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Monitoring of recombinant human insulin production by narrow-bore reversed-phase high-performance liquid chromatography, high-performance capillary electrophoresis and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry ☆

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Abstract

An analytical scheme for monitoring recombinant human insulin (rhI) production is suggested. The scheme includes high-performance separation micro-techniques (narrow-bore RP-HPLC, HPCE) based on different separation mechanisms and matrix-assisted laser desorption ionisation time-of-flight MS, and allows one to obtain unambiguous information about purity and primary structure of all intermediates of the rhI production. The use of this scheme at all production steps provided optimisation of certain technological parameters [conditions for a fusion protein (FP) refolding, temperature and duration of the FP cleavage with trypsin, conditions for carboxypeptidase B digestion of di-Arg$^{B31-B32}$-insulin] and achievement of a high purity of the end-product. The proposed scheme may be used for solving various problems in monitoring production of other recombinant proteins.

Keywords: Insulin; Proteins; Peptides

1. Introduction

Insulin is a mammalian cell hormone with known amino acid sequence and structural characteristics [1]. Insufficient insulin secretion in an organism leads to dangerous metabolism diseases and causes diabetes mellitus. The discovery of the insulin ability to decrease the blood sugar level has led to rapid development of insulin manufacture [2]. In the early years of insulin manufacture, only porcine and bovine insulins were available for therapy. However, both insulins cause undesirable allergic reactions in humans that induced the development of recombinant human insulin (rhI) manufacture. At present, several methods of large-scale rhI production by fermentation of *Escherichia coli* [3] or *Saccharomyces cerevisiae* yeast [4] are successfully employed. Nevertheless, efforts to develop more cost-saving production schemes are ongoing.

Today one of the most promising protocols of rhI

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production includes an enzymatic cleavage of the di-Arg$^{B31-B32}$-insulin from the whole molecule of fusion protein (FP) followed by carboxypeptidase B treatment of the di-Arg$^{B31-B32}$-insulin. Checking for purity and fractional conversion of intermediate products throughout the technological steps is important for creation of the correct technology, thus reducing production cost.

Separation of insulin and insulin-like peptides is a difficult task due to the very slight differences in their mass and charge [5,6], so analytical techniques such as high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE), which have high separation power, appear to be the methods of choice for analysis of peptides with a similar structure. Furthermore, the combined usage of HPLC and HPCE, differing in their separation mechanism, allow one to obtain more unambiguous information about the composition of a sample which is very important for the quality control of pharmaceuticals. However, to confirm a structure of analysed peptides and to identify impurities, other analytical methods such as mass spectrometry (MS) or amino acid sequencing, are needed. Thus, only the combination of the separation techniques and the identification methods can provide unambiguous structure and purity control of protein and peptide samples, and it should be used for solving various problems in monitoring of biotechnological procedures and in the analysis of biotechnological products.

This study is aimed at developing an analytical scheme, which includes narrow-bore HPLC, HPCE and MS analysis for the step-by-step control of rhI production.

2. Experimental

2.1. Reagents and chemicals

The reagents used were acetonitrile (MeCN), phosphoric acid, sodium dihydrogenphosphate, sodium sulfate, Tris, hydrochloric acid (HCl), sodium hydroxide (NaOH), lithium perchlorate, dithiothreitol (DTT) (all of the highest purity available); trifluoroacetic acid (TFA) and 12.5% trimethylamine (TMA) originated from Perkin-Elmer (Warrington, UK). Trypsin and carboxypeptidase B were obtained from ICN (USA). Purified water from a Milli-Q system (Millipore, Molsheim, France) was used throughout the experiments.

2.2. Instrumentation

2.2.1. Narrow-bore HPLC

HPLC analyses were performed on the narrow-bore high-performance liquid chromatograph Milichrom A-02 (Envirochrom A-02) (Chromatography Institute Eko-Nova, Novosibirsk, Russia) equipped with two syringe pumps, a thermostated column compartment, an autosampler, a photometric cell with a 1.2 μl volume, and a spectrophotometrical (190–360 nm) detector. The narrow-bore (75×2 mm I.D.) columns used were: Nucleosil C_{18} packed with a Macherey-Nagel (Germany) sorbent (120 Å, 5 μm) and Vydac C_4 packed with a sorbent (120 Å, 5 μm) of the Separation Group (USA). Column temperature was maintained from 35°C to 45°C as needed. Flow-rate was 0.1 ml/min in all analyses. ''MultiChrom for Windows'' program (Ampersend, Moscow, Russia) was used for data processing and calculation.

The elution systems used:

System 1: solvent A was 0.1% TFA in water (pH 2.2); solvent B was MeCN.

System 2: solvent A was 1% phosphoric acid in water (pH 2.35 adjusted with 12.5% TMA) containing 10% of MeCN; solvent B was A–1% phosphoric acid in water (pH 2.35 adjusted with 12.5% TMA) containing 60% MeCN.

System 3: solvent A was 0.2 *M* Na_2SO_4 in water (pH 2.30 adjusted with phosphoric acid); solvent B was MeCN.

System 4: solvent A was 0.2 *M* $LiClO_4$ in water (pH 2.30 adjusted with phosphoric acid); solvent B was MeCN.

All eluents were filtered through 0.45-μm membranes (Millipore) and degassed by helium for 3 min at 25°C.

2.2.2. HPCE

HPCE analyses were performed on the HPCE system Model 270 A (ABI, Foster City, CA, USA). All analyses were performed on two uncoated fused-silica capillaries (Perkin-Elmer). The first capillary

was 80 cm (60 cm effective length)×75 μm I.D. The second capillary was 122 cm (100 cm effective length)×50 μm I.D. Between injections, capillaries were conditioned for 5 min with 0.2 *M* NaOH and 10 min with a running buffer. The separation voltage was 20–30 kV, depending on the analysis. The temperature of the capillaries was kept constant at 25 or 30°C. The electropherograms were recorded on a Shimadzu Chromatopac C-R5A integrator (Shimadzu, Kyoto, Japan). The resolution (R_s) was calculated according to the formula: $R_s=(t_{m_i}-t_{m_j})/(W_2+W_1)$ where t_{m_i} and t_{m_j} are the migration times of the peaks of interest and W is the peak width, measured at the 0.607 peak height. The employed formula is identical to one which is used in program for chromatographic data processing and calculations (''MultiChrom for Windows''; Ampersend, Moscow, Russia) to obtain comparable results.

The buffer systems used:

System 1: 50 m*M* sodium phosphate, pH 11 (adjusted with 0.1 *M* NaOH).

System 2: 60 ml of 100 m*M* sodium phosphate, pH 9 (adjusted with 0.1 *M* HCl) was mixed with 30 ml of MeOH.

All buffers were filtrated through 0.45-μm membranes (Millipore) and degassed by helium for 3 min at 25°C.

2.3. Fusion protein folding

Washed inclusion bodies were solubilized by adding a solution of 8 *M* urea to a final concentration of protein ~20 mg/ml. After incubation at 25°C under gentle stirring for 4 h the mixture was centrifuged at 30 000 *g* for 20 min. The supernatant was decanted and two aliquots (10 μl) were taken before addition of DTT to a final concentration of 2 m*M*. After incubation at 25°C under stirring, the reaction mixture was diluted ~100 times and incubated under the same conditions for 24 h.

