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Determination of tricyclic antidepressants in human plasma by micellar electrokinetic capillary chromatography

Kong-Joo Lee*

Organic Analytical Laboratory, Korea Research Institute of Standards and Science, P.O. Box 3, Taejonsi Taeduk Science Town 305-606 (South Korea)

Jong Jin Lee and Dong Cheul Moon

College of Pharmacy, Chungbuk National University, Cheongju 303-763 (South Korea)

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ABSTRACT

The factors affecting the micellar electrokinetic chromatographic separation of structurally similar tricyclic antidepressants (imipramine, amitriptyline, desipramine, nortriptyline, doxepin and trimipramine as an internal standard) were investigated by changing the species of micelles, and adding an organic modifier (urea or methanol) to the micellar solution. The determination of tricyclic antidepressants in human plasma is demonstrated under the optimum separation conditions using 37.5 m*M* phosphate buffer (pH 8.0) containing 25 m*M* dodecyltrimethylammonium bromide and 2 *M* urea at −25 kV applied voltage.

INTRODUCTION

Capillary electrophoresis (CE) is a rapidly growing analytical technique for the separation of a variety of compounds, including peptides and proteins, DNA fragments, pharmaceuticals, other organic compounds, inorganic ions, etc. The application of CE was extensively broadened by the introduction of micellar electrokinetic capillary chromatography (MECC), which was first developed by Terabe *et al.* [1]. In MECC, selective partitioning of analytes into the micellar phase as a pseudo-stationary phase causes them to migrate at different rates from the elecrophoretic mobility. Therefore, the migration times of the analytes are determined by the hydrophobicity and ionic character of the solutes, the species and concentration of the micellar phase, and the pH of the micellar medium.

Extensive applications of MECC using sodium dodecyl sulphate (SDS) as a micellar phase have been reported [2–5]. Recently, several modifications of the MECC procedure have been developed to obtain better resolution for the separation of hydrophobic compounds or complicated mixtures (twenty amino acids): (1) the addition of methanol or acetonitrile to the micellar solution [6–8]; (2) the addition of urea to the micellar solution [9]; and (3) the use or the addition of cationic surfactant [10–12].

Measurement of many drugs in body fluids is important for therapeutic drug monitoring. The relationship between the concentrations of antidepressants in human plasma or serum and their

* Corresponding author.

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therapeutic effects has been extensively studied during the past fifteen years [13–15]. There is more therapeutic monitoring of tricyclic antidepressants (TCAs) in serum as an adjunct to the clinical management of patients with depression, because this measure can help to maximize therapeutic effectiveness and safety. High-performance liquid chromatography (HPLC) and immunoassay are analytical methods for antidepressant monitoring. HPLC techniques are applicable to a wide variety of drugs and metabolites that can be analysed simultaneously, but still requires a relatively high level of analytical skill. Immunoassays have become attractive for routine clinical TCA monitoring because of their ease of performance, speed of analysis and sensitivity. However, immunological methods analyse only one drug at a time, do not measure metabolites and in some cases are subject to problems with cross-reactive interferences. MECC could be an alternative analytical tool for quantifying drug levels in human serum or plasma [16–19]. Nakagawa *et al.* [19] tried direct injection of plasma containing cefpiramide in MECC. With this method, the sensitivity is not enough to detect most therapeutic drugs in human plasma. For therapeutic drug monitoring with MECC, human plasma was pretreated as in HPLC.

In this work, the factors affecting the separation of TCAs that are structurally similar have been investigated by changing the species of micelles, and adding an organic modifier (urea or methanol) to the micellar solution. The determination of TCAs in human plasma is demonstrated under the optimum separation conditions.

EXPERIMENTAL

Chemicals

The tricyclic antidepressants desipramine (DMI), nortriptyline (NT), doxepin (DX), imipramine (IMI), amitriptyline (AMI) and trimipramine (TMI) and sodium dodecyl sulphate (SDS), dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide (TTAB) and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma (St. Louis, MO, USA). Organic solvents including hexane and isoamyl alcohol were HPLC grade. Human sera were obtained as lyophilized form SRM 909 from the National Institute of Science and Technology (NIST, Gaithersburg, MD, USA).

