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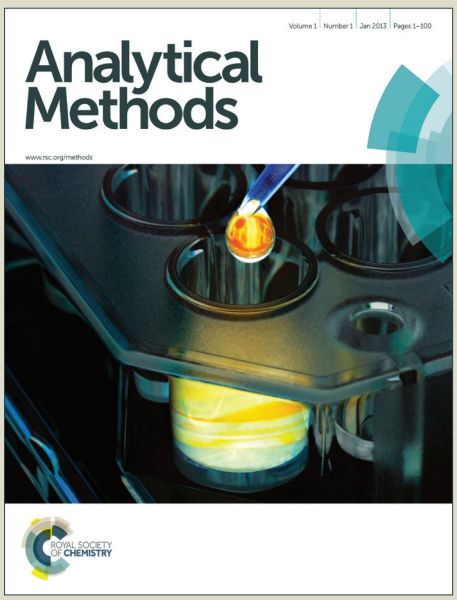

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Characterization of Degradation Products of Bambuterol Using LCMS-QTOF and NMR

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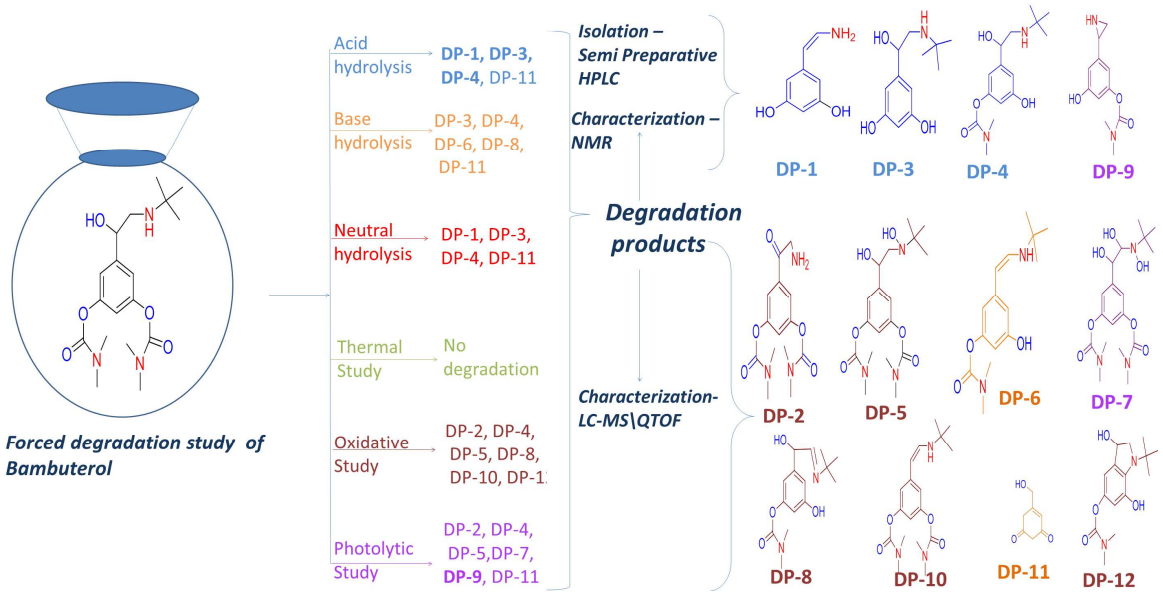
A systematic forced degradation study of bambuterol was carried out according to ICH guidelines

Twelve degradation products of bambuterol were identified and characterized. Plausible mechanisms of formation of degradation products are discussed.



Abstract:

Bambuterol was subjected to forced degradation studies as per International Conference on Harmonization (ICH) guidelines. Bambuterol was stable in thermal degradation conditions while it was found to be labile in acidic, basic, neutral, oxidative and photolytic stress conditions. In all, 12 degradation products (DP) were formed. Four degradation products were generated in both acid and neutral hydrolysis study (DP-1, DP-3, DP-4 and DP-11). Five degradation products (DP-3, DP-4, DP-6, DP-8 and DP-11) were formed in base hydrolysis study. Oxidative conditions gave six degradation products (DP-2, DP-4, DP-5, DP-7, DP-9 and DP-11). Photolytic study resulted in six degradation products (DP-2, DP-4, DP-5, DP-8, DP-10 and DP-12). Major degradation products (DP-1, DP-3, DP-4, DP-9) were isolated by semi-preparative high pressure liquid chromatography (SP-HPLC) and characterized by 1D (^{1}HNMR, ^{13}C-NMR, DEPT) and 2D-NMR studies (COSY). Characterization of the degradation products formed in extremely small quantities, were carried out using LCMS-QTOF and MS-MS fragmentation studies.

Keywords:

Bambuterol, Forced degradation studies, fragmentation pattern, characterization, HPLC-QTOF, isolation, NMR, Correlation spectra

Introduction

Stability characteristics of active pharmaceutical ingredient (API), forms the critical quality attribute of the medicinal drug. Intrinsic chemical stability of the molecule can be found by conducting forced degradation studies under a variety of conditions like pH, light, oxidation, dry heat. Forced degradation studies are particularly important for the drug which needs to be taken on a daily basis, and this study helps in identifying the ideal storage conditions and formulations for the drug.

Bambuterol hydrochloride [BH] (RS)-5-(2-tert-butylamino-1-hydroxyethyl)-m-phenylene bis (dimethyl carbamate) hydrochloride is a direct acting sympathomimetic prodrug with pre-dominantly adrenergic activity[1]. It is an ester pro-drug of beta-2 adrenergic agonist terbutaline[2]. The drug is used for prophylaxis and treatment of chronic asthma and chronic bronchitis in pediatrics.

Several analytical techniques reported for determining bambuterol[3-20]. Six impurities have been reported for bambuterol hydrochloride in British and European Pharmacopoeia[21-22]. Yet there is no systematic study on forced degradation behavior of bambuterol. So, there is a need to study the degradation behavior of the drug under different conditions according to ICH guidelines[23].

The aim of the present study was to investigate the intrinsic stability of the drug with the following objectives: 1) To conduct forced degradation study of the drug as per ICH guidelines, 2) Identification and characterization of degradation products which were formed in extremely small quantities using high resolution mass spectroscopy (HRMS), 3) To conduct liquid chromatography mass spectrometry (LCMS-MS) studies to establish fragmentation profiles of the drug and the degradation products, (4) Ascertaining the degradation pathway and mechanism of the drug, (5) Isolation of major degradation product by semi-preparative (SP) high pressure liquid chromatography (HPLC), (6) Characterization of major degradation products by ^{1}H, ^{13}C and 2D-NMR techniques.

Experimental

Materials and reagents

Bambuterol was obtained as gift sample from Sun Pharmaceuticals Advanced Research Company Limited (Baroda, India). Analytical reagent grade sodium hydroxide, conc. hydrochloric acid, glacial acetic acid and buffer salts were purchased from Merck, Mumbai, India. Hydrogen peroxide (30 %, AR grade) was obtained from S. D. Fine Chemical Limited, Mumbai, India. Highly purified water for HPLC obtained from Milli Q plus water purifying system, Millipore, Mumbai, India. Methanol and acetonitrile of HPLC grade were obtained from Fischer Scientific, Ahmedabad, India. Mobile phase was vacuum filtered through 0.22 μm poly tetrafluoroethylene (PTFE) filter membrane and degassed using sonicator to remove the dissolved gases.

Instrumentation

Radley's carousel multi reactor (Inkarp Company, Ahmedabad, India) was used for solution degradation studies (hydrolysis) and thermal studies. Thermo photo stability chamber (Thermo Lab, Thane, India) equipped with fluorescent lamps and UV lamps were used for photolytic degradation studies, in accordance with ICH guidelines[24]. A lux meter (Lutron LX101 A, Lutron Electronic Enterprise Co. Ltd, Taiwan) and a UV radiometer (UV-340, (Lutron Electronic Enterprise Co. Ltd, Taiwan) was used to measure visible illumination in visible and near UV region.

Analytical and semi-preparative HPLC experiments were performed using HPLC-PDA (Shimadzu, Kyoto, Japan) having LC-6AD pumps equipped with a SPDM20 (PDA) detector. The communication module used was Class VP software (6.14 SP1). A Phenomenex Luna C18 column (250 mm X 4.6 mm, 10 μm) and a Phenomenex semi-preparative column (250 mm X 10 mm, 10 μm) was used for analytical and semi-preparative analysis respectively.

Liquid Chromatography-Mass spectroscopy/ quadrupole time of flight (LC-MS/QTOF) studies were performed on LC hyphenated to Waters Micro TOF-Q spectrometer. This instrument is also connected to photo diode Array (PDA) detector. The outlet of PDA forms the inlet for QTOF detector through interface. A hexapole collision cell present between the two mass analyser, is used to induced fragmentation to study the structural investigations while using instrument in MS/MS mode. The LockSpray dual electrospray source enables exact mass measurement with an infused

internal lockmass from a second sprayer. The Mass Spectrometer is coupled with Waters 2795 HPLC having quaternary pumping configured for flow rates from 0.05- 5.0 mL/min. For internal calibration of the instrument, amino acid Leucine was used for both positive and negative ionization modes. The chromatograms represented in the figure were recorded using Waters-PDA /LC-QTOF instrument only.

Forced degradation studies

Forced degradation studies were carried out as per ICH prescribed conditions, i.e., hydrolysis (acid, base, and neutral), oxidation, photolysis and dry heat. Forced degradation conditions employed for the drug and the observations found during the experiment were given in the table 1. Figure 1 shows chemical structures of the drug and degradation products.

Preparation of samples for Analysis

Forced degradation samples were removed intermittently every 6 hours from the reaction mixture. 100 µL of the reaction mixture was taken and neutralized (in case of acid and base degradation studies) and made up to 1 mL. Samples from base mixture were taken for every half an hour, as the drug is more labile in basic conditions. In case of photolytic (dry) and thermal degradation studies, 1 mg of stressed solid sample was weighed and then made up to 1 mL. Final concentration used for injection was 100 µg/mL. All the solutions were filtered using 0.22 µm membrane before HPLC injections. Injection volume used is 20 µL, 100 µL, 5 µL in analytical, semi-preparative and LCMS respectively.

