Radioimmunoassay of Chlordiazepoxide in Plasma

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Abstract □ A simple, rapid, and sensitive radioimmunoassay was developed for the determination of chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide) in plasma. Antiserum capable of binding chlordiazepoxide-2-^{14}C was obtained from rabbits following immunization with an antigen prepared by coupling the reactive acyl azide of 7-chloro-5-(4-hydrazinocarbonylmethoxyphenyl) - 2 -methylamino-3*H*-1,4-benzodiazepine 4-oxide to bovine serum albumin. The radioimmunoassay allows for the specific determination of chlordiazepoxide directly in plasma without extraction and was compared with a differential spectrofluorometric assay for chlordiazepoxide. Both methods gave satisfactory agreement for the plasma levels of chlordiazepoxide in human subjects resulting from single and chronic oral doses of chlordiazepoxide hydrochloride.

Keyphrases □ Chlordiazepoxide—radioimmunoassay in plasma, compared to spectrophotofluorometric method □ Radioimmunoassay—chlordiazepoxide in plasma, compared to spectrophotofluorometric method

Chlordiazepoxide hydrochloride[1,2] (II), which was synthesized in the mid-1950's (1), was the first member of the 1,4-benzodiazepine class of compounds to be used clinically as an antianxiety agent in humans (2, 3) and is widely prescribed.

Three satisfactory methods have been developed for the determination of chlordiazepoxide in blood. In the most widely used technique, a spectrofluorometric assay (4) into which an earlier assay procedure (5) is incorporated, the level of chlordiazepoxide is obtained by subtraction of the fluorescence due to its metabolite, *N*-desmethylchlordiazepoxide, from the fluorescence due to both compounds. However, the determination of chlordiazepoxide is dependent on the accuracy of the assay of the metabolite. The method has a sensitivity limit of about 100 ng/ml using a 2-ml sample of plasma.

Recently, a polarographic method was developed which is specific for chlordiazepoxide (6). The technique involves plasma extraction and TLC isolation of chlordiazepoxide, which is then determined polarographically. The sensitivity limit is about 50 ng/ml using a 2-ml sample. An electron-capture GC technique has been reported (7) with a sensitivity limit of about 30 ng/ml using a 1-ml sample of plasma.

All of these methods require an extraction of chlordiazepoxide; in two instances, a chromatographic step is also employed. Such procedures limit the number of samples that may be analyzed in any one assay run because of the time required to carry out the assay.

The present report concerns the development of a radioimmunoassay for chlordiazepoxide directly in plasma without extraction. The method allows one person to analyze about 100 plasma samples over 2 days, using not more than a 0.1-ml sample of plasma and attaining a sensitivity limit of 20 ng/ml.

EXPERIMENTAL

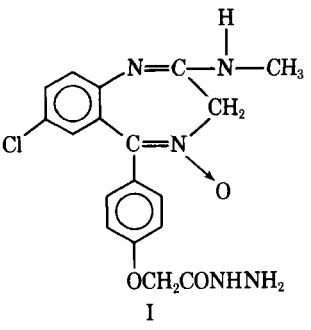
Preparation of Immunogen—7-Chloro-5-(4-hydrazinocarbonylmethoxyphenyl)-2-methylamino-3*H*-1,4-benzodiazepine 4-oxide (I) was converted to its reactive acyl azide and covalently coupled to bovine serum albumin[3] (8) in the following manner. Compound I (45 mg, 0.12 mmole), as a suspension in dimethylformamide (2 ml), was treated with 0.1 ml of 4.7 *N* hydrochloric acid in dioxane to give a pale-yellow solution. The mixture was cooled to −30°, and 0.16 ml of a 1:10 dilution of isoamyl nitrite (0.12 mmole) in dioxane was added. The solution was stirred at −30 to −40° for 30 min; then 0.1 ml of 0.5 *M* aqueous ammonium sulfamate was added and the stirring was continued for 10 min.

