J Fluoresc (2011) 21:1371–1384
DOI 10.1007/s10895-010-0819-6

ORIGINAL PAPER

# Conventional and First Derivative Synchronous Fluorometric Determination of Ethamsylate in Pharmaceutical Preparations and Biological Fluids. Application to Stability Studies

**Fathalla Belal · Amina El-Brashy · Nahed El-Enany · Manar Tolba**

Received: 31 July 2010 / Accepted: 29 December 2010 / Published online: 24 February 2011


**Abstract** Two simple, accurate and highly sensitive spectrofluorometric methods were developed for the determination of ethamsylate (ETM). Method I is based on measuring the native fluorescence of ethamsylate in water at 354 nm after excitation at 302 nm. The calibration plot was rectilinear over the range of 0.05–1 μg/mL for ETM with limits of detection and quantitation of 7.9 and 26 ng/mL, respectively. Method II involved synchronous and first derivative synchronous fluorometric methods for the simultaneous determination of ethamsylate (ETM) and hydroquinone (HQ) which is considered as an impurity and/or acidic degradation product. The synchronous fluorescence of both the drug and its impurity were measured in methanol at $\Delta\lambda$ of 40 nm. The peak amplitudes ($^1$D) were estimated at 293.85 or 334.17 nm for ETM and at 309.05 nm for HQ. Good linearity was obtained for ETM over the ranges 0.1–1.4 μg/mL and 0.1–1.0 μg/mL at 293.85 and 334.17 nm, respectively. For HQ, the calibration plot was rectilinear over the range of 0.01–0.14 μg/mL at 309.05 nm. Limits of detection were 20, 2.01 ng/mL and limits of quantitation were 60, 6.7 ng/mL for ETM and HQ by method II, respectively. Both methods were successfully applied to commercial ampoules and tablets. The results were in good agreement with those obtained by the reference method. Method I was utilized to study the stability of ETM and its degradation kinetics using peroxide. The apparent first-order rate constant, half-life times and activation energy of the degradation process were calculated. Method I was further extended to the in-vitro and in-vivo determination of ETM in spiked and real plasma samples. The mean% recoveries were 99.57±3.85 and 89.39±5.93 for spiked and real human plasma, respectively.

**Keywords** Ethamsylate · Hydroquinone · Synchronous fluorimetry · Human plasma · Stability study · Degradation-kinetics

F. Belal · A. El-Brashy · N. El-Enany (✉) · M. Tolba
Department of Analytical Chemistry, Faculty of Pharmacy, University of Mansoura,
35516 Mansoura, Egypt
e-mail: nelenany1@yahoo.com

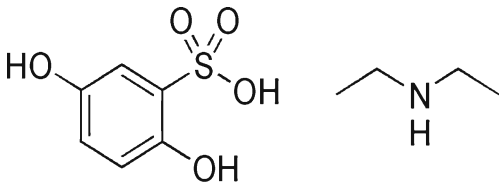
## Introduction

Ethamsylate (ETM); *N*-ethylethanamine 2,5-dihydroxybenzenesulphonate (Fig. 1), is the most widely prescribed drug for prophylaxis and control of haemorrhage from small blood vessels. It is a haemostatic that appears to maintain the stability of capillary walls and correct abnormal platelets adhesion [1]. The British Pharmacopoeia [2] recommended potentiometric titration method for the determination of ETM raw material, using cerium sulphate as a titrant and a TLC method for detection of its known impurity, hydroquinone (HQ) (Fig. 1). In addition, the acid degradation product of ETM was reported to be HQ by Kaul et al. [3].

Several analytical methods were developed for the assay of ethamsylate. These methods include; spectrophotometry [4–8], HPTLC [9, 10], HPLC [3, 11], electrochemical methods [12–15], chemiluminescence [16–19] and capillary electrophoresis [20].

To the best of our knowledge, no spectrofluorometric method has been yet reported for determination of ETM, neither in pharmaceutical preparations nor in biological fluids. Also, no fluorometric method has been described for the simultaneous determination of ETM and HQ.

In this work, two highly sensitive spectrofluorometric methods were developed for the determination of ETM




**Ethamsylate**


**Hydroquinone**

**Fig. 1** The structural formulae for ETM and HQ

either alone or in presence of HQ. The first method (I) is based on measuring the native fluorescence of ethamsylate in water and was utilized to investigate its stability and the kinetics of its degradation using peroxide. In addition, this method could be applied for the determination of ETM in spiked and real human plasma samples. The fluorescence spectra of ETM and HQ are greatly overlapped, so it was difficult to determine their contents by conventional fluorometry. Such problem was resolved by first derivative synchronous fluorometry (FDSF). The proposed method (II) allowed the separation and the quantitation of ETM and HQ as its impurity and/or its acidic degradation product with satisfied accuracy and precision. In addition, method II could be considered as stability indicating one. The proposed methods were successfully applied to commercial ampoules and tablets.

## Experimental

### Apparatus

- All fluorescence measurements were recorded with a Perkin-Elmer UK model LS 45 luminescence spectrometer, equipped with a 150 W Xenon arc lamp, grating excitation and emission monochromators and a Perkin Elmer recorder. The slit widths were 10 nm for both excitation and emission, and the photomultiplier voltage was set to auto. Quartz 1 cm cuvette was used. Derivative spectra were evaluated using fluorescence data manager software, FL WINLAB, Version 4.00.02, Copyright 2001, Perkin Elmer, Inc., UK.
- A Consort P-901 pH-meter was used for pH measurements.
- CAMAG UV-lamp, S/N 29000, dual wavelength 254/366 nm, 2×8 W (Switzerland) was used in the UV-degradation study.

### Materials and Reagents

All the chemicals used were of Analytical Reagent grade, and the solvents were of HPLC grade.

- Ethamsylate was kindly provided by Memphis Chemical Co. (Cairo, Egypt) and was used as received without further purification.
- Hydroquinone (El-Nasr Pharmaceutical Chemicals Company (ADWIC), Abu Zaabal, Egypt).
- Pharmaceutical preparations were purchased from commercial sources in the local market:

  ⇒ Dicynone® ampoules; batch # 9DE0636, labeled to contain 250 mg ETM/2 mL.
  ⇒ Dicynone® tablets; batch # 308521, labeled to contain 250 mg ETM.
  ⇒ Dicynone® 500 tablets; batch # 9DE0764, labeled to contain 500 mg ETM.
  ⇒ Dicynone® ampoules, Dicynone® 500 tablets (Minapharm, Egypt) and Dicynone® tablets (Memphis Co. for Pharma. & Chemical Ind., Cairo, Egypt) are manufactured under license of OM PHARMA (Meyrin, Geneva, Switzerland).

- Methanol (Sigma-Aldrich, Germany).
- Sodium hydroxide, Hydrogen peroxide (30% *w*/*v*) and hydrochloric acid (32%) (El-Nasr Pharmaceutical Chemicals Company (ADWIC), Abu Zaabal, Egypt).
- Human plasma was kindly provided by Mansoura University Hospitals (Mansoura, Egypt) and kept frozen until use after gentle thawing. Blood samples were obtained from a healthy volunteer (female, 40 years old).

### Standard Solutions

Stock solutions equivalent to 0.20 mg/mL of ETM were prepared in distilled water and methanol for methods I and II, respectively. For HQ, stock solution was prepared by dissolving 10.0 mg in 100 mL of methanol. Standard solutions were prepared by appropriate dilution of the stock solutions with the same solvent. The solutions were stable for at least 3 days without alteration when kept in the refrigerator and protected from light.

### Procedures

#### *Construction of Calibration Graphs*

Method I Aliquots of ETM standard solution were transferred into a series of 10 mL volumetric flasks so that the final concentration was in the

range of 0.05–1 μg/mL. Then, the volumes were completed to the mark with distilled water and mixed well. The fluorescence intensities of the solutions were measured at 354 nm after excitation at 302 nm. A blank experiment was performed simultaneously. The corrected fluorescence intensity (Δ FI) was then plotted against the final drug concentration in μg/mL to get the calibration graph. Alternatively, the regression equation was derived.

