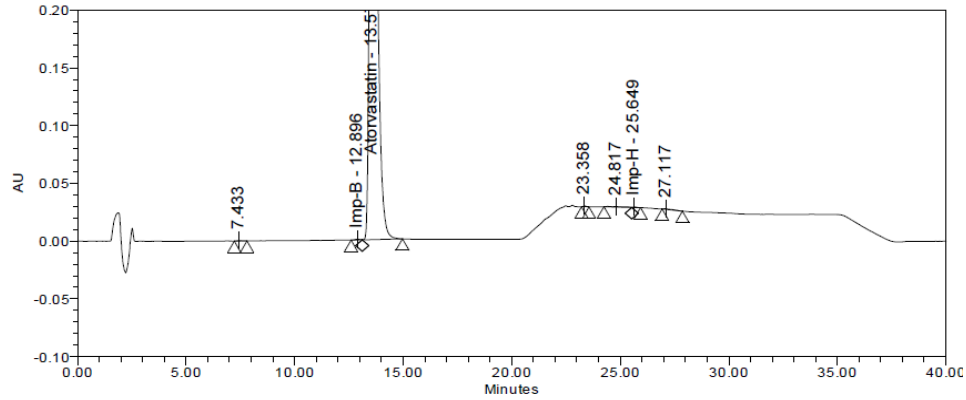


Fig. 12: Chromatogram represents photolytic condition

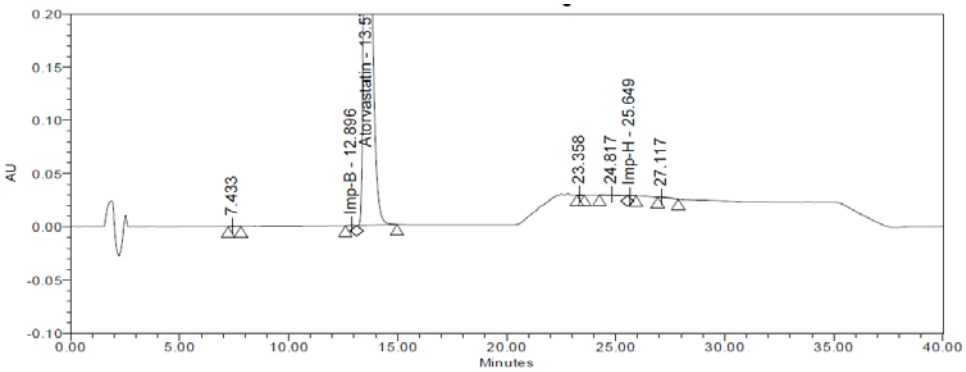


Fig. 13: Chromatogram represents humidity condition

Method precision: repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra-assay precision. The Precision of test method was evaluated by injecting six samples prepared by spiking test preparation with ATV impurities solution to get concentration at release specification level of 0.5% of Imp A, B, C, D, and H of ATV test concentration. The % relative standard deviation was found in between 1.5 to 3.5. The results are tabulated in table 8.

Table 8: Repeatability

Name of the impurity	Concentration (%)						Average	SD	% RSD
Imp A	0.461	0.449	0.442	0.454	0.456	0.467	0.455	0.01	1.9
Imp B	0.511	0.535	0.483	0.507	0.505	0.523	0.511	0.02	3.5
Imp C	0.522	0.500	0.505	0.505	0.513	0.512	0.510	0.01	1.5
Imp D	0.509	0.475	0.485	0.501	0.494	0.464	0.488	0.02	3.4
Imp H	0.536	0.541	0.525	0.532	0.545	0.547	0.538	0.01	1.6

Intermediate precision (Ruggedness)

The intermediate Precision was conducted by two different analysts, on different days using different columns. Three samples were prepared by spiking test preparation with ATV impurities solution to get concentration at release specification level of 0.5% of ATV test concentration at each condition.

The % relative standard deviation between all the three conditions was found in between 1.3 to 3.0. The results are tabulated in table 9.

Table 9: Intermediate precision

Name of the impurity	1	2	3	4	5	6	7	8	9	Average	SD	%RSD
	Concentration (%)											
	Analyst 1 Day 1 Instrument 1			Analyst 2 Day 2 Instrument 1			Analyst 1 Day 3 Instrument 2					
Imp A	0.461	0.449	0.442	0.451	0.462	0.459	0.478	0.477	0.472	0.461	0.01	2.7
Imp B	0.511	0.535	0.483	0.522	0.522	0.530	0.503	0.512	0.510	0.514	0.02	3.0
Imp C	0.522	0.500	0.505	0.517	0.515	0.513	0.510	0.508	0.509	0.511	0.01	1.3
Imp D	0.509	0.476	0.486	0.502	0.506	0.507	0.499	0.502	0.500	0.499	0.01	2.2
Imp H	0.536	0.541	0.525	0.521	0.530	0.525	0.505	0.510	0.510	0.523	0.01	2.4

Limit of detection (LOD) and limit of quantification (LOQ) from prediction linearity

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest quantity of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

A study to establish the LOD and LOQ for ATV (as unknown) and its related compounds was conducted. The LOD and LOQ was established by slope method from lowest level (10%) to 150% of target concentration.

