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Rapid and simple high-performance liquid chromatographic determination of tricyclic antidepressants for routine and emergency serum analysis

MARIA PIA SEGATTI*, GIUSEPPE NISI, FLAVIA GROSSI and MARIANGELA MANGIAROTTI
Laboratorio di Analisi Chimico Cliniche, Ospedale Infantile "Burlo Garofolo", Via dell'Istria 65/1, Trieste (Italy)
and
CLAUDIO LUCARELLI
Laboratorio Biochimica Clinica, Istituto Superiore di Sanità, Via le R. Elena 299, Rome (Italy)

ABSTRACT

An isocratic reversed-phase high-performance liquid chromatographic procedure is presented for the simultaneous detection of desipramine, nortriptyline, imipramine, amitriptyline and clomipramine in serum. Drugs are extracted after sample alkalinization and separated from each other on an octyl reversed-phase with *n*-butylamine as mobile phase modifier. Detection is achieved at 254 nm. The recovery of tricyclic antidepressants (92–110%) has good precision, with a relative standard deviation of less than 5%. Being rapid and simple, the method is suitable for the emergency clinical laboratory.

INTRODUCTION

Tricyclic antidepressants (TCAs) are commonly used for the treatment of depressive disorders, as their efficacy in alleviating depression has been well established [1–4]. Many studies have indicated that the clinical outcome for this group of compounds might be concentration dependent, and also should show a marked variation among subjects in achieving a steady-state dose [5–7]. Therefore, it is desirable to monitor the concentrations of these drugs and their active metabolites in serum in order to minimize side-effects and avoid toxic reactions [8].

Many methods have been used for the determination of TCAs, including immunoenzymatic techniques and gas chromatography [9–12], but high-performance liquid chromatography (HPLC) is now widely used in routine application owing to its sensitivity, specificity and low cost [13–16].

The aim of this work was to develop a method that allows the simultaneous determination of five commonly prescribed TCAs: desipramine (DESI), nortriptyline (NOR), imipramine (IMI), amitriptyline (AMI) and clomipramine (CLO). Even though these drugs are never administered together, the availability of a single chromatographic method, suitable for their simultaneous detection, is very useful for

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the clinical laboratory, as the maintenance of separate chromatographic methods for each drug would be more expensive.

Of the relevant HPLC methods reported in the literature, we considered only those concerning the above-mentioned TCAs. Proelss *et al.* [17] described a technique involving ion-pair chromatography, but the recoveries at a concentration of 75 ng/ml were unsatisfactory (68–76%). Koteel *et al.* [15] needed an automated sample processor for a precision assay. Likewise, Lensemeyer and Evenson [14] and Lin and Frade [18] employed a set of disposable solid-phase cyanopropyl columns and a vacuum-elution system, respectively. Moreover, none of them considered the detection of clomipramine (CLO). In Matsumoto *et al.*'s method [19], automated column switching is necessary, the analysis time is long (CLO retention time = 30 min) and the day-to-day relative standard deviations (R.S.D.) at the 500 ng/ml level were higher than values obtained at the lower level (100 ng/ml).

In this paper, we propose a reversed-phase HPLC method for resolving the above TCAs within 15 min. The measurement of TCA serum levels is based on peak-area ratios of drug to internal standard at 254 nm. The pretreatment is simple and rapid, involving a single-step solvent extraction.

EXPERIMENTAL

Materials

All reagents were of analytical-reagent grade. Acetonitrile was purchased from J. T. Baker (Deventer, The Netherlands), hexane, sodium dihydrogenphosphate and *n*-butylamine from Merck (Darmstadt, F.R.G.) and sodium borate, sodium hydroxide and phosphoric acid from Carlo Erba (Milan, Italy). Desipramine hydrochloride and clobazam were obtained from Chiesi Farmaceutici (Parma, Italy), maprotiline, clomipramine, desmethylclomipramine and imipramine hydrochloride from Ciba Geigy (Varese, Italy), nortriptyline hydrochloride from Recordati (Milan, Italy) and amitriptyline from Roche (Milan, Italy). TCA stock standards (1 g/l) were prepared by dissolving the drugs in methanol.

