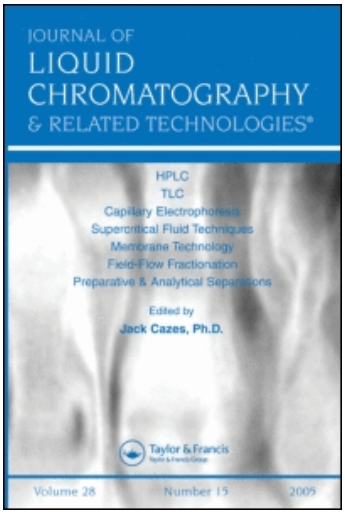

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 28 February 2001

To cite this Article Edafiogho, I. O. , Abdel-Hamid, M. E. , Hamza, H. and Scott, K. R.(2001) 'STABILITY STUDY OF AN ANTICONVULSANT ENAMINONE (E139) USING HPLC', Journal of Liquid Chromatography & Related Technologies, 24: 4, 565 — 577
To link to this Article: DOI: 10.1081/JLC-100103394
URL: http://dx.doi.org/10.1081/JLC-100103394

J. LIQ. CHROM. & REL. TECHNOL., 24(4), 565–577 (2001)

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ABSTRACT

A simple high performance liquid chromatography (HPLC) method has been developed for the stability study of an anticonvulsant enaminone (E139). Using a Chiral HSA column and a mobile phase of n-octanoic acid (5 mM) and isopropyl alcohol and disodium hydrogen phosphate solution 1:9 v/v at a flow rate of 1 mL/min., the chromatograms exhibited well resolved peaks at retention times of < 5 min. for the predominant diastereoisomer.

The stability study for E139 was carried out in acid, alkaline, neutral solution, and in a phosphate buffer solution of physiological pH. The results confirmed that the hydrolysis of E139 was fastest in acid medium, indicating that protonation of the enaminone system enhanced hydrolysis at a degradation rate constant (K_{deg}) of 0.044 $min.^{-1}$ and a degradation half-life ($t_{½}$) of 15.75 min. at room temperature (25°C). Deprotonation of E139 in alkaline solution also resulted in hydrolysis, but at a slower rate (K_{deg} of 0.017 $min.^{-1}$) and longer degradation half-life ($t_{½}$: 40.76 min.) at

25°C. The enaminone E139 was very stable in the buffer solution of physiological pH (K_{deg} of 0.001 hr^{-1}, and $t_{½}$ of 24 days at 25°C).

Analysis of the acid hydrolysis of E139 by liquid chromatography – mass spectrometry (LC-MS) revealed that the decarboxylated product of E139 was formed. This study offers a great potential for the application of HPLC and LC-MS methods in the bioassay of enaminone compounds.

INTRODUCTION

Enaminones are chemical compounds consisting of an amino group linked through a C=C to a keto group[1] and it has been reported by several workers that the enaminone system confers a variety of pharmacological properties to enaminone compounds.[2,3] Accordingly, enaminones have been known to possess cardiovascular,[4] anti-inflammatory,[5] histaminergic,[6] antimalarial,[7] and anticonvulsant activities.[8,9] However, the anticonvulsant enaminone (E139) has been reported to display high potency as an anticonvulsant compound and the racemate was evaluated.[9,10] Although enaminones are known to be stable compounds,[9,11–13] very little is known about the degradation kinetics and degradation products of enaminone analogues such as E139.

This paper describes the use of high performance liquid chromatography (HPLC) in an accelerated-stability testing of E139 at elevated temperatures to determine the degradation rate constant (K_{de}), and degradation half-life ($t_{½}$) of the compound in the designated solutions at various temperatures. Arrhenius plots were constructed to calculate the degradation kinetic parameters at room temperature. Recently, Abdel-Hamid employed an HPLC method for the analysis of antiepileptic agents,[14] and his method is modified for our stability study of E139. The liquid chromatography – mass spectrometry (LC-MS) method[14] is utilized to identify the degradation product.

EXPERIMENTAL

Materials

The anticonvulsant enaminone (E139) was prepared according to the method of Edafiogho, Scott, and co-workers.[10] The sodium salt of n-octanoic acid was purchased from Sigma Co. (St Louis, MO, USA). HPLC grade of isopropyl alcohol and ethanol were supplied by Fisher Scientific International Company, UK. Analytical grade of anhydrous disodium hydrogen phosphate (Fluka Chemie AG, Germany) was used. Water was purified by Milli-Q-System (Millipore Corporation, USA).

