Impurity Profiling: A Case Study of Ezetimibe

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Abstract: Impurity profiling includes a description of the identified and unidentified impurities present in new drug substances or drug products. Isolation and elucidation of the structures of degradation products are typically collaborative research involving knowledge of analytical, organic and physical chemistry with spectroscopic information. Stability testing guidelines issued by International Conference on Harmonization (ICH) require the reporting, identification and characterization of degradation products (DPs). The alkaline degradant was detected by high performance liquid chromatography (HPLC) at relative retention time (RRT) of 1.48 with respect to Ezetimibe. This 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. An understanding of the parts of the molecule that are susceptible to degradation can help in the design of more stable analogs. Determining the structures of the major degradation products can reveal whether or not a known carcinogen or toxic compound is or might possibly be formed.

Keywords: Degradant, Ezetimibe, Forced degradation study, Impurity Profile, Stability study, Structure elucidation.

1. INTRODUCTION TO IMPURITY PROFILING

Quality is the most important attribute of any pharmaceutical product. The source of pharmaceutical products varies greatly, from plants/ marine sources (natural resources), synthetic methods or recombinant DNA methods or a combination of any of these. This necessitates regulatory aspects as defining the standards for a quality product belonging to each of these categories would be a difficult task. The quality of a pharmaceutical product was determined by the content of active ingredient using appropriate techniques when advanced techniques were not available. In the past half a century, analytical techniques have undergone a revolutionary evolution which has enabled the detection of smaller and smaller quantities of any kind of analyte. These advancements have also resulted in far reaching changes in the regulatory aspects related to the development of pharmaceuticals. In the changed perspectives, not only the content of active ingredient is essential but also a complete detailing of the impurities present or likely to appear during the course of usage has become mandatory.

The Expert Working Group of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, commonly known as ICH has defined an impurity as "any compound of the medicinal product which is not the chemical entity defined as the active substance or as an excipient in the product". Similarly impurity profile has been defined as "a description of the identified and unidentified impurities present in the medicinal product" [1].

Chiral drugs constitute about 56 % of the drugs currently in use and about 88% of these chiral synthetic drugs are used therapeutically as racemates. The racemates can exhibit variable metabolic pathways and pharmacologic activity and under some special circumstances, enantiomers as well as polymorphs are also considered as impurities [2,3].

The identification of impurities and/or degradants in pharmaceuticals is critically important for reasons of both product efficacy and patient safety. The impurities and /or degradants may evoke any form of adverse response, either pharmacologic or toxicologic in patients undergoing medication. Hence to ensure patient safety, impurity profiling which can be defined as a group of analytical activities aimed at the detection, identification or structure elucidation and quantitative determination of organic and inorganic impurities as well as residual solvents in bulk drugs and pharmaceutical formulations is very essential. Complete identification of any impurity and /or degradation product present at > 0.1% is desirable for drugs dosed at < 2 g/day. For drugs dosed at > 2 g/day, the threshold for isolation and identification is lower at 0.05% according to ICH guidelines [4,5]

A degradant is the simplest unwanted constituent in a pharmaceutical agent; it may be formed by the degradation of the pharmaceutical agent itself or through an interaction or reaction of the active ingredient in a formulation with one of the other constituents in a dosage form, and is hence generally closely related structurally to the drug molecule. The most common degradation pathways encountered involve the oxidation or hydrolysis with/without thermal stress to the drug molecule. Generally, the level of a degradation product will increase with time. Impurities, in

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contrast to degradation products, might or might not have any relation to the drug molecule [6].

Forced degradation study (Stress testing) is a well established method to identify the possible degradation impurities. Stress testing is an important tool for the prediction of stability-related problems. Well-designed stress-testing studies can lead to a thorough understanding of the intrinsic stability characteristics of the drug molecule. Impurities generated during real stability study might be different from impurities generated during forced degradation study. Hence both forced degradation study and real stability study are necessary to identify actual degradation impurities in an active pharmaceutical ingredient (API) [7].

2. EZETIMIBE AND ITS IMPURITIES

Present study describes the impurity profiling of a drug substance taking into account the specific case of Ezetimibe. Ezetimibe is chemically designated as 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3-hydroxy propyl]-4(S)-(4 -hydroxy phenyl)-2-azetidinone [8,9] (Fig. **1**). It selectively inhibits the intestinal absorption of cholesterol and related phytosterols from dietary and biliary sources by blocking the transport of cholesterol through the intestinal wall by interacting with the Neimann-Pick-C_1 like (NPC_1L_1) transporter [10,11].

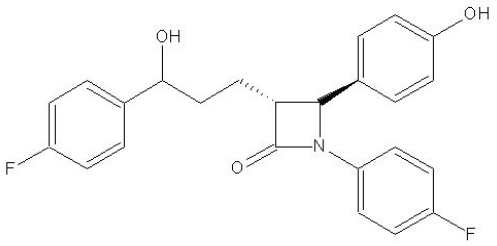


Fig. (1). Chemical structure of Ezetimibe.

