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Behaviour of substituted aminomethylphenol dyes in capillary isoelectric focusing with electroosmotic zone displacement

Jitka Caslavska[a], Sarah Molteni[a], Josef Chmelík[b], Karel Šlais[b], František Matulík[b], Wolfgang Thormann*,[a]

[a]*Department of Clinical Pharmacology, University of Berne, Murtenstrasse 35, CH-3010 Berne, Switzerland*
[b]*Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveri 97, CS-611 42 Brno, Czech Republic*

Abstract

The behaviour of six substituted aminomethylphenol dyes, having p*I* values between 5.3 and 10.4, in capillary isoelectric focusing with electroosmotic zone displacement is described. Using untreated fused-silica capillaries and different neutral capillary conditioners in the catholyte, the low-molecular-mass dyes are shown to focus and elute reproducibly in the order of decreasing p*I* values. In the absence of proteins, the detection times of the dyes are independent of the sampled amount. Hence these substances permit the characterization of the pH gradient produced in this capillary isoelectric focusing method. With concurrent focusing of dyes and test proteins, a macromolecular impact on detection times (reduction of electroosmosis) is revealed. The effect is shown to be dependent on the type and amount of proteins applied and has been observed with three different capillary conditioners. Nevertheless, mapping of the pH gradient with these dyes and determining the p*I* values of known proteins is shown to provide values in agreement with those in the literature. Hence the substituted aminomethylphenol dyes can be employed as p*I* markers in capillary isoelectric focusing with electroosmotic zone displacement. Further, focusing and separation of two of the six dyes by preparative recycling free fluid isoelectric focusing is described, illustrating that the substituted aminomethylphenol dyes are also applicable to other free fluid focusing methods.

1. Introduction

Isoelectric focusing (IEF) is an electrophoretic separation technique for amphoteric compounds in which separation is carried out in a pH gradient which increases from the anode to the cathode. Amphoteric compounds, including proteins, migrate until they align themselves at their isoelectric positions where a dynamic equilibrium between the electrokinetic concentrating and the dispersive processes, including diffusion, is established [1,2]. Traditionally, IEF on an analytical scale has been carried out in gels, requiring tedious, time-consuming preparation and protein staining procedures. In the past few years, however, attention has been focused on developing IEF into a more instrumental format by using gel-free capillaries as focusing columns [3–14]. The first approaches operated with minimized electroosmosis which required that after focusing, the IEF zone pattern had to be mobilized and swept past a stationary detector [3–6]. More recently, capillary isoelectric focusing (cIEF) in the presence of an electroosmotic

* Corresponding author.

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flow was described [7–14]. In this approach, mobilization of focused zones is unnecessary because these zones are displaced towards and across the point of detection by the electroosmotic flow. Using untreated fused-silica capillaries and the cIEF procedure investigated by ourselves [7–10], a typical experiment proceeds as follows. First the entire capillary is filled with the catholyte containing a small amount of a neutral polymer, *e.g.*, hydroxypropylmethylcellulose (HPMC), as capillary conditioner. Sample composed of carrier ampholytes and test compounds is introduced at the anodic end and occupies about 30–50% of the effective capillary length. After power application, the formation of the pH gradient, the separation of the sample compounds and the displacement of the entire ampholyte pattern towards the cathode occur simultaneously. Basic ampholytes and test substances reach the point of detection prior to neutral and acidic compounds [7]. This technique was recently reported to be an attractive and simple method for the analysis of normal and pathological haemoglobins [10].

In IEF, the determination of the isoelectric point of an amphoteric substance requires the determination of the pH value at the focusing position. Further, the knowledge of the pH gradient permits the characterization of the focusing process. Hence the pH course along the column has to be mapped, this being typically achieved by using reference substances referred to as p*I* makers. The pH gradient is simply visualized by plotting the focusing position against the p*I* values of these markers. Both high- [15–17] and low- [17–20] molecular-mass amphoteric compounds can be employed as p*I* markers. In gel IEF, coloured proteins are typically employed, such as those which are commercially available in kit form [21]. Suitable p*I* markers should be characterized by (i) a sufficiently high slope of mobility *vs*. pH around their p*I*, (ii) high solubility in ampholyte solutions at their p*I*, (iii) no interaction with analytes, (iv) long-term stability in solution and (v) with visual detection also a high colour intensity [15]. Coloured proteins do not meet all the above demands (*e.g.*, low solubility at p*I*, low stability in solution and they cannot be used in organic solvents or under other denaturing conditions). On the other hand, low-molecular-mass p*I* markers can provide most requirements, but are restricted for use in free fluids. The number of these compounds reported so far, however, is limited [17–20], and their relatively high hydrophobicity causes low solubility and undesired interaction with other compounds [20]. Recently, a new series of low-molecular-mass p*I* markers based on substituted aminomethylphenols was prepared and used for ion-exchange chromatography with pH gradient elution [22].

