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DETERMINATION OF 6-METHYLADENINE IN DNA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HIDETAKA YUKI and HIDEKI KAWASAKI

Toho University, School of Pharmaceutical Science, Funabashi, Chiba 274 (Japan)

AKEMI IMAYUKI

Kotaro Kampo Co. Ltd., Nakatsuhamadori, Oyodoku, Osaka (Japan)

and

TAKEHIKO YAJIMA

Toho University, School of Pharmaceutical Science, Funabashi, Chiba 274 (Japan)

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SUMMARY

A method for the determination of 6-methyladenine (6MA) by high-performance liquid chromatography (HPLC) has been developed. DNA bases were separated by using the strong cation-exchange resin Zipax SCX. Purine bases were obtained by hydrolysis and dialysis of DNA and analysed by HPLC. 6MA in DNA from *Escherichia coli* was determined by the proposed method. It is suggested that the method could be applicable to analyses of 6MA from other biological sources.

INTRODUCTION

It has been suggested that a relationship exists between the formation of methylated bases in nucleic acids by carcinogenic methylating agents and the subsequent neoplastic transformation[1]. In this connection, we initiated studies on the determination of minor bases in nucleic acids and have already developed methods for the determination of 5-methylcytosine, 3-methylcytosine[2] and 7-methylguanine[3] by high-performance liquuid chromatography (HPLC).

6-Methyladenine (6MA), one of minor bases found in deoxyribonucleic acid (DNA), has been determined by a radioisotope method[4] that involves labelling of the base with [$^{14}CH_3$]methionine and by a UV method[5] after its separation by paper chromatography. The application of the former method, however, is limited because it requires *in vivo* labelling prior to analysis, and in the latter method it is difficult to separate minor bases from major bases that are present in large amounts. On the other hand, HPLC provides a superior method for application to nucleic acid bases in terms of resolution, reproducibility and sensitivity. This paper deals with the development of a method for the determination of 6MA by HPLC.

EXPERIMENTAL

Chemicals and reagents

All nucleic acid bases were purchased from Sigma (St. Louis, Mo., U.S.A.). Deionized distilled water was used and hydrochloric acid was distilled after diluting reagent-grade concentrated hydrochloric acid to 6 *N*. All other chemicals were of reagent grade and were obtained from commercial sources. Sources of the DNA used are given in Table III.

Quantitative analysis

HPLC was carried out in a Shimadzu–DuPont 840 liquid chromatograph equipped with a column (1 m × 2.1 mm) packed with Zipax SCX. The column was eluted with 0.1 *M* potassium dihydrogen orthophosphate (KH_2PO_4)–dipotassium hydrogen orthophosphate (K_2HPO_4) solution at a pressure of 40 kg/cm^2 at room temperature. The eluate was monitored with a UV detector (254 nm) at 0.01 a.u.f.s.

Determination of 6-methyladenine in DNA

Fifty milligrams of DNA were hydrolysed under Chargaff's conditions[6], *i.e.*, the DNA was dissolved in 15 ml of water, the pH was adjusted to 1.6 with 0.1 *N* hydrochloric acid and the total volume was adjusted to 20 ml with water previously adjusted to pH 1.6 with hydrochloric acid. The resulting mixture was dialysed against 500 ml of dilute hydrochloric acid (pH 1.6) at 37° for 26 h and 300 ml of outside fluids* containing purine bases were evaporated to dryness under reduced pressure. The residue was dissolved by addition of a small volume of dilute hydrochloric acid and the volume was adjusted to 2 ml with water. Aliquots (5 μl) of the solution were analysed by HPLC. Adenine and 6MA were determined after identifying 6MA by co-chromatography with an authentic specimen.

