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Research Article

A VALIDATED UHPLC METHOD FOR THE DETERMINATION OF ATORVASTATIN ACETONIDE TERT-BUTYL ESTER AND 4-FLUORO-ALPHA-(2-METHYL-1-OXOPROPYL)-GAMMA-OXO-N,BETA-DIPHENYLBENZENE BUTANEAMIDE

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ABSTRACT

This study aimed at developing and validating an Ultra high performance liquid chromatography (UHPLC) method for determination of assay and chromatographic purity of Atorvastatin Acetonide tert-Butyl Ester (ATV-I). An high performance liquid chromatography (HPLC) method with Zorbax SB C-18, 4.6 mm x 250mm, 5µ, column, mobile phase of buffer and acetonitrile, a flow rate of 1.0 ml/min, a UV detector set at 210 nm, gradient flow rate and with analysis time of 60 min had shown good chromatographic separation and results. The development of this method in UHPLC, with Zorbax SB C-18, 2.1 mm x 100mm, 1.8µ column and chromatographic conditions like, mobile phase comprising of buffer and acetonitrile, a flow rate of 0.7 ml/min, UV detector set at 210 nm, gradient flow rate has shown a reduction in the analysis time to 12min, with equal or better chromatographic separation. The degree of linearity of the calibration curves, the percent recoveries of DKT-III, the limit of detection (LOD), and limit of quantization (LOQ) for the UHPLC method have been determined. The UHPLC method under study was found to be specific, precise, accurate, linear, and robust. The rapid UHPLC method is suitable for reaction monitoring as well as analysis of final ATV-I within good manufacturing practices (GMP) of the pharmaceutical industry.

Keywords: Atorvastatin Acetonide tert-Butyl Ester; Butaneamide; UHPLC; Method validation; Chromatographic purity; Assay.

INTRODUCTION

In pharmaceutical industry the reaction monitoring procedure and cleaning sample analysis is considered as one of the most critical tasks. The delay in the analysis may lead to generation of unexpected new impurities. Analytical methods used to determine residuals or contaminants should be specific and fast for the substance or the class of substances to be assayed and be validated prior to its intended use in routine reaction monitoring at manufacturing facility [2-5].

Atorvastatin Acetonide tert-Butyl Ester (ATV-I), (4R,6R)-6-[2-[2-(4-Fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrol-1-yl]ethyl]-2,2-dimethyl-1,3-dioxane-4-acetic Acid 1,1-Dimethylethyl Ester, Mol. Formula: $C_{40}H_{47}FN_2O_5$, Mol. Weight: 654.81 and the structure is as given in figure 1, is an intermediate in the synthesis of Atorvastatin API (Active pharmaceutical ingredient) which is a synthetic hydroxy methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitor that has been demonstrated to be efficacious in reducing both cholesterol and trigliceride. 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 nonfamilial) or combine hyperlipidemia [1].

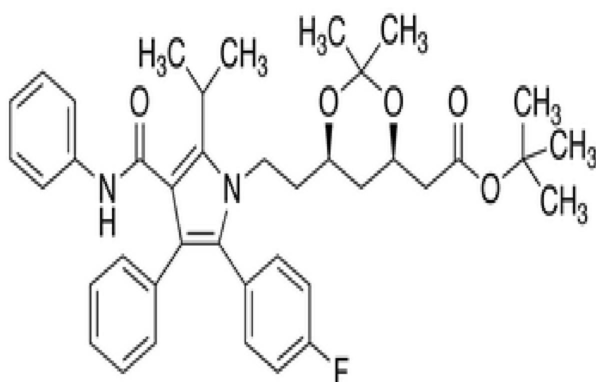

Figure 1: Structure of ATV-I

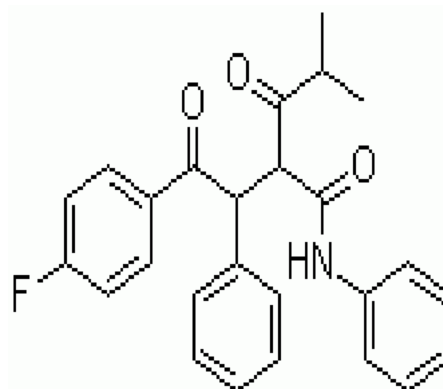

Figure 2: Structure of DKT-III

In accordance with Guidelines and recommendations thin layer chromatography (TLC), UV photometric, conductivity, gas chromatography (GC) and conventional high performance liquid chromatography (HPLC) methods for reaction monitoring are usually available at pharmaceutical manufacturing facilities [1,6].

Liquid chromatography–mass spectrometry (LC–MS) techniques applied in pharmaceutical reaction monitoring verification have the advantage of improved sensitivity, selectivity and general applicability even for UV-inactive compounds. However, these techniques are more expensive than the other techniques mentioned above and not widespread yet in reaction control analysis. Nowadays UHPLC–UV is the most commonly applied technique for cleaning control and validation [7-9]. In UHPLC, the analysis time can be reduced by using smaller columns packed with sub-1.8µm particles, which enhances the sensitivity compared to conventional HPLC as it has higher efficiency and smaller retention volume. However, extra column effects are more significant for scaled down separations, therefore it is essential to minimize extra column dispersion. A dedicated low dispersion system for ultra-high pressure separation (UHPLC) with the particle size of stationary phases reduced down to 1.8µm, small dwell and extra column volume is able to work up to 1000 bar (15,000 psi). In such a way

the analysis time could be reduced down from 60 min to 12 min, without the loss of resolution and sensitivity. In near future UHPLC systems with elevated pressure and/or temperature will replace the conventional HPLC gradually in all areas of liquid chromatography including pharmaceutical analysis.

