stretching frequencies at 1160 and 1340 cm^{-1}, a carbonyl stretching frequency at 1680 cm^{-1}, and an amino stretching frequency at 3405 cm^{-1}.

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Radioimmunoassay of the Anticonvulsant Agent Clonazepam

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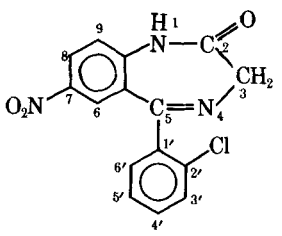
Abstract □ A simple and specific radioimmunoassay was developed for the determination of the anticonvulsant agent clonazepam directly in plasma without extraction. Antibodies to clonazepam were produced in rabbits after immunization with an immunogen prepared by covalently linking the 3-hemisuccinyloxy derivative of clonazepam to bovine serum albumin. When employing ^{3}H-clonazepam as the tracer, the radioimmunoassay has a limit of sensitivity of 5 ng/ml using a 0.1-ml sample of plasma. The antibodies exhibited a high degree of specificity for clonazepam; no cross-reactivity was observed with its 7-amino and 7-acetylamino metabolites nor with a number of other widely prescribed anticonvulsant agents that might be administered in conjunction with clonazepam. Satisfactory agreement was obtained for the plasma levels of clonazepam in humans when samples were assayed by the radioimmunoassay and an established electron-capture GC technique. By virtue of its simplicity, the radioimmunoassay offers a distinct advantage to the clinician for monitoring plasma clonazepam levels and the compliance of patients undergoing anticonvulsant therapy with the drug.

Keyphrases □ Clonazepam—radioimmunoassay, human plasma □ Radioimmunoassay—clonazepam, human plasma □ Anticonvulsants—clonazepam, radioimmunoassay, human plasma

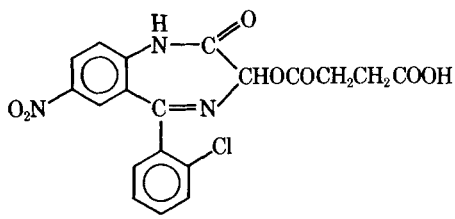
Clonazepam (I) is a member of the 1,4-benzodiazepine class of compounds which has recently been found to be clinically effective in controlling minor motor seizures (petit mal) in humans (1–4). Studies on the pharmacokinetics and metabolism of the drug also have been reported (5–7).

At the present time, only electron-capture GC methods have been reported for the determination of the nanogram levels of clonazepam that exist in the plasma of subjects undergoing anticonvulsant therapy (8–10). These electron-capture–GC methods have a limit of sensitivity in the order of 1 ng of clonazepam/ml of plasma using a 2-ml sample. However, apart from requiring skilled technical operation, these techniques are time consuming and thereby restrictive for the routine analysis of the numerous samples obtained during clinical studies.

The present report concerns the development of a specific and rapid radioimmunoassay for clonazepam in plasma. This assay allows the clinician to relate therapeutic response to plasma levels of the drug in individual patients.



I



II

EXPERIMENTAL

Tritium-Labeled Clonazepam—^{3}H-Clonazepam was prepared by tritium exchange using dimethylformamide–tritiated water (specific activity 100 Ci/g). The product was purified by silica gel column chromatography, yielding material with a specific activity of 4.23 mCi/mg. This method of introducing tritium probably provided exchange mainly at the C-3 position.

Preparation of Immunogen—The 3-hemisuccinyloxy derivative of clonazepam (II) was covalently coupled to bovine serum albumin using the mixed anhydride procedure of Erlanger *et al.* (11). After successive dialysis against dioxane–water (1:1), 0.05 *M* borate buffer (pH 9), and water, the immunogen was isolated by lyophilization.

On the basis of its absorbance at 365 nm in 0.1 *N* NaOH against a standard solution of II mixed with albumin, it was estimated that the immunogen consisted of 35 moles of II covalently coupled to 1 mole of albumin.

