Journal of Chromatography B, 746 (2000) 233–239

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of nortriptyline in human serum by fully automated solid-phase extraction and on-line high-performance liquid chromatography in the presence of antipsychotic drugs

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Received 4 February 2000; received in revised form 19 May 2000; accepted 31 May 2000

Abstract

A fully automated on-line method for determination of nortriptyline in human serum was developed using an ASPEC XL (Gilson) solid-phase extraction apparatus in combination with high-performance liquid chromatography. Solid phase extraction was performed on cyanopropyl cartridges. HPLC was carried out using a C_{18} column with a mobile phase of acetonitrile–0.01 *M* triethylamine (34:66 v/v) buffer, pH 3.0. UV detection was at 242 nm. The Inter-day CV% was $<5\%$. Comparison with liquid–liquid extraction of serum from patients treated with nortriptyline showed good agreement. Studies of analytical interference from coadministered psychoactive drugs revealed that only imipramine and a methotrimeprazine metabolite interfered. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Nortriptyline

1. Introduction

Nortriptyline (Fig. 1) belongs to the group of tricyclic antidepressant drug (TCA) introduced in the 1960s. Therapeutic drug monitoring of nortriptyline is a well-established practice [1,2]. It might have been anticipated that the introduction of the selective serotonin reuptake inhibitors (SSRIs') in the treatment of depression gradually would have finished the era of the tricyclic antidepressants, because the SSRIs' are less toxic to the heart and generally poses fewer unpleasant side effects. However, now more than five years after the arrival of the SSRIs, the number of nortriptyline determinations carried out at our department for therapeutic drug monitoring (TDM) has not decreased. The background may be that a considerable number of patients suffering from severe depression are non-responders or do not respond adequately to SSRI treatment, and many

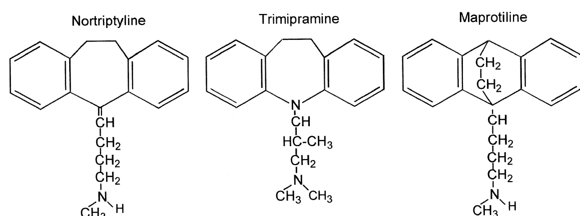

Fig. 1. Molecular structure of nortriptyline, trimipramine and maprotiline used as internal standards.

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PII: S0378-4347(00)00332-7

psychiatrists prefer nortriptyline as the second choice of drug.

Numerous methods for determination of TCA serum concentrations have been published, based on gas chromatography (GC), GC in combination with mass spectrophotometry (GC–MS) or the immunoassay principle [3–5]. In recent times, most methods for routine determination of TCA have been based on high-performance liquid chromatography (HPLC), inclusive semiautomatic methods using on-column extraction or column switching techniques [6–12]). A comprehensive review was published by Gupta 1992 [13].

The aim of the present study was to replace a manual liquid–liquid extraction method for nortriptyline in serum with a fully automated solid-phase extraction procedure. The liquid used for elution of the drug from the extraction cartridge should be compatible with the mobile phase allowing direct injection into a HPLC apparatus and thus making on-line determination possible. This required new HPLC conditions and as a consequence also renewed investigations of possible interfering drugs and metabolites, because polypharmacy is common within psychiatry.

2. Experimental

2.1. Chemicals

Nortriptyline, 3-(10,11-dihydro-5H-dibenzo[a,-b]-cycloheptene-5-ylidene)-*N*-methyl-1-propanamine was a gift from Lundbeck (Copenhagen, Denmark). Trimipramine and maprotiline (used as internal standards) and triethylamine were from Sigma (St. Louis, MO, USA). The molecular structures of the three compounds are shown in Fig. 1. HPLC grade methanol and acetonitrile (HiPerSolv) were from BDH (BDH Laboratory Supplies, Poole, UK). KH_2PO_4, K_2HPO_4,H_3PO_4 and NaOH were purchased from Merck (Darmstadt, Germany). Water was deionized and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Drug solutions