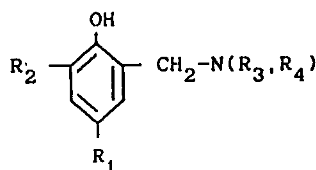
In this work, the cIEF of six substituted aminomethylphenol dyes (for structures and other characteristics see Table 1) was studied using uncoated capillaries and different dynamic capillary conditioners in the catholyte. Concurrent focusing of the dyes and model proteins was also investigated. Further, the characterization of two dyes using recycling isoelectric focusing [23] was examined.

2. Experimental

2.1. Chemicals

All chemicals were of research grade. The dyes were obtained from Tessek (Prague, Czech Republic). Preparation of the dyes has been described elsewhere [22]. Haemoglobin (Hb) samples were prepared as described previously [10]. Equine myoglobin from skeletal muscle (MYO; M_r = 17 800, p*I* = 6.8–7.0) was obtained from Serva (Heidelberg, Germany). Cytochrome *c* from horse heart (CYTC; M_r = 12 384, p*I* = 9.3), carbonic anhydrase from bovine erythrocytes (CA; M_r = 31 000, p*I* = 6.18), hydroxypropylmethylcellulose (HPMC), polyoxyethylene 23 lauryl ether (Brij 35) and methylcellulose {MC, with a viscosity of 4000 cP [2%(w/v) solution at 25°C]} were supplied by Sigma (St. Louis, MO, USA). Ampholine pH 3.5–10, Ampholine pH 5–8 and Pharmalyte pH 6.7–7.7 were obtained form Pharmacia–LKB (Uppsala, Sweden).

Table 1
Structures and characteristics of substituted aminomethylphenol dyes used in this work



Dye No.	R_1	R_2	$N(R_3,R_4)$	M_r	p*I*	$\lvert(dz/dpH)_{pI}\rvert$	λ_{max} (nm)	*A*	*C* (μg/ml)[a]
1	NO_2	$CH_2N(R_3,R_4)$	PIP	406	10.4	0.76	403	617	3.9
3	NO_2	$CH_2N(R_3,R_4)$	MPIPE	509	8.6	0.74	420	102	16.7
7	NO_2	H	PIP	273	8.0	0.02	392	661	2.8
13	CH_3	NO_2	MOR	289	7.2	0.15	416	162	10.9
18[b]	4-$CH_2N(R_3,R_4)$	2-Cl-6-NO_2	HPIPE	389	6.2	0.10	415	133	14.1
19[b]	4-$CH_2N(R_3,R_4)$	2-Cl-6-NO_2	MOR	309	5.3	0.12	409	142	12.1

According to ref. 22 with the exception of last data column. PIP = 1-piperidyl; MPIPE = 1-(4-methylpiperazinyl); HPIPE = 1-(4-hydroxyethylpiperazinyl); MOR = 4-morpholinyl; M_r = relative molecular mass; λ_{max} = wavelength of maximum absorption in aqueous solution at pH = p*I*; *A* = absorptivity of aqueous solution (1%) at pH = p*I*.

[a] Typical dye concentration employed in the sample. Concentration differences between dyes were chosen to obtain similar absorption responses after focusing.

[b] Aminomethyl group in position 4 and substituents R_1 and R_2 in positions 2 and 6, respectively.

2.2. Capillary isoelectric focusing

cIEF was performed in two instrumental setups. First a laboratory-made capillary electrophoretic analyser, which was described previously [7], was employed. Briefly, it features a 75 μm I.D. fused-silica capillary about 90 cm long (Product TSP/075/375; Polymicro Technologies, Phoenix, AZ, USA) together with a UVIS 206 PHD fast-scanning multi-wavelength detector and No. 9550-0155 on-column capillary detector cell (both from Linear Instruments, Reno, NV, USA) placed towards the cathodic end of the capillary. The effective separation distance was 70 cm. Sample application was by dipping the anodic capillary end into the sample vial and raising it *ca.* 34 cm for 2–4 min. Multi-wavelength data were read, evaluated and stored employing a Mandax AT 286 computer system and running the Model 206 detector software package version 2.0 (Linear Instruments) with Windows 286 version 2.1 (Microsoft, Redmont, WA, USA). The Model 206 detector was employed in the high-speed polychrome mode by scanning either from 200 to 360 nm (5-nm intervals) or from 380 to 500 nm (10-nm intervals). All runs were performed with 20 m*M* NaOH containing a small amount of a neutral polymer (HPMC or Brij 35) as catholyte and 10 m*M* H_3PO_4 as anolyte. Before each run, the capillary was rinsed for 10 min with catholyte. A constant voltage of 20 kV was applied. During an experiment, the current decreased from about 25–35 μA (beginning of experiment) to about 2–3 μA (time of detection of gradient), depending on the sampling time interval and the sample solution.

