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Characterization of degradation products of Ivabradine by LC-HR-MS/MS: a typical case of exhibition of different degradation behaviour in HCl and H_2SO_4 acid hydrolysis

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A validated stability-indicating HPLC method was established, and comprehensive stress testing of ivabradine, a cardiotonic drug, was carried out as per ICH guidelines. Ivabradine was subjected to acidic, basic and neutral hydrolysis, oxidation, photolysis and thermal stress conditions, and the resulting degradation products were investigated by LC-PDA and LC-HR-MS/MS. The drug was found to degrade in acid and base hydrolysis. An efficient and selective stability assay method was developed on Phenomenex Luna C18 (250 × 4.6 mm, 5.0 μm) column using ammonium formate (10 mM, pH 3.0) and acetonitrile as mobile phase at 30 °C in gradient elution mode. The flow rate was 0.7 ml/min and detection wavelength was 286 nm. A total of five degradation products (I-1 to I-5) were identified and characterized by LC-HR-MS/MS in combination with accurate mass measurements. The drug exhibited different degradation behaviour in HCl and H_2SO_4 hydrolysis conditions. It is a unique example where two of the five degradation products in HCl hydrolysis were absent in H_2SO_4 acid hydrolysis. The present study provides guidance to revise the stress test for the determination of inherent stability of drugs containing lactam moiety under hydrolytic conditions. Most probable mechanisms for the formation of degradation products have been proposed on the basis of a comparison of the fragmentation pattern of the drug and its degradation products. *In silico* toxicity revealed that the degradation products (I-2 to I-5) were found to be severe irritants in case of ocular irritancy. The analytical assay method was validated with respect to specificity, linearity, range, precision, accuracy and robustness. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: ivabradine; forced degradation; LC/ESI/MS/MS; accurate mass measurements; degradation products; *in silico* toxicity and stability assay

Introduction

Ivabradine (**IVA**), 3-[3-({[(7S)-3,4-dimethoxybicyclo[4.2.0]octa1,3,5-trien-7yl] methyl} (methyl) amino) propyl]-7,8 dimethoxy-2,3,4,5-tetrahydro-1H-3 benzazepin-2-one, is a cardiotonic agent prescribed for the symptomatic management of stable angina pectoris. It acts by reducing the rate of pacemaker activity in the sinoatrial node by selective inhibition of f-current (If) which is an important current involved in generating the early phase of spontaneous diastolic depolarisation in pacemaker cells, thereby reducing the frequency of action potential initiation and lowering heart rate.[1,2] Patients taking **IVA** medicine experience luminous phenomena, a sensation of enhanced brightness. This may occur due to blockage of Ih ion channels in the retina.[3]

Stress testing is a key aspect from view point of drug development and the regulatory approval perspective. International Conference on Harmonization (ICH) and other international agencies[4–7] issued guidelines on stability testing of drugs. These guidelines suggest that stress studies should be carried out on a drug to establish its inherent stability characteristics leading to identification of degradation products. Further, structure elucidation of unknown impurities and degradation products is also required to determine whether impurities and degradation products have toxicity. Organized research into the methods for the analysis of impurities and degradation products is of significant importance due to the stringent requirements on control of quality of drug products. Comprehensive details on the degradation behaviour of drugs could help to maintain its quality and pharmaceutical safety.[8,9]

The ICH guideline Q1A suggests the conditions to be employed to generate degradation products of the drug. However, the regulatory guidelines do not address the practical aspects related to stress testing of drugs. Singh *et al.*[10] proposed a practical scheme

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to help in the selection of the of stress condition *viz*., hydrolysis in acid, alkaline and neutral conditions, oxidation and photolysis. The stress condition used for the degradation of drugs in acid hydrolysis condition generally involves the use of hydrochloric acid (HCl) or sulphuric acid (H_2SO_4).[10] However, till date no single study has put forward the difference in the degradation behaviour of the drug in acid hydrolysis stress conditions. Degradation study of **IVA** is a typical example, where two chlorinated degradation products are absent in H_2SO_4 acid hydrolysis condition. The present studies provide guidance to revise the practical conduct of stress test for the determination of inherent stability of lactam containing drugs under hydrolytic conditions.

Over the years hyphenated techniques have been increasingly used for structural characterization of degradation products of drugs and impurities against the time consuming process involved in preparative isolation of degradation products followed by spectral characterization.[11–14]

A few analytical and bioanalytical methods have been reported in the literature for the determination of **IVA**. It includes estimation of **IVA** alone[15,16] or IVA and its metabolites[17,18] in biological matrices and in formulations.[19,20] Stability assay by HPLC[21] and HPTLC[22] is also reported. However, there is no systematic study on the degradation behaviour of **IVA** according to ICH guidelines. Hence, the purpose of the present study is to develop a stability indicating assay method for **IVA** and to characterize all the degradation products formed using LC/ESI/MS/MS and accurate mass measurements. In addition, useful information is provided for revising the practical conduct of stress studies under hydrolytic conditions for lactam containing drugs. *In silico* toxicity of all the proposed degradation products (DPs) was predicted using TOPKAT (toxicity prediction by computer-assisted technology) software. Some of the DPs may possess serious toxic effects which create an additional safety concern.

Experimental

Chemicals and reagents

Pure Ivabradine hydrochloride was purchased from Sigma Aldrich, India. HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck, India. HPLC grade water was prepared by filtrating through a Millipore Milli-Q-plus system (Millipore, Milford, MA, USA). Ammonium acetate and ammonium formate of HPLC grade were purchased from Finar Chemicals Pvt. Ltd. (Ahmedabad, India). All analytical grade reagents: formic acid, sodium hydroxide, hydrochloric acid, sulphuric acid and 30% hydrogen peroxide were purchased from Merck (Mumbai, India).

Instrumentation

The HPLC analysis was performed on Water's separation module e2695 series consisting of quaternary pump plus auto sampler and a Photo Diode Array (PDA) detector. The output signal was monitored and processed using Empower software. All pH measurements were done on a pH-meter, pH tutor (Eutech Instruments), and weighing was carried out on a Sartorius balance (CPA225D, Germany).

