Journal of Chromatography, 143 (1977) 499—512
Biomedical Applications

CHROMBIO. 054

DETERMINATION OF AMITRIPTYLINE AND SOME OF ITS METABOLITES IN BLOOD BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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(Received January 10th, 1977)

SUMMARY

Conditions for the determination of amitriptyline and some of its metabolites in serum on a reversed-phase material (C-8) by high-pressure liquid chromatography with UV detection at 254 nm were systematically investigated. The separation of tricyclic antidepressants is best carried out on a phase system consisting of C-8 bonded-phase material as the stationary phase and water—methanol—dichloromethane—propylamine as the mobile phase.

The precision and detection limit of the method and the extraction efficiency were established. A chromatogram of a serum extract from a patient treated with amitriptyline is shown. Serum levels of amitriptyline and its four main metabolites (nortriptyline, desmethylnortriptyline, *trans*-10-hydroxy-amitriptyline and *trans*-10-hydroxy-nortriptyline) in a patient receiving 150 mg of amitriptyline daily, are reported.

INTRODUCTION

Tricyclic antidepressants have been used for many years in order to treat psychiatric patients suffering from depression and amitriptyline, nortriptyline and protriptyline are the drugs most commonly prescribed for this purpose. In a number of papers [1—3], the relationship between the plasma concentration of tricyclic antidepressants and their clinical effect on depressive symptoms is discussed. Some workers maintain that there is no significant correlation between plasma concentrations and the improvement of depressive symptoms [1], while others found that the treatment of depression with nortriptyline is effective only in the concentration range 50—150 ng/ml in plasma [2,3].

It is known that tricyclic antidepressants are metabolized in the body [4], but present methods for the analysis of antidepressants, such as UV spectrometry [5,6], fluorimetry [7—9], thin-layer chromatography [10—12] and gas chromatography [11—17], are restricted mainly to the drugs themselves and cannot be applied to their metabolites. A more specific determination of antidepressants would be useful for metabolites with antidepressive properties (e.g., nortriptyline from amitriptyline) and to discriminate poor biological

availability from fast metabolic degradation (e.g., hydroxylation) in cases of low blood levels. Analytical procedures that permit the detection and quantitation of metabolites as well as the drug itself provide more reliable information about the concentration of active antidepressive compounds in body fluids and their clinical effect, more insight into the rate of the biotransformation of these drugs in the body, which can differ greatly individually, and the possibility of carrying out a more extensive pharmokinetic study.

High-pressure liquid chromatography (HPLC) has been shown to be a very powerful technique in biomedical analysis [18—22]. A few papers on the separation of some tricyclic antidepressants have been published, but metabolites were not included in these studies [23,24]. In this paper, the separation and determination of amitriptyline and some of its most important metabolites in plasma, using a reversed-phase system, by HPLC with UV detection is described.

EXPERIMENTAL

Apparatus

The liquid chromatographic system was constructed from a high-pressure reciprocating membrane pump (Orlita DMP 1515, Giesen, G.F.R.); a flow-through manometer acting as a pulse damper; a UV detector (Waters 440); an injection valve (Valco CV-6-UHP$_a$ or Rheodyne 70-10); a potentiometric recorder; and an electronic integrator (Autolab System I, Spectra Physics). The eluent reservoir was placed in a thermostated water-bath (25°).

In all experiments, stainless-steel 316 precision-bore columns with an I.D. of 3 mm and of different length were used. To connect the column to the detector and injection valve, terminators of modified Swagelok reducing unions assembled with 0.3-mm capillary tubing were used, guaranteeing minimal external peak broadening (Fig. 1). In order to prevent contamination of the analytical column by impurities in the eluent, a stainless-steel column (length 200 mm, I.D. 6 mm) was installed in front of the injection valve.

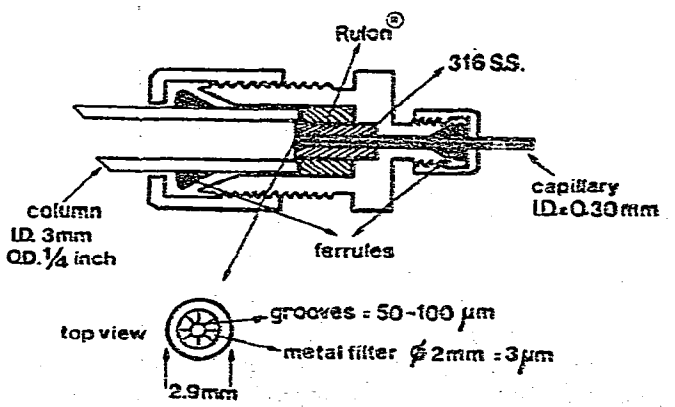


Fig. 1. Schematic representation of a column terminator.