The synthesis of Ezetimibe is a multi step process and hence there is a need to eliminate toxic and unstable intermediates from these synthetic steps. The process and degradation impurities likely to be present in Ezetimibe are described in Table **1** [12].

3. EXPERIMENTAL

Complete forced degradation study was performed on Ezetimibe taking into consideration the above mentioned aspects. Ezetimibe was subjected to thermolytic, photolytic, hydrolytic (acidic and alkaline) and oxidative stress conditions. The stressed and unstressed samples of Ezetimibe were injected into a developed and validated High Performance Liquid Chromatography (HPLC) method. In this method, a Hypersil C_{18} column (150 mm x 4.6 mm i.d., 5µm particle size) kept at a temperature of 40°C was used. Chromatographic separation was achieved under isocratic elution using a mobile phase consisting of a mixture of KH_2PO_4 buffer (pH 2.5; 0.05 M) – Methanol (45:55, v/v). UV detector was set at 242 nm. Flow rate was adjusted to 1.0 mL/min. Injection volume of standard and test solutions was kept as 10 µL. Water-methanol mixture (50:50, v/v) was used as the diluent [13].

Ezetimibe was found to be stable in acidic, oxidative, thermal and photolytic stress conditions, as shown in Fig. (**2**). Extensive degradation of Ezetimibe occurred only in alkaline hydrolytic conditions. Alkaline hydrolysis of Ezetimibe was performed by heating with 0.01 M aqueous sodium hydroxide solution at 60°C for 10 min. Major degradation product of alkali hydrolysis of Ezetimibe was found at RRT of 0.80 (Fig. **3**).

Hence, during forced degradation studies, it was observed that one major impurity was formed during alkaline degradation. The objective was now to identify this degradant and check its purity. This degradant was synthesized and further confirmed by isolation using preparative HPLC.

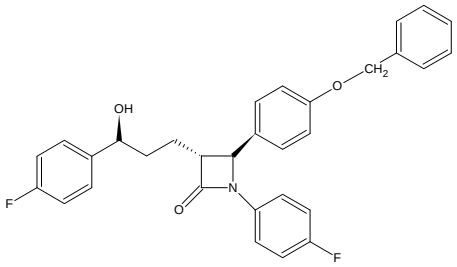
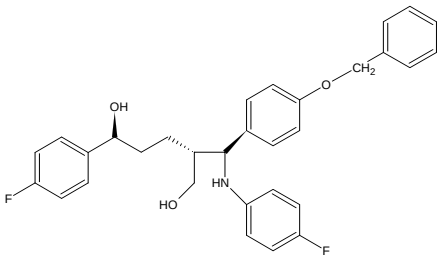
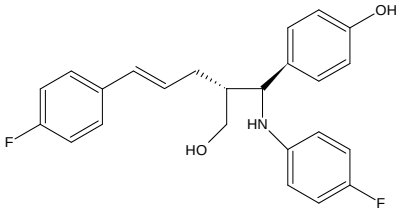
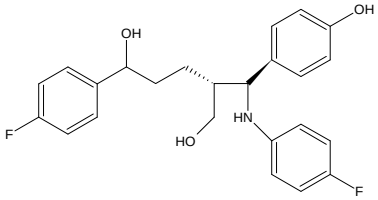
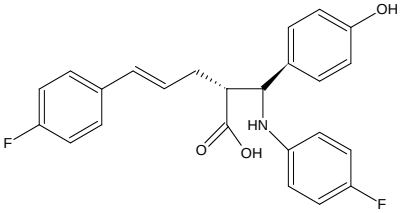
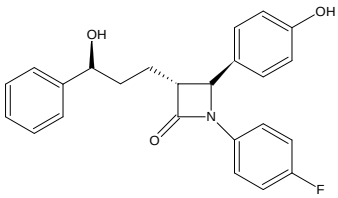
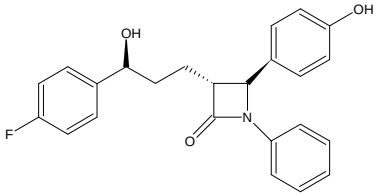
In the synthetic reaction, Ezetimibe was reacted with 0.1 M methanolic sodium hydroxide solution. Around 0.5 g of Ezetimibe drug substance was taken in 100 mL round bottom flask. About 50 mL of 0.1 M methanolic sodium hydroxide solution was added and the solution refluxed at 80°C on a constant temperature water bath (Matalab, India) for 15 min. The solution was allowed to cool to room temperature and neutralized with 1 M methanolic hydrochloric acid solution. The alkaline degradation solution of Ezetimibe was then concentrated on Buchi Rotavapor (R-124) with B-490 water bath under high vacuum to around 25 mL of Methanol. The solution was allowed to cool and around 50 mL of ice cold water was added. White solids precipitated out, were washed and dried in freeze dryer (Virtis Advantage, USA).

