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Open-tubular electrochromatography of organic phosphates on a sapphyrin-modified capillary

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Abstract

Sapphyrin coating of the inner wall of the capillary results in a distinct interaction of the phosphate residue-possessing compounds as proven by a seven-membered model mixture of nucleoside mono- and diphosphates and ATP. Modification of the inner surface of the capillary not only alters the endoosmotic flow (as would have been expected) but brings about an electrochromatographic effect based on the interaction of tested phosphate moiety-bearing solutes with the immobilized sapphyrin layer. Elution of the sample can be achieved by using either 25 m*M* borate–acetate buffer in which monophosphates are not only separated from each other, but also selectively separated from di- and triphosphates (ATP). With the other two buffer systems tested, i.e. borate–phosphate and Tris–HCl, better selectivity (though smaller interaction with the capillary coating) was observed. The coating is relatively stable (can be used for 20 subsequent runs at least), simple to materialize, and in spite of a strong UV absorbancy of sapphyrin at the wavelength used (254 nm), decreases the limit of detection by no more than one order of magnitude as compared to the untreated capillary. Resolution factors (calculated to the preceding peak) are in most cases better in the electrochromatographic separation mode as compared to the separation in the untreated capillary, which reflects both the decrease in the electroosmotic flow and the interaction with the capillary wall coating.

Keywords: Phosphates; Nucleoside phosphates; Sapphyrin; Electrochromatography

1. Introduction

Phosphorous oxo anions and their salts are important compounds with biological, agricultural and industrial chemical significance (for review see Ref. [1]). Capillary electrophoresis and capillary electrochromatography (CEC) represent some of the latest additions to the methods for analysis of inorganic and organic ions in solution [2]. Traditional electromigration separations are among the most powerful techniques for both inorganic and organic phosphate moiety-containing compounds.

The main feature of phosphate anions is that most contain both strong and weak acid groups; the weak acid groups are generally similar in strength with $pK_a \sim 7$ for the formation of a -2 charge on each phosphorous oxo group. The majority of work to date on CE determination of inorganic phosphorous-containing anions involves analyses of orthophosphate. Indirect UV detection, developed for small-ion analysis, has been the major detection mode in CE analyses for orthophosphate and inorganic phosphorous oxo anions as long as these compounds do not possess UV absorbance [3]. A lot of different buffer systems were used for CE separation of orthophosphates, involving chromate, pyromellitate or phthalate as probes allowing negative detection of the separated products. As the buffer pH decreases,

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orthophosphate mobility decreases due to the formation of monovalent $H_2PO_4^-$ ions. Common electroosmotic flow (EOF) modifiers are widely applied (hexadecyl-, tetradecyl- and dodecyltrimethyl ammonium bromides or hydroxides (CTAB/H, TTAB/H and DTAB/H, respectively)) to improve the separations in speciation analyses [4]. In the biomedical field the most frequently analyzed phosphorus-containing compounds are nucleoside mono- and polyphosphates and constituents of nucleic acids [5–9], the latter category being given considerable preference. So far there are only few papers dealing with the separation of labeled [^{32}P]ATP [10] or phosphonate analogues of uridine and adenosine [11,12] (for review see Ref. [13]).

The study of selective recognition of nucleobases and their derivatives by synthetic hosts sheds light on biological processes and contributes to the design of artificial receptor molecules useful in the analytical and medicinal field. In order to achieve a base-selective recognition it is necessary to endow the receptor molecules with binding sites capable of interacting with the nucleic bases or with the sugar moieties. Interaction with the former may be achieved by hydrogen bonding with sites capable of forming complementary hydrogen bonding patterns and/or by stacking with π-system of the host compounds. Of special interest as structural subunits are the planar heterocyclic dye molecules. Molecular recognition of nucleosides and nucleotides by a water-soluble cyclo-bis-intercaland receptor based on acridine subunits was reported. The interaction was followed by fluorescence change of dye molecule spectra after the addition of nucleotide or nucleoside [14]. On the other hand such compounds can be used as selectors for phosphate-bearing moieties in both electrodriven and chromatographic separations.

