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High performance liquid chromatographic determination of etofibrate and its hydrolysis products

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Abstract

High performance liquid chromatographic (HPLC) method is presented for the determination of etofibrate (EF) and its hydrolysis products. The method was based on HPLC separation of EF from its hydrolysis products using cyanopropyl column at ambient temperature with mobile phase consisting of acetonitrile–10 mM potassium dihydrogen phosphate, pH was adjusted to 4.1 using phosphoric acid (50:50, v/v). Quantitation was achieved with UV detection at 221 nm based on peak area. The flow rate was 1.5 ml min^{-1}. The proposed method was used to investigate the kinetics of acidic hydrolysis process of EF at different temperatures and the apparent pseudo first-order rate constant, half-life and activation energy were calculated. The kinetics of alkaline hydrolysis process of EF using 0.01 M sodium hydroxide at different temperatures cannot be studied as the drug is rapidly hydrolyzed in alkaline medium. The pH-rate profile of hydrolysis of EF in Britton–Robinson buffer solutions within the pH range 2–10 were studied.

Keywords: Etofibrate; HPLC; Kinetics of hydrolysis; pH-rate profile

1. Introduction

Etofibrate (EF), 2-(*p*-chlorophenoxy)-2-methylpropionic acid 2-(nicotinoyl-oxy)ethyl ester, a derivative of clofibrate and nicotinic acid, is a lipid regulating drug used in the treatment of hyperlipidaemias [1]. The literature survey reveals that EF was analyzed in environmental water samples using solid phase extraction followed by GC–MS or HPLC electrospray ionization MS–MS [2].

EF is an ester type drug susceptible to hydrolysis. However, no method has been reported for determination of EF in presence of its hydrolysis product. Therefore, it is necessary to study the stability of this drug towards acidic and alkaline hydrolysis process. The aim of this work was to develop simple stability indicating method for the determination of EF and its hydrolysis products using HPLC. The developed HPLC method was used to investigate the kinetics of the acidic hydrolytic process and to calculate the activation energy for the drug hydrolysis. The proposed HPLC method was used for pH-rate profile study of hydrolysis of EF in Britton–Robinson buffer solutions.

2. Experimental

2.1. Instrumentation

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12A Degasser, Rheodyne 7725i injector with a 20 μl loop and a SPD-10AVP UV–vis detector, separation and quantitation were made on a 250 mm × 4.6 mm (i.d.) Shim-pack cyanopropyl column (5 μm particle size). The detector was set at λ = 221 nm. Data acquisition were performed on class-VP software.

The IR spectrophotometer used was a Bruker Vector 22, Germany.

PMR spectra were recorded on a Varian Gemini 200 PMR spectrometer (200 mHz), USA.

Chromatoplates (20 cm × 20 cm, aluminium plates precoated with silica gel 60 F_{254}) were purchased from E. Merck (Darmstadt, Germany) and used in TLC studies with mobile phase

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consisted of ethyl acetate–methanol (70:30, v/v) for separation of EF and its hydrolysis products.

The spots were examined at 254 nm using UV source for TLC (Ultra Violet lamp, portable, UV-254/365 nm, model UVGL-25, USA) in CHROMATO-VUE cabinet, model CC-10, Upland, CA, USA.

2.2. Materials and reagents

Pharmaceutical grade of EF was kindly supplied by pharco pharmaceuticals, Egypt and certified to contain 99.98%. Acetonitrile used was HPLC grade (Scharlau, Barcelona, Spain), ethyl acetate used was HPLC grade (BDH, Poole, UK), methanol, and chloroform used were HPLC grade (Riedel-de Haën Laboratory Chemicals, Germany).

Sodium hydroxide, potassium dihydrogen phosphate, boric (Sigma–Aldrich, Inc., St. Louis, USA), hydrochloric (Merck, Darmstadt, Germany), phosphoric (BDH Laboratory Supplies Poole, England), and acetic (Riedel-de Haën Laboratory Chemicals, Germany) acids were analytical grade.

About 10 mM KH_2PO_4 was prepared by dissolving 1.36 g of KH_2PO_4 in 1 l of distilled water.

The commercial Lipo-Merz retard capsules (Batch No. 147) used was manufactured by pharco pharmaceuticals, Egypt under license of Merz + Co.GmbH & Co., D-6000 Frankfurt/M.1, Germany, labeled to contain 500 mg EF per capsule.

