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Analysis of antiepileptic drugs in human plasma using micellar electrokinetic capillary chromatography

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ABSTRACT

We describe a method for the simultaneous determination of antiepileptic drugs (ethosuccimide, phenytoin, primidone, phenobarbital, carbamazepine and valproic acid) by micellar electrokinetic capillary chromatography using sodium dodecyl sulphate as the micellar phase. Factors affecting the micellar electrokinetic separation were studied for the quantitative determination of these drugs in human plasma. The confirmation of the peaks and the specificity of the method were investigated by combining multiwavelength detection with micellar electrokinetic capillary chromatography.

INTRODUCTION

Measurements of many drugs in body fluids are important for therapeutic drug monitoring. For nearly 15 years the antiepileptics (Fig. 1) have been monitored in the plasma of patients, since a good correlation between their blood concentrations and clinical responses has been demonstrated and life-long multiple-drug therapy is necessary. Monitoring their concentrations in the body fluid, especially in plasma, is therefore essential for the optimization of pharmacotherapy. Analytical methods for the quantitation of antiepileptic drugs developed considerably in the late 1970s and early 1980s [1–3]. Recently immunological methods have become attractive for routine clinical monitoring during chronic therapy because of their ease of performance, speed of analysis and sensitivity. However, immunoassays analyse only one drug at a time, have a limited sensitivity for quantitation of drugs

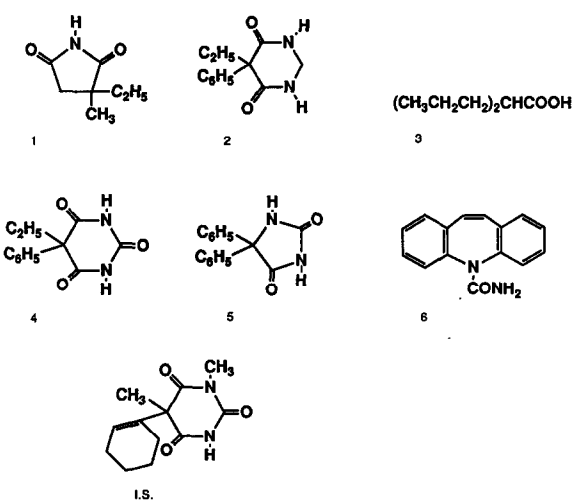

Fig. 1. Chemical structure of antiepileptic drugs. 1 = Ethosuccimide; 2 = primidone; 3 = valproic acid; 4 = phenobarbital; 5 = phenytoin; 6 = carbamazepine; I.S. = hexobarbital.

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following a single dose, do not measure metabolites, and in some cases are subject to problems with cross-reactive interferences. Chromatographic methods are still utilized as the standard reference methods for the antiepileptic drugs. High-performance liquid chromatographic (HPLC) methods have been applied for the determination of all antiepileptic drugs. However, they still require a relatively high level of analytical skill and are relatively high-cost procedures.

As newly developed analytical methods, capillary electrophoresis and micellar electrokinetic capillary chromatography (MECC) are perceived to be attractive tools for the analysis of pharmaceuticals because of their high separation efficiency, easy operation and low running cost [4–10]. MECC, first developed by Terabe *et al.* [11], utilized the same instrumental set-up as capillary electrophoresis except that it employs the micellar solution as the electrophoretic medium. Selective partitioning of the analytes into the micellar phase as a pseudo stationary phase causes them to migrate at rates different from their electrophoretic mobility.The analytes in MECC can be separated on the basis of either the hydrophobicity or the ionic character of solutes. Separation depending on the hydrophobicity of the solutes in MECC can be peformed by varying the micellar concentration, which has similar effects to changing in the surface structure in HPLC (*e.g.* changing from C_8 to C_{18}). Early work by Otsuka *et al.* [12] demonstrated the use of MECC for the analysis of neutral aromatic compounds. Applications of MECC have rapidly expanded to include neutral and charged molecules combining electrophoresis and chromatography.