Instruments

HPLC analyses were performed using an isocratic high performance liquid chromatograph (Waters 2690 Separation Module, USA) equipped with an autosampler (Waters, USA) and a variable UV detector (Waters 486 Tunable Absorbance Detector). Chromatographic separations were achieved at ambient temperature using Chiral-HSA, 100 × 4 mm, 5 μ column (ChromTech, Sweden). The mobile phase was prepared by dissolving 4.16 gm of n-octanoic acid (sodium salt) in 50 mL isopropyl alcohol and diluting the solution to 500 mL with 100 mM disodium hydrogen phosphate solution. The filtered and degassed mobile phase was pumped at a flow rate of 1 mL/min. The injection volume was 10 μL and the eluents were monitored by UV detector at 225 nm. Analytical data, such as retention time and peak area measurements, were processed by the instrument built-in Millennium software.

Liquid chromatography–mass spectrometry (LC-MS) analyses were performed using the LC-MS system which is comprised of an LC pump (Spectra System P 2000, USA) and an MS detector (Finnigan MAT, USA) with APCI as an ionization process. The APCI conditions were: vaporization temperature, 450°C; sheath gas flow, 80 mL/min.; discharge current, 5 μA; discharge potential, 4.38 kV, and capillary temperature 230°C. The mass spectrometer was programmed to detect the positive molecular and fragment ions of E139 in the range of m/z 100 – 500. Samples were injected directly into the MS detector using a 10 μL-loop size.

Elution of E139 was achieved using a mobile phase consisting of acetonitrile and 1% acetic acid solution in a ratio 4:1 at a flow rate of 1 mL/min. Analytical data were processed by the built-in LCQ software of the instrument.

Procedure

Stock Solution

An accurate weight of E139 powder was transferred into a 10-mL volumetric flask, dissolved, and diluted to volume with ethanol (1 mg/mL). The solution was freshly prepared for each study.

Stability Studies

Aliquots of 100 μL of the freshly prepared solution of E139 in ethanol (1 mg/mL) were accurately transferred into 1-mL glass vials and diluted to 1-mL with 0.1 M HCl solution or 0.1 M NaOH solution or phosphate buffer solution (pH 7.5) or ethanol/water 1:1 v/v. The solutions were properly vortexed and the vials placed

in a water bath (Techne Dri-Block) at 30 or 40 or 60°C for the appropriate periods of time. An accurate volume (10 μL) of each sample was automatically injected into HPLC and analyzed under the described chromatographic conditions.

Degradation Kinetics

The logarithmic values of the peak areas of E139 (retention time about 4.9 min.) at zero- and at different time-intervals were used to establish the degradation plots of E139 in 0.1 M HCl solution, 0.1 M NaOH solution, phosphate buffer solution (pH 7.5), and ethanol/water 1:1 v/v solution, respectively. The degradation kinetic parameters, such as the degradation rate constant (K_{deg}) and degradation half-life ($t_{½}$) at 30, 40, and 60°C, were derived from the first-order plots. The predicted kinetics for the degradation of E139 at 25°C were extrapolated from Arrhenius plots.

RESULTS AND DISCUSSION

Chemistry and Anticonvulsant Activity of E139

The chemical nomenclature of the examined enaminone compound is methyl 4-(4'-bromophenyl)amino-6-methyl-2-oxocylohex-3-en-1-oate. The designated number for the racemate in our laboratory was E139. Its ultraviolet, nuclear magnetic resonance spectra, and elemental (C,H,N,Br) analyses supported the assigned structure.[10] It has a melting point of 188–190°C, a CLOGP value of 3.383, and the molecular weight of 338.2 for the formula $C_{15}H_{16}NO_3Br$.

The enaminone (E139) was a Class 1 anticonvulsant agent in the Maximal Electroshock (MES) test.[10] In the phase 1 test, E139 had an anticonvulsant activity at a dose of 100 mg/Kg given intraperitoneally (i.p.), and protected mice from an electrically-induced seizure.[3] Enaminone E139 was evaluated further for oral (p.o) activity in a phase VIA test in rat at a dose of 50 mg/Kg, and the compound afforded a complete anti-MES protection to the rat without causing any neurotoxicity for periods up to 4 hours. In a quantitative evaluation in the rat,[10] E139 had a TD_{50} of 270 mg/Kg and an ED_{50} of 4 mg/Kg, affording a protective index of > 67. Thus, E139 was a potent anticonvulsant enaminone which was active by oral and parenteral routes.

