Bull. Fac. Fish. Hokkaido Univ.
39(2), 142-150. 1988.

Analysis of Purine Nucleotides by High Performance Liquid Chromatography: Its application for anadromous salmonid fishes

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Abstract

A very rapid and efficient high performance liquid chromatographic analysis technique using a hydrophilic polymer gel column (Asahipak GS-320) and UV monitor (260 nm) has been developed for measuring purine nucleotides. Satisfactory separation was achieved with a mobile phase of 200 mM sodium phosphate buffer, pH 3.25, flow-rate of 0.9 m*l*/min within 60 min. The linearity of the peak area and the amount of purine nucleotides was observed in a range of 0.1 to 10 nmol. . The potential application of this method for the quantitation of acid-soluble nucleotides and related compounds in the integument, liver, blood and serum from the anadromous salmonid fishes is described.

Introduction

It is well known that the silvery coloration in the integument of salmonid fishes fades during the anadromous migration. The integument of seawater salmon is silvery in color due to the presence of purines such as guanine and hypoxanthine (Folmar and Dickhoff, 1980), while that of freshwater fish becomes dark yellow or red in color due to the accumulation of carotenoids and melanin (Yamaguchi and Miki, 1981; Ando, 1986; Ando and Hatano, 1986). Hayashi (1970, 1971) has analysed in detail the acid-soluble purine nucleotides in the integument of salmon by means of ion-exchange column chromatography, but this analytical method is extremely time-consuming. A convenient method for quantitative analysis of purine nucleotides is necessary for the detailed examination of purine metabolism in the integument of salmon, because the integumental coloration affects the quality of the anadromous salmon (Hatano et al., 1987).

Anion-exchange (Nissinen, 1980; Miyatake et al., 1987) and reverse-phase (Walseth et al., 1980; Sugino et al., 1986) high performance liquid chromatography (HPLC) have been recently used for the separation and quantitation of purine nucleotides. We have successfully used a gel filtration column packed with hydrophilic polymer gel to analyse and determine purine nucleotides. In the present paper, we show that chromatography on a gel filtration HPLC column is a very rapid and efficient technique for the analysis of acid-soluble purine nucleotides in the integument, liver, blood and serum from anadromous salmon.

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Materials and Methods

Male and female adult chum salmon, *Oncorhynchus keta*, were caught at sea (pre-spawning stage) and in fresh water (spawning stage). The pre-spawning salmon were caught in Hakodate Bay, Hokkaido, in September～October 1987. The spawning fish were captured at Moheji River, near Hakodate, Hokkaido, in November 1987.

Spawning male and female adult pink salmon (*Oncorhynchus gorbuscha*) and masu salmon (*Oncorhynchus masou*) were captured in Yurappu River and Shiribetsu River, respectively, near Hakodate, Hokkaido, in September 1987.

Integuments and livers for analysis were excised from live salmon. Blood was collected from the caudal vasculature of live salmon and left at room temperature for several hours. The clotted blood was centrifuged to obtain the serum.

Chemicals

As authentic compounds, adenosine-5′-diphosphate (ADP), β-nicotinamide adenine dinucleotide (β-NAD), adenosine-5′-monophosphate (AMP), guanosine-5′-monophosphate (GMP), xanthosine-5′-monophosphate (XMP), adenine, adenosine, and guanosine were purchased from the Sigma Chemical Co. Adenosine-5′-triphosphate (ATP) and inosine-5′-monophosphate (IMP) were from Daiichi Pure Chemicals Co., Ltd. and Boehringer Mannheim GmbH, respectively. Inosine, guanine, hypoxanthine, uric acid, and xanthine were obtained from Wako Pure Chemical Industries, Ltd. All other chemicals were obtained from the usual commercial sources.

Extraction of acid-soluble purine nucleotides

The integuments, livers, blood, and sera from live salmon were extracted with 2 volumes of cold 10% perchloric acid. The extraction was followed by centrifugation at 3,000 rpm for 10 min. The combined extracts were neutralized with 10 N KOH and corrected to pH 6.5～7.5. The precipitate of $KClO_4$ was removed by centrifugation at 3,000 rpm for 10 min. The supernatant was filtered through a filter (0.45 μm pore size, Nihon Millipore Kogyo K.K.) and submitted to HPLC.

