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Structural elucidation of a process-related impurity in ezetimibe by LC/MS/MS and NMR

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ABSTRACT

A major process-related impurity associated with the synthesis of ezetimibe was detected by LC–MS. The isolated impurity was found not to have been previously reported. Based on LC/MS/MS studies and accurate mass data, the structure of that impurity was proposed to be 2-(4-hydroxybenzyl)-*N*,5-bis-(4-fluorophenyl)-5-hydroxypentanamide. The postulated structure was unambiguously confirmed with the help of the NMR and IR analyses of a synthetically obtained sample. The chemical shift of the labile proton of that new entity was assigned by a 2D-NOESY NMR experiment. A rationalization for the formation of this impurity is provided.

1. Introduction

Ezetimibe, designated as 1-(4-fluorophenyl)-3(*R*)-[3-(4-fluorophenyl)-3(*S*)-hydroxypropyl]-4(*S*)-(4-hydroxy phenyl)-2-azetidinone is the first in class of lipid-lowering drugs [1], which inhibits intestinal absorption of cholesterol and related phytosterols from the intestine lumen [2]. Ezetimibe helps in reducing elevated levels of sitosterol and campesterol in the treatment of homozygous familial sitosterolemia. It is also an effective option for treating patients with primary hypercholesterolemia and reduces the risk of coronary heart disease [3].

A quite few bioanalytical methods are reported in the literature for the determination of ezetimibe concentration in biological fluids [4–8]. Methods based on HPLC, HPTLC and UV are also reported in the literature for the analysis of ezetimibe and studies related to its degradation [9–13]. However, so far there is no published report, describing the complete characterization of related product or impurities in ezetimibe as active pharmaceutical ingredient (API).

Impurity profile of a drug substance is critical for its safety assessment and manufacturing process. It is mandatory to identify and characterize the impurities in the pharmaceutical product, if present above the accepted limits of 0.1% [14].

Phenolic groups must be protected during azetidine-2-one ring formation or further conversion to ezetimibe. Though there are few methods reported for synthesis of ezetimibe using various protecting group [15–19], benzylation of phenolic group is very facile, safe and easily scalable approach. This communication deals with the identification and structural elucidation of a process-related contamination which was found in the product (ezetimibe), when prepared by debenzylation of 1-(4-fluorophenyl)-3(*R*)-[3(*S*)-(4-fluorophenyl)-3-hydroxypropyl]-4(*S*)-[4(phenylmethoxy)phenyl]-2-azetidinone (hereinafter referred to as benzyl ezetimibe) (Fig. 1), a process reported to give high purity product [15]. However, the same does not give any details regarding the impurities. Gradient reverse phase HPLC method revealed the presence of two impurities in ezetimibe sample prepared by the above said process. In view of the fact that the impurity levels were above the accepted limits, a comprehensive study was carried out using spectrometric and spectroscopic techniques.

2. Experimental

2.1. Materials and reagents

Samples of ezetimibe and its intermediate were obtained from Chemical Research Division, Ipca Laboratories Ltd. (Mumbai, India). HPLC grade acetonitrile was purchased from Merck India Limited (Mumbai, India). Deionized water was prepared using MilliQ plus purification system (Millipore, Bradford, USA). Potassium bromide

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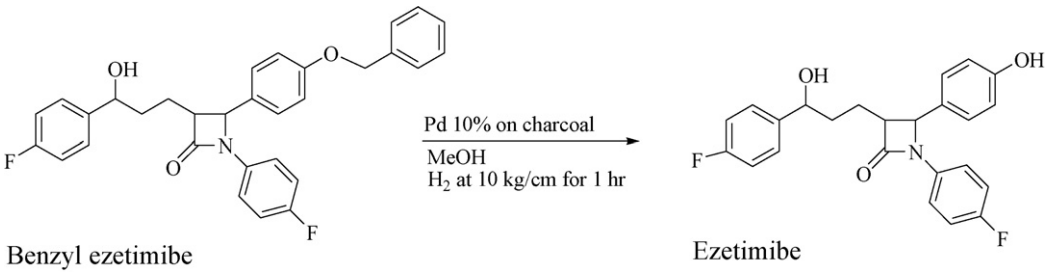


Fig. 1. Scheme of synthesis of ezetimibe.

