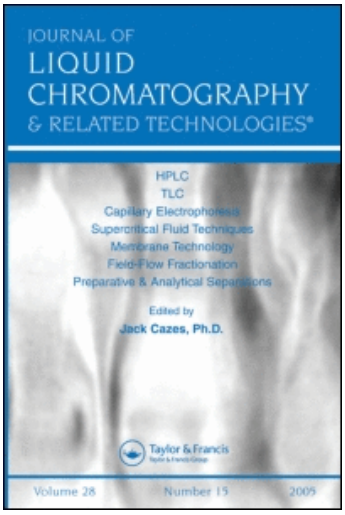

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A MODIFIED SIMPLE AND RAPID REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTIFICATION OF AMITRIPTYLINE AND NORTRIPTYLINE IN PLASMA

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ABSTRACT

A simple and rapid reversed-phase liquid chromatographic method for the determination of amitriptyline and nortriptyline in plasma is described. Protriptyline was used as internal standard. Plasma samples were extracted from alkaline pH with hexane-isoamylalcohol 98:2 v/v for 60 sec on a vortex apparatus. The organic phase was back extracted with HCl 0.1N and the aqueous phase was injected directly and monitored at 240nm. Samples were chromatographed on a 5 μm Kromasil 100-C8 column (25 cm x 4 mm id) using acetonitrile/0.04M KH_2PO_4 40:60 v/v as the mobile phase. The lower limit of detection was 5 ng/ml for amitriptyline and nortriptyline while the lower limit of quantification was 10 ng/ml for both compounds. Peak height and plasma amitriptyline or nortriptyline concentrations were linearly related from 25 to 300 ng/ml. No potential sources of interference have been identified. The methodology was used to monitor plasma amitriptyline or nortriptyline concentrations in patients receiving therapeutic doses of these drugs as well as for qualitative determination of several tricyclic antidepressants in plasma in cases of emergency toxicology.

INTRODUCTION

Tricyclic antidepressants are widely used in the treatment of patients suffering from depression. Therapeutic monitoring of tricyclic antidepressants plasma levels can be important in determining clinical efficacy, predicting side effects and monitoring compliance (1-4). Various methods have been described for the measurement of tricyclic antidepressants in plasma and have been extensively reviewed (5-7). High performance liquid chromatographic (HPLC) approaches for the determination of tricyclic antidepressants in plasma have been reported in the literature (8-23).

The most widely used tricyclic antidepressant is amitriptyline (AT). Its main metabolite nortriptyline (NT), formed by mono-N-demethylation, also contributes towards the pharmacological activity and is also marketed as a therapeutic agent.

Many analytical methods have been described for the determination of AT and NT, in plasma. Among these methods several are based on normal phase (10,11,24,25), reversed-phase (12-15,26,27) and ion-pair reversed-phase (16,28) high performance liquid chromatography as well as on gas chromatography (29-32).

This report describes a relatively simple, specific and accurate reversed-phase liquid chromatographic method, which allows quantification of AT and NT in plasma. The method developed modifies previously described methods (12-16,26) in several aspects offering convenience and rapidity in analysis. These modifications include the use of C-8 column with a simpler mobile phase, in which no pH adjustment or addition of amines are required , two rapid extraction steps and a direct injection of the aqueous acidic phase onto the HPLC system. Preliminary data suggests that the method is equally suitable for quantification of clomipramine in plasma as well as for qualitative determination of several tricyclic antidepressants and their metabolites in plasma, in cases of emergency toxicology.

MATERIALS AND METHODS

Apparatus

Chromatography was performed with an HPLC system consisting of a JASCO (Japan Spectroscopic Co. LTD) Model 880PU pump fitted with a model 880-02 Ternary Gradient Unit which was used under isocratic conditions on manual mode. The system

fitted with a Model 7125 manual injector (Cotati, Rheodyne, California, U.S.A.) and a 50 µl sample loop.

A Jasco Model 875 UV variable wavelength UV/VIS detector was operated at 240 nm. Samples were chromatographed on a 5 µm Kromasil 100-C8, 25 cm x 4 mm (i.d) reversed-phase column (MZ Analysentechnic D-6500 Mainz). A Hewlett Packard HP3394A integrator was used to record chromatograms, at peak height mode (chart speed 0.5 cm/min). A Millipore filtration system (Millipore, Bedford, M.A. USA) with type HV Millipore filters (pore size 0.45 µM) was used, for degassing mobile phase under vacuum.

