Rugged method for the determination of deamidation products in insulin solutions by free zone capillary electrophoresis using an untreated fused-silica capillary

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ABSTRACT

Addition of zwitterions and acetonitrile to the running buffer provided a successful separation of insulin and deamidation products in an untreated fused-silica capillary. A high efficiency and reproducible migration times were obtained without a base wash between each run and without thermostating of the capillary. A comparison was made of results achieved using capillary zone electrophoresis (CZE), ion-exchange chromatography (IEC) and reversed-phase high-performance liquid chromatography. A good correlation was observed between CZE and IEC.

INTRODUCTION

The aim of this study was to find a rugged and reproducible method for the determination of deamidation products in insulin solutions. The most common technique used for analysing the neutral and the acidic desamido compounds in insulin samples is disc electrophoresis [1]. Ion-exchange chromatography (IEC) is also a very useful method for the determination of desamido products in insulin, but a faster and less time-consuming, and also less expensive, method is desirable. As capillary zone electrophoresis (CZE) seemed to be a good alternative to the above two methods, experiments with CZE were started. Furthermore, CZE apparatus requires a minimum of maintenance and is easy to operate. A readily accessible and reproducible application of CZE was developed.

In this study human insulin (HI) was used as the main component. The molecular mass of HI is 5808 [2] and the isoelectric point is 5.5 [1–4]. The primary structure of HI was published by Nicol and Smith in 1960 [5], and the structure is shown in Fig. 1. HI consists of two peptide chains, A and B. The A-chain and B-chain contain 21 and 30 amino acids, respectively, connected through two interchain disulphide bridges. On standing, insulin in solution decomposes owing to deamidation at rates depending *inter alia* on the storage temperature. Desamido-insulin generated in acidic medium is hydrolysed in position A21 and monodesamido-A21-insulin is formed [1,7], which in the following is referred to as acidic desamido-insulin. The product formed at neutral pH is deamidated in the B-chain by hydrolysis of Asn-B3, and in the following is referred to as neutral desamido-insulin.

Several strategies have been developed to overcome the problems resulting from adsorption of proteins on the silanol surface of fused-silica capillaries. Of the numerous approaches to prevent the protein from sticking to the wall, this work was focused on changing the pH of the running buffer and using additives in the buffer. At near neutral pH,
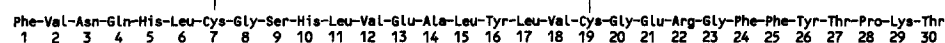
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Human insulin
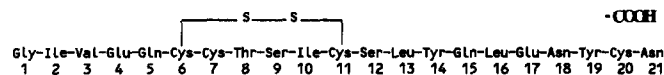
A-chain

Fig. 1. Primary structure of human insulin [6].

the negatively charged exposed silanol groups in the bare silica capillary often cause protein samples to adsorb on the column walls. This silanol–protein attraction results in low-efficiency separations. At pH below 3, the silanols are fully protonated and the negative charge of the capillary surface is removed, so the problem of adsorption can be reduced. Buffers with a very low pH are generally unsuitable because of their high conductivity, which limits the use of high voltages. Working at high pH alters the charge of the protein, so that no significant regions of positive charge exist.

In addition to the problems caused by the high conductivity of the electrolytes, there will often be problems with the stability of the proteins under extreme pH conditions. To maintain optimum stability of the analyte, a buffer system with a pH in a suitable range for the protein is required.

Zwitterions consisting of sulphonic acid and quaternary amino groups have previously been investigated as buffer additives for use in CZE [8,9]. Zwitterions could be substitutes for the cation and its anion. Becuase a zwitterion contains no net charge, it has no net conductivity. Zwiterions will not contribute to the conductivity of the operating buffer, but are able to associate with the negatively charged surface of the capillary and with charged sites of the protein. Therefore, zwitterions are effective in reducing the interaction of proteins with the surface of a fused-silica capillary at neutral pH.

In 1989, CZE and reversed-phase high-performance liquid chromatographic (RP-HPLC) studies of biosynthetic human insulin (BHI) and its degradation were reported to give similar information [10]. Using a tricine buffer with morpholine and sodium

chloride at pH 8, Nielsen *et al.* [10] separated BHI and acidic desamido-insulin. The buffer was replaced after every four to six runs. The smallest amount of acidic desamido-insulin determined by CZE was 9.7% of desamido compound as a percentage of total protein. This method was subjected to a critical examination in a parallel paper [11]. Later Lookabaugh *et al.* [12] described the determination of insulin using the same tricine buffer, with mixed results. The typical number of theoretical plates obtained with this application was 40 000.

The determination of insulin and related compounds has been accomplished with very reproducible results by RP-HPLC and IEC. The yield and purity were determined routinely at the different stages during the process of manufacturing insulin. In addition to large amounts of insulin-related substances, the separation of only the acidic desamido-insulin was obtained using RP-HPLC, whereas both acidic and neutral desamido-insulin of HI were separated by IEC. Addition of a different separation mechanism such as CZE gives valuable complementary information when validating the quality of a product. This paper presents a comparison of results obtained by the three different techniques.

EXPERIMENTAL

Materials

HI and acidic human desamido A-21 were obtained from Novo Nordisk (Bagsværd, Denmark). Sodium acetate, methanol, ethanol, triethylamine, ethanolamine, sodium hydroxide, sodium sulphate, phosphoric acid, acetic acid and hydrochloric acid

were purchased from, Merck (Darmstadt, Germany). CHES [2-(N-cyclohexylamino)ethanesulphonic acid], BES [N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid], AMPSO {3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid}, CAPSO [3-(cyclohexylamino]-2-hydroxy-1-propanesulphonic acid] and trizma base were obtained from Sigma (St. Louis, MO, USA). AccuPure Z1-Methyl (trimethylammonium propanesulphonate) was supplied by Waters (Waters Chromatography Division, Millipore, Milford, MA, USA). Acetonitrile was obtained from Rathburn (Walkerburn, UK).

Purified water obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA) was used to prepare all solutions.

Equipment

Most of the experiments were performed on Quanta CZE apparatus from Waters. The Quanta CZE apparatus was equipped with untreated fused-silica capillaries of total length 60 cm (52.5 cm top detection) and three different inside diameters. Hydrostatic injection was performed by raising the injection end 10 cm relative to the detector end for various intervals measured in seconds. UV absorption was measured at 214 nm using a time constant of 0.1–1.0 s. Data were collected at a rate of 5 data points per second. The electrophoretic data system was a Waters 860 Data system operated on a Micro-Vax 3300 computer (Digital Equipment, Maynord, MA, USA).