Antibody Production—Two New Zealand White female rabbits were immunized intradermally at multiple sites with 1 mg of the immunogen as an emulsion in Freund's complete adjuvant as previously described (12). The animals were then boosted intravenously with 50 μg of immunogen at monthly intervals, and serum was harvested 10–14 days after each administration. Both rabbits produced satisfactory titers of antibodies to clonazepam within 3 months following the initial immunization, and their serum was pooled. At a 1:2000 dilution, 1 ml of diluted serum bound approximately 60% of the ^{3}H-clonazepam used for the assay.

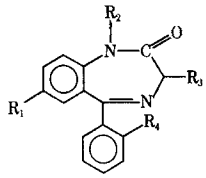


Table I—Cross-Reactivity of Substituted 1,4-Benzodiazepines

Compound	R_1	R_2	R_3	R_4	Cross-Reactivity, %[a]
Clonazepam	NO_2	H	H	Cl	100
Clonazepam 3-hydroxy derivative	NO_2	H	OH	Cl	24
Nitrazepam	NO_2	H	H	H	3.2
III	Cl	H	H	Cl	1.4
Oxazepam	Cl	H	OH	H	0.3
Desmethyldiazepam	Cl	H	H	H	0.06
Clonazepam 7-amino derivative	NH_2	H	H	Cl	0.04
Nitrazepam 7-amino derivative	NH_2	H	H	H	0.02
IV	H	H	H	Cl	0.02
Clonazepam 7-acetylamino derivative	CH_3CONH	H	H	Cl	<0.01
V	CH_3CONH	H	OH	Cl	<0.01
Diazepam	Cl	CH_3	H	H	<0.01

[a] Percentage cross-reactivity is defined as $100\ x/y$, where x is the mass of unlabeled clonazepam and y is the mass of competitor required to produce 50% inhibition of binding of ^{3}H-clonazepam by the antiserum.

Radioimmunoassay Procedure—The following stock solutions were prepared for the assay: standard solutions of 5, 10, 20, 50, 100, and 200 ng of clonazepam/ml of assay buffer (sodium phosphate, 0.01 *M*, pH 7.4, containing 0.9% sodium chloride); a solution of ^{3}H-clonazepam in buffer, 25,000 cpm/ml; and antiserum diluted 1:800 with buffer and containing 0.01% sodium azide. All stock solutions were stored at 4°.

To perform the assay, 0.1 ml of control plasma was added to 0.1 ml of each clonazepam standard in a 12 × 75-mm disposable tube to generate a calibration curve. Appropriate controls were included by adding the control plasma to 0.1 ml of buffer alone. Each unknown plasma sample (0.1 ml) was added to tubes containing 0.1 ml of buffer. Then 0.4 ml (10,000 cpm) of the ^{3}H-clonazepam solution followed by 0.4 ml of the diluted antiserum was added, and the contents (1.0 ml) of each tube were mixed and allowed to stand at room temperature for 30 min. Saturated ammonium sulfate (1 ml) was added to precipitate the globulin-bound ^{3}H-clonazepam and, after mixing, the tubes were allowed to stand for 15 min at 0° in a refrigerated centrifuge and were then centrifuged at 3000 rpm for 30 min.

Each supernate, containing the unbound ^{3}H-clonazepam, was decanted into a counting vial, and 10 ml of toluene scintillator[1] was added. The vials were then placed in a covered vial tray and shaken for 5 min on a reciprocating shaker with the tray in the vertical position. In this way, the ^{3}H-clonazepam was quantitatively extracted into the toluene scintillator. The samples were assayed for ^{3}H-activity in a liquid scintillation counter.

All samples including the standards, unknowns, and controls were assayed in duplicate, and the average of the tritium counts was used for calculation of percentage inhibition of binding (12). Quantitation of unknown samples was accomplished by interpolation from a logit–log plot of the percentage inhibition of binding *versus* the concentration of each standard (13).

RESULTS AND DISCUSSION

Sensitivity and Precision of Radioimmunoassay—Linearization of the calibration curve was obtained by plotting the percentage inhibition of binding of the ^{3}H-clonazepam *versus* the concentration of unlabeled clonazepam on logit–log transformation graph paper[2]. An almost perfect straight line was obtained in the range of 0.5–20 ng of unlabeled clonazepam added. The calibration lines obtained using 0.02, 0.05, and 0.1 ml of plasma were virtually superimposable. In repeated assays, the percentage inhibition of binding by 0.5 ng of clonazepam always exceeded 10%. However, since 0.1 ml of plasma was routinely assayed, the working limit of sensitivity was actually 5 ng/ml. This sensitivity is adequate for routine clinical determination of clonazepam (4).

