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Indirect laser-induced fluorescence detection of valproic acid in human serum by capillary electrophoresis

A capillary electrophoresis (CE) method with indirect laser-induced fluorescence detection for the analysis of valproic acid in human serum has been explored. The buffer system was optimized with 2.5 mM borate-phosphate at pH 8.4; fluorescein sodium was used to generate background signal at a concentration of 6 μM. Hexanoic acid was selected as internal standard. Serum sample was deproteinized by acetonitrile. Analysis was performed by direct injection of the supernatant. CE separation was carried out at 30 kV and the total analysis time was less than 15 min, including sample treatment and electrophoresis time. No interference from other common anticonvulsant drugs occurred under the experimental conditions used. The interference of human serum matrix was reduced by using a high ratio of acetonitrile to serum (minimum 5:1) for deproteinization. Interference of ionic components in serum could occur, depending on the sample source. The linear range of concentrations for standard drug was between 4.5–144.0 μg/mL ($r = 0.9947$). The limit of detection was 0.9 μg/mL at S/N ≥ 3; the limit of quantitation at S/N ≥ 20 was 3 μg/mL. The recoveries of valproic acid spiked into serum were 69.2% and 60.2% for concentration levels of 90 and 54 μg/mL, respectively. This CE method was shown to be successful in the analysis of valproic acid in standard solutions. However, interference from the matrix was observed in the analysis of this compound in serum samples. Additional work should be done to develop a highly selective sample preparation technique.

Keywords: Valproic acid / Anticonvulsant / Fluorescein / Laser-induced fluorescence / Capillary electrophoresis
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1 Introduction

Anti-epileptic drugs (AEDs) are applied in the treatment of patients with seizure disorders. Commonly prescribed AEDs include valproic acid (VPA), ethosuccimide (ESM), primidone (PRM), carbamazepine (CBZ), phenytoin (PHT) and phenobarbital (PB); see Fig. 1. VPA (2-propylpentanoic acid) is prescribed alone or in combination with other AEDs to treat epilepsy because of its wider spectrum of activity and less apparent central nervous system toxicity than other AEDs [1–4]. Chromatographic determination of VPA and other AEDs in blood, plasma or serum has been reported by high performance liquid chromatography (HPLC) [5–14], gas chromatography (GC) [15–19], and micellar electrokinetic capillary chromatography (MEKC) [20]. However, due to the poor ultraviolet absorption of VPA, an introduction of chromophore or fluorophore was usually adopted in most of the investigations to increase the detection sensitivity. It is generally known that derivatization often requires time-consuming preparation procedures, introduces sample contamination agents, produces side products, and causes changes in solute properties. Therefore, an alternative analytical technique capable of direct analysis of VPA is expected in order to carry out rapid analysis of VPA for emergency and routine use.

Charged substances not possessing a suitable detection property such as a chromophore or fluorophore are normally analyzed by indirect detection mode [21]. To date, indirect ultraviolet and fluorescence detection is the most commonly used indirect detection scheme, which shares the same displacement mechanism. Derivatization is obviated by this mode since the analyte does not have to respond to the detector. Recently, indirect fluorescence detection of octanoic acid (C8) [22] by capillary electrophoresis has been reported. VPA is also a C8 carboxylic acid with a branch in its molecular structure. In that report, fluorescein sodium was used to generate background sig-

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Abbreviations: AED, anti-epileptic drugs; **CBZ**, carbamazepine; **ESM**, ethosuccimide; **HA**, hexanoic acid; **IS**, internal standard; **PB**, phenobarbital; **PHT**, phenytoin; **PRM**, primidone; **VPA**, valproic acid

