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Reliable HPLC method for therapeutic drug monitoring of frequently prescribed tricyclic and nontricyclic antidepressants

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Abstract

A new high-performance liquid chromatography method is presented for the determination of 10 frequently prescribed tricyclic and nontricyclic antidepressants: imipramine, amitriptyline, clomipramine, fluoxetine, sertraline, paroxetine, citalopram, mirtazapine, moclobemide and duloxetine. The simple and accurate sample preparation step, consisted of liquid:liquid extraction with recoveries ranging between 72% and 86%, except for moclobemide (59%). Separation was obtained using a reverse phase Select B column under isocratic conditions with UV detection (230 nm). The mobile phase consisted of 35% of a mixture of acetonitrile/methanol (92:8, v/v) and 65% of 0.25 mol L^{-1} sodium acetate buffer, pH 4.5. The standard curves were linear over a working range of 2.5–1000 ng mL^{-1} for moclobemide, 5–2000 ng mL^{-1} for citalopram, duloxetine, fluoxetine, 10–2000 ng mL^{-1} for sertraline, imipramine, paroxetine, mirtazapine and clomipramine. The intra-assay and inter-assay precision and accuracy were studied at three concentrations (50, 200, and 500 ng mL^{-1}). The intra-assay coefficients of variation (CVs) for all compounds were less than 8.8%, and all inter-CVs were less than 10%. Limits of quantification were 2.5 ng mL^{-1} for moclobemide, 5 ng mL^{-1} for citalopram, duloxetine and amitriptyline, and 10 ng mL^{-1} for mirtazapine, paroxetine, imipramine, fluoxetine, sertraline, and clomipramine. No interference of the drugs normally associated with antidepressants was observed. The method has been successfully applied to the analysis of real samples, for the drug monitoring of ten frequently prescribed tricyclic and non-tricyclic antidepressant drugs.

Keywords: Therapeutic drug monitoring; Tricyclic; Nontricyclic antidepressants; HPLC UV detection

1. Introduction

Therapeutic drug monitoring (TDM) of antidepressants is necessary for an optimal supervision of patient drug regimen to avoid medical complications, intoxication, nonresponsiveness or noncompliance [1,2]. Pharmacological treatment for depression has advanced greatly since the development of the first therapies in the 1950s, with the introduction of monoamine oxidase inhibitors and tricyclic antidepressants (TCAs) [3]. Since the late 1980s, a whole new generation of chemically and neuropharmacologically unrelated agents has been introduced. These drugs appear to be safer and better tolerated [4,5] and include: selective serotonin reuptake inhibitors (SSRIs) [6–9], noradrenergic and specific serotonergic antidepressants [10,11], reversive and selective monoamino oxidase inhibitor [12], and a potent and balanced inhibitor of both serotonin and norepinephrine reuptake [13].

Several methods have been published for the determination of one or more antidepressants in biological fluids for therapeutic monitoring or in toxicologic purposes. These reports described the use of gas chromatography (GC) coupled with

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nitrogen–phosphorus [14,15] and mass spectrometry detection [16,17]. Liquid:liquid or solid-phase extraction have been used for sample clean-up followed, in some cases, by a derivatization step. The application of high-performance liquid chromatography (HPLC) to antidepressants quantification was first reported in 1975. The advantages of HPLC for antidepressants analysis are its versatility and simplicity of sample preparation, as well as a wide range linearity in detectors, thus making HPLC the method of choice for TDM of antidepressants [18–31]. Determination of one or more of these drugs has been described using HPLC with fluorescence or UV detection [18–27]. Because nontricyclic antidepressants drugs differ widely in their chemical

