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Spectrofluorimetric determination of 3-methylflavone-8-carboxylic acid, the main active metabolite of flavoxate hydrochloride in human urine

Hala E. Zaazaa [a,*], Afaf O. Mohamed [b,1], Maha A. Hawwam [b], Mohamed Abdelkawy [a]

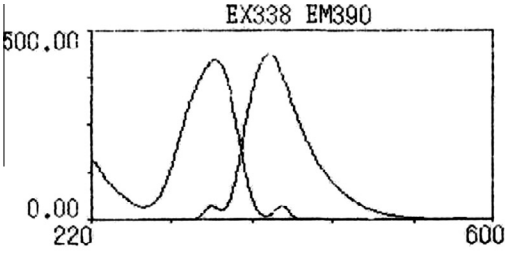
[a] Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, 11562 Cairo, Egypt
[b] National Organization for Drug Control and Research (NODCAR), 6-Abu Hazem Street, Pyramids Ave., P.O. Box 29, 35521 Giza, Egypt

HIGHLIGHTS

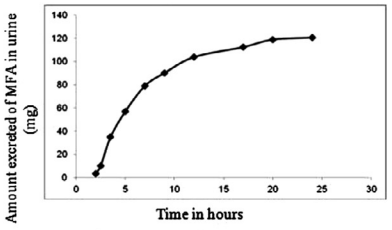
- Fluorimetric determination of flavoxate HCl via its metabolite and or degradation product; MFA.
- As MFA excreted in urine, so excretion studies of FLV can be performed via monitoring MFA in urine.
- The urinary excretion pattern has been calculated.
- The method was applied for the analysis of FLV in pharmaceutical formulation.

GRAPHICAL ABSTRACT

Excretion studies of flavoxate by monitoring the fluorescence character of its active metabolite (MFA) in urine.



Excitation and emission spectra of 4μg/mL **MFA** in methanol (at λ_{em}390 nm with λ_{ex}338 nm



Cumulative curve of amount excreted of MFA in urine after oral administration of commercial tablet containing 200 mg FX

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ABSTRACT

A simple, sensitive and selective spectrofluorimetric method has been developed for the determination of 3-methylflavone-8-carboxylic acid as the main active metabolite of flavoxate hydrochloride in human urine. The proposed method was based on the measurement of the native fluorescence of the metabolite in methanol at an emission wavelength 390 nm, upon excitation at 338 nm. Moreover, the urinary excretion pattern has been calculated using the proposed method. Taking the advantage that 3-methylflavone-8-carboxylic acid is also the alkaline degradate, the proposed method was applied to in vitro determination of flavoxate hydrochloride in tablets dosage form via the measurement of its corresponding degradate. The method was validated in accordance with the ICH requirements and statistically compared to the official method with no significant difference in performance.

Introduction

Flavoxate hydrochloride (FLV) Fig. 1, 3-methylflavone-8-carboxylicacid β-piperidinoethyl ester hydrochloride, belongs to a series of flavone derivatives which exhibits strong smooth muscle relaxant activity, especially on the urogenital tract [1]. 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 relief of vesicourethral [2]. A pharmacokinetic study in humans has shown that FLV, when given orally, is rapidly and completely absorbed and metabolized into 3-methylflavone-8-carboxylic acid (MFA) which has a direct action on smooth muscles of urinary tract through the inhibition of phosphodiesterase

* Corresponding author. Tel.: +20 01001543567.
E-mail addresses: hazaza@hotmail.com, hala.zaazaa@pharma.cu.edu.eg (H.E. Zaazaa).
[1] Present Address: Umm Al-Qura University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, P.O. Box 13174, Makkah, Saudi Arabia.

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(PDE) enzyme [3,4]. As MFA excreted in urine, so excretion studies of FLV can be performed via monitoring MFA in urine.

The literature survey reveals that FLV was analyzed in its pharmaceutical preparation by spectrophotometric [5,6], HPLC [7–11], TLC [11] voltammetric [12] and potentiometric [13] methods. Some methods are stability indicating, which analyzed FLV in the presence of MFA as degradation product [9–11,13], others focused on the analysis of MFA as active metabolite in biological fluids; these include HPLC [14,15], capillary electrophoresis [16] and TLC [17] methods. Additionally, FLV was analyzed in British Pharmacopeia in pure form by non-aqueous titration using perchloric acid as a titrant and by spectrophotometric method in tablets dosage form [18].

