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Analysis of sugar phosphates and related compounds using capillary zone electrophoresis with indirect UV detection

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Abstract

Sugar phosphates and related compounds can be rapidly separated using capillary zone electrophoresis and quantitated via indirect UV detection using potassium sorbate at 256 nm. Three buffers were examined at various pH values. The optimum separation conditions resulted with 6 m*M* potassium sorbate at pH 5.8. Pairs of regioisomers such as glucose-1-phosphate and glucose-6-phosphate or ribose-1-phosphate and ribose-5-phosphate were readily separated. A mixture of seven sugar phosphates and orthophosphate also was separated. The detection limits for samples of glucose-6-phosphate and glucose-1-phosphate were 0.14 m*M* and 0.26 m*M*, respectively.

Keywords: Buffer composition; Sugar phosphates

1. Introduction

As part of a program aimed at identifying methods for the phosphorylation of sugars in aqueous solution, we needed a method for the rapid separation and quantitation of sugar phosphates. A key requirement is the ability to analyze the sugar phosphates in the presence of a variety of other components, particularly large amounts of unreacted sugars and orthophosphate. Among the available methods, paper chromatography has been widely used [1–3]. Paper chromatography provides separation of analytes from crude samples, but the method is slow and not easily amenable to quantitation. ^{31}P NMR spectroscopy [4] enables clear distinction between sugar phosphates and orthophosphate, but distinction among various types of sugar phosphates is poor, and quantitation is difficult. Mass spectrometry is not well-suited to mixtures containing large amounts of orthophosphate. Our attention was directed towards capillary zone electrophoresis (CZE) for the separation of mixtures with indirect UV detection for quantitation. CZE with indirect UV detection has been used for analyzing condensed phosphates [5–7] and sugars [8–13], and CZE with direct UV detection has been used for the analysis of nucleotides [14,15]. In this paper, we describe the use of CZE with indirect UV detection for the analysis of 10 sugar phosphates and related compounds (Fig. 1). Most of the compounds selected are biologically relevant and play a central role in bioenergetics or metabolism. Characterizing the effects of experimental parameters on separation and quantitation required a survey of different buffers, pH values, ionic strengths, and UV chromophores. The lower limit of detection has been determined for selected sugar phosphates. In addition

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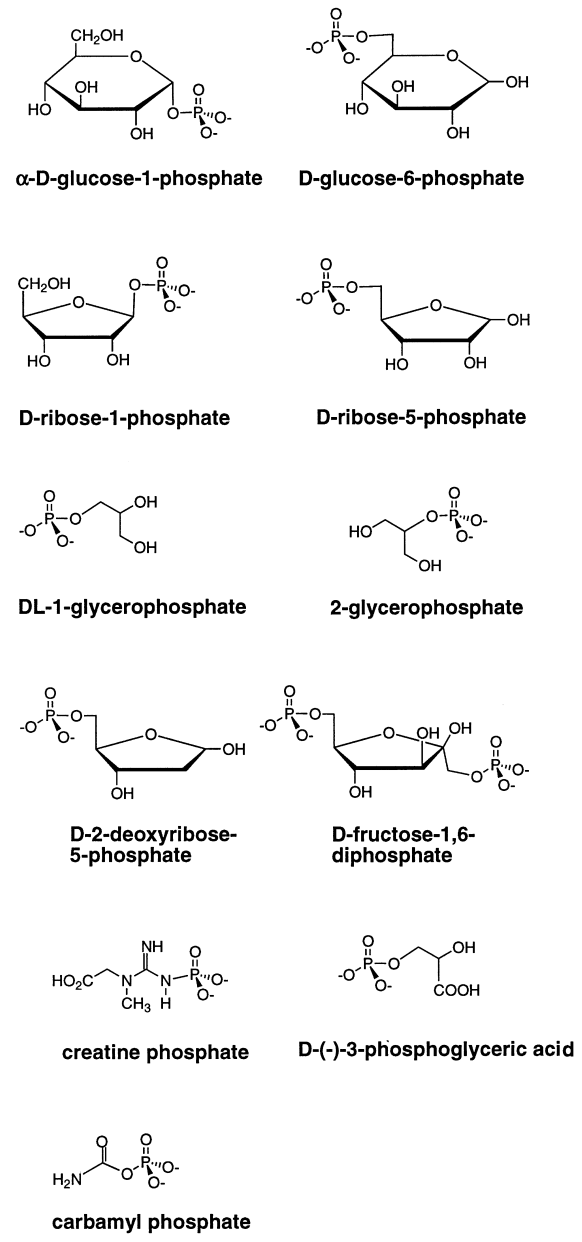

Fig. 1. Sugar phosphates and related compounds.

to our applications in studying phosphorylation chemistry, the ability to separate and quantitate various naturally-occurring sugar phosphates should be useful for biochemical studies.

