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PLASMA PROTEIN BINDING OF TRICYCLIC ANTIDEPRESSANTS IN MAN

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Abstract—The binding of various tricyclic antidepressants to human plasma and the effects thereupon of other drugs were studied by an ultrafiltration technique utilizing labeled compounds. At a total concentration of 0·29 μ/ml the percentage of unbound desmethylimipramine (DMI) was found to be 9·5 $\pm$ 1·4 in 41 individuals. The unbound fraction of DMI in plasma increased only twofold when the total concentration of the drug was increased a thousand times. The degree of binding over the entire range of therapeutic plasma drug concentration was relatively constant.

The binding of various tricyclic antidepressants was compared at a drug concentration of 1·1 μM. The percentage of unbound drug was for nortriptyline (NT) 5·5 $\pm$ 0·6, for amitriptyline (AT) 3·6 $\pm$ 0·8, for imipramine (I) 4·2 $\pm$ 0·8, for protriptyline (PT) 8·0 $\pm$ 0·6 (tested at a conc. of 7·7 μM) and for Leo 640, an imipramine analogue, 0·7 $\pm$ 0·7. The acetyl derivatives of DMI, NT and PT were much more bound than the parent compounds. The addition of NT, PT or AT in a "therapeutic" concentration of 0·2 μg/ml didnot displace DMI, nor did chlorpromazine in supratherapeutic concentration. Diphenylhydantoin was found to displace DMI, NT, PT, AT and particularly I.

THE PLASMA protein binding of acidic drugs has been extensively studied but relatively little knowledge of the binding of basic drugs is available. The present study was undertaken to evaluate the plasma protein binding of several tricyclic antidepressants, a group of highly lipophilic basic drugs. This investigation was brought about by the recent emphasis on the possible correlation between the total plasma level of psychotropic drugs and/or metabolites and their pharmacologic and toxic effects.[1] A major obstacle to this concept would be the occurence of marked individual differences in the protein binding of psychotropic drugs.

The significance of the plasma level of unbound drug was amply demonstrated in recent experiments by Aggeler *et al.*,[2] who reported an increased toxicity of warfarin in patients simultaneously treated with phenylbutazone. The latter displaced the anticoagulant from its binding sites on plasma albumin resulting in an increased concentration of unbound warfarin in the plasma without a significant change in the total plasma level. In the case of basic psychotropic drugs, which are often used in combinations, no systematic study of possible competition with other drugs for common protein binding sites has appeared.

We have studied the binding of various tricyclic antidepressants to human plasma (*in vitro*) and the effects thereupon of other drugs. Heparinized human plasma was

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used rather than albumin because it is not known to which molecular species these drugs are bound.

MATERIAL AND METHODS

Plasma. The plasma from blood of human volunteers was obtained by centrifugation and removal of the formed elements after addition of heparin (500 U/500 ml blood). The plasma was used fresh.

Drugs. The drugs used are described in Table 1 and Fig. 1. The radiochemical

TABLE 1. LABELED DRUGS USED IN THE STUDY

Drug (abbreviation)	Position of label	Specific activity mc/m-mole	Source	Thin-layer system
^{14}C-amitriptyline (AT)	*N*-methyl	4·2	Hoffmann-La Roche & Co. (gift)	a,b
^{3}H-desmethylimipramine (DMI)	10,11	14·7	AB Leo, Hälsingborg Sweden (gift)	c,d
Leo 640*	10,11	14·7	AB Leo, Hälsingborg Sweden (gift)	c,d
^{14}C-imipramine (I)	10,11	8·05	The Radiochemical Centre, Amersham, England	a,e
^{3}H-nortriptyline (NT)	*N*-methyl	88	Eli Lilly S.A. Geneva, Switzerland (gift)	a,e
^{14}C-protriptyline (PT)	5,10,11	0·75	Merck Sharp & Dohme (gift)	a,b

*An imipramine analogue with the systematic name *N*-methyl-*N*-(4-chloro-benzoyl-methyl)-3-(10, 11-dihydro-5-H-dibenz (b,f) azepin-5-yl) propylamine.

