ELSEVIER

Journal of Chromatography A, 807 (1998) 135–149

JOURNAL OF CHROMATOGRAPHY A

Capillary electrophoresis in biochemical and clinical laboratoriesp Selected attractive examples

Wolfgang Voelter[a,*], Jürgen Schütz[a], Ourania E. Tsitsiloni[a], Angelika Weiler[a], Gerald Grübler[a], Gerd Paulus[c], Stanka Stoeva[a], Rainer Lehmann[b]

[a]*Abteilung für Physikalische Biochemie, Physiologisch–Chemisches Institut, Universität Tübingen, Hoppe-Seyler-Strasse 4, D-72076 Tübingen, Germany*

[b]*Abteilung Innere Medizin IV, Endokrinologie, Stoffwechselerkrankungen und Klinische Chemie, Medizinische Klinik und Poliklinik, Universität Tübingen, Otfried-Müller-Strasse 10, D-72076 Tübingen, Germany*

[c]*Shimadzu Europe, Albert-Hahn-Strasse 6-10, D-47269 Duisburg, Germany*

Abstract

As demonstrated by selected examples from our laboratories, CE is a unique methodology for purity control of synthetic as well as natural tissue-isolated biopolymers, a prerequisite before reliable biotestings should be performed. A combination of rapid matrix-assisted laser desorption ionization mass and CE electrophoretic mobility determinations facilitates primary sequence determinations of enzymatic peptide digest mixtures often making costly Edman degradations unnecessary. The enormous separation efficiency and large variety of different possible separation modes in CE, allow detection of single components in complex mixtures which is demonstrated by the apolipoprotein A-I determination in human blood serum in this communication. © 1998 Elsevier Science B.V.

Keywords: Buffer composition; Peptides; Hormones; Proteins

1. Introduction

Minimal sample and buffer requirements in combination with rapid and efficient separation, made capillary electrophoresis (CE) [1–5] one of the most attractive tools for the analysis of biopolymers like peptides [6–9], proteins [10–13], glycoproteins [14,15] or oligonucleotides [16,17], as several selected references demonstrate. Very recently, pharmaceutical and clinical analysis laboratories started to develop routine CE methods for purity testing [18], quantitative determinations of the formulation content [19,20], chiral analysis [21], monitoring of drugs in body fluids [22] or reliable and precise analysis of blood serum and its fractions [23,24].

In capillary zone electrophoresis (CZE), the most commonly applied separation mode in CE, the solutes are separated in a single solution buffer. The ratio of charge to size of the analytes is the basis of separation, whereas the choice of the pH of the running buffer plays a crucial role, because it influences the charge of the sample component. Normally, CZE is performed in bare fused-silica capillaries, but for protein separations, for example, it is recommended that coated capillaries or buffer additives be used to suppress adsorptions to the capillary wall.

In this communication we would like to demonstrate the efficiency of CZE technology in checking the purity of thymus peptide (hormones), isolated

*Corresponding author.

0021-9673/98/$19.00
PII: S0021-9673(98)00178-2

from natural tissues and in supporting the identification of the peptide fragments of enzymatic digests via relationships between electrophoretic mobilities, charge and mass of the cleaved peptides. Furthermore, CZE methods for human serum proteins are elaborated showing substantial refinement in resolution compared to cellulose acetate membrane electrophoresis (CAME), allowing a far more unequivocal diagnosis compared to the conventional method. The development of a buffer system, allowing separation and quantification by CZE of apolipoprotein A-1 (Apo A-1) from more than 100 proteins after direct serum injection will be of prominent interest to clinical chemistry, as conventional assays are time-consuming, of high cost and often give erroneous results.

2. Materials and methods

2.1. Electrophoretic equipment

Separations of thymosins and Apo A-I were performed on a Bio-Rad (Munich, Germany) BioFocus 3000 CE system, equipped with an automatic constant volume sample injection system, a temperature control system for the capillary, sample and fraction collection compartment, a high sensitivity fast-scanning UV–Vis detector with wavelength programming and a dedicated computer system with a Microsoft Windows interface. During all runs, the capillary and the sample compartment was cooled to 15°C.

Separations of serum samples were also performed on a Dionex CE system CES I (Dionex, Idstein, Germany), equipped with an automatic constant volume sample injection system, a high sensitivity UV–Vis and fluorescence detector with wavelength programming and a dedicated computer system with a Dionex AC-interface and the Dionex AI 450 software. Samples were introduced hydrodynamically.

2.2. Electrophoretic and chromatographic running conditions

The electrophoretic and chromatographic running conditions are all mentioned in the legends of the figures.

2.3. CAME

Serum protein analysis on CAME was performed by the semiautomatic Fractosan electrophoresis system (Olympus, Hamburg, Germany). The cellulose acetate membrane, the sodium barbital–citrate buffer, pH 8.6 (1.1%, w/v, sodium barbital and 1%, w/v, citric acid), as well as the staining solution (1%, w/v, Ponceau S in 5%, v/v, acetic acid solution) were from Olympus. The intensity of the stained separation patterns were scanned by a densitometer at 520 nm. All procedures were according to the manufacturer's instructions.

2.4. Immunonephelometric assays (INA)

For the INA we used a Behring nephelometer (Behringwerke, Marburg, Germany) and fixed-time kinetic analysis. The Apo A-I nephelometric determinations were performed according to the procedures provided by the manufacturers. After adding an aliquot of diluted sample (100 μl) and antibody (40 μl) to a cuvette containing the reaction buffer (80 μl), a background reading (zero time) was taken. After 6 min, the net increase in scattered light was calculated from a second reading. The scattered light value is compared with those of a standard curve and the concentration calculated. The manufacturer states an assayable range from 18 to 580 mg/dl for Apo A-I, using a plasma or serum dilution of 1:20.

2.5. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)

For mass determinations, a laser desorption mass analyzer, Kratos Kompact MALDI I from Shimadzu (Duisburg, Germany) was applied. A 0.2 μl volume of the peptide sample was mixed with 0.3 μl of matrix solution directly on the sample slide. The droplet was allowed to dry and then the target was loaded for analysis into the instrument. α-Cyano-4-hydroxycinnamic acid from Sigma (Deisenhofen,

Germany), dissolved in 70% acetonitrile and 0.1% trifluoroacetic acid (TFA) in water, was used as matrix. The concentration of matrix ranges between the point of saturation and $\frac{1}{3}$ of that concentration.

2.6. Preparation of β-thymosin extracts

β-Thymosin extracts from bovine lung tissue were prepared according to Refs. [25,26] and references quoted therein.

2.7. Digestion of prothymosin α (ProTα)

ProTα was extracted from calf thymus according to the method of Haritos et al. [27]. The digestion of ProTα in 100 m*M* ammonium hydrogencarbonate, pH 7.5, was initiated by adding trypsin at a ratio of 1:50 (w/w) at room temperature, followed by another 1:50 addition 2 h later. After 4 h, the reaction was stopped by adding TFA up to 1%. The lyophilized digest mixture was dissolved in water for CE investigations or collection of the high-performance liquid chromatography (HPLC) fractions using an Eppendorf quaternary HPLC system of the series BT 9000 (Eppendorf-Biotronik, Hamburg, Germany) with a LiChrospher C_{18} (250×4 mm, 5 μm; Merck, Darmstadt, Germany) column. The applied gradient was 0% to 100% B over 60 min at a flow-rate of 1.0 ml/min (solvents, A, 0.1% TFA in water, B, 60% CH_3CN–0.8% TFA in water) applying a detection at 214 nm. Trypsin was obtained from Sigma.

2.8. Serum sample preparation

The serum samples were prepared from blood of clinical patients and allowed to clot. The serum was separated by low-speed centrifugation at 600 *g* for 7 min and used immediately after recovery or stored at −70°C until use.

3. Results and discussion

3.1. Thymosins

Thymosins are polypeptides, isolated from tissues of vertebrate species including man, structurally characterized during the last decade. Members of the thymosin family include ProTα (109 amino acid residues), thymosin α_1 (N-terminal fragment 1–28 of ProTα), parathymosin α (101 amino acid residues long and structurally partly homologous to ProTα), thymosin β_4 (43 residues long) and species-dependent analogues with significant homology to thymosin β_4 ([25,26] and references quoted therein).

