The Methylation of Nucleic Acids. The Analysis of Methylated Ribonucleosides by Means of High-performance Liquid ODS-Silica Gel[1] and Cation-exchange Chromatography

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The quantitative determination of adenosine, guanosine, inosine, cytidine, uridine, and their methylated derivatives was studied by means of a high-performance liquid chromatographic method using ODS-silica gel, cation-exchange, and anion-exchange resins as column packings. The method was applied to the analysis of yeast-RNA, which was methylated by trimethyl phosphate at pH 7 and 9.

Nucleic acids, especially tRNA and mRNA, contain a variety of modified nucleosides.[2,3] A mutagenic study of the action of alkylating agents on nucleic acids has shown them to give polymers with various alkylated nucleosides.[4] The rapid and quantitative analysis of such rare nucleosides has, therefore, been actively sought. Although two-dimensional thin-layer chromatography and ion-exchange chromatography,[2,5-7] which are coupled with a scintillation-counting technique, have frequently been employed, the chromatographic procedures are complex and time-consuming. These drawbacks may be avoided by utilizing high-performance liquid chromatography (HPLC). There have been reports on the HPLC of such major nucleosides as Ado, Guo, Cyd, and Urd.[8-11] However, only a limited number of papers have dealt with the HPLC of rare nucleosides of nucleic acids.[9,12]

In this paper, we wish to report the HPLC of a variety of methylated ribonucleosides, *etc.*, using ODS-silica gel, cation-exchange, and anion-exchange resins as column packings. The application of the HPLC method to the analysis of the enzymatic hydrolysates of methylated RNA will also be described.

Results and Discussion

The following nucleosides were studied for the HPLC analysis: Ado, m^{1}Ado, m^{6}Ado, Guo, m^{1}Guo, m^{6}Guo, m^{7}Guo, Ino, m^{1}Ino, Cyd, m^{3}Cyd, Urd, and m^{3}Urd.[13] The methylated substances include not only the constituents of tRNA, but also the products furnished easily upon the treatment of RNA with methylating agents. In addition to these compounds, 7-methylguanine and the imidazole ring-opened m^{7}Guo[14] were examined, since m^{7}Guo has been known to give the former by a depurination reaction, and the latter, by hydrolysis under weakly alkaline conditions.[15]

Although previous HPLC studies of the rare nucleosides of RNA used ion-exchange columns exclusively,[9-12] we found that a ODS-silica gel column was most useful in fractionating these compounds. Of the various solvent systems investigated as the mobile phase, a buffer [triethylamine–acetic acid–water (0.3–0.3–100 v/v)] containing 2% acetonitrile gave the optimum results. Figure 1a shows a typical elution

