Double-Antibody Enzyme Immunoassay for Nortriptyline

M. N. Al-Bassam, M. J. O’Sullivan, E. Gnemmi, J. W. Bridges, and V. Marks

β-D-Galactosidase (EC 3.2.1.23) from Escherichia coli was conjugated to desmethylnortriptyline by means of a bifunctional cross-linking reagent, dimethyl adipimiate, and used in a double-antibody immunoassay for nortriptyline. Eighty percent of the enzyme activity was retained after conjugation; 75% of the enzyme was conjugated to desmethylnortriptyline. In the final immunoassay the enzyme activity of the bound fraction was determined with o-nitrophenyl-β-D-galactopyranoside as substrate. The sensitivity, precision, and simplicity of the enzyme immunoassay compared favorably with that of a published radioimmunoassay method. Results for nortriptyline in plasma samples correlated well with those determined by either radioimmunoassay or gas-chromatography.

Additional Keyphrases: tricyclic antidepressants • monitoring therapy • drug assay • intermethod comparison

The tricyclic antidepressant drugs (TAD) such as amitriptyline, nortriptyline (a dibenzocycloheptadiene derivative), imipramine, desipramine (a dibenzazepine derivative), and other closely related compounds are very widely prescribed for treatment of depression. Their efficacy in alleviating depression has been well established, but published data are in conflict regarding the relationship between drug concentrations in plasma and optimal response (1–9). There are marked inter-individual differences in man in the concentrations attained in blood with individual TADs, and this is thought to be due, in part at least, to genetic factors (9).

A rapid, sensitive, reproducible method for measuring nortriptyline and related drugs would be extremely useful, both clinically and experimentally. There are several analytical techniques for measuring TAD in biological fluids (1): spectrophotometric (10), isotopic (11), and chromatographic procedures (12–14). Radioimmunoassays with use of 14C and 3H as labels for measuring TAD have recently been described (15, 16).

The technique of enzyme immunoassay has proved useful for measuring drugs in biological fluids (17) and is potentially an attractive technique to apply to the determination of TAD. Unfortunately, the range of reagents that have been successfully used to conjugate drugs to enzymes is small and many of the techniques involved are relatively crude, producing low yields of conjugates, loss in enzyme activity, and extensive polymerization of the enzyme. In consequence, unless very extensive purification procedures are embarked on, the “active” labeled molecules are diluted with many inactive labeled molecules and therefore the sensitivity of most of the previ-ously reported enzyme immunoassays, although adequate for the measurement of several substances of clinical importance, do not yet match the sensitivities of the comparable radioimmunoassays.

Bifunctional imidoesters are extremely useful as protein cross-linking agents (18, 19). In this paper we describe an improved procedure in which desmethylnortriptyline is conjugated to β-D-galactosidase by use of dimethyl adipimiate (DMA). Under the conditions used, 75% of the enzyme is conjugated to desmethylnortriptyline with little loss of enzyme activity.

The conjugate produced has been used as a label in a double-antibody enzyme immunoassay. The method is analogous to double-antibody radioimmunoassay. The binding of enzyme-labeled antigen to the antigen-specific first antibody is competitively inhibited by the unlabeled antigen to be assayed. Free antigen is then separated from antigen bound to the first antibody by precipitation with a second antibody directed against the first antibody. Enzyme activity is measured on the bound fraction in the precipitate.

Materials and Methods

Reagents

Desmethylnortriptyline hydrochloride was a generous gift from Dr. R. A. Braithwaite, Poisons Unit, Guy’s Hospital, London. Nortriptyline was a gift from Lilly Research Centre Ltd., Windlesham, Surrey, England. Prototype was a gift from Merck Sharp and Dohme Research Laboratories, Dorval, Quebec, Canada. Imipramine, desipramine, and carbamazepine were gifts from Geigy Pharmaceuticals Ltd., Basle, Switzerland. Amitriptyline was donated by Dr. A. C. Moffat, Home Office Central Research Establishment, Aldermaston, Berkshire, England. Dibenzepine was purchased from Wander Pharmaceuticals Ltd., Middlesex, England. β-D-Galactosidase (EC 3.2.1.23) from Escherichia coli was purchased from Boehringer Mannheim, Mannheim, West Germany, as a suspension in 2.2 mmol/liter ammonium sulfate. Tris(hydroxymethyl)aminoethane (Tris) was purchased from Sigma, London, England. Dimethyl adipimiate dihydrochloride (Pierce Chemical Co., Rockford, Ill.) was stored in 10-mg aliquots under reduced pressure at −20 °C. 2-Mercaptoethanol was from Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K. Sephadex G-25 gel (Pharmacia Fine Chemicals) and bovine serum albumin, a 300 g/liter solution from Armour Pharmaceutical Co., London, U.K., were also used. The nortriptyline antiserum (bleed 526, 10/6/76), supplied by Dr. G. W. Aherne (15) of this department, was raised in a sheep against a nortriptyline-bovine serum albumin conjugate prepared by use of N-4-(bromobutyl) phthalimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The donkey anti-sheep second antibody was purchased from Guildhay Antisera, Guildford, Surrey, England. Diluent buffer

Division of Clinical Biochemistry, Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 6XH, U.K.

