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HILIC-MS/MS for the Determination of Methylated Adenine Nucleosides in Human Urine

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Abstract

N^6-methyl-2′-deoxyadenosine (m^6dA) is a newly discovered DNA epigenetic mark in mammals. N^6-methyladenosine (m^6A), 2′-*O*-methyladenosine (A_m), N^6,2′-*O*-dimethyladenosine (m^6A_m), and N^6,N^6-dimethyladenosine ($m^6{}_2A$) are common RNA modifications. Previous studies illustrated the associations between the aberrations of these methylated adenosines in nucleic acids and cancer. Herein, we developed Fe_3O_4/graphene-based magnetic dispersive solid-phase extraction for the enrichment and hydrophilic interaction liquid chromatography–mass spectrometry (HILIC-MS/MS) for the measurements of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ in human urine samples. We found that malic acid could improve the HILIC-based separation of these modified nucleosides and markedly enhance the sensitivity of their MS detection. With this method, we accurately quantified the contents of these modified adenine nucleosides in urine samples collected from gastric and colorectal cancer patients as well as healthy controls. We

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c03829.
Detailed procedures for the synthesis of stable isotope-labeled internal standards; method validation; high-resolution ESI-MS^2 spectra; comparison of HLB cartridge-based SPE and Fe_3O_4/G-based MDSPE; comparison of HILIC-MS/MS and reversed-phase UPLC-MS/MS methods for urine sample analysis; MRM parameters; measured m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ levels in urine; and ROC curves (PDF)

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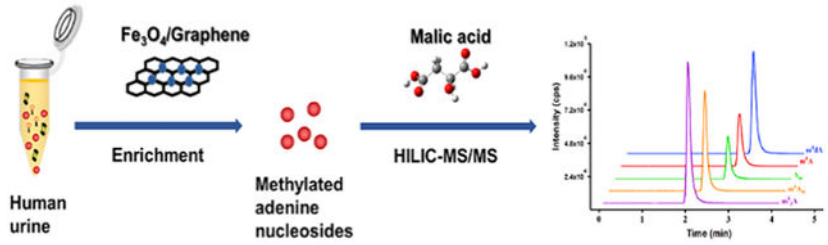
The authors declare no competing financial interest.

found that, relative to healthy controls, urinary m^6dA and A_m levels are significantly lower for gastric and colorectal cancer patients; while gastric cancer patients also exhibited lower levels of urinary m^6A, the trend was opposite for colorectal cancer patients. Together, we developed a robust analytical method for simultaneous measurements of five methylated adenine nucleosides in human urine, and our results revealed an association between the levels of urinary methylated adenine nucleosides and the occurrence of gastric as well as colorectal cancers, suggesting the potential applications of these modified nucleosides as biomarkers for the early detection of these cancers.

Graphical Abstract



INTRODUCTION

N^6-methyl-2′-deoxyadenosine (m^6dA) is commonly found in genomes of prokaryotes and plays vital roles in regulating a number of biological processes in bacteria.[1–3] Recently, m^6dA was also found to be present in genomic DNA of a number of eukaryotic species, including *Caenorhabditis elegans*, *Chlamydomonas reinhardtii*, *Drosophila melanogaster*, *Xenopus laevis*, pig, zebrafish, etc.[4–8] In addition, m^6dA was found to be present in the human genome[9,10] and plays important regulatory roles in gene expression, chromatin conformation, stress response, and tumorigenesis.[9–12] On the other hand, several other studies showed the failure to detect m^6dA in highly purified mammalian DNA; thus, there exists some controversy in the field about the mammalian origins of m^6dA.[13–15] In particular, the contamination of bacterial DNA, which carries abundant m^6dA, poses a significant challenge in accurately determining m^6dA in eukaryotic DNA.[16]

The content of m^6dA is dynamically regulated by methyltransferases (e.g., N6AMT1) and demethylases (e.g., ALKBH1), and several studies revealed that aberrant levels of m^6dA are associated with many human cancers, including glioblastoma[9] as well as liver,[10] gastric,[10] lung,[17] esophageal,[18] and breast cancers.[19] In addition, ALKBH1 is markedly upregulated in glioblastoma, and its modulation of m^6dA level could influence the survival of glioblastoma stem cells.[9]

Post-transcriptional modifications of RNA assume important roles in a variety of biological processes. To date, over 170 types of RNA modifications have been discovered, and some of them were found to regulate the structures and functions of RNA.[20] Among them, N^6-methyladenosine (m^6A) is the most prevalent internal modification in mRNA and has drawn tremendous attention in the past decade.[21] Accompanied by the discovery of methyltransferases (e.g., METTL3, METTL14), demethylases (e.g., ALKBH5, FTO),

and reader proteins (e.g., YTHDC1, IGF2BPs), the regulatory roles of m^6A in multiple biological processes have been revealed.[21–24] The content of m^6A is dynamic, and multiple lines of evidence indicate that the abnormal level of m^6A is tightly associated with tumor initiation and progression.[25,26]

Aside from nucleobase modification, the ribose in adenosine can be methylated at the 2′ oxygen to produce 2′-*O*-methyladenosine (A_m). In addition to monomethylated nucleosides, dimethylated adenosines, including N^6,2′-*O*-dimethyladenosine (m^6A_m) and N^6,N^6-dimethyladenosine ($m^6{}_2A$), also occur in RNA.[27] These modified nucleosides could influence the stabilities and translational efficiencies of mRNAs.[28]

Modified nucleosides arising from the degradation of nucleic acids are excreted into the urine. Urine samples are advantageous for biomarker discovery because they are readily accessible and their collection process is noninvasive. Compared with tissue DNA and RNA samples, urine sample analysis also obviates the needs of tedious processes of extraction or digestion of nucleic acids. Along this line, various modified nucleosides, e.g., alkylated and oxidized nucleosides, were detected in human urine.[29–32] As a novel epigenetic mark in eukaryotes, m^6dA is present in human genome and its levels vary in different tissues,[9,10,17] and m^6dA was also recently shown to be present in human urine.[33] In addition, while previous studies revealed the presence of A_m, m^6A_m, and $m^6{}_2A$ in human urine,[34,35] the levels of these modified nucleosides in urine samples have yet been accurately quantified.

Rapid advances have been made in the use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the highly sensitive and accurate analyses of modified nucleosides. Compared with commonly employed reversed-phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC) uses a high percentage of organic solvents as mobile phases, which can enhance analyte detection by improving desolvation and ionization efficiencies.[36] Additionally, we found recently that the addition of malic acid to mobile phase could improve the detection sensitivities in HILIC-MS/MS analysis of modified cytosine nucleosides.[37] Nevertheless, due to the low abundance of modified nucleosides and complex sample matrix of human urine, there remain substantial challenges in the determination of modified nucleosides in urine. Hence, appropriate enrichment methods are essential for the facile detection of modified nucleosides in human urine.