To investigate the influence of controlling the outside temperature of the capillary, a P/ACE System 2050 CZE apparatus (Beckman, Palo Alto, CA, USA) was used. The samples were introduced on to this CZE unit by pressure (0.5 psi) for 3–5 s. Detection was effected at 214 nm. The temperatures was varied from 5°C below room temperature to 50°C.

Capillary zone electrophoresis

Prior to use, a fused-silica capillary was prepared by washing with 0.5 M sodium hydroxide solution for 30 min and running buffer for 15 min. Before each run the capillary was flushed with running buffer for 2 min. Between series of runs (typically 30–50 runs) and before new buffers were introduced, the capillary was treated with series of sodium hydroxide solutions and hydrochloric acid. The

buffer in the electrode reservoirs was replaced after every 30–50 runs. When the capillary was stored for more than 24 h, it was flushed with 10% aqueous methanol.

The composition of the optimized running buffer was 50 mM sodium acetate and 0.85 M CHES in 10% aqueous acetonitrile adjusted to pH 7.8 with triethylamine.

The concentration of insulin in the introduced standards and samples was ca. 1 mg/ml.

Reversed-phase high-performance liquid chromatography

The RP-HPLC separation of HI and acidic desamido-insulin was performed on a C_{18} silica-based column packed with 5-μm, 120-Å particles. Eluent A consisted of 0.2 M sodium sulphate and 0.04 M phosphoric acid in 10% acetonitrile with the pH adjusted to 2.5 with ethanolamine. Eluent B was 50% acetonitrile. At approximately 28% acetonitrile the retention time of HI is 20 min. The temperature of the column was held at 50°C during the isocratic separation at a flow-rate of 1 ml/min. The components were detected at 214 nm. These conditions are used routinely in our in-process quality control of insulin.

Ion-exchange chromatography

HI and acidic and neutral desamido-insulin were separated using a Mono Q HR 5/5 anion-exchange column obtained from Pharmacia (Uppsala, Sweden). Eluent A was 18 mM Tris–4.5 mM acetic acid–53% ethanol (pH 8.4). Eluent B had the same composition as eluent A but with 0.33 M sodium acetate was added. The components were separated by gradient elution from 20 to 100% B in 30 min at a flow-rate of 1 ml/min with UV detection at 280 nm. The temperature of the column was ambient.

RESULTS AND DISCUSSION

Developing the running buffer

As mentioned previously, working with proteins using CZE in practice generally causes many problems. During the development phase of this application for insulin, several buffer systems such as phosphate, tricine and borate were tested. None of the buffers seemed to give the desired separation or reproducibility.

The effect of the cation and anion in the buffer on mobility in CZE was investigated by Atamna and co-workers [13,14]. When selecting a buffer for CZE studies, some of the issues to consider are the native pH of the solution and the conductivity. It has been reported that the migration time increases with increase in the atomic mass of the cation. The various anions turned out to give widely different currents at the same applied voltage. The observed current for sodium acetate was less than, e.g., that for potassium acetate or sodium phosphate. It is important to know the values of the current in order to control the heat generated in the capillary [15]. The combination of sodium as the cation and acetate as the anion showed values interesting for this study of insulin. Therefore, sodium acetate (NaAc) was chosen as the running buffer.

Experimental runs showed that buffers without additives do not prevent the interaction between the capillary wall and the protein. For this reason, the addition of several zwitterions to the electrolyte was tested. Electropherograms obtained using selected zwitterions are shown in Fig. 2. The choice of zwitterions depends on the following practical points:

(1) separation, (2) efficiency, (3) reproducible migration times, (4) solubility and (5) easy to reproduce with respect to pH and obtaining the same conductivity during the preparation of the running buffer. Observations regarding some of the zwitterions tested are listed in Table I. CHES was shown to fulfil the requirements mentioned, the other additives giving poor results with respect to at least one of the above points.

Adding organic solvents to the buffer improves the plate count considerably. The effect of adding methanol and acetonitrile to the buffer is shown in Table II, where the chromatographic data obtained are listed. Because of the improved efficiency (384 000 plates/m for HI) and resolution, acetonitrile was selected as the organic modifier. Adding acetonitrile to the buffer gave even better solubility of CHEs and also a more stable pH adjustment when preparing the electrolyte.

Optimization of the pH depends on the selectivity, resolution, efficiency and maximum acceptable current. Another important point to be aware of when optimizing pH is the sample matrix. First, the optimum pH for this buffer was determined to be
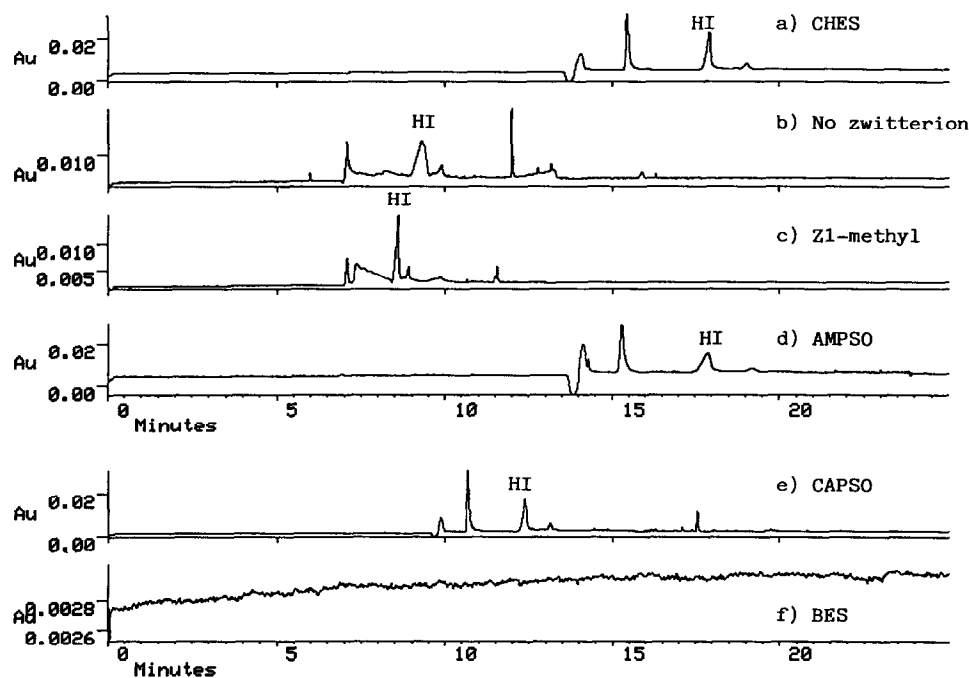

Fig. 2. Electropherograms of a formulation product of HI obtained using sodium acetate buffer with different additives. Detailed running conditions and information are given in Table II.

