Journal of Chromatography, 179 (1979) 229–232

CHROM. 12,205

Note

Separation of 3′:5′- and 2′:3′-cyclic ribonucleotides from their related mononucleotides by Amberlite XAD-4 column chromatography

TAKAYOSHI UEMATSU and TAKAYUKI SASAKI

Department of Chemical Hygiene, Hokkaido Institute of Pharmaceutical Sciences, 7-1 Katsuraoka, Otaru 047-02 (Japan)

(First received May 4th, 1979; revised manuscript received July 9th, 1979)

There is no report of a simultaneous separation of a mixture of 2′:3′-, 3′:5′-cyclic nucleotides and related mononucleotides. Existing column methods for the separation of cyclic nucleotides employ mostly ion-exchange resins[1–7]. Although these methods are excellent in the separation of two to five 3′:5′-cyclic nucleotides from each other or from a known simple mixture, some of them require such expensive equipments as high-pressure chromatographs, tedious prepurification and/or the removal of non-volatile electrolytes and in some cases problems are encountered when nucleotides are hydrolyzed by a low pH eluent.

To overcome these problems, Uematsu and Suhadolnik[8] were the first to report a unique application of Amberlite XAD-4 for the adsorption and separation of polar nucleosides and nucleotides as well as the bases, by coating the pulverized resin with volatile triethylammonium bicarbonate (TEA) and eluting with this buffer. There is no report of the use of Amberlite XAD-4 in cyclic necleotide research except that Amberlite XAD-2, analogous to XAD-4, was used for the isolation of 3′:5′-cyclic AMP from plant nucleotide extracts by a low pH buffer[9]. The present paper reports the applicability of this method to cyclic nucleotides.

EXPERIMENTAL

Materials

Amberlite XAD-4 beads (20–50 mesh) were obtained from Rohm & Haas (Philadelphia, Pa., U.S.A.). The 3′:5′-, 2′:3′-cyclic, 2′(3′)- and 5′-ribomononucleotides, except 3′:5′-cyclic CMP, were purchased from Seikagaku Kogyo (Tokyo, Japan) and Sigma (St. Louis, Mo., U.S.A.). The 3′:5′-cyclic CMP was synthesized by the method of Borden and Smith[10]. Yeast ribonucleic acid (RNA) and ribonuclease A (bovine pancreas) were obtained from Boehringer (Mannheim, G.F.R.). Cellophane tubing (MW cut off 3500, 0.45 in.) was obtained from A. H. Thomas (Philadelphia, Pa., U.S.A.). All other chemicals were of reagent grade purity.

Preparation of resin and column

Preparation and purification of Amberlite XAD-4 (100–200 mesh) from a coarse grade resin (20–50 mesh) were carried out as described in a previous paper[8].

The resin (6.6 g) was suspended in 200 ml of methanol. The very fine particles remaining in suspension were decanted and the resin slurry was poured into a column (22 × 1 cm). The column was treated successively with 500 ml of water and 200 ml of 0.05 *M* TEA buffer, pH 7.6. The sample was taken up in *ca.* 1 ml of 0.05 *M* TEA buffer and applied to the column. The column was developed with a linear gradient of TEA buffer (800 ml from 0.1 to 0.4 *M*, pH 7.6) unless otherwise stated. Fractions of 5–10 ml were collected at a flow-rate of 75 ml/h at room temperature.

Used columns were regenerated by passing 300 ml of methanol, followed by 500 ml of water and 200 ml of 0.05 *M* TEA buffer.

Ultraviolet absorbance was measured with a Shimazu double beam 200-S.

RESULTS AND DISCUSSION

In order to compare the elution characteristics of 5′-, 2′(3′)-, 2′:3′-cyclic and 3′:5′-cyclic ribonucleotides, a solution containing 2–5 μmoles of each nucleotide (in 1 ml of 0.05 *M* TEA buffer) was applied to the column. The column was developed with a linear gradient of TEA buffer (0.1 to 0.4 *M*; each of 400 ml) except for adenylates which were eluted with 1.2 l from 0.1 to 0.6 *M*. The elution patterns of a series of adenylates, guanylates, cytidylates and uridylates are shown in Figs. 1, 2, 3 and 4, respectively. Recovery of all the samples examined was quantitative.