HPLC analysis of purine nucleotides

A 10 μl portion of the above aqueous solution was injected into the HPLC system, a Hitachi 655 liquid chromatograph equipped with a Hitachi 655A variable-wavelength UV monitor and a Hitachi D-2000 Chromato-Integrator. The separation was performed on an Asahipak GS-320 column (7.6 mm i.d. × 500 mm) packed with a hydrophilic polymer gel and monitored at 260 nm. The mobile phase used was 10～200 mM sodium phosphate buffer at pH 3.07～3.27. The mobile phase was filtered through a filter (0.45 μm pore size, Nihon Millipore Kogyo K.K.), and its flow rate was maintained at 0.9 ml/min at room temperature throughout the separation process.

Results and Discussion

Separation conditions of authentic purine nucleotides and related compounds by HPLC

The separation conditions for authentic purine nucleotides, nucleosides and bases were investigated with an eluent of sodium phosphate buffer. The concentration of phosphate was varied from 10 mM to 200 mM, and the pH was varied from 3.07 to 3.27. As shown in Fig. 1, satisfactory separations could be attained at 200 mM sodium phosphate buffer (pH 3.25) within 60 min, although the overlapping peaks were observed in uric acid and guanosine.

The effect of pH (200 mM sodium phosphate buffer) on the retention time of

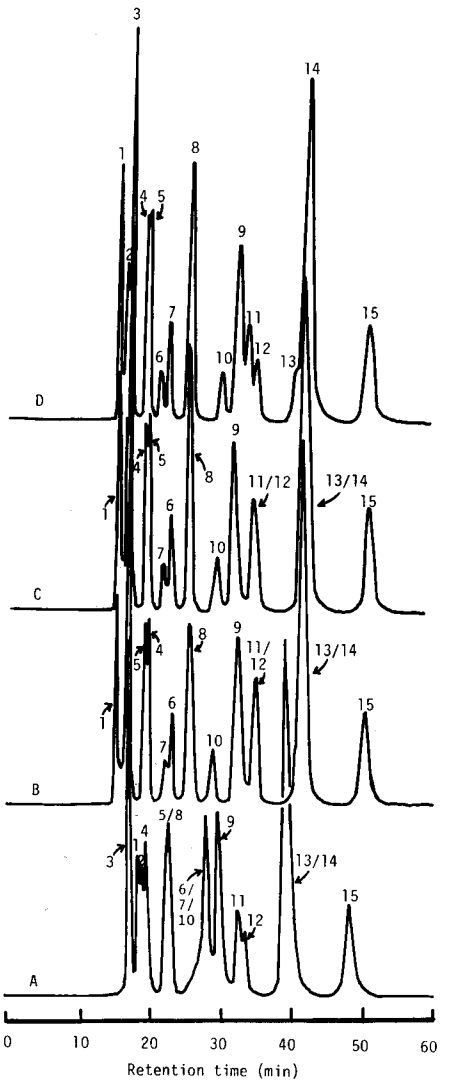

Fig. 1. Effect of the concentration of sodium phosphate buffer (pH 3.25) on the separation of purine nucleotides and related compounds.
A, 10 mM sodium phosphate buffer; B, 50 mM sodium phosphate buffer; C, 100 mM sodium phosphate buffer; D, 200 mM sodium phosphate buffer.
Column, Asahipak GS-320, 500×7.6 mm i.d.; flow-rate, 0.9 m*l*/min; detection, 260 nm; sample volume, 10 μl. Peaks: 1=ATP; 2=ADP; 3=β-NAD; 4=AMP; 5=IMP; 6=GMP; 7=XMP; 8=adenine; 9=adenosine; 10=inosine; 11=guanine; 12=hypoxanthine; 13=uric acid; 14=guanosine; 15=xanthine.

Table 1. Effect of pH (200 mM sodium phosphate buffer) on the retention time of purine nucleotides and related compounds

Compound	Retention time (min)				
	pH				
	3.07	3.15	3.18	3.25	3.27
ATP (1)*	15.24	15.29	15.31	15.33	15.38
ADP (2)	16.22	16.30	16.34	16.39	16.49
β-NAD (3)	—**	16.86	16.91	17.02	17.11
AMP (4)	18.46	18.70	18.83	19.18	19.63
IMP (5)	19.62	19.65	19.49	19.48	19.63
GMP (6)	22.30	21.38	21.30	21.21	—
XMP (7)	22.30	22.45	21.45	22.49	—
Adenine (8)	23.46	24.10	24.31	25.29	25.63
Adenosine (9)	—	29.08	29.56	31.96	32.84
Inosine (10)	29.75	29.96	29.56	29.82	30.22
Guanine (11)	29.75	31.26	31.50	33.45	34.52
Hypoxanthine (12)	33.94	34.44	33.72	34.62	35.24
Uric acid (13)	40.46	38.60	39.81	41.66	41.43
Guanosine (14)	—	38.60	39.81	41.66	42.81
Xanthine (15)	50.89	50.76	48.38	50.36	51.82

* Peak numbers on chromatogram.

