Bioactive macromolecules and ions: Study of equilibrium and kinetic processes influencing high performance analytical separations

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Final Report

1 Goal of the project

Analysis of biochemically active macromolecules and ionizable components has long been an analytical problem. In biological and environmental analysis, the most important and sometimes most difficult task is the separation of the complex mixture of molecules. The efficiency and selectivity of a chromatographic analysis depends on the equilibrium and kinetic processes take place in the column during elution. Development of comprehensive analytical solutions requires the understanding of these processes. Suitable theoretical models can support the development of efficient chromatographic separation methods and stationary phases. Accordingly, the aim of the project was the support of further development of bioanalytical liquid chromatography by investigating important fundamental aspects and developing data management algorithms.

2 Results

In the summary bellow, references in square brackets refer to the corresponding publications presented in the *List of Publications* attached to the *Final Report* as a separate document.

2.1 Separation of bioactive macromolecules

Cation exchange (CEX) chromatography of therapeutic monoclonal antibodies (mAbs) is generally performed with either salt gradient or using commercial pH gradient buffer. In our work we evaluated the applicability of combined pH and salt gradients for CEX separation of mAbs with buffers that offer high selectivity and acceptable peak shapes [3, 5, 8]. Among the new buffers that were tested, the (N-morpholino)ethanesulfonic acid (MES) / 1,3-diamino-2-propanol (DAP), and the citric acid / 2-(cyclohexylamino)ethanesulfonic acid (CHES) systems were particularly promising, especially when combining them with a moderate salt gradient of

NaCl. This two buffer systems provide equivalent or slightly better separation than the standard mobile phases for therapeutic mAbs. We also studied the pH profile of the effluent at the column outlet using a specific setup [9]. To make comprehensive observations of the phenomenon, four different mobile phase conditions and five cation exchange columns (weak and strong exchangers) were employed. The obtained pH responses were systematically compared to responses measured in the absence of the columns. Our results showed that both the column and mobile phase have significant effects on pH gradient chromatography and that their combination must be considered when developing a new method. Phase systems (column + mobile phase) providing linear pH responses are the most suitable for separating mAbs with different isoelectric points and, with them, it is possible to elute mAbs across wide retention time ranges and with high selectivity. We also investigated recently commercialized cation-exchanger stationary phases for their capabilities to separate therapeutic monoclonal antibodies [7]. It was demonstrated that the different combinations of stationary and mobile phases result in diverse retention, selectivity and efficiency. Hence, the whole phase system (combination of stationary and mobile phase) should be considered when developing a method. In addition, retention behavior is mAb dependent and should be individually optimized. It was observed that in cation exchange chromatographic separations of large proteins, the particle size of the columns impacted retention rather than efficiency, due to the non-porous particle structure and the ”bind and elute” retention mechanism of large solutes. The retention, efficiency and selectivity of the studied columns were quite different and strongly dependent on the elution mode (i.e. salt gradient, pH gradient or combined salt/pH gradient mode). Our results demonstrated that it is especially attractive to make use of short, narrow bore ion exchange columns that offer the possibility to perform 4-6 minutes long separations of both intact or partially digested antibodies.

2.2 Analysis of efficiencies of stationary phases with different structures

A mathematical treatment was developed for gradient mode of separation in order to understand the variation of apparent efficiency when serially coupling columns with the same chemistry, but with differences in their kinetic performance [2]. It was shown that the order of the columns was not indifferent, the chromatographic system is non-symmetrical regarding the column order. When the last column has high efficiency, the gradient band compression effect may outperform the competing band broadening caused by dispersive and diffusive processes (eddy dispersion, molecular diffusion, mass transfer). To validate the theory, experiments were carried out using various mixtures of compounds. The developed model allows the prediction of the apparent peak width for the combination of any column segments in any format. Based on these results, we also studied the applicability of particle size gradients in the chromatographic columns [11]. According to our calculations, in isocratic elution mode, the non-uniform column does not offer any advantage compared to the uniform column, regardless the type of

the particle size gradient. In gradient elution mode, however, extra band compression was found. For negative particle size gradients, the final physical bandwidth was found to be approximately 1–4% smaller than for uniform columns. This slight gain in efficiency in terms of bandwidth compression can be expanded to 5–8% by the optimization of the limiting particle sizes. Besides particle size gradients, we studied the separation efficiency of ion-exchange capacity gradient stationary phases combined with eluent concentration gradient [16]. It was found that for ions with same charges the gradient column offered only a marginal advantage. In the case of ions with different charges, however, the advantage of the gradient column was more significant. This was mainly due to the increased retention time difference of solutes. Ion-exchange capacity gradient columns may be a new way to separate ions more efficiently.

During our work, we used the general rate model (GRM) of chromatography for the analysis of efficiency of core-shell phases having two porous layers with different structures and/or surface chemistries [6]. The results demonstrated that bi-layer structures can offer higher separation power than that of the two layers alone if the inner layer has smaller surface coverage (retentivity), and the pore size and pore diffusion of the outer layer is either equal to or higher than that of the inner layer. Even in case of core-shell phases there is an increase in resolution by applying bi-layer structure, however, we can always find a mono-layer core-shell particle structure with a larger core size that provides better resolution. However, in case of the most-widely produced general purpose core-shell particles where the core is 70% of the particle diameter, 15-20% gain of resolution could be obtained by using well designed and optimized bi-layer core-shell phases.