Method II Aliquots of ETM or HQ standard solutions covering the working concentration range cited in Table 1 were transferred into a series of 10 mL volumetric flasks. The solutions were diluted to the mark with methanol and mixed well. The synchronous fluorescence spectra of the solutions were recorded by scanning both monochromators at a constant wavelength difference Δ λ=40 nm with scan rate of 600 $nm^{-1}$ using 10 nm excitation and emission windows. The first derivative synchronous fluorescence spectra of ETM and HQ were derived from the normal synchronous spectra using fluorescence data manager software. The peak amplitudes of the first derivative spectra ($^1D$) were estimated at 293.85 nm and 334.17 nm for ETM, and 309.05 nm for HQ. A blank experiment was performed simultaneously. The peak amplitude of the first derivative technique ($^1D$) of each drug was then plotted against the final drug concentration in μg/mL to get the calibration graph. Alternatively, the corresponding regression equations were derived.

*Analysis of ETM/HQ Synthetic Mixtures*

Aliquots of ETM and HQ standard solutions in different ratios were transferred into a series of 10 mL volumetric flasks, diluted to the mark with methanol and mixed well. The procedure described under "Construction of calibration graphs" for method II was then applied. The percentage recoveries were calculated by referring to the calibration graphs, or using the corresponding regression equations.

*Analysis of ETM in Pharmaceutical Preparations*

*Procedure for Ampoules* The contents of five ampoules (Dicynone®) were mixed well. An aliquot of the solution equivalent to 125.0 mg ETM was transferred into 25 mL volumetric flask. The solution was diluted to the mark with distilled water or methanol for method I or II, respectively. Then, 4 mL were transferred into 100 mL volumetric flask. Aliquots of these solutions were successively diluted with the appropriate solvent and the procedures described under "*Construction of calibration graphs*" were then applied. The nominal content of the ampoules was determined either from the previously plotted calibration graph or using the corresponding regression equation.

*Procedure for Tablets* Ten tablets (Dicynone® 250 mg or 500 mg) were accurately weighed, finely pulverized, and thoroughly mixed. An accurately weighed amount of powdered tablets equivalent to 20.0 mg of ETM was transferred into small conical flask and extracted with 3× 30 mL of distilled water for method I or methanol for method II. The extract was filtered into 100 mL volumetric flask. The conical flask was washed with few mLs of distilled water or methanol. The washings were passed into

**Table 1** Analytical performance data for the proposed methods

| Parameter | Method I (ETM) | Method II | | |
|---|---|---|---|---|
| | | ETM at 293.85 nm | ETM at 334.17 nm | HQ at 309.05 nm |
| Linearity range (μg/mL) | 0.05–1.0 | 0.1–1.4 | 0.1–1.0 | 0.01–0.14 |
| Intercept (*a*) | 2.271 | −2.034 | 0.188 | 0.580 |
| Slope (*b*) | 543 | 146 | 114 | 537 |
| Correlation coefficient (*r*) | 0.9999 | 0.9998 | 0.9998 | 0.9998 |
| S.D. of residuals ($S_{y/x}$) | 2.25 | 1.33 | 0.91 | 0.60 |
| S.D. of intercept ($S_a$) | 1.43 | 0.89 | 0.66 | 0.36 |
| S.D. of slope ($S_b$) | 2.55 | 1.07 | 1.10 | 4.24 |
| Percentage relative standard deviation,% RSD | 0.84 | 0.96 | 1.28 | 1.34 |
| Percentage relative error,% Error | 0.34 | 0.36 | 0.57 | 0.51 |
| Limit of detection, LOD (ng/mL) | 7.90 | 20 | 20 | 2.01 |
| Limit of quantitation, LOQ (ng/mL) | 26 | 60 | 60 | 6.70 |

the same volumetric flask and completed to the volume with the same solvent. Aliquots of these solutions were successively diluted with the appropriate solvent and the procedures described under "*Construction of calibration graphs*" were then performed. The nominal content of the tablets was determined either from the previously plotted calibration graphs or using the corresponding regression equations.

*Procedure for Spiked Human Plasma*

Aliquots of human plasma (1.0 mL) were transferred into a series of centrifugation tubes. The plasma samples were spiked with increasing aliquots of ETM standard solution (60 μg/mL) so that the final concentration was in the range of 0.2–1.0 μg/mL. The tubes were mixed well and then 4.0 mL of methanol was added. After vortex mixing for 5 min., the mixtures were centrifuged at 4000 rpm for 30 min. The upper layers were carefully aspirated. Then, 1.0 mL aliquots of the upper layers were quantitatively transferred into a series of 10 mL volumetric flasks and diluted to the volume with distilled water. A blank experiment was carried out simultaneously and the procedure described under "*Construction of calibration graphs*" for method I was then performed. The Δ FI was plotted versus the concentration of the drug in μg/mL.

*Procedure for Patient Samples*

A healthy volunteer (female, 40 years old) had been administered one Dicynone® 500 tablet after 10 h of fasting. A blood sample was taken from the volunteer before administration of the tablet as a blank. Then, blood samples were collected at several time intervals after oral administration. The samples were drawn into test tubes containing few drops of saturated solution of EDTA as anticoagulant and centrifuged at 4000 rpm for 30 min. The supernatant plasma samples were transferred into test tubes. Then, 1.0 mL aliquots of the supernatant plasma were transferred into a series of centrifugation tubes. The procedure described for spiked human plasma was then followed, but finally 2.0 mL of the upper clear layers were quantitatively transferred into a series of 10 mL volumetric flasks and diluted to the mark with distilled water.

*Stability Studies for ETM Using Method I*

*Alkali Degradation* A solution containing 0.2 mg/mL ETM was prepared in water. 2.5 mL aliquots of this aqueous solution were transferred into series of small conical flasks. Different volumes of 0.1 M NaOH were added to the flasks at room temperature. The solutions were then neutralized with 0.1 M HCl, transferred to 25 mL volumetric flask and diluted to the volume with distilled water. Aliquots of these solutions (0.5 mL) were transferred into a series of 10 mL volumetric flasks. The volume was completed to the mark with distilled water then, we proceeded as described under "*construction of calibration graphs*" for method I.

*Oxidative Degradation* 1.0 mL aliquots of aqueous ETM solution (10 μg/mL) were transferred into series of small conical flasks. 2.0 mL of 1% $H_2O_2$ (*w*/*v*) were added to each flask. The solutions were then heated in a thermostated controlled water bath at different temperatures (50, 60 and 70 °C) for different time intervals (5–30 min.), in addition to room temperature (25 °C). At the specified time intervals, the contents of each flask were cooled and transferred to 10 mL volumetric flask. The volume was completed to the mark with distilled water. The procedure described under "*construction of calibration graphs*" for method I was then proceeded.

*UV and Sun Light Degradation* A solution of ETM (0.2 mg/mL) was prepared in water. Aliquots of this solution (2.5 mL) were transferred into 25 mL volumetric flask. Then, the flask was exposed to UV-light at 254 nm for different time intervals (0.5–9 h) in a wooden cabinet, where the distance between the source and the sample solution was kept at 15 cm. Aliquots of the solution (0.5 mL) were transferred into a series of 10 mL volumetric flasks at the specified time interval. The volumes were completed to the mark with distilled water, and then proceed as described under *construction of calibration graph* using method I. Similarly, the procedure was applied for the sun light degradation study but the flask was exposed to sun light for the same time intervals.

## Results and Discussion

ETM exhibits strong native fluorescence in water at 354 nm after excitation at 302 nm, as shown in Fig. 2 (method I). This fact initiated the development of a simple and reliable method for the quantitation of ETM in raw material, pharmaceutical preparations and human plasma. It also was utilized to study the stability of ETM and the kinetics of its oxidative degradation.

