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Automated determination of tramadol enantiomers in human plasma using solid-phase extraction in combination with chiral liquid chromatography

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Abstract

A sensitive and automated method for the separation and individual determination of tramadol enantiomers in plasma has been developed using solid-phase extraction (SPE) on disposable extraction cartridges (DECs) in combination with chiral liquid chromatography (LC). The SPE operations were performed automatically by means of a sample processor equipped with a robotic arm (ASPEC system). The DEC filled with ethyl silica (50 mg) was first conditioned with methanol and phosphate buffer, pH 7.4. A 1.0-ml volume of plasma was then applied on the DEC. The washing step was performed with the same buffer. The analytes were eluted with 0.15 ml of methanol, and 0.35 ml of phosphate buffer, pH 6.0, containing sodium perchlorate (0.2 *M*) were added to the extract before injection into the LC system. The enantiomeric separation of tramadol was achieved using a Chiralcel OD-R column containing cellulose tris-(3,5-dimethylphenylcarbamate) as chiral stationary phase. The mobile phase was a mixture of phosphate buffer, pH 6.0, containing sodium perchlorate (0.2 *M*) and acetonitrile (75:25). The mobile-phase pH and the $NaClO_4$ concentration were optimized with respect to enantiomeric resolution. The method developed was validated. Recoveries for both enantiomers of tramadol were about 100%. The method was found to be linear in the 2.5–150 ng/ml concentration range [r^2=0.999 for (+)- and (−)-tramadol]. The repeatability and intermediate precision at a concentration of 50 ng/ml were 6.5 and 8.7% for (+)-tramadol and 6.1 and 7.6% for (−)-tramadol, respectively. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Tramadol

1. Introduction

Tramadol hydrochloride, (±)-*trans*-2-[(dimethylamino)methyl] - 1 - (3 - methoxyphenyl)cyclo - hexanol (cf. Fig. 1) is a centrally acting analgesic drug used in therapy as the racemate of the *trans*-isomer, the latter being more active than the *cis*-isomer [1]. On the other hand, it has also been demonstrated that the (+)-form of *trans*-tramadol is approximately ten-fold more active than the (−)-form [1].

The biotransformation of tramadol in man and animals was studied and the main metabolites of tramadol were found to be *N*-desmethyltramadol and *O*-desmethyltramadol [2] (cf. Fig. 1). Of these two

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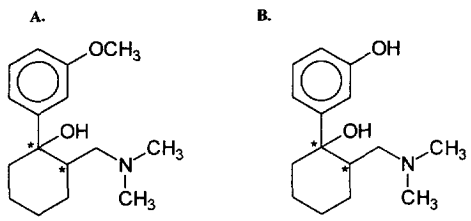

Fig. 1. Structure of tramadol (A) and *O*-desmethyltramadol (B). Stereogenic centres are marked with asterisks.

metabolites, only the *O*-desmethyl compound is pharmacologically active.

The methods described for the determination of tramadol in biological samples involve gas chromatography with a nitrogen-selective detector [3], gas chromatography–mass spectrometry [4] or liquid chromatographic (LC) methods coupled to UV [2,5] or fluorometric detection [6]. The determination of the enantiomers of tramadol and of its main metabolites in urine was also achieved by chiral LC, using two chiral stationary phases (Chiralpak AD and Chiralcel OD) in the normal-phase mode [5].

The sample clean-up of the biological samples was based in all cases on liquid–liquid extraction after alkalization [2–6]. In some methods, an aqueous acidic solution was used for back-extraction of tramadol from the organic phase [3,4]. Such an approach is often tedious and time-consuming and an interesting alternative consists in the isolation of the analytes by solid-phase extraction (SPE) using disposable extraction cartridges (DECs) [7–12].

Cellulose tris-(3,5-dimethylphenylcarbamate) (Chiralcel OD-R), which was selected as chiral stationary phase (CSP) in the present work, was previously used in the reversed-phase mode for the enantioseparation of various drugs by LC, with mixtures of aqueous buffer and organic modifier as mobile phases [13–19].

The fully automated method developed here for the determination of tramadol enantiomers in human plasma involves sample handling by SPE, on-line injection of the extracts into the LC system, and subsequent fluorometric detection of the analytes. The type of SPE sorbent and the minimum volume of eluent have been optimized with respect to selectivity and analyte recovery [12].

Different types of CSPs have been tested. The effects of mobile phase pH and sodium perchlorate concentration on chiral resolution have been more particularly investigated, using Chiralcel OD-R as CSP, in order to deduce the most suitable conditions for the LC enantioseparation of tramadol. Finally, the method has been validated.

