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Journal of Chromatography A, 807 (1998) 129–133

JOURNAL OF CHROMATOGRAPHY A

Short communication

Insulin stacking for capillary electrophoresis

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Abstract

Stacking methods are very important in overcoming the poor detection limits in capillary electrophoresis. Human insulin, a polypeptide, was concentrated on the capillary (stacked) based on three different and simple treatment methods to the sample: dilute buffers, high salt content, and acetonitrile (66%) were added to the sample to induce stacking. A dilute buffer in the sample caused a limited stacking, while acetonitrile treatment and high salt content in the sample caused much greater (~20-fold) stacking. High salt concentration in the sample caused stacking presumably by a transient isotachophoretic method. In addition to stacking, the acetonitrile treatment removed the excess proteins in the sample. Insulin did not denature or precipitate in 66% acetonitrile as confirmed by high-performance liquid chromatography (HPLC) and immunoassays. Acetonitrile treatment enabled one-third of the capillary to be loaded with sample thus increasing the detection signal greatly. The insulin peak after acetonitrile treatment and separation by capillary electrophoresis (CE) was confirmed by HPLC and by CE fraction collection followed by immunoassay. Based on acetonitrile treatment, insulin detection in pancreatic tissue homogenates is shown to be feasible. © 1998 Elsevier Science B.V.

Keywords: Sample stacking; Isotachophoresis; Insulin; Proteins

1. Introduction

Insulin analysis is important in many different areas such as checking the purity of pharmaceutical preparations, monitoring the extraction steps, study of the secretion of islets of Langerhans grown in tissue culture, etc. Capillary electrophoresis (CE) offers good separation but it suffers from poor detection limits owing to the short path-length of the absorbed light relative to high-performance liquid chromatography (HPLC). Simple stacking methods are crucial for enhancing the detection signal to render CE more useful in practical assays.

Previously, we have shown that the β-chain of insulin can be concentrated on the capillary ''stacked'' by dissolving it in acetonitrile–saline (66:34) mixtures [1,2]. Although we did not expect insulin itself to be soluble in 66% acetonitrile, insulin contains several hydrophobic amino acids, which bestows on this protein the characteristic of solubility in 60% ethanol [3]. Here, we show that insulin can be concentrated on the capillary by acetonitrile and furthermore the collected peak has an immunoreactivity against insulin antibodies. We show that insulin, under appropriate conditions, can be stacked in both diluted buffer solutions [4–6] and also, unexpectedly, in the presence of high concentrations of NaCl.

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0021-9673/98/$19.00
PII: S0021-9673(98)00055-7

2. Materials and methods

2.1. Chemicals

2.1.1. Human insulin

In most of the work Humulin insulin 100 U/ml (~4 mg/ml) (El:Lilly, Indianapolis, IN, USA) was used. However, in some experiments NPH Iletin I, Isophane insulin 100 U/ml (~4 mg/ml) (El:Lilly) and Novolin insulin (Nordis, Bagsaerd, Denmark) were used too and gave the same results as Humulin insulin.

Beef insulin (27 U/mg protein) was obtained from Sigma (St. Louis, MO, USA).

2.2. Electrophoresis buffer

Daily a fresh buffer 660 m*M* 2[N-cyclohexyl-amino]ethanesulfonic acid (CHES), 45 m*M* acetate, adjusted to pH 7.8 with triethanolamine and containing 10% acetonitrile was prepared [7].

2.3. Tissues

Pancreatic tissue (0.3 g) from autopsy was homogenized in 1 ml saline (1%) and centrifuged for 60 s at 14 000 *g*. An aliquot of 200 μl of the supernatant was mixed with 400 μl acetonitrile and centrifuged. The supernatant was evaporated under air to dryness and reconstituted with 50 μl of saline and 100 μl acetonitrile.

2.4. Acetonitrile treatment

From samples of insulin (diluted in 1% NaCl), 50 μl was mixed with 100 μl acetonitrile, vortex-mixed for 15 s and centrifuged.

