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Capillary isoelectric focusing with UV-induced fluorescence detection

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Abstract

Low-molecular-mass fluorescent compounds excitable in the near UV region with suitable acidobasic and electrophoretic properties are suggested as isoelectric point (p*I*) markers for isoelectric focusing (IEF) with UV photometric and UV excited fluorometric detection. The experimental set-up of capillary IEF with UV excited fluorometric detection and properties of new UV-induced fluorescent p*I* markers are given. The p*I* values of 18 new p*I* markers determined independently of IEF methods range from 2.1 to 10.3. The examples of separation of new p*I* markers together with derivatized proteins by capillary IEF with photometric or fluorometric detection are presented.

Keywords: Isoelectric focusing; Detection, electrophoresis; Fluorescence detection; Proteins

1. Introduction

Capillary isoelectric focusing (cIEF) [1,2] is a potent technique for the analysis of dilute protein samples. For the detection of the focused proteins in cIEF, UV photometric on-line detection is used most often. Since there are few chromophores in the moiety of most proteins, their specific absorbance, $A^{1\%}_{1\ cm}$, is typically about 10 around 280 nm [3]. Thus, the high absorption of current IEF carriers in the short wavelength region interferes with UV detection in cIEF. The derivatization of proteins for photometric or fluorometric detection at higher wavelength [4] can overcome this limitation [5,6]. Though the UV-excited fluorometric detection in capillary electrophoresis has emerged only recently [7], it is common in gel electrophoresis.

To determine the isoelectric point, p*I*, of an analyte, the pH at the place of its focusing should be known. This pH is evaluated with the help of the reference substances most often called isoelectric point markers (p*I* markers). In conventional IEF, proteins with known isoelectric points [8–10] are used as p*I* markers to calibrate the background pH gradient and for control of focusing process. The native proteins, however, have some distinct disadvantages for their use as p*I* markers. They include tendency to precipitate at pH close to p*I* and instability of the substances and their water solutions. Some protein standards comprise a mixture of the related proteins. Proteins have many possible reactive sites for a reactive dye and this might result in the formation of a heterogeneously labeled product with respect to p*I*.

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Thus, the search for non-protein ampholytes potentially applicable as the reference substances in IEF with photometric and fluorometric detection was initiated. One of the recent approaches uses the tryptophan-containing peptides for IEF with UV photometric detection [11]. The compounds prepared by dansylation of the carrier ampholytes [12] were examined by IEF with UV photometric detection. Their use for electrophoresis with UV-excited fluorometric detection, though possible in principle, was not reported until now. Further, the rhodamine-labeled peptides [13] were suggested for laser-induced fluorescence (LIF) detection in cIEF. However, the p*I* markers so far used do not seem to meet all the necessary demands. Disadvantages are mainly based either on their small number and range of p*I* values covered or complicated preparation and purifying of synthetic products. Some of above problems can be eliminated by low-molecular-mass (M_r<500) p*I* markers [14,15]. They can be prepared with no heterogeneity and greater stability than proteins and could potentially be useful p*I* markers in cIEF with UV detection [16–20].

Apart from their purity, solubility and detectability, the low-molecular-mass p*I* markers should behave as good ampholytes. It means that the dependence of their effective charge, *z*, on pH, [−dz/d(pH)], is sufficiently steep close to the marker p*I*. In the case of biprotic ampholytes, it implies that both pK_a values, i.e., pK_{a1}, pK_{a2}, must be close to one another. Thus, the condition for the good biprotic ampholyte can be formulated as [21,22]:

$$(pK_{a2} - pK_{a1}) < 4 \quad (1)$$

The relation of the tangent of the dependence of the effective charge on pH at the compound isoelectric point, $[-dz/d(pH)]_{pI}$, to pK_a values of a biprotic ampholyte is [22–24]:

$$[-dz/d(pH)]_{pI} = \ln 10/[1 + (K_{a1}/4K_{a2})]^{0.5} \quad (2)$$

Thus, the good ampholyte, and so the low-molecular mass p*I* marker, should have a $[-dz/d(pH)]_{pI}$ value above 0.045 [14,15].

Here we suggest fluorescent low-molecular-mass ampholytes with known isoelectric points as the UV photometric and fluorescent p*I* markers for IEF. To show the possibility of UV-induced fluorescence detection in IEF, new low-molecular-mass fluorescent compounds excitable in the near UV region were focused in cIEF with UV photometric or fluorometric detection. The experimental set-up of cIEF and properties of the new UV-induced fluorescent p*I* markers are given. The examples of IEF of native or derivatized proteins with suggested p*I* markers are presented.