The aim of this study was also to demonstrate the applicability of UHPLC to these purposes by developing, validating and applying an UHPLC/UV method to determine the residues of starting material such as 4-Fluoro-alpha-(2-methyl-1-oxopropyl)-gamma-oxo-N, beta-diphenylbenzene butaneamide (DKT-III), Mol. Formula: $C_{26}H_{24}FNO_3$, Mol. Weight: 417.47 and the structure is as given in figure 2, in support of good manufacturing facility of pharmaceutical.

As per our literature survey no articles can be found in the literature in which the HPLC or UHPLC determination of ATV-I are described and applied for reaction monitoring and analysis of final crystals of ATV-I.

EXPERIMENTAL

Reagents and solvents

Acetonitrile (Make-Spectrochem, Grade- HPLC) and Otho-phosphoric acid (Make-Rankem, Grade- HPLC). Ultra pure deionised water was freshly collected from a Milli-Q water purifier (Make-Millipore).

The reference materials and samples were produced by Biocon Ltd (Bangalore, India), having a purity of 99.8% and 99.9% for ATV-I and DKT-III respectively for reference materials and purity of ATV-I sample is of 99.0%. Agilent Zorbax SB-C18, 100 x 2.1mm, having 1.8µm particle size column was purchased from Agilent Ltd. The filters with pore size of 0.22µm (Millipore, USA) were used for the filtration of mobile phase and sample solution.

Equipment

High Performance Liquid Chromatography

Throughout the measurement an Agilent HPLC, 1200 series system equipped with quaternary gradient pump, auto sampler, column oven and variable wavelength detector (VWD) was employed Chromatographic data was acquired using Chemstation software (Agilent Ltd).

Ultra High performance liquid chromatography

An Agilent UHPLC system equipped with binary gradient pump, auto sampler, column oven and variable wavelength detector (VWD) was employed for analysis. Chromatographic data was acquired using Chemstation software Agilent Ltd.

Chromatographic conditions

Method 1

This method consisted of an Agilent SB-C18, 250×4.6 mm, 5µm column maintained at ambient temperature, with a flow rate of 1.5mL/min by varying the mobile composition at different time interval using gradient programme, with the mobile phase A consisted of 0.1% H_3PO_4 and B of acetonitrile and the gradient programme was as given in Table1. Injection volume was set to 10µL and the detector wavelength set at 210nm. The chromatogram is presented in figure 3.

Table 1: The gradient programme set in HPLC system

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	50	50
10	50	50
20	20	80
50	20	80
55	50	50
60	50	50

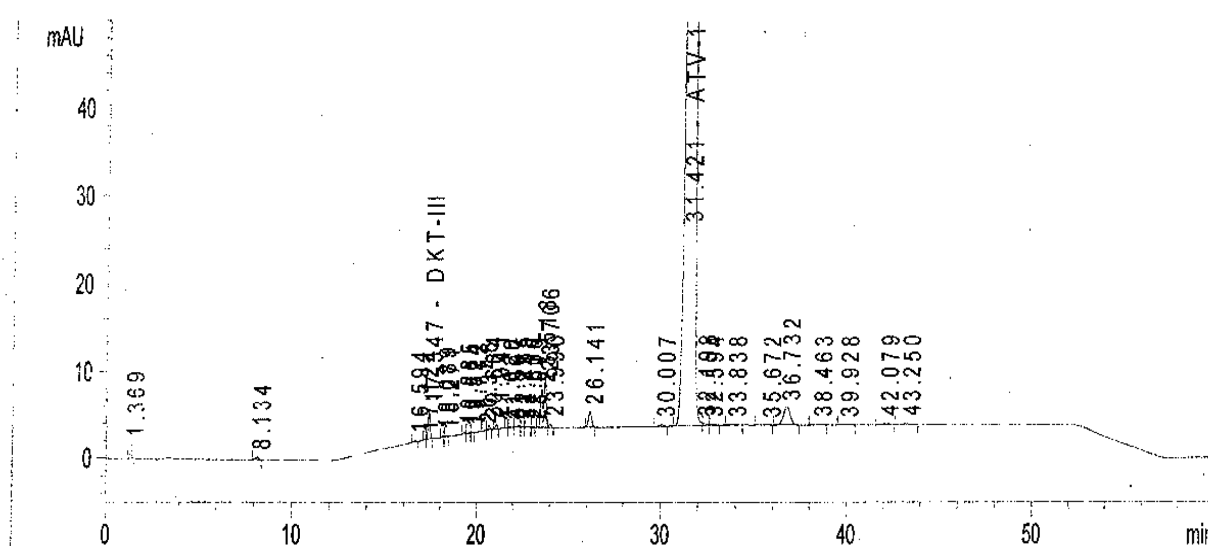

Figure 3: Chromatogram by HPLC.

