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Enantiomeric separation of tramadol hydrochloride and its metabolites by cyclodextrin-mediated capillary zone electrophoresis

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Abstract

The enantiomeric separation of tramadol hydrochloride and its major metabolites, *O*-demethyltramadol (M1) and *N*-demethyltramadol (M2) was studied using cyclodextrin (CD)-mediated capillary zone electrophoresis (CZE). Influence of the choice of type and concentration of CD, capillary temperature, length of capillaries, buffer pH and the addition of polymer modifier on the chiral separation of tramadol and its metabolites was evaluated. It was found that the drug and the metabolites can be baseline-separated simultaneously by using 50 m*M* phosphate buffer (pH 2.5) containing 75 m*M* methyl-β-CD, 220 m*M* urea and 0.05% (w/v) hydroxypropylmethyl cellulose. © 1998 Elsevier Science B.V.

Keywords: Enantiomer separation; Tramadol; Demethyltramadol

1. Introduction

Tramadol hydrochloride is a μ receptor agonist analgesic drug, supplied as a racemic mixture of the *trans* isomers (Fig. 1). Despite the preclinical pharmacology demonstrating an opioid mechanism of action, the clinical experience has proven tramadol to be unique among other centrally acting opioid analgesics. Unlike the typical opioid analgesics, the therapeutic use of tramadol has not been associated with clinically significant side effects such as respiratory depression, constipation (even after long term administration) or sedation [1,2]. In in-vitro studies, the (+)- and (−)-enantiomers of the drug were found to contribute to its analgesic effect through different, but complementary and interactive pharmacological mechanisms [3,4]. Data from antinociception tests suggested that tramadol produces antinociception via an opioid (predominantly μ) mechanism and also via a separate nonopioid mechanism, probably related to its ability to inhibit the neuronal uptake of norepinephrine and serotonin. Both mechanisms contribute to antinociception in vivo. The (+)-enantiomer exhibited a ten-fold higher analgesic activity than the

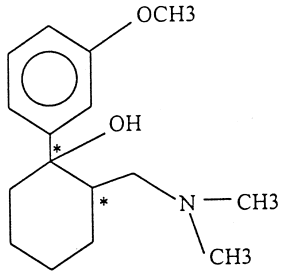

Fig. 1. Structure of tramadol. The chiral centres, near to the aromatic ring, are marked with asterisks.

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(−)-enantiomer as it has greater affinity at the μ receptor compared to the latter. The (+)-enantiomer is the more potent serotonin uptake inhibitor (Ki= 0.53 μm) and the (−)-enantiomer is the more potent norepinephrine uptake inhibitor (Ki=0.43 μm) [3,4].

Tramadol undergoes biotransformation in the liver via two main metabolic pathways to form the *N*- and *O*-demethylated compounds. The *O*-demethylated tramadol metabolite (M1) has been shown to have two- to four-times the potency of tramadol in mice [5]. Although the tramadol metabolite (M1) has been found to be more potent than the parent drug in animal study, inhibition of M1 formation by quinidine was not found to significantly alter the peak analgesic effect of a single dose of tramadol (100 mg) in healthy volunteers [6]. Therefore, the contribution of this metabolite to pain relief is still unknown. Due to the different pharmacological properties of the tramadol enantiomers and the unknown contribution of their metabolites to analgesia after single and multiple doses, it is desirable to establish a stereoselective assay for the clinical study of their pharmacokinetic and pharmacodynamic properties in humans.

