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Low-molecular-mass p*I* markers for isoelectric focusing

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ABSTRACT

Substituted aminomethylphenols are proposed as low-molecular-mass p*I* markers for the electrophoretic and chromatographic focusing of ampholytes. The important acid–base, spectroscopic and hydrophobic characteristics are presented for nineteen synthesized compounds. The low interaction between the suggested p*I* markers and proteins was verified by gradient ion-exchange chromatography of a mixture of some markers and alcohol dehydrogenase.

INTRODUCTION

Isoelectric focusing (IEF) is a technique for the separation, focusing and characterization of amphoteric analytes such as proteins [1,2]. The principle of the method involves the focusing of an amphoteric molecule at that point in the system where the pH value corresponds to its isoelectric point (p*I*).

To characterize the analyte, the pH at the place of its focusing should be known [1,3]. On gel plates, direct measurement by pH microelectrodes is possible. In preparative IEF variants, on-line or off-line pH measurement of the collected fraction can be considered. However, pH is most often evaluated with the help of reference substances. They may have different names, *e.g.*, p*I* markers [1,4], isoelectric point markers [5], IEF standards [6], IEF markers [7], pH markers [8,9], internal markers [10] or test substances for IEF. Their use may be universal: they are applicable on gel plates, in preparative channels and also in capillary modes of IEF. So far, native proteins have been used as p*I* markers [9,11,12]. Their p*I* values are determined mainly by IEF methods. For observing the focusing process, coloured proteins (*e.g.*, myoglobin, ferritin) or proteins stained with a suitable dye, *e.g.*, albumin stained with bromphenol blue [13], were used.

The native proteins, however, have some distinct disadvantages for use as p*I* markers. They tend to precipitate at pH values close to their p*I* and show instability as the substances themselves as aqueons solutions. Some protein standards consist of mixtures of related proteins; the high molecular mass of proteins makes their potential separation from the collected fractions of focused analytes difficult, and they cannot be used with reducing or denaturing agents such as urea, 2-mercaptoethanol or dithiothreitol [6]. So far, most analytical IEF has been carried out on gel plates; detection based on the protein staining is used almost exclusively in those methods. The low-molecular-mass analytes are washed out during the fixation step of this detection procedure. The newer capillary IEF method [14–18], preparative IEF methods and isoelectric focusing field flow fractionation (IEF FFF = IEF_4) [19]

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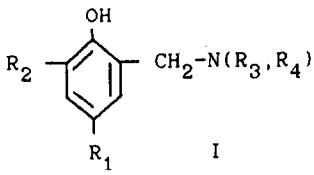
may also be applied to low-molecular-mass compounds. Detection methods based on the staining are not considered in such focusing methods. Further, in capillary IEF, off-line pH monitoring is possible using hydrodynamic or electroosmotic mobilization [20]; however, calibration with p*I* standards is necessary.

Suitable low-molecular-mass p*I* markers may help in avoiding some of the above disadvantages. A good p*I* marker should meet the following demands (see also ref. 9): it should be a good ampholyte [21–23], highly soluble in water and detectable by the method used, which in photometric detection usually means having a high absorptivity in the region where the focusing media are optically transparent and the analytes are detected, *i.e.*, at wavelengths of 270 nm and above.

Published tables [21–23] list few good low-molecular-mass ampholytes, only a few of them, including derivatives of tyrosine and 4-aminobenzoic acid, have satisfactory UV absorption at the wavelengths above 270 nm used for on-line detection of proteins and none of them absorbs in the visible region. As long as 20 years ago [8,10] the search for good low-molecular-mass ampholytes potentially applicable as reference substances in IEF was initiated. So far several have been reported, including methyl red [7,19], phenanthroline complexes [8] and some amphoteric triphenylmethane and azo dyes [10,19]. Methyl red has also been used for spectroscopic indication of the focusing solution pH [19] and for tracing the pH gradient in ion-exchange chromatography [24]. However, the low-molecular-mass p*I* markers so far used do not seem to meet all the necessary demands. Disadvantages are mainly based on their small number and the range of p*I* values covered. Moreover, their relatively high hydrophobicity causes their low solubility at the p*I* values, absorption on plastics and interactions with proteins; some of the dyes mentioned above have even been recomended for protein staining [25].

