International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 5, 2016

Original Article

INVESTIGATION OF FORCED DEGRADATION PRODUCTS OF ETODOLAC BY LC AND LC-MS/MS

DIVYA SAXENA[1*], SHAILESH DAMALE[2], AJIT DATAR[1]

[1]Guru Nanak Institute of Research and Development, Guru Nanak Khalsa College, Opp. Don Bosco School, Nathalal Parekh Marg, Matunga-east, Mumbai 400019, Maharashtra, India, [2]Shimadzu Analytical India Pvt. Ltd., Rushabh Chambers, Makwana Road, Marol, Andheri east, Mumbai 400059, Maharashtra, India
Email: divs.saxena12@gmail.com

Received: 28 Jan 2016 Revised and Accepted: 15 Mar 2016

ABSTRACT

Objective: The main objective of this study was to investigate the degradation products (DPs) of Etodolac (ETD) API under different stress conditions (acid hydrolysis, base hydrolysis, oxidation, thermal and photolysis) and to characterize the major DPs using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) and Infrared (IR) spectroscopy.

Methods: The proposed study describes a reversed-phase LC and LC-MS/MS method. Separation of ETD and its DPs was achieved on a Phenomenex C18 column. Structures of the major DPs were studied using a Triple Quadrupole Mass Spectrometer. A separate gradient LC-MS/MS method was developed for this purpose. The analysis was done using Shim-pack XR ODS column. Possible chemical reactions were predicted depending on the degradation type and the fragmentation data obtained from LC-MS/MS. Major DPs were isolated using preparative LC technique. These DPs were obtained in solid form using rotavap and lyophilizer and were then analyzed by IR spectroscopy to confirm their structural details.

Results: ETD was found to degrade completely under acid hydrolysis; it degraded 68 % under oxidation, 25 % after photolysis, 6 % in basic conditions and less than 1 % in thermal degradation. Four major DPs were characterized using LC-MS/MS and IR spectroscopy.

Conclusion: The DPs of ETD were investigated and characterized. Also, the tentative degradation pathways of ETD under different stress conditions were postulated.

Keywords: Etodolac, Forced degradation studies, Degradation products, LC, LC-MS/MS

INTRODUCTION

ETD, (*RS*)-2-(1, 8-Diethyl-4, 9-dihydro-3*H*-pyrano [3, 4-b] indol-1-yl) acetic acid is a non-steroidal anti-inflammatory drug (NSAID). This group of drugs has anti-inflammatory, analgesic and antipyretic activities. ETD is used worldwide to relieve inflammation, swelling, stiffness and joint pain in conditions like osteoarthritis and rheumatoid arthritis, as well as for general pain relief [1-3].

Forced degradation (FD) is a process whereby the natural degradation rate of a drug product or drug substance is accelerated by the application of an additional stress. FD studies or stress testing form a very important part of the drug development strategy in pharmaceutical industries under the guidelines of International Conference on Harmonization (ICH) and is carried out under more severe conditions than accelerated conditions [4]. The purpose of stress testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, light, etc. These studies serve to give information on a drug's inherent stability and assist in the validation of analytical methods to be used in stability studies. It also helps in determining the DPs and in estimating the tentative degradation pathway of the drug. Identification and qualification of these DPs is quite essential since it can cause undesirable side effects in patients, at times these side effects could also be fatal. Hence even though ICH and Food and Drug Administration (FDA) asks to include this study at Phase III of the clinical trial, it is recommended to start it as early as possible [5-12].

For the proposed study, ETD API (fig. 1) was subjected to degradation under different stress conditions like acid hydrolysis, base hydrolysis, oxidation, thermal and photolysis. Generally, during method development in pharmaceutical industries, a drug molecule is subjected to a maximum of 5-20 % degradation which is enough to provide a brief idea about its likely DPs [13, 14]. However, more harsh conditions were used in the present study to achieve complete or near to complete degradation of the drug substance so as to obtain a better indication of the DPs that are formed after maximum degradation and also to acquire relevant information regarding the degradation pathway of the molecule. Thus, the main purpose of this study was to identify and characterize the DPs formed after complete or maximum degradation of ETD, under different stress conditions using LC-MS/MS and IR spectroscopy techniques. A gradient LC method was developed for separation of ETD and its DPs. Simultaneously, a LC-MS/MS method was also developed to identify the DPs. An isocratic LC method was used on preparative LC to isolate the major DPs (which were found to be present ≥ 10 %). The fractions of DPs collected from preparative LC were processed through rotavap to evaporate the organic modifiers followed by lyophilization in order to obtain the DPs in solid form. IR spectra of these DPs were then obtained as a supporting data to LC-MS/MS results.

Literature survey revealed various analytical methods for the determination of ETD and few stability indicating assay methods. These included HPTLC [15-17], UV-Vis Spectrophotometry [18-23], RP-HPLC [24, 25], RP-UPLC [26] and a LC-MS method [27]. But, so far no detailed study on the DPs of ETD has been reported, especially using LC-MS/MS, to the best of our knowledge. Therefore, the present work was undertaken with the objective of developing a LC and LC-MS/MS method for studying the major DPs of ETD, to obtain the masses and the fragmentation pattern of these DPs formed after maximum degradation of the drug molecule and also to elucidate the tentative degradation pathway of ETD under different stress conditions, based on the MS and IR data. Thus, the novel contribution of this research paper was to investigate the DPs of ETD which are not yet reported, using LC-MS/MS and IR techniques.

