Short communication

Isolation and structure elucidation of major alkaline degradant of Ezetimibe

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ABSTRACT

This work presents the isolation and characterization of the alkaline degradant of Ezetimibe. Ezetimibe, a selective inhibitor of intestinal cholesterol absorption, was subjected to alkaline degradation. Ezetimibe was reacted with 0.1 M methanolic sodium hydroxide solution for 10 min at 80 °C to yield alkaline degradant to an extent of 90% of initial amount of the drug taken. This degradant was detected by high performance liquid chromatography (HPLC) at relative retention time (RRT) of 1.48 with respect to Ezetimibe. HPLC method involved an isocratic elution on a Waters Symmetry C_8 150 mm × 4.6 mm, 5 μm column using ammonium acetate buffer (pH 4.5, 50 mM) – acetonitrile (50:50, v/v) as the mobile phase at a flow rate of 1.0 mL/min and UV detection at 242 nm. The degradant was isolated by preparative HPLC. Purity of the isolated solid was found to be more than 99%. Structure of alkaline degradant was confirmed by LC–MS, ^{1}H and ^{13}C NMR and IR spectroscopy. On the basis of spectral data, the structure of the degradant was confirmed as 5-(4-fluorophenyl)-2-[(4-fluorophenyl amino)-(4-hydroxyphenyl)methyl]-pent-4-enoic acid. The route for the formation of this degradant is also proposed. Determining the structures of degradation products arouse during stress testing can be useful for preclinical discovery efforts.

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1. Introduction

Ezetimibe (EZE), a selective inhibitor of intestinal cholesterol and related phytosterol absorption, is designated as 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone [1,2] (Fig. 1(a)). It selectively prevents the absorption of cholesterol by binding to transport protein (NPC_1L_1), located in the wall of small intestine [3,4].

The stability indicating assays of EZE in single as well as combined dosage forms by HPLC [5–10]; HPTLC [11] and micellar electrokinetic chromatography [12] have been reported. Analysis of EZE in the presence of its alkaline degradation products by HPLC, TLC and derivative spectrophotometry is reported by Moghazy et al. [13]. The structural elucidation of a process-related impurity of EZE by LC–MS/MS and NMR is described by Raman et al. [14].

This paper elaborates alkali induced degradation of EZE to produce a major degradation product which is not reported in the literature till date. The present study illustrates the isolation and identification of the degradation product formed during alkaline degradation of EZE and proposes degradation pathway.

2. Experimental

2.1. Materials and reagents

Reference standard of EZE was gifted by Torrent Research Center, Gandhinagar, India with purity of 99.9%. Methanol and acetonitrile (both, HPLC grade) were purchased from Spectrochem (Mumbai, India). All other reagents and chemicals utilized in the study had analytical grade. Ammonium acetate and hydrochloric acid (35%, v/v) were procured from Ranbaxy Fine Chemicals (New Delhi, India), glacial acetic acid and sodium hydroxide pellets from Merck India Limited (Mumbai, India) and nylon syringe filters (0.45 μm) from Millex-HN, Millipore (Mumbai, India). Water was purified using Millipore system (Millipore Corp., Bangalore, India).

2.2. Chromatographic system (analytical HPLC)

A Shimadzu model LC-2010C (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) chromatograph with autoinjector, sample cooler, and UV–Visible and Photodiode array (PDA) detector (SPD 10 mA vp), connected to data processing system software (Class-VP 6.13 SP_2) was employed. In the developed method, a Waters Symmetry (Waters Corporation, USA) C_8 column (150 mm × 4.6 mm i.d., 5 μm particle size) was maintained at ambient temperature. Separation of EZE and its alkaline degradant was achieved under isocratic conditions using a mobile phase of ammo-

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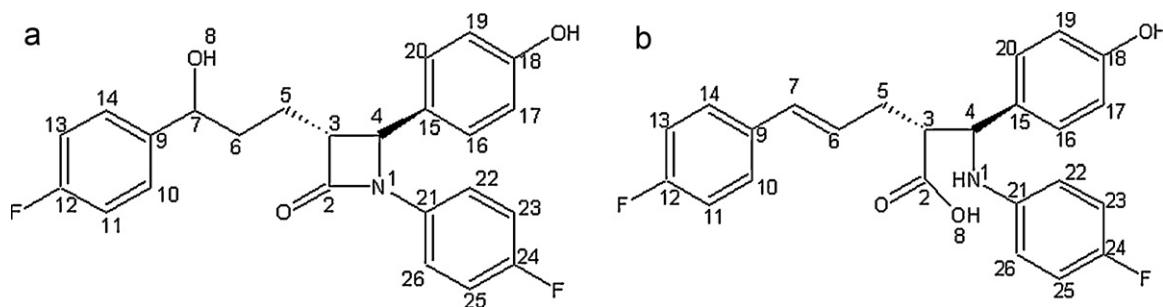 E-mail address: anuradha.gajjar@nirmauni.ac.in (A.K. Gajjar).