In spite of the high resolution of capillary electrophoresis, the problem of identifying peaks in the electropherogram remains to be solved. Capillary electrophoresis–mass spectrometry (CE–MS) is developing in many laboratories [13–15] for this purpose. Because of the technical difficulties and high cost of MS, the alternative method using multiwavelength detection with a photodiode-array detector is a suitable technique for monitoring in HPLC [16] and has recently been applied to capillary electrophoresis [10,17–20]. It allows the collection of absorption spectra of each peak in the electropherogram, and permits peak confirmation and determination of peak purity by comparison with standard absorption spectra. The purpose of this report is to describe the factors affecting separation of antiepileptic drugs with MECC, quantification with this method and identification of patient samples using a multiwavelength UV detector.

EXPERIMENTAL

Chemicals

The antiepileptic drugs phenytoin (PHT), phenobarbital (PB), primidone (PRM), carbamazepine (CBZ), ethosuccimide (ESM) and valproic acid (VPA) and sodium dodecyl sulphate (SDS) were purchased from Sigma (St. Louis, MO, USA). Sodium phosphate dibasic and sodium phosphate monobasic were from DukSan (Kyungkido, South Korea). Ethyl acetate was HPLC grade from Junsei (Japan). Human sera were obtained as lyophilized form SRM 909 from the National Institute of Science and Technology (NIST, Gaithersberg, MD, USA).

Capillary electrophoresis apparatus

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), 72 cm (50 cm to detector) × 50 μm I.D., was used as a separation column. On-column UV detection was measured at 210 nm, and the temperature of the column chamber was kept constant at 30°C unless specified otherwise. A D-502A integrator (Young-In Scientific, Seoul, South Korea) was used for recording the electropherograms and for quantification by peak area measurements. Prior to each run, the capillary was rinsed with 0.1 *M* sodium hydroxide and running buffer by the built-in vacuum system at 508 mmHg for 3–5 min. The capillary was filled with running buffer, mainly 50 m*M* SDS in 25 m*M* phosphate buffer (pH 8.0). The samples were introduced by the same vacuum system for 2.0 s.

The laboratory-made capillary electrophoresis system was constructed in a similar way to that described previously [21]. 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 capil-

lary. 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 Plexiglas 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 (Reno, NV, USA) which is controlled with 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 antiepileptic drug (1 mg/ml) were prepared in methanol, and a standard model mixture composed of six drugs (ESM, PRM, PB, VPA, PHT and CBZ) was prepared by diluting the standard stock solution with doubly distilled water to a certain concentration (7–100 μg/ml). Drug concentrations in human serum (NIST SRM 909) spiked with six antiepileptics and hexobarbital as an internal standard and in the plasma of patients treated with one of the antiepileptics were determined. Sample extractions were performed using ethyl acetate as described previously [22]. A 100-μl aliquot of standard solution, spiked serum or patient plasma was added to 900 μl of ethyl acetate in a glass tube. A known concentration of hexobarbital (15 μg/ml) was used as an internal standard. After vigorous vortex-mixing and centrifugation at 2060 *g* for 2 min, 500 μl of the organic layer were transferred to a conical glass tube, evaporated to dryness under a gentle stream of nitrogen gas and reconstituted with 50 μl of 5% methanol in distilled water. This reconstituted sample was injected for capillary electrophoresis.

RESULTS AND DISCUSSION

Optimization of MECC separation

Since antiepileptic drugs have wide spectrum of physical properties (solubility and pK_a values) [23], factors affecting the separation were investigated to determine the optimum separation conditions. The pH and micellar concentration of the running buffer mainly affect the resolution of solutes. When the pH was varied from 6.0 to 8.0 with 25 m*M* phosphate buffer containing 50 m*M* SDS, the migration times of six solutes stayed constant above pH 6.4. The migration time of the solutes increased with increasing SDS concentration, the degree of increase depending on the hydrophobicity of the molecules. The hydrophobic molecules CBZ and PHT show a dramatic increase in migration time depending on SDS concentration, and are followed by PB, PRM, ESM and VPA. This trend is consistent with previous findings by Terabe *et al.* [11]. The capacity factor, *k'*, defined and described previously [11], was calculated from the retention times of methanol as a neutral marker and Sudan III as a micelle marker depending on SDS concentration (Fig. 2). The capacity factors of all solutes except VPA increased linearly with increasing SDS concentration up to 80 m*M* SDS, which indicates that hydrophobic interaction between solute and micelle is the main interaction in the separation. However, negatively charged VPA, which is constant over the various SDS concentrations, is separated based on its

