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A THIN-LAYER METHOD FOR THE DETERMINATION OF AMITRIPTYLINE AND NORTRIPTYLINE IN PLASMA

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SUMMARY

A method for the simultaneous determination of amitriptyline and nortriptyline is described. Both drugs are isolated from biological material by means of a single extraction. Part of the organic phase is evaporated to dryness and the residue is dissolved in a small volume of ethanol containing a small amount of hydrochloric acid. Thin-layer chromatography (TLC) is used for the separation of amitriptyline and nortriptyline and their metabolites. The spots are rendered visible by immersing the TLC plate in dilute perchloric acid and subsequently heating it in a drying oven. A quantitative determination is carried out by measuring the fluorescent spots with a densitometer. Interpolation between two reference standards gives the concentration of the sample. Extensive studies on the recovery of amitriptyline and nortriptyline in the range 50–250 ng/ml have been performed. The results showed that this method gives a recovery of 104% for amitriptyline and 81% for nortriptyline, with a standard deviation of 8% in each instance. The sensitivity of the method is about 10 ng/ml; the method is sufficiently sensitive and specific for therapy control purposes and also requires relatively less investment. The time needed for the simultaneous analysis of both drugs is about 3 h.

INTRODUCTION

Toxicological analyses[1,2], therapy control[3] and pharmacokinetic studies on antidepressant drugs[4–7] have been the subject of many investigations and have included nortriptyline and its parent drug amitriptyline. The volume of literature that has been published demonstrates the lack of uniformity of the methods used and of the results obtained.

Several workers have described methods for the determination of amitriptyline. Hucker and Porter[8] and Wallace and Dahl[9] described spectrophotometric determinations. Hucker and Miller[10] demonstrated the possibility of determining tertiary amines, such as amitriptyline, by gas chromatography, but only an instrumental analytical method was described. Munksgaard[1] and Breyer and Remmer[2] examined the level of amitriptyline in poisoned persons. They extracted plasma or

homogenized tissue and, after thin-layer chromatography (TLC) on Kieselgel and elution of the spots, the amount of the drug present was measured by UV spectroscopy. The drug was present at a level of a few micrograms per millilitre or per gram, so the sensitivity of this method was very low. Westerlund and Borg[11] described a fluorimetric method for the determination of less than $2 \cdot 10^{-7}$ mole of ammonium ion ($\equiv$ 50 ng/ml of amitriptyline) with an acceptable precision. Breyer and Remmer[2] extracted amitriptyline from liver, kidneys, lungs and other organs under alkaline conditions with 1,2-dichloroethane or *n*-heptane containing 1.5% of isoamyl alcohol. The organic solvent was evaporated and the residue dissolved in a small volume of ethanol. The unknown solution was separated on a Kieselgel TLC plate. After separation, the spots were eluted and determined by a spectrophotometric method.

As the major metabolite of amitriptyline, nortriptyline, also shows antidepressant activity, several workers have studied its determination. Amundson and Manthey[12] described a UV spectrophotometric method for the quantitative determination of nortriptyline in urine, and a TLC system for the identification of nortriptyline and its metabolites in urine was also discussed. Hammer and Brodie[13] labelled secondary amines *in vitro* with radioactive [^{3}H]acetic anhydride, followed by quantitative determination by scintillation spectrometry. Sjöqvist *et al.*[14] applied the technique of *in vitro* labelling to the determination of nortriptyline. Both primary and secondary amines form derivatives with acetic anhydride, which is why other drugs and the metabolites interfere. In this way, 10–15% of desmethylnortriptyline is co-determined. Walle and Ehrsson[15] described a gas chromatographic determination, with electron capture detection, for primary and secondary amines, after acetylation with heptafluorobutyric anhydride. This method has a high sensitivity. Borgå *et al.*[16] further developed the quantitative determination of nortriptyline. After derivative formation with heptafluorobutyric anhydride, mass fragmentography was applied. A sample of the extract was injected into a gas chromatograph and after separation of the components of the sample, a mass spectrograph was used as a detector. Borgå and Garle[17], after reaction with heptafluorobutyric anhydride, determined nortriptyline quantitatively by gas chromatography, the sensitivity of the method being about 10 ng/ml.

Only a few methods for the simultaneous determination of nortriptyline and amitriptyline have been described. Eschenhof and Rieder[18] investigated the metabolism of amitriptyline, and used TLC for the separation of amitriptyline from nortriptyline and other metabolites. They extracted the sample with benzene at pH 9, the organic solvent was evaporated by means of a stream of nitrogen and the extract was concentrated. The concentrated benzene extract was separated on a Kieselgel TLC plate using pure amitriptyline as reference.

