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Study of interactions participating in the separation mechanism of chromatographic separation systems

The PhD Thesis Report

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Content

1. Introduction

High performance liquid chromatography (HPLC) is one of the preferently used separation techniques. Generally, this method provides good reproducibility and robustness. An advantage of the method is a possibility to influence separation not only by the type of stationary phase but also by composition of the mobile phase. A large range of structurally different compounds can be separated by HPLC and it can be used for qualitative as well as quantitative determination [1]. As various interaction types can participate in the retention mechanism of analytes in separation systems, the choice of the optimal separation system is often ambiguous. The selection of suitable separation conditions is very often made empirically. Such approach is rather time consuming therefore, it could be advantageous to utilize some physicochemical characteristics of the separation system.

The knowledge of retention and interaction mechanism is necessary for choice of the optimal separation condition, i.e. suitable stationary and mobile phases. In most cases the available technical information given by producers is not sufficient to select the optimum column for a particular separation [2]. Linear free energy relationship (LFER) is one of the comprehensive semiempirical methods that allows characterization and comparison of stationary phases/separation systems and yield to better understanding of the intermolecular interactions, which play role in the separation processes [3,4].

The optimization of separation processes can be disturbed or complicated by phenomena called system peaks. In the case of detectable system zone the resultant chromatogram includes more peaks than is the number of analytes in the sample. These additional signals are often misinterpreted since they are considered as analyte signals. A serious problem that can arise if an analyte interacts with the system zones is resonance. This phenomenon can disturb completely the separation results and make evaluation of the measured data impossible.

2. Aims of the thesis

The aim of the PhD thesis was investigation of interaction mechanisms that take part in separation processes in HPLC.

Partial goals can be divided as:

- characterization and comparison of interaction possibilities of three chiral stationary phases, namely teikoplanin, teikoplanin aglykon a methylated teikoplanin aglykon in various mobile phase compositions using LFER model; to characterize the interactions decisive in retention of analytes in studied separation systems and show prediction of new analytes retentions in these systems
- study of system peaks in RP HPLC, in order to describe their formation, appearance and also chromatographic behavior in the given separation system
- application of theoretical knowledge on separation of given analytes mixtures

3. Results and discussion

3.1 Characterization of separation system using the LFER model (publication I)

LFER is a semiempirical method that allows characterization and comparison of the intermolecular interactions possibilities of separation systems, which play a role in the separation processes. The main advantage of the LFER model lies in its ability to independently describe (qualify and quantify) the contributions of individual molecular interactions to the retention process. The principle of the LFER model is based on the correlation between the retention parameters determined for a representative series of analytes in a separation system (e.g. retention factor) and the solute parameters (descriptors) that characterize properties of the solute molecule [5]. The logarithm of the retention factor, log k, can be correlated with various fundamental solute properties, as seen in the general form of the LFER equation:

$$\log k = \mathbf{c} + \mathbf{r}.R_2 + \mathbf{s}.\pi_2^H + \mathbf{a}.\Sigma\alpha_2^H + \mathbf{b}.\Sigma\beta_2^H + \mathbf{v}.V_x \qquad (1)$$

The independent variables in Eq. (1) are solute descriptors and denote specific solute properties: V_x is the McGowan characteristic volume (hydrophobic interactions) [7], $\Sigma\alpha_2^H$ is the effective or overall hydrogen bond acidity [7], $\Sigma\beta_2^H$ is the effective or overall hydrogen bond basicity [6], π_2^H is the solute dipolarity/polarizability parameter (electrostatic interactions) [6] and R_2 is the excess molar refraction (dispersion interactions). The coefficients in Eq. (1) are determined by multivariate regression analysis and reflect the individual types of molecular interactions in the given separation system.

LFER model was used for characterization and comparison of three teicoplanin-based chiral stationary phases (CSPs) – native teicoplanin, teicoplanin aglycon (TAG) and methylated teicoplanin aglycon (MTAG) in four mobile phases differing in methanol (MeOH) and 1% triethylamine acetate buffer (TEAA), pH 4.20, ratio (content of MeOH in the mobile phase varied in the range of 20-80 volume percent). The possible interactions with silanol groups of silica gel support are eliminated using buffer at pH 4.20 with triethylamine; so retention of analytes is influenced only by interactions analyte/chiral selector and analyte/mobile phase. The series of test analytes covered a wide range of interactions, 34 solutes were structurally diverse and the distribution of individual descriptors was equal so that no interaction was preferred.

