Anal. Chem. **2009**, *81*, 6474–6480

Analytical Chemistry

A Fast Capillary Electrophoresis Method for Separation and Quantification of Modified Nucleosides in Urinary Samples

Yongqing Jiang and Yinfa Ma*

Department of Chemistry and Environmental Research Center, Missouri University of Science and Technology, Rolla, Missouri 65409

Modified nucleosides are formed at the post-transcriptional stage by chemical modification of normal nucleosides within the ribonucleic acid (RNA). These modified nucleosides cannot be reutilized or further degraded, but they are excreted in the urine as intact molecules. The elevated levels of modified nucleosides in the urine samples have served as potential cancer biomarkers in many studies. Although different analytical techniques have been reported for determining nucleosides levels, they are practically difficult to use as a routine tool for early cancer screening. In this paper, a novel method was developed to separate and quantify 10 nucleosides—adenosine, cytidine, guanosine, uridine, inosine, xanthosine, pseudouridine, N^2-methylguanosine, 1-methyladenosine, and N^2,N^2-dimethylguanosine—in urine samples using capillary electrophoresis with an ultraviolet (UV) detector (abbreviated as CE-UV) at a wavelength of 254 nm. A 50 μm (i.d.) × 38 cm (effective length) fused silica capillary was used for the separation, and a borate–phosphate buffer containing 25 mM cetyltrimethylammonium bromide (CTAB) at pH 9.50 was used as a background electrolyte. The separation was performed at 15 kV under reverse polarity and completed within 10 min. The linear range of the analytes was 5.0–500 μmol/L, and the limit of detection was <2.0 μmol/L. The effects of pH, buffer concentrations, CTAB concentration, and the operation voltages on the separation and quantification of the modified nucleosides were also investigated. The technique developed in this study is much simpler and faster, compared to previous studies, and can be used to quantify modified nucleosides in urine samples.

Nucleosides are the primary constituents of ribonucleic acids (RNAs). When RNAs are biotransformed, the normal nucleosides can either be metabolized or reutilized to synthesize nucleic acid. However, in particular cases, some RNAs are transformed to modified nucleosides, which can neither be further degraded nor reutilized, but are excreted intact in urine as end products, because of a lack of specific phosphorylases.[1] In cancer disease where cell proliferation ocurs, RNA metabolism increases dramatically and higher concentrations of excreted modified nucleosides will be observed. Consequently, the levels of modified nucleosides in urine can reflect RNA degradation in the organism; thus, they can be used as potential cancer biomarkers.

Modified nucleosides in human urine as possible cancer biomarkers have been of interest since the 1970s. Some studies reported that elevated levels of some nucleosides in the urine samples can serve as potential cancer biomarkers, and the urinary profile of modified and normal nucleosides have been widely studied as markers of leukemia,[2] breast cancer,[3–5] thyroid cancer,[6] uterine cervical cancer,[7] liver cancer,[8] lung cancer,[9] bladder and colorectal cancer,[10,11] head and neck cancer,[12] and the process of rheumatoid arthritis.[13] Profiles of urinary nucleosides also can be used to monitor the progress of the cancer disease and the response of individuals to an applied therapy.[14] Nevertheless, to date, no specific pattern has been discovered. Searching for the specific biomarker for specific cancer is very crucial for early cancer diagnosis. In many cases, an efficient separation and determination technique is required to assess the levels of these biomarkers.

* To whom correspondence should be addressed. Phone: 573-341-6220. Fax: 573-341-6033. E-mail: yinfa@mst.edu.

(1) Frickenschmidt, A.; Froehlich, H.; Bullinger, D.; Zell, A.; Laufer, S.; Gleiter, C. H.; Liebich, H.; Kammerer, B. *Biomarkers* **2008**, *13*, 435–449.

(2) Rasmuson, T.; Bjork, G. R. *Acta Oncol.* **1995**, *34*, 61–67.

(3) Bullinger, D.; Froehlich, H.; Klaus, F.; Neubauer, H.; Frickenschmidt, A.; Henneges, C.; Zell, A.; Laufer, S.; Gleiter, C. H.; Liebich, H.; Kammerer, B. *Anal. Chim. Acta* **2008**, *618*, 29–34.

(4) Sasco, A. J.; Rey, F.; Reynaud, C.; Bobin, J.-Y.; Clavel, M.; Niveleau, A. *Cancer Lett.* **1996**, *108*, 157–162.

(5) Zheng, Y.-F.; Kong, H.-W.; Xiong, J.-H.; Shen, L.; Xu, G.-W. *Clin. Biochem.* **2005**, *38*, 24–30.

(6) La, S.; Cho, J.; Kim, J.-H.; Kim, K.-R. *Anal. Chim. Acta* **2003**, *486*, 171–182.

(7) Kim, K. R.; La, S.; Kim, A.; Kim, J. H.; Liebich, H. M. *J. Chromatogr., B: Biomed. Sci. Appl.* **2001**, *754*, 97–106.

