Journal of Chromatography A, 1381 (2015) 64–73

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Combined effects of potassium chloride and ethanol as mobile phase modulators on hydrophobic interaction and reversed-phase chromatography of three insulin variants

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ARTICLE INFO

Article history:
Received 17 September 2014
Received in revised form
22 December 2014
Accepted 26 December 2014
Available online 7 January 2015

Keywords:
Hydrophobic interaction chromatography
Reversed-phase chromatography
Insulin
Combined modulator effect
Salting-in effect
Salting-out effect

ABSTRACT

The two main chromatographic modes based on hydrophobicity, hydrophobic interaction chromatography (HIC) and reversed-phase chromatography (RPC), are widely used for both analytical and preparative chromatography of proteins in the pharmaceutical industry. Despite the extensive application of these separation methods, and the vast amount of studies performed on HIC and RPC over the decades, the underlying phenomena remain elusive. As part of a systematic study of the influence of mobile phase modulators in hydrophobicity-based chromatography, we have investigated the effects of both KCl and ethanol on the retention of three insulin variants on two HIC adsorbents and two RPC adsorbents. The focus was on the linear adsorption range, separating the modulator effects from the capacity effects, but some complementary experiments at higher load were included to further investigate observed phenomena. The results show that the modulators have the same effect on the two RPC adsorbents in the linear range, indicating that the modulator concentration only affects the activity of the solute in the mobile phase, and not that of the solute–ligand complex, or that of the ligand. Unfortunately, the HIC adsorbents did not show the same behavior. However, the insulin variants displayed a strong tendency toward self-association on both HIC adsorbents; on one in particular. Since this causes peak fronting, the retention is affected, and this could probably explain the lack of congruity. This conclusion was supported by the results from the non-linear range experiments which were indicative of double-layer adsorption on the HIC adsorbents, while the RPC adsorbents gave the anticipated increased tailing at higher load.

1. Introduction

Since the first biopharmaceutical, a recombinant human insulin variant, was approved by the U.S. Food and Drug Administration in 1982, the market for these products has grown rapidly. Today, one out of every four new pharmaceuticals is based on recombinant therapeutic proteins, and hundreds of new candidates are being tested in clinical trials [1]. As this trend is unlikely to change, the production of biopharmaceuticals is expected to continue to increase. To enable this expansion, efficient purification methods for therapeutic proteins are needed.

Preparative chromatography has proved to be an excellent purification method for therapeutic proteins, and is therefore widely used in the pharmaceutical industry [2]. One commonly applied chromatographic mode for protein purification is hydrophobic interaction chromatography (HIC). In HIC, the hydrophobicity of the adsorbent is low, and the retention can be modulated by adjusting the salt concentration, making use of the salting-out effect. Thus, no organic modulator is required, and the risk of protein denaturing is low. In contrast, reversed-phase chromatography (RPC) adsorbents are highly hydrophobic, and elution requires the addition of an organic solvent, such as ethanol, to the mobile phase. RPC is thus more suitable for analytical purposes, but it can also be a powerful tool for the separation of proteins that can tolerate harsher conditions, e.g. insulin and peptides [3,4].

Both HIC and RPC are based on hydrophobic interactions between the ligands of the adsorbents and the proteins, but the

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http://dx.doi.org/10.1016/j.chroma.2014.12.081
0021-9673/

two modes differ in their degree of stationary phase hydrophobicity. Although the n-alkyl chains commonly used as ligands on HIC adsorbents (C_4–C_8) are shorter than those used on RPC adsorbents (C_4–C_{18}), the difference in hydrophobicity is not caused by the type of ligands, but by the ligand density [5,6]. Examples of reported values are 5–50 mmol/l gel for various Sepharose™ adsorbents (HIC) [5,7], and 200–700 mmol/l gel for Kromasil RPC adsorbents manufactured by AkzoNobel, Separation Products (Bohus, Sweden) [8,9]. Due to this difference in ligand density, solute–ligand interactions in HIC only involve one or a few ligands, while in RPC, the ligands form a more homogeneous hydrophobic phase, and the adsorption resembles, to some degree, a partition process.

As a consequence of the strong hydrophobicity of the stationary phases in RPC, salting-out has a very limited effect on the adsorption, and organic solvents such as ethanol, 2-propanol or acetonitrile are required to achieve elution. In HIC, elution can be promoted by organic solvents, but the effect of these is often too strong, and the retention is generally modulated by varying the ionic strength of the mobile phase. Nonetheless, the hydrophobic interactions between solute and ligand in both HIC and RPC can be manipulated by salts as well as organic solvents. However, few studies have been carried out on the effect of alcohols on HIC [10–13], and of inorganic salts on RPC [14,15], and those that have been performed were either focused on a certain separation problem [10,11,15], or on phenomena other than retention, such as protein unfolding during chromatography [14], or peak shape and carryover [12].

