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Sensitive and rapid method for the simultaneous quantification of five antidepressants with their respective metabolites in plasma using high-performance liquid chromatography with diode-array detection

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Abstract

A high-performance liquid chromatographic method with diode array detection (HPLC–DAD) has been developed for the simultaneous separation and quantification of imipramine, amitriptyline, maprotiline, fluoxetine, clomipramine and their respective metabolites using a 500-μl plasma sample and clovoxamine as the internal standard. The substances were eluted on a Symmetry C_{18} 5-μm column (250×4.6 mm, I.D.). Full UV spectra from 200 to 450 nm were recorded on-line during the entire analysis and were automatically compared to spectra stored in a library. The quantification was performed at 226, 254, and 400 nm. Peak height ratios were linear over a concentration range of 10–3000 ng/ml: the correlation coefficient (r) was better than 0.998 for all substances at each wavelength. Acceptable coefficients of variation are demonstrated for both within-run and day-to-day assays. The method is simple, highly specific and currently being used for drug monitoring and toxicological studies in children and adult patients. © 1997 Elsevier Science B.V.

Keywords: Imipramine; Amitriptyline; Maprotiline; Fluoxetine; Clomipramine

1. Introduction

Antidepressants are widely used in psychiatry. Tricyclic antidepressant (TCAs) are a reference treatment for depression [1,2], by acting mainly on the adrenergic system, and are responsible for many concentration dependent adverse effects.

The more recently used fluoxetine use is well tolerated and widely administered; it acts by inhibition of serotonin re-uptake [3]. However, the combined use of fluoxetine and TCAs has been associated with TCAs toxicity due to a marked elevation of their concentrations in plasma [4,5], presumably as result of inhibition of the ring hydroxylation of these drugs [6,7]. Moreover, heterogeneity of the hepatic metabolism, an associated disease and/or polymedication led to large inter-individual variations in the steady-state plasma concentrations observed in patients receiving equal oral dosage [8]. In addition to being a tool for maximising effectiveness and minimising adverse effects, monitoring TCAs in plasma serves as a guide for assessing patient compliance [9].

Of the techniques applicable to analysis of TCAs

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in biological fluids, high-performance liquid chromatography (HPLC) is the most preferred and widely advocated approach [10,11]. Numerous methods are published in the literature describing a specific dosage for each antidepressant. Methods with simultaneous assays of many antidepressants have been developed [12–19], but these HPLC methods are limited in identifying the different molecules by their retention time and the detection is set at one or two wavelengths. Furthermore, they do not allow the quantification of all antidepressants, due to poor resolution.

A screening method by HPLC is used in toxicology [20] for the simultaneous identification of 25 antidepressants. The eluted compounds are identified from their retention time and from their spectral properties with a diode-array detector (DAD). This detector dramatically improved the selectivity of HPLC and gave it some of the advantages of mass spectrometric detection [21,22].

We present in this paper a simple, rapid, sensitive and highly specific HPLC–DAD method for identification and simultaneous quantification of five antidepressants and their respective active metabolites in human plasma.

2. Experimental

2.1. Chemicals

All drugs except clovoxamine were obtained as hydrochloride salts. Fluoxetine and norfluoxetine were kindly supplied by Eli Lilly (Saint-Cloud, France). Clomipramine, desmethylclomipramine, imipramine, desipramine and maprotiline were obtained from Ciba-Geigy (Switzerland). Amitriptyline and nortriptyline were obtained from Roche (Neuilly-sur-seine, France). Clovoxamine base used as internal standard (I.S.) was kindly supplied by Duphar (Villeurbanne, France). Stock and working solutions of fluoxetine, norfluoxetine and I.S. were prepared in methanol. Stock and working solutions of TCAs were prepared with 0.01 *M* HCl. Stock solutions of all drugs were prepared by weighing 10.0 mg (as the free base equivalent) of pure drug and dissolving it in 10 ml of corresponding solvent. Stock solutions for all drugs and working solutions of TCAs drugs were stored at 4°C in the dark and found to be stable for at least 1 year.

