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Determination of reboxetine, a recent antidepressant drug, in human plasma by means of two high-performance liquid chromatography methods

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Abstract

Reboxetine is a new norepinephrine reuptake inhibitor (NRI) drug recently introduced in the therapy for depressed patients. It is effective in the treatment of severe depression and safer to use than traditional tricyclic antidepressants. In this paper an original high-performance liquid chromatography (HPLC) method with ultraviolet detection for the determination of reboxetine in human plasma is described. It uses a C_8 reversed-phase column and a mobile phase composed of acetonitrile and aqueous tetramethylammonium perchlorate. For the analysis of plasma samples containing very low levels of reboxetine, another HPLC method with fluorimetric detection was developed (limit of quantitation, LOQ=11 ng ml^{-1}; limit of detection, LOD=4 ng ml^{-1}). The fluorimetric method is based on precolumn derivatisation of reboxetine with 9-fluorenylmethyl chloroformate. An accurate sample pretreatment of human plasma samples has been implemented by means of solid-phase extraction (SPE) on Oasis HLB (hydrophilic–lipophilic balance) cartridges with very high extraction yields (>95%). Both methods were applied to the analysis of plasma samples from depressed patients undergoing therapy with reboxetine and gave satisfactory results in terms of precision (RSD<4.5%) and accuracy (mean recovery>94%).

Keywords: Pharmaceutical analysis; UV detection; Fluorimetric detection; Human plasma; Reboxetine

1. Introduction

Major depression is currently a very common illness, whose clinical relevance and dangerousness can be compared to those of hypertension and infective diseases. Current psychiatric knowledge considers this mental illness as a multifactorial disease which involves several neurotransmitter and neuroreceptor systems [1,2]. The therapy usually requires the use of several medications, each one of which is not universally effective and can cause significant side effects. The precise etiology of major depression is still unknown, however several studies indicate that the illness involves modifications in the activation of adrenergic neurons [3]. In fact, the drugs most frequently used for the treatment of depression are inhibitors of the reuptake of norepinephrine and/or serotonin [4,5].

Reboxetine, (*RS*)-2-[(*RS*)-α-(2-ethoxyphenoxy)-benzyl]morpholine (Fig. 1A), is a new antidepressant

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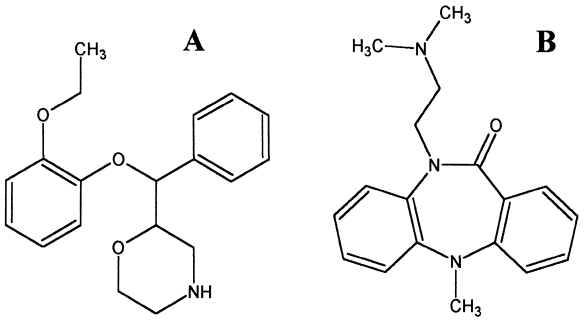

Fig. 1. Chemical structures of (A) reboxetine and (B) dibenzepine (I.S.).

drug recently introduced into the market. It seems to be as effective as traditional tricyclic drugs, and more safe.

The therapeutic properties of reboxetine are due to its activity of selective inhibition of the reuptake of norepinephrine [6–9]. With respect to tricyclic antidepressants (TCAs), it has a lower incidence of cholinergic side effects and seizures [10], because it has a low affinity toward cholinergic, α-adrenergic, hystaminergic and serotonergic receptors [11,12]. With respect to fluoxetine and other selective serotonin reuptake inhibitors it seems to have greater efficacy against the lack of confidence and motivation and to reduce the risk of suicide [13,14].

The therapeutic dosage of reboxetine is usually about 20 times lower than that of TCAs (4–8 mg day^{-1} as opposed to 150–200 mg day^{-1}) [15]. The most frequent adverse effects of reboxetine therapy are insomnia, diaphoresis, constipation, tachycardia and urinary retention [16]. These adverse effects only rarely require the discontinuation of treatment.

We recently proposed two methods (HPCE and derivative spectrophotometry) for the quality control of reboxetine in pharmaceutical formulations [17]. Some papers are reported in the literature which determine reboxetine in biological fluids. Frigerio et al. implemented an HPLC method with fluorimetric detection (FL) for the determination of reboxetine enantiomers in human plasma after derivatization with 9-fluorenylethyl chloroformate [18]. The same method was then used for pharmacokinetic studies in rat and dog plasma [19,20]. The method has been subsequently improved by substituting one liquid–liquid extraction step with a solid-phase extraction (SPE) step [21].These stereospecific methods are rather laborious and time-consuming. In the last few years three new papers have been published about the determination of reboxetine in human plasma by means of HPLC–UV methods [22–24]. The first one, however, uses a less common technique such as column-switching. The second and the third ones are more simple and feasible, however they have the disadvantage of using a rather low detection wavelength, which could lead to the detection of some interference. Very recently, another HPLC method with dual mass spectrometry detection has been published [25]. The aim of this investigation is the development of simple and reliable methods for the determination of reboxetine levels in the plasma of depressed patients treated with the drug. We chose to use a simple and widespread technique such as isocratic liquid chromatography with ultraviolet detection. Furthermore, another method with pre-column derivatization and fluorimetric detection has been developed and used for samples which required high sensitivity and selectivity. For these characteristics, and for the small amount of human plasma required, the HPLC–FL method can also be used for pharmacokinetic studies.

