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Determination of guanine and its nucleosides and nucleotides in human erythrocytes by high-performance liquid chromatography with postcolumn fluorescence derivatization using phenylglyoxal reagent

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Abstract

A high-performance liquid chromatographic method with on-line postcolumn fluorescence derivatization is described for the simple and sensitive determination of guanine and its nucleosides and nucleotides in human erythrocytes. After deproteinization of the biospecimen, guanine and its nucleosides and nucleotides were separated on a reversed-phase column (TSKgel ODS-120T) by gradient elution with methanol in the aqueous mobile phase consisting of tetra-*n*-propylammonium phosphate (pH 6.0) and phosphate buffer (pH 6.0). The compounds were then automatically converted into fluorescent derivatives by reaction with phenylglyoxal. This derivatization was selective for guanine-containing compounds. The present method permitted the reliable quantification of GDP and GTP in human erythrocytes. The detection limits (at a signal-to-noise ratio of 3) for guanine and its nucleosides and nucleotides were 3.2–10.0 pmol in a 20-μl injection volume. The concentrations of GDP and GTP in human erythrocytes were 17.2 ± 6.2 and 40.2 ± 5.8 nmol/ml, respectively.

1. Introduction

Guanine-containing nucleotides have been found to regulate several functions in a mammalian body. For example, GTP is a substrate of guanylate cyclase which catalyzes the formation of cGMP, which in its turn acts as an intercellular messenger [1]. The GTP and GDP concentrations in human erythrocytes change with different pathological states [2,3]. Therefore, quantitative analysis of guanine nucleosides and nucleotides is important in biomedical and physiological studies.

High-performance liquid chromatography (HPLC) has been the most suitable technique for the analysis of nucleic acid bases and their nucleosides and nucleotides in biological samples such as cell cultures, mammalian body fluids and tissues [2–7]. Most of the HPLC methods currently available for determination of the above-mentioned compounds depend on non-specific measurement of the ultraviolet (UV) absorption (254 nm) after chromatographic separation of the compounds [2–6].

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However, HPLC methods with electrochemical detection have been reported [8–10] for the selective determination of guanine and its nucleosides and nucleotides that are susceptible to electrochemical oxidation at a high applied potential. Although these methods can detect the compounds at pico- or subpicomole level, several problems in the detection exist, owing to interferences from many other biogenic substances and the unstable noise level depending on the composition of the HPLC eluent.

On the other hand, fluorogenic reagents that show a high molecular recognition specificity for one of the nucleic acid bases, nucleosides and/or nucleotides may be useful for a facile and sensitive determination of biogenic nucleosides and nucleotides. A few fluorogenic reagents (*e.g.* glyoxal hydrate trimer [11], chloroacetaldehyde [12] and bromoacetaldehyde [13]) have been reported from the selective determination of adenine and its nucleosides and nucleotides.

We recently reported that phenylglyoxal (PGO) reacts with guanine and its nucleosides and nucleotides to produce the corresponding fluorescent derivatives which can be separated by HPLC [14,15]. In this paper, the above fluorescence reaction with PGO was applied to post-column fluorescence derivatization HPLC for the selective and simultaneous determination of guanine and its nucleosides and nucleotides. The compounds were separated on a reversed-phase column and subsequently subjected to automated derivatization for fluorescence detection. The proposed method using 9-ethylguanine as an internal standard (I.S.) is discussed with regard to the quantification of guanine nucleosides and nucleotides in human erythrocytes, compared with conventional UV detection.

2. Experimental

2.1. Chemicals and solutions

Nucleic acid bases, nucleosides and nucleotides were purchased from Seikagaku Kogyo (Tokyo, Japan). Solutions (2.0 μmol/ml each) of guanine, guanosine and deoxyguanosine were prepared in methyl cellosolve and water (2:1, v/v). Other bases, nucleosides and nucleotides were dissolved in water. These solutions were diluted with water to 100 nmol/ml each, and then stored at −20°C. PGO monohydrate from Aldrich (Milwaukee, WI, USA) was recrystallized from water. PGO solution (60 m*M*) was prepared in methyl cellosolve–water (1:9, v/v), and degassed before use. The solution was stable for at least one week when stored at 4°C. Other chemicals were of the highest purity available, and were dissolved in water.

