Production and Characterization of Specific Antibody for Radioimmunoassay of Procainamide

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Abstract □ The production and characterization of a specific antibody for use in the radioimmunoassay of procainamide are described. Cross-reactivity was measured by a nonequilibrium competitive procedure. Procainamide analog concentrations resulting in 50% inhibition were: procainamide, 1.59 nmoles/ml; *N*-acetylprocainamide, 3.55 nmoles/ml; a propyl analog of procainamide, 398 nmoles/ml; procaine, 316 nmoles/ml; lidocaine, >8000 nmoles/ml; and practolol, >16,000 nmoles/ml. Variations in the ability to inhibit binding of labeled procainamide were related to structural similarities and differences. The affinity constant of the antibody for procainamide was 2.9×10^8 liters/mole as measured from a Scatchard plot. The assay allows the direct measurement of procainamide in a 0.1-ml aliquot of diluted serum. The advantages of this method over currently available techniques are its sensitivity, specificity, and simplicity. Furthermore, prior extraction of serum samples is not required. As little as 1 ng of drug/ml of serum can be detected by this method. The accuracy and precision were determined by adding known amounts of procainamide to human serum and then assaying five replicates of each concentration. The within-day and between-day coefficients of variation were 2 and 5%, respectively. The proposed method was used to determine the serum concentration after an intravenous dose of procainamide. A comparison of the radioimmunoassay results with values obtained by a GLC procedure showed excellent agreement.

Keyphrases □ Procainamide—radioimmunoassay in human serum □ Radioimmunoassay—procainamide in human serum □ Antiarrhythmic activity—procainamide, specific antibody production and characterization

Procainamide (I) is a widely used antiarrhythmic with a narrow therapeutic range (1). Since procainamide is very potent, the choice of the proper dosage is critical. Variation in the dose requirement of patients appears to be due mainly to individual differences in procainamide absorption, distribution, and elimination (2, 3).

Determination of plasma concentrations of I has been studied using spectrometric (4), GLC (5), and high-pressure liquid chromatographic (6) procedures. However, various shortcomings exist. A radioimmunoassay for I could provide more than a 1000-fold increase in sensitivity as well as much greater specificity over those levels offered by existing procedures. Radioimmunoassay is based on competition between antigenic determinants of the labeled and unlabeled antigens for a limited number of specific antibody binding sites. Anything that competes for such sites, or in any other way affects the binding of labeled antigen, will influence the results.

The establishment of a practical radioimmunoassay procedure for a particular compound of clinical significance depends, among other factors, on the production of a high affinity antiserum with little cross-reactivity with compounds of similar structure. An ideal antibody is one that reacts with only one species of the molecule (*i.e.*, the antigen). However, antibodies tend to cross-react with compounds other than the specific antigen if there are certain structural similarities. The greater the ability of an antibody to distinguish between minute structural differences, the less is the tendency to cross-react and the greater is the specificity of the antibody. A radioimmunoassay for I is described, with particular emphasis on antibody specificity.

EXPERIMENTAL

Hapten–Protein Condensation—Three drug–protein derivatives were prepared by coupling I[1] to bovine serum albumin[2], egg albumin[3], and rabbit serum albumin[2], respectively. Procainamide was conjugated mainly through the tyrosyl residues of bovine serum albumin *via* a diazotization and condensation procedure (7, 8). Compound I (150 mg) was dissolved in 2 ml of distilled water at 2°, and the pH was adjusted to 9 with 1 *N* NaOH.

Nitrous acid was prepared by mixing 40 mg of sodium nitrite, in 5 ml of ice-cold distilled water, with 1 ml of 1 *N* HCl. This solution was added immediately to the solution of I, and diazotization was allowed to proceed for 15 min with occasional stirring. The diazotized drug solution was added in 1-ml increments to the protein solution (1 g of protein in 50 ml of ice-cold 0.04 *N* NaOH), and the pH was adjusted to 8.5. The mixture was stirred to eliminate any local excess of acid. The pH of this mixture was checked at 15-min intervals for 2 hr and was finally adjusted to 8.5 with 0.1 *N* NaOH. The solution was incubated overnight at 5° to allow completion of the coupling reaction.