Method optimization using HPLC to UHPLC calculator

The UHPLC method was obtained by converting the HPLC method using the Method

Translator and Cost Saving Calculator (Agilent Technologies).

Injection Volume

To finalize on the injection volume we need to know the column volume, which is calculated using the formula, $\pi r^2 L$

Where, π = 3.14, r is the internal radius of the column and L is the length of the column. Hence to calculate the injection volume the formula used is (table 3).

Table 3: Injection volume.

	Dimensions	Radius (r in cm)	Length (L in cm)	Column Volume (mL)	Original Injection Volume	Target Injection Volume
HPLC Column	250×4.6 mm, 5µm	0.23	25	4.15	10µL	-----
UHPLC Column	100×2.1mm, 1.8µm	0.105	10	0.35	-----	0.8µL

Target Injection volume = Original Injection Volume X Target column volume
Original column volume

Table 4: Flow rate

	Dimensions	Radius (r in cm)	Diameter (d)	Original Flow rate (mL/min)	Target Flow rate (mL/min)
HPLC Column	250×4.6 mm, 5µm	0.23	0.46	1.5	NA
UHPLC Column	100×2.1mm, 1.8µm	0.105	0.21	NA	0.31

Flow Rate

To calculate the flow rate the formula used is (table 4).

$$\text{Target Flow rate} = \text{Original Flow rate} \times \frac{d^2\ \text{target}}{d^2\ \text{original}}$$

Where, d is the internal diameter of the column

Gradient Profiling

Express gradient duration in percent change per column volume (cv) units, Calculate each segment as a number of column volumes and calculate the time required to deliver the same column volumes to the target column at chosen flow rate. Table 5 depicts the original gradient programme.

Gradient program expressed as column volumes

Gradient volume = flow rate x time

Column volume = $\pi r^2 L$

$$\textbf{Gradient duration (cv)} = \frac{\text{Gradient volume}}{\text{Column volume}}$$

Table 5: Original gradient program

Step	Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow Rate	Segment duration (min)	Segment duration (cv)
Initial	0	50	50	1.5	0	0
2	10	50	50	1.5	10	3.6
3	20	20	80	1.5	10	3.6
4	50	20	80	1.5	30	10.8
5	55	50	50	1.5	5	1.8
6	60	50	50	1.5	5	1.8

Gradient program expressed as column volumes

Gradient volume = flow rate x time

Column volume = $\pi r^2 L$

$$\textbf{Gradient duration (cv)} = \frac{\text{Gradient volume}}{\text{Column volume}}$$

Scaling gradient step time

Original step 2 (original HPLC method gradient step 2) = 10min @ 1.5mL/min with duration of cv

Calculate target step 2 (Target UHPLC method gradient step 2) – Keep the duration as cv

Gradient step vol = Duration (cv) X Target column volume

$$\textbf{Gradient Step time} = \frac{\text{Gradient Step Volume}}{\text{Flow rate}}$$

Adjust time for same number of column volumes per gradient segment Scaled gradient profile (table 6)

Table 6: Scaled gradient profile

Step	Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow Rate	Segment duration (min)	Segment duration (cv)
Initial	0	50	50	0.31	0	0
2	3.87	50	50	0.31	3.87	5.29
3	7.87	20	80	0.31	4.00	5.47
4	19.87	20	80	0.31	12.00	16.41
5	21.87	50	50	0.31	2.00	2.74
6	23.87	50	50	0.31	2.00	2.74

Even though this theoretical approach is the tool for us to convert a method from HPLC to UHPLC but it was not considered exactly, several trials like change in column brand, change in flow rate and changes in the gradient programme were done to get the best separation and shorter run time as our main aim was to reduce the analysis time without affecting the quality of the analysis. Finally the method 2 was considered as the final method for our study and it is evident from the results of various validation parameters that the method is suitable for the intended purpose in routine analysis of the sample.

Method 2

This method consisted of an Agilent Zorbax SB-C18, 100×2.1mm, 1.8µm column maintained at ambient temperature, with a flow rate of 0.7mL/min by varying the mobile composition at different time interval using gradient programme, with the mobile phase A consisted of 0.1% H_3PO_4 and B of acetonitrile and the gradient programme was as given in Table 2. Injection volume was set to 2µL and the detector wavelength set at 210nm. The chromatogram is presented in figure 4.

Table 2: The gradient programme set in UHPLC system.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.00	50	50
3.5	20	80
10.0	0	100
10.5	20	80
11.0	50	50
12.0	50	50

CHROMATOGRAPHIC PURITY

Solution preparation

Standard stock solution

Individual standard stock solution of ATV-I and DKT-III was prepared separately by dissolving appropriate amounts of the compounds in acetonitrile (0.1mg/mL).

