

Determination of a Tricyclic Antidepressant, Clomipramine (Anafranil), in Plasma by a Specific Radioimmunoassay Procedure

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A radioimmunoassay is reported for the tricyclic antidepressant drug, clomipramine (Anafranil, Geigy). Antisera generated in rabbits to clomipramine conjugated to bovine serum albumin at positions 10 and 11 were specific, cross reacting <5% with the pharmacologically active metabolite, desmethylclomipramine. Specificity was confirmed by the good agreement in titres observed when the samples were assayed with and without a pre-assay thin-layer chromatographic purification. Intra- and inter-assay variations were less than 8 and 13%, respectively, with a sensitivity of 175 ng/liter. Results obtained agreed well with those reported by other groups using double radioisotope derivative assays. The method has a high throughput rate, one technician can assay 200 samples in duplicate in a working week; and it is sufficiently precise, sensitive, and specific for use in routine monitoring of the drug in plasma and for checking patient compliance with dosage regimen.

Tricyclic antidepressant drugs have been widely used in clinical practice for many years. However, there still are no analytical methods suitable for monitoring these drugs in plasma in large clinical trials. Such methodology would be valuable because it is well known that concentrations of tricyclic drugs in plasma vary widely among patients who are on the same drug regimen (1).

Although there is some controversy as to the correlation between clinical response and concentrations in plasma (2, 3), it is generally agreed that plasma monitoring would permit individual optimization of dosage and would probably increase clinical efficacy and reduce side-effects.

A gas-chromatographic-mass spectrometric technique has been described for measuring clomipramine (4); although this would be an excellent reference method, the high capital cost and comparatively slow turnover of samples render it unsuitable for routine

sample analysis. Satisfactory assay methods based on the double-radioisotope derivative technique have been reported (5, 6), but the need to remove a methyl group before forming the radiolabeled derivative so complicates the method that one technician can only assay 30 samples per week.

Radioimmunoassay (RIA) combines the possibilities of high sample turnover with the required specificity and sensitivity (7), but the reported methods for RIA of tricyclic drugs have shown disappointingly low specificity (8, 9).

The present paper describes a RIA for clomipramine. Suitable choice of antigen and use of a nonpolar solvent for sample extraction gives the assay good specificity. The assay incorporates use of a [³H]clomipramine tracer, with separation of "free" and "bound" tracer by use of dextran-coated charcoal.

Materials and Methods

Immunization Procedure

Four New Zealand white rabbits, four months old, were given 25 to 30 intradermal injections of an emulsion of Freund's complete adjuvant (7 ml) and a suspension of 0.5 mg of the immunogen in isotonic saline (3 ml). A total of 2 ml of the emulsion was injected at four- to five-week intervals into each rabbit; 0.5 ml of *Bordetella pertussis* (*Hemophilus pertussis*) antigen was given at the first injection only (10). All rabbits produced sera with adequate titre after the third and subsequent injections.

Synthesis of Immunogen

Succinic anhydride (60 mg) and 40 mg of 10/11-hydroxyclopmipramine¹ were dissolved in freshly distilled anhydrous pyridine and kept in the dark at room

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¹ "10/11" is intended to indicate that the compound was synthesized via the 10,11-dehydro compound. The compound obtained is probably a mixture of the 10-hydroxy and the 11-hydroxy compounds.

temperature for one month. Reaction was then judged complete by thin-layer chromatography and by field desorption mass spectrometry: molecular ions or (M + 1) peaks were seen for clomipramine hemisuccinate, succinic anhydride, and succinic acid, but not for clomipramine. Pyridine was removed under nitrogen and then over phosphorus pentoxide at reduced pressure. Bovine serum albumin was coupled to the residual gum by standard procedures (11, 12). Incorporation of clomipramine into the immunogen was 9.5 mol/mol, as assessed by ultraviolet spectrometry.

