THE RADIOIMMUNOASSAY OF TRICYCLIC ANTIDEPRESSANTS

G.W. AHERNE, E.M. PIALI & V. MARKS

Division of Clinical Biochemistry, Department of Biochemistry,
University of Surrey, Guildford, Surrey GU2 5XH

- 1 An antiserum to nortriptyline has been produced in a sheep against a nortriptyline-bovine serum albumin conjugate. The conjugate was prepared using the reagent N-(4-bromobutyl) phthalimide followed by a carbodiimide reaction to link the hapten to the carrier protein.
- 2 The antibodies produced were shown to be specific for the tricyclic group of drugs.
- 3 The sensitivity of the radioimmunoassay developed is currently limited by the lack of a high specific activity label. The possibilities of developing a radioimmunoassay for nortriptyline in plasma are discussed.

Introduction

Nortriptyline and amitriptyline from which it is formed, *in vivo*, are widely used in the treatment of depression. However, there is conflicting evidence as to the correlation of blood levels and clinical response (Marks, Lindup & Baylis, 1973; Burrows & Davies, 1974; Braithwaite & Goulding, 1975). Considerable variation in blood levels after oral dosing has been reported (Burrows, Scoggins, Turecek & Davies, 1974) and the metabolism of the tricyclic drugs can be affected by drugs such as barbiturates and phenothiazines (Gram & Overø, 1972). A rapid sensitive method for measuring nortriptyline and related drugs would be extremely useful both clinically and experimentally. Many methods for measuring tricyclic drugs in biological fluids exist (Marks *et al.*, 1973) including spectrophotometric (Wallace & Dahl, 1967), isotopic (Hammer & Brodie, 1967) and chromatographic determinations (Borgå & Garle, 1972; Faber, Mulder & Man in 't Weld, 1974). The technique of radioimmunoassay has, in recent years, proved useful for measuring drug concentrations in biological fluids (Butler, 1975) and is clearly an attractive alternative to presently available methods of measuring nortriptyline, especially if extraction of the drug could be avoided. The production of an antiserum is one of the prerequisites for the development of a radioimmunoassay. The present communication reports the production in a sheep immunized with a nortriptyline-BSA conjugate of an antiserum to nortriptyline suitable for use in a radioimmunoassay.

Methods

Nortriptyline-HCl and [14 C]-N-methylnortriptyline-HCl were gifts from the Lilly Research

Centre. Protriptyline was a gift from Merck, Sharp and Dohme Research Laboratories, dibenzipine from Wander Pharmaceuticals Limited, and carbamazepine from Geigy Pharmaceuticals Limited. Amitriptyline was donated by Dr A.C. Moffat, Home Office Central Research Establishment, Aldermaston. Samples of imipramine, desipramine and opipramol were purchased from their manufacturers. Bovine serum albumin (BSA) (Cohn Fraction V), N-(4-bromobutyl) phthalimide, ethyl (dimethylaminopropyl)- γ carbodiimide (EDC) and charcoal (Norit A) were purchased from Sigma Chemicals Limited, Dextran T-70 from Pharmacia Limited, BCG vaccine from Glaxo and pertussis vaccine from the Lister Institute, Elstree. All other reagents were from BDH Chemicals Limited.

Preparation of nortriptyline-BSA conjugate

N-4-aminobutyl nortriptyline was prepared according to the method of Cheng, Kim, Chung & Castro (1973) for methamphetamine. Nortriptyline-HCl (300 mg) and N-4-(bromobutyl) phthalimide (282 mg) were refluxed for 20 h in dry, absolute ethanol (15 ml). The reaction mixture contained a small quantity of [14 C]-N-methyl nortriptyline-HCl. One equivalent (50 mg) of 85% aqueous hydrazine was added and the mixture refluxed for a further 2 hours. Following acidification with a few drops of 2N HCl and centrifugation, the precipitate was extracted three times with chloroform. The pH of the aqueous phase was adjusted to pH 6.9, BSA (144 mg) and EDC (320 mg) were added to it and the mixture stirred at room temperature overnight. The nortriptyline-BSA conjugate was purified by dialysis against

Figure 1 The proposed structure of the nortriptyline-BSA conjugate.

distilled water and lyophilized under vacuum at 0°C. Measurement of tracer [^{14}C]-nortriptyline in the conjugate indicated that only two-three nortriptyline residues were coupled to each BSA molecule. The proposed structure of the conjugate is shown in Figure 1.

Immunization procedure

Nortriptyline-BSA (5 mg) and BCG vaccine (3 mg) in sterile saline (1 ml) were emulsified with 2 ml Marcol 52 adjuvant (Robinson, Morris & Marks, 1975) and injected into two sheep at six sites in the leg muscles. Pertussis vaccine (0.5 ml) was injected intradermally at the same time. The conjugate (2.5 mg) emulsified with the same adjuvant was injected at monthly intervals for 3 months. Venous blood samples were taken 10 days after each injection.