(8) Yang, J.; Xu, G.; Zheng, Y.; Kong, H.; Pang, T.; Lu, S.; Yang, Q. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2004**, *813*, 59–65.

(9) Wang, W.-z.; Zhao, X.-j.; Li, X.; Chen, J.; Li, F.-l.; Xu, G.-w. *Zhongguo Yixue Kexueyuan Xuebao* **2007**, *29*, 738–741.

(10) Feng, B.; Zheng, M.-H.; Zheng, Y.-F.; Lu, A.-G.; Li, J.-W.; Wang, M.-L.; Ma, J.-J.; Xu, G.-W.; Liu, B.-Y.; Zhu, Z.-G. *J. Gastroenterol. Hepatol.* **2005**, *20*, 1913–1919.

(11) Zheng, Y.-F.; Yang, J.; Zhao, X.-J.; Feng, B.; Kong, H.-W.; Chen, Y.-J.; Lv, S.; Zheng, M.-H.; Xu, G.-W. *World J. Gastroenterol.* **2005**, *11*, 3871–3876.

(12) Dudley, E.; Lemiere, F.; Van Dongen, W.; Langridge, J. I.; El-Sharkawi, S.; Games, D. E.; Esmans, E. L.; Newton, R. P. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1132–1136.

(13) Tebib, J. G.; Reynaud, C.; Cedoz, J. P.; Letroublon, M. C.; Niveleau, A. *Br. J. Rheumatol.* **1997**, *36*, 990–995.

(14) Dudley, E.; Tuytten, R.; Lemiere, F.; Esmans, E. E.; Newton, R. P. *Collect. Symp. Ser.* **2008**, *10*, 229–233.

10.1021/ac901216n CCC: $40.75
Published on Web 06/24/2009

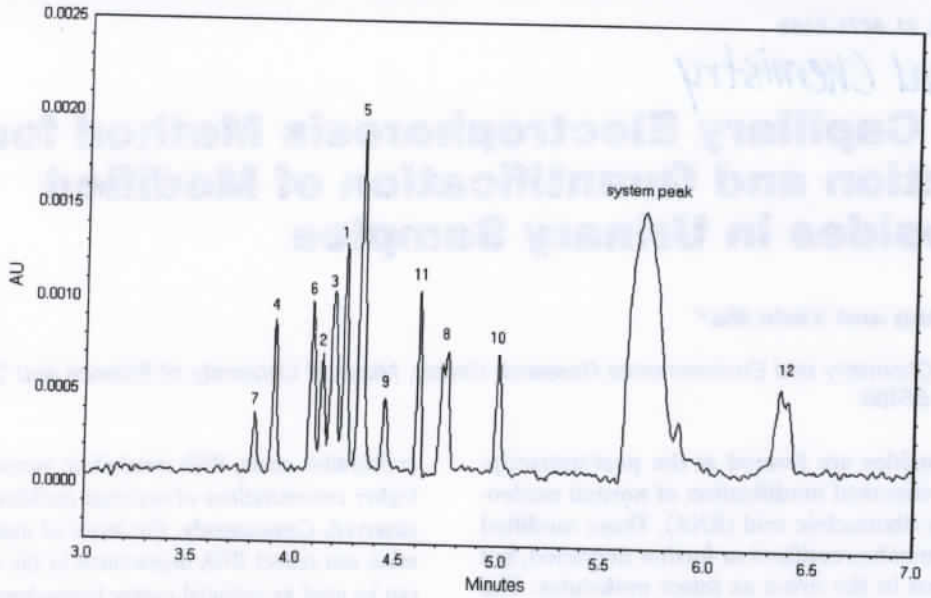

Figure 1. Electropherogram of 12 nucleoside standards under optimized conditions by HPCE with UV detection (HPCE-UV) at 254 nm. Capillary, 50 μm (i.d.) × 48 cm (38 cm to the detection window); applied voltage, −15 kV; temperature, 25 °C; sample injection, 0.5 psi for 10 s; running buffer, 25 mM $Na_2B_4O_7$ + 25 mM NaH_2PO_4 + 25 mM CTAB, pH 9.50. Legend used for peak identification: 1, adenosine (A); 2, cytidine (C); 3, guanosine (G); 4, uridine (U); 5, inosine (I); 6, xanthosine (X), 7; pseudouridine (Pseu); 8, N^2-methylguanosine (m^2G); 9, N^1-methyladenosine (m^1A); 10, 8-hydroxy-2′-deoxyguanosine; 11, 5-hydroxymethyl-2′-deoxyuridine; and 12, N^2,N^2-dimethylguanosine (m_2^2G).

