Bull. Environ. Contam. Toxicol. (1991) 46:713–719

Environmental Contamination and Toxicology

Comparison of Nucleoside Concentrations in Blood of Fish with and without Tumors

Douglas W. Kuehl,[1] Linda Eisenschenk,[2] Sandra Naumann,[2] Rodney D. Johnson,[1] Ronald Regal,[3] Phyllis Barnidge,[3] and James McKim, Jr.[4]

[1]U.S. EPA, Environmental Research Laboratory—Duluth, Duluth, Minnesota 55804, USA; [2]Center for Lake Superior Environmental Studies, University of Wisconsin—Superior, Superior, Wisconsin 54880, USA; [3]Department of Statistics, University of Minnesota—Duluth, Duluth, Minnesota 55812, USA, and [4]Department of Food Science and Technology, Oregon State University, Corvallis, Oregon, USA

Human cancer patients and tumor-bearing animals are known to have unusually high concentrations of modified purines and pyrimidines in their blood and urine. The source of these compounds appears to be subpopulations of transfer RNAs in tumors that exhibit a very high turnover rate (Borek et al. 1977). These compounds cannot be further used by cells because of the lack of appropriate kinases, which synthesize modified nucleoside triphosphates; consequently, they are excreted (Mandel et al. 1966). It has been proposed that the elevated nucleoside concentrations can therefore be used as biomarkers of tumor development in mammals, and possibly as a monitor of the regression of tumors following surgery or treatment (Gehrke et al. 1979; Speer et al. 1979; Birkmayer 1985; Fritsch 1985). The objective of this study was to develop and use HPLC based analytical methodology to characterize nucleosides in blood plasma and serum from fish with and without tumors, with a goal of determining if fish blood nucleoside concentrations could similarly be used as a bioindicator of tumor development in fish.

Our approach was to develop analytical methodology and quality assurance criteria for the analysis of nucleosides in fish blood, and to characterize nucleoside concentrations in blood of fish for which both healthy and tumor-bearing samples were available. Data would then be used to establish parameters with which tumor-bearing fish could be distinguished from healthy fish. Blood samples used to establish the diagnostic parameters were from control rainbow trout (Oncorhynchus mykiss) and those with tumors developed after exposure to aflatoxins. A second set of blood samples was from field collected black bullheads (Ictalurus melas).

MATERIALS AND METHODS

Fish exposed in the laboratory to aflatoxin B1 were obtained from the Marine and Freshwater Biomedical Center, Oregon State University, Corvallis, Oregon. Experimental details of exposure and histopathological examination are given in previous publications (Bailey 1984; Hendricks 1984). Two-mo-old fish were exposed to 0.4 mg/L aflatoxin B1 (afb1) in an aerated bath at 12.5°C. They were then removed after 30 min of exposure, and placed in grow-out tanks until sampling at 11 mo old. The average body weight at the termination of the experiment was 106 g. Blood (1 mL), collected by draining the fish (anesthetized with MS222) after severing the

Address reprint requests to D.W. Kuehl at the above address.

tail posterior to the anal fin, was allowed to clot overnight at 4°C. The clot was removed, and the serum was shipped on dry ice to ERL-Duluth where it was stored at -80°C until analyzed.

Two-yr-old black bullheads were collected in the lower Fox River near the mouth of the river at Green Bay, Wisconsin by the Wisconsin Department of Natural Resources using trap nets during census of June and September, 1988 (Johnson 1990). Fish were maintained in freshwater reservoirs on board the boats and transported to a sample preparation area on shore. Prior to drawing blood, the bullheads were anesthetized in a solution of MS222. Blood (3 mL) was drawn using a heparinized 3-mL syringe fitted with a #26 gauge needle by entering the ventral surface of the oral cavity and puncturing the Conus arteriosus. Samples were immediately transferred to polypropylene centrifuge tubes (15 mL), chilled on ice and centrifuged (5 min @ 1400 xg) within 30 min. Plasma (0.5 - 1.0 mL) was transferred to a clean polypropylene vial (3 mL), frozen, and stored on dry ice for transportation to the laboratory. All samples were maintained at -80°C until analysis.

The methodology for the isolation of the blood plasma nucleosides followed that reported by Gehrke (1978). An aliquot of blood plasma or serum (0.5 mL) was removed from a sample and spiked with 200 µL 5-iodocytidine (410 µg/mL) in a 1.5 mL microcentrifuge tube. Ice cold acetonitrile (0.5 mL) was added to the sample, which was vortexed and chilled on ice (15 min) to precipitate protein. The sample was then centrifuged (5 min @ 1400 xg), and the supernatant was quantitatively transferred to a second microcentrifuge tube. The precipitation procedure was repeated, and the sample was transferred to a third tube. The pH was then adjusted with the addition of 0.5 mL ammonium acetate (2.5 M, pH 9.5), thoroughly mixed and quantitatively transferred to an affinity gel column (Affi-Gel 601, BioRad Corp.*, Richmond, CA, 300 mg) with 1 mL of the ammonium acetate (0.25 M, pH 8.8). The column was washed with an additional 3 mL of ammonium acetate. Nucleosides, which were selectively retained by the gel, were eluted with 0.1 M formic acid (5 mL). The samples were lyophilized and reconstituted with distilled water (100 µL) for liquid chromatographic (LC) analysis.

