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HPLC analysis of the novel antidepressant duloxetine in human plasma after an original solid-phase extraction procedure

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Abstract

Duloxetine is the most recent serotonin and norepinephrine reuptake inhibitor (SNRI) drug introduced for the therapy of depression. Thus, it is evident that there is a need for having on hand new reliable analytical methods for the determination of duloxetine plasma levels in depressed patients. The present paper deals with the development of a rapid and sensitive high-performance liquid chromatographic method for duloxetine analysis in human plasma. The assays were carried out using a C8 reversed-phase column and a mobile phase composed of 60% aqueous phosphate buffer containing triethylamine at pH 3.0 and 40% acetonitrile. The UV detector was set at 230 nm and loxapine was used as the internal standard. An original pre-treatment of plasma samples was developed, based on solid-phase extraction (SPE) with mixed-mode reversed phase—strong cation exchange cartridges (30 mg, 1 mL). The extraction yields values were higher than 90%. Linearity was found in the 2–200 ng mL^{-1} duloxetine concentration range; the limit of quantitation was 2.0 ng mL^{-1} and the limit of detection was 0.7 ng mL^{-1}. The method was applied to plasma samples from depressed patients undergoing therapy with duloxetine. Precision data and accuracy results were satisfactory and no interference from other drugs was found. Thus, the method seems to be suitable for the therapeutic drug monitoring of duloxetine in depressed patients' plasma.

Keywords: Duloxetine; Antidepressant drug; High-performance liquid chromatography; Human plasma; Solid-phase extraction

1. Introduction

Duloxetine ((γ*S*)-*N*-methyl-γ-(1-naphthalenyloxy)-2-thiophenepropanamine, DLX, Fig. 1) is one of the most recent antidepressants introduced onto the market. It is used in the treatment of major depression [1], with or without melancholic symptoms [2], of diabetic peripheral neuropathic pain [3] and of stress urinary incontinence [4]. Like venlafaxine and milnacipran, it acts as a dual serotonin and norepinephrine reuptake inhibitor (SNRI) [5], with approximately equal potency at both transporters; DLX seems to be very efficient and to have a fast onset of action [6]. DLX has low affinity toward serotonergic, cholinergic, adrenergic and histamine receptors and this specificity of action accounts for its superior safety profile with respect to tricyclic antidepressants [7,8].

DLX (Cymbalta®, Xeristar®, Yentreve®, Ariclaim®) is administered as capsules containing 20, 30 or 60 mg of active principle in enteric-coated pellets. The most common doses for the treatment of major depression are 40–60 mg day^{-1} [9], with a maximum suggested dose of 120 mg day^{-1} [10,11]. After oral administration, maximum plasma levels (C_{max}) are reached at a median of 6 h and the drug elimination half-life is about 12 h [12].

DLX is mainly metabolised in the liver by the cytochrome P450 system (CYP) [13]. Some metabolites seem to possess a reuptake inhibition activity in vitro, however their activity in vivo and their therapeutic significance are still unclear [14]. Even though the side effect profile of DLX is certainly more benign than that of traditional antidepressants, DLX can cause several side effects, which in some cases can lead to discontinuation of therapy. The most common side effects associated with DLX

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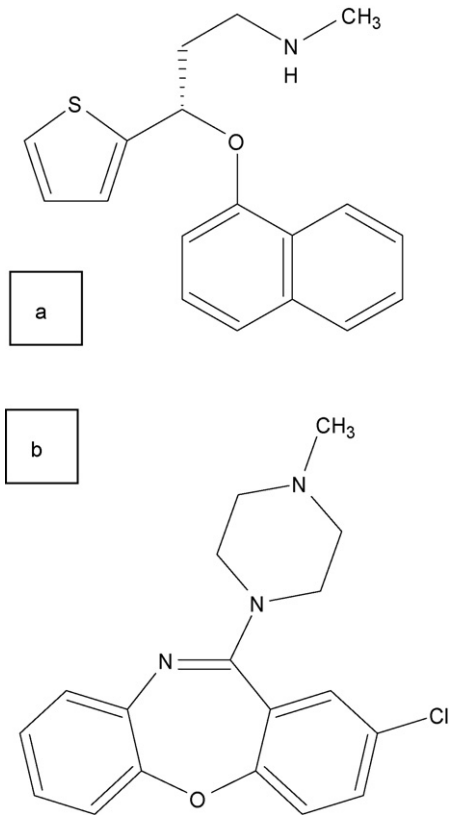

