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Assay of human platelet guanylate cyclase activity by high-performance liquid chromatography with fluorescence derivatization

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Abstract

A selective and sensitive high-performance liquid chromatography (HPLC) method with fluorescence derivatization for the assay of guanylate cyclase (GC) activity is described. GTP and cGMP, which are the substrate and the product of GC, respectively, and other guanine-containing compounds are selectively converted by the reaction with (3,4-dimethoxyphenyl)glyoxal to the fluorescent derivatives. The derivatives were separated by reversed-phase HPLC. The limit of detection at a signal-to-noise ratio of 3 for cGMP was 10 fmol on the column. The sensitivity of this method was less than that of the conventional radioisotopic method, but this method is simple and convenient. Human platelet GC activity was measured, and the effects of some compounds were investigated.

Keywords: Guanylate cyclase; Enzymes

1. Introduction

Cyclic GMP (cGMP), a second messenger like cyclic AMP (cAMP), is synthesized from GTP by guanylate cyclase (GC) and hydrolyzed to GMP by cyclic nucleotide phosphodiesterase (PDE) [1]. GC exists as soluble and membrane-bound forms [1,2]. The soluble GC is a heme protein and exists in the cytosol as a heterodimer with a molecular mass of 70–80 kDa. This form is activated by nitric oxide, which was identified as the endothelium-derived relaxing factor (EDRF) [3,4]. The membrane-bound form is a monomer or homodimer with a molecular mass of 120–180 kDa. This form is activated by peptides and hormones such as atrial natriuretic peptide (ANP) and adrenocorticotropic hormone (ACTH).

The most widely used methods for the assay of GC include radioisotopic methods using a radiolabeled substrate and the radioimmunoassay [5]. These methods possess high sensitivity, but with the use of radioisotopes, special facilities and substantial costs are involved. To avoid using radioisotopes and to simplify the procedure, high-performance liquid chromatographic methods monitoring nucleotides by UV absorption have been developed [6]. These methods are simple and safe, but they are relatively insensitive.

Using fluorescent detection, improvement in the sensitivity should be achieved. In a previous report, we have developed an HPLC determination of guanine and its nucleosides and nucleotides using (3,4-dimethoxyphenyl)glyoxal (DMPG), a fluorogenic reagent for guanine-containing compounds [7].

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In this paper, the fluorescent derivatization was applied to the assay of GC that synthesizes cGMP. Non-radiolabeled GTP was used as a substrate by GC, and the separation of the product and substrate derivatized with DMPG was measured by reversed-phase HPLC. Human platelets were used as a source of enzyme preparation. GC, in particular, the soluble form, is abundantly present in platelets [8,9] and regulates the cGMP level in platelets. When NO is released from endothelial cells or other tissues and reaches the platelets, it elevates the cGMP level and the activation of the platelets [10,11]. In fact, nitro-vasodilators, which elevate cGMP, are considered to have antiplatelet effects in addition to a vasodilative effects [9]. From these standpoints, platelets are one of the most interesting sources of GC activity.

2. Experimental

2.1. Reagents and solutions

GTP, GDP, GMP, cGMP, guanine and guanosine were purchased from Seikagaku Kogyo (Tokyo, Japan). 9-Ethylguanine, 8-azaguanine and 1H-[1,2,4]oxadiazolo[4,3-a]qunoxalin-1-one (ODQ) were purchased from Sigma (USA). DMPG was prepared as described previously [12]. Acetonitrile and tetrahydrofuran (THF) were of HPLC grade (Wako, Tokyo, Japan). Dimethylsulfoxide (DMSO) was of spectral analysis grade (Nacalai Tesque, Kyoto, Japan). Water was purified by a Milli-Q II system (Japan Millipore, Tokyo, Japan). All other chemicals were of reagent grade.

Standard solutions of guanine and its nucleosides and nucleotides (10 m*M* each) were prepared in methylcellosolve–water (7:3, v/v) and then stored at −20°C. The solutions were diluted to appropriate concentrations with water and used. DMPG was dissolved in DMSO–water (2:3, v/v) and then stored at 4°C.

