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DETERMINATION OF THE MAJOR URINARY METABOLITE OF FLURAZEPAM IN MAN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of N-1-hydroxyethylflurazepam, the major urinary metabolite of flurazepam, in human urine is described. Urine specimens were incubated enzymatically to deconjugate N-1-hydroxyethylflurazepam glucuronide (metabolite) and were then extracted at pH 9.0 to extract the metabolite. The extracts were chromatographed on a microparticulate silica gel column using automatic sample injection, isocratic elution at ambient temperature and UV monitoring at 254 nm. The internal standard was 7-chloro-5-(2'-chlorophenyl)-1,3-dihydro-1-2-dimethylaminoethyl-2H-1,4-benzodiazepine-2-one. The recovery from urine, in the 0.5—25.0 μg/ml range, was 96.5 ± 11.5% (S.D.), and the sensitivity limit was 0.5 μg/ml. The method was found to be specific for N-1-hydroxyethylflurazepam in the presence of intact flurazepam and other possible urinary metabolites of flurazepam. The method was successfully applied to urine specimens collected from human subjects following the administration of 30-mg single oral doses of flurazepam dihydrochloride.

INTRODUCTION

Flurazepam dihydrochloride, [I] · 2 HCl, a hypnotic of the 1,4-benzodiazepine class marketed as Dalmane®, undergoes extensive biotransformation in man [1—3] to form the mono-desethyl [I-A], the didesethyl [I-B], the N-1-hydroxyethyl [II], the N-desalkyl [III] and the N-desalkyl-3-hydroxy [IV] metabolites (Table I). The major urinary metabolite, [II], present largely as the glucuronide conjugate, accounted for 30—55% of an orally administered dose [4].

Methods for the quantitation of [I] and its metabolites in blood have included luminescence [4], electron-capture—gas—liquid chromatography [5—9],

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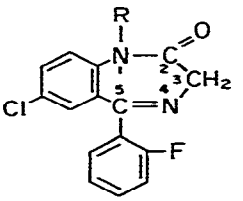
TABLE I

CHEMICAL STRUCTURES AND RELATIVE RETENTION TIMES OF FLURAZEPAM AND ITS MAJOR METABOLITES



	R		Relative retention time
Flurazepam	$-(CH_2)_2-N-(C_2H_5)_2$	[I]	0.88
Monodesethylflurazepam	$-(CH_2)_2-NH-C_2H_5$	[I-A]	0.55
Didesethylflurazepam	$-(CH_2)_2-NH_2$	[I-B]	2.43
N-1-Hydroxyethylflurazepam	$-CH_2-CH_2OH$	[II]	1.00
N-1-Desalkylflurazepam	-H	[III]	0.89
N-1-desalkyl-3-hydroxyflurazepam	-H	[IV], 3 > CHOH	3.75
Internal standard for HPLC	$-(CH_2)_2-N-(CH_3)_2$	[V], 2'-Cl	1.56

spectrofluorodensitometry [10], radioimmunoassay [II] and gas chromatography—mass spectrometry [12]. The pharmacokinetics of [I] and its metabolites [13] led to the consideration of utilizing the urinary excretion of the major metabolite, [II]-glucuronide, as a means of assessing bioavailability of [I] in man, since [II] accounts for more than 40% of a given dose over a 24-h excretion period [3,4]. Although bioavailability of dosage forms is commonly evaluated by measurement of unchanged drug in blood after the administration of a single dose of drug, utilization of urinary excretion data of unchanged drug or a major metabolite is accepted as a viable alternative [14].

Previously reported methodology utilizing enzymatic deconjugation, selective extraction and either spectrofluorometry [4] or differential pulse polarography [5] suggested the practicality of using the rate of excretion of [II] as a means of comparing bioequivalence of formulations of [I] · 2 HCl in man. In order to establish urinary excretion profiles following a single dose of [I] · 2 HCl in man, it is desirable to collect a series of specimens, over short time intervals, namely, 1—2-h intervals during the first 8 h, followed by 4-, 12-, and 24-h intervals up to 72 h post dose.

