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DETERMINATION OF CLORAZEPATE AND ITS MAJOR METABOLITES IN BLOOD AND URINE BY ELECTRON CAPTURE GAS–LIQUID CHROMATOGRAPHY

M. A. BROOKS, M. R. HACKMAN, R. E. WEINFELD and T. MACASIEB

Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, N.J. 07110 (U.S.A.)

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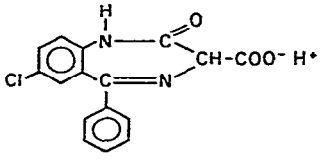
SUMMARY

A sensitive and specific blood level method employing differential extraction was developed for the determination of clorazepate and its N-desmethyldiazepam metabolite by electron capture gas–liquid chromatography (GLC–ECD). The assay requires the initial extraction of N-desmethyldiazepam, the major metabolite, into benzene–methylene chloride (90:10) from the biological sample made alkaline with 0.1 *N* NaOH. The sample is then acidified with 2 *N* HCl to decarboxylate clorazepate to N-desmethyldiazepam, which is then extracted into benzene–methylene chloride (90:10) after adjusting the pH to 12.8 with NaOH. The two extracts are evaporated, and the residues are dissolved in benzene which contains griseofulvin as the reference standard. These solutions are assayed by GLC–ECD. The overall recovery and sensitivity limit of the assay for clorazepate is 60 ± 5% (S.D.) and 4.0 ng/ml blood, respectively, while that for N-desmethyldiazepam is 95 ± 5% (S.D.) and 4.0 ng/ml blood, respectively. The urinary excretion of clorazepate was determined by the measurement of the levels of N-desmethyldiazepam and oxazepam, the major urinary metabolites of clorazepate, both prior to and after enzymatic deconjugation. These methods were applied to the measurement of clorazepate and its metabolites in blood and urine following a single 15-mg dose of clorazepate dipotassium.

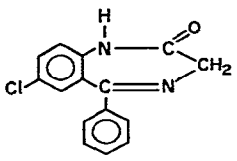
INTRODUCTION

Clorazepate dipotassium (7-chloro-1,3-dihydro-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-carboxylic acid, monopotassium salt, monopotassium hydroxide), synthesized by Schmitt *et al.*[1], is presently marketed as the antianxiety agent Tranxene[2–4]. Recent studies have measured the kinetics of the stability of the compound at 37° in various aqueous buffers and in whole blood[5] employing differential pulse polarographic analysis coupled with a differential extraction procedure. These studies demonstrated that clorazepate dipotassium degraded instantaneously to N-desmethyldiazepam below pH 4 but was relatively stable at pH 7.4 and above. The polaro-

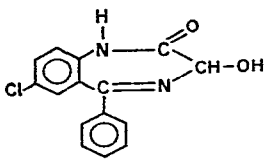
graphic procedure had a limit of sensitivity of 0.5–1 μg/ml solution and was sufficient to assess the "*in vitro*" stability of the compound. However, gas–liquid chromatography with electron capture detection (GLC–ECD) is required to measure the parent drug and its major metabolites in blood following single oral doses of clorazepate dipotassium to man. The procedure presented herein combines a modification of the differential extraction procedure used in the polarographic assay[5] with a GLC–ECD procedure described by de Silva *et al.*[6] to measure in blood and urine intact clorazepate (I) and its metabolites N-desmethyldiazepam (II) and oxazepam (III).



[I] Clorazepate



[II] Desmethyldiazepam



[III] Oxazepam

The differential extraction procedure used herein is similar to that described for the measurement of blood and urine levels of I and II by spectrophotometry[7] and GLC–ECD[8]. These assays require extraction of II from an alkalinized sample, followed by decarboxylation of I to II, and the subsequent extraction of II from the realkalinized sample. The reported assays[7,8], however, differ from the assay presently described in that they convert II to 2-amino-5-chlorobenzophenone (ACB) in both extracts by strong acid hydrolysis prior to quantitation. Thus any III present in the sample processed by these procedures[7,8] will also be converted to ACB, impairing the specificity of the assay. This problem is of particular concern in the analysis of urine samples where III is a measurable component. Specificity is attained in the present assay by analyzing II and III as their intact 1,4-benzodiazepines which are chromatographically resolved. The differential extraction of a single sample eliminates errors introduced in the quantitation of I by the extraction of two separate samples. A recent GLC–ECD report[9] which uses the ACB derivative requires one sample to measure the sum of I and II and a second sample to measure II selectively. The amount of I is then determined by the difference. Unfortunately the determination of I is the least accurate since it is obtained by subtraction of two relatively large values.

