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Simultaneous stereoselective analysis of tramadol and its main phase I metabolites by on-line capillary zone electrophoresis–electrospray ionization mass spectrometry

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Abstract

On-line combination of partial filling capillary electrophoresis and electrospray ionization mass spectrometry was demonstrated for the simultaneous enantioseparation of tramadol and its main phase I metabolites. The partial filling technique was efficient at avoiding MS contamination by the chiral selector. Different experimental factors were investigated, including the chiral selector nature and concentration, plug length as well as the separation temperature. The best enantioseparation of the investigated compounds was achieved with a coated polyvinyl alcohol capillary and a 40 m*M* ammonium acetate buffer, pH 4.0, adding sulfobutyl ether β-cyclodextrin (2.5 mg/ml) as the chiral selector. The charged cyclodextrin not only allowed enantioseparation of tramadol and its metabolites, but also improved the selectivity of compounds with the same molecular mass. Finally, CE–electrospray ionisation-MS was successfully applied to the stereoselective analysis of tramadol and its main metabolites in plasma after a simple liquid–liquid extraction. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Buffer composition; Partial filling capillary electrophoresis; Tramadol; Cyclodextrins

1. Introduction

(+/−)-*trans*-2-[(Dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride (tramadol) is a centrally acting drug which does not possess a pronounced opioid side-effect profile [1,2] and 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 [4–6].

After oral administration, tramadol is rapidly absorbed and reaches peak plasma concentration in a mean of 2 h. About 85% of a tramadol dose is metabolized by the liver [4,7,8]. As shown in Fig. 1, tramadol undergoes biotransformation in the liver through two main metabolic pathways to form *N*- and *O*-demethylated compounds (phase I reactions)

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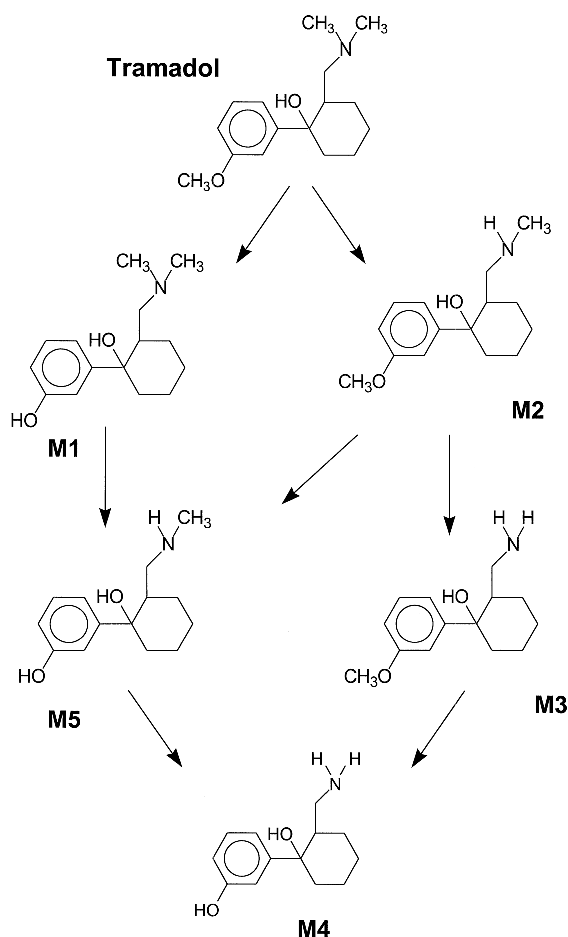

Fig. 1. Phase I metabolism of tramadol and structural formula, *O*-demethyl tramadol (M1), *N*-demethyl tramadol (M2), *N*-bis-demethyl tramadol (M3), *O*-demethyl-*N*-bis-demethyl tramadol (M4), *O*-demethyl-*N*-demethyl tramadol (M5).

which all 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) [7]. Up to now, only M1 has been shown to have a pharmacological activity contributing to the opioid action of tramadol [2,7,9,10].