Reagents

Rabbit antiserum to clomipramine, stored diluted 20-fold in 1-ml aliquots, at -20°C .

Tracer [10, 11- $^3\text{H}_2$]clomipramine (spec. act. 39 kCi/mol) was prepared by the Radiochemical Centre, Amersham, U.K., from 10,11-dehydroclomipramine supplied by Ciba-Geigy Ltd, Basel, Switzerland.

The following reagents, previously described for use in a testosterone radioimmunoassay (13), were used without modification:

Phosphate-buffered saline, pH 7.4.

Assay buffer: Phosphate-buffered saline containing 1 g of gelatin per liter.

Dextran-coated charcoal suspension.

Additional reagents used were:

Petroleum ether ($40-60^{\circ}\text{C}$), purchased from Eastman Kodak, Rochester, N.Y., was purified by washing first with concentrated sulfuric acid, then with distilled water, drying over calcium chloride, and refractionating.

Saline/ammonia solution: Sodium chloride (9 g) and (d_{25}^{25} 0.880) ammonia solution (20 ml) were diluted to 1 litre with distilled water.

Liquid scintillator: 2,5-Diphenyloxazole, 5 g, was dissolved in a mixture of 500 ml of the surfactant Triton X-100 and 1 litre of toluene. (All scintillation-grade reagents were from Koch-Light Laboratories, Colnbrook, U.K.).

Clomipramine stock standard, 50 mg/litre: Five milligrams of clomipramine hydrochloride was dissolved in absolute ethanol and diluted to 100-ml with ethanol. The solution was kept at room temperature overnight before use and subsequently was stored at 4°C , protected from light. This stock solution was stable for at least three months.

Clomipramine standards, in absolute ethanol, were prepared daily from stock. The stock standard was twice diluted 0.1 ml to 5 ml to provide a dilute stock standard with a concentration of $20\ \mu\text{g/litre}$. Serial dilution of this provided working standards, 2, 4, 6, 8, 10, 12, 16, and $20\ \mu\text{g/litre}$ (20, 40, 60, 80, 100, 120, 160, and 200 pg in the final assay tube).

Working tracer solution: Stock [^3H]clomipramine (50 μl) in ethanol was added to 15 ml of assay buffer. This solution was prepared daily.

Sample dilution. This will depend on the expected concentration in plasma. When subjects have been maintained on a high dose ($>200\ \text{mg/day}$), concentra-

tions in plasma may exceed $500\ \mu\text{g/litre}$ and the sample will be greatly diluted, e.g., 50 μl of plasma + 15 ml of saline/ammonia. Conversely, in a subject who is taking a placebo tablet and who should have zero values, a dilution of 200 μl of plasma + 0.5 ml of saline/ammonia is appropriate. When the sample protocol is inadequate, the sample is assayed at several dilutions.

Assay Procedure

To labeled $10 \times 75\ \text{mm}$ glass tubes, add plasma and dilute with the appropriate volume of ammonia/saline solution. Mix well and transfer 100- μl aliquots to fresh tubes. Add 1 ml of petroleum ether and mix on a multivortex-type shaker for 10 min. Transfer the tubes to a freezer for at least 30 min, then decant the upper phase into labeled tubes; discard the aqueous layer. Add 10- μl aliquots of each of the standard ethanolic solutions to labeled tubes, then add 1 ml of petroleum ether. Evaporate the solvent under nitrogen in a water bath at 30°C and add 100 μl of antiserum, diluted 400-fold, to all tubes. Cover, leave for 1 h at room temperature, then add 100 μl of clomipramine tracer to all tubes and incubate at 30°C for 1 h. Transfer all tubes to an ice-bath for 20 min and add 0.5 ml of dextran-coated charcoal suspension to all tubes. Mix well, and allow to stand for 15 min in the ice-bath. Centrifuge at 4°C and transfer 0.5 ml aliquots of the supernate to labeled scintillation vials. Add 5.0 ml of scintillation fluid and count the radioactivity in a suitable counter.

