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Potential of on-line isotachophoresis–capillary zone electrophoresis with hydrodynamic counterflow in the analysis of various basic proteins and recombinant human interleukin-3

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Abstract

Isotachophoresis–capillary zone electrophoresis with hydrodynamic counterflow was applied to the analysis of various basic model proteins and recombinant human interleukin-3. The use of coated capillaries and simple modifications of a commercial capillary zone electrophoresis (CZE) system allowed the detection of concentrations in the 10^{-8} *M* region. The limit of detection was decreased by a factor of 100 compared to conventional CZE. The separation efficiency was unaffected by the preceding isotachophoretic step. Our method is applicable to the analysis of proteins with an isoelectric point higher than 7.0.

Keywords: Isotachophoresis–capillary zone electrophoresis; Proteins; Interleukin-3

1. Introduction

Recent advances in recombinant DNA technology have led to the commercial availability of large quantities of proteins, like human insulin and cytokines, for pharmaceutical applications. Unglycosylated recombinant human interleukin-3 expressed in *E. coli* (rhIL-3) is a hematopoietic growth factor protein with a molecular mass of 13 kDa and a polypeptide chain length of 133 amino acids. Along with other cytokines, it possesses a growing clinical significance in the therapy of neutropenia induced by cytostatic agents in cancer chemotherapy [1]. Thus, there is a high demand for analytical methods for quality control of standard solutions and for the determination of therapeutic concentrations.

Due to the tremendous separation power of capillary zone electrophoresis (CZE), this new technique has proven to be an excellent tool for the analysis of proteins and peptides. In CZE, charged analytes migrating in an electrical field are separated according to differences in their charge-to-mass ratio. Separation is typically carried out in fused silica capillaries with inner diameters ranging from 10 to 100 μm. Usually, capillaries measuring an overall length from 10 to 100 cm are employed. During the separation process, electric field strengths in the range of 100 to 1000 V/cm are commonly applied [2].

CZE of proteins, however, suffers from non-spe-

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cific adsorption to the fused silica surface of the capillary, considerably reducing resolution, separation efficiency, peak shape and recovery [3]. To minimize interactions with the fused silica surface, CZE of proteins is usually carried out using buffers of extremely low or high pH values (<2.5 or >9, respectively) [3–6], or in buffers with high salt concentration [7]. The latter approach, however, limits the applicable electric field strength, as a consequence of significant Joule heating inherent to high currents during analysis, leading to severe peak shape deterioration. Furthermore, most proteins show decreased biological activity at the pH-extremes, due to changes in their specific three-dimensional folding which may also lead to a decreased solubility or even to precipitation. Therefore, it is desirable to handle proteins at a pH as close as possible to the physiological pH [8,9]. A possible means of counteracting adsorption is to use capillaries with a modified inner surface in order to avoid the above described effects [10–16]. The coating procedure established by Towns and Regnier [12] allows protein analysis to be carried out at pH 5.0.

Another challenging problem in protein analysis is their poor UV absorption, due to the relatively low number of chromophores in the molecules, leading to limits of detection around 10^{-6} *M* [3]. This is not sufficient for the analysis of therapeutic concentrations of many proteins in biological fluids. One approach allowing conventional UV absorbance detection is to increase the injection volume, which, with regard to the dimensions of the capillary used, ranges from 0.1 to about 100 nl in CZE [3].

Recently, several workers have successfully described the on-line coupling of electrophoretic preconcentration and CZE, thereby increasing the amount of sample that can be loaded and simultaneously improving the limit of detection by a factor of up to 1000 [isotachophoresis–CZE (ITP–CZE)] [17–29]. During the isotachophoretic step, focusing of the analytes appears due to local differences in the electric field strength, caused by a discontinuous electrolyte system formed by the leading electrolyte (LE), with high ionic mobility, and the terminating electrolyte (TE), with low ionic mobility. All ionic analytes exhibiting ionic mobility values between that of the leading and terminating ions simultaneously focus into distinct zones, migrating in order of their ionic mobilities in a steady-state at equal velocity. According to Kohlrausch [30], the analyte concentration at steady-state is adapted to the concentration of the LE. CZE with transient isotachophoretic preconcentration has been applied to protein analysis [23,26], but the reduced capillary length left for separation following the isotachophoretic step limits the possible injection volume. In order to overcome this problem, Reinhoud and co-workers [27,28] developed an automatic isotachophoretic preconcentration method, applying hydrodynamic counterflow during the isotachophoretic focusing step. This procedure prolongs the time span available for focusing and moves the focused zone towards the capillary inlet. Subsequently, the entire capillary can be used for electrophoretic separation. So far, this concept has been applied only to the analysis of low molecular mass compounds [27–29].