This report describes a more direct and rapid method than those previously reported [4, 5] for the quantitation of [II] in urine. The sample preparation is similar to a previously reported method [5], but is simplified by use of high-performance liquid chromatography (HPLC) for the separation of [II] from the other possible urinary metabolites of [I], thus eliminating the need for selective solvent extraction prior to enzymatic sample treatment. The method presented here involves enzymatic hydrolysis followed by solvent extraction at pH 9.0, followed by HPLC of the sample using a microparticulate silica gel column, with isocratic elution and UV detection at 254 nm. The limit of sensitivity of

the assay was 0.5 μg/ml of urine, and effectively resolves [II] in the presence of [I] and its metabolites, [I-A], [I-B], [III], and [IV]. In addition, the method was readily automated by using an automatic sample injector and includes use of 7-chloro-5-(2′-chlorophenyl)-1,3-dihydro-1-(2-dimethylaminoethyl)-2H-1,4-benzodiazepin-2-one, [V], (Table I), as the internal standard for HPLC analysis.

EXPERIMENTAL

Reagents

Reagent grade chemicals were used to prepare: phosphate buffer, 1 *M*, pH 5.4 (prepared by mixing 820 ml of 1 *M* potassium dihydrogen orthophosphate with 180 ml of 1 *M* dipotassium hydrogen orthophosphate and adjusting the mixture to pH 5.4); sodium hydroxide, 6 *N*; hydrochloric acid, 0.25 *N*. Other reagents included: ammonium hydroxide, (J.T. Baker, Phillipsburg, NJ, U.S.A.); diethyl ether, anhydrous (Mallinckrodt, St. Louis, MO, U.S.A.); methylene chloride and methyl alcohol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.).

The HPLC mobile phase was composed of: methylene chloride and a mixture of methanol—water—ammonium hydroxide (concentrated) (150:9:1, v/v) in a 500:25 (v/v) ratio, prepared fresh for each chromatographic run, and vacuum degassed for approximately 5 min with ultrasonic vibrating prior to use.

Standards. N-1-Hydroxyethylflurazepam, [II], [7-chloro-1-(2-hydroxyethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one], mol. wt. 332.76. Prepare a stock solution (A) by weighing 10.0 mg of [II] into a 10-ml volumetric flask and dissolving it in methanol to yield a 1 mg/ml solution. Prepare working solutions (B) and (C) to contain 100 μg and 10 μg [II] per ml respectively, in 10 ml of mobile phase by sequential dilution of stock solution (A).

Internal standard, [V]. 7-Chloro-5-(2′-chlorophenyl)-1,3-dihydro-1-(2-dimethylaminoethyl)-2H-1,4-benzodiazepin-2-one, mol. wt. 376.29. Prepare a stock solution (A′) containing 100 μg [V] per ml, by weighing 10.0 mg into a 100-ml volumetric flask and dissolving in the mobile phase. Prepare a working solution (B′) containing 5 μg/ml by transferring a 5-ml aliquot of (A′) into a 100-ml volumetric flask and diluting with mobile phase. This solution is used to reconstitute the extracted urine samples prior to injection into the HPLC.

Instrumentation

The liquid chromatographic system used consisted of the following components: a constant-flow high-pressure solvent delivery pump Model 6000A, and a fixed-wavelength dual-channel UV detector (254 nm) Model 440 (Waters Assoc., Milford, MA, U.S.A.); automatic sample injector with a 50-μl fixed-volume loop Model 725 Auto Injector (Micromeritics, Norcross, GA, U.S.A.); a microparticulate (10 μm) silica gel column, 25 cm × 4.6 mm I.D. Partisil PXS (Whatman, Clifton, NJ, U.S.A.) and a 10-mV dual-channel recorder Model 7130A (Hewlett-Packard, Avondale, PA, U.S.A.).

