Application of high performance separation techniques in the analysis of biologically active and drug-candidate molecules

Phd thesis

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1. Introduction

In 1984 Terabe et.al. were the fist to publish the method of micellar electrokinetic chromatography (MEKC), and it became one of the most common separation technique since then. With the application of capillary zone electrophoresis the separation of charged compounds can be performed, but this method is not suitable for the separation of neutral compounds. MEKC is an adequate method for the separation of neutral compounds, besides the charged analytes. The applied electrolyte solution contains some kind of anionic surfactant (between pH 6-10), exceeding the critical micelle concentration. At this concentration the surfactants form micelles. The inner side of the micelle is hydrophobic, the external side is negatively charged, and they form a pseudostationery phase.

The retention of the analytes in MEKC depends on the hydrophobic character of the test compounds. In the course of the separation there are three different groups.

One of them is where the compounds do not interact with the micelles, they remain in the aqueous phase during their migration time, and the only force that moves them in the capillary is the electroosmotic flow (EOF).the migration time of these compounds are equal with the migration time of the EOF (t_0).

In the second group, there are those highly hydrophobic compounds that remain inside the micelles, and therefore they move together with the micelles in the capillary. Accordingly the migration time (t_m) of these substances are equal with the migration time of the micelles (t_{mc}).

Those compounds that do not fall under the previous two groups, are spending one part of their migration time in the pseudostationery phase the other part in the aqueous phase according to their hydrophobicity. In the case of anionic surfactants it means that $t_0 \leq t_m \leq t_{mc}$.

The most commonly used surfactants are anionic tensides, and among them the most frequenly applied is sodium dodecyl sulphate (SDS).

In 1984 Terabe introduced the retention factor (k') on the analogy of HPLC. Since in MEKC both phases are moving, the corresponding component retention factors must be corrected. The equation applied for the calculation of retention factor is:

$$k' = \frac{t_m - t_0}{t_0\left(1 - t_m / t_{mc}\right)} = K * \frac{V_s}{V_M} \qquad \textbf{1}$$

where K is the partition coefficient, V_s is the volume of the micellar phase and V_M is the volume of the mobile phase.

Another quantity is the normalized retention factor (k") that has been introduced by our workgroup in 1996. This quantity can be calculated applying the following equation:

$$k" = \frac{t_m - t_0}{t_{mc} - t_0}$$

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If the test compound spends its whole migration time in the aqueous phase, that is to say $t_0=t_m$, and it means that k"=0. If the test compound spends its whole migration time in the micellar phase, that is to say $t_{mc}=t_m$, and it means that k"=1. If the test compound distributes between the micellar and the aqueous phase and spends some part of its migration time in the micellar the other part in the aqueous phase that is to say $t_0 < t_m < t_{mc}$, meaning that the value of k" falls between zero and one. One of the big advantages of the normalized retention factor is that its value stays in finite range.

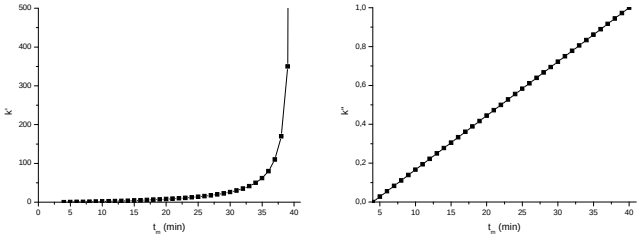

Figure 1.

Change of k' k" parameters in the function of migration time (t_0=4 min, t_{mc}=40 min)

The other advantage of the normalized retention factor is that utilizing this parameter the aqueous (t_{aq}) and the micellar (t_{mc}) phase residence times can be calculated. The migration time of the analyte is the sum of the phase residence times.

$$t_{mic} = t_{mc}\, k"$$

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$$t_{aq} = t_0 (1 - k")$$

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$$t_m = t_{mic} + t_{aq} = t_0(1 - k'') + t_{mc}k''$$

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2. Aim and objectives

Determination of the hydrophobicity of the selected compounds, applying the retention parameters obtained from a MEKC method.

Development of a suitable method, for the characterization of the hydrophobicity of the pseudostationery phases applied in MEKC and how they interact with the analytes. For this I use the normalized retention factor for the determination of these parameters. I intend to prove that the retention parameters originated from MEKC analysis, can be applied not only for the characterization of the hydrophobicity of the analytes, but with the help of the examined compounds the characterization of the interaction between the pseudostationary phase and the analyte is achievable. After these characterizations the pseudostationary phases become comparable.