Stability Study by HPLC

The HPLC chromatograms of freshly prepared solutions of E139 at zero time at 30, 40, and 60°C in neutral, physiological pH, acidic ,and alkaline media,

gave the reference retention times and areas under the curves for the peaks. The Chiral-HSA, 100 × 4 mm, 5 μ chromatographic column was used in this study in order to separate the peaks of the diastereoisomers of E139 using the described mobile phase at 225 nm. The applied HPLC procedure proved to be a stability-indicating assay, as it permitted detection of E139 in the presence of its acid- or base-induced degradation products. A decrease of the peak area of compound at different time intervals relative to the original peak area at zero time, was considered for establishment of the degradation profiles.

Figure 1 shows the chromatograms of the acid hydrolysis of E139 at 30°C. The four diastereoisomers were well separated with the one eluting at retention time of 4.978 min. being predominant (84%) at zero time, as shown in Figure 1(a). Figure 1(b) shows degradation progressing with the peak area of the predominant peak diminishing, while the putative product is increasing at retention time of 3.548 min. after hydrolyzing for 10 min. After 50 min of acid hydrolysis, the degradation product is predominant (84%), as shown in Figure 1(c). Since enaminones undergo protonation[13] in acid medium, the degradation specie would be protonated.

In a similar manner, hydrolysis of E139 occurred in alkaline solution, and after 40 min., almost all of E139 had been converted to the degradation product, presumably a deprotonated specie[13] which would exist as the sodium salt. Since polar compounds generally have shorter retention times, the value of 1.670 min. as retention time for the deprotonated product of E139 was expected.

In physiological pH solution (phosphate buffer pH 7.5), E139 did not change appreciably in the areas or the retention times for the peaks after standing at 40°C for 2 hr. The enaminone E139 showed only a slight change after standing for 3 hr. Similarly, E139 did not undergo any significant hydrolysis in a neutral solution (ethanol/water 1:1) after standing at 40°C for up to 3 hr. The compound E139 showed only a slight change after standing for 5 hr. Figures 2, 3, and 4 present the degradation plots of E139 in 0.1M HCl solution, 0.1M NaOH solution, phosphate buffer solution (pH 7.5), and ethanol/water solution at 30, 40, and 60°C, respectively.

As indicated, the hydrolysis of E139 followed first-order kinetics and the rate of hydrolysis is catalyzed by the presence of acid or base. Tables 1 and 2 show the calculated kinetic parameters of E139 at the above accelerated stability conditions.

To determine the degradation kinetics of E139 at 25°C, Arrhenius plots[16] were constructed by plotting the logarithmic values of the observed K_{deg} values, computed from the degradation plots, versus 1/T (exemplified in Tables 3 and 4). Using least squares regression, linear correlations with strong correlation coefficients (r: 0.9005–0.9559) were obtained for these plots.

As derived from Arrhenius plot, the degradation rate constant (K_{deg}) for the acid hydrolysis was 0.044 min^{-1}, and the degradation half-life ($t_{½}$) was 15.75 min. at room temperature (25°C). These data indicated that protonation of the

