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DETERMINATION OF AMITRIPTYLINE-N-OXIDE, AMITRIPTYLINE AND NORTRIPTYLINE IN SERUM AND PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the determination of amitriptyline-N-oxide, amitriptyline and nortriptyline in serum and plasma has been developed. After extraction from serum or plasma the drugs were analysed by high-performance liquid chromatography.

The detection limit was 10 ng/ml (2 ml serum or plasma actually used). The coefficient of variation for all three compounds was below 10%.

Amitriptyline-N-oxide was found in rat plasma after an oral dose (10 mg/kg) of amitriptyline-N-oxide.

INTRODUCTION

During recent years a number of methods for the determination of tricyclic antidepressants in biological fluids have been published. Amitriptyline and nortriptyline (Fig. 1) have been measured by gas—liquid chromatography using flame-ionization detection [1—6] or alkali flame-ionization detection [7—13], and gas chromatography with electron-capture detection has also been applied [1, 14—16].

Mass fragmentographic methods are specific and often more sensitive than gas chromatographic methods, and this technique has been used for the determination of amitriptyline and nortriptyline [17—19]. Owing to the expensive equipment, however, many investigators prefer other methods, such as thin-layer chromatography.

Thin-layer chromatography may be used for the determination of amitriptyline and nortriptyline either by scanning the plates in situ [20—23], or by cutting off the spots followed by detection by fluorimetry [24]. Furthermore,

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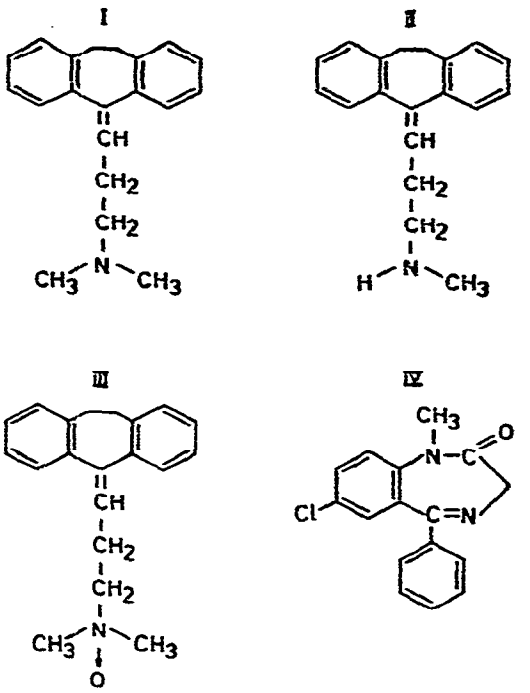

Fig. 1. Formulae of amitriptyline (I), nortriptyline (II), amitriptyline-N-oxide (III), and diazepam (IV).

it is possible to measure amitriptyline-N-oxide (Fig. 1) by thin-layer chromatography [25, 26].

Amitriptyline-N-oxide can also be determined by radioactivity measurements. Investigators have determined the drug in human plasma [27] and in plasma of rats and dogs [28] after a dose of ^{14}C-labelled amitriptyline-N-oxide. In other experiments amitriptyline-N-oxide was found as a metabolite in the urine of dogs [29] or of rats [30] after a dose of ^{14}C-labelled amitriptyline.

This paper describes a high-performance liquid chromatographic (HPLC) method for amitriptyline-N-oxide, amitriptyline and nortriptyline in serum and plasma. Several authors [31—39] have described HPLC methods for the determination of amitriptyline and nortriptyline in biological fluids. However, no such method for amitriptyline-N-oxide has been published so far.

EXPERIMENTAL

Materials and reagents

Hexane and dichloromethane were specially purified (nanograde) from Mallinckrodt (St. Louis, MO, U.S.A.). Acetonitrile (E. Merck, Darmstadt, G.F.R.) and methanol (Rathburn Chemicals, Walkerburn, Great Britain) were special HPLC grade. All other chemicals were analytical grade.