2.2. Sample preparation

Platelet GC samples were prepared according to the method of Radomski et al. [10]. Blood samples from healthy volunteers were collected into siliconized tubes containing 0.1 vol. of 3.13% (w/v) citrate (VENOJECT II, Terumo) and then centrifuged at 200 *g* for 10 min at 4°C. Platelet-rich plasma (PRP) was then removed, EDTA-2Na (7.7 m*M*) was added, and the PRP was centrifuged at 2000 *g* for 20 min at 4°C. The platelet pellets were washed in Hepes–Tyrode's buffer (129 m*M* NaCl, 8.9 m*M* $NaHCO_3$, 2.8 m*M* KCl, 0.8 m*M* KH_2PO_4, 0.8 m*M* $MgCl_2$, 5.6 m*M* glucose and 10 m*M* Hepes, pH 7.4) containing EDTA-2Na and then resuspended in 1 ml of 10 m*M* Hepes buffer (pH 7.4) containing 0.32 *M* sucrose followed by homogenization by sonication twice for 10 s. The homogenate was used as the source of GC.

The protein concentration in the enzyme sample was determined by the method of Smith et al. [13] with bovine serum albumin as the standard.

2.3. GC assay

The standard enzymatic reaction mixture contained 50 m*M* Tris–HCl buffer (pH 7.4), 5 m*M* $MnCl_2$, 1 m*M* GTP, 1 μ*M* 9-ethylguanine (internal standard) and 50 μl GC sample in a total volume of 200 μl. The enzymatic reaction was incubated at 37°C for 30 min, and the reaction was then stopped by heating in a boiling water-bath for 2 min. The protein precipitate was removed by centrifugation, and a 100-μl aliquot of the supernatant was used for the derivatization procedure.

2.4. Fluorescence derivatization

To the sample (100 μl) mentioned above, 100 μl of 100 m*M* DMPG solution was added and the mixture then incubated at 37°C for 60 min. A 20-μl portion of the final mixture was subjected to HPLC.

2.5. Apparatus and HPLC conditions

The HPLC system consisted of a JASCO PU-980 HPLC pump, an LG-980-02 Ternary Gradient Unit, equipped with a Rheodyne Model 7725i syringe-loading sample injection valve (20-μl loop) and a JASCO FP-920 fluorescence detector fitted with a 16-μl flow cell. The column was TSK-gel ODS-80TsQA (150×4.6 mm I.D., particle size 5 μm: Tosoh; Tokyo, Japan). The column was kept at ambient temperature. A stepwise gradient elution of acetonitrile (5–27%, v/v) in an aqueous mobile

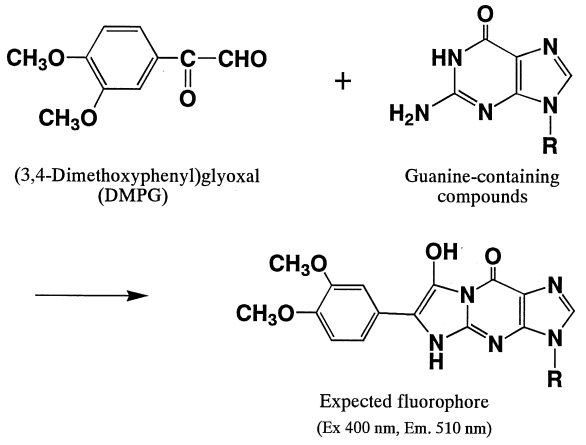

Fig. 1. Derivatization reaction of guanine-containing compounds with DMPG.