On the other hand, both ETM and HQ exhibit native fluorescence in methanol at 354 and 333 nm, after excitation at 308 and 295 nm, respectively, as shown in Fig. 2 (method II). The acid degradation product of ETM was reported to be HQ by Kaul et al. [3]. So, method II could be considered as stability indicating one as it permitted the quantitation and separation of ETM from its degradation product at Δ λ=40 nm.

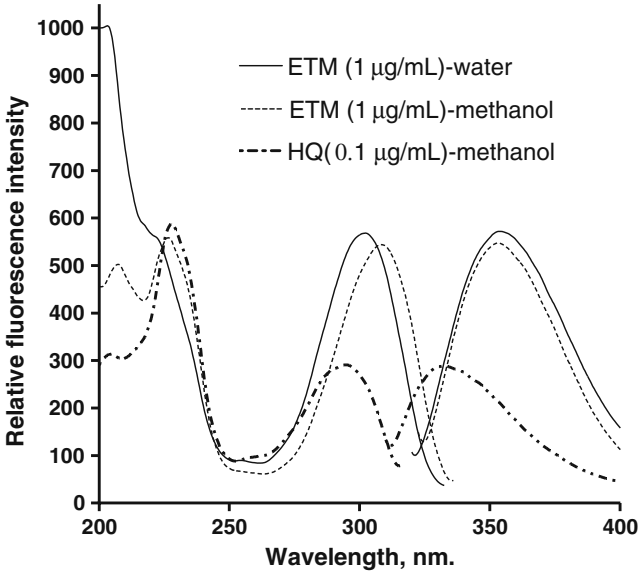



**Fig. 2** Excitation and emission fluorescence spectra of ETM (1 μg/mL) and HQ (0.1 μg/mL)

Figure 3(a) shows the synchronous fluorescence spectra of different concentrations of ETM at 309.68 nm in presence of HQ. While, Fig. 3(b) illustrates synchronous fluorescence spectra of different concentrations of HQ at 295 nm in presence of ETM. It is clear that, there is great overlapping between the two spectra. This led us to adopt first derivative synchronous fluorescence spectroscopy technique (FDSF) for the simultaneous determination of the two compounds. The fluorescence spectra of ETM and HQ were well separated with a zero-crossing point for each compound. Under the described experimental conditions, ETM could be separated by FDSF at 293.85 and 334.17 nm in presence of HQ (Fig. 4). Figure 5 illustrates FDSF of different concentrations of HQ at 309.05 nm in presence of ETM.

## Optimization of Experimental Conditions

The different experimental parameters affecting the fluorescence intensities of the studied compound and its impurity were carefully studied and optimized. Such factors were changed individually while others were kept constant. In method I, the experimental conditions affecting the fluorescence intensity of ETM were considered. However in method II, HQ (as impurity or degradation product) present in small concentration relative to ETM, so we should emphasize the experimental parameters affecting the fluorescence intensity of HQ.

### *Effect of pH*

The influence of pH either in method I or II was investigated using different kinds of buffers covering the whole pH range, such as 0.2 M acetate buffer (pH 3.6–5.6) and 0.2 M borate buffer (pH 7–10), in addition to 0.1 M HCl and 0.1 M NaOH (Fig. 6).

*Method I* The influence of pH on the native fluorescence intensity of ETM in water was studied. The results showed that, the native fluorescence intensity of ETM increased with increasing the pH of the buffer up to 7.0, then remained constant up to pH 7.5, after which the fluorescence intensity decreased. Therefore, borate buffer pH 7.0 could be used for ETM determination. However, it was found that constant and slightly higher fluorescence intensity was attained upon using water only as a diluting solvent so; addition of borate buffer was not incorporated in the standard procedure. Also, the effect of using 0.1 M HCl and 0.1 M NaOH instead of buffers was studied. It was found that using 0.1 M HCl resulted in slightly high fluorescence intensity nearly equal to the fluorescence intensity obtained by using 0.2 M acetate buffer with pH ranged from 5 to 5.6. However, using 0.1 M NaOH resulted in quenching of the fluorescence intensity of ETM which may be attributed to its degradation.

*Method II* The influence of pH on the synchronous fluorescence intensity of HQ in methanol was investigated. HQ is very close in behavior to ETM due to the high similarity in the chemical structures of both compounds. Where, increasing the pH of the buffer resulted in a gradual increase in the synchronous fluorescence intensity of HQ up to pH 7.0, then remained constant up to pH 8.0, after which the fluorescence intensity decreased gradually. However, no buffer was used throughout the study, since methanol alone resulted in higher fluorescence intensity compared to that of using methanol with buffer. Using 0.1 M HCl resulted in high fluorescence intensity equal to that of borate buffer of pH 7.0 and so; lower than that achieved by methanol alone. On the other hand, using 0.1 M NaOH resulted in complete quenching of the fluorescence intensity of HQ.

### *Effect of Diluting Solvent*

Dilution with different solvents such as water, methanol, ethanol, n-propanol, dimethyl sulphoxide (DMSO), dimethyl formamide (DMF) and acetone was attempted in both methods I and II. Each of DMSO and DMF decreased the fluorescence intensities of ETM and HQ, since they initiated intersystem crossing process (similar to heavy atom effect) [21]. Acetone resulted in complete quenching of the fluorescence intensities of both compounds. On the other hand, the fluorescence intensities of ETM and HQ were higher in water and methanol, respectively compared to

