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ORIGINAL RESEARCH

Direct determination of nucleosides in the urine of patients with breast cancer using column-switching liquid chromatography–tandem mass spectrometry

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ABSTRACT: We developed an analytical method for a simple, sensitive and simultaneous determination of oxidized nucleosides in urine using column-switching liquid chromatography–electrospray/tandem mass spectrometry (LC-ESI/MS/MS). We connected two columns through a six-way switching valve and effectively separated nucleosides in the urine from the interference by column-switching liquid chromatography. We monitored separated nucleosides using positive ionization tandem mass spectrometry in selective reaction monitoring (SRM) mode. The calibration ranges of nucleosides were 0.2–100 nmol/mL. The linearity of the method was 0.994–0.999, and the limits-of-detection (LOD) at a signal-to-noise (S/N) ratio of 3 were 0.1–0.2 nmol/mL. The coefficients of variation were in the range 2.28–11.74% for within-day variation and 4.36–11.15% for day-to-day variation, respectively. To explore the relationship between breast cancer and the nucleosides level in human urine, we measured the concentrations of nucleosides in female patients with breast cancer (n = 30) and in normal female subjects (n = 30). The concentration of nucleosides was significantly increased in patients with breast cancer when compared with the normal controls (1-methyladenosine; $p < 0.005$, N^2,N^2-dimethylguanosine; $p < 0.01$, 5-hydroxymethyl-2′-deoxyuridine; $p < 0.001$, 8-hydroxy-2-deoxyguanosine; $p < 0.001$). Therefore, the elevated levels of nucleosides could be used as an important biomarker for breast-cancer research. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: nucleosides; column-switching liquid chromatography; mass spectrometry; urine; breast cancer

INTRODUCTION

Oxidized nucleosides are generated post-transcriptionally by various modification enzymes during RNA turnover, and they circulate in the blood and are excreted in urine (Topp *et al*., 1993; Nakano *et al*., 1993). Their levels are increased in a number of malignant diseases such as those associated with tumors, aging, carcinogenesis, and neurodegeneration, so they can be used in clinical chemistry as biomarkers (Itoh *et al*., 1992; Thomale and Nass, 1983; Fischbein *et al*., 1983; Speer *et al*., 1979; Zambonin *et al*., 1999; Abe *et al*., 2002, 2003). During the last decade, many analytical methods for measuring and monitoring nucleosides in biological fluid have been reported. Some of those methods include the following: enzyme-linked immunoassay (Itoh *et al*., 1995), capillary electrophoresis (Inagaki *et al*., 2001), cathodic stripping (Paleek *et al*., 1985), voltametry, gas chromatography–mass spectrometry (GC-MS; Ravanat *et al*., 1999; Pouget *et al*., 2000; Evans *et al*., 1999), and high-performance liquid chromatography–mass spectrometry (HPLC-MS; Pouget *et al*., 2000; O'Donoghue *et al*., 1996; Dudley *et al*., 2000). The GC method and the HPLC method with MS detector were developed to obtain a profile of nucleosides. Those methods were sensitive and could give accurate and precise results. However, they also required a complex preparation process which included extraction, hydrolysis and derivatization steps. Thus, to simplify the process, we have reported a direct determination method of nucleosides in the urine using HPLC-ESI/MS/MS in our laboratory (Lee *et al*., 2004). However, this method could not detect 8-hydroxy-2′-deoxyguanosine (Li *et al*., 2001; Matsui *et al*., 2000; Bershtein *et al*., 2005) and 5-hydroxymethyl-2′-deoxyuridine (Djuric *et al*., 2001; Frenkel *et al*., 1998), which are known to be very important biomarkers in malignant diseases such as breast cancer, because of interference by components in the matrix. Therefore, we developed a highly sensitive column-switching liquid-chromatography–tandem mass spectrometry (LC-MS/

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Abbreviations used: ESI, electrospray ion; ISTD, internal standard; PEEK, poly ether ether ketone.

MS) method to quantify nucleosides in urine without interference. We directly injected urine to HPLC, and this method did not require any preparation process such as conventional solid-phase extraction or liquid–liquid extraction. However, we efficiently removed interference from the matrix using column-switching.

In this study, we detected the urinary concentration of nucleosides in female patients with breast cancer and age-matched female normal controls to explore the relationship between breast cancer and the nucleoside level in human urine.

