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Capillary electrophoretic profiling and pattern recognition analysis of urinary nucleosides from uterine myoma and cervical cancer patients

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Abstract

Capillary electrophoretic (CE) profiling analysis combined with pattern recognition methods is described for the correlation between urinary nucleoside profiles and uterine cervical cancer. Nucleosides were extracted from urine specimens by solid-phase extraction in affinity mode using phenylboronic acid gel. CE separation was carried out with an uncoated fused-silica capillary (570 mm×50 μm I.D.) maintained at 20°C, using 25 m*M* borate–42.5 m*M* phosphate buffer (pH 6.7) containing 200 m*M* sodium dodecyl sulfate as the run buffer under the applied voltage of 20 kV. A total of 15 nucleosides were positively identified in urine samples (2 ml) from eight uterine myoma (benign tumor group), 10 uterine cervical cancer (malignant tumor group) patients and 10 healthy females (normal group) studied. The star symbol plots drawn based on each mean concentration of nucleosides normalized to that in normal group enabled one to discriminate malignant and benign groups from normal group. In addition, canonical discriminant analysis performed on the nucleoside data of 28 individual urine specimens correctly classified into three separate clusters according to groups in the canonical plot.

Keywords: Nucleosides

1. Introduction

In recent years, clinical studies on the role of urinary modified nucleosides as the biochemical markers of various types of cancer have been actively undertaken [1–13]. Most of the urinary modified nucleosides are primarily originated by methylation of either the base part, the sugar hydroxyl part, or in some cases, both parts of the nucleosides in the course of biodegradation of transfer ribonucleic acid (tRNA) molecules [14–17]. Unlike major unmodified nucleosides, modified nucleosides are not the substrates for the salvage enzymes and thus are excreted in urine. Hence, the simultaneous detection of diverse modified nu-

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cleosides in urine in a single analysis became increasingly important for the study of their biochemical role in carcinogenesis as well as for the diagnosis at an earlier stage.

In the literature, high-performance liquid chromatography (HPLC) [3,4,10,13–15] has been extensively employed. However, recently, capillary electrophoresis (CE) in micellar electrokinetic capillary chromatography (MECC) mode is becoming popular in the multi-component metabolic profiling analysis of nucleosides as the complementary tool for HPLC [11,12,18–21]. It is because of its inherent higher resolution power and speed of analysis, the use of small sample amounts, less cumbersome in conditioning and regeneration of the uncoated CE capillary column and thereby less consumption of mobile carrier compared to HPLC as well documented elsewhere [18–21]. One of us has already shown that MECC has excellent linearity and reproducibility for the urinary nucleoside profiling analysis [11,12].

Among the various types of cancer, uterine cervical cancer is known to be one of the most prevalent in women and its early detection is thus desirable [22,23]. In our previous investigation of uterine tumor patients [24], urinary organic acid profiles were found to be correlated with uterine cervical cancer, since all 26 urine specimens studied were correctly classified into two separate clusters according to uterine cervical cancer or uterine myoma in the canonical plot. As the more important biochemical markers, measurements of urinary modified nucleosides combined with the previous canonical discriminant analysis will certainly provide another noninvasive diagnostic tool for the cervical cancer screening. It seems, therefore, of interest to investigate the effects of uterine cervical cancer on urinary nucleoside profiles. Attempts were, however, rarely made to measure the nucleoside levels of urine specimens from patients with uterine tumor patients to date.

This work discusses our recent investigation on the discrimination of changes in urinary modified nucleoside levels associated with uterine cervical cancer and myoma. In this study, 10 healthy female subjects were used as the normal control group together with 18 patients out of the same patient groups as those studied in the previous work [24]. Star symbol plotting [25] was examined for the effective visual comparison between groups. Canonical discrimination analysis was performed for classification of 28 urine specimens into three groups.

2. Experimental

2.1. Urine specimens

Mid-portions of the first morning urine specimens collected from eight uterine myoma patients, 10 uterine cervical cancer patients (aged from 33 to 69 years) and 10 healthy female volunteers (aged from 23 to 57 years) as the normal subjects were used for this study. All patients were hospitalized at Department of Obstetrics and Gynecology, Ajou University Hospital (Suwon, South Korea). The samples were immediately stored at -20°C until analyzed. The urinary concentration of creatinine was measured according to the Jaffe reaction [26] in triplicate.

2.2. Chemicals

The following 16 nucleoside standards were obtained from Sigma–Aldrich (Milwaukee, WI, USA): pseudouridine, dihydrouridine, uridine, cytidine, 5-methyluridine, 3-methyluridine, inosine, N^4-acetylcytidine, guanosine, 1-methylguanosine, adenosine, xanthosine, N^2,N^2-dimethylguanosine, N^2-methylguanosine, N^6-methyladenosine, and 3-deazauridine used as the internal standard (I.S.). Sodium dihydrogenphosphate ($NaH_2PO_4 \cdot 1H_2O$), phosphoric acid, borax ($Na_2B_4O_7 \cdot 10H_2O$) and sodium dodecyl sulfate (SDS) were also obtained from Sigma. Formic acid was purchased from Yakuri (Osaka, Japan), ammonium acetate from Junsei (Tokyo, Japan) and methanol from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were of analytical grade and used as received. Affi-gel 601 was obtained from Bio-Rad Labs. (Hercules, CA, USA).