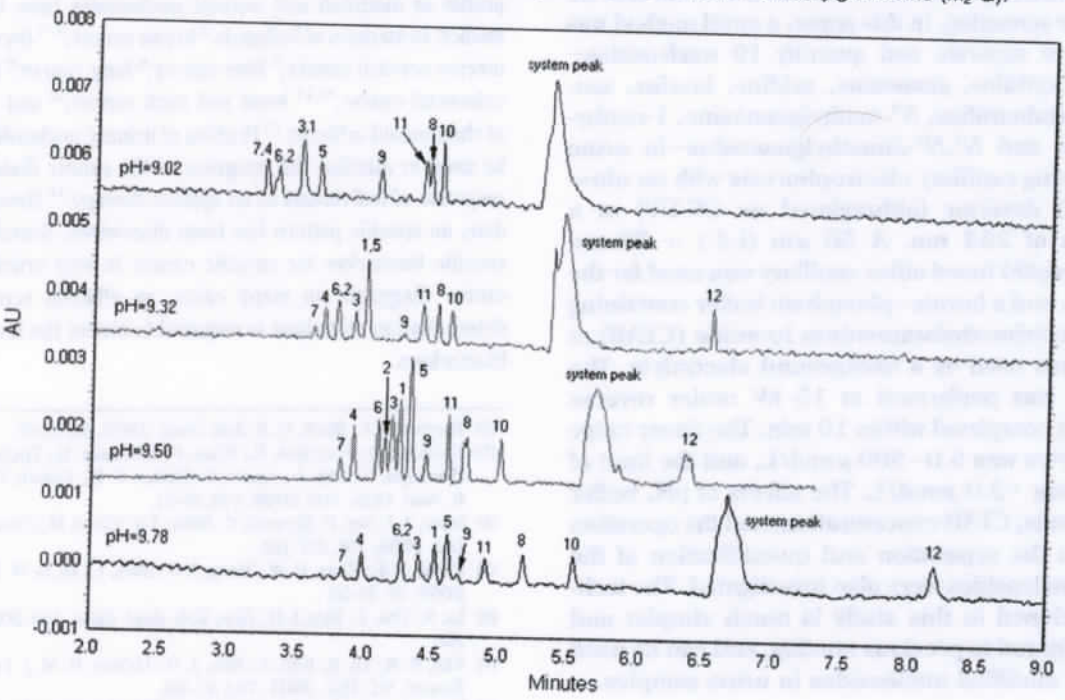

Figure 2. Effect of pH on the separation of 12 nucleoside standards. The experimental conditions were the same as those of Figure 1, except for the pH values of the running buffer. Peak identifications were the same as those in Figure 1.

Different analytical techniques have been reported for separating and determining normal and modified nucleosides in urine samples, including immunoassays,[15] high-performance liquid chromatography (HPLC) and capillary electrophoresis conjugated with UV detection (HPCE-UV),[16–21] photodiode-array detection,[18] and mass spectrometry (MS).[12,22–25] However, there is no routine cancer diagnostic tool based on levels of urinary nucleosides in clinical laboratories. One reason is that there is no specific nucleoside that can be used as a biomarker to a specific type of cancer. Another major reason is the lack of practicable analytical techniques that can be used to obtain levels of urinary nucleosides

(15) Moskal, J. R.; Grutsch, J. F. *Diagnostic modified-nucleoside detection assay*, World Patent WO/1999/051985, April 1, 1999, pp 1–27.
(16) Guowang, X.; Xu, G.; Lu, X.; Zhang, Y.; Lu, P.; Di Stefano, C.; Lehmann, R.; Liebich, H. *Sepu* **1999**, *17*, 97–101.
(17) Seidel, A.; Brunner, S.; Seidel, P.; Fritz, G. I.; Herbarth, O. *Br. J. Cancer* **2006**, *94*, 1726–1733.
(18) Seidel, P.; Seidel, A.; Herbarth, O. *Neural Networks* **2007**, *20*, 646–651.
(19) Szymanska, E.; Markuszewski, M. J.; Bodzioch, K.; Kaliszan, R. *J. Pharm. Biomed. Anal.* **2007**, *44*, 1118–1126.
(20) Xu, G.; Di Stefano, C.; Liebich, H. M.; Zhang, Y.; Lu, P. *J. Chromatogr., B: Biomed. Sci. Appl.* **1999**, *732*, 307–313.
(21) Zhao, X.; Zheng, Y.; Zhang, P.; Kong, H.; Xu, G. *Sepu* **2005**, *23*, 73–75.

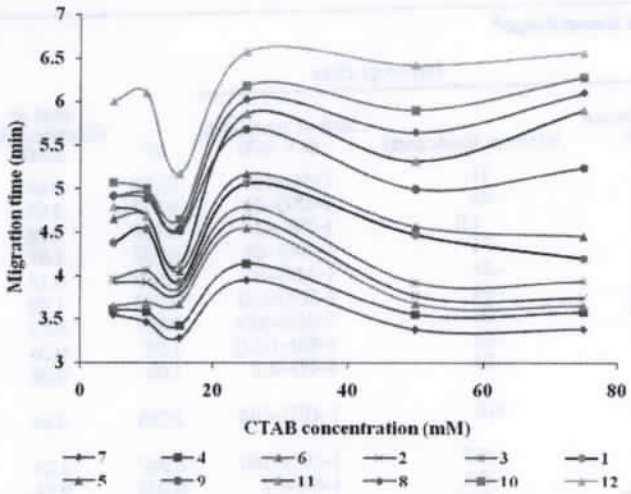

Figure 3. Effect of CTAB concentration on the separation of 12 nucleoside standards. The experimental conditions were the same as those of Figure 1, except for the CTAB concentrations.

conveniently. Although both immunoassay and HPLC have been demonstrated for the analysis of nucleosides, the procedures are tedious and the methods are time-consuming. In addition, HPLC consumes a large amount of organic solvents and the organic is not environmentally friendly.