2. Experimental

2.1. Chemicals and solutions

Reboxetine methansulfonate pure compound was kindly provided Pharmacia Upjohn (Milan, Italy). Novartis (Basel, Switzerland) kindly provided dibenzepine pure compound (Fig. 1B), which was used as the internal standard (I.S.).

Methanol, acetonitrile, perchloric acid, tetrahydrofuran, acetone, phosphoric acid, boric acid, 35% (m/m) ammonia, *n*-hexane and 2 *M* sodium hydroxide (all analytical grade) were purchased from Carlo Erba (Milan, Italy). Tetramethylammonium perchlorate, 9-fluorenylmethyl chloroformate (FMOC) and L-proline were supplied from Sigma (St. Louis, MO, USA).

Ultrapure water (18.2 MΩ cm) was obtained by means of a Millipore (Milford, MA, USA) Milli-Q apparatus.

The stock solutions of reboxetine (1 mg ml^{-1} of free base) were prepared by dissolving 26.12 mg of

reboxetine methansulfonate in 20 ml of methanol. The stock solutions of the I.S. (1 mg ml^{-1} of free base) were prepared by dissolving 21.6 mg of dibenzepine hydrochloride in 20 ml of methanol. The standard solutions were in an acetonitrile–pH 2.6, 11.3 m*M* aqueous tetramethylammonium perchlorate mixture (80:20, v/v) for the HPLC–UV assays, in 5 m*M* phosphoric acid for the HPLC–FL assays.

The L-proline solution was 100 m*M* in ultrapure water. The borate buffer was 0.8 *M* in ultrapure water, brought to pH 8.0 with NaOH. The FMOC stock solution was 18 m*M* in acetone; this solution was diluted with acetonitrile to obtain the 0.36 m*M* working solution.

All stock solutions were kept at −20°C and were stable for at least 2 months. The standard and working solutions were prepared daily from stock solutions and were kept at 4°C until use.

Several compounds commonly used in clinical practice were tested for interference with the HPLC–UV assay. The tested substances were: amitriptiline, benserazide, carbamazepine, chlorpromazine, clonazepam, clotiapine, clozapine, desmethylclozapine, fluoxetine, fluphenazine, flurazepam, fluvoxamine, gabapentin, haloperidol, imipramine, lamotrigine, loxapine, maprotiline, melatonin, norfluoxetine, olanzapine, orphenadrine, paroxetine, phenobarbital, protriptiline, risperidone, sertraline, zuclopentixol. Each stock solution (1 mg ml^{-1}) was prepared in methanol, and subsequently diluted with the mobile phase.

2.2. Apparatus and chromatographic conditions

2.2.1. HPLC–UV method

The HPLC apparatus consisted of a Jasco (Tokyo, Japan) PU-980 isocratic pump and UV-975 spectrophotometric detector.

The stationary phase was a Varian (Harbor City, CA, USA) reversed-phase ResElut C_8 column (150×4.6 mm I.D., 5 μm). The mobile phase was an acetonitrile–pH 2.6, 11.3 m*M* aqueous tetramethylammonium perchlorate mixture (40:60, v/v). The flow-rate was 1 ml min^{-1}. The injection loop was 20 μl and the detection wavelength was 230 nm.

2.2.2. HPLC–FL method

This chromatographic system was composed of a Varian 9012 pump and a Varian 9075 fluorimetric detector.

The stationary phase was a reversed-phase ResElut C_8 column (150×4.6 mm I.D., 5 μm). The mobile phase was a tetrahydrofuran–pH 7.5, 0.1 *M* phosphate buffer (50:50, v/v) mixture. The flow-rate was 1.3 ml min^{-1} and the fluorescence intensity was monitored at 314 nm, while exciting at 260 nm. The injection loop was 20 μl.

For both methods, data were acquired by means of a Star Chromatography 4.0 software (Varian), running on an IBM (IBM UK, Portsmouth, UK) 40486 microprocessor in Microsoft (Redmond, WA, USA) Windows 3.11 environment.

Both mobile phases were filtered through a Millipore membrane filter (47 mm diameter, 0.20 μm pore size) and degassed by sonication in a Helba (Singen, Gremany) Transsonic 310 apparatus.