2.3. Preparation of nucleoside standard and run buffer solutions

Each stock solution of nucleoside standards except for 3-deazauridine (I.S.) was made up at varied

concentrations in the range of 500 to 10 000 μg/ml in distilled water depending on nucleosides. The stock solutions were used to prepare mixed working standard solutions containing 15 nucleosides at the following different concentrations: 10 m*M* for pseudouridine, 1.5 m*M* for dihydrouridine, 1 m*M* for uridine, 0.6 m*M* for cytidine, 5-methyluridine, 3-methyluridine, inosine, N^4-acetylcytidine, guanosine and adenosine, 0.9 m*M* for 1-methylguanosine, 1.2 m*M* for xanthosine and N^2,N^2-dimethylguanosine, 0.5 m*M* N^2-methylguanosine and N^6-methyladenosine. I.S. solution was prepared at a concentration of 0.8 m*M* in distilled water. All the nucleoside solutions were kept frozen at −20°C.

A 50 m*M* borax–85 m*M* phosphate stock buffer was prepared by dissolving 0.954 g of borax and 0.288 ml of 85.0 wt.% phosphoric acid (density of 1.70 g/ml) in 40 ml of distilled water. The pH was then adjusted to 6.7 with 1.0 *M* NaOH and diluted to 50 ml with distilled water. A 500 m*M* SDS stock solution was prepared by dissolving 1.44 g of SDS in 10 ml of distilled water. All stock solutions used to prepare run buffer solution were stored at 4°C. A 10 ml volume of 25 m*M* borate–42.5 m*M* phosphate–200 m*M* SDS run buffer for daily use was prepared by mixing 5 ml of stock buffer, 4 ml of SDS stock solution and 1 ml of distilled water. The run buffer was filtered through a 0.45 μm membrane filter, followed by degassing in an ultrasonic bath (10 min) before use. Buffer solutions containing 50 or 42.5 m*M* phosphate using sodium dihydrogenphosphate instead of phosphoric acid were also prepared to test the resolution and analysis time.

2.4. Preparation of phenylboronic acid gel column

Each of luer-tipped polypropylene solid-phase extraction (SPE) tubes (3 ml) purchased from Supelco (Bellefonte, PA, USA) was packed with Affi-gel 601 (200 mg). They were then conditioned by washing sequentially with 0.1 *M* formic acid in 50% methanol and 0.25 *M* ammonium acetate (pH 8.6) prior to use as phenyl boronic acid (PBA) columns for SPE in affinity mode. All used PBA columns were regenerated by washing sequentially with 0.1 *M* formic acid in 50% methanol (10 ml×1), 50% methanol (10 ml×1) and 0.25 *M* ammonium acetate (pH 8.6; 15 ml×1) and then stored under 0.1 *M* NaCl solution.

2.5. Sample preparation

All 28 urine samples were individually processed in triplicate for nucleoside analysis according to the procedure described elsewhere [11,21] with a minor modification. Briefly, urine added with 25% ammonia (a few drops) was centrifuged. After addition of I.S. at a concentration of 24 μ*M* to the supernatant urine, an aliquot (2 ml) was loaded onto a preconditioned PBA column, followed by addition of 0.25 *M* ammonium acetate (pH 8.6; 0.5 ml). After standing for 5 to 10 min, the PBA column was successively rinsed with 0.25 *M* ammonium acetate (pH 8.6; 4.0 ml×1), 50% methanol (0.3 ml×2) and 0.1 *M* formic acid in 50% methanol (0.5 ml×1). The rinsed column was eluted with 0.1 *M* formic acid in 50% methanol (3 ml×1). The eluate (3 ml) was lyophilized at 30°C and the residue was reconstituted in distilled water (50 μl) for the direct analysis by CE.

For calibration works, pooled normal urine sample (2 ml), each spiked with six different amounts of nucleoside standards (0.9 to 4000 nmol) with a fixed amount of I.S. (48 nmol) were subjected to SPE in the same manner as described above. They were then analyzed for nucleosides measured at 210 nm for the construction of calibration curves.

