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Short communication

Methods for protecting silica sorbents used in high-performance liquid chromatography from strongly adsorbed impurities during purification of human recombinant insulin

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Abstract

One of the main stages of human recombinant insulin (HRI) production is the hormone purification by means of reversed phase high-performance liquid chromatography (RP HPLC). The optimization of this stage determines the increase of the total manufacturing yield. Therefore, the cost of the sorbent used in HPLC influences the cost of the manufacturing product, i.e. HRI substance. However, resolution between HRI and its admixtures decreases with time. The reason for this is the sorbent contamination with strongly adsorbed impurities (SAI) which are accumulated during elution. In the following research several methods for sorbent protection are studied. The opinion that SAI are mainly high-molecular weight compounds was examined using gel filtration. Different sorbent types were tested for the use in guard column. The results obtained were applied and improved at preparative level.

Keywords: Human recombinant insulin; Strongly adsorbed impurities; Reversed phase high-performance liquid chromatography; A21-desamidoinsulin; High-molecular weight compounds; Gel filtration; Guard column

1. Introduction

Human insulin is the polypeptide molecule that consists of 51 amino acid residues and has the molecular weight of about 5700. This hormone is the main remedy for the treatment of *diabetes mellitus* [1,2].

Human recombinant insulin (HRI) is the first hormone that was produced in a large scale. Its manufacturing takes about 25 000 kg per year world wide nowadays [3]. At present, several methods of HRI large-scale production by fermentation of *Escherichia coli* [4,5] or *Saccharomyces cerevisiae* [6] are employed. It is clear that the optimal of the HRI production influences the manufacturing profitability, that is why manufacturing optimization becomes very important for process economics.

It is well known that one of the main stages of the HRI production is the purification of the crude insulin isolated from its recombinant precursor by means of hydrolysis, with combined action of trypsin and carboxypeptidase. The purification step is usually performed by means of reversed phase high-performance liquid chromatography (RP HPLC) [7]. It was shown that RP HPLC is the very effective and selective method that gives good purification results with high yields [7,8]. RP HPLC makes it possible to separate insulin from its admixtures such as A21-desamidoinsulin (A21-DHI), insulin-like peptides, immunoreactive polypeptides and host cell-derived proteins [8–11].

Although reversed phase silica sorbents such as Kromasil are produced especially for chromatographic peptide purification, the quality of separation depends on the sorbent state, namely its total operating period and its contamination with strongly adsorbed impurities (SAI) which are accumulated during elution [12]. Although regeneration by means of acidic or basic mobile phase is preferred, in most cases, it cannot solve the contamination problem.

There is an opinion that SAI are mainly high-molecular weight compounds and gel filtration can be the good choice to separate them from insulin-containing solutions. The second

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way of the sorbent protection is using guard column in line with primary column.

This study is aimed to investigate the process of accumulating SAI, to find ways for the escape of the sorbent from SAI or for the sorbent guard, to study the sorbent regeneration and then to scale-up the received results for application in manufacturing technology.

2. Experimental

All chemicals were of analytical grade. The reagents used were acetonitrile, sodium sulphate, sodium chloride, orthophosphoric acid, citric acid, acetic acid, triethanolamine, L-arginine (L-Arg), 2-propanol, ethanol, tris and hydrochloric acid. All solutions were filtered through 0.22 μm filter and degassed by helium for 3 min at ambient temperature.

Human recombinant insulin used was obtained from its recombinant precursor (it was produced as fusion protein in *E. coli*-forming inclusion bodies) by means of enzymatic cleavage with trypsin and carboxypeptidase B (Experimental Biotechnological Pilot Plant, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of Russian Academy of Sciences, Russia). After hydrolysis of the recombinant preproinsulin, crude insulin was precipitated by adding sodium chloride and adjusting pH to 3.0. The supernatant was decanted and the precipitate was separated by centrifugation at 4500 × *g* for 10 min at 10 °C using centrifuge Heraeus Sepatech, Germany. The pellet was dissolved in 10 mM citric acid and pH was adjusted to 2.25. The crude insulin solution contained 87–89% of HRI, 7–9% of A21-DHI and insulin-like peptides (see Fig. 1). All crude insulin solutions were filtered through 0.45 μm filter.

