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High-Performance Liquid Chromatographic Determination of Flavoxate Hydrochloride and its Hydrolysis Product

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Liquid chromatographic method was presented for the determination of flavoxate hydrochloride (FX) and its hydrolysis product. The method was based on high-performance liquid chromatographic (HPLC) separation of FX from its hydrolysis product on CN column using a mobile phase consisting of acetonitrile-12 mM ammonium acetate (45:55, vol/vol, pH 4.0) with UV detection at 220 nm and flow rate of 1.5 mL min^{-1}. The proposed HPLC method for the determination of FX was utilized to investigate the kinetics of acidic hydrolytic process at different temperatures and to calculate its activation energy. In addition, the proposed HPLC method was used for pH-rate profile study of hydrolysis of FX in Britton–Robinson buffer solutions. The 3-methylflavone-8-carboxylic acid ethyl ester, as impurity of flavoxate hydrochloride, can be separated by the proposed HPLC method.

Keywords flavoxate hydrochloride; HPLC; kinetics of hydrolysis; pH-rate profile; Arrhenius plot

INTRODUCTION

Flavoxate hydrochloride (FX), 3-methylflavone-8-carboxylic acid (MFA) β-piperidinoethyl ester hydrochloride, belongs to a series of flavone derivatives, which exhibit strong smooth muscle relaxant activity, with selective action on the pelvic (Pedersen, 1977). It is used for the symptomatic relief of pain, urinary frequency, and incontinence associated with inflammatory disorders of the urinary tract. It is also used for the relief of vesico-urethral spasms resulting from instrumentation or surgery (Sweetman, 2002).

FX is readily absorbed from the gastrointestinal tract and rapidly metabolized in plasma to MFA (Cova & Setnikar, 1975). About 50–60% of a dose being excreted in the urine within 24 h as MFA (Sweetman, 2002). MFA did not display antispasmodic activity (Cazzulani et al., 1988).

The literature survey reveals that FX was analyzed in its pharmaceutical preparations by ultra-violet spectrophotometry (British Pharmacopoeia, 2005; Zheng, 1993) and high-performance liquid chromatography (HPLC) (Wang, Wang, & Zhang, 2002, Zarapkar et al., 1989). The official method for determination of FX is non-aqueous titration using perchloric acid as titrant in pure form and spectrophotometry in tablets (British Pharmacopoeia, 2005).

Several assay methods have been reported for the determination of MFA in biological fluids, including radiometric assay (Cazzulani, Panzarasa, Luca, Oliva, & Graziani, 1984), gas chromatography (Bertoli, Conti, Conti, Cova, & Setnikar, 1976; Pedersen, 1977), capillary electrophoresis (Zhang et al., 1993), and high-performance liquid chromatography (Sheu, Yeh, Keh, & Ho, 1958). Another HPLC method was developed for the determination of MFA in human urine using CN column with mobile phase consisting of acetonitrile-12 mM ammonium acetate (40:60, vol/vol) and calibration range of 0.3–25 μg mL^{-1} for complete separation of MFA from the endogenous components of urine (El-Gindy, Sallam, & Abdel-Salam, 2007).

FX is an ester-type drug susceptible to hydrolysis. Therefore, it was necessary to study the stability of FX toward hydrolysis process. However, no method has been reported for the simultaneous determination of FX and its hydrolysis products. The aim of this work was to develop an analytical method for the determination of FX and its hydrolysis product using HPLC methodology to investigate the kinetic of the acidic degradation process and to calculate the activation energy for FX.

EXPERIMENTAL

Instrumentation

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP

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system controller, DGU-12A Degasser, Rheodyne 7725i injector with a 20-μL loop, and a SPD-10AVP UV–VIS detector.

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

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

Materials and Reagents

Pharmaceutical grade of FX (Recordati, Milan, Italy) was used and certified to contain 99.7%. Acetonitrile and methanol used were HPLC grade (BDH, Poole, UK). Sodium hydroxide (Sigma-Aldrich, Inc., St. Louis, MO, USA), ammonium acetate (Sigma-Aldrich, Inc.), hydrochloric (Merck, Darmstadt, Germany), phosphoric (BDH Laboratory Supplies Poole, England), boric (Sigma-Aldrich, Inc.), and acetic acids (Riedel-de Haën Laboratory Chemicals, Germany) were used.

Commercial FX tablets (Genurin), batch no. 041037, were manufactured by Medical Union Pharmaceutical, Abu-Sultan, Ismailia, Egypt; Label claim was 200 mg FX per tablet with expiry date 10/2007.

HPLC Conditions

The HPLC separation and quantitation were made on a 250 × 4.6 mm (i.d.) Luna 5-μm CN column (Phenomenex, Cheshire, UK). The mobile phase was prepared by mixing acetonitrile and 12 mm ammonium acetate in a ratio 45:55 (vol/vol) and adjusted to the apparent pH 4.0 using acetic acid. The flow rate was 1.5 mL min^{-1}. All determinations were performed at room temperature. The injection volume was 20 μL. The detector was set at λ 220 nm for FX and its hydrolysis product. Data acquisition was performed on class-VP software.

Preparation of the Alkali-Induced Hydrolysis Product

One gram of FX was refluxed with 100 mL 0.1 M sodium hydroxide at 100°C for 30 min. Subsequently, the pH of the solution was adjusted to 3.4 using 1 M hydrochloric acid to precipitate the hydrolysis product of FX. The precipitate was filtered and dried under vacuum. The dried precipitate was analyzed by using IR and PMR and found to be 3-methyl flavone-8-carboxylic acid (MFA). The yield of MFA was 652 mg. The filtrate was washed three times, each with 10 mL chloroform. The washed aqueous extract was evaporated using rotary evaporator and dried under vacuum. The residue was analyzed by using IR and PMR and found to be 2-(1-piperidino) ethanol (PP). The yield of PP was 301 mg.

