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Development of guanine analyzer to measure activity of guanylate cyclase

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Abstract

A previous analyzer of adenine compounds by high-performance liquid chromatography was converted for the determination of guanine, its nucleoside and nucleotides by a post-column fluorescence derivatization with phenylglyoxal (PGO) in place of bromoacetoaldehyde. The gel filtration column (Asahipak GS-320H) was used for separation by a mobile phase consisting of 25 m*M* sodium citrate buffered (pH 4.0)–150 m*M* NaCl solution and CH_3CN (85:15, v/v) containing 15 m*M* PGO. The separated analytes reacted with flow through PGO in a reaction coil at 90°C into fluorescent derivatives. Those derivatives were detected fluorimetrically, highly selective and quantitatively. The activity of soluble guanylate cyclase (sGC) in the neuroblastoma N1E-115 cell was measured by tracing the peak height of cGMP synthesized from substrate GTP using this guanine analyzer. The sensitivity of the present method was lower than the radioisotope method. However, our modified method was simpler, safer and quicker than the radioisotope method. Furthermore, this method could trace other guanine compounds simultaneously, allowing measurement of guanine metabolizing enzymatic activity. Therefore, it will be useful for screening of effectors on sGC. © 1998 Elsevier Science B.V.

Keywords: Guanine analyzer; Derivatization, LC; Guanylate cyclase; Phenylglyoxal

1. Introduction

A variety of guanine-containing compounds are present in all organisms and undergo rapid enzymatic interconversion. GTP, GDP and GMP are all involved in energy exchange. cGMP produced from GTP by guanylate cyclase (GC) plays an important role as a second messenger in the transmission of hormone and nerve information between cells [1–3]. A systematic analysis of these guanines is relevant to the study of the transmission. Several systematic analyses of adenine-containing compounds were carried out using high-performance liquid chromatography (HPLC) with detection of UV absorption [4–8] or native fluorescence [9,10] of adenine base. Specifically, Yoshioka et al. developed an analyzer to measure adenine and their nucleosides and nucleotides by HPLC with pre-column or on-line post-column reaction with bromoacetoaldehyde as a fluorescent reagent [11]. Various adenines in biological samples were investigated by this method [12–14]. Despite our attempts, bromoacetoaldehyde, proved unsuitable for the detection of guanines.

Kai et al. originally reported a selective fluorescent reagent, phenylglyoxal (PGO) for pre-column derivatization of the guanines by HPLC [15]. Later, they improved this method by post-column derivatization with PGO. The guanines were well separated on a reversed-phase column under a gradient elution with two buffers and reacted with a PGO

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solution mixed by the other reagent delivery pump at 80°C. In this way, contents of GDP and GTP in human erythrocytes were measured [16,17]. We thought that their HPLC study was a prelude to measure guanine containing compounds which were usually present in biological materials in minute quantity. In this paper, we attempted to modify their HPLC method based on our adenine analyzer with a mobile phase containing a fluorescent reagent. The adenines were separated without gradient elution and reacted in a heated reaction coil. The fluorescent derivatives were detected by a fluorescence detector. This flow system was simplified by using a single pump [11]. For analysis of the guanines, we used PGO in place of bromoacetoaldehyde and obtained reasonable conditions. We would like to call this improved HPLC analyzer, a guanine analyzer, taking its potential versatility and popularization into consideration. Further, this analyzer was used to measure cGMP produced by soluble guanylate cyclase (sGC).

2. Experimental

2.1. Materials

Phenylglyoxal (PGO) monohydrate was purchased from Aldrich (Milwaukee, WI, USA). Citric acid monohydrate, trisodium citrate dihydrate, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), 3,4-dithiothreitol (DTT), manganese dichloride tetrahydrate, disodium creatine phosphate tetrahydrate, theophylline and L-arginine (L-Arg) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Sodium chloride was obtained from Nakarai Tesque (Kyoto, Japan). HPLC grade acetonitrile (CH_3CN) was obtained from J.T. Baker (Philipsburg, NJ, USA). Guanine, guanine 5′-GMP, 5′-GDP, creatine phosphokinase and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Trisodium 5′-GTP and monosodium 3′,5′-cGMP were kindly donated by Yamasa Shoyu (Choshi, Japan). N^G- Nitro-L-Arg (L-NA) was purchased from Nova Biochem (San Diego, CA, USA).

