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Identification of the degradation product of ezlopitant, a non-peptidic substance p antagonist receptor, by hydrogen deuterium exchange, electrospray ionization tandem mass spectrometry (ESI/MS/MS) and nuclear magnetic resonance (NMR) spectroscopy

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Abstract

The degradation product of ezlopitant was isolated from low specific activity material and identified by solution phase hydrogen/deuterium (H/D) exchange and electrospray ionization tandem mass spectrometry (ESI/MS/MS) to be an isopropyl peroxide analog of ezlopitant. The structure of the degradant was further confirmed by nuclear magnetic resonance (NMR) spectroscopy utilizing complete ^{1}H and ^{13}C assignments. Studies were also performed to identify the factors responsible for the oxidative degradation of ezlopitant, which included salt form, storage conditions and salt formation solvent. Of all the variable studies over a 3 weeks period, only a change in the salt form prevented this oxidative degradation.

Keywords: Ezlopitant; ESI/MS/MS; Degradation product identification; NMR; H/D exchange

1. Introduction

Ezlopitant, (2*S*,3*S*-*cis*)-2-(diphenylmethyl)-*N*-{(2-methoxy-5-isopropylphenyl)methyl}-1-azabicyclo[2.2.2]octan-3-amine, is a selective antagonist of the NK_1 tachykinin receptor which preferentially mediates the actions of substance P (SP). It is currently being investigated as a potential therapy for disorders in which SP is believed to play a role in the emetic pathway, the transmission of pain signals, and in preventing the cellular responses that are associated with chronic inflammation [1–8].

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As part of preclinical safety assessment, the synthesis of radiolabeled ezlopitant was completed [9] to aid in full absorption, distribution, metabolism, and excretion (ADME) studies. [^{14}C]-ezlopitant labeled with ^{14}C at the benzyl carbon of 5-isopropyl-2-methoxy moiety attached to the 2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-yl)- amine (Fig. 1) is quite stable in solution. However, when stored as a solid, [^{14}C]-ezlopitant degrades to one major degradation product over a relatively short time period. Therefore, the identification of this major degradation product was required. Factors responsible for the degradation of [^{14}C]-ezlopitant such as salt form, storage conditions and salt formation solvent were also studied.

Mass spectrometry has been regarded as one of the most important analytical tools in studies of drug metabolism, pharamcokinetics and biochemical toxicology. However, with the commercial introduction of new ionization methods such as those based on atmospheric pressure ionization (API) techniques and the combination of liquid chromatography-mass spectrometry (LC-MS), mass spectrometry has now become a truly indispensable technique in pharmaceutical sciences. LC-MS systems equipped with API sources (electrospray, ionspray or heated nebulizer interfaces) have been utilized extensively in studies of foreign compounds metabolism and identification of drug impurities due to their very high sensitivity, compatibility with reverse-phase chromatography, and their applicability to structural elucidation of polar and thermally-labile drug conjugates.

The determination of the number of active hydrogens such as OH, COOH, NH or SH by simple exchange of the sample in the presence of heavy water (D_2O) is widely used in mass spectrometry. The chemical incorporation of deuterium followed by measurement of the intact product by mass spectrometry has played a major role in the structural characterization of molecules, in gaining information on the mechanisms of chemical or biological reactions and in the interpretation of mass spectra [10–16]. Methods for producing these ions in mass spectrometry include electron impact (EI) [10], chemical ionization (CI) [17–19], fast-atom bombardment (FAB) [20–22], thermospray (TS) [23,24], particle beam (PB) [24] and electrospray ionization (ESI) [12,25–34].

