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Note

Simultaneous assay of cyclic AMP and cyclic GMP phosphodiesterase activities by anion-exchange column chromatography

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Although assay methods are available for the determination of the activity of a cyclic nucleotide phosphodiesterase, based on the determination of nucleoside monophosphate produced[1–8] or on direct measurement of proton released by a titrimetric method[9,10], none is suitable for the simultaneous assay of cyclic AMP and cyclic GMP phosphodiesterase activities. We have demonstrated[11,12] that AMP and cyclic AMP are easily separated by anion-exchange column chromatography using AG MP-1 resin. By a simple modification of the elution mode, the same column can be used to resolve GMP from cyclic GMP, AMP and cyclic AMP. This method is conveniently used to determine the activities of cyclic AMP and cyclic GMP phosphodiesterase(s) individually or simultaneously.

MATERIALS AND METHODS

Materials

AG MP-1 resin was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.); and the nucleotides from Sigma (St. Louis, MO, U.S.A.).

Enzyme preparation

A 1-g amount of rat brain was homogenized in 2 ml distilled water. The homogenate was centrifuged at 10,000 *g* for 30 min. The enzyme in the supernatant solution was precipitated by the addition of an equal volume of cold saturated ammonium sulfate. The precipitate was collected by centrifugation, and dissolved in 2 ml of 50 m*M* glycylglycine, pH 7.4.

Reaction mixtures

The reaction mixture in a final volume of 1 ml contained 50 m*M* glycylglycine (pH 7.4); 5 m*M* $MgCl_2$; 20–200 μM of cyclic AMP or cyclic GMP, or both; and the enzyme preparation. The reaction was started by the addition of the enzyme preparation, and after incubating the mixture at 37°C for 10 min, terminated by heating the tubes in a boiling-water bath for 5 min. After the solution was cooled in an ice bath, any precipitate was removed by centrifugation, and an aliquot was analyzed by column chromatography. A blank was prepared under similar conditions except that

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the enzyme preparation was added to the mixture while the tube was heated in the boiling-water bath.

Column chromatography

A column packed with AG MP-1 resin, 50 × 11 mm, was used. After an aliquot of the above reaction mixture was loaded, the column was stepwisely eluted with water, 0.0096 *M* hydrochloric acid, then 0.12 *M* hydrochloric acid. The effluent was continuously monitored at 257 nm, and each peak was quantitated according to the equation described previously[11]. The extinction coefficients are $15 \cdot 10^3$ mol^{-1} cm^{-1} for AMP and cyclic AMP; $13.2 \cdot 10^3$ mol^{-1} cm^{-1} for guanosine; and $12.2 \cdot 10^3$ mol^{-1} cm^{-1} for GMP and cyclic GMP.

RESULTS

Individual determination of cyclic AMP or cyclic GMP phosphodiesterase

Column chromatography of the reaction mixture containing 146 nmoles cyclic AMP and 10 µl of the enzyme preparation showed that a total of 33.2 nmoles of cyclic AMP were hydrolyzed in 10 min. Thus, 8.1 nmoles adenosine, produced due to the presence of 5′-nucleotidase activity in the enzyme preparation, were eluted with distilled water as a small peak near the void volume, and 25.2 nmoles of AMP were resolved from cyclic AMP by eluting with 0.0096 *M* hydrochloric acid.

When 164 nmoles of cyclic GMP were used as substrate instead of cyclic AMP, the chromatogram showed that a total of 30.4 nmoles of guanosine and GMP were produced. The former was eluted with distilled water as a small peak next to adenosine peak, while the latter was resolved from cyclic GMP in the 0.12 *M* hydrochloric acid fraction.

Simultaneous determination of cyclic AMP and GMP phosphodiesterase activities

The chromatogram of the reaction mixture containing 146 nmoles cyclic AMP and 164 nmoles cyclic GMP is shown in Fig. 1. All substrates and products were well separated by the elution mode indicated. From the figure the activities are calculated to be 12.6 nmoles of AMP and 18.6 nmoles of GMP produced in 10 min per 10 µl of the enzyme preparation.

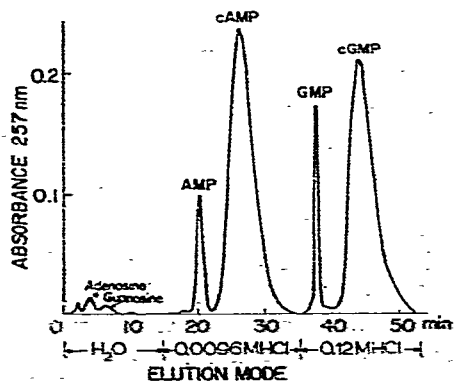
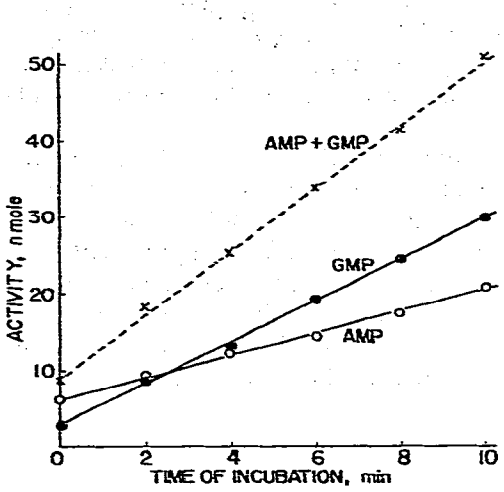


Fig. 1. Chromatogram of a reaction mixture containing cyclic AMP and cyclic GMP. The reaction mixture containing 146 nmoles/ml cyclic AMP and 164 nmoles/ml cyclic GMP was incubated with 10 µl/ml of the enzyme preparation for 10 min. A 200-µl aliquot was chromatographed.

