*Journal of Chromatography*, 383 (1986) 119—127
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3322

# DEVELOPMENT OF A RAPID EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION FOR AMITRIPTYLINE AND SIX BIOLOGICAL METABOLITES

JEFFREY S. KIEL

*Personal Products Division, Bausch & Lomb, 1400 N. Goodman Street, P.O. Box 450, Rochester, NY 14692 (U.S.A.)*

RUTH K. ABRAMSON

*William S. Hall Psychiatric Institute, Columbia, SC 29201 (U.S.A.)*

and

CYNTHIA S. SMITH and STEPHEN L. MORGAN*

*Department of Chemistry, University of South Carolina, Columbia, SC 29208 (U.S.A.)*

(First received October 16th, 1985; revised manuscript received July 9th, 1986)

## SUMMARY

The development of a rapid high-performance liquid chromatographic method for the determination of amitriptyline, amitriptyline-N-oxide, 10-hydroxyamitriptyline, 10-hydroxynortriptyline (*E* and *Z* isomers), nortriptyline and desmethylnortriptyline in plasma and liver tissue is described. A liquid—liquid extraction with hexane—butanol and back-extraction into phosphoric acid provides efficient extraction of amitriptyline-N-oxide along with amitriptyline and the other metabolites. A Supelcosil $C_8$ reversed-phase column with 5-$\mu$m packing and a methanol—sodium phosphate buffer—amine modifier mobile phase was used. The combination of mobile phase pH and amine modifier concentration for the best separation within a reasonable analysis time for all seven solutes plus an internal standard was determined using a factorial design coupled with a multi-factor window diagram technique. Ultraviolet detection at 214 nm provided limits of detection of approximately 1 ng/ml.

0378-4347/86/$03.50 

## INTRODUCTION

The tricyclic antidepressant amitriptyline (AMI) is widely prescribed for the treatment of major depressive disorders. Amitriptyline undergoes extensive biotransformations by N-demethylation pathways to nortriptyline (NOR) and desmethylnortriptyline (DES), by hydroxylation pathways to 10-hydroxy-amitriptyline (10-OH-AMI) and 10-hydroxynortriptyline (10-OH-NOR) and by N-oxidation to amitriptyline-N-oxide (AMI-N-O) [1–4]. The relative importance of each metabolic pathway appears to be species-dependent. For example, AMI-N-O is a significant product of amitriptyline metabolism in rat and mouse liver microsomes and is found in dog urine but has not been reported in human liver microsomes or human plasma [1, 5, 6]. AMI-N-O, which has recently undergone clinical trials for efficacy as an antidepressant, is itself metabolized to 10-OH-AMI and 10-OH-NOR (*E* and *Z* isomers) in humans [3].

The large number of reports describing high-performance liquid chromatographic (HPLC) methods for the determination of amitriptyline and its metabolites attest to the significance of this assay [7–16]. None of these papers, however, consider the simultaneous measurement of all of the metabolites of amitriptyline. Furthermore, methods for the measurement of the demethylated and hydroxylated metabolites of amitriptyline have not been able to quantitate AMI-N-O, perhaps because of the sample preparation method. A combined extraction and HPLC method has been developed for the quantitation of AMI-N-O [10], but the method is time-consuming and does not allow for the simultaneous measurement of the other amitriptyline metabolites.

In this paper, we report the development of an extraction method and reversed-phase HPLC separation for the simultaneous quantitation of amitriptyline and six biological metabolites in human plasma or animal tissue. Window diagram techniques, coupled with multi-factor experimental designs, are employed to define the mobile phase composition providing adequate separation of all components.

## EXPERIMENTAL

### *Apparatus*

A Waters HPLC system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 441 UV absorbance detector (operated at 214 nm), a Model 6000A solvent delivery system, a Model 710 autoinjector, a Model 720 integrator and a Model 730 system controller was employed. A Supelcosil $C_8$ reversed-phase column (250 × 4.6 mm with 5-$\mu$m packing) fitted with a $C_8$ guard column (50 × 4.6 mm with 40-$\mu$m packing) was used (Supelco, Bellefonte, PA, U.S.A.). The column was heated to 40°C in a Waters heating block with a Haake FJ water bath (Haake, Saddle Brook, NJ, U.S.A.).

