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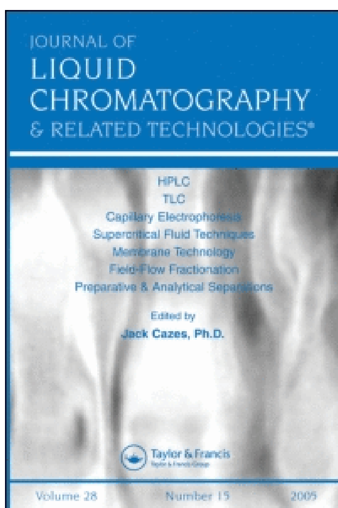

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Solid-Phase Extraction and High Performance Liquid Chromatographic Determination of Amitriptyline and Nortriptyline in Human Plasma

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SOLID-PHASE EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMITRIPTYLINE AND NORTRIPTYLINE IN HUMAN PLASMA

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ABSTRACT

A simple, rapid, cost-efficient solid-phase extraction and high performance liquid chromatographic method for the determination of amitriptyline and nortriptyline in human plasma is described. After conditioning of solid-phase sorbent diluted plasma samples were passed through the cartridge. Potentially interfering substances were washed, followed by elution of amitriptyline, nortriptyline and maprotiline (internal standard) from the sorbent. Eluates were collected, evaporated, reconstituted and injected directly and monitored at 215 nm. Samples were chromatographed on a 5 μm Supelcosil LC-PCN (150 x 4.6 mm) using 0.01 M dipotassium hydrogenphosphate (pH adjusted to 7 with 85% ortophosphoric acid)/ acetonitrile/

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methanol (15:60:25, v/v) as the mobile phase. Recoveries for amitriptyline and nortriptyline were 96% and 85%, respectively. The limit of detection and quantification for amitriptyline and nortriptyline were 1 ng/mL and 5 ng/mL, respectively.

The calibration curves for amitriptyline and nortriptyline in human plasma were linear over the range 5-500 ng/mL. The method was applied in single dose (75 mg) clinical pharmacokinetic studies of amitriptyline in depressed patients.

INTRODUCTION

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Tricyclic antidepressants continue to be one of the most widely used classes of psychotropic drugs employed in clinical practice on a long-term basis. In 1985 the American Psychiatric Association Task Force recommended therapeutic monitoring of tricyclic antidepressant drugs as a guideline for treating patients.[1] To achieve this goal various methods for the determination of tricyclic antidepressants in plasma have been developed and reviewed.[2-4]

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

Various methods have been described to determine the concentration of AT and NT in plasma including radioimmunoassay,[5] gas chromatography,[6,7] and high performance liquid chromatography-HPLC.[8-22] Gas chromatographic determination of AT is specific and sensitive but requires lengthy derivatization steps of the secondary amine formed. Among various analytical techniques used to measure AT and NT plasma levels in recent years, HPLC appears to be the most suitable for routine laboratory needs due to its versatility, availability, and reliability. Among these methods some are based on normal phase[9,11,13,18] or reversed phase,[8,14-17,19,20] and ion-pair reversed phase.[10,12]

The previous isolation techniques for AT and NT from plasma were generally accomplished by liquid-liquid extraction of an aqueous sample into an organic solvent, followed by back-extraction of the organic layer into another aqueous phase. The organic solvent is then, evaporated to dryness before reconstitution in the appropriate solvent. However, these methods are time-consuming and labor-intensive.

Therefore, the present paper will focus on simple, rapid, cost-efficient solid-phase extraction followed by a specific and accurate liquid chromatographic method which could be applied on a single dose clinical pharmacokinetic study of AT in depressed patients.

MATERIALS AND METHODS

Reagents and Chemicals

AT hydrochloride and NT hydrochloride were obtained from Lundbeck Co. (Amsterdam, The Netherlands), maprotiline hydrochloride was a gift from Pliva (Zagreb, Croatia). Ortophosphoric acid, dipotassium hydrogenphosphate and ammonium hydroxide (analytical grade) were obtained from Merck (Darmstadt, Germany). Methanol and acetonitrile HPLC grade (Licrosolv^R) were purchased from Merck (Darmstadt, Germany).