2.5. CE instrument

A Model 2000 CE (Beckman, Fullerton, CA, USA) was set at 16.5 kV and 214 nm. The sample was introduced hydrodynamically on a 42 cm×50 μm I.D. untreated silica capillary for 30 s (or as specified) and electrophoresed for 10 min.

2.6. HPLC

We used a pump Model 112 (Beckman) and a Model 342 detector set at 214 nm (Beckman). The sample, 25 μl was injected on a CO3-MP column (Aquapore 7 μm, RP 300, 100×4.6 mm; Brownlee, Sana Clara, CA, USA). The column was eluted

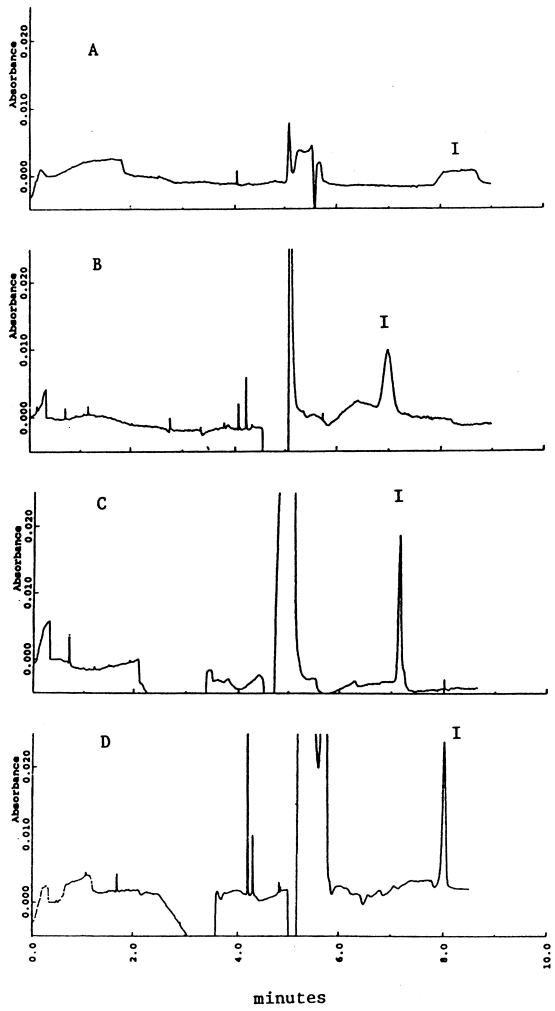

Fig. 1. Analysis of insulin (80 mg/l) by CE under different type of stacking conditions at injection of 10% of the capillary volume. (A) Nonstacking (the sample dissolved in the separation buffer). (B) Low ionic strength buffer stacking (the sample dissolved in a tentimes diluted separation buffer). (C) High ionic strength stacking (the sample dissolved in 1% NaCl). (D) Acetonitrile stacking (the sample dissolved in one volume of 1% NaCl and two volumes of acetonitrile); (I=insulin peak).

isocratically with 17% acetonitrile in 0.1% phosphoric acid.

2.7. Immunoassay

The analysis was performed on the automated instrument, IMX (Abbott, Deerfield, IL, USA), based on microparticle enzyme immunoassay technology. The samples were diluted in a serum pool low in insulin. The monoclonal antiinsulin coated microparticles were aspirated and mixed automatically with the diluted samples. The insulin bound to the antiinsulin coated particles is transferred to the glass fibers and washed. An alkaline phosphatase conjugate is added to the bound complex and the fluorescence of the substrate 4-methylumbelliferyl phosphate is finally measured. All these steps were performed automatically by the instrument.

2.8. Injection volume

The sample volume as % of the capillary total volume was determined as described earlier [8].

