Br. J. clin. Pharmac. (1981), **1**, 85–88

RADIOIMMUNNOASSAY FOR PERPHENAZINE IN HUMAN PLASMA

K.K. MIDHA, CINDY MACKONKA & J.K. COOPER

College of Pharmacy, University of Saskatchewan
Saskatoon, Saskatchewan S7N 0W0 Canada

J.W. HUBBARD & P.K.F. YEUNG

Faculty of Pharmacy, University of Manitoba
Winnipeg, Manitoba R3T 2N2 Canada

1 A new sensitive, specific and rapid radioimmunossay procedure for the determination of plasma concentrations of the neuroleptic drug perphenazine is described.

2 The antiserum developed for perphenazine did not cross-react with most of the major metabolites of perphenazine nor the tricyclic antidepressants and antianxiety agents commonly co-administered with the drug.

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 8% and therefore should be applicable for single dose pharmacokinetic studies, as well as therapeutic monitoring of the drug in patients.

Introduction

Perphenazine, a phenothiazine neuroleptic with a piperazine side chain, is often used in the treatment of schizophrenic disorders. Although treatment with perphenazine may be effective, large interindividual variations in steady state plasma levels have been observed (Hansen & Larsen, 1977). Monitoring of plasma perphenazine concentrations would permit dosage adjustment potentially improving therapeutic response. However, perphenazine being potent is given orally or intramuscularly as the enanthate in low doses and the resulting plasma drug concentrations are often below the range of standard assay techniques (Hansen, Christensen, Elley, Hansen, Kragh-Sorenson, Larsen, Naestoft & Hvideberg, 1976; Cooper, Albert, Dugal, Bertrand & Elie, 1979). Perphenazine 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 perphenazine sulfoxide, N-deshydroxyethyl perphenazine, piperazine ring degradation products and N-oxides, as well as ring hydroxylated metabolites (Breyer, Gaertner & Prox, 1974). Furthermore, the reported 'first pass effect' seen after oral administration also contributes to the low plasma concentrations (Hansen *et al.*, 1976).

A few gas-liquid chromatographic procedures based on extraction of the drug from plasma, followed by derivatisation to trimethylsilylethers or *n*-heptafluorobutyrates and determination using an electron capture detector (Larsen & Naestoft, 1973; 1975), are only sensitive to 5 ng/ml (Cooper *et al.*, 1979). The high performance liquid chromatographic procedure of Tjaden, Lankelma, Poppe & Muusze (1976), also requires extraction of the drug followed by measurement with a specialized anodic coulometric detector. Thus, the published procedures to this date may be specific, however, they are often insensitive, time consuming and cumbersome since they require extraction of the drug from plasma, followed by derivatisation. Therefore, the adoption of these procedures to routine clinical monitoring is not suitable.

Another important technique reported for antipsychotic drugs like chlorpromazine (Kawashima, Dixon & Spector, 1975; Midha, Loo, Hubbard, Rowe & McGilveray, 1979), flupenthixol (Jorgensen, 1978), and fluphenazine (Wiles & Franklin, 1979; Midha *et al.*, 1980) which is simple, sensitive and specific, requring no extraction or derivatisation and is readily applicable to routine analysis is radioimmunoassay (RIA). This technique developed and described here for perphenazine also offers the advantage of rapid sample turnover and the capacity to analyse large numbers of samples.

0306–5251/81/010085–04 $1.00

Methods

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 *0*-(3-carboxypropionyl)perphenazine employing the carbodiimide condensation reaction. This was followed by dialysis first against citrate buffer (pH 5), then saline (0.9%).

A blank was prepared in the same manner but without perphenazine. The number of hapten residues/mol of bovine serum albumin were determined to be 22 by the UV procedure of Erlanger, Borek, Beiser & Lieberman (1957, 1959).

The hapten (*o*-(3-carboxypropionly)perphenazine was prepared by reacting equimolar amounts of succinic anhydride and perphenazine in pyridine. The reaction was monitored by high performance liquid chromatography where the absence of the peak due to perphenazine indicated completion of the reaction.

Immunization

Eight New Zealand white rabbits, 4 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. All rabbits produced sera with adequate titer after the fourth and subsequent injections.

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 perphenazine, 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 distilled water before use.

Tracer solutions of tritiated trifluoperazine of perphenazine were prepared by catalytic exchange with tritium gas (Nuclear Research Center, Negev, Beer-Sheva, Israel). The specific activity of trifluoperazine and perphenazine were 12.8 and 10.3 Ci/mmol respectively.