Materials

All solvents were of analytical reagent grade and, with the exception of hexane, were used without further pre-treatment. The column materials used were commercially available C-8 (RP 8, mean particle size 5 μm; Merck, Darmstadt, G.F.R.) and C-18 (Nucleosil 10) modified silica. The antidepressants and metabolites were a gift from the St. Joris Gasthuis (Delft, The Netherlands). Their structures and the abbreviations used henceforth are given in Table I.

Procedures

Chromatography. The separation columns were packed by a pressurized balanced-slurry method [25] (the slurry liquid consisted of a mixture of chloroform and tetrabromoethane of specific gravity 1.82) and washed successively with 100 ml of acetone and 100 ml of eluent. The pre-column was packed by a dry-packing technique.

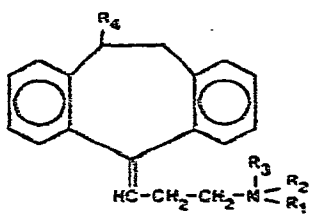
The capacity ratios (k'_i) of the solutes were calculated from their retention times and that of an unretained compound (4-aminotoluenesulphonic acid). The selectivity factors (r_{ji}) of pairs of compounds were expressed as the ratio of their capacity ratios.

The theoretical plate height of a compound was determined from its retention time and half the peak width at 0.6 of the peak height.

All solutions were prepared by mixing weighed amounts of solvents. The samples were dissolved in the eluent and injected by a valve, with a 25-μl sample loop, into the top of the column. Owing to the alkaline properties of the mobile phase, some of the column material at the top of the column becomes degraded after a few days, with the result that the column efficiency decreases.

TABLE I

STRUCTURES AND ABBREVIATIONS OF AMITRIPTYLINE AND ITS METABOLITES



Compound	Abbreviation	R_1	R_2	R_3	R_4
Amitriptyline	Ami	CH_3	CH_3	—	—
cis-10-Hydroxyamitriptyline	10-OH-Ami-C	CH_3	CH_3	—	OH
trans-10-Hydroxyamitriptyline	10-OH-Ami-T	CH_3	CH_3	—	OH
Amitriptyline N-oxide	N-ox	CH_3	CH_3	O	—
Nortriptyline	Nor	CH_3	H	—	—
cis-10-Hydroxynortriptyline	10-OH-Nor-C	CH_3	H	—	OH
trans-10-Hydroxynortriptyline	10-OH-Nor-T	CH_3	H	—	OH
Desmethylnortriptyline	Des	H	H	—	—

Removal of 3 mm of column packing and its replacement with fresh material usually restores the column efficiency completely. In all our experiments the top of the column was checked daily in order to ensure optimal performance.

Extraction. All of the glassware used for the extraction was treated successively with a solution of detergent, dilute nitric acid, ultrasonicated in acetone (10 min), washed with ethanol and finally washed with dichloromethane and dried at 50°. It was found to be necessary to use freshly distilled hexane for each extraction. The full extraction scheme for amitriptyline and some of its metabolites from plasma is outlined in Fig. 2.

The background in the chromatograms resulting from some sera can be improved by back-extraction using the following procedure. The collected hexane layers are shaken with 2 ml of 1 *M* hydrochloric acid, which extracts all of the antidepressants into the aqueous phase. The hexane layer is discarded and the aqueous phase neutralized, and the aqueous solution is then treated as the serum as outlined above.

RESULTS AND DISCUSSION

The choice of the optimal chromatographic and extraction conditions for the analysis of a mixture of compounds present at very low concentrations in a limited amount of sample by HPLC is based on a compromise between resolution and dilution on the one hand and between extraction yield and selectivity on the other. Large selectivity factors, medium retardation, minimal dispersion and short narrow-bore columns combined with a selective extraction procedure are the favourable conditions for obtaining sufficient resolution on one side and a low detection limit and precision on the other.