EXPERIMENTAL

Chemicals and reagents. Nucleosides were purchased from Sigma (St Louis, MO, USA): pseudouridine, cytidine, uridine, 1-methyladenosine, 5-methylcytidine, 5-methyl-2′-deoxycytidine, 5-hydroxymethyl-2′-deoxyuridine, guanosine, 3-methyluridine, 2′-deoxyguanosine, 8-hydroxy-2′-deoxyguanosine, adenosine, N^2,N^2-dimethylguanosine, 5-deoxyadenosine, and 1-hydroxy-isoguanine (internal standard, ISTD). Each standard stock solution of nucleosides was prepared at a concentration of 10 µmol/mL with 5 mM ammonium acetate. The solutions were diluted further with water to make the desired concentrations in the range 0.2–100 nmol/mL. The ISTD was prepared at a concentration of 10 µmol/mL with 5 mM ammonium acetate. All standard stock solutions were stored at –20°C. All chemicals were of analytical-reagent grade, and all solvents were of HPLC grade. All of the aqueous solutions were prepared with water purified using a Milli-Q water-purification system (Millipore, Milford, MA, USA).

Column-switching HPLC. The column-switching HPLC system used a Shiseido Nanospace SI-2 HPLC system model 3001 (Shiseido Co., Tokyo, Japan) equipped with an auto injector (200 µL loop; model 3023) and a dual, six-way switching-valve unit (model 3012). A flow diagram of the column-switching HPLC system is shown in Fig. 1. The two columns were

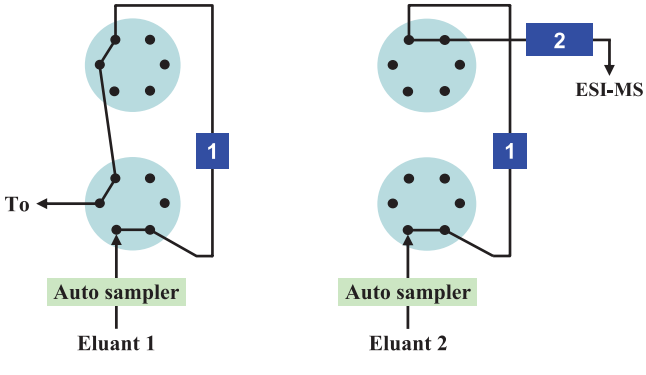

Figure 1. A flow diagram of the column-switching conditions in HPLC. **1**, Shodex MS Pak PK-2A (*n*-vinylacetamide copolymer, 2.0 mm i.d. × 10 mm length, Showa Denko K. K., Tokyo, Japan); **2**, Capcell Pak C_{18}, AQ (1.5 mm i.d. × 150 mm length, 5 µm particle size, Shiseido Fine Chemical Co., Tokyo, Japan). This figure is available in colour online at www.interscience.wiley.com/journal/bmc

connected through a six-way switching valve and poly ether ether ketone (PEEK) tubing of 0.13 mm i.d. was used for all of transfer lines. The system consisted of a first column, Shodex MS Pak PK-2A (*n*-vinylacetamide copolymer, 2.0 mm i.d. × 10 mm length, Showa Denko K. K., Tokyo, Japan), and a second column, Capcell Pak C_{18}, AQ (1.5 mm i.d. × 150 mm length, 5 µm particle size, Shiseido Fine Chemical Co., Tokyo, Japan). The first column was set up at room temperature, and the second column temperature was maintained at 40°C with a model 3004 column oven. The mobile phase consisted of eluant 1 (5 mM ammonium acetate, pH 7.5 adjusted with ammonia) and eluant 2 (5 mM ammonium acetate and 50% methanol, pH 4 adjusted with formic acid). We used eluant 1 to remove the interference in the urine and eluant 2 separate nucleosides. Eluant 1 was flushed at a flow rate of 500 µL/min for 10 min. The switching valve was changed, and eluant 2 was flowed to the first column to elute nucleosides at a flow rate of 100 µL/min. The eluted nucleosides were flowed directly through the second column to separate them. In the second analytical column we used the gradient system to the separate the nucleosides. The gradient was started with 20% B, and it was increased to 50% B at 6 min. It was increased again to 100% B at 15 min and held for 5 min. The column was then reequilibrated for 10 min with 20% B.