The aim of this study was to apply ITP with hydrodynamic counterflow to the CZE analysis of basic model proteins and rhIL-3, in order to enhance the sensitivity of this analytical method.

2. Experimental

2.1. Chemicals

Cytochrome c (horse heart, p*I* 10.2), myoglobin (horse heart, p*I* 7.3), lysozyme a (chicken egg white, p*I* 11.1), octadecyltrichlorosilane and Brij 35 were obtained from Sigma (St. Louis, MO, USA). Lysozyme b (hen egg white, p*I* 11.0) and ribonuclease A (bovine pancreas, p*I* 9.3) were obtained from ICN Pharmaceuticals (Meckenheim, Germany), recombinant non-glycosylated human interleukin-3 (p*I* 7.0) was kindly donated by Sandoz (Basle, Switzerland). Triethylamine (TEA), dichloromethane, β-alanine and acetic acid were purchased from Janssen Chimica (Brüggen, Germany). All chemicals used were of analytical grade quality, except the proteins which were of reagent grade purity.

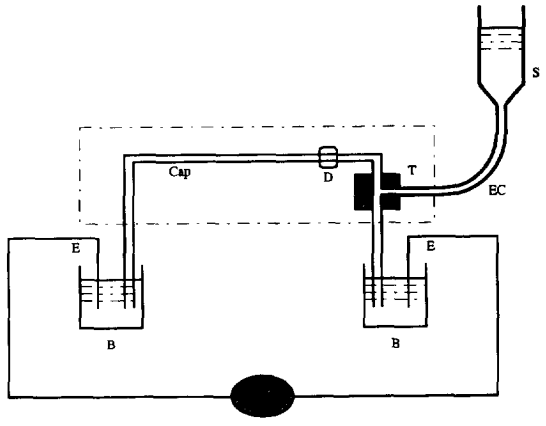
Samples were prepared by diluting stock solutions of 1 mg/ml of the proteins in water with terminating electrolyte (TE). RhIL-3 was obtained as a 4.3 mg/ml solution, and was diluted with TE to the desired

concentration. All solutions were prepared with water from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

All experiments were carried out on an automated P/ACE 2100 instrument (Beckman, Fullerton CA, USA), equipped with an UV-absorbance detector set at 200 nm and controlled by the System Gold software (Beckman). Fused-silica capillaries (GROM, Herrenberg, Germany), 57 cm total length, 50 cm to the detector, 75 μm I.D., were inserted into a modified P/ACE CE-cartridge (described below) with the liquid cooling system shortcut.

The fused-silica capillaries were coated following the procedure described by Towns and Regnier [12]. Prior to the coating procedure, a detection window was made by burning a 2 mm-long section of the external polyimide coating of the 57 cm separation capillary, 7 cm from the capillary outlet. This capillary was then cut 2.5 cm behind the detection window and both parts inserted into a laboratory-made polyethylene T-piece developed by Mazereeuw et al. [29], with the short part forming the outlet of the separation capillary (Fig. 1). One end of another 60 cm long coated capillary was inserted into the T-piece perpendicular to the separation capillary. The other end of this capillary was connected to a 10-ml plastic syringe, located outside the cartridge. The syringe was fixed to a vertically mobile clamp attached to a stative. All capillary parts outside the cartridge were isolated by a double layer of polyethylene tubing.



EC External capillary
Cap Separation capillary
T T-piece (Mazereeuw et al. [29])
S Syringe filled with leading electrolyte, vertically mobile to induce counterflow
E Platinum electrodes
HV High voltage power supply
D Detector
B Buffer vials

Fig. 1. Modification of the capillary cartridge (indicated by the dashed line) for the use of ITP–CZE with hydrodynamic counterflow.

2.3. Isotachophoresis

The isotachophoretic system consisted of TEA at concentrations ranging from 2 to 10 m*M* forming the leading electrolyte (LE) and β-alanine in equal concentration to the LE serving as the TE. All electrolytes contained 0.01% (w/v) Brij 35. The LE and the TE were adjusted to pH 5.0 with acetic acid. All buffers were filtered through a 0.45-μm membrane filter (Sartorius, Göttingen, Germany) and degassed in an ultrasonic bath, prior to use.