The cold azide solution was added slowly, dropwise with stirring, to a solution of bovine serum albumin (72 mg) in 4 ml of 0.16 *M* borate buffer (pH 8.5) at 0°. The pH was kept at 8.0–8.7 by the addition of 1 *N* NaOH. The resulting pale-yellow solution (pH 8.5) was kept at 4° for 36 hr and then dialyzed against 0.05 *M* tromethamine buffer (pH 8) for 3 days. After further dialysis for 2 days against distilled water, the immunogen was isolated by lyophilization.

Estimation of Number of Molecules of Hapten Coupled to Bovine Serum Albumin—A portion of the lyophilized hapten–protein conjugate was dissolved in buffer to give a solution of 1 mg/ml. A control solution of bovine serum albumin was similarly prepared. Each sample was then analyzed by differential pulse polarography (6) against a known amount of chlordiazepoxide. The control solution showed no peaks in its polarogram while the hapten–protein conjugate exhibited two peaks due to reduction of the *N*-oxide function and the azomethine bond in the $C_5{=}N_4$ position of the hapten molecule. On the basis of the peak heights relative to the standard of chlordiazepoxide, it was estimated that approximately eight molecules of the hapten had been covalently coupled to one molecule of the bovine serum albumin.

Immunization—The lyophilized immunogen was dissolved in 0.9% saline to give a solution of 2 mg/ml. After the addition to an equal volume of Freund's complete adjuvant[4], the mixture was emulsified to a thick paste using a blender[5] at 45,000 rpm.

Three New Zealand White female rabbits were injected intra-



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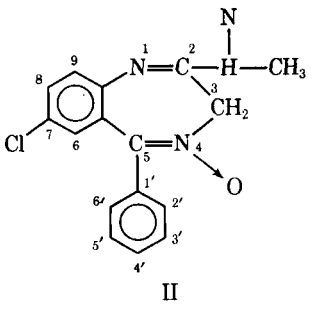

II

[1] The chemical names of the 1,4-benzodiazepines are: chlordiazepoxide, 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide; *N*-desmethylchlordiazepoxide, 7-chloro-2-amino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide; demoxepam, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide; diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one; *N*-desmethyldiazepam, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one; and clonazepam, 5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepin-2-one.

[2] Librium, containing chlordiazepoxide hydrochloride as its active ingredient, was administered throughout the study.

[3] Sigma Chemical Co., St. Louis, Mo.

[4] Grand Island Biological Co., Grand Island, N.Y.

[5] Virtis "S" 45, Virtis Co., Gardiner, N.Y.

Table I—Specificity of Antiserum

Compound	Cross-Reaction, %
Chlordiazepoxide	100
N-Desmethylchlordiazepoxide	5
Demoxepam	<1
N-Desmethyldiazepam	<1
Diazepam	<1
Clonazepam	<1

dermally on the flank with 0.1 ml of the emulsion at each of five separate sites; this process was repeated twice at 2-week intervals. Each animal then received 0.5 mg of the antigen subcutaneously as a solution in saline and bled from the ear artery 10 days later. Booster injections were given at monthly intervals.

Antiserums—The blood (20–30 ml) was allowed to clot at 37° for 2 hr and then kept at 4° overnight to allow for clot contraction. The serum was decanted, centrifuged at 3000 rpm, and divided into 1-ml aliquots. These aliquots were kept frozen at −20° until used.

Radioimmunoassay Method—The following stock reagents and materials were used: assay tubes[6] (12 × 75-mm disposable culture tubes); chlordiazepoxide-2-^{14}C (specific activity 200 μCi/mg)[7] in 0.001 *N* HCl to give 40,000 cpm/ml; antiserum diluted 1:50 with sodium phosphate buffer (0.01 *M*, pH 7.4) containing 0.1% sodium azide; and standard solutions (20, 50, 100, 200, 500, and 1000 ng/ml) of unlabeled chlordiazepoxide in 0.001 *N* HCl.