2. Experimental

2.1. Chemicals and solutions

All chemicals were reagent grade. The compounds α-D-glucose-1-phosphate disodium salt, D-glucose-6-phosphate monosodium salt, D-ribose-1-phosphate cyclohexylammonium salt, D-ribose-5-phosphate disodium salt, DL-1-glycerophosphate disodium salt, 2-glycerophosphate disodium salt, D(−)-3-phosphoglyceric acid barium salt, creatine phosphate disodium salt, 2-deoxyribose-5-phosphate disodium salt, D-fructose-1,6-diphosphate trisodium salt, sorbic acid, potassium sorbate and carbamyl phosphate dilithium salt were purchased from Sigma and used as received. The samples were stored at 5°C or below 0°C as required. Unless a specific anomer is indicated in Fig. 1, the sugars are assumed to be mixtures of the α-and β-anomers. Ammonium acetate and various sodium phosphates were purchased from Aldrich and used as received.

All buffers and samples were prepared with water purified with a Milli-Q system (Millipore, Bedford, MA, USA) and filtered through a 0.45-μm filter. For buffer system I, 6 m*M* potassium sorbate served as both the carrier electrolyte and the background absorber for indirect UV detection. A solution of 6 m*M* potassium sorbate was prepared and the pH was adjusted to 4.2–7.0 by titration with 6 m*M* sorbic acid (pH 3.3) at room temperature. For buffer system II, 15 and 20 m*M* NH_4OAc solutions were made by dissolving appropriate amounts of NH_4OAc in 6 m*M* potassium sorbate solution with addition of 0.25 m*M* acetic acid to achieve the required pH. For buffer system III, a phosphate solution (10, 15, or 20 m*M*) in 6 m*M* sorbic acid was prepared by dissolving the appropriate amount of Na_2HPO_4 in 6 m*M* sorbic acid solution, and by preparing a separate solution of NaH_2PO_4 with 6 m*M* sorbic acid, and then combining various amounts of the two solutions to obtain the desired pH. Buffer solutions were prepared fresh every day and were degassed with argon prior to CZE analysis.

2.2. CZE

The capillary electrophoresis apparatus was a homemade instrument comprised of a 0–30 kV high-

voltage power supply (Spellman Model SL30PN30, Hauppauge, NY, USA), high-voltage relay (Ross Engineering, Campbell, CA, USA), fused-silica capillaries of 50 μm I.D.×360 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA), 200 ml jacketed beakers (Ace Glass, Vineland, NJ, USA), variable-wavelength UV–visible detector (Model variable 500, Scientific System, State College, PA, USA), circulating controlled-temperature bath (Neslab RTE-100), analog converter (National Instruments), computer (Gateway 2000 Model G-6-200, North Sioux City, S.D., USA), and CZE data acquisition software written in Lab View (National Instruments). The capillary (untreated) was positioned inside Tygon tubing with the two ends of the capillary placed in glass vessels (~5 ml) containing buffer. The total length of fused-silica capillary was 64 cm, and the distance from the injection point to the UV detector was 46 cm. In some experiments, the two buffer reservoirs and the capillary were temperature-controlled with mineral oil (25°C) circulating in the tubing holding the capillary and in the 200 ml jacketed beakers. Samples were introduced by hydrostatic injection (20 s). Indirect UV detection was performed at 256 nm with a data sampling rate of 5 Hz. The capillary and the upstream buffer vessel were contained in a Plexiglas enclosure fitted with a positive safety lock and high-voltage relay for operator safety.

Each new capillary was flushed with 0.1 *M* NaOH for 30 min followed by water for 20 min, and finally equilibrated with the respective running buffer for 2–6 h. The same procedure was followed when switching from one buffer to another. The extensive equilibration time was required to obtain a stable background. Before each CZE experiment, the capillary was flushed with 0.1 *M* NaOH for 20 min, then with water for 10 min, and finally with the requisite buffer for 20 min. Between successive runs, the capillary was flushed with the buffer for 3–5 min. The capillary was stored in water overnight.