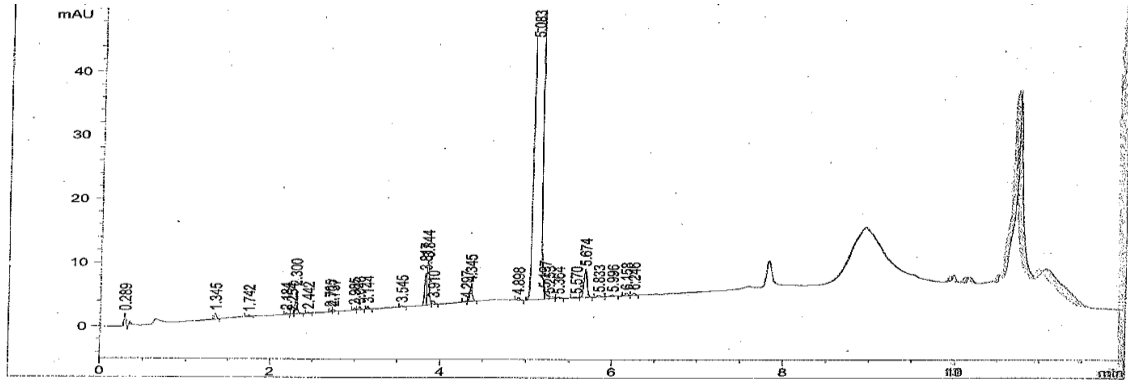

Figure 4: Chromatogram by UHPLC

System suitability solution

System suitability solution consisting of ATV-I and DKT-III was prepared by dissolving appropriate amounts in acetonitrile (0.001mg/mL) (Found to be stable for 48 hours if stored at 2°C-8°C).

Sample solution

ATV-I sample: Weigh accurately 0.025g of the ATV-I sample into 50mL volumetric flask dissolve and dilute to volume with acetonitrile.

Validation plan and execution

System suitability was checked by injecting the system suitability solution for parameter such as resolution and RRT. The specificity of the method was determined by injecting the diluent and matrix blanks to check the interference at the retention time of DKT-III and ATV-I.

Specificity

By the specificity study it was proved that the method for the determination of the active residues is selective and has no interfering effects. This was demonstrated by injecting the diluent blank, reference solution and matrix blanks leading to the conclusion that there is no interfering peak in the chromatogram obtained from diluent blank and matrix blank solution at the retention time corresponding to DKT-III and ATV-I

The retention time of DKT-III and ATV-I is about 2.3 minutes and 5.1 minutes respectively. The relative retention time of DKT-III with respect to ATV-I is about 0.45. Resolution between DKT-III and ATV-I should not be less than 35.0, resolution achieved is of 54.6. From the above discussion it is concluded that the method is specific.

Linearity of response

For each compound the linearity of responses was assessed by injecting standards from LOQ concentration to 200% which were prepared in acetonitrile. Working concentration for the ATV-I is 0.5mg/ml and target concentration DKT-III is 0.0025mg/ml as a limit level concentration.

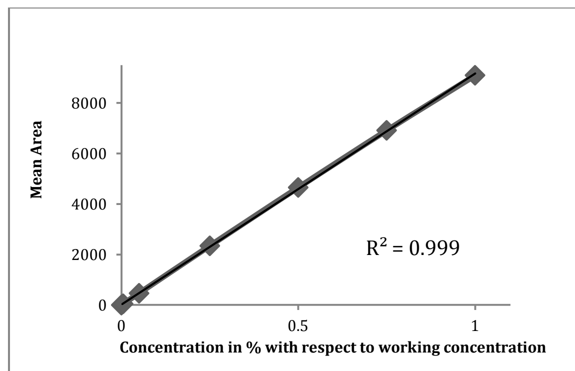

Figure 5: ATV-I Linearity

The results were analyzed by linear regression. The correlation coefficients, r^2, were found greater than 0.999 for both ATV-I (figure 5) and DKT-III (figure 6). %Y intercept at 100% concentration level for DKT-III is 0.4 and ATV-I is found to be 1.7 and ±5 criterion was successfully accomplished and the values were found to be satisfactory.

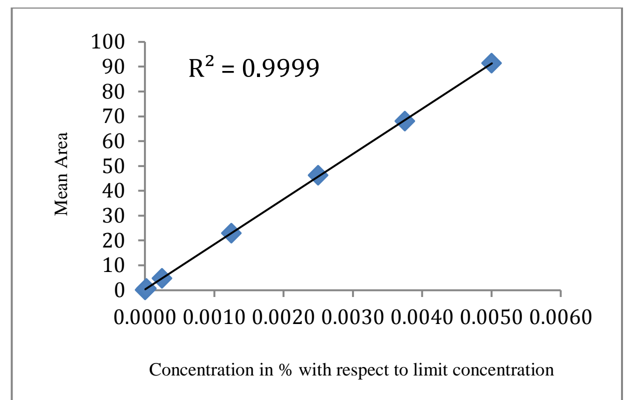

Figure 6: DKT-III Linearity

Range

Demonstration of the range at LOQ, 1%, 10%, 100% and 200% of ATV-I and DKT-III with respect to target/limit level concentration, different concentration of ATV-I and DKT-III was prepared and injected in 6 replicates (table 7).

Table 7: Concentration –LOQ, Linearity and Range. % RSD at each selected level.

ATV-I		DKT-III	
Concentration in % with respect to sample concentration	%RSD (NMT 5.0%)	Concentration in % with respect to sample concentration	%RSD (NMT 5.0%)
0.0125	2.693	0.0012	2.795
1.0	0.250	0.10	1.025
50.0	0.079	0.25	0.329
100.0	0.052	0.50	0.115
200.0	0.060	1.00	0.117

Accuracy

Known amount of sample was taken separately into nine different flasks and spiked with known quantities of DKT-III at three different levels, each in triplicate. The samples were analyzed by the proposed method and the amount of DKT-III recovered after makingcorrections for the amount already present were calculated. Data shown in Table 4 indicate that the method has an acceptable level of accuracy. (Acceptance criteria: Recovery should be in the range of 80%–120%).