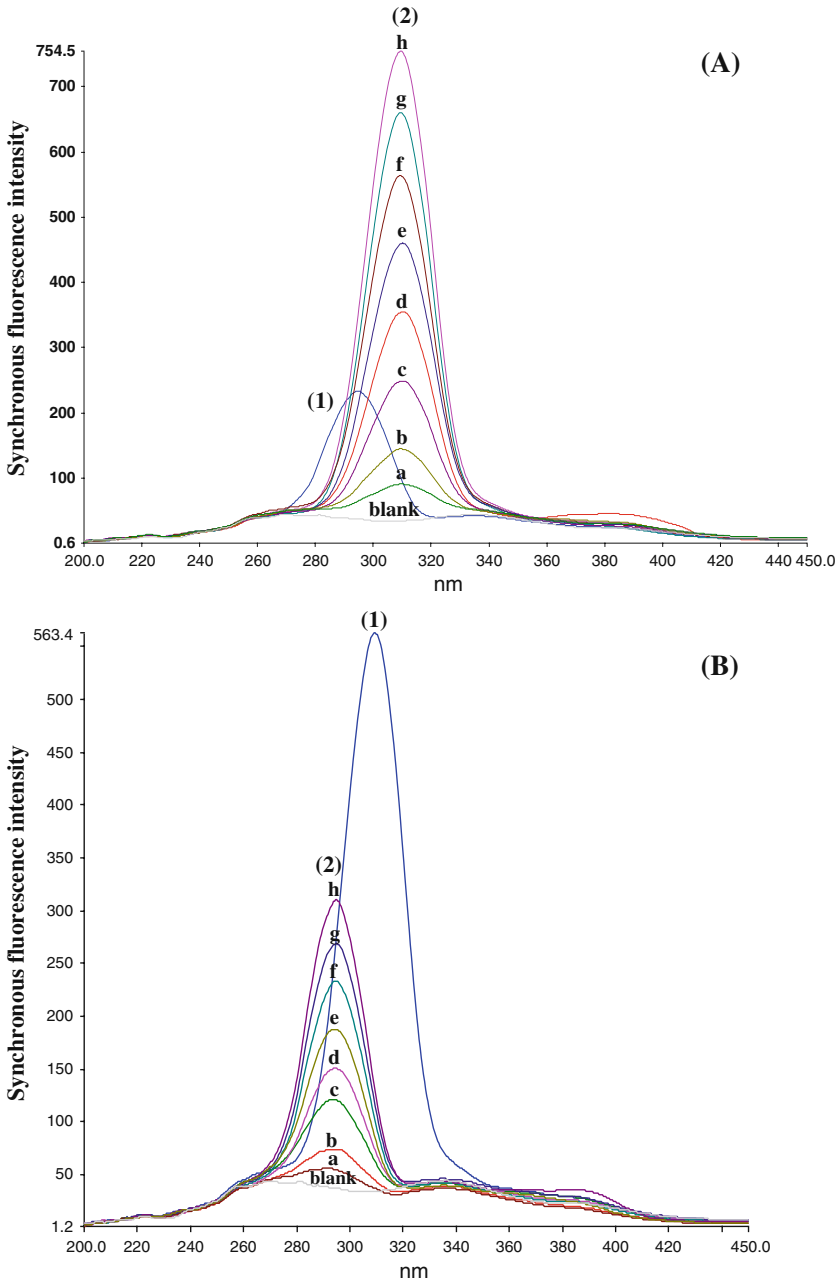



**Fig. 3** **a** Synchronous fluorescence spectra of (*1*) HQ (0.1 μg/mL) (*2*) (*a–h*) of ETM (0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4 μg/mL) at 309.68 nm. **b**. Synchronous fluorescence spectra of (*1*) ETM (1 μg/mL) (*2*) (*a–h*) of HQ (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12 and 0.14 μg/mL) at 295 nm

other solvents. Hence, in method I water was the best solvent for dilution as it gave the highest fluorescence intensity for ETM, the lowest blank reading and its use adds another advantage of this method. However in method II, methanol was the solvent of choice giving the highest fluorescence intensity for HQ. The results are summarized in Table 2.

*Effect of Surfactant (Method I)*

Different surfactants such as cetrimide (cationic surfactant), sodium dodecyl sulphate (anionic surfactant), β-cyclodextrin, β-hydroxy cyclodextrin, methyl cellulose and tween 80 (non ionic surfactants) were tried. All these surfactant caused a decrease in the fluorescence intensity of ETM.

*Selection of Optimum Δ λ (Method II)*

The synchronous fluorescence spectra of ETM with HQ were recorded using different Δ λ. The optimum Δ λ value is very important for performing synchronous fluorescence scanning technique concerning resolution, sensitivity and features. It can directly influence spectral shape, band width and signal value. For this reason a wide range of Δ λ (20–100 nm) was examined. It was found that the optimum Δ λ

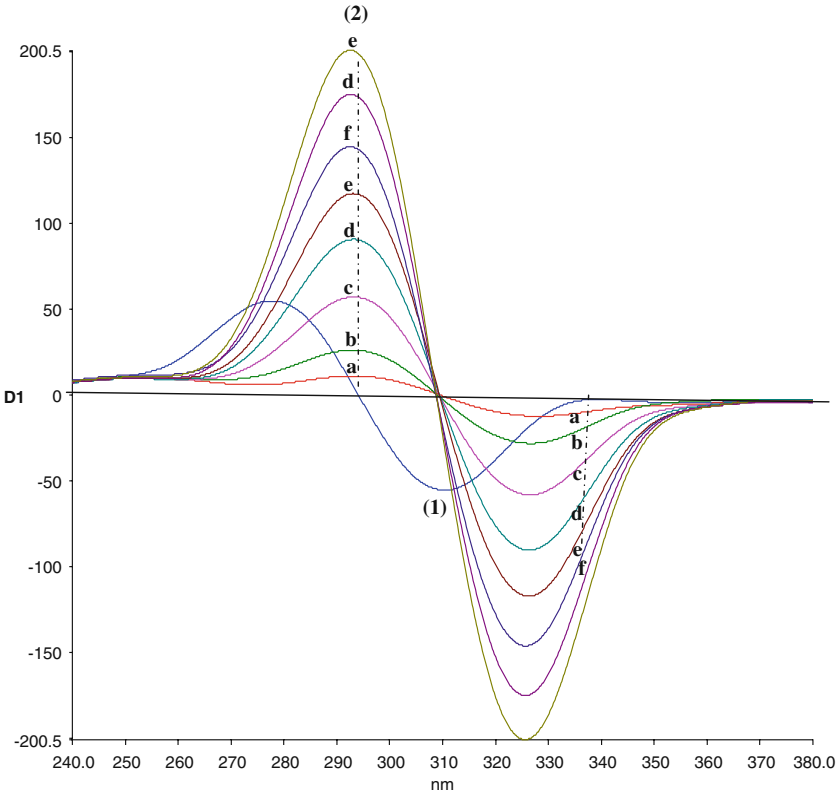



**Fig. 4** First derivative synchronous fluorescence spectra of: (*1*) HQ (0.1 μg/mL) (*2*) (*a–h*) of ETM (0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4 μg/mL) at 293.85 nm and (*a–f*) of ETM at 334.17 nm

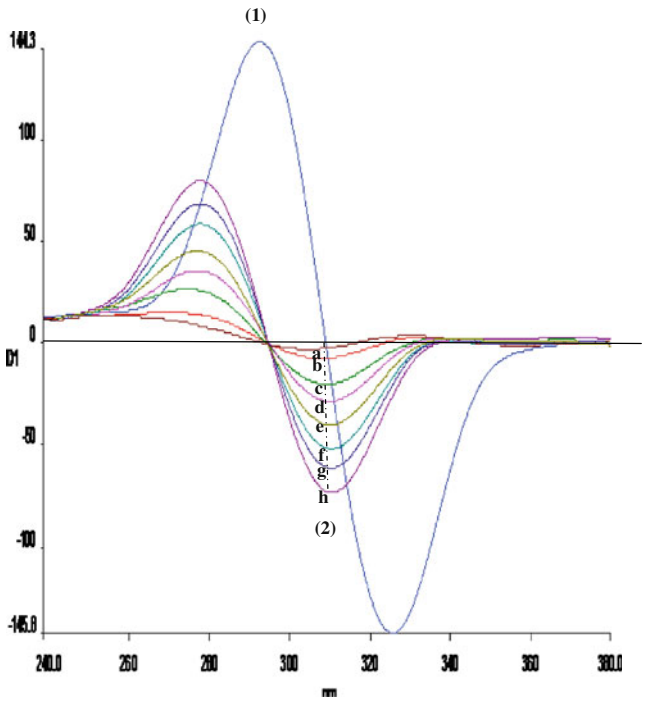



**Fig. 5** First derivative synchronous fluorescence spectra of (*1*) ETM (1 μg/mL) (*2*) (*a–h*) of HQ (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12 and 0.14 μg/mL) at 309.05 nm

for ETM and HQ was 45 and 40 nm, respectively. As HQ (impurity or degradation product) is present in small concentration relative to ETM, so the optimum condition was selected to increase its sensitivity. Therefore, Δ λ of 40 nm was chosen since it resulted in the highest fluorescence intensity for HQ and it gave two distinct peaks for both compounds with good shape. Lower and higher values of Δ λ than the optimum ones showed low fluorescence intensity for both compounds. However, very

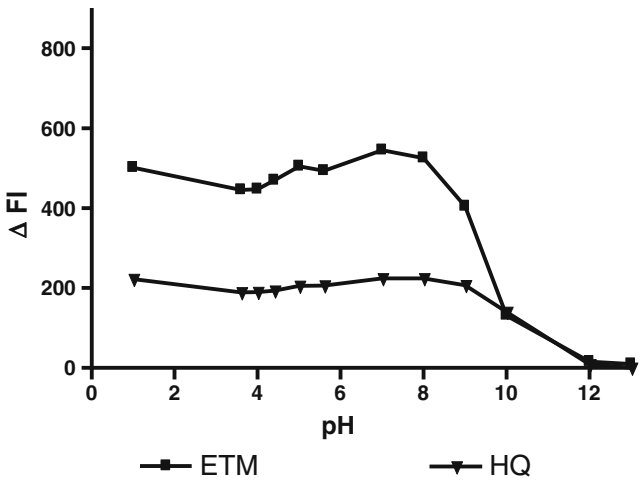



**Fig. 6** Effect of pH on the native and the synchronous fluorescence intensity of ETM (1 μg/mL) and HQ (0.1 μg/mL), respectively

**Table 2** Effect of solvents on the native fluorescence intensity of ethamsylate (1 μg/mL) and synchronous fluorescence intensity of HQ (0.1 μg/mL)

| Solvents | Fluorescence intensity | |
|---|---|---|
| | ETM (method I) | HQ (method II) |
| Water | 545 | 139 |
| Methanol | 520 | 233 |
| Ethanol | 528 | 234 |
| 1-propanol | 522 | 229 |
| Dimethyl formamide | 279 | 117 |
| Dimethyl sulfoxide | 361 | 140 |
| Acetone | 2 | −1 |

low and very high Δ λ values caused irregularities in the spectral shape.