So far, boronate affinity adsorbents, owing to their abilities in reacting with *cis*-diol compounds to yield cyclic esters, have been widely used for the enrichment of ribonucleosides.[38–40] In addition, other sorbents, including metal oxides (e.g., TiO_2, CeO_2)[41,42] and metal–organic frameworks,[43,44] were also utilized for selective enrichment of modified ribonucleosides. These methods, however, are not amenable to the enrichment of m^6dA, A_m, or m^6A_m due to their lack of *cis*-diol functional groups.

By capitalizing on the hydrophobicity of the methylated adenine nucleosides, herein, we developed a magnetic dispersive solid-phase extraction (MDSPE) strategy with the use of Fe_3O_4/graphene for the simultaneous enrichment of these nucleosides. We also established an HILIC-MS/MS method, in conjunction with the use of malic acid as a mobile

phase additive and the stable isotope-dilution technique, for the sensitive and accurate determination of these five modified adenine nucleosides in human urine samples collected from gastric and colorectal cancer patients as well as healthy controls.

EXPERIMENTAL SECTION

Material and Chemicals.

m^6dA was purchased from Alfa Aesar (Tewksbury, MA). $[D_3]m^6dA$, m^6A, $[D_3]m^6A$, m^6A_m, $[D_3]m^6A_m$, A_m, $m^6{}_2A$, and $[^{13}C_5]A$ were obtained from Toronto Research Chemical (Toronto, Canada). HPLC-grade methanol and acetonitrile were purchased from J.T. Baker (Radnor, PA) and Merck KGaA (Darmstadt, Germany), respectively. Formic acid and acetic acid were purchased from Fluka (Muskegon, MI). Graphene (G), nitrogen-doped graphene (NG), and graphene oxide (GO) were purchased from Aladin (Shanghai, China). The magnetic Fe_3O_4/G, Fe_3O_4/NG, and Fe_3O_4/GO were prepared following published procedures.[45] Unless otherwise specified, other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Syntheses of Stable Isotope-Labeled A_m and $m^6{}_2A$.

The chemical structures of isotope-labeled nucleosides utilized in the present work are illustrated in Scheme 1. $[^{13}C_5]A_m$ and $[^{13}C_5]m^6{}_2A$ were synthesized according to the established method with minor modifications.[46] $[^{13}C_5]A_m$ and $[^{13}C_5]m^6{}_2A$ were purified from the reaction mixture by HPLC and confirmed by high-resolution mass spectrometry (Figure S1). The detailed procedures for chemical syntheses and HPLC purifications are described in the Supporting Information.

Sample Collection.

The urine samples were collected from 45 healthy volunteers, 43 gastric cancer patients, and 31 colorectal cancer patients (Table S1) at The Second Affiliated Hospital, Zhejiang University School of Medicine (SAHZU). This study was approved by the ethical committee of SAHZU. All of the patients recruited for the present study were not treated with chemotherapy, radiotherapy, or surgery. It was found previously that nucleosides in urine samples may be susceptible to degradation if not stored at a low temperature; thus, we stored all of the aliquots of mid-stream early-morning urine samples at −80 °C immediately after collection. The urine samples were stored for about 3–4 months prior to the pretreatment and analysis. The level of creatinine in urine was also determined using a Beckman Coulter AU5831 automatic biochemical analyzer.

Optimization of MDSPE Procedures.

Pooled urine supernatant (100 μL) spiked with mixed standard solution (10 pmol each) was employed for the optimization of MDSPE procedures. The amounts of adsorbent, extraction time, elution solvent, the percentage of formic or acetic acid in the elution solvent, and elution volume were optimized to obtain the best extraction efficiency. The performance of the three types of magnetic nanoparticles was also assessed and compared.

Enrichment of Modified Nucleosides from Urine Samples.

The urine samples were thawed at room temperature and centrifuged at 13 000 rpm for 15 min at 4 °C. A 100 μL aliquot of the supernatant, which was spiked with $[D_3]m^6dA$ (250 fmol), $[D_3]m^6A$ (25 pmol), $[^{13}C_5]A_m$ (2 pmol), $[D_3]m^6A_m$ (5 pmol), and $[^{13}C_5]m^6{}_2A$ (250 fmol), was diluted with 300 μL of H_2O. Fe_3O_4/G (2 mg) was subsequently dispersed into the above mixture under sonication for 5 min. After incubating for 1 min, the supernatant was decanted by placing a magnet to the outside of the 1.5 mL sample tube. The adsorbent particles were then washed with 1.0 mL of H_2O, and the nucleosides were eluted using 0.6 mL of CH_3OH containing 0.1% CH_3COOH, followed by vortexing for 5 s. Another three replicates of elution were performed, and the total elution volume was 2.4 mL. The eluents were pooled and evaporated under vacuum. Finally, the residue was reconstituted in 100 μL of CH_3CN/H_2O (9:1, v/v) for subsequent HILIC-MS/MS measurement (Figure 1).

HILIC-MS/MS Analysis.

Chromatographic separation was performed using a Waters BEH HILIC column (2.1 × 100 mm, 1.7 μm) on an Acquity UPLC system (Waters, Milford, MA). The LC effluent was directed to a 4000 QTRAP mass spectrometer (AB SCIEX, Foster City, CA) equipped with an electrospray ionization source operated in the positive-ion mode. The mobile phase A was H_2O containing 0.2% acetic acid and 10 mM ammonium acetate, and B was acetonitrile containing 0.2% acetic acid, 2 mM ammonium acetate, and 0.05 mM malic acid. The flow rate was 0.25 mL/min, and an isocratic mode of 6% A and 94% B was used. The column was washed with 40% A for 3 min at a flow rate of 0.4 mL/min and equilibrated with 6% A for 5 min after the analyses of two samples. The flow rate was decreased to 0.25 mL/min and maintained for 10 min. A switching valve was utilized, and the eluent in the retention time range of 1.5–3.5 min was directed to the ion source of the mass spectrometer. The samples were maintained at 4 °C. Sample (5 μL) was injected, and each sample was analyzed twice. To enhance the detection sensitivity, other 11 mobile phase additives, including succinic acid, citric acid, maleic acid, fumaric acid, oxaloacetic acid, α-ketoglutaric acid, oxalic acid, propanedioic acid, phthalic acid, benzoic acid, and salicylic acid, were evaluated.

The modified nucleosides were quantified in the multiple-reaction monitoring (MRM) mode. The transitions monitored for these nucleosides and their stable isotope-labeled internal standards and optimized MRM parameters are listed in Table S2. The spray voltage and ion source temperature were set at 5.5 kV and 550 °C, respectively. The curtain gas and ion source gases 1 and 2 were set at 40, 50, and 50 psi, respectively.

