Characterization of Recombinant Cytokine Fragments Using Isotachophoresis-Capillary Zone Electrophoresis, Reversed-Phase High Performance Liquid Chromatography, and Mass Spectrometry

J. Gysler,[1] B. Helk,[2] S. Dambacher,[2] U. R. Tjaden,[1] and J. van der Greef[1,3]

Received September 9, 1998; accepted February 3, 1999

Purpose. Capillary zone electrophoresis with isotachophoretic sample preconcentration (ITP-CZE) and reversed-phase high performance liquid chromatography (RP-HPLC) with UV detection and on-line coupling to electrospray-ionization mass spectrometry were investigated for their potential to separate and identify fragments of recombinant human interleukin-6 formed during acidic stress of the parent protein.
Results. Based on the orthogonal separation principles governing ITP-CZE and RP-HPLC, different peak patterns were observed using both methods. The selectivity of ESI-MS allowed identification of several co-migrating compounds. Data obtained by on-line ESI-MS were compared to results from off-line investigations by MALDI-TOF-MS performed with single fractions collected from the RP-HPLC system. Cleavage of the protein backbone occurred preferably at acid-labile Asp-sites. The total amount of rhIL-6 needed for ITP-CZE-ESI-MS identification of all fragments was only in the upper femtomole range, while RP-HPLC required amounts of protein three orders of magnitude higher. On the other hand, the low CE sample volume opposes the collection of fractions to perform off-line analysis.
Conclusions. Growing acceptance of CE with on-line MS detection for pharmaceutical quality control of proteins is expected.

KEY WORDS: capillary zone electrophoresis; high performance liquid chromatography; isotachophoresis; mass spectrometry; proteins; degradation.

INTRODUCTION

Recombinant human interleukin-6 (rhIL-6) has potential clinical applications based on its function as pleiotropic cytokine (1). This drug accelerates patients' recovery from thrombocytopenia induced by cancer chemotherapy (2). During the course of infection and inflammation, rhIL-6 induces the formation of acute-phase proteins and β-fibrinogen-mRNA (3).

Proteins easily undergo chemical and physical degradation during single production phases as well as under storage conditions (4). Degradation may not only lead to loss of activity; but also to formation of toxic components, which is even more severe. Cleavage of peptide bonds in acidic media and deamidation in alkaline media, or oxidation, are some of the most frequently observed chemical instabilities, while aggregation and denaturation have physical origins. Various analytical techniques are commonly employed for protein analysis. Besides activity-based techniques like ELISA, RIA, or bioassays which give information on the overall activity of a sample, chromatographic (HPLC) and electrophoretically based methods are applied mainly to separate protein mixtures (for review see Ref. 5). The high price of the final product and the limited sample availability during the phase of drug development demand analytical procedures which minimize sample consumption, while providing sufficient selectivity and sensitivity to separate products, and be able to detect degradation products either formed during the production process or during storage of the final pharmaceutical formulation. During the last ten years chromatographic and electrophoretic techniques have experienced miniaturization to the capillary format. The different modes of capillary electrophoresis (CE) have proved to be highly efficient tools in protein analysis (6,7). While separation in reversed-phase HPLC (RP-HPLC) is governed by partition and adsorption processes between a mobile and a stationary phase, capillary zone electrophoresis (CZE), the most frequently used CE mode, exploits the differences in mobility of analytes in an electric field thus following an orthogonal separation mechanism. Proteins are predestined for CZE analysis due to their amphotheric features giving them pH-dependent electrophoretic mobility (6). Since a flat zone profile is generated in contrast to the parabolic flow in HPLC, higher efficiencies are obtained with CZE. Sensitivity problems occurring with CZE analysis of proteins have reasonably been overcome by on-column isotachophoretic sample preconcentration allowing for 100 to 1000-fold increased sample loadability (ITP-CZE) (8–11). The ITP step takes place in a discontinuous electrolyte system consisting of a highly mobile leading electrolyte (LE) and a terminating electrolyte (TE) exhibiting low mobility (12). Ionic sample components with a mobility between the LE and TE will be selectively focused from a large injection volume to form distinct, sharp, and narrow zones migrating at equal velocity when the ITP equilibrium is reached. According to the Kohlrausch equation (13) the concentration of the sample ions is adapted to the leading electrolyte. They are separated in the following CZE step. Nevertheless, the lack of preparative working capacity due to the limited sample capacity is still regarded as one of the major drawbacks of CE over larger scale HPLC.