Utilizing the MEKC parameters obtained for a homologous series of alkyl-benzene I intend to determine the role of the methylene selectivity of the applied pseudostationary phases. I also intend to confirm that the newly determined parameter, the normalized retention factor is applicable for the determination of methylene selectivity.

3. Materials and Methods

3.1 Analytes

The investigated compounds are the followings:

- alkyl benzenes: benzene, toluene, ethylbenzene, propylbenzene, butylbenzene
- alkyl phenones: propiophenone, butyrophenone, valerophenone, heptanophenone
- alcohols: pentan-1,5-diol, butan-1-ol, pentan-3-ol, pentan-1-ol
- miscellaneous aromatic compounds: aniline, o-toluidine, benzaldehyde, methylbenzoate, naphthalene
- MDR peptides

MDR peptides

The following di-, tri-, and tetra-peptides were synthesized and analyzed in our workgroup. They are tested against multidrug resistance, and are potential reversine molecules. After the synthesis the protecting group and the free carboxyl and amino groups were not removed.

- $(Z\text{-}Pro)_2$-Lys-OMe
- $[BOC\text{-}Glu(OBzl)]_2$-Lys-OMe
- [BOC-Asp(OBzl)-Lys(Z)]-OtBu
- $[BOC\text{-}Pro\text{-}Glu(OBzl)]_2$-Lys-OMe
- $[BOC\text{-}Pro\text{-}Pro\text{-}Glu(OBzl)]_2$-Lys-OMe
- $[BOC\text{-}Asp(OBzl)\text{-}Glu(OBzl)]_2$-Lys-OMe
- $FMOC\text{-}Glu[Lys(Z)OtBu]_2$

Abbreviations: Boc: tert-butyloxycarbonyl-; tBu, tert-butyl-; Bzl: benzyl-; FMOC: 9-fluorenylmethyloxycarbonyl; Me: methyl; Z, benzyloxycarbonyl-

3.2 Pseudostationer phases

As pseudostationary phases six different tensides were chosen for investigation. Among these were both anionic and cationic ones, structurally similar ones (there is two methylene group difference between them), and a perfluorinated one too. The designated pseudostationary phases were the following: sodium dodecyl sulphate (SDS), sodium deoxycholate (SDC), sodium cholate (SC), tetradecyltrimethylammonium bromide (TTAB), hexadecyltrimethylammonium bromide (HTAB) and lithium perfluoro octanesulphonate (LiPFOS).

3.3 Equipments

3.3.1 ISCO Model 3850 Capillary Electropherograph

In the case of MDR peptides the micellar electrokinetic chromatographic separations were performed with an ISCO Model 3850 Capillary Electropherograph (Lincoln, NE, USA). The separations were performed on an uncoated fused silica capillaries, with 50 μm inner diameter (Polymicro Technologies, Phoenix, AZ, USA), total length 60 cm, 40 cm to the detector window.

Detection was performed at 205 nm with 0,02 AUFS sensitivity. Analyses were performed at 21 kV with positive polarity (anodic end on the injector site). The injection was performed with the use of a built-in splitter in 1: 1000 ratio (it means that 1/1000 of the injected solute has reached the capillary). The collection of data was performed with the help of ISCO ChemResearch data handling system.

3.3.2 Beckman P/ACE System 5500

Except the MDR peptides the measurements were achieved on a Beckman P/ACE System 5500 with an UV diode array detector, in Barcelona by Roses and co. workers. Capillary was fused-silica (40 cm effective length, 50 mm ID); the applied voltage was +15 kV for anionic surfactants and -15 kV for cationic surfactants and the detection was performed at 214 nm.

The capillary was conditioned between each surfactant change according to the followings: 5 min wash with water, 20 min wash with 1 M base, 10 min wash with water, 10 min wash with 0,1 M base, 20 min wash with the separation buffer. Before each run the capillary was rinsed with the separation buffer for 5 minutes.

3.4 Buffers

The running buffer used at the separation of MDR peptides was 0,1 M Na-borate buffer (pH=7,70) containing 75 mM SDS. Na-borate buffer was prepared by dissolving 0,1 M H_3BO_3 and 0,05 M NaOH in 1000 ml water; the final pH of the buffer was adjusted to 7,70 with 0,1 M HCl solution.

For LiPFOS (40 mM) the separation buffers were prepared by solving the surfactants in water, adding H_3PO_4 , and neutralizing with LiOH up to pH 7,0.