2. Experimental

2.1. Chemical and reagents

Tramadol hydrochloride was kindly supplied by the Research and Development Department of the Pharmaceutical Company SMB (Brussels, Belgium). The pharmacologically active metabolite *O*-desmethyltramadol hydrochloride and the (+)- and (−)-tramadol enantiomers as hydrochloride salts were a gift from Prof. G. Blaschke (University of Münster, Münster, Germany).

Sodium dihydrogenphosphate dihydrate, disodium hydrogenphosphate dihydrate, sodium hydroxide, phosphoric acid, sodium perchlorate monohydrate were all of analytical grade from Merck (Darmstadt, Germany). Methanol and acetonitrile were of HPLC grade from Fischer Scientific (Loughborough, UK). Water used in all experiments was of Milli-Q quality (Millipore, Bedford, MA, USA). Bond Elut DECs (1-ml capacity) filled with 50 mg of ethyl silica (C2) were obtained from Varian (Harbor City, CA, USA). Other Bond Elut DECs filled with 50 mg of octadecyl (C18), octyl (C8), cyanopropyl (CN) or phenyl silica (Ph) were also tested.

The chiral stationary phase used for the enantioseparation of tramadol was a Chiralcel OD-R column (250×4.6 mm I.D.) packed with cellulose tris-(3,5-dimethylphenylcarbamate) coated on silica (10 μm), from Daicel Chemical Industries (Tokyo, Japan). The latter was preceded by a LiChroCart guard column (4×4 mm I.D.), packed with Lichrospher 100 DIOL (5 μm) from Merck.

The other CSPs tested in this study were an Ultron ES-OVM column (150×4.6 mm I.D.) containing chicken ovomucoid chemically bonded to aminopropylsilica (5 μm) (Shinwa, Kyoto, Japan) and a Chiradex column (250×4 mm I.D.) from Merck in which β-cyclodextrin is covalently bonded to silica gel (5 μm). These two CSPs were also preceded by a

guard column from Merck, packed with a diol-bonded phase.

2.2. Apparatus

The liquid chromatographic system consisted of a Model 305 pump (Gilson, Villiers-le-Bel, France), a Model 200 programmable wavelength UV detector from Spectra-Physics (San Jose, CA, USA) and a Model F-1050 fluorescence detector from Merck–Hitachi equipped with a mercury–xenon lamp (200 W). The UV detector was set at 220 nm and the fluorometer was set at an excitation wavelength of 230 nm and an emission wavelength of 295 nm [6].

The ASPEC system (Automated Sample Preparation with Extraction Cartridges) from Gilson consisted of an automatic sampling injector module, a Model 401 dilutor-pipettor and a set of racks and accessories for handling DECs, plasma samples and solvents [9,10]. The analytical columns were maintained at 30±0.1°C in a Model 12 B/VC Julabo water-bath (Seelbach, Germany). An IBM-compatible computer (CPU type 80486) equipped with GME-715 version 1.3 (HPLC system controller) and GME-718 version 1.1 (sample manager) software from Gilson was used to control the LC and ASPEC systems, respectively.

2.3. Chromatographic technique

All chromatographic experiments were carried out in the isocratic mode. For the determination of suitable conditions for the enantiomeric separation of tramadol, the mobile phases consisted of mixtures of 50 m*M* phosphate buffer and methanol or acetonitrile. When the Chiralcel OD-R column was used, sodium perchlorate was added to the phosphate buffer, the pH of which was adjusted with sodium hydroxide.

The mobile phase used for the separation of the enantiomers of tramadol and *O*-desmethyltramadol consisted of a mixture of a 50 m*M* phosphate buffer, containing sodium perchlorate (0.2 *M*) adjusted to pH 6.0 with sodium hydroxide solution, if necessary, and acetonitrile (75:25 v/v).

Before use, all mobile phases were degassed for 15 min in an ultrasonic bath. The flow-rate was 0.6 ml/min using Chiralcel OD-R as CSP and 0.9 ml/min with Chiradex and Ultron ES-OVM columns.

2.4. Standard solutions

2.4.1. Solutions used for method development

A stock solution of tramadol hydrochloride racemate was prepared by dissolving 5 mg of the compound in 50 ml of water. The solution used for method development was prepared by diluting the stock solution in order to obtain a final concentration of 2 μg/ml for racemic tramadol. The stock solution of *O*-desmethyltramadol racemate was prepared by dissolving 5 mg of the compound in 50 ml of water.

