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Capillary zone electrophoresis and packed capillary column liquid chromatographic analysis of recombinant human interleukin-4

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ABSTRACT

Capillary zone electrophoresis (CZE) and packed capillary column liquid chromatography (micro-LC) have been applied to the analysis of the recombinant human protein interleukin-4 (rhIL-4). Separations for both the parent protein and its enzymatic digest were developed for the purpose of characterizing protein purity and identity. CZE separations of the intact protein were investigated over the pH range of 4.5 to 8.0 using uncoated fused silica capillaries. Gradient reversed-phase micro-LC was performed using 0.32 mm packed capillary columns at flow-rates of 5–6 μl/min. Emphasis was placed on the ability of these methods to separate close structural variants and degradation products of the protein. Peptide mapping of the tryptic digest of rhIL-4 using a combination of CZE and micro-LC provided complimentary high resolution methods for establishing protein identity. Reproducible separations were achieved using sub-picomol amounts of sample. The advantages and problems encountered with these two techniques for characterizing rhIL-4 were assessed.

INTRODUCTION

The characterization of recombinant DNA derived protein products intended for therapeutic purposes has created significant challenges in the field of bioanalytical chemistry. The need to detect subtle structural differences such as posttranslational modification of individual amino acid residues, genetic variants, degradation products and other protein impurities places significant demands on the efficiency and quality of analytical separations. In many cases, an additional constraint is imposed by a limited quantity of sample available for analysis, a situation that is often encountered in characterizing low-level impurities or new protein species. In such instances, the amount of sample available may be in the low microgram to nanogram range.

HPLC and electrophoresis have attained prominent positions as analytical tools for characterizing proteins and peptides [1,2].The potential for higher-resolution separations along with the reduced sample requirements associated with the capillary analogues of these techniques, capillary zone electrophoresis (CZE) and packed capillary liquid chromatography (micro-LC), has resulted in a greater utilization of these reduced-scale separation techniques in bioanalytical chemistry. The separation mechanisms associated with these techniques (charge-based for CZE and hydrophobicity-based for reversed-phase micro-LC) are quite different providing complementary methods for separating close structural protein analogs or complex protein digests. A added advantage of these reduced-scale separation techniques is their compatibility with a mass spectrometer [3,4], which can provide additional selectivity and structural information for the analysis of unknowns.

Investigations comparing the utility of RP-HPLC and CZE for characterizing biopolymers have been demonstrated for recombinant proteins [5], small peptides [6] and tryptic digests of proteins [7]. However, in most cases the investigations in-

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volved the comparison of CZE with conventional HPLC using 4.6 mm I.D. columns with a notable exception being the work by Cobb and Novotny [8] in which a comparison was made using packed capillary columns for peptide mapping. The rapid development and influx of commercial CZE instrumentation in the last couple of years has resulted in a dramatic increase in the utilization of this technology. However, the commercialization of micro-LC instrumentation and packed capillary LC columns has lagged behind the developments in CZE instrumentation. This has hindered this technique from achieving the same level of acceptance in bioanalytical chemistry. Nevertheless, the advantages of micro-LC enumerated earlier makes this an attractive technique, in many instances, for biopolymer analysis.

The present work focuses on the use of CZE and micro-LC for characterizing the recombinant human protein interleukin-4 (rhIL-4), a cytokine which has been investigated for cancer therapy. This M_r 15 400 monomeric protein contains three intra-chain disulfide bonds and possesses a p*I* value of 9.2. Separations of structural variants and degradation products of the parent protein as well as peptide mapping on its tryptic digest have been accomplished using both techniques. The relative merits of these techniques in terms of selectivity, efficiency and reproducibility for the analysis of rhIL-4 have been assessed. The utilization of the complimentary nature of these techniques to improve the quality of tryptic digest separations will also be demonstrated.

EXPERIMENTAL

Materials

Trifluoroacetic acid (TFA) was HPLC/Spectro grade (Pierce, Rockford, IL, USA). 1,3-Diaminopropane was purchased from Aldrich (Milwaukee, WI, USA). Perchloric acid, *n*-propanol and acetonitrile (HPLC grade) were obtained from EM Science (Cherry Hill, NJ, USA). Trypsin (sequencing grade) was from Boehringer Mannheim (Indianapolis, IN, USA). rhIL-4 was from Immunex/Sterling. Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were reagent grade and obtained from J. T. Baker (Phillipsburg, NJ, USA).

Preparation of tryptic digest of rhIL-4

To 370 μg of rhIL-4 in 40 μl of 100 m*M* pH 7.4 Tris buffer were added 7.4 μg of trypsin (20 μl) plus 63 μl of 100 m*M* pH 8.6 Tris buffer. The sample was incubated at 37°C for 20 h after which the pH was adjusted below 3 with dilute HCl. The sample was stored frozen.

