GLC Determination of Plasma Drug Levels after Oral Administration of Clorazepate Potassium Salts

D. J. HOFFMAN and A. H. C. CHUN ˣ

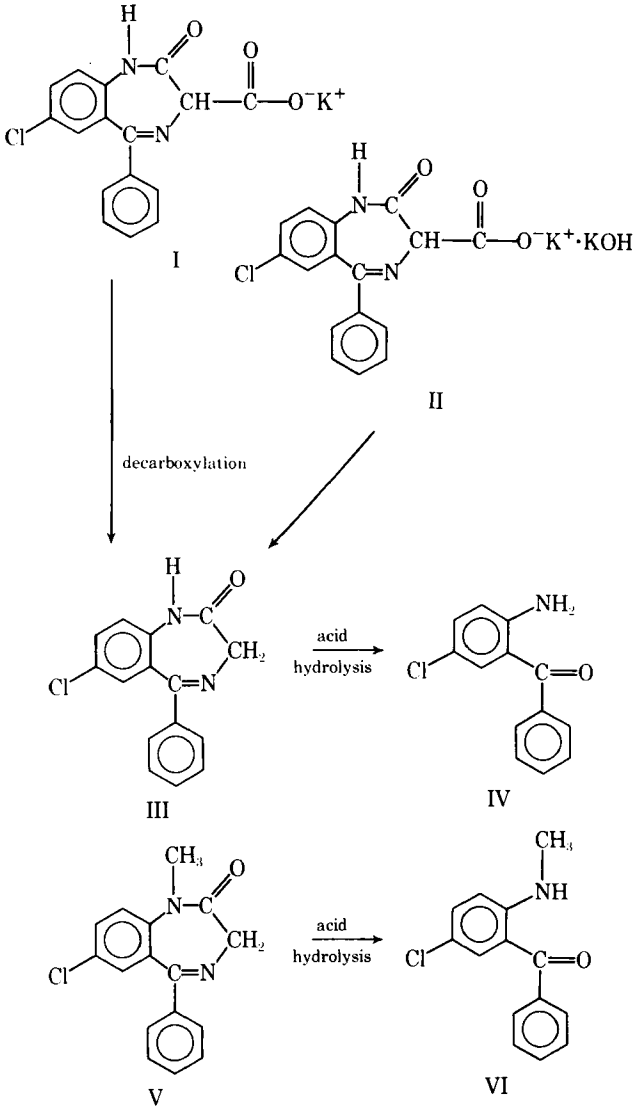
Abstract □ Plasma nordiazepam levels resulting from the oral administration of clorazepate potassium salts were determined by a sensitive GLC assay. Nordiazepam and the internal standard (diazepam) were selectively extracted into ether at pH 9.2, hydrolyzed to their respective benzophenones, and quantified by electron-capture detection. The assay was used in a comparative bioavailability study of single equimolar oral doses of monopotassium and dipotassium salts of clorazepate in dogs. Both clorazepate salts were rapidly absorbed and exhibited mean peak total drug levels after 1 hr. Clorazepate levels accounted for about 50% of the total drug levels present. No statistical difference in the plasma drug levels of clorazepate mono- and dipotassium salts and the metabolite was found in dogs.

Keyphrases □ Clorazepate monopotassium and dipotassium salts—determination of plasma levels after oral administration, dogs, GLC analysis of nordiazepam metabolite □ Nordiazepam—GLC analysis in plasma as clorazepate metabolite, dogs □ GLC—analysis, nordiazepam as clorazepate metabolite after oral administration, determination of clorazepate plasma levels

Clorazepate potassium salts (I and II) are new 1,4-benzodiazepine antianxiety agents (1–4). These salts are highly water soluble and rapidly decarboxylate (Scheme I) to nordiazepam (III) at a low pH. Decarboxylation also occurs at physiological pH but at a much slower rate[1].

