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Metabolic Fates of Flurazepam. I. Gas Chromatographic Determination of Flurazepam and Its Metabolites in Human Urine and Blood Using Electron Capture Detector

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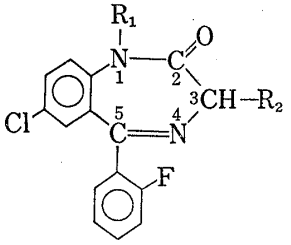
A rapid, specific and highly sensitive electron-capture gas chromatographic method for the determination of flurazepam and its four metabolites in human urine, serum and plasma was developed. The metabolites in blood specimens and in β-glucuronidase treated urines were cleaned up using porous polymer resin and selective extraction, and were derivatized to trimethyl silylate (TMS), methyl derivatives and methyl ester, respectively. The recoveries were almost quantitative and the sensitivity was enough for therapeutic dose levels (15—30 mg). The sensitivity limits were 0.3—2.0 ng/ml of each metabolites in serum and plasma. The method was applied to urine and serum specimens of single dose or chronic dose of 30 mg ot flurazepam, and the metabolic properties of the drug were also discussed.

Flurazepam[7-chloro-1-(2-diethylaminoethyl)-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one, F in Chart 1][2] monohydrochloride is a new central nervous system agent and has been reported to have pharmacological properties similar to those of the other benzodiazepines. Clinically it is used as a unique hypnotic which has little effect on rapid eye movement (REM) sleep,[3] and therefore can induce natural sleep-like sleep. A few studies on metabolism in humans[4] and animals[5] have been reported for this drug.



name	R_1	R_2
F (flurazepam):	$CH_2CH_2N(C_2H_5)_2$	H
II:	CH_2CH_2OH	H
III:	H	H
IV:	H	OH
V:	CH_2COOH	H

Chart 1. Structures of Flurazepam and Its Metabolites

The determination of flurazepam and its four metabolites (II, III, IV and V, Chart 1) in human urine and blood was first achieved by de Silva, *et al.* by the spectrophotometric and spectrofluorometric method developed by them.[6]

Unfortunately their method involved somewhat complicated assay procedures, so that it was very much time consuming and seemed to require skillfullness to obtain sufficient

1) Location: *3-6-6, Asahi-cho, Machida-shi, Tokyo.*
2) Flurazepam is the product of joint development with Nippon Roche Co., Ltd. in Japan.
3) J. Kales, A. Kales, E.O. Bixler and E.S. Slye, *Clin. Pharmacol. Therap.*, **12**, 691 (1971); S. Fujii, *Psychiat. Neurolog. Jap.*, **75**, 545 (1973).
4) M.A. Schwartz, F.M. Vane, and E. Postoma, *J. Med. Chem.*, **11**, 770 (1968); M.A. Schwartz and E. Postoma, *J. Pharm. Sci.*, **59**, 1800 (1970).
5) T. Nishikawa, K. Mineura, and H. Takahira, *Yakugaku Zasshi*, **93**, 226 (1973); T. Nishikawa, K. Mineura, and H. Takahira, *ibid.*, **93**, 232 (1973).
6) *a*) J.A.F. de Silva and N. Strojny, *J. Pharm. Sci.*, **60**, 1303 (1971); *b*) S.A. Kaplan, J.A.F. de Silva, M.L. Jack, K. Alexander, N. Strojny, R.E. Weinfeld, C.V. Puglisi, and L. Weissman, *ibid.*, **62**, 1932 (1973).

reproducibility. Moreover, the recoveries of these metabolites from biological specimens were conciderably low. Thus the method did not seem to meet the routine analysis.[7]

We describe here a simple and rapid gas chromatographic method using an electron capture detector, which has enough sensitivity to determine these metabolites in urine and serum or plasma specimens at therapeutic dose levels of flurazepam (15—30 mg per body). The recoveries were greatly improved and the reproducibility was also satisfactory. This method meets the clinical usage dealing with a large number of samples. We also describe the application of the present method to urine and serum specimens following single or chronic oral administration of flurazepam, and discuss the metabolic properties of the drug.