For LC-MS analysis, an Agilent 1290 series LC instrument (Agilent Technologies, USA) coupled to a quadrupole time of flight mass spectrometer (Q-TOF LC/MS) 6540 series, Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source was used. The data acquisition was under the control of Mass Hunter workstation software. The typical operating source conditions for MS scan of **IVA** in positive ESI mode were optimized as follows: the fragmentor voltage was set at 144 V, the capillary at 3500 V and the skimmer at 65 V, and nitrogen was used as the drying (325 °C, 10 l/min) and nebulizing (40 psi) gas. For collision-induced dissociation (CID) experiments, keeping MS1 static, the precursor ion of interest was selected using the quadrupole analyser, and the product ions were analysed using a TOF analyser. Ultra high pure nitrogen gas was used as collision gas. All the spectra were recorded under identical experimental conditions and were an average of 20–25 scans.

Photolytic studies were carried out in a photostability chamber (Osworld OPSH-G-16-GMP series, Osworld Scientific Equipments Pvt. Ltd. India) set at 40 ± 5 °C/75% ±3% RH (relative humidity) and equipped with an illumination bank on inside top, consisting of a combination of two black light UV lamps and four white fluorescent lamps in accordance with option two of the ICH guideline Q1B. Assessment of *in silico* toxicity was carried out by using TOPKAT (Discovery Studio 2.5, Accelrys, Inc., San Diego, CA, USA) software.

Forced degradation studies

Stress studies were carried out on the bulk drug under ICH recommended conditions of hydrolysis, photolysis, oxidation and dry heat. For all the stress studies, the concentration of **IVA** was kept 1.0 mg/ml. Acidic, basic and neutral hydrolytic degradations were carried out by refluxing the drug in 1 N HCl and 1 N H_2SO_4, 3 N NaOH and water at 80 °C, respectively. For oxidative degradation, the drug was subjected to 30% H_2O_2 at room temperature for 7 days. Photo degradation study was done by exposing the drug in solution (0.1 N HCl, 0.1 N NaOH and water) as well as in solid form to 1.2×10^6 lux h of fluorescent light and 200 W h m^{-2} UV light in a photostability chamber. For thermal stress study, the drug was kept in an oven at 70 °C for 7 days. The optimized stress conditions of **IVA** are given Table 1.

Sample preparation

All the stressed samples (hydrolytic, oxidative, thermal and photolytic stress) were neutralized and diluted with mobile phase and filtered through 0.22-μm membrane filter before LC and LC/MS analysis.

Chromatographic conditions

The chromatographic conditions were optimized using Phenomenex C18 (250 × 4.6 mm, 5.0 μm) column with a mobile phase composed of ammonium formate buffer pH 3 (Solvent A) and acetonitrile (Solvent B) in gradient mode. The gradient programme was set as follows: (T_{min}/% proportion of solvent B): $_{0–2}$/20, $_{2–7}$/33, $_{7–13}$/33, $_{13–15}$/70, $_{15–18}$/20 and $_{18–21}$/20. The injection volume and detection wavelength were 10 μl and 286 nm, respectively. Column temperature was kept 30 °C. For LC/MS analysis, conditions such as nebulizing gas flow, capillary voltage, drying gas temperature, skimmer voltage, drying gas flow and spray voltage were optimized to get maximum ionization of **IVA** and all the DPs.

Method validation

The method was validated as per ICH guideline Q2B.[23]The specificity of the method was established by determining peak purity for IVA in a mixture of stressed samples using LC-PDA and LC-MS. To establish linearity, solution containing 1 mg/ml of IVA was diluted to six different concentrations within the range of 5–200 μg/ml. All the samples were analysed in triplicates. The accuracy was

Table 1. Stress degradation conditions for Ivabradine

Stress conditions	Concentration of stressor	Exposed conditions	Duration	% degradation
Hydrolysis				
Acid	1 N HCl	80 °C	1 h	56.01
	1 N H_2SO_4	80 °C	1 h	12.54
Base	3 N NaOH:ACN (60:40 % *v/v*)	80 °C	48 h	4
Neutral	H_2O	80 °C	48 h	0
Oxidation	30% H_2O_2	RT	7 days	0
Photolysis	1.2×10^{-6} lux h (fluorescent) 200 W h/m^2 (UV)			
Solid		40 °C,	2 days	0
Neutral	H_2O	75%RH		0
Acid	0.1 N HCl			2.60
Base	0.1 N NaOH			1.06
Thermal				
Solid		100 °C	7 days	0

determined at three different concentrations (10, 50 and 100 μg/ml) in triplicate analysis, and the recoveries of the added drug were obtained from the difference between peak areas of fortified and unfortified degraded samples. The intra- and inter-day precisions were determined at three different concentrations 10, 50 and 100 μg/ml, on the same day ($n = 3$) and consecutive days ($n = 3$). Robustness of proposed method was determined by purposely changing the flow rate (0.6–0.8 ml/min), column temperature (30 ± 5 °C) and pH of mobile phase (3.0 ± 0.2) at three different concentrations (10, 50 and 100 μg/ml). Each sample was injected in triplicate ($n = 3$), and peak areas obtained were used to calculate means and % RSD values.

Results and discussion

Degradation behaviour of the drug

The degradation behaviour of **IVA** was studied using HPLC and LC-MS under various forced degradation conditions. Sufficient degradation was observed in acid and base hydrolytic conditions whereas it was stable under oxidative, neutral hydrolysis and photolytic conditions. A total of five degradation products were identified and characterized by using LC-ESI-QTOF-MS/MS experiments and accurate mass measurements. Based on the structure of the drug and the *m/z* values of their $[M + H]^+$ ions of the DPs, most plausible structures have been proposed for all the DPs. The proposed structures and their elemental compositions are given in Scheme 1 and Table 2, respectively. The overlay of HPLC chromatograms of all stress degradation samples of **IVA** is given in Fig. 1.

Hydrolysis

A total of five DPs (**I-1** to **I-5**) were formed in acid hydrolysis at 80 °C for 1 h when conducted with HCl, whereas in the presence of H_2SO_4 three degradation products (**I-1**, **I-3** and **I-4**) were formed (Fig. 2). Only one DP (**I-1**) was detected in base hydrolysis (Fig. 1B). **IVA** was found to be stable in neutral hydrolytic conditions (Fig. 1C).

Oxidative

The drug was found to be stable in oxidative condition after subjecting to 30% H_2O_2 at room temperature for 7 days (Fig. 1D).

Photolytic degradation

On exposing the solid drug sample and neutral drug solution at 1.2 million lux hours and 200 W h/m^2 for two days, no DPs were formed (Fig. 1E and 1F). Drug solution in acidic (Fig. 1G) and basic (Fig. 1H) solution showed formation of one DP **I-1**.