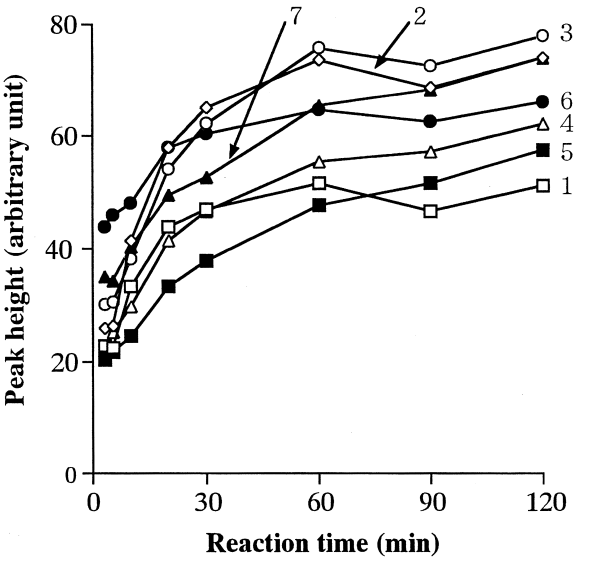

Fig. 2. Effect of reaction time on the formation of fluorescent derivatives with DMPG. The value of each point was the mean value ($n=2$) of determinations. Reaction conditions: Buffer, Tris–HCl (pH 7.6); Temperature, 30°C. Curves: 1=GTP; 2=GDP; 3=GMP; 4=cGMP; 5=guanine; 6=guanosine; 7=9-ethylguanine.

phase containing tetrahydrofuran (3%, v/v), 50 m*M* sodium phosphate buffer (pH 6.0) (25%, v/v) and water (67–45%) was carried out during 20 min at a flow-rate of 1.0 ml min^{-1} (for the gradient curve, see in Fig. 1(A)). The fluorescence intensity of the column eluate was monitored at 510 nm with excitation at 400 nm. Peak areas were obtained by a JASCO 807-IT integrator.

2.6. Determination of K_m value

The Michaelis constant (K_m) was determined using eight concentrations of cGMP from 0 to 1000 μ*M* and was obtained from double-reciprocal Lineweaver–Burk plots.

3. Results and discussion

We previously described the use of sodium phosphate buffer (pH 7.0) and incubation at 37°C for 5 min for the derivatization of guanine and its nucleoside and nucleotides using DMPG [7], whereas Tris–HCl buffer is commonly used in GC assay. For a simple procedure for GC assay, we also used Tris–HCl buffer for the fluorescence derivatization. Fig. 2 shows the effect of derivatization time. The peak heights from the derivatives of guanine and its seven nucleosides and nucleotides reach maxima at 60–120 min. Thus, 60 min was used for derivatization.

Fig. 3 shows the chromatograms obtained with a standard mixture of guanine and its nucleoside and nucleotides (A), a standard (B) and a blank (C) enzyme reaction mixture. The fluorescence derivatives of eight guanine-containing compounds were separated by reversed-phase HPLC with stepwise and gradient elution of acetonitrile and eluted within 20 min (Fig. 3 -(A)). Fig. 3-(B) shows typical chromatogram obtained from human platelet sample. cGMP, the product of GC from GTP, was detected. When the substrate or enzyme sample was not subjected to the enzyme reaction mixture, cGMP could not be detected.

The calibration curve of cGMP was linear in the range 25–500 pmol/assay tube ($r=1.000$). The limit of detection ($S/N=3$) of cGMP was 10 fmol on the column. The sensitivity of this method was lower than that of the radioisotopic or radioimmunoassay method but higher than that of the HPLC–UV method.

