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 (NH₄HCO₃) 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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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 ShmC in human urine. By the developed approach, we for the first time demonstrate the presence and the abundance of ShmC in human urine.

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

EXPERIMENTAL SECTION

Chemicals. ShmC, 5fC, and SmC were obtained from Berry & Associates (Dexter, MI). ScaC and ShmU were gifts from Dr. Guoliang Xu at the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. 5-Hydroxymethylcytosine-d5-2-deoxyctydine ([D5]5mC) and 5-methyl-d5-2-deoxyctydine ([D5]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 x 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, ShmC, 5fC, ScaC, and ShmU 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 5shmC (5 eV); m/z 261 → 145 for [D3]5mC (5 eV); m/z 256 → 140 for 5fC (5 eV); m/z 272 → 156 for ScaC (5 eV); m/z 259 → 143 for ShmU (5 eV). These 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 ShmC (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/
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. ShmU, 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. The mobile phase consisted of solvents A and B.

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 ShmC and 5mC standards of varying concentrations (2.0, 4.0, 8.0, 16.0, 32.0, and 64.0 nM) mixed with 80 nM [D3]5mC and 40 nM [D3]5C (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 ShmC and 5mC to the corresponding stable isotopic standards against the concentration of the added nonisotopic ShmC and 5mC.

To calculate the recovery of the method, known amounts of ShmC and 5mC (10 and 20 nM) were added to the urine samples, respectively. After addition of 40 nM [D3]5mC and 80 nM [D3]5C, each sample was passed through a HLB cartridge (3.0 mL, 60 μg per cartridge) and analyzed by HPLC-MS/MS. The recovery (R) was determined according to the formula shown below.

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

C_{\text{measured}} and C_{\text{background}} represent the measured concentrations of ShmC or 5mC in spiked and nonspiked urine samples, respectively; C_{\text{added}} is the added amounts of ShmC and 5mC standards. The intraday and interday precision was estimated by triplicate quantification of ShmC 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 [D3]5mC of 40 nM (final concentration) and [D3]5C of 80 nM (final concentration), were loaded; the cartridges were washed with 1.0 mL of methanol/water of 1:9 (v/v) 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 50 μL of pure H2O.

**RESULT AND DISCUSSION**

Ammonium Bicarbonate Enhances the MS Detection of 5mC, ShmC, 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. 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 (ShmC 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, ShmC 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 5mC (2.6–2.7 min), ShmC (4.4–5.1 min), and 5fC (7.6 min) were significantly improved.

![Figure 1](image-url)
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 ≥ 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 | ShmC (nM) (mean ± SD) | SmC (nM) (mean ± SD) | ShmC vs SmC (SD) |
|--------------|-----------------------|----------------------|------------------|
| no. 11       | 20.6 ± 1.8 (8.9)       | 100.0 ± 2.9 (2.9)    |                  |
| no. 12       | 10.9 ± 0.7 (6.8)       | 4.8 ± 0.5 (10.6)     |                  |
| no. 13       | 28.2 ± 1.6 (5.6)       | 24.5 ± 1.0 (4.2)     |                  |

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

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

ingredients are Na⁺, Cl⁻, K⁺, and urea, the levels of which range from 4.6 to 22.5 mM/MM creatinine. 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 5hmC 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 5fC 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 [D3]ShmC and [D3]SmC 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 [D3]ShmC) = 0.0152 × (concn nM) − 0.0025) and SmC (y (peak area ratio of SmC to [D3]SmC) = 0.0347 × (concn nM) + 0.1329) was achieved in the concentration range from 2.0 to 64.0 nM with the correlation coefficient of R² ≥ 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 ± 4.1% for 10 nM ShmC, 103.5 ± 2.4% for 20 nM ShmC, 70.2 ± 0.9% for 10 nM SmC, and 89.9 ± 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, S/N ≥ 3) are estimated to be 25 amol for ShmC and 250 amol for ShmC, and the limits of quantification (LOQs, S/N ≥ 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, 5fC, 5caC, and ShmU could not be 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.

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 ([D3]ShmC). Consistent with the nonisotope ShmC standard (Figure 4C), urinary ShmC also generates a transition with the second highest abundance (m/z 258 → 124) (Figure 4B). Moreover, the signal ratio of the primary ion transition (m/z 258 → 142) to the secondary transition (m/z 258 → 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, the normalized value of ShmC is about 5.2 ± 5.0 nmol/mmol creatinine, which is consistent with the previous work.

Previous work reported that the level of ShmC is much less than that of its precursor SmC in mammalian tissues. In mouse tissues, the highest levels of ShmC are found in genomic DNA from brain tissues, including cortex, brain stem, and...
and just account for 7.0–16.3% of SmC. 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 SmC. The average ratios of ShmC vs SmC in human tissues are also calculated: 2.9 ± 0.8% for lung (n = 18) and 21.1 ± 2.9% for brain (n = 6).18 Moreover, the ratios are much less in human lung (1.4 ± 0.4%, n = 24) and brain tumors (5.5 ± 4.3%, n = 35).18

The molar concentration ratio of ShmC to SmC (from urinary samples) is about 0.21–4.67 (Table 2). The level of ShmC is even higher than that of SmC in four samples (nos. 6, 8, 12, and 13), and the molar concentration ratio of ShmC to SmC is about 1.15–4.67. On average, the measured molar concentration ratio of ShmC to SmC is about 1.08. Compared with the ratio of tissue ShmC to SmC (0.007–0.16), the molar concentration ratio of ShmC to SmC 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 SmC). If urinary ShmC and SmC result from genomic DNA metabolism, the observed higher ratio of ShmC to SmC may indicate that ShmC has a more rapid turnover than SmC 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 SmC and its iterative oxidation products (ShmC, 5fC, and 5caC) and ShmU in human urine. The use of NH₄HCO₃ significantly enhanced the ESI-MSMS detection of SmC, ShmC and 5fC by 1.8–14.3 times. Even though all four deoxynucleosides (ShmC, 5fC, 5caC, and ShmU) are intermediates in DNA demethylation, only ShmC could be detected in human urine. Unexpectedly, the molar ratio of ShmC vs SmC in urine is found to be much higher than that in genomic DNA of mammalian tissues, indicating a more rapid turnover of ShmC than SmC in mammals.

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### Notes

The authors declare no competing financial interest.
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