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Microemulsion electrokinetic chromatography applied for separation of levetiracetam from other antiepileptic drugs in polypharmacy

Microemulsion electrokinetic chromatography was applied for the separation of levetiracetam from other antiepileptic drugs (primidone, phenobarbital, phenytoin, lamotrigine and carbamazepine) that are potentially coadministered in therapy of patients. The influence of the composition of the microemulsion system (with sodium dodecyl sulfate as charged surfactant) was investigated, modifying the kind of cosurfactant (lower alcohols from C3 to C5), the pH (and salinity) of the aqueous background electrolyte, and the ratio of aqueous phase to organic constituents forming the microdroplets of the oil-in-water emulsion. Separation selectivity was depending on all these parameters, resulting even in changes of the migration sequence of the analytes. Only moderate correlation was observed for the microemulsion system compared with a micellar system, both consisting of the aqueous borate buffer (pH 9.2) and SDS as micelle former (linear correlation coefficient for analyte mobilities is 0.974). The sample solvent plays an important role on the shape of the resulting chromatograms: methanol at concentrations higher than 35% impairs peak shape and separation efficiency. The microemulsion method (with 93.76% aqueous borate buffer (pH 9.2, 10 mM), 0.48% *n*-octane, 1.80% SDS, 3.96% 1-butanol, all w/w) is suitable for the determination of levetiracetam in human plasma (combined with a sample pretreatment based on solid-phase extraction).

Keywords: Antiepileptic drugs / Microemulsion electrokinetic chromatography / Polypharmacy
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1 Introduction

Levetiracetam (Fig. 1), is an oral anticonvulsant introduced recently onto the drug market (it has been approved in December, 1999). It seems to be effective in patients with complex partial seizures and generalized seizures [1, 2]. Until now it has been approved by FDA [3] only as adjunctive therapy in the treatment of partial onset seizures in adults. Recently, one clinical trial hypothesized that levetiracetam may be effective as monotherapy as well [4]. Glauser *et al.* [5], observed good results for the pediatric therapy of epilepsy.

Epileptic patients generally require chronic therapy, often with two or more antiepileptic drugs at high doses. Levetiracetam has the advantage to give no interaction with concomitantly administered antiepileptic drugs also at very high dosage. Levetiracetam has minimal adverse effects: the most frequent side effects are somnolence, vertigo, headache and asthenia. Daily dosage of levetiracetam is in the 250–5000 mg range. The drug has a wide therapeutic index, with good separation between therapeutic and toxic doses [6]. Levetiracetam has a very favorable pharmacokinetic profile. It is rapidly and almost completely absorbed (absolute oral bioavailability is about 100%); the plasma peak concentration appears in about 60–90 min. Steady state is reached after 48 h of twice-daily repeated dosing. Levetiracetam exhibits linear and dose-proportional steady-state pharmacokinetics [7] and is not extensively metabolized. The unchanged drug excreted in the urine is about 66% of the administered dose, while 27% is excreted as inactive metabolites [8]. Mean elimination half-life is about 7 h. Levetiracetam has minimal plasma protein binding, nor does it affect the protein binding of other drugs.

To our knowledge, only three papers dealing with the analysis of levetiracetam in biological fluids have been published in the literature. Vertmeij *et al.* [9] reported two

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Abbreviations: CBZ, carbamazepine; **MEEKC**, microemulsion electrokinetic chromatography

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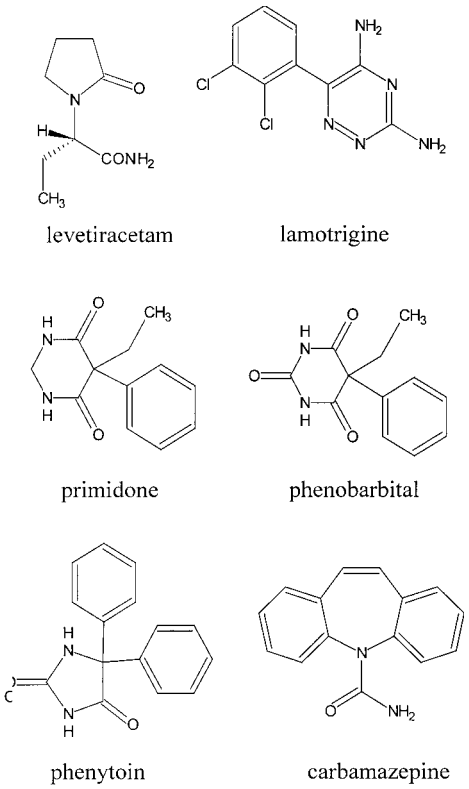