The conjugation mixture was dialyzed against distilled water over 2–3 days, thus allowing for the removal of unreacted low molecular weight materials. The purified drug–protein solution was lyophilized and stored frozen in small quantities. The spectrometric analysis of the conjugate (I–bovine serum albumin) indicated that the molar ratio of procainamide to bovine serum albumin was between 20 and 22.

Radioiodination—Radioiodinated antigen was prepared by conjugation of I to rabbit serum albumin with subsequent iodination of the complex by the chloramine-T procedure (9) using 2 mCi of carrier-free sodium [^{125}I]iodide[4]. The I–rabbit serum albumin (2 mg in 0.5 ml of phosphate buffer, pH 7.5) was added to 2 mCi of sodium [^{125}I]iodide (in 0.5 ml of phosphate buffer) and mixed with 2 mg of chloramine-T[5] in 0.5 ml of iodination buffer. After 2 min of incubation, 4 mg of sodium metabisulfite in 0.5 ml of phosphate buffer was added to reduce both chloramine-T and any unreacted iodine. Carrier potassium iodide (5 mg in 0.5 ml of buffer) then was added.

This mixture was incubated at room temperature for 2 min and then was purified by gel filtration in a 0.9 × 15-cm G-25 Sephadex column[6] equilibrated with the iodination buffer. The total iodination mixture (2.5 ml) was added to the column with a plastic disposable syringe, and the column eluate was collected in 0.5-ml fractions, which were counted to determine the radioactivity profile. The radiochemical purity and percent labeling yield were measured, and the specific activity of ^{125}I-labeled I–rabbit serum albumin was calculated (1.90 Ci/mmole of I).

Preparation of Anti-I Serum—The drug–protein conjugate (I–bovine serum albumin) was antigenic when female albino rabbits were given several injections of 0.5–2 mg of complex dissolved in phosphate-buffered saline (pH 7.5) emulsified in a 1:2 ratio of Freund's complete adjuvant[7].

Booster shots were given at 4–5 week intervals, and blood samples were collected 10 days after each booster *via* the marginal ear vein. Development of the drug-specific antibody was followed qualitatively and quantitatively by use of a microgel immunodiffusion technique (10, 11). After 5–6 months, antiserum dilutions of 1:500 and 1:1000 yielded 50% binding of 4 ng of I.

[1] E. R. Squibb & Sons, Princeton, N.J.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Fisher Scientific Co., Fair Lawn, N.J.
[4] New England Nuclear, Boston, Mass.
[5] J. T. Baker Chemical Co., Phillipsburg, N.J.
[6] Pharmacia Laboratories, Piscataway, N.J.
[7] Difco Laboratories, Detroit, Mich.

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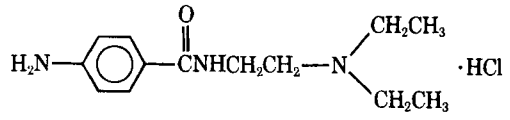

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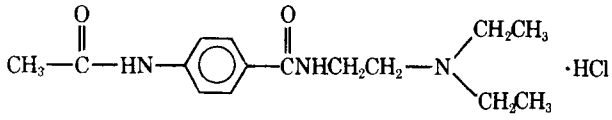

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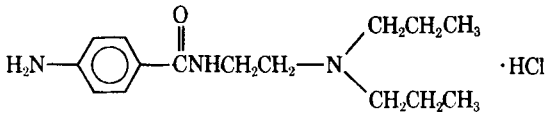

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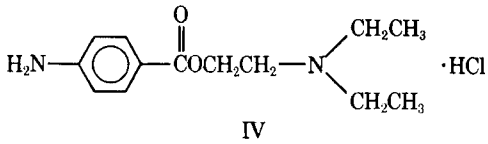

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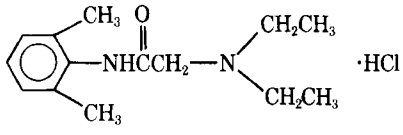

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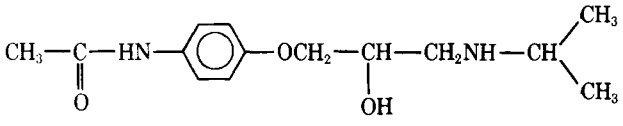

VI

Specificity Analysis—Several antiarrhythmic and cardiac depressants were selected as potential cross-reacting compounds on the basis of their structural similarity with I or because they are coprescribed with I. The compounds selected (shown as their hydrochlorides for I–V) were: *N*-acetylprocainamide[8] (II), a propyl analog of procainamide[1] (III), procaine[2] (IV), lidocaine[9] (V), and practolol[10] (VI).