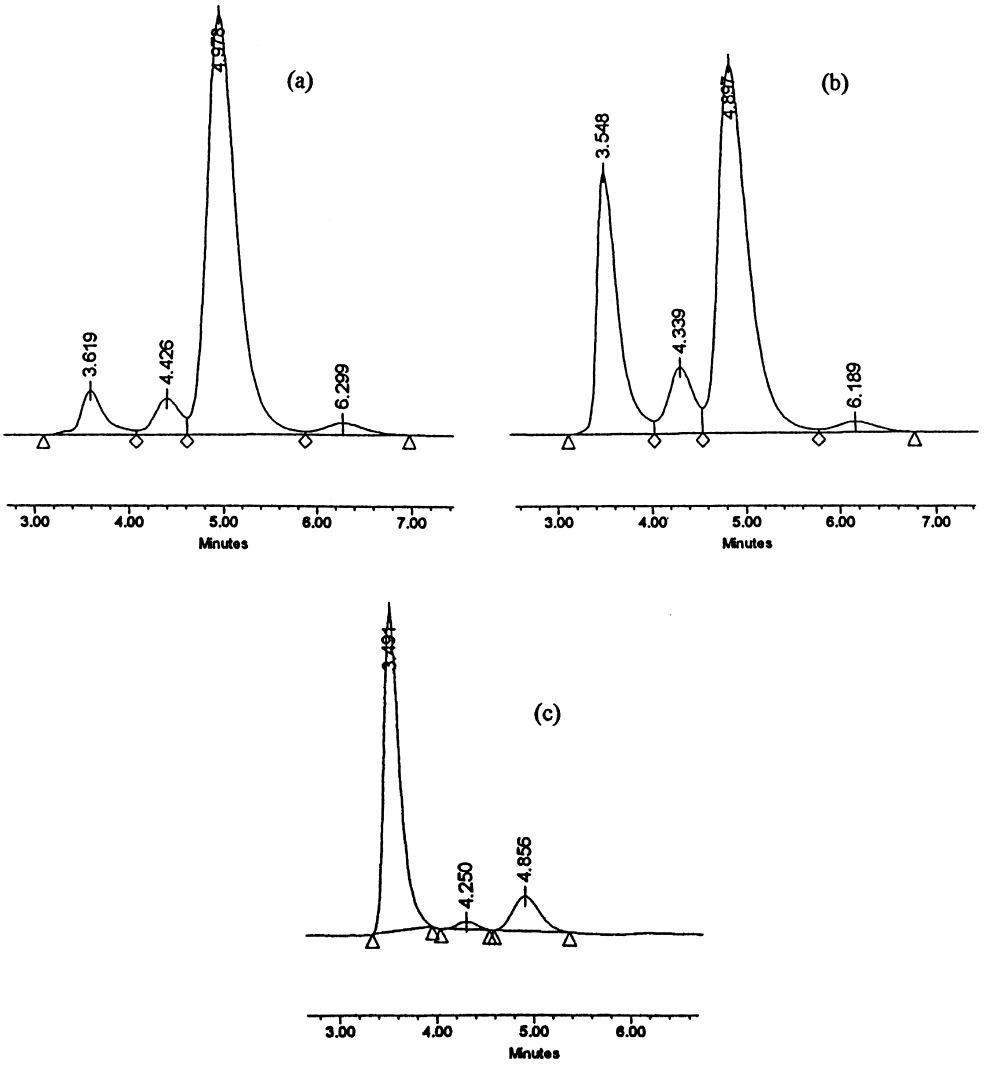

Figure 1. Chromatograms of acid hydrolysis of E139 after standing at 30°C for (a) zero time, (b) 10 min., and (c) 50 min.

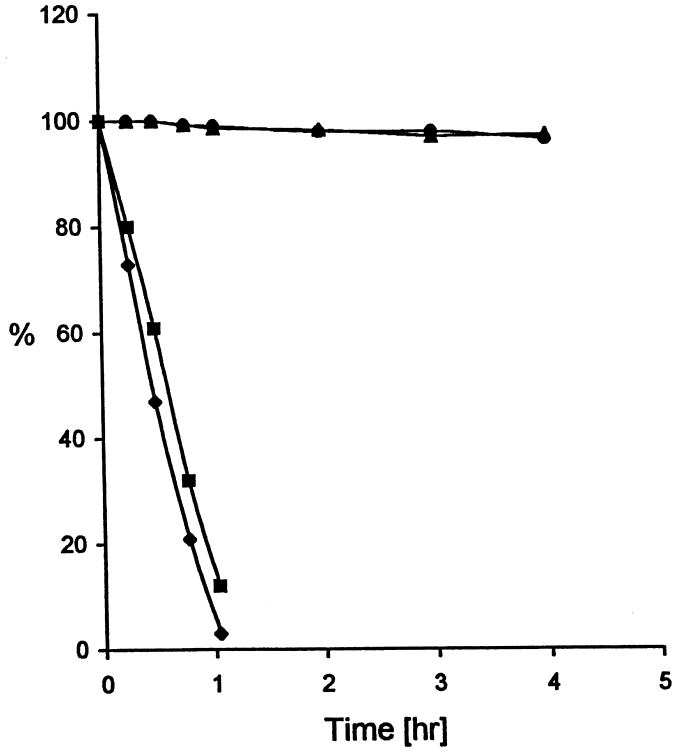

Figure 2. Degradation profile of E139 in ◆ 0.1 M HCl solution, ■ 0.1 M NaOH solution, ▲ phosphate buffer solution (pH 7.5) and ● ethanol/water solution at 30°C.