2.3. HPLC conditions

The mobile phases were prepared by mixing acetonitrile–10 mM potassium dihydrogen phosphate, pH was adjusted to 4.1 using phosphoric acid (50:50, v/v). The flow rate was 1.5 ml min^{-1}. All determinations were performed at room temperature. The injection volume was 20 μl.

2.4. Preparation of the alkali-induced hydrolysis product

One gram of EF was firstly dissolved in 10 ml methanol and refluxed with 100 ml 0.01 M sodium hydroxide at 100 °C for 30 min. Subsequently, the solvent was evaporated, the residue was dried under vacuum and protected from air and light.

TLC of the dried precipitate indicated the occurrence of two components, namely 2-(*p*-chlorophenoxy)-2-methyl-propionic acid (clofibric acid) (CA) with $R_f = 0.63$ and 3-pyridinecarboxylic acid (nicotinic acid) (NA) with $R_f = 0.34$ using the above discussed TLC system.

Glass column (35 cm × 3 cm) packed with silica gel 60 (0.063–0.200 mm particle size) (E. Merck, Darmstadt, Germany) and mobile phase consisted of ethyl acetate to elute CA, then ethyl acetate:methanol (80:20, v/v) to elute NA. All fractions were monitored using TLC system. Each of the two components were separated from the mobile phase by vacuum distillation, further purification of the obtained two hydrolysis products (CA and NA) were carried out using glass column (35 cm × 3 cm) packed with sephadex LH-20 (Sigma–Aldrich, Inc., St. Louis, USA) and mobile phase consisted of methanol:chloroform (1:1) for CA, or methanol for NA.

2.5. Preparation of the acid-induced hydrolysis product

One gram of EF was firstly dissolved in 10 ml methanol and refluxed with 100 ml 0.1 M hydrochloric acid at 100 °C for 12 h. Subsequently, the solvent was evaporated, the residue was dried under vacuum and protected from air and light.

TLC of the dried precipitate indicated the occurrence of two components, namely CA and NA using the above discussed TLC system. Separation of CA and NA was done using the same procedures described previously under preparation of the alkali-induced hydrolysis product.

2.6. Standard solutions and calibration graphs

Stock solutions were prepared by separately dissolving EF, CA, and NA in methanol to obtain concentration of 1000 μg ml^{-1} for each compound. The standard solutions were prepared by dilution of the stock solutions with the HPLC mobile phase to reach concentration ranges of 1–30 μg ml^{-1} for EF, CA, and NA. Triplicate 20 μl injections were made for each concentration and chromatographed under the specified HPLC conditions described previously. The peak area of each concentration was plotted against the corresponding concentrations to obtain the calibration graph. Linear relationship was obtained for each compound (Table 1).

2.7. Sample preparation

The contents of 20 Lipo-Merz retard® capsules were weighed and finely powdered. Accurately weighed portion of the powder equivalent to about 50 mg of EF was extracted and diluted to 50 ml with methanol. The sample solution was filtered, the filterate was diluted with HPLC mobile phase to reach concentration range of (1–30 μg ml^{-1}) for EF.

2.8. Kinetic investigation

Accurately weighed 75 mg of EF was dissolved in 100 ml methanol. Separate 2 ml aliquots of this solution were transferred into separate stoppered conical flasks and mixed with 2 ml of 0.2 M hydrochloric acid. The flasks were placed in a thermostatic oven at different temperatures (60, 70, 80, 90, and 95 °C) for different time intervals. At the specified time the contents of the flasks were adjusted to pH 7.0 using predetermined volumes of 0.1 M sodium hydroxide solution. The contents of the flasks were transferred into 50 ml volumetric flasks and diluted to volume with HPLC mobile phase. Aliquots of 20 μl of each solution were chromatographed under the HPLC conditions described above and the concentrations of the remaining EF were calculated at each temperature and time interval.

The hydrolysis of EF in 0.01 M NaOH was studied and found to be extremely labile to alkaline hydrolysis.

