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High-performance liquid chromatography of guanine and its nucleosides and nucleotides by pre-column fluorescence derivatization with phenylglyoxal reagent

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ABSTRACT

A pre-column fluorescence derivatization method for the high-performance liquid chromatographic determination of guanine and its nucleosides and nucleotides is described. The compounds are converted into fluorescent derivatives by reaction with phenylglyoxal in a phosphate buffer (pH 6.0) at 37°C for 15 min. The derivatives are separated on a reversed-phase column (TSKgel ODS-120T) by gradient elution of acetonitrile in mobile phase containing 5 m*M* phosphate buffer (pH 6.5), and then detected fluorimetrically. The derivatization results in the guanine-containing compounds eluting as single fluorescent peaks. The method is highly selective and sensitive; the limits of detection for the compounds tested are 140–720 fmol per 100-μl injection volume.

INTRODUCTION

A sensitive quantification method for nucleic acid-related compounds is becoming increasingly important in studies of cellular proliferation and metabolism [1–3]. Nucleic acid bases and nucleos(t)ides have generally been measured by methods based on high-performance liquid chromatography (HPLC) with UV detection [4–8] or native fluorescence detection [9,10] using ion-exchange or reversed-phase columns. However, to measure these compounds in complex biological samples such as mammalian body fluids and tissues using such methods is difficult because of their limited sensitivity and low selectivity and because many other biogenic substances interfere with the detection in the chromatography.

An HPLC method with dual-electrochemical detection has been reported for the sensitive determination of guanine-containing nucleotides [11,12]; this detection system is operated at a high applied potential (+0.95 V *vs.* Ag/AgCl) and permits the nucleotides to be detected at the picomole level. However, there are problems in the detection, owing to the disturbance by many detectable biogenic substances at the high voltage and also the unstable noise level depending on composition of the mobile phase for HPLC.

Selective fluorimetric detection is possible for the HPLC determination of nucleos(t)ides,

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provided that the fluorogenic reagent used for the derivatization shows a high molecular recognition specificity to one of the nucleic acid bases; chloroacetaldehyde [13–15] and bromoacetaldehyde [16,17] have been used for the selective detection of adenine nucleos(t)ides in HPLC, which permits their quantification in pico- and subpicomole quantities on-column [14,15,17].

We reported previously that phenylglyoxal (PGO) reacts with guanine-containing compounds to produce fluorescent derivatives and the reaction is applicable to the manual spectrofluorimetric determination of the compounds [18]. However, the structures of the fluorescent derivatives remained unknown, and the derivatives yielded from some of the guanine-containing compounds resulted in multiple peaks when they were subjected to reversed-phase HPLC.

This paper describes the derivatization reaction conditions that can produce single fluorescent peaks from guanine and its nucleos(t)ides in the HPLC of their derivatives, and an HPLC method with pre-column derivatization utilizing these reaction conditions for the selective and sensitive determination of these compounds.

EXPERIMENTAL

Chemicals and solutions

Nucleic acid bases and nucleos(t)ides were purchased from Seikagaku Kogyo (Tokyo, Japan). PGO monohydrate was from Aldrich (Milwaukee, WI, USA). Water was deionized and then distilled prior to use. Other chemicals were of analytical reagent grade.

A guanine solution (1.0 μmol/ml) was prepared by dissolving guanine hydrochloride in 0.1 *M* sodium hydroxide and diluting twenty-fold with water. The solution was diluted with water to 100 nmol/ml, and then stored in a refrigerator (−20°C). The stock solutions (100 nmol/ml each) of other nucleic acid bases and nucleos(t)ides were prepared with water. A mixture of guanine and its nucleos(t)ides (5 nmol/ml each) and/or a 5 nmol/mol solution of each compound were used within a day for the experiments of the derivatization and HPLC separation. PGO solution (0.1 *M*) was prepared in dimethyl sulphoxide (DMSO).

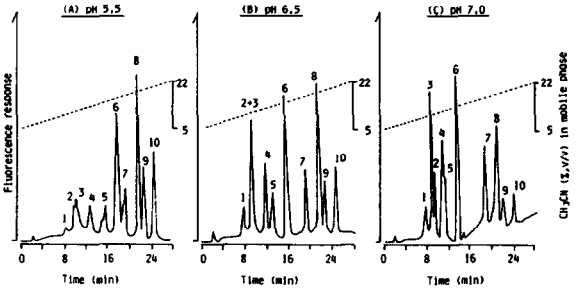

Fig. 1. Chromatograms of a standard mixture of guanine and its nucleos(t)ides (250 pmol of each per injection volume). HPLC conditions were as described in the Experimental section, but the pH of the phosphate buffer in the mobile phase was changed to (A) 5.5, (B) 6.5 and (C) 7.0. Peaks: 1 = GTP; 2 = GDP; 3 = dGTP; 4 = dGDP; 5 = GMP; 6 = dGMP; 7 = cGMP; 8 = guanine; 9 = guanosine; 10 = deoxyguanosine.