Table 8: Accuracy for DKT-III

Recovery level	DKT-III Amount added mg/mL	Amount obtained mg/mL	Recovery %
Level-1 Rec-1	0.000255	0.000246	98.5
Level-1 Rec-2	0.000251	0.000242	96.5
Level-1 Rec-3	0.000252	0.000240	95.3
Level-2 Rec-1	0.002515	0.002465	98.0
Level-2 Rec-2	0.002502	0.002407	96.2
Level-2 Rec-3	0.002509	0.002449	97.6
Level-3 Rec-1	0.005026	0.005005	99.6
Level-3 Rec-2	0.005015	0.004930	98.3
Level-3 Rec-3	0.005006	0.004936	98.6

Precision

System precision

Standard mixture: 0.1 mg/ml of ATV-I and DKT-III was prepared and injected into the chromatograph in 6 replicates. Using the statistical analysis %RSD of area responses of ATV-I and DKT-III were calculated. The % R.S.D. of results (n=6). For DKT-III and ATV-I was found to be 0.10.

Method precision

The method precision was demonstrated by estimating the % content of DKT-III, any individual impurity and total impurity from 6 replicate preparations and duplicate injections of a homogenised sample of the same batch prepared with a concentration of 0.5mg/mL (Figure 7). In ATV-I intermediate stage the DKT-III, any individual impurity and total impurities was found to be 0.063%, 0.235% and 0.909% with %RSD values of 1.353, 0.265 and 0.144 respectively.

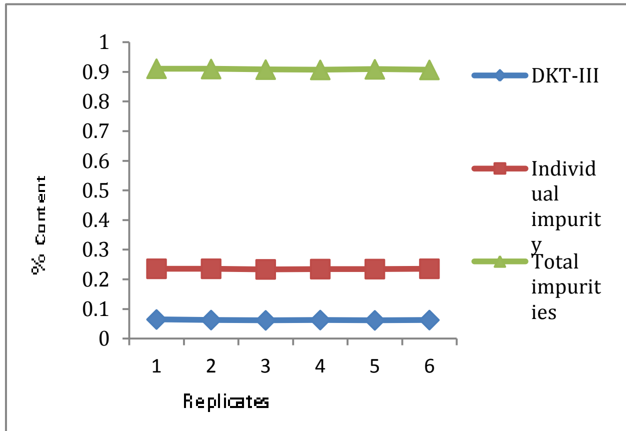

Figure 7: A trend chart for the method precision.

Limit of quantitation and Limit of detection

Limit of Quantitation

Concentration of DKT-III and ATV-I where S/N is 10 are 0.0012% with respect to sample concentration and 0.0125% with respect to sample concentration respectively.

Precision was demonstrated at LOQ concentration at which for DKT-III and ATV-I, by injecting replicates (n=6). %RSD of DKT-III and ATV-I were 2.795 and 2.693 respectively. The %RSD was well within the limit of 10.

Limit of Detection

Concentration of DKT-III where S/N 3 was 0.00038% with respect to sample concentration and concentration of ATV-I where S/N is 3 was 0.0038% with respect to sample concentration. Quantitation limits (LOQ) were determined by the %RSD of six repeated injections of standard solutions. And detection limits (LOD) were another term for LOD – in the laboratory – it is preferred to be lower than the 50% of analytical limit. The sensitivity of the method is proved to be sufficient for each compound.

Solution Stability

Standard Solution Stability

Standard preparation was injected at different time intervals during the course of experiment and recorded the peak area responses of ATV-I standard. Result is compiled as a trend chart of the % Cumulative RSD at different time intervals with that of the initial system precision values for the standard solution stability (Figure 8).

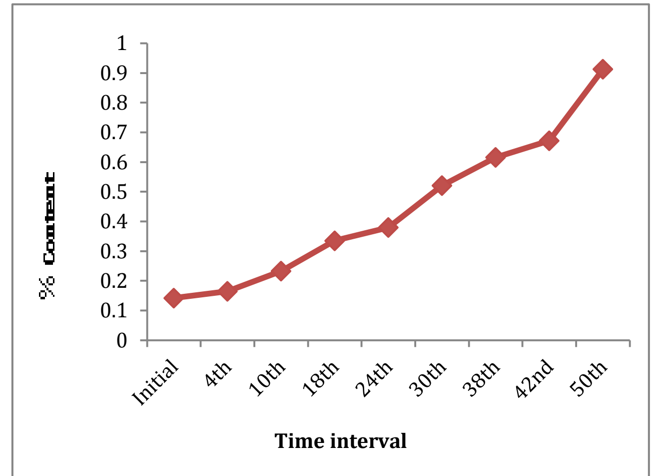

Figure 8: A trend chart of the % Cumulative RSD at different time intervals with that of the initial system precision values for the standard solution stability.

Sample Solution Stability

Prepared a fresh sample as per methodology and studied at different time intervals during the course of experiment and recorded the chromatograms. A trend chart was compiled for the % content of DKT-III, individual impurity and total impurity at different time intervals (figure 9).

