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GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF FLURAZEPAM AND ITS MAJOR METABOLITES IN PLASMA WITH ELECTRON-CAPTURE DETECTION*

SAM F. COOPER* and DANIEL DROLET

Centre de Recherche en Sciences de la Santé, Institut National de la Recherche Scientifique, Université du Québec, 7401 rue Hochelaga, Montréal, Québec H1N-3M5 (Canada)

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SUMMARY

A sensitive and selective gas—liquid chromatographic method, using the electron-capture detector for the quantitative determination of flurazepam and its major blood metabolites is described. After extraction and back-extraction steps, flurazepam (I) is well separated from its main metabolites, N-1-hydroxyethylflurazepam (metabolite II) and N-1-desalkylflurazepam (metabolite III). Metabolite II is quantitated after forming its stable *tert.*-butyldimethylsilyl derivative by reaction with *tert.*-butyldimethylchlorosilane—imidazole reagent. The procedure permits the rapid and selective routine determination of flurazepam and its metabolites (II and III) in plasma with a detection limit of 3 ng/ml for flurazepam (I), 1 ng/ml for metabolite II and 0.6 ng/ml for metabolite III. The procedure is linear over the range of concentrations encountered after administration of a single oral therapeutic dose. No interference from the biological matrix is apparent. The suitability of the method for the analysis of biological samples was tested by studying the variation with time of flurazepam and its metabolites' plasma concentrations in normal human volunteers after a single, therapeutic 30-mg oral dose of flurazepam.

INTRODUCTION

Flurazepam (dihydrochloride) is a 1,4-benzodiazepine derivative (Fig. 1, I) utilized as the most widely prescribed hypnotic agent for the treatment of insomnia in North America [1]. Studies on the biotransformation of flurazepam [2] showed that the drug is extensively metabolized in both man and dog. The major metabolites in human plasma are N-1-hydroxyethylflurazepam (Fig. 1, metabolite II) and N-1-desalkylflurazepam (Fig. 1, metabolite III). Several methods for the quantitative determination of flurazepam and its major plasma and urinary metabolites in biological samples exist in the recent literature;

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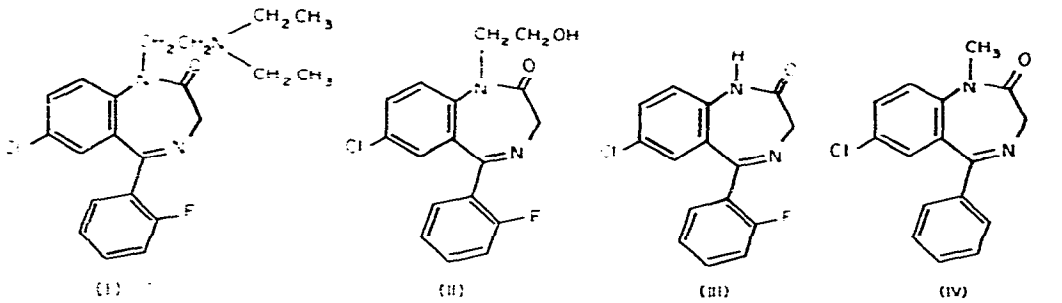

Fig. 1. Structural formulae of flurazepam (I), N-1-hydroxyethylflurazepam (II), N-1-desalkylflurazepam (III) and diazepam (IV).

employing such diverse techniques as spectrofluorometry [3,4], spectrofluorodensitometry [5], radioimmunology [6], differential pulse polarography (DPP) [7], electron-capture—gas—liquid chromatography (EC—GLC) [7—10], high-performance liquid chromatography (HPLC) [11], and gas chromatography—mass spectrometry (GC—MS) [12].

