Rapid Radioisotopic Procedure for Determination of Nortriptyline in Plasma

K. P. Maguire,1 G. D. Burrows,1 J. P. Coghlan,2 and B. A. Scoggins2

With the widespread use of tricyclic antidepressant drugs, the relationship between the concentration of the drug in the plasma and the therapeutic response is of considerable interest. We describe a double-isotope derivative dilution procedure for measuring plasma nortriptyline. In the method, [14C]nortriptyline is used for estimating procedural losses and [3H]acetic anhydride for derivative formation. The assay is rapid and adequately specific, sensitive, precise, and reproducible for routine clinical use. We used it to investigate the variation in steady-state drug concentrations in plasma of persons who were on a 150 mg/day dose of nortriptyline. Intra-individual variation from day to day was 10–14%. This variation was not significantly affected by the dosage schedule, the time of sampling after an oral dose, or the storage of the plasma samples. For 19 patients on 150 mg of nortriptyline per day, the mean concentration in plasma was 181 ± 22 (SE) μg/liter, a value that compares well with our previous findings and those of other groups.

We report a rapid double-isotope derivative dilution assay for measuring nortriptyline, based on a modification (1) of an isotope-derivative dilution assay (2). In the method [14C]nortriptyline is used to correct for procedural losses and [3H]acetic anhydride for derivative formation. Chromatography of the radiolabeled derivative has been included to improve specificity.

The double-isotope derivative dilution assay has advantages over the isotope-derivative assay. Because each sample has its own recovery indicator, a standard curve for each assay is unnecessary, as is also the duplicate analysis of samples. Moreover, the method has advantages over other currently available procedures such as gas–liquid chromatography (3–5) and massfragmentography (6, 7), both of which are more time-consuming and require expensive, sophisticated equipment. Suitable radioimmunoassay procedures have yet to be described.

Measurement of plasma nortriptyline is important in monitoring an individual patient’s therapeutic response because there can be large inter-individual differences for patients who are on the same dosage regimen.

Materials and Methods
Purification of [14C]Nortriptyline

[14C]Nortriptyline (18 mCi/g) was purified every 14 days by thin-layer chromatography in the system benzene/dioxane/ethanol/diethylamine, 50/40/5/5 by volume (8). Purified material was stored in ethanolic solution in the dark at 4°C.

Extraction of Plasma Samples

Plasma samples (acidified with 1/5-volume of 0.1 mol/liter HCl) were stored at −20°C until assay. Siliconized 7-ml glass-stoppered tubes were used in all steps.

After addition of [14C]nortriptyline (1.8 × 10−4 μCi), 1-ml plasma samples were alkalinized with 0.4 ml of 1 mol/liter NaOH. Treated samples were gently extracted with 3 ml of n-heptane by rotation for 15 min. Any emulsions were broken by freezing the samples in liquid nitrogen and allowing them to thaw. A 2-ml aliquot of each heptane extract was transferred to clean tubes for acetylation.

Derivative Formation

Anhydrous pyridine, 100 μl, and 50 μl of 0.02% vacuum-distilled [3H]acetic anhydride (330 Ci/mol) (The Radiochemical Centre, Amersham, U. K.) in anhydrous benzene were added to each sample. The method of purification and determination of specific activity of the [3H]acetic anhydride has been described previously (9). After thorough mixing of their contents, the stoppered tubes were placed in a water bath at 60°C for 1 h.

Purification of the Derivative

Hydrolysis and re-extraction. The solvent was evaporated in an air stream and 2 ml of 0.1 mol/liter NaOH was added per sample. The stoppered tubes were returned to the 60°C water bath for 15 min, then cooled to room temperature. n-Heptane, 2 ml, was added to each tube and the acetylated derivative extracted by rotation for 10 min. After phase separation (about 10 min), the aqueous phase was aspirated. A further 2 ml of 0.1 mol/liter NaOH was added and the samples were rotated again for 10 min. After phase separation, 1-ml aliquots of the heptane phase were transferred to clean tubes.

