ELSEVIER

Journal of Chromatography B, 695 (1997) 417–425

JOURNAL OF CHROMATOGRAPHY B

Micellar electrokinetic capillary chromatography for therapeutic drug monitoring of zonisamide

Kazutaka Makino, Yoko Goto, Masanori Sueyasu, Koujiro Futagami, Yasufumi Kataoka, Ryozo Oishi*

Department of Hospital Pharmacy, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-82, Japan

Received 3 December 1996; revised 3 March 1997; accepted 17 March 1997

Abstract

The simultaneous determination of zonisamide, a new type of antiepileptic drug, and the typical antiepileptic drugs phenobarbital, phenytoin and carbamazepine in human serum was developed using micellar electrokinetic capillary chromatography (MECC) with a diode array detector. A high correlation was revealed between the zonisamide levels in human serum obtained by MECC and those obtained by high-performance liquid chromatography (r=0.981). The serum levels of phenobarbital, phenytoin and carbamazepine determined by MECC were almost equal to those obtained by fluorescence polarization immunoassay. The reproducibility of separation and quantification with MECC analysis was appropriate for the intra- and inter-day assay coefficients. Therefore, the MECC method established here could provide a simple and efficient therapeutic drug monitoring method for antiepileptic drugs in patients, especially those treated with a combination of zonisamide and other antiepileptic drugs. © 1997 Elsevier Science B.V.

Keywords: Zonisamide; Phenobarbital; Phenytoin; Carbamazepine

1. Introduction

Zonisamide, a 1,2-benzisoxazole derivative, was developed as a new type of antiepileptic drug in Japan, being effective in the treatment of partial and generalized seizures [1]. Measurements of antiepileptic drugs in serum, including zonisamide, are essential for therapeutic drug monitoring to decide the optimum doses and to prevent side effects or intoxication in antiepileptic pharmacotherapy [2,3].

Immunological analytical techniques have been applied in clinical laboratories, based on their simplicity of analysis, efficiency in time and performance, and sensitivity of detection. Immunoassays for some drugs, such as phenobarbital, carbamazepine and vancomycin, possess cross-reactivity to various analogs or metabolites [4] and are also incapable of analyzing several drugs at the same time. For monitoring zonisamide, an analytical kit is available only for an enzyme immunoassay, not for a fluorescence polarization immunoassay (FPIA), which is the most popular method among the various immunoassays [5]. High-performance liquid chromatography (HPLC) can separate several drugs, including zonisamide and its metabolites [6]. This method also has some disadvantages compared with immunoassays: It can be troublesome to maintain the system, the assay is time-consuming and the use of

*Corresponding author.

PII S0378-4347(97)00185-0

organic solvent as the mobile phase is expensive and causes environmental pollution.

Attention has recently been focused on micellar electrokinetic capillary chromatography (MECC) for the analysis of pharmaceuticals. The benefits of MECC, including the simultaneous measurement of several drugs, specificity of the separation, low running costs and the avoidance of the use of organic buffers, are favorable for the determination of serum drug concentrations [7–10].

Zonisamide is often used in combination with other antiepileptic drugs in the treatment of seizures. A number of interactions have been reported between zonisamide and other antiepileptic drugs. In addition to the non-linear pharmacokinetics of zonisamide, such interactions produce an increase or a decrease in the serum concentration of zonisamide, an event which may lead to toxicity or inadequate effect [1]. Clinical trials have raised the possibility that the application of zonisamide could expand into the prevention of post-operative and post-traumatic seizures and to the alleviation of trigeminal neuralgia. These pharmacokinetic properties and potential multiple uses of zonisamide in pharmacotherapy accentuate the importance of developing a convenient method for analysing zonisamide, for therapeutic drug monitoring in patients. This study was therefore aimed at developing the simultaneous measurement of zonisamide and other typical antiepileptic drugs in human serum with MECC.

2. Experimental

2.1. Reagents

The standard drugs used were as follows: Zonisamide was from Dainippon Pharmaceutical (Osaka, Japan). Primidone was from Wako Pure Chemical Industries (Osaka, Japan). Ethosuximide and carbamazepine-10,11-epoxide were from Sigma (St. Louis, MO, USA). The calibrators for the calibration of the phenobarbital, phenytoin and carbamazepine assay were from Abbott Laboratories (Abbott Park, IL, USA). Human serum was from Bio-Rad (Anaheim, CA, USA). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries.