All stock solutions were prepared by dissolving pure substances in ethanol:nortriptyline or trimipramine in a concentration of 1.50 mg/ml and 2.05 mg/ml, respectively (5 m*M* of each) and maprotiline 2.22 mg/ml (8 m*M*). For further dilution of stock solutions, ethanol–water (50:50, v/v) was used for nortriptyline, and methanol–water (50:50, v/v) was applied for trimipramine and maprotiline. Serum-based calibrators and controls containing known amounts of nortriptyline were prepared by spiking serum from healthy drug-free blood donors to obtain final concentrations of nortriptyline in the range 0–1802 ng/ml. Controls, calibrators and serum samples from patients were stored frozen at −20°C until analysis. After thawing, the sera were inspected for precipitations and if present the sample was centrifuged before analysis to avoid obstruction of the extraction cartridge.

2.3. Solid-phase extraction

ASPEC XL (Gilson, Villiers le Bell, France) automatic solid-phase extraction apparatuses were used. A detailed description of the function of the programmable extraction has been published previously [14]. The XL model treats one sample at a time and therefore, in connection with a HPLC apparatus, was used for on-line determinations. The extraction cartridge was an Isolute SPE column from International Sorbent Technology LTD (Hengoed, Mid Glamorgan, UK), which contains 100 mg cyanopropyl-bonded silica gel with a reservoir volume of 1 ml. The solvents used for dilution of samples, conditioning of the cartridge sorbent, washing and elution from the SPE column were designated A, B, C and D, respectively. A: 0.1*M* potassium phosphate buffer pH 8.0, B: Methanol, C: 9% methanol in water and D: A mixture of an aqueous solution containing 31 m*M* triethylamine and 290 m*M* phosphoric acid and acetonitrile (70:30 v/v). The reservoir contained 2% methanol in water, and the internal standard was 6.2 μg/ml of trimipramine in methanol–water (50:50, v/v). If nothing else is mentioned, the flow-rate used for dispensing of fluid was set to 10 ml/min, and the flow-rate for aspiration of fluid to 3 ml/min. The air push volume was 1 ml. The sample, serum blind, serum calibrator and serum control (1.5 ml each) were placed in the appropriate racks, and the automatic extraction proceeded in the following way: (1) 1.2 ml serum was mixed with 2.45 ml solvent A and 50 μl internal

standard solution. (2) The cartridge was conditioned with 1 ml B followed by 2 ml D and 2 ml A at a flow rate of 20 ml/min. (3) 3.1 ml mixed sample corresponding to 1 ml serum was passed through the extraction column at a dispenser flow rate of 1.00 ml/min. (4) The inside and outside of the dispensing needle was rinsed with 2% methanol solution from the reservoir, 4.0 ml and 2.0 ml, respectively. (5) The cartridge was washed with 3.0 ml A, followed by 2 times 2.0 ml C, and finally 0.2 ml D. (6) The needle rinse was repeated (see point 4). (7) The analytes were subsequently eluted with 0.3 ml D and 125 μl of the eluate was injected into the HPLC apparatus. The optimum volume used for eluting nortriptyline was found by gradually increasing the elution volume from 200 to 800 μl, while keeping the injection volume constant. In the same way, the initially discharged volume (0.2 ml under point 5) of the fluid used for elution (D) was determined by gradually increasing the volume until a significant reduction of the recovery occurred.

2.4. Stability of nortriptyline in serum

Sera from patients treated with nortriptyline were divided into two parts. One part was frozen immediately and stored at −20°C until analysis. The other part was stored at room temperature for 48 h before freezing. For the two series of samples, nortriptyline concentrations were compared.

2.5. Liquid–liquid extraction

In 12 ml centrifuge tubes, 1 ml serum was mixed with 500 μl 1*M* NAOH and 50 μl internal standard solution containing 89 μg/ml of maprotiline. Three ml heptane/isoamylalcohol (98.5:1.5, v/v) was added, and the mixture was shaken for 5 min at 250 shakings/min on a HS 500 shaking apparatus (Janke & Kunkel, Staufen, Germany). After centrifugation at 1500 *g* for 5 min, the aqueous layer was frozen by immersing the tubes into a cooling bath consisting of dry ice and ethanol. The organic layer was decanted into centrifuge tubes and evaporated to dryness at 60°C under a gentle stream of nitrogen. The residue was dissolved in 100 μl mobile phase, and 75 μl was injected into the chromatograph.