Although chromatographic methods have a high degree of specificity, yet, sample clean up and instrumentation limitations preclude their use in routine clinical studies. This led us to study fluorescence characteristics of FLV and MFA as an attempt to develop a simple, sensitive and reliable method for their determination. The native fluorescence of MFA initiated the present study that aimed to monitoring the excretion of FLV via its metabolite, MFA, in human urine. Furthermore, MFA is also reported to be the degradation product of FLV [8,9,11], thus the method was extended to the analysis of FLV in its dosage form via MFA measurement. The method is a good alternative to HPLC methods, and the results obtained were satisfactorily accurate and precise.

Experimental

Instrumentation

- The fluorescence intensities were measured using Shimadzu RF – 1501 spectrofluorimeter equipped with 20 KW Xenon lamp, excitation, emission grating monochromators and a 1 cm quartz cell. Centrifuge (Memmert-Germany). A pH-meter, Digital pH/MV/TEMP/ATC meter, Jenco Model-5005 (USA).

Materials and reagents

Pharmaceutical grade of FLV (Recordati, Milan, Italy) was used and certified to contain 99.7% according to the official BP method, which is non-aqueous titration using perchloric acid as titrant. Commercial Genurin tablets were manufactured by Medical Union Pharmaceutical, Abu-Sultan, Ismailia, Egypt, labeled to contain 200 mg FLV per tablet.

Standard solutions

Stock standard solution MFA (derived from complete alkaline hydrolysis of 100.0 mg/mL FLV): prepared by refluxing 100 mg of FLV with 50 mL of 1 M NaOH for 3 h then the hydrolyzed solution was neutralized with 1 M HCl, evaporated nearly to dryness and then the obtained residue was dissolved in methanol, filtered into 100 mL volumetric flask and the volume was completed with methanol to obtain a solution of MFA derived from complete degradation of 1 mg/mL FLV.

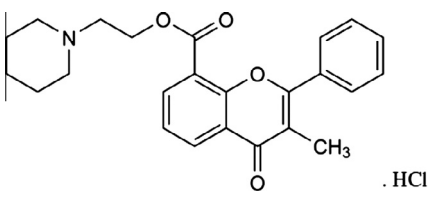

Fig. 1. Chemical structure of flavoxate hydrochloride.

MFA working solution 50 μg/mL: prepared by transferring 2.5 mL of standard stock solution of MFA (1 mg/mL) into 50-mL volumetric flask and the volume was completed to the mark with methanol.

Stock standard solution of FLV 50 μg/mL: was prepared by dissolving 5.0 mg of FLV in 10 mL methanol, then diluting to 100 mL with methanol.

Solvents and chemicals

Methanol used were HPLC grade (BDH Chemicals Ltd., England), Dioxan (BDH Chemicals Ltd., England), Acetonitrile, acetone (lab. scan-Ireland), Hydrochloric acid (Loba-Chemie Indoustranal Co., India, 0.1 M aqueous solution), Sulphuric acid (Fischer Scientific, UK, 0.1 M aqueous solution), Sodium hydroxide (Adwic Co., Egypt, 0.1 M aqueous solution), Cetylpyridinium bromide (Sigma Aldrich, St. louis, USA), Sodium lauryl sulphate (Sigma Aldrich, St. louis, USA), Tween 40 (Merck, Munich, Germany), Cetyltrimethyl ammonium bromide (Sigma Aldrich, St. louis, USA).

Calibration graph

Different volumes of the working standard solution of MFA (50 μg/mL) were transferred into a series of 10-mL volumetric flask and diluted to volume with methanol to obtain concentration range of 0.5–5.0 μg/mL.

The relative fluorescence intensity was measured at 390 nm after excitation at 338 nm versus a blank of equivalent concentration of intact FLV. The calibration graph was obtained by plotting the fluorescence intensities versus the final concentrations of MFA and the regression equation was computed.

Procedure for commercial tablets

Ten tablets of Genurin® were weighed and finely powdered. A portion of the powdered tablets equivalent to 200 mg of FLV were weighed and refluxed with 1 M NaOH for 3 h till complete degradation then neutralized with 1 M HCl, evaporated nearly to dryness then the residue was dissolved in 100 mL methanol. Different dilutions were prepared to suite the procedure as under Section 'Calibration graph'.