Recent methods for the determination of tramadol in biological sample employed gas chromatography (GC) with nitrogen selective detector [7] and GC–mass spectrometry (MS) [8]. However, these GC methods required tedious recrystallisation, synthetic and purification processes and they were not fabricated to separate the enantiomers. Chiral high-performance liquid chromatography (HPLC) has been used for determination of enantiomeric ratios of tramadol and its metabolites in urine [9]. However, this method is incapable of separating the compounds mentioned simultaneously. In the last decade, capillary electrophoresis (CE) has become a powerful technique as an alternative to chiral chromatographic methods. This analytical technique is fast, inexpensive, and usually does not require the labour intensive pre- or post-column derivatisation step [10–14]. In addition, CE requires relatively small volumes of sample and buffer (in nl and μl, respectively) for the assay and this greatly reduces the cost of each run and it also eliminates the problems related to solvent disposal. Expensive chiral columns can also be avoided because chiral selector can be easily added to the background electrolyte (BGE). Cyclodextrins (CDs) are the chiral selectors commonly used for enantiomeric separation of drugs [15]. According to Armstrong et al. [16], CDs are especially useful as chiral selectors for aromatic chiral drugs with the asymmetric atom situated close to the aromatic ring system. Based on the abovementioned, chiral separation of tramadol and metabolites by using CDs as the chiral selectors seems promising.

In this communication, we report the successful enantiomeric separation of tramadol hydrochloride, *O*-demethyltramadol (M1) and *N*-demethyltramadol (M2) by using capillary zone electrophoresis (CZE) with CDs as the chiral selectors.

2. Experimental

2.1. Apparatus

Experiments were carried out on BioFocus 3000 CE system (Bio-Rad Labs., Hercules, CA, USA). This system is equipped with a high voltage supply and voltages at 15, 20 and 25 kV were used to drive the separation. On-column detection was performed at the cathode with the UV absorbance detector set to either 200 nm or 272 nm. A built-in coolant system was also designed to minimise the Joule heat, thus allowing the CE system to use high electrical fields and to achieve very low band dispersion. Four liquid polyacrylamide (LPA) coated fused-silica capillaries (65 cm×50 μm I.D., 50 cm×50 μm I.D., 36 cm×50 μm I.D., 24 cm×25 μm I.D., respectively; Bio-Rad Labs.) were used as separation tubes for the method development. A pH meter (Accumet Model 15, Fisher Scientific, Leicestershire, UK) was used for pH measurements.

2.2. Chemicals

α-CD, methyl-β-CD (substitution value of 1.8) and γ-CD were obtained from Wacker (Munich, Germany). β-CD and DM-β-CD (heptakis 2,6-dimethyl-β-CD, substitution value of 2) were purchased from Sigma (St. Louis, MO, USA) and Tokyo Kasei Organic Chemicals (Tokyo, Japan), respectively. HP-β-CD (hydroxypropyl-β-CD, substitution value of 4.5) was obtained from Nihon

Shokunin Kakoco (Tokyo, Japan). Potassium dihydrogenorthophosphate (KH_2PO_4) was purchased from BDH Labs. (Poole, UK), while urea was obtained from Bio-Rad Labs. HPMC (hydroxypropylmethyl cellulose) was obtained from Shin-Etsu (Tokyo, Japan). Racemate tramadol hydrochloride, enantiomers of (+)- and (−)-tramadol, metabolites M1 and M2 were kindly supplied by Grünenthal (Stolberg, Germany). Trimethylamine (TMA), 12.5% (w/v) stock solution, was purchased from Perkin-Elmer (CT, USA). All other chemicals were of analytical-reagent grade and Milli-Q reagent water was used in the study.

2.3. Procedures

Stock solutions of 50 m*M* KH_2PO_4 buffer were prepared and the pH was adjusted with H_3PO_4 or NaOH to obtain values of 2.5, 3.0, 5.0, 7.0 and 8.5, respectively. These phosphate buffers (50 m*M*) were used as background electrolytes for studies of the effects of different buffer pH values on separation. An appropriate amount of the different types of CDs was then added to the phosphate buffers mentioned herein. Urea and HPMC were added when required in the study. All run buffers were freshly prepared at the beginning of each study day. The contents were vortexed for 2 min, degassed by sonication for 10 to 15 min and filtered through a 0.45-μm pore size filter before use. The racemic mixture and the enantiomers of tramadol, and its metabolites were stored in air-tight dessicator and kept in the dark to prevent degradation. All sample solutions were prepared in Milli-Q reagent water, stored at 4°C, diluted to the desired concentrations and carefully degassed by sonication before use.