The spectrofluorometric [3,4] and spectrodensitometric methods [5] are however time-consuming and lack the specificity necessary for assaying the drug and its metabolites in a clinical context. The reported immunological method [6] describes the determination of only intact flurazepam (I) in human plasma. The DPP technique [7] was used to quantitate the major urinary metabolites of flurazepam (I), which were present in the submicrogram range in several subjects. De Silva et al. [7] developed an EC—GLC assay to measure flurazepam (I) and its metabolites (II and III) in man. Metabolite II gave an asymmetrical peak due to the polarity of the hydroxyl group. Hasegawa and Matsubara [8] studied the metabolic fate of flurazepam (I) and its several metabolites in human blood and urine by EC—GLC. Their method is time-consuming as the plasma samples were subjected to enzymatic hydrolysis, purification by Amberlite XAD resin and then derivatization of metabolites (II and III) with two different reagents. Aderjan et al. [9] investigated the plasma levels of flurazepam and its metabolites (II and III) in volunteers. Metabolite II was estimated only after enzymatic hydrolysis of the plasma samples. Riva et al. [10] reported a quantitative assay of flurazepam (I) and metabolite III in human plasma by EC—GLC but their method was not applied to human studies. Weinfeld and Miller [11] developed a method to determine only metabolite II by HPLC in human urine. Miwa and Garland [12] reported a specific and sensitive GC—MS technique to determine flurazepam (I) alone in human plasma.

The majority of the above published techniques, however, are either too time-consuming for routine use, too insensitive for the measurement of therapeutic concentrations, too non-specific or too specific and extraordinarily sensitive (e.g. GC—MS) to cause technical problems while developing the assay. The method described in this paper assays the drug and its major plasma metabolites in the absorption phase and over an interval of several biological half-lives after single-dose administration, as is necessary in pharmacokinetic and physiological availability studies in man. Metabolite II is estimated for the first time quantitatively in human plasma as the *tert.*-butyldimethylsilyl

derivative using *tert.*-butyldimethylchlorosilane—imidazole (t-BDMS) as a silylation reagent.

MATERIALS AND METHODS

Flurazepam dihydrochloride (I), its two authentic metabolites (II and III) and diazepam (IV) for chromatographic standards were supplied by Hoffman-la Roche (Vaudreuil, Canada). Diazepam was used as an internal standard for GLC analysis.

Reagents and materials

The following reagents were used: GLC-spectrometric quality benzene and toluene (J.T. Baker, Phillipsburg, NJ, U.S.A.), analytical grade methylene chloride, acetone (J.T. Baker) and methanol (Mallinckrodt, Montreal, Canada). All the above reagents were distilled before use. Analytical grade anhydrous diethyl ether (J.T. Baker) containing less than 1% peroxides, must be used from a can opened not more than five days previously. *tert.*-Butyldimethylchlorosilane—imidazole reagent (t-BDMS) was obtained from Applied Science (State College, PA, U.S.A.). The inorganic reagents were made up in double distilled water. Borate buffer solution (1 *M*) was prepared according to De Silva and Puglisi [13].

Gas—liquid chromatography

A Hewlett-Packard Model 5830A gas chromatograph equipped with a ^{63}Ni (15 mCi) electron-capture detector (ECD) was used in this study; the instrument was linked to a digital integrator (HP 18850A).

The chromatographic conditions for flurazepam (I) and its major metabolites (II and III) in plasma were as follows: a 1.8 m × 2 mm I.D. coiled glass column was packed with 3% OV-17 on 100—120 mesh Gas-Chrom Q (Chromatographic Specialties, Brockville, Canada). In order to avoid adsorption losses of the compounds on the active sites of the column, it was deactivated by injecting 25 μl of REJUV-8 (Supelco, Bellefonte, PA, U.S.A.) daily before use. Column, injector and ECD temperatures were 255°C, 275°C and 300°C, respectively. The argon—methane (95:5) carrier gas flow-rate was 22 ml/min. Under these conditions, the relative retention times of metabolite III, flurazepam (I) and t-BDMS derivative of metabolite II to the internal standard (diazepam) were 1.17, 2.02 and 2.46, respectively (Figs. 2 and 3).