3. Results and discussion

Mandrup [7] has determined the optimum conditions for insulin analysis by CE. Among the different buffers they found acetate with CHES as a zwitterion, and acetonitrile as a modifier, yielded a high plate number for the assay. This buffer in addition to controlling the pH has several additional advantages, such as decreases in the protein binding to the capillary walls, and favors stacking in general. Thus, we used the same buffer in this study. Under nonstacking conditions, using small injection volume

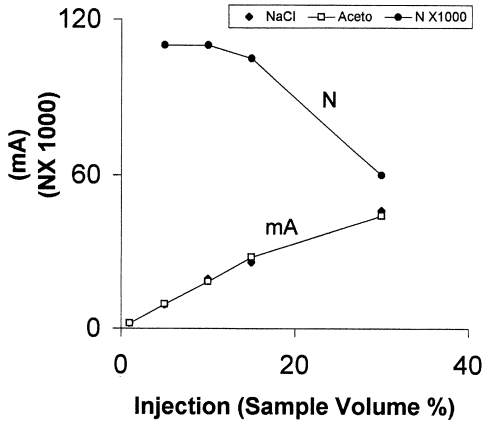

Fig. 2. Effect of sample volume on absorbance (mA) and plate number (*N*) (samples in 1% NaCl or acetonitrile).

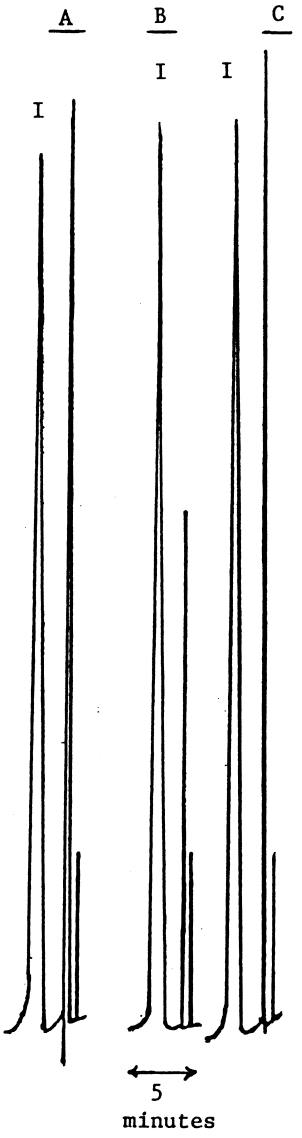

Fig. 3. Analysis of insulin by HPLC. The sample (80 mg/l) was dissolved in: (A) 1% NaCl (one volume) and acetonitrile (two volumes); (B) 1% NaCl; and (C) water.

(1% of the capillary), insulin yields a good peak shape, but the signal-to-noise ratio is very poor. Increasing the sample size tenfold caused sample overloading with a poor peak shape (Fig. 1A). Stacking by diluting the sample in a weak buffer [5,6] was slightly but not much better, (Fig. 1B). Unexpectedly, dissolving the insulin in 1% NaCl gave much better stacking (Fig. 1C). High ionic strength in the sample causes band diffusion except where a transient isotachophoretic step is involved [9–11], which may occur accidentally [12]. The chloride in the sample seems to act, for a short period, as a leading ion.

Proteins precipitate in acetonitrile, while many small molecules including the peptide chain of β-insulin stack well [1]. Surprisingly, if one volume of insulin solution (dissolved in 1% NaCl) is mixed with two volumes of acetonitrile the insulin peak also stacks well (Fig. 1D) similar to that for the peptides and the small molecules [1,2]. The sharpness of the insulin peak (N=~120 000) in the untreated capillary resembles that of a small molecule rather than a protein. We were able to load about one-third of the capillary with sample Fig. 2. The NaCl present in the sample can be replaced by other small ions such as NaBr or NaI. The ability of insulin to withstand acetonitrile without precipitation was not expected and even suspicious especially since insulin solutions are not that pure.