The capillaries were subjected to preparation cycles (pre-rinse and washing cycles) programmed in between runs to ensure a clean and equilibrated surface for separations. They were rinsed with 50 m*M* phosphate buffer pH 2.5, 0.1 *M* NaOH, Milli-Q reagent water, washing buffer and running buffer for 120, 120, 60, 120 and 120 s, respectively. The washing and running buffers were of the same buffer that was contained in two different inlet vials designated for washing and running purposes. This was to prevent possible contamination of the running buffer during the preparation cycles. For method development, the samples were injected by displacement or electrophoretic injection at the anode; and the CZE operations were run under various voltages and at different capillary temperatures.

Resolution (*R*) between the enantiomers was evaluated by the following equation:

$$R = (X_2 - X_1)/[0.5(W_1 + W_2)]$$

where X_1 and X_2 are the positions of the zone centres for peaks 1 and 2, respectively, and W_1 and W_2 are the widths measured at the base of the peaks. When *R* is greater or equal to 1.5, the separation of the two bands is regarded to be complete, and the peaks are said to be baseline resolved.

3. Results and discussion

3.1. Effect of CD type

In this study, α-CD, β-CD, γ-CD, methyl-β-CD, DM-β-CD and HP-β-CD were investigated for the chiral separation of the drug and its metabolites, M1 and M2. Fig. 2 shows the electropherograms of the enantiomeric separation of tramadol using 10 m*M* of each methyl-β-CD, HP-β-CD and γ-CD in 50 m*M* phosphate buffer, pH 2.5, at a capillary temperature of 20°C. The peaks were detected and integrated at 272 nm (UV_{max} of tramadol). Chiral resolution was only observed with methyl-β-CD, but not with other CDs. The run was repeated with methyl-β-CD, DM-β-CD and β-CD under similar conditions. Chiral separation was again observed with methyl-β-CD but not with the other two CDs in the same run. Although baseline resolution was not achieved, the racemic drug was clearly enantioseparated with methyl-β-CD under the selected conditions.

3.2. Selection of buffer pH

Fig. 3 shows the electropherograms of the enantiomeric separation of tramadol at five different pH values with 5.0 m*M* methyl-β-CD in the running buffer. The conditions selected were able to separate the enantiomers at the acidic pH values of 2.5 and 3.0. When the buffer pH was increased to the higher pH values, no resolution of the peak was observed.

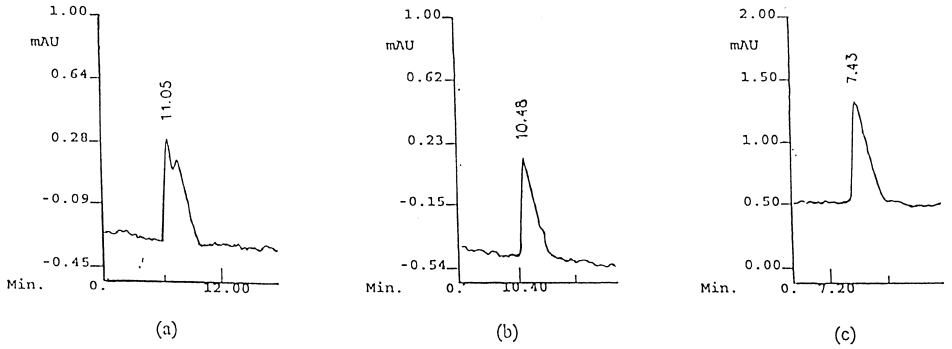

Fig. 2. Electropherograms of the enantiomeric separation of a racemic mixture of tramadol hydrochloride (0.2 mg/ml), using (a) 10 m*M* methyl-β-CD, (b) 10 m*M* HP-β-CD and (c) 10 m*M* γ-CD in 50 m*M* phosphate buffer at pH 2.5. Temperature: 20°C. Injection mode: displacement injection at pressure of 6 p.s.i. s. Applied voltage: 6 kV. Column: 24 cm×25 μm LPA-coated capillary. Detection wavelength: 272 nm.

