[Chem. Pharm. Bull. 25(11)2827—2830(1977)]

UDC 547.854.81.08 : 543.554.061

Determination of Cytosine, 3-Methylcytosine, and 5-Methylcytosine in Nucleic Acids by High Performance Liquid Chromatography

Hidetaka Yuki,[1a] Hideki Kawasaki, Toshiko Kobayashi, and Akira Yamaji[1b]

Faculty of Pharmaceutical Sciences, Osaka University[1a] *and Department of Pharmacy, Osaka University Hospital*[1b]

(Received December 25, 1976)

A sensitive and selective analytical method for cytosine, 3-methylcytosine, and 5-methylcytosine in nucleic acids by high performance liquid chromatography was developed. This method involves hydrolysis of deoxyribonubleic acid (DNA) or ribonucleic acid (RNA) with perchloric acid at 100° for 1 hr, removal of the acid by weakly basic anion exchange resin (CG-4B, OH^- form), separation of cytosine, 3-methylcytosine, and 5-methylcytosine from other bases by treatment with Dowex 1×2 column ($HCOO^-$ form), and the analysis of the eluate by high performance liquid chromatography. Recoveries were more than ninety percent. This method was applied to several nucleic acids, and molar ratio of 3-, and 5-methylcytosine to cytosine was obtained.

Keywords——3-methylcytosine; 5-methylcytosine; nucleic acid; minor component; high performance liquid chromatography

5-Methylcytosine (5MC) was found in both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA),[2,3] and 3-methylcytosine (3MC) in RNA as one of the minor components, and it was demonstrated that these components were produced from cytosine by the action of adenosylmethionine and methylase after nucleic acid chain was synthesized.[4–8] Methylation of DNA was suggested to play an important role in the process of neoplasia and differentiation, although its definite role still remains unclarified.[3]

5MC content in DNA differs from source to source.[3,9] The studies of its function require sensitive, and selective analytical method. Determination of 5MC after separation by paper or column chromatography[10–12] is not sensitive and consumes much sample. Analysis using ^{14}C-methionine[13] is sensitive, but it is not always reliable because the other route of methylation is possible, and labelled methyl group may be metabolized and incorporated into the heterocyclic ring of newly formed bases, as Vanyushin, *et al*, pointed out.[14] Deutsch, *et al.*[15] reported the analysis of 5MC in DNA by mass spectrometry basing upon its parent peak.

1) Location: *a) 133–1 Yamadakami, Suita-shi, Osaka; b) 1–1–50 Fukushima, Fukushima-ku, Osaka.*

2) G.R. Wyatt, *Biochem. J.*, **48**, 548 (1951).
3) B.F. Vanyushin, S.G. Tkacheva, and A.N. Belezersky, *Nature* (London), **225**, 948 (1970).
4) J. Doscocil and F. Sorm, *Biochem. Biophys. Acta*, **55**, 953 (1962).
5) R. Solomon and A.M. Kaye, *Biochem. Biophys. Acta*, **204**, 340 (1970).
6) B. Sheid, P.R. Srinivasan, and E. Boreck, *Biochemistry*, **7**, 280 (1968).
7) F. Kalusek and N.R. Morris, *J. Biol. Chem.*, **243**, 2440 (1968).
8) F. Kalusek and N.R. Morris, *J. Biol. Chem.*, **244**, 1157 (1969).
9) B.F. Vanyushin, A.L. Mazin, V.K. Vasilyev, and A.N. Belozersky, *Biochim. Biophys. Acta*, **299**, 397 (1975).
10) K. Randerath, *Nature* (London), **205**, 908 (1965).
11) E.D. Rubery and A.A. Newton, *Anal. Biochem.*, **42**, 149 (1971).
12) W.E. Cohn in E. Chargaff, and J.N. Davidson (Editors), "The Nucleic Acids," Vol 1, Academic Press, New York, 1955, p. 211
13) T.W. Sneider, *J. Biol. Chem.*, **246**, 4774 (1971).
14) B.F. Vanyushin, A.N. Belozersky, N.A. Kokurina, and D.X. Kadirova, *Nature* (London), **218**, 1066 (1968).
15) J. Deutsch, A. Razin, and J. Sedat, *Anal. Biochem.*, **72**, 586 (1976).