The instrumental parameters used to obtain the separation were: flow-rate, 1.5 ml/min; operating pressure, 45 bar; ambient column temperature; detector

sensitivities, 0.05 and 0.5 a.u.f.s.; recorder chart speed, 0.5 in./min.

Under these conditions of analysis, 150 ng of [II] injected gave nearly full-scale response and 250 ng of [V] injected gave 60% of full-scale response at 0.05 a.u.f.s. The retention times of [II] and [V] were 4.5 and 7.0 min, respectively (Fig. 1).

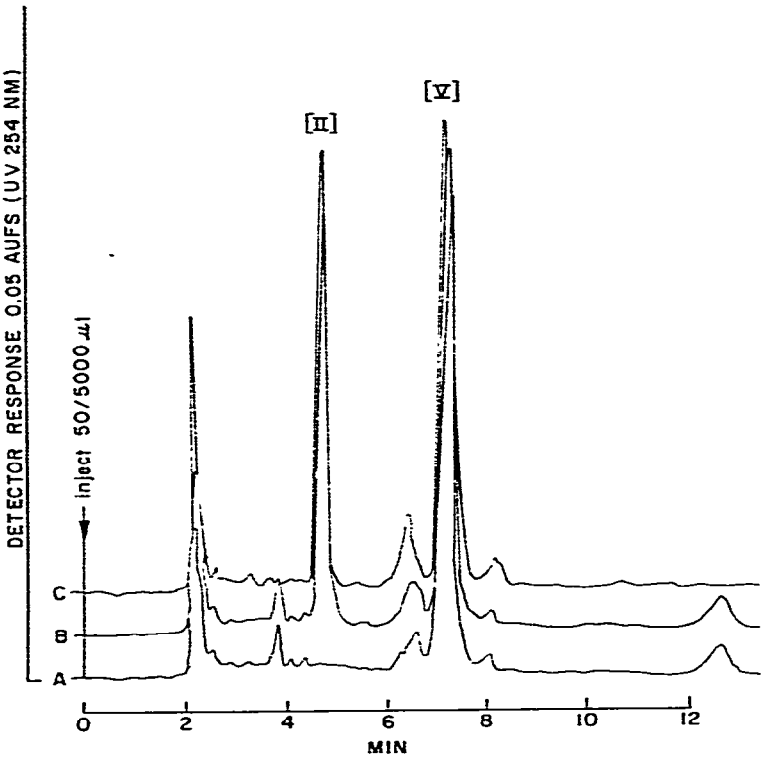


Fig. 1. Chromatograms of urine extracts of urine specimens post-glusulase incubation: (A) control urine spiked with internal standard [V]; (B) control urine spiked with 10 µg [II] per ml; (C) urine specimen from a subject following oral administration of 30 mg [I] · 2 HCl, 2—3 h collection, 1 ml urine assayed.

Preparation of samples for automatic injection into the HPLC instrument

Aliquots of 1.0 ml of urine were transferred into 50-ml glass centrifuge tubes using a pipetting device Pipetman, Model P1000D (Gilson, Middleton, WI, U.S.A.). The pH values of the samples were measured with a Model 125 digital pH meter, using a micro combination electrode (Corning Glass Works, Corning, NY, U.S.A.) and adjusted to pH 5.4 by dropwise addition of 0.25 *N* hydrochloric acid. Then 2 ml of 1 *M* phosphate buffer, pH 5.4, were added and the sample mixed well. Enzyme preparation: 0.10 ml Glusulase® (Endo Labs., Garden City, NY, U.S.A.) was added to each sample using a 1-ml glass hypodermic syringe, fitted with a stainless-steel needle. The samples were then placed in a rack, loosely stoppered with cotton and incubated overnight (about 16 h) at 37°C in a mechanical incubation shaker (Dubnoff, Precision Scientific, Chicago, IL, U.S.A.).