The relative retention time of underivatized metabolite II to the internal standard was 2.21. This indicates that metabolite II was separated in both derivatized and underivatized forms from flurazepam (I) and metabolite III.

Gas chromatography—mass spectrometry

Mass spectra were recorded using a Hewlett-Packard dual electron impact/chemical ionization source gas chromatograph—mass spectrometer (Model 5982A) linked to a laboratory data system (Hewlett-Packard Model 5933A). Operating conditions were as follows: a 1.2 m × 2 mm I.D. coiled glass column was packed with 3% OV-17 on 80—100 mesh Gas-Chrom Q (Chromatographic Specialties). The oven temperature was maintained at 230°C for 2 min then

programmed at 16°C/min to a final temperature of 270°C and held for 6 min. The membrane separator was also programmed at 16°C/min and the transfer line temperature was 240°C. The apparatus was operated in the electron impact mode. Source temperature was 170°C, electron energy 70 eV and trap current 0.35 mA.

Extraction procedure

The extraction procedure in plasma was established using a modification of the extraction procedures described by De Silva and co-workers [7,14] for determination of flurazepam (I) and its major metabolites (II and III) in whole blood by GLC. To 3 ml of plasma in a 15-ml tube with a PTFE-lined cap were added 100 μl of the methanolic solution of diazepam (400 ng/ml) as the internal standard. The plasma sample was made alkaline by the addition of 3 ml of 1 *M* borate buffer solution (pH 9.0). The mixture was agitated with vortex action for 15 sec.

The sample was then extracted with 6.0 ml of benzene—methylene chloride (90:10) for 5 min on an Eberbach mechanical agitator. After centrifuging at 600 *g* for 15 min (Model TJ-6, rotor TH-4, Beckmann, Palo Alto, CA, U.S.A.), the organic layer was transferred to a 15-ml tube with screw cap and the aqueous layer was discarded. The organic layer was back-extracted with 3.0 ml of 4.0 *N* hydrochloric acid for 10 min. The sample was centrifuged for 10 min at 400 *g* and the organic layer was removed and discarded. The aqueous layer was washed with 5 ml of diethyl ether by shaking for 10 min and centrifuging for 10 min. The organic layer was removed by aspiration and discarded. The sample was cooled in an ice-bath and made alkaline by slowly adding 3.5 ml of 4.0 *N* sodium hydroxide solution. The tube was vortexed for 15 sec. The aqueous phase was extracted twice with 2.5 ml of diethyl ether by shaking for 10 min and centrifuging 10 min. The ether extracts were combined in a 7-ml tube, dried with sodium sulfate, then transferred and evaporated off in a 3-ml conical glass-stoppered tube by successive evaporation at 35—40°C with a slow stream of nitrogen. The walls of the tube were rinsed with 200 μl of methanol by vibrating with a Vortex mixer for 1 min. The solution was evaporated to dryness as before and the residue was redissolved in 100 μl of benzene—acetone—methanol (85:10:5). The precise volume of 5.0 μl of the solution was injected into the gas chromatograph for the estimation of flurazepam (I) and metabolite III.

Derivatization of metabolite II

The remainder of the solution was used for the determination of metabolite II. The solution was evaporated to dryness at 60°C with a slow stream of nitrogen. To the residue were added 5 μl of t-BDMS reagent. The mixture was vortexed well for 30 sec in order to rinse the walls of the conical tube and then heated at 60°C for 10 min. To this mixture were added 200 μl of toluene. The solution was vortexed again for 30 sec and the precise volume of 5.0 μl was injected into the gas chromatograph.