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Thin-layer chromatography. After addition of 10 μg of nortriptyline acetate (prepared by reacting nortriptyline with unlabeled acetic anhydride in a similar manner to that described above) as ultraviolet marker, we evaporated the solvent. The samples were applied to aluminum sheets precoated with silica-gel (20 cm × 20 cm × 0.25 mm, Silica-Gel GP254/5554; Merck) in three successive 0.2-ml portions of methanol in methylene chloride (40/60 by vol). Twelve samples were applied per sheet. The samples were developed at room temperature in the ascending system benzene/acetone 1/1 by vol (2). The samples were located by the absorption of the added marker at 254 nm, and the appropriate area of the silica gel was scraped with the help of vacuum into glass “transpettes” (Clay-Adams) plugged with glass wool. Samples were eluted into glass vials by passing 10 ml of the methanol/methylene chloride mixture through the pipettes. The solvent was evaporated and 10 ml of a phosphor solution was added (4 g PPO and 40 mg dimethyl POPOP/liter of toluene, Packard Instrument Co.). (The thin-layer chromatography step can be omitted, in which case, after hydrolysis and reextraction, the 1-ml aliquots of the solvent phase are transferred to glass vials, the solvent is evaporated, and phosphor solution added.)

Determination of Mass

The radioactivity of samples was counted for 20 min in a liquid scintillation spectrometer (Packard). Standard vials for the determination of machine background, ³H and ¹⁴C counting efficiencies, specific activity of the [³H]acetic anhydride, recovery of [¹⁴C]nortriptyline indicator, and leakage between channels were counted with each assay.

Equations for the estimation of the mass of double-labeled samples by use of isotope derivative dilution techniques have been described previously (10, 11). We followed the latter method (11) in estimating nortriptyline.

Results

Analytical Variables

Precision. Plasma pools of known nortriptyline concentration, 100 and 200 μg/liter, were used to evaluate the precision of the assay. In most patients treated clinically, concentrations of the drug in plasma lie between these values. The precision (CV) of the assay at the two concentrations was ±7%. Analysis of a quality-control sample (about 100 ng of nortriptyline per milliliter of water) in each assay gave a result of 90.4 ± 7.0 μg/liter (mean ± SD; n = 34) and a CV of 8%. Reproducibility was further demonstrated by re-assay of 19 samples. Results for first assay were 158 ± 65 μg/liter (mean ± SD) and on second assay after 120 days, 159 ± 70 μg/liter.

Analytical recovery. Accuracy was reflected by a linear recovery of nortriptyline over the range 25-200 μg/liter. Recovery of the [¹⁴C]indicator through the method was 23.9 ± 3.5% (mean ± SD; n = 43). Losses during the early extraction steps predominantly accounted for the low recovery. The chromatography step accounted for 7% of losses, because the recovery without chromatography was 31.3 ± 3.0% (mean ± SD; n = 43).

Interferences. Inclusion of the chromatography step eliminated interference by the major metabolite of nortriptyline, 10-hydroxy-nortriptyline. Rf values for the acetylated derivatives were 0.32 (metabolite) and 0.37 (nortriptyline).

Salicylic aldehyde was used by Gram and Fredricson-Overo (12) to block primary amines; hence desmethyl-nortriptyline could not interfere in the acetylation reaction. Assay of samples by this method and by the present double-isotope procedure lead to no significant difference in the plasma levels obtained (P > 0.9, Student's t-test).

Non-specific interference was also decreased significantly (P < 0.05) by inclusion of the chromatography step. The non-specific blank of the assay was 14.6 ± 10.9 μg/liter (mean ± SD) without, and 2.7 ± 2.9 μg/liter with chromatography (n = 42).

Comparison with Other Methods

Close correlation was obtained between the isotope-derivative assay used previously (1) and the new assay. Samples analyzed by both methods resulted in a mean of 158 ± 17 (old assay, ±SE) and 181 ± 22 (new assay) μg/liter. These were not significantly different (n = 19, P > 0.05).

Our results for persons on a daily dose of 150 mg compare favorably with other published data (1, 14-16). In our most recent study by use of our method, we found a mean drug concentration in plasma of 181 ± 15 μg/liter (±SE) for 22 patients [2 men, 20 women; mean age, 41.6 ± 9.7 y (±SD)] (13). This compares with mean values of 171 ± 19 (±SE; n = 32) and 171 ± 13 (n = 65) mg/liter obtained in two previous studies (1, 14).

Results reported by other groups also compare well. Lyle et al. (15) found a mean plasma nortriptyline concentration of 170 ± 24 μg/liter (±SE, n = 14, on 150 mg/day) and Braithwaite (16) found a mean of 166 μg/liter (n = 10, 80-200 mg/day); both used the gas-chromatographic method of Braithwaite and Widdop (3).