There are basically two approaches to investigate the binding affinity of phosphate-bearing molecules to the ligand molecules. Either they can be used as micellar pseudophase modifiers or they can serve as modifiers of the inner capillary wall surface. While the interpretation of the results obtained in the micellar electrokinetic mode can bring about some difficulties, the interpretation of the latter approach (open tubular capillary electrochromatography) is much more straightforward (for review see Ref. [15]).

Electromigration methods were not the only techniques used for separation of nucleotides on modified solid support. For instance a sapphyrin-modified silica gel support for use in HPLC was reported [16,17]. It was proven that anionic and phosphorylated substrates are bound to the protonated macrocyclic sapphyrin core via close contacts involving the pyrrolic hydrogens and the phosphate oxyanion. In the solid state sapphyrin has been shown to complex monobasic phosphate and phenylphosphate [18]. Evidence for sapphyrin-based phosphate anion binding in solution has come from a variety of transport experiments [19–23].

It was the aim of this study to create a system of open tubular capillary electrochromatography capable of specific binding of organic phosphates that contain a UV detectable chromophore. A set of seven nucleoside mono-, di- and triphosphates was used as the test mixture.

2. Experimental

2.1. Instrumentation

All experiments were performed using SpectraPhoresis 504 apparatus from Thermo Separation Products (TSP, Riviera Beach, FL, USA), controlled with PC 1000 Version 2.6 software (supported on OS2 2.1), equipped with the on-line, variable-wavelength detector set to 254 nm. The separations were run in two types of capillaries. The first type was an untreated fused-silica capillary, I.D. 50 μm, fitted into the TSP cartridge. Total capillary length was 70 cm (62.5 cm to the detector) if not stated otherwise.

The second one was the capillary with sapphyrin-modified inner wall surface (for the formula of sapphyrin see Fig. 1). Other characteristics of the modified capillary were the same as specified above. The separations were run at 10–20 kV (20–60 μA) and the analysis time was 30–60 min; detection was done at 254 nm. It is noticeable that coverage of the inner capillary wall with the sapphyrin modifier did not distort the possibility of UV detection in spite of the fact that sapphyrin solution exerts considerable absorbance of 254 nm (the wavelength used for the detection of nucleoside phosphates), which reflects the small thickness of the capillary coating. (After

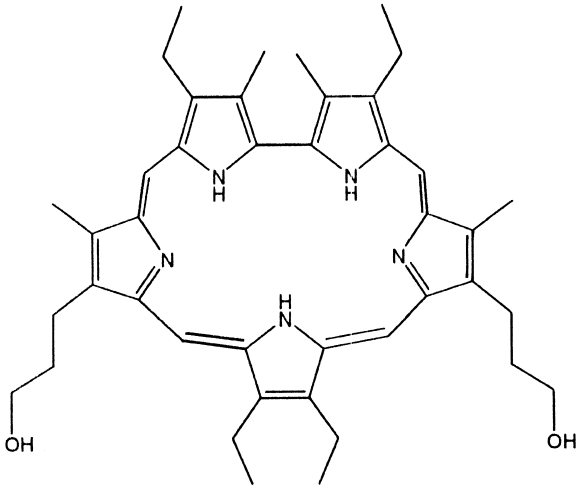

Fig. 1. The structure of the sapphyrin molecule.

coating the limit of detection was decreased about 10 times.)

An uncoated silica capillary was conditioned by sequentially washing it for 5 min with water, 10 min with 1 mol/l NaOH, 5 min with water and finally 5 min with the running buffer. This procedure was also used to recondition the capillary after each run.

The modified capillary was conditioned by sequentially washing it with water for 5 min and the running buffer for 10 min.