TABLE I

PRACTICAL POINTS OBSERVED WITH DIFFERENT ADDITIVES IN RUNNING BUFFER

Running buffer: 50 mM sodium acetate–10% acetonitrile.

Curve in Fig. 2	Additive	Parameter[a]						Comments
		I	II	III	IV	V	VI	
a	0.85 M CHES	6.53	2.42	7.76	3.01	15	48	Buffer easy to prepare Reproducible electropherograms
b	None	8.20	3.39	7.76	3.50	15	54	Irreproducible electropherograms
c	0.85 M Z1-methyl	7.96	2.92	7.78	3.21	15	45	Very sensitive to pH adjustment Irreproducible electropherograms
d	0.85 M AMPSO	5.65	2.28	7.76	3.38	15	53	Irreproducible electropherograms
e	0.50 M CAPSO	6.11	3.03	7.80	3.33	15	44	Low solubility Irreproducible electropherograms
f	0.85 M BES	5.67	3.01	7.79	14.51	5	66	No peaks

[a] I = pH in native solution; II = conductivity (mS) in native solution; III = pH after adjustment with triethylamine–acetic acid; IV = conductivity (mS) after pH adjustment; V = applied voltage (kV); VI = current (μA).

6.5. At this pH there is a good resolution between HI and desamido-insulin in a standard solution and the current is low. Also, very little pH adjustment in the buffer is necessary. When introducing a sample on to the capillary, additional peaks between the main peak of interest and the electroosmotic flow (EOF) front were discovered. To obtain a good separation of all the peaks, the pH was adjusted to pH 7.8. Fig. 3 shows the electropherograms of a formulation product obtained with buffers in the pH range 2–9.

Different concentrations of sodium acetate and CHES in the running buffer were examined. Electropherograms obtained under the various conditions are exhibited in Fig. 4. The content of the buffer ion is proportional to the resolution of the peaks. In addition, an increasing content of sodium acetate results in increased migration times. Concentrations of sodium acetate of 75 and 100 mM give very good separations of the components, but at the same time also more noise on the baseline, due to the high current (only obvious at an enlarged range). Increased amounts of CHES enhance the efficiency and give longer migration times. At 1 M concentration, CHES is difficult to dissolve in the electrolyte.

The buffer for the separation of HI and at least two forms of desamido-insulin was finally optimized to contain 50 mM sodium acetate, 0.85 M CHES and 10% acetonitrile (pH 7.8). A typical set

of electropherograms for a formulation product of insulin and the corresponding reference standards of HI and acidic desamido-insulin is presented in Fig. 5. In the case of the sample solution the peak eluting near the EOF front is attributable to a preservative which was added to the the formulation.

Influence of base washing and replication of injections

It is often pointed out that base washing between runs is necessary to obtain reproducible migration times with untreated fused-silica capillaries [16]. The ruggedness of this method was investigated by several consecutive injections of a sample on to the capillary.

Using the P/ACE unit the temperature was set at 30°C. Several injections were made without base washing of the capillary, followed by nine injections made with base washing between each injection (2 min with 0.1 M sodium hydroxide solution). Results showing the reproducibility are presented for 36 successive runs of a formulation product using the optimized buffer. As shown in Table III, the method is highly reproducible with respect to the migration time, also without base washing. In spite of the large number of injections the relative standard deviation (R.S.D.) of the migration times is excellent (below 1%). Considering the number of injections, the decrease in efficiency of the HI peak is acceptable (from 503 000 to 265 000 plates/m).

TABLE II

EFFECT OF ADDING ORGANIC MODIFIER TO ELECTROLYTE

Electrolyte: 50 mM sodium acetate–0.85 M CHES (pH 7.8).

Organic modifier	Voltage (kV)	Current (μA)	HI migration time (min)	Plate count (10^3 N/m)[a]			Resolution[c]			Selectivity[d]		
				HI	Acidic des.[b]	Neutral des.	HI	Acidic des.	Neutral des.	HI	Acidic des.	Neutral des.
None	15	46	12.79	121	377	425	2.166	1.780		1.032	1.017	
None	25	103	5.88	78	43	353	–	–		–	–	
10% methanol	15	43	18.63	272	343	192	3.591	1.655		1.044	1.020	
10% methanol	25	97	8.03	189	109	553	2.971	1.258		1.049	1.020	
10% acetonitrile	15	46	15.26	384	576	146	4.846	2.187		1.047	1.018	
10% acetonitrile	25	102	6.67	169	–		–	–		1.051	1.016	

[a] Plate count per metre: $N = 5.54 (t/w_{1/2})^2$. Dimensions of capillary: 60 cm × 75 μm I.D., 52.2 cm to detection.

[b] des. = desamido-insulin.

[c] Resolution: $R = 2(mt_2 - mt_1)/(w_2 - w_1)$, where mt = migration time, w = peak width at baseline using tangent lines drawn through 50th percentile points until they intercept the baseline.