2.4.2. Solutions used for method validation

A stock solution of (+)-tramadol hydrochloride was prepared by dissolving 5 mg of this enantiomer in 10 ml of water. This solution was then diluted with water to obtain a final concentration of 1 μg/ml. The solution of (−)-tramadol hydrochloride was prepared in the same way.

Two mixed solutions of tramadol and *O*-desmethyltramadol hydrochloride (racemate) were prepared by diluting stock solutions of racemic tramadol and *O*-desmethyltramadol with water to achieve concentrations of 10 and 1 μg/ml, respectively. These two solutions were used to spike plasma samples (1.5 ml) for calibration curves (from 2.5 to 150 ng/ml for each enantiomer).

2.5. Sample preparation

The plasma sample was centrifuged at 4500 rpm for 15 min and a 1.5-ml volume of plasma was transferred manually into a vial on the appropriate rack of the ASPEC system. All the other operations were then executed automatically by the sample processor.

2.5.1. DEC conditioning (flow-rate, 6 ml/min; air volume, 0.5 ml)

The DEC holder was located above the drain cuvette (front position). The DEC was first con-

ditioned with 1.0 ml of methanol and then with 1.0 ml of phosphate buffer, pH 7.4.

2.5.2. Loading with plasma sample (flow-rate, 0.18 ml/min; air volume, 1.0 ml)

A 1.0-ml volume of plasma sample was aspirated by the autosampler needle from the corresponding vial and dispensed onto the DEC.

2.5.3. Washing (flow-rate, 1.5 ml/min; air volume, 1.0 ml)

A 1.0-ml volume of phosphate buffer, pH 7.4, was dispensed onto the DEC.

2.5.4. Elution (flow-rate, 1.5 ml/min; air volume, 0.5 ml)

The DEC holder was pushed by the needle over the collection rack. A 0.15-ml volume of methanol was applied onto the DEC and the eluate was collected in the tube positioned under the DEC.

2.5.5. Addition of buffer (flow-rate, 1.5 ml/min; air volume, 0.5 ml)

A 0.35-ml volume of phosphate buffer, pH 6.0, containing $NaClO_4$ (0.2 *M*) was passed through the DEC. The DEC holder was then replaced in its front position.

2.5.6. Mixing

The resulting eluate was successively aspirated and dispensed in the collection tube by the needle. These operations were repeated twice.

2.5.7. Injection

The whole volume of the final extract was aspirated from the collection tube and dispensed in the loop filler port. By switching of the injection valve, 200 μl of the final extract is injected into the LC column, the excess being directed to the waste.

The minimum dispensing flow-rate available (0.18 ml/min) was automatically selected for the sample loading step in order to avoid detrimental effects from the strong binding of the analytes to plasma proteins [8]. Each plasma sample was prepared individually during the LC analysis of the previous sample (concurrent mode).

3. Results and discussion

3.1. Chiral LC conditions

Three chiral stationary phases were tested in order to separate tramadol enantiomers: a β-cyclodextrin-bonded phase (Chiradex), a protein-based CSP using chicken ovomucoid (OVM) (Ultron ES-OVM) and a cellulose tris-(3,5-dimethylphenylcarbamate)-based CSP (Chiralcel OD-R). All these three CSPs could be used in the reversed-phase mode, with mixtures of aqueous buffers and methanol or acetonitrile as mobile phases, i.e. under conditions well suited to bioanalytical applications. No enantiomeric separation for tramadol was achieved on the Chiradex phase by varying the mobile phase pH and the concentration of methanol or acetonitrile. However, a partial separation ($\alpha=1.17$ and $R_s=1.1$) of the enantiomers of this compound was obtained on the Ultron ES-OVM column using a mobile phase consisting of a mixture of 50 m*M* phosphate buffer, pH 5.0, and acetonitrile (97:3, v:v) (cf. Fig. 2A). On the other hand, the resolution of tramadol enantiomers was more easily obtained on the Chiralcel OD-R column (cf. Fig. 2B) and further optimization was only performed with this CSP.

The effect of pH on the enantioseparation of tramadol was investigated (Table 1) in the range from 3 to 7. The mobile phases consisted either of a 50 m*M* phosphate buffer and acetonitrile (75:25) (Table 1A) or of a 50 m*M* phosphate buffer containing sodium perchlorate (0.2 *M*) and acetonitrile (75:25) (Table 1B). With the mobile phases without sodium perchlorate, changes in pH caused significant modifications in the retention and separation of tramadol enantiomers. Both retention and enantioselectivity were increased with increasing pH and a partial enantioseparation of tramadol was only obtained at pH 6–7 ($R_s<0.7$).