Instrumentation

Micro-LC analyses were conducted using an Isco (Lincoln, NE, USA) microbore HPLC system composed of two Micro-LC-500 pumps connected to a ChemResearch interface box and controlled using a Compaq Desk Pro 286E PC. Several modifications to the standard system were made to improve gradient performance at the low flow-rates used in this work. The standard 3-μl Upchurch mixing tee was replaced by a 10-μl Lee (Westbrook, CT USA) micro-mixer tee followed immediately by a second inline Lee 10-μl micro-mixer. All tubing connections downstream of the mixers were made using minimum lengths of 0.004 in. (1 in. = 2.54 cm) I.D. stainless-steel tubing. All tubing, mixers, injector and column were insulated with 1 cm thick foam insulation. A 30 cm × 0.32 mm Delta Pack C_{18}, 5 μm, 300 Å packed capillary column (LC Packings, San Francisco, CA, USA) was connected directly to a Valco Model C14W injector with a 200-nl injector rotor. The outlet of the column was connected directly to an Isco Micro-LC-10 UV detector equipped with a 2-mm microbore flow cell. Data were collected and analyzed with a PENelson Model 6000 data system (PENelson, Cupertino, CA, USA) operated off a VAX computer system.

Three different CZE systems were used for this work. System A was a modular system constructed in-house which has been described elsewhere [9]. System B was a Pace 2000 from Beckman (Palo Alto, CA, USA) and system C was a Spectra-Phoresis 1000 from Spectra-Physics (San Jose, CA, USA). All capillary tubing (50 μm I.D. × 375 μm O.D.) was purchased from Polymicro Technologies Phoenix, AZ, USA). Capillary cartridges for the commercial systems were prepared in-house using the respective manufacturers' cartridge kits. Capillaries were conditioned by rinsing with 1 *M* NaOH for 30 min followed by operating buffer for about 10 h. All samples were injected from 100-μl vials. In between injections, capillaries were rinsed with the operating

buffer. All data was collected and analyzed using the PENelson Model 6000 data system.

RESULTS AND DISCUSSION

Purity analysis of rhIL-4

One of the more critical tasks in characterizing a protein pharmaceutical is the determination of purity. This has typically been achieved using a variety of techniques including electrophoresis, chromatography and immunoassays. While no one technique is able to detect all possible impurities that might be encountered, HPLC and more recently CZE, have proven to be particularly effective in detecting close structural analogues and protein degradation products due to the high resolving power of these techniques.

As a result of the basic character of rhIL-4 (p*I* 9.2) [10], the development of suitable CZE separations for this protein using uncoated fused-silica capillaries was problematic. Irreversible protein adsorption occurred when working in the pH range 2.5–10 using standard separation protocols. Although analysis could be performed below pH 2.5 or above pH 10, resolution was limited at low pH values and protein stability was poor at high pH values. We have found that a combination of an amine modifier with alkali metal salts to be effective in reducing protein–capillary wall interactions for basic proteins [11]. Using this strategy, several useful separations were developed for rhIL-4 in the pH range from 4.5 to 8.0.

Fig. 1 shows a series of electropherograms obtained at pH 7.0 on 3 different samples of rhIL-4 possessing different impurity profiles (typical bulk drug substance sample, acid-stressed sample and sample stressed at room temperature/neutral pH). The high resolution of this technique is evident from an examination of the electropherogram of the acid stressed sample. As many as 24 components are partially to totally resolved in this sample. Although the exact nature of all of these peaks has not been elucidated, experiments were conducted to demonstrate that these are real components rather than separation artifacts. Since aggregation/oligomerization has been identified as a major degradation pathway for rhIL-4 drug product [12] it is possible that this is, in part, responsible for some of these peaks. The sample of rhIL-4 bulk drug substance and the sample stressed at pH 7.4/room temperature for several months were found to contain several percent oligomeric impurities using size exclusion HPLC. The peak labeled 2 (analogue 1) in