Laboratory prepared mixtures containing different ratios of flavoxate HCl and its degradation product (MFA)

Aliquots (0.9–0.1 mL) of MFA were accurately transferred from its working standard solution (50 μg/mL) equivalent to (45–5 μg) into a series of 10-mL volumetric flasks. Aliquots (0.1–0.9 mL) of FLV stock standard solution (50 μg/mL) equivalent to (5–45 μg) were added, and then the volume was completed with methanol to prepare mixtures containing 10–90% of FLV.

Procedure for spiked human urine samples

Aliquots equivalent to 5.0–40 μg of MFA were accurately transferred into 10-mL volumetric flask. After the addition of 1 mL of blank urine, the solutions were diluted to 10 mL with methanol, centrifuged at 3000 rpm for 15 min and then filtered through 0.45 μm disposable membrane filters. The relative fluorescence intensities were measured against a blank treated at the same manner but containing 5.0–40 μg of FLV instead of MFA. The relative fluorescence intensity of each concentration was plotted against the corresponding concentration to obtain the calibration graph of MFA and the regression equation was computed.

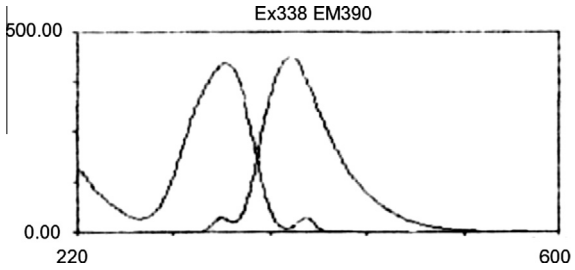

Fig. 2. Excitation and emission spectra of 4 μg/mL MFA in methanol (at λ_{em} 390 nm with λ_{ex} 338 nm).

Table 1
Effect of solvent on the fluorescence-intensity of 5 μg/mL MFA.

Solvent	Fluorescence intensity
Dioxan	500.0
Acetonitrile	476.0
Methanol	493.0
Acetone	480.0
Water	157.0
0.1 M H_2SO_4	27.6
0.1 M NaOH	32.0
0.1 M HCl	78.7

Table 2
Effect of surfactants on the fluorescence intensity of 5 μg/mL MFA.

Surfactant	Fluorescence intensity
Without surfactant	490.0
Cetylpyridinium bromide	167.0
Sodium lauryl sulphate	181.0
Tween 40	237.0
Cetyltrimethyl bromide	170.0

Sample preparation (in vivo procedure)

To check the ability of the proposed method for monitoring MFA excretion in human urine at low concentrations, it was applied to monitor the concentration of analytes after an oral administration of a Genurin® formulation containing 200 mg FLV to a normal, healthy (normal liver, kidney functions and electrocardiogram), female, informed adult volunteer (26 years, 80 kg, 169 cm height), with no past history of allergic reaction to FLV. The volunteer was instructed to abstain from all medications for 2 weeks before administration and also during study. Also, the volunteer was instructed to be sure of evacuating her bladder as thoroughly as possible exactly before the administration of one FLV tablet (200 mg) with about 250 mL of water. The 0-h urine sample was collected as blank. Urine samples were collected at 0–2.0, 2.0–2.5, 2.5–3.5, 3.5–5.0, 5.0–7.0, 7.0–9.0, 9.0–12.0, 12.0–17.0, 17.0–20.0, and 20.0–24.0 h. The volume of urine specimen was measured and recorded after each collection; 20 mL aliquots were stored at 4 °C until determination.

Suitable volume of the urine specimen from each sampling point was alkalinized with equal volume of 0.1 M sodium hydroxide and centrifuged at 3000 rpm for 15 min. The mixture was neutralized by addition of 0.1 M hydrochloric acid. The solution was filtered through 0.45 μm membrane filter. Then suitable volume of this solution was diluted to 10 mL with methanol and its fluorescence intensity was measured versus blank urine treated at the same manner.

The nominal content of MFA was determined from the corresponding regression equation.

Table 3
Results of assay validation obtained by applying the proposed spectrofluorimetric method for the determination of flavoxate HCl (via its degradate) in its pure powdered form and in spiked human urine.

Parameters	Drug substance	Spiked urine
Validation of regression equation		
Slope	100.80	30.24
Intercept	14.58	8.86
Correlation coefficient	0.9998	0.9996
Validation of response		
Linearity range (μg/mL)	0.5–5.0	0.5–4.0
Accuracy[a]		
Mean ± R.S.D%	99.43 ± 0.504	99.33 ± 0.920
Precision		
Repeatability[b] R.S.D%	0.507	0.814
Intermediate precision[b] R.S.D%	0.510	0.635
Selectivity	99.85 ± 0.470	–
LOQ (μg/mL)	0.24	0.28
LOD (μg/mL)	0.08	0.09

The LOD and LOQ were calculated using the following equations: LOD = 3.3 (SD/S) and LOQ = 10 (SD/S) where SD is the standard deviation of response and S is the slope of the graph.
[a] $n = 6$.
[b] $n = 3 \times 3$.