Cross-reactivity of the antibody was measured by a nonequilibrium competitive procedure: 100 μl of the cross-reacting antigen in the range of 10^{-8}–10^{-3} *M* was mixed with 50 μl of the properly diluted antiserum in a 0.5-ml disposable polypropylene test tube[11] and incubated for 0.5 hr at 5°. Then 10 μl of the radioiodinated antigen (equivalent to 4 ng of the tracer) was added, and the mixture was incubated for 1 hr at 5°. Finally, 100 μl of the second antibody[12] (goat anti-rabbit immunoglobulin G) was added and the tubes were incubated for an additional hour at 5°.

The tubes then were centrifuged for 15 min at 3000 rpm, and the free (supernatant) and bound (precipitated) fractions were separated. The percentage of the iodinated antigen bound to the antibody was calculated in the presence (% B_p) and absence (% B_0) of the unlabeled cross-reacting molecules (12). The percentage of inhibition was calculated from:

$$\text{percentage of inhibition} = \left(\frac{\%\ B_0 - \%\ B_p}{\%\ B_0}\right) \times 100 \qquad \text{(Eq. 1)}$$

The percentage of inhibition was plotted as a function of the logarithm of the cross-reactant concentration (Figs. 1 and 2). The concentration resulting in 50% inhibition of binding with 4 ng of the radiolabeled antigen in a total volume of 260 μl was measured from the graph.

Radioimmunoassay—Procainamide standards were prepared by dilution of a 0.2-mg/ml stock solution in 0.05 *M* phosphate-buffered saline, pH 7.5. Duplicate standards, 0–200 ng, were prepared in the phosphate-buffered saline.

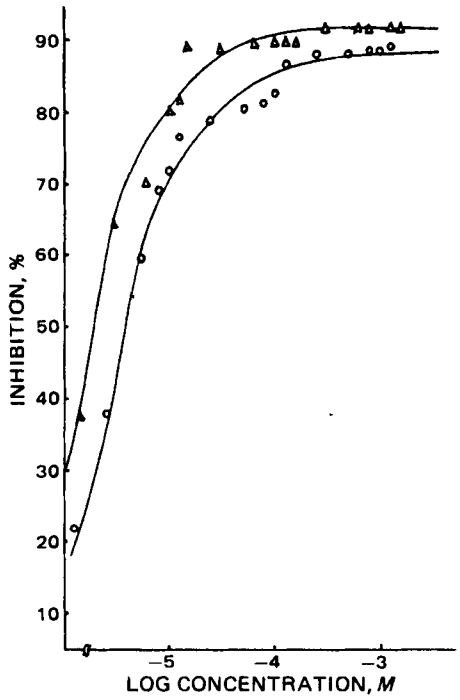

Figure 1—*Competitive inhibition of binding of 4 ng of radioiodinated procainamide by the prior addition of an increasing concentration of unlabeled I (△) or II (○).*

Antiserum was diluted (1:500–1:1000) with the assay buffer to yield 40–60% binding with labeled hapten. Then a 0.1-ml aliquot of diluted antibody was used per tube for the assay. The radioiodinated antigen also was diluted in 0.05 *M* phosphate-buffered saline so that a 0.05-ml aliquot had an activity of 35,000–40,000 cpm. Radioisotopic purity was checked periodically by paper chromatography, using 75% methanol for development. An appropriate dilution of goat anti-rabbit immunoglobulin G was used as the precipitating antibody (0.15 ml/tube). An equilibrium assay procedure was used.