Buffer pH is an important parameter in CZE. Alterations in pH can affect the solute charge, depending on the solute properties, and change the electroosmotic flow (EOF), which generally increases as the pH is increased, thus influencing the resolution. However, tramadol hydrochloride which has a pK_a of 8.3 [15] would be essentially ionised throughout the pH range used in this study. One possible reason for the observed pH effect on the enantioseparation could be that our coated capillaries though are expected to have presumably no EOF, may still have some residual EOF. The low buffer pH would help to remove any of these residual EOF effects.

3.3. Effect of CD concentration

Fig. 4 shows the influence of the concentration of methyl-β-CD on the enantioseparation of tramadol hydrochloride. Obviously, there is an optimum concentration at which chiral resolution reaches a maximum value for tramadol. Such a relationship between resolution and CD concentration has also been reported by other authors [10,14]. The difference in the apparent mobility between the two enantiomers will reach a maximum at a particular chiral selector concentration which depends on the affinity of the enantiomers for the selectors [17]. Based on these

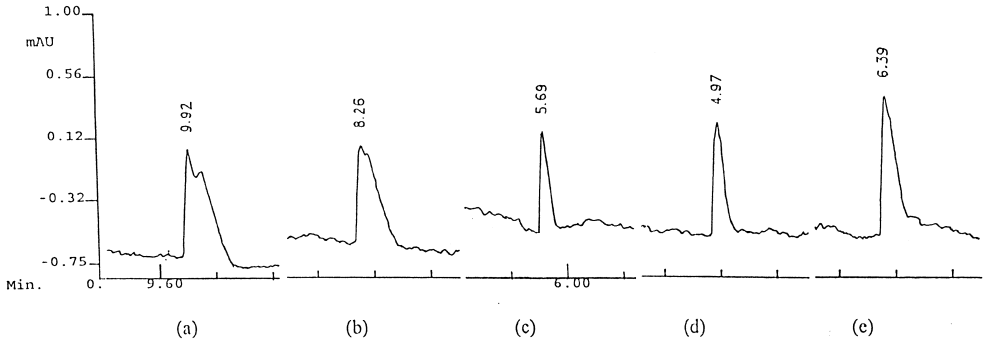

Fig. 3. Electropherograms of the enantiomeric separation of a racemic mixture of tramadol hydrochloride (0.2 mg/ml) at pH (a) 2.5, (b) 3.0, (c) 5.0, (d) 7.0 and (e) 8.5, with 5.0 m*M* methyl-β-CD in 50 m*M* phosphate buffer. Temperature: 20°C. Injection mode: displacement injection at pressure of 6 p.s.i. s. Applied voltage: 6 kV. Column: 24 cm×25 μm LPA-coated capillary. Detection wavelength: 272 nm.

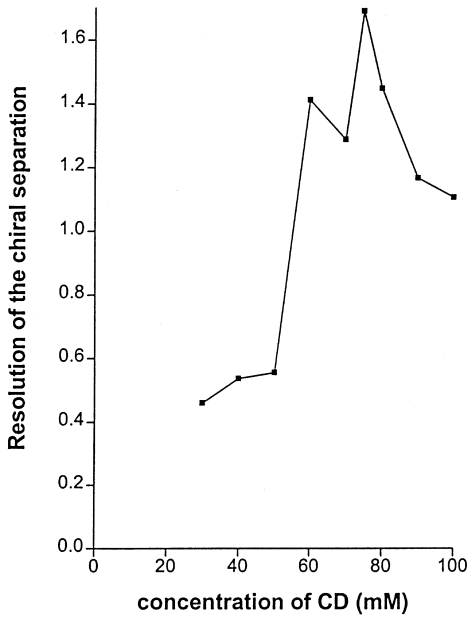

Fig. 4. Influence of the concentration of methyl-β-CD on the chiral resolution of a racemic mixture of tramadol hydrochloride (2.5 μg/ml). Buffer: 50 m*M* phosphate buffer at pH 2.5, containing 6 *M* urea and 15 m*M* trimethylamine. Temperature: 15°C. Injection mode: electrophoretic injection at 30 kV for 6 s. Applied voltage: 20 kV. Column: 65 cm×50 μm LPA-coated capillary. Detection wavelength: 272 nm.

