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Stability study of the anticonvulsant enaminone (E118) using HPLC and LC–MS

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Abstract

The stability of the new chemical synthetic enaminone derivative (E118) was investigated using a stability-indicating high-performance liquid chromatography (HPLC) procedure. The examined samples were analyzed using a chiral HSA column and a mobile phase (pH 7.5) containing n-octanoic acid (5 mM), isopropyl alcohol and 100 mM disodium hydrogen phosphate solution (1:9 v/v) at a flow rate of 1 ml min^{-1}. The developed method was specific, accurate and reproducible. The HPLC chromatograms exhibited well-resolved peaks of E118 and the degradation products at retention times < 5 min. The stability of E118 was performed in 0.1 M hydrochloric acid, 0.1 M sodium hydroxide, water/ethanol (1:1) and phosphate buffer (pH ~ 7.5) solutions. E118 was found to undergo fast hydrolysis in 0.1 M hydrochloric acid solution. The decomposition of E118 followed first order kinetics under the experimental conditions. The results confirmed that protonation of the enaminone system in the molecule enhanced the hydrolysis of E118 at degradation rate constant of 0.049 min^{-1} and degradation half-life of 14.1 min at 25 °C. However, E118 was significantly stable in 0.1 M sodium hydroxide, physiological phosphate buffer (pH 7.5) and ethanol/water (1:1) solutions. The degradation rate constants and degradation half-lives were in the ranges 0.0023–0.0086 h^{-1} and 80.6–150.6 h, respectively. Analysis of the acid-induced degraded solution of E118 by liquid chromatography–mass spectrometry (LC–MS) revealed at least two degradation products of E118 at m/z 213.1 and 113.1, respectively.

Keywords: Enaminone; E118; HPLC; LC/MS

1. Introduction

Enaminones [1] have been recently recognized by several co-workers as compounds of interesting pharmacological activities [2,3]. Accordingly, enaminones have been reported to possess cardiovascular [4] anti-inflammatory [5], histaminergic [6], antimalarial [7] and anticonvulsant activities [8–10]. An overview of the structural characterization and stability of enaminones using UV spectrometry was reported [9,11–13]. As the described method lacked specificity, it was not possible to determine the degradation kinetics of the examined enaminones. Recently, the kinetic parameters for the degradation of an enaminone (E139) were

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first established by the authors using a stability-indicating high-performance liquid chromatography (HPLC) assay [14]. Preliminary studies proved a promising antiepileptic activity of E118 and encouraged the conduction of pharmacokinetic studies to evaluate the absorption, metabolic and excretion behaviors of E118 in animals. However, before running the in vivo studies, it is necessary to elucidate the in vitro stability of E118 under simulated physiological conditions. To achieve this goal, a stability-indicating HPLC assay was proposed. This presentation describes the utility of HPLC in an accelerated-stability study to determine the degradation kinetics of E118 under simulated gastric (pH ~ 1) and intestinal (pH ~ 7.5) conditions at different temperatures. Furthermore, the paper reports on the potential of liquid chromatography–mass spectrometry technique (LC/MS) in elucidating the chemical structure of the degradation products.

2. Experimental

2.1. Chemicals and reagents

The examined enaminone (E118) 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).

2.2. 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, 5μ column (ChromTech, Sweden). The mobile phase was prepared by dissolving 4.16 g 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 (pH ~ 7.5). The filtered and degassed mobile phase was pumped at a flow rate 1 ml min^{-1}. The injection volume was 10 μl and the eluents were monitored at 225 nm. Analytical data such as retention time and peak area measurements were processed by the instrument in-built Millennium software.

Liquid chromatography–mass spectrometry analyses were performed using LC–MS system, which was comprised of LC pump (Spectra System P 2000, USA) and MS detector (Finnigan MAT, USA) with APCI as an ionization source. The APCI conditions were, vaporization temperature, 430 °C; sheath gas flow, 60 ml min^{-1}; discharge current, 5 μA; corona discharge, 4.38 kV and capillary temperature 150 °C. The mass spectrometer was programmed to detect the positive molecular and fragment ions of E118 in the range of m/z 100–300. Samples were directly injected into the MS detector using a 20 μl loop size. Elution of E118 was achieved using a mobile phase consisting of acetonitrile and 20 mM ammonium acetate solution in a ratio 4:1 at a flow rate of 1 ml min^{-1}. Analytical data were processed by the in-built LCQ software of the instrument.

