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Note

Gas chromatographic analysis of hypoxanthine and guanine

HIROSHI IWASE, TOSHIKO KIMURA, TAEKO SUGIYAMA and ASAO MURAI
Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Kanagawa (Japan)
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Many methods for the analysis of nucleic acid components have been developed, including ion-exchange[1,2], paper[3], thin-layer[4,5] and gas chromatography[6–13].

Gas chromatography has been used for the analysis of many substances because of its simplicity, rapidity, accuracy and sensitivity. As nucleic acid components are not sufficiently volatile to permit direct analysis, they must be converted into suitable volatile derivatives prior to gas chromatography. Since the gas chromatographic analysis of nucleic acid components was reported by Miles and Fales[6] in 1962, a number of derivatization methods have been investigated by many workers. However, gas chromatography could not be used for the routine analysis of nucleic acid components because experimental conditions for the preparation of sufficiently volatile derivatives of nucleic acid components have not been investigated in detail. This paper deals with the experimental conditions for the preparation of trimethylsilylated derivatives with six different silylating agents at room temperature and the effect of moisture and cations on the trimethylsilylation of hypoxanthine and guanine.

EXPERIMENTAL

Apparatus and conditions

An F & M Model 402 gas chromatograph equipped with dual flame ionization detectors linked with a Honeywell recorder was used, with a glass column (4 ft. × ¼ in. O.D.) packed with 4% SE-30 (Applied Science Labs., State College, Pa., U.S.A.) on Diatoport S (Hewlett-Packard, Avondale, Pa., U.S.A.). The column temperature was 205° and helium was used as the carrier gas at the flow-rate of 60 ml/min.

Reagents and material

Hypoxanthine and guanine were obtained from Ajinomoto Co. (Kawasaki, Japan). N,O-Bis(trimethylsilyl)acetamide (BSA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), N-trimethylsilyldimethylamine (TMSDMA) and N-trimethylsilyldiethylamine (TMSDEA) were purchased from Pierce (Rockford, Ill., U.S.A.). N-Methyl-N-trimethylsilylacetamide (MSA), N-trimethylsilylimidazole (TSIM) and trimethylchlorosilane (TMCS) were purchased from Tokyo Kasei (Tokyo, Japan). Pyridine was obtained from Koso Chemical (Tokyo, Japan) and was dried over sodium hydroxide pellets. *n*-Nonadecane was obtained from Koso Chemical. Hypovial was purchased from Pierce.

Preparation of trimethylsilylated derivatives

A reaction mixture containing 5 mg of hypoxanthine and guanine, 1 ml of each silylating reagent, 1 ml of pyridine containing 2 mg of *n*-nonadecane as an internal standard (I.S.) and a few drops of TMCS (as catalyst) was stirred occasionally at room temperature. A 3-μl portion of this solution was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Reaction time

In order to examine the difference of reaction time in trimethylsilylation between hypoxanthine and guanine, and the necessity for heating, periodical changes in the solution were studied by gas chromatography. The reaction time for the formation of the trimethylsilylated derivatives with each silylating agent was tested. Evaluation of the optimum reaction time was made by comparing the peak heights of hypoxanthine and guanine relative to that of *n*-nonadecane. The results are shown in Figs. 1–5 and the gas chromatogram of hypoxanthine and guanine is shown in Fig. 6.