Following incubation, the samples were removed from the water bath, allowed to equilibrate to room temperature, and adjusted to pH 9.0 by the addition of 6 *N* sodium hydroxide (usually only 3—4 drops required), mixed well and checked on a pH meter. The samples were then extracted twice with 12-ml portions of anhydrous diethyl ether (sealing these tubes using PTFE stoppers) by shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.), and then centrifuging for 10 min at 2500 rpm (1000 *g*) at 5°C in a refrigerated centrifuge (Model PR 6000, Rotor No. 253, IEC/Damon, Needham Heights, MA, U.S.A.). Following each centrifugation the ether extracts were transferred to 15-ml glass centrifuge tubes and evaporated to dryness under a gentle stream of nitrogen in a water bath at 40°C.

The residues were then reconstituted in solution (B′) containing 5 μg/ml of internal standard [V] in the mobile phase. The volume of solution used varied from 1.5 to 5.0 ml depending on the concentration of [II] in the unknowns. Aliquots (about 500 μl) of the samples were then transferred to vials (Micromeritics; capacity about 750 μl) and capped for automatic injection using a 50-μl loop. Each sample vial in the autoinjector carousel was separated by a wash vial containing mobile phase, programmed not to be injected.

Typical chromatograms following automatic sample injection are shown in Fig. 1 for (A) control urine extracts, (B) control plus added standards, and (C) an extract from a subject following the administration of 30 mg [I] · 2 HCl.

Quantitation of [II] in the unknown samples

Along with each set of unknowns to be determined, a set of standards of [II] added to control (drug-free, predosing) urine was assayed. Concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, and 25.0 μg [II] per ml of urine (prepared by evaporation of the appropriate aliquot of standard solution (B) or (C) were processed along with the unknowns. These calibration standards were used to establish a least squares linear regression curve, using the peak height ratio of [II]/[V] for each concentration of standard, for the measurement of the unknowns. The equation of a typical calibration curve from 0.5—25.0 μg [II] per ml was $y = 2.842x + 0.004$ and the coefficient of correlation was 0.9999.

RESULTS

Recovery, reproducibility, linearity and sensitivity limits

The recovery of [II] was found to be 96.5 ± 11.5% (S.D.) over the concentration range 0.5—25.0 μg [II] per ml urine. The sensitivity limit ranged from 0.5 to 1.0 μg [II] per ml depending on the endogenous material in control urine. The precision of the autoinjector was 1.9%.

Specificity of the method

The other minor metabolites of [I] (Table I) were chromatographed as pure standards to determine their relative retention times compared to [II]. Interferences from endogenous compounds in the urine extracts were minimized or eliminated by injecting only 1—3% of the reconstituted extracts (50 μl from 1.5—5.0 ml of reconstituted extract).

Substances other than [I] and its metabolites, and the internal standard [V],

were not determined in this system for potential interference, since no other drugs were co-administered in these controlled human studies. However, other benzodiazepines and/or their metabolites could potentially interfere with the specificity of this method since they are also amenable to HPLC analysis [15].

Application of the method for bioavailability assessment

The urinary excretion profiles of [II] were determined in two normal volunteers following the administration of single 30-mg oral doses of [I] · 2 HCl. Urine specimens were collected over the following time intervals: −12—0 h (pre-dosing control), 0—1, 1—2, 2—3, 3—4, 4—6, 6—8, 8—12, 12—24, 24—48,

TABLE II

URINARY EXCRETION DATA OF THE N-1-HYDROXYETHYLFLURAZEPAM LEVELS IN TWO SUBJECTS WHO RECEIVED 30-MG ORAL DOSES OF FLURAZEPAM DIHYDROCHLORIDE*

Subject no. 1: age, 20 years; sex, male; weight, 59.47 kg; dose, 0.50 mg [I] · 2 HCl per kg.
Subject no. 2: age, 51 years; sex, male; weight, 61.29 kg; dose, 0.49 mg [I] · 2 HCl per kg.