The analytical methods used so far for the determination of tramadol in different biological fluids include gas chromatography with nitrogen-selective detection [11], gas chromatography–mass spectrometry [10,12–14] or high-performance liquid chromatography with UV [15,16], fluorimetric [8,16,17] or amperometric detection [18]. Enantioseparation of tramadol and some of its metabolites has also been reported by HPLC with chiral stationary phases [15,16].

Because of its high efficiency, flexibility and very high resolution, capillary electrophoresis (CE) has revealed an enormous separation potential for the determination of drugs in biological fluids [19,20]. In particular, CE exhibits excellent results in the field of optical isomer separation, proving to be a very attractive and challenging analytical tool with respect to other commonly used chromatographic techniques [21–24]. CE enantioseparation is generally performed in the direct separation mode, requiring the addition of a chiral selector to the background electrolyte (BGE). Among the great number of selectors reported in the literature, cyclodextrins (CDs) are now the most widely used in chiral CE [25–27]. Neutral and charged CDs derivatives, with various functional groups, have been developed to induce different stereoselective interactions and enhance enantioselectivity. Recently, CE was used for the separation of *cis* and *trans* tramadol isomers [28] and for the stereoselective determination of tramadol [29] and its metabolites [30–32].

However, one of the main disadvantages of CE with on-column UV detection is its relatively low sensitivity due to the short optical path-length afforded by the small internal diameters (I.D.) of the capillaries [33]. Moreover, with spectroscopic detectors, peak identity is generally confirmed by migration times only. However, this information is often insufficient to unequivocally identify compounds of interest because of the possible variation of the electroosmotic flow (EOF). On-line coupling of chiral CE with electrospray mass spectrometry (CE–ESI-MS) is a promising combination of two analytical techniques. While CE provides high separation efficiency per unit of time, MS affords high sensitivity and selectivity as well as molecular structural elucidation. Excellent reviews on CE–MS have been published and can be consulted for a more systematic coverage of the field [34–36]. Application of CE–MS to the enantioseparation of chiral drugs has also been demonstrated [37–41]. The main problem encountered when coupling CE with MS is the risk of the MS source being contaminated by non-volatile additives such as CDs [42]. The partial

filling technique is generally recommended to avoid the chiral selector appearing in the MS ion source and having a detrimental effect on the method sensitivity. This technique involves filling a discrete portion of the capillary with a separation buffer containing a suitable amount of chiral selector to achieve enantioseparation [43,44]. In the case of basic compounds, negatively charged CDs are generally used; application of the electric field results in a counter current process in which the chiral selector and the drug enantiomers migrate in opposite directions [37].

This paper describes the successful application of CE–ESI-MS to the simultaneous enantioseparation of tramadol and its five phase I metabolites. For this purpose, the use of negatively charged cyclodextrins combined with the partial filling technique was investigated to avoid MS instrument contamination and achieve MS detection.

2. Materials and methods

2.1. Chemicals

Both (+) and (−) tramadol enantiomers, racemic tramadol and racemic metabolites: M1, M2, *N*-bisdemethyltramadol (M3), *O*-demethyl-*N*-bisdemethyltramadol (M4) and M5 as hydrochloride salts were a gift from Grünenthal (Stolberg, Germany).

Sulfobutyl ether β-cyclodextrin (CD) (SBE-β-CD-I) with a substitution degree of 7.0 was a gift of Dr. P. Morin (Institute of Organic and Analytical Chemistry, Orléans University, France). Sulfobutyl ether β-CD (SBE-β-CD-II) was purchased from Cydex (KS, USA). Sulfated β-CD was obtained from Aldrich (Buchs, Switzerland). γ-CD phosphate sodium salt and carboxymethyl β-CD (CMB) were purchased from Cyclolab (Budapest, Hungary). Ammonium acetate, potassium carbonate, acetic acid, formic acid, phosphoric acid, hydrochloric acid and isopropanol were of analytical-reagent grade and purchased from Fluka. Hexane, ethyl acetate and methanol were purchased from BDH (Poole, UK). Ultrapure water was supplied by a Milli-Q RG unit from Millipore (Bedford, MA, USA).

