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Reversed-phase high-performance liquid chromatographic investigation of urinary normal and modified nucleosides of cancer patients

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Abstract

Post-transcriptional modifications in RNA give rise to free modified ribonucleosides circulating in the blood stream and excreted in urine. Due to their abnormal levels in conjunction with several tumor diseases, they have been suggested as possible tumor markers. The developed RP-HPLC method has been applied to analyze the urinary nucleosides in 34 urinary samples from 15 kinds of cancer patients. The statistical analyses showed the urinary nucleoside excretion, especially modified nucleoside levels, in cancer patients were significantly higher than those in normal healthy volunteers. Factor analysis was used to classify the patients with cancer and normal healthy humans. It was found that using 15 urinary nucleoside levels or only five modified nucleoside levels as data vectors the factor analysis plot displayed two almost separate clusters representing each group.

Keywords: Pattern recognition; Nucleosides

1. Introduction

RNA contains modified nucleosides synthesized post-transcriptionally by modification enzymes. Normal RNA turnover creates free modified nucleosides excreted intact in urine. Consequently, the levels of urinary modified nucleosides reflect RNA degradation in the organism. Therefore, any disorder of RNA turnover alters the levels of modified nucleosides. A number of studies have correlated abnormal, generally high, concentrations of urinary modified nucleosides with carcinogenesis [1,2] and proposed them as potential tumor markers [1,3–15].

Although a capillary electrophoretic method developed in our laboratory [16,17] and immunoassays [18–21] have allowed the separation and quantification of nucleosides in urine, reversed-phase high-performance liquid chromatography (RP-HPLC) following concentration using boronate gel [3–6] has been the main analytical method reported. In this paper we modified the previous RP-HPLC method [22] to study the urinary modified nucleosides from cancer patients. The data were analyzed by factor analysis method to distinguish between cancer patients and normal healthy humans. The results

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showed that they could be separated into two distinct clusters representing each group.

2. Experimental

2.1. Chemicals

Formic acid was purchased from Riedel-de Häen (Germany). Ammonium acetate, methanol, ammonia, potassium dihydrogenphosphate were from Merck (Germany). Nucleoside standards were obtained from Sigma. Affi-gel 601 was obtained from Bio-Rad (Munich, Germany). All reagents were used directly without further purification.

2.2. Collection of urine samples

Spontaneous urine samples were collected from a five-year-old healthy child and 34 cancer patients from the Radiologische Klinik, Abteilung Strahlentherapie, Tübingen, Germany. After collection the samples were frozen immediately and stored at −20°C. For the analysis of the ribonucleosides the samples were thawed at room temperature. The patients were between 43 and 77 years of age (61.3±8.7, mean±SD). Fourteen kinds of cancer included breast, bronchial, oesophagus, rectum, hypopharynx, prostate, thigh, follicular, anaplastic, bladder, endocrine, floor of mouth cancer, as well as glioblastoma and oligodendroglioma. While urine samples were collected, most of the patients were receiving chemotherapy and/or radiation treatment. Some patients were operated on several months or years ago, but the carcinoma recurred and existed at collection time.

2.3. Extraction of nucleosides from urine and RP-HPLC method

A 10-ml volume of spontaneous urine with 0.5 ml of isoguanosine in water (0.25 m*M*) was treated on a phenylboronic acid column as described earlier [2,5]. After evaporation of the eluate, the residue was dissolved in 1 ml of 25 m*M* potassium dihydrogenphosphate (KH_2PO_4) buffer (pH 4.65–4.70), prior to HPLC separation. KH_2PO_4 buffer was prepared by dissolving KH_2PO_4 in water and adjusted to pH 4.65–4.70 by using 0.1 *M* NaOH.

To make the concentrations of nucleosides in the standard solutions more similar to those in urine, in this work, by diluting and mixing the concentrated stock aqueous solution of individual nucleosides the concentrations of 16 nucleosides in the standard aqueous solution were prepared as follows: dihydrouridine (Dhu) 0.32 m*M*, pseudouridine (Pseu) 1.28 m*M*, cytidine (C) 0.008 m*M*, uridine (U) 0.016 m*M*, 1-methyladenosine (m1A) 0.16 m*M*, inosine (I) 0.032 m*M*, 5-methyluridine (m5U) 0.032 m*M*, guanosine (G) 0.008 m*M*, xanthosine (X) 0.032 m*M*, 3-methyluridine (m3U) 0.016 m*M*, 1-methylinosine (m1I) 0.064 m*M*, 1-methylguanosine (m1G) 0.032 m*M*, 2-methylguanosine (m2G) 0.032 m*M*, adenosine (A) 0.032 m*M*, 6-methyladenosine (m6A) 0.032 m*M* and 5′-deoxy-5′-methylthioadenosine (MTA) 0.016 m*M*.

