Journal of Chromatography, 190 (1980) 212–216

CHROM. 12,508

Notes

Detection and quantitation of diadenosine tetraphosphate by high-performance liquid chromatography

DAVID W. NIERENBERG, ALFONSO L. POGOLOTTI, Jr and DANIEL V. SANTI

Departments of Medicine, Biochemistry and Biophysics, and Pharmaceutical Chemistry, University o California, San Francisco, Calif. 94143 (U.S.A.)

(Received October 26th, 1979)

Diadenosine-5′,5′-P^1,P^4-tetraphosphate (Ap4A) is one of a growing number of unusual dinucleotides of biochemical interest[1–4]. Recent reports indicate that Ap4A levels parallel the proliferative activity of mammalian cells and may represent a positive pleiotypic mediator[3,4]. Further, it has been reported that certain bacterial aminoacyl-tRNA synthetases catalyze the formation of Ap4A from ATP and the cognate amino acid[1,2]. Previously reported methods of measuring Ap4A require the use of radioactive precursors and tedious multiple separations by column and thin-layer chromatography. In this report we describe one high-performance liquid chromatographic (HPLC) system which separates Ap4A from other adenine-containing nucleotides, and a second system which separates Ap4A from all UV-absorbing acid-soluble intracellular components. Ap4A may be rapidly and directly quantitated by UV absorbance at levels of 30 pmol applied, and the use of radioisotopes would enhance this sensitivity.

EXPERIMENTAL

Materials

Nucleotides 5′-AMP, ADP, and ATP were obtained from Sigma (St. Louis, Mo., U.S.A.); Ap4A was obtained from P-L Biochemicals (Milwaukee, Wisc., U.S.A.). Standard solutions of nucleotides were prepared in 10 m*M* K_2HPO_4, pH 7.0. All other reagents were the highest grades available. Double-distilled deionized water was used in all solvents, which were filtered through 0.45 μm pore-size cellulose filters. Alkaline phosphatase, pyrophosphatase (Worthington Biochemical, Freehold, N.J., U.S.A.) and snake venom phosphodiesterase (Boehringer, Mannheim, G.F.R.) were stored at 4°. Homogenous *E. coli* isoleucyl-tRNA synthetase was kindly provided by Dr. P. Schimmel.

HPLC

Chromatography was performed on a Hewlett-Packard 1084A liquid chromatograph equipped with a fixed wavelength (254 nm) UV detector. Peaks were identified by retention time, and quantitated by comparison with standard solutions of either peak areas (accurate for amounts greater than 100 pmol) or peak heights

(accurate as low as 30 pmol)[5]. Reversed-phase chromatography was performed on a 25 cm × 4.6 mm column containing a C_{18} bonded-phase (LiChrosorb C_{18}, Altex) at 30°, and a flow-rate of 3.0 ml/min. Buffer A contained 100 m*M* KH_2PO_4–5 m*M* tetrabutylammonium hydroxide, pH 5.0, with a linear gradient in acetonitrile from 5% at 0 min to 15% at 12 min. Anion-exchange chromatography was performed on a 25 cm × 4.6 mm Partisil (PXS) 10/25 SAX column (Whatman) at 50°, flow-rate = 1.5 ml/min. Buffer B was an isocratic buffer of 0.32 *M* $NH_4H_2PO_4$, pH 3.28.

Preparation of cell extracts

Acid-soluble cell extracts were obtained using 0.6 *M* trichloroacetic acid by the method of Khym[7].

Enzymatic reactions

E. coli isoleucyl-tRNA synthetase catalyzed formation of Ap4A was performed under conditions similar to those described by Zamecnik *et al.*[1]. Incubations were performed at 37° for 21 h, in 0.25-ml aliquots containing 50 m*M* Tris HCl (pH 7.4), 8 m*M* $MgCl_2$, 2 m*M* ATP, 5 m*M* 2-mercaptoethanol, 3 m*M* L-isoleucine, pyrophosphatase 10 μg/ml, and isoleucyl-tRNA synthetase 150 μg/ml. After 21 h, 40-μl aliquots were removed and diluted 1:6 with 50 m*M* Tris, pH 7.4, and either loaded directly on the reversed-phase column, or treated with either alkaline phosphatase (final concentration 0.8 units/ml) or phosphodiesterase (final concentration 0.01 units/ml) for 30 min at 37°.