The first aliquot was diluted with 1% TFA before HPLC analyses to a final concentration of TFA of 0.1% in the sample. The second aliquot was diluted in 10 times with deionised water and applied to analysis by HPCE.

The sample of refolded FP for the HPLC and HPCE analyses was prepared by dissolving of 2 mg of lyophilised substance in 1 ml of 0.01 *M* HCl.

2.4. Enzymatic cleavages

2.4.1. Proteolysis of FP with a trypsin

The suspension of FP was dissolved in 50 m*M* Tris–HCl buffer (pH 8) containing 1 m*M* $CaCl_2$ to a protein concentration of ~2 mg/ml. Trypsin, dissolved in the same buffer, was added to FP in an enzyme/substrate ratio of 1:1000 (w/w). The temperature was kept at 37, 25 and 10°C. Aliquots were taken at different time intervals and digestion was stopped by addition of 10% TFA to pH~3.

2.4.2. Cleavage of di-Arg$^{B31-B32}$-insulin with carboxypeptidase B

Lyophilised di-Arg$^{B31-B32}$-insulin (2 mg) was dissolved in 1 ml of 50 m*M* Tris–HCl buffer, pH 7.3 containing 1 *M* urea. Carboxypeptidase B, dissolved in same buffer, was added to the enzyme/substrate ratio 1:1500 (w/w). The temperature was maintained at 37°C throughout the reaction.

Aliquots were taken at different time intervals (as is specified in the captions to Figs. 8 and 9) and digestion was stopped by adding of 10% TFA to pH~3 before HPLC analysis. The samples for HPCE analysis were prepared as follows: 15 μl trichloroacetic acid (10%, w/w) and 15 μl acetone (Merck, Darmstadt, Germany) were added to each aliquot (50 μl). Aliquots were kept at 4°C for 15 min and centrifuged at 10 000 *g* using an Eppendorf 5410 (Eppendorf, Germany) centrifuge for 10 min. The supernatant was decanted and the precipitate was washed with acetone (200 μl), dried by air and dissolved in 50 μl of 0.01 *M* HCl.

3. Results and discussion

rhI manufacture consists of several technological steps (Fig. 1). During the technological process protein transformations lead to the formation of semi-products, which significantly differ in charge or hydrophobicity, so an employment of analytical technique with a proper separation mechanism (HPCE or HPLC) could provide an effective separation of all species at any stage of the technological process.

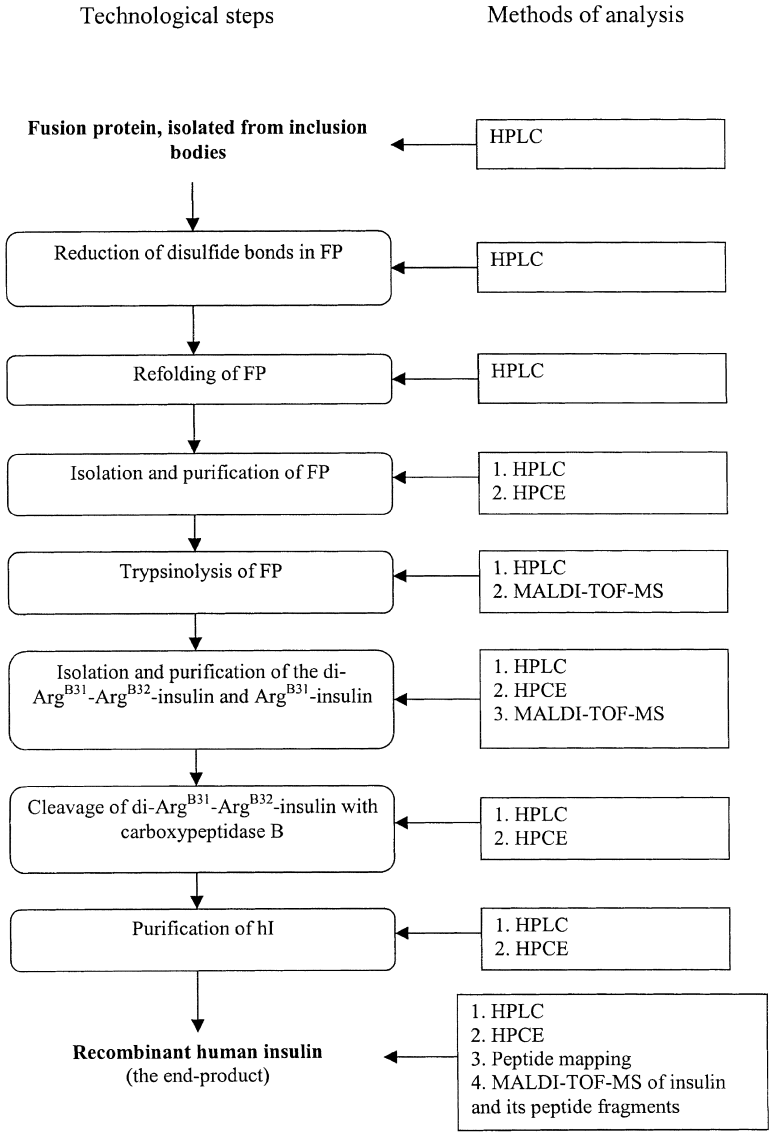

Fig. 1. Main technological steps of rhI production and supposed scheme of analytical control.

3.1. Fusion protein folding

The FP, expressed at the first stage, consists of human proinsulin, linked to the leader protein (synthetic IgG-binding domain of A-protein from *Staphylococcus aureus* and peptide linker His_6–Gly–Ser–Arg) with an Arg residue [7]. The current gene-engineering construction of the FP provides an optimal conformation of the protein globule for proper disulfide bridge formation, so the particularity of the present production scheme lies in the absence of a proinsulin route used in the scheme described earlier [8].

However, the FP, isolated from inclusion bodies, had a number of conformations probably corresponding to different variety of disulfide bridge arrangements (Figs. 2A and 3A). These proteins were effectively separated by HPLC (Fig. 2A) due to the different allocation of the amino acids on the surface of the protein globule, so it could affect the