(FT-IR grade), deuterated dimethylsulfoxide and D_2O were purchased from Merck KGaA, Darmstadt, Germany.

2.2. High performance liquid chromatography

Samples were analyzed on Waters Alliance 2690 HPLC system (Milford, MA, USA) equipped with Waters 2487 UV detector. A Kromasil C18 column (250 mm × 4.6 mm, 5 μm Akzo Nobel, Bohus, Sweden) was used for chromatographic separation. The mobile phase consisting of A: water and B: acetonitrile, with timed gradient programme *T* (min)/B (%): 0/50, 5/50, 15/20, 25/20, 30/50, 35/50 with flow rate of 1.0 ml per minute was used. The injection volume was 20 μl and the detector wavelength was fixed at 232 nm.

2.3. Mass spectrometry

The LC–ESI/MS and MS/MS analysis was carried out on LCQ-Advantage (Thermo Finnigan, San Jose, CA, USA) ion trap mass spectrometer. The LC unit consisted of an Agilent 1100 series quaternary gradient pump with a degasser and auto sampler. The chromatographic condition described in Section 2.2 has been used for the analysis. The source voltage was kept at 3.0 kV and capillary temperature at 250 °C. Nitrogen was used as both sheathe and auxiliary gas. Mass range was kept at *m*/*z* 100–500. MS/MS studies were carried out by maintaining normalized collision energy at about 30% with the mass range *m*/*z* 100–500.

Q-TOF Micromass spectrometer with mass resolution 6000 (Waters, Milford, MA, USA) was used for accurate mass determination. Leucine enkephalin ($C_{28}H_{37}N_5O_7$) was used as a lock mass (556.2771 Da). The source block and desolvation lamp were kept at 150 °C and 300 °C, respectively. The nebulizer and desolvation gas flows were 20 l/h and 450 l/h. The instrument parameters in positive mode were: capillary voltage 3000 V, cone at 25 V, extractor at 2 V and MCP at 2700 V. Data acquisition and processing was done using masslinks (version 4.0) software.

2.4. NMR

The ^1H, ^{13}C and DEPT measurements were recorded on a Bruker AVANCE 400 NMR spectrometer (Fallanden, Switzerland) instrument at 300 K. The exchangeable protons were identified by D_2O exchange experiment. The phase sensitive double quantum filtered correlation spectroscopy (DQF-COSY), heteronuclear single quantum correlation (HSQC) and nuclear Overhauser effect spectroscopy (NOESY) was also performed using the same instrument. The ^1H and ^{13}C chemical shift values were reported on the δ scale in ppm relative to dimethyl sulfoxide (2.49 ppm) and (39.5 ppm), respectively.

2.5. IR spectroscopy

The IR spectrum of isolated impurity was recorded in the solid state KBr powder dispersion using a Spectrum-One FT-IR spectrometer (PerkinElmer, Beaconsfield, UK).

2.6. Sample preparation

2.6.1. Ezetimibe sample preparation

An intermediate, benzyl ezetimibe was dissolved in MeOH. After the addition of palladium catalyst (Pd 10% on charcoal) the mixture was subjected to hydrogenation at ambient temperature in the autoclave at a hydrogen pressure of about 10.0 kg/cm^2 for 1 h to obtain ezetimibe. The resulting mixture was filtered through highflow and the solvent was evaporated. By keeping the same experiments conditions three such experiments were conducted.

2.6.2. Enrichment of impurity-I (imp-I) in ezetimibe sample

Ezetimibe sample (chromatographic purity > 99.0%) prepared by synthetic process mentioned in Section 2.6.1, was dissolved in methanol with palladium catalyst (Pd 10% on charcoal) and the mixture was subjected to autoclave at ambient temperature and under hydrogen pressure of 5.0 kg/cm^2 for 6–8 h. The resulting mixture was filtered through highflow and the solvent was evaporated.