Instrumentation

Both commercial and laboratory-made capillary electrophoresis systems were used. The commercial instrument was a Model 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA). For the experiments, a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), 71.5 cm (50 cm to detector) × 50 μm I.D., was used as a separation column. On-column UV detection was measured at 254 nm, and the temperature of the column chamber was kept constant at 30 or 40°C unless specified otherwise. A D-502A integrator (Young-In Scientific, Seoul, South Korea) was used to record the electropherograms and for quantification by peak- area measurements. Prior to each run, the capillary was rinsed with 1 *M* sodium hydroxide and running buffer by the built-in vacuum system at 508 mmHg for 3 min each. The capillary was filled with running buffer, mainly 25 m*M* DTAB and 2 *M* urea in 37.5 m*M* phosphate buffer (pH 8.0). Running buffers were modified for best separation by adding urea or methanol. The samples were introduced by the same vacuum system for 7 s.

The laboratory-made capillary electrophoresis system was constructed in a similar way to that described previously [20]. A high-voltage power supply (0–40 kV, Glassman, Whitehouse Station, NJ, USA; Model PS/EH 40R 2.5 CTZR) was used to drive the electrophoretic process across the capillary. The platinum wires connected to the anode and the cathode of the power supply were immersed in 3-ml buffer chambers. This system was isolated in a Plexiglass box for operator safety. A longer capillary with the same I.D. as the commercial instrument was used as a separation tube. Detection was performed by on-column measurement of UV absorption by a Linear Model 206HR variable-wavelength detector (Re-

no, NV, USA), which was controlled by the 206 SOFT software. Throughout the work the Model 206 detector was employed in the high-speed polychrome mode by scanning from 195 to 320 nm at 5-nm intervals.

Sample preparation

Standard stock solutions of each tricyclic antidepressant (1 mg/ml) were prepared in doubly distilled water, and a standard model mixture of five drugs (DMI, NT, DX, IMI and AMI) was prepared by diluting the standard stock solution with doubly distilled water to a certain concentration (10–20 μg/ml). Drug concentrations in human serum (NIST SRM 909) spiked with five TCAs and TMI as an internal standard (I.S.) and in the plasma of patients treated with one of the TCAs were determined. Sample extractions were carried out using hexane–isoamyl alcohol in a similar way to the method described previously [21]. A 1-ml aliquot of standard solution, human serum or patient plasma was spiked with 50 μl of the I.S. (10 μl/ml), 1 ml of 2 *M* NaOH was added to raise the pH at which the free bases of TCAs can be extracted into the organic solvent, and 5 ml of hexane–isoamyl alcohol (99:1, v/v) were used to extract these free-base TCAs. After vigorous vortex-mixing and centrifugation at 2500 *g* for 5 min, 4 ml of the organic layer were transferred to a conical glass tube. The organic layer was acidified by adding 100 μl of 0.1 *M* HCl, and at the acidic pH, the base could be re-extracted into an aqueous phase. The aqueous layer (60 μl) was dried in a speed vacuum concentrator, and reconstituted in 20 μl of distilled water for CE analysis.

RESULTS AND DISCUSSION

For the determination of TCAs with CE, the factors affecting the separation of six TCAs (Fig. 1 and Table I) were examined. TCAs are secondary or tertiary amines with similar structures and are basic compounds ($pK_a > 9.0$).

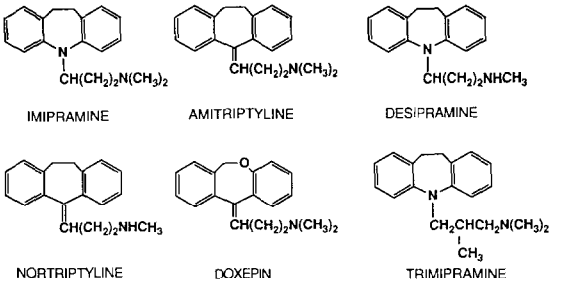

Fig. 1. Structures of TCAs.