2. Experimental

2.1. Equipment

The cIEF experiments were carried out using a laboratory-made apparatus. The electroosmotic flow (EOF) technique of mobilization could bring some problems with linearity and reproducibility over a wide p*I* range for individual fused-silica capillaries [2]. Therefore, the EOF was reduced and a suitable linear velocity of focused zones was achieved hydrodynamically. The hydrodynamic flow was accomplished by siphoning action obtained by elevating the inlet (anolyte reservoir), relative to the outlet (catholyte reservoir) (see Fig. 1). Here, the height difference of the reservoirs for a focusing run, Δh_I, can be adjusted in range 50 to 100 mm. The sample was injected in the same way, see Fig. 1, with height differences of the reservoirs for sampling, Δh_{II}, adjustable in range 150 to 300 mm for 35 to 40 s.

All measurements were made at constant voltage (–) 19 kV supplied by a Spellman CZE 1000 R high-voltage unit (Plainview, NY, USA). During the experiments, the current decreased from 30 to 40 μA at the beginning of the experiment to 3 to 5 μA at the time of detection, depending on the sampling time interval and the sample solution. The length of the fused-silica capillaries (Lachema, Brno, Czech Republic), was 500 mm, 400 mm to the detector. Their I.D. was 0.072 mm and the O.D. was 0.36 mm. The ends of the separation fused-silica capillary were dipped in 3-ml glass vials with the buffer (anolyte or catholyte) and electrodes.

Three detectors were used in IEF instrumental set-ups. For single-wavelength on-column UV detection, a UV–Vis detector LCD 2082 (Ecom, Prague, Czech Republic) was connected to the

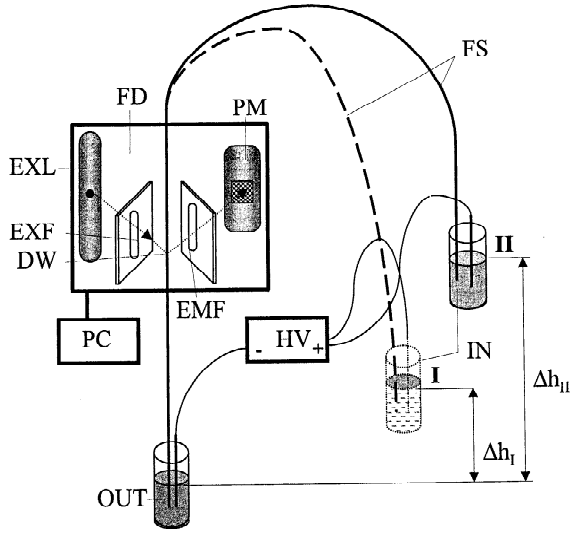

Fig. 1. Schematic representation of the laboratory-made apparatus for the cIEF with hydrodynamic mobilization and fluorometric detection. HV = High-voltage unit; PC = personal computer; FS = fused-silica; DW = detection window; FD = fluorometric detector; PM = photomultiplier; EXL = excitation lamp; EXF = excitation filter; EMF = emission filter; IN = inlet, anolyte reservoir in position I or II; OUT = outlet, catholyte reservoir; Δh_I or Δh_{II} = height differences of the reservoirs for the hydrodynamic mobilization of the focused zones or for the siphoning injection, respectively.

detection window of the separation fused-silica (FS) capillary by optical fibers with a core diameter 0.2 mm (Polymicro Technologies, Phoenix, AZ, USA). The analytical signal was collected by a personal computer equipped with the CSW data handling software (DataApex, Prague, Czech Republic).

For multi-wavelength UV detection, a fast-scanning UV–Vis Spectra Focus optical detector (Thermo Separation Products, CA, USA) with the detection cell No. 9550-0155 was used. Throughout this work, scanning from 250 to 360 nm at 10-nm intervals was performed. The analytical signal was collected by the personal computer equipped with the PC 1000 Ver. 3.0 software from Thermo Separation Products.

For on-column UV fluorometric detection we adapted a Kratos FS 950 fluorometer (Schoeffel Instruments, Westwood, NJ, USA) [25] see Fig. 1. The maximum intensity range of the excitation was 330–375 nm, the chosen cut-off wavelengths of filters were 365 nm for excitation and 418 nm for emission. Similarly to single-wavelength UV photometric detection, the analytical signal was collected by the personal computer equipped with the CSW data handling software.