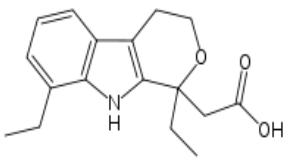


Fig. 1: Chemical structure of etodolac

MATERIALS AND METHODS

Chemicals and reagents

ETD API was procured from Hetero Chemical Lab (Hyderabad, India) with a purity of 99.4 % w/w.

HPLC grade-Acetonitrile, Formic acid, water; Analytical grade-Hydrochloric acid (HCl), Sodium Hydroxide (NaOH), Hydrogen Peroxide (H_2O_2)–30 % v/v and LC-MS grade-Formic acid were purchased from Merck (Mumbai, India).

Sample preparation

ICH guidelines were referred for carrying out forced degradation studies [4, 28]. Since the aim was to obtain maximum degradation of the molecule, the conditions were modified accordingly.

(A) For LC and LC-MS/MS analysis

Diluent

Water: ACN (1:1, v/v)

Blank

Diluent was used as blank

Standard

12.5 mg of ETD API was weighed and transferred to 25 ml volumetric flask; dissolved and volume made up with the diluent (500 ppm).

Acid degradation

12.5 mg of ETD API was weighed in a 25 ml volumetric flask. To this 5.0 ml of the diluent was added to dissolve the API followed by 5.0 ml of 5 M HCl. The solution was kept in water bath at 60 °C for 8 h; it was then cooled at room temperature, neutralized with 5 M NaOH and volume was made up with the diluent (500 ppm). This solution was filtered through 0.45 µ Millipore membrane before injecting into the system.

Blank was given the same treatment as that of the sample.

Alkali degradation

12.5 mg of ETD API was weighed in a 25 ml volumetric flask. To this 5.0 ml of the diluent was added to dissolve the API followed by 5.0 ml of 5 M NaOH. The solution was kept in water bath at 80 °C for 8 h; it was then cooled at room temperature, neutralized with 5 M HCl and volume was made up with the diluent (500 ppm). This solution was filtered through 0.45 µ Millipore membrane before injecting into the system.

Blank was given the same treatment as that of the sample.

Oxidative degradation

12.5 mg of ETD API was weighed in a 25 ml volumetric flask. To this 5.0 ml of the diluent was added to dissolve the API followed by 5.0 ml of 30 % H_2O_2 (v/v). The solution was kept in water bath at 80 °C for 8 h. It was cooled at room temperature and volume was made up with the diluent (500 ppm). This solution was filtered through 0.45 µ Millipore membrane before injecting into the system.

Blank was given the same treatment as that of the sample.

Thermal degradation

100 mg of ETD API was kept in controlled oven at 105 °C for 7 d. From this, 12.5 mg of the API was weighed in a 25 ml volumetric flask, dissolved and volume made up with the diluent (500 ppm). This solution was filtered through 0.45 µ Millipore membrane before injecting into the system.

Photolysis

12.5 mg of ETD API was weighed in a 25 ml volumetric flask, dissolved and volume made up with the diluent. This solution was exposed to UV radiation for 1.2 million Lux hours. It was filtered through 0.45 µ Millipore membrane before injecting onto the system.

(B) Preparative LC

Acid degradation

1 g of ETD API was weighed and dissolved in 15.0 ml of the diluent, to this 5.0 ml of 5 M HCl was added. The solution was kept in water bath at 60 °C for 8 h; it was then cooled at room temperature and neutralized with 5 M NaOH. This was filtered through 0.45 µ Millipore membrane before injecting into the system.

Oxidation degradation

1 g of ETD API was weighed and dissolved in 15.0 ml of the diluent, to this 5.0 ml of 30 % H_2O_2 (v/v) was added. The solution was kept in water bath at 80 °C for 8 h; it was then cooled at room temperature. This was filtered through 0.45 µ Millipore membrane before injecting into the system.

LC analysis

HPLC (Shimadzu Prominence Binary Gradient System, Shimadzu Corporation, Japan) equipped with a binary pump (20 AP), degasser, an autosampler (SIL-20AC), a temperature controlled column compartment and photodiode array detector (SPD-20A) was used. Chromatographic data was acquired using Lab solutions software. The analysis was done using Phenomenex C18 column (250 mm x 4.6 mm, 5 µ). The mobile phase comprised of-(A) water (pH 3.0 adjusted with formic acid) (B) acetonitrile, in a gradient mode. The gradient program is given in table 1. The flow rate was maintained at 1.5 ml/min; injection volume was 20 µl and the column temperature was maintained at 30 °C. Run time for the analysis was kept 32 min. The chromatograms were monitored at 225 nm.

