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Validated Stability Indicating Chromatographic Methods for Determination of IvabradineHydrochloride in the Presence of Its Acidic Degradation Product

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Abstract

Two sensitive, accurate, precisestability indicating methods were developed and validated for the determination of IvabradineHCl (Iva) in the presence of its acid degradate, in bulk powder and in pharmaceutical formulations. Forced degradation was performed using 2N HCl. The degradation product was identified by infrared spectroscopy and mass spectrometry, and the pathway of degradation was illustrated. The first methodwas based on thin-layer chromatographic (TLC) separation of Iva from its degradation product, followed by densitometric measurement of the intact drug band at 286 nm. The separation was carried out on aluminium sheet of silica gel 60 F_{254} using methanol:chloroform:water (8:1:1 V/V) as the mobile phase, over a concentration range of 0.5–9 µg/band and mean percentage recovery of99.88 ± 0.994. The second method was based on reversed-phase high-performance liquid chromatography (HPLC), on C_{18} column using methanol:acetonitrile:phosphate buffer pH 3 (50:40:10 V/V) as the mobile phase at a flow rate of 1mL/min. Quantification was achieved with UV detection at 230 nm over a concentration range of 0.5–200 µg/mLwith mean percentage recovery of 99.98 ± 0.765.The developed methods were validated according to the ICH guidelines and were applied for bulk powder and dosage forms.

Keywords: Ivabradine hydrochloride, HPLC, TLC-densitometry, Stability indicating method, Degradation

1. Introduction:

IvabradineHydrochloride(Iva),3-(3-{[((7S)-3,4-Dimethoxybicyclo[4,2,0]octa-1,3,5-trien-7-yl) methyl]methylamino}propyl)-1,3,4,5-tetrahydro-7,8-dimethoxy-2H-3-benzazepin-2-one,hydrochloride (Figure 1) is a heart rate reducing agent. So it is used for symptomatic management of stable angina pectoris. IvabradineHydrochloride is a specific heart rate lowering agent, acting by reducing the rate of cardiac pacemaker activity in the sinoatrial node[1-3].

The literature survey reveals several methods for determination of Iva in biological fluids, in pharmaceutical dosage forms , in combination with other drugs including high performance liquid chromatography[4-21] , spectrophotometric methods[7,16] , Thin layer chromatography [22-25].However all these methods lack the achievement of complete degradation of Iva and identification of the degradation product which is a point of analysis interest as the structural characterization allow establishment of the degradation pathway .In the present work the degradation pathway of Iva was illustrated and accurate, simple, sensitive and rapid stability-indicating chromatographic methods for the determination of Iva in the presence of its acidic degradate was also developed.

2. Experimental:

2.1. Apparatus

The thin-layer chromatography (TLC) was performed using a Camag 3S/N/30319 TLC scanner with win CATS software; an ultraviolet (UV) lamp with a short wavelength at 254 nm (Desaga, Wiesloch, Germany), a CamagLinomatauto sampler (Muttenz, Switzerland), a Camagmicro syringe (100 µL) and;

and TLC plates pre coated with silica gel $G.F_{254}$ 10X20 cm, 0.25 mm thickness (Merck, Darmstadt, Germany).

The HPLC was performed using an Agilent pump with different flow rates (model 1100 series; Agilent, Germany) equipped with a variable wavelength detector and a 20 µL injection loop. A Zorbax ODS column (250 mm, 4.6 mm, i.d.5µm) was used as stationary phase. The samples were injected with a 50 µL Hamilton analytical syringe. An infrared (IR) spectrophotometer (Shimadzu, Kyoto, Japan) and Ultra-performance liquid chromatography mass spectrophotometer (LC/MS/MS) (Waters, Milford, USA) were used for infrared and mass spectrophotometric analysis.

2.2. Materials

- Pure Ivabradine hydrochloride was kindly supplied by Global Napi pharmaceuticals, its purity is certified to be 100.12%±0.463 according to a reported method [8]
- Procoralan® tablets, Batch No. 919844 and 947159 labeled to contain 7.5 mg/tab and 5 mg/tab respectively, manufactured by Servier (Cairo, Egypt) were purchased from local market.