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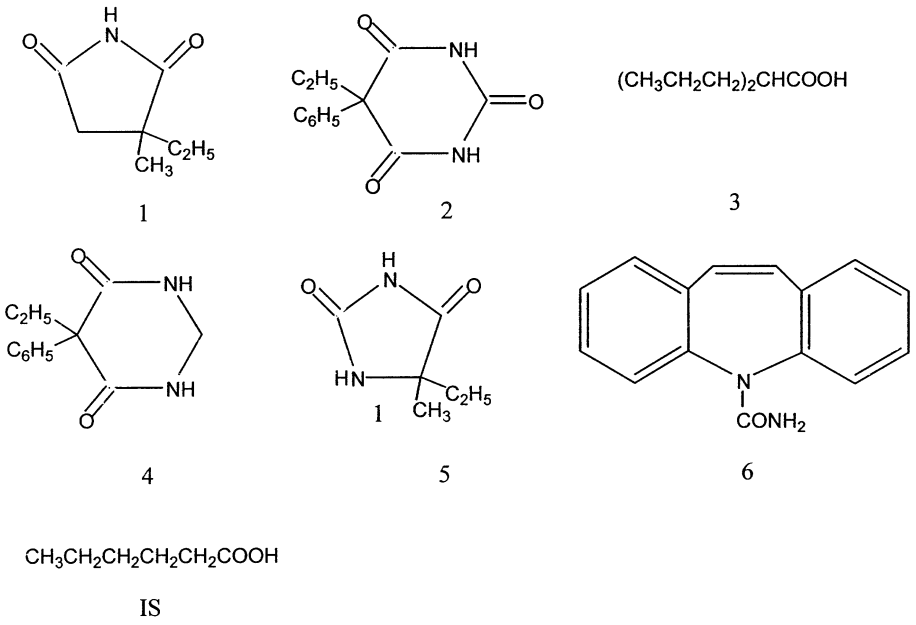

Figure 1. Structures of anticonvulsant drugs. (1) Ethosuccimide; (2) phenobarbital; (3) valproic acid; (4) primidone; (5) phenytoin; (6) carbamazepine; (IS) hexanoic acid.

nal in 5 mM sodium tetraborate buffer at pH 9.2 with a fluorescein concentration of 10^{-5} M. Serum sample treatment procedure is important for the determination of VPA as it may evaporate during sample treatment due to its relatively high volatility. Recently, deproteinization of serum with acetonitrile has been reported in some CE studies [23–26]. The success of the method depends on removing serum proteins with acetonitrile followed by direct injection of a larger volume of the sample with an increase in sensitivity which is produced by the special stacking effect of acetonitrile.

The purpose of this study was to investigate the feasibility of indirect laser-induced fluorescence detection of VPA in human serum by capillary electrophoresis. Another aim was to develop a rapid alternative technique for the analysis of this type of compound. Fluorescein sodium was used to produce background signal. Hexanoic acid was selected as internal standard (IS). Buffer electrolyte concentration, fluorescein concentration and buffer pH were optimized. Linearity and reproducibility were examined. Interference from other AEDs was removed by optimizing the buffer pH. Serum deproteinization with acetonitrile was investigated in a bid to minimize the matrix interference. The method developed in this study could be applicable for the determination of VPA in serum in therapeutic drug monitoring.

2 Materials and methods

2.1 Chemicals

Six AEDs, *i.e.*, valproic acid, phenobarbital, ethosuccimide, phenytoin, carbamazapine, and primidone, were purchased from Sigma (St. Louis, MO, USA). Hexanoic acid and nonanoic acid were obtained from Aldrich (Milwaukee, WI, USA). Fluorescein sodium was bought from the British Drug Houses (Poole, England). Sodium tetraborate anhydrous and sodium dihydrogen phosphate dihydrate were the products of Fluka (Buchs, Switzerland). Methanol was supplied by Fisher Scientific (Fairlawn, NJ, USA). Acetonitrile was from J. T. Baker (Phillipsburg, NJ, USA). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2 Apparatus

The CE-LIF was performed with a Prince CE system (Prince Technologies B. V., Emmen, The Netherlands), a laser-induced fluorescence detector (ZETA Technology, Ramonville, France) and an argon ion laser system (Omnichrome, Chino, CA, USA). The indirect fluorescence detection was performed with excitation at 488 nm and emission at 520 nm. The excitation power at 488 nm from an argon ion laser was set at 3 mW. A capillary of 50 μm ID with an effective/total length of 56.2/74.7 cm was used as separation column. A new capillary was conditioned by 0.1 M NaOH for 30 min. Capillary conditioning before each analysis was carried out by flushing successively with 0.1 M NaOH, H_2O and buffer for 2 min each at 1000 mbar. Sample was injected at 75 mbar for 0.05 min.