In order to determine the optimal chromatographic conditions for the analysis of amitriptyline and its metabolites by HPLC, a number of experiments were carried out. Initially, a C-18 bonded-phase material (Nucleosil 10 C-18) was tested as the stationary phase with water—organic solvent mixtures as the mobile phase. The effects of the type and amount of organic solvent added to the aqueous phase and the pH of the eluent on k'_i, r_{ji} and the theoretical plate

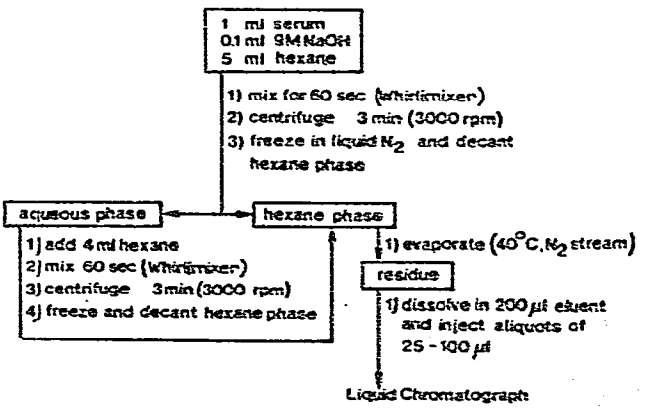


Fig. 2. Extraction scheme for amitriptyline and some of its metabolites.

height and peak form were investigated. From these primary experiments, it could be concluded that:

(i) the column efficiency of the C-18 bonded-phase material is far from ideal (tailing peaks) and is not suitable for trace analysis;

(ii) the capacity ratios and selectivity factors can be adjusted by the amount of organic solvent added to the mobile phase (methanol or acetonitrile);

(iii) the capacity ratios and elution sequence change irregularly with the pH of the mobile phase;

(iv) in acidic media the peak shape of the more retained compounds is asymmetrical;

(v) in alkaline media the peak shape of all substances is more symmetrical and favourable selectivity factors can be obtained.

To improve the column efficiency, a C-8 bonded-phase material (RP 8) with a mean particle size of 5 μm was chosen, and the possibilities of adjusting the retardation and selectivity as well as the optimal column efficiency were investigated in more detail.

Column efficiency

On the basis of the earlier results obtained with C-18 material and by means of some repeated experiments on C-8 (such as the effect of pH on k_i' and peak shape), it was decided that for precise quantitative analysis the elution of the substances as the free bases (i.e., using an alkaline mobile phase) was far more favourable with respect to column efficiency and peak shape. Fig. 3 shows a plot of the theoretical plate height, H, versus the mobile phase velocity, u, for two compounds (Nor, $k' = 9.52$; N-ox, $k' = 2.50$) on C-8 silica as the stationary phase and water—methanol, containing 1% (v/v) of propylamine, as the mobile phase. The curves are rather flat, indicating rapid mass transfer, in contrast to the results obtained on the C-18 bonded-phase material. Owing to the small particle size, the convective mixing is very favourable. The larger plate height of N-ox compared with that of Nor must be attributed to the larger contribution of the external peak broadening, mainly caused by the detector, to the overall dispersion of this compound (the external variance was found to be 325 μl^2).

Fig. 3 demonstrates that, with C-8 bonded-phase material, highly efficient narrow-bore columns can be packed that are suitable for the determination of amitriptyline and its metabolites at low concentrations.

Composition of the mobile phase

In order to optimize the composition of the mobile phase with respect to retardation and selectivity, the effects of methanol content and the type and amount of the base added to the mobile phase and the addition of dichloromethane to the water—methanol mixtures on the capacity ratios and selectivity factors were investigated.

Influence of methanol

Fig. 4 shows the effect of the methanol content of the mobile phase on k_i' and r_{ji}. An increase in the methanol content decreases the capacity ratios, changes the elution sequence and lowers the selectivity factors. A medium methanol content (ca. 60%, v/v) seems to be an appropriate choice with respect to selectivity. Such a phase system, however, is less favourable with