Thus, in order to prove that the observed peak is indeed insulin we used different approaches. We injected insulin treated with acetonitrile on the HPLC (Fig. 3A). The peak has the same migration and height as insulin diluted in saline or water (Fig. 3B and C) indicating that insulin survives the acetonitrile treatment with recovery close to 100%. Secondly we treated insulin (one volume) with acetonitrile (two volumes) and analyzed it by immunoassay directly. We were also surprised again to see the insulin did not just simply survive the acetonitrile but it had about twice the activity. It seems the acetonitrile treatment exposed some of the antigenic sites making it more accessible for the antibody. Thirdly we injected insulin 280 mg/l on the capillary and

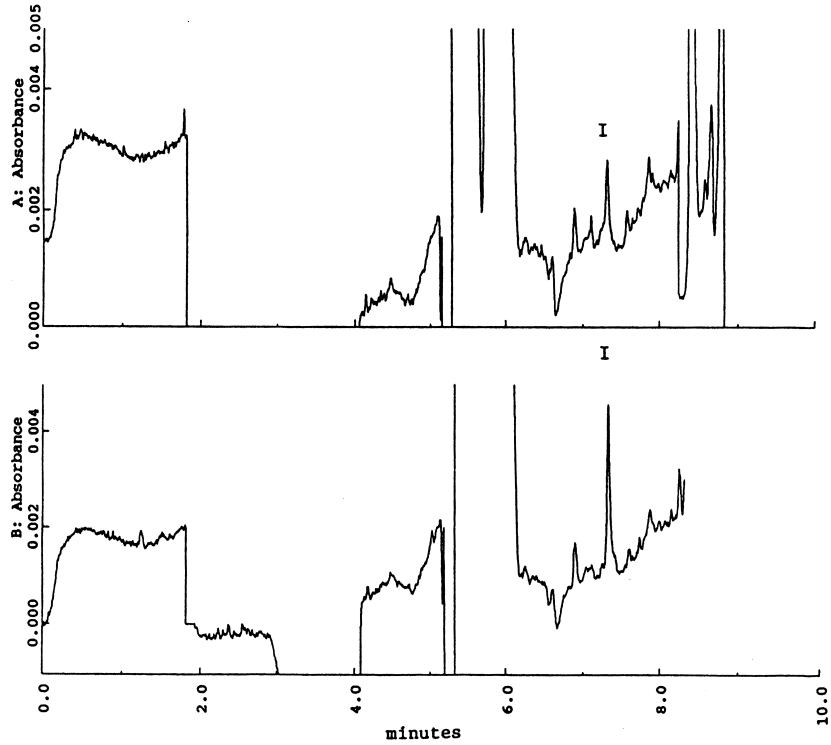

Fig. 4. (A) Analysis of insulin in pancreatic tissue homogenate; (B) after spiking with insulin 8 mg/l; (I=insulin peak).

collected the peak. We were able to account for 78% of the injected insulin in the peak. Fortunately the immunoassay is so sensitive that the tiny collected CE fractions have such a high activity that it required further dilution before the assay. Pure bovine insulin, dissolved in saline solutions, comigrated with human insulin. It is also stacked in NaCl solutions. Together, these data indicate that the detected peak is indeed insulin. Thus, human insulin survives acetonitrile treatment and can be stacked in CE behaving as a peptide.

Water, saline and acetonitrile all caused insulin stacking (Fig. 2). By using acetonitrile or saline in the sample about one-third of the capillary can be loaded giving about a 20-fold increase in detection signal. Acetonitrile treatment is the most useful method for stacking from a practical aspect since it removes the excess proteins in biological fluids or tissue. Insulin concentrations are very low in tissues and especially serum. To illustrate the potential of the stacking by acetonitrile, insulin was detected in homogenized human pancreas tissues Fig. 4. Based on the peak height calculation, the pancreas tissue contained by CE about 0.032 mg/g wet mass tissue while by the enzyme immunoassays it contained about 0.080 mg/g wet mass. The difference between the two calculations can be related to better solubilization (high dilution by immunoassays) and detection of proinsulin and other related peptides. Based on Fig. 4, the limit of detection for insulin is about 4 mg/l, which is better than that of nonstacking and closer to that reported for HPLC [13]. This study illustrates the different methods of stacking which can enhance detection in CE for insulin and peptides in general.

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