Acetonitrile (Lichrosolv, Merck, Darmstadt, Germany), methanol (Uvasol, Merck) and *n*-hexane (Carlo Erba, Milan, Italy) were UV grade. Potassium dihydrogenphosphate buffer (KH_2PO_4) (Fluka, Buchs, Switzerland), orthophosphoric acid (H_3PO_4) 85% (Merck) sodium carbonate monohydrate (Na_2CO_3) (Sigma, St. Louis, MO, USA) were analytical-reagent grade.

2.2. Apparatus and chromatographic conditions

A HPLC system (Thermo Separation Product: TSP) consisting of a 100-μl loop volume automatic sample-injection module (Model AS3000) set for a run time of 20 min/sample, a solvent delivery pump (Model P1000) and a UV–Vis diode-array spectrophotometer (Spectra Focus Model) set at a wavelength range from 200 to 450 nm and a spectral resolution of 2 nm was used.

The system was monitored by computer, with software (TSP PC1000) which allowed the creation of a personal library of compounds and automatic comparison of current analytical data (retention time and UV spectra) with references previously stored in a library.

Separation was achieved at room temperature using a reversed-phase C_{18} Symmetry column (250× 4.6 mm I.D.; particle size 5 μm) purchased from Waters Millipore (Milford, MA, USA).

The mobile phase consisted of 0.067 *M* KH_2PO_4 buffer (adjusted to pH 3.0 with H_3PO_4 and filtered through a 0.22-μm filter Durapore GVWP 047 purchased from Millipore, Bedford, MA, USA) and acetonitrile (65:35, v/v), then degassed ultrasonically. The flow-rate was set at 1.2 ml/min with an average operating pressure of 120 bar. At the end of each chromatographic session the column was washed with 200 ml of acetonitrile–deionized water (50:50, v/v).

2.3. Extraction procedure

In a 15-ml silicone tube (Venoject, Terumo, Belgium), 500 μl of plasma (from human heparinized blood) were alkalinized with 250 μl of 2 *M* sodium carbonate. The plasma was then extracted

with 10 ml of *n*-hexane after addition of 100 μl of the working solution (1 μg/ml) of I.S. The tube was capped, shaken horizontally for 30 min, then centrifuged for 10 min at 3000 *g*. The tube was then placed in a dry ice–acetone bath, the lower aqueous layer was frozen and the entire upper organic layer was transferred to a clean tube. A 200-μl volume of 0.03% diluted H_3PO_4 was added to the tube containing the organic layer for back extraction. Following shaking for 10 min and centrifugation (as described above), the upper organic layer was aspirated to waste. A 100-μl volume of the acidic solution was injected into the LC system.

2.4. Preparation of the calibration curve

A calibration curve based on peak-height ratio was constructed for each assay by adding known amounts of each drug to drug-free human plasma. Concentrations of each drug equivalent to 10, 20, 50, 100, 200, 300, 400 and 500 ng/ml as base were assayed. Each spiked plasma was processed as described previously.

3. Results and discussion

3.1. Quantification, separation and plasma interferences

Molecular quantification was done at three wavelengths: 226, 254 and 400 nm; 226 nm corresponding to a maximum absorption in UV for fluoxetine and its metabolite [23], 254 nm corresponding to the most common wavelength for antidepressant determination [13] and 400 nm corresponding to maximum absorption in the visible spectrum for all antidepressants identified in this method (Fig. 1). Chromatograms obtained from drug-free human plasma, spiked plasma and patient

