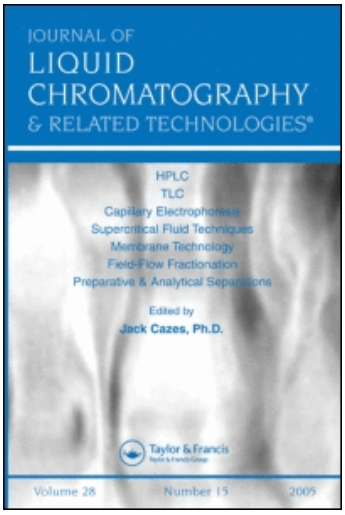


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# Separation of Cyclic GMP AND Cyclic AMP from Other Nucleotides by Reverse Phase Hplc

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# SEPARATION OF CYCLIC GMP AND CYCLIC AMP FROM OTHER NUCLEOTIDES BY REVERSE PHASE HPLC

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## ABSTRACT

An accurate, specific and sensitive method to identify and quantify both cyclic GMP and cyclic AMP by HPLC is described and applied to standards mixtures and lichen extracts. Separation is achieved by reverse phase HPLC on a MCH-10 column by using methanol:water:acetic acid ( 80:19.5:0.5 v/v), isocratically, as mobile phase. Selectivity and resolution coefficients are always higher than 1.0. Cyclic nucleotides conveniently separate from GMP, AMP and adenosine. Recovery of cyclic GMP from biological samples has been estimated as about 93% after extraction procedure whereas that of cyclic AMP varies from 92.25% to 55.61% depending on phosphodiesterase activity.

By using this method, cyclic nucleotides have been identified from lichen species, *Himantormia lugubris* and *Usnea aurantiaco-atra*. Whereas cyclic AMP is the main cyclic nucleotide contained in the first species, cyclic GMP is the most abundant in the second one. In addition, apothecia of *Usnea* seem to contain an active phosphodiesterase which hydrolyzes mainly cyclic AMP.

## INTRODUCTION

Many physiological functions of both cyclic GMP and cyclic AMP have been studied in plants during the last years. Cyclic GMP stabilizes RNA by inhibiting specific nucleases[1] and regulates the function of ion channels through its binding to specific G proteins of *Lemna*[2]. The discovery of cholinergic-like systems during lichen morphogenesis implies cyclic GMP in G protein-mediated responses[3]. Cyclic AMP reverses catabolite repression of several enzymes in eukaryote plant cells, even that produced by urea[4], in contrast to that found for urea-sensitive operons in prokaryotes[5]. Cyclic AMP induces swelling of etiolated oat protoplasts[6] and facilitates diffusion of some organic anions across the plasma membrane[7]. In addition, the nucleotide produces eli-citation of phytoalexins in carrot cells[8] and activates protein kinases other than protein kinase C, which is specifically activated by diacylglycerol[9]. Hence the importance to investigate accurate and simple methods to identify and quantify these cyclic nucleotides in plants.

Traditionally, radioimmunoassay of 2'-***O***-acetyle-cyclic AMP derivatives is the most usual method to estimate cyclic AMP in both plant and animal extracts[10,11]. Sometimes, HPLC procedures have been described including sample pre-treatment and separation as complicated as it can be suspected considerable losses of metabolites during processing[12]. In addition, cyclic nucleotides have chromatographic characteristics of both nucleotides and nucleosides. On anion-exchange columns, their retention time is intermediate between their associated mono- and diphosphates[13]. For example, cyclic AMP elutes between ADP and ATP from an APS-Hypersil column using 80 mM $KH_2PO_4$, pH 2.8, as mobile phase.

In this paper, we describe a simple procedure to separate both cyclic GMP and cyclic AMP by reverse phase HPLC from standard mix-tures and from two different lichen extracts.

## EXPERIMENTAL

*Plant Material*

*Himantormia lugubris* ( Hue ) Lamb, collected on soil in King George Island ( Antarctica ) and *Usnea aurantiaco-atra* (Jacq.) Bory, growing on soil in Coppermine ( Antarctica ), were used as biological material. Samples were air-dried and stored in polythene bags, in the dark, at 5° C, until required. Samples of 0.5 g of air-dried thalli, or 0.1 g of *Usnea* apothecia, were rehydrated with distilled water for 4 min at room temperature before extraction.