[d] Selectivity: $a = (mt_2 - v)/(mt_1 - v)$, where v = void volume. Note: as the selectivity is compared relatively, v is taken as a constant.
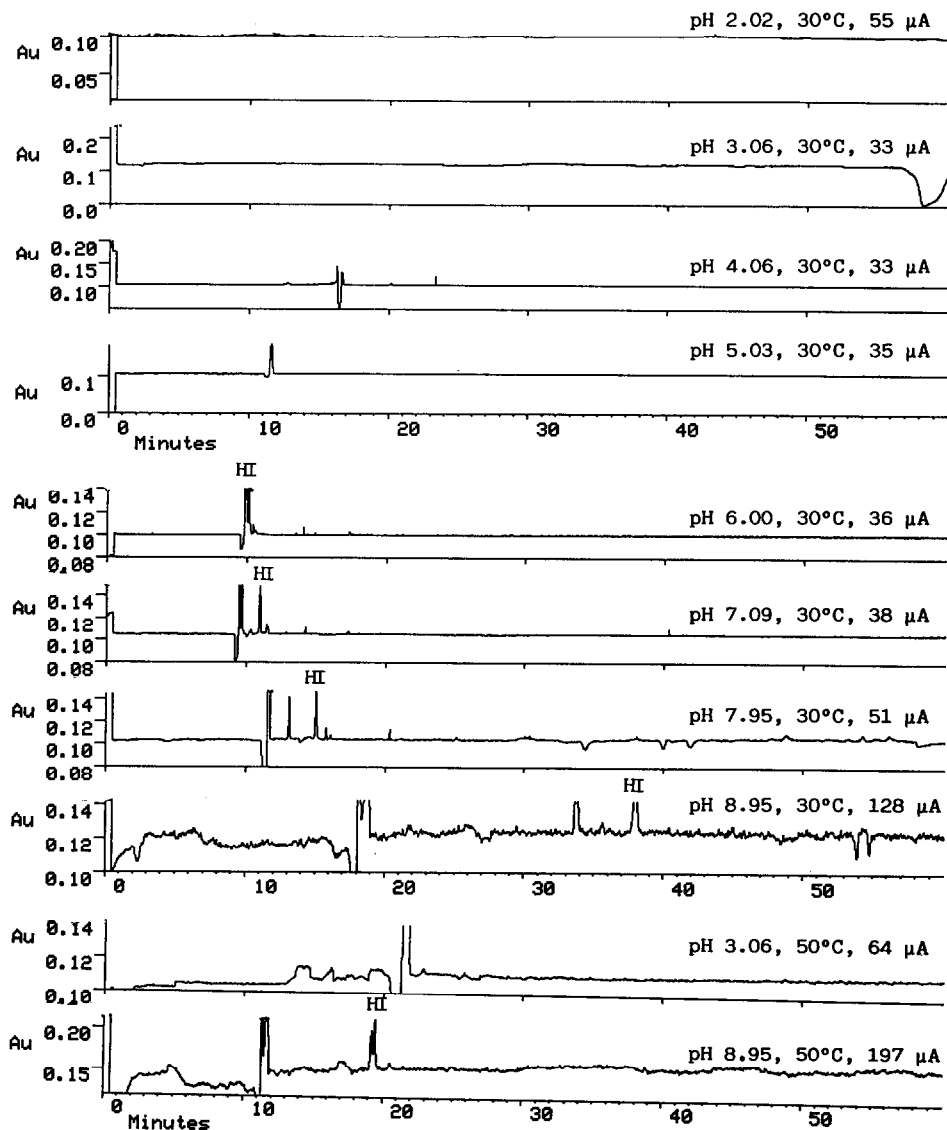
Fig. 3. Electropherograms showing the effect of pH in running buffer (50 mM NaAc–0.85 M CHES–10% CH$_3$CN). The applied voltage is 15 kV in all instances. The electropherograms were obtained with the P/ACE 2050 unit at 30 and 50°C.

With the Quanta CZE appratus eighteen continuous injections were made. This experiment was carried out without control of the external temperature of the capillary and without flushing with base between each run. The statistical data are given in Table IV. Also under these conditions the R.S.D. for the migration time is excellent (0.38%), especial-

ly in view of the number of injections executed.

The ruggedness of this method is underlined by these experiments on two different types of CZE apparatus. The migration times exhibited an R.S.D. in the order of 1% or less even without base washing and without thermostating the capillary.
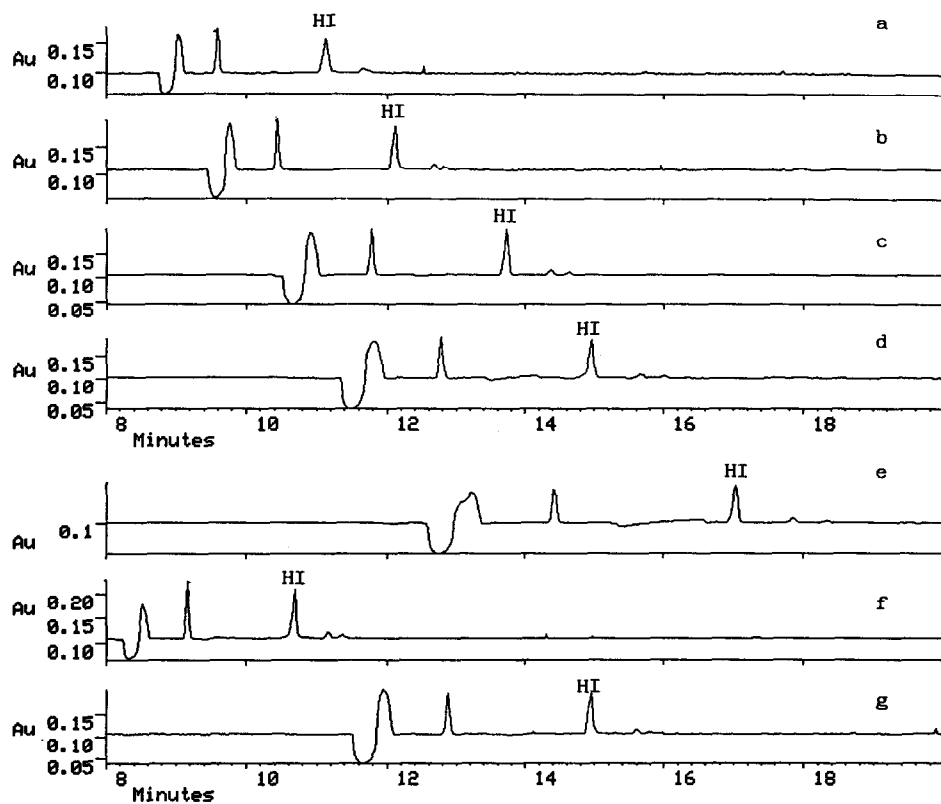
Fig. 4. Analysis of a formulation product of HI using various running buffers. Applied voltage, 15 kV; temperature, 30°C (P/ACE 2050). (a) 10 mM NaAc–0.85 M CHES–10% CH$_3$CN (pH 7.77) (17 μA); (b) 25 mM NaAc–0.85 M CHES–10% CH$_3$CN (pH 7.79) (27 μA); (c) 50 mM NaAc–0.85 M CHES–10% CH$_3$CN (pH 7.79) (45 μA); (d) 75 mM NaAc–0.85 M CHES–10% CH$_3$CN (pH 7.76) (60 μA); (e) 100 mM NaAc–0.85 M CHES–10% CH$_3$CN (pH 7.77) (81 μA); (f) 50 mM NaAc–0.5 M CHES–10% CH$_3$CN (pH 7.80) (48 μA); (g) 50 mM NaAc–1 M CHES–10% CH$_3$CN (pH 7.73) (43 μA).
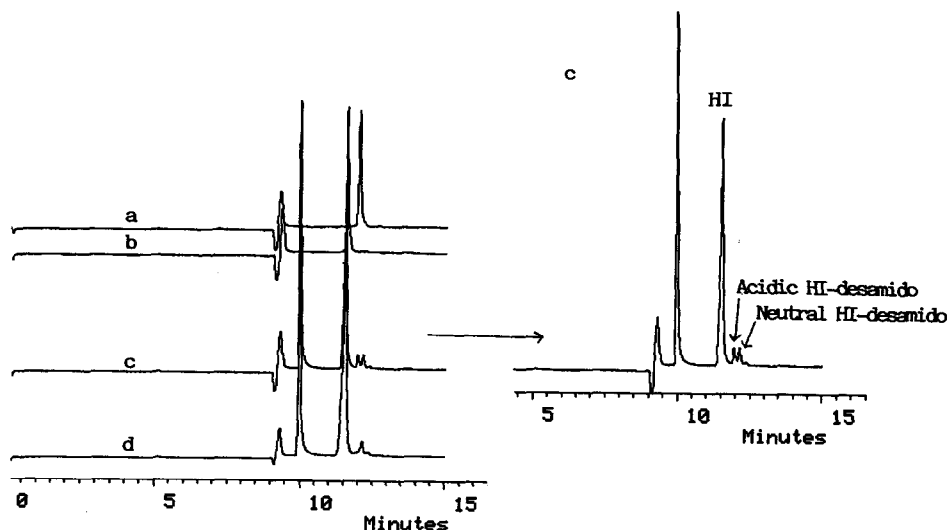

