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RADIOIMMUNOASSAY FOR TRIFLUOPERAZINE IN HUMAN PLASMA

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1 A new sensitive and rapid radioimmunoassay procedure for the determination of plasma concentrations of the neuroleptic drug trifluoperazine is described.

2 The antiserum developed for trifluoperazine cross-reacted with *N*-desmethyltrifluoperazine and 7-hydroxytrifluoperazine to the extent of 26 and 24% respectively but its cross-reactivity with commonly co-administered tricyclic antidepressants and antianxiety agents tested was negligible.

3 The assay, based on the above antiserum, enabled the quantitation of 50 pg of the drug in 200 μl of plasma with a coefficient of variation of about 2% and therefore should be applicable for single dose pharmacokinetic and bioavailability studies. It should be applicable to therapeutic monitoring of the drug in patients.

Introduction

Trifluoperazine, a phenothiazine neuroleptic with a piperazinopropyl side chain, is widely used in the treatment of schizophrenic disorders. Study of the correlation of plasma concentrations of this drug with clinical efficacy has not been possible due to the lack of sensitive and specific analytical procedures. Like other piperazine phenothiazines, such as fluphenazine and perphenazine, trifluoperazine is administered orally to patients in far smaller doses than chlorpromazine, and thus the resulting plasma drug concentrations are generally considered to be below the range of standard assay techniques (Cooper *et al.*, 1979; Wiles & Gelder, 1979; Curry *et al.*, 1979; Hansen *et al.*, 1976).

Trifluoperazine undergoes extensive metabolism, metabolic attack occurring at both the side chain and the phenothiazine ring system, resulting in a large number of metabolites. These include *N*-desmethylderivatives, piperazine ring degradation products, sulphoxides, and *N*-oxides, as well as ring-hydroxylated products such as 7-hydroxy metabolites and their conjugates (Breyer, Gaertner & Prox, 1974). Furthermore, animal experimentation of trifluoperazine has demonstrated that it undergoes a very pronounced 'first pass effect' (Schmalzing, 1977; Schmalzing & Breyer, 1978) like chlorpromazine (Loo, Midha & McGilveray, 1980), perphenazine (Hansen *et al.*, 1976) and fluphenazine (Curry *et al.*, 1979). This 'first pass effect' also contributes to the low plasma concentration of trifluoperazine.

As far as the present authors are aware there are no analytical procedures reported for the quantitative determination of trifluoperazine from plasma which are sensitive down to 1 ng/ml of the drug. Breyer & Schmalzing (1977) in studying the metabolism and disposition of trifluoperazine in rats, extracted basified tissue homogenates with di-isopropyl ether. Following the removal of the bulk lipids, trifluoperazine, *N*-desmethyltrifluoperazine and 7-hydroxytrifluoperazine were separated by thin-layer chromatography and measured by ultraviolet reflectance photometry on the plates. The sensitivity limit for the quantitative determination of the drug and the metabolites was about 0.1 nmol (approximately 41 ng) per extract. Thus, this and other published procedures (West & Vogel, 1975) do not have the sensitivity required to study the single and multiple dose pharmacokinetics of trifluoperazine.

Because of rapidity, simplicity, sensitivity and ease of adoption to routine clinical monitoring, numerous radioimmunoassays (RIA) are being developed for antipsychotic drugs including chlorpromazine (Kawashima, Dixon & Spector, 1975; Midha *et al.*, 1979), flupenthixol (Jørgensen, 1978), fluphenazine (Wiles & Franklin, 1978; Midha, Cooper & Hubbard, 1980) and perphenazine (Midha, Mackonka *et al.*, 1980). The RIA procedure developed and de-

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scribed here for trifluoperazine also offers the advantage of rapid sample turnover and the capacity to analyze large numbers of samples.