3.1.1. β-Thymosins

β-Thymosins, originally thought to be thymus peptide hormones, have been shown to occur almost ubiquitously in different types of mammalian cells and are widespread in the animal kingdom. Recently, thymosin β_4 has become of interest as an actin-sequestering peptide and as a precursor molecule for Ac–Ser–Asp–Lys–Pro–OH, a regulator of the hematopoietic system. We have established efficient isolation procedures to make this important class of peptides available for investigations on structure as well as biological activities and nowadays CE provides an integrated tool facilitating this kind of work as can be seen from the following by isolating and performing the structure elucidation of β-thymosins and ubiquitin from bovine lung tissue ([25,26] and references quoted therein).

CE combines the instrumental control and quantification features of HPLC with the separation power of electrophoresis, and in the following we would like to demonstrate, how a combination of HPLC–CE spiking, Edman degradation, amino acid analysis and MALDI-MS facilitates elucidation of the primary structure of β-thymosins.

At least two different highly homologous β-thymosins are expected to be produced in bovine lung tissue, one of which should be $T\beta_4$ as the main isomer. However, under the conditions applied, these two homologous peptides could not be separated in a satisfactory way from the crude bovine lung extract using a semipreparative LiChrospher 100 RP-18 column (250×4.6 mm, 5 μm) and a gradient elution of 5–90% B within 30 min (A: 0.05% TFA in water and B: 60% CH_3CN–0.05% TFA in water; data not shown). Besides, ubiquitin, another peptide first isolated from bovine tissue, causes a broad peak and can often only be removed from the column after several washing steps. With a minute amount of sample, the electropherogram of the crude bovine

lung extract, however, allows an analysis within about 6 min and all three ''thymus peptides'' can be detected with much better resolution. As seen from Fig. 1B, three clearly separated peaks correspond to the three components of the investigated mixture. Furthermore, by changing the absorption wavelength from 214 nm to 200 nm, the detection sensitivity can be increased by a factor of three. This rapid and sensitive CE analysis gave valuable information for the development of effective HPLC conditions (column, gradient etc.) to isolate the three ''thymus peptides'' with sufficient yield for their structural determination.

An optimized HPLC protocol (see legend to Fig. 1A), allowed separation of the homologous β-thymosins and ubiquitin, now sufficiently homogeneous for sequencing and MALDI-MS as indicated by control with CE (data not shown). As compared to HPLC (Fig. 1A; 70 min), only about 7 min and amounts of 2–5 n*M* are needed for CE analysis (Fig. 1B). The minute sample quantities required and short analysis time make CE also an attractive tool for following, e.g., the kinetics of enzymatic digestions.

3.1.2. ProTα

ProTα was first isolated from rat thymus as a precursor molecule of thymosin α_1 [27] and earlier proposed to function as a ''thymic hormone'' promoting lymphocyte maturation, but its high evolutionary conservation and its wide tissue distribution [28] suggest that it plays a more fundamental role in the cell. Although the exact function of ProTα has not yet been defined, it has been shown to be active in vitro, enhancing T lymphocyte proliferative responses in patients with autoimmune diseases and also restoring depressed cytotoxicity in patients with cancer [29].

In order to determine the active sites of ProTα in different bioassays, ProTα was isolated from calf thymus and finally purified by HPLC. Due to the better separation efficiency of CE, the purified samples are checked with this method and eventually rechromatographed. Then, the protein sample is subjected to tryptic digestion. The enzymatic fragments are collected by HPLC, their purity controlled with CE and then tested in different bioassays [e.g. human lymphocyte-derived cytotoxic responses against human tumor cell lines such as K562 (chronic myelogenous leukemia)]. The primary structure of the purified tryptic fragments could be achieved efficiently by combining methods like N-terminal Edman degradation and MALDI with several theoretical approaches of CE, focusing on relationships between certain physical parameters of proteins and peptides and the observed electrophoretic mobility. The models should provide useful means for predicting the approximate electrophoretic mobilities of peptides and thereby facilitating peak identification in electrophoretic separations of tryptic fragments. The considered correlations were described by Grossman et al. [30], who have proposed a semiempirical model which incorporates charge and the number of amino acid residues to describe the effect of the peptide's size. Jokl [31] correlated in the 1960s the ratio of mobility to charge, obtained from paper electrophoresis for a series of small ionic species, to $M^{-1/2}$. A few years later, Offord [32] related mobilities of peptides at pH 1.9 and 6.5 to $M^{-2/3}$. Paper electrophoretic mobilities of more than 100 peptides vs. the molecular weight were plotted on a log–log graph, with the slopes of the series of straight lines corresponding to almost exactly $\frac{2}{3}$. Rickard et al. [33] reviewed these early results and established the ability to correlate electrophoretic mobility in CE with physicochemical properties of various analytes and separation buffers.

In order to determine the active sites of ProTα in different bioassays, ProTα is isolated from calf thymus and finally purified by HPLC, applying conditions as given in the legend of Fig. 1A. The chromatogram shows only one single broad peak at a retention time of 40 min (data not shown). The molecular weight of the main HPLC peak was investigated by MALDI (Fig. 2), yielding the expected relative molecular mass of 11 982.9 for ProTα.

The electropherogram (Fig. 3) of the collected HPLC fraction allows an analysis within a few minutes, indicating more impurities as observed by HPLC. Therefore, the raw material had to be rechromatographed before subjecting it to tryptic digestion. The enzymatic fragments were separated again by HPLC (Fig. 4) and identified by Edman degradation, and MALDI (Fig. 5 and Table 1), thereby confirming the primary structure and cleavage sites of ProTα. An electropherogram (Fig. 6) of