Fig. 1. Chemical structures of (a) duloxetine (DLX) and (b) loxapine (IS).

treatment are nausea, dry mouth, fatigue, insomnia, sedation, dizziness, constipation, increased sweating, decreased appetite and body weight [15]. Less frequent are cardiovascular effects, such as increased blood pressure [16]. A detailed DLX overdose case has recently been reported [17]: in a suicidal attempt the patient took 540 mg of DLX as well as other antidepressants and showed signs of altered mental status, cardiovascular alterations with hypotension, sinus bradycardia and prolonged QTc interval.

The importance of having on hand accurate, selective and feasible analytical methods for the determination of DLX in plasma is thus evident.

To the best of our knowledge, only three papers can be found in the literature, which specifically deal with the analysis of DLX in biological fluids. The first one [18] describes an HPLC method with fluorescence detection (HPLC-FL), after derivatisation with dansyl chloride, to determinate DLX and its main metabolite in plasma. The second one [19] describes a gas chromatographic method with mass spectrometric detection (GC–MS), which has been used for the toxicological analysis of DLX in post-mortem specimens. Another paper regarding DLX pharmacokinetics [12] briefly describes the use of a validated HPLC-MS method for the determination of DLX. However, the HPLC-FL method requires analyte derivatisation, which is a complicated and time-consuming procedure; the GC–MS and HPLC-MS methods require very expensive instrumentation, which is not always readily available in clinical analysis laboratories. Finally, two papers [20,21] regard the chiral resolution of some drugs (including DLX) by capillary electrophoresis, however these methods have not been applied to biological fluids.

The present paper describes the development and the validation of a rapid HPLC method with UV detection, which is certainly less complicated and requires less expensive instrumentation than other available methods, while being equally reliable, precise and accurate. The developed method is based on the employment of an original solid-phase extraction (SPE) procedure for the plasma pre-treatment. With respect to the liquid–liquid extractions used by other authors, this SPE procedure gives higher extraction yields and a good plasma sample purification.

2. Experimental

2.1. Chemicals and solutions

DLX, reference pure compound, was kindly provided by Eli Lilly (Indianapolis, IN, USA). Loxapine (2-chloro-11-(4-methyl-1-piperazinyl)dibenz[b,f][1,4]oxazepine, Fig. 1), used as the internal standard (IS), was kindly donated by Lederle Laboratories (Gosport, Hampshire, UK). Acetonitrile and methanol HPLC grade, 85% (w/w) phosphoric acid, 25% (w/w) ammonia and 0.1N HCl, all pure for analysis were purchased from Carlo Erba (Milan, Italy). Triethylamine pure for analysis was purchased from Fluka (Buchs, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, USA).

Stock solutions of the analyte and the IS (1 mg mL^{-1}) were prepared by dissolving suitable amounts of each pure substance in methanol. Standard solutions were obtained by diluting stock solutions with the mobile phase and were directly injected into the HPLC. Stock solutions were stable for at least 2 months when stored at −20 °C (as assessed by HPLC assays); standard solutions were prepared fresh every day.

2.2. Instrumentation and chromatographic conditions

The chromatographic system was composed of a Jasco (Tokyo, Japan) PU-980 chromatographic pump and a Jasco UV-975 spectrophotometric detector set at 230 nm.