HPLC method

The mobile phase used for LC-MS/PDA is eluent A; methanol: acetonitrile: ammonium acetate pH (6.0) in the ratio of 5 : 5 : 90 (v: v: v) and in eluent B; methanol: acetonitrile: ammonium acetate pH (6.0) in the ratio of 20 : 40 : 20 (v: v: v). The time program for the gradient run: Time (minutes): B. Concentration (%): 0.01-1.00 : 10; 1.01-20.00 : 80; 20.01-25 : 10; 25-30 : 10.

The mobile phase condition used for semi-preparative separation is as follows: eluent A; methanol: acetonitrile: ammonium acetate pH (6.0) in the ratio of 25: 25 : 200 (v: v: v) and in eluent

B; methanol: acetonitrile: ammonium acetate pH (6.0) in the ratio of 20 : 40 : 20 (v: v: v). The time program for the gradient run: Time (minutes): B. Concentration (%): 0.01-8.00 :10, 8.01-10.00 : 20, 10.01-14.00 : 40, 14.01-16.00 : 60, 16.01-25.00 : 80, 25.01-28.00 : 60, 28.01-30.00 : 40, 30.01-32.00 : 20, 32.01-40.00 : 10.

Results and discussion

Degradation behavior of the drug

The drug showed significant degradation under acidic, basic and neutral conditions (refer figure 2) while it remained unaffected to thermal stress. In the chromatogram of base degradation studies, DP-1 is formed in large quantities. However, in MS it was found out that the single peak splitted into multiple peaks. Bambuterol being labile in base conditions, it would have formed multiple degradation products. The degradation products could have a similar molecular scaffold, which made it impossible to visualize the difference in ultra violet (UV) spectra in PDA detector. But in MS, each ion will have an unique m/z value and being polar, the molecules might have eluted together, resulting in splitting pattern. Hence, the first peak of base degradation studies was not considered. In acid, neutral and photolytic chromatogram, the number of peaks obtained in both PDA and LCMS are the same. In peroxide study, DP-7, DP-9 and DP-11 are observed only in LCMS chromatogram and not in PDA. This can be attributed to the sensitivity of the LCMS detector at lower concentrations as compared to the PDA detector.

A total of twelve degradation products (DP 1-12) were formed during the degradation studies and they were numbered as per their elution order. Base hydrolysis (0.1 N NaOH, 50 ^{o}C, 3 hours) gave five degradation products (DP-3, DP-4, DP-6, DP-8 and DP-11). Acid and neutral hydrolysis study resulted in four degradation products (DP-1, DP-3, DP-4 and DP-11). Six degradation products (DP-2, DP-4, DP-5, DP-8, DP-10 and DP-12) emanate from photolytic studies. Oxidative degradation studies (refer figure 2) resulted in six degradation products (DP-2, DP-4, DP-5, DP-7, DP-9 and DP-11). Drug was found to be stable in thermal degradation conditions.

Spectral analysis

Mass fragmentation pathway for bambuterol and its degradation products were laid out using MS-TOF and MS/MS fragmentation studies with the help of electrospray ionization (ESI) in positive mode. Fragmentation pattern for each degradation product was individually achieved by subjecting base peak to fragmentation studies. The above study for LCMS was performed in two steps: 1. To run the degradation mixture sample in LCMS and identifying the base peak for each prominent peak obtained in the chromatogram. 2. In the second run, noted m/z values was fed into the software and was subjected to fragmentation techniques.

To understand the complicated degradation pathway of the drug, it was necessary to understand its fragmentation and NMR splitting pattern. The spectral behavior of the degradation products are further deduced based on behavior of bambuterol. Supporting information like expanded spectra of bambuterol, NMR spectra of degradation products and their LCMS spectra and fragmentation pattern were schematically represented in the supplementary section.

NMR Spectra of the drug:

Spectra (^{1}H-NMR and ^{13}C-NMR) of the drug are shown in figure 3 and figure 4. The prominent signals includes (i) one singlet at δ 1.10 ppm corresponding to three methyl groups (p,q,r). (ii) Methyl groups (NMe_2CO group, (g, i)) present cis to oxygen (attached to the benzene ring) appeared upfield. Methyl groups (NMe_2CO group, (h,j)) which are cis to carbonyl oxygen appeared at downfield[25] as compared to simple aliphatic methyl group. This confirmed the fact that two methyl groups on the nitrogen were present in magnetically non-equivalent and highly electronegative environment. (iii) Methylene protons (m) appeared at 2.94 and 3.02 ppm as doublet of doublet due to the presence of nonequivalent protons at position k and n. (iv) The proton on benzylic carbon (k) appeared at 4.66 ppm as doublet of doublet instead of triplet as the neighbouring protons of the carbon (l) are diastereotopic. (v) Aromatic protons (4 & 6) appeared at 6.99 ppm as doublet due to the proton- proton coupling occurred in the meta-position (i.e. C-2) viz. the singlet splits as doublet. Proton in C-2 appeared as triplets in 6.81 ppm due to the presence of equivalent protons at positions 4 and 6. Expanded ^{1}H NMR spectra is represented in Sfigure 1.

A total of 10 different carbon signals were found in the ^{13}C NMR spectra of the drug.

Methylene carbon is differentiated from methyl and methine carbon by the use of DEPT-135 measurement. (i) Carbonyl carbons (c and d) appeared at 153.72 ppm. (ii) Aromatic carbon (1 and 3) appeared downfield at 151.33 ppm due to presence of carbamate functional group. Aromatic carbon (4 & 6) appeared at 115.99 ppm and Aromatic carbon (2) appeared at 114.34 ppm. Aromatic carbon (5) appeared at 146.07 ppm. (iii) Methyl carbon (g, h, i and j) of -CO-N-Me_2 appeared downfield at 36.09 and 36.29 ppm. (iv) Methine carbon (k) appeared at 70.35 ppm downfield as it was attached to the aromatic ring. (v) Methylene carbon (m) appeared at 49 ppm and in the downward direction in DEPT- 135 (Sfigure 2) confirming the fact that it was a CH_2 group.(vi) The tert-butyl carbon(o) appeared at 51.65 ppm downfield as this was attached to electronegative nitrogen atom. Methyl carbons (p, q and r) of tert-butyl group appeared at 27 ppm as a singlet. In COSY spectra of bambuterol, correlation occured because of spin spin coupling. There was correlation between the benzylic protons and the protons of the carbon (m). Correlation was also found between m and n but this looks very mild in the spectra as represented in figure 5. NMR shift of bambuterol and its degradation products were given in the table 4.

Mass spectra of the drug

Mass fragmentation pattern of the drug was ascertained by performing MS/ TOF studies. Fragmentation data for the drug as well as degradation products were given in the table 2. The open source software mMass was used to calculate the accurate mass for each fragment. The fragmentation value for bambuterol, its degradation products and the difference in masses (predicted and experimented value) were represented in table 3.

The MS/TOF line spectrum of the drug along with the fragmentation pathway is schematically represented in the figure 6. As depicted in the figure, the molecular ion (m/z: 368) lost the fragment $C_{15}H_{22}N_2O_4$, to form dimethyl amino methylidyne oxonium ion. Dimethyl amino methylidyne oxonium ion was a common fragment found if the skeletal structure has a carbamate[26]. The molecular ion (m/z: 368) formed the ion of m/z 312 by losing a tert-butyl radical. The ion with m/z 294, was formed from m/z 312 by losing water. Dimethyl amine is sequentially lost from the ion 294. Dimethyl amine is lost from ion of m/z 294 resulted in the formation of ion of m/z 249. The

latter ion then lost dimethyl amine to form ion of m/z 205. Ammonia lost from m/z 205 resulted in the formation of the ion of m/z 162. The smallest fragment of m/z 136 is derived from 162 by losing CO.

Characterization of degradation products

Structural elucidation of the degradation products was achieved by high resolution mass spectroscopy mass fragmentation studies. As the degradation products were either hydrolyzed or oxidative products of the drug, their structure resembled closely to the drug or to the metabolite terbutaline. Degradation products (DP-1, 3, 4 and 9) were characterized by NMR (^{1}H, ^{13}C, DEPT and COSY) studies.

DP-1 was formed in acid and neutral degradation studies (approx. 12%). This molecule was isolated from acid degradation studies and characterized by NMR. When 1000 mg of drug subjected to forced degradation studies, 70-80 mg of the DP-1 was recovered.

(1) The proton NMR spectra of DP-1 (refer Sfigure 3) reveal the following facts. In total, nine protons appeared in the ^{1}H NMR spectrum. (i) Aromatic protons (4,6) appeared at 6.67 ppm and the proton (2) appeared at 6.52 ppm. The aromatic protons of DP-1 appeared upfield as compared to the drug (refer table 4) confirming the loss of electronegative group in the molecule (-NMe_2CO). (ii) Proton (k,m) appeared as doublet at 6.12 ppm and 5.32 ppm respectively. (iii) The amine protons (n) appeared downfield at 8.6 ppm due to resonance with double bond which is in conjugation with the benzene ring. (iv)Disappearance of methyl signals in the region 1.1 - 3.00 ppm confirmed hydrolysis of carbamate (-$OCONMe_2$), resulting in the formation of resorcinol derivative.

(2) In ^{13}C spectra, totally six different carbon signals are reported. (i) Aromatic carbon (1,3) appeared at 157.94 ppm whereas aromatic carbon (2,5) appeared at 103.93 ppm and 138.94 ppm respectively. Aromatic carbon (4,6) appeared at 104.7 ppm. Aromatic carbon (2, 4, 6) appeared upfield due to the presence of electron releasing group (-OH) increasing π elctron densities in ortho and para positions, thereby inducing shielding[27] in the ring.(ii) carbon

(k,m) appeared at 110.76 ppm and 133 ppm respectively (refer Sfigure 4). The disappearance of signals in region 20- 40 ppm emphasis the loss of methyl group in the molecule. Both ^{13}C and DEPT-135 show similar spectra as there were no CH_2 in the molecule.

