Identification and Validation of Genotoxic Impurity in Ezetimibe by Reverse Phase High-Performance Liquid Chromatography

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ABSTRACT

The method emphasizes identification and validation of potential Genotoxic impurity in pharmaceutical drug substances of Ezetimibe by Reverse Phase High-Performance Liquid Chromatography (HPLC). The impurity was separated by using the Zorbax Rx Octylsilane (C8) HPLC column with 250 cm length and internal diameter of 4.6mm with pore size 5 µm. The partition of impurity was operated at a significant pH 3.0 was maintained by buffer of 10% potassium dihydrogen phosphate and Acetonitrile with 80:20 ratio and the mobile phase is Acetonitrile with a gradient inflow of 1.5 mL/minute. The UV absorption maximum were observed at 258nm. The proposed approach shows the results of linear boundaries in between 0.16 µg/g to 7.5 µg/g with correlation coefficient is lower than 0.999. The method was further evident by accuracy results are in the region of 98.82% to101.04% for Genotoxic impurity of (5R, 6S)-1-(4-fluorophenyl)-5-((S)-3-(4-fluorophenyl)-3-hydroxypropyl)-3-(2-hydroxy-1-phenylethyl)-6-(4hydroxyphenyl)di-hydropyrimidine-2,4(1H,3H)-dione. The approach was shown acceptable results as per International Council of Harmonisation (ICH) guidelines and the method was operated even at lower concentrations.

**Keywords:** Genotoxic impurity, Ezetimibe, HPLC, ICH guidelines, Cholesterol related disorders.

INTRODUCTION

Ezetimibe is an active pharmaceutical ingredient was used as a cholesterol penetration blockage and then it was segregated a new classification of its kind. Its chemical name is (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone and its structure was shown in Fig. 1. The most significant function is dietary and biliary cholesterol absorption potentially inhibits by Ezetimibe[1] from human intestine without affecting vitamins present in the fat[2]. The intake of cholesterol leads to malfunctions of intestinal lumen cholesterol evacuation and lower the absorption of small intestinal enterocyte[3]. The supported clinical trial results evident for the treatment for several cholesterol related disorders like hypercholesterolemia, homozygous familial and homozygous sitosterolemia[4,5]. This drug was commercially available in different forms of

either individual or combinations other biological ingredients at various concentrations manufactured by different pharma companies.
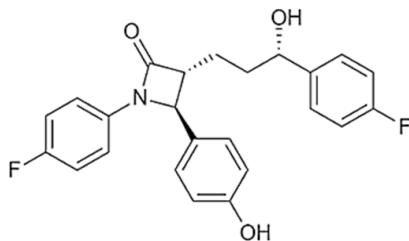
(3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)azetidin-2-one

Fig. 1. Ezetimibe structure with IUPAC name

As per the recent release guidelines of ICH M7 emphasizes the risk assessment of Genotoxic impurities in drug substances and drug products makes more caution towards their usage in several treatment. The word Genotoxicity is very familiar to pharma industries and regulatory authorities because of toxic nature when it was reacted with cells in the body it may leads to several change in biological reactions including mutations does not lead to heritability modifications. The significant importance was given to mutagenicity testing of any drug sample of API enlightened information on various genetic changes in cells, this information further useful to regulatory authorities for their approvals. In the Present investigation describes the presence of one Genotoxic impurity in Ezetimibe drug substance usually formed during their synthetic process.

There were various analytical methods[6-12] were available for the estimation of assay and related compounds of Ezetimibe in both active pharmaceutical ingredient and drug substances by using Liquid Chromatography connected with Mass Spectrometry[13], High-Performance Thin Layer Chromatography[14], High-Performance Liquid Chromatography, Ultra Performance Liquid Chromatography[15] and Ultra-Performance Liquid Chromatography Mass Spectrometer[16] (UPLC-MS/MS) & Liquid Chromatography tandem Mass spectrometric[17] (LC-MS/MS) techniques. The pharmacopeia[18] directed researchers for the identification and quantification for the various impurities found in the drugs and the process related impurities including degradation impurities. Some of the literature studies related impurities by HPLC and LCMS[19-21] reported in Ezetimibe drug

substance. There was no reported analytical method for identified Genotoxic impurity in Ezetimibe Active Pharma Ingredient (API) by using High Performance Liquid Chromatographic technique.