Separations were obtained on a Jones Chromatography (Hengoed, UK) Genesis C8 reversed-phase column (150 mm × 4.6 mm I.D., 5 μm) kept at room temperature. The mobile phase was composed of a mixture of acetonitrile (40%, v/v) and a pH 3.0, 20 mM phosphate buffer containing 0.3% (v/v) triethylamine (60%, v/v). The mobile phase was filtered through a Phenomenex (Torrance, CA, USA) membrane filter (47 mm membrane, 0.2 μm, NY) and degassed by an ultrasonic bath. The flow rate was 1 mL/min and the injections were carried out through a 50-μL loop. Data processing was handled by means of a Varian (Walnut Creek, USA) Star Chromatography 4.0 software.

Solid-phase extraction was carried out by means of a VacElut (Varian) apparatus.

A Crison (Barcelona, Spain) Basic 20 pHmeter and a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge were used.

2.3. Sample collection and preparation

The blood samples were collected from patients of the Division of Psychiatry of the Department of Neurosciences (University of Parma, Italy) subjected to therapy with DLX for at least 2 weeks at constant daily doses. Blood samples were usually drawn 12 h after the last drug administration. Blood was stored in glass tubes containing EDTA as the anticoagulant, then centrifuged (within 2 h from collection) at 1400 × *g* for 15 min; the supernatant (plasma) was then transferred into polypropylene test tubes and stored at −20 °C until HPLC analysis. "Blank" plasma was obtained in the same way from blood drawn from healthy volunteers not subjected to any pharmacological treatment.

The solid-phase extraction procedure was carried out on Waters (Milford, MA, USA) Oasis mixed-mode reversed phase—strong cation exchange (MCX) cartridges (30 mg, 1 mL). Cartridges were conditioned by passing 1 mL of methanol through the cartridge two times and then equilibrated by passing 1 mL of ultrapure water two times. To 450 μL of plasma, 1 mL of pH 3.0, 20 mM phosphate buffer and 50 μL of IS standard solution were added and the resulting mixture loaded onto a conditioned cartridge. The cartridge was then washed twice with 1 mL of 0.1 N HCl and once with 50 μL of methanol. The analytes were then eluted with 1 mL of an ammonia/water/methanol (5/15/80, w/w/v) mixture. The eluate was dried under vacuum (rotary evaporator) and redissolved with 150 μL of mobile phase. An aliquot of 50 μL of this solution was injected into the HPLC system.

2.4. Method validation

2.4.1. Calibration curves

Aliquots of 50 μL of analyte standard solutions (prepared daily) at seven different concentrations containing the IS at a constant concentration were added to 450 μL of blank plasma. The resulting mixture was subjected to the previously described SPE procedure and injected into the HPLC. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios (pure numbers) obtained were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}) and the calibration curves set up by means of the least-square method. The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated according to USP [22] and "Crystal City" [23] guidelines as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

2.4.2. Extraction yield (absolute recovery)

The procedure was the same as that described under "Calibration Curve" above, except the points were at 3 different concentrations, corresponding to the upper limit, lower limit and middle point of each calibration curve. The analyte/IS peak area ratios were compared to those obtained by injecting standard solutions at the same theoretical concentrations and the extraction yield values were calculated.

2.4.3. Precision

The assays described under "Extraction yield" were repeated six times within the same day to obtain repeatability (intraday precision) and six times over six different days to obtain intermediate precision (interday precision) [23], both expressed as RSD% values.

2.4.4. Selectivity

Blank plasma samples from six different volunteers were subjected to the SPE procedure and injected into the HPLC; the resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was that no interference peak is to be higher than an analyte peak corresponding to its LOD. Furthermore, standard solutions of several different drugs active on the Central Nervous System were injected at concentrations higher than the respective therapeutic levels; if the resulting chromatograms contained any interference peak, the potentially interfering compounds were then subjected to the SPE and injected to ascertain if they could be extracted.

