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Direct Analysis of 5-Methylcytosine and 5-Methyl-2′-deoxycytidine in Human Urine by Isotope Dilution LC-MS/MS: Correlations with N-Methylated Purines and Oxidized DNA Lesions

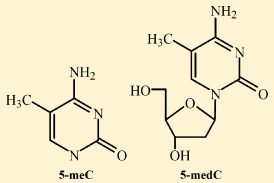
Chiung-Wen Hu,[†] Hung-Hsin Liu,[‡] Yi-Jie Li,[‡] and Mu-Rong Chao*[,‡,§]

[†]Department of Public Health and [‡]Department of Occupational Safety and Health, Chung Shan Medical University, Taichung 402, Taiwan

[§]Department of Occupational Medicine, Chung Shan Medical University Hospital, Taichung 402, Taiwan

Supporting Information

ABSTRACT: Recent evidence suggests that active DNA demethylation involves base excision repair (BER) and nucleotide excision repair (NER) pathways. We hypothesized that the resulting excision products could be further excreted and present in urine. A highly specific and sensitive liquid chromatography−tandem mass spectrometric (LC-MS/MS) method was first developed for simultaneously measuring urinary 5-methylcytosine (5-meC) and 5-methyl-2′-deoxycytidine (5-medC). With the use of isotope internal standards and online solid-phase extraction (SPE), the detection limits of 5-meC and 5-medC were estimated to be 1.2 and 0.3 pg, respectively. This method was applied to measure urinary samples of 376 healthy males. Urinary samples were also measured for methylated and oxidized DNA lesions, namely, N7-methylguanine (N7-meG), N3-methyladenine (N3-meA), 8-oxo-7,8-dihydroguanine (8-oxoGua), and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), using reported online SPE LC-MS/MS methods. Results showed that mean urinary levels of 5-meC and 5-medC were 28.4 ± 14.3 and 7.04 ± 7.2 ng/mg creatinine, respectively, supporting the possible presence of DNA demethylation through BER and NER mechanisms. Urinary levels of 5-meC were significantly positively correlated with N7-meG, N3-meA, and 8-oxodG. Good correlations between 5-meC and methylated and oxidized DNA lesions may have implied the underlying linkage between genetic (DNA lesions) and epigenetic (DNA methylation) alterations derived from exogenous exposure and/or from endogenous cellular processes in human and require further investigation.



INTRODUCTION

Methylation of the C-5 position of cytosine in genomic DNA is a central mammalian epigenetic control mechanism that impacts gene expression, genome stability, genetic imprinting, and cellular differentiation.[1] Aberrant methylation of certain genes, either hypomethylation or hypermethylation, has been associated with various diseases, especially cancers.[2,3] The level and pattern of 5-methylcytosine (5-meC) are determined by both DNA methylation and DNA demethylation processes. DNA methylation is catalyzed by DNA methyltransferases, with *S*-adenosylmethionine (SAM) as a methyl donor. DNA methylation can be removed enzymatically by several mechanisms including base excision repair (BER), nucleotide excision repair (NER), and hydrolysis.[4−8] Also, the recent studies demonstrated that 5-meC may be hydroxymethylated to 5-hydroxymethylcytosine (5-hmeC) and then further oxidized to 5-formylcytosine and 5-carboxycytosine, providing an alternative mechanism for active DNA demethylation.[9−11] It is generally believed that the resulting repair products are released into the bloodstream and consequently appear in the urine. Previously, several attempts were made to measure 5-methyl-2′-deoxycytidine (5-medC) in human urine, presumably through excision repair in DNA,[12−14] although its underlying mechanism has not been fully explored. Nevertheless, it was of interest that there was absolutely no information on the urinary 5-meC in the literature, which resulted from a more explicit mechanism, namely, the BER pathway initiated by DNA glycosylase.[4,5,8]