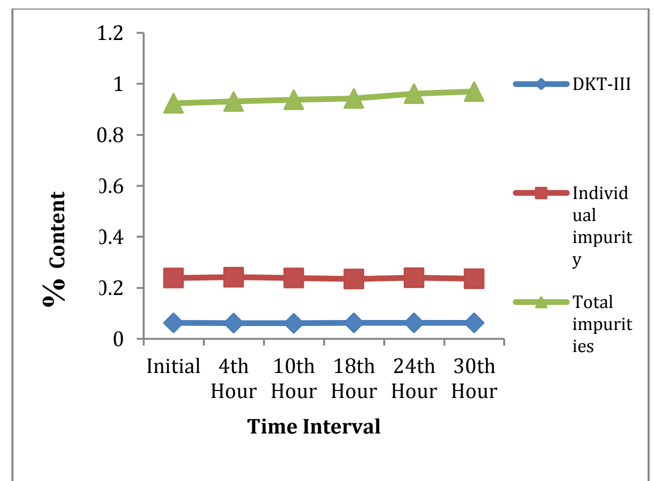

Figure 9: A trend chart for the sample solution stability.

Mobile Phase Solution Stability

Blank and resolution solutions were injected at different interval, and recorded the chromatogram. Resolution between DKT-III and ATV-I at different time intervals for the mobile phase is been recorded and compiled in a trend chart (Figure 10).

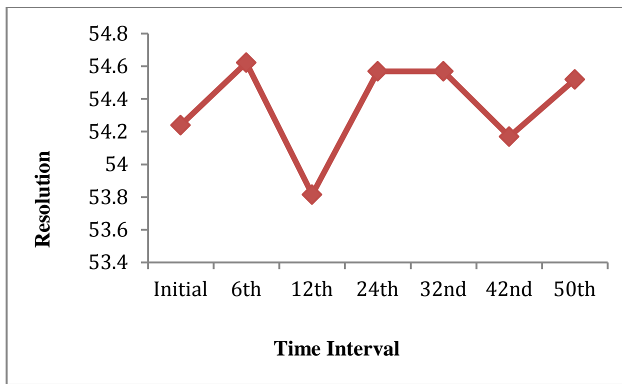

Figure 10: A trend chart of the resolution at different time intervals for the mobile.

Phase solution stability

Robustness

In order to check the robustness of the UHPLC method some deliberate variations were done in method parameters like change in flow rate to 0.6mL/min and 0.8mL/min from 0.7mL/min, change in the temperature of the column oven thermostat to 23°C and 27°C from 25°C. The parameters like system suitability and precision were evaluated during the Robustness study under all the deliberate variations. Figure 11 is the trend chart of the retention time of ATV-I and DKT-III and Figure12 for the trend chart of the %RSD for the area response of ATV-I standard for different robustness conditions and also Figure 13 is a trend chart for the % Cumulative RSD of Initial method precision values with values at different robustness conditions.

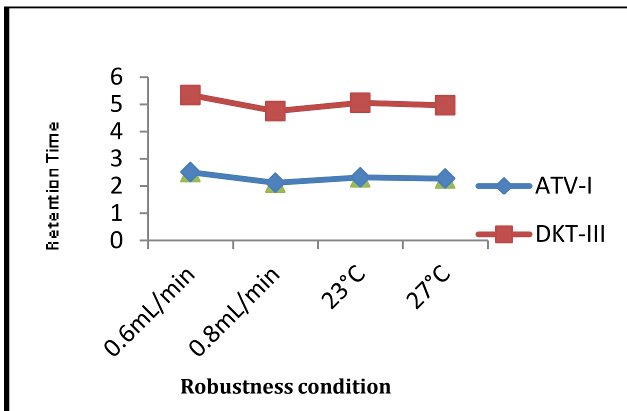

Figure 11: A trend chart of the retention time of ATV-I and DKT-III for different robustness conditions.

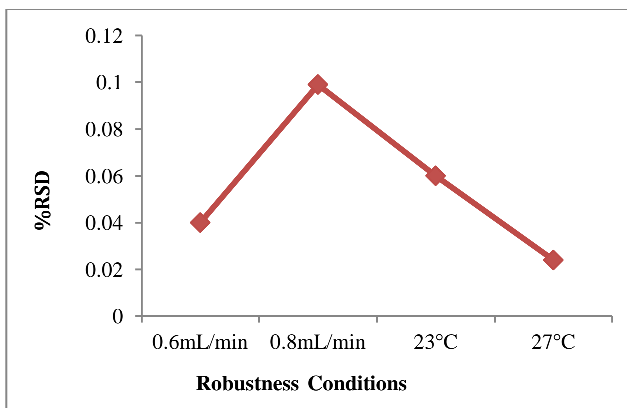

Figure 12: A trend chart of the %RSD for the area response of ATV-I standard for different robustness conditions.