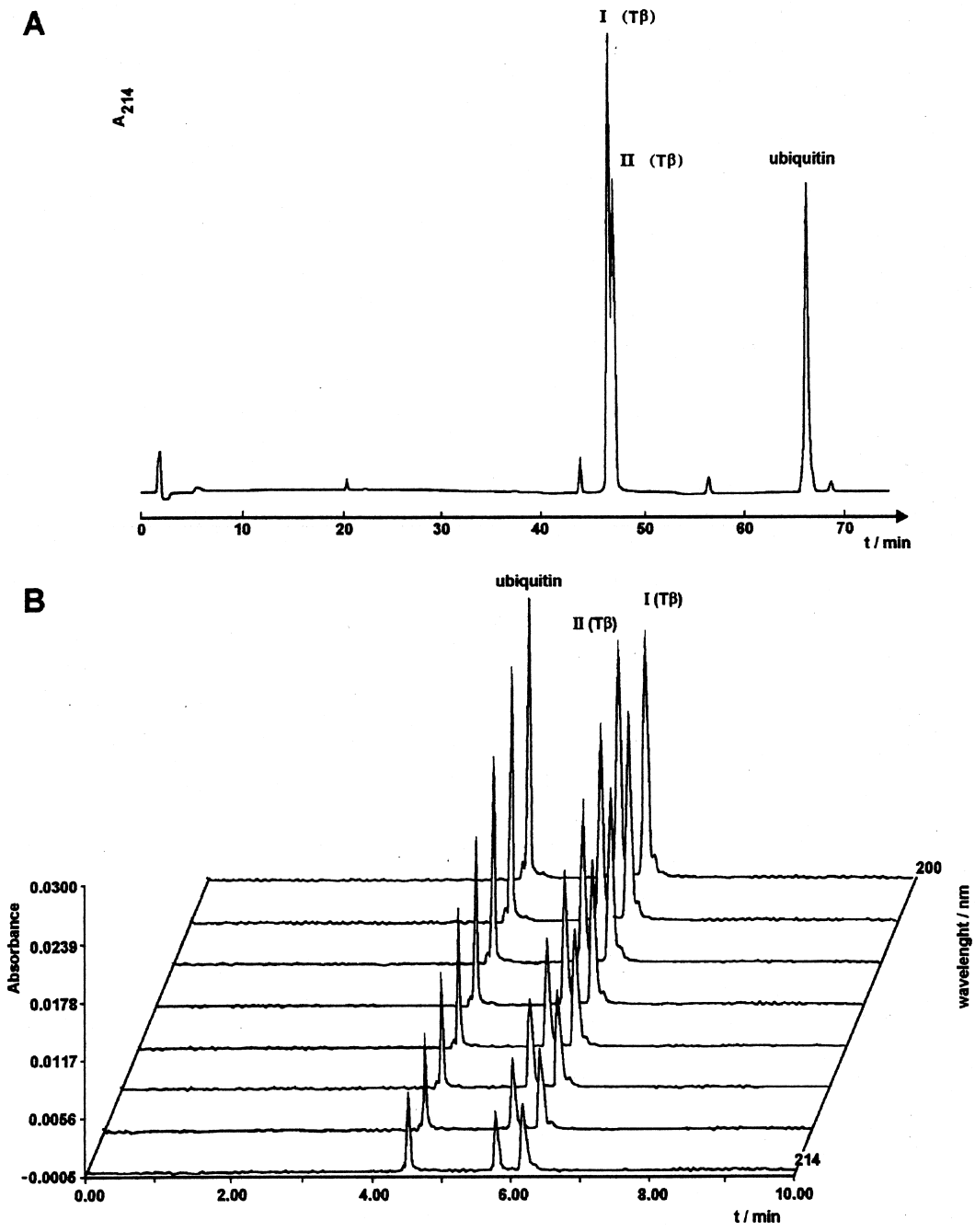

Fig. 1. (A) HPLC of purified ''thymus peptides''. Chromatographic conditions: column, LiChrospher C_{18} (125×4.0 mm, 5 μm; Merck); gradient, 0–90% B in 90 min; solvents, A: 0.1% TFA in water, B: 60% CH_3CN–0.08% TFA in water; flow-rate, 1 ml/min; injection volume, 10 μg peptide mixture in 20 μl A; detection, UV at 214 nm; equipment, Eppendorf quarternary HPLC system of the series BT 9000 (Eppendorf-Biotronik). (B) Multiwavelength capillary electropherogram of purified ''thymus peptides''. Electrophoretic conditions: capillary, coated (25 cm×50 μm I.D.); loading, $3.44 \cdot 10^7$ Pa s; running conditions, 10 kV; buffer, Bio-Rad phosphate buffer (pH 2.5); detection, UV at 200–214 nm; equipment, Bio-Rad CE 3000, BioFocus 3000.

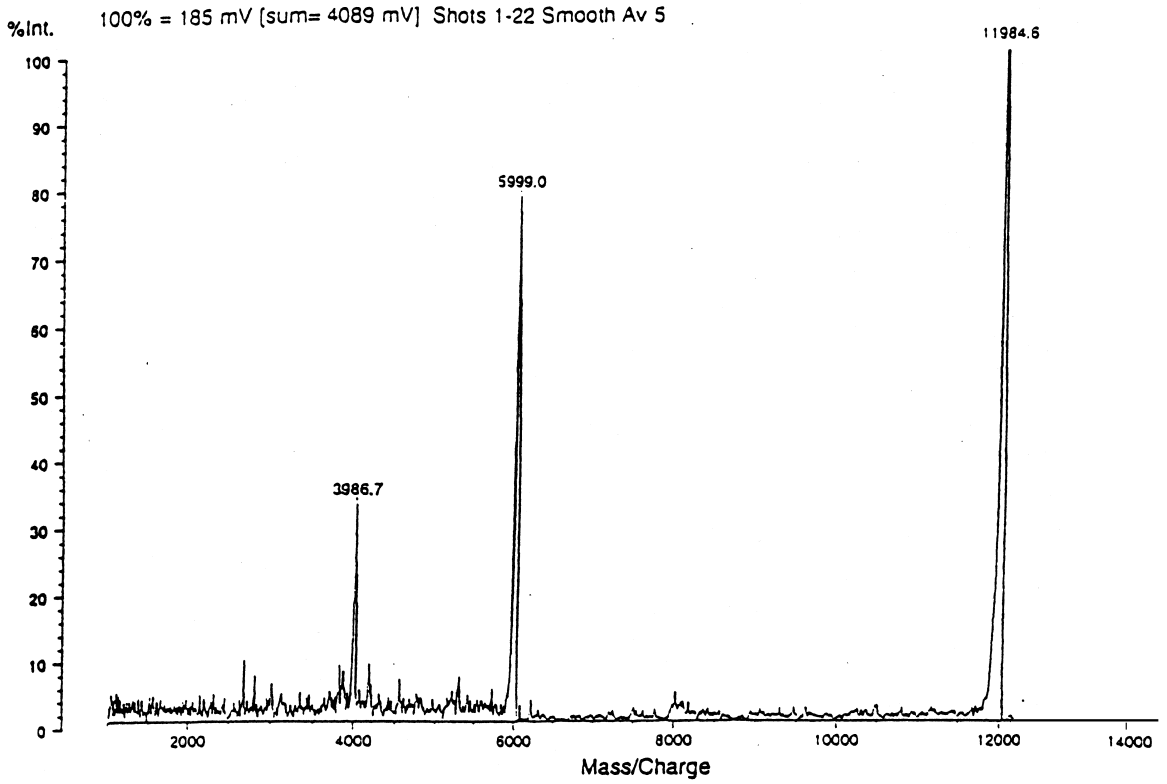

Fig. 2. MALDI spectrum of ProTα, isolated by HPLC (conditions see Fig. 1A). Running conditions: wavelength, $\lambda = 337$ nm; matrix, α-cyano-4-hydroxycinnamic acid; peak at the relative molecular mass 11 984.6: $[M+H]^+$, 5999.0: $[M+2H]^{2+}$, 3986.7: $[M+3H]^{3+}$ of ProTα; equipment, Kratos Kompact MALDI 1 (Shimadzu).

the total digest mixture of ProTα is also recorded. Spiking experiments with collection of single HPLC fractions, as done in former studies [34], can be used to identify the CE peaks; another possibility is the comparison of the measured electrophoretic mobilities with those from theoretical approaches. The electrophoretic mobilities of the digest components are calculated from the experimentally determined migration times according to the following equation:

$$\mu_{EP} = \frac{v_{EP}}{E} = \frac{L_{EFF}/t_M}{V/L_{TOTAL}} \quad (1)$$

where μ_{ep} denotes the electrophoretic mobility, v_{EP} the electrophoretic velocity of the analyte, which is calculated by the ratio of the effective capillary length L_{EFF} (''sample end'' to detector) to the migration time t_M of the peptide; the applied voltage V, divided by the capillary length L_{TOTAL}, results in the electric field strength E. Exact calculations should include the influence of the electroosmotic flow (EOF), but the use of a coated capillary practically eliminates the EOF and any solute–wall interactions. Additionally, a buffer pH of 2.5 supports the latter effect [35]. Consequently, the correction of the measured mobility due to the EOF can be omitted.

The following relationships were tested for their applicability in facilitating peak identification of tryptic fragments. Grossman et al. [30] showed that mobilities μ of peptides at pH 2.5 follow the correlation of:

$$\mu \sim \frac{\ln(q+1)}{N^{0.43}} \quad (2)$$

where N is the number of amino acid residues and q

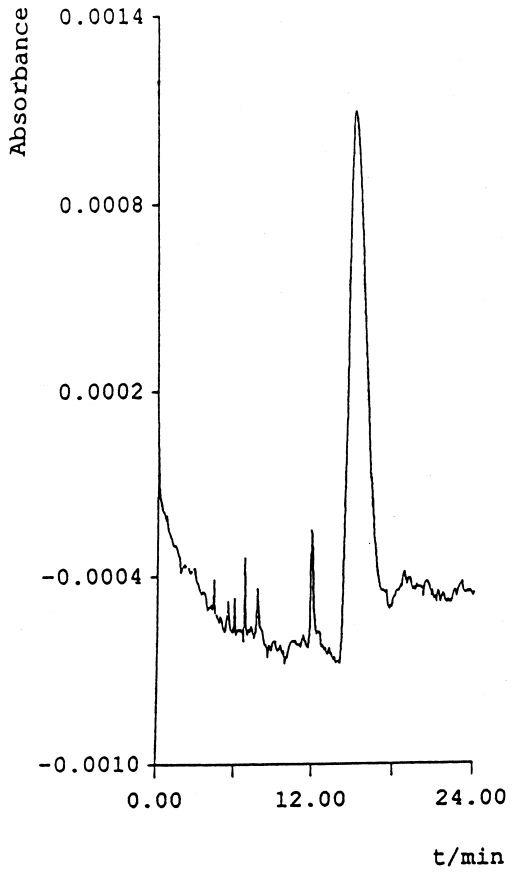

Fig. 3. Electropherogram of isolated ProTα. Electrophoretic conditions: capillary, 25 cm×50 μm, coated; loading, $3.44 \cdot 10^{7}$ Pa s; running conditions, 10 kV; buffer, Bio-Rad phosphate buffer, pH 2.5; detection, 200 nm; equipment, BioFocus 3000 (Bio-Rad).