Calibration curves

To 3 ml of heparinized blank human plasma in 15-ml tubes were added

(1) 25, 50, 75 or 100 μl of methanolic solution of flurazepam base (400 ng/ml), (2) 10, 20, 30, 40, 50 or 60 μl of methanolic solution of metabolite II (2 μg/ml), (3) 25, 50, 75, 100, 125 or 150 μl of methanolic solution of metabolite III (400 ng/ml) and (4) 100 μl of diazepam solution in methanol (400 ng/ml). The samples were then carried through the complete extraction procedure described above. Quantitation was achieved using the ratio of the peak areas of flurazepam (I), and metabolites II and III to that of the internal standard, diazepam.

Peak area ratios were plotted against weight to obtain the calibration curves. The response of ECD to flurazepam (I) was linear over the range of 3.3—13.3 ng per ml plasma, to metabolite III from 3.3—20 ng per ml of plasma, and to metabolite II from 6.3—38 ng per ml of plasma.

Human studies

Flurazepam dihydrochloride (I, 30-mg capsules) was administered separately to twenty healthy volunteers under controlled conditions. Blood samples (10 ml) were withdrawn from the antecubital vein using heparinized evacuated tubes (Venoject, Kimble-Terumo, Elkton, MD, U.S.A.) at fifteen appropriate time intervals after dosing. The blood samples were centrifuged at 600 *g* for 10 min and the plasma was transferred to another tube and frozen at −20°C until analyzed.

RESULTS AND DISCUSSION

Selectivity

Analytical studies indicate that extracts from blank human plasma do not show peaks that could interfere with the quantitative determination of flurazepam (I) and its major metabolites (II and III) in plasma. Typical chromatograms are shown in Figs. 2 and 3. Although metabolite II could be identified by ECD without derivatization, as indicated by De Silva et al. [7], the detector response of this compound was very low, and in addition, showed tailing due to the polarity of the hydroxyl group. In this procedure, metabolite II was quantitated in plasma as *tert*.-butyldimethylsilyl ether with t-BDMS as a silylation reagent. The main advantages of t-BDMS ethers are: (1) they are 10^4 times more stable to hydrolysis than trimethylsilyl ethers and (2) their mass spectra which usually have their base peak at M^+—57, are less complicated.

De Silva et al. [7] prepared the trimethylsilyl derivative of metabolite II with bistrimethylsilylacetamide (BSA) in pyridine. The resultant silyl ether eluted as a sharp symmetrical peak with a five-fold increase in sensitivity but its retention time was identical to that of metabolite III, the major metabolite of flurazepam in plasma. Owing to this interference, the derivatization of metabolite II with BSA was rendered impractical.

Hasegawa and Matsubara [8] used bistrimethylsilyltrifluoracetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) as a silylation reagent for metabolite II. These authors divided the plasma extract into two parts. One part was methylated to convert metabolite III to N-1-methyl derivative with diazomethane. The other part was silylated to convert metabolite II to its silyl ether. We have observed that the methylation of metabolite III was unnecessary due to its high sensitivity and resolution from other interfering