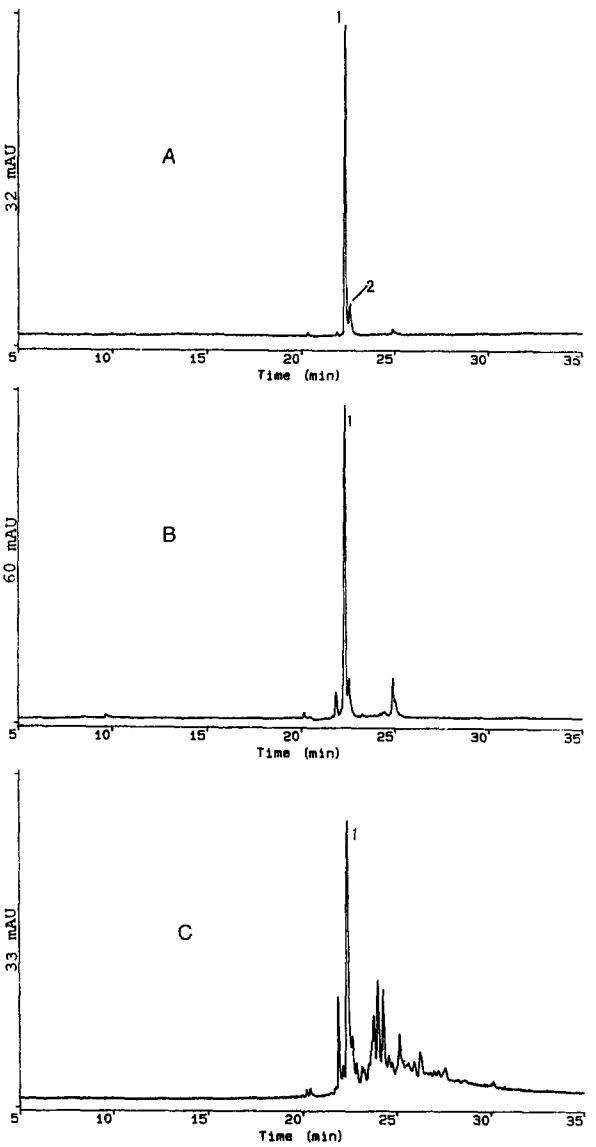

Fig. 1. CZE separations of (A) rhIL-4 bulk drug substance, (B) rhIL-4 degraded at pH 7.4/room temperature and (C) rhIL-4 degraded at pH 2. CZE system B: Capillary, 57 cm (50 cm separation distance) × 50 μm I.D.; buffer, 50 m*M* 1,3-diaminopropane, 0.04 *M* Na_2SO_4, pH 7.0 with H_3PO_4; voltage, 17 kV (70 μA); temperature, 25°C; detection, 200 nm, 3 s pressure injection of a 0.8 μg/μl protein solution. Peaks: 1 = rhIL-4; 2 = analogue 1.

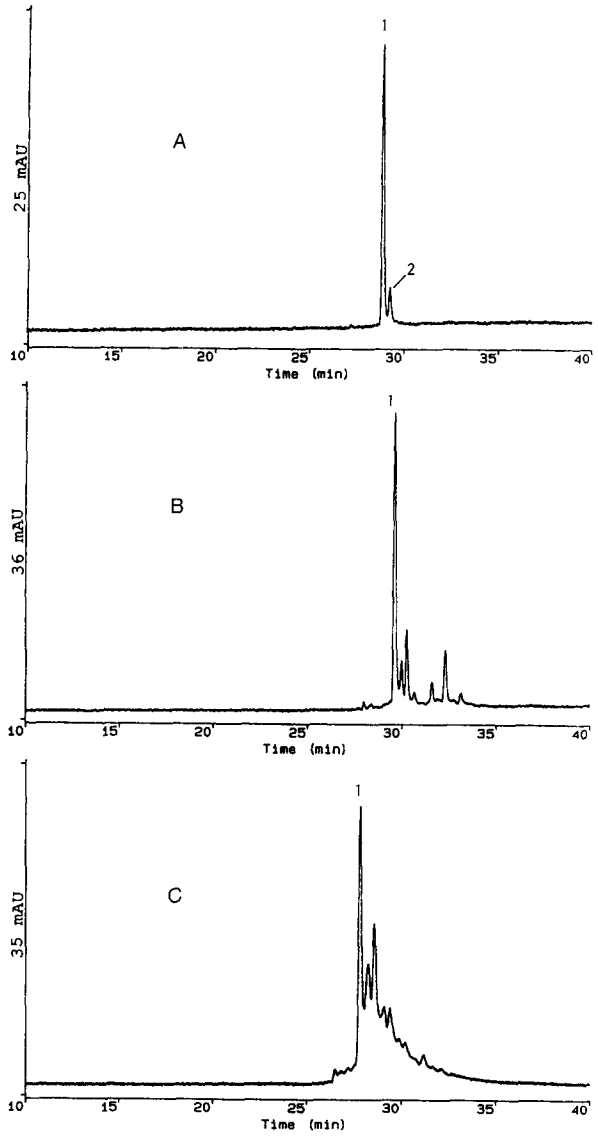

Fig. 2. CZE separations of rhIL-4 in a pH 4.5 buffer, CZE conditions and sample identities as in Fig. 1 with the following changes: Buffer, 50 m*M* 1,3-diaminopropane, 0.04 *M* Na_2SO_4, 10 m*M* formic acid, 0.01 *M* NaH_2PO_4, pH 4.5 with H_2SO_4; voltage, 15 kV (68 μA). Peaks: 1 = rhIL-4; 2 = analogue 1.

