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A novel malic acid-enhanced method for the analysis of 5-methyl-2'-deoxycytidine, 5-hydroxymethyl-2'-deoxycytidine, 5-methylcytidine and 5-hydroxymethylcytidine in human urine using hydrophilic interaction liquid chromatography-tandem mass spectrometry

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Abstract

5-Methyl-2'-deoxycytidine (5-mdC), 5-hydroxymethyl-2'-deoxycytidine (5-hmdC), 5-methylcytidine (5-mrC) and 5-hydroxymethylcytidine (5-hmrC) are epigenetic marks of DNA and RNA, and aberrant levels of these modified nucleosides were found to be associated with various cancers. Urine is a preferred source of biological fluid for biomarker discovery because the sample collection process is not invasive to patients. Herein, we developed a novel malic acid-enhanced hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) method for sensitive and simultaneous quantification of the modified cytosine nucleosides in human urine. Malic acid markedly increased the detection sensitivities of all four cytosine nucleosides, with the limits of detection (LODs) for 5-mdC, 5-hmdC, 5-mrC and 5-hmrC being 0.025, 0.025, 0.025 and 0.050 fmol, respectively. By using this method, we demonstrated, for the first time, the presence of 5-hmrC in human urine, and we successfully quantified 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine samples collected from 90 patients with colorectal cancer (CRC) and 90 healthy controls. We found that the levels of 5-mdC, 5-hmdC, 5-mrC and 5-Malic acid Colorectal cancer hmrC in urine were all substantially decreased in CRC patients, suggesting that these modified nucleosides might have great potential to be noninvasive biomarkers for early detection and prognosis of CRC. Together, we established a novel and sensitive method for

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Appendix A. Supplementary data

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detecting 5-methylated and 5-hydroxymethylated cytosine nucleosides in human urine and the results from this study may stimulate future investigations about the regulatory roles of these cytosine derivatives in the initiation and development of CRC.

GRAPHICAL ABSTRACT

Keywords

DNA and RNA modification; 5-Methylated and 5-hydroxymethylated; cytosine nucleosides; Hydrophilic interaction liquid; chromatography-tandem mass; spectrometry

1. Introduction

DNA methylation (5-methyl-2'-deoxycytidine, 5-mdC) plays vital roles in numerous biological processes, including embryo-genesis, regulation of gene expression, genomic imprinting, and X-chromosome inactivation [1]. Maintenance of proper DNA methylation status is critical for the normal functions of cells [2]. Numerous diseases including cancer may be attributed to aberrant DNA methylation [3,4]. Previous studies revealed that 5-mdC could be oxidized to 5-hydroxymethyl-2'-deoxycytidine (5-hmdC) by ten-eleven translocation (Tet) proteins in mammals [5,6]. 5-hmdC is also involved in various biological processes, including cellular differentiation and epigenetic regulation [7,8]. In addition, the level of 5-hmdC was found to be significantly decreased in several types of cancers, suggesting that 5-hmdC might be involved with tumor formation and development [9–13].

RNA modification has attracted increasing attention and RNA epigenetics/epitranscriptomics has been proposed [14–16]. More than 140 distinct types of modified nucleosides are known to exist in RNA and these modifications modulate the functions of RNA [17]. Among these RNA modifications, 5-methylcytidine (5-mrC) is commonly present in all RNA species and influences the structure and functions of RNA [18]. Recently, it has been found that Tet enzymes can also oxidize 5-mrC to 5-hydroxymethylcytidine (5-hmrC) in RNA [19]. 5-hmrC mainly exists in mRNA and is hardly detected in other types of RNA [20]. Additionally, the content of 5-hmrC was dramatically decreased in colorectal cancer and hepatocellular carcinoma, suggesting that 5-hmrC may play vital roles in cancer development [20].