Table I is an outline of the radioimmunoassay protocol. The procedure was as follows. The standards and unknowns were mixed with the radioiodinated antigen, buffer, and the antiserum in 6 × 50-mm disposable culture tubes[13], which were placed inside regular 12 × 75-mm disposable

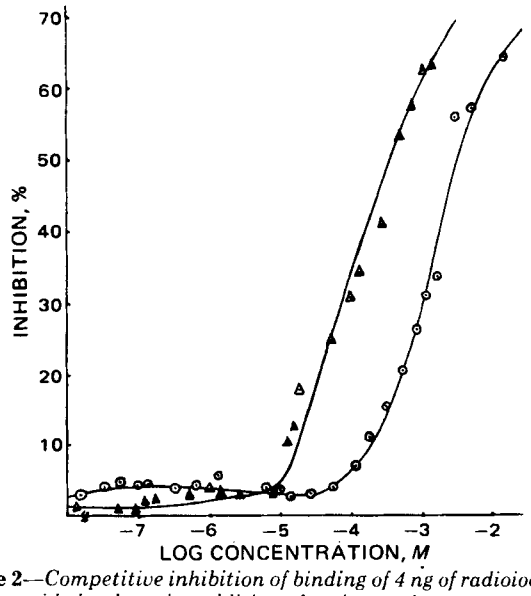

Figure 2—*Competitive inhibition of binding of 4 ng of radioiodinated procainamide by the prior addition of an increasing concentration of unlabeled III (△) or IV (⊙).*

[8] Arnar-Stone Laboratory, Mount Prospect, Ill.
[9] Astra Pharmaceutical Products, Worcester, Mass.
[10] Stuart Pharmaceutical Co., Wilmington, Del.
[11] Brinkmann Instruments, Westbury, N.Y.
[12] Miles Research Products, Elkhart, Ind.
[13] Corning Glass Works, Corning, N.Y.

Table I—Procainamide Radioimmunoassay Protocol and Volumes of Reagents Used

Tube Number	Description	Cold Procainamide, ml	Buffer, ml	Iodinated Procainamide, ml	Antiserum, ml	Second Antibody, ml	Total Volume, ml
1, 2	Zero standard	0.1	0.1	0.05	0.1	0.15	0.4
3, 4	10-ng standard	0.1	—	0.05	0.1	0.15	0.4
5, 6	20-ng standard	0.1	—	0.05	0.1	0.15	0.4
7, 8	40-ng standard	0.1	—	0.05	0.1	0.15	0.4
9, 10	60-ng standard	0.1	—	0.05	0.1	0.15	0.4
11, 12	80-ng standard	0.1	—	0.05	0.1	0.15	0.4
13, 14	100-ng standard	0.1	—	0.05	0.1	0.15	0.4
15, 16	120-ng standard	0.1	—	0.05	0.1	0.15	0.4
17, 18	140-ng standard	0.1	—	0.05	0.1	0.15	0.4
19, 20	160-ng standard	0.1	—	0.05	0.1	0.15	0.4
21, 22	200-ng standard	0.1	—	0.05	0.1	0.15	0.4
23, 24	Total count	—	0.350	0.05	—	—	0.4
25, 26	"Nonspecific binding"	—	0.200	0.05	—	0.15	0.4
27, 28	Unknowns (0.1 ml)	—	—	0.05	0.1	0.15	0.4

culture tubes for convenience in handling and centrifugation. All tubes were vortexed for 2–3 sec and then incubated for 3 hr at 25°. Then 0.15 ml of the second antibody was added to all tubes, which were vortexed again for 3 sec.

After centrifugation of the tubes at 3000 rpm for 20 min, the supernate (free fraction) was separated from the precipitate (bound fraction) using a disposable pasteur pipet[14]. The activity of the free and bound fractions was counted using a γ-scintillation spectrometer[15]. The procainamide concentration was plotted as a function of the total-to-bound radioactivity ratio. However, the data also can be processed by use of a computer program (13). The unknown concentrations were measured by interpolation.

