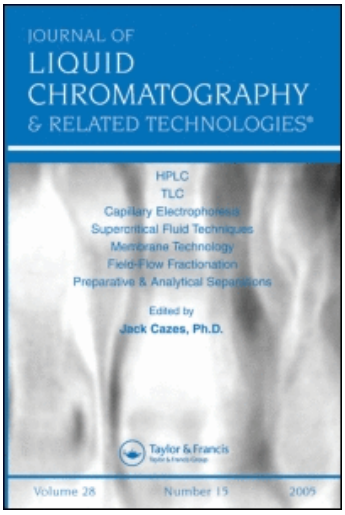

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Analysis of Guanylate Cyclase Activity by High-Pressure Liquid Chromatography

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To cite this Article Mehta, Shashikant , Dunlap, Diane , Lapeyre, Jean-Numa and Maizel, Abby(1980) 'Analysis of Guanylate Cyclase Activity by High-Pressure Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 3: 10, 1517 — 1535
To link to this Article: DOI: 10.1080/01483918008062793
URL: http://dx.doi.org/10.1080/01483918008062793

JOURNAL OF LIQUID CHROMATOGRAPHY, 3(10), 1517-1535 (1980)

ANALYSIS OF GUANYLATE CYCLASE ACTIVITY BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for the assay of guanylate cyclase which permits the correction of concurrent phosphatase and phosphodiesterase reactions has been developed using HPLC. The method, based on the conversion of tritium labelled guanosine triphosphate to tritium labelled cyclic guanosine monophosphate, uses [^{14}C]-cGMP as the internal standard to account for the degradative and procedural losses. Radiolabelled reaction products are isolated by high pressure liquid chromatography on a Partisil SAX column with a single step isocratic elution using 12.5 mM potassium phosphate buffer (pH 3.25). Since column recovery of the nucleotides is virtually quantitative and complete purification is achieved, the method possesses a high degree of accuracy and precision.

INTRODUCTION

Several methods have been developed to measure the activity of guanylate cyclase from mammalian tissue (1-9). The methodologies are usually based on the quantitation of the enzyme end product, cGMP, following incubation of the crude or semi-purified enzyme with labelled GTP as substrate. The isolation and purification of the cGMP product has been accomplished by a variety of

chromatographic methods including ion-exchange resins followed by alumina chromatography and assorted paper chromatographic or thin layer chromatographic systems (for review see 10). The separated cGMP is subsequently recovered and quantified by scintillation counting of the radiolabelled product. The above mentioned techniques involve the use of several separate methodological steps to effectively separate cGMP from other guanine-derived nucleotides.

Rapid and highly specific purification of nucleotides by HPLC can be achieved by several current methods (11-19). The presently developed assay uses a [^{14}C]-cGMP internal standard in the reaction mix to specifically measure the turnover of cGMP that is formed de novo from a tritiated precursor. The guanine-containing nucleotides were isolated by a modification of HPLC protocol developed by Hartwick and Brown (11) for the separation of nucleotides in biological samples. Conditions for the separation of the compounds of interest were optimized so that the elutions could be performed isocratically at room temperature.

MATERIALS AND METHODS

Crude Enzyme Preparation

Guanylate cyclase was assayed using a cytoplasmic fraction prepared from tissue derived from 150-200 g Timco rats. Following cervical dislocation, rat spleen or liver was extirpated and immediately placed at 4°C. All subsequent procedures were performed at 0-4°C. The tissue was finely minced in 0.15 M NaCl, 10 mM Tris-HCl (pH 7.8) and twice washed in this buffer. The tissue was

homogenized (0.5 g wet tissue in 10 ml homogenization medium) with several strokes of a Teflon-glass homogenizer in 10 mM Tris-HCl, pH 7.8. Cell disruption was monitored by light microscopy following staining with 0.1% solution of toluidine blue. The resultant broken cell suspensions was spun at 2000 rpm in a Beckman J21C centrifuge to sediment the nuclei. The supernatant solution was then spun in a Beckman 50 Ti rotor at 105,000 x g for one hour. The post-ribosomal supernatant from the ultracentrifugation was used as the source of soluble guanylate cyclase in the assay.