(3) The correlation between "m" and "k" and between "m" and "n" is clearly represented in COSY spectrum (Sfigure 5).

(4) LC- MS/TOF of DP-1 shows the base peak at m/z 152.0761. Mass fragmentation pattern of the molecule gave rise to line of m/z 136, which was obtained by losing ammonia[28]. DP-1 could rearrange to seven membered ring and loses methaniminium to form ion of m/z 125. Small molecule of m/z 107 is formed from 125, which loses water. Fragmentation pattern of DP-1 and the spectra is clearly represented in Sfigure 6.

(5) From all the above data, DP-1 structure can be deduced as 5-(2- amino vinyl) benzene -1,3-diol.

DP-2, proved to be derivative of acetophenone and was formed in both photolytic and oxidative conditions (approx. 7 %). The molecular ion 309 lost two molecules of dimethyl amine to gave an ion of m/z 221, which further lost CO and ammonia to give product ion of m/z 180. The ion of m/z 180 rearranged to a seven membered ring to form ion of m/z 124 (refer Sfigure7) [29]. The fragment ion of m/z 235 is formed from ion of m/z 309 by loss of methaniminium ion and dimethyl amine. The plausible structure of DP-2 is 5- (2-aminoacetyl)-1,3- phenylene bis (dimethyl carbamate).

DP-3 was formed in acid, base and neutral hydrolysis studies. This molecule terbutaline, is the metabolite of bambuterol formed in appreciable good amounts (approx. 7.4 %) and this was isolated and characterized. Approximately 60 mg of DP-3 was recovered from 1000 mg of forced degradation mixture.

(1) DP-3, the metabolite of bambuterol, is formed from acid, base and neutral degradation studies. Total number of protons present in DP-3 is nineteen as per $^{1}HNMR$ spectrum.

Methine proton (k) appeared at 4.76 ppm as multiplet and methylene proton (m) appeared as doublet of doublet at 2.74 ppm and 2.61 ppm (diastereotopic protons). NH proton at position (n) appeared at 1.82 ppm as singlet and the methyl protons (p, q, r) appeared at 1.36 ppm as singlet of 9 protons. Protons (4,6) present in the benzene ring appeared at 7.01 ppm and proton (2) of benzene ring appeared at 6.84 ppm. (refer Sfigure 8 and table 4). Loss of methyl signals around 3 ppm in comparison with the drug indicates that hydrolysis of carbamate group.

(2) In ^{13}C, 8 different types of carbon are found. Equivalent carbons (4, 6 and 2) appeared at 109.72 ppm and 107.93 ppm respectively (refer Sfigure9). Equivalent carbons (1, 3) appeared at 157.94 ppm and the carbon (5) appeared at 145.48 ppm. Carbon(k) appeared downfield at 70.02 ppm. Methylene carbon (m) appeared at 49.22 ppm and downwards in DEPT-135 (Sfigure10). Quartenary carbon (o) appeared at 52.94 ppm and the methyl carbons of the tert-butyl group (p,q,r) appeared at 26.75 ppm[30]. COSY spectra clearly indicated the relationship between the benzylic proton and the protons of the carbon (m) and m and amino protons as represented in Sfigure 11.

(3) Fragmentation pattern of DP-3 confirmed the structure as follows: LC-QTOF shows the m/z of its molecular ion as 226.1355. (1) The molecular ion(M+H) ion peak lost tert-butyl radical to form an ion of m/z 170, which lost water and DP-1 was yielded. DP-1 fragments in the same pattern (refer Sfigure 12). Terbutaline lost water to form the ion of m/z 208. The latter also lost a tert-butyl free radical to form DP-1 .

(4) A collection of all the above facts revealed the structure of DP-3 as 5-(2-(tert-butylamino)-1-hydroxyethyl) benzene- 1,3-diol.

DP-4 was formed in acid, base, neutral, oxidative and photolytic conditions (approx. 11 %). Approximately 100 mg of DP-4 was recovered from 1000 mg of forced degradation mixture.

(1) DP-4, monocarbamate derivative, both ^{1}H and ^{13}C spectra are found to be more similar compared to the drug. All the protons in the benzene ring are in different environment and

this is clearly given by the NMR. There are twenty five protons present in DP-4. The major difference between drug and DP-4 is that, all the protons present in the benzene rings (4,6,2) of DP-4 appeared at different values 6.6, 6.5 and 6.4 ppm as they are all chemically non-equivalent. Presence of six protons instead of 12 protons at 3.0 ppm and 2.70 ppm indicate clearly the hydrolysis of one carbamate group of the drug to gave rise to DP-4. Methylene and methine protons appeared at 4.7 and 2.8 ppm respectively(refer Sfigure13, table 4).

(2) In ^{13}C, C(1), C(d), C(3) and C(5) are found at 157.94, 153.94, 152, 145.48 ppm. Equivalent Carbon (4,6) appeared at 109 ppm and carbon (2) appeared at 107 ppm.(refer Sfigure 14 , Sfigure15 and table4). Other carbon (k,o,m,g,h,i,p,q,r) values does not show significant difference from the drug. COSY spectra clearly indicate the relationship between the k and m and also between m and n (refer **Sfigure 16**).

(3) The LCMS-QTOF value for DP-4 is 297.1595(refer Sfigure17). The chromatogram of all the degradation studies shows a common peak at retention time 5.2. Fragmentation pattern of DP-4 (refer Sfigure 17)followed three routes: Route A: The molecular ion peak (m/z 297) sequentially lost tert-butyl radical and water to yield a fragment of m/z 223. The latter further lost dimethyl amine and carbonyl and then ammonia to give product ion of m/z 136; Route B: The molecular ion(m/z 297) could lose water and tert-butyl radical to give ion of m/z 223. The ion of m/z 152 formed by loss of tert-butyl ion and follows similar pattern like 152[30]. The plausible structure of DP-4 is 3-(2-(tert-butylamino)-1-hydroxyethyl)-5-hydroxyphenyl dimethyl carbamate.

DP-5, a N-oxide of bambuterol was found to be a common degradation product of oxidative and photolytic studies. (i) The ion (m/z 384) lost a tert-butyl radical and formed m/z 328, which lost water to form a Schiff base molecule of m/z 310[31-33]. The ion of m/z 310 loses dimethyl carbonium ion and water to form ion of m/z 123(refer Sfigure 18 and Sfigure19). (ii) The ion of m/z 384 lost tert-butyl hydroxylamine and NMe_2CO to form ion of m/z 223. (iii)The ion having m/z 310 formed

fragment of m/z 223 by losing dimethyl formamide and water.(iv) DP-5 lost water and dimethyl carbonyl ion to form ion of m/z 292[34]. The plausible structure for DP-5 could be written as 5-(2-(tert-buty(hydroxyl)amino)-1-hydrosyethyl)-1,3- phenylene bis dimethyl carbamate.

DP-6 was formed in base degradation study, lost tert-butyl radical and gave a product ion of m/z 223. The latter ion lost methaniminium ion and dimethyl formamide ion to form the tropolone derivative (refer Sfigure20). The plausible structure predicted could be written as 3-(2-(tert-butylamino)vinyl)-5- hydroxyl phenyl dimethyl carbamate.

DP-7, is an unique degradation product formed only in oxidative studies. It is fragmented in two ways : (1) It lost a molecule of dimethyl amine, tert-butyl amine and carbonyl to give ion of m/z 240. The latter ion lost formadehyde and dimethyl formamide to form ion of m/z 140. (2) DP-7 lost a molecule of water to reduce to ion of m/z 382, which then lost tert-butyl amine and water to form ion of m/z 293. The latter ion further lost dimethylamine and carbonyl to form tropolone derivative of m/z 124 (refer Sfigure 21). The plausible structure for DP-7 is 5-(2-(tert-butyl(hydroxyl)amino)-1,2-dihydroxy ethyl)- 1,3- phenylene bis (dimethyl carbamate).

DP-8 was formed in photolytic and base degradation conditions. DP-8 lost N-methylene t-butylamine and dimethyl amine and carbonyl to form ion of m/z 140. The latter ion rearranged and lost water to give m/z 140, which is followed by loss of water to form ion of m/z 124. DP-8 could lose a molecule of dimethyl amine to form ion of m/z 249 and a carbonyl and tert-butyl radical to give ion of m/z 166 (refer Sfigure22 and table 4). The plausible structure is (3-(2-tert-butylimino)-1-hydroxyethyl)-5-hydroxy phenyl dimethyl carbamate.

DP-9 similar to DP-4, also has one carbamate. One of the carbamate hydrolysed and also even the tert-butyl group is not present in the molecule as compared to the structure of Bambuterol. The values were more or less similar to DP-4. All the protons of the benzene ring (4, 6, 2) gave separate signals at 6.95, 6.84 and 6.66 ppm as singlet as they were present in non equivalent environment. Protons at carbon (k) appeared at 2.6ppm and protons at carbon (m) appeared at 2.35 ppm and protons at carbon(n) appeared at 1.83 ppm[35] (refer Sfigure 23 and table 4). The significant

difference in the structure of the molecule was that it formed the aziridine ring[36].

In ^{13}C NMR, , C(1), C(d), C(3) and C(5) appeared at 157, 153, 151 and 145 ppm and Carbon (4,6,2) appeared at 116,115,114 ppm(refer Sfigure24). The methyl groups (i,h) appeared at 36ppm and k and m appeared at 26 and 22 ppm. In COSY spectra (refer Sfigure 25), the relationship between the benzylic proton and the protons of the carbon(m) and also between m and n were clearly represented.

DP-9 formed in oxidative conditions lost both dimethyl amine and CO, thus forming fragment of m/z 152 (refer Sfigure 26). The plausible structure for DP-9 is 3-(aziridin-2-yl)-5-hydroxy phenyl dimethyl carbamate.

