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Note

Quantitation of amitriptyline and nortriptyline in human serum

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Amitriptyline and nortriptyline, its N-desmethyl metabolite, are widely used in the treatment of depression. Large individual differences in steady-state plasma concentrations of these drugs were observed in patients receiving the same dose, and several studies indicate an optimum clinical response to these tricyclic antidepressants may be associated with a relatively narrow range of steady-state plasma concentrations [1—4]. Safe and effective drug therapy can only be accomplished by monitoring amitriptyline and nortriptyline concentrations in serum; hence, there is need for methods which can be performed routinely in clinical chemistry laboratories.

Numerous methods for the determination of tricyclic antidepressants have been reported, however, none appears to have gained widespread routine use due to complexity or lack of specificity and sensitivity. Hammer and Brodie [5] assayed desmethylimiprimine and nortriptyline by labelling these secondary amines with [^{3}H]acetic anhydride; however, this method is not applicable to measurement of tertiary amines including amitriptyline. Hucker and Stauffer [6] and Braithwaite and Widdop [7] used gas—liquid chromatography (GLC) with flame ionization detection to measure amitriptyline and the N-trifluoroacetyl derivative of nortriptyline. These methods, however, require large volumes of serum, are subject to interference from endogenous compounds, and are not readily applicable to routine monitoring. Borgå and Garle [8] reported an electron-capture—gas chromatographic procedure for the N-heptafluorobutyric acid derivative of nortriptyline. Similarly, Walle and Ehrsson [9] developed a very sensitive electron-capture detection method for this derivative of nortriptyline. Neither of these procedures, however is applicable to the determination of amitriptyline.

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Recently a method for measuring various tricyclic antidepressants by means of gas chromatography—mass fragmentography has been reported [10]. Although this method is specific, it requires a large volume of serum (4 ml) and the instrument is expensive and not yet widely available in clinical chemistry laboratories.

Recently several methods using GLC with nitrogen sensitive detection have been described for the quantitative determination of tricyclic antidepressants. Gifford and co-workers [11] used an alkali flame ionization detector and a unique backflush system to determine various tricyclic antidepressants without need for derivatization. Jorgensen [12] determined amitriptyline and the N-acetyl derivative of nortriptyline, however, it is our experience that the latter compound chromatographs poorly. Bailey and Jatlow [13] recently described a method for amitriptyline and nortriptyline which did not require derivatization of the secondary amine, but used 3 ml of serum.

We present a GLC method utilizing nitrogen sensitive detection for the simultaneous determination of amitriptyline and nortriptyline as the N-trifluoroacetyl derivative which has been developed for routine use in our Therapeutic Drug Monitoring Laboratory. Although formation of this derivative requires an additional step in the procedure, the resultant compound has much better chromatographic characteristics; hence, sensitivity of the method is improved and the analytical procedure is less susceptible to changes in column conditioning.

MATERIALS AND METHODS

Chemicals

Acetone, ethyl acetate, hexane and isopropanol, all nanograde, were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.); boric acid, sodium hydroxide, and sulfuric acid were obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.); heptane (chromatoquality) was obtained from Matheson, Coleman & Bell, (East Rutherford, N.J., U.S.A.); potassium chloride was obtained from Fisher Scientific (Fairlawn, N.J., U.S.A.). Amitriptyline HCl was provided by Merck, Sharp & Dohme Research Lab. (West Point, Pa., U.S.A.); doxepin HCl was provided by Pfizer Pharmaceutical (Brooklyn, N.Y., U.S.A.); maprotiline HCl was provided by Ciba-Geigy (Basle, Switzerland); nortriptyline HCl was provided by Eli Lilly (Indianapolis, Ind., U.S.A.); tri(butoxyethyl)phosphate was obtained from ICN Life Sciences Group (Plainview, N.Y., U.S.A.) and trifluoroacetic anhydride (99+%) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.).