cGMP Assay Conditions

The incubation mixture for the assay of guanylate cyclase was a modification of the procedure of Krishnan and Krishna (7). The assay mixture contained 3.3 mM $MnSO_4$, 10 mM theophylline, 1 mM cold cGMP containing 8.3 nCi[^{14}C]-cGMP (55 mCi/mmol), 40 mM Tris-HCl, pH 7.4 and 0-60 µg of cytosol protein. The reaction was initiated by the addition of GTP (250 µM) which contained [^{3}H]-GTP to a final specific activity of 110 cpm/pmole. The GTP concentration thus formulated was five times the reported apparent Km value for guanylate cyclase (7). The reactions were maintained at 37°C for 0-20 minutes, then terminated by cooling to 0°C and adding perchloric acid (PCA) to a final concentration of 0.15 N PCA. The mixture was subsequently centrifuged in a Beckman microcentrifuge for one minute and 10 µl aliquots of the acid soluble supernatant used for subsequent quantitation by high-pressure liquid chromatography (HPLC).

High-Pressure Liquid Chromatography

The HPLC system used was a modified System 1 from Glenco, Inc. (Houston, TX) in which the ultraviolet (U.V.) monitoring system was from Pharmacia, Inc. (Upsala, Sweden). The HPLC procedure utilized was a modification of the original procedures of Hartwick and Brown (11). The acid soluble material derived from the assay mixture after PCA precipitation was injected onto a 25 x 0.4 cm Whatman Partisil 10/25 SAX column (strong anion exchanger). The samples were eluted isocratically at a flow rate of 0.8 ml/min (500-550 PSI) at room temperature with 12.5 mM potassium phosphate buffer (pH 3.25). Elution was monitored by U.V. absorption of the column effluent at 254 nm. Nucleotide di- and triphosphates as well as oligonucleotides not eluted by the low ionic strength buffer were eluted from the column with 1.0 M potassium phosphate buffer (pH 3.25). This step was included to recycle the column after multiple assay runs. The nucleoside and nucleotide peaks of interest were collected directly into scintillation vials and counted in a Beckman LS8100 liquid scintillation counter using ACS cocktail (Amersham). The [^{14}C]-3',5'-cGMP included in the reaction mix served as an internal standard for cGMP elution and to correct for procedural and metabolic losses of newly synthesized cGMP.

Thin Layer Chromatography

Thin layer chromatography on PEI cellulose of cGMP and other guanine derived nucleotides was performed as described previously (20).

Materials

Guanosine, GTP, GDP, 5'-GMP and 3,5'-cGMP were purchased from Sigma; [^{3}H]-GTP (16.7 Ci/mmol), [^{3}H]-cGMP (8 Ci/mmol), [^{14}C]-GTP (55 mCi/mmol) and [^{14}C]-cGMP (55 mCi/mmol) were purchased from Amersham. Pre-coated plastic backed polygram CEL 300 PEI plates were purchased from Brinkman Instruments. All other chemicals were of reagent quality or better.

RESULTS

HPLC Separation of Guanine Compounds

A representative chromatographic separation of guanosine and assorted guanine nucleotides using a Partisil SAX column may be seen in Figure 1. The column was eluted isocratically with 12.5 mM potassium phosphate buffer (pH 3.25), at 0.8 ml/min. After elution of 5'-GMP, the buffer was changed to 1.0 M potassium phosphate for the subsequent elution of GDP and GTP. Assay samples were routinely processed one after the other with the column equilibrated only with 12.5 mM potassium phosphate buffer (pH. 3.25). Each sample was run until 5'-GMP was eluted (approximately 40 min.) after which time another sample could be injected. Under these conditions, GDP and GTP were bound to the strong anion exchange matrix and did not interfere with subsequent analyses. After multiple assays had been completed, GDP and GTP were removed with 1.0 M potassium phosphate elution (pH 3.25) and the column subsequently re-equilibrated with 12.5 mM buffer.

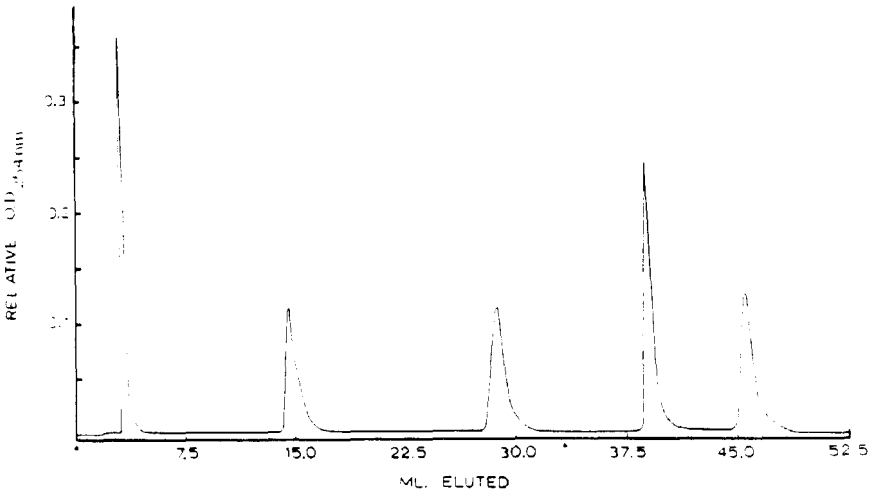

Figure 1.