2.2. MECC

MECC analysis was performed on an HP^{3D} capillary electrophoresis system (Hewlett-Packard, Wilmington, DE, USA) with a diode array UV detector. The monitoring wavelength was set at 210 nm and spectral data acquisition was performed at 190–400 nm. MECC data were collected and analyzed by the HP^{3D} CE ChemStation software. The samples were injected by the vacuum system at 50 mm Hg for 2.0 s. In all experiments, a constant voltage of 30 kV was applied and the temperature was set at 30°C.

A fused-silica extended light path capillary (Hewlett-Packard) with a 50 μm internal diameter (total length, 64.5 cm; effective length, 56 cm) was used for all MECC separations. The extended light path capillary had an internal diameter of 150 μm at the detection window [11]. The capillary was rinsed with running buffer at 630 mm Hg for 5 min before each analysis. The composition of the electrophoresis running buffer was 10 m*M* phosphate buffer (pH 8.0) containing 50 m*M* sodium dodecyl sulfate (SDS). The buffer was filtered through a 0.45-μm membrane and was ultrasonically degassed before use.

2.3. Preparation of standard solution and serum samples

Stock solutions of zonisamide, other antiepileptic drugs and *n*-propyl *p*-hydroxybenzoate (internal standard) were prepared in methanol and stored at −20°C. Various concentrations of standard solution were prepared by diluting the stock solution with human serum. Patients' samples were obtained for routine drug assays and were stored at −20°C until analysis.

2.4. Extraction

Liquid–liquid extraction was performed with ethyl acetate [8]. A 0.2-ml volume of serum and 1.0 ml of ethyl acetate, containing *n*-propyl-*p*-hydroxybenzoate solution (5 μg/ml), were placed in a plastic microtube. Vigorous vortex-mixing for 30 s was followed by centrifugation at 13 400 *g* for 2 min. A 500-μl volume of the organic layer was transferred to other plastic microtube and evaporated to dryness

under a gentle stream of nitrogen gas. The residue was reconstituted in 50 μl of 5% methanol in deionized water for capillary electrophoresis.

In the case of HPLC assay for zonisamide, the extraction procedure was the same as above, except that zonisamide was extracted from serum with acetonitrile and the organic layer obtained after centrifugation was injected into the HPLC system (20 μl).

2.5. FPIA and HPLC

The FPIA assay was performed using a TDx analyzer (Dainabot Laboratories, Tokyo, Japan). HPLC analysis was performed on a system consisting of a pump (LC10AD), a UV detector set at 210 nm (SPD-10A) and an integrator (C-R6A; Shimadzu, Kyoto, Japan). The analytical column was TSK gel ODS-80Tm (75×4.6 mm I.D.; Tosoh, Tokyo, Japan). The mobile phase was 5 m*M* KH_2PO_4 (pH 4.7)–acetonitrile (69:31, v/v) filtered through a 0.45-μm membrane and ultrasonically degassed. The flow-rate was 1.0 ml/min in all experiments.

3. Results and discussion

For solute identification, sample zones were characterized by their migration time behavior, and absorption spectra were obtained by the diode array detector. No interfering peak was observed in human serum samples extracted with ethyl acetate (Fig. 1).

Fig. 1A shows a typical electropherogram of the extraction of human serum spiked with standard drugs, including ethosuximide, primidone, zonisamide, phenobarbital, phenytoin, carbamazepine-10,11-epoxide, carbamazepine and internal standard. Ethosuximide and primidone are infrequently administered to epileptic patients; they were also well separated from the typical antiepileptic drugs. The peak of carbamazepine-10,11-epoxide, a main metabolite of carbamazepine, did not disturb any peak of zonisamide or of the other antiepileptic drugs. All drugs and internal standard in this study were separated within 10 min. However, the patients' samples did not always show the peak of this metabolite, probably due to its low concentration.