Fig. 1. Chemical structure of (a) EZE and (b) alkaline degradant.

nium acetate buffer (pH 4.5; 50 mM) – acetonitrile (50:50, v/v). pH of the buffer solution was measured using a PHAN, LABINDIA pH meter (Mumbai, India). The UV detector was set at 242 nm, as both EZE and its alkaline degradant showed good response at this wavelength. The flow rate was adjusted to 1.0 mL/min. Injection volume was set as 20 μL for both standard and test solutions.

2.3. Alkaline degradation of EZE

EZE was reacted with 0.1 M methanolic sodium hydroxide solution to produce around 90% of the major degradant and this degradant was detected by HPLC at relative retention time (RRT) of 1.48 (Fig. 2). About 0.5 g of EZE drug substance was taken in 100 mL standard volumetric flask. Methanolic sodium hydroxide solution (50 mL, 0.1 M) was added and refluxed at 80 °C on a constant temperature water bath (Matalab, India) for 10 min. The solution was cooled to room temperature and neutralized with 1 M methanolic hydrochloric acid solution. The solution was loaded into a preparative HPLC and isolated the alkaline degradant present at RRT of 1.48.

2.4. Preparative HPLC

Waters preparative HPLC (Delta Prep) 4000 system (Waters Corporation, Milford, MA, USA) with high pressure unit of 4000 psi equipped with fraction collector was used. It was operated through Empower software. Symmetry (Waters) C_{18} (100 mm × 30 mm i.d.) preparative column packed with 8 μm particle size was employed for separation and isolation of the degradant. The mobile phase consisted of ammonium acetate buffer (pH 4.5; 50 mM) – acetonitrile

(50:50, v/v). Flow rate was kept at 30 mL/min and UV detection at 242 nm.

2.5. Isolation of degradant by preparative HPLC

The methanolic alkaline degradation solution of EZE was concentrated on a Buchi rotavapor R-124 (Buchi Labortechnik, Switzerland) and loaded on the preparative column using conditions mentioned in Section 2.4. The isolated fractions were collected and analyzed by analytical HPLC using conditions mentioned in Section 2.2. The isolated fractions having purity greater than 99.0% were pooled together and methanol was then evaporated using rotavapor with B-490 water bath to get solid impurity. The solid obtained was then dried using freeze dryer (Virtis Advantage, USA).

2.6. Mass spectrometry

The mass spectra of EZE and its alkaline degradant were recorded on API 2000 Perkin Elmer SCIEX-mass spectrometer (Perkin Elmer, Beaconsfield, United Kingdom) equipped with a turboionspray interface at 375 °C. Detection of ions was performed in Electrospray ionization, positive ion mode. Data acquisition and processing was done using MassLynx (Version-4) software.

2.7. NMR spectrometry

The ^{1}H NMR and ^{13}C NMR experiments were performed on AVANCE DPX 400 Bruker NMR spectrophotometer (Fallanden, Switzerland). DMSO-d_6 was used as solvent and tetramethylsilane was used as internal standard.
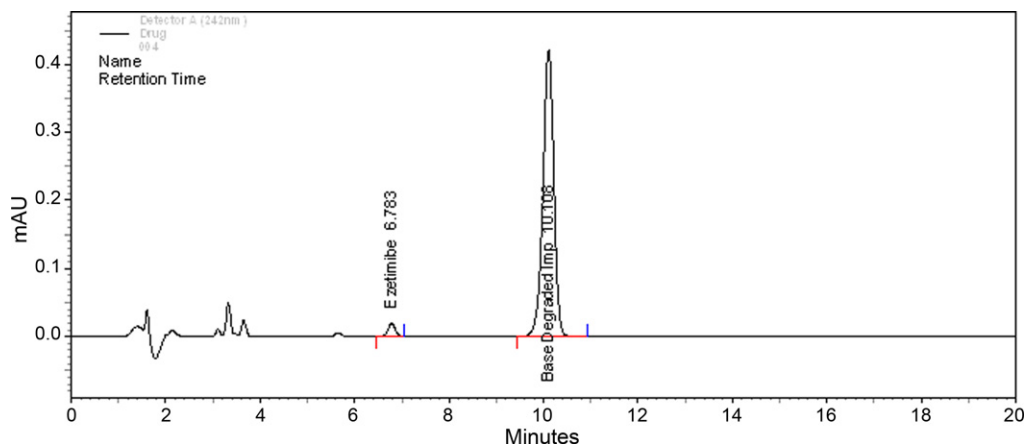