cIEF was also performed on an automated ABI 270A-HT capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a fused-silica capillary of 75 μm I.D., and 39 cm effective length and 58 cm total length (Polymicro Technologies). Data collection and evaluation were carried out with a data acquisition system consisting of a PC Integration Pack version 3.0 (Kontron Instruments, Zürich, Switzerland) together with a Mandax AT 286 computer system. NaOH (20 m*M*) containing 0.015% (w/v) MC and H_3PO_4 (10 m*M*) were used as catholyte and anolyte, respectively. The

carrier ampholyte solution consisted of a mixture of Ampholine pH 3.5–10 and Pharmalyte pH 6.7–7.7 (1:2, v/v) in a total concentration of 4.5% (w/v), a blend which was optimized for the analysis of Hb variants [10]. Samples were injected hydrodynamically for 90 s (initial sample zone length *ca.* 25% of the effective column length) and a voltage of 20 kV was applied. Hbs and dyes were detected at 415 nm. Between runs the capillary was rinsed with NaOH (0.1 *M*), water and catholyte for 5 min each.

2.3. Recycling isoelectric focusing

Preparative IEF was performed in a Model RF3 recycling free-flow focusing apparatus (Protein Technologies, Tuscon, AZ, USA; distributor Rainin Instruments, Woburn, MA, USA), an instrument that has been described previously [24,25]. A separation cell of 20 cm length and 4 cm width having a fluid layer thickness of 0.75 mm and providing 30 fractions was used. The electrolyte chambers were separated from the separation channel by an anion-exchange (cathodic side) and a cation-exchange (anodic side) membrane (Ionics, Watertown, MA, USA). Electrode buffer reservoirs of 60 ml were used and filled with 0.1 *M* NaOH (catholyte) and 0.1 *M* H_3PO_4 (anolyte). The total processing volume was about 130 ml. The outlet temperature was monitored to be about 21–24°C (cooling bath 3–6°C) with a recycling pump rate of 40% and an applied constant power of 150 W. First the instrument was filled with a 4% (w/v) solution of Ampholine pH 3.5–10 containing 10% (v/v) glycerol, then a constant power of 150 W was applied for 2 h. The end voltage and current after this prefocusing period were about 1230 V and 121 mA, respectively. The sample, composed of 60 μl of dye solution (1 mg/ml each in water), dissolved in 1 ml of carrier ampholyte solution and 10% (v/v) glycerol, was slowly injected into the processing fluid stream inside the separation cell through the loading tube using a syringe. After sample injection, power was reapplied at a constant 150 W for 60 min, *i.e.*, until no voltage (about 1280 V) and current (about 117 mA) changes were observed for at least 30 min. Collected fractions were analysed for pH using a Model 720 pH meter and a Ross Model 8103 SC pH electrode (both from Orion Research, Cambridge, MA, USA) and for conductivity with a Model 101 conductivity meter (Orion Research) equipped with a PW 9510/65 cell (Philips, Eindhoven, Netherlands). The absorbance was measured at 430 nm with a Lambda 15 UV–Vis spectrophotometer (Perkin-Elmer, Überlingen, Germany).

3. Results and discussion

cIEF with electroosmotic zone displacement performed in uncoated fused-silica capillaries (as used in this work) has been described previously in detail [7–10]. The conditions selected for focusing of the dyes in the two instrumental set-ups were based on this previous work, including optimization of sample composition and electrolytes (catholyte and anolyte), sample introduction, power application and capillary geometry.

Three-dimensional cIEF data (absorbance *vs.* detection time *vs.* wavelength) of all six dyes and the carrier ampholytes are presented in Fig. 1A. At low wavelengths, the characteristic absorption profile of the carrier compounds is monitored [7], whereas between about 260 and 360 nm the dyes are measured. This is more clearly illustrated with the data in Fig. 1B, which represent a section of those in Fig. 1A which are drawn on elongated absorbance and time scales. Further, the absorption characteristics of dyes 3, 7, 13, 18 and 19 between 380 and 500 nm are presented in Fig. 1C. These data were obtained in a separate experiment with the detector settings changed for monitoring in the visible range. It is clear from the data in Fig. 1 that the six substituted aminomethylphenols are separated according to differences in p*I* values and detected in the order of decreasing p*I* values (Table 1). As expected, dye 1 with a p*I* of 10.4 is detected first, at the very edge of the pH gradient, and dye 19 with a p*I* of 5.3 is seen last but still within the pH gradient (Fig. 1A). According