To obtain standard solutions with different concentrations, four different volumes (0.625, 1.25, 1.563 and 2.5 ml) of the stock aqueous solution were mixed with 0.5 ml of the internal standard isoguanosine (0.25 m*M*) and diluted with water to 10 ml. These solutions were extracted separately like urine samples and were used for the calibration of the HPLC system.

The HPLC system (Merck–Hitachi) used was composed of an L-6200 pump, an L-3000 photodiode array detector, a 655A-40 column oven and a D-6000 interface. The separation of the isolated urinary nucleosides was performed on a 250×4 mm, 5 μm LiChrospher 100 C_{18} column (Merck) at 30°C using a gradient comprising 25 m*M* KH_2PO_4 buffer, pH 4.65–4.70, and 60% methanol (MeOH) in water (Table 1). The nucleosides were detected by two channels set at 260 and 280 nm and were quantified using the internal standard method. The calibration curves were established set at 260 nm and 280 nm and allowed the determination of the urinary nucleoside concentrations in nmol/ml, which were transformed into nmol/μmol creatinine. Urinary creatinine levels were determined by a modified Jaffe method [23].

2.4. Data analysis method

The mean excretion and standard errors of urinary nucleosides have been calculated using the MS Excel program. A specially developed software in the authors' laboratory has been used to handle the

Table 1
Gradient composition for the RP-HPLC separation

Time (min)	KH_2PO_4 (%)	MeOH/water (%)	Flow (ml/min)
0	100	0	1.5
1	99	1	1.5
5	99	1	1.5
15	90	10	1.5
40	40	60	1.4
42	40	60	1.3
45	100	0	1.4
50–75	100	0	1.5

urinary nucleoside data based on the factor analysis method given in the literature [24,25]. The oblique rotational method used in the software is the Promax method [24].

3. Results and discussion

Up to now, we have been able to separate 17 normal and modified nucleosides including the internal standard isoguanosine. The calibration curves, with a mean correlation coefficient of 0.99, were obtained for 14 nucleosides at both wavelengths and, additionally, for dihydrouridine and pseudouridine, at 260 nm only. At 280 nm, dihydrouridine was not detected and pseudouridine is out of detection range. Table 2 shows the analytical characteristics of RP-HPLC method used.

Five portions of a pool of spontaneous urine from a normal healthy child were analyzed separately to evaluate the relative standard deviation in series (Table 2). The data show that the reproducibility of HPLC determination is adequate. The recoveries of nucleosides are in the range of 70~124% with an average of 105%.

Gehrke et al. [6] and Borek's [5] investigation showed that the ratio of nucleosides to creatinine in random urine samples was the same as that in 24-h

Table 2
The analytical characteristics of HPLC method and relative standard deviation (RSD, %) in series determined in normal pool spontaneous urine of a five-year-old child[a]