Here we propose the use of the aminomethylated nitrophenols of general formula **I** as p*I* markers. These compounds were prepared by aminomethylation of the phenolic substrates [26,27]. The purity of the reaction products was



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determinated by ion-exchange chromatography with a pH gradient and UV–Vis spectrophotometer with diode-array detection [28]. The acid–base properties were determined by potentiometric titration and numerical evaluation of the titration curves obtained.

THEORETICAL BACKGROUND FOR SELECTION OF SUGGESTED STRUCTURES

It is necessary for the sharp IEF of a low-molecular-mass amphoteric compound that it be a good ampholyte or, in other words, all the p*K* values adjacent to the p*I* value must be close to one another. The condition for the good ampholyte can be formulated as [22,29]

$$(pK_2 - pK_1) < 4 \quad (1)$$

For calculation of the width of the focused zone, it is more convenient to use the steepness of the dependence of the effective charge on pH at the isoelectric point of the compound, $[-dz/d(pH)]_{pI}$ [30,31]. Its relationship to the difference in p*K* is [29]

$$[-dz/d(pH)]_{pI} = \ln 10/[1 + (K_1/4K_2)^{0.5}] \quad (2)$$

A good ampholyte (and hence the proposed p*I* marker) then should have a $[-dz/d(pH)]_{pI}$ value above 0.045. The variance in the length units of the focused ampholyte zone, σ^2, is then [30,31]

$$\sigma^2 = RT/\{FE[-dz/d(pH)]_{pI}\, d(pH)/dx\} \quad (3)$$

where R, T and F have their usual meanings, E is the intensity of the electrical field and $d(pH)/dx$ is the steepness of the pH gradient.

For the design of the p*I* marker formula it is convenient if the p*K* values of both acidic and basic ionizable groups vary independently in a broad range. The phenolic group and aliphatic amino groups suit this concept. The interaction

of the respective groups within the molecule should also be considered when conbining the pK_a values of isolated groups [32,33]. Owing to the low molecular mass, the presence of two relatively independent charged groups at the p*I* of the compound and the presence of at least one hydrophilic amino group, the solubility of the compound in water can be expected to be sufficiently high even at pH values close to the p*I*. The presence of a nitro group leads to a high absorptivity also in the visible region.

EXPERIMENTAL

Materials

Compounds **1–19** (see Table I) were prepared from the commercially available nitrophenols by means of the Mannich reaction [26,27]. The appropriate amine (50 mmol) was added portionwise with cooling to 37% aqueous formaldehyde (60 mmol) in 25 ml of ethanol. After addition of substituted phenol (50 mmol), the reaction mixture was heated under reflux for 10 h [27,34]. The aqueous ethanol was removed under reduced pressure, the residue dissolved in 25 ml of methanol and to the resulting solution 5 ml of concentrated hydrochloric acid were added portionwise. After cooling, the products were separated by filtration or removal of the solvent under reduced pressure. The isolated hydrochlorides were recrystallized from methanol or aqueous ethanol. The purity of all compounds was checked by TLC and ion-exchange liquid chromatography with a pH gradient and UV–Vis diode-array detection [28].

Liquid chromatography

The conditions for ion-exchange chromatography with a wide pH gradient range were described previously [28]. A PU 4100M liquid chromatograph (Philips, Cambridge, UK) equipped with a Model 7125 injection valve (Rheodyne, Cotati, CA, USA) and a PU 4021 multi-channel detector (Philips) were used. The data collection and postrun evaluation were controlled by PU 6003 v. 3.0 diode-array detec-