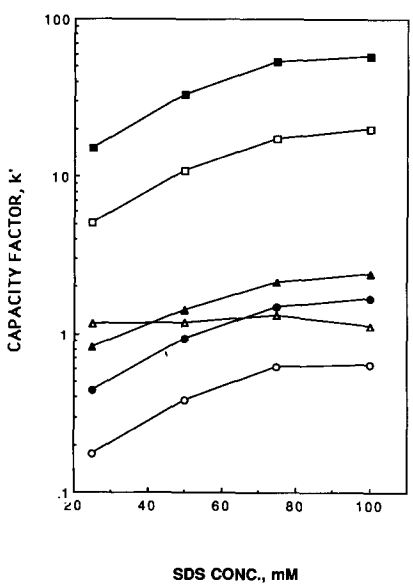

Fig. 2. Effect of SDS concentration of *k'* using a standard mixture of antiepileptics. Conditions: an Applied Biosystem capillary electrophoresis system was used with running buffer (25 m*M* phosphate buffer containing various concentration of SDS at pH 7.0). Detection at 210 nm, run at 30 kV at 30°C in a 50 μm I.D. capillary. Symbols: ○ = ethosuccimide; ● = primidone; △ = valproic acid; ▲ = phenobarbital; □ = phenytoin; ■ = carbamazepine.

electrophoretic mobility and does not interact with the micelle.

For the quantitative analysis of solutes by MECC, the reproducibility of the migration and peak area is important. Temperature is a crucial factor in the reproducibility. In this experiment, ambient temperature was kept constant.

With the optimization of the resolution and the separation time, the running conditions chosen were 25 m*M* phosphate buffer containing 50 m*M* SDS at 30°C with 30 kV applied voltage. Under these conditions, the antiepileptic drugs with capacity factors ranging from 0.2 (ESM) to 40 (CBZ) were well resolved in less than 13 min. With electrophoresis, antiepileptics and the internal standard all exhibited symmetrical peaks (Fig. 3). Fig. 3A shows a typical chromatogram for the drug standard without extraction [VPA and ESM (125 μg/ml each), PHT, PRM and hexobarbital (15 μg/ml each), PB (10 μg/ml) and CBZ (7 μg/ml)]. No interfering peak was observed when the blank human serum spiked with internal standard hexobarbital was extracted with ethyl acetate and analysed (Fig. 3B). When the same standard drug mixture as in Fig. 3A was spiked in human serum and extracted with ethyl acetate as described in the Experimental section, the peak of volatile VPA completely disappeared and the ESM peak was remarkably reduced (Fig. 3C). This indicates that the extraction procedure is not appropriate for the quantitative analysis of VPA and ESM. Fig. 3D–F shows electropherograms of the plasma of a patient taking PB, PHT and CBZ as