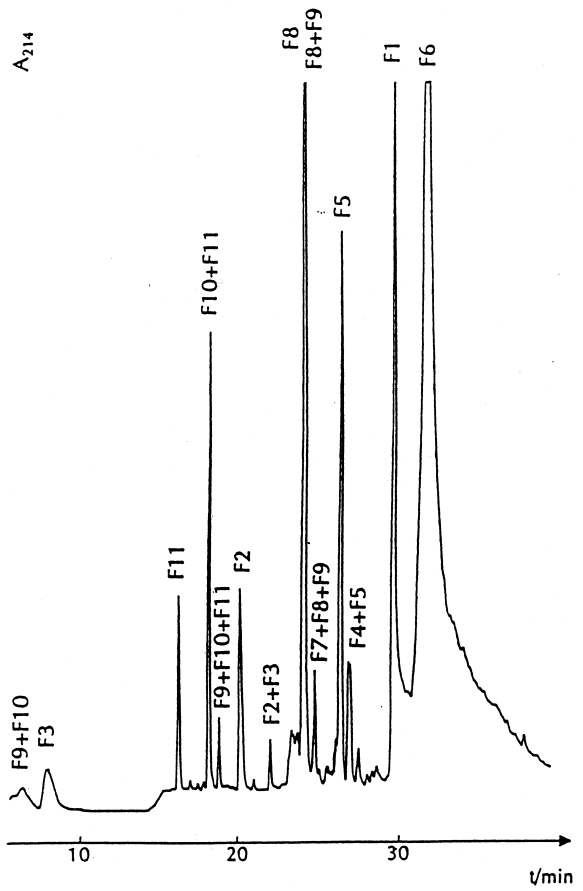

Fig. 4. HPLC of tryptic digest of ProTα. Fraction numbering according to the fragment position in the ProTα sequence (see Fig. 7); identified fragments: F9+F10, F3, F2+F3, F2, F9+F10+F11, F4+F5, F7+F8+F9, F8+F9, F10+F11, F5, F8, F11, F1, F6. Chromatographic conditions: column, LiChrospher C_{18} (250×4 mm, 5 μm; Merck); gradient, 0–100% B in 60 min (A, 0.1% TFA in water; B, 60% CH_3CN–0.08% TFA in water); flow-rate, 1 ml/min; detection, UV at $\lambda = 214$ nm; separation equipment, Eppendorf quarternary HPLC system of the series BT 9000 (Eppendorf-Biotronik).

the net charge of the molecule. Rickard et al. [33] examined electrophoretic mobility as a function of q/M^x, whereby x has different values:

$$\mu \sim \frac{q}{M^{1/2}} \tag{3}$$

$$\mu \sim \frac{q}{M^{1/3}} \tag{4}$$

$$\mu \sim \frac{q}{M^{2/3}} \tag{5}$$

The method used for the calculation of the theoretical charges on the enzymatic fragments is a model presented by Skoog and Wichman [36] for computation of protein isoelectric points based on the pK_a value of the component amino acid side chain and terminal residues. The ionization constants for isolated amino acids are well known and their use assumes the charge on each residue to be independent from that contributed by other groups in the molecule. Rickard et al. [33] illustrated that the pK_a values are shifted to larger values for all species compared to those of single amino acids and proposed a table with adjusted pK_a values. Therefore,

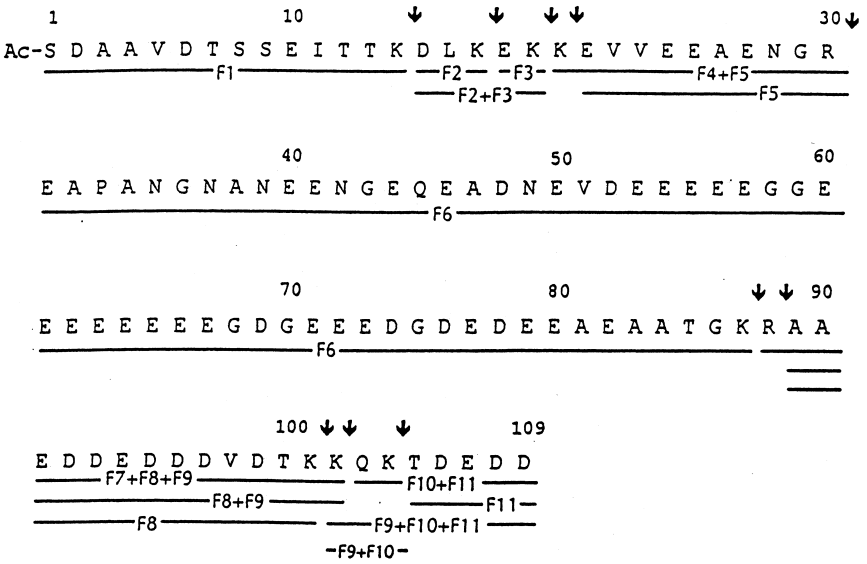

Fig. 5. Primary structure of prothymosin α. Tryptic cleveage sites are marked with arrows. The characterized peptide fragments are underlined.

linearities were developed for all relationships using pK_a values from the table given in Ref. [33].

The observed mobility values are plotted against the predicted ones. The corresponding results for the correlations obtained using Eqs. (2)–(5) are given in Fig. 7. The plots show a linear trend, with the best correlation coefficients for Eqs. (2) and (5) (r= 0.994 and r=0.995, respectively). Eqs. (3) and (4) are inappropriate for peak identification, confirming previous findings of a relationship $\mu \sim q/M^x$ of Rickard et al. [33]. The migration order of many fragments does not correlate. The plots derived from Eqs. (2) and (5) are shown in Fig. 7(A and D), the peak identification resulting in expected migration orders for most of the fragments. Exceptions in Fig. 7(A and D) are observed for fragment F5: Eq. (2)

Table 1
Identified fragments from a tryptic digest of prothymosin α, their relative molecular masses, number of amino acid residues and sequences, experimentally determined electrophoretic mobilities μ_{exp} and charge q at pH 2.5, calculated on the basis of adjusted pK_a values [33]

Fragment number	Rel. mol. mass	Amino acid residues (N)	μ_{exp} (10^{-6} cm^2/Vs)	Charge q at pH 2.5	Amino acid sequence
F9+F10	402.47	3	156.74	2.83	KQK
F3	275.30	2	135.87	1.82	EK
F2+F3	631.71	5	116.82	2.73	DLKEK
F2	374.43	3	106.38	1.74	DLK
F9+F10+F11	977.16	8	88.65	2.36	KQKTDEDD
F4+F5	1259.23	3	84.32	2.79	KEVVEEAENGR
F7+F8+F9	1721.70	15	82.37	3.27	RAAEDDEDDDVDTKK
F8+F9	1565.52	14	67.84	2.27	AAEDDEDDDVDTKK
F10+F11	849.80	7	65.45	1.36	QKTDEDD
F5	1131.17	10	59.67	1.79	EVVEEAENGR
F8	1437.36	13	46.04	1.27	AAEDDEDDDVDTK
F11	593.51	5	35.64	0.36	TDEDD
F1	1466.54	14	23.39	0.64	Ac-SDAAVDTSSEITTK
F6	6143.79	57	23.39	1.03	EAPANGNANEENGEQEADNEVDEEEEEGG-EEEEEEEEGDGEEEDGDEDEEAEAATGK