2.3. Standard solution analysis

2.3.1. HPLC–UV method

The standard solutions for the HPLC–UV method were injected directly into the HPLC.

2.3.2. HPLC–FL method

The standard solutions for the HPLC–FL method were derived and extracted as follows. To 250 μl of standard solution, 250 μl of pH 8 borate buffer and 250 μl of FMOC working solution (0.36 m*M*) were added. The mixture was left to react for 15 min, then 250 μl of L-proline solution were added. After 2 min the mixture was extracted with 3.0 ml of *n*-hexane (vortex-mixing for 1 min), then the aqueous phase was discarded and the organic phase was back-extracted with 1.0 ml of acetonitrile. The *n*-hexane was discarded. After washing with 1.0 ml of *n*-hexane and discarding the washing, the acetonitrile solution was evaporated to dryness at 40°C and redissolved with 250 μl of the mobile phase. This solution was injected into the HPLC.

2.4. Human plasma sampling

The assays were carried out on plasma samples from depressed patients of the Psychiatric Clinic of the University of Bologna. They were at steady state,

subjected to treatment with Edronax tablets at a constant daily dose for at least 2 weeks. Blood samples were obtained 12 h from the last dose.

Blood samples were drawn into test tubes, containing EDTA as an anticoagulant, and centrifuged at 1400 *g* for 20 min. The supernatant plasma was transferred into test tubes and frozen at −20°C until analysis, usually within 1 week. This procedure was also used to separate plasma from the blood of healthy volunteers (''blank'' plasma).

2.5. Stability of plasma samples

From preliminary assays, reboxetine resulted to be stable. In fact, the same biological samples have been analysed several times at different intervals of time, even 2 months apart and after thawing and freezing, and no substantial difference in the analytical results was noticed.

2.6. Sample pretreatment

2.6.1. HPLC–UV method

For the SPE procedure Oasis HLB cartridges (30 mg, 1 ml) from Waters (Milford, MA, USA) were used.

The cartridges were activated with 1 ml of methanol and conditioned with 1 ml of water, then loaded with 500 μl of plasma diluted with 500 μl of water and added with 100 ng ml^{-1} of I.S.

The cartridge was then washed with 1 ml of water and the analyte finally eluted with 250 μl of modified mobile phase, i.e. an acetonitrile–pH 2.6, 11.3 m*M* aqueous tetramethylammonium perchlorate mixture (80:20, v/v).

2.6.2. HPLC–FL method

The Oasis cartridges were activated and conditioned as described for the UV method. The cartridges were loaded with 250 μl of plasma diluted with 500 μl of water, then washed twice with 1 ml of water. Reboxetine was eluted by means of 1.5 ml of methanol.

The resulting eluate was evaporated to dryness, then redissolved in 250 μl of 5 m*M* phosphoric acid and subjected to the same derivatisation and extraction procedure already described for standard solutions.

2.7. Method validation

The method validation assays for both methods were carried out according to the ''Crystal City'' [26] guidelines. Calibration curves were set up on blank plasma, by adding known amounts of reboxetine standard solution to known volumes of blank plasma and subjecting the resulting mixture to the SPE procedure (and derivatisation and extraction for the HPLC–FL method) and HPLC analysis. The calibration curve was constructed plotting the height of the reboxetine peak against the concentration of reboxetine added and applying the least-squares method. The detection limit (LOD) and quantitation limit (LOQ) were determined as 3 and 10 times the baseline noise, respectively, following the United States Pharmacopeia [27] and ''Crystal City'' [26] guidelines.

The extraction yield (or absolute recovery) was obtained preparing and analysing six different samples of blank plasma containing the same amount of reboxetine standard solution, then calculating the percentual recovery of reboxetine. The relative standard deviation (RSD) values of these assays on the same day assessed the repeatability, on different days assessed the inter-day precision. This procedure was repeated for three different concentrations of reboxetine added, namely 100, 500 and 750 ng ml^{-1} (which correspond to 200, 1000 and 1500 ng ml^{-1} of reboxetine injected) and 100 ng ml^{-1} of I.S. for the HPLC–UV method, and 25, 500 and 1000 ng ml^{-1} for the HPLC–FL method.

Accuracy data were obtained by adding known amounts of reboxetine standard solution to patient plasma samples, in order to obtain additions of 100, 500 and 750 ng ml^{-1} (corresponding to 200, 1000 and 1500 ng ml^{-1} of added reboxetine injected) and 100 ng ml^{-1} of I.S. for the HPLC–UV method, and additions of 25, 500 and 1000 ng ml^{-1} for the HPLC–FL method. The resulting solutions were then analysed in triplicate and the percentage recovery of the added reboxetine calculated.