Experimental

Materials

All reagents were obtained from commercial source and used without further purification except methanol (MeOH) used for derivatization of metabolites, which was redistilled and dried over magnesium. Porous polymer resin Diaion HP-10 (Amberlite XAD-like resin, Mitsubishi Kasei Kogyo Co., Ltd.) was thoroughly washed with MeOH until no back ground peaks originated from the resin impurities became detectable on gas chromatograms. Flurazepam monohydrochloride and its four authentic metabolites were generous gifts from F. Hoffmann-La Roche Co. (Basle).

Apparatus and Conditions

Yanagimoto model G-800 gas chromatograph equipped with electron capture detector (ECD) (^{63}Ni as a β-ray sources) was employed. All chromatography were carried out on a glass column (2.0 m × 3.0 mm i.d.) packed with 2% (W/W) silicone OV-17 on 100—200 mesh acid-washed Chromosorb W at 253°. The injection port was kept at 290°, and the inlet pressure of carrier gas (N_2) was adjusted to 1.2 atm so that the t_R of Diazepam used as the internal standard was 6.0 min.

The structures of the derivatives of metabolites were determined with Varian T-60 NMR spectrometer using $CDCl_3$ as a solvent.

Assay Procedures

Preparation of Samples——Urine specimens were analyzed after β-glucuronidase treatment. To determine the free form–conjugate ratios in urine and blood, enzymatically treated serum and non-treated urine specimens were also analyzed.

Frozen urine specimens were thawed just prior to the analysis. Aliquots of 5 ml of the samples were taken in 20 ml test tube with 1 ml of 1M sodium acetate–acetic acid buffer (pH 5.0). After brief incubation at 38°, 1.56U of β-glucuronidase/arylsulphatase (Boehringer Mannheim) in 1 ml of 0.2M acetate buffer (pH 5.0) was added, and the mixtures were incubated shaking reciprocally (120 cpm) at 38° for 120 min. The reaction mixtures were then directly applied to short columns packed with 1 ml of Diaion HP-10 resin (2.0 × 0.8 cm i.d.). After washing out non or weakly adsorptive materials with 20 ml of deionized water and 20 ml of 20% MeOH successively, F and its metabolites (II, III, IV and V) were eluted with MeOH. The eluates (25 ml) to which 2 ml of deionized water was added, were then evaporated under vacuo to remove MeOH. The resultant aqueous residues were transferred to 30 ml separatory funnels with 5 ml of 0.01M boric acid–Na_2CO_3 buffer (pH 9.0). F, II, III and IV were then extracted twice with 16 ml (8 ml × 2) of ethylacetate (EtOAc) mechanically shaking for 10 min. The combined organic phases were filled up to 20 ml with EtOAc and dried over anhydrous Na_2SO_4 (hereafter called "pH 9 extract"). To the remaining aqueous phases, 5 ml of 1M citric acid–sodium citrate buffer (pH 3.0) saturated with NaCl and 2.2 g of NaCl were added, and the mixtures were extracted once with 15 ml of EtOAc shaking for 15 min. The organic phase was dried over anhydrous Na_2SO_4 (hereafter called "pH 3 extract").

Two-four milliliters of serum or plasma specimens were applied to HP-10 columns directly or after β-glucuronidase treatment, and then cleaned up in the same way as described for urine specimens.

Preparation of Derivatives——Unchanged form (F); Aliquots of 3 ml of the pH 9 extracts were taken in small (1 cm × 12 cm) glass-stoppered test tubes and evaporated to dryness under vacuo, followed by addition of 0.1 ml of EtOAc containing 1 ppm of Diazepam (IS solution). One microliter aliquots were applied to GLC. Metabolite II; Aliquots of 0.5 ml of diluted pH 9 extracts (10—20 times with EtOAc) were taken with 0.1 ml of the IS solution, and evaporated to dryness. To the residues 0.1 ml of BSTFA[bis(trimethylsilyl)trifluoroacetoamide, Pierce Chemical Co.] containing 1% (V/V) of TMCS (trimethylchlorosilane, Tokyo

7) Recently, in preparation of this report, they reported a gas chromatographic method using ECD for blood specimens and polarographic determination of the urinary metabolites after separation by thin-layer chromatography (TLC). But their method lacked in sensitibity for the major metabolite (II) in blood, and III, IV and V in urine [J.A.F. de Silva, C.V. Puglisi, M.A. Brooks, and M.R. Hackman, *J. Chromatog.*, **99**, 461 (1974)].