Derivatization procedure

A 200-μl portion of sample solution was placed in a test tube, to which were added 100 μl each of 0.1 *M* PGO and 50 m*M* phosphate buffer (pH 6.0). The mixture was warmed at 37°C for 15 min. A 100-μl portion of the final reaction mixture was used for HPLC.

HPLC system and its operating conditions

The HPLC system consisted of a Hitachi 638-30 chromatograph equipped with a programming controller for gradient elution, a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Hitachi F-1000 spectrofluorimeter fitted with a 25-μl flow cell. The column was a TSKgel ODS-120T (particle size 5 μm; 150 mm × 4.6 mm I.D.; Tosoh, Japan). The column temperature was ambient (24 ± 4°C). For the separation of the fluorescent derivatives, a gradient elution of acetonitrile (5–22%, v/v) in an aqueous mobile phase containing 5 m*M* phosphate buffer (pH 6.5) was carried out over 28 min at a flow-rate of 1.0 ml/min (see Fig. 1). The fluorescence intensity of the eluate was monitored at 510 nm (emission) with excitation at 360 nm.

RESULTS AND DISCUSSION

HPLC separation

A standard mixture of guanine, guanosine, deoxyguanosine, GMP, cGMP, dGMP, GDP,

dGDP, GTP and dGTP (ten species in all) was subjected to the fluorescence derivatization and the derivatives were then separated by reversed-phase HPLC. The derivatives were retained on the ODS column and eluted within 28 min by a linear gradient elution of acetonitrile in the mobile phase (Fig. 1). All of the eluates from their peaks showed almost identical fluorescence excitation (maxima, around 360 nm) and emission (maxima, around 510 nm) spectra.

The pH of the buffer in the mobile phase affected the retention time and peak heights (Fig. 1). At the investigated pH in the range 3.5–8.5, the complete separation of the derivatives of the ten species of guanine and its nucleos(t)ides could not be attained. With increasing pH of the buffer, the derivatives of the nucleotides were eluted earlier than those of guanine and nucleosides. A relatively good separation was achieved at pH 6.5 or 7.0 (Fig. 1B and C). The fluorescent peaks resulting from the nucleotides were higher at higher pH than at lower pH. In contrast, the peaks due to guanine and its nucleosides fluoresced more intensely at weakly acidic pH.

At pH 6.5, higher concentrations of the phosphate buffer in the range 2.5–10 m*M* caused early elution of all the derivatives, though no significant change in the separation profile was found at different concentrations of buffer. However, the maximum peak heights were attained at phosphate buffer concentration 2.5–5.0 m*M*. In this study, 5 m*M* phosphate buffer (pH 6.5) in the mobile phase was employed for the HPLC separation.

Fluorescence derivatization

The optimum conditions that could afford a single fluorescent derivative from each of the guanine-containing compounds were different from those previously reported for the manual spectrofluorimetric method [18]. Under the previously described conditions, *i.e.* heating with PGO in 11 m*M* maleate buffer (pH 4.0) at 60°C for 30 min, the phosphate moieties of the nucleotides were partially hydrolysed, and two or more fluorescent peaks from each nucleotide were observed in the chromatogram. For example, derivatization of GTP as described previously [18] resulted in three peaks due to GTP, GDP and GMP.

However, modification of the reaction conditions to milder ones resulted in single fluorescent peaks for each guanine-containing compound. The optimum derivatization conditions were reaction with PGO at 37°C for 15 min in 12.5 m*M* phosphate buffer. In this experiment, the derivatization of GTP and dGTP (5 nmol/ml solution) resulted in minor peaks corresponding to GDP and dGDP, respectively, as well as the major peaks due to the nucleotides. The heights of these minor peaks were approximately 3% of the height of the major peaks of GTP and dGTP. This may be the result of spontaneous degradation of the nucleotides during sample preparation and storage, because the 100 nmol/ml solutions of GTP and dGTP, after storage at −20°C for 1 week, contained the corresponding nucleotide diphosphates at the 3% level, as determined when the non-derivatized samples were subjected to reversed phase ion-pair HPLC [8] with UV detection (254 nm).

Under the above-mentioned conditions, the pH of the reaction mixture had greatly affected the fluroescence production (Fig. 2). Maximum and stable peak heights were attained at phosphate buffer pH 6.0. The concentration of the phosphate buffer (pH 6.0) also influenced the fluorescence derivatization (Fig. 3). Most peak heights were greatest at a concentration range of 10–15 m*M* in the reaction mixture. Thus, 50 m*M*

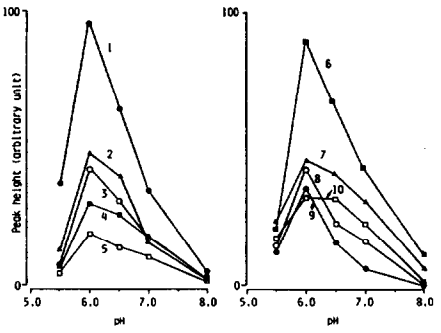

Fig. 2. Effect of the pH of the phosphate buffer in the reaction mixture on the fluorescence derivatization. HPLC conditions were as in Fig. 1B. Curves: 1 = guanine; 2 = GDP; 3 = cGMP; 4 = GMP; 5 = GTP; 6 = dGMP; 7 = dGDP; 8 = deoxyguanosine; 9 = guanosine; 10 = dGTP.