MATERIAL AND METHODS

Materials

The chemicals used in this method are acetonitrile (HPLC grade, Purity-99.9%) and Ortho phosphoric acid (GR grade, Purity-85.0%) purchased from Merck Ltd., India. Ultra-pure water is used for this method from Milli-Q water purification system (Millipore USA). The samples of standard of Impurity-A and Ezetimibe were purchased from Toronto research chemicals, Canada were used for this entire study. All dilutions were performed in the standard volumetric flask.

Conditions of chromatographic method

For determination of Ezetimibe Genotoxic impurity from the experimental study on Agilent manufactured high performance liquid chromatograph equipped with Sample manager, Quaternary solvent manager, column heating compartment & variable wavelength detector (VWD), with a Empower control software. The chromatographic requirements are Zorbax RxC8 HPLC column is used with dimensions; 0.25mX4.6mmX5µm. All quantitative samples are weighted on Sartorius semi micro analytical balance along with Bandelin sonicator and Thermo pH meter, were used for dissolving the standard sample & buffer pH adjustments and finally, Hermle centrifuge machine was used for centrifuging of turbidity components.

Mobile phase standard and sample solutions preparations
Preparation of Buffer

2.7 g of Monopotassium phosphate (KH_2PO_4) dissolved (weigh to accuracy of 0.001 g) into half-filled with water of 1000 mL beaker and then completely dissolved with remaining portion with water; adjust the volume to pH 3.0±0.05 with diluted 10% phosphoric acid. Filter the solution through cartridge filter for removal of undissolved particles and degas the solution for 10 min in sonicator instrument.

Mobile phase preparation
Mobile phase Preparation-A

Mix Acetonitrile and Buffer in composition of 20:80(v/v) respectively.

Mobile phase Preparation-B
Pure Acetonitrile as used.

Preparation of diluent
　All solutions have been prepared by acetonitrile as a diluent.

Standard stock solutions preparation
　Weighed and transferred about 10.0 mg of Imp-A standard into a 100 mL flask and dissolved in 50mL of diluent by sonication, then diluted volume with diluent. Took 2.5 mL of this solution fed in to 50 mL of volumetric flask and further make up to the mark with diluent whose concentration is 5 µg/mL of Imp-A.

Solution of system checks standard preparation
　Pipetted out 5mL of above aliquots (standard stock solution) and transferred into 50mL flask. Diluted and makeup with diluent up to the mark. The resulted system checks solution concentration was 0.5µg/mL.

Sample solution preparation
　Accurately weighted 0.1 mg of Ezetimibe sample and transferred into 5 micro litter flask and added 2.5 micro litter of diluent and dissolved the components for 10 min by sonication. Later, this solution makeup with diluent up to mark.

RESULTS AND DISCUSSIONS

Method development
　Based on the route of synthesis of Ezetimibe drug substance and analysed the purity by using agencies published monograph HPLC method but there are no additional peaks were observed. As part of internal development works, a short run time HPLC method has been developed and which gave the clue for peaks in chromatogram. A new peak has been observed and it was well-separated from main peak, labelled as impurity-A. During sample analysis the missing assay was observed. For impurity-A structure confirmation (Fig. 2) and LC-MS analysis has been performed and the base peak of m/z 572 showed in mass spectra with positive mode of ESI-MS (Electro Spray Ionization Mass Spectrometry) technique are shown in Fig. 3 and Fig. 4 shows the possible way of fragmentation for the formation of various ions.
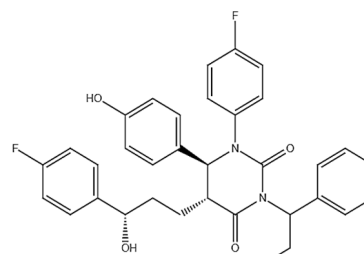
(5R, 6S)-1-(4-fluorophenyl)-5-((S)-3-(4-fluorophenyl)-3-hydroxypropyl)-3-(2-hydroxy-1-phenylethyl)-6-(4hydroxyphenyl)di-hydropyrimidine-2,4(1H,3H)-dione