DP-10 was formed only in photolytic degradation studies. LCMS-QTOf shows m/z to be 350. It lost tert-butyl radical to form fragment of m/z 294, which further lost ammonia to form ion of m/z 276. The ion of m/z 205 is formed from m/z 276 by loss of N,N-dimethyl formamide. The ion of m/z 205 lost acetylene to form ion of m/z 181, which then lost dimethylamine to form m/z 139 (refer Sfigure 27). The plausible structure is 5-(2-(tert-butylamino)vinyl)-1,3-phenylene bis dimethyl carbamate.

DP-11 was a degradation product found in all degradation studies except photolytic. The base peak ion lost hydroxyl radical to form ion of m/z 124 (M+1)(refer Sfigure28). The plausible structure is 5-(hydroxymethyl)cyclohex-4-ene-1,3-dione.

DP-12 was a degradation product formed only in photolytic degradation studies. It lost dimethyl amine yielding a fragment of m/z 249, which then lost a tert-butyl radical to form ion of m/z 166. The latter ion rearranged to seven membered ring to give ion of m/z 124 (refer Sfigure29). The plausible structure is 1-(tert-butyl)-3,7-dihydroxyindolin-5yl dimethyl carbamate.

Proposed degradation pathway of Bambuterol

Mechanism of the degradation pathway of bambuterol is mostly based on the hydrolysis of carbamate.(i) In basic hydrolysis, hydrolysis of the carbamate resulted in the formation of DP-4 and DP-3[37]. Dehydration reaction on DP-4 gave the product DP-6. The driving force for formation of

Schiff base enabled the formation of DP-8 from DP-4 (refer Figure 7). (ii) In acid hydrolysis study, hydrolysis of one of the carbamate resulted in the formation of DP-4. DP-4 on further hydrolysis formed DP-3 which undergoes keto-enol tautomerism to form DP-11. Both dehydration and hydrolysis leads to the formation of DP-1[38] (refer figure 8).(iii) Attack of hydroxyl free radical during oxidative studies on the carbonyl leads to hydrolysis and formed DP-4 which undergoes keto-enol tautomerism to form DP-11. The presence of free radical enabled the formation of N-oxide (DP-5). Drug undergoes dehydration and alkene is formed. The alkene on hydrolysis formed the pinacol N-oxide (DP-7). The drug on oxidation by peroxide formed DP-2, which rearranged to form the aziridine derivative DP-9[39]. (iv) Bambuterol undergoes photolytic reactions (refer figure 10) upon irradiation of UV and visible radiation in aqueous solution. The molecule can undergo dehydration reaction to form DP-10 or oxidation reaction to form DP-2. Presence of air and oxygen in photolytic conditions leads to the formation of superoxide[40], that paved the way to the formation of DP-5, which is N-oxide. Dehydration reaction resulted in the formation of DP-10. A simple hydrolysis reaction resulted in the formation of DP-4. DP-4 exhibited keto-enol tautomerism under photolytic conditions forms DP-11. DP-5 lost water formed the schiffs base DP-8, which undergo ring cyclization to form DP-12[41].

Conclusion

Twelve degradation products of bambuterol hydrochloride were formed during forced degradation studies and were duly characterized using LC-MS/MS. Degradation pathway and the mechanism of formation of degradation products were laid out. The developed analytical method proved to be convenient and effective, since it provided efficient separation of bambuterol from its degradations products. Structures of the detected degradation products were characterized based on the mass shift from the drug. The drug was found to be stable in thermal conditions. The ideal conditions for storage of the drug could be at room temperature in a cool place devoid of humid conditions.

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Figure Captions

Figure 1 : Chemical structure of the drug and its degradation products

Figure 2: Chromatogram of the drug under A) Basic hydrolysis-PDA detector B) Basic hydrolysis-QTOF-MS detector C) Neutral hydrolysis- PDA detector D) Neutral hydrolysis –QTOF-MS detector E) Acidic hydrolysis - PDA detector F) Acidic hydrolysis - QTOF-MS detector G) Oxidative study- PDA detector H) Oxidative study - QTOF-MS detector I) Photolytic Study - PDA detector J) Photolytic Study - QTOF-MS detector

Figure 3 : ^{1}HNMR spectra of Bambuterol

Figure 4: ^{13}CNMR spectra of Bambuterol

Figure 5: COSY spectra of Bambuterol

Figure 6: HPLC-QTOF Spectra and fragmentation pattern of Bambuterol

Figure 7: Schematic representation of base hydrolysis mechanism

Figure 8: Schematic representation of Acid hydrolysis mechanism

Figure 9: Schematic representation of Oxidative degradation mechanism

Figure 10: Schematic representation of Photolytic degradation mechanism

Table captions

Table 1 : Forced degradation conditions employed to study the degradation behavior of the drug Bambuterol

Table 2 : Mass data obtained from MS^n Fragmentation of drug Bambuterol and its degradation products

Table 3 : MS/TOF, MS^n data for the drug Bambuterol and its degradation products

Table 4: Comparison of proton NMR signals of Bambuterol and its degradation products

Supplementary

Sfigure1: Proton NMR expanded spectra of Bambuterol

Sfigure2: DEPT-135 spectra of Bambuterol

Sfigure3: Proton NMR spectra of DP-1

Sfigure4: ^{13}C-NMR & DEPT-135 spectra of DP-1

Sfigure5: COSY spectra of DP-1

Sfigure6: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-1

Sfigure7: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-2

Sfigure8: Proton NMR spectra of DP-3

Sfigure9: ^{13}C-NMR spectra of DP-3

Sfigure10: DEPT spectra of DP-3

Sfigure11: COSY spectra of DP-3

Sfigure12: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-3

Sfigure13: Proton NMR spectra of DP-4

Sfigure 14: ^{13}C-NMR spectra of DP-4

Sfigure15: DEPT-135 spectra of DP-4

Sfigure16: COSY spectra of DP-4

Sfigure17: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-4

Sfigure18: LCQTOF-MS/MS spectra of DP-5

Sfigure 19: Fragmentation pattern of DP-5

Sfigure20: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-6

Sfigure21: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-7

Sfigure22: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-8

Sfigure23: Proton NMR spectra of DP-9

Sfigure24: ^{13}C-NMR spectra of DP-9

Sfigure25: COSY spectra of DP-9

Sfigure26: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-9

Sfigure27: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-10

Sfigure28: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-11

Sfigure29: Fragmentation pattern and LCQTOF-MS/MS spectra of DP-12

Stress conditions	Time	Observations
Base hydrolysis (0.1N NaOH, 50^{o}C)	6 hours	Five degradation products observed (DP-3, DP-4, DP-6, DP-8, DP-11)
Neutral hydrolysis (water, 50^{o}C)	2 weeks	Four degradation products observed (DP-1, DP-3, DP-4, DP-11)
Acid hydrolysis (1NHCl, 50^{o}C)	24 hours	Four degradation products observed (DP-1, DP-3, DP-4, DP-11)
Photolysis (dry & neutral study) (Photostability chamber)	1 month	DP-3 is major degradation product. Degradation products formed in very small quantities are DP-2, DP-4, DP-5, DP-8, DP-10, DP-12.
Thermal (70^{o}C, Oven)	2 weeks	No degradation
Peroxide (3% H2O2, 60^{o}C)	2 weeks	DP-9 is major degradation product. Degradation products formed in very small quantities are DP-2, DP-4, DP-5, DP-6, DP-7, DP-11.

Table 1: Forced degradation conditions employed to study the degradation behavior of Bambuterol

Compound	Stress condition	Retention Time	molecular weight	m/z fragmentation
Drug	-		368	311, 294, 276, 249, 205, 163, 72
DP-1	Acid, neutral	3.30	152	136, 125, 107
DP-2	Photolytic, oxidative	4.57	309	235, 221, 180, 152, 124
DP-3	Acid, base , neutral	4.62	226	208, 170, 152, 125, 107
DP-4	Acid, base , neutral, photolytic, oxidative	5.23	297	279, 241, 223, 152, 136, 124, 107
DP-5	Photolytic, Oxidative	5.64	384	328, 310, 292, 223, 140, 123
DP-6	Base	6.02	279	223, 193, 124
DP-7	Oxidative	7.05	400	382, 293, 240, 180, 140, 124
DP-8	Base, Photolytic	7.72	294	249, 207, 166, 140, 124
DP-9	Oxidative	9.19	223	180, 152
DP-10	Photolytic	13.02	350	312, 294, 276, 180, 140
DP-11	Base, acid, neutral, oxidative	13.01	140	124
DP-12	Photolytic	14.39	294	249, 166, 124

Table 2 : Mass data obtained from MS^n Fragmentation of drug Bambuterol and its degradation products

