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Short communication

High performance liquid chromatographic determination of 3-methylflavone-8-carboxylic acid, the main active metabolite of flavoxate hydrochloride in human urine

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Abstract

High performance liquid chromatographic (HPLC) method was presented for the determination of 3-methylflavone-8-carboxylic acid as the main active metabolite of flavoxate hydrochloride (FX) in human urine. The proposed method was based on using CN column with mobile phase consisting of acetonitrile–12 mM ammonium acetate (40:60, v/v) and adjusted to apparent pH 4.0 with flow rate of 1.5 ml min^{-1}. Quantitation was achieved with UV detection at 220 nm. The proposed method was utilized to the determination of dissolution rate for tablets containing flavoxate hydrochloride. The urinary excretion pattern has been calculated using the proposed method.

Keywords: Flavoxate hydrochloride; HPLC; Urine; 3-Methylflavone-8-carboxylic acid

1. Introduction

Flavoxate hydrochloride (FX), 3-methylflavone-8-carboxylic acid β-piperidinoethyl ester hydrochloride, belongs to a series of flavone derivatives, which exhibit strong smooth muscle relaxant activity, with selective action on the pelvic [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 the relief of vesico-urethral spasms resulting from instrumentation or surgery [2].

Flavoxate is rapidly metabolized in plasma to 3-methylflavone-8-carboxylic acid, MFA [3]. MFA has a direct action on smooth muscles of urinary tract through the inhibition of phosphodiesterase (PDE) enzyme [4].

A pharmacokinetic study in humans [5] has shown that flavoxate, when given orally, is rapidly and completely absorbed and metabolized into MFA, which was excreted in the urine, so excretion studies of flavoxate can be performed by monitoring MFA in urine.

The literature survey reveals that FX was analyzed in its pharmaceutical preparations by Ultra-violet spectrophotometry [6,7] and high performance liquid chromatography (HPLC) [8,9]. The official method for determination of FX is non-aqueous titration using perchloric acid as titrant in pure form and spectrophotometry in tablets [7].

Several assay methods have been reported for determination of MFA in biological fluids, including radiometric assay [10], gas chromatography [1,5], capillary electrophoresis [11], and HPLC in plasma [12].

However, the radiometric method lacks specificity and the specific GC method involves extraction and methylation of samples before injection. The calibration curve of MFA in the capillary electrophoresis method [11] was linear in the range of 1–50 μg ml^{-1} and the detection limit was 0.2 μg ml^{-1}. The specific HPLC method [12] involves extraction of plasma sample and using nifedipine as internal standard with run time up to 18 min.

No HPLC method was reported for determination of MFA in human urine. The proposed HPLC method has been developed for direct injection of urine samples without extraction and without internal standard, so the procedure of analysis was greatly simplified. Complete separation of MFA from the endogenous

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components of urine was obtained within 3 min. The calibration curve of MFA in the developed method was linear in the range of 0.3–25 μg ml^{-1} and the detection limit was 0.056 μg ml^{-1}).

2. Experimental

2.1. Instrumentation

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12A Degasser, Rheodyne 7725i injector with a 20 μl loop and a SPD-10AVP UV–VIS detector.

Dissolution tester model TDD.6 (S.B.S. instruments, Barcelona, Spain) was used.

2.2. Materials and reagents

Pharmaceutical grade of FX (Recordati, Milan, Italy) was used and certified to contain 99.7%. MFA was prepared by hydrolysis of FX and recrystalized four times with chloroform. MFA was identified for purity by NMR and IR analysis.

Acetonitrile and methanol used were HPLC grade (BDH, Poole, UK). Sodium hydroxide (Sigma–Aldrich, Inc., St. Louis, USA), ammonium acetate (Sigma–Aldrich), and acetic acid (Riedel-de Haën Laboratory Chemicals, Germany) were used.

Commercial Genurin tablets (Batch No. 041037) used was manufactured by Medical Union Pharmaceutical, Abu-Sultan, Ismailia, Egypt, labeled to contain 200 mg FX per tablet.

2.3. HPLC conditions

The HPLC separation and quantitation were made on a 250 mm × 4.6 mm (i.d.) Luna 5 μm CN column (Phenomenex, UK). The mobile phase was prepared by mixing acetonitrile and 12 mM ammonium acetate in a ratio 40:60 (v/v) 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 ambient temperature. The injection volume was 20 μl. The samples were also filtered using 0.45-μm disposable filters. The detector was set at λ 220 nm. Data acquisition was performed on class-VP software.

2.4. Standard solutions and calibration graph (spiked urine)

Stock solution was prepared by dissolving MFA in methanol to obtain a concentration of 50 μg ml^{-1}.

