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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC INVESTIGATION OF MUCOSAL NUCLEOSIDES AND BASES AND URINARY MODIFIED NUCLEOSIDES OF GASTROINTESTINAL CANCER PATIENTS

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SUMMARY

Reversed-phase high-performance liquid chromatography (HPLC) was used to determine the levels of nucleosides, bases and their metabolites in perchloric acid extracts of gastrointestinal mucosa. By comparing the levels of these compounds in the normal portion with the neoplastic portion of mucosa resected from malignant cancer patients, it was found that there was significant elevation of the uracil level in the neoplastic mucosa of all eight patients with colorectal cancer (2.7-fold in normal mucosa), but only in the neoplastic mucosa of one out of four patients with gastric cancer. The levels of hypoxanthine and uridine in the colorectal cancer mucosa samples and the inosine in gastric cancer samples were also significantly higher than those in normal mucosa.

The urinary modified nucleosides were prefractionated with a boronate affinity gel column, and their levels were determined by the same HPLC method. There was no significant difference in the concentrations of pseudouridine, 1-methylguanosine, N^2-methylguanosine and N^2,N^2-dimethylguanosine between urine samples taken before and after surgery from eight patients with malignant colorectal cancer. Contrary to other reports, no significant differences in modified nucleoside levels were observed between urine samples from patients with colorectal cancer and those from normal subjects.

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INTRODUCTION

The analysis of nucleosides and bases in physiological fluid is of primary importance for the understanding of metabolic disorders of nucleic acid and related pathological states. During the last decade, the development of high-performance liquid chromatography (HPLC) has made it possible to determine accurately and with high resolution the naturally occurring nucleotides, nucleosides, bases and their metabolites in very small samples of biological fluids (for reviews, see refs. 1—3).

Thus, the determinations of these compounds in serum or plasma [4—9], urine [10, 11], saliva [12], cerebrospinal fluids [13], and blood cells [8, 9] have been performed using several HPLC methods. HPLC techniques have also been applied to the determination of nucleotides, nucleosides and bases in acid extracts of rat liver [14], skin fibroblasts [15, 16], rat cardiac myocytes suspension [17], cat spleen perfusates [18] and rabbit kidney perfusates [19].

However, less information has been reported on the levels of nucleosides, bases and their metabolites in tissues. So far, the endogenous compounds in acid extracts of gastrointestinal (GI) mucosa have never been studied by the HPLC method. A comparison of profiles of ultraviolet (UV)-absorbing compounds in the normal portion with the neoplastic portion of mucosa resected from cancer patients could provide important information on the nucleic acid metabolism in mucosa of malignant cancer patients.

The urinary modified nucleosides derived from the enzymatic degradation of transfer RNA have been investigated for their clinical significance as biochemical markers in cancer detection [20—23]. In recent studies, these nucleosides have been prefractionated using a boronate affinity gel column, and then determined by reversed-phase HPLC [24, 25].

In most of the previous investigations, the elevations of the urinary nucleoside levels in cancer patients have been studied using the comparative data for normal subjects and cancer patients. However, the change in nucleoside levels in urine taken before and after surgery on the same patient have not been examined.

In this work, reversed-phase HPLC was used to determine the UV-absorbing compounds in perchloric acid (PCA) extracts of normal and neoplastic mucosa from GI cancer patients. Further, modified nucleosides in urines of colorectal cancer patients and normal subjects were determined by the same HPLC method, and the change in these nucleoside levels in the same patient pre- and post-operative was examined.

EXPERIMENTAL

Chemicals and chromatographic standards

The phosphate buffer, methanol (HPLC grade), enzymes, ammonium acetate buffer, formic acid and perchloric acid were obtained from Wako (Osaka, Japan). Standard compounds, such as creatinine (Crt), uracil (Ura), pseudouridine (Ps-Urd), uric acid (UA), hypoxanthine (Hyp), xanthine (Xan), uridine (Urd), 1-methyladenosine (1-m-Ado), 7-methylguanosine (7-m-Guo), inosine (Ino), guanosine (Guo), 4-thiouridine (4-S-Urd), 1-methylinosine (1-m-Ino),

1-methylguanosine (1-m-Guo) and N^2-methylguanosine (N^2-m-Guo), were obtained from Sigma (St. Louis, MO, U.S.A.) and Wako. N^2,N^2-Dimethylguanosine (N^2-dm-Guo) was purchased from Vega Biochemicals (Tucson, AZ, U.S.A.).