The plates were immersed in perchloric acid (70%) and heated at 120° for 10 min. After cooling, the unknown and the known amitriptyline spots were estimated at 350 nm. The sensitivity of this semi-quantitative method was 50 ng; no recovery experiments were carried out. By comparison with a known reference amount of amitriptyline, the amount of amitriptyline and nortriptyline present were determined. The sensitivity represented a spot containing about 50 ng. Braithwaite and Widdop[19] used trifluoracetic anhydride, which was added to the extract. An anhydride reacts with primary and secondary amines but not with tertiary amines. After the formation of a derivative of nortriptyline but not of amitriptyline, the two drugs were deter-

mined by gas chromatography. The sensitivity of the method was 20 ng/ml. The recovery in the range 50–250 ng/ml was 86 ± 6% for amitriptyline and 83 ± 4% for nortriptyline.

In order to establish the optimum and safe dosage regimen of amitriptyline and nortriptyline, we studied the possibility of using fluorodensitometry[20–22] for the determination of both compounds. In this paper, a method is described for the simultaneous determination of amitriptyline and nortriptyline.

EXPERIMENTAL

Materials and reagents

The TLC plates were Kieselgel 60 DC-Fertigplatten of dimensions 20 × 20 cm (E. Merck, Darmstadt, G.F.R.). A 100-μl Hamilton syringe (with a PTFE-coated plunger and a PTFE gasket tip) with a Hamilton repeating dispenser was used. The chromatography tank was obtained from Desaga, the Vibromixer was a Vortex-Genie mixer (Wilten & Co.), the centrifuge was a Christ Model UJ 1, with a maximum speed of 3200 rpm and the TLD-100 densitometer was obtained from Vitatron (Dieren, The Netherlands).

Toluene p.a. (min. 99.5%), isobutanol p.a. (min. 99%), chloroform p.a. (99–99.5%), acetic acid p.a. (min. 99.8%) and hydrochloric acid p.a. (min. 37%) were obtained from Merck. Methanol (min. 99.5%), ethanol (99.5%) and perchloric acid (70–72%) were obtained from J. T. Baker Chemicals, Deventer, The Netherlands. Nortriptyline hydrochloride was obtained from H. Lundbeck & Co., Amsterdam, The Netherlands, and amitriptyline hydrochloride from Merck, Sharp & Dohme, Haarlem, The Netherlands.

The immersion liquid consisted of 500 ml of perchloric acid, 600 ml of demineralized water and 600 ml of ethanol.

The stock solution of nortriptyline and amitriptyline was prepared by dissolving 56.4 mg of nortriptyline hydrochloride and 56.8 mg of amitriptyline hydrochloride in 100 ml of ethanol.

A standard solution was prepared by diluting 5 ml of the stock solution to 50 ml with ethanol and adding 4 ml of this solution to 46 ml of ethanol.

Extraction

A 4-ml volume of plasma is shaken for 2 min with 5 ml of toluene (containing 3% of isobutanol) in a 10-ml glass-stoppered centrifuge tube and the mixture is centrifuged for 10 min at 3200 rpm. Then 4 ml of the supernatant are evaporated to dryness using a rotary evaporator or a stream of dry air. The residue is dissolved in 0.3 ml of ethanol containing $1.2 \cdot 10^{-2}$ *M* hydrochloric acid.

Chromatography

With a Hamilton syringe, three 20-μl volumes of the unknown solution (X) are applied on a thin-layer plate, together with 8 ng (S_1) and 24 ng (S_2) of an ethanolic solution of amitriptyline and nortriptyline, respectively. The sequence of application is S_1, X, S_2, S_1, X, S_2, S_1, X, S_2.

The centres of the spots are 2 cm apart and 2 cm from the edge of the plate. The eluent used is chloroform–methanol–acetic acid (8:1:1), the development being

carried out in a saturated tank with an elution time of about half an hour. The elution distance is 10 cm and the plate is dried under a stream of air. The TLC plate is immersed in 20% perchloric acid in ethanol–water (1:1) and subsequently, after wiping off the excess of liquid with filter paper, it is heated in a drying oven at 120° for 7 min. This process converts amitriptyline and its metabolites into fluorescent compounds.