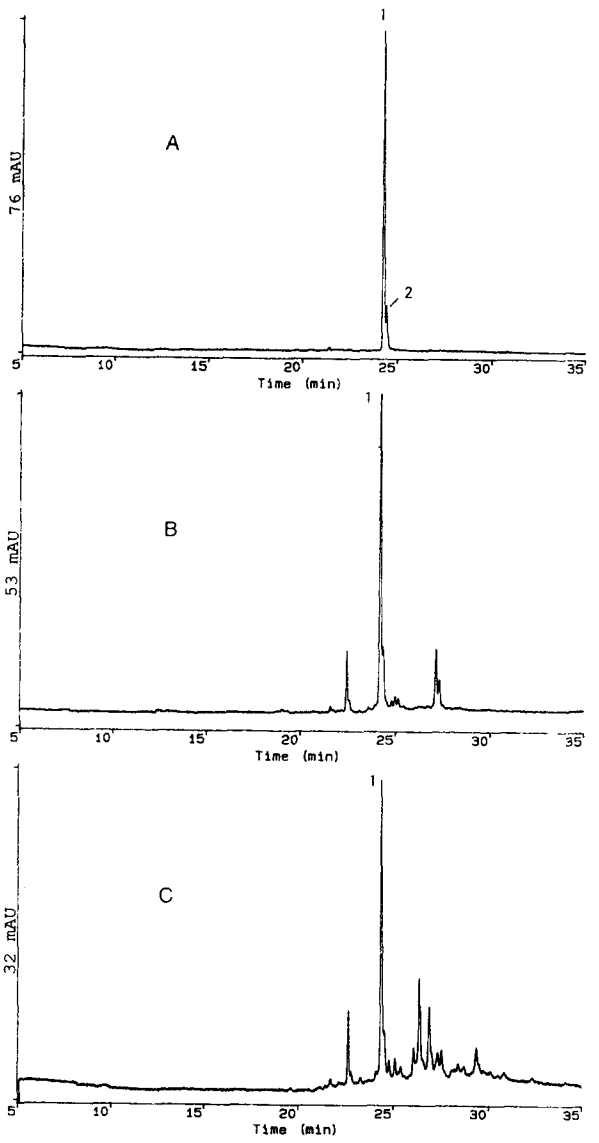

Fig. 3. CZE separations of rhIL-4 in a pH 8.0 buffer. CZE conditions and sample identities as in Fig. 1 with the following changes: Buffer, 50 m*M* 1,3-diaminopropane, 0.08 *M* NaH_2PO_4 pH 8.0; voltage, 17 kV (59 μA). Peaks: = rhIL-4; 2 = analogue 1.

the typical bulk drug substance sample has been identified as an active analogue of rhIL-4 in which one of the three intrachain disulfide linkages has been cleaved. Several impurities are resolved in a sample of rhIL-4 stressed at pH 7.4/room temperature for several months, clearly demonstrating the utility of CZE for detecting likely degradation products formed under normal storage or handling conditions.

The flexibility of CZE for optimizing or altering the selectivity of a separation is demonstrated with Figs. 2 and 3. These electropherograms were obtained at two different pH values (4.5 and 8.0) using the same set of three samples depicted in Fig. 1.

Both resolution and selectivity (peak migration order) could be altered by changing the buffer pH and composition. This flexibility of CZE is clearly an advantage when attempting to resolve a series of close structural analogues. While separations at pH 7.0 and 8.0 were highly reproducible within day and over different days, separations at pH 4.5 were more variable from day to day. This is apparently due to the pronounced pH hysteresis exhibited by fused-silica in the pH range from 4–6 [9]. Thus, although there is an apparent better overall resolution of impurities in sample B when analyzed at pH 4.5, this is at the expense of the precision of the separation.

Fig. 4 contains a series of reversed-phase micro-LC chromatograms of these same samples of rhIL-4. A 0.32-mm column packed with a 5-μm C_{18} phase was used at a flow-rate of 5 μl/min. A perchloric acid–acetonitrile gradient was used in an attempt to reduce background absorbance typically encountered with TFA at low wavelengths.

Although there are examples in the literature demonstrating the successful separation of close structural analogues of other interleukins using HPLC [13,14], for the present application, RP-HPLC was not able to provide anywhere near the same quality of separation achieved using CZE. Only in the acid stressed sample were any significant impurities separated and detected. Using a very shallow gradient, the active analogue 1 of rhIL-4 could be partially resolved from the main peak (data not shown). Since the peak response of the intact rhIL-4 peak decreases after stressing, it is possible that the decomposition products are selectively and irreversibly adsorbed to the column. In addition to the poorer resolution and/or lack of recovery of impurities, these chromatograms tended to be contaminated with several "blank" peaks (peaks marked by an asterisk in Fig. 4) and a rising baseline resulting from the gradient process. These artifacts tend to reduce sensitivity and can possibly obscure impurity peaks.