2.2. Guanine analyzer

The original adenine analyzer consisted of a mobile phase containing bromoacetoaldehyde, an anion-exchange column, a reaction coil and a fluorescence detector. For the measurement of guanines, it was modified so that the Hitachi gel 3013N ion-exchange column was replaced with a Asahipak GS-320H column (250×7.6 mm I.D.) (Showadenko, Tokyo) for gel filtration, which resulted in better separation of cGMP from a large amount of substrate GTP. The fluorescent reagent was replaced with PGO. The final conditions of the system were as follows. The flow-rate was 0.5 ml/min. The wavelengths of emission and excitation were 515 nm and 365 nm, respectively. The length and temperature of the reaction coil were 15 m×0.25 mm I.D. and 90°C. The column temperature was 16°C. The mobile phase consisted of 25 m*M* sodium citrate buffered (pH 4.0)–150 m*M* NaCl solution and CH_3CN (85:15, v/v) containing 40 m*M* PGO.

2.3. Determination of sGC activity

A radioisotope method described by Deguchi and Yoshioka [18] was modified in the following way. In the assay medium, [8-^{3}H]GTP was replaced with cold GTP. The enzymatic reaction mixture was not passed through an alumina column to remove [8-^{3}H]GTP from [8-^{3}H]cGMP. The other conditions were similar to the previous ones.

Neuroblastoma N1E-115 cells were used, because of their high sGC content, and L-Arg was first identified by using them as an endogenous activator to sGC from rat brain by Deguchi and Yoshioka [18]. The cells were kindly donated by Dr. Takehiko Amano, Mitsubishi-Kagaku Institute of Life Sciences and were grown in 260- ml tissue culture flasks in 25 ml of Dulbecco-Vogt modified Eagles minimal essential medium (DMEM) supplemented with 9% fetal calf serum (JRH Biosciences, Lenexa, USA) in a humidified atmosphere of 8% CO_2 and 92% air at 37°C. The minimal essential medium was changed every three days, and the cells were usually grown for 7 days. The confluent cells were collected and washed 3 times with 0.1 *M* phosphate buffered (pH 7.4)–150 m*M* saline (PBS) and then stored at −80°C until use.

The frozen cells were thawed and then suspended in approximately 5 volumes of 5 m*M* Tris–HCl buffer (pH 7.6) containing 1 m*M* DTT, and homogenized. The homogenate was centrifuged at

700 *g* for 15 min and 20 000 *g* for 30 min at 4°C. The supernatant fraction was used as a crude sGC fraction. A protein concentration at 9.54 mg/ml of the crude sGC fraction was determined by the method of Bradford using bovine serum albumin as standard.

The reaction mixture in a total volume of 100 μl consisted of 50 m*M* Tris–HCl buffer (pH 7.4), 5 m*M* creatine phosphate, 3 m*M* $MnCl_2$, 8 m*M* theophylline, 167 μg/ml creatine kinase, 0.5 m*M* GTP and 50 μl of the crude sGC fraction at the final concentration. To the reaction mixture, 0.1 m*M* L-Arg or 0.02 μ*M*–0.2 m*M* L-NA was added. The enzyme reaction was carried out at 37°C for 10 min, and terminated by heating at 94°C for 3 min and then put on ice. Thereafter, it was ultradialyzed to eliminate molecules larger than 10 kDa. To see the nonenzymatic reaction, the crude sGC fraction was heated at 94°C for 3 min and added to the reaction mixture.

The activity of the enzyme in 20 μl of the mixture was calculated as a peak height of cGMP synthesized from GTP by the analyzer.

