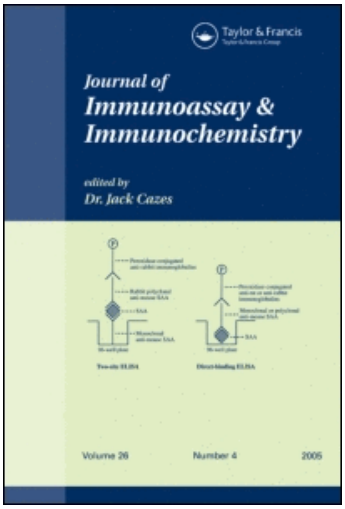

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A RADIORECEPTOR ASSAY FOR NEUROLEPTIC DRUGS IN PLASMA

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ABSTRACT

A simple and rapid method for measuring neuroleptic drugs in plasma by the estimation of dopamine receptor blocking activity is described. The assay has the advantages that it can be used without modification for all neuroleptic drugs and measures both parent compound as well as pharmacologically active metabolites.

Intra-assay and inter-assay coefficients of variation are 20% and 30% respectively and sensitivity is adequate to measure drug activity in plasma from patients treated with oral neuroleptics but not for those receiving depot injections.

Preliminary clinical investigations indicate that the ranges of dopamine receptor blocking activity in plasma vary for different drugs. Interference in the assay has been seen with some samples from patients receiving amitriptyline and nortriptyline, but not with other psychotropic drugs.

The receptor assay is considered suitable for routine monitoring of neuroleptic drug therapy.

INTRODUCTION

Routine monitoring of drug therapy by measurement of plasma concentrations is becoming recognised as a valuable aid to clinical management. To be suitable for routine use, assay methods need to be both simple and specific for the pharmacologically active substances; such procedures

are now available for a number of drugs including digoxin, gentamicin, lithium and anticonvulsants. Similar approaches to the control of therapy with neuroleptic (antipsychotic) drugs, however, have been complicated by the large number of drugs in use and the even larger number of metabolites produced from them in the body (1). The metabolic fate of any particular drug varies considerably between subjects; some metabolites are biologically inert, some even more potent than the parent compound, so that measurement of the latter alone is of relatively little value in most instances.

The most commonly used techniques for the determination of the neuroleptic drugs in serum and plasma are gas chromatography, gas-liquid chromatography and fluorimetry, although more recently radioimmunoassay methods have been described. The former methods are complex and time-consuming, allowing only a small throughput of samples, and being highly selective the methods must be modified to measure different compounds. The structurally-related specificity of fluorimetry and radioimmunoassay techniques, on the other hand, makes it impossible to distinguish between active and inactive metabolites, both of which are equally likely to interfere in the assay.

A novel approach to measurement of the neuroleptics, suggested by Creese and Snyder (2), is based on the ability of these drugs and their pharmacologically active metabolites to bind to dopamine receptors in the brain. Both Creese et al. (3) and Seeman et al. (4) have shown that the relative potency of the neuroleptic drugs in producing dopamine receptor blockade in vitro is highly correlated with their pharmacological potency in vivo assessed from behavioural experiments in animals and from the effective clinical dosage in man. It is inferred that the in vitro dopamine receptor blocking activity of plasma, which is related to the concentration of circulating drug and active metabolites, should reflect the quantity of drug reaching the receptor sites in the brain.

The present paper describes a modification of the radioreceptor assay of Creese and Snyder (2). The principle of the assay is competition between

the neuroleptic drug in the patient's plasma sample and a radiolabelled drug for binding to dopamine receptors in calf caudate membranes. The tracer of choice is ^{3}H-spiperone which binds to the receptor with high affinity and thus allows an assay of adequate sensitivity for most clinical requirements.

MATERIALS

Caudates from the brains of 12 - 16 week old calves were obtained from Froxfield Research Suppliers, Petersfield, Hampshire, England. The caudates were frozen and stored at -20°C within twelve hours of slaughter.