Reagents and chemicals

Acetonitrile and water were HPLC grade (Lichrosolv®) and were obtained from Merck. Hexane and isoamylalcohol were analytical grade and were obtained from Ferak-Berlin. Amitriptyline, nortriptyline and protriptyline were gifts from local representatives.

Chromatographic Conditions

The mobile phase consisted of acetonitrile/0.04M KH_2PO_4 40:60 v/v. A flow rate of 1.0 ml/min was used at ambient temperature, resulting in a pressure of about 130 kg/cm^2. Mobile phases were degassed by vacuum through filtration, after mixing. Analysis was performed at 240 nm with the detector set at 0.004 absorbance units full-scale.

Standards for calibration graphs

Stock standard solutions of AT, NT, and protriptyline (Pro), which was used as an internal standard, were prepared in methanol to give final concentrations of 1 mg/ml. Standards stored at 4°C have been stable for 8 months. Standard solutions of final concentration of 100 µg/ml were prepared by diluting the stock standards 10-fold with distilled water. An aqueous reference solution containing both AT and NT to final concentration 10 µg/ml was prepared from a 100 µg/ml standard solution of each compound. Working solutions were prepared containing 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5,

3.0 μg/ml of AT and NT by appropriate dilutions of the reference solution with water. Plasma standards for calibration curves were prepared by spiking 1.0 ml aliquots of pooled drug free plasma with 100 μl of the above mentioned working solutions, to make AT and NT plasma standards ranging from 25 to 300 ng/ml. A working solution of internal standard (0.3 μg/ml) was prepared by dissolving 300 μl of a 10 μg/ml aqueous solution into 10ml of distilled water. Calibration graphs of the recovered standards were prepared for each day of analysis to establish linearity and reproducibility of the HPLC system. Graphs were constructed of the peak-height ratio of each compound to internal standard against drug concentration.

Extraction procedure

In 10-ml glass conical tube with glass stopper, 1.0 ml of plasma, 100 μl of internal standard aqueous solution 0.3 μg/ml (30ng), and 200 μl of 1.5M solution NaOH were added and mixed briefly for 15 sec on vortex. Each sample was extracted with 6.0 ml of hexane-isoamylalcohol 98:2 v/v on vortex for 60 sec at speed 4 (Vortex-Genie, Model K-550 GE, Scientific Ind. Spingfield Mass 01103). The sample tube was centrifuged for 5 min at 2000rpm. The upper (organic) layer was then transferred into a 10-ml conical glass tube and reextracted with 200 μl HCl 0.1N on vortex for 60 sec at speed 4 and then centrifuged for 5 min at 2000rpm. An aliquot of about 80 μl of the aqueous phase was injected onto the HPLC system.

Analytical variables

Absolute recovery was calculated at 50, 150, 300 ng/ml spiked plasma samples by comparing the peak heights from extracted samples with those obtained from a direct injection of the corresponding unextracted aqueous standards.

Relative recovery was calculated at 50, 150, 300 ng/ml spiked plasma samples by comparing peak heights of extracted analytes with peak heights of extracted internal standards.

Within run and between run precision was determined by extracting plasma supplemented with AT and NT to 50, 150, 300 ng/ml.