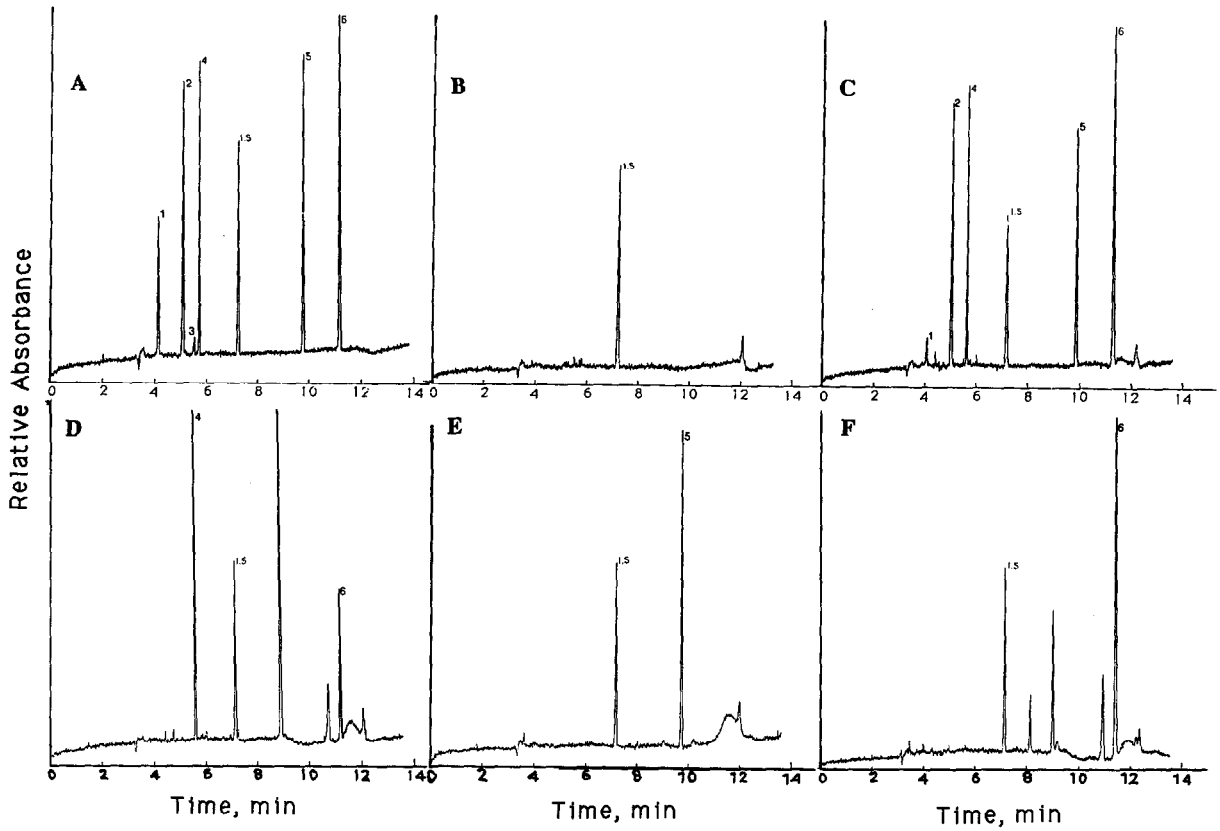

Fig. 3. Electropherograms obtained from (A) a standard mixture of ethosuccimide (1), primidone (2), valproic acid (3), phenobarbital (4), hexobarbital (internal standard, I.S.), phenytoin (5) and carbamazepine (6) without extraction; (B) extracted blank human serum spiked with internal standard; (C) extracted human serum spiked with antiepileptics and internal standard; (D–F) extracted plasma from an epileptic patient taking phenobarbital (D), phenytoin (E) or carbamazepine (F). Conditions were the same as in Fig. 2 except the SDS concentration was 50 m*M*.

confirmed by immunoassay. Fig. 3D, from a patient taking PB, shows the extra peaks which were not detected in the other patient taking PB. This indicates that the extra peaks might be the other medications. Human plasma from a patient taking PHT shows clear peaks of PHT and hexobarbital (Fig. 3E). However, electropherograms from two patients taking CBZ show the same pattern (Fig. 3F). The extra peaks besides CBZ and hexobarbital in Fig. 3F are presumed to be the metabolites of CBZ, *i.e.* carbamazepine-10, 11-diol and carbamazepine-10,11-epoxide. Further studies should be performed to identify metabolites.

To confirm the peak and check the peak purity, a Linear Model 206HR variable-wavelength detector, which permits automatic recording of the spectra of the peaks during analysis, was used. The three-dimensional electropherograms depicted in Fig. 3 represent the absorbance *vs.* retention time *vs.* wavelength relationships for human serum spiked with antiepileptics (Fig. 4C) and the plasma of a patient taking PB (Fig. 4D), PHT (Fig. 4E) or CBZ (Fig. 4F). The retention times in Fig. 4 are higher than those in Fig. 3 because in this case the laboratory-made capillary system with a longer capillary than that used in Fig. 3 was used with a multiwavelength UV detector. The absorption spectrum of each peak can be extracted from the gathered data points as a so-called time slice using detector software. The absorption spectra between 195 and 320 nm of the peak, which are assumed to be PB (Fig. 5D), PHT (Fig. 5E) or CBZ (Fig. 5F) as in Fig. 4D–F are compared with those of standard drug (Fig. 5d–f). The excellent agreement between standard and sample indicates that MECC separation of these drugs in human plasma is not subjected to interference by other components in plasma, and multiwavelength scanning of peak permits a quick and reliable confirmation of the drugs.