** Not determined.

authentic purine nucleotides and related compounds is shown in Table 1. At lower pH, the peaks for inosine and guanine overlapped. Furthermore, we found that the elution orders of inosine and adenosine were reversed at pH 3.18. From these results, we decided to use 200 mM sodium phosphate, pH 3.25 as a preferred mobile phase for separating the purine nucleotides, especially inosine, guanine and hypox-

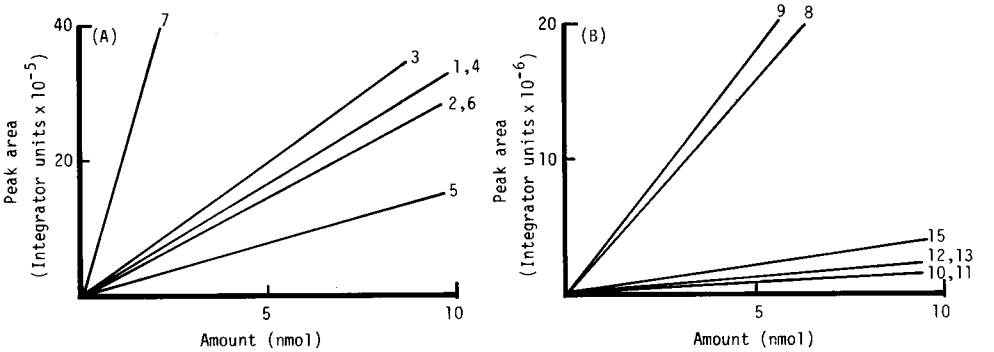

Fig. 2. Linearity between the peak area and injected amount of purine nucleotides (A) and related compounds (B).
1, ATP; 2, ADP; 3, β-NAD; 4, AMP; 5, IMP; 6, GMP; 7, XMP; 8, adenine; 9, adenosine; 10, inosine; 11, guanine; 12, hypoxanthine; 13, uric acid; 15, xanthine.

anthine, which were markedly observed in the integument of salmon (Markert and Vanstone, 1966; Johnston and Eales, 1967; Hayashi, 1970, 1971; Fujii et al., 1971).

Figure 2 shows calibration curves for each authentic purine nucleotide and related compound. The linearity of the peak area and the amount of each authentic standard was observed in a range of 0.1 to 10 nmol.

Acid-soluble purine nucleotides and related compounds of integuments from salmonid fishes

Typical chromatograms of integumental acid-soluble purine nucleotides from pre-spawning male chum salmon and spawning male masu salmon are shown in Fig. 3. Guanine and hypoxanthine were dominant compounds in the integument of chum salmon, while an unknown peak which retention time was 21 min, as well as guanine and hypoxanthine, was dominant in the integument of masu salmon. The chromatograms of spawning chum salmon and pink salmon were similar to those of pre-spawning chum salmon, although the amounts of guanine and hypoxanthine were decreased (Table 2).

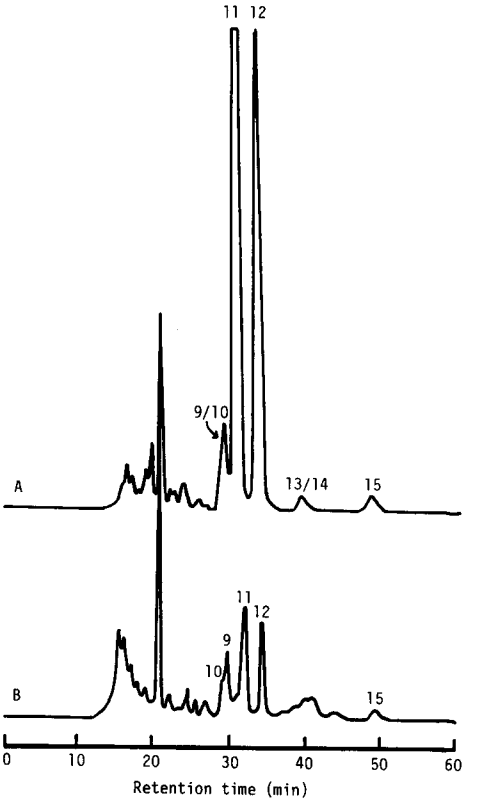

Fig. 3. HPLC separation of integumental acid-soluble purine nucleotides and related compounds from pre-spawning male chum salmon (A) and spawning male masu salmon (B).
HPLC conditions are described in the text.