This paper describes an assay procedure for nordiazepam (III) in plasma. The procedure is a modification of the GLC method of de Silva *et al.* (5), which converts III to 2-amino-5-chlorobenzophenone (IV) with subsequent quantification by GLC using electron-capture detection (Scheme I). Diazepam (V), which is converted by hydrolysis to 2-methylamino-5-chlorobenzophenone (VI), is used as the internal standard.

Plasma clorazepate levels were calculated from the difference between the total measured nordiazepam levels, by converting the residual parent drug (clorazepate) to nordiazepam, and the nordiazepam levels measured after immediate extraction. The method was applied to the determination of the bioequivalence of a single equimolar oral dose of clorazepate mono- and dipotassium salts in dogs.



Scheme I

EXPERIMENTAL

Drug Administration to Dogs—Twelve female beagle dogs of approximately equal weights were used in this crossover study to determine the bioequivalency of these two compounds after oral administration. Six dogs were randomly assigned 6.5-mg doses of the monopotassium salt or 7.5-mg doses of the dipotassium salt during the first period of the study. The administration of formulations to the dogs was reversed during the second period 1 week later. These doses were equivalent on a molar basis to 5.8 mg of the free acid. The capsule formulations were administered to the dogs in the morning after an overnight fast, and 30 ml of water then was given to each dog.

Blood samples were taken in 5-ml heparinized vacuum tubes before dosing and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 hr after dosing. Samples (3 ml) were cooled and centrifuged within 3 min after collection. One-half milliliter of the 0.5-, 1-, 1.5-, 2-, 3-, and 4-hr plasma samples was placed into tubes containing buffer and ether, and these samples were immediately extracted after collection to minimize conversion of clorazepate to nordiazepam (Scheme I). The remaining portion of the plasma samples was frozen until they were assayed.

Instrumental Conditions—Analysis was performed with a gas chromatograph[2] equipped with a ^{63}Ni electron-capture detector, containing a 2 mCi ^{63}Ni β-ionization source, and an electronic inte-

[1] R. C. Sonders and D. J. Anderson, Abbott Laboratories, North Chicago, IL 60064, 1974, unpublished data.

[2] Hewlett-Packard model 7620A.

Table I—Reproducibility of Plasma Nordiazepam Assay

Sample (0.15 μg/ml)	Peak Area IV	Peak Area VI	Peak Area Ratio
1	8491	6673	0.786
2	11320	8680	0.767
3	12150	9842	0.810
4	11760	9633	0.819
5	12750	9983	0.783
6	13450	11650	0.866
7	10570	9042	0.855
8	12910	10700	0.823
		Mean ± *SD*	0.813 ± 0.035
		Coefficient of variation = 4.31%	

grator[3]. Argon–methane[4] (95:5) was used as the carrier gas, and the column head pressure was preset at 40 psig. A 1.56-m (6-ft), 2-mm i.d. borosilicate helical glass column was packed with 3% Poly A-103 on Gas Chrom Q[5] and conditioned for 15 hr at 270° with the carrier gas flowing at 25 ml/min. Column life was 2–3 months when the carrier gas was passed through an oxygen trap[6].

The temperature settings were as follows: oven, 250°; injection port, 265°; and detector, 325°. The electrometer[7] range was set at 10^3, the output attenuation of the integrator was set at 2, and the recorder[8] chart speed was 0.6 cm (0.25 in.)/min. The pulse interval for the electron-capture detector was 150 μsec. Under these conditions, IV (hydrolysis product of III) and VI (5) (hydrolysis product of V; internal standard) had retention times of 5.0 and 3.9 min, respectively. By using the following procedure, the minimum measurable amount of III is 0.01 μg/ml of plasma.

