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Note

Liquid chromatographic analysis of nucleosides, purine base-modified adenosine derivatives and 3',5'-cyclic AMP

Biochemical tests with modified adenine nucleotides necessitate the critical purity control of commercial or synthesized material. So-called nucleoside analyzers have recently been described[1,2] in which conventional cation-exchange column chromatography is used. Modern high-pressure liquid chromatography provides better resolution as well as greater sensitivity. Although the time of analysis could be shortened considerably by the use of a pellicular ion exchanger having a small capacity[3,4], it has been shown that highly efficient separations can be achieved with a conventional exchanger in a reasonable amount of time in a simple and relatively inexpensive manner. This allows micro-scale trace analysis and preparative work.

Experimental

A flow diagram of the apparatus is shown in Fig. 1.

The eluent was pumped through the column by a commercially available pump, Model LDP 13 A (Labotron Messtechnik, 8191 Gelting, G.F.R.). The effluent was monitored with a Zeiss PMQ II photometer with a flow cell of 8 μl volume and

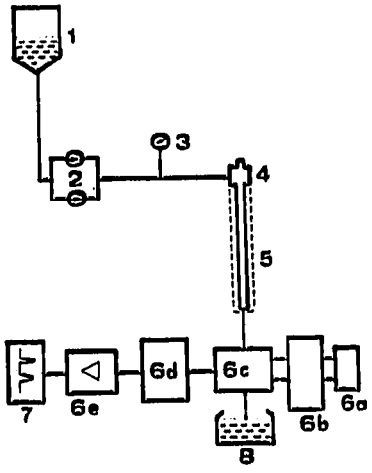

Fig. 1. Flow diagram of the apparatus. 1 = Tank of mobile phase, 2 = LDP 13A labotron pump, 3 = pressure gauge, 4 = injection port, 5 = thermostated modular column, 6 a–c = Zeiss PMQ 11 photometer, 7 = Philips recorder, 8 = waste.

10 mm path length, the feed line being modified according to HUBER[5]. The other components, including the modified column, were manufactured in our workshop as described elsewhere[6,7].

The separating conditions were as follows: inlet pressure, 40 atm; flow-rate,

8 ml/h; column, 2.3 × 300 mm at 34°, packed with Beckmann M 81 cation-exchange resin (particle size range 8–40 μm); eluent, 0.4 *M* ammonium formate, pH 4.65; attenuation, 0.2 absorbance units full-scale. The resin was suspended in reagent-grade methanol and freed from fines by sedimentation. The column was filled by pressure filtration, thus achieving a stable and highly pressure-resistant, uniformly packed bed, using a method similar to that already described[6]. Although the costly pellicular ion-exchange resin allows faster separations, a conventional ion-exchange resin was selected so as to provide maximum capacity.

Products to be separated. The nucleosides and 3',5'-AMP were purchased from Boehringer, Mannheim, G.F.R. 1-N-methyl- and 6-N-methyl-adenosine monophosphate were obtained from Terra Marine Bio-Research, La Jolla, Calif., U.S.A. 1-N-oxide-ATP was prepared as described by VON DER HAAR *et al.*[8]. 8-C-bromo-ATP