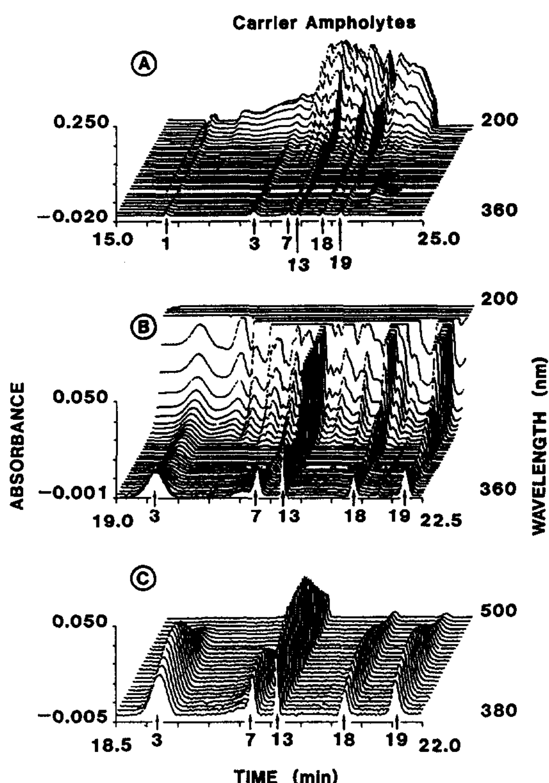


Fig. 1. Three-dimensional cIEF data for all six dyes, the graphs displayed representing (A) the UV absorbance (200–360 nm) data for the complete gradient, (B) a section of the UV data in (A) and (C) a section of the Vis (380–500 nm) data obtained in a second experiment. The pH gradient was established using 1% Ampholine pH 3.5–10 and 2% Ampholine pH 5–8. Sample application was for 4 min and 0.06% HPMC was used in the catholyte. The current dropped from 29 to 2 μA. For concentration, characteristics and assignment of dyes, see Table 1.

to the listed pI values, dye 7 was expected to be found somewhere in the centre between dyes 3 and 13. This was not the case, however, with dye 7 focusing fairly close to dye 13 (Fig. 1B and C). Further, it appears that this dye is not completely focused at the time of detection (Fig. 1B and C). According to the data summarized in Table 1, dye 7 has a much lower dz/dpH value compared with the other compounds investigated. Therefore, this dye is indeed expected to focus more slowly. Differences encountered with dye 7 will be dealt with in more detail below.

For the better characterization of the focusing of all six dyes, experiments were performed under a range of different conditions using the laboratory-made instrumental set-up. Selected single-wavelength electropherograms obtained at 410 nm are depicted in Fig. 2. For the data shown in Fig. 2A–C, sampling was carried out for 2 min and the catholyte contained 0.06% (w/v) of HPMC. The sample composition in terms of non-dye additives, however, was different. Electropherogram A was obtained with 2% of Ampholine pH 3.5–10 and 10% (v/v) of glycerol. With omission of glycerol, a similar electropherogram was obtained (Fig. 2B). The run time, however, was shorter, indicating that glycerol decreases the electroosmotic flow. Even earlier elution was noted with an increase in the carrier ampholyte concentration (Fig. 2C with 4% Ampholine pH 3.5–10), which is in agreement with the data published previously [8]. Further, with increase in the carrier concentration, a decrease in resolution was observed.

The data presented in Fig. 2D, E and F were all obtained with a sampling period of 4 min. For Fig. 2D the same sample as for Fig. 2B and HPMC (0.06% w/v) as dynamic capillary conditioner were used. Compared with the data in Fig. 2B, improved resolution and sharper peaks were noted. Not surprisingly, separation of the dyes was even more pronounced on employing a blend of two different carrier ampholytes, 1% Ampholine pH 3.5–10 and 2% Ampholine pH 5–8 (Fig. 2E and data in Fig. 1; HPMC in catholyte). It is evident that the narrow-range product (pH 5–8) is flattening the pH gradient such that dyes 7, 13, 18 and 19 become better separated. Finally, the experiment depicted in Fig. 2E was repeated with a different capillary conditioner, namely Brij 35 (0.4%, w/v) instead of HPMC (Fig. 2F). Comparison of the data in Fig. 2E and F reveals that Brij 35 affects cIEF of the dyes differently. This is seen in the detection times of dyes 1 and 19, and also in the peak shape of dye 3. Electroosmotic pumping, however, appears to be comparable with the two capillary conditioners.

According to theoretical considerations, a good ampholyte is characterized with a $|(\mathrm{d}z/\mathrm{dpH})_{pI}|$ value >0.045 [22]. According to the data