Fig. 2. Chromatogram representing the separation of EZE and alkaline degradant.
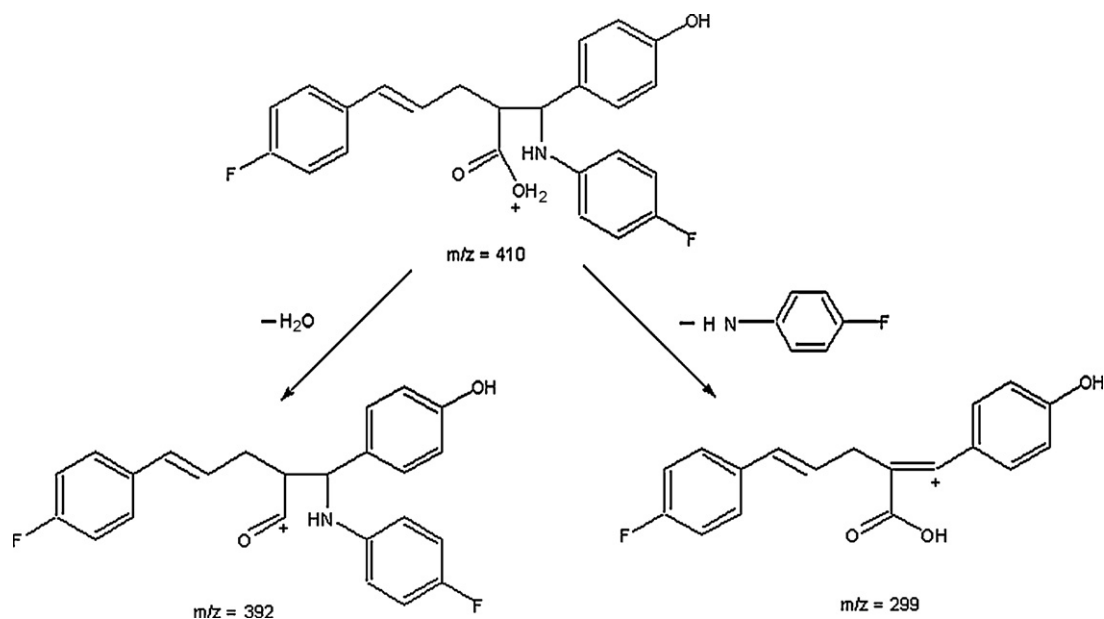
Fig. 3. Mass fragmentation of alkaline degradant of EZE.

2.8. FT-IR spectrometry

The IR spectra of EZE and its alkaline degradant were recorded in solid state as KBr dispersion using Bruker (TENSOR 27) FT-IR spectrophotometer (Ettlingen, Germany).

3. Results and discussion

3.1. Isolation of major alkaline degradant of EZE

EZE was subjected to alkaline degradation as discussed in Section 2.3 to generate 90% of major degradant, which was detected at relative retention time (RRT) of 1.48 with respect to EZE (Fig. 2). This degraded solution was subjected to preparative chromatography (Section 2.4) to enable the isolation of the degradant. The purity of this solid was assessed to be more than 99%. The degradant was then characterized by spectroscopic techniques like NMR (^{1}H and ^{13}C) and FT-IR while the molecular weight was determined by mass spectrometry.

3.2. Structure elucidation of alkaline degradant of EZE

The electrospray ionization (ESI) mass spectrum of degradant exhibited a molecular ion peak at m/z of 410 [M+H]$^+$ and an ion at 432 [M+Na]$^+$ supporting a molecular weight of 409 for major degradant of EZE, which was same as that of EZE (molecular ion peak at m/z of 410 [M+H]$^+$). The mass spectrum of the degradant shows an ion at m/z of 392 amu corresponding to loss of H_2O from the [M+H]$^+$ ion and an ion at m/z of 299 amu corresponding to loss of 4-fluoroaniline group. Raman et al. [14] have shown mass spectrum and fragmentation pattern for EZE. The fragmentation pattern of major alkaline degradant is shown in Fig. 3.

