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Chiral HPLC Analysis of Milnacipran and Its FMOC-Derivative on Cellulose-Based Stationary Phases

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ABSTRACT The HPLC enantioseparation of the last generation antidepressive drug milnacipran (±)-**1** was investigated on different cellulose-based chiral stationary phases (CSPs). On carbamate-type columns, Chiralcel OD and OD-H (±)-**1** could be separated with α value about 1.20 but the resolution was quite low because of the tailing of the peaks. Direct determination of (±)-**1** with high selectivity and resolution was obtained on Chiralcel OJ in normal phase mode elution. Precolumn derivatization of milnacipran with Fmoc-Cl gave compound (±)-**2** which was enantioseparated on all the investigated CSPs and allowed enhanced UV or fluorimetric detection. The Chiralpak IB, that could be considered the immobilized version of Chiralcel OD-H, was found completely ineffective in the chiral recognition of (±)-**1** and moderately efficient in the separation of (±)-**2**. *Chirality 20:63–68, 2008.*

KEY WORDS: milnacipran; chiral drug; enantioselective HPLC; cellulose-based chiral stationary phase; Fmoc-derivative

INTRODUCTION

Milnacipran [(±)-*Z*-diethylaminocarbonyl-2-aminomethyl-1-phenylcyclopropane, (±)-**1**] is an antidepressive drug, belonging to the class of selective serotonin reuptake inhibitors, developed and marketed as the racemic hydrochloride salt by Pierre Fabre Medicament (Ixel®, Dalcipran®). It shows an equipotent inhibitory action on serotonin and noradrenaline neuronal reuptake systems[1] and a total lack of affinity for neurotransmitter receptors, thus giving a similar efficacy to the triciclic antidepressant in the treatment of clinical depression but with fewer side effects.[2] The therapeutical potential of (±)-**1** could be related to its activity as *N*-methyl-D-aspartate receptor antagonist,[3] and recently it has also been found effective in relieving the chronic pain associated with fibromyalgia.[4]

Although some synthetic methodologies for the preparation of enantiomerically enriched **1** have been reported,[5–8] pharmacokinetic and bioavailability studies of milnacipran have been mainly carried out on the racemic form.[9] The importance of pharmacokinetic studies on the separate enantiomers of a chiral drug, also in cases where a given drug is marketed as racemate, is well documented[10,11] and there is a continuous demand for the development of suitable enantioselective analytical and/or preparative methods.[12,13]

The achiral determination of milnacipran in plasma has been previously carried out by reversed-phase HPLC analyses on C_{18} columns using UV or fluorimetric detection[14–16] or by micellar electrokinetic capillary chromatography.[17] In some articles dealing with the asymmetric synthesis of milnacipran, the optical purity of the target product has been indirectly determined from chiral HPLC analysis of its lactone precursor.[6,8] To date, the only reported enantioselective separation of (±)-**1** has been obtained by capillary electrophoresis[18] and we decided to develop a chiral HPLC method as a useful alternative.

The present article deals with the investigation of the direct enantioseparation of (±)-**1** as well as its Fmoc-derivative, (±)-**2** (Figure 1), on different cellulose-based chiral stationary phases (CSPs). Beyond the analysis of biological samples from patients (or animals) administered with the drug, this study can be useful in all the cases where it is necessary to check the optical purity of milnacipran (e.g., asymmetric synthesis, analysis of enriched formulation of the drug) or for semipreparative purpose.

EXPERIMENTAL PROCEDURES

Chromatographic Conditions and Procedures

Chromatography was performed on a Dionex Summit HPLC equipped with a P680A LPG/4 pump, a UVD 170U UV/Vis 4-channel detector, a TCC-thermostatted column compartment, and ASI-100 autosampler. Typical injection volume was 20 μl. The analyses were carried out at constant temperature of 25°C with simultaneous detection at λ = 220, 235, 250, and 266 nm and the chromatographic

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data were collected on a computer running Chromeleon CHM-1 version 6.7 software. The Chiralcel OJ and Chiralcel OD (both 250 × 4.6 mm I.D., 10 μm) columns and Chiralcel OD-H and Chiralpak IB (both 250 × 4.4 mm I.D., 5 μm) columns were purchased from Daicel Chemical Industries (Tokyo, Japan).