3. Results and discussion

3.1. Detection of impurity by HPLC

The product obtained by process described in Section 2.6.1 was analyzed by HPLC method described in Section 2.2 without any further purification. The analysis revealed the presence of two peaks marked as impurity-I at retention time (RT) of 8.4 min (ranging from 0.1% to 0.5%) and impurity-II (imp-II) at RT of 18.9 min (ranging from 0.04% to 0.12%) in the chromatogram by area normalization method, apart from ezetimibe peak at RT of 10.2 min. The imp-II was identified as precursor of ezetimibe and is confirmed by co-injection of authentic sample of the same.

3.2. Structural elucidation of imp-I by mass spectrometry

Mass spectra obtained by subjecting the peak of imp-I, using conditions described in Section 2.3, showed pseudomolecular ion peak $[M+H]^+$ at *m*/*z* 412. The main peak, as expected, gave pseudomolecular ion peak $[M+H]^+$ at *m*/*z* 410, confirming it to be ezetimibe. Based on the HPLC and LC/MS spectral data, the imp-I is inferred to be unknown. The sample containing ezetimibe and imp-I was subjected to LC/ESI/MS (positive mode) and MS/MS analysis. MS/MS study of imp-I showed three product ion peaks at *m*/*z* 394, *m*/*z* 301 and *m*/*z* 283 which were obtained from parent pseudomolecular ion peak $[M+H]^+$ at *m*/*z* 412, whereas MS/MS spectrum obtained for ezetimibe showed two product ion peaks at *m*/*z* 392 and *m*/*z* 299 resulted from parent pseudomolecular ion peak $[M+H]^+$ at *m*/*z* 410 (molecular mass of ezetimibe is 409). Further MS^3 showed no significant fragments from these peaks.

Before characterization of imp-I, it is logical to understand the LC/MS/MS fragmentation pattern of the parent drug molecule, ezetimibe. The MS spectral data showed pseudomolecular ion peak at *m*/*z* 410 and base peak at *m*/*z* 392 (Fig. 2a). The MS^2 spectra of