The spots are quantified by measuring the fluorescence directly with a Vitatron TLC-100 densitometer. The operating conditions are as follows. Light source, mercury lamp; mode, In II (÷); level, f; coarse zero, 7; damping, 2; span, 9–10; excitation filter, UVB filter (260–340 nm); emission filter, 565 nm; diaphragm, 0.5; swing, 5; scanning speed, 1 cm/min; paper speed, 0.5 cm/min; integrator, 8.

RESULTS AND DISCUSSION

Linearity

It appeared that there was a linear relationship up to 50 ng between the amounts of amitriptyline hydrochloride and nortriptyline hydrochloride and the corresponding fluorescence (integrated peak area). To confirm this finding a number of duplicate determinations were carried out on solutions containing 10, 20, 30, 40 and 50 ng amounts of the drugs. After statistical analysis[20] of the results, it appeared that the deviation from linearity was not statistically significant at a threshold value of 5%.

Calculation of the unknown concentration

From two different amounts (8 and 24 ng) of both amitriptyline hydrochloride and nortriptyline hydrochloride, a calibration graph was constructed. The concentration of the sample was obtained by interpolation of the two reference standards. In Fig. 1, integrated values of the standards S_1 and S_2 and of the unknown sample X are

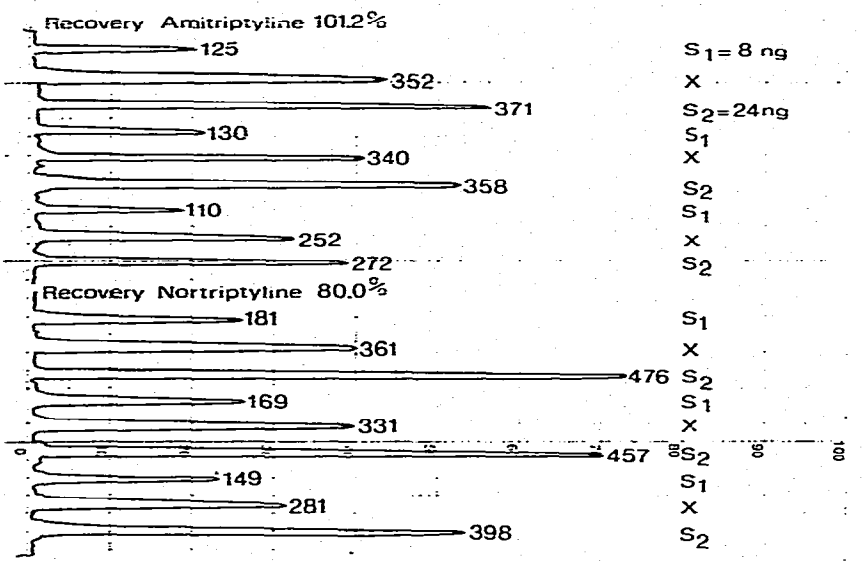

Fig. 1. Densitogram of different amounts of pure amitriptyline hydrochloride and nortriptyline hydrochloride and of amitriptyline hydrochloride and nortriptyline hydrochloride isolated from plasma.

shown in a densitogram. Average values for S_1, S_2 and X were taken; with the two known amounts of substance the calibration graph was constructed and, from the number of pulses of the unknown sample of amitriptyline hydrochloride and/or nortriptyline hydrochloride, the amount in nanograms can be calculated directly from the calibration graph.

Recovery

An ethanolic solution of amitriptyline hydrochloride and nortriptyline hydrochloride was added to blank plasma with a 100-μl constriction pipette. Table I summarizes the results of a series of recovery experiments in the concentration range 50–250 ng per millilitre of plasma. In Fig. 1, the results of a recovery experiment for amitriptyline hydrochloride and nortriptyline hydrochloride from the same sample are shown in a densitogram.

TABLE I

RECOVERY OF AMITRIPTYLINE AND NORTRIPTYLINE FROM PLASMA ON CONSECUTIVE DAYS

The overall recovery (n = 18) for amitriptyline is 104 ± 7.4%, with a range of 91–120%. The overall recovery (n = 18) for nortriptyline is 81 ± 7.4%, with a range of 72–95%.