2.3. Procedures

2.3.1. Synthesis of 3-(4′-chlorophenyl)aminocyclohex-2-en-1-one (E118)

To a solution of cyclohexane-1,3-dione (4.26 g, 40 mmol) in absolute ethanol (80 ml), was added 4-chloroaniline (5.2 g, 40 mmol) and refluxed for 2 h. The reaction mixture was evaporated and the crude product was crystallized from ethyl acetate–ethanol 95% to obtain E118 (m.p. 190–190.5 °C). The yield was 5.93 g (67%). ^{1}H-NMR analysis of E118 in $CDCl_3$ gave δ values of 2.49 (t, $J = 6.25$ Hz, 2H, CH_2), 2.04 (quintet, $J = 6.25$ Hz, 2H, CH_2), 2.36 (t, $J = 6.25$ Hz, 2H, CH_2), 5.52 (s, 1H, =CH–), 6.46 (s, 1H, NH), 7.06–7.40 (m, 4H, phenyl). Elemental analysis ($C_{12}H_{12}$

NOCl) gave, C, 64.87; H, 5.54; N, 6.21; Cl, 15.99% (theoretical: C, 65.01; H, 5.42; N, 6.32; Cl, 16.03%).

2.3.2. Stock solution of E118

An accurate weight of E118 powder (10 mg) was transferred into a 10-ml volumetric flask, dissolved and diluted to volume with ethanol (1 mg ml^{-1}). The solution was freshly prepared for each study.

2.3.3. Calibration curves using HPLC

Aliquots of 10–100 μl of the stock solution were transferred into 1-ml vials and diluted to 1 ml with ethanol. An accurate volume (10 μl) of each solution was injected into HPLC using autosampler and analyzed under the described chromatographic conditions. The peak area (PA) of E118 was automatically calculated by the instrument software. Calibration curves were constructed by plotting PA values versus concentrations of E118.

2.3.4. Accelerated stability studies

Aliquots of 100 μl of the prepared solution of E118 in ethanol (1 mg ml^{-1}) were 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). The vials were placed in a thermostatically controlled water bath at 30 or 40 or 60 °C for the appropriate periods of time and an accurate volume (10 μl) of each sample was injected into HPLC.

2.3.5. Calculation of the degradation kinetics

The logarithmic values of percentages of the remaining concentrations of E118 (R_t ~3.3 min) at zero- and at different time-intervals were used to establish the degradation plots of E118 in 0.1 M HCl solution, 0.1 M NaOH solution, phosphate buffer solution (pH 7.5), and ethanol/water (1:1) solution, respectively. The degradation kinetic parameters such as the degradation rate constant (K_{deg}) and degradation half-life ($t_{1/2}$) at 30, 40 and 60 °C were derived from the plots. The predicted kinetics for the degradation of E118 at 25 °C were extrapolated from Arrhenius plots.

2.3.6. Preparation of the acid-induced degradation products

In 10-ml tube, an aliquot of 1 ml solution of E118 in acetonitrile (~10 μg ml^{-1}) was mixed with few drops of 1 M HCl solution and heated in a water bath at 60 °C for 1 h. After cooling and neutralizing the solution, ~10 μl aliquot was injected into LC/MS spectrometer and analyzed using MS and MS/MS-SIM scanning modes.

3. Results and discussion

3.1. Chemistry and anticonvulsant activity of E118

The chemical nomenclature of the examined enaminone compound is 3-(4′-chlorophenyl) aminocyclohex-2-en-1-one (Scheme 1). The designated number for the compound was E118. Spectroscopic analysis including ultraviolet, nuclear magnetic resonance spectra, and elemental (C, H, N, O, Cl) analyses supported the assigned structure [10,11].1 The prepared compound has a melting point of 190–191.5 °C and the molecular mass ion m/z 222.3. The CLOGP value [3] for E118 was 2.938. The enaminone (E118) was a class 1 anticonvulsant agent in the maximal electroshock (MES) test [10]. In phase 1 test, E118 had an anticonvulsant activity at a dose of 100 mg kg^{-1} given (I.P) and protected mice from electrically-induced seizure.