2.6. Capillary electrophoresis

CE analyses were performed with a Beckman P/ACE 5500 CE system interfaced to a Gold version 8.12 data station and equipped with a photo diode array detector, an automatic injector and an uncoated fused-silica capillary [570 mm (effective length 500 mm)×50 μm I.D.] installed inside a fluid-cooled column cartridge (Beckman-Coulter, Fullerton, CA, USA). Samples (ca. 4 nl) were introduced in pressure injection mode for 4 s at 0.5 p.s.i. to the capillary maintained at 20°C (1 p.s.i.=6894.76 Pa). The capillary was rinsed between runs sequentially for 3 min with water and 0.1 *M* NaOH, and then for 2 min with water and buffer solution. The run buffer was

25 m*M* borate–42.5 m*M* phosphate (pH 6.7) containing 200 m*M* SDS. The applied voltage along the capillary was 20 kV (70~80 μA), within the linear region of Ohm's plot. Simultaneous measurements of UV absorbances at 210 and 260 nm in dual-channel mode and absorption spectra from 190 to 390 nm were performed. Peaks in urine samples were identified on the basis of migration time, co-electrophoresis of nucleoside standards and comparison of UV spectral patterns with those of nucleoside standards.

2.7. Calculation and pattern recognition analysis

The limit of detection for each nucleoside was calculated based on the concentration giving a signal three times the peak-to-peak noise of the background signal. Peak areas measured at 210 nm were used to calculate peak area ratios relative to that of the I.S. Least-squares regression analysis was performed on the peak area ratios against increasing amounts in nmol to plot calibration curves for the direct determination of nucleoside concentrations in urine samples. Due to incomplete resolution between N^2-methylguanosine and N^2,N^2-dimethylguanosine, their responses were summed up in the calculations. Each nucleoside concentration was then corrected for creatinine to transform into nmol/μmol creatinine.

Each corrected mean concentration of 14 nucleosides was plotted in bar graphical form to produce histograms. And each corrected mean concentration in malignant and benign groups was normalized to the corresponding value in normal group. Subsequently, the percentage normalized concentration of each nucleoside was plotted as a line radiating from a common central point and the far ends of the lines were joined together to produce a star plot for each group average using the MS Excell program.

The mean peak area ratios (corrected for creatinine) of nucleosides from 28 individual urine samples as the data vectors were subjected to canonical discriminant analysis by means of the statistical software package SAS. Canonical plots were then drawn on the basis of the first canonical discriminant function (CAN1) against the second canonical discriminant function (CAN2) of the nucleosides for each urine specimen.

3. Results and discussion

3.1. Optimal electrophoretic conditions

The present CE system employed for this study provides coolant fluid-cooling of the capillary wall to 20°C and thus overheating of the capillary due to the increased current was prevented. Under the previous CE conditions described elsewhere [11,21], the overall peak resolution was poor and the last N^6-methyladenosine was eluted later than 60 min. Hence, a number of preliminary experiments were performed to optimize CE condition for improving their resolutions in a shorter time. When the con-

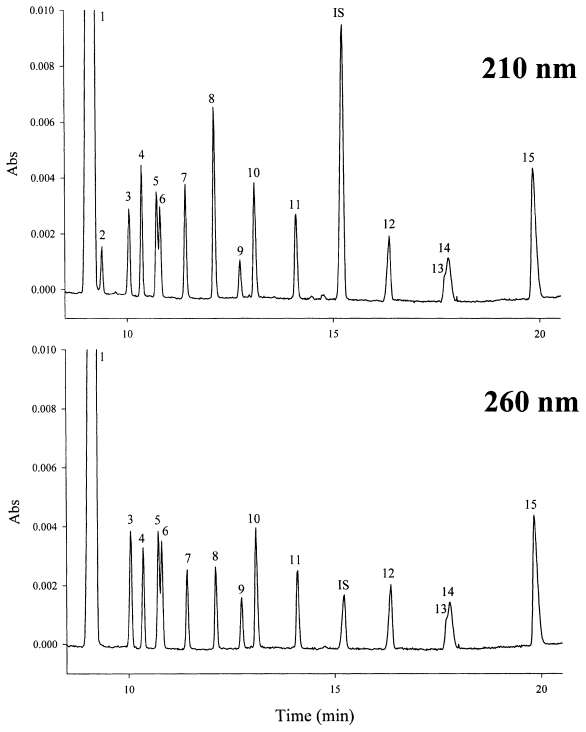

Fig. 1. Electropherograms of nucleoside standard mixture separated on an uncoated fused-silica capillary [570 mm (effective length 500 mm)×50 μm I.D.] maintained at 20°C using 200 m*M* SDS–25 m*M* borate–42.5 m*M* phosphate (pH 6.7) as the run buffer under the applied voltage of 20 kV after 4-s injection at 0.5 p.s.i., with simultaneous detection at 210 and 260 nm in dual-channel mode. Peaks: 1=pseudouridine; 2=dihydrouridine; 3=uridine; 4=cytidine; 5=5-methyluridine; 6=3-methyluridine; 7=inosine; 8=N^4-acetylcytidine; 9=guanosine; 10=1-methylguanosine; 11=adenosine; 12=xanthosine; 13=N^2-methylguanosine; 14=N^2,N^2-dimethylguanosine; 15=N^6-methyladenosine; I.S.=3-deazauridine.