2.3. Chemicals and Reagents

- All chemicals used throughout this work were of analytical grade, and solvents were of HPLC grade.
- Methanol, Acetonitrile& Chloroform are from sigma-Aldrish.
- Hydrochloric acid & Sodium hydroxide are from Adwic, El-Nasr Pharmaceutical Chemicals Co. Cairo, Egypt.
- Phosphate buffer (pH 3) was prepared according to British Pharmacopoeia [26]
- Double distilled deionized water is from Egypt Otsuka Pharmaceutical Co. Cairo, Egypt.

2.4. Standard solutions

- Stock solutions of Iva and its acid degradate (1mg/mL) were prepared in methanol.
- Working solutions of Iva and degradate(200 µg/mL) were prepared from their corresponding stock solutions in methanol for HPLC method.

2.5. Procedures:

2.5.1. Preparation of Acid Degradation Product

50 mg of Iva was dissolved in the least amount of distilled water, refluxed with 50 mL of 2N HClin a 250 mL round-bottom flask for 1 hour, and tested for complete degradation by TLC using methanol:chloroform:water (8:1:1, V/V) as the mobile phase. Only one spot was observed not corresponding to Iva.

The degraded solution was cooled and neutralized with an amount of base equivalent to that of the previously added till pH was 7. The solution was nearly evaporated to dryness, cooled and transferred into a 50 mL volumetric flask with methanol. Then the volume was completed to the mark with methanol to obtain solution with final concentration equivalent to 1 mg /mL.The structure of the degradation product was elucidated using IR and mass spectrometry.

2.5.2. TLC-Densitometric Method

Aliquots equivalent to0.5– 9µLofIva standard stock solution(1 mg/mL) were applied to the TLC plates. The plates were developed to a distance of approximately 9.5 cm by the ascending technique with methanol: chloroform:water (8:1:1 V/V) as the mobile phase. The plates were removed, air-dried, and the spots were visualized under a UV lamp at 254 nm. The chromatogram was scanned at 286 nm.The calibration curve representing the relationship between the recorded area under the peak and the corresponding concentration of the drug in micrograms per band was plotted, and the regression equation was computed.

2.5.3. HPLC Method

Aliquots equivalent to 5 –2000 µg of Iva were accurately transferred from the working solution into a set of 10 mL volumetric flasks; the volumes were completed with the mobile phase. A 20 µL aliquot of each solution was injected into a Zorbax ODS column (250 X 4.6 mm,i.d.5µm) using the mobile phase methanol: acetonitrile:phosphate buffer pH 3 (50:40:10V/V)at a flow rate 1.0 mL/ min and with detection at 230 nm.The sample and the mobile phase were filtered through a 0.45 µmmillipore membrane filter. The mobile phase was degassed for about 15 min in an ultrasonic bathprior to use, The calibration curve was constructed by plotting the peak area and the corresponding concentrations of the drug (0.5– 200) in micrograms per milliliter, and the regression equation was computed. The system suitability parameters, retention time, tailing factor, theoretical plate count (N), height equivalent to theoreticalplate (HETP), separation of Iva peak and its degradate peak (resolution) and column capacity were studied.

2.5.4. Application to Pharmaceutical Formulations

The film coat was removed using methanol and the contents of ten tablets were accurately weighed and powdered. An accurate weight of the mixed sample was transferred into a 100 mL volumetric flask, complete to the mark with methanol and sonicated for 1 hour. The solution was filtered into a 100 mL volumetric flask. Aliquots from this solution were transferred to 10 mL volumetric flasks and diluted with methanol to prepare tablet solutions. For the HPLC method, the last solution was further diluted by transferring aliquots to 10 mL volumetric flasks and completing the volumes with the mobile phase. The general procedure previously described for each method was followed to determine the concentration of Iva in the prepared dosage form solutions.