3. Results and discussion

3.1. HPLC–UV method

Reboxetine is a recent basic drug active on the central nervous system (CNS). Our previous paper on the determination of several CNS drugs in human plasma by means of an HPLC–UV method [28] gave us the starting point for the development of this method.

With respect to the previous method, the acetonitrile–aqueous perchlorate ratio in the mobile phase has been slightly adjusted from 37:63 to 40:60, and the SPE procedure for the purification of plasma samples has been also modified. Furthermore, an internal standard for the control of retention times was introduced. Dibenzepine, a tricyclic antidepressant currently not commercially available in Italy, was found particularly suitable for this purpose.

The modification of the mobile phase allowed for shorter analysis times, while maintaining good resolution of the reboxetine and dibenzepine peaks.

The optimal leading conditions were: stationary phase, a reversed-phase ResElut C_8 column (150× 4.6 mm I.D., 5 μm); mobile phase, an acetonitrile–pH 2.6, 11.3 m*M* aqueous tetramethylammonium perchlorate mixture (40:60, v/v); flow-rate, 1 ml min^{-1}; injection loop, 20 μl and detection wavelength, 230 nm.

Under these conditions reboxetine is detected as a neat chromatographic peak at retention time 7.8 min, while the I.S. (dibenzepine) is detected at 6.5 min.The peaks are symmetrical and the separation is good.

3.2. Method validation

The new SPE procedure uses the same Oasis cartridges but a different solvent from the one we previously used [28] to elute the analytes from the cartridges. Now, the optimal elution solvent is constituted by the mobile phase of the HPLC method modified to contain 80% acetonitrile and 20% aqueous tetramethylammonium perchlorate, instead of methanol [28]. This different elution requires a smaller volume of solution to obtain an almost complete recovery of reboxetine from human plasma, and a 2-fold concentration of the analyte is thus obtained during the SPE step.

The chromatogram of a blank plasma sample spiked with 100 ng ml^{-1} of I.S. and subjected to the SPE procedure is shown in Fig. 2A; no interference from the matrix is apparent. The chromatogram of

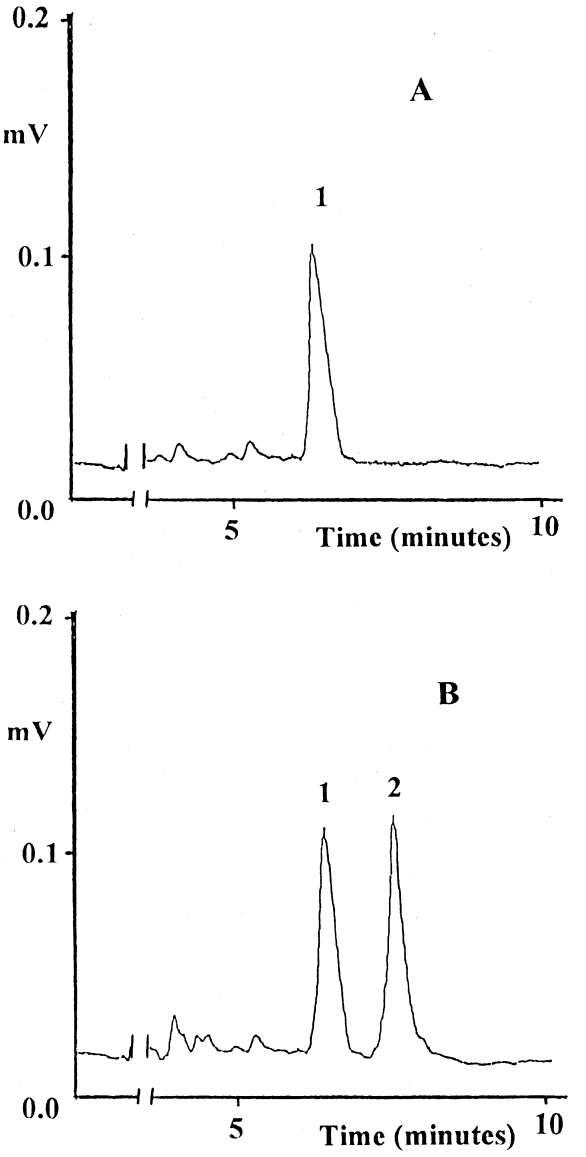

Fig. 2. Chromatograms of (A) a blank plasma sample spiked with 100 ng ml^{-1} of the I.S. and (B) the same blank plasma sample spiked with 100 ng ml^{-1} of the I.S.and 500 ng ml^{-1} of reboxetine, analysed by HPLC–UV. Peak identification: 1=I.S.; and 2= reboxetine.

the same blank plasma sample spiked with 500 ng ml^{-1} of reboxetine and 100 ng ml^{-1} of I.S. is shown in Fig. 2B; the peak of reboxetine has a t_R of 7.8 min, that of the I.S. has a t_R of 6.5 min. These peaks are almost identical to those obtained injecting standard solutions containing reboxetine and the I.S.