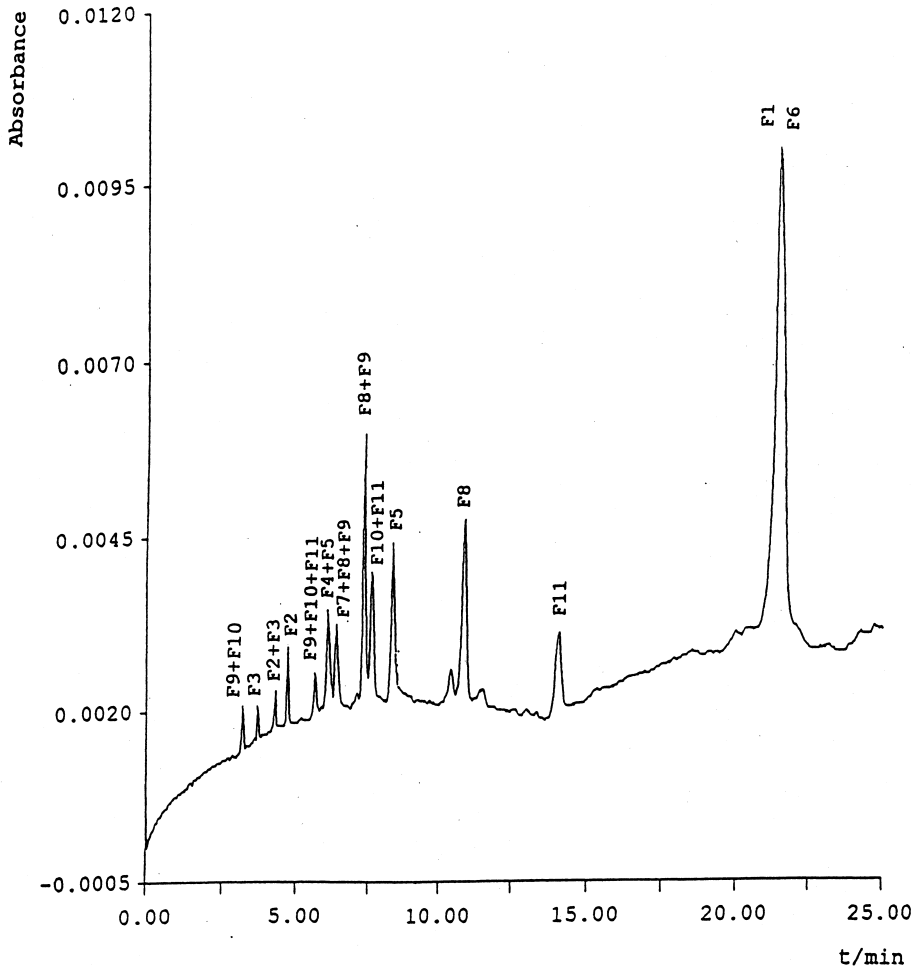

Fig. 6. Electropherogram of tryptic digest of ProTα. The peak numbering is performed according to the fragment position in the ProTα sequence. Identified fragments: F9+F10, F3, F2+F3, F2, F9+F10+F11, F4+F5, F7+F8+F9, F8+F9, F10+F11, F5, F8, F11, F1, F6; for other details see legend to Fig. 3.

(Fig. 7A) predicts a too high mobility. In Fig. 7D (Eq. (5)), the exchanged peak order for F5 and F10+F11 is caused by a higher predicted value for F5 and/or a too low mobility for F10+F11. Furthermore, the measured mobilities for F1 and F6 are surprising. These fragments result in one peak and could not be separated by varying the CE conditions (capillary length, buffer pH, ionic strength etc.). Both fragments have unusual properties in comparison to the other ones. F1 is N-terminally blocked, and F6 with 57 amino acid residues consists of the longest peptide chain of all investigated fragments with only one basic side chain. In Fig. 7(A and D) it can be seen that the equations predict similar mobility values for F1 and F6. Also, for F11 a mobility in the range of the values of F1 and F6 is predicted, but in the electropherogram of Fig. 6 the peak of F11 appears 7.35 min before the signal of F1 and F6, respectively, Consequently, the experimentally determined mobility for F11 differs clearly from the values of F1 and F6, respectively. Furthermore F8 and F8+F9 are coeluted under the used HPLC conditions (Fig. 4), but are clearly separated with CE (Fig. 6). The plots in Fig. 7(A and D) always show the same peak order: F8+F9 is followed by F8. These considerations show that the electrophoretic

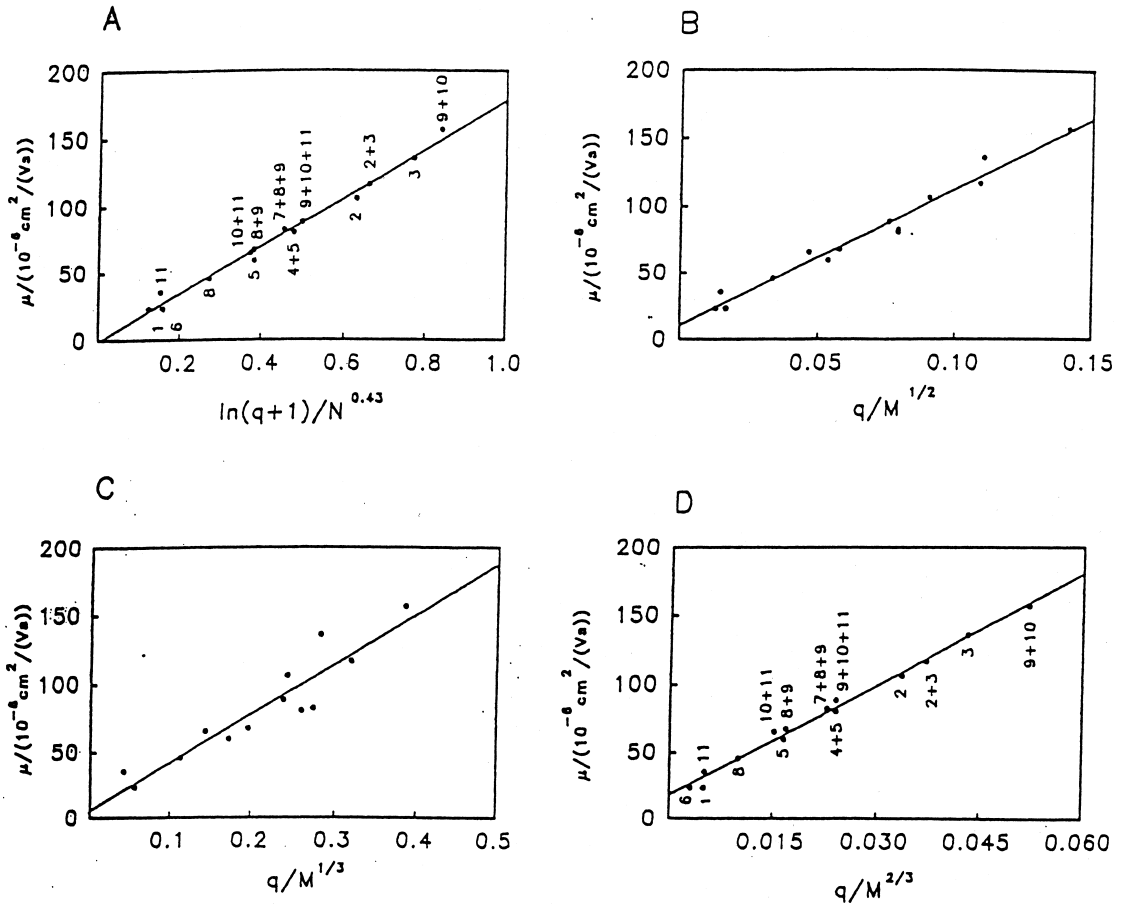

Fig. 7. Fit of electrophoretic mobility μ vs. (A) ln $(q+1)/N^{0.43}$, (B) $q/M^{1/2}$, (C) $q/M^{1/3}$, (D) $q/M^{2/3}$ for tryptic digest peptides of prothymosin α separated in pH 2.5, Bio-Rad phosphate buffer. Numbering of the data points corresponds to the name of the fragments. Charge calculation is based on the adjusted pK_a values as described in Ref. [33]. The following correlation coefficients were obtained: (A) $r=0.994$, (B) $r=0.987$, (C) $r=0.945$, (D) $r=0.995$.

mobility of nine from 14 tryptic fragments of ProTα could be related successfully to the values obtained from the correlations ln $(q+1)/N^{0.43}$ and $q/M^{2/3}$. Regarding the theoretical models, the latter mentioned correlations are the most useful ones for supporting peak identification in CE, thus reducing the necessity of spiking experiments with single collected HPLC fractions. Only incongruous data require correction by a spiking experiment.

