High-Performance Liquid-Chromatographic Determination of Guanosine 3',5'-Cyclic Monophosphate in Human Urine with Fluorescence Detection Using 3,4-Dimethoxyphenylglyoxal

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A sensitive and selective method for the determination of guanosine 3',5'-cyclic monophosphate in human urine is described. The method uses high-performance liquid chromatography with fluorescence detection. Guanosine 3',5'-cyclic monophosphate and other guanine nucleotides in human urine, after a clean-up with an anion exchanger cartridge (TOYOPAK DEAE S), gave fluorescence products by a reaction with 3,4-dimethoxyphenylglyoxal in a neutral medium. The formed derivatives were separated using reversed-phase high-performance liquid chromatography. The limit in the detection ($S/N=3$) of guanosine 3',5'-cyclic monophosphate is 4.4 pmol on the column.

Keywords Guanosine 3',5'-cyclic monophosphate, human urine, 3,4-dimethoxyphenylglyoxal, high-performance liquid chromatography, fluorescence detection

There are many metabolites of nucleic acid bases and their nucleos(t)ides in urine. These metabolites are derived from ribonucleic acid metabolism *in vivo*, and increased urinary excretion of these products in patients with cancer have been reported.^{1,2} Guanosine 3',5'-cyclic monophosphate (cGMP), which was produced by an enzymatic reaction of guanylate cyclase from guanosine 5'-triphosphate (GTP) as a substrate and plays important roles in the human body, was also increased in the urine of patients with cancer.^{3,4} A radioimmunoassay was used to assay cGMP because of its high sensitivity and selectivity; however, it is restricted in usage.

Generally, nucleic acid bases and their nucleos(t)ides in biological tissues and fluids have been measured by methods based on high-performance liquid chromatography (HPLC) with spectrophotometric detection using an ion-exchange or reversed-phase column.^{5,6} There are some difficulties using these methods due to low sensitivity and selectivity; also, biological samples contain many different types of metabolic end products that interfere with the chromatographic detection. HPLC methods with electrochemical detection have been reported for the determination of guanine and its nucleos(t)ides based on the electrochemical oxidation at a high applied potential.^{7,8} Although this method can detect these compounds at a pico- or subpicomole level,

there are some problems due to a disturbance with detectable biogenic compounds and the unstable noise level, depending on the composition of the mobile phase used in the HPLC. On the other hand, HPLC methods with fluorometric detection were useful because of their high sensitivity and high selectivity; some fluorogenic reagents (*e.g.* bromoacetaldehyde, chloroacetaldehyde) that showed a high molecular recognition specificity for adenine and its nucleosides and/or nucleotides were reported.⁹⁻¹¹

We previously reported alkoxyphenylglyoxals, especially 3,4-dimethoxyphenylglyoxal (DMPG), as a highly sensitive and selective fluorogenic reagent for guanine and its nucleos(t)ides, and fluorescence derivatization with a DMPG reagent applied for detection in reversed-phase HPLC separation.¹²⁻¹⁴ In this paper we described a new HPLC method for the quantification of cGMP and some other guanine nucleotides in human urine, after solid-phase extraction using an anion-exchanger resin cartridge (TOYOPAK DEAE S), that are converted into the corresponding fluorescent derivatives by a reaction with the DMPG reagent.

Experimental

Reagents and solutions

Nucleic acid bases, nucleosides and nucleotides, such as guanosine 5'-triphosphate (GTP), guanosine 5'-diphosphate (GDP), guanosine 5'-monophosphate (GMP), 2'-deoxyGTP (dGTP), 2'-deoxyGDP (dGDP), 2'-deoxyGMP (dGMP), cGMP, guanine, guanosine and

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deoxyguanosine, were purchased from Seikagaku Kogyo (Tokyo, Japan) and 9-ethylguanine was purchased from Sigma (USA). The standard solutions ($2.0 \mu\text{mol ml}^{-1}$ each) of guanine and its nucleos(t)ides 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 within a day. DMPG was prepared as previously described.¹⁵ DMPG was dissolved in dimethyl sulfoxide-water (2:3, v/v). All other chemicals were of analytical reagent grade. A TOYOPAK DEAE S cartridge (anion exchanger, diethylaminoethyl resin; Tosoh, Tokyo) was washed with 2 ml of water before use.