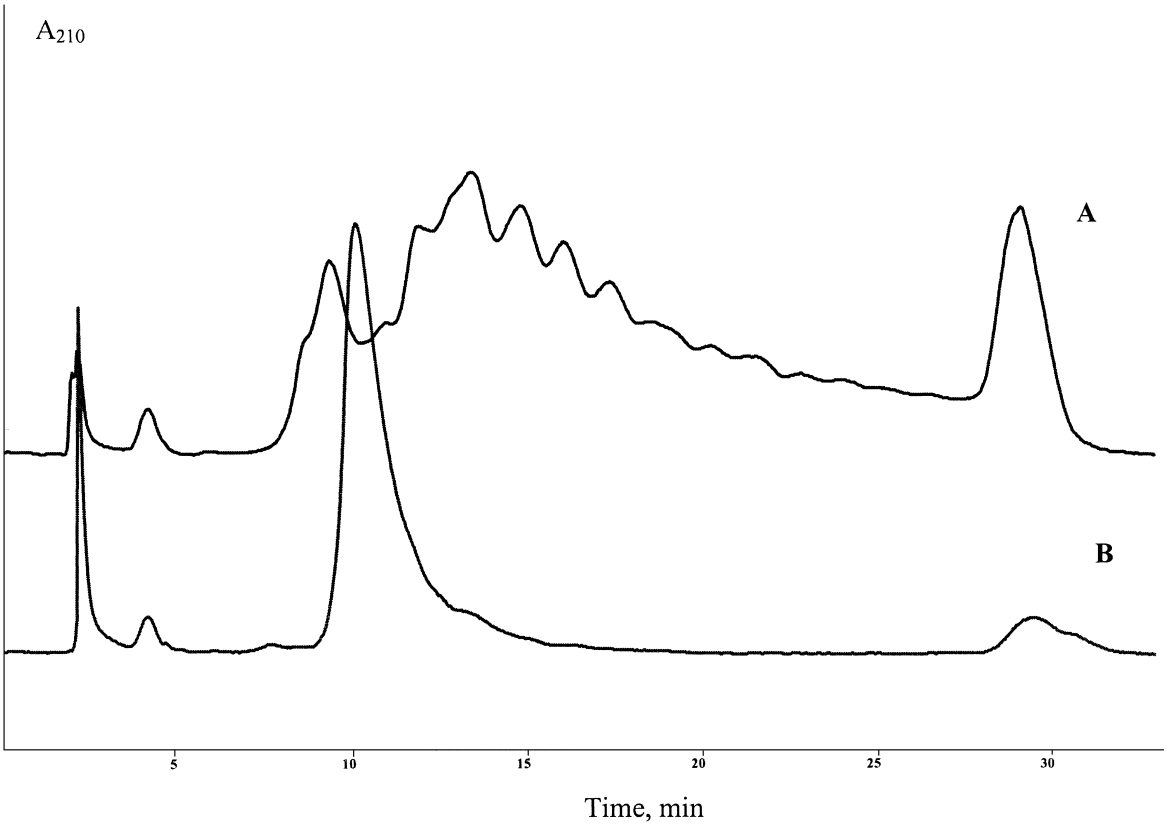

Fig. 2. Narrow-bore HPLC of unfolded (A) and folded FP (B). Column: Vydac C_4-300 5 μm (75×2 mm). Mobile phase: A=1% H_3PO_4 (pH 2.3), B=MeCN. Gradient elution: 0 min–26%→40% B for 25 min, 25 min: 40%→100% B for 7 min. Protein concentration: ~2 mg/ml. Injection volume: 5 μl. Temperature: 35°C.

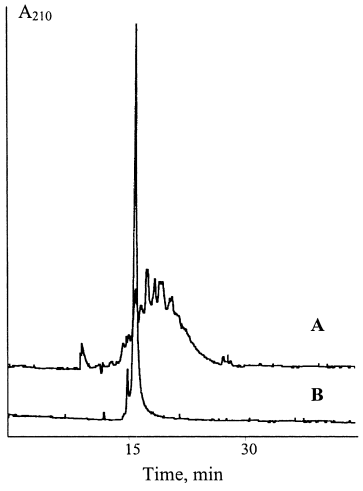

Fig. 3. HPCE of unfolded (A) and folded FP (B). Capillary: 80 cm (60 cm to the detector)×75 μm I.D. Buffer: 50 m*M* phosphate, pH 11. Protein concentration: ~2 mg/ml. Injection: 150 mbar s. Voltage: 20 kV. Current: ~40 μA. Temperature: 30°C.

hydrophobicity and the retention time of FP molecules with different disulfide bond arrangements. At the same time, all conformations of FP should have a similar charge and mass, but they, probably, significantly differ in size and could be separated by HPCE also, but with significantly less efficiency (Fig. 3A).

Since in this case the efficiency of separation by HPCE is lower than could be achieved with HPLC, the kinetics of FP refolding were monitored with the latter technique. The study of the FP refolding allowed one to optimise some technological parameters (type and amount of reducing agent, temperature and time of each reaction) and to achieve a high yield of properly renatured FP (~85%).

Under the conditions found, the reduction of disulfide bonds followed by the oxidising of SH groups led to the proper disulfide bridge formation in the FP molecule and after completion of FP folding a

homogenous peak on HPCE and HPLC was observed (Figs. 2B and 3B).

3.2. Control of FP trypsinolysis

An enzymatic cleavage of the renatured FP with a trypsin was the next step of rhI production. The preferable formation of di-Arg$^{B31-B32}$-insulin is caused by the fact that the Arg^{B32}–Glu^{B33} bond in proinsulin is more sensitive to trypsin than Lys^{B29}–Thr^{B30} [9] but an increase in hydrolysis time leads to the considerable amount of des-Thr^{B30}-insulin which is an undesirable side-product. Searching for the optimal cleavage conditions, which allows a decrease in the side-product formation is the main technological problem at this step.

The mechanism of FP cleavage with trypsin and confirmation of FP primary structure were described in detail in our previous report [10]. The kinetics of the FP trypsinolysis were studied by HPLC. All peptides were collected and identified by MS analysis.