3.2. Serum protein analysis

Electrophoresis of human serum samples began with the moving boundary experiments in the 1930s by Tiselius, separating albumin from α-, β- and γ-globulins [37]. Clinical routine analysis of serum samples started in the 1950s using paper strips, replaced a few years later by microporous acetate membranes which are still in use in many clinical laboratories. Since the 1970s, gel-supported media were also introduced in diagnostic laboratories. After membrane- or gel-supported electrophoretic separations, followed by a staining and destaining procedure, five distinct zones (albumin, α_1-, α_2-, β-, and γ-globulins) can be scanned by a densitometer for quantitative estimation. The clinical interpretation of the electropherograms is based on variation in their content. Each fraction is composed of many in-

dividual proteins, except the single pattern of albumin.

Since the introduction of commercial CE instruments in the late 1980s, quite a few research groups published their results on CZE separations of serum proteins with liquid-filled capillaries [38–43]. These results demonstrate that CZE profiles show higher resolution, thus yielding more detailed diagnostic information, compared to the conventional and still officially adopted agarose gel electrophoresis or CAME. Most of these reports are dealing only with optimization procedures of the separation profile [41] or qualitative comparisons [38,39,42]. One communication compared CZE with agarose gel electrophoresis using a very low number of samples [40], and another one, dealing with 1000 specimens, evaluated only paraprotein correlations in the β-, and γ-region and not all five protein fractions [43].

Our interest was to test and compare the CZE results with those obtained with the well established CAME. We used the optimized CZE serum protein analysis under daily routine conditions, which have been recently developed in our laboratory [42].

In the following the clinical evaluation of CZE vs. CAME is demonstrated. Moreover, the analytical power of the CZE method and the practicability for daily use in a central laboratory of a medical center is assessed.

In Fig. 8, the capillary zone electropherogram of a normal serum protein sample is compared with the result obtained by routinely used CAME. Fig. 8A shows a scan of the conventional CAME electropherogram and Fig. 8B the corresponding separation by CZE. The total running time of CZE is 18 min (including all regeneration procedures) and that of CAME around 45 min (including staining and scanning). Performing the CZE under alkaline conditions, the serum proteins are negatively charged, resulting in a migration order of the fractions opposite to that in CAME. During separation, the peaks are migrating toward the anode, however, the velocity of the electroosmotic flow toward the cathode dominates. Proteins of lower charge migrate with lower mobility and will therefore appear first, while peaks of higher mobility appear later. In CAME also the albumin is the fastest protein peak, but the order of migration is reversed compared to that observed in CZE (see Fig. 8).

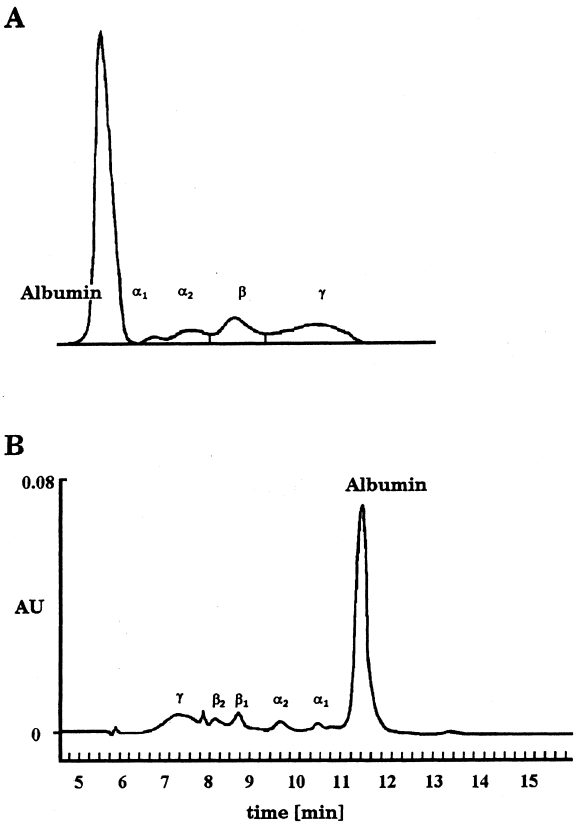

Fig. 8. (A) Electropherogram of normal serum sample on cellulose acetate membrane. Electrophoretic conditions: 100 V; buffer, 1.1% sodium barbital–1.0% citric acid, pH 8.6; the cellulose acetate membrane was stained with Ponceau S. (B) Capillary electropherogram of the same sample. Electrophoretic conditions: capillary, fused-silica (50 cm×50 μm I.D.); running conditions, 35 μA; buffer, 30 m*M* borate, pH 10.0; detection, UV at 195 nm; equipment, capillary electrophoresis system CES I (Dionex).

Each of the serum protein fractions can clearly be distinguished in the CE method, but it is obvious that the resolving power of CZE is superior to that of CAME (Figs. 8 and 9).

The CZE pattern of the α_1-, β-, and γ-fractions shows substantial refinement in resolution. Especially the β-fraction is separated in two distinct peaks, also in the heterogeneous γ-fraction one single peak is detectable, which is described as C_3-complement in the literature [39]. Two facts of serum protein electrophoresis are particularly important [44]: (1) changes in the distribution of specific proteins that reflect pathophysiological conditions and (2) the

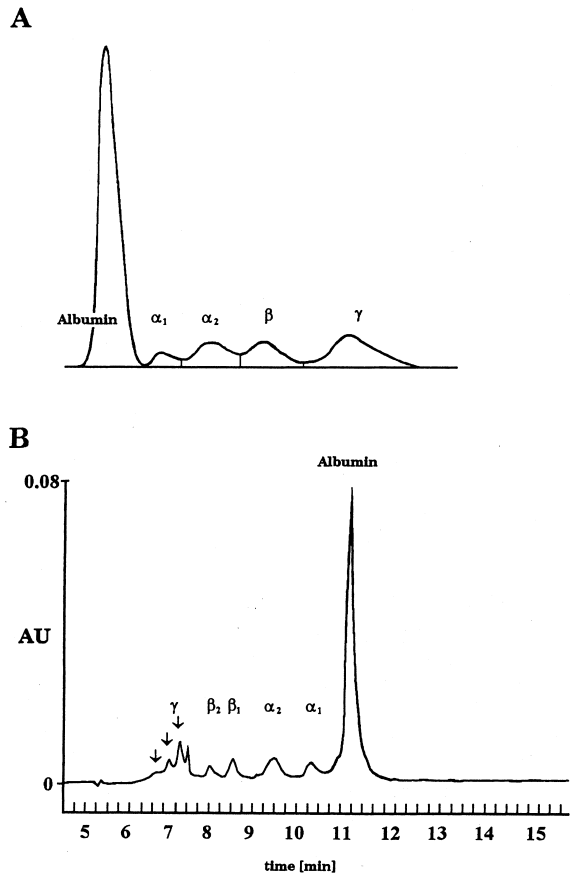

Fig. 9. (A) Electropherogram of serum proteins from an IgG λ myeloma patient run on cellulose acetate membrane. Same conditions as in Fig. 8A. (B) Capillary electropherogram of an identical sample, but separation conditions as in Fig. 8B (the monoclonal IgG peaks are marked with arrows).

ability to detect proteins of abnormal mobility at elevated as well as lower concentrations. Both criteria are fulfilled by the CZE method.

Fig. 9 demonstrates the power of CZE in supplying additional information of valuable diagnostic use: Fig. 9A shows an electropherogram obtained in the daily routine electrophoresis laboratory on cellulose acetate membrane, with an inconspicuous pattern, except the shoulder in the peak of the γ-fraction. Also, the visual control on the membrane did not give any pathological indication. From the CZE pattern, shown in Fig. 9B, a monoclonal gammopathy is indicated, based on the separation observed for the γ-globulins, as shown by two prominent and one minor plus the C_3-complement peaks in the electropherogram. The diagnosis, suggested from the CZE results, could be confirmed by agarose gel immunofixation electrophoresis to be a gammopathy of type IgG λ. Similarly, the CZE electropherograms of sera from patients with, e.g., α_1-antitrypsin deficiency or iron deficiency allow a far more unequivocal diagnosis compared to the conventional CAME electropherograms, due to the much better resolved globulin fractions (data not shown).