2.2. Chemicals

All chemicals were analytical grade. The fluorescent p*I* markers were from Fluka (Buchs, Switzerland). The relevant acidobasic and optical properties of the fluorescent UV p*I* markers summarized in Table 1 include rounded p*I*, p*I* calculated from pK_{a1}, pK_{a2}:

$$pI = \frac{pK_{a1} + pK_{a2}}{2} \quad (3)$$

$[-dz/d(pH)]_{pI}$, λ_{max}, the specific absorption, $A^{1\%}_{1\ cm}$, at λ_{max}, $A^{1\%}_{1\ cm}$ at 280 nm and λ_{em}. The compound p*I* as well as pK_{a1}, pK_{a2}, $[-dz/d(pH)]_{pI}$ were determined independently of IEF, i.e., by potentiometric titration and spectrophotometry of pure compounds in aqueous 0.1 *M* phosphate buffer at 25°C. The optical properties given are measured in 0.1 *M* phosphate buffer of pH equal to the p*I* of the respective compound. Cytochrome *c* from horse heart (M_r 12 400, p*I* 9.4], bovine serum albumin (M_r 67 000, p*I* 4.8) and hydroxypropylmethylcellulose (HPMC) (H 7509) were from Sigma (St. Louis, MO, USA); myoglobin from skeletal muscle (M_r 17 800, p*I* 6.8–7.0) was from Serva (Heidelberg, Germany). Ribonuclease A from bovine pancreas (M_r 13 700, p*I* 8.9 [9]) was from Reanal (Budapest, Hungary), the solution of synthetic carrier ampholytes (Ampholine, pH 3.5–10.0) was obtained from Pharmacia (Uppsala, Sweden). 5-(Dimethylamino)naphthalene-1-sulfonylchloride (dansyl chloride, Dns-Cl) was from Merck (Darmstadt, Germany). Other chemicals were obtained from Lachema.

2.3. Preparation and conditioning of the fused-silica capillaries

The EOF in cIEF could flush the ampholytes from the capillary before focusing is complete so the EOF needs to be reduced or eliminated. The reduction of EOF and protein adsorption can be accomplished by the use of suitable coatings. It was found that the

Table 1
Acidobasic and optical properties of fluorescent UV p*I* markers

p*I*, rounded	p*I*, $pK_{a1}+pK_{a2}/2$	pK_{a1}	pK_{a2}	$[-dz/dpH]_{pI}$	λ_{max} (nm)	$A^{1\%}_{1\ cm}$ at λ_{max}	$A^{1\%}_{1\ cm}$ at 280 nm	λ_{em} (nm)
2.1	2.10	1.50	2.70	0.77	340	235	14	408
3.0	3.02	1.69	4.35	0.20	349	117	29	416
3.5	3.45	4.00	2.90	0.83	300	78	47	402
4.0	4.00	3.20	4.80	0.55	308	90	39	400
4.4	4.43	3.86	5.00	0.80	329	149	32	402
5.1	5.12	3.94	6.30	0.27	324	130	22	402
5.5	5.45	3.85	7.05	0.11	325	126	17	402
6.1	6.15	5.35	6.95	0.55	389	68	64	475
6.6	6.60	5.80	7.40	0.55	391	77	66	475
6.8	6.80	6.05	7.55	0.60	314	104	28	400
7.0	6.95	6.40	7.50	0.83	314	114	30	400
7.2	7.15	6.20	8.10	0.42	347	54	119	500
7.6	7.55	6.80	8.30	0.60	350	52	121	500
8.1	8.05	6.80	9.30	0.23	338	142	17	400
8.6	8.60	7.60	9.60	0.38	383	95	147	500
9.0	8.95	8.05	9.85	0.46	385	59	75	500
9.5	9.50	8.70	10.30	0.55	325	169	19	398
10.3	10.30	9.95	10.65	1.08	385	142	65	500

coating of the inner surface of the fused-silica capillary with γ-glycidoxypropyltrimethoxysilane (GOPTMS) [26,27] greatly suppressed EOF [26]. The capillary was rinsed with methanol and then with a 5% (v/v) solution of GOPTMS in methanol for 2 h slowly forced through the capillary. Then the column was purged with N_2 gas at 80°C for 2 h. After this procedure capillary was washed with 2 ml of methanol and water. Newly prepared capillaries were filled overnight and then before each injection for 15 min with a carrier mixture of solution 2% (w/v) of ampholyte and 0.25% (w/v) of HPMC and then back-flushed with catholyte.