Optimization of MECC separation

pH effect. The pH dependence of the separation was examined with 25 m*M* phosphate buffer or borate buffer with and without micelles over the pH range 8.0–11.0 (Fig. 2). Without micelles, five TCAs were comigrated below pH 9.0 and were relatively well separated at pH 7.0, but not sufficiently for quantitative analysis.

Addition of micelles. Anionic surfactant SDS micelles, which are extensively used for MECC, were added to separate the TCAs based on the hydrophobicity in addition to the ionic character. As shown in Fig. 2, it is impossible to separate TCAs with SDS because the ionic interaction of amine group in TCAs with the anionic surface of SDS micelles is much stronger than the hydrophobic partitioning of tricyclic part into hydrophobic core of micelles. In order to remove this ionic interaction, cationic surfactants such as long-chain alkyltrimethylammonium bromide were used to reverse the electroosmotic flow, as noted previously [10]. Fig. 3 shows the effect on

TABLE I

pK_a VALUES AND THERAPEUTIC RANGE OF TCAs [22]

Compound	pK_a	Therapeutic range (μg/ml)
Desipramine	10.2	0.05–0.15
Nortriptyline	9.7	0.05–0.15
Doxepin	9.0	0.02–0.15
Imipramine	9.5	0.1
Amitriptyline	9.4	0.1–0.2

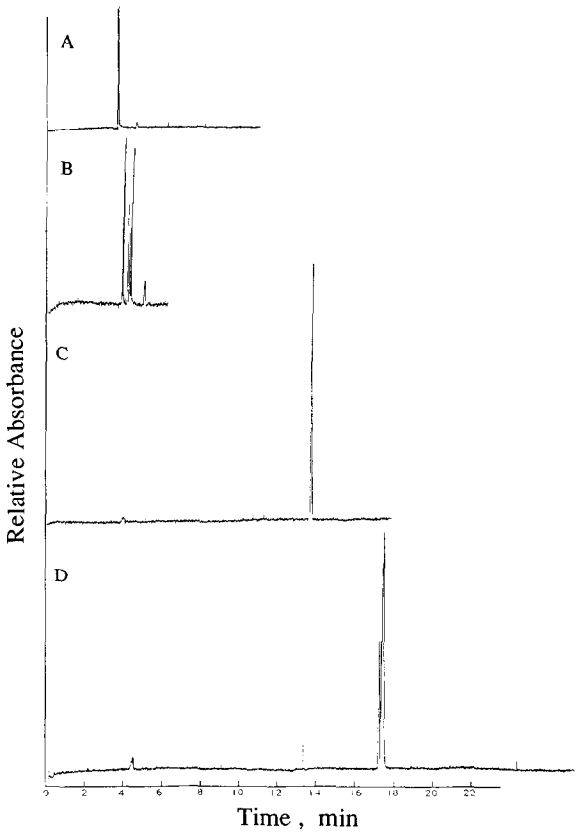

Fig. 2. Electropherograms of five TCAs (DMI, NT, DX, IMI, AMI) obtained using (A) 25 m*M* phosphate buffer (pH 8.0), (B) 25 m*M* borate buffer (pH 9.3), (C) 25 m*M* phosphate buffer (pH 8.0) containing 50 m*M* SDS, and (D) 25 m*M* phosphate buffer (pH 11.1) containing 50 m*M* SDS at 30 kV applied voltage with a fused-silica capillary (715 mm × 0.05 mm I.D.). Detection was at 254 nm.

the electroosmotic flow of the alkyl chain length of the cationic surfactants. As the alkyl chain length increases, the electroosmotic flow changes rapidly; it can be kept constant at higher concentrations of surfactants. Optimum resolutions with various concentrations (0.025–50 m*M*) of cationic surfactants were obtained at 1.0 m*M* CTAB, 2.5 m*M* TTAB and 25 m*M* DTAB (Fig. 4). The order of solute elution depends on the hydrophobicity of the solute. The most hydrophilic compound, DMI, emerged out first, followed by less hydrophilic NT, DX, DMI and AMI. With CTAB and TTAB, the peaks were