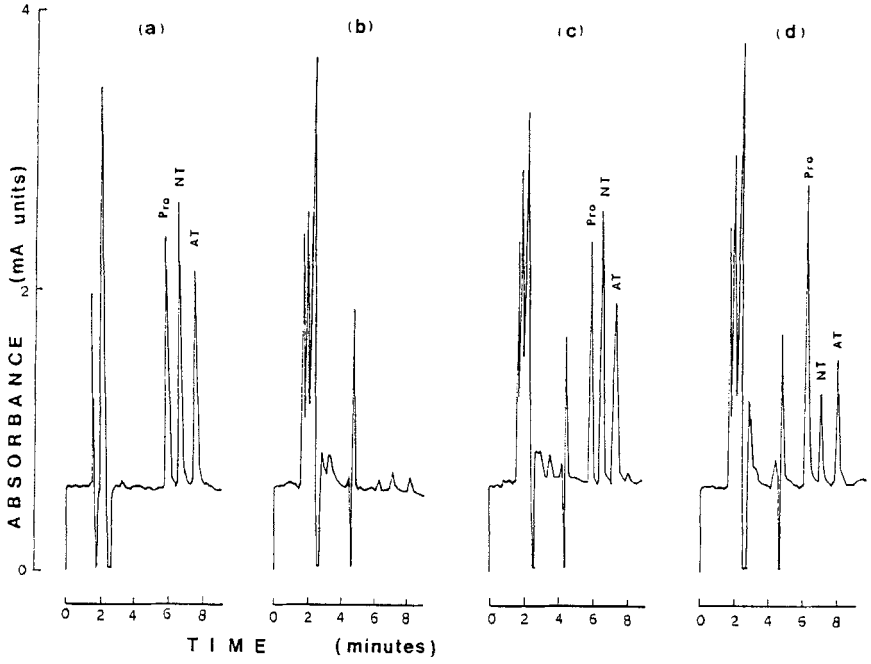

Figure 1: Chromatograms obtained from:
(a)a direct injection of an aqueous test solution containing 1µg/ml of AT,NT and 0.15µg/ml of Pro. Injected volume 50µl, corresponding to 50ng AT,NT and 7.5ng Pro
(b)an extracted drug free plasma
(c)an extracted drug free plasma supplemented with AT,NT,Pro to 200, 200, 30ng/ml respectively and
(d)an extracted plasma sample of a patient receiving 150mg amitriptyline daily for 5 days.
AT: 101.7ng/ml NT: 42.8ng/ml
Amitriptyline (AT), Nortriptyline (NT), Protriptyline-Internal standard (Pro).

Assay versatility

The method was also evaluated for qualitative analysis of several tricyclic antidepressants and their metabolites in plasma, in cases of emergency toxicology (screening method). Preliminary data suggests that the method is equally suitable for quantification of clomipramine in plasma.

Analysis of plasma samples

The overall precision of the assay was evaluated by analysing plasma samples spiked by three different concentrations of AT and NT, as well as in plasma samples of 10 patients who were receiving different oral doses of SAROTEN® (amitriptyline) daily. Patients samples were centrifuged immediately after collection and plasma stored at -20°C until analysis.

RESULTS

Retention times for Pro (internal standard), NT and AT were 5.87, 6.64, and 7.53 minutes, respectively. Figure 1a shows a chromatogram obtained from a direct injection of an aqueous test solution containing 1 μg/ml of AT and NT and 0.15 μg/ml Pro (injected volume 50 μl corresponding to 50 ng AT, 50 ng NT and 7.5 ng Pro). Figure 1b shows a chromatogram obtained from an extracted drug free plasma, while Figure 1c of an extracted drug free plasma supplemented with AT, NT to 200 ng/ml each and internal standard to 30 ng/ml. Figure 1d shows a chromatogram obtained from extracted plasma of a patient receiving AT for five days (150 mg/day). No interfering peaks were observed in several samples of drug free plasma. Figure 2 shows chromatograms obtained from a direct injection of an aqueous solution containing a mixture of several tricyclic antidepressants and their metabolites (a) and an extracted drug free plasma spiked with this mixture of compounds (b).

Recovery

Absolute and relative extraction recovery data from plasma samples supplemented with AT and NT to 50, 150, 300 ng/ml are referred in Tables 1 and 2 respectively (means of five experiments).

Linearity and Sensitivity

The peak height ratios for AT/internal standard and NT/ internal standard were linearly related to plasma concentrations of AT and NT, respectively, from 25 to at least 300 ng/ml.

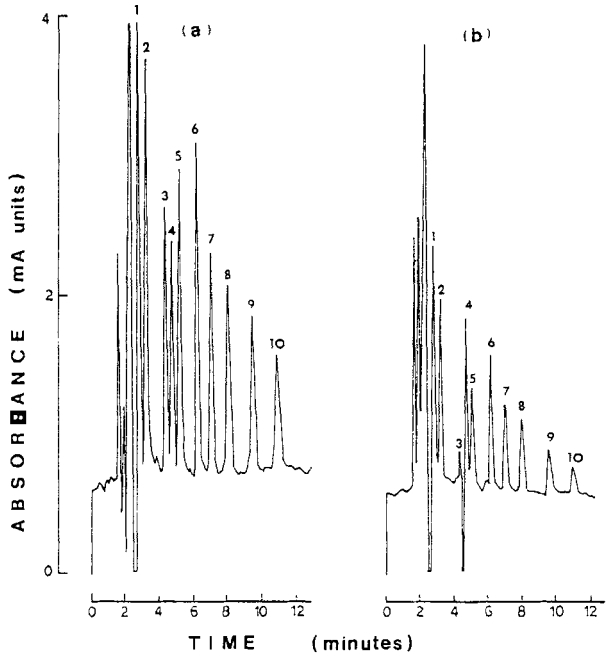

Figure 2. Chromatograms obtained from a direct injection of an aqueous solution containing 1µg/ml of compounds 1,2,3,4,5,7,8,9,10 and 0.20µg/ml of coumpound 6 (injected volume 50µl, corresponding to 50ng of each of compounds 1,2,3,4,5,7,8,9,10 and 10ng of compound 6) [a] and an extracted drug free plasma supplemented with compounds 1,2,3,4,5,7,8,9,10 to 100ng/ml and compound 6 to 20ng/ml [b].