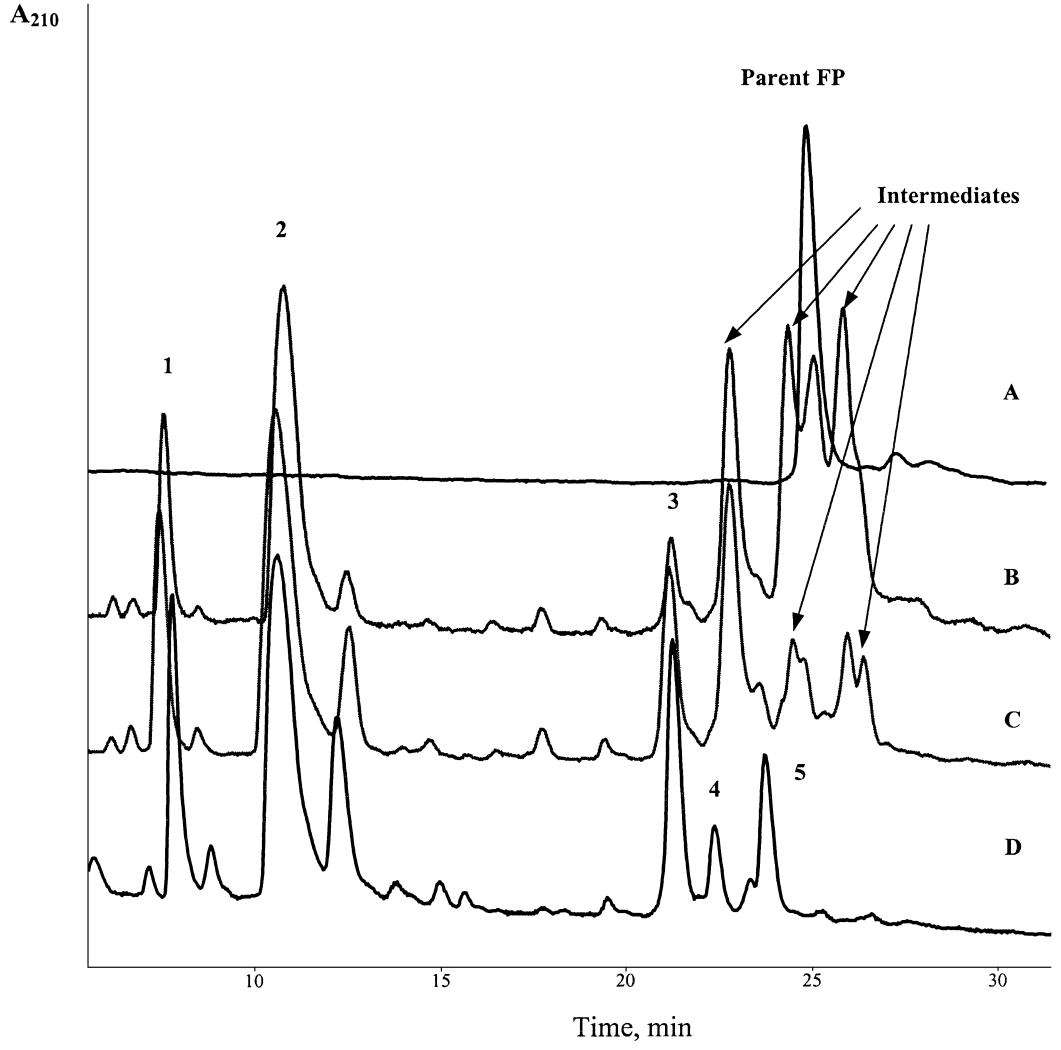

Fig. 4. Narrow-bore HPLC. The kinetics of FP tryptic digestion at 25°C. Chromatograms A–D correspond to the cleavage time of 0, 5, 20 and 40 min, respectively. Numbered peaks correspond to C-peptide (peak 1), leader protein (peak 2), di-Arg$^{B31-B32}$-insulin (peak 3), Arg^{B31}-insulin (peak 4) and des-Thr^{B30}-insulin (peak 5). Column: Vydac C_4-300 5 μm (75×2 mm). Mobile phase: A=0.1% TFA, B=MeCN. Protein concentration: ~2 mg/ml. Injection volume: 5 μl. Linear gradient: 23%→32% B in 32 min. Temperature: 40°C.

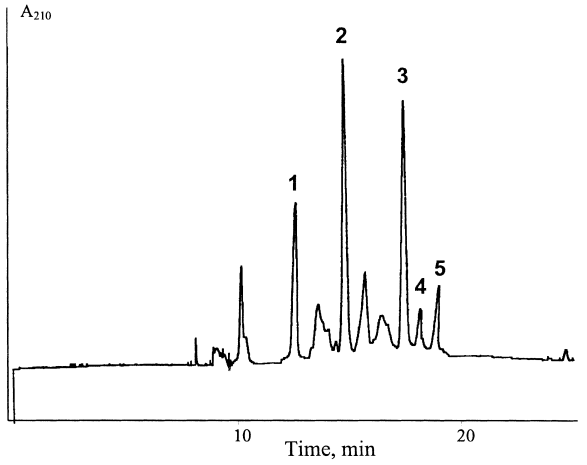

Fig. 5. Tryptic cleavage of the FP. Peak details as in Fig. 4. Capillary: 80 cm (60 cm to the detector)×75 μm I.D. Buffer: 50 m*M* phosphate, pH 9 containing 10% methanol. Protein concentration: ~2 mg/ml. Injection: 150 mbar s. Voltage: 30 kV. Current: ~30 μA. Temperature: 30°C.

Tryptic cleavage of the renatured FP leads to the formation of C-peptide (Figs. 4 and 5, peak 1), leader protein (Figs. 4 and 5, peak 2), di-Arg$^{B31-B32}$-insulin (Figs. 4 and 5, peak 3), Arg^{B31}-insulin (Figs. 4 and 5, peak 4) and by-side product, viz. des-Thr^{B30}-insulin (Figs. 4 and 5, peak 5), which significantly differ in charge and hydrophobicity, so its separation both by HPCE (Fig. 5) and HPLC (Fig. 4C) is not difficult. However, the FP primary structure contains nine lysine and five Arg residues (see Ref. [7]) and the appearance of the great number of intermediate products during the reaction was theoretically expected.

Fig. 4A–D outline the kinetics of the FP cleavage with trypsin at 25°C. After 30 min of hydrolysis some intermediates are still present in the mixture. Their peaks are superimposed to the peak of des-Thr^{B30}-insulin and prevent quantitative analysis. In

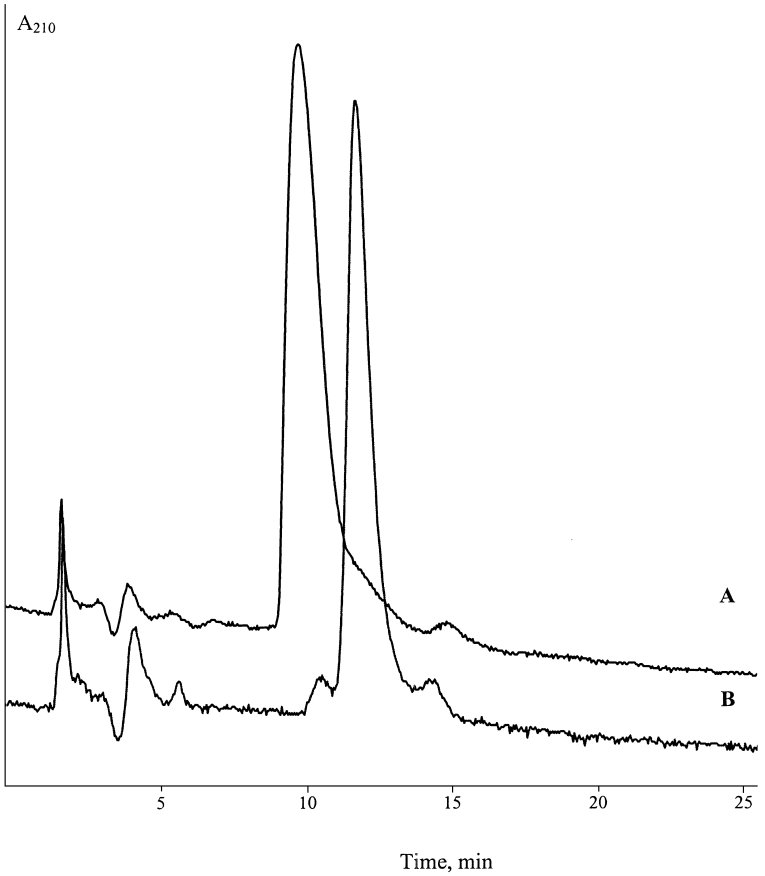

Fig. 6. Narrow-bore HPLC. Di-Arg$^{B31-B32}$-insulin (A) and Arg^{B31}-insulin (B) isolated from reaction mixture. Column: Vydac C_4-300 5 μm (75×2 mm). Mobile phase: A=1% H_3PO_4 (pH 2.3), B=MeCN. Protein concentration: ~1.5 mg/ml. Injection volume: 7 μl. Linear gradient: 25%→27% B in 25 min. Temperature: 40°C.

this case, the extent of FP conversion is better measured by the amount of the C-peptide which is formed in the equimolar amount with respect to the original FP and is not cleaved with trypsin, so its content does not change after the complete conversion of FP. This period of time (~40 min) corresponds to the moment when the reaction must be stopped.