Laser-induced fluorescence spectra were collected using a LabRam system (Dilor) equipped with external Ar^+-ion laser (Melles Griot). The 488-nm line was used for excitation. The power on the head of the laser was 25 mW. An objective (×10) (Olympus) was used to focus the laser beam on the sample placed on an *x–y* motorized sample stage. The scattered light was analyzed by spectrograph with holographic grating (1800 gr/mm), slit width was 130 μm and confocal hole was opened to 1000 μm. Peltier-cooled charge coupled device (CCD) detector (1024×256 pixels, ca. −70°C) detected the dispersed light. The adjustment of the system was regularly checked using a sample of silicon and by the measurement in the zero-order position of the grating. The time of acquisition of a particular spectral window was 120 s. Two accumulations were merged to obtain a spectrum. The spectrometer and the positioning of a sample were controlled via personal computer (Pentium, Dell) with Labspec v. 2.08 (Dilor) software. The same software was used for treatment of the data obtained.

2.2. Reagents

Potassium dihydrogen phosphate, sodium tetraborate and thiourea were obtained from Lachema (Brno, Czech Republic) and were of p.a. purity. Adenosine-5′-mono-, di- and triphosphate, guanosine-5′-monophosphate, uridine-5′-mono- and diphosphate, cytidine-5′-monophosphate and Tris were purchased from Sigma (St. Louis, MO, USA) and were of 97% purity grade.

Sapphyrin derivative was synthesized according to the literature [24] and was characterized by ^1H NMR, MS and IR.

Samples for analysis were prepared by dissolving 5–10 mg of each nucleotide in 1 ml of MilliQ water and stored below 4°C. Electroosmotic flow marker (thiourea) was prepared by dissolving 2 mg of thiourea in 1 ml of the run buffer.

Then, 150 μl of each standard stock solution and the EOF marker were pipetted into the electrophoretic vial and made up to the total volume of 1.5 ml with MilliQ water.

The final concentration of each nucleotide in the sample was 0.5 mg/ml. All samples were injected hydrodynamically at 10 kPa underpressure.

2.3. Coating of the capillary wall

A new fused-silica capillary was sequentially washed with water, 1 *M* NaOH and water, each step for 5 min. Next it was dried in the air stream and washed with methanol, the solution of sapphyrin in dichloromethane (2 mg/ml) and dried by flushing air (each step 5 min). Then the excess of sapphyrin was removed by washing the capillary with methanol and water. Final washing was made with the running buffer. Each step took 5 min. To remove less tightly bound excess of sapphyrin and to prevent leaking of the capillary wall modifier, the capillary was flushed for 5 min with absolute ethanol. To stabilize the coating the capillary was run in the separation buffer at 20 kV for a period of 60 min, and this procedure was repeated until the baseline drift was eliminated. (For the scheme of the coating procedure see Table 1.)

Table 1
Schematic representation of capillary modification with the sapphyrin modifier

Operation step	Treatment of the capillary	Duration (min)
1	MilliQ water wash	5
2	1 *M* NaOH wash	5
3	2 mg/ml sapphyrin in dichloromethane wash	5
4	Drying of the capillary by air flushing	5
5	Removing of the less tightly bound sapphyrin by ethanol wash	5
6	Final stabilization of the coating by running the capillary in the separation buffer at 20 kV	60

2.4. Buffer systems

The first electrolyte tested was 25 m*M* borate–acetate buffer at the concentration 25 m*M* at pH 7.0. In this buffer both the early studies of migration times of each nucleotide, and the separation of the test mixture of seven nucleotides were done. The other background electrolytes tested were Tris–HCl (25 m*M*; pH 7.0) and borate–phosphate buffer with the same concentration and pH as the former two. Separations were done in both the unmodified and sapphyrin-modified capillaries and the results were compared.

