Detection of Human Urinary 5-Hydroxymethylcytosine by Stable Isotope Dilution HPLC-MS/MS Analysis

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ABSTRACT: The sixth DNA base 5-hydroxymethylcytosine (5hmC) is the major oxidation product of the epigenetic modification 5-methylcytosine (5mC), mediating DNA demethylation in mammals. Reduced 5hmC levels are found to be linked with various tumors and neurological diseases; therefore, 5hmC is an emerging biomarker for disease diagnosis, treatment, and prognosis. Due to its advantages of being sterile, easily accessible in large volumes, and non-invasive to patients, urine is a favored diagnostic biofluid for 5hmC analysis. Here we developed an accurate, sensitive, and specific assay for quantification of 5mC, 5hmC, and other DNA demethylation intermediates in human urine. The urinary samples were desalted and enriched using off-line solid-phase extraction, followed by stable isotope dilution HPLC-MS/MS analysis for 5hmC and 5mC. By the use of ammonium bicarbonate (NH_4HCO_3) as an additive to the mobile phase, we improved the online-coupled MS/MS detection of 5mC, 5hmC, and 5-formylcytosine (5fC) by 1.8–14.3 times. The recovery of the method is approximately 100% for 5hmC, and 70–90% for 5mC. The relative standard deviation (RSD) of the interday precision is about 2.9–10.6%, and that of the intraday precision is about 1.4–7.7%. By the analysis of 13 volunteers using the developed method, we for the first time demonstrate the presence of 5hmC in human urine. Unexpectedly, we observed that the level of 5hmC (22.6 ± 13.7 nmol/L) is comparable to that of its precursor 5mC (52.4 ± 50.2 nmol/L) in human urine. Since the abundance of 5hmC (as a rare DNA base) is 1 or 2 orders of magnitude lower than 5mC in genomic DNA, our finding probably implicates a much higher turnover of 5hmC than 5mC in mammalian genomic DNA and underscores the importance of DNA demethylation in daily life.

The well-characterized epigenetic modification 5-methylcytosine (5mC) plays important roles in regulation of gene expression,^{1–3} genomic imprinting,⁴ and X-chromosome inactivation.^{5,6} Recently, 5mC was found to be oxidized by Tet (ten eleven translocation) family dioxygenases to form 5-hydroxymethylcytosine (5hmC) in mammals.^{7,8} As the “sixth” DNA base, 5hmC is involved in a variety of physiological processes, e.g., DNA demethylation,^{9,10} somatic cell reprogramming,¹¹ and embryonic development.^{12–14} Recently, our group and other laboratories discovered that some nutrient or environmental factors (vitamin C,¹⁵ redox-active quinones,¹⁶ heavy metals¹⁷) can alter the genome-wide distribution of 5hmC in the mammalian genome. It is also noted that the level of 5hmC significantly decreases in various cancers,^{18–23} including the tumors of lung, brain, liver, kidney, skin, small intestine, prostate, breast, and colon, melanomas, and hepatocellular carcinomas, suggesting that 5hmC plays an important role in cancer development.^{22–25} In addition, aberrant 5hmC distribution has also been found in myelodysplastic syndrome,²⁶ Huntington’s disease,²⁷ Alzheimer’s disease,²⁸ and psychosis.²⁹ All of these findings prompt us to propose that 5hmC is an emerging biomarker for disease diagnosis, treatment, and prognosis.

5hmC is present with the highest abundance in brain and at low levels in lung, heart, breast, and spleen.^{30–34} Although 5hmC levels have been detected in various tissues, information on urinary 5hmC is lacking. Since it is sterile, easily accessible in large volumes, and noninvasive to patients, urine has long been considered as a favored diagnostic biofluid in clinical practice. Therefore, urinary 5hmC analysis should be a first choice for clinical diagnosis, disease treatment, and prognosis. Compared with tissue 5hmC analysis, urinary 5hmC analysis does not require genomic DNA extraction and digestion. Several biological processes (e.g., apoptosis, erythropoiesis, and lens cell development^{35–37}) can cause DNA degradation into deoxynucleosides. Therefore, it is reasonable to believe that DNA degradation or metabolism-caused 5hmC in the form of single deoxynucleosides could be excreted into urine through the circulation system. In fact, the 5hmC precursor 5mC was successfully identified and quantified in human urine.^{38–42}