Received Mar. 6, 1978; accepted May 17, 1978.
contained sodium phosphate (5 mmol/liter, pH 7.4), magnesium chloride (10 mmol/liter), 2-mercaptoethanol (10 mmol/liter), sodium chloride (100 mmol/liter), and bovine serum albumin (1 g/liter) in distilled water. All other reagents were purchased from BDH Chemicals Ltd., Parkstone, England.

The Tris buffer was prepared from Tris (50 mmol/liter, adjusted with acetic acid to pH 7.5) and containing, per liter, magnesium chloride, 10 mmol, sodium chloride, 100 mmol, and 2-mercaptoethanol, 10 mmol. Sodium bicarbonate buffer (100 mmol/liter, pH 9.9) also contained magnesium chloride (10 mmol/liter) and 2-mercaptoethanol (10 mmol/liter).

The β-D-galactosidase substrate solution contained o-nitrophenyl-β-D-galactopyranoside (2.3 mmol/liter), sodium phosphate buffer (100 mmol/liter, pH 7.0), magnesium chloride (1 mmol/liter), and 2-mercaptoethanol (10 mmol/liter).

Plasma samples collected from patients receiving treatment with nortriptyline were assayed by gas-liquid chromatography (13) by Dr. R. A. Braithwaite and by radioimmunoassay, with use of tritiated imipramine as label, by Dr. G. Mould (16). The results were compared with those obtained on the same samples by the method described below.

Procedure

The purity of each β-D-galactosidase preparation was ascertained by electrophoresing 50 μg of the material on 50 g/liter polyacrylamide gel in Tris/glycine buffer, pH 8.3. The gels were stained for protein with Coomassie Blue and for β-D-galactosidase activity by using 6-bromo-2-naphthyl-β-D-galactopyranoside. To examine further the purity of the commercial enzyme, we applied a 2-μg enzyme sample to a Sepharose 6B column (90 × 0.9 cm) and eluted with sodium phosphate buffer (10 mmol/liter, pH 7.0) containing sodium chloride (100 mmol/liter) and magnesium chloride (10 mmol/liter). The absorbance of the eluate was monitored at 280 nm and β-D-galactosidase activity was assayed using o-nitrophenyl-β-D-galactopyranoside and measuring the absorbance at 420 nm. All commercial preparations, which were used without further purification, contained 65 000 units of activity per milligram (where 1 unit is the amount of enzyme that hydrolyses 1 nmol of o-nitrophenyl-β-D-galactopyranoside in 1 min at 28 °C in the diluent buffer described above).

Preparation of the Desmethylnortriptyline-β-D-Galactosidase Conjugate

Desmethylnortriptyline (570 μg) and DMA (488 μg) were dissolved in dry methanol (0.4 ml) containing 45.5 g of N-ethylmorpholine per liter, at 20 °C. A 100-μl aliquot was removed after 30 min and added to 100 μg of β-D-galactosidase in 1 ml of the bicarbonate buffer. This solution was maintained for 90 min at 20 °C, when the reaction was terminated by adding 1 ml of the Tris buffer. To separate compounds of low relative molecular mass from the enzyme, we applied the solution to a Sephadex G-25 column and eluted with Tris buffer. The fractions containing enzyme activity were collected, pooled, and diluted to a final volume of 10 ml with Tris buffer, sodium azide being added to give a 200 mg/liter solution. The solution was stored at 4 °C until required.

Immunoreactivity of the β-D-Galactosidase-Desmethylnortriptyline Conjugate

The percentage of β-D-galactosidase bound to desmethylnortriptyline was determined by incubating a portion of the enzyme conjugate (equivalent to 6.5 enzyme units) with excess nortriptyline antiserum (0.1 ml, 20-fold dilution of antiserum).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Total binding tube</th>
<th>Non-specific binding tube</th>
<th>Zero tube</th>
<th>Std. or sample tube</th>
<th>Volume, μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent buffer</td>
<td>500</td>
<td>400</td>
<td>300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard or sample</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Label</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>First antiserum</td>
<td>100</td>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Incubate for 90 min at 20 °C then add second antibody (100 μl), leave overnight at 4 °C, separate, and wash the precipitate once with diluent buffer. Add 1 ml of substrate solution to the precipitate, mix, and leave for 2 h at 20 °C. To stop the reaction, add carbonate buffer (1.5 ml, 0.2 mol/liter), centrifuge, and measure the absorbance of the supernate at 420 nm.