Unconjugated N-desmethyldiazepam is not a urinary product in man[10]. The GLC–ECD assay of urine used in this study measures levels of unconjugated N-desmethyldiazepam which result from spontaneous transformation of intact clorazepate to N-desmethyldiazepam in the acidic environment of the urine. The assay also employs enzymatic deconjugation followed by GLC–ECD to measure levels of conjugated N-desmethyldiazepam and oxazepam, the major identifiable metabolites of clorazepate.

The GLC–ECD assays were applied to the measurement of clorazepate and its metabolites in blood and urine following a single 15-mg dose of Tranxene (clorazepate dipotassium).

EXPERIMENTAL

Column

The column is a U-shaped 4 ft. × 4 mm I.D. borosilicate glass column containing 3% OV-17 on 60–80 mesh Gas-Chrom Q (Applied Science Labs., State Park, Pa., U.S.A.). The column is conditioned as previously described[11].

Instrumental parameters

A MicroTek MT-220 gas chromatograph (Tracor Instruments, Austin, Texas, U.S.A.) equipped with a 15-mCi ^{63}Ni electron capture detector is used. The carrier gas is argon–methane (90:10) (Matheson Gas Products, East Rutherford, N.J., U.S.A.; oil pumped and dry). The column head pressure is set at 40 p.s.i.g., the flow-rate is 70 ml/min, and the detector purge is 20 ml/min.

The temperature settings for the injection port and detector are 275° and 350°, respectively. The oven temperature for the blood assay is 240°, resulting in retention times of 6.2, and 14.2 min for II and IV (griseofulvin, the reference standard), respec-

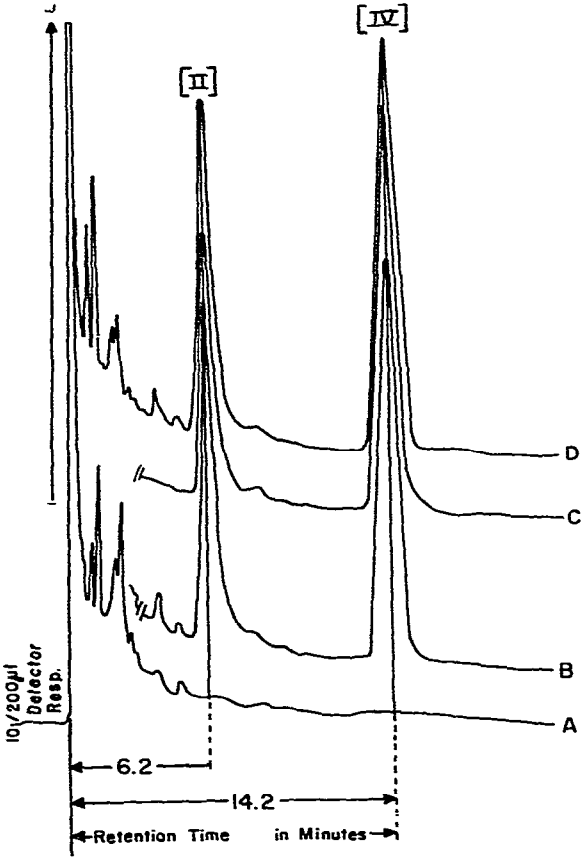


Fig. 1. Chromatograms of benzene–methylene chloride (90:10) extracts of (A) control blood, (B) authentic standards of II and IV, (C) control blood containing added II, and (D) patient blood 24 h post oral dose of 15 mg of clorazepate dipotassium.

tively (see Fig. 1). Under these conditions III has a retention time of 3.2 min. The oven temperature for the urine assay is 230°, resulting in retention times of 4.3, 8.2, and 18.0 min for III, II, and IV, respectively (see Fig. 2). The conditions of column head pressure, flow-rate and oven temperature must be adjusted accordingly to obtain the above retention times in their respective assays.