Table 1
Linearity of CE responses to nucleoside standards extracted from spiked urine samples[a]

Nucleoside	Regression line[b]		R^2
	m	*b*	
Pseudouridine	0.0116	7.4842	0.9901
Dihydrouridine	0.0081	1.6117	0.9898
Uridine	0.019	0.3387	0.9978
Cytidine	0.045	0.057	0.9952
5-Methyluridine	0.0227	0.0654	0.9937
3-Methyluridine	0.0244	0.2301	0.9990
Inosine	0.0341	0.1817	0.9991
N^4-Acetylcytidine	0.0242	0.3421	0.9991
1-Methylguanosine	0.0385	0.8447	0.9988
Adenosine	0.0547	0.5949	0.9952
Xanthosine	0.02	0.4542	0.9959
N^2-Methylguanosine+N^2,N^2-dimethylguanosine	0.0256	2.195	0.9992
N^6-Methyladenosine	0.074	0.1615	0.9995

[a] Separation conditions: uncoated fused-silica capillary [570 mm (effective length 500 mm)×50 μm] maintained at 20°C, 25 m*M* borate–42.5 m*M* phosphate buffer (pH 6.7)–200 m*M* SDS. 20 kV, 4-s pressure injection at 0.5 p.s.i. and monitored at 210 nm.
[b] *m*=Slope=mean peak area ratio of nucleoside/amount of nucleoside (nmol); *b*=*y*-intercept.

centrations of phosphate and SDS in the run buffer containing 25 m*M* borate were decreased from 50 to 42.5 m*M* and from 300 to 200 m*M*, respectively, and concentrated phosphoric acid was used rather than sodium dihydrogenphosphate as the phosphate source, the voltage applied along the capillary maintained at 20°C could be increased up to 20 kV (351 V/cm) within the linear region of Ohm's plot. As demonstrated in Fig. 1, CE separation of 15 nucleoside standards detected simultaneously at 210 and 260 nm completed in less than 25 min. The resolution between dihydrouridine and pseudouridine was much more improved and a partial resolution (resolution factor of 0.72) between 5-methyluridine and 3-methyluridine was achieved, which were not resolved in the previous works [11,21]. An additional minimal resolution between N^2-methylguanosine and N^2,N^2-dimethylguanosine was obtained.

As seen in the dual-channel electropherograms, the absorbances of most nucleosides at the two wavelengths were similar except for dihydrouridine, N^4-acetylcytidine and I.S. Therefore, peaks were measured at 210 nm for the quantitative analysis to include dihydrouridine. The precision of migration times and peak area ratios ranged from 0.9 to 2.6% and from 5.2 to 8.5%, respectively. The limits of detection ranged from 1.8 to 7.1 μ*M*, which are in good agreement with the previous reports [11,21], thus being suitable for the quantitative analysis.

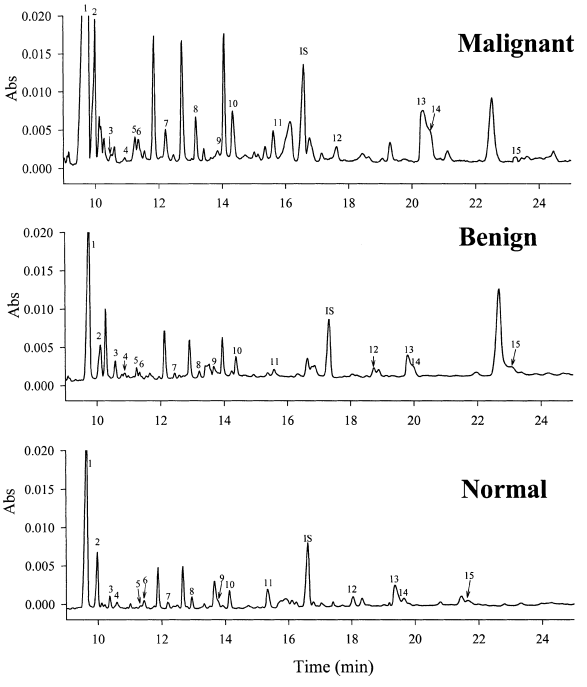

Fig. 2. Electropherograms of urinary nucleosides from a uterine cervical cancer patient (malignant), a myoma patient (benign) and a healthy subject (normal). CE conditions as described in Fig. 1. Peaks: 1=pseudouridine; 2=dihydrouridine; 3=uridine; 4=cytidine; 5=5-methyluridine; 6=3-methyluridine; 7=inosine; 8=N^4-acetylcytidine, 9=guanosine; 10=1-methylguanosine; 11=adenosine; 12=xanthosine; 13=N^2-methylguanosine; 14=N^2,N^2-dimethylguanosine; 15=N^6 -methyladenosine; I.S.=3-deazauridine.

3.2. Urinary nucleoside profiles

Prior to CE analysis, the SPE procedure using a PBA column in affinity mode [11,21] was employed to recover nucleosides from urine with minor modification. In this study, urine volume was reduced to 2 ml to shorten the overall sample work-up time. The residue after lyophilization was reconstituted in 50 μl of water, thereby concentration of urine by a factor of 40 was achieved as compared to the original urine.