Phenyl-4-^{3}H spiperone (spiroperidol), specific activity 21 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, England.

Haloperidol, pure drug, was kindly supplied by Janssen Pharmaceutical Ltd.

METHODS

Preparation of Reagents

The receptor preparation was usually prepared from calf caudates stored frozen for less than four weeks although satisfactory batches were made using caudates which had been stored at -20°C for up to 6 months.

For a typical batch of material, 40 g of calf caudates were homogenised for 30 seconds in a Silverson Homogeniser, Model L2R, in 500 ml 200 mM phosphate buffer pH 7.4. ^{3}H-spiperone, 1.9 nmol, diluted into 200 ml phosphate buffer was added to the homogenised caudate and the final volume adjusted to 1250 ml. The resulting suspension was mixed on a magnetic stirrer for at least 30 minutes at room temperature before being aliquotted and lyophilised. After reconstitution of the dried material with

four times the original volume of deionised water the concentration of caudate tissue was 8 mg/ml and tracer 0.38 pmol/ml. The freeze-dried reagent (referred to as tissue-tracer mixture) was stored at 4°C; after reconstitution it could be stored at 4°C for up to 2 days or at -20°C for up to 6 months.

Haloperidol standards were prepared by dilution of a 100 μM solution of the drug in 1 per cent acetic acid into pooled normal human serum to yield a series of concentrations ranging from 15 to 3000 nM. The standards were aliquotted, freeze dried and stored at 4°C. Reconstituted standards could be stored at -20°C for at least one month and frozen and re-thawed up to 5 times without loss of activity.

Neuroleptic and other drugs for cross-reactivity studies were dissolved in appropriate solvents at mM concentrations and stored at 4°C in dark bottles. Before each assay they were diluted in pooled normal human serum and protected from light where possible.

Patient samples for assay were collected with a plastic syringe into sodium heparin tubes, E.D.T.A. tubes or plain glass tubes for serum (all obtained from Path Lab Supplies, Gillingham, Kent, U.K.) and protected from light. Plasma was separated within four hours and stored in aliquots at -20°C. Samples from normal subjects were collected into lithium heparin tubes (Searle, U.K.) E.D.T.A. tubes (Seward, U.K.) or plain glass bottles.

Clinical samples

Sodium heparin plasma samples were collected from 100 patients receiving neuroleptic treatment to determine the ranges of dopamine receptor blocking activity for different drugs. All samples were collected ten to fifteen hours after the last dose i.e. before the morning dose of drug. Plasma samples from patients receiving antidepressants, anticonvulsants, lithium carbonate and benzodiazepines were also screened in the assay.

Assay Procedure

The assay was carried out in 12 x 55 mm polypropylene tubes (Walter Sarstedt U.K. (Ltd.)). Triplicate tubes were set up for measuring non-specific binding in the presence of 100 μM haloperidol, total binding, haloperidol standards and patient samples. The incubate consisted of 100 μl standard or sample and 500 μl reconstituted tissue-tracer mixture (4 mgm caudate tissue and 0.19 pmol ^{3}H spiperone per tube). The final concentration of tracer in the incubate was 0.3 nM. After mixing, tubes were incubated in a water bath at 37 °C for 30 minutes. 2.5 ml cold saline (4 °C) was added to the tubes which were then centrifuged for 20 minutes at 4 °C at not less than 1000g. The supernatant was decanted and the tissue pellet dissolved in 1.5 ml scintillation fluid (Lumagel, L.K.B.). Tubes were counted for 5 minutes in a Corumatic 200 (I.C.N.) or more recently, a Rackbeta liquid scintillation counter (LKB Instruments Ltd.).

Dopamine receptor blocking activity of drug in plasma samples was calculated from a log-logit calibration and results expressed as neuroleptic units (N.U.) in terms of the haloperidol standard. One neuroleptic unit of dopamine receptor blocking activity was defined as that activity produced by 100 μl of a 1 nM solution of haloperidol in the assay as described.