A wide range of possible detection techniques is currently available for CZE and HPLC, with UV detection finding most widespread use. However, when structural information is required for the identification of analytes, the sole evaluation of a UV track is inadequate.

Off-line sequencing for the determination of the primary structure of proteins is widely performed with fractions collected from chromatographic separation systems. Biomedical

[1] Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, P.O. Box 9502, 2300 RA Leiden, The Netherlands.
[2] Novartis Pharma AG, Biotechnological Development & Production, 4002 Basel, Switzerland.
[3] To whom correspondence should be addressed. (e-mail: gysler@chem.leidenuniv.nl)

ABBREVIATIONS: CE, capillary electrophoresis; CZE, capillary zone electrophoresis; ESI, electrospray ionization; ITP, isotachophoresis; LE, leading electrolyte; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; rhIL-6, recombinant human interleukin-6; RP-HPLC, reversed-phase high performance liquid chromatography; TE, terminating electrolyte; TOF, time of flight.

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mass spectrometry (MS), using soft ionization like matrix-assisted laser desorption/ionization (MALDI), and electrospray ionization (ESI) have become increasingly popular methods used to obtain the desired structural information (14). While the coupling of liquid chromatographic separation devices to MS can be regarded as well-established, the coupling of CE systems to MS is far from being routine (15,16). Recently, our group demonstrated the feasibility of on-line CZE-ESI-MS in conjunction with size exclusion chromatography to characterize rhIL-6 dimer formation in acidic media (in press). During ESI proteins form characteristic envelopes of multiply charged protein ions. Final identification of proteins is carried out by calculation of their molecular mass from their characteristic ion pattern with assistance of computer software utilizing mass deconvolution algorithms.

Low flow rates in the nl range, insufficient for the formation of a stable electrospray are observed with CZE of proteins usually carried out in coated fused silica capillaries in order to suppress analyte adsorption and to reduce the electroosmotic flow (EOF) (17). In the sheath-flow interface design which appears most popular for the on-line connection of HPLC and CE system to ESI-MS an external make up flow in the microliter range is added to the capillary effluent and also provides electrical contact essential for the electrospray process (18,19).

The aim of this report was to evaluate the suitability of CZE and HPLC coupled on-line to ESI-MS systems to separate and identify chemical degradation products of rhIL-6 with regard to the complementary separation principles governing these separation techniques. Data received by on-line-MS-coupled systems were compared with results obtained from off-line investigation of fractions collected from the HPLC system, e.g. protein sequencing and MALDI-TOF-MS.

MATERIALS AND METHODS

ITP-CZE

All capillary electrophoresis experiments were carried out with a programmable injection system for CE (PrinCE, Lauerlabs, Emmen, The Netherlands) equipped with a UV detector with a custom-made detection cell for CE (Spectra Physics, San Jose, California, USA). High voltage was delivered by a CZE 100R power supply (Spellman, Plainview, New York, USA). Fused silica capillaries, such as, ID 75 μm and OD 360 μm (SGE, Ringwood, Victoria, Australia) were cut to a total length of 80 cm and coated with linear polyacrylamide to suppress protein adsorption and to minimize the electroosmotic flow (EOF) (20). The ITP-CZE procedure was similar to that published earlier (8). The leading electrolyte (LE) was 20 mM ammonium acetate adjusted to pH 4.2 with acetic acid; 10 mM acetic acid was used as terminating electrolyte (TE). After initial filling of the entire capillary with LE, 500 nl of a sample dissolved in TE was pressure injected. ITP focusing was performed at 7 kV over 2 min with the capillary inlet and outlet dipping in TE and LE, respectively. The CZE step took place at 30 kV utilizing LE as continuous background electrolyte. UV detection (200 nm) was carried out through a detection window formed by removal of 2 mm of the outer polyimide coating at 12 cm from the capillary outlet. Signals were recorded with a model BD 40 recorder (Kipp & Zonen, Delft, The Netherlands). For MS detection the outlet end of the capillary was connected to the electrospray ionization (ESI) interface of the mass spectrometer.