Apparatus and HPLC conditions

The HPLC system consisted of two Yokogawa LC100P liquid-chromatograph pumps equipped with a Rheodyne Model 7125 syringe-loading sample injection valve (20- μl loop) and a Tosoh FS-8010 spectrofluorometer fitted with a 18- μl flow cell. A TSK-ODS 120T (150 $\times$ 4.6 mm i.d., particle size 5 μm ; Tosoh, Tokyo) was used. The column was kept at ambient temperature ($24\pm 4^\circ\text{C}$). A stepwise and gradient elution of acetonitrile (2–25%, v/v) in an aqueous mobile phase containing tetrahydrofuran (3%, v/v), 50 mM sodium phosphate buffer (pH 6.0) (25%, v/v) and water (69–47%) was carried out for 30 min at a flow-rate of 1.0 ml min^{-1} . The fluorescence intensity of the column eluate was monitored at 510 nm emission and 400 nm excitation. The peak areas, which were obtained using a Hitachi D-2500 integrator, were used for quantification.

Urine sample preparation

Human urine was collected without a preservative and frozen at -20°C , if not immediately used. Aliquots of urine were filtered and a 0.2-ml volume of urine was mixed with 0.2 ml of 20 mM HCl. The urine-HCl mixture was applied to a TOYOPAK DEAE S cartridge (pretreated with 2 ml of water). The cartridge was washed with 0.6 ml of water and then eluted with 0.2 ml of 0.5 M NaCl. The eluate was used for the fluorescence derivatization.

The creatinine concentration in urine was enzymatically determined with reagents from Wako-chemical (Tokyo, Japan).

Fluorescence derivatization

To the eluate sample (50 μl) mentioned above, 50 μl each of 30 mM sodium phosphate buffer (pH 7.0), 0.1 M DMPG solution and water were added. The mixture was warmed at 37°C for 5 min. A 20- μl portion of the final reaction mixture was used for an HPLC analysis.

Results and Discussion

Figure 1 shows typical chromatograms obtained from urine, which was diluted with water (1:1, v/v) and then

Fig. 1 Chromatograms of (A) urine and (B) its tissue blank without a clean-up procedure. A portion (50 μl) of urine-H₂O (1:1, v/v) was directly treated with DMPG. Peaks: 1, cGMP; others, unknown compounds.

Fig. 2 Chromatograms obtained with (A) urine and (B) the same sample spiked with guanine-containing compounds (5 pmol on column, each) treated by solid-phase extraction using a TOYOPAK DEAE S cartridge. Peaks: 1, GDP; 2, dGDP; 3, GMP; 4, dGMP; 5, cGMP.

directly treated with DMPG, and its reagent blank. Since the direct derivatization of a urine sample gave a high background interference derived from unknown intrinsic fluorescent compounds and some unknown guanine analogs, sample purification was necessary. Therefore, an anion-exchange column was used for this purpose.

Typical chromatograms obtained with urine treated using a solid-phase extraction with an anion-exchange

Fig. 3 Influence of the pH in the urine sample solution on the elution from a TOYOPAK DEAE S cartridge. Peaks: 1, GDP; 2, GMP; 3, cGMP; 4, 9-ethyl-guanine; 5, guanosine; 6, dGDP; 7, dGMP; 8, guanine; 9, deoxyguanosine.

Fig. 4 Effect of (A) the volume and (B) concentration of the NaCl solution on the elution from a TOYOPAK DEAE S cartridge. Peaks: 1, cGMP; 2, GDP; 3, dGMP; 4, dGDP; 5, GMP.

cartridge (TOYOPAK DEAE S cartridge), and the same sample spiked with a standard mixture are shown in Fig. 2. The peaks were identified on the basis of their retention times compared with standard compounds, and also by co-chromatography of the standards and samples. In addition, the shaded peaks in the chromatograms were formed only by the derivatization procedure with DMPG reagent. The fluorescent derivatives corresponding to GDP, dGDP, GMP, dGMP and cGMP could be detected. Other cartridges such as the TOYOPAK ODS S and Bondelut C18 (Varian) cartridges, which were reversed-phase type cartridges, could not adsorb guanine and its nucleos(t)ides.