The whole sample preparation procedure including PBA column extraction and lyophilization was applied to pooled normal urine samples spiked with 15 nucleosides at six different levels. Upon the CE analysis measured at 210 nm for the construction of calibration curves, linear responses in peak area ratios against increasing amounts in nmol of the nucleosides were obtained with correlation coefficients varying from 0.9898 to 0.9995 (Table 1) with the exception of guanosine. It was eluted as a shoulder peak of a prominent interfering compound co-extracted from normal urine. Hence, it was excluded in the quantitative works. And the responses of N^2-methylguanosine and N^2,N^2-dimethylguanosine were summed up due to the incomplete resolution.

When applied to the first morning urine specimens from eight uterine myoma, 10 uterine cervical cancer patients and 10 normal female individuals, a total of 15 nucleosides were positively identified. As demonstrated in typical electropherograms (Fig. 2) of urinary nucleosides from a uterine cervical cancer patient, a myoma patient and a healthy subject, the present method was found to be useful for producing good CE profiles with a small volume of urine (2 ml) within 25 min.

The excretion values of nucleosides identified from triplicate runs of each urine sample were expressed as nmol/μmol creatinine (Tables 2–4). Cervical cancer patients (Table 2) were served as the malignant tumor group (M1–M10), myoma patients (Table 3) as the benign tumor group (B1–B8), and healthy subjects (Table 4) as the normal group (N1–N10). Large variations in the levels of nucleosides from individual to individual even within each group were observed. The levels of most nucleosides were found to be lower compared with the values reported previously [11,21]. Both in mean and median values, the most abundant nucleoside in all groups was pseudouridine, followed by dihydrouridine and N^2-methylguanosine+N^2,N^2-dimethylguanosine. Pseudouridine levels were elevated above the normal values plus 2SD in the 40% of cervical cancer

Table 2
Levels of nucleosides found in urine samples from 10 uterine cervical cancer patients (malignant tumor group)

Nucleoside	Amount (nmol concentration/μmol creatinine)[a]										Mean[b]	Median[c]
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10		
Pseudouridine	13.73	18.44	13.97	67.28	22.19	93.51	19.80	14.00	18.19	23.57	30.47	19.12
Dihydrouridine	3.73	5.45	4.29	18.52	6.24	29.08	5.05	2.31	3.11	6.53	8.43	5.25
Uridine	0.16	0.19	0.31	0.54	0.39	0.63	0.27	0.17	0.37	0.80	0.38	0.34
Cytidine	0.02	0.06	0.04	0.17	0.08	0.08	0.25	0.09	0.09	0.31	0.12	0.09
5-Methyluridine	0.16	0.35	0.29	0.55	0.37	1.63	0.15	0.08	0.13	0.24	0.39	0.26
3-Methyluridine	0.08	0.14	0.15	0.00	0.18	1.12	0.20	0.39	0.33	0.48	0.31	0.19
Inosine	0.39	0.31	0.30	1.21	0.65	1.15	0.27	0.12	0.26	0.47	0.51	0.35
N^4-Acetylcytidine	0.40	0.54	0.47	1.69	0.52	2.73	0.18	0.41	0.10	0.00	0.70	0.44
1-Methylguanosine	0.35	0.62	0.43	1.65	0.51	2.25	0.48	0.51	0.21	0.84	0.79	0.51
Adenosine	0.08	0.11	0.10	0.73	0.00	0.70	0.71	0.26	0.31	0.63	0.36	0.28
Xanthosine	0.38	0.50	0.38	1.42	0.52	2.37	0.48	0.71	0.52	0.99	0.83	0.52
N^2-Methylguanosine+ N^2,N^2-dimethylguanosine[d]	1.54	2.18	1.83	7.22	2.26	9.78	1.69	1.62	1.41	2.37	3.19	2.01
N^6-Methyladenosine	0.02	0.20	0.07	0.00	0.05	0.84	0.14	0.11	0.06	0.58	0.21	0.09

[a] Mean concentration from triplicate runs of each urine was corrected for the creatinine amounts in 2 ml of urine.
[b] Mean concentrations of 10 patients.
[c] Median concentrations of 10 patients.
[d] N^2-Methylguanosine and N^2,N^2-dimethylguanosine were summed up due to incomplete resolution.

Table 3
Levels of nucleosides found in urine samples from eight uterine myoma patients (benign tumor group)

