Journal of Chromatography, 579 (1992) 291–297
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6447

On-line solid-phase extraction and high-performance liquid chromatographic determination of nortriptyline and amitriptyline in serum

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(First received February 7th, 1992; revised manuscript received May 15th, 1992)

ABSTRACT

An isocratic reversed-phase high-performance liquid chromatographic method with on-line solid-phase extraction for the simultaneous determination of amitriptyline and nortriptyline in serum has been developed. A 250-μl serum sample is injected directly onto a commercially available CN cartridge and, after a washing step, the retained solutes are backflushed onto a bonded-phase CN column using a column-switching technique and a mobile phase composed of acetonitrile (26%) and 0.05 *M* phosphate buffer with diethylamine. Serum is diluted with 0.1 *M* sodium lauryl sulphate and centrifuged before the injection. Detection at 210 nm ensures sufficient sensitivity. The recovery is almost quantitative and the relative standard deviation ranges from 2.8 to 8.0% for concentrations of 200–40 ng/ml. Being rapid and simple, the method is convenient for routine use.

INTRODUCTION

There is a need for measurements of the serum concentration of tricyclic antidepressants (TCAs) commonly administered in the treatment of depressive illnesses. Various methods have been utilized for the routine monitoring of the concentration of TCAs, the first choice being chromatographic and immunoassay techniques [1,2], and especially high-performance liquid chromatography (HPLC). A number of HPLC methods for determining TCAs and their metabolites have been developed and reviewed [2–4]. These methods differ with respect to the mode of HPLC (normal-phase, reversed-phase or ion-pair chromatography), the detection method (UV at 254 or at 205–215 nm, fluorescence or electrochemical detection) and sample preparation [solvent extraction and solid-phase extraction (SPE) in the off-line or on-line mode].

The problem of sample pretreatment, in particular, has attracted much attention in recent years, as this procedure has often been a rate-limiting step in HPLC analyses of biological fluids. Instead of tedious traditional solvent extraction procedures with low recovery, modern SPE techniques in off-line and on-line modes have been increasingly used. In addition to off-line SPE in the HPLC analysis of TCAs [5–9], a few applications of on-line SPE with column switching allowing direct injection of serum have been reported [10–13].

The aim of this work was to devise an HPLC method suitable for routine monitoring of amitriptyline and its demethylated metabolite, nortriptyline, in serum samples from paediatric

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0378-4347/92/$05.00

psychiatric patients. In an attempt to avoid multiple extraction and transfer steps in the sample pretreatment, on-line SPE with column switching was chosen.

Dry-packed, short precolumns are mostly used for on-line SPE [10–13]. In this application, the use of commercially available, densely packed, plastic precolumn cartridges (Guard-Pak) is described. Such precolumns can be readily and conveniently replaced, and if a large numer of serum injections on each precolumn are performed, the sample pretreatment is even cost-effective.

EXPERIMENTAL

Reagents and chemicals

Amitriptyline · HCl (AMI) and nortriptyline · HCl (NOR) were obtained from H. Lundbeck (Copenhagen, Denmark). Diethylamine, *n*-amylamine and lauryl sulphate were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (Merck, Darmstadt, Germany) was of spectroscopy grade and water was passed through a Nanopure Barnstead water purificiation system (W. Werner, Berg. Gladbach, Germany). All other chemicals were of analytical-reagent grade from Lachema (Brno, Czechoslovakia).

Preparation of standards

Stock standard solutions of both drugs were separately prepared in methanol to give a concentration of 500 μg/ml. Working standard solutions were prepared in the concentration range 4–40 μg/ml by appropriate dilution with water–methanol (2:1). HPLC injection standards and spiked serum standards were then prepared by adding 5 μl of the working standard solution of each drug to 500 μl of water–acetonitrile (3:1) and of serum, respectively.

Preparation of serum samples

A 500-μl volume of serum to be analysed was mixed with 250 μl of 0.1 *M* lauryl sulphate and centrifuged at 2500 *g* for 8 min. A 250-μl aliquot was injected onto the precolumn.