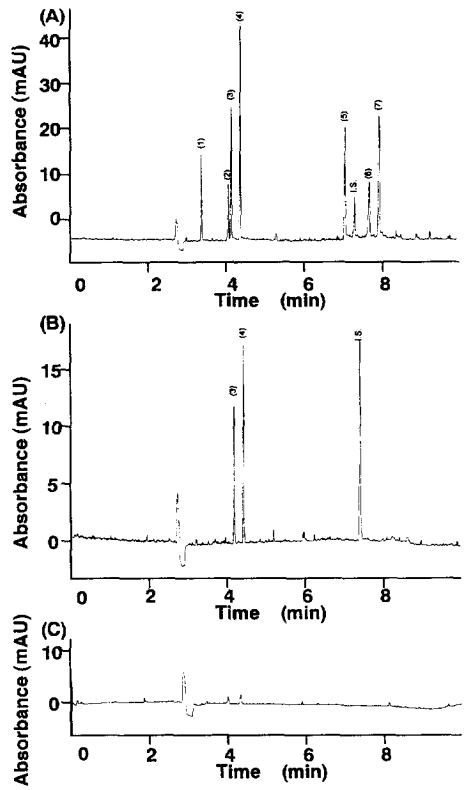

Fig. 1. Electropherograms obtained from (A) the extraction of serum spiked with standard mixtures of ethosuximide (128 μg/ml; 1), primidone (16 μg/ml; 2), zonisamide (32 μg/ml; 3), phenobarbital (48 μg/ml; 4), phenytoin (32 μg/ml; 5), *n*-propyl *p*-hydroxybenzoate (as the internal standard), carbamazepine-10,11-epoxide (10 μg/ml; 6) and carbamazepine (12.8 μg/ml; 7). (B) The extraction of serum from an epileptic patient taking zonisamide (3), phenobarbital (4) and sodium valproate. (C) The extraction of blank serum.

Careful determination of this metabolite in patients' serum with MECC is now under investigation.

Fig. 1B shows a representative current electropherogram of the extraction of serum from a patient who was taking zonisamide, phenobarbital and sodium valproate. The sodium valproate peak was not detected because it did not absorb in the UV region. There were no substrates interfering with the peak of any drug in the extraction of serum from any patient examined in this study.

The qualitative characterization of the peak of each drug was carried out using a diode array detector. Fig. 2 represents the UV spectra between

