Metabolic *N*-Demethylation of Chlordiazepoxide

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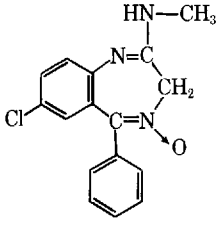
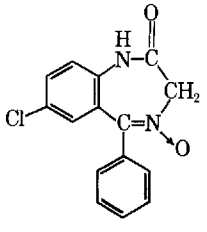
Chlordiazepoxide was *N*-demethylated to 7-chloro-2-amino-5-phenyl-3H-1,4-benzodiazepin-4-oxide (metabolite D-M) when incubated aerobically with either rat or dog liver preparations. Pretreatment of rats with phenobarbital resulted in an approximately threefold increase in the chlordiazepoxide metabolized to metabolite D-M after 2 hr. incubation with the liver microsome fraction. A fluorometric assay for metabolite D-M was developed which when used in conjunction with the Koechlin and D'Arconte fluorometric assay for chlordiazepoxide allowed for a specific determination of each compound in blood. The application of this assay to the study of chlordiazepoxide metabolism in man is illustrated.

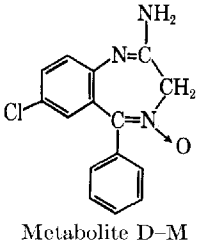
CHLORDIAZEPOXIDE,[1] 7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepin-4-oxide, is metabolized in the dog and man to 7-chloro-1,3-dihydro - 5 - phenyl - 2H - 1,4 - benzodiazepin-2-one-4-oxide ("lactam") (1) which is then metabolized to "opened lactam" metabolites (2). These metabolites have not as yet been detected in the rat which metabolized chlordiazepoxide to unidentified products (2). The present work, which was initiated to obtain information on the site and mechanism of the metabolic conversion of chlordiazepoxide to "lactam," has resulted in the discovery of a new chlordiazepoxide metabolite, 7-chloro-2-amino-5-phenyl-3H-1,4-benzodiazepin-4-oxide (metabolite D-M), which is common to man, dog, and rat. The structures are given below.



Chlordiazepoxide



"Lactam"



Metabolite D-M

METHODS AND MATERIALS

In Vitro Studies.—Chlordiazepoxide hydrochloride (100 mcg. in 0.1 ml. of water) was incubated for 2 hr. at 37° with liver slices (450–490 mg.) or liver homogenate (0.3 ml. of a 1:4 homogenate in 1.15% KCl) prepared from 200–300 Gm. male rats. Also used as enzyme sources were 9000 × g supernatants (0.3 ml.) prepared as described by Fouts and Brodie (3) from 1:3 liver homogenates in 1.15% KCl. Liver supernatants prepared from four control rats were pooled as were those prepared from four rats given i.p. injections of 37.5 mg./Kg. of phenobarbital twice a day for 4 days to stimulate the drug-metabolizing enzymes of the liver microsomes. In addition, a liver supernatant from a dog who had been pretreated with phenobarbital (2 daily oral 20 mg./Kg. doses followed by 10 daily oral doses of 10 mg./Kg.) and which had been stored at −20° for 3 months was used. Besides substrate and enzyme source, the incubation mixture contained the cofactors described by Conney *et al.* (4): 0.2 ml. of 0.01 *M* ATP, 0.1 ml. of 0.003 *M* NADP, 0.1 ml. of 0.003 *M* NAD, 0.1 ml. of 0.6 *M* nicotinamide, 0.2 ml. of 0.03 *M* glucose-6-phosphate, 0.1 ml. of 0.1 *M* $MgCl_2$, 0.1 ml. of 2 *M* KCl, 1.0 ml. of 0.1 *M* potassium phosphate buffer, pH 7.4, and sufficient water to bring the total volume to 3.0 ml.