Different volumes of the stock standard solution were transferred to 25-ml volumetric flask, 1 ml of blank urine was added and the solutions were diluted to 25 ml with the mobile phase to obtain concentration range of 0.3–25 μg ml^{-1} for MFA. The solutions were filtered through 0.45-μm disposable membrane filters. Triplicate 20 μl injections were made for each concentration of 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 MFA.

2.5. Sample preparation (in vivo procedure)

An excretion study of MFA was carried out on a normal, healthy (normal liver, kidney functions and electrocardiogram), male, informed adult volunteer (25 years, 80 kg, 178 cm height), with no past history of allergic reaction to FX. 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 his bladder as thoroughly as possible exactly before the administration of one FX 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 sonicated for 15 min. The mixture was neutralized by addition of 0.1 M hydrochloric acid. Suitable dilution was carried out to 25 ml with the mobile phase. The solution was filtered through 0.45 μm membrane filter. A 20 μl was injected into HPLC, in triplicate for each solution and chromatographed under the conditions described above. The peak area was used for determination of MFA at each specified time using the calibration graph.

2.6. Dissolution rate study

Dissolution rate study was carried out on six tablets using dissolution apparatus. Each tablet of FX was dissolved in 900 ml of 0.1 M hydrochloric acid with basket and the apparatus was set at 55 rev. The dissolution was carried out at 37 ± 0.5 °C and the dissolved amounts of FX were determined at 5, 15, 30, 45, 60, and 90 min using the proposed method.

After each specified time, a portion of the solution under test was filtered and suitably diluted with the HPLC mobile phase. A 20 μl was injected into HPLC in triplicate for each solution and chromatographed under the conditions described above. The peak area was used for determination of dissolved FX at each specified time using the calibration graph with FX concentration ranged from 2.5 to 20 μg ml^{-1}.

3. Results and discussion

HPLC method has been developed for a rapid, simple, accurate, reproducible, and sensitive assay for monitoring the excreted MFA in human urine. A satisfactory separation of MFA from biological endogenous components in urine was obtained. Separation and quantitation were carried out using a Phenomenex, CN column (5 μm) at ambient temperature with mobile phase consisting of acetonitrile and 12 mM ammonium acetate in a ratio (40:60, v/v, pH 4.0). The flow rate was 1.5 ml min^{-1}. Quantitation was achieved with UV detection at 220 nm. Cyanide column was used to overcome tailing that occurred on ODS column, and improve the sharpness of the MFA peak. The specificity of the HPLC method is illustrated

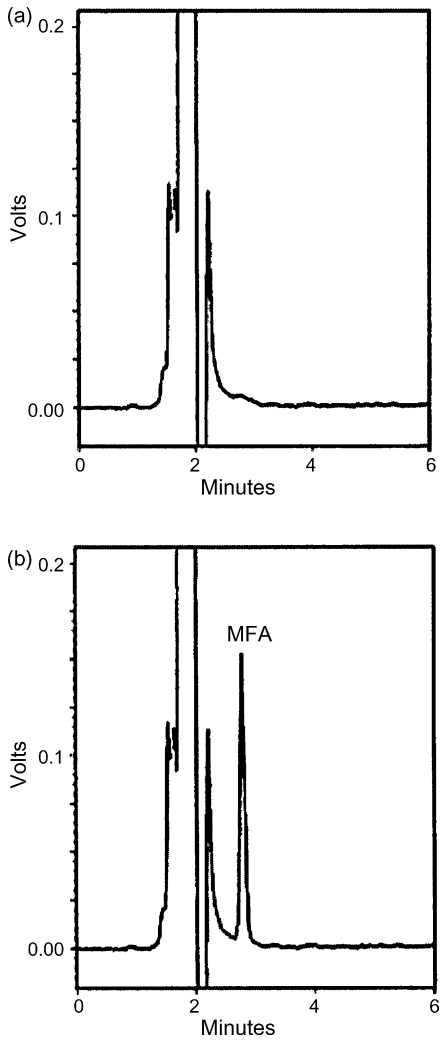

Fig. 1. HPLC chromatograms of 20 μl injection of urine without MFA treatment (a) and urine spiked with 10 μg ml^{-1} MFA (b).

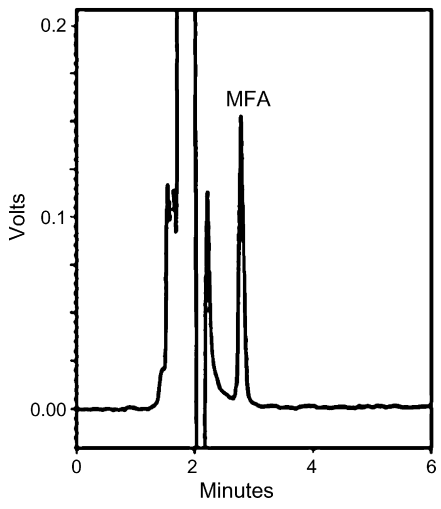

Fig. 2. HPLC chromatogram of urine sample containing MFA.