SDS (40 mM), SC (80 mM), TTAB (20 mM) and HTAB (20 mM) separation buffers were prepared by solving the surfactants in sodium phosphate buffer at pH 7.0, and SDC (40 mM) in sodium phosphate– sodium tetraborate buffer at pH 8.0. All separation solutions were 20 mM in buffer.

3.5 Samples

3.5.1 MDR-peptides

Peptide samples were dissolved in acetonitrile/water (50:50 v/v) at a concentration of 1 mg/ml. Dissolved samples were filtered through Spartan 13 (Schleicher and Schuell, Dassel, Germany) disposable filters (0.45 μm).

Acetone and Sudan III were utilized to determine migration times corresponding to the electroosmotic flow, EOF (t_0) and to the micelles (t_{mc}), respectively. Sudan III was dissolved in acetonitrile at a concentration of 1 mg/ml.

3.5.2 Test compounds

Test solutes (2 mg/ml) were dissolved in methanol (used as EOF marker) and the solutes contained ca. 2 mg ml of dodecanophenone as micellar marker. All solutions were filtered through Albet nylon syringe filters (0.45 mm).

3.5.3 Hydrophobicity

Software-predicted hydrophobicity (CLOGP) of the compounds analyzed was calculated with the program CLOGP accessible via Internet (www.daylight.com/daycgi/clogp) working with the Hansch-Leo's "fragment constant" method on the basis of the chemical structure of the compound processed.

4. Results and conclusion

4.1 CLOGP-t_{mic}

The relationship between the micellar phase residence time and the hydrophobicity of the analyte in the case of alkyl benzenes and alkyl phenones had been investigated on the SDS pseudostationary phase earlier.

In the present work the investigation has been extended to other pseudostationary phases including cationic and anionic ones. CLOGP values and micellar phase residence times of the molecules were investigated on six different pseudostationary phases (SDS, TTAB, HTAB, SC, SDC, LiPFOS).

A good linear correlation between the log t_{mic} and CLOGP values of the analytes was found for all pseudostationary phases applied. The comparison was made for the different subgroups of the analytes, and a similar good linear correlation was found in all cases.

4.2 CLOGP-$t_{prop,mic}$

The good correlation between CLOGP and t_{mic} obtained on the pseudostationary phases supported that the t_{mic} could be applied to characterize experimentally the hydrophobicity of the analyte. Since the absolute time values (t_{mic}) are difficult to compare, similarly to HPLC, introduction of a relative quantity seemed to be reasonable. The micellar proportion ($t_{prop,mic}$) can be defined as:

$$t_{prop,mic} = \frac{t_{mic}}{t_m} \qquad \textbf{6}$$

The $t_{prop,mic}$ values have been determined in all of the pseudostationary phases applied in this work for the alkyl benzenes, alkyl phenones, alcohols, MDR peptides and these values have been compared with the CLOGP values of the same analytes.

Both t_{mic} and $t_{prop,mic}$ show likewise a good linear correlation with CLOGP values, and are therefore suitable to characterize the hydrophobicity of the analytes, with $t_{prop,mic}$ as a relative quantity being a more advantageous parameter for comparative purposes. (Figure 2.)

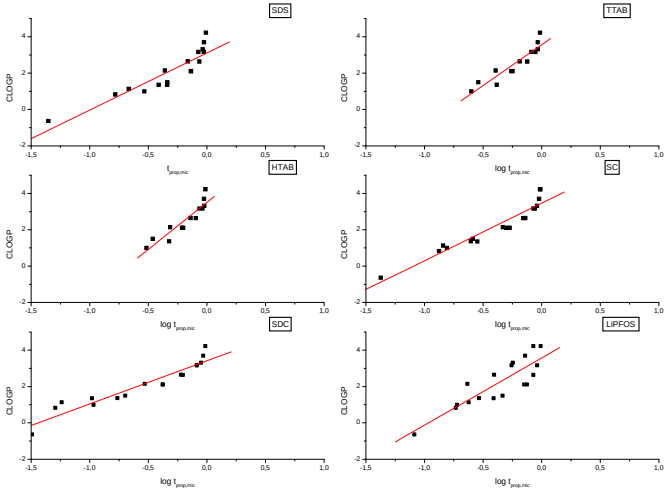

Figure 2.