Kasei Kogyo Co.) was added. After standing at room temperature for 90 min, 1 μl of the reaction mixtures was injected into gas–liquid chromatography (GLC). Metabolite III and IV; These compounds were analyzed as N_1-methyl and N_1, O-dimethyl derivatives respectively. To the evaporated residues of 3 ml aliquots of pH 9 extracts, 0.2 ml of dry MeOH and 0.2 ml of diazomethane in ether were added, and the mixtures were allowed to stand for 90 min at 0°. After removal of excess reagent with gentle stream of N_2 gas, 0.1 ml of the IS solution was added, and 1 μl aliquots were injected into GLC. Metabolite V; Aliquots of 3 ml of pH 3 extracts were evaporated to dryness, to which 0.2 ml of diazomethane in ether was added. After standing for 1 min at room temperature, excess reagent was removed with gentle N_2 gas stream, and then 0.1 ml of the IS solution was added. Aliquots of 1 μl were applied to GLC.

For the serum or plasma specimens, sampling sizes from extracts and injection sizes into GLC were larger than those for urine specimens, but the derivatization conditions were the same. For the determination of F, 6 ml aliquots of pH 9 extracts were taken and 5 μl aliquots with the internal standard were injected. Metabolite II was analyzed using 2 ml aliquots of pH 9 extracts, and 2—4 μl aliquots of the silylated mixtures were applied to GLC. To determine III and IV, 6 ml aliquots of pH 9 extracts were taken and methylated. The methylation products were dissolved into 0.1 ml of the IS solution and their 5 μl aliquots were injected to GLC. V was determined using 5 ml aliquots of pH 3 extracts. The esters in 0.1 ml of the IS solution were applied to GLC at 5 μl injection size.

For these blood analysis, 0.1 ppm of Diazepam in EtOAc was used as the IS solution.

Drug Administration to Humans

Single Dose——Six adult healthy male volunteers were given two 15 mg-capsules of flurazepam monohydrochloride 2 hr after breakfast. Serum specimens were collected just before drug administration and at 0.5, 1, 3, 8, 24, 48 and 72 hr postadministration. Urine specimens were collected in the following periods; 0—1 hr, 1—3 hr, 3—8 hr, 8—24 hr and 24—48 hr postadministration.

Chronic Dose——Two adult male volunteers were given 30 mg of flurazepam monohydrochloride for 8 days. Serum specimens were obtained just before drug administration, 1 hr postadministration (Days 1—8) and after drug withdrawal (Days 9 and 10). Urine specimens in 0—8 hr period postadministration were collected in the same days as described above.

All of these samples were frozen immediately and stored at −20° until analysis.

Results and Discussion

Enzymatic Hydrolysis of Conjugates

It has been known that metabolites II, IV and V were excreted mostly as the glucuronides in humans.[4] de Silva, *et al.*[6] hydrolyzed these conjugates with 4N HCl and obtained respective benzophenones which were then separated by TLC. By their method, 3-hydroxy metabolites (*e.g.* IV) could not be distinguished from not hydroxylated metabolites (*e.g.* III). Therefore they employed selective extraction before hydrolysis on the bases that the urinary excreted forms of III and IV were different, the former was excreted as free form and the latter as the glucuronide.[6]

In this work, to simplify the assay procedures and to improve the specifficity, enzymatic hydrolysis of these conjugates was studied. As this method maintained the benzodiazepine structures of the metabolites, it was possible to determine III and IV separately by single extraction and injection into GLC. The efficiency of this method was clearly shown by the fact that a new 3-hydroxy metabolite was found in urine specimens (See below and Fig. 6), which could not be distinguished from II by the chemical hydrolysis.