The entire capillary system was rinsed with LE for 15 min before the first analysis of the day. Three min rinses with LE were carried out between the individual electrophoretic runs. Five kV were applied to the capillary filled with LE and the resulting current was registered. In all experiments including an isotachophoretic preconcentration step, samples were injected for 6 s at a pressure of 1380 mbar, corresponding to an injection volume of 1.3 μl. Following injection, the anodic buffer vial was changed to TE. Then, 5 kV were applied for 2 min to make the analytes migrate away from the capillary inlet. Next, the syringe filled with LE was elevated to a height of 50 cm, corresponding to a pressure of 50 mbar, to induce the counterflow. During the application of hydrodynamic counterflow, free flow through the capillary outlet was hindered by closing it with a porous septum made from a polyurethane tube (M. Christ, Osterode, Germany). The increase of the current was monitored until it reached 95% of the value measured with the capillary filled only with LE (7.5–8.5 min). The voltage was then switched off and the syringe lowered until the liquid level was equal to the buffer vials in the P/ACE. Afterwards, the anodic buffer vial was changed to LE and a

constant voltage of 24.5 kV (425 V/cm) was applied for CZE separation.

3. Results and discussion

3.1. Protein separation without ITP

To obtain a good separation of the proteins, we selected the coating procedure established by Towns and Regnier [12], because the non-ionic character of the coating and the surfactant added to the buffer solutions did not interfere with the isotachophoretic focusing step. The applied coating chemistry reduced the electroendosmotic flow (EOF) but did not eliminate it, resulting in reasonable analysis times. Fig. 2 shows a typical electropherogram obtained using this coating procedure and a protein concentration of 1 mg/ml, with the proteins dissolved in TE. The sample was injected by applying a pressure of 34.5 mbar for 3 s, corresponding to an injection volume of about 18 nl. All proteins were well separated and the migration times of the proteins correlated well with their isoelectric points (Fig. 3). The correlation is described by $t_m = -1.0928 \cdot pI + 16.517$ ($r = 0.9835$), where t_m is the migration time and pI is the isoelectric point. This correlation might help to predict the migration times of other proteins not under investigation, thus facilitating the selection of suitable isotachophoretic conditions.

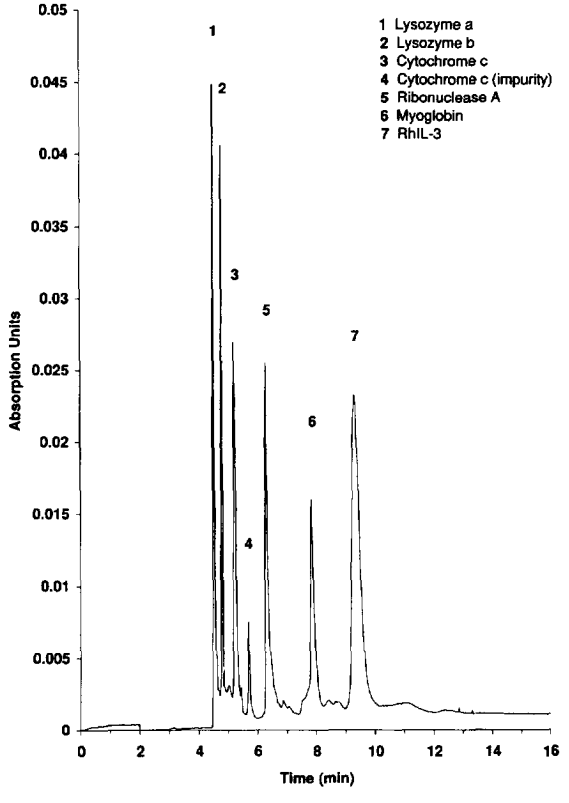


Fig. 2. CZE separation of basic model proteins and rhIL-3 (1 mg/ml each). Separation conditions: coated fused-silica capillary, total length 57 cm (50 cm to the detector), I.D. 75 μm, O.D. 360 μm; 24.5 kV constant voltage; 3 s low pressure injection (34.5 mbar). Buffer: 10 mM triethylamine–acetate, 0.01% (w/v) Brij 35, pH 5.0.