In the past decade, continuing efforts have been devoted toward characterizing 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in cultured human cells and human tissues [21–24], and these

studies suggested that these modified cytosine nucleosides have great potential to be biomarkers for tumor diagnosis and prognosis. However, these analyses require tedious processes of nucleic acid extraction and digestion. Moreover, the sample collection process is invasive, rendering it difficult for large-scale clinical investigations. Because it is noninvasive to obtain and readily available, urine is considered a favorable diagnostic biofluid in clinical practice [25,26]. 2'-Deoxynucleosides and ribonucleosides derived from degradation or metabolism of DNA and RNA could be excreted into urine. In this vein, 5-mdC, 5-hmdC and 5-mrC were previously identified in human urine [27–29]. However, there has been no report about the detection of 5-hmrC in human urine. Exploration of the potential of urinary 5-mdC, 5-hmdC, 5-mrC and 5-hmrC as noninvasive biomarkers in clinical practice calls for sensitive methods for the simultaneous quantification of these epigenetic modifications in urine.

Great progress has been achieved toward the development of analytical methods for the determination of these modified cyto-sine derivatives [30]. Owing to its great advantages in sensitivity and accuracy, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the most frequently used approach for such analysis [31,32]. However, quantification of these modified cytosine nucleosides in urine samples is challenging due to their low levels of presence and relatively poor ionization efficiency. Conjugation with an easily ionizable moiety through chemical derivatization has been employed to improve the sensitivity for the detection of modified nucleosides [33] and various chemical derivatization methods were also developed for the detection of 5-mdC, 5-mrC and their oxidation products [12,20,24,34]. Moreover, hydrophilic interaction liquid chromatography (HILIC), which employs high contents of organic solvent as the mobile phase, was found to improve the ionization efficiency of analytes [11,35]. Furthermore, selective enrichment is another strategy to enhance sensitivity [36,37]. Recently, we successfully enriched and quantified other two intermediates involved in DNA demethylation, 5-formyl-2'-deoxycytidine (5-fodC) and 5-carboxyl-2'-deoxycytidine (5-cadC) in human urine by magnetic hyper-cross-linked microporous polymer material [38]. Moreover, mobile phase additive can also enhance the signal response of analytes in MS [28,39], and a recent study demonstrated that NH_4HCO_3 can significantly improve the determination of 5-mdC, 5-hmdC and 5-fodC [28]. This strategy exhibits great potential in detection of these modified cytosine nucleosides since it obviates complicated chemical derivatization and specific enrichment materials.

Herein, we developed a malic acid-enhanced ionization method for the sensitive and simultaneous quantifications of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in human urine by HILIC-MS/MS. We first assessed the effects of different additives on the MS detection of these modified cytosine nucleosides. Our results demonstrated that malic acid could enhance, by 20–40 fold, the detection sensitivities of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC. When malic acid was used, the limits of detection of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC could reach 0.025, 0.025, 0.025 and 0.050 fmol, respectively. Moreover, a solid-phase extraction (SPE) method was established for pretreatment of urine samples. Using the established analytical method, we revealed, for the first time, the presence of 5-hmrC in human urine and quantified 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine from patients with colorectal cancer (CRC) and healthy controls. We demonstrated that the contents of urinary 5-mdC, 5-hmdC, 5-mrC and 5-hmrC were substantially lower in CRC patients than healthy

controls, suggesting that these modified cytosine nucleosides may have great potential to be noninvasive biomarkers for early detection and prognosis of CRC.

2. Material and methods

2.1. Chemicals

Water was purified using a Milli-Qpurification apparatus (Millipore, Milford, MA, USA). HPLC-grade acetonitrile (CH_3CN) was purchased from Merck KGaA (Darmstadt, Germany). Formic acid (HCOOH), ammonium formate (HCOONH_4), malic acid, oxaloacetic acid, succinic acid, citric acid, fumaric acid, α -ketoglutaric acid, phthalic acid, oxalic acid, propanedioic acid, and 5-methylcytidine (5-mrC) were purchased from Sigma-Aldrich (St Louis, MO, USA). 5-Methyl-2'-deoxycytidine (5-mdC), 5-hydroxymethyl-2'-deoxycytidine (5-hmdC), 5-hydroxymethylcytidine (5-hmrC), 5-methyl-D₃-2'-deoxycytidine ($[\text{D}_3]5\text{-mdC}$), 5-hydroxymethyl-D₃-2'-deoxycytidine ($[\text{D}_3]5\text{-hmdC}$) and 5-hydroxymethyl- $^{13}\text{CD}_2$ -cytidine ($[\text{D}_3]5\text{-hmrC}$) were purchased from Toronto Research Chemical (Toronto, Canada). 5-Methyl- $^{13}\text{C}_5$ -cytidine ($[\text{D}_3]5\text{-mrC}$) was synthesized previously [22]. Stock solutions of standards and isotope-labeled internal standards (IS) were prepared at a concentration of 1 mM in water and stored at -80°C until use.