Elution profile of guanine-derived compounds on Partisil SAX: Standards were solubilized in 0.15 N PCA, injected onto the column and initially eluted with 12.5 mM potassium phosphate buffer. At the indicated point (arrow), the buffer was changed to 1.0 M. phosphate. The peaks represent, in order of elution, guanosine, cGMP, 5'-GMP, GDP and GTP, respectively.

The reproducibility of nucleotide elution on Partisil SAX was assessed using multiple independent samples (Table 1). All samples were solubilized in 0.15 N PCA prior to injection to reproduce the standard assay conditions. The reported relative standard errors of mean of the determinations were less than 2.5% indicative of the precision of the separation achieved. Furthermore, complete separation of guanine, cGMP, 5'-GMP, GDP and GTP was achieved with no overlap between the compound detectable by either optical density or radiochemical measurements.

TABLE 1

HPLC of Guanine-Derived Compounds.

	Elution[1]	
	Time (Minutes)	Volume (ml)
Guanosine[2]	4.3 ± 0.10	3.3 ± 0.10
Cyclic-GMP	19.1 ± 0.05	14.9 ± 0.05
5'-GMP	37.9 ± 0.10	29.5 ± 0.10
GDP	50.9 ± 0.05	39.7 ± 0.05
GTP	59.8 ± 0.05	46.6 ± 0.05

[1]The column was run initially with 12.5 mM potassium phosphate buffer, pH 3.25 at a flow rate of 0.8 ml/min. Routinely, 3.5 min. following the elution of 5'-GMP, the buffer was changed to 1.0 M phosphate buffer. The time refers to the total continuous time of chromatography and the volume refers to total fluid volume passed through the column.

[2]The results represent the mean ± S.E.M. of five independent chromatographic separations. Samples were prepared for chromatography in 0.15 N PCA to reproduce routine assay conditions.

Guanylate Cyclase Assay Characteristics

Utilizing cytosol derived from rat liver or spleen, the activity of guanylate cyclase was assayed as described in Materials and Methods. In Figure 2, the rate of formation of cGMP was determined with a constant amount of cytosol from rat spleen. The time course of cGMP formation was linear up to 20 min., demonstrating a constant proportionality between the rate of cGMP formation and the amount of incubated enzyme.

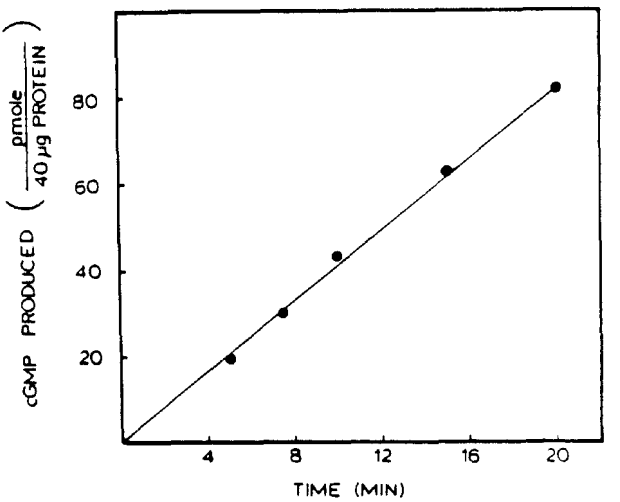

Figure 2.

Time course of cGMP formation: 40 µg of cytosol from rat spleen was incubated, according to Materials and Methods, for variable periods of time as indicated. Following incubation, samples were precipitated with perchloric acid, injected onto the column and the cGMP fraction collected. The data represents the amount of cGMP formed, per 40 µg cytosol protein, in the specified time intervals.

Therefore, a 20 min. time point was chosen to maximize the quantity of cGMP produced during the assay.