The kinetics of the FP trypsinolysis were monitored at three temperature points (10, 20, 37°C). It was found that the decrease in hydrolysis temperature leads to a lower yield of des-Thr^{B30}-insulin. At 37°C and 25°C the formation of 45% and 23% of des-Thr^{B30}-insulin was observed after completion of the reaction (15 and 40 min, respectively). At 10°C the formation of only 13% of des-Thr^{B30}-insulin (after 4 h of hydrolysis) was observed. So the monitoring of the reaction allowed one to optimise the conditions of FP trypsinolysis and to decrease the yield of undesirable by-product.

3.3. Control of isolation and purification of di-Arg$^{B31-B32}$-insulin from the reaction mixture

In the present technological scheme the isolation of di-Arg$^{B31-B32}$-insulin from the reaction mixture was carried out by ion-exchange chromatography. Employment of the current technique allows one to separate di-Arg$^{B31-B32}$-insulin, Arg^{B31}-insulin and des-Thr^{B30}-insulin because of differences in their charge, caused by presence of different number of the charged Arg residue in the target products. So the determination of the purity of the isolated peptides allows one to estimate the efficacy of the selected purification method.

Collected peptides were identified by MS analysis and checked for the purity by HPLC (Fig. 6) and HPCE (Fig. 7). As can be seen from Figs. 6 and 7 the combined usage of HPLC and HPCE provides unambiguous information about the purity of the fractions. In particular, the purity of Arg^{B31}-insulin, determined by HPLC (Fig. 6B), appears to be 90%, while the purity, determined by HPCE, was only 87% (Fig. 7B). The purities of di-Arg$^{B31-B32}$-insulin (Figs. 6A and 7A) were found to be 95 and 98% (HPCE and HPLC, respectively); the contamination of the Arg^{B31}-insulin was determined as 2%, which confirms the high efficiency of ion-exchange chromatography for isolation and purification of di-Arg$^{B31-B32}$-insulin from the reaction mixture.

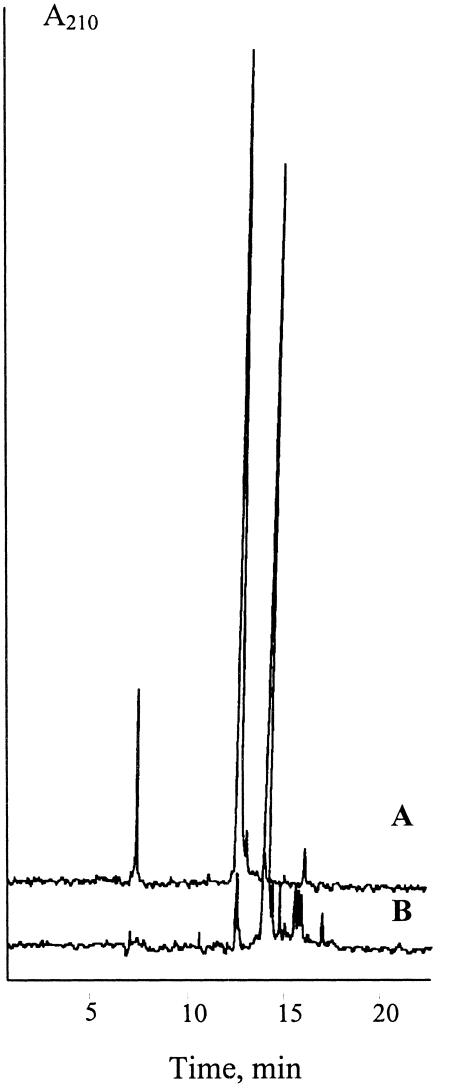

Fig. 7. HPCE. Di-Arg$^{B31-B32}$-insulin (A) and Arg^{B31}-insulin (B) isolated from reaction mixture. Capillary: 80 cm (60 cm to the detector)×75 μm I.D. Buffer: 50 m*M* phosphate, pH 11. Protein concentration: ~1.5 mg/ml. Injection: 250 mbar s. Voltage: 30 kV. Current: ~90 μA. Temperature: 25°C.

3.4. Control of di-Arg$^{B31-B32}$-insulin cleavage with carboxypeptidase B

Enzymatic cleavage of di-Arg$^{B31-B32}$-insulin with

carboxypeptidase B at the last step of rhI production leads to rapid and quantitative conversion of the raw material to insulin. High speed and selectivity of the reaction is exploited for one-step conversion of refolded insulin precursor into insulin by simultaneous treatment of the protein with trypsin and carboxypeptidase B [11]. Enzymatic cleavage of the charged amino acids from the insulin molecule