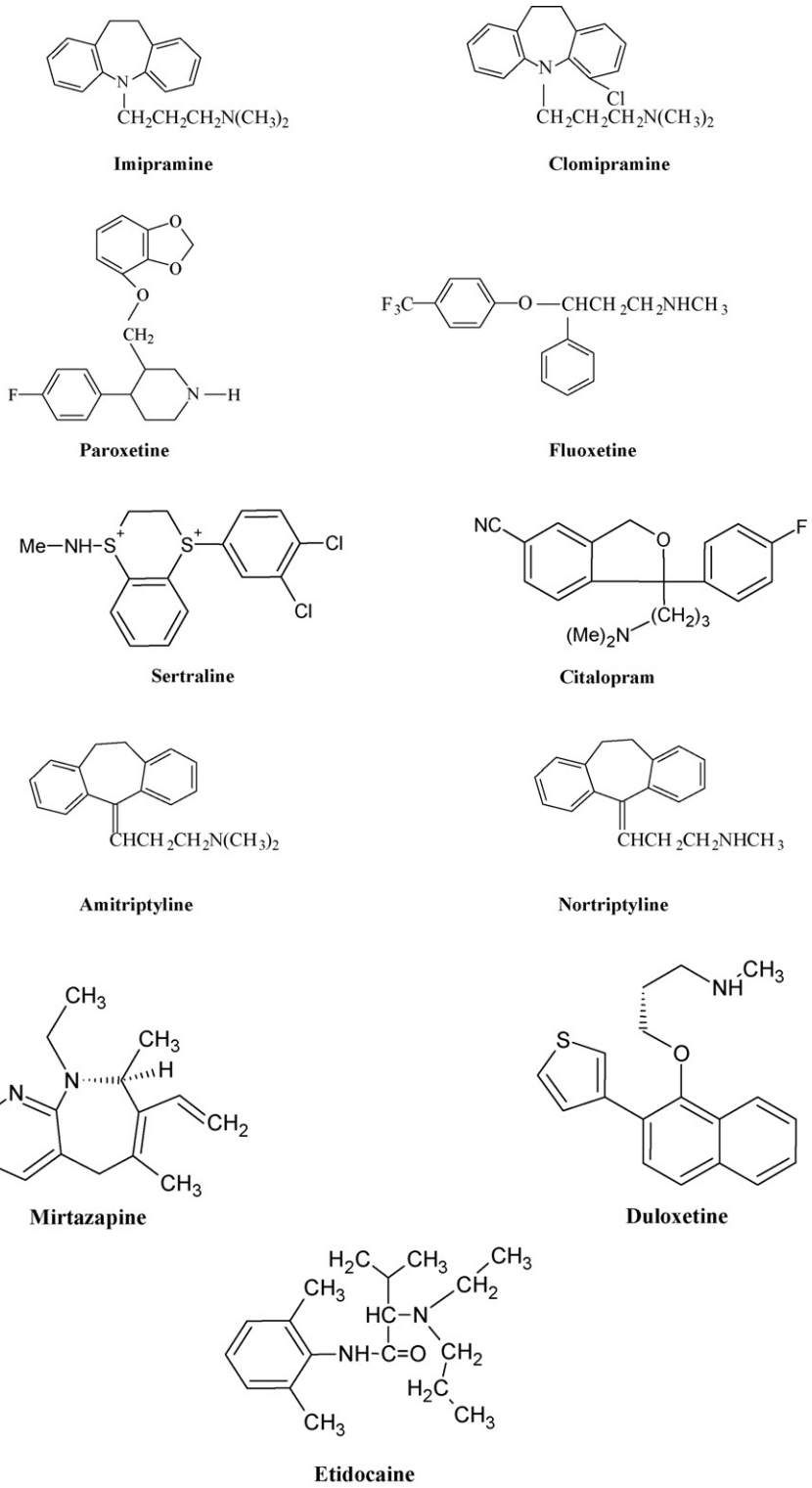

Fig. 1. Chemical structures of the antidepressants.

structure, analytic methods for their quantitative determination in biologic matrices have been developed for each drug individually [17–22]. Furthermore, methods have been reported allowing the simultaneous determination of tricyclic [23] and nontricyclic [24–27] antidepressants drugs. More recently multi-drug methods for screening or quantification of antidepressants have been described, but several of these methods use solid-phase extraction [28] and photodiode-array UV detection [25,26] or LC/MS with ionic spray ionization [29] or LC–MS (MS) [30,31]. These methods provide a high selectivity and sensitivity in combination with a good precision and accuracy over a wide dynamic range, allowing the development of very rapid and efficient analytical methods. Therefore, these assays, especially the newer ones [30,31], are costly and not widely available. The recent trend in TDM or quick analysis of intoxication aims to develop accurate, simple, rapid and low cost methods to determine several drugs simultaneously. Duloxetine has recently proved effective and well-tolerated in the treatment of major depressive disorder (MDD) [32,33], painful neuropathies [34] and in urinary stress incontinence [35], so it is important to have a sensitive method for TDM especially in older patients. Thus we designed a method using HPLC–UV for simultaneous determination of the 10 main antidepressants prescribed in Brazil: amitriptyline, imipramine, clomipramine fluoxetine, sertraline, paroxetine, citalopram, mirtazapine, moclobemide, duloxetine, using etidocaine as the internal standard (Fig. 1). The assay described here requires liquid:liquid extraction, is sensitive, selective and fully validated.