While the gradient elution process is responsible for the problems documented above, it is also an advantage of HPLC that the elution strength can conveniently be varied using a solvent gradient. This fact is demonstrated in Fig. 5 which compares a reveresed-phase micro-LC chromatogram with a CZE electropherogram of a crude sample of rhIL-4.

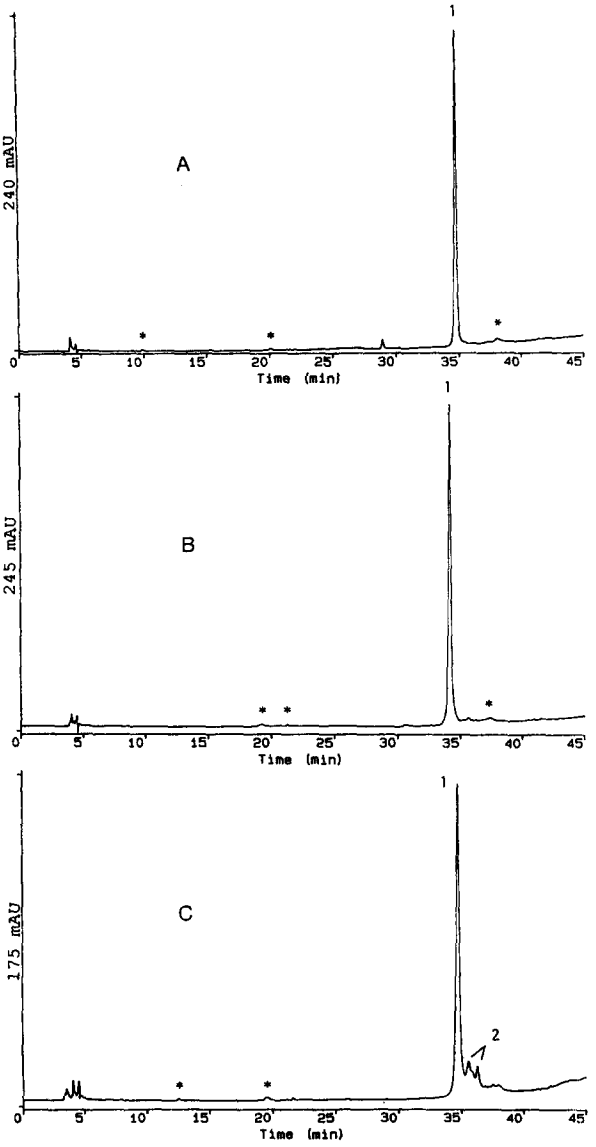

Fig. 4. Reversed-phase micro-LC chromatograms of rhIL-4. Sample identities as in Fig. 1. Column, 30 cm × 0.32 mm Deltabond C_{18}; 300 Å; flow-rate, 5 μl/min; mobile phase, (A) water–acetonitrile–*n*-propanol (92:5:3)–0.05 *M* $HClO_4$–$NaClO_4$ pH 2.0, (B) water–acetonitrile–*n*-propanol (15:82:3)–0.05 *M* $HClO_4$–$HClO_4$; linear gradient from 10–80% B over 40 min; injection volume, 200 nl (100 ng) rhIL-4; detection, 214 nm. Peaks: 1 = rhIL-4; 2 = degradation peaks; for peaks marked *, see text.

Here, gradient elution allows for the separation of many impurity peaks of widely differing hydrophobicities in a reasonable amount of time. CZE also provides good resolution of these impurities. How-

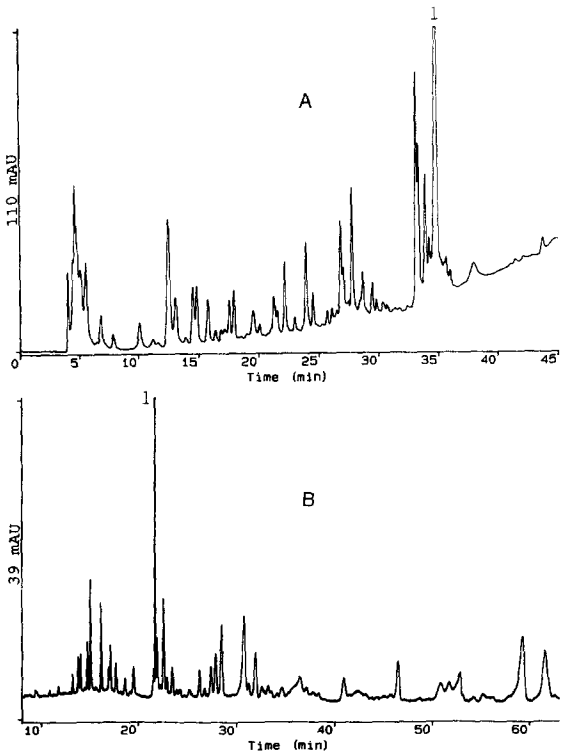

Fig. 5. Comparison of (A) reversed-phase micro-LC and (B) CZE separations of a crude sample of rhIL-4. CZE conditions as in Fig. 1. Reversed-phase micro-LC conditions as in Fig. 4 with a gradient of 5–80% B over 45 min and detection at 200 nm. Peak 1 = rhIL-4.

ever, due to the apparent wide variation in charge to size ratios for these components, the electropherogram is spread out over a longer time window. The orthogonality of these two techniques is evidenced from the difference in the relative position of the main rhIL-4 peak in each separation pattern. By HPLC rhIL-4 is nearly the last peak to elute while in CZE it is one of the earlier peaks to elute.