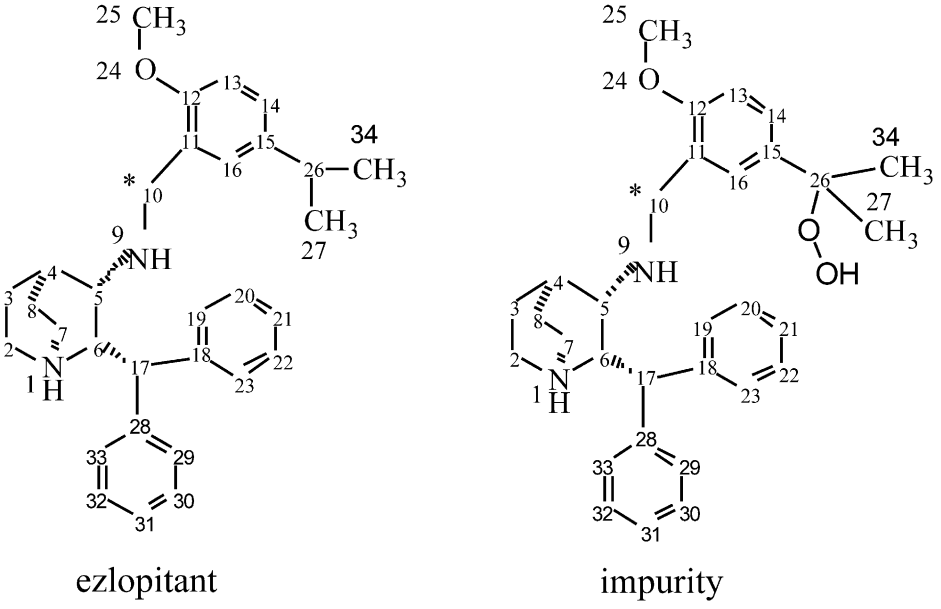

Fig. 1. Structures of ezlopitant and its isolated impurity. The asterisks denote the site of ^{14}C incorporation. Numbering of ezlopitant and its impurity from ISIS data base and complies with IUPAC system.

Nuclear magnetic resonance (NMR) spectroscopy in conjunction with liquid chromatography and mass spectrometry have been successfully employed in pharmaceutical analysis including quantitative and qualitative determinations of bulk drug impurities [35–45]. Such determinations are essential and required to comply with regulatory guidelines and to assess biological activity.

This paper describes the detection, isolation, and structure elucidation of the degradation product of [^{14}C]-ezlopitant and determines the factors responsible for its formation.

2. Experimental

2.1. Chemicals and materials

Ezlopitant, (2*S*,3*S*-*cis*)-2-(diphenylmethyl)-*N*-{(2-methoxy-5-isopropylphenyl)methyl}-1-azabicyclo[2.2.2]octan-3-amine (Fig. 1), was synthesized by Dr K. Zandi, Pfizer Global Research and Development, Groton, CT, USA. Methanol (CH_3OH), acetonitrile (ACN) acetone, hexane, tetrahydrofuran (THF), *tert*-butylated hydroxytoluene (BHT), chloroform ($CHCl_3$) and deionized water (HPLC-grade) were obtained from J.T. Baker (Phillisburg, NJ, USA). Glacial acetic acid (CH_3COOH), ammonium hydroxide (NH_4OH), and ammonium acetate (NH_4OAc), HPLC-grade, were obtained from Fisher (Fair Lawn, NJ, USA). All deuterated compounds, ≥ 99 at.% D, were obtained from Aldrich Chemical Company (Milwaukee, WI, USA): acetic-d_3 acid-d_1 (CD_3COOD), methanol-d_1 (CH_3OD), chloroform-d_1 ($CDCl_3$) and deuterium oxide (D_2O).

2.2. Isolation and purification of the degradation product

After being stored for 3 months at 5 °C, a sample of low specific activity [^{14}C]-ezlopitant (2.2 g, 0.8 mCi, 0.2 mCi/mmol) contained 3% of the degradant by HPLC analysis was purified by reverse phase Biotage chromatography (ACN/0.05 M NH_4OAc 55:45 v/v) and extracted with chloroform to give 175 mg (71 μCi) [^{14}C]-ezlopitant contaminated with 20% degradation product. This material was then purified by silica gel chromatography (2% methanol/chloroform with 0.1% NH_4OH) to give 40 mg (15.7 μCi) material, consisting of 78% degradation product contaminated with 16% [^{14}C]-ezlopitant. Finally, this material was purified by silica gel chromatography (20% acetone/hexane) to give 21 mg (9.3 μCi) carbon-14 labeled degradation product.

2.3. Hydrogen/deuterium exchange, sample preparation and introduction for mass spectrometry analysis

Solution phase hydrogen/deuterium (H/D) exchange method was utilized to study the fragmentation pathways in the mass spectra of [^{14}C]-ezlopitant degradation product. The residue from the purified sample (~ 2 mg) was dissolved in MeOH (~ 2 ml) to give a stock solution with a concentration of 1 mg/ml. A 0.5 ml aliquot of the stock solution was transferred into a clean tube and mixed with 0.5 ml of 1% HOAc in water to give a working solution I with a concentration of 0.5 mg/ml. A 0.5 ml aliquot of the stock solution was evaporated to dryness under N_2 stream and the residue was dissolved in 1 ml CH_3OD/1% CD_3COOD in D_2O (1:1) to give a working solution II with a concentration of 0.5 mg/ml. Working solutions I and II were used to obtain the collision induced dissociation (CID) mass spectra of the non-deuterated and deuterated species, respectively. Recent work from these laboratories showed that the addition of 1% CH_3COOH to the mobile phase caused a significant increase in the sensitivities of the ESI mass spectra of a series of tetracyclines and antiviral agents in the positive ion mode [46,47]. Samples were infused into the electrospray interface using a Harvard syringe pump (South Natick, MA) at a flow rate of 10 μl/min.