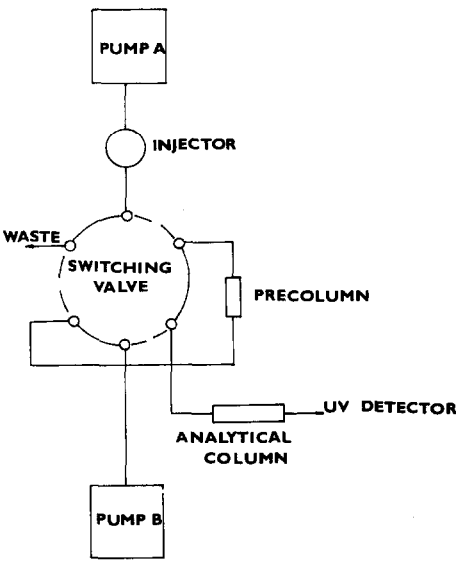

Fig. 1. Schematic representation of the column-switching system. The switching valve is in position $V = 2$.

Instrumentation

All measurements were performed with HPLC apparatus consisting of Waters Models 590 and 510 HPLC pumps, a Waters U6K injector, a Waters 990+ diode-array detector and a six-port automatic switching valve (Waters Assoc., Milford, MA, USA). Automatic operation of the valve was accomplished with the aid of a Model 590 programmable pump. The whole column-switching system is depicted in Fig. 1.

Chromatographic conditions

A Waters Guard-Pak precolumn module with a cartridge packed with Resolve CN (10 μm) was used as a precolumn. The analytical column was 150 mm × 3 mm I.D. Separon SGX CN (7 μm) (Tessek, Prague, Czechoslovakia).

The washing solvent (pump A) was water–acetonitrile (97:3, v/v). The mobile phase (pump B) was prepared by mixing 0.05 *M* phosphoric acid–0.05 *M* ammonium phosphate containing 28 m*M* diethylamine (pH 2.55) and acetonitrile (usually 26%, v/v). The mixture was passed through a 0.22-μm Millipore filter before use.

Column switching procedure

Step 1 (V= 1; 0 min). A sample of serum is injected.

Step 2 (V = 1; 0–6 min). The sample is swept onto the precolumn with washing solvent from pump A. The precolumn is washed and proteins and other polar substances are eluted to waste.

Step 3 (V = 2; 6–10 min). The mobile phase from pump B passes through the precolumn in the backflush mode and elutes retained substances onto the analytical column, where they are separated. Detector data acquisition starts.

Step 4 (V = 1; 10–16 min). The mobile phase passes only through the analytical column, on which the separation continues. The precolumn is washed and prepared for the next injection.

After every eight injections of the serum samples, the precolumn was purged separately in the backflush mode with acetonitrile–water (7:3, v/v) for 10 min.

RESULTS

Optimization of the chromatographic system

CN-modified silica gel was chosen as the stationary phase for reversed-phase HPLC separation of the drugs. The mobile phase, composed of acetonitrile and aqueous phosphate buffer with alkylamine, was optimized in terms of the pH of the aqueous part, acetonitrile content and the type and amount of the added alkylamine.

The resolution of both drugs and the symmetry of their peaks were the same for pH in the range 2.0–7.9. However, at low pH, a lower percentage of acetonitrile in the mobile phase was needed, which is why an acidic mobile phase was used.

The main factors influencing the separation were the content of acetonitrile and the amount of alkylamine added. Two types of alkylamine, diethylamine and pentylamine, were tested, both of them improving the peak shapes even at low concentrations. Pentylamine lowered the retention of AMI and NOR more significantly than did diethylamine, which seems to be of advantage as a lower content of the organic modifier in the mobile phase was needed. However, coupling such a chromatographic system to the SPE system posed difficulties as a negative system peak occurred and interfered with the peak of NOR. Therefore, the mobile phase with diethylamine was used. The dependence of the capacity factors of the drugs on the diethylamine concentration is shown in Fig. 2.

By changing the content of acetonitrile and diethylamine in the mobile phase, a reasonable retention for NOR and AMI (capacity factors in the range 4.5–8.0) was obtained. During the use of the analytical column a slow decrease in the drug retention was observed and the content of acetonitrile, or even of diethylamine, in the mobile phase had to be lowered. After four weeks the mobile phase usually contained only 21–23% instead of the initial 26–28% of acetonitrile in order that the same capacity factors for the drugs might be obtained. Such a loss of retention was observed even when HPLC injection standards only were analysed. No comparison with other CN columns was made, but poor stability of CN columns used as a weakly retentive reversed phase in combination with aqueous mobile phases has often been noted.

