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Stability-Indicating RP-HPLC Method for the Determination of Ezogabine and Identification of Its Degradation Products

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Abstract

In this stability-indicating, reversed phase high performance liquid chromatographic method for Ezogabine, forced degradation has been employed and the formed degradants were separated on a Enable C18 column with a 40:60% v/v mixture of water containing 0.1% (v/v) triethylamine; the pH was adjusted to 8 with ortho phosphoric acid(OPA):methanol. The flow rate was 1 mLmin−1 and the photodiode array detection wavelength was 221 nm. A sharp peak was obtained for Ezogabine at a retention time of 10.2 min. Forced degradation studies was carried out under acidic, basic, thermal, photolytic and oxidative conditions. Chromatographic peak purity data indicated no co-eluting peaks with the main peaks. This method resulted in the detection of two degradation products. Two major degradation products from acidic and basic hydrolysis were identified and characterized by ^{1}H-NMR, Bruker Alpha-T IR and mass spectral data. The method was validated as per International Conference on Harmonization guidelines (Q2). Linear regression analysis data for the calibration plot showed there was a good linear relationship between response in the concentration range of 5 to 25 µg mL^{-1} with regression coefficient of 0.999. The relative standard deviations for intra and interday precision were below 2%. The specificity of the method is suitable for the stability-indicating assay.

Key words: Ezogabine; Stability: RP HPLC; Identification: Spectral studies

INTRODUCTION

Ezoganine (Retigabine) is a novel drug acts as neuronal potassium channel opener for the adjunctive anticonvulsant treatment of partial onset seizures in refractory epilepsy. It is chemically known as N-[2-Amino-4-(4-fluorophenylmethylamino)-phenyl] carbamic acid ethyl ester [Figure 1]. Ezogabine acts primarily by targeting voltage gated potassium channels in the brain like valproic acid [1-3]. A detailed literature survey reveals that there are very few methods reported for the estimation of Ezogabine by UV Spectrophotometer [4], Colorimeter [5] and RP-HPLC methods [6-12] were reported for estimation of ezogabine. But there is no report on the degradation profile, nor characterization of the degradation products under stress conditions. Hence the present study was undertaken to develop a new stability-indicating RP-HPLC method for the estimation of Ezogabine and to identify the major degradants.

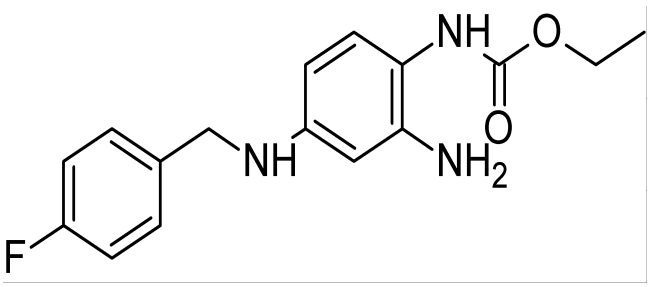


Figure 1: Structure of Ezogabine

MATERIALS AND METHODS

Chemicals and reagents

Ezogabine was obtained as gift sample from M/s Torrent Pharmaceuticals, Ahmedabad, India. All chemicals, reagents of analytical grade and solvents used were of HPLC grade, purchased from Himedia Chemicals, India.

Instrumentation and analytical conditions

Chromatographic analysis of Ezogabine was carried out on a UFLC system (Shimadzu,Japan) equipped with a reverse phase Enable C_{18} column (250 X 4.6 mm, 5 µm), a LC- 20 AD isocratic pump, manual injector with loop volume of 20 µl and a photo diode array detector and running on LC solution Software version. Isocratic elution with 0.1% triethylamine in water (pH 8 adjusted using ortho phosphoric acid: Methanol 40:60 (v/v) was used as mobile phase and UV detection was done at 221 nm with a flow rate of 1 mLmin^{-1}. Injection volume was 20 µl with ambient temperature, run time was 15 min and retention time was 10.2 min. The mobile phase was prepared freshly and degassed and sonicated for 5 min before use. A preparative HPLC equipped with Enable C_{18} column and PDA detection (using the software lab solution) was used for the isolation and purification of degradation products. ^{1}H-NMR was recorded on the Varian Unity Inova at 400 MHz (using TMS as internal standard and DMSO-d_6 as solvent), IR was recorded on the Bruker Alpha-T IR and mass spectral studies were performed on the API 3000 ABPCIES instrument.