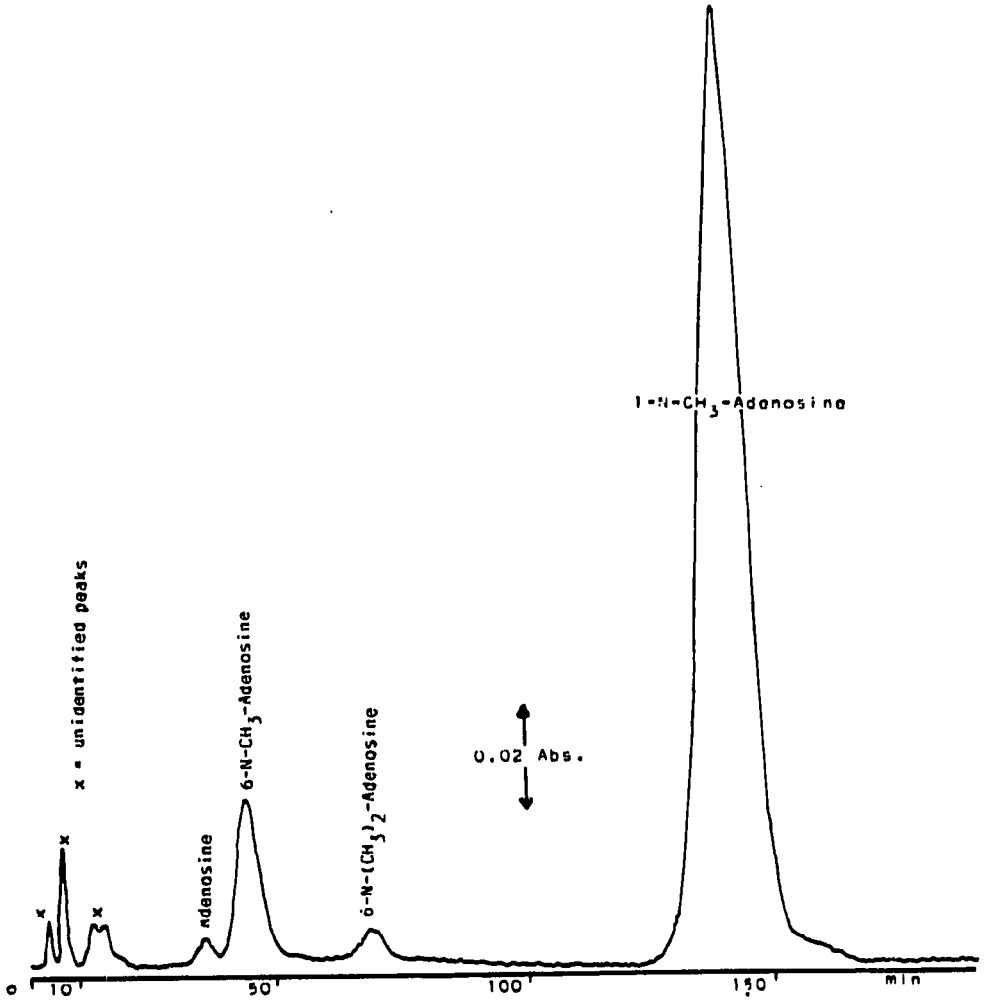

Fig. 2. Chromatogram showing purity control of 1-N-methyl-AMP after enzymatic digestion.