3. Results and discussion

3.1. Detection of sugar phosphates

Sugar phosphates absorb very weakly in the UV and this presents challenges to their detection in CZE. A similar problem is encountered when investigating unphosphorylated carbohydrates. Sorbic acid, which has been used for various sugar compounds [8–11], was chosen as the background electrolyte and chromophore for the indirect UV detection at 256 nm for the following reasons [9]; (a) Sorbate has a high molar absorption coefficient ($e_{256\ nm}$=27 800 M^{-1} cm^{-1}); (b) Sorbate is soluble in water and in various buffers, and does not interact with the analytes or the capillary surface; (c) Sorbate carries a single negative charge, thus ensuring a good transfer ratio (defined as the number of chromophore molecules displaced by one analyte molecule); (d) The effective mobility of sorbate matches that of carbohydrates, thus avoiding band broadening. Here we report that sorbic acid also works well for sugar phosphates and related compounds.

3.2. Separation of sugar phosphates from sugar and orthophosphate

Sugar phosphates have very different pK_a values from sugars (Table 1), and therefore require different pH buffer systems for separation. The pK_a values for sugars are usually >12, therefore, the running buffer must be very basic to cause ionization. Such highly alkaline media can cause rearrangements of sugars

Table 1
pK_a values for sugars, sugar phosphates and related compounds

Compounds	pK_a	Ref.
Glucose	12.46	[17]
Glucose-1-phosphate	1.10, 6.13	[18,19]
Glucose-6-phosphate	0.94, 6.11	[18,19]
Ribose	12.22	[17]
Ribose-1-phosphate	not available	
Ribose-5-phosphate	not available	
Fructose	12.27	[17]
Fructose-1,6-diphosphate	1.48, 6.1	[18]
2-Deoxyribose	12.61	[17]
2-Deoxyribose-5-phosphate	6.7, 13.05	[20]
1-Glycerophosphate	1.40, 6.44	[19]
2-Glycerophosphate	1.37, 6.34	[19]
3-Phosphoglyceric acid	1.42, 3.42	[19]
Creatine phosphate	2.7, 4.58	[18]
Carbamyl phosphate	5.2	[21]

[16]. For sugar phosphates the pK_a values are usually around 6. At a buffer pH of about 6, the sugar phosphates, but not the sugars, will be ionized. Therefore the sugars elute with the electroosmotic flow and cannot be determined. For example, CZE of a sample solution of glucose-1-phosphate and glucose-6-phosphate (0.8 m*M* each) with a 100-fold excess of NaH_2PO_4 and of glucose (using 6 m*M* potassium sorbate as the carrier electrolyte and chromophore) gave peaks at 3.7 min (glucose 6-phosphate), 3.8 min (glucose-1-phosphate), and 4.5 min (NaH_2PO_4). No peak was observed for glucose. This result shows that small amounts of sugar phosphate can be separated and detected in the presence of large amounts of glucose and ortho-phosphate, a common feature encountered with various phosphorylation chemistries.

3.3. Effect of pH on effective mobility

Separation in CZE is based on differences in the electrophoretic mobilities (μ_{ep}) of the analytes [22]. Because the electrophoretic mobility depends mainly on the pH of the separation system, optimization of the buffer pH is important for reaching the optimum separation conditions. The influence of pH on the electrophoretic mobility of glucose-6-phosphate in 6 m*M* potassium sorbate is shown in Fig. 2. We also examined the separation of various mixtures of sugar phosphates as a function of pH. At pH near 7, separation can be achieved but with fronting peaks (N=7 820), while at pH 4.2, the peaks were sharper but tailing was observed (N=21 200). We found pH 5.8 with potassium sorbate to be effective in affording separation with symmetrical peaks (N=49 700). The results are quite sensitive to pH. Indeed, when glucose-6-phosphate was examined at pH 6.1 (a pH equal to the literature pK_a value for glucose-6-phosphate and a number of other sugar phosphates; see Table 1), the electropherogram was noisy and a fronting peak was observed (N=20 900). Others have noted that the ideal pH for separation with CZE can differ from the literature pK_a value because the experimental conditions are different [23].