A number of specific technologies for tissue 5hmC analysis have been developed, including thin layer chromatography

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(TLC)- ^{32}P labeling,^{7,25,43} immunoassays,^{24,25,31,44} and liquid chromatography–mass spectrometry (LC-MS).^{15,16,32,33,45–50} TLC- ^{32}P labeling method is very sensitive due to the use of radioactive ^{32}P labeling, but this method is limited to the determination of 5hmC in restriction enzyme-dependent DNA sequences and requires special safety settings. Immunoassays using antibodies against 5hmC⁴⁴ or cytosine 5-methylenesulfonate²⁴ have been used to specifically detect 5hmC in genomic DNA without the need for DNA digestion. Compared with the above two methods, LC-MS/MS is of interest due to its advantages in sensitivity, specificity, and structural characterization.^{45,46} However, the ionization efficiency of 5mC and its oxidation products is poor, and the corresponding sensitivity of LC-MS/MS analysis is unsatisfactory. To improve LC-MS/MS analysis, one solution is to use hydrophilic-interaction liquid chromatography (HILIC), by which organic solvents of high concentration can be exploited to promote solvent evaporation and protonation of the analytes during electrospray ionization.^{47,48} HILIC can also be used to enrich glucosylated 5hmC.⁴⁹ To the best of our knowledge, no methods have been reported so far for the detection of 5mC oxidation products (including free 5hmC nucleosides) in human urine.

In this work, we describe in detail an enhanced ionization method specifically for detection of 5mC and DNA demethylation intermediates (Scheme 1). Moreover, we

Scheme 1. Possible Pathways of Tet-Mediated Active DNA Demethylation

developed a solid-phase extraction (SPE) approach for desalting and enriching the nucleosides from human urine. By off-line coupling of SPE with stable isotope dilution HPLC-MS/MS, we developed an approach for accurate determination of 5mC and 5hmC in human urine. By the developed approach, we for the first time demonstrate the presence and the abundance of 5hmC in human urine.

EXPERIMENTAL SECTION

Chemicals. 5hmC, 5fC, and 5mC were obtained from Berry & Associates (Dexter, MI). 5caC and 5hmU were gifts from Dr. Guoliang Xu at the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. 5-Hydroxymethylcytosine-*d*₃-2-deoxycytidine ([D₃]5hmC) and 5-methyl-*d*₃-2-deoxycytidine ([D₃]5mC) were purchased from Toronto Chemical Research (Toronto, Canada). Formic acid (HCOOH) and ammonium acetate (CH₃COONH₄) were ordered from Acros Organics (Geel, Belgium) and Sinopharm Chemical Reagent Co. (Beijing, China), respectively. Ammonium formate (HCOONH₄) and NH₄HCO₃ were purchased from Sigma-Aldrich (St Louis, MO).

HPLC-MS/MS Analysis. The HPLC-MS/MS analysis was performed on an Agilent 1290 UHPLC system coupled with an ESI-triple quadrupole mass spectrometer (G6410B or G6495, Agilent Technologies, Santa Clara, CA). A NUCLEOSHELL RP 18 column (2.0 × 100 mm, 2.7 μm particle size, Macherey-Nagel, Düren, Germany) was employed for separation of single deoxynucleosides. The mobile phase consisted of two solvents: 2.0 mM NH₄HCO₃, pH 8.5, in water (A), and 100% methanol (B). The deoxynucleosides were separated with an optimized gradient elution: 0–5.0 min, 5% B; 5.0–8.0 min, 15% B; 8.0–12.0 min, 100% B; 12.0–18.0 min, 5% B. The flow-rate was 0.25 mL/min. To optimize the MS detection of deoxynucleosides, four volatile additives to the mobile phase were tested, including HCOOH, HCOONH₄, NH₄HCO₃, and CH₃COONH₄.