Reagents

Prepare stock solutions (100 μg/ml) of amitriptyline (AMI), nortriptyline (NOR), doxepin (DOX) and maprotiline (MAPRO) by dissolving 10 mg of each compound in methanol to a volume of 100 ml. Prepare working solutions (1 μg/ml) of AMI and NOR by diluting 1 ml of their respective stock solutions to 100 ml with methanol, prepare an internal standard working solution (40

ng/ml) by evaporating to dryness 400 μl of DOX and MAPRO stock solution and dissolving the residue in heptane—isopropanol (99:1) to a volume of 1 l. Prepare a pH 10 borate buffer by dissolving 24.7 g of boric acid, 29.8 g of potassium chloride and 14.1 g of sodium hydroxide in distilled water to a volume of 1 l [14]. Prepare 0.1 *N* sulfuric acid by diluting 2.8 ml of concentrated sulfuric acid with distilled water to a volume of 1 l. Prepare 1 *N* sodium hydroxide by dissolving 40 g of sodium hydroxide in distilled water to a volume of 1 l.

Preparation and extraction of standards, controls and patient samples

Prepare serum standards (0, 50, 100, 200 and 300 ng/ml) by adding appropriate amounts (0, 50, 100, 200 and 300 μl) of AMI and NOR working solutions to 16 × 125 mm screw-top culture tubes and evaporating them to dryness under nitrogen at room temperature. Add 1.0 ml of pooled serum to each standard and 1.0 ml of control and patient serum to their respective 16 × 125 mm screw-top culture tubes. To each sample, add 1.0 ml of pH 10 borate buffer and vortex briefly. Add 7.0 ml of internal standard working solution to each tube using a Repipette from Lab. Industries (Berkeley, Calif., U.S.A.) and close with PTFE-lined screw caps.

Extract the samples by shaking on an Eberbach shaker for 15 min; then centrifuge for 10 min at 2000 rpm. Transfer the heptane layer (top) to clean tubes, containing 1.3 ml of 0.1 *N* sulfuric acid. Cap, extract as above for 10 min and centrifuge for 10 min.

Aspirate the organic layer (top), add 500 μl of 1 *N* sodium hydroxide to the aqueous layer and vortex each briefly. Add 5.0 ml of hexane to each, extract and centrifuge the samples as described above.

Transfer the hexane (top) layer to 5.0 ml mini-vials (Applied Science Labs., State College, Pa., U.S.A.) and evaporate the hexane to dryness (being careful not to overdry) under nitrogen at room temperature. To each residue, add 100 μl of ethyl acetate and vortex briefly. Add 30 μl of trifluoroacetic anhydride to each vial and cap them with aluminium foil. Incubate the vials in a heating block at 50—55° for 20 min. Allow the samples to cool to room temperature, then evaporate them to dryness under nitrogen at room temperature, again being careful not to overdry.

Prior to analysis, add 20 μl of acetone to the residue, vortex for 20 sec and inject 4.0 μl into the gas chromatograph.

Gas—liquid chromatography

A Model 3920 Perkin-Elmer dual-column gas chromatograph with dual nitrogen—phosphorus detectors and an all-glass receiver system (Perkin-Elmer, Norwalk, Conn., U.S.A.) was used. A Hitachi—Perkin-Elmer Model 165 recorder was operated at 1 mV full scale and a chart speed of 10 mm/min. The glass columns were 138 cm × 2 mm I.D., configured for the all-glass version utilizing heated on-column injectors. The column packing was 3% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science Labs).

The injection port temperature was 230°, the detector temperature was

270°, and the oven temperature was programmed from 220—275° at 8°/min. The carrier gas (nitrogen) flow-rate was 35 ml/min; air, 100 ml/min; and hydrogen, 4 ml/min.

The detector was operated in the nitrogen/phosphorus mode with the jet potential control in position 3. After flow-rates of the support gases were set, the potentiometer controlling the bead temperature was increased from zero until the recorder deflection caused by background was 15% full-scale with the electrometer attenuation set at × 10.

RESULTS AND DISCUSSION

In preliminary studies we prepared several derivatives of the desmethyl compounds nortriptyline and maprotiline. Of the compounds studied, the mono-trifluoroacetyl derivatives of nortriptyline and its internal standard maprotiline exhibited chromatographic peaks which were not subject to interference by substances normally encountered in serum. Similarly, the tertiary amines, amitriptyline and doxepin, which are not derivatized were well resolved from each other. We selected doxepin and maprotiline as internal standards for amitriptyline and nortriptyline respectively because these compounds would not normally be administered concurrently with these drugs.