Figure 1. Structures of levetiracetam and coadministered drugs.

analytical methods for the drug determination in serum, based on liquid chromatography with spectrophotometric detection (HPLC-UV), and gas chromatography (GC) with flame ionization detection. Sample pretreatment is carried out by means of solid-phase extraction (SPE). Ratnaraij *et al.* [10] published another HPLC-UV method, with liquid-liquid extraction as sample pretreatment. More recently, Isoherranen *et al.* [11] implemented an enantioselective GC method with mass spectrometric detection for the determination of levetiracetam and its enantiomer using a chiral cyclodextrin column.

Microemulsion electrokinetic chromatography (MEEKC) is an alternative to RP-HPLC; both are based on the differential partitioning of the analytes between a lipophilic and a hydrophilic phase which are moving relative to each other [12–18]. In case of MEEKC the lipophilic phase is formed from microdroplets consisting of a very unpolar compound like an alkane, a tenside (*e.g.*, SDS) implementing charges to the droplets, and a cosurfactant (*e.g.*, a lower alcohol). The aqueous mobile phase moves by the electroosmotic flow, the pseudostationary phase formed by the droplets migrates electrophoretically in addition. Although the experimental setup of this method, especially the composition of the microemulsion system, is apparently complex, the method is seemingly robust in practice. It was, therefore, the goal of the present paper to work out MEEKC conditions for the separation of levetiracetam from drugs potentially administered in polytherapy. Adjustment of the conditions can be carried out mainly by variation of the composition of the microemulsion system. The results were briefly compared with those obtained by usual micellar electrokinetic chromatography (MEKC). Applicability of the method for determination of levetiracetam on the therapeutical level in human plasma after sample pretreatment by SPE was examined as well.

2 Materials and methods

2.1 Materials

Levetiracetam (1-pyrrolidineacetamide, α-ethyl-2-oxo-, (αS)) was from UCB Pharma (Brussels, Belgium); carbamazepine (5-carbamoyl-5*H*-dibenz[*b*,*f*]azepine, CBZ) from Novartis Pharma (Basel, Switzerland); lamotrigine (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine) was from GlaxoSmithKline (Uxbridge, UK). Primidone (4,6(1*H*,5*H*)-pyrimidinedione, 5-ethyldihydro-5-phenyl-), phenobarbital (2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione, 5-ethyl-5-phenyl-) and phenytoin (2,4-imidazolidinedione, 5,5-diphenyl-) were purchased from Sigma Chemicals (St. Louis, MO, USA). The organic solvents, boric acid and sodium hydroxide were all analytical grade (E. Merck, Darmstadt, Germany). SDS was from Sigma-Aldrich (St. Louis, MO, USA); dodecylbenzene from Fluka (Buchs, Switzerland). Water was double-distilled from a quartz apparatus prior to use. The solutes were dissolved in water.

2.2 Instrumentation and procedures

2.2.1 CE

Capillary electrophoresis was carried out with an instrument (model Capel-105, Lumex Ltd., St. Petersburg, Russia) equipped with UV-vis detector and a liquid cooling system. Uncoated fused-silica capillaries (Supelco, Bellefonte, PA, USA) of 50 cm total length (effective length, 41 cm; 50 μm ID, 360 μm OD) were used for separation. Injection was from the anodic side of the capillary at 80 mbar·s. The detector was operated at 214 nm. New capillaries were rinsed with 1 M NaOH for 10 min, 0.1 M NaOH for 10 min, distilled water 10 min and BGE for 10 min. At the beginning of every working day the capillary was flushed with water (1 min), 0.1 M NaOH (1.5 min), water (2 min) and background electrolyte (BGE) 2 min. Between the runs the capillary was

washed with 0.1 M NaOH (3 min), with water (2 min) and BGE (3 min). At the end of the day the capillary was washed with water (1 min), 0.1 M NaOH (3 min), with water (3 min).