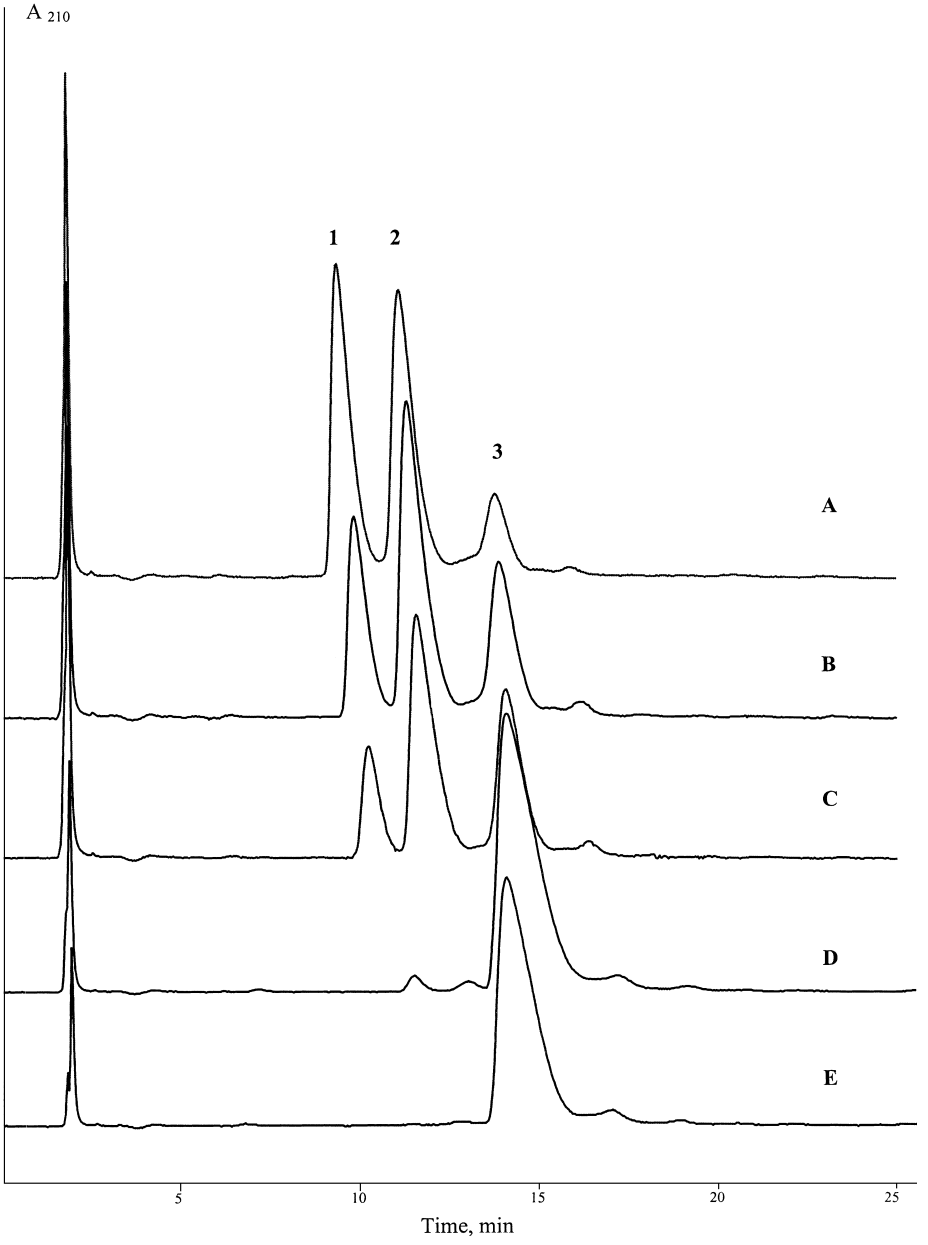

Fig. 8. Narrow-bore HPLC. Cleavage of di-Arg^{B31}–Arg^{B32}-insulin with carboxypeptidase B (A – 5 min, B – 8 min, C – 10 min, D – 30 min, E – 120 min). Peaks 1, 2, 3 correspond to di-Arg^{B31}–Arg^{B32}-insulin, Arg^{B31}-insulin and insulin, respectively. Column: Vydac C_4-300 5 μm (75×2 mm). Mobile phase: A=1% H_3PO_4 (pH 2.3), B=MeCN. Protein concentration: ~2 mg/ml. Injection volume: 4 μl. Linear gradient: 25%→27% B in 25 min. Temperature: 40°C.

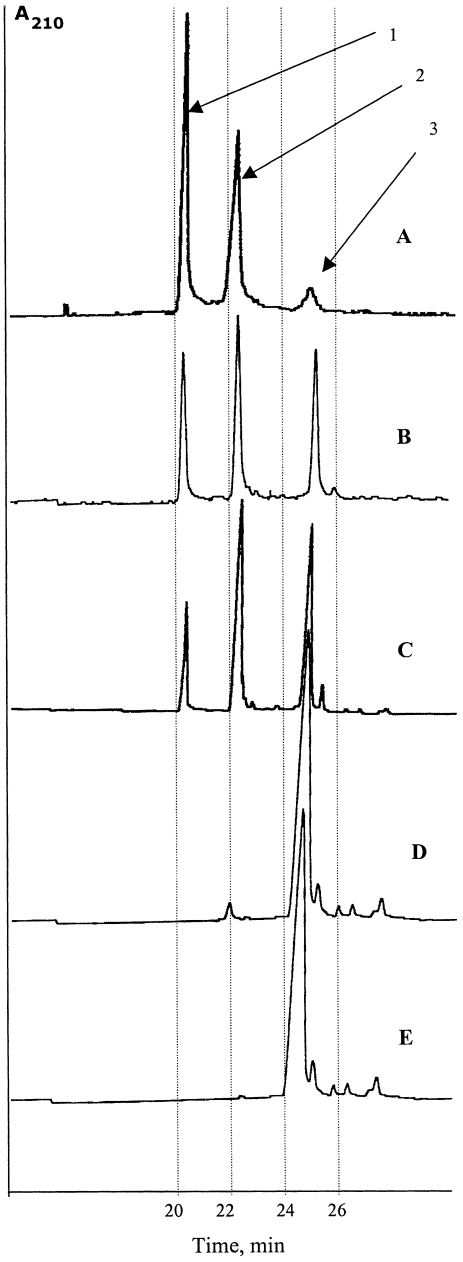

Fig. 9. HPCE. Cleavage of Arg^{B31}–Arg^{B32}-insulin with carboxypeptidase B (A – 2 min, B – 7.5 min, C – 12 min, D – 30 min, E – 120 min). Peak details as in Fig. 8. Capillary: 122 cm (100 cm to the detector)×50 μm I.D. Buffer: 50 m*M* phosphate, pH 11. Protein concentration: ~2 mg/ml. Injection: 200 mbar s. Voltage: 30 kV. Current: ~ 45 μA. Temperature: 25°C.

results in significant changes in charge-to-mass ratio, so more effective separation of di-$Arg^{B31-B32}$-insulin, Arg^{B31}-insulin and insulin by HPCE is to be expected.

Figs. 8 and 9 represent the kinetics of di-$Arg^{B31-B32}$-insulin cleavage with carboxypeptidase B monitored by HPLC (Fig. 8) and HPCE (Fig. 9). As can be seen from Figs. 8 and 9, higher resolution of di-$Arg^{B31-B32}$-insulin (Figs. 8 and 9, peak 1), Arg^{B31}-insulin (Figs. 8 and 9, peak 2) and insulin (Figs. 8 and 9, peak 3) was achieved by HPCE ($R_{s_{1,2}}$=6.7 and $R_{s2,3}$=8.6) than by HPLC ($R_{s1,2}$=1.8 and $R_{s2,3}$=2.2). Nevertheless, both techniques allowed one to obtain identical quantitative results and could be employed for control of di-$Arg^{B31-B32}$-insulin cleavage with carboxypeptidase B.

The almost quantitative conversion of the raw material via the intermediate Arg^{B31}-insulin takes about 10 min but it should be taken into account that an increase in the reaction time up to 2 h is needed to achieve complete conversion of Arg^{B31}-insulin (Figs. 8 and 9, peak 2) into insulin (Figs. 8 and 9, peak 3). We found that in contrast to the tryptic cleavage, an increase in the reaction time with carboxypeptidase B entails no undesirable by-products.

3.5. Control of the insulin purification

Insulin obtained at the previous stage was purified by ion-exchange chromatography. For an estimation of the rhI purity in three collected fractions we employed HPCE, which, as described in the previous section, appears to be more effective for analysis of charged insulin derivatives than HPLC.

As can be seen from Fig. 10, the content of Arg-insulin [incompletely converted Arg^{B31}- and (or) Arg^{A0}-insulin] achieved its maximum in fraction A (Fig. 10) and decreases A→C, while the content of rhI desamido forms increases in the same order. It was shown, that the purity of rhI in each obtained fraction does not exceed 92% (fraction C) so further steps of insulin purification are needed.