Fig. 2. Impurity-A structure with IUPAC name
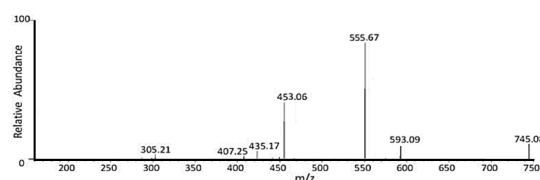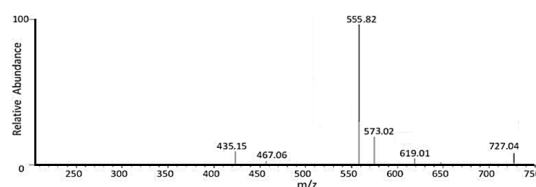
Fig. 3. Mass spectra and MS-MS spectra of Impurity-A
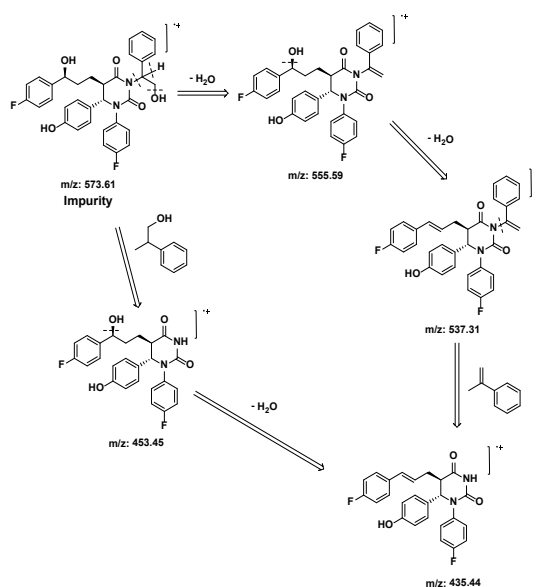
Fig. 4. Mass fragmentation pathway of Impurity-A

　The analytical method was optimized (Table 1) to estimate the Genotoxic impurity of Impurity-A in the Ezetimibe drug substance by using HPLC equipment. The key parameters like injection volume, column oven temperature, selection of wave length, diluent, HPLC column, flow rate and mobile phase.

Table 1: Optimized Chromatographic Conditions

Chromatographic Conditions	
Column	ZorbaxRxC8 HPLC column
Column oven temperature	37°C
Flow rate for instrument	1.5 mL/min
Injection volume	30 µL
Wavelength	258 nm
Run time	50 min
Buffer solution	For pH=3 optimized by combination of Potassium dihydrogen phosphate with water & 10% phosphoric acid
Mobile-phase A	Buffer 80%:Acetonitrile 20%
Mobile-phase B	Acetonitrile
Diluent	Acetonitrile

Wavelength selection

Impurity-A standard solution was prepared by using diluent and the obtained solution concentration was~0.5 µg/mL. This solution was injected by using High-performance liquid chromatographic instrument with variable detector of Photo Diode Array detector and absorption maxima of impurity-A was shown about 258.88nm. Hence, we quantified this impurity at 258nm in Ezetimibe drug substance. The obtained spectrum of impurity-A was presented in Figure 5.
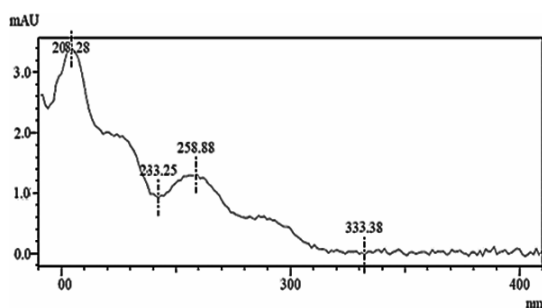

Fig. 5. Absorption spectrum of Impurity-A

Column selection

Based on the packing material, internal diameter, length and particle size of the column, various experimental trails were made for column selection. Finally, good peak separation was achieved in ZorbaxRxC8 HPLC column.

