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Note

High-performance liquid chromatographic method for separation of dinucleotides

EBERHARD HAGEMEIER, SIEGMAR BORNEMANN, KARL-SIEGFRIED BOOS and ECKHARD SCHLIMME*

Laboratorium für Biologische Chemie der Universität (GH) Paderborn, Warburger Strasse 100, D-4790 Paderborn (G.F.R.)

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During the last decade, dinucleotides linked via a 5′,5″-phosphate bridge have become of particular interest, as nucleotides of this unusual type were found to function as 5′-terminal cap structures in the majority of eucaryotic and viral messenger RNA[1,2] and on the monomer level as regulators in the biosynthesis of macromolecules[3].

For analytical investigations in the biochemical field and for reaction and purity control monitoring in chemical synthesis work with 5′,5″-bridged dinucleotides and structurally related compounds highly efficient chromatographic separation techniques are required. In this paper we report three aspects of the high-performance liquid chromatographic (HPLC) analysis of nucleotides and dinucleotides: (1) efficient separation of cap-structured dinucleotides and related compounds with respect to resolution, sensitivity and shortness of separation time; (2) monitoring of the time-dependent progress of dinucleotide synthesis; and (3) purity control of naturally occurring and synthesized compounds.

EXPERIMENTAL

Equipment

HPLC separations were carried out on two different systems, as follows, with a column temperature of 24°C in each instance.

(A) A Serva two-piston pump, a home-made separating column[4] (300 × 2.3 mm I.D.) packed with Nucleosil 10 SB (10 μm) (Macherey & Nagel, Düren, G.F.R.) with a diaphragm-sealed injection system was used. The effluent was monitored continuously with an ISCO Model UA 5 absorbance detector (Instrumentation Specialties, Lincoln NE, U.S.A.) at 254 nm.

(B) Two Altex Model 110 A pumps (Altex, CA, U.S.A.) controlled by a Model 420 microprocessor, a Rheodyne Model 7125 loop injector for sample introduction and a Kontron Uvicon Model 725 spectrophotometer (Kontron, Eching, G.F.R.) to monitor the UV absorbance of the effluent at 266 nm throughout the study were used. The column was an Altex Partisil PAC (10 μm) (250 × 3.2 mm I.D.).

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Mobile phases

The mobile phase for system A was a 0.1 *M* KNO_3 potassium nitrate–0.02 *M* potassium dihydrogen orthophosphate buffer adjusted to pH 2.6 with dilute orthophosphoric acid. The flow-rate of the mobile phase was constant at 8 ml h^{-1} and the inlet pressure was 500 p.s.i. The mobile phases for system B were alternatively (B1) 0.8 *M* ammonium formate buffer; (B2) 0.4 *M* ammonium formate buffer; both buffers were adjusted to pH 4.1 with 85% formic acid; (B3) a 6-min linear gradient from water up to 0.8 *M* ammonium formate, pH 4.1. The flow-rate of the mobile phase in each instance was 2 ml min^{-1} at an inlet pressure of 2000 p.s.i. All buffers were prepared with doubly distilled water and degassed in an ultrasonic bath.

Materials

5'-AMP, 5'-ADP, 5'-ATP, 5'-GMP, 5'-GDP, 5'-GTP, 5'-IMP, 5'-IDP and 5'-ITP were purchased from Boehringer (Mannheim, G.F.R.). [^{14}C]5'-AMP$(NH_4)_2$ was a product from New England Nuclear (Boston, MA, U.S.A.). All other dinucleotides investigated were prepared in micromolar amounts[5].

RESULTS AND DISCUSSION

Separation of 5',5''-phosphate-bridged dinucleotides

5',5''-Phosphate-bridged dinucleotides represent groups of similar compounds as there are dinucleotide 5',5''-di-, -tri-, -tetra- and -pentaphosphates which differ in anionic charge with respect to the length of the intramolecular phosphate chain and, in addition, to their heterocyclic bases. Depending on these molecular differences, especially due to anionic charges, it was possible to find a suitable anion exchanger and a mobile phase for the analysis of such similar groups of compounds under both isocratic and gradient elution conditions.

Under isocratic separation conditions it was possible to separate completely up to eighteen 5',5''-linked dinucleotides. Although the separation was efficient with respect to resolution and sensitivity, dinucleotides bearing a tri-, tetra- and pentaphosphate bridge could not be eluted from the column in a reasonable time with system A (Table I).

Concerning the retention times of the different groups of dinucleotides, systems B1 and B2 are more convenient. The resolution of compounds of the same group, on the other hand, is not as efficient as that obtained with system A. However, with systems B1 and B2 a complete separation of the different groups of dinucleoside di-, tri-, tetra- and pentaphosphates is possible (Table I).

A very rapid and selective separation, however, of all nucleotides investigated in the course of this work was achieved by the use of system B with linear gradient elution (B3). This was done by programming a 6-min linear gradient from water up to 0.8 *M* ammonium formate (pH 4.1), followed by a "hold" at the final concentration of 0.8 *M* ammonium formate until all nucleotides were completely eluted (Table I).

Fig. 1 shows the separation of a synthetic mixture of chemically synthesized dinucleotides with system B3.