*Preparation of Plant Extracts for HPLC Analysis*

After rehydration, thallus samples were washed with distilled water, gently dried with filter paper, and macerated in a mortar with 25 ml chloroform:acetonitrile ( 60:40 v/v ) for 15 min at room temperature to remove lichen phenolics[14] . Homogenates were filtered through Wahtman No 3 filter paper and solid residues were dried in air flow. Dry residues were macerated again with 4.0 ml distilled water and centrifuged at 27,000 x *g* for 30 min at 2° C. When indicated, 1.6 ug cyclic AMP or 64 ug of cyclic GMP or other standards were added to residues before water extraction. Supernatants were stored and precipitates were extracted again as above. Mixtures of both supernatants were precipitated with equal volume of pure acetonitrile. Precipitates were removed by centrifugation at 27,000 x *g* for 30 min at 2° C and supernatants dried in air flow.

Solid residues were resuspended in 2.0 ml pure methanol, filtered through Millipore GS filters ( 0.22 um pore diameter ), and injected onto the chromatographic column.

Alternatively, mixed supernatants were incubated at 37° C for 30 min with one unit of activator-insensitive phosphodiesterase 3':5'-cyclic nucleotide, from Sigma Chemical Co., before acetonitrile addition. From this step, extraction procedure was performed as above.

*Chromatographic Analysis*

Samples and standards were chromatographed by using a Spectra Physics SP8800 liquid chromatograph equipped with a SP 4290 computer. Chromatographic conditions were: reverse phase column ( 300 mm x 4 mm i.d. ) packed with MicroPak MCH-10, from Varian; loading, 10 ul; mobile phase, methanol:acetic acid:water ( 80:0.5:19.5 v/v ) isocratically; pressure, 101 bars; temperature, 20° C; flow rate, 1.0 ml $min^{-1}$; absorbance units at full scale, 0.005; detector, UV set at 254 nm.

*Standards*

Adenosine, cyclic AMP, cyclic GMP, GMP and AMP, from Sigma Chem. Co., were used as standards. To 2.0 mg of each one, 2.0 ml chloroform:acetonitrile ( 60:40 v/v ) were added. Suspensions were dried in air flow, resuspended in 2.0 ml distilled water on which 2.0 ml acetonitrile were added, and dried again. Dry residue was redissolved in 5.0 ml pure methanol, filtered through Millipore GS filters and injected onto the column.

## RESULTS

*HPLC Separation of Cyclic AMP and its Analogues*

A flow rate of 1.0 ml $min^{-1}$ has been revealed as adequate to separate cyclic and non cyclic nucleotides ( Fig. 1 ). Retention times for each one nucleotide, separately

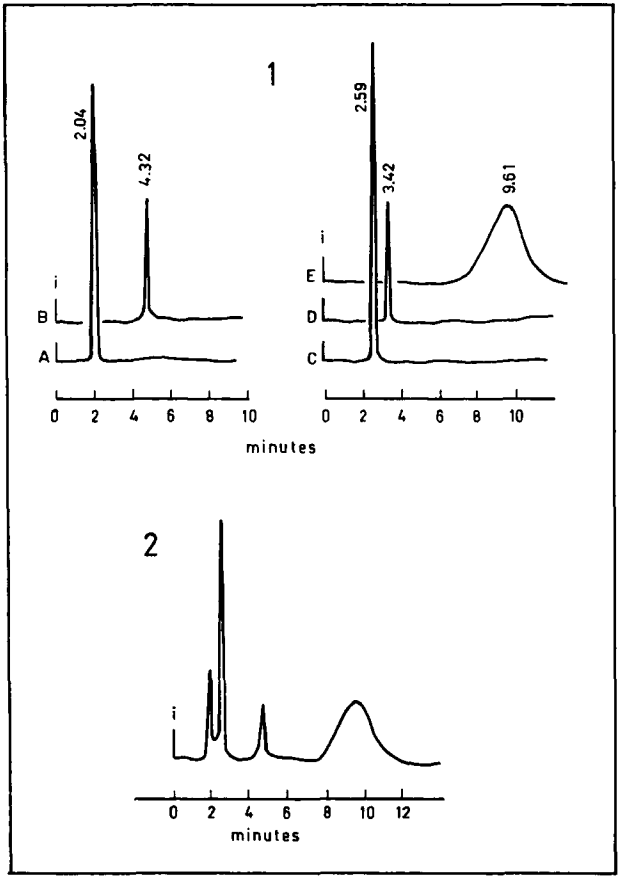


Figure 1. Chromatographic traces in HPLC for (1) single standards, where A) is cyclic GMP, B) GMP, C) cyclic AMP, D) adenosine, and E) AMP, or for (2) a equimolar mixture of cyclic GMP, cyclic AMP, adenosine and AMP. Number near the peaks indicate the retention time in minutes.