Table 2. Content (μmol/g) of integumental acid-soluble purine nucleotides and related compounds from salmonid fishes

Compound	Pre-spawning chum salmon		Spawning chum salmon		Pink salmon		Masu salmon	
	Male	Female	Male	Female	Male	Female	Male	Female
Inosine/ Adenosine	0.259±0.061	0.286±0.117	0.156±0.032	0.144±0.025	0.164	0.186	0.198	0.412
Guanine	9.444±2.036	13.350±6.999	5.277±2.716	5.381±2.801	2.789	9.100	0.618	1.702
Hypoxanthine	3.596±1.066	5.076±1.629	1.433±0.476	1.760±0.848	1.317	2.790	0.476	0.780
Uric acid/ Guanosine	0.136±0.040	0.170±0.047	0.124±0.014	0.146±0.018	0.135	0.235	tr	tr
Xanthine	0.071±0.012	0.069±0.032	0.027±0.009	0.028±0.004	0.014	0.044	0.044	0.039

Values represent the mean±standard deviation of five chum salmon.

Acid-soluble purine nucleotides and related compounds of livers from salmonid fishes

Typical chromatograms of liver acid-soluble purine nucleotides from spawning male and female chum salmon are shown in Fig. 4. Various purine nucleotides and related compounds were found in the liver from chum salmon, pink salmon and masu salmon (Table 3).

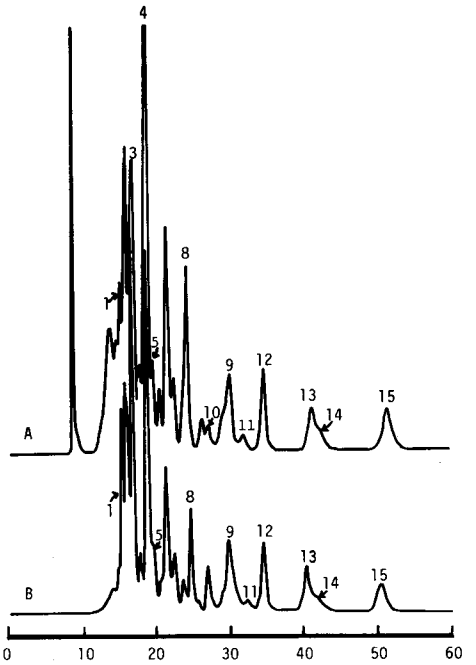

Fig. 4. HPLC separation of liver acid-soluble purine nucleotides and related compounds from spawning male (A) and female (B) chum salmon.
HPLC conditions are described in the text.

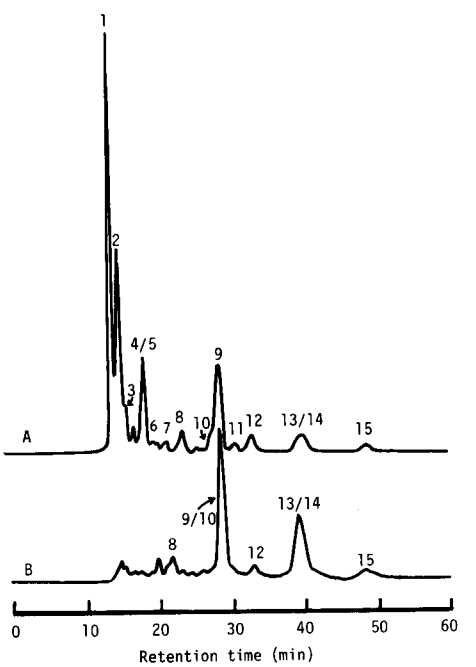

Fig. 5. HPLC separation of blood (A) and serum (B) acid-soluble purine nucleotides and related compounds from spawning male chum salmon.
HPLC conditions are described in the text.

Table 3. Content (μmol/g) of liver acid-soluble purine nucleotides and related compounds from salmonid fishes