Mass spectrometry. ThermoFinnigan LCQ Advantage mass spectrometry (ThermoFinnigan, San Jose, CA, USA) was used with an electrospray ion source (ESI) fitted with a pneumatically assisted electrospray probe. The orthogonal electrospray interface allowed the entire column effluent from the HPLC to be directed into the source without flow-splitting, which contributed to the greatly enhanced sensitivity. We operated the mass spectrometer in the positive-ionization mode with the following operating conditions: ion spray voltage at 5 kV; capillary voltage at 10 V; and tube lens offset at 15 V; capillary temperature at 250°C. Nitrogen was used as the sheath gas at 50 arbitrary units. Helium was used as both a damping and collision gas. We performed quantitative analysis of the nucleosides by monitoring them in MS/MS mode using the SRM (selective reaction monitoring). The HPLC-MS/MS parameters for nucleosides are given in Table 1.

Subjects and sample-collection. We obtained urine samples of the female patients with breast cancer (n = 30, age 47.37 ± 7.37 years) from the Samsung Hospital, and College of Medicine, Hanyang University, Seoul, Korea. All of the control subjects (n = 30, age 47.26 ± 7.95 years) were obtained from healthy women and matched with the breast cancer patients in age. All of the urine samples were collected in early morning and kept frozen at –20°C until analysis.

Sample preparation. The frozen samples were thawed in cold storage prior to analysis. A 100 µL aliquot of urine was centrifuged at 14000 rpm for 4 min, and the supernatant was filtered through a PVDF filter (Milles-GV, 0.2 µm pore size, Millipore, Korea Co., Seoul, Korea) to remove urine protein. A 5 mL of ISTD was added in a 50 µL of the filtered urine, and the mixtures were transferred to autosampler vials. The vials were capped and mixed with vortexing for 30 s. A 50 µL amount of the final solution was injected into the HPLC-MS/

Table 1. Collision-induced dissociation of the protonated molecules of the analytes in positive ionization mode

Analyte	$[M+H]^+$	Isolation width	Collision energy (%)	MS/MS fragmentation
Pseudouridine	245	2	20	209
Cytidine	244	2	22	112
Uridine	245	2	20	113
1-Methyladenosine	282	2	25	150
5-Methylcytidine	258	2	18	126
5-Methyl-2′-deoxycytidine	259	2	15	143
5-Hydroxymethyl-2′-deoxyuridine	242	2	16	126
Guanosine	284	2	18	152
3-Methyluridine	259	2	15	127
Adenosine	268	2	25	136
2′-Deoxyguanosine	268	2	15	152
8-Hydroxy-2′-deoxyguanosine	284	2	20	168
N^2,N^2-Dimethylguanosine	312	2	25	180
5-Deoxyadenosine	252	2	27	136
1-Hydroxyisoguanine (ISTD)	168	2	6	168

MS system. The urinary creatinine values were measured using the Jaffé method.

Method validation. We prepared the calibration curves to quantify nucleosides in the concentration range of 0.2–100 nmol/mL. The calibration samples were prepared in water instead of urine because all urine has nucleosides. We injected the prepared standard solutions to HPLC for validation. We determined intra-day variations by analyzing four samples at each concentration in one batch, and we analyzed inter-day variations on four separate days.

RESULTS AND DISCUSSION

Column-switching HPLC

In order to eliminate interferences from the urine, we used column-switching HPLC. We directly injected the urine onto the first column (pre-column) and removed the interferences from the urine during the flushing of eluant 1 (5 mM ammonium acetate, pH 7.5 adjusted with ammonia). We used a higher pH of the eluant 1 to increase the interactions of the polar analytes with reversed-phase material. We changed the path for eluant 2 using a switching valve in HPLC, and then we flushed samples onto the secondary column (analytical column) with eluant 2 (5 mM ammonium acetate and 50% methanol, pH 4.5 adjusted with formic acid). We passed eluant 2 through the secondary column using a gradient to increase the elution strength. The nucleosides were separated by this HPLC condition.