The pH of a sample which was applied to the TOYOPAK DEAE S cartridge influenced the adsorption of guanine and its nucleos(t)ides (Fig. 3). A standard mixture of 11 guanines and its nucleos(t)ides, and Briton-Robinson buffer (pH 2.0–10.0) were used for this discussion. GTP, dGTP, GDP, dGDP, GMP, dGMP and cGMP, which contains the 5'-tri-, di-, mono- or 3',5'-cyclic monophosphate group, were adsorbed in an acidic medium, and eluted by an NaCl solution. On the other hand, guanine, guanosine, deoxyguanosine, and 9-ethyl-guanine, which do not have a phosphate group, were adsorbed on a TOYOPAK DEAE-S in an alkaline medium. When a urine sample was applied to the

Table 1 Recoveries of guanine-containing compounds added to urine

Compound	Recovery (%, mean± SD, n=5)	Compound	Recovery (%, mean± SD, n=5)
GTP	—	dGTP	—
GDP	79±3	dGDP	75±3
GMP	72±4	dGMP	77±2
cGMP	67±3	Guanine	8±5
Guanosine	—	Deoxyguanosine	3±5
9-Ethylguanine	5±4		

The amounts added to 200 µl of urine, 0.5 nmol each.

cartridge, however, it was difficult to adjust the pH to 5.0 using a Briton–Robinson buffer. Accordingly, 20 mM HCl was used to adjust pH at 5.0 in urine samples.

The volume and concentration of the NaCl solution utilized for eluting cGMP and some guanine nucleotides from the cartridge were examined (Fig. 4). NaCl did not affect the fluorescence derivatization reaction. A smaller volume (100 µl) of NaCl was used for elution, and maximum fluorescence intensities were obtained (Fig. 4A). However, the measured values of guanine nucleotides for the same sample were widely scattered when 100 µl of an NaCl solution was used for elution; 200 µl was employed in the procedure. When NaCl in the concentration range of 0.4–1.0 M was used, guanine nucleotides were effectively eluted, and could produce maximum fluorescence intensities; 0.5 M was finally selected (Fig. 4B).

The recoveries of the solid-phase anion-exchange extraction, checked at a concentration of 5 nmol ml⁻¹ each for the 11 guanine and its nucleos(t)ides, were 67–79% for guanine nucleotides and 0–8% for guanine and its nucleosides (Table 1).

The calibration curves for guanine and its nucleosides were linear over the concentration range of 12.5–250 pmol per 20 µl injection volume and the correlation coefficients were between 0.995 and 0.998. The limits of detection at a signal-to-noise ratio of 3 for the compounds were in the range of 4–14 pmol per 20 µl injection volume. The concentrations of cGMP and some guanine nucleotides in healthy human urine (n=5) are shown in Table 2. We determined these concentrations using a standard additional method. The concentrations measured in this method agreed well with previously reported data.^{3,4,16}

In conclusion, the present method is simultaneously, sensitive and highly selective for the determination of cGMP and some guanine nucleotides in human urine. The method is also useful for exploring these compounds in other biological samples.

Table 2 Urinary excretion of guanine-containing compounds from healthy persons

Sex ^a	Age	Urinary level (µmol/g creatinine)				
		GDP	dGDP	GMP	dGMP	cGMP
M	27	0.734	ND ^b	0.864	ND	0.535
M	25	0.373	ND	0.559	ND	0.599
M	24	0.307	ND	ND	0.307	0.516
M	22	0.220	ND	0.652	ND	0.696
Mean		0.409		0.692	0.307	0.587
SD		0.196		0.128		0.070

a. M, male.

b. ND, not detected.

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