Chromatographic equipment

High-performance liquid-chromatographic equipment (Hitachi 638-30; Hitachi, Tokyo, Japan) with a multi-wavelength UV monitor (Hitachi 635-M) was used. Peak areas and retention times were measured at 260 nm with a Chromatopack C-R2A (Shimadzu, Kyoto, Japan). Another recorder was used to monitor the 280-nm signal and to calculate peak height ratios (280/260 nm). UV absorption spectra of fractionated HPLC peaks were measured with a spectrophotometer (Hitachi 200-10).

Chromatographic conditions

The UV-absorbing compounds and nucleoside fractions in PCA extracts of GI mucosa and urinary nucleosides of colorectal cancer patients and normal subjects were separated on commercially available columns (250 × 4.6 mm I.D.) packed with 5-μm Develosil ODS-5 (Nomura Chemicals, Nagoya, Japan). A pre-column (50 × 4.0 mm I.D.; Chemuco, Osaka, Japan) packed with 15—30-μm Develosil ODS (Nomura Chemicals) was used to protect the analytical column. A filter (1-μm mesh size; Nomura Chemicals) was fitted between the injector and pre-column.

For the separation, linear gradient elution from 0.02 *M* potassium dihydrogen phosphate (pH unadjusted; pH 4.53) to 40% methanol—water (3:2, v/v) in 35 min was used. The flow-rate was 1.2 ml/min and the column temperature was ambient. All eluents were degassed by purging with helium. The injection volume was 100 μl unless stated otherwise.

Boronate gel affinity chromatography

The boronate affinity gel column technique was used to fractionate nucleosides in 1-ml PCA extracts of mucosa homogenate and 0.5-ml urine samples. Affi-gel 601 polyacrylamide—boric acid gel resin (Bio-Rad Labs., Richmond, CA, U.S.A.) was packed in a plastic column (60 × 9 mm I.D.) to a height of 13 mm (bed volume 0.83 ml). The nucleosides were isolated according to the method developed by Gehrke et al. [24] except for the elution of nucleosides and the following steps. A 4-ml volume of 0.2 *M* formic acid was used for the elution. The eluate was evaporated under reduced pressure and redissolved in 0.5 ml of the starting buffer of HPLC analysis.

Sample collection

Samples of normal and neoplastic mucosa of colorectum and stomach were obtained by the surgical operation on eight patients (four male, four female) with malignant colorectal cancer (colon, 1; sigmoid colon, 3; rectum, 4), and four patients (three male, one female) with malignant gastric cancer, respectively. The histological stages [26] of these cases were classified as follows: stage II, 3; stage III, 3; stage IV, 2 for colorectal cancer, and stage III, 3; stage IV, 1 for gastric cancer. In the preliminary experiments, normal and neoplastic

mucosa from another three patients with malignant colorectal cancer (colon, 1; rectum, 2) were used. Resected mucosa were frozen immediately after the operation in a deep-freezer at −70° C until used.

Urine samples one day before and one week after surgical operation for eight patients (three male, five female) with malignant colorectal cancer (colon, 1; rectum, 7) were collected in the morning after a 12-h fasting period. The histological stages of these cases were as follows: stage II, 2; stage III, 3; stage IV, 1; recurrence, 2. Urine samples from sixteen normal subjects (eight male, eight female) were obtained from the PL Osaka Health Control Centre.

Sample preparation

Each mucosa sample (1.25 g wet weight) was chopped off from the normal portion and the neoplastic portion of mucosa of the same patient using scissors. The chopped mucosa were immediately minced with scissors to facilitate the following homogenization. After the addition of 3 ml of cold water, the minced mucosa samples were homogenized using a micro Waring blender (NK Micronizer; Nihon Seiki, Tokyo, Japan). After a stainless-steel blender vessel had been washed with 1 ml of cold water, 5 ml of cold PCA (5%, w/v) were added to the homogenized mucosa and the mixture was vortexed vigorously for about 3 min. After standing in ice about 30 min, the mixture was centrifuged at 1500 *g* for 10 min and the supernatant was adjusted to approximately pH 5 with 10 *M* potassium hydroxide. The pH-adjusted sample was centrifuged to remove precipitable perchlorate and the supernatant fluids were stored at −20° C until HPLC analysis.