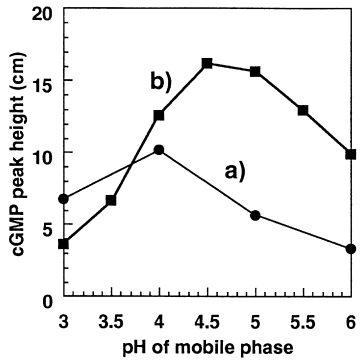

Fig. 1. Effect of pH of mobile phase on cGMP peak height. The mobile phase consisted of 25 m*M* sodium citrate buffered (a) or maleate buffered–150 m*M* NaCl solution and CH_3CN (85:15, v/v) containing 5 m*M* PGO. The flow-rate of the mobile phase was 0.5 ml/min. 2 nmol of cGMP in 20 μl was injected. The column temperature was 45°C. The length and temperature of the reaction coil were 15 m×0.25 mm I.D. and 80°C. The wavelengths of emission and excitation were 515 nm and 365 nm, respectively.

3. Results

For the fluorescent derivatization, pH of a buffer in the mobile phase was critical to the reaction of PGO with the guanines. The peak height of cGMP on-line reacted with PGO was measured in the various pH values of the two buffers as shown in Fig. 1. In the citrate buffer, the peak was highest at pH 4.0. The peak was higher in the maleate buffer at the same pH. The separation of the guanines in the citrate buffer, however, was better than that observed in the maleate buffer (data not shown). Thus, the citrate buffer at pH 4 was adopted. The maximum wavelengths of emission and excitation of the fluorescent peak of cGMP were measured without the column of the analyzer. When the excitation was fixed at 365 nm, the emission wavelength was scanned from 485 to 545 nm at intervals of 10 nm each. The maximum emission wavelength was found to be 515 nm. The maximum wavelength of the excitation was 365 nm at the emission maximum of 515 nm as described by Kai et al. [15] (data not shown).

The temperature of the reaction coil was examined as shown in Fig. 1. An 8.7-fold increase in peak height of cGMP was observed upon increasing the temperature from 60 to 90°C, while an 10% decrease in peak height was observed by increasing the temperature to 95°C assumed to the degradation of the derivative. Separation of GTP, GDP, GMP, cGMP, guanosine and guanine were examined at various temperatures of the column as shown in Fig. 2. There was a big negative shock around 20 min of retention time due to the solvent front in the gel filtration and made difficult to determine cGMP. The shock depth increased parallel to the temperature, the reason why was not explained. On the other hand, the retention of cGMP became shorter parallel to the column temperature, while its peak did sharper and did not resolve from the shock. The peak height was highest at 16°C. Thus, the optimum temperature of the column was decided as 16°C. The peak height of cGMP increased linearly up to 40 m*M* PGO as shown in Fig. 3.

Under these optimized conditions, a standard mixture of guanine, guanosine, cGMP, GMP, GDP and GTP was injected into the analyzer.

Six compounds were well separated in the reversed order within 27 min as shown at 16°C in Fig. 2. Their working curves were linear in the range 5–100 pmol as shown in a curve for cGMP as shown