3.2. Development and validation of HPLC method

HPLC separation of E118 and its degradation products was achieved using a chiral HSA column and a mobile phase (pH 7.5) containing n-octanoic acid (5 mM), isopropyl alcohol and 100 mM disodium hydrogen phosphate solution (1:9 v/v) at a flow rate of 1 ml min^{-1}. The chromatographic conditions were previously used for evaluating the stability of E139 [14]. The chromatograms exhibited well-resolved peaks at retention times < 5 min for the enaminone E118 and the degradation products (Fig. 1). These peaks were monitored at 225 nm using UV detec-

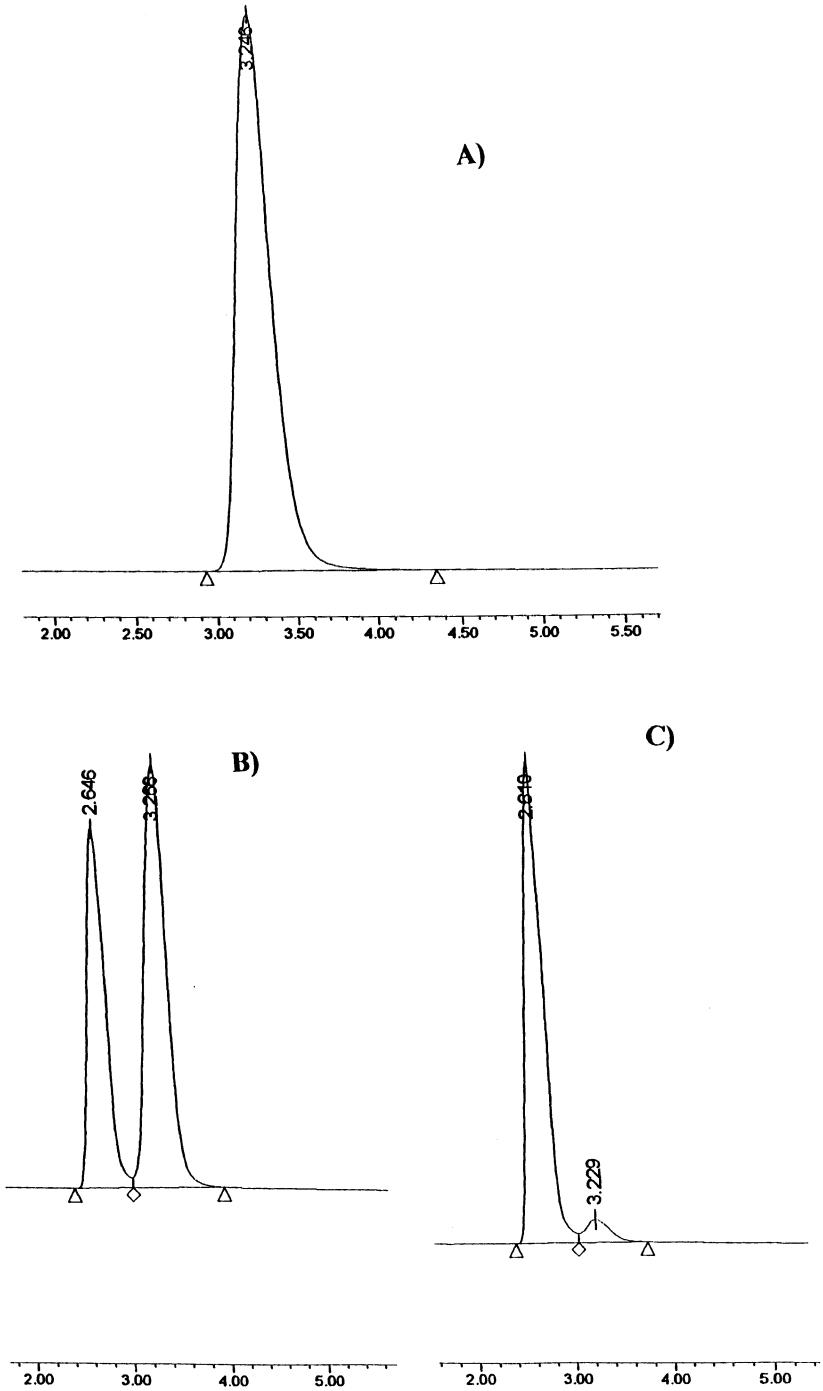


Fig. 1. HPLC chromatograms of the acid hydrolysis of E118 at 30 °C at (A) zero time; (B) 10 min; and (C) 50 min.

tion. Linear correlation between the PA and various concentrations of E118 in the range10–100 μg ml^{-1} was obtained. Using least square regression, the linear equation was, $PA \times 10^{-5} = 2.46(\pm 0.09) + 1.75\ (\pm 0.05)\ C$ ($r = 0.9971 \pm 0.0015$), using three determinations. The R.S.D. values for the intercept, slope and regression coefficient were, 3.66, 2.80 and 0.15%, respectively. The limit of detection was 2 μg ml^{-1}. The reproducibility and accuracy of HPLC method were evaluated by determining R.S.D.% and % deviation from the mean (%Dev.) of samples containing E118 at concentration 100 μg ml^{-1}, as in stability studies. The R.S.D. and %Dev. were 2.64 and 3.95%, respectively.

3.3. Stability study by HPLC