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Apparatus

HPLC system Hewlett Packard (Avondale, PA, USA) model 1050 was used, equipped with a diode-array UV detector Hewlett Packard and an integrator model HP 3396A (Hewlett Packard, Avondale, PA, USA). A model 7125 sample injector and six-port switching valve (Rheodyne, Cotati, CA, USA) were used. The guard column (20 x 4.6 mm) packed with 3 μm Supelguard LC-CN (Supelco, Bellefonte, PA, USA) and analytical column (150 x 4.6 mm) packed with 5 μm Supelcosil LC-PCN (Supelco, Bellefonte, PA, USA) were used.

For sample preparation, we used 1 mL Supelclean LC-WCX SPE tubes and VISI^{TM}-1 Single SPE tube processor.

Chromatographic Conditions

The mobile phase consisted of 0.01 M dipotassium hydrogenphosphate (pH adjusted to 7 with 85 % ortophosphoric acid)/acetonitrile/methanol (15:60:25, v/v). A flow rate of 2 mL/min was used at ambient temperature. The mobile phase, after mixing, was filtered through a 0.45 μm membrane filter by vacuum and degassed in an ultrasonic bath prior to use. Analysis was performed at 215 nm.

Standards for Calibration Graphs

AT hydrochloride (0.00226 g) and NT hydrochloride (0.00227 g) were weighed out exactly and dissolved in a 100 mL volumetric flask with mobile phase to give final concentration of 20 mg/L. Working solutions were prepared by appropriate dilutions of the stock solution with mobile phase to give final concentrations of 5, 25, 50, 100, 200, 400 and 500 ng/mL AT and NT. Plasma standards for calibration curves were prepared by spiking 1 mL aliquots of pooled drug free plasma with appropriate volumes of the above mentioned working solutions to make AT and NT plasma standards ranging from 5 to 500 mg/L . Internal standard - maprotiline (MT) (10 μL of 20 mg/L) was added in plasma standards. Calibration graphs of the recovered standards were prepared for each day of analysis to establish linearity and reproducibility of the HPLC system. Calibration curves were obtained by plotting the ratio of peak area of AT and NT to internal standard against drug concentrations.

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Sample Preparation

AT and NT were isolated from human plasma by solid-phase extraction with 1 mL Supelclean LC-WCX SPE tubes. The solid-phase sorbent was conditioned with 500 μL of 0.5 M ortophosphoric acid followed by 1 mL of deionized water. 500 μL of plasma samples containing 10 μL of MT (20 mg/L) mixed with 500 μL of deionized water, then, passed through the sorbent. Analytes (AT, NT and MT) were bound onto the sorbent, and interfering substances were washed with an appropriate solvent system (1 mL of deionized water, 500 μL of 1 M ammonium hydroxide in water, twice with 1 mL of 5 % methanol in water). Analytes were , then eluted on-line from the sorbent twice with 1 mL of 0.22 M ammonium hydroxide in methanol.

The eluate was collected in silanized glass vial, evaporated to dryness under nitrogen and reconstituted in 250 μL of HPLC mobile phase. Throughout the procedure silanized glassware was used, due to irreversible adsorption of AT and NT by untreated borosilicate glass.

Analytical Variables

Extraction efficiency was studied by adding known amounts of AT and NT to drug free plasma. After extraction and injection into the chromatograph, the ratio of peak area of AT and NT to MT obtained was compared with the

corresponding ratio obtained with standard solution of AT and NT in mobile phase. The precision and accuracy of the method were evaluated by repetitive analysis of plasma spiked with AT and NT.

Analysis of Plasma Samples

The HPLC procedure was used to study clinical pharmacokinetics of AT and NT in adult depressive patients on AT monotherapy. AT and NT plasma concentrations were determined after single dose administration of 75 mg of AT. Blood samples (5 mL) were withdrawn just before drug administration (0 hr) and at 1, 2, 3, 4, 6, 9, 12, 24, 36, and 48 hr after single oral dose. At the steady state (on the 14 th day of therapy) five blood samples were obtained.

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RESULTS

Under the chromatographic conditions described, the retention times for AT, NT and MT were 3.7 , 7.5 and 10.5 minutes, respectively. Figure 1A shows the chromatogram obtained from an extracted drug free plasma. No peaks corresponding to the drug, its metabolite or internal standard retention times, were found. Figure 1 B shows the chromatogram obtained from extracted plasma of a patient receiving AT monotherapy. Good selectivity of the method was indicated by the lack of interfering peaks in plasma.