Selection of mobile phase

Initial trials, we have started with combination of water and methanol as a mobile phase in the different ratio, in all trails the interested component of impurity-A peak was not eluted. Later modified the organic phase in different proportions with acetonitrile. The experiment was continued and it was observed that impurity-A peak has more tailing factor and peak shape was broad. For improving of impurity-A peak shape, milli-Q water was substituted with buffer of phosphate and experiment was continued with different portions of acetonitrile. Mixture of mobile phase composition was finalized after various experiments and as the gradient programme contain mobile phase A mixture of acetonitrile and buffer pH3.0±0.05 (2.7 g of Potassium monophosphate in 1000 mL water, adjust pH3.0 with 10% phosphoric acid) of 20:80(v/v) respectively and mobile phase B acetonitrile.

Selection of column oven temperature

After several trials with different column oven temperatures, we found that the optimum temperature to attain accuracy results at 37±1°C column temperature and also suite for the well separated and the reproducible results.

Selection of flow rate

For efficient separation with good resolution of chromatograms the optimized the mobile phase inoculation rate is in between 0.5 mL/min to 2.0 mL/minute. The correct rate of inflow was set at 1.5 mL/min was gave separation chromatogram.

Selection of injection volume

For good chromatogram, optimized the injection volume from 5-50µL. finally, we found that 30µL was gave separation chromatogram.

Selection of diluent

The Ezetimibe drug substance and Imp-A solubility were checked in different solvents like methanol, deionized water, buffer solution of mobile phase-A, acetonitrile and the aquas combinations of all above said solvents in different proportions. Good solubility was observed in acetonitrile. So, Acetonitrile was selected as diluent for the optimization of analytical method.

Analytical methodology and validation

A High-performance liquid chromatographic with Reverse phase approach was established and the method was validated for the quantification of Imp-A in Ezetimibe API was validated based on ICH and USP guidelines. Specific validation parameters were experimentally evaluated by Ezetimibe sample solution injected along with imp-A standard solution.

System suitability

The standard solutions of impurity-A was prepared at a strength of 0.5 µg/mL and took 30 µL of this solution was inoculated in to the HPLC system for about six times repetitions to check the correctness the approach by studying their results are tailing factor, theoretical plate counts and %RSD

are in acceptance with ICH guidelines and these are tabulated in Table 2 and their chromatogram is shown in Figure 6.

Specificity

The developed method for specificity was established by injecting blank, impurity standard solution, Ezetimibe drug substance, and impurity spiked solution. The observed chromatograms are no interference peak for blank, an elution was observed at 15.621 min for impurity, at below 10 min elution for Ezetimibe sample and elution at 15.520 min for spiked samples. Specificity parameter executed chromatograms were presented in Figures 7 to 10.
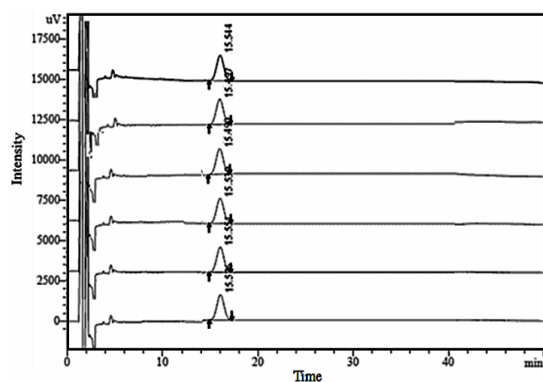