enaminone system enhanced the acid hydrolysis of E139. Greenhill[15] estimated that acid hydrolysis is usually completed for enaminones of dimedone within 15 min. For this anticonvulsant enaminone (E139), complete hydrolysis took longer than 15 min., probably due to the ester group in the molecule. The chemical stability of enaminones is highly sensitive to the structure of the 1,3-diketone from which it is formed.[12]

Deprotonation of E139 in 0.1 M NaOH solution resulted also in hydrolysis, but at a relatively slower rate (K_{deg}: 0.017 min^{-1}) and longer degradation half-life of 40.76 min. compared to acid hydrolysis. In phosphate buffer solution of physiological pH 7.5, E139 had K_{deg} of 0.001 hr^{-1}, and $t_{1/2}$ of 693 hr (24 days) at room temperature (25°C). In neutral solution (ethanol/water 1:1), E139 had K_{deg} of 0.0007hr^{-1}, and $t_{1/2}$ of 990 hr. (41 days) at room temperature (25°C). The data suggested that E139 is significantly stable in these solutions.

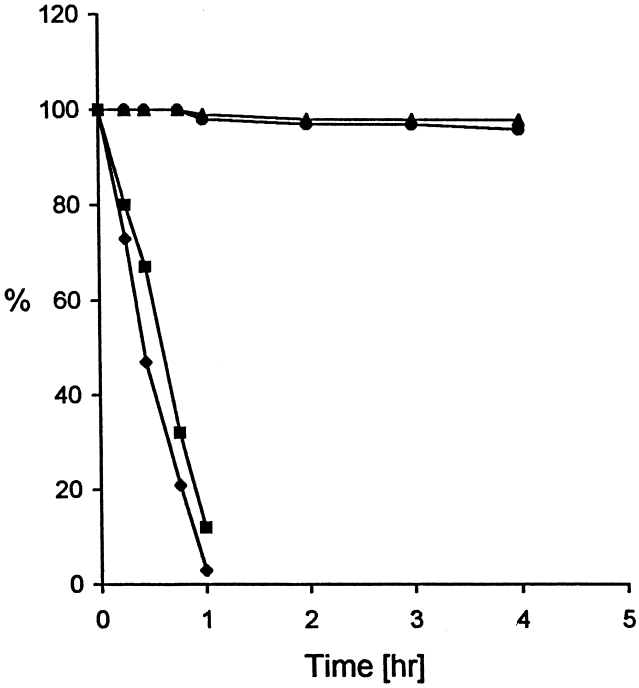

Figure 3. Degradation profile of E139 in ◆ 0.1 M HCl solution, ■ 0.1 M NaOH solution, ▲ phosphate buffer solution (pH 7.5) and ● ethanol/water solution at 40°C.

Table 5 summarizes the predicted degradation rate constants (K_{deg}), degradation half-lives ($t_{1/2}$) and degradation shelf-lives (t_{90}) of E139 in the respective solutions at room temperature (25°C). Additionally, the values of the activation energy (E_a) were calculated from Arrhenius plots and included in Table 5. An activation energy of 1044.3 kcal/mol is required for acid hydrolysis, 2991.9 kcal/mol for alkaline hydrolysis, 8090.7 kcal/mol for hydrolysis in phosphate buffer solution, and 6699.1 kcal/mol for hydrolysis in ethanol/water solution.

The collected $t_{1/2}$, t_{90}, and E_a data proved that the anticonvulsant enaminone (E139) was unstable in strongly acidic or basic solutions, but highly stable in physiological solution of pH 7.5, as well as in neutral ethanolic solution at room temperature.