RP-HPLC

The HPLC system consisted of two pumps (model 2150, LKB, Bromma, Sweden), a controller (model 2152, LKB, Bromma, Sweden), and a 100×4 mm Nucleosil™ 300-C4 column, particle size 5 μm (Macherey-Nagel, Düren, Germany). Solvents A and B were acetonitrile-water-TFA 27-72.9-0.1 and 90-9.9-0.1% (v/v), respectively. Binary gradient elution was performed as follows: in 20 min from 0% B to 71% B (1.0 ml/min), in 0.5 min from 71% B to 92% B (1.3 ml/min), over 1.5 min 92% B (1.3 ml/min), in 0.5 min from 92% B to 0% B (1.3 ml/min), and finally over 3 min 0% B (1.0 ml/min). A solvent mixer with a magnetic stirrer (Pharmacia, Sweden) was used to avoid air bubble formation. The UV detector (Spectra Physics, San Jose, California, USA) was set to 215 nm. Samples were injected using a Rheodyne™ model 7125 injector (Cotati, California, USA), equipped with a 20 μl injection loop.

A mixture of propionic acid and isopropanol (75–25% v/v) was added post-column in a volume ratio of 1:2 by a third HPLC pump in order to reduce the negative influence of TFA on the ionization efficiency, using the procedure of Kuhlmann and co-workers (21). Post-column flow splitting served to reduce the flow rate towards the MS to about 10 μl/min.

On-Line Mass Spectrometry

The above described ITP-CZE- and RP-HPLC separation systems were connected on-line to a double focusing mass spectrometer MAT 900 via an ESI interface (Finnigan MAT, San Jose, California, USA). The mass spectrometer was equipped with a 20 kV conversion dynode-secondary ion multiplier slit detector (SEM). All mass spectra were obtained in the positive ionization mode at full accelerating voltage (5 kV) with a scan duration of 2 s. The electron multiplier voltage was set to 2.5 kV. We selected entrance and exit slit settings of 350 and 300 μm, respectively. The sampling capillary was held at 200°C. Electrical contact at the tip of the electrospray needle was provided by a sheath liquid consisting of methanol - 5% acetic acid (80-20 v/v) added at a flow rate of 2 μl/min by means of a syringe pump (Harvard Apparatus, South Natick, Massachusetts, USA). The polyimide was removed 1 cm from the end of the capillary tip to aid wetting. During the HPLC experiments nitrogen was added as an additional sheath gas at 6 l/min to perform nebulizer-assisted ion spray. The electrospray voltage was set to +3 kV. The mass spectrometer was calibrated and optimized by constant infusion of lysozyme (100 μg/ml). We carefully polished the tip of the fused silica capillary to a conical shape and positioned it at a distance of 0.5 mm from the spray needle tip to ensure stability of the electrospray.

Experimental masses of the proteins were calculated from the full-scan mass spectra with the BIOMASS™ deconvolution software (Finnigan MAT, San Jose, California, USA). The amino acid sequence of detected rhIL-6 fragment peptides was deduced with help of the GPMAW™ software (vers. 3.06, Lighthouse data, Odense, Denmark) using the rhIL-6 primary structure (Swiss Prot™ data bank).

Off-Line Analysis

Fractions collected from the RP-HPLC apparatus were freeze-dried and mixed with sinapinic acid in a ratio of 1 to 5000 and further analyzed using a Bruker Biflex™ MALDI-TOF-MS system (Bruker Franzen GmbH, Bremen, Germany).

Furthermore, the RP-HPLC fractions were analyzed by automated Edman sequencing using an automated 473A™ protein sequencing system (Applied Biosystems, Foster City, USA) which was controlled by 6110A data analysis software (Applied Biosystems).

Incubation and Sampling

Over a period of 48 h, 300 μl of rhIL-6 stock solution (4.5 mg/ml) were mixed with 600 μl of 25 mM HCl and incubated at 45 °C. Aliquots of this solution were kept at −30°C until analysis. Without further preparation, 20 μl samples were injected into the HPLC system. For ITP-CZE analysis 10 μl aliquots were mixed with 10 μl of an aqueous solution of cytochrome c and lysozyme (500 μg/ml each). The sample volume was adjusted to a final volume of 500 μl with 10 mM acetic acid also serving as terminating electrolyte during the ITP focusing step. Five hundred nanoliter of this solution were finally injected into the CE system.