Following the 2-hr. incubation each sample was extracted twice with 6 ml. of ether. The ether extracts were evaporated to dryness, brought to 0.1 ml. with ethanol, and 0.025 ml. of the ethanol solutions were examined by thin-layer chromatography (TLC) using fluorescent silica gel (Camag Kieselgel DF-5) plates and a solvent system of ethyl acetate–ethanol (90:10) (system *1*). In this system the chlordiazepoxide R_f varied from 0.2–0.4 while the R_f values of "lactam" were 0.7–0.8. A second solvent system which proved useful for identifying metabolite D-M was heptane–chloroform–ethanol (1:1:1) (system *2*). Under shortwave U.V. light compounds on the plate appeared as dark areas against a fluorescent background. The plates were then sprayed with 10% sulfuric acid and viewed under long-wave U.V. light to detect compounds which were fluorescent against a dark background.

A quantitative determination of the amount of chlordiazepoxide metabolized to metabolite D-M was obtained by using as substrate chlordiazepoxide-2-^{14}C (specific activity of 1690 dpm/mcg.) which was purified by sublimation as previously described (2). The ^{14}C which was extracted from the incubation media by ether and which migrated on TLC as chlordiazepoxide and metabolite D-M was determined by the liquid scintillation techniques with the POPOP–PPO fluors previously described (5) and a Nuclear-Chicago Mark I liquid scintillation spectrometer equipped with a ^{133}Ba external standard. The instrument was programmed so that the ratio of the external standard counts in channel B/channel A was convertible from a standard curve into counting efficiency which when divided into the

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[1] Marketed as Librium by Hoffmann-LaRoche, Inc., Nutley, N. J.

channel C sample counts yielded the sample ^{14}C content in dpm.

Fluorometric Determination of Metabolite D-M, Chlordiazepoxide, and "Lactam." —The development of the fluorometric determination of metabolite D-M is given under *Results*. This assay has been incorporated into a slightly modified Koechlin and D'Arconte (1) assay for chlordiazepoxide and "lactam" so as to yield a fluorometric determination of each compound when all three are present in a single blood sample. All fluorometric determinations were made in a Farrand spectrofluorometer.

Reagents

A 1.0 *M* potassium phosphate buffer, pH 7.0–7.2, and 0.2 *M* potassium phosphate buffer, pH 6.7–6.8; 0.10 *N* H_2SO_4 and 7 *N* H_2SO_4, 5 *N* NaOH and 0.10 *N* NaOH, and 0.15 *M* diethanolamine aqueous solution.

Procedure

A 2-ml. control blood or plasma (blank) and 2 control bloods (or plasmas) containing internal standards are run with each set of unknowns. Five micrograms of chlordiazepoxide in 0.1 ml. of ethanol and 5 mcg. of "lactam" in 0.1 ml. of ethanol are added to one control blood, while 5 mcg. of metabolite D-M in 0.1 ml. of ethanol is added to the other; each control containing internal standard is taken through the entire procedure. Specimens of whole blood (heparinized or oxalated) and plasma (separated immediately after drawing the blood) must be kept frozen if the assay is not performed within 24 hr.

Two milliliters of blood is first diluted with 2.0 ml. of water and 4.0 ml. of 1.0 *M* phosphate buffer, pH 7.0–7.2, and is then extracted with 15 ml. of ether. A 12-ml. aliquot of the ether phase is extracted with 4 ml. of 0.1 *N* NaOH to remove "lactam" but not chlordiazepoxide or metabolite D-M from the ether. Two 5.0-ml. aliquots (to be used for the metabolite D-M and chlordiazepoxide assays) are then removed from the remaining ether phase.

Analysis A for "Lactam."—The 0.1 *N* NaOH extract is washed by extraction with 5 ml. of ether and is then placed 8 10 in. from a Pyro-Lux R-57 lamp (Luxor Lighting Products, Inc., New York, N. Y.) and light-exposed for 1 hr. This photochemical reaction may also be accomplished by exposure to bright sunlight for 1 hr. The fluorescence is then determined at 380 mμ excitation and 460 mμ emission.