Relationship between the hydrophobicity (CLOGP) and micellar proportion time ($t_{prop,mic}$) on the six different pseudostationary phases

4.3 Comparison of the pseudostationary phases

Not only the hydrophobicity of the analytes, but also the interaction between the pseudostationary phase and the analyte can be characterized utilizing the $t_{prop,mic}$ value. Information about the hydrophobicity of the pseudostationary phase (its willingness to interact with the probe molecule) can be gained by considering the micellar proportion of an analyte of known hydrophobicity (probe molecule) in the given pseudostationary phase. This interaction is the solubilization of the analyte in the micelles. The higher the micellar proportion of a hydrophobic probe analyte, i.e., the greater the proportion of its whole migration time spent in the pseudostationary phase, the stronger the willingness of this phase is to interact with this probe molecule, resulting in higher retentivity.

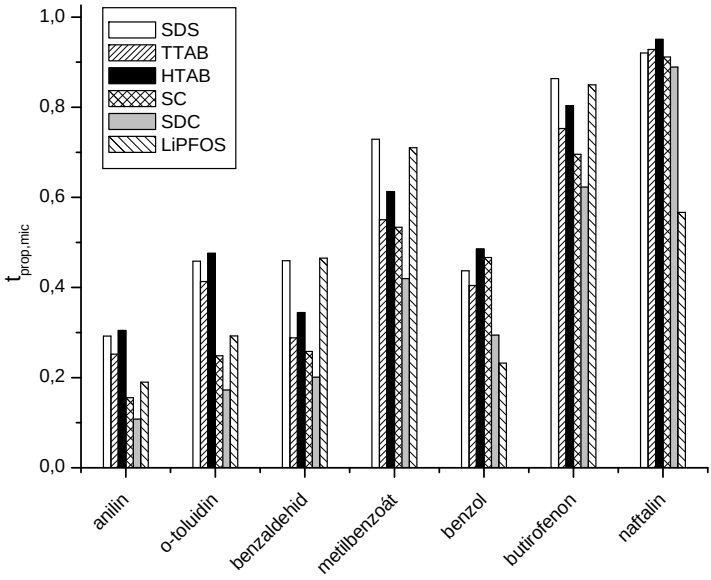

Figure 3.

The micellar proportion of the test compounds on the six different pseudostationary phases

The micellar proportion obtained with a probe analyte is suitable to compare the stationary phases applied in MEKC. However, more reliable experimental data can be gained by applying not only one probe molecule but a set of probe analytes to characterize the strength of interaction between the pseudostationary phase and the analytes. Therefore, comparison of the pseudostationary phases has been performed with a set of molecules covering a wide range of hydrophobicity and representing different chemical structures and nature (basic, neutral polar, highly or less hydrophobic molecules). The selected compounds are: aniline, o-toluidine, benzaldehyde, methylbenzoate, naphthalene, benzene and butyrophenone. (Figure 3.)

4.3.1 The $CLOGP_{50}$ value

Utilizing the good correlation between the CLOGP and the micellar proportion the $CLOGP_{50}$ value can be defined. It is the hydrophobicity of a virtual molecule of which the $t_{prop,mic}$ value is equal to 0.5, meaning that it spends exactly 50% of its migration time in that pseudostationary

phase. The suggested parameter $CLOGP_{50}$ corresponding to the equal distribution in the hydrophilic aqueous and in the hydrophobic micellar phase characterizes the retention power of the given pseudostationary phase. The low $CLOGP_{50}$ represents a highly effective micellar phase to interact with the analyte, while for a micellar phase characterized by a high value of $CLOGP_{50}$ a strong interaction can be expected only for highly hydrophobic analytes. The lower the $CLOGP_{50}$ value of a given pseudostationary phase is, the greater its interaction with the analytes of hydrophobic nature (i.e. its hydrophobicity) is.

The capability of the pseudostationary phases to interact with the test analytes decreases in the order: SDS > HTAB > TTAB > SC > LiPFOS > SDC. This finding is in accordance with the results of Yang and Khaledi giving the decreasing hydrophobicity order of the three phases investigated (SDS, LiPFOS and SC) as follows: SDS > SC > LiPFOS.

This experimentally determinable numerical data may be applicable for the comparison of the pseudostationary phases applied in MEKC.

4.4 Methylene selectivity

Hydrophobic or methylene selectivity is generally interpreted as the relative retention of the adjacent members of homologous series differing only in one methylene group. This parameter depends on the hydrophobic interaction between the stationary phase and the test compounds. According to the Martin equation, a homologous series with increasing number of carbon atoms shows a linear relationship between the log retention factor and the number of carbon atoms. Methylene selectivity of the pseudostationary phases applied in MEKC can also be characterized applying the micellar proportion values.