Chemicals

Vinyltriacetoxysilane, N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium acetate, ammonium persulfate, cytochrome c from horse heart, (M_r 12240, pI 9.7), and lysozyme from chicken egg white (M_r 14306, pI 11.0) were obtained from Sigma (Deisenhofen, Germany). Acetic acid and propionic acid were purchased from J.T. Baker (Deventer, The Netherlands), and hydrochloric acid and trifluoroacetic acid (TFA) were purchased from Merck, (Darmstadt, Germany). Acetonitrile, isopropylic alcohol, and ammonia were purchased from Rathburn (Walkerburn, UK). Water from a Milli-Q™ UF Plus water purification system (Millipore, Bedford, Massachusetts, USA) was used for all solutions. Solvents and buffers were filtered through 0.2 μm pore filters (Gelman Nylon Acrodisc, Ann Arbor, Michigan, USA) and degassed with helium for 5 min prior to use. Unglycosylated recombinant human interleukin-6 (rhIL-6, M_r 20977, pI 6.3) and interleukin-3 (rhIL-3, M_r 15080, pI 7.0) from E.coli were received from Novartis (Basel, Switzerland) and were provided as stock solutions containing 4.5 mg/ml rhIL-6 or rhIL-3, respectively. Polymethylvinylsiloxanediol was synthezised according to Ref. 22.

RESULTS AND DISCUSSION

ITP-CZE

Acidic cleavage of peptide bonds within the rhIL-6 molecule was predicted to create fragments exhibiting different mass-to-charge ratios and therefore different electrophoretic mobility than the parent protein. ITP-CZE electropherograms with UV detection before and after incubation of rhIL-6 with diluted HCl are shown in Fig. 1.

Several fragments formed during incubation, that originally gave only a single peak, could now be separated from rhIL-6. Good separation efficiency was achieved for all investigated proteins; several impurities of the two basic model proteins lysozyme and cytochrome c could be baseline separated. Compared to simple CZE separations the sensitivity could be increased by approximately two orders of magnitude by ITP sample preconcentration, thus, even minor impurities were still detectable in samples originally containing 30 μg/ml rhIL-6. Using ITP-CZE concentration determination limits of 1 μg/ml could be attained. The focusing power of ITP permits dilution of samples with TE. Highly mobile salts present in the incubation mixture which reduced CZE separation efficiency were diluted to result in a nearly ideal final sample composition.

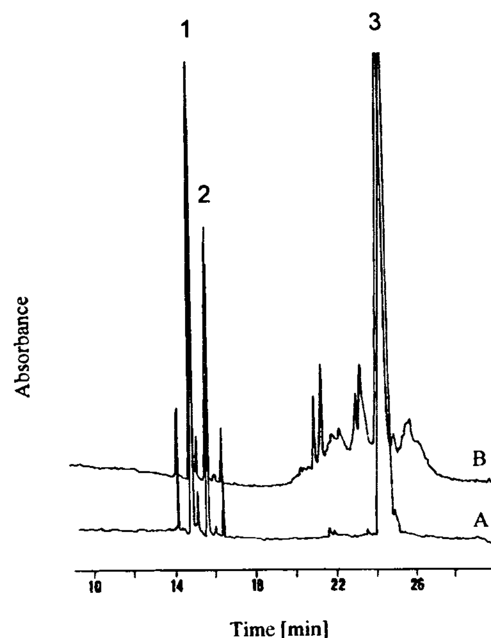

Fig. 1. ITP-CZE electropherograms with UV detection at 200 nm of (1) 10 μg/ml lysozyme, (2) 10 μg/ml cytochrome c, and (3) 30 μg/ml rhIL-6 dissolved in terminating electrolyte. The lower trace (A) contained native rhIL-6 while (B) shows a number of additional peaks originating from incubation of rhIL-6 in 25 mM HCl at 45°C over 48 h. 20 mM ammonium acetate buffer served as leading electrolyte, the terminating electrolyte was 10 mM acetic acid. The injection volume was 500 nl. Only rhIL-6 was subjected to incubation, the other proteins were added later and served as model compounds.

Unfortunately, UV absorption alone did not provide adequate information on the chemical identity of the fragments. Furthermore, no reference material of the expected rhIL-6 fragments was available for identification of single peaks via the highly laborious and time consuming standard addition technique.