concepts and the results obtained, enantiomers of tramadol display the greatest difference in mobility at 75 m*M* of methyl-β-CD. This is a relatively large concentration of methyl-β-CD used and therefore 6 *M* of urea was added to improve its solubility in the 50 m*M* phosphate buffer, pH 2.5. Such a high optimum CD concentration also implies a relatively low binding affinity between these enantiomers and the CD. Later, we found that 220 m*M* of urea was sufficient for the purpose of improving the solubility of CD in the buffer.

3.4. Effect of capillary temperature on separation

Changes in the capillary temperature can lead to changes on chiral resolution. The electropherograms in Fig. 5 show the chiral separation of racemic tramadol under similar experimental conditions except that the capillary temperature was maintained at 20°C and 15°C, respectively. At 15°C, the enantiomers were resolved to a greater extent than at 20°C. This observed reduction of resolution at a higher temperature was due to a decrease in the formation constant between the analytes and the chiral selectors and an increase in the solutes' diffusion. The capillary temperature was therefore subsequently maintained at 15°C to optimise the enantioseparation of the racemic drug.

3.5. Effect of capillary length on separation

The effect of capillary length on the enantioseparation of the racemic tramadol and the migration time of the peaks was studied using capillaries of length 24 cm, 36 cm, 50 cm and 65 cm, respectively. In Fig. 6, it was clear that the 50 cm capillary

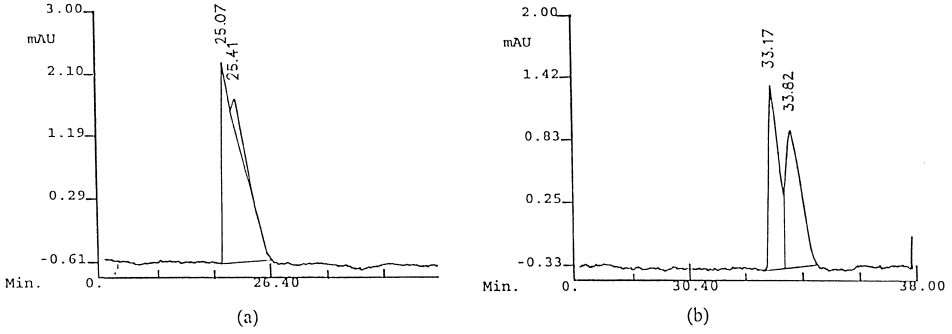

Fig. 5. Electropherograms of the enantiomeric separation of a racemic mixture of tramadol hydrochloride (0.1 mg/ml) at (a) 20°C and (b) 15°C with 10.0 m*M* methyl-β-CD in 50 m*M* phosphate buffer at pH 2.5. Injection mode: displacement injection at pressure of 6 p.s.i. s. Applied voltage: 9.6 kV. Column: 50 cm×50 μm LPA-coated capillary. Detection wavelength: 272 nm.