The identity of individual peaks was confirmed by injection of the single components. Cytochrome c was not found to be homogeneous, as indicated by an additional peak appearing just after the major component.

When, however, the analyte solution was diluted with TE to a concentration of 40 μg/ml for each protein, the resolution between the lysozyme species was lost and we observed that the ratio of peak heights was not very reproducible and was not comparable to that obtained with higher analyte concentrations. No signal from rhIL-3 could be detected at this concentration (Fig. 4). The selective disappearance of selected protein peaks at 40 μg/ml and the poor reproducibility might be attributable to residual non-specific adsorption to the capillary wall, thereby preventing quantification at this low concentration level.

3.2. Protein separation with ITP

Fig. 5 demonstrates the separation of the lysozyme species a and b, cytochrome c, ribonuclease A and myoglobin at a concentration of only 40 μg/ml each, but after a preceding isotachophoretic focusing step, using a discontinuous buffer system consisting of 10 mM LE and TE (see Section 2.3. The analysis was performed without rhIL-3, since the resolution between cytochrome c and both lysozyme species was found to be rather poor in the presence of rhIL-3 (data not shown). We attributed this observation to unknown basic additives present in the rhIL-3 solution, as received from the supplier, that may interfere with proper zone formation of the proteins with

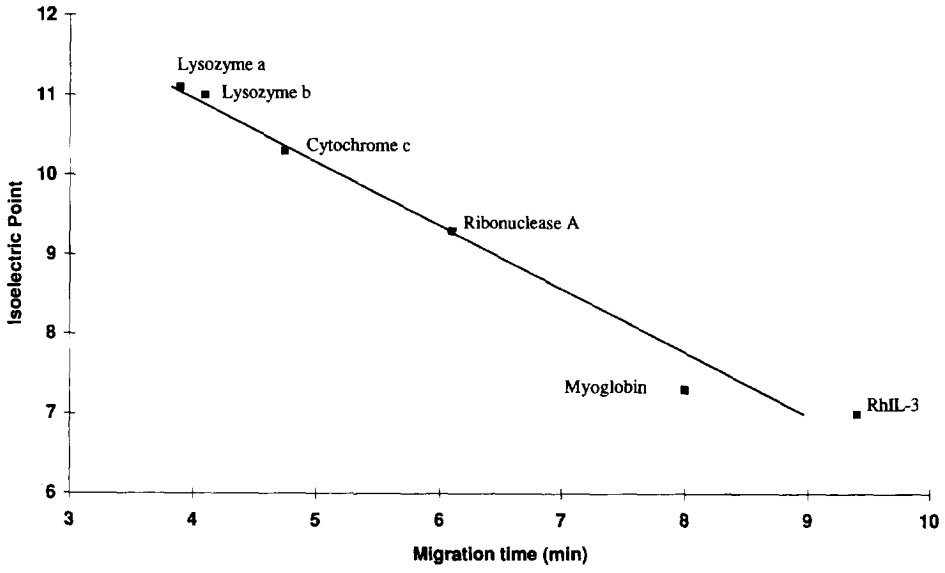


Fig. 3. Correlation plot of isoelectric points and migration times of basic model proteins and rhIL-3.

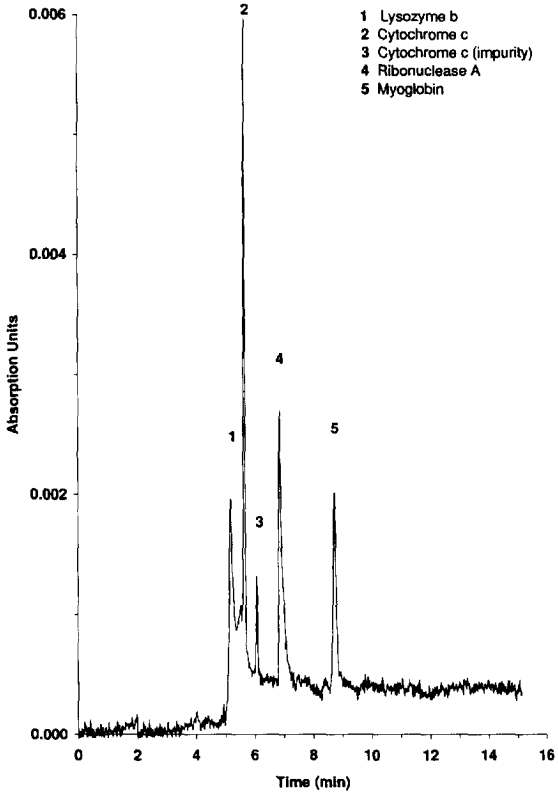


Fig. 4. CZE separation of basic model proteins and rhIL-3 (40 μg/ml each). For separation conditions see Fig. 2.