RESULTS AND DISCUSSION

Fig. 1 illustrates retention times of AMP, ADP, ATP, and Ap4A on the reversed-phase column as a function of percent acetonitrile in the eluting buffer, which also contained 100 m*M* KH_2PO_4 and 5 m*M* tetrabutylammonium hydroxide, at pH 5.0. As shown, decreasing concentration of acetonitrile results in increasing retention times and efficiency of separation. Buffer A, a 5–15% linear gradient of

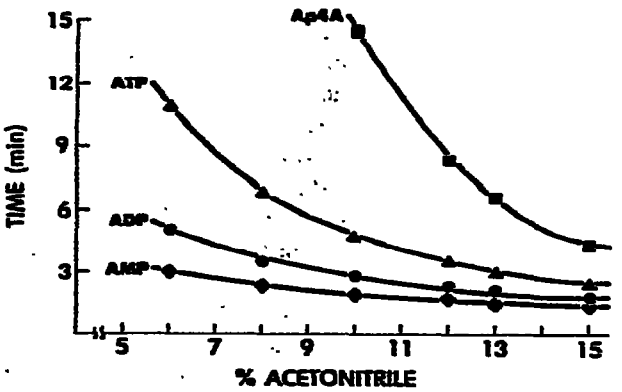

Fig. 1. Retention times of adenine nucleotides on LiChrosorb C_{18} column as a function of % acetonitrile in elution buffer. Flow-rate = 3.0 ml/min. Buffer also contained 5 m*M* tetrabutylammonium hydroxide, 100 m*M* KH_2PO_4, pH 5.0.

acetonitrile in the ion-paired buffer, was designed for optimal and rapid separation of these 4 adenine-containing nucleotides (Fig. 2A). Reproducibility of retention times on twelf consecutive runs was excellent, with standard deviations of retention times being less than 0.05 min. With this system, integrated peak areas were proportional to the amount of nucleotide applied to the column between 100 and 10,000 pmol; using peak heights, as low as 30 pmol could be quantitated.

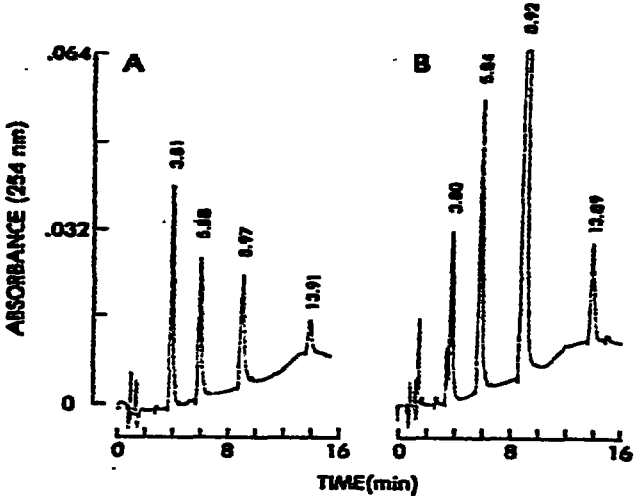

Fig. 2. Separation of AMP, ADP, ATP, and Ap4A on LiChrosorb C_{18} column, buffer A. (A): Mixture of nucleotide standard solutions. Peaks, with mean retention times ± S.D.: AMP (1400 pmol), retention time 3.76 ± 0.03 min; ADP (1140 pmol), 5.83 ± 0.03 min; ATP (1025 pmol), 8.90 ± 0.05 min; Ap4A (165 pmol), 13.85 ± 0.05 min. (B) Mixture of nucleotides after incubation of ATP with isoleucyl-tRNA synthetase. Peaks: AMP (1100 pmol); ADP (2420 pmol); ATP (7150 pmol); Ap4A (540 pmol).

One application of this system is illustrated in Fig. 2B, which shows the mixture of nucleotides formed when ATP and isoleucine are incubated with isoleucyl-tRNA synthetase. As shown, after 21 h ATP is converted to AMP (9.8%), ADP (21.6%), and Ap4A (4.8%); no Ap4A was formed in the absence of the synthetase. The identity of Ap4A was verified by retention time, its resistance to alkaline phosphatase (100% recovery with destruction of 80% of ATP), and degradation by snake venom phosphodiesterase (97% converted to AMP). Previously reported assays for the lysyl-tRNA synthetase catalyzed formation of Ap4A required the use of radioisotopes, separation by thin-layer chromatography, and subsequent quantitation[1–4]. The HPLC assay described here, which requires less than 15 min, has obvious advantages in terms of time, cost, and effort.

Because of recent interest in Ap4A as a possible regulator of mammalian cell growth, a simple and direct method for its quantitation in cell extracts was desirable. The methods previously described require prelabeling of cells with radioactive precursors, two chromatographic steps for separation of nucleotides, and comparison of activity in Ap4A and ATP fractions[3,4].