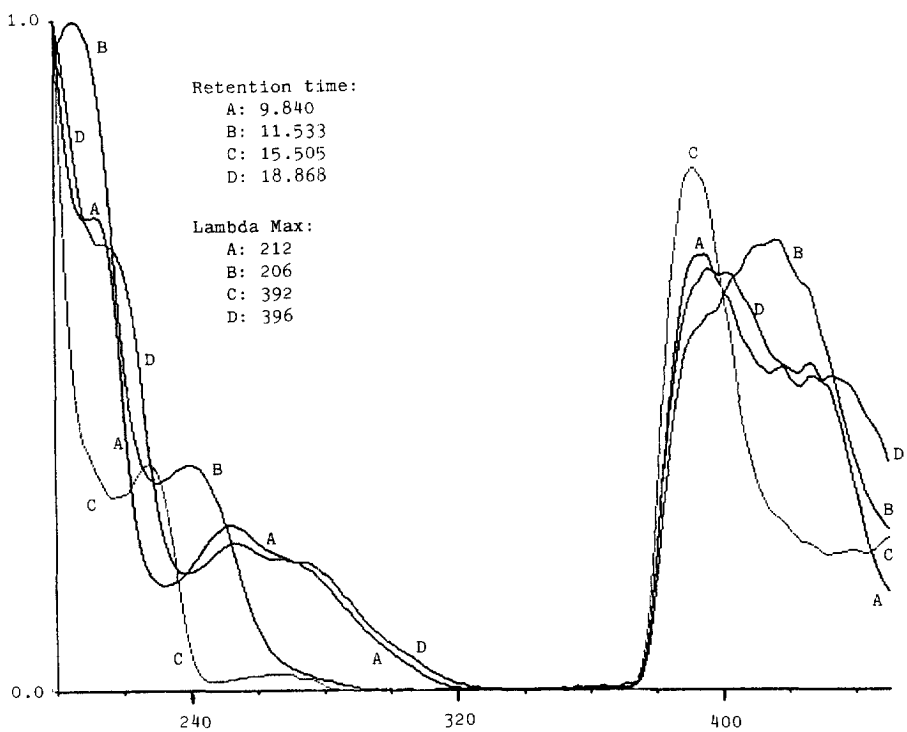

Fig. 1. Retention time and spectral data of some antidepressants in UV and visible wavelengths.

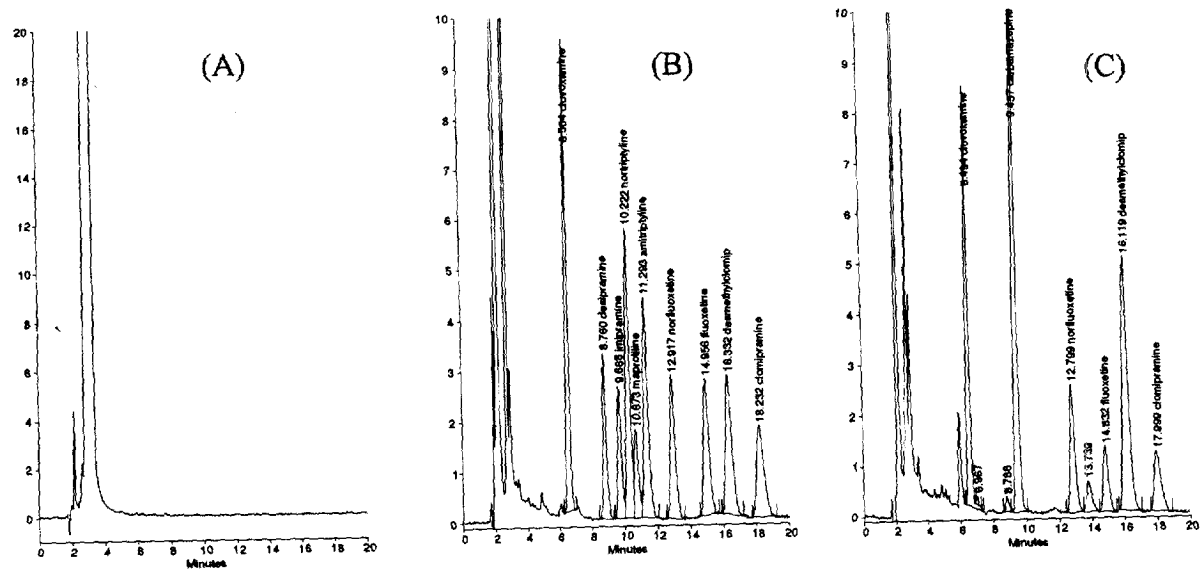

Fig. 2. Representative chromatograms at 226 nm of (A) drug-free human plasma, (B) spiked plasma with 100 ng/ml of five antidepressants with their respective metabolites, and (C) patient plasma sample with fluoxetine (46 ng/ml), norfluoxetine (81 ng/ml), clomipramine (63 ng/ml) and desmethylchlomipramine (162 ng/ml).

plasma sample receiving fluoxetine, clomipramine and carbamazepine are shown in Fig. 2. The ten peaks were adequately resolved without any interference from endogenous compounds.