2.4.5. Accuracy

Accuracy was evaluated by means of recovery assays. The assays described under "Extraction yield" were carried out adding standard solutions of the analytes and the IS to real plasma samples taken from depressed patients subjected to therapy with DLX. The assays were repeated three times during the same day to obtain mean recovery and SD data.

3. Results and discussion

3.1. Choice of the experimental conditions

Our previous experience with the analysis of second generation antidepressants [24–27] and in particular of the antidepressant fluoxetine [27] prompted us to study DLX starting from the same chromatographic conditions. However, using this mobile phase (a 50/50, v/v mixture of acidic phosphate buffer containing triethylamine and acetonitrile) and a C8 column, DLX was scarcely retained; thus, a 60/40 (v/v) buffer/acetonitrile mixture was tried: this last mobile phase resulted suitable for the analysis of DLX. After spectrophotometric measurements (Fig. 2) the UV detector was set at 230 nm. Loxapine was chosen as the internal standard: in fact, under these conditions it is eluted in a short time; furthermore, it is not commercialised in Italy, thus interference from co-administration is not possible.

The chromatogram of a standard solution containing DLX and the IS is reported in Fig. 3a. As can be seen, the peaks are neat, symmetric and well separated.

3.2. Analysis of standard solutions

Seven-point calibration curves were set up in the 6–600 ng mL^{-1} concentration range. Good linearity ($r^2 = 0.9997$) was obtained, with a limit of quantitation of 6 ng mL^{-1} and a limit of detection of 2 ng mL^{-1}.

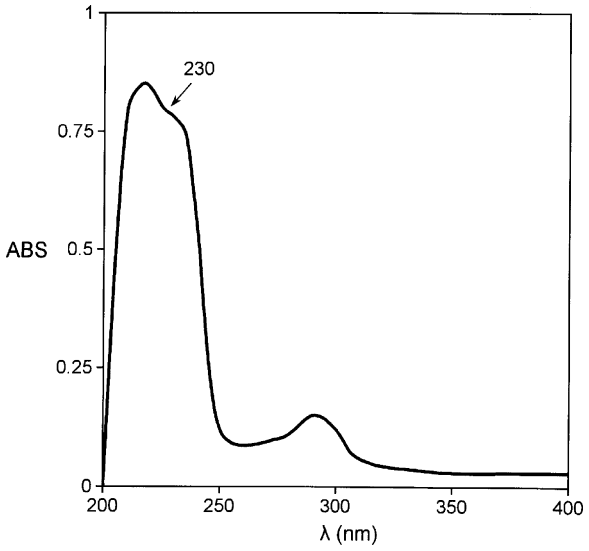

Fig. 2. UV spectrum of a 5 μg mL^{-1} DLX solution, prepared in a pH 3.0 phosphate buffer/acetonitrile 60/40 (v/v) mixture.

Precision was evaluated at three concentrations (6, 300 and 600 ng mL^{-1}), with the following results: RSD values were always lower than 3.2% for repeatability (intraday precision) and lower than 3.6% for intermediate precision (interday precision).

3.3. Development of the solid-phase extraction procedure

The sample pre-treatment procedure is a critical step of the analysis. It was decided to use SPE for this purpose, because it confers high selectivity to the method and gives good plasma sample purification and extraction yields while being fast, feasible and using small amounts of biological sample. Different kinds of sorbents were tried, such as hydrophilic–lipophilic balance (HLB), cyanopropyl (CN), C2 and C8. The CN sorbent gave low extraction yields (32%) of the analyte. The C2 and C8 sorbents, while providing better extraction yields (76 and 63%, respectively), gave unsatisfactory sample purification. The best, albeit not completely satisfactory, results were obtained with the HLB sorbent (still some interference, 80% extraction yield). Thus, it was decided to use the same sorbent base polymer, but containing different functionalities: in particular, mixed-mode reversed phase—strong cation exchange (MCX) cartridges were tried. This kind of sorbent allows taking advantage of the two different mechanisms for the selective retention and subsequent elution of the analytes. As a consequence, it gave much better results in terms of sample purification, while also granting higher extraction yields of the analyte and the IS. Thus, the MCX sorbent was chosen for the SPE procedure. The washing step was initially carried out with 0.1N HCl, to keep the analytes protonated, while eliminating hydrophilic interference. In order to obtain better purification, another washing step with a small volume (50 μL) of methanol was added. Finally, analyte elution was obtained with 1 mL of ammonia/methanol mixture: this mixture is sufficiently basic to suppress DLX ionic interactions with the sorbent and sufficiently lipophilic to suppress the lipophilic ones. The eluate was then dried under vacuum and redissolved with 150 μL of mobile phase: in this way, DLX is concentrated three times with respect to the original plasma level.