2.4. Electrolyte systems and sample preparation

For all the experiments presented here, 20 m*M* sodium hydroxide and 100 m*M* orthophosphoric acid were used as the catholyte and anolyte, respectively. 0.001% and 0.25% (w/v) HPMC was added to the catholyte or anolyte, respectively. The sample components (p*I* markers and/or proteins, 25 μg ml^{-1}) were dissolved in 2–3% (w/v) Ampholine without the addition of HPMC. The amount of the single protein or p*I* marker injected into the capillary was 0.25 or 0.50 ng, respectively. For the fluorometric detection, the proteins were converted to their fluorescent dansyl (Dns) derivatives. Dns derivatization was carried out under conditions similar to those used previously [28–31] with the solution of Dns-Cl in acetonitrile (1.5 mg ml^{-1}) and 40 m*M* aqueous solution of Li_2CO_3 as the buffer. The ratio of Dns-Cl to buffer was 1:2; the ratio of Dns-Cl to proteins was 15:1. The proteins mixture in the vial was treated with the Dns-Cl solution and mixed vigorously for 20 s. The vial was heated at 100°C for 2 min and allowed to cool. The fluorescent Dns derivatives of proteins were prepared anew daily.

3. Results and discussion

The record obtained by cIEF with UV detection at 340 nm is shown in Fig. 2. In the bottom panel, the cIEF record of 10 markers covering the p*I* range from 3.5 to 10.3 can be seen. In the upper panel, the marker p*I* vs. migration time is plotted. The graph shows acceptable linearity which indicates the possibility of using the p*I* markers for determination of the p*I* of unknown ampholyte by simple linear interpolation also in cIEF, providing the EOF is reduced. In our case the electroosmotic migration is decreased so that the analysis time would be excessively long. Therefore, additional hydrodynamic

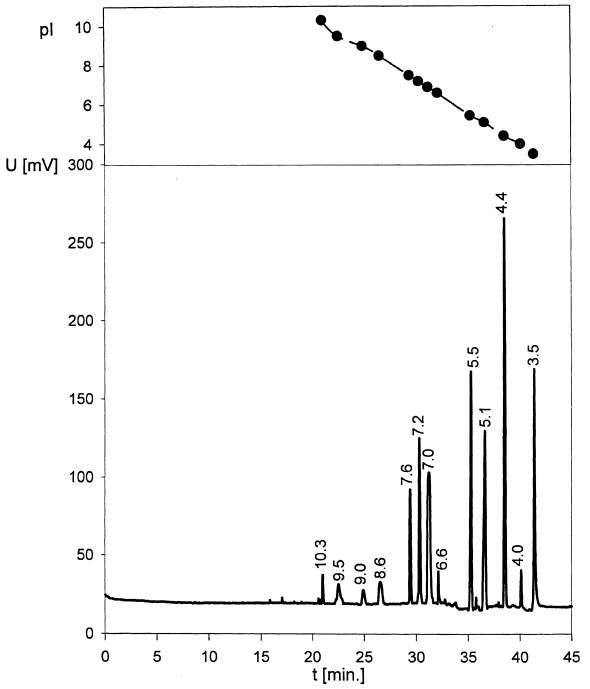

Fig. 2. Calibration curve of the background pH gradient and the electropherogram of the separation of the focused p*I* markers with UV detection at 340 nm. Conditions: FS 0.072 mm I.D., length 500 mm (400 mm to the detection window); coated with γ-glycidoxypropyltrimethoxysilane; anolyte: 100 m*M* H_3PO_4, 0.25% (w/v) HPMC; catholyte: 20 m*M* NaOH, 0.001% (w/v) HPMC, Δh_I = height difference of the reservoirs at the hydrodynamic mobilization of the focused zones, 50 mm; Δh_{II} as Δh_I at the siphoning injection, 300 mm; injection time 35 s; applied voltage, (–) 19 kV; the current values decreases from about 30 μA to 3 μA; detection wavelength, 340 nm; p*I* markers: 10.3–3.5 (25 μg ml^{-1}) in 2% (w/v) Ampholine.

mobilization by siphoning (see Fig. 1, Δh_I) is used to decrease the analysis time by about one half.