2.3 Buffer and sample preparation

2.5 mM borate-phosphate buffer was prepared by mixing equimolar sodium tetraborate and phosphate to the desired pH. AEDs were weighed and dissolved in meth-

anol as stock solutions; the stock solution was further diluted with buffer or acetonitrile to obtain desired sample concentrations. Serum was deproteinized by the following procedure: 50 μL serum or buffer was supplemented with 50 μL VPA in acetonitrile (54 or 90 μg/mL), 50 μL hexanoic acid in acetonitrile (51.5 μg/mL) and 150 μL acetonitrile. After vortexing for 15 s, the deproteinized serum was centrifuged for 3 min at 5000 rpm. The supernatant was injected for electrophoresis.

3 Results and discussion

3.1 Optimization of separation conditions

Separation conditions for C8 [22] were modified and applied to VPA analysis. As the minimum buffer concentration with buffer capacity for borate was 2.5 mM [22], this concentration was chosen to obtain optimum sensitivity since sensitivity of the detector response is generally greater at smaller ionic strength values [22]. Further, VPA signals generated in 2.5 and 5 mM buffers were compared; 2.5 mM borate buffer proved to produce more sensitive yet reproducible detection of VPA. Because the fluorescent intensity of fluorescein generally increased with buffer pH and reached a plateau at pH ≥ 9 [28], pH 9.2 was selected initially as buffer pH. Although pH 9.2 was appropriate for standard VPA, PB (pK_a 7.2) comigrated with VPA and interfered with detection. However, an adjustment to pH 8.4 made VPA and PB separable. PHT (pK_a 8.3 or 8.6) migrated away from VPA and PB and did not show interference. ESM (pK_a 9.3) and two neutral CBZ and PRM also did not interfere with detection of VPA because of less ionization at pH 8.4 or neutrality. Fluorescein concentrations ranging from 6–15 μM in 2.5 mM borate-phosphate buffer at pH 8.4 were compared and 6 μM were found to be appropriate to generate sufficient background fluorescence without overloading problems. It is known that the concentration limit of detection (C_{LOD}) of a given indirect detection scheme can be represented by Eq. (1):

$$C_{LOD} = C_M/(TR \times DR) \quad (1)$$

where C_M is the concentration of the electrolyte generating the detector background signal; and *TR* and *DR* are the transfer ratio and the dynamic reserve, respectively. According to the equation, the lower the concentration of the electrolyte (C_M) generating the detector background signal, the lower the C_{LOD}. This is based on the assumption that *TR* and *DR* remain constant and are independent of C_M. The IS was selected between hexanoic acid (C6) and nonanoic acid (C9). Experiments showed that C6 was a suitable internal standard which migrated next to VPA. Experimental conditions, therefore, were optimized as 2.5 mM borate-phosphate buffer at pH 8.4 with 6 μM fluorescein. Hexanoic acid (HA) was selected as the IS. Electrophoresis was carried out at 30 kV and ambient temperature.

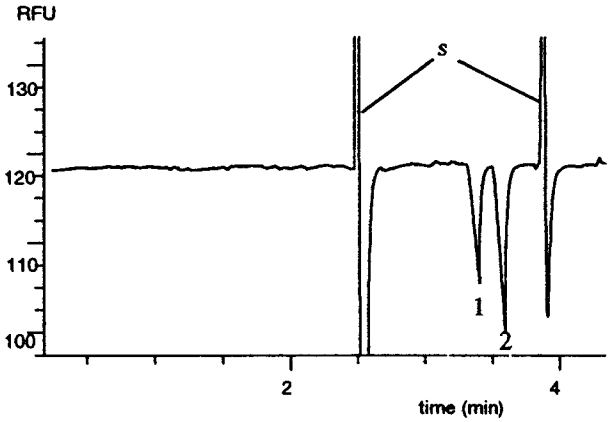

Figure 2. Electropherogram of VPA with HA (IS). Electrophoretic conditions: 2.5 mM borate-phosphate buffer, pH 8.4, 6 μM fluorescein; 30 kV. Peak 1, VPA (63.0 μg/mL); 2, HA (IS, 51.5 μg/mL), s, system peak.