In order to shorten the incubation time, the enzyme of relatively high units was added. As shown in Fig. 1, hydrolysis of a urine specimen of 0—8 hr postadministration of 30 mg of the drug reached plateau at 2 hr incubation. There was no increase of hydrolysis by the addition of the excess units of the enzyme, indicating that the hydrolysis was completed.

Adsorption on HP-10 Resin and Selective Extraction

The direct injection of the urine extracts and the most of the serum extracts gave interfering multiple back ground peaks. Moreover, the direct extraction of blood specimens formed stable emulsions caused by blood proteins.

Preliminary screening of the clean up procedures showed that F and the all of its known metabolites were adsorbed strongly on porous polymer resin Diaion HP-10. They could

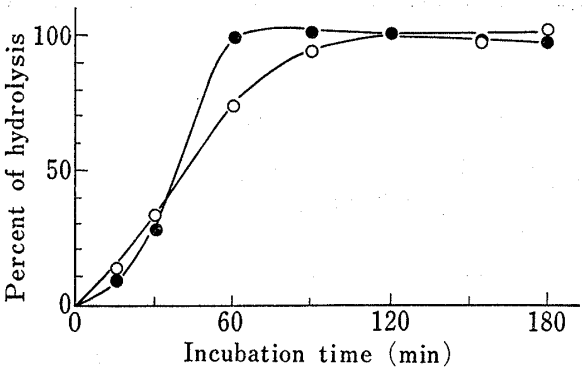


Fig. 1. Enzymatic Hydrolysis of Urinary Conjugated II and IV

5 ml of urine specimen in the period 0—8 hr. Postdose was treated with 1.56 U of β-glucuronidase/aryl sulphatase, and liberated II and IV were analyzed by GLC. The peak areas at each interval were compared with those at 2 hr incubation by percentage. ○ :II, ● :IV

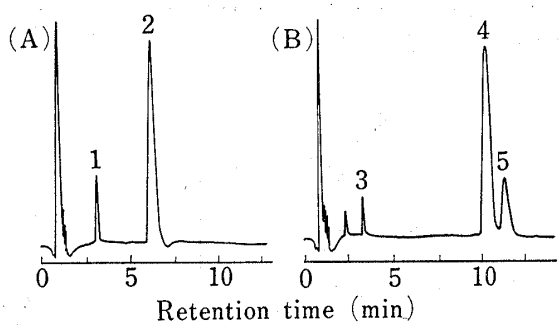


Fig. 2. Gas Chromatograms of Methylated III and IV

5 μg of authentic samples of III and IV were methylated with CH_2N_2 and dissolved in 0.1 ml of EtOAc. 1 μl aliquots of the solution were injected into GLC.
(A) methylation products of III
(B) methylation products of IV

be eluted quantitatively with MeOH after washing out non and weakly adsorptive materials. Through this step, gas chromatographic interfering materials, high concentration of proteins from the glucuronidase sources and blood could be effectively removed.

As metabolite V had almost the same t_R (11.2 min) as that of F (11.5 min), they were separated before GLC analysis by the selective extraction into pH 3 and pH 9 extracts, respectively.

These clean up procedures showed their effectiveness giving no interfering peaks in chromatograms.

Derivatization of Extracted Metabolites

Preliminary examination showed that the authentic samples of F, III and IV, but II and V gave clear peaks in gas chromatograms at respectively suitable oven temperatures without derivatization. Thus, F, III and IV could be chromatographed simultaneously if temperature programming was employed (*e.g.*, 210—255° using silicone OV-17). But in concerning that the temperature programming was not suited to ECD causing the decreasing alteration of its sensitivity, preferable derivatives having desirable t_R's at one oven temperature should be chosen with careful selection of stationary phases. The selected derivatives here were silylated II, methylated III and IV, and methyl ester of V.