LC-MS/MS analysis

HPLC system (Shimadzu Prominence Binary Gradient System, Shimadzu Corporation, Japan) equipped with a binary pump (LC-30AD), autosampler (SIL-30ACMP), a temperature controlled column compartment (CTO-30A) and photodiode array detector (SPD-M20A) was used. Chromatographic data was acquired using Lab solutions software. The analysis was done using Shim-pack XR ODS column (100 mm x 2 mm, 3 µ). The mobile phase comprised of-(A) water (pH 3.0 adjusted with formic acid) (B) acetonitrile, in a gradient mode. The gradient program is given in table 2. The flow rate was maintained at 0.4 ml/min; injection volume was 5 µl and the column temperature was maintained at 40 °C. Run time for the analysis was kept 10 min. The chromatograms were monitored at 225 nm. The structure elucidation of DPs was done using triple quadrupole mass spectrometer LCMS-8040 equipped with the electrospray ionization (ESI) source, operated in positive mode. Nitrogen gas was used at flow rates of 2 l/min for nebulization, 15 l/min for heating and as a curtain gas at 2.4 l/min. Argon was used as a collision gas. The collision energy was optimized and set to-30.0 V. Mass spectra were acquired over m/z range of 100-300. Event time was off 0.03 seconds.

Table 1: Gradient program used for liquid chromatography analysis

Time	A (%)	B (%)
0.01	50	50
14.0	50	50
22.0	30	70
25.0	30	70
27.0	50	50
32.0	50	50

Table 2: Gradient program used for liquid chromatography-tandem mass spectrometry

Time	A (%)	B (%)
0.01	80	20
5.00	10	90
7.00	10	90
7.10	80	20
10.0	80	20

Preparative LC analysis

HPLC (Shimadzu Prominence Binary Gradient System, Shimadzu Corporation, Japan) equipped with binary pump (20 AP), degasser, injector (SIL-10AP), fraction collector (FRC 10A) and photodiode array detector (SPD-20A) was used. Chromatographic data was acquired using Lab solutions software. Analysis was done using Phenomenex C18 column (250 mm x 21 mm, 5 µ). The mobile phase comprised of-(A) water (pH 3.0 adjusted with formic acid) (B) acetonitrile (1:1, v/v). The flow rate was maintained at 30.0 ml/min; injection volume was 2.0 ml and column was maintained at room temperature (around 25 °C). The chromatograms were monitored at 225 nm.

IR analysis

IR analysis was performed on 'IR Affinity-1' instrument with diamond ATR accessory, manufactured by Shimadzu, Shimadzu Corporation, Japan.

RESULTS AND DISCUSSION

The main objective of this study was to investigate the DPs of ETD under different stress conditions. For this purpose, ETD was subjected to base hydrolysis, acid hydrolysis, oxidation, thermal and photolytic degradation. ETD was found to degrade completely under acidic conditions; it degraded 6 % under basic conditions, 68 % under oxidative conditions, 25 % after photolysis and less than 1 % under thermal stress conditions.

The results indicated that ETD is most degradable under acidic conditions followed by oxidative and photolytic conditions while it is most stable under thermal conditions followed by basic conditions. The degradation conditions and amount of degradation obtained are mentioned in table 3. Few degradation studies of ETD have been carried out in the past at much milder conditions than mentioned in this study; however no specific DPs were obtained or identified [29-31]. A complete, similar degradation study of ETD has not been reported in the literature as yet.

A gradient LC method was developed and optimized for separation of ETD and its DPs (table 1). There were two major DPs obtained under acid hydrolysis at a retention time (RT) of 4.4 min and 23.8 min, one major DP under oxidative hydrolysis at 3.5 min, two major DPs under photolysis at 2.9 min and 3.6 min; while there were no major DPs obtained under base hydrolysis and thermal degradation. The RT of ETD in this method was found to be around 11 min. The representative chromatograms are given in fig. 2.

Table 3: Percentage of degradation observed in each of the stress conditions

Degradation type	Degradation condition	Amount degraded (%)
Acid hydrolysis	5 M HCl, 60 ^{0}C, 8 h	100.00
Base hydrolysis	5 M NaOH, 80 ^{0}C, 8 h	5.74
Oxidation	30 % H_2O_2, 80 ^{0}C, 8 h	68.22
Photolysis	1.2 million Lux h	24.73
Thermal	105 °C, 7 d	0.70

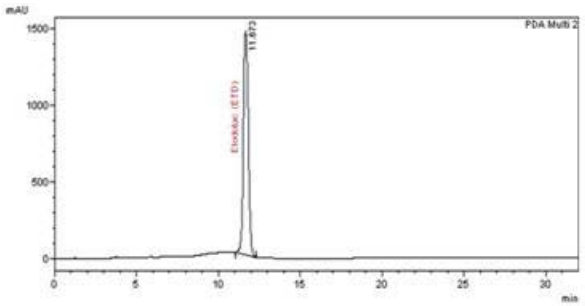
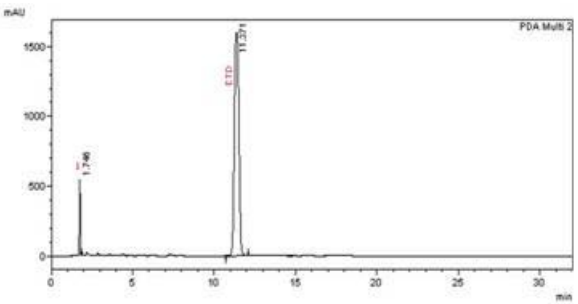