Methods

Experimental

Synthesis of drug protein conjugates Antiserum was raised in New Zealand white female rabbits to an immunogen which was prepared by covalent linkage of bovine serum albumin (BSA) to *N*-(2-carboxyethyl)desmethyltrifluoperazine employing the mixed anhydride condensation reaction (Erlanger *et al.*, 1957, 1959). This was followed by dialysis first against bicarbonate buffer (pH 8.0) followed by citrate buffer (pH 5), then saline (0.9%).

A blank was prepared in the same manner but without *N*-desmethyltrifluoperazine. The number of hapten residues per mol of bovine serum albumin were determined to be 27 by the UV procedure of Erlanger *et al.* (1957, 1959).

The hapten *N*-(2-carboxyethyl)desmethyltrifluoperazine was prepared by alkaline hydrolysis of *N*-(2-methoxycarbonylethyl)desmethyltrifluoperazine a compound synthesized by refluxing equimolar amounts of methyl acrylate and *N*-desmethyltrifluoperazine as reported for *N*-desmethylchlorpromazine (Hubbard *et al.*, 1978). The addition of *N*-desmethyltrifluoperazine to methyl acrylate was monitored by gas-liquid chromatography where the absence of the peak due to *N*-desmethyltrifluoperazine indicated completion of addition.

Immunization

Eight New Zealand white rabbits, four months old, were each given one intradermal injection of 1.0 mg of the immunogen emulsified with 0.25 ml of Freund's complete adjuvant and 0.25 ml of isotonic saline. Thereafter, the rabbits were immunized at 2 week intervals with the same amount of immunogen emulsified with incomplete adjuvant, rather than complete. Five rabbits produced sera with adequate titre after the fourth injection and subsequent injections. In the remaining three, sera with adequate titre could not be produced even after the eighth injection and then they were sacrificed without collecting the antisera.

Scintillation counting was carried out using a LKB Rackbeta Liquid Scintillation Counter, Model number 1215, equipped with an automatic quench compensation AQC (Fisher Scientific Company, Canada).

Rabbit antiserum to *N*-desmethyltrifluoperazine, in 0.25 ml aliquots was lyophilized in glass vials and stored at −20°C. The contents of each vial were reconstituted with 100 ml of double distilled water before use. A tracer solution of tritiated trifluoperazine was prepared by catalytic exchange with tritium gas (Nuclear Research Center, Nagev, Beer-Sheva, Israel). The specific activity of trifluoperazine was 12.8 Ci/mmol.

The following reagents were used without modification: phosphate buffer, pH 7.2, 0.2 mol/l, dextran-coated charcoal suspension, containing 0.2 gl of BSA (Bio RIA, Montreal Canada). For liquid scintillation counting, PCS II (Amersham Corporation, Arlington Heights, Illinois, U.S.A.) was used.

An aqueous stock solution of trifluoperazine was prepared weekly by dissolving the dihydrochloride salt with double distilled water. Appropriate dilutions of the stock solutions were made in pooled plasma obtained from the Red Cross Blood Bank. Calibration curves were constructed by using the equation:

$$\text{Logit y} = \text{Log}_e \frac{B/Bo}{1-B/Bo} = M \text{Log}_{10} C + I$$

where B is percentage bound; Bo, percentage bound at zero concentration; M, slope; C, concentration (ng/ml); I, intercept.

The cross reactions (50% inhibition of binding at zero drug concentration) for the metabolites and other drugs were determined by the criteria of Abraham (1969).

A 5 mg dose of trifluoperazine (Stelazine®, SKF) was given orally with water (50 ml) to a healthy male volunteer (65 kg). Blood samples were collected over a 48 h period in evacuated glass tubes (Vacutainers, Becton Dickinson Co., Mississauga, Ontario, Canada), centrifuged, and separated plasma was stored at −4°C for a maximum period of 7 days. During collection of the venous samples, care was taken to avoid contact of the blood with the rubber stopper of the evacuated tube.