Table 1
Characteristic parameters for the regression equations of the proposed HPLC method for determination of EF, CA and NA

Parameters	EF	CA	NA
Calibration range ($\mu g\,ml^{-1}$)	1–30	1–30	1–30
Detection limit ($\mu g\,ml^{-1}$)	5.35×10^{-3}	6.95×10^{-3}	7.3×10^{-3}
Quantitation limit ($\mu g\,ml^{-1}$)	1.78×10^{-2}	2.32×10^{-2}	2.43×10^{-2}
Regression equation (Y)[a]			
Slope (b)	4.02×10^{4}	3.11×10^{4}	4.15×10^{4}
Standard deviation of the slope (S_b)	0.92×10^{2}	0.92×10^{2}	1.29×10^{2}
Relative standard deviation of the slope (%)	0.23	0.30	0.31
Confidence limit of the slope[b]	3.96×10^{4} to 4.09×10^{4}	3.04×10^{4} to 3.17×10^{4}	4.06×10^{4} to 4.24×10^{4}
Intercept (a)	8.45×10^{3}	-7.84×10^{3}	4.56×10^{3}
Standard deviation of the intercept (S_a)	1.67×10^{3}	1.64×10^{3}	2.21×10^{3}
Confidence limit of the intercept[b]	-0.29×10^{4} to 1.98×10^{4}	-1.90×10^{4} to 3.30×10^{3}	-1.05×10^{4} to 1.96×10^{4}
Correlation coefficient (r)	0.9999	0.9999	0.9999
Standard error of estimation	7.19×10^{3}	7.09×10^{3}	10.116×10^{3}

[a] $Y = a + bC$, where C is the concentration of EF, CA and NA in $\mu g\,ml^{-1}$ and Y is the peak area.
[b] 95% confidence limit.

2.9. pH-rate profile

Accurately weighed 150 mg of EF was transferred into 100 ml volumetric flask, dissolved and diluted to volume with methanol. Separate 1 ml aliquots of EF solution and 2 ml of the Britton–Robinson buffer solutions were transferred into stoppered conical flasks. The pH values of Britton–Robinson buffer solutions [3] used for the measurement of the pH-rate profile of the hydrolysis of EF were as follows: pH 2, 2.8, 3.6, 4.4, 5.2, 6, 6.8, 8, 9 and 10. The pH values of these buffer solutions were checked before and after the reaction, and were unchanged. The flasks were placed in a thermostatic oven at 75 °C for different time intervals. At the specified time interval the contents of the flasks were adjusted to pH 7.0 using 1 M sodium hydroxide or 1 M hydrochloric acid solutions. The contents of the flasks were transferred into 50 ml volumetric flasks and diluted to volume with HPLC mobile phase. Aliquots of 20 μl of each solution were chromatographed under the HPLC conditions described above and the concentrations of the remaining EF were calculated at each pH value and time interval.

3. Results and discussion

3.1. Identification of the hydrolysis products

When EF was firstly dissolved in 10 ml methanol and refluxed with 100 ml 0.01 M NaOH for 30 min or 0.1 M hydrochloric acid at 100 °C for 12 h, CA and NA were formed.

The assignments of the hydrolysis products CA and NA, were based on the comparison of the IR and PMR spectral data of the purified specimens, separated from the hydrolysis reaction, with those of the intact EF.

However, during the beginning of the hydrolysis in alkaline or acidic condition two peaks appear in the HPLC chromatogram (Fig. 1) in addition to the peaks of CA and NA. These two peaks disappear rapidly. So, there isolation from the medium was found to be extremely difficult.

These two peaks suggested to be CE and NE as 2-hydroxyethyl 2-(*p*-chlorophenoxy)-2-methylpropanoate and 2-hydroxyethyl nicotinate, respectively, as represented in Scheme 1.

The PMR spectrum of EF in dimethylsulphoxide was characterized by the appearance of the protons of the ethyl chain at δ 4.454–4.534 ppm (singlet, 4H, O–CH_2–CH_2–O), 2-methylpropianoate chain at δ 1.508 ppm (singlet, 6H, CH_3–C(CH_3)–COO), benzene ring at δ 6.786–7.187 ppm; and pyridine ring at δ 7.538–9.001 ppm.

By contrast the PMR spectrum of CA in the same solvent lacked the characteristic protons signals of ethyl chain, pyridine ring and showed protons signals of 2-methylpropianoate chain at δ 1.501 ppm (singlet, 6H, CH_3–C(CH_3)–COO) and benzene ring at δ 6.766–7.323 ppm.