3. Results and discussion

Modification of the inner surface of the capillary was proven by measuring laser-induced fluorescence immediately after coating and conditioning the column and after 20 runs performed in the borate–acetate buffer at 20 kV (Fig. 2). As expected, coverage of the inner capillary wall resulted in a distinct change of the endoosmotic flow. (The peak of the endoosmotic flow marker occurred at 8.1 min in the treated capillary while it was 5.9 min in the capillary, which was not treated by the modifier in the borate–acetate buffer.) While the individual

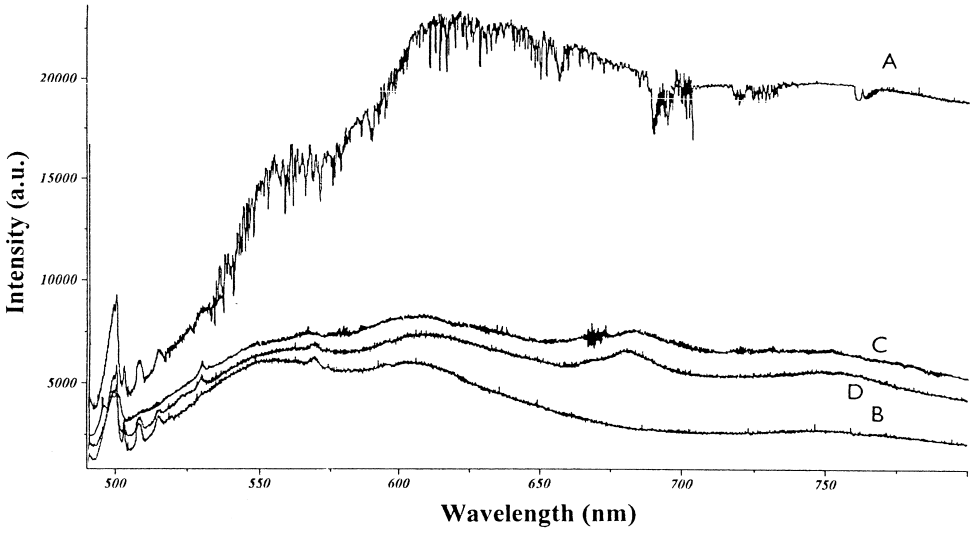

Fig. 2. Laser-induced resonance spectra of the treated capillary immediately after coating before washing away the excess of sapphyrin (A), untreated capillary (B), after conditioning of the capillary for the first run (C), and after 20 runs in 25 m*M* borate–acetate buffer at pH 7.0 (D).

components of the test mixture were eluted in borate–acetate buffer at 17 kV between 13 and 18 min on the untreated capillary (Fig. 4), the elution in the sapphyrin-modified capillary took considerably longer. As can be seen from Table 2, the anodic migration speed (retention) in the sapphyrin-modified capillary in the borate–acetate buffer was considerably higher for ATP and the nucleoside diphosphates than it would have been expected from the decrease of the electroosmotic flow proving thus the interaction of the test solutes with the capillary modifier. As a matter of fact di- and triphosphates could not be brought in front of the detector window at all (run voltage 17 kV, Fig. 3b) unless the capillary is considerably shortened (43×35.5 cm) and left unmodified (Fig. 3a). If the voltage is increased to 20 kV at least partial separation of the diphosphates and ADP can be observed (see Fig. 4b). As also demonstrated in Table 2, the retardation owing to the solute-capillary wall modifier interaction was distinct with ATP and nucleoside diphosphates tested. To prove further that such an interaction really occurred borate–phosphate run buffer was used. As predicted the presence of phosphate in the run buffer decreased the additional retardation of the analytes (as compared to the borate–acetate buffer) and the resulting separation was comparable to that obtained with the unmodified capillary. However, if the run was done at 20 kV a considerably better separation of UDP from the incompletely resolved peaks of CDP and ADP was achieved (Fig. 5) (higher than 1.0 as compared to incompletely resolved peaks in untreated capillary under the same conditions). An unexpected effect was observed when Tris–HCl buffer was used. In this situation the endoosmotic flow was faster and the resolution was similar to that observed with the uncoated capillary. In this case it appears feasible to assume that the inner wall of the

Table 2
Effective mobility of nucleoside phosphates in the uncoated and sapphyrin-modified capillary (20 kV per 70 cm capillary except for borate–acetate where the separations were run at 15 kV)