Thermal degradation

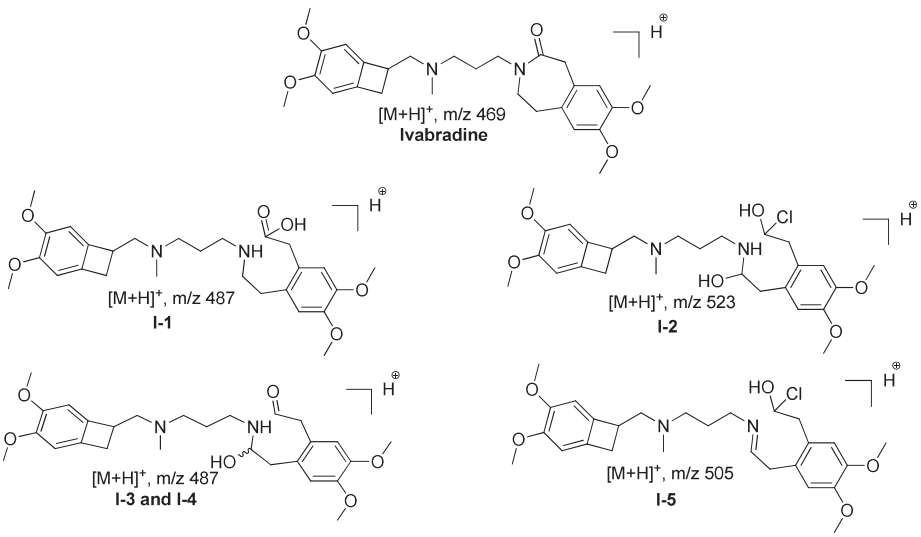
The drug was found to be stable in thermal conditions after subjecting the drug at 100 °C in oven for 7 days (Fig. 1I).

LC/ESI/MS/MS study of IVA and its degradation products

The developed HPLC method was successfully transferred to LC-MS for identification of degradation products.

MS/MS of IVA

The MS/MS spectrum of protonated **IVA** (3-[3-({[(7S)-3,4-dimethoxybicyclo [4.2.0]octa1,3,5-trien-7yl] methyl} (methyl) amino) propyl]-7,8 dimethoxy-



Scheme 1. Proposed structures of protonated degradation products of Ivabradine.

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Table 2. High resolution mass spectrometry (HRMS) data of Ivabradine and degradation products along with their Elemental composition and major fragments

	Retention time (min)	Molecular formula	Observed *m/z*	Calculated *m/z*	Error
IVA	12.19	$C_{27}H_{37}N_2O_5^+$	469.2693	469.2697	0.85
		$C_{15}H_{20}NO_3$+	262.1436	262.1438	0.76
		$C_{12}H_{16}NO_2$+	206.1168	206.1176	3.88
		$C_{11}H_{13}O_2^+$	177.0902	177.0910	4.52
		$C_9H_{11}O_2$+	151.0757	151.0759	1.32
		$C_{10}H_{10}O^{+\cdot}$	146.0724	146.0726	1.37
		$C_8H_9O^+$	121.0642	121.0648	4.96
		$C_{10}H_{12}NO^+$	162.0911	162.0919	4.87
		$C_{10}H_{12}N^+$	146.0964	146.0969	3.42
		$C_6H_{15}N_2O$+	131.1182	131.1184	1.53
		$C_8H_9O^+$	121.0642	121.0648	4.96
		C_8H_7+	103.0554	103.0547	−6.79
		$C_7H_7^+$	91.0535	91.0542	7.69
		$C_6H_5^+$	77.0394	77.03912	−3.63
		$C_3H_8N^+$	58.0658	58.0657	−1.72
I-1	5.88	$C_{27}H_{38}N_2O_6$+	487.2800	487.2803	0.62
		$C_{27}H_{37}N_2O_5^+$	469.2658	469.2697	8.31
		$C_{15}H_{22}NO_4^+$	280.1533	280.1543	3.57
		$C_{15}H_{22}NO_2$+	248.1632	248.1645	5.24
		$C_{13}H_{20}NO_2$+	222.1479	222.1489	4.50
		$C_{10}H_{10}O^+$	146.0731	146.0726	−3.42
I-2	8.59	$C_{27}H_{40}ClN_2O_6^+$	523.2561	523.2575	2.68
		$C_{27}H_{38}ClN_2O_5^+$	505.2439	505.2469	5.94
		$C_{27}H_{38}N_2O_6$+	487.2749	487.2803	11.08
		$C_{15}H_{22}NO_4^+$	280.1536	280.1543	2.50
		$C_{15}H_{20}NO_3$+	262.1430	262.1438	3.05
		$C_{13}H_{20}NO_2$+	222.1487	222.1489	0.90
I-3	10.77	$C_{27}H_{39}N_2O_6$+	487.2787	487.2803	3.28
		$C_{15}H_{22}NO_4^+$	280.1540	280.1543	1.07
		$C_{15}H_{20}NO_3$+	262.1430	262.1438	3.05
		$C_{12}H_{16}NO_2$+	206.1169	206.1176	3.40
		$C_{11}H_{13}O_2^+$	177.0914	177.0910	−2.26
I-4	11.76	$C_{27}H_{39}N_2O_6$+	487.2783	487.2808	5.13
I-5	14.39	$C_{27}H_{38}N_2ClO_5$+	505.2457	505.2464	1.39

2,3,4,5-tetrahydro-1H-3 benzazepin-2-one) ($[M+H]^+$, *m/z* 469; retention time (Rt) = 12.19) display abundant product ions at *m/z* 262 (loss of $C_{12}H_{17}NO_2$), *m/z* 177 (loss of $C_{16}H_{24}N_2O_3$) and low abundance ions at *m/z* 305 (loss of $C_{10}H_{12}O_2$), *m/z* 206 (loss of $C_{15}H_{21}NO_3$), *m/z* 162 (protonated 4,5-dihydro-benzoazepin-2-one), *m/z* 151 (loss of C_2H_2 from *m/z* 177), *m/z* 146 (loss of 2(HCHO) from *m/z* 206), *m/z* 121 (loss of HCHO from *m/z* 151), *m/z* 103 (protonated cyclobutabenzene), *m/z* 91 ($C_7H_7^+$) and *m/z* 77 ($C_6H_5^+$) (Fig. 3(a), Scheme 2). It can be noted that the fragment ions at *m/z* 305, 162 and 262 are characteristic of the benzazepin-2-one skeleton in **IVA** while the fragment ions at *m/z* 206, 146 and 177 are the diagnostic ions of 4, 5-dimethoxy-1-methylcyclobutabenzene skeleton in **IVA**. The elemental compositions of all these ions have been confirmed by accurate mass measurements (Table 2).