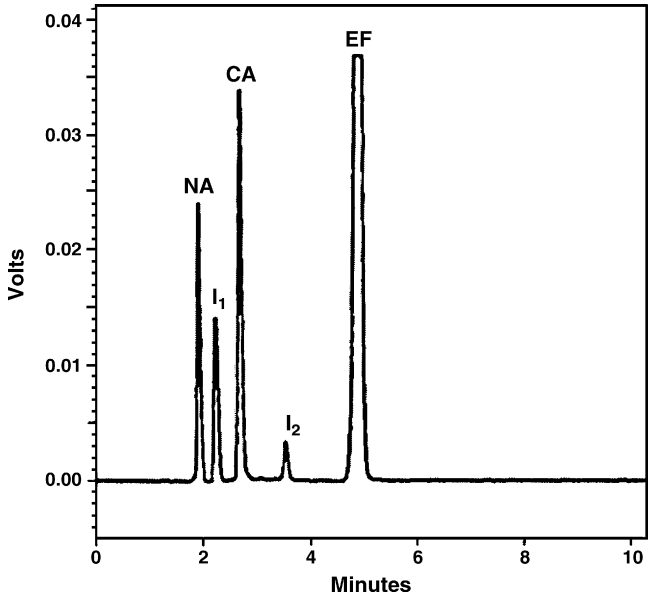
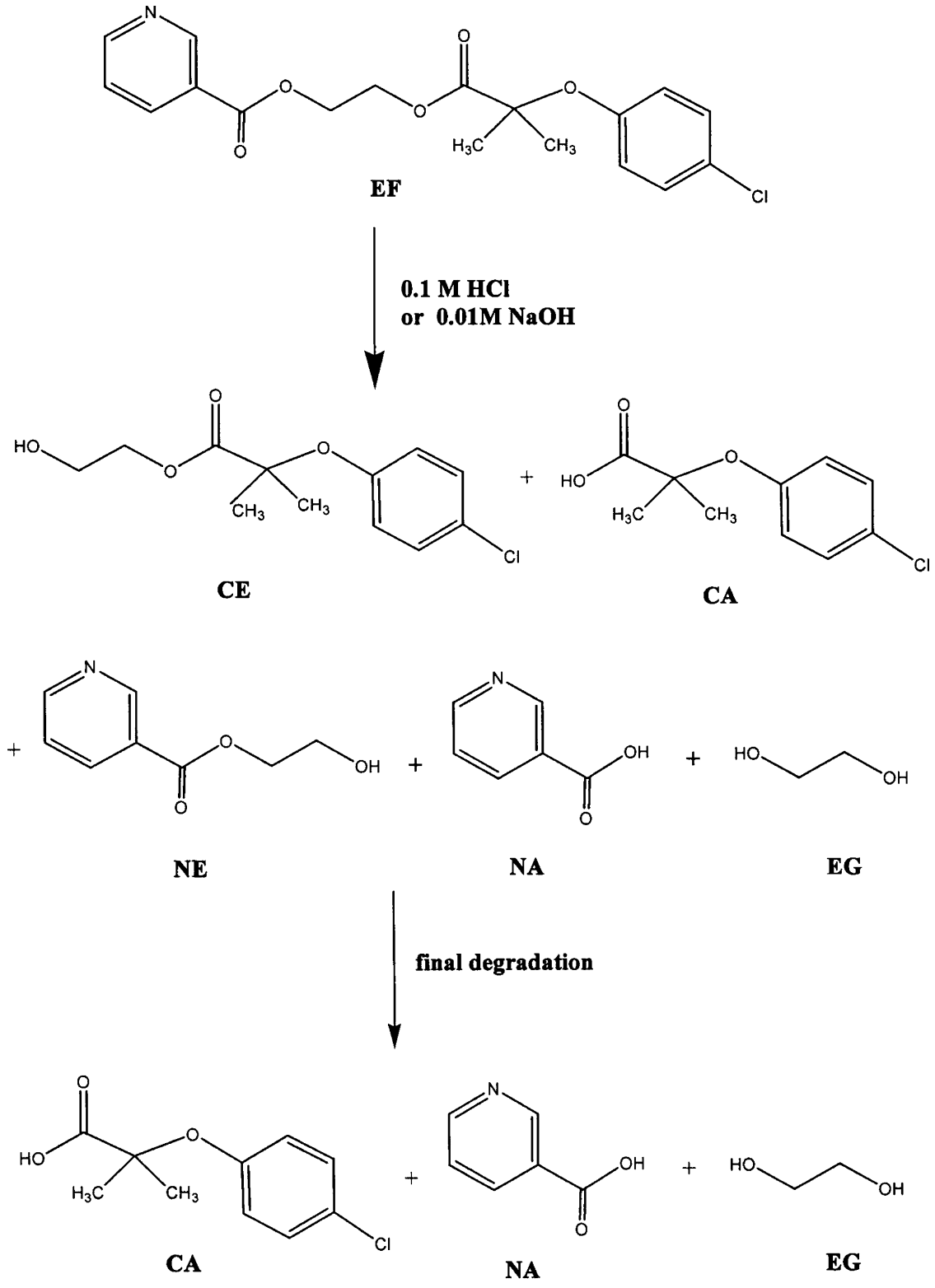


Fig. 1. HPLC chromatogram of 20 μl injection containing EF and its hydrolysis products NA, I_1 (intermediate 1), CA and I_2 (intermediate 2).