Buffer used	Nucleoside phosphate	Non-modified capillary	Sapphyrin-modified capillary	Relative change in effective mobility (modified vs. non-modified capillary) (%)	Retardation of the tested solutes owing to the interaction with the modified capillary (%)
		Effective mobility ($cm^2\ V^{-1}\ s^{-1}$)			
(a) Borate–phosphate	Thiourea	6.191×10^{-4}	6.000×10^{-4}	96.92%	3.08%
	GMP	-3.043×10^{-4}	-2.908×10^{-4}	95.56%	4.44%
	AMP	-3.101×10^{-4}	-2.975×10^{-4}	95.93%	4.07%
	UMP	-3.227×10^{-4}	-3.112×10^{-4}	96.46%	3.54%
	ATP	-3.786×10^{-4}	-3.670×10^{-4}	96.92%	3.08%
	ADP	-3.938×10^{-4}	-3.817×10^{-4}	96.92%	3.08%
	CDP	-3.965×10^{-4}	-3.838×10^{-4}	96.80%	3.20%
	UDP	-3.994×10^{-4}	-3.913×10^{-4}	97.96%	2.04%
(b) Tris–HCl	Thiourea	4.606×10^{-4}	5.000×10^{-4}	108.56%	−8.56%
	GMP	-2.611×10^{-4}	-2.639×10^{-4}	101.08%	
	AMP	-2.716×10^{-4}	-2.759×10^{-4}	101.59%	No significant
	UMP	-2.942×10^{-4}	-2.992×10^{-4}	101.67%	change
	ATP	-3.508×10^{-4}	-3.678×10^{-4}	104.86%	
(c) Borate–acetate	Thiourea	8.122×10^{-4}	6.020×10^{-4}	74.12%	25.88%
	GMP	-4.611×10^{-4}	-3.680×10^{-4}	79.80%	20.20%
	AMP	-4.761×10^{-4}	-3.870×10^{-4}	81.27%	18.73%
	UMP	-4.993×10^{-4}	-3.905×10^{-4}	78.21%	21.79%
	ATP	-7.408×10^{-4}	-4.340×10^{-4}	58.58%	41.42%
	ADP	-7.477×10^{-4}	-4.386×10^{-4}	58.66%	41.34%
	CDP	-7.494×10^{-4}	-4.482×10^{-4}	59.81%	40.19%
	UDP	-7.518×10^{-4}	-4.530×10^{-4}	60.26%	39.74%