Tandem mass spectrometry

We monitored separated nucleosides and ISTD using positive-ionization tandem mass spectrometry (MS/MS) in the SRM mode (Fig. 2). The positive mass spectra of nucleosides were identified in advance to search the MS/MS spectra and to select adequate product ion for quantification. All of the nucleosides appeared to be a protonated precursor ion, $[M+H]^+$. For the generation of MS/MS spectra, we fragmented protonated precursor ions were at an optimal collision energy shown in Table 1. The most-abundant product ions of MS/MS were that of the protonated bases $[BH_2]^+$ moiety, which was generated from the loss of a ribose moiety $[MH-132]^+$ (cytidine, uridine, 1-methyladenosine, 5-methylcytidine, guanosine, 3-methyluridine, adenosine, N^2,N^2-dimethylguanosine) or 2′-deoxyribose moiety $[MH-116]^+$ (5-methyl-2′-deoxycytidine, 5-hydroxymethyl-2′-deoxyuridine, 2′-deoxyguanosine, 8-hydroxy-2′-deoxyguanosine, 5-deoxyadenosine), except for pseudouridine. Pseudouridine did not produce protonated bases $[BH_2]^+$ moiety because of the stable carbon–carbon (C–C) glycosidic bond. The major fragmentation ion of pseudouridine is the loss of two water molecules as $[MH-2H_2O]^+$.

Validation

In order to validate the developed method, we prepared a calibration curve of nucleosides and quantified the urinary concentration in MS/MS mode using abundant product ions. The calibration curves showed good linearity in the range 0.2–100 nmol/mL. The correlation coefficients (R^2) were higher than 0.994 in all of the nucleosides, and the limit-of-detection (LOD) for the nucleosides was 0.1–0.2 nmol/mL. The results of calibration data are shown in Table 2. The coefficient-of-variation (CV, %) for the within-day variation was in the range 2.28–11.74% and that for day-to-day variation was in the range of 4.36–11.15% (Table 3).

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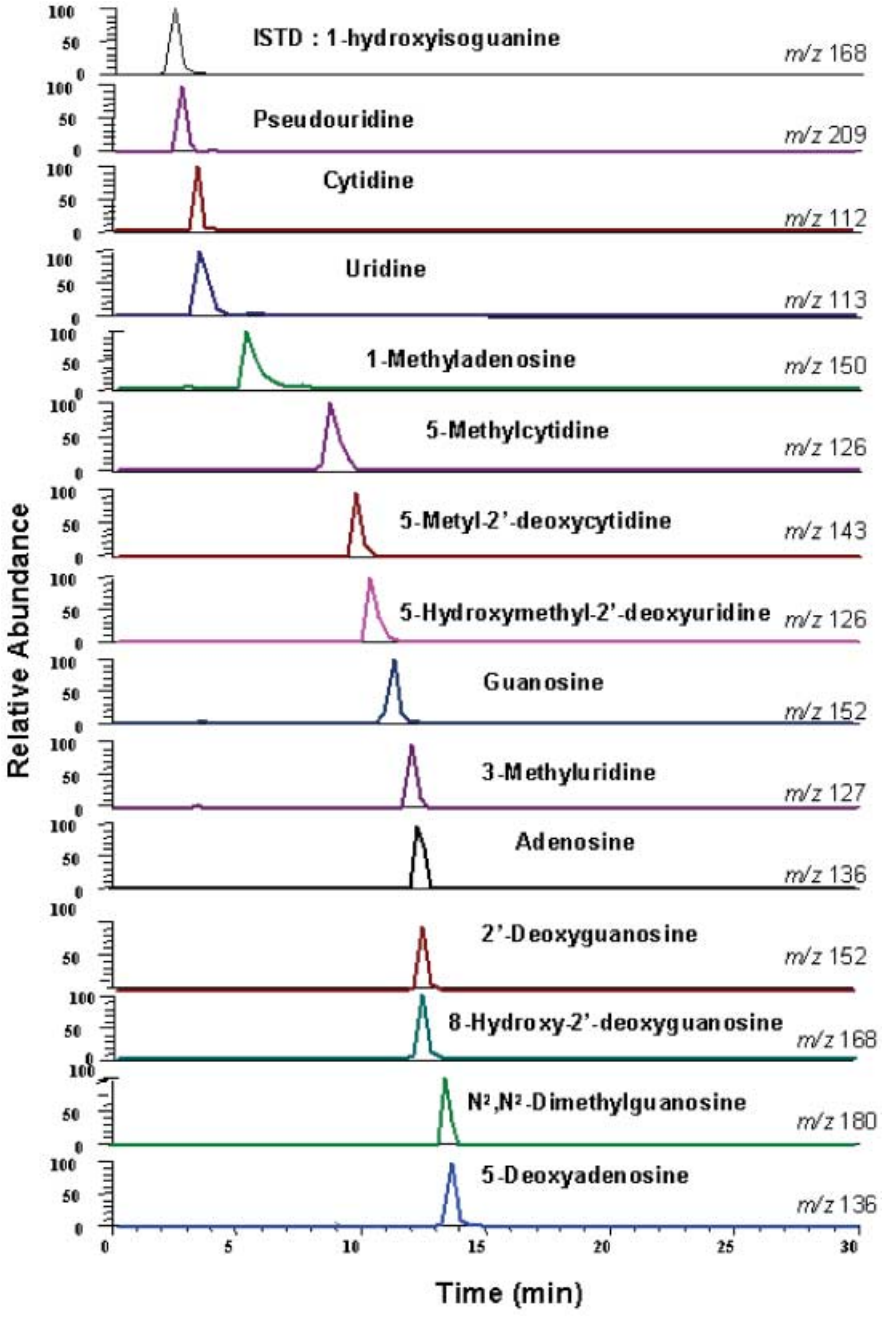