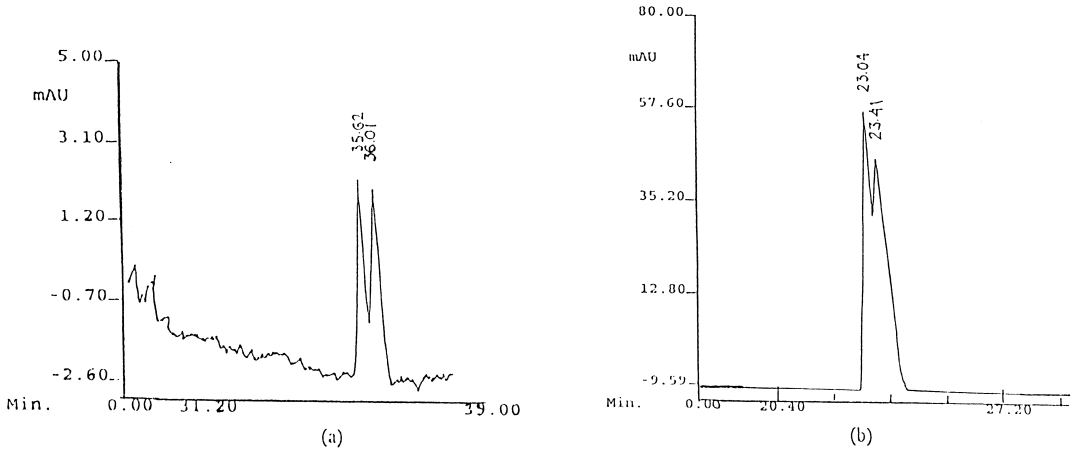

Fig. 6. Electropherograms of the enantiomeric separation of a racemic mixture of tramadol hydrochloride (10.0 μg/ml), using (a) 50 cm×50 μm and (b) 36 cm×50 μm LPA-coated capillaries. Buffer: 50 m*M* phosphate buffer at pH 2.5, containing 220 m*M* urea. Temperature: 15°C. Injection mode: electrophoretic injection at 5 kV for 6 s. Applied voltage: 15.0 kV. Detection wavelength: 200 nm.

allowed better resolution of the peaks compared to the 36 cm capillary. However, the improvement in resolution was achieved at the cost of a longer migration time, for instance, from 33.42 min for the 36 cm capillary to 36.01 min for the 50 cm capillary. These results were expected since a longer capillary would give analytes a longer interaction time with the CD and therefore, improving the resolution. Generally, longer capillaries give better resolution because they allow the uses of higher voltages. Fig. 7 illustrates that chiral separation of tramadol and its metabolites was achieved by applying 25 kV across a 65 cm×50 μm LPA-coated capillary. It may be noted that in these experiments detection of peaks was made at 200 nm, the wavelength which gives the maximum sensitivity for detection of tramadol. Trimethylamine was also added to the running buffer in the latter experiment. The addition of this compound

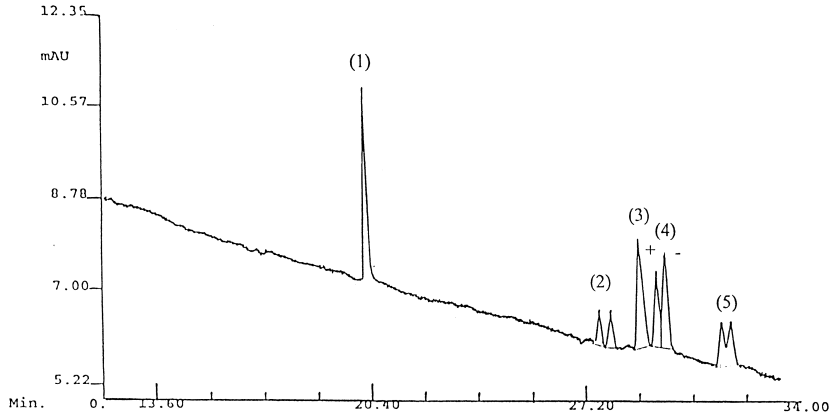

Fig. 7. Electropherogram of the separation of morphine sulphate (1), (±)-*O*-demethylated tramadol hydrochloride (M1) (2), pethidine hydrochloride (3), (+)- and (−)-tramadol hydrochloride (4) and (±)-*N*-demethylated tramadol hydrochloride (M2) (5). Morphine sulphate was in 5 μg/ml and the others were in 1 μg/ml. Buffer: 50 m*M* phosphate buffer at pH 2.5, containing 75 m*M* methyl-β-CD, 220 m*M* urea and 15 m*M* trimethylamine. Temperature: 15°C. Injection mode: electrophoretic injection at 5 kV for 18 s. Applied voltage: 25 kV. Column: 65 cm×50 μm LPA-coated capillary. Detection wavelength: 200 nm.

appeared to reduce the problem of tailing peaks probably by improving the mobility matching of the analytes and co-ions.