The tubes used for evaporation of hexane (tube I) and dichloromethane (tube II) were treated with a solution of 0.1% triethylamine in hexane and dried, and only used once.

The mobile phase for HPLC was membrane filtered (pore size 0.45 μm) and kept in an ultrasonic bath for 15 min immediately before use.

Amitriptyline and nortriptyline were available as hydrochlorides and amitriptyline-N-oxide as the base. All three compounds were synthesised by Synthesis Laboratories of A/S Dumex.

HPLC conditions

The apparatus consisted of a solvent delivery system, Model 6000 A (Waters Assoc.), a loop injection system U 6 K (Waters Assoc.), and a UV detector, Model 440, fixed wavelength of 254 nm (Waters Assoc.). The column was μBondapak C_{18} (30 cm × 3.9 mm, particle size 10 μm). Generally, the mobile phase was a mixture of acetonitrile and potassium dihydrogen phosphate (0.6% w/v) adjusted to pH 3 with phosphoric acid.

The mobile phase for the amitriptyline-N-oxide analysis was a mixture of 60% acetonitrile in 0.6% potassium dihydrogen phosphate. The flow-rate was 1.0 ml/min.

For the amitriptyline—nortriptyline analysis the mobile phase consisted of 50% acetonitrile in 0.6% potassium dihydrogen phosphate. The flow-rate was 0.9 ml/min.

Printing of the chromatograms and calculation of the peak areas were performed by an electronic integrator from Hewlett-Packard, Avondale, PA, U.S.A. (No. 3080). The peak heights of amitriptyline-N-oxide were measured and the peak areas for amitriptyline and nortriptyline were used.

Procedure

The extraction procedure is illustrated in Fig. 2. The internal standard (diazepam) was dissolved in dichloromethane before use.

The residue was dissolved in the mobile phase by shaking it in a whirlimixer immediately before injection into the chromatograph.

On the basis of calibration graphs constructed from control serum (plasma) containing known amounts of amitriptyline-N-oxide, amitriptyline and nortriptyline, the concentration of the three drugs in unknown samples was calculated.

Sampling

Male rats (Sprague—Dawley, weighing about 275 g) were fasted from the day before, but had free access to drinking water during the experiment.

A suspension of amitriptyline-N-oxide was given orally to the rats. The dose was 10 mg/kg. The rats were killed 0.5, 1, 2, 3, 4, 7, or 24 h after administration and heparinized blood was collected. The plasma was centrifuged off and kept at −18°C until analysed.

RESULTS

The calibration graph of amitriptyline-N-oxide in serum (serum concentration 50—250 ng/ml) was found to be satisfactory, but the best result was obtained using an internal standard (diazepam). The correlation coefficient was $r = 0.9559$ when the internal standard was not used and $r = 0.9828$ when the internal standard was used.