2. Experimental

2.1. Standards and reagents

Certified standards of duloxetine, clomipramine, paroxetine, mirtazapine, fluoxetine, norfluoxetine, didesmethylcitalopram, desmethylcitalopram were purchase from Pharmacopéia® C.I.L. (São Paulo, Brazil), sertraline, imipramine (Ciba-Geigy, Brazil), amitriptyline, citalopram (Sigma, St. Louis, MO, USA), moclobemide (Roche, Brazil) and the internal standard etidocaine (Galena, Brazil). Methanol, HPLC grade, was obtained from J.T. Baker (Phillipsburg, USA), acetonitrile, hexane and isoamyl alcohol, HPLC grade were purchased from Merck (Darmstadt, Germany). The reagents used for drug extraction were analytical grade and were purchased from Merck (Darmstadt, Germany). Water was deionised and filtered with a Milli-Q water processing system.

2.2. Chromatographic system

The analysis was performed on an HPLC system consisting of a Shimadzu Model LC 10 AD pump, a Shimadzu Model SPD 10 A ultraviolet detector, a chromatopac C-R6A integrator (Shimadzu) and a Rheodyne injector with a 100 μL loop. Chromatographic separation was achieved isocratically, at room temperature, on a LiChrospher 60 RP-select B column (250 mm × 4 mm, 5 μm particle size (Merck)). The mobile phase consisted of 35% a mixture of acetonitrile: methanol (92:8, v/v) and 65% of 0.25 M L^{-1} sodium acetate buffer, pH 4.5. Flow-rate was 1.0 mL min^{-1}. The ultraviolet detector was set at 230 nm.

2.3. Calibration curve

Separated stock solutions of each antidepressant were prepared by dissolving accurately weighed amounts of each reference compound in methanol to yield a 1 mg mL^{-1} drug concentration. A 1 mg mL^{-1} stock solution of etidocaine, internal standard (IS), in methanol was prepared and further diluted 1/50 in methanol to give a 20 μg mL^{-1} working solution. All stock solutions were stored at −20 °C and were stable for 6 months.

Routine daily calibration curves were prepared by the addition of 25 μL of each standard solution at concentrations of 0.1, 0.2, 0.4, 2.0, 4.0, 8.0, 20.0, 40.0 μg mL^{-1} of methanol to 1 mL of blank plasma (plasma from a patient not exposed to any drug for at least 2 months) aliquots, resulting in plasma drugs concentrations of 2.5, 5, 10, 50, 100, 200, 500, and 1000 ng mL^{-1}. Each spiked plasma sample was processed as described in sample preparation.

2.4. Sample preparation

The extraction consisted of the addition of 25 μL of etidocaine (IS), 200 mg NaCl, 50 μL of sodium hydroxide 1.5 M, and 5 mL hexane-isoamyl alcohol (99:1, v/v) to 1 mL of plasma. After being shaken in a vortex-type shaker for 1 min and centrifuged at 1800 × *g* for 5 min, an aliquot (4.4 mL) of the organic phase was transferred to conic tubes and evaporated under a constant air flow at room temperature. The mixture was reconstituted in 150 μL of the mobile phase and 100 μL of hexane. After shaking for 10 s in a vortex-type shaker, 100 μL of the mobile phase was chromatographed.

2.5. Validation of the method

The recovery of the drugs was determined at three different concentrations in blank plasma. Plasma samples with the drugs were extracted in triplicated according to the procedure proposed. The concentrations of these samples were calculated on the basis of calibration curves constructed from the data for the drugs not submitted to the extraction procedure.

To determine the intra-assay precision, aliquots ($n = 10$) of blank plasma containing the standards solution of the drugs at three concentrations were analysed by the method proposed. To determine the inter-assay precision, blank plasma samples containing the standard solution at the same concentrations were analysed on 10 consecutive days. Linearity was obtained by analysing blank plasma samples ($n = 3$) containing standard solutions of drugs at concentrations of 20–2000 ng mL^{-1}. The concentration range was estimated on the basis of the regression curve ($y = ax + b$) and correlation coefficient (r^2).