Preparation of the Acid-Induced Hydrolysis Product

One gram of FX was refluxed with 100 mL 0.1 M hydrochloric acid at 100°C for 3 h. Subsequently, the pH of the solution was adjusted to 3.4 using 1 M sodium hydroxide to precipitate the hydrolysis product of FX. The same procedure for separation of MFA and PP described under preparation of the alkali-induced hydrolysis product was followed.

The hydrolysis of FX was carried out using two different hydrolysis methods, acid-induced and alkali-induced, to ensure that the different type of hydrolysis process given the same hydrolysis products.

Both of MFA and PP manufactured by acid-induced hydrolysis and alkali-induced hydrolysis were characterized by IR and PMR.

The samples were prepared for IR analysis by mixing 2 mg of each sample with 300 mg of finely powdered and dried potassium bromide, and compressed in the form of disc of 10 mm diameter.

The samples were prepared for PMR analysis by dissolving 3 mg of each sample in 5 mL dimethylsulfoxide and transferring to the PMR specimen tube. The specimen tube is placed in a probe located in the magnetic field of PMR spectrometer.

Standard Solutions and Calibration Graphs

Stock solutions were prepared by separately dissolving FX and MFA (white crystals came from alkaline hydrolysis, with melting point 237°C) in methanol to obtain a concentration of 50 μg mL^{-1}. The standard solutions were prepared by diluting the stock solutions with the HPLC mobile phase to reach the concentration range of 2.5–20 μg mL^{-1} for FX and 1–20 μg mL^{-1} for MFA.

Triplicate 20-μL injections were made for each concentration of FX and MFA and chromatographed under the conditions described above. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph of each compound. Linear relationship was obtained for each compound.

Sample Preparation

The contents of 20 Genurin® tablets were weighed and finely powdered. Accurately weighed portion of the powder equivalent to about 50 mg of FX was extracted and diluted to 100 mL with methanol. The sample solution was filtered.

Further dilutions of the filtrated tablet sample were carried out with the HPLC mobile phase to reach concentration range 2.5–20 μg mL^{-1} for FX. The general procedures described under calibration were followed.

Recovery Test Method

Recovery test was performed by addition of known amounts of FX and MFA to a known concentration of the commercial tablets. The recovery was assessed using nine determinations over three concentration levels, covering the specified calibration range of FX and MFA. The percentage of recovery by the assay of the known added amount of FX and MFA in each sample was calculated.

Kinetic Investigation

Accurately weighted 50 mg aliquots of FX were dissolved in 250 mL 0.1 M hydrochloric acid. Separate 5 mL aliquots of this solution were transferred into separate durable stoppered conical flasks for high temperatures up to 100°C. The flasks were placed in a thermostatic oven at different temperatures (90, 80, 75, 60, and 50°C) for different time. At each 15 min, the pH of the content of each flask was adjusted to pH 7.0 using predetermined volume of 0.2 M sodium hydroxide solution. The contents of the flasks were transferred into 50 mL volumetric flasks and diluted to volume with mobile phase.

Aliquots of 20 µL of each solution were chromatographed under the conditions described above and the concentration of the remaining FX was calculated at each temperature and time interval.

pH-Rate Profile

Accurately weighted 50 mg aliquots of FX were transferred into 250 mL volumetric flasks and diluted to volume with Britton–Robinson buffer solutions. The pH values of Britton–Robinson buffer solutions (Brezina & Zuman, 1958) used for the measurement of the pH-rate profile of the hydrolysis of FX were as follows: 2.0, 3.0, 4.0, 5.0, 5.8, 6.7, 8.0, 9.0, 10.7, and 12.3. Separate 5 mL aliquots of the buffer solutions containing FX were transferred into separate durable stoppered conical flasks for high temperatures up to 100°C. The flasks were placed in a thermostatic oven at 75°C for different time. At each 10 min, the pH of the content of each flask was adjusted to pH 7.0 using 1 M sodium hydroxide or 1 M hydrochloric acid. The contents of flasks were transferred into 50 mL volumetric flasks and diluted to volume with the mobile phase. Aliquot of 20 µl of each solution was chromatographed under the conditions described above and the concentration of the remaining FX was calculated at each pH value and time interval.

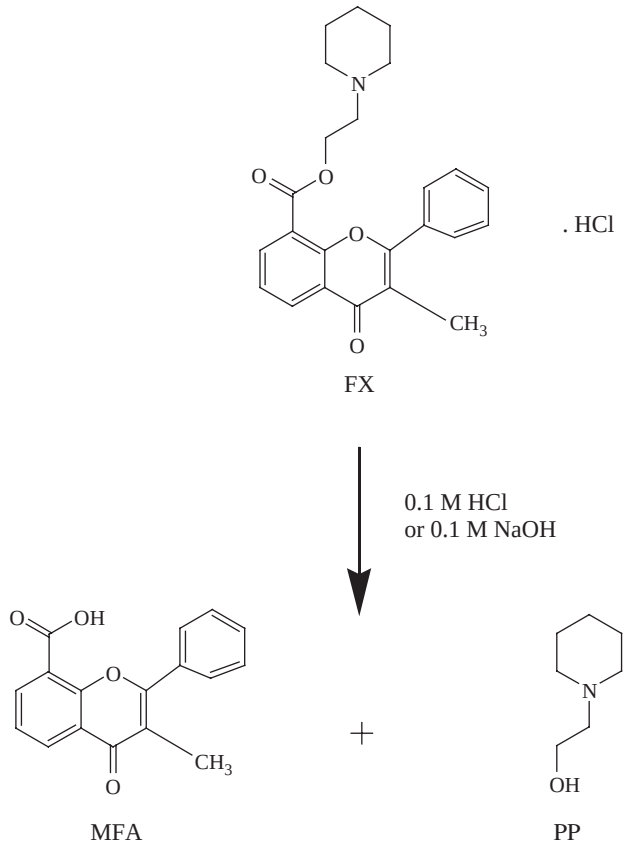
RESULTS AND DISCUSSION

Identification of the Hydrolysis Products

When FX was boiled with 0.1 M sodium hydroxide for 30 min or 0.1 M hydrochloric acid for 3 h, MFA and PP could be isolated from the reaction mixture as hydrolysis products of FX. The suggested pathway reaction equation for the hydrolysis of FX in 0.1 M sodium hydroxide and 0.1 M hydrochloric acid is presented in Scheme 1.