However, when sodium perchlorate was added to the mobile phase, the retention of tramadol enantiomers was only slightly increased with pH, and no significant changes in enantioselectivity were ob-

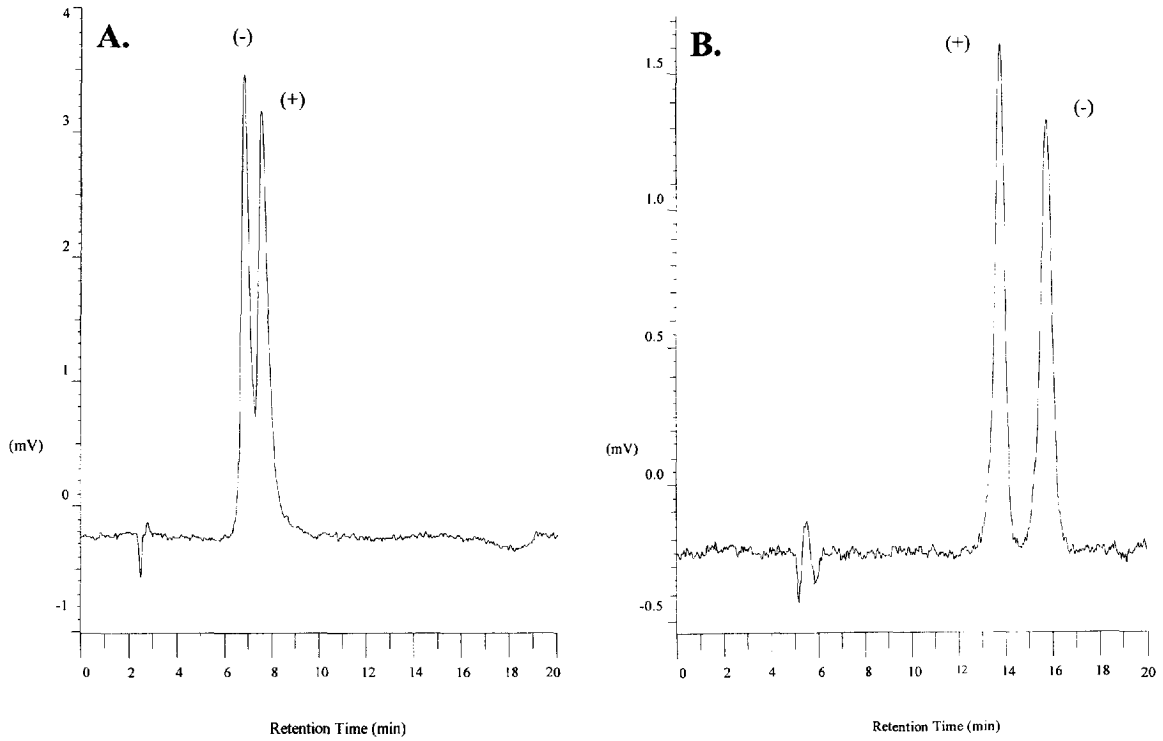

Fig. 2. Enantiomeric separation of tramadol on Ultron ES-OVM and Chiralcel OD-R CSPs. (A) Column, Ultron ES-OVM (150×4.6 mm I.D.); mobile phase, 50 m*M* phosphate buffer (pH 5.0)–acetonitrile (97:3 v/v); flow-rate, 0.9 ml/min. (B) Column, Chiralcel OD-R (250×4.6 mm I.D.); mobile phase, 50 m*M* phosphate buffer, pH 6.0, containing $NaClO_4$–acetonitrile (75:25 v/v): flow-rate, 0.6 ml/min. Temperature, 27°C; detection, UV at 220 nm; injection, 50 μl; sample, aqueous solution of racemic tramadol (2 μg/ml).

Table 1
Influence of mobile-phase pH on the retention and separation of tramadol enantiomers with Chiralcel OD-R as CSP

Mobile phase	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0
(A)					
k'_1	0.18	0.20	0.23	0.32	0.61
k'_2	0.18	0.20	0.23	0.35	0.70
α	–	–	–	1.11	1.14
R_s	–	–	–	<0.7	<0.7
(B)					
k'_1	1.44	1.44	1.47	1.61	1.67
k'_2	1.75	1.74	1.78	1.96	2.01
α	1.21	1.21	1.21	1.22	1.20
R_s	2.2	2.2	2.2	2.3	2.2

k'_1 and k'_2, capacity ratios of the first (+) and second (−) eluting enantiomer; α, selectivity factor; R_s, resolution; –, no visible enantioselectivity or resolution.