MS/MS of degradation products

On line LC/ESI/MS/MS experiments were performed to characterize all the DPs ((**I-1** to **I-5**) formed under stress conditions. Most

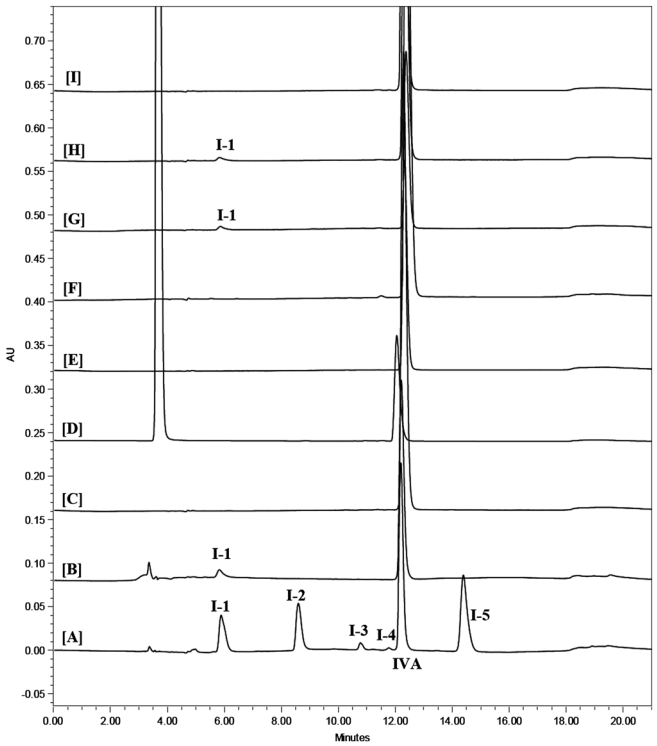


Figure 1. Chromatograms showing the separation of degradation products in optimized conditions of stress [A] Acid degradation, [B] Base degradation, [C] Neutral degradation, [D] Oxidative degradation, [E] Photo degradation of Solid sample, [F] Photo degradation of solution, [G] Photo degradation sample in 0.1 N HCl, [H] Photo degradation sample in 0.1 N NaOH and [I] Thermal degradation.

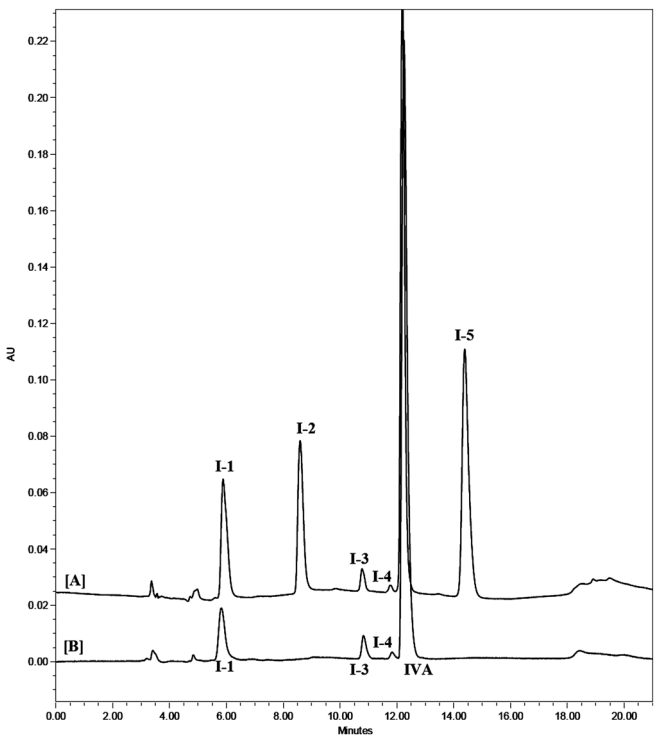


Figure 2. Chromatograms of acid hydrolytic degradations [A] HCl hydrolysis (1 N HCl reflux for 1Hr) [B] H_2SO_4 hydrolysis (1 N H_2SO_4 reflux for 1 h).