The detector is operated in the "constant current pulsed mode" using a Tracor Model No. 114460 ECD linearizer with the following parameters: standing current, 0.3×10^{-9} A; attenuator 8; relative pulse width, 0.18 setting (corresponding to 0.75 μsec). A 1.0-mV Hewlett-Packard Model 7127A strip chart recorder at a chart speed of 0.25 in./min is used. Under these conditions 4.0 ng of II and 10.0 ng of III give nearly full-scan responses on the 1.0-mV recorder.

Preparation of standard solutions

Weigh out 12.99 mg of clorazepate dipotassium (7-chloro-1,3-dihydro-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-carboxylic acid, monopotassium salt, monopotas-

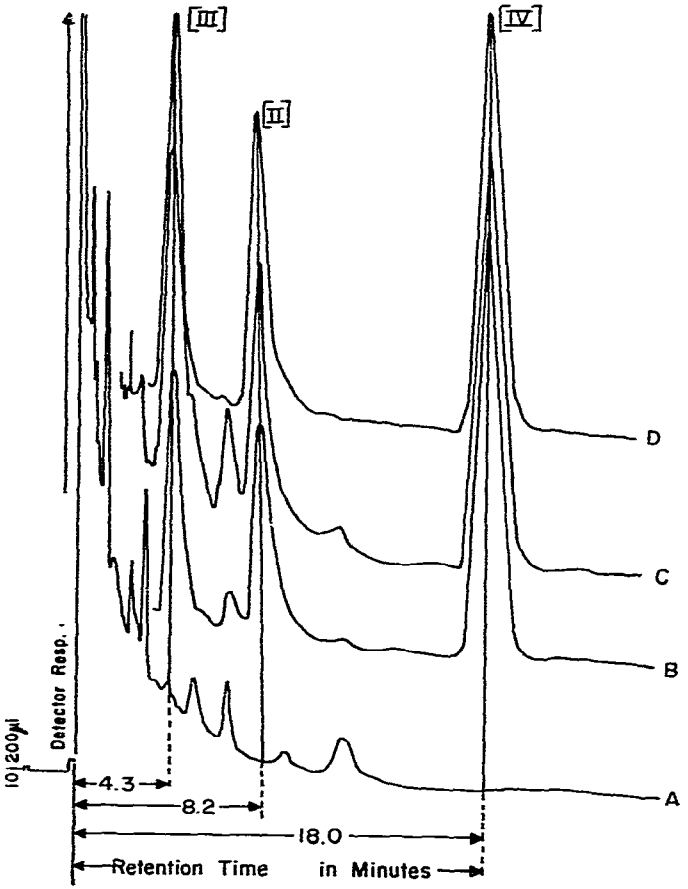


Fig. 2. Chromatograms of benzene–methylene chloride (90:10) extracts of (A) control urine, (B) authentic standards of II, III, and IV, (C) control urine containing added II and III, and D patient urine pool 0–24 h post oral dose of 15 mg of clorazepate dipotassium.

sium hydroxide, $C_{16}H_{11}O_4N_2ClK_2$, mol. wt. = 408.93) equivalent to 10.00 mg of the free acid of I, into a 10-ml volumetric flask and dissolve in 0.1 *N* NaOH. This stock solution contains 1.0 mg/ml and is used to prepare serial dilutions in 0.1 *N* NaOH. Make serial dilutions of the stock solution to yield respective working solutions containing 100, 200, 300, and 400 ng I/ml. 100-μl aliquots of each standard solution of I (equivalent to 10, 20, 30, and 40 ng I/ml blood) are added directly to 1 ml of control blood as internal standards for recovery determination and for the construction of an internal standard curve from which the concentrations in the unknowns are determined by interpolation. All solutions of I should be prepared fresh daily.

Weigh out 10.00 mg of N-desmethyldiazepam (II) (7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one, $C_{15}H_{11}N_2OCl$, mol. wt. = 270.74) and oxazepam (III) (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one, $C_{15}H_{11}ClN_2O_2$, mol. wt. = 286.72) into two separate 10-ml volumetric flasks and dissolve in benzene. These stock solutions contain 1.0 mg/ml and are used to prepare serial dilutions in benzene. Make serial dilutions of the stock solutions of II to yield respective working solutions containing 100, 200, 300, and 400 ng II/ml. Make serial dilutions of the stock solutions of III to yield respective working solutions containing 500, 1000, 1500, and 2000 ng III/ml. 50-μl aliquots of these standard solutions (equivalent to 5, 10, 15, and 20 ng II/ml sample and 25, 50, 75, and 100 ng III/ml sample, respectively) are added directly to the control urine or alkaline control blood (do not evaporate solvent) as internal standards for per cent recovery determination and for the construction of an internal standard curve from which the concentrations in the unknowns are determined directly.