Enzyme conjugate bound by nortriptyline antibody was precipitated by a second antiserum against the first antibody. β-D-Galactosidase activity was determined in the precipitate after washing it once with diluent buffer.

Determination of Nortriptyline Concentration

The assay protocol is illustrated in Table 1. All dilutions of antiserum, label, standards, and samples were made with diluent buffer. The first antiserum was used at a dilution of 300-fold. The enzyme-labeled desmethylnortriptyline was diluted to give 6.5 enzyme units per tube. The standards were prepared by dissolving 1 mg of nortriptyline in 10 ml of diluent buffer. This was further diluted to give a standard of 100 μg/liter, which was further diluted to give the range of concentrations, 1 to 20 μg/liter, used in the assay. The reagents were added to the tubes in the order indicated in the protocol. Calibration curves were plotted as the ratio of the fraction of the enzyme-label nortriptyline bound at a given nortriptyline concentration to the fraction bound when no nortriptyline is present, i.e., (B/T)/(B0/T) = B/B0, where B = enzyme activity bound at given concentration, B0 = enzyme activity bound at zero concentration, and T = total enzyme activity.

The effect of human plasma and serum on nortriptyline determinations was studied by carrying out the assay over a wide range of nortriptyline concentrations and using 2 to 50 μl of plasma or serum. The precision and the reproducibility of the assay were assessed by making repeated assays on several serum samples over a wide range of nortriptyline concentrations (30–250 μg/liter). We also compared our enzyme immunoassay procedure for nortriptyline with results by radioimmunoassay and gas-liquid chromatography. The specificity of the nortriptyline antiserum was assessed by investigating the ability of other drugs to interfere in the nortriptyline enzyme-immunoassay.

Results

Purity of β-D-Galactosidase

On staining with Coomassie Blue after polyacrylamide gel electrophoresis of the commercially available β-D-galactosidase, one major and six relatively faint protein bands were visible. Most of the β-D-galactosidase activity was located in the same position as the major protein band. Four minor β-D-galactosidase bands were also detected, which were aligned with four of the minor protein bands. The elution profiles of protein and β-D-galactosidase activity from the Sepharose 6B column coincided very closely; no separate protein peaks were present. These results indicate that the commercially available β-D-galactosidase preparation contained at most only very little impurities. The preparations were therefore used without further purification.
Preparation of Enzyme-Desmethylnortriptyline Conjugate

Antibody dilution curves for several different conjugates are shown in Figure 1. These results indicate that as the time of incubation of desmethylnortriptyline and DMA increases the incorporation of desmethylnortriptyline into the enzyme increases but the enzyme activity decreases. The label formed during a 30-min incubation period has 75% of the enzyme bound to desmethylnortriptyline, with minimum loss of enzyme activity. This label was used to set up the standard curves.

Nortriptyline Standard Curve

The standard curve (Figure 2) of enzyme-labeled nortriptyline bound at a given nortriptyline concentration to the fraction of label bound at zero concentration illustrates that 1 μg of nortriptyline per liter inhibits binding of the label by 15%.

Table 2. Cross Reaction of Various Tricyclic Antidepressant Drugs in Enzyme- and Radioimmunoassay (15) a

<table>
<thead>
<tr>
<th>Hapten</th>
<th>Percentage cross reaction b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nortriptyline</td>
<td>100</td>
</tr>
<tr>
<td>Amtriptyline</td>
<td>153</td>
</tr>
<tr>
<td>Imipramine</td>
<td>89</td>
</tr>
<tr>
<td>Desmethylnortriptyline</td>
<td>45</td>
</tr>
<tr>
<td>Protriptyline</td>
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</tr>
<tr>
<td>Desipramine</td>
<td>24</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Dibenzepine</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

a Same batch of antiserum used in each technique.

b Cross reaction expressed as (amount of nortriptyline that displaces 50% of enzyme activity/amount of hapten that displaces 50% of enzyme activity) X 100.

Table 3. The Effect of Various Amounts of Plasma on Recovery of Nortriptyline

<table>
<thead>
<tr>
<th>Conc. of nortriptyline, μg/liter plasma</th>
<th>Plasma volume in assay, μl</th>
<th>Percentage recovery of nortriptyline ± SD a</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>50</td>
<td>80 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>100 ± 4</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>96 ± 4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>98 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>250</td>
<td>8</td>
<td>99 ± 4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>100 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>100 ± 3</td>
</tr>
</tbody>
</table>

a Mean of three measurements.

