*Journal of Chromatography*, 145 (1978) 445—451
*Biomedical Applications*


CHROMBIO. 146

# DETERMINATION OF AMITRIPTYLINE AND NORTRIPTYLINE IN HUMAN PLASMA BY QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

P. HAEFELFINGER

*Biological Pharmaceutical Research Department, F. Hoffmann—La Roche & Co., Ltd., Basel (Switzerland)*

(Received November 10th, 1977)

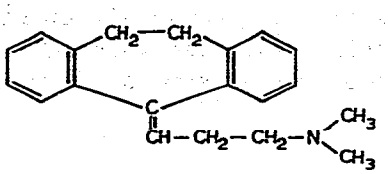
## SUMMARY

A thin-layer chromatographic method for simultaneous determination of amitriptyline (AT) and nortriptyline (NT) in human plasma is described. Both substances are extracted from biological material by means of a single extraction. The extract is evaporated until dry and the residue quantitatively applied to a silica gel thin-layer plate. AT and NT are separated from interfering plasma components by chromatography. The spots are visualized by nitration, reduction and coupling with N-(1-naphtyl)ethylenediamine on the plate. The intensity of the azo-dyes formed can be measured densitometrically. Using 1 ml of plasma, the sensitivity limit was 0.5 ng/ml for both substances. About 10—15 plasma samples can be analysed per day. The method is applicable to pharmacokinetic studies after a single oral dose of 25 mg AT as hydrochloride in man.

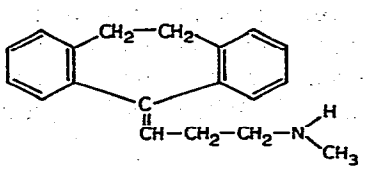
## INTRODUCTION

In recent years several methods have been published for the determination of amitriptyline (AT) and nortriptyline (NT) in human plasma. Jørgensen [1] and Bailey and Jatlow [2] used a gas chromatographic procedure with a nitrogen detector. The sensitivity of this procedure was about 5 ng AT and NT/ml of serum. Wallace et al. [3] determined AT, after oxidation to a polyaromatic carbonyl derivative, by gas—liquid chromatography with an electron capture

AMITRIPTYLINE



NORTRIPTYLINE



detector. In this case no differentiation between AT and NT was possible. The sensitivity of the method was about 2 ng/ml of serum. A similar procedure is described by Hartvig et al. [4]. Prior to oxidation they separated the extracted AT and NT by column chromatography. A quantitative thin-layer chromatography (TLC) method for the determination of AT in human plasma has also been developed by Faber et al. [5], with a sensitivity of about 10 ng/ml of plasma.

All these methods have sensitivity limits which do not allow pharmacokinetic studies to be carried out after a normal single oral dose of 25 mg AT-hydrochloride in which case concentrations of as little as 0.5—1 ng AT per ml of plasma should be measurable.

We therefore tried to develop a sufficiently sensitive TLC method using direct densitometric measurement. This procedure was similar to the method published recently [6] for the determination of chlorpheniramine and codeine. By changing the reaction conditions and the reagents the necessary sensitivity for AT and NT was achieved. The procedure is described in this paper.

## METHOD

AT and NT are extracted from alkalized plasma with pentane—isobutanol. The extract is concentrated and applied to a silica gel thin-layer plate. After separation, AT and NT are nitrated with nitric acid, reduced with sodium dithionite to the corresponding aromatic amines which are diazotized and coupled with N-(1-naphthyl)ethylenediamine. The intensities of the azo-dyes are directly proportional to the concentrations of AT and NT and can be measured densitometrically on the TLC plate.

### *Evaluation of the test conditions*

#### *Extraction*

At the nanogram level the extraction of AT and NT from plasma is critical. To reduce the extraction of interfering plasma constituents it is advantageous to use apolar organic solvents. But it was observed that in apolar solvents the substances to be determined were partially adsorbed at the glass surfaces. Even siliconisation of the surfaces did not eliminate this effect. However, by adding 1% of isobutanol to pentane, the adsorption of AT and NT was practically eliminated.

The extraction yield of AT and NT from an aqueous solution is independent of the quantity of NaOH added. This quantity is however critical for extraction of these substances from plasma. A minimum concentration of NaOH is necessary to compensate for the buffer capacity of the plasma. We expected that the quantities of NaOH could be varied arbitrarily above this minimum level. We found however, that an optimal concentration exists above which the extraction yield diminishes considerably. We cannot explain this phenomenon. The highest recoveries from plasma (more than 90%) were found after addition of 0.2 ml of 2.5% NaOH to 1 ml of plasma.

*Thin-layer chromatography*

From a large number of solvents examined, the following systems were found to be the most suitable (vapour saturation of the jar): chloroform—ethanol—acetic acid (5:1:1.5), $R_F$ values: AT ca. 0.3, NT ca. 0.45; chloroform—methanol (4:1), $R_F$ values: AT ca. 0.35, NT ca. 0.1.

*Detection*

*Principle of spraying.* The best results were obtained by spraying the reagents. The layer should be completely and homogeneously wet, but without drops. For spraying, we used the commercial glass sprayers without modification.

