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QUANTITATION OF FLUPIRTINE AND ITS ACTIVE ACETYLATED METABOLITE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUOROMETRIC DETECTION

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SUMMARY

A simple, selective and sensitive procedure is described for the quantitation of flupirtine maleate (FLU) and its active acetylated metabolite (Met. 1) in plasma and urine. Using a 0.5-ml sample, a sensitivity of 10 ng/ml is easily achieved with a reversed-phase octadecylsilane (C_{18}) column, and a high-performance liquid chromatographic system with fluorescence detection. Quantitation from plasma involves addition of an internal standard, protein precipitation with acetonitrile and a sample concentrating step, while for urinalysis the samples are taken through a single extraction with methylene chloride. Analytical recoveries of FLU and Met. 1 from plasma averaged $\geq$ 95%, while from urine only 60 and 50%, respectively, could be recovered. The overall, inter- and intra-day variability for both FLU and Met. 1 averaged 6, 5 and 3%, in plasma, respectively. Standard calibration plots in plasma were linear ($r \geq 0.99$) for FLU (range: 0.01—10.0 μg/ml) and Met. 1 (range: 0.5—25 μg/ml) over the extended range. A slightly modified elution system was employed for quantitation of FLU and Met. 1 in urine.

INTRODUCTION

Flupirtine® [2-amino-3-carbethoxyamino-6-(*p*-fluorobenzylamino)pyridine maleate] (Carter-Wallace Labs., Cranbury, NJ, U.S.A.), is a centrally acting analgesic with a long duration of action and apparently a low addiction and tolerance liability [1]. Its activity appears not to be affected significantly by naloxone and it may therefore lack opiate activity. It is currently being evaluated by the Epilepsy Branch of the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) at the Clinical Center of the National Institutes of Health as an anti-epileptic agent. A sensitive assay was

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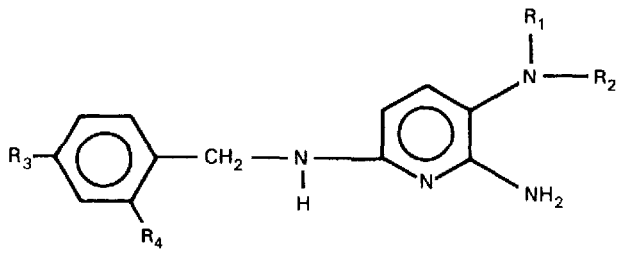

$R_1 = H$	$R_2 = -C(=O)-O-C_2H_5$	$R_3 = F$	$R_4 = H$	(Flupirtine)
$R_1 = H$	$R_2 = -C(=O)-CH_3$	$R_3 = F$	$R_4 = H$	(Metabolite 1)
$R_1 = H$	$R_2 = -C(=O)-O-C_2H_5$	$R_3 = -CH_3$	$R_4 = -CH_3$	(Internal Standard)

Fig. 1. Flupirtine and related substituted pyridine analogues.

desired in order to study its disposition following single oral doses in epileptic patients.

Earlier quantitative procedures for flupirtine maleate (FLU) in biological fluids included measurement of radiolabelled drug [1], thin-layer chromatography [1] and measurement of absorbance via spectrophotometric analysis [1]. These procedures used in preclinical studies lack sensitivity and specificity, and thereby analytical measurements are usually non-specific.

The drug undergoes biotransformation to two primary metabolites which have been identified: (1) an acetylated product and (2) *p*-fluorohippuric acid. The acetylated metabolite [Met. 1: 2-amino-3-acetylamino-6-(*p*-fluorobenzylamino)pyridine] has been shown to possess almost 20—30% analgesic activity of the parent compound. Valid quantitation procedures evaluating the disposition of FLU should be specific enough to resolve the parent drug from any of the metabolites present. Fig. 1 shows the parent compound and other related chemical entities employed in this procedure.

A rapid, reproducible and sensitive assay is described for the quantitation of FLU and its active metabolite (Met. 1) in biological fluids by reversed-phase high-performance liquid chromatography (RP-HPLC) using fluorometric detection, suitable both for routine therapeutic monitoring and/or detailed disposition studies.

MATERIALS AND METHODS

Flupirtine maleate and its dimethyl derivative [2-amino-3-carbethoxyamino-6-(2,4-dimethylbenzylamino)pyridine] used as internal standard (I.S.) were obtained from the Epilepsy Branch of the NINCDS (National Institutes of Health, Bethesda, MD, U.S.A.). Met. 1 was generously supplied by Carter-Wallace Labs.