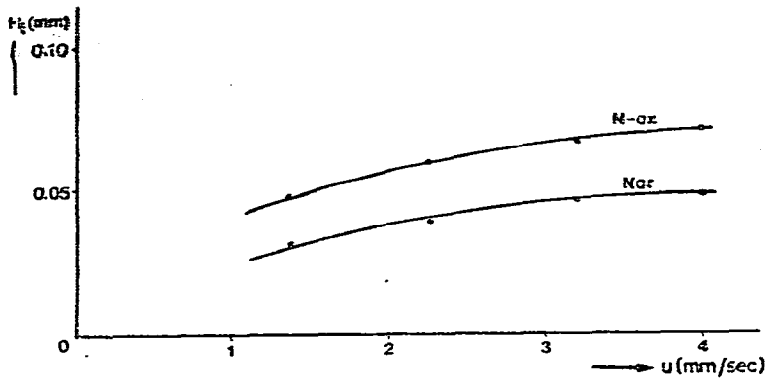


Fig. 3. H versus u curve for nortriptyline and amitriptyline N-oxide on a reversed-phase column. Stationary phase: C-8 bonded silica (RP 8). Mobile phase: water—methanol—dichloromethane (13:8:3, v/v) + 1% (v/v) of propylamine.

respect to the separation time and detection limit. The separation time of a mixture is determined by the solute pair with the smallest selectivity factor ($r_{j,i}$) and the capacity ratio of the most retained compound, and is expressed by

$$t_{R\text{min.}} = \frac{1}{(r_{j,i}-1)^2} \cdot R^2 \left(\frac{k'_i+1}{k'_i}\right)^2 (k'_{\text{max.}}+1)\,\frac{H}{u} \qquad (1)$$

where

$r_{j,i}$ = the selectivity factor of the solute pair that is the most difficult to separate, i.e., $\frac{1}{r_{ji}-1} \cdot \frac{k'_i+1}{k'_i}$ is minimal;

R = resolution;

k'_i = capacity ratio of compound i;

$k'_{\text{max.}}$ = capacity ratio of the most retained compound;

H_i = theoretical plate height of compound i;

u = linear fluid velocity.

The maximum concentration of a solute in the mobile phase at the end of the column ($<c^m_i>_{\text{max.}}$) as function of the amount injected, Q_i, is expressed by [26]

$$<c^m_i>_{\text{max.}} = \frac{Q_i}{\sqrt{2\pi}\,\epsilon_m A(1+k'_i)\,(H_i L)^{1/2}} \qquad (2)$$

where

ϵ_m = porosity of the mobile phase;

A = cross-sectional area of the column;

L = column length.

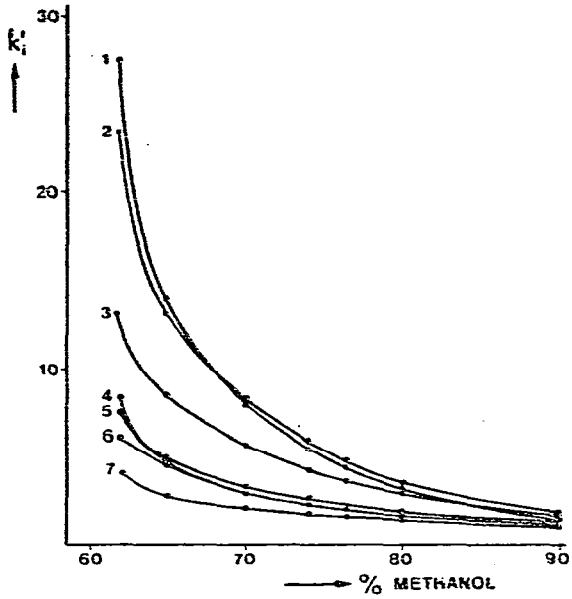


Fig. 4. Influence of the methanol content of the mobile phase on the capacity ratio (k_i'). Stationary phase: C-8 bonded silica. Mobile phase: water—methanol + 1% (v/v) of propylamine. Curves: 1 = amitriptyline; 2 = nortriptyline; 3 = desmethylnortriptyline; 4 = *trans*-10-hydroxyamitriptyline; 5 = *trans*-10-hydroxynortriptyline; 6 = *cis*-10-hydroxynortriptyline; 7 = amitriptyline N-oxide.

According to eqns. 1 and 2, large capacity ratios result in long separation times and poor detection limits.

Influence of dichloromethane

In an attempt to decrease the capacity ratios and to maintain the sufficiently large selectivity factors obtained with a medium methanol content, a more apolar organic solvent (dichloromethane) was added to a water—methanol—propylamine mixture used as the mobile phase.