Proton Magnetic Resonance (PMR) spectroscopy has been used for structure elucidation of the unknown specimens using chemical shift multiplicity of the signals and number of protons under each signal. The PMR analysis usually coupled with other analytical techniques such as infra red (IR).

The assignments of the hydrolysis product MFA as 3-methylflavone-8-carboxylic acid was based on the comparison of the IR and PMR spectral data of the purified specimen, separated from the hydrolysis reaction, with this of the intact FX.



SCHEME 1. Mechanism of hydrolysis of FX in 0.1 M hydrochloric acid and 0.1 M sodium hydroxide.

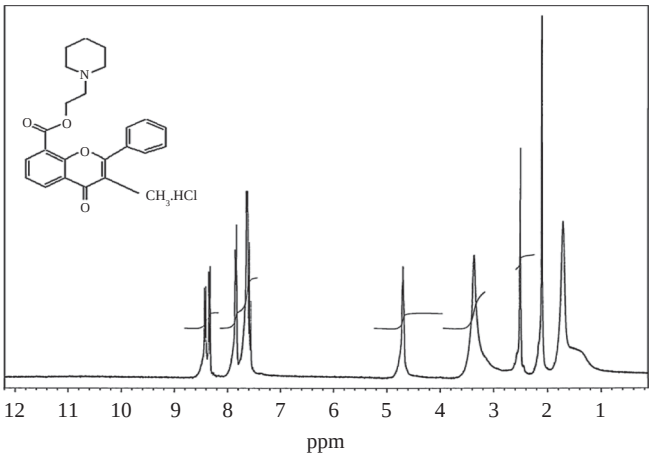


FIGURE 1. PMR spectrum of FX in dimethylsulfoxide.

The PMR spectrum of FX (Figure 1) in dimethylsulfoxide was characterized by the appearance of the protons of methyl

group attached to the flavone ring at δ 2.090 ppm (singlet, 3H, CH_3–flavone); protons of piperidine ring at δ 1.70 ppm (multiplet, 6H, piperidine C_3-H_2, C_4-H_2, C_5-H_2), δ 2.493 ppm (multiplet, 4H, piperidine C_2-H_2, C_6-H_2). Also, the appearance of protons of ethyl chain attached to the piperidine ring at δ 3.349 ppm (multiplet, 2H, $N-CH_2-CH_2-$), δ 4.683 ppm (multiplet, 2H, $N-CH_2-CH_2-$) and appearance of aromatic protons at δ 7.555–8.425 ppm.

By contrast, the PMR spectrum of MFA (Figure 2) in the same solvent lacked the characteristic protons of piperidine ring and ethyl chain attached to piperidine ring and showed protons signals of methyl group attached to the flavone ring at δ 2.111 ppm (singlet, 3H, CH_3-flavone) and appearance of aromatic protons at δ 7.529–8.302 ppm.

On the contrary, the PMR spectrum of PP (Figure 3) in the same solvent lacked the characteristic aromatic protons but showed protons of piperidine ring at δ 1.662 ppm (multiplet, 6H, piperidine C_3-H_2, C_4-H_2, C_5-H_2) and δ 2.493 ppm (multiplet, 4H, piperidine C_2-H_2, C_6-H_2). Also, the appearance of protons of 2-ethyl chain attached to the piperidine ring at δ 2.823 ppm (multiplet, 2H, $-N-CH_2-CH_2-O$) and δ 3.029 ppm (multiplet, 2H, $N-CH_2-CH_2-O$).

Table 1 represents the expected and found PMR spectral assignment for FX, MFA, and PP.

The IR spectrum (KBr) of FX (Figure 4) was characterized by the absorption frequency of C=O ester band at 1717 and C−N stretching band at 1205 cm^{-1}. On the contrary, the IR spectrum (KBr) of MFA (Figure 5) revealed the OH association, C=O stretching, and in-plane C−O bending of the COOH at 2750–3500, 1617, and 1334 cm^{-1}, respectively. Moreover, the spectrum lacked the characteristic ester C=O stretching band of FX. Moreover, the IR spectrum (KBr) of PP (Figure 6) showed the appearance of band at 3446 cm^{-1} corresponding to the alcoholic O−H stretching, whereas the FX lacked this band.

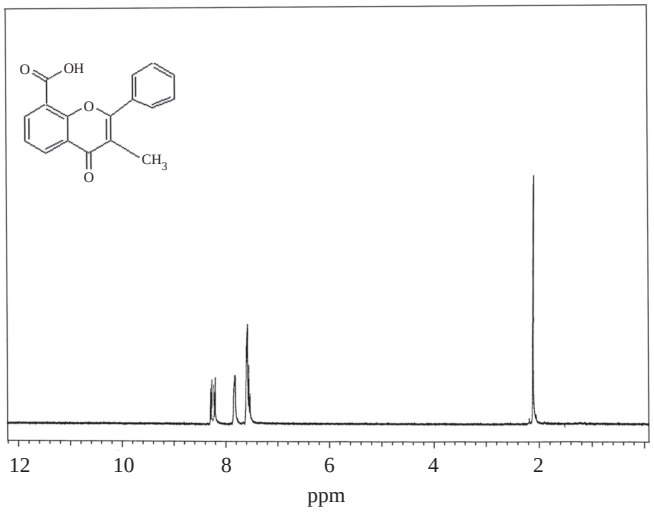


FIGURE 2. PMR spectrum of MFA in dimethylsulfoxide.