In order to further maximize the sensitivity without compromising the accuracy of the assay, the effect of the quantity of acid soluble reaction products on column performance and recovery of cGMP was examined. Cytosol was incubated under the assay conditions previously described and aliquots containing increasing amounts of the acid-soluble reaction products obtained after PCA precipitation were injected on the column. Figure 3 shows that the quantity of eluted cGMP

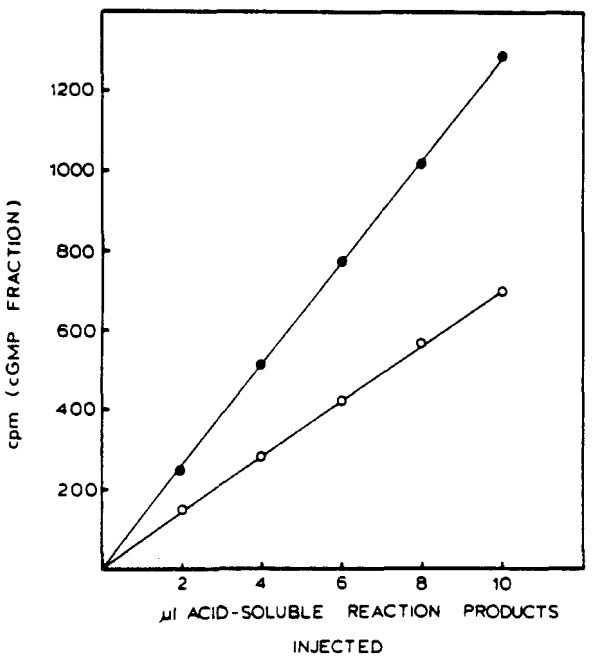

Figure 3.

Column recovery of cGMP: 40 µg of splenic cytosol protein was incubated as in standard assay conditions. The reaction was terminated by the addition of perchloric acid to a final concentration of 0.15 N PCA. The acid soluble material was aliquoted into samples of increasing volume and subsequently injected onto the column. The cGMP peak was collected and cpm determined in the [^{3}H] and [^{14}C] populations. [^{3}H]-GTP was used as the cGMP precursor and [^{14}C]-cGMP was used as the internal standard. ●-● [^{3}H] counts in the cGMP fraction; O-O [^{14}C] counts in the cGMP fraction. All samples were corrected for background activity.

was directly proportional to the quantity of injected reaction product throughout the range of volume encountered in the assay. Consequently, the injected volume of acid soluble

supernatant was not critical and could be varied to accommodate samples having low levels of activity. Routinely, 10 µl of the reaction acid-soluble supernatant was sufficient to accurately measure cGMP levels and guanylate cyclase activity.

Proportionality between cGMP formation and amount of incubated cytosol protein in the assay was next examined. This was done to determine whether the ratio of cGMP formation to incubated protein was linear over the range of protein concentrations encountered in the assay. Figure 4 shows that linearity was obtained up to 60 µg of cytosol protein.

Column Recovery of cGMP

Measurement of guanylate cyclase in biological preparations is hampered by the competing reactions lowering the GTP concentrations by phosphatase and cGMP concentration by phosphodiesterase In addition to metabolic losses, procedural losses inherent to the methodology must be accounted for. In order to determine the magnitude of these losses, the column recovery of purified cGMP on Partisil SAX was subsequently determined. [^{14}C]-cGMP was purified by thin-layer chromatography on PEI cellulose (20); and, a known amount was injected onto a Partisil column. The recovery of the cGMP from the column was seen to be greater than 98%. Thus, in the assay, to account for any biological losses, each sample always contained known amounts of [^{14}C]-cGMP as a marker and sub-

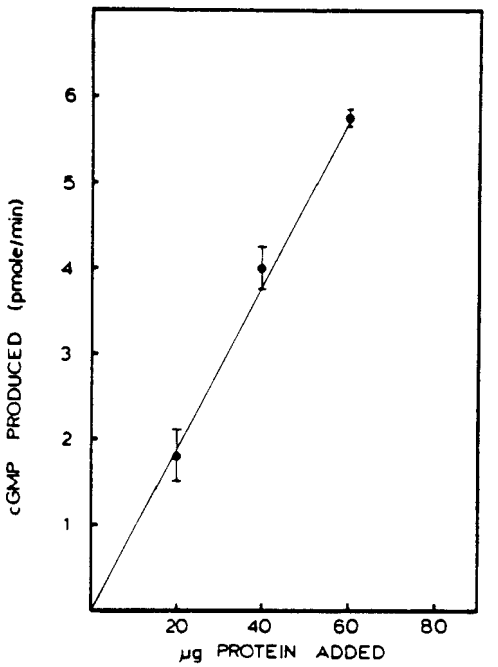

Figure 4.

cGMP production as a function of amount of protein incubated: Amount of cGMP formed, under routine assay conditions, was determined as a function of increasing concentrations of cytosolic protein as indicated in the figure. The results represent the mean ± S.E.M. of four independent cytosol preparations.

strate to measure cGMP conversion and/or breakdown. Control incubations which contained [^{14}C]-cGMP and either no enzyme protein or heat denatured protein (100°C for 2 min.) were run to compensate for background incorporation and to serve as a standard for degradative loss determinations.