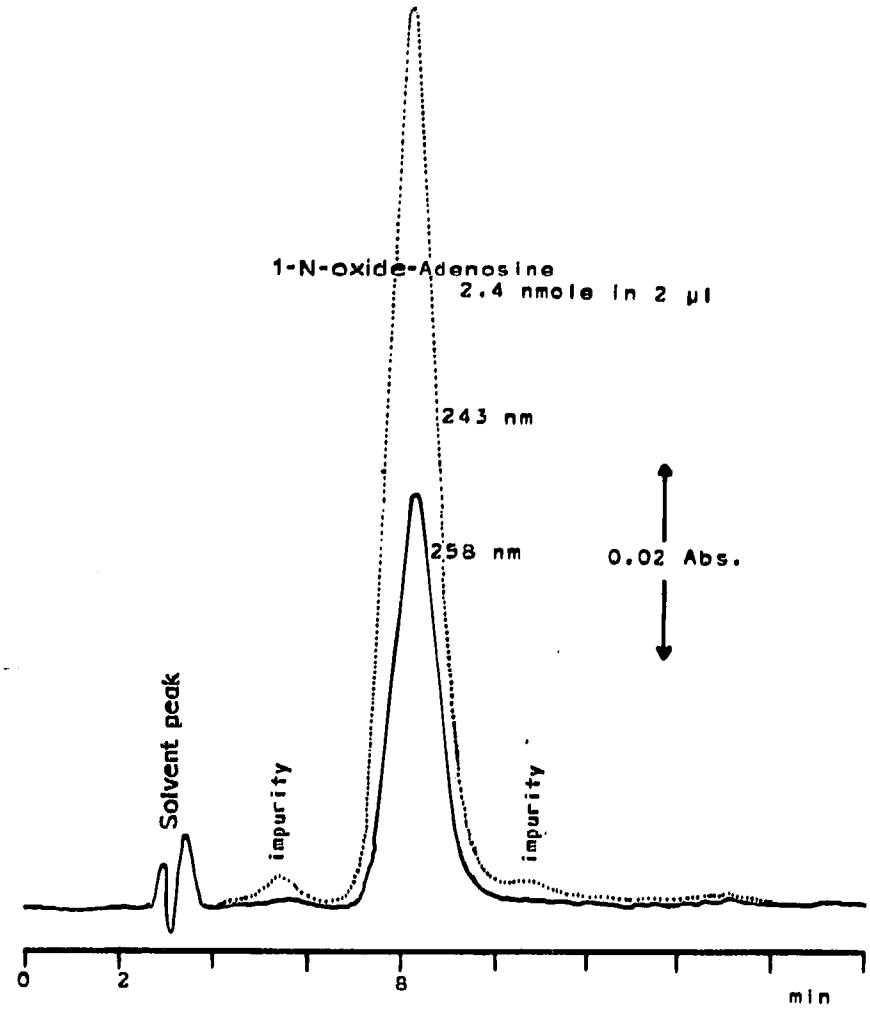

Fig. 3. Chromatogram showing purity control of 1-N-oxide-ATP after enzymatic digestion.

was synthesized by a method similar to that described by REIST *et al.*[9]. Imidazo-ATP* was prepared according to BARRIO *et al.*[10].

Nucleotides were hydrolyzed prior to sample application in 0.3-ml plastic tubes (Beckmann Co., Catalogue No. 314326 B) with a maximum sample volume of 10 µl and a maximum total volume of 50 µl by incubation with 5 µl of alkaline phosphatase (E.C. 3.1.3.1, Boehringer, 1 mg/ml = 25 U) at 37° for 3 h. The incubation medium was 10 µl of 0.1 *M* $MgCl_2$ and 25 µl of Tris buffer, pH 8.0. The samples were injected into the column with a 5-µl Hamilton high-pressure syringe.

Results and discussion

Fig. 2 shows the purity control of 1-N-methyl-AMP, containing adenosine, 6-N-methyladenosine and 6-N-dimethyladenosine impurities. The first three peaks are unidentified.

* 3-β-D-Ribofuranosyl [5'-O-triphosphate]imidazo[2,1-*i*]purine.