Fig. 2 (a)



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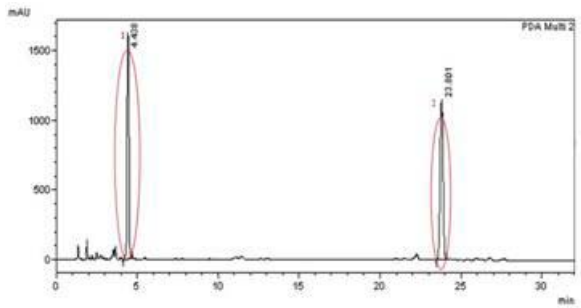


Fig. 2 (b)

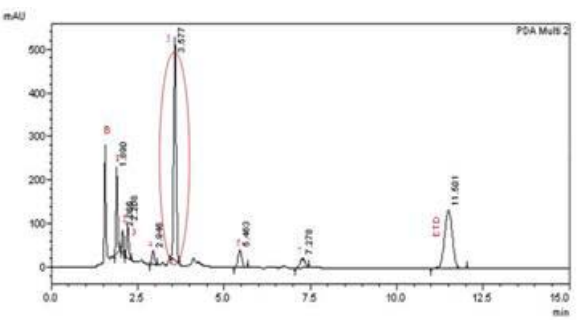


Fig. 2 (d)

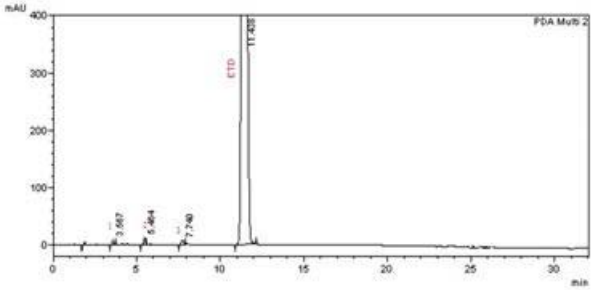


Fig. 2 (e)

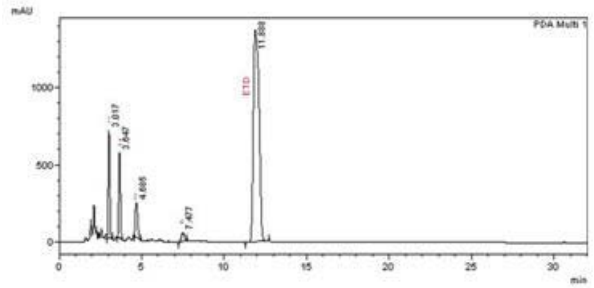


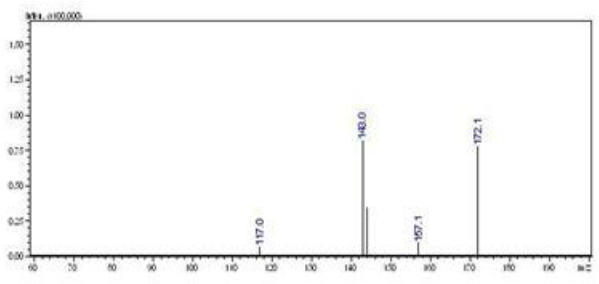
Fig. 2 (f)

Where, B=blank; Numbers= degradation products obtained under each stress conditions; Encircled peaks= peaks that were isolated on preparative liquid chromatography

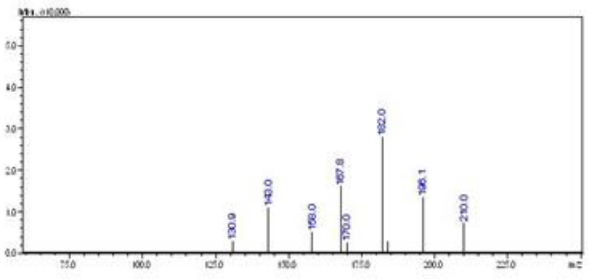
Fig. 2: Representative chromatograms of forced degradation study (a) etodolac (b) acid degradation (c) base degradation (d) oxidation degradation (e) thermal degradation (f) photolysis

The major DPs of acid hydrolysis and oxidative hydrolysis were isolated using preparative LC technique. From the fractions collected, organic modifiers were distilled out using rotavap and water was removed by lyophilization techniques to obtain the DPs in solid form. The yield of DPs obtained after lyophilization was very less. Also, no product was obtained for the second DP of acid degradation (RT 23.8 min), and so it was not possible to perform its IR. The reason for not obtaining any product for this DP could be its instability. IR analysis of the remaining two DPs was performed.