Analysis B for Metabolite D-M.—One 5-ml. ether aliquot is extracted with 3.5 ml. of 7 *N* H_2SO_4 and the ether is removed by aspiration. After standing for 1 hr. (no light exposure required) the fluorescence of the acid extract is determined at 370 mμ excitation and 460 mμ emission.

Analysis C for Chlordiazepoxide.—The other 5-ml. ether aliquot is extracted with 1.5 ml of 0.1 *N* H_2SO_4. To the acid extract 0.5 ml. of 0.2 *M* phosphate buffer, pH 6.7–6.8, and 1 ml. of 0.15 *M* diethanolamine solution are added and the mixture is kept in a boiling water bath for 2 hr.[2] After cooling, 0.2 ml. of 5 *N* NaOH is added, and the sample is then exposed to light for 1 hr. and its fluorescence determined exactly as described above for the "lactam" determination.

Calculations

Analysis A ("Lactam").—The fluorescence reading (F) for the "lactam" internal standard (5 mcg.) corrected for the blood blank fluorescence (B) is divided by 5 to yield F-B per microgram of "lactam." This specific fluorescence value divided into the corrected fluorescence (F-B) obtained with each unknown yields microgram "lactam" per 2 ml. of blood and final division by 2 then gives the "lactam" concentration in mcg./ml.

Analysis B (Metabolite D-M).—The metabolite D-M internal standard fluorescence is used to get the specific fluorescence (F-B/mcg.) and the calculations proceed as described above.

Analysis C (Chlordiazepoxide.)—Since both chlordiazepoxide and metabolite D-M yield fluorescence in this part of the method, a correction for metabolite D-M fluorescence must be made before the fluorescence in an unknown due to chlordiazepoxide can be determined. The specific fluorescence for metabolite D-M in analysis *C* is determined. This value multiplied by the micrograms of metabolite D-M found in each unknown in analysis *B* yields the fluorescence in analysis *C* which is due to metabolite D-M. Subtraction from the total analysis *C* fluorescence (F-B of each unknown) yields the F-B value of each unknown which is due only to chlordiazepoxide. The remainder of the calculations are identical to those of the above analyses: the F-B per mcg. of chlordiazepoxide (obtained from the fluorescence of the chlordiazepoxide internal standard) is divided into the F-B of each unknown attributed to chlordiazepoxide to get the chlordiazepoxide content per 2 ml. of blood.

Comments

The sensitivity of this assay is 0.2–0.3 mcg. of each compound per ml. of blood or plasma.

The chlordiazepoxide assay is the least accurate of the three because it involves the subtraction of one fluorescence from another. An error in the determination of metabolite D-M will cause an error in the chlordiazepoxide determination.

RESULTS

In Vitro Metabolism of Chlordiazepoxide.—Aerobic incubation of chlordiazepoxide with rat liver slices, rat liver homogenate, and the 9000 × g supernatant of liver from a dog pretreated with phenobarbital all gave essentially the same results when the ether extracts were chromatographed in system *1*. A metabolite (metabolite D-M), which was not completely separated on the plate from nicotinamide (a constituent of the incubation medium which was also extracted by ether), was seen under shortwave U.V. light. The metabolite migrated 54–58% of the distance chlordiazepoxide migrated in this system. When the plates were sprayed with 10% sulfuric acid and examined under long-wave U.V. light this metabolite was seen more clearly because it fluoresced while nicotinamide did not. The metabolite was not detected in control samples incubated without chlordiazepoxide or in complete samples which were extracted immediately with

[2] This hydrolysis of chlordiazepoxide to "lactam" is a modification of the Koechlin and D'Arconte procedure suggested by M. Roth and J. Rieder, Hoffmann-LaRoche and Co., Basle, Switzerland.

ether without prior incubation. Furthermore, when chlordiazepoxide was incubated anaerobically (in evacuated Thunberg tubes) with rat liver homogenate the metabolite was not found, indicating that it was formed by an oxidative process. In none of these *in vitro* experiments, however, was any conversion of chlordiazepoxide to "lactam" detected.