On-line solid-phase extraction

First, the retention of NOR and AMI on the precolumn cartridge and their backflush elution with the suggested mobile phase were investigated using HPLC injection standards. No elution of the drugs adsorbed on the precolumn was found if the precolumn was washed for 10 min

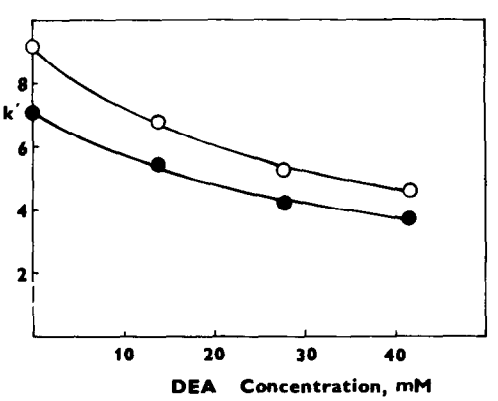

Fig. 2. Dependence of capacity factors (k') of (○) AMI and (●) NOR on the concentration of diethylamine (DEA).

with water containing up to 6% (v/v) of acetonitrile. With the use of the analytical mobile phase, complete elution of both drugs in the backflush mode was achieved.

The optimum washing time for complete removal of polar, UV-absorbing components of serum from the precolumn was determined by connecting the precolumn system directly to the detector, injecting a blank serum sample and monitoring the purge process. With the use of a washing solvent composed of water–acetonitrile (97:3, v/v) and a washing flow-rate of 1.0 ml/min, the clean-up process on a new precolumn was accomplished in 2 min. However, after four or five injections of serum samples, this period increased to 5 min and did not change any more (Fig. 3a). Hence, 6 min was set as the washing time.

Fig. 3a and b illustrate the effect of cleaning of the precolumn by purging it with acetonitrile–water (7:3, v/v). The components of serum samples that remained adsorbed on the precolumn after each analysis and that caused a gradually increasing background absorbance were effectively removed in this purging step, which was inserted in the column-switching procedure before every ninth injection of a serum sample.

Sample pretreatment

Direct injection of serum was tried at first but after seven to ten injections the back-pressure in the system increased markedly and the precolumn could not be used for further injections.

Dilution of serum with a buffer did not prevent the back-pressure from increasing. Protein precipitation with acetonitrile prior to injection improved the stability of the column-switching system but the sensitivity was lowered owing to the dilution, and sometimes interfering peaks appeared on the chromatograms.

The best results were obtained when 0.1 *M* lauryl sulphate was added to serum prior to the injection. Except for one case, no build-up of pressure in the system was observed, even after injecting 55 samples of 250 μl each. Chromatograms without interfering peaks were obtained, the peak of serum being negligible (with a new precolumn) or reasonably large (with a precolumn used for more than fifteen injections of serum).

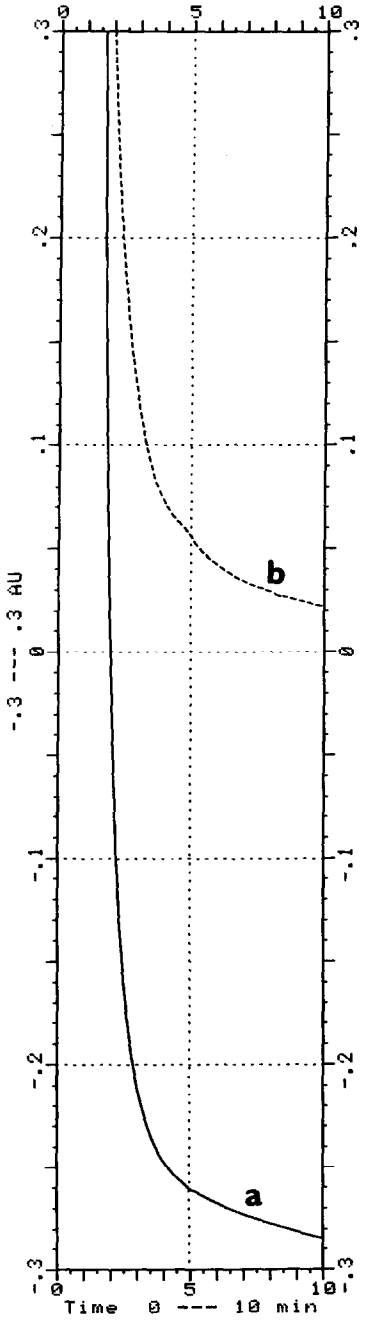

Fig. 3. Washing process after injection of 250 μl of diluted blank serum onto (a) the precolumn purged with acetonitrile–water (7:3, v/v) and (b) the precolumn used for on-line SPE of six serum samples without any purging. The precolumn was connected directly to the detecor. Detection at 210 nm.