No.	Compound	a	b	S_a	S_b	r	Rec. (%)	Conc.	±SD	RSD
1	Dhu	$1.71\cdot10^{-2}$	0.41	$7.80\cdot10^{-3}$	$1.5\cdot10^{-2}$	0.999	98	6.20	±0.22	3.5
2	Pseu	0.83	2.60	$6.93\cdot10^{-2}$	$3.3\cdot10^{-2}$	1.000	70	30.3	±1.32	4.3
3	C	$7.97\cdot10^{-3}$	5.75	$7.28\cdot10^{-3}$	0.56	0.991	115	0.30	±0.05	17.7
4	U	$2.97\cdot10^{-2}$	4.72	$8.47\cdot10^{-3}$	0.32	0.995	–	0.29	±0.01	4.9
5	m1A	0.12	5.45	$2.37\cdot10^{-2}$	$9.1\cdot10^{-2}$	1.000	124	1.68	±0.11	6.7
6	I	$5.00\cdot10^{-2}$	7.28	$1.54\cdot10^{-2}$	0.30	0.998	–	0.38	±0.02	5.5
7	m5U	$1.43\cdot10^{-2}$	3.38	$1.48\cdot10^{-2}$	0.28	0.993	–	–	–	–
8	G	$-5.44\cdot10^{-4}$	9.49	$6.66\cdot10^{-3}$	0.51	0.997	116	0.09	±0.01	8.2
9	X	$6.47\cdot10^{-2}$	1.17	$1.07\cdot10^{-2}$	0.20	0.97	83	0.63	±0.04	6.3
10	m3U	$3.68\cdot10^{-3}$	6.18	$1.04\cdot10^{-2}$	0.40	0.996	–	0.10	±0.03	25.9
11	m11	$7.62\cdot10^{-2}$	10.8	$3.46\cdot10^{-2}$	0.33	0.999	120	1.11	±0.01	1.2
12	m1G	$3.42\cdot10^{-2}$	9.69	$2.32\cdot10^{-2}$	0.44	0.998	108	0.55	±0.02	2.7
13	m2G	$6.75\cdot10^{-2}$	8.38	$3.23\cdot10^{-2}$	0.62	0.995	–	1.17	±0.07	5.8
14	A	$1.53\cdot10^{-2}$	8.67	$9.29\cdot10^{-3}$	0.18	1.000	116	0.45	±0.06	13.4
15	m6A	$7.18\cdot10^{-2}$	8.52	0.13	2.48	0.92	108	–	–	–
16	MTA	$-2.26\cdot10^{-2}$	9.09	$3.20\cdot10^{-2}$	1.23	0.98	94	0.13	±0.03	24.5

[a] Linear relationship ($y=a+bx$) was got at 260 nm by the linear regression of four data from different concentrations for each analyte, where y is the relative area of nucleosides to internal standard, x is the relative concentration of nucleosides to the internal standard. S_a is the standard deviation of intercept (a), S_b is the standard deviation of slope (b), r is the related coefficient. Rec. is the mean recovery of nucleosides detected at 260 nm and 280 nm and was determined by spiking 160 nmol of Pseu and 40 nmol of other nucleosides to 10 ml of normal urine. Conc.: mean concentration (nmol/μmol creatinine) of five determinations at 260 nm.

samples. This means we could use spontaneous urine samples instead of 24-h collections in the study of the nucleoside excretion in cancer patients. Typical chromatograms of normal and modified nucleosides extracted from a spontaneous urine are given in Fig. 1. Based on the developed HPLC methods, nucleoside concentrations from 34 urine samples including 14 kinds of cancers were determined at 260 nm and 280 nm.

The mean levels of urinary nucleosides for normal individuals were described previously [22] and were used as the reference for comparison with cancer patients. Fig. 2 gives the mean values of urinary nucleoside excretion levels from 34 cancer patients and 18 normal healthy humans. It is shown that the levels of nucleosides in urine from cancer patients are elevated, and the increase of six modified nucleosides (Dhu, Pseu, m1A, m1I, m1G and MTA) is