High-performance capillary electrophoresis (HPCE) has proven to be a rapid and simple technique for separating charged biomolecules with very high resolution. Its unique advantages, such as a relatively short time of analysis and a high separation efficiency with consumption of a minimal amount of sample and buffer solutions, make HPCE a valuable technique for the determination of urinary nucleosides. Micellar electrokinetic chromatographic (MEKC) methods with a sodium dodecyl sulfate (SDS)−borate−phosphate buffer have been applied for the determination of urinary nucleosides by many researchers.[6,7,19,26] However, it was found that the method had a long separation time and the SDS in the background electrolyte (BGE) caused poor reproducibility, because of the frequent generation of air bubbles in the separation column. In this paper, we have developed a simple and fast HPCE method for the determination of urinary nucleosides using $Na_2B_4O_7$−NaH_2PO_4 as the BGE and cetyltrimethylammonium bromide (CTAB) as a BGE additive. The reproducibility and migration time were greatly improved. The optimized method has been used for the analysis of 12 important urinary nucleosides from both normal and breast cancer-carrying subjects. The main goal of this study was to develop a fast and reproducible HPCE method to study modified nucleosides that are present in urine samples from both cancer-carrying patients and healthy controls, so that it can be used to provide nucleoside profile information for potential early cancer screening.

MATERIALS AND METHODS

Chemicals. Twelve nucleoside standards, including adenosine, cytidine, guanosine, uridine, inosine, xanthosine, pseudouridine, N^2-methylguanosine, 1-methyladenosine, N^2,N^2-dimethylguanosine, 8-hydroxy-2′-deoxyguanosine, 5-hydroxymethyl-2′-deoxyuridine, in addition to creatinine (for normalization purposes), were purchased from Sigma−Aldrich (St Louis, MO). All other chemicals that were used for BGE preparation and capillary rinsing, such as sodium phosphate, sodium tetraborate, CTAB, methanol, sodium hydroxide, and hydrochloric acid, were also obtained from Sigma−Aldrich (St. Louis, MO). Deionized water (18.2 MΩ) from the Millipore Simplicity 185 system (Millipore, MA) was used to prepare standard solutions, the BGE, and other solutions.

Preparation of BGE. The BGE solution, which contained 25 mM $Na_2B_4O_7$, 25 mM NaH_2PO_4 and 25 mM CTAB, was prepared with deionized water and the pH was adjusted to 9.50 by adding 1.0 M NaOH in the buffer before diluting it to the final volume. The buffer solution was filtered through the 0.45-μm membrane filter before use.

Preparation of Standard Nucleoside Solutions. The 10 mM stock solutions of adenosine and xanthosine were prepared in 1.0 M NaOH and guanosine solutions were prepared in 50% formic acid. The 10 mM stock solutions of other nucleosides were prepared in deionized water. All stock solutions were kept at −20 °C. The working nucleoside solutions were prepared by diluting the stock solutions with deionized water to the concentration range of 1−5000 μM (1, 5, 10, 50, 100, 500, 1000, and 5000 μM).

Preparation of Urine Samples. Spontaneous urine samples from one healthy adult and one breast cancer patient were collected from the Ellis Fischel Cancer Center (Columbia, MO). After collection, the samples were frozen and stored at −80 °C immediately. Before the analysis, the samples were thawed at room temperature. Each urine sample was prepared for both nucleosides and creatinine analysis.

Nucleosides were extracted from urine samples, using affinity chromatography gel, as described in the literature.[7] Basically, each 3-mL extraction (SPE) tube purchased from Supelco (Bellefonte, PA) was packed with Affi-gel 601 (200 mg). The gel was conditioned by washing sequentially with 15 mL of 0.1 M formic acid in 50% methanol and 0.25 M ammonium acetate (pH 8.6) prior to use as phenylboronic acid (PBA) columns for SPE in affinity mode. An ammonia solution (25%) was added to urine samples to adjust pH to the range of 8.2−8.6 and then centrifuged. For the analysis of nucleosides, 1 mL of supernatant was loaded to a preconditioned PBA column, followed by the addition of 0.5 mL of 0.25 M ammonium acetate (pH 8.6) and being allowed to stand for 5−10 min. The PBA column was then successively rinsed with 4.0 mL of 0.25 M ammonium acetate (pH 8.6), 0.3 mL of 50% methanol twice, and 0.5 mL of 0.1 M formic acid in 50% methanol. The rinsed column was eluted with 3 mL of 0.1 M formic acid in 50% methanol. The eluent was evaporated to dryness in the Turbovap LV evaporator (Zymark, Hopkinton, MA) at 50 °C, and then the residue was dissolved in 200 μL water for HPCE analysis.

