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SIMULTANEOUS DETERMINATION OF FLURAZEPAM AND FIVE METABOLITES IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO PHARMACOKINETIC STUDIES IN RATS

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SUMMARY

A reversed-phase high-performance liquid chromatographic method is described which allows the quantification of flurazepam and five of its metabolites with a single, isocratic determination. In addition, it has the advantage of possessing a low detection limit and high precision. A 2 mm I.D. column was used to minimize sample size (50 μl), increase sensitivity and reduce solvent consumption. The method was used to demonstrate that N-1-desalkylflurazepam, the major metabolite, has a short half-life in the rat in contrast to its prolonged life in humans.

INTRODUCTION

Flurazepam dihydrochloride (Dalmane®), 7-chloro-1-(2-diethylaminoethyl)-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one dihydro chloride (I·2HCl, see Table I), is used as a hypnotic agent for the treatment of insomnia [1–3]. It is biotransformed to didesethylflurazepam (Ib), N-1-desalkyl-3-hydroxyflurazepam (Ie), monodesethylflurazepam (Ia), N-1-hydroxyethylflurazepam (Ic), N-1-desalkylflurazepam (Id), flurazepam-N-1-acetaldehyde (If), flurazepam-N-1-acetic acid (Ig) and N-1-hydroxyethyl-3-hydroxyflurazepam (Ih) [4–8]. The metabolic fate of flurazepam has been studied extensively in the dog [5] and humans [4–8], partially in the cat [9–11] and mouse [12], but to the best of our knowledge, there are no reports of studies in the rat. We believe this to be the first report on the pharmacokinetics of flurazepam in the rat.

Spectrofluorometric [13], polarographic [14,15] and thin-layer chromatographic [16] methods have been used to determine flurazepam and some of its metabolites, but these methods were either too time-consuming for routine use

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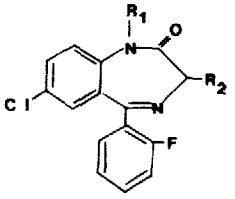
TABLE I

CHEMICAL STRUCTURES OF FLURAZEPAM AND ITS METABOLITES



Compound		R_1	R_2
I.	Flurazepam	$-CH_2CH_2N(C_2H_5)_2$	H
Ia.	Monodesethylflurazepam	$-CH_2CH_2NHC_2H_5$	H
Ib.	Didesethylflurazepam	$-CH_2CH_2NH_2$	H
Ic.	N-1-Hydroxyethylflurazepam	$-CH_2CH_2OH$	H
Id.	N-1-Desalkylflurazepam	-H	H
Ie.	N-1-Desalkyl-3-hydroxyflurazepam	-H	OH
If.	Flurazepam-N-1-acetaldehyde	$-CH_2CHO$	H
Ig.	Flurazepam-N-1-acetic acid	$-CH_2COOH$	H
Ih.	N-1-Hydroxyethyl-3-hydroxyflurazepam	$-CH_2CH_2OH$	OH

or too insensitive for the measurement of therapeutic concentrations. Sensitive gas chromatographic methods with electron-capture detection have also been used [17–21], but on-column thermal degradation of some of the metabolites (Ib, Ie, Ig) [8] renders this method unsuited for analysis.

Most high-performance liquid chromatographic (HPLC) methods [21–27] either need two eluents to separate quantitatively flurazepam from its metabolites or are developed to measure only Ic, the major urinary metabolite, and not flurazepam or the serum metabolites. An HPLC method using reversed-phase ion pairing [28] can separate I, Ic and Id with one eluent, but the method lacks sensitivity. We describe here a simple, isocratic HPLC method capable of simultaneous, quantitative analysis of flurazepam and five of its metabolites in small serum samples (50 μl). Sample size is a critical consideration when the animal species used is small, especially when repeated blood sampling is necessary to trace the temporal changes in drug and metabolite levels of individual animals.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a dual-piston Model 510 pump (Waters Assoc., Milford, MA, U.S.A.), a Model 7010 sample injection valve, a Model 7011 loop filler port equipped with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.), a Model 163 variable-wavelength detector (Beckman Instruments, San Ramon, CA, U.S.A.) operated at 230 nm and an LCI-100 integrator (Perkin-Elmer, Norwalk, CT, U.S.A.). The separation was performed on an Ultrasphere C_{18} column (5 μm

particle size, 150×2.0 mm I.D., Altex, San Ramon, CA, U.S.A.). A 2-μm pre-column filter (Waters Assoc.) was also used.

