3 and 4 and the recent work of Chien and coworkers (1, 2).

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Metabolic Fate of Flurazepam II: A New Potent Metabolite Obtained by *In Vitro* Liver Drug-Metabolizing Enzyme System

Keyphrases □ Flurazepam—isolation of new metabolite using *in vitro* liver drug-metabolizing enzyme system □ Metabolism—flurazepam, isolation of potent metabolite, *in vitro* liver drug-metabolizing enzyme system

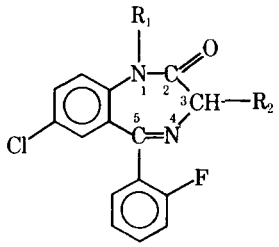
To the Editor:

Although a few investigations on the metabolism of flurazepam (I), 7-chloro-1-[2-(diethylamino)ethyl]-5- (*o*-fluorophenyl) -1,3-dihydro-2*H*- 1,4-benzodiazepin-2-one, in humans (1,2) and experimental animals (1, 3, 4) have been reported, its detailed *in vitro* metabolism is still unknown. This communication describes a new potent metabolite found using an *in vitro* liver drug-metabolizing enzyme system[1].

Reaction mixtures contained the following: 0.5 ml of male DDY mouse or Wister rat liver 9000×*g* supernate or the microsomal fraction (150 mg as fresh liver), NADPH generating system (1 μmole of NADP, 30 μmoles of glucose-6-phosphate, 25 μmoles of nicotinamide, 37.5 μmoles of magnesium chloride, 1.4 units of glucose-6-phosphate dehydrogenase), 5 μmoles of substrate, and 225 μmoles of tromethamine–hydrochloric acid buffer in 2.75 ml of total volume. After incubation at 37° for 90 min, the reaction mixtures were adjusted to pH 9.0 and were extracted once with 10 ml of ethyl acetate. Organic phases were concentrated *in vacuo*, and aliquots of the concentrated organic phases were applied to TLC plates [Kieselgel GF_{254}, benzene–methanol–acetic acid (90:10:10)].

Besides some of the known major metabolites, *e.g.*, II–IV, a minor unknown spot was detected under UV light when I was used as the substrate. Since this metabolite (V) was the predominant metabolite of IV, which is one of the known metabolites of I, 90 mg of authentic IV was then metabolized using mice liver enzyme for the preparation of V.

The metabolite was purified by preparative TLC (Kieselgel GF_{254}) followed by alumina (Woelm, neutral) column chromatography to yield 11.7 mg of oily material. Purified V gave a single spot with several TLC solvent systems, and its chromatographic behavior differed from that of the known metabolites. The IR spectrum of the metabolite showed the increased strength of the OH stretching band at 3400 cm^{-1} and the appearance of a CO stretching band at 1130 cm^{-1}, suggesting that the introduction of the secondary OH to the parent compound (IV) had occurred.



I: $R_1 = CH_2CH_2N(C_2H_5)_2$, $R_2 = H$
II: $R_1 = CH_2CH_2NHC_2H_5$, $R_2 = H$
III: $R_1 = CH_2CH_2NH_2$, $R_2 = H$
IV: $R_1 = CH_2CH_2OH$, $R_2 = H$
V: $R_1 = CH_2CH_2OH$, $R_2 = OH$

The mass spectrum (25 ev) of V showed a weak, but apparent, molecular ion at *m/e* 348 (11%) followed by other prominent fragments at *m/e* 320 (21), 319 (100), 301 (33), 287 (14), 275 (28), and 260 (13). This increment of molecular weight by 16 mass units from that of IV also indicated that one oxygen atom had been introduced to the substrate (IV), but the lack of an M − 16 ion suggested that it might not be any oxide form. The molecular ion of acetylated V (*m/e* 432) and the successive split of two ketenes, giving fragments at *m/e* 390 and 348, were additional supports for the idea.

The proton NMR spectrum of V run in $CDCl_3$,

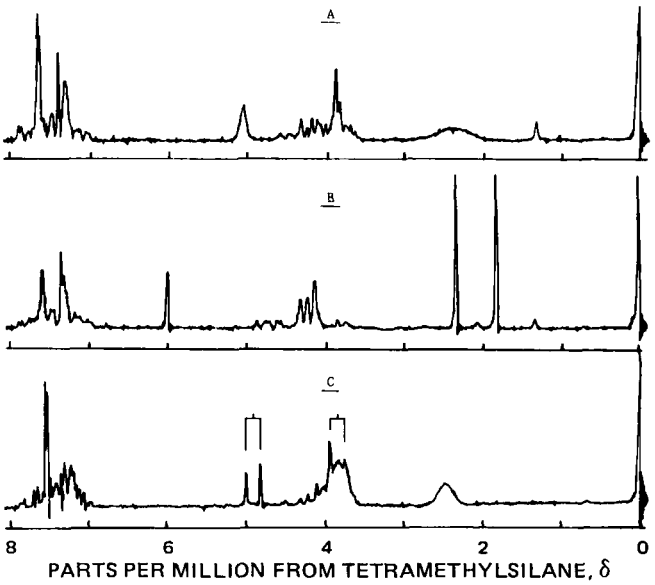


Figure 1—*Proton NMR spectra of V, V-acetate, and authentic parent compound IV in $CDCl_3$: Key: A, V; B, V-acetate; and C, authentic IV (two methylene protons of C_3 at 3.84 and 4.90 ppm are specified).*