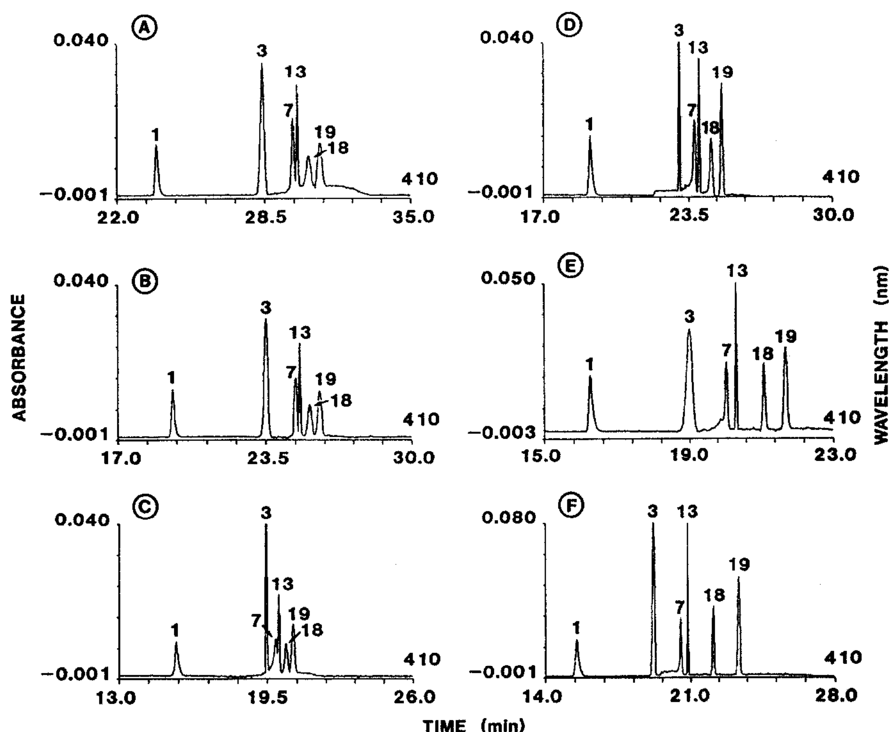


Fig. 2. Single-wavelength electropherograms of the six dyes. The dye concentrations in the sample are those listed in Table 1. Other conditions used are summarized in Table 2.

in Table 1, this criterion is well fullfilled for all dyes except dye 7. However, sharp focusing was observed for all six dyes when working with the conditions used to generate the data presented in Fig. 2B. Dye 7 also produced a sharp peak in the presence of glycerol (Fig. 2A). In all other cases studied (Fig. 2C–F) dye 7 was found not to be completely focused, this essentially being attributed to its low $|(dz/dpH)_{pI}|$ value which makes it focus much more slowly than the other compounds. Hence this compound cannot be recommended as a pI marker (see below). Depend-

Table 2
Experimental configurations employed to generate the data in Fig. 2

Fig. 2	Injection time (min)	Capillary conditioner[a]	Carrier ampholyte	Current (μA)	
				Initial	End
A	2	HPMC	3.5–10 (2%)[b]	31	2
B	2	HPMC	3.5–10 (2%)	32	2
C	2	HPMC	3.5–10 (4%)	39	4
D	4	HPMC	3.5–10 (2%)	31	3
E	4	HPMC	3.5–10 + 5–8 (1% + 2%)	29	2
F	4	Brij 35	3.5–10 + 5–8 (1% + 2%)	25	3

[a] The concentrations of HPMC and Brij 35 were 0.06 and 0.4% (w/v), respectively.
[b] The sample also contained 10% (v/v) of glycerol.

ing on the experimental conditions, differences in the sharpness of the foci, particularly for dye 3, were noted. Dye 3 is characterized by a broad peak in Fig. 2A, B and E (most pronounced in Fig. 2E), whereas the same compound is sharply focused under the conditions employed to produce the data shown in Fig. 2C, D and F. Because dye 3 has a high $|(dz/dpH)_{pI}|$ value of 0.74, these differences can only be attributed to differences in the pH gradient. Hence, in cIEF with electroosmotic zone displacement, not only the properties of amphoteric substances but also proper selection of the experimental conditions is important.

Next, the impact of proteins on the focusing behaviour of the dyes was investigated. Typical multi-wavelength cIEF data obtained with three dyes (dyes 1, 3 and 18) and two model proteins (CYTC and MYO) are presented in Fig. 3. The configuration of the data in Fig. 2D, having a 4-min injection with 2% Ampholine pH 3.5–10 and HPMC (0.06%) in the catholyte, was employed. Both the UV (Fig. 3A and B) and the Vis (Fig. 3C) data reveal clearly the focusing of CYTC between dyes 1 and 3 and of MYO between dyes 3 and 18. This is in complete agreement with the sequence of pI values of these compounds. However, addition of the proteins increased the residence time of all dyes, but not their focusing characteristics, as is revealed by comparing these data with those in Fig. 2D. For further data evaluation, detection times of the dyes were plotted against the pI values (Table 1). Typical graphs are depicted in Fig. 4.