2.2. Sample collection

Patients with colorectal cancer (CRC) were pathologically confirmed and had not been treated with surgery, chemotherapy or radiotherapy. Healthy volunteers were free of any types of cancer. All of these individuals were recruited from The Second Affiliated Hospital, Zhejiang University School of Medicine (SAHZU). 90 healthy volunteers (44 males and 46 females with a mean age of 50.0 ± 9.3 years, range 29–73 years, 16 smokers and 74 non-smokers) and 90 CRC patients (45 males and 45 females with a mean age of 58.0 ± 10.2 years, range 26–79 years, 21 smokers and 69 non-smokers) were included (Table S1). Individuals were required to provide mid-stream early-morning urine samples. After collection, these specimens were stored at -80°C . Urinary creatinine was also assayed (Department of Laboratory Medicine, SAHZU) to provide a correction factor for urine concentration [40].

2.3. Solid-phase extraction

Two types of cartridges (Oasis HLB and Oasis MCX) were evaluated and compared for extraction efficiency. The HLB and MCX cartridges were obtained from Waters Corporation (Milford, MA, USA). The HLB cartridge (1.0 mL, 30 mg) was preconditioned with 1.0 mL of CH_3CN followed by 1.0 mL of H_2O , and a mixture of a 0.2 mL solution of nucleoside standards (100 nM each) and 0.4 mL of H_2O was loaded. The cartridge was washed with 1.0 mL of H_2O and eluted with 1.0 mL of CH_3CN . The MCX cartridge (1.0 mL, 30 mg) was preconditioned with 1.0 mL of CH_3CN followed by 1.0 mL of H_2O , and followed by loading with a mixture of a 0.2 mL solution of nucleoside standards (100 nM each) and 0.4 mL of 2% HCOOH in H_2O . The cartridge was then washed sequentially with 1.0 mL of H_2O and 1.0 mL of CH_3CN , and the nucleosides were eluted from the cartridge with 1.8 mL of $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{NH}_4\text{OH}$ (90:10:5, v/v/v). The collected SPE fractions were dried under

vacuum and the solid residue was redissolved in 0.2 mL of CH₃CN/H₂O (9:1, v/v) for HILIC-MS/MS analysis to assess the performance of the two types of SPE cartridges.

The urine samples were centrifuged at 16242 g for 15 min at 4 °C after thawing at room temperature on the day of extraction. The resulting supernatant (0.2 mL) was diluted with 0.4 mL of 2% HCOOH in H₂O, and then spiked with isotope-labeled internal standard [D₃]5-mdC (4 pmol), [D₃]5-hmdC (2 pmol), [¹³C₅]5-mrC (2 pmol) and [¹³CD₂]5-hmrC (2 pmol). The samples were pretreated using MCX cartridge. Each cartridge was preconditioned as mentioned above and urine was subsequently loaded. The cartridges were then washed and the analytes eluted following the aforementioned procedures. The eluted fractions were evaporated and reconstituted in 0.4 mL of CH₃CN/H₂O (9:1, v/v) for HILIC-MS/MS detection.

2.4. HILIC-MS/MS analysis

HILIC-MS/MS analysis was carried out on an Acquity UPLC system (Waters, Milford, MA, USA) coupled with a 4000 QTRAP mass spectrometer (AB SCIEX, Foster City, CA, USA). Chromatographic separation of these nucleosides was conducted on a Waters BEH HILIC column (2.1 mm × 100 mm, 1.7 μm) at 40 °C. The mobile phase was (A) acetonitrile containing 0.2% formic acid, 2 mM ammonium formate and 0.06 mM malic acid, and (B) H₂O containing 0.2% formic acid and 10 mM ammonium formate. Mobile phase A without addition of malic acid was used for comparison. An isocratic mode of 94% A and 6% B was utilized, and the flow rate was 0.4 mL/min. To minimize contamination of the mass spectrometer, a six-way injection valve was used and the eluent in the retention time range of 2.5–4.2 min was subjected to MS/MS analysis. The temperature of sample chamber was 4 °C. Each sample was measured three times with 5 μL of injection volume. Other additives including oxaloacetic acid, succinic acid, citric acid, fumaric acid, α-ketoglutaric acid, phthalic acid, oxalic acid and propanedioic acid were evaluated for detection sensitivity.