3.6. Optimisation of peak shapes and resolution by the addition of polymer modifier

Although chiral separation of tramadol and its metabolites was achieved in Fig. 7, the peak shapes and resolution appear not to be fully optimised. It is known that the resolution of peaks is inversely proportional to the electrophoretic mobility of compounds. The electrophoretic mobility of compounds is in turn inversely proportional to the buffer viscosity and the square root of the buffer concentration [18]. An increase in buffer concentration and viscosity would therefore lead to an improvement in peak shapes and resolution. In our further study, phosphate buffer with higher concentration (100 m*M*, pH 2.5) was used as the background electrolyte for separation. The use of this buffer, however, was found to cause noisy and unstable baseline. This unstable baseline could be due to the excessive joule heating that resulted from the increased electrical conductivity in the more concentrated buffer. In the light of this, we decided to alter the second parameter, viscosity, by adding HPMC of two different viscosities (3 cps and 15 cps) as polymer modifier to the buffer. The effect of viscosities of the HPMC used on chiral separation was then compared. Fig. 8 shows that peak shapes and resolution were significantly improved when 0.05% (w/v) of HPMC of either 3 cps and 15 cps was included in the buffer. This improvement was observed even with a larger sample loading. The difference in the resolution (*R*) of peaks between the 3 cps and 15 cps HPMC appeared to be minimal. Nonetheless, the use of the lower viscosity HPMC (3 cps) is preferred because it gives relatively shorter migration times. With such improvement in peak shapes and resolution, addition of trimethylamine in the running buffer and a 65 cm capillary were no longer required. A capillary of 50 cm long was found to be sufficient for the optimal peak separation. However, peak resolution was compromised when a shorter capillary of 25 cm was used.

4. Conclusions

Only recently, a nonstereospecific HPLC determination of tramadol in human serum was reported [19]. The inaccuracy of the pharmacokinetic data obtained from such nonenantiospecifc method should

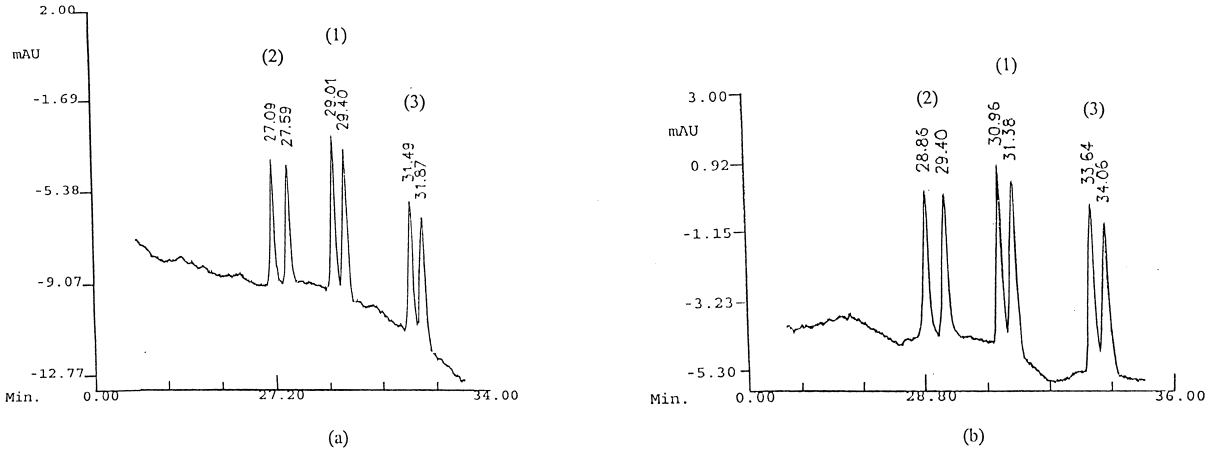

Fig. 8. Electropherograms of the enantiomeric separation of a racemic mixture of 5.0 μg/ml of each of (+)- and (−)-tramadol hydrochloride (1), (±)-*O*-demethylated tramadol hydrochloride (M1) (2) and (±)-*N*-demethylated tramadol hydrochloride (M2) (3). Buffer: 50 m*M* phosphate buffer at pH 2.5, containing 75 m*M* methyl-β-CD, 220 m*M* urea and 0.05% (w/v) hydroxypropylmethyl cellulose of (a) 3 cps and (b) 15 cps viscosity. Temperature: 15°C. Injection mode: electrophoretic injection at 5 kV for 7 s. Applied voltage: 25 kV. Column: 50 cm×50 μm LPA-coated capillary. Detection wavelength: 200 nm.