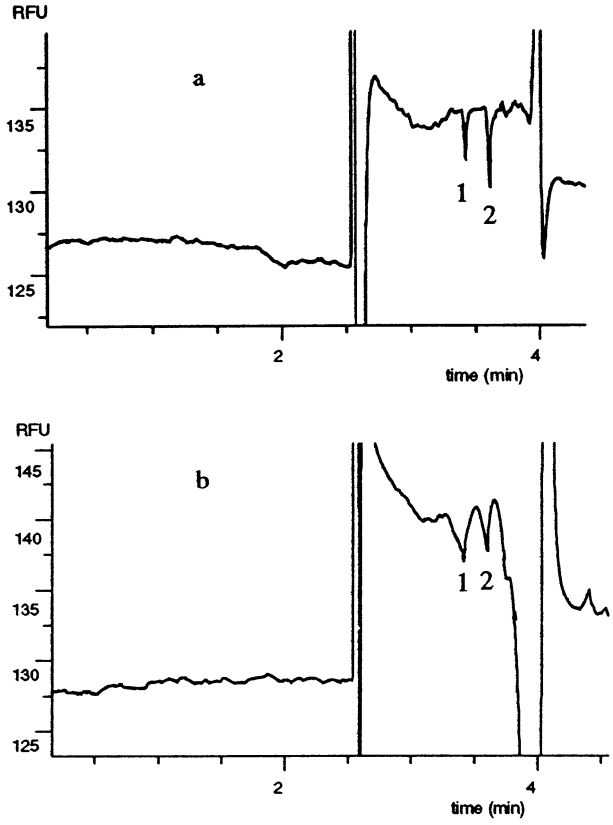

Figure 3. Comparison of electropherograms of VPA and HA (IS) spiked into (a) buffer and (b) serum. Serum to acetonitrile ratio, 1:5. Electrophoretic conditions as given in Fig. 2. Peak 1, VPA (54.0 μg/mL); 2, HA (IS, 51,5 μg/mL) – because of dilution actual concentrations determined for VPA and HA were 9 and 12.8 μg/mL, respectively.

3.2 Serum deproteinization

The ratio of serum to acetonitrile for deproteinization was reported to be appropriate at 2:3 for removing serum proteins, especially albumin [26], in capillary electrophoresis. Our previous work [27] proved that a 1:2 ratio was sufficient for removing interferences in a direct UV detection mode. In this study, a 1:2 serum to acetonitrile ratio was initially tried and found to be insufficient for removing a broad negative peak that interfered with VPA and IS detection. However, further increasing the serum to an acetonitrile ratio of 1:5 or above suppressed the interfering peak and alleviated the interference. It was noted that other ionic components interfered with the analysis of VPA. These interfering ionic components only occurred in certain serum samples but not in all. The drawback of this sample treatment procedure was that the sample was diluted six times. Increasing the injection amount did not improve the peak shape in this case.

3.3 Electropherograms for optimum conditions

Figure 2 shows a typical electropherogram of standard VPA with HA (IS) carried out at the optimized conditions. A comparison of electropherograms of VPA and HA (IS) spiked into serum and in standard sample (Fig. 3) shows that the peak shapes of VPA and HA (IS) in the standard sample were sharp and symmetrical while the peak shapes of VPA and HA (IS) spiked into serum were not as sharp as those in standard sample. It appears that quantitative analysis of VPA in serum can be problematic depending on the nature of interfering compounds.

3.4 Analytical variables

3.4.1 Precision

The reproducibility of migration time and relative peak area for standard VPA was evaluated by five successive experiments at different concentrations ranging from 4.5 to 144 μg/mL. The coefficients of variation for migration time and relative peak area were less than 1% and 8%, respectively, at different concentrations (Table 1).

3.4.2 Recovery

Acetonitrile containing standard VPA and HA (IS) was added to equal volumes of serum and buffer. After vortexing and centrifugation, the supernatants were injected for electrophoresis. Absolute analytical recovery was calculated by comparing the relative peak area of VPA in serum with that of standard VPA in buffer prepared and measured by the same procedure. Absolute recoveries for VPA at concentration levels of 90.0 and 54.0 μg/mL were 69.2% and 60.2%, respectively. The low recovery values of VPA could be attributed to the unsymmetrical peak shapes obtained, which may mean that the measurement of peak area is not a true reflection of the actual values of VPA.