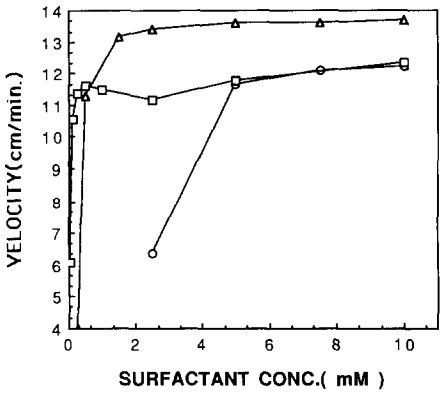

Fig. 3. Effect of cationic surfactants on electroosmotic mobility. (○) DTAB; (△) TTAB; (□) CTAB in 37.5 m*M* phosphate buffer (pH 8.0) at −30 kV. Other conditions as in Fig. 2.

broadened and the best separation efficiency was obtained with 25 m*M* DTAB.

Additives. The addition of urea and methanol to 25 m*M* DTAB solution improved the resolu-

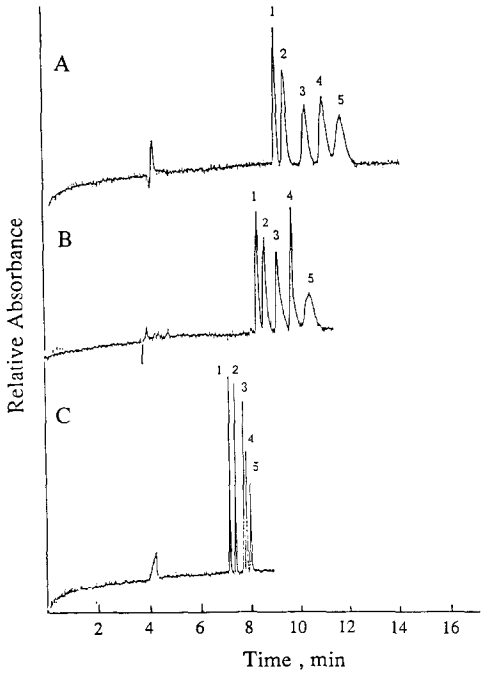

Fig. 4. Electropherograms of TCAs with different micellar species. (A) 1.0 m*M* CTAB; (B) 2.5 m*M* TTAB; (C) 25 m*M* DTAB in 37.5 m*M* phosphate buffer (pH 8.0). Peaks: 1 = DMI; 2 = NT; 3 = DX; 4 = IMI; 5 = AMI. Other conditions as in Fig. 3.

tion of TCAs (Fig. 5). Factors affecting the resolution were examined by observing the dependence of the retention time on the concentration of urea or methanol (Fig. 6). The capacity factors, k', could not be calculated because a suitable DTAB marker could not be found. When Sudan III, which is proper marker for SDS micelles, was used with DTAB micelles, Sudan III was eluted in between solutes. This means that Sudan III is not a proper marker of DTAB micelles. As shown in Fig. 6A, the electroosmotic velocity did not alter significantly with increasing urea concentration, and the resolution was a maximum with 2–3 M urea addition into DTAB solution. Except for the most hydrophobic AMI, the velocities of the solutes were slightly increased by raising the urea concentration.