The mass spectrometer was operated under positive ionization mode with electrospray voltage (capillary voltage) of 3500 V. The molecular ions of 5mC, 5hmC, 5fC, 5caC, and 5hmU prefer to lose the deoxyribose moiety (MW: 116 Da) in the process of collision-induced dissociation. Thus, multiple reaction monitoring (MRM) transitions of [M + H]⁺ → [M + H – 116]⁺ were chosen to quantify the tested deoxynucleosides: *m/z* 242 → 126 for 5mC (collision energy, 5 eV); *m/z* 245 → 129 for [D₃]5mC (5 eV); *m/z* 258 → 142 for 5hmC (5 eV); *m/z* 261 → 145 for [D₃]5hmC (5 eV); *m/z* 256 → 140 for 5fC (5 eV); *m/z* 272 → 156 for 5caC (5 eV); *m/z* 259 → 143 for 5hmU (5 eV). Those daughter ions with the second highest intensities were used as qualitative ions: *m/z* 242 → 109 for 5mC (30 eV); *m/z* 258 → 124 for 5hmC (30 eV). The fragmentation voltage for all the MRM transitions were set at 90 V to allow efficient transit of precursor ions. Nitrogen gas was used for nebulization and desolvation. The nebulization gas pressure was set at 40 psi, and the temperature and the flow rate of desolvation gas were set at 300 °C and 9.0 L/min, respectively. High purity nitrogen (99.999%) was used as collision gas. Each sample was analyzed at least three times with an injection volume of 5.0 μL.

Solid-Phase Extraction. To obtain satisfactory extraction efficiency, two types of SPE cartridges (C18 and HLB) were first tested using HPLC-UV analysis. The C18 and HLB cartridges with equal amounts of packing (6.0 mL, 500 mg per cartridge) were purchased from Agela Technologies (Tianjin, China) and Waters Corporation (Milford, MA), respectively. Each cartridge was first preconditioned with 10.0 mL of methanol followed by 6.0 mL of water. Then a 1.0 mL premixed deoxynucleoside standard solution (final concentration of each deoxynucleoside: 1.0 μM) was loaded into the preconditioned cartridges. The cartridges were washed with 5.0 mL of H₂O and successively eluted with 5.0 mL of methanol/

Figure 1. Effect of mobile-phase additives on the HPLC separation and MS detection sensitivity of 5mC, 5hmC, and 5fC. Left panel represents typical chromatograms of 5mC, 5hmC, and 5fC that were obtained using four different additives to the mobile phase. The peak area ratios of the target analytes obtained by ammonium salt vs HCOOH are summarized in the table to the right. The mobile phase consisted of solvents A and B (pure methanol). Solvent A was 2.0 mM ammonium salt (HCOONH_4 , $\text{CH}_3\text{COONH}_4$, and NH_4HCO_3) or 0.1% formic acid in water. An isocratic elution of 95% A and 5% B was used, and the flow was set at 0.25 mL/min. The final concentrations of 5mC, 5hmC, and 5fC standards were 10 nM, and the injection volume was 5.0 μL .

water 1:9 (v/v) and 5.0 mL of methanol/water 3:7 (v/v). When methanol was removed by nitrogen gas at room temperature, the eluted fractions from each step were frozen at -80°C and then lyophilized at -50°C under a vacuum of 0.01 mbar. The residues were centrifuged at 12 000 rpm for 10 min and redissolved in 50 μL of H_2O .

To evaluate the SPE protocol, the collected SPE fractions were further analyzed using a Shimadzu LC-20AD HPLC system equipped with an SPD-20A UV detector. 5hmU, 5mC, and its oxidation products were separated with a Venusil MP C18 column (4.6×100 mm, 5.0 μm , Agela Technologies, Tianjin, China) using an isocratic elution of methanol/water (1:9) at 0.8 mL/min. The detection wavelength was set at 280 nm, and the injection volume was 5.0 μL .

To fit to HPLC MS/MS analysis, the collected urine samples were also pretreated using solid-phase extraction. However, a smaller HLB cartridge (3.0 mL, 60 mg per cartridge) was tailored for this application.