A series of eight dilutions with increasing concentrations were injected onto the HPLC system. A graph of concentration vs. area was plotted. The LOD and LOQ were calculated based on the standard deviation of response and slope (refer table 10).

Table 10: LOD and LOQ

Compound name	LOD, %	LOQ, %
ATV as unknown	0.01	0.04
Imp A	0.01	0.04
Imp B	0.01	0.04
Imp C	0.01	0.04
Imp D	0.02	0.05
Imp H	0.01	0.04

Precision and accuracy at LOQ level

The precision of ATV having its impurities at about the LOQ was conducted. Six test preparations of ATV and its impurities at the quantification level were prepared and injected onto the HPLC system. The % RSD of spiked concentrations for six replicate were found between 3.2 to 7.8. The precision of the ATV was spiked at the quantification level on placebo equivalent to test concentration and were injected onto the HPLC system. The % RSD was found to be 1.5 (refer table 11).

The accuracy of ATV having its impurities at about the limit of quantification level was conducted. Impurities were spiked at the quantification level using ATV drug product equivalent to test concentration. Six preparations were prepared and injected onto the HPLC system. The % recovery of ATV impurities at the quantification were calculated and found to be in between 94 to 108. Results are tabulated in the table 11.

Accuracy of the ATV at Quantification limit was conducted. ATV stock was spiked at quantification limit on placebo equivalent to test concentration. Six preparations were prepared and injected onto the HPLC system. The % recovery of the ATV at the quantification limit were calculated and found to be in between 101 to 105. Results are tabulated in the table 12.

Table 11: Precision at LOQ

Name of the component	Concentration (%)						Average	SD	% RSD
ATV as unknown	0.0382	0.0375	0.0382	0.0386	0.0389	0.0375	0.0382	0.001	1.5
Imp A	0.0396	0.0414	0.0411	0.0412	0.0389	0.0385	0.0401	0.001	3.2
Imp B	0.0372	0.0391	0.0371	0.0396	0.0371	0.0361	0.0377	0.001	3.6
Imp C	0.0369	0.0374	0.0317	0.0352	0.0402	0.0374	0.0365	0.003	7.8
Imp D	0.0540	0.0566	0.0577	0.0525	0.0521	0.0561	0.0548	0.002	4.2
Imp H	0.0400	0.0365	0.0355	0.0421	0.0372	0.0372	0.0381	0.002	6.5

Table 12: Accuracy at LOQ

Name of the component	% Recovery						Average	SD	% RSD
ATV	103.3	101.4	103.4	104.3	105.2	101.4	103.2	1.5	1.5
Imp A	103.7	108.5	107.7	107.8	101.8	100.8	105.1	3.4	3.2
Imp B	98.9	104.0	98.6	105.3	98.7	96.0	100.3	3.6	3.6
Imp C	101.0	102.6	86.8	96.4	110.1	102.5	99.9	7.8	7.8
Imp D	106.0	111.0	113.2	103.0	102.3	110.1	107.6	4.5	4.2
Imp H	98.7	90.0	87.4	103.8	91.7	91.7	93.9	6.1	6.5

Linearity

The linearity of an analytical procedure is it's ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Five solutions with concentration ranging from quantification limit to 150% of the specification of ATV impurities (0.5% of Imp-A, B, C, D, H and 0.2% maximum unknown impurity) were prepared.

The Linearity was established by plotting a graph of concentration versus area response of ATV, impurities and the correlation coefficient was determined.

The linearity of impurities was obtained and the correlation coefficient was found to be more than 0.999 for all impurities (refer table 13).

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

The Accuracy study of ATV and its impurities was performed by spiking impurities on test preparation. Samples were prepared in triplicate by spiking at the levels of 50%, 100%, and 150% to get the target concentration of ATV impurities solution [0.5% of ATV test concentration (4 ppm)].

Accuracy analysis was also performed for ATV by spiking ATV stock solution on placebo preparation (equivalent to test concentration) with 50%, 100%, and 150% of the target concentration of ATV

[target concentration is 0.2% (1.6 ppm)]. Each sample was prepared in triplicate by spiking test preparation with respective levels of the target impurity concentration. % Recoveries were found in between 97 to 106 for ATV and its impurities (refer table 14).