Figure 2. SRM product ion chromatograms for 14 nucleosides in the positive-ionization mode. This figure is available in colour online at www.interscience.wiley.com/journal/bmc

Patient studies

We directly measured nucleosides in the urine from patients with breast cancer of pre- and post-operation with that of female, age-matched normal controls using column-switching HPLC-MS/MS. We detected nucleosides in urine samples within 30 min. The levels of nucleosides in the urine were significantly different between pre-operative patients with breast cancer and the controls (Table 4, Fig. 3). Nucleosides in the urine were significantly increased (1-methyladenosine; $p < 0.005$, N^2,N^2-dimethylguanosine; $p < 0.01$, 5-hydroxymethyl-2′-deoxyuridine; $p < 0.001$, 8-hydroxy-2-deoxyguanosine; $p < 0.001$) in the pre-operative patients. Previous researchers reported that transfer RNA (tRNA) in malignant tumors differed from the tRNA of normal tissue (Fischbein *et al*., 1983; Borek *et al*., 1983). In particular, the modifications related to methylation were enhanced in malignant tissue (Borek and Kerr, 1972). Researchers observed that the significantly increased nucleosides in breast-cancer patients were methylated compounds, except for 8-hydroxy-2-deoxyguanosine.

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Table 2. Calibration data for nucleosides (linear regression equation: $y = ax + b$)

Analyte	Regression line		Linearity (R^2)	Calibration range (nmol/mL)	LOD (nmol/mL)
	Slope, *a*	Intercept, *b*			
Pseudouridine	0.241	0.117	0.995	0.2–300	0.2
Cytidine	0.151	0.273	0.997	0.2–300	0.2
Uridine	0.193	0.261	0.998	0.2–300	0.1
1-Methyladenosine	0.980	0.157	0.999	0.2–300	0.1
5-Methylcytidine	1.031	3.210	0.994	0.2–300	0.1
5-Methyl-2′-deoxycytidine	0.753	1.352	0.995	0.2–300	0.2
5-Hydroxymethyl-2′-deoxyuridine	0.247	0.214	0.997	0.2–300	0.2
Guanosine	0.332	0.033	0.997	0.2–300	0.2
3-Methyluridine	0.174	0.792	0.996	0.2–300	0.1
Adenosine	0.851	0.063	0.997	0.2–300	0.1
2′-Deoxyguanosine	0.322	0.153	0.995	0.2–300	0.1
8-Hydroxy-2′-deoxyguanosine	0.452	1.893	0.995	0.2–300	0.1
N^2,N^2-Dimethylguanosine	0.632	2.011	0.996	0.2–300	0.2
5-Deoxyadenosine	1.331	0.715	0.997	0.2–300	0.2