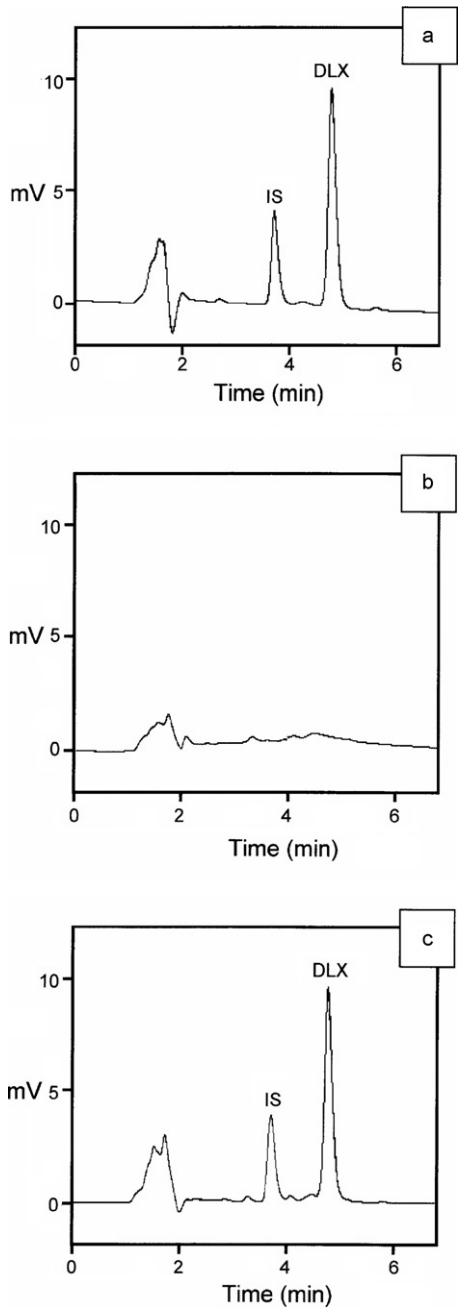

Fig. 3. Chromatograms of (a) a standard solution containing 100 ng mL^{-1} of DLX and of the IS; (b) a blank plasma sample from a healthy volunteer and (c) the same blank plasma sample spiked with 100 ng mL^{-1} of DLX and of the IS (concentrations in the injected solution).

Using this SPE procedure, good extraction yields of the analytes and the IS were obtained, while eliminating all endogenous interference. Fig. 3b reports the chromatogram of a blank plasma sample after SPE, while Fig. 3c reports the chromatogram of the same blank plasma sample spiked with a known amount of DLX and the IS and subjected to the SPE procedure. No interference can be detected near the retention times of the compounds of interest; furthermore, peak shapes and resolution are good.

Table 1
Extraction yield and precision assays

Compound	Concentration (ng mL^{-1})	Mean extraction yield (%)[a]	Repeatability (RSD%)[a]	Intermediate precision (RSD%)[a]
DLX	2	91	4.8	5.0
	100	92	3.2	3.8
	200	93	2.0	2.9
IS	100	96	2.1	3.0

[a] $n = 6$.