Fig. 6. Overlay Chromatograms of six replicate standard injections

Table 2: System suitability of six replicate standard injections

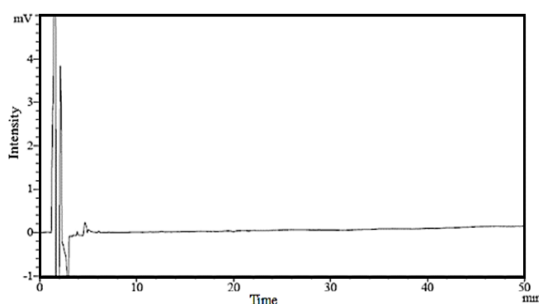| Injection S. No	Retention time in minutes	Area
1	15.524	43872
2	15.554	43541
3	15.532	42986
4	15.492	43128
5	15.497	44301
6	15.544	43674
	Mean	43584
	Standard deviation	484.2564
	%RSD	1.1111
	Tailing factor	1.06

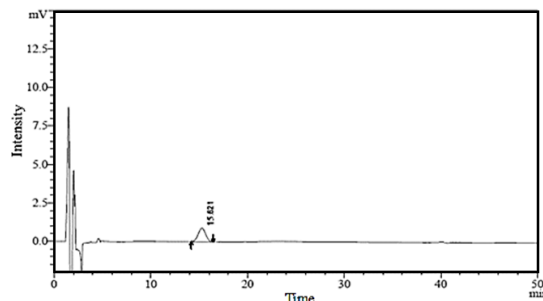**Fig. 7. Blank chromatogram**

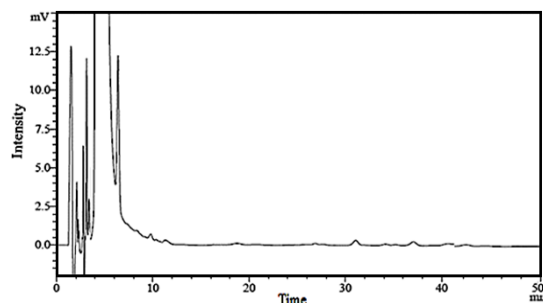**Fig. 8. Impurity-A chromatogram**

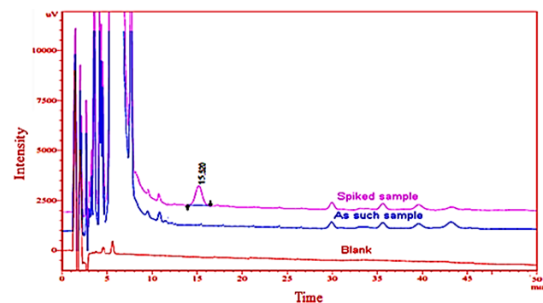**Fig. 9. Ezetimibe 100% sample chromatogram**

Fig. 10. Overlay chromatograms of Blank, Ezetimibe and Spiked samples Limit of impurity

Detection & Quantification (LOD & LOQ)

For impurity-A, LOD and LOQ was performed by S/N ratio method and preparing various concentrations of impurity-A solution and evaluated with HPLC instrument. The LOD concentration of the impurity (0.167 µg/g) was determined by observing that the s/n (signal-to-noise) ratio was approximately 3:1 shows 3.15. The LOQ concentration of the impurity-A (0.506 µg/g) was determined by observing an S/N ratio of approximately 10:1 shows 10.63 and their respective chromatograms are Figure 11 and 12.

Precision at LOQ level

Impurity-A solution was prepared at LOQ concentration level for determination impurity precision and six replicates were injected into developed method conditions. The calculated %RSD for area of six injections and calculated values were summarized in Table 3.
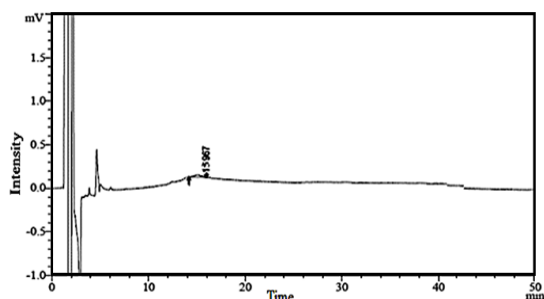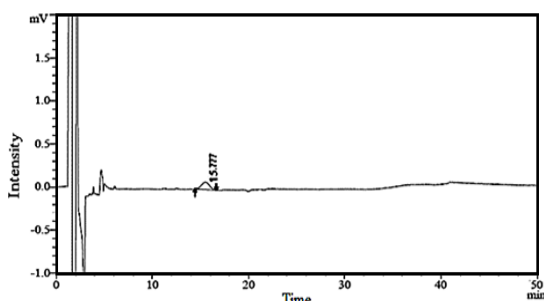
Fig. 11. Chromatogram of Limit of detection