Mass spectrometry with electrospray ionization was selected to provide the desired information. Fig. 2 depicts full scan mass analysis of rhIL-6 before (Fig. 2A) and after incubation with 25 mM HCl at 45°C over 48 h (Fig. 2B). Protein solutions were infused into the ESI-MS system at a flow rate of 1 μl/min. While only a single envelope of signals representing differently highly charged ions of intact rhIL-6 appeared in Fig. 2A, three other proteins could be identified in Fig. 2B by computer assisted deconvolution of their real masses from their characteristic ion patterns.

On one hand, the simultaneous identification of several proteins demonstrates the impressive selectivity of ESI-MS

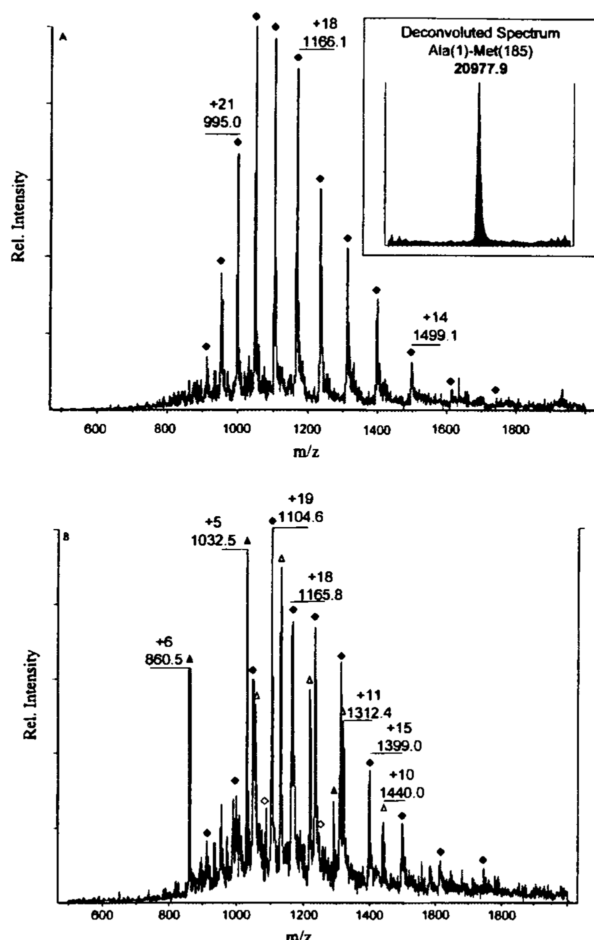

Fig. 2. Constant infusion full-scan mass analysis of rhIL-6 (A) before and (B) after 48 h incubation in 25 mM HCl at 45°C. Several distinct envelopes corresponding to the following peptides were detected. (♦) intact rhIL-6 (M_r 20977), (Δ) Ala (1)-Asp(141) (M_r 15833), (◇) Asp(11)-Gln(184) (M_r 19872), (▲) Pro(142)-Met(185) (M_r 5161).

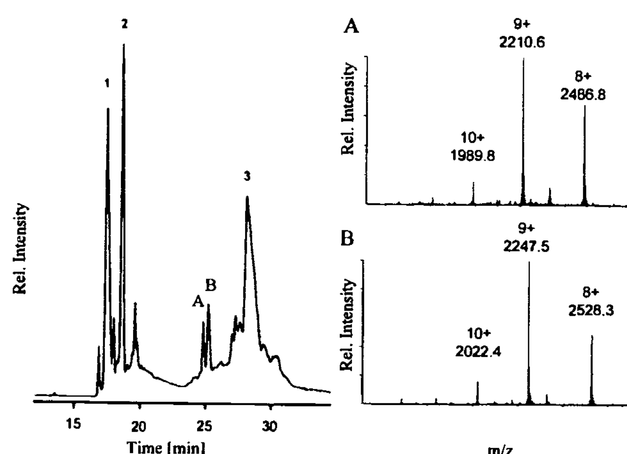

Fig. 3. Total ion electropherogram of ITP-CZE-ESI-MS of a sample (1) 10 μg/ml lysozyme, (2) 10 μg/ml cytochrome c, and (3) 30 μg/ml rhIL-6 incubated 48 h with 25 mM HCl at 45°C. The two characteristic artifact peaks labeled (A) and (B) were identified as rhIL-6 fragment peptides Val(12)-Met(185) and Ser(9)-Met(185), respectively (see table 1). The full-scans at the respective migration times of (A) and (B) are depicted next to the electropherogram.

allowing for identification of several substances in a mixture without any previous separation. On the other hand, insufficient peak resolution limits the number of substances that can be determined simultaneously using this approach.