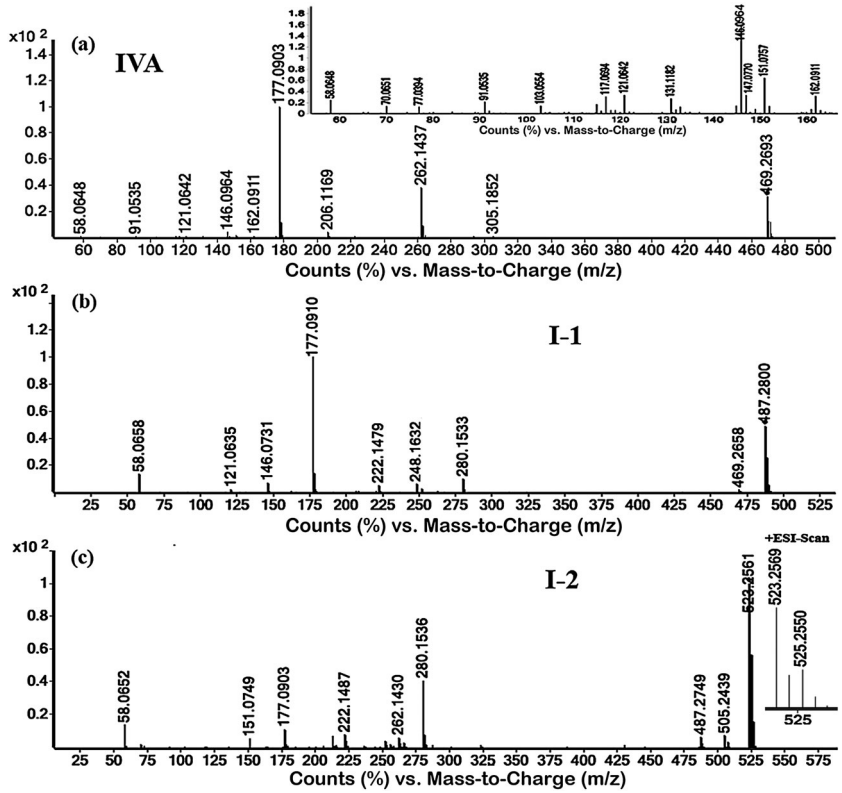
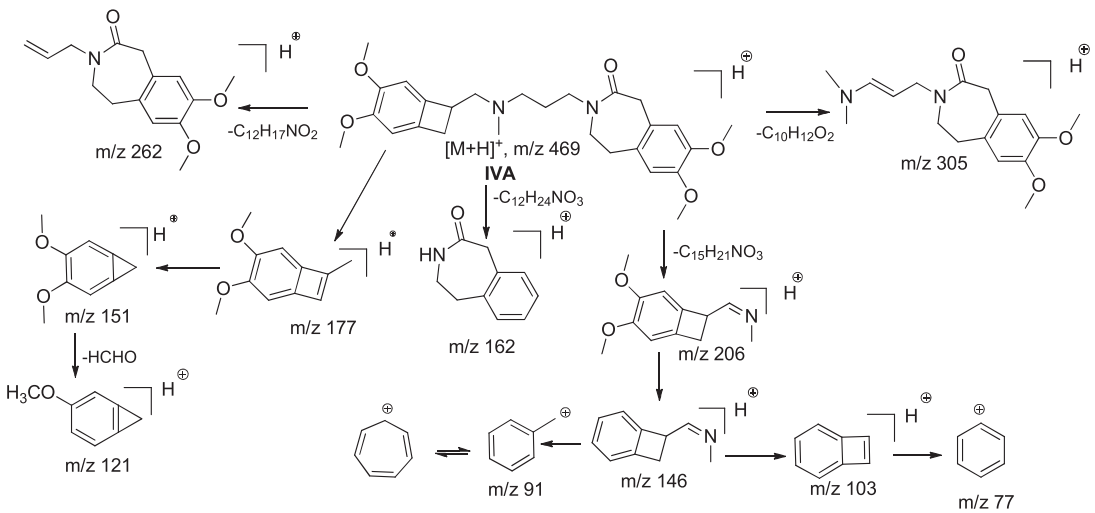


Figure 3. ESI/MS/MS spectrum of (a) **IVA** (*m/z* 469) at 20 eV, (b) **I-1** (*m/z* 487) at 20 eV and (c) **I-2** *m/z* 523 at 20 eV.



Scheme 2. Proposed fragmentation pathway of protonated Ivabradine.

plausible structures have been proposed for all the DPs of **IVA** based on the *m/z* values of their $[M+H]^+$ ions and the MS/MS data in combination with elemental compositions derived from accurate mass measurements as discussed below.

The ESI/MS/MS spectrum of $[M+H]^+$ ion of **I-1** at *m/z* 487.2800 with an elemental composition of $C_{27}H_{38}N_2O_6$ eluted at Rt = 5.88 min.. The increase of 18 Da in molecular weight as compared to that of the drug can be attributed to the addition of a water molecule to the drug. To elucidate the structure of **I-1**, the MS/MS spectrum of protonated **I-1** was examined. The formation of the ion at *m/z* 280 and the absence of *m/z* 262 clearly indicates the hydrolysis of ε-lactam ring of **IVA**. This is similar to hydrolysis of ε-caprolactum.

In comparison with the LC/MS/MS spectrum of protonated **IVA**, it can be noted that the peak at *m/z* 262 is shifted to m/z 280 in the LC/MS/MS spectrum of protonated **I-1** due to the conversion of carbonyl group to –COOH. Formation of the characteristic fragment ions at *m/z* 469 (loss of H_2O from *m/z* 487 to form protonated **IVA**, *m/z* 280 (loss of $C_{12}H_{17}NO_2$ from *m/z* 487) and *m/z* 248 (loss of $C_{12}H_{17}NO_4$ from *m/z* 487) are explained in Scheme 3a. All these data are highly compatible with the proposed structure, 2-(2-(2-(3-(((4, 5-dimethoxy-1, 2-dihydro cyclobutabenzen-1-yl) methyl) (methyl) amino) propyl amino) ethyl)-4, 5-dimethoxyphenyl) acetic acid for **I-1** (Scheme 1). The elemental compositions of product ions have been confirmed by accurate mass measurements (Table 2). A