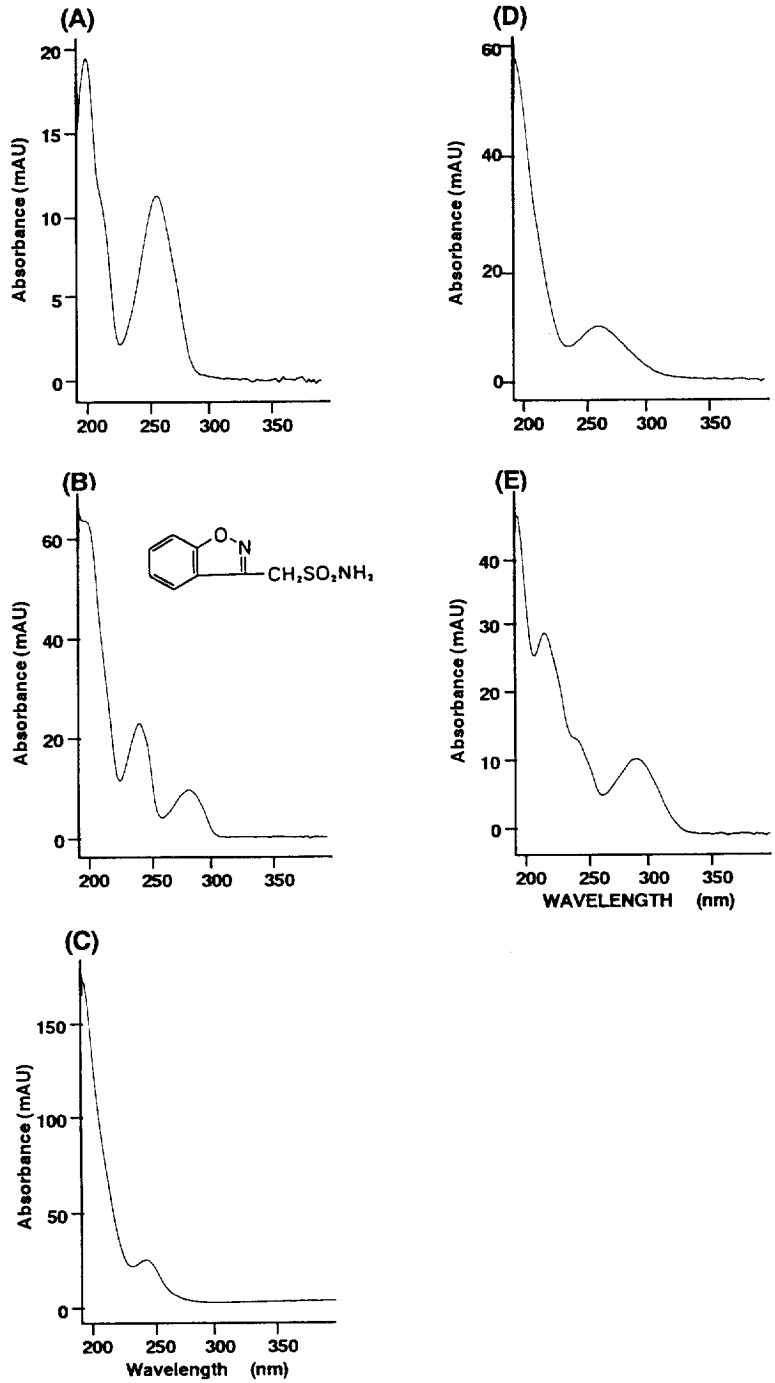

Fig. 2. Normalized spectra of (A) *n*-propyl *p*-hydroxybenzoate (as the internal standard), (B) zonisamide, (C) phenobarbital, (D) phenytoin and (E) carbamazepine, obtained from the data in Fig. 1A. Inset in (B) shows the chemical structure of zonisamide.

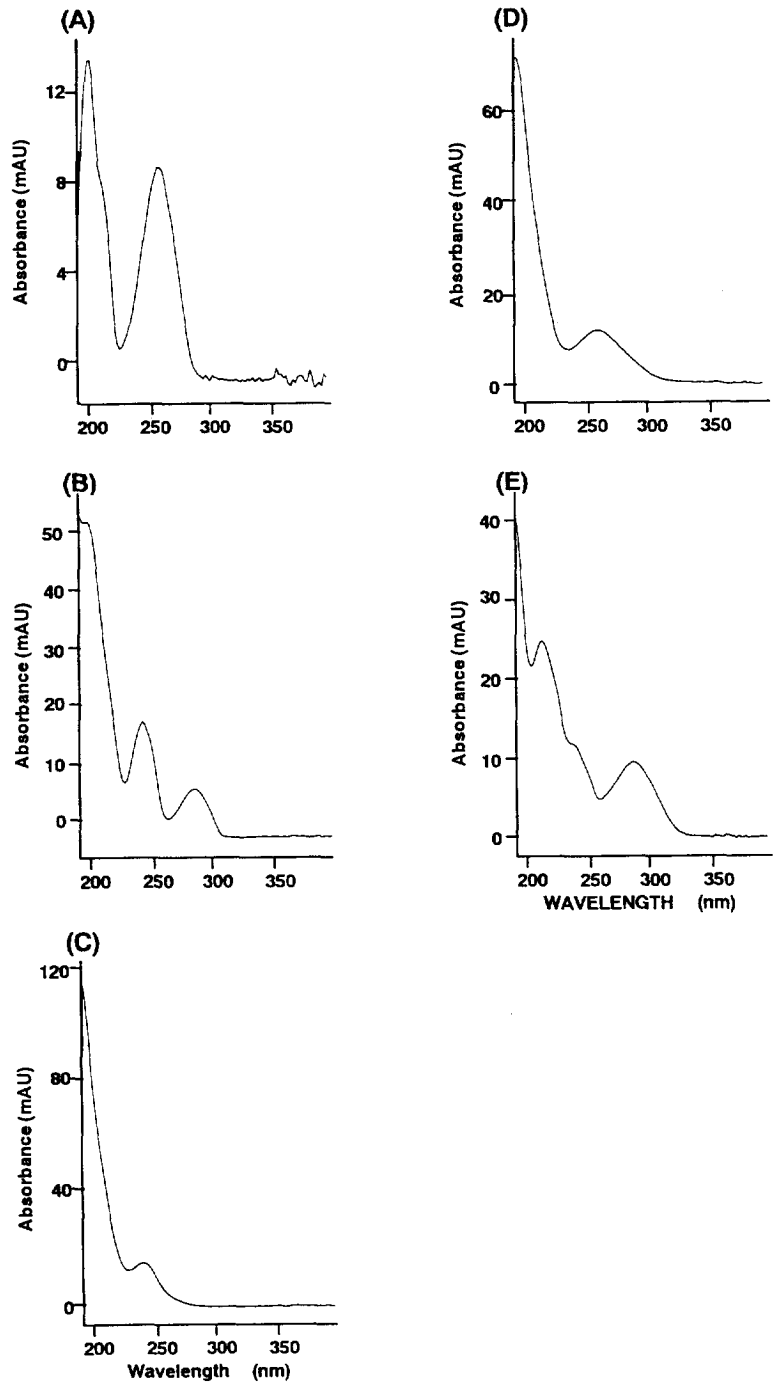

Fig. 3. Normalized spectra of (A) *n*-propyl *p*-hydroxybenzoate (as the internal standard), (B) zonisamide, (C) phenobarbital, (D) phenytoin and (E) carbamazepine, obtained from the extraction of patients' serum.