As is shown with the data presented in Fig. 4A, the rate of transport of the entire gradient system through the capillary is dependent on the carrier ampholytes and other additives employed (as discussed above for Fig. 2), but essentially independent on the amount of dyes sampled. The graph labelled 2B′ was obtained with a tenfold smaller dye load compared with the data in Figs. 1 and 2, the experimental conditions being otherwise identical with those employed to produce the data shown in Fig. 2B. Differences between the detection time *vs*. pI graphs labelled 2B and 2B′ were found not to be larger than

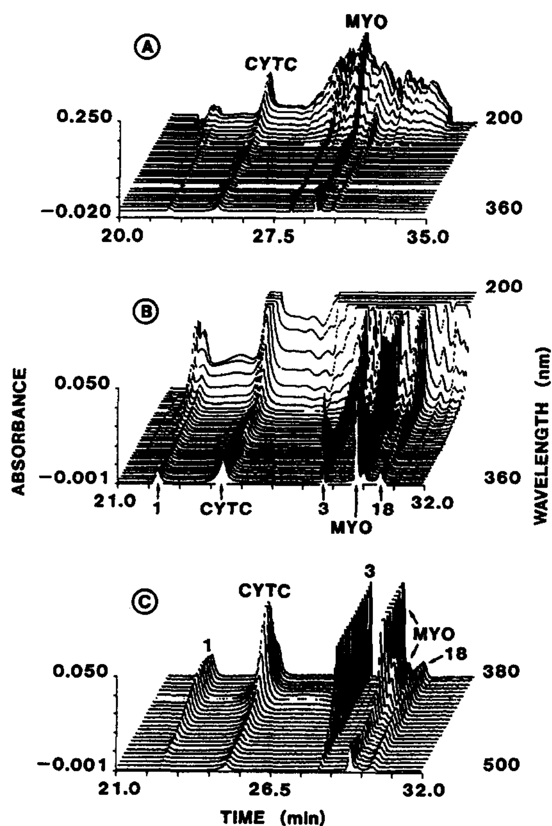


Fig. 3. Three-dimensional cIEF data for three dyes and two proteins, the graphs displayed representing (A) the UV absorbance (200–360 nm) data for the whole gradient system, (B) a section of the UV data in (A) and (C) a section of the Vis (380–500 nm) data obtained in a second experiment. The pH gradient was established using 2% Ampholine pH 3.5–10. Sample application was effected within 4 min and 0.06% HPMC was employed as capillary conditioner. The current dropped from 31 to 2 μA.

those observed when the same sample was measured twice (see Fig. 4C). The impact of the addition of CYTC and MYO as test proteins (as in Fig. 3) is depicted by the graphs presented in Fig. 4B. In the absence of proteins, the pH gradient was mapped with the data in Fig. 2D. Addition of the two proteins with a sample load of about 0.22 mg/ml each employing dyes 1, 3 and 18 only (Fig. 3) resulted in a significant increase in the detection times of all three dyes. Hence the two proteins appear to decrease the electroosmotic pump rate and HPMC is thereby