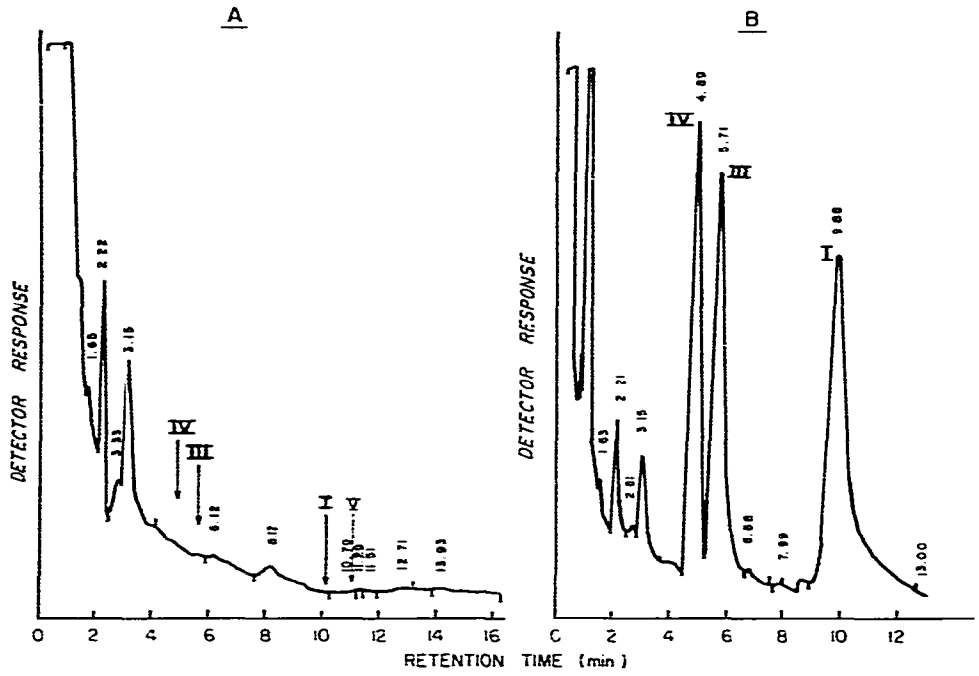

Fig. 2. (A) Chromatogram obtained from an extract of 3 ml of blank human plasma. The arrows show the absence of signals at the retention times of the internal standard (IV), N-1-desalkylflurazepam (III), flurazepam (I) and N-1-hydroxyethylflurazepam (V). (B) Chromatogram obtained from an extract of 3 ml of human plasma containing the compounds IV, III and I.

peaks, whereas, with t-BDMS reagent, the silyl derivative of metabolite II was completely separated from metabolite III (Fig. 3).

Flurazepam (I) and metabolite III were assayed before metabolite II because t-BDMS reagent decreased the sensitivity of compound I. The stationary phase 3% OV-17 gave a good resolution for flurazepam (I), its two major plasma metabolites (II and III) and the internal standard (IV).

Recovery studies

The recoveries of flurazepam (I) and its metabolites (II and III) from 3 ml of spiked plasma were determined using the same internal standardization method as described above. The peak area ratio of each compound and the internal standard (IV) was used as the index of detector performance and overall effiency of the analytical procedure. The reproducibility and recovery results of compounds (I, II and III) are given in Table I. The overall coefficient of variation (C.V.) is below 10%.

Sensitivity

The lower limits for accurate determination of flurazepam (I) and its major plasma metabolites (II and III) were established by spiking 3 ml of blank plasma with dilute methanolic solutions of compounds I, II and III in the range of 10—60 ng, 10—60 ng and 20—120 ng respectively followed by the previously described extraction procedure using diazepam (IV) as an internal standard.