Chromatographic conditions. Stationary phase: Chiralcel OD-R (10 μm); flow-rate, 0.6 ml/min; UV detection, 220 nm; sample, aqueous solution of racemic tramadol (2 μg/ml). Mobile phases: (A) 50 m*M* phosphate buffer–acetonitrile (75:25 v/v); (B) 50 m*M* phosphate buffer containing $NaClO_4$ (0.2 *M*)–acetonitrile (75:25 v/v).

Table 2
Influence of sodium perchlorate concentration on the retention and separation of tramadol enantiomers with Chiralcel OD-R as CSP

	Concentration (*M*)						
	0	0.01	0.02	0.05	0.1	0.2	0.5
k'_1	0.32	0.53	0.69	0.92	1.22	1.63	2.08
k'_2	0.35	0.63	0.81	1.09	1.47	2.00	2.55
α	1.11	1.17	1.18	1.19	1.20	1.22	1.23
R_s	<0.7	1.1	1.3	1.7	2.0	2.4	2.5

k'_1 and k'_2, capacity ratios of the first (+) and second (−) eluting enantiomer; α, selectivity factor; R_s, resolution.
Chromatographic conditions. Stationary phase: Chiralcel OD-R (10 μm). Mobile phase: phosphate buffer, pH 6.0, containing $NaClO_4$–acetonitrile (75:25 v/v); flow-rate, 0.6 ml/min; UV detection, 220 nm; sample, aqueous solution of racemic tramadol (2 μg/ml).

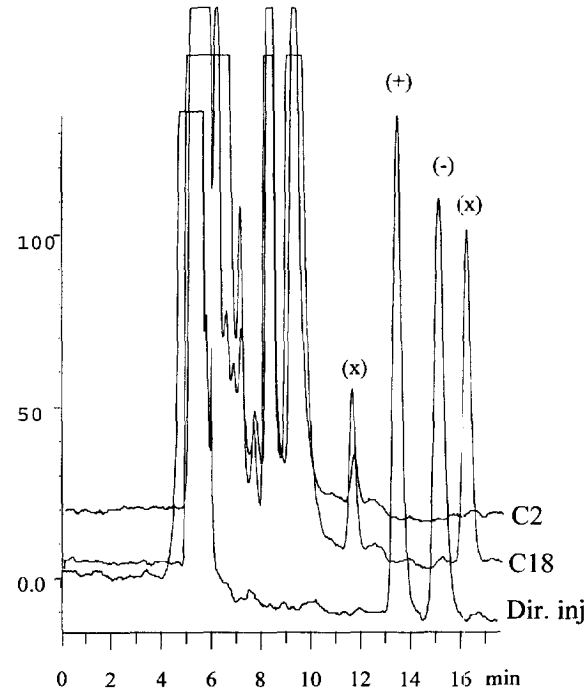

Fig. 3. Selection of SPE sorbent. Chromatograms of blank plasma obtained after clean-up on C18 and C2 DECs. Comparison with direct injection of aqueous standard solution of tramadol. x, endogenous compound.

served. A pH value of 6 was finally selected because it gave rise to slightly higher resolution.

As can be seen from Table 2, an increase in sodium perchlorate concentration led to an increase in the capacity ratios of tramadol enantiomers but also to a significant improvement in enantioselectivity and resolution. Since almost no further enhancement in resolution was obtained at sodium perchlorate concentrations higher than 0.2 *M*, the latter concentration was selected. A similar behaviour on Chiralcel OD-R by addition of sodium perchlorate was reported earlier for other chiral cationic compounds [13,18,19]. This favourable effect seems to be related to an ion-pairing process with perchlorate [13].

Besides the better enantiomeric resolution obtained with Chiralcel OD-R (cf. Fig. 3), the mobile phase used with this CSP was also more convenient for an on-line combination with the solid-phase extraction procedure because of its higher content in acetonitrile (25%). Indeed, if the concentration of organic modifier in the LC mobile phase is higher, the volume of buffer added to the methanolic extract to reduce its eluting strength can be lowered. A higher fraction of the final extract can then be injected into the LC system, which can lead to an increase in detectability [10].

Even if a baseline enantiomeric resolution of the active metabolite *O*-desmethyltramadol could not be completely achieved under these conditions, the separation was sufficient to obtain information about the relative proportion of the two enantiomers.