3. Results and Discussion:

3.1. Degradation of Iva

Iva being an amide is liable to hydrolysis. It was degraded by refluxing in 2N HCl and the degradation process was monitored by spotting on TLC plates and developing using methanol: chloroform:water (8:1:1 V/V) as a developing solvent. Different factors were studied such as concentrations of hydrochloric acid, heating time and mobile phase solvents such as methanol, ethyl acetate, chloroform, acetone, toluene, benzene with different ratio. The optimum conditions were found to reflux with 2N HCl for 1 hours using methanol: chloroform:water (8:1:1, V/V) as the mobile phase.

It was found that complete degradation of Iva occurs afterrefluxing for 1 hrwith 2N HCl giving one degradation product. The degradate was separated and its structure elucidated by IR and mass spectrometry. The IR spectra of intact Iva and its degradate (Figures 2A and 2B) show that the characteristic band at 1640 to 1690 cm^{-1} corresponding to C=O of amidegroup which is present in the spectrum of intact Iva (Figure 2A) disappeared in the IR spectrum of its degradate (Figure 2B).The proposed structure of acid degradate was further confirmed by mass spectrometrywhere the mass spectrum shows mass ion peak at m/z = 487 corresponding to itsmolecular weight ,(Figure 3) .The increase of molecular weight of degraded Iva (m/z=487) compared to the drug (m/z=469) is attributed to the addition of water molecule to the drug indicating the hydrolysis of lactam ring of intact Iva. The degradation pathway is illustrated in scheme 1.

3.2. TLC – Densitometric Method

Several trials were conducted to develop the optimum chromatographic conditions for the sufficient separation of the intact drug from its degradate. The results of the TLC system were satisfactory when using methanol: chloroform: water (8:1:1 V/V) as the mobile phase. The chromatogram was scanned at 286 nm. The method is based on the difference in the Rf values of Iva (Rf = 0.32) and the degradate (Rf = 0.11), Figure4.Chromatographic separation allows the determination of Iva without any interference from its degradate. A polynomial relationship was found to exist between the integrated area under the peak of the separated spots at the selected wavelength (286nm) and the corresponding concentration

ofIva in the range of 0.5 – 9µg/band. Scanning profile of different concentrations of Iva at 286 nm was shown in Figure 5. The regression equation wascomputed and found to bethe following:

$A = -117.98C^2 + 3861.8C + 1637.6 \quad r^2 = 0.9999$

where A is the area under the peak, C is the concentration in µg/band, and $r^2$is the correlation coefficient.

3.3. HPLC Method

To optimize the proposed reversed-phase HPLC method, the experimental conditions were investigated. Concerning the mobile phase, different systems were tried for chromatographic separation of the drug from its degradate such as methanol, water, acetonitrile. The best resolution was achieved when using a mobile phase consisting of methanol:acetonitrile:phosphate buffer pH3 (50:40:10V/V).Also the effect of pH was studied by using buffers of different pH values.
Finally, the optimum stationary/mobile phase matching trials for the HPLC system were achieved by using a Zorbax ODS column (250X4.6 mm,i.d. 5µm) with a mobile phase consisting of ethanol: acetonitrile:phosphate buffer pH 3 (50:40:10V/V) at flow rate of 1mL/min, followed by UV detection at 230 nm (Figure 6).Chromatographic separation allows the determination of Iva without any interference from its degradate. An HPLC calibration curve was constructed, representing the relationship between the peak areasand their corresponding concentrations.
The linear regression equation was found to be the following:

$A = 33.681\ C + 13.691 \quad r^2 = 1$

Where A is the peak area, C is the concentration in µg/mL, and r^2isthe correlation coefficient.

The proposed methods were validated according to ICH guidelines (Table 1). Validity parameters of the methods are satisfactory, including linearity, range, accuracy,intermediate precision and repeatability. The two methods were linear in the range from 0.5 – 9µg/band and 0.5 – 200 µg/mL for TLC - densitometric method and HPLC method respectively. An overall system suitability testing[27,28] was calculated (Table 2). Satisfactory system suitability parameters including retention time, resolution, tailing factor, capacity factor, selectivity factor, column efficiency, and height equivalent to theoretical plates were obtained for the proposed TLC and HPLC methods. The proposed methods were successfully applied forthe determination of Iva in Procoralan® tablets, and the standard addition technique was applied to assess the validity of the proposed methods, Table 3.Finally, the results obtained by applying the proposed methods for the determination of Iva were statistically compared with the reported method[8] and showed no significant difference regarding both accuracy and precision (Table 4).