3.4.3 Linearity and sensitivity

The concentration and relative peak area for standard VPA correlated linearly in the concentration range from 4.5 to 144.0 μg/mL with a correlation coefficient r = 0.9947 (Fig. 4). The limit of detection was 0.9 μg/mL at S/N ≥ 3; the limit of quantitation at S/N ≥ 20 was 3 μg/mL.

Table 1. Reproducibility of migration time and relative peak area for standard VPA

VPA concentration (μg/mL)	Migration time (min)	Relative peak area
4.5	3.38 ± 0.01 (0.30)	0.0283 ± 0.00 (1.08)
13.5	3.40 ± 0.01 (0.29)	0.14 ± 0.01 (7.70)
22.5	3.41 ± 0.02 (0.59)	0.26 ± 0.02 (7.69)
31.5	3.42 ± 0.03 (0.88)	0.33 ± 0.01 (3.13)
40.5	3.41 ± 0.02 (0.58)	0.43 ± 0.03 (5.94)
54.0	3.43 ± 0.02 (0.58)	0.55 ± 0.02 (3.64)
63.0	3.43 ± 0.03 (0.87)	0.66 ± 0.02 (3.43)
72.0	3.45 ± 0.02 (0.58)	0.78 ± 0.03 (3.51)
85.5	3.45 ± 0.03 (0.87)	0.99 ± 0.03 (3.03)
94.5	3.48 ± 0.03 (0.86)	1.15 ± 0.05 (4.22)
103.5	3.46 ± 0.01 (0.29)	1.28 ± 0.04 (3.12)
117.0	3.52 ± 0.03 (0.85)	1.40 ± 0.04 (2.80)
126.0	3.51 ± 0.03 (0.85)	1.51 ± 0.02 (1.30)
135.0	3.50 ± 0.02 (0.57)	1.59 ± 0.04 (2.51)
144.0	3.53 ± 0.03 (0.85)	1.65 ± 0.04 (2.24)

Values are mean ± SD (CV), n = 5

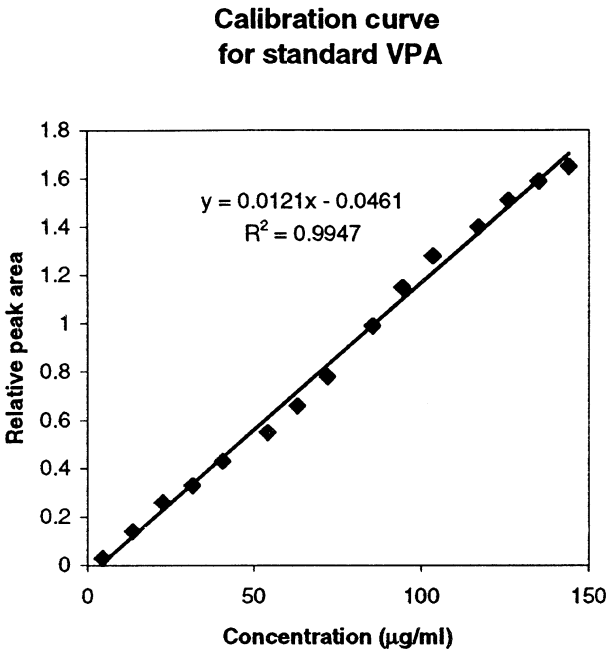

Figure 4. Calibration curve for standard VPA.