Table 3 : MS/TOF, MS^n data for the drug Bambuterol and its degradation products

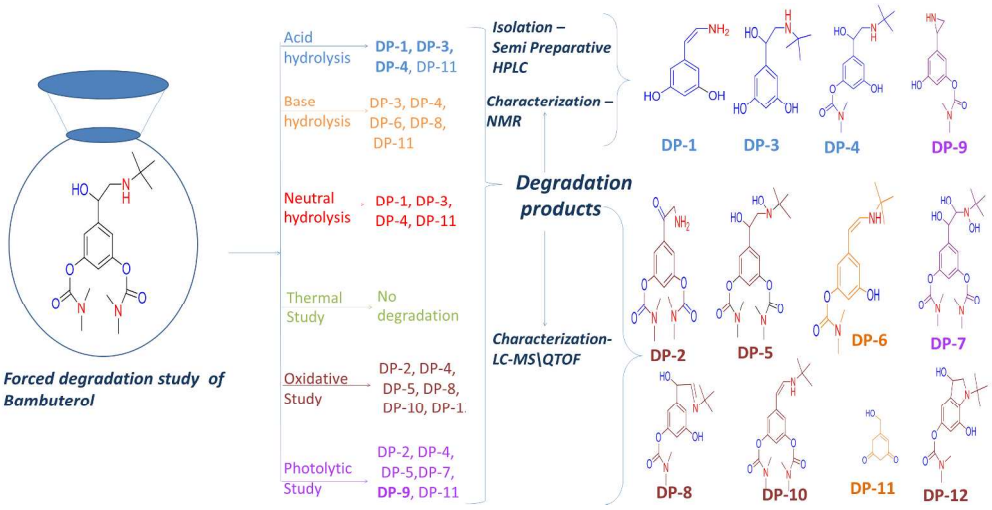
Peak	MS/TOF data	Molecular formula	Exact mass	Error in mu	Difference From previous fragment	Possible molecular formula lost
	368.2022	$C_{18}H_{30}N_3O_5^+$	368.2185	0.0163	-	-
	72.0449	C_3H_6NO+	72.0449	0.0000	296.1736	H_2, $C_{15}H_{22}N_2O_4$
	312.1455	$C_{14}H_{22}N_3O_5^+$	312.1559	0.0104	56.0626	Me_3C.
	294.1366	$C_{14}H_{20}N_3O_4^+$	294.1454	0.0088	18.0105	H_2O
Drug	249.1585	$C_{12}H_{13}N_2O_4^+$	249.0875	-0.071	45.0579	$NHMe_2$
	204.0415	$C_{10}H_7NO_4^+$	205.0375	-0.996	44.05	NMe_2
	162.1154	$C_9H_6O_3^+$	162.0317	-0.0837	43.0058	CO&NH_3
	136.9433	$C_8H_8O_2^+$	136.0524	-0.8909	26.9871	CO
	152.0738	$C_8H_{10}NO_2^+$	152.0712	-0.0026	-	
DP-1	136.9433	$C_8H_7O_2^+$	136.0524	-0.8909	17.0266	NH_3
	125.0753	$C_7H_9O_2^+$	125.0603	0.015	27.0109	CH_2=NH_2
	107.0165	$C_7H_7O^+$	107.0497	-0.0332	18.0106	H_2O
	309.1849	$C_{14}H_{19}N_3O_5^+$	309.1325	-0.0524	-	
	235.1050	$C_{11}H_9NO_5^+$	235.0481	-0.0569	74.0855	CH_2=NH_2,NMe_2
DP-2	221.0928	$C_{10}H_7NO_5^+$	221.0324	-0.0604	88.1001	$2NMe_2$
	180.9863	$C_9H_8O_4^+$	180.0423	-0.944	40.9901	CO&NH_3
	152.0808	$C_8H_8O_3^+$	152.0473	-0.0335	27.995	CO
	124.0414	$C_7H_8O_2^+$	124.0524	-0.011	27.9949	CO
	226.1355	$C_{12}H_{20}NO_3^+$	226.1443	-0.0088		
	208.1374	$C_{12}H_{18}NO_2^+$	208.1337	0.0037	18.0106	H_2O
DP-3	170.0870	$C_8H_{12}NO_3^+$	170.0817	0.0053	56.0625	Me_3C
	152.0738	$C_8H_{10}NO_2^+$	152.0712	0.0026	18.0106	H_2O
	125.0753	$C_7H_9O_2^+$	125.0603	0.015	27.0109	CH_2=NH_2
	107.0165	$C_7H_7O^+$	107.0497	0.0332	18.0106	H_2O
	297.1463	$C_{15}H_{25}N_2O_4^+$	297.1814	0.0351		
	279.1676	$C_{15}H_{23}N_2O_3^+$	279.1709	0.0033	18.0105	H_2O
	241.1110	$C_{11}H_{17}N_2O_4^+$	241.1188	-0.0078	56.0626	Me_3C.
	223.0884	$C_{11}H_{15}N_2O_3^+$	223.1083	-0.0199	18.0105	H_2O
DP-4	152.0738	$C_8H_{10}NO_2^+$	152.0712	-0.0026	71.0371	NMe_2CO
	136.9433	$C_8H_8O_2^{+2}$	136.0524	-0.8909	16.0188	NH_3
	124.0389	$C_7H_8O_2^+$	124.0524	0.0135	28.0188	CH_2=NH_2
	107.0165	$C_7H_7O^+$	107.0497	0.0332	45.0215	NH_3 & H_2O
	384.2004	$C_{18}H_{30}N_3O_6^+$	384.2135	0.0131		
	328.1540	$C_{14}H_{22}N_3O_6^+$	328.1509	-0.0031	56.0626	Me_3C.
DP-5	310.1447	$C_{14}H_{20}N_3O_5^+$	310.1403	-0.0044	18.0105	H_2O
	292.1281	$C_{15}H_{20}N_2O_4^+$	292.1423	0.0142	92.0712	NMe_2CO, H_2O
	223.1085	$C_{11}H_{13}NO_4^+$	223.0845	-0.024	161.0926	$2NMe_2CO$, OH
	140.0157	$C_7H_8O_3^{2+}$	140.0473	0.0316	170.093	$2NMe_2CO$, CH_2NH_2
	279.1604	$C_{15}H_{23}N_2O_3^+$	279.1709	0.0105		
DP-6	223.1006	$C_{11}H_{15}N_2O_3^+$	223.1083	0.0077	56.0626	Me_3C.
	193.9733	$C_{10}H_{12}NO_3^+$	194.0817	0.1084	29.0266	CH_2NH_2
	124.0429	$C_7H_8O_2^+$	124.0524	0.0095	70.0293	NMe_2CO
	400.1948	$C_{18}H_{30}N_3O_7^+$	400.2084	0.0136		
	382.1988	$C_{18}H_{28}N_3O_6^+$	382.1978	-0.001	18.0106	H_2O
	293.1329	$C_{14}H_{17}N_2O_5^+$	293.1137	-0.0192	89.0836	Me_3CNH, OH
DP-7	240.9795	$C_{11}H_{14}NO_5^+$	240.0872	0.8923	159.0901	NMe_2CO, Me_3CNH
	180.9924	$C_9H_8O_4^+$	180.0423	-0.9501	113.0714	NMe_2CO, NMe_2
	140.0148	$C_7H_8O_3^+$	140.0473	0.0325	70.0293	NMe_2CO
	124.0412	$C_7H_8O_2^+$	124.0524	0.0112	15.9944	2CO

Peak	MS/TOF data	Molecular formula	M+H/M	Error in mu	Difference	Possible molecular Formula lost
	294.1104	$C_{15}H_{22}N_2O_4^+$	294.1580	0.0476	-	-
DP-8	249.0889	$C_{13}H_{15}NO_4^+$	249.1001	0.0112	45.0579	NMe_2
	207.1134	$C_{12}H_{17}NO_2^+$	207.1259	0.0125	86.9742	NMe_2CO, OH
	166.5555	$C_8H_8NO_3^+$	166.0504	0.5051	83.0497	$Me_3C.$, CO
	140.0160	$C_7H_8O_3^+$	140.0473	0.0313	154.1112	NMe_2CO, $Me_3CN{=}CH_2$
	124.0422	$C_7H_8O_2^+$	124.0524	0.0102	15.9944	OH
	223.1012	$C_{11}H_{15}N_2O_3^+$	223.1083	0.0071	-	
DP-9	180.9935	$C_9H_{10}NO_3^+$	180.0661	0.9274	43.0422	NMe_2
	152.0738	$C_8H_{10}NO_2^+$	152.0712	0.0026	27.9949	CO
	350.1981	$C_{18}H_{28}N_3O_4^+$	350.2080	0.0099	-	
DP-10	294.1384	$C_{14}H_{20}N_3O_4^+$	294.1454	0.007	56.0626	$Me_3C.$
	276.7374	$C_{14}H_{16}N_2O_4^+$	276.1110	0.6164	18.0344	$-NH_3$
	180.9892	$C_9H_{11}NO_3^+$	181.0739	-0.0847	95.0371	C_2H_2
	140.0174	$C_7H_7O_3^+$	139.0395	0.9779	41.9984	$2NMe_2$ CO
DP-11	140.0128	$C_7H_8O_3$	140.0473	0.0345	-	
	124.0394	$C_7H_7O_2$	124.0524	0.013	15.9954	OH
	294.1104	$C_{15}H_{22}N_2O_4^{+\cdot}$	294.1580	0.0476		
DP-12	249.0889	$C_{13}H_{15}NO_4^{+\cdot}$	249.1001	0.0112	45.0579	NMe_2
	166.5555	$C_8H_8NO_3$	166.0504	-0.5051	83.0497	$Me_3C.$, CO
	124.0422	$C_7H_8O_2$	124.0524	0.0102	41.998	CO, CH_2NH_2

Table 3 : MS/TOF, MS^n data for the drug Bambuterol and its degradation products

	Drug		DP-1		DP-3		DP-4		DP-9	
Positions	1HNMR	13C	1HNMR	13C	1HNMR	13C	1HNMR	13C	1HNMR	13C
1	-	151.35	-	157.94	-	157.94	-	157.94		151.44
2	6.815(1H, t, ph)	114.34	6.524(1H,s)	103.93	6.846(1H,s,ph)	107.93	6.489(1H, s, ph)	107.93	6.667(1H,s, ph)	114.61
3	-	151.35	-	157.94	-	157.94	6.593(1H, s, ph)	152.00	6.849(1H, s, ph)	157.43
4	6.995(2H, d, ph)	115.99	6.679(1H,s)	104.72	7.012(2H,s,ph)	109.76	6.667(1H, s, ph)	109.72	6.953(1H, s, ph)	115.23
5	-	146.07	-	138.94	-	145.48	-	145.48	-	145.43
6	6.991(2H, d, ph)	115.99	6.679(1H,s)	104.72	7.012(2H,s,ph)	109.76	-	109.76	-	116.06
a	-	-	-	-	-	-	-	-	-	-
b	-	-	-	-	-	-	-	-	-	-
c	-	153.72	-	-	-	-	-	153.94	-	153.72
d	-	153.72	-	-	-	-	-	-	-	-
e	-	-	-	-	-	-	-	-	-	-
f	-	-	-	-	-	-	-	-	-	-
g	2.903(6H, s, NMe_2)	36.29	-	-	-	-	3.009(3H, s, NMe_2)	36.12	3.029(3H,s, NMe_2)	36.31
h	2.903(6H, s, NMe_2)	36.29	-	-	-	-	-	-		
i	3.028(6H, s, NMe_2)	36.29	-	-	-	-	- 2.891(3H, s, NMe_2)	36.12	2.904(3H,s, NMe_2)	36.11
j	3.028(6H, s, NMe_2)	36.29	-	-	-	-	-	-		
k	4.668(1H, dd, CH(OH))	70.35	6.127 (1H, d, CH))	110.76	4.652(1H,t,CH(OH))	70.02	4.656(1H, t,CH(OH))	69.12	2.661(1H,t)	26.65
l	-	-	-	-	-	-	-	-	-	-
m	2.63,2.78 (2H, d,CH_2)	49.43	5.327 (1H,d, CH_2)	133.00	2.879, 2.750(2H,t, CH_2)	49.22	2.659,2.637 (2H,t, CH_2)	49.13	2.359,2.190 (2H,t, CH_2)	22.50
n	1.829(1H,s,NH)	-	8.614(2H,s, NH_2)	-	1.822(1H,s,NH)	-	1.822(1H, s,NH)	-	1.833(1H,s, NH)	-
o		51.65	-	-	-	52.94	-	52.74	-	-
p	1.107(9H, s, CMe_3)	27.49	-	-	1.363(9H,s, CMe_3)	26.75	1.161(9H, s, CMe_3	26.66	-	-
q	1.107(9H, s, CMe_3)	27.49	-	-	1.363(9H,s, CMe_3)	26.75	1.161(9H, s, CMe_3)	26.66	-	-
r	1.107(9H, s, CMe_3)	27.49	-	-	1.363(9H,s, CMe_3)	26.75	1.161(9H, s, CMe_3)	26.66	-	-