Hydrophobic interactions (described by regression coefficient **v**) exhibited a clearly defined trend, i.e., increase with the buffer contents in the mobile phase. The highest value of the **v** coefficient was obtained on the MTAG column, on which we expected the highest hydrophobicity due to methylation of the carboxylic acid and phenolic groups of TAG CSP. The hydrophobicity of these CSPs clearly decreases in the sequence MTAG > TAG > T in all the mobile phases used.

The ability to interact with solute *n* and π-electrons (described by coefficient **r**) also increases with the contents of the aqueous buffer in the mobile phase. However, lower values of the **r** coefficient than of the former one show that the contribution of this type of interaction is smaller than that of the hydrophobicity. The tendency cannot be generalized, as the **r** values are not significant under certain mobile phase compositions. Nevertheless, it can be seen that better accessible aromatic structures or carbonyl groups on the TAG and MTAG CSPs result in a higher portion of these *n*- and π -electron interactions in the retention mechanism there, than on the T CSP.

The hydrophobic interactions (and *n*- and *π* -electron interactions) are substituted by polar interactions (described by regression coefficiens **s**) in mobile phases with increased MeOH content. The polar interactions are significant for almost all the studied separation systems because many polar and polarizable groups are available on the teicoplanin-based CSPs.

The regression coefficients **a** and **b**, reflecting a disposition of the separation system to interact through hydrogen bonding interactions, are negative. This means that these interactions are preferred in the mobile phase.

3.2 Occurrence and behavior of system peaks in RP HPLC with solely aqueous mobile phases (publication II)

System peaks are important but often also disturbing phenomena that complicate optimization processes occurring in separation systems. Signals of system peaks can cause serious problems in evaluation of separation results - they are often misinterpreted since they are considered as analyte signals [8]. On the other hand these peaks have not only negative or disturbing properties they can be used for example for calculation of column void volumes and retention factors or determination of adsorption isotherms [9].

Behavior of system peaks was studied in reversed phase high performance liquid chromatography (RP HPLC) systems consisting of an RP Amide C16 column and aqueous solutions of organic acids with alkaline metal hydroxides as mobile phases without any organic modifier. Binary mobile phases were composed of aqueous solutions of benzoic acid with various concentrations of hydroxides – LiOH or CsOH. The ternary mobile phase used contained LiOH, benzoic acid, and tropic acid (3-hydroxy-2-phenylpropionic acid) in different concentrations. Binary mobile phases yielded two system peaks; ternary mobile phases yielded three system peaks after injection of disturbance of LiOH. The first system peak was stationary while the second or second and third moved with changes of concentration of the buffer components. The injection of higher/lower concentration of LiOH and the same concentration of benzoic acid leads to the positive/negative first system peak and the negative/positive second system peak in binary mobile phases, respectively. The injection of higher/lower concentration of benzoic acid causes only one positive/negative second system peak (the stationary peak is missing) – see Fig. 1.

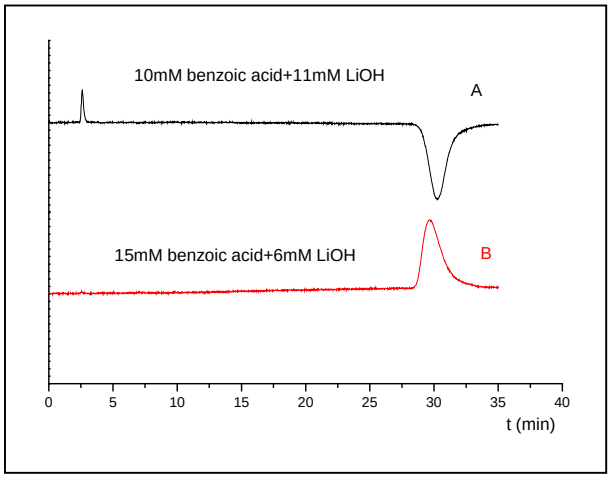

Fig. 1. Chromatograms obtained by injections of a disturbance of: A) LiOH, B) benzoic acid. Compositions of injected disturbances are given in the figure. Mobile phase: 10 mM benzoic acid + 6 mM LiOH. Indirect UV detection: 290 nm; column and sample temperature: 25 °C; flow rate: 0.2 ml/min; injected volume: 10 µl.