Linearity, Accuracy, and Precision. The standard solutions of 5hmC and 5mC standards of varying concentrations (2.0, 4.0, 8.0, 16.0, 32.0, and 64.0 nM) mixed with 80 nM $[\text{D}_3]5\text{hmC}$ and 40 nM $[\text{D}_3]5\text{mC}$ (as internal standards) were prepared and then analyzed using the HPLC-MS/MS method described above. Calibration curves were constructed by linearly plotting the peak area ratios of 5hmC and 5mC to the corresponding stable isotopic standards against the concentration of the added nonisotopic 5hmC and 5mC.

To calculate the recovery of the method, known amounts of 5hmC and 5mC (10 and 20 nM) were added to the urine samples, respectively. After addition of 40 nM $[\text{D}_3]5\text{mC}$ and 80 nM $[\text{D}_3]5\text{hmC}$, each sample was passed through a HLB cartridge (3.0 mL, 60 mg per cartridge) and analyzed by HPLC-MS/MS. The recovery (R) was determined according to the formula shown below.

$$R = \frac{C_{\text{measured}} - C_{\text{background}}}{C_{\text{added}}} \times 100\%$$

C_{measured} and $C_{\text{background}}$ represent the measured concentrations of 5hmC or 5mC in spiked and nonspiked urine samples, respectively; C_{added} is the added amounts of 5hmC and 5mC

standards. The intraday and interday precision was estimated by triplicate quantification of 5hmC and 5mC in human urine samples (nos. 11–13) per day for three consecutive days.

Collection and Preparation of Human Urine Samples.

The urine samples were collected from 13 healthy and young volunteers (age 23–30, five males and eight females). One female volunteer (no. 1) was pregnant in the 25th week at the time of urine collection. All volunteers were nonsmokers. All collected urine samples were stored at -80°C before they were subjected to any pretreatment and analysis. The samples were fully thawed and centrifuged at 12 000 rpm for 5.0 min. The collected urine samples were pretreated using HLB cartridges (3.0 mL, 60 mg per cartridge). Each cartridge was preconditioned with 3.0 mL of methanol followed by 3.0 mL of water; then urine samples of 200 μL , each mixed with $[\text{D}_3]5\text{mC}$ of 40 nM (final concentration) and $[\text{D}_3]5\text{hmC}$ 80 nM (final concentration), were loaded; the cartridges were washed with 1.0 mL of H_2O , followed by the final elution using 1.0 mL of methanol/water of 3:7 (v/v). The eluted fractions were evaporated with nitrogen gas, lyophilized, and redissolved in 200 μL of pure H_2O .

RESULT AND DISCUSSION

Ammonium Bicarbonate Enhances the MS Detection of 5mC, 5hmC, and 5fC. Our recent work demonstrates that ammonium bicarbonate can significantly improve the ESI-MS/MS detection of acrolein–deoxyguanine adducts by suppressing the formation of MS signal-deteriorating metal complexes.⁵¹ However, we do not know whether ammonium bicarbonate enhances the LC-ESI-MS/MS detection of 5mC and DNA demethylation intermediates. Here we examined the possibility to improve LC-ESI-MS/MS detection of 5mC and its oxidation products (5hmC and 5fC) using ammonium bicarbonate.

First, we tested four additives to the mobile phase, including ammonium bicarbonate, ammonium acetate, ammonium formate, and formic acid. As shown in the bottom trace of Figure 1, 5hmC migrates out at 1.7 min and is well separated from 5mC (2.6 min) and 5fC (5.9 min) by the use of 0.1% HCOOH as the additive to the mobile phase. Evidently, for all three tested ammonium salts (2.0 mM), the retentions of 5hmC (2.6–2.7 min), 5mC (4.4–5.1 min), and 5fC (7.6 min)

Figure 2. Typical ESI-MS spectra of 5hmC when four additives were used. An isocratic elution of 95% A (2.0 mM ammonium salt or 0.1% formic acid) and 5% B (pure methanol) was used, and the flow was set at 0.25 mL/min. A 10 μ M 5hmC standard solution (final concentration) was used, and the injection volume was 5.0 μ L.