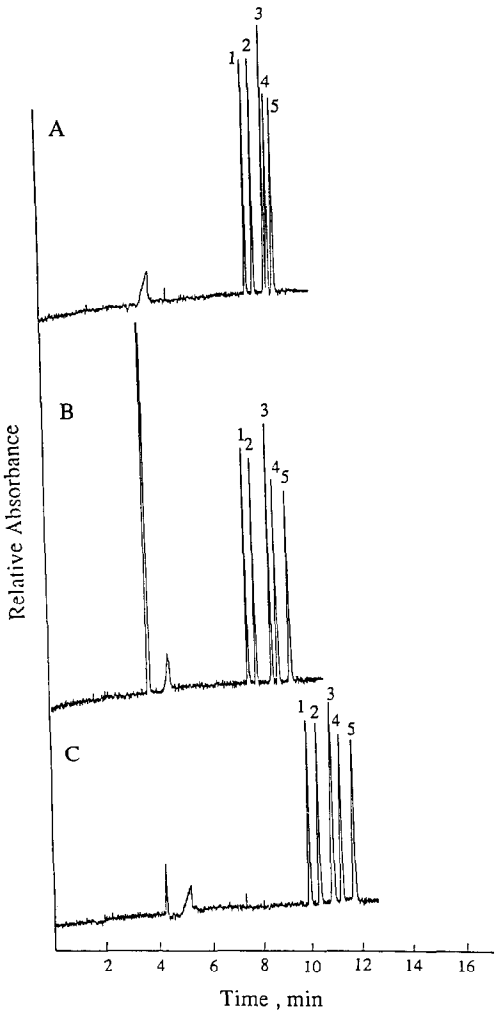

Fig. 5. Effect of additives on TCA separation. (A) Without additives; (B) with 2 M urea; (C) with 10% methanol. Other conditions as in Fig. 3.

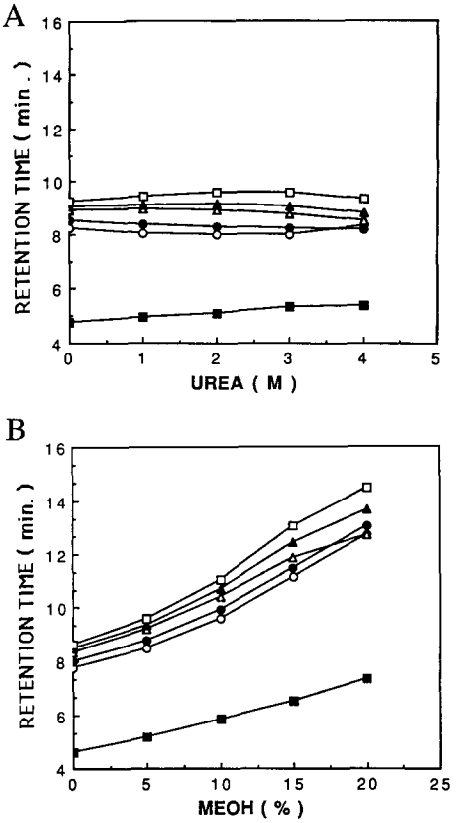

Fig. 6. Effect of additive concentration of (A) urea and (B) methanol, on the TCA mobility: (○) DMI; (●) NT; (△) DX; (▲) IMI; (□) AMI; (■) electroosmotic flow.

The effect of urea on MECC in terms of thermodynamic quantities, *i.e.* micellar solubilization and micelle formation, has been discussed elsewhere [9]. It is not easy to find a simple explanation for the observed effect of urea. However, it is clear that urea increases the solubility of most TCAs in water by diminishing the water structure around the tricyclic group, and consequently the partitioning of TCAs to DTAB micelles is significantly reduced. The solubility change affects the capacity factor and selectivity, and the migration-time window broadens without significant

alteration of the analysis time. A wide migration-time window is beneficial for high resolution.

The addition of methanol to the buffer containing DTAB micelles improved the resolution of TCAs (Fig. 5). The better resolution was demonstrated by raising the methanol concentration to 20% and arised from a decrease of the electroosmotic and electrophoretic flow (Fig. 6B). The decrease in the electroosmotic flow following the addition of methanol has been discussed previously [6–8,23] and is associated with the change in the zeta potential that arises from the changes in the viscosity and the dielectric constant of the buffer. Salomon *et al.* [6] have demonstrated that the reduction in the thickness of the compact layer next to the interface is another factor responsible for the decrease in the electroosmotic flow. The decrease of the electrophoretic flow beyond electroosmotic flow reduction is shown in Fig. 6B. Part of the decrease could arise from the increase of viscosity in the presence of methanol. Another factor reducing the electrophoretic flow was the decreasing dielectric permittivity of the water–methanol as the percentage of methanol increased. A lower dielectric permittivity would favour the deprotonated amines in the equilibrium between the protonated amines and their neutral bases. Such a decrease in the concentration of the protonated amines would possibly reduce the electrophoretic mobility. A high resolution of TCAs was obtained by the reduction of the electroosmotic and electrophoretic flow; however, the separation efficiencies of TCAs were dramatically decreased following the addition of methanol.