Identification of Degradation Product by LC-MS

The experiments with liquid chromatography-mass spectrometry (LC-MS)[17] revealed that E139 was predominantly one compound with a molecular ion

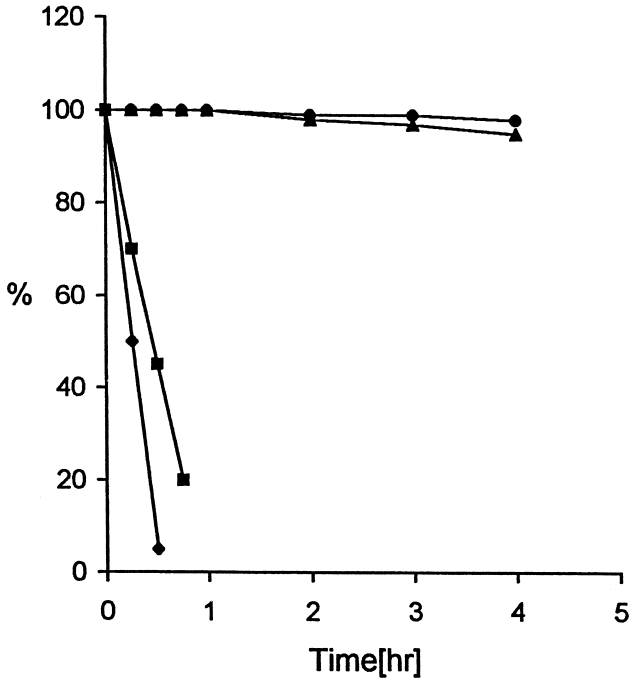

Figure 4. Degradation profile of E139 in ◆ 0.1 M HCl solution, ■ 0.1 M NaOH solution, ▲ phosphate buffer solution (pH 7.5) and ● ethanol/water solution at 60°C.

(M^{2+}) peak at m/z 340.1. The calculated molecular weight of E139 ($C_{15}H_{16}NO_3Br$) is 338.2. To explain the LC-MS of E139 in neutral solution, protonation at the amino- and keto- groups during mass spectrometry would yield the molecular ion (I) at m/z 340.1. However, the LC-MS for the acid-hydrolysis of E139 showed two peaks with molecular ion peaks of m/z 280.8 for degradation product, and m/z 340.4 for E139.

Table 1. Kinetic Parameters for the Degradation of E139 in Acid and Alkaline Solutions at 30, 40, and 60°C, as Derived from First-Order Plots

Temperature	0.1 *M* HCl		0.1 *M* NaOH	
	K_{deg} (min^{-1})	$t_{½}$ (min)	K_{deg} (min^{-1})	$t_{½}$ (min)
30°C	0.051	13.60	0.017	40.76
40°C	0.071	9.80	0.039	17.77
60°C	0.115	6.03	0.053	13.08

Table 2. Kinetic Parameters for the Degradation of E139 in Physiological pH (pH 7.5) and Ethanol/Water Solutions (1:1) at 30, 40, and 60°C as Derived from First-Order Plots

Temperature	Phosphate Buffer (pH 7.5)		Ethanol/Water (1:1 v/v)	
	K_{deg} (h^{-1})	$t_{1/2}$ (h)	K_{deg} (h^{-1})	$t_{1/2}$ (h)
30°C	0.0044	157.50	0.0019	368.6
40°C	0.0076	91.18	0.0084	82.50
60°C	0.0760	9.12	0.0250	27.72

Table 3. Arrhenius Plot of E139 in 0.1 *M* HCl Solution[a]

T[b]	$1/T$	K_{deg} (min^{-1})	Log K_{deg}
303	0.0033	0.051	−1.252
313	0.0032	0.071	−1.149
333	0.0030	0.115	−0.939

[a]Arrhenius plot: log $K_{deg} = 2.154 - 1044.3/T$ ($R = 0.9559$).
[b]$T = 273 + t$ (°C).

Table 4. Arrhenius Plot of E139 in 0.1 *M* NaOH Solution[a]

T[b]	$1/T$	K_{deg} (min^{-1})	Log K_{deg}
303	0.0033	0.017	−1.769
313	0.0032	0.039	−1.409
333	0.0030	0.053	−1.276

[a]Arrhenius plot: log $K_{deg} = 3.28 - 1503.5/T$ ($R = 0.9005$).
[b]$T = 273 + t$ (°C).