190 and 400 nm for the standard zonisamide, phenobarbital, phenytoin, carbamazepine and internal standard, corresponding to each peak of the electropherogram shown in Fig. 1A. The spectrum of each drug and internal standard extracted from the serum of patients was almost the same as that of each standard (Figs. 2 and 3). Therefore, the diode array detector facilitates the identification of each peak of four drugs, even at a concentration that is at the lower limit of detection.

The calibration curves for each drug, ranging from therapeutic to toxic levels, are demonstrated in Fig. 4. The coefficients of correlation (r) of zonisamide, phenobarbital, phenytoin and carbamazepine were 0.991, 1.000, 0.996 and 0.997 for UV detection at 210 nm, respectively, demonstrating excellent linear correlations between the concentration and the relative peak area-to-internal standard in the calibration range. When we calculated the detection limit of each drug by setting the signal-to-noise ratio (S/N) to 3, the values of zonisamide, phenobarbital, phenytoin and carbamazepine were 3.0, 2.5, 0.8 and 0.6 μg/ml, respectively.

The intra-day and inter-day precision data, obtained by repeated MECC analyses, are shown in Table 1. As for the intra-day reproducibility, the coefficients of variation of the migration time were less than 1.1% for all drugs. The peak-area values were less than 4.5% for all drugs, except for that of low concentrations of carbamazepine. These findings indicate that separation and quantification with the MECC system are reproducible for the measurement

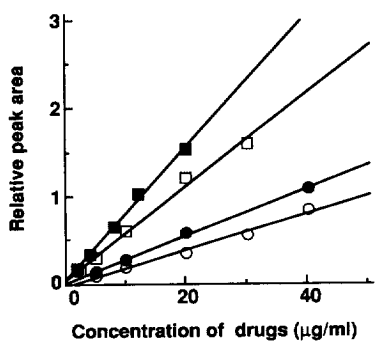

Fig. 4. Calibration curves for zonisamide (○, $y=2.36\cdot10^{-2}+2.07\cdot10^{-2}x$, $r=0.991$), phenobarbital (●, $y=1.27\cdot10^{-2}+2.71\cdot10^{-2}x$, $r=1.000$), phenytoin (□, $y=5.01\cdot10^{-2}+5.34\cdot10^{-2}x$, $r=0.996$) and carbamazepine (■, $y=4.32\cdot10^{-2}+7.61\cdot10^{-2}x$, $r=0.997$).

of zonisamide levels in human serum. In the inter-day assay, the coefficients of variation of the migration time were less than 7.3%, although carbamazepine, which was the last to be separated, had a larger variation than that of the other drugs. The coefficients of variation of the peak area were less than 10.0%, except for those of the lowest concentrations of zonisamide and carbamazepine (16.5 and 19.2%, respectively). A low concentration of carbamazepine examined to test the reproducibility of MECC analysis (Table 1) showed a broad peak. A low concentration of zonisamide was in the range near the detection limit (3.0 μg/ml). The absolute analytical recoveries of drugs from serum were not so high (about 70%). These events may contribute to the larger variations on the intra- and inter-day assays for low levels of carbamazepine and zonisamide. Consequently, the inter-day assay showed larger variations than those of the intra-day assay, concerning reproducibility of separation and peak area. Attention should be paid to this matter in monitoring the levels of zonisamide and other antiepileptic drugs in human serum with the MECC method.

The absolute analytical recoveries of zonisamide, phenobarbital, phenytoin and carbamazepine from serum were examined by adding the standards to serum or to the final solution (5% methanol). The recoveries of zonisamide, phenobarbital, phenytoin and carbamazepine were 73.3% ($n=9$, 20 μg/ml), 73.6% ($n=9$, 30 μg/ml), 63.7% ($n=9$, 20 μg/ml) and 70.1% ($n=7$, 8 μg/ml), respectively. The coefficients of variation were small in all recoveries of the four drugs (4.13–8.51%). These results show that liquid–liquid extraction with ethyl acetate is appropriate and stable, although it is not as efficient as desired.