The mass spectrometer equipped with an electrospray ionization source (TurboSpray) was operated in the positive-ion mode. Firstly, collision-induced dissociation (CID) experiments [41] of the [M+H]⁺ ions of these nucleosides and the corresponding internal standards were carried out. Upon collisional activation, protonated 5-mdC, 5-hmdC, 5-mrC and 5-hmrC preferentially eliminate a deoxyribose or ribose moiety. Thus, the quantification of these modified nucleosides were carried out in multiple-reaction monitoring (MRM) mode by monitoring the transitions of *m/z* 242.1→126.0 (5-mdC), *m/z* 258.1→142.0 (5-hmdC), *m/z* 258.1→126.0 (5-mrC) and *m/z* 274.1→142.0 (5-hmrC). Moreover, the fragment ions of the second highest abundance were employed as qualitative ions: 242.1→117.0 (5-mdC), *m/z* 258.1→124.0 (5-hmdC), *m/z* 258.1→133.0 (5-mrC) and *m/z* 274.1→124.0 (5-hmrC). For isotope-labeled internal standard, the transitions of *m/z* 245.1→129.0 ([D₃]5-mdC), *m/z* 261.1→145.0 ([D₃]5-hmdC), *m/z* 263.1→126.0 ([¹³C₅]5-mrC) and *m/z* 277.1→145.0 ([¹³CD₂]5-hmrC) were monitored for quantification. The MRM parameters for the analyses of these nucleosides were optimized and listed in Table S2. The mass spectrometer parameters were also optimized. The ion source temperature, the spray voltage, and the curtain gas were set at 550 °C, 5.5 kV, and 40 psi, respectively. Ion source gases 1 and 2 were both set at 50 psi.

2.5. Method validation

The standard solutions of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC at different concentrations (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, 100.0 nM), which were mixed with internal standards [D_3]5-mdC (10 nM), [D_3]5-hmdC (5 nM), [$^{13}C_5$]5-mrC (5 nM) and [$^{13}CD_2$]5-hmrC (5 nM), were prepared and analyzed. Calibration curves were established with nine appropriate concentrations and described as $y = ax + b$, where x denotes the concentration of analyte and y designates the peak area ratio of the analyte to the corresponding internal standard. The limit of detection (LOD) and limit of quantification (LOQ) were measured by analyzing standard solutions at concentrations that gave signal-to-noise ratios of 3.0 and 10.0, respectively.

Mixtures of standard solutions at three different concentrations of 5-mdC (5, 30, 90 nM), 5-hmdC (2, 10, 60 nM), 5-mrC (4, 20, 80 nM) and 5-hmrC (2, 10, 60 nM) were prepared in triplicate as the quality control samples (QC) to evaluate the precision and accuracy. The intra-day results were obtained by measuring each sample for three times within one day and thus each concentration level was analyzed nine times. The inter-day results were obtained by measuring the samples in three consecutive days. The accuracy was determined by comparing the measured value to the theoretical concentration.

For evaluating the recovery of solid-phase extraction, known amounts of standards at three different concentrations of 5-mdC (20, 60, 120 nM), 5-hmdC (10, 30, 90 nM), 5-mrC (15, 50, 100 nM) and 5-hmrC (10, 30, 90 nM) were spiked to the urine, and then internal standards were added. Urine samples were pretreated with a MCX cartridge and measured as mentioned above. The recovery (R) was calculated by using the formula of $R = (\text{concentration in spiked sample} - \text{concentration in unspiked sample}) / \text{spiked concentration} \times 100\%$.