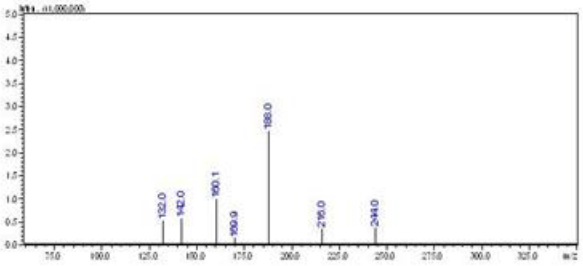
A separate gradient LC method (table 2) was also developed for analyzing the degradation samples of ETD and its isolated DPs on LC-MS/MS. The molecular weight of ETD is 287 and that of the protonated ion in the positive ion mode is 288. The masses of the major DPs obtained for acid degradation were m/z 190 and m/z 244, oxidation degradation-m/z 304 and photolysis-m/z 304 and m/z 320. The DP with m/z of 304 was common in oxidation and photolysis. These major DPs were further subjected to product ion scan at different collision energies.



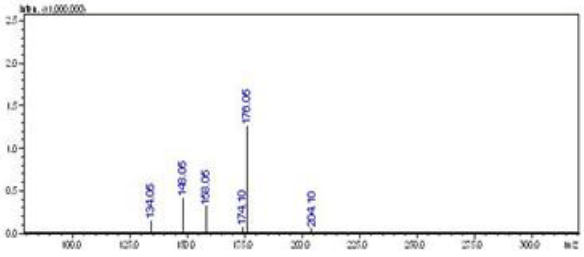
(A-i) m/z 190



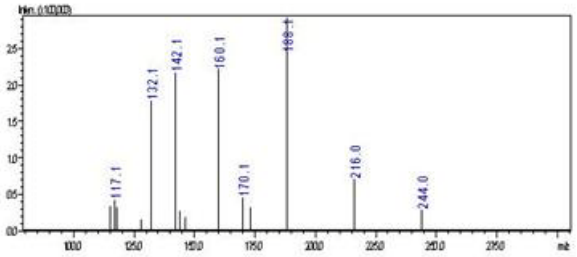
(A-ii) m/z 244



(B-i) m/z 304



(C-i) m/z 320



(C-ii) m/z 304

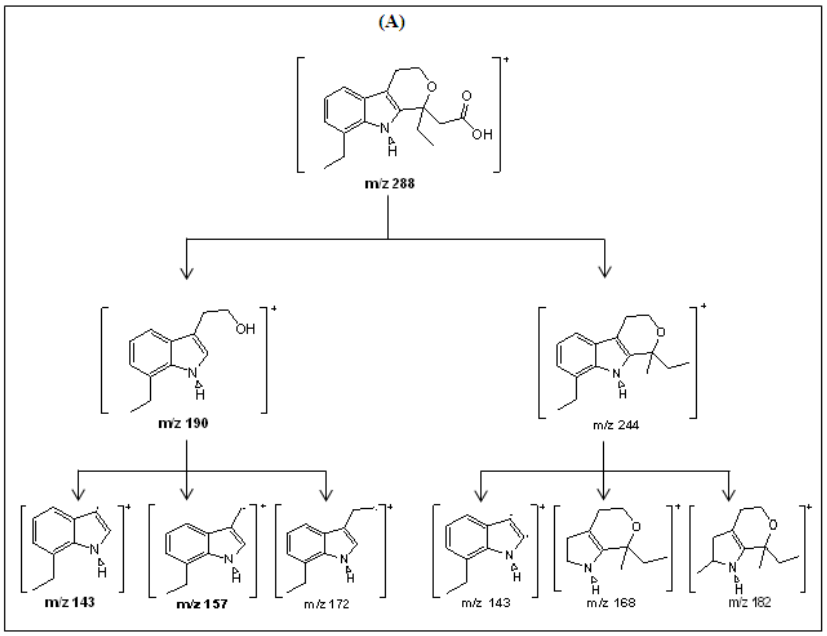
Fig. 3: Product ion scan of major degradation products of etodolac obtained under different stress conditions (A) acid degradation: (i) m/z-190 (ii) m/z 244 (B) oxidation degradation: (i) m/z-304 (C) photolysis: (i) m/z–320 (ii) m/z 304

