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High-Performance Liquid Chromatographic Determination of 3-Methylcytosine in Deoxyribonucleic Acid Treated with Carcinogenic Methylating Agents *in Vitro* and *in Vivo*

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A high-performance liquid chromatographic method is described for the determination of 3-methylcytosine (m^3Cyt) in deoxyribonucleic acid (DNA). Calf thymus DNA was methylated with *N*-methyl-*N*-nitrosourea or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine *in vitro*. Methylated rat liver DNA was obtained by administration of dimethylnitrosamine to rats. The DNA samples were hydrolyzed with perchloric acid at 100 °C for 1 h, and after the removal of perchloric acid by the use of anion exchange resin, the filtrate was concentrated and loaded on a Dowex 1 × 2 anion exchanger. The eluted cytosine derivatives were collected and injected into a liquid chromatograph. The content of m^3Cyt in methylated DNA was successfully determined by this method. The amount of m^3Cyt increased with the dosage of methylating agent *in vitro*, but showed no significant dose-dependent increase *in vivo*.

Keywords——HPLC; 3-methylcytosine; *N*-methyl-*N*-nitrosourea; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; dimethylnitrosamine; deoxyribonucleic acid; methylation; modified base

Since Magee and Barnes reported the development of malignant liver tumors in rats as a result of administration of dimethylnitrosamine (DMN) in 1956,[1] the carcinogenic properties of methylating agents have been studied by various workers.[2,3] Although chemical modification of cellular nucleic acids by these agents is thought to play a critical role in the initiation of tumor induction, the details remain unclear. The major sites in nucleic acids that are attacked by the agents are the nitrogen atoms at position 7 of guanine, positions 1 and 3 of adenine and position 3 of cytosine, and the oxygen atom at position 6 of guanine.[4] In these studies, determination of modified bases has been carried out by radioisotope methods[5,6] with satisfactory results, except that the methods involve time-consuming column chromatographic operations and the possibility of biohazard caused by the radioisotopes. Recently many reports have dealt with the separation and determination of modified nucleic acid bases by high-performance liquid chromatography (HPLC)[7,8] in place of the radioisotope methods. The authors have already reported the determination of 5-methylcytosine (m^5Cyt), 3-methylcytosine (m^3Cyt), and cytosine (Cyt) in nucleic acids by HPLC.[9] Under the chromatographic conditions described in the previous report, m^3Cyt and 1-methyladenine (m^1Ade) were eluted so close together that large amounts of m^1Ade interfered with the precise quantitation of m^3Cyt. In the present work, the authors have established conditions for baseline separation of m^1Ade and m^3Cyt, and applied this method to the determination of m^3Cyt in deoxyribonucleic acids (DNAs) treated with such methylating agents as *N*-methyl-*N*-nitrosourea (MNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and DMN.

Materials and Methods

Reagents——Adenine was obtained from Kohjin Co. (Tokyo, Japan). 3-Methyladenine (m^3Ade) was purchased from Fluka AG Chemische Fabrik (Buchs, Switzerland). O^6-Methylguanine was synthesized in our laboratory by the method of Balsiger.[10] Other nucleic acid major bases and modified bases were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). m^3Cyt and m^5Cyt were provided as HCl salts. The methylating agents, MNU, MNNG and DMN were products of Nakarai Chemicals Ltd. (Kyoto, Japan). DNA was Type I grade of Sigma Chemical Co. All other reagents were of reagent grade quality. Deionized and glass-distilled water was used throughout the present experiments.

Chromatographic Conditions——The liquid chromatograph consisted of a Shimadzu LC-3A delivery system, a Shimadzu SIL-1A injection valve, and two 500 × 2.1 mm i.d. analytical columns, joined in series, and packed with Zipax SCX. The detection system comprised a Shimadzu UVD-2 photometric detector set at a wavelength of 254 nm, and a Shimadzu Chromatopac C-R1A plotter-integrator. The mobile phase for the determination of m^3Cyt was 0.035 M KH_2PO_4–K_2HPO_4 buffer solution (pH 6.8), and the mobile phase for Cyt was 0.1 M H_3PO_4–KH_2PO_4 buffer solution (pH 4.5). Elution was performed at ambient temperature at a flow-rate of 0.4 ml/min. The detector was used in the range of 0.01 absorbance unit full scale, and determination was performed by the peak area method. Standard curves were prepared by the conventional method.