2.2. Sample preparation

Erythrocytes were separated from 5 ml of healthy human blood. The erythrocytes were successively washed three times with 5 ml of ice-cold saline and then quickly packed by centrifuging at 1400 *g* for 10 min at 4°C. To 100 μl of the erythrocyte layer were added 400 μl of water, 100 μl of 3.0 *M* perchloric acid and 20 μl of 200 nmol/ml 9-ethylguanine as I.S. The mixture was homogenized and centrifuged at 1400 *g* for 10 min. The supernatant was neutralized to pH 6.0 with approximately 190 μl of 1.0 *M* potassium bicarbonate. The mixture was passed through an ODS-minicartridge (Toyopak ODS-M, Tosoh Co., Tokyo, Japan) in order to remove hydrophobic substances. The first eluate (20–50 μl) from the cartridge was used for HPLC.

2.3. Chromatographic system and its operation

Fig. 1 shows a schematic diagram of the HPLC system constructed for the analysis of guanine and its nucleosides and nucleotides. Guanine and its nucleosides and nucleotides in the sample solution were separated by gradient elution with methanol in the mobile phase on a reversed-phase column, TSKgel ODS-120T (150 × 4.6 mm I.D., particle size 5 μm, Tosoh Co.). The column temperature was ambient (24 ± 4°C). Eluents A and B used for the gradient elution were methanol–10 m*M* tetra-*n*-propylammonium phosphate (pH 6.0)–50 m*M* sodium phosphate buffer (pH 6.0)–water (0:17:20:63 and

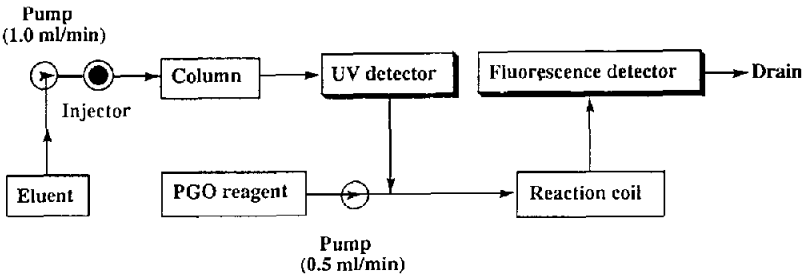

Fig. 1. Schematic diagram of on-line postcolumn fluorescence derivatization HPLC using the PGO reagent. The system and its operating conditions are described in the Experimental section.

20:17:20:43%, v/v, for A and B respectively). The mobile phase was pumped at a flow-rate of 1.0 ml/min by a Hitachi 638-30 high-pressure pump fitted with electronic valves for the gradient elution.

The column eluate was first monitored with a UV Tosoh spectrometer and then directed to the derivatization system. In the system, 60 m*M* PGO solution was added to the eluate-stream with a Hitachi 633 reagent-delivery pump at a flow-rate of 0.5 ml/min. The mixture was heated at 80°C in a reaction coil (3 m × 0.5 mm I.D., stainless-steel tube). The fluorescence in the final eluate was monitored at 515 nm (emission) with excitation at 365 nm by a Hitachi F-1000 spectrofluorometer fitted with a 12-μl flow-cell.

3. Results and discussion

3.1. HPLC separation and postcolumn derivatization conditions

Nucleic acid bases, nucleosides and nucleotides have been separated on reversed-phase columns by elution with an aqueous mobile phase containing methanol or acetonitrile, phosphate buffer (pH 6–7) and an ion-pairing agent [2–4,7]. Therefore, the separation of nucleic acid bases, nucleosides and nucleotides and their related compounds (31 species in all) was performed by ion-pairing reversed-phase HPLC. Fig. 2 shows chromatograms of a standard mixture of the compounds obtained with UV detection at 254 nm and obtained with fluorescence detection after the present postcolumn derivati-