Fig. 5. Sample with corresponding reference standards. (a) Acidic desamido-insulin; (b) HI; (c) formulation product of HI (stored at 4°C for 15 months) containing standard of acidic desamido-insulin; (d) same sample as (c) but without acidic desamido-insulin added.

TABLE III

REPLICATION OF INJECTIONS AT 30°C (P/AGE 2050).

Statistical values for HI. Running buffer: 50 mM sodium acetate–0.85 M CHES–10% acetonitrile (pH 7.75) (15 kV, 45 μA).

Base wash between runs	Injection No.	Migration time (min)		Plate count ($10^3 \cdot N/m$)[a]		
		Mean	R.S.D. (%)	Mean	R.S.D. (%)	Range
No	1–27	12.413	0.61	382	14.69	503–322
No	1–9	12.452	0.17	466	7.62	503–399
No	10–18	12.410	0.98	363	2.90	376–349
No	19–27	12.377	0.20	351	5.77	385–322
Yes	28–36	12.457	0.51	288	6.65	317–265

[a] Plate count per metre: $N = 5.54(t/w_{1/2})^2$.

Additional optimization aspects

The influence of temperature control as a separation parameter was briefly studied. Fig. 6 shows electropherograms of a sample analysed at 30 and 50°C. Except for a shorter time of analysis at the higher temperature, no other advantages were obtained by raising the temperature. In this application no difference in selectivity was observed at the two temperatures. A higher current was observed with the higher temperature.

Capillaries with of I.D. 50, 75 and 100 μm were tested and the results obtained are shown in Table V. When deciding which inside diameter to use, the demands on sensitivity, need for resolution and efficiency, current, etc., should be considered.

As also observed in Table V, the amount of compound injected (which in this instances is directly proportional to the time of injection) is important when evaluating efficiency. A 1 mg/ml concentration of HI was introduced into the capillary (75 μm I.D.) for 1–20 s. The volume introduced into the

capillary by hydrostatic injection was determined as ca. 1.4 nl/s (calculated using eqn. E3.7 in ref. 17). Depending on the injection volume, the range of the amount of HI injected was 1.4–28 ng. Fig. 7 shows the relationship between the time of injection and the plate count achieved. The efficiency decreases with increasing amount of sample applied to the capillary. The correlation between time of injection and efficiency of the HI peak shows linearity with r^2 = 0.9510, whereas r^2 = 0.9985 for the correlation between time of injection and peak area.

In order to achieve optimum utilization of the analysis time, four injections of a sample were made at 10-min intervals. The electropherogram is shown in Fig. 8. As shown, four samples are analysed in 50 min instead of four times 20 min.

Quantitative aspects

As described earlier, the precision of multiple injections was excellent for the reproducibility of migration times. Also, the R.S.D. of the peak area of

TABLE IV

REPLICATION OF EIGHTEEN INJECTIONS WITHOUT A BASE WASH BETWEEN RUNS AND WITHOUT TEMPERATURE CONTROL

Statistical values for HI. Running buffer: 50 mM sodium acetate–0.85 M CHES–10% acetonitrile (pH 7.76) (15 kV, 45 μA).

Migration time (min)		Plate count ($10^3 \cdot N/m$)[a]			R.S.D. (%)	
Mean	R.S.D. (%)	Mean	R.S.D. (%)	Range	Area	Height
13.177	0.38	333	2.39	346–325	1.75	1.79

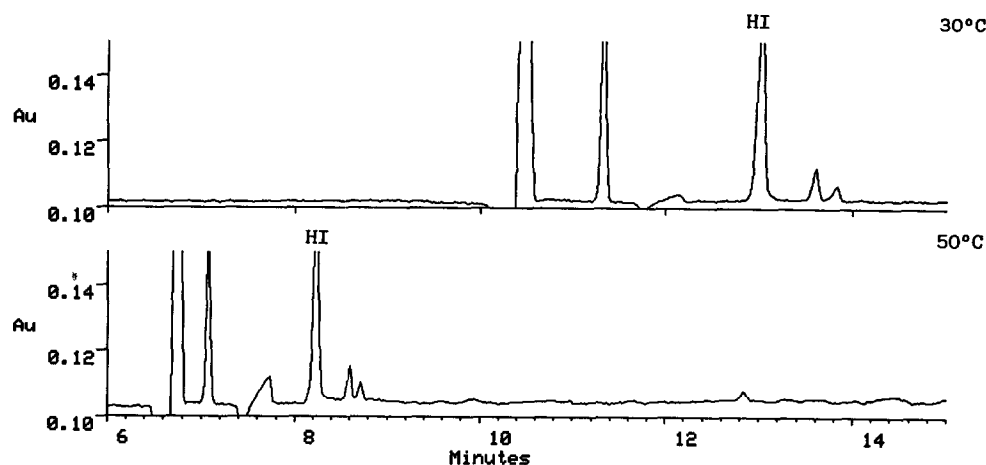[a] Plate count per metre: $N = 5.54(t/w_{1/2})^2$.