Forced Degradation Studies

Forced degradation of the Ezogabine was performed under acid, base, oxidative, thermal and photolytic stress conditions. In all stress conditions, the drug concentration used was 1000 µgmL−1. After degradation, samples were diluted with mobile phase to a concentration of 10 µgmL−1 and injected under optimized conditions with the appropriate blank. Blank solutions for each hydrolysis were prepared at the same time with the preparation of stock solutions.

Preparation of Stock Solution for Stress Studies

In all of the stress studies, 10 mg of the Ezogabine was accurately weighed and transferred to a 10 mL volumetric flask, dissolved with methanol, and diluted up to the mark with methanol. The same procedure was adopted to prepare the sample solutions used for acid hydrolysis, base hydrolysis, thermal, photolytic and oxidation, respectively, with HCl (0.1 M), NaOH (0.1 M), and H_2O_2 (3%). For acid degradation, the initial degradation trials were performed in 0.1 M HCl. Thermal degradation was carried out on the solid substance by means of heating the samples over a period in a hot air oven, at 85°C. Photo degradation was carried out on the solid sample according to the procedure described in the following section. Hydrolysis Stock solutions (1000 µgmL−1) were prepared in methanol at room temperature. Samples (100 µL) were withdrawn at different intervals and diluted to 10 mL with mobile phase (10 µgmL−1). Samples from the acidic hydrolysis were neutralized with 0.1 M NaOH and samples from the basic hydrolysis were neutralized with 0.1 M HCl. Oxidation Samples (1 mL) were withdrawn at different times, diluted to 10 mL with mobile phase, and injected under the optimized conditions for analysis. The blank sample was prepared with the same concentration of hydrogen peroxide and analyzed in the same way. Thermal Degradation to assess the solid-state stability, the sample was heated as a thin layer on a Petri dish at 85°Cfor one week, 10 mg of the heated sample was weighed, dissolved in methanol, diluted with mobile phase to 10 µgmL−1 and analyzed. Photo degradation studies were conducted by exposing a solution of the sample to UV radiation at 1.2 million lux-h for one week. After degradation, the sample was dissolved in methanol, diluted with mobile phase to 10 µgmL−1 and analyzed under the optimized conditions. In all of the degradation studies, the percentage degradation of Ezogabine was calculated using the response factor. Peak area was used to calculate the response factor as well as the amount of degradation product formed. The formed degradation product was isolated and purified on a Enable C_{18} column by using preparative HPLC, then evaporated in a rotary vacuum evaporator. Two degradation products from hydrolysis were isolated and characterized by IR, ^{1}H-NMR and mass spectrometry.

RESULTS AND DISCUSSION

Method Development and Optimization of the Chromatographic Conditions

In preliminary experiments, the drug was subjected to the reversed-phase mode using a Enable C_{18} column (250 x 4.6 mm, 5µ) and mobile phases consisting of water (pH 8.0 adjusted with ortho phosphoric acid) and methanol by varying the % aqueous phase from 10% to 40%. Ezogabine was retained on the column, but the peak shape was not good. It was noted that increasing the % aqueous phase in the mobile phase composition increases the retention time of Ezogabine. Based on the suitable retention time for SIAM, the 40% aqueous phase was optimized. To reduce the tailing effect, 0.1% triethylamine (TEA) was added and the pH was adjusted to 8.0 with ortho phosphoric acid and the corresponding retention time of Ezogabine was 10.2 min. Finally, the mobile phase of 0.1% v/v TEA (pH-adjusted to 8.0 with OPA) and methanol in the ratio of 40:60% v/v was optimized at a flow rate of 1.0 mLmin−1. The injection volume was 20 µL and the PDA detection wavelength was at 221 nm. The chromatogram obtained in the optimized condition is shown in [Figure 2]. Two degradation products were formed with retention times 8.1 and 8.2 min for acid(0.1M) and base(0.1M) hydrolysis. The chromatographic resolution among all of the peaks was more than 2. The % degradation was about 5–30% depending on stress conditions.

Validation Parameters

The method was validated as per ICH (Q2) guidelines with respect to linearity, accuracy, precision, robustness, limit of detection, and limit of quantification [11].

Specificity

Specificity is the ability of the analytical method to measure the analyte concentration accurately in presence of all potential DPs. Specificity of the method towards the drug was studied by determination of purity for drug peak in stressed sample using a PDA detector. The study of resolution factor of the drug peak from the nearest resolving DP was also done. Both drug and DP peaks were found to be pure from peak purity data. Also, the resolution factor for the drug from degradation peak was greater than 3. The method was proven to be specific by separating the degradation products formed under the stress conditions.