2.4. Sample preparation and introduction for NMR analysis

HPLC purified samples of [^{14}C]-ezlopitant and its degradation product were used for NMR analysis. These samples were converted into the free base and typically 10–15 mg of sample was

dissolved in 1 ml of acid-free deuterated chloroform. The deuterium exchange experiments were performed by adding 100–200 μl of either H_2O or 50:50 H_2O/D_2O, shaking the sample briefly, and allowing the two phases to separate. NMR data was then acquired on the chloroform phase of the material.

2.5. Mass spectrometry

Mass spectral analyses were performed with a Sciex API-III^{plus} atmospheric pressure ionization (API) triple quadrupole mass spectrometer with a mass range to 2400 μ (Thornhill, Ont., Canada). The mass spectrometer was equipped with an ion spray (IS) interface set at a nebulizer gas pressure (nitrogen) of 60 psi. The nitrogen curtain gas was adjusted to a constant flow rate of 1.2 l/min. Positive ions formed at atmospheric pressure were sampled into the quadrupole mass filter via a 0.0045 in. diameter aperture. Collisionally induced dissociation (CID) studies were performed using argon gas at a thickness of 2×10^{15} atoms cm^2 and a collision energy of 35 eV. Signals for $[M+H]^+$ and fully exchanged species, $[M_D+D]^+$, ions were dissociated by collision in Q2 with argon. The quadrupole power supply was set for unit mass resolution with a 2 ms dwell time and the signal was averaged over ten scans.

2.6. Nuclear magnetic resonance

NMR experiments were performed at 500 MHz (^{1}H) on a Bruker DMX-500 spectrometer (Billerica, MA) equipped with temperature control and z-axis pulsed field gradients. An inverse broadband probe, with the inner coil tuned to proton nuclei and the outer coil tuned to carbon nuclei, was used for all experiments. The temperature was set to 298 K throughout the analysis. Typical 1D proton experiments were performed over the spectral range 0–8 parts per million (ppm) by acquiring 16 384 total points using quadrature detection. Eight to thirty two scans were sufficient to give adequate signal to noise for proton experiments. Carbon experiments were performed over the range 20–160 ppm with proton decoupling employed, and a short (total of 1 s) recycle delay was used. Eight thousand one hundred and ninety two scans were used to give adequate sensitivity for both ezlopitant and its degradant. Distortionless enhancement by polarization transfer (DEPT) experiments were acquired using the same carbon spectral width and 1024 transients. Gradient correlation spectroscopy (COSY) experiments were acquired with 2 scans per increment and 512 increments using the same 0–8 ppm spectral width as that used for 1D proton experiments. Heteronuclear multiple-quantum coherence experiments (HMQC) were run gradient enhanced employing 8 scans per increment and 512 increments. Long-range HMQC experiments were performed by changing the spin-coupling refocus time to 83 ms to optimize for 6 Hz couplings. These experiments were run using 32 scans per increment and 512 increments.

2.7. Factors affecting the degradation product of [^{14}C]-ezlopitant

[^{14}C]-ezlopitant is chemically stable when stored as a solution in water or ethanol. However, when stored as a solid, [^{14}C]-ezlopitant degrades to one major product over relatively short periods of time. Therefore, experiments were carried out in an effort to identify factors responsible for the degradation of ezlopitant which included storage conditions (Table 1), salt formation solvent (Table 2), and salt form (Table 3). [^{14}C]-ezlopitant was stored as a solid ($\sim$25 mg, 0.4 mCi/mg) or as a solution (1 mg/ml) and the purity of each sample was evaluated weekly by HPLC analysis.

3. Results and discussion

3.1. Mass spectrometry

A key step in elucidating degradant structure is to understand the fragmentation pattern of ezlopitant. The CID mass spectrum of $[M+H]^+$ of ezlopitant is shown in Fig. 2. The degradation product of ezlopitant was identified as the isopropyl peroxide derivative of parent drug. The assignments of fragment ions in the CID mass spectrum of $[M+H]^+$ were made possible and confirmed by

Table 1
Percent of the degradation product of ezlopitant as a function of storage atmosphere and temperature

Storage condition	Storage atmosphere	Temperature (°C)	Percentage of degradation	
			1 week	3 weeks
Ethanol	Air	5	< 0.020	< 0.020
Water	Air	5	< 0.020	< 0.020
Solid	Air	5	0.157	0.455
Solid	Nitrogen	5	0.12	0.397
Solid	Argon	5	0.136	0.475
Solid	Air	25	0.182	0.544

Table 2
Percent of the degradation product of ezlopitant as a function of salt formation solvent (THF with or without BHT stabilizer)

Salt formation solvent	Storage condition	Percentage of degradation	
		1 week	3 weeks
THF + BHT	Ethanol	< 0.020	< 0.020
	Water	< 0.020	< 0.020
	Solid	0.038	0.338
THF	Ethanol	< 0.020	0.022
	Water	0.043	0.063
	Solid	0.174	0.496

