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Recombinant human insulin VI☆. Determination of recombinant human proinsulin by capillary zone electrophoresis: optimization of efficiency and selectivity

Dmitry M. Koulich*, Igor V. Nazimov, Konstantin V. Maltsev, Andrey N. Wulfson

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, GSP V-437, Mikluho-Maklaya str. 16/10, 117871 Moscow, Russian Federation

Abstract

The optimization of the separation of recombinant human insulin (rhI) and recombinant human proinsulin (rhP) by free-solution capillary zone electrophoresis in uncoated fused-silica capillaries with ionic and zwitterionic buffers was carried out. The relationship between the selectivity and pH of the buffer was established. The effects of pH and ionic strength of the buffer on protein adsorption on the capillary walls and Taylor diffusion was investigated. The separation of rhI and rhP with an efficiency of 200 000 theoretical plates was achieved using 20 m*M* Na_2HPO_4–NaOH buffer (pH 11.2). The proposed method allows the simultaneously determination of rhP and some rhI degradation products (which are also included in pharmacopoeias) in pharmacopoeially limited amounts in rhI.

1. Introduction

Capillary zone electrophoresis (CZE) has proved to be a useful method for the high-resolution determination of proteins [1–3]. CZE is especially effective for the control and monitoring of recombinant protein production, where such features of CZE as high speed, low operating cost and reliability of equipment are important [4].

Recombinant human insulin (rhI) is an important pharmaceutical protein. In rhI biotechnological production, its certification according to the requirements of pharmacopoeias is necessary [5]. Recombinant human proinsulin (rhP) is an impurity whose concentration is strictly limited to 0.1% [6]. The contents of other impurities are between 1 and 3%. RP-HPLC separates rhI and all its pharmacopoeial impurities with high selectivity but low efficiency (ca. 10 000 theretical plates) [5]. The determination of minor species is invalid because of the low signal-to-noise ratio, which results in low statistical validity. For this reason, for pharmacopoeial purposes rhP must be determined using a more sensitive method. Today, radioimmunoassay and poly[illegible]amide gel electrophoresis (PAGE) with an overloaded track, which are labour consuming and slow, are used for this purpose.

The aim of this research was to explore the applicability of CZE to the determination of the rhP in rhI during the final purification and certification during rhI biotechnological product-

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* Corresponding author.

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ion. Uncoated capillaries were used. Although coated capillaries are promising for improving efficiency, they have some disadvantages that make uncoated capillaries more practical at present [7].

2. Experimental

2.1. Chemicals

Recombinant proteins were prepared in the Laboratory of Biotechnology of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, according to a scheme reported previously [5].

All chemicals were of analytical-reagent grade from Merck (Darmstadt, Germany) or Sigma (Munich, Germany). Reagent-grade water obtained from a Milli-Q purification system from Millipore (Bedford, MA, USA) was used to prepare all solutions.

2.2. Apparatus

A Model 270 A instrument (ABI, Santa Clara, CA, USA) was used, with a fused-silica uncoated capillary (52 cm × 100 μm I.D.) supplied by Beckman Instruments.

Buffer conductivity was determined using an LF 2000 microprocessor conductivity meter (WTM, Weilheim, Germany).

2.3. Buffers

Citrate, acetate, phosphate, borate, tricine {N-[tris(hydroxymethyl)methyl]glycine} and CAPS [3-(cyclohexylamino)-1-propanesulphonic acid] buffers were used in the pH optimization study. Concentrations were adjusted to the required ionic strength. Appropriate salts were dissolved and adjusted to required pH with 0.1 *M* NaOH or 0.1 *M* HCl during preparation of the buffer. All buffers were degassed and filtered through a 0.45-μm pore-size membrane (Millipore) prior to use.

2.4. Sample preparation

Samples were prepared by dissolving rhI and rhP in 20 m*M* acetate buffer or running buffer. All samples were centrifuged for 10 min at 5000 *g* before analysis.

2.5. Separation conditions

CZE was carried out at a voltage between 5 and 25 kV and a current between 20 and 120 A. The capillary was thermostated at 28°C. It was regenerated with 0.1 *M* NaOH prior to each run. If anomalous noise and baseline drift occurred, rinsing with potassium dichromate was used. Proteins were monitored spectrophotometrically at 214 nm. The sensitivity of the detector was 0.0004 absorbance.