The following reagents were used without modification: Phosphate buffer, pH 7.2, 0.2 mol/l. Dextran coated charcoal suspension, containing 0.2 g of BSA/l (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 perphenazine was prepared weekly by dissolving the dihydrochloride salt with 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 6 mg dose of perphenazine tablets (Shering, Trilafon^R, 4 mg and 2 mg tablet) was given orally with an aqueous solution (50 ml) to a healthy male volunteer (82 kg). Blood samples were collected over a 48-h period in evacuated glass tubes (Vacutainers, Becton Dickinson Co., Mississauga, Ontario) centrifuged, and separated plasma was stored at −4°C for 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 5 μl of the tritiated tracer (trifluoperazine or perphenazine) and 300 μl of 0.2 M phosphate buffer (pH 7.2) and the tube was mixed (Vortex, 10 s). The antiserum (0.25%, 200 μl) was added and the tube was mixed (Vortex, 10 s) once more and incubated at 4°C for 30 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 15, 30 and 60 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 30 min at 4°C and the Bo was 40%.

Concentrations of unknown samples were estimated by running a calibration curve with each set of unknown samples. If the 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 = 67) curve covering the range 0.5 ng/ml to 5 ng/ml which is defineable by the following equation.

Logit y = $-2.477 \text{ Log}_{10} \times + 1.292$ ($r^2 = 0.994$)

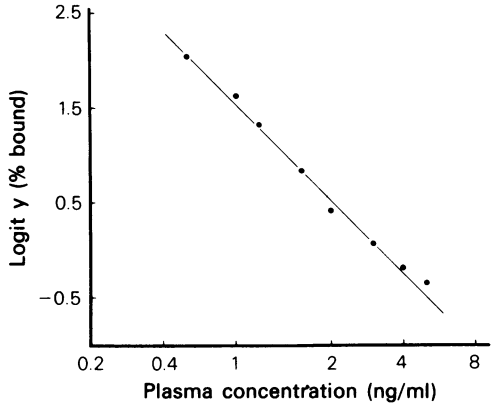

Figure 1 Composite standard curve for perphenazine

Specificity

The cross reactivity of available metabolites of perphenazine and other psychotropic drugs assessed by the criteria of Abraham (1969) is shown in Table 1. Except for N-deshydroxyethyl perphenazine, i.e. N-desmethyl-prochlorperazine, the other available metabolite tested did not cross-react significantly with perphenazine. Drugs closely related to perphenazine in structure such as prochlorperazine and fluphenazine cross-reacted sufficiently enough to be assayed using the same antiserum. Such assays are under development. Of the tricyclic antidepressants examined, amitriptyline and nortriptyline cross-reacted very weakly giving flat-response curves. The benzodiazepines tested were not recognized by the antiserum.

Table 1 Cross reactions of perphenazine antiserum

Compounds tested	*Cross-reaction %*
Perphenazine	100
Perphenazine sulfoxide	<1
7,8-Dihydroxyperphenazine	<1
Prochlorperazine	59.5
7-Hydroxyprochlorperazine	2.4
N-Desmethylprochlorperazine	13.8
Fluphenazine	100
Chlorpromazine	23.4
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 GLC-ECD methods.

Precision

The inter- and intra-assay variation was determined by using plasma standards in accordance with the procedure described. The data calculated according to Rodbard (1974) are summarized in Table 2. Note that intra-assay variances do not differ greatly from inter-assay variances.

Table 2 Intra- and inter-assay variance

Intra-assay				
Mean (ng)	0.5	0.75	2	5
s.d. (ng)	0.04	0.07	0.18	0.09
n	4	4	5	4
CV%	7.51	10.37	7.28	1.59
Inter-assay				
Mean (ng)	0.5	5.0		
s.d. (ng)	0.04	0.19		
n	4	3		
CV%	8.56	4.23		

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 15 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 perphenazine or trifluoperazine were added to plasma samples containing perphenazine 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 of 0.5, 1.0 and 4.0 ng were 96%, 97% and 95% respectively. It should be stressed that by using the decanting technique, small amounts of the sample adhere to the side of the tube, thus actual recoveries may be higher. In another experiment, radioactive tracer was added directly to the polystyrene tube withoug using plasma, it was found that at least half the radioactive tracer was adsorbed on the surface of the tube. Clearly, 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 6 mg dose of perphenazine are illustrated in Figure 2. Note that the assay can detect the drug in specimens collected as late as 24 h after a single oral dose.

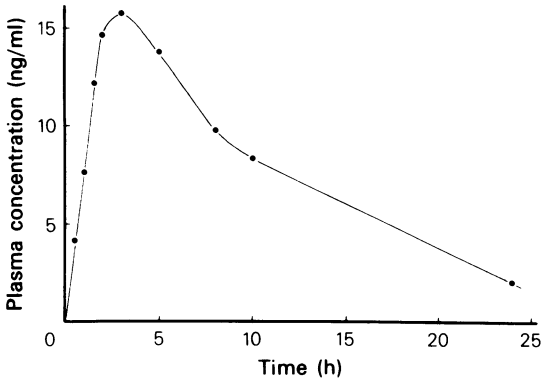

Figure 2 Perphenazine concentrations in the plasma of a human volunteer (82 kg) following a single oral dose of 6 mg perphenazine