3.4. Separation of mixtures of sugar phosphates

The conditions for analysis of glucose-6-phosphate were applied to other sugar phosphates. Fig. 3 shows the separation of three pairs of compounds including glucose-1-phosphate and glucose-6-phosphate, ribose-1-phosphate and ribose-5-phosphate, and 1-glycerophosphate and 2-glycerophosphate. In each case, the separation was achieved in under 5 min. These separations are significant in that each pair of compounds consists of regioisomers with nearly identical pK_a values.

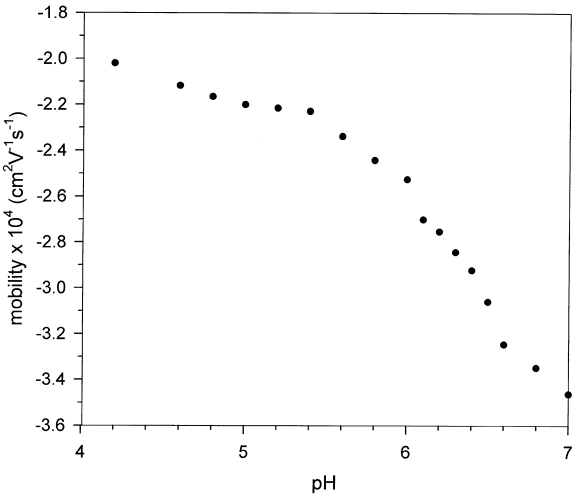

Fig. 2. Electrophoretic mobilities of glucose-6-phosphate (2.1 m*M*) as a function of pH. Running conditions: 25°C with 6 m*M* sorbate adjusted to different pH values; for further conditions see Section 2.

We also examined different buffer systems for the separation of glucose-1-phosphate and glucose-6-phosphate (Fig. 4). Buffer system II, consisting of 15 m*M* NH_4OAc with 6 m*M* potassium sorbate, provides resolution comparable to that with potassium sorbate alone. This buffer is attractive for later combination of CZE with mass spectrometry (rather than indirect UV detection), where the volatilizable NH_4OAc would be used alone. The best separation is achieved with 15 m*M* phosphate and 6 m*M* sorbic acid at pH 6.3 (buffer III).

The three buffer systems were applied to a variety of sugar phosphates and the results are listed in (Table 2). The results with the pair of ribose phosphates resembled those with the glucose phosphates in the three buffer systems (entries 1 and 2), though greater resolution of the former was achieved using buffers II and III than with buffer I. The

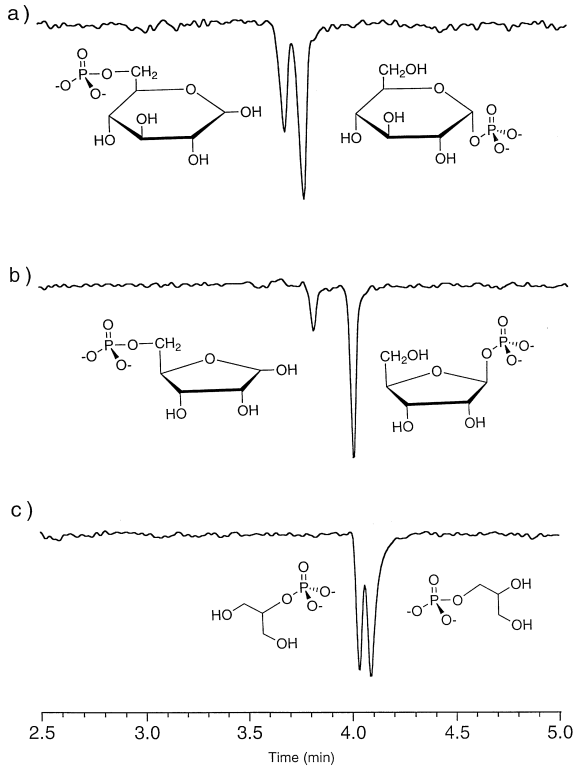

Fig. 3. Separation of the solutes in a carrier electrolyte of 6 m*M* sorbate at pH 5.8. (a) glucose-6-phosphate (1.0 m*M*) and glucose-1-phosphate (1.2 m*M*); (b) ribose-5-phosphate (0.8 m*M*) and ribose-1-phosphate (1.3 m*M*); (c) 2-glycerophosphate (1.2 m*M*) and 1-glycerophosphate (1.2 m*M*). For further experimental conditions, see Section 2 and Table 2.

