Novel $^{125}$I-Labeled Nortriptyline Derivatives and Their Use in Liquid-Phase or Magnetizable Solid-Phase Second-Antibody Radioimmunoassays

R. S. Kamel, J. Landon, and D. S. Smith

Nortriptyline derivatives prepared by reaction with fluorescein isothiocyanate or conjugation to N-acetyl-L-histidine were radiiodinated and the products purified with Sephadex LH-20 columns to obtain two novel nortriptyline radioligands. Antisera were raised in rabbits by immunization with nortriptyline conjugated to succinylated ovine albumin. By use of the iodinated fluorescein derivative we developed a liquid-phase second-antibody radioimmunoassay that gives results correlating closely ($r = 0.98$) with those by an established radioimmunoassay of similar specificity in the assay of apparent total amitriptyline and its metabolite nortriptyline in serum or plasma from patients being treated with these drugs. With the iodinated N-acetyl-L-histidine derivative we developed a magnetizable solid-phase second-antibody radioimmunoassay. The cross reactivities of amitriptyline and nortriptyline could be made equal by performing the assay at pH 9.0, which makes it possible to measure true total active drug concentrations in patients receiving amitriptyline.

Additional Keyphrases: amitriptyline • antidepressants • therapeutic drug monitoring • drug assay

Nortriptyline, a useful member of the large group of tricyclic antidepressant drugs, is also important as the active demethylated metabolite of the more frequently prescribed amitriptyline. Steady-state circulating concentrations vary widely among patients receiving similar doses of these drugs (1). For this reason, the relationship between concentrations in plasma and therapeutic effectiveness or side-effects has been investigated extensively (1–4). Although no consistent conclusions have emerged, a therapeutic concentration for total amitriptyline and nortriptyline ranging between about 80 and 200 µg/L was suggested as the result of a recent extensive study (4).

Existing assay methods include gas–liquid chromatography (5) and “high-performance” liquid chromatography (6). These methods are reliable, and with them both amitriptyline and nortriptyline may be measured in the same sample. However, they require relatively large sample volumes and are too time-consuming and complex for the routine processing of many specimens. Several radioimmunoassay (RIA) methods involving tritiated ligands have been reported (7–9), but have the disadvantages of all assays in which this beta-emitting isotope is used, such as the need for expensive liquid scintillant, relatively long counting times, and potential quenching problems when biological specimens are assayed. An enzyme immunoassay has also been developed (10), but again the end-point determination is comparatively complex and prolonged.

We describe the preparation of $^{125}$I-labeled derivatives of nortriptyline and their use in simple and convenient RIA systems.

Materials and Methods

Reagents

Fluorescein isothiocyanate isomer I (FITC), N-acetyl-L-histidine (NALH), 1-ethyl-3-(3-dimethylaminopropyl)carboodiimide, bovine albumin type A4503, and ovine albumin type A4885 were obtained from Sigma, Poole, Dorset BH17 7NH, U. K.; Chloramine T, sodium metabisulphite, sodium azide, chloroform, dimethylformamide, diethyl ether, and methanol (all solvents AR grade) from BDH Chemicals, Poole, Dorset BH12 4NN, U. K.; triethylamine and Tween 20 from Koch-Light, Colnbrook, Bucks SL3 0BZ, U. K.; carrier-free Na$^{125}$I (IMS30) from The Radiochemical Centre, Amersham, Bucks HP7 9LL, U. K.; and donkey anti-rabbit precipitating serum from Wellcome Reagents, Beckenham, Kent BR3 3SB, U. K. Sheep anti-(rabbit immunoglobulin G) serum was a product of Technia Diagnostics, City Road, London ECIV 1JX.

Microgranular cellulose type CC 31 and chromatography paper No. 3MM were obtained from Whatman, Maidstone, Kent ME14 2LE, U. K.; silica gel thin-layer chromatography sheets DC-Alufolien Kieselgel 60 F$_{254}$ Art. 5554 from E. Merck, Darmstadt, G.F.R.; and Sephadex LH-20 from Pharmacia, Hounslow, Middlesex TW3 1NE, U. K.