Sample Preparation——Methylated DNA was hydrolyzed with perchloric acid at 100 °C for 1 h as described in a previous paper.[9] Perchloric acid was removed by addition of anion exchange resin, Amberlite IR-45 (OH^- form). The solution was filtered and the filtrate was evaporated to dryness. The residue was dissolved by addition of the mobile phase and an aliquot of the solution was injected into the liquid chromatograph. In the analysis of *in vivo* methylated DNA, the above procedure was scaled up ten times, and followed by fractionation with Dowex 1 × 2 as described previously.[9] The fraction containing cytosine derivatives was evaporated to dryness and the residue was dissolved in the mobile phase. An aliquot of the solution was injected into the chromatograph.

Methylation of DNA *in Vitro*——Calf thymus DNA (60 mg) was dissolved in 12 ml of Tris–HCl buffer solution (pH 8.0). MNU was dissolved in ethanol and added to give a designated molar ratio with respect to nucleotide of DNA. The reaction was carried out at 37 °C with stirring in the dark until evolution of N_2 was no longer observed (about 30 min). The solution was cooled, and 12 ml of 2.5 M sodium acetate was added. The methylated DNA was precipitated in fibrous form with 50 ml of ethanol, washed three times with 1 ml of ethanol, and dried in a vacuum desiccator ($CaCl_2$). MNNG was dissolved in dimethylformamide and reacted in the same manner.

Methylation of DNA *in Vivo*——A physiological saline solution of DMN was administrated intraperitoneally to Wistar albino male rats (10 rats/group, 300 g average body weight, 10 weeks of age) at a single dose from 20 to 250 mg/kg body weight. The animals were sacrificed at 5 h after administration of DMN. The livers were removed and placed on solid CO_2 immediately, then DNA was extracted by Kirby's method.[11]

Results and Discussion

Conditions for HPLC

Figure 1 shows the separation of m^1Ade and m^3Cyt under the optimal conditions. As the pK_a values of the majority of modified bases ranged from 3 to 5, we used phosphate buffers of pH 4.5 to 5.0 as a mobile phase. Under these conditions, the bases were suitably protonated for retention and separation on Zipax SCX. However, as the pK_a values of m^1Ade and m^3Cyt are 7.2 and 7.4, respectively, an exceptional mobile phase was necessary to obtain base-line separation of these two bases. A systematic study of retention *versus* pH and ionic strength on the column showed that effective separation could be obtained with 0.035 M KH_2PO_4–K_2HPO_4 (pH 6.8). Table I shows the retention times of the nucleic acid bases under these conditions. Other bases listed in the table did not interfere with the peak of m^3Cyt, and separation of a hydrolyzate of DNA was achieved in less than 30 min. The calibration plot for m^3Cyt was linear from 2.0 to 130 ng ($r = 0.9995$). The limit of detection was 1.7 ng for m^3Cyt. Repeated injections ($n = 10$) of 20 ng of m^3Cyt gave an average relative standard deviation of 2.5% for peak area and 1.1% for retention time.

Sample Preparation

The new chromatographic conditions enabled us to determined the content of m^3Cyt in DNA without fractionation of the cytosine derivatives on Dowex 1 × 2, as required in the previous method. After the removal of perchloric acid from the hydrolyzate with Amberlite

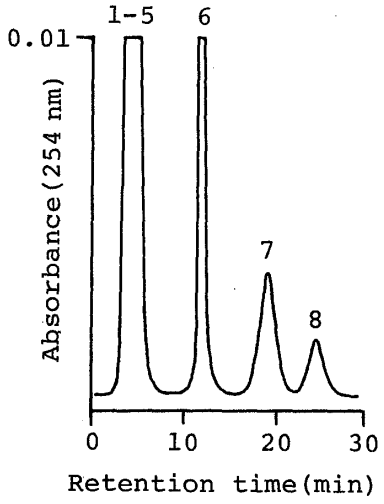

Fig. 1. Chromatogram of Nucleic Acid Bases

1, adenine; 2, guanine; 3, cytosine; 4, thymine; 5, O^6-methylguanine; 6, 3-methyladenine; 7, 1-methyladenine; 8, 3-methylcytosine.