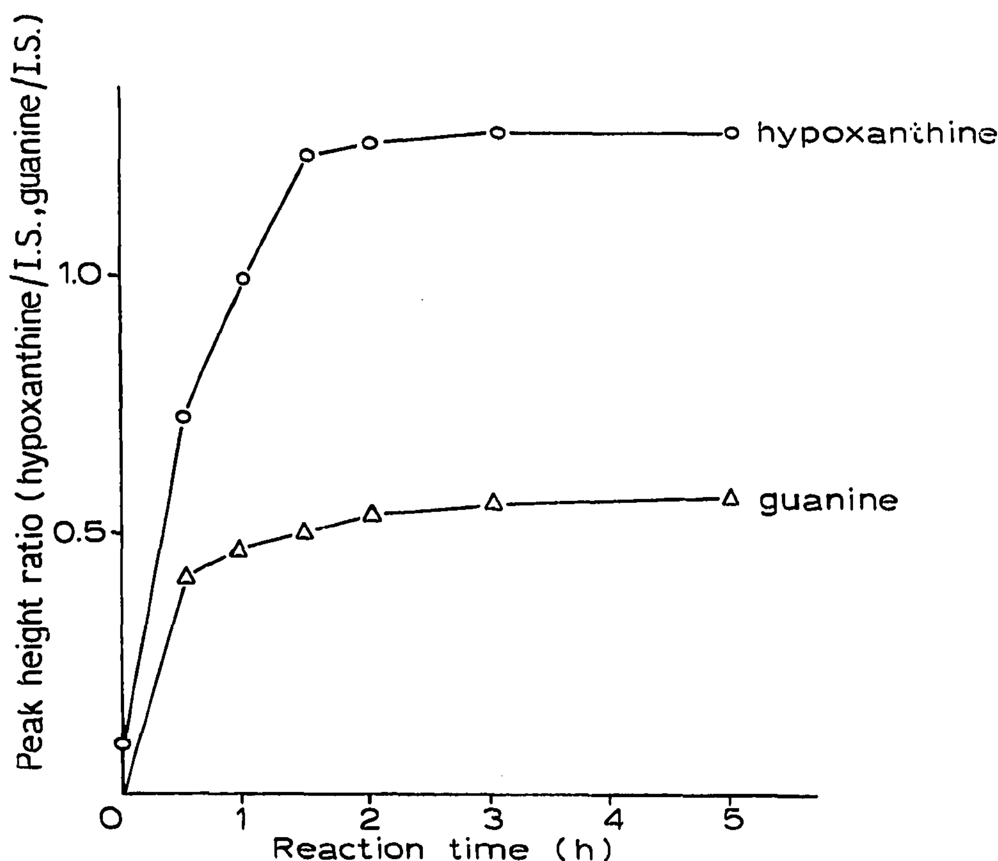
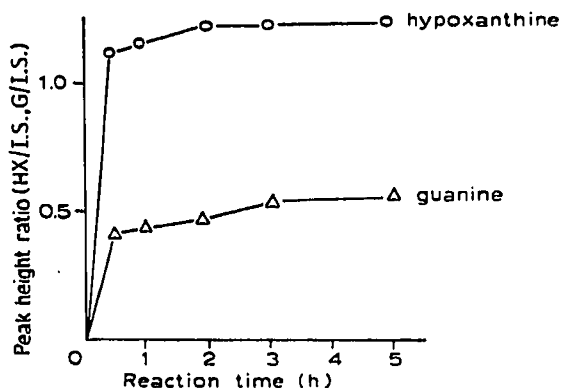


Fig. 1. Trimethylsilylation of hypoxanthine and guanine with BSA at room temperature. I.S. = Internal standard.



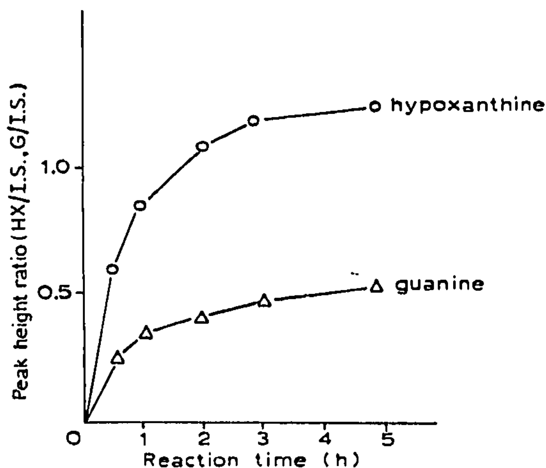
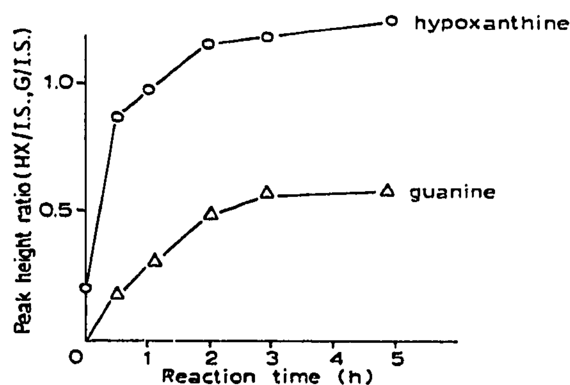


Fig. 2. Trimethylsilylation of hypoxanthine and guanine with TMSDEA at room temperature.

Fig. 3. Trimethylsilylation of hypoxanthine and guanine with BSTFA at room temperature.



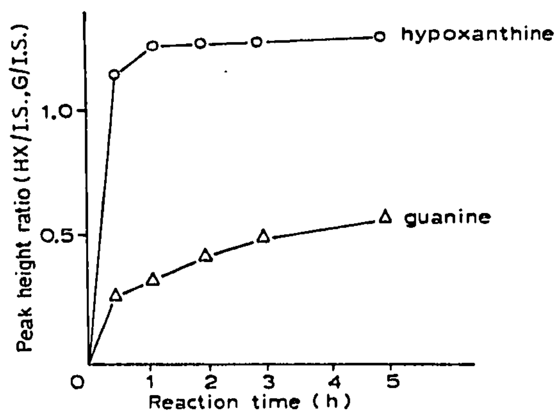


Fig. 4. Trimethylsilylation of hypoxanthine and guanine with MSA at room temperature.

Fig. 5. Trimethylsilylation of hypoxanthine and guanine with TMSDMA at room temperature.