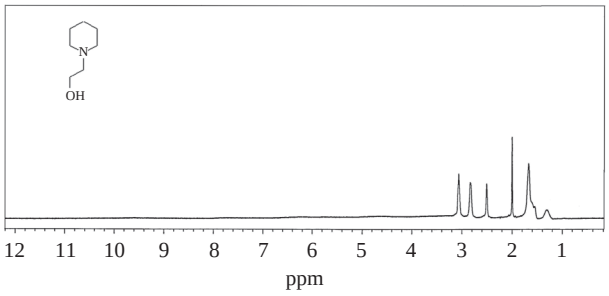


FIGURE 3. PMR spectrum of PP in dimethylsulfoxide.

Assay Parameters

HPLC Method

Figure 7 shows the UV absorption of FX and its hydrolysis products MFA and PP.

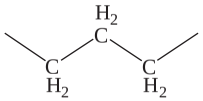
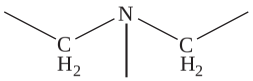
As can be seen from the figure, the UV absorption bands of FX and MFA are extensively overlapped, whereas the UV absorption spectrum of PP is too weak to be determined.

The simultaneous determination of FX and MFA by conventional, derivative, and derivative ratio spectrophotometric methods is hindered by spectral overlap throughout the wavelength range.

Therefore, HPLC method was necessary for such determination because of the presence of this spectral interference.

To optimize the HPLC assay parameters for their determination, the effect of acetonitrile composition and the apparent pH of the mobile phase on the capacity factor (K′) were studied. A satisfactory separation for FX and MFA was obtained with a mobile phase consisting of acetonitrile and 12 mm ammonium acetate (45:55, vol/vol, pH 4.0) at ambient temperature (Figure 8). Increasing acetonitrile concentration to more than 60% led to inadequate separation of FX and its hydrolysis product MFA. At lower acetonitrile concentration (less than 30%), separation occurred but with excessive tailing and increased retention time for FX peak. Variation of apparent pH of the mobile phase resulted in maximum K′ value at pH 7.0 with loss of peak symmetry for FX. At lower pH values (1.5–2.5), bad resolution for FX and MFA was observed. At pH 3.5–4.5, improved resolution of the two peaks was observed with optimal resolution and retention time observed at pH 4.0. The average retention time ± *SD* for FX and MFA were found to be 4.0 ± 0.007 and 2.6 ± 0.005 min, respectively, for 10 replicates. The peaks obtained were sharp and have clear base line separation. The void volume was found to be at 0.9 min. The system suitability test results of the developed method are summarized in Table 2.

TABLE 1
Expected and Found PMR Spectral Assignment for FX, MFA, and PP

Chemical Shift										
FX Expected	MFA Expected	PP Expected	FX Expected	MFA Expected	PP Found	Multiplicity Expected	Multiplicity Found	Number of Protons	Number of Protons Found	Assignment
1.500	—	1.500	1.700	—	1.662	Multipet	Multipet	6	6	
1.930	1.930	—	2.090	2.111	—	Singlet	Singlet	3	3	—CH_3
2.740	—	2.740	2.493	—	2.493	Multipet	Multipet	4	4	 Alicyclic of pipredine
2.555	—	2.555	3.349	—	2.823	Multipet	Multipet	2	2	=N—$\underline{CH_2}$
3.630	—	3.360	4.683	—	3.029	Triplet	Multipet	2	2	=N—$\underline{CH_2}$—$\underline{CH_2}$
7.210:8.240	7.210:8.240	—	7.555:8.425	7.529:8.302	—	Multipet	Multipet	8	8	Aromatic protons