Linearity

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within suitable level of precision, accuracy and linearity Five points graph was constructed covering a concentration range 5 to 25 μgmL^{-1}. Linear relationships between the peak area and concentration of Ezogabine was recorded and evaluation was performed [Table 1] and calibration curve was shown in [Figure 3]

Table 1: Linearity range of Ezogabine

Concentration(μgmL^{-1})	Peak area*
5	1021319
10	1915140
15	2746719
20	3699405
25	4631698

*average of 5 determinations

Table 2: Intra-day and inter-day precision of the developed method

Concentration (µgmL^{-1})	Measured concentration (µgmL^{1})			
	Intraday(n=6)	%RSD	Interday (n=3)	%RSD
10	10.40	1.661	10.35	1.114
15	14.96	1.457	15.03	0.010
20	20.47	1.601	19.75	1.020

Table 3: LOD and LOQ determination of ezogabine

Parameter	Concentration
LOD	0.625 µgmL^{-1}
LOQ	1.895 µgmL^{-1}

*average of 3 determinations

Table 4: Accuracy (Recovery studies) results of the developed method

Concentration(µgmL$^{-1)}$	Spike Level (%)	Percentage Recovery	%RSD*
10	50	100.32	1.519
	100	100.33	0.517
	150	98.00	1.622

*average of 5 determinations

Table 5: **Robustness results for the developed method**

S.No	Parameter	Level	Retention time	T_f	% RSD
1	Flow rate 0.9 mLmin^{-1}	-1	11.4	1.03	0.502
2	Flow rate mLmin^{-1}	+1	9.2	1.10	1.391
3	Mobile phase composition (38:62)	+2	9.4	1.02	0083
4	Mobile phase composition (42:58)	-2	12.0	1.10	0.519
5	Wavelength 223 nm	-2	10.3	1.06	1.49
6.	Wavelength 219 nm	+2	10.3	1.06	1.49

Table 6: System Suitability

S No.	Parameters	Ezogabine
1	Theoretical plate	12320
2	HETP	12.17
3	Tailing factor	1.068
4	LOD	0.625 µgmL^{-1}
5	LOQ	1.895 µgmL^{-1}

Table 7: Degradation data of Ezogabine under stress studies

Degradation studies	Time	Assay of Ezogabine % of peak area	Remarks
Acid hydrolysis (0.1M HCl, 80^{0}C)	2h	78.68	Degradation product I observed
Base hydrolysis (0.1M NaOH,80^{0}C,)	2h	87.65	Degradation product I observed
Oxidation (3%H2O2,80^{0}C,)	2h	83.0	Degradation observed
Photolytic condition	1 Week	95.57	Degradation observed
Thermal condition(85^{0}C)	1 Week	79.43	Degradation observed

Precision

Intraday Precision

Intraday precision was found out by carrying out the analysis of the Ezogabine in the linearity range for six times on the same day. Each concentrations were applied in duplicate and percentage RSD was calculated which is found to be within acceptable criteria of not more than 2 [Table 2].

Interday Precision

Inter day precision was found out by carrying out the analysis of the Ezogabine in the linearity range for three days for six times and the percentage RSD was calculated which is found to be within acceptable criteria of not more than 2 [Table 2]. Low values of standard deviation denoted very good repeatability of the measurement. Thus it was showing that the equipment used for the study was

correctly and hence the developed analytical method is highly repetitive.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of the method, determined by the standard deviation method, which indicated the method, can be used for detection and quantification of Ezogabine over a very wide range of concentrations [Table 3].

Accuracy

The recovery of the method, determined by spiking a previously analyzed solution with additional standard Ezogabine solution. The values of recovery (%), RSD (%) in [Table 4] indicated that the method is accurate.

Robustness

There was no significant change in the retention time of Ezogabine with the change in flow rate and composition of the mobile phase. The low values of the RSD, shown in [Table 5], indicated that the method is robust.

System suitability

The system suitability parameters like capacity factor, asymmetry factor, tailing factor and number of theoretical plates were also calculated and the values are found to be within the limits. The statistical evaluation of the proposed method was revealed its good linearity, reproducibility and its validation for different parameters. The results are furnished in [Table 6].