Fig. 6. Sample analysed at 30 and 50°C (P/ACE 2050). Running buffer: 50 mM NaAc–0.85 M CHES–10% CH$_3$CN (pH 7.75).

HI was satisfactory (Table IV). In addition to validation of precision, other important performance characteristics such as linearity and detection limit for the application were evaluated.

The linearity of response for HI was evaluated by varying the amount of material introduced into the capillary. First, the time of injection was held at 5 s while the concentration of the standard ranged from *ca.* 0.1 to 4 mg/ml, corresponding to an absolute amount from 0.7 to 28 ng. A plot of the peak area of HI *versus* amount of HI introduced gave good linearity ($r^2 = 0.9998$). The plate count ranged from 141 000 to 319 000 plates/m for 28 and 0.7 ng, respectively. In the second linearity experiment the injection time was 15 s, corresponding to 2.1–84

ng using the same concentrations of HI as in the previous test of linearity. This gave an excellent linearity ($r^2 = 0.9999$). With this test, the plate count ranged from 62 000 tot 294 000 plates/m for 84 and 2.1 ng, respectively. Data obtained from the two linearity experiments were combined into one calibration graph. As the experiments were carried out on two different days the normalized peak areas (area/migration time) were used. The linearity was acceptable ($r^2 = 0.9986$) for the range 0.7–84 ng of HI.

The detection limit was determined to be 80 pg of acidic desamido-insulin when introducing a standard 0.005 mg/ml for 30 s (signal-to-noise ratio = 8) or 0.0025 mg/ml for 60 s (signal-to-noise ratio = 3).

TABLE V

PERFORMANCE CHARACTERISTICS OBTAINED WITH CAPILLARIES WITH DIFFERENT INSIDE DIAMETERS

Electrolyte: 50 mM sodium acetate–0.85 M CHES–10% acetonitrile (pH 7.8).

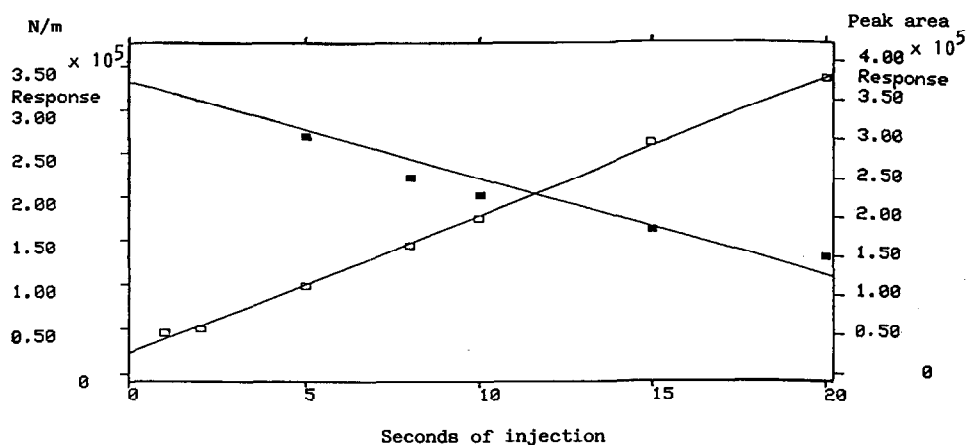| I.D. (μm)	Injection time (s)	Voltage (kV)	Current (μA)	Migration time (min)	HI area ($\times 10^{-3}$)	Plate count (10^3 N/m)	Resolution Acidic des.[a]	Neutral des.
75	5	15	45	13.150	103	344	4.093	1.525
50	5	15	22	16.116	38	415	5.476	2.093
50	5	20	31	11.414	27	389	4.777	1.863
50	30	20	31	10.975	122	283	2.736	1.484
100	5	15	92	13.306	389	104	1.917	0.731
100	3	15	92	13.250	242	154	2.667	1.049

[a] des. = Desamido-insulin.

Fig. 7. Correlation between time of injection of HI in a standard solution and (■) efficiency (plate count per meter) and (□) peak area.

The above results concerning quantitative aspects were tested using standard solutions of HI and acidic desamido-insulin. The precision of the method was confirmed during tests with samples. Three lots of formulation products were tested on five different days with two replicates per day (except for day 7 with one replicate). The results obtained from analysing the three samples are presented in Table VI. Acidic desamido-insulin was not detected in any of the lots. The R.S.D. of the area percentage of neutral desamido-insulin increases with decreasing content of this compound, owing to the values approaching the detection limit.

In order to cover all the quantitative aspects the accuracy of the method was also tested on three different samples of formulation products. In the samples amounts corresponding to 4.5% of acidic desamido-insulin were added on four different days of analysis. As shown in Table VII, the results are satisfactory when the uncertainty of the weighing and the volume of addition of acidic desamido-insulin are taken into account.