Compound	Pre-spawning chum salmon		Spawning chum salmon		Pink salmon		Masu salmon	
	Male	Female	Male	Female	Male	Female	Male	Female
ADP	0.355±0.191	0.344±0.195	0.244±0.022	0.280±0.046	0.444	0.570	0.747	0.429
β-NAD	—*	—	—	—	0.085	0.102	0.334	0.300
AMP	0.536±0.208	0.712±0.192	0.676±0.069	0.456±0.087	0.527	0.696	0.261	0.141
IMP	0.209±0.153	0.154±0.018	0.145±0.037	0.144±0.033	0.178	0.199	0.033	0.159
Adenine	0.186±0.028	0.213±0.064	0.151±0.049	0.141±0.031	0.072	0.102	0.447	0.515
Inosine/ Adenosine	0.298±0.108	0.141±0.030	0.170±0.047	0.176±0.017	0.427	0.341	0.488	0.558
Guanine	0.066±0.026	0.056±0.025	0.064±0.017	0.059±0.021	0.084	0.113	0.117	0.208
Hypoxanthine	0.186±0.155	0.153±0.027	0.240±0.074	0.230±0.029	0.281	0.233	0.431	0.696
Uric acid/ Guanosine	0.270±0.073	0.204±0.038	0.239±0.020	0.290±0.028	0.208	0.243	0.366	0.359
Xanthine	0.101±0.037	0.077±0.012	0.080±0.013	0.085±0.013	0.036	0.047	0.098	0.152

* Not determined.

Values represent the mean±standard deviation of five chum salmon.

It was notable that the peak eluted at the void volume (retention time 9 min) of column was observed in only spawning male salmon. The component eluted at the void volume was characterized by macro molecules with UV absorption, ninhydrin positivity and sugar positivity. The nature of this compound will be described elsewhere.

Acid-soluble purine nucleotides and related compounds of blood and sera from salmonid fishes

Typical chromatograms of blood and serum acid-soluble purine nucleotides

Table 4. Content (μmol/ml) of blood acid-soluble purine nucleotides and related compounds from salmonid fishes

Compound	Pre-spawning chum salmon		Spawning chum salmon		Pink salmon		Masu salmon	
	Male	Female	Male	Female	Male	Female	Male	Female
ATP	0.296±0.126	0.230±0.083	0.713±0.182	0.705±0.249	0.101	0.165	0.295	0.417
ADP	0.389±0.059	0.302±0.070	0.534±0.104	0.682±0.091	0.097	0.112	0.286	0.305
AMP	0.181±0.054	0.142±0.018	0.101±0.033	0.132±0.039	0.015	0.006	0.163	0.109
IMP	0.128±0.058	0.089±0.039	0.206±0.200	0.086±0.032	0.076	0.092	0.141	0.096
Adenine	0.158±0.118	0.173±0.115	0.055±0.003	0.069±0.011	0.069	0.085	0.132	0.108
Inosine/ Adenosine	0.387±0.054	0.323±0.054	0.375±0.174	0.280±0.049	0.085	0.150	0.202	0.158
Guanine	0.193±0.138	0.209±0.135	0.074±0.008	0.084±0.010	0.103	0.114	0.169	0.136
Hypoxanthine	0.105±0.017	0.099±0.020	0.072±0.046	0.057±0.013	0.097	0.187	0.102	0.021
Uric acid/ Guanosine	0.374±0.135	0.295±0.094	0.309±0.087	0.295±0.068	0.059	0.184	0.191	0.103
Xanthine	0.008±0.003	0.009±0.002	0.016±0.011	0.016±0.011	0.016	0.012	0.019	0.006

Values represent the mean±standard deviation of five chum salmon.

Table 5. Content (μmol/ml) of serum acid-soluble purine nucleotides and related compounds from salmonid fishes

Compound	Pre-spawning chum salmon		Spawning chum salmon		Pink salmon		Masu salmon	
	Male	Female	Male	Female	Male	Female	Male	Female
Inosine/ Adenosine	0.342±0.065	0.281±0.068	0.116±0.056	0.294±0.051	0.318	0.229	0.123	0.224
Guanine	0.022±0.010	0.018±0.016	0.012±0.007	0.019±0.007	tr	tr	tr	tr
Hypoxanthine	0.072±0.031	0.046±0.012	0.058±0.051	0.096±0.035	0.396	0.311	0.067	0.118
Uric acid/ Guanosine	0.280±0.103	0.224±0.055	0.307±0.075	0.364±0.093	0.207	0.202	0.105	0.132
Xanthine	0.007±0.002	0.006±0.002	0.017±0.018	0.015±0.004	0.045	0.025	0.012	0.017

Values represent the mean±standard deviation of five chum salmon.

from spawning male chum salmon are shown in Fig. 5. ATP and ADP were dominant compounds in the blood, while adenosine, inosine, uric acid and guanosine were dominant in the serum (Tables 4 and 5). The chromatograms of pre-spawning chum salmon, pink salmon and masu salmon were similar to those of spawning chum salmon.

Acknowledgments

The authors are indebted to Dr. M. SANEYOSHI for his valuable discussion, and to Dr. K. YAMAUCHI for kindly use of UV monitor.

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