This method would not distinguish 5MC from other methylated cytosine, *i.e.* 3MC, and high resolution mass spectrometer is not widely used in laboratories. Recently, Breter, *et al.*[16] reported the determination of 5MC in DNA as 5-methyldeoxycytidine by use of high performance liquid chromatography (HPLC), which involves enzymatic hydrolysis of DNA by DNase I and alkaline phosphatase and quantitation of the resultant deoxyribonucleosides by HPLC. However, resolution between deoxycytidine and 5-methyldeoxycytidine was rather poor, and these were eluted at very end of each run, which leads to broadening of the peaks and makes quantitation less accurate. Analysis of 5MC in RNA was not possible by this method. Specific analytical method for 3MC has not been reported yet. The authors developed simultaneous analytical method for 3MC and 5MC together with cytosine in both DNA and RNA by HPLC. These were reported below.

Materials and Methods

Seventy percent perchloric acid, potassium hydrogen phosphate, and potassium dihydrogen phosphate were purchased from Wako Chemicals. Co. Ltd., and adenine and guanine were products of Kojin Co. Ltd. Other nucleic acid bases, calf thymus DNA, herring sperm DNA, baker's yeast RNA, and calf liver RNA were products of Sigma Chemical Co. Ltd. Rat liver DNA was extracted by Kirby's method,[17] and rat liver *t*RNA was prepared by Brunngraber's method.[18] Deionized distilled water was used throughout these experiments.

Du Pond 840 liquid chromatograph equipped with a steel column (100×0.21 cm) packed with a strong cation exchanger (Zipax SCX) and 254 nm ultraviolet (UV) detector was used. Elution was carried out with phosphate buffer solution (0.2 M, pH 5.50) at a pressure of 50 kg/cm² at ambient temperature.

Determination of 3MC and 5MC in DNA and RNA——Five milligrams of DNA or RNA were heated in 0.1 ml of 70% perchloric acid at 100° for 1 hr. After cooling, small volume of water was added, and the solution was neutralized by addition of approximately 1 ml anion exchange resin CG-4B (OH^- form). The mixture was passed through a cotton filter, and the resin was washed with 100 ml water. The filtrate and washings were combined and evaporated to dryness. The residue was dissolved by addition of 1 ml ammonium formate buffer solution (0.01 M, pH 12.0), and the solution was loaded on Dowex 1×2 column ($HCOO^-$ form, 7×0.6 cm). After washing of the column with 5 ml of the same buffer solution, elution of cytosine, 3MC, and 5MC was carried out by 50 ml of pH 10.2 ammonium formate buffer solution (0.02 M). The eluate was evaporated to dryness, and the residue was dissolved by addition of 1 ml pH 5.5 phosphate buffer solution (0.2 M). The aliquot of the solution was injected into liquid chromatograph. Calibration curves of cytosine, 3MC, and 5MC were prepared by injecting 5 μl of solution containing 2, 5, 10, 20, 30, and 40 ng/μl of authentic samples, and plotting the points obtained by average of five runs.

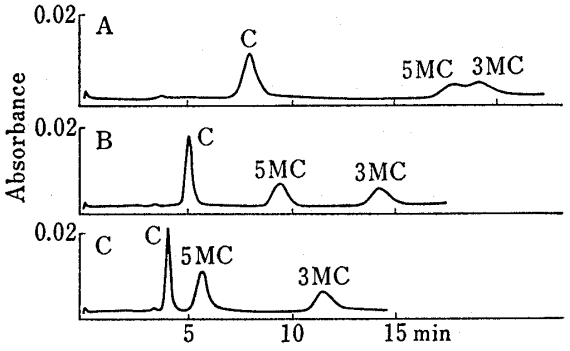

Fig. 1. Chromatograms of a Mixture of Cytosine, 3MC, and 5MC

HPLC conditions: column, Zipax SCX (1m); pressure, 50 kg/cm²; mobile phase, 0.2M KH_2PO_4-K_2HPO_4; pH, (A) 5.00, (B) 5.50, (C) 5.93.

Table I. Retention Times of Nucleic Acid Bases

Bases	Retention times[a]
Uracil	3.89(0.74)
Guanine	3.93(0.75)
5-Hydroxymethylcytosine	4.19(0.80)
Thymine	4.30(0.82)
Adenine	4.48(0.85)
Cytosine	5.24(1.00)
6-Methyladenine	7.85(1.50)
5-Methylcytosine	9.42(1.80)
3-Methylcytosine	14.26(2.72)
N^6,N^6-Dimethyladenine	22.48(4.29)

a) Retention times are minutes. The values in parenthesis show relative retention times to cytosine.
HPLC conditions: column, Zipax SCX (1m); pressure, 50 kg/cm²: mobile phase, 0.2M KH_2PO_4–K_2HPO_4(pH 5.50).