*Effect of Stability Time*

The effect of time on the development and stability of the fluorescence intensity of each compound was studied in either method I or II. It was found that the fluorescence readings in both methods remained stable for more than 2 h.

Validation of the Methods

*Linearity*

Under the above described experimental conditions, a linear relationship was established by plotting either the corrected fluorescence intensity or the peak amplitude ($^1$D) against the drug concentration. For method I, the calibration plot of ETM was rectilinear over the range of 0.05–1 μg/mL. In method II, The concentration ranges were found to be 0.1–1.4 and 0.1–1 μg/mL for ETM at 293.85 and 334.17 nm, respectively. For HQ, the calibration plot was rectilinear over the range of 0.01–0.14 μg/mL at 309.05 nm using method II. Linear regression analysis of the data gave the following equations:

Method I $\Delta FI = 2.271 + 543C$ (r = 0.9999) for ETM at 354 nm

Method II $^1D = -2.034 + 146C$ (r = 0.9998) for ETM at 293.85 nm

$^1D = 0.188 + 114C$ (r = 0.9998) for ETM at 334.17 nm

$^1D = 0.580 + 537C$ (r = 0.9998) for HQ at 309.05 nm

Where: Δ FI is the corrected fluorescence intensity, $^1$D is the peak amplitude in the first derivative synchronous fluorescence mode, C is the concentration of the drug in μg/mL and r is the correlation coefficient.

Statistical analysis [22] of the data gave high value of the correlation coefficient (r) of the regression equation, small values of the standard deviation of residuals ($S_{y/x}$), of intercept ($S_a$), and of slope ($S_b$), and small value of the percentage relative standard deviation and the percentage relative error (Table 1). These data proved the linearity of the calibration graph.

*Limit of Quantitation (LOQ) and Limit of Detection (LOD)*

LOQ and LOD were calculated according to ICH Q2R1 recommendations using the following equation [23]:

$$LOQ = 10\,S_{a/b} \text{ and } LOD = 3.3\,S_{a/b}$$

**Table 3** Accuracy and precision data for the determination of ethamsylate by the proposed methods

| Amount taken (μg/mL) | % Found | % RSD | % Error |
|---|---|---|---|
| Method I | | | |
| Intraday | | | |
| 0.4 | 101.35±1.02 | 1.01 | 0.58 |
| 0.6 | 101.08±0.29 | 0.29 | 0.17 |
| 0.8 | 99.39±0.34 | 0.34 | 0.20 |
| Interday | | | |
| 0.4 | 100.00±1.32 | 1.32 | 0.76 |
| 0.6 | 100.89±1.38 | 1.37 | 0.79 |
| 0.8 | 99.79±0.31 | 0.31 | 0.19 |
| Method II | | | |
| At 293.85 nm | | | |
| Intraday | | | |
| 0.4 | 100.36±0.67 | 0.67 | 0.39 |
| 0.6 | 100.81±0.11 | 0.11 | 0.06 |
| 0.8 | 99.60±0.45 | 0.45 | 0.26 |
| Interday | | | |
| 0.4 | 99.84±0.70 | 0.70 | 0.40 |
| 0.6 | 98.97±1.05 | 1.06 | 0.61 |
| 0.8 | 99.24±0.98 | 0.99 | 0.57 |
| At 334.17 nm | | | |
| Intraday | | | |
| 0.4 | 99.32±1.06 | 1.07 | 0.62 |
| 0.6 | 99.26±0.89 | 0.90 | 0.52 |
| 0.8 | 100.29±0.82 | 0.82 | 0.47 |
| Interday | | | |
| 0.4 | 98.19±0.48 | 0.49 | 0.28 |
| 0.6 | 98.93±1.40 | 1.42 | 0.82 |
| 0.8 | 99.85±1.38 | 1.38 | 0.80 |

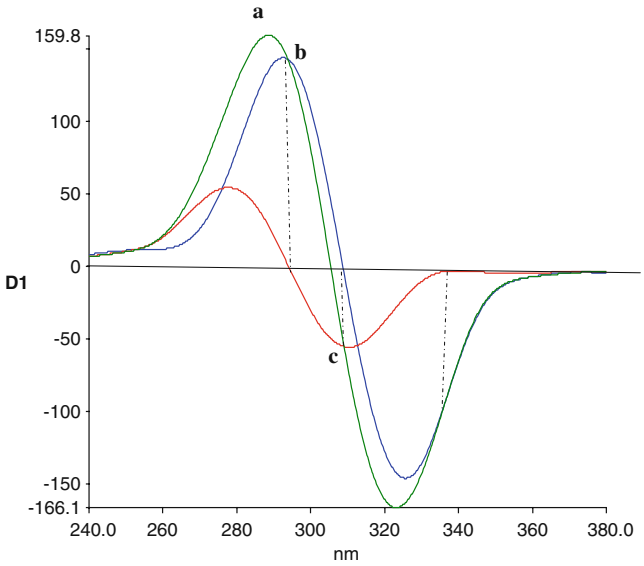



**Fig. 7** First derivative synchronous fluorescence spectra of: (*a*) Mixture of 1 μg/mL ETM and 0.1 μg/mL HQ (*b*) 1 μg/mL ETM (*c*) 0.1 μg/mL HQ

Where $S_a$ = standard deviation of the intercept of the calibration curve and b = slope of the calibration curve. The values of LOD and LOQ for ETM and HQ are abridged in Table 1.

*Accuracy and Precision*

To prove the accuracy of the proposed method, the results of the assay of ETM were compared with those of the reference method [6]. Statistical analysis of the results using Student's *t*-test and variance ratio *F*-test [22] revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively.

The reference method [6] based upon oxidation of ETM with 3-methyl-2-benzothiazolinone hydrazone hydrochloride in presence of cerium (IV) ammonium sulfate at room temperature for 20 min. The absorbance of the reaction product was measured at 514 nm.

For both methods I and II, intraday and interday precisions were assessed using three concentrations and three replicates of each concentration. The relative standard deviations were found to be very small indicating reason-

**Table 4** Application of the proposed method II for the determination of ethamsylate in presence of hydroquinone

| Sample | Amount taken (μg/mL) | | Amount found (μg/mL) | | % Found | |
|---|---|---|---|---|---|---|
| | ETM | HQ | 293.85 nm | 334.17 nm | 293.85 nm | 334.17 nm |
| ETM and HQ synthetic mixture | 1.0 | 0.04 | 0.978 | 0.981 | 97.80 | 98.10 |
| | | 0.05 | 1.025 | 1.029 | 102.50 | 102.90 |
| | | 0.06 | 0.998 | 1.008 | 99.80 | 100.80 |
| | | 0.07 | 0.995 | 1.007 | 99.50 | 100.70 |
| | | 0.08 | 1.021 | 1.026 | 102.10 | 102.60 |
| | | 0.09 | 0.990 | 1.003 | 99.00 | 100.30 |
| | | 0.10 | 0.991 | 1.000 | 99.10 | 100.00 |
| $(\bar{x})$ | | | | | 99.97 | 100.77 |
| ± SD | | | | | 1.71 | 1.62 |
| % RSD | | | | | 1.71 | 1.61 |
| % Error | | | | | 0.65 | 0.61 |
| | 0.5 | 0.020 | 0.492 | 0.490 | 98.40 | 98.00 |
| | | 0.025 | 0.503 | 0.493 | 100.60 | 98.60 |
| | | 0.030 | 0.510 | 0.496 | 102.00 | 99.20 |
| | | 0.035 | 0.499 | 0.494 | 99.80 | 98.80 |
| | | 0.040 | 0.494 | 0.512 | 98.80 | 102.40 |
| | | 0.045 | 0.505 | 0.504 | 101.00 | 100.80 |
| | | 0.050 | 0.506 | 0.505 | 101.20 | 101.00 |
| $(\bar{x})$ | | | | | 100.26 | 99.83 |
| ± SD | | | | | 1.32 | 1.59 |
| % RSD | | | | | 1.32 | 1.59 |
| % Error | | | | | 0.50 | 0.60 |