*Nitration.* AT and NT can be nitrated at high temperature on silica gel thin-layer plates using nitric acid. The best results were obtained with a mixture of nitric acid (65%)—methanol (1:1). Two factors are critical for nitration on thin-layer plates. The temperature of 125—130° has to be reached in a short time and should be homogeneous over the whole plate. We achieved this by placing the thin-layer plate on a preheated aluminium block with a high heat capacity in a drying oven. A more complex problem was ventilation of the drying oven. We found that an intensive air-circulation and draining off of the nitric-acid vapours gave the highest nitration yield.

The structures of the nitration products were not identified, since this is of secondary importance for our project aim. The optimal nitration conditions were: temperature 125—130°; intensive air circulation and sucking-off the nitric acid vapours with a waterjet pump; reaction time 15 min.

*Reduction.* The reduction of aromatic nitro compounds to the corresponding amino compounds is possible using several reducing reagents. Titanium(III) chloride and thin(II) chloride have been used [6, 7]. However, for the reduction of the nitroderivatives of AT and NT sodium dithionite gave advantageous results. The reduction with this reagent on thin-layer plates depends mainly on the pH of the solution used. The best results were obtained at pH 6.5 using a 0.5 *M* sodium-phosphate buffer as solvent for sodium dithionite. The optimum reaction temperature was 80—85°. Air circulation had to be omitted since it reduced the reduction yield. The reaction period had to be limited to 8 min, longer periods led to lower recoveries.

The amino-compounds of AT and NT showed an intensive fluorescence under long-wave UV light (366 nm). Due to bad reproducibility, however, these fluorescences were found to be unsuitable for quantitative measurement.

*Diazotizing and coupling.* The procedure according to the Bratton—Marshall reaction was used with a 2% solution of sodium nitrite in 1 *M* HCl and a 1% solution of N-(1-naphthyl)ethylenediamine dihydrochloride in methanol. After spraying the plate with sodium nitrite it was necessary to completely dry the silica gel layer with a stream of cold air. Neglection of this step led to a reduction in sensitivity of the method.

*Densitometry on the thin-layer plate*

The intensity of the AT and NT spots can be measured with the commercial chromatogram spectrophotometers. We used the Zeiss chromatogram-spectralphotometer model PMQ II.

## EXPERIMENTAL

### *Reagents*

The following reagents were used: Sodium hydroxide (2.5%) in distilled water; equal parts of analytical-grade methanol and analytical-grade 65% nitric acid are mixed carefully and cooled thereafter; 4% sodium dithionite ($Na_2S_2O_4$) in 0.5 *M* sodium phosphate buffer pH 6.5 (this solution remains stable for about 1 h); 2% sodium nitrite in 1 *M* hydrochloric acid, prepared immediately before use; 1% N-(1-naphthyl)ethylenediamine dihydrochloride in analytical-grade methanol. This solution can be used for a few days; analytical-grade solvents: acetic acid, chloroform, ethanol, isobutanol, methanol, *n*-pentane.

### *Standards*

Solution I: dissolve 10.0 mg of AT hydrochloride in 10 ml of methanol.

Solution II: dissolve 10.0 mg of NT hydrochloride in 10 ml of methanol.

Solution III: mix 1 ml of solution I and 0.25 ml of solution II and dilute with methanol to 10 ml.

Solution IV: 0.5 ml of solution III are diluted with chloroform to 100 ml. This solution contains 5 ng of AT hydrochloride and 1.25 ng of NT hydrochloride in 10 $\mu$l.

Solution V: 1 ml of solution III is diluted with distilled water to 100 ml.

Plasma standard I: 0.5 ml of solution V are diluted with a blank plasma to 25 ml (20 ng AT/5 ng NT/ml). Starting with this plasma standard the following plasma standards are prepared by dilution: plasma standard II; 10 ng AT/2.5 ng NT/ml, plasma standard III; 5 ng AT/1.25 NT ng/ml and plasma standard IV; 2.5 ng AT/0.625 ng NT/ml.

### *Materials for TLC*

The following materials are used: Merck (Darmstadt, G.F.R.) silica gel 60, $F_{254}$ 0.25 mm precoated thin-layer plates, 20 × 20 cm; 10 $\mu$l automatic capillary-dosage pipettes from Desaga (Heidelberg, G.F.R.). Chromatographic tanks for 20 × 20 cm plates; Zeiss chromatogram-spectral photometer PMQ II.

### *Procedure*

*Extraction.* Mix well 1 ml of plasma and 0.2 ml of 2.5% sodium hydroxide in a 15 ml centrifuge tube. Add 5 ml of pentane—isobutanol (100:1) and shake for 5 min on a reciprocating shaker. Centrifuge the samples for 5 min at 700 *g*. Transfer 4.5 ml of the organic phase into a 10—15-ml glass tube. Evaporate the extracts in a shaking thermostat at a temperature of 55—60°. Process 1 ml of each of the plasma standards I—IV along with the samples.