2.6. Chromatography and calculations

The chromatographic system consisted of an 805 manometric module, a 306 isochratic pump, a 118 UV–VIS detector and Unipoint software from Gilson (Villiers le Bell, France). When liquid–liquid extraction was used, the HPLC apparatus was from Perkin-Elmer (Norwalk, CT, USA) consisting of an isochratic LC 200 pump, ISS-100 autosampler and an UV/VIS LC-75 detector. Chromatograms were recorded on Turbochrom Navigator software (PE Nelson, Perkin-Elmer). For separation of the analytes extracted by the Aspec system, we used a Luna $C_{18,}$ 3μ particle size, 150×4.6 mm column equipped with a C_{18} guard column (Phenomenex, Torrance, CA, USA). The mobile phase was acetonitrile–0.01 *M* triethylamine adjusted to pH 3.0 with $H_3PO_{4,}$ (34:66 v/v) [9]. The flow-rate was 0.85 ml/min. For liquid–liquid extracted samples, a Sperisorb S5 CN, 5μ, 150×4.6 mm (Waters, Taunton, MA, USA) was used, and the mobile phase was acetonitrile–methanol–concentrated ammonia water, 950:50:7 (v/v). The flow-rate was 1.3 ml/min. Both photometers were set at 242 nm.

From recorded peak heights, the ratios of nortriptyline to internal standards were calculated. One-point calibration was performed on the basis of serum spiked with known amounts of nortriptyline.

3. Results

3.1. Chromatography

A chromatogram of a serum sample from a healthy drug-free donor is shown in Fig. 2A. The procedure in question is automatic solid-phase extraction. In Fig. 2B, the same serum was extracted after addition of nortriptyline and the internal standard trimipramine. After the solvent front, an unknown peak was present at 7.2 min, but the peak did not influence the heights of nortriptyline or trimipramine with retention times of 7.8 min and 9.7 min, respectively. The unknown peak was also present, when pure water was extracted instead of serum. The chromatogram shown in Fig. 2C is serum extracted from a patient receiving nortriptyline as the only medication, while the patient in Fig. 2D was comedi-