The amount of cGMP formed was linear at least

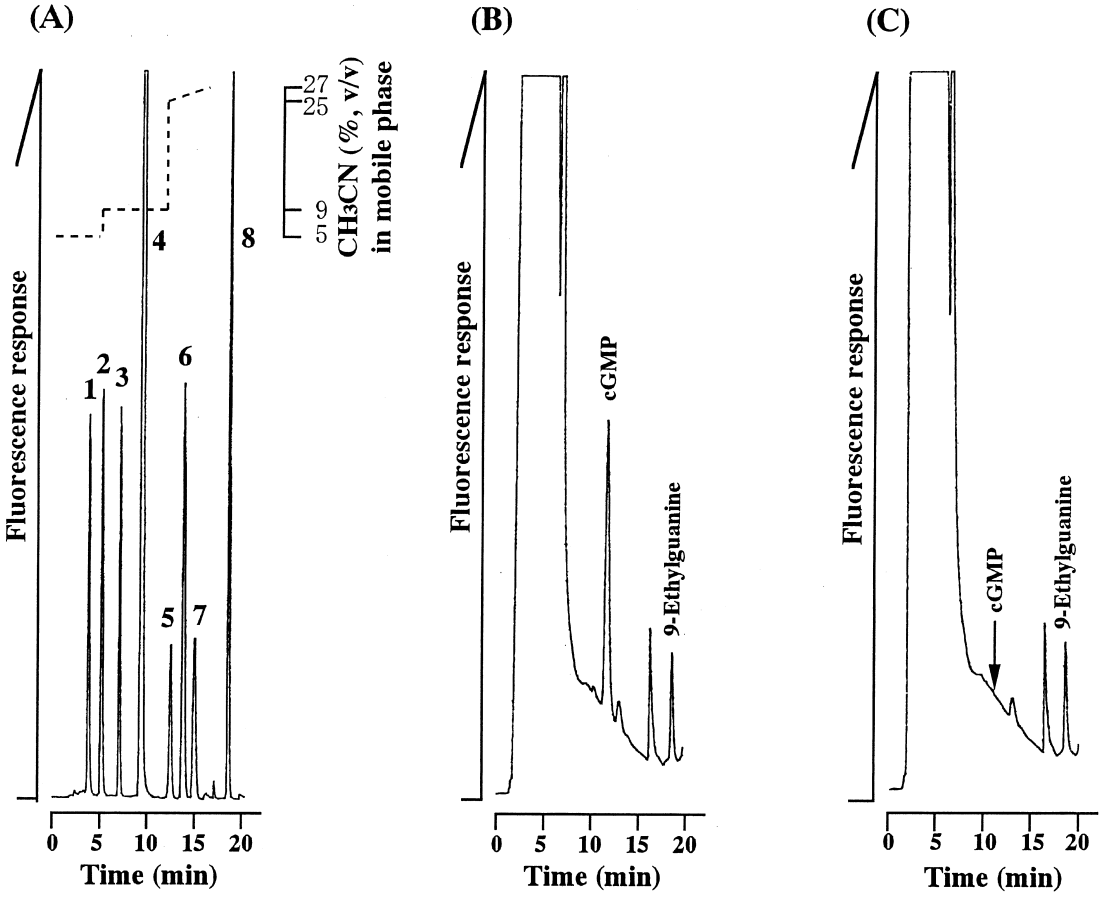

Fig. 3. Chromatograms obtained with (A) a standard mixture of guanine and its nucleos(t)ides, and (B) an enzyme reaction mixture and (C) the enzyme blank. The enzyme blank was prepared by heating the enzyme in a boilng water bath for 2 min before the enzyme reaction. Peaks: 1=GTP; 2=GDP; 3=GMP; 4=cGMP; 5=8-azaguanine; 6=guanine; 7=guanosine; 8=9-ethylguanine.

up to 60 min (Fig. 4). Thus, the enzyme reactions were stopped after incubation for 30 min. The amount of cGMP increased linearly with protein concentration up to 20 μg (Fig. 5).

GC requires a divalent cation for activity. The effects of Mn^{2+} and Mg^{2+} were examined. The maximum activity was obtained at 5 m*M* Mn^{2+}. When Mg^{2+} was used, production of cGMP was about 40% of the amount produced when Mn^{2+} was used; 5 m*M* $MgCl_2$ was used for the enzyme reaction.

Fig. 6 shows the effect of GTP concentration (0–1 m*M* in the reaction mixture) on the velocity of the enzyme reaction. When the GTP concentration was increased, the GC activity showed a saturable kinetic pattern. In these assays, 1 m*M* GTP in the reaction mixture was used. The K_m value for GTP was estimated from Lineweaver–Burk plots and obtained with homogenates of platelets as 51±37 μ*M* (mean±SD) (Fig. 6). The value corresponded to the affinity of the soluble form for GTP (K_m=5–150 μ*M* [1]).