Comparison of CZE, IEC and RP-HPLC

A formulation product of HI, which had been stored for at least 15 months at 15°C, was analysed
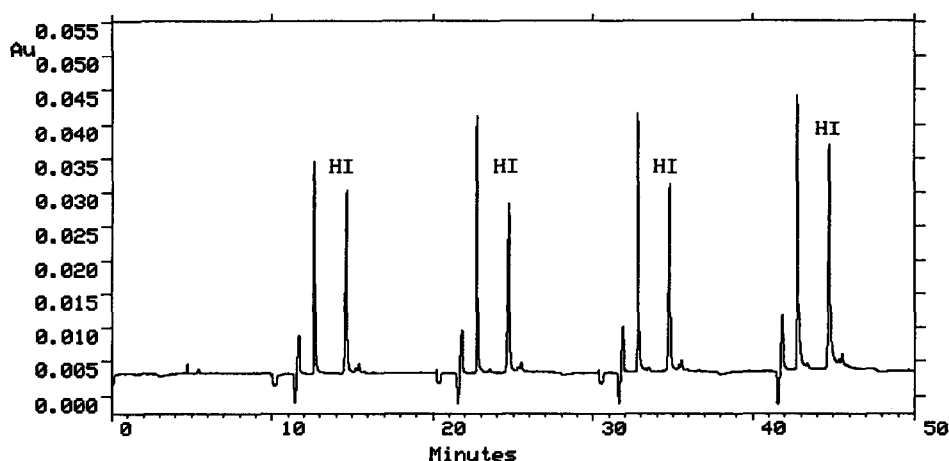

Fig. 8. Formulation product of HI introduced into the capillary at intervals of 10 min. Running buffer: 50 mM NaAc–0.85 M CHES–10% CH$_3$CN (pH 7.78) (15 kV, 48 μA).

TABLE VI

PERCENTAGE OF MAIN COMPONENT AND NEUTRAL DESAMIDO-INSULIN IN THREE DIFFERENT LOTS OF FORMULATION PRODUCTS

Lot No.	Day	Main component		Mean area %	S.D. (abs.) area %	R.S.D. (%)	Neutral desamido-insulin		Mean area %	S.D. (abs.) area %	R.S.D. (%)
		Area %					Area %				
1	1	91.36	91.16				7.40	7.55			
	2	90.64	90.59				8.36	8.39			
	3	90.60	90.00	90.68	0.39	0.4	8.15	8.61	8.06	0.43	5.3
	4	90.51	90.59				8.19	8.26			
	5	90.67	–				7.61	–			
2	1	98.73	98.65				1.27	1.35			
	2	98.51	98.49				1.10	1.00			
	3	98.60	98.70	98.63	0.17	0.2	1.40	1.30	1.27	0.20	15.7
	4	98.74	98.89				1.26	1.11			
	5	98.32	–				1.68	–			
3	1	98.97	99.21				1.03	0.79			
	2	–	–				–	–			
	3	99.17	99.35	98.79	0.58	0.6	0.83	0.65	0.99	0.24	24.1
	4	97.98	98.07				1.32	1.15			
	5	98.85	–				1.15	–			

by RP-HPLC, IEC and CZE. The patterns of the elution curves obtained with the three techniques are depicted in Fig. 9.

Comparing the electropherogram and the chromatogram obtained from IEC several observations can be made. The elution order of acidic and neu-

TABLE VII

ACCURACY OF DETERMINATION OF ACIDIC DESAMIDO-INSULIN ADDED TO THREE SAMPLES OF FORMULATION PRODUCTS

Lot No.	Day	Acidic desamido-insulin added (%)	Found (%)	Recovery (%)
1	1	4.5	3.86	86
	2	4.5	4.01	89
	3	4.5	4.30	96
	4	4.5	4.33	96
2	1	4.5	4.05	90
	2	4.5	3.69	82
	3	4.5	3.55	79
	4	4.5	3.65	81
3	1	4.4	4.03	92
	2	4.4	4.39	100
	3	4.4	4.14	94
	4	4.4	4.39	100

tral desamido-insulin is reversed. Second, the resolution of neutral desamido-insulin and the efficiency are improved when using CZE. Further, the analysis period is reduced from 60 min using IEC (including gradient and equilibration to the starting composition) to a maximum of 20 min using CZE. The preservative in this particular sample migrates together with the EOF front in the electropherogram and elutes with the solvent front in the chromatogram from IEC. As mentioned earlier, only the acidic desamido-insulin is isolated from HI by RP-HPLC, while the neutral desamido-insulin co-elutes with HI. The peak at 10 min is the preservative in the sample.

Comparative data obtained by CZE, IEC and RP-HPLC of two different samples are presented in Table VIII. The qualitative determination of the two deamidation products showed a close resemblance between CZE and IEC. In order to obtain comparative results for the purity of HI, the integration of the chromatogram from RP-HPLC does not include the peak of the preservative.

A more thorough investigation of the relationship between results achieved by CZE and IEC was made. Eight samples were analysed for neutral and acidic desamido-insulin by CZE and IEC using dual
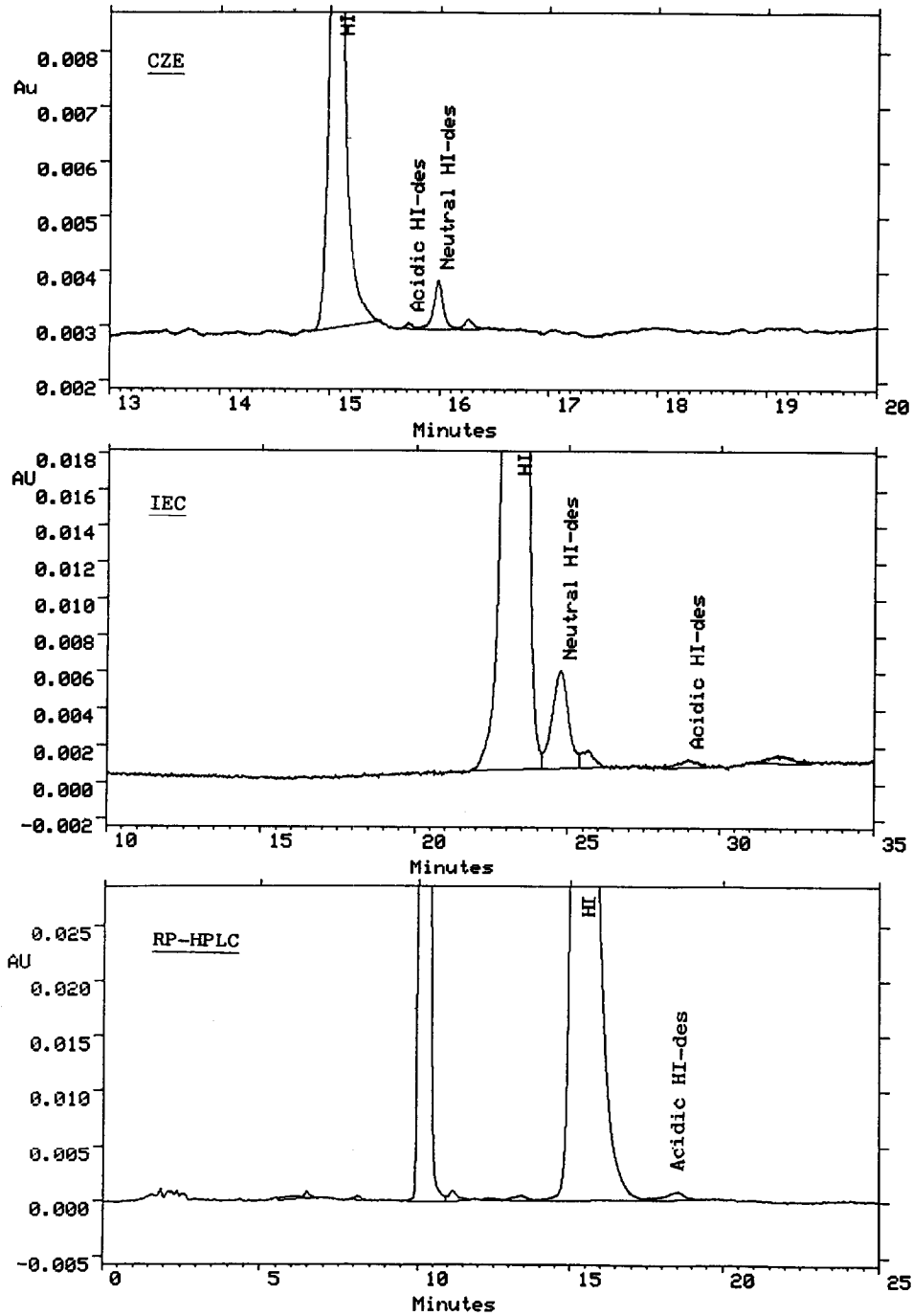
Fig. 9. Formulation product of HI stored at 15°C. Comparison of electropherogram and chromatograms obtained using CZE, IEC and RP-HPLC. Analytical conditions as described in the procedures for CZE, IEC and RP-HPLC under Experimental.