The regression parameters can be described with the following equation:

$$\log t_{prop,mic} = A \cdot \log Z + B \qquad \textbf{7}$$

where Z is the carbon number of the alkyl chain. These parameters have been determined for an alkyl-benzene homologous series for the six different pseudostationary phases. Slope A of equation 7 characterizing the methylene selectivity has been compared for the different pseudostationery phases.

The decreasing methylene selectivity order of the pseudostationery phases is: LiPFOS> SDC > TTAB > SDS > SC > HTAB. LiPFOS proved to be the pseudostationary phase with the highest, while HTAB the lowest methylene selectivity.

5. Conclusions

A good correlation between the experimentally determined micellar proportion ($t_{prop,mic}$) and the calculated hydrophobicity (CLOGP) of the analyte has been demonstrated for six different anionic and cationic pseudostationary phases. The higher the hydrophobicity of the analyte is, the higher its micellar proportion. This relation was found to be valid for all pseudostationary phases applied. Consequently, an experimentally obtainable numerical value, $t_{prop,mic}$ characterizing the hydrophobicity of the analytes can be obtained by MEKC.

On the other hand, $t_{prop,mic}$ can be utilized to characterize the interaction between the analyte and the pseudostationary phase. Applying a set of probe molecules with known hydrophobicity, the $CLOGP_{50}$ value (showing the value of hydrophobicity of a virtual analyte spending exactly 50% of its migration time in the pseudo-stationary phase) has been calculated for each of the pseudostationary phases applied. This experimentally determinable numerical value ($CLOGP_{50}$) is characteristic for the given pseudostationary phase and allows to compare their hydrophobicity and hence retention ability. The hydrophobicity order of the pseudostationary phases (SDS > HTAB > TTAB > SC >. LiPFOS > SDC) obtained by using $CLOGP_{50}$ was in agreement with the results of earlier investigations suggesting the possible application of $CLOGP_{50}$ to further pseudostationary phases.

The $t_{prop,mic}$ values obtained for the homologous series of alkyl benzenes were applied to compare methylene selectivity of the pseudostationary phases as well.

Publications

List of own publications related to the dissertation

Zs. Dobos, É. Kiss, B. Hallgas, Gy. Kéri, M. Idei: Micellar proportion: A parameter to compare the hydrophobicity of the pseudostationary phases or that of the analytes in micellar electrokinetic chromatography, **Electrophoresis** 2005, 26, 849-857

A. Varga, M. Huszár, ***Zs. Dobos***. É. Kiss, A. Horváth, M. Idei: Characterisation of mixed lithium dodecylsulphate / lithium perfluorooctanesulphonate pseudo-stationary phases in MEKC **Electrophoresis** 2009, 30, 1923–192

List of own publications not related to the dissertation

M. Idei, É. Kiss, ***Zs. Dobos***, B. Hallgas, Gy. Mészáros, Gy. Kéri, F. Hollósy: Separation of anti-tumor peptides by capillary electrophoresis in organic solvent containing background electrolytes. **Electrophoresis** 2003. pp. 829-833.

B. Hallgas, T. Patonay, A. Kiss-Szikszai, ***Zs. Dobos***, F. Hollósy, D. Erős, L. Őrfi, Gy. Kéri, M. Idei: Comparison of measured and calculated lipophilicity of substituted aurones and related compounds, **Journal of Chromatography B**, 2004 Volume 801, pp 229-235.

Zs. Dobos, T. Lóránd, F. Hollósy, B. Hallgas, D. Erős, Gy. Mészáros, Gy. Kéri,M. Idei: Determination of the basicity of Mannich ketones by capillary electrophoresis. **Journal of Chromatography B** 2004 Volume 799, pp 179-183.

B. Hallgas, ***Zs. Dobos***, E. Ősz, F. Hollósy, R.E. Schwab, E.Z. Szabó, D. Erős, M. Idei, Gy. Kéri and T. Lóránd: Characterization of lipophilicity and antiproliferative activity of E-2-arylmethylene-1-tetralones and their heteroanalogues, **Journal of Chromatography B**, 2005 Volume 819, pp 283-291

B. Hallgas, ***Zs. Dobos***, A. Agócs, M. Idei, Gy. Kéri, T. Loránd, Gy. Mészáros: Lipophilicity and antiproliferative activity profiling of 2-benzylidencycloalkanones **Journal of Chromatography B**, Volume 856, Issues 1-2, 1 September 2007, Pages 148-155