3.2. Selection of SPE sorbent

Five different kinds of DECs containing bonded silicas with different polarities were tested. Aqueous solutions of racemic tramadol were used as samples and the corresponding recoveries of the drug enantiomers were determined (cf. Table 3).

The conditioning and washing steps were carried out using a pH 7.4 phosphate buffer, which was found to be effective in other methods developed earlier for the determination of drugs in plasma [8–12]. The elution step was first performed with 0.35 ml of methanol and 0.65 ml of the same buffer as used in the LC mobile phase.

Under these preliminary conditions, analyte recoveries were close to 100%, except with cyano-

Table 3
Types of sorbents used in the disposable extraction cartridges (DECs)

Sorbent	Recovery of (+)-tramadol (%)	Recovery of (±)-tramadol (%)
CN	37	40
C_2	103	102
C_8	102	101
Ph	101	95
C_{18}	102	101

Conditions. DECs: Bond Elut (50 mg); conditioning, methanol–buffer, pH 7.4 (1.0 ml of each); washing, buffer, pH 7.4; elution, 0.35 ml of methanol; buffer addition, 0.65 ml of buffer (pH 6.0) containing $NaClO_4$; sample, aqueous standard solution of racemic tramadol (2 μg/ml); other conditions as given in Section 2.

propyl silica (cf. Table 3). Low recoveries observed for tramadol enantiomers with the CN phase can be explained by analyte losses during the washing step. Indeed, recoveries were found to increase with the pH of the washing solution: 70% at pH 8 and 81% at pH 9.

The selectivity of the different sorbents was evaluated by comparing chromatograms of plasma samples obtained at 220 nm under the same sample preparation conditions. Fig. 3 shows chromatograms obtained with octadecyl silica and ethyl silica as SPE sorbents. No interferences were observed for the analytes when the C2 phase was used. On the contrary, chromatograms obtained with more hydrophobic sorbents such as the C18 phase show the presence of a potential interfering peak in the vicinity of the peaks of tramadol enantiomers. Taking into account sorbent selectivity and analyte recovery, DECs filled with ethyl silica were finally selected.

3.3. Elution step

Methanol was selected as solvent for the elution step. In order to improve detectability for tramadol enantiomers, the minimum volume of methanol that still gives a satisfactory elution of these compounds was determined [12].

Fig. 4 shows the influence of methanol volume on the elution of tramadol enantiomers. Volumes equal to or higher than 150 μl gave rise to a total recovery of tramadol (absolute recovery higher than 95%). A 0.15-ml volume of methanol was therefore selected for the elution of the analytes from the DEC. After addition of 350 μl of phosphate buffer, pH 6.0, containing $NaClO_4$ (0.2 *M*) in order to obtain the same eluting strength as in the LC mobile phase, the analyte concentration in the extract is twice as high as in the plasma sample due to the reduction of the total volume from 1.0 to 0.5 ml.

3.4. Fluorometric detection

Another way of increasing detectability for tramadol enantiomers in plasma is the use of fluorometric detection [6]. With this detection mode, it was possible to reach concentrations of about 1 ng/ml for tramadol.

3.5. Validation

3.5.1. Selectivity

Fig. 5 shows a typical chromatographic trace of a plasma extract containing racemic tramadol and racemic *O*-desmethyltramadol. Under the conditions selected for the LC separation of tramadol enantiomers, the mean retention times were 7.8 and 8.2 min for the *O*-desmethyltramadol enantiomers and 13.3 and 14.9 min for (+)-tramadol and (−)-tramadol ($n=20$). The absence of interfering endogenous components at the retention times of the enantiomers of tramadol is also demonstrated in Fig. 5. The order of elution of tramadol enantiomers was determined by separately injecting solutions of each enantiomer. The order of elution of *O*-desmethyltramadol could not be determined as single enantiomers of this metabolite were not available.