The assay was done in subdued light. To a 12 × 75 mm polystyrene tube containing 50–200 μl plasma sample (standard or from dosed volunteer) diluted to 200 μl with drug free plasma, were added 300 μl of 0.2M phosphate buffer (pH 7.2) containing the tritiated trifluoperazine as a tracer and the tube was mixed (Vortex, 10 s). Two hundred μl of the antiserum (0.25%) was added and the tube was mixed (Vortex, 10 s) once more and incubated at 4°C for 60 min. To this incubated solution was added 1.0 ml of a cold dextran coated charcoal solution (4°C); the tube was mixed and incubated for 10 min at 4°C. The sample was then centrifuged at 1720 g for

15 min at 4°C. The supernatant was decanted into a scintillation vial containing 18 ml of PCS II cocktail, then counted.

Results

The amount bound at zero concentration of the drug was determined at incubation times of 30, 60 and 90 min and at temperatures of 4°, 22° and 37°C for the first incubation step in the assay. From the results of these experiments, the optimum conditions for the assay were found to be an incubation time of 60 min at 4°C and the Bo was 40%.

The concentration of unknown samples were estimated by running a calibration curve with each set of unknown samples. If the concentrations of the unknown samples were outside the calibration range, appropriate dilutions with plasma blanks were made to bring them into the linear range.

Figure 1 shows a typical standard ($n = 61$) curve

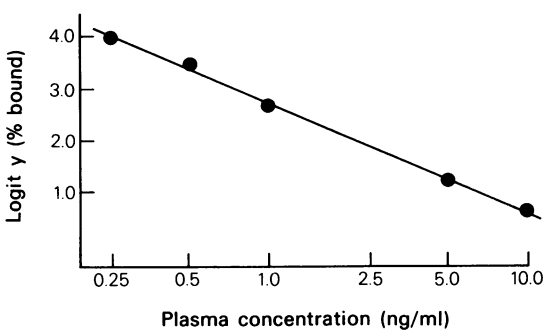

Figure 1 Composite standard curve for trifluoperazine Logit y = $-2.161 \log x + 1.764$ ($r^2 = 0.999$).

covering the range 0.5 ng/ml to 10 ng/ml, which is definable by the following equation:

$$\text{Logit } y = -2.161 \text{ Log}_{10}x + 1.764 \ (r^2 = 0.999)$$

Specificity: The cross-reactivity of available metabolites of trifluoperazine and other psychotropic drugs assessed by the criteria of Abraham (1969) is illustrated in Table 1.

Perphenazine, fluphenazine and prochlorperazine as expected did cross react, however, a structurally related long acting neuroleptic flupenthixol and commonly co-administered drugs such as amitriptyline, nortriptyline, diazepam and *N*-desalkylflurazepam, the active metabolite of flurazepam, did not cross react. A major inactive metabolite of trifluoperazine, i.e. trifluoperazine sulphoxide, did not cross-react, however, 7-hydroxytrifluoperazine and *N*-desmethyltrifluoperazine cross-reacted to an extent of 24 and 26% respectively. The significant cross-reactivity of *N*-desmethyltrifluoperazine (26%) was not surprising since the antisera was raised to a drug protein conjugate prepared by coupling to this very same secondary amine site. However, the cross-reactivity of the active 7-hydroxy metabolite was unexpected.

Table 1 Cross-reactions of trifluoperazine antiserum

Compounds tested	*Cross-reaction (%)*
Trifluoperazine	100
Fluphenazine	130
Perphenazine	130
Prochlorperazine	52
Chlorpromazine	21
Trifluoperazine sulphoxide	<1
N-Desmethyltrifluoperazine	26
7-Hydroxytrifluoperazine	24
Flupenthixol cis isomer	<1
Flupenthixol trans isomer	<1
Flupenthixol decanoate	<1
N-Desalkylflupenthixol	<1
Amitriptyline	<1
Nortriptyline	<1
Diazepam	0
N-Dealkylflurazepam	0

Sensitivity

The detection limit is less than 50 pg, which corresponds to 250 pg/ml if 200 µl of plasma sample is used; this sensitivity is far greater than the existing methods.