In Table II, the numbers of theoretical plates (N) of TCAs with running buffer containing 25 mM DTAB micelles were calculated with or

TABLE II

EFFECTS OF UREA AND METHANOL ON THE SEPARATION EFFICIENCY OF TCAs

Values are numbers of theoretical plates.

Compound	No additive	Urea (2 M)	Methanol (10%)
Desipramine	$1.43 \cdot 10^5$	$1.43 \cdot 10^5$	$0.83 \cdot 10^5$
Nortriptyline	$1.17 \cdot 10^5$	$1.41 \cdot 10^5$	$0.84 \cdot 10^5$
Doxepin	$1.65 \cdot 10^5$	$1.24 \cdot 10^5$	$0.64 \cdot 10^5$
Imipramime	$1.69 \cdot 10^5$	$1.21 \cdot 10^5$	$0.84 \cdot 10^5$
Amitriptyline	$1.92 \cdot 10^5$	$1.15 \cdot 10^5$	$0.72 \cdot 10^5$

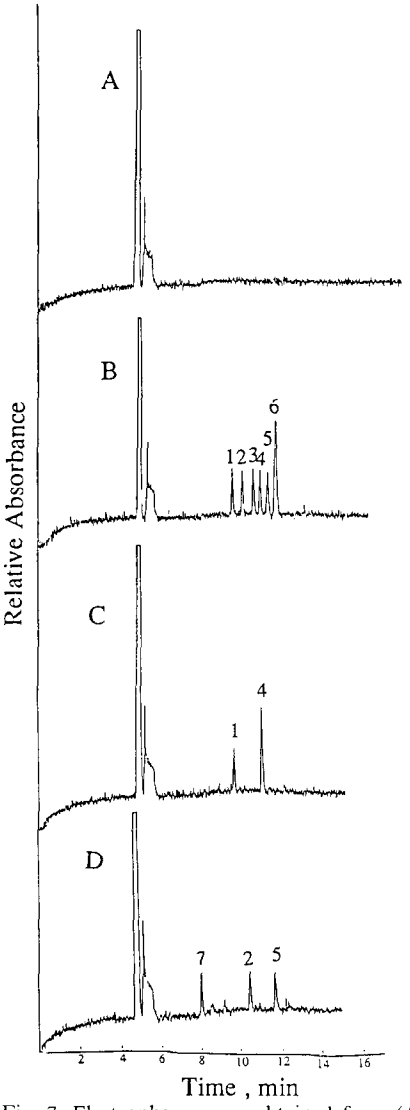

Fig. 7. Electropherograms obtained from (A) extracted blank human serum, (B) extracted human serum spiked with TCAs (final concentration 100 ng/ml each) and the I.S., (C and D) extracted plasma from a patient taking IMI (C, 100 mg) or AMI (D, 175 mg). Peaks: 1 = DMI; 2 = NT; 3 = DX; 4 = IMI; 5 = AMI, 6 = TMI. Peak 7 is assumed to be *trans*-10-hydroxynortriptyline. The analytes contained 37.5 mM phosphate buffer (pH 8.0) with 25 mM DTAB and 2 M urea at −25 kV and other conditions were as in Fig. 2.

without additives (2 *M* urea and 10% methanol). The separation efficiencies after the addition of 2 *M* urea were similar to those without additives; on the other hand, values of *N* in the presence of methanol were significantly reduced.

