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ASSAY OF HUMAN PLASMA FOR NORTRIPTYLINE BY RADIOACETYLATION AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A highly specific and sensitive thin-layer chromatographic method for determining nortriptyline levels in plasma is presented. The procedure involves extracting nortriptyline, acetylating it with radioactive acetic anhydride, resolving acetylnortriptyline by thin-layer chromatography, and measuring its radioactivity.

INTRODUCTION

Nortriptyline is a classical example of a drug which attains vastly different plasma levels in different people. These interindividual differences, which are considered to be under polygenic control, extend over a 25-fold range even after establishing a steady state by repeated drug administration[1]. The additional observation that the compound expresses antidepressive activity only within a range of plasma concentration[1] adds importance to the accuracy of nortriptyline assay.

Both the specificity and the sensitivity of nortriptyline assay have been investigated. Hucker and Stauffer[2] improved the specificity of the gas chromatographic procedure published by Braithwaite and Widdop[3], but did not increase sensitivity beyond 20–25 ng/ml of plasma. Similarly, Faber *et al.*[4] developed a highly specific thin-layer chromatographic (TLC) assay, but found the lower limit of assay to be 50 ng/ml. Fredricson Overø[5] acetylated nortriptyline with radiolabeled acetic anhydride, extracted the derivative, and counted its radioactivity. This method excluded the concurrent assay of interfering primary amines by prior reaction with salicylic aldehyde, but the publication does not provide complete experimental details and does not state the sensitivity of the assay. The method described below is also a modification of the Hammer and Brodie procedure[6]. Hammer and Brodie extracted desipramine from plasma, acetylated with radioactive acetic anhydride, extracted the acetyldesipramine, and counted its radioactivity. This procedure is unsatisfactory for nortriptyline because radioactive contaminants are extracted with the acetylated drug. Hammer and Brodie used distilled water, not plasma, to generate their reagent blank. They reported that normal plasma contained material which increased their desipramine assays by 3–4 ng/ml. This may accurately reflect their observations, but we found normal plasma to contain far more material which interfered with nor-

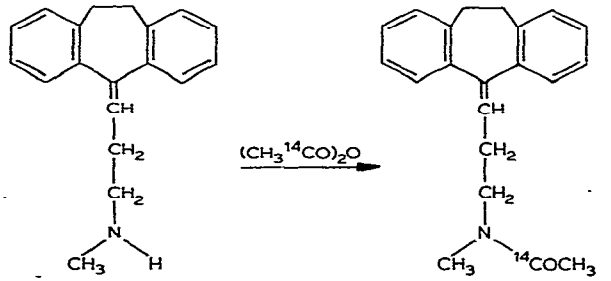
triptyline assay. Additionally, we observed wide variations in the quantity of interfering material present in different plasma samples.

To obviate the lack of specificity and the quantitative problem discussed above, TLC was employed to separate labeled acetylnortriptyline from other labeled substances (presumably acetylated plasma components). The balance of the procedure involves locating acetylnortriptyline by radiochromatogram scanning, removing it from the plate, and counting it by liquid scintillation spectrometry.

EXPERIMENTAL

Reagents

The simple reaction used in this assay is:



Nortriptyline Acetylnortriptyline

Nortriptyline hydrochloride was obtained from Eli Lilly (Indianapolis, Ind., U.S.A.). [1-^{14}C]Acetic anhydride (0.25 mCi, 5.1 mg) was received in a sealed ampoule from New England Nuclear (Boston, Mass., U.S.A.). The reagent was transferred to 10 ml of benzene which had been dried over metallic sodium. This solution was stored in a stoppered tube in a desiccator under refrigeration. Before opening the tube, the desiccator was allowed to reach room temperature. The solvents employed in this study were reagent grade.

Apparatus

Used to examine reaction mixtures was a Perkin-Elmer Model 900 gas chromatograph fitted with a 6 ft. × 2 mm I.D. coiled glass column packed with 3% SE-30 on 100–120 mesh Gas-Chrom Q. The carrier gas, nitrogen, was used at a flow-rate of 45–50 ml/min. The oven, injection and manifold temperatures were 205°, 230°, and 230°, respectively.

The ^{14}C content of radioactive samples was determined with a Packard TriCarb Model 3320 liquid scintillation spectrometer using a fluorophore containing 100 g of naphthalene, 7.0 g of 2,5-diphenyloxazole (PPO), and 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (dimethyl-POPOP) in 1.0 l of dioxane. The external standarization method of quench correction was used.

The other equipment used were 5 cm × 20 cm silica gel GF plates (Analtech, Newark, Del., U.S.A.), a UV lamp (Ultra-Violet Products, San Gabriel, Calif., U.S.A.), and a Packard Model 7201 radiochromatogram scanner.

Identification of acetylated nortriptyline

One milligram of nortriptyline hydrochloride was placed into a conical-bottomed 1-ml reaction vial, and 0.25 ml of pyridine, 0.65 ml of dry benzene, and two molar equivalents of unlabeled acetic anhydride in dry benzene were added. The vial was capped and incubated at 60° for 1 h. Aliquots of the reaction mixture were examined by gas–liquid chromatography (GLC) and by TLC. The solvent for TLC was cyclohexane–1,4-dioxane (1:1). The plates were inspected under short-wave UV light and submitted to radiochromatogram scanning.