The standard curve was obtained, and the concentration of clomipramine in unknown samples was calculated by using the four-parameter-fit model of Rodbard and Hutt (14). This method has several advantages: it does not depend on linearity in the log-logit plot, the output is in both numerical and graphical form, and the parameters have real physical significance, which can be of great value when trouble-shooting.

Results

Analytical Variables

The standard curve is shown in Figure 1. Replicate assays ($n = 20$) established that the precision was satisfactory at all fixed points. Logit transformation resulted in a nearly linear response to clomipramine from 10 to 200 pg, with a correlation coefficient of -0.99 .

Specificity. The cross reactivity of available metabolites of clomipramine and of certain other tricyclic antidepressants was assessed by the criteria of Abraham (15) and the data are shown in Table 1.² The significant cross reaction of other antidepressant drugs, such as amitriptyline, is not a practical difficulty because these

² Other metabolites of clomipramine are ring-hydroxylated phenolic derivatives and, to a lesser extent, compounds hydroxylated at positions 10 or 11. The ring-hydroxylated compounds are not available to us to measure the cross-reaction. Studies with the available derivatives of imipramine have demonstrated that, as expected, these phenolic compounds are separated by the thin-layer chromatographic system described below. The good agreement in titres achieved with and without this thin-layer chromatographic system indicates that these phenolic metabolites do not interfere in the assay.

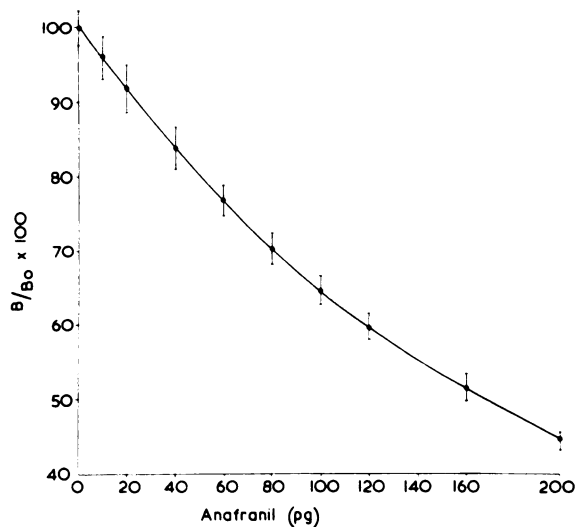


Fig. 1. Composite standard curve for 20 successive assays
The CV at each point (n = 20) is indicated

drugs are unlikely to be given concomitantly. The cross reaction of certain drugs commonly co-administered with clomipramine, such as chlordiazepoxide, nitrazepam, and diazepam, was negligible. The antisera showed the desired specificity with respect to the side-chain metabolites; cross reaction of desmethyl-clomipramine (4.1%) was low and that of didesmethyl-clomipramine (0.74%) negligible.

Sensitivity. The assay sensitivity is less than 10 pg, corresponding to a concentration in plasma of less than 0.2 µg/litre. The RIA appears to be 25-fold as sensitive as is the double-radioisotope derivative assay. Plasma samples from subjects not taking clomipramine invariably showed no detectable apparent clomipramine.

Precision. Three plasma pools of high, medium, and low titres were established. Aliquots (n = 30) of each pool were titred in one assay and these data used to assess intra-assay variance (Table 1). These pools were

Table 1. Cross Reactions of Anti-clomipramine Serum R₃b₃

Compound (and trade name)	Cross-reaction %
Clomipramine (Anafranil)	100
10/11-Hydroxyclopmipramine	114
Desmethylclomipramine	4.1
Didesmethylclomipramine	0.74
Imipramine (Tofranil)	20
2-Hydroxyimipramine	10.6
Desmethylimipramine (Pertofran)	0.55
2-Hydroxydesmethylimipramine	0.16
Prothiaden	19
Maprotiline (Ludiomil)	0.34
Amitriptyline (Lentizol)	7.0
Nortriptyline (Aventil)	0.55
Chlorpromazine (Largactil)	13.0
Chlordiazepoxide (Librium)	<0.02
Carbamazepine (Tegretol)	<0.02
Nitrazepam (Modadon)	<0.02
Diazepam (Valium)	<0.02