Validation Of The Method

Recovery

Extraction recovery data from plasma samples spiked with known amounts of AT and NT are presented in Table 1.

Linearity And Sensitivity

A typical calibration curves of AT and NT from plasma were linear in the range of 5-500 ng/mL.

The average regression equation and regression coefficient for AT were $y= 0.0056\ x + 0.043$ (where y= peak area ratio of AT to MT and x= plasma concentration of AT) and $r= 0.9996$, respectively.

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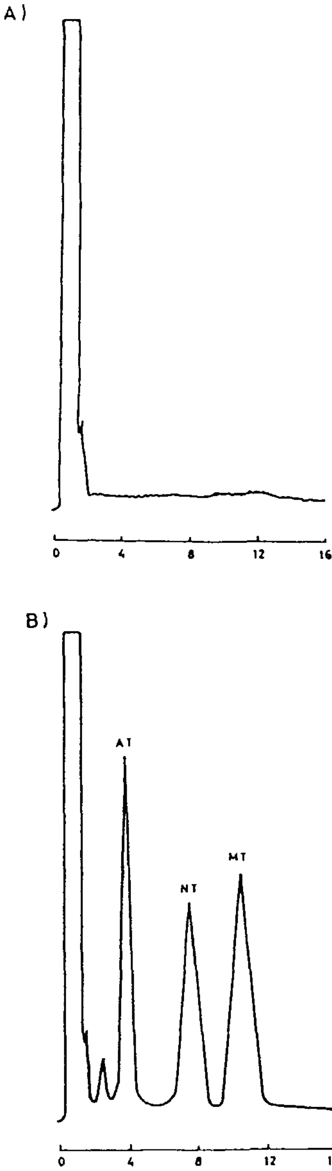

Figure 1. Chromatograms of extracted drug free plasma A) and extracted plasma of a patient on AT therapy B).

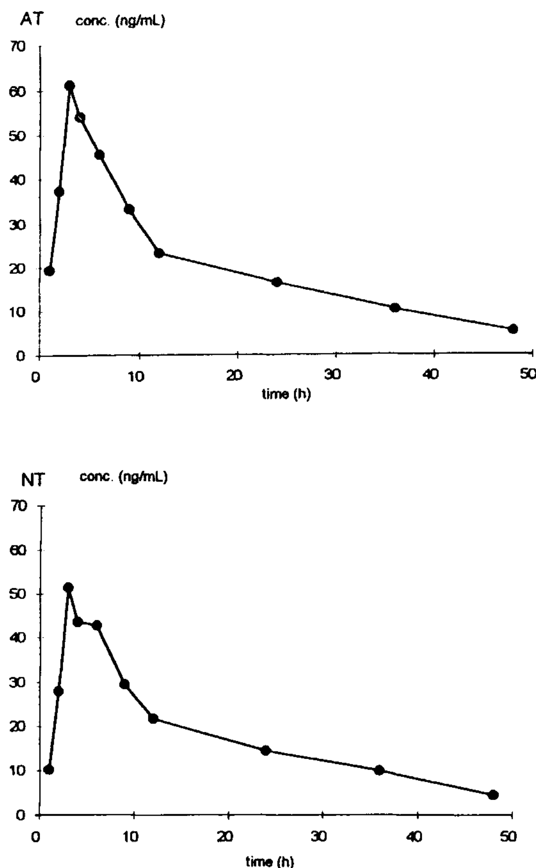

Figure 2. Mean plasma concentrations of AT and NT B) in 9 depressed patients on AT monotherapy after single dose adminsitraiton (D = 75 mg).

The average regression equation and regression coefficient for NT were $y=0.0057\ x + 0.11$ (where y= peak area ratio of NT to MT and x=plasma concentration of NT) and $r=0.9994$, respectively.

The limit of detection, arbitrarily defined as 5 times baseline noise, was 1 μg/L for AT and NT while the limit of quantification was 5 μg/L for both compounds.