Sensitivity and Detectability—Sensitivity was defined by Horwitz (14) as the amount of measured response per unit of stimulus (*i.e.*, the slope of the response curve). Sensitivity should be evaluated from a linear response plot, because a linear dose–response curve gives a constant value for sensitivity. Sensitivity of the procainamide radioimmunoassay system was calculated as the reciprocal of the slope of the dose–response plot (Fig. 3). The detectability or detection limit of a radioimmunoassay procedure is defined as the minimum concentration of the unlabeled antigen that provides a measurable response under defined conditions (14). The detection limit of the system, estimated as the reciprocal of the affinity constant, was 4.8×10^{-9} *M*.

Precision—The precision of the system was determined by addition of known amounts of I to procainamide-free human serum and then assay of five replicates of each concentration (Table II).

Human Study—A healthy, 26-year-old male subject was given an intravenous dose of I (461 mg/30 ml) over 10 min by use of an infusion pump[16]. Blood samples were collected at several postinfusion intervals (Table III). The serum was separated and kept frozen until it was analyzed.

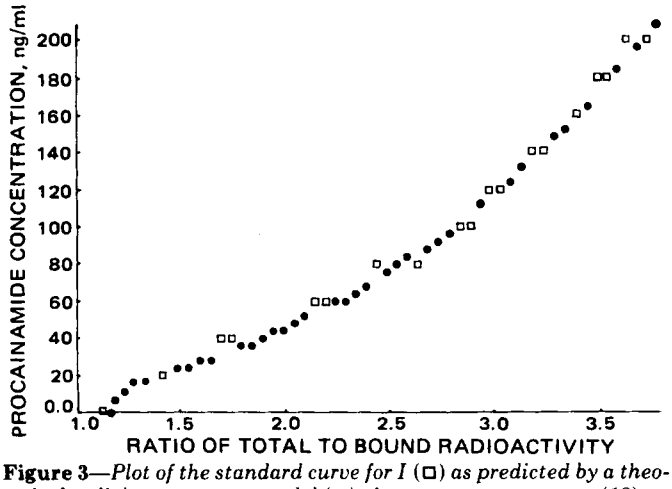

Figure 3—*Plot of the standard curve for I* (□) *as predicted by a theoretical radioimmunoassay model* (●) via *a computer program* (*13*).

[14] Fisher Scientific Co., Pittsburgh, Pa.
[15] Model 5220, Packard Instrument Co., Downers Grove, Ill.
[16] Harvard Apparatus, Hillis, Mass.

Table II—Estimation of Procainamide in Human Serum by Radioimmunoassay

Procainamide Added, ng	Procainamide Measured, ng	Mean ± *SE*	Ratio of Assayed to Added
5.00	4.85	4.92 ± 0.063	0.984
	4.72		
	4.96		
	5.10		
	4.95		
10.0	9.82	9.96 ± 0.102	0.996
	10.2		
	9.65		
	10.2		
	9.96		
15.0	14.5	14.9 ± 0.102	0.993
	15.1		
	14.9		
	14.9		
	15.0		
20.0	20.1	19.9 ± 0.081	0.998
	19.8		
	19.9		
	20.2		
	19.8		
25.0	24.8	24.8 ± 0.136	0.992
	25.1		
	24.5		
	24.6		
	25.2		

GLC Assay—Procainamide was extracted from the serum with methylene chloride as described by Elson *et al.* (15) and was analyzed by the GLC[17] method of Simons and Levy (16). Each serum sample then was analyzed for procainamide concentration by the radioimmunoassay. The results were compared with values obtained from the GLC procedure (Table III).

RESULTS AND DISCUSSION

The results of the cross-reactivity analysis of the procainamide antibody are summarized in Table IV. Inhibition plots of the cross-reacting analogs are shown in Figs. 1 and 2.

Since procainamide is a low molecular weight drug, *i.e.*, <500, the antiserum usually recognizes the whole structure rather than parts of it. Therefore, the antibody can detect slight changes in molecular structure from that of the immunizing antigen (I). This characteristic of the antibody is more pronounced in the nonequilibrium competitive procedure (Table IV). The results of the cross-reactivity experiments indicate that any modifications in the basic benzamide structure of I (*i.e.*, procaine and lidocaine) diminish the antibody binding stimulated by the I–bovine serum albumin complex. Also, the presence of the tertiary nitrogen and the carbon chain length on this nitrogen influence the immunoreactivity of the molecule.