The limit of quantification was determined by analysing blank plasma samples enriched with decreasing concentrations of all

drug standard solutions. The limit of quantification was considered, the lowest concentration quantified with a coefficient of variation less than 10%, obtained for five determinations. The selectivity of the method was evaluated by analysing several drugs normally combined with the antidepressants. The drugs that presented retention times close to those of the drugs under study were submitted to the extraction procedure and rechromatographed.

2.6. Blood samples

Plasma samples from patients submitted to treatment with antidepressants were collected after filling out a protocol containing the patient's name, sex, age, weight, prescribed medication, dose and combined medications. Blood samples from patients in steady-state plasma concentrations of antidepressants were obtained in the morning with heparin (Liquemine®) immediately before drug administration. After centrifugation, plasma samples were stored at −20 °C until analysis. Stability studies carried out directly on plasma indicated that samples were stable for at least 3 months when stored at −20 °C.

Pooled blank plasma samples used for development and validation of the procedure were obtained from a local blood bank.

The principles embodied in the Helsinki Declaration were adhered to, and the Ethics Committees at the University of São Paulo in Ribeirão Preto, Brazil, approved the study.

3. Results and discussion

Because the majority of patients attending the Research Center for Mood Disorder at our hospital are treated with these 10 antidepressants, their plasma levels were determined in the framework of a therapeutic drug monitoring study.

The procedure yielded excellent separation and symmetrical peaks for each antidepressants. Representative chromatograms of blank plasma and plasma spiked with 100 ng mL^{-1} of each antidepressant drug are presented in Fig. 2A and B, respectively. Under the described chromatographic conditions, retention times were 4.3 min (moclobemide), 7.3 min (internal standard), 9.6 min (mirtazapine), 11.3 min (citalopram), 13.0 min (paroxetine), 16.0 min (duloxetine), 18.0 min (imipramine), 19.8 min (amitriptyline), 22.3 min (fluoxetine), 23.2 min (sertraline), and 27.6 min (clomipramine). The complete elution was obtained in less than 30 min.

Calibration curves were linear in the range of 2.5–1000 ng mL^{-1} for moclobemide, 5–2000 ng mL^{-1} for imipramine, paroxetine, mirtazapine, citalopram, duloxetine, amitriptyline, 10–2000 ng mL^{-1} for fluoxetine, sertraline and clomipramine, all of them with correlation coefficients $r^2 > 0.997$. Limits of quantification ranged from 2.5 to 10 ng mL^{-1} (Table 1). The precision and accuracy of the assay are summarized in Table 2. The inter-assay coefficients of variation (CVs) for all compounds were less than 8.5%, and all intra-assay CVs were less than 8.8%. The intra-assay and inter-assay accuracies for all compounds were found to be within 92.6% and 101.8% at 50 ng mL^{-1} and within 98.4% and 99.7% at 500 ng mL^{-1}.

Limits of quantification (LOQs) (Table 1) represent an improvement with respect to previous methods in which LOQs ranged from 10 to 50 ng mL^{-1} [22,24,26,27]. Few of them were more sensitive than the present method, but they did not include