Figure 3. HPLC-UV analysis of each SPE fraction obtained from the mixture of 5hmC, 5hmU, and 5fCs pretreated with C18 (A) and HLB cartridges (B). A 1.0 mL premixed solution (final concentration of each deoxynucleoside: 1.0 μ M) was loaded into the preconditioned cartridges. Each SPE fraction was lyophilized and redissolved in 50 μ L of H₂O. The concentrations of 5hmC, 5hmU, and 5fC standards were 20 μ M. The injection volume was 5.0 μ L.

increase. More importantly, compared with other three additives (HCOOH, HCOONH₄, and CH₃COONH₄), NH₄HCO₃ increased the MS/MS signals of 5mC, 5hmC, and 5fC by 1.8–14.3 times (right panel, Figure 1). However, the MS intensity of 5caC and 5hmU (data not shown) decreased when using NH₄HCO₃ compared with HCOOH. They probably prefer to deprotonate in basic mobile phase (pH $\geq$ 8).

Second, we compared the ionization complex distribution of 5hmC using four additives to the mobile phase. The HPLC fractions of 5hmC were directly scanned from m/z 100 to 300 under the positive ionization mode by an ESI-triple quadrupole mass spectrometer. HCOOH, HCOONH₄, or CH₃COONH₄

as the mobile-phase additive promotes 5hmC to form abundant [5hmC+Na]⁺ and [5hmC+K]⁺ complexes. Using normalizing intensity of [5hmC+H]⁺ as 1.0, the relative abundance of metal–5hmC complexes is about 19.1% to 202.9% (Figure 2). Interestingly, when using NH₄HCO₃ as the additive to the mobile phase, neither the [5hmC+Na]⁺ nor the [5hmC+K]⁺ complex forms (Figure 2). These data suggest that NH₄HCO₃ partially improves the ionization efficiency of 5hmC by suppressing the formation of metal–5hmC complexes during the ESI process.

Optimization of SPE Columns. There are more than 3000 chemicals found in human urine, and the 4 most abundant

Table 1. Precision of the HPLC-MS/MS Method

urine sample	interday mean $\pm$ SD, nM (RSD, %)		intraday mean $\pm$ SD, nM (RSD, %)	
	ShmC	SmC	ShmC	SmC
no. 11	20.6 $\pm$ 1.8 (8.9)	100.0 $\pm$ 2.9 (2.9)	19.9 $\pm$ 1.2 (5.8)	97.9 $\pm$ 1.4 (1.4)
no. 12	10.9 $\pm$ 0.7 (6.8)	4.8 $\pm$ 0.5 (10.6)	10.3 $\pm$ 0.3 (3.2)	4.3 $\pm$ 0.1 (2.7)
no. 13	28.2 $\pm$ 1.6 (5.6)	24.5 $\pm$ 1.0 (4.2)	27.1 $\pm$ 2.1 (7.7)	23.3 $\pm$ 0.8 (3.4)

ingredients are Na^+ , Cl^- , K^+ , and urea, the levels of which range from 4.6 to 22.5 mM/mM creatinine.⁵² To avoid possible ionization source contamination and ion suppression caused by coexisting ingredients in urine, two types of SPE cartridges (C18 and HLB) were tested for the cleanup and enrichment of the target deoxynucleosides. We found that the C18 cartridges were unable to effectively adsorb ShmC during cartridge washing by pure water, and approximately 95% of ShmC and ShmU were washed away (Figure 3A). In comparison, the HLB cartridges, filled with a water-wettable stationary phase mixed with the immobilized hydrophilic ligands and lipophilic ligands, show a much better performance in the separation and enrichment of all the targets from urine (Figure 3B). Less than 20% ShmC is lost during washing with water, and most of ShmC, ShmU, and Sfc are retained in the HLB cartridges until they are eluted with 10–30% methanol (Figure 3B). Of note, we could observe a matrix effect even when we used HLB cartridges to enrich ShmC from urinary samples. The MS signals of ShmC and SmC enriched from urinary samples were repressed over 17 times and 2.7 times (data not shown), respectively. However, the accurate quantification of urinary ShmC and SmC is obtained by adding stable isotopic standards of $[\text{D}_3]\text{SmC}$ and $[\text{D}_3]\text{ShmC}$ in known amounts to the urinary samples.