The accuracy and intraassay precision of the radioimmunoassay were determined by the addition of 10, 50, and 100 ng of clonazepam to separate 1.0-ml aliquots of a clonazepam-free plasma pool obtained from 20 patients receiving other anticonvulsants. Six 0.1-ml replicates of each of the three concentrations were assayed. The mean values and coefficients of variation obtained were 9.62 ± 7.1, 49.7 ± 5.2, and 99.7 ng/ml ± 4.9%, respectively. The interassay precision was determined by assaying unknown samples from four different subjects who had received 5–29 mg of clonazepam/day in conjunction with other anticonvulsants. The samples were stored at 4° and assayed on five different occasions over 2 weeks. The mean values for clonazepam and the coefficients of variation were 21.4 ± 4.2, 36 ± 2, 69.6 ± 2.4, and 625 ng/ml ± 4%. The sample with a mean value of 625 ng/ml was diluted 1:10 with control plasma prior to analysis.

The foregoing data, apart from showing adequate intra- and interassay precision for routine clinical analysis, indicate no decomposition of clonazepam in plasma when stored at 4°.

Specificity—In designing the radioimmunoassay for clonazepam, several points were considered regarding a suitable hapten. As in any radioimmunoassay procedure, antibody specificity was of the utmost importance. In the case of clonazepam, this specificity centered on the ability of the antibodies to recognize clonazepam alone in the presence of its known major metabolites in plasma: the 7- amino and 7-acetylamino derivatives (5). Recent studies[3] (7) have shown that the 7-amino metabolite is present in plasma in almost equal concentration to clonazepam on chronic administration. The 7-acetylamino metabolite appears to be present in considerably lower concentrations (9). Another consideration was that other benzodiazepines might be administered concomitantly to subjects receiving clonazepam. For these reasons, II was chosen as a possible hapten to fulfill the specificity requirements of the antibodies. By coupling at the C-3 position to the protein, the 7-nitro and 2′-chloro groups are relatively free to act as antigenic determinants.

To determine which functional groups on the hapten influenced the specificity of the antibodies obtained to clonazepam, various benzodiazepines with different substituents in the 1-, 2′-, 3-, and 7-positions were examined for their extent of cross-reactivity. The greatest cross-reactivity (24%) occurred with the 3-hydroxy derivative of clonazepam (Table I). This is not surprising, since the hapten was coupled to the protein at the C-3 position. Although the 3-hydroxy derivative of clonazepam has been isolated as a conjugated metabolite of clonazepam in urine (5), it has not been detected in plasma.

Nitrazepam, which differs from clonazepam by the absence of the 2′-chloro group, showed only 3.2% cross-reactivity. This result illustrates the effect of the 2′-chloro group on the immunogen as an antigenic determinant. However, when rabbits were immunized with an albumin conjugate of the 3-hemisuccinyloxy derivative of nitrazepam, the resulting antibodies recognized both nitrazepam and clonazepam almost equally well[3]. The most significant lack of cross-reactivity was observed with compounds that did not have a nitro group but had some other substituent at the 7-position. The latter included the 7-amino and 7-acetylamino metabolites of clonazepam, which showed 0.04 and <0.01% cross-reactivity, respectively.

[1] Omnifluor, New England Nuclear Corp., Boston, MA 02118.
[2] TEAM, Box 25, Tamworth, NH 03886.

[3] Data on file, Hoffmann–La Roche Inc., Nutley, NJ 07110.

Table II—Specificity of the Radioimmunoassay for Clonazepam in Plasma Containing 20 ng of Clonazepam/ml in the Presence of High Concentrations of Various Potential Competitors

Competitor	Competitor Concentration, ng/ml	Clonazepam Found, ng/ml
Clonazepam 7-amino derivative	10,000	21
Diazepam	10,000	19
Desmethyldiazepam	10,000	23
Acetazolamide	20,000	19
Phenytoin	50,000	20
Phenobarbital	50,000	19
Primidone	50,000	20
Ethosuximide	100,000	21
Trimethadione	100,000	21

To evaluate further the specificity of the radioimmunoassay in the clinical situation, several drugs commonly used for seizure control that might be administered with clonazepam were checked for their possible interference. Control plasma containing 20 ng of clonazepam/ml was used as the reference standard, and aliquots were spiked with each compound listed in Table II. The concentration of each potential competitor added was judged to be greater than the concentration that might be encountered in plasma during routine seizure control therapy.