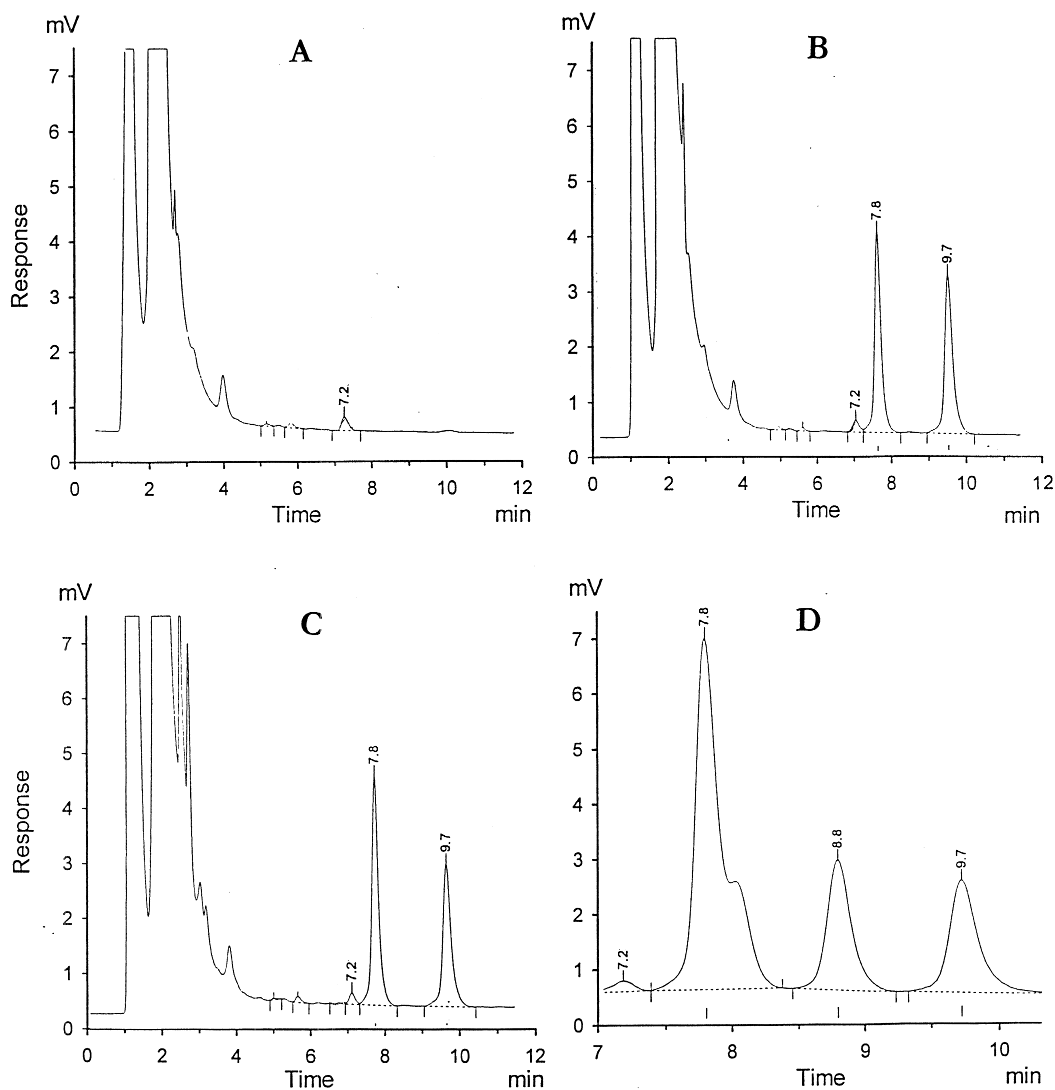

Fig. 2. Chromatograms of serum samples subjected to automated solid-phase extraction and analysis. (A) A blank serum sample. An unknown peak is present at 7.2 min. (B) Serum blank spiked with 90 ng/ml of nortriptyline (t_R=7.8) and the internal standard trimipramine (t_R=9.7). (C) Serum from a patient treated with nortriptyline 75 mg/day. The measured concentration of nortriptyline was 102 ng/ml. (D) Serum from a patient treated with 75 mg/day of nortriptyline and 100 mg/day of methotrimeprazine. The expanded chromatogram shows methotrimeprazine (t_R=8.8 min) and an unknown methotrimeprazine metabolite as a shoulder on the nortriptyline peak.

cated with methotrimeprazine. In the latter case, it appears from the expanded chromatogram that a shoulder was present at the nortriptyline peak, which significantly influenced the result of the quantitation. We were not able to identify the interfering metabolite, but demethyl-methotrimeprazine and di-demethyl-methotrimeprazine could be excluded by adding these metabolites to a nortriptyline serum standard before extraction.

3.2. Recovery and linearity

The absolute recovery was calculated by comparing the peak height after injection of nortriptyline

and trimipramine dissolved in mobile phase with the peak heights obtained after extraction of the same amounts of the compounds from serum. The absolute recoveries of nortriptyline and trimipramine were (mean±C.V., $n=8$) 75±13% and 51±8%, respectively. Blank serum spiked with 0–1802 ng/ml of nortriptyline was analyzed, and the equation describing the relation between nortriptyline serum concentration (x) and ratio (y) between the heights of nortriptyline and the internal standard was: $y=0.0052x+0.0095$ ($r=0.999$; $n=10$).