Figure 5 shows a typical chromatographic profile of the reaction products formed with spleen cytosol. In panel A, measurement

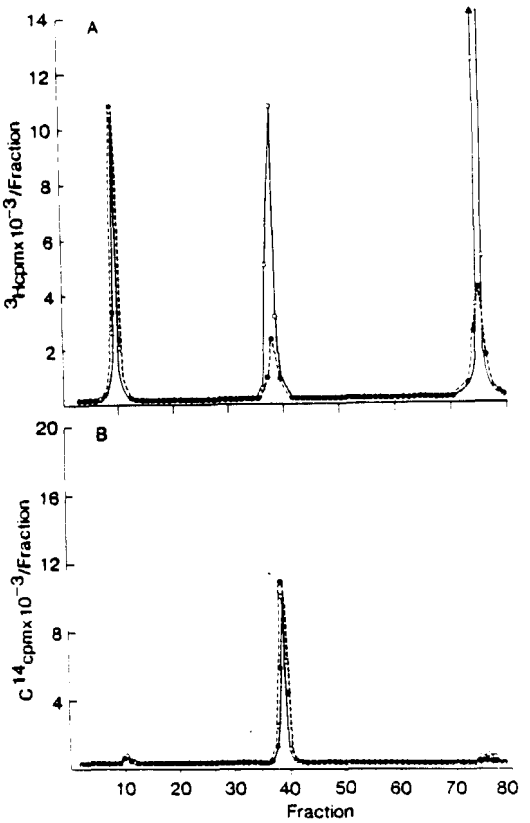

Figure 5.

HPLC profile of guanylate cyclase assay products: Panel A, a profile of [^{3}H] components found after assay incubation. Assay contained initially 2.95x10^6 cpm [^{3}H]-GTP, 17,500 cpm [^{14}C]-cGMP and 60 µg of spleen cytosol. In the sample incubation denoted by 0-0, 16,000 cpm and 650,000 cpm were recovered in cGMP and GMP peaks, respectively, after subtracting the background. The background from incubation with heat inactivated cytosol is denoted by ●-●. Panel B, profile of [^{14}C] components found after assay incubation. In the sample incubation, 3.5% of [^{14}C]-cGMP was converted to GMP after 20 min. For this particular assay, the specific activity of the [^{3}H]-GTP was 195 cpm/pmole.

of the uncorrected guanylate cyclase activity is determined by the formation of [^{3}H]-cGMP from the [^{3}H]-GTP precursor. The background under the cGMP peak in the control incubation is approximately .001 to .0015 of the counts initially present in the reaction mix. During the reaction, the formation of a sizeable [^{3}H]-GMP peak ascribed to either phosphatase activity acting on the GTP precursor or phosphodiesterase breakdown of de novo formed cGMP is observed. Addition of [^{14}C]-cGMP permits the determination of subsequent losses of de novo synthesized cGMP during the reaction. In panel B, such a chromatographic profile is shown demonstrating that the phosphodiesterase activity in spleen is not completely blocked by the presence of 10 mM theophylline. Varying degrees of efficacy in the inhibition of further breakdown of cGMP in various tissues and cell lines with theophylline has been noted using the HPLC assay. In a 20 min. incubation, approximately 3.5% conversion of cGMP standard is noted in spleen while in transformed lymphocytes (CCRF-CEM), this value exceeds 12% (data not shown). For this reason, a correction for the metabolic loss of [^{14}C]-cGMP is applied to the observed uncorrected guanylate cyclase activity shown in panel A.

Authenticity of cGMP Recovered

The authenticity of the collected cGMP in an enzymatically active sample was confirmed using PEI thin-layer chromatography. The putative [^{3}H]-cGMP fractions were collected and subsequently spotted on a PEI plate and developed (20). All the tritium counts, corrected for recovery of known standards on PEI, were found in

the cGMP region (R_f = 0.48). Conversely, when the labelled cGMP was first chromatographed on PEI, the cGMP fraction eluted and subsequently run on a Partisil SAX column, all of the tritiated counts were again found to elute from the column in the cGMP position.