RESULTS AND DISCUSSION

Conditions for HPLC analysis

Nucleic acid bases are retained on cation-exchange resins to extents that depend on the pK_a values of each base and the hydrogen ion and buffer concentrations of the mobile phase. In this investigation, the strong cation-exchange resin Zipax SCX was employed and various buffer concentrations and pH values were tested using potassium phosphate buffer solution. Optimal conditions for the separation of 6MA from other bases were established by measuring the retention times of major nucleic acid bases and their derivative minor bases under various conditions. It was found that the best resolution was achieved at a buffer concentration of 0.1 *M* and pH 5.0. As shown in Fig. 1, 6MA was separated completely from other bases. As the content of adenine and guanine in the hydrolysate of DNA exceeded that of 6MA, a mixture of authentic adenine, guanine and 6MA in the proportions 100:100:1 was employed as a model hydrolysate for HPLC analysis. The results showed that the separation of 6MA from the other two bases even under such conditions was satisfactory.

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* The fluids present outside the dialysis bag after dialysis.

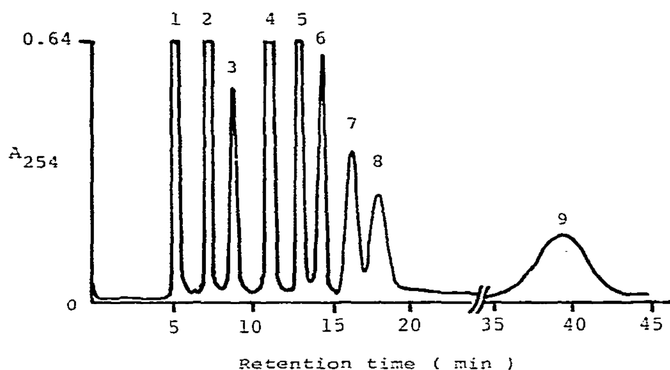

Fig. 1. Chromatogram of nucleic acid bases. Chromatograph, DuPont LC 840; column, Zipax SCX (1 m); mobile phase, 0.1 M KH_2PO_4–K_2HPO_4, pH 5.0; temperature, ambient; pressure, 40 kg/cm^2; detector, UV (254 nm); sensitivity, 0.64 a.u.f.s. Peaks: 1 = adenine; 2 = cytosine; 3 = O^6-methylguanine; 4 = N^6-methyladenine; 5 = 2-methyladenine; 6 = 5-methylcytosine; 7 = 1-methyladenine; 8 = 3-methylcytosine; 9 = N^6,N^6-dimethyladenine.

Calibration graphs

Average peak heights in HPLC for various concentrations of 6MA were obtained by injecting five 5-μl volumes each of solutions containing 60, 50, 40, 25, 8 and 0.8 ng of 6MA. As shown in Fig. 2, a straight line was observed for these six concentrations. 6MA was determined by using the least-squares line derived from the calibration graph. For the quantitation of adenine, the integrator count method was employed because of the different scales involved due to the presence of a large amount of this compound in DNA in comparison with 6MA. Five microlitres of solutions containing 750, 500, 250, 100 and 20 ng of adenine were subjected to HPLC in the same manner as for 6MA and a linear calibration graph was obtained.

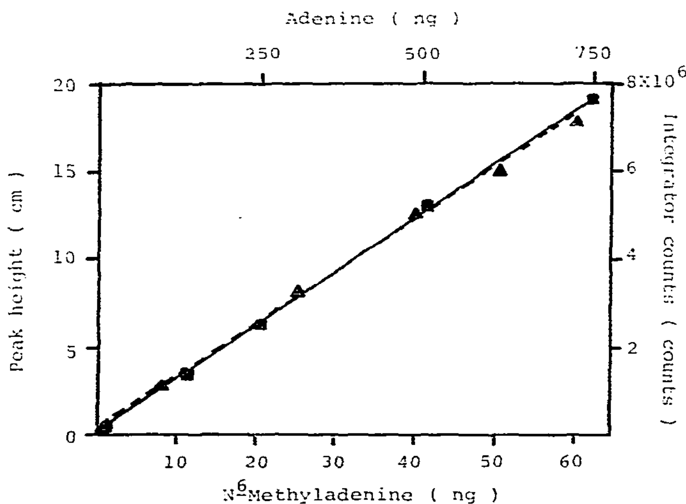

Fig 2. Calibration graph for 6MA (▲---▲) and adenine (■—■). 6MA was measured by the peak-height method and adenine by the integrator method.

Recovery of 6MA

Amounts of 5 mg each of 6MA and adenine were subjected to the entire process and their recoveries are given in Table I.