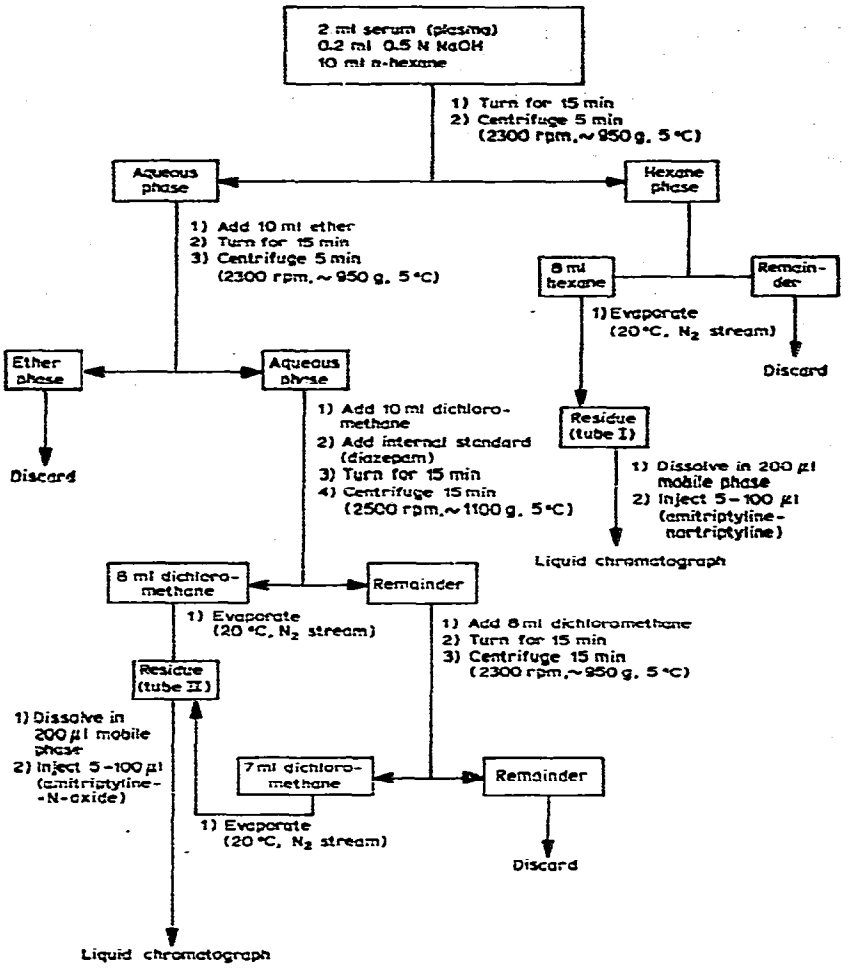

Fig. 2. Extraction scheme for amitriptyline-N-oxide, amitriptyline and nortriptyline.

Plotting the calibration graph of nortriptyline (50—400 ng/ml of serum) produced a correlation coefficient of $r = 0.9960$. Similarly, the correlation coefficient of amitriptyline was found to be $r = 0.9970$ (50—400 ng/ml of serum).

Fig. 3 shows a chromatogram of control serum (A) and of control serum with amitriptyline-N-oxide (200 ng in 2 ml) added (B). The retention time for amitriptyline-N-oxide was 5.2 min, and for diazepam (internal standard) 7.6 min.

Chromatograms of control serum (A) and of control serum with amitriptyline and nortriptyline (50 ng/ml) added (B) are shown in Fig. 4. The retention times were 6.1 min (amitriptyline) and 5.6 min (nortriptyline).

The reproducibility of the methods was tested for all three drugs (Table I). The coefficient of variation was found to be below 10%.

Extracted samples and standard solutions of amitriptyline-N-oxide were injected into the chromatograph, and the recovery of amitriptyline-N-oxide was found to be 75—80%.

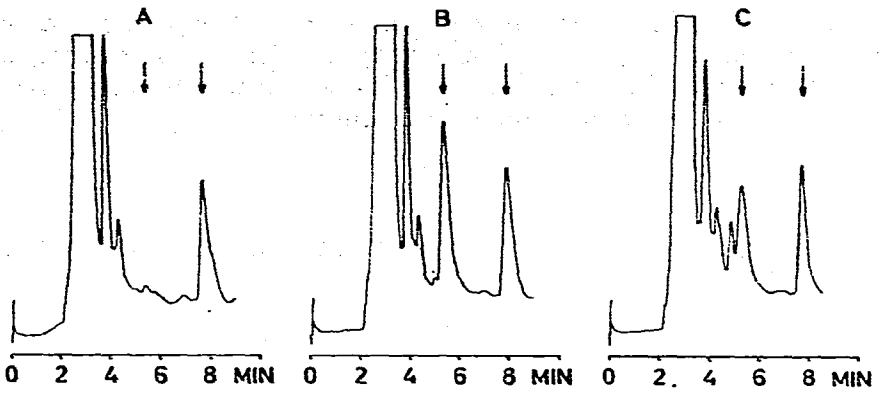

Fig. 3. Chromatograms of: (A) control serum; (B) control serum with amitriptyline-N-oxide (retention time 5.2 min) (100 ng/ml) added; and (C) rat plasma collected 30 min after an oral dose of amitriptyline-N-oxide (10 mg/kg). Internal standard: diazepam (retention time 7.6 min).