### *Reagents*

HPLC-grade hexane, methanol, 1-butanol, phosphoric acid and isopropanol were obtained from Fisher Scientific (Atlanta, GA, U.S.A.) and reagent-grade potassium hydroxide, dibasic sodium phosphate, propylamine and methyl-

amine hydrochloride were obtained from Aldrich (Milwaukee, WI, U.S.A.). A 4.5 *M* solution of potassium hydroxide containing 0.1% triethylamine was prepared and stored at room temperature. A 3 *M* solution of phosphoric acid was prepared and stored at room temperature. A mixture of hexane—1-butanol (95:5) was stored in a glass container at room temperature.

*Mobile phase*

The isocratic mobile phase for routine analysis was prepared by mixing 600 ml of methanol and 300 ml of water, and then adding sufficient dibasic sodium phosphate and methylamine hydrochloride to make final concentrations in the mobile phase of 25 and 250 m*M*, respectively. The pH was adjusted to 7.25 by dropwise addition of concentrated phosphoric acid. Other mobile phase combinations were prepared as needed. All mobile phases were degassed by ultrasonic vibration prior to use. Column mobile phase flow-rate was 2.0 ml/min.

*Standards*

A 0.5 g/l standard stock solution of the following pure drugs was prepared in isopropanol: amitriptyline, nortriptyline, desmethylnortriptyline, *trans*-10-hydroxyamitriptyline, *trans*-10-hydroxynortriptyline (*E*-10-OH-NOR), *cis*-10-hydroxynortriptyline (*Z*-10-OH-NOR) and amitriptyline-N-oxide. All drug standards were provided by Merck Sharp & Dohme (Rahway, NJ, U.S.A.). A small amount of triethylamine (0.1%) was added to prevent adsorption of drugs to the glass container. Working standards were made by serial dilution of the stock standard with water and stored at 4°C.

Imipramine was chosen as the internal standard because of its structural similarities to amitriptyline and because it elutes in a region of the chromatogram well separated from the solutes of interest. A stock internal standard containing 1 g/l imipramine (USV Labs., Tuckahoe, NJ, U.S.A.) was prepared in isopropanol. The working internal standard was prepared by a 500-fold dilution of the stock internal standard with water to a final concentration of 2 mg/l with 0.1% triethylamine added to prevent adsorption to the glass container.

*Samples*

Patient blood samples were collected in Royal Blue stoppered vacutainers (Bectin-Dickinson, Rutherford, NJ, U.S.A.) and centrifuged within 2 h of sampling to obtain the plasma. The plasma was then stored at −15°C in a clean Royal Blue vacutainer tube until analyzed.

Rat and mouse liver homogenates were prepared as described by Abramson and Hutton [17] for assay of microsomal metabolites of amitriptyline. Substrate metabolism was measured after incubation for 20 min at 37°C in a system containing microsomes (0.2 ml liver homogenate) in 50 $\mu M$ Tris buffer (pH 9.0), 0.5 $\mu$g NADPH, 5 $\mu$mol glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase and 3 $\mu$mol magnesium chloride (0.8 ml) to which 50 $\mu$g amitriptyline were added. The reaction was quenched with 1 ml of acetone and samples were stored at −70°C until analyzed.

*Sample preparation*

Plasma (2 ml) or tissue homogenate was transferred to a 150 × 15 mm borosilicate disposable glass test tube. Into this tube were added 100 μl of working internal standard, 250 μl of 25% sodium carbonate solution, and 5 ml of the hexane—1-butanol (90:10) solution. Each tube was vortexed rapidly for 30 s and then centrifuged for 3 min at 500 *g* to break the emulsion. The aqueous layer was then removed by aspiration and the organic layer was transferred to a 15-ml glass conical centrifuge tube. A 100-μl aliquot of 3 *M* phosphoric acid was added and the tube vortexed rapidly for 30 s. The organic layer was discarded and 75 μl of 4.5 *M* sodium hydroxide were added to neutralize the acid layer. A 100-μl aliquot of the neutralized aqueous layer was injected onto the column.