Because these four nucleosides are endogenous metabolites and blank urine samples are difficult to obtain, the matrix effect was evaluated by using a slope comparison method. Eleven aliquots of urine extracts were spiked with standard solutions at different concentrations (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, 100.0 nM) and internal standards, and the standard addition calibration curve was subsequently established. Its slope ratio to the standard solutions calibration curve was calculated as the matrix effect.

2.6. Statistical analysis

SPSS 20.0 software (IBM, Armonk, NY, USA) was utilized for statistical analyses. The differences in nucleoside concentrations between CRC patients and healthy volunteers were evaluated by employing two-tailed Student's t -test, where $p < 0.05$ was considered to have statistical significance.

3. Results and discussion

3.1. Malic acid enhances the MS detection of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC

Analysis of the four modified cytosine nucleosides (5-mdC, 5-hmdC, 5-mrC and 5-hmrC, Fig. S1) may allow for the evaluation of their potential application as biomarkers for tumor

detection. Accurate quantification of 5-hmrC in urine samples, however, has not been achieved perhaps due to its poor ionization efficiency and/ or its trace level of presence in urine.

The intriguing roles of mobile phase additive in MS detection have attracted great attention [42–44]. Tricarboxylic acid cycle, an important and evolutionarily conserved metabolic pathway, involves several carboxylic acids including malic acid, oxaloacetic acid, citric acid, succinic acid, α -ketoglutaric acid and fumaric acid. However, whether these carboxylic acids can enhance the HILICMS/MS detection of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC is unknown. Herein, we selected these six carboxylic acids, together with oxalic acid, propanedioic acid and phthalic acid, as additives to explore whether they could improve the sensitivity for the detection of these modified cytosine nucleosides.

Nine types of mobile phase A containing carboxylic acid additives were prepared and tested for HILIC-MS/MS analyses of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC. The peak area ratios for the individual nucleosides were calculated between mobile phases with and without carboxylic acid additives (Table S3). As shown in Fig. 1A, most carboxylic acids enhanced the detection of the four modified cytosine nucleosides, where the use of malic acid as additive elevated the MRM signals of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC by 14.9, 25.7, 18.3 and 9.4 fold, respectively. In addition, the signal-to-noise ratios for the detection of these modified nucleosides were increased when malic acid was utilized. Owing to the excellent separation efficiency of UPLC system, these four compounds could be well resolved by HILIC column in less than 5 min. The retention time of these nucleosides was not changed when malic acid was utilized, indicating that malic acid could be employed in the LC method using the formic acid/ammonium formate buffer system. Therefore, malic acid was chosen as the mobile phase additive for subsequent analysis.

We next optimized the concentration of malic acid used for the HILIC-MS/MS analysis. Mobile phase A containing malic acid at several different concentrations including 0.02, 0.04, 0.06, 0.08 and 0.10 mM were prepared and examined. The results showed that the signal response increases as the malic acid concentration rises from 0.02 to 0.06 mM (Fig. 1B). When malic acid concentration was 0.08 mM, the signal responses of 5-hmdC and 5-hmrC were slightly improved, whereas there is no signal enhancement for 5-mdC or 5-mrC. In contrast, the signal responses for all four compounds decrease at a higher malic acid concentration (i.e. 0.10 mM). In addition, high concentrations of malic acid may induce blockage of the HILIC column and contamination of the ionization source of the mass spectrometer. Hence, the optimized concentration of malic acid additive for HILIC-MS/MS analysis of these modified cytosine nucleosides was 0.06 mM.

The main purpose of using mobile phase additive is to improve the sensitivity of the HILIC-MS/MS method for the analyses of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC. The MRM chromatograms of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC with or without malic acid in mobile phase A are illustrated in Fig. 2A D. Obviously, the addition of malic acid significantly enhances the signal intensity. Compared with the results obtained without malic acid, the detection sensitivities were increased by approximately 20-fold for 5-mdC and 5-hmrC, and about 40-fold for 5-hmdC and 5-mrC (Table 1). In the presence of malic acid, the limits of

detection (LODs) of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC reached 0.025, 0.025, 0.025 and 0.050 fmol, respectively. These LODs values were better than what were previously reported [12,20,28], demonstrating the excellent sensitivity of the developed method.