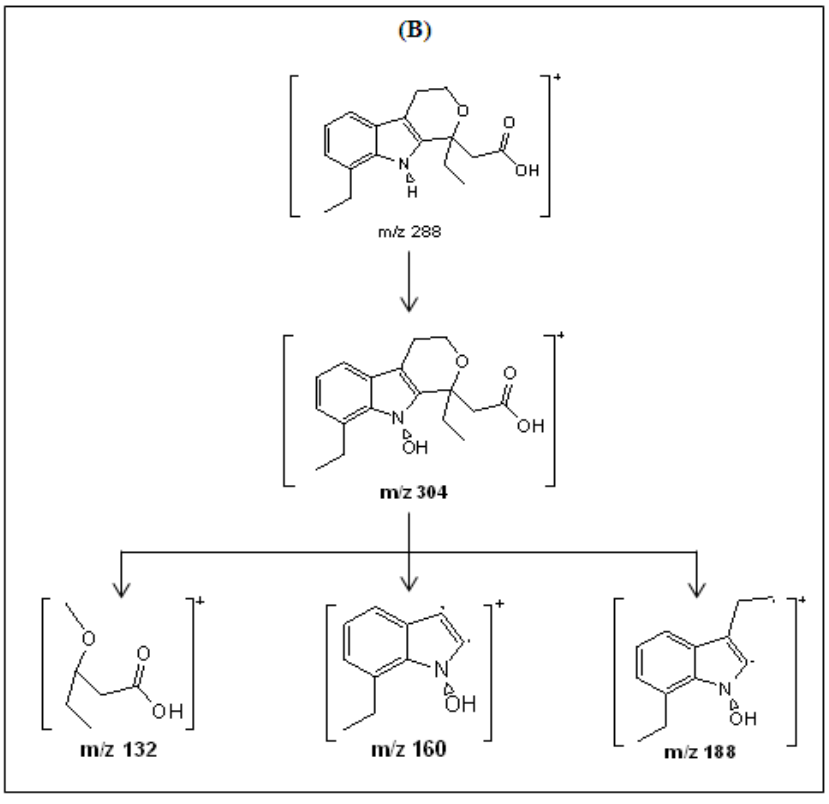
Best fragmentation was obtained at collision energy of-30.0V. The production scan of the major DPs of ETD under different stress conditions is given in fig.3; while table 4 shows the precursor ions and their respective product ion scan. The acid DPs of ETD have been reported in two similar studies [32, 33]. The masses of both the acid DPs are also specified in one of the study [32] and are in agreement with the present study. The other DPs obtained in the present study are not reported in the literature. So far, no other study has provided the masses and fragmentation pattern of the DPs of ETD.

Table 4: Precursor ions and their respective product ion scan obtained from liquid chromatography-tandem mass spectrometry study

Stress conditions	Precursor ions (m/z)	Collision energy used	product ion scan obtained (m/z)
Acid	190	-30 V	117,143,157,172
	244		131,143,158,168,170,182,196,210
Oxidation	304		132,142,160,170,188,216,244
Photolysis	304		117,132,142,160,170,188,216,244
	320		134,148,158,174,176,204

Structural elucidation of the DPs and the tentative degradation pathway of ETD (fig.4) were predicted based on the fragmentation pattern of the DPs and the type of degradation. The neutral losses of the fragments are given in table 5.