**TABLE 1.** Chromatographic parameters for the separation of standards

| Pair | Selectivity | Resolution |
|---|---|---|
| Cyclic AMP - Cyclic GMP | 1.27 | 1.10 |
| GMP - Cyclic AMP | 1.84 | 2.72 |
| AMP - GMP | 2.01 | 1.73 |

injected, exactly corresponded to that recorded after separation of a mixture containing all of them. Both selectivity and resolution coefficients were always higher than 1 for each pair of nucleotides, selected according to their elution order ( Table 1 ). Baseline correction was always applied. This was constructed from the start of the first peak ( internal standard ) to the lowest valley point at the end of the same peak and, in this way, after a baseline segment was constructed, the area of the peak was corrected. The result was stored in a time and area file and then, next baseline segment was calculated for the included peak. Each successive baseline segment started at the end of the preceding one, to include all the peaks[15]. Cyclic AMP quantitation, giving the highest response in the detector, showed to be linear in a range of concentrations from 0 to 1.0 ug of mass injected whereas cyclic GMP quantitation showed to be linear from 0 to 2.0 ug of mass injected ( Fig. 2 ).

Peaks with retention time identical to that of standard cyclic GMP, cyclic AMP and adenosine were obtained from *H. lugubris* thalli rehydrated in distilled water for 4 min in the dark ( Fig. 3A ). These peaks only increased when lichen samples were loaded, before aqueous extraction, with 1.6 ug cyclic AMP or 64 ug of standard cyclic GMP or adenosine ( Figs. 3B, C and D ). Anyway, cyclic AMP seemed to be the main free nucleotide in this lichen species.

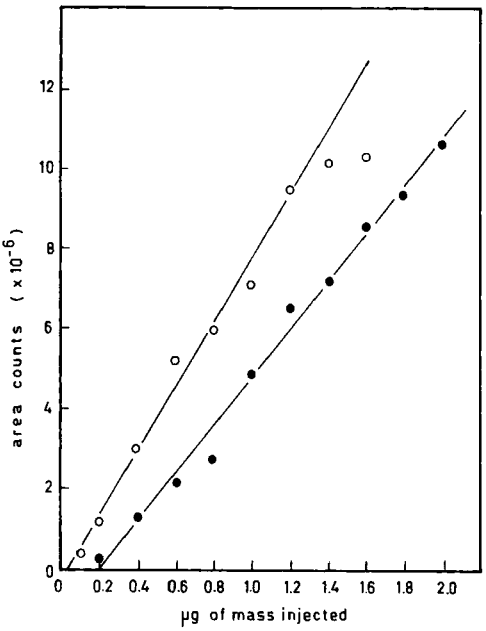


Figure 2. Direct calibration lines for both cyclic AMP ( ) and cyclic GMP ( ) where y = 8240x - 448; $r^2$ = 0.99, for cyclic AMP, and y = 5947x - 1201; $r^2$ = 0.99, for cyclic GMP.

In contrast, *U. aurantiaco-atra* mainly contained cyclic GMP and only traces of GMP, although the small amount of cyclic AMP could be correlated to the net recovering of both adenosine and AMP ( Fig. 4 ). Each peak selectively increased after loading thallus samples with the corresponding nucleotide or nucleoside.