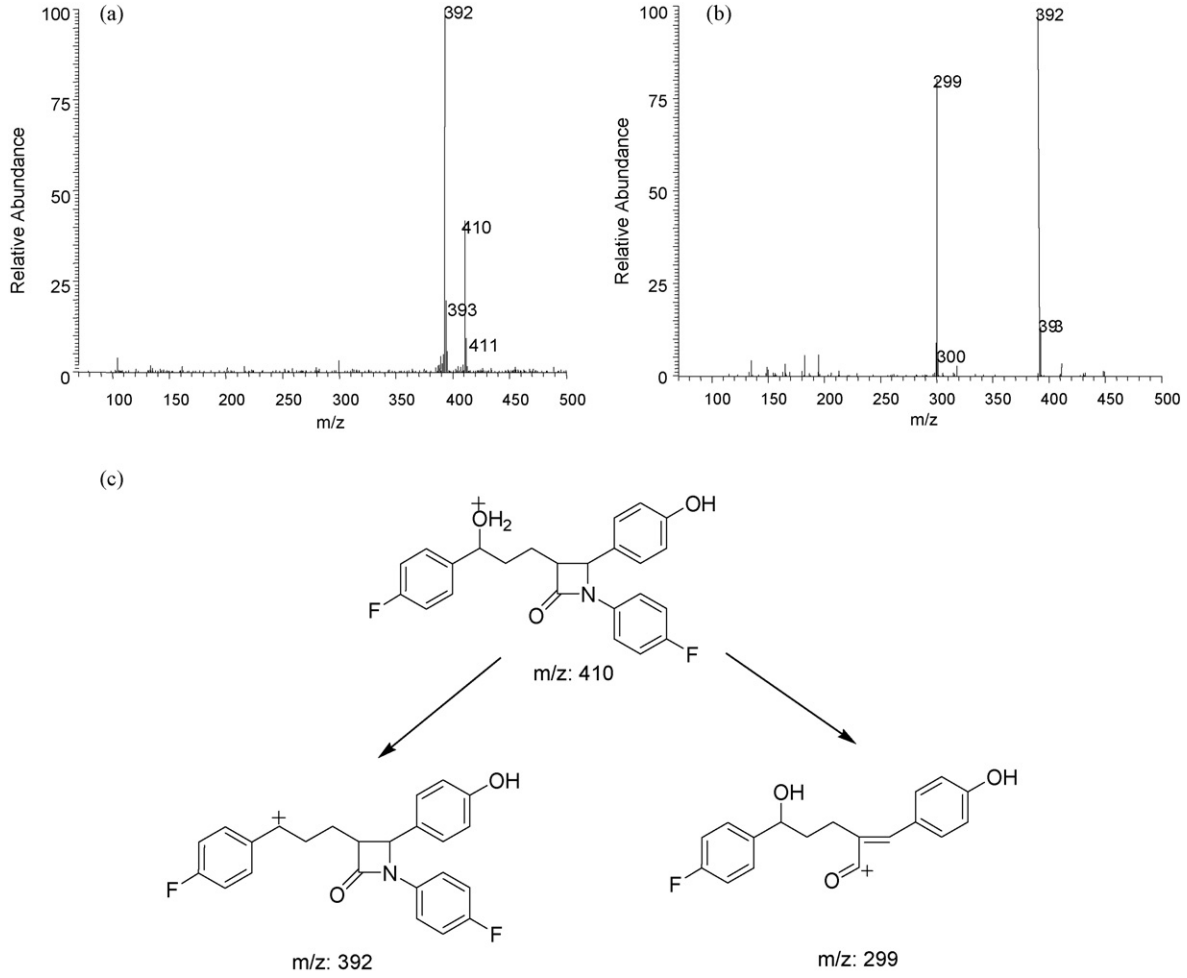


Fig. 2. MS and MS/MS data of ezetimibe. (a) Mass spectrum of ezetimibe, (b) MS/MS spectrum of parent ion at *m*/*z* 410 and (c) mechanism of formation of fragment ions with *m*/*z* 410.

parent ion peak at *m*/*z* 410 showed two prominent peaks at *m*/*z* 392 and *m*/*z* 299 (Fig. 2b). Formation of fragment ion at *m*/*z* 392 can be attributed to the loss of water molecule (−18 Da) and formation of daughter ion peak at *m*/*z* 299 can be attributed to the loss of 4-fluoroaniline group (−111 Da). The mass fragmentation mechanism is depicted herein (Fig. 2c).

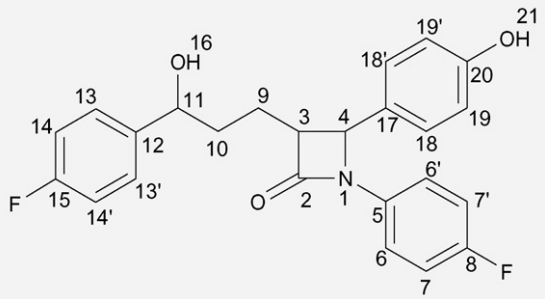
The accurate mass of imp-I, measured by Q-TOF instrument was 412.1719 Da. In order to determine the molecular formula of unknown impurity, these figures of measured masses were plugged into elemental composition calculator setting the reasonable limits as carbon 0–30, hydrogen 0–30, nitrogen 0–5, oxygen 0–5, and fluorine 0–3. The search revealed several theoretically possible molecular formulae. The closest possible molecular formula for pseudomolecular ion of imp-I ($C_{24}H_{24}F_2NO_3^+$) was selected on the basis of the lowest difference in mass (milli Dalton) between theoretical (412.1709 Da) and observed (412.1719 Da) values.

The mass spectral data obtained for imp-I showed pseudomolecular ion peak at *m*/*z* 412 and base peak at *m*/*z* 394 (Fig. 3a). An odd molecular mass (411 Da) of imp-I implies the existence of odd number of nitrogen atoms. A daughter ion peak at *m*/*z* 394 in MS^2 shown in mass spectral data can be attributed to the loss of water molecule (−18 Da). Formation of product ion peak at *m*/*z* 301 is due to the loss of neutral leaving moiety 4-fluoroaniline group (−111 Da) and *m*/*z* 283 is due to the loss of 4-fluoroaniline group along with water molecule (−129 Da) (Fig. 3b). Based on LC/MS/MS and LC/Q-TOF/MS analysis, the structure of imp-I can be rationalized as 2-(4-hydroxybenzyl)-*N*,5-bis-(4-fluorophenyl)-5-hydroxypentanamide. The proposed fragmentation mechanism is given in Fig. 3c.