Fig. 12. Chromatogram of Limit of Quantitation

Table 3: Limit of Quantitation results (LOQ)

Injection serial No.	Retention time in minutes	Area
LOQ injection-1	15.778	4865
LOQ injection-2	15.789	4689
LOQ injection-3	15.795	4763
LOQ injection-4	15.769	4986
LOQ injection-5	15.785	4892
LOQ injection-6	15.772	4941
	Mean	4856
	Standard deviation	111.3912
	Relative Standard Deviation in %	2.2939

Linearity and Range

Established the linearity validation parameter by preparing the standard solutions through quantitative dilutions of the impurity standard stock solution to 150%, 120%, 100%, 80%, 50% and LOQ.

Analyzed each diluted concentration and have been recorded area responses at 250nm. Draw the linearity plot between peak vs concentration and calculated the correlation coefficient of the regression line, slope, intercept and sum of squares. By injecting the impurity-A solution at the range of upper (150%) and lower (LOQ) level of target concentration linearity was evaluated. The obtained linearity results tabulated in Table 4 and linearity curve represented in Fig. 13 for impurity. Experimental Linearity and range analytical chromatograms were showed in Figure 14.

Table 4: Linearity parameter results

Concentration Level	Concentration in µg/g	Area
Solution at LOQ	0.5035	4596
Solution at 50 %	2.5175	21678
Solution at 80 %	4.0280	35289
Solution at 100 %	5.0350	44632
Solution at 120 %	6.0420	52369
Solution at 150 %	7.5525	66043
	Slope	8728.2489
	Y-Intercept	79.7769
	Correlation Coefficient	0.9999
	Residual Sum Square	0.9997

Study of precision

Repeatability and intermediate precision studies were performed on the precision of the developed analytical method.

Repeatability

Six sample solutions of Ezetimibe were prepared with spiking the Imp-A analyzed in the proposed instrument conditions and examine the precision of the analytical method. The mean of impurity-A, standard deviation and the %RSD of outcomes were found to be within the limits.
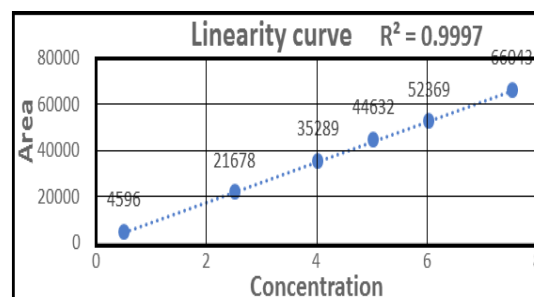

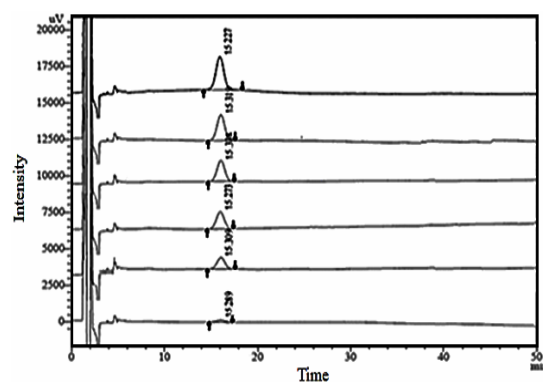**Fig. 13. Linearity graph of Impurity-A**

Fig. 14. Overlay chromatogram Linearity of Impurity-A (LOQ to 150%)

Intermediate precision

By performing the intermediate precision

analysis by different operators on two days by using different system. The average area of impurity-A, standard deviation and the relative standard deviation of outcomes were reveals to be meet the criteria. Table 5 & Table 6 represent the repeatability and intermediate precision data.