Preparation of standard curves

Solutions of nortriptyline hydrochloride in ethanol were pipetted into 15-ml centrifuge tubes. Duplicate tubes containing 50, 25, 12.5, 6.25, and 3.125 ng of nortriptyline (as the base) were prepared. The ethanol was evaporated under nitrogen and 1 ml of human plasma was added to each tube. The tubes were thoroughly shaken to effect solution. Then 0.5 ml of 1 *N* NaOH was mixed with the contents of each tube. Five milliliters of hexane were added to each tube; the tubes were capped and shaken gently for 10 min. The tubes were centrifuged and the hexane phase was removed with pipettes which had been rinsed with isoamyl alcohol and thoroughly drained. Then the extraction process was repeated using the same pipettes without rinsing. The two extracts per tube were pooled, concentrated, transferred to a vial, and evaporated with nitrogen to dryness. Added to each vial were 0.25 ml of pyridine, 0.65 ml of dry benzene, and 100 μl of the [^{14}C]acetic anhydride solution. The vials were capped and heated at 60° for 1 h. After the solutions had evaporated with nitrogen under a closed hood, 2 ml of 0.1 *N* NaOH were added and the mixture was heated at 95° for 15 min to hydrolyze excess acetic anhydride. The tubes were cooled to room temperature and extracted twice with 5 ml of heptane. The extracts from duplicate tubes were combined, concentrated, and transferred with washings to 1-ml conical-bottomed tubes. The solutions were concentrated under nitrogen to about 50 μl and streaked on TLC plates in narrow bands about 25 mm long. The vials were rinsed three times with heptane and each rinse was applied to the plate. Backwashing the sample on the plate was found to be unnecessary when the solutions were streaked in a straight line. The plates were developed in cyclohexane–1,4-dioxane (1:1) to a distance of 15 cm from the origin. After drying each plate was scanned, and the area of the plate containing [^{14}C]acetylnortriptyline was scraped off, transferred to a scintillation vial, and shaken gently for 30 min with 1 ml of 50% methanol. Then, scintillation fluid was added and the vial was counted for radioactivity for 50 min.

Eight variables in the procedure were evaluated. These included the number of hexane and heptane extractions, the volumes of extraction solvents, the rinsing of transfer pipettes, and the interval between opening and using vials of [^{14}C]acetic anhydride.

RESULTS AND DISSCUSION

Under the GLC conditions described, nortriptyline and acetylnortriptyline gave sharp, well-defined peaks with retention times of 4.0 min and 14.0 min, respectively. Examination of the reaction mixture after nortriptyline acetylation showed the presence of no detectable nortriptyline by GLC. The completeness of acetylation

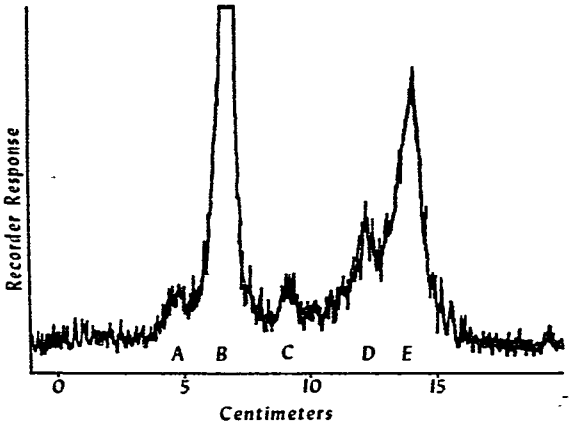


Fig. 1. Representative TLC radioscan showing [^{14}C]acetylnortriptyline (B) and four radioacetylated plasma components (A, C, D, and E).

was confirmed by TLC; acetylnortriptyline (R_F 0.44) but no nortriptyline (R_F 0.04) was observed on chromatograms which were inspected under UV light and scanned for radioactivity. As illustrated in Fig. 1, however, there was extensive acetylation of several plasma components. It would be interesting to identify these extractable endogenous compounds and to determine the suitability of the present methodology for their assay.

An evaluation of the variables showed that the assay procedure described above in detail is the most sensitive. It is capable of measuring nortriptyline levels down to 5 ng/ml of plasma (Fig. 2). Optimal use of this procedure requires the dilution of plasma specimens which contain nortriptyline concentrations greater than 50 ng/ml. A major factor in the sensitivity of the assay is the purity of the radioactive acetic anhydride. This reagent is hydrolyzed so readily, even under very favorable storage conditions, that approximately 25% of the assay sensitivity is lost for each week that the vial is kept open. Another important point is that transfer pipettes must be rinsed with isoamyl alcohol (or a substitute) to avoid the adsorption of

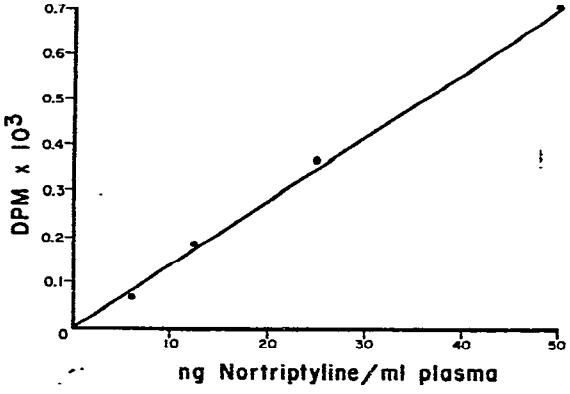


Fig. 2. Standard curve for nortriptyline assay.

nortriptyline to the glass. Omission of this simple rinsing operation can cause losses as high as 80%. It is noteworthy that recovery was not improved by increasing the number of extractions with hexane to collect nortriptyline or with heptane to collect acetylnortriptyline.

A point to be emphasized is that all variations of the assay gave straight-line calibration curves which intersected both the abscissa and ordinate at 0 and extended to a nortriptyline concentration of 50 ng/ml of plasma. This assay method is considerably more sensitive than those previously reported[1–3] and may serve to determine plasma levels of nortriptyline after single doses; data of this kind are presently unavailable.

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