2.3 Study of retention mechanism

A theoretical model was developed for the estimation of axial gradient of thermodynamic state properties and velocity of water and methanol eluents in insulated (2 cm foam insulation) chromatographic columns [1]. The isenthalpic pressure-temperature (P-V) plots of eluents were generated by a software written in-house applying the Python wrapper of CoolProp scientific library. From the isenthalpic P-V plots, the total changes of eluent temperatures ($\Delta\Delta$) were estimated at different eluent conditions. The calculated ΔT values were compared to experimental data. The density, viscosity and local velocity of the eluent were calculated from the isenthalpic data. The model needs further verification before publication.

We developed a complex retention model for the description of retention behavior of carboxylic acid anions on cryptand stationary phases [10]. The effect of binary mixed eluent (Li/Na, Li/K) on the retention behavior and peak shape of carboxylic acids were also discussed in view of the proposed theory. It was shown that the effects of binary aqueous mobile phases, held isocratically behave very similar to the step gradient mode. The ”internal gradient” separation system had advantages over traditional step gradient mode. The predicted and measured

retention data were in good agreement.

Based on theoretical considerations, we studied the chromatographic efficiencies under pressure-induced gradient conditions [15]. It was shown that components with the same retention time can have different migration patterns. The width of the initial band after injection is affected by the pressure gradient, providing significantly thinner initial bands for compounds with higher pressure sensitivity. In addition to classical band broadening phenomena, the influence of pressure gradients on band broadening is remarkable, the zones are significantly wider at the end of the column if the change of molar volume of solute during adsorption is large (e.g. peptides, proteins). The high release velocity of the bands somewhat counteracts the extra band broadening effect, however, it can not offset it perfectly. As a result, under UHPLC conditions, the extent of apparent efficiency loss can reach up to 50% compared to the intrinsic efficiency of the column.

We have developed an inverse method that gives results of similar accuracy to the frontal analysis without assuming the equation of the isotherm [17]. The overloaded peaks were calculated using a spline fitted to data points instead of the derivative of the isotherm. The distribution of the isotherm points were optimized for minimizing the difference between the measured and calculated overloaded peaks. The accuracy of the developed method was verified with synthetic benchmark peaks and by the determination of isotherm of buthyl-benzoate under real conditions. The results confirmed that the accuracy of the developed method is similar to that of Frontal Analysis. In addition, we developed an expectation-maximization algorithm to separate overlapping peaks numerically [14]. The method makes the peaks narrower keeping the areas and retention times. As a result, even highly overlapping peaks can be separated.

2.4 HPLC method development for small, bioactive molecules

During the project work, several analytical methods has been developed for the analysis of small, bioactive solutes. We developed an ultra-high performance hydrophilic interaction liquid chromatographic method for the separation of oligomers of Triton X-100 octylphenol-polyethoxylate non-ionic surfactant [4]. LC-MS was used to identify the Triton X-100 compounds. A quadratic retention model was applied for the estimation of retention of the examined non-ionic surfactant and the optimization of gradient elution conditions. The optimized method was suitable for the baseline separation of 28 Triton X-100 oligomers in five minutes.

The applicability of a multifactorial liquid chromatographic optimization software was studied for the analysis of apixaban pharmaceutical ingredient and its pollutants [12]. A mixture of route of synthesis of apixaban was analyzed on short narrow bore column (50 $\times$ 2.1 mm, packed with sub-2 μm particles) resulting in short analysis time. The aim of the study was to cover a relatively narrow range of method parameters (t G , T, pH) in order to find a robust working point (zone). The results of the virtual (modeled) robustness testing were sys-

tematically compared to experimental measurements and Design of Experiments (DoE) based predictions.

Cilostazol is a commonly used active pharmaceutical ingredient (API) to treat and reduce the symptoms of intermittent claudication in peripheral vascular disease. Recently, it was found to be a potential medicine in the effective treatment of COVID-19. We developed a method for the determination of sodium azide (as azide anion) in cilostazol API at 7.5 ppm limit level by using ion chromatography (IC) and liquid–liquid extraction (LLE) sample preparation [13]. The developed method was validated in accordance with the relevant guidelines.

N-bromosuccinimide (NBS) is used as a brominating agent in the synthesis of some active pharmaceutical ingredients. Its determination is difficult due to its high reactivity. During the project, we developed a high-performance ion chromatographic method for the determination of NBS through the analysis bromide produced as a result of NBS hydrolysis. Two different types of active pharmaceutical ingredients (API), i.e., prasugrel and favipiravir, were chosen to test the developed method and sample preparation. For both APIs, sample preparation was performed in a vial and consists of liquid–liquid extraction with an alkaline reagent. The method was validated at the limit value level.

3 Outcomes beyond direct scientific results

In addition to the scientific significance of the results, four doctoral dissertations directly related to the project theme were written during the project period. Of these, three PhD students have already successfully defended their theses, and the fourth submitted his thesis for defense in January 2023. In addition to these, 12 BSc and MSc theses were written during the duration of the project. As a result of the grant, a research group with young, enthusiastic, and talented researchers has been formed successfully.