Further confirmation of the alkaline degradant was done by isolation using preparative HPLC. Waters preparative HPLC (Delta Prep) 4000 system (Waters Corporation, Milford, MA, USA) with high pressure unit of 4000 psi, operated through Empower software equipped with fraction collector was used. Waters Symmetry C_{18} (100 mm × 30 mm.) preparative column packed with 8 µm particle size was employed. 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 was at 242 nm. Methanolic alkaline degradation solution of Ezetimibe was concentrated on Buchi rotavapor R-124 (Buchi Labortechnik, Switzerland) and loaded on the preparative column for the isolation of alkaline degradant. The isolated fractions were collected and methanol was then evaporated using rotavapor with B-490 water bath to get the solids, which were then dried using freeze dryer.

For the identification of the degradant by MS detector, an LC-MS compatible HPLC method was developed. The solids were then checked for purity, which was found to be more than 99.0%. HPLC method involved an isocratic elution on a Waters Symmetry C_8 150 x 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 the flow rate of 1.0mL/min and UV detection at 242nm. Solids obtained by the two methods were established to be the same, looking at the RRT of 1.48.

The structure of this alkaline degradant was elucidated using spectroscopic techniques like MASS, NMR and IR spectroscopy. The degradant was identified as 5-(4-fluorophenyl)-2-[(4-fluorophenyl amino)-(4-hydroxyphenyl) methyl]-pent-4-enoic acid (Fig. **4**) [14].

Table 1. Known Impurities of Ezetimibe

Sr. No.	Name of Impurity	Structure	IUPAC Name
1	Benzyl Ezetimibe Impurity		(3R,4S)-1-(4-Fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxyphenyl]-4-(4-benzyloxyphenyl)-2-azetidinone
2	Benzyl Ezetimibe Diol Impurity		4-[4-benzyloxyphenyl)-[4-Fluorophenylamino]-methyl]-1-(4-fluorophenyl)-pentane-1,5-diol
3	Lactam cleaved alcohol Impurity		4-(5-Fluorophenyl)-1-[(4-fluorophenylamino)-2-hydroxymethyl-pent-4-enyl]-phenol
4	Ezetimibe Diol Impurity		(3R,4S)-1-(4-Fluorophenyl)-(4-hydroxymethyl)-5-(4-hydroxyphenyl)-5-N-(4-fluorophenylamino)-pentanol
5	Lactam cleaved acid Impurity		5-(4-fluorophenyl)-2-[(4-fluorophenylamino)-(4-hydroxyphenyl)methyl]-pent-4-enoic acid
6	Hydroxyl related desfluoro Impurity		1-(4-fluorophenyl)-4(S)-(4- hydroxyphenyl)-3(R)-(3-hydroxy-3-phenyl-propyl)-2-azetidinone
7	Lactam related desfluoro Impurity		3-[3-(4-fluorophenyl)-3- hydroxypropyl]-4-(4-hydroxyphenyl)-1-phenyl-2-azetidinone