RESULTS

Binding characteristics of assay

Under the conditions described the percentage binding of ^{3}H-spiperone to caudate tissue in the absence of unlabelled drug was 27.2 ± 1.8 (S.D.) (mean of 30 consecutive assays carried out over a period of 5 months). The non-specific component of binding measured in the presence of excess unlabelled haloperidol was 6.9 ± 0.8 per cent. The proportion of spiperone binding specifically associated with dopamine receptors was thus approximately 75 per cent.

Early experiments were carried out using a more concentrated tissue-tracer mixture (8 mg caudate tissue and 0.38 pmol ^{3}H spiperone per assay tube); the more dilute mixture was eventually favoured as it allowed greater sensitivity without loss of precision (Table 1).

TABLE 1

Precision of Dopamine Receptor Assay: Quality Control Samples

	Within assay		Between assay	
	C.V.%	(d.f.)	C.V.%	(d.f.).
8 mg tissue/tube: 23 assays				
All values N.U.	19.8	(167)	29.0	(86)
Values from 50 - 200	22.1	(78)	27.9	(43)
Values from 200 - 500	17.4	(44)	31.2	(22)
Values > 500	17.8	(45)	28.7	(21)
4 mg tissue/tube: 8 assays				
All values N.U.	16.7	(43)	22.7	(20)
Values from 0 - 50	16.4	(14)	26.9	(6)
Values from 50 - 200	17.5	(10)	22.6	(7)
Values from 200 - 500	8.8	(6)	-	
Values > 500	19.1	(13)	17.3	(7)

Between Assay Reproducibility: results for individual samples

Control Serum	n	Mean content (N.U.)	S.D.	C.V. per cent
1	23	97.4	25.6	26.3
2	23	170.9	50.2	29.4
3	23	392.8	124.4	31.7
4	23	711.4	204.6	28.8
5	8	22.9	6.2	27.0
6	8	195.5	46.0	23.5
7	8	1628.5	237.0	14.5

Precision

Within assay precision and between assay reproducibility were assessed by the inclusion of quality control samples in routine assays. The results are summarised in Table 1. Precision was also assessed from clinical samples which had been assayed on two separate occasions; within and between assay variances were pooled for all samples within the stated ranges (Table 2).

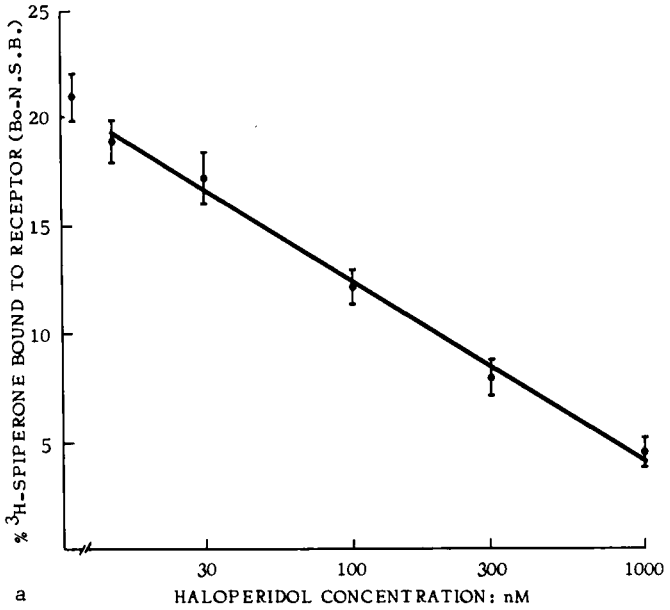
TABLE 2

Precision of Dopamine Receptor Assay: Clinical Samples

	Within assay		Between assay	
	C.V.%	(d.f.)	C.V.%	(d.f.)
8 mg tissue/tube : 119 samples				
Values from 0 - 50 N.U.	33.5	(103)	32.3	(34)
Values from 50 - 200 N.U.	30.6	(147)	29.7	(46)
Values from 200 - 500 N.U.	30.3	(53)	28.1	(19)
Values > 500 N.U.	14.5	(59)	23.1	(21)
4 mg tissue/tube: 26 samples				
Values from 0 - 50 N.U.	37.8	(15)	20.7	(4)
Values from 50 - 200 N.U.	18.9	(40)	25.7	(10)
Values from 200 - 500 N.U.	18.5	(28)	15.2	(7)
Values > 500 N.U.	24.3	(20)	20.4	(5)