Silylation of the hydroxy group of II was carried out using BSTFA containing TMCS as a catalyst which had been used for amino acids by Gehrke, *et al.*[8] This reagent did not stain the detector unlike the other silylation agents. Methylation of III and IV was accomplished with diazomethane in dry MeOH. Methylation of III gave two spots on TLC that could be detected under UV light. The main spot, the structure of which was confirmed by NMR to be N_1-methylated III (Fig. 3, A) after separation by preparative TLC, corresponded to the larger peak (peak 2) in Fig. 2, A. This peak was used for quantification of III. In the case of IV, three spots on TLC and correspondent three peaks in gas chromatogram were obtained (Fig. 2, B). The main peak (peak 4) which was determined to be N_1,O-dimethyl IV by NMR (Fig. 3, B) after isolation by TLC, was employed to determine metabolite IV. The gas chromatographic peak areas of these two main products of III and IV were well proportional to the original amounts of III and IV. The structures of compounds giving peaks 1, 3 and 5 respectively are not obvious at the present. Metabolite V was easily and quantita-

8) C.W. Gehrke, H. Nakamoto, and R.W. Zunwalt, *J. Chromatog.*, **45**, 24 (1968).

tively esterified with diazomethane. The structure of the ester was also confirmed by NMR (methyl group of the ester=δ 3.81 ppm).

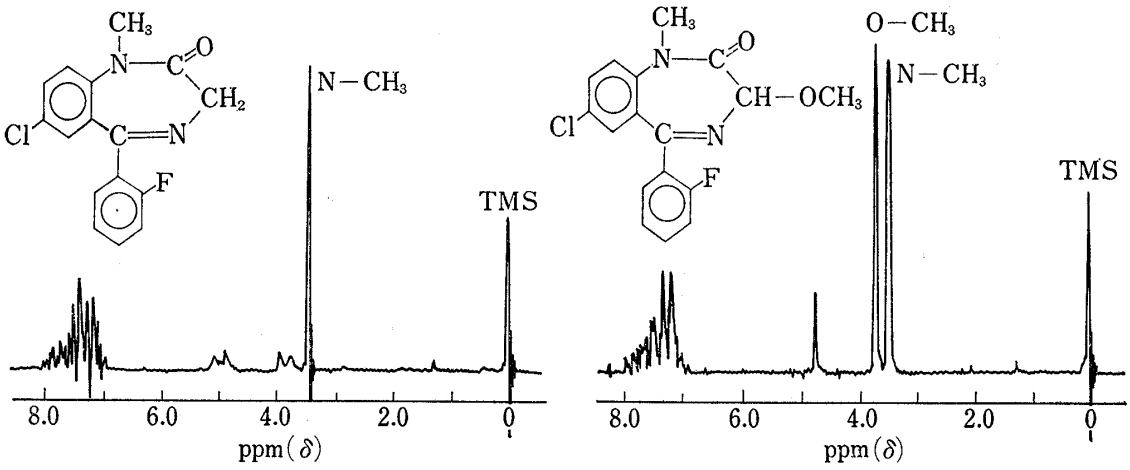


Fig. 3. Proton NMR Spectra in $CDCl_3$ of Main Methylation Products of III and IV Used for Analysis

10 mg of III and IV were methylated, and the products were separated on TLC (Kieselgel G, benzene–MeOH–AcOH=80: 10: 10). Main spots that corresponded to peak 2 and peak 4 (Fig. 2) respectively were eluted with MeOH and their structures were examined by NMR.
(A) main methylation product of III
(B) main methylation product of IV

Gas Chromatography

It was shown that silicone OV series were suitable and that OV-17 gave good separation and symmetrical peaks. To avoid the column bleeding, lightly coated packings were chosen.

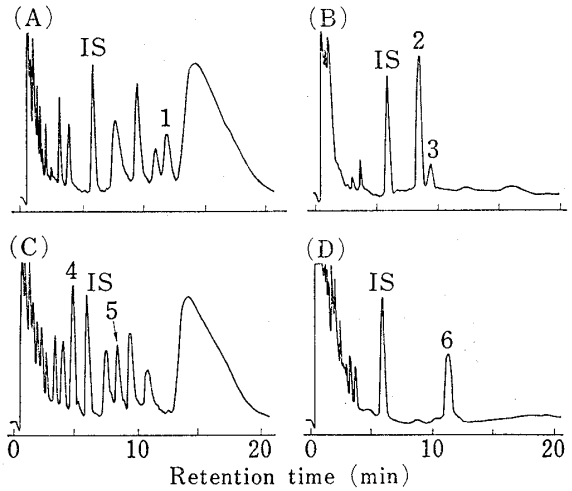


Fig. 4. Gas Chromatograms of a Urine Specimen in 3—8 hr Period Postadministration of 30 mg Oral Dose of Flurazepam HCl