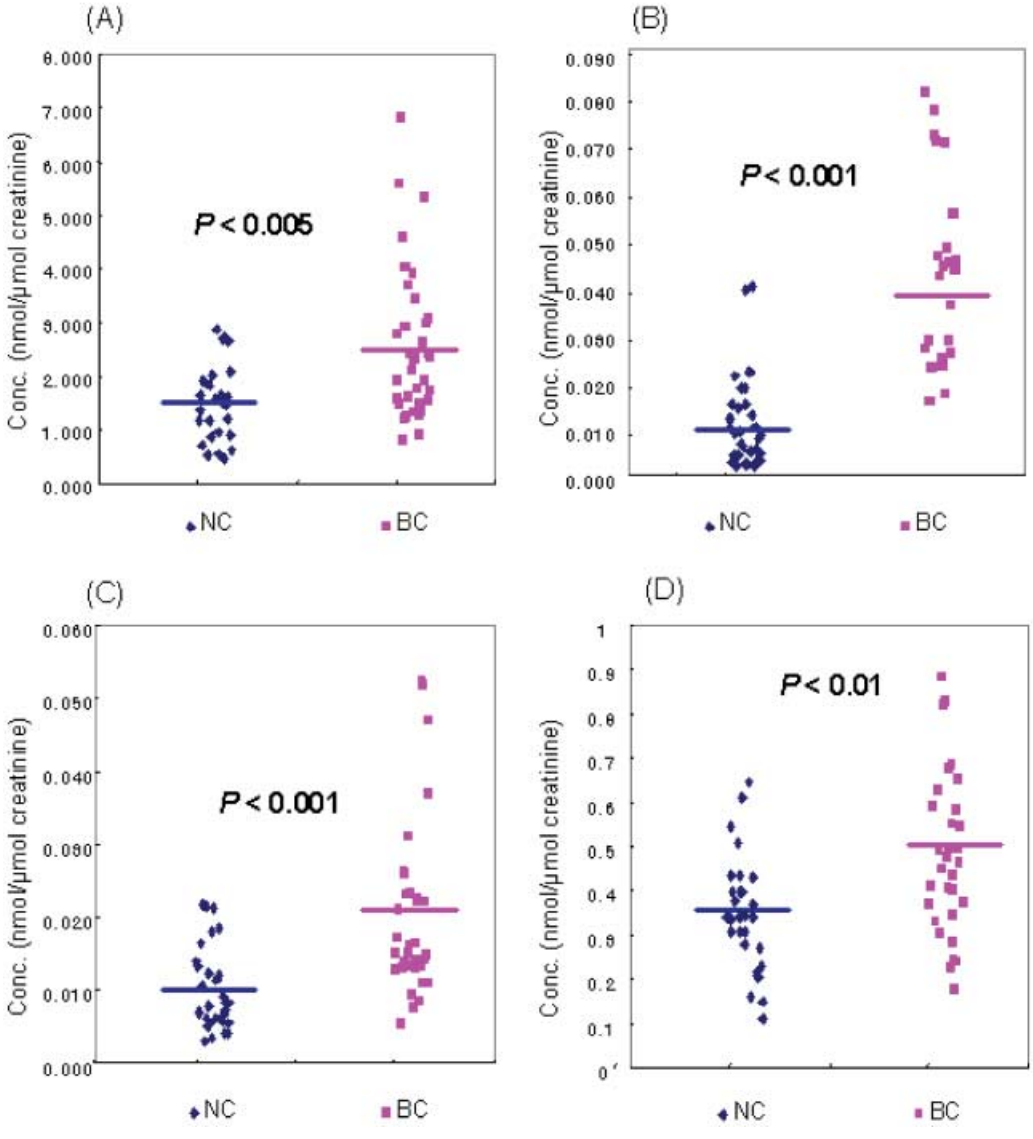

Figure 3. Distributions of (A) 1-methyladenosine, (B) 5-hydroxymethyl-2′-deoxyuridine, (C) 8-hydroxy-2′-deoxyguanosine, (D) N^2,N^2-dimethylguanosine in urine of patients with breast cancer (BC) and normal controls (NC). This figure is available in colour online at www.interscience.wiley.com/journal/bmc

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Table 3. Within-day and day-to-day variations of nucleosides