In the IR spectrum of degradant, the characteristic absorption frequency at 3310 cm^{-1} confirms the presence of secondary amine (–NH stretch) which is not observed in the IR spectrum of EZE confirming the breakage of the lactam ring to secondary amine and carboxylic acid through alkaline hydrolysis. Significant peak assignment of IR spectra of both the EZE and the degradant are shown in Table 1.

During NMR study, 21 hydrogens and 24 carbons were observed from the ^{1}H NMR and ^{13}C NMR spectra respectively, in both EZE and its alkaline degradant. Presence of –NH group in the degradant was again confirmed by the signal at $\delta = 9.31$ ppm in ^{1}H NMR spectrum of the degradant which was not observed in EZE. In ^{13}C NMR spectrum of degradant, the signal at 167.37 ppm corresponding to C=O (amide) in EZE was absent and a new signal appeared with downfield shift at 174.41 ppm indicating the presence of C=O (carboxylic acid). Signal at 71.25 ppm of EZE was shifted upfield to 52.99 ppm in the spectrum of degradant confirming loss of –OH on C_7 and the formation of double bond between C_6 and C_7. ^{1}H NMR and ^{13}C NMR spectral assignments of both EZE and its major alkaline degradant are presented in Table 2. The structure of the degradant is elucidated as 5-(4-fluorophenyl)-2-[(4-fluorophenylamino)-(4-hydroxy phenyl)methyl]-pent-4-enoic acid (Fig. 1(b)).

3.3. Proposed alkaline degradation pathway

The degradation pathway of EZE in alkaline conditions is proposed by the pathway, shown in Fig. 4. The four membered azetidinone ring of EZE is a highly strained system and the ring is cleaved upon alkaline hydrolysis to give the intermediate product and simultaneous heating of this intermediate leads to the formation of degradant, with the loss of a water molecule.

Table 1
Spectral details of FT-IR spectra of EZE and alkaline degradant.

Sr. no.	Peak assignments (KBr dispersion)	Peak observed in (cm^{-1})	
		EZE	Degradant
1	–OH (phenolic) stretch	3256 (broad)	3268 (broad)
2	Carboxylic acid O–H stretch	–	2847
3	C–H stretch in aromatic ring	2962	2976
4	Aliphatic C–H stretching	2914	2931
5	2^0 amine –NH stretch	–	3310
6	C=O stretching	1717 (amide)	1778 (acid)
7	C=C stretching in aromatic ring	1511	1510
8	–OH bending	1356	1364
9	C–O stretching	1220	1214
10	C–N stretching	1068	1108
11	C–H bending in aromatic ring	828	844
12	C–H bending in alkane	1403	1408
13	N–H bending	–	1546

Table 2
^{13}C and ^{1}H NMR spectral assignments of EZE and alkaline degradant.

| Sr. no. | ^{13}C NMR | | | ^{1}H NMR | | |
| | Assignments | Chemical shift (ppm) | | Assignments | Chemical shift (ppm)/multiplicity | |
		EZE	Degradant		EZE	Degradant
1	C-2	167.37	174.42	OH (1H) – on C$_{18}$	9.50 (s)	9.79 (s)
2	C-12	158.67	164.63	NH (1H)	–	9.31 (s)
3	C-24	157.45	161.91	H-22,23,25,26,10,11,13,14,16,20 (10H)	7.05–7.32 (m)	7.04–7.44 (m)
4	C-18	155.67	158.43	H-17,19 (2H)	6.72–6.76 (d)	6.65–6.67 (d)
5	C-15	142.09	140.427	H-4 (1H)	5.25 (m)	4.60 (m)
6	C-9	134.04	135.61	OH (1H) – on C$_7$	4.77–4.78 (d)	–
7	C-21	127.94	133.12	OH (1H) – on C$_2$	–	4.64 (s)
8	C-10,14	127.62	129.52	H-7 (1H)	4.47–4.49 (m)	2.087–2.116 (d)
9	C-16,20	127.52	128.95, 128.89	H-3 (1H)	3.07–3.24 (m)	2.64–2.71 (m)
10	C-22,26	118.37, 118.22	123.79, 123.73	H-5,6 (4H)	1.72 (m)	–
11	C-11,13,17,19,23,25	114.46–115.99	115.90–116.33	H-5 (2H)	–	1.96–2.02 (m)
12	C-7	71.25	52.99	H-6 (1H)	–	1.48–1.59 (m)
13	C-4	59.85	83.34			
14	C-3	59.59	80.78			
15	C-6	36.48	34.20			
16	C-5	24.66	29.40			