TABLE I

REPRODUCIBILITY OF RETENTION TIME AND PEAK AREA OF ANTIEPILEPTICS

Values are mean ± S.D. (n = 5); values in parentheses are coefficients of variation (%).

Compound	Concentration (μg/ml)	Retention time (min)	Relative peak area
Ethosuccimide	25	4.45 ± 0.01 (0.10)	0.25 ± 0.01 (4.71)
	75	4.41 ± 0.14 (0.33)	0.58 ± 0.04 (8.27)
	100	4.44 ± 0.01 (0.32)	1.03 ± 0.06 (6.30)
	200	4.41 ± 0.01 (0.08)	1.84 ± 0.08 (2.54)
Primidone	5	5.47 ± 0.01 (0.16)	0.53 ± 0.03 (5.81)
	20	5.39 ± 0.01 (0.10)	1.46 ± 0.03 (2.41)
	30	5.45 ± 0.02 (0.38)	2.21 ± 0.03 (1.59)
	40	5.42 ± 0.01 (0.35)	2.80 ± 0.03 (1.31)
Phenobarbital	5	6.03 ± 0.01 (0.20)	0.54 ± 0.01 (2.57)
	20	5.87 ± 0.01 (0.16)	1.79 ± 0.03 (1.93)
	40	5.82 ± 0.01 (0.29)	3.57 ± 0.10 (2.94)
	60	5.98 ± 0.03 (0.52)	5.15 ± 0.09 (1.86)
Phenytoin	5	10.58 ± 0.03 (0.28)	1.04 ± 0.01 (1.52)
	20	10.40 ± 0.01 (0.16)	3.51 ± 0.14 (4.12)
	30	10.39 ± 0.05 (0.50)	3.13 ± 0.07 (2.46)
	40	10.55 ± 0.05 (0.55)	2.96 ± 0.06 (2.11)
Carbamazepine	5	12.09 ± 0.03 (0.32)	1.77 ± 0.01 (0.39)
	20	11.88 ± 0.02 (0.19)	6.13 ± 0.23 (3.82)
	30	11.81 ± 0.06 (0.53)	9.90 ± 0.39 (4.01)
	40	12.10 ± 0.06 (0.53)	13.23 ± 0.32 (1.32)

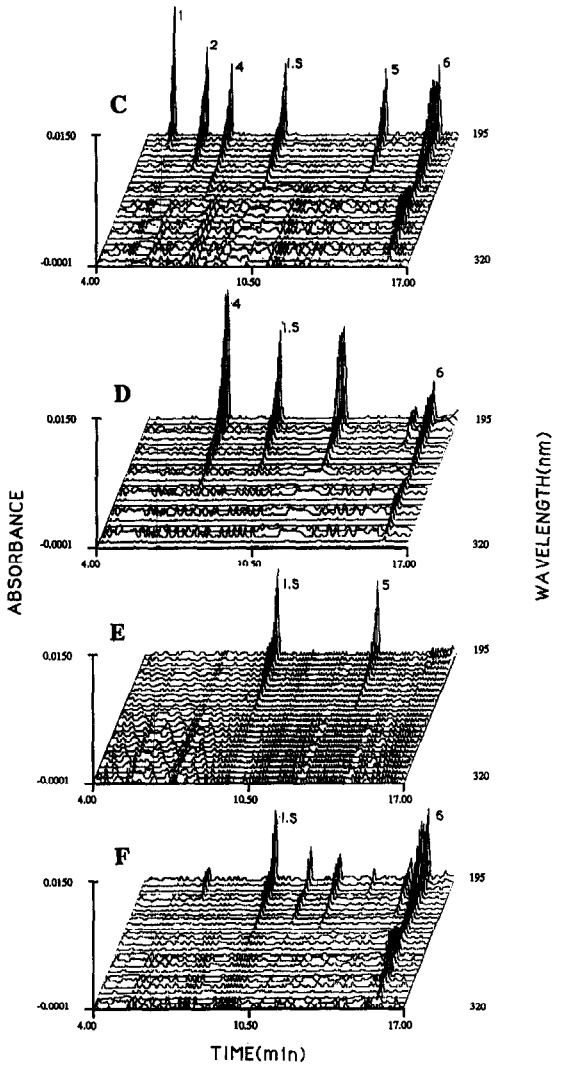

Fig. 4. Three-dimensional electropherograms of the same samples as in Fig. 3C–F. Conditions are the same as in Fig. 3 except that a laboratory-made capillary electrophoresis system with a variable-wavelength detector and a longer capillary column that that used in Fig. 3 was utilized.