Quantitation

The relative quantitative aspects of these techniques were evaluated by measuring peak migration/retention time and area responses for multiple injections of a typical sample of rhIL-4 drug substance. For CZE (pH 7.0 buffer) hydrodynamic injections were made using a 0.8 ng/nl protein solution. For the hydrodynamic injections (3 s) the amount of protein injected was estimated to be 3.6 nl [15] or 2.88 ng. For the micro-LC evaluation, a 200-nl injection of a 0.5 ng/nl solution, corresponding to 100 ng, was used.

TABLE I

PRECISION OF rhIL-4 PEAK MIGRATION TIMES AND AREA RESPONSES BY CZE

CZE conditions as in Fig. 1 (sample A).

	Migration time	Peak area
Mean (n = 8)	22.67 min	34 620
S.D.	0.06468 min	1 061
R.S.D. (%)	0.285	3.06

Table I contains the CZE data and Table II the analogous reversed-phase micro-LC data. The precision of peak migration times was slightly better by CZE whereas the precision of peak area responses was comparable for the two techniques. The strategy used in developing the CZE separation was to carefully optimize the buffer composition to eliminate protein adsorption. By eliminating protein adsorption, highly reproducible separations were achieved at pH 7.0 and 8.0. The absence of temperature control and the difficulty in delivering gradients at 5 μl/min flow-rates is responsible for the poorer precision in retention times by micro-LC compared to what is typically achieved using standard-bore LC. It is possible that the precision of the micro-LC system could be improved by operating the pumps at a somewhat higher flow-rate where the precision of solvent delivery is better and splitting the flow at a tee prior to the injector to achieve

TABLE II

PRECISION OF rhIL-4 PEAK RETENTION TIMES AND AREA RESPONSES BY REVERSED-PHASE MICRO-LC

Chromatographic conditions as in Fig. 4 (sample A).

	Retention time	Peak area
Mean (n = 7)	34.68 min	4847
S.D.	0.2002 min	142.7
R.S.D. (%)	0.577	2.94

TABLE III

PRECISION DATA FOR THE PURITY DETERMINATION OF rhIL-4 BY CZE

CZE conditions as in Fig. 1 (sample A).

	rhIL-4	Impurity 1	Impurity 2	Impurity 3	Impurity 4
Mean (n = 8) (peak area %)	79.75%	1.16%	1.72%	11.95%	5.42%
S.D. (peak area %)	0.6724	0.132	0.457	0.651	0.815
R.S.D. (%)	0.843	11.4	26.6	5.45	15.0

the desired flow-rate through the column, although the precision was deemed suitable for this application.

Since the ultimate goal of these methods is the determination of purity, the peak responses for the impurities were determined for the CZE experiments using a typical sample of rhIL-4 bulk drug substance (Fig. 1A). The data compiled in Table III shows fairly good precision for overall purity estimation (R.S.D. 0.843%), although R.S.D.s for individual impurity peaks were much higher. This is due to the fact that some of the impurities are at or just above the limit of detection. Improvements in the concentration sensitivity and dynamic linear range of the detection scheme are needed to realize improvements here. No comparison can be made with micro-LC in this instance since no significant impurity peaks were resolved in this sample with this method.

The estimated limit of detection (mass sensitivity) for rhIL-4 by CZE (pH 7.0 buffer system) of 18 pg ($1.2 \cdot 10^{-15}$ mol) is more than an order of magnitude better than that found by micro-LC (400 pg, $2.6 \cdot 10^{-14}$ mol). Poor recovery of low concentrations of rhIL-4 from the reversed-phase column (a problem that is typically encountered with proteins and is not unique to rhIL-4), the presence of blank peaks and the gradient induced rising baseline contributed to the poorer sensitivity by micro-RP-HPLC. However, this detection limit considers only the amount of sample actually being injected onto the columns. With micro-LC a much greater percentage of the available sample could be injected (approximately 50%) compared to CZE where several microliters of solution were required to make an injection of a few nanoliters. Thus, while CZE exhibits a much lower mass sensitivity, in a true sample limited situation this advantage is offset by the poorer concentration sensitivity and the fact that a much larger volume of sample can be loaded onto the micro-LC column.