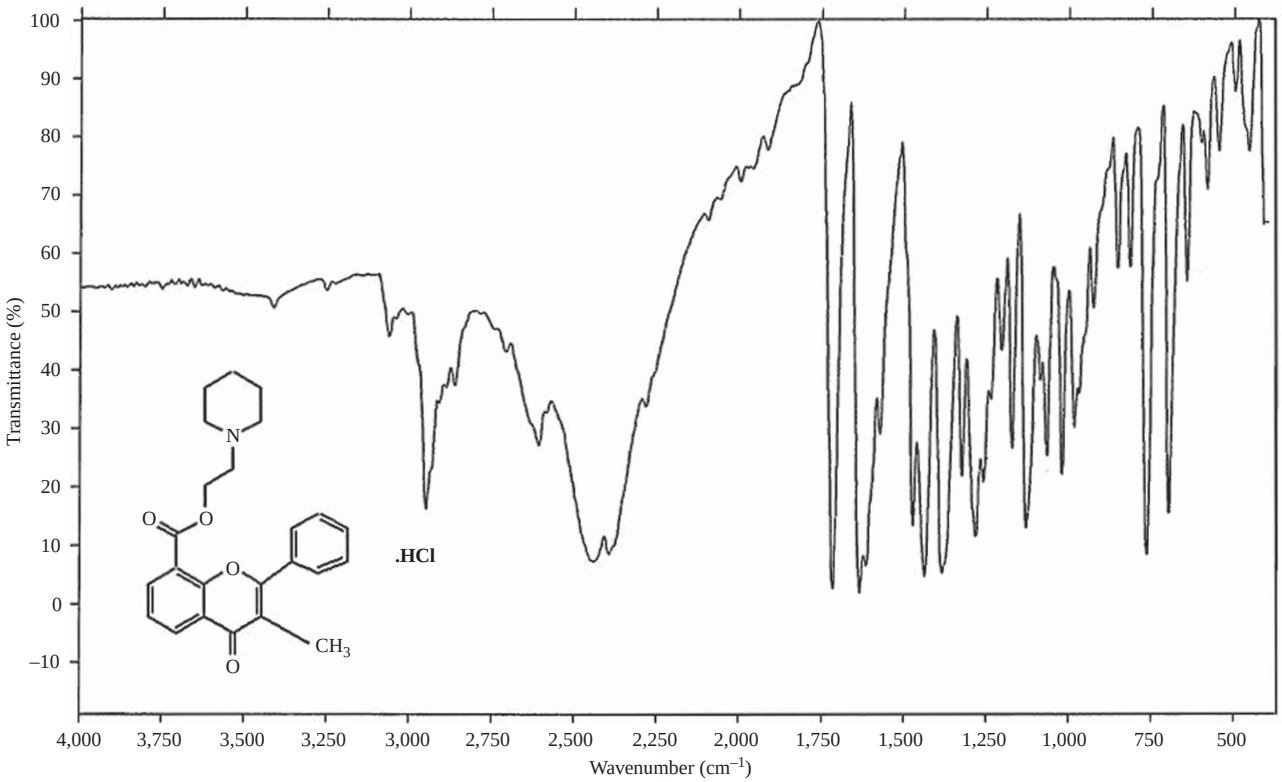


FIGURE 4. IR spectrum of FX in KBr.

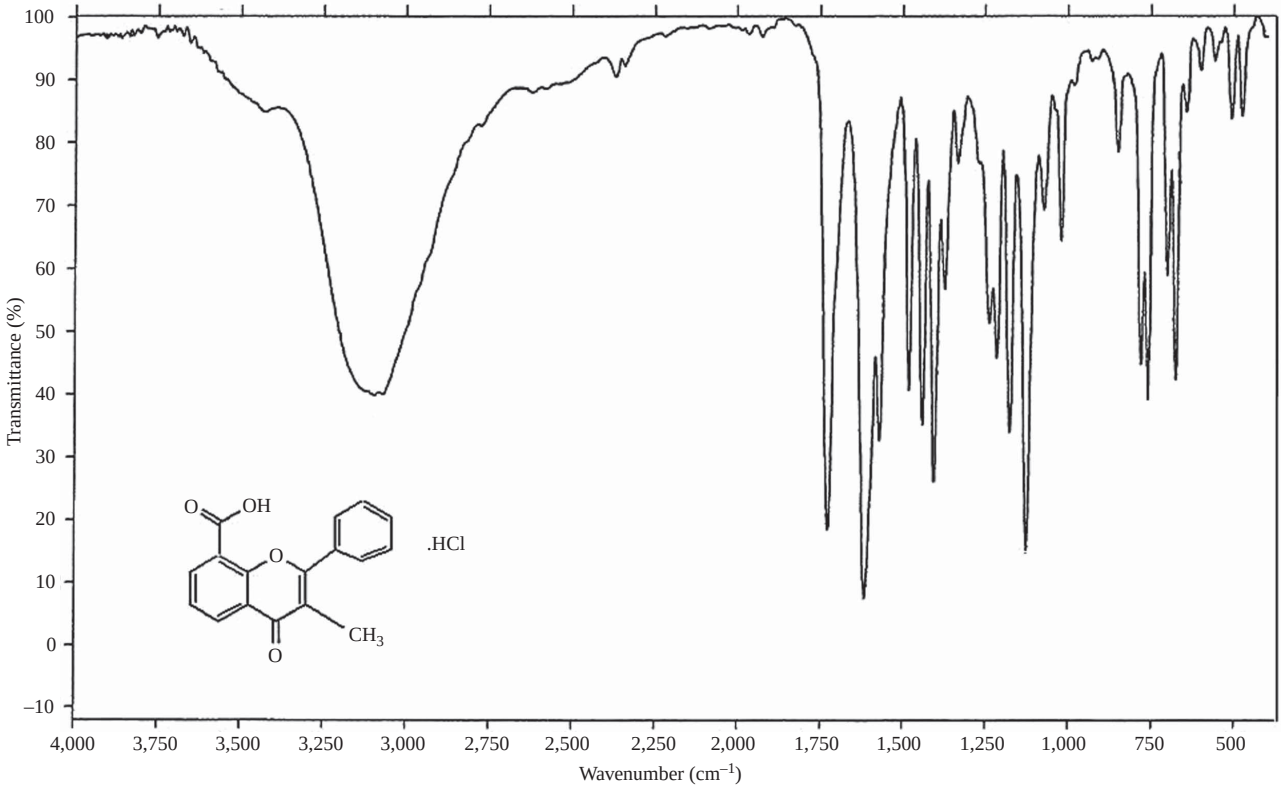


FIGURE 5. IR spectrum of MFA in KBr.