Preparation of Nortriptyline Immunogen

Dissolve 1 g of ovine albumin in 50 mL of sodium phosphate buffer (100 mmol/L, pH 7.0) at room temperature. Add solid succinic anhydride in small amounts (50–100 mg), with continuous stirring, keeping the pH of the reaction mixture be-

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between 6.7 and 7.3 by adding NaOH (1 mol/L solution). After each addition of anhydride, use the fluorescamine method (11) to estimate protein amino groups. We detected no unmodified amino groups after adding 1 g of anhydride. Dialyze the mixture against distilled water, at 4 °C, and lyophilize the dialysate to obtain the exhaustively succinylated ovine albumin product.

To 12.5 mL of a 40 g/L aqueous solution of succinylated ovine albumin add 12.5 mL of a 2.63 g/L aqueous solution of nortriptyline hydrochloride. Add 84.3 mg of solid carbodiimide and stir the mixture at room temperature. Monitor the progress of the coupling reaction by removing small aliquots and adding nine volumes of perchloric acid (330 mmol/L), centrifuging (1000 × g, 10 min) them, and estimating the amount of uncoupled nortriptyline remaining in the supernates by measuring their absorbance at 240 nm. (In control experiments we found that protein was completely precipitated by the perchloric acid, and that less than 30% of the nortriptyline was precipitated from the reaction mixture before addition of carbodiimide. After 2 h of reaction we could detect no remaining uncoupled nortriptyline.) Dialyze the mixture against distilled water at 4 °C, and lyophilize the dialysate to obtain the nortriptyline immunogen.

Anti-Nortriptyline Sera

We immunized four rabbits, following a recommended method (12). Booster immunizations were given monthly. We obtained the antiserum used in the studies described below after the sixth boost of one animal.

Magnetizable Second-Antibody Solid-Phase

Whole sheep anti-(rabbit immunoglobulin G) serum was coupled to cellulose/iron oxide particles as described elsewhere (13). The second-antibody solid-phase was stored at 4 °C in sodium phosphate buffer (100 mmol/L, pH 7.5) containing, per liter, 1 g of bovine albumin, 2.5 mL of Tween 20 detergent, and 1 g of sodium azide.

Preparation of Fluoresceinithiocarbamyl Nortriptyline (FTC-NT)

Dissolve separately FITC (2.97 g/L, 36 mL) and nortriptyline hydrochloride (6.7 g/L, 12 mL) in a medium of equal volumes of methanol and aqueous (10 mL/L) triethylamine. Mix the solutions and, after 2 h at room temperature, add 48 mL of ammonium acetate buffer (200 mmol/L, pH 4.0), to precipitate the reaction products. Collect the products by centrifugation, wash the centrifugate with 96 mL of distilled water, and dissolve it in 20 mL of aqueous ammonium bicarbonate (50 mmol/L, pH about 8) with the aid of added aqueous ammonia. Apply the solution to a column (2.35 × 90 cm) of microgranular cellulose equilibrated with ammonium bicarbonate buffer (50 mmol/L, pH 9.0), and elute (10 mL/h) with this same buffer. Collect 4-mL fractions, and pool and lyophilize those incorporating the major colored peak. The product appeared free of unreacted or degraded FITC by chromatography on paper developed with sodium bicarbonate buffer (50 mmol/L, pH 9.0). Rf values (determined relative to the solvent front) were: FITC 0.50, FTC-NT 0.25.

Preparation of NALH Conjugate of Nortriptyline (NALH-NT)

Dissolve NALH (10 mg), nortriptyline hydrochloride (13.9 mg), and carbodiimide (44 mg) simultaneously in 2 mL of an equimolar mixture of dimethylformamide and distilled water. After 18 h at room temperature, isolate the conjugated product by thin-layer chromatography on silica gel developed with chloroform/diethyl ether/methanol (85/15/20 by vol). Under short-wave ultraviolet, a single major band with Rf 0.46 will be visible on the plate. (The Rf of nortriptyline in this system is 0.34.) Scrape the corresponding area from the plate, and elute the scrapings with methanol, evaporate the decanted methanol under a stream of nitrogen, and re-dissolve the residue in methanol to give a concentration of 1 g/L; store this solution at −20 °C.

Preparation of 125I-Labeled Nortriptyline Derivatives

Use 100 mmol/L sodium phosphate buffer (pH 7.5) throughout.

FTC-NT. To 2 µg of FTC-NT in 30 µL of buffer add 10 µL (1 mCi) of Na125I. Initiate iodination by adding 20 µL of a 1 g/L solution of Chloramine T; terminate the process by adding 10 µL of a 6 g/L solution of sodium metabisulfite.