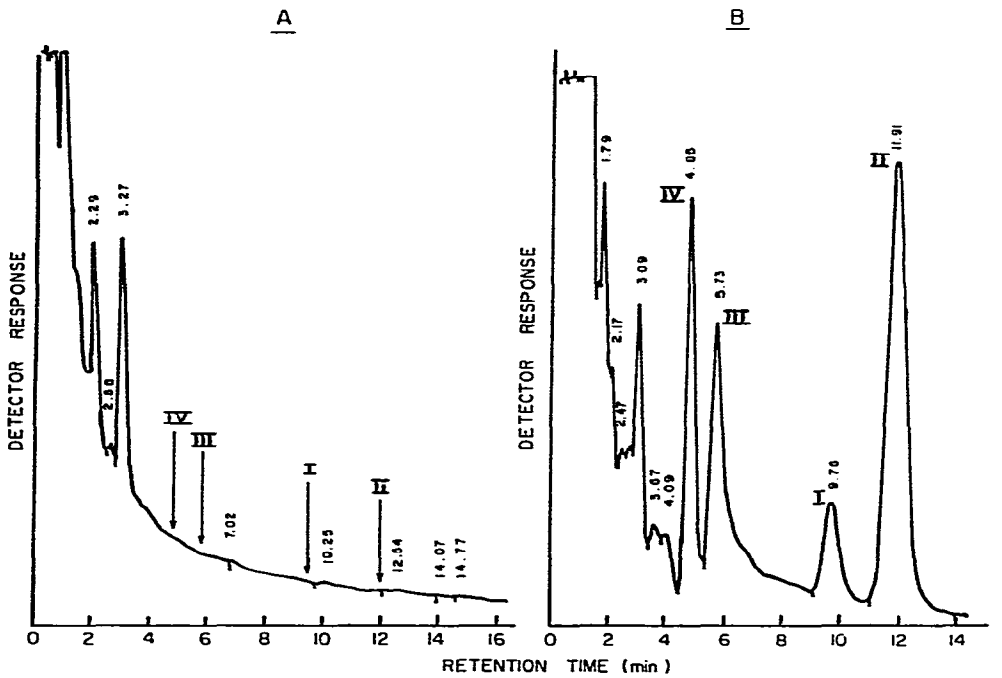

Fig. 3. (A) Chromatogram obtained from an extract of 3 ml of blank human plasma derivatized with t-BDMS. The arrows show the absence of signals at the retention times of the internal standard (IV), N-1-desalkylflurazepam (III), flurazepam (I) and t-BDMS derivative of N-1-hydroxyethylflurazepam (II). (B) Chromatogram obtained from an extract of 3 ml of human plasma containing the compounds IV, III, I and II derivatized with t-BDMS.

The lower detection limit was fixed to the minimum response of ECD to the respective compounds with peak areas up to 40,000 counts. It was found that the lowest detection limits of flurazepam (I), metabolites II and III were 3, 1 and 0.6 ng/ml of plasma respectively with a signal-to-noise ratio of 5.

Precision and accuracy

The ECD response to flurazepam (I) was linear over the range of 3.3—13.3 ng/ml of plasma, to metabolite III from 3.3—20 ng/ml of plasma, and to metabolite II from 6.3—38 ng/ml of plasma. Standard curves constructed by plotting the peak area ratio (y) against the amount (ng added) of I, II and III (x) coincided with the line $y = mx + b$. The linear regression values are: (I), $m = 0.121$, $b = -0.286$, $r = 0.9951$; (II), $m = 0.099$, $b = -0.090$, $r = 0.9980$; (III), $m = 0.097$, $b = 0.027$, $r = 0.9997$. These plots indicate the validity of the peak area ratio method of quantitation using diazepam (IV) as the internal standard.

Gas chromatography—mass spectrometry

The GC—MS data of flurazepam and its major metabolites were first reported by Clatworthy et al. [15]. The mass spectrum of *tert.*-butyldimethylsilyl derivative of metabolite II is shown in Fig. 4 using the electron impact mode. The major ion in the spectrum is at m/z 389 (M^+—57) corresponding to the loss of the *tert.*-butyl group along with the ^{37}Cl isotope peak at 391.

TABLE I

GLC ESTIMATION OF FLURAZEPAM (I) AND METABOLITES II AND III ADDED TO PLASMA

Compound	Compound added (ng per 3 ml plasma)	Compound recovered (ng)	Recovery* (%)	C.V. (%)
Flurazepam (I)	10	8.52	85.2	4.65
	20	16.76	83.8	5.18
	30	25.98	86.6	3.06
	40	32.70	81.8	4.28
Metabolite II**	19	14.40	75.8	5.49
	38	27.88	73.4	4.03
	57	43.80	76.8	4.05
	76	54.16	71.3	3.40
	95	66.72	70.2	2.41
	114	78.68	69.0	2.05
Metabolite III	10	7.86	78.6	4.08
	20	15.32	76.6	6.03
	30	23.04	76.8	5.32
	40	29.04	72.6	4.50
	50	38.40	76.8	4.82
	60	44.28	73.8	4.11

*Each value is the mean of five determinations.
**Determined as *tert.*-butyldimethyl derivatives.