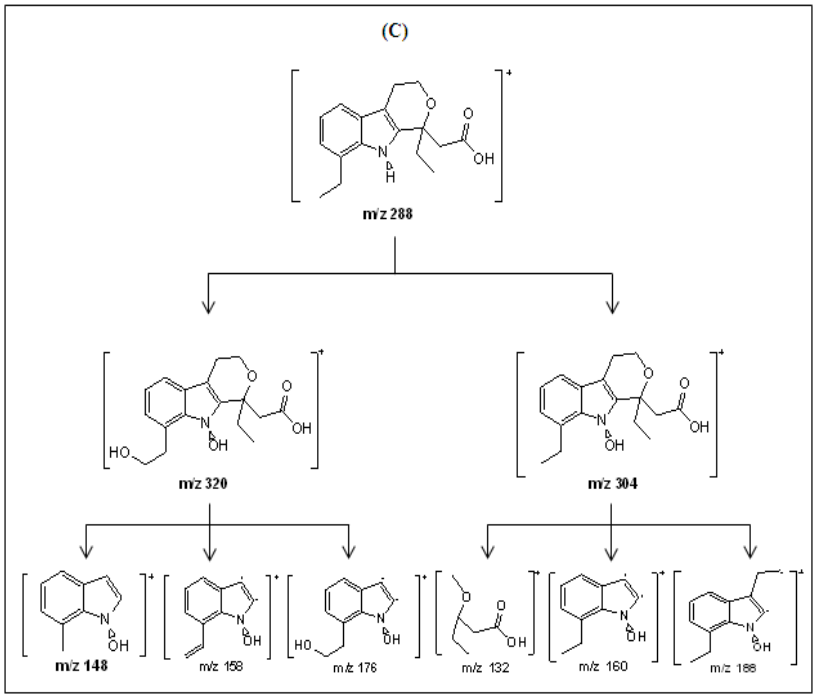


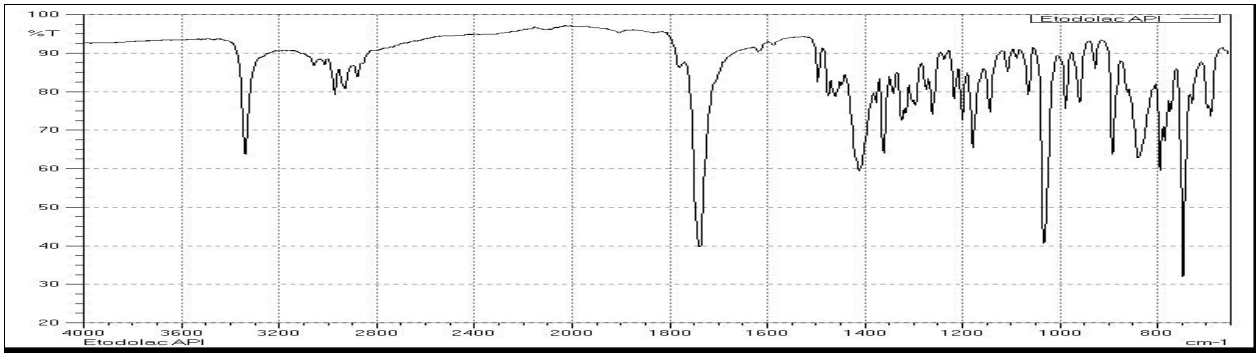
Fig. 4: Tentative degradation pathway of etodolac under different stress conditions, based on the degradation type and the fragmentation pattern of the degradation products obtained by mass spectrometry data (A) acid (B) oxidation(C) photolysis

Table 5: Neutral losses for the degradation pathways of etodolac

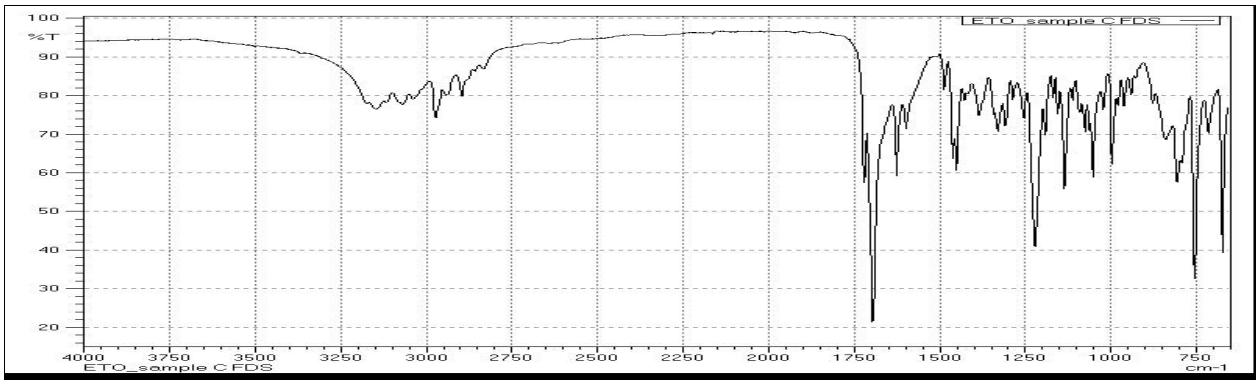
Degradation type	m/z of major degradation products (precursor ions) and their *mass difference (neutral loss/gain)	m/z of product ion scan and their #neutral loss
Acid	190 ($C_{12}H_{15}NO$)	143 ($C_{10}H_{10}N^{\bullet}$)
	Neutral loss of 98 units ($C_5H_6O_2$)	Neutral loss of 45 units ($C_2H_5O^{\bullet}$)
		157 ($C_{11}H_{12}N^{\bullet}$)
		Neutral loss of 31 units ($CH_3O^{\bullet}$)
		172 ($C_{12}H_{14}N^{\bullet}$)
		Neutral loss of 17 units ($^{\bullet}OH$)
	244 ($C_{16}H_{21}NO$)	143 ($C_{10}H_9N^{\bullet\bullet}$)
	Neutral loss of 44 units(CO_2)	Neutral loss of 100 units ($C_6H_{12}O^{\bullet\bullet}$)
		168 ($C_{10}H_{17}NO$)
		Neutral loss of 76 units (C_6H_4)
		182 ($C_{11}H_{19}NO$)
		Neutral loss of 62 units (C_5H_2)
Oxidation	304 ($C_{17}H_{21}NO_4$)	132 ($C_6H_{12}O_3$)
	Neutral gain of 16 units ($O^{\bullet\bullet}$)	Neutral loss of 172 units ($C_{11}H_9NO^{\bullet\bullet}$)
		160 ($C_{10}H_9NO^{\bullet\bullet}$)
		Neutral loss of 144 units ($C_7H_{12}O_3^{\bullet\bullet}$)
		188 ($C_{12}H_{13}NO^{\bullet\bullet}$)
		Neutral loss of 116 units ($C_5H_8O_3^{\bullet\bullet}$)
Photolysis	320 ($C_{17}H_{21}NO_{5)}$	148 (C_9H_9NO)
	Neutral gain of 32 units($2O^{\bullet\bullet}$)	Neutral loss of 172 units ($C_8H_{12}O_4$)
		158 ($C_{10}H_7NO^{\bullet\bullet}$)
		Neutral loss of 162 units ($C_7H_{14}O_4^{\bullet\bullet}$)
		176 ($C_{10}H_9NO_2^{\bullet\bullet}$)
		Neutral loss of 144 units ($C_7H_{12}O_3^{\bullet\bullet}$)
	304	Productions and neutral loss–same as mentioned
	(as mentioned under the oxidative condition)	under the oxidative condition)