Method Validation.

The developed method was evaluated for parameters including selectivity, linearity, limits of detection (LODs), limits of quantification (LOQs), accuracy, precision, recovery, matrix effect, and carryover, according to the guidelines of the European Medicines Agency (EMA). The detailed validation procedures are provided in the Supporting Information.

Statistical Analysis.

Statistical analysis of data was carried out using SPSS 20.0 software (IBM, Armonk, NY). The concentration data were presented as mean ± standard deviation (SD). Two-tailed Student's *t*-test was employed to evaluate the differences between the groups, and a *p* value of less than 0.05 was employed to indicate statistical significance.

RESULTS AND DISCUSSION

The overall objectives of the present study were to establish a robust analytical method for measuring simultaneously methylated adenine nucleosides in human urine and to assess whether the levels of these nucleosides may serve as biomarkers for gastric and colorectal cancer.

Optimization of MDSPE Procedures for the Simultaneous Enrichment of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ from Urine Sample.

We first optimized the experimental conditions, i.e., the amount of adsorbent, extraction time, elution solvent, the percentage of acid in elution solvent, elution volume, and type of adsorbent for the extraction of target nucleosides. A pooled urine sample spiked with 10 pmol each of the five methylated adenine nucleosides was employed for the optimization.

We compared the extraction efficiencies with the use of different amounts of Fe_3O_4/G adsorbent. The results showed that the adsorption of these five methylated adenine nucleosides increased gradually with increasing amounts of adsorbent up to 2 mg, whereas a lower extraction efficiency was obtained with 4 mg of adsorbent (Figure 2A). In this vein, it is worth noting that our adsorption isotherms experiments showed that 2 mg of Fe_3O_4/G could adsorb a maximum of 9–10 nmol of each of the five methylated adenine nucleosides. Therefore, 2 mg of adsorbent was employed for subsequent experiments. We also optimized the extraction time in the range of 1–20 min, and our results revealed that these nucleosides could be effectively adsorbed within 1 min, indicating that Fe_3O_4/G exhibited a high enrichment efficiency for the modified adenine nucleosides (Figure 2B).

We next optimized the types of elution solvents to achieve high analyte recovery. Our results revealed that, among the several solvents tested (methanol, acetonitrile, dichloromethane, ethyl acetate, and acetone), methanol displayed the highest desorption capacity toward these five methylated nucleosides (Figure 2C). Furthermore, the addition of formic acid or acetic acid into methanol could further improve the desorption of the target analytes from the adsorbent (Figure 2D). Hence, 0.1% acetic acid in methanol was chosen as the elution solvent. We further optimized the elution volume and found that, in the range of 0.6–3.0, 2.4 mL of 0.1% acetic acid in methanol provided the best analyte recovery (Figure 2E).

We also assessed the performance of Fe_3O_4/G, Fe_3O_4/NG, and Fe_3O_4/GO on the extraction of these five methylated adenine nucleosides from urine samples under the optimized adsorption and desorption conditions. As illustrated in Figure 2F, Fe_3O_4/NG and Fe_3O_4/GO gave lower extraction efficiency than Fe_3O_4/G. This could be ascribed to that the adsorption mainly relies on hydrophobic and π–π stacking interaction between these methylated adenine nucleosides and the graphene surface, whereas the existence of nitrogen atom,

hydroxyl, or other hydrophilic groups on the surface of Fe_3O_4/NG and Fe_3O_4/GO weakened such interactions.

We also compared the intensities of these five adenine nucleosides extracted from the same urine sample using HLB cartridge-based SPE[47] or Fe_3O_4/G-based MDSPE. Our results showed that the latter SPE method provided higher signal intensities and more efficient measurements of these analytes than the former (Figure S2). This is particularly beneficial when a large number of samples are handled. Considering the above factors, we chose the Fe_3O_4/G-based MDSPE for urine sample pretreatment in this study.

Malic Acid Improves the MS Detection of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$.

Our recent study revealed that the use of malic acid as a mobile phase additive could markedly enhance the HILIC-MS/MS determination of modified cytosine nucleosides.[37] Thus, we next examined whether malic acid could enhance the HILIC-MS/MS detection of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$. To this end, we tested 12 aliphatic and aromatic carboxylic acids as additives for HILIC-MS/MS detection of these nucleosides.

As shown in Figure 3A, about half of carboxylic acids tested could improve the detection of these nucleosides. Moreover, compared with other additives, malic acid exhibited the best performance in enhancing the detection sensitivity, where the MRM signals were elevated by 2.9- to 6.3-fold. Further experiments with different concentrations of malic acid in the mobile phase showed that the MS response elevates as the malic acid concentration increases from 0.01 to 0.05 mM; no obvious signal enhancement, however, was observed when 0.075 or 0.10 mM malic acid was utilized (Figure 3B). Therefore, we chose 0.05 mM malic acid for the subsequent experiments.

Compared with the MRM chromatograms of these nucleosides without the utilization of malic acid (Figure 3C), the peaks were narrower and sharper, and the separation efficiencies of these nucleosides were improved when malic acid was employed (Figure 3D). Additionally, malic acid can pronouncedly improve the MS detection of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$, by 7.5-, 20-, 5-, 6.7-, and 10-fold, respectively (Table S3). In the presence of malic acid, the LODs of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ reached 0.01, 0.01, 0.015, 0.0075, and 0.01 nM, respectively. On the basis of our previous study,[37] the notable improvement in detection sensitivities achieved by the utilization of malic acid can be ascribed to the suppression of the formation of alkali metal-ion adducts and promotion of the protonation of these nucleosides during the ionization process.

Method Validation.

We utilized UPLC separation followed by MS/MS in the MRM mode to ensure that the analyte peaks could be easily distinguished from those of interference(s). We constructed calibration curves for the five methylated adenine nucleosides, which exhibited excellent linearities, with correlation coefficients (R^2) being >0.999 (Table S4). Additionally, the matrix effect was between 102.4 and 106.0%, suggesting that it is negligible. The accuracy observed in intra- and interday assays fell in the ranges of 92.9–109.1 and 93.0–109.1%, respectively (Table S5), demonstrating the excellent accuracy of the method. The intra- and interday precision values, as reflected by relative standard deviations (RSDs), were

in the ranges of 1.2–6.1 and 0.9–6.0%, respectively (Table S5), indicating that excellent reproducibility was achieved. The recoveries of the established method varied from 92.7 to 109.8% at three different spiking levels under the optimized MDSPE conditions (Table S6), demonstrating the excellent performance of the method in the enrichment of these five methylated adenine nucleosides from urine samples by Fe_3O_4/G. No residual signals for the analytes or internal standards were detectable when a blank sample was analyzed after injection of a high concentration of standards, indicating the lack of analyte carryover in our method. During the course of analysis, the results of monitored parameters, including retention time and accuracy of the quantification data for QC samples, revealed good system stability.