3.3. Apolipoprotein in clinical samples

Apo A-I is the major protein constituent of human high density lipoprotein (HDL). Decreased Apo A-I levels in human serum are indicative for arteriosclerotic processes, acute hepatitis and hepatic cirrhosis. The median serum concentration for Apo A-I were determined to be 145 mg/dl for men and 160 mg/dl for women [45]. Today, the INA is the most common method applied in clinical laboratories for routine analysis of Apo A-I concentrations in human serum, other methodologies used for the quantification of Apo A-I include radial immunodiffusion, radioimmunoassay, electroimmunoassay, enzyme-linked immunosorbent assay or immunoturbidimetric assay (ITA). The major drawbacks of immunological determination methods of Apo A-I are caused by the inhomogeneity of HDL [46] and that masking lipids prevent antigenic sites to be expressed [47]. Furthermore, a serious problem of any INA or ITA is that hyperlipemic samples may disturb the Apo A-I determination. A reliable Apo A-I assay to be performed via a simple serum sample work up procedure and applying the advantages of CE, is therefore of enormous interest for the clinical chemist and will be presented in the following.

To determine Apo A-I concentrations in serum directly, a whole series of buffer systems with different additives were tested with the result that the Bio-Rad LLV buffer allows a specific quantification of Apo A-I in blood samples. Fig. 10 shows electropherograms of a diluted serum sample (1:30), spiked with 0.25 mg/ml Apo A-I recorded at wavelengths from 220 to 195 nm and performed with the Bio-Rad LLV buffer. By adding an Apo A-I standard to the normal serum sample, the well separated peak

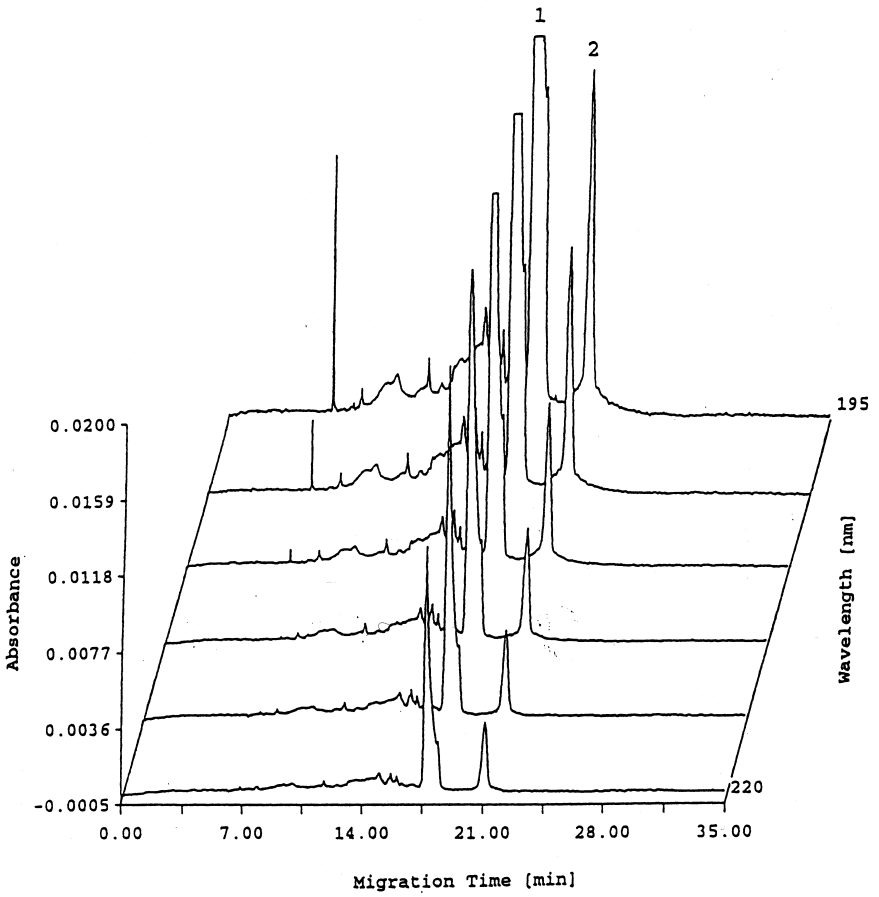

Fig. 10. Multiwavelength electropherogram of a normal human serum sample (dilution 1:30) spiked with 0.25 mg/ml Apo A-I. Electrophoretic conditions: capillary, fused-silica (50 cm×50 μm I.D.); loading, $2.75 \cdot 10^7$ Pa s; running conditions, 20 kV; buffer, Bio-Rad evaluation LLV buffer; equipment, Bio-Rad CE 3000, BioFocus 3000; peaks: 1=albumin; 2=Apo A-I.

at a migration time of 21.10 min could be identified. As the sensitivity could be drastically increased by using a recording wavelength at 195 nm instead of 200 to 220 nm, which is commonly in use, all further runs were performed at the lowest wavelength. Fig. 11 demonstrates that the electrophoretic conditions discussed before allow a clear-cut separation of Apo A-I from serum proteins and quantitative peak evaluation even in highly lipemic serum samples, while nephelometry gives doubtful results (see above). A plot of relative peak areas vs. the amounts of Apo A-I standard (coinjected with a serum sample) and obtained under the conditions given in the legend of Fig. 10 shows a high linearity from 3 to 800 mg/dl. As clinical relevant Apo A-I serum concentrations are within 5 to 300 mg/dl, the detection limit and range of the CE method are well suitable for routine analysis.

In Table 2 nephelometrically and capillary electrophoretically determined Apo A-I concentrations in sera of different patients are compared with each other. If only the main Apo A-I peak fraction is considered, the concentrations, determined by CE, are about 50% too low compared to the nephelometric results. If the peak areas of the polymorphic Apo A-I forms are also included, there is a good coincidence between nephelometry and CE, though on the average, about 10% higher values are found with the CE and this may be caused by the drawbacks (mentioned above) that nephelometry suffers from.

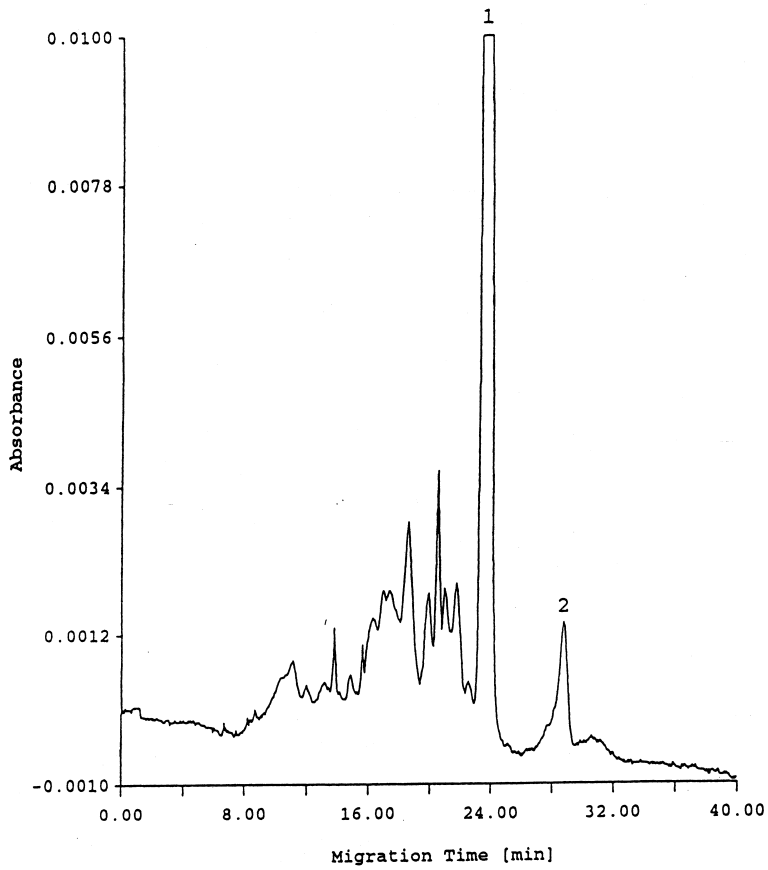

Fig. 11. Electropherogram of a lipemic serum sample from a patient (triglycerides: 967 mg/dl; cholesterol: 298 mg/dl). For separation conditions see legend to Fig. 10; detection, UV at 195 nm. Peaks: 1 = albumin; 2 = Apo A-I. Results for quantitative determination of Apo A-I: CE, 167 mg/dl; nephelometry, 147 mg/dl.