Other Analytical Variables

Specificity. The cross reactivity of some other drugs is shown in Table 2. Cross reactivity ranged from 153% for amtriptyline and 89% for imipramine to <3% for carbamazepine and dibenzepine. The cross reactivities as determined by enzyme immunoassay and radioimmunoassay are very similar.

Analytical recovery of nortriptyline. Table 3 shows the effect of plasma volume on the recovery of nortriptyline added to plasma samples. All the added nortriptyline was accounted for when as much as 25 μl of plasma was present. The presence of 50 μl of plasma diminished the recovery of nortriptyline by as much as 20%.

Precision and reproducibility. Within-assay variation was determined by measuring the same sample several times. Samples from nortriptyline-treated patients having low, medium, and high (30, 125, and 250 μg/liter) nortriptyline concentrations were used. The coefficients of variation were <5% in all cases. The between-assay variation was estimated by assaying a number of samples on at least four different occasions. The coefficients of variation were <8%.

Stability of reagents. The activity of the enzyme label decreased by <10% when it was stored for six months at 4 °C in Tris buffer containing, per liter, 1 g of bovine serum albumin.
and 200 mg of sodium azide. The immunoreactivity of the label decreased by <1% during this period.

Accuracy. The enzyme immunoassay method was compared with a radioimmunoassay in which 3H-labeled imipramine is used (16) and a gas–liquid chromatographic method in which a flame-ionization detector is used (13). We assayed by each of the three procedures a set of 21 plasma samples from patients being treated with nortriptyline. For the two immunoassay techniques we used the same batch of nortriptyline antiserum. There was good agreement among results of all three methods. The correlation coefficients (r) for enzyme immunoassay vs. radioimmunoassay and enzyme immunoassay vs. gas–liquid chromatography were 0.98 and 0.97, respectively, with a confidence limit of 99% (P < 0.001). The regression lines were (y = 0.9x + 15.5) and (y = 0.9x + 11.6), respectively, where y = results obtained with enzyme immunoassay. Scattergrams are shown in Figures 3 and 4.

Discussion

Imidoesters react in alkaline solutions with amines to form imidoamides. Of the many reactive groups in proteins, only amino groups will react, and the products, like the amino groups they replace, are protonated at physiological pH (22). The imidoamides are stable in neutral or acidic solutions, but slowly hydrolyze at high pH values (23). DMA, a bifunctional imidoester, has been used to form crosslinkages in several proteins (18). Here, we have used DMA to link desmethyl-nortriptyline to β-D-galactosidase.

Interestingly, when nortriptyline itself reacted with the enzyme under the same conditions used to conjugate desmethyl-nortriptyline, only 15% of the enzyme was bound by the specific antiserum. This difference in reactivity of nortriptyline as compared with desmethyl-nortriptyline is explained by the fact that the former contains a secondary amine while the latter contains a reactive primary amine.

Using the enzyme immunoassay, 1 μg of nortriptyline per liter could be detected, which is comparable to the limit of detection for the corresponding radioimmunoassay. Therapeutic concentrations of nortriptyline reportedly lie between 50 to 250 μg/liter plasma (5, 24–26). Thus the enzyme immunoassay is more sensitive enough to measure clinically significant concentrations of nortriptyline. The analytical recovery of nortriptyline in the presence of various amounts of plasma likely to be used in practice indicates that nortriptyline measurements are unaffected by natural constituents of plasma. The assay has a precision which is within acceptable immunoassay standards, with an intra-assay CV of less than 5% and an inter-assay CV of less than 8%.

The enzyme label was still usable after storage in solution at 4 °C for at least six months.

The performance of the enzyme immunoassay was compared with that of the radioimmunoassay and a gas–liquid chromatography method. In each case the results correlated well.

Thus the present method would appear to be suitable for the routine measurement of nortriptyline in plasma samples from patients undergoing nortriptyline treatment if they were not simultaneously receiving another tricyclic antidepressant drug capable of reacting with the antiserum. Previous experiments (16) from this department have shown that the antiserum does not cross react with the major metabolites of nortriptyline—e.g., 10-hydroxynortriptyline (27)—or with other tricyclic antidepressants in which the nucleus itself is altered.

We thank Dr. G. W. Aberne for many helpful discussions, for the generous gifts of the nortriptyline antiserum, and for supplying many of the nortriptyline analogs used in this work. We are indebted to Dr. R. A. Braithwaite for assaying nortriptyline in plasma samples by gas liquid chromatography and for the generous gift of desmethyl-nortriptyline. We also thank Dr. G. Mould for assaying nortriptyline in plasma samples by RIA. M.N.A.-B. thanks the University of Baghdad College of Medicine for providing financial support during this project.

References