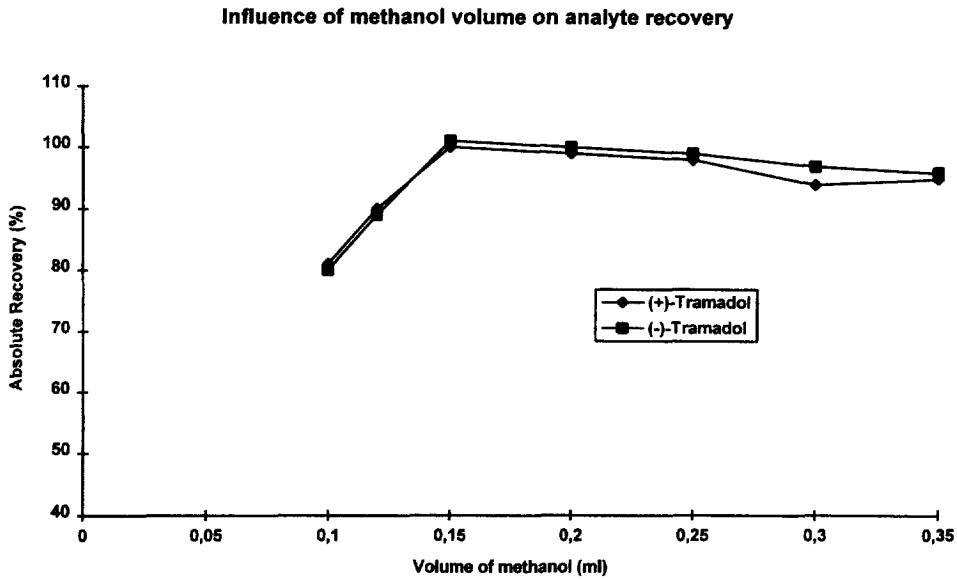

Fig. 4. Minimum volume of methanol for the elution step. DEC, Bond Elut C2 (50 mg); other conditions as given in Section 2.

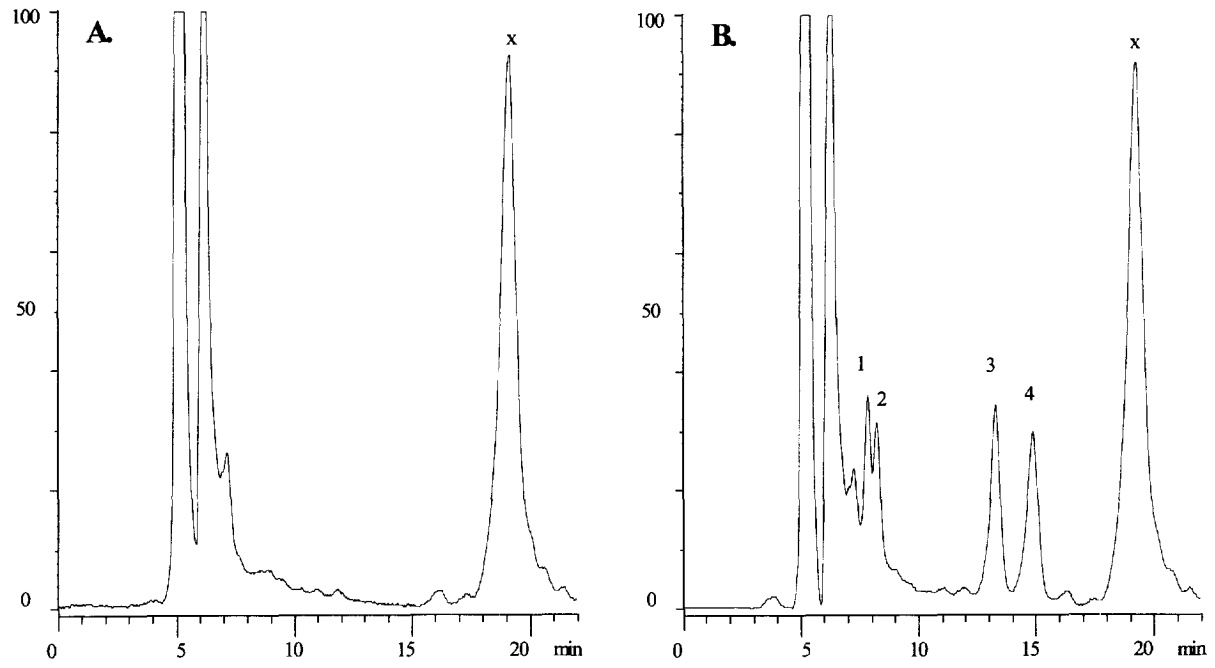

Fig. 5. Typical chromatograms obtained by using SPE extraction coupled to LC. (A) Chromatogram of blank plasma. (B) Chromatogram of plasma spiked with tramadol and *O*-desmethyltramadol (10 ng/ml for each enantiomer). SPE and LC conditions as described in Section 2, with fluorescence detection (excitation wavelength, 230 nm; emission wavelength, 295 nm). Peaks: (1) *O*-desmethyltramadol (enantiomer 1); (2) *O*-desmethyltramadol (enantiomer 2); (3) (+)-tramadol; (4) (−)-tramadol; x, endogenous peak.