In the series of the purine nucleotides, the elution order is 5′-, 2′(3′)-, 2′:3′-cyclic and 3′:5′-cyclic monophosphates. On the other hand, the pyrimidine nucleotides are separated in the order: 5′-, 2′:3′-cyclic, 2′(3′)- and 3′:5′-cyclic monophosphates. There was a tendency for the pyrimidine nucleotides to be eluted faster than the purine nucleotides; in particularly all the adenylates required higher concentrations of TEA and more eluent as compared with the corresponding phosphate esters of the other three kinds of nucleotides. This might indicate that non-polar

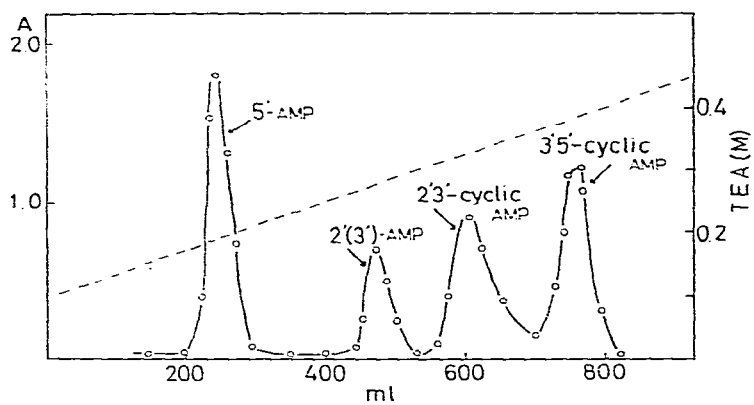


Fig. 1. Separation of adenylates on Amberlite XAD-4 resin. Experimental conditions: 2 μmoles of each compound were added to 6.6 g of pulverized resin. The column (diameter, 1.0 cm) was pretreated with 0.05 *M* TEA, and then developed with a linear gradient of 0.1 to 0.6 *M* TEA buffer (pH 7.6, each of 600 ml). Fractions of 10 ml were collected; flow-rate, 75 ml/h. Recovery, 96–104%. Absorbance was measured at 260 nm.

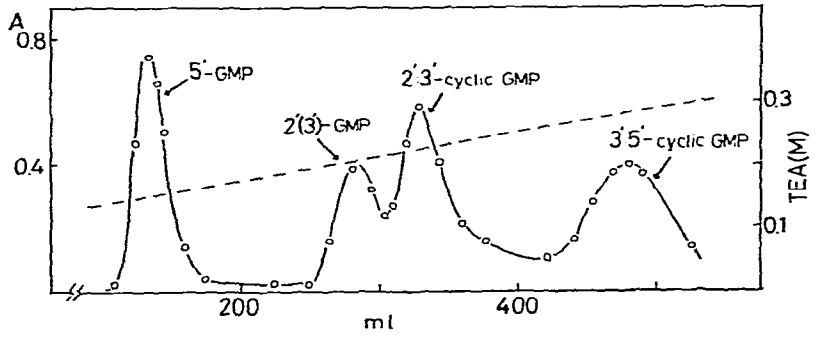


Fig. 2. Separation of guanylates on Amberlite XAD-4 resin. The column was developed with a linear gradient of TEA (800 ml from 0.1 to 0.4 *M*, pH 7.6). Other conditions as in Fig. 1.

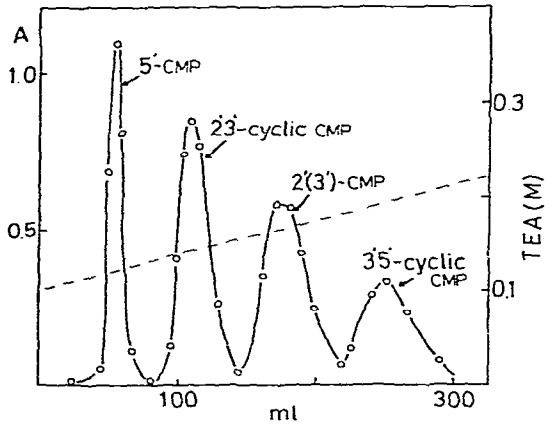
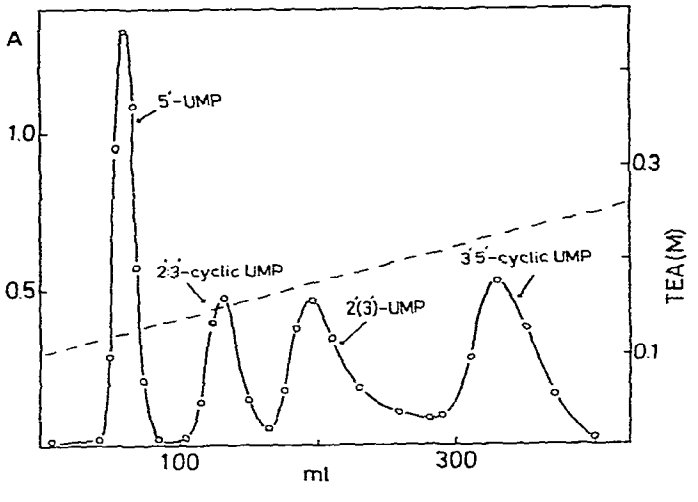