TABLE I

STRUCTURES OF SUGGESTED p*I* MARKERS OF GENERAL FORMULA I

No.	R_1	R_2	$N(R_3, R_4)$[a]	M_r
1	NO_2	$CH_2N(R_3, R_4)$	PIP	406
2	$CH_2N(R_3, R_4)$	NO_2	PIP	406
3	NO_2	$CH_2N(R_3, R_4)$	MPIPE	509
4	$CH_2N(R_3, R_4)$	NO_2	MPIPE	509
5	$CH_2N(R_3, R_4)$	NO_2	HPIPE	569
6	NO_2	H	DEA	261
7	NO_2	H	PIP	273
8	NO_2	$CH_2N(R_3, R_4)$	MOR	435
9	CH_3	NO_2	MPIPE	338
10	CH_3	NO_2	HPIPE	368
11	$CH_2N(R_3, R_4)$	NO_2	MOR	410
12	Cl	NO_2	MPIPE	359
13	CH_3	NO_2	MOR	289
14	Cl	NO_2	HPIPE	389
15	NO_2	H	MOR	275
16	Cl	NO_2	MOR	309
17[b]	4-$CH_2N(R_3, R_4)$	2-Cl-6-NO_2	MPIPE	359
18[b]	4-$CH_2N(R_3, R_4)$	2-Cl-6-NO_2	HPIPE	389
19[b]	4-$CH_2N(R_3, R_4)$	2-Cl-6-NO_2	MOR	309

[a] PIP = 1-piperidyl; MPIPE = 1-(4-methylpiperazinyl); HPIPE = 1-(4-hydroxyethylpiperazinyl); DEA = diethylamino; MOR = 4-morpholinyl.

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

tor software (Philips). The actual pH profile of the column effluent was monitored by a capillary flow-through pH electrode (Model OP-0745P; Radelkis, Budapest, Hungary) connected to a Model OP-208/1 pH meter (Radelkis) and a line recorder. A 150 × 2 mm I.D. Separon HEMA-BIO 1000 Q ion-exchange column (Tessek, Prague, Czech Republic) was used as received.

The alkaline buffer (A) was an aqueous solution of 10 m*M* each piperazine, L-histidine, ethylenediamine, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 20 m*M* ammonia solution. The pH of buffer A was adjusted to 10.0 with 2 mol l^{-1} potassium hydroxide solution. The acidic buffer (B) was 0.83 mol l^{-1} formic acid. Chemicals used for buffer preparation were obtained from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany).

Alcohol dehydrogenase (yeast) No. 01106 (Reanal, Budapest, Hungary) was sampled in the alkaline buffer. In a sample volume of 10 μl, 0.8–1.2 mg of protein was loaded on the column.

Determination of absorptivity, $A_{1\,cm}^{1\%}$

The absorptivities of compounds **1–19** in aqueous buffer solutions with pH corresponding to the marker p*I* value were determined with a Varian Techtron Series 634 UV–Vis spectrophotometer.

Determination of p*K*, p*I and* $[-dz/d(pH)]_{pI}$

The acid–base properties of the prepared compounds were evaluated by potentiometric titration using a Model MS 22 pH meter (Laboratory Instruments, Prague, Czech Republic), equipped with a Model 01-29 combined glass pH electrode (Crytur, Turnov, Czech Republic). The instrument was calibrated by means of commercial standard buffer solutions (Institute of Sera and Vaccines, SEVAC, Prague, Czech Republic). The temperature during titration was kept at 23°C. The titration curves obtained were evaluated both graphically and numerically to obtain p*K*, p*I* and $[-dz/d(pH)]_{pI}$ values of the p*I* marker. For the curve-fitting procedure, the program Eureka V. 1.0 (Borland, Scotts Valley, CA, USA) was used. The whole procedure for p*K* determination was verified by the determination of the p*K* of L-histidine monohydrochloride (Renal) as a standard. The differences between the determined and tabulated [35] p*K* values were less than 0.1 pH unit.

In fact, the titration enables one to calculate the isoionic point which might be different from the isoelectric point owing to the difference in mobilities of cationic and anionic forms of the ampholyte, which can amount to up to 5% [36,37]. It can be estimated that such a difference can make a difference between the isoionic and isoelectric point of only up to a few hundredths of a pH unit.

Determination of log P_{OW}

The partition coefficient between 1-octanol and water, P_{OW}, was determined spectroscopically by the shake-flask method as described previously [38]. The values presented correspond to the pH of the water-rich phase equal to the p*I* value of the respective marker. The pH of the water rich phase was adjusted with 0.1 mol l^{-1} phosphate buffer. The absorptivity of the water-rich phase was determined at its λ_{max} in the visible spectrum: the solution was equilibrated for 3 h at 23°C with a known amount of water-saturated 1-octanol and the absorptivity of the aqueous phase was measured again.