Reagents and standards

Spectranalyzed® diethyl ether, HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Springfield, NJ, U.S.A.). All other chemicals were reagent grade. The 1 *M* borate–sodium carbonate–potassium chloride buffer (pH 9.0) was prepared as described by De Silva and Puglisi [29].

Flurazepam dihydrochloride, the five metabolites (Ia–e) and desmethyldiazepam were gifts from Hoffmann-La Roche (Nutley, NJ, U.S.A.). A set of six stock methanolic solutions (A) containing 1.0 mg/ml each (weight as free base) of flurazepam and the five metabolites was prepared using 10-ml volumetric flasks. Solution B was made by pipetting 10 μl of the six stock solutions (A) into a volumetric flask and making up the volume to 10 ml with methanol to give 10 μg/ml of each compound. Working standards (0.05, 0.1, 0.25, 0.5, 0.75 and 1.0 μg/ml) containing the six compounds were prepared by appropriate dilutions of solution B.

The mobile phase was acetonitrile–methanol–0.034 *M* sodium acetate buffer adjusted to pH 2.9 with 40% phosphoric acid (25:15:60, v/v/v). The flow-rate was set at 0.3 ml/min and normally operated at a pressure of 138 bar (2000 p.s.i.).

Animals

Four male, albino rats (Holtzman strain) were housed individually in a temperature-regulated room with a 12-h light–dark cycle. They were maintained at 80% of their free-feeding, adult body weights (mean 383.1 g, range 380–386 g) by limiting food (Purina Laboratory Chow) rations. Water was continuously available. The experimental protocol was reviewed and approved by the Rutgers Animal Care and Facilities Committee.

Serum sampling

Flurazepam dihydrochloride was dissolved in distilled water and administered to animals intraperitoneally. Tail blood samples were obtained at various times between 15 min and 7 h by cutting approximately 1 mm from the tip of the tail with a guillotine. Subsequent samples were obtained by removing a suture that had been tied around the tail tip. Blood samples were centrifuged in an Autocrit Ultra 3 centrifuge (Clay-Adams, Towson, MD, U.S.A.) for 5 min at 13 700 *g*. The clear serum layer (50 μl) was used for analysis.

Sample preparation

Standards and serum samples were prepared as previously described [30]. Briefly, 25 μl of the internal standard (desmethyldiazepam, 1 μg/ml) and 50 μl of a working standard were evaporated to dryness in a 15-ml conical centrifuge tube. A 50-μl blank serum sample, 100 μl of the 1 *M* borate buffer and 2.5 ml of diethyl ether were added, mixed and centrifuged. The ether layer was evaporated to dryness and the residue was resuspended in 50 μl of the mobile phase. Hexane (100 μl) was added, mixed, centrifuged, removed immediately, and the resus-