Precision

The coefficient of variation for the replicate analysis of various concentrations of trifluoperazine from 0.25 to 10 ng/ml is shown in Table 2. The coefficient of variation of 0.25 ng/ml ($n = 7$) was around 2%.

Influence of plasma volume on standard curves: standard curves were prepared by use of 50 and 200 µl volumes of plasma. The slopes and intercepts derived were identical thereby suggesting that plasma volumes did not affect the assay. Plasma was drawn from 12 different subjects and the Bo values were estimated in triplicate; no differences were observed which indicated that endogenous constituents in plasma did not interfere with the assay.

Analytical recovery

Tritiated trifluoperazine was added to plasma samples containing trifluoperazine and incubated with buffer in accordance with the procedure described. The solutions were then decanted into scintillation fluid and the radioactivity measured. The percentage recoveries at 0.25, 1.0 and 10.0 ng were 93.7%,

Table 2 Calibration curve data for trifluoperazine[a]

Concentration (ng/ml)	n	*Mean B/Bo (%)*	*s.d. (ng)*	*CV (%)*
0.25	7	95.39	1.83	1.92
0.5	15	92.44	1.88	2.03
1.0	15	84.67	2.93	3.46
5.0	12	54.89	1.68	3.06
10.0	12	40.56	0.82	2.02

[a]Mean CV = 2.49%; y = mx + b where m = −2.161, b = 1.764; $r^2 = 0.999$

95.2% and 96.8% respectively. It should be stressed that by using the decanting technique, small amounts of the sample adhere to the sides of the tube, thus actual recoveries may be greater. In another experiment, radioactive tracer was added directly to the polystyrene tube without using plasma, it was observed that more than 40% of the radioactive tracer was absorbed onto the surface of the tube. Thus, all standards at low nanogram range should be prepared in plasma.

Capacity of the procedure

One technician can assay 50 samples in triplicate in a normal working day. Concentrations in plasma measured in a healthy volunteer after administration of a 5 mg dose of trifluoperazine is illustrated in Figure 2.

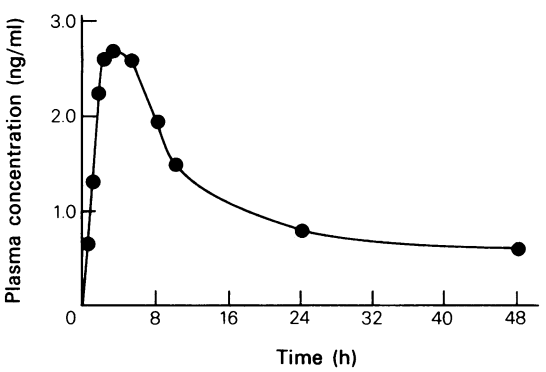

Figure 2 Trifluoperazine concentrations in the plasma of a human volunteer (65 kg) following a single oral dose of 5 mg trifluoperazine.

Note that the assay can detect the drug in specimens collected as late as 48 h after a single oral dose.

Discussion

Sensitive, specific and reproducible procedures are required for pharmacokinetic and bioavailability studies of trifluoperazine. Rapidity, high sample turnover and low cost would be added advantages of an assay, so aiding the clinician in monitoring the individual patient. So far, the lack of such methods for antipsychotic drugs such as trifluoperazine, fluphenazine, and perphenazine has been a major limiting factor in studying the possible correlations of the plasma concentrations of these drugs with clinical response. In general, these radioimmunoassay procedures developed for antipsychotic drugs (Kawashima *et al.*, 1975; Jørgensen, 1978; Wiles & Franklin, 1978, Midha *et al.*, 1979; Midha, Cooper *et al.*, 1980; Midha, Mackonka *et al.*, 1980) have provided the sensitivity, rapidity and specificity required to monitor the plasma concentrations of these drugs.

The radioimmunoassay procedure described here should be suitable for both single dose pharmacokinetic and bioequivalence studies, and for the routine clinical monitoring of plasma concentrations of trifluoperazine.

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