2.2.2 Preparation of microemulsions

All microemulsions were prepared on a w/w basis. They had the following composition. With 1-propanol and 1-butanol as cosurfactants: 93.76% aqueous borate buffer (10 mM total borate, pH 9.2, if not otherwise stated), 0.48% *n*-octane, 1.80% SDS, 3.96% alcohol. With 1-pentanol: 96.41% aqueous borate buffer, 0.50% *n*-octane, 2.07% SDS, 1.02% alcohol. With 1-hexanol: 96.87% aqueous borate buffer, 0.51% *n*-octane, 2.10% SDS, 0.52% alcohol. The components for the preparation of the microemulsions were mixed and ultrasonicated for 30 min. All samples and BGEs were filtered (0.45 μm, Minisart, Sartorius) prior to use.

2.2.3 MEKC and SPE

MEKC was carried out using a BGE consisting of borate buffer (pH 9.2, 30 mM total borate concentration) with 50 mM SDS. Mobilities were determined in duplicate, with the EOF and the micelle mobility in the same run. The EOF mobility was calculated from the water dip, the micelle mobility from the peak of dodecylbenzene. SPE of levetiracetam from human plasma was carried out similar to that described recently [19]. Briefly, to 500 μL plasma 50 μL internal standard solutions were added. Varian Bond Elut C18 cartridges (100 mg, 1 mL) were activated with 5×1 mL methanol and conditioned with 5×1 mL ultrapure water. Then the cartridge was rinsed with 5×1 mL phosphate buffer (pH 7.3, 20 mM). The samples were loaded, and the cartridges were washed with 1 mL phosphate buffer. Elution was carried out with 1 mL methanol. After evaporation to dryness in a rotary evaporator at 37°C the residue was redissolved in 500 μL of water-methanol mixture, and directly injected.

3 Results and discussion

3.1 Separation by MEEKC

3.1.1 Variation of cosurfactant

The cosurfactant is added normally in order to decrease the surface tension between the microdroplet and the aqueous phase (see, *e.g.*, [12, 15, 16, 18]). As the phases of the microheterogeneous system are in equilibrium, the cosurfactant determines the chemical environment in both phases. It is thus not surprising that this additive affects the partition coefficients of the analytes, and has an impact on migration and separation selectivity. In the present work, the microemulsion system was modified by adding the homologue series of lower alcohols, from 1-propanol to 1-hexanol. The resulting chromatograms are shown in Fig. 2. It can be seen that in all systems levetiracetam is, although least retained, well separated from the EOF marker. CBZ is the strongest retarded compound. Between these extremes, we can distinguish two groups of compounds with similar retention: primidone and phenobarbital on the one hand, phenytoin and lamotrigine on the other hand. It is known that retention is governed by the lipophilicity of the analytes. Relating roughly the number of aromatic rings in the molecule to the lipophilicity of the present analytes, this overall sequence is consistent with their structural properties: levetiracetam has a nonaromatic ring, phenobarbital and primidone have one, lamotrigine and phenytoin have two, and CBZ has a tricyclic system with a large delocalized π-electron system. Primidone and phenobarbital reverse their migration order with pentanol and hexanol compared to propanol and butanol, a clear example for the influence of the cosurfactant on selectivity. However, it is obvious that – due to the complexity of the solvent systems – there is no simple relation between the selectivity changes and the modification of the composition.

3.1.2 Variation of pH and salinity

The BGE with *n*-butanol as cosurfactant was chosen to investigate the effect of pH because of its favorable selectivity and good peak efficiency (plate number for, *e.g.*, CBZ is larger than 300 000). It should be noted that upon increase of the pH of the borate buffer used here the ionic strength increases as well, nearly by one order of magnitude (from 1 to 8 mM between pH 8.3 and 9.7 for the buffer with 10 mM total concentration). From Fig. 3 it can be seen that the migration order of levetiracetam, primidone, phenobarbital, lamotrigine and CBZ remains independent of pH, but phenytoin becomes less and less retained with increasing pH. In this context it should be pointed out that carbamazepine, primidone and levetiracetam (as amides) are very weakly basic, whereas phenobarbital and phenytoin are weakly acidic. The pK_a values of the latter compounds are 7.4 and 8.3, respectively [20]. This means that except these latter two analytes the compounds are neutral under the moderate to high pH conditions, which is in accordance with their pH-independent migration behavior. Phenobarbital