In the preliminary experiments, homogenized mucosa were fractionated using the differential centrifugation method. PCA-soluble fractions of 105 000 *g* supernatant were used as the sample for HPLC analysis.

Urine samples were stored at −20° C and centrifuged at low speed to remove the precipitable compounds before use.

Peak identification

The UV-absorbing compounds in the PCA extracts of GI mucosa were identified using the combined data for retention time, co-injection of standards, peak height ratios, UV-absorption spectra of fractionated HPLC peaks and the enzymatic peak shift technique [4, 27]. In addition, the nucleosides were confirmed by the HPLC separation of samples prefractionated on a boronate affinity gel column.

RESULTS

Chromatography of standard compounds

The chromatogram in Fig. 1 shows the separation of 0.7—3.5 nmol each of sixteen nucleosides, bases and their metabolites using the chromatographic conditions described under Experimental. The chromatographic conditions developed by Hartwick et al. [28] were slightly modified to improve the separation at the front part of the chromatogram of the serum profile [4].

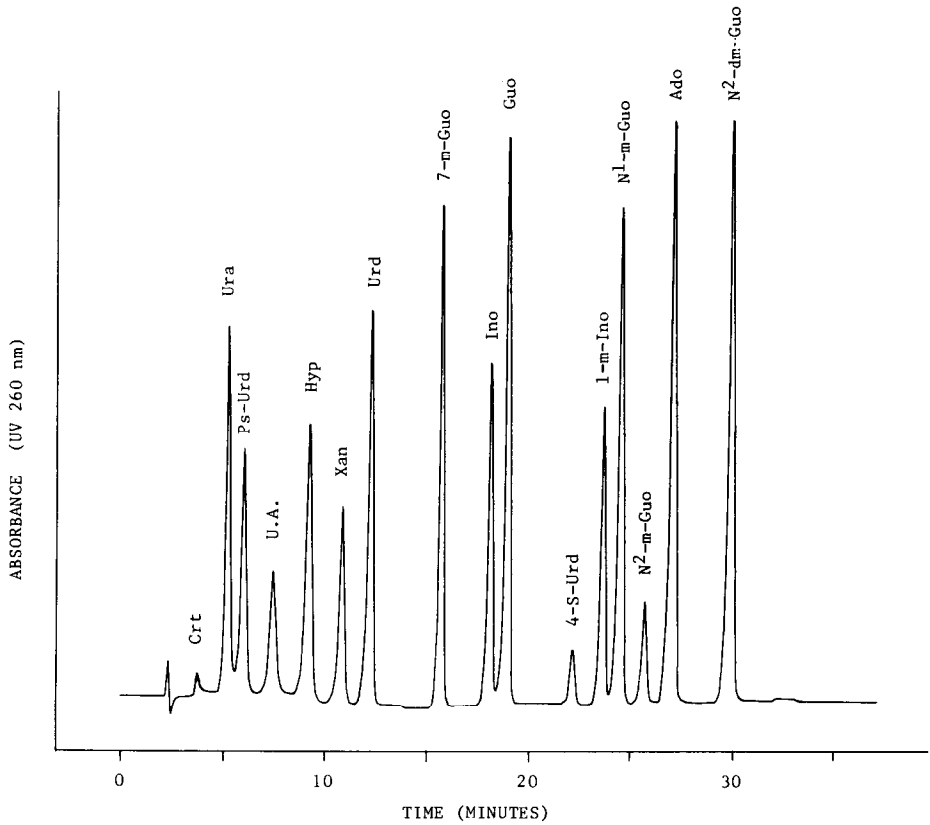

Fig. 1. HPLC separation of a standard solution of nucleosides, bases and their metabolites. Column: Develosil ODS-5 (5-μm, 250 × 4.6 mm I.D., Nomura Chemicals). Eluents: A, 0.02 *M* potassium dihydrogen phosphate, pH 4.53; B, methanol—water (3:2, v/v); linear gradient from A to 40% B in 35 min; flow-rate, 1.2 ml/min. Detector: UV at 260 nm (0.16 a.u.f.s.). Temperature: ambient. Injection volume: 70 μl of a solution $5 \cdot 10^{-5}$ mol/l in each standard except $2.5 \cdot 10^{-5}$ mol/l of 4-thiouridine and $1 \cdot 10^{-5}$ mol/l of N^2-methylguanosine.