As LiOH does not adsorb on the stationary phase the position of the first system peak is constant and does not depend on the mobile phase composition therefore, it can be used as a void volume marker. The position of the second system peak can be affected by mobile phase composition. The position of the second system peak shifts to higher retention times with reduced concentration of the buffer or with increasing dissociation of benzoic acid (e.g. with higher LiOH content). The shifting can be caused in both cases by lower coverage of the stationary phase with non-dissociated benzoic acid (only this form of benzoic acid adsorbs on the stationary phase) as the mobile phase constituent; thereby the competition for binding sites on the stationary phase is diminished.

The resonance phenomenon ("interaction" of analyte and system zones) was obtained by injection of 1-pentanol as a sample into one component mobile phase – 10 mM benzoic acid – see Fig. 2. The increase of the response when the analyte peak approaches the system peak is evident. Moreover, the peak of hexane-3-ol is larger than that of hexane-2-ol because the former is closer to the system peak (concentrations and injected volumes were the same for the both analytes). In addition, reverse responses (peak signs) of the analyte peak eluted before (propane-1-ol and butane-1-ol) and behind (hexane-3-ol and hexane-2-ol) the position of the system peak were observed.

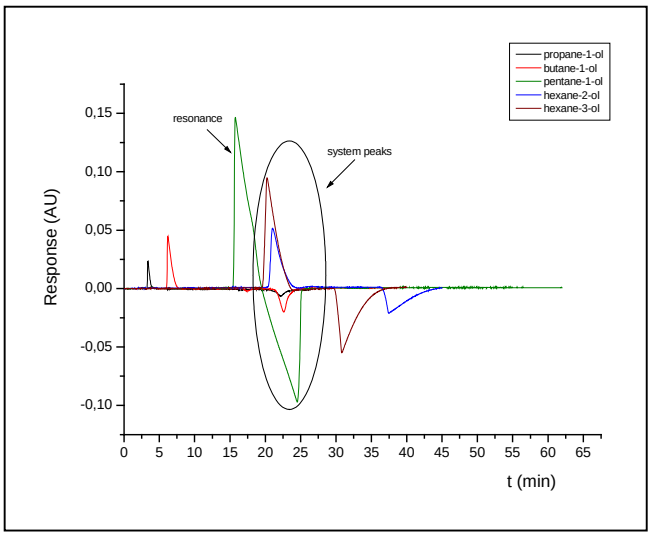

Fig. 2. Resonance phenomenon and responses in indirect detection. Mobile phase: 10 mM benzoic acid. The composition of injected samples is shown in the chromatogram. Injected samples: 0.5% solutions of alcohols dissolved in the used mobile phase. Indirect UV detection 290 nm, injected volume 20 µl.

The collected data were used for development of a mathematical model that describes positions and amplitudes of system peaks. The chromatographic model was incorporated into the computer program Simul 5 originally designed for simulations in capillary electrophoresis. The computer program can simulate time depending positions and amplitudes of system peaks also under various initial compositions.

3.3 Examples of optimized separation systems and their application

3.3.1 Achiral separation systems

3.3.1.1 HPLC separation of estrogens (publication III)

This work deals with the development and optimization of a simple HPLC method for determination of five estrogen derivatives, namely estrone, 17α-estradiol, estriol, 17-α-ethinylestradiol and metranol. RP HPLC method was developed with C18 stationary phase (Supelcosil TM LC-18-DB (250 x 4.6 mm, particle size 5 µm)) and mobile phase composed of just acetonitrile (ACN) and deionised water. For isocratic elution the optimized mobile phase composition found was ACN/deionised water 40/60 (*v*/*v*). A baseline resolution of all five compounds was achieved but analysis time was too long for practical application. The reason was strong hydrophobic interaction of mestranol with the stationary phase - its retention time was 85.8 min. The next step was optimization of gradient elution. ACN/deionised water 40/60 (*v*/*v*) with a linear gradient to 100 vol. % of acetonitrile applied from 16^{th} to 17^{th} minutes provided separation of all the analytes within 22 minutes. The

mobile phase flow rate was kept at a constant value of 1.3 ml/min. Shorter retention and better peak shape and efficiency were obtained when gradient elution was employed.

A shorter column Symmetry ® C18 (150 x 4.6 mm, particle size 5 μm) with principally analogous stationary phase type was tested in order to decrease the analysis time and improve peak symmetry. When gradient elution with this shorter column was performed, the optimized mobile phase composition found was ACN/water 40/60 (*v/v*) with the linear gradient to 100 vol. % of acetonitrile applied from 8^{th} to 9^{th} minutes. In addition a linear gradient of flow rate from 2 ml/min to 3 ml/min was applied between 8^{th} – 9^{th} minutes. Shorter retention, better peak symmetry and higher separation efficiency were obtained. Baseline resolution of all five compounds was achieved and the analysis time did not exceed 11 min.