Chromatography

The HPLC equipment consisted of a Model S2 pump (Perkin-Elmer, Norwalk, CT, U.S.A.), a Rheodyne Model 7105 injection valve, fitted with a 175-μl sample loop, and a Perkin-Elmer LC 75 UV spectrophotometric detector, set at 254 nm and 0.04 a.u.f.s. The analytical column was reversed-phase C_8 (150 mm × 4.6 mm I.D.), particle size 5 μm, connected to a 2-cm long Pelliguard LC-8 guard column with 40-μm packing, both from Supelco (Bellefonte, PA, U.S.A.).

The mobile phase was a modification of that proposed by Gill and Wanagho [20], consisting of acetonitrile–phosphate buffer (pH 3) in a 50:50 (v/v) mixture at a flow-rate of 1 ml/min. The buffer was obtained by adding 1.2 ml/l of butylamine to 0.01 *M* aqueous sodium dihydrogenphosphate and then adjusting the pH to 3 with phosphoric acid.

Extraction

To a screw-capped glass tube containing 1 ml of serum were added 3 μl of working internal standard solution (clobazam, 20 ng/ml), 1 ml of saturated sodium

borate (pH adjusted to 11 with 6 *M* sodium hydroxide) and 5 ml of *n*-hexane. The contents of the tube were mixed for 2 min and centrifuged for 10 min at 3000 *g*.

The organic phase was separated and evaporated to dryness under a stream of helium at 30°C. The residue was reconstituted in 20 μl of mobile phase and 10 μl were injected into the HPLC system.

RESULTS

Table I lists the capacity factors (k') of antidepressants most commonly used in the treatment of mood disorders in Italy and those of benzodiazepines, which may be administered simultaneously with TCAs in medical practice. Even if benzodiazepines had been extracted in a basic medium, as our method recommends, we observed no interference among benzodiazepines and the group of TCAs considered.

Table II gives the regression equations and correlation coefficients for the TCAs tested. The calibration graph was obtained by the plotting peak-area ratios (drug/internal standard) *versus* concentrations (ng/ml) of the drugs after analysing serum samples spiked with various amounts of TCAs (25–1000 ng/ml) and a fixed amount of internal standard (clobazam, 3 μl of 20 ng/ml solution). The relationship was linear over the range considered. The detection limit was *ca.* 10 ng/ml, sufficiently below the lowest therapeutic concentration (50 ng/ml). Nevertheless, it is possible to improve this limit by increasing the detector sensitivity or analysing a larger sample (>2 ml).

Table III shows the results of an analytical recovery study in which known amounts of drugs and internal standard were added to 1 ml of drug-free serum and carried out through the entire procedure. Reproducibility was assessed by reporting within- and between-assay results. The latter were calculated in a 5-day sequence over a 1-month period. As shown in Table IV, the within-day R.S.D. was <7% at 100 ng/ml, <4% at 250 ng/ml and <3% at 500 ng/ml. The between-assay R.S.D. ranged from 9.8% at 100 ng/ml to 3.9% at 500 ng/ml.

Fig. 1 illustrates chromatograms of a blank serum used for recovery and precision studies and the same serum supplemented with TCA standard (100 ng/ml). Fig. 2 shows representative chromatograms obtained from a child who had

TABLE I

RETENTION BEHAVIOUR OF TCAs AND OTHER DRUGS COMMONLY PRESCRIBED IN PSYCHIATRIC DISORDERS: INTERFERENCE STUDY AT 254 nm

Drug	k'	Drug	k'
Nitrazepam	0.831	Diazepam	1.830
Lorazepam	0.853	Haloperidol	1.976
Clonazepam	0.916	Desipramine	2.282
Triazolam	1.009	Nortriptyline	2.460
Desmethylclomipramine	1.092	Maprotiline	2.613
Flunitrazepam	1.185	Imipramine	3.025
Alprazolam	1.200	Amitriptyline	3.333
Clobazam	1.344	Clomipramine	4.085

TABLE II

LINEAR REGRESSION AND CORRELATION PARAMETERS DETERMINED BETWEEN 25 AND 1000 ng/ml

y = Peak area ratio drug/internal standard; x (ng/ml) = concentration of TCA.