Representative chromatograms of serum extracted and derivatized according to our procedure are presented in Fig. 1. Chromatogram A with the internal standards omitted was obtained from the serum of a normal individual not receiving amitriptyline or nortriptyline. Chromatogram B was obtained with normal serum and the internal standards included in the extraction procedure. Chromatogram C was obtained from the serum of a normal individual to which amitriptyline and nortriptyline (100 and 150 ng/ml, respectively) were added. Chromatogram D was obtained from the serum of an individual receiving amitriptyline at a dose of 150 mg daily. The resultant levels were 121.0 ng/ml amitryptiline and 65.0 ng/ml nortriptyline. The drugs and their internal standards are well separated from each other and are free from interference from endogenous serum substances. The observed differences in retention times between amitriptyline and doxepin, and between nortriptyline and maprotiline were 0.35 min and 1.1. min, respectively.

A typical standard curve was obtained with this procedure. A linear relation between the ratio of peak height of drugs to internal standard, and the concentration of each drug in serum over a range of 0—300 ng/ml was found. The sensitivity, which was calculated from the minimum detectable amount at twice the noise level under normal operating conditions, was determined to be 5 ng/ml for both amitriptyline and nortriptyline when the sample volume was 1 ml.

The recovery of amitriptyline, doxepin, nortriptyline and maprotiline from serum over a range of 0—300 ng/ml is presented in Table I. The recoveries were determined in the following manner: 1 ml of drug-free serum was added to separate tubes containing 50, 100, 200 and 300 ng of each drug. These samples were extracted as described above; however, all volume transfers were quantitated. A second group of non-extracted standards containing the same amount

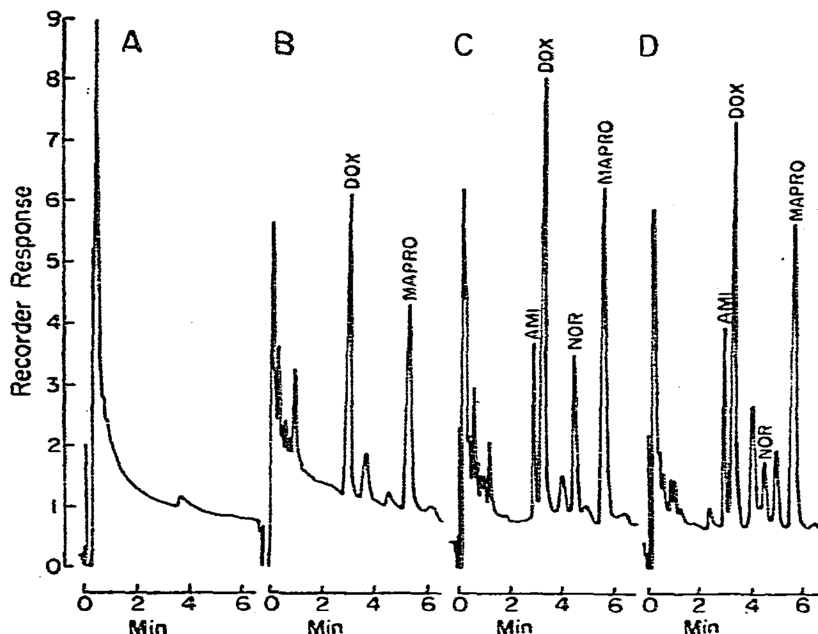

Fig. 1. Chromatograms obtained by the analysis of 1 ml of normal serum. Temperature was programmed from 220—275°, at 8°/min. Column packing was 3% OV-17 on 100—120 mesh Gas-Chrom Q. AMI = amitriptyline, NOR = nortriptyline, DOX = doxepin, MAPRO = maprotiline. (A) Drug-free serum, internal standards omitted; (B) serum to which doxepin and maprotiline (300 ng/ml) were added; the internal standards were included in the extraction; (C) serum to which amitriptyline (100 ng/ml) and nortriptyline (150 ng/ml) were added, internal standards included; (D) serum obtained from a patient receiving 150 mg amitriptyline daily; concentrations of amitriptyline and nortriptyline were determined to be 121 ng/ml and 65 ng/ml, respectively.

TABLE I

ABSOLUTE RECOVERIES OF AMITRIPTYLINE, DOXEPIN, NORTRIPTYLINE AND MAPROTILINE

Each value is the result of a single determination at the concentration indicated.