The sensitivities achieved here do not approach those obtained for other compounds by other investigators using modified UV detection [16,17] or alternative detection schemes such as laser-induced fluorescence [18,19]. Instead, the results reported here reflect what might typically be achieved for underivatized proteins using presently available commercial instrumentation with UV detection.

Tryptic mapping

Peptide mapping of enzymatic digests of proteins is a well established technique for confirming protein identity and potentially detecting protein modifications. Fig. 6 shows a reversed-phase micro-LC

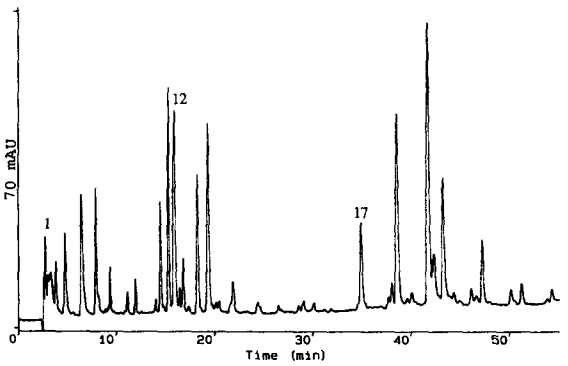

Fig. 6. Reversed-phase micro-LC map of tryptic digest of rhIL-4. Flow-rate, 5 μl/min; (A) 0.05% TFA in water, (B) 0.05% TFA in acetonitrile–water (50:50); gradient, 2–30% B in 10 min, 30 to 70% B at 50 min; detection, 210 nm; sample, 200 nl of a 1 ng/nl solution of rhIL-4 tryptic digest. For numbered peaks, see text.

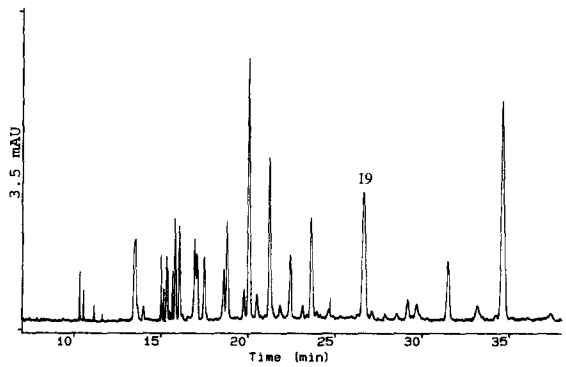

Fig. 7. CZE map of rhIL-4 tryptic digest. CZE system A: Capillary, 100 cm (80 cm separation distance) × 50 μm I.D.; buffer, 20 m*M* 1,3-diaminopropane–phosphate, pH 3.20; voltage, 30 kV (8 μA); detection, 200 nm; sample, 3 mg/ml rhIL-4 tryptic digest, hydrodynamic injection by raising the inlet capillary a height of 7 cm for 5 s. For numbered peak, see text.

map of a tryptic digest of rhIL-4 and Fig. 7 a CZE of the same digest. Due to the different separation mechanisms, the selectivity of these separations are quite different, a point that has been demonstrated by other investigators applying CZE to peptide separations [6–8]. This difference can be used as an aid in developing a suitable separation by either technique. By isolating RP-HPLC peaks and subsequent analysis by CZE, it was determined that HPLC peak 12 contained two peptides. Similarly, two peptides that were well separated by HPLC (peaks 1 and 17) coeluted by CZE (peak 19). Using