*Yield of the Extraction Procedure and Accuracy and Specificity of the Method*

Aqueous homogenates of *U. aurantiaco-atra* thalli, after chloroform extraction, were supplied with 64 ug of cyclic GMP or 1.6 ug of cyclic AMP, chromatographically estimated, and the final extracts were analyzed by HPLC. Cyclic GMP quantitation indicated that the overall yield could be estimated as about 93.2% There is no quantitative increase of the peak corresponding to GMP after loading and no significant

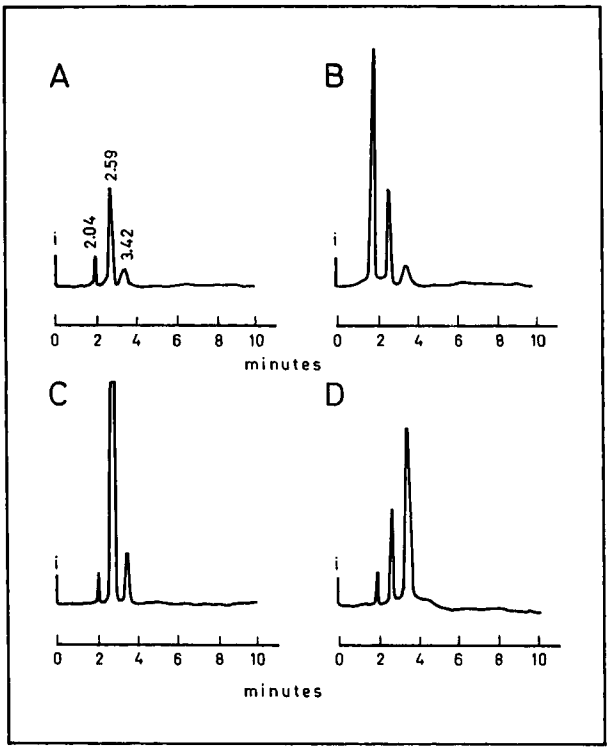


Figure 3. Chromatographic traces of nucleotide separation by using extracts of the lichen *H. lugubris* (A), loaded with cyclic GMP (B), cyclic AMP (C), or adenosine (D). Numbers near the peaks indicate retention time in minutes.

differences were observed by using apothecia instead of thallus samples. Cyclic AMP quantitation in thallus extracts indicated that the overall yield could be estimated as about 92.25% and about 7.75% of the total supplied was lost after acetonitrile precipitation and subsequent centrifugation, possibly bound to protein precipitate. However, only 55.61% of the loaded cyclic AMP was recovered from apothecia whereas the peak corresponding to adenosine increased about 38% over that obtained from unloaded tissues ( Table 2 ).

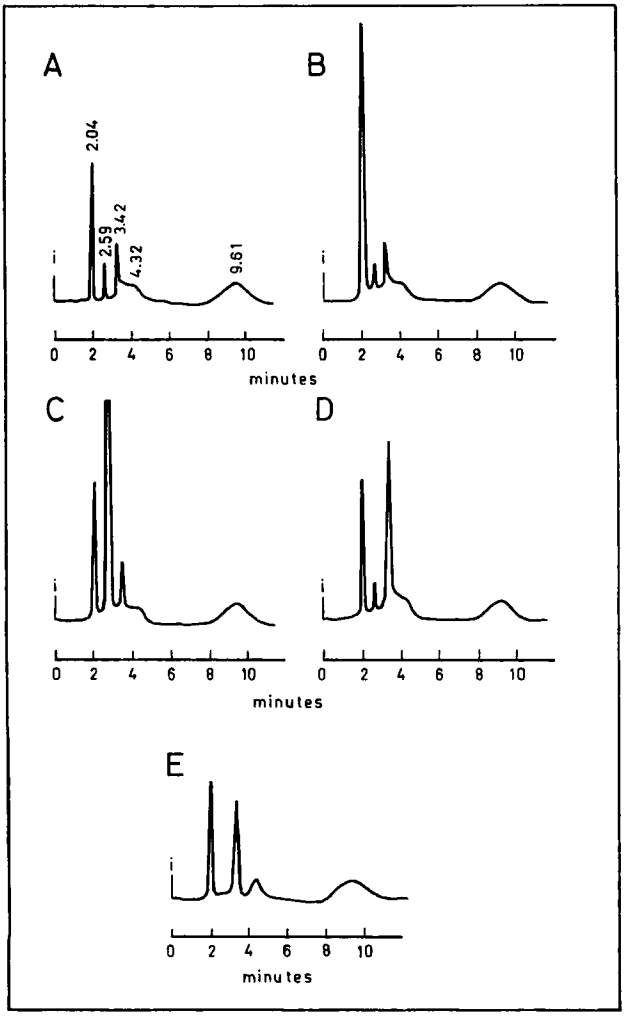


Figure 4. Chromatographic traces of nucleotide separation by using extracts of the lichen *U. aurantiaco-atra* (A), loaded with cyclic GMP (B), cyclic AMP (C), adenosine (D) or incubated, before acetonitrile precipitation, with cyclic phosphodiesterase. Numbers near the peaks indicate retention time in minutes.