Drug (ng/ml)	Recovery (%)			
	AMI	DOX	NOR	MAPRO
50	67.8	89.2	69.1	85.1
100	81.2	92.8	68.1	80.0
200	91.0	99.1	78.1	86.4
300	98.7	85.0	85.5	91.8
Mean	86.9	91.5	75.2	85.8

of the drugs was prepared. All samples were derivatized as described in the methods section and exactly 4.0 µl of each sample was chromatographed. The peak heights of the extracted samples were then corrected for solvent transfers. Absolute recoveries of the compounds were calculated by comparing the ratio of the corrected peak height of the extracted samples to that of the non-extracted standards. The average recoveries of these compounds were: amitriptyline, 86.9%; doxepin, 91.5%; nortriptyline, 75.2%; and maprotiline 85.8%.

The within-run coefficient of variation of our assay at a serum concentration of 100 ng/ml amitriptyline and 150 ng/ml nortriptyline was 2.1% and 2.5% (n=12) respectively. Over a period of four months the between-run precision of this assay at these concentrations was 3.0% for amitriptyline and 3.8% for nortriptyline (n=20).

Fig. 2 illustrates chromatogram A obtained from a sample collected in a B-D vacutainer (Becton-Dickinson, Rutherford, N.J., U.S.A.) and chromatogram B, from a sample collected in a J-Vac (Jelco Laboratory, Raritan, N.J., U.S.A.) evacuated blood collection tube, each from a patient not receiving amitriptyline or nortriptyline therapy. Chromatogram C was obtained from a patient receiving nortriptyline, whose blood was collected in a B-D vacutainer. In contrast to the chromatograms obtained from the samples collected in the B-D vacutainers, the chromatogram of the sample collected in the J-Vac tube does not exhibit a large peak with the retention of 3.7 min which interferes with the

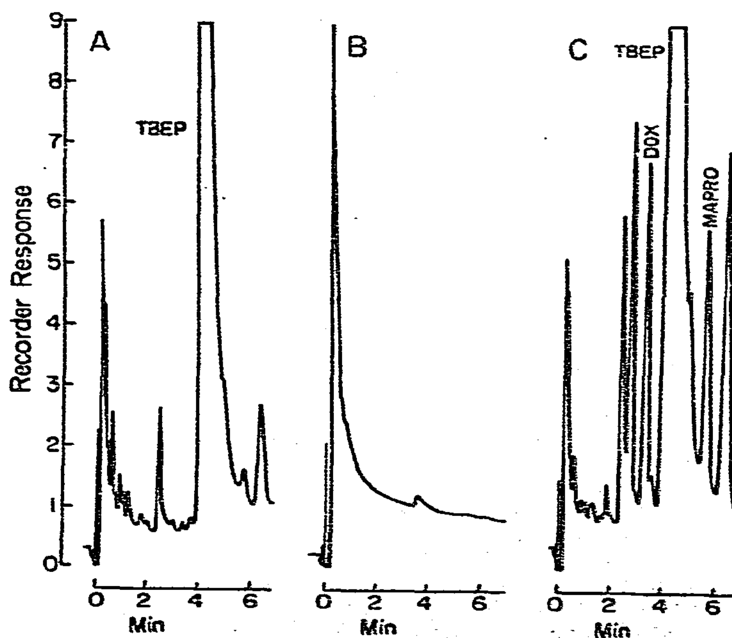

Fig. 2. Chromatograms obtained by the analysis of 1 ml of serum. (A), serum collected in a B-D vacutainer and (B), serum collected in a J-Vac evacuated blood collection tube, each obtained from patients not receiving amitriptyline or nortriptyline drug therapy; (C), 1 ml of serum collected in a B-D vacutainer from a patient receiving nortriptyline. TBEP = tri-2-butoxyethylphosphate.

quantitation of nortriptyline. This peak has been tentatively identified as tri-2-butoxyethyl-phosphate, a plasticizer used in the manufacture of the rubber B-D vacutainer stoppers. Authentic tri-2-butoxyethylphosphate had the same retention time as the interfering compound.

We have analyzed serum from patients receiving therapeutic doses of other drugs which are often administered concurrently with tricyclic antidepressants and have not encountered any chromatographic interference with our procedure. Although imiprimine and its N-desmethyl metabolite had the same retention times as doxepin and nortriptyline, respectively, it is highly unlikely that these compounds would be given concomitantly with amitriptyline or nortriptyline.

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