THF, tetrahydrofurane; BHT, *tert*-butylated hydroxytoluene.

the mass shift from the corresponding CID mass spectrum of the fully exchanged species. The degradation product showed a protonated molecular ion at *m*/*z* 487, 32 mass units higher than the drug, suggesting the addition of two oxygen atoms. The increase from *m*/*z* 487 for $[M+H]^+$ of ezlopitant impurity to 490 for the fully exchanged species, M_DD^+, corresponding to two exchangeable hydrogens (plus the added proton/deuteron). The CID mass spectra of these two species at a sufficiently high collision energy to cause extensive fragmentation (CE = 35 eV) are shown in Fig. 3. The prominent fragment ion at *m*/*z* 455 in the CID mass spectrum from *m*/*z* 487 is shifted to *m*/*z* 458 from the fully exchanged ion at *m*/*z* 490-retaining all of the exchanged hydrogens (including the added D) and indicated a very fascile loss of O_2 to form the parent drug. Loss of dioxygen (O_2) has been observed in the fragmentation pathways of organic and bicyclic peroxides [48,49]. A proposed mechanism for the loss of O_2 from the protonated ezlopitant impurity to form the fragment ion at *m*/*z* 455 is shown in Fig. 4. The initial site of protonation is based on the ionization constants calculated by ZPARC pK_a program [50].

Table 3
Percent of the degradation product of ezlopitant as a function of salt form of [^{14}C]-ezlopitant

Salt form	Storage condition	Percentage of degradation		
		1 week	3 weeks	60 days
HCl	Ethanol	< 0.020	< 0.020	n/a
	Water	< 0.020	< 0.020	n/a
	Solid	0.157	0.455	n/a
Citrate	Ethanol	< 0.020	< 0.020	n/a
	Water	< 0.020	< 0.020	n/a
	Solid	< 0.020	< 0.020	< 0.020[a]

n/a, Not applicable.
[a] Material stored at 25 °C after initial 3 weeks.

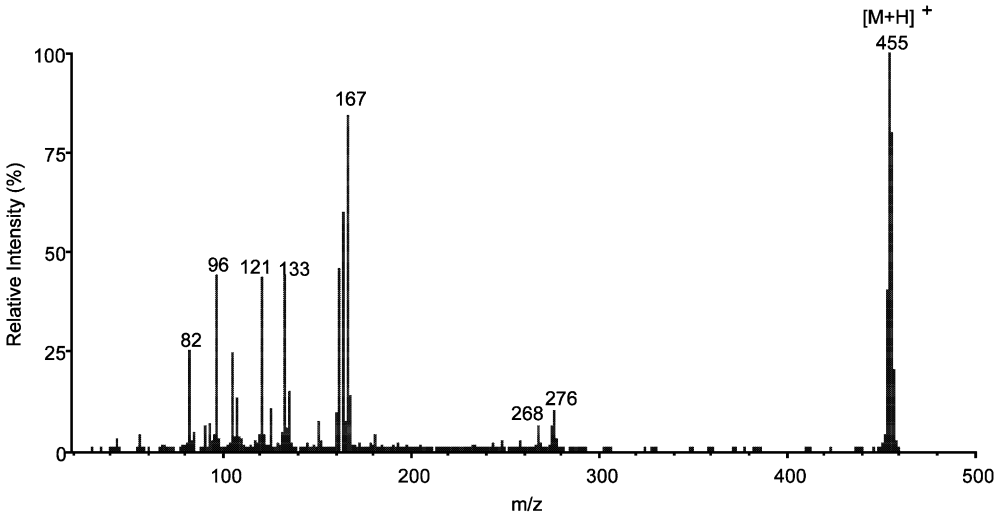

Fig. 2. CID product ion mass spectrum of $[M+H]^+$ of ezlopitant at *m/z* 455.

The fragment ion at *m/z* 167 in the CID mass spectrum from *m/z* 487 contains no exchangeable hydrogens since there is no shift in its mass from the CID mass spectrum of the fully exchanged species at *m/z* 490 and indicate that there is no substitution at the benzhydryl moiety. The fragment ion at *m/z* 276 in the CID mass spectrum from *m/z* 487 is shifted to *m/z* 277 from the fully exchanged ion at *m/z* 490 and is identical to that of the parent drug suggesting that the addition of the 32 amu had occurred remote from the benzhydryl-quinuclidine moiety. The fragment ion at *m/z* 195, 32 daltons higher than that of the parent drug, indicated the presence of two hydroxylations at the anisole moiety. However, the fragment ion at *m/z* 195 in the CID mass spectrum from *m/z* 487 is shifted to *m/z* 196 from the fully exchanged ion at *m/z* 490 suggesting that this fragment ion contains only one exchangeable hydrogen and the possibility of two hydroxylations at the anisole moiety was ruled out. The fragment ion at *m/z* 121 contains no exchangeable hydrogens since there is no shift in its mass from the CID mass spectrum of the fully exchanged species and indicates that the addition of 32 Da had occurred at the isopropyl moiety and consistent with peroxide addition.