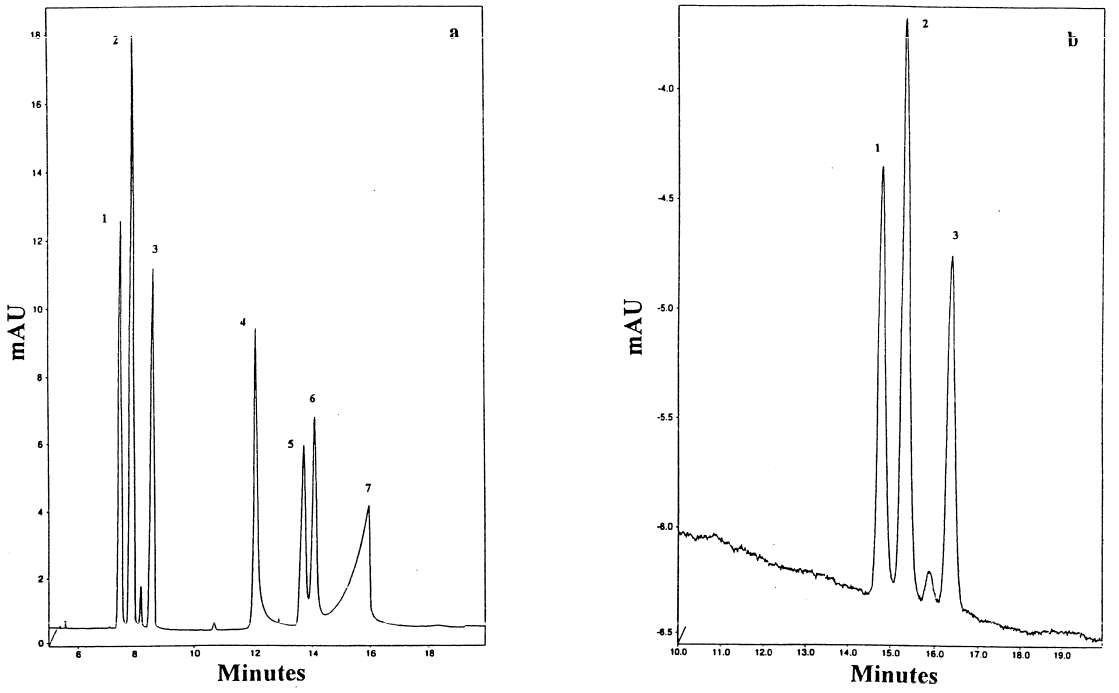

Fig. 3. Separation of the test mixture in the (a) unmodified and (b) sapphyrin-modified capillary in 25 m*M* borate–acetate buffer pH 7.0; voltage 17 kV per capillary. Peak identification: 1, GMP; 2, AMP; 3, UMP; 4, ATP; 5, ADP; 6, CDP; 7, UDP. Note that di- and triphosphates are not brought in front of the detection window in sapphyrin-modified capillary before 20 min running time.

sapphyrin-modified capillary exerted a similar (a bit higher) negative charge comparable to the untreated column resulting in a similar profile as obtained with the uncoated capillary. It can be explained by binding of the Cl^- ions to the protonated sapphyrin core thus resulting in more negative charge on the capillary wall and higher EOF.

The advantage of using the sapphyrin-modified capillary can be visualized in the selective separation of monophosphates from di- and triphosphates as the system can be run in such a mode that these solutes are not brought before the detector window at all. On the other hand by the washing procedure used this fact does not prevent the coated capillary from being re-used.

It is noticeable that by washing the sapphyrin-modified capillary with the modifier solvent (dichloromethane) bound sapphyrin cannot be eluted. On the other hand, the mixture of the solvent (dichloromethane and trifluoracetic acid) effectively removes bound sapphyrin off the wall.

In order to elute diphosphates and ATP from the sapphyrin-coated capillary the separation must be run either in borate–acetate buffer (Fig. 4, Table 2) or in the borate–phosphate (Fig. 5, Table 2); however, the voltage applied must be 20 kV per capillary in both cases. The running times are considerably increased as compared to the results obtained with the unmodified capillary. Migration times seen at different voltages used with sapphyrin-modified capillaries represent apparent values only as they are influenced both by the change in the endoosmotic flow and the interaction between nucleoside phosphates and the sapphyrin coating. This is particularly true for ATP and diphosphates in the borate–acetate system. The selectivity of the system for monophosphates in borate–acetate buffer is clearly higher in the sapphyrin-modified capillary (see Table 3). With the phosphate-containing background electrolyte the separation of AMP from GMP is about the same as in the uncoated capillary and the resolution of CDP from ADP is incomplete in both types of capillaries used, however the resolution of all the other components is better in the sapphyrin-modified capillary.

