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Recombinant human insulin V
Optimization of the reversed-phase high-performance liquid chromatographic separation ☆

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Abstract

The applicability of reversed-phase high-performance liquid chromatography (HPLC) to the analysis of the products of recombinant insulin was studied. The influence of several mobile phases in reversed-phase and ion-pair HPLC on selectivity, resolution and sensitivity was investigated. Optimum conditions for the separation of insulin-related proteins on commercial and laboratory-made supports were established by means of three-dimensional optimizations of selectivity and resolution as a function of pH and ionic strength (μ). A mechanism for the separation of proteins with a mobile phase containing a high salt concentration and a pH near the isoelectric point of proteins is proposed. The questions of scaling up are considered. The proposed techniques allow the analysis of the main impurities and ensures a high quality of active insulin production.

1. Introduction

A number of papers concerning the HPLC of insulin have been published [2–4]. The main reasons for the increased interest in this protein are twofold. First, insulin is an important protein and its pharmaceutical production is complex, hence the search for new and more effective methods for the isolation and purification of recombinant human insulin and its close analogues of animal origin continues [5,6]. Second, if insulin is injected into human blood, minor impurities may generate a powerful immune response or toxicosis. In order to avoid this, the quality-control system must be sophisticated. This is now possible, in most instances, by using HPLC.

The separation of insulin-related proteins can be achieved efficiently by reversed-phase (RP) HPLC. In spite of small differences in the structure and sequence of animal and human insulin, RP-HPLC ensures the separation of these proteins and their derivatives and precursors [7–9]. The flexibility of this method is demonstrated by the quality of the separation of insulin, proinsulin and desamidoinsulin peaks [10]. One of the ways of obtaining insulin biosynthetically is by biotransformation of pig into human insulin, by the exchange of alanine in the chain B-30 to threonine [11]. As this takes place,

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by-products similar to these of recombinant human insulin are generated [3]. In spite of the similarities between human recombinant and animal insulin-related proteins, it is impossible to transpose the results of RP-HPLC separations from one matrix to the other. HPLC is an effective method not only for analytical separations, but also for the preparative isolation of insulin with high purity [4,12].

The aim of this work was to study the application of specially designed supports for the RP-HPLC of proteins, based on synthetic silica with a narrow distribution of pore size (Armsorb-Si-300 [1]), to compare data obtained on the Armsorb column with those of its commercial analogues; and to investigate the influence of ionic strength (μ) and pH on selectivity, resolution and column capacity during the separation of the insulin-related proteins.

We tested the column efficiency with a series of mobile phases in reversed-phase and ion-pair (IP) HPLC. The dependence of the selectivity (α), resolution (R_s) [13] and sensitivity on the mobile phase composition was investigated. The recommended system is suitable for the analysis of impurities according to the requirements of current pharmacopoeias [14,15].

2. Experimental

The columns used for HPLC were Vydac C_4, 15–20 μm (15 × 0.8 cm I.D.) in the Z-module (Millipore, Milford, MA, USA), Nucleosil 300-7 Protein RP (15 × 0.4 cm I.D.), Nucleosil 5 C_8 (15 × 0.4 cm I.D.), (Machery–Nagel, Düren, Germany), TSK Gel-120T (30 × 0.4 cm I.D.), (Tosoh, Japan), Armsorb-Si-300-C_8P (15 × 0.4 cm I.D.), Armsorb-Si-300-C_8P (25 × 2.2 cm I.D.) Armsorb-Si-500-C_4-Protein (15 × 0.4 cm I.D.), Armsorb-Si-300-C_8-Protein (25 × 0.4 cm I.D.) and Armsorb-Si-500-C_{16}-Protein (25 × 0.4 cm I.D.), (ErONEM, Armchrom, Yerevan, Armenia). Chromatography was carried out with a Model 510 pump, a U6K injector, a Model 490E spectrophotometer and a Model 740 integrator (all from Waters).

Insulin, proinsulin and desamidoinsulin were obtained in the Laboratory and Biotechnology with Experimental Pilot Division, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (Moscow, Russian Federation) (see below) for identification and a human insulin standard specimen from Chemie- und Handelsgesellschaft (Heidelberg, Germany). Acetonitrile, methanol, sodium hydroxide, sodium sulphate, sodium phosphate, phosphoric acid and acetic acid were of analytical-reagent grade. Water was purified with a Milli-Q system (Millipore).