TABLE I

RECOVERY OF 6-METHYLADENINE AND ADENINE

Experiment No.	*Recovery (%)*	
	6MA	*Adenine*
1	99.2	95.1
2	90.2	100.1
3	92.4	85.5
4	87.1	96.7
5	97.1	93.9
Mean	93.2	94.3
Standard deviation	4.43	4.85
Coefficient of variation	4.75	5.14

Recovery of 6MA added to DNA

Known amounts of 6MA were added to 50 mg of calf thymus DNA and the mixture was subjected to the proposed procedure. A satisfactory recovery of 6MA (94.5%) was obtained, as shown in Table II, and the procedure was therefore applied to the determination of natural 6MA in DNA.

TABLE II

RECOVERY OF 6-METHYLADENINE ADDED TO DNA

6MA added (μg)	*6MA found (μg)*		*Recovery (%)*
0	—		—
92.3	82.5		89.4
96.0	96.0		100.0
96.0	94.7		98.6
96.0	94.7		98.6
96.0	82.3		85.7
		Mean:	94.5
		Standard deviation:	5.78
		Coefficient of variation:	6.12

Determination of 6MA in various DNA sources

Fifty milligrams of calf thymus DNA gave two large peaks for guanine and adenine in HPLC after hydrolysis. No peak corresponding to pyrimidines was observed under the condition employed. For *Escherichia coli* DNA, a peak corresponding to 6MA was observed (Fig. 3) and it was identified as 6MA by co-chromatography with an authentic sample. The amount of 6MA was determined to be 1.4% of that of adenine. No conversion of 1-methyladenine into 6MA was expected as the analysis was carried out under acidic conditions. It was also found that under the

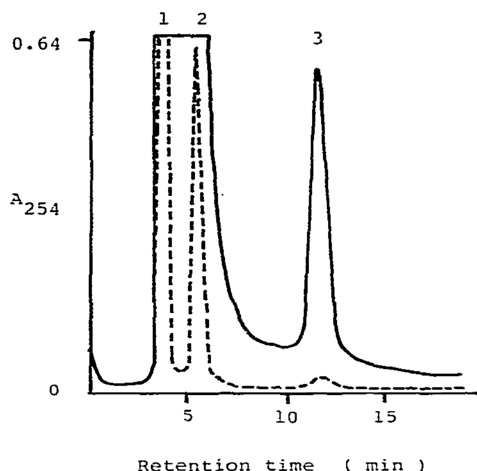

Fig. 3. Chromatogram of *E. coli* DNA purine bases. Chromatographic conditions as in Fig. 1, except sensitivity of detector: solid line, 0.01 a.u.f.s.; broken line, 0.64 a.u.f.s. Peaks: 1 = guanine; 2 = adenine; 3 = 6MA.

conditions employed 6MA was not detectabele in DNAs from the following sources: normal rat liver; rat liver administered intraperitoneally with carcinogenic methylating agents, dimethylnitrosamine (DMN) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG); rat hepatoma induced by 3'-dimethylaminoazobenzene (3'-DAB); and Ehrlich solid tumour (Table III). DNA from these sources was obtained by Kirby's method[7].

TABLE III

6-METHYLADENINE CONTENT IN VARIOUS DNAs

Origin of DNA	*6MA (% relative to adenine)*
Calf thymus (Sigma)	ND***
DMN-treated rat liver*	ND
MNNG-treated rat liver*	ND
Herring sperm (Sigma)	ND
Normal rat liver	ND
E. coli (Sigma)	1.4
Ehrlich solid tumour	ND
3'-DAB-induced rat hepatoma**	ND

* 30 mg/kg of DMN and MNNG were administered intraperitonealy to five male rats. After 8 and 3 h, respectively, the rats were killed.

** A diet containing 0.06% of 3'-DAB was fed to rats for 6 months.

*** ND = not detected (the detection limit of 6MA was 0.03% relative to adenine).

CONCLUSION

The determination of 6MA in DNA by HPLC has advantages over conventional methods in terms of high sensitivity, simplicity and rapidity. In addition, studies are in progress concerning the possibility of reducing the amount of samples further and also of applying the HPLC analysis to other minor bases.

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