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Note

Isocratic reversed-phase liquid chromatographic separation of 3′,5′-cyclic ribonucleotides

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The importance of the naturally occurring 3′,5′-cyclic ribonucleotides is well known [1]. Of the methods used in cyclic nucleotide research, the liquid chromatographic techniques appear to be the most advantageous [2]. However, the time required to achieve separations of the five naturally occurring cyclic nucleotides with the previously published methods is too long for routine work [2—4]. Furthermore, a simultaneous separation of the cyclic nucleotides along with corresponding nucleosides, nculeotides and bases, which is essential in studies of cAMP and cGMP metabolism, has been difficult to achieve. Therefore, we developed conditions for the individual 3′,5′-cyclic ribonucleotides from a mixture of the five naturally occurring cyclic nucleotides, and for the simultaneous separation of cAMP and cGMP in a mixture of nucleosides, nucleotides and bases. The proposed method utilizes the reversed-phase mode of high-performance liquid chromatography and isocratic elution with a totally aqueous buffer eluent which facilitates automated procedures for repetitive analyses. In addition, this system alleviates the need for gradient elution which can hamper the detection of compounds at very low levels due to baseline fluctuations.

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EXPERIMENTAL

The chromatographic system was a DuPont 8820 (DuPont Instruments, Wilmington, DE, U.S.A.) equipped with a controlled-temperature column compartment, 254-nm UV detector, HP 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) and strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). The column (250 × 4.6 mm I.D.) was packed with 6-μm spherical octadecyl silica (Zorbax-ODS, DuPont Instruments) and was heated to 35°C.

The eluent was 4.0 m*M* $(NH_4)_2HPO_4$ and 4.0 m*M* $(NH_4)H_2PO_4$, pH 3.0 (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Standards were obtained from Sigma (St. Louis, MO, U.S.A.) and were dissolved in the mobile phase at ca. 100 μM.

RESULTS AND DISCUSSION

The liquid chromatographic separation of the five naturally occurring 3',5'-cyclic ribonucleotides has been reported using isocratic or gradient elution [2—4]. Using our conditions, excellent resolution of these compounds was obtained in 10 min (Fig. 1). The sensitivity of this system is in the 5—10-pmol range. Sample-to-sample reproducibility was excellent and there was no appreciable loss of efficiency or resolution due to the use of relatively high flow-rate. In addition, the reproducibility between different batches of the Zorbax-ODS columns was good.

Since the cyclic nucleotides are often present in extremely low concentrations compared to the levels of the structurally similar nucleosides, nucleotides and bases, a chromatographic system is needed which will separate the cyclic

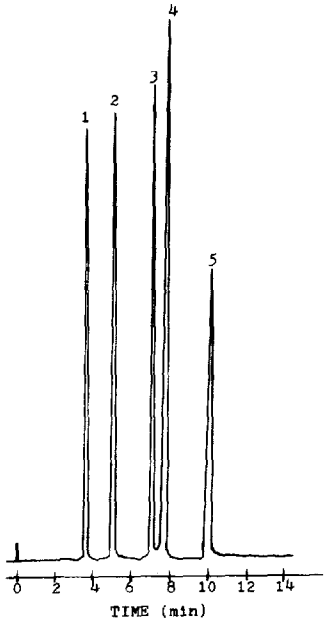

Fig. 1. Isocratic separation of the five naturally occurring 3',5'-cyclic ribonucleotides. Flow-rate: 2.5 ml/min; other conditions as given in text. Peaks: 1 = cCMP, 2 = cUMP, 3 = cGMP, 4 = cIMP and 5 = cAMP.

nucleotides from their congeners. In addition, studies of cAMP (or cGMP) metabolism require the determination of the cyclic nucleotide with its metabolites [5]. Using a flow-rate of 1.5 ml/min (all other conditions remaining the same), a simultaneous separation of the cyclic nucleotides, cAMP and cGMP, and their nucleosides, nucleotides and bases was achieved (Fig. 2). The time required for the separation of the thirteen purine compounds is under 20 min. Resolution of the slightly retained compounds was improved by operating at ambient temperature, however analysis time is increased and retention times were not as reproducible.

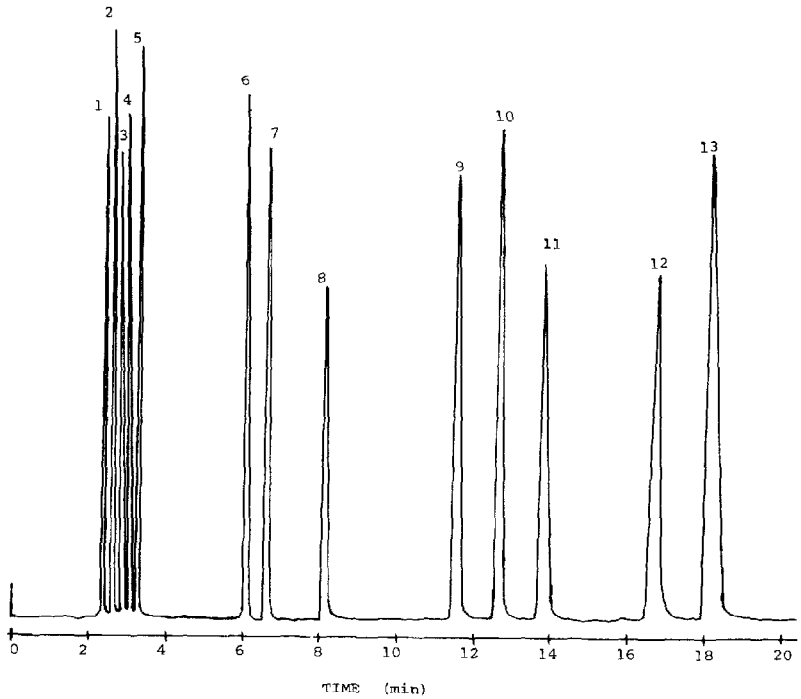

Fig. 2. Isocratic separation of cAMP and cGMP, and associated nucleosides, nucleotides and bases. Conditions are the same as in Fig. 1, except flow-rate is 1.5 ml/min. Peaks: 1 = ATP (GTP), 2 = GDP, 3 = ADP, 4 = GMP, 5 = AMP, 6 = Gua, 7 = Hyp, 8 = Ade, 9 = cGMP, 10 = Guo, 11 = Ino, 12 = cAMP and 13 = Ado.

An isocratic system employing a simple aqueous eluent is ideal for routine work in biomedical research or the clinical laboratory. The separations shown here can be used to determine the concentrations of cAMP and cGMP in cellular or tissue extracts, as well as the activities of the enzymes in the metabolic pathways [5, 6].

REFERENCES

1 T. Bartfai, Trends Biochem. Sci., 3 (1978) 121.
2 A.M. Krstulovic, R.A. Hartwick and P.R. Brown, Clin. Chem., 25 (1979) 235.
3 J.X. Khym, J. Chromatogr., 151 (1978) 421.
4 S.P. Dutta, A. Mittelman and G.B. Chheda, Anal. Bicohem., 75 (1976) 409.
5 H. Kizaki and T. Sakurada, J. Chromatogr., 211 (1981) 409.
6 Z. Schneider, D. Leszczyńska and J. Socha, J. Chromatogr., 229 (1982) 77.