2.6. Calculations

The selectivity was calculated using the equation

$$\alpha = \frac{(t_I - t_{EO})}{(t_P - t_{EO})}$$

where t_I is the migration time of rhI, t_P is the migration time of rhP and t_{EO} is the migration time of the electroosmotic flow marker.

The efficiency was calculated using the equation

$$N = 5.54 \cdot \frac{t^2}{W_{1/2}^2}$$

where t is the migration time of a peak and $w_{1/2}$ is the peak width at half-height.

3. Results and discussion

3.1. Optimization of selectivity

rhP is chemically similar to rhI and both proteins have isoelectric points in the pH region 5.3–5.5. The structure of rhP differs from that of rhI by a C-peptide, but it does not give rise to significant differences in Stokes radii or effective

charges because of the compact tertiary structure of the molecules.

General ways for optimizing the selectivity of CZE with uncoated capillaries are screening of the buffer pH and the use of different methods of physical and chemical modification of separated compounds or the hydrodynamics of the capillary [8].

Grossman et al. [4] and Nielsen and co-workers [9,10] used a zwitterionic buffer to increase the selectivity of the CZE separation of insulin-containing proteins. Addition of an ionic salt to the buffer was needed in order to diminish the protein adsorption on the capillary walls [11], which results in high Taylor diffusion [12] manifested by peak fronting. Kosinov et al. [13] used the addition of organic solvents to the buffer for the same purpose, but peak broadening was also significant. Both of the above methods improve the selectivity but worsen the efficiency of the CZE separation of insulin-containing proteins. However, a high efficiency is necessary for the determination of a minor component such as rhP in rhI.

Other approaches to physical or chemical modifications do not appear to have been reported. Therefore, we chose screening of the buffer pH as the main method for the optimization of selectivity.

Fig. 1a shows the relationship between buffer pH and selectivity of the CZE separation of rhI and rhP. These data agree well with the titration curves for rhI and rhP [14]. The sequence of migration of proteins changes at their isoelectric point: rhP moves ahead of rhI below pH 5, but rhI precedes rhP in basic buffers.

The profile of the electroosmotic flow-rate coincides with that of the migration time of proteins (Fib. 1b and c). The difference in the electroosmotic flow-rates in buffers with similar pH values, but different ionic strengths, is slight and does not affect the selectivity. Therefore, electroosmotic flow is not essential for the optimization of selectivity.

Dramatic increases in electroosmotic flow-rate, migration times of proteins and, therefore, selectivity occur at pH 11.5 (Fig. 1b and c). This phenomenon is not of practical importance,

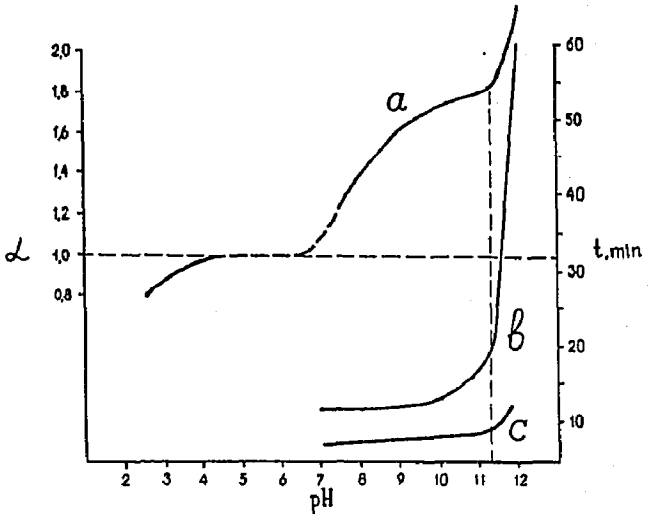

Fig. 1. Influence of buffer pH on (a) selectivity of CZE separation of rhI and rhP, (b) migration time of rhI as the last peak and (c) migration time of electroosmotic flow marker. Voltage, +10 kV; buffer conductivity, 4 mS/cm. The dashed line at pH 11.2 indicates a region of strong adsorption and conglomeration.

because there is corresponding decrease in efficiency. It could be due to physical or chemical modification of the protein, capillary hydrodynamics or buffer properties. For example, Kasicka and Prusik [15] predicted a dramatic increase in the effective negative charge of rhI in highly basic conditions.