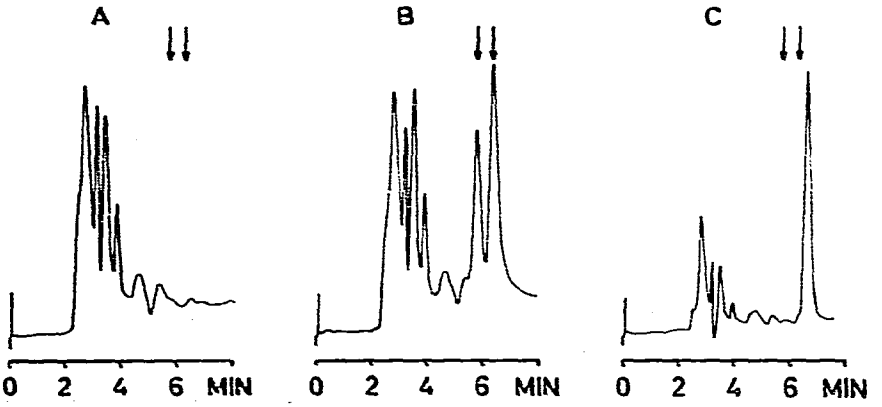

Fig. 4. Chromatograms of: (A) control serum; (B) control serum with amitriptyline (retention time 6.1 min) and nortriptyline (retention time 5.6 min) (50 ng/ml) added; and (C) rat plasma collected 30 min after an oral dose of amitriptyline-N-oxide (10 mg/kg). Peak with retention time 6.4 min: unknown.

The minimal concentration that can be determined is 10 ng/ml (2 ml actually used) for amitriptyline-N-oxide, amitriptyline and nortriptyline.

Various drugs which might interfere with the analysis were injected into the chromatograph; the retention times relative to amitriptyline-N-oxide are given in Table II.

Rat plasma

Fig. 3C shows a chromatogram of a rat plasma sample taken 30 min after an oral dose of amitriptyline-N-oxide, 10 mg/kg. The content of amitriptyline-N-oxide was calculated to be 270 ng/ml (500 μl of plasma were used).

The mean concentrations of amitriptyline-N-oxide in the rat plasma samples are illustrated in Fig. 5.

Fig. 4C shows a chromatogram of rat plasma, extracted and chromatographed as described for amitriptyline and nortriptyline. The plasma sample was drawn 30 min after an oral dose of amitriptyline-N-oxide, 10 mg/kg. No amitriptyline or nortriptyline was detectable in this sample.

TABLE I

REPRODUCIBILITY OF THE DETERMINATIONS OF AMITRIPTYLINE-N-OXIDE, AMITRIPTYLINE AND NORTRIPTYLINE

	No. of determinations	Added to 2 ml of serum (ng)	Found Mean (ng)	Found C.V.* (%)
Amitriptyline-N-oxide	6	90	96	8
	6	100	97	7
	6	150	153	6
	5	200	188	9
Amitriptyline	6	100	98	7
	5	150	146	8
	6	200	204	3
Nortriptyline	6	100	98	6
	5	150	146	8
	6	200	202	4

*C.V., coefficient of variation.

TABLE II

RELATIVE RETENTION TIMES FOR SOME COMPOUNDS

The chromatographic system for amitriptyline-N-oxide was used.