The reason for exchanging the precolumn was a deterioration of the performance of the column system. This serum pretreatment enabled us to carry out 45–55 analyses with each precolumn, the maximum decrease in efficiency being 20%. The lifetime of the analytical column was the same as in analyses of HPLC injection standards only.

Quantitative analysis of serum samples

Typical chromatograms of drug-free serum, serum spiked with AMI and NOR and serum from a child treated with amitriptyline are presented in Fig. 4a, b, and c, respectively. For quantification, the external standard method was used.

The linearity of the method was tested by analysing serum spiked with AMI and NOR at four concentration levels ranging from 40 to 400 ng/ml. The relationship between peak height and concentration was linear over the entire range for both drugs (the correlation coefficient of the regression lines was better than 0.998).

Recovery was studied by comparing the peak heights of both drugs determined in spiked serum samples with those determined in HPLC injection standards injected directly onto the analytical column (without on-line SPE). As the recovery data for two concentrations in Table I show, AMI and NOR were recovered in the range 94.9–100.9%.

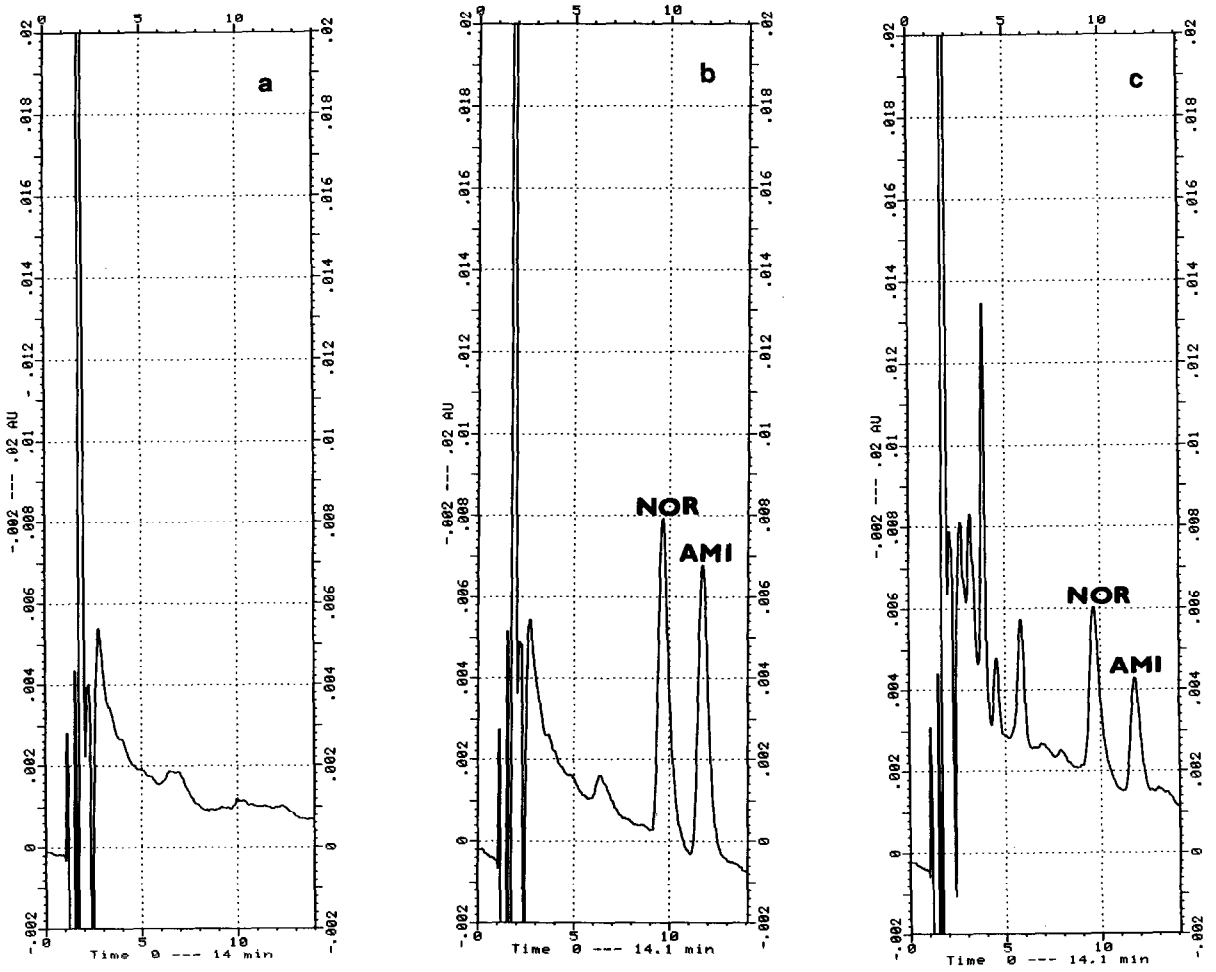

Fig. 4. Chromatograms of (a) a drug-free serum, (b) a spiked serum standard (195 ng/ml AMI and 206 ng/ml NOR) and (c) serum from a child who had received oral doses of 50 mg of NOR and 25 mg of AMI. Detection at 210 nm.

TABLE I

ANALYTICAL RECOVERY AND PRECISION FOR SERUM SPIKED WITH AMI AND NOR

Drug	Concentration added (ng/ml)	R.S.D. (n = 5) (%)		Recovery (mean ± S.D., n = 4) (%)
		Within-day	Day-to-day	
NOR	48.0	5.2	6.8	100.9 ± 10.2
	192.2	2.8	3.5	98.0 ± 8.3
AMI	46.7	5.8	8.0	94.9 ± 8.3
	186.7	3.4	4.0	97.2 ± 4.0

The precision of the method was determined at two concentration levels. The within-day precision was determined by analysing each concentration five times on the same day. The day-to-day precision was obtained by analysing the samples on five days over a period of two weeks, using a separate calibration for each day. The precision data expressed as relative standard deviations (R.S.D.) are presented in Table I. They ranged from 2.8 to 8.0% (n = 5).