2.1. HPLC analytics

HPLC analyses were performed on the high-performance liquid chromatograph Agilent 1100 (Agilent Technologies, USA) equipped with two syringe pumps, a thermostated column compartment, an autosampler and UV detector (detection was performed at 214 nm). The column used was Phenomenex Luna C18-2, 5 μm (4.6 × 250.0 mm), USA. Flow rate was 1.5 ml/min. Column temperature was maintained as 40 °C. "Multichrom for Windows" software (Ampersand, Russia) was used for data processing and calculation. The elution system used was as follows:

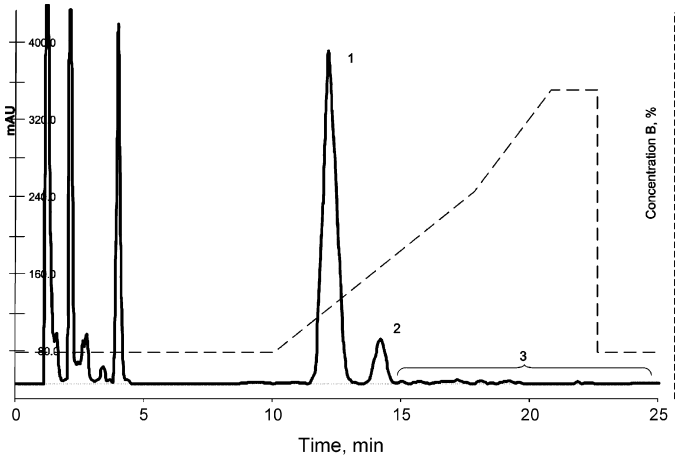

Fig. 1. HPLC analysis of crude insulin preparation. (1) HRI (87–89%), (2) corresponds to A21-DHI (7–9%), (3) insulin-like peptides. Column Phenomenex Luna C18-2, 5 μm, size 4.6 × 250.0 mm. Flow rate 1.5 ml/min. Mobile phase A: 10% acetonitrile in water, 0.1 M sodium sulphate, 0.02 M orthophosphoric acid, 0.25 ml/l triethanolamine; mobile phase B: 50% acetonitrile in water.

- mobile phase A: 10% acetonitrile in water, 0.1 M sodium sulphate, 0.02 M orthophosphoric acid, 0.025% triethanolamine;
- mobile phase B: 50% acetonitrile in water.

2.2. SEC analytics

Size-exclusion chromatography (SEC) analyses were performed on the high-performance liquid chromatograph Agilent 1100 (Agilent Technologies, USA) equipped with two syringe pumps, a thermostated column compartment, an autosampler and UV detector (detection was performed at 214 nm). The column used was Waters Insulin HMWP (7.8 × 300.0 mm), USA. Flow rate was 1.0 ml/min. Column temperature was maintained as 20 °C. "Multichrom for Windows" software (Ampersand, Russia) was used for data processing and calculation. The elution system used was as follows:

- mobile phase: 70% water, 15% acetic acid, 15% acetonitrile, 0.7 mg/ml L-Arg.

2.3. Gel-filtration experiments

Gel filtration of insulin solutions was performed on the chromatograph Äkta Basic (GE Healthcare, Sweden). The column used (20.0 × 450.0 mm) was packed with sephadex G-25 (GE Healthcare, Sweden). Flow rate was 1.0 ml/min. "Unicorn" software (GE Healthcare, Sweden) was used for data processing and calculation. The elution system used was as follows:

- mobile phase: 0.5 M acetic acid.

2.4. Experiments with guard columns

Guard columns (4.6 × 1.5 mm) were packed with following sorbents: Kromasil C18 100 Å 25 μm (Eka-Chemicals, Sweden), Polygoprep C18 100 Å 30 μm (Macherey-Nagel, Germany), PLRP-S 100 Å 30–50 μm (Polymer Laboratories Ltd., UK). The experiments with guard columns were performed on the high-performance liquid chromatograph Beckman System Gold (Beckman, USA) equipped with two syringe pumps, an autosampler and UV detector (detection was performed at 280 nm). Flow rate varied in the 0.1–1.5 ml/min range. The elution system used was as follows:

- mobile phase A: 20% ethanol in water, 10 mM citric acid, 70 mM sodium sulphate, pH 2.25 adjusted with 4 M HCl;
- mobile phase B: 50% ethanol in water, 10 mM citric acid, pH 2.25 adjusted with 4 M HCl.

The cleaning process was performed with 60% 2-propanol in water, 10 mM tris.