Calibration Curves, Recovery, and Precision. Calibration curves were obtained according to Experimental Section. Excellent linearity for ShmC (y (peak area ratio of ShmC to $[\text{D}_3]\text{ShmC}$) = $0.0152 \times (\text{concn nM}) - 0.0025$) and SmC (y (peak area ratio of SmC to $[\text{D}_3]\text{SmC}$) = $0.0347 \times (\text{concn nM}) + 0.1329$) was achieved in the concentration range from 2.0 to 64.0 nM with the correlation coefficient of $R^2 \geq 0.999$.

The recovery was measured by spiking human urine samples with known amounts of ShmC and SmC (10.0 and 20.0 nM, final concentration). The estimated recovery is about $101.3 \pm 4.1\%$ for 10 nM ShmC, $103.5 \pm 2.4\%$ for 20 nM ShmC, $70.2 \pm 0.9\%$ for 10 nM SmC, and $89.9 \pm 0.6\%$ for 20 nM SmC.

Three urine samples (nos. 11–13) were used for evaluating the precision of the HPLC-MS/MS method, and the results are summarized in Table 1. The interday precision values shown by relative standard deviations (RSD) vary from 2.9% to 10.6%, and the intraday precision values vary from 1.4% to 7.7%.

Given the presence of the matrix repression effect, the limits of detection (LODs, $\text{S/N} \geq 3$) are estimated to be 25 amol for SmC and 250 amol for ShmC, and the limits of quantification (LOQs, $\text{S/N} \geq 10$) are 75 amol for SmC and 760 amol for ShmC. The detection sensitivity of SmC and ShmC was measured using the latest series of Agilent triple quadrupole mass spectrometers (G6495).

Identification and Quantification of ShmC in Human Urine. By the developed off-line SPE-coupled stable isotope dilution HPLC-MS/MS method, we further examined ShmC and the other DNA intermediates in human urine. We collected morning urine samples from 13 healthy volunteers. SmC and ShmC could be detected in all the collected urine samples (Table 2). However, Sfc, ScaC, and ShmU could not be

Table 2. Quantification of ShmC and SmC in Human Urine Samples

sample	gender	ShmC (nM) (mean $\pm$ SD)	SmC (nM) (mean $\pm$ SD)	ShmC vs SmC
1	female	51.4 $\pm$ 3.2	112.4 $\pm$ 2.6	0.46
2	male	17.8 $\pm$ 0.4	19.0 $\pm$ 0.2	0.94
3	female	36.9 $\pm$ 1.7	41.0 $\pm$ 1.4	0.90
4	female	12.9 $\pm$ 1.6	41.4 $\pm$ 2.1	0.31
5	male	16.0 $\pm$ 1.9	18.8 $\pm$ 0.4	0.85
6	female	5.4 $\pm$ 0.1	1.2 $\pm$ 0.2	4.67
7	male	17.9 $\pm$ 0.7	55.0 $\pm$ 1.4	0.32
8	male	6.7 $\pm$ 0.3	4.6 $\pm$ 0.1	1.45
9	male	35.7 $\pm$ 1.8	161.5 $\pm$ 1.0	0.22
10	female	33.9 $\pm$ 2.0	97.0 $\pm$ 1.4	0.35
11	female	20.6 $\pm$ 1.8	100.0 $\pm$ 2.9	0.21
12	female	10.9 $\pm$ 0.7	4.8 $\pm$ 0.5	2.27
13	female	28.2 $\pm$ 1.6	24.5 $\pm$ 1.0	1.15
average		22.6 $\pm$ 13.7	52.4 $\pm$ 50.2	1.08

detected in all the urine samples. This is reasonable since those undetectable DNA intermediates display an abundance 1 or 2 orders of magnitude lower than that of ShmC.^{15,33,45}

As shown in Figure 4A, the peak of ShmC from human urine (no. 2) (2.7 min, blue line) completely overlaps that of the added internal stable isotope standard ($[\text{D}_3]\text{ShmC}$). Consistent with the nonisotope ShmC standard (Figure 4C), urinary ShmC also generates a transition with the second highest abundance (m/z 258 $\rightarrow$ 124) (Figure 4B). Moreover, the signal ratio of the primary ion transition (m/z 258 $\rightarrow$ 142) to the secondary transition (m/z 258 $\rightarrow$ 124) for urinary ShmC is almost the same as that of the nonisotope ShmC standard (~ 3.2 , evaluated by peak height). These results suggest that urinary ShmC has the same chromatographic retention and the same MS fragmentation pattern as the ShmC standard, consistently confirming the presence of ShmC in human urine.