The optimum separation of TCAs with the best resolution and separation efficiency was achieved with 37.5 m*M* phosphate buffer (pH 8.0) containing 25 m*M* DTAB and 2 *M* urea at an applied voltage of −30 kV. Under these conditions, no interfering peak was observed when the blank human serum was extracted with hexane–isoamyl alcohol, as described in Experimental (Fig. 7A). When the standard drug mixture, including TMI as an I.S., was spiked human serum (final concentration 100 ng/ml) and extracted, each peak was well resolved (Fig. 7B). Fig. 7C and D shows the electropherograms of the plasma of a patient taking IMI (100 mg) or AMI (175 mg), respectively, as confirmed by immunoassay. Fig. 7C shows an extra peak, which is presumed to be desipramine, the demethylated metabolite of IMI. IMI and desipramine in Fig. 7C are quantitated as 166 and 106 ng/ml, respectively. In Fig. 7D, the extra peaks besides the parent drug AMI are assumed to be nortriptyline, a demethylated metabolite of AMI, and *trans*-10-hydroxynortriptyline, a hydroxylated metabolite of nortriptyline, which were identified by HPLC [24]. In Fig. 7D, the AMI peak is determined as 100 ng/ml and nortriptyline as 104 ng/ml. The parent drug and its metabolites in plasma can be simultaneously determined with MECC.

To confirm the peak and to check the peak purity, a variable-wavelength detector, which permits the automatic recording of the spectra of the peaks during analysis, was used. The three-dimensional electropherograms in Fig. 8 represent the absorbance *vs.* retention time *vs.* wavelength relationships for human serum spiked with TCAs (Fig. 8). The retention times in Fig. 8 are higher than those in Fig. 7, because the laboratory-made capillary system used with the multiwavelength UV detector had a longer capillary than that used in Fig. 7. There was no difference between spectra obtained with and without urea in the running buffer. Multiwavelength scanning of peaks permits a quick and reliable confirmation of the drug peaks.

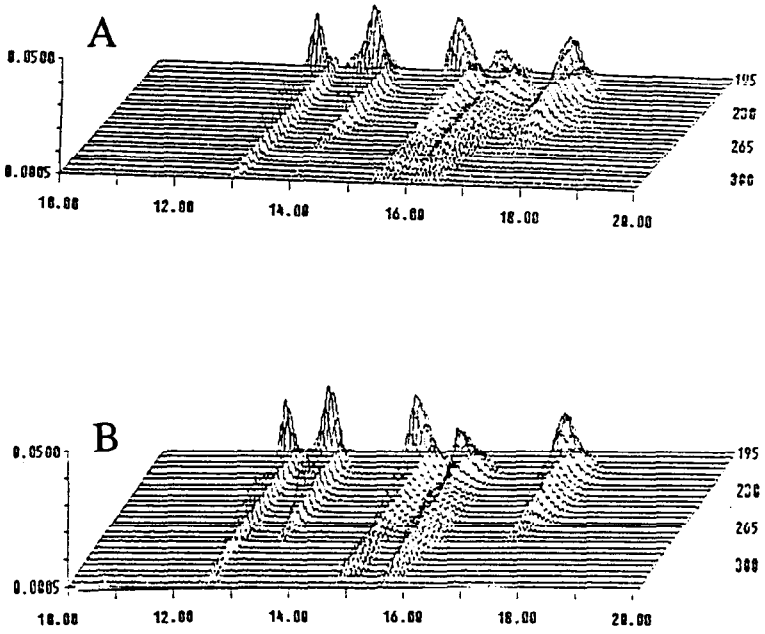

Fig. 8. Three-dimensional electropherograms of standard TCAs (A) without 2 *M* urea and (B) with 2 *M* urea. Conditions as in Fig. 3, except that a laboratory-made capillary electrophoresis system with a variable-wavelength detector and a longer capillary column were used.