Assay Procedure—*Preparation of Plasma Standards*—Prepare a nordiazepam primary standard of 100 μg/ml in ethanol. Use

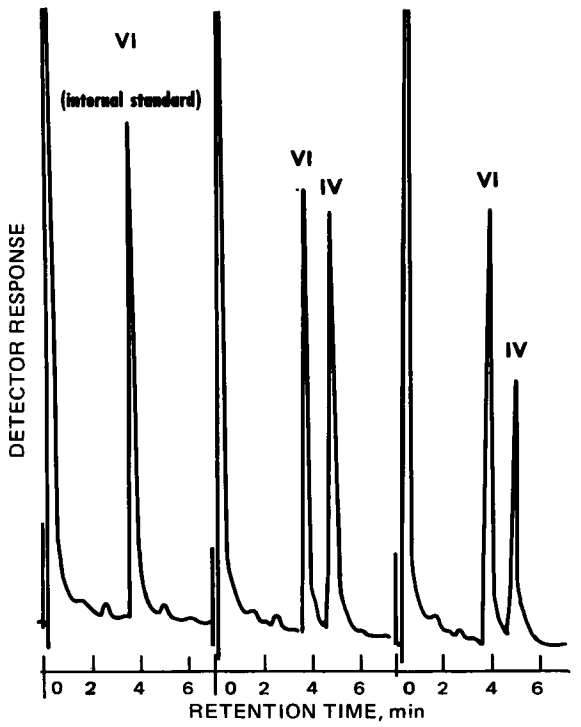


Figure 1—*Gas–liquid chromatograms (electron-capture detector) of plasma extracts: left, normal plasma specimen with internal standard; middle, plasma specimen after a single oral dose of a clorazepate salt; and right, plasma standard containing 0.15 μg/ml of nordiazepam.*

[3] Hewlett-Packard model 3370A.
[4] Matheson, oil pumped and dry.
[5] Applied Science.
[6] Altech Associates.
[7] Model 7650A.
[8] Model 7128A.

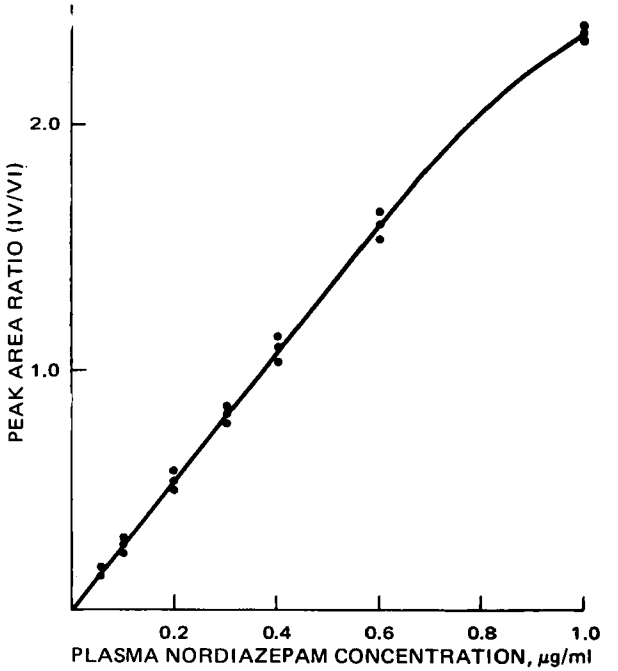


Figure 2—*Electron-capture detector calibration curve using the peak area ratio of IV/VI* versus *the micrograms per milliliter of nordiazepam in the plasma.*

1.0 ml of the ethanolic standard to prepare 100 ml of plasma standard at a concentration of 1.00 μg/ml. Further dilute the plasma to 0.50, 0.25, and 0.125 μg/ml with plasma.

Determination of Nordiazepam—Immediately centrifuge (within 3 min) freshly collected heparinized blood in a refrigerated centrifuge set at 2°. Transfer 0.5 ml of plasma to a 20-ml screw-capped test tube containing 5 ml of cold (5°) 0.1 *M* K_2HPO_4 buffer (pH 9.2) containing 0.10 μg of diazepam/ml (freshly prepared). Add ether[9] (7 ml) immediately, shake the tube on a reciprocal shaker at 250 cpm for 10 min, and then separate by centrifugation for 10 min at 2000 rpm. Transfer 5 ml of ether to a 20-ml screw-capped tube containing 5 ml of 6 *N* HCl. After shaking and centrifuging as already described, discard the ether layer by aspiration. Also aspirate off a small amount of the acid layer to assure complete removal of ether.