Time interval (h)	Concentration** (μg/ml)	mg per time interval**	mg per h**	Percentage of dose excreted***
Subject no. 1				
−12—0	—	—	—	—
0—1	67.65	1.759	1.759	8.12
1—2				
2—3	38.39	2.284	1.142	10.55
3—4	11.24	0.354	0.354	1.63
4—6	6.49	0.454	0.227	2.10
6—8	2.34	0.367	0.184	1.69
8—12	0.64	0.685	0.171	3.16
12—24	0.85	0.481	0.040	2.22
24—48	0.10	0.261	0.011	1.21
48—72	0.05	0.121	0.005	0.56
0—72	—	6.766	—	31.24
Subject no. 2				
−12—0	—	—	—	—
0—1	13.94	0.523	0.523	2.42
1—2	26.21	1.979	1.979	9.14
2—3	5.47	1.001	1.001	4.62
3—4	1.62	0.365	0.365	1.69
4—6	4.14	0.513	0.257	2.37
6—8	1.38	0.221	0.111	1.02
8—12	0.58	0.293	0.073	1.35
12—24	0.62	0.397	0.033	1.83
24—48	0.25	0.465	0.019	2.15
48—72	0.17	0.196	0.008	0.19
0—72	—	5.953	—	27.50

* 30 mg flurazepam dihydrochloride administered equivalent to 25.25 mg flurazepam free base.
** All expressed in terms of N-1-hydroxyethylflurazepam.
*** Expressed in terms of flurazepam free base.

and 48—72 h post dose. The total urine volume voided in each interval was recorded, and 50-ml aliquots were stored at −17°C for analysis.

The urinary concentrations of [II] determined in specimens collected from the two subjects are shown in Table II. The urinary excretion rate data (mg per h) for [II] are shown in Table II and plotted in Fig. 2. The percentages of dose excreted at each interval and cumulatively, expressed in terms of [I] free base are also shown in Table II. These excretion profiles were shown to be reproducible within subjects and consistent between subjects.

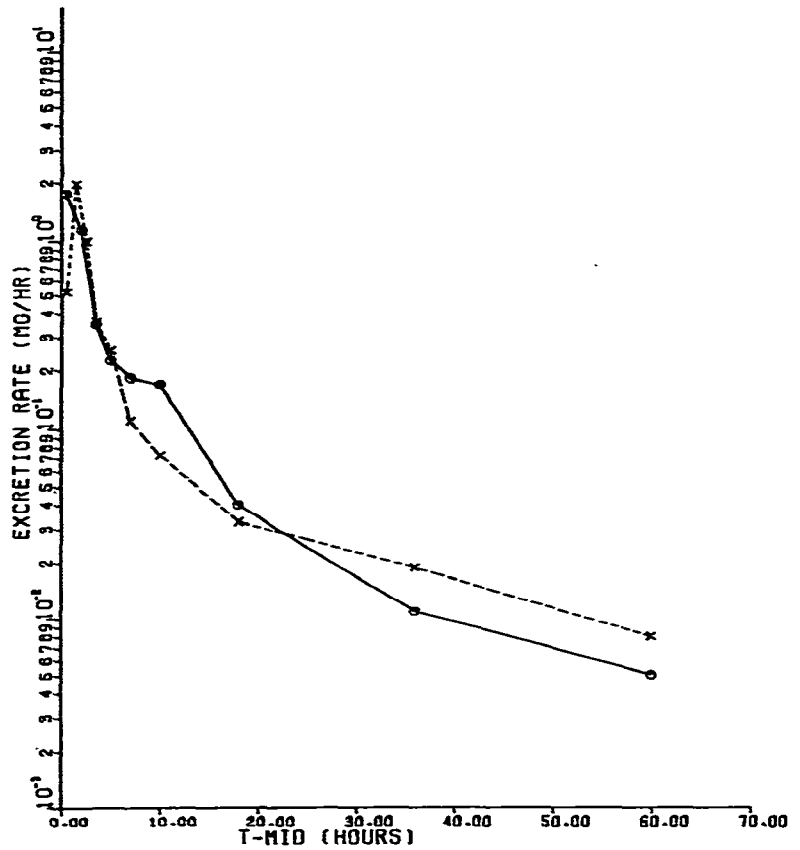


Fig. 2. Urinary excretion rate—time profiles of N-1-hydroxyethylflurazepam following oral administration of 30-mg doses of flurazepam dihydrochloride capsules to two human volunteers. o——o, Subject 1; x — — — x, Subject 2.

