Journal of Chromatography, 237 (1982) 506–510
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,565

Note

Capillary tube isotachophoretic separation of nucleotides using complex-forming equilibria

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(Received November 20th, 1981)

Many papers have described the separation of nucleotides using various methods, *e.g.*, liquid chromatography[1–7], electrophoresis[8–12] and isotachophoresis[13–16].

Beckers and Everaerts[13] studied the separation of nucleotides by isotachophoresis and showed that the separation of complex mixtures was difficult and the use of some other techniques, *e.g.*, a counter-flow of electrolyte, might be necessary. We found that the differences among the effective mobilities of nucleoside triphosphates were small in comparison with those of di- or monophosphates.

The use of complex-forming equilibria in capillary tube isotachophoresis is a powerful technique for improving the separability. Inorganic anions[17], organic acids[18], some EDTA complexes[19], alkaline earth metal cations[20] and lanthanide cations[21] have been separated using this technique. In this paper we show that nucleotides can be effectively separated by capillary tube isotachophoresis using complex-forming equilibria between magnesium(II) ion and the nucleotides.

EXPERIMENTAL

Isotachopherograms were recorded with a Model IP-1B capillary tube isotachophoretic analyser with a PGD-1 potential gradient detector (Shimadzu, Kyoto,

TABLE I

LEADING AND TERMINATING ELECTROLYTE SYSTEMS

Electrolyte	*Parameter*	*Value*
Leading	Leading ion	10.4 mmol dm^{-3} NO_3^- [added as nitric acid and magnesium(II) nitrate]
	Complexing agent	2.86 mmol dm^{-3} Mg^{2+} (added as nitrate)
	Additive	5% ethanol
		0.005% poly(vinyl alcohol)
	Buffering counter ion	Adenosine
	pH	3.00
Terminating	Terminating ion	10 mmol dm^{-3} 2,2-dimethylpropanoic acid
	pH	3.5

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Japan). The length of the capillary tube (PTFE, I.D. 0.5 mm) was 20 cm. Measurements of pH were made with a Model F-7ss expanded-scale pH meter (Horiba, Japan).

Adenosine and 5'-nucleotides were obtained from Boehringer (Mannheim, G.F.R.). The other chemicals used were of the highest grade commercially available and deionized water was used. The leading and terminating electrolyte systems are shown in Table I. The nitric acid solution and the magnesium(II) ion solution were standardized by acid–base and chelatometric titration, respectively. In all experiments metal ions were added as nitrate and the leading ion (*i.e.*, nitrate) concentration was kept constant.

RESULTS AND DISCUSSION

Fifteen 5'-nucleotides (AMP, CMP, GMP, IMP, UMP, ADP, CDP, GDP, IDP, UDP, ATP, CTP, GTP, ITP and UTP)* were used as sample constituents. All experiments were carried out in a low pH region of the leading electrolyte because the differences among the effective mobilities of the nucleotides were larger than those in

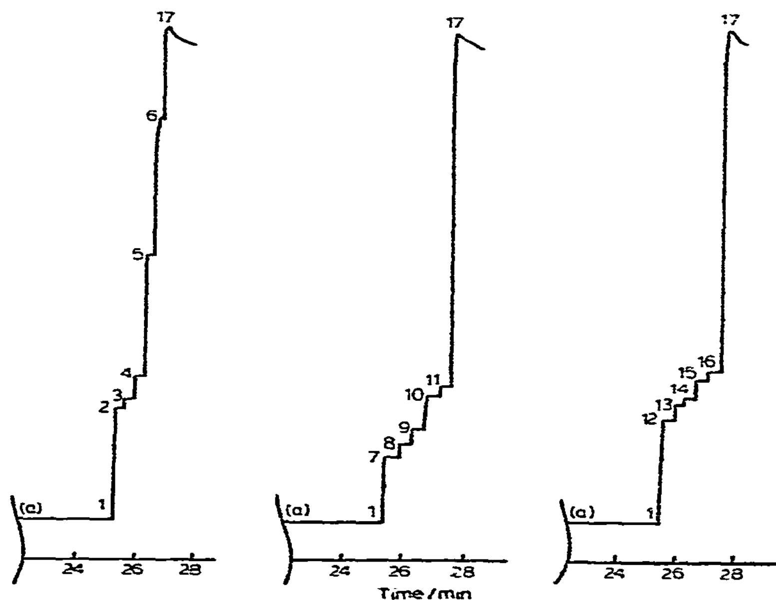

Fig. 1. Isotachopherograms of nucleoside mono-, di- and triphosphates. Conditions as in Tables I and II except chart speed, 10 mm min^{-1}. Differential potential gradient curves are not shown, to simplify the figures. (a) Potential gradient. 1 = Nitrate; 2 = UMP; 3 = IMP; 4 = GMP; 5 = AMP; 6 = CMP; 7 = UDP; 8 = IDP; 9 = GDP; 10 = ADP; 11 = CDP; 12 = UTP; 13 = ITP; 14 = GTP; 15 = CTP; 16 = ATP; 17 = 2,2-dimethylpropanoic acid.