Refer structural formula for numbering (Fig. 1a for EZE and Fig. 1b for degradant); s, singlet; d, doublet; m, multiple.
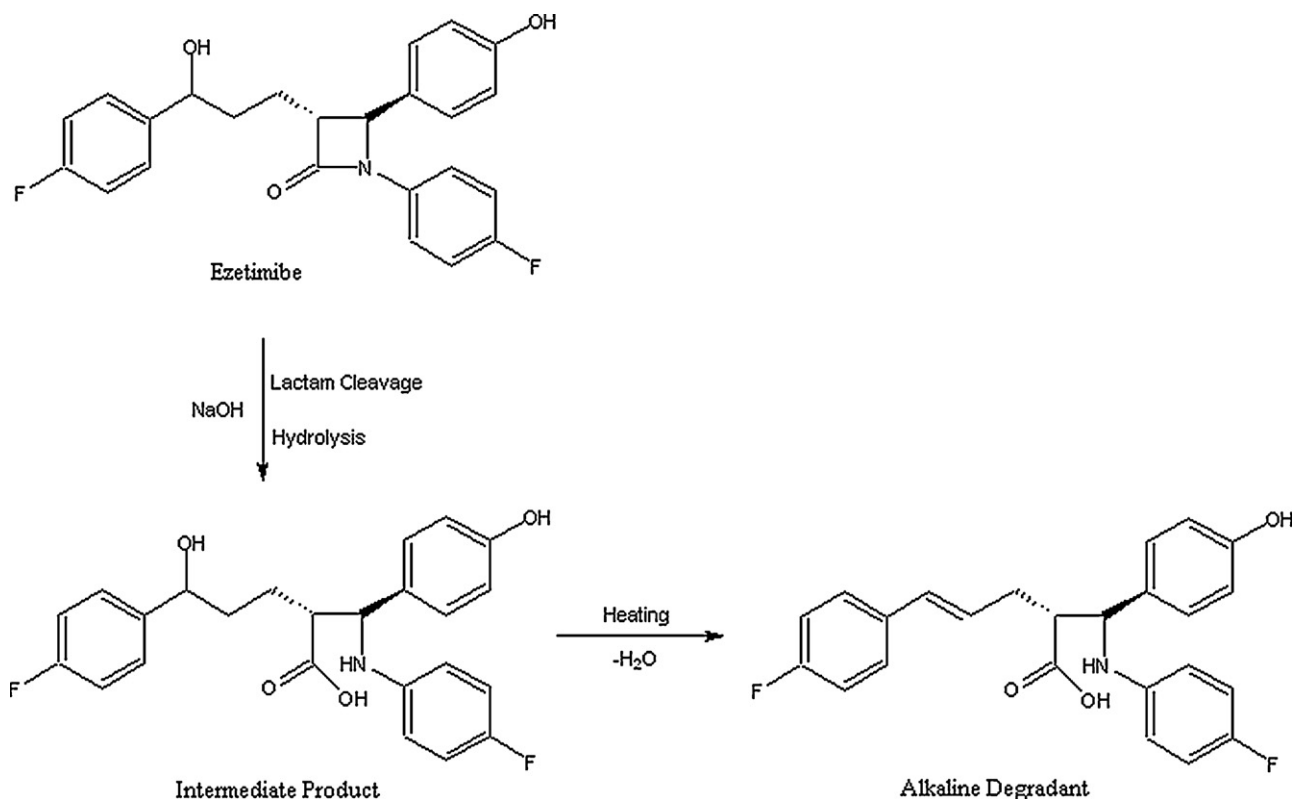

Fig. 4. Proposed pathway for alkaline degradation of EZE.

4. Conclusions

Determining the structures of degradation products arising in the course of stress testing can be useful for preclinical discovery efforts during structure–activity relationship investigations. EZE undergoes degradation in alkaline conditions, which was detected by HPLC and isolation of alkaline degradant was achieved using preparative chromatography. The structure of this degradant was characterized by IR, NMR (^{1}H and ^{13}C) and mass spectroscopic studies and elucidated as 5-(4-Fluorophenyl)-2-[(4-fluorophenylamino)-(4-hydroxyphenyl) methyl]-pent-4-enoic acid. The alkaline degradation pathway of EZE is also proposed.

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