(22) Bond, A.; Dudley, E.; Lemiere, F.; Tuytten, R.; El-Sharkawi, S.; Brenton, A. G.; Esmans Eddy, L.; Newton Russell, P. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 137–150.
(23) Kammerer, B.; Frickenschmidt, A.; Gleiter, C. H.; Laufer, S.; Liebich, H. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 940–947.
(24) Mao, Y.; Zhao, X.; Wang, S.; Cheng, Y. *Anal. Chim. Acta* **2007**, *598*, 34–40.
(25) Wang, S.; Zhao, X.; Mao, Y.; Cheng, Y. *J. Chromatogr., A* **2007**, *1147*, 254–260.
(26) Liebich, H. M.; Lehmann, R.; Xu, G.; Wahl, H. G.; Haring, H. U. *J. Chromatogr., B: Biomed. Sci. Appl.* **2000**, *745*, 189–196.

Table 1. Linearity and Detection Limit of Nucleosides and Creatinine[a]

		Slope Data		Intercept Data			
nucleoside	linear range (μM)	slope (peak area/μM)	limit of confidence (p = 95%)	intercept (peak area)	limit of confidence (p = 95%)	R^2	limit of detection, LOD (μM)
Pseu	50–5000	30	27–34	11	(–84)–107	0.994	0.67
U	10–400	66	61–71	–46	(–187)–94	0.997	1.06
X	5–500	82	81–84	1.6	(–39)–42	0.999	0.78
C	5–400	71	66–76	–74	(–209)–60	0.998	1.67
G	5–250	88	85–92	–44	(–144)–56	0.999	0.77
A	5–500	95	88–101	–66	(–254)–121	0.998	1.09
I	10–400	128	117–138	–53	(–345)–239	0.997	0.56
m^1A	10–500	89	89–89	–59	(–65)–(–54)	1.00	0.76
5-hydroxymethyl-2′-deoxyuridine	10–500	75	74–76	–20	(–44)–3.3	1.00	0.98
8-hydroxy-2′-deoxyguanosine	10–500	117	96–137	118	(–467)–703	0.996	0.55
m^2G	10–1000	74	67–81	–7.7	(–196)–180	0.997	1.26
m^2_2G	5–500	79	76–82	–8.4	(–89)–72	0.984	0.74
creatinine	5–5000	0.068	0.065–0.071	–0.99	(–7.0)–5.1	0.999	0.89

[a] The experimental conditions were the same as those of Figure 1.

Table 2. Reproducibility and Recovery for Nucleosides and Creatinine[a]

Nucleosides	relative standard deviation, RSD (%)	recovery (%)
Pseu	3.6	108
U	7.2	104
X	7.9	82.6
C	6.4	113
G	6.8	83.8
A	5.7	92.9
I	8.3	81.2
m^1A	7.2	89.4
5-hydroxymethyl-2′-deoxyuridine	N/A[b]	0
8-hydroxy-2′-deoxyguanosine	N/A[b]	0
m^2G	5.1	105
m^2_2G	6.7	108
creatinine	4.1	95.6

[a] The experimental conditions were the same as those of Figure 1. [b] Not applicable.

For the creatinine analysis, a method developed by Zheng et al.[27] was used with minor modification. Briefly, the urine supernatant was diluted 8-fold in deionized water and then injected into a CE column for direct analysis.

Instrumentation. All CE experiments were performed on a Beckman Coulter P/ACE MDQ instrument (Beckman Instruments, Fullerton, CA) that was equipped with a UV-absorbance detector. Electrophoretic data were acquired and analyzed by 32 Karat software (version 4). Separations were performed in fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with dimensions of 50 μm (i.d.) × 38 cm (effective length). New capillaries were conditioned by rinsing with methanol for 15 min, deionized water for 5 min, and 1.0 M HCl for 5 min, followed by deionized water for 5 min again, then 1.0 M NaOH for 20 min and deionized water for 5 min. The capillary was rinsed with deionized water for 5 min and then prerun with BGE for 20 min under −15 kV every morning to obtain the best reproducibility. Samples were injected into the capillary at 0.5 psi for 10 s. After each analysis, the capillary was rinsed successively with 0.1 M NaOH for 1.0 min and then deionized water and BGE, for 2.0 min each. Nucleosides separation was conducted at −15 kV at 25 °C, and the wavelength of the UV detector was set at 254 nm. For the analysis of creatinine, 30 mM phosphate at pH 6.0 was used as the BGE and the separation was completed under 15 kV with a detection wavelength of 214 nm. Other conditions were the same as those of the nucleosides.