Variation

Within-individual. In a study of two individuals, plasma nortriptyline was measured daily for 10 days. Variations of ±10% and ±12% were observed in their steady-state values (Table 1). Patient a was studied for a further 30 days and a variation of ±14% was observed for 23 samples taken during the total period.

Variation in steady-state concentrations in plasma. Some factors that could contribute to the variation observed in steady-state plasma values were examined. As shown in Figure 1, the time of sampling after the last oral dose did not appear to contribute importantly, because no significant change occurred during an 8-h period.

When persons were on different dosage schedules, all
Table 1. Between-day Variation in Steady-state Concentrations of Nortriptyline in Plasma of Two Patients

<table>
<thead>
<tr>
<th>Day</th>
<th>Drug concn, μg/liter</th>
<th>(a)</th>
<th>(b)</th>
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<tbody>
<tr>
<td>1</td>
<td>251</td>
<td>70</td>
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</tr>
<tr>
<td>2</td>
<td>269</td>
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<td></td>
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<td>3</td>
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<td>10</td>
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<td>74</td>
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Mean ± SD 257 ± 26 69 ± 8
CV 10.2% 12.2%

Both patients were treated with 150 mg/day for 21 days before and during this study. Patient \(b\) was also receiving 0.25 mg of digoxin, 0.5 mg of navi- drex, 1 tab. of slow-release K, 5–10 mg of nitrazepam, and 1 tab. Glycerol trinitrate when necessary.

totalling 150 mg of nortriptyline per day, little difference was found in plasma concentrations, and they varied similarly for the four such schedules (Figure 2). This has important clinical implications, because a once-nightly dose minimizes side-effects and takes full advantage of the sedative effects of the drug.

Effects of storage. The method of storage of the plasma samples may be important. It appears that after separation of the plasma, the nortriptyline is stable for several days at room temperature (Table 2). If kept frozen, no measurable change occurs during 120 days.

Discussion

For routine clinical use, an assay must be specific, sensitive, precise, and reproducible. If possible, it should be simple, quick, and inexpensive. The described double-isotope derivative dilution assay fulfills most of these requirements.

Specificity was achieved by inclusion of a chromatography step, which adequately separates nortriptyline from its major plasma metabolites, 10-hydroxy-nortriptyline and demethylnortriptyline (17). We found a close correlation between results obtained by this new assay and the isotope-derivative assay (1), further indicating the specificity of both techniques.

The lower limit of sensitivity of the assay is 5 μg/liter, as determined primarily by the low specific activity of the \([^{14}C]\)nortriptyline. If this could be brought closer to the theoretical (65 Ci/mol) then the sensitivity would be greatly improved. Reduction in the nonspecific blank value by further chromatography would also improve sensitivity. However, the sensitivity obtained with this assay is more than adequate for routine clinical use.

Our results obtained with this assay compare favorably with those from our earlier studies and those from the workers who used gas-chromatography. The one exception is the Scandinavian group, who observe lower values for plasma in their studies by gas-chromatography–mass-spectrometry (18–20). The reasons for the differences between these studies and others have been discussed (21).

A double-isotope derivative dilution method for measuring concentrations in plasma of a similar antidepressant, maprotiline ("Ludronil"; Ciba-Geigy) has been published (22), but the author did not take full advantage of the principle of double-isotope dilution because standards were used in calculating the mass of the drug in unknown plasma samples.

Once the assay is established, two important considerations when measuring plasma nortriptyline are the between-day variations within individuals on a fixed dose and the within-day within-individual variations
in the steady-state drug concentration in plasma. The latter is largely (83%) genetically determined as a result of genetic control over the metabolism of the drug (23, 24). Alexanderson (24) also found that only 17% of the variation in nortriptyline plasma levels was due to between-day (13%) and within-day (4%) fluctuations. The oral dose is obviously an important factor (13) as also may be other drugs administered at the same time (12, 25, 26). The time of sampling after an oral dose, the dosage schedule on a fixed regimen, and the method of handling plasma do not appear to contribute significantly to the variations observed in steady-state values for plasma drug concentration.

A between-day, within-individual variation of 10–14% has been found, which is close to the results of Alexanderson (24). Because the amount of variation likely to occur in steady-state values has been established, daily measurement of plasma nortriptyline is probably unnecessary. Weekly or twice-weekly samples would be quite satisfactory. Frequent sampling is necessary when studying pharmacokinetics or drug interactions.

For routine clinical use, the double-isotope assay without the chromatography step is satisfactory. Although some sensitivity and precision is lost, less time is required to complete each assay and more samples can be analyzed per assay.

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References