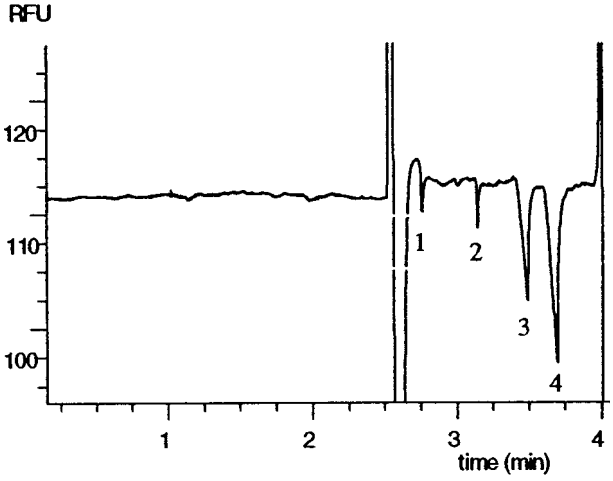

Figure 5. Electropherogram of PHT, VPA, PB and HA (IS) in one run. Electrophoretic conditions as given in Fig. 2. Peak 1, PHT (32 μg/mL); 2, PB (22 μg/mL); 3, VPA (45 μg/mL); 4, HA (IS, 51.5 μg/mL).

3.4.4 Interference

Five of the AEDs listed in Fig. 1, which may be administered concurrently with VPA, did not interfere with detection of VPA under the conditions developed in this study. The electropherogram of PHT, PB and VPA with HA (IS) in one run is shown in Fig. 5. ESM did not even interfere at a concentration of 500 μg/mL because it has a pK_a of 9.3 and the ionization fraction at pH 8.4 is small. Since PRM and CBZ are neutral compounds they migrated with the EOF. The interference of the human serum matrix was reduced by deproteinization at low serum to the acetonitrile ratio. Figure 6 shows the effect of serum deproteinization at serum to acetonitrile ratios of 1:3 and 1:5. Interference of ionic components in serum is difficult to predict, as these may exist in one matrix but not in the others.

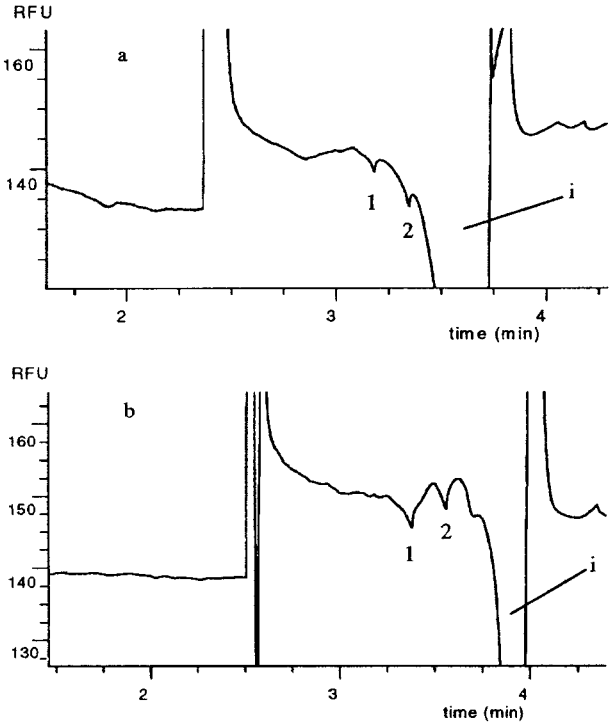

Figure 6. Serum deproteinization with acetonitrile at different ratios of serum to acetonitrile (a) 1:3, broad interference (i) peak; (b) 1:5 suppressed interference (i) peak.

4 Concluding remarks

CE with indirect laser-induced fluroescence detection of VPA in standard solutions carried out without derivatization was feasible. Serum sample deproteinization with acetonitrile was simple and rapid. The indirect detection scheme of VPA developed in this study is rapid because derivatization commonly adopted in GC or HPLC was omitted. In addition, an electrophoretic run was finished within 5 min, faster or comparable to GC or HPLC run times. Furthermore, direct injection of deproteinized serum sample by acetonitrile was accomplished to minimize sample loss due to volatility. The total analysis time was less than 15 min, including sample treatment and electrophoresis time. However, due to the use of a low ratio of serum to acetonitrile (*i.e.*, high ratio of acetonitrile to serum) to minimize the interference from serum matrix, sample dilution was unavoidable. Moreover, the interfering ionic components that may exist in certain serum could not be removed by single deproteinization. The method developed in this study has been demonstrated

to be successful in the analysis of VPA in standard solutions. However, interference from the matrix was observed in the analysis of this compound in serum samples. Additional work should be done to develop a sufficiently selective sample preparation step.