NALH-NT. To 10 µL of the solution of NALH-NT in methanol add 10 µL of buffer and 10 µL (1 mCi) of Na125I. Initiate iodination by adding 10 µL of a 5 g/L solution of Chloramine T; terminate the process by adding 10 µL of a 30 g/L solution of sodium metabisulfite.

Use the same procedure for the purification of 125I-labeled FTC-NT and NALH-NT. Add buffer (500 µL) to the reaction mixture to facilitate its transfer to an 0.8 × 8 cm column of Sephadex LH-20 equilibrated in buffer. Elute the column with buffer and collect 20 to 30 500-µL fractions. Then apply methanol to the column to displace the labeled nortriptyline product, which appears between the fourth and ninth fractions after the change of eluent. Store the pooled product at −20 °C.

Assay Standards

Amitriptyline hydrochloride or nortriptyline hydrochloride (dissolved in methanol) was added to pooled normal human serum.

Radioimmunoassay Procedures

As diluent buffer, use sodium phosphate (100 mmol/L, pH 7.5) or sodium borate (100 mmol/L, pH 9.0), both containing 20 g of bovine albumin per liter. Carry out assays at room temperature. Process all samples in duplicate.

125I-labeled FTC-NT used. Prepare working reagents as follows, using the phosphate buffer.

Labeled nortriptyline: dilute 100-fold.

Anti-nortriptyline serum: dilute 400-fold.

Non-specific rabbit (carrier) serum: dilute 200-fold.

Anti-rabbit precipitating serum: dilute 20-fold.

Add 100 µL of labeled nortriptyline to 25 µL of serum or plasma sample, followed by 100 µL of anti-nortriptyline serum. After 1 h, add 50 µL of carrier serum, followed by 100 µL of anti-rabbit serum. After 1 h, centrifugate (2000 × g, 30 min, 4 °C), aspirate the supernates, and count the precipitates for 20 s.

125I-labeled NALH-NT used. Prepare working reagents as follows, using the phosphate or the borate buffer throughout.

Labeled nortriptyline: dilute 800-fold.

Anti-nortriptyline serum: dilute 600-fold.

Anti-rabbit magnetizable solid-phase: suspend to 8 g/L.

Dilute 50 µL of serum sample with 200 µL of the appropriate buffer. Add 100 µL of labeled nortriptyline followed by 100 µL of anti-nortriptyline serum. After 1 h, add 100 µL of anti-rabbit solid-phase and vortex-mix. After 15 min, add 1 mL of buffer and place the rack containing the assay tubes on the flat surface (approx. 15 × 20 cm) of a multipolar ferrite magnet (Magnet Applications, City Road, London EC1V 1LL, U. K.) for about 5 min to sediment the solid-phase. Aspirate the supernates and count the sedimented particles for 20 s.
Fig. 1. Proposed reaction scheme of the carbodiimide condensation between NALH and nortriptyline

Results

Immunogen and Antisera

The hapten/cARRIER molar ratio of the nortriptyline immunogen was 16, an estimate based on the assumption of complete succinylation of 60 lysine residues (14) of ovine albumin and complete coupling of nortriptyline in the carbodiimide reaction.

After a six-month course of booster immunizations, all the rabbits produced anti-nortriptyline sera of similar titer.

Preparation of NALH-NT

Figure 1 shows the proposed reaction scheme and structure of NALH-NT. The expected molecular ion at m/e 442 was found by mass spectrometry.

125I-Labeled Nortriptyline Derivatives

The iodination of FTC-NT was accompanied by a change in the faint color of the reaction mixture from yellow-green to orange-pink.

The column purification of both 125I-labeled FTC-NT and NALH-NT proceeded similarly. A non-immunoreactive impurity peak (presumably unreacted iodide) was eluted during the buffer wash. After the methanol elution step, no significant amount of radioactivity remained on the LH-20 gel. Table 1 summarizes yield and immunoreactivity of the products. In the studies described below, we used 125I-labeled FTC-NT prepared with a 20-s iodination time.

The stored methanolic solutions of the 125I-labeled nortriptyline derivatives showed no loss of immunoreactivity over at least three months.

Radioimmunoassays

Bovine albumin, 20 g/L, was included in the diluent buffers to obviate serum or plasma sample-matrix effects, and to decrease nonspecific binding of the 125I-labeled nortriptyline derivatives to the walls of polystyrene assay tubes to less than 3.5% as assessed by substituting a nonspecific rabbit serum for the anti-nortriptyline serum in each assay system.

