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Note

Rapid analytical separation of the 3′,5′-cyclic ribonucleotides on a conventional anion exchanger with mixed counter-ion elution

J. X. KHYM

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830 (U.S.A.)

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Now that it has been established that there are at least five naturally occurring 3′,5′-cyclic ribonucleotides[1–6], refinements in cyclic nucleotide methodology are needed to distinguish one from another in mixtures. Previously published chromatographic methods for assaying the cyclic nucleotides (for concise summaries of these procedures see refs. 7–10) pertain mainly to the separation of the then known simple binary mixtures (*e.g.*, cAMP from cGMP); however, some of these probably could be modified to handle more complex mixtures.

Rapid separation and ease of quantitation appear to be the advantages of high-performance columnar methods as compared to other methods used in cyclic-nucleotide research. Brooker[8,11–13] was the first to demonstrate this when he used sophisticated columnar methods to separate mixtures of cAMP and cGMP. With gradient elution, and using a similar system, Dutta *et al.*[6] separated the five known naturally occurring ribonucleoside 3′,5′-cyclic monophosphates. Both of the sesystems utilize pellicular anion exchangers. Although very efficient separations can be obtained, the pellicular packings are not without their drawbacks. Compared with conventional exchangers, the pellicular resins have capacities that are several hundredfold less, require more-specialized high-pressure equipment, and are more easily contaminated by impurities in eluent solutions. By modifications of the ion-exchange procedures[14–16] used in this laboratory to separate noncyclic nucleotides, the rapid analytical separation on Aminex A-27 of the five naturally known 3′,5′-cyclic ribonucleotides has been accomplished.

RESULTS

As shown in Fig. 1, the ribonucleoside 3′,5′-cyclic monophosphates are separated in about 40 min on a short column (5.2 × 0.62 cm) of Aminex A-27 at relatively low pressure (40 p.s.i.), with eluent composed of mixed counter-ions (*i.e.*, citrate and perchlorate). The presence of dilute perchlorate in the eluent is necessary so that the cyclic nucleotides are eluted in sharp, distinct peaks with short retention times. When perchlorate is absent, the cyclic nucleotides tend to be irreversibly sorbed to the citrate form of the Aminex exchanger[14]; the reason for this anomaly remains unsolved. The citrate–perchlorate form of Aminex A-27 used in the column (Fig. 1) can be obtained

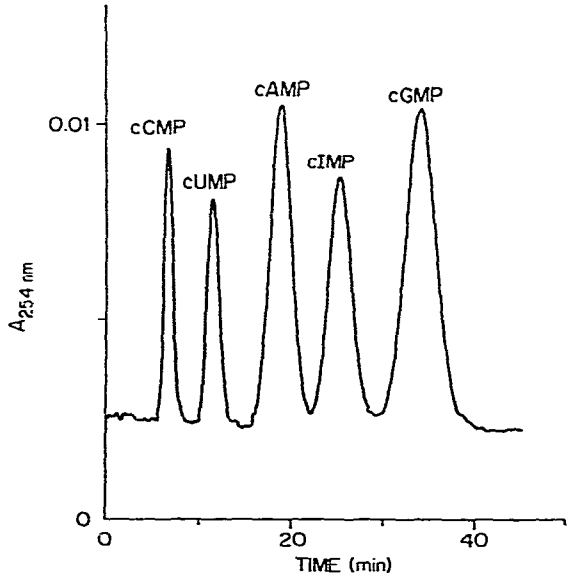

Fig. 1. Separation of the 3',5'-cyclic ribonucleotides was done on a 5.2 × 0.62-cm column of Aminex A-27 converted to the citrate–perchlorate form (see text). When freshly packed, and at start-up time each day, the column was equilibrated for *ca.* 2 h with eluent composed of 25 m*M* sodium citrate + 1 m*M* potassium perchlorate (pH 7.5), and also containing 0.3 m*M* NaN_3 to prevent bacterial growth; flow-rate was 0.7 ml/min; column temperature, 70°; operating pressure, 40 p.s.i. Sorbed material was approximately 1.0 nmole of each cyclic nucleotide contained in 5 μl of eluent. The sample was applied to the column by an off-column septum-type injector with a Hamilton syringe without interrupting the flow of eluent, recovery was quantitative and the column was monitored with a Model UA-5 ISCO UV analyzer (10-mm path-length flow-cell of 19-μl capacity).

by equilibrating, batchwise, either the citrate or the perchlorate form of the A-27 exchanger with the mixed eluent of the composition given in the figure legend.

Even though this is a mixed counter-ion system, it behaves well chromatographically. Retention times and other data can be reproduced from different columns prepared from different batches of the resin. Also, each cyclic nucleotide gives a linear response between peak height and sample load as measured in the nmole and μmole ranges. Identification of the elution peaks of Fig. 1 was made on the basis of their retention times as determined individually, and by the bandwidth constancy of the respective elution peaks as measured at one-half peak height[16].

The method reported here, in addition to all other chromatographic procedures utilized in cyclic nucleotide work, is essentially useless unless structurally similar components are removed prior to the determination of the cyclic nucleotides found in cellular extracts. Analytical difficulties are compounded by the fact that the cyclic nucleotides are present at extremely low concentrations compared with the higher levels of interfering components such as the noncyclic nucleotides and the nucleotide sugars. We have been investigating both chemical and enzymic methods to convert interfering noncyclic nucleotide compounds to components (*e.g.*, to nucleosides and/or bases) that are easily separated from the cyclic nucleotides. Preliminary accounts of this work have been reported[17,18].

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