Identification and Quantification of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ in Human Urine.

By employing the aforementioned optimized experimental conditions for nucleoside enrichment using Fe_3O_4/G-based MDSPE and quantification using the malic acid-improved HILIC-MS/MS method, we measured the levels of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ in human urine samples. In addition, we utilized a stable isotope-dilution technique so as to unambiguously identify and accurately quantify these nucleosides in human urine. The results demonstrated that the retention times of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ were identical to those of their corresponding isotope-labeled internal standards (Figure 4A–E), demonstrating the presence of these five methylated adenine nucleosides in human urine samples. Moreover, the performance of the UPLC-MS/MS method using a reversed-phase C18 column for the determination of these modified nucleosides in human urine was evaluated and compared with that of the established HILIC-MS/MS method. However, urinary m^6dA and $m^6{}_2A$ were barely detectable with the reversed-phase UPLC-MS/MS (Figure S3), which is attributed to the limited sensitivity of the reversed-phase UPLC-MS/MS method (Table S3).

Having developed a robust analytical method for measuring these methylated adenine nucleosides, we next measured their levels in urine samples collected from cancer patients and healthy controls. In total, we analyzed 119 urine samples from 31 colorectal cancer patients, 43 gastric cancer patients, and 45 healthy controls.

Because 24 h urine samples were not available, we collected urine samples in the morning. Although there are some limitations, urinary creatinine was commonly utilized to normalize the determined levels of metabolites in urine.[48] Thus, we normalized the concentrations of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ against the level of urinary creatinine. The quantification results revealed that the measured concentrations of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ in human urine fell in the ranges of 0.002–0.054, 0.25–52.54, 0.32–7.07, 1.54–22.80, and 0.003–0.105 nmol per mmol creatinine, respectively. The mean concentrations of these adenine nucleosides in urine in these three groups are summarized in Table S7, and the detailed concentrations of urine samples from each individual are listed in Table S8.

We next evaluated whether there were concentration differences of these methylated adenine nucleosides between cancer patients and healthy controls. The results demonstrated that the contents of urinary m^6dA and A_m were significantly lower in colorectal cancer patients than healthy controls ($p < 0.01$ for both m^6dA and A_m, Figure 5A,C), whereas the level of

m^6A in urine was significantly elevated in colorectal cancer patients than healthy controls ($p < 0.05$, Figure 5B). On the other hand, the levels of urinary m^6dA, m^6A, and A_m were significantly lower in gastric cancer patients than healthy controls ($p < 0.01$ for m^6dA and m^6A, $p < 0.05$ for A_m, Figure 5A–C). However, there was no significant difference in concentrations of urinary m^6A_m or $m^6{}_2A$ between healthy controls and cancer patients ($p > 0.05$, Figure 5D–E). Furthermore, we analyzed the data according to the stages of disease in these patients; there was, however, no apparent association between the levels of these modified nucleosides and disease stages.

Receiver operator characteristic (ROC) curve analysis was performed to evaluate the potential of these methylated nucleosides to differentiate cancer patients from healthy controls. As shown in Figure S4, the area under the curve (AUC) is in the range of 0.63–0.71 with using m^6dA, m^6A, and A_m for the detection of colorectal cancer and gastric cancer. Hence, our results revealed an association between the levels of these methylated nucleosides and cancer occurrence, suggesting the potential applications of these nucleosides as biomarkers for early cancer detection.

As a newly discovered epigenetic modification in eukaryotes, m^6dA has drawn increasing attention in the past few years.[4–19] Although the m^6dA content in genomic DNA of human tissues was seldom quantified previously, a recent study revealed that the m^6dA content in genomic DNA was diminished in gastric cancer tissues compared to tumor-adjacent normal tissues.[10] In this study, we accurately quantified, for the first time, the levels of m^6dA in human urine using the stable isotope-dilution technique, and its level was significantly lower in urine samples from gastric cancer and colorectal cancer patients than healthy controls. Nevertheless, we were not able to discern whether urinary m^6dA is from human or microbial DNA since m^6dA is present in nucleoside form in the urine samples.

Among the methylated ribonucleosides, m^6A has attracted substantial attention and its regulatory functions have been widely investigated in the last decade.[21–26] It has been revealed that m^6A plays disparate roles (i.e., promoting or suppressing cancer occurrence and progression) in different types of cancer.[49] Interestingly, we demonstrated that gastric cancer patients had diminished the m^6A level in urine, whereas colorectal cancer patients had an elevated m^6A content in urine, compared with healthy controls. This indicates the different roles of m^6A in the initiation and development of gastric and colorectal cancers.

We also measured, for the first time, the level of A_m in human urine, and its level is consistently diminished in gastric cancer patients and colorectal cancer patients, relative to healthy controls. We also reported the presence of m^6A_m and $m^6{}_2A$ in human urine, although there was no significant difference in contents of these two dimethylated adenine nucleosides between cancer patients and healthy controls.

CONCLUSIONS

In the present study, we developed an MDSPE-HILIC-MS/MS method, in combination with the stable isotope-dilution method for sensitive and accurate determination of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ in human urine. By employing this method, we realized the

simultaneous enrichment of these five methylated adenine nucleosides from human urine samples. We also demonstrated that the use of malic acid as a mobile phase additive led to an improved separation of these nucleosides and enhanced detection sensitivities in MS. Moreover, we were able to unambiguously identify the presence of these methylated nucleosides and accurately measure their levels in human urine using the stable isotope-dilution technique. We further confirmed the presence of m^6dA, A_m, and m^6A_m in human urine. To the best of our knowledge, this is the first report about the accurate levels of m^6dA, A_m, m^6A_m, and $m^6{}_2A$ in human urine. Moreover, in light of the alterations in the levels of urinary m^6dA, m^6A, and A_m in gastric and colorectal cancer patients, our results suggest that these methylated nucleosides may serve as noninvasive indicators for early detection of these cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

(1). Ratel D; Ravanat JL; Berger F; Wion D Bioessays 2006, 28, 309–315. [PubMed: 16479578]

(2). Wion D; Casadesus J Nat. Rev. Microbiol 2006, 4, 183–192. [PubMed: 16489347]

(3). Loenen WAM; Dryden DTF; Raleigh EA; Wilson GG; Murray NE Nucleic Acids Res. 2014, 42, 3–19. [PubMed: 24141096]

(4). Greer EL; Blanco MA; Gu L; Sendinc E; Liu J; Aristizabal-Corrales D; Hsu CH; Aravind L; He C; Shi Y Cell 2015, 161, 868–878. [PubMed: 25936839]