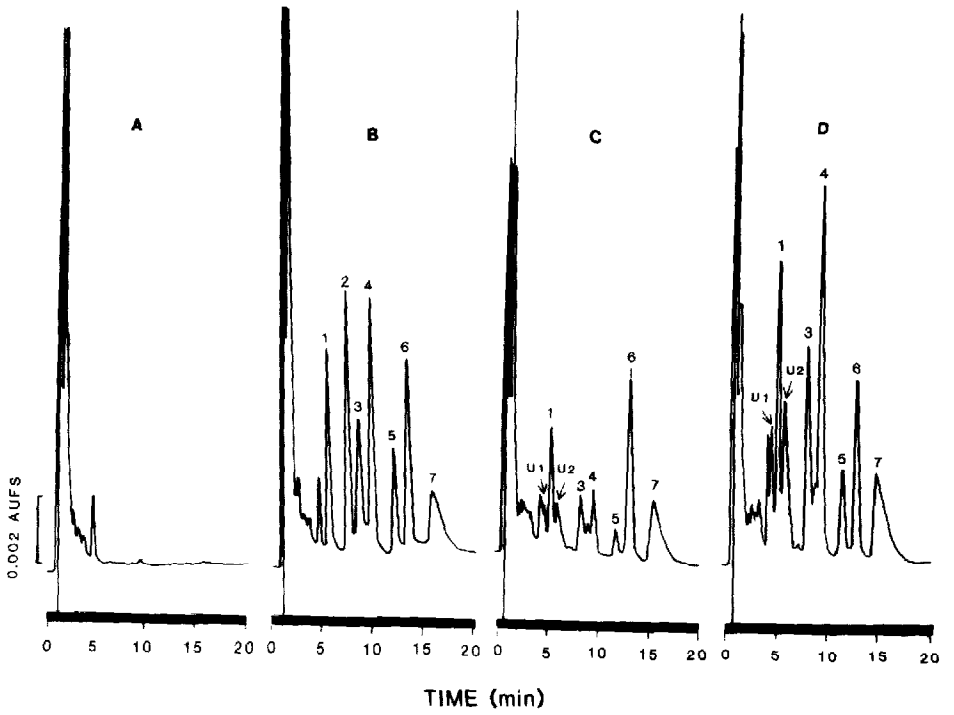


Fig. 1. Chromatograms of (A) rat serum blank, (B) rat serum containing 0.5 μg/ml flurazepam and five of its metabolites (Ia–e) taken through the extraction procedure, (C) a 50-μl rat serum sample obtained 15 min after a 16 mg/kg I·2HCl intraperitoneal injection and (D) same as (C) but after a 32 mg/kg I·2HCl intraperitoneal injection. Peaks: 1 = Ib; 2 = Ie; 3 = Ia; 4 = Ic; 5 = Id; 6 = internal standard; 7 = I; U1, U2 = unknowns.

pended residue was injected for analysis. Samples for serum drug analysis were prepared identically except drug working standards were not added. Instead, serum samples (50 μl) were added after the internal standard was initially evaporated to dryness.

RESULTS

Method evaluation

Fig. 1 shows the chromatogram of a serum blank (A), a spiked serum sample containing 0.5 μg/ml working standard and internal standard (B), and a rat serum sample obtained 15 min after intraperitoneal injection of either 16 mg/kg (C) or 32 mg/kg (D) flurazepam dihydrochloride. This method identifies simultaneously flurazepam and four of its metabolites (Ia, Ib, Ic and Id) in rat serum samples; Ie was not detected. Chromatograms C and D indicate that the peak heights for flurazepam and the four metabolites were dose-related. There are two dose-related peaks identifying unknowns present in C and D which did not occur in the serum blank (A). With continued use the column loses efficiency which results in a decreased resolution between Ia and Ic. The resolution can normally be

TABLE II

PRECISION DATA FOR FLURAZEPAM AND ITS METABOLITES IN SERUM

Compound	Within-day ($n=6$)		Between-day ($n=6$)	
	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)
I	0.051 ± 0.003	5.9	0.050 ± 0.005	10.0
	0.494 ± 0.036	7.3	0.486 ± 0.035	7.2
	1.000 ± 0.047	4.7	1.026 ± 0.063	6.1
Ia	0.050 ± 0.003	6.0	0.049 ± 0.005	10.2
	0.517 ± 0.032	6.2	0.508 ± 0.040	7.9
	0.996 ± 0.071	7.1	1.012 ± 0.090	8.9
Ib	0.050 ± 0.005	10.0	0.051 ± 0.005	9.8
	0.516 ± 0.043	8.3	0.520 ± 0.053	10.2
	1.006 ± 0.085	8.5	0.989 ± 0.097	9.8
Ic	0.051 ± 0.002	3.9	0.050 ± 0.002	4.0
	0.497 ± 0.019	3.8	0.504 ± 0.016	3.2
	1.008 ± 0.031	3.1	1.020 ± 0.041	4.0
Id	0.049 ± 0.002	4.1	0.053 ± 0.003	5.7
	0.493 ± 0.031	6.3	0.501 ± 0.006	1.2
	1.000 ± 0.049	4.9	0.987 ± 0.033	3.3
Ie	0.050 ± 0.001	2.0	0.051 ± 0.003	5.9
	0.515 ± 0.035	6.8	0.491 ± 0.041	8.4
	1.003 ± 0.038	3.8	0.974 ± 0.055	5.7

restored by increasing the molarity of the sodium acetate buffer used in the mobile phase.