Table 5. Summary of the Predicted K_{deg}, $t_{½}$, t_{90}, and E_a of E139 in Various Solutions as Derived from Arrhenius Plots at 25°C

Solution	*K*deg	$t_{½}$[a]	t_{90}[b]	E_a(kcal/mol)
0.1 *M* HCl	0.044 min^{-1}	15.75 min	2.39 min	1044.3
0.1 *M* NaOH	0.017 min^{-1}	40.76 min	6.18 min	2991.9
Phosphate buffer	0.001 h^{-1}	693.0 h	105.5 h	8090.7
Ethanol/water 1:1	0.0007 h^{-1}	990.0 h	150.0 h	6699.1

[a]$t_{½} = 0.693/K_{deg}$.
[b]$t_{90} = 0.105/K_{deg}$.

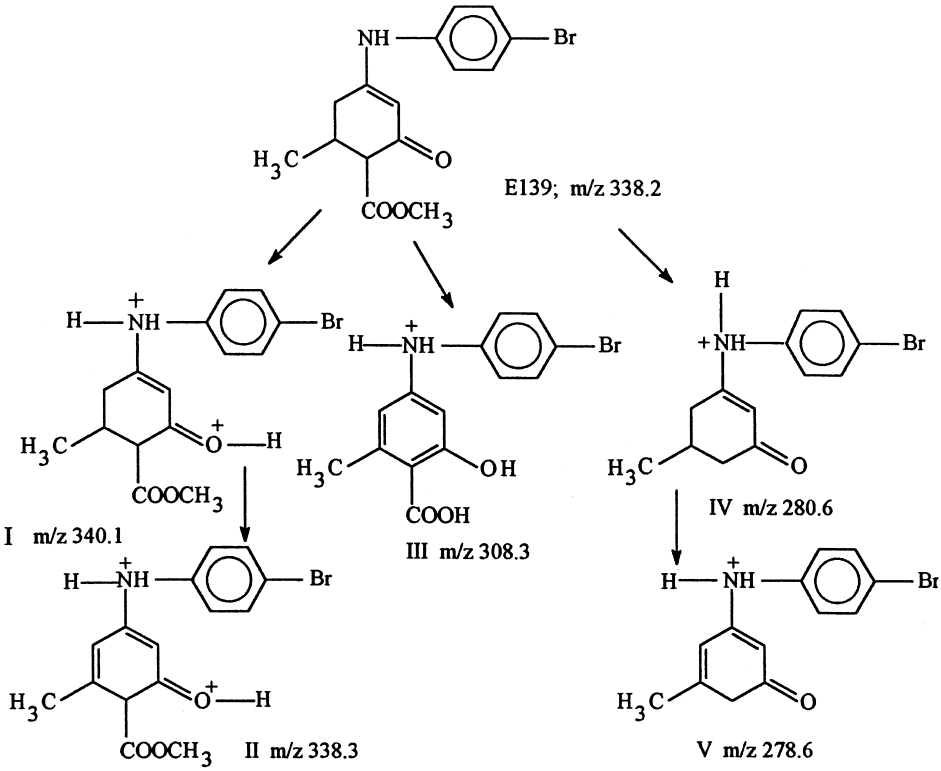

Figure 5. Scheme of the proposed fragment ions and molecular ions of E139 in liquid chromatography – mass spectrometric (LC-MS) analysis.

The molecular ions I and II, and the fragment ions III, IV, and V would satisfy the fragmentation pattern of E139, as shown in the scheme in Figure 5. Accordingly, it is proposed that the decarboxylated product IV resulted from the hydrolysis of E139. Therefore, the results from this stability study support the potential biotransformation pathway in which the decarboxylated product was obtained after esterase hydrolysis[10] of the chloro analog of E139.

CONCLUSION

A stability-indicating HPLC method has been developed for the stability study of an anticonvulsant enaminone E139. The various kinetic parameters for the hydrolysis of E139 in acid, alkaline, neutral, and physiological pH media were determined from the first-order plots.

As indicated from Arrhenius plots, E139 undergoes very fast hydrolysis in acidic solution at 25°C, whereas in neutral and physiological pH solutions, E139 is significantly stable. The data suggested that HPLC and LC-MS can be used for bioanalysis of enaminone compounds in biological fluids.

ACKNOWLEDGMENTS

This work was supported by the Kuwait University Research Grant FDC 119. The cooperation of Ms. Shorook Al Molohy, Mubarak Hospital is appreciated.

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Received July 8, 2000 Manuscript 5342
Accepted September 7, 2000