DISCUSSION

The UV absorbances of [I] and its metabolites, [I-A], [I-B], [II], [III] and [IV] in the HPLC mobile phase at 254 nm were sufficient to detect as little as 10 ng of pure standards injected at a detector sensitivity of 0.01 a.u.f.s. It has been reported that following single oral 90-mg doses of [I] · 2 HCl in man, about 0.5% of the dose could be accounted for in a 24-h excretion period as unconjugated [I], [II], [III], and [IV] while significant amounts of uncon-

jugated [I-A] and [I-B] (0.49—2.98% and 3.63—13.1% of the dose, respectively) and conjugated [II] (29.7—32.1% of the dose) were found [4].

Attempts to quantitate [I-A] and [I-B] by the HPLC method described were unsuccessful due to the presence of endogenous interfering substances. Modification of the method to resolve this problem was not attempted since no interferences were encountered for the analysis and quantitation of [II], the compound of interest.

A suitable HPLC procedure was developed for the quantitation of [II] in human urine following the administration of single 30-mg oral doses of [I] · 2 HCl and was used to determine the rate of excretion of conjugated [II] as a facile non-invasive means of determining the bioavailability of [I] · 2 HCl. The feasibility of this approach is demonstrated by the data shown in Table II and graphically in Fig. 2 for two subjects. The data are in agreement with previously reported results for percent of dose recovered as [II] in 24 h [3, 4]. The importance of urine sample collections over short excretion intervals to assess both rate and extent of bioavailability was shown in the data tabulation (Table II). If too few collection intervals are obtained, however, the only evaluation that could be made would be the extent of recovery of dose.

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REFERENCES

1 M.A. Schwartz, F.M. Vane and E. Postma, J. Med. Chem., 11 (1968) 770.
2 J.V. Earley, R.I. Fryer, D. Winter and L.H. Sternbach, J. Med. Chem., 11 (1968) 774.
3 M.A. Schwartz and E. Postma, J. Pharm. Sci., 59 (1970) 1800.
4 J.A.F. de Silva and N. Strojny, J. Pharm. Sci., 60 (1971) 1303.
5 J.A.F. de Silva, C.V. Puglisi, M.A. Brooks and M.R. Hackman, J. Chromatogr., 99 (1974) 461.
6 M. Hasegawa and I. Matsubara, Chem. Pharm. Bull., 23 (1975) 1826.
7 J.L. Ferguson and D. Couri, J. Anal. Toxicol., 1 (1977) 171.
8 J.P. Cano, R. Brandt, C. Aubert, G. Wendt and A. Viala, Bull. Med. Legale. Toxicol. Med., 21 (1978) 357.
9 M.A. Peat and L. Kopjak, J. Forensic Sci., 24 (1979) 46.
10 J.A.F. de Silva, I. Bekersky and C.V. Puglisi, J. Pharm. Sci., 63 (1974) 1837.
11 W. Glover, J. Earley, M. Delaney and R. Dixon, J. Pharm. Sci., 69 (1980) 601.
12 A.J. Clatworthy, L.V. Jones and M.J. Whitehouse, Biomed. Mass Spectrom., 4 (1977) 248.
13 S.A. Kaplan, J.A.F. de Silva, M.L. Jack, K. Alexander, N. Strojny, R.E. Weinfeld, C.V. Puglisi and L. Weissman, J. Pharm. Sci., 62 (1973) 1932.
14 Department of Health, Education and Welfare, Food and Drug Administration, Federal Register, 42 (5) (1977) 1624—1653.
15 N. Strojny, C.V. Puglisi and J.A.F. de Silva, Anal. Lett., B11 (1978) 135.