HPLC monitoring of dinucleotide synthesis

Fig. 2 shows schematically the HPLC monitored reaction course of a Gp_3A

TABLE I

RETENTION TIMES OF 5′,5″-PHOSPHATE-BRIDGED DINUCLEOTIDES

Type of compound	*Compound*	*Retention time (min)*			
		A	*B1*	*B2*	*B3*
Nucleotides	5′-AMP	12	0.92	1.02	3.12
	5′-ADP	22	1.06	1.64	5.17
	5′-ATP	58	1.33	3.46	6.82
	5′-GMP	18	0.88	1.01	3.25
	5′-GDP	42	1.02	1.54	5.61
	5′-GTP	–	1.24	3.15	7.39
	5′-IMP	21	0.86	0.96	3.14
	5′-IDP	–	0.98	1.43	5.34
	5′-ITP	–	1.16	2.33	7.12
Dinucleoside diphosphates	Ap_2A	19	0.99	1.25	4.17
	rroAp$_2$rroA	14	1.28	1.53	4.66
	Gp_2A	25	0.96	1.24	3.90
	Ip_2A	30	–	–	–
	2′dGp$_2$A	31	–	–	–
	2′,3′ddGp$_2$A	42	–	–	–
	Ip_2I	74	–	–	–
Dinucleoside triphosphates	rroGp$_3$rroA	29	1.19	2.26	6.58
	Gp_3A	96	1.15	2.17	6.16
	2′dGp$_3$A	108	1.26	2.64	6.75
	2′-O-mGp$_3$A	109	1.26	2.42	6.97
	2′,3′ddGp$_3$A	117	1.93	4.68	7.99
	2′dGp$_3$2′dG	152	1.35	3.34	7.13
	Gp_3G	160	.–	.–	.–
	Ip_3G	146	1.04	2.10	5.44
	Ip_3A	160	1.10	2.14	5.96
	m^7-Gp$_3$A	–	1.01	1.46	5.13
Dinucleoside tetra- and pentaphosphates	Gp_4G	360	1.43	6.36	8.07
	2′dGp$_4$2′dG	304	1.76	7.81	9.20
	Ap_5A	–	2.00	.–	10.23
	Ip_5I	–	1.60	.–	9.71
	2′dGp$_5$2′dG	–	2.53	.–	11.92

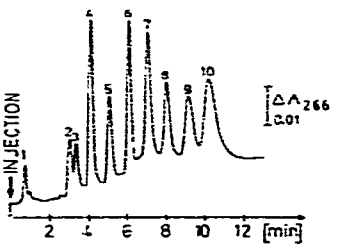
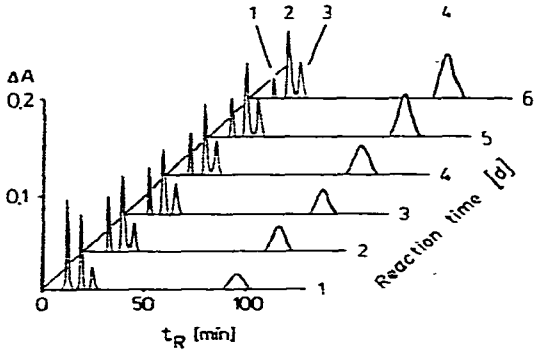


Fig. 1. HPLC separation of a synthetic mixture of chemically synthesized dinucleotides. Peaks: 1 = adenosine, guanosine; 2 = AMP; 3 = GMP; 4 = Ap_2A; 5 = m^7-Gp$_3$A; 7 = 2′dGp$_3$2′dG; 8 = Gp_4G; 9 = 2′dGp$_4$2′dG; 10 = Ap_5A.

synthesis. This application allows convenient reaction control by analysing the time-dependent decrease of the starting material AMP, the increase of the side-product Gp_2A and the increase of the desired product Gp_3A. Ap_2A turned out to be constant over the monitored time range owing to its generation in a preceding reaction step[5].



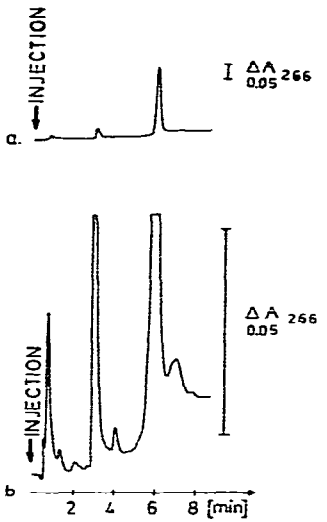


Fig. 2. Results of HPLC analysis of 5-μl aliquots of a Gp_3A synthesis reaction mixture (17 ml) with system A. Peaks: 1 = AMP (retention time, t_R = 12 min); 2 = Ap_2A (t_R = 19 min); 3 = Gp_2A (t_R = 25 min); 4 = Gp_3A (t_R = 96 min). GDP (t_R = 42 min) is present in excess and not shown for graphical reasons.

Fig. 3. Analysis of an aliquot of a homogeneous product peak (Gp_3A), obtained by DEAE-cellulose chromatography, using two different detector sensitivities with system B3.

Purity control

A homogeneous product peak of a Gp_3A synthesis eluted from DEAE-cellulose was analysed for purity and yield (Fig. 3). A 75-fold increase in sensitivity leads to the appearance of five additional impurities and decreases the overall yield by 10%. This comparison shows that the highest available detector sensitivity should be used in order to obtain reasonable purity and yield.

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