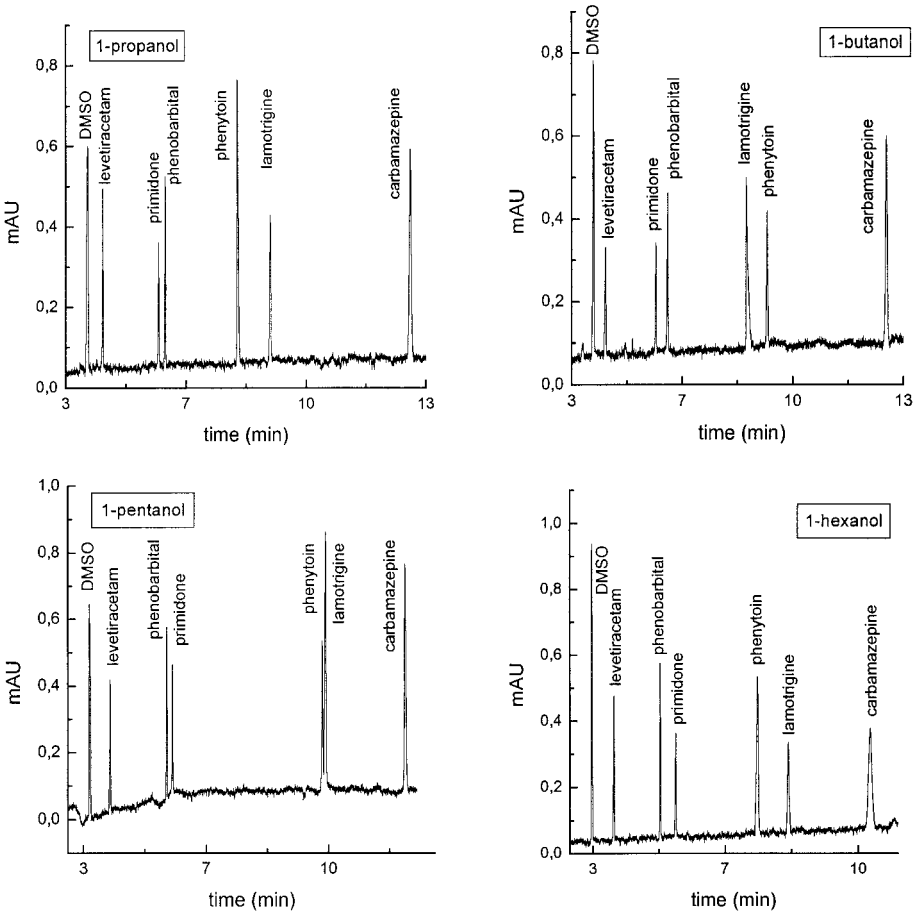

Figure 2. MEEKC of the drugs with different alcohols as cosurfactants. The aqueous buffer is borate pH 9.2, total concentration 10 mM. Composition of the microemulsion: with 1-propanol and 1-butanol as cosurfactants: 93.76% aqueous borate, 0.48% *n*-octane, 1.80% SDS, 3.96% alcohol. With 1-pentanol: 96.41% aqueous borate buffer, 0.50% *n*-octane, 2.07% SDS, 1.02% alcohol. With 1-hexanol: 96.87% aqueous borate buffer, 0.51% *n*-octane, 2.10% SDS, 0.52% alcohol. Conditions: bare fused-silica capillary, 50.0/41.0 cm length, 50 μm ID; voltage, 20 kV; temperature, 25°C; injection, 80 mbar·s; detection at 214 nm.

is anionic in this pH range, and migrates predominantly by its own electrophoretic mobility without significant partitioning into the microdroplet. Phenytoin is only half-dissociated at pH 8.3, its neutral fraction migrating thus electrokinetically upon partitioning, its ionic fraction electrophoretically as well. Increasing the pH shifts the acid-base equilibrium to the dissociated species, which is distributed to a lower extent into the microdroplets, and is more and more approaching the electrophoretic mobility of the charged form. This explains its special behavior upon variation of the pH.

3.1.3 Changes in phase ratio

Changing the phase ratio between the aqueous phase and the microdroplets should lead to a change in the magnitude of the k' values, but not necessarily to a variation in separation selectivity. However, we have observed recently for CBZ, oxcarbazepine and their metabolites that changes in selectivity might occur [21]. This effect is observed for the present analytes, too. It can be seen from the plot of apparent mobility *vs.* composition (Fig. 4; apparent mobilities are the measured mobilities corrected by that of the EOF marked with DMSO) that phenobarbital and primidone are better separated at high and low concentration of the microemulsion, and phenytoin and lamotrigine even change migration order. Although the effect in the apparent mobilities is small, it can clearly be recognized in the chromatograms (not shown).