Scheme 1. Suggested pathway for the hydrolysis of EF in 0.01 M NaOH or 0.1 M hydrochloric acid.

Also, the PMR spectrum of NA in the same solvent lacked the characteristic protons signals of ethyl chain, 2-methylpropianoate chain and benzene ring and showed protons signals of pyridine ring at δ 7.518–9.067 ppm.

The IR spectrum (KBr) of EF characterized by the absorption frequency of two C=O ester band at 1716 and at 1741 cm^{-1}. By contrast, the IR spectrum (KBr) of CA revealed the OH association, C=O stretching and in-plane C–O bending of the COOH

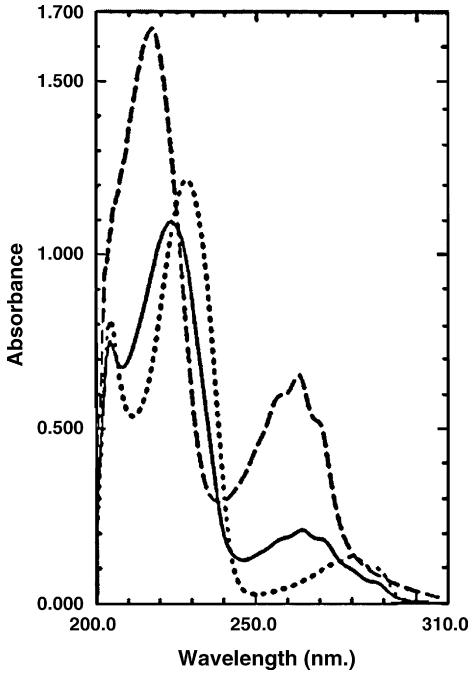


Fig. 2. UV absorption spectra of 20 μg ml^{-1} of EF (—), 20 μg ml^{-1} of CA (· · · ·), 20 μg ml^{-1} of NA (- - - -) in methanol.

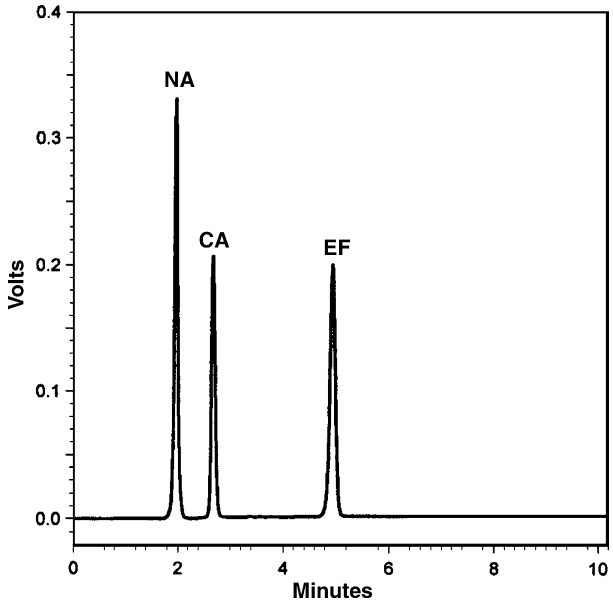


Fig. 3. HPLC chromatogram of 20 μl injection of laboratory-prepared mixture of 30 μg ml^{-1} of EF, 30 μg ml^{-1} of CA, 30 μg ml^{-1} of NA.

at 2572–3080, 1707 and 1488 cm^{-1}, respectively. Moreover, the spectrum lacked the characteristic two ester C=O stretching band of EF.

While, the IR spectrum (KBr) of NA revealed the OH association, C=O stretching and in-plane C–O bending of the COOH at 2435–3070, 1709 and 1417 cm^{-1}, respectively. Moreover, the spectrum lacked the characteristic two ester C=O stretching band of EF.