The effect of the amount of dichloromethane added to the mobile phase on k_i' and r_{ji} is shown in Fig. 5. As could be expected, owing to its stronger elution strength in reversed-phase chromatography, the capacity ratios decrease with increasing dichloromethane content. At the saturation point (ca. 18.2% of dichloromethane), a small increase in k_i' occurs, which can be explained by a "spontaneous" loading effect as described earlier for straight-phase adsorption systems [27] (i.e., the solid support is loaded with the co-existing less polar phase).

On the addition of dichloromethane, the selectivity factors also decrease. Compared with the water—methanol system (Fig. 3), however, more favourable selectivity factors are obtained. This result indicates that the addition of a less polar third component such as dichloromethane or diethyl ether to water—methanol mixtures, as a type of modifier, is a useful means of adjusting retention and selectivity in reversed-phase chromatography.

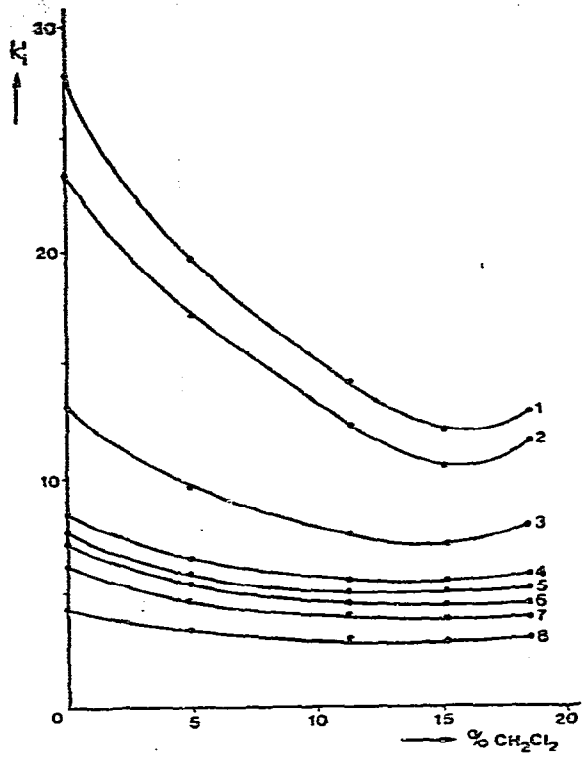


Fig. 5. Influence of the dichloromethane content of the mobile phase on the capacity ratio. Stationary phase: C-8 bonded silica. Mobile phase: water—methanol (62:38, v/v) + dichloromethane + 1% (v/v) of propylamine. Curves: 1 = amitriptyline; 2 = nortriptyline; 3 = desmethylnortriptyline; 4 = *trans*-10-hydroxyamitriptyline; 5 = *trans*-10-hydroxynortriptyline; 6 = *cis*-10-hydroxyamitriptyline; 7 = *cis*-10-hydroxynortriptyline; 8 = amitriptyline N-oxide.

Influence of the type of base

The influence of the type of base on the retention, selectivity and column efficiency was investigated by dissolving different types of bases in the mobile phase (fixed concentration) and by measuring the capacity ratios of the components (Fig. 6). Almost no difference in behaviour can be noticed between ethylamine and propylamine. The capacity ratios are smaller with hexylamine, probably owing to a stronger competition with the substances for occupation of adsorption sites, as would be expected for the hydrophobic hexalyamine.

With ammonia as the base, a drastic increase in the capacity ratios and a completely different elution sequence occur. This anomalous behaviour shows that the more hydrophobic bases also act as a modifier. According to Fig. 6, ammonia is by far the best choice with respect to selectivity but, especially for the most retained compounds, the peak shapes are less symmetrical. Propylamine seems to be a good compromise, because on the one hand the peak symmetry is reasonable while on the other the elution sequence is favourable for the determination of the metabolites, which are usually present at lower concentrations than that of the main drug.

Influence of propylamine concentration

The addition of propylamine to the mobile phase improves the column efficiency and peak shape considerably, but also affects the capacity ratio and selectivity factors, as is shown in Fig. 7. It can be seen that the amount of propylamine significantly influences the capacity ratio, selectivity factors and elution sequence. The capacity ratios decrease sharply with increasing amount of propylamine and then tend to become constant. According to Fig.7, a small content of propylamine (ca. 0.2—0.6%) seems to be favourable for the separation of these substances. Unfortunately, the column efficiency and peak shape at low propylamine contents is poor. A content of 1% seems to be a good choice.