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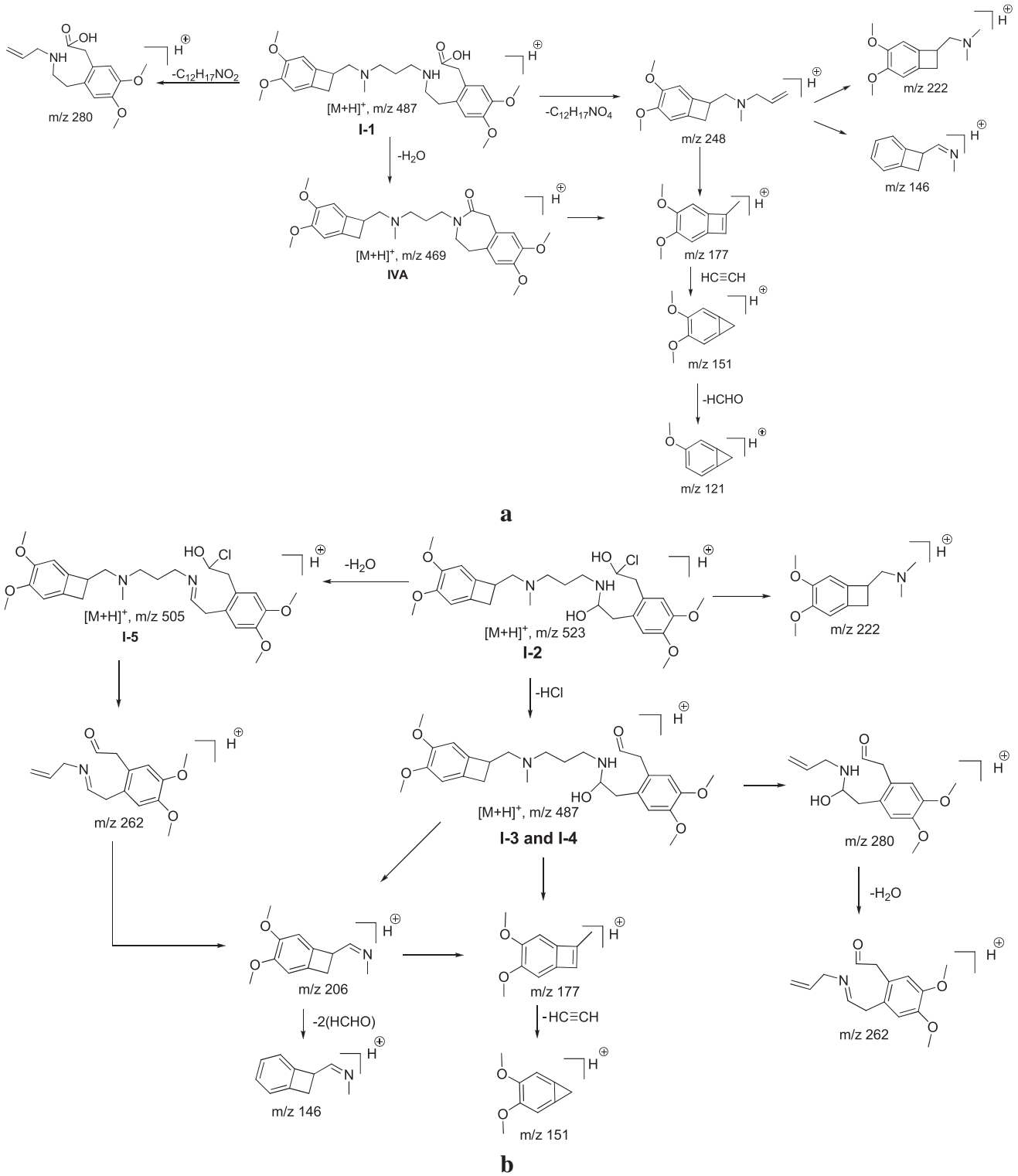
plausible mechanism of formation of **I-1** from **IVA** in acid hydrolysis (HCl and H_2SO_4) may involve protonation of nitrogen followed by an opening of the ε-lactam ring of **IVA** and further addition of –OH to the carbonyl group. Whereas, base hydrolysis may involve nucleophilic attack by –OH on the ε-lactam carbonyl group followed by formation of the tetrahedral intermediate and cleavage of the C–N bond[24] (Scheme 4).

The degradation product **I-2** at *m/z* 523.3561 ($[M+H]^+$; $C_{27}H_{40}ClN_2O_6$) was eluted at Rt = 8.59 min. It is formed only in HCl hydrolysis of **IVA** by addition of Cl + H_2O. The intensities of $[M+H]^+$ ion

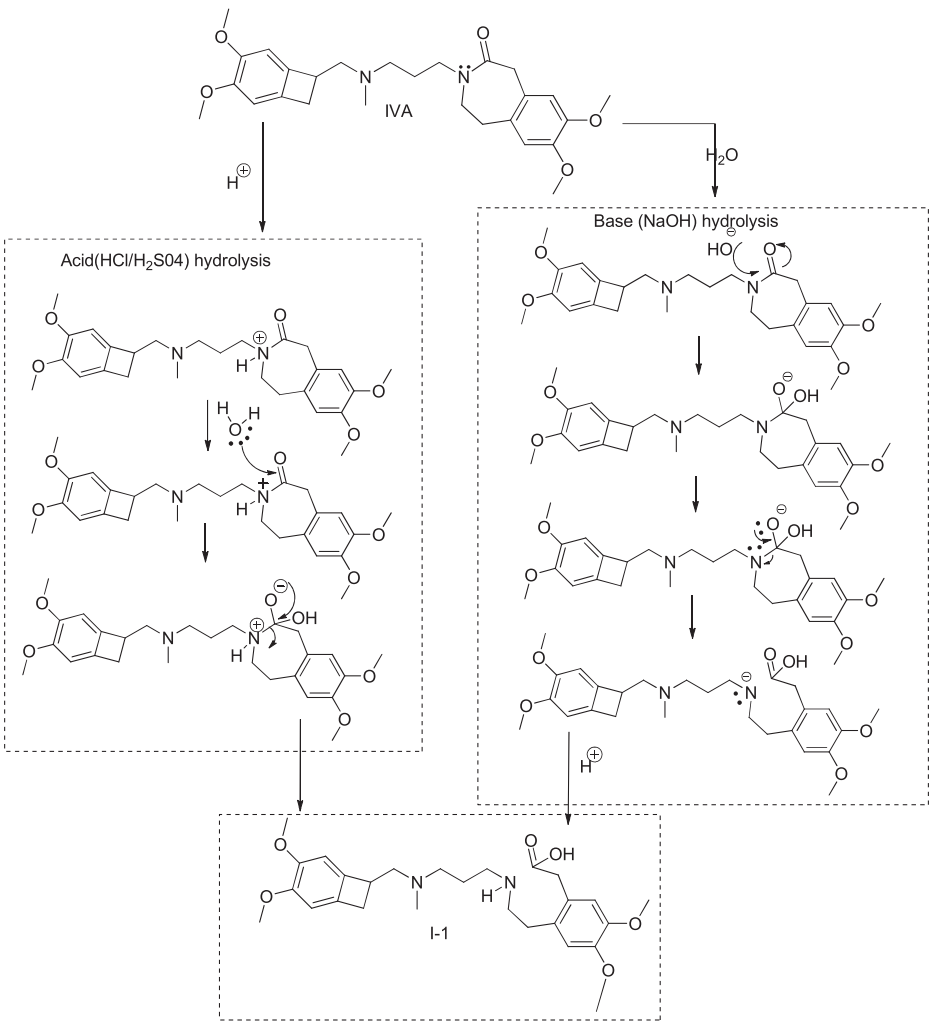


Scheme 3. a) Proposed fragmentation pathway of protonated degradation product I-1, and b) Proposed fragmentation pathway of protonated Ivabradine degradation products (I-2 to I-5).