4. Conclusion:

The proposed methods are found to be simple, accurate, and preciseand can easily be applied as stability-indicating methods for the determination of Iva. They could be applied for routine analysis of pure drug or in its pharmaceutical formulation in quality control laboratories without any preliminary separation step.

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Table1.Assay and Validation Parameters for the Determination of Iva by the Proposed Methods

Parameter	TLC	HPLC
Range	0.5 – 9 µg/band	0.5 – 200 µg/mL
Slope	Slope 1* = -117.98 Slope 2* =3861.8	33.681
Intercept	1637.6	13.691
Standard error of the slope	0.0044	0.0009
Standard error of the intercept	0.0233	0.0912
Mean of recovery of calibration	99.88	99.98
SD of recovery of calibration	0.994	0.765
Correlation coefficient (r)	0.9999	1.0000
Accuracy	100.53 ± 1.134	100.26 ± 0.126
Repeatability**	100.09±0.877	99.47±0.293
Intermediate precision**	99.33±1.135	100.70±0.768

* Slope 1 and 2 are the coefficients of X^2 and X, respectively. Following a polynomial regression $A = ax^2 + bx + c$ Where, A is the peak area, x is the concentration of Iva (µg/band), a and b are coefficients 1 and 2, respectively and c is the intercept.
**RSD (%) of three concentrations of Iva (2, 4 and 6 µg/band for TLC and 40, 100 and 160 µg/mL for HPLC) analyzed intra-daily in triplicate (repeatability) and on three successive days (intermediate precision)

Table2. Parameters Required for System Suitability Tests of TLC–Densitometric and HPLC Methods

	TLC		HPLC		Reference values [27,28]
	IvabradineHCl	Degradation Product	IvabradineHCl	Degradation Product	
t_R (min)	-------	-------	2.920	5.343	---------
R_f	0.32	0.11	-------	------	--------
Resolution (R_s)	3.50		3.79		R_s> 1.5
Tailing factor (T)	1.14	1.07	1.17	1.13	T = 0.8–1.2
Capacity factor (K)	-------	-------	2.24	4.94	1 < K< 10
Selectivity factor (α)	2.91		2.20		α > 1
Column efficiency (N)	--------	---------	852.64	1983.22	Increase with efficiency of the separation
Height equivalent to theoretical plate (cm)	--------	-------	0.03	0.01	The smaller the value the higher the column efficiency

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Table3.determination of Iva in its dosage form and application of standard addition technique using the proposed methods

	TLC	HPLC
	%Found ± S.D.*	
Procoralan® 5 mg	101.22 ± 0.750	98.40 ± 0.468
Recovery of standard added %	100.84 ± 0.943	100.28 ± 0.313
Procoralan® 7.5 mg	101.10 ± 0.805	99.07 ± 0.380
Recovery of standard added %	100.37 ± 1.097	99.96 ± 0.894
*Average of three determinations.		

Table 4.Statistical comparison for the results obtained by the proposed methods and the reported method for the analysis of Iva

	TLC	HPLC	Reported method*
Mean	99.88	99.98	100.12
S.D.	0.994	0.765	0.463
Variance	0.988	0.585	0.215
n	10	14	6
Student's t-test**	0.657 (2.132)	0.507(2.132)	
F value**	4.60 (4.77)	2.72(4.66)	

* HPLC method using C_{18} column, methanol:25 mM phosphate buffer (60:40 v/v) as a mobile phase and UV detection at 285 nm
** Figures between parentheses represent the corresponding tabulated values of t and F at P = 0.05