The dead time (t_0) was obtained from the elution time of the unretained 1,3,5-tri-*iso*-propylbenzene and the retention factor was determined as $k_i = (t_{Ri} - t_0)/t_0$. The resolution was calculated from the USP formula $R_s = 2(t_{R2} - t_{R1})/(w_2 + w_1)$ where t_{Ri} and w_i are the retention time and the baseline width of each enantiomer. A conditioning time of 90 min at least, applied whenever the eluent composition or the temperature were changed, was found essential to obtain data reproducibility. Retention times were mean values of three replicate determinations.

On Chiralcel OJ, eluting with *n*-hexane:EtOH 90:10 at flow rate 1.0 ml/min, the linearity of the response was assessed in the range 1.5 μg/ml−1.0 mg/ml concentration of each enantiomer using nine different concentrations and three replicates for each point. The limit of detection (LOD) and quantification (LOQ) were determined by calibration curve method.[19] Solutions containing each enantiomer in the range 0.5–1.0 μg/ml were analyzed in triplicate and the average areas plotted against concentration. The slope S of the obtained curve and the calculated residual variance σ due to the regression were then applied in the equations: $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$.

Precision of the method was determined as injection repeatability at three assay concentrations (30,125, and 500 μg/ml of each enantiomer).

For the percentage recovery determination, samples of human plasma were spiked with known amounts of (±)-**1** hydrochloride on three concentration levels and extracted according to a procedure specifically described for milnacipran.[16]

Sample Preparation

In a mortar, tablets of the pharmaceutical product were pulverized and the powder suspended in 2 N NaOH aqueous solution and stirred for 30 min. The suspension was then extracted with AcOEt and the organic phase dried on Na_2SO_4. After filtration, the solution was taken to dryness to give (±)-**1** as pale yellow oil, with analytical purity 95%. Purity >98% was achieved after column chromatography on silica gel (AcOEt:MeOH 1:1). Standard solutions, prepared by dissolving 3–5 mg of analyte in 10 ml of chromatographic eluent, were filtered through a 0.45 μm membrane filter before the injection.

The extraction of (±)-**1** hydrocloride, required to spike human plasma samples in the recovery experiments, was carried out by suspending the pulverized tablets of the pharmaceutical product in MeOH, stirring the suspension for 30 min, and removing the solid by filtration. The solution was then taken to dryness affording (±)-**1** hydrocloride as white solid with >95% purity.

For the derivatization with 9-fluorenyl-methoxycarbonyl chloride (Fmoc-Cl), a sample of (±)-**1** (1.0 mg, 4 μmol) was dissolved in 200 μl of a 2:1 v/v mixture dioxane: saturated $NaHCO_3$ aqueous solution and the reagent (1.2 mg, 4.4 μmol) was added. The immediate formation of a white precipitate was observed and after 5 min the mixture was diluted with saturated NH_4Cl and extracted with AcOEt (3 × 1.0 ml). The organic phase was collected and dried over Na_2SO_4. The solvent was then removed under a stream of nitrogen and the residue dissolved in the HPLC eluent.

In a separate batch, (±)-**2** was purified on silica gel column (*n*-hexane:AcOEt 7:3) and its identity confirmed by ^{1}H-NMR (400.13 MHz, $CDCl_3$): δ 0.85 (3H, t, J = 7.2 Hz), 1.14 (4H, bt), 1.54 (1H, m), 1.59 (1H, m), 2.84 (1H, ddd, J = 3.2, 8.9 and 14.2 Hz), 3.33 (2H, m), 3.49 (2H, m), 3.86 (1H, ddd, J = 4.5, 8.7 and 14.2 Hz), 4.24 (1H, m), 4.36 (2H, m), 6.21 (1H, bd), 7.22–7.40 (9H, m), 7.63 (2H, m), 7.76 (2H, d, J = 8.0 Hz).^{13}C-NMR (100.03 MHz, $CDCl_3$): δ 12.42 (CH_3), 12.97 (CH_3), 17.55 (CH_2), 28.85 (CH), 33.99 (C), 39.36 (CH_2), 41.80 (CH_2), 42.98 (CH_2), 47.29 (CH), 66.68 (CH_2), 119.83 (CH), 125.18 (CH), 125.65 (CH), 126.59 (CH), 126.96 (CH), 127.52 (CH), 128.73 (CH), 140.45 (C), 141.24 (C), 144.08 (C), 156.54 (CO), 170.38 (CO).