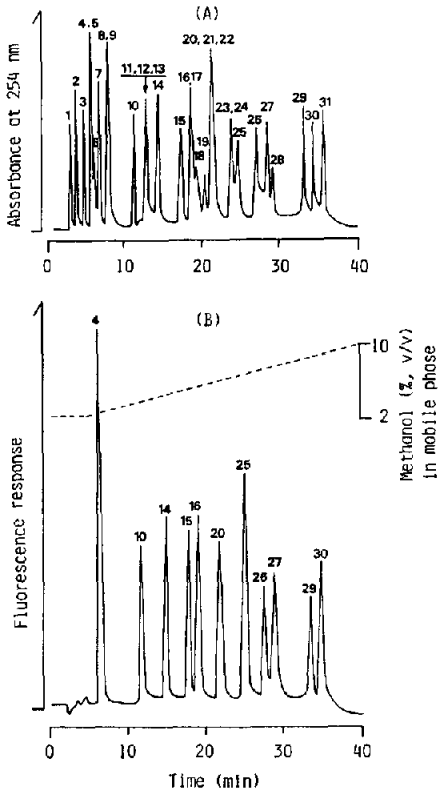

Fig. 2. Chromatograms obtained with (A) UV and (B) fluorescence detection of a standard mixture of various nucleic acid bases, nucleosides, nucleotides and related compounds. A mixture (50 μl) of the compounds (20 nmol/ml each) was injected onto the chromatograph. Peaks: 1 = cytosine; 2 = uracil; 3 = cytidine; 4 = guanine; 5 = hypoxanthine; 6 = CMP; 7 = uridine; 8 = thymine; 9 = UMP; 10 = GMP; 11 = adenine; 12 = xanthosine-s'-monophosphate (XMP); 13 = ionsine; 14 = guanosine; 15 = deoxyguanosine; 16 = GDP; 17 = thymidine; 18 = cAMP; 19 = AMP; 20 = dGMP; 21 = NAD; 22 = CTP; 23 = cCMP; 24 = UTP; 25 = 9-ethylguanine; 26 = GTP; 27 = dGDP; 28 = adenosine; 29 = cGMP; 30 = dGTP; 31 = ATP.

zation. By the linear gradient elution with methanol (2–10%) in the presence of 10 m*M* phosphate buffer (pH 6.0) and 1.7 m*M* tetra-*n*-propylammonium phosphate (pH 6.0) as the paired ions in the mobile phase, however, not all the tested compounds that are considered to be present in biospecimens could be completely separated (Fig. 2A).

On the other hand, fluorescence detection after the postcolumn derivatization permitted the selective detection of only guanine and its nucleosides and nucleotides (Fig. 2B). The fluorescence derivatization conditions were studied in order to construct an on-line postcolumn reactor system. We previously found that the PGO reaction proceeds in weakly acidic maleate [14] and neutral phosphate [15] buffers.

The mobile phase that has been widely used for the separation of various nucleosides and nucleotides did not interfere with the fluorescence reaction in the reactor system. After HPLC separation, guanine and its nucleosides and nucleotides in the mobile phase could be converted to fluorescent derivatives by adding 60 m*M* PGO solution followed by heating at 80°C in a reaction coil for approximately 24 s.

A reaction temperature of 75–90°C was required to obtain maximum fluorescence (Fig. 3).

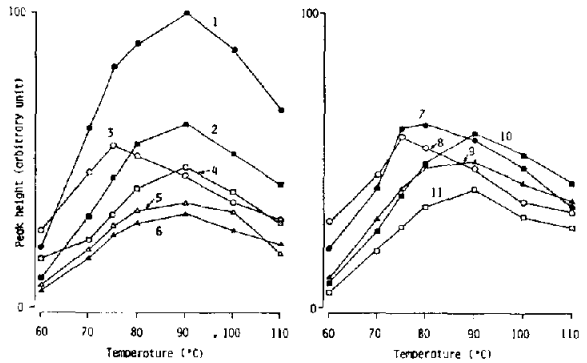

Fig. 3. Effect of the temperature of the reaction coil (3 m × 0.5 mm I.D.) on the postcolumn fluorescence derivatization of guanine and its nucleosides and nucleotides. A mixture (50 μl) of the compounds (10 nmol/ml each) was injected onto the chromatograph. Curves: 1 = guanine; 2 = GDP; 3 = guanosine; 4 = GMP; 5 = GTP; 6 = cGMP; 7 = 9-ethylguanine; 8 = deoxyguanosine; 9 = dGTP; 10 = GDP; 11 = dGDP.

The peak heights were maximal at 75°C for guanosine, deoxyguanosine and 9-ethylguanine, and at 90°C for the other compounds. As a compromise a temperature of 80°C was selected for the reactor system.

A high concentration of PGO (20–90 m*M*) was required to obtain highly fluorescent compounds. However, PGO is slightly soluble in water (approximately 0.2% solubility at room temperature). Methyl cellosolve or dimethyl sulfoxide was used as solvent (15–25%, v/v in the reaction mixture) in the manual derivatization reaction [14,15]. However, higher concentrations (>10%, v/v) of these solvents in the PGO solution resulted in an increased base line noise in the chromatograms. Therefore, we used a mixture of methyl cellosolve and water (1:9, v/v) as the solvent with which we could prepare a 60-m*M* PGO solution (nearly maximum solubility). The reactor system did not detect the following commercial substances (1.0 nmol each on column); 20 different L-α-amino acids, sugars (glucose, galactose, galactosamine, N-acetylgalactosamine), vitamins (nicotinamide, nicotinic acid, ascorbic acid), steroids (estrone, cholesterol), and amines (histamine, spermine, spermidine).

