Note

Simultaneous Analysis of 5'-Mononucleotides and Nucleosides in Saline Solution by High Performance Liquid Chromatography

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Guanosine 5'-monophosphate (5'-GMP) and inosine 5'-monophosphate (5'-IMP) are widely used as flavoring agents in foods and the industrial production by enzymatic degradation of RNA was established by Kuninaka *et al.*¹⁾ and Ogata *et al.*²⁾

Kamekura and Onishi³⁾ found an extracellular halophilic nuclease (nuclease H) which decomposed RNA to 5'-mononucleotides exonucleolytically, from a moderate halophile, *Micrococcus varians* subsp. *halophilus* (ATCC21971). A halophilic 5'-nucleotidase was also found in the culture supernatant of this organism.^{4,5)} Kamekura *et al.* reported the commercial production of 5'-GMP using the culture supernatant of the bacterium in a batch system with selective inhibition of the 5'-nucleotidase.⁵⁾ A convenient method for quantitative analysis of each nucleotide and nucleoside during the course of RNA degradation is necessary for the detailed examination of the 5'-nucleotide production by halophilic enzymes.

Anion-exchange^{6~9)} and reverse-phase^{10,11)} high performance liquid chromatography (HPLC) had been used for the separation and quantitation of nucleotides and nucleosides; a gel filtration column packed with hydrophilic polymer gel (Asahipak GS-320), was used for the separation and quantitation of low molecular weight components of nucleic acid.¹²⁾ However, none of these methods dealt with the simultaneous analysis of 5'-mononucleotides and nucleosides in saline solution, and none could be used to examine the RNA degradation process by halophilic enzymes without troublesome desalting treatment as previously described.⁵⁾ This paper describes the convenient analysis of 5'-mononucleotides and nucleosides in saline solution by HPLC with Asahipak GS-320.

Chromatography was done with a HPLC (Hitachi 655A-11; Hitachi Ltd.) with a UV monitor (Hitachi 655A-21; Hitachi Ltd.) operated at 260 nm and a integrator-recorder (Sic intelligent integrator Model 7000A; System Instruments, Co.). An Asahipak GS-320 column (7.6 mm i.d. × 500 mm; Asahi Chemical Industry Co., Ltd.) packed with a hydrophilic polymer gel was used at room temperature.

As authentic compounds, adenosine 5'-monophosphate (5'-AMP), guanosine 5'-monophosphate (5'-GMP), adenosine, guanosine, cytidine, and uridine were purchased from Wako Pure Chemical Industries, Ltd. Cytidine 5'-monophosphate (5'-CMP) and uridine 5'-monophosphate (5'-UMP) were obtained from the Sigma Chemical Co.

The separation conditions for authentic 5'-mononucleotides and nucleosides dissolved in distilled water were investigated with the eluent of sodium phosphate buffer. The concentration of phosphate was varied from 5 mM to 500 mM, and the pH was varied from 3.0 to 9.0. As shown in Fig. 1(a), the eight compounds were separated fairly well by 6.0 mM sodium phosphate buffer (pH 3.2) within 20 min. Next, the separation of these compounds dissolved in 2 M NaCl and 2% MgSO₄·7H₂O solution was examined for application of the method to RNA degradation with the halophilic nuclease H in the presence of 2 M NaCl and 2% MgSO₄·7H₂O. The result in Fig. 1(b) showed the

FIG. 1. Separation of 5'-Mononucleotides and Nucleosides.

Column, Asahipak GS-320, 500 × 7.6 mm i.d.; eluent, 6.0 mM sodium phosphate buffer (pH 3.2); column pressure, 60 kg/cm²; flow-rate, 2.0 ml/min; temperature, ambient; detection, 260 nm; detection intensity, 0.02 AUFS; sample volume, 10 µl.

(a) Chromatogram of 100 mg/l of each compound dissolved in distilled water. Peaks: 1 = cytidine; 2 = 5'-CMP; 3 = 5'-AMP; 4 = uridine; 5 = 5'-UMP; 6 = adenosine; 7 = 5'-GMP; 8 = guanosine (b) Chromatogram of 100 mg/l of each compound dissolved in 2 M NaCl and 2% MgSO₄·7H₂O.

FIG. 2. Effects of Dilution on the Separation from the 2M NaCl Sample Solution of 5'-Mononucleotides and Nucleosides.

Column conditions are the same as in Fig. 1 except for detection intensity.

(a) Dilution rate, 1/10; 1.28 AUFS.

(b) Dilution rate, 1/50; 0.64 AUFS.

(c) Dilution rate, 1/100; 0.16 AUFS; peak numbers are equal to Fig. 1.

FIG. 3. Separation of 5'-Mononucleotides and Nucleosides.

Column, Asahipak GS-320, 500 × 7.6 mm i.d.; eluent, 6.5 mM sodium phosphate buffer (pH 3.25); column pressure, 60 kg/cm²; flow-rate, 2.0 ml/min; temperature, ambient; detection, 260 nm; detection intensity, 0.16 AUFS; sample volume, 10 μl. Peaks: 1 = cytidine; 2 = 5'-CMP; 3 = 5'-AMP; 4 = uridine; 5 = 5'-UMP; 6 = adenosine; 7 = 5'-GMP; 8 = guanosine. Sample amount, 25 mg/l of each compound dissolved in 20 mM NaCl solution.

TABLE I. RETENTION TIME AND RECOVERY OF 5'-MONONUCLEOTIDES AND NUCLEOSIDES

Compound	Retention time (min)	Recovery (%)
5'-AMP	10.4	101
5'-GMP	16.3	101
5'-CMP	8.0	98
5'-UMP	12.6	100
Adenosine	13.9	99
Guanosine	18.0	99
Cytidine	7.1	101
Uridine	11.5	100

separation was unsatisfactory, disturbing the chromatogram by NaCl and MgSO₄. Since the addition of ethylenediamine tetraacetic acid in the eluate or authentic solution to avoid the interference of Mg²⁺ had no effect on the recovery of separation, 2 M NaCl was supposed to be the main disturber. The disturbance of separation was avoided when more than 250 mM NaCl was added to 6.0 mM sodium phosphate buffer (pH 3.2) or the concentration of phosphate was higher than 250 mM. However, under these conditions, the 5'-mononucleotides and nucleosides could not be simultaneously separated because of the mutual overlap of peaks (data not shown). The disturbance of separation by salts disappeared when the authentic solution in 2 M NaCl and 2% MgSO₄ · 7H₂O was diluted to 1/100 with distilled water, i.e., less than 20 mM NaCl (Fig. 2). Figure 3 shows the best separation of the authentic solution containing 25 mg/l of each compound in 20 mM NaCl with 6.5 mM sodium phosphate buffer (pH 3.25). The order of elution was as follows: cytidine, 5'-CMP, 5'-AMP, uridine, 5'-UMP, adenosine, 5'-GMP, and guanosine. The linearity of the peak area and the concentration was observed in a range of 0.1 mg/l to 25 mg/l, and the recovery was good in all compounds (Table I).

This analytical method can be used for samples with high NaCl concentration after dilution to less than 20 mM NaCl, and takes 20 min.

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