RESULTS AND DISCUSSION

Nucleosides are suitable to be analyzed by capillary electrophoresis,[19] because of their negative charges in a wide range of pH values, diverse molecular weight, and hydrophilic property. The quality of the electrophoretic separation and the time required for this separation are dependent on several analytical parameters, such as running buffer composition and pH, buffer concentration and additives, the applied voltage, the length and diameter of the capillary, and the sample size introduced, and so on. All these conditions were investigated systematically in this study to get the optimum separation conditions. Figure 1 showed the separation of 12 nucleoside standards under optimal conditions using HPCE. The running buffer was composed of 25 mM $Na_2B_4O_7$ + 25 mM NaH_2PO_4 + 25 mM CTAB (pH 9.50), and the separation voltage was −15 kV with UV detection at 254 nm.

Effect of Buffer Concentration. Tetraborate, which can form a complex network with hydroxyl groups, has been used to improve the separation of catechols, carbohydrates, and nucleosides.[16,28,29] After preliminary experiments, an electrolyte containing tetraborate, phosphate, and CTAB was selected as the running buffer. Five different tetraborate concentrations (15, 25, 50, 75, and 100 mM) were examined to compare the separation efficiency and peak-to-peak resolutions of 12 nucleosides. With the increase of the tetraborate concentration above 25 mM, the migration time became longer and longer without any improvement of the

(27) Zheng, Y.-F.; Xu, G.-W.; Liu, D.-Y.; Xiong, J.-H.; Zhang, P.-D.; Zhang, C.; Yang, Q.; Shen, L. *Electrophoresis* **2002**, *23*, 4104–4109.

(28) Taverna, M.; Baillet, A.; Biou, D.; Schluter, M.; Werner, R.; Ferrier, D. *Electrophoresis* **1992**, *13*, 359–366.

(29) Landers, J. P.; Oda, R. P.; Schuchard, M. D. *Anal. Chem.* **1992**, *64*, 2846–2851.

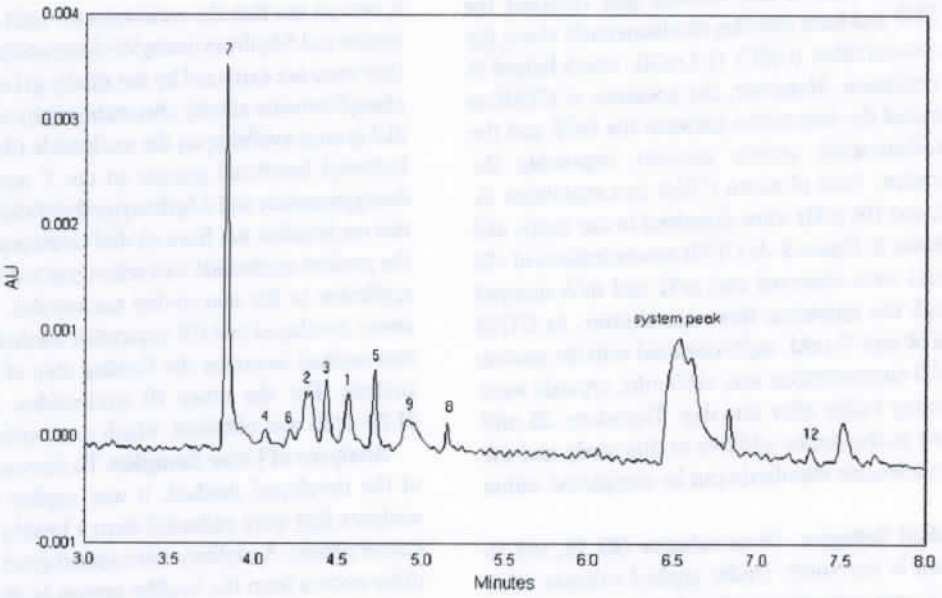

Figure 4. Representative electropherogram of nucleosides in a urine extract of a healthy subject. The experimental conditions and peak identifications were the same as those of Figure 1.

Table 3. Average Nucleoside Levels Excreted in Urine Samples from a Normal Subject and a Breast-Cancer-Carrying Patient

	Average Nucleoside Level (nM nucleoside/μM creatinine)		
nucleoside	normal subject mean ± SD	cancer patient mean ± SD	literature data for normal subject[5,7,13,26,32–34]
Pseu	24.0 ± 3.2	48.2 ± 2.4	13.0–42.0
U	1.01 ± 0.10	1.22 ± 0.08	0.21–0.99
X	0.76 ± 0.04	1.30 ± 0.09	0.24–0.86
C	0.57 ± 0.29	7.55 ± 0.62	0.01–0.78
G	10.0 ± 0.1	11.7 ± 1.0	0.01–10.70
A	3.51 ± 0.21	6.09 ± 0.18	0.18–4.70
I	7.38 ± 0.59	12.3 ± 0.2	0.09–8.80
m^1A	3.01 ± 0.23	3.34 ± 0.31	2.02–2.90
m^2G	0.59 ± 0.01	2.46 ± 0.14	0.26–2.00
m^2_2G	0.32 ± 0.01	2.03 ± 0.32	0.36–1.74