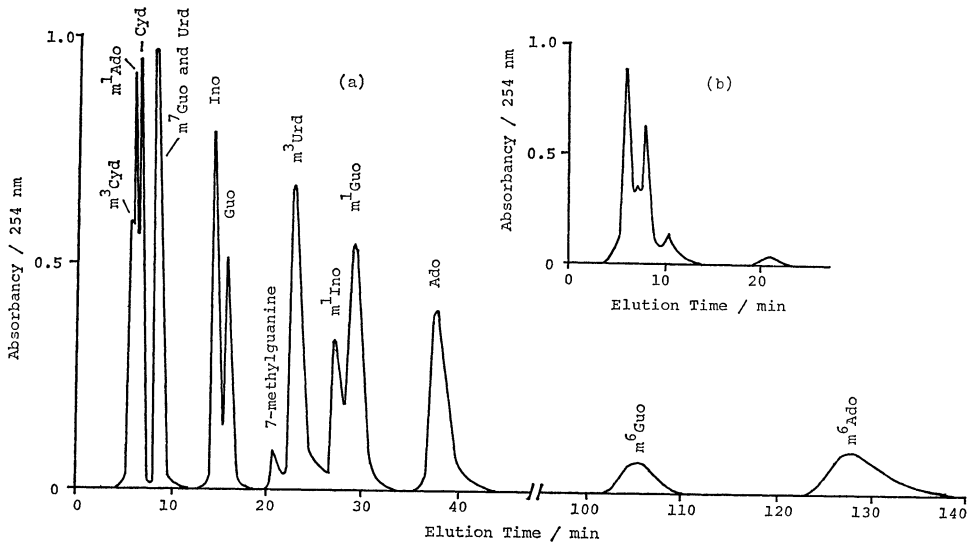

Fig. 1. (a): ODS silica gel HPLC of a mixture of nucleosides and 7-methylguanine; Ado; 3.33; m^{1}Ado, 2.10; m^{6}Ado, 1.81; Guo, 1.77; m^{1}Guo, 2.95; m^{6}Guo, 1.68; m^{7}Guo, 3.00; 7-methylguanine, 1.40; Ino, 3.33; m^{1}Ino, 1.94; Cyd, 3.33; m^{3}Cyd, 3.50; Urd, 3.33; m^{3}Urd, 4.36 (mmol/l). Injected volume, 15 μl. Solvent, triethylamine–acetic acid–water–acetonitrile= 0.3—0.3—100—2 v/v; pH 8.9; flow rate, 0.73 ml/min; column pressure, 40 kg/cm^2.
(b): ODS silica gel HPLC of imidazole ring opened m^{7}Guo (15 mmol/l). Injected volume, 15 μl. For chromatographic conditions see Fig. 1a.

pattern. The addition of acetonitrile to the buffer decreased the retention times considerably and resulted in a higher sensitivity (or height of the peaks) than the same solvent system without the organic solvent. The increase in the content of the amine in the solvent (for instance, triethylamine–acetic acid–water=0.5–0.8–0.3–100 v/v, 2% acetonitrile) resulted in a decreased resolution, especially for Urd, m^3Urd, m^1Guo, and Guo. The imidazole ring-opened m^7Guo may exist as a mixture of various compounds,[14] and its elution pattern overlaps with the peaks of Cyd, m^3Cyd, m^1Ado, m^7Guo, *etc.* (Fig. 1b).

This drawback in the silica gel column was, however, solved by the use of a cation-exchange column. The gradient chromatography achieved by changing the solvent was not employed here because of the time-consuming process needed for the reequilibration of the column after each sample application. As the mobile phase, an ammonium formate buffer (0.05 mol dm^{-3} with respect to the HCO_2^- concentration, pH 4.2) gave good results. A typical elution pattern furnished by the cation-exchange column is shown in Fig. 2a using the sample used in Fig. 1a. It is apparent that the ion-exchange column complemented