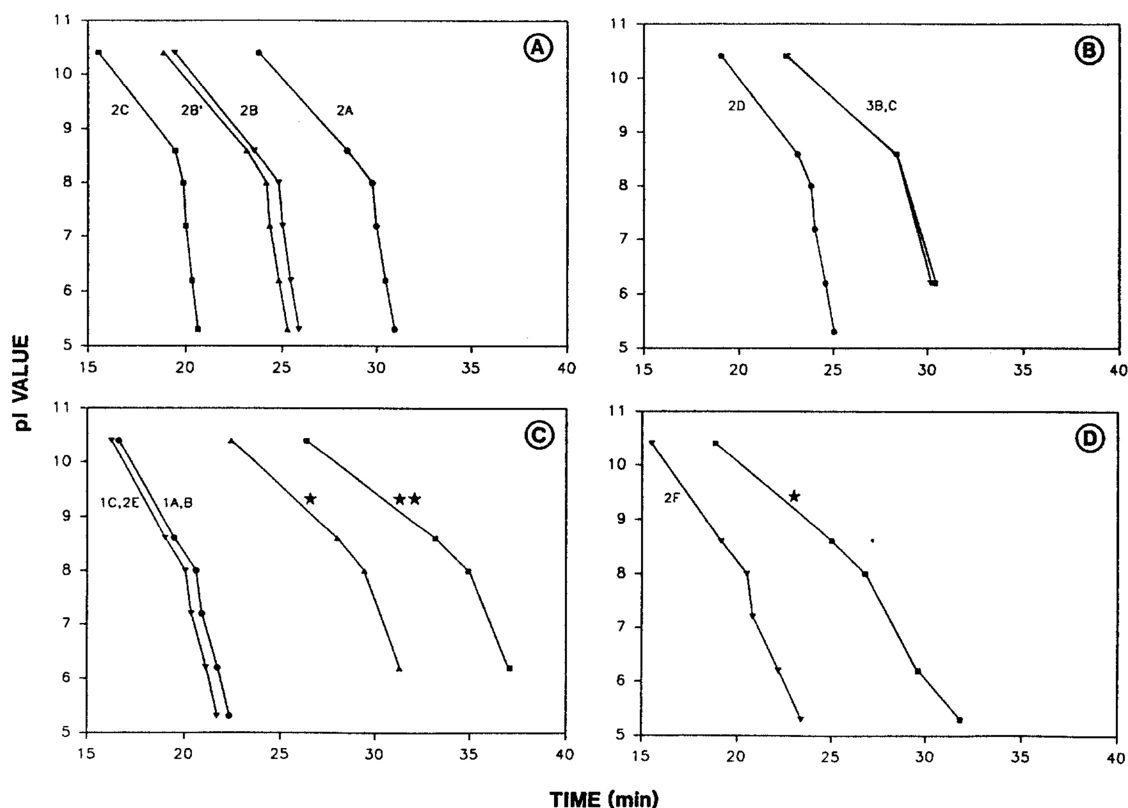


Fig. 4. Graphs representing detection time *vs.* p*I* of the dyes and proteins with (A) 2-min injection and 2% Ampholine pH 3.5–10 (data from Fig. 2A–C; HPMC in catholyte), (B) 4-min injection and 2% Ampholine pH 3.5–10 (data from Figs. 2D and 3B and C; HPMC in catholyte), (C) 4-min injection and a mixture of 1% Ampholine pH 3.5–10 and 2% Ampholine pH 5–8 with HPMC (data from Fig. 2E; graphs marked with asterisks were obtained with proteins and are explained in text) and (D) with the same pH gradient as in (C) but using Brij 35 as conditioner (data from Fig. 2F; protein data marked with asterisk).

proved not to abolish protein–wall interactions completely. Further, differences in the initial sample zone length due to differences in sample viscosity, and the establishment of two fluid elements with increased viscosity (the two protein foci), are other potential contributing factors affecting the detection times of solutes in cIEF. So far, the magnitudes of these effects are unknown.

Employing the three dyes as p*I* markers for an approximate mapping of the pH gradient, the p*I* values of CYTC and MYO were calculated to be 9.6 and 7.1, respectively. These values are in agreement with those reported in the literature (*cf.*, Experimental). The impact of CYTC and MYO on the retention behaviour of the dyes was also investigated for the experimental configuration used to produce the data shown in Figs. 1 and 2E. Corresponding graphs of detection time *vs.* p*I* are presented in Fig. 4C. Here, data for two different protein sample loads are depicted, the first (marked with a single asterisk) being produced with about 0.13 mg/ml of each protein, the second (marked with two asterisks) with about 0.29 mg/ml each. Similar data were also obtained with three proteins, CYTC, MYO and CA (data not shown). Hence detection times are shown to be affected by the amount of proteins sampled. Further, the retarding effect of proteins was also observed when Brij 35 instead of HPMC was employed as the capillary conditioner. The relationships between detection time and p*I* for the data presented in Fig. 2F and for data produced in a comparable configuration

but with five dyes and three proteins (CYTC, MYO and CA, about 0.009, 0.01 and 0.013 mg/ml, respectively) in the sample are depicted in Fig. 4D.

Working with the automated instrument, changes in detection times on addition of proteins was also noted. The data presented in Fig. 5A were obtained with all six dyes, having a pH gradient established with a mixture of two carrier ampholyte preparations, a blend which is different to that used for generation of the data in Figs. 1 and 2E. Also, MC was used instead of HPMC. The focusing pattern of the dyes, however, was found to be very similar to those in Figs. 1 and 2E. With the addition of an Hb sample (Fig. 5B), the detection times of all dyes were found to be slightly higher compared to those of Fig. 5A. This is in agreement with the data obtained with MYO, CYTC and CA (see above), and also with the data discussed elsewhere, in which just one dye was employed as a p*I* marker [10]. However, with Hb the magnitude of the protein retarding effect is smaller than that observed with the mixtures of the other model proteins. Further, the data presented in Fig. 5B and C reveal that the Hbs elute faster in the presence of the dyes (Fig. 5B) than without the dyes (Fig. 5C). Hence there is no doubt that addition of the dyes decreases the protein–wall interactions, which leads to an increase of electroosmotic pumping. This, in turn, provides faster elution and decreased resolution for a given set of experimental parameters. The latter effect is clearly seen for the separation of Hb A and Hb A_{1c}, two proteins that are separated in the data presented in Fig. 5C but not in the data in Fig. 5B. With automatic sampling, reproducibility evaluations were performed as described previously [10]. For consecutive injection of the same sample, the calculated R.S.D. values for detection time and peak area were determined to be 1.5–5% ($n = 5$) and 3–6% ($n = 5$), respectively, the lower values being obtained with optimized conditioning only. R.S.D. values for detection time, peak area and relative peak area (peak area divided by detection time) of a marker dye (dye 18) added to different pathological Hb samples were higher, namely 7–8%, 8–12% and 7–8%, respectively.