2.5. Preparative-scale insulin purification

Preparative-scale purification was performed on the high-performance liquid chromatograph Knauer-250 (Knauer, Germany) equipped with two syringe pumps and UV detector (detection was performed at 280 nm). The column (100.0 × 600.0 mm) used was packed with Kromasil C8 100 Å 16 μm (Eka-Chemicals, Sweden). Flow rate varied in the 100–200 ml/min range. The elution system used was as follows:

- mobile phase A: 20% ethanol in water, 10 mM citric acid, 70 mM sodium sulphate, pH 2.25 adjusted with 4 M HCl;
- mobile phase B: 50% ethanol in water, 10 mM citric acid, pH 2.25 adjusted with 4 M HCl.

The concentration gradient used (CV = volume of sorbent bed in main column):

1st step	0.0 CV	0% B
2nd step	0.5 CV	40% B
3rd step	2.0 CV	42% B
4th step	5.0 CV	48% B

Steps 1–3 are a preliminary gradient mode (peptides desorption from guard column). The final step is the main gradient mode (peptide desorption from main column).

The cleaning-in-place (CIP) process was performed with 60% 2-propanol and 40% buffer of 10 mM tris.

3. Results and discussion

3.1. Gel filtration

To study the influence of gel filtration the following strategy was chosen: (a) the test column (4.6 × 250.0 mm) packed with Kromasil C8-100 Å-16 μm was loaded with crude insulin; (b) the reference column (4.6 × 250.0 mm) packed with the same sorbent under the same conditions was loaded with insulin purified from high-molecular weight compounds by means of gel filtration; (c) after a number of chromatographic cycles (equilibration–loading–elution–regeneration) the resolution between HRI and A21-DHI peaks was compared. It is well known that after a long working time the separation features of the sorbent deteriorate and the resolution between compounds decreases. The reason for this deterioration may mainly be accumulation of SAI on the sorbent.

The gel-filtration mainstream (see Fig. 2) of HRI that has to be loaded on the reference column was purified from high-molecular weight compounds and their concentration was decreased more than three times (see Fig. 3).

Each cycle consisted of column equilibration with 100% mobile phase A; loading of 10 mg of crude insulin; elution in isocratic mode (50% mobile phase A); regeneration with 100% mobile phase B. After 100 chromatographic cycles using test column the resolution between HRI and A21-DHI became worse

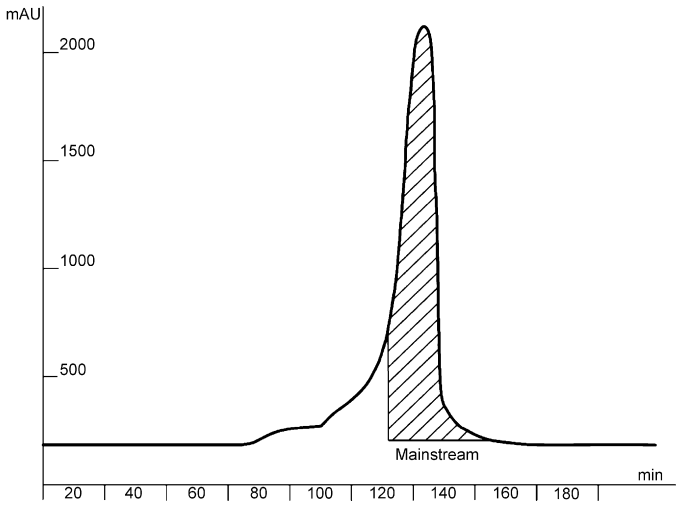

Fig. 2. HRI purification from high-molecular compounds by means of gel filtration: sephadex G-25, 0.5 M acetic acid as eluent, loading capacity = 1.5 mg of insulin/ml sorbent; loading volume does not exceed 7% of column volume.