3.2. Optimization of efficiency

The main causes of peak broadening in CZE are [11,12,16,17] adsorption of proteins on capillary walls, which depends on ionic strength and pH, and Taylor diffusion, which depends on ionic strength.

The efficiency of separations in buffers with acidic pH was extremely low (Fig. 2). Protein adsorption on capillary walls results in strong tailing of peaks.

Fig. 3 displays the relationship between buffer pH and ionic strength and the efficiency of the CZE determination of rhP (that of rhI is much lower). Data were obtained at a constant voltage of +10 kV. The injection length of the sample was minimized by using he shortest time of voltage injection available instrumentally, in

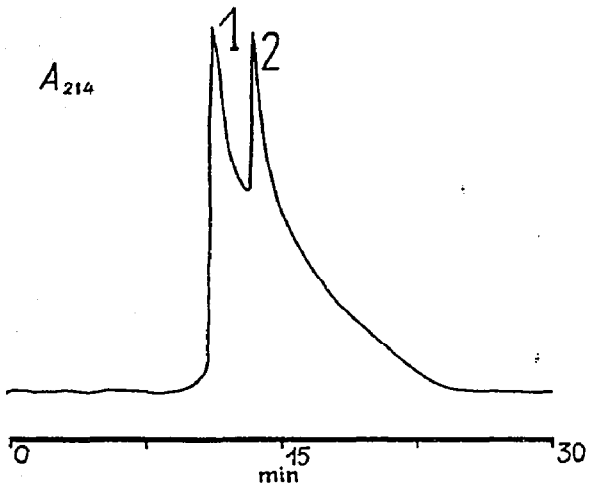

Fig. 2. CZE of (1) rhI and (2) rhP under acidic conditions. 20 m*M* citrate buffer (pH 2.5); voltage +10 kV.

order to increase the efficiency. Na_2HPO_4 was used for preparing buffers in the pH range 10.5–12 rather than Na_3PO_4, which was a higher ionic strength at this pH. The maximum of the surface area in Fig. 3 corresponds to the conditions for optimum separation efficiency. Under these conditions efficiency losses due to adsorption and diffusion are minimal. Efficiency losses due to adsorption prevail at pH or ionic strength values lower then optimum and losses due to Taylor diffusion prevailing in the opposite conditions. The dramatic decrease in efficiency at pH 11.5 is due to an increase in the migration times of proteins, which results in an increase in losses due to Taylor diffusion.

The choice of the optimum buffer for a 20 kV voltage results in a decrease in the optimum ionic strength and allows the efficiency to be increased up to 200 000 theoretical plates and the separation time to be decreased (Fig. 4). Further voltage increases with buffer dilution result in a decrease in efficiency because of increased adsorption.

3.3. Optimum conditions

The conditions for maximum efficiency and those for maximum selectivity coincide for the CZE separation of rhI and rhP. They are optimum for the determination of rhP in rhI.

The determination of the low content of rhP in a real sample of rhI requires an increase in the time of injection. This results in a lower efficiency but it does not prevent a precise determination (Fig. 5). Some other impurities reported in the pharmacopoeia could also be determined simultaneously.

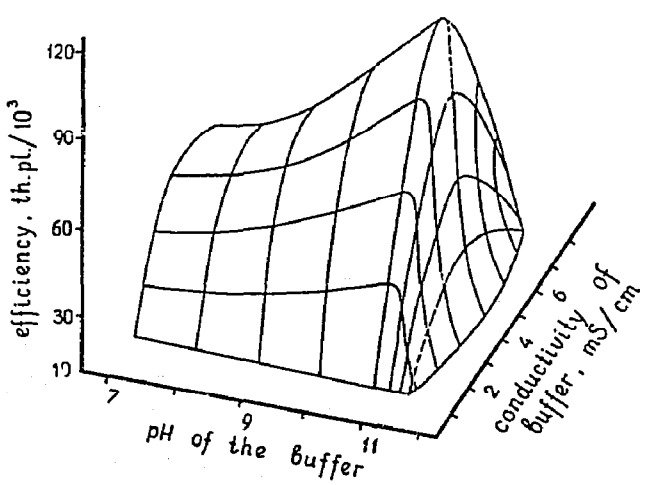

Fig. 3. Influence of buffer pH and ionic strength on the efficiency of the CZE determination of rhP. Voltage, +10 kV; sample concentration, 3 mg/ml; voltage injection, 0.1 s at +5 kV.