The retention time and capacity factors of selected drugs checked for potential interference and which could be co-administered with antidepressants are listed in Table 1.

Table 1
Retention time (t_R) and capacity factors (k') of some compounds tested for interference

Compound	t_R (min)	k'	Compound	t_R (min)	k'
Desmethylvenlafaxine	2.73	0.27	Imipramine	9.84	0.80
Zopiclone	2.75	0.28	Fluvoxamine	10.00	0.80
Sulpiride	2.78	0.29	Nortriptyline	10.45	0.81
Viloxazine	2.80	0.29	Maprotiline	10.92	0.82
Zolpidem	3.25	0.39	Levomepromazine	11.51	0.83
Venlafaxine	3.50	0.43	Amitriptyline	11.53	0.83
Clozapine	4.45	0.55	Trimipramine	13.14	0.85
Chlordiazepoxide	4.63	0.57	Norfluoxetine	13.32	0.85
Mianserine	5.66	0.65	Lorazepam	13.70	0.86
Doxepine	6.48	0.69	Chlorpromazine	14.90	0.87
Amineptine	6.55	0.70	Clonazepam	15.37	0.87
Clovoxamine	6.66	0.70	Fluoxetine	15.50	0.87
Loxapine	7.11	0.72	Desmethylclomipramine	16.91	0.88
Haloperidol	7.22	0.73	Clomipramine	18.87	0.89
Desipramine	8.92	0.78	Clorazepate dipotassium	19.34	0.90
Desmethylmaprotiline	9.23	0.78	Flunitrazepam	24.10	0.92
Cyamemazine	9.43	0.79	Diazepam	ND[a]	–
Carbamazepine	9.46	0.79	Valproic acid	ND	–

[a] Non-detectable during the 20 min run under the described conditions.

This shows an interference between carbamazepine–imipramine, fluvoxamine–imipramine, desmethylmaprotiline–desipramine, levomepromazine–amitriptyline, norfluoxetine–trimipramine and between fluoxetine–clonazepam–chlorpromazine. In addition to the nine substances already mentioned, we can assay doxepine, trimipramine as well as fluvoxamine in the absence of imipramine. Viloxazine as well as venlafaxine and desmethylvenlafaxine, which are eluted in the solvent front, can also be assayed with a slight modification of the proportion of acetonitrile in the mobile phase.

3.2. Linearity, recovery and limit of quantification

Calibration curves were linear over the range 10–3000 ng/ml at the three wavelengths and for all molecules assayed in this method ($r>0.998$, $n=4$), except for clomipramine where the range is linear between 20 and 3000 ng/ml at 254 nm. Our method can be applied to therapeutic drug monitoring as well to overdosing since the assay is linear up to very high concentrations.

The method was found to be reproducible, as indicated by the low value obtained for the coefficient of variation (C.V.) at the three wavelengths (Table 2). The extraction recoveries (Table 2) determined by comparing peak-height ratios of the extracts with those obtained by direct injection of the same amount of drug was higher than 67% for all molecules at each concentration, except for clomipramine for which the extraction recovery is 52%, constant over the whole range of concentrations.

The metabolites generally have a higher extraction recovery than the parent compound: due to their higher polarity, they are extracted more efficiently during back extraction. In spite of the fact that extraction recovery is higher than 80% for all molecules after the first extraction with *n*-hexane without back extraction, this procedure has not been retained because of the extractum which is likely to obstruct the column and because a lower signal-to-noise ratio related to interferences with unknown endogenous compounds occurred.