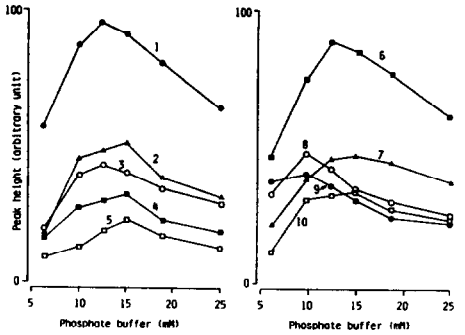

Fig. 3. Effect of the concentration of phosphate buffer (pH 6.0) in the reaction mixture on the fluorescence derivatization. HPLC conditions were as in Fig. 1B. For curves, see Fig. 2.

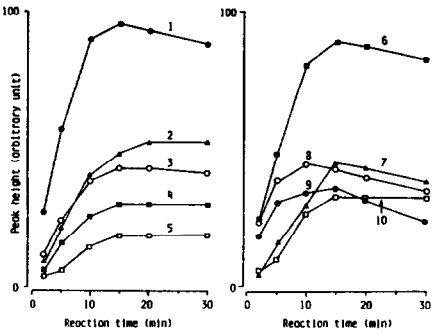

Fig. 4. Effect of reaction time at 37°C on the fluorescence derivatization. HPLC conditions were as in Fig. 1B. For curves, see Fig. 2.

phosphate buffer (pH 6.0) was used for the derivatization reaction.

The reaction temperature of 37°C was effective in obtaining single fluorescent peaks from the compounds and the reaction time of 15 min generally provided the compounds with the highest peaks (Fig. 4). On the other hand, higher temperatures in the range 20–60°C allowed the derivatization to proceed more rapidly, and at 60°C the maximum peak heights were achieved by heating for 5 min. However, the peak heights were approximately 60% of these obtained by heating at 37°C for 15 min.

The organic solvent used to dissolve PGO also influenced the production of the fluorescent derivatives (Table I). Of the solvents tested, DMSO resulted in guanine-containing compounds with the highest peaks. A DMSO concentration of 25% (v/v) in the reaction mixture served to give the greatest production of the fluorescent derivatives.

Other nucleic acid bases and nucleos(t)ides (500 pmol each on-column; adenine, cytosine, uracil, thymine, hypoxanthine, xanthine, adenosine, cytidine, uridine, thymidine, ATP, CTP, UTP, ADP, AMP, CDP, CMP, dATP, dADP and dATP) did not provide any fluorescent derivatives when treated under the present derivatization conditions.

TABLE I

EFFECT OF ORGANIC SOLVENT USED TO DISSOLVE PGO ON THE FORMATION OF THE FLUORESCENT DERIVATIVES OF GUANINE AND ITS NUCLEOS(T)IDES

Compound	Peak height				
	DMSO	N,N-Dimethyl-formamide	Acetonitrile	Methyl cellosolve	Methanol
GTP	19	0	1	3	7
dGTP	34	1	1	7	14
GDP	51	3	3	10	18
dGDP	48	3	14	14	19
GMP	31	4	7	21	14
dGMP	93	51	34	51	31
cGMP	45	6	17	25	14
Guanine	100[a]	62	68	64	45
Guanosine	37	12	5	38	27
Deoxyguanosine	45	8	12	42	29

[a] The peak height for guanine obtained with DMSO was taken as 100.

Reproducibility, calibration graph and detection limit

The reaction products of guanine and its nucleos(t)ides were relatively stable because the peak heights (90–98%) of the derivatives did not change when the final reaction mixture was kept for 1 h in an ice-water bath after the reaction. The relative standard deviations of the peak heights for the compounds (50 pmol each on each column) were 4.0–6.0% ($n = 10$ in each instance).

Peak heights were used for the quantification. The calibration graphs of the guanine-containing compounds (0–250 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 140–720 fmol per 100-μl injection volume.

CONCLUSIONS

The present method is highly selective for guanine-containing compounds, and offers the necessary sensitivity to permit the determination of biogenic guanine-containing nucleos(t)ides in mammalian tissues. In addition, fluorescence derivatization with PGO should be useful for the fluorescence labelling of high-molecular-mass nucleic acids, DNA and RNA. This work is now in progress in our laboratories.

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