RESULTS AND DISCUSSION

Direct Enantioseparation of (±)-1

Milnacipran (±)-**1** was extracted as free base from the commercial drug formulation, and its identity and purity were checked by ^{1}H- and ^{13}C-NMR analyses. Among the commercially available cellulose-based CSPs, the tris-(3,5-dimethylphenylcarbamate)cellulose derivative (Chiralcel OD) and the tris-(4-methylbenzoate)cellulose-derivative (Chiralcel OJ), both coated with silica gel, are the leader chiral selectors allowing the resolution of a large variety of racemic samples. The recently marketed Chiralpak IB, where the same polymer of Chiralcel OD is chemically bonded to a silica support, has also been included in this study to compare the performances of these two CSPs and gain information on the recognition mechanism. Furthermore, due to the polysaccharide immobilization, on Chiralpak IB it is possible to use a broader range of solvents as mobile phases thereby introducing additional parameters for the optimization of the enantioseparation.[20]

On Chiralcel OD column, the elution with *n*-hexane:2-PrOH mixtures gave a partial separation of milnacipran enantiomers with concomitant low resolution due to the tailing of the peaks, which was found unaffected by the increase in the flow rate and variation of temperature. Changing the alcohol modifier in the mobile phase with EtOH was detrimental for the separation since (±)-**1** was eluted as a very broad single peak. (Table 1, entries 1–2).

Comparable unsatisfactory results were also achieved on Chiralcel OD-H column with *n*-hexane:2-PrOH mixture, despite of the smaller (5 μm) particle size with respect to Chiralcel OD (entry 3). The use of diethylamine (DEA) up to 0.2% as additive in the eluent to suppress the strong interaction of the analyte with the CSP did not improve the separation and resolution (entries 4–5). Furthermore, higher concentration of a basic additive in the eluent was not allowed due to the sensible increase in the background level which led to severe limitation in the detection of the analyte, which has low UV absorption at λ > 220 nm.

TABLE 1. Chromatographic parameters for the separation of (±)-1 on cellulose-based CSPs[a]

Entry	Column	Eluent	Flow	k_1	α	R_s
1	Chiralcel OD	*n*-hexane:2-PrOH 90:10	0.7	3.91	1.20	0.63
2		*n*-hexane:EtOH 90:10	0.7	2.29	1.00	–
3	Chiralcel OD-H	*n*-hexane:2-PrOH 90:10	0.7	3.85	1.23	0.97
4		*n*-hexane:2-PrOH 90:10 (+0.1% DEA)	0.7	3.94	1.21	0.83
5		*n*-hexane:2-PrOH 90:10 (+0.2% DEA)	0.7	4.05	1.22	0.84
6		*n*-hexane:EtOH 98:2	1.0	15.09	1.26	0.98
7		*n*-hexane:EtOH 98:2	1.5	12.54	1.24	1.32
8	Chiralcel OJ	*n*-hexane:2-PrOH 90:10	1.0	1.42	3.43	5.97
9		*n*-hexane:EtOH 90:10	1.0	1.12	2.65	6.39
10		*n*-hexane:2-PrOH 95:5	1.0	3.55	3.76	7.11
11		*n*-hexane:EtOH 95:5	1.0	2.53	3.03	7.51

[a]All the analyses were carried out at 25°C and the UV detection set at 220 nm.

However, on the Chiralcel OD-H column using EtOH as alcohol modifier a separation of (±)-**1** was obtained and more symmetrical peaks were observed. The separation profile was found sensitive to changes in the flow rate and good performances were obtained with *n*-hexane:EtOH 98:2 at 1.5 ml/min flow rate (Table 1, entries 6–7).