Table 5: Repeatability results

Preparation	Weight(mg)	Area	Impurity(%)	Impurity(µg/g)
Sample solution-1	500.42	47053	0.0005104	5.104
Sample solution-2	500.98	46592	0.0005219	5.219
Sample solution-3	499.81	46359	0.0005148	5.148
Sample solution-4	500.14	47287	0.0005242	5.242
Sample solution-5	500.42	47693	0.0005229	5.229
Sample solution-6	500.95	46894	0.0005340	5.340
Mean				5.214
SD				0.0817
%RSD				1.5668

Table 6: reported results of precision at intermediate level

Preparation	Weight(mg)	Area	Impurity(%)	Impurity(µg/g)
Sample solution-1	501.25	46872	0.0005187	5.187
Sample solution-2	500.37	46269	0.0005208	5.208
Sample solution-3	500.94	47013	0.0005234	5.234
Sample solution-4	500.72	46980	0.0005368	5.368
Sample solution-5	500.43	47630	0.0005236	5.236
Sample solution-6	499.86	46183	0.0005385	5.385
Mean				5.270
SD				0.0849
%RSD				1.6105
Combined %RSD of Repeatability and Intermediate Precision				1.6146

Accuracy

By spiking the impurity-A into the sample at upper and lower concentrations and analyzed in the method conditions. The spiked and recovered amount of impurity-A was calculated the percentage of recovery was tabulated in Table 7. The obtained recovery values are in between 98% to 102% and which provides the recovery nature of method.

Solution stability

Impurity-A stability was established by using the precision sample and it was kept in RT for a period of 24 h and analyzed in an interval of 4 h increment up to four hours and then analyzed with an interval of 4 h up to 24 hours. The chromatograms were evaluated and the absolute variation was calculated with impurity content initial with each time point. The complete results are summarized in below Table 8.

Table 7: Accuracy parameter results

Strength	Weight of the compound(mg)	Amount added(µg/g)	Response	Amount found(µg/g)	Recovery(%)	Average recovery(%)	% 1of RSD
At LOQ	500.61	0.527	4763	0.521	98.86	98.82	1.40
	500.46	0.532	4896	0.530	97.42		
	499.89	0.544	4968	0.545	100.18		
50 %	500.04	2.601	24698	2.543	97.77	99.04	1.44
	501.08	2.582	24796	2.597	100.58		
	500.53	2.608	25731	2.576	98.77		
100%	501.84	5.214	48468	5.302	101.69	100.52	1.12
	500.63	5.328	47638	5.298	99.44		
	500.24	5.293	47196	5.316	100.43		
150%	499.91	7.635	67642	7.845	102.75	101.04	1.64
	500.27	7.721	68432	7.792	100.92		
	500.86	7.694	69367	7.690	99.45		

Table 8: Solution stability results

Interval	Area	Content (µg/g)	Absolute variation
Initial	45628	5.268	--
4 Hours	45123	5.263	0.005
8 Hours	46798	5.259	0.009
12 Hours	47014	5.256	0.012
16 Hours	44989	5.258	0.010
20 Hours	45289	5.261	0.007
24 Hours	45916	5.254	0.014

CONCLUSION

The proposed method was used for the identification of genotoxic impurity (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone in Ezetimibe active pharmaceutical ingredient by RP-HPLC, UV method was advised and validated. This method was developed to be simple, precise, linear, accurate, and low-cost approach. As a result, this technology can be utilised in the quality control department to analyse low-level

of impurity-A in pharmaceutical compound of Ezetimibe on a regular basis.

University, Visakhapatnam for proving necessary facilities to do this research work.

Conflict of interest

All the authors are declared to publish this work. There was no conflicts of interest.

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