Isolated blood plasma nucleosides were identified using a reversed phase microbore C-18 HPLC column operated isocratically (3% ethanol/water) with UV detection (254 nm). First, a comparison of the relative retention volume of known nucleoside standards to unknown peaks in fish blood samples was made. Relative retention volumes were based upon the retention volume of 5-iodocytidine relative to formic acid, an unretained compound. Second, a portion of sample extract was spiked with a small amount of nucleoside thought to be present, and the sample was rechromatographed. An increased relative height of the tentatively identified peak indicated the presence of the selected nucleoside. Detector response was monitored using a dual pen (2 mV and 10 mV) strip chart recorder.

Adenosine, cytidine, guanosine, n-methylguanosine, inosine, pseudouridine, thymidine, uridine and xanthosine were purchased from Sigma Chemical Co. (St. Louis, MO). N,N-Dimethylguanosine was a generous gift from Dr. Girish B. Chheda, Roswell Memorial

*The use of trade names or manufacturer's names does not imply U.S. Government endorsement of commercial products.

Institute, Buffalo, New York. Ethanol was obtained from Fisher Scientific (Chicago, IL). Ammonium acetate and formic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Quantification of the isolated nucleosides was accomplished with the use of an external calibration curve. Nucleoside UV response peak height was measured and normalized to 5-iodocytidine, a surrogate analyte added to the sample prior to sample preparation. Samples were analyzed in sets of 12, including one blank, one sample duplicate (when possible due to limited amount of sample), one procedure blank spiked with a nucleoside analytical standard mixture, and nine samples. Quantification was done using the same LC conditions described above.

The following criteria were used to assess the quality of analytical measurements: 1. Retention volume of 5-iodocytidine in any sample was within ± 0.2 min of the value obtained in the quantification standard; 2. UV detector response for any analyte was not greater than the response obtained for the highest concentration standard; 3. UV detector response for any analyte in the blank sample was less than 3:1 s/n; 4. Quantification of spike sample nucleosides were within ± 10% of expected; 5. Quantification of the highest concentration analyte in duplicate analyses was within 10%; and 6. The recovery of 5-idocytidine was greater than 50% in each sample.

Discrimination between fish with and without tumors on the basis of nucleoside concentrations was attempted by comparing individual nucleosides in the two groups of fish and by applying multivariate statistical methods to the suite of six nucleoside values. The concentrations of a single nucleoside in the two groups were compared by viewing histograms and computing two-sample t-tests. The concentrations for each pair of nucleosides were displayed on scattergrams with different symbols for the two groups. Principal component analysis (Gnanadesikan 1977) was used to reduce the six nucleoside measurements to a linear two-dimensional projection that was as close as possible to the original six-dimensional points. The first two principal component scores were then plotted again assigning different symbols to the two groups. In addition canonical discriminant analyses or discriminant coordinates (Gnanadesikan 1977) were used to determine if any linear combination of the nucleoside concentrations displayed a significant distinction between the groups of fish with and without tumors. Canonical discriminant analysis finds and evaluates the significance of the linear combination that produces the largest T statistic for comparing the groups. The principal component analyses were performed using SAS (1985) procedure PRINCOMP, and the canonical discriminant analyses were performed with SAS procedure CANDISC.

RESULTS AND DISCUSSION

Results from the quantification of blood plasma nucleosides in four control and 14 aflatoxin exposed rainbow trout and in 25 bullhead are presented in Tables 1 and 2, respectively. It was observed that inosine was the major nucleoside in all of the rainbow trout samples. This is in contrast to human blood where pseudouridine was generally found to be the major nucleoside (Salvatore 1983). Guanosine was most often the nucleoside at the second highest concentration in the trout samples; however, the concentration of xanthosine exceeded guanosine in two of the toxicant exposed fish. The average concentration of guanosine was 11% that of inosine in the trout. Cytidine, pseudouridine, and

Table 1. Nucleoside concentrations in trout blood serum, μg/mL. Trout 1-14 exposed to aflatoxin B1.