(a) Benzene/ethyl acetate/ethanol/conc. ammonia, 50:50:10:5. Adsorbent: $Si0_2GF_{254}$
(b) Ethylene dichloride/ethanol, 5:1, Adsorbent: $Al_2O_3GF_{254}$
(c) Diethyl ether/ethylene dichloride/diethylamine. 12:12:1. Adsorbent: SiO_2GF_{254}
(d) Chloroform/acetone/diethylamine, 5:4:1. Adsorbent: SiO_2GF_{254}
(e) Methanol/2N ammonia, 4:1. Adsorbent: SiO_2GF_{254}

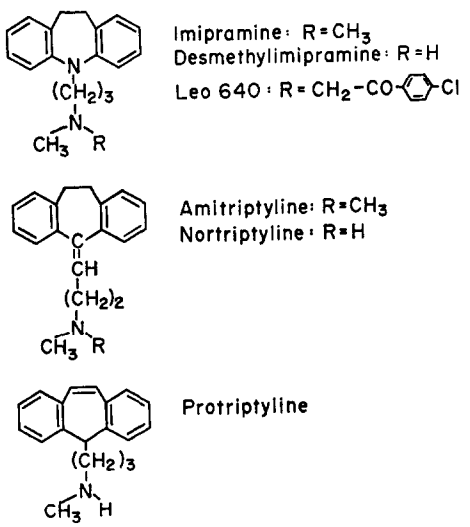

FIG. 1. Structural formulae of the tricyclic antidepressants used in the study.

purity was established for each of the labeled drugs using at least two different thin-layer chromatographic systems. The adsorbents and solvent mixtures used are also summarized in Table 1. The location of the radioactive spots on the plates was ascertained either by scraping off zones of adsorbent into vials for assay in a Packard liquid scintillation spectrometer or by scanning the plates in a Packard chromatogram scanner. Stock solutions of the drugs were prepared in 0·1 N HCl.

N-(^{3}H-acetyl)-amide derivatives of desmethylimipramine, nortriptyline and protriptyline were synthesized merely by scaling up the analytical procedure by Hammer and Brodie[3] for determination of desmethylimipramine (DMI) in body fluids which utilizes an *in vitro* acetylation with ^{3}H-acetic anhydride (S.A. 100 mC/m-mole). The products had a S.A. of 50 mC/m-mole and were found to be radiochemically pure in the thin layer chromatographic systems used by theses authors.

Ultrafiltration

Plasma was incubated at room temperature (24–26°) with varying amounts of isotope labeled tricyclic antidepressants for 60 min. In the experiments where the displacing effects of other drugs were studied, the drug to be tested was added immediately before or after the labeled drug. All drugs were added in the smallest possible volume, usually 10–25 μl, to minimize the dilution of the plasma (the pH of the plasma was not affected by adding the drugs). Twenty-five cm lengths of Visking dialysis tubing (24 mm folded width) were soaked in water for 30 min. The excess water was removed by blotting, and one end of the bag securely knotted. Ten ml of the incubated plasma was placed in the bag which was then inserted in a 50 ml centrifuge tube. The upper end of the dialysis tubing was folded over the lip of the test tube so that the bottom of the bag was approximately 3 cm above the bottom of the tube. The bag was held in place by inserting a rubber stopper in the mouth of the tube. The tube was then centrifuged for 10 min at 800 *g* to remove the water from the pores of the dialysis tubing. The tubing was removed, wiped free of moisture on the external surface and replaced in a clean, dry centrifuge tube. This tube was again centrifuged at 800 *g* for 45–60 min, which was sufficient to collect 600–800 μl of ultrafiltrate. The centrifugation caused a temperature increase of 2–3°. and a change in plasma pH from 7.6 to around 8.1. This procedure is similar to that described by Schanker and Morrison.[4]

Assay of labeled drugs

Following centrifugation, two 250 μl aliquots of the ultrafiltrate were pipetted directly into polyethylene scintillation vials, 250 μl 2·5 N NaOH and 10 ml scintillation mixture (4 g PPO and 0·5 g POPOP dissolved in 1 l. of toluene) added, and the vials shaken for 30 min. Aliquots of the plasma inside the dialysis tubing were treated similarly. The radioactivity was determined in a Packard liquid scintillation spectrometer. With this procedure the drugs were extracted with the same recovery from plasma and ultrafiltrate and the resulting solutions showed no quenching compared to the original scintillation mixture.

Assay of non-labeled drugs

In similar experiments with unlabeled nortriptyline (NT) or protriptyline (PT), the drugs were determined in 500 μl aliquots by the method of Hammer and Brodie.[3]

Calculations

The percentage of unbound drug was calculated as 100 times the ratio between drug concentration in the ultrafiltrate and the plasma inside the dialysis bag *after* centrifugation. No corrections were found necessary for the volume occupied by the proteins in the plasma (between 6 and 7 per cent),[5, 6] or for the increase in concentration in the dialysis bag due to the decrease in volume (6–8 per cent) during the ultrafiltration. The approximate correction factors are namely 1/1·07 and 1·07 respectively and thus (in this particular case) cancel out each other.