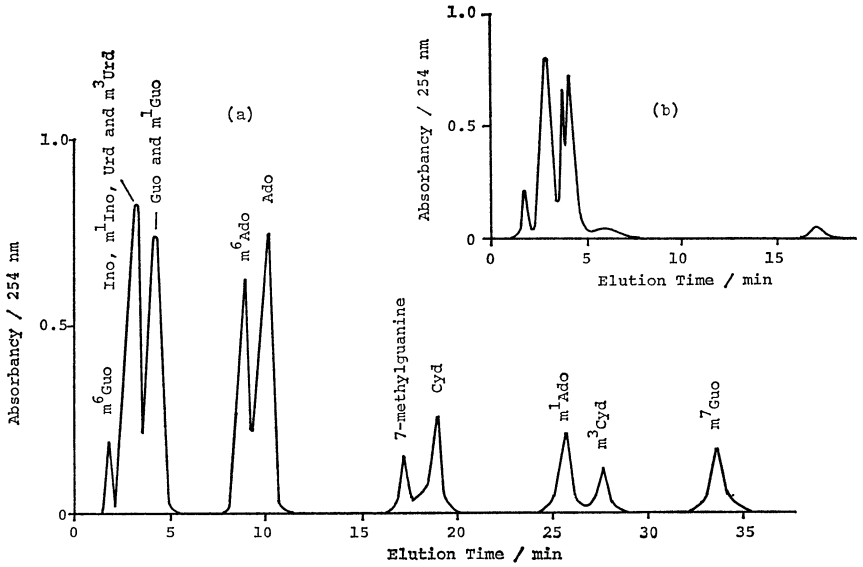

Fig. 2. (a): Cation-exchange HPLC of a mixture of nucleosides and 7-methylguanine. See Fig. 1a for the sample. Injected volume, 10 μl. Solvent, ammonium formate buffer (0.05 M, pH 4.2); flow rate, 0.67 ml/min; column pressure, 75 kg/cm².
(b): Cation-exchange HPLC of imidazole ring-opened m^7Guo (15 mmol/l). Injected volume, 10 μl. For chromatographic conditions see Fig. 2b.

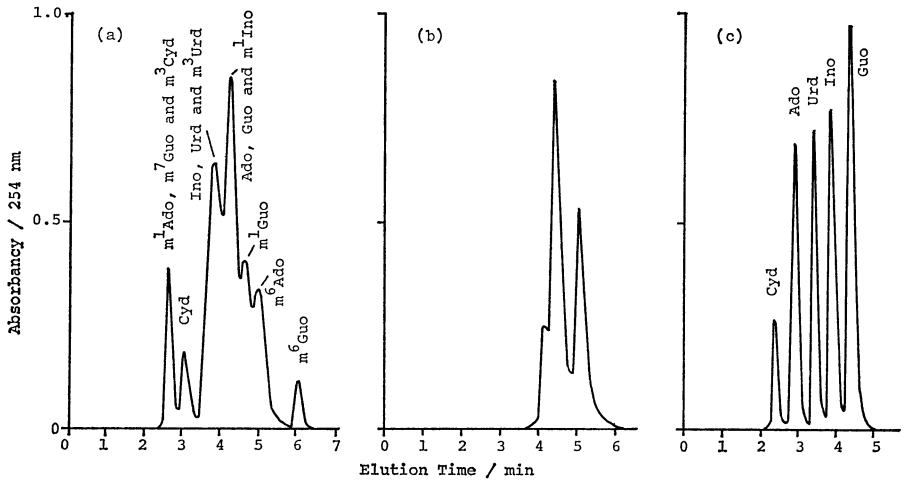

Fig. 3. (a): Anion-exchange HPLC of a mixture of nucleosides and 7-methylguanine. See Fig. 1a for the sample. Injected volume, 7 μl. Solvent, potassium phosphate buffer (0.03 M, pH 3.9); flow rate, 1.00/min; column pressure, 50 kg/cm².
(b): Anion-exchange HPLC of imidazole ring-opend m^7Guo (15 mmol/l). Injected volume, 5 μl. For chromatographic conditions see Fig. 3a.
(c): Anion-exchange HPLC of a mixture of Ado (0.83 mmol/l), Guo (1.37), Ino (0.95), Cyd (0.85) and Urd (1.00). Injected volume, 25 μl. Solvent, potassium phosphate buffer (0.03 M, pH 2.9); flow rate, 0.53 ml/min; column Pressure, 23 kg/cm².

Table 1. Retention data of nucleosides and 7-methylguanine[a)]

