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Simultaneous stereoselective analysis by capillary electrophoresis of tramadol enantiomers and their main phase I metabolites in urine

Serge Rudaz[a,b], Jean Luc Veuthey[b], Claudia Desiderio[a], Salvatore Fanali[a,*]

[a]*Istituto di Cromatografia del C.N.R., Area della Ricerca di Roma, P.O. Box 10, 00016 Monterotondo Scalo (Rome), Italy*
[b]*Laboratory of Pharmaceutical Analytical Chemistry, University of Geneva, 20 Boulevard d'Yvoy, 1211 Geneva 4, Switzerland*

Abstract

Capillary zone electrophoresis was successfully applied to the enantiomeric resolution of racemic tramadol and its six phase I metabolites using carboxymethylated β-cyclodextrin (CMB) added to the background electrolyte (BGE). Baseline resolution of tramadol and its metabolites was obtained in less than 30 min using a 50 m*M* phosphate buffer (pH 2.5) containing 5 m*M* of CMB. Chiral determinations of tramadol and its main three metabolites, *O*-demethyltramadol (M1), *N*-demethyltramadol (M2) and *O*-demethyl-*N*-demethyltramadol (M5), were performed in urine after a simple double liquid–liquid extraction of 200 μl of biological material. In the tested concentration range (0.5–20 μg/ml, except for M2: 0.5–10 μg/ml) coefficients of correlation superior than 0.994 were obtained. Within-day variation determined on three different concentrations for each enantiomers showed accuracies ranging from 95.4% to 103.2%. The relative standard deviation (RSD) of these assays was determined to be less than 10.0%. Day-to-day variation presented accuracies ranging from 96.3% to 106.5% with a RSD less than 9.0%. After oral administration of 100 mg of tramadol hydrochloride to an healthy volunteer, the urinary excretion was monitored during 30 h. About 15% of the dose was excreted as unchanged tramadol. The enantiomeric ratios of all the excreted analytes, T, M1, M2 and M5, were found to be very different to 1.0, showing that a stereoselective metabolism of tramadol clearly occurred.

Keywords: Enantiomer separation; Tramadol

1. Introduction

(±)-*trans*-2[(Dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride (tramadol) is a centrally acting drug that possesses an analgesic action with a potency ranging between weak opioid and morphine. Recent clinical studies have shown that tramadol does not have a pronounced opioid side-effect profile, due to the methyl group presence on the phenolic moiety of the molecule [1]. Thus, little or no respiratory depression and no analgesic tolerance after repeated administration were observed with tramadol [2]. Experimental data suggest that tramadol may exert part of its analgesic effect through activity on the monoaminergic system [3]. Tramadol is formulated as a racemic mixture, where each enantiomer displays different binding properties for various receptors: (+)-tramadol preferentially inhibits serotonin reuptake whereas (−)-tramadol mainly inhibits noradrenalin reuptake [4–6]. This dual mode of action of tramadol, opioid and non-opioid, may contribute to its efficiency in certain chronic pain [1].

After oral administration, tramadol is rapidly absorbed reaching peak plasma concentration at mean of 2 h. About 85% of a dose of tramadol is

*Corresponding author. Tel.: +39-6-9062-5328; fax: +39-6-906-72269.
E-mail address: fanali@nserv.icmat.mlib.cnr.it (S. Fanali)

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metabolized by the liver and essentially excreted by the kidney [4,7,8]. As shown in Fig. 1, tramadol undergoes biotransformation in the liver by two main metabolic pathways to form *N*- and *O*-demethylated compounds (phase I reactions). The *O*-demethylated metabolites are further conjugated by phase II reactions [7]. All the phase I metabolites possess a chiral center. *O*- and *N*-demethylation of tramadol was found to be highly stereoselective [5]. The main metabolites are: *O*-demethyltramadol (M1), *N*-demethyltramadol (M2) and *O*-demethyl-*N*-demethyltramadol (M5). The cumulative renal excretion of *N*-bis-demethyl tramadol (M3) and *O*-demethyl-*N*-bis-demethyltramadol (M4) is inferior to 1% of the oral administered dose [7].

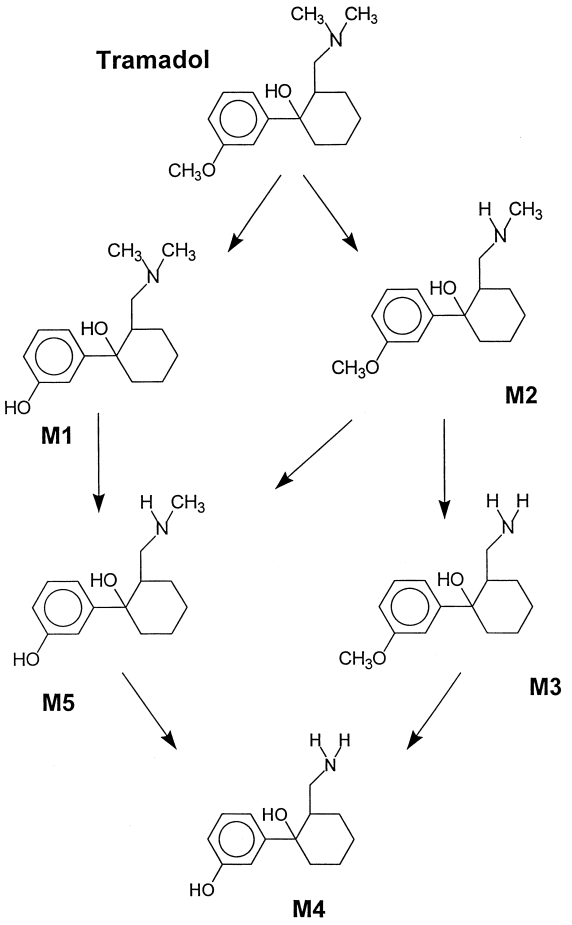

Fig. 1. Phase I metabolism of tramadol and structural formula, *O*-demethyl tramadol (M1), *N*-demethyl tramadol (M2), *N*-bis-demethyl tramadol (M3), *O*-demethyl-*N*-bis-demethyl tramadol (M4), *O*-demethyl-*N*-demethyl tramadol (M5).