Table 2. Intra- and Inter-assay Variance in High-, Medium-, and Low-Concentration Quality-Control Samples

	High	Medium	Low
<i>Intra-assay</i>			
Mean	125.9	72.09	28.87
SD	7.48	5.13	8.42
n	30	30	30
CV%	5.94	7.12	29.1
<i>Inter-assay</i>			
Mean	131.4	70.56	24.98
SD	16.22	8.58	8.24
n	46	48	47
CV, %	12.3	12.2	33.0

Table 3. Analytical Recovery of [³H]Clomipramine from Plasma

Anafranil (pg/assay tube)	Recovery (% ± SD)
10	96.6 ± 2.1 (n = 11)
250	95.5 ± 2.2 (n = 12)

subsequently split into aliquots, stored at -20 °C, and used as quality controls for subsequent routine assays, giving the inter-assay variance shown in Table 2. The standard deviation is approximately constant, resulting in a higher coefficient of variance for the lower-titre samples. Samples having this titre are re-assayed at a higher concentration.

Analytical recovery. Tritium-labeled clomipramine was added to samples with known titres corresponding to both ends of the standard curve, and the samples were incubated overnight. Recovery on extraction was excellent (Table 3).

Comparison with another assay technique. Samples from a previous clinical trial, for which results from the double-radioisotope derivative technique were available, were re-assayed by the present method. The correlation is shown in Figure 2. The small but significant trend to lower values in the RIA may equally well reflect losses on storage rather than a true assay difference, because more than 12 months elapsed between the double radioisotope derivative assay and the subsequent RIA.

Further Validation

The effect of a pre-assay thin-layer chromatography step was assessed in samples from a patient who had been on a high dosage regimen for 14 days and would be expected to have high metabolite concentrations in his plasma. Plasma extracts, containing [³H]clomipramine to monitor recovery, were run on silica pre-coated glass plates (E. Merck) in the solvent system benzene/ethyl acetate/triethylamine (7/2/1 by vol) in which clomipramine had an R_F of 0.72. Areas corresponding to clomipramine were scraped off into glass tubes, 1 ml of saline/ammonia was added, and the mixture was extracted with 3 ml of ethyl acetate. The extract was

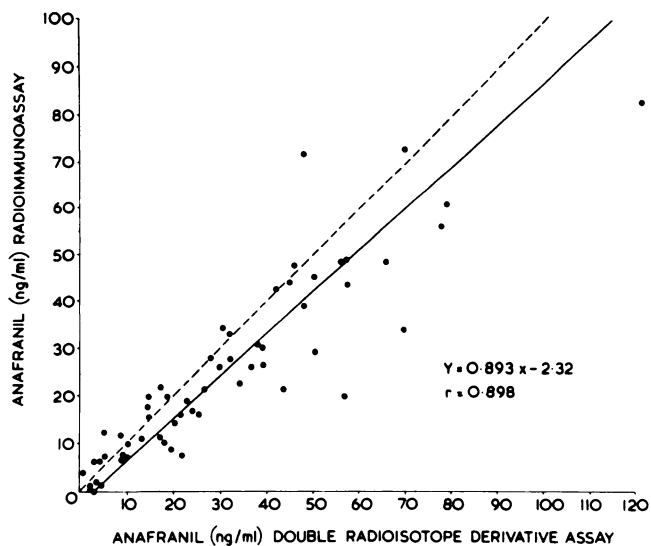


Fig. 2. Comparison of results for clomipramine obtained by the present RIA method and by a double radioisotope derivative method
Lines of equivalence (---) and best fit (—) are shown