(5). Fu Y; Luo GZ; Chen K; Deng X; Yu M; Han D; Hao Z; Liu J; Lu X; Dore LC; Weng X; Ji Q; Mets L; He C Cell 2015, 161, 879–892. [PubMed: 25936837]

(6). Zhang G; Huang H; Liu D; Cheng Y; Liu X; Zhang W; Yin R; Zhang D; Zhang P; Liu J; Li C; Liu B; Luo Y; Zhu Y; Zhang N; He S; He C; Wang H; Chen D Cell 2015, 161, 893–906. [PubMed: 25936838]

(7). Koziol MJ; Bradshaw CR; Allen GE; Costa ASH; Frezza C; Gurdon JB Nat. Struct. Mol. Biol 2016, 23, 24–30. [PubMed: 26689968]

(8). Liu J; Zhu Y; Luo GZ; Wang X; Yue Y; Wang X; Zong X; Chen K; Yin H; Fu Y; Han D; Wang Y; Chen D; He C Nat. Commun 2016, 7, No. 13052. [PubMed: 27713410]

(9). Xie Q; Wu TP; Gimple RC; Li Z; Prager BC; Wu Q; Yu Y; Wang PC; Wang YS; Gorkin DU; Zhang C; Dowiak AV; Lin K; Zeng C; Sui Y; Kim LJY; Miller TE; Jiang L; Lee CH; Huang Z; Fang X; Zhai K; Mack SC; Sander M; Bao S; Kerstetter-Fogle AE; Sloan AE; Xiao AZ; Rich JN Cell 2018, 175, 1228–1243. [PubMed: 30392959]

(10). Xiao CL; Zhu S; He M; Chen; Zhang Q; Chen Y; Yu G; Liu J; Xie SQ; Luo F; Liang Z; Wang DP; Bo XC; Gu XF; Wang K; Yan GR Mol. Cell 2018, 71, 306–318. [PubMed: 30017583]

(11). Li X; Zhao Q; Wei W; Lin Q; Magnan C; Emami MR; Wearick-Silva LE; Viola TW; Marshall PR; Yin J; Madugalle SU; Wang Z; Nainar S; Vagbo CB; Leighton LJ; Zajaczkowski EL; Ke K; Grassi-Oliveira R; Bjoras M; Baldi PF; Spitale RC; Bredy TW Nat. Neurosci 2019, 22, 534–544. [PubMed: 30778148]

(12). Beh LY; Debelouchina GT; Clay DM; Thompson RE; Lindblad KA; Hutton ER; Bracht JR; Sebra RP; Muir TW; Landweber LF Cell 2019, 177, 1781–1796. [PubMed: 31104845]

(13). Schiffers S; Ebert C; Rahimoff R; Kosmatchev O; Steinbacher J; Bohne AV; Spada F; Michalakis S; Nickelsen J; Muller M; Carell T Angew. Chem., Int. Ed 2017, 56, 11268–11271.

(14). O'Brown ZK; Boulias K; Wang J; Wang SY; O'Brown NM; Hao Z; Shibuya H; Fady PE; Shi Y; He C; Megason SG; Liu T; Greer EL BMC Genomics 2019, 20, No. 445. [PubMed: 31159718]

(15). Douvlataniotis K; Bensberg M; Lentini A; Gylemo B; Nestor CE Sci. Adv 2020, 6, No. eaay3335. [PubMed: 32206710]

(16). Liu B; Liu X; Lai W; Wang H Anal. Chem 2017, 89, 6202–6209. [PubMed: 28471639]

(17). Xiong J; Ye TT; Ma CJ; Cheng QY; Yuan BF; Feng YQ Nucleic Acids Res. 2019, 47, 1268–1277. [PubMed: 30517733]

(18). Chen L; Zhang M; Guo M Discovery Med. 2020, 29, 85–90.

(19). Sheng X; Wang J; Guo Y; Zhang J; Luo J Front. Oncol 2020, 10, No. 616098. [PubMed: 33614498]

(20). Boccaletto P; Machnicka MA; Purta E; Piatkowski P; Baginski B; Wirecki TK; Crecy-Lagard V; Ross R; Limbach PA; Kotter A; Helm M; Bujnicki JM Nucleic Acids Res. 2018, 46, D303–D307. [PubMed: 29106616]

(21). Jia G; Fu Y; Zhao X; Dai Q; Zheng G; Yang Y; Yi C; Lindahl T; Pan T; Yang YG; He C Nat. Chem. Biol 2011, 7, 885–887. [PubMed: 22002720]

(22). Luo S; Tong L Proc. Natl. Acad. Sci. U.S.A 2014, 111, 13834–13839. [PubMed: 25201973]

(23). Liu J; Yue Y; Han D; Wang X; Fu Y; Zhang L; Jia G; Yu M; Lu Z; Deng X; Dai Q; Chen W; He C Nat. Chem. Biol 2014, 10, 93–95. [PubMed: 24316715]

(24). Meyer KD; Jaffrey SR Annu. Rev. Cell Dev. Biol 2017, 33, 319–342. [PubMed: 28759256]

(25). Huang HL; Weng HY; Chen JJ Cancer Cell 2020, 37, 270–288. [PubMed: 32183948]

(26). Fang ZH; Hu YQ; Hu JJ; Huang YQ; Zheng S; Guo C Cell Biosci. 2021, 11, No. 72. [PubMed: 33836813]

(27). Limbach PA; Crain PF; McCloskey JA Nucleic Acids Res. 1994, 22, 2183–2196. [PubMed: 7518580]

(28). Mauer J; Luo X; Blanjoie A; Jiao X; Grozhik AV; Patil DP; Linder B; Pickering BF; Vasseur JJ; Chen Q; Gross SS; Elemento O; Debart F; Kiledjian M; Jaffrey SR Nature 2017, 541, 371–375. [PubMed: 28002401]

(29). Chao MR; Hsu YW; Liu HH; Lin JH; Hu CW Chem. Res. Toxicol 2015, 28, 997–1006. [PubMed: 25825822]

(30). Raćkowska E; Bobrowska-Korczak B; Giebultowicz JJ Chromatogr. B 2019, 1128, No. 121775.

(31). Zhang YF; Qi CB; Yuan BF; Feng YQ Anal. Chim. Acta 2019, 1081, 103–111. [PubMed: 31446947]

(32). Guo C; Chen Q; Chen JN; Yu JK; Hu YQ; Zhang SS; Zheng SJ Chromatogr. B 2020, 1136, No. 121931.

(33). Gątarek P; Joanna Kaluzna-Czaplinska J; Pawelczyk M; Jastrzebski K; Giebultowicz J; Glabinski A; Bobrowska-Korczak B Molecules 2020, 25, No. 4959.