To elucidate the enhancement mechanism of malic acid, we compared the ionization complex distributions of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC between mobile phases with and without malic acid. A solution of nucleoside standards (at 10 μ M each) was prepared and the UPLC fraction was analyzed by ESI-MS in full-scan mode. In the absence of malic acid, the $[M + Na]^+$ ion and, to a lesser degree, the $[M + K]^+$ ion were produced in high abundance, whereas the formation of the $[M+H]^+$ ion was less favorable (Fig. S2A). With the addition of malic acid, the generation of the $[M + H]^+$ ion was greatly favored, which was accompanied with the marked suppression of the $[M + Na]^+$ and $[M + K]^+$ ions (Fig. S2B). The peak area ratios $[M + H]^+ / ([M + Na]^+ [M + K]^+)$ for the four individual nucleosides were calculated and displayed in Table S4, all of the ratios tend to increase when malic acid was used as additive in the eluent. Furthermore, the transition of the $[M+Na]^+$ ion was also monitored. Taking 5-mdC as an example, the intensity of the transition of m/z 264.1 $\rightarrow$ 148.0 decreased when malic acid was utilized (Fig. S3). These findings indicate that malic acid enhances the ionization efficiency of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC by impeding the formation of nucleoside alkali ion adducts during the ionization process.

3.2. Optimization of SPE procedures

Urine samples contain numerous organic compounds and inorganic salts, which may result in ionization source contamination, ion suppression and interference of MS detection. Hence, we employed SPE for sample cleanup prior to HILIC-MS/MS detection, where we assessed two types of SPE cartridges (HLB and MCX). We found that 5-hmdC and 5-hmrC could not be effectively retained on the HLB cartridge during the sample loading process. Additionally, all four cytosine nucleosides could be washed off the cartridge with H_2O . Consequently, only part of 5-mdC and 5-mrC were retained on the cartridge (Fig. 3A). Considering the existence of cytosine base and the alkaline property of these nucleosides, we next assessed the performance of the MCX cartridge, which was filled with mixed-mode polymeric sorbents and exhibited higher selectivity for extracting basic compounds with cation-exchange groups. It turned out that none of the four analytes displayed substantial loss during the sample loading, or washing with H_2O or CH_3CN (Fig. 3B). These nucleosides were retained in the MCX cartridge until they were eluted with $CH_3CN/H_2O/NH_4OH$ (90:10:5, v/v/v). In addition, the use of internal standards allowed for the correction of the loss of analytes during sample preparation [45], which facilitated accurate quantification of these cytosine modifications in urine.

3.3. Method validation

Calibration curves were obtained according to the aforementioned procedures. Excellent linearities were achieved in the range of 0.1–100.0 nM, and the equations are shown in Table S5 with correlation coefficients (R^2) being greater than 0.999. The slope ratio values were between 95.4% and 126.3% (Table S5), indicating the lack of obvious matrix effect. Our results showed that subfemtomole levels of LOQs could be attained for these modified nucleosides (Table S6).

By comparing the measured concentrations of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in QC samples with their actual concentrations, the accuracy of the developed method was assessed. The accuracy of the method, as represented by percent recoveries from intra- and inter-day assays were in the range of 98.2%–111.5% and 98.2–111.7%, respectively (Table S7).

As shown in Table S7, the intra-day precision values ranged from 0.5% to 2.6%, and the inter-day precision values varied from 0.2% to 1.4%, demonstrating that the method offered excellent reproducibility. The recoveries of the developed method ranged from 91.9% to 115.8%, with RSD values being less than 10.0% at three different addition levels (Table S8).

To monitor the stability of the equipment after hundreds of injections, a QC sample was analyzed in every twenty urine samples. Parameters such as retention time and accuracy were found to be stable during the course of analysis of a large batch of samples. These results revealed that the developed malic acid-enhanced HILIC-MS/MS method was sensitive, reproducible, accurate, and robust for the analyses of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in human urine samples.

3.4. Simultaneous quantification of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine from CRC patients

By employing the developed malic acid-enhanced HILIC-MS/MS method, we determined the contents of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine collected from 90 CRC patients and 90 healthy volunteers. These four nucleosides could be detected in all urine samples (Fig. 4A–D, Table S1).