However, when ESI-MS was coupled on-line with the ITP-CZE separation, we were able to resolve nine different artifacts of rhIL-6. A typical total ion electropherogram (TIE) using ITP-CZE-ESI-MS is presented in Fig. 3.

The TIE demonstrates the loss of separation efficiency occurring during ESI can be effectively controlled by proper geometry of the interface. The peaks labeled (A) and (B) refer to two selected fragments, the corresponding full-scan mass spectra are exemplarily shown next to the electropherogram. These degradation products could undoubtedly be identified as rhIL-6 fragments originating from cleavage of the peptide backbone at position Val(12) and Ser(9), respectively. This matches our predictions since cleavage of peptide bonds are preferably observed in direct neighborhood of Asp residues (4). Loss of amino acids with acidic side chains lead to increased electrophoretic mobility and made separation of the peptides from the parent compound possible. Those deconvoluted protein masses and their deduced sequences that could be identified are summarized in Table 1.

The data confirmed our expectations towards detection of further Asp-connected fragments. Again, the selectivity of ESI-MS enabled the detection of several partially co-migrating peptides and proteins, because all of the artifacts could not be separated from the parent compound with equal efficiency. For this reason, we dispensed with investigating alternative ITP-CZE buffer systems that might have lead to increased resolution of some of the co-migrating compounds.

Table 1. Masses Deconvoluted from Multiply Charged Protein Ions Observed at Different Migration Times (t_m) during ITP-CZE Analysis of rhIL-6 After Acidic Incubation (Conditions see Fig. 2)

t_m [min:s]	Deconvoluted mass [M]	Deduced sequence
16:59	14289.5	Lysozyme - H_2O
17:33	14306.2	Lysozyme
18:14	14306.3	Lysozyme
18:53	12240.3	Cytochrome c (major peak)
19:57	12246.7	Cytochrome c (minor peak)
24:24	19872.1	Asp(11)-Gln(184)
25:03	20192.3	Asp(8)-Gln(184)
25:37	19886.5	Val(12)-Met(185)
26:02	20217.0	Ser(9)-Met(185)
27:59	20960.5	rhIL-6-H_2O
28:34	20977.6	Ala(1)-Met(185) (rhIL-6)
28:00-28:50	5161.0	Pro(142)-Met(185)
28:00-28:58	5759.0	Ala(136)-Met(185)
27:37-28:34	15239.0	Ala(1)-Asp(135)
28:34-32:03	15833.0	Ala(1)-Asp(141)

Note: Molecular masses were deconvoluted from the spectra using the BIOMASS software. Deduction of amino acid sequence was accomplished with the GPMAW software. n.id. not identified.

RP-HPLC

Besides CZE, RP-HPLC was carried out as an alternative analytical procedure to separate the parent protein from fragments. Two chromatograms representing RP-HPLC separations of HCl-stressed rhIL-6 applying (A) UV and (B) ESI-MS detection are depicted in Fig. 4. While intact rhIL-6 eluted as a single peak with a retention time of 12.5 min (not shown), five peaks could be well separated following HCl incubation. Compared to the electropherograms obtained using ITP-CZE separation a totally different peak pattern was observed.

This finding was in accord with our expectations since both techniques are based on orthogonal separation principles, with RP-HPLC taking advantage of differences of hydrophobicity among the analytes. Again, a severe loss in efficiency was not caused by the additional ESI-MS interface (Fig. 4B). The post-column addition of propionic acid proved very effective regarding the ESI-efficiency, since propionic acid counters the ion pairing effect of TFA anions with positively charged proteins (21). Although a smaller overall number of peaks could be resolved by RP-HPLC in comparison to ITP-CZE; two groups of fragments exhibiting masses around 15 and 5 kDa nearly co-migrating in ITP-CZE were well-resolved by RP-HPLC and accessible to identification by deconvolution of the full-scan mass spectra at the respective retention times. On the other hand the two fragments whose identification could easily be achieved using ITP-CZE (see Fig. 3) co-eluted with intact rhIL-6. A summary of all deconvoluted masses and the respective theoretical sequences found in the individual RP-HPLC fractions is given in Table 2.