*Experimental design and modelling*

A two-factor 3 × 4 factorial experimental design was performed over mobile phase combinations of 100, 300 and 500 m*M* amine modifier and pH 6.9, 7.2, 7.5 and 7.8. This factorial design was performed for both of the two amine modifiers investigated (methylamine and propylamine). The retention time for each of the eight solutes was measured and capacity factors ($k'$) were calculated. A full second-order model was fitted to the $k'$ data versus pH (factor $x_1$) and amine modifier concentration (factor $x_2$):

$$k' = b_0 + b_1 x_1 + b_{11} x_1^2 + b_2 x_2 + b_{22} x_2^2 + b_{12} x_1 x_2$$

The parameters of these linear models for each solute were estimated by a matrix least-squares program written in BASIC on a Model 85A computer (Hewlett-Packard, Palo Alto, CA, U.S.A.) [18]. The fitted models were plotted as a function of the two factor levels using a pseudo-three-dimensional plotting program on the HP-85A interfaced to a Model 7225B graphics plotter (Hewlett-Packard).

Regions of maximum relative retention over the factor space investigated were defined using a two-dimensional window diagram. A BASIC computer program calculated the relative retention of each pair of peaks (28 combinations are possible from the 8 solutes) by ratioing predicted $k'$ values over a 20 × 20 grid (400 points) in the factor space examined. At each of these grid points, the smallest relative retention (the relative retention for the worst separated pair of peaks) was plotted by the pseudo-three-dimensional plotting program.

## RESULTS

*Chromatography*

The pH of the mobile phase and the type of amine modifier added to the mobile phase have previously been shown to be crucial factors in obtaining adequate separation and peak symmetry for tricyclic antidepressants [19, 20]. Other factors, such as mobile phase sodium ion concentration, also affect retention of high $pK_a$ amines [20]. In this study, sodium ions were added to the mobile phase to make a fixed concentration of 50 m*M* (as sodium phosphate). Methanol was employed as the organic modifier (instead of aceto-

nitrile or tetrahydrofuran) to improve the peak symmetry of the AMI-N-O peak.

Several amine modifiers were examined, but only the primary amine modifiers (e.g. methylamine and propylamine) were found to produce both acceptable peak symmetry and separation of all seven solutes. The level of pH and the amine modifier concentration in the mobile phase were found to greatly affect the peak separation of the solutes. Initial experiments indicated that a mobile phase pH below 6.9 resulted in poor peak symmetry for the AMI-N-O peak, except at very low pH (less than 3.0) where coelution of desmethylnortriptyline and nortriptyline occurred. The addition of amine modifier (methylamine or propylamine) to the mobile phase in a concentration of 100 m*M* was necessary to provide adequate peak symmetry.

The $k'$ plot for the last eluting solute, amitriptyline, with methylamine as the amine modifier is shown in Fig. 1. The $k'$ response surfaces for the other solutes were similar in appearance; the plot for amitriptyline is shown because it is this solute that determines the overall analysis time. Window diagrams for chromatographic systems employing the two different amine modifiers,

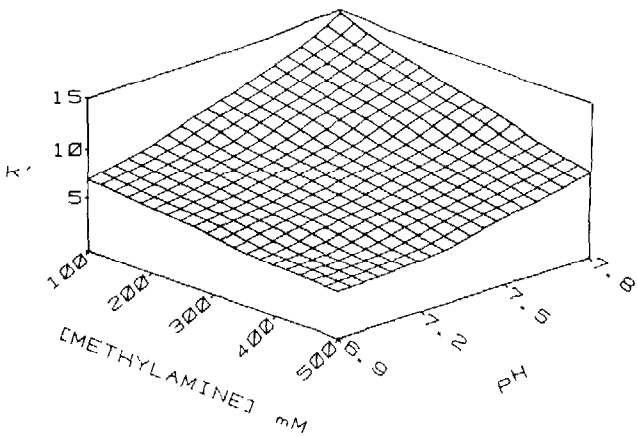



Fig. 1. Plot of the effects of pH and mobile phase methylamine concentration on the capacity factor ($k'$) for amitriptyline.