Good linearity was found from 50 to 1000 ng of reboxetine per ml of plasma (which correspond to a 100–2000 ng ml^{-1} range of solutions of reboxetine injected into the HPLC) and the linear regression equation was $y=2.330+0.247x$ ($r_c=0.997$), where y is the peak height, expressed as arbitrary units, and x is the reboxetine concentration, expressed as ng ml^{-1}. LOQ and LOD values, calculated according to USP 24 [27] and ''Crystal City'' [26] guidelines, were 45 and 15 ng ml^{-1} of plasma reboxetine, respectively.

The extraction yields and the respective precisions were calculated for plasma samples spiked with 100, 500 or 750 ng ml^{-1} of reboxetine. The results (reported in Table 1) were very good: the extraction yield is always high, being the mean value 96.6%, and the repeatability and inter-day precision are always better than 3.8%.

3.3. Interference studies

Since UV detection is not very selective, interference from coadministered drugs could sometimes be a problem.

For this reason, several compounds were tested for interference. These substances are all active on the CNS and often were coadministered together with reboxetine to patients of the Psychiatric Clinic. The examined drugs belong to different therapeutic classes, such as: antiepileptics (carbamazepine, gabapentin, lamotrigine, phenobarbital), antidepressants (amitriptiline, fluoxetine, fluphenazine, fluvoxamine, imipramine, maprotiline, paroxetine, protriptiline, sertraline), antipsychotics (chlorpromazine, clotiapine, clozapine, haloperidol, loxapine, olanzapine, risperidone, zuclopentixol), anxiolytics and hypnotics (clonazepam, flurazepam, lorazepam), one anticholinergic antiparkinson agent (orphenadrine) and two selected metabolites (desmethylclozapine and norfluoxetine). The results of the investigation are reported in Table 2. As can be seen, none of the tested compounds interferes with the analysis of reboxetine.

3.4. Application to patient plasma samples

Having thus validated the method, it was then applied to plasma samples obtained from patients suffering from major depression who were treated with Edronax at the Psychiatric Clinic of the University of Bologna.

A chromatogram obtained from the analysis of authentic human plasma of a subject receiving 8 mg day^{-1} of reboxetine orally (administered in two doses) is reported in Fig. 3A. The chromatogram shows several peaks, of which that at retention time 7.8 min corresponds to reboxetine. The plasma reboxetine concentration in this sample was 274 ng ml^{-1}; it was found by interpolating in the calibration curve. This patient was undergoing therapy with orphenadrine, flurazepam, clotiapine and betanecol, in addition to reboxetine. These drugs are eluted later than the analyte (Table 2), therefore they are not shown in the chromatogram. However, the other peaks found in Fig. 3 are presumably metabolites of the drugs. Nevertheless, a peak interferes with the I.S. peak, however this is not a problem because the I.S. was used for the control of retention times only.

Table 1
Assay characteristics (HPLC–UV method)

Parameter	Reboxetine concentration (ng ml^{-1})		
	100	500	750
Extraction yield (%)[a]	96.3	96.8	97.2
Repeatability, RSD (%)[b]	3.0	2.5	2.1
Interday precision, RSD (%)[b]	3.8	3.3	2.4
Accuracy, % recovery ($n=3$)	95.6±4.9	96.5±4.2	96.8±3.9

[a] Each value is the mean of six independent assays.
[b] Each value is obtained from six independent assays.

Table 2
Compounds tested for interference in the reboxetine analysis (HPLC–UV method)

Compound	Retention time (min)	Compound	Retention time (min)
Antiepileptics		Antipsychotics	
Carbamazepine	4.2	Chlorpromazine	n.d.
Gabapentin	n.d.	Clotiapine	12.8
Lamotrigine	4.0	Clozapine	6.1
Phenobarbital	2.7	Haloperidol	11.5
Antidepressants		Loxapine	11.7
Amitriptiline	16.9	Olanzapine	4.3
Dibenzepine (I.S.)	*6.5*	Risperidone	9.6
Fluoxetine	17.2	Zuclopentixol	19.6
Fluphenazine	16.6	Anxiolytics	
Fluvoxamine	11.1	Clonazepam	5.1
Imipramine	n.d.	Flurazepam	10.3
Maprotiline	14.3	Lorazepam	4.4
Paroxetine	11.2	Antiparkinson	
Protriptiline	12.5	Orphenadrine	12.2
Reboxetine	*7.8*	Metabolites	
Sertraline	19.5	Desmethylclozapine	4.8
		Norfluoxetine	13.6

The identity of the reboxetine peak was also confirmed by adding a known amount of reboxetine standard solution (250 ng ml^{-1}) to the sample. The resulting chromatogram is reported in Fig. 3B.