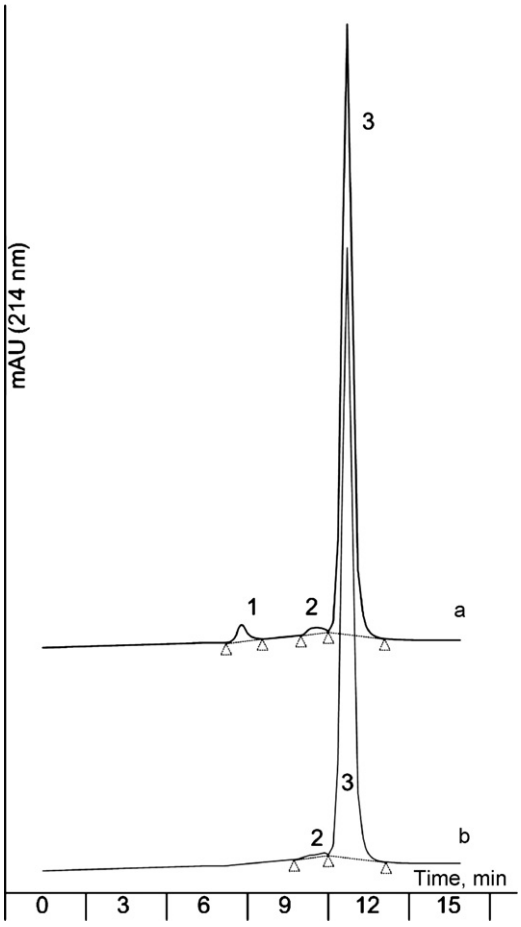

Fig. 3. SEC analysis of insulin solution before and after gel filtration. Peaks 1 and 2 correspond to high-molecular impurities (summary percentage is about 4.3% before gel filtration (a) and 1.3% after gel filtration (b)), peak 3 corresponds to HRI. Column: Insulin HMWP Waters 7.8 × 300.0 mm, eluent: 70% water, 15% acetic acid, 15% acetonitrile, 0.7 g/l L-Arg.

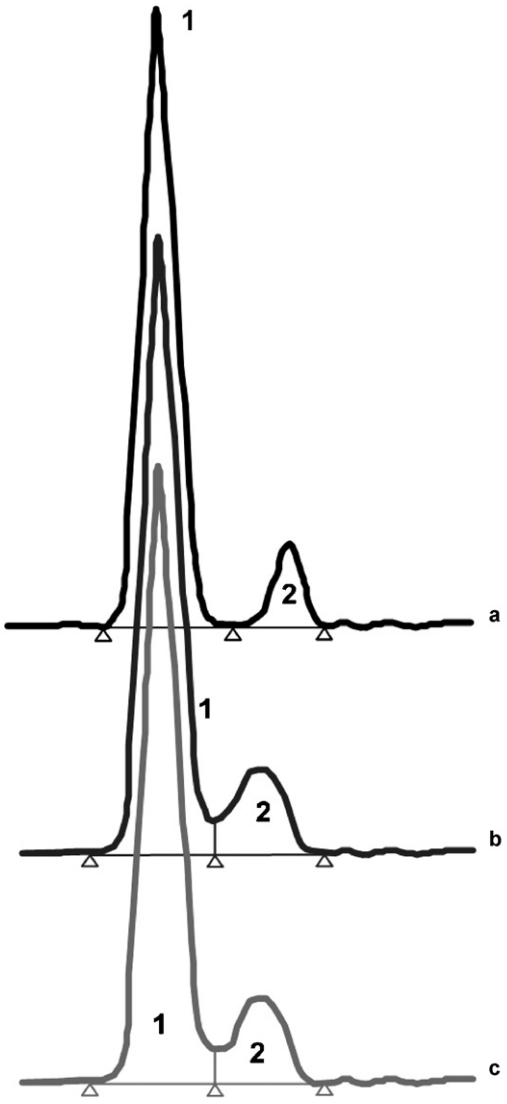

Fig. 4. Chromatograms of crude insulin initially (a) and after 100 chromatographic cycles for (b) test column and (c) reference column. Peak 1 corresponds to HRI; peak 2 corresponds to A21-DHI. Columns 4.6 × 250.0 mm packed with Kromasil C8-100 Å-16 μm in both cases; elution mode: 50% B ((A) 20% ethanol, 10 mM citric acid, 70 mM ammonium sulphate, pH 2.2; (B) 50% ethanol, 10 mM citric acid, pH 2.2).

compared with initial resolution (see Fig. 4). However, in case reference column was used, results were similar. Thus, it can be concluded that gel filtration of the crude insulin cannot protect the sorbent from SAI pollution satisfactorily.

3.2. Guard column

The using of guard column in line with primary column is considered to be a powerful method for sorbent protection at the analytical level because the most part of SAI is sorbed by the guard-column sorbent. Furthermore, it seems that the using of guard column may be able to protect primary column at the preparative level too. The only weakness of such technique is the necessity to change guard-column sorbent bed from time to time.