Metabolite D-M was suspected of being *N*-desmethyl chlordiazepoxide because this compound would be a logical intermediate in the metabolism of chlordiazepoxide to "lactam" (see introduction for formulas). Authentic 7-chloro-2-amino-5-phenyl-3H-1,4-benzodiazepin-4-oxide (*N*-desmethyl chlordiazepoxide) was therefore spotted on TLC plates alongside an aliquot of the ether extract obtained after incubation of chlordiazepoxide with the 9000 × g supernatant of dog liver. *N*-Desmethyl chlordiazepoxide and metabolite D-M in the extract migrated identically in system *1* (R_f 0.25) and also in system *2* (R_f 0.53). The authentic *N*-desmethyl chlordiazepoxide also fluoresced under long-wave U.V. light after the plates were sprayed with 10% sulfuric acid. It was therefore concluded that metabolite D-M was *N*-desmethyl chlordiazepoxide.

The effect of phenobarbital pretreatment of rats on the metabolism of ^{14}C-labeled chlordiazepoxide by the liver microsome fraction (9000 × g supernatant) is shown in Table I. The first two flasks demonstrated the validity of the quantitation technique in that incubation of chlordiazepoxide-2-^{14}C in the absence of enzymes resulted in 95 and 94% of the ^{14}C being extracted by ether and 98% of the extracted ^{14}C being recovered on TLC as intact chlordiazepoxide. When labeled chlordiazepoxide was incubated with pooled liver supernatant from control rats (flasks 3 and 4) 90 and 92% of the ^{14}C was ether-extracted but only 74% of the extracted ^{14}C was recovered as unmetabolized chlordiazepoxide while 20% was found as metabolite D-M. Incubation with the pooled liver supernatant from phenobarbital-treated rats (flasks 5 and 6) resulted in a striking increase in chlordiazepoxide metabolism. Only 78% of the ^{14}C was extracted by ether indicating that significant quantities of polar metabolites were now formed. In addition the ether-soluble ^{14}C was mostly present in metabolite D-M (77 and 66%) while 22 and 23% was present as chlordiazepoxide and a significant amount of ^{14}C (5 and 6%) was now found at the origin. Calculation of the chlordiazepoxide-derived ^{14}C which was actually recovered as metabolite D-M yielded 18% for flask 3 and 56% as an average for flasks 5 and 6; this demonstrates that *N*-demethylation had been stimulated at least threefold (the metabolites not extracted by ether may also be *N*-demethylated) by pretreatment of the rats with phenobarbital. The stimulation of over-all chlordiazepoxide metabolism was similarly estimated to be approximately fourfold.

TABLE I.—EFFECT OF PHENOBARBITAL PRETREATMENT ON *In Vitro* METABOLISM OF CHLORDIAZEPOXIDE-2-^{14}C BY 9000 × g SUPERNATANT OF RAT LIVER[a]

Flask	Source of 9000 × g Supernatant	^{14}C Extracted by Ether After 2 hr. Incubation, %	^{14}C of Ether Extract in[b] Chlordiaz., %	Met. D-M, %	Origin, %
1	None, no enzyme	95	98	1	1
2	None, no enzyme	94			
3	Control rats	90	74	20	1
4	Control rats	92			
5	Phenobarb-treated rats	78	23	77	5
6	Phenobarb-treated rats	78	22	66	6

[a] Labeled chlordiazepoxide (100 mcg.) was incubated with the cofactors described under *Methods and Materials* and each designated enzyme source for 2 hr. at 37°. The 9000 × g supernatants of liver from four phenobarbital-treated rats were pooled as were the supernatants from four control rats. The pooled supernatant was run in duplicate as enzyme source. [b] Distribution of ether-extracted ^{14}C determined by TLC in system *1* and counting of the silica gel segments containing chlordiazepoxide, metabolite D-M, and origin. Chlordiazepoxide and metabolite D-M were spotted as internal standards with each extract.