16) H.J. Breter, G. Seibert, and R.K. Zahn, *J. Chromatog.*, **118**, 242 (1976).
17) K.S. Kirby, *Biochem. J.*, **64**, 405 (1956).
18) E.F. Brunngraber, *Biochem. Biophys. Res. Commn.*, **8**, 1 (1962).

Results and Discussion

Fig. 1 shows the effect of pH of eluting agent (0.2 M phosphate buffer solution) on the retention times of cytosine, 3MC, and 5MC. The peaks of 3MC and 5MC overlapped for the most part when eluted at pH 5.0. Elution at pH 5.95 allowed 5MC peak to come near cytosine peak. After all, elution with pH 5.5 buffer solution gave the best separation. Table I represents the retention times of several nucleic acid bases on this condition. 5MC and 3MC have so longer retention times than major bases, that these bases will not interfere with the analysis of 3MC and 5MC. The interference, however, due to 6-methyladenine, which has close retention time to 5MC is possible. 6-Methyladenine is contained in most *t*RNAs and DNAs, so it was required to remove 6-methyladenine prior to HPLC analysis of 3MC and 5MC. Cohn[19] separated cytosine from uracil, adenine, and guanine by eluting with pH 10.2 ammonium formate buffer solution from Dowex 1×2 column ($HCOO^-$ form). Previously, we reported that 5MC was eluted together with cytosine by pH 10.2 buffer solution.[20] In the present study, it was found that 3MC was also eluted by the same buffer solution. 6-Methyladenine was not eluted till pH of the eluting agent was lowered down to 6.0. Thus, elution with pH 10.2 buffer solution successfully separated cytosine, 3MC, and 5MC from other nucleic acid bases. Fig. 2 shows chromatograms of the neutralized nucleic acid hydrolysate before and after the treatment with Dowex column. Treatment with Dowex column gave cleaned up chromatogram particularly on cytosine peak enabling its quantitation.

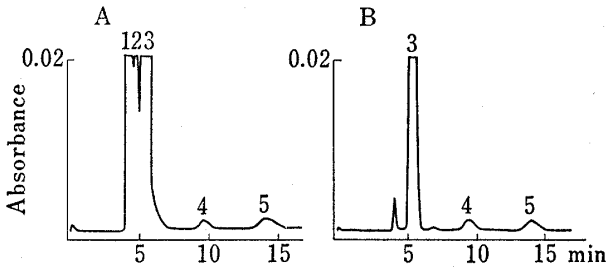

Fig. 2. Chromatograms of the Hydrolysate of tRNA before and after the Treatment with Dowex Column

*t*RNA was hydrolyzed with 70% $HClO_4$, and the hydrolysate was neutralized with CG-4B (OH^- form). A: The neutralized solution was injected into HPLC. B: The neutralized solutions was treated with Dowex 1×2 ($HCOO^-$ form), and the eluate of pH 10.2 buffer solution was injected into HPLC.

1, uracil and guanine; 2, adenine; 3, cytosine; 4, 5MC; 5, 3MC.

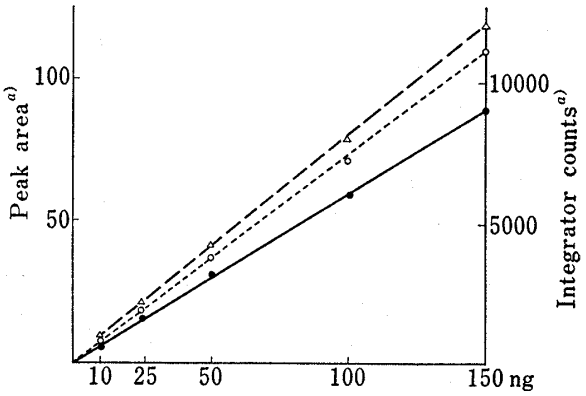

Fig. 3. Calibration Curves of Cytosine, 3MC, and 5MC

a) Peak area of 3MC and 5MC was measured by weight method, and that of cytosine was measured by integrator method.

-----: cytosine,
------: 3MC,
——: 5MC.