TABLE VIII

COMPARISON OF QUALITATIVE RESULTS OBTAINED USING CZE, IEC AND RP-HPLC

Technique	Sample No.	Area %		
		HI	Acidic desamido-insulin	Neutral desamido-insulin
CZE	1	98.41	0.24	1.35
	2	95.53	0.29	3.69
IEC	1	97.90	0.25	1.82
	2	95.36	0.30	3.65
RP-HPLC	1	99.89[a]	0.11	—
	2	99.84[a]	0.16	—

[a] Integration starting from the main peak.

determination. The comparative results are shown in Table IX. The determination of neutral desamido-insulin showed a good agreement between the results obtained by CZE and IEC. In most of the samples the content of acidic desamido-insulin was close to or below the detection limit using both CZE and IEC. The low content of acidic desamido-insulin explains the poor correlation between CZE and IEC. It should also be mentioned that during analysis by IEC the samples were stored at 5°C in the autosampler, whereas the CZE apparatus still does not have the capability to keep the samples cooled. The formation of deamidation products increases at higher temperatures.

TABLE IX

COMPARATIVE RESULTS OF SAMPLES ANALYSED BY CZE AND IEC

Sample No.	Desamido-insulin	CZE			IEC			Mean$_{CZE}$:Mean$_{IEC}$[a]
		Area %		Mean$_{CZE}$ (area%)	Area %		Mean$_{IEC}$ (area%)	
1	Neutral	0.96	0.96	0.96	0.78	0.78	0.78	1.21
	Acidic	0.07	0.15	0.11	0.11	0.11	0.11	1.00
2	Neutral	0.26	0.32	0.29	0.27	0.28	0.28	1.04
	Acidic	0.12	0.08	0.10	0.10	0.12	0.11	0.91
3	Neutral	10.20	9.86	10.08	10.21	10.30	10.26	0.98
	Acidic	0.48	0.12	0.30	0.16	0.17	0.17	1.76
4	Neutral	7.85	7.97	7.91	8.65	8.56	8.61	0.92
	Acidic	0.74	0.55	0.65	0.24	0.23	0.24	2.71
5	Neutral	0.68	0.68	0.68	0.66	0.62	0.64	1.06
	Acidic	0.38	0.51	0.45	0.18	0.21	0.20	2.25
6	Neutral	3.11	2.77	2.94	2.89	2.99	2.94	1.00
	Acidic	0	0	0	0.09	0.11	0.10	—
7	Neutral	16.11	15.23	15.67	15.59	15.48	15.54	1.01
	Acidic	0	0	—	0.07	0.07	—	—
8	Neutral	4.69	4.89	4.79	4.42	4.44	4.43	1.08
	Acidic	0	0	0	0.08	0.09	0.09	—

[a] Neutral desamido-insulin: mean (mean$_{CZE}$:mean$_{IEC}$) = 1.04, S = 0.09–8.3% (n = 8). Acidic desamido-insulin: mean (mean$_{CZE}$:mean$_{IEC}$) = 1.73, S = 0.78–45.2% (n = 5).

It is important to note that among the eight samples were formulation products consisting of three different species of insulin. The main component in the different samples was either HI, porcine insulin or bovine insulin. Because of the small differences in charge between the three species of insulin, they all have the same retention time in IEC and the same migration time in CZE. Likewise, there is no separation between the desamido products of the three species of insulin in either CZE or IEC. However, human, procine and bovine insulin and their desamido products are easily separated by the RP-HPLC method described.

CONCLUSIONS

This work has shown that CZE is a complementary technique which may facilitate discrimination between the main protein component and closely related impurities or degradation products.

A method for the determination of acidic and neutral desamido-insulin in HI was developed. The method is reproducible even without a base wash between each run and without controlling the outer temperature of the capillary. Under these conditions the R.S.D. of the migration times is excellent (below 1%). The detection limit is 80 pg for acidic desamido-insulin and the calibration graph for HI is linear up to 84 ng (correlation coefficient 0.999 based on peak-area evaluation).

An excellent correlation was observed between CZE and IEC. Although the desamido components can be separated by IEC, CZE gives a better resolution in a shorter analysis time. The excellent separation efficiencies obtained indicate that CZE is complementary to RP-HPLC and IEC in the analysis of insulin. CZE adds another dimension to chromatographic separation techniques and therefore enhances the confidence level in the analytical results. The high speed and impressive separation power make this technique very valuable.

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