Amount (ng/ml)	*Day*	*Amitriptyline*		*Nortriptyline*	
		Recovery (%)	*Blank (%)*	*Recovery (%)*	*Blank (%)*
50	1	107	8	73	4
	2	108		84	
	3	115		72	
	4	106		72	
	5	120		75	
100	1	91	4	75	2
	2	103		81	
	3	105		75	
	4	101		77	
	5	113		91	
	6	101		80	
	7	106		74	
	8	107		86	
250	1	101	0	95	0
	2	101		94	
	3	93		83	
	4	97		85	
	5	95		81	
		x_m = 104%		x_m = 81%	

For the recovery of 250 ng/ml, 10 μl of the extract were applied: the blank (also 10 μl) did not give any response. For the recovery of 50 and 100 ng/ml, 20 μl of the extract were applied: the blank corresponded to 4 ng/ml of amitriptyline and to 2 ng/ml of nortriptyline, *i.e.*, at a recovery of 100 ng/ml the blank caused a systematic error of 4% (amitriptyline) and 2% (nortriptyline) and at 50 ng/ml an error of 8% (amitriptyline) and 4% (nortriptyline).

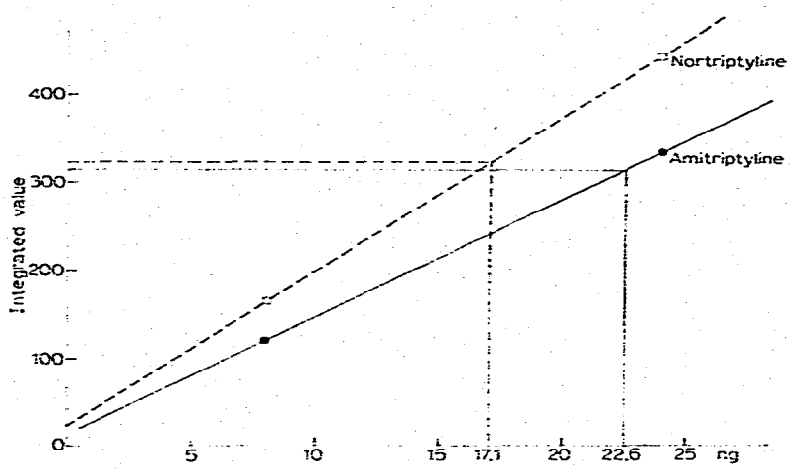

Fig. 2. Calculation of the unknown concentration of amitriptyline hydrochloride and nortriptyline hydrochloride.

The sensitivity of the method is about 10 ng/ml, if one considers that the sensitivity is three times the blank.

Specificity of the method

According to McMahon *et al.*[23], Cassano *et al.*[24], Amundson and Manthey[12] and Eschenhof and Rieder[18], the principle metabolites of the two drugs are 10-hydroxyamitriptyline and 10-hydroxynortriptyline. These metabolites differ in their R_F values from amitriptyline and nortriptyline (Table II).

TABLE II

R_F VALUES AND FLUORESCENCE OF AMITRIPTYLINE, NORTRIPTYLINE AND THEIR MAJOR METABOLITES AND OF SOME OTHER COMPOUNDS

Compound	R_F	*Fluorescence colour*
Amitriptyline	0.40	Orange-yellow
Nortriptyline	0.49	Orange-yellow
10-Hydroxyamitriptyline	0.23	Orange-yellow
10-Hydroxynortriptyline	0.32	Orange-yellow
Chlordiazepoxide	0.87	Pink
Diazepam	0.77	Yellow-green
Medazepam	0.50	Blue
Nitrazepam	0.74	Brown-green*
Promazine	0.33	Pink-brown*
Chlorpromazine	0.38	Pink-brown*

* In these cases we cannot speak of fluorescence, but we can still distinguish a colour.

As these tricyclic antidepressants are combined with hypnotics and tranquillizers, a specific and, if possible, a simultaneous method for the determination of amitriptyline and nortriptyline is needed, especially when amitriptyline is prescribed because of the active metabolite nortriptyline. In order to evaluate the selectivity of the determination of amitriptyline and nortriptyline, it was necessary to include a

study to determine the R_F values and fluorescence of some hypnotics and tranquillizers administered together with the antidepressant drugs. It appeared that some of them had almost the same R_F values, while the fluorescence was different or there was no fluorescence, and moreover it was not certain they were extracted completely Only a few of these compounds in therapeutic dosage can interfere in the present method (Table II). Limbritol (a combination of amitriptyline and chlordiazepoxide) did not seem to give rise to any difficulties. In special cases of drug combinations, another solvent system can be used, and Table II gives only some basic information that might be useful in a particular treatment with different drugs when it is required to establish the correlation between clinical effects and plasma levels of the drugs.

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