In each centrifugation one sample was included with the studied drug added to phosphate buffer (0·1 M, pH 7·4) to measure the binding of the drug to the dialysis tubing. Corrections were made for this binding effect. The decrease in the ultrafiltrate concentration of DMI was 6 ± 3 per cent (mean ± S.D.). It was independent of concentration over the range 25–500 mμg/ml. For the other drugs the "bag binding" was as follows (n = 5): NT 6 ± 3 per cent; Leo 640 6 ± 2 per cent; imipramine (I) 4 ± 2 per cent; amitriptyline (AT) 2 ± 1 per cent and PT 1 ± 0·5 per cent. The correction for a x per cent bag binding was done by multiplying "per cent unbound" with the correction factor 100/100-x.

RESULTS

Effect of protein concentration on the binding of DMI and NT

The amount of DMI and NT bound to plasma proteins was determined in different dilutions of plasma in 0·1 M phosphate buffer, pH 7·4, keeping the concentration of drug (1·1 μM and 3·8 μM respectively) constant (Table 2). Plotting the ratio

TABLE 2. BINDING OF DMI AND NT AT DIFFERENT CONCENTRATIONS OF PLASMA

plasma (%)	unbound DMI (%)	unbound NT (%)
10	61·2	42·9
25	38·7	23·4
50	23·2	14·8
75	15·1	9·0
100	11·3	5·4

between the unbound and bound DMI (or NT) against the reciprocal of the concentration of the plasma gives a straight line through the origin. This indicates that at this very low drug concentration, where the probability of binding more than one drug molecule per molecule of protein is low, and the concentration of free (non-occupied) protein is approximately equal to the total protein concentration, a very simple correlation exists namely;

$$\frac{(\text{DMI})_{\text{unbound}} \cdot \ (\text{protein})}{(\text{DMI})_{\text{bound}}} = \text{constant}$$

Effect of temperature on the binding of DMI

The binding of DMI (1·1 μ M) was investigated at temperatures of 4, 20, and 30° and was found to decrease moderately with an increase in temperature (Table 3).

TABLE 3. EFFECT OF TEMPERATURE ON THE BINDING OF DMI

Temperature (°C)	4	20	30
% unbound DMI (± S.D)*	8·0 ± 0·5	9·5 ± 0·4	10·4 ± 0·7

*Each value is the mean of five determinations with five different plasmas.

Binding at different concentrations of DMI

The unbound fraction of DMI in plasma was found to increase only twofold when the total concentration of the drug was increased a thousand times from 0·286 μg/ml to 296 μg/ml (Table 4, plasma A).

Figure 2 shows a Scatchard plot of these data, where the ratio r/D has been plotted against r. D designates the unbound DMI concentration and r moles of DMI bound

TABLE 4. BINDING OF DMI AT SUPRATHERAPEUTIC (PLASMA A) AND THERAPEUTIC (PLASMA B) CONCENTRATIONS

	Plasma A						Plasma B						
Conc. DMI (μg/ml)	0·286	0·572	3·25	29·9	74·3	296	0·033	0·066	0·145	0·290	0·725	1·45	2·90
% unbound DMI	8·8	9·0	9·9	12·1	14·3	16·3	8·9	8·9	8·4	9·2	9·4	9·7	9·8

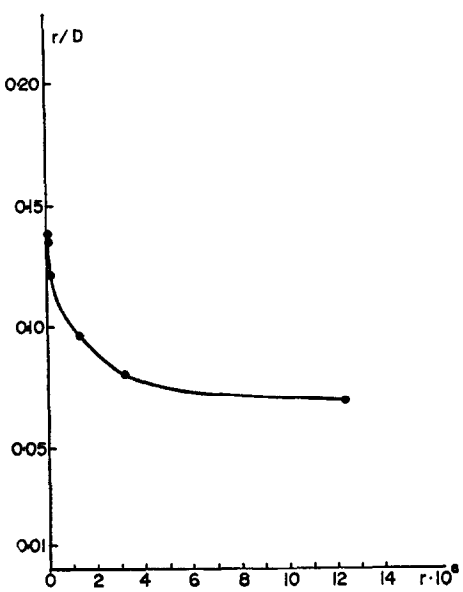

FIG. 2. Plasma protein binding of desmethylimipramine (DMI) at different concentrations. Scatchard plot of one of two experiments with similar results.
r = moles of DMI bound per gram of protein.
D = molar concentration of unbound DMI.

per gram of total protein* in the plasma. (For a solution of a pure protein with a molecular weight of 10^5, an r-value of $1 \cdot 10^{-5}$ corresponds to 1 mole of drug bound per mole of this protein.) The curve can be interpreted to indicate that there are several binding sites available with different affinities for DMI.