2.2. Instrumentation

2.2.1. Capillary electrophoresis

CE data were generated by a Hewlett-Packard $HP^{3D}CE$ capillary electrophoretic system (Waldbronn, Germany) equipped with an on-column diode-array detection system, an autosampler and a power supply able to deliver up to 30 kV. A CE CHEMSTATION (Hewlett-Packard) was used for instrument control, data acquisition and data handling. A Hewlett-Packard polyvinyl alcohol (PVA) coated capillary (70 cm total length×50 μm I.D.) was employed, from which 20 mm of the polyimide coating had been removed from the MS end. All experiments were carried out in the cationic mode (anode at the inlet and cathode at the outlet). During sample analysis, a constant voltage (25 kV) with an initial ramp voltage for 0.2 min was applied, unless otherwise stated. The capillary was thermostated at 20°C. Samples were kept at ambient temperature in the autosampler and injected by applying a pressure of 500 mbar s (12.3 nl corresponding to 0.9% of the total capillary length).

At the beginning of each working day, the PVA capillary was sequentially purged with water, 0.1 *M* phosphoric acid, water and BGE for 5 min each.

The partial filling technique involves filling a portion of the capillary with a separation buffer containing an appropriate amount of chiral selector. Before the injection, the capillary was rinsed with the running buffer at 3 bar for 3 min; then the running buffer containing a charged CD as chiral selector was introduced (90% of the capillary length). The BGE consisted of a 40 m*M* ammonium acetate solution adjusted to pH 4.0, unless otherwise stated.

When not in use, the capillary was sequentially washed with water, 10 m*M* phosphoric acid and water for 5 min each and then dry stored. Before use, electrolyte solutions were filtered through a 0.45-μm pore size filter (Millipore, Milford, MA, USA) and degassed in an ultrasonic bath for 10 min.

2.2.2. Mass spectrometry

Electrospray mass spectrometry measurements were carried out in the positive ionization mode and were performed in a single quadrupole HP Series 1100 MSD system (Hewlett-Packard, Palo Alto, CA,

USA), which has an upper mass limit of 3000 u. In order to interface the $HP^{3D}CE$ instrument with the mass spectrometer, a CE–MS adapter kit interface from Hewlett-Packard was applied. This triple tube ESI-MS interface provides both a coaxial sheath liquid make-up flow and a nebulization gas to assist droplet formation.

The drying gas and the nebulization gas were both nitrogen. The coaxial sheath liquid, consisting of isopropanol–water (50:50, v/v) in presence of 0.5% formic acid, was delivered by a Harvard Model 22 syringe pump (South Natick, MA, USA) at 3 μl/min. MS detection was carried out in the selected ion monitoring (SIM) mode for the positive molecular ion. The selected masses were acquired with a dwell time of 68 ms on each mass.

2.3. Standard and sample solutions

2.3.1. Standard solutions

Stock standard solutions of (+) tramadol, (−) tramadol, racemic tramadol and racemic metabolites were prepared by dissolution of each compound in methanol (1 mg/ml) and stored frozen at −20°C until use. Standard solutions at desired concentration were daily prepared by appropriate dilution in water of the stock solution.

2.3.2. Liquid–liquid extraction

To 1 ml of plasma, 0.5 ml of 1 *M* K_2CO_3 was added. The mixture was shaken for 15 min after the addition of 5 ml of the hexane–ethyl acetate (80:20, v/v). The tube containing lower plasma and upper organic layers was centrifuged at 1000 rpm for 5 min. After centrifugation, the tubes were stored in a freezer. The organic layer was saved and 100 μl 5 *M* HCl in methanol added. The organic solution was evaporated to dryness under a gentle stream of nitrogen and the dry residue redissolved in 100 μl 0.01 *M* HCl.