While the system using buffer A described above suffices for analysis of Ap4A

in relatively simple systems, it does not have sufficient resolving power for detection of Ap4A in the presence of the numerous UV absorbing components present in acid-soluble cell extracts. However, buffer B and anion-exchange chromatography allow separation of Ap4A from all UV-absorbing components of cell extracts.

With this system there is a "window" between ATP (retention time 18.38 min) and GTP (31.13 min) which has no significant UV absorbance in cell extracts (Fig. 3A). When S-49 tissue culture cell extracts are spiked with Ap4A (retention time 26.29 min), as little as 120 pmol may be detected with baseline separation from the peaks due to large excesses of ATP and GTP (Fig. 3B). In a preliminary experiment employing the acid-soluble cell extract from regenerating rat liver, a peak with the same retention time as Ap4A was detected (Fig. 3C). This peak was resistant to alkaline phosphatase (> 62% recovery), while > 96% ATP was destroyed.

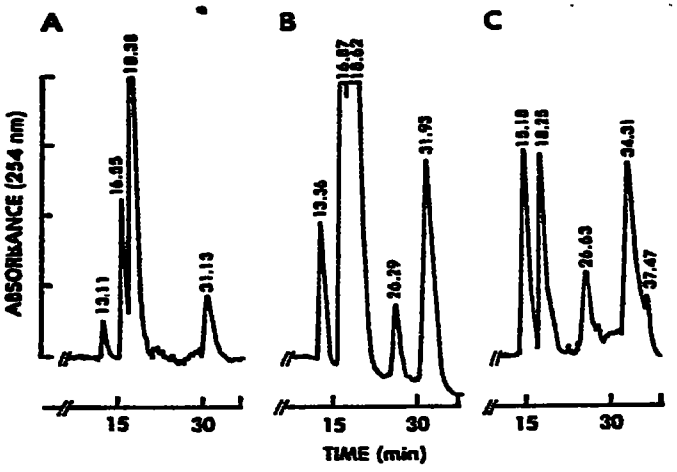

Fig. 3. Anion-exchange chromatography of acid-soluble cell extracts. Buffer B, flow-rate 1.5 ml/min, temperature 50°. (A) Extract of S-49 cells, revealing CTP (13.11 min), UTP (16.55 min), ATP (18.38 min, ≈1580 pmol), and GTP (31.13 min, ≈210 pmol). 0.008 a.u.f.s. (B) Extract of S-49 cells spiked with Ap4A (26.29 min, ≈120 pmol) in presence of ATP (18.62 min, ≈6650 pmol) and GTP (31.93 min, ≈ 1230 pmol). CTP, UTP also present. (C) Extract of regenerating rat liver after digestion by alkaline phosphatase. ATP (10.25 min, ≈5450 pmol), Ap4A (26.63 min, ≈76 pmol). 0.008 a.u.f.s. (13.0–17.5 min), 0.064 (17.5–24.0 min), 0.002 (24.0–33.0 min), 0.016 (33.0–38.0 min).

In summary, two HPLC systems are described which permit rapid, single-step detection and separation of Ap4A. Reversed-phase ion-pair chromatography is ideal for separation of Ap4A from other adenine-containing nucleotides and is currently being used to study enzymology of Ap4A. Anion-exchange chromatography separates Ap4A from all other UV-absorbing components of cell extracts and is being used to study the role of Ap4A in rapidly proliferating cells. Using a UV absorbance detector, as little as 30 pmol Ap4A may be quantitated, and the sensitivity could be greatly enhanced (if necessary) by the use of radioisotopes. The HPLC methods described here have obvious advantages over previously described methods of detection and quantitation of Ap4A.

ACKNOWLEDGEMENTS

This work was supported by USPHS Grant CA 14266 from the National Cancer Institute. D.V.S. is the recipient of an NIH Career Development Award.

REFERENCES

1 P. C. Zamecnik *et al.*, *Biochem. Biophys. Res. Commun.*, 24 (1966) 91–97.
2 P. C. Zamecnik and M. L. Stephenson, in A. San Pietro, M. R. Lambois and F. T. Kenney (Editors), *Regulatory Mechanisms for Protein Synthesis in Mammalian Cells*, Academic Press, New York, 1968, pp. 3–16.
3 E. Rapaport and P. C. Zamecnik, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 3984–3988.
4 F. Grummt, *Proc. Nat. Acad. Sci. U.S.*, 75 (1978) 371–375.
5 P. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651–662.
6 N. C. Bucher and N. Swaffield, *Biochim. Biophys. Acta*, 129 (1966) 445–459.
7 J. X. Khym, *Clin. Chem.*, 21 (1978) 1245–1252.