Both methanol and acetonitrile (Burdick and Jackson Labs., Muskegon, MI,

U.S.A.) used in the assay were glass-distilled and of HPLC grade. Water was double distilled in a glass-distillation apparatus. All HPLC solvents were filtered through a 0.45-μm filter and then degassed prior to use. Stock solutions of FLU, Met. 1 and I.S. were stored at 4°C. Solutions of FLU showed no degradation over a period of three months when kept refrigerated, covered with aluminium foil. The internal standard solution was freshly prepared every two weeks from the stock solution.

Assay method

Quantitation of FLU and Met. 1 from plasma. Plasma, 0.5 ml, was placed in an 4-ml screw cap disposable glass vial (Fisher Scientific, Silver Spring, MD, U.S.A.) which contained 50 μl of I.S. (2.0 μg/ml in methanol). To this, 1 ml of acetonitrile was added using a Repipet® dispenser (Lab Industries, Berkeley, CA, U.S.A.). Each vial was mixed using a vortex mixer for 5 sec and centrifuged for 5 min at 2000 *g*. The supernate was then decanted into another 4-ml disposable screw cap glass vial and evaporated under a gentle stream of air at 37°C. The residue was reconstituted with 150 μl of mobile phase, mixed for 1 min and centrifuged for 2 min at 2000 *g*. A 20-μl aliquot of a sample was injected into the HPLC system.

Quantitation of FLU and Met. 1 from urine. Urine, 0.5 ml, was placed in a 8-ml screw cap disposable glass vial (Fisher Scientific) which contained 50 μl of I.S. and 50 μl of ammonium hydroxide (10% solution of ammonia). The sample was mixed on a vortex mixer for 5 sec and 5 ml of methylene chloride (Burdick and Jackson Labs.) was added using a Repipet dispenser. The vials were then shaken on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 5 min and centrifuged for another 5 min at 2000 *g*. Approximately 4 ml of the organic layer was drawn and transferred to another disposable vial. The organic phase was slowly evaporated to dryness under a gentle stream of air at 37°C. The residue was treated the same way as with plasma, above.

Chromatographic conditions

The chromatographic analysis of the samples was performed using an isocratic system. An Altex Model 110A single-pump system equipped with a Schoeffel variable-wavelength fluorometric monitor (FS-970, Schoeffel Instrument, Westwood, NJ, U.S.A.) was employed for quantitation of FLU. The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were set at 323 and 370 nm, respectively. The cut-off emission filter used (λ_{em} = 370 nm) has a greater than 89% transmittance at the wavelength. The analysis was performed on a 25 cm × 4.6 mm I.D. Altex Ultrasphere octadecylsilane (ODS) analytical column with a 5-μm particle size (Beckman Instruments, Berkeley, CA, U.S.A.). Preceding the analytical column was a 7 cm × 2.3 mm I.D. guard column packed with Co:Pell ODS (30—38 μm particles) (Whatman, Clifton, NJ, U.S.A.). The mobile phase of methanol—acetontrile—0.005 *M* phosphate buffer (32:32:36, v/v) adjusted to pH 6.7 was used at a flow-rate of 1.4 ml/min as an eluent for plasma samples. The back pressure ranged from 190—210 bar. Standards for FLU and Met. 1 were prepared by spiking plasma and/or urine with FLU and Met. 1 stock solutions to obtain final concentrations ranging from 0.01 to 2.0 μg/ml for FLU and 0.5 to 15.0 μg/ml for Met. 1.

The concentrations of FLU and/or Met. 1 were estimated by comparing the peak area ratios of the drug to the internal standard with area ratios of a simultaneously run standard calibration curve. Analytical recoveries of FLU and Met. 1 were determined by comparison of direct injection of standard aqueous solutions with the injection of the same standards run through the assay procedure.

Inter-day variation was computed by measuring precision in the concentration determined from two plasma quality control standards (0.15 and 1.0 μg/ml) and reproducibility in measured concentration and slope of the standard calibration curves (n = 9) over a three-month period. The intra-day variability was estimated by performing replicate analyses (n = 10) plasma standard containing 0.15 μg/ml of FLU and 4.0 μg/ml of Met. 1 and measuring precision based on peak area ratios of drug and/or Met. 1 to internal standard. Computation of statistical parameters, e.g. means, standard deviation, coefficient of variation (%) and 95% confidence intervals was performed using standard programs available in statistical package software of Hewlett-Packard 85 microcomputer (Hewlett-Packard, Corvallis, OR, U.S.A.).

Selectivity of the assay was also evaluated by checking for possible interference of other anti-epileptic agents that patients might be taking concomittantly.