Effect of Radioactive Precursor

The method is dependent upon the conversion of [^{3}H]-GTP (labelled in the 8 position) to [^{3}H]-cGMP by guanylate cyclase. The possibility of tritium loss by exchange reactions at the 8 position was examined by using [^{14}C]-ring labelled GTP in place of the tritiated precursor. When assay conditions were duplicated with a reaction mix containing [^{14}C]-GTP at a specific activity of 110 cpm/pmole, the measured guanylate cyclase activity was within a single standard deviation of that observed with the tritium precursor. For this reason, the utilization of the inexpensive tritiated GTP precursor rather than [^{14}C]-GTP was preferred.

Enzymatic Determinations

Determinations of guanylate cyclase activity using the HPLC methodology described above compares favorably with previously reported values in splenic and liver cytosol (Table II) (21,22). In the case of the liver, a determination was included where the cytosol preparation was preincubated with sodium azide since this compound has been shown to increase the activity of soluble guanylate cyclase in crude protein preparations (21). Consistent

TABLE 2.

Guanylate Cyclase Activity.

	pmole cGMP formed/mg protein/min[1]
Splenic cytosol	89.8 ± 3.4
Liver cytosol	48.4 ± 1.8
Liver cytosol[2] +NaN_3	196.7 ± 4.6

[1]The results represent the mean ± the standard error of the mean of multiple independent samples. The results are expressed as the pmole of cyclic-GMP formed per mg of cytosol protein per minute.

[2]The sodium azide treatment involves incubating the cytosol in 1 mM NaN_3 for 15 min. prior to assay incubation. This procedure was done according to the methodology of Kimura et al. (21).

with previously reported data, the level of guanylate cyclase activity significantly increased after sodium azide exposure. The data found in Table II also demonstrate the high degree of reproducibility in the entire assay as determined by the magnitude of the standard errors. The values found in Table II were compiled from assays performed on multiple independent enzyme preparations. To further examine the reproducibility of the entire assay, a cytosol fraction of rat splenic tissue was aliquoted into three samples. These three aliquots were then treated as independent entities and assayed. The mean ± S.E.M. of these samples was 94.4 ± 2.2 pmole cGMP/mg/min. The magnitude of the standard error indicates a high degree of precision and compares favorably with the precision seen previously in terms of the high pressure liquid chromatography of standard compounds.

DISCUSSION

In this communication, an assay for guanylate cyclase activity based on the conversion of tritiated guanosine triphosphate to tritiated cyclic-guanosine monophosphate was accomplished with HPLC. The parameters involved in optimizing the HPLC nucleotide separation procedure of Harwick and Brown (11) for an enzyme assay were determined. Our use of low ionic strength isocratic elutions where GDP and GTP are firmly bound to the column has eliminated the need to re-equilibrate the column after each determination. The use of this single isocratic elution also results in marked separation of cGMP from other guanine based compounds ensuring its purity for the determinations. The HPLC elution profiles achieved with the present methodology also may be adapted to the simultaneous determination of both cGMP formation and degradation. The necessary end-products may be collected in a quantitative manner with a high degree of purity. Inclusion of [^{14}C]-cGMP in the HPLC assay allows for the determination of cGMP breakdown and for the determination of what degradation products have been formed.

Previously, HPLC separation techniques have been utilized for enzymatic determinations (14,15,23-25). Those involved in the determination of guanylate cyclase were burdened by large column dimensions and/or assorted environmental restraints (14,15). The present HPLC procedure utilizes a small and efficient anion exchange column run at ambient temperature to achieve separation characteristics seen with much more cumbersome systems. The methodology is considerably simplified; it requires a minimum of sample handling

before the elution of the radiolabelled cGMP or its degradation products. Since the recovery of the reaction end products is nearly quantitative, the assay has the advantage of a high degree of reproducibility and accuracy. Adoption of the high-pressure liquid chromatographic method with automated sample injection systems for enzymatic analysis of nucleosides and nucleotides involved in GTP metabolism should therefore provide a precise tool for those interested in this branch of cyclic nucleotide research.

ACKNOWLEDGEMENT

We would like to gratefully acknowledge Lynette Weissman and Julie Collins for preparation of the manuscript. This investigation was supported in part by Grant Number CA 21927 awarded by the National Cancer Institute, Department of Health, Education and Welfare.

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