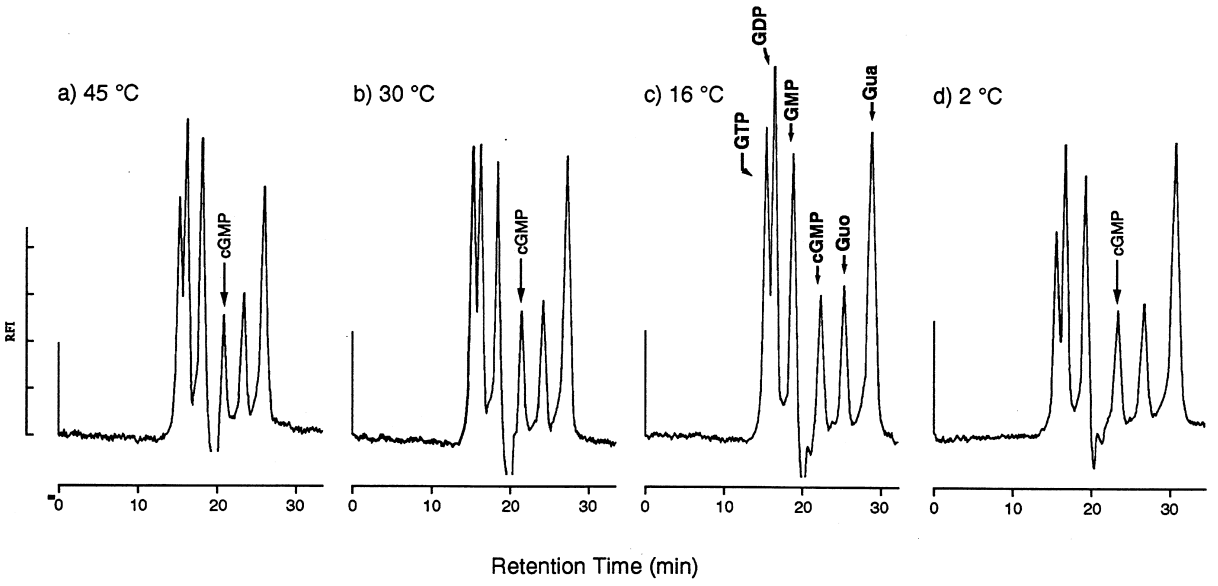

Fig. 2. Effect of the column temperature on cGMP peak height. The column temperature was 45°C (a), 30°C (b), 16°C (c) or 2°C (d). 150 pmol of guanines in 20 μl were injected. The temperature of the reaction coil was 90°C. Other conditions as in Fig. 1.

in Fig. 4. The lowest detection limit for each was 5 pmol ($S/N=5$).

cGMP production with the crude sGC fraction, the 20 000 *g* supernatant prepared from the homogenate of neuroblastoma N1E-115 cells, was measured with the analyzer under the fixed conditions (Fig. 5). A cGMP peak corresponding to 114 pmol appeared but the heat denatured enzyme gave a base line of cGMP. Therefore, its total activity was calculated as 1.14 nmol/min/ml and its specific activity was 0.12 nmol/min/mg protein/ml. The effect of L-NA, a

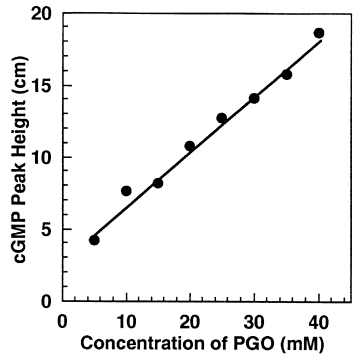

Fig. 3. Effect of concentration of PGO in the mobile phase on cGMP peak height. The mobile phase consisted of 25 m*M* sodium citrate buffered (pH 4.0)–150 m*M* NaCl solution and CH_3CN (85:15, v/v) containing 5–40 m*M* PGO. 80 pmol of cGMP in 20 μl was injected. The column temperature was 16°C. Other conditions as in Fig. 2.

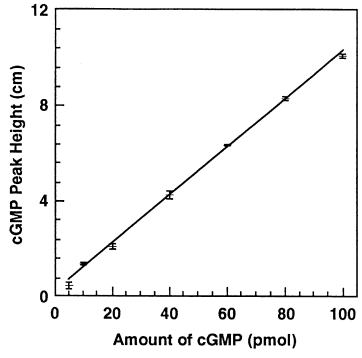

Fig. 4. Working curves of standard cGMP. The mobile phase consisted of 25 m*M* sodium citrate buffered (pH 4.0)–150 m*M* NaCl solution and CH_3CN (85:15, v/v) containing 15 m*M* PGO. 5–100 pmol cGMP was injected. Duplicate samples were used and their mean values were figured. The average standard deviation was 0.09. Other conditions as in Fig. 3. $F(x)=0.101x+0.159$, $R=0.999$.

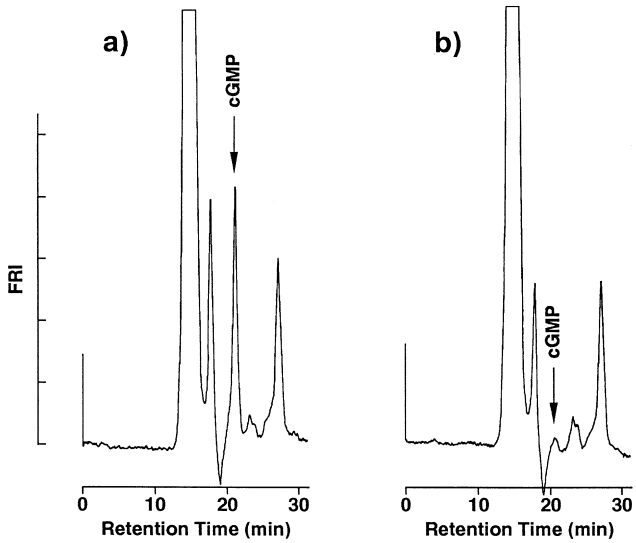

Fig. 5. Chromatogram of enzymatic products. The crude sGC fraction was heated (b) or not (a).