In Fig. 3, the records obtained by cIEF in combination with fast scanning UV photometric detection are displayed. Here, the sample comprises seven p*I* markers together with unmodified albumin. The three-dimensional record in Fig. 3A indicates the marked dependence of the detector response intensity on the wavelength in the near UV range. While above 300 nm only the ladder of markers is detected, the protein is well detectable at lower wavelengths where the detection of some markers is suppressed. The detection sensitivity dependence on the wavelength used is more obvious from normalized spectra extracted from the three-dimensional record shown in Fig. 3B and C. Due to the differences in the chemical structures of the p*I* markers, the spectral properties are different, see Table 1. Some p*I* markers have strong absorption around 280 nm where the most proteins are detected, see, e.g., the spectra of p*I* markers with p*I* 6.6, 7.2, 7.6 and 10.3 in Fig. 3B and Table 1. Such compounds can be advantageous for single-wavelength detection of both native proteins and p*I* markers. The other p*I* markers have absorption maxima around 330 nm while their detectability around 280 nm is strongly decreased, see the spectra of markers with p*I* 3.5, 5.5 and 9.5 in Fig. 3C and Table 1. Such properties make them suitable for dual- or multi-wavelength UV detection. In this mode of operation, two records of both the p*I* ladder and proteins can be obtained from a single cIEF run. In this way, certain parallels to the two-lane gel bed operation can be seen.

As can be seen from Table 1, all the p*I* markers listed in Table 1 are fluorescent with emission in the visible part of the spectrum and with a Stokes shift of more than 50 nm. Thus, their optical properties make them useful for UV-excited fluorescence detection. The examples of the records obtained by cIEF equipped with the modified simple two-filter fluorometric detector (see Fig. 1) are shown in Fig. 4. In the bottom panel, the record of 11 p*I* markers shows the ladder of p*I*. The upper panel shows the record of a focusing run of five p*I* markers + four dansylated proteins. The records indicate the feasibility of cIEF with UV-induced fluorescence detection.

4. Conclusions

The optical properties of the suggested p*I* markers make them detection compatible with the products of protein derivatization with, e.g., Dns-Cl, fluorescamine, *o*-phthaldialdehyde and coumarine moieties [4] which are used for the enhancement of protein detection both by UV photometric and UV-excited fluorometric detectors. Good focusing ability, easy spectroscopic identification and stability of these p*I* markers together with their fluorescence facilitate the p*I* determination of proteins using both capillary and slab gel electrophoresis.