(34). Hirschhorn R; Ratech H; Rubinstein A; Papageorgiou P; Kesarwala H; Gelfand E; Roegner-Maniscalco V Pediatr. Res 1982, 16, 362–369. [PubMed: 6980397]

(35). Wu Q; Wu D; Guan Y Anal. Chem 2014, 86, 10122–10130. [PubMed: 25264947]

(36). Spagou K; Tsoukali H; Raikos N; Gika H; Wilson ID; Theodoridis GJ Sep. Sci 2010, 33, 716–727.

(37). Guo C; Xie C; Chen Q; Cao XJ; Guo MZ; Zheng S; Wang YS Anal. Chim. Acta 2018, 1034, 110–118. [PubMed: 30193624]

(38). Liu Z; He H Acc. Chem. Res 2017, 50, 2185–2193. [PubMed: 28849912]

(39). Chen Y; Huang AL; Zhang YN; Bie ZJ Anal. Chim. Acta 2019, 1076, 1–17. [PubMed: 31203952]

(40). Li HY; He H; Liu Z TrAC, Trends Anal. Chem 2021, 140, No. 116271.

(41). Wang ST; Huang W; Lu W; Yuan BF; Feng YQ Anal. Chem 2013, 85, 10512–10518. [PubMed: 24059655]

(42). Chu JM; Qi CB; Huang YQ; Jiang HP; Hao YH; Yuan BF; Feng YQ Anal. Chem 2015, 87, 7364–7372. [PubMed: 26086917]

(43). Pan YN; Guo XM; Li SS; Liu XY; Zhang HX New J. Chem 2018, 42, 2288–2294.

(44). Zhang RQ; Wang Z; Wang TF; Su P; Yang Y Anal. Chim. Acta 2020, 1106, 42–51. [PubMed: 32145854]

(45). Wu QH; Zhao GY; Feng C; Wang C; Wang ZJ Chromatogr. A 2011, 1218, 7936–7942.

(46). Yamauchi K; Nakagima T; Kinoshita MJ Org. Chem 1980, 45, 3865–3868.

(47). Chen Q; Hu Y; Fang Z; Ye M; Li J; Zhang S; Yuan Y; Guo C Front. Chem 2020, 8, No. 606495. [PubMed: 33392149]

(48). Wu Y; Li LJ Chromatogr. A 2016, 1430, 80–95.

(49). He L; Li H; Wu A; Peng Y; Shu G; Yin G Mol. Cancer 2019, 18, No. 176. [PubMed: 31801551]

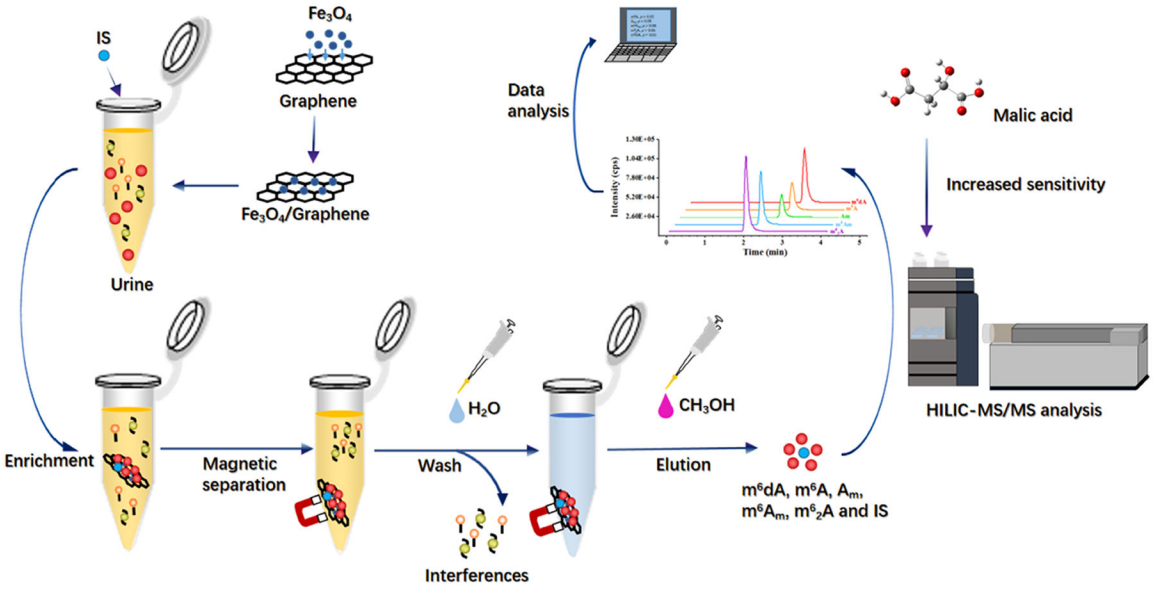


Figure 1.

Schematic diagram to illustrate the analytical procedures for the determination of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ in human urine. The analytes were enriched using Fe_3O_4/G and measured by HILIC-MS/MS with the use of malic acid as a mobile phase additive and with the stable isotope-dilution method.

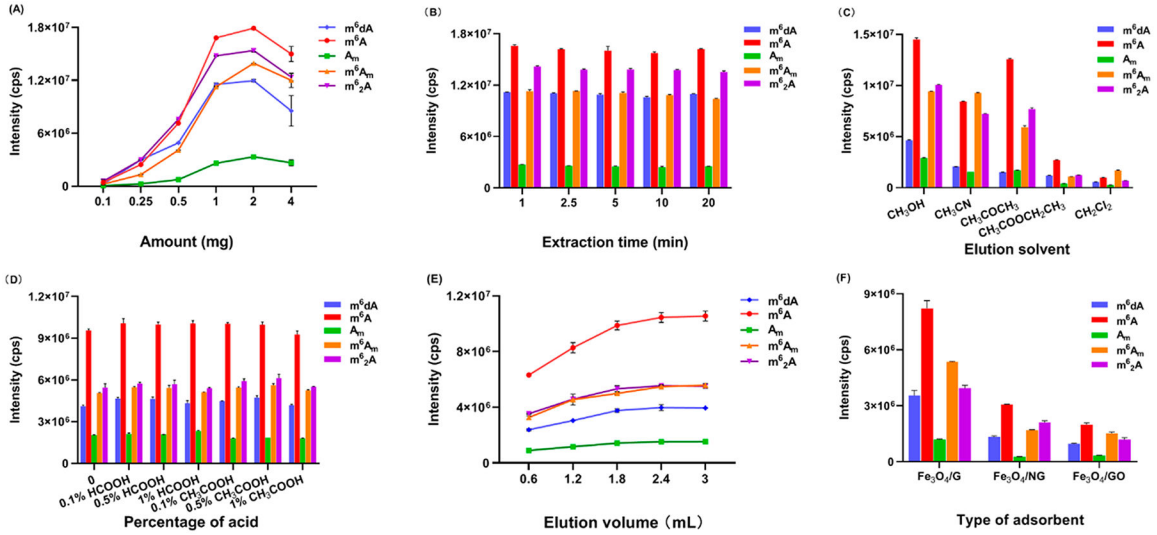


Figure 2.