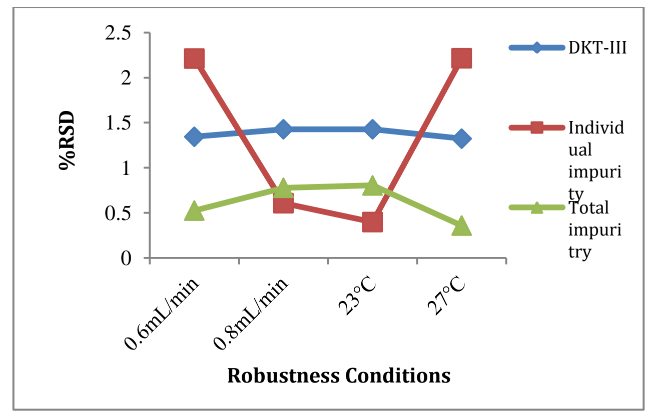

Figure 13: A trend chart for the % Cumulative RSD of Initial method precision values with values of different robustness conditions

ASSAY

The chromatographic conditions for the assay method stand same as that of the chromatographic purity method.

Solution preparation

System suitability solution

System suitability solution consisting of ATV-I and DKT-III was prepared by dissolving appropriate amounts in acetonitrile (0.0001mg/mL). (Found to be stable for 48 hours if stored at 2°C-8°C.)

Standard solution

Standard solution of ATV-I prepared by dissolving appropriate amounts of the compounds in acetonitrile (0.5mg/mL).

Sample solution

Weigh accurately 0.025g of the ATV-I sample into 50mL volumetric flask dissolve and dilute to volume with acetonitrile.

Validation plan and execution

System suitability was checked by injecting the system suitability solution for parameter such as resolution and RRT. The specificity of the method was determined by injecting the diluent and matrix blanks to check the interference at the retention time of DKT-III and ATV-I.

Method validation

The method validation was performed in accordance with the ICH guidelines [7].

Specificity

By the specificity study it was proved that the method for the determination of assay is selective and has no interfering effects.

Linearity of response

As the chromatographic conditions for assay and chromatographic purity are same, the linearity performed under chromatographic purity (from LOQ to 200% with respect to working concentration) covers the range of 80% to 120% with respect to working concentration as per assay sample concentration also.

Precision

System precision

ATV-I standard (0.5mg/mL) was prepared and injected into the chromatograph in 6 replicates. The peak area response was evaluated statistically by calculating the %RSD for 6 replicate injections and was found to be 0.097%.

Method precision

The method precision was demonstrated by estimating the content assay of ATV-I from 6 replicate preparations and duplicate injections of a homogenised sample of the same batch prepared with a concentration of 0.5mg/mL.

The content assay of ATV-I was found to be 99.0% with %RSD value of 0.124, Figure 14 shows the trend chart of the results of replicate sample preparations.

Solution Stability

Assay of sample was monitored up to about 30 hour. Refer figure 15 for the trend of assay content at different intervals and figure 16 for % cumulative RSD of assay at different time intervals with respect to initial values.

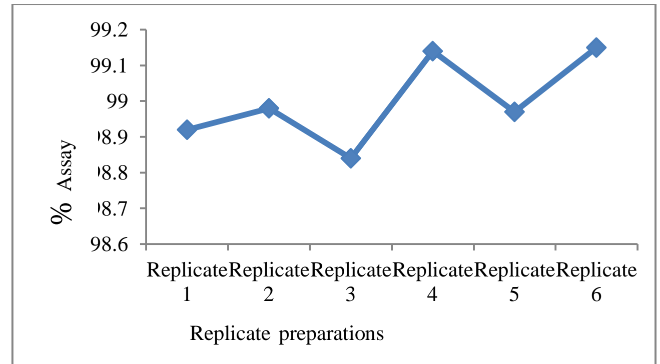

Figure 14: A trend chart for the content assay of ATV-I for replicate sample preparations

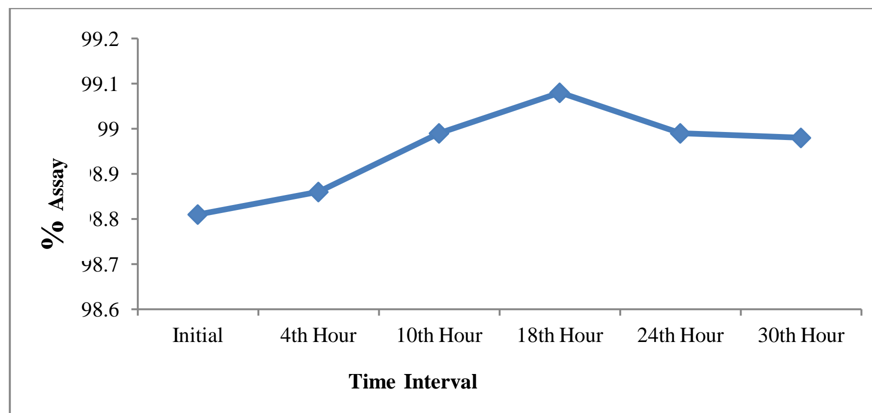

Figure 15: A trend chart for the content assay of ATV-I at different time interval- the sample solution stability.