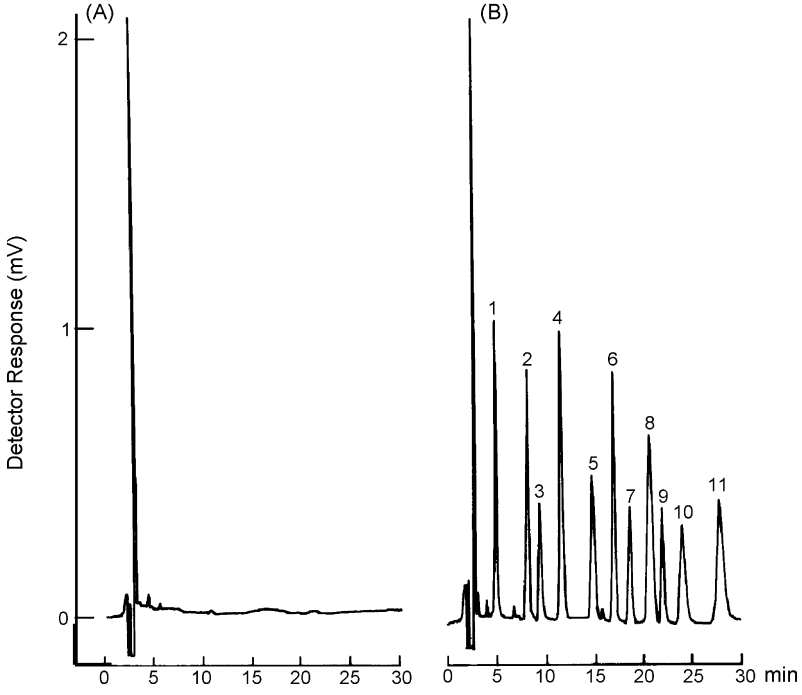

Fig. 2. (A) Chromatogram obtained from a blank (without internal standard). (B) Chromatogram obtained from a plasma spiked with 200 ng mL^{-1}: (1) moclobemide, (2) internal standard (etidocaine), (3) mirtazapine, (4) citalopram, (5) paroxetine, (6) duloxetine, (7) imipramine, (8) amitriptyline, (9) fluoxetine, (10) sertraline, and (11) clomipramine.

Table 1
Linearity, limit of quantification and recovery of the antidepressants in plasma samples

Drugs concentrations range	Linear regression, r^2	Limit of quantification (ng mL^{-1})	Recovery	
			Evaluated concentration (ng mL^{-1})	Results (%) ($n=5$)
Moclobemide (2.5–1000 ng mL^{-1})	$y=0.06057x+0.00335$ **0.999**	2.5	400 50 2.5	58.2 59.0 55.0
Mirtazapine (10–2000 ng mL^{-1})	$y=-0.00236x+0.00108$ **0.998**	10	500 50 10	83.7 85.9 83.2
Citalopram (5–2000 ng mL^{-1})	$y=0.00764x+0.00283$ **0.999**	5	500 50 5	76.3 75.6 74.9
Paroxetine (10–2000 ng mL^{-1})	$y=0.02585x+0.00115$ **0.999**	10	500 50 10	75.9 75.6 73.3
Duloxetine (5–2000 ng mL^{-1})	$y=0.02774x+0.00233$ **0.998**	5	500 50 5	75.2 74.6 73.3
Imipramine (10–2000 ng mL^{-1})	$y=0.04896x+0.00107$ **0.999**	10	500 50 10	81.9 81.2 80.8
Amitriptyline (5–2000 ng mL^{-1})	$y=0.105x+0.00148$ **0.999**	5	500 50 5	86.3 85.2 84.8
Fluoxetine (10–2000 ng mL^{-1})	$y=0.03189x+0.00109$ **0.999**	10	500 50 10	73.6 72.9 72.2
Sertraline (10–2000 ng mL^{-1})	$y=0.0187x+0.00078$ **0.999**	10	500 100 10	80.1 79.9 79.2
Clomipramine (10–2000 ng mL^{-1})	$y=0.03943x+0.000933$ **0.999**	10	500 100 10	83.1 82.8 82.3

duloxetine [32–35] and they used HPLC with photodiode-array UV detection [25] and HPLC/MS [28–30].

Liquid:liquid extraction was used because of its high efficiency, selectivity, and simplicity. Despite differences in chemical structure among the ten compounds, the extraction recoveries were satisfactory for nine of them (Table 1). Moclobemide showed a low but acceptable extraction recovery (59%) due to the extraction procedure used, which was first developed for SSRIs. The polarity of the extraction solvent, i.e., the proportion of isoamyl alcohol in the mixture with hexane, has been shown to be the most important variable influencing extraction. We preferred hexane: isoamyl alcohol (99:1, v/v), a procedure guaranteeing high recoveries (72–86%) and ensuring quantification limits of 2.5–10 ng mL^{-1}, including moclobemide (2.5 ng mL^{-1}). Analysis by HPLC necessarily involves one or more steps of biological sample preparation. Therefore, the organic phase was evaporated under an air flow, and the residue was reconstituted with 150 μL of the mobile phase and 100 μL of hexane after extraction. This procedure was used to purify the biological sample and was based on the differential partition between the mobile phase and *n*-hexane. This is a simple procedure for the purification of biological samples that is more readily reproducible by various technicians in the laboratory [36].

After the extraction procedure there was no interference of the drugs normally associated with antidepressants in psychiatric patients: haloperidol, chropromazine, risperidone and several benzodiazepines (diazepam, lorazepam, flurazepam, triazolam, clonazepam and alprazolam). The same was true for methyldopa, captopril, phenobarbital, primidone, furosemide, hydrochlorothiazide, acetaminophen, caffeine, salicylic acid, cimetidine, ranitidine and prednisone that were evaluated for possible interference with the antidepressants. Among tested drugs diazepam was the only compound co-extracted and showed the peak around the retention time (19.6 min). However, we could separate the peaks of amitriptyline and diazepam when we used a different mobile phase, 35% of acetonitrile–methanol (87:13, v/v) and 65% of 0.25 mol L^{-1} sodium acetate buffer at pH 4.5.