The HPLC chromatograms of E118 in 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and physiological phosphate buffer (pH 7.5) solutions, at 30, 40, and 60 °C gave the reference retention times and peak areas of E118 and the degradation products. The remaining concentrations following degradation were determined from the linear regression equation. Fig. 1A–C show the HPLC chromatograms of E118 in 0.1 M hydrochloric acid solution at 30 °C. The examined enaminone E118 and the degradation products were well separated at retention times 3.27 and 2.65 min, respectively (Fig. 1B). As the degradation process was fast, the peak area of the predominant peak of E118 was rapidly diminishing while the putative product was progressively increasing (Fig. 1C). After 60 min of the acid hydrolysis, the degradation product became predominant at approximately 94%. Since enaminones undergo protonation [1] in acid medium, the degradation species would be protonated and would be appeared at shorter retention time. On the contrary, E118 concentrations did not change appreciably even when heated at 60 °C for 3 h in 0.1 M sodium hydroxide solution or physiological phosphate buffer solution or aqueous solution of water/ethanol (1:1). This inference was drawn from the insignificant changes of the peak areas and absence of the degradation peaks in the chromatograms. Plotting of the logarithmic values of the remaining concentrations of E118, expressed as percentages, versus time indicated that the hydrolysis of E118 followed first-order kinetics (Fig. 2) which was distinctly catalyzed by the

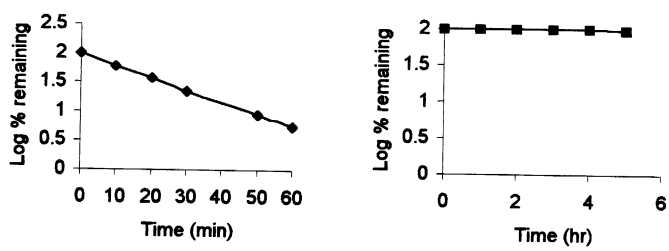


Fig. 2. First-order plots of E118 at 30 °C in (◆) 0.1 M HCl solution; (■) phosphate buffer solution (pH 7.5).