Results and discussion

Due to the ease and convince of spectrofluorometric method, where analysis can be done within a short period of time by the use of less sophisticated equipments in comparison with the reported chromatographic methods, our work aimed to developed simple, selective and reliable method for direct determination of MFA. It was observed that MFA has an intense fluorescence at 390 nm emission wavelength upon excitation at a maximum of 338 nm as shown in Fig. 2, while a similar concentration of FLV has no emission, using the same excitation wavelength. Thus; this idea has been used to develop a sensitive stability indicating spectrofluorimetric method for determination of FLV via its metabolite and monitoring the excreted MFA in human urine for its analysis. Furthermore, MFA is also reported to be the degradate product of FLV [8,9,11], thus the method was extended to the analysis of FLV in its dosage form after subjecting to complete alkaline hydrolysis to obtain equivalent amount of MFA.

Optimization of the method

Effect of diluting solvent

Dilution with different solvents such as: dioxan, acetonitrile, methanol, acetone, water, 0.1 M H_2SO_4, 0.1 M NaOH and 0.1 M

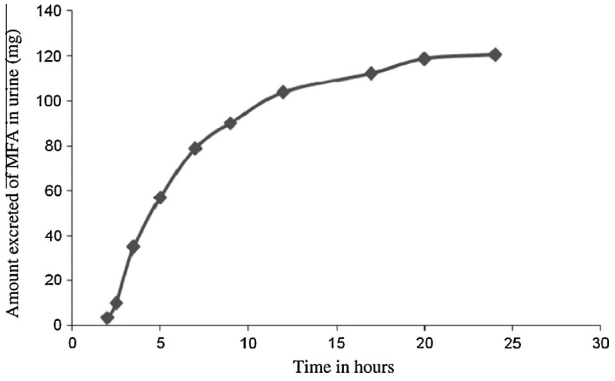

Fig. 3. Cumulative curve of amount excreted of MFA in urine after oral administration of commercial tablet containing 200 mg FX.

Table 4
Quantitative determination of flavoxate HCl (via its degradate) in pharmaceutical formulation by the proposed spectrofluorimetric method and the result of application of standard addition technique.

Pharmaceutical formulation	Found %[a] of claimed amount ± R.S.D	Standard added (μg/mL)	Recovery % [a] of standard added
Genurin tablets (200 mg FLV/tab) B.N. 21081	100.3 ± 0.364%	0.5	99.81
		1.5	100.20
		2.5	100.52
Mean ± R.S.D			100.2 ± 0.35%

Claimed amount (2.5 μg/mL).
[a] Average of three different determinations.

Table 5
Statistical comparison between results obtained by applying the proposed spectrofluorimetric method and the official method for determination of FLV (via its degradate) in pure powdered form.

Item	Spectrofluorimetric method	[a]Official method [18]
Mean	99.43	99.04
SD	0.504	0.594
R.S.D%	0.507	0.600
Variance	0.255	0.353
n	5	5
t-Test (2.306)[b]	1.112	
F-test (6.400)[b]	1.384	

[a] Nonaqueous titration using perchloric acid as a titrant.
[b] Figures in parentheses are the corresponding tabulated values at $p = 0.05$.

HCl was studied. Although fluorescence intensity was maxima upon using dioxan as shown in Table 1, but its usage was omitted due to its toxicity being classified as human carcinogen [19]. The use of methanol was the best regarding sensitivity and safety.

Effect of surfactant

The influence of concentration of different surfactants on the absorbance of the reaction product was studied hopefully they may enhance the absorbance. It was found that by using different concentrations of Cetylpyridinium bromide, Sodium lauryl sulphate, Tween 40 and Cetyltrimethyl bromide decreases the absorbance; therefore the study was performed omitting any surfactant Table 2.

Choice of the excitation wavelength

Several wavelengths were tested and the use of $\lambda_{excitation}$ 338 nm was the best regarding the sensitivity and reproducibility.