Compound	Relative retention time
Pindolole	0.52
Nortriptyline	0.77
Carbamazepine	0.78
Imipramine	0.81
Cyproheptadine	0.82
Amitriptyline	0.85
Nitrazepam	0.86
Imipramine-N-oxide	0.91
Amitriptyline-N-oxide	1.00
Desmethyldiazepam	1.00
Diazepam	1.32
2-Amino-5-nitrobenzophenone	1.42

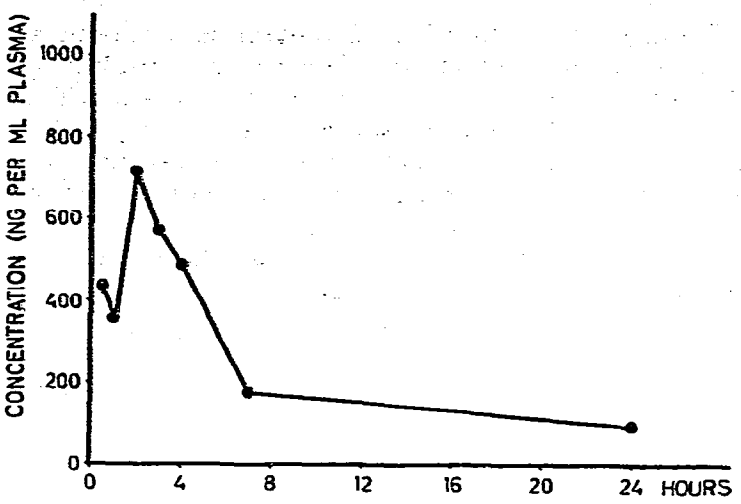

Fig. 5. Mean plasma concentration (n=3) of amitriptyline-N-oxide after an oral dose of amitriptyline-N-oxide (10 mg/kg) to rats.

DISCUSSION

In the present study amitriptyline, nortriptyline and amitriptyline-N-oxide were determined by HPLC without previous derivatization of the compounds.

The recovery of amitriptyline-N-oxide was found to be approximately 80% when the extracted drug was compared with standard solutions injected directly. About 10% was not extracted from the serum (checked by ^{14}C-labelled amitriptyline-N-oxide). The remainder (10%) may be lost by adsorption to glassware used during the analysis. Pretreatment with triethylamine to prevent this phenomenon is described by Jørgensen [10], who found a recovery of 83% (amitriptyline and nortriptyline). In the present study only the tubes containing the last residue were treated with triethylamine. Breyer-Pfaff et al. [26] found recoveries of 86% and 84% for amitriptyline-N-oxide and amitriptyline, respectively.

The reproducibility of the method for amitriptyline-N-oxide, amitriptyline and nortriptyline expressed as the coefficient of variation was found to be below 10%, which is comparable with the values reported by Breyer-Pfaff et al. [26], namely 4% (amitriptyline-N-oxide) and 9% (amitriptyline). The coefficient of variation was calculated by Jørgensen [10] to be 7.6% (amitriptyline and nortriptyline) and by Mellström and Braithwaite [37] to be 10% (amitriptyline) and 5% (nortriptyline).

In the present study the detection limit was 10 ng/ml of serum for all three compounds.

Santagostino et al. [25] 500 ng/ml the detection limit of amitriptyline-N-oxide in the urine to be reported. Breyer-Pfaff et al. [26] obtained a higher sensitivity (amitriptyline-N-oxide 15 ng/ml, amitriptyline 12 ng/ml), but they used 4 ml of plasma for the analysis. Jørgensen [10] stated that the lowest detectable concentration of amitriptyline was 5 ng/ml and of nortriptyline 10—15 ng/ml (2 ml plasma used), and Brodie et al. [32] reported a sensitivity of 5 ng/ml (amitriptyline and nortriptyline) using 4 ml of plasma.

For the determination of amitriptyline-N-oxide, diazepam was chosen as internal standard. It is separated from amitriptyline-N-oxide and suffers no interference from peaks of control serum. The internal standard was added immediately before the extraction with dichloromethane, and any possible diazepam content in the serum of patients was removed by hexane and ether before adding the internal standard. Desmethyldiazepam, if present, is also removed at this stage of the extraction procedure.

In the present study amitriptyline-N-oxide was found in rat plasma after an oral dose (10 mg/kg) of amitriptyline-N-oxide.

Amitriptyline-N-oxide is reported to be metabolised extensively in the rat [28], but neither amitriptyline nor nortriptyline were found in any of the plasma samples. An unknown peak, having a longer retention time than that of amitriptyline, may be a metabolite.

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