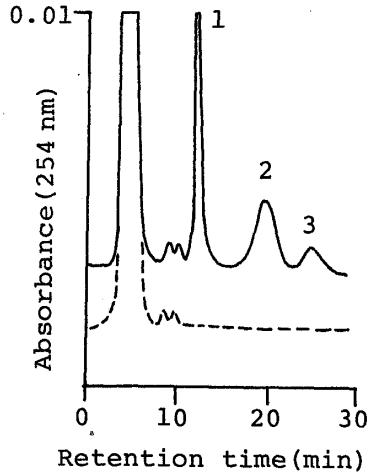

Fig. 2. Chromatograms of Bases from DNA Treated with MNU (1 : 100) *in Vitro*

1, 3-methyladenine; 2, 1-methyladenine; 3, 3-methylcytosine. ----, untreated.

TABLE I. Retention Times of Various Bases

Base	Retention time (min)
Adenine	4.11
Cytosine	4.25
Guanine	4.03
Thymine	4.18
Uracil	4.06
1-Methyladenine	18.52
3-Methyladenine	11.45
N^6-Methyladenine	4.40
N^6, N^6-Dimethyladenine	5.99
N^6-Isopentenyladenine	12.12
3-Methylcytosine	23.99
5-Methylcytosine	4.85
1-Methylguanine	4.08
O^6-Methylguanine	4.31
7-Methylguanine	4.11
3-Methyluracil	4.06

Mobile phase, 0.035 M KH_2PO_4–K_2HPO_4 (pH 6.8).

IR-45, the sample solution was concentrated and injected into the liquid chromatograph. In the case of the analysis of rat liver DNA methylated *in vivo* the amount of m^3Cyt was so small that scale-up of the procedure was needed for the detection of m^3Cyt. Consequently the fractionation with Dowex 1 × 2 was necessary to avoid interference by the peaks of major bases on the chromatogram. To determine the recovery, authentic m^3Cyt was subjected to the same procedure. The recoveries of m^3Cyt from Amberlite IR-45 and Dowex 1 × 2 were 96.1 and 97.1%, respectively (averages of three experiments).

Methylation of DNA *in Vitro*

The methylation of DNA with MNU or MNNG was performed by the method of Lawley.[14-16] Figure 2 shows a typical chromatographic elution profile of a hydrolyzate of

TABLE II. The Contents of 3-Methylcytosine in DNA Treated with MNU or MNNG *in Vitro*

Methylating agent	(nucleotide : agent)	Mole % of 3-methylcytosine to cytosine
MNU	1 : 1	Not detected
	1 : 10	0.15
	1 : 100	1.68
MNNG	1 : 1	Not detected
	1 : 10	0.16
	1 : 100	0.85

The values are each the average of four determinations. Detection limit, 0.025% of cytosine.

TABLE III. The Contents of 3-Methylcytosine in Liver DNA of Rats Treated with DMN *in Vivo*

DMN dose (mg/kg body weight)	Mole % of 3-methylcytosine to cytosine
250	0.034
200	0.020
150	0.024
50	0.030
20	0.031
Control	Not detected

Detection limit, 0.008% of cytosine.

methylated DNA. The peaks of major bases appeared within 10 min, followed by three peaks of m^3Ade, m^1Ade, and m^3Cyt. No peak of methylated base was found from control calf thymus DNA. Table II summarizes the results of the determination of m^3Cyt. The values are averages of four determinations. The amount of m^3Cyt increased with increase in the ratio of the methylating agent to DNA nucleotide unit. No peak of m^3Cyt was detected by this method at the ratio of 1 : 1. There was no significant difference between MNU and MNNG in the degree of methylation. DMN did not give any methylated bases *in vitro*.

Methylation of DNA *in Vivo*

DMN was administrated to rats in a manner similar to that described by Pegg.[17] The content of m^3Cyt in extracted rat liver DNA was determined by HPLC. The peak of m^3Cyt appeared over the whole range of dosage from 20 to 250 mg/kg. There was no marked change in the content of m^3Cyt over this dose range, in accordance with our previous report.[18] The reason for this is not clear. It is known that human cancer patients excrete in the urine elevated levels of modified nucleosides and bases originating from ribonucleic acid.[19] We are now investigating the application of the present methods to the determination of modified bases in urine of normal and tumor-bearing humans.

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