(A) not treated pH 9 extract for F (peak 1), (B) silylated pH 9 extract for II (peak 2), (C) methylated pH 9 extract for III (peak 4) and IV (peak 5), (D) methylated pH 3 extract for V (peak 6)
IS; internal standard (diazepam), peak 3; new metabolite (see Fig. 6) The large peaks that appeared in (A) and (C) at t=15 min are those of free form of II.

titative, reproducible recovery in each step, extraction of eluates, and the derivatization.

Diazepam was employed as the internal standard which had the same relative molar sensitivity to ECD as the flurazepam metabolites and the desirable t_R.

Gas chromatograms of a urine specimen postadministration of 30 mg of the drug were shown in Fig. 4.

Recoveries and Sensitivities

Known amounts of the authentic compounds in MeOH were added to control urine and serum or plasma, and the overall recoveries were determined. The results of this experiment which are summarized in Table I showed that almost all of the metabolites except V in blood specimens were recovered nearly quantitatively. Even if the recovery of V from blood specimens was somewhat low, these recoveries were greatly improved when they were compared with the fluorimetric method.[6a] This may be caused by much simpler procedures and by quan- *i.e.*, the HP-10 treatment, the selective

Table I. Overall Recovery, Assay Sensitivity and Linear Dynamic Range of Calibration Curves

Compounds	Urine		Serum[a]			Linear dynamic range of calibration curve (ng per injection)
	Recovery (%)	Level[b] (μg/ml)	Recovery (%)	Level[b] (ng/ml)	Lower detection limit (ng/ml sample)	
F	99±3.0	0.1	94±6.0	4.0	0.5	0.1 —4.0
II	98±5.0	2.0	100±7.0	10	0.3	0.01—3.0
III	95±4.0	0.2	93±5.0	10	2.0	0.1 —3.0
IV	93±6.0	0.2	96±6.0	10	2.0	0.2 —4.0
V	93±2.0	0.1	88±5.0	5.0	0.3	0.05—1.0

a) Almost the same results were obtained for blood plasma.
b) Authentic compounds were added to control urine or serum to give these concentrations.

Practical assay sensitivities were also shown in Table I with the linear dynamic ranges of the calibration curves per injection. The lower detection limits were also improved and were enough for the therapeutic dose levels of flurazepam (15—30 mg).

Application of Method

Single Dose——The urinary excretion data in 0—48 hr period following the oral administration of 30 mg dose of the drug for six volunteers were summarized in Table II. The excretion profiles of the two of them were shown in Fig. 5. Free form-conjugate ratios were measured for one of the volunteers (N.N., Table II). About 30—60% of the dose amount was eliminated in the period, the greater part (more than 70%) of which was excreted in 0—8 hr postadministration. The major urinary metabolite was II, which was excreted for the most part as a conjugated form (glucuronide), accounting for more than 85% of the total excreted. Small but easily measurable amounts of III, IV and V were also detected, the sum of which was about 4.5% of the dose amounts. As shown in Table II, the most part of IV and a part of III were excreted as glucuronides, but V was excreted as a free form. Only trace amounts of F were observed even in earlier periods, and about 0.1—0.2% of the dose amounts was recovered in the period.

Table II. Urinary Excretion Data of Flurazepam and Its Metabolites in 0—48 hr. Following Single Oral Dose of 30 mg Flurazepam HCl

Subjects (ages)	Excreted compounds in urine (μg)[a]					Total excreted[a]	% of dose recovered
	F	II	III	IV	V		
N.N. (32)	35.4	10100	483	993	77.8	11700	39.0
(N.N., free form)[b]	(35.0)	(0.572)	(343)	(17.8)	(76.9)	(473)	(1.58)
T.T. (41)	67.5	10600	397	595	72.0	11700	39.0
K.I. (30)	23.5	16600	398	595	77.4	17700	59.0
K.K. (24)	25.5	13300	153	622	125	14200	47.3
T.Y. (23)	27.8	8730	423	696	131	10000	33.3
F.O. (24)	43.1	12700	534	632	126	14000	46.7
% of dose recovered	0.1—0.2	29.1—55.3	0.5—1.8	2.0—3.3	0.2—0.4	33.3—59.0	—

a) μg equivalent as flurazepam HCl
b) Enzymatically not treated urine was assayed.