at *m/z* 523 and 525 clearly indicate the isotope pattern of mono chlorinated compound (Fig. 3). When H_2SO_4 was used for acid hydrolysis of **IVA**, I-2 was not formed. This clearly shows that **I-2** can be formed only when HCl is used as a stress induced solvent. The LC/MS/MS spectrum of $[M+H]^+$ ion of **I-2** displays diagnostic product ions at *m/z* 505 (loss of H_2O) and *m/z* 487 (loss of HCl) (Scheme 3b). However, the loss of H_2O and loss of HCl from *m/z* 523 substantiate the adduct formation of chlorine with the drug through covalent bond formation. The presence of an abundant product ion *m/z* 280 and absence of *m/z* 469 in the LC/MS/MS spectrum of **I-2** indicate that CO is not converted to –COOH as in the case of I-1. Hence, the structure of *m/z* 262 in **I-2** should be different from that of **IVA**. The elemental compositions of product ions have been confirmed by accurate mass measurements (Table 2). Based on all these data **I-2** was identified as 1-chloro-2-(2-(2-((3-(((3,4-dimethoxybicyclo[4.2.0] octa-1,3,5-trien-7-yl) methyl) (methyl) amino) propyl) amino)-2-hydroxyethyl)-4,5-dimethoxyphenyl)ethanol (Scheme 1). The formation of I-2 may involve a nucleophilic attack by Cl^- on the carbonyl group leading to the formation of chloro hydrin with the cleavage of the lactam ring. This may undergo further addition of a hydroxyl group as shown in Scheme 5.

The $[M+H]^+$ ions of **I-3** (Rt = 10.77 min) and **I-4** (Rt = 11.76 min) have accurate masses, i.e. *m/z* 487.2783 and *m/z* 487.2787 and identical MS/MS spectra suggesting that they could be diastereomers. (Fig. 4, Table 2). The spectra showed peaks at *m/z* 280 (loss of $C_{12}H_{17}NO_2$), *m/z* 262 (loss of H_2O from *m/z* 280), *m/z* 206 (loss of $C_{15}H_{23}NO_4$) and *m/z* 177 (4, 5-dimethoxy-1-methylcyclobutabenzene) (Scheme 3b). The elemental compositions of product ions have been confirmed by accurate mass measurements (Table 2). It can be noted that these spectra are different from that of I-1 in the way that *m/z* 469 is absent in the spectra of I-3 and I-4. This suggests that structure of I-1 is different from those of I-3 and I-4. The absence of H_2O loss indicates that hydroxylation does not occur at the carbonyl carbon of dihydrobenzoazepin-2-one of **IVA**. Based on these data, **I-3** and **I-4** were identified as diastereomers of 2-(2-(2-(3-(((4, 5-dimethoxy-1,2-dihydrocyclobutabenzen-1-yl) methyl) (methyl) amino) propyl amino)-2-hydroxyethyl)-4,5-dimethoxyphenyl) acetaldehyde. Probable mechanisms of formation of I-3 and I-4 may involve cleavage of lactam ring and α-hydroxylation as shown in Scheme 5. Alternatively, I-3 and I-4 may be formed by loss of HCl from **I-2**.

The degradation product **I-5** at *m/z* 505.2457 ($[M+H]^+$, $C_{27}H_{37}N_2ClO_5$) was detected at Rt = 14.39 min. The mass difference between **IVA** and **I-5** is 36 Da indicating that it is a chlorinated degradation product. The intensities of $[M+H]^+$ ions at *m/z* 505 and 507 clearly indicate the isotope pattern of chlorinated compound (Fig. 4). The ESI/MS/MS spectrum of protonated **I-5** shows product ions at *m/z* 262 (loss of $C_{12}H_{18}ClNO_2$), *m/z* 206 (loss of $C_{15}H_{22}ClNO_3$) and *m/z* 177 (loss of $C_{16}H_{22}N_2O_3Cl$ from *m/z* 505) (Scheme 3b). All



Scheme 4. Probable mechanisms of formation of **I-1**.

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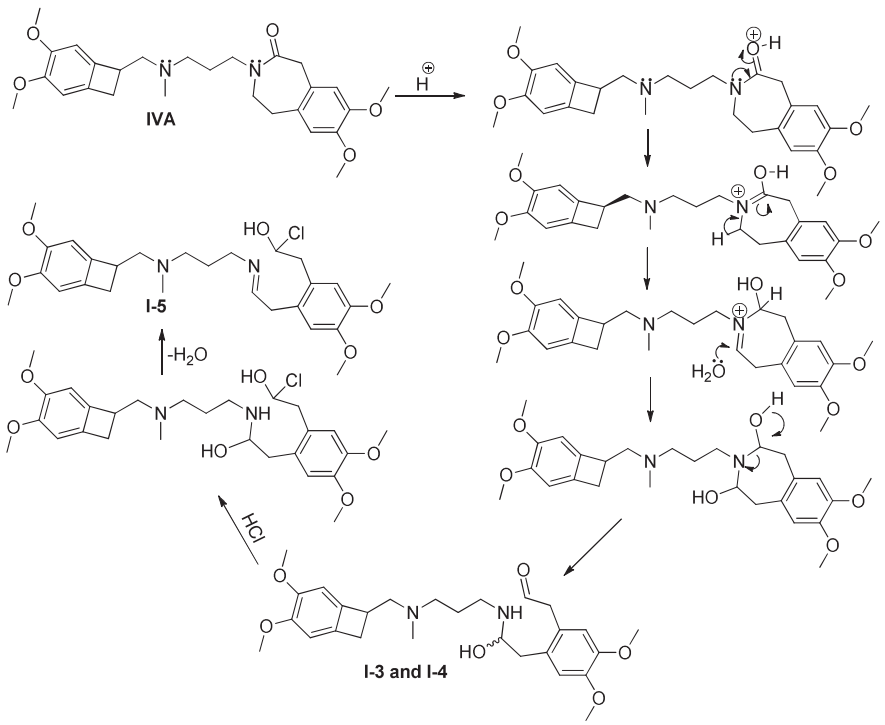

Scheme 5. Probable mechanisms of formation of **I-2**, **I-3**, **I-4 and I-5**.