Radioimmunoassay procedure

The protocol adopted for both antiserum dilution curves and standard curves is shown in Table 1. Dilution and additions were made either using Oxford^R dispensers or with a Compu-pet^R (Warner). The diluent used throughout was 0.05 M phosphate buffer, pH 7.4 containing 1 g/litre gelatine and 6 g/litre NaCl. For antiserum dilution

curves the serum was doubly diluted with diluent buffer and that dilution which bound 50% of the added label was subsequently used for establishing standard curves. Nortriptyline-HCl standards and cross-reacting substances were dissolved in buffer and diluted immediately before use. [^{14}C]-N-methyl nortriptyline-HCl (2.13 mCi/mmol) was diluted with absolute ethanol and stored at -20°C as a stock solution containing 220 $\mu\text{mol/litre}$. This solution was diluted with buffer immediately before use so that 0.22 nmol (66 ng) nortriptyline was added to each assay tube. Dextran coated charcoal was prepared by mixing overnight at 4°C a 50 g/litre suspension of charcoal (Norit A) with an equal volume (200 ml) of a 5 g/litre suspension of Dextran T-70. The fines were removed by centrifugation and the charcoal resuspended in buffer (400 ml) and stored at 4°C. The reagents were added, in the order indicated, to LP3 plastic tubes (Luckham), the contents of the tubes mixed and incubated at room temperature for 50 min followed by 10 min in iced water. Dextran-coated charcoal was added, and the tubes mixed again and after 10 min centrifuged at 2500 rev/min for 5 min. An aliquot (200 μl) of the supernatants was taken for scintillation counting in a Packard Tricarb (2425) or an LKB Ultrabeta liquid scintillation counter.

Results

Both sheep immunized with nortriptyline-BSA responded by producing antibodies but only one (HP/S/1) produced antisera with sufficient binding of [^{14}C]-nortriptyline to warrant further investigation. Preimmunization sera did not bind the radiolabel. The titre of the anti-nortriptyline antiserum steadily increased during the period of observation and reached 1:86 (final dilution) after the third booster injection, and this antiserum was used for the work described.

Table 1 Radioimmunoassay procedure

	Total counts	Non-specific binding	Volume of reagents (μl)		
			Maximum binding	Zero or antiserum dilution curve	Standard or displacement curve
Diluent buffer	500	500	100	400	300
Nortriptyline or cross-reactant	—	—	—	—	100
Antiserum	—	—	400	100	100
[^{14}C]-nortriptyline-HCl	100	100	100	100	100
Diluent buffer	100	—	—	—	—
Dextran coated charcoal	—	100	100	100	100
Aliquot for counting	200	200	200	200	200

The displacement of [^{14}C]-nortriptyline label from antibody binding sites by nortriptyline standard is shown in Figure 2. The average affinity constant of the antibodies, as determined by a Scatchard plot of the data was 8.5×10^8 litre/mol at an antibody concentration of 3.3×10^{-5} mol/litre. The inhibition of the binding

of [^{14}C]-nortriptyline by other tricyclic drugs is shown in Table 2. The binding of [^{14}C]-nortriptyline label to antibody sites was not affected by the addition of $10 \mu\text{g}$ of several structurally unrelated drugs, i.e. diazepam, prednisolone, codeine, lignocaine, amphetamine, tetracycline, ephedrine and digoxin.

Table 2 The inhibition of [^{14}C]-nortriptyline binding by tricyclic drugs

Drug	Structure	50% inhibition of zero binding (ng)	% cross-reactivity

Nortriptyline	R: $-\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCH}_3$	98	100
Amitriptyline	R: $-\text{CH}_2\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$	64	153
Protriptyline		235	42

Desipramine	R: $-\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCH}_3$	410	24
Imipramine	R: $-\text{CH}_2\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$	110	89

Opipramol	R: $-\text{CH}_2\text{CH}_2-\text{CH}_2-\text{N}(\text{CH}_2)_4\text{N}-\text{CH}_2-\text{CH}_2\text{OH}$	240	41
Carbamazepine	R: $-\text{CONH}_2$	>3200	<3
Dibenzipine		>3200	<3

Figure 2 The displacement of [^{14}C]-nortriptyline HCl binding by nortriptyline HCl. Final dilution of antiserum HP/S/1 = 1 : 86. [^{14}C]-nortriptyline (0.22 nmol, 66 ng) was added to each tube.

Discussion

The nortriptyline-BSA conjugate used in these experiments was immunogenic in both animals immunized though each BSA molecule contained only two-three nortriptyline residues. This is a considerably lower level of derivatization than we have previously found to produce optimum immunogenicity (Robinson *et al.*, 1975). It is suggested that in the present case the nortriptyline residues became attached to the carrier protein through a 4 carbon chain link produced by the reagent N-(4-bromobutyl) phthalimide and that this was responsible for the increased immunogenicity.