Fig. 3. Separation of cytidylates on Amberlite XAD-4 resin. Column conditions as in Fig. 2. Fractions of 5 ml were collected.



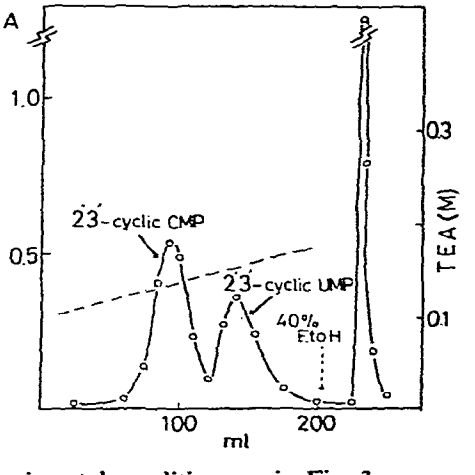


Fig. 4. Separation of uridylates on Amberlite XAD-4 resin. Experimental conditions as in Fig. 3.

Fig. 5. Separation of dialyzate of yeast RNA treated with ribonuclease A on Amberlite XAD-4 resin. Column conditions as in Fig. 3.

interaction between the purine rings of adenylates or guanylates and the divinylbenzene cross-linked polystyrene matrix contributes to the increased retention of these nucleotides in comparison with their pyrimidine counterparts. This observation agrees with the previous result[8] not only in a series of non-cyclic nucleotides but also in that of nucleosides and the bases. The present data indicate that the order of elution of each series of nucleotides is related to the location of the phosphate group on the ribose rather than to the number of dissociable hydrogen atoms in the phosphate functions. The fact that all the 3′:5′-cyclic nucleotides in each series showed the greatest adsorption might imply that these nucleotides have the most favoured configuration for the binding to the coated resin.

An example demonstrating the utility of this method is shown in Fig. 5. A mixture of yeast RNA (100 mg), ribonuclease A_2 (36 μg, 2 U) and 5 ml of 0.05 *M* TEA buffer, pH 7.6 in a cellophane tubing was dialyzed against 70 ml of water at 20° for 3 h with stirring [11]. The dialyzate was concentrated to dryness *in vacuo*. The residue was dissolved in 1 ml of 0.05 *M* TEA buffer and applied to a column. Only two peaks occurred in the range of TEA gradient elution, which were identified as 2′:3′-cyclic CMP and 2′:3′-cyclic UMP. The column showed the existence of additional materials eluted with 40% ethanol, which were probably oligonucleotides, judging from paper chromatographic behaviour before and after the alkaline hydrolysis.

Thus, this coated resin can be usefully applied to the separation of mixtures in cyclic nucleotide research.

REFERENCES

1 G. Schultz, E. Böhme and J. G. Hardman, *Methods Enzymol.*, 38 (1974) 9.
2 G. Booker, *Methods Enzymol.*, 38 (1974) 20.
3 A. A. White, *Methods Enzymol.*, 38 (1974) 41.
4 S. P. Dutta, A. Mittelman and G. B. Chheda, *Anal. Biochem.*, 75 (1976) 649.
5 H. Wombacher, *FEBS Lett.*, 85 (1978) 77.
6 J. X. Khym, *J. Chromatogr.*, 151 (1978) 421.
7 G. Brunius, *J. Chromatogr.*, 170 (1979) 486.
8 T. Uematsu and R. J. Suhadolnik, *J. Chromatogr.*, 123 (1976) 347.
9 R. H. Nieman, D. L. Pap and R. A. Clark, *J. Chromatogr.*, 161 (1978) 137.
10 R. K. Borden and M. Smith, *J. Org. Chem.*, 31 (1966) 3241.
11 R. Markham, *Methods Enzymol.*, 3 (1957) 806.