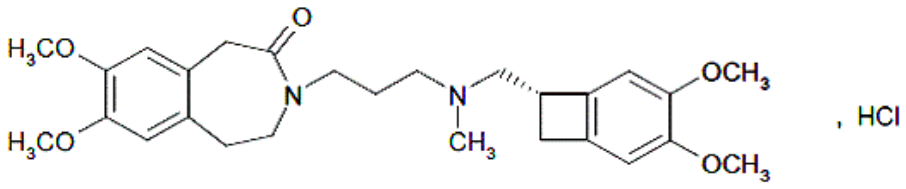

Figure 1.Chemical structure of Ivabradine Hydrochloride

ISSN:2249-1236
Joliana F. Farid et al. IJRRPAS,(2016) Vol.6 Issue.1 Pg:1370-1380
International Journal of Research and Reviews in Pharmacy and Applied sciences

(A) (B)

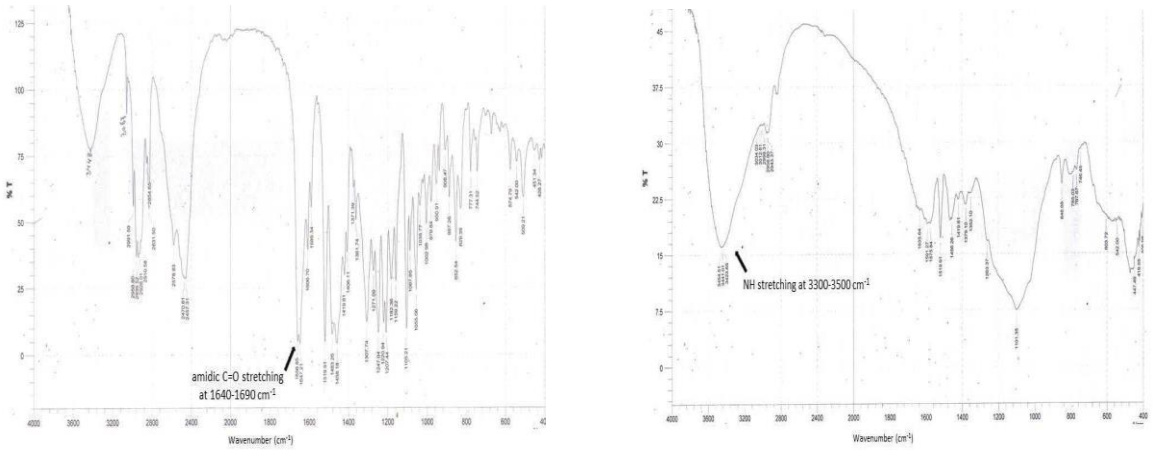

Figure 2.IR-spectra of (A) intact Iva and (B) its degradation product.