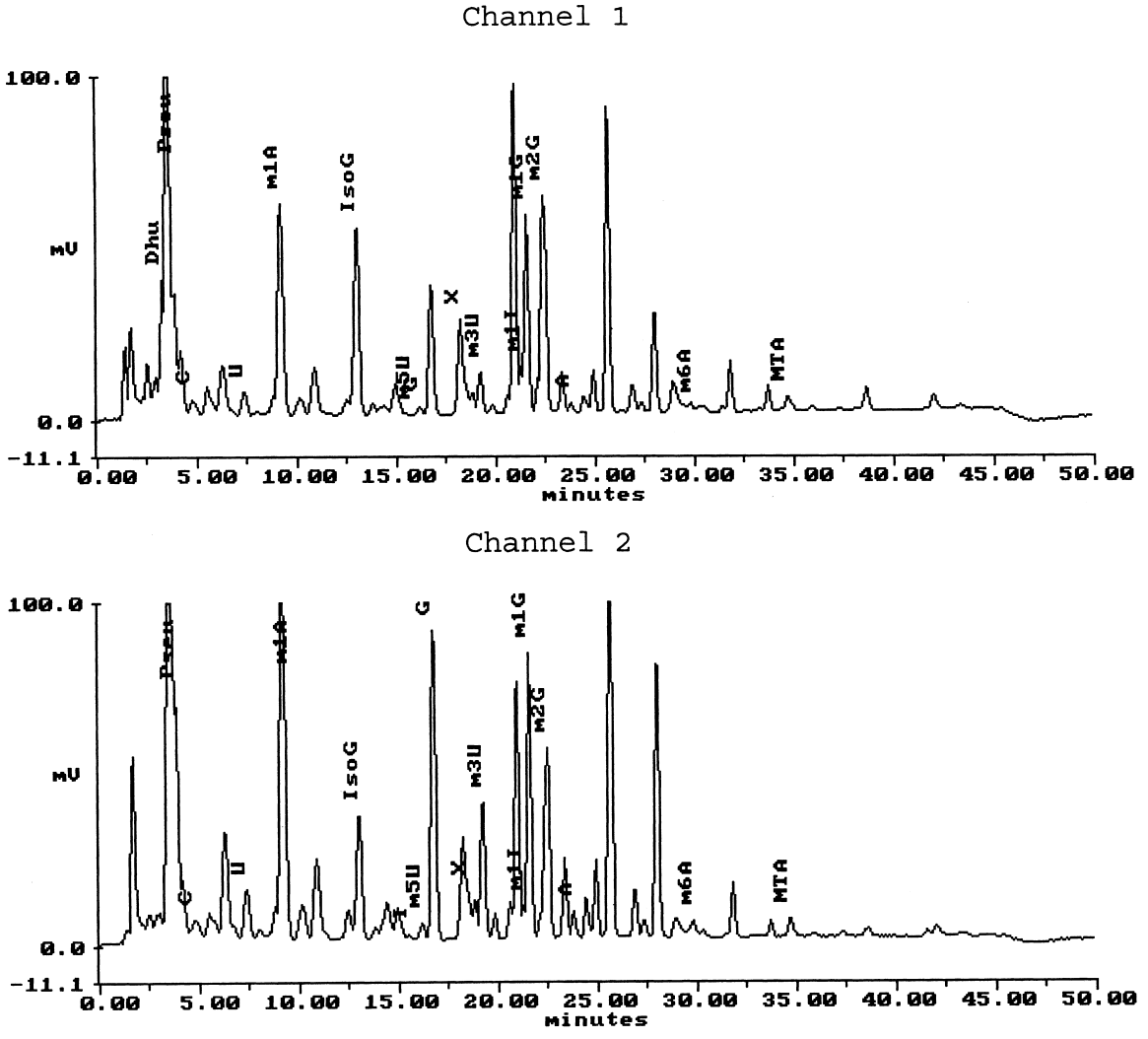

Fig. 1. Chromatograms of RP-HPLC separation of nucleosides in urine. Column: 250×4 mm, 5 μm LiChrospher 100 C_{18}, mobile phase: with a gradient beginning with 100% 25 m*M* KH_2PO_4, pH 4.67, and changing to 60% methanol–water (3:2, v/v) over 40 min; UV detection: at 260 nm for channel 1 and 280 nm for channel 2.

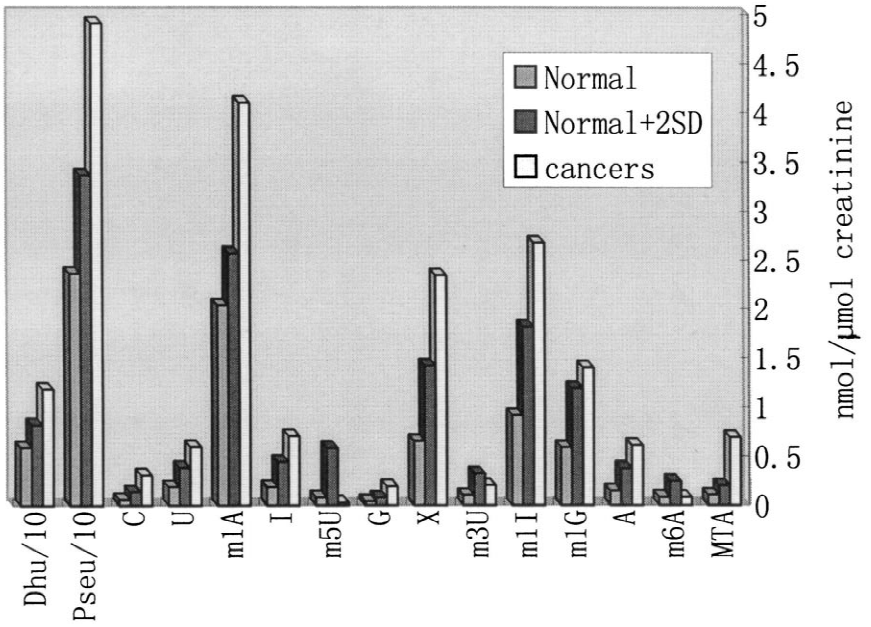

Fig. 2. Mean excretion of normal and modified nucleosides in urine from normal healthy volunteers and patients with cancer. Urinary nucleoside mean excretion levels (Normal) by healthy volunteers from previous work [22]. Normal+2 SD means normal mean excretion+2×standard error.

more pronounced than that of normal nucleosides, which is similar to the results obtained by capillary electrophoresis [17].