Chromatogram of PCA extracts of mucosa

Fig. 2 shows chromatograms for PCA extracts of normal and neoplastic mucosa of rectum surgically resected from a rectal cancer patient. The peaks that were present in the majority of the GI mucosa samples are numbered. The chromatograms are typical of the profiles that were observed for most mucosa. Based on the combined data from the identification techniques, the endogenous compounds present in GI mucosa were identified as uracil, uric acid, hypoxanthine, xanthine, uridine, inosine and guanosine. The levels of uracil, hypoxanthine, xanthine and uridine were markedly increased in the neoplastic mucosa compared with the normal sample.

These chromatograms contained the same components as found in serum [4] and saliva [12]. Compared with the profiles of serum and saliva, the profile of GI mucosal extracts showed relatively high levels of hypoxanthine, xanthine and especially uracil, and a low level of uric acid, as shown in Fig. 2.

The uracil peak (peak 1) was confirmed as uracil from the characteristic change (bathochromic shift) in the UV absorption spectra at alkaline pH and from the retention time, co-injection of a standard and the peak-height ratio.

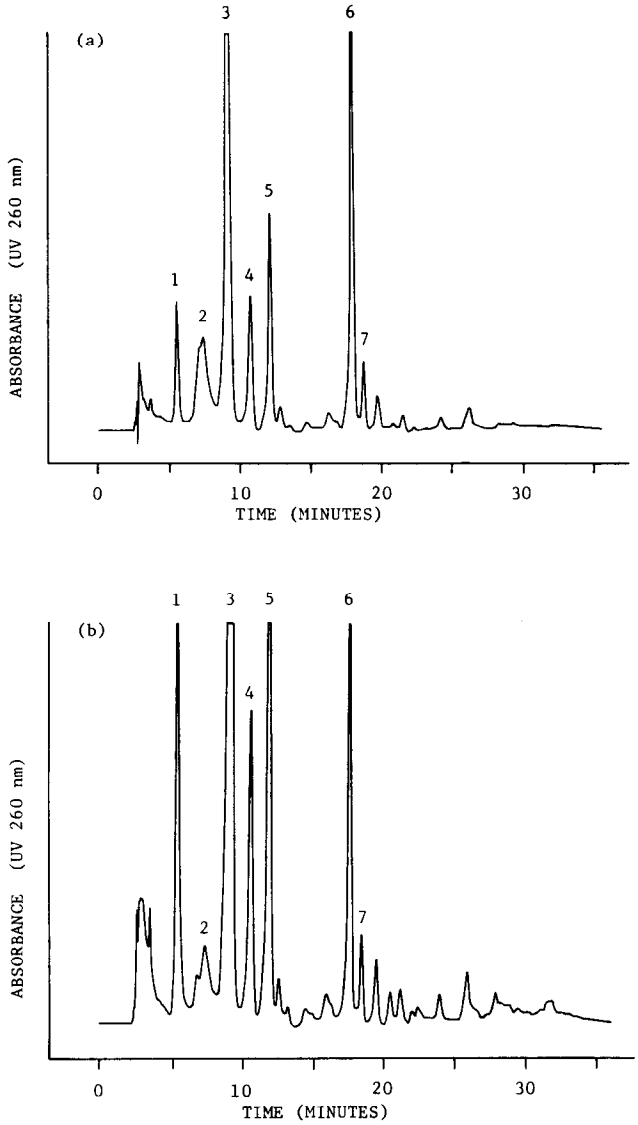

Fig. 2. Chromatograms of a PCA extract of (a) normal and (b) neoplastic rectal mucosa from a patient with malignant rectal cancer. Injection volume: 100 μl, corresponding to 13.9 mg of wet mucosa. Chromatographic conditions as in Fig. 1. Peaks: 1 = uracil; 2 = uric acid; 3 = hypoxanthine; 4 = xanthine; 5 = uridine; 6 = inosine; 7 = guanosine.