Sensitivity

The theoretical least concentration of haloperidol which could be distinguished from "zero drug" was 13 nM (5 ng/ml) estimated from a standard curve with ten replicates at each point (Fig. 1). The mean and standard deviation of the zero standard were calculated and the point on the dose response curve corresponding to the mean minus 2 standard deviations was considered to be the detection limit of the assay.



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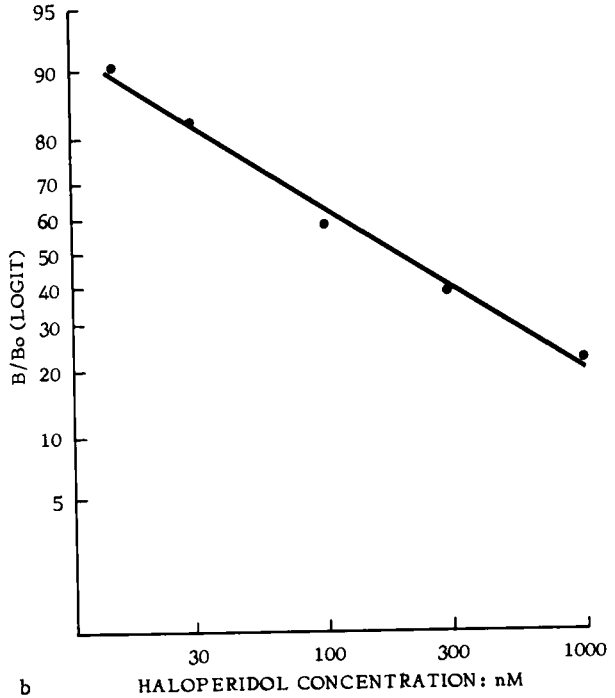

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Figure 1 a) Haloperidol standard curve with ten replicates at each point; the bars indicate ± 2 S.D. The detection limit of haloperidol derived from this data was 13 nM. b) The same data plotted as log-logit showing linear transformation of the standard curve.

A more practical assessment of sensitivity was based on the dose of drug producing 15 per cent inhibition of binding on the logit-log dose response curve. In 20 routine assays the mean dose of haloperidol at 15 per cent inhibition was 19.2 nM, S.D. 3.3 (7 ng/ml). Doses of other neuroleptic drugs at 15 per inhibition calculated from log-logit dose response curves (Fig. 2) were chlorpromazine 80 nM (26 ng/ml), thioridazine 700 nM (280 ng/ml) and fluphenazine 7 nM (3.6 ng/ml).

Specificity

The potencies of neuroleptic and other psychotropic drugs (Table 3) relative to haloperidol were calculated from IC_{50} values (drug concentration, nM, displacing 50% of specific binding) derived from log-logit dose-response curves, some of which are illustrated in Fig. 2.