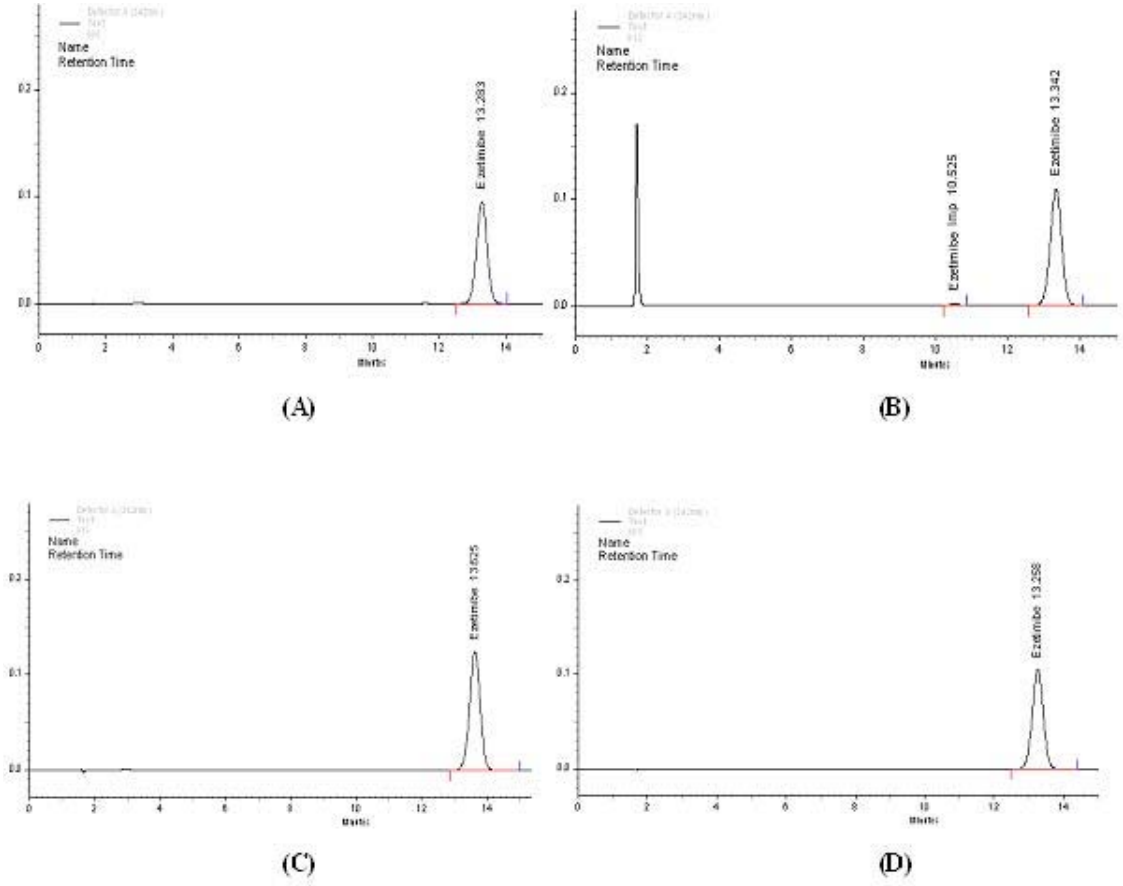


Fig. (2). Chromatograms representing (**A**) Acidic stress, (**B**) Oxidative stress, (**C**) Thermal Stress, (**D**) Photolytic stress for Ezetimibe.

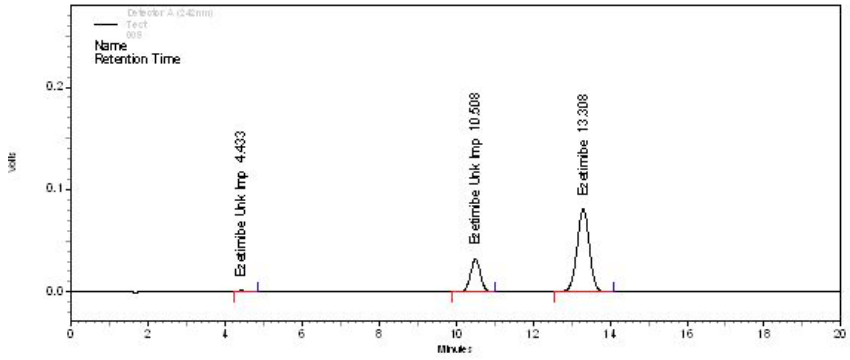


Fig. (3). Chromatogram representing alkali stress for Ezetimibe.

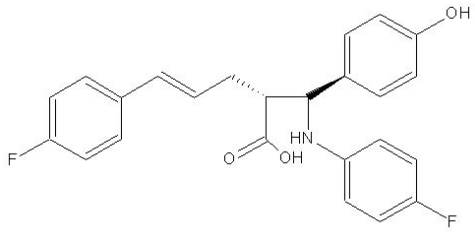


Fig. (4). Structure of alkaline degradant of Ezetimibe.

CONCLUSIONS

Isolation and elucidation of the structures of degradation products are typically collaborative research involving analytical, organic and physical chemistry knowledge combined with spectroscopic information. When this process is performed at an early stage, there is ample time to address various aspects of drug development to prevent or control the production of impurities and degradation products well before the regulatory filing and thus ensure production of a high-quality drug product.

Determining the structures of degradation products, arising during stress testing, can also be useful for preclinical discovery efforts during structure-activity relationship investigations. An understanding of the parts of the molecule that are susceptible to degradation can help in the design of more stable analogs. The development of a stable

formulation is also aided by an understanding of the reactive parts of the drug molecule.

Knowledge of the structures of the major degradation products of a drug compound is prerequisite for understanding the degradation pathways, which allows an assessment to be made of the sites in the compound that are susceptible to degradation under different conditions; essential to an understanding of the "intrinsic stability" characteristics of a drug compound. Hence, it can be concluded that structure elucidation of degradation impurities can be helpful in formulation development to comply with the regulatory requirements for impurity limits. [15]

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ABBREVIATIONS

RRT = Relative Retention Time

NMR = Nuclear Magnetic Resonance

IR = Infrared

min = minute

HPLC = High Performance Liquid Chromatography

mM = millimolar

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