3.4. Method validation

Satisfactory linearity ($r^2 = 0.9995$) was obtained over the 2–200 ng mL^{-1} concentration range (plasma concentrations; the concentration range for the injected solutions is 6–600 ng mL^{-1} due to the threefold concentration introduced by the sample pre-treatment procedure). LOQ and LOD values were equal to 2.0 ng mL^{-1} and 0.7 ng mL^{-1}, respectively; this LOD value corresponds to an injected DLX amount of 105 pg. The linearity equation was: $y = 0.0012 + 0.0235x$, where x is the DLX concentration, expressed as ng mL^{-1}, and y is the DLX/IS peak area ratio (a pure number).

Extraction yield (absolute recovery) and precision assays were carried out on blank plasma spiked with analyte concentrations corresponding to the lower limit, middle point and upper limit of the calibration curves (i.e., 2, 100 and 200 ng mL^{-1}). The results of these assays are reported in Table 1. As one can note, mean extraction yields were always higher than 90% (96% for the IS). Precision results were also satisfactory: RSD values were always lower than 4.9% (2.1% for the IS) for repeatability and lower than 5.1% (3.0% for the IS) for intermediate precision.

Selectivity was evaluated by injecting into the HPLC standard solutions of several drugs, most of which are commonly co-administered during psychiatric therapy: other antidepressants, antipsychotics and anxiolytics-hypnotics. The complete list of the tested drugs and their retention times are reported in Table 2. As can be seen, none of them causes any interference in the analysis. Furthermore, six blank plasma samples were injected after SPE and none of them produced peaks from endogenous compounds, which could interfere with the determination. Therefore, the method has demonstrated to be selective.

3.5. Analysis of patient plasma samples

Having thus validated the method, it was applied to the analysis of plasma samples from some depressed patients of the Department of Neurosciences (University of Parma, Italy) undergoing therapy with DLX (Cymbalta®). Fig. 4a shows a plasma sample from a patient undergoing therapy with 60 mg day^{-1} of DLX and who was also taking 2.5 mg day^{-1} of lorazepam (LRZ). The DLX concentration found in this real blood sample, drawn 12 h after the last drug administration, was 15 ng mL^{-1}. As expected, the co-administered drug (LRZ) did not interfere with the determination, thus confirming the selectivity of the method.

Table 2
Drugs tested for selectivity assays

Therapeutic class	Compound	t_R (min)
(Analyte and IS)	DLX	4.8
	Loxapine (IS)	3.8
Antidepressants	Amitriptyline	7.1
	Clomipramine	6.9
	Fluoxetine	6.5
	Mirtazapine	n.r.[a]
	Paroxetine	5.6
	Sertraline	6.1
	Trazodone	2.5
	Venlafaxine	n.r.
Antipsychotics	Amisulpride	n.r.
	Chlorpromazine	8.5
	Clotiapine	6.3
	Clozapine	n.r.
	Haloperidol	5.2
	Levomepromazine	6.5
	Promazine	7.9
	Quetiapine	2.3
	Risperidone	n.r.
Anxiolytics-hypnotics	Clonazepam	6.6
	Delorazepam	9.4
	Diazepam	8.4
	Flurazepam	n.r.
	Lorazepam	6.1

[a] n.r. = not retained.

Accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations (2, 50 and 100 ng mL^{-1}) and of the IS at a constant concentration (100 ng mL^{-1}) were added to plasma samples containing known amounts of DLX (i.e., samples which had already been analysed). Then, the recovery of the added analyte was calculated, as well as the standard deviation of the assays. Fig. 4b corresponds to a plasma sample from the same patient as in Fig. 4a, after spiking with DLX. The results of the accuracy assays are reported in Table 3: mean recovery values were always higher than 89%. Thus, method accuracy is satisfactory.