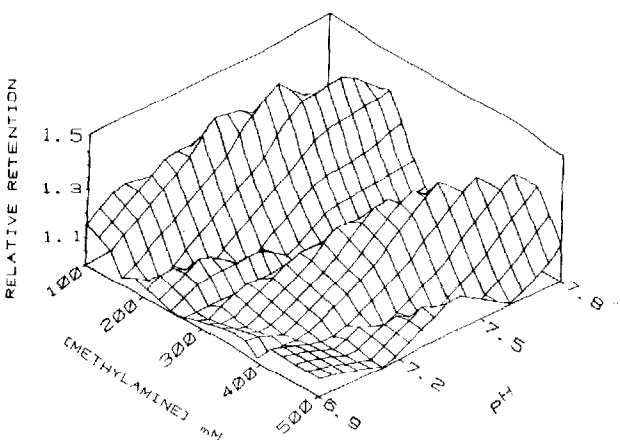



Fig. 2. Two-factor window diagram showing the effects of pH and mobile phase methylamine concentration on the relative retention of all eight solutes.

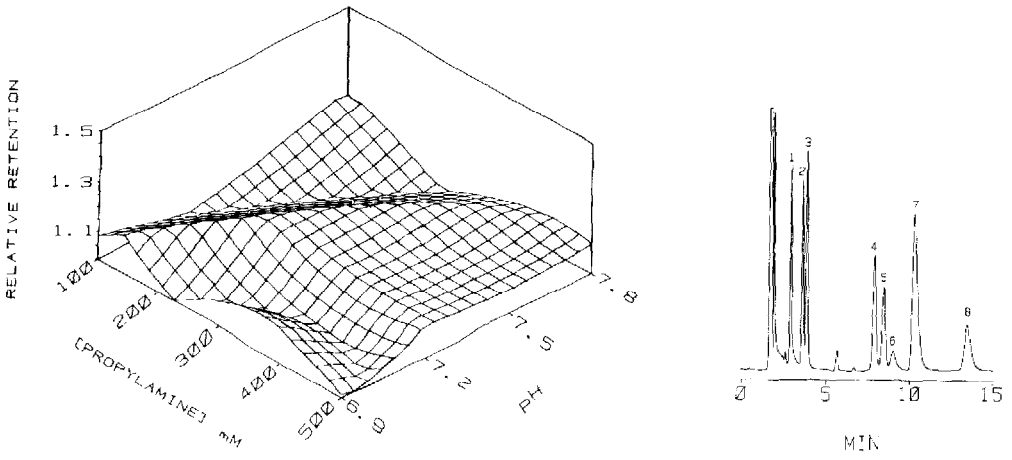



Fig. 3. Two-factor window diagram showing the effects of pH and mobile phase propylamine concentration on the relative retention of all eight solutes.

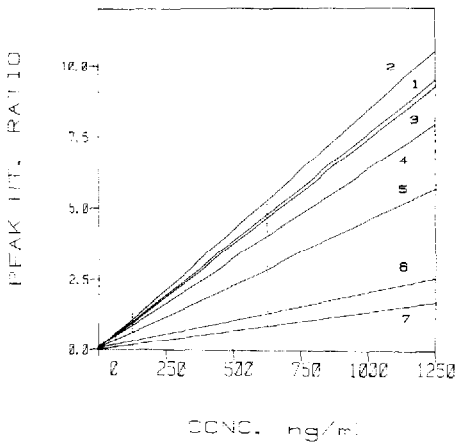
Fig. 4. Chromatogram produced at optimum mobile phase conditions of the seven solutes and the internal standard, imipramine. Peaks: 1 = *trans*-10-hydroxynortriptyline; 2 = *cis*-10-hydroxynortriptyline; 3 = *trans*-10-hydroxyamitriptyline; 4 = desmethylnortriptyline; 5 = nortriptyline; 6 = amitriptyline-N-oxide; 7 = internal standard; 8 = amitriptyline.

methylamine and propylamine, are shown in Figs. 2 and 3. Examination of these plots reveals several regions in which each of the solutes can be separated. The jagged tops of the ridges in Fig. 2 are due to the resolution of the plot and are actually sharp smooth ridges.