Calibration of II and III by GLC–ECD

Fifty-microliter aliquots of the eight standard solutions of II and III prepared above are taken to dryness under nitrogen and redissolved in 100 μl of benzene containing 200 ng IV/ml. A 10-μl aliquot of these solutions is assayed to establish external calibration curves of peak area ratio *versus* concentration of II or III. The ECD response to the compounds is directly proportional to the amount injected, and is linear over a wide range of concentration (1000-fold) by employing the constant-current pulsed mode (EC linearizer). The external standard calibration curve is not required for daily operation, but several standards should be routinely processed to monitor detector stability and to measure per cent recovery of the internal standards.

Reagents

All inorganic reagents are of analytical grade and are prepared in deionized, distilled water. The reagents include 0.1, 4.0, and 6.0 *N* NaOH and 2.0 and 6.0 *N* HCl. The organic reagents include benzene and methylene chloride (Mallinckrodt, St. Louis, Mo., U.S.A.; nanograde) and diethyl ether (Mallinckrodt; anhydrous). The reference standard solution is 200 ng griseofulvin/ml benzene (Calbiochem, Los Angeles, Calif., U.S.A.). Enzymatic deconjugation is performed using Glusulase reagent (enzyme preparation containing 100,000 units of glucuronidase and 50,000 units of sulfatase per milliliter; Endo Labs., Garden City, Long Island, N.Y., U.S.A.).

Samples

Ten-milliliter blood samples were drawn, oxalated, and 1 ml of 1 *N* NaOH

was added. The samples were frozen until analysis. Urine specimens were collected from 0–24 h in bottles containing 1 ml of toluene. The volume of urine voided was recorded, and a 50-ml aliquot was frozen (without the addition of alkali) for subsequent analysis.

Analysis of blood samples for I and II

Procedure. Into a 50-ml stoppered-centrifuge tube add 1.0 ml of the alkaline whole blood, and 3.0 ml of 0.1 *N* NaOH, and mix well. Add a 10-ml aliquot of benzene–methylene chloride (90:10), sealing the tube with a PTFE stopper and immediately shaking briefly by hand. The samples are extracted by shaking for 10 min on a reciprocating shaker and then centrifuged for 5 min at 1207 *g* in a refrigerated centrifuge at 10°. Remove the upper organic layer, transfer to a clean 15-ml stoppered conical centrifuge tube, and evaporate to dryness under nitrogen at 65°. Repeat the extraction with a second 10-ml aliquot of benzene–methylene chloride (90:10) and combine the extracts. (This extract contains the major metabolite II.) Wash the remaining aqueous phase with a 10-ml portion of benzene–methylene chloride (90:10), shaking on a reciprocating shaker for 5 min and centrifuging at 1207 *g* for 5 min. Remove the organic phase by aspiration and discard.

Adjust the pH of the aqueous phase to approximately 1 by the addition of 1 ml of 2 *N* HCl and allow to stand at room temperature for 30 min. At the end of this interval make strongly alkaline with 1 ml of 4 *N* NaOH and extract with two 10-ml portions of benzene–methylene chloride (90:10) as described above. (This extract contains II, which was formed as a result of decarboxylation of the free acid of I in the acidified specimen.)

The residues of the benzene–methylene chloride (90:10) extracts are vacuum desiccated for 15 min over $CaSO_4$ (Drierite) and dissolved in 100 μl (or a suitable aliquot) of benzene containing 200 ng of griseofulvin/ml. Ten microliters are analyzed by GLC–ECD using the conditions described above.

Along with the samples, a specimen of control blood made alkaline with 1 *N* NaOH (taken preferably from the subject prior to medication) and four specimens of alkaline control blood containing 10, 20, 30, and 40 ng I and 5, 10, 15, and 20 ng II, respectively, are run as internal standards. These standards are used to establish an internal standard calibration curve for the direct quantitation of the unknowns.