Table 4: Comparison of proton NMR signals of Bambuterol and its degradation products



762x381mm (96 x 96 DPI)

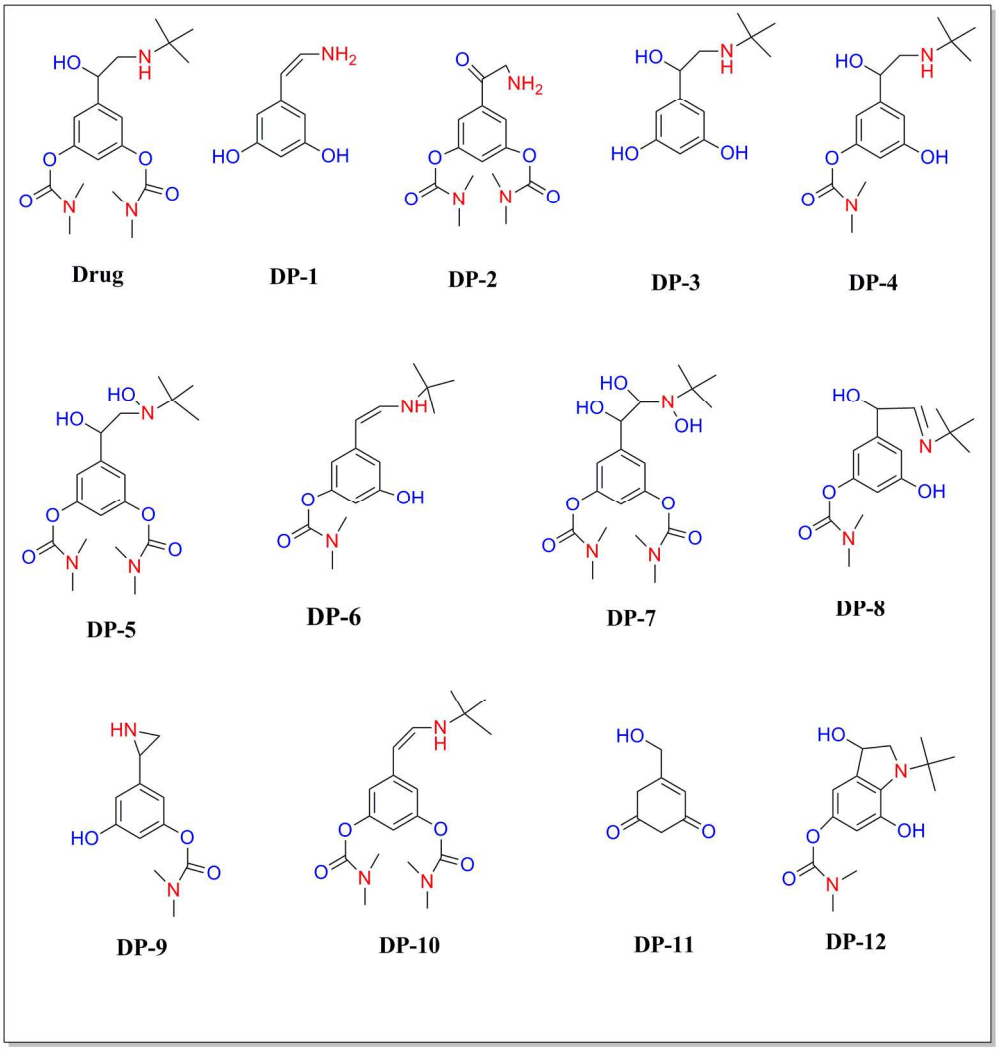


Figure 1: Chemical structure of bambuterol and its degradation products

Chemical structure of the drug and its degradation products
183x202mm (300 x 300 DPI)

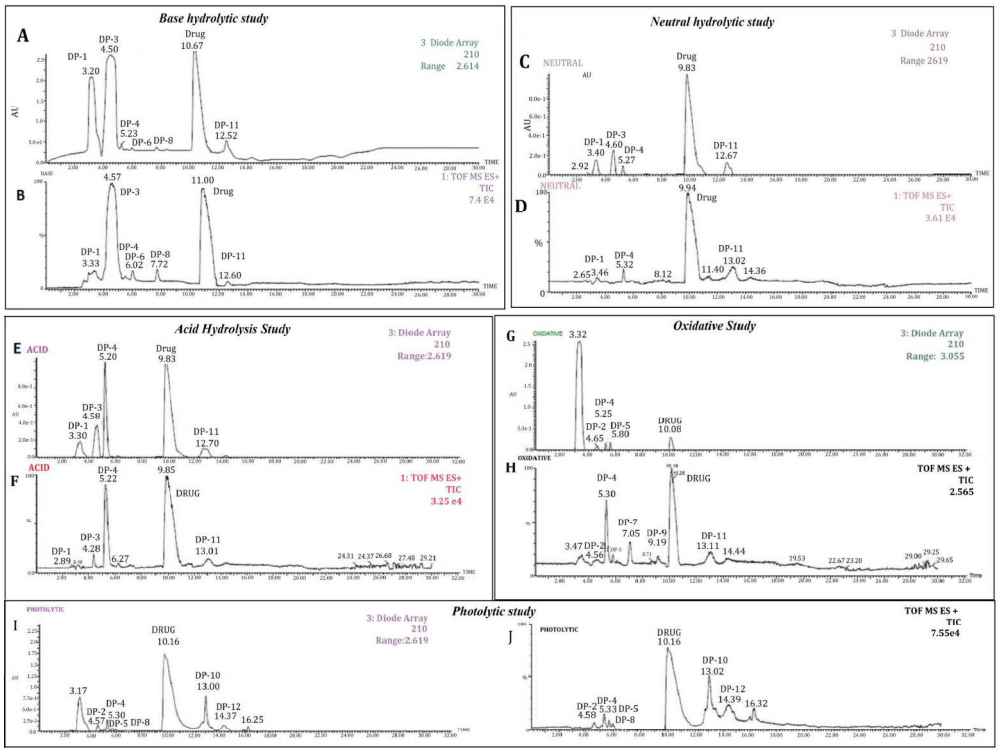


Figure 2

Chromatogram of the drug under A) Basic hydrolysis- PDA detector B) Basic hydrolysis - QTOF MS detector C) Neutral hydrolysis- PDA detector D) Neutral hydrolysis- QTOF-MS detector E) Acid hydrolysis- PDA detector F) Acid hydrolysis- QTOF-MS detector G) Oxidative study- PDA detector H) Oxidative study- QTOF-MS detector I) Photolytic study- PDA detector J) Photolytic study- QTOF-MS detector

Figure 2: Chromatogram of the drug under A) Basic hydrolysis-PDA detector B) Basic hydrolysis-QTOF-MS detector C) Neutral hydrolysis- PDA detector D) Neutral hydrolysis –QTOF-MS detector E) Acidic hydrolysis - PDA detector F) Acidic hydrolysis - QTOF-MS detector G) Oxidative study- PDA detector H) Oxidative study - QTOF-MS detector I) Photolytic Study - PDA detector J) Photolytic Study - QTOF-MS detector