Detection of Metabolite D-M in Blood.—The possibility that metabolite D-M was present in blood following the administration of chlordiazepoxide was investigated in three species. Blood (2 ml.) drawn from a squirrel monkey 4 hr. after an oral dose of 5 mg./Kg. of chlordiazepoxide was first mixed with equal volumes of water and 0.1 *M* potassium phosphate buffer, pH 7.4, and then extracted twice with 3 vol. of ether; the combined ether extracts were concentrated for TLC. In a similar manner ether extracts for TLC were prepared from rat blood (5 ml.) obtained 3.5 hr. after i.p. doses of 7.5 mg./Kg. and 15 mg./Kg. of chlordiazepoxide and from human blood (5 ml.) obtained from a woman who had taken an overdose of chlordiazepoxide. TLC in system *1* of each of the above concentrated ether extracts followed by inspection of the plates under U.V. light before and after acid treatment revealed the presence of both chlordiazepoxide and metabolite D-M. These findings established the need for an assay for metabolite D-M in blood.

Development of Metabolite D-M Assay.—Further studies revealed that the Koechlin and D'Arconte fluorometric determination of chlordiazepoxide (1) did not discriminate between chlordiazepoxide and metabolite D-M; 5 mcg. of metabolite D-M added to 1 ml. of blood and analyzed by this method resulted in approximately the same fluorescence as that obtained from 5 mcg. of chlordiazepoxide per ml. of blood. This made imperative the development of an assay specific for one or the other of these two compounds. Such an assay was developed for metabolite D-M using as the starting point the observed fluorescence of this compound on thin-layer plates which had been sprayed with 10% sulfuric acid. The compound was found to fluoresce in 10% sulfuric acid solution and an excitation wavelength of 370 mμ and emission wavelength of 460 mμ yielded the highest fluorescence reading. The fluorescence of 5 mcg./ml. of metabolite D-M increased sharply from 66 units after standing in the acid for 15 min. to 111 units after 1 hr. and then rose only to 119 units after 2 hr. It was further found that this was not a photochemical reaction; the same fluorescence was obtained whether metabolite D-M in 10% sulfuric acid was kept for 1 hr. in the dark or in daylight. Investigation of the effect of acid concentration on fluorescence revealed that metabolite D-M developed maximum fluorescence in 7 *N* H_2SO_4. Accordingly, 7 *N* H_2SO_4 was used to obtain data on the relationship of fluo-