These investigations demonstrate that the developed buffer system makes direct determination of Apo A-I concentrations in clinical human serum samples possible. In contrast to nephelometry, CE also allows detection of different polymorphic Apo A-I forms, the clinical relevance of which is now investigatable with this method. The ease of serum sample preparation, a good linearity of relative peak areas vs. Apo A-I concentrations, possible automation of the technique and low-cost reagent consump-

Table 2
Comparison of nephelometrically and capillary electrophoretically determined Apo A-I concentrations (mg/dl) in sera of different patients

	Nephelometry	CE main peak		CE both peaks	
Patient 1	128	83	(−54%)	131	(+2%)
Patient 2 lipemic	147	97	(−52%)	167	(+12%)
Patient 3	149	92	(−63%)	173	(+14%)
Patient 4	139	85	(−63%)	136	(−2%)
Patient 5 lipemic	244	170	(−43%)	301	(+19%)

The CE values are calculated on basis of a peak area–concentration plot.
For conditions, see legend to Fig. 10.

tion are attractive advantages to applying this method in routine clinical laboratories in the future.

References

[1] H. Engelhardt, W. Beck, J. Kohr, T. Schmitt, Angew. Chem. 105 (1993) 659.
[2] W. Voelter, G. Grübler, H. Straubinger, H. Zimmermann, S. Stoeva, H. Echner, T. Kaiser, G. Paulus, LABO 25 (1994) 40.
[3] C.A. Monnig, R.T. Kennedy, Anal. Chem. 66 (1994) 280.
[4] H. Engelhardt, W. Beck, T. Schmitt, Kapillarelektrophorese, Vieweg, Braunschweig, Wiesbaden, 1994.
[5] J.P. Landers, Handbook of Capillary Electrophoresis, CRC Press, Boca Raton, FL, 1994.
[6] J.S. Green, J.W. Jorgenson, J. Chromatogr. 478 (1989) 63.
[7] E. Yildiz, G. Grübler, S. Hörger, H. Zimmermann, H. Echner, S. Stoeva, W. Voelter, Electrophoresis 13 (1992) 683.
[8] H.-J. Gaus, A.G. Beck-Sickinger, E. Bayer, Anal. Chem. 65 (1993) 1399.
[9] T. Kaiser, S. Stoeva, G. Grübler, H. Echner, A. Haritos, W. Voelter, in R.S. Hodges, J.A. Smith (Editors), Proceedings of the 13th American Peptide Symposium, Edmonton, Escom, Leiden, 1994, p. 224.
[10] P.D. Grossman, J.C. Colburn, H.H. Lauer, R.G. Nielsen, R.M. Riggin, G.S. Sittampalam, E.C. Rickard, Anal. Chem. 61 (1989) 1186.
[11] N.A. Guzman, M.A. Trebilcock, J.P. Advis, Anal. Chim. Acta 249 (1991) 247.
[12] M.A. Strege, A.L. Lagu, Anal. Biochem. 210 (1993) 402.
[13] W. Voelter, G. Grübler, S. Stoeva, T. Kaiser, H. Liebich, R. Lehmann, in Abstracts of the 2nd Symposium on Capillary Electrophoresis, Göttingen, Bio-Rad, Richmond, CA, 1994, p. 8.
[14] S. Honda, A. Makino, S. Suzuki, K. Kakehi, Anal. Biochem. 191 (1990) 228.
[15] J.P. Landers, R.P. Oda, B.J. Madden, T.C. Spelsberg, Anal. Biochem. 205 (1992) 115.
[16] D. Demorest, R. Dubrow, J. Chromatogr. 559 (1991) 43.
[17] W. Voelter, G. Grübler, H. Straubinger, T.H. Al-Tel, A. Tippelt, M. Geiger, W. Reinig, Minerva Biotechnol. 5 (1993) 162.
[18] S. Fanali, M. Cristalli, A. Nardi, L. Ossicini, S.K. Shukla, Farmaco 45 (1990) 693.
[19] G. Grübler, H. Mayer, W. Voelter, Bioforum 3 (1992) 50.
[20] H. Wätzig, C. Dette, H. Uhl, Pharmazie 48 (1993) 527.
[21] T. Schmitt, H. Engelhardt, J. High Resolut. Chromatogr. 16 (1993) 525.
[22] W. Thormann, S. Molteni, J. Caslavska, A. Schmutz, Electrophoresis 15 (1994) 3.
[23] O.W. Reif, R. Lausch, R. Freitag, Adv. Chromatogr. 34 (1994) 1.
[24] R. Lehmann, H. Liebich, G. Grübler, W. Voelter, in Abstracts of Electrophoresis Forum 1994, Munich, Technical University, Munich, 1994, p. II/15.
[25] W. Voelter, J. Schütz, F.P. Armbruster, H. Echner, G. Grübler, A. Kapurniotu, S. Stoeva, O.E. Tsitsilonis, G. Paulus, H. Faulstich, R. Stoll, T.A. Holak, GIT Int. Ed. 1 (1997) 48.
[26] M. Mihelic, W. Voelter, Amino Acids 6 (1994) 1.
[27] A.A. Haritos, G.J. Goodall, B.L. Horecker, Proc. Natl. Acad. Sci. USA 81 (1984) 1008.
[28] A.A. Haritos, O. Tsolas, B.L. Horecker, Proc. Natl. Acad. Sci. USA 81 (1984) 1391.
[29] C.N. Baxevanis, G.J. Reclos, M. Papamichail, Int. J. Cancer 53 (1993) 264.
[30] P.D. Grossman, J.C. Colburn, H.H. Lauer, Anal. Biochem. 179 (1989) 28.
[31] V. Jokl, J. Chromatogr. 13 (1964) 451.
[32] R.E. Offord, Nature 5049 (1966) 591.
[33] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197.
[34] A. Weiler, S. Stoeva, R. Lehmann, G. Grübler, G. Paulus, W. Voelter, Electrophoresis 17 (1996) 518.
[35] P.D. Grossman, K.J. Wilson, G. Petrie, H.H. Lauer, Anal. Biochem. 173 (1988) 265.
[36] B. Skoog, A. Wichman, Trends Anal. Chem. 5 (1986) 82.
[37] A. Tiselius, Trans. Faraday Soc. 33 (1937) 524.
[38] F.-T.A. Chen, C.-M. Liu, Y.-Z. Hsieh, J.C. Sternberg, Clin. Chem. 37 (1991) 14.
[39] F.-T.A. Chen, Clin. Chem. 38 (1992) 1651.
[40] J.W. Kim, J.H. Park, J.W. Park, H.J. Doh, G.S. Heo, K.-J. Lee, Clin. Chem. 39 (1993) 689.
[41] V. Dolník, J. Chromatogr. A 709 (1995) 99.
[42] R. Lehmann, H. Liebich, G. Grübler, W. Voelter, Electrophoresis 16 (1995) 998.
[43] M.A. Jenkins, E. Kulinskaya, H.D. Martin, M.D. Guerin, J. Chromatogr. B 672 (1995) 241.
[44] L.Thomas, Eiweisselektrophorese, Grundlagen, Technik, Fehlermöglichkeiten und Bewertung der Eiweisstrennung auf Celluloseacetatfolie, Urban und Schwarzenberg, Munich, Wien, Baltimore, 1981.
[45] F. Dati, M. Lammers, A. Adam, D. Sondag, L. Stienen, Lab. Med. 13 (1989) 87.
[46] R.F. Atmeh, J. Shepherd, C.J. Packard, Biochim. Biophys. Acta 751 (1983) 175.
[47] S.J.T. Mao, J.P. Miller, A.M. Gotto Jr., J.T. Sparrow, J. Biol. Chem. 255 (1980) 3448.