1:E-10-OH-nortriptyline(Rt=2.67), 2:Z-10-OH-nortriptyline(Rt=3.11), 3:8-OH-desmethylclomipramine.HCl(Rt=4.19), 4:8-OH-Clomipramine(Rt=4.57), 5:Doxepine(Rt=5.04), 6:Protriptyline(Rt=6.04), 7:Nortriptyline(Rt=6.86), 8:Amitriptyline(Rt=7.84), 9:Desmethylclomipramine.HCl(Rt=9.32), 10:Clomipramine(Rt=10.71)

TABLE 1.

Absolute Extraction Recoveries and Coefficient of Variation(CV) of nortriptyline, amitriptyline and protriptyline from spiked plasma samples.

	Nortriptyline		**Amitriptyline**		**Protriptyline***	
Spiked plasma standards (ng/ml)	Rec% ± SD	CV	Rec% ± SD	CV	Rec% ± SD	CV
50 (n = 5)	88.7 ± 7.7	8.7	93.4 ± 11.8	12.6	96.0 ± 4.3	4.5
150 (n = 5)	95.7 ± 4.2	4.4	89.4 ± 4.2	4.7	95.8 ± 2.5	2.6
300 (n = 5)	87.0 ± 7.6	8.7	83.8 ± 7.3	8.7	92.5 ± 6.0	6.5

* All spiked plasma standards contained protriptyline (internal standard) to 30ng/ml.

TABLE 2.

Relative Extraction recoveries and Coefficient of variation (CV) of nortriptiline and amitriptyline from spiked plasma samples

	Nortriptyline		Amitriptyline	
Spiked plasma standards(ng/ml)	PHR ± SD	CV	PHR ± SD	CV
50 (n=5)	0.29 ± 0.01	3.4	0.24 ± 0.01	4.2
150 (n=8)	0.82 ± 0.04	4.9	0.56 ± 0.03	5.4
300 (n=5)	1.59 ± 0.05	3.1	1.11 ± 0.04	3.6

PHR: Peak height of extracted analyte to peak height of extracted internal standard.

The slopes of 15 calibration curves of AT in plasma, prepared over a period of four months, had a CV of 7.96%. The average regression equation was: $y = 0.00345x + 0.044$, where y = peak height ratio of AT /internal standard and x = plasma concentration of AT (ng/ml). The correlation coefficients for each standard curve constructed invariably exceeded 0.996.

The slopes of 15 calibration curves for NT in plasma over a period of four months had a CV of 5.63%. The average regression equation was: $y = 0.00519x + 0.064$ where y = peak height ratio of NT/internal standard and x = plasma concentration of NT (ng/ml). Correlation coefficients for each standard curve exceeded 0.994.

The lower limit of detection was 5 ng/ml for AT and NT while the lower limit of quantification was 10 ng/ml for both compounds.

Reproducibility and Accuracy

Within-run reproducibility and accuracy data for AT and NT are referred in Table 3. Between-run CVs were 7.3% and 8.5% for AT and NT respectively at 150 ng/ml spiked plasma (n=15), over a period of 4 months.

Interferences

Twenty compounds were studied for possible interference, including several drugs that might be administered to anxious or depressed patients (Table 4). Propoxyphene

TABLE 3.

Within-run accuracy and reproducility data of amitriptyline and nortriptyline from spiked plasma samples.

	Amitriptyline			Nortriptyline		
Conc Added (ng/ml)	50	150	300	50	150	300
Conc Found g/ml(x)	54.18	151.72	307.60	41.41	145.64	293.50
SD	6.38	9.95	12.90	5.02	6.88	9.53
CV%	11.77	6.56	4.19	12.12	4.72	3.24
Rr (Rel.range)	0.31	0.20	0.12	0.34	0.14	0.10
Er (Rel.error)	0.08	0.01	0.02	-0.17	-0.03	-0.02
n	5	10	5	5	10	5

TABLE 4.

Compounds studied for interference.