TABLE III

REPRODUCIBILITY OF RETENTION TIME AND PEAK AREA OF TCAs

Concentration (ng/ml)	Coefficient of variation (%)									
	Retention time					Peak area				
	DMI	NT	DX	IMI	AMI	DMI	NT	DX	IMI	AMI
Within-run (n = 6)										
50	0.85	0.80	0.51	0.49	0.46	6.74	6.35	5.81	5.35	2.53
100	0.64	0.59	0.38	0.41	0.41	4.83	3.23	3.12	2.70	2.25
150	0.52	0.54	0.53	0.50	0.53	2.45	3.05	3.17	1.88	1.93
200	0.41	0.40	0.36	0.40	0.42	2.35	2.13	1.08	2.31	0.69
Between-run (n = 4)										
50	1.04	1.02	1.14	1.13	1.24	4.54	7.87	6.84	8.27	6.26
100	2.47	2.53	2.55	2.64	2.43	6.01	6.08	7.62	7.92	6.49
150	1.16	1.20	2.20	1.22	1.22	6.03	8.89	7.29	6.23	3.56
200	1.16	1.19	1.67	1.27	1.33	5.80	4.81	6.78	5.86	3.85

TABLE IV

RECOVERY OF TCAs DURING THE ANALYTICAL PROCEDURE

Concentration (ng/ml)	Recovery, (mean ± S.D., n = 4) (%)	C.V. (%)
Desipramine		
50	78.7 ± 3.4	4.3
100	67.3 ± 5.1	7.6
150	78.0 ± 6.0	7.7
200	75.6 ± 5.3	7.0
Nortriptyline		
50	79.7 ± 3.4	4.4
100	75.9 ± 4.8	6.3
150	80.7 ± 3.3	4.1
200	77.1 ± 5.8	7.5
Doxepin		
50	76.4 ± 4.8	6.3
100	78.1 ± 3.0	3.9
150	84.1 ± 4.3	5.1
200	91.4 ± 2.5	2.8
Imipramine		
50	79.7 ± 4.1	5.1
100	84.4 ± 4.8	5.6
150	91.0 ± 1.8	4.0
200	90.2 ± 4.1	4.5
Amitriptyline		
50	88.0 ± 2.7	3.1
100	80.0 ± 3.7	4.6
150	94.7 ± 3.0	3.1
200	88.1 ± 3.0	3.4

Analytical variables

Precision. We assessed the precision of the method by repeated analyses of plasma specimens containing known concentrations of the drugs being investigated. As shown in Table III, the coefficients of variations (C.V.) of the retention time were less than 0.8% for the within-run precision, and less than 2.0%, for the between-run precision. Those of the peak areas were less than 6–8% both within and between runs.

Recovery. The absolute analytical recovery from plasma of the five drugs was measured by adding a known concentration of drugs and the I.S. to drug-free human serum (Table IV). These were extracted and analysed by our method. The absolute recoveries were calculated from the peak-area ratio of the drug to the I.S. The recoveries were 81–92% for DMI, 80–87% for NT, 94–106% for DX, 94–108% for IMI and 90–97% for AMI, respectively.

Detection limit. The detection limits for quantitative determination were 5–10 ng/ml through the whole procedure including concentration process during extraction.

Linearity. The calibration curves for TCAs are linear ($r > 0.995$) within a therapeutic range from 50 to 250 ng/ml, as shown in Fig. 9.

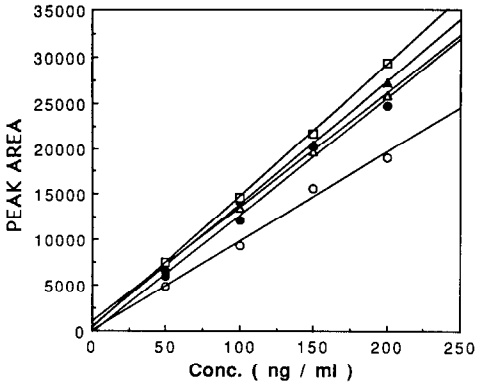

Fig. 9. Calibration curve for TCAs. Symbols as in Fig. 6.

CONCLUSION

MECC optimization for the separation of TCAs by quantitative analysis was demonstrated by examining the effects of additives (urea, methanol) and various micelle species. The proposed method provides good sensitivity and reproducibility for the MECC analysis of DMI, NT, DX, IMI and AMI. These quantitative data prove the feasibility of the method for the monitoring of the drugs in body fluids.

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