Table 3
Accuracy assays

DLX concentration added (ng mL^{-1})	Mean concentration found (ng mL^{-1})	Mean recovery (%)	SD
2	1.8	90	3.0
50	45.5	91	2.7
100	93.2	93	2.0

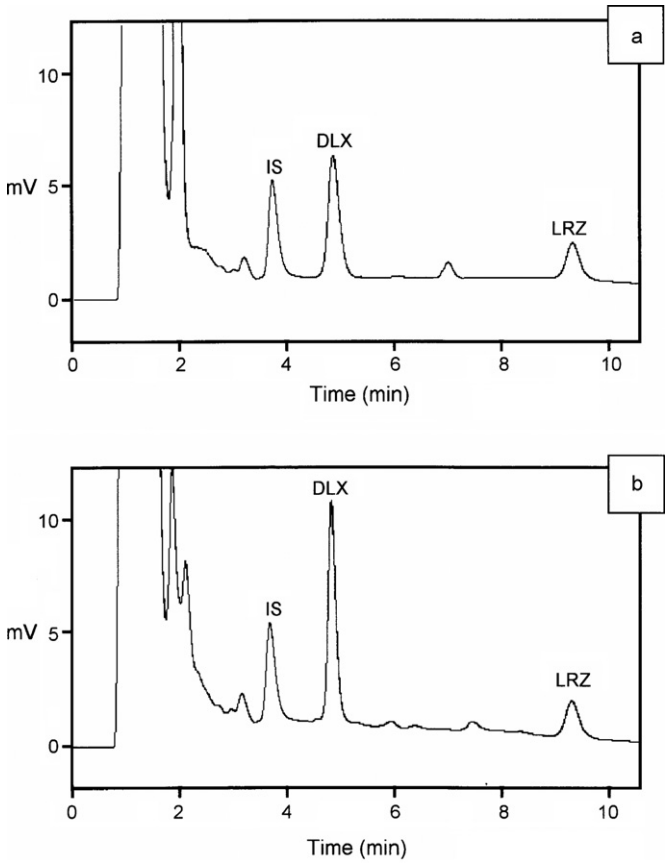

Fig. 4. Chromatograms of (a) a plasma sample from a patient who was subjected to treatment with 60 mg day^{-1} of DLX and (b) the same plasma sample after spiking with 15 ng mL^{-1} of DLX (plasma concentration). The patient was also taking 2.5 mg day^{-1} of LRZ.

4. Conclusion

The HPLC method presented here for the analysis of DLX is feasible and rapid: a chromatographic run lasts less than five minutes. The SPE procedure implemented for the sample pre-treatment, based on MCX cartridges, gives good extraction yields (>90%) and satisfactory precision (RSD% < 5.1%). The method is also selective: neither endogenous compounds nor any of the central nervous system drugs tested has produced any interference in the analysis of DLX in depressed patients' plasma. The use of SPE poses several advantages with respect to the liquid–liquid extraction procedures used by other authors [18,19]: in fact, the SPE procedure is faster and requires lower volumes of organic solvents. The proposed method is also advantageous for other reasons: it has high accuracy and a wide linearity range, which allows the determination of the analyte not only at therapeutic doses but also in overdose cases and when administered at sub-therapeutic doses (e.g., scarce patient compliance). Compared to the GC–MS method [19], the proposed method has higher extraction yields (>90% versus 75%) and better sensitivity (LOQ = 2 ng mL^{-1} versus 50 ng mL^{-1}). When compared to the HPLC-FL method [18], the proposed method is certainly much more feasible, since it does not require any time-consuming and expensive derivatisation step, and uses lower volumes of plasma (450 μL instead of 1 mL), while reaching a better sensitivity level (LOQ = 2 ng mL^{-1} versus 5 ng mL^{-1}). The HPLC-MS method briefly described by Lantz et al. [12] has better sensitivity (0.5 ng mL^{-1}), however it requires more expensive instrumentation. The proposed method has demonstrated that it possesses sufficient sensitivity for the analysis of DLX in plasma of patients undergoing therapy with the drug and is a significant improvement with respect to currently available procedures.

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