	Rt(min)		Rt(min)
Lorazepam (a)	-	Phenobarbital (c)	4.49
Oxazepam (a)	-	Chlorpromazine (c)	9.10
Bromazepam (a)	-	Chlordiazepoxide(c)	8.66
Triazolam (a)	-	Chlorpheniramine(c)	3.61
Diazepam (a)	-	Propanolol (c)	4.25
Nitrazepam (a)	-	Propoxyphene (b)	7.62
Alprazolam (a)	-	Maprotiline (b)	7.60
Artane (a)	-	Imipramine (b)	7.20
Theophylline (a)	-	Phenytoin (b)	6.75
Pseudoephedrine (a)	-		
Haloperidol (a)	-		

(a) = Not detected
(b) = Interfered, see text.
(c) = Detected but not interfered.

produced peak at 7.62 min; resolution was insufficient for quantification of AT. Phenytoin peak (Rt=6.75) was overlapping with that of NT. Maprotiline and imipramine (Rt 7.60 and 7.20 respectively) cannot be separated from AT by the described method, but in clinical practice they are not administered with AT.

Most of the benzodiazepines, which are commonly coadministered to depressed patients, were studied in spiked plasma samples and did not interefere to the analysis since they are not coextracted through the procedure described in this methodology. All the other compounds shown in Table 4 were tested for possible interference as aqueous solutions.

DISCUSSION

A number of analytical methods using reversed-phase high performance liquid chromatography have been reported for quantification of AT and NT in plasma (12-15,26,27). The methodology developed in this report modifies previously reported methods (12-15,26).

In this study several solvent mixtures were tested for the extraction of AT, NT and Pro (internal standard) from plasma samples. Hexane-isoamylalcohol 98:2 v/v, which has been used for the extraction procedure in previously reported methods (11,14,16,25) showed satisfactory extraction recovery (Tables 1,2). Since both solvents of the extraction mixture have relatively high boiling points, analysis is prolonged because of the time which is required for the evaporation step. For this reason, back extraction of the organic phase into 200 µl HCl 0.1N and direct injection of the aqueous phase onto the HPLC system was found preferable, making the analysis more rapid. Furthermore, in the present study, plasma samples were extracted in both steps of the extraction procedure, on a vortex apparatus for only 60 sec, while previously reported methods suggested gentle shaking for 10 min (15) or 60 min (14) and rotate-mix for 5 min (16). Our efforts to increase sensitivity using 100 µl instead of 200 µl HCl 0.1N for the back extraction of the organic phase proved to be unsuccessful because extraction recovery of the analytes was decreased.

During the preliminary phase of this study C-18 and C-8 columns (25 cm x 4 mm i.d., 5 µm) were tested for the analysis at ambient temperature. When the

C-18 (Lichrospher RP-18, MZ Analysentechnic D-6500 Mainz) column was tested, which has been used in previous studies (14-16,26) and the aqueous acidic phase was injected directly onto the HPLC system, unsatisfactory chromatograms were obtained, in regard to peak shape and resolution, after 50 injections of extracted plasma samples. On the contrary, the C-8 material was far more favourable with respect to column efficiency and peak shape. Moreover the C-8 column proved to be more resistant in our hands, being used for the analysis of more than 20 plasma samples per day. The use of acetonitrile / 0.04M KH_2PO_4 40:60 v/v as the mobile phase resulted in an excellent separation of the substances which were studied. In this methodology, pH adjustment of the mobile phase, used in other methods (13-16) has been avoided. Furthermore, the addition of aliphatic amines in the eluent, as competing bases for silica sites to decrease chemisorption which causes peak tailing, reported in other studies (13,26), has also been avoided. Therefore, it can be concluded that the method developed is more convenient, in comparison with previously reported ones.

The methodology used in this study, proved to be precise and accurate for the determination of AT and NT in spiked plasma samples (Table 3). Moreover plasma AT and NT levels in 10 patients included in this study, who received different oral doses of AT daily, are in accord with those reported in relevant studies (1,2).

Although the method has not been extensively used for the determination of clomipramine and its metabolites desmethylclomipramine, 8-OH-clomipramine and 8-OH-desmethylclomipramine, it appears to have sufficient specificity, sensitivity and precision using protriptyline as internal standard.

The methodology developed in this study can also be used as a screening procedure for the qualitative determination of several tricyclic antidepressants and their metabolites in plasma (Fig.2), being extremely useful in cases of emergency toxicology, as a part of the general toxicological analysis.

In conclusion, the analytical methodology developed in this report is simple, rapid, accurate, sensitive and specific. It can be used for monitoring plasma AT and NT steady-state concentrations as well as the subtherapeutic concentrations encountered during the initial start-up phase of AT therapy. The present methodology also provides the possibility for qualitative determination of several tricyclic antidepressants in plasma; this is certainly of value in cases of emergency toxicology.

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