RESULTS AND DISCUSSION

Referring to the general structure **I** and the related structures in Table I, it is obvious that the compounds prepared include all the important groups necessary to meet the key properties of a good p*I* marker, namely the acid–base behaviour, hydrophilicity and light absorptivity. The hydrophilic amino groups are similar to those of common Good's buffers and the molecular masses are similar to those of the polyampholytic buffers used in IEF. The variation of the groups and their positions in formula **I** leads to changes in the acid–base properties of both phenolic and amino groups [39]. Consequently, the p*I* values of the prepared compounds cover a wide pH range (see Table II). Variations in the p*K* of ionizable groups also influence the $[-dz/d(pH)]_{pI}$ values of the respective compounds. It follows from Table II that except for compounds **6** and **7**, all of them can be considered as good

TABLE II

IEF, SPECTRAL AND LIPOPHILIC CHARACTERISTICS OF PROPOSED p*I* MARKERS

No.	p*I*	$\lvert(dz/dpH)_{pI}\rvert$	λ_{max} (nm)[a]	$A^{1\%}$ [b]	Log P_{OW}[c]
1	10.4	0.76	403	617	1.08
2	10.1	0.60	412	374	0.64
3	8.6	0.74	420	102	−0.02
4	8.5	0.72	419	131	−0.78
5	8.4	0.60	417	80	−1.30
6	8.1	0.01	392	744	0.38
7	8.0	0.02	392	661	0.62
8	7.9	0.45	403	698	0.43
9	7.9	0.27	425	165	0.79
10	7.7	0.19	423	119	0.31
11	7.5	0.43	416	115	−0.19
12	7.4	0.17	428	156	0.58
13	7.2	0.15	416	162	1.05
14	7.0	0.14	423	139	0.02
15	6.6	0.15	400	526	0.49
16	6.5	0.07	421	142	0.88
17	6.4	0.09	416	131	−0.94
18	6.2	0.10	415	133	−2.18
19	5.3	0.12	409	142	−0.16

[a] Wavelength of absorption maximum in UV–Vis spectrum of aqueous buffer solution at pH equal to the p*I* value.
[b] Absorptivity of a 1% aqueous buffer solution at pH equal to the p*I* value.
[c] Partition coefficient between 1-octanol and water at 25°C.

ampholytes. Nevertheless, even **7** can give a sharp peak in capillary IEF [40].

The suggested markers have satisfactory light absorptivity in both the UV and visible regions of the spectrum (see Table II). Therefore, a small amount of the marker in its focused zone is sufficient for its reliable detection and the pH gradient need not be influenced by the presence of the marker.

The p*I* markers can also be used for the approximate tracing of the pH gradient in ion-exchange chromatography (see Fig. 1). Here, compounds **4**, **11**, **17** and **19** (see Tables I and II) were chromatographed in a gradient decreasing from pH 10 to 4 on a strong anion exchanger. In Fig. 1a, the detection wavelength was close to the absorbtion maxima in the visible region of the marker spectra. In Fig. 1b, the wavelength common for detection of the proteins, *i.e.*, 280 nm, was selected. It should be noted that the pH of elution in ion-exchange chromatography need not always correspond to the isoelectric point of the analyte as interactions other than purely electrostatic ones can occur between the analyte and the stationary phase [41].

The important property of a suitable p*I* marker is its lowest possible interaction with the analytes, namely proteins. This property was checked by the ion-exchange chromatography of a mixture of some markers with proteins. Fig. 2 presents the chromatograms of compounds **4** and **11** (see Tables I and II) and alcohol dehydrogenase. Two peaks of the markers and a group of peaks corresponding to the enzyme was observed with detection at 280 nm (see Fig. 2b). With detection at the wavelength where only markers are detectable, *i.e.*, at 430 nm, almost no peaks can be detected in the elution range corresponding to the elution of the enzyme (see Fig. 2a); this means that less than 1% of marker can be bound to the sampled protein. This observation supports the statement that the tested markers are not irreversibly bound to the alcohol dehydrogenase proteins.