When the method is applied in TDM of patients in therapy with Celexa®(citalopram) (Fig. 3A), Paxil® (paroxetine) (Fig. 3B), Prozac® (fluoxetine) (Fig. 3C) and Amitryl®

Table 2
Intra- and inter-day precision and accuracy of the antidepressants in plasma samples

Drugs (ng mL^{-1})	Precision intra-assay CV (%), $n = 10$	Precision inter-assay CV (%), $n = 5$	Accuracy error (%)
Moclobemide			
500	6.3	6.5	0.4
200	5.2	6.8	1.0
50	7.6	6.3	0.2
Mirtazapine			
500	1.7	2.8	1.3
200	4.8	8.5	3.7
50	8.8	8.0	2.2
Citalopram			
500	2.4	4.5	1.2
200	4.7	7.1	6.6
50	8.5	8.4	7.2
Paroxetine			
500	1.9	7.2	1.3
200	5.9	5.0	6.5
50	8.3	3.7	9.4
Duloxetine			
500	2.4	6.8	1.7
200	6.1	5.3	6.5
50	8.0	5.1	6.8
Imipramine			
500	2.6	6.1	1.7
200	6.0	5.3	5.8
50	7.8	5.3	2.4
Amitriptyline			
500	2.4	5.3	1.6
200	5.7	7.5	8.0
50	6.9	6.1	4.2
Fluoxetine			
500	2.7	5.6	1.5
200	6.2	7.8	6.9
50	8.3	7.2	2.6
Sertraline			
500	2.7	5.9	1.6
200	5.1	6.8	8.3
50	7.7	6.8	6.2
Clomipramine			
500	2.1	6.0	0.7
200	5.4	7.1	7.9
50	8.5	8.1	5.4

CV: coefficient of variation.

(amitriptyline) (Fig. 3D), UV detection at 230 nm disclosed the drug metabolites in the chromatograms. TDM should also determine, especially for TCAs like whose metabolite nortriptyline is active and has an established therapeutic-range (Fig. 3D).

Sertraline was the drug most prescribed for elderly patients in our hospital. We also noted that amitriptyline is used in the treatment of depression and is administered in small doses (25 mg/day) in fibromyalgia or painful neuropathies (Table 3). A significant variability of drug concentrations in plasma was observed among the depressed patients (Table 4). This can partly be explained by the polymorphism in the genes encoding some of the CYP450 isoenzymes implicated in their metabolization. These genetic differences have been well characterized mainly in the case of CYP2D6 and CYP2C19, and this study implemented the recommendations on individual dose adjustment [37]. Many commonly used drugs are potent inhibitors of CYP2D6, including certain antiarrhythmics, neuroleptics, and SSRIs (paroxetine and fluoxetine). Thus CPY2D6 is the source of clinically important drug–drug interactions resulting from the combined intake of a substance and an inhibitor. A neuroleptic in combination with an SSRI or with a tricyclic antidepressants represent clinically important examples of CYP2D6 interactions. Dose reduction by TDM is often necessary. This highlights the importance of developing a simple, accurate and cost-effective method for the quantitative determination of such compounds.