Nucleoside	Amount (nmol concentration/μmol creatinine)[a]								Mean[b]	Median[c]
	B1	B2	B3	B4	B5	B6	B7	B8		
Pseudouridine	20.18	17.97	16.56	25.20	10.07	24.79	5.89	13.37	16.75	17.26
Dihydrouridine	6.23	5.36	4.76	4.85	2.93	8.17	1.91	4.25	4.81	4.80
Uridine	0.23	0.14	0.29	0.10	0.14	0.46	0.09	0.22	0.21	0.18
Cytidine	0.08	0.07	0.07	0.07	0.06	0.27	0.08	0.09	0.10	0.08
5-Methyluridine	0.25	0.19	0.24	0.36	0.17	0.26	0.05	0.22	0.22	0.23
3-Methyluridine	0.17	0.14	0.13	0.28	0.22	0.57	0.11	0.23	0.23	0.19
Inosine	0.26	0.20	0.31	0.26	0.18	0.42	0.06	0.27	0.25	0.26
N^4-Acetylcytidine	0.63	0.44	0.43	0.48	0.25	0.51	0.09	0.25	0.38	0.44
1-Methylguanosine	0.93	0.43	0.34	0.54	0.24	0.65	0.17	0.39	0.46	0.41
Adenosine	0.19	0.23	0.17	0.28	0.11	0.38	0.04	0.25	0.21	0.21
Xanthosine	0.38	0.40	0.25	0.49	0.15	1.11	0.22	0.39	0.42	0.38
N^2-Methylguanosine+ N^2,N^2-dimethylguanosine[d]	1.76	2.87	1.94	2.57	1.26	2.93	0.71	1.61	1.96	1.85
N^6-Methyladenosine	0.00	0.03	0.06	0.09	0.02	0.24	0.12	0.09	0.08	0.07

[a] Mean concentration from triplicate runs of each urine was corrected for the creatinine amounts in 2 ml of urine.
[b] Mean concentrations of eight patients.
[c] Median concentrations of eight patients.
[d] N^2-Methylguanosine and N^2,N^2-dimethylguanosine were summed up due to incomplete resolution.

patients, while 25% of uterine myoma patients was elevated. Each mean nucleoside level in malignant group was higher compared to the value in normal group, while levels of xanthosine and 3-methyluridine in benign group were lower than in normal group as demonstrated in histogram (Fig. 3).

When each mean level of nucleosides both in malignant and benign groups was normalized to the

Table 4
Levels of nucleosides found in urine samples from 10 healthy females (normal group)

Nucleoside	Amount (nmol concentration/μmol creatinine)[a]										Mean[b]	Median[c]
	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10		
Pseudouridine	14.19	11.27	10.99	15.54	12.49	22.52	17.64	14.47	14.69	14.20	14.80	14.33
Dihydrouridine	2.61	2.76	1.53	3.36	4.07	6.58	5.31	4.07	3.84	4.17	3.83	3.96
Uridine	0.17	0.16	0.11	0.30	0.27	0.50	0.20	0.17	0.15	0.23	0.23	0.19
Cytidine	0.17	0.14	0.10	0.11	0.07	0.19	0.05	0.07	0.04	0.04	0.10	0.09
5-Methyluridine	0.15	0.11	0.17	0.13	0.11	0.13	0.23	0.16	0.12	0.06	0.14	0.13
3-Methyluridine	0.26	0.26	0.04	0.32	0.26	0.32	0.47	0.32	0.30	0.36	0.29	0.31
Inosine	0.00	0.11	0.00	0.18	0.12	0.20	0.08	0.05	0.13	0.06	0.09	0.09
N^4-Acetylcytidine	0.00	0.04	0.00	0.23	0.26	0.48	0.38	0.29	0.30	0.28	0.23	0.27
1-Methylguanosine	0.16	0.29	0.03	0.28	0.31	0.58	0.49	0.47	0.40	0.30	0.33	0.30
Adenosine	0.15	0.28	0.08	0.24	0.32	0.35	0.27	0.18	0.26	0.15	0.23	0.25
Xanthosine	0.41	0.47	0.33	0.39	0.44	1.03	0.88	0.72	0.85	0.73	0.62	0.59
N^2-Methylguanosine+ N^2,N^2-dimethylguanosine[d]	1.59	1.75	0.95	1.31	1.46	3.19	1.88	2.14	1.68	1.47	1.74	1.64
N^6-Methyladenosine	0.13	0.12	0.08	0.07	0.09	0.06	0.00	0.00	0.00	0.00	0.05	0.06

[a] Mean concentration from triplicate runs of each urine was corrected for the creatinine amounts in 2 ml of urine.
[b] Mean concentrations of 10 patients.
[c] Median concentrations of 10 patients.
[d] N^2-Methylguanosine and N^2,N^2-dimethylguanosine were summed up due to incomplete resolution.