3.2 Comparison of MEEKC with SDS-MEKC

We have compared the separation obtained by MEEKC with that in a micellar system consisting of the same buffering BGE and micelle former (but without alkane and cosurfactant). The resulting apparent mobilities in MEEKC show only moderate dependence with those in the MEKC system; the linear correlation coefficient is 0.9737 (Fig. 5). Especially phenytoin deviates from the migration order compared to lamotrigine, indicating a certain difference in selectivity between the two systems. However, from this comparison no general conclusion is made, as it is based on only one MEKC system without taking account the possible selectivity changes upon addition of organic solvents.

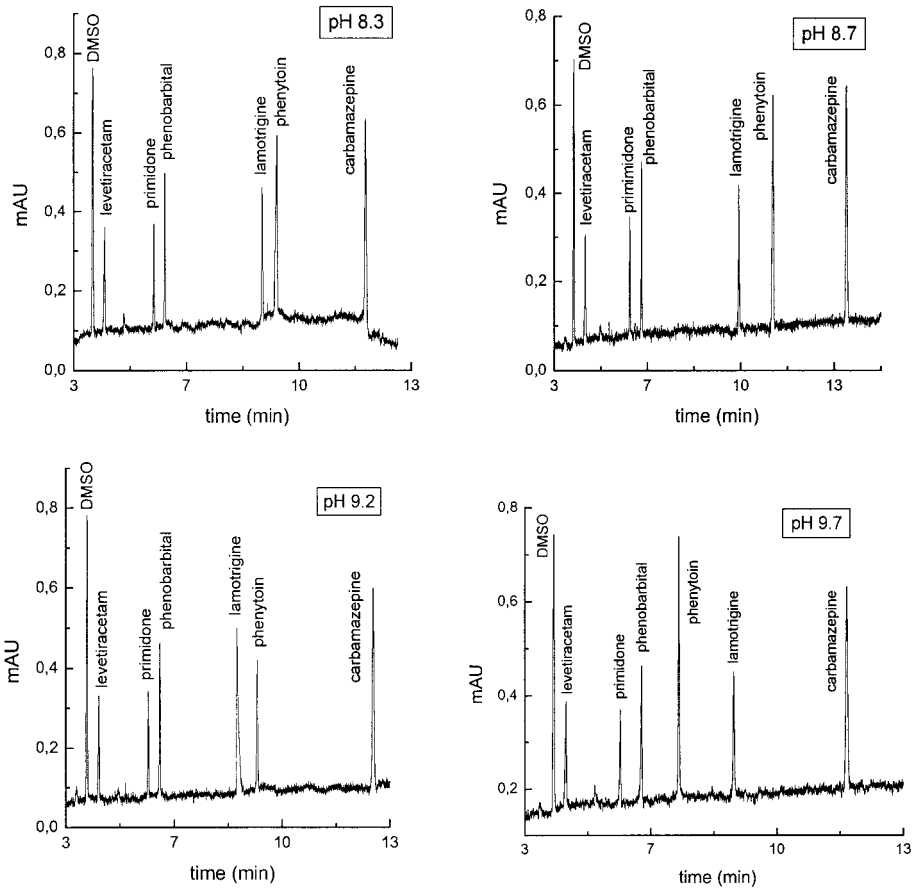

Figure 3. MEEKC of the drugs at different pH (and salinity) of the aqueous buffer (borate, 10 mM total concentration). Composition of the microemulsion: 93.76% aqueous borate, 0.48% *n*-octane, 1.80% SDS, 3.96% 1-butanol. Other conditions as in Fig. 2.