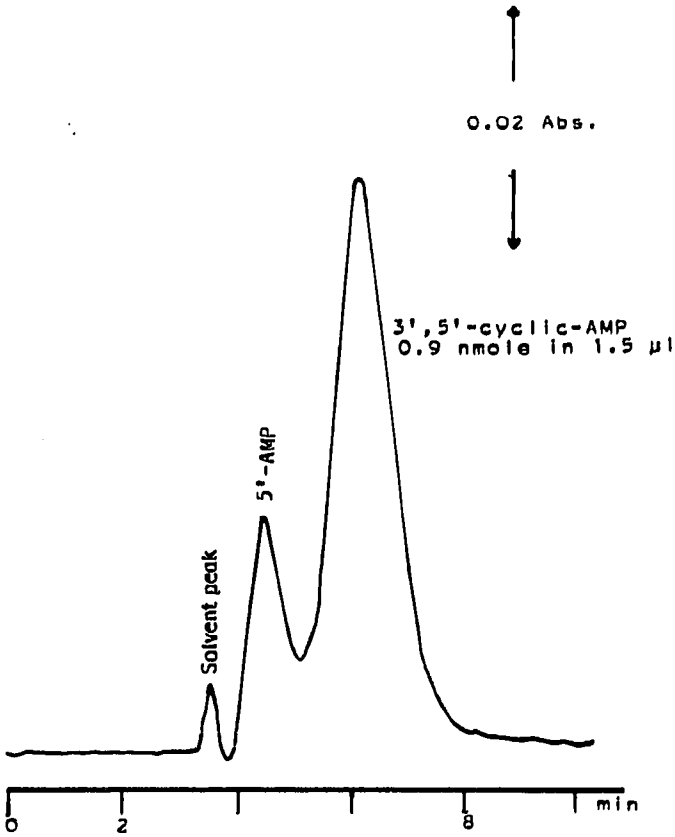

Fig. 4. Chromatogram showing separation of 3',5'-cyclic AMP from 5'-AMP.

Fig. 3 shows the purity control of 1-N-oxide-ATP recorded at both 258 and 243 nm. The absorbance of the eluent does not enable a measurement to be made at 232 nm, the maximum extinction of 1-N-oxide-ATP.

Fig. 4 shows the separation of 3',5'-cyclic-AMP from 5'-AMP.

Tables I and II give the chromatographic properties of some nucleosides (I),

TABLE I

CHROMATOGRAPHIC PROPERTIES OF NUCLEOSIDES

Nucleoside	*Property*[a]						
	t_R	$t_R - t_{R0}$	$w_{\frac{1}{2}}$	N_0	N	H_0	H
Uridine	7.94	4.99	0.76	239.05	605.24	1.26	0.5
Inosine	13.85	10.90	1.5	292.81	472.75	1.03	0.64
Guanosine	22.55	19.60	2.5	340.84	451.16	0.88	0.67
Adenosine	35.50	32.55	3.2	573.74	682.45	0.52	0.44
Cytidine	48.70	45.75	3.3	1065.79	1207.66	0.28	0.25

[a] t_R = retention time (min). t_{R0} = retention time of a component that is not retained (min). $w_{\frac{1}{2}}$ = peak width at half the peak height. $N = 8 \ln 2 \, (t_R/w_{\frac{1}{2}})^2$ = number of theoretical plates. $H = 300/N$ = height equivalent of the theoretical plate (mm). N_0, H_0 = effective values of N and H, taking t_{R0} into account.

TABLE II

CHROMATOGRAPHIC PROPERTIES OF 3',5'-CYCLIC-AMP AND SUBSTITUTED ADENOSINES

Compound	Property[a]						
	t_R	$t_R - t_{R_0}$	$w_{\frac{1}{2}}$	N_0	N	H_0	H
3',5'-Cyclic-AMP	5.92	2.97	1.1	40.42	160.61	7.42	1.87
1-N-O-Adenosine	8.25	5.30	1.1	128.73	311.92	2.33	0.96
6-N-CH_3-Adenosine	44.80	41.85	5.0	388.48	445.18	0.77	0.67
8-C-Br-Adenosine	59.60	56.65	8.6	240.61	266.32	1.25	1.13
Imidazoadenosine[b]	67.00	64.05	5.4	780.13	853.65	0.39	0.35
6-N-$(CH_3)_2$-Adenosine	71.00	68.05	5.5	848.88	924.07	0.35	0.33
1-N-CH_3-Adenosine	144.00	141.05	9.4	1248.55	1301.32	0.24	0.23

[a] See footnote to Table I.
[b] 3-β-D-Ribofuranosylimidazo [2-1-i] purine

3',5'-cyclic-AMP and some substituted adeninosines(II). The H_0 values decrease with increasing retention time, with only one marked exception to this expected result, *viz.*, 8-C-bromo-adenosine. This exception could be explained by the existence of the *syn* and *anti* conformational forms. Adenosine is known to exist in the *anti* form, whereas bromination leads to the *syn* product, as evaluated from molecular structure studies of 8-C-bromo-adenosine crystals by TAVALE AND SOBELL[11]. The partial conformational change from *syn* to *anti* may be caused during incubation with alkaline phosphatase. Evidence of this was established by other experiments[12].

In such work, modern liquid chromatography will prove to be a useful tool for both chemical and conformational analysis.

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