Comparison of the two-factor window diagrams indicates that higher values of relative retention (for the worst separated pair of peaks) are found on the methylamine surface, which has two local optima. The ridge at lower methylamine concentration has a greater relative retention than the ridge at higher methylamine concentration; however, the retention time of the last eluting peak (amitriptyline) is much longer at lower methylamine concentrations (Fig. 1). The overall best separation is thus found along the lower ridge with the lowest mobile phase pH. A mobile phase with 250 m*M* methylamine and pH 7.25 was selected on the basis of this data as providing the best separation of all components within the shortest run time. Fig. 4 shows a chromatogram produced at these optimum chromatographic conditions.

*Applications of the method*

A calibration curve is presented in Fig. 5 for each of the drugs at seven different levels of concentration over a range of 1—1250 ng/ml. Each of the standards at the different concentration levels was assayed in triplicate. The calibrations were all found to be linear with no significant lack of fit at the 95% level of confidence over the concentration range examined. Reproducibility of replicate analyses were typically in the range of 4—6% relative standard deviation. The limit of detection for each of the peaks was 1 ng/ml. The analytical recovery of each of the components was determined by comparing chromatograms of extracted standards with chromatograms of standards injected directly into the HPLC system. The recovery at two different concentration levels is



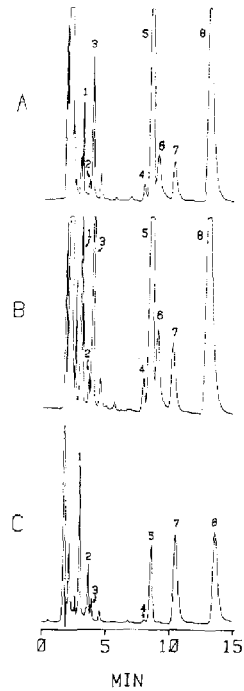




Fig. 5. Linear calibration plots for each of the seven drugs. 1 = *cis*-10-hydroxynortriptyline; 2 = *trans*-10-hydroxynortriptyline; 3 = *trans*-10-hydroxyamitriptyline; 4 = desmethylnortriptyline; 5 = nortriptyline; 6 = amitriptyline; 7 = amitriptyline-N-oxide.

Fig. 6. Representative chromatograms of (A) mouse liver homogenate, (B) rat liver homogenate and (C) human plasma. Peaks: 1 = *trans*-10-hydroxynortriptyline; 2 = *cis*-10-hydroxynortriptyline; 3 = *trans*-10-hydroxyamitriptyline; 4 = desmethylnortriptyline; 5 = nortriptyline; 6 = amitriptyline-N-oxide; 7 = internal standard; 8 = amitriptyline.

TABLE I

ANALYTICAL RECOVERY OF THE TRICYCLIC ANTIDEPRESSANT AMITRIPTYLINE AND SIX METABOLITES

| Compound | Recovery (%) | |
|---|---|---|
| | 1250 ng/ml | 125 ng/ml |
| Amitriptyline | 60.4 | 50.0 |
| Nortriptyline | 56.9 | 49.0 |
| Desmethylnortriptyline | 52.2 | 44.9 |
| 10-Hydroxyamitriptyline | 63.2 | 60.6 |
| *trans*-10-Hydroxynortriptyline | 52.3 | 49.7 |
| *cis*-10-Hydroxynortriptyline | 62.0 | 66.5 |
| Amitriptyline-N-oxide | 53.3 | 46.1 |

presented in Table I. With the reasonable assumption that imipramine extracts similarly to the drug components of interest, the use of an internal standard compensates for the rather low recovery values.

Analysis of plasma samples from patients undergoing routine amitriptyline therapy were performed using the method as described. A representative chro-

matogram of an extracted human plasma sample is shown in Fig. 6C. Amitriptyline and all of its metabolites, except for AMI-N-O, can be seen in this chromatogram. Mouse and rat liver homogenates were also prepared as described above; representative results are shown in Fig. 6A and B. Amitriptyline and all six metabolites were present in these samples. Qualitative differences observed in these samples suggests the potential for further quantitative studies of drug metabolism using HPLC [21].