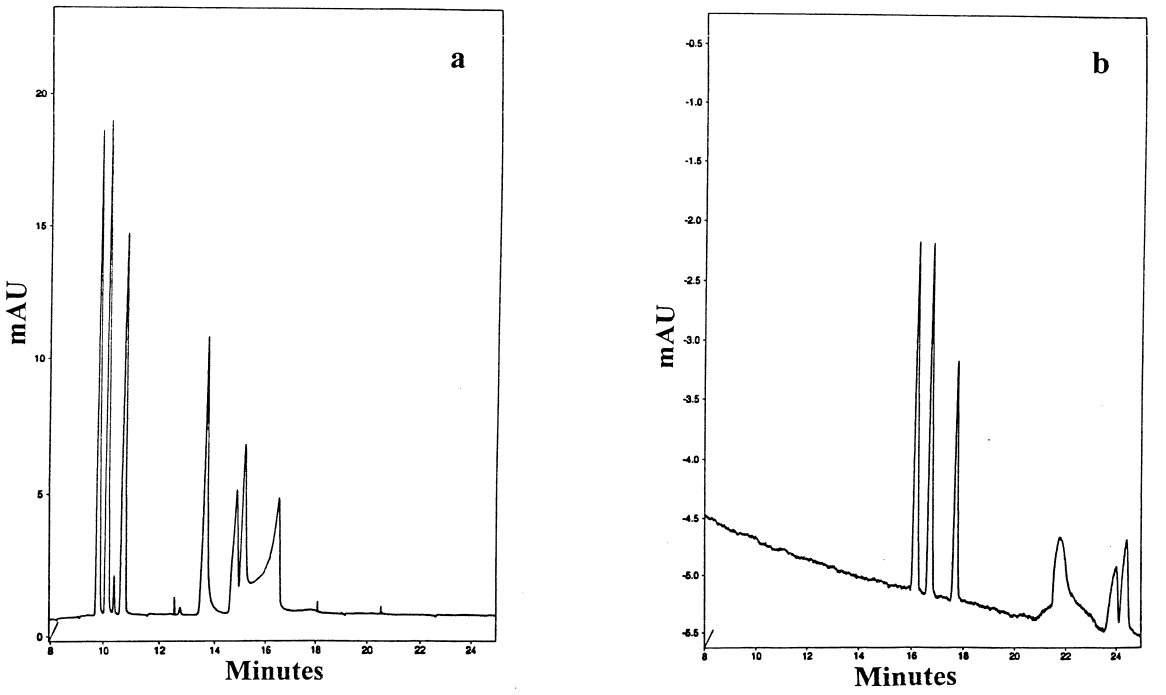

Fig. 4. Separation of the test mixture in 25 m*M* borate–acetate buffer pH 7.0, voltage 20 kV per capillary; (a) untreated capillary, (b) sapphyrin-coated capillary. Peak sequence as in Fig. 3.

4. Conclusion

The following conclusions can be drawn from the results obtained.

1. Sapphyrin coating of the internal wall of the capillary results in an interaction with the diphosphates and the triphosphate (ATP) provided that borate–acetate buffer is used as the background electrolyte.
2. The quality of separation for monophosphates as judged from the resolution factors is better in the coated capillary no matter whether borate–acetate or borate–phosphate was used as the run electrolyte. For the other members of the test system the selectivity of the borate–phosphate system is better with the coated capillary; however, even under these conditions ADP–CDP are incompletely resolved. In the borate–acetate system the critical pair appears to be ATP–ADP.
3. Because of slowing down of the electroosmotic flow in the borate–phosphate and particularly in the borate–acetate buffers and because of the solutes–capillary wall interaction the running times of all compounds tested were increased in the sapphyrin-modified capillary. However, in Tris–HCl buffer the endoosmotic flow was (on the contrary) increased reflecting possibly the interaction of the Cl^- ions with the capillary coating, and no significant changes in separation were observed with the solutes tested.
4. Under appropriate conditions with borate–acetate as background electrolyte the possibility to separate selectively nucleoside-monophosphates from diphosphates and the triphosphate (ATP) is possible.
5. No change was observed in the elution sequence of the individual phosphates tested.
6. Coating of the inner wall of the capillary decreases the limit of the detection by about one order of magnitude as compared to the uncoated capillary which reflects the absorbance of the sapphyrin layer at the wavelength used for detection (254 nm).
7. The coating procedure is quite simple (it can be