The accuracy of the method was determined by means of recovery assays. These assays gave good results, as can be seen in Table 1. In fact, the values of recovery are always above 95%.

3.5. HPLC–FL method

In order to improve the sensitivity and the selectivity of the assay, another HPLC method with fluorimetric detection was implemented. Reboxetine does not possess native fluorescence, so a derivatisation step with a fluorophore was necessary. FMOC is a well-known fluorescent compoud which reacts in basic conditions with primary and secondary amines [29], according to the scheme reported in Fig. 4.

The resulting derivative compound has very different chromatographic properties with respect to reboxetine alone. The previous chromatographic system had thus to be changed, in order to allow for the determination of the derivative. The reaction conditions and the mobile phase composition are similar (but not identical) to those reported by other authors who used 1-(9-fluorenyl)ethyl chloroformate (FLEC) to derivatise and separate reboxetine enantiomers [18]. Since our purpose was to determine racemic reboxetine, we used FMOC, which is a much cheaper reagent. Keeping the same stationary phase already used for the HPLC–UV method (namely a C_8 ResElut column, 150×4.6 mm), the optimal mobile phase was a tetrahydrofuran–pH 7.5 phosphate buffer (50:50, v/v) mixture flowing at 1.3 ml min^{-1}. The detection wavelength was 314 nm and the excitation wavelength was 260 nm.

Under these leading conditions, reboxetine–FMOC is eluted as a neat peak at t_R=14.3 min.

The derivatization step was applied to blank plasma spiked with reboxetine. However many components of plasma possess native fluorescence and/or react with FMOC in the reported conditions. Thus a sample pretreatment before the derivatization was necessary, in order to eliminate interference from the biological matrix. The previous paper [18] proposed a liquid–liquid extraction procedure which is very laborious. A subsequent paper [21] proposes an equally laborious SPE procedure, with rather low

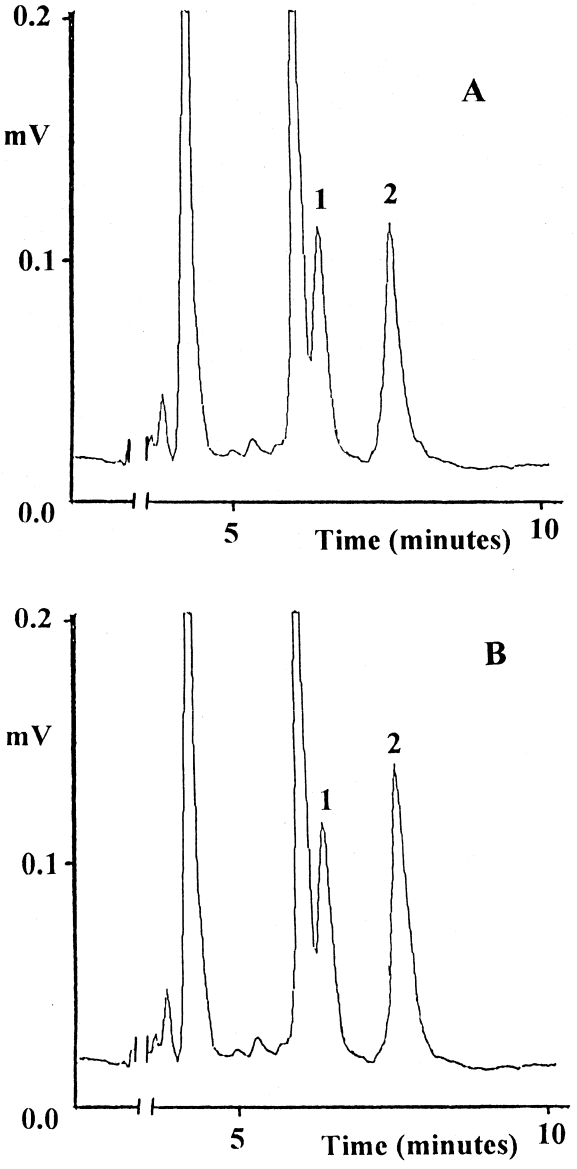

Fig. 3. Chromatograms of (A) a patient plasma sample spiked with 100 ng ml^{-1} of the I.S. and (B) the same plasma sample spiked with 100 ng ml^{-1} of the I.S.and 250 ng ml^{-1} of reboxetine, analysed by HPLC–UV. Peak identification: 1=I.S.; and 2= reboxetine.

extraction yields. We obtained very good results using Oasis HLB cartridges and slightly modifying the SPE procedure already implemented for the HPLC–UV method, as reported in the Experimental section.