The final choice of the mobile phase composition and column length for the determination of amitriptyline and some of its metabolites is a column length of 125 mm and an eluent composition of water—methanol—dichloromethane (8:13:3, v/v) containing 1% (v/v) of propylamine. The efficiency of this system in the separation of amitriptyline and its metabolites is illustrated by Fig. 8, which shows the separation of amitriptyline and five of its metabolites in about 10 min. On the column used, some pairs of metabolites (10-OH-Nor-T/10-OH-Ami-C and 10-OH-Nor-C/N-ox) are not well resolved owing to the limited number of theoretical plates. In practice, however, the metabolites 10-OH-Ami-C, 10-OH-Nor-C and N-ox are usually less important and are present in plasma at such low concentrations that no disturbance of the determination of the other metabolites occurs. If one is particularly interested in these metabolites, longer columns have to be used or the phase system has to be modified (e.g., by using ammonia instead of propylamine). In both instances one has to accept larger capacity ratios and higher detection limits.

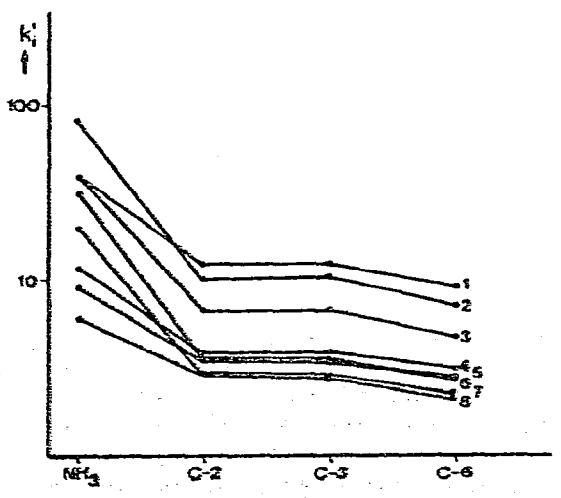


Fig. 6. Influence of the type of base added to the mobile phase on the capacity ratio. Stationary phase: C-8 bonded silica. Mobile phase: water—methanol—dichloromethane (13:8:3, v/v + 0.12 *M* of the base. Base: C-2 = ethylamine; C-3 = propylamine; C-6 = hexylamine; NH_3 = ammonia. Curves as in Fig. 5.

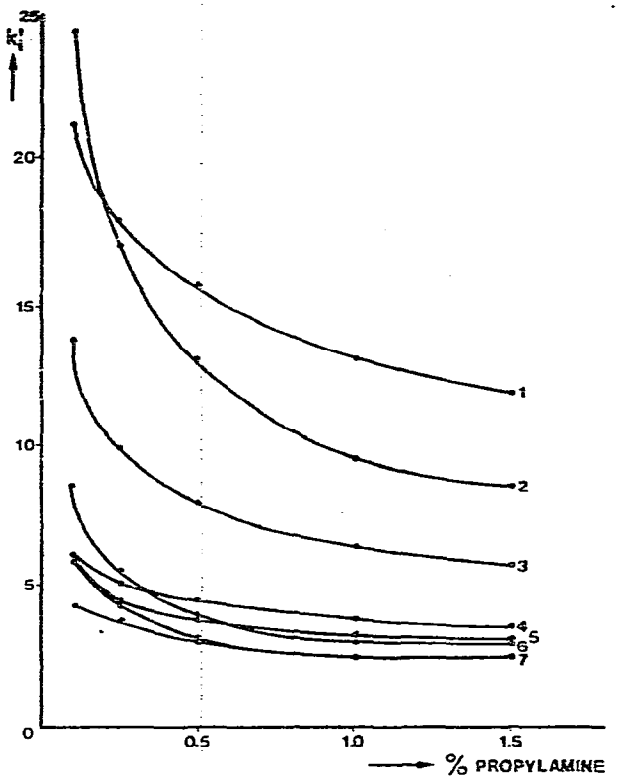


Fig. 7. Influence of the amount of propylamine on the capacity ratio. Stationary phase: C-8 bonded silica. Mobile phase: water—methanol—dichloromethane (13:8:3, v/v) + propylamine. Curves as in Fig. 4.