The amount of 125I-labeled nortriptyline derivative used was such as to provide at least 20,000 total counts in each assay tube. Results were expressed as the percentage of total counts bound.

125I-labeled FTC-NT used. With nortriptyline serum standards, the liquid-phase second-antibody RIA covered the clinically important range of serum or plasma nortriptyline concentration (Figure 2). Logit transformation (15) did not linearize the dose-response curve.

From the results for 20 replicates, the standard deviation of the response for zero dose was found and used to estimate assay sensitivity as recommended by Rodbard (16). We found the least detectable concentration of serum nortriptyline to be 2.8 µg/L at the 95% confidence level (the smallest detectable dose of nortriptyline was 68 pg).

We tested structurally related tricyclic drugs and some metabolites for cross reactivity relative to nortriptyline (Table 2). Other drugs that might be used in combination with amitriptyline or nortriptyline, but which showed less than 10% cross reactivity included chlordiazepoxide, chlorpromazine, cyprophedrine, diazepam, dihydroergotamine, and flupenthixol.

Two serum specimens from patients receiving nortriptyline were used to assess within-assay precision (20 replicates), giving mean results 40.7 and 83.2 µg/L, with CVs of 4.1 and 3.1%, respectively. Three specimens were measured in 10 different assays, giving mean results 41.6, 71.4, and 145 µg/L, with between-assay CVs of 10.4, 6.9, and 6.9%, respectively.

125I-labeled NALH-NT used. At pH 7.5, the magnetizable solid-phase second-antibody RIA gave satisfactory standard curves for serum amitriptyline and nortriptyline (Figure 3). Amitriptyline had a cross reactivity of 225% relative to nortriptyline. A study of the pH-dependence of assay specificity showed that amitriptyline was more effective than nortriptyline in competing for antibody binding between pH 5 and pH 9, the reverse being true at pH 9 to pH 12. At pH 9.0,

![Graph showing RIA standard curve (pH 7.5) for serum nortriptyline with 125I-labeled FTC-NT and liquid-phase second-antibody separation.](graph.png)

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**Table 1. Preparation and Properties of 125I-Labeled Nortriptyline Derivatives**

<table>
<thead>
<tr>
<th>Iodinated derivative</th>
<th>Iodination time, s</th>
<th>Yield of methanol-eluted product, %</th>
<th>Immunoreactivity of product, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTC-NT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20</td>
<td>36</td>
<td>65</td>
</tr>
<tr>
<td>FTC-NT</td>
<td>60</td>
<td>91</td>
<td>80</td>
</tr>
<tr>
<td>NALH-NT</td>
<td>45</td>
<td>84</td>
<td>85</td>
</tr>
</tbody>
</table>

<sup>a</sup> in terms of incorporation of radioactivity.

<sup>b</sup> Percent bound in the presence of excess antibody.

<sup>c</sup> FTC-NT, fluorescein thiocarbamyl nortriptyline; NALH-NT, N-acetyl-L-histidine conjugate of nortriptyline.
Table 2. Cross Reactivities in Nortriptyline RIA Systems

<table>
<thead>
<tr>
<th>Substance</th>
<th>Labeled FTC-NT used, pH 7.5</th>
<th>Labeled NALH-NT used, pH 9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nortriptyline</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>130</td>
<td>100</td>
</tr>
<tr>
<td>10-Hydroxyamitriptyline</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Butriptyline</td>
<td>54</td>
<td>33</td>
</tr>
<tr>
<td>Cyclobenzaprine</td>
<td>59</td>
<td>30</td>
</tr>
<tr>
<td>Protriptyline</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Doxepin</td>
<td>22</td>
<td>36</td>
</tr>
<tr>
<td>Dothiepin</td>
<td>62</td>
<td>35</td>
</tr>
<tr>
<td>Imipramine</td>
<td>53</td>
<td>55</td>
</tr>
<tr>
<td>Desipramine</td>
<td>40</td>
<td>52</td>
</tr>
<tr>
<td>2-Hydroximipramine</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>2-Hydroxydesipramine</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>10-Hydroxyimipramine</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Imipramine-N-oxide</td>
<td>54</td>
<td>55</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Opipramol</td>
<td>38</td>
<td>52</td>
</tr>
<tr>
<td>Trimipramine</td>
<td>36</td>
<td>23</td>
</tr>
</tbody>
</table>

* The amount of each substance required to displace 50% of specifically antibody-bound labeled nortriptyline was determined, and results are expressed relative to nortriptyline.

standard curves obtained using either amitriptyline or nortriptyline serum standards were virtually superimposable (Figure 3). Logit transformation (15) did not linearize any of the standard curves.