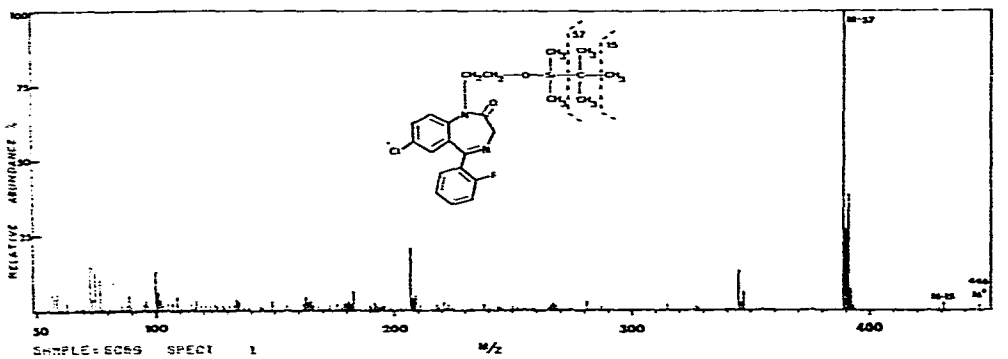

Fig. 4. Mass spectrum of *tert.*-butyldimethylsilyl derivative of metabolite II using the electron impact mode.

The molecular ion is present at *m/z* 446. The ion at *m/z* 431 (M^+—15) indicates the loss of a methyl group.

Application of the method to human studies

The lower limit of sensitivity of the procedure as described permits the quantitation of flurazepam (I), whose mean apparent half-life was found to be 2.9 h as reported elsewhere [6,9,12], metabolite II (mean half-lives α-phase 1.9 h and β-phase 14.1 h) as observed by Aderjan et al. [9] and metab-

olite III (mean half-life 93.1 h) as found by Greenblatt et al. [16], after oral administration of a single therapeutic dose. Flurazepam (I) and metabolites II and III could be detected as long as 7, 24 and 264 h post administration, respectively. The average plasma concentrations (as a function of time) of flurazepam (I) and its metabolites (II and III) found in twenty volunteers after a single 30-mg oral dose of flurazepam dihydrochloride are illustrated in Figs. 5, 6 and 7. Plasma levels of flurazepam (I) and its metabolites (II and III) vary considerably between subjects. The pharmacokinetic data of this study are reported elsewhere [17]. This variability in plasma concentrations of in-

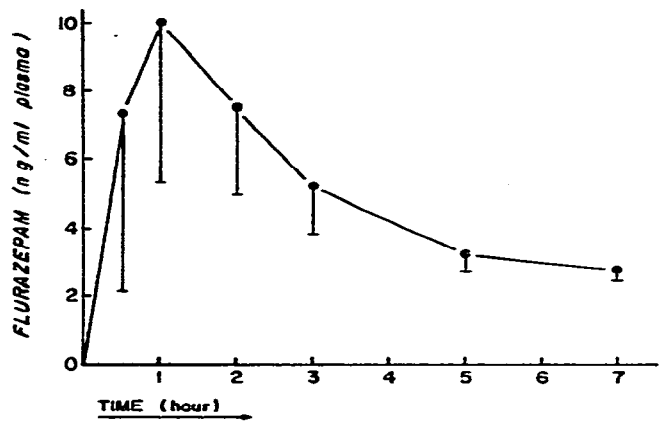

Fig. 5. Average plasma concentration—time profile of flurazepam (I) in 20 volunteers after administration of 30 mg of flurazepam dihydrochloride. Bars indicate 1 S.D.