Optimization of the MDSPE conditions, including Fe_3O_4/G amount (A), extraction time (B), elution solvent (C), percentage of acid in eluent solvent (D), elution volume (E), and type of adsorbent materials (i.e., Fe_3O_4/G, Fe_3O_4/NG, and Fe_3O_4/GO) (F).

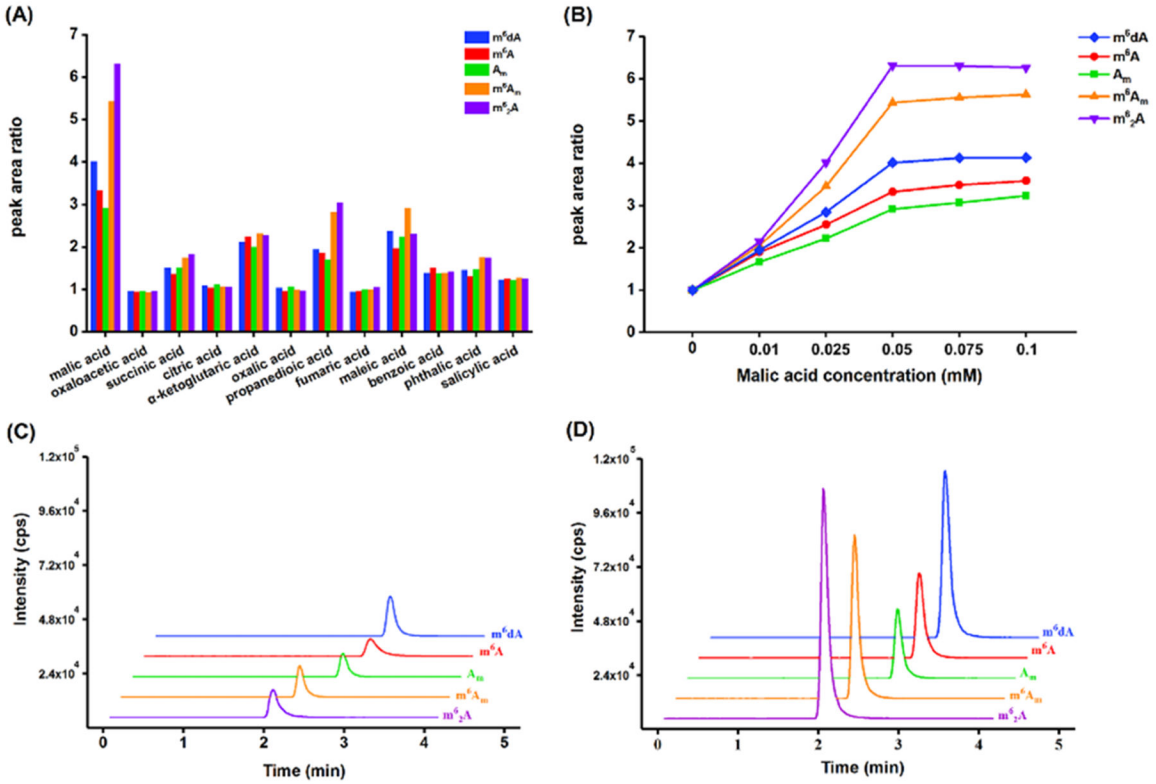


Figure 3.

Optimization of the mobile phase additives. (A) Comparison of various additives for the detection of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$. (B) Optimization of malic acid concentration. MRM chromatograms of these modified nucleoside standards without (C) and with (D) the use of malic acid as a mobile phase additive. The peak area ratios in (A) and (B) were obtained from the use of mobile phase with carboxylic acid vs mobile phase without carboxylic acid, and mobile phase with different concentrations of malic acid vs mobile phase without malic acid, respectively. The concentration of modified nucleosides was 10 nM each.

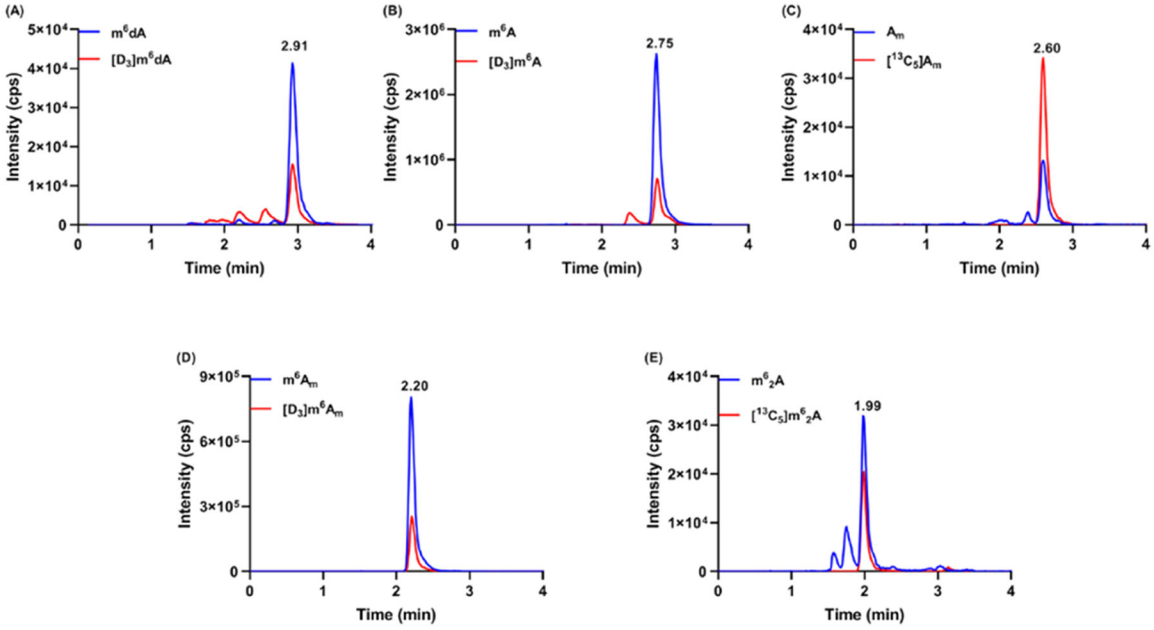


Figure 4.

Identification of m^6dA, m^6A, A_m, m^6A_m, and $m^6{}_2A$ in human urine. MRM chromatograms of (A) m^6dA, (B) m^6A, (C) A_m, (D) m^6A_m, (E) $m^6{}_2A$, and their corresponding stable isotope-labeled internal standards in human urine sample.