Each sample was assayed for clonazepam, and Table II shows the percent response for clonazepam in the presence of each compound studied as compared to the control sample containing 20 ng/ml of clonazepam alone (100% response). No competitor, including the 7-amino metabolite of clonazepam, which was in a 500–5000-fold excess, caused any serious error in the clonazepam determination, and it is unlikely that a false positive reaction would be encountered. These studies are further substantiated when one considers the almost quantitative response previously obtained for clonazepam when added to the pool of plasma from patients receiving other anticonvulsants. In the latter instance, the plasma pool contained all anticonvulsants listed in Table II and their respective plasma metabolites.

Comparison of Radioimmunoassay with Electron-Capture GC Determination of Clonazepam—Sixteen plasma samples, obtained from subjects receiving clonazepam either alone or with concomitant anticonvulsants, were assayed for clonazepam using the radioimmunoassay and the electron-capture GC method[4] of de Silva *et al.* (8). The joint determinations were subjected to straight-line analysis by the method of Wald (14) using a 95% confidence ellipse[5]. The fitted intercept and slope (−0.56 and 0.94, respectively) were not significantly different from 0 and 1 over a range of 7–130 ng of clonazepam/ml. This result shows that the radioimmunoassay can measure clonazepam as precisely as the electron-capture GC method.

Plasma Clonazepam Levels during Routine Anticonvulsant Therapy—To obtain data regarding the range of plasma levels of clonazepam *versus* dose that might be encountered during routine anticonvulsant therapy, plasma samples from subjects who had received various doses of clonazepam in conjunction with other anticonvulsants were assayed by the radioimmunoassay. Figure 1 shows the steady-state plasma levels of clonazepam *versus* dose for 15 subjects, each of whom had been treated chronically with at least two different doses of the drug. Each subject was also receiving concomitantly at least one anticonvulsant listed in Table II, and 10 subjects were receiving two or three of the latter anticonvulsants in various combinations. There was as much as a fivefold variation in the plasma levels at steady state with the same chronic dose

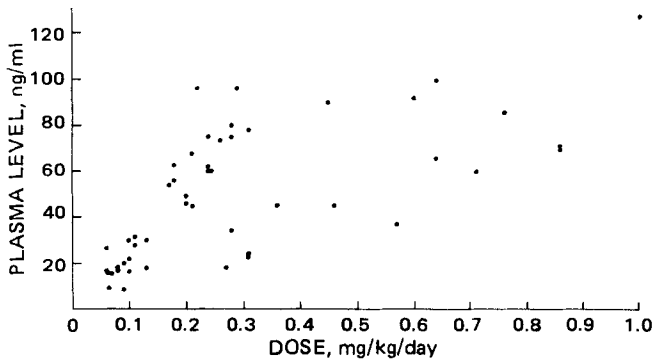


Figure 1—*Steady-state plasma levels of clonazepam in 15 subjects, each of whom had been treated chronically with at least two different doses of the drug.*

of clonazepam. Several factors probably contributed to this result. First, it was not known at what time the blood samples were drawn relative to the time the patient took the last dose of clonazepam. Although the half-life of clonazepam ranges from 19 to 39 hr, somewhat elevated plasma levels would exist for a few hours following each dose (6). Second, every patient was receiving concomitant anticonvulsants, and their effect on the disposition of clonazepam is unknown. Third, the compliance of each patient for the prescribed dosage regimen was unknown.

Although there is undoubtedly some correlation between plasma level and dose, no attempt has been made to define it statistically in this instance since such a definition could be misleading in view of the lack of controlled experimental conditions. The data, however, realistically illustrate the range of plasma clonazepam levels that may be encountered in the clinical situation.

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[5] Statistical analyses were performed by Mr. T. Lewinson, Hoffmann-La Roche Inc., Nutley, NJ 07110.