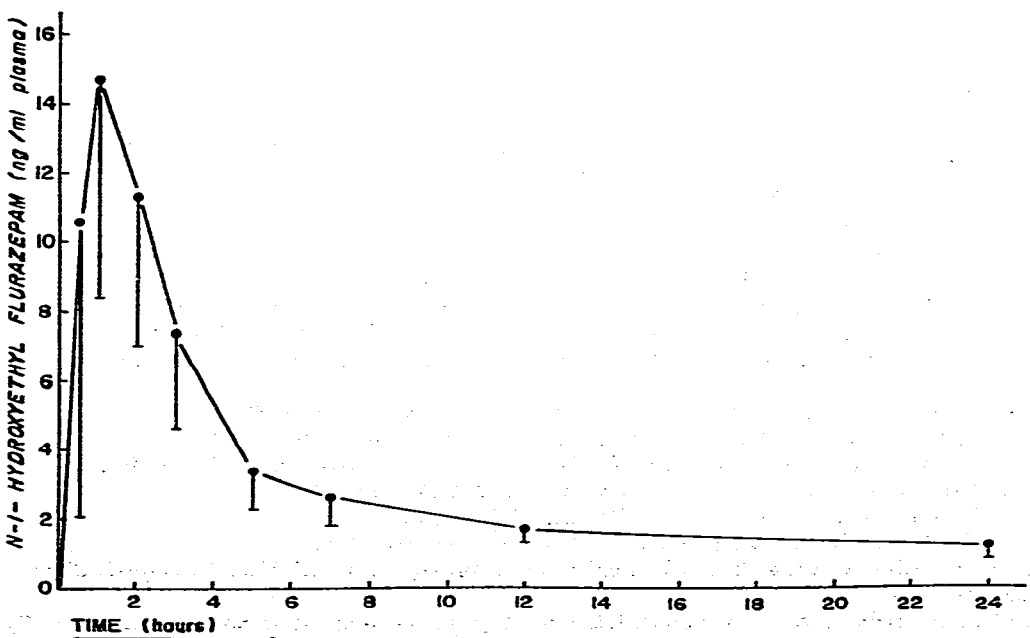

Fig. 6. Average plasma concentration—time profile of metabolite II in 20 volunteers after administration of 30 mg of flurazepam dihydrochloride. Bars indicate 1 S.D.

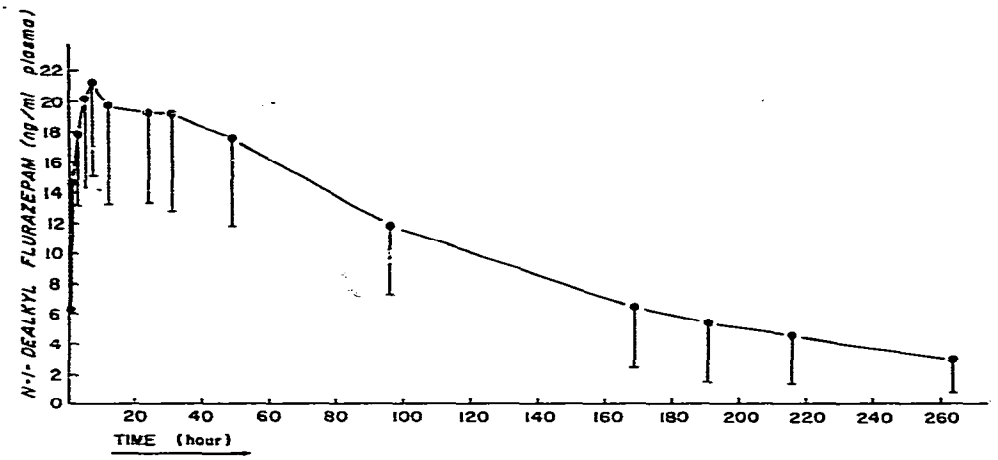

Fig. 7. Average plasma concentration—time profile of metabolite III in 20 volunteers after administration of 30 mg of flurazepam dihydrochloride. Bars indicate 1 S.D.

dividuals under controlled conditions has been found and reported for diazepam [18] and lorazepam [19]. These variations may be ascribed to individual differences in the metabolic rate of elimination.

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