N. B. Each result is the average of three separate determinations

**Table 5** Assay results for the determination of ethamsylate in dosage forms by the proposed and reference methods

| Preparation | Amount taken (μg/mL) | Method I | | Method II | | | | Reference method [6] |
|---|---|---|---|---|---|---|---|---|
| | | Amount found (μg/mL) | % Found | Amount found (μg/mL) | | % Found | | % Found |
| | | | | 293.85 nm | 334.17 nm | 293.85 nm | 334.17 nm | |
| 1-Dicynone® ampoules (250 mg ETM/2 mL) Batch # 9DE0636 | 0.2 | 0.201 | 100.50 | 0.199 | 0.197 | 99.50 | 98.50 | 99.59 |
| | 0.4 | 0.401 | 100.25 | 0.392 | 0.397 | 98.00 | 99.25 | 99.13 |
| | 0.6 | 0.595 | 99.17 | 0.599 | 0.606 | 99.83 | 101.00 | 100.71 |
| | 0.8 | 0.798 | 99.75 | 0.790 | 0.792 | 98.75 | 99.00 | |
| Mean | | | 99.92 | | | 99.02 | 99.44 | 99.81 |
| S.D. | | | 0.59 | | | 0.82 | 1.09 | 0.81 |
| *t*-test | | | 0.12 (2.57) | | | 1.26 (2.57) | 0.48 (2.57) | |
| *F*-test | | | 1.88 (9.55) | | | 1.02 (19.16) | 1.81 (19.16) | |
| 2-Dicynone® tablets (250 mg ETM/tablet) Batch # 308521 | 0.2 | 0.202 | 101.00 | 0.201 | 0.199 | 100.50 | 99.50 | 99.18 |
| | 0.4 | 0.399 | 99.75 | 0.394 | 0.396 | 98.50 | 99.00 | 98.58 |
| | 0.6 | 0.600 | 100.00 | 0.592 | 0.593 | 98.67 | 98.83 | 100.11 |
| | 0.8 | 0.796 | 99.50 | 0.785 | 0.801 | 98.13 | 100.13 | |
| Mean | | | 100.06 | | | 98.93 | 99.38 | 99.29 |
| S.D. | | | 0.66 | | | 1.06 | 0.56 | 0.77 |
| *t*-test | | | 1.41 (2.57) | | | 0.91 (2.57) | 0.16 (2.57) | |
| *F*-test | | | 1.36 (9.55) | | | 1.90 (19.16) | 1.76 (9.55) | |
| 3-Dicynone® 500 tablets (500 mg ETM/tablet) Batch # 9DE0764 | 0.2 | 0.202 | 101.00 | 0.204 | 0.202 | 102.00 | 101.00 | 100.98 |
| | 0.4 | 0.395 | 98.75 | 0.403 | 0.399 | 100.75 | 99.75 | 101.13 |
| | 0.6 | 0.597 | 99.50 | 0.602 | 0.613 | 100.33 | 102.17 | 102.00 |
| | 0.8 | 0.801 | 100.13 | 0.798 | 0.799 | 99.75 | 99.88 | |
| Mean | | | 99.85 | | | 100.71 | 100.70 | 101.37 |
| S.D. | | | 0.96 | | | 0.95 | 1.13 | 0.55 |
| *t*-test | | | 2.42 (2.57) | | | 1.06 (2.57) | 0.93 (2.57) | |
| *F*-test | | | 3.05 (19.16) | | | 2.98 (19.16) | 4.22 (19.16) | |

All the pharmaceutical preparations are manufactured under license of OM PHARMA, Switzerland

*N.B.* Each result is the average of three separate determinations

[a] Figures between parentheses are the tabulated *t* and *F* values, respectively at $p=0.05$ [22]

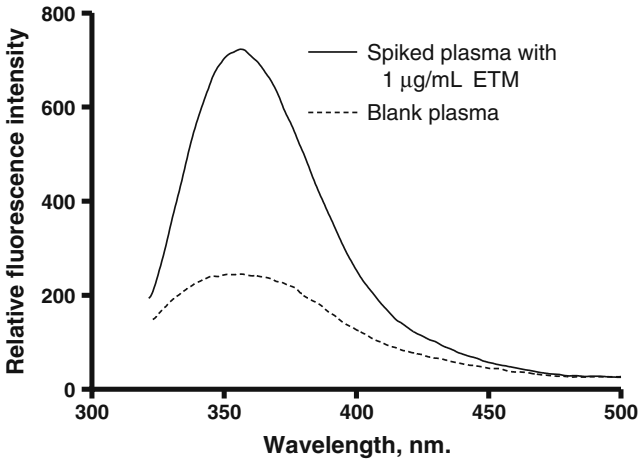



**Fig. 8** Application of method I for the determination of ETM (1 µg/mL) in spiked human plasma

able repeatability and intermediate precision of the proposed methods (Table 3).

*Selectivity*

The selectivity of the methods was investigated by observing any interference encountered from common tablet excipients and ampoule. It was shown that these compounds did not interfere with the results of the proposed methods.

## Applications

*Analysis of ETM/HQ Synthetic Mixtures*

Method II was applied to the simultaneous determination of ETM and HQ in synthetic mixtures containing different ratios of both compounds (Fig. 7). The concentrations of both compounds in the synthetic mixture were calculated according to the linear regression equations. The results indicate the accuracy of the proposed method as shown in Table 4.

*Dosage Form Analysis*

The proposed methods were successfully applied to the determination of EM in commercial tablets and ampoules. The results shown in Table 5 are in good agreement with those obtained using the reference method [6]. Statistical analysis of the results obtained using Student's *t*-test and variance ratio *F*-test [22] revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively.

*Biological Application*

Ethamsylate is slowly absorbed from the gastrointestinal tract when given orally. After oral administration of 500 mg ethamsylate, maximum plasma level; 15 µg/mL is reached at 4 h, but bioavailability is not known [24]. The high sensitivity of method I allowed the in-vitro and in-vivo determination of ETM in spiked and real human plasma. Figure 8 shows ETM fluorescence spectrum obtained from spiked plasma by method I. Table 6 shows the results obtained from spiked plasma. Under the above described experimental conditions, a linear relationship was established by plotting the corrected fluorescence intensity against the drug concentration.