3.3.1.2 HPLC method for separation of 1,4-benzodiazepines (publication in progress)

Optimization of separation conditions of seven drugs from 1,4-benzodiazepine group – bromazepam, oxazepam, nitrazepam, chlordiazepoxide, flunitrazepam, lormetazepam and diazepam was also carried out in RP HPLC system. The first column tested was Zorbax SB-C8 (4.6 x 150 mm, particle size 5 μm) packed with an encapped octyl-silica gel stationary phase. The most suitable mobile phase composition found was ACN/deionised water with acetic acid (HAc), pH 3.0, 30/70 (*v/v*). Baseline resolution of all seven analytes was achieved and the analysis time was 25 minutes. Acidic mobile phase component was applied to enhance peak symmetry if compared with pure deionized water used as the mobile phase constituent. Using column with more hydrophobic stationary phase (octadecyl-silica gel SP) – Zorbax SB-C18 (4.6 x 150 mm, particle size 5 μm) the analysis time was substantially reduced. Then a mobile phase with higher ACN content (40 volume percent) had to be applied for the separation. The baseline resolution of all seven analytes was achieved within 7 min – see Fig. 3.

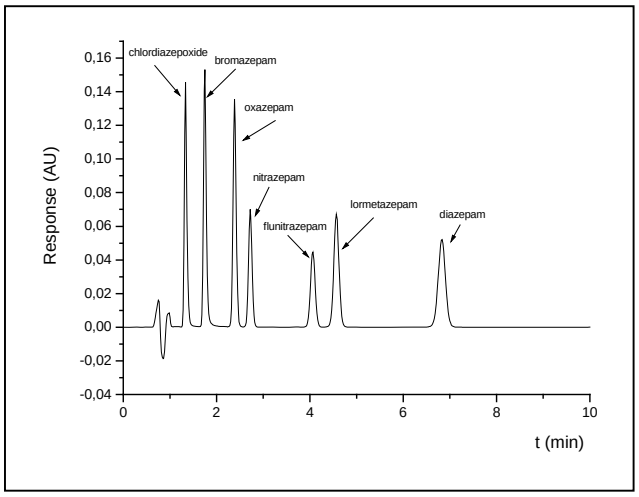

Fig. 3. Chromatogram of the separation of 1,4-benzodiazepines mixture. Column: Zorbax SB-C18; mobile phase: ACN/deionised water with acetic acid, pH 3.0, 40/60 (*v/v*); flow rate: 2ml/min; column temperature: 25 °C; UV detection: 240 nm.

The LOD and LOQ values were established for all the analytes under optimized separation conditions (see caption to Fig. 3) as shown in Table 1.

Table 1. LOD and LOQ values determined for the individual 1,4-benzodiazepines.

	LOD (ng/ml)	LOQ (ng/ml)
chlordiazepoxide	8.68	28.92
bromazepam	7.89	26.28
oxazepam	8.91	29.69
nitrazepam	17.05	56.83
flunitrazepam	26.70	89.00
lormetazepam	17.77	59.23
diazepam	22.76	75.86

3.3.2 Chiral separation systems (publication IV)

A significant number of natural substances are chiral – they can be present in enantiomeric or diastereomeric forms [10]. Chirality can be considered the basic characteristic of living organisms. Therefore, monitoring of stereoselective behavior/effect of chiral compounds, especially drugs, agrochemicals and also food components, is very important.

Acquaintance of chirality of natural and synthetic compounds is a significant factor in evaluation of food quality. Individual enantiomers or enantiomeric ratios of food components do not influence only flavour and aroma but affect also nutritional values. As a consequence of processing of foodstuffs chiral components can racemize. The distribution of enantiomers can change also during storage, e.g. owing to bacterial contamination, or due to adulteration with synthetic additives. Determination of enantiomeric ratios in food and beverages can

therefore provide valuable information on food quality control (QC). A basic review of chiral food components and their significance in food QC is given in publication IV.