Table 1
Kinetic parameters for the degradation of E118 in 0.1 M HCl solution and phosphate buffer solution (pH ~7.5) at 30, 40 and 60 °C, as derived from first-order plots

Temperature (°C)	0.1 M HCl		Phosphate buffer (pH ~7.5)	
	K_{deg} (min^{-1})	$t_{1/2}$ (min)	K_{deg} (h^{-1})	$t_{1/2}$ (h)
30	0.048	14.40	0.003	231.0
40	0.113	6.10	0.005	138.6
60	0.142	4.90	0.013	53.3

Table 2
Arrhenius plots of E118 in different solvents

Solvent	Arrhenius plot
0.1 M HCl	$\log K_{deg} = 3.447–1416.4/T$ ($r = 0.9712$)
0.1 M NaOH	$\log K_{deg} = 1.81–1155.7/T$ ($r = 0.9527$)
Phosphate buffer (pH ~7.5)	$\log K_{deg} = 4.46–2116.4/T$ ($r = 0.9988$)
Ethanol/water (1:1)	$\log K_{deg} = 1.88–1257.1/T$ ($r = 0.9769$)

Table 3
Summary of the predicted K_{deg}, $t_{1/2}$, t_{90} of E118 in various solvents as derived from Arrhenius plots at 25 °C

Solvent	K_{deg}	$t_{1/2}$	t_{90}
0.1 M HCl	0.0490 min^{-1}	14.10 min	2.14 min
0.1 M NaOH	0.0086 h^{-1}	80.60 h	12.20 h
Phosphate buffer (pH 7.5)	0.0023 h^{-1}	301.30 h	45.65 h
Ethanol/water 1:1	0.0046 h^{-1}	150.6 h	22.83 h

presence of acid and was significantly enhanced by elevation of temperature. Table 1 displays the calculated kinetic parameters of E118 in 0.1 M hydrochloric acid and phosphate buffer (pH 7.5) solutions. The data showed higher values of the degradation rate constants with shorter half-lives in 0.1 M HCl solution compared with physiological phosphate buffer solution. Furthermore, the degradation was progressively enhanced by increase of temperature.

To determine the degradation kinetics of E118 at 25 °C, Arrhenius plots [15] were constructed by plotting the logarithmic values of the observed K_{deg} values, computed from the degradation plots at different temperatures, versus $1/T$. Using least squares regression, linear relationships with correlation coefficients ($r = 0.9527–0.9988$) were obtained (Table 2). As derived from Arrhenius plots, the degradation rate constant (K_{deg}), half-life ($t_{1/2}$) and shelf -life (t_{90}) for the acid-catalyzed hydrolysis of E118 were 0.049 min^{-1}, 14.1 and 2.14 min, respectively, whereas in physiological phosphate buffer solution, the values of K_{deg}, $t_{1/2}$ and t_{90} were 0.0023 h^{-1}, 301.3 and 45.65 h, respectively (Table 3). As concluded from Table 3, the calculated kinetic parameters proved that E118 was unstable in strongly acidic medium (simulated gastric fluid), but highly stable at physiological phosphate buffer solution (simulated intestinal fluid).