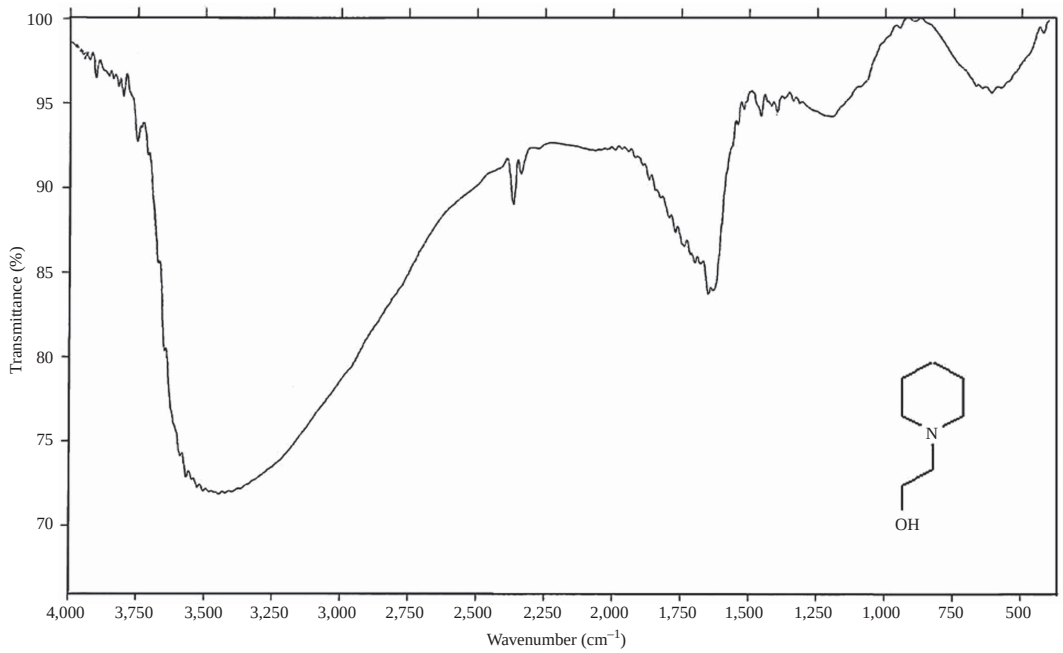


FIGURE 6. IR spectrum of PP in KBr.

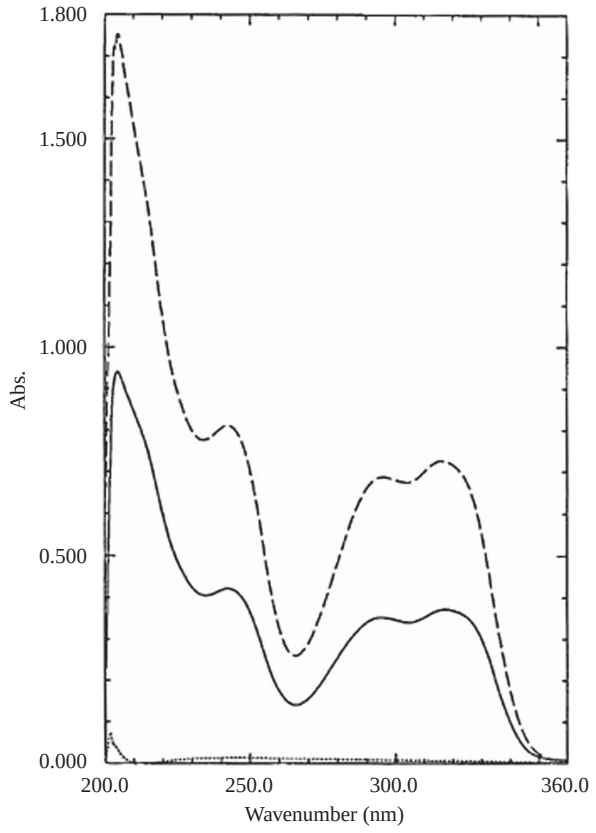


FIGURE 7. UV absorption spectra of 10 μg mL^{-1} of FX (——), 10 μg mL^{-1} of MFA (– – – –), and 10 μg mL^{-1} of PP (------) in 0.1 M hydrochloric acid.

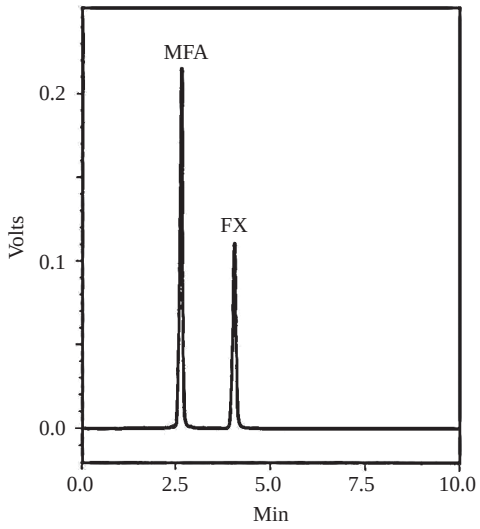


FIGURE 8. Typical HPLC of 20 μL injection of laboratory-prepared mixture of 10 μg mL^{-1} FX and 10 μg mL^{-1} of its hydrolysis product MFA.

Pharmaceutical Product Analysis

The proposed method was applied to the determination of FX in its commercial tablets. Nine replicate determinations were made. Satisfactory results were obtained in a good agreement with the label clams (Table 3). The results for the determination of FX were compared with the official spectrophotometric method depending on

TABLE 2
The System Suitability Test Results of the Developed Method

Compound	Retention Time (min)	Capacity Factor (K′)	Selectivity α	Resolution R_s	Tailing Factor
MFA	2.6	1.7	2.02	1.83	1.21
FX	4.0	3.44	—	—	1.17

TABLE 3
Determination of FX in Laboratory-Prepared Mixtures and Commercial Tablets Using the Proposed Methods

	Mean Found ± *SD*[a]	
Laboratory-Prepared Mixtures	HPLC	Official Method [6]
FX	99.04 ± 3.21	
MFA	99.85 ± 0.50	
Genurin® tablets[b]		
FX	99.70 ± 0.52	99.50 ± 0.54
T	0.67	(2.18)[c]
F	1.27	(4.28)[c]
Recovery[d]		
FX	99.99 ± 0.52	
MFA	100.03 ± 0.50	

[a]Mean and *SD* for nine determinations, percentage recovery from the label claim amount.
[b]Batch no.: 041037.
[c]Theoretical values for *T* and *F*.
[d]For standard addition of different concentrations of FX and MFA.

measuring the UV absorbance at 293 nm (British Pharmacopoeia, 2005). Statistical comparison of the results was performed with regard to accuracy and precision using Student's *t*-test and the F-ratio at 95% confidence level (Table 3). There is no significant difference between the proposed and the official method with regard to accuracy and precision.

Expired commercial FX tablets (trade name: Genurin), batch no. 010355, manufactured by Medical Union Pharmaceutical, Abu-Sultan, Ismailia, Egypt, stored at ambient temperature and humidity and protected from light, were analyzed by the proposed method. The percentage of declared content of FX was found to be 87.5%, and the concentration of MFA was found to be 16 mg/tablet. The HPLC chromatogram of expired commercial tablets of FX showed the peak of the hydrolysis product of MFA (Figure 9).