RESULTS AND DISCUSSION

Typical HPLC chromatograms obtained from control blank (A), spiked (B), and patient plasma (C) are shown in Fig. 2. The eluted peaks are clean, sharp and symmetric, the control or blank plasma did not show any interfering peaks at or near the elution volumes for drug and the internal standard. No attempt

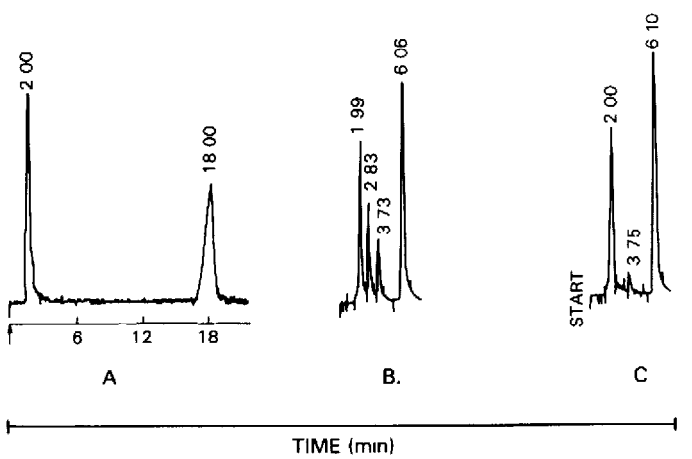

Fig. 2. Representative HPLC chromatograms from plasma samples taken through the assay procedure. (A) Blank plasma; (B) calibration standard containing 0.2 and 5.0 μg/ml of FLU and Met. 1, respectively; (C) patient plasma sample (estimated FLU concentration ca. 0.04 μg/ml). Peaks with retention times relative to the I.S. (retention times in min are in parentheses): solvent front, 0.32 (2.00); Met. 1, 0.46 (2.83); FLU, 0.61 (3.73) and I.S., 1.00 (6.08).

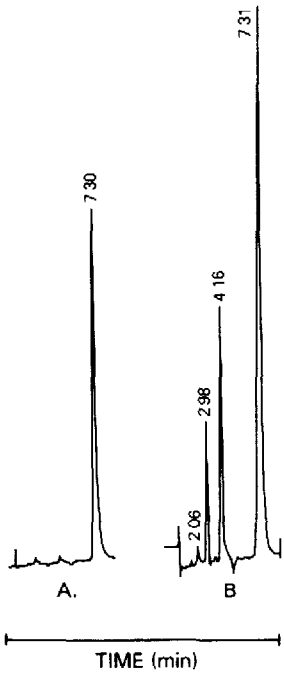

Fig. 3 Representative chromatograms from urinalysis following extraction. (A) Blank urine with I.S.; (B) calibration standard containing 0.8 and 8.0 μg/ml of FLU and Met. 1, respectively. Peaks with relative retention (actual retention times in min are in parentheses): solvent front, 0.29 (2.06); Met. 1, 0.42 (2.98); FLU, 0.57 (4.16) and I.S., 1.00 (7.30).

was made to identify the peak that eluted at approximately 18 min (Fig. 2A from some plasma samples). Retention times for Met. 1, FLU and I.S. were 2.83, 3.73 and 6.08 min, respectively. The relative retention of these compounds is given in Fig. 2.

Fig. 3 shows typical chromatograms obtained from urinalysis following the extraction step of blank urine containing I.S. (A) and a calibration (spiked) standard (B) containing both FLU and Met 1. The elution is clean, peaks are symmetric and sharp. Retention times for Met. 1, FLU and I.S. were 2.98, 4.16 and 7.30 min, respectively. Relative retention times are also shown in Fig. 3.

Fig. 4 shows a mean standard curve (n = 6) in plasma over a two-month period, where peak area ratios are plotted as a function of spiked FLU and Met. 1 concentration. The inset in Fig. 4 shows an excellent linearity when peak height ratio (FLU/I.S.) is regressed as a function of FLU concentration (range 0.01—0.05 μg/ml). This validates linearity at the low range and suggests that either the peak height or peak area ratios may be used to obtain standard calibration plots.