The measured concentration of ShmC in human urine is about 5.4–51.4 nM, and the average concentration is about 22.6 ± 13.7 nM ($n = 13$). The average concentration of ShmC in male and female urine samples is about 18.8 ± 10.5 ($n = 5$) and 25.0 ± 15.5 nM ($n = 8$), respectively. There is no significant difference in ShmC levels between male and female samples (student t test, $p = 0.45$).

To evaluate the metabolism activity of ShmC in genomic DNA, SmC as an intrinsic reference is detected and quantified in all urine samples. The average concentration is about 52.4 ± 50.2 nM ($n = 13$). Considering the normal concentration of creatinine in adult human urine (>18 years old) is about 10 mM,⁵² the normalized value of SmC in our urine samples is about 5.2 ± 5.0 nmol/mmol creatinine, which is consistent with the previous work.⁴²

Previous work reported that the level of ShmC is much less than that of its precursor SmC in mammalian tissues.^{18,32–34} In mouse tissues, the highest levels of ShmC are found in genomic DNA from brain tissues, including cortex, brain stem, and

Figure 4. Identification of ShmC in no. 2 urine sample by HPLC-MS/MS. Chromatograms of urinary ShmC and $[D_3]$ ShmC (internal standard) are shown in A; the overlapped chromatograms for the two most intensive MRM transitions of 5hmC obtained from no. 2 urine samples and 5hmC standards are shown in B and C, respectively. The concentration of 5hmC standard used in C was 5 nM. The optimized method was used, and the details are described in Experimental Section.

cerebellum,^{32,33} and just account for 7.0–16.3% of 5mC. In other mouse tissues, e.g., kidney, lung, heart, pancreas, liver, spleen, thymus, muscle, bladder, and testes,^{33,34} the level of ShmC is relatively low and accounts for 0.7–3.7% of 5mC. The average ratios of ShmC vs 5mC in human tissues are also calculated: $2.9 \pm 0.8\%$ for lung ($n = 18$) and $21.1 \pm 2.9\%$ for brain ($n = 6$).¹⁸ Moreover, the ratios are much less in human lung ($1.4 \pm 0.4\%$, $n = 24$) and brain tumors ($5.5 \pm 4.3\%$, $n = 35$).¹⁸

The molar concentration ratio of ShmC to 5mC (from urinary samples) is about 0.21–4.67 (Table 2). The level of ShmC is even higher than that of 5mC in four samples (nos. 6, 8, 12, and 13), and the molar concentration ratio of ShmC to 5mC is about 1.15–4.67. On average, the measured molar concentration ratio of ShmC to 5mC is about 1.08. Compared with the ratio of tissue ShmC to 5mC (0.007–0.16), the molar concentration ratio of ShmC to 5mC in urine is astonishingly high (0.21–4.67).

It is not known how and why ShmC has a higher ratio in urine than in various tissues (in reference to 5mC). If urinary ShmC and 5mC result from genomic DNA metabolism, the observed higher ratio of ShmC to 5mC may indicate that ShmC has a more rapid turnover than 5mC in genomic DNA. Since ShmC is linked to passive and active DNA demethylation, our observation probably suggests that DNA demethylation is an indispensable and important physiological process involved in the daily life of mammals and humans.

CONCLUSIONS

We developed an off-line SPE-stable isotope dilution HPLC-MS/MS method for accurate quantification of 5mC and its iterative oxidation products (ShmC, Sfc, and ScaC) and ShmU in human urine. The use of NH_4HCO_3 significantly enhanced the ESI-MS/MS detection of 5mC, ShmC and Sfc by 1.8–14.3 times. Even though all four deoxynucleosides (ShmC, Sfc, ScaC, and ShmU) are intermediates in DNA demethylation, only ShmC could be detected in human urine. Unexpectedly, the molar ratio of ShmC vs 5mC in urine is found to be much higher than that in genomic DNA of mammalian tissues, indicating more rapid turnover of ShmC than 5mC in mammals.

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Notes

The authors declare no competing financial interest.

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