The fragment ions at *m/z* 82 and 96 in the CID spectrum from *m/z* 487 are shifted to *m/z* 83 and 97, respectively, from the fully exchanged ion at *m/z* 490 and are quinuclidine-derived ions. The fragment ion at *m/z* 412 in the CID spectrum from *m/z* 487 is shifted to *m/z* 414 from the fully exchanged ion at *m/z* 490 and further indicated that the modification had occurred at the isopropyl moiety and consistent with peroxide addition.

3.2. Nuclear magnetic resonance

The structure identification of the impurity was accomplished by detailed NMR investigations. First, complete ^1H and ^{13}C chemical shifts were required as an initial measure of whether the degradant was similar to, or widely different from, the parent compound [^{14}C]-ezlopitant. Second, both the standard sample of ezlopitant and the degradant were completely assigned using standard NMR proton–proton and proton–carbon connectivity tools. Finally, the effect of H/D exchange on the carbon spectrum of the degradant was measured to detail the number of directly attached functional groups with exchangeable protons (one, the exocyclic amine).

The ^1H and ^{13}C NMR spectra of ezlopitant and its impurity showed that the largest and most significant chemical shift changes occurred near the site of attachment of the isopropyl in ezlopitant and the two compounds were generally closely related. The aliphatic ^{13}C spectra of ezlopitant

