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Note

High-performance liquid chromatographic quantitation of amitriptyline and nortriptyline in dialysate from plasma or serum using on-line solid-phase extraction

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In therapeutic drug monitoring of psychoactive drugs, their total concentration is determined in plasma. However, determination of the unbound concentrations may be preferable [1,2]. Existing methods for the determination of the unbound concentration of amitriptyline and nortriptyline involves either the use of radioactive tracer substances or organic extraction with subsequent high-performance liquid chromatographic (HPLC) or gas chromatographic determination of a protein-free dialysate or filtrate [3–5]. The use of radioactive tracers permits, as a rule, the determination of only one substance in each sample. There may also be problems in obtaining tracer substances of satisfactory purity and stability. A method involving an extraction procedure often gives a low yield when the concentrations are low: the procedures are usually tedious and time-consuming, and the use of organic solvents may involve health hazards for laboratory personnel. For plasma samples there are methods in which the samples are processed by a solid-phase extraction before it is injected into a liquid chromatograph [6,7]. A method has been reported in which the sample is processed on a precolumn directly connected to the analytical column [8].

We have developed a method of analysing an aliquot of the dialysate directly with HPLC by combining a column valve and an enrichment column on-line with the analytical column. The method allows a quick, simple and accurate determination of the rather low drug concentrations found in a plasma dialysate. The HPLC system used also has the advantage that only one pump is required. The method may be used for determining free plasma concentrations in pharmacokinetic studies and for developing analytical techniques for therapeutic drug monitoring of free plasma concentration.

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EXPERIMENTAL

Chemicals

Amitriptyline and nortriptyline standards were obtained from H. Lundbeck (Copenhagen-Valby, Denmark). Clomipramine, the internal standard, was obtained from Ciba Geigy (Basel, Switzerland). Stock solutions of each drug were prepared in 99.5% ethanol at a concentrations of 1 m*M* and stored at 4°C. Aliquots of the stock solutions were diluted to a concentration of 5 μM for preparation of calibration standards.

To make the dialysis buffer, 3.998 g of disodium hydrogenphosphate dihydrate 0.775 g of sodium dihydrogenphosphate monohydrate and 2.250 g of sodium chloride were dissolved in distilled water to a final volume of 1 l. Then 0.055 g of mercuric nitrate was added to each litre of solution to prevent bacterial growth. The pH of the buffer was 7.4. These chemicals were of analytical grade.

Serum samples (3 ml) from patients with amitriptyline or nortriptyline medication were adjusted to pH 7.4 and dialysed against 5 ml of dialysis buffer for 4 h at 37°C in PTFE dialysis chambers, as described by Nyberg and Mårtensson [3].

High-performance liquid chromatography

The HPLC system consisted of a Constametric III pump (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) and a Model 7120 injector (Rheodyne, Berkeley, CA, U.S.A.). The loop of the injector was exchanged against an enrichment column (10.0 mm × 6.0 mm I.D.) packed with column material (40 μm) from a Bond Elut cartridge, originally developed for cannabis analysis from Analytichem International (Cat. No. 620303, Harbor City, CA, U.S.A.) (Fig. 1). The analytical column was a reversed-phase Spherisorb ODS Superpac cartridge, 3 μm, 100 mm×4.0 mm I.D. (LKB, Bromma, Sweden), connected to a Spectro Monitor III variable-wavelength UV detector (LDC/Milton Roy). The absorbance was followed with a recorder (Linear Instruments).

A 1-ml syringe, Hamilton gastight No. 1001 (Hamilton Bonaduz, Bonaduz Switzerland) was used. The mobile phase was water–85% phosphoric acid–triethylamine–acetonitrile (50:0.225:0.225:49.55, v/v), all of HPLC-grade. The so-

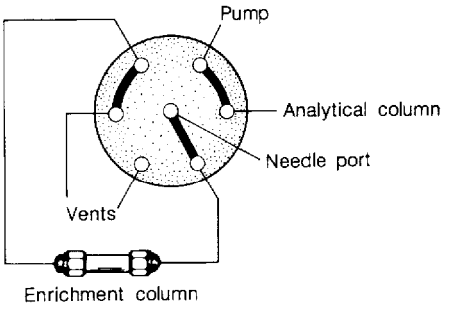

Fig. 1. Schematic diagram of the injection valve and enrichment column with the connection to the analytical column and pump. The position for injection onto the enrichment column is shown.

lution was degassed by stirring under reduced pressure produced by a water suction pump. The flow-rate of the mobile phase was 0.65 ml/min at 20 °C. The absorbance was measured at 238 nm.

The column valve was set in "load" position. To wash out any remaining drug substance from the preceding sample on the enrichment column, each injection of a dialysed sample was preceded by the injection of 1 ml of the mobile phase, which was washed from the column with 1 ml of water. Then 2 ml of dialysate were injected, followed by 1 ml of water. Finally the enrichment column was washed with 1 ml of acetonitrile–water (1:1, v/v). The column valve was then turned to the "inject" position for 30 s and then back to "load".