Analyte	Concentration added (nmol/mL)	Within-day (n = 3) Amount found (mean ± SD[a])	Within-day (n = 3) Precision (% CV[b])	Day-to-day (n = 3) Amount found (mean ± SD)	Day-to-day (n = 3) Precision (% CV)
Pseudouridine	0.20	0.21 ± 0.02	9.52	0.21 ± 0.02	9.52
	1.00	1.10 ± 0.10	9.09	1.10 ± 0.10	9.09
	10.00	10.31 ± 1.21	11.74	10.21 ± 1.08	10.57
	50.00	50.83 ± 4.84	9.52	51.34 ± 4.88	9.51
	100.00	112.01 ± 10.84	9.68	112.07 ± 10.98	9.80
Cytidine	0.20	0.21 ± 0.02	7.14	0.21 ± 0.01	6.77
	1.00	0.99 ± 0.08	8.08	1.11 ± 0.09	8.11
	10.00	10.08 ± 0.81	8.04	11.37 ± 1.03	9.06
	50.00	50.07 ± 1.25	2.50	50.11 ± 2.52	5.03
	100.00	101.08 ± 7.81	7.73	105.08 ± 7.11	6.77
Uridine	0.20	0.21 ± 0.02	7.62	0.20 ± 0.02	10.00
	1.00	1.01 ± 0.07	7.22	1.10 ± 0.08	7.27
	10.00	10.03 ± 0.74	7.38	11.32 ± 1.08	9.54
	50.00	50.09 ± 1.16	2.28	50.52 ± 2.22	4.36
	100.00	103.03 ± 8.74	8.48	102.02 ± 9.11	8.91
1-Methyladenosine	0.20	0.20 ± 0.02	10.00	0.20 ± 0.02	10.00
	1.00	1.10 ± 0.06	5.90	1.12 ± 0.11	9.82
	10.00	11.00 ± 0.65	5.91	9.98 ± 0.82	8.22
	50.00	50.10 ± 1.20	2.40	43.83 ± 4.28	9.77
	100.00	110.00 ± 5.65	5.14	107.11 ± 7.83	7.60
5-Methylcytidine	0.20	0.21 ± 0.02	10.33	0.20 ± 0.02	8.57
	1.00	1.08 ± 0.08	7.12	0.78 ± 0.07	8.97
	10.00	10.89 ± 0.78	7.16	9.83 ± 0.89	9.05
	50.00	50.05 ± 1.44	2.88	45.62 ± 4.44	9.73
	100.00	107.89 ± 8.78	8.14	101.72 ± 8.71	8.57
5-Methyl-2′-deoxycytidine	0.20	0.20 ± 0.02	10.00	0.19 ± 0.02	7.72
	1.00	0.99 ± 0.67	6.56	1.02 ± 0.08	7.84
	10.00	10.07 ± 0.63	6.26	11.96 ± 1.09	9.11
	50.00	50.03 ± 1.54	3.06	43.00 ± 4.42	10.28
	100.00	106.07 ± 7.63	7.19	101.05 ± 8.32	8.23
5-Hydroxymethyl-2′-deoxyuridine	0.20	0.20 ± 0.02	8.04	0.20 ± 0.02	10.00
	1.00	0.98 ± 0.06	6.02	1.31 ± 0.11	8.40
	10.00	10.77 ± 0.69	6.41	9.56 ± 0.89	9.31
	50.00	50.06 ± 1.28	2.56	42.34 ± 4.43	10.16
	100.00	107.77 ± 7.69	7.14	99.62 ± 7.71	7.72
Guanosine	0.20	0.21 ± 0.02	9.52	0.21 ± 0.02	9.52
	1.00	1.03 ± 0.01	0.97	1.10 ± 0.08	7.27
	10.00	10.89 ± 0.43	3.95	8.97 ± 1.00	11.15
	50.00	50.08 ± 1.34	2.68	51.01 ± 5.00	9.80
	100.00	108.89 ± 6.43	5.91	99.47 ± 6.22	6.28
3-Methyluridine	0.20	0.21 ± 0.02	9.52	0.21 ± 0.02	9.52
	1.00	1.02 ± 0.08	7.54	1.11 ± 0.09	8.11
	10.00	10.65 ± 0.67	6.29	10.99 ± 0.99	9.01
	50.00	40.99 ± 1.27	3.10	54.11 ± 4.83	8.93
	100.00	101.65 ± 5.67	5.58	100.72 ± 6.05	6.01
Adenosine	0.20	0.20 ± 0.02	10.00	0.21 ± 0.02	9.52
	1.00	0.99 ± 0.08	8.08	1.21 ± 0.11	9.09
	10.00	10.98 ± 0.81	7.38	12.32 ± 1.18	9.58
	50.00	50.08 ± 1.33	2.66	48.01 ± 4.73	9.85
	100.00	106.98 ± 8.81	8.24	102.83 ± 10.00	9.73
2′-Deoxyguanosine	0.20	0.20 ± 0.02	10.00	0.20 ± 0.02	10.00
	1.00	0.98 ± 0.07	7.43	0.98 ± 0.09	9.18
	10.00	10.88 ± 0.74	6.80	9.53 ± 0.88	9.23
	50.00	49.89 ± 1.36	2.72	48.13 ± 4.44	9.19
	100.00	106.88 ± 6.74	6.31	103.05 ± 7.36	7.14

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Table 3. (*Continued*)