3.2. Assay parameters

The UV absorption spectra of EF and its hydrolysis products (CA and NA) in methanol are overlapped (Fig. 2). The simultaneous determination of EF, CA and NA by conventional, derivative ratio spectrophotometry is hindered by strong spectral overlap through the wavelength range. The method used to resolve such compounds is mainly HPLC. To optimize the HPLC assay parameters, the effect of the mobile phase composition was studied. A satisfactory separation was obtained with a mobile phase consisting of acetonitrile–10 mM potassium dihydrogen phosphate, pH was adjusted to 4.1 using phosphoric acid (50:50, v/v) at ambient temperature. Increasing acetonitrile concentration to more than 65% led to inadequate separation between EF hydrolysis products. At lower acetonitrile concentration (<30%) separation occurred but with excessive tailing and increased retention time for EF. Variation of pH of the 10 mM potassium dihydrogen phosphate resulted in maximum capacity factor (K') value at pH 5; however, at pH 4.1 optimum resolution with reasonable retention time was observed. Quantitation was achieved with UV detection at 221 nm based on peak area. The specificity of the HPLC method is illustrated in (Figs. 1 and 3) where complete separation of EF and its hydrolysis products was noticed.

The average retention time ± S.D. for NA, I_1 (intermediate product 1), CA, I_2 (intermediate product 2), and EF were found to be 1.96 ± 0.008, 2.24 ± 0.012, 2.69 ± 0.011, 3.55 ± 0.007, and 4.91 ± 0.006 min, respectively, for 10 replicates. The system suitability test results of the developed method are presented in Table 2.

3.3. Analysis of pharmaceutical product

The proposed HPLC method was applied to the determination of EF in freshly prepared capsules. Seven replicates determinations were made. Satisfactory results were obtained for EF in a

Table 2
Chromatographic characteristics of NA, I_1 (intermediate product 1), CA, I_2 (intermediate product 2), and EF

Compound	Retention time (min)	Capacity factor (K')	Selectivity, α	Resolution, R_s	Tailing factor
NA	1.96	1.57	1.27 (a1)	1.87 (b1)	1.17
I_1	2.24	1.99	1.31 (a2)	2.27 (b2)	0.95
CA	2.69	2.6	1.43 (a3)	3.10 (b3)	1.05
I_2	3.55	3.73	1.49 (a4)	2.68 (b4)	1.14
EF	4.91	5.54			0.9

*a*1, *b*1: α and R_s calculated for NA–intermediate product 1 (I_1); *a*2, *b*2: α and R_s calculated for intermediate product 1 (I_1)–CA; *a*3, *b*3: α and R_s calculated for CA–intermediate product 2 (I_2); *a*4, *b*4: α and R_s calculated for intermediate product 2 (I_2)–EF.

Table 3
Determination of EF, CA and NA in laboratory-prepared mixtures and commercial pharmaceutical product using the proposed HPLC method

	Mean ± S.D.[a]		
	EF	CA	NA
Laboratory-prepared mixtures	100.45 ± 0.57	99.83 ± 0.58	100.48 ± 0.68
Lipo-Merz retard capsules	100.11 ± 0.63		
Recovery[b]	100.08 ± 0.65	100.02 ± 0.55	99.83 ± 0.69

[a] Mean ± S.D., percentage recovery from the label claim amount.
[b] For standard addition of different concentrations of EF, CA and NA.

good agreement with the label claims (Table 3). Typical chromatogram obtained for the quantitative analysis of the studied compounds in freshly prepared capsules was very similar to that presented in Fig. 1, except that the hydrolysis products could not be detected.

Expired batch of Lipo-Merz retard® capsules stored at ambient temperature under normal conditions was analyzed by the proposed HPLC method. The CA and NA as hydrolysis products of EF were found clearly (Fig. 4). The mean percentage of EF ± S.D. ($n = 7$) was found to be 89.44 ± 0.61% and the mean concentration ±S.D. ($n = 7$) of CA and NA were found to be 34.85 ± 0.28 and 33.60 ± 0.33 mg/capsule, respectively.

3.4. Kinetic investigation

The kinetic of acidic hydrolysis of EF was investigated in 0.1 M hydrochloric acid, while alkaline hydrolysis in 0.01 M sodium hydroxide was very rapid to obtain a reliable kinetic data. So, the kinetic investigation was carried in acidic medium only (0.1 M hydrochloric acid). A regular decrease in the concentration of intact EF with increasing time intervals was observed for the acidic hydrolysis process. At the selected temperatures (60, 70, 80, 90, and 95 °C), the hydrolytic processes followed pseudo first-order kinetics (Fig. 5). From the slopes of the straight lines it was possible to calculate the apparent first order hydrolysis rate constant and the half-life at each temperature for acidic hydrolysis processes (Table 4). Plotting log K_{obs} values versus $1/T$, the Arrhenius plot (Fig. 6) was obtained, which was found to be linear in the temperature range 60–95 °C for acidic hydrolysis of EF. The activation energy was calculated for EF and found to be 10.48 kcal mol^{-1} for acidic hydrolytic process.