3. Results and discussion

3.1. Conventional separation

In previous applications, capillary zone electrophoresis coupled to UV detection was successfully applied to the enantiomeric resolution of racemic tramadol and its five phase I metabolites using negatively charged chiral selectors added to a phosphate buffer [31]. Although the latter provided excellent separation power, it is not amenable to electrospray mass spectrometry. More volatile buffers, such as formic acid, acetic acid, ammonium acetate, ammonium formate and ammonium carbonate are often recommended for CE–MS coupling. Therefore, prompted by the good results obtained at acidic pH, ammonium acetate, titrated at pH 4 with (10%, v/v) acetic acid, was selected as running buffer. Following our previous investigations [45–47], the sheath liquid composition consisted of isopranol–water (50:50, v/v) in the presence of 0.5% formic acid and the flow-rate was maintained at 3 μl/min. These conditions allowed a high signal-to-noise ratio as well as a stable electrospray current.

The chemical structure of tramadol and its phase I metabolites (Fig. 1), makes it possible to differentiate them as *O*-methylated compounds (T, M2, M3) and *O*-demethylated ones (M1, M5, M4). As shown in Fig. 2, using a 20 m*M* ammonium acetate buffer without chiral selector, the separation of *O*-methylated compounds from their *O*-demethylated homologues was not possible. Nevertheless, the use of single ion monitoring (SIM) mode allowed to distinguish metabolites by molecular mass differences (demethylations on the phenyl moiety).

In function of the amine group methylation, another classification can be made: tertiary amines, such as *N*-methylated analytes (T, M1), secondary amines such as *N*-mono-demethylated analytes (M2, M5) and primary amines which are *N*-bis-demethylated analytes (M3, M4). As shown in Fig. 2 and already observed [31], the highest mobility was observed for primary amines (M3, M4) whereas tertiary amines (M1, T) had the lowest mobility. These findings are in accordance with the CE theory, where the electrophoretic mobility is mainly governed by the charge-to-size ratio.

3.2. Enantioseparation

Enantioseparation by CE requires the addition of a suitable chiral selector to the BGE. Thus, different negatively charged CDs, including sulfated, sulfobutyl ether (SBE-β-CD), phosphated and carboxy-