Within-day and between-day precisions were established on three different concentrations (0.05, 0.5 and 1.0 μg/ml) for flurazepam and five of its metabolites by adding these six compounds to blank serum. The coefficients of variation (C.V.) for these compounds ranged from 2.0 to 10.0% for within-day and 1.2 to 10.2% for between-day precision (Table II).

Calibration curves for flurazepam and the metabolites observed are linear within the ranges (0.05–1.0 μg/ml) examined. For each of the six regression lines the correlation coefficients are all greater than 0.994. The coefficients of variation of the slopes ($n=10$) of the regression lines ranged from 5.8 to 8.1% with intercepts all close to zero (Table III).

Mean recoveries of flurazepam and the five metabolites were greater than 90%, while those for Ib and Id were 76.98 and 82.85%, respectively. These values were calculated at three concentrations (0.05, 0.5 and 1.0 μg/ml) for each of the six compounds. The detection limit was 0.2 ng (10 ng/ml) for flurazepam and 0.1 ng (5 ng/ml) for each of the five metabolites studied. All six compounds were linear to 5.0 μg/ml.

To determine the specificity of the method, the retention times of other benzodiazepines and drugs that might be used as co-medications with flurazepam

TABLE III

MEAN OF TEN CALIBRATION EQUATIONS FOR FLURAZEPAM AND FIVE OF ITS METABOLITES OVER THE CONCENTRATION RANGE 0.05–1.00 μg/ml

y = peak-height ratio (compound/internal standard); x = concentration of each compound.

Compound	Equation	Correlation coefficient	C.V. of slope (%)
I	$y=0.5287(\pm0.0428)x+0.0102(\pm0.0279)$	0.994	8.1
Ia	$y=0.9426(\pm0.0618)x-0.0191(\pm0.0147)$	0.996	6.6
Ib	$y=1.7830(\pm0.1319)x-0.0407(\pm0.0370)$	0.994	7.4
Ic	$y=2.1453(\pm0.1610)x-0.0259(\pm0.0252)$	0.998	7.5
Id	$y=1.2101(\pm0.0795)x-0.0146(\pm0.0114)$	0.999	6.6
Ie	$y=2.1036(\pm0.1228)x-0.0053(\pm0.0340)$	0.997	5.8

were determined. None of the compounds tested interfered (Table IV). The retention times of clonazepam and oxazepam partially overlapped with that of Ic, but not with either flurazepam or its other metabolites.

Pharmacokinetic results

Fig. 2 shows the mean serum concentration–time profiles for flurazepam and four of its metabolites after a single intraperitoneal dose of 16 mg/kg flurazepam dihydrochloride to four rats. The elimination rate constants were determined by the slope of the linear regression lines for the log-linear portion of the curve. The half-lives were obtained from the elimination rate constants and the values were: I, 1.69 h; Ia, 1.88 h; Ib, 1.74 h; Ic, 1.01 h; Id, 1.12 h.

TABLE IV

RELATIVE RETENTION TIMES (k') OF SEVERAL BENZODIAZEPINES, THEIR METABOLITES AND SOME OTHER COMMON DRUGS UNDER CHROMATOGRAPHIC CONDITIONS DESCRIBED IN TEXT

N.D. = peak not observed within 20 min.