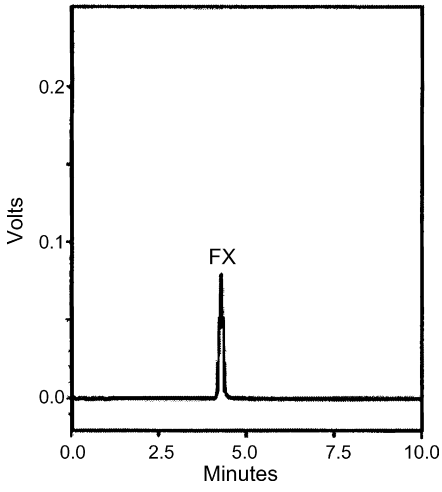

Fig. 3. Typical HPLC chromatogram of 20 μl injection of 10 μg FX.

in Fig. 1, where complete separation of MFA from biological endogenous components in the urine was noticed and no interfering peaks were observed in the blank urine. The average retention time ± S.D. for 10 replicates of MFA was found to be 2.8 ± 0.01 min.

As MFA is present primarily in the conjugated form in human urine [11], and because of the lack of a conjugate MFA reference standard, hydrolysis of the conjugate form to the free form is needed for the precise determination of the total MFA found in urine. Various hydrolysis conditions were investigated, including hydrolysis time, and the concentration of acid or alkali. It was found that, alkaline hydrolysis was preferable to the acidic hydrolysis as, alkaline hydrolysis is faster. Hydrolysis of the conjugated MFA was completed by using 0.05 M sodium hydroxide within 15 min at ambient temperature (Fig. 2).

Also, the proposed method was applied for the determination of FX in tablets using the same specified conditions. The average retention time ± S.D. for 10 replicates of FX was found to be 4.5 ± 0.02 min (Fig. 3). The percentage content of FX in commercial tablets was found to be 99.7%.

3.1. Dissolution rate study of FX tablets

The proposed method was applied for the determination of the dissolved amount of FX from its pharmaceutical tablets. The dissolution profile of FX was studied in 0.1 M hydrochloric acid. The amount of FX dissolved in 90 min was found to be 95.0% (Fig. 4).

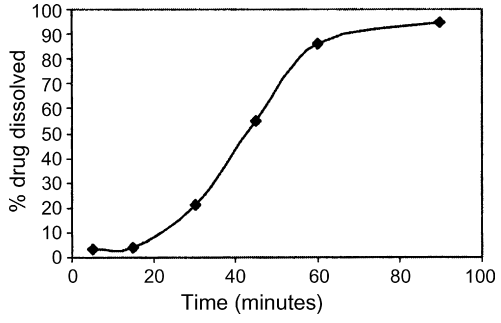

Fig. 4. Dissolution rate profile for commercial tablets containing 200 mg FX.

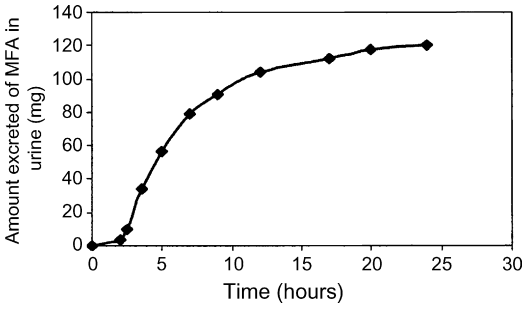

Fig. 5. Cumulative curve of amount excreted of MFA in urine after oral administration of commercial tablet containing 200 mg FX.

Table 1
Cumulative amount of MFA excreted in urine

Excretion profile	
Time (h)	Cumulative amount (mg)
0.0	0.00
2.0	3.4
2.5	10.1
3.5	34.5
5.0	56.6
7.0	78.8
9.0	90.3
12.0	103.7
17.0	112.1
20.0	117.7
24.0	120.1

3.2. Urinary excretion pattern of MFA

Since FX is rapidly and completely absorbed and metabolized into MFA, which excreted in human urine. The proposed analytical method was applied for determination of the cumulative amount of MFA excreted in urine after oral administration of 200 mg FX tablet, which reflect the bioavailability of FX. The cumulative amount of MFA excreted in urine was found to be 120.1 mg after 24 h following oral administration of one tablet containing 200 mg FX (Fig. 5) and (Table 1).