Nucleosides in PCA extracts of mucosa

The nucleosides in PCA extracts of GI mucosa were prefractionated using the boronate gel affinity chromatographic procedure described under Experimental. Fig. 3 shows the HPLC separation of nucleoside fractions obtained from the same mucosal sample as described in Fig. 2. The major peaks were identified as uridine, inosine and guanosine.

Pseudouridine and adenosine, which were confirmed in serum nucleosides [29], were not observed in detectable amounts in PCA extracts of mucosa.

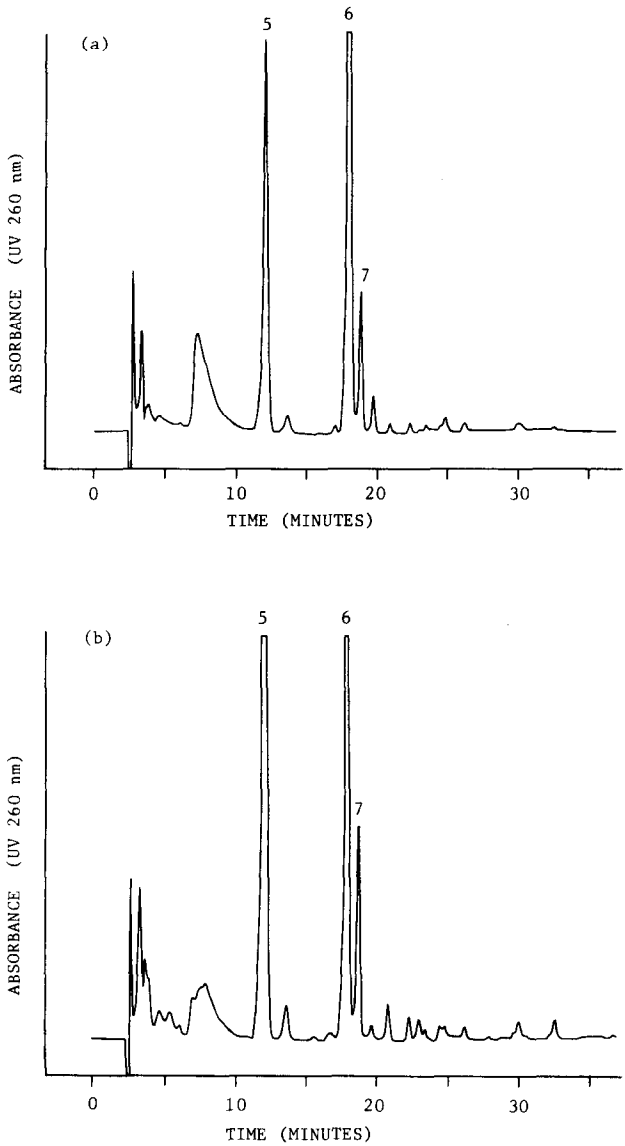

Fig. 3. Chromatograms of nucleosides in a PCA extract of (a) normal and (b) neoplastic rectal mucosa from the same patient as in Fig. 2. Injection volume: 100 μl, corresponding to 27.8 mg of wet mucosa. Chromatographic conditions as in Fig. 1 and peaks as in Fig. 2.

An unknown peak with a retention time of about 7.5 min was suggested to contain nucleotides such as ATP and ADP from the retention behaviour.

Chromatograms of PCA extracts of muscle of gastrointestinal tissue showed almost the same profile as those for mucosa. In muscle, a comparatively large peak corresponding to nucleotides was observed, as expected.