We determined the concentrations of zonisamide in the serum from patients using both the MECC and HPLC methods (Fig. 5A, Table 2). The correlation of zonisamide levels between MECC and HPLC was excellent ($r=0.981$, $n=25$), although the levels obtained with MECC were slightly lower than those found with HPLC (slope=0.869). The agreement between the two sets of data was evaluated by plotting the difference versus the mean of each pair of data points (Fig. 5B). This approach provides the bias (mean of the difference) and identifies outliers

Table 1
Reproducibility of migration time and peak area of antiepileptic drugs

Compound	Concentration (μg/ml)	Migration time (min)	Relative peak area
A. *Intra-day reproducibility*			
Zonisamide	5	4.123±0.024 (0.58)	0.123±0.005 (4.43)
	20	4.141±0.019 (0.45)	0.397±0.005 (1.26)
	30	4.184±0.023 (0.55)	0.582±0.014 (2.45)
	40	4.210±0.012 (0.28)	0.960±0.038 (3.93)
Phenobarbital	7.5	4.366±0.027 (0.62)	0.188±0.005 (2.64)
	30	4.376±0.021 (0.47)	0.625±0.015 (2.39)
	45	4.431±0.026 (0.60)	0.923±0.034 (3.70)
	60	4.468±0.016 (0.35)	1.448±0.029 (1.97)
Phenytoin	5	7.011±0.048 (0.69)	0.285±0.007 (2.44)
	20	7.051±0.049 (0.70)	0.995±0.012 (1.21)
	30	7.109±0.066 (0.93)	1.422±0.027 (1.87)
	40	7.259±0.037 (0.51)	2.040±0.030 (1.50)
Carbamazepine	2	7.920±0.081 (1.03)	0.160±0.015 (9.22)
	8	7.953±0.053 (0.80)	0.566±0.019 (3.43)
	12	8.089±0.086 (1.06)	0.800±0.031 (3.82)
	16	8.154±0.050 (0.61)	1.227±0.017 (1.39)
B. *Inter-day reproducibility*			
Zonisamide	5	4.178±0.080 (1.91)	0.116±0.019 (16.45)
	20	4.157±0.077 (1.86)	0.386±0.032 (8.40)
	30	4.192±0.072 (1.72)	0.584±0.054 (9.29)
	40	4.237±0.155 (3.67)	0.881±0.082 (9.28)
Phenobarbital	7.5	4.417±0.089 (2.00)	0.175±0.018 (10.26)
	30	4.397±0.085 (1.93)	0.597±0.060 (10.04)
	45	4.437±0.077 (1.74)	0.913±0.102 (11.14)
	60	4.487±0.171 (3.81)	1.359±0.134 (9.86)
Phenytoin	5	7.079±0.210 (2.96)	0.266±0.014 (5.26)
	20	7.055±0.186 (2.64)	0.933±0.043 (4.56)
	30	7.143±0.196 (2.74)	1.435±0.046 (3.23)
	40	7.301±0.488 (6.68)	2.050±0.149 (7.28)
Carbamazepine	2	8.070±0.253 (3.13)	0.149±0.029 (19.23)
	8	8.002±0.239 (2.99)	0.518±0.030 (5.75)
	12	8.131±0.252 (3.10)	0.816±0.052 (6.32)
	16	8.320±0.608 (7.31)	1.178±0.112 (9.54)

Value are mean±S.D. ($n=5$): Values in parentheses are the coefficients of variation (%).

Table 2
Linear regression analysis data of comparative drug concentrations in patients' sera

Compound	*x*-axis	*y*-axis	*n*	Slope	*y*-intercept (μg/ml)	*r*
Zonisamide	HPLC	MECC	25	0.869	1.521	0.9810
Phenobarbital	FPIA	MECC	18	1.059	−1.339	0.9916
Phenytoin	FPIA	MECC	14	1.038	−0.738	0.9983
Carbamazepine	FPIA	MECC	14	1.083	−0.356	0.9863