3.3. Validation of the method

3.3.1. Linearity

The linearity of the HPLC method for determination of MFA and FX was evaluated by analyzing a series of different concentrations. In this study, five concentrations were chosen, ranging between 2.5 and 20 $\mu g\,ml^{-1}$ for FX and 0.3 and 25 $\mu g\,ml^{-1}$ for MFA. The calibration graphs were constructed by plotting peak area against concentrations of MFA and FX. The linearity of the calibration graphs were validated by the high values of the correlation coefficient, and the intercept value, which was not statistically ($p = 0.05$) different from zero (Table 2).

3.3.2. Precision

The within-day precision was evaluated by replicate analysis of urine samples spiked with known concentrations of MFA at five concentration levels. Similarly, the between-day precision was evaluated in several days up to 5 days (Table 3). Every day, calibration graph was constructed and the results were calculated in comparison with the calibration graph. The results in both cases indicated high precision, as the CV% did not exceed 2%.

3.3.3. Range

The calibration range was established taken in consideration the practical range necessary to give accurate, precise and linear results. The calibration ranges of the proposed HPLC method was given in Table 2.

3.3.4. Detection and quantitation limits

According to FDA's guidance for bioanalytical method validation [13], the detection and quantitation limits were determined (Table 2).

Table 2
Characteristic parameters for the regression equations of the proposed HPLC method for determination of FX and MFA

Parameters	HPLC	
	FX	MFA
Calibration range ($\mu g\,ml^{-1}$)	2.5–20	0.3–25
Detection limit ($\mu g\,ml^{-1}$)	4.9×10^{-2}	5.6×10^{-2}
Quantitation limit ($\mu g\,ml^{-1}$)	2.5	0.3
Regression equation (Y)[a]		
Slope (b)	1.06×10^{3}	1.98×10^{3}
Standard deviation of the slope (S_b)	18.39	39.7
Relative standard deviation of the slope (%)	1.72	2.00
Confidence limit of the slope[b]	10.5×10^{2}–10.8×10^{2}	19.4×10^{2}–20.21×10^{2}
Intercept (a)	-1.5×10^{3}	4.9×10^{2}
Standard deviation of the intercept (S_a)	2.0×10^{2}	4.4×10^{2}
Confidence limit of the intercept[b]	(-16.8×10^{2})–(-12.8×10^{2})	69.9–91.6×10^{1}
Correlation coefficient (r)	0.9998	0.9997
Standard error of estimation	1.0×10^{2}	3.1×10^{2}

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

Table 3
Within-day and between-day precision for the assay of MFA in urine

MFA concentration ($\mu g\,ml^{-1}$)	Within-day precision		Between-day precision	
	Mean measured[a] concentration ± S.D.	CV%	Mean measured[b] concentration ± S.D.	CV%
1	1.01 ± 0.01	1.14	1.02 ± 0.02	1.96
3	2.92 ± 0.06	1.95	3.1 ± 0.06	2.00
5	4.99 ± 0.07	1.49	4.9 ± 0.08	1.61
10	9.99 ± 0.13	1.28	9.8 ± 0.15	1.56
20	19.98 ± 0.09	0.46	20 ± 0.14	0.68

[a] Mean of five urine samples for each concentration.
[b] Mean of five days results for each concentration.

Table 4
Determination of MFA in human urine from different three volunteers

MFA concentration ($\mu g\,ml^{-1}$)	Volunteer 1 (%recovery)	Volunteer 2 (%recovery)	Volunteer 3 (%recovery)
1	99.9	99.8	99.8
3	100.5	101.3	99.5
5	100.4	101.1	98.6
10	99.2	100.7	100.3
20	100.3	100.8	100.1
Mean	100.06	100.74	99.66
S.D.	0.53	0.58	0.67

3.3.5. Selectivity

Method selectivity was achieved by analyzing MFA in three urine samples from different persons, at five different levels of concentration within the linearity range. Satisfactory results were obtained in Table 4, indicating the high selectivity of the proposed method for determination of MFA in urine.

3.3.6. Robustness

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

3.3.7. Analytical solutions stability

MFA and FX analytical solutions in mobile phase or methanol exhibited no chromatographic changes for 6 h when kept at ambient temperature and for 4 days when stored refrigerated at 5 °C.

4. Conclusion

The proposed HPLC method provide simple, accurate, sensitive and direct quantitative analysis for the assay of MFA, the main active metabolite of FX in human urine without any extraction procedure prior to the separation. The urinary excretion pattern of the metabolite was easily established. Also, the proposed method can be used for determination of dissolution profile of FX commercial tablets.

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