Calculations. The concentrations of I and II in the unknowns are determined from the internal standard calibration curves of peak areas ratio of I or II/IV *vs.* concentration of I and II, respectively. All values must be multiplied by 1.11 to correct for dilution which resulted from the addition of 1 ml of 1.0 *N* NaOH to each 10-ml blood sample.

Per cent recovery, sensitivity limit and specificity of blood assay. The recoveries of I and II are calculated by comparing the average peak area ratio of II/IV per ng in the internal and external standards. The recovery of I must be multiplied by the molecular weight ratio of I/II (314.73/270.72 = 1.16) to obtain the correct per cent recovery.

The recoveries of I and II, using the blood assay procedure, are 60 $\pm$ 5% (S.D.) and 95 $\pm$ 5% (S.D.), respectively, using 1 ml of blood per assay. Experiments with ^{14}C-labelled I demonstrated that the low recovery of I results from the formation of non-extractable products which are formed upon treatment of I with 2 *N* HCl.

Samples larger than 1 ml cannot be analyzed using this extraction procedure due to endogenous impurities extracted from the biological sample. The addition of 1 ml of 1 *N* NaOH to the 10-ml blood sample serves to prevent decomposition of I to II. The decomposition may also be prevented by freezing the sample immediately after collection. However, when defrosting these samples preserved without alkali, they must be thawed at room temperature and processed within an hour to prevent significant (>5%) decomposition. The sensitivity limit of the assay for I and II is 4.0 ng/ml of blood.

The specificity of the blood assay for II was established by the absence of any interfering peaks occurring in the region of II following extraction of control (drug-free) human blood from different sources and subjects, and by chromatographic separation under the conditions described above. Authentic standards of III (a possible blood metabolite) added to blood were recovered to approximately 70%, and were completely resolved from II (R_t of 6.2 min) with a retention time of 3.2 min.

Analysis of urine samples for II and III

Determination of intact I as II. Acidify 1.0 ml urine with 1 ml 6 *N* HCl in a 50-ml stoppered centrifuge tube, mix and allow to stand for 30 min at room temperature. (This procedure will hydrolyze any I remaining in the sample to II.) Along with the samples process a specimen of control urine and two 1-ml specimens of control urine containing 50.0 and 100.0 ng of II added as internal standards. Make the sample alkaline by addition of 1 ml 6 *N* NaOH and adjust to pH 9.0. Add 10 ml of benzene–methylene chloride (90:10), seal the tube with a PTFE stopper, and extract by shaking for 10 min on a reciprocating shaker. Centrifuge the sample for 10 min at 997 *g* in a refrigerated centrifuge (10°). Transfer the organic extract to a clean 50-ml stoppered centrifuge tube. Repeat this extraction procedure with a second 10-ml aliquot of benzene–methylene chloride (90:10) and combine the extracts. Save the aqueous phase for the determination of conjugated II and III.

Add 5 ml of 6 *N* HCl to the combined extracts, seal the tubes with a PTFE stopper, and back-extract by shaking for 10 min on a reciprocating shaker. Centrifuge at 997 *g* for 5 min at 10°, aspirate the organic phase, and discard. Wash the acid phase once with 10 ml of benzene–methylene chloride (90:10) by shaking, centrifuging, and discarding the organic phase as described above. Adjust the acid phase to approximately pH 9.0 by the addition of 5 ml of 6 *N* NaOH, add 10 ml of benzene–methylene chloride (90:10), and extract as previously described, transferring the organic phase to a 15-ml stoppered conical centrifuge tube. Evaporate the extract to dryness under a nitrogen stream in a 60° water-bath. Repeat the extraction with a second 10-ml aliquot of solvent and combine with the residue of the first extract. Evaporate the solvent as before and vacuum desiccate the sample for 15 min over $CaSO_4$ (Drierite). The residue is redissolved in 200 μl of benzene containing 200 ng griseofulvin/ml and analyzed by GLC–ECD using the previously described parameters.