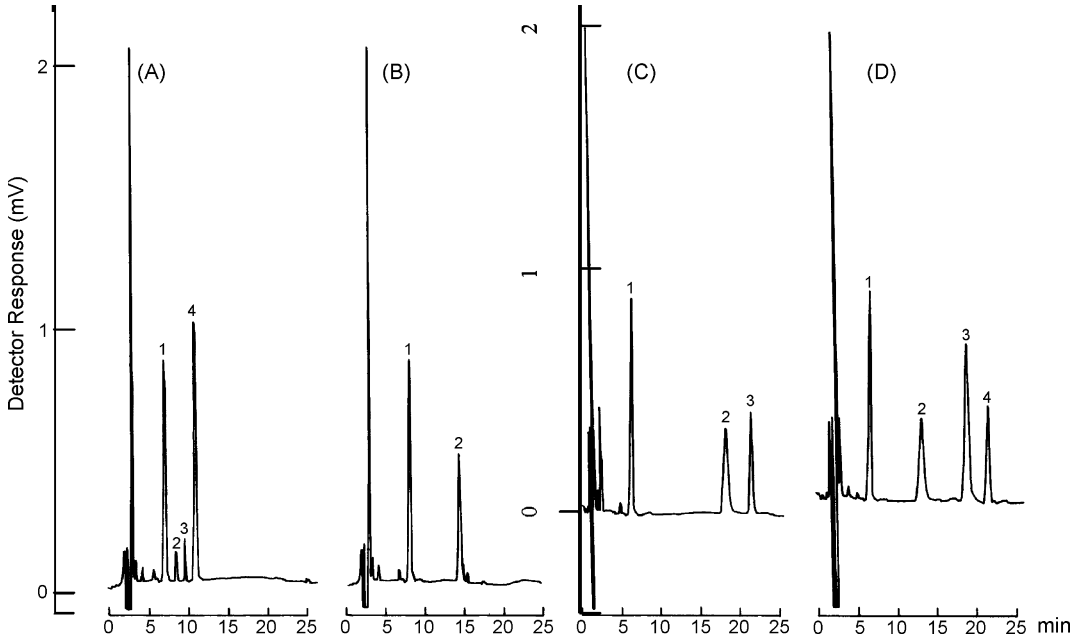

Fig. 3. Clinical plasma samples containing: (A) (1) IS (etidocaine), (2) didesmethylcitalopram, (3) desmethylcitalopram, and (4) Citalopram. (B) (1) IS (etidocaine) and (2) paroxetine. (C) (1) IS (etidocaine), (2) norfluoxetine, and (3) fluoxetine. (D) (1) IS (etidocaine), (2) nortriptyline, (3) amitriptyline, and (4) diazepam.

Table 3
Clinical characteristics of samples

Characteristic	Treatment conditions				
	Sertraline ($n=45$)	Fluoxetine ($n=42$)	TCA ($n=20$)	Amitriptyline ($n=35$)	Paroxetine ($n=10$)
Age, years	74 ± 3.6	42 ± 10	65 ± 10	40 ± 20	30 ± 10
Sex, M/W	8/27	2/40	45/55	16/4	2/8
Race, white/black	43/2	38/4	58/12	18/2	10/0
MDD	19	4	70	–	–
Depressive symptoms	25	38	–	20	10
Fibromyalgia	–	–	–	20	–
Dose (mg/day)	75 ± 25	30 ± 10	200 ± 50	25	40

MDD: major depressive disorder; TCA: tricyclic antidepressants; *n*: number of patients.

Table 4
TDM of patients receiving antidepressants pharmacotherapy

Drug	Dose (mg/day)	*n*	Plasma concentration (ng mL^{-1})		CV (%)
			Parent drug	Metabolite	
Amitriptyline	25	13	38.6 ± 15.4	31.8 ± 11.1	53.3; 34.6
	50	15	55.8 ± 23.4	32.8 ± 12.5	41.9; 38.5
	75	7	66.8 ± 18.3	22.4 ± 11.6	27.4; 51.9
	100	1	512.0		–
Imipramine	25	5	36.5 ± 10.38	20.4 ± 3.8	28.4; 18.8
	50	6	48.8 ± 17.66	46.7 ± 23.8	36.6; 50.1
	75	10	140.2 ± 103.3	63.1 ± 46.9	73.7; 74.1
	100	1	111.0		
	250	25	194.5 ± 114.7	213.72 ± 196.3	58.8; 92.2
Nortriptyline	20	1	31.3		–
	25	1	34.3		–
	50	2	147.0 ± 79.2		53.9
Fluoxetine	20	20	49.8 ± 18.7	42.7 ± 17.6	37.5; 41.1
Paroxetine	20	10	159 ± 103		56.8

TDM: therapeutic drug monitoring; *n*: number of patients; CV: coefficient of variation.

4. Conclusions

The HPLC method described herein allows the simultaneous detection and quantification of ten frequently prescribed tricyclic and nontricyclic antidepressant drugs including duloxetine. The method was validated, and successfully applied to the analysis of real samples from depressed patients. The limit of quantification, and the good selectivity make this rapid and feasible method suitable for routine therapeutic drug monitoring.

The usefulness of TDM to optimize drug management the pharmacotherapy was strongly supported by the significant variability of plasma drug concentration among the depressed patients.

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