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Table 1

Recoveries of AT and NT from Spiked Plasma Samples (N = 5)

Cond. Added (ng/mL)	Conc Found (ng/mL)		Recovery (%)	
AT/NT	AT	NT	AT	NT
5	4.75	4.25	95	85
25	24.08	22.10	96	88
50	49.11	44.54	98	89
100	96.13	86.73	96	87
200	188.53	164.13	94	82
400	392.14	328.63	98	82
500	465.34	405.12	93	81
		mean:	96	85
		+/-SD:	2	3

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Table 2

Within-run (CV) and Day-to-day (CV*) Coefficient of Variation of AT and NT from Spiked Plasma Samples (N = 5)

Conc. (ng/mL)	CV(%)		CV* (%)	
AT/NT	AT	NT	AT	NT
5	7.1	6.3	8.2	8.8
25	4.3	8.1	6.1	9.2
50	3.7	7.1	4.1	8.2
100	2.6	4.1	3.9	5.6
200	5.1	6.3	4.3	5.8
400	6.2	7.7	5.1	6.6
500	7.1	7.9	6.2	6.8
mean:	5.2	6.8	5.4	7.3
+/- SD:	1.7	1.4	1.5	1.4

Accuracy and Precision

The accuracy of the method is indicated by the error of assayed samples relative to their spiked concentrations. The method demonstrated superior accuracy in that all errors were less than 10 %. The method was also shown to be precise in studies of replicate assays. Study demonstrated coefficient of variation within-run (CV) and day-to-day (CV*) less than 10 % which demonstrates good reproducibility of the method (Table 2).

Application

The mean plasma concentration-time curves of AT and NT obtained after oral administration of a single dose of 75 mg of AT (N=9) in depressed patients are presented in Figure 2.

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DISCUSSION

Because large variations in the steady state concentration of AT can occur in patients receiving the same oral dose,[23] successful therapy using AT depends upon the reliable monitoring of plasma levels.[24] The analysis of AT (and NT) from plasma has been accomplished using various analytical procedures including radioimmunoassay, gas chromatography, HPLC based on normal phase, reversed phase, and ion-pair reverse phase.

Previous extraction techniques for AT have been time-consuming and labor-intensive. For routine investigations a simple and rapid sample preparation procedure with a reproducible and effective extraction of AT and NT is desirable.

In recent years, investigators have demonstrated the merits of solid-phase sorbent extractions over conventional liquid-liquid techniques for drug extractions. Cleaner extracts, lower solvent consumption, and reduced analysis times have been reported advantages.

In the current method, solid-phase extraction is used. After conditioning of solid-phase sorbent diluted plasma samples were passed through the cartridge. Potentially interfering substances were washed, followed by elution of AT, NT and MT from sorbent. To ensure optimum drug recovery a slow dropwise flow rate was maintained during the sample addition and sample elution steps of the extraction procedure. Rapid flow rates reduced recovery for 15-25%. Dilution of the sample with deionized water prior to extraction, by

reducing the concentration and ionic strength of unwanted plasma components, allowed them to pass more easily through the tube. More concentrated solutions of ammonium hydroxide used for packing wash, or larger wash volumes would remove AT and NT from packing, reducing drug recovery for 20-30 %. Due to irreversible adsorption of AT and NT by untreated borosilicate glass we used silanized glassware throughout the procedure.

The current method was found to reduce errors due to sample manipulation, which provided improved reproducibility as well as superior recovery. The sorbent extraction is therefore more convenient, and much less time is required for sample processing. The saving in the time for the analysis using solid-phase extraction is the most important favorable factor for this method. Since elution solvent volume is low, evaporations presents no problem, and selective elution procedures yield final samples with fewer endogenous impurities than are usually obtained in liquid-liquid extraction methods.

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Also the chromatographic system provided a fast establishment of equilibrium between the mobile and the stationary phase, a stable column performance even after prolonged usage, as well as a relatively short run time for assay.

Small sample volume (0.5 mL) combined with simple, rapid, cost-efficient solid-phase extraction, short chromatographic time and minimal source of interference has been achieved in the method described here. The combination of obtained features (the simplicity, selectivity, sensitivity and cost effectiveness of solid-phase extraction) has allowed the development of a method that is accurate, precise, sensitive, and ideally suited for monitoring therapeutic and/or toxic levels of AT and NT. The method presented in this paper has also proved to be useful in single-dose clinical pharmacokinetic studies of AT in depressed patients.

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