The following mobile phases were used: CH_3CN–0.1% TFA (pH 2), CH_3CN–0.1% H_3PO_4 (pH 2), CH_3CN–0.1% H_2SO_4, (pH 2), CH_3OH–0.2 *M* $AcONH_4$ ($3 < pH < 7$), CH_3CN–0.1–2 *M* $AcONH_4$ ($3 < pH < 7.5$), C_2H_5OH–0.2 *M* $AcONH_4$ ($3 < pH < 7.5$), $(CH_3)_2CHOH$–0.1% TFA (pH 2), CH_3CN–0.1–1% $(C_2H_5)_3NOAc$ ($3 < pH < 7$) and $(CH_3)_2CHOH$–0.1–1% $(C_2H_5)_3NOAc$ ($3 < pH < 7$). Before chromatography, the mobile phases were filtered through nitrocellulose and GVWP filters (pore diameter 0.45 μm) (Millipore) and degassed for 20 min. For three-dimensional optimization Surfer and Grapher software for an IBM PC AT (Golden Software) were used.

3. Results and discussion

3.1. Analysis

The separation of insulin, its close analogues and proinsulin in the RP mode is based on the differences in the hydrophobic properties of these proteins on RP columns. These proteins were eluted on well known commercial supports such as C_4, C_8, C_{18} and on the specially designed supports Armsorb C_4, C_8, C_{16}.

We maximized the selectivity in the separation of insulin and its impurities and then optimized the resolution. The column efficiency was also optimized. For experimental verification of the general column efficiency, model proteins and their mixtures (insulin, proinsulin, lysozyme, albumin, ovalbumin, cytochrome *c*, myoglobin and others) were eluted under different con-

ditions in the gradient mode and the best conditions for protein separation were selected from the results obtained. Then, isocratic separation of insulin and proinsulin was performed on each column in the range of optimum concentrations of organic modifiers so that the range of capacity factors (k') was 1–15.

In RP-HPLC, solvation of the support by an active compound of the mobile phase occurs, followed by displacement of solvating molecules by the sorbate. The logarithm of retention time varies linearly with the logarithm of the concentration of organic modifier and salts [13,16]. In order to explain the mechanism of protein separation under these conditions, the following equation may be used [13,17]:

$$\log k' = a - b \log C \quad (1)$$

where a = support hydrophobicity parameter, C = active compound (organic modifier) concentration and b = number of molecules of organic solvent removed from the mobile phase due to sorption of protein molecules. According to Eq. 1, log k' depends linearly on log C. The lines that connect the points of log k' values for different proteins eluted on the same column tended to cross (for a given range of k').

The dependences of log k' on C and log k' on log C are shown in Figs. 1 and 2. The plot of log k' vs. C is more suitable for demonstrating column hydrophobic properties. The concentration of organic modifier in the mobile phase is plotted on the abscissa. However, the log k' vs. log C plots, are close to linear and therefore more suitable for data handling.

For the separation of insulin-related proteins, the different eluent systems given under Experimental were used. For each column, conditions were chosen so as to obtain the maximum selectivity for insulin and proinsulin peaks. The dependence of log k' of insulin and proinsulin on the organic modifier concentration and its logarithm is shown in Figs. 1 and 2. The separation of insulin and proinsulin was carried out in the isocratic mode. First, commercial columns (Fig. 1) and Armsorb (Fig. 2) were tested. The difference between log k' of insulin and proinsulin, eluted from the same column with the