Analyte	Concentration added (nmol/mL)	Within-day (n = 3) Amount found (mean ± SD[a])	Within-day (n = 3) Precision (% CV[b])	Day-to-day (n = 3) Amount found (mean ± SD)	Day-to-day (n = 3) Precision (% CV)
8-Hydroxy-2′-deoxyguanosine	0.20	0.21 ± 0.02	7.69	0.20 ± 0.02	10.00
	1.00	1.08 ± 0.03	3.15	0.89 ± 0.08	8.99
	10.00	10.09 ± 0.44	4.36	8.91 ± 0.88	9.88
	50.00	49.92 ± 1.26	2.52	42.12 ± 3.83	9.09
	100.00	102.09 ± 5.44	5.32	102.78 ± 8.41	8.18
N^2,N^2-Dimethylguanosine	0.20	0.21 ± 0.02	9.52	0.21 ± 0.02	9.52
	1.00	1.09 ± 0.05	4.67	1.00 ± 0.09	9.00
	10.00	10.13 ± 0.38	3.75	9.99 ± 0.98	9.81
	50.00	50.08 ± 1.12	2.24	51.13 ± 4.88	9.54
	100.00	107.13 ± 4.38	4.09	101.50 ± 9.26	9.12
5-Deoxyadenosine	0.20	0.21 ± 0.02	9.52	0.20 ± 0.02	10.00
	1.00	1.09 ± 0.05	4.67	0.99 ± 0.08	8.08
	10.00	10.25 ± 0.72	7.02	11.37 ± 1.09	9.59
	50.00	50.06 ± 1.14	2.28	54.34 ± 4.88	8.98
	100.00	105.25 ± 6.72	6.38	100.39 ± 9.36	9.33

[a] Standard deviation.
[b] Coefficient of variation.

Table 4. Measurement of urinary nucleosides in patients with breast cancer and normal controls

Analyte	Concentration[a] Normal control (NC, n = 30)	Concentration[a] Patient with breast cancer (BC, n = 30)	p-value NC:BC
Pseudouridine	13.28 ± 7.31	18.17 ± 11.56	NS[b]
Cytidine	0.11 ± 0.07	0.11 ± 0.08	NS
Uridine	0.20 ± 0.11	0.20 ± 0.12	NS
1-Methyladenosine	1.62 ± 0.91	2.54 ± 1.41	<0.005
5-Methylcytidine	9.09 ± 6.23	9.76 ± 5.40	NS
5-Methyl-2′-deoxycytidine	0.13 ± 0.12	0.13 ± 0.08	NS
5-Hydroxymethyl-2′-deoxyuridine	0.01 ± 0.01	0.04 ± 0.02	<0.001
Guanosine	0.07 ± 0.04	0.09 ± 0.10	NS
3-Methyluridine	0.20 ± 0.13	0.36 ± 0.22	NS
Adenosine	0.23 ± 0.13	0.24 ± 0.20	NS
2′-Deoxyguanosine	0.02 ± 0.01	0.02 ± 0.02	NS
8-Hydroxy-2′-deoxyguanosine	0.01 ± 0.01	0.02 ± 0.01	<0.001
N^2,N^2-Dimethylguanosine	0.34 ± 0.17	0.49 ± 0.17	<0.01
5-Deoxyadenosine	0.06 ± 0.06	0.06 ± 0.10	NS

[a] Concentration was expressed as nmol/μmol creatinine (mean ± SD).
[b] NS, not significant.

This result is consistent with the previous results (Borek and Kerr, 1972). It means that the levels of nucleosides could be a useful tool in diagnosing and monitoring breast cancer.

CONCLUSIONS

We developed the column-switching LC-ESI/MS/MS method for directly determining nucleosides in urine. We separated nucleosides within 20 min by column-switching using a pre-column for clean-up and an analytical column for the complete separation. We monitored the separated nucleosides with positive electrospray ionization mass spectrometry. This method is quite simple and rapid and does not require clean-up step, such as solid-phase extraction or liquid–liquid extraction. This assay also provides a linearity over 0.994, reliable accuracy and precision, and the limits-of-detection (LOD) of 0.1–0.2 nmol/mL. When we compared the urinary concentration of nucleosides in patients with breast cancer with the age-matched

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normal controls, the levels of nucleosides in the patients were significantly increased (1-methyladenosine; $p < 0.005$, N^2,N^2-dimethylguanosine; $p < 0.01$, 5-hydroxymethyl-2′-deoxyuridine; $p < 0.001$, 8-hydroxy-2-deoxyguanosine; $p < 0.001$). Therefore, we conclude that this method could be adapted to understand the pathogenesis of breast cancer.

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