Linearity of the standard curve was established over an extended range of up to 10.0 μg/ml FLU and 25.0 μg/ml Met. 1 in plasma. The mean slope of the standard curves was 1.165 for FLU and 0.053 for Met 1. The variability in the slope of the standard calibration curves (n = 6) for FLU and Met. 1 over a two-month period was 6.2% and 7.8%, respectively. The correlation

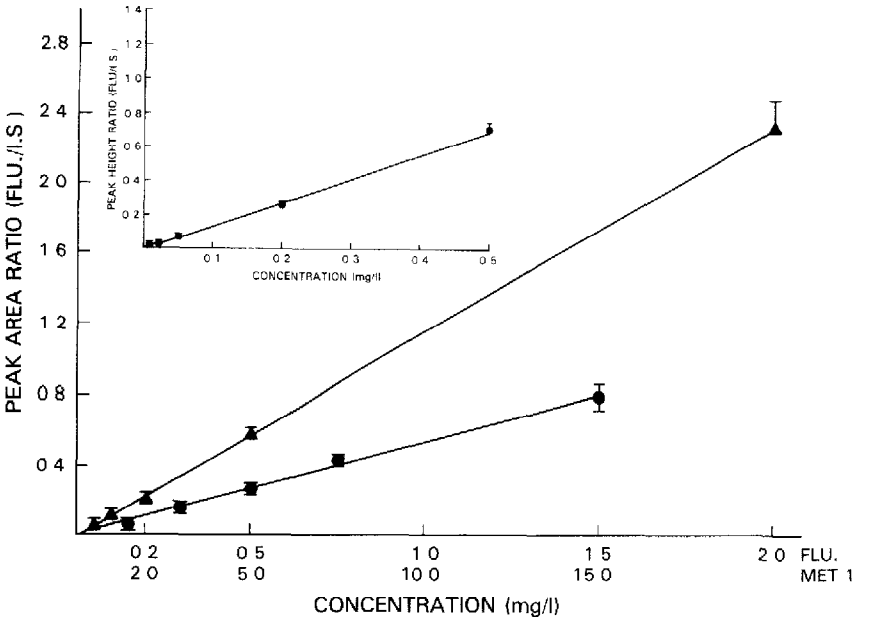

Fig. 4. Mean standard calibration curves for FLU and its active metabolite in plasma over a two-month period. Each datum represents a mean (± S.D.) of six determinations. Key with regression parameters: (▲) FLU (y = 0.011 + 1.165x; r = 0.996) and (●) Met. 1 (y = 0.002 + 0.053x; r = 0.992). Inset: mean (n = 7) standard curve for FLU in plasma over the low concentration range (0.01—0.5 mg/l).

TABLE I

ASSAY REPRODUCIBILITY OF NINE REPLICATES OVER A THREE-MONTH PERIOD FROM PLASMA

	Actual concentration of standards (mg/l)	Measured concentration		
		Mean	S.D	C.V. (%)
Flupirtine	0.05	0.0496	0.005	10.0
	0.10	0.1031	0.006	6.1
	0.20	0.1840	0.008	4.6
	0.50	0.5082	0.015	2.9
	2.00	1.9656	0.072	3.6
Controls	0.15	0.140	0.006	4.3
	1.00	1.067	0.060	5.6
Met. 1	1.5*	1.335	0.129	9.7
	3.0	2.975	0.099	3.3
	5.0	5.113	0.180	3.5
	7.5	8.067	0.359	4.5
	15.0	14.846	0.299	2.0
Controls	4.0	3.812	0.174	4.6
	10.0	10.546	0.616	5.8

*n = 8 in this case.

coefficients (r) generated by linear regression of peak area ratios of both FLU/I.S. and Met. 1/I.S. on concentration in all cases were 0.99 or better.

The assay sensitivity for a 20-μl injection is at least 10 ng/ml for FLU and 200 ng/ml for Met. 1. This was determined by observing the lowest concentration that maintained an arbitrary signal-to-noise ratio of 3.0. The assay sensitivity for both Met. 1 and FLU can be increased several fold by increasing the injection volume, sample size or decreasing the reconstitution volume.

The assay reproducibility for FLU and Met. 1 determined from nine replicate standard calibration curves in plasma over a three-month period is shown in Table I. As can be seen the overall coefficient of variation (C.V.) for the quantitation procedure was less than 6% for both FLU and Met. 1. Reproducibility data for FLU computed from seven replicate analyses in plasma over the lower concentration range indicate an average variation of 7% (Table II) which is consistent with the data in Table I.

The inter-day variability determined by measuring precision in the measured concentration for two (a low and a high) quality control standards, 0.15 and 1.00 μg/ml for FLU, and 4.0 and 10.0 μg/ml for Met. 1, was approximately 5% for both the parent compound and its metabolite (Table III). The intra-day variation computed similarly by measuring precision in the peak area ratios of FLU/I.S. and Met. 1/I.S. for ten replicates averaged 2.2% and 2.7%, respectively.