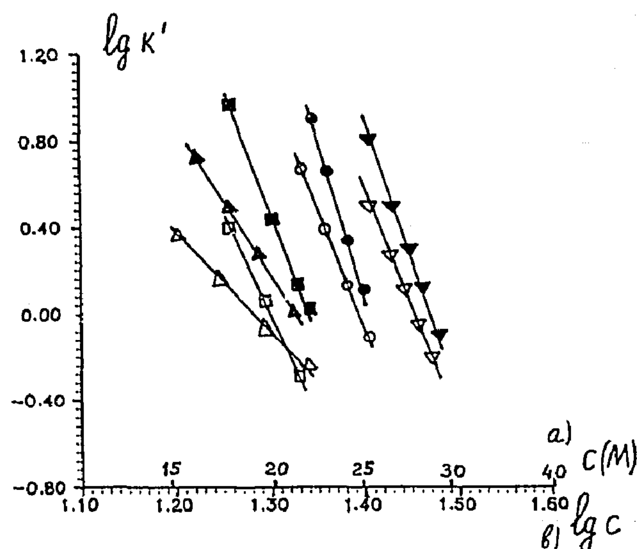


Fig. 1. Relationship between logarithm of capacity factor and (a) concentration and (b) logarithm of concentration or organic modifier in the mobile phase in the separation of insulin and proinsulin on the following columns: △,▲ = Vydac C_4, 15–20 μm, in Z-module, flow-rate 2.0 ml/min, □,■ = Nucleosil 300-7 Protein RP, flow-rate 0.8 ml/min, ○,● = Nucleosil 5 C_8, flow-rate 0.8 ml/min, and ▽,▼ = TSK Gel-120T, flow-rate 0.8 ml/min. Mobile phase: A = 10% CH_3CN–0.1% TFA, B = 100% CH_3CN–0.1% TFA. Closed symbols, insulin; open symbols, proinsulin.

same mobile phase, is a function of the selectivity of the separation of these two proteins. The greater the distance between the peaks at the same value of k', the higher is the selectivity of a given separation of proteins. The projection on the abscissa of the imaginary points where lines corresponding to the same protein intersect and the angle of these lines are components of the parameters of the column hydrophobicity a (Eq. 1) [18].

Thus, each mobile phase system and column can be characterized and the best combination can be found. In particular, in Fig. 1 we can see the dependence of the support hydrophobicity on the alkyl chain length (C_4–C_{18}). This dependence may not be constant as the mobile phase components are changed (such as the organic modifier, Fig. 2). For example, Armsorb-Si-300-C_8-P (silica with pore size 300 Å, modified by γ-glycidyl with phase C_8) exhibits a stronger hydrophobicity in the mobile phase system

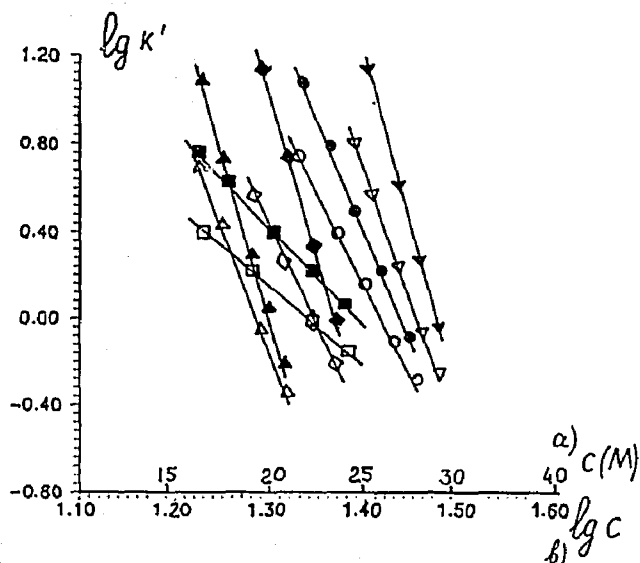


Fig. 2. Relationship between logarithm of capacity factor and (a) concentration and (b) logarithm of concentration of organic modifier in the mobile phase in the separation of insulin and proinsulin on the following columns: △,▲ = Armsorb-Si-300-C_8-P, □,■ = Armsorb-Si-500-C_4-Protein, ○,● = Armsorb-Si-300-C_8 protein and ▽,▼ = Armsorb-Si-500-C_{16}-Protein. Mobile phase: A = 10% CH_3CN–0.1% TFA, B = 100% CH_3CN–0.1% TFA. ◇,◆ = Armsorb-Si-300-C_8P. Mobile phase: A = 10% CH_3CN–1 *M* $AcONH_4$, B = 50% CH_3CN–1 *M* $AcONH_4$ (pH 5.5). Flow-rate, 0.8 ml/min. Closed symbols, insulin; open symbols, proinsulin.