The GC activities in the platelet samples from seven healthy persons were determined by the present method. All the samples examined had GC activity, with a mean±SD of 504±104 pmol/min/mg protein. The values of these results are within the range of published data [8,9,14,15].

To characterize the type of GC activity being measured, the effect of some GC modulators on the

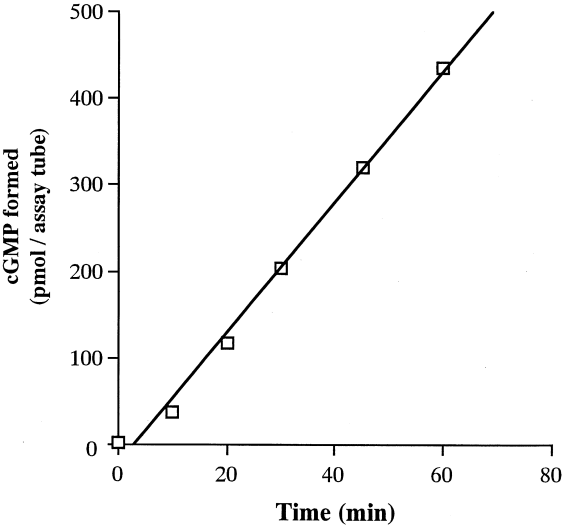

Fig. 4. Relationship between enzyme reaction time and the formation of cGMP in the enzyme reaction mixture. The value of each point was the mean value (n=2) of determinations. Enzyme reactions were performed as recommended with various reaction times.

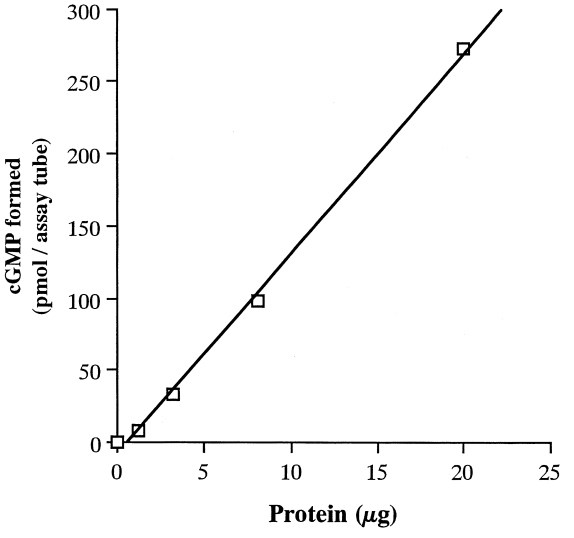

Fig. 5. Relationship between protein concentration and the formation of cGMP in the enzyme reaction mixture. The value of each point was the mean value (n=2) of determinations. Enzyme reactions were performed as recommended with various concentrations of protein.