The limit of quantification was estimated under the described conditions at a signal-to-noise ratio of 3 and with a C.V. lower than 20% for the precision and accuracy. At wavelengths of 226 nm and 400 nm, the limit of quantification was 5 ng/ml for desipramine, imipramine, nortriptyline, amitriptyline, maprotiline, fluoxetine, norfluoxetine, desmethylclomipramine and 7 ng/ml for clomipramine. At a wavelength of 254 nm, the limit of quantification was 7 ng/ml for desipramine, imipramine, amitriptyline, nortriptyline, desmethylclomipramine and 10 ng/ml for clomipramine. At a wavelength of 254 nm the sensitivity is poor compared with that at 226 and 400 nm and does not allow quantification of maprotiline, fluoxetine and norfluoxetine. Although it is frequently claimed that diode-array detectors have lower sensitivity than UV spectrophotometers, our quantification limits were found to be adequate for identification of all drugs tested under steady-state therapeutic blood levels.

3.3. Stability

All compounds tested were stable in human plasma controls (50, 250 and 500 ng/ml) for more than 3 months at −22°C. Spiked plasma samples extracted following the described procedure then stored at 4°C remain stable in the injection solvent for at least 24 h without significant degradation.

3.4. Spectral analysis

In addition to the molecular quantification at the three wavelengths, the molecular spectra corresponding to retention times of the molecule to be dosed is compared to the reference one previously stored in the library. A good correlation is defined as a value higher than 950 (scale: 0–1000).

The molecular spectra and the retention time of the compounds identified in the patient (Fig. 2) were comparable to the reference.

A value lower than 950 indicates a spectra mixture, composed of the molecules to be dosed and the spectrum of an endogenous or exogenous compound (other drugs: see Table 1) which suggest an analytical interference.

Unlike DAD, which allows a spectral comparison in addition to the quantification at one or more wavelength, the use of a UV spectrophotometer does not identify any analytical interference.

The use of a DAD improves the specificity since it makes it possible to evaluate the purity of a peak.

Table 2
Accuracy, precision and recovery data

Drug	Recovery (%)	Precision ($n=10$)					
		Within-assay Mean (c.v.%)			Day-to-day Mean (c.v.%)		
		226 nm	254 nm	400 nm	226 nm	254 nm	400 nm
20 ng/ml							
Desipramine	90	21.1 (5.5)	20.8 (4.4)	21.4 (4.2)	21.7 (4.8)	21.1 (5.1)	20.3 (6.2)
Imipramine	77	21.0 (8.3)	22.0 (12.0)	22.2 (5.0)	20.7 (6.2)	20.8 (4.3)	21.0 (5.1)
Nortriptyline	90	20.7 (4.7)	21.0 (5.9)	21.5 (4.3)	21.1 (5.2)	21.2 (4.7)	21.2 (4.7)
Maprotiline	81	21.0 (8.7)	–	20.8 (6.2)	21.3 (4.1)	–	20.9 (5.3)
Amitriptyline	70	20.4 (9.0)	21.5 (7.8)	21.5 (7.9)	20.1 (7.9)	20.4 (4.2)	21.1 (6.3)
Norfluoxetine	78	22.1 (6.7)	–	23.0 (5.9)	21.9 (7.2)	–	21.7 (6.9)
Fluoxetine	84	20.8 (8.0)	–	22.1 (7.3)	20.0 (6.1)	–	21.6 (7.2)
Desmethylclomipramine	87	20.2 (9.5)	20.1 (9.4)	21.2 (7.7)	20.7 (6.2)	19.7 (5.7)	20.7 (6.8)
Clomipramine	55	20.9 (8.0)	20.5 (13.0)	21.3 (7.5)	20.2 (3.7)	20.1 (6.2)	21.1 (6.3)
100 ng/ml							
Desipramine	85	101.5 (2.3)	101.1 (1.7)	102.1 (1.9)	96.4 (3.7)	95.1 (1.7)	104.7 (4.2)
Imipramine	71	104.1 (3.9)	103.2 (3.8)	104.1 (3.4)	103.2 (4.1)	100.6 (2.1)	103.2 (5.1)
Nortriptyline	88	100.3 (2.7)	100.4 (2.9)	101.3 (3.0)	101.2 (3.1)	93.6 (2.3)	101.7 (2.7)
Maprotiline	78	99.5 (1.9)	–	99.8 (2.5)	98.7 (5.7)	–	96.9 (3.1)
Amitriptyline	68	100.7 (6.5)	99.3 (4.2)	99.4 (3.9)	101.1 (7.0)	94.2 (4.7)	100.3 (2.9)
Norfluoxetine	75	109.0 (7.1)	–	99.8 (2.7)	107.0 (6.2)	–	97.2 (2.3)
Fluoxetine	80	108.0 (5.4)	–	98.2 (2.9)	106.2 (5.5)	–	99.7 (3.1)
Desmethylclomipramine	87	101.5 (3.3)	100.7 (3.8)	100.6 (4.6)	102.7 (4.1)	102.1 (5.1)	100.9 (4.7)
Clomipramine	52	96.9 (5.2)	101.8 (5.7)	98.2 (5.0)	99.2 (5.7)	98.5 (4.8)	99.1 (4.2)
500 ng/ml							
Desipramine	86	497.5 (0.9)	497.0 (1.0)	496.0 (1.0)	475.8 (1.7)	474.8 (0.7)	474.5 (0.6)
Imipramine	71	502.7 (0.5)	503.0 (0.6)	501.0 (0.7)	513.3 (2.1)	502.8 (3.8)	503.6 (3.7)
Nortriptyline	86	495.8 (1.2)	495.1 (0.9)	493.6 (1.2)	486.0 (2.2)	484.3 (1.3)	484.5 (1.2)
Maprotiline	78	503.7 (1.3)	–	502.4 (1.2)	501.4 (1.9)	–	482.8 (1.1)
Amitriptyline	67	498.0 (0.9)	486.9 (0.6)	495.5 (0.8)	460.8 (1.8)	458.7 (1.5)	452.8 (1.2)
Norfluoxetine	75	512.6 (6.9)	–	499.7 (2.8)	451.3 (2.2)	–	465.1 (1.9)
Fluoxetine	80	509.4(2.2)	–	508.2 (2.1)	452.0 (2.3)	–	460.2 (2.3)
Desmethylclomipramine	85	504.5 (1.5)	503.9 (1.8)	502.4 (1.6)	496.8 (1.7)	472.7 (2.8)	473.8 (2.4)
Clomipramine	52	496.9 (1.5)	494.3 (1.8)	496.6 (1.4)	520.2 (2.1)	510.1 (1.7)	495.2 (1.4)