*Application to the TLC-plates.* The cold extraction-residues are dissolved in 30 $\mu$l of chloroform. Apply these solutions to a silica gel TLC plate (20 × 20 cm) heated to about 60°, with 10 $\mu$l pipettes. The distance from the bottom and side edges should be 1.5 cm and between the spots 1.2 cm. Rinse the tubes carefully with 30 $\mu$l of chloroform and apply the rinsings to the plate. For localisation of AT and NT after separation place 5 $\mu$l of solution III on the boundary points.

*Chromatography.* The chromatograms are developed in a jar, lined with filter-paper to achieve vapour saturation. The tank is preconditioned with the solvent, either chloroform—ethanol—acetic acid (5:1:1.5) or chloroform—methanol (4:1), about 10 min before use. The migration distance of the solvent from the bottom edge is 10 cm. Dry the plate and mark the zones of AT and NT under short-wave UV light (254 nm).

*Detection.* Spray the plate with the nitration-mixture until the layer is uniformly wet. After the liquid has completely penetrated into the silica gel, place the TLC plate in a drying oven with a temperature of 125—130°. To achieve a high nitration yield, the plate is located on a preheated aluminium block, intensive air circulation is maintained and the acid vapours are sucked off by a powerful waterjet pump. After 15 min cool the plate and spray with the dithionite reagent. The reduction is performed in a drying oven at a temperature of 80—85° for 8 min without ventilation. After cooling, AT and NT show under long-wave UV light a pale-yellow fluorescence. Spray the plate with a fresh nitrite solution, dry for 10 min with a stream of cold air (hair drier) and spray with the N-(1-naphthyl)ethylenediamine dihydrochloride reagent. After 1 min dry with air at a temperature of about 50—60° (hair drier). As already mentioned, all spraying must be performed very carefully.

*Scanning.* The operating conditions for the Zeiss chromatogram-spectral photometer PMQ II are as follows: tungsten lamp 550 nm; entrance diaphragm 3.5 mm; slit width 0.5 mm; ordinate extended 4 times; damping 1; scanning speed 10 cm/min; paper speed 10 cm/min.

*Calculation.* Up to 20 ng of AT or NT hydrochloride per spot, the peak heights are directly proportional to the quantity chromatographed. Therefore, the peak heights of the standards are divided by the corresponding plasma concentrations resulting in the height of 1 ng/ml of plasma. The mean value of all the plasma standards applied to the plate is compared with the height of the signal of the samples to be analysed.

$$\frac{H_S}{H_{PS}} \text{ ng/ml} = \text{concentration of substances in plasma (ng/ml)}$$

where $H_S$ = height of the signals of the samples

$H_{PS}$ = mean height of the plasma standards corresponding to 1 ng/ml in plasma

## RESULTS

*Linearity.*

As already mentioned, a linear relationship between the heights of the peaks and the amounts on the thin-layer plate was observed up to 20 ng of AT and NT hydrochloride. For levels higher than about 20 ng/ml the plasma has to be diluted in such a manner that 1 ml of the solution contains not more than 20 ng.

## DISCUSSION

On comparison of the results from Jørgensen [1] with our data, the levels of AT are seen to be somewhat lower than expected. But the differences are explained by the different test set-up and the variations between individuals. The concentrations of NT are rather low after a single oral dose of AT and the sensitivity of most published methods is not sufficient to determine these quantities. However, the described method is sensitive enough, and highly specific for AT and the metabolite NT. The procedure is simple and not very time-consuming, but requires some experience. The sensitivity of the procedure is sufficient for pharmacokinetic studies after a single dose of 25 mg AT as hydrochloride. The determination of nanogram amounts of drugs in biological materials is always susceptible to contamination. In general, repeated sophisticated cleansing of the glass-ware and reagents is necessary. It is therefore very surprising that in order to determine AT and NT in nanogram quantities no similar problems were encountered. The normal analytical grade solvents, normal distilled water and the conventional reagents were used. The only drawback of the procedure is the necessity of scanner use for thin-layer plates. However, we agree with other authors who use this technique that such an instrumentation will become standard in the near future, because of the great number of potential applications, available for direct spectrodensitometry or measurement after derivatization, to assays in clinical laboratories. The same procedure can probably be applied to other tricyclic substances of similar structure.

## ACKNOWLEDGEMENT

The author is grateful to Mr. B. Hess for his excellent technical assistance.

## REFERENCES

1 A. Jørgensen, Acta Pharmacol. Toxicol., 36 (1975) 79.
2 D.N. Bailey and P.J. Jatlow, Clin. Chem., 22 (1976) 777.
3 J.E. Wallace, H.E. Hamilton, L.K. Goggin and K. Blum, Anal. Chem., 47 (1975) 1516.
4 P. Hartvig, S. Strandberg and B. Näslund, J. Chromatogr., 118 (1976) 65.
5 D.B. Faber, C. Mulder and W.A. Man in 't Veld, J. Chromatogr., 100 (1974) 55.
6 P. Haefelfinger, J. Chromatogr., 124 (1976) 351.
7 P. Haefelfinger, J. Chromatogr., 111 (1975) 323.