On the immobilized carbamate-type CSP Chiralpak IB (±)-**1** was highly retained, so that a 20% at least alcohol concentration in the mobile phase was required, without selectivity; a single broad peak was observed in all the tested conditions using *n*-hexane/alcohol (2-PrOH or EtOH) eluents. Since in both Chiralcel OD, Chiralcel OD-H, and Chiralpak IB the cellulose is derivatized with the same carbamate group, the hydrogen-bond interactions between the selectand and the selector, that are considered the main factor in determining the analyte enantiodifferentiation,[21] should be the same and give a comparable selectivity. However, it is evident that other factors, not easily understood due to the unknown exact chemical structure of the immobilized selector, are responsible for the observed differences in selectivity.

Nonconventional solvent mixtures of 20% EtOH with *tert*-butyl methyl ether or chloroform as well as the recently developed polar-mode elution[22] were also unsuccessful in the enantioseparation of (±)-**1** on Chiralpak IB. Furthermore, some eluent mixtures developed high back pressure in the column giving some limitation in the allowable flow rate.

Large values of selectivity and resolution were found on the benzoate-type CSP Chiralcel OJ and the separation profiles were influenced by the nature of the alcohol in the mobile phase; higher selectivities were obtained using 2-PrOH whereas better resolution values were measured with EtOH, which is more efficient in suppressing the peak tailing (Table 1, compare entries 8–9 and 10–11). The absolute configuration (1*R*, 2*S*) of the first eluting enantiomer (−)-**1** was determined by collecting single enantiomers and measuring their optical activity.[5]

The enantioseparation of (±)-**1** on Chiralcel OJ was found linear in the range 1.5 μg/ml − 1 mg/ml concentration of each enantiomer, the calibration curves showing excellent linearity for both (+)-**1** and (–)-**1** with correlation coefficient (R^2) >0.999. LOD = 0.21 and 0.23 μg/ml and LOQ = 0.64 and 0.70 μg/ml were determined for (−)-**1** and (+)-**1** respectively. Since the analyses were carried out with a 20 μl injection volume, the found LOQs correspond to 13 ng for (−)-**1** and 14 ng for (+)-**1** as absolute amount. Precision (% RSD) of injection repeatability was 0.31 for (–)-**1** and 0.41 for (+)-**1**.

The averaged extraction recovery from human plasma was found 75% for both enantiomers of milnacipran, confirming the data obtained in the achiral quantification of this drug.[16]

Representative chromatograms of the different retention behavior of (±)-**1** on the four CSPs here investigated are shown in Figure 2.

Enantioseparation of (±)-2

Because of the high values of α and R_s observed on Chiralcel OJ an easy scale up of the direct separation of

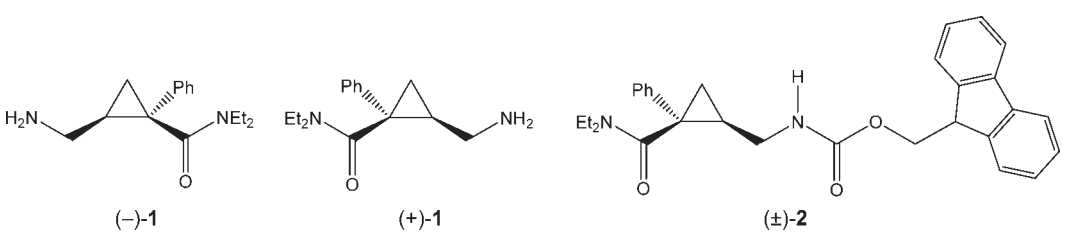

Fig. 1. Chemical structures of milnacipran enantiomers and the Fmoc-derivative.