higher pI. Before the ITP focusing was started, a 1.3-μl volume of sample was injected. The separation efficiency and the migration order of the basic model proteins were found to be unchanged after the preceding ITP preconcentration step (Fig. 2 and Fig. 5). Again the analytes were well separated (Fig. 5) and the correlation of migration times and isoelectric points was comparable to the results described for the separation without ITP. The correlation is described by $t_m = -1.2235 \cdot pI + 17.48$ ($r = 0.992$). This result demonstrates that the electrophoretic migration behaviour is not affected by the ITP step or by the application of hydrodynamic counterflow prior to CZE separation. All peak heights of the major components exceeded 0.1 AU. In contrast to the analysis of the same sample without ITP, we observed good separation efficiency for the lysozyme species at 40 μg/ml. The sample concentration detection limit was about 1 μg/ml for both lysozyme species, 400 ng/ml for cytochrome c and 800 ng/ml for ribonuclease A, with this buffer system. The respective molar concentrations are presented in Table 1.

Under these conditions, the analysis of the two proteins with lower pI, myoglobin and rhIL-3, was not satisfactory. Myoglobin gave a second, large, slowly migrating peak representing an artefact

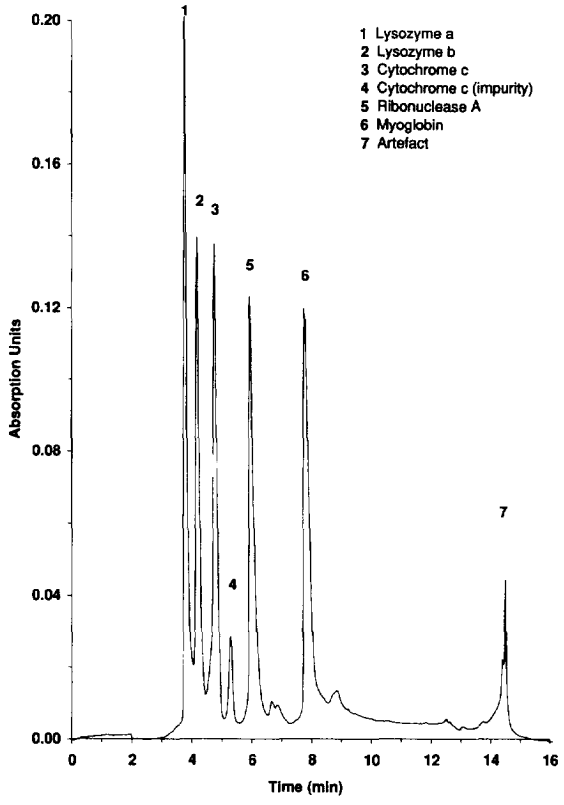


Fig. 5. ITP–CZE of basic model proteins (40 μg/ml each in TE) with hydrodynamic counterflow capillary: see Fig. 2. *ITP*: LE: 10 m*M* triethylamine–acetate, 0.01% (w/v) Brij 35, pH 5.0. TE: 10 m*M* β-alanine–acetate, 0.01% (w/v) Brij 35, pH 5.0; injection of 1.3 μl in TE; 5 kV constant voltage; hydrodynamic counterflow until current reached 4.1 μA (about 8 min). *CZE*: 24.5 kV constant voltage. For additional information see Section 2.

formed during the isotachophoretic preconcentration of the sample solution. Artefact formation was confirmed by conventional CZE of myoglobin (1 mg/ml) without sample preconcentration which resulted in a single peak (data not shown). The identity of the second peak could not be verified, although its irreproducible shape, height and migration time led us to assume that precipitation may occur during isotachophoretic preconcentration, despite the high solubility of myoglobin. After decreasing the protein concentration, the artefact was still present since only the length but not the concentration of the final ITP sample plug is determined by the sample concentration [18,19]. On analyzing a mixture of basic model proteins at a concentration of 15 μg/ml, no signal could be detected at the migration time predicted for myoglobin (Fig. 6). In contrast, the artefact was still observed. When rhIL-3 was analyzed under these conditions, it exhibited badly reproducible peak shape and migration time that were comparable with the observations made for myoglobin (data not shown). Whether or not this observation can be attributed to solubility problems remains to be evaluated.