Place the samples in a water bath and heat to boiling with occasional shaking of the samples. Cap the tubes tightly and leave them in the boiling water bath for 1 hr to convert completely III to IV and V to VI. After cooling samples in an ice bath, add 5.5 ml of

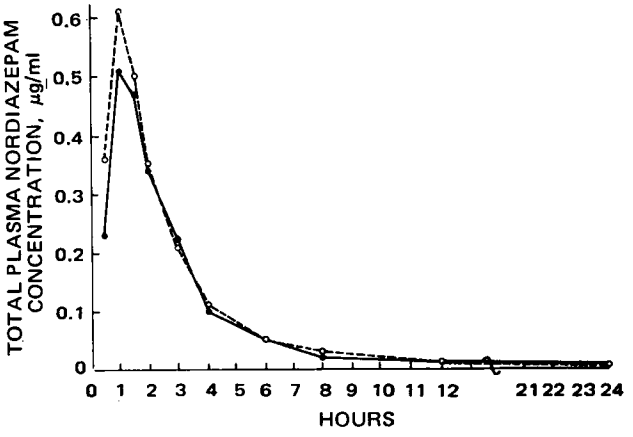


Figure 3—*Mean total plasma nordiazepam level curves in dogs (12 dogs/compound) following the oral administration of clorazepate salts. Key: ●, 6.5-mg dose of I; and ○, 7.5-mg dose of II.*

[9] Mallinckrodt, anesthetic grade.

Table II—Mean Plasma Nordiazepam (Immediate Extraction) Levels after Oral Administration of 6.5 mg of Clorazepate Monopotassium and 7.5 mg of Clorazepate Dipotassium to Dogs (12 Dogs/Compound)

Compound	Plasma Nordiazepam[a], μg/ml							Area under Curve[a], (0–4 hr)
	0 hr	0.5 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	
Clorazepate monopotassium								
Mean	0	0.10	0.29	0.26	0.19	0.11	0.06	0.61
SD		±0.12	±0.15	±0.09	±0.06	±0.06	±0.04	±0.22
Range		0.00–0.39	0.04–0.53	0.04–0.41	0.03–0.26	0.02–0.19	0.01–0.11	0.10–0.88
Clorazepate dipotassium								
Mean	0	0.14	0.31	0.24	0.19	0.12	0.07	0.64
SD		±0.12	±0.13	±0.11	±0.08	±0.08	±0.06	±0.30
Range		0.01–0.36	0.08–0.48	0.06–0.41	0.05–0.34	0.05–0.28	0.01–0.20	0.22–1.22

[a] No significant differences were found at the 0.05 level.

Table III—Mean Calculated Plasma Clorazepate Levels after Oral Administration of 6.5 mg of Clorazepate Monopotassium and 7.5 mg of Clorazepate Dipotassium to Dogs (12 Dogs/Compound)

Compound	Calculated Plasma Clorazepate[a], μg/ml							Area under Curve[a] (0–4 hr)
	0 hr	0.5 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	
Clorazepate monopotassium								
Mean	0	0.14	0.22	0.21	0.14	0.11	0.04	0.52
SD		±0.18	±0.25	±0.21	±0.14	±0.08	±0.04	
Clorazepate dipotassium								
Mean	0	0.22	0.30	0.25	0.16	0.09	0.04	0.62
SD		±0.13	±0.21	±0.19	±0.13	±0.06	±0.04	

[a] No significant differences were found at the 0.05 level.

6 *N* NaOH and 6 ml of ether[9]. Shake and centrifuge the samples as previously described.

Transfer 5 ml of the ether phase to a 15-ml conical screw-capped tube and evaporate to dryness at 50° with a stream of air. Dissolve the residue in 1.0 ml of benzene and inject 2 μl into the GLC. This procedure results in GLC tracings essentially free of coextracted impurities (Fig. 1).

Determination of Clorazepate—Plasma clorazepate concentrations[10] were determined by subtracting immediately extracted nordiazepam levels from the total drug levels obtained after hydrolysis and multiplying the difference by 1.16 to correct for the molecular weight difference.