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5 References

[1] Bruni, J., Wilder, B. J., *Arch. Neurol.* 1979, *36*, 393–398.

[2] Delgado-Escueta, A. V., Treiman, D. M., Walsh, G. O., *N. Engl. J. Med.* 1983, *308*, 1576–1578.

[3] Coulter, D. L., Wu, H., Allen, R. J., *J. Amer. Med. Ass.* 1980, *244*, 785–789.

[4] Kupferberg, H. J., in Levy, R., Mattson, R., Meldrum, B., Penry, J. K., Dreifuss, F. E., *Anti-Epileptic Drugs*, Third Edition, Raven Press, New York 1989, pp. 577–582.

[5] Kushida, K., Ishizaki, T., *J. Chromatogr.* 1985, *338*, 131–139.

[6] Farinotti, R., Pfaff, M. C., Mahuzier, G., *Ann. Biol. Clin.* 1978, *36*, 347–353.

[7] Van Der Wal, S. J., Bannister, S. J., Dolan, J. W., Snyder, L. R., *Clin. Chem.* 1980, *26*, 1006.

[8] Lucarelli, C., Villa, P., Lombaradi, E., Prandini, P., Brega, A., *Chromatographia* 1992, *33*, 37–40.

[9] Gupta, R. N., Keane, P. M., Gupta, M. L., *Clin. Chem.* 1979, *25*, 1984–1985.

[10] Sutheimer, C., Fretthold, D., Sunshine, I., *Chromatogr. Newslett.* 1979, *7*, 1–4.

[11] Alric, R., Cociglio, M., Blayac, J. P., Puech, R., *J. Chromatogr.* 1981, *224*, 289–299.

[12] Kline, W. F., Enagonio, D. P., Reeder, D. J., May, W. E., *J. Liq. Chromatogr.* 1982, *5*, 1697–1709.

[13] Moody, J. P., Allan, S. M., *Clin. Chim. Acta* 1983, *127*, 263–269.

[14] Nakamura, M., Kondo, K., Nishioka, R., Kawai, S., *J. Chromatogr.* 1984, *310*, 450–454.

[15] Kumps, A., Mardens, Y., *Clin. Chem.* 1980, *26*, 1759.

[16] Nishioka, R., Kawai, S., *J. Chromatogr.* 1983, *277*, 356–360.

[17] Andrealini, F., Borra, C., Corcia, A. D., Camperi, R., *J. Chromatogr.* 1984, *310*, 208–212.

[18] Nakamura, M., *J. Chromatogr.* 1985, *342*, 89–96.

[19] Yu, D., Gordon, J. D., Zheng, J., Panesar, S. K., Riggs, K. W., Rurak, D. W., Abbott, F. S., *J. Chromatogr. B* 1995, *666*, 269–281.

[20] Lee, K. J., Heo, G. S., Kim, N. J., Moon, D. C., *J. Chromatogr.* 1992, *608*, 243–250.

[21] Yeung, E. S., *Acc. Chem. Res.* 1989, *22*, 125–130.

[22] Desbene, A. M., Morin, C. J., Mofaddel, N. L., Groult, R. S., *J. Chromatogr. A* 1995, *716*, 279–290.

[23] Shihabi, Z. K., Oles, K. S., *Clin. Chem.* 1994, *40*, 1904–1908.

[24] Garcia, L. L., Shihabi, Z. K., Oles, K., *J. Chromatogr.* 1995, *669*, 157–162.

[25] Shihabi, Z. K., Hinsdale, M. E., Bleyer, A. J., *J. Chromatogr. B* 1995, *669*, 163–169.

[26] Shihabi, Z. K., *J. Chromatogr.* 1993, *652*, 471–475.

[27] Jin, L. J., Wang, Y., Xu, R., Go, M. L., Lee, H. K., Li, S. F. Y., *Electrophoresis* 1999, *20*, 198–203.

[28] Desbene, P. L., Morin, C. J., Desbene Monvernay, A. M., Groult, R. S., *J. Chromatogr. A* 1995, *689*, 135–148.