Sample	Sex	Tumor	Inosine	Guanosine	Pseudouridine	Cytidine	Uridine	Xanthosine
1	F	+	248	184	1.73	13.7	8.55	ND (.02)
2	M	+	179	19.6	0.93	ND (.01)	7.08	ND (.02)
3	M	+	175	34.0	0.28	1.10	ND (.01)	14.8
4	M	+	377	23.5	ND (.01)	7.82	ND (.01)	22.0
5	F	+	346	23.4	ND (.01)	15.8	ND (.01)	17.7
6	F	+	208	24.2	ND (.02)	1.17	ND (.02)	ND (.02)
7	F	+	261	15.5	0.29	1.20	8.78	24.2
8	F	+	251	18.8	1.00	6.20	ND (.02)	ND (.02)
9	M	+	266	8.15	ND (.02)	ND (.01)	ND (.02)	13.5
10	F	+	966	85.3	ND (.06)	2.38	6.29	1.97
11	F	0	252	30.2	ND (.02)	0.62	ND (.02)	ND (.02)
12	F	0	803	81.3	0.28	5.09	ND (.16)	4.22
13	F	+	811	78.1	ND (.06)	2.23	ND (.16)	1.85
14	F	+	925	76.5	ND (.06)	4.06	ND (.16)	3.36
Control A	--	0	833	86.1	0.20	ND (.16)	8.49	ND (.26)
Control B	--	0	799	75.2	0.18	2.36	15.9	1.95
Control C	--	0	351	53.4	ND (.02)	48.2	ND (.02)	ND (.02)
Control D	--	0	142	25.1	.22	2.30	3.32	20.7

+ = tumor present; 0 = tumor not detected; -- not determined; ND = not detected.

Table 2. Nucleoside concentration in twenty-five bullhead blood plasma samples, μg/mL. Fish were collected from lower Fox River, at the mouth of the river near Green Bay of Lake Michigan, Green Bay, Wisconsin. No tumors were present.

Sample	Sex	Inosine	Guanosine	Pseudouridine	Cytidine	Uridine	Xanthosine
1	F	5.5	ND (1.0)	0.04	2.15	0.11	ND (.26)
2	F	17.7	6.3	0.03	0.44	1.08	1.67
3	F	44.0	4.6	0.21	2.19	0.97	2.27
4	M	84.0	53.5	0.06	0.27	1.20	ND (.26)
5	F	304	18.4	0.03	0.63	0.68	ND (.26)
6	M	26.7	2.8	0.31	3.82	ND (.08)	4.95
7	M	12.5	1.9	0.07	0.57	1.40	0.81
8	M	175	23.1	ND (.07)	0.61	0.99	0.32
9	F	124	9.0	ND (.07)	0.70	0.71	ND (.26)
10	F	16.1	3.4	0.05	0.15	0.60	0.50
11	M	27.2	3.3	0.33	0.59	ND (.08)	0.80
11*	M	26.8	1.2	0.06	0.58	0.28	0.49
12	F	41.7	6.4	0.05	0.76	0.56	0.35
13	M	151	9.5	0.06	1.21	0.82	0.17
14	M	540	231	0.13	1.80	0.84	0.30
14*	M	479	195	ND (.07)	3.09	0.79	0.46
15	M	23.0	9.5	0.04	4.26	0.11	ND (.26)
15*	M	26.5	11.8	ND (.07)	2.47	0.08	ND (.26)
16	M	64.3	45.0	0.36	0.57	0.71	ND (.26)
17	M	10.5	2.5	0.33	1.11	2.56	ND (.26)
18	F	21.1	5.4	0.10	2.94	ND (.08)	0.96
19	M	72.8	79.7	0.11	0.89	1.39	ND (.26)
20	F	371	387	0.18	ND (.13)	ND (.08)	ND (.26)
21	M	9.0	2.1	0.57	3.56	0.07	2.80
22	F	20.6	17.5	0.12	0.26	0.70	ND (.26)
23	F	12.8	4.4	0.41	2.79	0.61	ND (.26)
24	M	8.5	6.1	0.39	3.49	ND (.08)	0.11
25	M	9.1	11.5	1.09	4.51	0.65	ND (.26)

* Mean value of duplicate analysis; ND = not detected.

uridine were also identified as minor nucleosides. The same six nucleosides were identified in bullhead samples, and again inosine was identified as the major nucleoside in most of the samples. The concentration of guanosine exceeded inosine in three bullhead samples. The concentrations of inosine and guanosine in trout (#1-14) were bimodal with values less than 380 μg/mL and more than 800 μg/mL, and less than 35 μg/mL and more than 75 μg/mL for each compound respectively. The fish in the high range of concentrations were the same (#10, 11, 13 and 14) for each compound, and were female, indicating a concentration bias may possibly be influenced by the sex of the fish. A similar bimodal distribution was observed in the bullhead data, where concentrations less than 175 μg/mL and greater than 300 μg/mL, and less than 80 μg/mL and more than 195 μg/mL for inosine and guanosine, respectively, were measured. Fish in the high range of concentrations included two females and one male (inosine) and one female and one male (guanosine) indicating that although bimodal, it did not appear to be influenced by the sex of the bullheads.