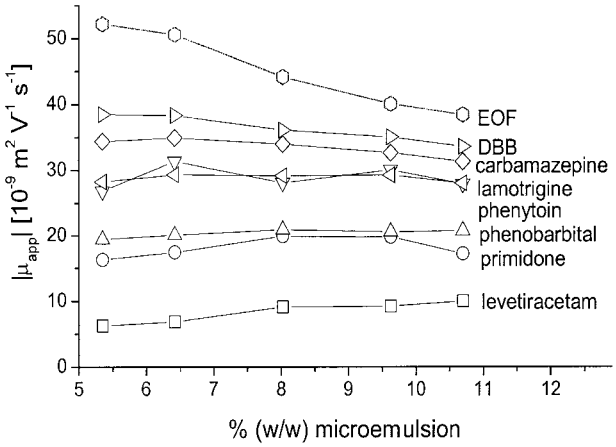

Figure 4. Apparent mobility, μ_{app}, as a function of the total content of the organic constituents forming the microemulsion. DBB: dodecylbenzene used as marker for the microemulsion droplets. Temperature, 20°C. The data are the average of duplicate measurements. The relative span is typically smaller than 1%.

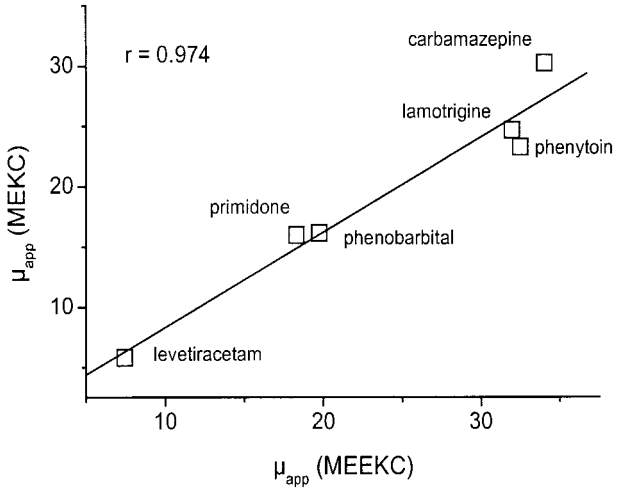

Figure 5. Apparent mobility, μ_{app} (in 10^{-9} $m^2V^{-1}s^{-1}$) of the analytes in the MEEKC and the MEKC system, respectively, both with the same pH of the aqueous BGE, and with SDS as micelle former. Composition of the microemulsion: 93.76% aqueous borate (pH 9.2, 10 mM), 0.48% *n*-octane, 1.80% SDS, 3.96% 1-butanol (all w/w). Micellar system: borate buffer (pH 9.2, 30 mM), 50 mM SDS.

3.3 Application to real samples

3.3.1 Influence of sample solvent

The composition of the solvent in which the sample is dissolved could have an eminent effect on the shape of the chromatogram in MEEKC. Especially organic solvents might strongly influence the resulting separation. This assumption follows from the conditions the microdroplets in the BGE are encountering when they are electrophoretically migrating against the EOF into the interior of the initial sample zone. Given that this zone forms an environment where the microemulsions are not stable (*e.g.*, consisting of methanol or acetonitrile, common solvents for biological extracts), the droplets could disrupt. SDS, the one constituent of the droplets, is still ionic under these circumstances, but the cosurfactants and the alkanes are not. This means that upon disruption of the microemulsion their neutral constituents would remain in the sample zone, whereas SDS will migrate out of it. Anyway, partitioning of the neutral analytes is disturbed due to the lack of microdroplets. In this segment of the separation system in the strict sense MEEKC will thus not take place. Therefore, it can be assumed that the sample solvent can have a decisive influence on the chromatographic result. The effect of the composition of the sample solvent is shown in one example in Fig. 6. It was found that the shape of the chromatograms at varying methanol concentration of the sample solvent is unaffected up to 35% methanol, whereas at higher alcohol content the peaks are increasingly distorted. Note the loss in efficiency, especially for CBZ.

3.3.2 Determination of levetiracetam in plasma after sample pretreatment

It is obvious that direct analysis of levetiracetam in plasma by UV detection will hardly be successful due to the low extinction coefficient of levetiracetam, as the molecule contains neither an aromatic ring nor conjugated double bonds. Therefore sample pretreatment, favorably with preconcentration, is a necessity in practice. We have recently developed [19] a suitable pretreatment procedure of plasma samples containing levetiracetam based on SPE. 500 μL of plasma were loaded on a hydrophilic-lipophilic balance cartridge, after suitable washing (see Section 2.2.3) the analytes were eluted with 1 mL methanol. The eluate is then dried and the sample redissolved in 500 μL water-methanol mixture. This procedure, slightly modified from those previously used for CBZ, oxcarbazepine and their metabolites [22–25], seems to be well suited also for levetiracetam even in the presence of other antiepileptic drugs. This can be concluded from the chromatogram shown in Fig. 7, obtained from a real human plasma sample spiked with 50 ppm drug. It is seen that no interference is present; the drug can be quantified. Rough estimation of the limit of quantitation (which is about 10 ppm for the present method) leads to the summary that in practice this limit should be improved say by a factor of 5. This goal could be reached by applying a larger volume of plasma and/or by reducing the final volume of the sample after SPE and prior to MEEKC. However, this is not the topic of the present paper.