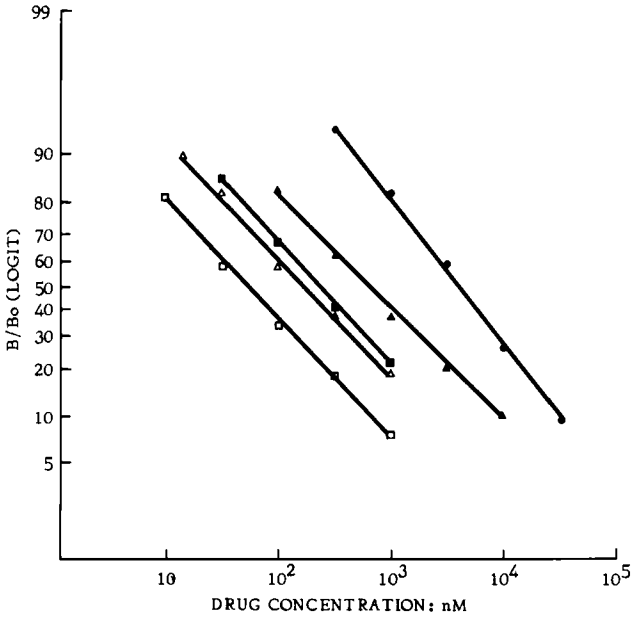


Figure 2

Dose response curves of various neuroleptic drugs derived from three assays using 4 mg caudate tissue/tube; the log-logit plots represent inhibition of ^{3}H-spiperone binding to caudate tissue. △ Haloperidol, □ fluphenazine, ■ trifluperazine, ▲ chlorpromazine, ● thioridazine.

TABLE 3

Relative Potencies of Neuroleptic and other Psychotropic Drugs

Drug		IC_{50} (nM) Mean	S.D.	Potency relative to Haloperidol
Neuroleptics				
Haloperidol	(9)	61.9	13.2	100
Spiperone	(4)	1.86	0.5	3330
Fluphenazine	(3)	17.5	1.9	357
Perphenazine	(3)	37.5	3.7	164
Trifluperazine	(2)	47.4	6.6	130
Pimozide	(5)	76.3	24.5	81.3
α Flupenthixol	(4)	148.8	8.0	41.7
Chlorpromazine	(4)	338	81.4	18.3
β Flupenthixol	(5)	499	151	12.4
Thioridazine	(4)	1361	217	4.5
Promazine	(1)	2357	-	2.6
Clozapine	(5)	3571	712	1.7
Reserpine	(1)	>4285	-	<1.4
Tricyclic Antidepressants				
Amitriptyline	(2)	2072	303	2.2
Nortriptyline	(3)	6048	951	0.94
Imipramine	(1)	4286	-	1.4
Desmethyl-imipramime	(2)	6429	-	0.75
Miscellaneous Drugs				
Metoclopramide	(4)	1332	271	4.7
Methysergide	(2)	1286	202	4.4
Cyclizine	(1)	15714	-	0.4

IC_{50} values (drug concentration, nM, displacing 50% of specific binding) were calculated from log-logit dose response curves.
Figures in parenthesis indicate number of assays.
This data was derived from assays using 8 mg caudate membranes per tube.

Metabolites of chlorpromazine, thioridazine and fluphenazine were also examined Those known to be pharmacologically active showed potencies in some cases as high or higher than the parent drug, while the relatively inactive ring sulphoxides had potencies less than 10 per cent of the parent compound (data to be published). Several tricyclic antidepressant drugs

tested in the assay produced dose response curves parallel to those of the neuroleptic drugs with potencies similar to some of them. No interference in the assay was seen with benzodiazepines or anticholinergic drugs (orphenadrine, procyclidine and benzhexol).

Effects of serum and plasma on assay

Increasing the volume of drug-free serum added to the incubate (up to a maximum of 300 µl) progressively reduced the binding of tracer to receptor. To eliminate this source of variation the sample volume was standardised at 100 µl, which gave adequate sensitivity for measurement of clinical drug levels with minimal non-specific effect on tracer binding. Under these conditions the between-sample effect on tracer binding at zero drug concentration was of the order of 1.3 per cent of the total counts. In normal subjects and 5 patients under treatment with neuroleptic drugs no significant difference was seen between the response observed with matched heparin plasma, EDTA plasma and serum samples. (Table 4).

TABLE 4

Comparison of Tracer Binding in the Presence of Heparin Plasma (A), EDTA Plasma (B) and Serum (C).