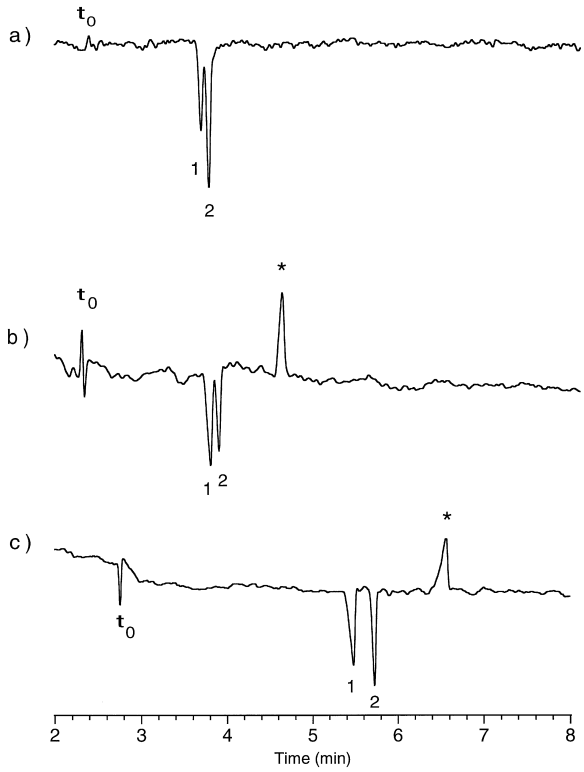

Fig. 4. Separation of glucose-6-phosphate (peak 1) and glucose-1-phosphate (peak 2) in various buffers. (a) Buffer I (6 m*M* potassium sorbate) at pH 5.8. (b) Buffer II (15 m*M* NH_4OAc with 6 m*M* potassium sorbate) at pH 5.8. (c) Buffer III (15 m*M* sodium phosphate with 6 m*M* sorbic acid) at pH 6.3. The * indicates a ghost peak.

glycerophosphates were examined only in buffer I, which afforded good separation (entry 3 and Fig. 3). 3-Phosphoglyceric acid and creatine phosphate each gave good separation and sharp peaks in buffers II and III ($N>30\,000$), but a broad peak in buffer I ($N<5\,000$) (entries 4, 5). 2-Deoxyribose-5-phosphate and fructose 1,6-diphosphate each gave sharp peaks in each of the buffers examined (entries 6, 7).

Carbamyl phosphate, a high-energy organic phosphate compound that has been shown to phosphorylate AMP and ADP [24] as well as acetic acid [21], was analyzed effectively with the three buffer systems (entry 8, Table 2). Carbamyl phosphate has been identified by ^{31}P NMR [21] spectroscopy and has been quantitatively analyzed by alkaline hydrolysis followed by molybdate complexation [24] of the liberated phosphate. Because carbamyl phosphate is easily hydrolyzed in aqueous solution [25], the short analysis times and small sample requirements are superior features of CZE.

The best results achieved with the sugar phosphates generally involved use of buffer I, with the exceptions of 3-phosphoglyceric acid and creatine phosphate. A mixture was prepared of seven sugar phosphates and orthophosphate with concentrations ranging from 1.2–1.5 m*M* (entry 9, Table 2). As shown in Fig. 5, separation of the mixture was accomplished in 6 min and afforded sharp peaks. The solvent (water) peak was well separated from the peaks of sugar phosphates.

3.5. Quantitation

In indirect UV–Vis detection, the linear response

Table 2
Conditions for analysis of various sugar phosphates and related compounds