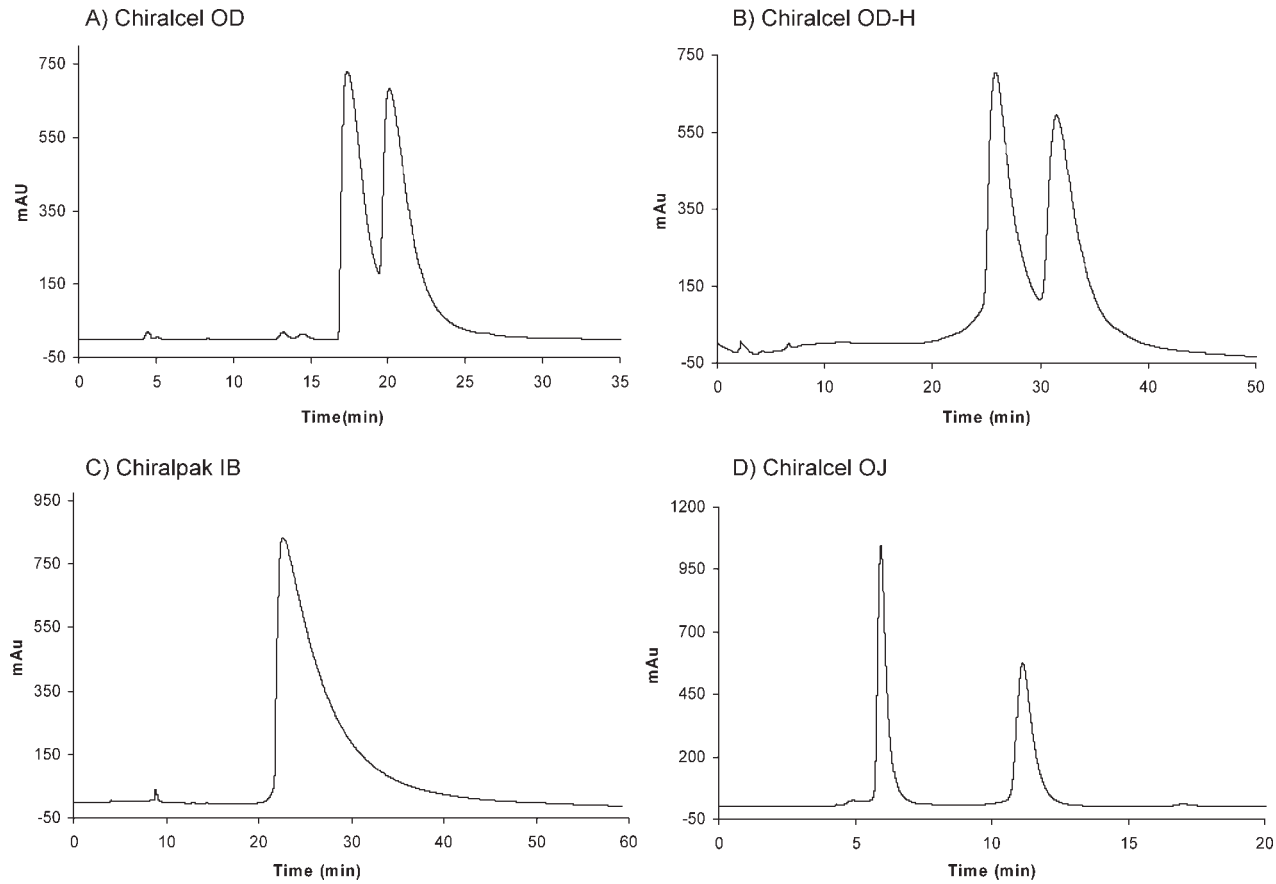

Fig. 2. HPLC chromatograms of the analysis of **(±)-1** on different columns. (**A**) *n*-hexane:2-PrOH 90:10, flow rate 0.7 ml/min; (**B**) *n*-hexane:EtOH 98:2, flow rate 1.5 ml/min; (**C**) *n*-hexane:EtOH 80:20, flow rate 0.7 ml/min; (**D**) *n*-hexane:EtOH 90:10, flow rate 1.0 ml/min.

(±)-**1** on a semipreparative column seems reasonable; on the contrary quantitative detection of the milnacipran enantiomers in biological samples, as required in the pharmacokinetic studies, could be limited by the UV absorption features of the analyte.