4 Concluding remarks

MEEKC based on an oil-in-water system exhibits pronounced selectivity for the separation of the antiepileptic drugs levetiracetam, primidone, phenobarbital, phenytoin, lamotrigine and CBZ. Expectedly, retention of the different drugs is related to their lipophilicity, in the present case to the number of aromatic rings the molecules consist of. Selectivity depends on the choice of the alcohol used as cosurfactant, and on the pH (and ionic strength) of the aqueous phase (for all systems SDS was the surfactant). Even more interesting, the sequence of

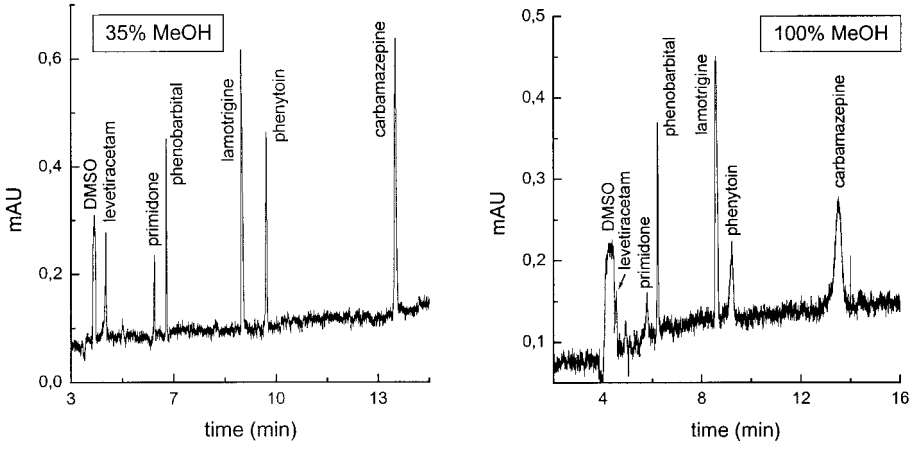

Figure 6. Effect of the sample solvent composition on peak shape in MEEKC. Sample was dissolved in a mixture of water and methanol as indicated, and injected. Composition of the microemulsion: 93.76% aqueous borate (pH 9.2, 10 mM), 0.48% *n*-octane, 1.80% SDS, 3.96% 1-butanol. Other conditions as in Fig. 2.

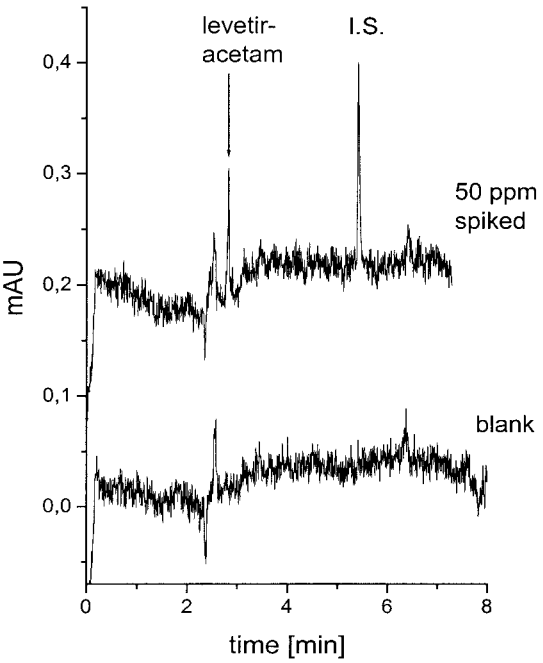

Figure 7. MEEKC of a human plasma sample spiked with 50 ppm levetiracetam. The sample was treated with SPE prior to chromatography as detailed in the text. I.S.: internal standard (melatonine). Sample solvent, water-methanol (80:20 w/w). Composition of the microemulsion: 93.76% aqueous borate (pH 9.2, 10 mM), 0.48% *n*-octane, 1.80% SDS, 3.96% 1-butanol. Other conditions as in Fig. 2.

migration of lamotrygine and phenytoin depends also on the percentage of organic constituents of the microemulsion. However, due to the complex composition of the microemulsion system, selectivity can hardly be predicted in detail. Comparison of the MEEKC system with MEKC (with borate buffer of the same pH 9.2, and SDS as tenside) also shows reversal of migration order for the two drugs mentioned. The MEEKC method enables to determine levetiracetam in human plasma after sample pretreatment based on SPE in RP columns in the lower tens ppm level without scooping out the potential of SPE to improve the method determination limit.