Entry	Compounds	Buffer components		pH	Field strength (V m^{-1})	Current (μA)	Separation efficiency (*N*)	Migration time (min)
1	Glucose-6-phosphate, glucose-1-phosphate	I	Potassium sorbate [6 m*M*][a]	5.8	469	3	84 300 88 800	3.7 3.8
		II	NH_4OAc [15 m*M*], potassium sorbate [6 m*M*]	5.8	469	25	39 500 93 600	3.9 4.0
		III	Phosphate [15 m*M*], sorbic acid [6 m*M*]	6.3	313	30	55 400 147 000	5.5 5.7
2	Ribose-5-phosphate, ribose-1-phosphate	I	Potassium sorbate [6 m*M*][a]	5.8	469	3	312 000	3.8
		II	NH_4OAc [20 m*M*], potassium sorbate [6 m*M*]	6.4	469	32	24 800 65 300	6.7 7.6
		III	Phosphate [15 m*M*], sorbic acid [6 m*M*]	6.3	313	30	43 600 35 400	7.1 8.0
3	2-Glycerophosphate, 1-glycerophosphate	I	Potassium sorbate [6 m*M*][a]	5.8	469	3	222 000 120 000	4.0 4.1
4	3-Phosphoglyceric acid	I	Potassium sorbate [6 m*M*]	5.8	469	3	4 400	7.3[b]
		II	NH_4OAc [15 m*M*], potassium sorbate [6 m*M*]	6.0	469	24	50 600	4.2
		III	Phosphate [20 m*M*], sorbic acid [6 m*M*]	7.6	391	45	119 000	6.2
5	Creatine phosphate	I	Potassium sorbate [6 m*M*][a]	5.8	469	3	3 100	5.6[b]
		II	NH_4OAc [20 m*M*], potassium sorbate [6 m*M*]	6.4	469	32	88 600	9.6
		III	Phosphate [15 m*M*], sorbic acid [6 m*M*]	6.3	469	35	34 500	6.5

6	2-Deoxyribose-5-phosphate	I	Potassium sorbate [6 m*M*][a]	5.8	469	3	108 000	3.9
		II	NH_4OAc [20 m*M*], potassium sorbate [6 m*M*]	6.4	469	32	121 000	6.8
7	Fructose-1,6-diphosphate	I	Potassium sorbate [6 m*M*][a]	5.8	469	3	12 400	5.2
		II	NH_4OAc [15 m*M*], potassium sorbate [6 m*M*]	6.3	469	18	54 100	8.4
		III	Phosphate [20 m*M*], sorbic acid [6 m*M*]	7.6	391	45	83 700	5.9
8	Carbamyl phosphate	I	Potassium sorbate [6 m*M*]	6.3	469	4	14 500	6.9
		II	NH_4OAc [15 m*M*], potassium sorbate [6 m*M*]	6.0	469	25	40 900	4.3
		III	Phosphate [15 m*M*], sorbic acid [6 m*M*]	6.3	313	30	120 000	5.6
9	Glucose-6-phosphate, glucose-1-phosphate, 2-deoxyribose-5-phosphate, ribose-1-phosphate, 2-glycerophosphate, 1-glycerophosphate, NaH_2PO_4, fructose-1,6-diphosphate	I	Potassium sorbate [6 m*M*][a]	5.8	469	3	47 800 84 000 115 000 332 000 248 000 118 000 51 000 16 700	3.8 3.9 4.0 4.1 4.2 4.3 4.8 5.5

[a]With temperature control at 25°C. [b]Broad peak.

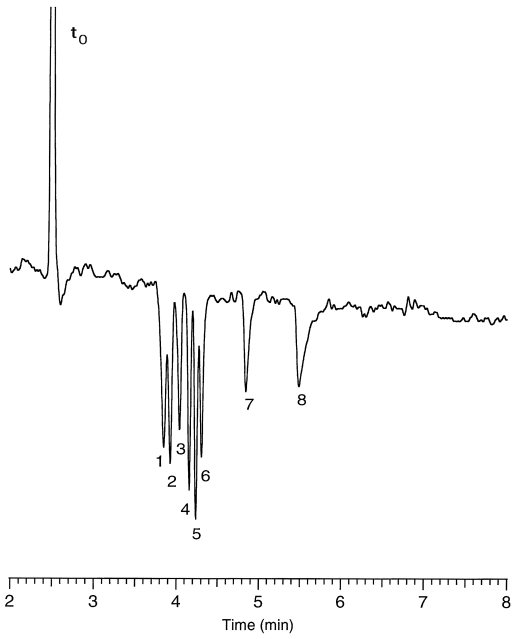

Fig. 5. Separation of seven sugar phosphates and orthophosphate (1.2–1.5 m*M* each) in a carrier electrolyte of 6 m*M* sorbate at pH 5.8. Peaks: t_0=water, 1=glucose-6-phosphate, 2=glucose-1-phosphate, 3=2-deoxyribose-5-phosphate, 4=ribose-1-phosphate, 5=2-glycerophosphate, 6=1-glycerophosphate, 7=NaH_2PO_4, 8=fructose-1,6-diphosphate. For further experimental conditions, see Section 2.

range is limited both by the concentration of the chromophore and by the degree of dissociation of the analyte. We determined working curves using glucose-6-phosphate (0.27–8.64 m*M*) and glucose-1-phosphate (0.51–6.12 m*M*). Each working curve showed quite good linearity and a *y*-intercept near zero. Within the range of linearity, least-square linear regression analysis provided the following regression equations and coefficients for glucose-6-phosphate and glucose-1-phosphate, respectively: $y=0.0003x+7\times10^{-5}$ ($R^2=0.994$), $y=0.0003x+5\times10^{-5}$ ($R^2=0.998$).