Compound	pK_a	Retention volume/ml (time/min)		
		Silica gel pH 8.9	Cation resin pH 4.2	Anion resin pH 3.9
Ado	3.5	27.9 (38.2)	6.4 (10.0)	4.2 (4.2)
m^{1}Ado	8.8	4.3 (5.9)	17.3 (25.8)	2.6 (2.6)
m^{6}Ado	4.0	93.4 (128)	5.9 (8.8)	5.0 (5.0)
Guo	1.6, 9.2	11.3 (15.5)	2.9 (4.4)	4.2 (4.2)
m^{1}Guo	2.4	21.3 (29.3)	2.9 (4.4)	4.6 (4.6)
m^{6}Guo	2.4	78.1 (107)	1.3 (2.0)	6.2 (6.2)
m^{7}Guo	7.0	5.7 (7.8)	22.5 (33.6)	2.6 (2.6)
Ring-opened m^{7}Guo	—	4.1—15.3 (5.6—21)	1.3—4.2, 11.7 (2.0—6.2, 17.4)	4.0—4.9 (4.0—4.9)
7-Methylguanine	3.5, 9.9	15.3 (21.0)	11.7 (17.4)	4.8 (4.8)
Ino	8.8, 1.2	10.2 (14.0)	2.1 (3.2)	3.9 (3.9)
m^{1}Ino	—	20.1 (27.6)	2.1 (3.2)	4.1 (4.1)
Cyd	4.2	4.5 (6.1)	12.1 (18.0)	3.1 (3.1)
m^{3}Cyd	8.7	3.9 (5.4)	18.5 (27.6)	2.6 (2.6)
Urd	9.2	5.7 (7.8)	2.1 (3.2)	3.6 (3.6)
m^{3}Urd	—	17.0 (23.3)	2.1 (3.2)	3.8 (3.8)

a) See the Experimental section for chromatographic conditions.

the silica gel column in separating characteristics. Thus, the group of compounds which eluted simultaneously with the imidazole ring-opened m^{7}Guo in the silica gel column (*vide supra*) were resolved fairly well by the cation-exchange column except for Urd. Although the peaks of m^{6}Guo, m^{1}Guo, Guo, and m^{3}Urd were overlapped by the complex peaks of the imidazole ring-opened m^{7}Guo, as is shown in Fig. 2b, the silica gel column separated these compounds well (Fig. 1a).

As is to be expected from the pK_a values (Table 1) of the nucleosides examined, the increase in the pH value of the buffer to 4.4—4.6 in the cation-exchange column resulted in the peak-coherence of m^{6}Ado with Ado, of Cyd with m^{7}Guo, and of m^{1}Ado with m^{3}Cyd.

An anion-exchange column, on the other hand, was not as useful as the silica-gel column and the cation-exchange column. The elution patterns of various nucleosides are shown in Figs. 3a—3c, using a potassium phosphate buffer (0.03 mol dm^{-3} with respect to the $H_2PO_4^-$ concentration). The retention volume (R_v)-difference between the initial peak and the last peak was small (*ca.* 3.7 ml) in comparison with the R_v of the initial peak (2.6 ml). It thus appeared that the anion-exchange column was suitable for the analysis of a mixture of major nucleosides of RNA (Fig. 3c).

The lowest concentration of samples for quantitative determination was found to be better than *ca.* 0.5 μmol/l (R_v 3—12 ml)—*ca.* 8 μmol/l (R_v 15—28 ml) for the ODS–silica gel column, *ca.* 0.5 μmol/l (R_v 1.3—7 ml)—5 μmol/l (R_v 11—23 ml) for the cation-exchange column, and *ca.* 0.5 μmol/l for the anion-exchange column. The retention data are summarized in Table 1.

As an application of the present HPLC method, yeast RNA was methylated with trimethyl phosphate at pH 7 and 9, and its enzymatic hydrolysates were analyzed. Table 2 shows the relative distribution of products against each major nucleosides.[16)] The values were obtained by dividing the peak areas by the respective molar extinction coefficients at 254 nm in the eluting buffer and by normalizing the data to 100% for each major nucleoside (see the Experimental section). Although the methylating conditions were vigorous, the distribution of the products agreed with

Table 2. Product distributions in the enzymatic hydrolysates of methylated yeast RNA[a,b)]