The assay procedure involved the addition of a constant volume of 20–100 μl of control human plasma to 0.1 ml of each standard of chlordiazepoxide to generate a calibration curve of 2–100 ng/tube. The same volume of the unknown plasma samples was added to tubes containing 0.1 ml of 0.001 *N* HCl. Two blanks were included by adding the control plasma to 0.1 ml of 0.001 *N* HCl. Then 0.1 ml (4000 cpm) of the chlordiazepoxide-2-^{14}C solution was added to each tube, followed by 0.1 ml of the antiserum solution to all tubes except one blank. The volume in each tube was brought to 1 ml with buffer. After mixing on a vortex, each tube was immersed in an ice water bath for 1 hr.

An equal volume (1 ml) of saturated ammonium sulfate was then added to precipitate globulin-bound chlordiazepoxide-2-^{14}C. After thorough mixing on a vortex and standing at 4° for 15 min, the tubes were centrifuged at 3000 rpm for 30 min at 4°. The supernate containing unbound chlordiazepoxide-2-^{14}C was decanted into a counting vial, and 10 ml of toluene scintillator[8] was added. The vial was then vortexed for 10 sec to extract the radioactive material into the organic phase and each sample was counted[9]. When many samples were to be analyzed, it was convenient to carry out the extraction on a reciprocating shaker for 10 min with the vials in a covered vial tray in the vertical position.

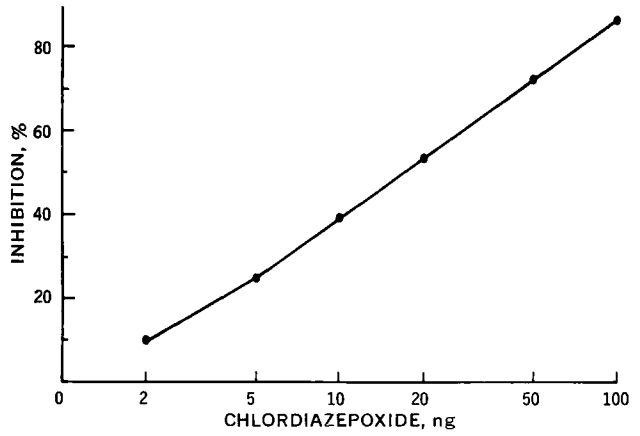

Figure 1—*Standard curve of chlordiazepoxide in 20 μl of human plasma.*

[6] diSPo, Scientific Products, McGaw Park, Ill.
[7] Supplied by Dr. A. Liebman, Hoffmann-La Roche Inc., Nutley, N.J.
[8] Omnifluor, New England Nuclear, Boston, MA 02118
[9] Packard Tri-Carb model 3380.

Table II—Chlordiazepoxide Plasma Levels in Different Subjects at Random Periods after Administration of Chlordiazepoxide Hydrochloride, Determined by the Radioimmunoassay and Spectrofluorometric Methods

Radioimmunoassay Method, μg/ml	Spectrofluorometric Method, μg/ml
Single Dose (30 mg)	
1.35	1.54
1.12	0.97
1.00	1.3
0.65	0.56
0.65	0.52
0.40	0.32
0.30	0.55
0.15	0.30
0.70	1.00
Chronic Dose (10 mg tid)	
1.85	1.50
1.35	1.23
0.95	1.11
0.23	n.m.[a]
0.80	0.90
0.95	0.85
1.25	1.47
0.78	1.0

[a] n.m. = not measurable.

All determinations, including the standards, were done in duplicate, and the average of each set of counts was used in the calculation.

RESULTS

Antibody Production—Each rabbit produced antiserum capable of binding chlordiazepoxide-2-^{14}C when compared to serum from nonimmunized rabbits. Almost identical and usable titers of antibody were obtained from each rabbit at the first bleeding. All data reported in this article were obtained with this first batch of antiserum, where 1 ml of a 1:500 dilution was capable of binding about 60% of the 4000 cpm of the tracer. After four monthly booster immunizations, it became possible to work with 1:1000 dilution of the antiserum.