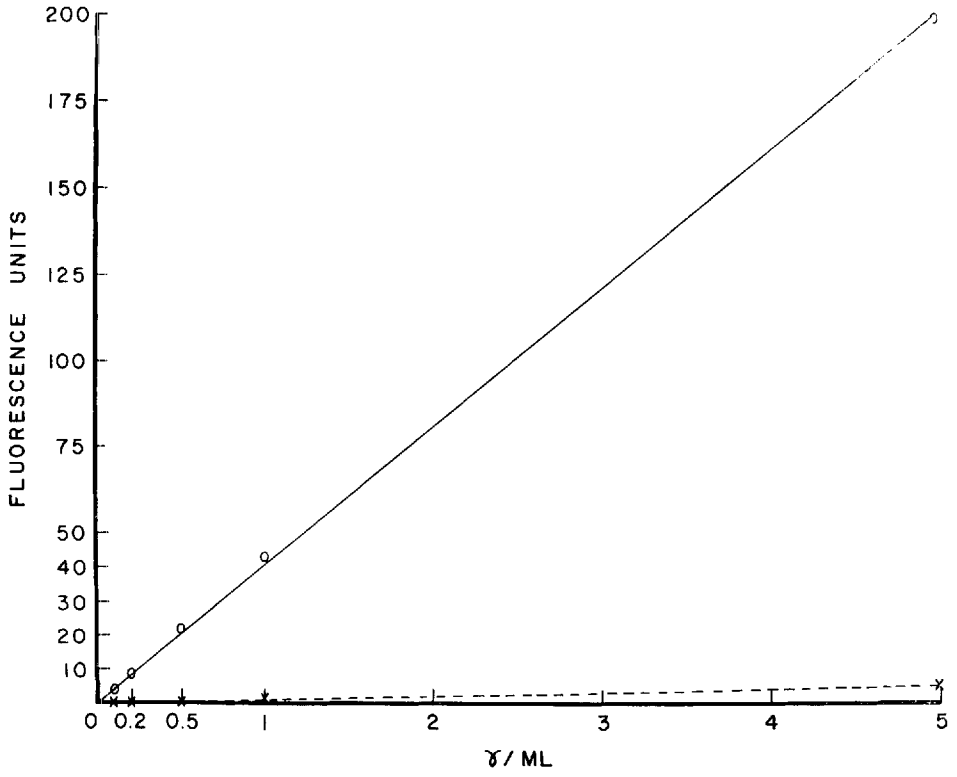

Fig. 1.—Relationship of fluorescence to concentration of metabolite D-M and chlordiazepoxide in 7 *N* sulfuric acid. After standing 1 hr. at room temperature, the solutions were read at 370 mμ excitation and 460 mμ emission. Key: O, metabolite D-M; X, chlordiazepoxide.

TABLE II.—RECOVERY OF CHLORDIAZEPOXIDE, "LACTAM," AND METABOLITE D-M IN BLOOD AND PLASMA CONTAINING ALL THREE COMPOUNDS[a]

		Recovery of Each Compd.								
		"Lactam"			Metabolite D-M			Chlordiazepoxide		
Expt.	Sample	Added, mcg.	Found, mcg.	Recovery, %	Added, mcg.	Found, mcg.	Recovery, %	Added, mcg.	Found, mcg.	Recovery, %
1	Human plasma	5.0	5.4	108	5.0	4.8	96	5.0	5.1	102
	Human plasma	3.0	2.7	90	2.0	2.0	100	4.0	3.5	88
2	Dog blood	4.0	4.1	103	6.0	6.1	102	2.0	1.9	95
	Dog blood	4.0	3.6	90	2.0	2.2	110	6.0	6.2	103
3	Dog plasma	5.0	4.8	96	5.0	5.4	108	5.0	4.5	90
	Dog plasma	3.0	3.1	103	2.0	2.3	115	4.0	3.9	98

[a] The method for metabolite D-M was incorporated into the Koechlin and D'Arconte assay to yield the combined method described under *Methods and Materials*.

rescence to metabolite D-M concentrations. As seen from Fig. 1 the fluorescence of metabolite D-M was a linear function of the concentration from 0.5 mcg./ml. The fluorescence obtained from 0.1–1 mcg./ml. of chlordiazepoxide under these conditions was barely measurable while the fluorescence from 5 mcg./ml. of chlordiazepoxide was about the same as that obtained from 0.1 mcg./ml. of metabolite D-M.

This metabolite D-M assay was therefore of sufficient specificity so that it could be used together with the Koechlin and D'Arconte assay to achieve specific determinations of chlordiazepoxide, "lactam," and metabolite D-M. This combined assay (described under *Methods and Materials*) was tested by determining the recoveries of each compound added to the same blood sample. It is seen from Table II that good recovery of each compound from blood and plasma was obtained in a series of recovery experiments in which the ratio of one compound to another in the added mixture was varied.

Applications of the Combined Assay for Chlordiazepoxide, Metabolite D-M, and "Lactam."—The plasma levels of each compound following 100 mg. of chlordiazepoxide hydrochloride given intravenously to each of two subjects are shown in Fig. 2. In both subjects the half-life of plasma chlordiazepoxide was roughly 16 hr. In subject No. 1 metabolite D-M appeared before "lactam," reached higher levels, and fell off faster; in subject No. 2

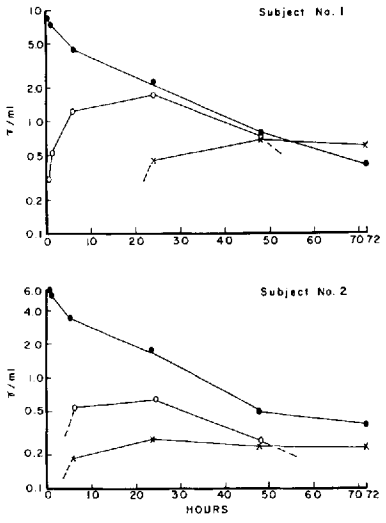

Fig. 2.—The plasma levels of chlordiazepoxide (●), metabolite D-M (O), and "lactam" (×) plotted logarithmically as a function of time. Each of the two subjects received a 100-mg. intravenous dose of chlordiazepoxide hydrochloride. The broken lines denote that the levels have either risen from or descended to levels which were below the sensitivity of the assay.