Detection limits of the method for AMI and NOR are 20–25 and 15–20 ng/ml, respectively (signal-to-noise ratio = 3:1), depending on the operational conditions and the efficiency of a given column.

DISCUSSION

The commercially available cartridge precolumns with a 10-μm packing were preferred for on-line SPE. The increase in back-pressure in the system (observed after direct injections of serum due to the small size of the precolumn particles) was completely prevented by dilution of the serum samples with sodium lauryl sulphate, which has the ability to solubilize proteins. The 2:1 dilution of serum with 0.1 M sodium lauryl sulphate followed by centrifugation was found to be sufficient for the direct serum injection without pressure problems. Volumes of 11–14 ml of the diluted serum can be injected and cleaned up on one precolumn if the precolumn is regularly recovered after eight analyses by being purged with acetonitrile–water (7:3, v/v) in the backflush mode.

The washing of an injected serum sample on the precolumn takes 6 min. No peak broadening due to this washing time was observed, as has been reported for precolumns with larger packing particles [10,12].

HPLC methods with traditional preparation of biological samples use an internal standard to minimize inaccuracies caused especially by sample pretreatment involving several steps. Column-switching techniques are reproducible even in the absence of internal standards. Low levels of variability were measured even at low drug concentrations with the suggested method using external standardization. The recoveries of AMI and NOR from serum were almost quantitative.

The proposed method with on-line SPE is well suited for routine application in the clinical laboratory because it is simple and fast in terms of sample handling and is reproducible and sufficiently sensitive. The direct injection of diluted serum eliminates tedious sample preparation, lowers the amount of serum needed and gives better recoveries. The inserts for the commercially available cartridge precolumn can be simply and quickly exchanged, their use being economic, as 45–55 analyses of serum samples can be performed with one insert.

An HPLC method for determining maprotiline, imipramine, dosulepin and their demethylated metabolites with the use of on-line SPE is now under investigation.

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