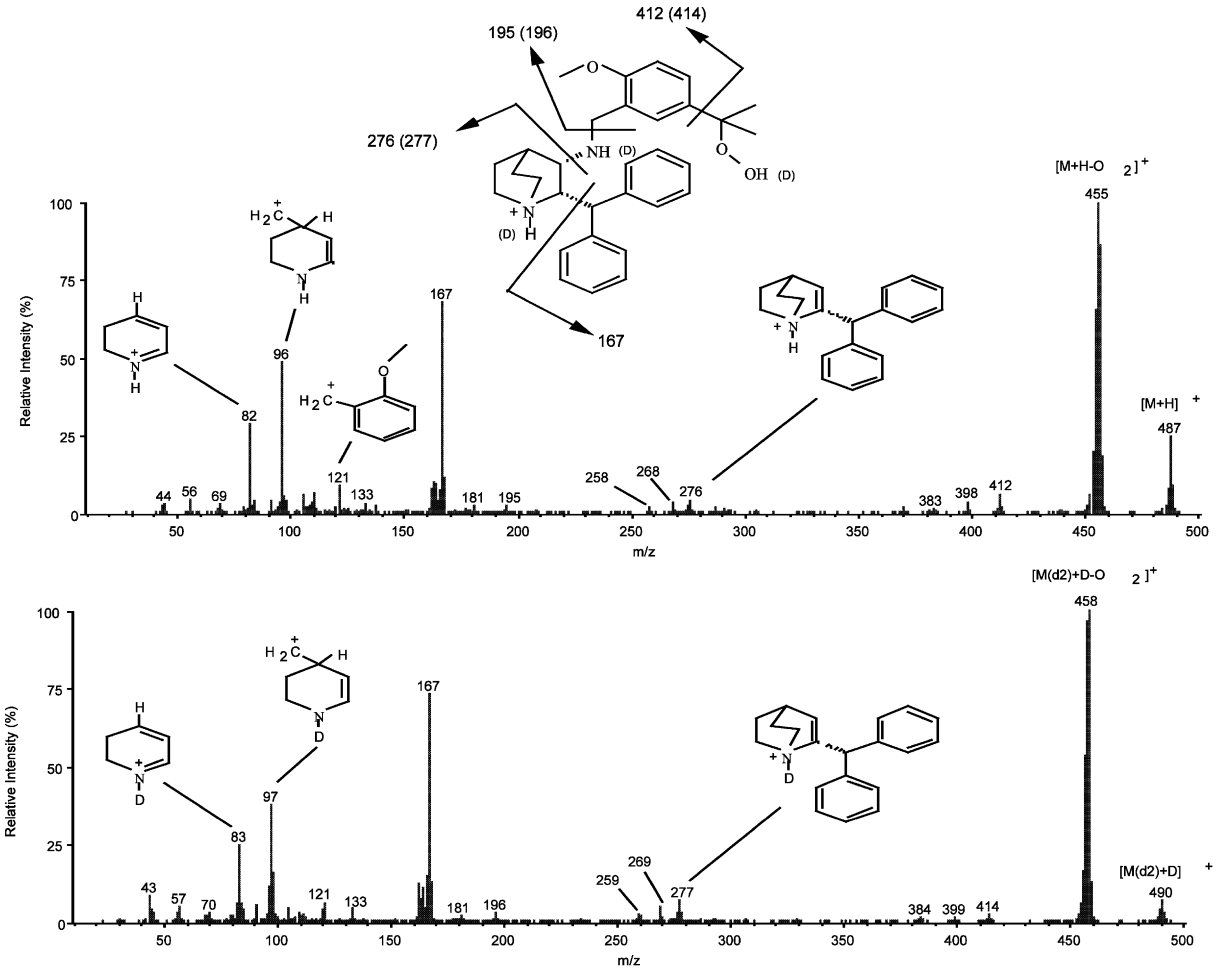

Fig. 3. Positive ion ESI mass spectra of ezlopitant impurity (MW = 486): (a) CID product ion spectrum of $[M+H]^+$ at m/z 487 (b) CID product ion spectrum of the fully exchanged $[M(d2)+D]^+$ at m/z 490 at a CE of 35 eV. Deuteration was achieved by solution phase H/D exchange method. MS and MS^2 experiments were performed on a Sciex API-III^{plus} triple quadrupole mass spectrometry. Numbers in parentheses refer to deuterated fragment ions.

(bottom) and its degradant (top) are shown in Fig. 5. Note the appearance of a single quarternary carbon at 83.7 ppm in the top spectrum, this is the isopropyl peroxide carbon, which starts out as an isopropyl carbon in ezlopitant with a chemical shift of approximately 33.8 ppm. Several of the carbons below 34 ppm are impurity peaks. The ^{1}H NMR spectra of ezlopitant (bottom) and its degradant (top) are shown in Fig. 6. The isopropyl protons change from doublets to singlets and shift downfield about 0.3 ppm, consistent with loss of the isopropyl methine proton and concomitant addition of an oxygen or nitrogen.

The proton–proton connectivities were established using a combination of COSY, DEPT, and HMQC. The individual proton–proton connectivities were recorded in a gradient-enhanced COSY experiment; the identity of the carbon directly attached to each proton is read from the HMQC map; and the ^{13}C DEPT data indicate the multiplicity of each carbon. We observed two types of connectivities: in the aromatic region one connec-

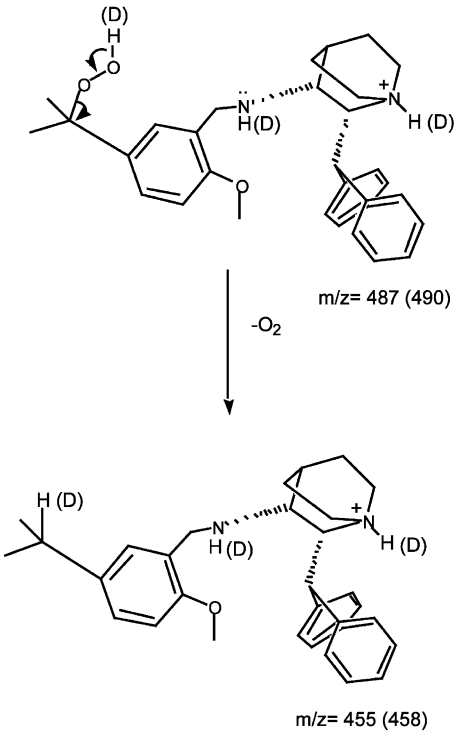

Fig. 4. Proposed initial reaction and CID fragmentation pathway mechanism for the loss of O_2 from the protonated ezlopitant impurity at m/z 487 determined from both H/D exchange patterns and MS^2 experiments. Numbers in parentheses refer to deuterated fragment ions. Initial site of protonation is based on ionization constants calculated using ZPARC pK_a program. 3-D structure of ezlopitant is obtained from ISIS data

tivity trace is seen for each of the three aromatic rings; in the aliphatic region there are two methylene pair connections (carbon 2–3 and carbon 7–8) and a string of four methine carbons which are COSY-connected, corresponding to carbons 4, 5, 6 and 17

The heteronuclear connectivities in the degradant are complete with the exception of the three-bond H–C connection to the proton of carbon 16 (in the aromatic ring; this proton linewidth is unusually broad), and between carbons 18 and 28 and the adjacent aliphatic sites (carbon 6 and 17). There is, however, a definitive three-bond heteronuclear connectivity between carbon 26 and the proton of carbon 14, which serves to position the isopropyl peroxide. Connectivities between carbon 10 and protons on carbons 13 and 5, and connectivities between carbon 17 and protons on carbons 29, 33 and 19, 23 show that the basic architecture of the molecule is unperturbed.