The ability of other tricyclic drugs to cross-react with the antiserum to a variable extent is difficult to explain. There appear to be two major antigenic determinants responsible for the cross-reactivity. The tricyclic structure is clearly important since molecules with the same side chain as nortriptyline but a slightly different ring

structure, e.g. protriptyline and desipramine had reduced ability to react with the antibody. Amitriptyline, on the other hand, with exactly the same ring structure, had a greater ability to displace [^{14}C]-nortriptyline from antibody than nortriptyline itself. Likewise, imipramine cross-reacted more than desipramine. Whereas carbamazepine did not react with the antiserum at all, opipramol, which has an identical ring structure, showed 40% cross-reactivity. We suggest, therefore, that the chemical grouping

is also an important determinant in the antibody : antigen reaction. Dibenzipine, which contains the closely related N-dimethylethylamine

group is not recognized by the antibody. Enough has been said to indicate that more extensive cross-reactivity studies are necessary to define all of the antigenic determinants.

The present work provides the basis for a rapid radioimmunoassay for nortriptyline in plasma during treatment with the drug. The cross-reactivity of the antiserum with other tricyclic drugs does not preclude its use for this purpose as it is unlikely that more than one tricyclic drug will be administered simultaneously. In order to obtain clinically meaningful results it will be necessary to determine how metabolites of nortriptyline affect the assay. The major metabolites of nortriptyline are desmethylnortriptyline and 10-hydroxy-nortriptyline (McMahon, Marshall, Culp & Miller, 1963). Neither has as yet been made available for testing and it would be unwise to predict how they would react with the antiserum.

The method, as described, is insensitive (approximately 200 ng/ml) compared with most radioimmunoassay techniques due to the use of a low specific activity ^{14}C -label—the only radio-labelled form of nortriptyline currently available to us. By using a higher specific activity label either by tritiation or by radioiodination we expect to be able to increase the sensitivity of the assay at least one hundred times. This would make it 5-10 times as sensitive as reported chromatographic methods (Faber *et al.*, 1974; Borgâ & Garle, 1972; Braithwaite & Widdop, 1971). A high specific activity radiolabel will also enable the amount of antiserum used per assay to be reduced considerably.

References

- BORGA, O. & GARLE, M. (1972). Application of isotope derivative technique to assay of secondary amines: estimation of desipramine by acetylation with [^3H]-acetic anhydride. *J. Chromatogr.*, **68**, 77-88.
- BRAITHWAITE, R.A. & GOULDING, R. (1975). Effective dosage of tricyclic antidepressants. *Br. med. J.*, **1**, 206.
- BRAITHWAITE, R.A. & WIDDOP, B. (1971). A specific gas-chromatographic method for the measurement of 'steady state' plasma levels of amitriptyline and nortriptyline in patients. *Clin. chim. Acta*, **35**, 461-472.
- BURROWS, G.D. & DAVIES, B., (1974). Variability in response to drugs. *Br. med. J.*, **4**, 533.
- BURROWS, G.D., SCOGGINS, B.A., TURECEK, L.R. & DAVIES, B. (1974). Plasma nortriptyline and clinical response. *Clin. Pharmac. Ther.*, **16**, 639-644.
- BUTLER, V.P. (1975). Drug immunoassays. *J. Immunol. Methods*, **7**, 1-24.
- CHENG, L.T., KIM, S.Y., CHUNG, A. & CASTRO, A. (1973). Amphetamines: new radioimmunoassay. *FEBS Letter*, **36**, 339-342.
- FABER, D.B., MULDER, C. & MAN IN 'T VELD, W.A. (1974). A thin-layer method for the determination of amitriptyline and nortriptyline in plasma. *J. Chromatogr.*, **100**, 55-61.
- GRAM, L.F. & OVERØ, K.F. (1972). Drug interaction: inhibitory effect of neuroleptics on metabolism of tricyclic antidepressants in man. *Br. med. J.*, **1**, 463-465.
- HAMMER, W. & BRODIE, B.B. (1967). A gas chromatographic method for the quantitative determination of nortriptyline and some of its metabolites in human plasma and urine. *J. Pharmac. exp. Ther.*, **157**, 503-508.
- MARKS, V., LINDUP, W.E. & BAYLISS, E.M. (1973). Measurement of therapeutic agents in blood. *Adv. clin. Chem.*, **16**, 47-109.
- McMAHON, R.E., MARSHALL, F.J., CULP, H.W. & MILLER, W.M. (1963). The metabolism of nortriptyline-N-methyl- ^{14}C in rats. *Biochem. Pharmac.*, **12**, 1207-1217.
- ROBINSON, J.D., MORRIS, B.A. & MARKS, V. (1975). Development of a radioimmunoassay for etorphine. *Res. Comm. Chem. Path. Pharmac.*, **10**, 1-8.
- ROBINSON, J.D., MORRIS, B.A., PIALI, E.M., AHERNE, G.W. & MARKS, V. (1976). The use of rats in the screening of drug-protein conjugates for immunoreactivity. In *Radioimmunoassay and Related Techniques in Clinical Biochemistry*, pp. 101-111. London: Heydon.
- WALLACE, J.E. & DAHL, E.V. (1967). The determination of amitriptyline by ultraviolet spectrophotometry. *J. Forensic Sci.*, **12**, 484-489.

(Received November 27, 1975)