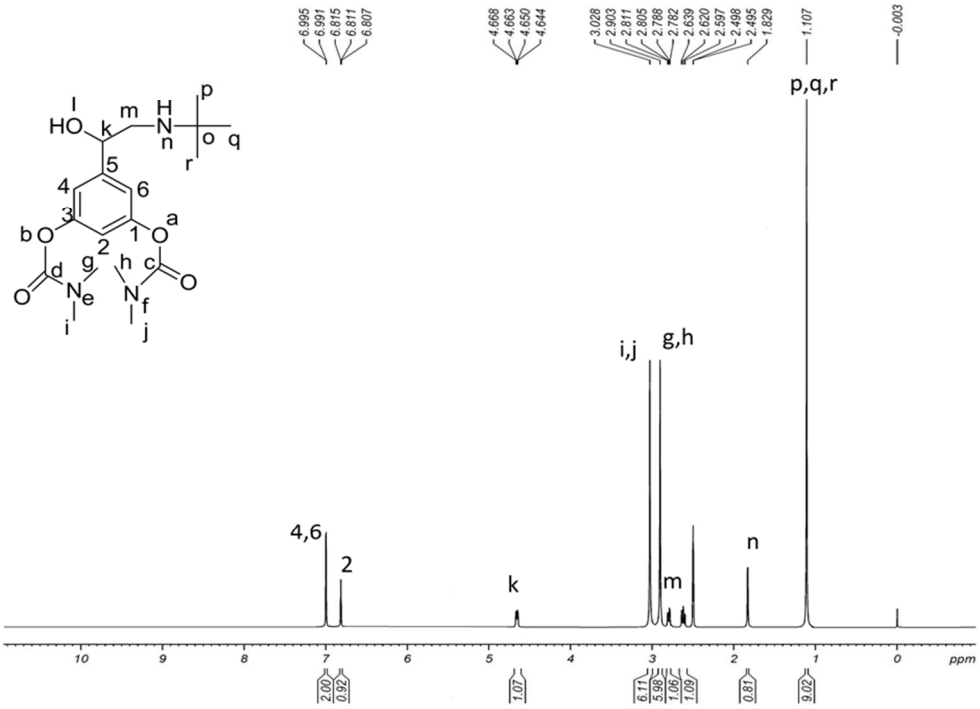


Figure 3 : 1H NMR spectra of Bambuterol

1 H NMR spectra of bambuterol
254x190mm (96 x 96 DPI)

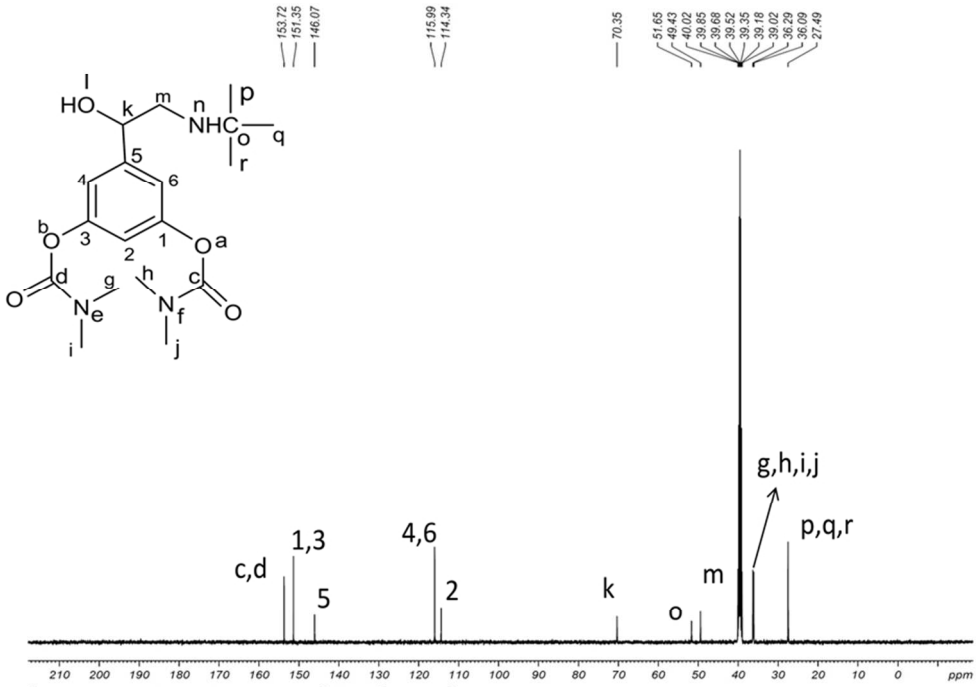
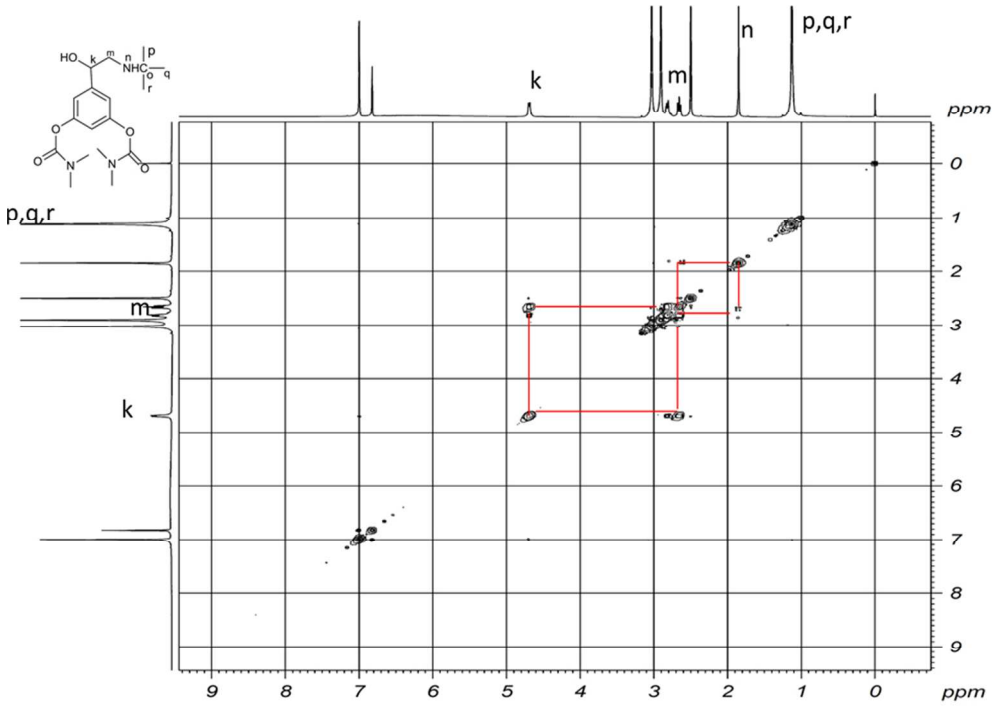


Figure 4 : 13C NMR spectra of Bambuterol

13 C NMR spectra of bambuterol
254x190mm (96 x 96 DPI)



COSY- Bambuterol

Figure 5: COSY spectra of Bambuterol

COSY Spectra of bambuterol
254x190mm (96 x 96 DPI)