The details degradations of Ezogabine is shown in [Table 7] and chromatogram are shown from [Figure 4 to 8]. All degradation products were adequately separated, thus the method was found to be more selective and specific. The peak purity was more than 0.999. The study revealed that Ezogabine was more sensitive to all stress conditions. No degradation product was observed after 15 min. Ezogabine was more labile to degradation in the presence of base and acid.

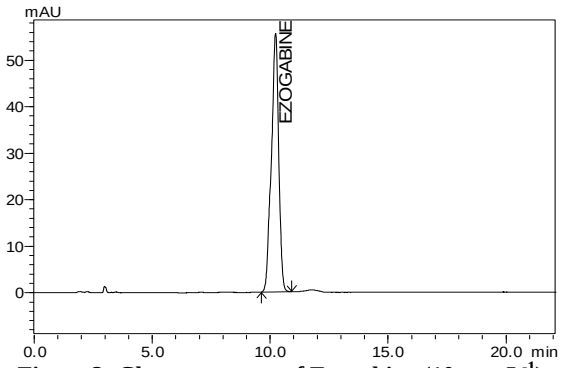


Figure 2: Chromatogram of Ezogabine (10 µgmL^{-1})

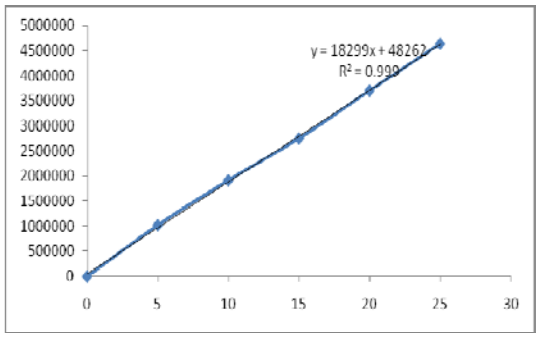


Figure 3: Calibration curve of Ezogabine

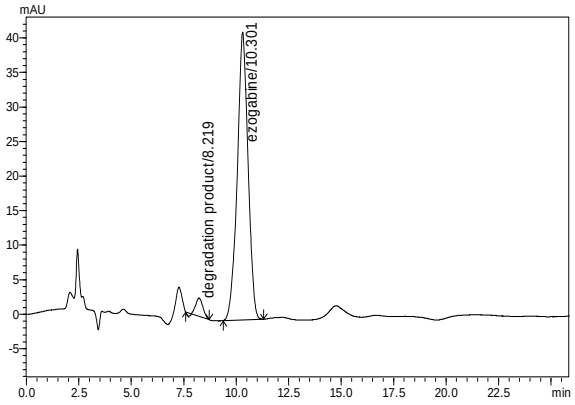


Figure 4: Acid (0.1M HCl) degradation chromatogram of Ezogabine

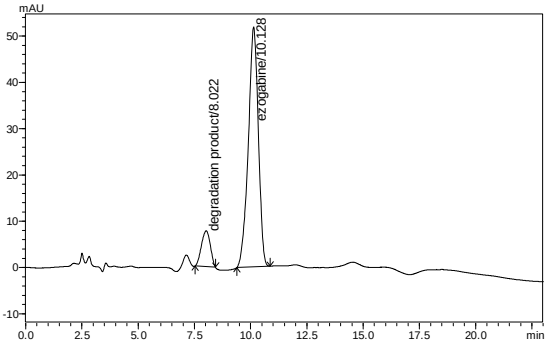


Figure 5: Base (0.1M NaOH) degradation chromatogram of Ezogabine

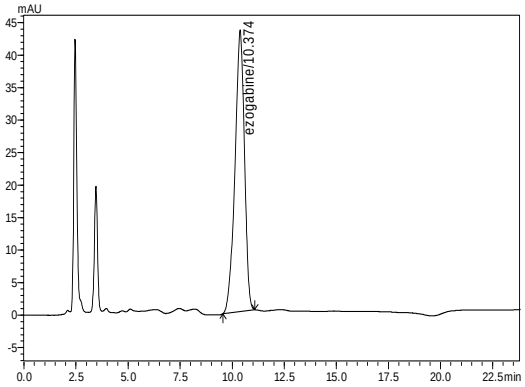


Figure 6. Peroxide (3% H_2O_2) degradation chromatogram of Ezogabine

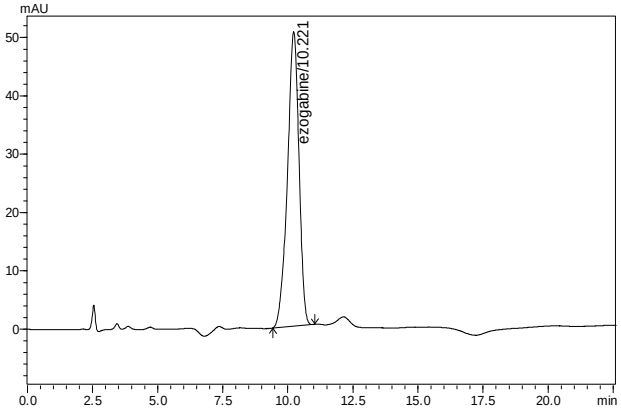


Figure 7: Thermal degradation chromatogram of Ezogabine

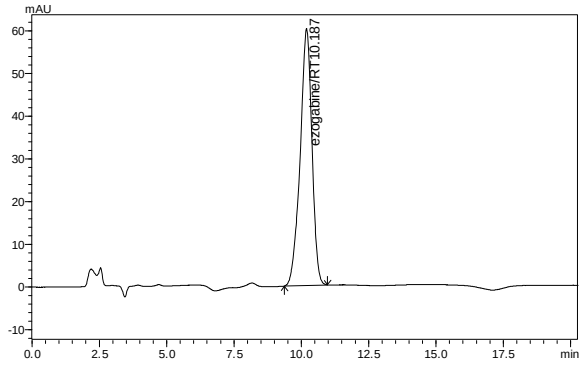
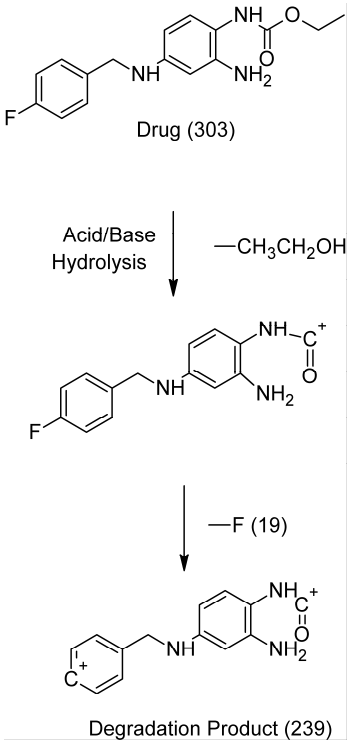


Figure 8: Photolytic degradation of Ezogabine



Scheme 1: Mechanism of formation of degradation product from the drug

Identification of Degradants