The presence of 5-hmrC in urine was further confirmed. As illustrated in Fig. 5A, the retention time of 5-hmrC in urine is consistent with that of the spiked isotope-labeled internal standard ($[^{13}\text{CD}_2]$ 5-hmrC). Additionally, urinary 5-hmrC produces a transition (m/z 274.1 $\rightarrow$ 124.0) with the second highest abundance (Fig. 5B), which is in accordance with the 5-hmrC standard (Fig. 5C). The signal ratio between the quantitative and qualitative ion transitions was also assessed, and the value for urinary 5-hmrC is almost identical to that of 5-hmrC standard (~ 2.4 , evaluated by peak height). Hence, urinary 5-hmrC exhibits the same chromatographic retention and the identical dissociation behavior as the 5-hmrC standard, thereby supporting the identification of this modified nucleoside in human urine. To the best of our knowledge, this is the first time that 5-hmrC was detected in human urine.

The concentrations of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine were normalized against the urinary creatinine concentration. The detailed concentrations of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC are listed in Table S1. The measured average concentrations of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine from healthy controls were 4.45 ± 2.75 , 1.04 ± 0.41 , 4.42 ± 2.66 and 2.09 ± 0.92 nmol/ mmol creatinine, respectively ($n = 90$), whereas the measured average concentrations of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine from CRC patients were consistently lower, at 3.41 ± 2.29 , 0.85 ± 0.40 , 3.41 ± 2.64 and 1.65 ± 0.76 nmol/mmol creatinine, respectively ($n = 90$). In previous studies, the measured concentrations of 5-mdC were 3.30 ± 3.80 nmol/mmol creatinine [27], 5.20 ± 5.00 nmol/mmol creatinine [28] and 4.97 ± 26.96 nmol/ mmol creatinine [46], and the measured concentrations of 5-hmdC were

2.26 ± 1.37 nmol/mmol creatinine [28] and 1.23 ± 4.62 nmol/ mmol creatinine [46]. Our results were in reasonably good agreement with the previously reported results. The results demonstrated that the levels of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine were significantly decreased in CRC patients compared to healthy controls ($p < 0.01$ for 5-mdC, $p < 0.01$ for 5-hmdC, $p < 0.05$ for 5-mrC, $p < 0.001$ for 5-hmrC, Fig. 6A–D).

The effects of smoking status on the levels of these modified cytosine nucleosides were also evaluated. The results indicate that smoking status did not significantly affect the levels of the four modified nucleosides except for 5-hmrC, whose level was lower in smokers than non-smokers ($p < 0.05$) of CRC patients (Fig. S4). Additionally, the concentration of 5-hmrC in males was lower than that in females ($p < 0.05$), whereas no significant differences in the levels of the other three modified nucleosides were found between males and females (Fig. S5).

As the oxidized product of 5-mdC and 5-mrC, 5-hmdC and 5-hmrC were found to be of much lower levels than its precursor in mammalian tissues. Taking the CRC tumor tissue as an example, the mean levels of 5-mdC and 5-mrC were $3.5 \pm 0.6/10^2$ dG and $5.1 \pm 0.7/10^3$ G, respectively; however, the mean frequencies of 5-hmdC and 5-hmrC were $27.0 \pm 6.4/10^5$ dG and $17.9 \pm 2.9/10^6$ G, respectively [12,20]. In the present study, the concentrations of 5-hmdC and 5-hmrC in urine samples were found to be only 2–4 fold lower than those of 5-mdC and 5-mrC, respectively. This indicates that the concentration ratios of 5-hmdC to 5-mdC and 5-hmrC to 5-mrC were much higher in urine samples than those in tissues. Provided that urinary 5-mdC, 5-hmdC, 5-mrC and 5-hmrC are derived from DNA and RNA metabolism, the higher ratios of 5-hmdC to 5-mdC and 5-hmrC to 5-mrC in urine suggest that 5-hmdC and 5-hmrC have more rapid turnover than 5-mdC and 5-mrC in DNA and RNA, respectively. It is also possible that 5-hmdC and 5-hmrC arise from oxidation of the corresponding methylated nucleosides after their removal from DNA and RNA, respectively. In this vein, malondialdehyde-induced DNA adduct, M₁dG, is known to undergo oxidative metabolism to yield 6-oxo-M₁dG [47].