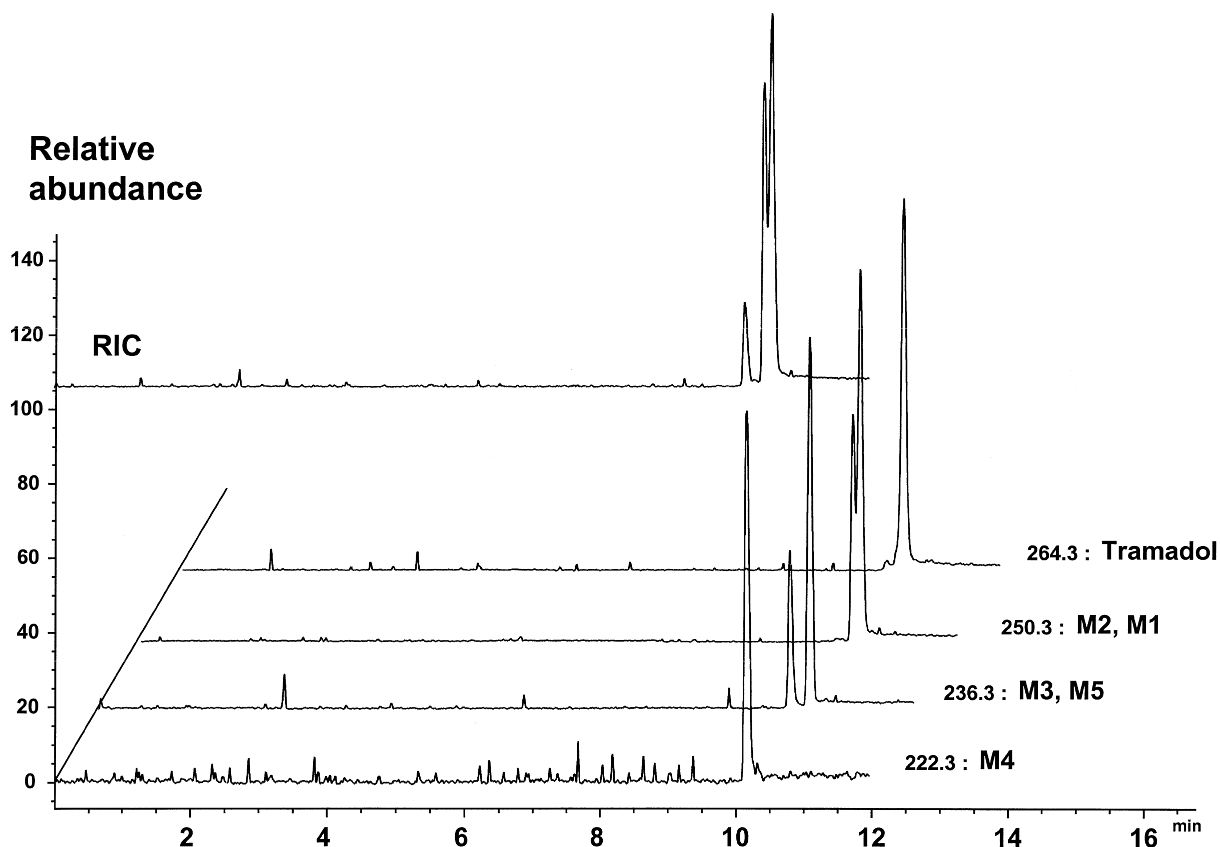

Fig. 2. Electropherogram of a standard solution of 100 ng/ml tramadol (T) and its phase I metabolites (M1–M5). Experimental conditions; capillary: PVA, 70 cm×50 μm ID. CE conditions: running buffer, 20 m*M* ammonium acetate, pH 4; PVA coated capillary, 70 cm total length×50 μm I.D.; pressure injection, 50 mbar for 10 s; applied voltage, 30 kV; temperature, 20°C. MS conditions: SIM positive ion mode (4 ions); capillary voltage, 3 kV; fragmentor, 70 V; drying gas N_2 flow and temperature, 6 l/min and 150°C; nebulizer pressure 4 p.s.i. (1 p.s.i.=6894.76 Pa); sheath liquid, 0.5% formic acid in water–isopropanol (50:50, v/v); sheath flow, 3 μl/min, RIC=Reconstructed ion chromatogram..

methylated CDs, were investigated for the chiral separation of tramadol and its phase I metabolites. Additionally, the partial filling technique was applied to avoid the chiral selector entering the electrospray chamber and suppressing the analyte signal. Since the chiral selector was negatively charged, it migrated towards the anodic compartment and did not move into the MS in absence of EOF. In order to reduce the EOF and decrease the interactions between analytes and the capillary, a PVA-coated capillary was used. Among the tested chiral selectors, SBE-β-CD achieved the best enantioseparation of the investigated drugs (data not shown) and was thus selected for subsequent investigations.

It is well known that SBE-β-CD is a mixture of structurally related compounds such as positional and regional isomers [48–51]. Therefore, the SBE-β-CD origin and concentration, the separation temperature as well as the plug length (part of the separation capillary effectively filled with the chiral selector) were systematically investigated.

Modifying the temperature (15–25°C) induced no significant improvement of the enantioseparation, probably due to the fact that an important part of the capillary is not thermostated when CE is coupled with ESI-MS. Thus, the separation temperature was fixed at 20°C as a compromise for a good enantioseparation as well as an acceptable analysis time.