TABLE II

ASSAY REPRODUCIBILITY OF SEVEN REPLICATES OVER A TWO-WEEK PERIOD FROM PLASMA

Actual concentration of standard (mg/l)	Peak height ratio		
	Mean	S.D.	C.V. (%)
0.01	0.0146	0.0012	8.2
0.02	0.0260	0.0028	10.7
0.05	0.0700	0.0045	6.4
0.2	0.2530	0.0142	5.6
0.5	0.7130	0.0319	4.5
Mean			7.1
S.D.			2.4

TABLE III

INTER-DAY AND INTRA-DAY VARIABILITY FROM PLASMA

Control	Concentration (mg/l)		Variation (%)	
	FLU	Met. 1	FLU	Met 1
Inter-day variability (n = 9)				
A	0.15	4.00	4.3	4.6
B	1.00	10 00	5.6	5 8
Intra-day variability (n = 10)				
A	0.15	4.0	2.2	2.7

TABLE IV

ANALYTICAL RECOVERY DATA FROM URINE (n = 4)

Compound	Percent recovery		
	Mean	S.D.	C.V (%)
FLU	60.3	2.3	3.9
Met. 1	49.2	3.9	8.0
I.S.	63.8	1.5	2.3

Analytical recovery from plasma for both FLU and Met. 1 was > 95%. However, the recoveries of Met. 1 and FLU for urinalysis, following extraction with methylene chloride, had a mean of 49.2% and 60.3% (n = 4), respectively. Overall variation in analytical recovery of Met. 1 was approximately two-fold that of FLU (Table IV). The extraction efficiency for the internal standard had a mean of 64%. The standard calibration plots for both FLU and Met. 1 were linear in the range of 0.5–50.0 μg/ml in urine. Sensitivity limit (lower limit of detection) for FLU and Met. 1 in urine was 30 ng/ml and 500 ng/ml, respectively. Extraction efficiencies were not completely optimized since these sensitivities were sufficient for urinalysis.

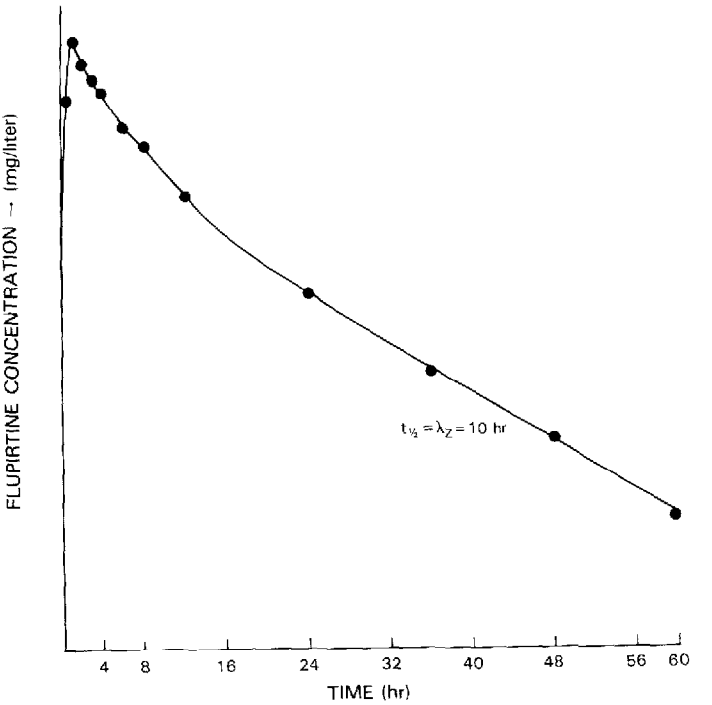

Fig 5. A representative disposition profile of FLU in epileptic patient over 2.5 days following oral administration of flupirtine maleate

Selectivity of assay

Carbamazepine and its epoxide, phenytoin, valproic acid, diazepam, clonazepam, aspirin and methylprednisolone were evaluated for possible interference with the assay procedure. None of the compounds chromatographed interfered with FLU and/or Met. 1 peaks. However, at concentrations above 15—20 μg/ml carbamazepine, Met. 1 peak eluted as a shoulder to carbamazepine peak. Therefore, the assay appears to be selective for the quantitation of both FLU and Met. 1 in epileptic patients taking other anti-convulsants concomitantly.

The assay procedure has been successfully used in evaluating the disposition kinetics of FLU in epileptic patients following oral dosing. Fig. 5 shows a representative profile for FLU disposition in one such patient. After relatively quick absorption, the drug decline from plasma appears to be biexponential. The terminal-phase half-life ($t_{1/2}$) in this patient was determined to be approximately 10 h.

The data in the paper clearly demonstrate that the assay is sensitive, rapid, selective, requires only 0.5 ml of the biological sample and should prove useful in further investigations of FLU pharmacokinetics in both man and animals.

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