Good water solubility, high hydrophility and low interaction of the markers with the proteins

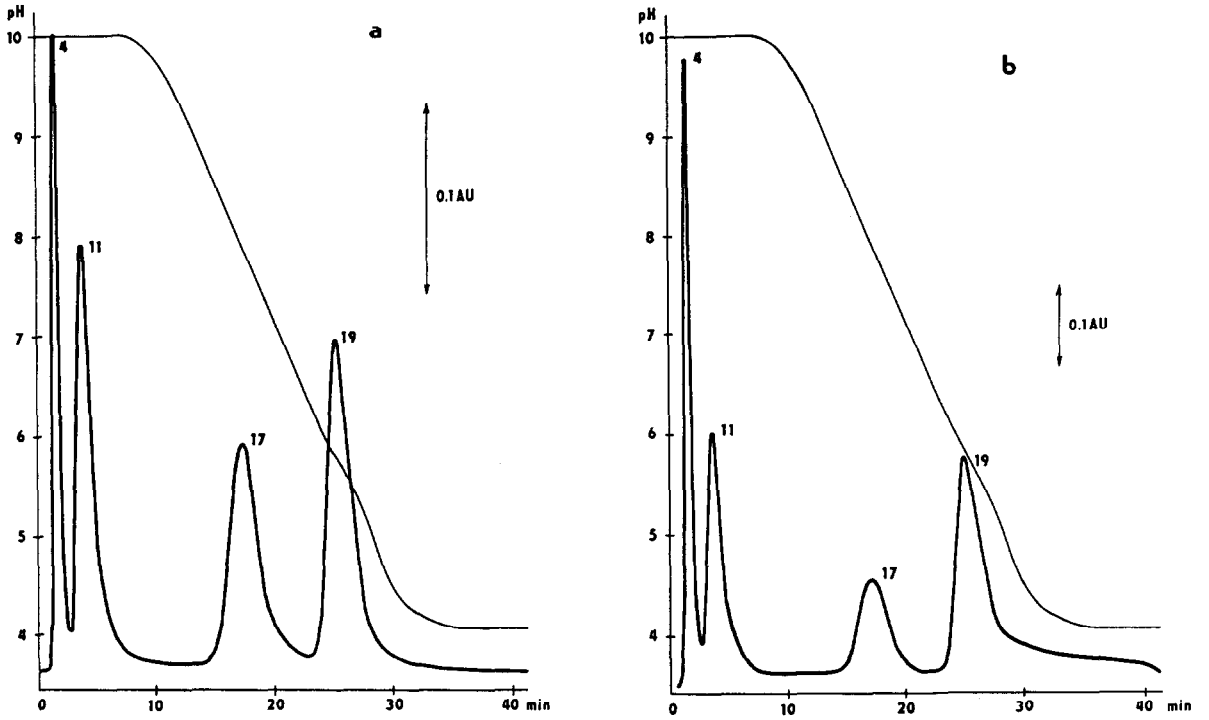

Fig. 1. Separation of selected p*I* markers by anion exchange liquid chromatography with a pH gradient. Column: 150 × 2 mm I.D. glass cartridge packed with Separon HEMA-BIO 1000 Q anion exchanger (Tessek); pH gradient from 0% of acidic buffer B in A (pH 10.0) to 13% B in A (pH 4.0) in 30 min. Detection wavelength (a) 430 nm and (b) 280 nm. Peak numbers correspond to the compound numbers in Tables I and II.

can further be illustrated by their behaviour during the focusing on the gel plates, *e.g.*, in the Pharmacia PhastSystem. When focusing the suggested markers with proteins, the development of visible sharp zones of the markers is possible. During the staining procedure, the nitrophenol markers are eluted from the plate in such a way that they are not detectable after staining. At the same time, the positions of the protein standards are the same irrespective whether they are sampled with or without the nitrophenol markers. The behaviour of the prepared compounds in the capillary and preparative electrophoretic focusing methods will be described elsewhere [40,42].