Compound	k'	Compound	k'
Barbital	<2	Oxazepam	8.68
Caffeine	<2	N-1-Desalkylflurazepam	10.88
Flumazepil (Ro 15-1788)	<2	Desmethyldiazepam	12.10
Phenobarbital	2.38	Midazolam	12.13
Desmethylchlordiazepoxide	3.17	Temazepam	13.25
Didesethylflurazepam	4.39	Flurazepam	14.07
Demoxepam	4.64	Chlorodesmethyldiazepam	15.69
Hexobarbital	5.02	Diazepam	23.14
Chlordiazepoxide	5.06	Chlorpromazine	N.D.
Buspirone	5.86	Cocaine	N.D.
Hexobarbital	5.89	Meprobamate	N.D.
N-1-Desalkyl-3-hydroxyflurazepam	6.29	Methamphetamine	N.D.
Monodesethylflurazepam	7.22	Reserpine	N.D.
Clonazepam	8.34	4′-Chlorodiazepam (Ro 5-4864)	N.D.
N-1-Hydroxyethylflurazepam	8.59	Tybamate	N.D.

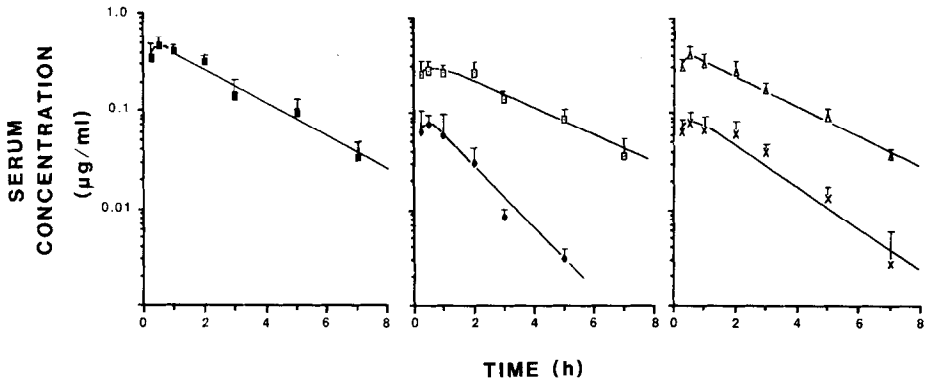


Fig. 2. Mean (S.E.) serum concentration-time profiles of flurazepam and four of its metabolites after a single 16 mg/kg flurazepam dihydrochloride intraperitoneal injection ($n=4$). Key: ■ = I; □ = Ia; ♦ = Ic; △ = Ib; × = Id.

DISCUSSION

The major advantage of the present method lies in the simultaneous quantification of flurazepam and five of its metabolites in the same sample with a single, isocratic mobile phase elution. Other methods [21,23,25] require, in separate determinations, one eluent for the hydrophobic flurazepam, which contains large hydrocarbon moieties, and another eluent for its polar metabolites. A possible factor which allows our method to use a single elution is the lower pH of the mobile phase. Our sodium acetate buffer had a pH of 2.9 while others have used buffers of neutral pH (7.2–7.5). When our buffer was shifted to 7.5, the first four metabolites in the chromatogram (Fig. 1B) were not resolved. Phosphoric acid was selected over acetic acid to adjust the pH of the sodium acetate buffer since phosphoric acid is more transparent at 230 nm. Using this mobile phase, the UV absorbance maxima of the compounds studied (I, Ia–e) all fell at 230 nm, in contrast to the 254 nm commonly used by other investigators [24–26]. When 254 nm was used in conjunction with the present method, detection sensitivity was actually minimized; the sensitivity loss was 41%. Another advantage of this method is the sensitivity achieved with small sample size. By using a direct injection method without the loop, sample size can be further reduced without compromising sensitivity.

The heights of the two unidentified peaks in the chromatogram appear to be dose-related. It is quite possible that they are two of the three metabolites (If–h) previously identified in the blood [4,7,8]. Neither identification nor quantification of these unknowns was possible since we were unable to obtain metabolites If–h.

The present results show that, in the rat, both flurazepam and its major metabolite, desalkylflurazepam, have short half-lives of 1.69 and 1.12 h, respectively. By contrast, although the blood concentration of flurazepam is short-lived in humans [20,31–33], desalkylflurazepam has a half-life ranging from 40 to 200 h

[16,18,20,34]. A similar species difference exists in the pharmacokinetics of diazepam and its major metabolite (desmethyldiazepam), except that in humans the parent compound itself also has an extended half-life [35–40].

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