Three different types of sorbents which can be applied as a guard-column sorbent were studied: regular (spherical silica particles) silica sorbent (Kromasil C18-100 Å-25 μm), non-spherical silica sorbent (Polygoprep C18-100 Å-30 μm) and poly(styrene-divinylbenzene) sorbent (PLRP-S, 30–50 μm). To identify the best solution for preparative purposes, following criteria were chosen as being important: (1) sorbent price; (2) maximum possible loading capacity for each type of sorbent; (3) number of theoretical plates per column (NTP); (4) maximum working pressure; (5) value of concentration of organic modifier (ethanol) needed for insulin desorption with retention time not exceeding two column volumes (CV) and (6) extreme pH value for sorbent regeneration. The obtained results are given in Table 1.

Thus, from these criteria, it can be concluded that polymer sorbent is not suitable for protecting the silica sorbent in the main column because the insulin retention by poly(styrene-divinylbenzene) sorbent was much greater than this one by silica sorbent in the main column.

Also, one can see that the results for spherical and non-spherical sorbents are similar, but using of spherical sorbent is preferred since the back pressure is slightly less in case of spherical Kromasil.

It was necessary to investigate the quality of main column protection from SAI by means of guard column. The crude insulin contains a number of impurities with retention times greater than those of HRI and A21-DHI (see Fig. 5a). After the elution from guard column the content of these impurities was slightly decreased and amounted about 2.5% in comparison with initial 4.0% (see Fig. 5b). After passing through the guard column there were almost no insulin-like peptides with retention times

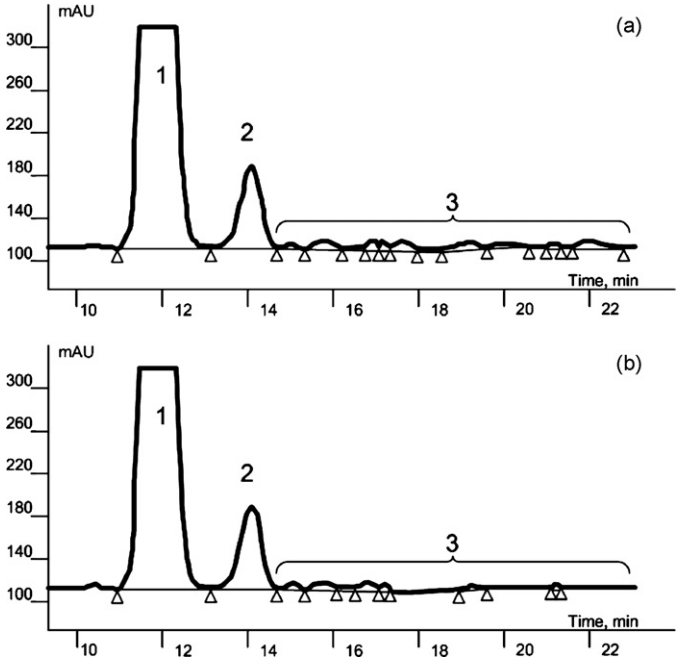

Fig. 5. HPLC analysis of the effect of using guard column: (a) crude insulin (almost 4.0% of insulin-like peptides correspond to 3rd peak), (b) insulin purified by means of guard column (quantity of insulin-like peptides corresponding to 3rd peak ranged as 1.8–2.3%). Column and elution mode are similar as in Fig. 1.

Table 1
Comparison of different sorbents used in guard column

Criterion	Type		
	Regular silica sorbent	Nonspherical silica sorbent	Polymeric sorbent
Sorbent	Kromasil C18 100 Å 25 μm	Polygoprep C18 100 Å 30 μm	PLRP-S 100 Å 30–50 μm
Column size (mm)	4.6 × 1.5	4.6 × 1.5	4.6 × 1.5
Price, arbitrary unit	2.93	1.0	3.52
Maximum possible assay loading, mg of insulin per mg of sorbent	30.0	23.3	42.0
NTP (same loading (10 mg of insulin per mg of sorbent), same retention times, same rates (0.1 ml/min))	1600	596	1721
Back pressure for overloaded column (kpsi)	1.3	2.0	1.6
Back pressure after regeneration (kpsi)	1.4	2.8	1.6
Concentration of ethanol for insulin desorption with retention time about two CV[a]	33.6	34.2	41.0
Permissible pH range during 50 CV	1.8–11.8, passed	1.8–11.8, passed	1.8–11.8, passed

[a] The less the concentration of ethanol in mobile phase the better, since the organic modifier concentration for insulin desorption from guard column has to be less than 1 for insulin desorption from main column. The ethanol concentration in mobile phase for insulin desorption from main column was 34.2–34.4%.