Plasma nordiazepam concentrations were determined after immediate extraction under conditions that minimized *in vitro* conversion of clorazepate to nordiazepam. Another aliquot of the plasma sample was incubated at 40° overnight and extracted as previously described to determine the total drug concentration (nordiazepam and clorazepate). Incubation converts essentially all (>99%) of the clorazepate to nordiazepam[11].

Calculations—The peak area ratios of IV to VI are plotted against known standards of nordiazepam, expressed as micrograms per milliliter of plasma. Values for unknown concentrations of nordiazepam in plasma are read directly from the calibration curve (Fig. 2).

RESULTS AND DISCUSSION

Assay Reproducibility, Sensitivity, and Specificity—The analysis of eight plasma samples at 0.15 μg/ml gave a coefficient of variation of 4.31% (Table I). Comparable reproducibility was found with samples at other concentrations. Under the described assay conditions, the lower limit of the quantitative detection of nordiazepam in plasma is 0.01 μg/ml. This value is based on a sample signal equivalent to about 2% of full-scale response. A linear relationship between peak area ratios and concentration was obtained over the 0.01–0.80-μg/ml range. Quantification from a calibration curve was used (Fig. 2).

The method is not specific for nordiazepam. If significant levels of other 1,4-benzodiazepines, such as oxazepam, are present, they also are hydrolyzed to IV (6). Oxazepam, a metabolite of nordiazepam, is found almost exclusively in urine (7, 8) with small but insignificant amounts in plasma (9).

Serum or plasma samples for human subjects receiving clorazepate salts do not require incubation since little, if any, clorazepate is present[12].

Plasma Nordiazepam Levels in Dogs—Mean (±*SD*) nordiazepam peak levels of 0.29 ± 0.15 and 0.31 ± 0.13 μg/ml occurred 1 hr after oral administration of equimolar doses of the monopotassium and dipotassium salts, respectively. These levels declined to about 0.07 μg/ml at 4 hr (Table II). There were no significant differences by crossover analysis of variance ($p < 0.05$) in the nordiazepam levels at all sampling times and in the 0–4-hr area under the plasma level curve between the two compounds.

Plasma Clorazepate Levels—Plasma clorazepate levels were calculated based on the difference between total nordiazepam levels (parent drug plus metabolite) and nordiazepam levels (Table III). About one-half of the total nordiazepam level was present as clorazepate, although large variations (10–90% clorazepate) existed between dogs. However, no significant differences in clorazepate levels were obtained with both salts (Table IV).

Total Drug[13] Plasma Levels—Total plasma drug levels expressed as nordiazepam were determined after converting plasma clorazepate to nordiazepam and measuring the combined concentrations as nordiazepam. The mean total nordiazepam levels are given in Table IV. Both clorazepate salts were rapidly absorbed, with mean (±*SD*) peak total plasma nordiazepam levels of 0.51 ± 0.24 μg/ml for the monopotassium salt and 0.61 ± 0.24 μg/ml for the dipotassium salt (Fig. 3). Total levels declined to about 0.01 μg/ml at 12 hr and were undetectable after 24 hr.

There were no significant differences ($p < 0.05$) in plasma levels between the two clorazepate salts at all sampling times. Also, no significant differences were found between the areas under the plasma level curve for various time intervals (Table III). The total levels of combined parent drug and metabolite levels are more representative than either species alone in the assessment of bioavailability of these two potassium salts of clorazepate.

[10] Clorazepate concentrations are based on the free ionized form since both potassium salts would exist as such at a physiological pH.

[11] Clorazepate has an *in vitro* half-life of 70 min in dog plasma at 38°. This value was determined by the rate of formation of ether-soluble radioactivity from ^{14}C-clorazepate.

[12] A. H. Chun, Abbott Laboratories, North Chicago, IL 60064, 1974, unpublished data.

[13] "Total drug levels" is the term used for the combined levels of parent drug and metabolite measured as nordiazepam.