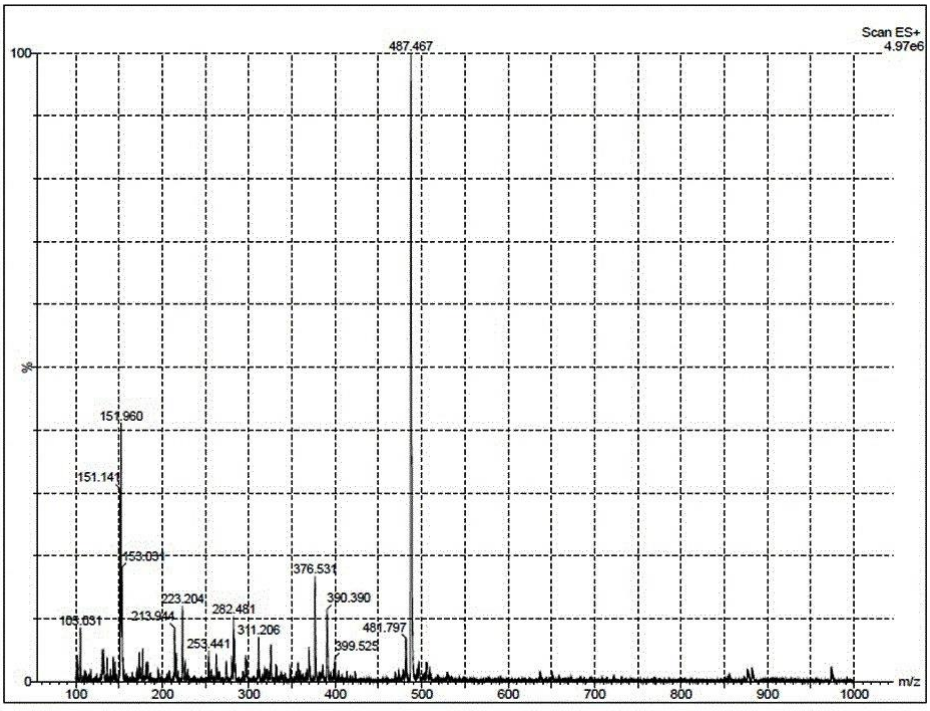

Figure 3.Mass spectrum of Iva degradation product.

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International Journal of Research and Reviews in Pharmacy and Applied sciences

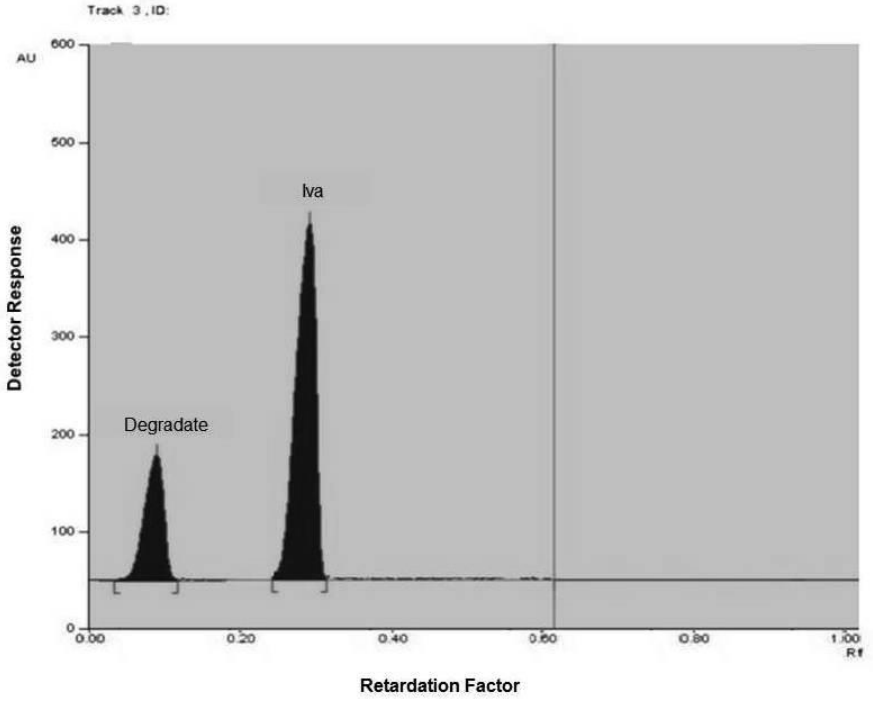


Figure 4.TLC chromatogram of Iva (Rf= 0.32) and its degradate (Rf= 0.11) using a mobile phase of methanol: chloroform:water (8:1:1 v/v) and detection at 286 nm

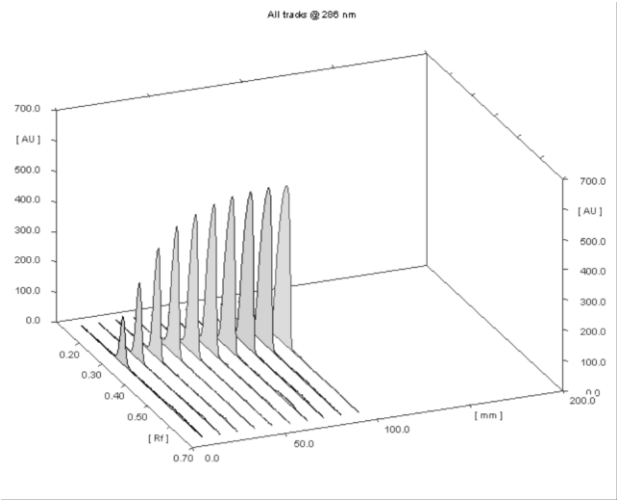


Figure 5.Scanning profile of the TLC chromatogram of Iva (0.5 – 9 µg/band) at 286 nm.