not be disregarded. A chiral HPLC method achieved chiral separations of tramadol and M2 by using one chiral column (ChiralPak AD) and M1 by using another chiral column (Chiralcel OD) [9]. Recently, a study also reported the determination of tramadol enantiomers in human plasma using chiral liquid chromatography [20]. This method however, was not designed to effect enantiomeric separation of the two major metabolites, *O*-demethyltramadol (M1) and *N*-demethyltramadol (M2). In this study, we have successfully resolved the enantiomers of tramadol hydrochloride and its two metabolites simultaneously by using CD-mediated CZE. This will definitely be an advantage in the study of the stereoselective effect on the pharmacokinetic and pharmacodynamic properties of tramadol and its metabolites.

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References

[1] H. Müller, M. Stoyanov, G. Brähler, G. Hempelmann, Anaesthesist 31 (1982) 604.
[2] M.D. Vickers, Ó. Flaherty, S. Szekely, M. Read, J. Yoshizami, Anaesthesia 47 (1992) 291.
[3] R.B. Raffa, E. Fridericks, W. Reimann, R.P. Shank, E.E. Codd, J.L. Vaught, J. Pharmacol. Exp. Ther. 260 (1991) 275.
[4] R.B. Raffa, E. Fridericks, W. Reimann, R.P. Shank, E.E. Codd, J.L. Vaught, H.I. Jacoby, N. Selve, J. Pharmacol. Exp. Ther. 267 (1993) 331.
[5] H.H. Hennies, E. Friderichs, J. Schneider, Arzneim.-Forsch. Drug Res. 38 (1988) 877.
[6] L. Collart, C. Luthy, P. Dayer, Clin. Pharmacol. Ther. 53 (1993) 223.
[7] R. Becker, W. Lintz, J. Chromatogr. 397 (1986) 213.
[8] W. Lintz, H. Uragg, J. Chromatogr. 341 (1985) 65.
[9] B. Elsing, G. Blaschke, J. Chromatogr. 612 (1993) 223.
[10] I. Bjørnsdottir, S.H. Hansen, Chirality 7 (1995) 409.
[11] A. Guttman, B. Sandra, C. Jurado, N. Cooke, Chirality 7 (1995) 409.
[12] B. Chankvetadze, G. Endresz, G. Blaschke, Electrophoresis 15 (1994) 804.
[13] A. Guttman, Electrophoresis 16 (1995) 1900.
[14] I. Bechet, P. Paques, M. Fillet, P. Hubert, J. Crommen, Electrophoresis 15 (1994) 818.
[15] V.E. Frankus, E. Friderichs, S.M. Kim, G. Osterloh, Arzneim.-Forsch. 28 (1978) 114.
[16] D.W. Armstrong, Y. Tang, T.J. Ward, R.D. Armstrong, T.E. Beesley, Science 232 (1986) 1132.
[17] S.A.C. Wren, R.C. Rowe, J. Chromatogr. 603 (1992) 235.
[18] H.J. Issaq, G.M. Janini, K.C. Chan and Z.E. Rassi, in P.R. Brown and E. Grushka (Editors), Advances in Chromatography, Vol. 35, Marcel Dekker, NY, 1995, Ch. 3, p. 101.
[19] M. Nobilis, J. Pastera, P. Anzenbacher, D. Svoboda, J. Kopecky, F. Perlik, J. Chromatogr. B 681 (1996) 177.
[20] A. Ceccato, P. Chiap, Ph. Hubert, J. Crommen, J. Chromatogr. B 698 (1997) 161.