In these forced degradation studies, a total of two degradation products were observed for Ezogabine, (acidic and basic hydrolysis), separated and purified by preparative HPLC chromatography. The obtained degradation products were characterized by IR, ^{1}H-NMR, and mass spectral data. The retention time for degradation products were identified as 8.1 and 8.2 min for acid and base hydrolysis respectively.

Degradant1: [((3-amino-4-[(oxomethylinmyl) amino] phenyl) amino) methyl] phenylium. Formula: $C_{14}H_{13}N_3O$, molecular weight: 239.15, ^{1}H-NMR (CDCl3, δ ppm): 4.24 (2H, CH_2), 6.1-7.5 (m, 7, Ar-H), 10.22 (s, 1H, NH), 10.38 (s, 1H, NH), 10.7 (bs, 2H, NH_2), IR (KBr): 1637(C=C), 2937 (Ar-H), 3414 (primary amine), 2741, 2679(NH), 1637(C=O).

Degradant2: [((3-amino-4-[(oxomethylinmyl) amino] phenyl) amino) methyl] phenylium. Formula: $C_{14}H_{13}N_3O$, molecular weight: 239.15, ^{1}H-NMR (CDCl3, δ ppm): 4.12 (2H, CH_2), 6.1-7.5 (m, 7, Ar-H), 10.118 (s, 1, NH). IR (KBr): 1645(C=C), 2925 (Ar-H), 3413(primary amine), 2925(NH), 1645(C=O). The probable structure of degradation product is given in scheme 1.

CONCLUSION

Forced degradation studies on ezogabine, carried out according to ICH guidelines, provided information regarding degradation behaviour of the drug. The drug was susceptible to acidic and basic hydrolytic degradation. one degradation product from acidic and basic hydrolysis condition was formed and was separated in a single run by an isocratic LC-DAD method. The method proved to be simple, accurate, precise, specific and robust. The degradation product was identified with the help of IR, NMR and mass spectral data.

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