Linear regression analysis of the data gave the following equation:

$$\Delta FI = 27.55 + 449.50\ C \quad (r = 0.9993)$$

**Table 6** Assay results for the determination of ethamsylate in spiked and real human plasma samples using method I

| Parameter | Amount taken (µg/mL) | Amount found (µg/mL) | % Found |
|---|---|---|---|
| Spiked human plasma | 0.20 | 0.190 | 95.00 |
| | 0.40 | 0.417 | 104.25 |
| | 0.80 | 0.789 | 98.63 |
| | 1.0 | 1.004 | 100.40 |
| $\bar{x}$ | | | 99.57 |
| ± SD | | | ± 3.85 |
| % RSD | | | 3.87 |
| % Error | | | 1.94 |
| Real human plasma | 0.6 | 0.526 | 87.29 |
| | | 0.579 | 96.09 |
| | | 0.511 | 84.80 |
| $\bar{x}$ | | | 89.39 |
| ± SD | | | 5.93 |
| % RSD | | | 6.63 |
| % Error | | | 3.83 |

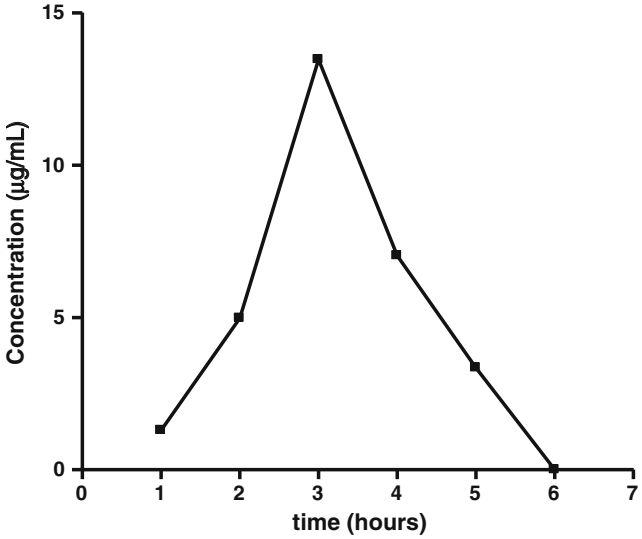



**Fig. 9** Monitoring of the blood level of the studied drug in patient plasma samples at different time intervals using method A

Where: Δ FI is the corrected fluorescence intensity, C is the concentration of ETM in μg/mL and r is the correlation coefficient.

*Real human Plasma* The plasma samples obtained from the volunteer were investigated using the previously obtained calibration graph or regression equation of the spiked human plasma and the results obtained are shown in Fig. 9. Practically, the maximum plasma level reached after 3 h and was found to be 13.41 μg/mL. Hence, method I allows the therapeutic monitoring of the drug level in plasma.

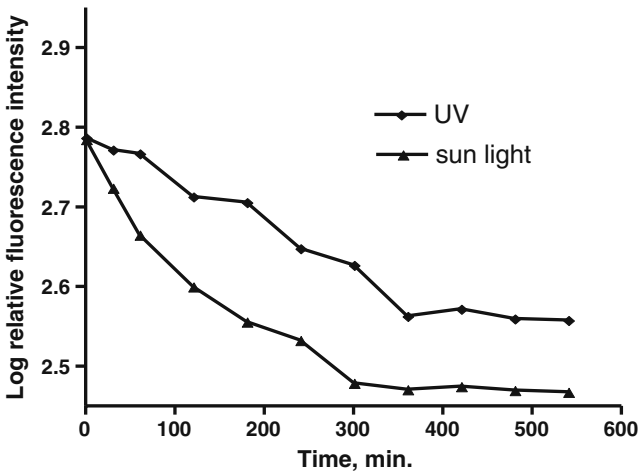



**Fig. 10** A plot of log relative fluorescence intensity of ETM (1 μg/mL) vs time (min.) upon exposure to UV lamp and sun light

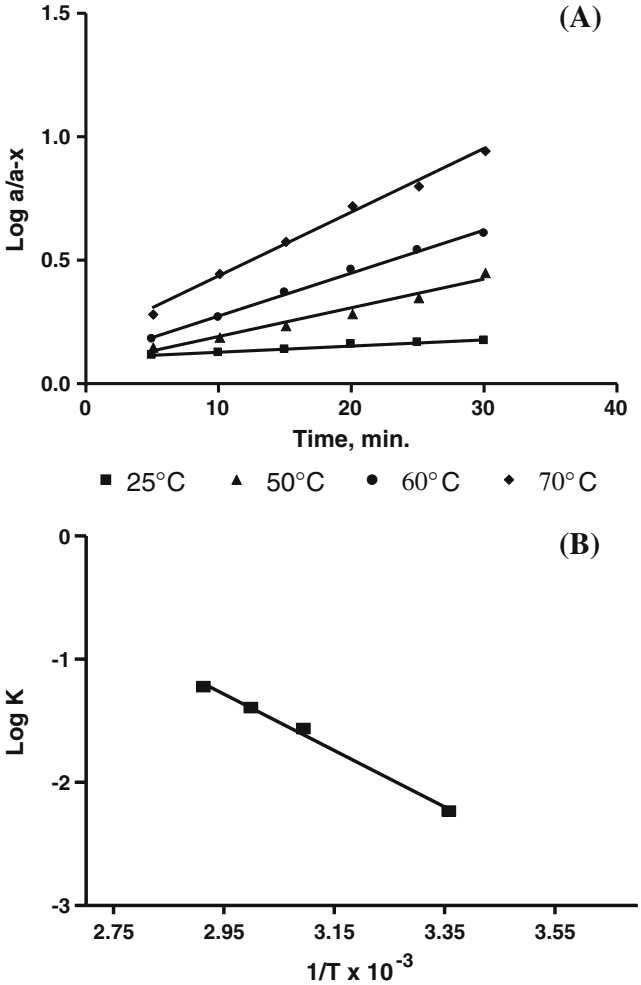



**Fig. 11** **a** Semi logarithmic plots of ETM (1 μg/mL) vs different heating times with 1% $H_2O_2$ (*w*/*v*) at different temperatures. **b** Arrhenius plot for the degradation of ETM (1 μg/mL) with 1% $H_2O_2$ (*w*/*v*)

The nominal content of ETM in real plasma (Table 6) was determined according to previous report [25] using the following equation:

$$\text{Recovery in vivo} = \text{Delivery in vivo} \times \text{Recovery in vitro}/\text{Delivery in vitro}$$

Where: Recovery in vivo is % recovery for the drug in real human plasma, Delivery in vivo is concentration of the drug in real plasma, Recovery in vitro is % recovery in spiked plasma, Delivery in vitro is concentration of the drug in spiked plasma

*Stability Studies for Method I*

Ethamsylate was found to be susceptible to alkaline degradation. It was found that, the fluorescence intensity of ETM

**Table 7** Effect of temperature on the kinetic parameters of ethamsylate (1 μg/mL) using 1% $H_2O_2$ (*w*/*v*) (method I)

| Temperature (°C) | K ($min^{-1}$) | $t_{1/2}$ (min) | $E_a$ (Kcal. $mol^{-1}$ Kelvin) |
|---|---|---|---|
| 25 | $5.78\times10^{-3}$ | 119.90 | 11.80 |
| 50 | $2.69\times10^{-2}$ | 25.76 | 8.47 |
| 60 | $4.00\times10^{-2}$ | 17.32 | 8.95 |
| 70 | $5.94\times10^{-2}$ | 11.67 | $\overline{X} = 9.74$ |

decreased with increasing the volume of NaOH. Leaving the drug in contact with 7 mL of 0.1 M NaOH at room temperature, 91% of the drug was degraded immediately.

The effect of UV and sun light on the stability of ETM was studied. Upon exposure of an aqueous solution of the drug to UV lamp and sun light for 6 h, 40% and 51% of ETM was degraded, respectively. After 6 h, no more degradation was observed for neither UV nor sun light. A plot of log relative fluorescence intensity vs time is shown in Fig. 10. The linear decrease in the RFI with time indicates that, the degradation reaction is first order. The rate of degradation of ETM was determined kinetically [26]. The reaction rate constants (K) and the half-life times ($t_{1/2}$= 0.693/K) were calculated. The reaction rate constants (K) were found to be $1.4\times10^{-3}$ and $2\times$10-3 $min^{-1}$, while the half-life times ($t_{1/2}$) were 8.2 and 5.7 h for UV and sun light, respectively.

*Degradation Kinetic Study Using Hydrogen Peroxide*
Oxidative degradation with hydrogen peroxide was also studied. Treatment of ETM with 1% $H_2O_2$ (w/v) was accompanied by a gradual decrease in the fluorescence intensity. It was found that, heating ETM with 1% $H_2O_2$ (*w*/*v*) at 70 °C for 30 min. resulted in about 89% degradation of the original sample.