17 normal subjects:		A	B	C
Mean per cent bound		21.71	21.27	21.36
S.D.		1.29	1.31	1.20
Matched pairs "t" test:		**A v. B**	**A v. C**	**B v. C**
17 normal subjects	t	1.77	0.87	1.53
	p	0.09	0.59	0.14
5 patients	t	0.62	0.35	0.04
	p	0.57	0.74	0.97

Recovery of haloperidol and thioridazine and comparison with gas chromatography

Known concentrations of haloperidol were added to four samples of human serum and E.D.T.A. plasma and assayed on 2 separate occasions. The mean recovery of added drug was 93% and no significant differences were observed between recoveries from serum or plasma (Table 5).

Thioridazine was added in known concentrations to pooled human serum and assayed by both gas chromatography and the receptor assay against thioridazine standards. The percentage recovery in the receptor assay of samples at concentrations of 4, 2 and 1 µg/ml was 106, 94.7 and 95 respectively while that by gas chromatography was 88, 95 and 110.

TABLE 5

Recovery of Haloperidol added to Serum and Plasma

Added (nM)	Recovered* in: serum	(S.D.)	plasma	(S.D.)	Mean % recovery
300	255	(76)	281	(83)	89
150	144	(45)	149	(57)	98
75	60.9	(27)	76.8+	(7.4)	92

Serum v. plasma differences were not significant by "t" test ($p>0.2$).

* 24 replicate determinations were made at each point except where indicated.

+ 6 replicates (one sample, two assays) only at this point.

Paired serum and plasma samples were collected from each of four donors. Measured amounts of haloperidol were added to each sample. Each recovery sample was assayed twice with triplicate analyses on each occasion. No obvious between sample differences were observed and data for all four samples (serum or plasma) at each concentration were pooled for statistical purposes.

Clinical studies

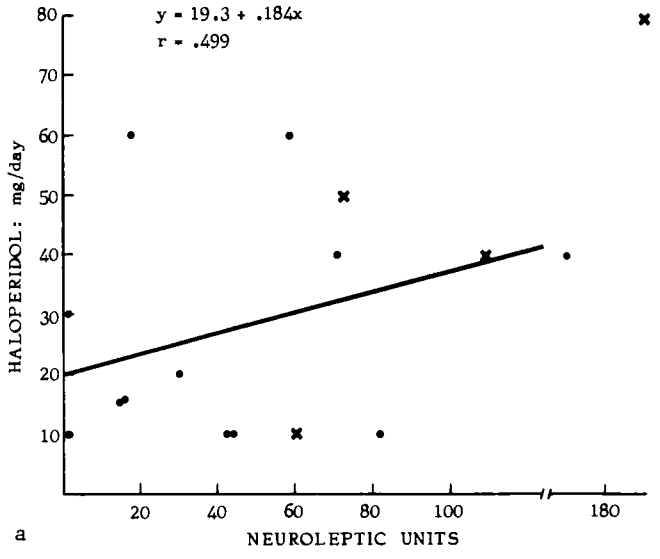
The ranges of dopamine receptor blocking activity in plasma from patients receiving a single neuroleptic drug (haloperidol, chlorpromazine or thioridazine) plotted against the daily dose of drug are seen in Fig. 3. While the values for haloperidol and chlorpromazine generally fall below 200 N.U. (i.e. equivalent to less than 200 nM (66 ng/ml) for haloperidol and 1100 nM (320 ng/ml) for chlorpromazine) those for thioridazine vary between 100 and 1000 N.U. (2.2 to 22 μM, 0.8 to 8 μg/ml). Low levels of dopamine receptor blocking activity (< 40 N.U.) were detected in only one third of samples from patients receiving depot injections of phenothiazines.

Seven of nineteen samples from patients treated with nortriptyline or amitriptyline showed dopamine receptor blocking activity up to 120 N.U. (range 31 - 123). Plasma samples from patients receiving imipramine, benzodiazopines, lithium carbonate or various anticonvulsant drugs (phenytoin, phenobarbitone, primidone, carbamazepine) had no activity in the receptor assay.