The filling of a small portion of the capillary (<50%) with a high CD concentration was found to generate excessive electric current (>50 μA) for the CE–ESI-MS instrument. Therefore, the use of a longer plug-length with a lower chiral selector concentration was found more appropriate to maintain the enantioseparation at reduced electric current.

The effect of the buffer concentration was also studied. With a low ammonium acetate concentration, poor resolution was observed. The use of a relatively high buffer concentration (>40 m*M*) improved efficiency as expected, even if a high ionic strength of the BGE is not recommended in ESI-MS [52].

Enantioseparation was also performed in the presence of two SBE-β-CDs from different manufacturers. As illustrated in Fig. 3, a good enantioseparation of this complex drug metabolite mixture was achieved by filling 90% of the capillary with a 40 m*M* ammonium acetate buffer, pH 4.0, and containing SBE-β-CD-I at a concentration of 2.5 mg/ml. Under the same conditions, SBE-β-CD (SBE-β-CD-II), from another manufacturer, exhibited also satisfactory results but a lower enantioseparation was observed with M4 and M2 (data not shown).

Under the optimized conditions, (+)-tramadol was determined to migrate first. The order of migration of T and its five metabolites in presence of SBE-β-CD was: M1<T<M5<M4<M2<M3. It is noteworthy that high peak efficiencies (*N*~200 000) were obtained for all the tested analytes. Indeed, in spite of the non-thermostated part of the capillary and the