## CONCLUSION

A method for the simultaneous measurement of amitriptyline and six of its metabolites (nortriptyline, desmethylnortriptyline, amitriptyline-N-oxide, *trans*-10-hydroxyamitriptyline, *cis*-10-hydroxynortriptyline and *trans*-10-hydroxynortripytline) in human plasma, rat liver homogenates and mouse liver homogenates has been described. The drugs are extracted from biological tissues with a rapid liquid–liquid extraction technique requiring less than 8 min to complete. This extraction scheme also provides greater recovery for amitriptyline and nortriptyline compared to our previously reported extraction method [19].

Combinations of mobile phase pH and amine modifier (methylamine or propylamine) concentration providing best separation of the worst separated pair of components within a reasonable analysis time were explored with a two-factor experimental design. To visualize the relative retention response surface, a two-factor window diagram was produced using chromatographic retention models of the data obtained from the factorial experiments. Computer graphics enables the chromatographer to visualize the appropriate response surfaces and make informed judgments concerning experimental regions giving best operating conditions. With the optimized chromatographic conditions, the reversed-phase separation of the seven solutes plus the internal standard can be completed within 15 min. The HPLC method presented in this report is being applied in our laboratory to determine concentrations of amitriptyline and its different metabolites in a variety of biological samples.

## REFERENCES


1 B. Mellström and C. von Bahr, Drug Metab. Dispos., 9 (1981) 565–568.
2 J.L. Bock, E. Giller, S. Gray and P. Jatlow, Clin. Pharmacol. Ther., 31 (1982) 609–616.
3 I. Midgley, D.R. Hawkins and L.F. Chasseaud, Arzneim.-Forsch./Drug Res., 28 (1978) 1911–1916.
4 R.R. Brodie, L.F. Chasseaud, D.R. Hawkins and I. Midgley, Arzneim.-Forsch./Drug Res., 28 (1978) 1908–1910.
5 H.B. Hucker, A.J. Balletto, J. Demetriades, B.H. Arison and A.G. Zacchei, Drug Metab. Dispos., 5 (1977) 132–142.
6 M.H. Bickel, Arch. Biochem. Biophys., 148 (1971) 54–62.
7 J.C. Kraak and P. Bijster, J. Chromatogr., 143 (1977) 499–512.
8 B. Mellström and R. Braithwaite, J. Chromatogr., 157 (1978) 379–385.
9 S.R. Biggs, L.F. Chasseaud, D.R. Hawkins and I. Midgley, Drug Metab. Dispos., 7 (1979) 233–236.
10 K.M. Jensen, J. Chromatogr., 183 (1980) 321–329.
11 S.H. Preskorn, K. Leonard and C. Hignite, J. Chromatogr., 197 (1980) 246–250.

12 R.F. Suckow and T.B. Cooper, J. Chromatogr., 230 (1982) 391—400.
13 G.A. Smith, P. Schulz, K.M. Giacomini and F. Blaschke, J. Pharm. Sci., 71 (1982) 581—583.
14 P.M. Edelbroek, J.M. de Haas and F.A. de Wolff, Clin. Chem., 28 (1982) 2143—2148.
15 T. Visser, M.C.J.M. Oostelbos and P.J.M.M. Toll, J. Chromatogr., 309 (1984) 81—93.
16 P.P. Rop, A. Viala, A. Durand and T. Conquy, J. Chromatogr., 338 (1985) 171—178.
17 R.K. Abramson and J.J. Hutton, Cancer Res., 35 (1975) 23—29.
18 S.N. Deming and S.L. Morgan, Clin. Chem., 25 (1979) 840—855.
19 J.S. Kiel, R.K. Abramson, S.L. Morgan and J.C. Voris, J. Liq. Chromatogr., 6 (1983) 2761—2773.
20 J.S. Kiel, S.L. Morgan and R.K. Abramson, J. Chromatogr., 320 (1985) 313—323.
21 C.S. Smith, R.K. Abramson and S.L. Morgan, J. Liq. Chromatogr., 9 (1986) 727—745.