nucleoside separations. When the concentration was decreased to 15 mM, the resolution worsened and several peaks merged together and could not be separated. Meanwhile, the current increased significantly at higher buffer concentrations and the Joule heating generated affected the separation. Therefore, the optimized tetraborate concentration was maintained at 25 mM. Phosphate was used as a co-ion, because of its UV transparency at 254 nm. After a series of experimental studies, 25 mM was chosen as the optimal concentration. Under this condition, the current was ~60 μA at 25 °C, which did not produce a significant amount of Joule heating. The running buffers were replaced every 12 runs to keep the ionic strength balanced on both sides of the running buffer, so that reproducible data can be obtained.

Effect of Buffer pHs. The most crucial parameter for the nucleoside separation using HPCE is the pH of the running buffer. It not only affects the solute charge, but it also influences the capillary wall surface and will cause a concomitant change in electro-osmatic flow (EOF). Most nucleosides, with *cis*-diol structures, will bind with boric acid at high pHs and form negatively charged complex.[30] Therefore, four different pH values (9.02, 9.32, 9.50, and 9.78) were examined for the separation of 12 nucleosides. The results are shown in Figure 2. Clearly, the HPCE separation of nucleosides was very sensitive to pHs. Under pH 9.02, Pseu and U, X and C, and G and A co-eluted with each other, respectively. In addition, m_2^2G coeluted with the system peak. As the pH increased to 9.32 or above, m_2^2G eluted after the system peak. With the increase of the pH to 9.50, the migration time became longer but the resolution also increased and all 12 nucleosides can be well-separated. When the running buffer pH increased to 9.78, X and C could not be well-separated. For the examined pHs, these 12 nucleosides can only be separated at 9.50. The nucleosides clearly have different mobilities at pH ~9.50, under the present separation conditions. Therefore, the optimal pH for the separation of these 12 nucleosides was set at 9.50.

Effect of Additive Concentrations. Additives are widely used in HPCE separations, acting as solubilizing agents for hydrophobic solutes or as wall modifiers. For the separation of nucleosides, SDS has been used as a surfactant at 300 mM in most of the studies to help improve the separations. However, several problems have been encountered by adding SDS in the running buffer:

(1) Bubbles were easily generated at such a high surfactant concentration,

(2) Relatively long separation time (normally 30−40 min) and pH shifting were observed, and

(3) Poor reproducibility after three runs and the noisy baseline were often obtained.

Without additives, these nucleosides could not be well-separated, because of their close isoelectric points (pIs). Therefore, CTAB was used in our study to help the separation. CTAB, which is a cationic surfactant, was used to cover the silanol groups and make the capillary wall positively charged, to prevent positively charged particles from adsorbing on the wall. In

(30) Tuytten, R.; Lemiere, F.; Esmans, E. L.; Herrebout, W. A.; Van der Veken, B. J.; Maes, B. U. W.; Witters, E.; Newton, R. P.; Dudley, E. *Anal. Chem.* **2007**, *79*, 6662–6669.

addition, the use of CTAB in this method also reversed the direction of the EOF and form micelles simultaneously above the critical micelle concentration (CMC) (1.3 mM), which helped in the nucleoside resolution. Moreover, the presence of CTAB as an additive enhanced the interaction between the BGE and the nucleosides (predominantly anionic species), improving the nucleoside separation. Total of seven CTAB concentrations (5, 10, 15, 25, 50, 75, and 100 mM) were examined in our study, and the results are shown in Figure 3. At CTAB concentrations of <25 mM, only 11 peaks were observed and m^2G and m^1A merged together, although the migration time was shorter. At CTAB concentrations of 50 and 75 mM, m_2^2G coeluted with the system peak. When CTAB concentration was >100 mM, crystals were found in the running buffer after one day. Therefore, 25 mM CTAB was chosen as the buffer additive in this study and the separation of 12 nucleoside standards can be completed within 7 min.

Effect of Applied Voltages. Three voltages (20, 18, and 15 kV) were examined in our study. Under applied voltages of 20 and 18 kV, X and C cannot be separated and merged as a single peak and m^1A also was hidden in I. Although the migration time was longer at 15 kV, the peak resolution was greatly improved within the reasonable time. Therefore, 15 kV was selected as the optimized separation voltage in our study.