Contamination of desamido-insulins in fraction C was determined by HPCE using a second buffer system (Fig. 11). Several procedures for analysis of the human insulin sample by HPCE was described earlier [12,13], but our system appears to be more

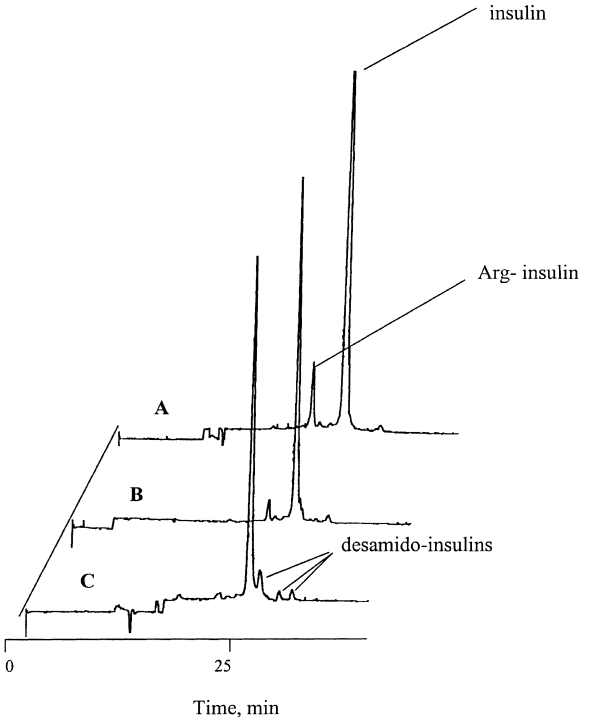

Fig. 10. HPCE. Control of insulin purity in fractions, obtained after ion-exchange chromatography. (A) Front of peak, (B) central part, (C) tail of peak. Capillary: 122 cm (100 cm to the detector)× 50 μm I.D. Buffer: 50 m*M* borate, pH 10.6. Protein concentration: ~1.6 mg/ml. Injection: 260 mbar s. Voltage: 30 kV. Current: ~55 μA. Temperature: 25°C.

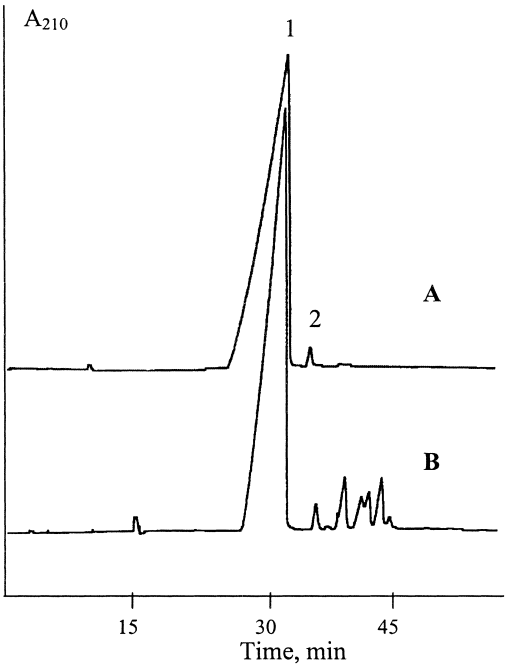

Fig. 11. CZE. (A) Purified rhI, (B) rhI before purification. Peaks 1 and 2 correspond to insulin and desamido-Asn^{A21}-insulin, respectively. Capillary: 80 cm (60 cm to the detector)×75 μm I.D. Buffer: 100 m*M* phosphate–30% MeOH (pH 9). Insulin concentration in each sample: ~2 mg/ml (dissolved in 0.01 *M* HCl). Injection: 260 mbar s. Voltage: 30 kV. Current: ~15 μA. Temperature: 25°C.

selective. Analysis of the rhI sample by HPCE revealed the impurities, which could not be detected with HPLC and, probably, corresponds to unidentified desamido forms of the insulin.

The sample of the refined rhI was also checked for impurities (Fig. 11B) The conditions used provided the very good separation of insulin (peak 1) and desamido-Asn^{A21}-insulin (peak 2), identified by degradation of insulin under acidic conditions [14]. Determination of insulin purity in all fractions after each step of purification provided developing an effective procedure for insulin purification, which allowed one to obtain rhI with high purity (99.2% determined by HPCE).

3.6. Analysis of insulin preparation

Two of the major concerns of pharmaceutical companies are the evaluation of the purity of the substance and the confirmation of primary structure of proteins used as drugs. Effective procedure, based on peptide mapping of proteins with off-line matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) MS analysis of separated fragments for the evaluation of the primary structure of insulins from different species was described elsewhere [15].

rhI may be accompanied by impurities formed by proteolysis, desamidation, covalent polymer formation, uncompleted reactions, etc. These contaminants may cause allergic reactions in humans or display a toxic effect, so that their content must not exceed the specified levels. Various modifications of the HPLC and HPCE systems were described for analytical separation of insulin, monodesamido-insulins, proinsulin, C-peptide, insulin A and B chains from each other and from the recombinant fusion proteins [18–20]. As a rule, confirmation of purity of the insulin