ISSN:2249-1236
Joliana F. Farid et al. IJRRPAS,(2016) Vol.6 Issue.1 Pg:1370-1380
International Journal of Research and Reviews in Pharmacy and Applied sciences

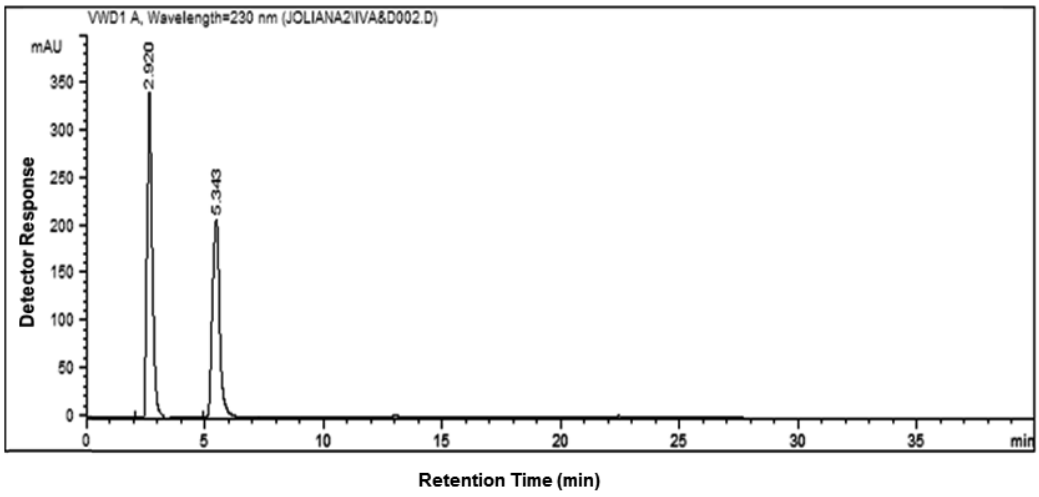
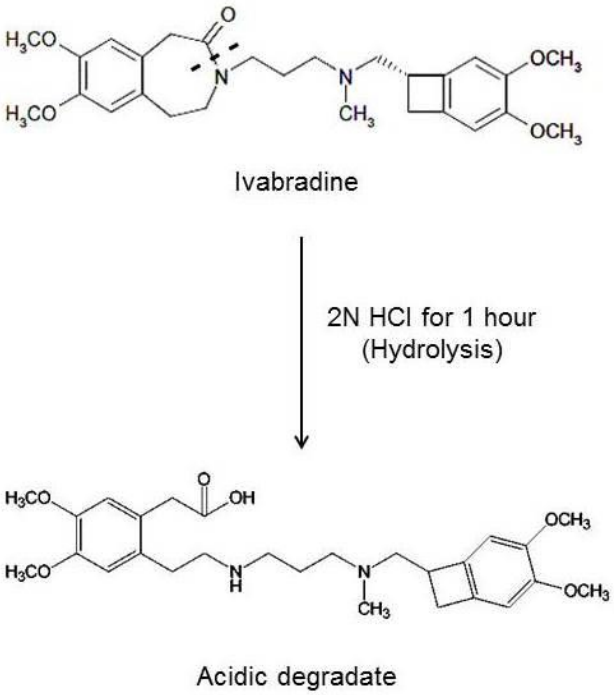


Figure 6.HPLC chromatogram of Iva (tR= 2.920) and its degradate (tR =5.343) using a Zorbax ODS column (250X4.6 mm, 5µm i.d.), mobile phase of methanol:acetonitrile:phosphate buffer pH 3 (50:40:10 v/v) at flow rate of 1mL/min and detection at 230 nm



Scheme1. Suggested pathway for acidic degradation of Iva.

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