Phosphodiesterase activities of rat brain enzyme

The rat enzyme catalyzed the hydrolysis of cyclic GMP as well as cyclic AMP. Both activities were also linearly related to the enzyme concentration.

In the presence of a constant concentration of the enzyme preparation, the phosphosdiesterase activities were linearly proportional to the time of incubation. When the activities on cyclic AMP and cyclic GMP were determined individually, the enzyme tended to hydrolyze preferably cyclic AMP to cyclic GMP. When the activities on cyclic AMP and cyclic GMP were determined simultaneously as shown in Fig. 2, the combined activity was equivalent to the cyclic AMP phosphodiesterase activity determined individually, and the individual activity was linearly proportional to the time of incubation. However, in contrast to the result of individual determination, the cyclic GMP phosphodiesterase activity was higher than the cyclic AMP phosphodiesterase activity.



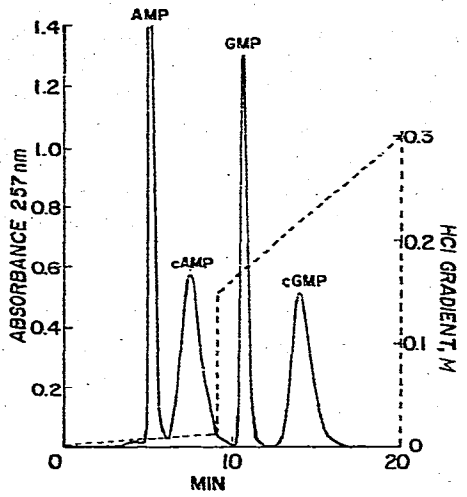


Fig. 2. Activities of cyclic AMP and cyclic GMP phosphodiesterasese determined simultaneously *versus* incubation time. The summation of the two activities is shown by the broken line.

Fig. 3. Separation of the nucleotides by gradient elution. A mixture containing 100 nmoles each of AMP, cyclic AMP, GMP and cyclic GMP was loaded on the column, and the separation was accomplished by a hydrochloric acid gradient as shown by the broken line.

Modification of elution mode to facilitate column chromatography

The time for the column chromatographic separation required 50 min using the stepwise elution. It could be shortened to 20 min by using gradient elution as shown in Fig. 3. The hydrochloric acid gradient indicated on the figure was established by using a Gilson Mixograd gradient former from two solutions, distilled water and 0.3 *M* hydrochloric acid.

DISCUSSION

With the method described both the products and the substrates can be determined, as well as the activities of cyclic AMP and cyclic GMP phosphodiesterase(s) individually or simultaneously. Since the AMP and GMP peaks are sharper

than those of their respective substrates, a concentration as low as 100 pmoles can be easily measured. Therefore, the sensitivity of this method is compatible to other methods which have been reviewed by Frielle *et al.*[10], except the method utilizing radioisotope substrates.

The method also detects the presence of 5′-nucleotidase which catalyzes the hydrolysis of AMP and GMP. Since adenosine and guanosine are eluted separately by water, the whole chromatographic procedure can be simplified by merely measuring the nucleosides as the final products if excess amount of 5′-nucleotidase is added to the reaction mixture to complete the hydrolysis of AMP and GMP. Thus, this not only facilitates the assay procedure but also can further tailor it to a most sensitive method utilizing radioisotope substrates.

The presence of deaminases, however, will interfere with the experimental results. Since under the conditions used, inosine will co-elute with AMP, and IMP with GMP, the presence of deamines will result in lower activity for cyclic AMP phosphodiesterase but in a higher value for cyclic GMP phosphodiesterase activity. In order to test the possibility of this interference, separation of inosine from AMP, and IMP from GMP can be accomplished by a proper modification of the elution mode according to the principle reported previously[12].

The total time for the chromatography requires 50 min to complete the separation of the four peaks. However, the time can be shortened in two ways: One is to eliminate the steps of elution with water and elution of cyclic GMP. Thus, it takes only 20 min to eluted out the GMP peaks, thereafter the column is washed with 1 *M* hydrochloric acid for a few min and then with water for 5 min for reuse. The other is to use a gradient method as shown in Fig. 3. Complete separation of the four peaks requires less than 20 min. After the run, the column is washed with water for 5 min and another sample is applied. The latter procedure can be easily automated for a continuous run.

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