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 I, the coefficients of variation (C.V.s) for within-day precision of retention time are less than 0.5% and those of peak area are less than 4%, except for ESM, whose C.V.s vary from 2.5 to 8.27%.

Recovery. The absolute analytical recovery from plasma of the five drugs was measured by adding a known concentration of drugs and internal standard to drug-free plasma, as shows in Table II. This plasma was then analysed by our method. Absolute recoveries were calculated from the drug to internal standard peak-area ratio. The recoveries of PRM, PB, PHT and CBZ were 93–106% and those of ESM 77–92%, while the recovery of VPA was impossible to measure because of its volatility.

Linearity and sensitivity. Concentration and peak area ratio correlated linearly with each other for the

TABLE II

RECOVERY OF ANTIEPILEPTICS DURING THE ANALYTICAL PROCEDURE

Values are mean ± S.D. (n = 5); values in parentheses are coefficients of variation (%)

Compound	Concentration (μg/ml)	Recovery (%)
Ethosuccimide	25	77.09 ± 7.98 (10.35)
	50	89.64 ± 9.85 (10.99)
	75	85.76 ± 7.05 (8.22)
	100	90.38 ± 6.99 (7.73)
	200	92.91 ± 7.64 (8.22)
Primidone	5	98.62 ± 8.11 (8.22)
	10	107.03 ± 5.69 (5.31)
	20	91.05 ± 2.85 (3.13)
	30	106.52 ± 5.64 (5.30)
	40	93.19 ± 1.71 (1.84)
Phenobarbital	5	94.21 ± 2.87 (3.05)
	10	105.72 ± 4.25 (4.02)
	20	94.01 ± 5.81 (6.20)
	40	106.65 ± 4.92 (4.61)
	60	97.82 ± 3.96 (4.05)
Phenytoin	5	97.28 ± 3.73 (3.83)
	10	98.70 ± 4.89 (4.95)
	20	93.05 ± 7.14 (7.67)
	30	81.01 ± 6.88 (8.49)
	40	68.17 ± 8.27 (12.14)
Carbamazepine	5	93.49 ± 3.21 (3.44)
	10	98.58 ± 4.65 (4.72)
	20	93.90 ± 4.33 (4.61)
	30	102.63 ± 3.84 (3.75)
	40	93.39 ± 2.75 (2.95)