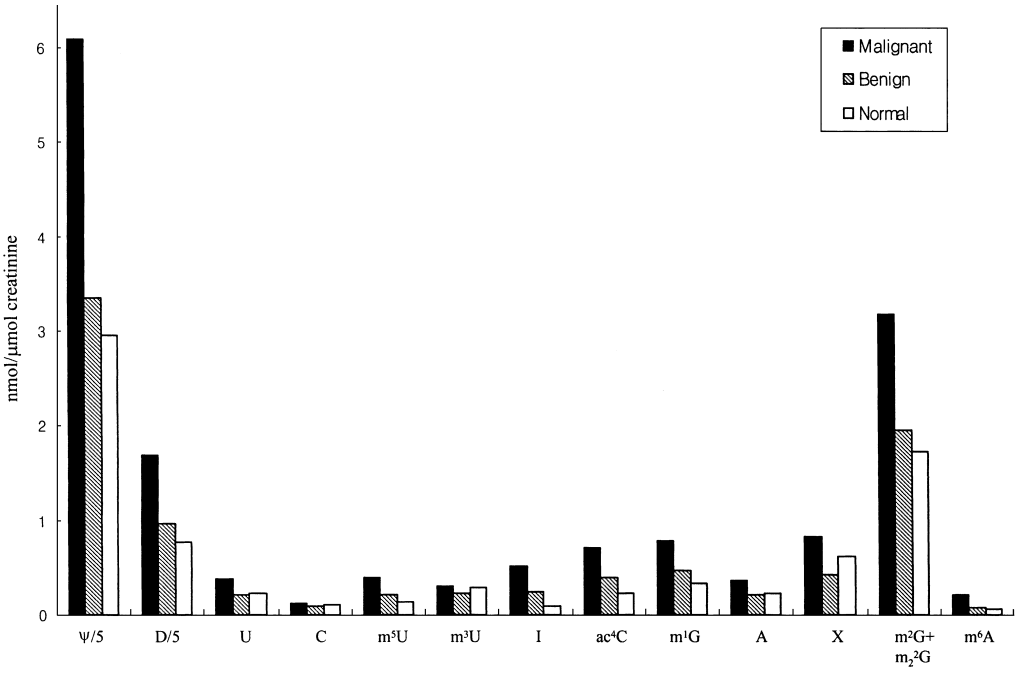

Fig. 3. Bar graphical plots of each mean level of nucleosides in malignant, benign and normal groups. Bars: ψ=pseudouridine; D=dihydrouridine; U=uridine; C=cytidine; m^5U=5-methyluridine; m^3U=3-methyluridine; I=inosine; $ac^4C=N^4$-acetylcytidine; m^1G=1-methylguanosine; A=adenosine; X=xanthosine; $m^2G+m_2^2G=N^2$-methylguanosine+N^2,N^2-dimethylguanosine; $m^6A=N^6$-methyladenosine.

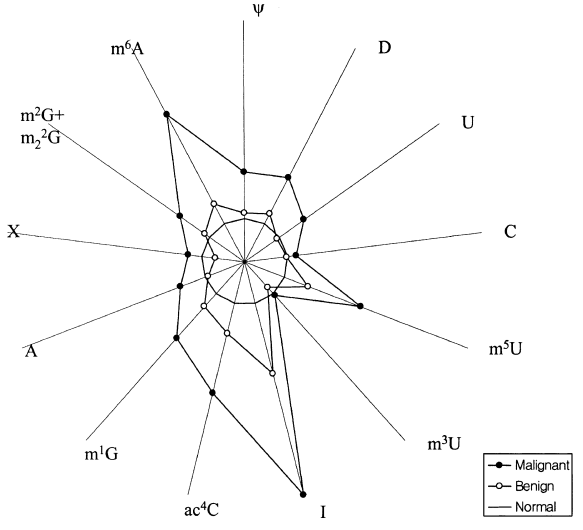

Fig. 4. Star symbol plots of malignant, benign and normal group averages drawn based on each mean level of nucleosides normalized to the value of each corresponding nucleoside in normal group. Rays: ψ=pseudouridine; D=dihydrouridine; U=uridine; C=cytidine; m^5U=5-methyluridine; m^3U=3-methyluridine; I=inosine; $ac^4C=N^4$-acetylcytidine; m^1G=1-methylguanosine; A=adenosine; X=xanthosine; $m^2G+m_2^2G=N^2$-methylguanosine+N^2,N^2-dimethylguanosine; $m^6A=N^6$-methyladenosine.

value of each corresponding nucleoside in normal group and used as the variables to draw star graphs composed of 13 rays, visual comparison between groups was much easier than the conventional histogram (Fig. 4). Overall star patterns of the tumor patient groups look similar, but malignant group is about twice as large as benign group. And their patterns are very different from the circular shape of normal group. Minor modified nucleosides in their excretion levels such as inosine, N^6-methyladenosine, N^4-acetylcytidine and 5-methyluridine were found to excel the major pseudouridine and dihydrouridine in discriminating malignant group from normal group. The circular shape of normal group serves well as the standard pattern both for the malignant and benign groups.