Linearity, Detection Limit, Reproducibility, and Recovery Study. Completely study of linearity, detection limit, reproducibility, and recovery of modified nucleosides of this HPCE method was conducted and the data were summarized in Tables 1 and 2. Because this method can also be used to determine the nucleoside levels in other biological samples, CE and SPE methods were validated separately. In the linearity study, nucleoside concentrations selected were based on the expected concentration range in the urine samples. The following concentrations in deionized H_2O were used to construct the calibration curves: 0, 5, 10, 25, 50, 100, 250, 400, 500 μM for A, C, G, U, X, I, m_2^2G, and m^1A; 0, 10, 25, 50, 100, 250, 500, and 1000 μM for m^2G, 8-hydroxy-2′-deoxyguanosine, and 5-hydroxymethyl-2′-deoxyuridine; 0, 50, 100, 200, 400, 1000, 2000, and 5000 μM for Pseu; and 5, 20, 100, 1000, 2000, and 5000 μM for creatinine. The regression parameters (such as linearity range, slope, intercept, and correlation coefficients) are presented in Table 1. The averages of the correlation coefficients for six injections were in the range of 0.9843−1.0000, which indicates good linearity. The limit of detection (LOD) of this method was defined as the concentration where the signal-to-noise ratio was 3. As listed in Table 1, the LOD of this method is in the range of 0.55−1.67 μM, which is sensitive enough for nucleoside detection in urine samples.

The reproducibility of relative peak areas, which is expressed as a percentage of relative standard deviation (RSD), was determined by six consecutive analyses of the normal urine sample. As shown in Table 2, the developed method was reproducible and these nucleosides were very stable during the analysis. The good reproducibility is not only due to the usage of CTAB as additive, but also due to the capillary rinsing procedure at the beginning of each day and the one between runs.

The recoveries of the nucleosides were determined by spiking the standards into a 1.0 mL urine sample. From the data in Table 2, we can see that the recoveries for both 8-hydroxy-2′-deoxyguanosine and 5-hydroxymethyl-2′-deoxyuridine were 0, which means they were not extracted by the affinity gel used in this study. While phenylboronate affinity chromatography selectively binds the *cis*-diol groups available on the nucleoside ribose sugar, the missing hydroxyl functional groups on the 2′ position for 8-hydroxy-2′-deoxyguanosine and 5-hydroxymethyl-2′-deoxyuridine makes these two nucleosides not have *cis*-diol structures anymore. Therefore, the present nucleoside extraction method from urine may not be applicable to the non-*cis*-diol nucleosides. Therefore, using this newly developed fast CE separation method, the sample preparation method becomes the limiting step of the entire nucleoside analysis. For the other 10 nucleosides, a recovery range of 81.2%–113% was obtained, which is acceptable.

Analyses of Urine Samples. To demonstrate the applicability of the developed method, it was applied to two urine sample analyses that were collected from a healthy person and a breast cancer patient. A capillary electropherogram of nucleosides in the urine extract from the healthy person is shown in Figure 4. The average nucleoside levels excreted in urine samples from a non-cancer-carrying person and a cancer patient are shown in Table 3. The peaks were identified by comparing migration times of the unknown peaks with those of the standard nucleosides eluted under the same conditions, and by spiking the urine sample with pure single nucleoside standards. The levels of the urinary nucleosides were calculated using standard calibration curves and then were transformed to units of nM nucleoside/μM creatinine. Creatinine has been used in many clinical studies as an internal standard, because its concentration strictly corresponds to urine dilution. Many studies have shown that the excretion of urinary nucleosides from human beings is affected only slightly by diet, and, when normalized to urinary creatinine, the daily excretion rate is remarkably constant in an individual.[20,31] The results obtained through our newly developed HPCE-UV method were comparable to those of the previous CE-UV and HPLC methods. More urine samples will be analyzed using this newly developed method for potential early cancer screening, using nucleosides as biomarkers.

CONCLUSION

A simple and fast high-performance capillary electrophoresis (HPCE) method was developed for the separation and quantitation of 10 modified nucleosides in urine samples. The separation time was reduced dramatically and the reproducibility was significantly improved, compared to previous capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) methods, which is very valuable for clinic diagnosis. The factors that affect separation efficiency, such as pH, voltages, and buffer composition and concentration, have been systematically investigated and optimized. This method can be used for urinary nucleoside

(31) Sander, G.; Topp, H.; Heller-Schoech, G.; Wieland, J.; Schoech, G. *Clin. Sci.* **1986**, *71*, 367–374.
(32) Lee, S. H.; Jung, B. H.; Kim, S. Y.; Chung, B. C. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 973–977.
(33) Liebich, H. M.; Di Stefano, C.; Wixforth, A.; Schmid, H. R. *J. Chromatogr., A* **1997**, *763*, 193–197.
(34) Vidotto, C.; Fousert, D.; Akkermann, M.; Griesmacher, A.; Muller, M. M. *Clin. Chim. Acta* **2003**, *335*, 27–32.

determination for early cancer screening in which nucleosides are used as biomarkers.

ACKNOWLEDGMENT

This research was supported by Environmental Research Center and startup funds awarded to Y.M. from Missouri University of Science and Technology. The authors sincerely thank the Ellis Fischel Cancer Center (Columbia, MO) for providing urine samples from breast-cancer-carrying patients.

Received for review April 23, 2009. Accepted June 7, 2009.

AC901216N