3.3.2.1 Optimization and validation of HPLC method for enantioseparation of (±)-cloprostenol (publication V)

This work is focused on the development of a HPLC method for separation and quantification of (±)-cloprostenol enantiomers. In our preliminary experiments we examined the possibility to utilize CSPs based on macrocyclic antibiotics but they were not suitable for the enantioseparation. Suitable alternatives to macrocyclic antibiotics CSPs are polysaccharide CSPs. Chiralcel OD-RH column (cellulose tris(3,5-dimethylphenylcarbamate) CSP) was proved to be more convenient for separation of cloprostenol enantiomers than CSPs used before. The optimized separation conditions, as the compromise between resolution and analysis time, were found: Chiralcel OD-RH column; mobile phase, ACN/sodium dihydrogenphosphate (pH 3.0; 20 mM) (33/67, *v/v*); flow rate 0.7 ml/min; column temperature 20 °C; detection wavelengths 274 nm and 210 nm. The chromatogram is shown in Fig. 4.

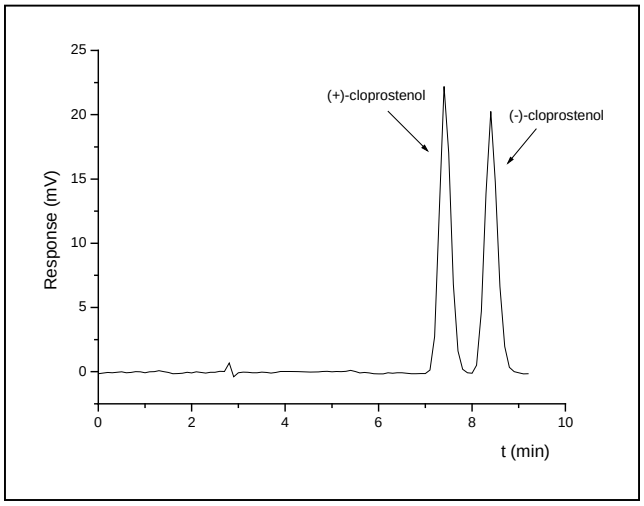

Fig. 4. Chromatogram of enantioseparation of (±)-cloprostenol on the Chiralcel OD-RH column. Mobile phase: ACN/sodium dihydrogenphosphate (pH 3.0; 20 mM) (33/67, *v/v*); flow rate: 0.7 ml/min; column temperature: 20 °C; UV detection: 274 nm.

Basic validation parameters (stability of sample solution, precision, linearity, LOD, LOQ, robustness) have been evaluated under optimized separation conditions. The method is aimed for enantiomeric purity control but it should be easy switched to a semipreparative mode.

4. Conclusions

The PhD Thesis combines the theoretical part focused on better understanding of separation processes and interaction mechanisms with the experimental applications for specific analytical purposes.

LFER model was used for comparison and characterization of interaction abilities of three teicoplanin-based chiral stationary phases in four mobile phases. Interactions resulting in the retention mechanism were evaluated and also the influence of mobile phase composition on the distribution and quantity of individual interaction types was taken into account. Obtained results can help with choice of suitable separation system for analysis of compounds of interest.

Occurrence and behavior of system peaks in RP HPLC composed of Discovery® RP Amide C16 column and aqueous buffers as mobile phases were described. It was found and also confirmed by CZE that alkaline metal cations do not interact with the stationary phase so they are suitable for determination of column void volume. On the other hand organic acids in non-dissociated form interact with stationary phase and their position in the chromatogram can be affected by their dissociation or buffer dilution. The collected data were used for development of a mathematical model that describes positions and amplitudes of system peaks.

RP HPLC methods for separation of selected estrogens and 1,4-benzodiazepines were developed. The best gradient conditions found for separation of estrogens were: Symmetry® C18, mobile phase ACN/deionised water 40/60 (*v/v*) with the linear gradient to 100 vol. % of acetonitrile applied from 8^{th} to 9^{th} minutes, and linear gradient of flow rate from 8^{th} to 9^{th} minutes from 2 ml/min to 3 ml/min that were applied simultaneously. The baseline separation of all five analytes was obtained within 11 min. The optimized isocratic conditions found for separation of 1,4-benzodiazepines were: Zorbax SB-C18 column, mobile phase ACN/deionised water with HAc, pH 3.0, 40/60 (*v/v*), flow rate 2 ml/min. The baseline resolution of all compounds was achieved and the analysis time did not exceed 8 min.

The method for enantiomeric purity control of the veterinary drug (±)-cloprostenol was developed and validated. The optimized chromatographic conditions were: Chiralcel OD-RH column, mobile phase ACN/sodium dihydrogenphosphate (pH 3.0; 20 mM) (33/67, *v/v)*, flow rate 0.7 ml/min. The baseline resolution within 9 min was achieved. The method is aimed for enantiomeric purity control but it should be easy switched to a semipreparative mode.