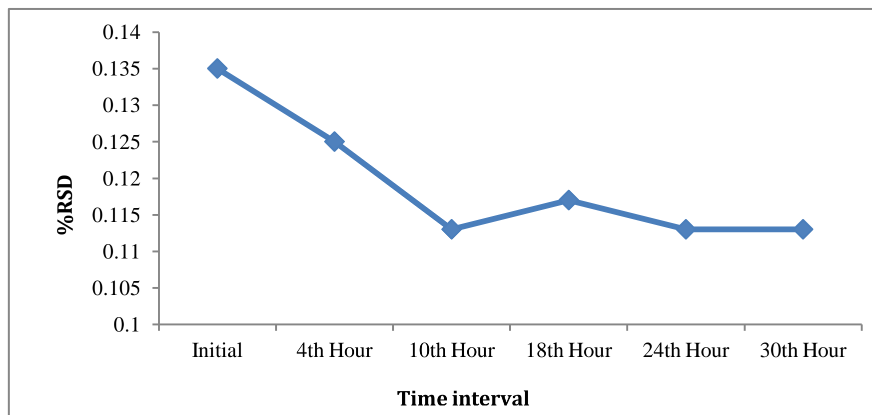

Figure 16: A trend chart of the % Cumulative RSD at different time intervals with that of the initial method precision values for the sample solution stability.

Robustness

In order to check the robustness of the UHPLC method some deliberate variations were done in method parameters like change in flow rate to 0.6mL/min and 0.8mL/min from 0.7mL/min, change in the temperature of the column oven thermostat to 23°C and 27°C from 25°C. The content assay was evaluated during the Robustness study under all the deliberate variations. Refer figure 17 for the trend chart for the content assay of Initial method precision values with values various robustness conditions.

Results and discussion

The migration of assay and chromatographic purity determination in ATV-I from HPLC to UHPLC was successful based on the facts and results of the various validation parameters considered.The entire exercise is considered as successful because of the impact of the method on the decrease in the time for results release and consumption of the solvents for the analysis. As the UHPLC method consists of the column with smaller particle size the resolution and the peak symmetry is increased which raises the efficacy of the results.

The data evidently shows that the method adopted to estimate the assay of ATV-I, % content of DKT-III, any individual impurity, and total impurity in ATV-I sample is specific for intended purpose.

Data from the precision exercise indicates that the method shows consistent repeatability.

The linearity of the method is determined in the range of LOQ to 200% with respect to working concentration using different concentrations of ATV-I, the Linearity regression co-efficient for ATV-I was found to be 0.999.

It is observed that the method is specific and precise even though there is change is flow rate (±0.1ml/min) and column oven temperature (±2°C).

Stability of sample solution, standard solution and resolution solutions was evaluated for a time period of up to 50 hours but at the 30th hour two unknown impurities were found at the RRT at 0.899 and 0.917 with respect to ATV-I in the sample but the assay of ATV-I was unaffected, so it was concluded that the solutions were stable up to 24 hours without any increase in degradation product

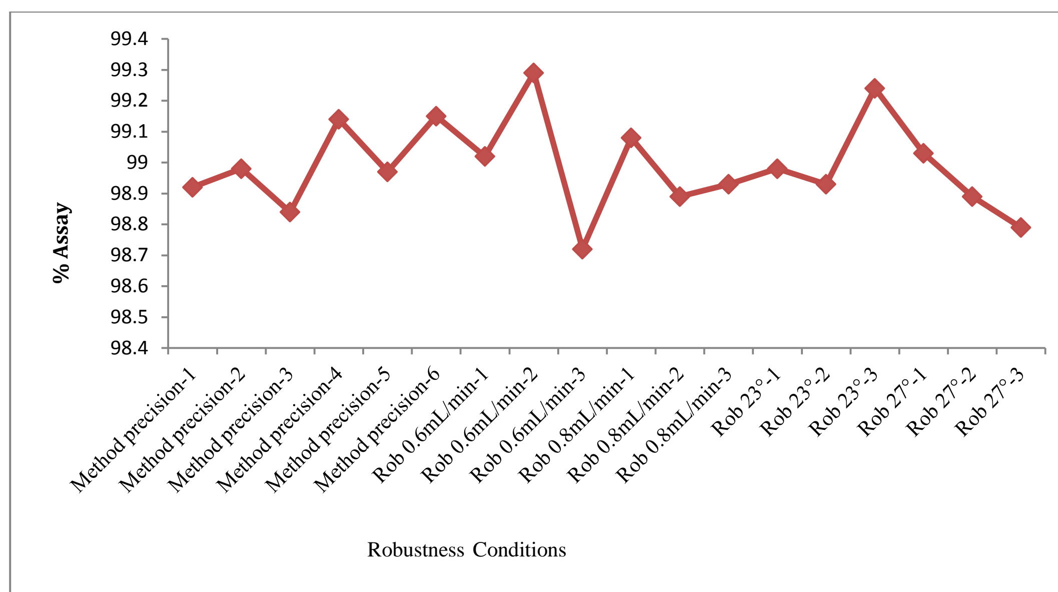

Figure 17: A trend chart for the content assay of Initial method precision values with values of various robustness conditions.

CONCLUSION

The elaborative study leads us to a conclusion that the UHPLC method for determination of assay and process related impurities of ATV-I is practical in quantifying the API intermediate and its residues. The saving of time and solvents makes this UHPLC method more advantageous than conventional HPLC technique. The enhanced sensitivity of the UHPLC–UV method compared to conventional HPLC does not necessitate the use of a mass spectrometry detector, which is expensive and not widespread in quality control analysis. Good agreement was seen in the assay results by developed method. We concluded that the proposed method is a good approach for obtaining reliable results and found to be suitable for the routine analysis of ATV-I.

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