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5 References

[1] Shorvon, S. D., *Eur. J. Neurol.* 1998, *13*, 553.

[2] Patsalos, P. N., in: Bowman, W. C., Fitzgerald, J. D., Taylor, J. B., (Eds.), *Emerging drugs: The Prospect for Improved Medicines*, Ashley, London 1999, Vol. 4, pp. 87–106.

[3] http://www.fda.gov/cder/foi/label/1999/21035lbl.pdf.

[4] Welty, T. E., Gidal, B. E., Ficker, D. M., Privitera, M. D., *Ann. Pharmacother.* 2002, *35*, 296–304.

[5] Glauser, T. A., Pellock, J. M., Bebin, E. M., Fountain, N. B., Ritter, F. J., Jensen, C. M., Shields, W. D., *Epilepsia* 2002, *43*, 518–524.

[6] Klitgaard, H., Matagne, A., Gobert, J., Wülfert, E., *Eur. J. Pharmacol.* 1998, *353*, 191–206.

[7] Patsalos, P. N., *Pharmacol. Therapeut.* 2000, *85*, 77–85.

[8] Perucca, E., Bialer, M., *Clin. Pharmacokinet.* 1996, *31*, 29–46.

[9] Vermeij, T. A., Edelbroek, P. M., *J. Chromatogr. B* 1994, *662*, 134–139.

[10] Ratnaraj, N., Doheny, H. C., Patsalos, P. N., *Ther. Drug Monit.* 1996, *18*, 154–157.

[11] Isoherranen, N., Roeder, M., Soback, S., Yagen, B., Schurig, V., Bialer, M., *J. Chromatogr. B* 2000, *745*, 325–332.

[12] Watahari, H., *J. Chromatogr. A* 1997, *780*, 93–102.

[13] Miola, M. F., Snowden, M. J., Altria, K. D., *J. Pharm. Biomed. Anal.* 1998, *18*, 785–797.

[14] Altria, K. D., *J. Chromatogr. A* 1999, *844*, 371–386.

[15] Pedersen-Bjergaard, S., Gabel-Jensen, C., Honore Hansen, S., *J. Chromatogr. A* 2000, *897*, 375–381.

[16] Altria, K. D., *J. Chromatogr. A* 2000, *892*, 171–186.

[17] Gabel-Jensen, C., Hansen, H. S., Pedersen-Bjergaard, S., *Trends Anal. Chem.* 2001, *20*, 614–618.

[18] Gabel-Jensen, C., Honore Hansen, S., Pedersen-Bjergaard, S., *Electrophoresis* 2001, *22*, 1330–1336.

[19] Pucci, V., Raggi, M. A., Kenndler, E., 2nd S.A.Y.C. *Symposium*, Riccione, Italy, October 7–8, 2002.

[20] Albert, A., Serjeant, E. P., *The Determination of Ionization Constants – A Laboratory Manual*, Chapman and Hall, London 1984.

[21] Ivanova, M., Marziali, E., Raggi, M. A., Kenndler, E., *J. Sep. Sci.* 2002, *25*, 863–871.

[22] Mandrioli, R., Albani, F., Casamenti, G., Sabbioni, C., Raggi, M. A., *J. Chromatogr. B* 2001, *762*, 109–116.

[23] Raggi, M. A., Pucci, V., Maurizio, A., Muzikar, J., Kenndler, E., *J. Chromatogr. B* 2002, *770*, 217–225.

[24] Mandrioli, R., Ghedini, N., Albani, F., Kenndler, E., Raggi, M. A., *J. Chromatogr. B* 2003, *783*, 253–263.

[25] Pucci, V., Kenndler, E., Raggi, M. A., *Biomed. Chromatogr.* 2003, in press.