TABLE III.—PLASMA CHLORDIAZEPOXIDE, METABOLITE D-M, AND "LACTAM" IN A SUBJECT WHO RECEIVED 10 mg. OF CHLORDIAZEPOXIDE HYDROCHLORIDE q.i.d. FOR 14 DAYS

Time,[a] Days	Plasma Levels of— Chlordiazepoxide, mcg./ml.	Metabolite D-M, mcg./ml.	"Lactam," mcg./ml.
2	1.69	0.47	Nil
4	3.13	1.17	0.70
6	1.61	1.27	1.06
10	3.13	1.17	1.41
12	1.90	1.27	1.41
14	3.42	1.17	1.27
22	Nil	Nil	(0.24)[b]

[a] Blood was drawn on each day listed immediately before the first of the 4 doses was administered. Day 22 values represent residual plasma levels after 7 days of no drug.
[b] This value is at the limit of sensitivity of the assay.

both metabolites appeared at 6 hr., but the metabolite D-M levels were more than twice the "lactam" levels and metabolite D-M again fell off faster.

Table III presents the plasma levels of each compound in a subject who received chlordiazepoxide hydrochloride chronically. The levels of the metabolites are of particular interest because it is again seen that metabolite D-M is detectable in the blood before "lactam" and that after drug administration was stopped for 1 week both metabolite D-M and chlordiazepoxide were no longer detectable while a very slight amount of "lactam" appeared to be still present.

The limitation of the combined assay was illustrated when 30 mg. of chlordiazepoxide hydrochloride was administered intravenously to a subject. The chlordiazepoxide blood levels declined from 2.3 mcg./ml. 15 min. after the dose to approximately 0.4 mcg./ml. at 48 hr.; metabolite D-M was not seen until the eighth hour and from 8–48 hr. low levels at the sensitivity threshold of the assay appeared to be present; and "lactam" was not detected in any of the blood samples. In this subject the blood levels of metabolite D-M were too low for accurate measurement, and since the determination of blood chlordiazepoxide is dependent on an accurate assay of metabolite D-M, it was not possible to get an accurate measurement of chlordiazepoxide.

DISCUSSION

The *in vitro* studies demonstrate that rat and dog liver are capable of producing metabolite D-M by oxidative *N*-demethylation of chlordiazepoxide. This metabolite is also formed *in vivo* as evidenced by its detection in the blood of chlordiazepoxide-treated rats, squirrel monkeys, and humans. In previous studies with ^{14}C-chlordiazepoxide (2) most of the radioactivity excreted as neutral or basic urinary metabolites by two humans was identified as "lactam." It is possible that a small but significant amount of metabolite D-M was excreted but escaped detection. In the rat the basic metabolite fraction of the urine contained at least two labeled metabolites which were not identified. Studies on the characterization of chlordiazepoxide metabolites in rat urine have been reinstituted to determine if metabolite D-M is one of these excretion products.

Although the structure of metabolite D-M makes this compound a logical intermediate in the formation of "lactam" from chlordiazepoxide, only indirect evidence for this pathway is presently available. In all human blood level studies to date, including those reported above, the appearance of "lactam" in the blood of chlordiazepoxide-treated subjects has never preceded the appearance of metabolite D-M indicating that this latter compound may be the immediate precursor of "lactam" in man and dog. It must be noted, however, that *in vitro* experiments with dog liver aimed at demonstrating "lactam" formation from either chlordiazepoxide or metabolite D-M have been unsuccessful.

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