Determination of conjugated II and III. To the remaining aqueous urine phase add 10 ml anhydrous diethyl ether to remove any residual amounts of nonconjugated II and benzene–methylene chloride (90:10). Shake and centrifuge as described above and discard the organic phase. Place the tube in a 50° water-bath under a stream of nitrogen to expel any residual ether in the sample. Equilibrate the sample to room

temperature and adjust to pH 5.3 by the addition of 5 ml of pH 5.3 phosphate buffer (check on pH meter). Transfer the two internal standard samples to two tubes containing the residue of: (1) 50 ng II and 250 ng III and (2) 100 ng II and 500 ng III, respectively. These samples will serve as the internal standards for the determination of conjugated II and III. Add 0.1 ml Glusulase reagent, mix, stopper loosely with cotton, and incubate overnight in a Dubnoff metabolic incubating shaker at 37°, at moderate speed. After incubation adjust to pH 9.0 with 6 *N* NaOH and extract with two 10-ml portions of benzene–methylene chloride (90:10). From this point the procedure is identical to that described for unconjugated II.

Calculations. The concentrations of nonconjugated II and conjugated II and III in μg/ml urine are determined by direct comparison to the concentration of internal standards of II and III included in the assay. The total amount of drug excreted, expressed as clorazepate dipotassium, is calculated from the concentration in μg/ml found in the urine pool multiplied by the appropriate molecular weight factor. (The conversion factors for clorazepate dipotassium/II and clorazepate dipotassium/III are 1.51 and 1.43, respectively.

Per cent recovery, sensitivity limit, and specificity of urine assay. The recoveries of II and III are calculated by comparison of the average peak areas for the two compounds in internal and external standards. The recoveries of II and III using the urine assay procedure are 98 ± 5% (S.D.) and 50 ± 5% (S.D.), respectively. The sensitivity limits of the assay for II and III are 5 and 25 ng/ml urine, respectively, using 1 ml of urine per assay. Samples larger than 1 ml cannot be analyzed using this extraction procedure due to endogenous impurities extracted from the biological sample.

The specificity of the urine assay for II and III was established by the absence of any interfering peaks occurring at the retention times of II and III upon chromatography of control (drug-free) human urine extracts (Fig. 2).

RESULTS AND DISCUSSION

Application of the method to biological specimens

Blood levels of clorazepate and its N-desmethyl metabolite are presented for

TABLE I

BLOOD LEVELS (ng/ml) OF I AND II FOLLOWING A SINGLE ORAL DOSE OF 15 mg CLORAZEPATE DIPOTASSIUM

n.m. = Non-measurable < 4 ng I/ml and < 4 ng II/ml.

Time (h)	*Subject 1*		*Subject 2*	
	I	*II*	*I*	*II*
0	n.m.	n.m.	n.m.	n.m.
0.5	19.0	119	22.4	131
1	11.8	175	22.5	139
2	13.4	108	22.5	106
4	7.9	64.6	15.8	82.3
12	n.m.	62.5	8.6	68.4
24	n.m.	46.6	n.m.	44.3
48	n.m.	39.6	n.m.	40.4
72	n.m.	24.1	n.m.	27.8

TABLE II

URINE LEVELS OF II AND III FOLLOWING A SINGLE DOSE OF TRANXENE

The dose was one 15-mg capsule of clorazepate dipotassium and the urine collection period was 0–24 h post dosing.

Subject	*% of dose**			
	*Unconjugated II***	*Conjugated II*	*Conjugated III*	*Total*
3	0.52	n.m.	4.9	5.42
4	1.32	n.m.	6.2	7.52

* Expressed as clorazepate dipotassium.
** Equivalent to clorazepate dipotassium.

two human subjects following a single oral 15-mg dose of the clorazepate dipotassium (Table I). Blood levels of I were measurable from 0.5 to 12 h and showed maximum levels of approximately 20 ng/ml. Such levels were previously not reported. Blood levels of II were measurable up to 72 h post dosing with maxima of 175 and 139 ng/ml at 1 h, in the two subjects, declining to approximately 25 ng/ml at 72 h (Table I). III was nonmeasurable (<10 ng/ml) throughout the experimental period.

Urine levels of unconjugated II (equivalent to the intact drug) and conjugated II and III were measured in two other subjects following a single oral 15-mg dose of clorazepate dipotassium. Total levels of drug related metabolites in the 0–24 h collection period were 5.42 and 7.52%, with conjugated III being the major components (see Table II). Levels of unconjugated III were nonmeasurable in these samples. These urinary levels are consistent with those previously reported[10] following administration of a gelatin capsule of clorazepate dipotassium.

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