[1] For part I, see M. Hasegawa and I. Matsubara, *Chem. Pharm. Bull.*, in press.

with tetramethylsilane as the internal standard, is shown in Fig. 1 along with the spectra of V-acetate and IV. The signals of the methylene protons at the C_3 position of IV at δ 3.84 (doublet, 1H) and 4.90 (doublet, 1H) ppm turned into a singlet signal (1H), shifting toward lower field to 5.05 ppm, by the metabolic transformation to V. The integration of the broad signal of V at 2.30 ppm, which could be replaced by deuterium adding CD_3OD, counted as two protons. These facts indicated that the structural change, *i.e.*, hydroxylation, had occurred at C_3 of IV. Further evidence was that the spectrum of V-acetate showed two acetyl signals at δ 1.83 and 2.26 ppm; the former was assigned to the N_1-2-acetoxyethyl group and the latter to the C_3-acetoxy group. The signal of the C_3 proton of V shifted toward lower field to 6.00 ppm by acetylation. Thus the structure of the metabolite was determined to be as shown in V.

This compound was then synthesized in a separate experiment, and its psychotropic activity was studied. Its CNS-depressant activity in DDY strain male mice was at the 50-mg/kg dose level (oral), which was approximately the same potency as unchanged I. It is of interest that V was found to be one of the metabolites in humans, and details of this work will be reported.

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(3) T. Nishikawa, K. Mineura, and H. Takahira, *Yakugaku Zasshi*, **93**, 226(1973).

(4) *Ibid.*, **93**, 232(1973).

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Pithed Rat Blood Pressure in the Assay of Norepinephrine

Keyphrases □ Norepinephrine—analysis in plasma and cerebrospinal fluid of rats by pithed blood pressure method □ Sympathomimetics—norepinephrine, assay, pithed rat blood pressure

To the Editor:

Measurement of rat blood pressure is a standard method in biological estimations of acetylcholine (1–5), angiotensin (6), norepinephrine (6, 7), epinephrine (6, 7), and vasopressin (8). Preparations reported for norepinephrine assay show a sensitivity of 1–2 × 10^{-7} g/ml. The pithed rat blood pressure in 250 preparations, with some modifications used in our study for the estimation of norepinephrine in plasma (9) and cerebrospinal fluid (10), was sensitive from 1 × 10^{-9} to 5 × 10^{-10} g/ml. The changes followed in these preparations are described here since a highly sensitized preparation could be mounted with the additional operations.

Albino Wistar rats of either sex, 200–350 g, were used. The rats, after being fasted overnight, were injected intraperitoneally with 0.4 ml (0.4% in 0.85% saline) of atropine sulfate[1] 20 min before anesthetization. Anesthesia was induced by spraying ethyl chloride into the glass funnel initially and was maintained with anesthetic ether until the rat became unconscious.

The anesthetized rat was opened for tracheotomy, and a polyethylene cannula with a side opening was immediately introduced into the trachea and tied in position. The rat was then turned on its back to one side and pithed as described previously (6). Once the brain and spinal cord were destroyed, as seen by spasticity of contralateral muscles, the rat was immediately ventilated mechanically by connecting the tracheal cannula to a mini-respiratory pump. The regulator was adjusted to ensure sufficient ventilation so that inflation and deflation of the chest and heart rate were 30–40 strokes/min.

Both carotid arteries were ligated at the central end. Then a laparotomy was done, and the viscera were removed from the esophagus to the rectum, including the spleen; the liver was left *in situ* as per the method of Venkatakrishna-Bhatt and Haranath (3), and the bleeding points were carefully ligated. The left carotid artery and the right femoral vein were cannulated and connected to a manometer[2] and saline stand, respectively. Heparin[3], 0.5 mg, was injected into the femoral vein to prevent clotting. Then 1–3 ml of saline was injected to compensate for the loss of blood volume during evisceration.

Initially, the response with a low concentration of norepinephrine[4] (10^{-11} g/ml) was recorded on a kymograph. Sensitivity increased on repeated injections of standard norepinephrine into the femoral vein at regular intervals. Thus, when the blood pressure reached a steady level, usually after 10–15 trials, the test solution was injected. The volume of fluid injected was always kept constant, usually being 0.1 ml followed by 0.1 ml of saline. Care was taken to prevent leakage or passing of air bubbles from the syringe or leaking points.

This preparation was frequently found to be sensitive to a concentration of 10^{-9}–10^{-10} g/ml of norepinephrine when prepared in saline with 5 × 10^{-6} g/ml of ascorbic acid. Ascorbic acid in saline as a control, when injected in a way similar to the test and standard norepinephrine solutions, did not elicit a peak response as did the test solutions. Although skill and precision are essential for handling the preparation,

[1] E. Merck.
[2] Condon.
[3] Biological Evans, India.
[4] Fluka.