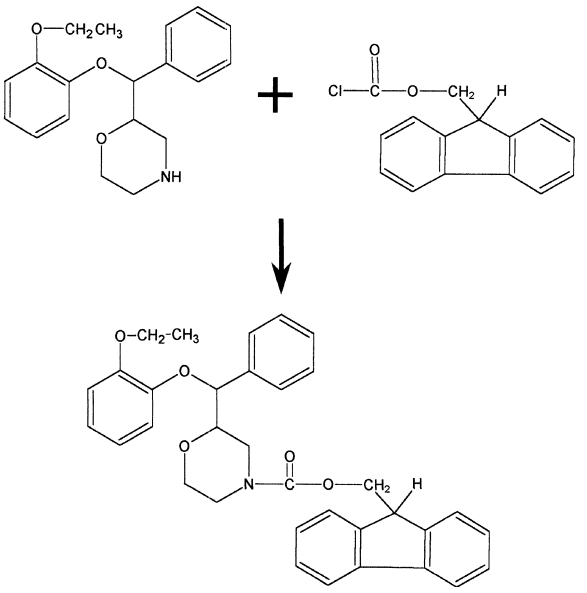

Fig. 4. Scheme of the reaction between reboxetine and FMOC.

Under these experimental conditions, the chromatogram of a blank plasma sample (Fig. 5A) does not present any interference near the retention time of the reboxetine–FMOC derivative. The chromatogram of a blank plasma sample spiked with 300 ng ml^{-1} of reboxetine is reported in Fig. 5B. As can be seen, the peak is neat and again no interference is present.

Good linearity was found in the 20–2000 ng ml^{-1} of reboxetine concentration range. The calibration equation, obtained by means of the least-squares method, was $y=-4029+15510x$ ($r_c=0.9997$), where y is the peak area, expressed as arbitrary units, and x is the reboxetine concentration, expressed as ng ml^{-1}. LOQ and LOD values, calculated according to USP 24 [27] and ''Crystal City'' [26] guidelines, were 11 and 4 ng ml^{-1}, respectively. This method has LOQ and LOD values about four times lower than those of the HPLC–UV method, and is thus suitable even for very low levels of the analyte.

The extraction yield (absolute recovery) and the precision values were obtained on 25, 500 and 1000 ng ml^{-1} additions of reboxetine standard solutions to blank plasma samples. The extraction yield data were found by analysis of extracted plasma samples subsequently derivatized according to the procedure

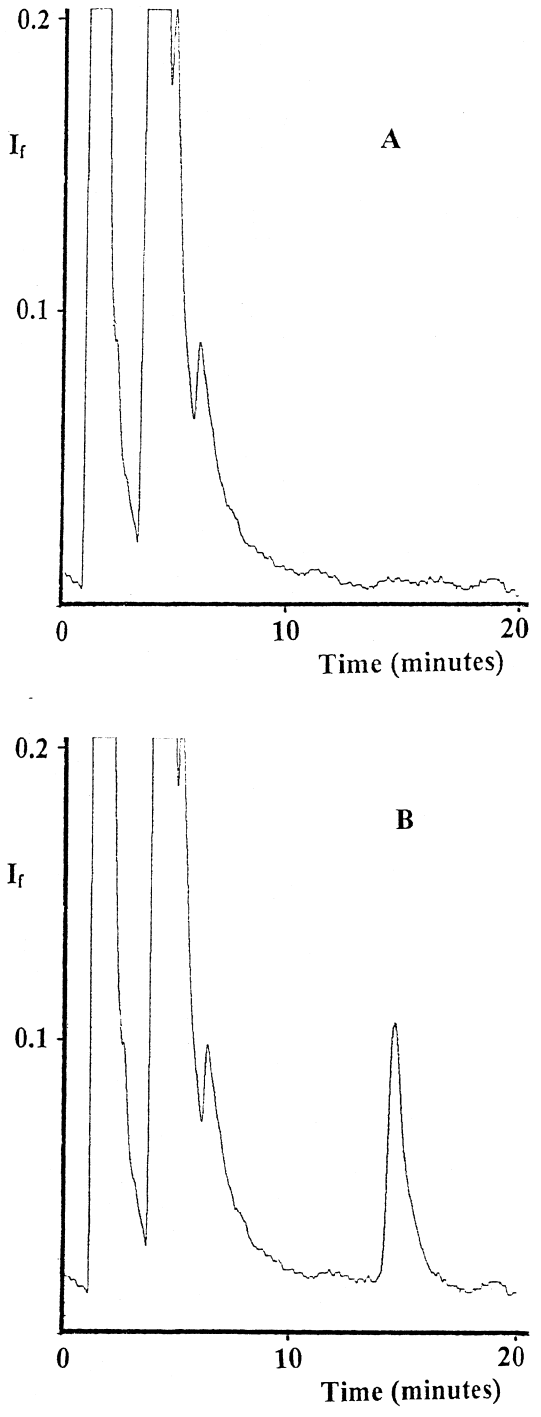

Fig. 5. Chromatograms of (A) a blank plasma sample and (B) the same blank plasma sample spiked with 300 ng ml^{-1} of reboxetine, analysed by means of the HPLC–FL method. Reboxetine retention time: 14.3 min. I_f=fluorescence intensity.

described; percentual values were calculated with respect to directly derivatized samples containing reboxetine standard solution in phosphoric acid. The results obtained are reported in Table 3. These results are very satisfactory, because the extraction yield is always better than 95%. The precision was also satisfactory.