Using ESI-MS detection the mass of unknown proteins can only be identified via their characteristic ion pattern. Selected ion monitoring mode (SIM), which offers higher sensitivity compared to full-scan analysis, however, is not suitable for the identification purposes. Therefore all ESI-MS investigations were performed in the full-scan mode.

Off-Line Analysis

In order to demonstrate the complementary features of ITP-CZE and RP-HPLC, fractions collected from the HPLC column were re-injected into the CE system. Fig. 5 shows the respective electropherogram (UV-detection) of the fraction eluting homogeneously from the column after 12.5 min. Besides the intact rhIL-6 separation of several other components, it could be accomplished using their electrophoretic mobility revealing the heterogeneity of the RP-HPLC main fraction. In this case, cleavage of a short N-terminal amino acid chain did not lead to sufficient differences in hydrophobicity resulting in co-elution with intact rhIL-6.

Additionally, all of the HPLC fractions were further analyzed by off-line MALDI-TOF-MS and N-terminal protein sequencing analysis. Data were compared to the results obtained using on-line RP-HPLC-ESI-MS. The findings of these investigations are summarized in Table 2.

One selected MALDI-TOF mass spectrum representing the peak eluting 13.2 min was shown in Fig. 6. Generally, off-line derived data confirm the results acquired using on-line RP-HPLC-ESI-MS However, several fragments whose presence was undoubtedly confirmed with on-line MS, remained undetected in the MALDI-TOF mass spectra. N-terminal sequencing data confirmed the heterogeneity of some of the RP-HPLC fractions. The results were in accordance with the off-line and on-line MS data, however, the presence of protein fragments beside intact rhIL-6 in fraction #4 could not be proven.

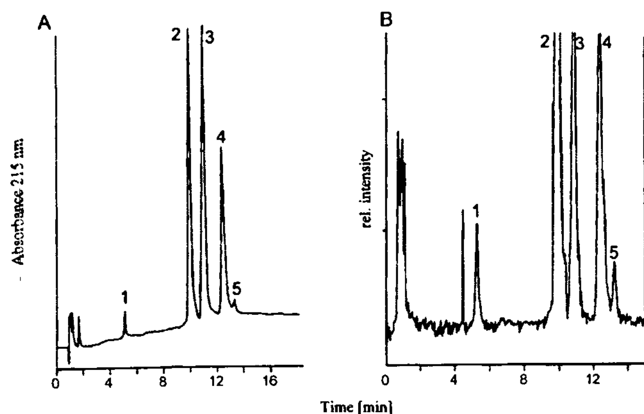

Fig. 4. Typical chromatograms obtained from RP-HPLC analysis of rhIL-6 after 48 h incubation in 25 mM HCl at 45°C. Chromatogram (A) shows the UV track while (B) is the reconstructed ion chromatogram from the ESI-MS detection mode. Apparently no severe loss of separation efficiency was caused in the ESI-interface. For identification of peaks (1–5) see Table 2.

CONCLUSIONS

ITP-CZE and RP-HPLC separation with on-line coupling to mass spectrometric detection proved to be fast and highly efficient analytical tools for detection and identification of protein fragments formed during acidic incubation of a recombinant protein drug. Due to the high selectivity of ESI-MS it is not obligatory to entirely separate all substances of a mixture. Several examples were given where unequivocal identification of substances hidden in overlapping or co-migrating peaks could be accomplished which stands in clear contrast to the interpretation of the respective UV tracks where co-migrating substances might remain undetected.

Our results revealed the orthogonal separation principles with differences is charge-to-mass ratio and hydrophobicity governing CZE and RP-HPLC, respectively. Electropherograms and chromatograms of rhIL-6 after acidic degradation showed completely different peak patterns. ITP-CZE has clear benefits over large scale RP-HPLC regarding injection volume and absolute limit of detection. Only 7×10^{-13} mol of rhIL-6 were required for ITP-CZE while an amount of about 10^{-9} mol was needed for on-line identification with RP-HPLC-ESI-MS. Miniaturization of the RP-HPLC method should be taken into consideration regarding the high price of recombinant protein drugs at the cost of reduced preparative potential. Neither of the separation techniques can be entirely replaced by the other. The combination of analytical information gained with both techniques increases information about fragments derived by acidic protein cleavage. Our data reveals cleavage of the protein backbone occurs preferable in the neighborhood of Asp-residues.