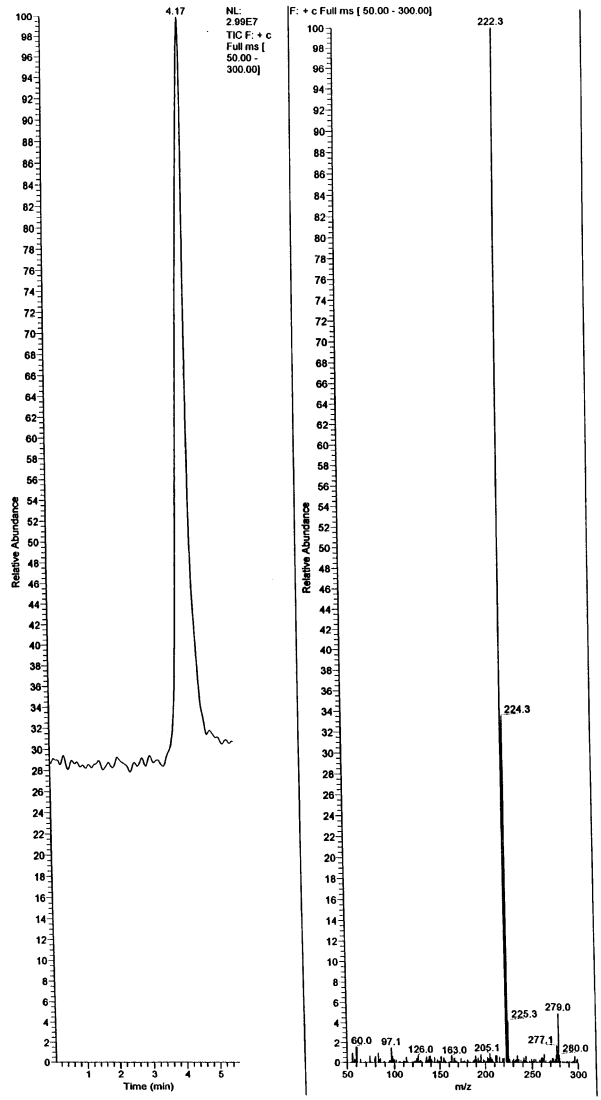


Fig. 3. LC–MS spectrum of E118.

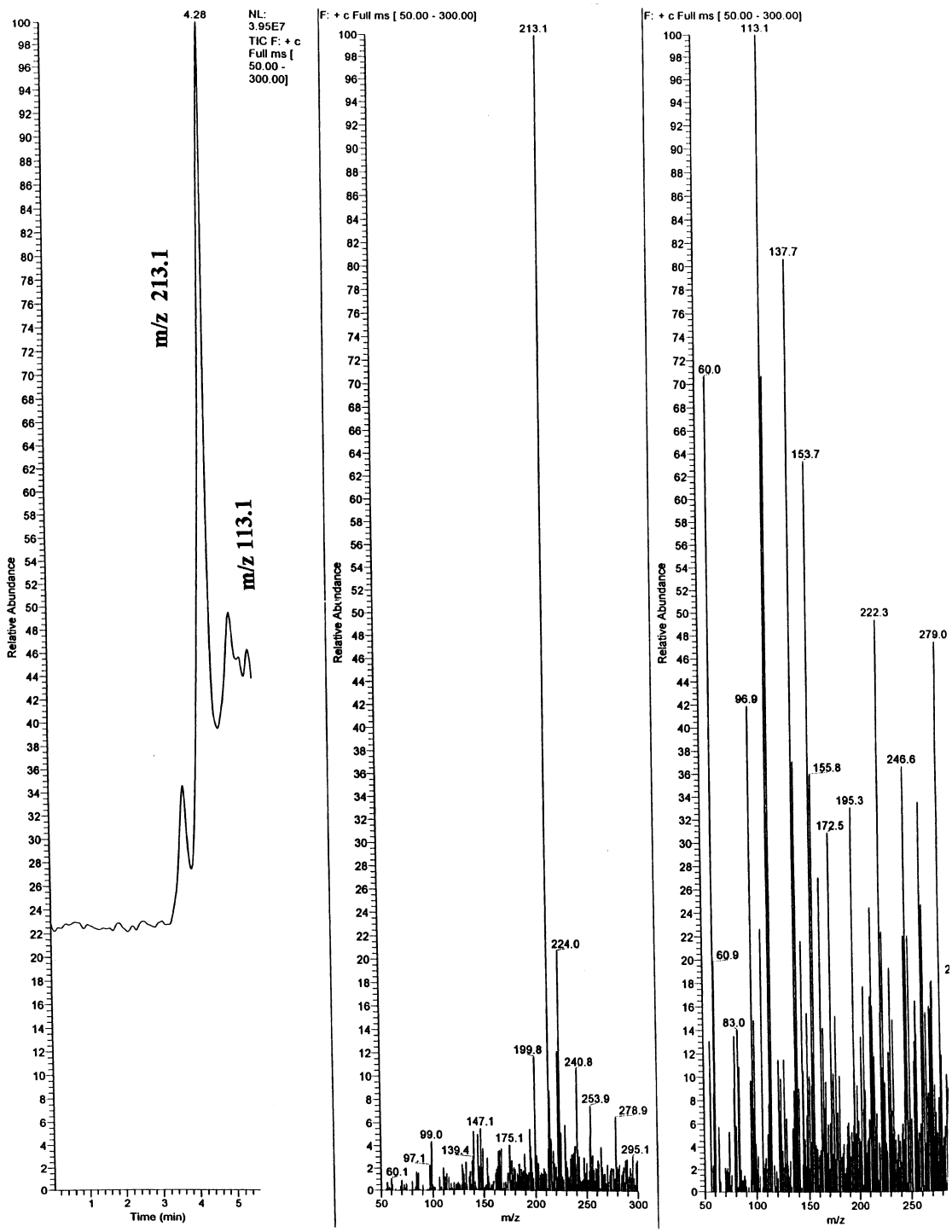


Fig. 4. LC–MS spectrum of the acid-induced degradation products of E118.