Quantitation of the compounds in PCA extracts of mucosa

The concentrations of endogenous compounds in PCA extracts of GI mucosa

TABLE I

COMPARISON OF PCA EXTRACT COMPOUND LEVELS BETWEEN NORMAL AND NEOPLASTIC MUCOSA FROM COLORECTAL CANCER PATIENTS

Patients* (cancer)	Mucosa	Level of compounds (nmol/g wet weight of mucosa)						
		Ura**	UA	Hyp***	Xan	Urd***	Ino	Guo
38 (F, 66)	Normal	152	221	760	703	151	167	11.9
(colon)	Neoplastic	757	583	1188	1141	199	143	10.5
100 (F, 72)	Normal	100	474	564	333	153	579	58.2
(sigmoid)	Neoplastic	205	371	771	357	155	563	48.5
106 (M, 52)	Normal	400	428	1216	716	346	231	45.2
(sigmoid)	Neoplastic	715	152	2045	1212	657	409	67.4
111 (M, 66)	Normal	374	306	969	818	285	303	33.9
(sigmoid)	Neoplastic	787	483	1684	708	409	923	97.2
101 (F, 56)	Normal	187	393	712	269	210	671	66.1
(rectum)	Neoplastic	584	817	1276	399	207	515	23.1
105 (F, 63)	Normal	155	790	1010	297	229	584	46.0
(rectum)	Neoplastic	557	512	1969	679	584	549	62.8
107 (M, 49)	Normal	84.6	102	727	333	213	531	10.9
(rectum)	Neoplastic	490	408	1976	822	601	512	65.2
108 (M, 65)	Normal	315	287	904	705	275	296	29.8
(rectum)	Neoplastic	598	448	1236	596	288	548	44.1
Mean	Normal	221	375	858	522	233	420	37.8
(S.D.)		(124)	(206)	(207)	(232)	(66.9)	(191)	(20.0)
	Neoplastic	587	472	1518	739	388	520	52.4
		(186)	(189)	(467)	(311)	(203)	(214)	(27.3)

*Sex (F, female; M, Male) and age are given in parentheses.
**1% significant with the paired t-test.
***5% significant with the paired t-test.

were determined by the HPLC method described above. Table I shows the levels of these compounds in normal and neoplastic mucosa from the same patient. The mean level of uracil in neoplastic mucosa from eight patients with colorectal cancer was found to be 2.7-fold higher than that in normal mucosa. This difference was statistically significant in the paired t-test ($P < 0.01$). The elevation of the uracil level in neoplastic mucosa to the same extent was also observed in another three patients with colorectal cancer examined in preliminary experiments. However, an increase in uracil levels in neoplastic mucosa was found in only one out of four patients with gastric cancer, as shown in Table II.

In neoplastic mucosa, the levels of hypoxanthine and uridine in the samples from patients with colorectal cancer and inosine in the patients with gastric cancer were significantly higher than those in normal mucosa by the paired t-test ($P < 0.05$).

Urinary nucleosides analysis

Urinary nucleosides were isolated using a boronate affinity gel column, then separated and quantitated by the same HPLC system as described above. Samples equivalent to 200 μl of urine were used for each HPLC analysis, and chromatograms for such analyses are shown in Fig. 4.

TABLE II

COMPARISON OF PCA EXTRACT COMPOUND LEVELS BETWEEN NORMAL AND NEOPLASTIC MUCOSA FROM GASTRIC CANCER PATIENTS

Patients* (cancer)	Mucosa	Level of compounds (nmol/g wet weight of mucosa)						
		Ura	UA	Hyp	Xan	Urd	Ino**	Guo
102 (M, 41)	Normal	169	349	1095	251	248	512	66.3
(stomach)	Neoplastic	139	368	647	209	94.8	457	52.0
104 (M, 58)	Normal	544	1643	630	1311	379	100	10.5
(stomach)	Neoplastic	420	625	1940	424	434	579	77.9
109 (F, 63)	Normal	325	352	934	697	418	255	27.9
(stomach)	Neoplastic	319	71.3	776	164	344	948	5.81
110 (M, 81)	Normal	315	470	869	882	393	299	16.4
(stomach)	Neoplastic	662	599	1474	656	249	1268	120
Mean	Normal	338	704	882	785	360	292	30.3
(S.D.)		(155)	(629)	(193)	(439)	(76.1)	(170)	(25.1)
	Neoplastic	385	416	1209	363	280	813	63.9
		(218)	(257)	(608)	(226)	(145)	(368)	(47.8)

*Sex (F, female; M, male) and age are given in parentheses.
**5% significant with the paired *t*-test.

Urine samples were collected before and after surgery from each colorectal cancer patient. In many instances, the levels of the urinary nucleosides in the pre- and post-operative samples showed considerable differences (maximum ten-fold).