For individual urine samples, even for the same kind of cancer, the situation of increase of each nucleoside concentration is different. Based only on Fig. 2, it is very difficult to deduce whether someone has cancer or not. To classify cancer and non-cancer, factor analysis software using the oblique rotational Promax method was developed. The concentrations of 15 nucleosides were used as the data vectors. Fig. 3 shows that the normal healthy humans are clustered in a very narrow area and the patients with cancer and normal healthy humans are separated into two almost distinct clusters representing each group. Only three data points (8.8%) from the cancer patient group are located in the normal human group.

From Fig. 2, it is observed that the modified nucleosides are changed more than the normal nucleosides when a tumor exists. Therefore, we can pay more attention to the modified nucleoside excretion. Because MTA elutes as a small wide peak at a long retention time it is not easy to determine correctly small changes in concentrations. Therefore, we suggest selecting five urinary modified nucleosides (Dhu, Pseu, m1A, m1I and m1G) as possible tumor markers. The same factor analysis method was used to classify cancer patients and normal healthy humans (Fig. 4). It is observed that

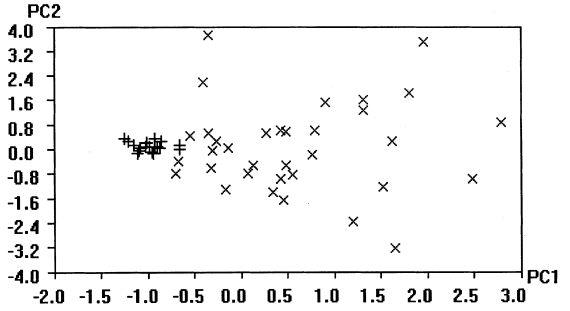

Fig. 3. Factor analysis based on 15 urinary normal and modified nucleosides from cancer patients (+ normal healthy volunteers, × cancer patients).

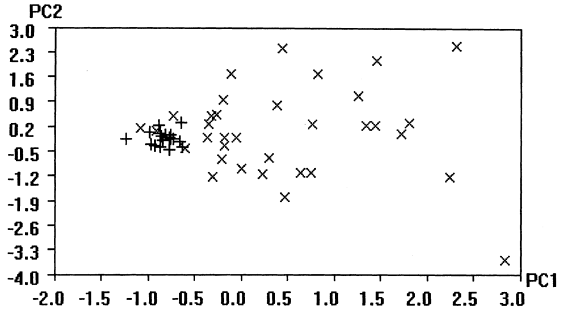

Fig. 4. Factor analysis based on five urinary modified nucleosides (Dhu, Pseu, m1A, m1I and m1G) from cancer patients (+: normal healthy volunteers, ×: cancer patients).

the result is similar to that when 15 urinary nucleosides were used as data vectors. Only four data points (11.8%) from cancer patient group are in the normal human group area. This means that in the practical clinical application, instead of 15 nucleosides, clinical chemists can use the concentrations of five urinary modified nucleosides to fast-screen the cancer disease if the nucleosides were used as tumor markers. These results are in agreement with the suggestion of several previous studies [13,14,17,26].

In summary, the results presented here give further credence to previous observations [17,22], patterns of urinary excretion of modified nucleosides may serve as a useful diagnostic tool for malignant disease, especially when a pattern recognition method (the factor analysis method in this paper) is applied. A major advantage for using the nucleosides as possible biomarkers lies in the HPLC method. All of them can be measured accurately and quickly per individual urine sample in one HPLC run. Although further studies are needed, the use of modified nucleosides in combination with other biomarkers, e.g., carcinoembryonic antigen (CEA), the carbohydrate antigen CA-50 etc., may eventually have potential application as an adjunct to the clinical assessment of each patient. Because the samples studied were limited in number, it is not yet possible to exactly correlate the nucleoside levels with the clinical data. This study is being continued to evaluate the clinical usefulness of modified nucleosides as tumor markers.

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