Sensitivity of Radioimmunoassay—A typical standard curve obtained with chlordiazepoxide in 20 μl of normal human plasma is shown in Fig. 1. The percentage inhibition of binding was calculated by expressing the decrease in binding of the tracer due to unlabeled chlordiazepoxide as a percentage of the total amount bound in the absence of unlabeled chlordiazepoxide. The method appears readily applicable over a range of 2–100 ng/tube. Thus, the sensitivity limit is 20 ng/ml using a 0.1-ml sample of plasma.

Specificity—Several benzodiazepines were tested for their ability to compete with labeled chlordiazepoxide for the antibody. The competitors were tested at a concentration of 200 ng—10 times the concentration of chlordiazepoxide required to produce a 50% inhibition of binding (Fig. 1). The percentage cross-reaction was expressed as ng of chlordiazepoxide/200 ng × 100%, in which the amount of standard chlordiazepoxide and the 200 ng of competitor were read at the same percentage inhibition of binding. The results (Table I) indicate that the highest cross-reaction was 5% with *N*-desmethylchlordiazepoxide while demoxepam, *N*-desmethyldiazepam, diazepam, and clonazepam showed less than 1%.

Comparison of Radioimmunoassay with Spectrofluorometric Determination of Chlordiazepoxide in Plasma—Samples of plasma were obtained from subjects who had received both single and chronic oral doses of chlordiazepoxide hydrochloride. Duplicate 20-μl aliquots were analyzed for chlordiazepoxide by the radioimmunoassay procedure; single 1-ml aliquots were assayed by the spectrofluorometric method of Schwartz and Postma (4) (Table II). The joint determinations were subjected to a straight-line analysis. The fitted intercept and slope (−0.005 and 0.967, respectively) were not significantly different from 0 and 1 at the 0.05 level.

Precision—Since no extraction procedures were involved in the radioimmunoassay, thereby avoiding differences in extraction recoveries, the precision depended on the accuracy with which the

Table III—Reproducibility of Radioimmunoassay for Plasma Chlordiazepoxide

Sample[a]	Chlordiazepoxide, μg/ml[b]		
	Assay 1	Assay 2	Assay 3
1	0.98	1.1	1.0
2	0.62	0.64	0.65
3	0.44	0.40	0.38
4	1.30	1.35	1.25
5	1.2	0.93	0.95
6	0.27	0.23	0.22

[a] Samples obtained at random times from subjects receiving single and chronic doses of chlordiazepoxide hydrochloride. [b] Analyzed on three separate occasions several weeks apart.

operator pipetted the samples and reagents and decanted the "free" radioactivity into the counting vial. The mean coefficient of variation for six replicates at each point of the calibration curve was 4%. Six samples of plasma from subjects who had received chlordiazepoxide hydrochloride were analyzed for chlordiazepoxide on three separate occasions several weeks apart (Table III). The pooled standard deviation was 0.072 μg/ml (12 degrees of freedom) over a range of 0.22–1.35 μg/ml.

Chlordiazepoxide Plasma Levels in Humans—Table IV shows the plasma levels of chlordiazepoxide, as determined by radioimmunoassay, in a subject who received a single 30-mg oral dose of chlordiazepoxide hydrochloride.

DISCUSSION

A radioimmunoassay, developed for the determination of chlordiazepoxide directly in plasma, is simple, rapid, and easy to perform and it may be carried out using as little as 20 μl of plasma. The technique thus lends itself to the analysis of large numbers of samples. The radioimmunoassay might be employed in monitoring the blood levels of chlordiazepoxide in patients receiving the drug chronically and/or determining whether, in fact, the patient is taking the drug at all. Such monitoring of blood levels may help the practitioner in the optimization of drug therapy with chlordiazepoxide.