The limit of detection was calculated as the concentration that gave a signal three times greater than the baseline noise. The limit of detection is 0.26 m*M* for glucose-1-phosphate, and 0.14 m*M* for glucose-6-phosphate. Assuming a 20 s injection takes up about 4 nl of analyte solution, then the absolute detectable amounts of the glucose phosphates are 1.0 and 0.6 pmol, respectively. In contrast, ^{31}P NMR spectroscopy requires solutions that are ~20 m*M* and sample volumes of ~0.5 ml. Thus, CZE with indirect UV-detection is superior for the rapid separation and quantitation of dilute solutions of sugar phosphates.

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References

[1] M. Halmann, R.A. Sanchez, L.E. Orgel, J. Org. Chem. 34 (1969) 3702.
[2] R.S. Wright, H.G. Khorana, J. Am. Chem. Soc. 78 (1956) 811.
[3] I.M. Hais, K. Macek (Ed.), Paper Chromatography, Czechoslovak Academy of Sciences, Prague, and Academic Press, New York, 1963, pp. 793 and 819.
[4] D.G. Gorenstein (Ed.), Phosphorus-31 NMR, Principles and Applications, Academic Press, Orlando, FL, 1984.
[5] F.S. Stover, S.S. Keffer, J. Chromatogr. A 657 (1993) 450.
[6] T. Wang, S.F.Y. Li, J. Chromatogr. A 723 (1996) 197.
[7] S.A. Shamsi, N.D. Danielson, Anal. Chem. 67 (1995) 1845.
[8] A.E. Vorndran, P.J. Oefner, H. Scherz, G.K. Bonn, Chromatographia 33 (1992) 163.
[9] P.J. Oefner, A.E. Vorndran, E. Grill, C. Huber, G.K. Bonn, Chromatographia 34 (1992) 308.
[10] X. Xu, W.T. Kok, H. Poppe, J. Chromatogr. A 716 (1995) 231.
[11] A. Klockow, A. Paulus, V. Figueiredo, R. Amado, H.M. Widmer, J. Chromatogr. A 680 (1994) 187.
[12] B. Lu, D. Westerlund, Electrophoresis 17 (1996) 325.
[13] A. Zemann, D.T. Nguyen, G. Bonn, Electrophoresis 18 (1997) 1142.
[14] A.L. Nguyen, J.H.T. Luong, C. Masson, Anal. Chem. 62 (1990) 2490.
[15] R. Takigiku, R.E. Schneider, J. Chromatogr. 559 (1991) 247.
[16] J.F. Robyt, Esssentials of Carbohydrate Chemistry, Springer-Verlag, New York, 1998.
[17] A. Albert, E.P. Serjeant, The Determination of Ionization Constants, Chapman and Hall, New York, 3rd Edition, 1984, p. 143.
[18] R.M.C. Dawson, D.C. Elliott, W.H. Elliott, K.M. Jones (Eds.), Data for Biochemical Research, Oxford University Press, 2nd Edition, 1969, pp. 108, 110 and 113.
[19] G.D. Fasman (Ed.), Handbook of Biochemistry and Molecular Biology, CRC Press, Boca Raton, FL, 1976, p. 308.

[20] H.A. Sober (Ed.), Handbook of Biochemistry; Selected Data for Molecular Biology, Chemical Rubber Co., CA, 1968, p. J-126.
[21] Ö. Saygin, Origins of Life 13 (1983) 43.
[22] .A. Weston and P.R. Brown, HPLC and CE Principles and Practice, Academic Press, San Diego, CA, 1997, p. 154.
[23] M.G. Khaledi, S.C. Smith, J.K. Strasters, Anal. Chem. 63 (1991) 1820.
[24] Ö. Saygin, Naturwissenschaften 68 (1981) 617.
[25] M.E. Jones, F. Lipmann, Proc. Natl. Acad. Sci. USA 46 (1960) 1195.