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* Abbreviations: AMP = adenosine monophosphate; CMP = cytidine monophosphate; GMP = guanosine monophosphate; IMP = inosine monophosphate; UMP = uridine monophosphate; ADP = adenosine diphosphate; CDP = cytidine diphosphate; GDP = guanosine diphosphate; IDP = inosine diphosphate; UDP = uridine diphosphate; ATP = adenosine triphosphate; CTP = cytidine triphosphate; GTP = guanosine triphosphate; ITP = ionsine triphosphate; UTP = uridine triphosphate.

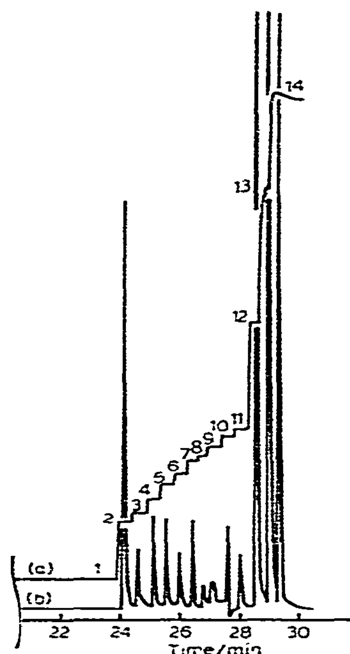

Fig. 2. Isotachopherogram of eleven nucleotides and phosphate. Conditions as in Fig. 1 except sample volume, 6.0 µl. (a) Potential gradient; (b) differential potential gradient. 1 = Nitrate; 2 = phosphate; 3 = UDP; 4 = IDP; 5 = GDP; 6 = UTP; 7 = ITP; 8 = GTP; 9 = CDP; 10 = CTP; 11 = ATP; 12 = AMP; 13 = CMP; 14 = 2,2-dimethylpropanoic acid.

a high pH region. In the initial experiments several metal cations were tested as complex-forming reagents with the nucleotides. It was found that magnesium(II) was one of the most effective ions and calcium(II) had almost the same effect as magnesium(II).

The effective mobilities of the nucleotides decreased with increasing magnesium(II) ion concentration in the leading electrolyte and the differences among the mobilities of some nucleotides were increased. To obtain a sufficient separation of nucleotides it was preferable to make the magnesium(II) ion concentration high. Such conditions, however, make the buffering ability of the leading electrolyte low because the amounts of the buffering agent, *i.e.*, adenosine, decrease with increase in the magnesium(II) ion concentration to keep the pH of the leading electrolytes constant.

As the stability constants of metal complexes are higher in organic than in aqueous media[22], ethanol was added to the leading electrolyte to enhance the interaction between magnesium(II) ion and the nucleotides. This effect appeared when several percent of ethanol were added. Not only were the differences among the mobilities of some nucleotides increased and the separabilities improved by adding ethanol to the leading electrolyte, but also the isotachopherograms obtained were better than those obtained in experiments with no ethanol: the sloping steps obtained with some sample constituents were improved to a flat shape and the drift of the potential gradient of the leading ion became very small.

As shown in Fig. 1, five kinds of nucleoside mono-, di- and triphosphates could

TABLE II

PR VALUES AND ZONE LENGTHS

The PR value (potential gradient ratio) is the ratio of the potential gradient of the leading ion to that of the sample ion (PG_L/PG_S), which corresponds to the ratio of the mobility of sample ion to that of the leading ion (m_S/m_L)[16]. Conditions as in Table I; other conditions, migration current, 75 μA; chart speed, 40 mm min^{-1}; sample, about 4 nmol; sample volume 2.0 μl.

Compound	*PR value**	*Relative standard deviation (%)*	*Zone length (mm)**	*Relative standard deviation (%)*
Phosphate	0.427	1.9	14.9	2.7
AMP	0.135	2.0	14.4	2.0
CMP	0.0293	2.0	12.2	1.2
GMP	0.225	2.3	14.7	1.2
IMP	0.257	1.9	12.6	1.7
UMP	0.272	2.0	14.3	2.0
ADP	0.246	2.7	22.2	2.7
CDP	0.233	2.9	17.7	2.1
GDP	0.308	2.4	17.3	2.3
IDP	0.344	2.3	16.6	2.1
UDP	0.387	2.5	21.0	1.7
ATP	0.216	1.4	20.1	2.1
CTP	0.226	1.5	16.8	2.2
GTP	0.251	1.6	16.2	1.7
ITP	0.261	1.7	12.9	1.7
UTP	0.287	1.3	16.8	1.8

* Average of six determinations.

be separated into individual constituents. Furthermore, twelve sample constituents, including five nucleoside triphosphates, four nucleoside diphosphates, two nucleoside monophosphate and phosphate, could be separated simultaneously, as shown in Fig. 2. The complexing agent was 2.86 mmol dm^{-3} magnesium(II) ion and 5% of ethanol was added to the leading electrolyte. Table II shows that the relative standard deviations of the PR values of the nucleotides and phosphate are 1.3–2.9% and those of the zone lengths are 1.2–2.7% with about 4 nmol of sample constituents.

The effective mobilities of the nucleotides were very sensitive to the ethanol and magnesium(II) ion concentrations in the leading electrolyte. In other words, suitable conditions for given samples can be chosen by adjusting the ethanol or magnesium(II) ion concentration in the leading electrolyte. The mobilities of the nucleotides were also sensitive to the pH of the leading electrolyte. Satisfactory separations of the nucleotides could not be obtained at pH 5.3 and 8.5.

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