Comparison of ^{13}C NMR spectra of ezlopitant and its impurity (Table 4) showed three significant carbon chemical shift changes of the impurity relative to ezlopitant at positions 15, 16 and 26. The chemical shift changes at 15 and 16 are less than 5 ppm. The carbon at position 26 is a methine carbon in the parent with a chemical shift of approximately 34 ppm which becomes a quaternary carbon with a chemical shift of approximately 84 ppm (this chemical shift is consistent with

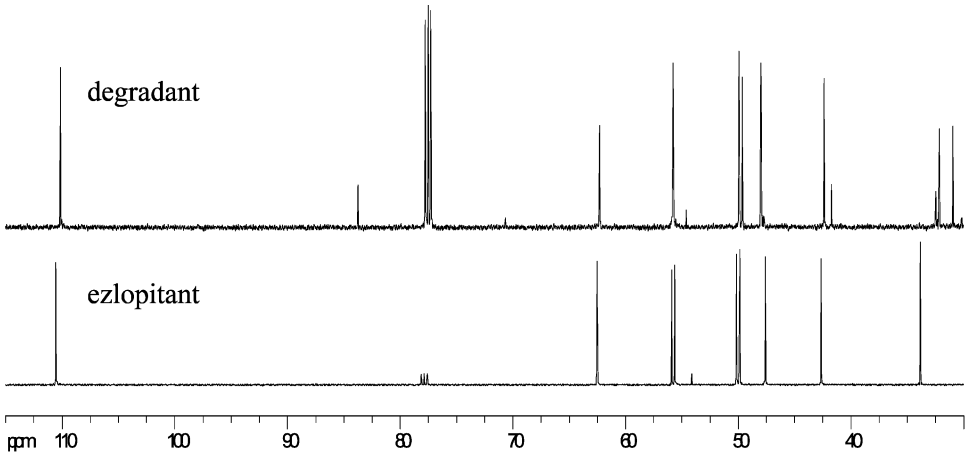

Fig. 5. Aliphatic region of the ^{13}C NMR spectra of the parent drug ezlopitant (bottom) and the major degradation product (top).

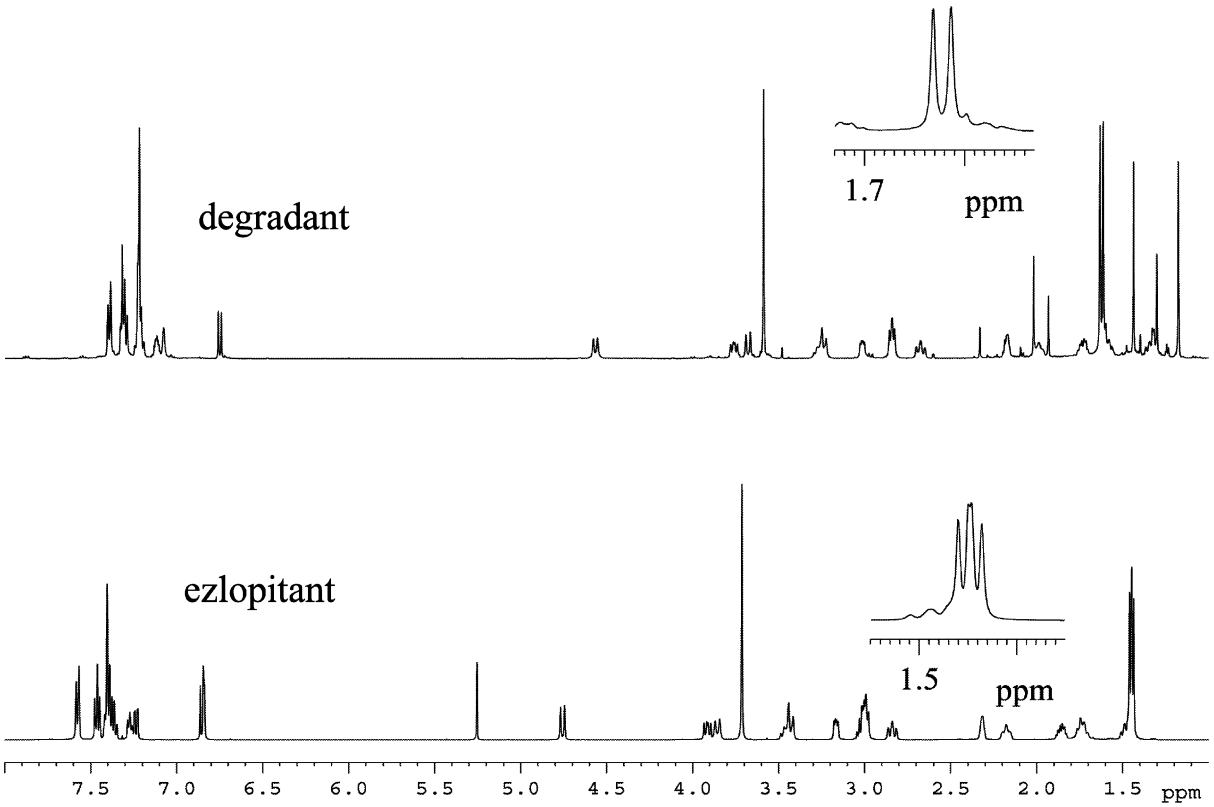

Fig. 6. ^{1}H NMR spectra of the parent drug ezlopitant (bottom) and the major degradation product (top).

peroxide addition at this site) in the impurity. The chemical shifts of methine and quaternary carbons have been reported [51,52].