An unknown peak which appeared in gas chromatograms of silylated pH 9 extracts (Fig. 4. B, peak 3) was determined by our separate experiment to be one of new metabolites of flurazepam, 3-hydroxy II (Fig. 6). The details of this experiment will be reported elsewhere.

Blood level fall-off curves of the two volunteers (Fig. 7) show that the major metabolites in free forms were II and III, and that high concentration of conjugated II and relatively low

levels of conjugated III were also present in blood. Free form of II reached maximum concentrations of 38 and 50 ng/ml at 1 hr, and declined with half lives of about 3.2 and 3.4 hr. Only small but measurable amount of II was detected up to 24 hr. Metabolite III reached a maximum concentration of about 17 ng/ml at 1—3 hr postadministration, and then declined slowly up to the last sampling time (72 hr) when low but still measurable levels (3.9 and 2.2 ng/ml) of III were observed. The levels of unchanged form (F) were extremely low, which reached the maximum levels of about 1—2 ng/ml at 1 hr postadministration and declined over the next 7 hr. Only trace amounts of IV (less than 2.0 ng/ml) and V (less than 0.3 ng/ml) were detected at the earlier periods (1—8 hr).

Chronic Dose——Table III summarizes the urinary excretion data for 0—8 hr postdosing periods for chronic administration at a daily dose of 30 mg of the drug. There were no significant changes in the daily excretion of F, II, IV and V with exception of that of III which in-

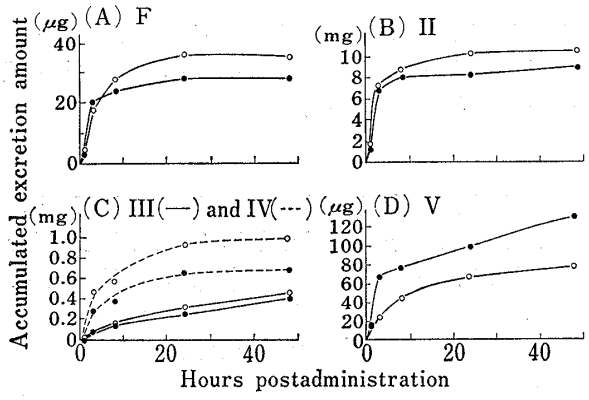


Fig. 5. Urinary Excretion Profiles of Flurazepam and Its Metabolites Following Single Oral Dose of 30 mg Flurazepam HCl

○ : subject N.N., ● : subject T.Y.

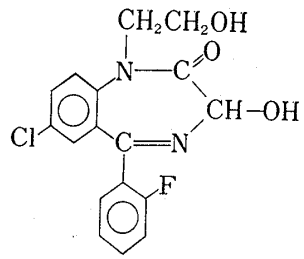


Fig. 6. Structure of Newly Found Metabolite in Human Urine

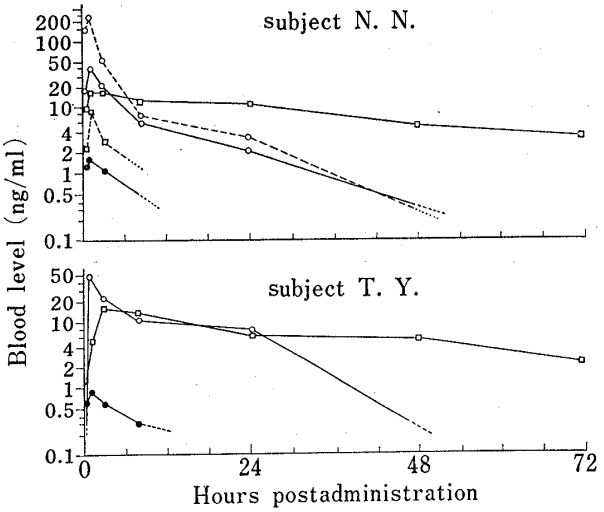


Fig. 7. Blood Level Fall-off Curves of Flurazepam and Its Metabolites Following Single 30 mg Oral Dose of Flurazepam HCl