Compound	Buffer A[c)]		Buffer B[c)]	
	pH 7	pH 9	pH 7	pH 9
Ado (Ino)[d)]	85%	90%	93%	95%
m^{1}Ado	10	tr	tr	nd
m^{6}Ado[e)]	2	8	4	tr
Guo	70	50	85	83
m^{1}Guo	nd	25	nd	3
m^{6}Guo	nd	3	nd	nd
m^{7}Guo	13	tr	3	nd
Ring-opened m^{7}Guo	13	20	12	14
7-Methylguanine	4	2	tr	tr
Cyd	75	86	87	95
m^{3}Cyd	23	10	11	3
Urd	nc	nc	nc	nc
m^{3}Urd	tr	80	tr	11

a) Anion-exchange HPLC showed that the starting yeast RNA contained Ado, Guo, Cyd and Urd in the molar ratio of 0.95 : 1.37 : 0.84 : 1. b) Percentages are based on the amounts of products against the respective major nucleosides in the starting RNA. Tr, nd, and nc indicate "a trace yield," "a compound was not detected," and "a yield was not calculated" respectively. c) The reaction media; buffer A, KH_2PO_4-NaOH (0.03 M); buffer B, Tris-HCl (0.05 M). d) See Ref. 16. e) See Ref. 18.

the general order of the increasing reactivity of the alkylation sites of the nucleoside residues in RNA:[4,17] *e.g.* at pH 7, m^7Guo>m^3Cyd>m^1Ado, m^6Ado[18]> m^3Urd, and at pH 9, m^3Urd>m^1Guo>m^3Cyd> m^1Ado, m^6Ado. It was found that the methylation of RNA in the phosphate buffer occurred more smoothly than the methylation in the tris buffer. Possibly, tris(hydroxymethyl)aminomethane (tris) in the buffer scavenged trimethyl phosphate by reacting the methylating agent at a rate faster than RNA. It is also noteworthy that the extent of the formation of m^6Guo, which may be an adequate measure of the mutagenic activity of the methylating agents,[3] was very small when trimethyl phosphate was used.

In conclusion, a variety of methylated nucleosides can be analyzed rapidly and easily by the HPLC method. ODS–silica gel and cation-exchange columns are especially useful for the study of methylated RNA.

Experimental

HPLC was run by means of a Toyo-Soda 803 liquid chromatograph with a 254-nm UV flow-cell detector and a Milton Roy model 396/2396 piston pump (Riviera Beach, Florida). All the measurements were carried out at the ambient temperature.

An ODS-silica gel column (stainless steel, 0.4 cm×30 cm, LS-410-ODS), a cation-exchange column (stainless steel, 0.4 cm×30 cm, IEX-510-SP), and an anion-exchange column (stainless steel, 0.4 cm×30 cm, IEX-260) were purchased from the Toyo-Soda Co., Shinnanyo-shi, Hiroshima.

The most frequently employed solvent systems were as follows: triethylamine–acetic acid–water–acetonitrile (0.3–0.3–100–2 v/v), an ammonium formate buffer (0.05 mol dm^{-3} with respect to the HCO_2^- concentration, pH 4.2), and a potassium phosphate buffer (0.03 mol dm^{-3} with respect to the $H_2PO_4^-$ concentration, pH 4.5, 3.9, and 2.9).

The major nucleosides (Ado, Guo, Cyd, Urd, and Ino) were commercially available. The methylated nucleosides (m^1Ado, m^6Ado, m^1Guo, m^6Guo, m^7Guo, the imidazole ring-opened m^7Guo, m^1Ino, m^3Cyd, and m^3Urd) and 7-methylguanine were prepared in previous experiments[17] or according to the literature.[15] The yeast RNA was obtained from the Sigma (U.S.A.) Chemical Co. (lot R6750). The ribonuclease (bovine pancrease) was from Miles Laboratory (U.S.A.) (code 36-511, batch 228). The phosphodiesterase (P-6877, type II) and RNAse T_2 (lot 57c-9510) were obtained from the Sigma (U.S.A.) Chemical Co. The alkaline phosphatase (36—482) was purchased from Miles-Seravac (Cape Town, South Africa).