While RP-HPLC-ESI-MS has already found widespread use in many protein laboratories we expect growing future acceptance of CE-based techniques with on-line ESI-MS detection in the field of pharmaceutical quality control of proteins.

Table 2. Summary of Results Obtained by On-Line RP-HPLC-MS, Off-Line MALDI-TOF-MS and Protein Sequencing Analysis (First Six N-Termial Amino Acids) of Protein-Containing RP-HPLC Fractions 1–5 (Conditions see Fig. 4)

		Mass			Sequence	
Fraction	t_R [min]	MALDI-TOF-MS (off-line)	RP-HPLC-MS (on-line)	Theoretical (GPMAW)	Theoretical (GPMAW)	Sequencing (N-terminal)
1	4.7	7949.1	7948.6	7949.0	Ala(1)-Asp(72)	Ala-Pro-Val-Pro-Pro-Gly
2	9.5	15836.9	15833.0	15835.0	Ala(1)-Asp(141)	"
		15237.4	15231.6	15236.3	Ala(1)-Asp(135)	"
		—	15069.1	n. id.	n. id.	—
		—	11950.7	n. id.	n. id.	—
3	10.6	5160.0	5159.0	5161.0	Pro(142)-Met(185)	Pro-Thr-Thr-Asn-Ala-Ser
		5758.0	5760.1	5758.7	Ala(136)-Met(185)	Ala-Ile-Thr-Thr-Pro-Asp
			7904.4	7906.0	Asp(72)-Pro(140)	—
4	12.4	20981.9	20977.6	20977.9	rhIL-6	Ala-Pro-Val-Pro-Pro-Gly
		—	20218.1	20218.1	Ser(9)-Met(185)	—
		—	19890.1	19887.8	Val(12)-Met(185)	—
5	13.2	13047.6	13156.0	13046.0	Gly(73)-Met(185)	Gly-xxx-Phe-Gln-Ser-Gly
		—	13025.1	13024.6	Ala(1)-Val(116)	—

Note: The theoretical masses of detected rhIL-6 fragments were calculated using the GPMAW software. n.id.: not identified.

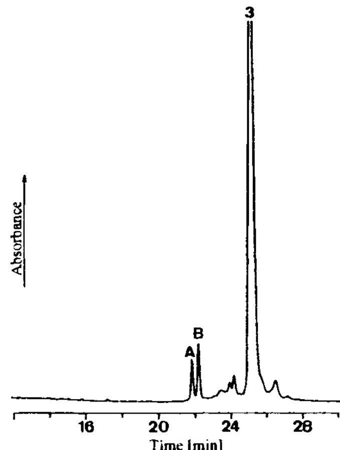

Fig. 5. Electropherogram with UV detection showing the ITP-CZE reanalysis of the RP-HPLC fraction (4) eluting after t_R 12.5 min. ITP-CZE revealed that several rhIL-6 fragments co-eluted with intact rhIL-6 (main peak) during RP-HPLC analysis. This finding confirms the results obtained from on-line RP-HPLC-ESI-MS analysis and demonstrates the orthogonal separation principles governing RP-HPLC and ITP-CZE.

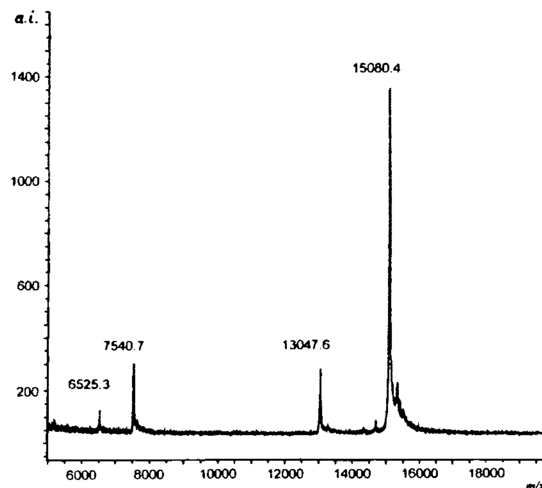

Fig. 6. Off-line MALDI-TOF mass spectrum of the RP-HPLC fraction #5 (see Fig. 4). RhIL-3 (M_r 15080) was added as an internal calibration standard.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert technical assistance of S. Sigrist. We would like to thank M. Barnes for her help with the protein sequencing analysis. A. J. P. Hofte is gratefully acknowledged for his assistance with the HPLC system.

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