Up to now, only M1 has been shown to have pharmacological activity and none of the others metabolites – phase I and II – are pharmacologically active [2,7]. It is interesting to note that the M1 production rate is carried out by the isoenzyme cytochrome called CYP 2D6 [4] and the polymorphism of this enzyme is the basis of a larger interindividual variability in the metabolism process of different drugs [8]. Tramadol appeared to be a better analgesic in extensive metaboliser than in poor metaboliser which do not form the active *O*-demethylated metabolite, M1, due to the lack of CYP 2D6 [2]. In fact, the coexistence of opioid and non-opioid mechanisms of tramadol might be explained by the pharmacological properties of M1. It has been shown that racemic tramadol or its pure enantiomers bound with weaker affinity than M1 enantiomers to cloned human (μ)-opioid receptors [9]. M1 therefore, might contribute to (or be a major source of) the opioid component of tramadol effect and thus to the therapeutic efficacy of tramadol [10].

Analytical methods so far used for the determination of tramadol include gas chromatography with nitrogen-selective nitrogen–phosphorous detection (NPD) [11], gas chromatography–mass spectrometry (GC–MS) [10,12–14] or high-performance liquid chromatography (HPLC) with UV [15,16] or fluorimetric detection [8,16,17]. Tramadol enantiomers and some of its metabolites have been separated by HPLC using chiral stationary phases [15,16]. Recently, capillary electrophoresis (CE) was used for the separation of *cis* and *trans* tramadol isomers [18] and for the stereoselective determination of tramadol [19,20].

In the last decade CE has been shown to be a powerful separation technique in a number of analytical fields, and particularly for the determination of drugs in biological fluids [21,22]. Its high resolution power and its high efficiency present several advantages in comparison with chromatographic techniques such as HPLC, particularly for the separation of complex drug metabolites mixtures [23,24]. Moreover, CE exhibited excellent results in the field of optical isomer separation, demonstrating to be a very attractive and challenging analytical tool with

respect to the other commonly used techniques [25–28].

For the enantiomeric separation, the simple method used in CE is the addition of chiral selectors such as native or derivatised cyclodextrins (CDs) into the background electrolyte (BGE) selectively modifying the mobility of the two enantiomers to form labile diastereoisomeric complexes [29–31]. In a previous study, it has been shown that the carboxymethylated β-cyclodextrin (CMB) was the most appropriate cyclodextrin for the enantiomeric determination of tramadol in commercial formulations [20]. In this study, the stereoselective determination of tramadol is extended to its phase I metabolites in order to analyze urine samples of a healthy volunteer after administration of racemic tramadol.

2. Materials and methods

2.1. Chemicals

CMB with a degree of substitution of 3.5 was a gift from Cyclolab (Budapest, Hungary). (+)- and (−)-tramadol enantiomers, racemic tramadol and racemic metabolites: *O*-demethyltramadol (M1), *N*-demethyltramadol (M2), *N*-bis-demethyltramadol (M3), *O*-demethyl-*N*-bis-demethyltramadol (M4), *O*-demethyl-*N*-demethyltramadol (M5) as hydrochloride salts were a gift from Professor P. Dayer (Clinical Pharmacology Division, Geneva University Hospital, Switzerland). Tris(hydroxymethyl)aminomethane (Tris), concentrated hydroxide ammonium solution (33% water solution of NH_3), hexane and hydrochloric acid were purchased from Carlo Erba (Milan, Italy). Phosphoric acid (85%), *tert*.-butyl methyl ether were purchased from Fluka (Buchs, Switzerland) and ethyl acetate was purchased from BDH (Poole, UK). All other reagents used were of analytical or HPLC grade. Double distilled water was used for the preparation of all solutions.

2.2. Apparatus

Electrophoretic experiments were performed in a Biofocus 3000 automatic electrophoresis apparatus (Bio-Rad, Hercules, CA, USA) equipped with a multi-wavelength UV–visible detector set at 195 nm. A thermostating liquid was used to maintain the capillary temperature at 25°C. Analysis were carried out in a fused silica capillary polyacrylamide coated 40.5 cm (effective length 36.0 cm)×50 μm I.D. prepared in our laboratory following the procedure of Schutzner et al. [32]. The BGE used for all experiments contained 50 m*M* of phosphoric acid adjusted to pH 2.5 with Tris and the appropriate concentration of chiral selector. Before use, all solutions were filtered through a 0.45-μm pore size filter (Millipore, Milford, MA, USA) and degassed in a ultrasonic bath for 10 min. At the beginning of working day, the capillary was washed with water 10 min and with buffer without chiral selector for 10 min. Between runs, capillary was treated successively with water for 100 s, with buffer without chiral selector for 200 s and running buffer containing the chiral selector for 100 s. Injection were done by pressure (5 p.s.i.= 34 473.9 Pa) as followed: (i) injection of the sample (1 s), (ii) injection of the running buffer (1 s). Vials containing anode and cathode buffer were changed before each analysis.

2.3. Standard solutions

Internal standard (I.S.): stock solutions of (+)-ephedrine hydrochloride in water containing 10 mg/ml were weekly prepared and stored at 4°C. Further dilutions of the stock solutions in water to obtain final internal standard concentration of 5 μg/ml were made daily.