Drug	Regression equation	r
Desipramine	$y = 0.0132x - 0.105$	0.98
Nortriptyline	$y = 0.0127x - 0.050$	0.99
Imipramine	$y = 0.0235x - 0.011$	0.99
Amitriptyline	$y = 0.0138x - 0.104$	0.99
Clomipramine	$y = 0.0196x - 0.033$	0.99

accidentally ingested an unknown number of clomipramine pills. The chromatograms correspond to two blood samples collected 2 h (left) and 5 h (right) after the pills had been swallowed. The results were obtained 30 min after the child was admitted to the emergency room.

DISCUSSION AND CONCLUSIONS

The method proposed for TCA determination involves a one-step clean-up and a resolution on a C_8 reversed-phase column with a binary mixture of acetonitrile and a phosphate buffer (pH 3) and *n*-butylamine as mobile phase modifier. For sample pretreatment, a single-step liquid–liquid extraction was preferred to a solid-phase extraction [15,18,19] because it does not require any special apparatus. Several efforts were made using different combinations of extractants; *n*-hexane–ethyl acetate or *n*-hexane–isoamyl alcohol were evaluated in order to improve the efficiency and avoid

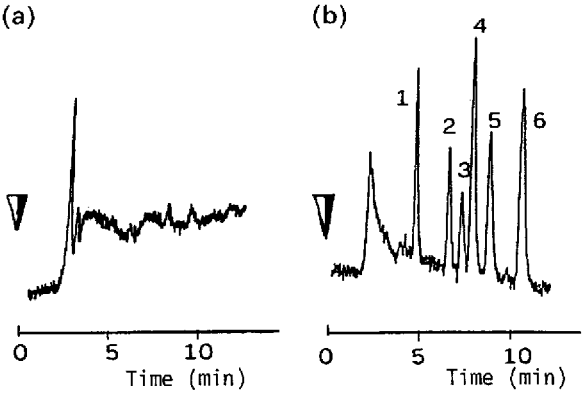

Fig. 1. Chromatogram of 10 ll of (a) drug-free human serum extract and (b) extract of serum, containing 100 ng/ml of each the tricyclic antidepressants analysed. Peaks: 1 = clobazam (internal standard); 2 = desipramine; 3 = nortriptyline; 4 = imipramine; 5 = amitryptyline; 6 = clomipramine. Mobile phase, acetonitrile–phosphate buffer (pH 3) (3:2); column, RP-C_8, 150 mm × 4.6 mm I.D.; flow-rate, 1 ml/min; detection, 254 nm (0.04 a.u.f.s.).

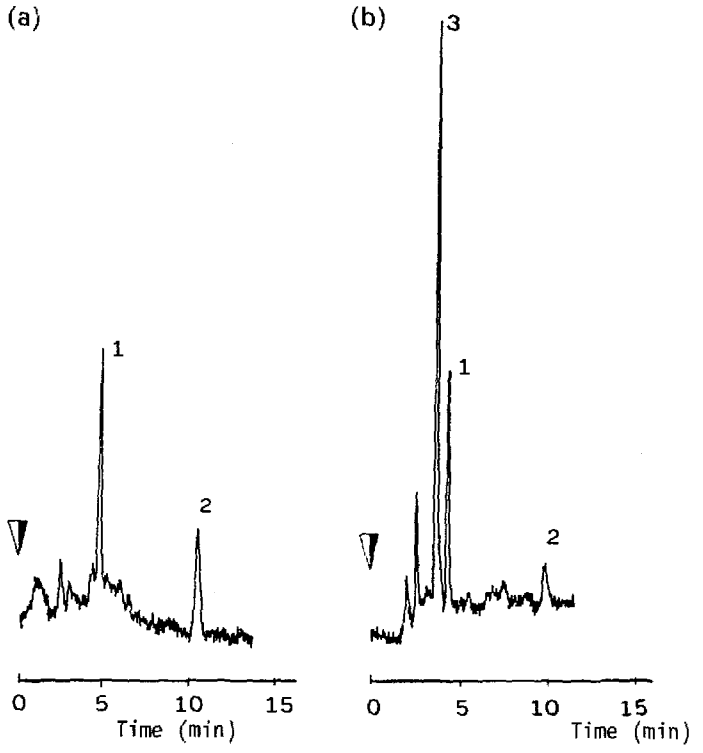

Fig. 2. Chromatograms of a serum extract obtained from a child who had accidentally ingested clomipramine. Sampling time: (left) 2 h and (right) 5 h after drug ingestion. Peaks: (a) 1 = clobazam (I.S.); 2 = clomipramine (50 ng/ml); (b) 1 = clobazam (I.S.); 2 = clomipramine (14.5 ng/ml); 3 = desmethylclomipramine. HPLC conditions as in Fig. 1.