The degradation was found to be temperature dependent (Fig. 11a). The apparent first order degradation rate constant, half-life time at each temperature and the activation energy Ea were calculated (Table 7). By plotting log $K_{obs}$ values versus 1/T, Arrhenius plot was obtained (Fig. 11b). Arrhenius equation [26] was found to be:

$$\text{Log}\,K = 5.468 + 2.289 \times 103/T$$

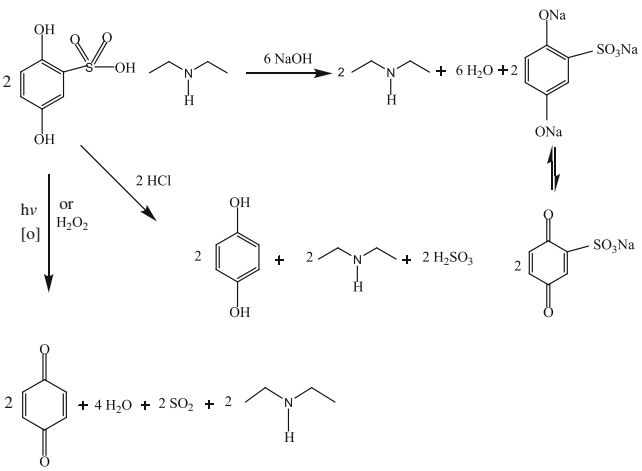
Where; K is the specific reaction rate, T is the absolute temperature.

*Pathway of Degradation*

ETM undergoes alkaline degradation and is proposed to be converted to the corresponding phenolate (sodium salt) which in turn is changed to quinone. In acidic condition, ETM is proposed to be decomposed to hydroquinone (HQ) as reported by Kaul et al. [3]. Upon heating ethamsylate ETM with 1% $H_2O_2$ (*w*/*v*) or exposure to UV or sun light, it undergoes oxidative degradation with the formation of quinone. The proposal of the degradation pathway is postulated in the following Scheme 1.

## Conclusion

The present study described fully validated and accurate spectrofluorometric methods for the determination of ETM either alone or in presence of HQ with enhanced sensitivity and specificity. The proposed methods don't require elaborate treatment for the plasma sample or tedious procedure for the extraction. As well as, the methods are sensitive enough for the analysis of lower concentration of ETM as low as 0.026, 0.06 μg/mL for methods I and II, respectively. Both methods could be applied to the analysis of ETM in pharmaceutical preparations. Method I, by virtue of its high sensitivity, is good analytical tool for the analysis of ETM in spiked and real human plasma. In addition, it was utilized to study the stability of ETM and its degradation kinetics using peroxide. And as Method II was used for the simultaneous determination of ETM and its acidic degradation product (HQ), so it could be considered as stability indicating method.




**Scheme 1** The proposed pathways of alkaline, acidic, UV and sun light degradation of ethamsylate

## References


1. Sweetman SC (ed) (2007) Martindale, the complete drug reference, 35th edn. Pharmaceutical Press, London, p 959
2. The British Pharmacopoeia (2007) The Stationery Office: London; Electronic version
3. Kaul N, Agrawal H, Kakad A, Dhaneshwar SR, Patil B (2005) Stress degradation studies on etamsylate using stability-indicating chromatographic methods. Anal Chim Acta 536(1–2):49–70
4. Zhang M, Zhang Y, Li Q (2010) A novel visible spectrophotometric method for the determination of ethamsylate in pharmaceutical preparations and biological samples. Spectrochim Acta A 75(3):1013–1017
5. Goyal A, Singhvi I (2008) Spectrophotometric estimation of ethamsylate and mefenamic Acid from a binary mixture by dual wavelength and simultaneous equation methods. Indian J Pharm Sci 70(1):108–111
6. El-Enany N, Belal F, Rizk M (2007) Kinetic spectrophotometric determination of ethamsylate in dosage forms. J AOAC Int 90 (3):679–685
7. El-Shabrawy Y, El-Enany N, Salem K (2004) Sensitive kinetic spectrophotometric determination of captopril and ethamsylate in pharmaceutical preparations and biological fluids. Farmaco 59 (10):803–808
8. Xu ZC, Li XY, Shi J, Yao XJ, Liu AZ, He ZS (1994) Determination of Dicynone [ethamsylate] in gastric mucosa by ultra-violet spectrophotometry. Fenxi Huaxue 22(4):420
9. Jaiswal YS, Talele GS, Surana SJ (2005) Quantitative analysis of ethamsylate and mefenamic acid in tablets by use of planar chromatography. J Planar Chromatogr Mod TLC 18(106):460–464
10. Jaiswal YS, Talele GS, Surana SJ (2005) A simple and sensitive HPTLC method for quantitative analysis of ethamsylate in tablets. J Planar Chromatogr Mod TLC 105(18):380–383
11. Ma J, Liu Y (1984) Quantitative analysis of etamsylate by h.p.l.c. Yaowu Fenxi Zazhi 4(4):209–211
12. Wang SF, Xu Q (2007) Electrochemical parameters of ethamsylate at multi-walled carbon nanotube modified glassy carbon electrodes. Bioelectrochemistry 70(2):296–300
13. Wang ZH, Li ZG, Zhou SP (2005) Voltammetric behavior of embedded super-thin carbon film electrode and its application. Fenxi Huaxue 33(4):523–526
14. Zhang XH, Wang SF (2005) Determination of ethamsylate in the presence of catecholamines using 4-amino-2-mercaptopyrimidine self-assembled monolayer gold electrode. Sens Actuators B 104 (1):29–34
15. Wang ZH, Zhang D, Zhang Y, Zhou SP (2001) Voltammetric behaviour of Dicynone at a poly(4-aminopyridine) film-modified electrode and its determination by adsorptive stripping voltammetry. Fenxi Huaxue 29(1):83–86
16. Yang FZ, Zhang C, Baeyens WRG, Zhang XR (2002) Determination of ethamsylate in pharmaceutical preparations based on an auto-oxidation chemiluminescence reaction. J Pharm Biomed Anal 30(3):473–478
17. Li YH, Du JX, Lu JR (2002) Chemiluminescence reaction of luminol-[potassium ferricyanide-potassium ferrocyanide]-ethamsylate system. Fenxi Huaxue 30(6):742–744
18. Du JX, Li YH, Tang Y, Lu JR (2002) Flow-injection chemiluminescence determination of ethamsylate based on permanganate oxidation. Anal Lett 35(3):463–472
19. Zhang CX, Huang JC, Feng ML, Zhang ZJ (1998) Flow injection chemiluminescence determination of etamsylate with electrogenerated hypochlorite. Anal Lett 31(11):1917–1928
20. Li J, Ju H (2006) Simultaneous determination of ethamsylate, tramadol and lidocaine in human urine by capillary electrophoresis with electrochemiluminescence detection. Electrophoresis 27 (17):3467–3474
21. Skoog DA, Holler FJ, Crouch SR (2007) Principles of instrumental analysis, 6th edn. Thomson, Belmont, p 406
22. Miller JN, Miller JC et al (2005) Statistics and chemometrics for analytical chemistry. Pearson Education Limited, Harlow, pp 39–73, 107–149, 256
23. ICH Harmonized Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology, Q2(R1), Current Step 4 Version, Parent Guidelines on Methodology Dated November 6 1996, Incorporated in November 2005. http://www.ich.org/LOB/media/MEDIA417.pdf (accessed February 15, 2008).
24. http://www.ompharma.com/dicynone/pharmacokinetics.html
25. Sun Y, Nakashima MN, Takahashi M, Kuroda N, Nakashima K (2002) Determination of bisphenol A in rat brain by microdialysis and column switching high-performance liquid chromatography with fluorescence detection. Biomed Chromatogr 16:319–326
26. Martin A, Bustamante P (1993) Physical pharmacy, 4th edn. Lea and Febiger, Pheladelphia, p 295