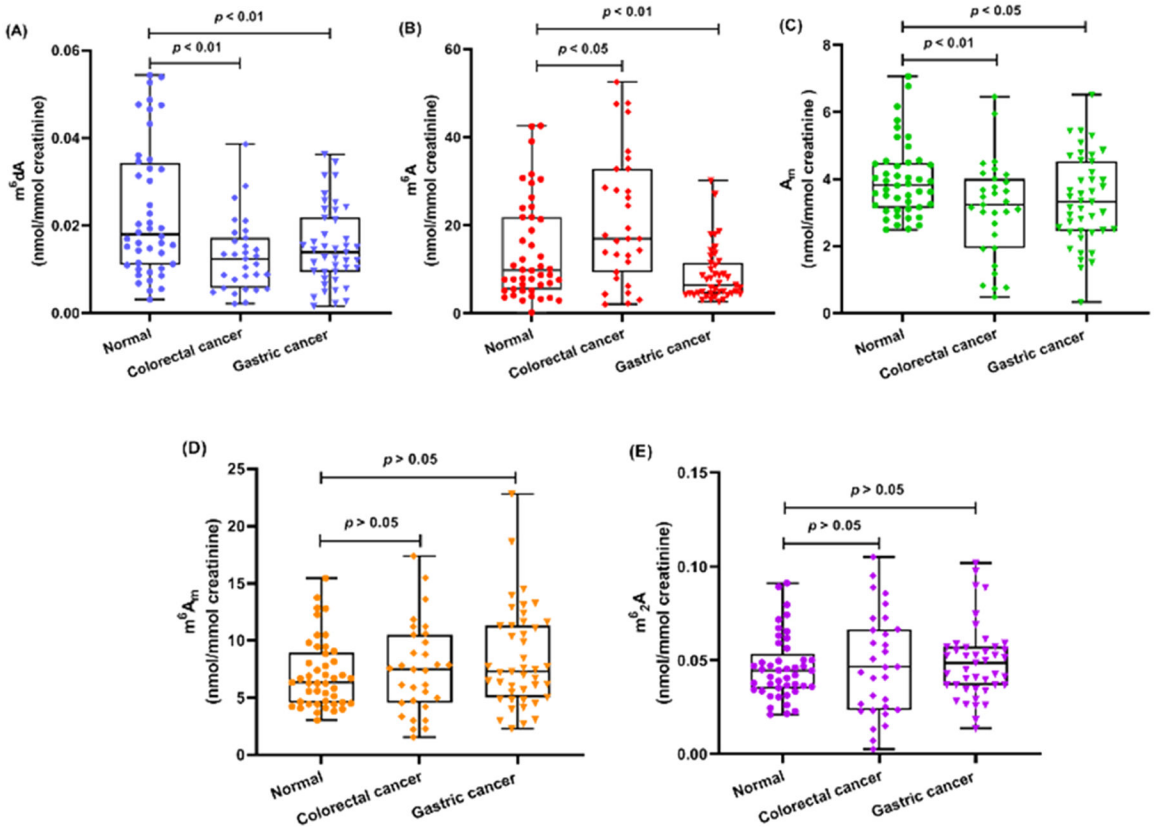
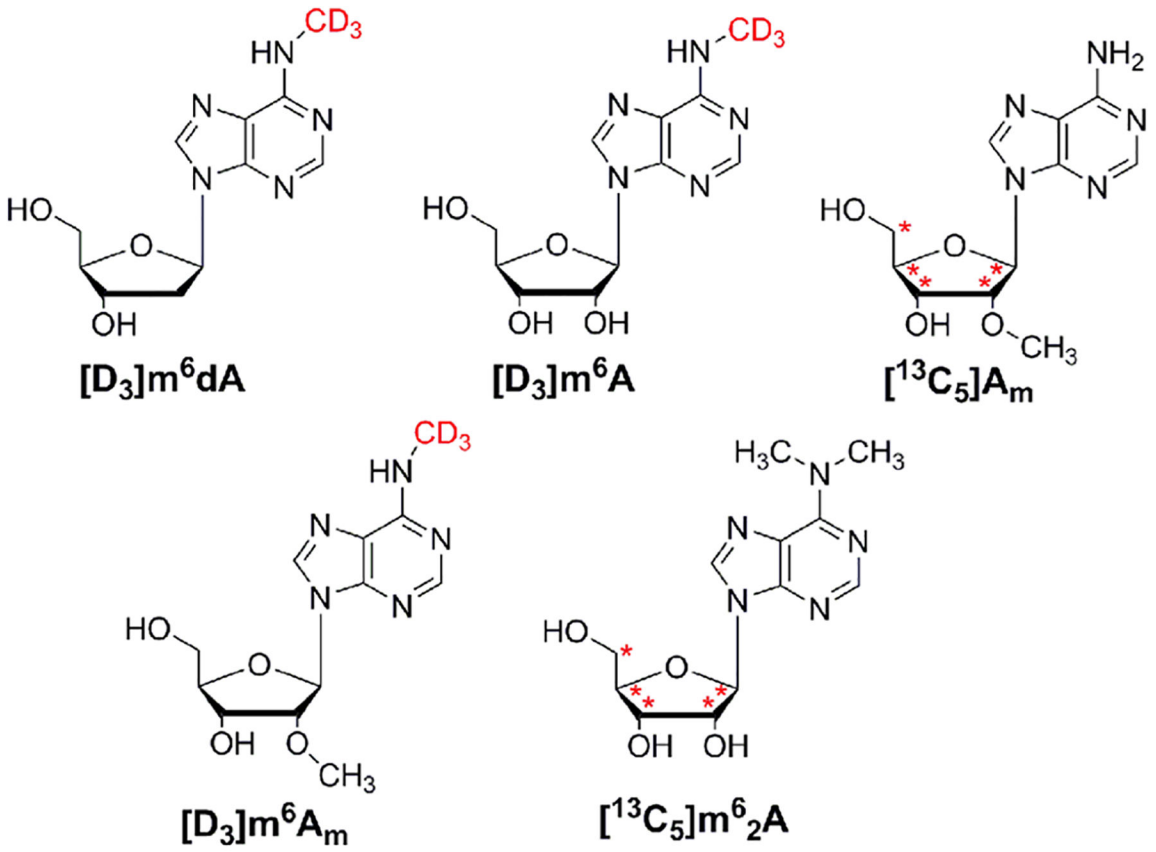


Figure 5.

Quantification results of (A) m^6dA, (B) m^6A, (C) A_m, (D) m^6A_m, and (E) $m^6{}_2A$ in urine from cancer patients and healthy controls.



Scheme 1.

Chemical Structures of the Stable Isotope-Labeled Nucleosides[a]

[a]Asterisk (*) designates the site of ^{13}C labeling.