The degree of binding over the entire range of therapeutic plasma drug concentrations was relatively constant (Table 4, plasma B).

Binding of DMI in different individuals

Unbound DMI in human plasma with a total concentration of 0·29 μg/ml (1·1 μM) was found to be approximately 10 per cent in 41 plasmas. The mean ± S.D. was 9·5 ± 1·4. (The analytical method has a S.D. of ± 0·35.) Therefore, there is little inter-individual variability in the plasma protein binding of DMI.

Comparative binding study of different tricyclic antidepressants

Each comparison was performed using the same human plasma at a drug concentration of 1·1 μM (Table 5) except in the experiments with unlabeled PT and NT

TABLE 5. PLASMA PROTEIN BINDING OF VARIOUS TRICYCLIC ANTIDEPRESSANTS

Drug	Unbound drug % ± S.D.	Number of plasmas	Conc. μM
^{14}C-imipramine	4·2 ± 0·8	5*	1·1
^{3}H-desmethylimipramine	8·4 ± 0·9	5*	1·1
^{14}C-amitriptyline	3·6 ± 0·8	5*	1·1
^{3}H-nortriptyline	5·5 ± 0·6	5*	1·1
nortriptyline	6·1 ± 1·1†	4	4·3
^{3}H-labeled Leo 640	0·7 ± 0·7	5*	1·1
^{14}C-protripyline	8·0 ± 0·6	4	7·7
protriptyline	7·8 ± 1·8†	3	4·3

*The same five plasmas were used for the different drugs.
†Determined by the method of Hammer and Brodie.

and with ^{14}C-labeled PT (low specific activity) where higher concentrations were necessary to achieve adequate levels of the drugs for analysis. The results obtained with the two assay methods as well as with duplicate samples were in good agreement.

The tertiary amines (I, AT and Leo 640) bind to a greater extent than the corresponding secondary amines (DMI and NT). The differences in binding were significant on the 0·1 per cent (DMI — Leo 640), 1 per cent (DMI — I) and the 5 per cent level (NT — A). The substitution of a hydrogen in one of the methyl groups in I for a *p*-chlorobenzoyl group (Leo 640), which decreases the basic strength of the compound and at the same time makes it more lipophilic, increased the binding markedly. This finding initiated an investigation of the binding properties of the acetyl derivatives of the secondary amines being studied. The ^{3}H-acetyl derivatives of DMI, NT and PT were synthesized and their binding investigated (Table 6). The introduction of an acetyl group, which yields a neutral but structurally very similar compound, markedly increased the protein binding. The binding to the dialysis membrane was found to be negligible for these derivatives.

*Work is under way to determine to which molecular species DMI is bound in human plasma.

TABLE 6. PLASMA PROTEIN BINDING OF ACETYLATED DMI, NT AND PT

Compound	Unbound (%)	Conc. (μM)
N-(^{3}H-acetyl)-desmethylimipramine	1·0, 1·1	2·2
N-(^{3}H-acetyl)-nortriptyline	0·8, 0·8	2·2
N-(^{3}H-acetyl)-protriptyline	0·6, 1·2	2·2

Effects of other drugs

The effects of various acidic, basic, and neutral drugs on the binding of DMI are summarized in Table 7. The concentrations used were considered either therapeutic or supratherapeutic in humans.

The addition of NT, PT or AT in a concentration of 0·2 μg/ml did not displace DMI to any important extent. The same was true for chlorpromazine at a concentra-

TABLE 7. THE EFFECT OF OTHER DRUGS ON THE BINDING OF DMI TO PLASMA PROTEINS

Drug	Concentration (μg/ml)	n	Increase in % unbound DMI ± S.D.	Significance test P<
acetylsalicylic acid, (a)	70*	6	2·7 ± 1·0	0·001
aminopyrine, (b)	20*	7	2·8 ± 1·2	0·001
amitriptyline, (b)	0·2*	8	0·7 ± 0·7	0·05
atropine, (b)	500	5	2·4 ± 1·0	0·01
chlorophenoxy*iso*butyric acid, (a)	200	6	0·1 ± 1·3	n.s.
chlorpromazine, (b)	2	5	0·2 ± 0·3	n.s.
diphenylhydantoin, (a)	11*	6	3·3 ± 1·6	0·01
lithium carbonate	37*	5	−0·2 ± 0·5	n.s.
meprobamate, (n)	100*	6	1·9 ± 0·4	0·001
mecamylamine, (b)	200	6	1·4 ± 1·5	n.s.
nortriptyline, (b)	0·2*	6	0·5 ± 0·5	0·05
phenacetine, (n)	10*	6	1·6 ± 0·8	0·01
phenobarbital, (a)	80*	5	0·2 ± 0·5	n.s.
phenylbutazone, (a)	200	7	2·6 ± 1·1	0·001
protriptyline, (b)	0·2*	6	−0·1 ± 0·2	n.s.
scopolamine, (b)	500	7	3·4 ± 1·3	0·01

The % unbound DMI was measured in the same plasma with and without addition of the drug to be tested. The significance of the observed difference was tested.

n.s. = not significant (P > 0·05).