The calibration graphs of guanine and its nucleosides and nucleotides (20, 50, 100, 250 and 500 pmol on column) were all linear. The limits of detection at a signal-to-noise ratio of 3 for the compounds were in the range 3.2–10.0 pmol per 20-μl injection volume. The correlation coefficients (r) of the calibration curves for the compounds were all greater than 0.996 ($n = 2$ each plot).

3.2. Quantitative analysis in biological samples

Fig. 4 shows typical chromatograms obtained from a human erythrocyte sample. Peaks corresponding to GDP, GTP and I.S. in the sample were detected fluorimetrically (Fig. 4B). The peaks were identified on the basis of their retention times compared with the standard compounds and also by co-chromatography of the standards and the samples. In addition, the

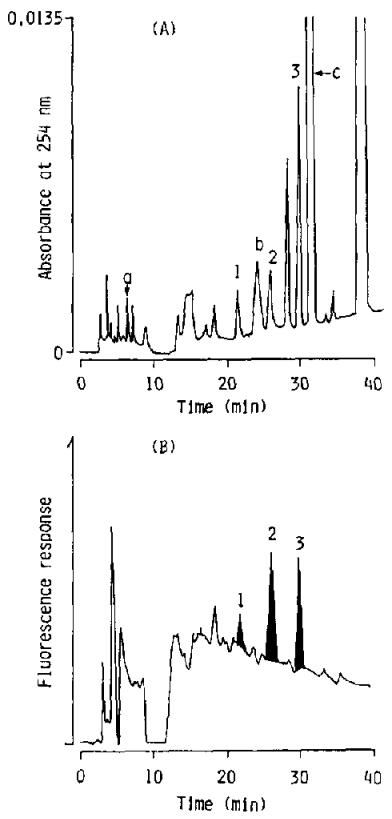

Fig. 4. Chromatograms obtained with (A) UV and (B) fluorescence detection in the HPLC of human erythrocytes. Peaks (concentration, nmol/ml): 1 = GDP (9.6); 2 = I.S., (40.0); 3 = GTP (36.8). Peaks a, b and c were eluted at the same retention times as those of guanine, dGMP and dGDP, respectively.

shaded peaks in the chromatogram were found only when the PGO reagent was added to the column eluate in the reactor system. Therefore, other peaks in the chromatogram were probably substances in the sample with native fluorescence. Biogenic 9-ethylguanine was not detected in the erythrocyte sample, and thus this compound was used as an I.S.

On the other hand, many peaks were found in the chromatogram obtained with UV detection of the same sample (Fig. 4A). Peaks a, b and c were detected at the retention times of guanine, dGMP and dGDP, respectively. However, these peaks were not found with the postcolumn fluorescence detection; and thus the UV-detectable peaks do not correspond to the guanine nucleosides and nucleotides. The proposed fluorescence detection allowed the quantitative determination of the nucleotides without interferences from biomatrices.

The concentrations of GTP and GDP in healthy human erythrocytes ($n = 8$) were determined by the present postcolumn detection method (Table 1). The results for the determination of GDP were in good agreement with published data [16,17]. However, the values determined for the GTP concentration were slightly lower than the published data [16,17]. A plot of the ratios of the peak heights of the fluorescent guanine nucleotides added to the erythrocyte sample to that of the I.S. *versus* their concentrations was linear in the range 1.0–100 nmol/ml in the sample. Recoveries of the compounds (250 pmol each) added to 100 μl of sample were 90–100%.

The sensitivity of the proposed HPLC method was comparable to that of other HPLC methods with UV detection. However, the present method has the selectivity necessary for the facile quantification of guanine nucleotides in human erythrocytes. This study provides the first practical HPLC method based on postcolumn fluorescence derivatization for the determination of biogenic nucleotides.

Table 1
Concentrations of GDP and GTP in erythrocytes from healthy persons

Sex[a]	Age (year)	Concentration (nmol/ml)	
		GDP	GTP
M	28	20.8	44.0
M	28	11.2	43.2
M	27	28.0	52.0
M	26	10.4	35.2
M	24	23.2	40.8
F	29	9.6	36.8
F	24	16.0	32.8
F	22	18.4	36.8
Mean ± S.D.		17.2 ± 6.2	40.2 ± 5.8

[a] M = male; F = female.

4. Acknowledgement

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