Standard solution of tramadol and metabolites: stock solutions of (+)-tramadol, (−)-tramadol, racemic tramadol and racemic metabolites containing 1 mg/ml compound concentration were prepared in methanol and stored at 4°C. Standard solution of tramadol were daily prepared by appropriate dilution in water of the stock solution.

2.4. Urine samples

A blank pool urine collected at different times of the day from an healthy volunteer was used to prepare spiked urine samples. Spiked urine samples

were prepared by adding known amounts of racemic tramadol, M1, M2 and M5 to blank human urine in order to obtain a calibration series of racemic tramadol and racemic metabolites concentrations of 1.0, 2.0, 5.0, 10.0, 20.0 and 40.0 μg/ml.

A single oral dose of 100 mg of tramadol hydrochloride (two 50 mg capsules of Tramal, Grünenthal, Stolberg, Germany, equivalent to 87.85 mg of tramadol) was administered to an healthy volunteer for the urinary excretion evaluation. Urine was collected for 30 h after tramadol administration during the following intervals: 0–1, 1–2, 2–4, 4–6, 6–8, 8–10, 10–12, 12–14, 14–24 and 24–30 h. The volume and the pH of the urine were noticed and the samples were stored at −20°C until analysis.

For each interval of excretion, quantitative determination of T and its main phase I metabolites were done. Amounts of excreted compounds were obtained by multiplication of the calculated concentration by the measured excreted urine volume during the time interval. Cumulative excretion curves were obtained by addition of the amount of compounds previously obtained related to the administered dose (%). Enantiomeric ratio was calculated as the concentration ratio of the second migrated enantiomers by the first detected enantiomer.

2.5. Extraction procedure

Twenty μl of concentrated ammonium hydroxide solution, 100 μl of I.S. solution and 1 ml of an ethyl acetate–hexane (20:80, v/v) solution were added to a 200 μl aliquot of human urine in a 1.5 ml eppendorf tube. The sample was vortex mixed for 10 min. The tube containing lower urine and upper organic layer was centrifuged at 1000 rpm during 5 min then the organic layer containing analytes was removed into another clean eppendorf tube. A second extraction step was conducted by addition of 1 ml of *tert*.-butyl methyl ether on the sample, which was vortex mixed for 10 min. After centrifugation (1000 rpm, 5 min), the organic layer was removed and combined with the previous one. After evaporation to dryness under a gentle stream of nitrogen, the dry residue was redissolved in 50 μl of 0.01 *M* HCl by vortex mixing during 5 min. This extraction procedure was applied for both spiked urine sample and collected sample after drug administration.

2.6. Testing and statistical evaluation of the analytical procedure

Linearity was obtained with calibration curves constructed by analysing spiked urine sample of known concentration of racemic tramadol (T) and racemic metabolites (M1, M2 and M5). Six-point calibration ($k=6$) with six analysis ($n=6$) at each concentration levels was performed over the range 0.5 to 20 μg/ml for each enantiomers of T, M1 and M5. Five-level calibrations ($k=5$) with six analysis ($n=6$) at each concentration level were performed over the range 0.5 to 10 μg/ml for each enantiomers of M2. Normalised peak-area (area divided by migration time) ratio of tramadol and metabolite enantiomers/internal standard were measured and plotted against the theoretical concentration of the spiked standards. Least-square linear regression analysis were performed to determine correlation coefficients, slopes and intercepts. Within-day variation was assessed by six replicate determinations of three concentrations over the tested range (1.00, 5.00 and 10.00 μg/ml). Within-day accuracy was expressed as the mean of the assays relative to the theoretical values (%). The within-day precision of the method was expressed as the relative standard deviation (RSD) of the assays made for within-day accuracy. Day-to-day variation was assessed by analysing replicates of standards with the same concentration on three separate days. Day-to-day accuracy was expressed as the mean of the assays relative to the amount added (%). The day-to-day precision of the method was expressed as the RSD of the assays made for day-to-day accuracy. The recovery of the extraction was determined by comparing the normalised peak areas for tramadol and its metabolites extracted from urine and a standard solution in 0.01 *M* HCl at the expected concentration. The limit of detection (LOD), given by a tramadol enantiomer signal-to-noise ratio of 3:1, and the limit of quantification (LOQ), given by a tramadol enantiomer standard solution signal-to-noise ratio of 10, was determined from three electropherograms. The LOD

and the LOQ were determined for each enantiomer to be 0.100 μg/ml and 0.300 μg/ml, respectively.

3. Results and discussion

Due to its high efficiency and resolution power, CE seemed to be the most suitable analytical technique to obtain the simultaneous enantiomeric resolution of this complex drug metabolites mixtures with a fast method development. On the basis of previous obtained results for the enantiomeric separation of tramadol in pharmaceutical formulations, CMB was chosen as chiral selector [20].

As shown in Fig. 1, T and its phase I metabolites present structural differences which consist in a series of demethylations on the phenyl moiety or on the amine group of the parent drug. Observing the chemical structure of these analytes, it is possible to divide them as *O*-methylated compounds (T, M2, M3) and *O*-demethylated compounds (M1, M5, M4). Another classification can be done in function of the amine group methylation. In this case, couple of compounds are obtained: *N*-methylated analytes (T, M1) which are tertiary amines, *N*-mono-demethylated analytes (M2, M5) which are secondary amines and *N*-bis-demethylated analytes (M3, M4) which are primary amines. Consequently, M1 is the *O*-demethylated homologue of T, M5 the *O*-demethylated homologue of M2 and M4 the *O*-demethylated homologue of M3.