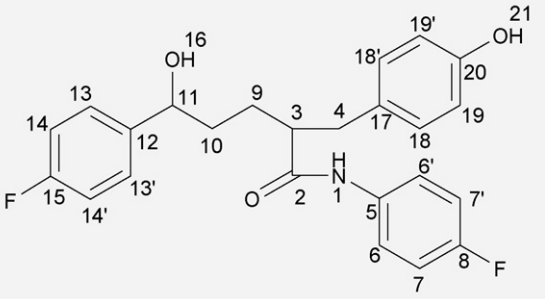
3.3. Structural confirmation of imp-I by NMR and IR

It is imperative to understand the ^1H and ^{13}C NMR assignment of ezetimibe, the parent drug. The complete assignment was established based on ^1H, ^{13}C, DEPT (Table 1), ^1H–^1H (DQF-COSY) and ^1H–^{13}C (HSQC) experiment. Sample obtained by process described in Section 2.6.2 was used for imp-I structural elucidation without any further purification. The ^1H NMR spectral data obtained for imp-I revealed extra signals at 2.74 ppm and 9.82 ppm. The signal at 9.82 ppm is not observed with ezetimibe, corresponds to an exchangeable proton and is spatially close to H6 and 6′ therefore it is assigned as –NH group. ^{13}C and DEPT NMR spectral data of imp-I confirmed the presence of fourteen methine, three methylene and seven quaternary carbon atoms. This concludes that the imp-I have an extra $-CH_2$ group at 38.1 ppm as compared to that of ezetimibe. ^1H–^1H correlation (DQF-COSY) and ^1H–^{13}C (HSQC) experiments revealed that the C4 is now $-CH_2$ and linked to two protons at 2.74 ppm and 2.48 ppm and no more CH as it is in ezetimibe. Whereas the signals at 5.22 ppm, 9.16 ppm does not show correlation with any carbon atom and are confirmed as exchangeable proton by D_2O analysis. Further, in order to identify and confirm the chemical shift position of phenolic and hydroxyl (–OH) labile protons of imp-I, 2D-NOESY NMR experiment was performed. The results obtained from this experiment showed that the proton at

Table 1
NMR assignment for ezetimibe and ezetimibe imp-I.



(a) Ezetimibe



(b) Ezetimibe imp-I

Position	Integration	^{1}H (chemical shift in ppm)	Multiplicity, (J, Hz)[a]	^{13}C (chemical shift in ppm), (J, Hz)[b]	DEPT[c]
1	–	–	–	–	–
2	–	–	–	167.8	–
3	1H	3.06	m	59.8	CH
4	1H	4.80	d (2.3)	60.0	CH
5	–	–	–	142.6 (2.9)	–
6, 6′	2H	7.09	m	118.7 (8.1)	CH
7, 7′	2H	7.09	m	116.4 (22.7)	CH
8	–	–	–	158.4 (240.0)	–
9	2H	1.67	m	24.9	CH_2
10	2H	1.87	m	36.8	CH_2
11	1H	4.47	m	71.5	CH
12	–	–	–	134.4, (2.6)	–
13, 13′	2H	7.28	m	127.9, (7.3)	CH
14, 14′	2H	7.09	m	115.1, (20.5)	CH
15	–	–	–	161.5, (241.5)	–
16 (OH)	1H	5.29	d (4.6)	–	–
17	–	–	–	128.3	–
18, 18′	2H	7.09	d (8.5)	128.0	CH
19, 19′	2H	6.74	d (8.5)	116.1	CH
20	–	–	–	157.8	–
21 (OH)	1H	9.55	s	–	–