CH_3CN–1 *M* $AcONH_4$ (pH 5.5) than in CH_3CN–0.1% TFA. This is due to the pH value being close to the isoelectric point and the high ionic strength. Stronger hydrophobic interactions occur under these conditions and proteins elute from the column at higher concentrations of the organic modifier. In Fig. 2 one can see that Armsorb-Si-500-C_4-Protein (with decreased hydrophobicity) and Armsorb-Si-300-C_8-P (with a long lifetime) columns have high selectivity. The highly efficient separation of insulin, proinsulin and desamidoinsulin is especially important in the final purification stage for detecting small amounts of impurities.

An efficient separation of insulin-related proteins was obtained on the Armsorb-Si-300-C_8-P column with a CH_3CN–1 *M* $AcONH_4$ (pH 5.5) mobile phase. A three-dimensional optimization of the selectivity (Fig. 3) and resolution (Fig. 4) of insulin and proinsulin peaks was carried out on this support at different values of the ionic

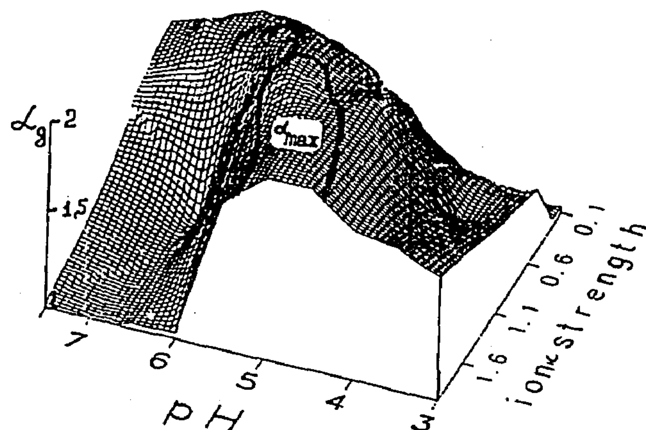


Fig. 3. Dependence of selectivity of insulin and proinsulin separation on pH and ionic strength as three-dimensional surface. The zone of maximum value of α (α_{max}) is indicated. The Column: Armsorb-Si-300-C_8P. Mobile phase: A = 10% CH_3CN–0.1–2 *M* $AcONH_4$; B = 50% CH_3CN–0.1–2 *M* $AcONH_4$ (pH 3–7.5); initial concentration of buffer B, 15%; gradient, 2% B/min. Flow-rate: 0.8 ml/min.

strength and pH in the same organic modifier gradient. The optimization was performed by the net method [19]. The dependences of α_g and R_{Sg} as three-dimensional surfaces and three-dimensional topographic isolines were obtained. In the gradient mode the numerical values of α and R_S are difficult to calculate from the chromatogram [20]. Because of this, in Figs. 3 and 4, the values of α_g and R_{Sg} are taken from the values obtained in the isocratic mode at the same pH and ionic strength of the mobile phase at $k' = 8$. In this

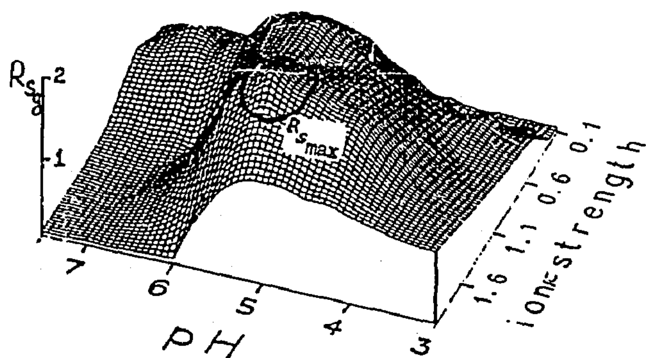


Fig. 4. Dependence of resolution of insulin and proinsulin separation on pH and ionic strength as three-dimensional surface. The zone of maximum value of R_S ($R_{S,max}$) is indicated. For other conditions, see Fig. 3.

case, the shape of three-dimensional surfaces remains unchanged, but the values of the selectivity and resolution correlate with real isocratic conditions. As shown in Fig. 3, maximum selectivity is obtained at pH 4.7–5.5 and a concentration of 0.5–2 M $AcONH_4$. As can be seen in Fig. 4, maximum resolution was obtained at pH 4.8–5.5 and 1–1.5 M $AcONH_4$.