3.5.2. Absolute recovery

The absolute recovery was determined by comparing peak areas obtained from freshly prepared sample extracts and those found by direct injection of an aqueous standard solution at the same concentration, using the same autosampler equipped with the same loop of 200 μl [20]. The absolute recovery for both enantiomers of tramadol was found to be about 100% (cf. Table 4).

3.5.3. Linearity

The linear regression analysis for tramadol enantiomers was made by plotting peak area (y) versus analyte concentration (x) in ng/ml in the concentration range from 2.5 to 150 ng/ml. This range seems to be appropriate, according to plasma concentrations found for racemic tramadol in pharmacokinetic studies (15–270 ng/ml) [6]. The following equations were obtained:

(+)-tramadol: $y = 2273.76x - 2426.09$ $r^2 = 0.9990$

(−)-tramadol: $y = 2125.70x - 2595.86$ $r^2 = 0.9985$

The linearity of the relationship between peak area and concentration is demonstrated by the determination coefficients (r^2) obtained for the regression lines in the case of both enantiomers of tramadol. Moreover, an analysis of variance (ANOVA) was carried out on calibration curves in order to confirm the linearity (F_1) and to test the quality of the fitting (F_2) [21]. The linearity was assessed for (+)- and (−)-tramadol with $F_{calc} \gg F_{(0.95;\ 1,\ 19)}$ (4.38), as well as the fitting, with $F_{calc} < F_{(0.95;\ 5,\ 14)}$ (2.96) (cf. Table 4).

3.5.4. Detectability

The limits of detection (LOD) and quantitation (LOQ) were determined as analyte concentrations giving rise to signal-to-noise ratios of 3 and 10, respectively. The LODs and LOQs for both enantiomers were found to be 0.5 and 1.5 ng/ml, respectively.

3.5.5. Precision

The precision of the automated bioanalytical method was determined by measuring the repeatability and intermediate precision for both enantiomers of

Table 4
Validation of the automated LC method for the determination of tramadol enantiomers in plasma

Validation criterion	(+)-Tramadol	(−)-Tramadol
Absolute recovery (mean±S.D., $n=7$)	102.3±2.0%	99.7±2.9%
Linearity ($n=7$, $k=3$) 2.5–150 ng/ml	$y=2273.76x-2426.09$ $r^2=0.9990$	$y=2125.70x-2595.86$ $r^2=0.9985$
F-test for the slope	$F_1=10256.18$	$F_1=8763.71$
F-test for fitting	$F_2=0.45$	$F_2=0.43$
LOD	0.5 ng/ml	0.5 ng/ml
LOQ	1.5 ng/ml	1.5 ng/ml
Repeatability ($n=6$; 3 days)		
2.5 ng/ml	4.7%	6.0%
50 ng/ml	6.5%	6.1%
150 ng/ml	5.0%	5.6%
Mean	5.4%	5.9%
Intermediate precision ($n=6$; 3 days)		
2.5 ng/ml	7.0%	9.1%
50 ng/ml	8.7%	7.6%
150 ng/ml	5.8%	5.6%
Mean	7.1%	7.4%
Overall accuracy ($n=18$)		
t-test for the slope	0.63	0.21
t-test for the origin	0.43	0.66

tramadol at three concentration levels, ranging from 2.5 to 150 ng/ml [21]. The mean values for repeatability and intermediate precision were 5.4 and 7.1% for the (+)-enantiomer and 5.9 and 7.4% for the (−)-enantiomer, respectively.

3.5.6. Accuracy

The overall accuracy of the procedure was assessed by plotting the analyte amount found versus the amount spiked in the plasma sample at three concentration levels ($n=6$) ranging from 2.5 to 150 ng/ml ($r^2=0.998$ for (+)- and (−)-tramadol). *t*-Tests indicated that the slopes of the regression lines were not significantly different from unity [calculated *t* values were 0.96 and 1.13 for (+)- and (−)-tramadol, respectively] and that intercepts were not significantly different from zero [calculated *t* values were 0.56 and 0.54 for (+)- and (−)-enantiomers, respectively]. The critical *t* value was 2.12 ($P=0.05$).

The automated LC procedure developed for the determination of tramadol enantiomers in human plasma using solid-phase extraction as sample preparation can therefore be considered as accurate within the concentration range investigated.

Acknowledgments

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