In designing the radioimmunoassay, several points were considered regarding the preparation of a suitable antigen. The ultimate goal of these investigations was to obtain antibodies of sufficient specificity to obviate the necessity of extracting chlordiazepoxide from plasma for its determination. Thus, the antibodies would have to be specific for chlordiazepoxide in the presence of all of its known plasma metabolites in humans, namely *N*-desmethylchlordiazepoxide (4), demoxepam (5), and *N*-desmethyldiazepam (9). Therefore, the antibodies would be required to distinguish structural changes occurring only in the 1,4-diazepine ring or "fingerprint" region. It is well recognized that antibody specificity for a particular fingerprint region of a hapten is enhanced by coupling the hapten to the protein at a position in the hapten well removed from the fingerprint region. For this reason, the 4′-position of the 5-phenyl group in chlordiazepoxide was chosen for linkage to the protein, thereby having the functional groups in the 1,4-diazepine ring relatively free to confer immunological specificity. This goal was achieved by immunization of rabbits with an immunogen prepared by covalently coupling an active acyl-azide at this 4′-position to the ε-amino groups of lysine on albumin (8). The antiserum obtained had a high degree of specificity for chlordiazepoxide. *N*-Desmethylchlordiazepoxide showed only about 5% cross-reactivity, while demoxepam and *N*-desmethyldiazepam showed less than 1%. This finding would suggest that the C-2 methylamino group was a critical antigenic determinant.

Since neither diazepam nor clonazepam cross-reacted, chlordiazepoxide apparently can be determined in the plasma of subjects receiving chlordiazepoxide in conjunction with diazepam and/or clonazepam.

The fact that the metabolite, *N*-desmethylchlordiazepoxide, cross-reacted with the antibody to about 5% did not significantly affect the accuracy of the assay for chlordiazepoxide. The plasma levels of *N*-desmethylchlordiazepoxide, as determined by the spectrofluorometric method (4), only sometimes exceeded those of chlordiazepoxide, by at the most a factor of two, at 36–48 hr after a single oral dose[10]. Thus, the maximum error in the determination of chlordiazepoxide due to *N*-desmethylchlordiazepoxide at these late periods of time cannot exceed a 10% overestimation.

Table IV—Plasma Levels of Chlordiazepoxide in Human Subject following a Single 30-mg Oral Dose of Chlordiazepoxide Hydrochloride

Hours	Chlordiazepoxide, μg/ml
0.5	0.35
1	2.30
2	1.80
3	1.65
4	1.55
6	1.30
8	1.22
12	0.84
24	0.48
30	0.37
48	0.19

In subjects receiving 10 mg tid or 30 mg/day of chlordiazepoxide hydrochloride, the average steady-state levels of *N*-desmethylchlordiazepoxide are about 50% of the chlordiazepoxide levels[10], thereby reducing the error in the overestimation of the steady-state levels of chlordiazepoxide by the radioimmunoassay to around 2.5%. The less than 1% cross-reactivity of demoxepam and *N*-desmethyldiazepam becomes insignificant since their levels have been found to be considerably less than those of chlordiazepoxide at all times following either single or chronic doses of chlordiazepoxide hydrochloride[10].

The specificity of the radioimmunoassay for chlordiazepoxide also was tested by comparison with an established spectrofluorometric method (4). Satisfactory agreement was obtained for the levels of chlordiazepoxide in subjects who had received both single and chronic doses of chlordiazepoxide hydrochloride. The greatest differences were noted with low levels of chlordiazepoxide. This finding is not too surprising when one considers that the sensitivity limit of the radioimmunoassay (100 ng/ml using a 20-μl sample of plasma) was twice that of the spectrofluorometric method (200 ng/ml using a 1-ml sample of plasma). Also, in the spectrofluorometric assay the level of chlordiazepoxide is dependent on the accuracy with which *N*-desmethylchlordiazepoxide is determined.

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[10] Data on file, Hoffmann–La Roche Inc., Nutley, N.J.