Standards

Standards were made of dialysis buffer to which equal amounts of amitryptiline and nortriptyline were added to give concentrations of 2.5–50 n*M*; this is approximately the concentration range in plasma dialysate from amitriptyline-treated patients [9]. Standard curves of chromatographic peak height as a function of concentration were calculated according to a straight linear model.

RESULTS

Injection technique

There was a linear relationship between the volume injected and the peak height in the range 1–5 ml. The injected sample was a pooled dialysate from patients' sera with the concentration of 25 n*M* nortriptyline and 13 n*M* amitriptyline. Injection of 5 ml of dialysate made it possible to detect concentrations of 0.5 n*M* nortriptyline and 1 n*M* amitriptyline.

The length of time for which the column valve is in the "injection" position determines how long the mobile phase takes to pass through the enrichment column. This should be long enough only to elute the drugs from the enrichment column. If it is too long, interfering components are eluted. Volumes of 2 ml of dialysis buffer containing 25 n*M* amitriptyline and 25 n*M* nortriptyline were repeatedly injected with the column valve in the injection position, for different

TABLE I

PEAK HEIGHT OF ANALYSED SUBSTANCE IN RELATION TO TIME IN INJECTION POSITION

Time in injection position (s)	Peak height (mm)	
	Nortriptyline	Amitriptyline
20	67	38
25	94.5	60
30	97	68
40	97	70.5
45	96	69

TABLE II

RETENTION TIMES OF DRUGS TESTED FOR INTERFERENCE

Flow-rate = 0.65 ml/min.

Drug	Retention time (min)
Phenytoin	Not detected
Lorazepam	Not detected
Carbamazepine	Not detected
Oxazepam	Not detected
Nitrazepam	Not detected
Clonazepam	Not detected
Zimelidine	2.80
Flunitrazepam	2.90
Perphenazine	3.10
Thioridazine side-chain sulphone	3.15
Haloperidol	3.20
Zuclophenthixol	3.30
Fluphenazine	3.35
Desmethylimipramine	3.45
Protriptyline	3.50
Thioridazine side-chain sulphoxide	3.50
Alprazolam	3.60
Thioridazine ring sulphoxide	3.70
Nortriptyline	3.85
Maprotiline	3.90
Promethazine	4.10
Imipramine	4.20
Diazepam	4.25
Desclomipramine	4.75
Trimipramine	4.75
Levomepromazine	4.80
Amitriptyline	4.85
Chlorpromazine	5.80
Clomipramine	5.95
Chlorprothixene	6.55
Thioridazine	8.35

TABLE III

STANDARD CURVES FOR DIALYSATE AND DIALYSIS BUFFER WITH AND WITHOUT INTERNAL STANDARDS

Sample	Nortriptyline		Amitriptyline	
	Slope	Intercept	Slope	Intercept
Dialysate*	3.88	4.85	2.79	0.65
Dialysis buffer*	3.86	3.74	2.76	3.73
Dialysate with I.S.**	0.070	0.057	0.051	−0.011
Dialysis buffer with I.S.**	0.076	−0.161	0.054	−0.096

*The regression analysis compared the concentration (nM) of nortriptyline and amitriptyline (x-axis) with the peak height (mm) (y-axis).

**The regression analysis compared the concentration (nM) of nortriptyline and amitriptyline (x-axis) with the ratio of peak height of compound to internal standard (I.S.) (y-axis).