Quality of data was evaluated by measuring the change in relative retention volume, a study of the overall recovery of each identified nucleoside, and an assessment of the spiked samples and the duplicate associated with each analysis. It was found that the relative retention volume was reproducible to ± 0.01 with formic acid = 0.0 and 5-iodocytidine = 1.0. An evaluation of the overall analytical performance is provided in Table 3, a study of the quantification of each identified nucleoside in spiked blank procedure samples.

Table 3. Quantification of Nucleosides in Spiked Blanks (μg/mL) N = 6

	Nucleosides					
	Pseu.	Cyt.	Urid.	Xan.	Ind.	Guan.
Analyte concentration	2.36	1.32	1.36	0.197	1.59	1.15
Mean measured value	2.95	1.43	2.06	0.277	2.31	1.85
Standard deviation	0.52	0.19	0.25	0.038	0.21	0.15

Trout with tumors could not be distinguished from trout without tumors by comparing the concentration of any individual nucleoside. In addition, no differences were observed when plotting scattergrams from all possible pairs of nucleosides. Neither was it possible to determine a higher dimensional distinction between the two groups using multivariate relationships determined by the techniques of principal component and canonical discriminant analysis. These methods were applied to the nucleosides both on the original scale and in the log scale. The below detectable observations were replaced with 0.1 μg/mL for log calculations. The p-value in the log scale for instance, for testing the significance of canonical discrimination between the groups was p = 0.86, or essentially no indication of any difference between the groups.

The lower Fox River/Green Bay water way was chosen as the field sampling site because of the likely success of observing a high frequency of tumors in its bullhead population. Therefore the determination of the concentration of nucleosides in bullhead blood plasma and the evaluation of liver tumors proceeded simultaneously. Unfortunately, although numerous lip papilloma

and bacterial infections were observed, no liver tumors were observed in any of the more than 100 bullheads examined (Johnson 1990). These data however can serve as an indication of the background concentrations of nucleosides in blood of bullheads collected from a contaminated environment. Further, the analytical methods and quality assurance criteria presented here can be used for routine monitoring of blood nucleosides in large numbers of fish.

REFERENCES

Bailey GS, Hendricks JD, Nixon JE, Pawlowski NE (1984) The sensitivity of rainbow trout and other fish to carcinogens. Drug Metab 15(4):725-750

Birkmayer GD (1985) Optimizing of tumor markers for patient surveillance and therapy control. Bull Mol Med 10:81-99

Borek E, Baliga BS, Gehrke CW, Kuo CW, Belman S, Troll W, Waalkes TP (1977) High turnover rate of transfer RNA in tumor tissue. Cancer Res 37:3362-3366

Fritsche HA (1985) Clinical application of tumor markers. Bull Mol Biol Med 10:9-23

Gehrke CE, Kuo KC, Davis GE, Suits RD, Waalkes TP, Borek E (1978) Quantitative high-performance liquid chromatography of nucleosides in biological materials. J Chromatogr 150:455-476

Gehrke CW, Kuo KC, Waalkes TP, Borek E (1979) Patterns of urinary excretion of modified nucleosides. Cancer Res 39:1150-1153

Gnanadesikan, R (1977) Methods for statistical data analysis of multivariate observations. Wiley, New York

Hendricks JD, Meyers TR, Casteel JL, Nixon JE, Loveland PM, Bailey GS (1984) Rainbow trout embryos: Advantages and limitations for carcinogenesis research. Cancer Inst Monogr 65:129-137

Johnson RD, Mac MJ, Tietge JE, Stokes GN, Zhang F (1990) Lack of liver tumors in bullheads collected from the lower Fox River and lower Green Bay, Wisconsin. Personal Communication

Mandel LR, Srinivasan PR, Borek E (1966) Origin of urinary methylated purines. Nature 209:586-588

Salvatore F, Savoria M, Russo T, Sachetti L, Cimino F (1987) Pseudouridine in biological fluids of tumor bearing patients. In F Cimino, GD Birkmayer, JV Klavins, E Pimentel and F Salvatore (eds). Human Tumor Markers, pp 451-462 Berlin: Walter de Guyter and Company

SAS Institute Inc (1985) SAS user's guide: statistics, version 5 edition. SAS Institute Inc, Cary, North Carolina

Speer J, Gehrke CW, Kuo KC, Waalkes TP, Borek E (1979) tRNA breakdown points as markers for cancer. Cancer 44:2120-2123

Received September 28, 1990; Accepted November 15, 1990.