**TABLE 2.** Recovery of standards after loading biological samples prepared from *Usnea aurantiaco-atra.*

| | Area counts* for | | | |
|---|---|---|---|---|
| | cyclic AMP | | cyclic GMP | |
| Sample | observed | expected | observed | expected |
| Unloaded thalli | 190871 | -------- | 414730 | -------- |
| Loaded thalli** | 1002381 | 1086551 | 906025 | 972130 |
| Unloaded apothecia | 85965 | -------- | 337602 | -------- |
| Loaded apothecia** | 545893 | 981645 | 814452 | 895002 |

*Values are the mean of three replicates. Standard error was no higher than 7%.

**Loading consisted of 8.0 ng cyclic AMP or 32 ng cyclic GMP in the injected volume.



The nature of peaks with retention times of 2.04 min and 2.59 min was confirmed by incubating aqueous extracts of lichen samples with cyclic phosphodiesterase for 1 h at 30° C. The reaction was stopped by adding acetonitrile to the mixtures and methanolic extracts were chromatographed. Only the peak previously identified as cyclic AMP completely disappeared and a peak with a retention time of 3.42 min, identified as adenosine, quantitatively increased, as it is shown in Fig. 4E. The peak eluted at 2.04 min was was slightly, but significantly decreased and a short peak at 4.32 min appeared.

## DISCUSSION

Although anion-exchange HPLC is well suited for separating cyclic nucleotides from standard mixtures[13], several attempts to use reverse phase HPLC have been described, specially when biological samples are the subject of analysis. Both cyclic GMP

and cyclic AMP were analyzed from rat brain tissue by using a simple isocratic separation on an ODS column and $KH_2PO_4$, pH 3.7, and methanol as mobile phase[16]. Retention time were estimated as about 20 min for cyclic GMP and 25 min for cyclic AMP and the corresponding peaks disappeared after incubation of extracts with cyclic phosphodiesterase. However, reaction products did not appear by this procedure in the chromatographic trace. Cyclic AMP has also been detected by reverse phase HPLC from incubation media where *Neurospora crassa* was growing by using a linear gradient from 0.1% (v/v) acetic acid to 0.1% (v/v) acetic acid containing 20% methanol on an C-18 Ultrasphere-ODS column[17]. Retention time of cyclic AMP was estimated as about 12 min, that diminished to 6 min when cyclic AMP was eluted isocratically from *N. crassa* extracts.



According to our results, extraction procedure drastically diminishes losses impossed by other more complicated methods[12,17] and cyclic nucleotides are eluted in 3 min, before the corresponding nucleotides ( Table II and Fig. 1 ). Separation between cyclic GMP and cyclic AMP is good, even from biological samples ( Figs. 3 and 4 ), since selectivity is higher than 1. Resolution coefficient can be improved by decreasing flow rate to 0.5-0.6 ml $min^{-1}$, but the gain in the separation ( $R_s$ = 1.3 ) does not compensate the increase in the retention time for both cyclic nucleotides. Linearity of the relatioship dose-reponse ( Fig. 2 ) is in the range of cyclic nucleotide concentrations found in lichen systems[7,18] and, in this way, the method described here seems to be adequate to be applied to biochemical and physiological studies involving cyclic nucleotides. On the other hand, the use of reverse-phase column avoids the baseline rise that, often, derives from the use of phosphate buffers as mobile phase[19].

Identification of cyclic AMP by the disappearance of its chromatographic peak after incubation of the extract with commercial cyclic phosphodiesterase ( Fig. 4 ) implies that the enzyme contains an associated phosphatase which produces adenosine from AMP. This has also been described for many purified phosphodiesterases[20] for which separation of phosphatase activity is a very difficult problem. Any way, cyclic GMP seems to be a very poor substrate for this commercial enzyme, since it is hydrolyzed in a very little extent and, in addition, associated phosphatase is not able to produce guanosine from GMP ( Fig. 4 ).



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