Position	Integration	^{1}H (chemical shift in ppm)	Multiplicity, (J, Hz)[a]	^{13}C (chemical shift in ppm), (J, Hz)[b]	DEPT[c]
1 (NH)	1H	9.82	s	–	–
2	–	–	–	173.9	–
3	1H	2.48	m	49.3	CH
4	1Ha	2.48	m	38.1	CH_2
	1Hb	2.74	m		
5	–	–	–	135.9, (2.2)	–
6, 6′	2H	7.52	m	121.4, (7.3)	CH
7, 7′	2H	7.07	m	115.6, (22.0)	CH
8	–	–	–	158.4, (239.3)	–
9	2H	1.43	m	29.0	CH_2
10	2H	1.65	m	37.6	CH_2
11	1H	4.45	m	72.1	CH
12	–	–	–	142.8, (2.9)	–
13, 13′	2H	7.25	m	128.0, (7.3)	CH
14, 14′	2H	7.07	m	115.1, (21.2)	CH
15	–	–	–	161.5, (241.5)	–
16 (OH)	1H	5.22	d (4.6)	–	–
17	–	–	–	130.4	–
18, 18′	2H	6.95	d (8.5)	130.2	CH
19, 19′	2H	6.62	d (8.5)	115.4	CH
20	–	–	–	156.0	–
21 (OH)	1H	9.16	s	–	–

s: singlet; d: doublet; and m: multiplet.

[a] ^{1}H–^{1}H coupling constants.

[b] ^{13}C–^{19}F coupling constants.

[c] Hybridization (degree of bonding) of carbon atoms.