3.3. Precision and accuracy

The day-to-day and within-day precision and accuracy were evaluated by analysing blank serum samples spiked with 15–180 ng/ml nortriptyline. In our TDM-service, we do not quantitate levels below 15 ng/ml. The therapeutic interval for nortriptyline is 60–180 ng/ml. Table 1 shows, that within the therapeutic range for nortriptyline, the inter-day variation was less than 4.5%. The lower level of detection (equal to two times the SD of a low sample) was 0.8 ng/ml. The lower level of quantitation (equal to five times the SD of a low sample) was 2.0 ng/ml. The accuracy ranged from 93 to 104%.

3.4. Stability of nortriptyline in serum

Sera from 16 patients treated with nortriptyline were divided into two parts. One part (A) was frozen immediately and stored at −20°C until analysis. The other part (B) was stored at room temperature for 48 h before freezing. The means with SE in parenthesis of series A and B were 157 (21) and 154 (20) ng/ml, respectively. The difference between means was not significant (paired *t*-test).

3.5. Comparison with liquid–liquid extraction

In serum from 101 patients treated with various doses of nortriptyline, the concentration of nortriptyline in serum was determined using ASPEC XL for extraction and liquid–liquid extraction, respectively. The inter-day CV% of the liquid–liquid extraction method was 2.5% at 105 ng/ml ($n=85$) and 2.7% at 210 ng/ml ($n=86$). The response of the liquid–liquid extraction method was linear up to a nortriptyline concentration of at least 1300 ng/ml. The results obtained by the two methods were highly correlated (Fig. 3). A Deming regression analysis showed that the slope (1.04) was not significantly different from one, and the intercept (3.7 ng/ml) was not significantly different from zero [15].

3.6. Analysis for interference

Serum specimens from patients receiving drugs, which may be used in combination with nortriptyline, were analysed in order to study possible interference. Additionally, pure substances and metabolites of the drugs in question were added to serum spiked with nortriptyline and analysed according to the method described. Table 2 shows that of the compounds tested, only imipramine and an unknown methotrimeprazine metabolite (see Fig. 2D) interfered with the quantitative determination of nortriptyline. The therapeutic effects of imipramine are very similar to the effects of nortriptyline, which means that the two drugs are almost never administered simultaneously.

Table 1
Precision and accuracy of the determination of automatically extracted nortriptyline from spiked serum

Concentration (ng/ml)	n	Inter-day		Intra-day	
		C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)
	10			2.6	104
22.5	10	2.2	99	2.2	95
45	10	3.1	98	1.6	95
90.	10	4.3	94	1.5	97
180.2	10	3.9	93	1.3	93

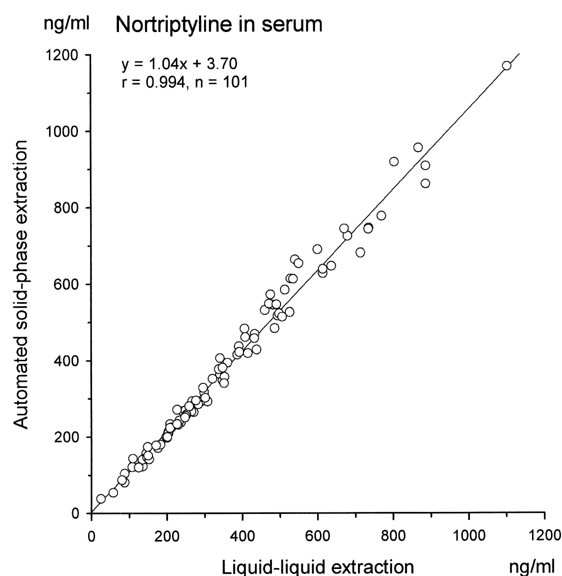

Fig. 3. Comparison between the concentrations of nortriptyline in serum from 101 different patients determined by the liquid–liquid extraction method (x) and the automated solid-phase extraction method (y).