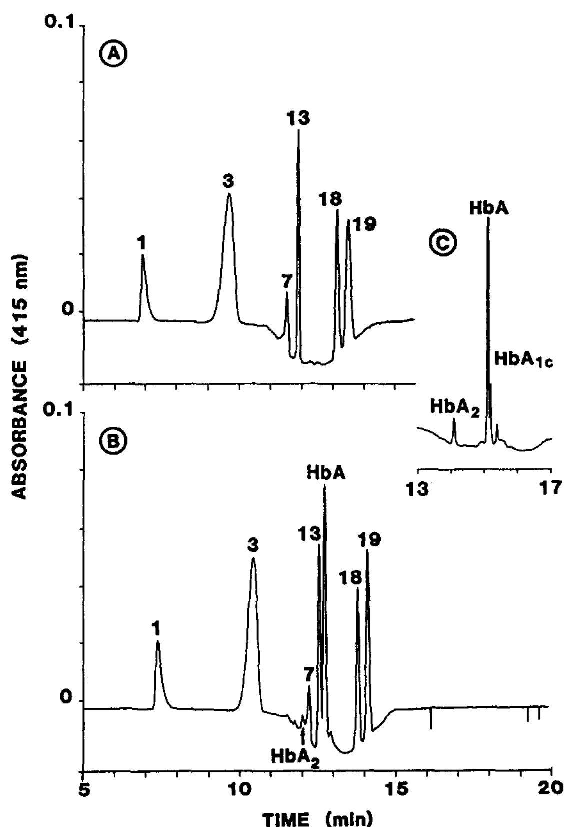


Fig. 5. Electropherograms monitored on the automated instrument with (A) the six dyes, (B) the dyes and an Hb sample from a normal adult and (C) the Hb sample without dyes. For conditions, see Experimental.

For the characterization of the dyes under free fluid preparative conditions, dyes 1 and 7 were separated by recycling IEF. The data presented in Fig. 6 represent absorbance (at 430 nm), pH and conductivity levels measured on the collected fractions. The conductivity and pH profiles obtained are typical for this kind of focusing experiments. Based on the pH and absorbance data, the p*I* of dye 7 was determined to be about 7.8, which is in fair agreement with the value of 8.0 in Table 1. This determination was straightforward, because dye 7 focused near the centre of the gradient. Calculation of the p*I* of dye 1, however, was found to be more difficult. This compound focused at the cathodic edge of the gradient, *i.e.*, near the membrane, and a small

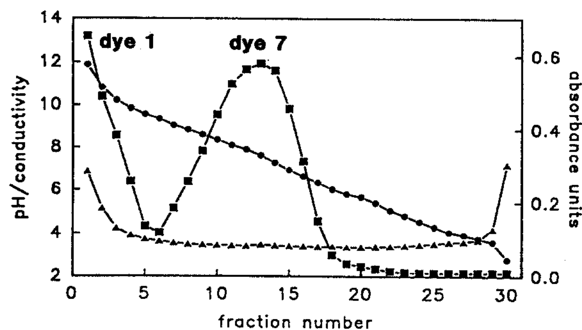


Fig. 6. (■) Absorbance (430 nm), (●) pH and (▲) conductivity data obtained on the collected fractions after recycling IEF of dyes 1 and 7. For data presentation, the conductivity values (S/m) were increased by a constant 3.3 S/m. For experimental conditions, see Experimental.

part of it might have been lost via penetration into the electrode compartment. Based on these data it can be concluded only that dye 1 has a p$I \geq 10$, which is in reasonable agreement with the pI of 10.4 in Table. 1.

4. Conclusions

The cIEF data presented here reveal that the substituted aminomethylphenol dyes focus sharply and that, in the absence of proteins, their presence in small amounts does not influence the electroosmotic pump rate. Hence they can be used to characterize the pH gradient and its transport along the capillary column. With addition of proteins, the electroosmotic pump rate is diminished, the magnitude of the decrease in total transport through the capillary column being dependent on the nature of the proteins applied, and also on the protein load. This effect was observed using three different capillary conditioning agents, namely HPMC, MC and Brij 35. For a given experimental configuration, however, good reproducibility was noted. Hence, with the exception of dye 7, which focuses too slowly, the substituted aminomethylphenol dyes used in this work can be recommended for employment as low-molecular-mass pI markers.

The dyes were applied in concentrations between 10^{-4} and 1 mg/ml. The detector responses were comparable to the noise at the lowest concentration and the peaks were too high and broad at the highest concentration. A reasonable concentration range of these dyes for use in cIEF is between 10^{-3} and 0.05 mg/ml, and with a concentration of 0.01 mg/ml the total load of a dye in the IEF capillary is about 10 ng. Further, the data presented here demonstrate that low-molecular-mass aminomethylphenol dyes can also be used in preparative-scale free fluid IEF, such as recycling IEF. Hence they may also be applicable in focusing field flow fractionation [26] and in other capillary isoelectric focusing techniques including those using polymer-coated capillaries [6,16]. The use of such low-molecular-mass compounds as marker dyes will permit a clear assessment of the pros and cons, and also most importantly the limitations, of the various cIEF methods, methods which essentially differ in terms of the nature of the capillary walls and the characteristics of buffer flow and/or electromigration along the column.

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