Discussion

Sensitive, specific and rapid assays for perphenazine as an aid to the clinician in determining drug dosage for the individual patient are needed. In the ten reported clinical studies of this drug, the electron-capture gas chromatographic procedure of Larsen & Naestoft (1973) or its modification (Larsen & Naestoft, 1975) have been used. However, several problems have been noted (Cooper *et al.*, 1979; Hansen *et al.*, 1976) when this method has been applied to routine determinations. The radioimmunoassay procedure described here is suitable for the routine clinical monitoring of plasma concentrations of perphenazine in the presence of its major metabolites (Breyer *et al.*, 1974) formed by sulfoxidation, but not in the presence of its N-deshydroxyethylated metabolite. However, the concentration of this metabolite in patients' plasma may not be significant enough to influence the clinical interpretation of data involving concentrations in plasma.

The authors gratefully acknowledge the Medical Research Council of Canada for Development Grant No. DG222 (to K.K.M.). Drs S.M. Wallace and E.M. Hawes are thanked for helpful discussions and Miss Shirley Fournier is acknowledged for excellent technical assistance. Dr McSheffrey of the Red Cross Society is also gratefully acknowledged for donating units of plasma.

References

ABRAHAM, G.E. (1969), Solid-phase radioimmunoassay of estradiol-17B. *J. clin. Endocrinol. Metab.*, **29**, 866–870.

BREYER, U., GAERTNER, H.J. & PROX, A. (1974). Formation of identical metabolites from piperazine and dimethylamino-substituted phenothiazine drugs in man, rat and dog. *Biochem. Pharmac.*, **23**, 313–322.

COOPER, S., ALBERT, J.-M., DUGAL, R., BERTRAND, M. & ELIE, R. (1979). Gas chromatographic determination of amitriptyline, nortriptyline and perphenazine in plasma of schizophrenic patients after administration of the combination of amitriptyline with perphenazine. *Arzneim.-Forsch.*, **29**, 148–161.

ERLANDER, B.F., BOREK, F., BEISER, S.M. & LIEBERMAN, S. (1957). Steroid protein conjugates, I. Preparation and characterization of conjugates of bovine serum albumin with testosterone and cortisone. *J. biol. Chem.*, **228**, 713–727.

ERLANGER, B.F., BOREK, F., BEINSER, S.M. & LIEBERMAN, S. (1959). Steroid protein conjugates, II. Preparation and characterization of conjugates of bovine serum albumin with progesterone, deoxycorticosterone and estrone. *J. biol. Chem.*, **234**, 1090–1094.

HANSEN, C.E., CHRISTENSEN, T.R., ELLEY, J., HANSEN, L.B., KRAGH-SORENSEN, P., LARSEN, N.-E., NAESTOFT, J. & HVIDBERG, E.F. (1976). Clinical pharmacokinetic studies of perphenazine. *Br. J. clin. Pharmac.*, **3**, 915–923.

HANSEN, L.B. & LARSEN, N.-E. (1977). Plasma concentrations of perphenazine and its sulfoxide metabolite during continuous oral treatment. *Psychopharmacology*, **53**, 127–130.

JORGENSEN, A. (1978). A sensitive and specific radioimmunossay for cis (Z)-flupenthixol in human serum. *Life Sci.*, **23**, 1533–1542.

KAWASHIMA, K., DIXON, R. & SPECTOR, S. (1975). Development of radioimmunoassay for chlorpromazine. *Eur. J. Pharmac.*, **32**, 195–202.

LARSEN, N.-E. & NAESTOFT, J. (1973). Determination of perphenazine and fluphenazine in whole blood by gas chromatography. *Med. Lab. Techn*, **30**, 129–132.

LARSEN, N.-E. & NAESTOFT, J. (1975). Determination of perphenazine and its sulphoxide metabolite in human plasma after therapeutic doses by gas chromatography. *J. Chromatogr.*, **109**, 259–264.

MIDHA, K.K., LOO, J.C.K., HUBBARD, J.W., ROWE, M.L. & McGILVERAY, I.J. (1979). Radioimmunoassay for chlorpromazine in plasma. *Clin. Chem.*, **25**, 166–168.

MIDHA, K.K., COOPER, J.K. & HUBBARD, J.W. (1980). Radioimmunoassay for fluphenazine in human plasma. *Comm. Psychopharmac.* (in press).

RODBARD, D. (1974). Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assay. *Clin. Chem.*, **20**, 1255–1270.

TJADEN, U.R., LANKELMA, J., POPPE, H. & MUUSZE, R.G. (1976). Anodic coulometric detection with a glassy carbon electrode in combination with reversed-phase high-performance liquid chromatrography. Determination of blood levels of perphenazine and fluphenazine. *J. Chromatogr.*, **125**, 275–286.

WILES, D.J. & FRANKLIN, M. (1978). Radioimmunoassay for fluphenazine in human plasma. *Br. J. clin. Pharmac.*, **5**, 265–268.

(Received April 17, 1980)