Validation of the method

Range and linearity

Aliquots equivalent to 5–50 μg (in bulk powder) and 5–40 μg (in spiked urine) of MFA were transferred accurately from its working standard solution (derived from complete degradation of 50 μg/mL of FLV) into a series of 10-mL volumetric flasks and the volume was completed to the mark with methanol. A blank was prepared by transferring aliquots equivalent to 5–50 μg of intact FLV from standard stock solution (50 μg/mL) were transferred accurately into a series of 10-mL volumetric flasks and the volume was completed to mark with methanol to assure that no interference was exerted by the intact drug [20,21]. The difference in the fluorescence intensity between the test experiment and blank (which has no emission upon excitation at the selected wavelength) was measured at λ_{em} 390 nm with λ_{ex} 338 nm, and plotted versus the corresponding concentrations, and the regression equations were calculated, Table 3.

Accuracy

The accuracy was assessed from three replicate determinations of three different solutions containing different concentrations of MFA. The proposed method was successfully applied for the determination of the drug (via its degradate) in bulk powder and in spiked human urine at λ_{em} 390 nm with λ_{ex} 338 nm, Table 3.

Precision

Repeatability. It was evaluated by analyzing freshly prepared solutions in triplicate at concentrations of 2, 3, 4 μg/mL and 2.5, 3, 3.5 for bulk powder and spiked urine respectively using the previously mentioned procedure under linearity. The percentage recovery and the relative standard deviation were then calculated, Table 3.

Intermediate precision. It was evaluated by assaying the above mentioned concentrations in triplicate on three successive days. The percentage recovery and the relative standard deviation were calculated, Table 3.

Selectivity

Methods selectivity was achieved by preparing several laboratory-prepared mixtures of the studied compounds at various concentrations within the linearity range. The laboratory-prepared mixtures were analyzed according to the previous procedure described under the proposed method. Satisfactory results were obtained as indicated in Table 3.

Detection and quantitation limits

According to ICH's guidelines method validation [22], the detection and quantitation limits were determined, Table 3.

Urinary excretion pattern of MFA

Flavoxate hydrochloride is rapidly and completely absorbed and metabolized into MFA, which is excreted in human urine. As MFA is present primarily in the conjugated form in human urine, so alkaline hydrolysis of the conjugate form to the free form is needed [15] for the precise determination of the total MFA found in urine. The proposed analytical method was applied for determination of

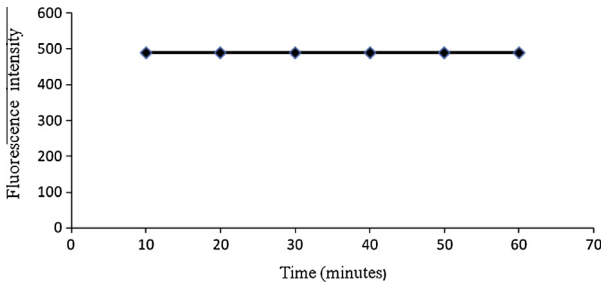

Fig. 4. Effect of the time on the stability of the fluorescence intensity of 5 μg/mL MFA at 390 nm.

the cumulative amount of MFA excreted in urine after oral administration of 200 mg FLV formulated in Genurin tablet, which reflect the bioavailability of FLV. The cumulative amount of MFA excreted in urine was found to be 120.6 mg after 24 h following oral administration of one tablet containing 200 mg FLV as shown in Fig. 3.

The proposed method has been successfully applied to assay FLV via MFA in Genurin tablets where the extracted FLV was quantitatively converted into MFA following the alkaline hydrolysis process. The validity of the proposed method was further assessed by applying the standard addition technique for the analysis of Genurin tablet, Table 4.

The results obtained by applying the proposed method for the analysis of the studied drug via MFA in pure form were statistically compared with those obtained by applying the official method [18] for FLV. The values of the calculated t and F are less than the tabulated ones which reveals that there is no significant difference with respect to accuracy and precision as shown in Table 5.

The stability of MFA solutions in methanol exhibited no spectrofluorimetric changes for 6 h when kept at ambient temperature for 2 days when stored in refrigerator at 4 °C as shown in Fig. 4.

Conclusion

The proposed spectrofluorimetric method provides simple, accurate, sensitive and direct quantitative analysis of MFA, the main active metabolite of FLV in human urine without any extraction procedure prior to determination. The urinary excretion pattern of the metabolite was easily established. Also, the proposed method can be used for determination of FLV in bulk powder and dosage form via MFA measurement thus the method presents a good alternation to other reported HPLC methods.

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