In the concentration range 1.5–2 M $AcONH_4$ the peaks became asymmetric and the resolution decreased. At low values of μ (0.1–0.25) and at pH 3–3.5, there is virtually no separation between the insulin and proinsulin peaks and α_g is close to 1 and R_{Sg} to 0. At high values of μ (1–2) and at pH 6–7, acetonitrile does not mix with the highly concentrated solution of $AcONH_4$, and a separation with this mobile phase is also impossible ($\alpha_g = 1$ and $R_{Sg} = 0$).

The separation of insulin, desamidoinsulin and proinsulin was performed after these optimizations on the Armsorb-Si-300-C_8-P analytical column (Fig. 5b). In Fig. 5b one can see that a higher resolution was obtained than with a traditional CH_3CN–TFA system. The increase in selectivity, and consequently resolution, between insulin-related protein peaks with this mobile phase can be explained by the complete interaction of proteins with the mobile and stationary phases. It is known from the classical mechanisms of interactions in RP-HPLC that, at a high salt concentration in a mobile phase, the retention time of proteins on an RP column may be increased (salting-out effect). Hydrophobic interaction chromatography (HIC) is based on this principle. However, in this study, a high concentration of $AcONH_4$ did not influence the chromatographic process. A high ionic strength is maintained, the acetic acid is a slightly hydrophobic reagent, which increases the solubilization of hydrophobic proteins, and exhibits polar interactions towards unmodified silica. Thus the desorption mechanism of proteins from the support surfaces has more hydrophobic character than in known systems such as CH_3CN–TFA, phosphate, sulphate and other buffers.

Ammonium ions also have a dual character. They can act as cations and as a weak ion-pair agent, thus increasing the retention time of

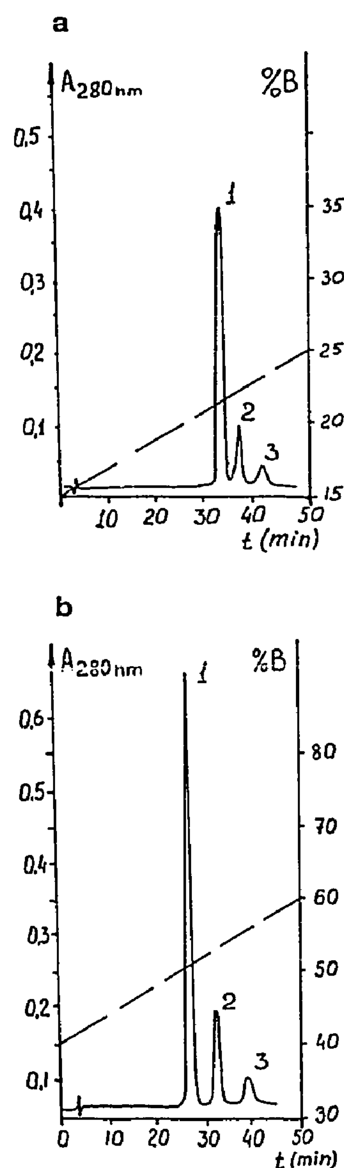


Fig. 5. Separation of insulin (1), desamido-(A_{21})-insulin (2) and proinsulin (3) on the Armsorb-Si-300-C_8P column. Mobile phase: (a) A = 10% CH_3CN–0.1% TFA, B = 100% CH_3CN–0.1% TFA; (b) A = 10% CH_3CN–1 M $AcONH_4$, B = 50% CH_3CN–1 M $AcONH_4$ (pH 5.5). Flow-rate: 0.8 ml/min.

proteins on the column and maintaining the increase in selectivity by an ion-pair mechanism.

Lastly, at pH values close to the isoelectric point, proteins generally exhibit non-specific adsorption or precipitate on the column. In this

instance these hydrophobic proteins dissolve in the mobile phase because of the high concentration of $AcONH_4$ and CH_3CN.

Taking into account the interaction mechanism between proteins and the mobile and stationary phases, and the exchange of mobile phase parameters, we obtain a method for multi-factor control of the protein separation selectivity.