4. Discussion

The presented method allows unattended on-line determination of nortriptyline in human serum. The time used for automatically extraction of nortriptyline from serum was 15 min, and the time necessary for chromatography was 11 min, but in order to avoid that late eluting peaks from comedicated drugs appeared in the next chromatogram the time was extended to 17 min. If off-line extraction is preferred, an ASPEC XL4 model can be used and four samples extracted simultaneously (detailed apparatus settings are available upon request). However, we have found that in order to avoid splitting of the air volume, which separates the solvent volumes, it was necessary to reduce the aspirating and dispensing flow-rates in some of the extraction steps. This means that the time for off-line extraction of four samples was 30 min or 7.5 min per sample. Automated solid-phase extraction methods have been described for analysis of other antidepressant such as citalopram and fluoxetine, but these methods were not operating on-line [16,17].

Table 2
Retention times relative to the internal standard trimipramine (Rt_R) of drugs and and metabolites. Interfering compounds are underlined. The mean t_R of trimipramine was 9.70 min

Compound	Rt_R	Compound	Rt_R
Tricyclic antidepressants		Mianserin	0.44
Nortriptyline	**0.80**	Mianserin metabolite	0.40
N-demethylnortriptyline	0.70	Sertraline	1.22
(E)-10-OH-nortriptyline	<0.40	*Antipsychotics*	
(Z)-10-OH-nortriptyline	<0.40	Chlorprothixene	1.57
Trimipramine (IS)	**1.00**	Chlorprothixene metabolite	1.40
Amitriptyline	0.90	Clozapine	<0.40
(E)-10-OH-amitriptyline	<0.40	Demethyl-clozapine	<0.40
(Z)-10-OH-amitriptyline	<0.40	Haloperidol	0.60
Clomipramine	1.55	Methotrimeprazine	0.93
Demethyl-clomipramine	1.37	*N*-demethyl-methotrimeprazine	0.84
Doxepine	0.51	Didemethyl-methotrimeprazine	0.73
Demethyl-doxepine	0.45	Methotrimeprazine sulphoxide	<0.40
Imipramine	0.81	Methotrimeprazine metabolite	0.81
Desipramine	0.74	Olanzapine	<0.40
Other antidepressants		Perphenazine	1.11
Citalopram	0.49	Risperidone	<0.40
Demethyl-citalopram	0.44	9-OH-risperidone	<0.40
Fluoxetine	1.21	Zuclopenthixol	1.33
Norfluoxetine	1.02	*Other drugs*	
Fluvoxamine	0.71	Carbamazepine	0.86

When on-line methods are developed, the greatest challenge is to compose a fluid suitable for eluting the drugs from the cartridges. An acceptable recovery should be obtained without disturbing the following chromatography or lead to a rapid deterioration of the analytical column. Elution with mobile phase gave insufficient recovery, and methanol with addition of acetic acid or triethylamine broadened the peaks or significantly altered the retention times. An increase of the ion strength of the mobile phase, while keeping the pH constant, solved the problems. The optimum volume used for elution was now determined by injection of a fixed volume into the chomatograph, while we gradually increased the total volume used for elution. The next step was to discharge some of the initial eluent in order to remove fluid from the dead volume and at the same time get rid of some impurities, which shortened the HPLC column life-time. The compromise chosen resulted in some decrease of the recovery and also contributed to the analytical variation. However, the use of an internal standard kept the analytical variation at a low level. Using the optimized extraction procedure, the original column has resisted more than a thousand samples analyzed during the past half year. The much less expensive pre-column has been changed once during the same period.

The cost of a cartridge is ca. $1.5, but this expense shall be outweighed against the benefits of automation and on-line determination. The presented method allows unattended operation and a more continuous workflow in the laboratory. Compared to the liquid–liquid extraction method, the manual work is reduced and the quantity of expensive organic fluids diminished. Furthermore, freezing of the aqueous phase and evaporation of organic solvents are avoided.

Acknowledgements

This work was supported by the Novo Nordic Foundation, Albert Nielsen's Foundation, Geert-Jorgensen's Foundation, Petersen's Foundation, Marshall's Foundation, Psychiatric Research Foundation and the Foundation of 1870.

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