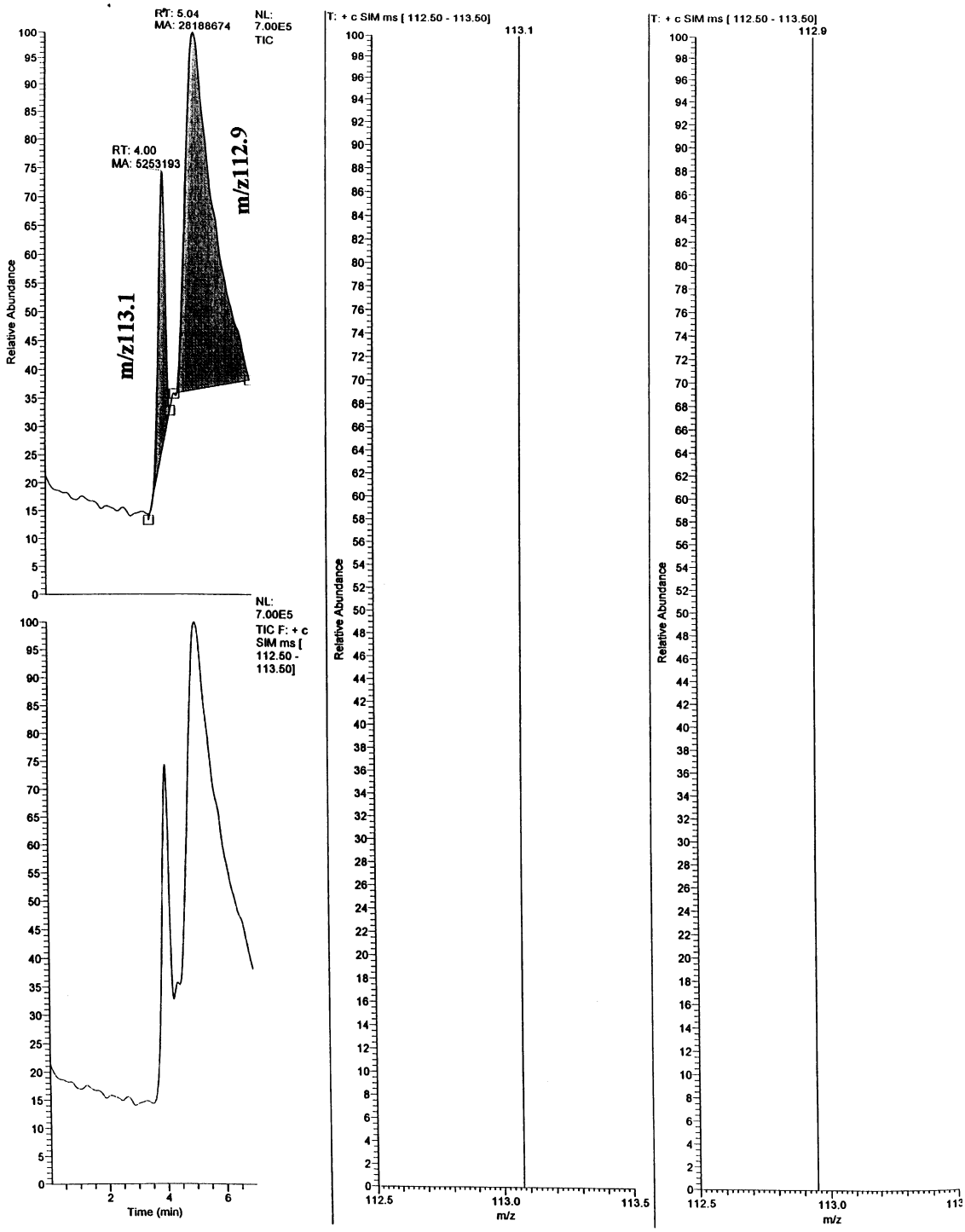
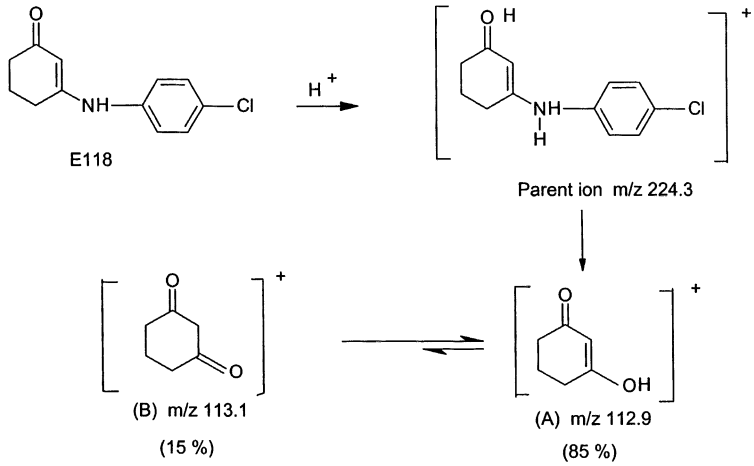