The pH rate profile of hydrolysis of EF in Britton–Robinson buffer solutions was studied at 75 °C (Fig. 7). Britton–Robinson buffer solutions were used throughout the entire pH range in order to avoid possible effects of different buffer species. The apparent first order hydrolysis rate constant and the half-life

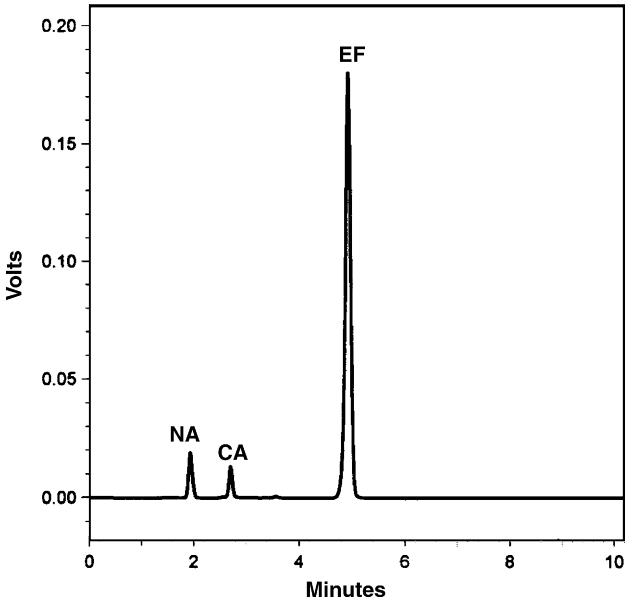


Fig. 4. HPLC chromatogram of expired EF capsule solution containing EF and its two hydrolysis products NA and CA.

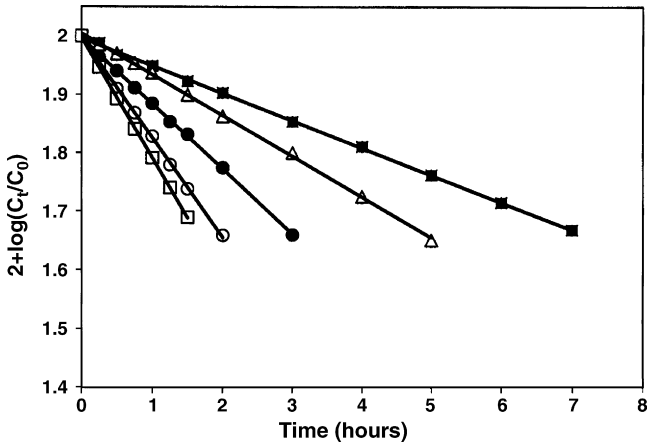


Fig. 5. Pseudo first-order plots for the hydrolysis of EF in 0.1 M hydrochloric acid at various temperatures using HPLC method. Key: 60 (■); 70(△); 80 (●); 90 (◯), and 95 (□), C_t, concentration at time t, and C_0, concentration at zero time.

Table 4
Hydrolysis rate constant (K_{obs}) and half-life ($t_{1/2}$) for EF in 0.1 M hydrochloric acid

Temperature (°C)	K_{obs} (h^{-1})	$t_{1/2}$ (h)
60	0.108	6.430
70	0.161	4.311
80	0.257	2.696
90	0.394	1.758
95	0.475	1.459

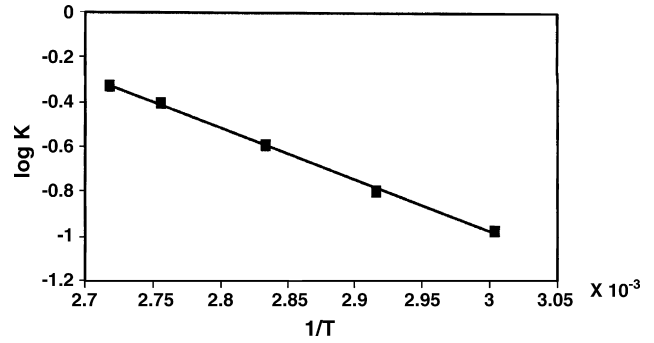


Fig. 6. Arrhenius plot for the hydrolysis of EF in 0.1 M hydrochloric acid.