When canonical discriminant analysis was applied to all nucleosides as data vectors, 28 urine specimens were well separated into three distinct clusters (Fig. 5). This result was in good agreement with our previous studies on the urinary organic acids [24]. Both of the urinary nucleoside and organic acid profiling analysis methods appear to be complements

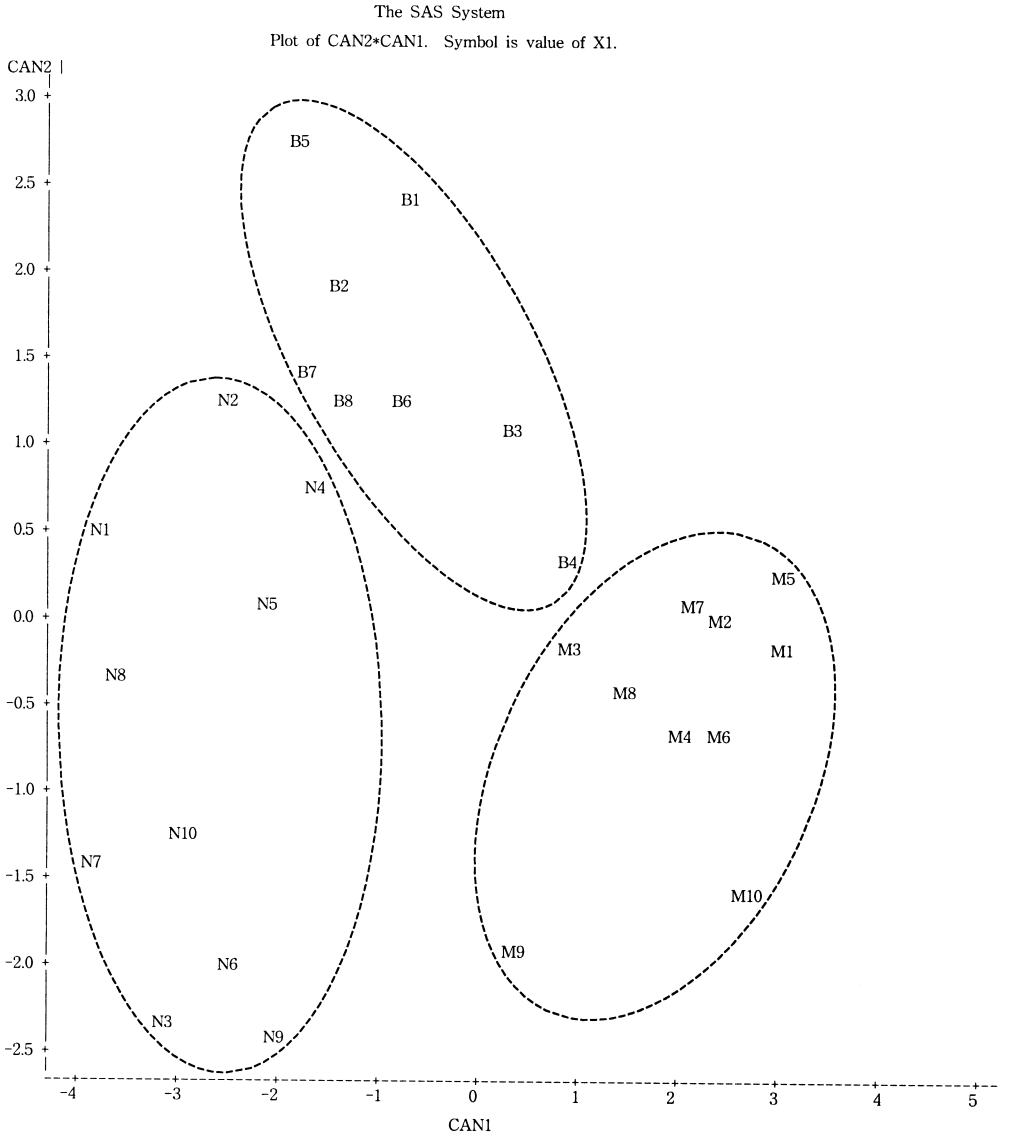

Fig. 5. Plot of the first and second canonical functions of the 14 nucleoside variables for the 10 uterine cervical cancer patients (each represented by M), 8 uterine myoma patients (each represented by B), and 10 normal subjects (each represented by N). Variables: pseudouridine, dihydrouridine, uridine, cytidine, 5-methyluridine, 3-methyluridine, inosine, N^4-acetylcytidine, 1-methylguanosine, adenosine, xanthosine, N^2-methylguanosine, N^2,N^2-dimethylguanosine, N^6-methyladenosine.

each of the other as the diagnostic tools for the uterine cervical tumors.

4. Conclusions

The present sample preparation with subsequent CE analysis was suitable for the profiling and screening for nucleosides in a small volume of urine within 25 min. A total of 15 nucleosides were positively identified in urine samples from eight uterine myoma (benign tumor group), 10 uterine cervical cancer (malignant tumor group) patients and 10 normal female subjects (normal group) studied. Star symbol plotting provided easier comparison by showing differences in levels of both minor and

major nucleosides between groups. Minor modified nucleosides in their excretion levels such as inosine, N^6-methyladenosine, N^4-acetylcytidine and 5-methyluridine were found to excel the major pseudouridine and dihydrouridine in discriminating malignant group from normal group in star graphs. The circular shape of normal group serves well as the standard pattern both for the malignant and benign groups. Canonical plot produced by canonical discriminant analysis performed on CE data from 28 urine samples displayed three separate clusters representing each group. An extension of the present method to other types of cancer is under way to examine its suitability as the early diagnostic tool for cancer.

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