● : F, ○ : II, □ : III, —— : free form, ------ : conjugated form

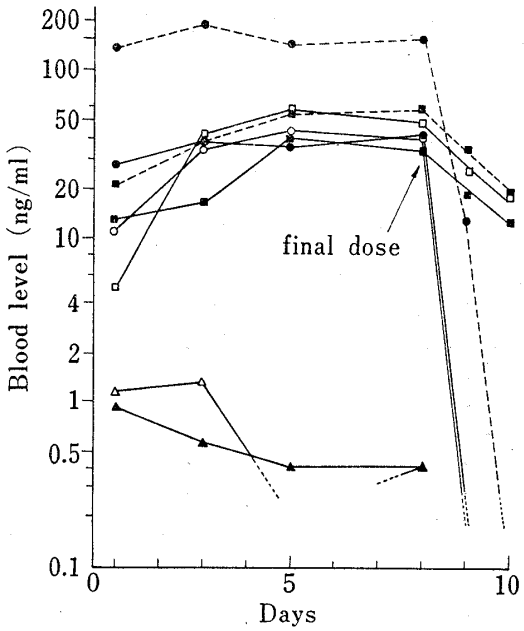


Fig. 8. Effects of Chronic Dose of Flurazepam HCl on the Blood Levels of Flurazepam and Its Metabolites

subject H.Y. (△ : F, ○ : II, □ : III)
subject J.Y. (▲ : F, ● : II, ■ : III)
—— : free form, ------ : conjugated form

Table III. Effects of Chronic Daily 30 mg Dose of Flurazepam HCl on Excretion of Flurazepam and Its Metabolites in 0—8 hr Periods of Postadiministration

Experimental days	Excreted compounds in urine (μg)[a]					Total excreted
	F	II	III	IV	V	
			Subject H. Y.			
Days 1	20.5	7350 (0.486)[b]	102 (44.7)	554 (7.28)	45.6 (43.7)	8070 (117)
3	15.0	8100	137	524	44.7	8820
5	13.5	8760 (0.686)	178 (77.9)	475 (8.73)	56.4 (54.3)	9480 (155)
8[c]	27.5	11000 (0.452)	169 (106)	672 (23.0)	52.3 (50.4)	11900 (207)
9	trace	1130 (trace)	81.9 (57.0)	253 (7.85)	11.2 (10.3)	1480 (75.2)
10	N.D.[d]	139 (trace)	45.8	135	2.27	322
			Subject J. Y.			
1	18.1	8870	63.4	546	48.0	9550
3	16.0	9480	102	557	79.1	10200
5	21.0	9410	163	642	60.7	10300
8	18.8	9460	181	507	38.3	10200
9	trace	90.0	114	76.3	—[e]	—
10	—	—	—	—	—	—

a) μg equivalent as flurazepam HCl
b) Enzymatically not treated urine was assayed.
c) final dose day
d) not detected
e) not asayed

creased gradually in the period Days 1 to 5, and thereafter seemed to reach plateau. The free form-conjugate ratios of F, II and III were not affected by the chronic dose.

Blood level profiles of F, II and III were shown in Fig. 8. The free forms of II and III, especially that of the latter increased gradually up to Days 5, but adding the conjugates, the former showed little alterlation in the level. The increasing levels of III reached plateau on Days 5—8 with declining fall-off curves with half lives of 32—42 hr after the final dose day. Blood levels of F were below 1.5 ng/ml throughout this experiment ranging between 0.2 and 1.3 ng/ml.

These blood level profiles were in good accordance with the urinary excretion profiles. These data suggested that the biotransformation of flurazepam in humans is very fast.

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