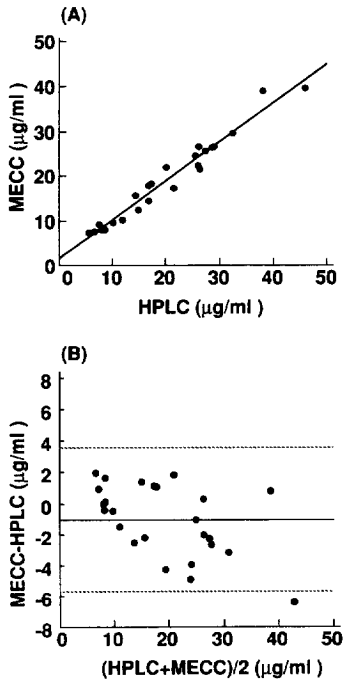

Fig. 5. Comparative zonisamide serum levels in the sera from 25 patients, determined by MECC and HPLC. (A) MECC versus HPLC by linear regression analysis. (B) Bias analysis defined by the difference versus the mean of comparative drug levels. The solid line in panel A is a regression line (data as in Table 2). In panel B, the solid line represents the mean of the differences (−1.07 mg/ml) and the broken lines represent this mean±2 S.D.(+3.54/−5.68 mg/ml).

[12]. Of the 25 samples, 24 were found to be within the range defined by the mean of the differences±2 S.D. Zonisamide was extracted from serum with ethyl acetate and acetonitrile for MECC and HPLC analysis, respectively. This difference in sample pretreatment may be interpreted as being responsible for the bias between MECC and HPLC results of up to more than 10%.

When the concentrations of phenobarbital, phenytoin and carbamazepine, determined by MECC, were compared with the levels obtained by FPIA in our routine assay, the correlation coefficients were 0.992, 0.998 and 0.986, respectively (Table 2). An analysis using the bias method for serum levels of other drugs revealed that the differences between FPIA data and MECC data were almost within the mean of the differences±2 S.D. (phenobarbital, 16 of 18; phenytoin, 14 of 14; carbamazepine, 13 of 14 samples). The drug levels determined by the MECC method were shown to be in good accord with those obtained with the HPLC or FPIA methods, thereby suggesting that the MECC method is useful for the therapeutic drug monitoring of zonisamide and other antiepileptic drugs in patients.

Based on these findings, the MECC assay appears to be complementary to the widely employed immunoassays in clinical laboratories. MECC may become a more attractive method for therapeutic drug monitoring, because of its specificity of separation, automation of procedure, ease of method development, the low cost of capillaries, the small amounts of aqueous buffer needed, the speed of analysis and the small injection volumes (1–50 nl) required.

4. Conclusions

The MECC method established here could provide a simple and efficient therapeutic drug monitoring method for antiepileptic drugs in patients, especially those treated with a combination of zonisamide and other antiepileptic drugs.

Acknowledgments

We thank H. Yokoo for technical assistance.

References

[1] D.H. Peters, E.M. Sorkin, Drugs 45 (1993) 760–787.
[2] J.K. Penry, M.E. Newmark, Ann. Intern. Med. 90 (1979) 207–218.
[3] A.J. Wilensky, P.N. Friel, L.M. Ojemann, C.B. Dodrill, K.B. McCormick, R.H. Levy, Epilepsia 26 (1985) 212–220.
[4] H. Morishige, H. Shuto, I. Ieiri, K. Otsubo, R. Oishi, Ther. Drug Monit. 18 (1996) 80–85.
[5] K. Kaibe, S. Nishimura, H. Ishii, N. Sunahara, S. Naruto, S. Kurooka, Clin. Chem. 36 (1990) 24–27.
[6] K. Furuno, R. Oishi, Y. Gomita, K. Eto, J. Chromatogr. B 656 (1994) 456–459.
[7] W. Thormann, P. Meier, C. Marcolli, F. Binder, J. Chromatogr. 545 (1991) 445–460.

[8] K.-J. Lee, G.S. Heo, N.J. Kim, D.C. Moon, J. Chromatogr. 608 (1992) 243–250.
[9] A. Schmutz, W. Thormann, Ther. Drug Monit. 15 (1993) 310–316.
[10] K.E. Ferslew, A.N. Hagardorn, W.F. McCormick, J. Forensic Sci. 40 (1995) 245–249.
[11] D.N. Heiger, P. Kaltenbach, H.-J.P. Sievert, Electrophoresis 15 (1994) 1234–1247.
[12] J.M. Bland, D.G. Altman, Lancet 1 (1986) 307–310.