known nitric oxide (NO) synthase inhibitor [19,20] on cGMP production by sGC was examined (Fig. 6a). L-NA inhibited cGMP production in a dose dependent manner from 0.02 μ*M* up to 0.02 m*M*. At 0.2 m*M* of L-NA, cGMP production was 39.7% of control. Further, L-Arg the substrate to NO synthase, reversed the inhibition of cGMP production by L-NA in a dose dependent manner as shown in Fig. 6b. The partial inhibition was due to an ingredient of L-Arg in crude sGC.

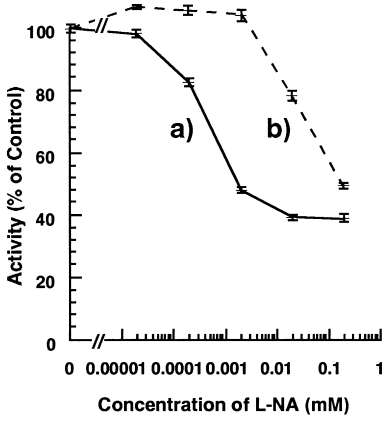

Fig. 6. Effect of L-NA on cGMP production in the crude sGC fraction. In curve a and b, no and 0.1 m*M* L-Arg were used, respectively. The experiments were repeated twice. The average standard deviation ($n=3$) was 1.13.

4. Discussion

The present guanine analyzer was convenient and had a simple flow system, in which a single pump was sufficient to deliver the mobile phase and PGO. The fluorescent reagent in the mobile phase did not affect the separation of the column. Separation was carried out without gradient elution. The eluent was heated in the reaction coil to quantitatively make the fluorescent derivatives. The gel filtration column separates guanine, guanosine, its ribonucleotides and cGMP in the enzymatic reaction mixture containing a large amount of GTP as the substrate. For further application, it could be replaced by ion-exchange columns so that this analyzer could be applicable to various samples containing guanine compounds. Although the fluorescent reagent was specific for guanine base as described by Kai et al. [15], its reaction and separation condition of particular guanines using different columns in separation principle will require specific optimization for reliable measurement.

It was possible to measure cGMP produced from

GTP. The activity with L-Arg by the present method was comparable to the one by the radioisotopic method [18]. In the radioisotope methods, ^{3}H-labeled GTP was used as a substrate and produced ^{3}H-cGMP was separated from ^{3}H-GTP by the alumina column and counted. Depending on the radioactivity of ^{3}H-GTP, the radioisotopic method is 50 times higher in sensitivity than the present method. ^{3}H-GTP was not stable and apt to be contaminated with its degradation products. Manipulation of the radioisotopes is limited by law, therefore, the radioisotopic analysis of cGMP is inconvenient.

As described, cGMP plays important roles as second messengers to hormonal and neuronal information. GC is classified into two subtypes, soluble and particulate. Particulate GC is present in membrane of cells and corresponds to hormones such as human atrial natriuretic peptide. Soluble GC is present in cells. We have been involved in investigating effectors to sGC [21]. When the muscarinic receptor on the surface of neural cells such as neuroblastoma N1E cells used in this experiment, Ca^{2+}–calmodulin complex is produced and activates NO synthase which synthesizes NO from L-Arg. The synthesized NO activates sGC [19,20]. The crude sGC used consists of NO synthase and sGC itself. Thus, L-Arg reversed the inhibition with L-NA as expected.

The conditions of the analyzer were established and its applicability as well was confirmed. The present analyzer will be useful in screening for effectors of NO–cGMP signal transduction. Further applications will be described elsewhere.

Acknowledgements

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