3.2. Capacity of supports

For the efficient scale-up of the separation of insulin and its impurities, the three-dimensional optimization of capacity *versus* pH and ionic strength (as in the previous analytical experiment) for the Armsorb-Si-300-C_8P column (15 × 0.4 cm I.D.) was performed. Amounts of 0.02, 0.5 and 2 mg of insulin (95% purity) were injected sequentially on to the column. The column capacity changed in the same range of mobile phase pH and μ as in the previous experiments and was defined by measuring peak broadening. The area with the smallest peak broadening is shown in Fig. 6. This optimum condition was checked on a semi-preparative Armsorb-Si-300-C_8P column (25 × 2.2 cm I.D.). Fig. 7a shows the separation of insulin and desamidoinsulin. The analysis of the main fraction on the analytical column showed that the purity was more than 99% (Fig. 7b). The output of the separation increased up to 90% compared with the separation in the previous system (70%). From Fig. 6 it can be seen that the

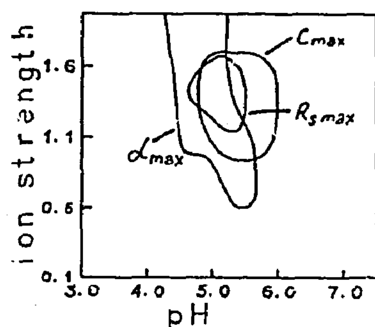
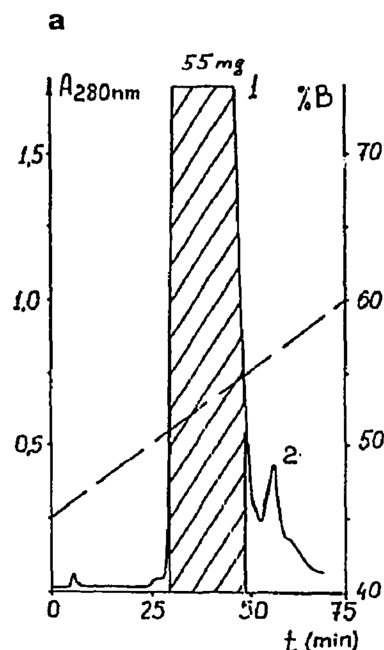


Fig. 6. Combination of three maxima: selectivity (α), resolution (R_S) and capacity (C) for the separation of insulin and proinsulin using $AcONH_4$ ($3 < pH < 7.5$ and $0.1 < \mu < 2$). Column: Armsorb-Si-300-C_8P. Flow-rate: 0.8 ml/min.

a



b

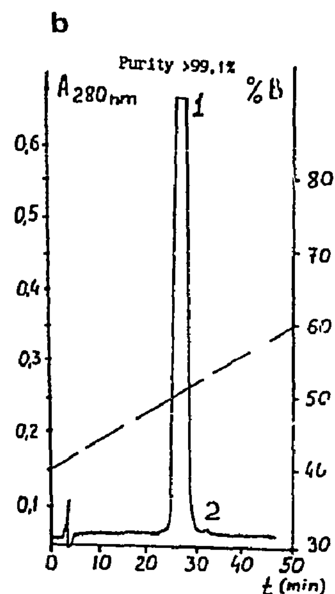


Fig. 7. (a) Semi-preparative separation of insulin (1), desamido-(A_{21})-insulin (2) and proinsulin (3) on the Armsorb-Si-300-C_8P column (25 × 2.2 cm I.D.). Mobile phase: A = 10% CH_3CN–1 *M* $AcONH_4$, B = 50% CH_3CN–1 *M* $AcONH_4$ (pH 5.5). Flow-rate: 2 ml/min. Main fraction is hatched. (b) Analysis of the main fraction under the same conditions as in Fig. 5.

combination of three maxima, selectivity (α), resolution (R_S) and capacity (C), permit not only optimum analysis, including cases of overloading, but also scale-up of the separation of the main products and impurities in production technology.

4. Conclusions

We compared well known commercial and newly designed (Armsorb) RP columns for the separation of a model mixture of insulin and its precursors and derivatives, which usually accompany the recombinant human and semi-synthetic insulin production. The optimization of the selectivity, resolution and capacity on an analytical scale by net techniques and computer graphics was carried out. The analytical and semi-preparative separation of insulin, desamidoinsulin and proinsulin with a mobile phase with a high ionic strength and a pH close to the isoelectric point of these proteins is described. A mechanism of protein interaction with the mobile and stationary phases is proposed.

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