Kinetic Investigation

It was difficult to study the kinetics of hydrolysis of FX in 0.1 M sodium hydroxide because of the complete and instant hydrolysis of FX.

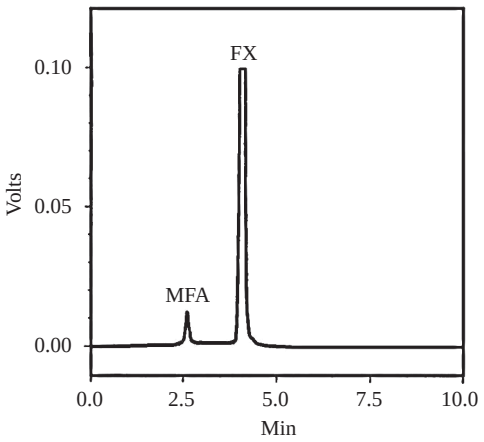


FIGURE 9. Typical HPLC of expired Genurin tablets containing FX and its hydrolysis product MFA.

The kinetics of hydrolysis of FX was investigated in 0.1 M hydrochloric acid, because the hydrolysis rates of FX at lower strengths of hydrochloric acid were too slow to obtain reliable kinetic data. A regular decrease in the concentration of intact FX with increasing time intervals was observed. At the selected temperatures (50, 60, 75, 80, and 90°C); the hydrolytic process followed pseudo first-order kinetics (Figure 10). From the slopes of the straight lines, it was

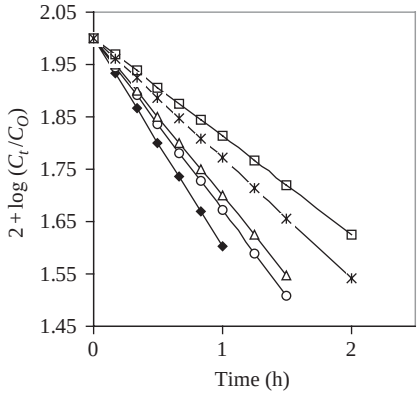


FIGURE 10. Pseudo first-order plots for the hydrolysis of FX in 0.1 M hydrochloric acid at various temperatures. Key: 50 (□); 60 (×); 75 (Δ); 80 (○); and 90 (■); C_t, concentration at time *t*, and C_0, concentration at zero time.

possible to calculate the apparent first-order hydrolysis rate constant and the half-life at each temperature for acidic hydrolytic process (Table 4). Plotting log K_{obs} values versus $1/T$; the Arrhenius plot (Figure 11) was obtained, which was found to be linear in the temperature range 50–90°C. The activation energy was calculated for FX and found to be 4.343 kcal mol^{-1}.

The pH-rate profile of hydrolysis of FX in Britton–Robinson buffer solutions were studied at 75°C (Figure 12). 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 were calculated for each pH value (Table 5). FX was found to be most stable at a pH of 4.

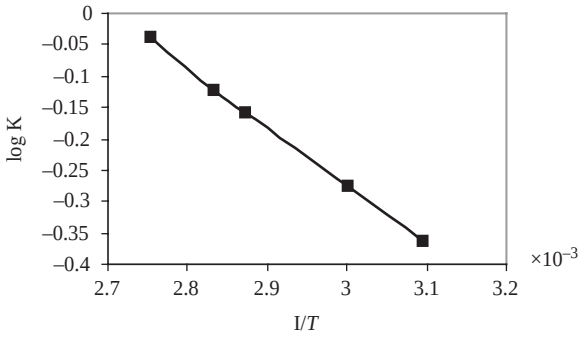


FIGURE 11. Arrhenius plot for the hydrolysis of FX in 0.1 M hydrochloric acid.

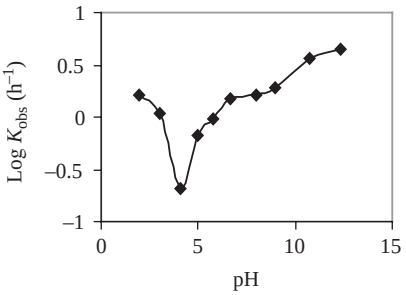


FIGURE 12. pH-rate profile for the hydrolysis of FX in Britton–Robinson buffer at 75°C.

Validation

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in samples within a given range.

The linearity of the proposed method was evaluated by analyzing seven concentrations of FX, and MFA ranging between 2.5–20 and 1–20 μg mL^{-1}, respectively. Each concentration was repeated three times. The assay was performed according to experimental conditions previously established. The linearity of the calibration graphs and adherence of the system to Beer's law were validated by the high value of the correlation coefficient and the intercept value, which was not statistically ($p = 0.05$) different from zero (Table 6).

Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation of a series of measurements.

Precision may be a measure of the degree of intermediate precision and repeatability of the analytical method under normal operating conditions. Intermediate precision expresses within-laboratory variation, as on different days within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment.

