Abstract

The development of a chromatographic method often involves a trial-and-error approach until the set criteria are met. The advantage of such a tactic is the development of an adequate method in a relatively short period of time. However, the lack of a systematic approach may lead us in a local optimum and unaware of the critical parameters that affect separation. This severely hinders our ability to troubleshoot when the performance of the method falls out of the accepted range. The educational guesses of chromatography experts can solve many problems encountered when separating on a column with a single retention mode. Unfortunately, such knowledge is not sufficient to decipher the optimal path to improve separation on a mixed mode stationary phase. The presence of multiple retention mechanisms in combination with very complex molecules such as proteins and other biomolecules renders such predictions extremely difficult. In this case, a good optimization path is the Quality by Design approach. Therefore, the aim of this study is to understand the principles of the governing factors. Our work began with screening the most suitable stationary phase chemistry for the separation of seven insulin variants commonly used in the treatment of diabetes mellitus. Before optimizing the composition of the mobile phase and gradients of acetonitrile content, buffer concentration, and pH value, we focused on the often neglected effects of temperature and pressure on separation efficiency. These effects were studied separately on appropriate single mode columns, as the selected mixed mode column included a reversed-phase and anion exchange mechanism. The effect of temperature on the separation of insulin is opposite to that of small molecules on both columns up to 55 °C. At higher temperature, the separation of insulin on the anion exchange column shows a similar trend as before. On the reversed phase and temperatures above 55 °C, insulin retains like a small molecule. The effect of pressure was observed only on the reversed-phase and the mixed mode column. In these cases, the retention of insulins increased significantly even when the column inlet pressure was increased by 100 bar. The retention of small molecules was only slightly affected. This was not observed for separations on an anion exchange column due to the non-denaturing mobile phase and thus the stability of the insulin molecule. This pressure effect on an anion exchange column was further studied with a probe molecules (oligonucleotides of different lengths), larger proteins (BSA and thyroglobulin) and a plasmid DNA molecule. A significant increase in retention time was observed for isocratic and gradient separations, which was dependent on the size and flexibility of the molecules. To investigate the adsorption mechanism, these separations were described using stoichiometric displacement and linear gradient elution models. A pressure and ionic strength dependence of distribution constant was developed and derived to obtain partial molar volume changes. Analysis of the calculated parameters indicated a compression of the macromolecules towards the stationary phase upon adsorption. This enabled the molecules to have more interactions with the stationary phase.

Finally, we conducted a systematic study of the influence of mobile phase composition on the separation efficiency of seven insulin variants and two excipients on a mixed mode column. In addition, an SPE purification procedure was developed to remove interferences present in the formulations. Two separation methods were developed, each suitable for the separation of nine molecules on HPLC systems with either binary or quaternary solvent delivery system. The methods enable the quantification of human insulin and the six most commonly used therapeutic analogues in formulations or pharmaceutical raw materials.