[17] Series 2400, Varian Aerograph, Palo Alto, Calif. The gas chromatograph was equipped with a flame-ionization detector and had a 0.914-m glass column packed with 10% OV-7 on 100–120-mesh WH-P. The temperatures of the injector, column, and detector were 260, 245, and 280°, respectively.

Table III—Comparison of Procainamide Levels as Measured by Radioimmunoassay and GLC

Postinfusion Time, min	Serum Procainamide Levels, μg/ml	
	Radioimmunoassay	GLC
3	6.95	6.9
11	5.61	5.3
43	4.22	4.4
53	3.98	3.8
68	3.76	4.2
82	3.70	3.6
97	3.55	3.5
112	3.42	3.8
142	3.35	3.3
290	1.69	1.6

Table IV—Summary of Nonequilibrium Competitive Cross-Reactivity of Procainamide Antibody [a] with Structurally Related Compounds

Compound	Concentration for 50% Inhibition of Binding, $M \times 10^{-5}$	Concentration Ratio[b]	Molecular Weight
I	0.159	1.00	272.0
II	0.355	2.30	313.5
III	39.8	250	300.0
IV	316	1987	273.0
V	>800	>5000	272.0
VI	>1600	>10000	266.0

[a] Antibody raised against I-bovine serum albumin. [b] Compared to I.

Differences in 50% inhibition values for the cross-reacting compounds were indicated by inhibition curves with differing slopes. The antibody developed against the hapten differentiates between the antigen and its *N*-acetyl metabolite (II). Although the values of 50% inhibition for these two compounds differ only slightly, twice the concentration of II is required to compete equally with I for the antibody binding sites. In addition, the antibody recognizes the molecular differences between the immunizing antigen and its propyl analog. This analog concentration must be 250 times that of I to inhibit binding by 50%. In the case of procaine, this value is ~2000. The antibody appears to be very sensitive to changes in molecular structure.

The increasing value of 50% inhibition of II–VI parallels the degree of difference between the molecular structure of these compounds and I. Furthermore, the antibody was characterized with respect to its affinity constant by a Scatchard analysis. The calculated value was 2.9×10^8 liters/mole. The radioimmunoassay parameters were adjusted to optimum values. The standard curve showed excellent linearity over the range of 0–100 ng of I/ml. Therefore, patient samples should be diluted properly (about 100-fold) to fall within this concentration range. Dilution also reduces the degree of nonspecific binding. Correction for nonspecific binding was made by subtracting the activity of a nonspecific binding blank from both standards and unknowns.

The proposed method was characterized by evaluation of the sensitivity and detectability of the system, which were 0.04 division/ng of I and 1.3 ng of I/ml, respectively. The precision of the assay is good since the standard error of replicates was ≤1% of the mean value for each concentration measured (Table II). The ratios of the assayed procainamide to that added indicate an acceptable recovery.

The within-day and between-day precision were evaluated by duplicate measurements of identical samples on the same day and on consecutive days. The coefficient of variation, *CV*, was estimated from (17):

$$CV = \sqrt{\frac{\sum d^2}{2n}} \qquad \text{(Eq. 2)}$$

where:

$$d = \left[\frac{\text{highest value of each duplicate}}{\text{lowest value of each duplicate}} - 1\right] \times 100 \qquad \text{(Eq. 3)}$$

and n is the number of duplicate determinations. For the procainamide antibody, the within-day coefficient of variation ranged from 1.4 to 4.2% with a mean of 2.2%. The between-day coefficient of variation ranged from 2.9 to 9.1% with a mean of 5.6%. The coefficient of variation was measured over the concentration range of 0–200 ng/ml.

The validity of the assay was established further by comparing the values obtained by radioimmunoassay to values obtained from a GLC assay. The results (Table III) showed excellent agreement.

In summary, the described method is a sensitive and relatively simple microanalytical procedure, which can be performed directly on an aliquot of serum without prior extraction or purification. This radioimmunoassay procedure permits the simultaneous analysis of a large number of samples.

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