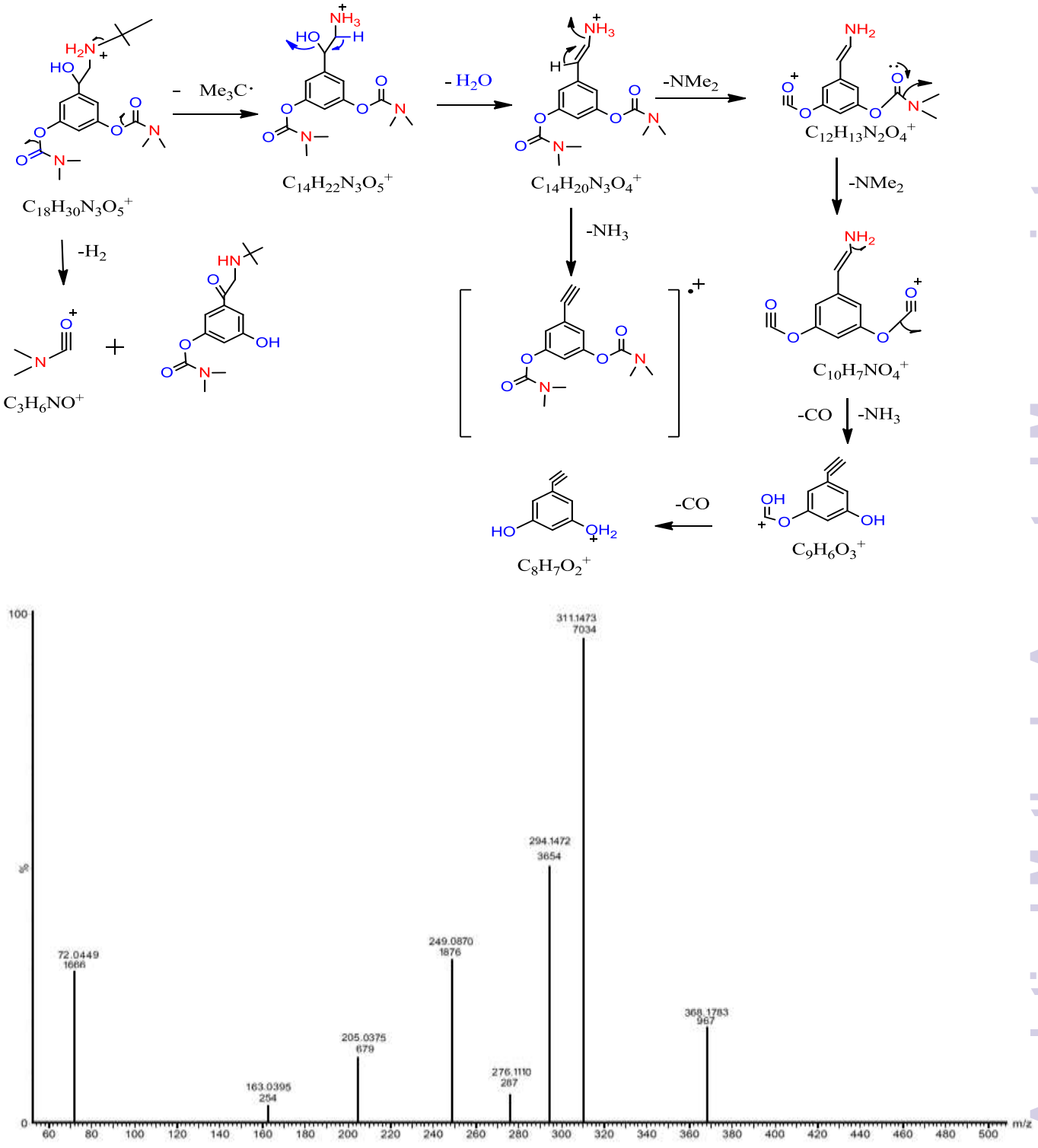
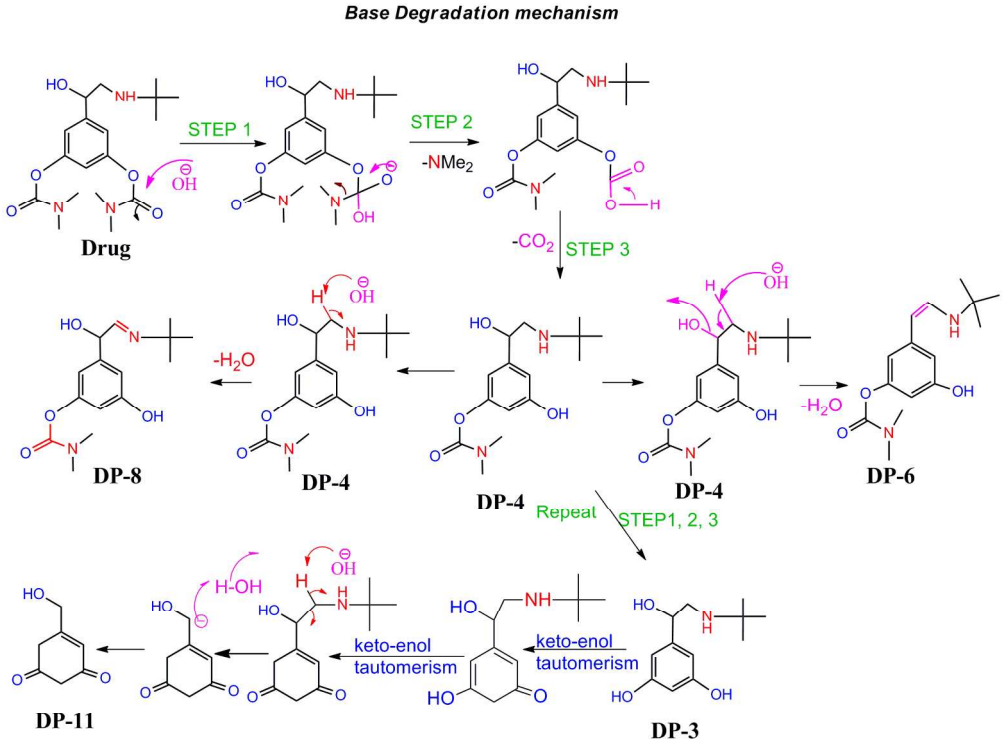
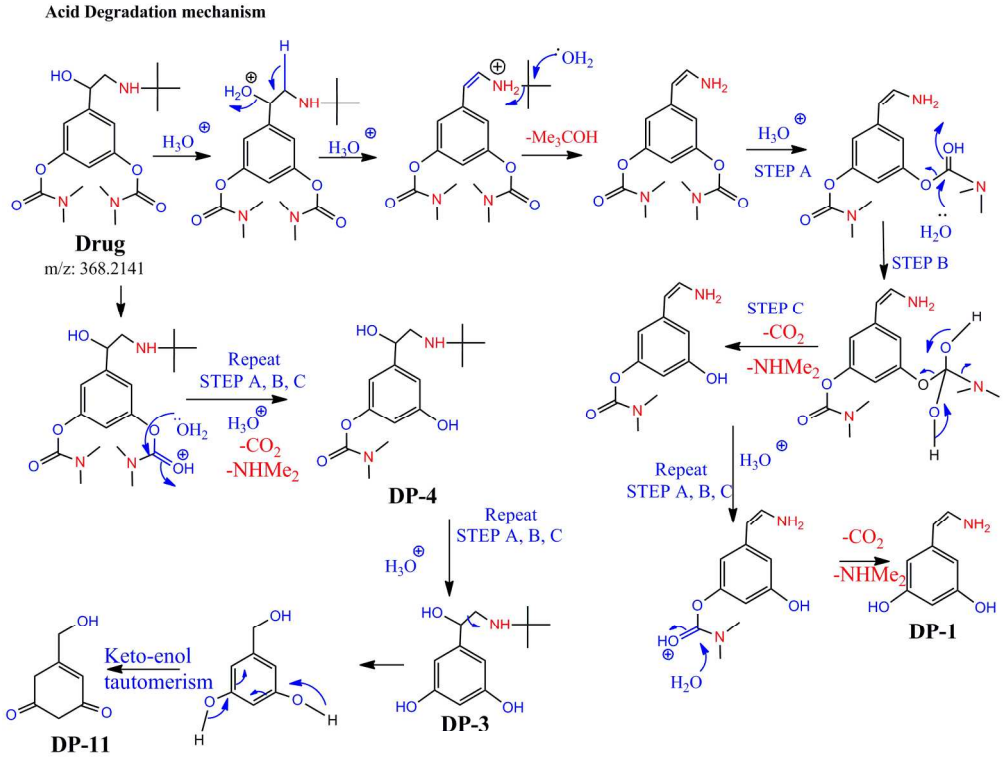


Figure 6: Schematic representation of fragmentation pattern of Bambuterol and LC-QTOF /MS /MS spectra



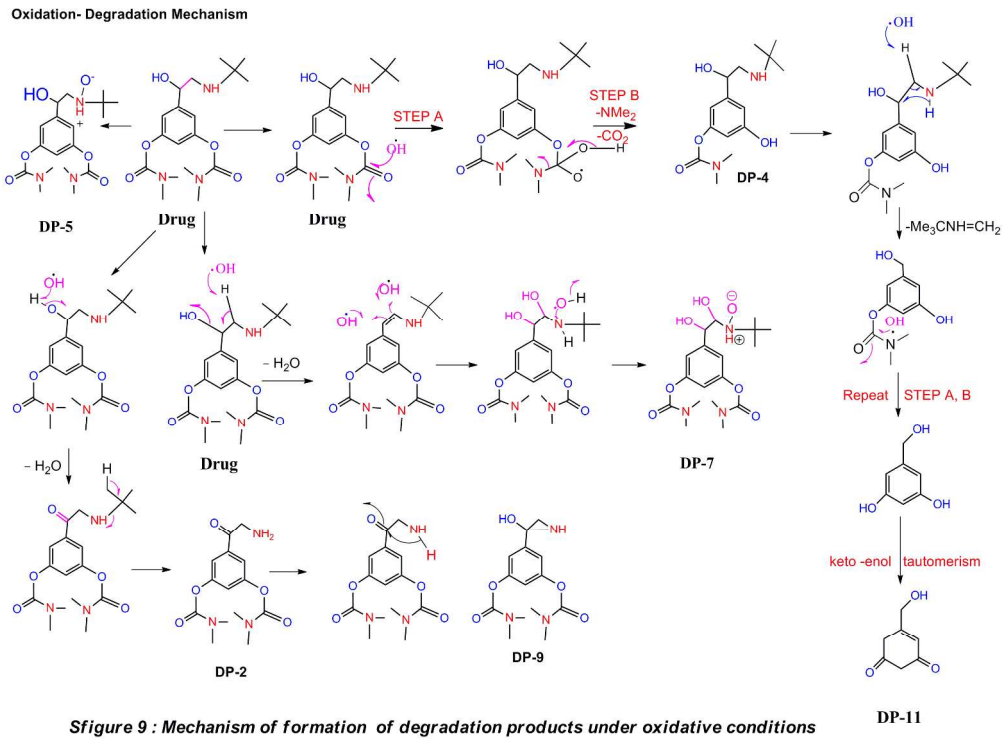
Sfigure 7 : Base degradation mechanism of bambuterol

Schematic representation of base hydrolysis mechanism
188x153mm (300 x 300 DPI)



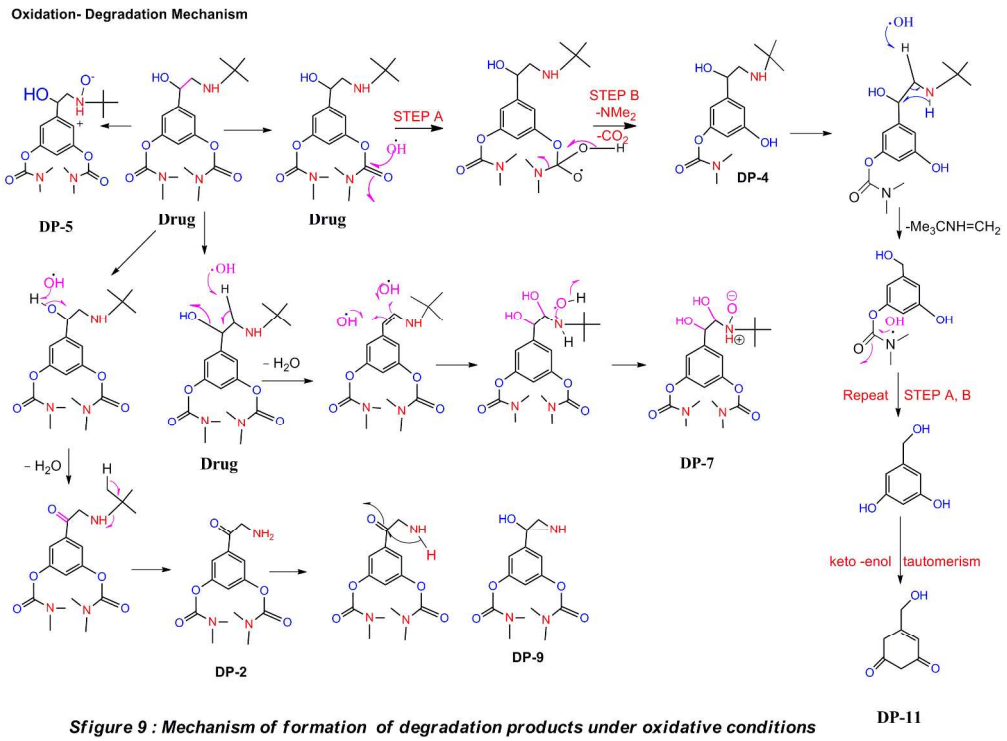
Sfigure 8 : Acid degradation mechanism of bambuterol

Schematic representation of acid hydrolysis mechanism
186x156mm (300 x 300 DPI)



Sfigure 9 : Mechanism of formation of degradation products under oxidative conditions

Schematic representation of oxidative mechanism
249x183mm (300 x 300 DPI)



Sfigure 9 : Mechanism of formation of degradation products under oxidative conditions

Schematic representation of photolytic mechanism
249x183mm (300 x 300 DPI)