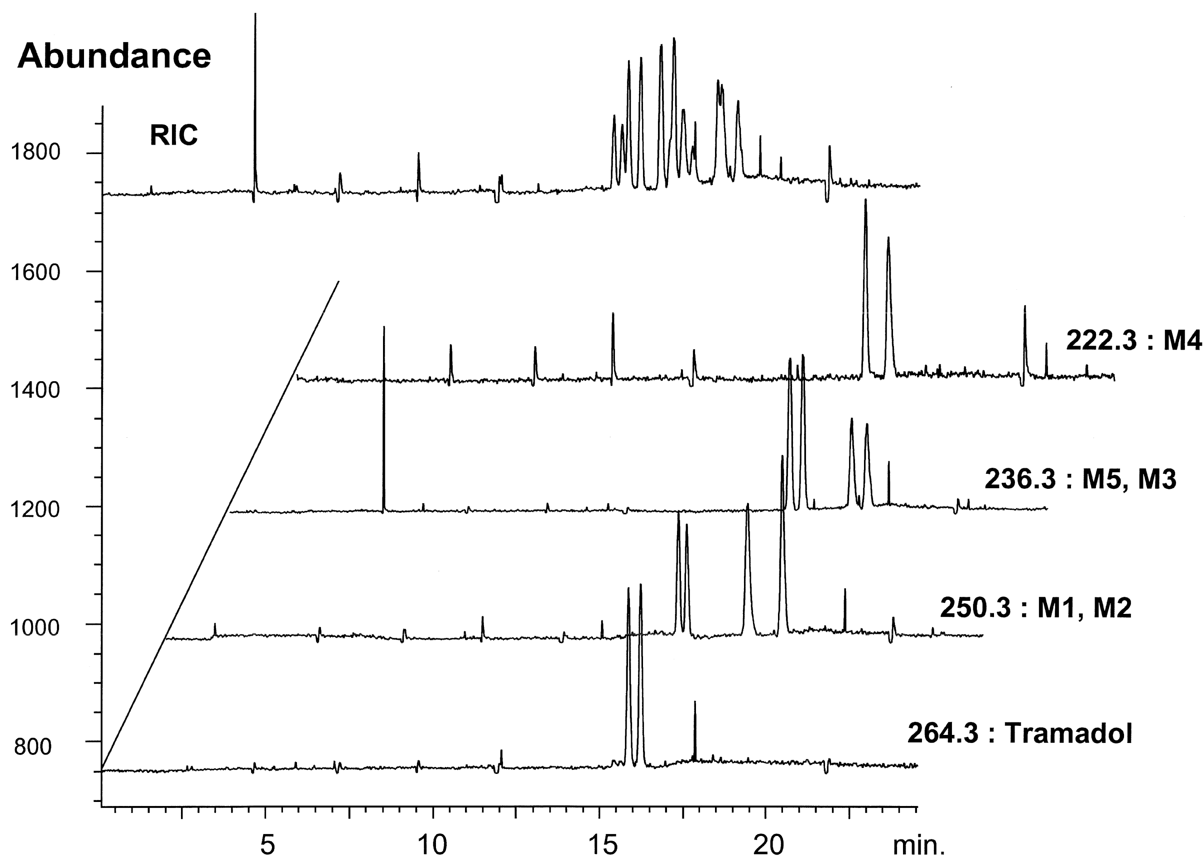

Fig. 3. CE–ESI-MS enantioseparation of tramadol and its phase I metabolites (M1–M5) in presence of a negatively charged chiral selector (SBE-β-CD). CE conditions: running buffer, 40 m*M* ammonium acetate, pH 4, in the presence of SBE-β-CD (2.5 mg/ml); partial filling of the capillary (90%); PVA-coated capillary, 70 cm total length×50 μm I.D.; sample concentration, 100 ng/ml; pressure injection, 50 mbar for 10 s; applied voltage, 25 kV; temperature, 20°C. ESI-MS conditions as in Fig. 2.