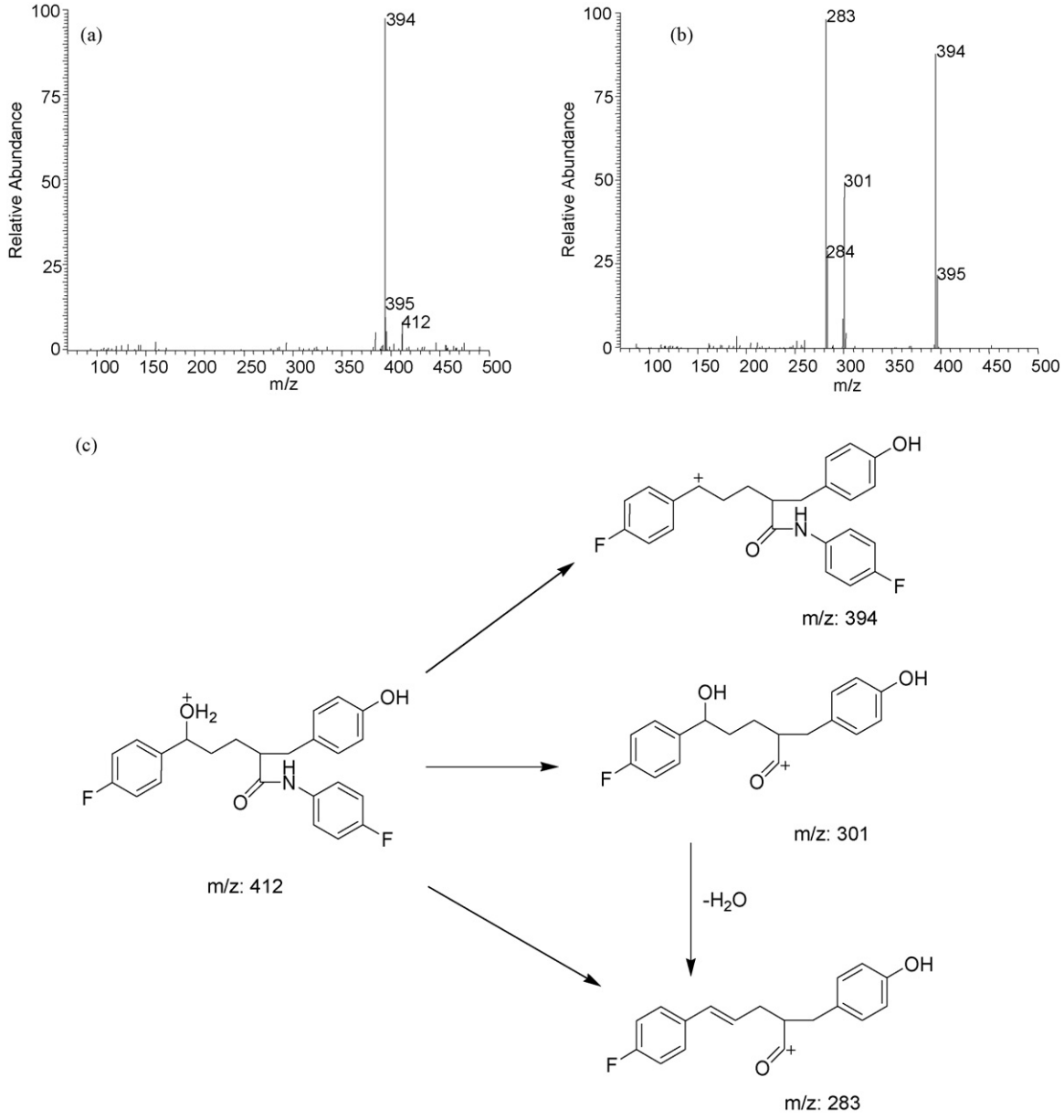


Fig. 3. MS and MS/MS data for ezetimibe imp-I. (a) Mass spectrum of imp-I and (b) MS/MS spectrum of parent ion at *m/z* 412.

5.22 ppm is in the proximity of fluorobenzene and spatially close to H10, 11, 13 and 13′. The proton at 9.16 ppm is spatially close to H19 and 19′ of phenyl group. The complete NMR spectral assignment of imp-I is tabulated (Table 1).

The ezetimibe molecule contains one lactam ring. The –C=O stretching vibration of lactam is at 1719 cm^{-1}. Whereas IR spectral data of imp-I showed strong peak at 1650 cm^{-1} for –C=O stretching.

Taken together the above LC/MS/MS, NMR and IR spectral data, it was concluded that the imp-I contains an amide (–NH–C=O) group along with an additional $–CH_2$ group as compared to that of ezetimibe. This suggests that the tertiary amine bond of lactam ring in ezetimibe at position N1–C4 is cleaved and leading into formation of $–CH_2$ and amide group at the same position. This kind of β-lactam N1–C4 bond cleavage is well established [20].

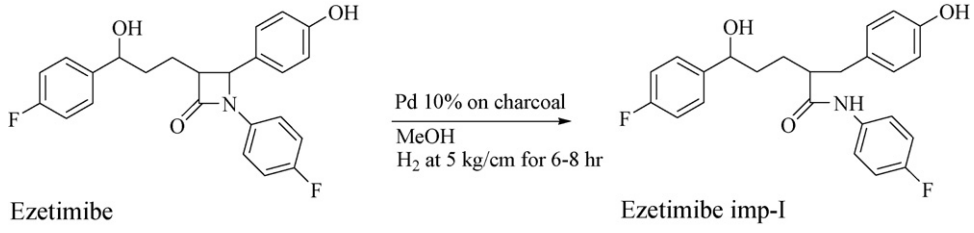


Fig. 4. Plausible mechanism of formation of ezetimibe imp-I.

3.4. Formation of impurity

During synthesis of ezetimibe, an intermediate benzyl ezetimibe is subjected to debenzylation to give ezetimibe. Simultaneously, the opening of ezetimibe β-lactam is also taking place and parallelly yielded the imp-I. The plausible mechanism of formation of impurity is depicted (Fig. 4).

4. Conclusion

The present investigation revealed a major process-related impurity while analyzing ezetimibe through the gradient reverse phase HPLC method. LC/MS/MS and LC/Q-TOF/MS provided the molecular formula and the sub-structural information of this impurity. NMR and IR spectroscopic analyses confirmed the structure as 2-(4-hydroxybenzyl)-*N*,5-bis-(4-fluorophenyl)-5-hydroxypentanamide.

The present study therefore, clearly indicates that an intermediate benzyl ezetimibe is subjected to debenzylation to give ezetimibe during synthesis. Ezetimibe β-lactam ring is also opened parallelly yielding the imp-I, at levels beyond the ICH guidelines. Elucidation of the structure of such impurities can certainly be very effective in the pharmaceutical development processes to comply the regulatory norms for impurity levels.

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