Table II. Recovery Test of Bases

	After hydrolysis and neutralization with CG-4B resin (%)	After separation with Dowex resin (%)	Overall procedure (%)
Cytosine	98.3	96.9	95.3
3MC	99.1	97.1	96.2
5MC	97.7	97.2	95.0

0.1 ml solution containing 50 μg of base was evaporated to dryness and then subjected to the same procedure described in the text.

Values are average of three experiments.

19) W.E. Cohn, *J. Am. Chem. Soc.*, **72**, 1471 (1950).
20) H. Yuki, A. Yamaji, and S. Fujiwara, *Yakugakuzasshi*, **96**, 1184 (1976).

For recovery test of bases from the resins, authentic samples of the bases were subjected to the same procedure. Recoveries from CG-4B and Dowex column, and the overall procedure were shown in Table II. Washing of CG-4B resin with 40 ml water gave recovery of 93% for 3MC and 87% for 5MC, but washing with 100 ml water gave satisfactory recovery. Elution from Dowex column with 50 ml pH 10.2 buffer solution gave complete recovery of cytosine, 3MC, and 5MC. Breter, *et al.*[16] have chosen the enzymatic hydrolysis of DNA to avoid the decomposition of certain bases. However, more than 97% of cytosine, 3MC, and 5MC were recovered by the procedure of perchlorate hydrolysis and treatment with CG-4B resin. So, we decided to use this method which was applicable to both DNA and RNA. When cytosine (150 μg), 3MC (25 μg), and 5MC (25 μg) were added to calf thymus DNA (5 mg), recoveries in five experiments were 91.6±5.5, 90.4±5.8, and 93.1±4.4 (mean(%)±S.D.), respectively.

Fig. 3 shows calibration curves for cytosine, 3MC, and 5MC. Straight lines were observed from 10 to 150 ng for each base (80—1200 pmole for 3MC and 5MC). The peak areas of 3MC and 5MC were measured by weight method, but that of cytosine was measured by integrator count method because of its overscale out of the chart. The limit of detection was 2.5 ng (20 pmol) for 3MC and 5MC.

Table III. Content of Cytosine, 3MC, and 5MC in Various DNAs and RNAs

	Cytosine μg/mg	3MC		5MC	
		μg/mg	mol % to cytosine	μg/mg	mol % to cytosine
Calf thymus DNA[a]	64.3	—	—	5.4	7.5 (lit. 6.1[2], 8.1[21], 9.2[22], 5.3[23])
Herring sperm DNA[b]	73.8	—	—	11.5	13.9 (lit. 10.4[24], 9.2[2])
Rat liver DNA[b]	34.2	—	—	1.8	4.6 (lit. 4.5[9])
Baker's yeast sRNA Type III[a]	84.5	9.8	10.3	5.6	5.8
Calf liver RNA[a]	98.5	3.3	3.0	2.3	2.1
Rat liver *t*RNA[b]	33.8	1.3	3.4	1.6	4.3

a) Sample quantities were measured by UV method as 0.020 OD_{260}=1 μg DNA/ml.
b) Sample quantities were measured by weight method.

Cytosine, 3MC, and 5MC in some DNA and RNA were analyzed by this method. Results were shown in Table III. 5MC was found in both DNA and RNA, but 3MC was detected only in RNA. Five milligrams of DNA and RNA were used in the present experiment, but even one milligram would be enough to measure the amounts of 3MC and 5MC for the high sensitivity of this analytical method. Previously, the authors reported a gas chromatographic analysis of 5MC,[20] but it was impossible to analyze 3MC and experimental procedure was more complicated for derivatization of base to a volatile substance. By the present method, cytosine, 3MC, and 5MC were analyzed simultaneously, and mole percentage of 3MC and 5MC to cytosine was easily obtained. Mole percentage of 5MC to cytosine in DNA, thus obtained, showed nearly the same values as that of literatures.[2,9,21–24] As Wyatt[2] stated, alteration of 5MC content among species and organs is very interesting, so we are now investigating the relationship between tumor growth and the contents of 3MC and 5MC in nucleic acids.

21) R.L. Sinsheimer and J.F. Koener, *J. Biol. Chem.*, **198**, 293 (1952).
22) S.G. Laland, W.G. Overend, and M. Webb, *J. Chem. Soc.*, 3224 (1952).
23) R.O. Hurst, A.M. Marco, and G.C. Butler, *J. Biol. Chem.*, **204**, 847 (1953).
24) R.K. Zahn, *Z. Physiol. Chem.*, **313**, 77 (1958).