Finally, the carbon spectrum of the impurity (which had been pretreated by addition of 0.1 ml D_2O, shaken (not stirred) and allowed to phase separate) was compared with the carbon spectrum of the impurity after a 50:50 mixture of H_2O/D_2O had been added. The chemical shift of a carbon which is close to an exchangeable proton is known to undergo an isotope shift [53,54] when the sample is changed from hydrogen to deuterium of approximately 0.1 ppm for two-bond connections (e.g. C–OH will have a chemical shift about 0.1 ppm upfield of C–OD). The only significant changes in carbon chemical shift occur at carbons 5 and 10, on opposite sides of the exocyclic nitrogen (the chemical shift change is approximately 0.05 ppm in each case).

Therefore, the NMR data of the impurity indicates that the only obvious change from ezlopitant is the loss of the isopropyl methine proton, change of the chemical shift of the corresponding carbon to a chemical shift consistent with peroxide addition (the carbon shifts too far downfield to be either a sulfur or nitrogen addition), with the preservation of the proton on the exocyclic nitrogen. The carbons around the ring nitrogen are essentially unperturbed compared with ezlopitant, so addition at this site is quite unlikely. The NMR data is consistent with isopropyl peroxide formation and consistent with the observation of a molecular mass of 32 amu higher than ezlopitant from mass spectral analysis.

Table 4
NMR chemical shift data of ezlopitant and its impurity

Carbon atom number	Ezlopitant		Impurity	
	^{13}C NMR δ_C (ppm)	^{1}H NMR δ_H (ppm)	^{13}C NMR δ_C (ppm)	^{1}H NMR δ_H (ppm)
2	50.15	2.99, 2.99	49.91	2.97, 2.97
3	26.22	1.87, 1.74	25.99	1.85, 1.71
4	25.52	2.31	25.35	2.29
5	55.62	3.17	55.79	3.14
6	62.5	3.92	62.29	3.88
7	42.63	3.46, 2.84	42.36	3.39, 2.80
8	20.6	2.18, 1.48	20.27	2.11, 1.45
10	47.6	3.86, 3.43	47.96	3.80, 3.35
11	128.39	N/A	127.57	N/A
12	156.12	N/A	157.19	N/A
13	110.51	6.85	110.12	6.86
14	125.7	7.23	125.69	7.44
15	140.92	N/A	136.5	N/A
16	128.39	6.84	127.57	7.19
17	49.86	4.76	49.61	4.68
18	146.35	N/A	145.95	N/A
19, 23	128.19	7.4	127.97	7.34
20, 22	128.91	7.41	128.78	7.34
21	126.4	7.27	126.3	7.23
25	55.89	3.71	55.79	3.71
26	33.82	3.02	83.73	N/A
27	25	1.44	26.76	1.74
28	143.99	N/A	143.67	N/A
29, 33	128.21	7.57	128	7.51
30, 32	129.55	7.46	129.39	7.42
31	126.96	7.36	126.82	7.32
34	24.85	1.45	26.46	1.75

N/A, not applicable, quaternary carbon.

3.3. Factors affecting the oxidative degradation of [^{14}C]-ezlopitant

Storage of solid [^{14}C]-ezlopitant under inert atmosphere (nitrogen and argon) or at lower temperatures (5 °C) does not significantly slow the degradation as shown in Table 1. The presence of the antioxidant BHT in the tetrahydrofuran, solvent used for the hydrochloride salt formation step, slows degradation by ~22% after 3 weeks (see Table 2). To avoid the solid state stability issues associated with [^{14}C]-ezlopitant, the citrate salt was prepared (citric acid, isopropanol). As shown in Table 3, the citrate salt of [^{14}C]-ezlopitant is much more resistant to oxidative degradation in the solid state than that of the hydrochloride salt.

4. Conclusions

The degradation product of ezlopitant was identified by solution phase H/D exchange and electrospray ionization tandem mass spectrometry (ESI/MS/MS) to be an isopropyl peroxide analog of ezlopitant. H/D exchange and mass spectrometry allowed easy determination of the number of replaceable hydrogens in the degradant, aided in the interpretation of the collisionally induced decomposition reactions of the protonated species and was useful to investigate the origin of fragment ions in the CID mass spectra. The structure of the degradant was further confirmed by NMR spectroscopy utilizing complete ^{1}H and ^{13}C assignments. Of all the variable studies to determine factor(s)

responsible for the formation of this degradant, only a change in the salt form from hydrochloride to citrate prevented this oxidative degradation.

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