The precolumn derivatization of primary amines to give a fluorescent compound is a well established protocol in liquid chromatography to enhance the detection limit. Derivatization of (±)-**1** also appeared as a useful means for improving the determination of this drug on the carbamate-type CSP here investigated, whose low-selective interaction with the analyte could be due to the free aminic group present in (±)-**1**. So, although we did not have a fluorimetric detector, the enantioseparation of the Fmoc derivative of milnacipran, the compound (±)-**2**, was evaluated using UV-detection.

Our choice was based on the good stability of (±)-**2** in organic solvent and the hypothesis that the conversion of an aminic group into a carbamate one, structurally able to form complementary hydrogen-bonds with the carbamate-derivatized cellulose, should give a more enantiodifferentiated analyte on this CSP.

Indeed, a baseline separation of the enantiomers of (±)-**2** was obtained on all the tested columns with *n*-hexane:EtOH mixtures in the optimized conditions reported in Table 2 and a remarkable increase in selectivity and resolution was observed on Chiralcel OD changing the alcohol modifier with 2-PrOH. However, also in this case the immobilized carbamate-type Chiralpak IB was found to be sensibly less efficient with respect to the coated analogue Chiralcel OD (see Fig. 3).

Compound (±)-**2** was quantitatively formed in 5 min reaction and the excess of reagent as well as the side product Fmoc-OH did not interfere in the determination of milnacipran.

To date, there is only one report describing the enantiomer resolution of some Fmoc-α-aminoacids derivatives on Chiralcel columns.[23]

TABLE 2. Chromatographic parameters for the separation of (±)-2 on cellulose-based CSPs

Column	Eluent	k_1	α	R_s
Chiralcel OD	*n*-hexane:2-PrOH 80:20[a]	12.95	1.51	4.52
	n-hexane:EtOH 80:20[a]	4.50	1.13	1.59
Chiralpak IB	*n*-hexane:EtOH 80:20[b]	3.23	1.07	1.30
Chiralcel OJ	*n*-hexane:EtOH 90:10[b]	3.87	1.57	3.11

[a]Flow rate 0.7 ml/min.
[b]Flow rate 1.0 ml/min.