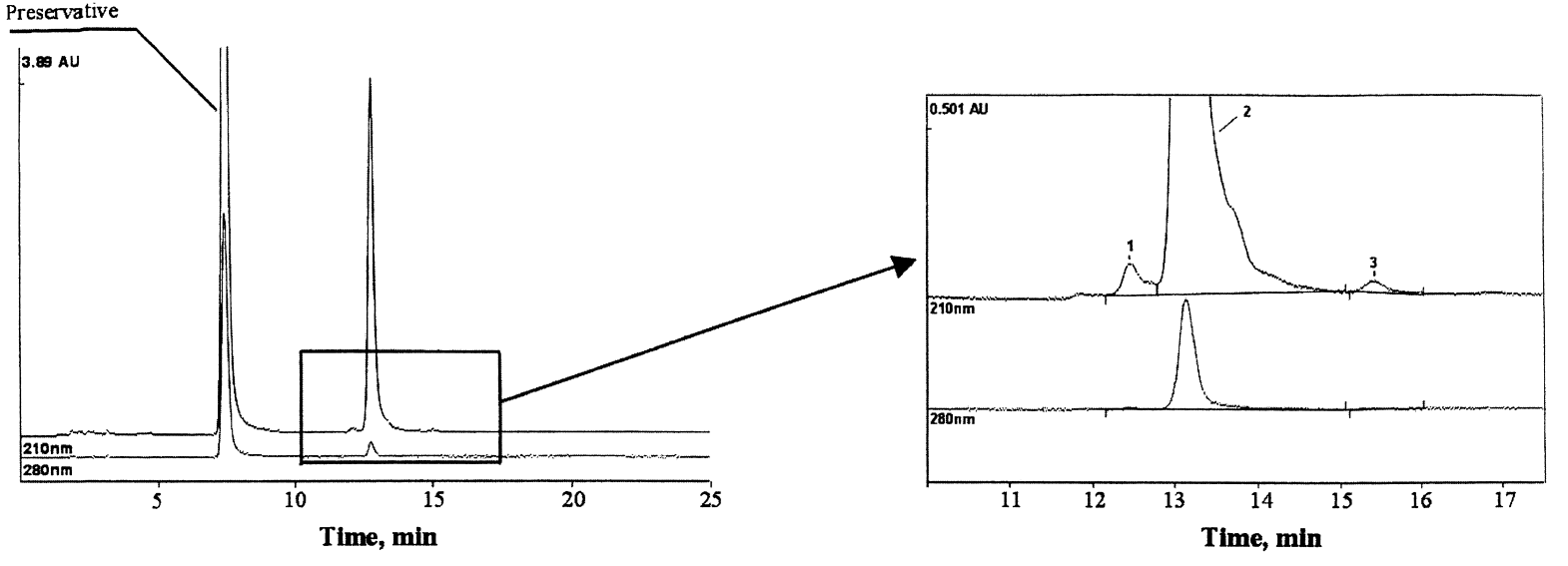

Fig. 12. Narrow-bore HPLC of rhI preparation. Peaks 1–3 correspond to Arg-insulin, rhI and desamido-insulin, respectively. Column: Vydac C_4-300 5 μm (75×2 mm). Mobile phase: A=0.2 *M* $LiClO_4$ (pH 2.35); B=MeCN. Protein concentration: ~1.8 mg/ml. Injection volume: 6 μl. Linear gradient: 28%→35% B in 25 min. Two-channel detection: 210 nm and 280 nm. Temperature: 40°C.

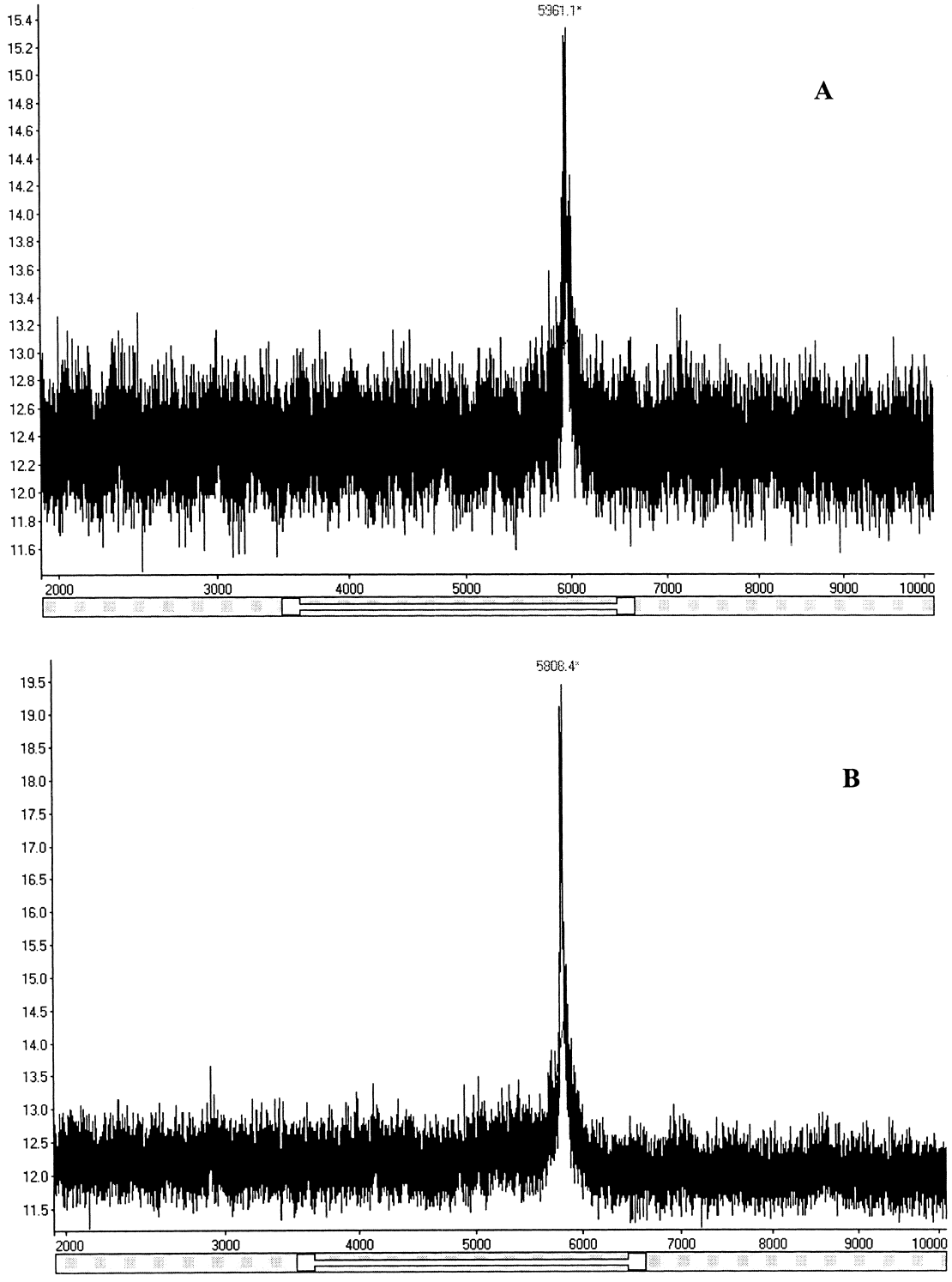

Fig. 13. MALDI-TOF-MS spectra of collected fractions 1 (A) and 3 (B) from Fig. 12. *x*-Axis: *m*/*z*; *y*-axis: intensity (%).

substance or preparation is performed by HPLC using known conditions as described in the Pharmacopoeias [16,17]. In the study we planned to test the ability of narrow-bore HPLC (75×2 mm column) to reliably determine the purity of rhI. Since the elution system described in the US Pharmacopoeia [16] could not provide satisfactory separation of insulin and desamido-insulin on the short column, we examined three systems of eluents: 0.1% TFA–MeCN, 0.2 *M* Na_2SO_4–MeCN and 0.2 *M* $LiClO_4$–MeCN to analyse hI; a higher resolution was achieved with the third one.

We found that the column with the wide-pore butyl stationary phase allowed one to reach a higher resolution of insulins than the column with the octadecyl support. Analysis of rhI is shown in Fig. 12. The impurity marked as peak 1 on Fig. 12 has a molecular mass of 5961.1 (Fig. 13A) and probably corresponds to the Arg^{B31}-insulin (calculated M_r= 5963.3). Peak 3 (Fig. 12) contains peptide, which has a molecular mass of 5808.4 (Fig. 13B) and corresponds to a desamido-insulin. It should be noted that the molecular masses of the insulin and the monodesamido-insulin differ only by 1 u and it is impossible to identify minor quantities of monodesamido-insulin in insulin sample only by MS analysis.

These results provide further evidence of the high efficacy of HPLC coupled with on (off)-line MALDI-TOF-MS for identification of micro-impurities in samples of recombinant proteins.

4. Conclusion

Thus, during the study, a high-resolution, highly sensitive, analytical scheme including HPLC, HPCE and MS, for the step-by-step control of rhI production was developed. Taken together, the use of HPLC, HPCE and MS analyses is shown to be efficient for determining both the purity and the primary structure of peptides and proteins by using microquantities of the samples (less than 10 nmol for all the analytical procedures) and may be recommended for quality monitoring of any recombinant peptide during its production. The scheme may become an integral part of Pharmacopoeia standards describing insulin and be applied for quality control in the large-scale production of rhI.

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