* Mass difference w. r. t. t parent drug molecule (m/z 288), #Neutral loss w. r. t. t corresponding precursor ions

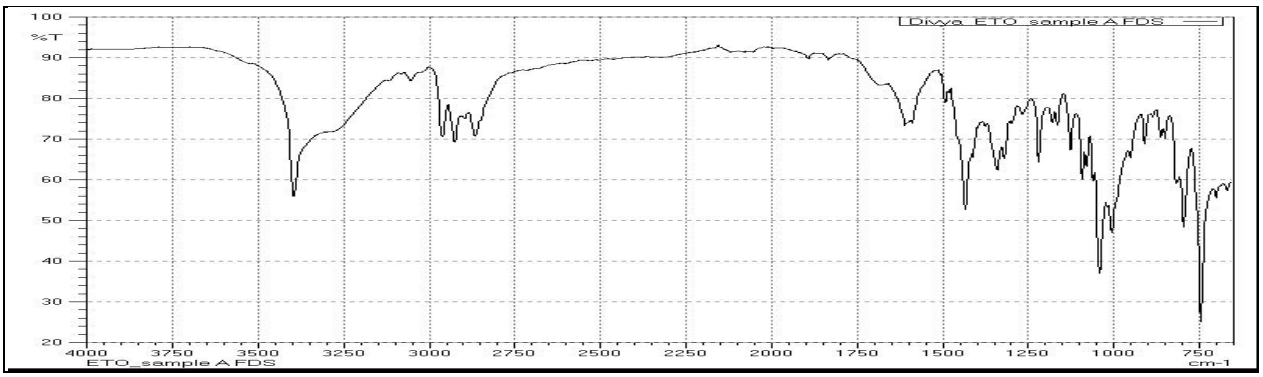
The major functional groups of the DPs were also specified in IR analysis. The main frequencies of IR which support the inferences drawn from the MS/MS data are: presence of 1720.5 band representing C= O group and absence of the–NH group, in the oxidation DP (m/z 304); presence of 3394.7 band representing–NH group and absence of C=O group, in the acid DP (m/z 190). The IR spectra are given in fig.5 and their interpretations in table 6.



(a)



(b)



(c)

Fig. 5: Infrared spectra of (a) etodolac (b) oxidation degradation product (m/z 304) (c) acid degradation product (m/z 190)

Table 6: Interpretation for infrared spectroscopy

(A) Oxidation degradation product (m/z–304)

S. No.	Frequency range	Actual band obtained	Functional group
1)	1700-1720 S	1720.5	**C=O**
2a)	1210-1310 S	1220.9, 1253.7, 1286.5,1309.6	**C-O-C**
2b)	1010-1050 M	1022.27	
2c)	1170-1250 S	1192.0, 1220.9	
3a)	2500-3100	2833.4, 2897.0,2937.6, 2972.3,3068.8	**-OH**
3b)	875-960 M	879.5	

(B) Acid degradation product (m/z–190)

S. No.	Frequency range	Actual band obtained	Functional group
1)	3100-3400	3394.7	Pyrole (-H bonded)
2)	3400(Approx.) M	3394.7	Aromatic secondary Amine (ArNHR)
3a)	2500-3100 S	2866.2, 2926.0, 2962.6	-OH
3b)	1280-1380 M	1321.2, 1340.5	

The IR spectra of ETD in its pure as well as in combined form (along with other drugs and excipients) are available in the literature [34, 35]; however, the IR spectra of the DPs are not yet reported.

In the future, a larger amount of DPs can be isolated on preparative LC and NMR analysis can be performed for an additional confirmation of structures.

CONCLUSION

From the stress studies carried out, it was found that ETD is highly degradable under acidic conditions (100 %), followed by oxidative (68 %) and photolytic conditions (25 %); while it's most stable under the thermal conditions (<1 %) followed by the basic conditions (6 %).

A simple gradient LC method was successfully developed and optimized for the separation of ETD and its DPs. Also, a fast LC-MS/MS method was effectively developed to identify the DPs; being a fast method, it saves lot of time and resources. The study was helpful in characterizing four major DPs of ETD-based on the LC-MS/MS and IR data. The masses of these major DPs of ETD were found to be: acid-190 and 244; oxidation-304, photolysis-304 and 320.

A tentative degradation pathway of ETD was also postulated under different chemical stress conditions.

ACKNOWLEDGEMENT

The authors are grateful to Shimadzu Analytical India Pvt. Ltd (Mumbai, India) for letting the LC-MS/MS, IR and preparative LC work be carried out at their lab. We appreciate the efforts put in by Ms. Sampada Khopkar of Shimadzu Analytical India Pvt. Ltd (Mumbai, India) for IR analysis.

The authors are thankful to Guru Nanak Institute of Research and Development, Guru Nanak Khalsa College, Matunga, Mumbai, India for providing all the research related facilities, required in accomplishing the present research work.

We are also grateful to Department of Science and Technology (DST), India for providing the INSPIRE fellowship.

CONFLICT OF INTERESTS

Declared none

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