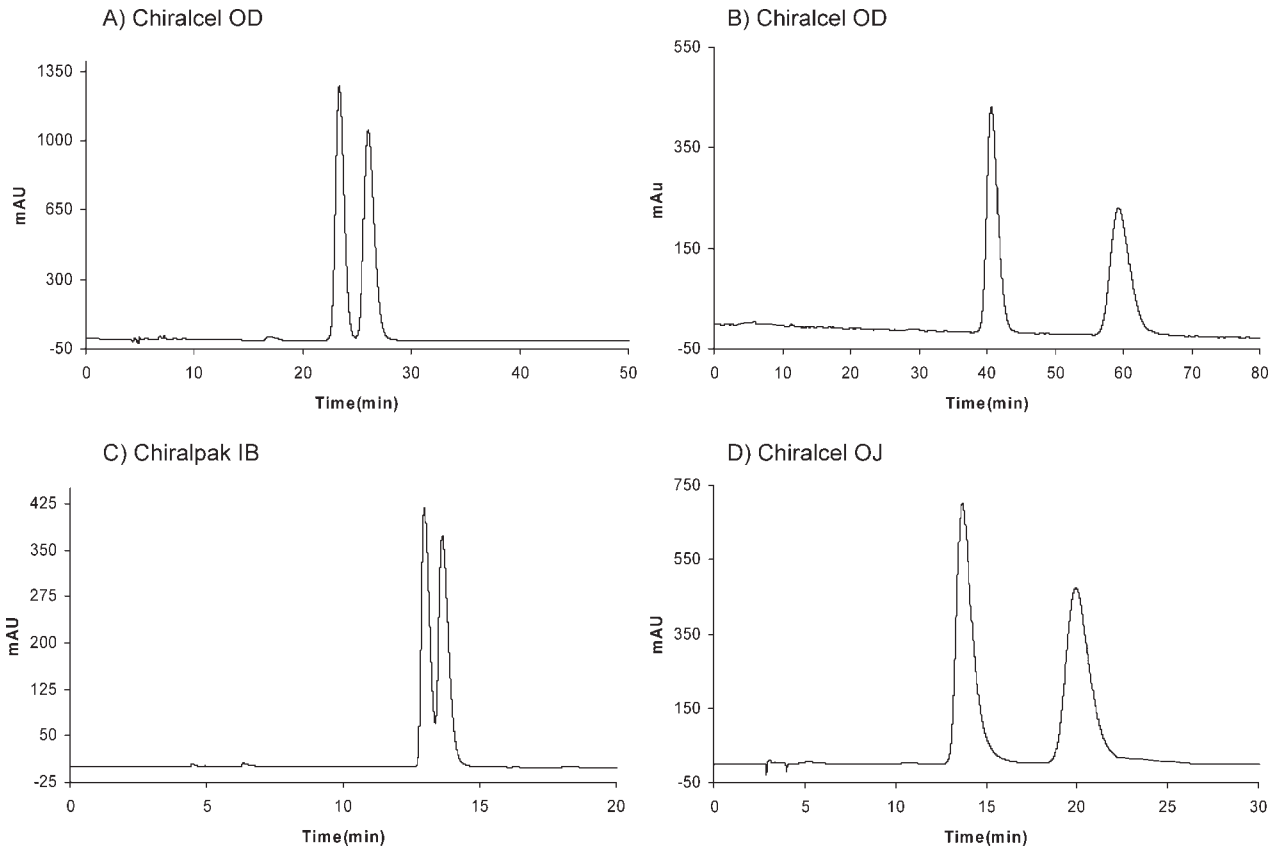

Fig. 3. HPLC chromatograms of the enantioseparation of (±)-**2** on different columns. **(A)** *n*-hexane:EtOH 80:20, flow rate 0.7 ml/min; **(B)** *n*-hexane:2-PrOH 80:20, flow rate 0.7 ml/min; **(C)** *n*-hexane:EtOH 80:20, flow rate 0.7 ml/min; **(D)** *n*-hexane:EtOH 90:10, flow rate 1.0 ml/min.

Although maximum sensitivity in the determination of (±)-**2** could be obtained via fluorimetric detection, from the comparison of the UV spectra of (±)-**1** and (±)-**2** reported in Figure 4, it seems evident that the analysis of milnacipran as Fmoc-derivative can be advantageously carried out also by UV-detection at $\lambda = 220$ nm, with about a two-fold increase of the molar extinction coefficient, or $\lambda = 266$ nm where interferences of biological matrix should be minimized.

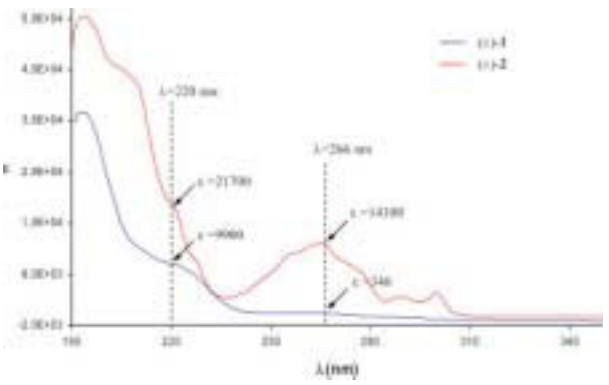

Fig. 4. UV spectra of milnacipran (±)-**1** and its Fmoc-derivative (±)-**2**. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CONCLUSIONS

The first enantioselective HPLC method for the direct determination of the enantiomers of the antidepressive drug milnacipran can be considered developed. On Chiralcel OJ column, eluting with *n*-hexane-alcohol mixtures without any basic additive, high selectivity and resolution were obtained in short analysis time and the method was found simple, linear, and precise.

Precolumn derivatization of (±)-**1** with Fmoc-Cl and subsequent analysis shows advantageous enhanced sensitivity of the assay with UV- or fluorescence detection and allows good enantioseparation also on carbamate-type Chiralcel columns, which were ineffective in the direct determination of (+)- and (−)-**1**. The structural features of the Fmoc group could be generally exploited for the enantioseparation of amines without any additive addition in the mobile phase.

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