In absence of chiral selector into the BGE, it was impossible to separate the *O*-methylated compounds from their *O*-demethylated homologues. Even if a difference of molecular mass, due to the methyl presence or absence, exists into each couple of analytes, the analysis of all the compounds revealed only three peaks, corresponding to the amine group methylation. The highest mobility was observed for the primary amines (M3, M4) whereas the tertiary amines (M1, T) had the lowest mobility. The secondary amines (M2, M5) were found to have an intermediate mobility. Thus, in a non stereoselective environment, the mobilities of the analytes were depending on the *N*-methylation. In fact, the methyl are electrodonor groups and probably decreased the cationic charge of the nitrogen.

3.1. Effect of CMB on stereoselective separation

On the tested range (0–6 m*M*), the increase of CMB concentration into the BGE enhanced the separation between all compounds as well the enantiodiscrimination for each of them. At a concentration of chiral selector lower than 4 m*M*, only a partial chiral separation of M1 was obtained. Furthermore, a co-migration of the enantiomers of M4 and M2 occurred until the concentration of the chiral selector reached 5 m*M* (see Fig. 2). This concentration allowed to obtain the simultaneous enantioseparation of the tramadol and its five phase I metabolites. As indicated in Fig. 2, at a concentration of 6 m*M* of CMB in the BGE, a co-migration of M4 and M2 occurred again.

The order of migration of T and its five metabolites in presence of chiral selector was as follows: M1<T<M5<M4<M2<M3. At any concentration of chiral selector, the *N*-methylated/*O*-demethylated metabolite, M1, exhibited the shortest analysis time and the lowest enantiomeric resolution. The *N*-bis-demethylated/*O*-methylated metabolite, M3, was the slowest analyte with the highest optical isomer separation.

The *O*-methylated compounds (T, M2, M3) exhibited a stronger CD complexation than their *O*-demethylated homologues (M1, M5, M4), demonstrating the important role of *O*-methylation in the interaction with the CMB. This effect can be explained by the more hydrophobic character of the molecules due to the methyl presence on the phenolic moiety. This hydrophobicity probably induced a more stable inclusion complex with the CMB, since chiral selector cavity is known to have hydrophobic properties. On the other hand, the methyl group presence on the amine function was appeared to be important in the stereoselective recognition by the CMB for the analysed compounds. In both series of *O*-methylated compounds (T, M2, M3) or *O*-demethylated homologues (M1, M5, M4), the migration time as well as the resolution increased with the *N*-demethylation. From the previous observations, it can be concluded that the *N*-demethylation promoted secondary interaction between the CMB and the analytes, probably by the formation of hydrogen bonds, producing more stable diastereoisomeric com-

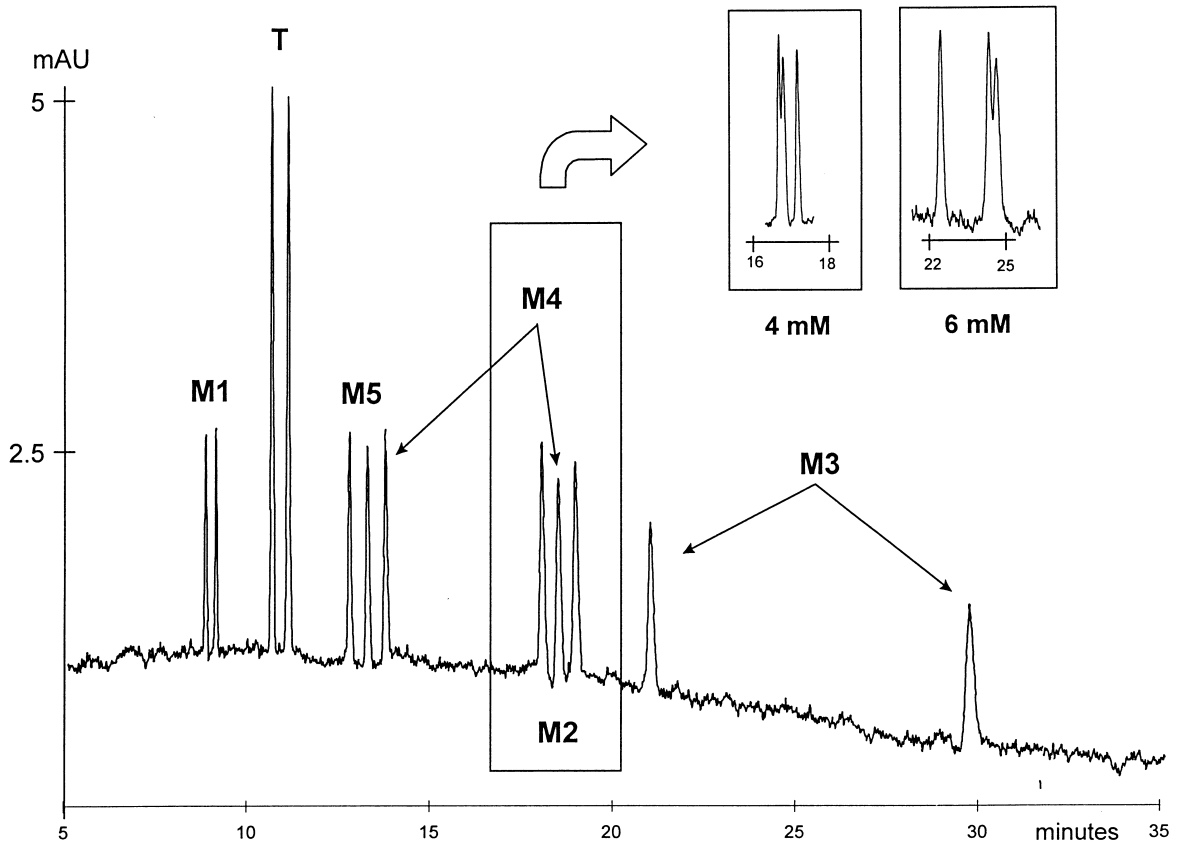

Fig. 2. Electropherogram of a standard solution of tramadol (T) and its phase I metabolites (M1, M2, M3, M4, M5) using carboxymethylated β-cyclodextrin (CMB) as chiral selector. Experimental conditions; capillary: coated, 40.5 (36.0 effective length) cm×50 μm I.D.; 50 m*M* phosphate buffer, pH 2.5 containing 5 m*M* of CMB, applied voltage: 20 kV. Temperature: 25°C. UV detection: 195 nm. M4 and M2 separation is also shown at 4 m*M* and 6 m*M*. For other experimental conditions see text.