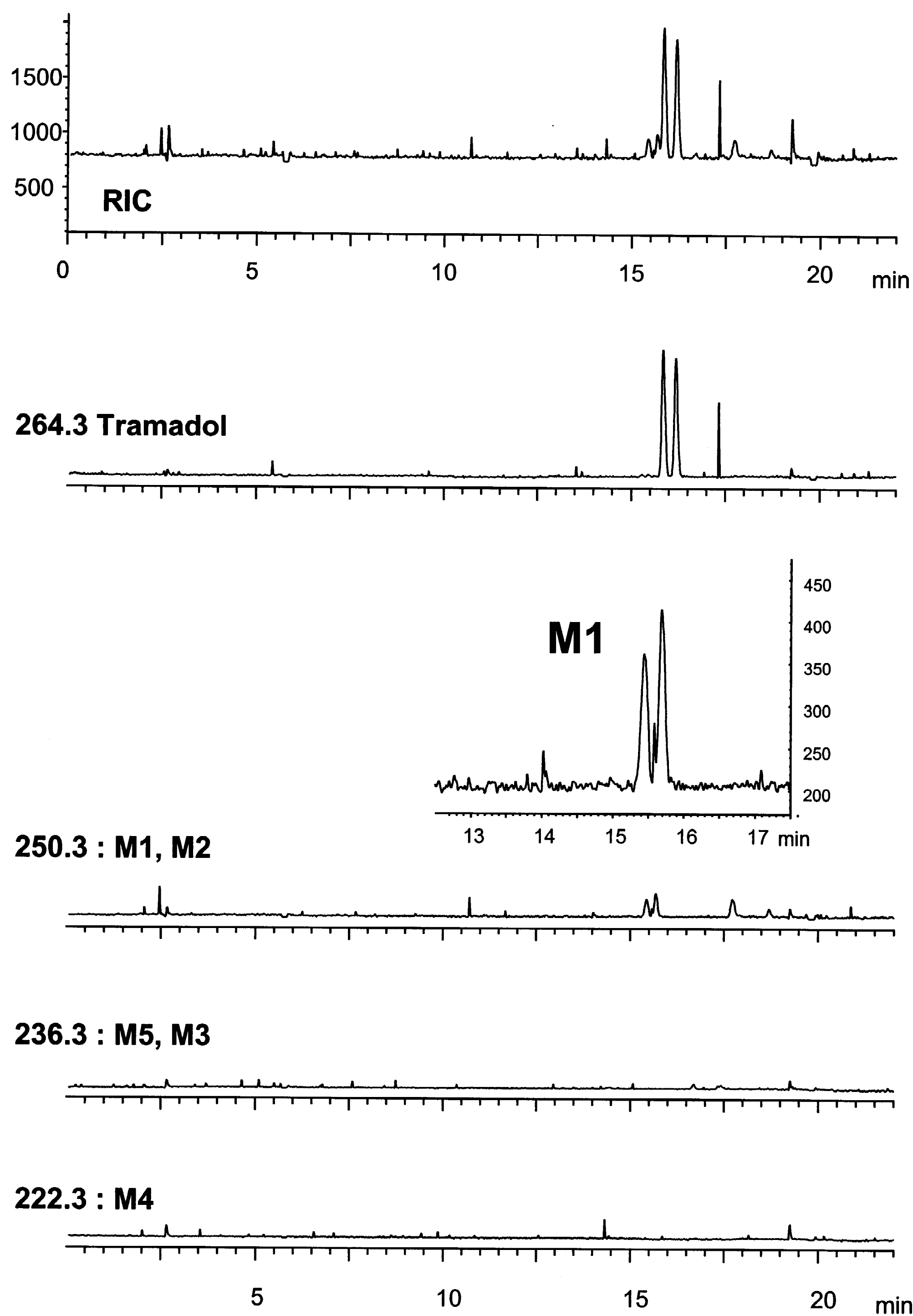

Fig. 4. Typical CE–ESI-MS electropherogram obtained after liquid–liquid extraction of a healthy volunteer's serum, collected 2 h after an administered dose of 100 mg of tramadol hydrochloride. Sample concentration (estimated); tramadol 300 ng/ml, M1 75 ng/ml; other conditions as in Fig. 3.

coupling of CE with ESI-MS, no significant band broadening was observed as already reported [37].

Although peak overlapping was observed in the RIC electropherogram (Fig. 3), the recording of selected masses allowed an unambiguous determination of each analyte, which demonstrates the high selectivity of MS in comparison to conventional detectors [38]. Indeed, all compounds were baseline resolved in $<$20 min. Furthermore, the use of charged CDs not only allowed the enantioseparation of investigated drugs, but also improved the selectivity of metabolites possessing the same molecular mass, such as M1 and M2 as well as M3 and M5.

3.3. Application

Finally, a plasma sample was collected from a healthy volunteer 2 h after oral administration of 100 mg of tramadol hydrochloride (two 50 mg capsules of Tramal, Grünenthal, Stolberg, Germany, equivalent to 87.85 mg of racemic tramadol). A liquid–liquid extraction process was applied prior to the CE–ESI-MS analysis in order to concentrate and purify the plasma sample. As reported in Fig. 4, both tramadol and its active metabolite, M1, were present in the plasma extract, while other metabolites were not detected under the optimized conditions. In comparison to conventional detectors, the use of the SIM mode resulted in sensitivity and selectivity improvement because metabolites could comigrate, as previously observed [31].

4. Conclusion

A simultaneous enantioseparation of tramadol and its five phase I metabolites (M1, M2, M3, M4 and M5) was achieved by CE–ESI-MS, using negatively charged cyclodextrins combined with the partial filling technique which avoids MS instrument contamination. The migration behavior as well as the stereoselective recognition of tramadol and its metabolites were found to be critically affected by the structural differences of the compounds.

Among the investigated negatively charged chiral selectors, SBE-β-CD was found the most appropriate. The SBE-β-CD origin showed to have a significant influence on the enantioresolution of some metabolites. Moreover, the use of this selector not only allowed enantioseparation of the investigated drugs, but also improved the selectivity of metabolites possessing the same molecular mass, such as M1 and M2, as well as M3 and M5. The described method was finally applied to the stereoselective analysis of tramadol and its main metabolites in a plasma sample.

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