As shown in Figs. 1–5, trimethylsilylation proceeds when the mixture is left at room temperature for 3–5 h in all silylating agents except for TSIM, and progress of the reaction is slightly faster in hypoxanthine than in guanine. The time required until the curves in Figs. 1–5 reach a constant value corresponds to the time at which the crystal in the reaction solution dissolves completely. At this point, heating of the reaction solution does not increase the peak heights. It may be considered that the reaction has been completed when the crystals dissolve completely. If the pyridine solution is heated to boiling, crystals dissolve in 5–10 min and trimethylsilylation is completed in that time.

Comparison of the ratio of trimethylsilylation showed that the silylating reagents of TMS-amines reacted faster than those of the TMS-amides, except for the rate of trimethylsilylation of guanine with BSA, in a short time (30 min).

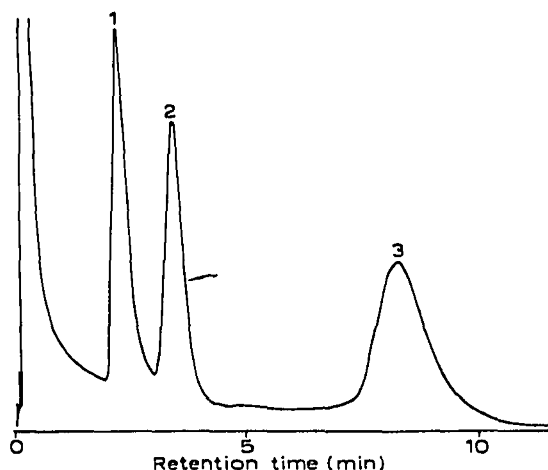


Fig. 6. Gas chromatogram of trimethylsilylated hypoxanthine and guanine with BSA. 1, Hypoxanthine; 2, internal standard (*n*-nonadecane); 3, guanine. Column, glass (4 ft. × ¼ in.), packed with 4% SE-30 on Diatoport S; column temperature, 205°; carrier gas, helium at the flow-rate of 60 ml/min.

The crystals in the sample solution treated with TSIM were dissolved completely, while peaks of hypoxanthine and guanine were not observed. It may be considered that the trimethylsilylated derivatives of hypoxanthine and guanine cannot be formed with TSIM.

Effect of moisture

The effect of moisture on the trimethylsilylation of hypoxanthine and guanine with BSA and TMCS system was examined. Amounts of water were added to samples containing 5 mg each of hypoxanthine and guanine, and trimethylsilylation was carried out for 10 min until the pyridine solution was boiled. The results are shown in Table I.

It can be seen in Table I that moisture has an effect on trimethylsilylation. It is necessary that the trimethylsilylation of hypoxanthine and guanine be carried out under anhydrous conditions. When 100 μl of water were added to the sample, peaks of hypoxanthine and guanine were not detected. However, by addition of a further

TABLE I

EFFECT OF MOISTURE ON TRIMETHYLSILYLATION

Water added		*Peak height ratio*	
Added as	*Volume (μl)*	*Hypoxanthine/I.S.*	*Guanine/I.S.*
None	—	1.28	0.57
Water	10	1.24	0.54
Water	30	0.92	0.26
Water	100	0	0
Ammonia solution (28%)	30	1.13	0.34
Hydrochloric acid (35%)	30	1.05	0.32

1 ml of BSA to the sample followed by heating for 5–10 min until the pyridine solution was boiled, peaks of hypoxanthine and guanine appeared and they had almost the same peak heights as those of the sample treated under anhydrous conditions.

Effect of cations

The effect of several cations on the formation of trimethylsilylated hypoxanthine and guanine with BSA and TMCS was examined. The trimethylsilylation was carried out as follows: 1 ml of an aqueous solution containing 0.2 mg/ml of cation added as chloride was added to samples containing accurately weighed, 5-mg amounts of each of hypoxanthine and guanine, followed by addition of 2–3 ml of methylene chloride to the samples and repeated evaporation of any remaining water *in vacuo* to an azeotrope. Then 1 ml of pyridine containing *n*-nonadecane, 1 ml of BSA and a few drops of TMCS were added to the samples. The samples were heated at 115° in a mantle heater for about 10 min under anhydrous conditions, then cooled to room temperature. The results are shown in Table II.

TABLE II

EFFECT OF CATIONS ON TRIMETHYLSILYLATION

Cation	*Peak height ratio*	
	Hypoxanthine/I.S.	*Guanine/I.S.*
None	1.28	0.57
Na^+	1.15	0.35
K^+	1.18	0.49
NH_4^+	1.28	0.58
Ca^{2+}	0.38	0.12
Mg^{2+}	0	0
Cu^{2+}	0.05	0.04

Table II indicates that cations, except for ammonium, have an effect on the trimethylsilylation. This is probably due to two factors: (1) reduced volatilization of hypoxanthine and guanine, and (2) reduced response due to chelation. Therefore, the trimethylsilylation of hypoxanthine and guanine should be carried out after removal of cations.

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