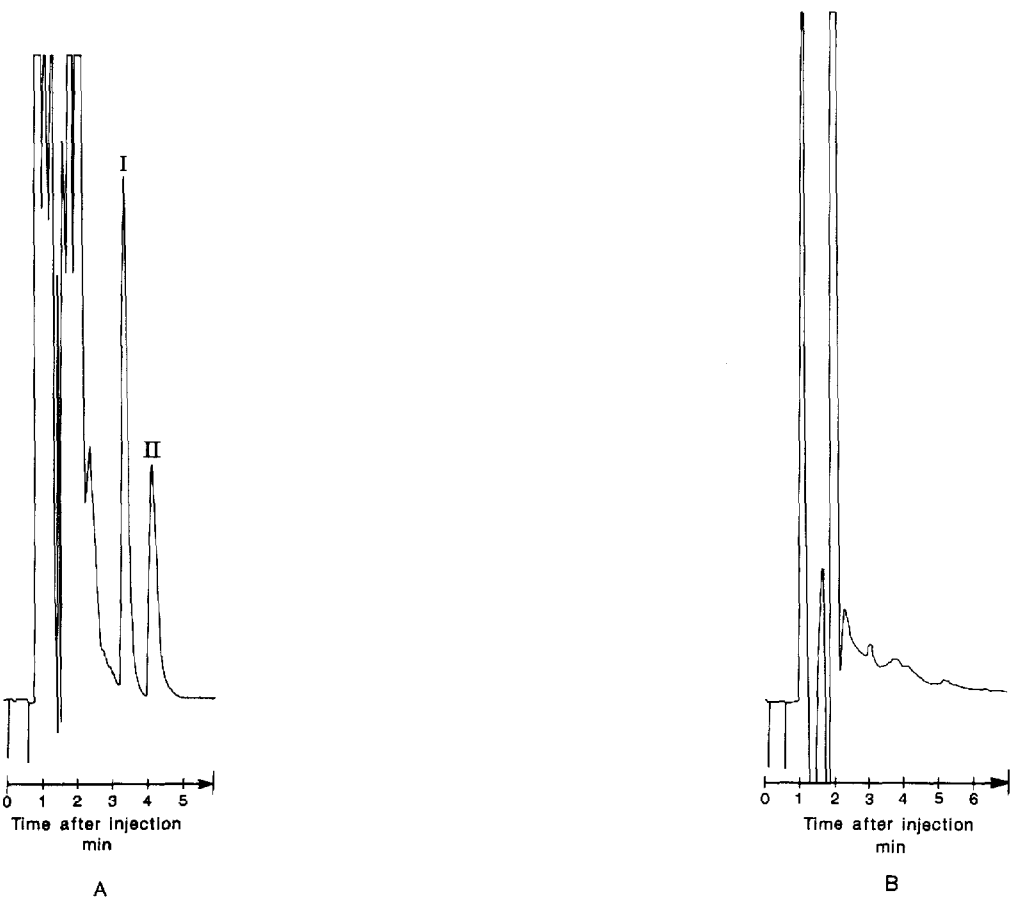

Fig. 2. Chromatograms of (A) dialysate, containing 20 n*M* nortriptyline and 15 n*M* amitriptyline from pooled human serum from a patient on amitriptyline medication and (B) dialysate from drug-free serum. Peaks: I = nortriptyline; II = amitriptyline.

periods of time. It appeared that 30 s were sufficient to elute the drugs from the enrichment column (Table I).

When the column valve remained in the injection position until the next sample was injected, impurities in the samples interfered with the baseline.

Precision and accuracy

The within-run precision of the analysis was tested by repeated injections of a dialysate with a concentration of 26 n*M* nortriptyline and 13 n*M* amitriptyline and with 25 n*M* clomipramine added as internal standard ($n=10$). The coefficient of variation (C.V.) when the absolute peak height was measured was 3.3% for nortriptyline and 4.6% for amitriptyline; the C.V. when the ratio between the peak height for amitriptyline and nortriptyline and the internal standard was measured was 2.5% for nortriptyline and 3.5% for amitriptyline. When samples containing 50 pmol of nortriptyline and 50 pmol of amitriptyline dissolved in dialysis buffer were injected via the enrichment column directly onto the analyt-

ical column, the peak heights of nortriptyline and amitriptyline became, on average, 98 and 97% of the peak heights obtained when the same amounts of drugs were injected directly onto the analytical column via a 100-μl loop.

Selectivity

Twenty-nine drugs were tested for possible interference. The retention times of these drugs are shown in Table II. Maprotiline, promethazine and thioridazine ring sulphoxide interfere with nortriptyline. Desmethylclomipramine, trimipramine and levomepromazine interfere with amitriptyline.

Standards

Standards made of dialysis buffer were compared with standards made of dialysate from dialysis of plasma from a healthy volunteer. Standards were also made with clomipramine added as internal standard. The standards curves are tabulated in Table III.

The curves obtained with dialysate and buffer are very close, but at concentrations at the lower range of detection the differences in calculated concentration can be as large as 15–20%. For this reason the dialysate standard is preferable.

Fig. 2 shows a chromatogram of a dialysate from a pooled serum from patients on amitriptyline medication, together with a chromatogram of a dialysate from a blank serum.

DISCUSSION

With the present method, amitriptyline and its metabolite nortriptyline can be determined in a dialysate from patients' blood plasma by HPLC without prior extraction with organic solvents. The method is highly precise, easy to perform and seems suitable for determining the free fraction of the drug in plasma. The very low concentrations of the drugs make it necessary to inject sample volumes of at least 2 ml. This was possible with an enrichment column connected to the column valve.

The particle size of the enrichment column was chosen to give a back-pressure that still allowed the use of a syringe for the injections. The enrichment column was connected to the column valve in such a way that when the column valve was switched to the "inject" position, there was a backflush of the drugs to the analytical column; the peak width is thus not influenced by the large particle size of the enrichment column.

Because the mobile phase was passed through the enrichment column for only 30 s, impurities in the dialysate were retained in the enrichment column and not eluted into the analytical column. To obtain maximum detectability and separation, the particle size in the analytical column was only 3 μm. The enrichment column was washed with water before dialysate was injected to prevent the precipitation of salts from the dialysate. Contamination of the dialysate by impurities results in a peak at almost the same retention time as the amitriptyline peak. When the enrichment column was washed with acetonitrile–water (1:1) this contamination disappeared.

The fact that a total of five injections was necessary for each sample does not preclude a rapid handling of each sample, since a sample may be injected on the enrichment column at the same time as the preceding sample is eluted from the analytical column. By this technique it is possible to analyse twelve samples per hour, provided there has been no co-administration of interfering drugs.

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