The incidence of CRC is rather high worldwide and early detection is vitally necessary. Previous work revealed that the amounts of 5-hmdC and 5-hmrC are both dramatically decreased in CRC tumor tissues. No such difference, however, was observed for 5-mdC and 5-mrC [12,20]. To the best of our knowledge, this is the first report about the significantly lower levels of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine from CRC patients than healthy controls. The results suggested that the decrease of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine might be a general characteristic of CRC, and these modified nucleosides may have great potential to be noninvasive biomarkers for early detection and prognosis of CRC.

4. Conclusions

In this work, we developed a novel malic acid-enhanced HILICMS/MS method for sensitive quantifications of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in human urine. The detection sensitivities of these modified cytosine nucleosides were increased by 20e40 fold with the assistance of malic acid. Using the established method, we were able to reveal, for the first time, the presence of 5-hmrC in human urine, and successfully quantify 5-mdC, 5-hmdC, 5-

mrC and 5-hmrC in urine from 90 CRC patients and 90 healthy volunteers. The results revealed that the amounts of urinary 5-mdC, 5-hmdC, 5-mrC and 5-hmrC were significantly lower in CRC patients than healthy volunteers. The present study is the first report about significantly diminished levels of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC in urine from CRC patients, suggesting that these modified nucleosides may serve as potential noninvasive biomarkers for early detection and prognosis of CRC. Furthermore, the decrease of these modified cytosine nucleosides in urine may be a general characteristic of CRC and this may stimulate the future investigation about the regulatory roles of cytosine methylation and demethylation in the formation and development of CRC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- A novel method was reported for analysis of 5-methylated and 5-hydroxymethylated cytosine nucleosides in urine.
- Enhancement of detection sensitivity was achieved with the assistance of malic acid.
- Accurate quantification was realized by using stable isotope dilution method.
- 5-hydroxymethylcytidine was firstly discovered in human urine.
- The modified cytosine nucleosides are present in significantly lower levels in urine of colorectal cancer patients.

Fig. 1.

(A) The effects of different additives on the MS signal responses of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC. Due to the low solubility of citric acid in acetonitrile or methanol, the concentration of citric acid in mobile phase A was 0.02 mM. For other additives, the concentrations were 0.06 mM. (B) Optimization of malic acid concentration. The concentration of each nucleoside standard was 10 nM, and the injection volume was 5.0 μ L.

Fig. 2.

The MRM chromatograms of (A) 5-mdC, (B) 5-hmdC, (C) 5-mrC and (D) 5-hmrC standards with or without the addition of malic acid to the mobile phase. The concentration of each nucleoside standard was 10 nM, and the injection volume was 5.0 μ L.

Fig. 3.
The MRM chromatograms of each SPE fraction obtained from the mixture of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC (100 nM each) pretreated with (A) HLB cartridge and (B) MCX cartridge.

Fig. 4. The MRM chromatograms of (A) 5-mdC, (B) 5-hmdC, (C) 5-mrC and (D) 5-hmrC obtained for a human urine sample.

Fig. 5.

Identification of 5-hmrC in human urine by HILIC-MS/MS. (A) Chromatograms of urinary 5-hmrC (m/z 274.1 $\rightarrow$ 142.0) and internal standard [$^{13}\text{CD}_2$]5-hmrC (m/z 277.1 $\rightarrow$ 145.0). 5-hmrC (m/z 274.1 $\rightarrow$ 142.0) and the qualifier ion (m/z 274.1 $\rightarrow$ 124.0) obtained for (B) a human urine sample and (C) standard.

Fig. 6. Quantification and statistical analysis of the levels of (A) 5-mdC, (B) 5-hmdC, (C) 5-mrC and (D) 5-hmrC in urine from CRC patients and healthy volunteers.

Table 1

Limits of detection (LODs) of 5-mdC, 5-hmdC, 5-mrC and 5-hmrC with or without assistance of malic acid.

	LOD (without malic acid, fmol)	LOD (with malic acid, fmol)	Fold increase in sensitivity
5-mdC	0.5	0.025	20
5-hmdC	1	0.025	40
5-mrC	1	0.025	40
5-hmrC	1	0.050	20