The nature of the hydrophobic phenomena in HIC and RPC, and the effects of mobile phase modulators are still being debated, despite many proposed explanations [13,16–23]. Further investigations are therefore required to elucidate the mechanisms, and the similarities between the two modes suggest that a possible route toward a better understanding of HIC and RPC is to treat them as two sides of the same coin. Only one such combined study of HIC and RPC was found in the literature [6], and it was mainly concerned with the differences in selectivity and the influence of ligand density on resolution and retention. Thus, to the best of the authors' knowledge, the combined effect of salts and organic solvents on HIC and RPC has not yet been investigated.

The aim of this study was thus to investigate and describe the common factors and dividers for the two modes of chromatography, HIC and RPC. Three insulin variants were separated using four different adsorbents; two HIC adsorbents and two RPC adsorbents. We studied the linear range, in order to elucidate the influence of mobile phase composition on the adsorption equilibrium, without causing any adsorption capacity effects. A number of additional experiments were performed at higher column loads, in order to further investigate observed non-idealities.

2. Hydrophobic effects in HIC and RPC

Since the discovery that the adsorption of biomolecules onto nonpolar adsorbents could be enhanced by high salt concentrations [24], and impaired by high concentrations of nonpolar solvents [25,26], many attempts have been made to explain the hydrophobic phenomena governing HIC and RPC [27]. Earlier suggestions regarding the driving force for adsorption in HIC were based on the gain in entropy when water molecules, arranged around the hydrophobic solute and ligands, are displaced into the less ordered bulk upon adsorption [28,29]. The first comprehensive theory of the hydrophobic interactions in HIC and RPC was the application of Sinanoğlu's solvophobic theory to chromatography by Horváth, Melander and coworkers in the late 1970s [16,17,30]. According to this theory, the change in free energy associated with the transfer of a solute into solution, during dissolution or desorption, is dominated by cavity formation and electrostatic forces. In RPC, the electrostatic forces are negligible, and the retention depends on the surface tension of the mobile phase [16,30].

Since both the surface tension and the electrostatic forces are directly related to the salt concentration in HIC, a relationship between the retention and the salt molality of the solvent was suggested. At high salt concentrations, the relationship simplifies to an exponentially increasing dependence of the retention time on the salt molality [17]. A similar phenomenon is the decrease in protein solubility caused by most inorganic salts at high concentrations, generally referred to as the salting-out effect. The opposite phenomenon, salting-in, occurs at relatively low salt concentrations, and results in a concomitant increase in protein solubility with the salt concentration. In combination, the salting-out and salting-in effects give a maximum in protein solubility at a certain salt concentration. In a study on the effect of salt on hydrophobic interactions [17], Melander and Horváth compared precipitation and protein chromatography, as a decrease in protein solubility generally results in an increase in chromatographic retention of the protein. This adaptation of the solvophobic theory is probably the most well-known and widely used theory to describe the hydrophobic effects in chromatography [31–34]. However, it has been shown that the effect of salts on protein retention cannot be explained by their effect on surface tension alone [20,22,27,35].

Another theory that has become popular for the modeling of HIC [36–38] is the preferential interaction theory developed by Arakawa and Timasheff in the 1980s [18,39]. As in the solvophobic theory, the retention is dependent on the salt molality, but the effect of a certain salt depends on whether the interaction with the protein is preferential or not. If the interaction is not preferential, the protein is hydrated and does not interact with the salt ions. Adsorption is thermodynamically favorable since it reduces the hydrophobic surface area of the protein that is in contact with the surrounding water molecules. If the interaction is preferential, ions cluster around the protein, thus increasing the solubility and decreasing the retention. Arakawa [20] and Perkins et al. [19] successfully adapted the preferential interaction theory to describe retention in HIC.

An HIC model that is valid from salt-free conditions up to high ionic strengths was suggested by Staby and Mollerup in the 1990s [21]. Here, the retention is correlated to the protein activity coefficients in the mobile and stationary phases. The former is modeled according to the Debye–Hückel theory, while the latter is described by an empirical expression. The final model is a function of the ionic strength and pH of the mobile phase. In a later study [13], Mollerup et al. presented a modified version of the model, assuming that changes in mobile phase composition only affect the activity coefficient of the solute when it is in solution and in the adsorbed state. The modulator effects are described by Kirkwood's theory of salting-in and salting-out of macromolecules. Experimental data support this, as a change in stationary phase only causes a parallel shift of the curves of the logarithm of the retention factor, when plotted against the ionic strength of the mobile phase. This theory treats precipitation and the chromatographic separation of proteins as two manifestations of the same basic thermodynamic phenomenon, and the same model is used to describe both effects.

In a study on the effect of various salts on the binding of proteins to two different HIC adsorbents, Oscarsson [22] found that some of the results could be correlated to the surface tension increment, while others were totally uncorrelated. He suggested that the conformation of a protein is affected by both the type of salt present in solution and by the stationary phase, and that changes in conformation could explain the deviations from the solvophobic theory.

In 2000, Lin et al. [23] proposed that the adsorption mechanism in HIC could be divided into five steps: (i) dehydration or