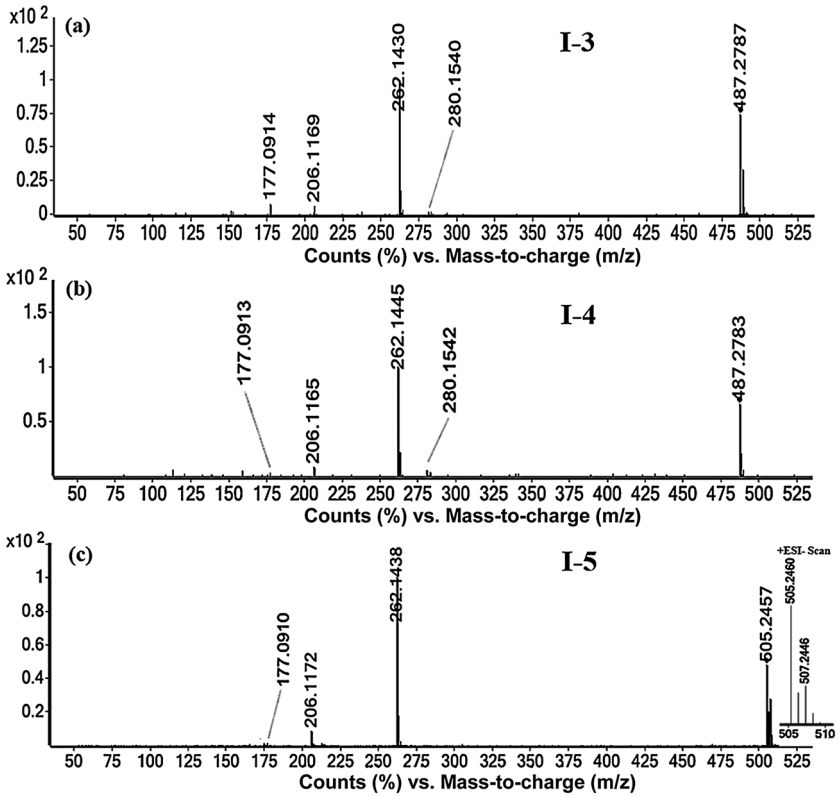

Figure 4. ESI/MS/MS spectrum of (a) **I-3** (*m/z* 487) at 20 eV, (b) **I-4** (*m/z* 487) at 20 eV and (c) **I-5** (*m/z* 505) at 20 eV.

these data are consistent with the proposed structure, 1-chloro-2-(2-(2-(3-(3,4-dimethoxybicyclo [4.2.0] octa-1,3,5-trien-7-yl) methyl) (methyl) amino) propyl) imino) ethyl)-4,5-dimethoxyphenyl) ethanol. The elemental compositions of product ions have been confirmed by accurate mass measurements (Table 2). The mechanism of formation of **I-5** may involve loss of H_2O from **I-2** (Scheme 5).

In silico toxicity prediction

The potential toxicity of **IVA** and its degradation products were assessed by using TOPKAT (Toxicity Prediction by Komputer Assisted Technology) software. TOPKAT uses 2D molecular structure of the drug as an input and a range of robust, cross-validated,

Quantitative Structure-Toxicity Relationship (QSTR) models for assessing specific toxicological endpoints. It estimates the toxicity of a compound quantitatively using structural, electronic (charge, electro density, residual electronegativity and effective polarizability), topological and electrotopological molecular descriptors. TOPKAT computes a probable value of toxicity (0.0 to 1.0) for a submitted chemical structure from a QSTR equation. If probable value is between 0.0 and 0.3 the compound is considered as non-toxic, if it is between 0.3 and 0.7 the result is indeterminate, and if it is between 0.7 and 1, the compound is toxic. Supplementary table S1 (see Supporting Information) shows predicted toxicity and carcinogenicity in different animal models for five compounds. The probabilities of carcinogenicity for five compounds in all the models was found very less (<0.7). In detail, the compounds were found carcinogenic with moderate probability value in TOPKAT_Mouse_Male_NTP model (degradation product **I-2** and **I-3**) and TOPKAT_Rat_Male_NTP model (compound **I-1** and **I-5**). The compound 4 was predicted non-toxic with very less probabilities of carcinogenicity in all the models (<0.3), except Developmental Toxicity Potential (DTP) model. All the five compounds were found to be toxic in Developmental Toxicity Potential (DTP) model. In case of ocular irritancy, the compounds (I-2 to I-5) were found to be severe irritant, while in case of skin irritancy all compounds were found to be mild or non-irritant. However, the overall predicted higher toxicity could not be considered significant in view of the qualification threshold of 0.5% for degradation impurities in drug products.[25]

Method validation

The specificity of the method was established by determining peak purity for **IVA** in a mixture of stressed samples using a photodiode array (PDA) detector and evaluation of the resolution factor. It was also demonstrated by subjecting all the degradation samples to LC-MS. The mass detector showed an excellent purity for **IVA** and every degradation product, which unambiguously proves the specificity of the method. The response for the drug was linear in the investigated concentration ($r^2 = 0.9996$) and the %RSD for each investigated concentration was <0.15%. The linearity data are given in supplementary table S2 (see Supporting Information). Supplementary table S3 (see Supporting Information) shows accuracy data at three different concentrations in triplicate analysis. The recoveries of the added drug were obtained from the difference between peak areas of fortified and unfortified degraded samples. The recovery of **IVA** in the presence of degradation products ranged from 99.80 to 100.75%. Table S3 (see supporting information) shows that the %RSD for intra and intermediate precision was <0.71 % and 0.97 %, respectively, indicating that the method was sufficiently precise. Robustness was evaluated for change of flow rate (0.6–0.8 ml/min), column temperature (30 ± 5 °C) and pH of mobile phase (3.0 ± 0.2) at three different concentrations (10, 50 and 100 μg/ml). The %RSD was <1%. No significant changes in assay value were observed by changing these chromatographic conditions which confirms the robustness of the method.

Conclusion

Stress degradation of ivabradine a cardiotonic drug leads to the formation five degradation products. An HPLC method has been developed for the separation of all the degradation products from the drug as well as from each other under various conditions. The degradation products have been characterized using online LC-ESI-MS/MS experiments combined with accurate mass measurements. The proposed structures of the degradation products have been rationalized by appropriate mechanisms. Interestingly, the drug underwent distinct transformation to form two chlorinated degradation products under HCl hydrolytic conditions and these were absent when H_2SO_4 was used. *In silico* toxicity prediction for all degradation products was carried out using TOPKAT software. The degradation products (**I-2** to **I-5**) were found to be severe irritant in case of ocular irritancy. However, the overall predicted higher toxicity could not be considered significant in view of the qualification threshold of 0.5% for degradation impurities in drug products. The HPLC method was validated as per regulatory requirements and can be used for routine analysis and stability studies.

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