3.3. Impact of buffer concentration

Assuming that precipitation is the underlying problem for the observed artificial peaks found when analyzing proteins with lower p*I*, we decreased the concentration of the LE as well as of the TE to 3 m*M*, in order to reduce the protein concentration in the sample plug resulting from the isotachophoretic focusing step. The concentration of the isotachophoretically migrating analyte zones adapts to that of the leading ions (TEA) in the LE, according to the equation of Kohlrausch [30].

$$c_A = c_L \cdot \frac{\mu_A}{\mu_L} \cdot \frac{\mu_L + \mu_R}{\mu_A + \mu_R} \quad (1)$$

where c_A is the analyte concentration in the isotachophoretic steady-state and c_L is the concentration of the leading ions. μ_A and μ_L are the mobilities of the analyte ions and the leading ions, respectively and μ_R is the mobility of the counter ion. When μ_A is close to μ_L, c_A will approximate to c_L.

For this reason, all further experiments with rhIL-3 and myoglobin were carried out in 3 m*M* buffer solutions. The TE was simultaneously diluted in order to create a sufficient difference in the electric field strength between the LE and the TE. This dilution step led to efficient reduction of artefact formation following myoglobin injection (Fig. 7). In addition, analysis of rhIL-3 with regard to peak shape and efficiency was considerably improved (Fig. 7). The detection limits for myoglobin and rhIL-3 were 800 ng/ml and 1 μg/ml, respectively. The respective molar concentrations are presented in Table 1. Further dilution of the buffer solutions to less than 3 m*M* led to poor separation efficiency and peak shape for all proteins investigated. With de-

Table 1
Sensitivity enhancement factors (*EF*) of peak height by isotachophoretic preconcentration with hydrodynamic counterflow, prior to CZE of basic model proteins and rhIL-3

Protein	Peak height		*EF*	Sample concentration detection limit (*M*) ITP–CZE
	CZE 1 mg/ml	ITP–CZE 40 μg/ml		
Lysozyme a	0.045	0.20	111.1	4.0×10^{-8}
Lysozyme b	0.036	0.14	97.2	4.3×10^{-8}
Cytochrome c	0.028	0.13	116.1	1.3×10^{-8}
Cytochrome c (impurity)	0.007	0.03	107.1	–[b]
Ribonuclease A	0.025	0.12	120.0	3.3×10^{-8}
Myoglobin	0.024	0.12	125.0	4.7×10^{-8}
RhIL-3	0.021	0.02[a]	95.2	7.7×10^{-8}

[a]10 μg/ml.
[b]Molecular mass unknown.
[c]The results are the average of three injections

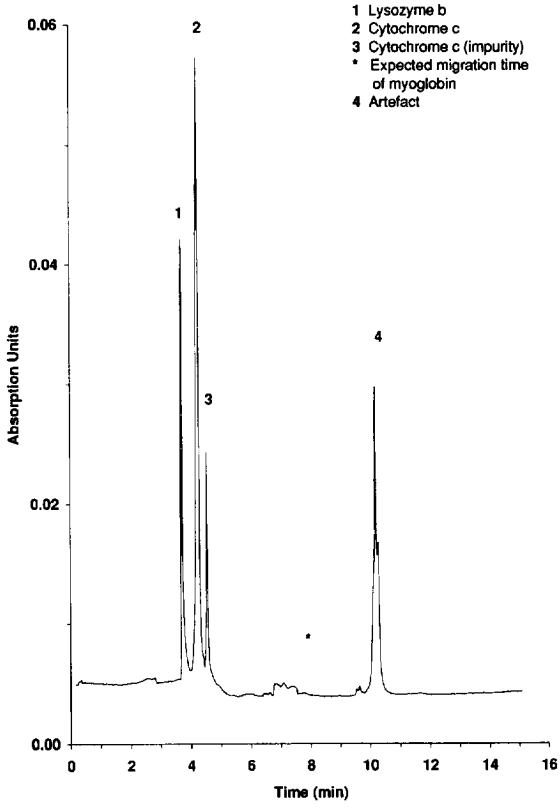


Fig. 6. ITP–CZE of lysozyme b, cytochrome c and myoglobin (15 μg/ml each in TE). For separation conditions see Fig. 5. The asterisk marks the anticipated migration time for myoglobin.