Based on the combined data from the identification techniques and the results reported by Gehrke and co-workers [24, 25], the nucleosides in the majority of urine samples were identified as pseudouridine, 1-methylguanosine, N^2-methylguanosine and N^2,N^2-dimethylguanosine. Two peaks with retention times of 13 min (peak 2) and 23.5 min (peak 3) were observed as the migration peaks of 1-methyladenosine + uridine and 1-methylinosine + an unknown compound, respectively.

The recovery for the boronate affinity gel column was determined by processing 0.5 ml of a standard mixture on the affinity column. The average recoveries of two runs were 93.9% for pseudouridine, 105.0% for uridine, 98.5% for inosine, 93.3% for guanosine, 96.7% for 1-methylinosine, 93.8% for 1-methylguanosine, 97.2% for N^2-methylguanosine, 85.0% for adenosine and 97.3% for N^2,N^2-dimethylguanosine. The recoveries of the nucleosides were slightly lower than that reported by Gehrke et al. [24].

Quantitation of urinary modified nucleosides

The level of modified nucleosides in urine samples taken before and after surgery from the eight patients with colorectal cancer and from sixteen normal subjects was determined by the HPLC method, and then reduced using the urinary creatinine level of each sample.

Table III shows a comparison of mean levels of urinary modified nucleosides among pre- and post-operative cancer patients and normal subjects. Contrary to

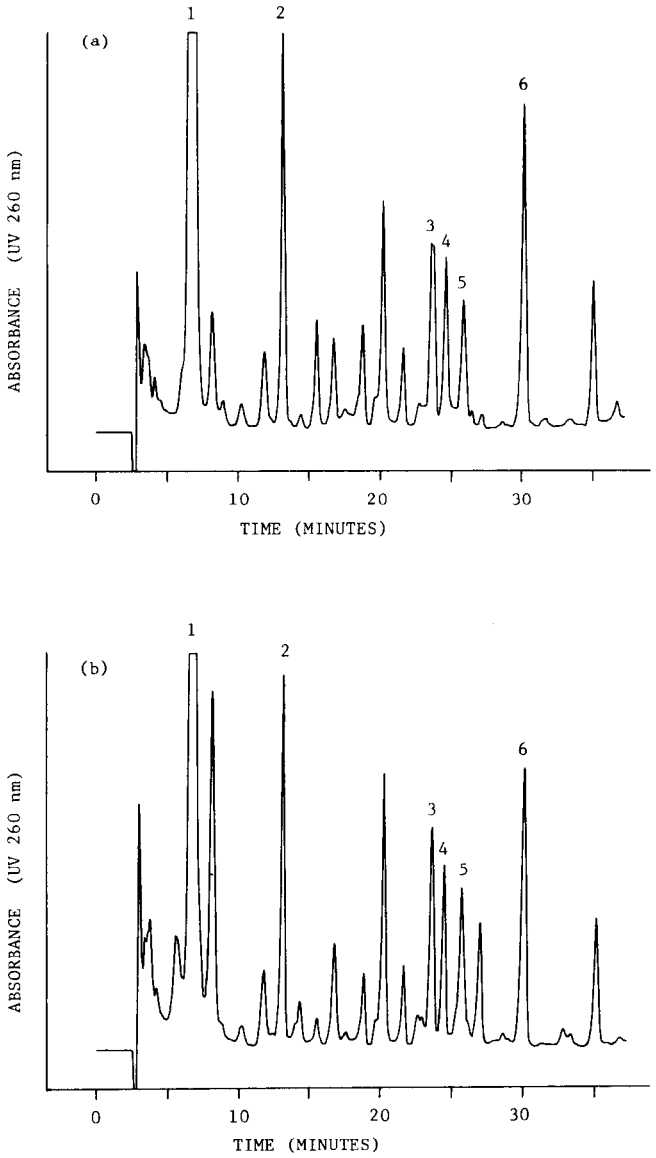

Fig. 4. Chromatograms of nucleosides in urine taken (a) before and (b) after surgical operation from a patient with malignant rectal cancer. Injection volume: 200 μl, equivalent to the same volume of urine. Chromatographic conditions as in Fig. 1. Peaks: 1 = pseudouridine; 2 = uridine + 1-methyladenosine; 3 = 1-methylinosine + unknown compound; 4 = 1-methylguanosine; 5 = N^2-methylguanosine; 6 = N^2,N^2-dimethylguanosine.

the other reports [21—23], the present results did not show an elevation of modified nucleoside levels in urine from colorectal cancer patients. The low creatinine level in post-operative patients, which was not statistically significant compared with that of pre-operative patients, seems to cause the relatively high nucleosides/creatinine level in these patients.