plexes. As previously mentioned, the nitrogen charge is influenced by the electrodonor methyl groups and moreover the steric hindrance of the methyl groups can also affect the formation of secondary interaction with the chiral selector. At a concentration of 5 m*M* of CMB, the *N*-bis-demethylated analytes (M3, M4) were found to be particularly well resolved into their enantiomers (R_s>12).

Further optimization experiments for the separation after extraction of tramadol and its metabolites from urine were conducted in absence of M3 and M4 according to both their pharmacology inactive profile and their low determined concentration levels in urine after oral administration (<1%) [7]. Only tramadol was available as pure enantiomers and (−)-tramadol was determined to elute first. For the metabolite enantiomer migration order, an indice (1) was assigned to the first detected enantiomer and an indice (2) was assigned to the second one.

3.2. Extraction

In order to obtain both clean electrophoretic sample profile and good extraction yields of tramadol and its three metabolites, M1, M2 and M5, different organic solvent mixtures as well as different extraction procedures were tested. The two-step extraction, described in the experimental section, was found to be optimal to recover sufficient amounts of the four analytes expected in the urine sample, without co-extracted endogenous interfering substances. The extraction of sample conduced with

Table 1
Recovery percentage (RSD,%) obtained for the enantiomers of tramadol (T), *O*-demethyltramadol (M1), *N*-demethyltramadol (M2) and *O*-demethyl-*N*-demethyltramadol (M5) after liquid–liquid extraction of an urine aliquot of 200 μl (for experimental conditions: see text)

Concentration	T (−)	T (+)	M1 (1)	M1 (2)	M2 (1)	M2 (2)	M5 (1)	M5 (2)
1.0 μg/ml	95.1% (16.9%)	93.7% (16.4%)	88.9% (17.3%)	93.1% (16.6%)	70.7% (14.5%)	68.9% (13.3%)	75.7% (17.3%)	72.4% (15.9%)
5.0 μg/ml	95.7% (24.1%)	92.5% (22.1%)	92.7% (19.6%)	93.3% (21.6%)	73.6% (18.2%)	72.6% (15.8%)	79.4% (29.5%)	77.6% (23.3%)
10.0 μg/ml	95.2% (14.0%)	95.3% (9.7%)	92.2% (12.9%)	92.1% (12.3%)	69.2% (12.0%)	70.3% (11.6%)	73.8% (12.4%)	70.3% (13.8%)

only the hexane–ethyl acetate solution showed recoveries for T, M1, M2 and M5 of about 85%, 85%, 60% and 45%, respectively. Clearly, the *N*-methylated compounds (T, M1) were extracted in a higher extend than the *N*-demethylated compounds (M2, M5). M5 exhibited a particular lower solubility in this extraction mixture probably due to its two demethylations which confer a less hydrophobic character to the molecule. To increase the extraction yields of the *N*-demethylated compounds, especially of M5, a second more polar extraction step was following and *tert*.-butyl methyl ether was found to be the most appropriate solvent. As indicated in Table 1, with the help of this second extraction step, recoveries were found to be greater than 68% for the *N*-demethylated compound (M2, M5) and greater than 90% for the *N*-methylated compound (T, M1). In order to prevent any variability in the extraction and injection process, an internal standard was used for the quantitative work. 0.01 *M* HCl (pH 1.9) was chosen as reconstitution solution instead of double distilled water or diluted BGE in order to improve the resolubilisation of the analytes in the dried extracted urine.

3.3. Quantitative analysis

Table 2 presents the linearity data from the analysis of the tramadol, M1, M2 and M5 enantiomers. The linearity assays showed coefficients of correlation greater than 0.994 for all compounds. Hence the normalised peak-area ratios of compounds were linearly related to the amount of tramadol, M1, M2 or M5 added. This was confirmed by statistical analysis and for each calibration curve, the intercept was not statistically different from zero (Student's *t*-test, $p=0.05$).

As shown in Table 3, within-day variation, assessed by performing six replicate determinations of three spiked standards (1.00, 5.00 and 10.00 μg/ml), gave accuracies ranging from 95.4% to 104.5% at the lower tested concentration, from 96.3% to 103.2% at the intermediate concentration and from 98.3% to 100.6% at 10.00 μg/ml of each enantiomers. The within-day precision expressed as the RSD of the accuracy assays was found to be satisfactory with values less than 10.0% for all compounds at the three tested concentrations.

Day-to-day variation of the method was assessed by analysing replicates of these standards on three separate days. Results are shown in Table 3. Day-to-day accuracy for urine spiked sample at a concentration of 1.0 μg/ml was found to be ranging from 95.0% to 106.5% with a RSD less than 10.0%. At 5.0 μg/ml of each enantiomers, the day-to-day accuracies were ranging from 96.3% to 102.2% with a RSD less than 2.5%. At the highest tested concentration of enantiomers, accuracy did not differ by more than 1.0% of the expected values with a RSD less than 2.0%.