Table 13: Linearity

Name of the Component	Correlation coefficient	Slope	Intercept	Bias at 100%
ATV as unknown	0.9996	17774	1004	3.6
Imp A	0.9999	1206	14897	1.9
Imp B	0.9999	680	14940	1.1
Imp C	0.9998	1583	14349	2.8
Imp D	0.9996	1845	10794	4.0
Imp H	0.9995	180	14540	0.3

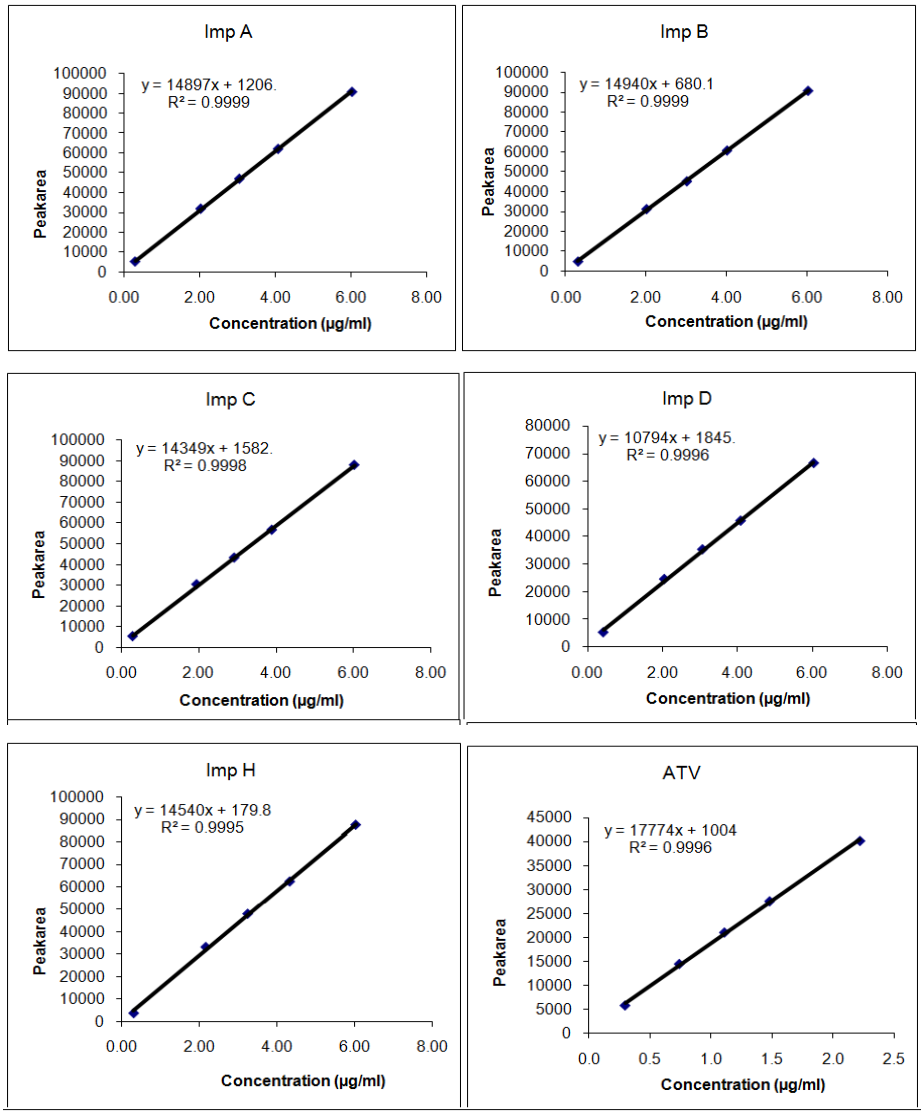


Fig. 14: Linearity graphs

Table 14: Accuracy

Name of the component	Mean recovery of 50%	Mean recovery of 100%	Mean recovery of 150%	Average	SD	% RSD (precision)
ATV as unknown	103.1	105.4	104.2	104.2	1.2	1.1
Imp A	99.9	99.0	103.7	100.9	2.5	2.5
Imp B	100.7	101.6	102.3	101.5	0.8	0.8
Imp C	97.9	99.2	102.1	99.7	2.2	2.2
Imp D	107.6	110.4	99.5	105.8	5.7	5.3
Imp H	95.6	97.8	106.5	100.0	5.8	5.8

Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including those concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The overall data of the quantification limit, accuracy and linearity were reflecting the range of the method.

CONCLUSION

The developed method was validated with respect to linearity, precision, accuracy and specificity. The specificity of the method was established by performing forced degradation under different stress conditions. Hence, this stability indicating method is useful for the determination of related substances in ATV bulk drug substance and in the dosage form.

ACKNOWLEDGEMENT

The authors express their gratitude towards analytical research and development department, Ashland India Pvt Ltd, Hyderabad, Telangana for the support in terms of research facilities and samples.

CONFLICT OF INTERESTS

Declared None

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