Quantitative aspects of the method

Precision and linearity. The precision of the determination of amitriptyline and its metabolites by HPLC and the linear range were determined by injection of a constant volume (25 μl) of solutions of the solutes at different concentrations (20—4000 ng/ml) and peak-area measurements. The correlation coefficient of the linear regression of peak area versus amount of amitriptyline injected was found to be 0.9999, indicating a high degree of linearity. The relative standard deviation was about 0.6% for 4000 ng/ml and 15% for 20 ng/ml (i.e., 100 and 0.5 ng injected, respectively). The sensitivity of the whole system, defined as the slope of the peak area versus amount of amitriptyline injected and calculated by linear regression, was found to be 1425 IU/ng (1 IU = 1 μV · sec).

The standard deviation of the baseline noise, measured during the same period of time as the peak integral, was determined to be 150 IU, corresponding to 0.1 ng of amitriptyline. The detection limit of amitriptyline for a signal-to-noise ratio of 3 is about 0.3 ng. For all metabolites the detection limit falls within the range 0.3—0.6 ng.

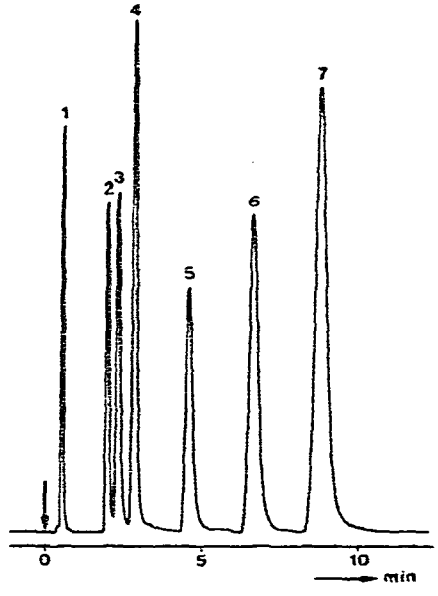


Fig. 8. Separation of a test mixture of amitriptyline and five of its metabolites. Stationary phase: bonded silica; column length, 125 mm. Mobile phase: water—methanol—dichloromethane (13:8:3, v/v) + 1% of porpylamine. Peaks: 1 = unretained; 2 = *cis*-10-hydroxynortriptyline; 3 = *trans*-10-hydroxynortriptyline; 4 = *trans*-10-hydroxyamitriptyline; 5 = desmethylnortriptyline; 6 = nortriptyline; 7 = amitriptyline.

Protriptyline, with a k'_i value between those of Nor and Des, can be used as an internal standard.

Recovery and reproducibility of the extraction. The recovery and reproducibility of the extraction procedure were determined by HPLC and by extraction of known amounts of amitriptyline and some metabolites added in different amounts of distilled water and blank serum. In order to check that no interferring substances are co-extracted from water and serum, some blank extractions were carried out. As shown in Fig. 9, clean extracts were obtained, showing little interference even at the most sensitive detector attenuation (0.005 absorbance units full-scale). It was found that under the chosen extraction conditions, the most polar metabolite (N-ox) was not extracted. The recoveries of amitriptyline and the polar metabolite 10-OH-Nor-T from both water and serum were found to be 98% and 79%, respectively. The recoveries of the other metabolites (except N-ox) were between these two values.

The reproducibility of the extractions was about 4% at 500 ng/ml (n = 3) and 7% at 80 ng/ml (n = 3).

Fig. 10 shows the separation of amitriptyline and four of its most important metabolites (about 150 ng of each) added to 1 ml of blank serum and extracted as described above.