Table 2
Linearity data from the chiral determination of tramadol (T), *O*-demethyltramadol (M1), *N*-demethyltramadol (M2) and *O*-demethyl-*N*-demethyltramadol (M5) (for experimental conditions: see text)

Linearity	T (−)	T (+)	M1 (1)	M1 (2)	M2 (1)	M2 (2)	M5 (1)	M5 (2)
r[a]	0.9977	0.9969	0.9984	0.9982	0.9944	0.9965	0.9954	0.9945
Slope	0.0316	0.0309	0.0302	0.0304	0.0207	0.0207	0.0229	0.0217
Intercept	0.0024	−0.0002	0.0031	0.0020	0.0037	0.0028	0.0038	0.0023

[a] r=Coefficient of correlation.

Table 3
Within- and between-day variation data from the chiral determination of tramadol (T), *O*-demethyltramadol (M1), *N*-demethyltramadol (M2) and *O*-demethyl-*N*-demethyltramadol (M5) (for experimental conditions: see text)

	1.00 μg/ml		5.00 μg/ml		10.00 μg/ml	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Within-day variation						
T (−)	100.5	7.4	96.1	9.3	100.6	3.4
T (+)	104.5	4.7	96.3	9.6	98.3	1.9
M1 (1)	96.1	9.8	98.6	9.6	100.9	3.3
M1 (2)	95.5	6.0	98.7	9.6	100.0	2.6
M2 (1)	96.2	9.3	99.1	9.4	99.7	5.2
M2 (2)	96.4	5.4	99.5	9.4	99.7	3.9
M5 (1)	95.4	6.4	102.4	6.1	99.3	3.7
M5 (2)	97.5	9.8	103.2	7.7	99.1	5.1
Between-day variation						
T (−)	102.7	4.4	97.4	2.4	100.5	0.5
T (+)	106.5	3.9	96.3	2.5	100.0	1.6
M1 (1)	99.3	6.0	99.0	2.3	100.4	0.7
M1 (2)	98.9	5.5	99.1	2.1	100.1	0.5
M2 (1)	106.0	8.1	97.2	1.9	100.5	0.7
M2 (2)	103.8	6.3	98.1	1.4	100.3	0.5
M5 (1)	95.0	5.9	102.2	2.2	99.4	0.5
M5 (2)	98.7	1.1	101.3	1.7	99.6	0.5

Using relative migration time of enantiomers as the ratio of the effective migration time by the I.S. migration time, RSD did not exceed 2.8% at the middle of the tested range ($n=6$). Precisions of less than 0.6% were obtained for the relative migration time of enantiomer (1) on enantiomer (2) indicating a good consistency of selectivity during the injection sequence.

3.4. Analysis of urine

Urine is the biological matrix of choice used to monitor the tramadol metabolism and its pharmacokinetic properties. Moreover, the concentration of tramadol in urine was found to be at least one-order of magnitude greater than blood [10] which make possible to work with a very small volume of biological material as previously described. The liquid–liquid extraction method allowed one to obtain clear electropherograms as shown in Fig. 3. A blank urine spiked only with the internal standard did not show any interfering peaks due to co-extracting substances. A spiked urine at a concentration of 2.5 μg/ml of each enantiomers and an extract of a healthy volunteer urine sample collected during 6–8 h after an administered dose of 100 mg of tramadol hydrochloride are presented where enantiomeric differences are clearly obtained.

3.4.1. Total excretion

The cumulative renal excretion of unchanged tramadol, M1, M2 and M5 reached approximately the 50% of the oral dose administered. Regarding the parent drug, 16% of unchanged tramadol were found (see Fig. 4), suggesting that approximately 84% of the dose was metabolised, which is in accordance with the literature data [4,7,8]. M1 and M5 were found to be excreted in the same proportion than tramadol and about 16% of the dose were detected as M1 and about 15% as M5. Minor amount of the *N*-demethylated metabolite was found to be excreted by the healthy volunteer and only 2.2% of the oral dose was determined as M2. As indicated in Section 1, part of the phase I metabolites are further conjugated by phase II reactions, explaining a cumulative excretion substantially less than 100% of the administered dose.

3.4.2. Enantiomeric ratio

The metabolism of T was found to be largely

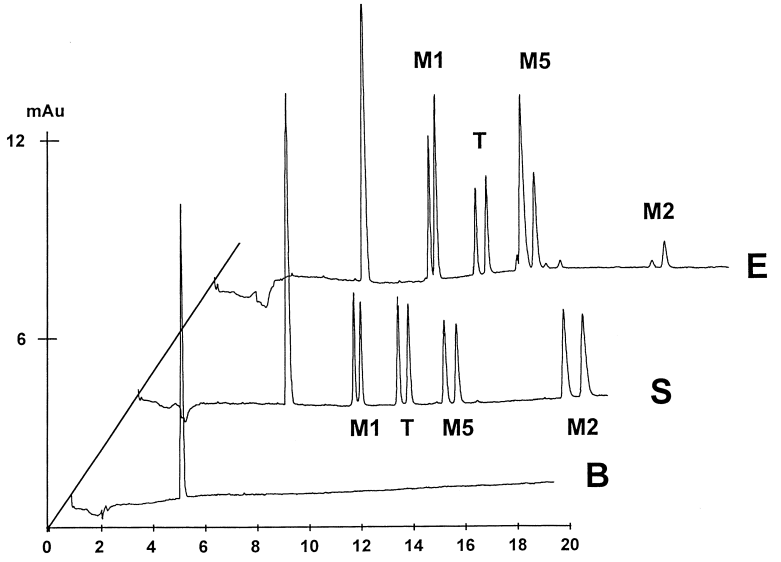

Fig. 3. Electropherograms obtained after liquid–liquid extraction of a blank urine spiked with (+)-ephedrine as internal standard (B), a blank urine spiked with 2.5 μg/ml of each enantiomers of tramadol (T) and its main phase I metabolites (M1, M2, M5) (S) and a healthy volunteer urine sample collected during 6–8 h after an administered dose of 100 mg of tramadol hydrochloride (E). Experimental conditions; capillary: coated, 40.5 (36.0 effective length) cm×50 μm I.D.; 50 m*M* phosphate buffer, pH 2.5 containing 5 m*M* of CMB, applied voltage: 20 kV. Temperature: 25°C. UV detection: 195 nm. For other experimental conditions see text. *x*-Axis: Time in min.