Methylation of Yeast RNA. RNA (6 mg) was dissolved in a KH_2PO_4–NaOH buffer (1.5 ml, pH 7.0 or 9.0, 0.05 mol dm^{-3} with respect to the $H_2PO_4^-$ concentration) or in a Tris-buffer (1.5 ml, pH 7.0 or 9.0, 0.05 mol dm^{-3}). The solution was mixed with trimethyl phosphate (0.2 g, 1.7 mmol) and kept at 37 or 55 °C for 12 h. The homogeneous solution was then concentrated under −10 °C to give a residue, which was subsequently washed with ether and dissolved in water (0.8 ml). Ethanol was poured into the solution until a cloudy, turbid solution appeared. Sometimes a few drops of sodium acetate (3 mol dm^{-3}) were added to the solution in order to make the precipitation complete. After repeating the precipitation procedure thrice, the precipitate was dried and subjected to an enzymatic hydrolysis, which was carried out in a manner similar to the method previously reported.[6]

Quantitative Analysis of the Enzymatic Hydrolysate. The hydrolysate was dissolved in 2 ml of water, and then 10—20 μl of the solution was injected into the chromatograph. The sample peaks of the HPLC chart were cut and weighed. They were then divided by the respective molar extinction coefficient at 254 nm in the eluting buffer in order to obtain the molar ratios of the methylated nucleosides against the respective major nucleosides. The yields of the products were calculated from the ratio; they are shown in Table 2.

Since the imidazole ring-opened m^7Guo showed a complex elution pattern, its content (%) in the mixture of products was estimated by means of the following equation: 100—[yields (%) of m^7Guo (cation-exchange column), of 7-methylguanine (cation-exchange column), of m^1Guo (ODS-silica gel column), of m^6Guo (ODS-silica gel column), and of unreacted Guo (ODS-silica gel column)].

The yield of m^3Urd could not be determined by the aforementioned method because neither the ODS-silica gel column nor the cation-exchange column gave an isolated peak for Urd. The yield of m^3Urd was, therefore, obtained by means of this equation: 100·a/the amount of Urd in the starting RNA, where a is the amount of m^3Urd in the hydrolysate. This last quantity was determined as follows. The ODS-silica gel HPLC was run before and after the addition of a known amount of m^3Urd to the hydrolysate; a=the added amount of m^3Urd×(the original area of m^3Urd/the increased area of m^3Urd).

References

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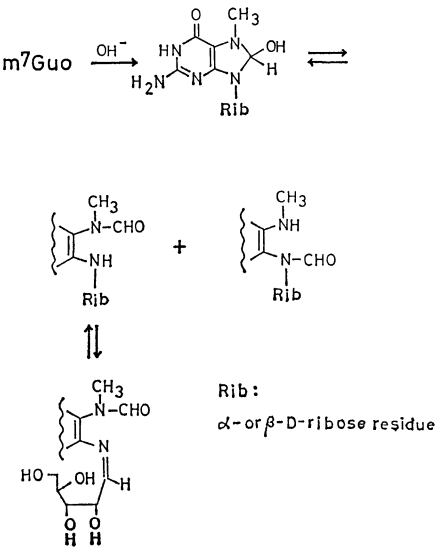
13) Abbreviations used are: Ado: adenosine; Guo: guanosine; Ino: inosine; Cyd: cytidine; Urd: Uridine; m^1Ado: 1-methyladenosine; m^6Guo: O^6-methylguanosine; m^3Cyd: 3-methylcytidine. Similar connotations are used for the other methylated nucleosides.

14) The imidazole ring of m^7Guo is opened easily under alkaline conditions. The reaction may give the various compounds through the following postulated pathway.



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16) Since adenosine deaminase, which was present as an impurity in RNAse-T_2 and spleen phosphodiesterase, converted Ado substantially into Ino, the elution peak of Ado was always very small or was completely absent. The observed yield of Ino was, therefore, considered to be the yield of Ado.

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18) m^1Ado has been known to undergo a Dimroth rearrangement reaction to produce m^6Ado. Trimethyl phosphate does not methylate the 6-NH_2 of Ado. The combined extents of m^1Ado and m^6Ado may, therefore, be considered to represent the extent of methylation at the N-1 position of the adenosine residues in RNA.

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