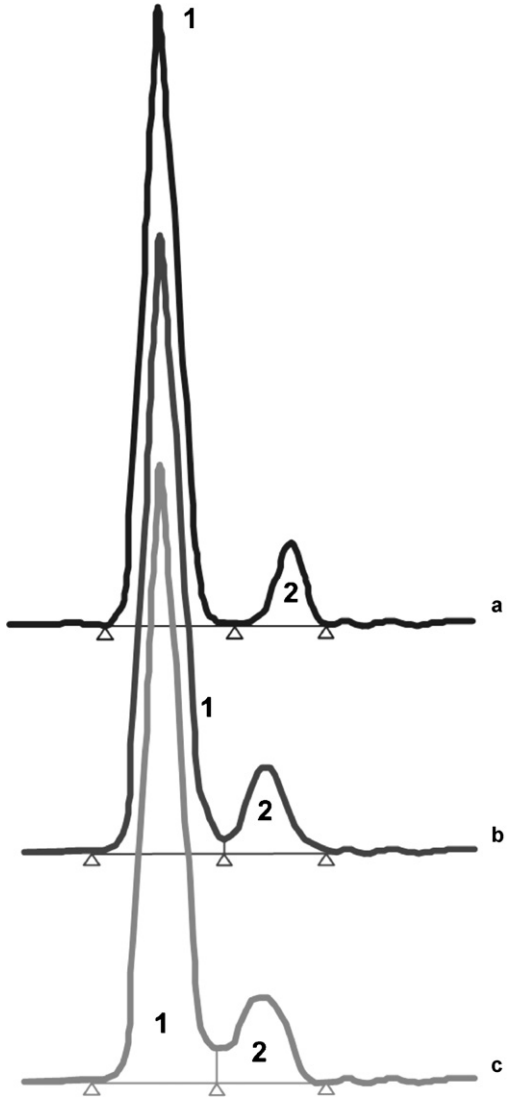

Fig. 6. Chromatograms of crude insulin initially (a) and after 100 chromatographic cycles for (b) test column using guard column in line and (c) reference column. Peak 1 corresponds to HRI; peak 2 corresponds to A21-DHI. Column and elution mode are similar as in Fig. 4.

exceeding 18 min. Hence, the guard column seems to be a good method for main column protection.

After 100 chromatographic cycles using guard column in line with main column the resolution between HRI and A21-DHI became slightly worse compared with initial resolution but much better compared with reference column (see Fig. 6). Consequently, it can be concluded that guard column protects sorbent from SAI satisfactorily in this elution system and that it is a technique advantageous to use in the manufacture.

3.3. Preparative scale insulin purification

The guard column was used in line with the main column, and results received were compared with reference case (insulin purification without guard column). After several chromatographic cycles the resolution between HRI and A21-DHI became worse and, therefore, the CIP procedure was performed. It is necessary to note that preparative column is usually used in an overloaded state that makes the cleaning procedure essential. When the column was used without any protection the resolution between HRI and A21-DHI became worse after 3–4 cycles (that corresponds to purification of about 120–160 g of crude insulin with average 70% yield and average purity of 98.5%) and cleaning procedure was performed after each 3–4 cycles. However, in case of using guard column in line with main column the resolution of the constituents pointed above became worse only after 14–16 cycles (this corresponds to purification of about 560–640 g of crude insulin with average 75% yield and average purity of 98.5%).

4. Conclusions

It was mentioned above that RP HPLC is one of the key stages of the HRI manufacturing. Therefore, optimization of the HPLC purification is essential for production economics. The problem is that the sorbent contamination by SAI results in poor resolution between HRI, A21-DHI and other insulin-like admixtures. Although cleaning procedure with either acidic or basic mobile

phase is preferred in most cases, it cannot solve the contamination problem. The gel filtration of insulin solution before HPLC does not protect silica sorbent efficiently. Hence, it can be concluded that SAI are not high-molecular weight compounds. The precise nature of SAI remains unknown. On the contrary, using of the guard column in line with main column results in sufficient protection from SAI. Thus, using of the guard column seems to be a good method for silica sorbent protection. Among different types of sorbents, the spherical silica sorbent is a good choice.

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