TABLE III

URINARY MODIFIED NUCLEOSIDE LEVELS OF COLORECTAL CANCER PATIENTS (PRE- AND POST-OPERATION) AND NORMAL SUBJECTS

Urinary modified nucleosides*	Colorectal cancer patients (n = 8)		Normal (n = 16)
	Pre-operative	Post-operative	
Pseudouridine	33.9 ± 5.17	43.9 ± 9.36	35.6 ± 5.00
1-Methylguanosine	1.21 ± 0.411	1.49 ± 0.704	1.07 ± 0.325
N^2-Methylguanosine	1.06 ± 0.258	1.38 ± 0.586	0.805 ± 0.193
N^2,N^2-Dimethylguanosine	2.20 ± 0.399	2.70 ± 0.764	2.09 ± 0.315
Urinary creatinine**	105.1 ± 89.6	60.9 ± 53.4	101.2 ± 44.7

*Units: nmol/μmol creatinine. Mean and S.D. were calculated from the data for eight patients with colorectal cancer and sixteen normal subjects.
**Creatinine level (units: mg/dl) was analysed by the Jaffe method using a Griner selective analyser II C (Griner, Langental, Switzerland).

DISCUSSION

Previously, little information has been reported on the determination of nucleosides, bases and their metabolites in human tissue. In this study, reversed-phase HPLC was applied to the determination of the endogenous compounds in PCA extracts of GI mucosa. The concentration of these compounds in the normal and neoplastic portions of mucosa resected from the patients with GI cancer was determined, and a remarkable elevation of uracil levels in the neoplastic mucosa of colorectal cancer patients was found.

In 1954, Horrigan [30] reported an increased excretion of urinary uracil in patients with chronic myelocytic leukaemia (approximately twice the normal excretion). This observation was confirmed in patients with chronic and acute leukaemia by Adams et al. [31] and in a child with a malignant tumour of the brain by Berglund et al. [32].

In the salvage pathway of pyrimidine metabolism, dihydrouracil dehydrogenase (DHUDH, E.C. 1.3.1.2) is a rate-limiting enzyme of the degradation system of uridine and thymidine. A decrease in DHUDH activity has been observed in rat hepatoma [33], embryonic liver [33] and human leukaemia [34]. The elevated level of uracil in neoplastic mucosa of colorectal cancer patients may be related to the decrease in DHUDH activity.

The activity of xanthine oxidase, the rate-limiting enzyme of IMP catabolism, has been observed to decrease in hepatoma and in other tumours [33]. The increased level of hypoxanthine and xanthine in neoplastic mucosa may be caused by a decrease in this enzyme activity.

As can be seen in Table I, all seven compounds show an increased level in neoplastic mucosa compared with normal mucosa. The cell density per unit wet weight of mucosa was not examined in this study. The increased level of these compounds in neoplastic mucosa might be related to the cell density. To clarify these problems, further investigations are needed.

The urinary nucleosides pre-fractionated using a boronate affinity gel

column were analysed by reversed-phase HPLC. Several investigators have reported an elevation of the modified nucleoside levels in urine from cancer patients [21—23]. Recently, Tamura et al. [35] reported that the urinary pseudouridine level of patients with lung cancer was significantly higher than those of controls. However, our results showed no elevation of modified nucleosides in the pre-operative urine of colorectal cancer patients compared with post-operative urine and normal urine.

As the mean urinary creatinine level of the post-operative patients was low compared with that of the pre-operative patients, the results shown in Table III may merely indicate that the change in urinary compound levels is caused by changes in the physiological condition of the patients between pre- and post-operation and there appears to be little difference in the modified nucleoside levels in the urines of pre-operative cancer patients and those in normal urines.

The mean level of urinary pseudouridine/creatinine in normal subjects in the present study (see Table III) was slightly higher than those reported by Speer et al. [22] and Tamura et al. [35], but lower than those reported by Evans et al. [11]. These differences might be caused by problems of quality control in microanalysis and differences in physiological environments, including nutrition.

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