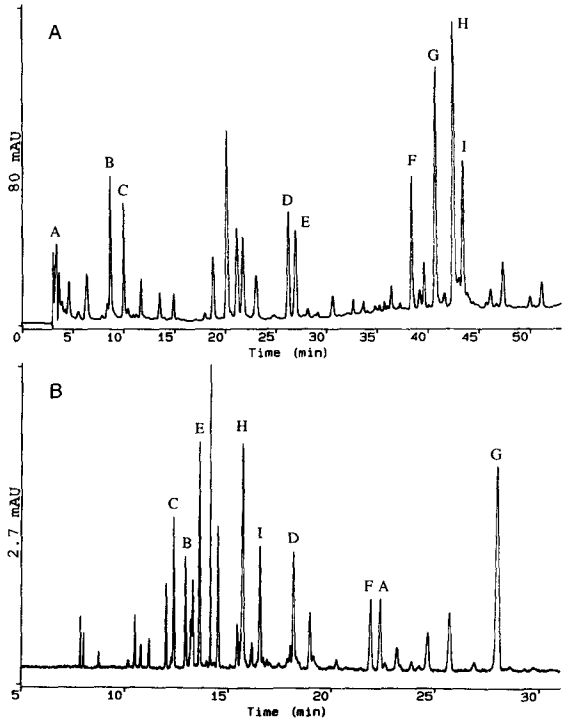

Fig. 8. Optimized reversed-phase micro-LC (A) and CZE (B) maps of rhIL-4 tryptic digest. Micro-RP-HPLC conditions as in Fig. 7 with a flow-rate of 6 μl/min, (a) 0.05% TFA in water, (B) 0.05% TFA in acetonitrile–water (50:50); linear gradient from 0–18% B in 8 min, 18–38% B at 25 min, 38–53% B at 30 min, 53–70% B at 50 min. CZE conditions: CZE system C: Capillary, 70 cm (63 cm separation distance) × 50 μm I.D.; buffer, 25 m*M* 1,3-diaminopropane–phosphate, pH 3.35; temperature, 25°C; voltage 25 kV (31 μA); detection, 200 nm; sample 1-s vacuum injection of a 2.3 mg/ml solution of rhIL-4 tryptic digest. For peaks, see text.

TABLE IV

PRECISION OF rhIL-4 TRYPTIC PEPTIDE MIGRATION TIMES BY CZE

CZE conditions as in Fig. 8.

	Peak migration times of tryptic peptides (min)									
	1	2	3	4	5	6	7	8	9	10
Mean (n = 8)	10.50	11.97	12.91	13.61	14.51	15.73	18.24	22.02	25.95	28.26
S.D.	0.0226	0.0774	0.0421	0.0362	0.0325	0.0484	0.0456	0.0880	0.123	0.142
R.S.D. (%)	0.216	0.647	0.326	0.266	0.224	0.308	0.250	0.399	0.476	0.502

TABLE V

PRECISION OF rhIL-4 TRYPTIC PEPTIDE RETENTION TIMES BY REVERSED-PHASE MICRO-LC

Chromatographic conditions as in Fig. 6.

	Peak retention times of tryptic peptides (min)									
	1	2	3	4	5	6	7	8	9	10
Mean (n = 6)	6.59	10.55	12.38	18.45	23.28	38.42	41.96	44.89	46.29	54.22
S.D.	0.365	0.3263	0.2657	0.2265	0.3206	0.3845	0.3619	0.3415	0.3262	0.2973
R.S.D. (%)	5.54	3.09	2.15	1.23	1.38	1.00	0.863	0.761	0.705	0.548

this information, the two methods were further optimized resulting in the separations depicted in Fig. 8. As an indication of the complementary separation mechanisms, letters have been assigned to some of the peaks indicating identical peptides separated by the two techniques. The overall resolution and efficiency of CZE and reversed-phase micro-LC were found to be comparable for this particular application. A notable exception is the ability of CZE to better resolve the most hydrophilic peptides, some of which elute in or just after the void volume in reversed-phase micro-LC.

Precision of tryptic maps

The reproducibility of rhIL-4 tryptic peptide migration times by CZE and retention times by reversed-phase micro-LC was evaluated for replicate injections made over the course of a day. Table IV contains the CZE data and Table V the reversed-phase micro-LC data for selected peaks in these maps. Overall, precision of migration times were better by CZE (mean R.S.D. of 0.361% *versus* 1.73% by micro-LC). It should be noted that to obtain this level of precision by CZE, a new buffer vial was required for each injection. If the same buffer vial was used for multiple injections, peak migration times and in some cases migration orders changed significantly from injection to injection. This was attributed to ion depletion/pH changes that occurred in the buffer vial during the course of a run. The precision of the peptide retention times using micro-LC improved steadily over the course of a single run. Apparently, the gradient process tends to compensate for minor flow-rate differences that occur from run to run leading to better precision for later eluting peaks. Peptides eluting in the first 10 min are being eluted under near isocratic conditions and the retention times are more susceptible to flow-rate fluctuations in either pump. The overall poorer precision by reversed-phase micro-LC could be traced, in part, to a lack of suitable column and solvent temperature control. In fact, changes in retention times from injection to injection correlated to changes in the laboratory temperature over the course of the day. It is anticipated that improvements in this aspect of the micro-LC instrumentation will improve the overall precision.

CONCLUSIONS

CZE and micro-LC were evaluated for characterizing the purity and identity of rhIL-4. CZE was found to be superior to reversed-phase micro-LC for separating close structural analogues and degradation products from the parent protein due to its high efficiency and ability to manipulate the selectivity and resolution of the separation by changing the buffer composition/pH. Reversed-phase micro-LC, due to its gradient elution capabilities, may have advantages when separating sample components of widely differing structures and properties such as in crude protein isolates. The quantitative aspects of these techniques, in terms of precision, approach what can typically be achieved using standard-scale HPLC with the added advantage of improved mass sensitivity. The combination of these two techniques provides a powerful set of tools for characterizing protein purity and identity. This is

especially apparent for the analysis of protein digests, where the complementary nature of the separation mechanisms can be utilized to aid in the development process for peptide mapping.

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