The smallest detectable concentration of serum nortriptyline in the pH 9.0 assay system was 3.6 µg/L (the smallest detectable dose was 180 pg). By use of the anti-nortriptyline serum at a working dilution of 2500-fold, a more sensitive assay was obtained, with a minimal detectable concentration of 360 ng/L serum nortriptyline.

We made a detailed study of assay specificity at pH 9.0 (Table 2). Three serum specimens from patients receiving nortriptyline were used to assess within-assay precision (20 replicates) at pH 9.0, giving mean results 29.5, 108, and 194 µg/L, with CVs of 6.4, 4.8, and 4.9%, respectively. Three specimens were measured in 20 different assays, giving mean results 41.1, 69.4, and 137 µg/L, with between-assay CVs of 9.5, 7.5, and 6.6%, respectively.

Amitriptyline and nortriptyline were both added to pooled normal human serum in various amounts. Using the pH 9.0 assay system with nortriptyline serum standards, we determined the apparent total nortriptyline concentration. Analytical recovery of total active drug was good (Table 3).

In preparing 125I-labeled NALH-conjugated ligand and using it in these RIA systems, we found that deamethylnortriptyline could be substituted for nortriptyline with no difference in results.

Patients’ Specimens

Seventeen specimens of serum or plasma from patients receiving amitriptyline or nortriptyline (possibly in combination with other drugs) were assayed by the RIA involving 125I-labeled FTC-NT and, in an independent laboratory, by a previously established RIA (7). Nortriptyline serum standards were used, and results expressed as apparent total nortriptyline concentration (µg/L). A correlation coefficient of 0.98 was found. The regression line, calculated as recommended by Lloyd (17) with the assumption that the two methods had equal precision, was given by y = 0.96x - 2 (present method y-axis; established method x-axis).

Discussion

The amino groups of ovine albumin were succinylated in order to reduce protein–protein reactions in the subsequent carbodiimide coupling with nortriptyline. The latter reaction proceeded easily and completely to give an immunogenic product that was freely water-soluble in spite of its high content of hydrophobic tricyclic residues.

Nortriptyline was 125I-labeled by preparing suitable derivatives, which were then radioiodinated. FITC will react with secondary amines to give the corresponding fluorescein-carbamyl derivative, as demonstrated and illustrated, for example, for the fluorescent derivatization of chlorpromazine metabolites (18). The fluorescein group may be easily iodinated, presumably at positions on the xanthene ring structure as shown by Gabel and Shapiro (19), and this property has recently been shown to have useful applications in radiiodine-labeling methodology (19, 20). We found that the characteristic color change upon introduction of iodine into the fluorescein nucleus provided an unusual and convenient visual check of the progress of the radioiodination reaction.

As an alternative approach to the labeling of the amines nortriptyline and desmethylnortriptyline, the carboxylic compound NALH was used (a reversal of the familiar chemistry involving histamine in the labeling of carboxyl-functionalized haptens). Iodination of NALH-NT presumably occurs at the carbon atoms of the imidazole ring (21).

A column technique, previously used in the preparation of 125I-labeled methotrexate derivatives (19), was adopted for

Table 3. Analytical Recovery of Total Amtriptyline and Nortriptyline from Serum by Magnetizable Second-antibody Solid-phase RIA

<table>
<thead>
<tr>
<th>Substance</th>
<th>Conc., µg/L</th>
<th>50</th>
<th>50</th>
<th>100</th>
<th>100</th>
<th>200</th>
<th>200</th>
<th>200</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amtriptyline</td>
<td></td>
<td>25</td>
<td>25</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td></td>
<td>75</td>
<td>98</td>
<td>98</td>
<td>97</td>
<td>98</td>
<td>98</td>
<td>104</td>
<td>112</td>
</tr>
<tr>
<td>Apparent total nortriptyline</td>
<td>measured</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery of total active drug</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td>98</td>
<td>97</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>104</td>
<td>112</td>
</tr>
</tbody>
</table>

* With 125I-labeled NALH-NT, pH 9.0 system.
purifying radiiodination reaction products. The method is safer and simpler than are commonly-used thin-layer chromatographic procedures. It takes advantage of the affinity shown by many organic species, notably phenolic compounds and their iodinated derivatives, for cross-linked dextran gels (22). We obtained $^{125}$I-labeled NAH-NT in good yield and with high immunoreactivity. With FTC-NT, however, a compromise had to be made between yield and immunoreactivity of the product.