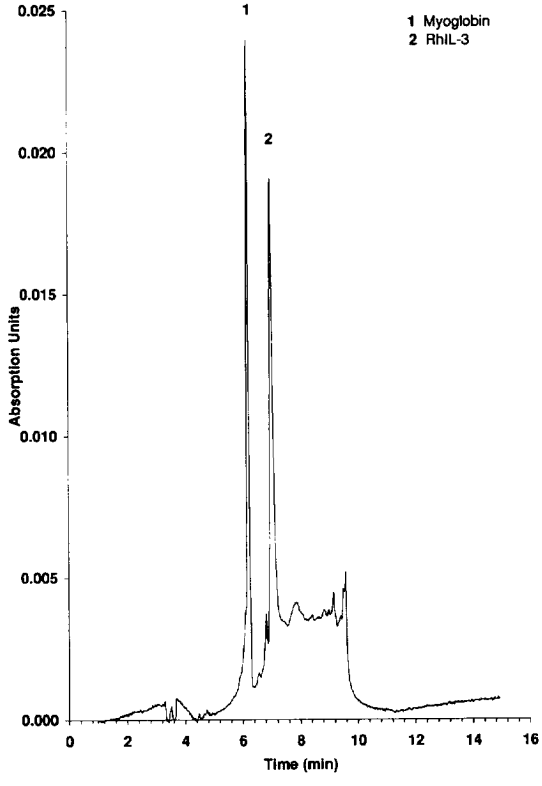


Fig. 7. ITP–CZE of myoglobin and rhIL-3 (10 μg/ml each in TE). For separation conditions see Fig. 5, except that the concentration of all electrolytes was 3 m*M*.

creasing electrolyte concentration, the determination of the end of the isotachophoretic step becomes more difficult, due to the low measurable currents and the

low absolute difference between the conductivities of the LE and the TE.

3.4. Sensitivity enhancement

Sensitivity enhancement obtained by on-line ITP compared to CZE without a preceding isotachophoretic step was assessed by calculation of an enhancement factor (*EF*) which is defined as follows:

$$EF = \frac{S_{ITP}}{C_{ITP}} \cdot \frac{C_{dir}}{S_{dir}} \quad (2)$$

where S_{ITP} is the peak height following isotachophoretic focusing, S_{dir} is the peak height obtained by direct CZE determination, C_{ITP} and C_{dir} are the analyte concentrations in the samples analyzed by ITP–CZE and by direct CZE, respectively.

The results are summarized in Table 1. Average values of the peak heights determined in three injections were used. We found *EF*s ranging from 95.2 for rhIL-3 to 125 for myoglobin. All values were obtained by comparing the peak heights found after conventional CZE separation of a sample containing 1 mg/ml of each protein and those obtained after ITP–CZE of a solution containing 40 μg/ml or 10 μg/ml, respectively. Apparently, there is no relationship between the p*I* of the proteins and their calculated *EF*, indicating that no artefacts are formed when lower buffer concentrations were used for the proteins with lower p*I*.

From our results, it is evident that the sensitivity enhancement is limited to about two orders of magnitude using the described ITP–CZE setup. This limitation can be explained by electrophoretic band broadening occuring with high analyte concentrations at the beginning of the CZE step, because of a conductivity mismatch between the sample zone and the LE now acting as a background electrolyte. This phenomenon has been described in detail elsewhere [27].

4. Conclusions

The feasibility of CZE, following cationic isotachophoresis combined with hydrodynamic counterflow to decrease the detection limit for basic model proteins and rhIL-3 by a factor of about 10^2 (Table 1), was demonstrated. We were able to show the suitability of a commercial instrument for this analytical discipline after slight modification of its capillary cartridge.

The physicochemical characteristics, particularly the isoelectric point, of each individual protein have to be taken into account when selecting the isotachophoretic parameters. Our method proved to be generally applicable for the analysis of basic proteins with a pI>8. When proteins with a pI<8 are to be analyzed at pH 5.0, the buffer concentration should be carefully selected because of possible artefact formation. More acidic proteins cannot be focused in the cationic mode at pH 5.0, as they require anionic ITP pretreatment at basic working pH.

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