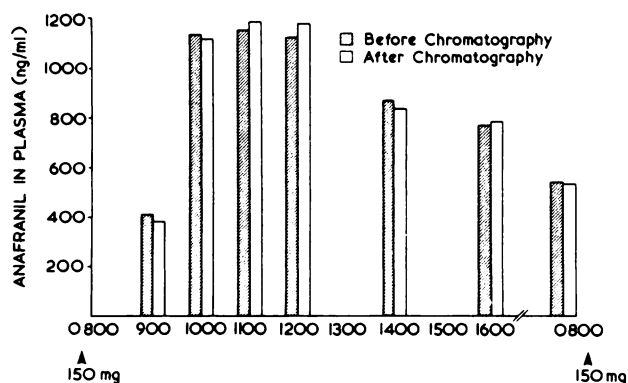


Fig. 3. Effect of a pre-assay thin-layer chromatographic purification on clomipramine (Anafranil) titres found in plasma
Subject was a 54-year-old man on a regimen of 150 mg/day. Numbers on the abscissa are clock time of sample collection

centrifuged to remove silica particles and aliquots taken for RIA and to monitor recovery (routinely $\geq 75\%$). From the data (Figure 3) it is apparent that there is no significant decrease in titre after the chromatographic step, confirming the specificity of the method.

Capacity of the Procedure

One technician can comfortably perform two runs of 20 samples in duplicate per day, giving the assay a routine capacity of at least 200 samples per week.

Discussion

Tricyclic drugs such as clomipramine are lipophilic bases and thus are more concentrated in organs such as the lung, and concentrations in plasma are correspondingly low. The required assay sensitivity, combined with high sample throughput, is well suited to a radioimmunoassay procedure with a specific antiserum, which obviates the need for pre-assay purification procedures. Highly specific antisera are obtained only by the correct choice of immunogen. Because desmethylclomipramine is known to be a major me-

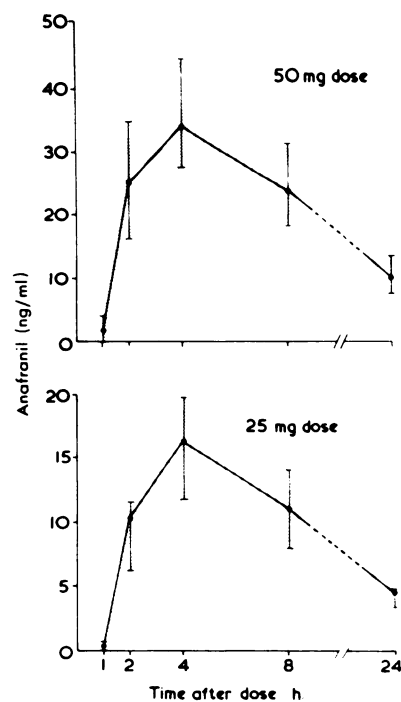


Fig. 4. Plasma clomipramine (Anafranil) concentration ($\mu\text{g/litre}$) attained after a single dose of 25 or 50 mg
Mean and first and third quartiles are shown

tabolite with pharmacological activity, it was necessary to conjugate clomipramine to an immunogenic protein through a site distal to the side chain. The excellent specificity achieved by antisera for clomipramine-10/11-hemisuccinate/bovine serum albumin conjugate indicated the correctness of this approach. The alternative approach, to conjugate the drug through a ring hydroxyl group, was less attractive because ring hydroxylation is thought to be a major metabolic route. Concentrations in serum, measured in healthy volunteers after administration of 25 or 50 mg of clomipramine hydrochloride (Figure 4), agree well with previous studies of the pharmacokinetics of clomipramine (16-18).

The assay allows determination of clomipramine in plasma during the course of treatment, but its unprecedented sensitivity further allows it to be determined at the low concentrations appearing in plasma after a single dose.

The assay has been in use for over six months. No technical difficulties have appeared.

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