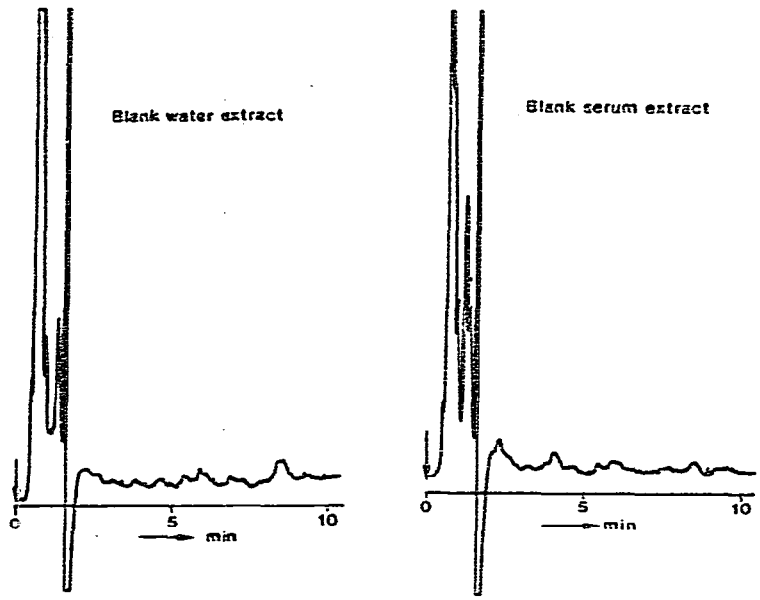


Fig. 9. Background of blank water and serum extracts. Conditions as in Fig. 8. Detector attenuation, 0.005 absorbance units full-scale. Injection volume, 50 μl.

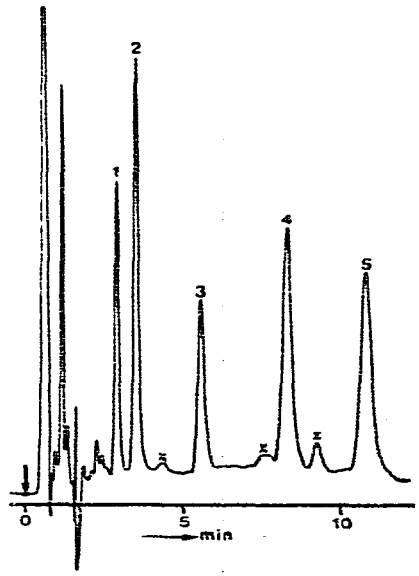


Fig. 10. Analysis of amitriptyline and four metabolites (ca. 150 ng of each) extracted from a spiked serum. Conditions as in Fig. 8. Peaks: 1= *trans*-10-hydroxynortriptyline; 2= *trans*-10-hydroxyamitriptyline; 3= desmethylnortriptyline; 4= nortriptyline; 5= amitriptyline; x= unknown compounds.

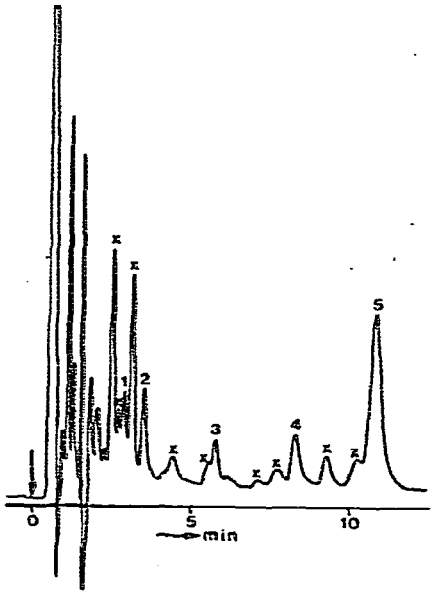


Fig. 11. Analysis of a serum extract from a patient after a daily oral administration of 150 mg of amitriptyline for more than 2 weeks. Conditions as in Fig. 8. Peaks as in Fig. 10.

Determination of amitriptyline and its metabolites in serum

The procedure described above was applied to the determination of amitriptyline and some of its metabolites in a sample of serum from a psychiatric patient supposed to receive only one of the tricyclic drugs.

Fig. 11 shows the chromatogram of a serum extract of a patient who had been receiving 150 mg of amitriptyline daily for more than 2 weeks. The large number of peaks possibly indicate either co-medication or the residues from such treatment. At least five peaks could be identified positively: Ami, Nor, Des, 10-OH-Ami-T and 10-OH-Nor-T. The serum levels found for this patient were: Ami 181; Nor 35; Des 25; 10-OH-Ami-T 16; and 10-OH-Nor-T 84 ng/ml.

CONCLUSION

The studies described show that reversed-phase adsorption chromatography is very suitable for the determination of a drug and its more polar metabolites in blood at very low concentrations. Future work will be devoted to the determination of the very polar metabolites of amitriptyline and to the determination of antidepressants in the presence of co-medicaments.

ACKNOWLEDGEMENT

The authors thank Dr. R.G. Muusze for his stimulating and valuable discussions during the preparation of the manuscript.

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