TABLE III

ANALYTICAL RECOVERY OF TRICYCLIC ANTIDEPRESSANTS IN FORTIFIED SERUM AFTER EXTRACTION (n = 30)

Drug	Level (ng/ml)	Recovery (%)	R.S.D. (%)
Desipramine	100	94.6	6.4
Nortriptyline		93.2	6.7
Imipramine		95.4	5.7
Amitriptyline		96.1	7.5
Clomipramine		91.7	8.4
Desipramine	250	97.1	3.8
Nortriptyline		96.8	5.3
Imipramine		96.4	4.8
Amitriptyline		97.5	5.1
Clomipramine		102.6	4.1
Desipramine	500	108.2	4.0
Nortriptyline		105.5	3.8
Imipramine		98.9	3.4
Amitriptyline		104.5	4.5
Clomipramine		110.6	4.1

TABLE IV

PRECISION

Data pertain to a serum supplemented with three different amounts of TCAs.

Drug	Concentration added (ng/ml)	Within-day (n = 6)		Between-day (n = 5)	
		Concentration found (ng/ml)	R.S.D. (%)	Concentration found (ng/ml)	R.S.D. (%)
Desipramine	100	93.4	4.2	95.1	5.2
	250	257.3	2.8	234.6	4.2
	500	537.9	2.0	554.1	4.7
Nortryptiline	100	92.8	4.0	93.6	6.0
	250	248.9	2.7	235.2	5.9
	500	536.3	1.8	512.4	4.2
Imipramine	100	94.6	4.5	95.0	7.1
	250	225.6	3.1	236.4	5.2
	500	502.3	2.1	489.2	3.9
Amitriptyline	100	96.9	3.8	95.1	6.9
	250	251.2	2.9	237.3	4.7
	500	501.5	2.0	537.4	4.0
Clomipramine	100	92.1	5.3	91.3	9.8
	250	269.2	3.8	225.7	4.6
	500	547.1	2.9	569.2	5.1

emulsions or endogenous impurities. *n*-Hexane alone was found to be adequate, the recovery being close to 100% at several widely different concentrations. We found a discrepancy with a previously published paper [17], in that we did not notice any losses of the analysed drugs if isoamyl alcohol was absent from the extraction procedure. This was also confirmed by the high recoveries. Extracts prepared from TCA-free serum yielded no endogenous peaks with retention times similar to those of the antidepressant tricyclic drugs. Consequently, further purification as described by other workers [17] is not required.

In the elution of TCAs, the strong hydrogen-bonding interaction between the amino side-chain of TCAs with the possible free silanol group of the silica support of the reversed-phase packing broadens the peaks. The use of *n*-butylamine in the mobile phase improves the resolution and peak shape. Variables of the composition of the mobile phase, such as pH, buffer strength, concentration of amine added and acetonitrile percentage were considered in order to determine their effectiveness in recovering the TCAs. Retention of drugs and their separation from interfering substances on the C_8 column were not influenced by the pH or buffer strength of the eluent mixture, but the concentration of acetonitrile in particular had a very significant effect on k'. We found that the resolution of TCA also increased as the concentration of *n*-butylamine increased.

The choice of wavelength required a compromise between sensitivity and baseline noise, since at 254 nm the background noise, due to the mobile phase modifier,

is less than that at lower wavelengths, whereas the sensitivity is sufficiently high to determine serum TCAs at therapeutic levels. There is no interference from drugs, such as benzodiazepines or TCA metabolites, an observation made also by other workers [18]. In developing the assay, it was difficult to obtain an internal standard that would behave in a manner similar to TCAs in the extraction procedure and yet have acceptabe absorption at 254 nm and chromatographic retention times. As benzodiazepines were suitable for this purpose, we propose clobazam as an internal standard. For clinical use, when clobazam is simultaneously administered as medication, an alternative internal standard may be used, such as another TCA, as situations in which more than one TCA is administered at the same time are rare.

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