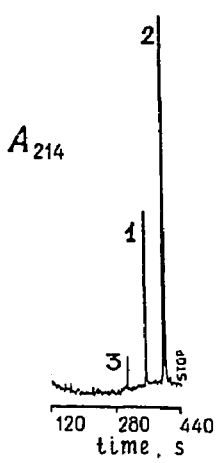

Fig. 4. CZE of (1) rhP and (2) rhI under optimum conditions; 3 = electroosmotic flow marker. 20 m*M* Na_2HPO_4 buffer (pH 11.2); voltage, +20 kv; rhI concentration, 3 mg/ml; rhP concentration, 0.5 mg/ml; voltage injection, 0.1 s at +5 kV.

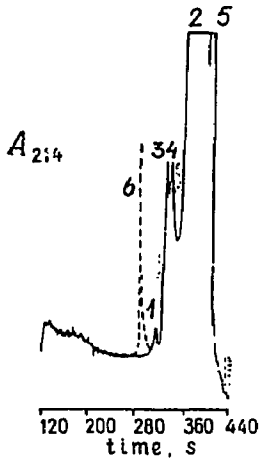

Fig. 5. CZE determination of 0.1% of (1) rhP in (2) rhI. 3, 4 = Unknown peaks coinciding in migration with peaks that were identified by Nielsen et al. [8] as diarginine (B31–B32) insulin and arginil(AO)insulin; 5 = desamido(A21)insulin; 6 = unidentified degradation product formed within 2 h of sample dissolution. Buffer and voltage as in Fig. 4; voltage injection, 8 s at + kV.

3.4. Dynamics of protein degradation under proposed conditions

The extreme pH value of the buffer proposed in this technique raises questions about protein resistance under these conditions. We therefore investigated the dynamics of the formation of degradation products in a sample of rhI in the above-mentioned buffer. Changes in the initial mixture give rise to an unidentified peak that migrates more slowly then rhP (Fig. 5), and negligible formation of other degradation products. By 2 h after dissolution or buffer changing of rhI in the buffer, the content of the unidentified peak reached 0.5% (Fig. 5). After 48 h it reached 1.5%, desamidoinsulin reached 3% and other degradation products reached 1%. Obviously rhI is relatively stable under these conditions. This is not exceptional and has been observed for some other proteins [16,17].

3.5. Comparison of proposed technique with recently used methods

As was mentioned in the Introduction, the main methods used at present for the precise determination of rhP in rhI are RP-HPLC [5], radioimmunoassay (RIA) [6] and PAGE with an overloaded track [8]. All these methods have a higher selectivity than that obtained by CZE in this work, but have significant disadvantages.

RIA is extremely labour consuming and expensive, although more precise. Therefore, this method should be used in complex cases. PAGE with an overloaded track is also labour consuming and does not ensure precise quantification. However, it is widely available and also the possibility of the simultaneous analysis of several samples has proved useful.

High-performance techniques such as HPLC and high-performance capillary electrophoresis (HPCE) are preferable in many instances owing to their substantial automation, which results in rapid analyses with precise quantification using different detection methods. As HPLC is not suitable for the determination of low contents of rhP in rhI (see Introduction), CZE is the method of choice for this purpose. In addition, the following specific features of CZE make this method more practical: high reliability of HPCE equipment owing to the absence of complicated, fragile and expensive units such as pumps, detector cells and column adaptors; low operating costs as capillaries are stable and inexpensive and the reagents used are also inexpensive and the amounts used are extremely low; and ecology, as CZE does not require any toxic compounds.

4. Conclusions

The proposed method allows the pharmacopoeially limited amounts of rhP in rhI to be determined significantly faster and more easily than hitherto. The high separation efficiency, selectivity, mass sensitivity and low operating costs make CZE promising for application in protein biotechnology, especially for pharmacopoeial determinations.

The relationship between buffer pH and the selectivity of the CZE separation of rhI and rhP obtained in this work is useful for separations of these proteins using both different modes of

zone electrophoresis and other methods exploiting differences in charge. Further, the optimum relationship between buffer pH and ionic strength and the efficiency of CZE determination of rhP allows the existence of such optima to be proposed for other proteins. The search for such optima may become a general approach to the optimization of CZE efficiency.

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