Non-specific binding of the $^{125}$I-labeled nortriptyline derivatives to plastic tubes was minimized by the use of a relatively high concentration of bovine albumin in the assay buffers, a measure also adopted in an RIA for phentoin, another hydrophobic hapten (23).

The within- and between- assay precision of our RIA systems is similar to the precision of previously reported immunoassays for tricyclic antidepressants (7-10). Although lower CVs have been obtained by chromatographic methods (5, 6), the precision of the immunoassays is sufficient for therapeutic monitoring purposes. Because therapeutic concentrations of these drugs are comparatively high, we de-sensitized our assays by the use of relatively high concentrations of anti-nortriptyline serum. This may account for the non-linearity of logit-log plots, since the condition that the antibody be near saturation (15) was probably not satisfied in the low-dose region of the standard curves. The sensitivity of our RIA systems, as optimized for routine therapeutic monitoring, was comparable with that of gas-liquid chromatography (5), "high-performance" liquid chromatography (6), and immunoassays involving tritium (8, 9) or enzyme (10) labels. It was possible, however, by virtue of the superior specific activity of the $^{125}$I label, to extend assay sensitivity below concentrations attained by any previous method.

The cross reactivity of other drugs (Table 2) was most affected by changes in the tricyclic nucleus, with alterations in side-chain structure having less effect. Ring-hydroxylated metabolites showed low cross reaction. Previously reported immunoassays have shown similar specificity (7-10). With $^{125}$I-labeled FTC-NT and liquid-phase second-antibody separation, apparent total amitriptyline and nortriptyline concentrations in patients' serum or plasma specimens correlated well with those reported with use of the existing RIA of similar specificity (7).

The overall assay time for the liquid-phase second-antibody RIA was about 3 h, with total technician time between 30 min and 1 h. The use of second-antibody coupled to magnetizable solid-phase enabled the time required for the incubation and separation steps to be reduced by a total of about 1 h. The magnetic-separation technique (13, 24) removed the need for a centrifuge and allowed the entire assay to be performed at the bench, with consequent saving in technician time. Supernatants were easily and reliably aspirated, with the solid-phase firmly held in the assay tubes by the magnet.

Reversal of the relative cross reactivities of amitriptyline and nortriptyline was noted when an RIA system involving tritiated ligand was examined at pH 7.4 and pH 10.6 (7). In a comprehensive study we showed that at pH 9.0 the two drugs were equally effective (on a weight basis) in competing with $^{125}$I-labeled NAH-NT for antibody binding over the entire working range of the solid-phase second-antibody assay, presenting the possibility of obtaining true values for total amitriptyline and nortriptyline concentrations in patients receiving amitriptyline—previously reported immunoassays (7-10) would give only a weighted apparent result for total drug concentration in such circumstances, because amitriptyline and nortriptyline, although of roughly equal clinical effectiveness, cross reacted to a different extent.

No simple immunoassay system could enable the determination of both amitriptyline and nortriptyline in the same sample, as can be done in a single test by gas-liquid chromatography (5) or "high-performance" liquid chromatography (6). At present, such specific quantitation has only been possible after selective extraction of the individual drugs (9), which is not a practical routine procedure. However, reliable measurement of total active drug might suffice for therapeutic monitoring in most cases (4).

Pure drugs (chiefly as hydrochlorides) used in these studies were kind gifts obtained as follows: nortriptyline from Lilly Research Centre, Windlesham, Surrey GU20 6PH, U. K.; desmethylnortriptyline from Dr. R. A. Braithwaite, Poisons Unit, New Cross Hospital, London SE14 5ER, U. K.; amitriptyline and its metabolite, and nortriptyline from Merck Sharp and Dohme Research Laboratories, Hoddesdon, Herta. EN11 9BU, U. K.; imipramine and desipramine, and their metabolites from Geigy Pharmaceuticals, Maclesfield, Cheshire SK10 2LY, U. K.; :fluoxetine from Lundbeck, Luton, Bedfordshire LU1 5BB, U. K.; others from their respective U. K. suppliers.

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