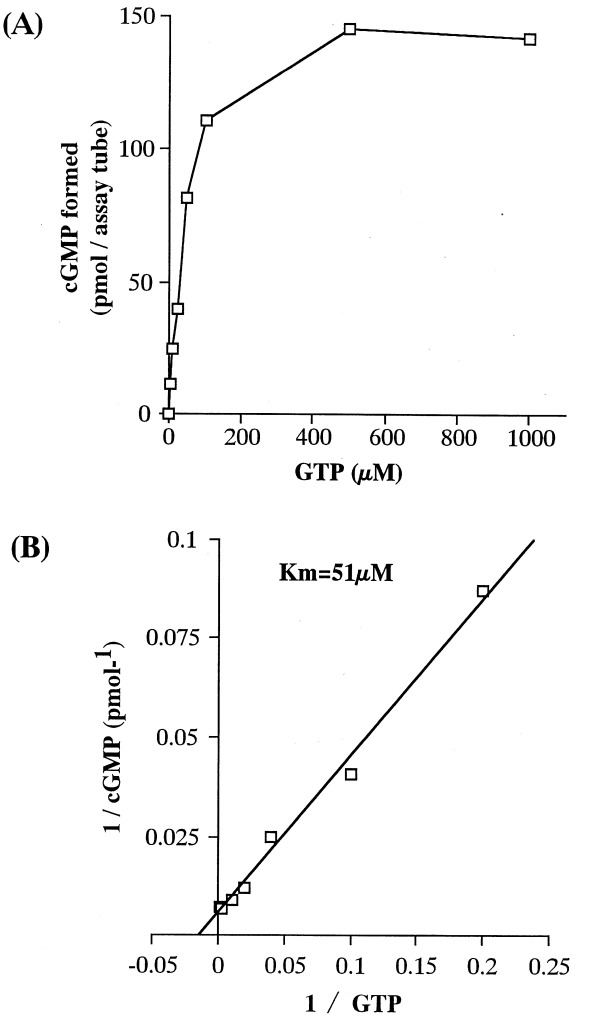

Fig. 6. (A) Effect of substrate concentration on the formation of cGMP by GC in the incubation mixture (B) Lineweaver–Burk plots for GTP. The value of each point was the mean value (n=2) of determinations.

GC activity were investigated. Sodium nitroprusside (SNP) activates soluble GC through the release of nitric oxide (NO) [16]. Fig. 7 shows the effect of SNP on the human platelet GC activity. SNP stimulated the GC activity in a dose-dependent manner. ODQ (1 H-[1,2,4]oxadiazolo[4,3-a]qunoxalin-1-one) is a soluble GC inhibitor [17] and inhibited the GC activity (Fig. 8). These data indicate that the samples contain the soluble form of GC, and this corresponds to the report that the soluble GC is abundantly present in platelets [8,9].

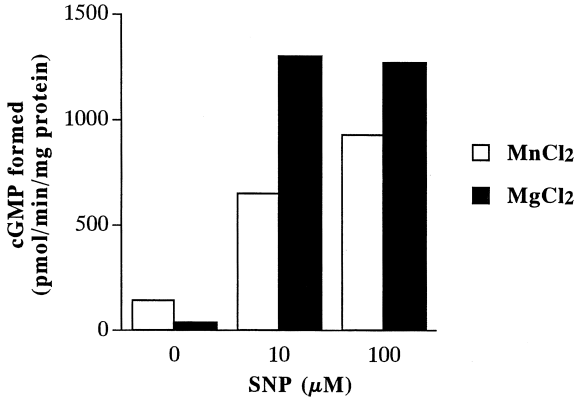

Fig. 7. Effect of SNP on the formation of cGMP. The value of each point was the mean value ($n=2$) of determinations. Enzyme reactions were performed as recommended with various concentrations of SNP, and $MnCl_2$ or $MgCl_2$.

In conclusion, using this assay, the GC activity in human platelets can be determined, and the stimulative or inhibitory effect of modulators on GC activity can be investigated. This method is simpler and more convenient than radioisotopic methods because it does not require a special substrate such as radioactive compounds. This method should be useful for the determination of the GC activities in other biological tissues and the search for novel modulators of GC.

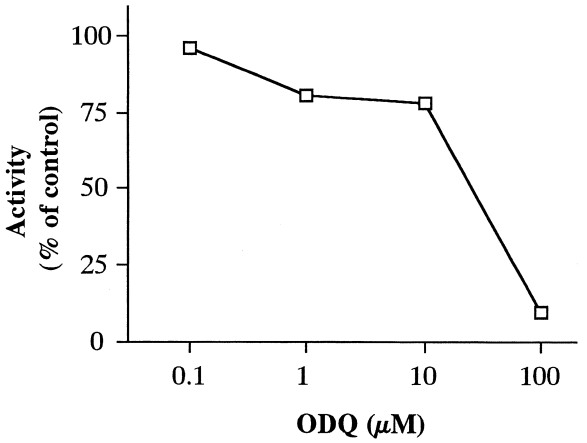

Fig. 8. Effect of ODQ concentration on the formation of cGMP. The value of each point was the mean value ($n=2$) of determinations. Enzyme reactions were performed as recommended with various concentrations of ODQ.

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