Fig. 5. LC/MS-SIM spectrum of the acid-induced degradation product of E118.



Scheme 1. Proposed degradation pathway of E118 according to LC/MS analysis.

3.4. Identification of the acid-induced degradation products of E118 by LC–MS

Although, the degradation of E118 in 0.1 M hydrochloric acid solution was proven using kinetic studies, however, it was not possible to identify the chemical structure of the degradation products by HPLC. For this purpose, LC/MS was selected. The full scan LC/MS spectra of E118 and its acid-induced degradation product were measured in the mass range m/z 100–300. E118 displayed a single peak at 4.17 min, which corresponds to the molecular mass ion m/z 222.3 (Fig. 3). The TIC chromatogram of the degraded solution of E118 showed at least three peaks at 4.0 (weak), 4.28 (strong) and 4.95 min (broad and splitted). These peaks referred to the residual E118, degradation product I (m/z 213.1) and degradation product II (m/z 113.1), respectively (Fig. 4). Based on LC/MS data, the chemical structure of the degradation product I, could not be identified. However, LC/MS-SIM analysis was used to identify the degradation product II at m/z 113.1. As shown in Fig. 5, the peaks at 4.00 and 5.04 min indicated that the degradation product II was a mixture of two compounds of almost identical molecular masses (m/z 112.9 and 113.1). The structures A and B would satisfy the tautomers of cyclohexane-1,3-dione as a degradation product (Scheme 1). Due to stabilization of the tautomer A by intermolecular and intramolecular hydrogen bonding, it would be expected to be predominant over tautomer B. Based on the previous data, it was confirmed that E118 was hydrolyzed to at least two degradation products when heated in hydrochloric acid solution.

4. Conclusions

The stability of the new enaminone (E118) is investigated using a stability-indicating HPLC procedure. This method permits detection and quantitation of E118 in the presence of its degradation products. The kinetic studies indicate that E118 undergoes fast degradation by hydrolysis in 0.1 M hydrochloric acid solution, whereas in physiological phosphate buffer solution (pH $\sim$ 7.5), E118 was highly resistant to hydrolysis. The rate of hydrolysis in acid medium followed first-order kinetics and was enhanced by elevation of temperature. LC/MS analyses of the acid-induced degraded solution of E118 indicated at least two degradation products of E118 at m/z 213.1 and 113.1, respectively. The degradation product at m/z 113.1 was identified as a mixture of tautomers of cyclohexane-1,3 dione in a ratio 5:1. These results should be taken into consideration during pharmacokinetic studies in animals in which E118 is administered by oral route.

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