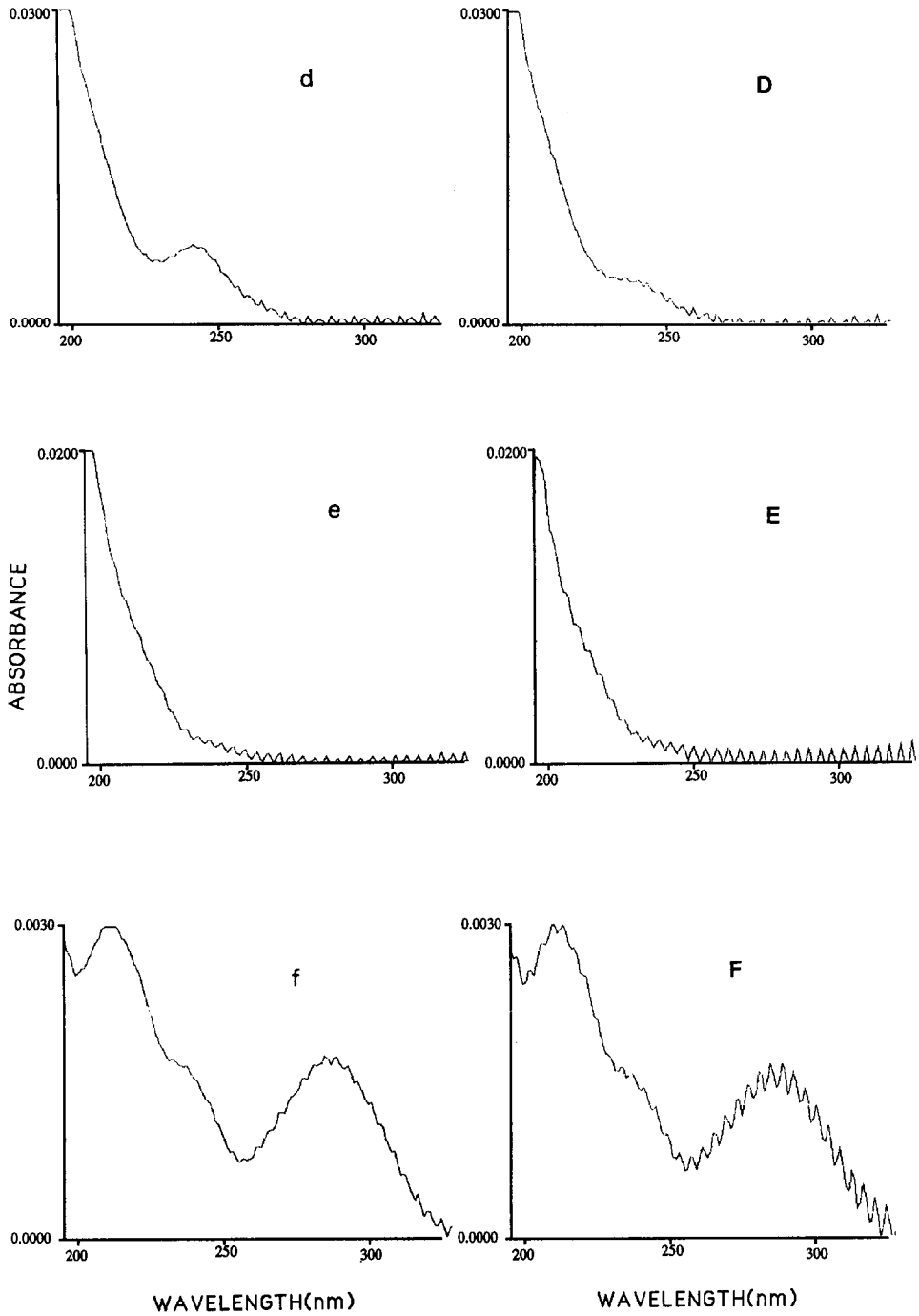

Fig. 5. Normalized time slices of (D) phenobarbital, (E) phenytoin and (F) carbamazepine of the data in Fig. 4 are compared with standard phenobarbital (d), phenytoin (e) and carbamazepine (f).

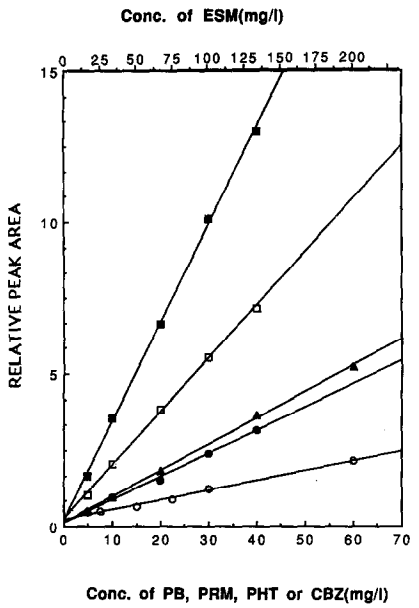

Fig. 6. Calibration curves for ethosuccimide (○), primidone (●), phenobarbital (▲), phenytoin (□) and carbamazepine (■) in a standard mixture.

drugs examined in Fig. 6. Detection ranges are reasonable for the monitoring of the therapeutic concentration.

Interference. To determine the potential clinical usefulness of the method, we examined the absorption spectra of the peak and compared then with the standard sample in Fig. 5. None of components in plasma showed potential interference with the method.

In conclusion, the monitoring of antiepileptic drugs in human plasma with MECC is demonstrated. Identification of solutes was performed by characterizing the sample peak in terms of the retention time and absorption spectra. Capillary electrophoresis with multiwavelength detection is a new and powerful technique for confirmation of the analysis. The detection methodology and the quantitative analysis data prove the feasibility of the method for the monitoring of the drugs in body fluids.

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