The compounds can be considered to belong to the three main groups: acids, bases and neutral compounds. (a) = acid, (b) = base, (n) = neutral compound.

* Concentration within the therapeutic range.

TABLE 8. EFFECT OF DIPHENYLHYDANTOIN ON THE PLASMA PROTEIN BINDING OF VARIOUS TRICYCLIC ANTIDEPRESSANTS

Drug	Drug concentration (μM)	% unbound without DPH	% unbound with DPH	Change in % unbound
^{14}C-amitriptyline	1·1	5·0	8·6	+3·6
^{14}C-imipramine	1·1	4·2	10·3	+6·1
^{3}H-nortriptyline	1·1	5·3	8·1	+2·8
^{14}C-protriptyline	7·7	8·2	10·9	+2·7

The figures are based on duplicate determinations with one single plasma. The DPH concentration was 11 μg/ml.

tion of 2 μg/ml. This finding is in agreement with the finding that raising the concentration of DMI itself from 0·033 μg/ml to 2·9 μg/ml increases the unbound DMI levels less than 1 per cent (Table 4).

Acetylsalicylic acid, aminopyrine, phenacetine and meprobamate, added in concentrations achieved in therapy, displaced DMI to a small but statistically significant extent. The displacing effect of DPH was more pronounced. DPH also displaced the other tricyclics in the study particularly the tertiary amines, I and AT (Table 8).

DISCUSSION

In a previous study, species differences of plasma protein binding of DMI were reported.[7] The present data further substantiate that the tricyclic antidepressants are extensively bound to human plasma proteins. The degree of binding of DMI is approximately the same over the entire therapeutic range (0·033–0·290 μg/ml) of plasma concentrations. The degree of binding in different humans also appears to be relatively constant. Only one of 41 plasmas showed an unbound level outside the 7–13 per cent range. It is therefore meaningful to relate pharmacologic effects of this class of drugs in man to their total plasma level.[8] NT was bound to approximately 94 per cent *in vitro*. These data agree well with our finding *in vivo* that, under steady-state conditions the concentration of NT in cerebro-spinal fluid (CSF) was 3–11 per cent (mean 6·6 per cent) of the plasma level (unpubl. data). At equilibrium the drug concentration in CSF, which can be considered to be practically free of protein, should be equal to the unbound concentration in the blood.

In vivo, the therapeutic plasma level of tricyclic antidepressant drug seldom exceeds 0·3 μg/ml[9]. At this concentration of DMI few of the other drugs tested (in therapeutic concentrations) displaced the antidepressant from plasma proteins to any appreciable extent. The number of binding sites available for DMI at therapeutic concentrations seems to be so great that a displacement is not likely to occur upon addition of a drug up to concentrations of 1–2 μg/ml even if this drug is binding to the same sites with greater affinity than DMI. Modern psychoactive drugs such as chlorpromazine[10] and tricyclic antidepressants[9] do not achieve therapeutic plasma levels of that order of magnitude.

It is interesting to observe that DPH was the most potent displacer of the tricyclic antidepressants. DPH is also known to displace competitively thyroxine from thyroxine binding globulin.[11] The potent antidepressants DMI and PT were the least bound of the drugs studied. The unbound concentration of I was equal to that of DMI in the presence of DPH. It would be interesting to find out whether the latter drug can be used to potentiate I, since nothing is known about the importance of the free level for the pharmacologic effects of this class of compounds.

The binding of tricyclic antidepressant drugs seems to differ from the acidic drugs such as warfarin and phenylbutazone, which achieve much higher therapeutic blood levels and compete for similar binding sites. Displacement of these acidic drugs results in a marked increase in the level of unbound drug as well as pharmacologic effect.[2, 12] For the tricyclic antidepressants, drug interactions of this type may not be a great clinical or toxicological concern. It should be pointed out, however, that it is also important to study the effect of displacement from tissue binding sites since a much greater quantity of these antidepressants is non-specifically bound to tissue compared to plasma proteins.

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