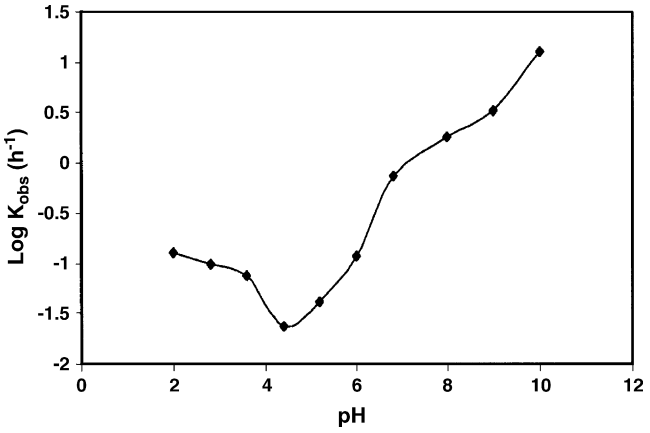


Fig. 7. pH-rate profile for the hydrolysis of EF in Britton–Robinson buffer at 75 °C.

were calculated for each pH value (Table 5). EF was found to be most stable at pH of 4.4.

3.5. Validation of the method

3.5.1. Linearity

The linearity of the HPLC detector response for determination of EF, CA and NA was evaluated by analyzing a series of different concentrations of each compound. In this study seven concentrations were chosen, ranging between 1 and 30 μg ml^{-1} for EF, CA and NA. Each concentration was repeated three times, this approach will provide information on the variation in peak area between samples of same concentration. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value that was not statistically ($P < 0.05$) different from zero. Characteristic parameters for regression equations obtained by least squares treatment of the results are given in Table 1.

3.5.2. Precision

For evaluation of the precision estimates, repeatability and intermediate precision were performed at three concentration levels for EF, CA and NA. The data for each concentration level were evaluated by one-way ANOVA. An 8 days × two replicate design was performed. Statistical comparison of the results was performed using the *P*-value of the *F*-test. Three univariate analyses of variance for each concentration level were made. Since the *P*-value of the *F*-test is always greater than 0.05, there is no statistically significant difference between the mean results obtained from one level of day to another at the 95% confidence level.

Table 5
Hydrolysis rate constant (K_{obs}) and half-life ($t_{1/2}$) for EF in Britton–Robinson buffer at different pH values and 75 °C

pH	K_{obs} (h^{-1})	$t_{1/2}$ (h)
2	0.128	5.422
2.8	0.099	6.982
3.6	0.076	9.119
4.4	0.023	29.5
5.2	0.041	16.72
6	0.118	5.866
6.8	0.731	0.947
8	1.844	0.376
9	3.331	0.208
10	12.75	0.054

3.5.3. Range

The calibration range was established through consideration of the practical range necessary, according to each compound concentration present in pharmaceutical product, to give accurate, precise and linear results. The calibration range of each compound is given in Table 1.

3.5.4. Detection and quantitation limits

According to ICH recommendations [4], the approach based on the S.D. of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were assessed practically and are given in Table 1.

3.5.5. Selectivity

Method selectivity was achieved by preparing different laboratory-prepared mixtures of EF, CA and NA at various concentrations within the linearity range. The laboratory-prepared mixtures were analyzed. Satisfactory results were obtained (Table 3), indicating the high selectivity of the proposed method for determination of the studied compounds.

The proposed method is highly selective towards EF, CA and NA. Regarding ethylene glycol (EG) it is assumed that its ultraviolet absorption characteristics are relatively low to be detected by the assay conditions used in this work.

3.5.6. Accuracy

This study was performed by addition of known amounts of EF and its hydrolysis products CA and NA to a known concentration of the commercial capsule (standard addition method). The resulting mixtures were assayed and the results obtained for added compounds were compared with the expected results. The excellent recoveries of standard addition method (Table 3) suggest that good accuracy of the proposed method.

3.5.7. Robustness

Variation of the organic strength of the mobile phase by ±2% or pH of the 10 mM potassium dihydrogen phosphate by ±0.2 did not have a significant effect on chromatographic resolution of the EF and its hydrolysis products.

3.5.8. Analytical solution stability

The analytical solution stability of the EF, CA and NA in mobile phase exhibited no chromatographic changes for 3 h when kept at room temperature, and for 1 day when stored refrigerated at 5 °C.

4. Conclusion

The proposed HPLC method provides simple, accurate and reproducible quantitative analysis for the determination of EF and its hydrolysis products (CA and NA).

It was found that EF is rapidly degraded in alkaline medium, while it is more stable in acidic medium. The most stability of EF was found to be at pH 4.4.

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