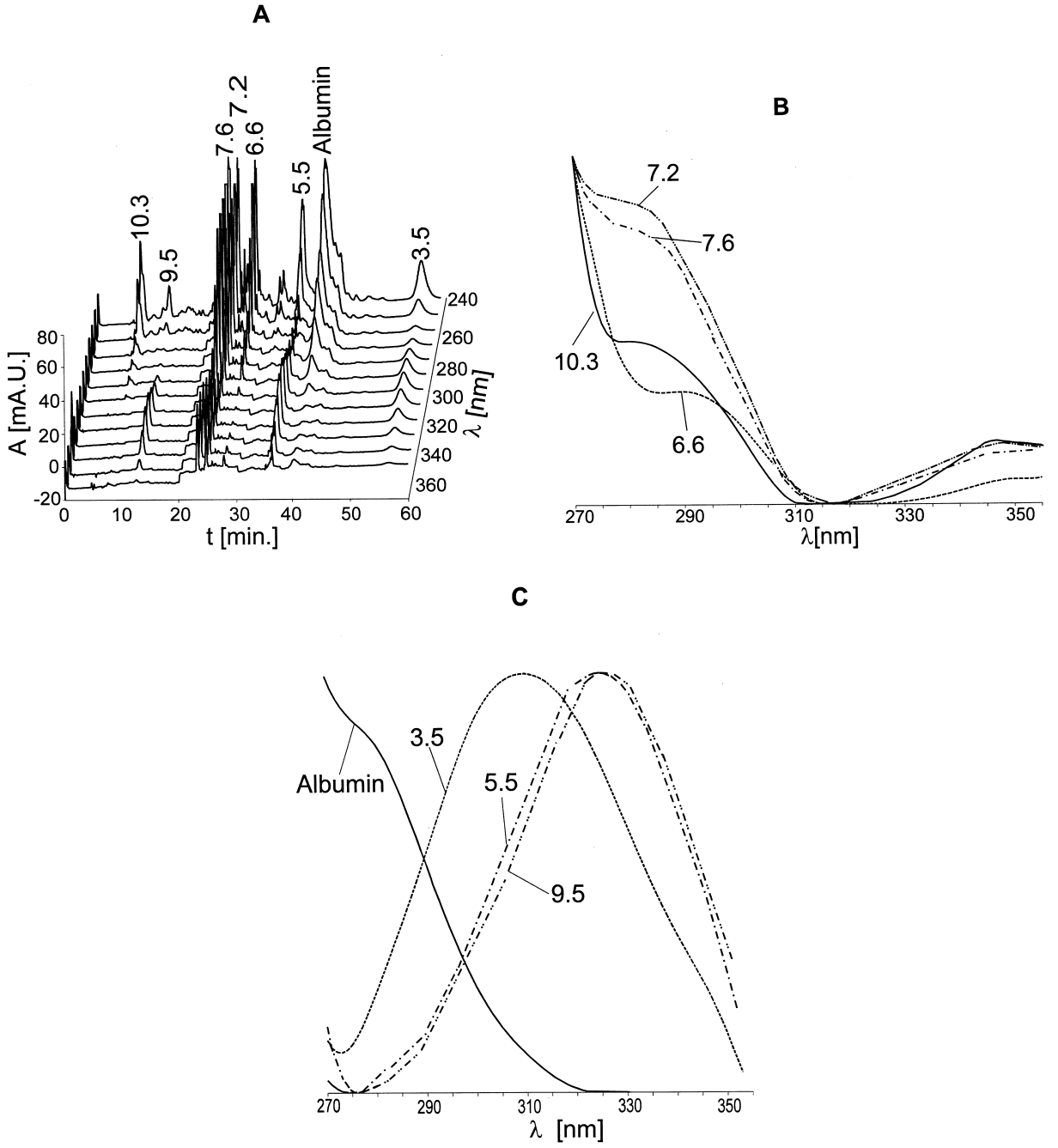

Fig. 3. Three-dimensional data plot electropherograms of p*I* markers and albumin and their normalized spectra. Conditions and designations, see Fig. 2. $\Delta h_{I} = 100$ mm; $\Delta h_{II} = 150$ mm, injection time 40 s; multi-wavelength UV detector, scanning from 250 to 360 nm at 10-nm intervals; sample solution, 3% (w/v) Ampholine, albumin (25 μg ml^{-1}); (A) three-dimensional date plot; (B, C) normalized spectra for p*I* markers and albumin from 270 to 355 nm.

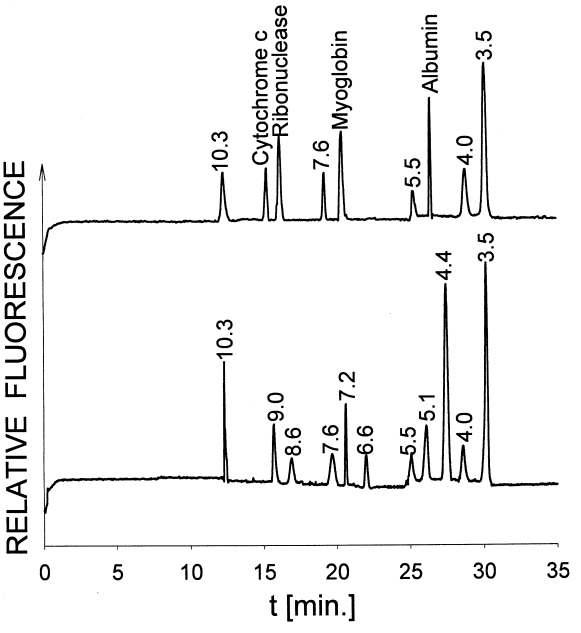

Fig. 4. The electropherograms of the p*I* markers and fluorescent Dns derivatives of proteins with fluorimetric detection. Conditions and designations, see Fig. 2. $\Delta h_{I} = 100$ mm; $\Delta h_{II} = 150$ mm, injection time 40 s; the current values decreases from about 40 μA to 5 μA; the maximum intensity range of the excitation lamp, 330–375 nm, the filters: for the excitation wavelength 365 nm, the emission cut-off 418 nm; Dns derivatives of proteins, expected concentration after dansylation and dilution, 25 μg ml^{-1}.

Acknowledgements

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