3.6. Application to patient plasma samples

The proposed HPLC method with fluorescence detection was applied to the analysis of plasma samples from patients undergoing therapy with reboxetine tablets. The chromatogram of a plasma sample from a patient who was taking 4 mg day^{-1} of reboxetine (as well as lorazepam and doxazosin) is shown in Fig. 6. A neat peak corresponding to reboxetine appears at t_R=14.3 min, and no interference from the matrix or other coadministered drugs is present. The reboxetine concentration found in this sample, obtained by interpolating on the blank plasma calibration curve, was 143 ng ml^{-1}.

Plasma samples from treated patients were spiked with standard solutions of reboxetine (final addition: 25, 500 and 1000 ng ml^{-1}), then subjected to the extraction and derivatisation procedure and to HPLC analysis. The accuracy results are very satisfactory (94–95%).

It can be noted that no internal standard was used for the HPLC–FL assays. In fact, the high accuracy and reproducibilty of the method made the presence of an I.S. unnecessary.

4. Conclusion

The method based on HPLC with UV detection is fast and simple and has been validated for the determination of reboxetine in human plasma with good results in terms of accuracy and precision.

The sample pretreatment by means of an original SPE procedure grants good extraction yields and concentrates the analyte, thus enhancing the sensitivity of the method. The overall sensitivity is rather good, bearing in mind that the method uses a UV detection.

The method based on HPLC with fluorimetric detection is not so fast, however it is more sensitive

Table 3
Assay characteristics (HPLC–FL method)

Parameter	Reboxetine concentration (ng ml^{-1})		
	25	500	1000
Extraction yield (%)[a]	96.1	95.2	95.3
Repeatability, RSD (%)[b]	3.3	2.4	1.7
Interday precision, RSD (%)[b]	4.5	3.2	3.0
Accuracy, % recovery (n=3)	94.1±5.3	94.6±4.3	95.0±4.1

[a] Each value is the mean of six independent assays.
[b] Each value is obtained from six independent assays.

and selective, for this reason suitable for the determination of small amounts of reboxetine. In comparison with other papers with fluorimetric detection found in the literature, it is more simple and feasible. Moreover, it has the advantage of better extraction yields (>95% instead of 75% found in other papers [21]) and needs only a small amount of plasma for one complete analysis (250 instead of 1000 μl [18]).

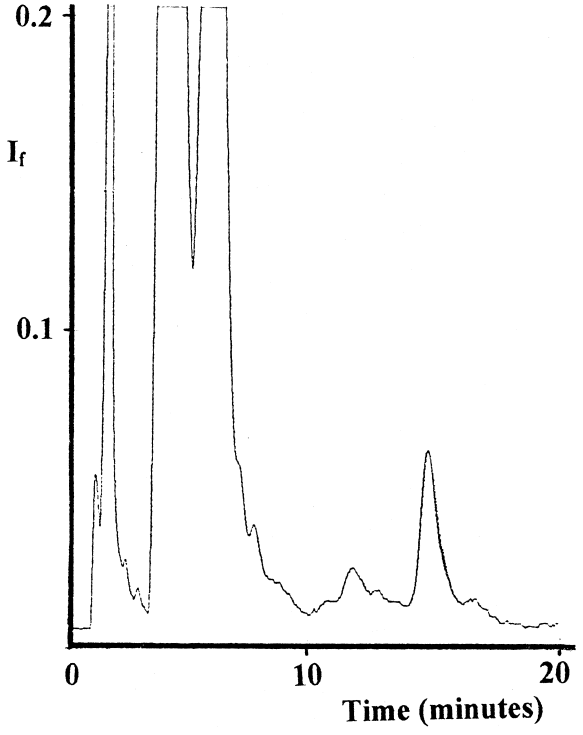

Fig. 6. Chromatogram of a patient plasma sample, analysed by means of the HPLC–FL method. Reboxetine retention time: 14.3 min. I_f=fluorescence intensity.

Further assays are in progress, in order to investigate the chemical–clinical correlations of reboxetine treatment, and to apply the methods to the clinical monitoring of depressed patients. The fluorimetric detection method, due to its high sensitivity, will be used to carry out pharmacokinetic studies.

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