Spectral analysis is of particular interest for plasma samples from patients receiving (in addition to their usual antidepressant treatment) medication belonging to the neuroleptic and/or benzodiazepine category likely to create exogenous interferences (see Table 1) during assay [12,13,23].

Spectral analysis is also useful for plasma samples from patients with renal or hepatic insufficiency as their plasma often contains endogenous molecules likely to interfere with the assay.

Tracqui et al. [20] show a HPLC–DAD screening of 25 antidepressants. However, the plasma sample volume of 2 ml is too large in drug monitoring; we propose a minimal plasma sample of 500 μl with an comparable limit of detection; furthermore their resolution is rather poor and the 25 antidepressants cannot be assayed simultaneously. In the method proposed here the commonly prescribed antidepressants can be assayed. Their UV spectra are 'flat', starting from 340 nm to 400 nm, whatever the antidepressant. However, it should be pointed out that spectra in the visible region are more intense than those in the middle UV region, common to all antidepressants and set at 400 nm wavelength, which greatly improves sensitivity and allows a more detailed spectral analysis.

El-Yazigi et al. [12] describe the simultaneous assay of fluoxetine, amitriptyline, imipramine and their respective metabolites at 220 nm, but their run time is 35 min vs. 20 min. Furthermore clomipramine has not been assayed since the retention time is very close to that of imipramine. This point is actually a major problem as clomipramine is one of the most prescribed antidepressants in Europe (as well as fluoxetine). With DAD, our limit of quantification is comparable to theirs, using the same volume of plasma. The use of doxepine as I.S. could be a problem for those patients receiving both drugs.

4. Conclusion

The assay described here is one of the first assays that simultaneously measures fluoxetine, TCAs and their active metabolites in plasma with DAD detection. This method is simple, rapid, highly specific and currently being used for drug monitoring and toxicological studies in children and adult patients.

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