The high hydrophility of the compounds prepared follows from the low values of their partition coefficients between 1-octanol and water, P_{OW} (see Table II). Here, log P_{OW} of the p*I* markers are given as determined at the pH of the water-rich phase corresponding to p*I* of the marker; for methyl red (not included in Table II), an approximate value of log P_{OW} was calculated to be 3.5. For comparison, log P_{OW} values reported for some other ampholytes [43] are alanine −2.94, N-phenylglycine 0.62, 4-aminobenzoic acid 0.68 and 2-aminobenzoic acid 1.21.

The high hydrophility of the suggested markers can also be supported by the observation that common plastics (*e.g.*, PVC, Perspex) and skin are not observably coloured by aqueous solutions of the suggested markers.

CONCLUSION

Coloured ampholytes based on aminomethylnitrophenols are suggested as p*I* markers for free fluid formats of IEF. The suggested general formula offers wide variations of acid–base properties of the compounds. It can be expected that the utilization of other amino groups and/or other substituents on the aromatic ring can further increase the scope of p*I* values.

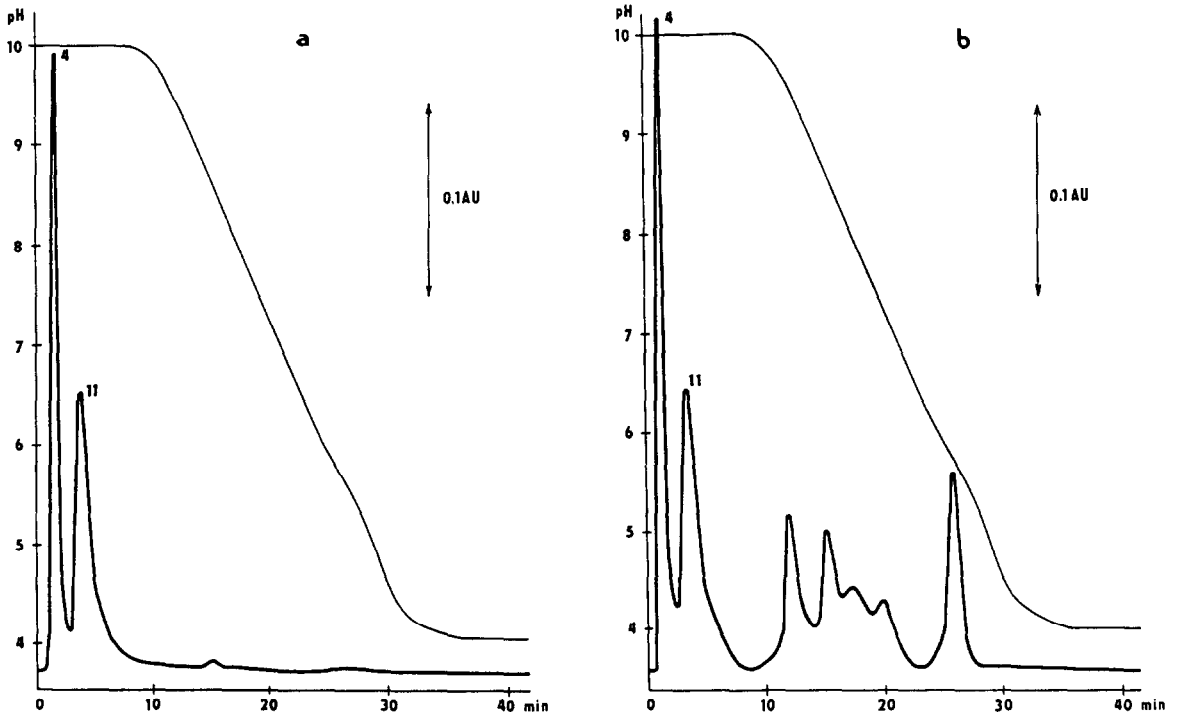

Fig. 2. Separation of a mixture of p*I* markers with alcohol dehydrogenase by anion-exchange liquid chromatography with a pH gradient. Detection wavelength: (a) 430 nm and (b) 280 nm. Sample amount: alcohol dehydrogenase 300 μg, p*I* markers 6 μg each. Experimental conditions as in Fig. 1.

A change in the spectroscopic properties of the marker would undoubtedly need a profound modification of the structure. A more detailed specification and testing of these compounds as p*I* markers in IEF are in progress.

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