Table IV—Mean Total Plasma Drug Levels (Expressed as Nordiazepam) after Oral Administration of 6.5 mg of Clorazepate Monopotassium (I) and 7.5 mg of Clorazepate Dipotassium (II) to Dogs (12 Dogs/Compound)

Compound	Plasma Nordiazepam[a], μg/ml											Area under Curve[a]	
	0 hr	0.5 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	12 hr	24 hr	0–4 hr	0–12 hr
I													
Mean	0	0.23	0.51	0.47	0.34	0.22	0.10	0.05	0.02	0.01	0	1.12	1.41
SD		±0.21	±0.24	±0.19	±0.13	±0.09	±0.05	±0.03	±0.02	±0.02		±0.43	±0.56
Range		(0.00 −0.69)	(0.24 −1.16)	(0.28 −0.94)	(0.17 −0.58)	(0.07 −0.36)	(0.02 −0.20)	(0.01 −0.10)	(0.01 −0.07)	(0.00 −0.05	0.00 −0.01	0.60 −2.10	0.71 −2.80
II													
Mean	0	0.36	0.61	0.50	0.35	0.21	0.11	0.05	0.03	0.01	0	1.26	1.56
SD		±0.15	±0.14	±0.17	±0.13	±0.09	±0.06	±0.03	±0.02	±0.01		±0.34	±0.53
Range		(0.10 −0.62)	(0.42 −0.85)	(0.25 −0.84)	(0.16 −0.60)	(0.08 −0.36)	(0.02 −0.21)	(0.01 −0.11)	(0.00 −0.08)	(0.00 −0.04)	0.00 −0.00	0.64 −1.82	0.68 −2.34

[a] No significant differences were found at the 0.05 level.

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Hansch Analysis of Interaction of Haptens with Benzylpenicilloyl Antibodies

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Abstract □ Hansch analysis is applied to inhibition data obtained from the interaction of penicilloic and penilloic acid haptens with benzylpenicilloyl specific antibodies. Significant regressions with partition coefficient and molar volume parameters indicate the importance of hydrophobic and steric effects in these interactions.

Keyphrases □ Benzylpenicilloyl antibodies—interaction with penicilloic and penilloic acid haptens, Hansch analysis of inhibition data, hydrophobic and steric effects □ Penicilloic and penilloic acid haptens—interaction with benzylpenicilloyl specific antibodies, Hansch analysis of inhibition data □ Hansch analysis—interaction of haptens with benzylpenicilloyl antibodies □ Structure–activity relationships—interaction of penicilloic and penilloic acid haptens with benzylpenicilloyl specific antibodies

Munro *et al.*[1] studied the cross-reaction with benzylpenicilloyl specific antibodies of the penicilloic (I) and penilloic (II) acids obtained from several penicillins (III). They determined the extent of this interaction by measuring the inhibition by the haptens of agglutination of sensitized erythrocytes caused by the antibodies.

[1] A. C. Munro, M. G. Chainey, and S. R. Woroniecki, to be published.

In this paper the data so obtained are subjected to Hansch quantitative structure–activity analysis to evaluate the role of various physicochemical properties of the haptens in their interaction with the antibodies. Published applications of Hansch analysis to hapten antibody interactions appear to be confined to a single paper in which Kutter and Hansch (1) showed the importance of steric factors in such interactions.

EXPERIMENTAL

The term C in log $1/C$ in Table I is the molar concentration of hapten required to give 50% inhibition of hemagglutination by benzylpenicilloyl specific antibodies.

Hydrophobic effects are modeled by log P, where P is the octanol–water partition coefficient of the parent penicillins in their unionized form. Values of P are not available for the penilloic or penicilloic acids, but the variation of P with changes in the side chain is expected to be the same for these compounds as for the parent penicillins. Log P for penicillin G (benzylpenicillin), cloxacillin, methicillin, phenethicillin, penicillin V (phenoxymethyl penicillin), and propicillin are measured values from Bird and Marshall (2). The log P values for ampicillin and carbenicillin are calculated from those for penicillin G with the appropriate hydrophobic frag-