DISCUSSION

Creese et al. (3) and Seeman et al. (4) have shown that measurement of the in vitro inhibition of binding of tritiated haloperidol or spiperone to caudate tissue receptors correlated well with published values for biological activity in a range of neuroleptic drugs and related compounds. Biological activity had been measured in several different animal models and was also estimated, albeit probably less reliably, by calculation of "average clinical dose" in man. The crucial question of whether in vitro measurement of plasma activity correlates well with anti-psychotic or toxic effects in man requires a great deal of clinical investigation, in an area where patient response is both difficult to assess and influenced by many factors other than drug treatment.

With this caveat, the receptor assay described has advantages over existing methods for measuring neuroleptic drugs which make it well suited



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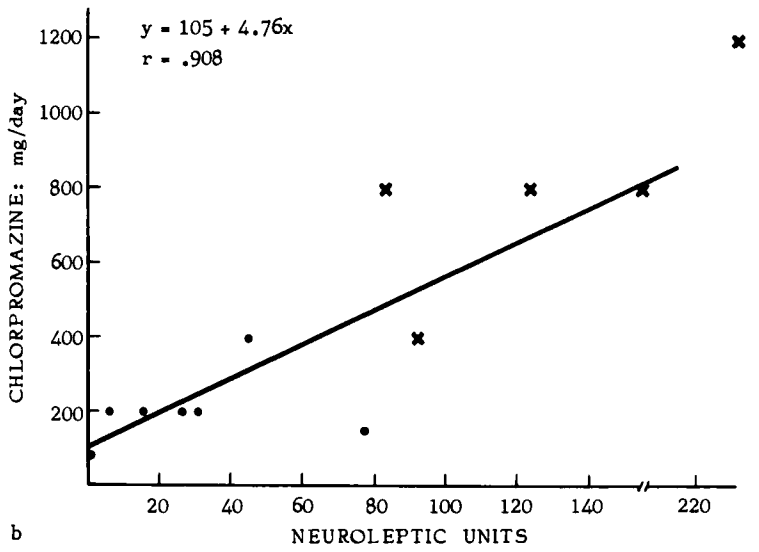

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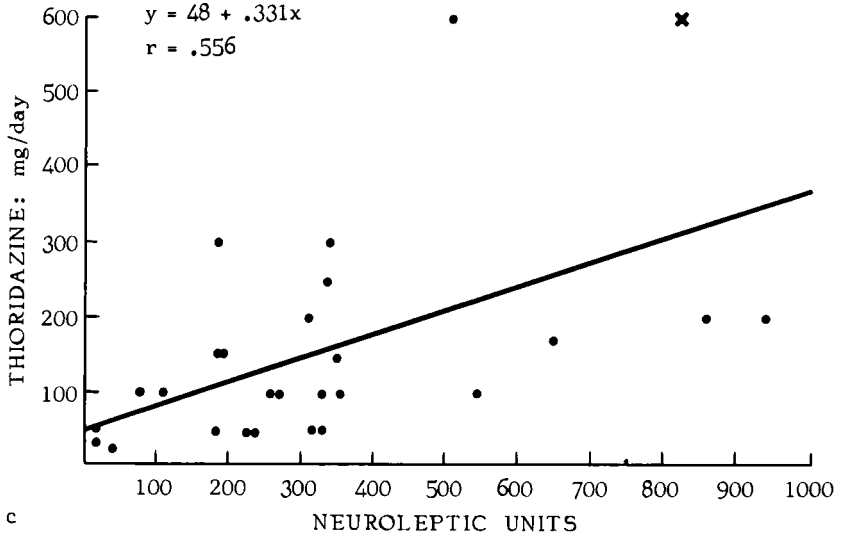

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Figure 3

Ranges of dopamine receptor blocking activity (N.U.) in plasma samples from patients receiving a) haloperidol, b) chlorpromazine, c) thioridazine. Regression equations for daily dose of drug against dopamine receptor blocking activity are shown. ✖ Extrapyramidal signs present.

for use as a routine monitoring procedure. It is technically simple to perform, requires only a small volume of plasma and yields results within a working day. Although it does not have the highest levels of sensitivity and reproducibility sometimes attained with gas chromatographic and radioimmunoassay methods, the observed performance may be considered adequate for most clinical requirements particularly as the therapeutic range observed with neuroleptic drugs is so wide. As with the method of Creese and Snyder, from which it is derived, potencies observed in this assay correlate well with biological activities for the range of drugs so far tested.