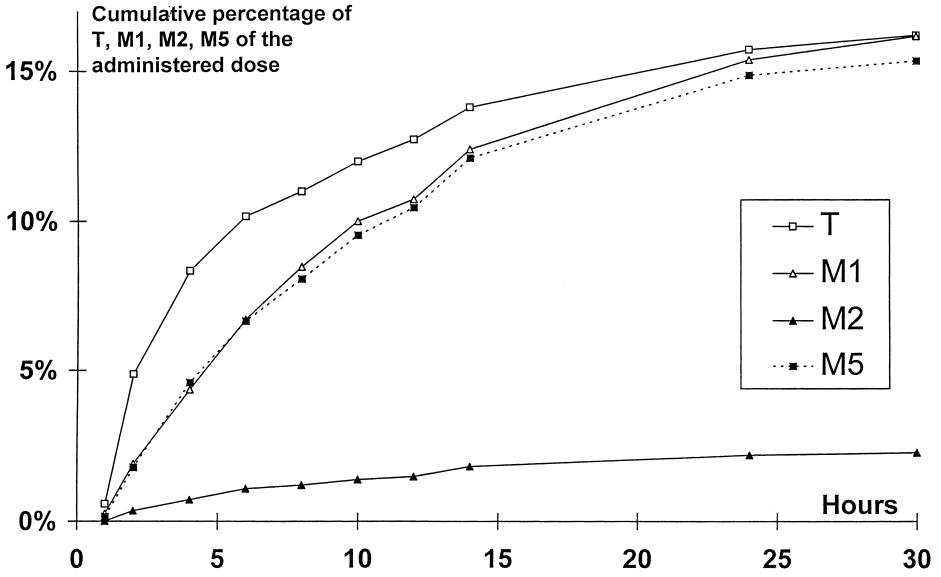

Fig. 4. Cumulative excretion curves of tramadol (T), *O*-demethyltramadol (M1), *N*-demethyltramadol (M2) and *O*-demethyl-*N*-demethyltramadol (M5) of a healthy volunteer during 30 h after oral administration of 100 mg of tramadol hydrochloride.

Table 4
Enantiomeric ratio of tramadol (T), *O*-demethyltramadol (M1), *N*-demethyltramadol (M2) and *O*-demethyl-*N*-demethyltramadol (M5) of a healthy volunteer during 30 h after oral administration of 100 mg of tramadol hydrochloride [obtained as the ratio of the second migrated enantiomers concentration by the first detected enantiomer concentration, (2)/(1), except for T: (+)/(−)]

Time (h)	0–1	1–2	2–4	4–6	6–8	8–10	10–12	12–14	14–24	24–30
T	1.11	1.13	1.14	1.18	1.15	1.26	1.55	1.39	1.46	1.58
M1	1.16	1.01	1.26	1.32	1.49	1.63	1.76	1.88	1.82	1.80
M2	nd[a]	2.76	2.85	3.31	3.50	3.50	nd	nd	5.56	nd
M5	0.60	0.51	0.57	0.55	0.45	0.42	0.44	0.39	0.38	0.29

[a] nd=Not determined.

stereoselective. As presented in Table 4, the enantiomeric ratio (defined as the ratio of the second enantiomer obtained concentration on the first one: (2)/(1), (+)/(−) for tramadol) were determined to be very different to 1.0. For T, M1 and M2, the second enantiomer was detected to a higher extent than the first enantiomer. Thus, enantiomeric ratios greater than 1.0 were obtained for these three compounds during the excretion study. On the other hand, the enantiomeric ratios of M5 were found to be less than 1.0, due to a M2 (2) concentration always lower than that of M2 (1). For all compounds, a time-dependent increase of the initial enantiomeric ratio found were observed during the excretion study, as already described elsewhere [15]. In some cases, both enantiomers of M2 were not detected in the calibration probably due to the large urinary volume excreted during these time intervals.

As presented in Table 5, after 30 h of urinary excretion, 49.9% of the taken dose of tramadol was excreted as M1, M2 and M5 metabolites or as unchanged tramadol. The more abundant excreted enantiomer of tramadol metabolism was found to be M5 (1) with 10.5% of the administered dose. The other enantiomer M5 (2) was approximately detected in the half proportion (4.8%). M1 (2) was found to represent 9.6% of the dose while M1 (1) was calculated counting for 6.5%. M2, previously indicated as the less present analytes, was in fact detected as 1.6% of M2 (2) and only 0.7% of M2 (1). As indicated above, 16.2% of unchanged tramadol was quantified and particularly (+)-tramadol represented 8.9% while (−)-tramadol was presented as 7.3%, which represents a mean excretion enantiomeric ratio of 1.22. (−)-Tramadol was clearly more metabolised by the healthy volunteer. As described by Paar et al. [5], the *O*-demethylation of T, conducing to the M1 formation was determined to be two-fold greater for the (−) enantiomer of tramadol than that of the (+) enantiomer. On the other hand, *N*-demethylation, conducing to M2, was determined to be considerably faster after incubation of the (+) enantiomer as compared with the (−) enantiomer of the racemate. According to the fact that (−)-tramadol was found to be more metabolised than (+)-tramadol and that the *N*-demethylated metabolite was detected to a lesser extent than the *O*-demethylated metabolite, M1, it could be suspected that the tramadol metabolism of the healthy volunteer was preferentially excerced via the *O*-demethylation.