TABLE 4
Hydrolysis Rate Constant (K_{obs}) and Half-Life ($t_{1/2}$) for FX in 0.1 M Hydrochloric Acid

Temperature (°C)	K_{obs} (h^{-1})	$t_{1/2}$ (h)
50	0.431	1.608
60	0.528	1.313
75	0.693	0.999
80	0.755	0.918
90	0.916	0.756

TABLE 5
Hydrolysis Rate Constant (K_{obs}) and Half-Life ($t_{1/2}$) for FX in Britton–Robinson Buffer at Different pH Values and 75°C

pH	K_{obs} (h^{-1})	$t_{1/2}$ (h)
12.3	4.531	0.153
10.7	3.642	0.190
9.0	1.871	0.370
8.0	1.602	0.433
6.7	1.508	0.460
5.8	0.944	0.734
5.0	0.669	1.035
4.0	0.211	3.289
3.0	1.090	0.636
2.0	1.597	0.434

TABLE 6
Characteristic Parameters for the Regression Equations of the Proposed High-Performance Liquid Chromatography (HPLC) Method for The Determination of FX and MFA

	HPLC	
Parameters	FX	MFA
Calibration range (μg mL^{-1})	2.5–20	1–20
Detection limit (μg mL^{-1})	4.0×10^{-2}	5.5×10^{-2}
Quantitation limit (μg mL^{-1})	1.3×10^{-1}	1.8×10^{-1}
Regression equation (Y)[a]: Slope (b)	1.5×10^{3}	3.2×10^{3}
Standard deviation of the slope (S_b)	19.4	56.1
Relative standard deviation of the slope (%)	1.3	1.8
Confidence limit of the slope[b]	$14.9 \times 10^{2} - 15.3 \times 10^{2}$	$31.0 \times 10^{2} - 32.0 \times 10^{2}$
Intercept (a)	(-2.5×10^{3})	(-1.3×10^{3})
Standard deviation of the intercept (S_a)	2.1×10^{2}	6.2×10^{2}
Confidence limit of the intercept[b]	$(-27.0 \times 10^{2}) - (-22.8 \times 10^{2})$	$(-19.2 \times 10^{2}) - (-71.9 \times 10^{1})$
Correlation coefficient (r)	0.9999	0.9998
Standard error of estimation	1.1×10^{2}	3.4×10^{2}

[a]$Y = a + bC$, where C is the concentration of FX and MFA in μg mL^{-1} and Y is the peak area.
[b]95% confidence limit.

TABLE 7
Within-Day and Between-Day Precision Values for Determination of FX and MFA

Within-Day Precision			Between-Day Precision	
Concentration (μg mL^{-1})	Mean Measured Concentration ± SD	Percentage of RSD	Mean Measured Concentration ± SD	%RSD
FX				
2.5	2.50 ± 0.04	1.42	2.45 ± 0.04	1.63
5	4.92 ± 0.07	1.53	4.89 ± 0.09	1.84
10	9.93 ± 0.09	0.89	9.82 ± 0.15	1.50
15	14.93 ± 0.21	1.42	14.95 ± 0.19	1.25
20	19.9 ± 0.08	0.41	19.93 ± 0.22	1.10
MFA				
1	0.98 ± 0.02	1.89	0.98 ± 0.01	1.02
5	4.93 ± 0.09	1.82	4.97 ± 0.09	1.81
10	9.82 ± 0.12	1.17	9.95 ± 0.19	1.88
15	14.93 ± 0.16	1.09	14.92 ± 0.19	1.30
20	19.98 ± 0.15	0.74	19.97 ± 0.19	0.93

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range of the procedure (i.e., three concentrations and three replicates of each concentration).

For evaluation of the precision estimates, repeatability and intermediate precision were performed at five concentration levels for FX and MFA. The data for each concentration level were evaluated by 5-replicate analysis for FX and MFA. Similarly, the between-day precision was evaluated in several days up to 5 days (Table 7). Every day, calibration graph was constructed and the results were calculated in comparison with the calibration graph. The results of FX and MFA indicated high precision, as the RSD did not exceed 2%.

Range

The range of the method is validated by verifying that analytical method provides acceptable precision, accuracy, and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

The calibration range was established through consideration of the practical range necessary, according to FX concentration present in pharmaceutical product, to give accurate, precise,

and linear results. The calibration range of the proposed method is given in Table 6.

Detection and Quantitation Limits

Detection limit is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions, whereas the quantitation limit is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

According to ICH recommendations (ICH Guidance, 1996), the approach based on the *SD* of the response and the slope was used for determining the detection and quantitation limits. The theoretical values were assessed practically and given in Table 6.

Selectivity

Method selectivity was achieved by preparing different laboratory-prepared mixtures of FX with MFA within the linearity range concentration in the ratio ranged from 1:0.05 to 1:8 for FX : MFA. The laboratory-prepared mixtures were analyzed according to the previous procedures described under the proposed method. Satisfactory results were obtained (Table 3), indicating the high selectivity of the proposed method for determination of the drug and its hydrolysis product.

Extreme hydrolysis of FX in aqueous solution was achieved by acid and alkaline hydrolysis. No peak interfering with the elution of FX was observed.

The influence of the commonly used tablet excipients (lactose, pregelatinized starch, magnesium stearate, sodium lauryl sulfate, and crospovidone) was investigated before the determination of FX in its pharmaceutical tablets. No interference could be observed with the proposed method.

Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. The accuracy of an analytical method should be established across its range. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample.

This study was performed by the addition of known amounts of FX and MFA to a known concentration of the commercial tablets (standard addition method). The resulting mixtures were assayed and the results obtained for FX and its hydrolysis product MFA were compared with the expected results. The excellent recoveries of standard addition method (Table 3) suggested the good accuracy of the proposed method.

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Variation of the pH of the mobile phase by ±0.1 and its organic strength by ±2% did not have a significant effect on HPLC resolution.

The proposed method was applied by a second analyst using another HPLC instrument. No significant differences in the results were obtained.

Analytical Solution Stability

The stability of FX and MFA analytical solutions in mobile phase or methanol was tested at room temperature for 24 h and at refrigerator at 5°C for 4 days. No chromatographic changes were observed for 24 h at room temperature and for 4 days at 5°C.

CONCLUSION

The proposed HPLC method provides simple, accurate, and reproducible quantitative analysis for the simultaneous determination of FX, and its hydrolysis product MFA in its pharmaceutical tablets, without any interference from tablet excipients. It was found that FX is rapidly hydrolyzed in alkaline medium, whereas FX exhibits moderate stability in acidic medium. The most hydrolytic stability of FX was found to be at pH 4.0. The proposed method for the simultaneous determination of FX and MFA was completely validated and suitable for quality control laboratories.

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