The major advantage of the receptor assay is that the technique can be applied without modification to all presently available neuroleptic drugs. The dopamine receptor blocking activity measured in the assay represents the total effect of parent drug and active metabolites; this activity can be expressed in terms of any reference neuroleptic drug. Haloperidol was selected for use as a standard as it is widely prescribed and is chemically more stable than the phenothiazines.

The assay is unaffected by other drugs which may be administered with the neuroleptics with the exception of some tricyclic antidepressants. The interference seen with amitriptyline and nortriptyline may be entirely non-specific or may suggest some binding to dopamine receptors by these drugs at high concentrations.

The clinical samples assayed in this preliminary study were collected from a heterogeneous population of patients suffering from schizophrenia, manic-depressive psychosis and senile dementia. No formal clinical ratings were made and so no attempt has been made to relate therapeutic response to the measured dopamine receptor blocking activity. The presence of extra-pyramidal signs was, however, noted and it is interesting that those patients in whom it was observed had generally higher plasma levels. Further clinical data are required to clarify whether the therapeutic ranges are indeed different for individual neuroleptic drugs and to confirm whether unwanted side effects are associated with higher levels of dopamine receptor blocking activity. It is apparent, however, that the test would be unlikely to give quantitatively useful information in patients receiving mixtures of neuroleptic drugs with widely differing therapeutic ranges.

No differences between paired serum and plasma samples, from normal subjects or patients under treatment, have been found in the present study. In relation to other assays, of basic, lipophilic drugs however, concern has been expressed that components of the blood taking system may exert a variety of undesirable effects on the assay, in particular by altering the distribution of drug between plasma and red cells (5, 6, 7). A recent

study (Rosenberg, B. and Patzke, J., personal commmunication) comparing methods of blood sample collection, which has been reproduced in this laboratory, indicated that samples collected into green, red and lavender stoppered Vacutainers (Becton, Dickinson and Co.) gave lower results than did the same samples collected into blue stoppered Vacutainer tubes (both plain and heparin containing types) or plain glass syringes. In view of this, it is recommended that plasma or serum samples be collected by a consistent method which should preferably be tested first to show freedom from interfering effects with blood from normal subjects. Plasma was preferred in the present study because it allows faster sample preparation.

The clinical problems for which the assay is most likely to yield useful information are those of monitoring drug compliance and assessing poorly responding patients. Several recent publications have indicated that poor therapeutic response may be associated with either very low or very high plasma neuroleptic concentrations, thus implying that an optimum therapeutic range exists (8). Smith (9), for example, described a group of chronic non-responding patients treated with butaperazine in whom plasma concentrations were two to seven times lower than those in a group of patients showing better clinical response. Garver and his associates (10) measuring butaperazine concentrations in plasma and red blood cells described patients whose clinical condition deteriorated as the drug concentrations rose to high levels when the dose of drug was increased. Similarly, a therapeutically optimal concentration range for thioridazine has been proposed by Axelsson and Mårtensson (11) who studied the clinical status of patients with acute paranoid psychosis in relation to plasma concentrations of the drug and its major metabolites.

Neuroleptic drug monitoring may also be used to prevent the appearance of unwanted side effects. Firstly, the clinical practice of increasing neuroleptic dosage until the appearance of extrapyramidal signs as an indication of adequate dosage may be obviated. Secondly, the association of tardive dyskinesia with prolonged high dosage of neuroleptic drugs argues

for maintaining the plasma concentration of drug at the lowest effective level (12).

While further validation of the receptor assay is required, both technically and clinically, it provides for the first time a simple technique for investigation of the relationship between plasma neuroleptic drug activity and therapeutic response.

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