Table 5
Enantiomeric distribution percentage of tramadol (T), *O*-demethyltramadol (M1), *N*-demethyltramadol (M2) and *O*-demethyl-*N*-demethyltramadol (M5) of a healthy volunteer up to 30 h after oral administration of 100 mg of tramadol hydrochloride

Compound	Enantiomeric distribution percentage (%)
T (−)	7.3
T (+)	8.9
M1 (1)	6.5
M1 (2)	9.6
M2 (1)	0.7
M2 (2)	1.6
M5 (1)	10.5
M5 (2)	4.8

4. Conclusions

Simultaneous enantiomeric baseline resolution of tramadol and its five phase I metabolites (M1, M2, M3, M4 and M5) were obtained using CMB as chiral selector into the BGE. The structural differences between the tramadol and its metabolites consist in a

series of demethylations on the phenyl moiety or on the amine group of the parent drug. These modifications were found too selectively influenced both the migration behaviour of these structural analogues during the electrophoretic process as well as the stereoselective. *O*-Methylation mostly influenced the analyte migration behaviour whereas the *N*-methylation played a leading role for the enantiomeric resolution of the analytes. A liquid–liquid extraction process was developed in order to pre-concentrate and clean the urine sample prior the chiral capillary zone electrophoretic analysis. The optimised procedure, using particularly small amounts of biological material, exhibited recoveries higher than 70% for tramadol and its main phase I metabolites, M1, M2 and M5. After validation of the method, analysis of urine sample of an healthy volunteer have demonstrated the usefulness of the developed method to investigate some of pharmacological parameters involved during tramadol metabolism.

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References

[1] P. Dayer, L. Collart, J.A. Desmeules, Drugs 47 (1994) 3.
[2] L. Poulsen, L. Arendt-Nielsen, K. Brosen, S.H. Sindrup, Clin. Pharmacol. Ther. 60 (1996) 636.
[3] J.A. Desmeules, V. Piguet, L. Collart, P. Dayer, Br. J. Clin. Pharm. 41 (1996) 7.
[4] P. Dayer, J.A. Desmeules, L. Collart, Drugs 53 (1997) 18.
[5] W.D. Paar, P. Frankus, H.J. Dengler, Clin. Invest. 70 (1992) 708.
[6] R.B. Raffa, E. Friderichs, W. Reinman, R.P. Shank, E.E. Codd, J.L. Vaught, H.I. Jacoby, N. Selve, J. Pharmacol. Exp. Ther. 267 (1993) 331.
[7] C. Rhoda Lee, D. McTavish, E.M. Sorkin, Drugs 46 (1993) 313.
[8] W.D. Paar, P. Frankus, H.J. Dengler, J. Chromatogr. B 686 (1996) 221.
[9] J. Lai, S. Ma, F. Porreca, R.B. Raffa, Eur. J. Clin. Pharmacol. 316 (1996) 369.
[10] B. Levine, V. Ramchatirar, J.E. Smialek, Forensic Sci. Int. 86 (1997) 43.
[11] R. Becker, W. Lintz, J. Chromatogr. 377 (1986) 213.
[12] W. Lintz, H. Uragg, J. Chromatogr. 341 (1985) 65.
[13] M. Mersalvic, L. Zupancic-Kralj, J. Chromatogr. B 693 (1997) 222.
[14] K.E. Goeringer, B.K. Logan, G.D. Christian, J. Anal. Tox. 21 (1997) 529.
[15] B. Elsing, G. Blaschke, J. Chromatogr. 612 (1993) 223.
[16] A. Ceccato, P. Chiap, P. Hubert, J. Crommen, J. Chromatogr. B 698 (1997) 161.
[17] M. Nobilis, J. Pastera, P. Anzenbacher, D. Svoboda, J. Kopecky, F. Perlik, J. Chromatogr. B 681 (1996) 177.
[18] W. Guo, Q. Zhan, Y. Zhao, L. Wang, Biomed. Chromatogr. 12 (1998) 13.
[19] G. Blaschke, presented at the 9th International Chiral Symposium, Nagoya, October 1997.
[20] S. Rudaz, C. Desiderio, J.-L. Veuthey, S. Fanali, Electrophoresis, in press.
[21] F. VonHeeren, W. Thormann, Electrophoresis 18 (1997) 2415.
[22] W. Thormann, C. Zhang, A. Schmutz, Ther. Drug Monit. 18 (1996) 506.
[23] S. Naylor, L.M. Benson, A.J. Tomlinson, J. Cap. Electrophoresis 1 (1994) 181.
[24] W. Thormann, S. Molteni, J. Caslavska, A. Schmutz, Electrophoresis 15 (1994) 3.
[25] S. Fanali, J. Chromatogr. A 735 (1996) 77.
[26] M.M. Rogan, K.D. Altria, D.M. Goodall, Chirality 6 (1994) 25.
[27] K.D. Altria, J. Chromatogr. 646 (1993) 245.
[28] R. Kuhn, S. Hoffstetter-Kuhn, Chromatographia 34 (1992) 505.
[29] S. Fanali, C. Desiderio, Z. Aturki, J. Chromatogr. A 772 (1997) 185.
[30] B. Chankvetadze, J. Chromatogr. A 792 (1997) 269.
[31] W. Lindner, B. Böhs, V. Seidel, J. Chromatogr. B 697 (1995) 549.
[32] W. Shützner, G. Caponecchi, S. Fanali, A. Rizzi, E. Kenndler, Electrophoresis 15 (1994) 769.