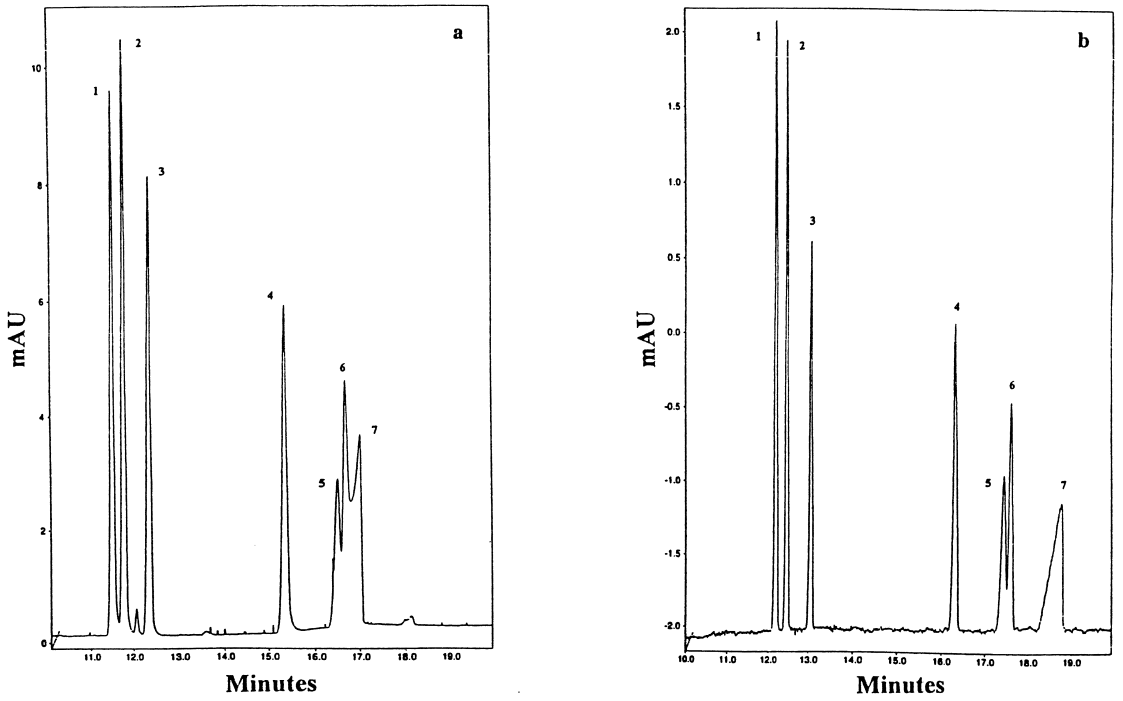

Fig. 5. Separation of the test mixture in 25 m*M* borate–phosphate buffer at pH 7.0 and 20 kV, (a) untreated capillary, (b) sapphyrin-coated capillary. Peak identification as in Fig. 3.

Table 3
Resolution of the test solutes in borate–phosphate and borate–acetate buffer using uncoated and sapphyrin-coated capillary. Separation in borate–acetate buffer at 10 kV allows selective separation of monophosphates from di- and triphosphates in sapphyrin-coated capillary

	Resolution from the preceding peaks		
	Solute	Coated capillary	Uncoated capillary
Borate–phosphate buffer	GMP	52.77	22.10
(20 kV per capillary)	AMP	2.08	2.0
	UMP	5.70	2.56
	ATP	22.07	9.20
	ADP	5.67	3.88
	CDP	0.89	0.88
	UDP	1.96	4.58
Borate–acetate buffer	GMP	109.8	43.83
(10 kV per capillary)	AMP	4.50	4.28
	UMP	10.75	4.19
	ATP	Not eluted	13.65
	ADP	Not eluted	4.26
	CDP	Not eluted	0.78
	UDP	Not eluted	0.43

materialized by flushing the capillary with 2 mg/ml solution of sapphyrin in dichloromethane) with subsequent stabilization of the coating by drying the capillary by an air flush. Loosely adsorbed sapphyrin can be removed by an ethanol wash (5 min), and stabilization of the capillary by running in the separation buffer at 20 kV.

8. The capillary modification materialized by the procedure described in Section 2 is stable for 20 subsequent runs at least. As a matter of fact no baseline drift (indicating the sapphyrin leaking from the capillary) was observed and the once modified capillary could not be brought to the original state even by prolonged washing with dichloromethane.

Acknowledgements

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