There is increasing evidence indicating multiple connections between DNA methylation and methylated DNA lesions. Previous studies have shown that SAM, being the major methyl donor for DNA methylation, was able to methylate DNA nonenzymatically to produce the same putative promutagenic and procarcinogenic lesions (e.g., O^6-methylguanine or less potent N7-methylguanine and N3-methyladenine) formed by carcinogenic chemical methylating agents.[15,16] Meanwhile, the precursor of SAM, methionine (Met), is readily oxidized by reactive oxygen species (ROS) to form methionine sulfoxide (MetO)[17] that can further react with hydroxyl radicals to produce methyl radicals and ultimately nonenzymatically methylate cytosine and guanine residues in DNA.[18] Among these, the hydroxyl radical is well-known to cause oxidatively generated damage to nucleic acids and produce the most common oxidized lesion 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). In addition to the endogenous cellular processes, exogenous exposure has also been shown to induce both methylated DNA lesions and chemical DNA methylation. For

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example, *tert*-butyl hydroperoxide (tBOOH), an environmental tumor promoter, can generate carbocation ions (CH_3^+) and methyl radicals to produce N7-methylguanine (N7-meG) and C8-methylguanine (8-meG), respectively.[19,20] Because the C-5 position of cytosine is an active site for free radical reaction, the formation of 5-meC was recently observed in DNA by the tBOOH treatment.[21,22] However, there was little information on the possible associations between 5-meC (or 5-medC) and those methylated and oxidized DNA lesions in vivo.

Several methods were previously developed for the determination of 5-medC in human urine, such as immunoassay and high-performance liquid chromatography with UV detection (HPLC-UV).[12,13] Recently, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has become a powerful technology that can overcome the sensitivity and selectivity issues in analysis of modified DNA bases. Accurate quantitation of modified bases at extremely low concentrations has frequently relied on the use of nonradioactive isotope-labeled standards to compensate for the loss of analyte during sample preparation, which has been the most critical step to eliminate the matrix effect for analysis of modified bases by mass spectrometry.[23] Furthermore, the online sample extraction using a column-switching device is an extremely useful technique to prepare biological samples automatically for LC-MS methods.[24] Its advantages include less ion suppression and relatively short run times, as well as higher sensitivity and selectivity, especially for urine samples containing a considerable amount of coeluting interferences.

In this study, we first developed an isotope dilution LC-MS/MS method coupled with an online solid-phase extraction (SPE) for a direct and simultaneous analysis of urinary 5-meC and 5-medC. This method was further applied to investigate the possible associations between urinary 5-meC (or 5-medC) and the methylated as well as oxidized DNA lesions. Urinary methylated DNA lesions including N7-meG and N3-methyladenine (N3-meA) and oxidized lesions including 8-oxo-7,8-dihydroguanine (8-oxoGua) and 8-oxodG were determined, respectively, using our previously reported online SPE LC-MS/MS methods.[25−27]

EXPERIMENTAL PROCEDURES

Chemicals. Solvents and salts were of analytical grade. Reagents were purchased from the indicated sources: 5-meC from Alfa Aesar; 5-medC from Tokyo Chemical Industry; N7-meG from Merck; N3-meA, 8-oxoGua, and 8-oxodG from Sigma-Aldrich; d_3-5-methyl-2′-deoxycytidine (d_3-5-medC) from Toronto Research Chemicals Inc.; and d_3-N3-methyladenine (d_3-N3-meA) and $^{15}N_5$-8-oxo-7,8-dihydro-2′-deoxyguanosine ($^{15}N_5$-8-oxodG) from Cambridge Isotope Laboratories. The internal standards, $^{15}N_5$-N7-methylguanine ($^{15}N_5$-N7-meG) and $^{15}N_5$-8-oxo-7,8-dihydroguanine ($^{15}N_5$-8-oxoGua) were synthesized as described previously.[25,27] The internal standard, d_3-5-methylcytosine (d_3-5-meC), was obtained by hydrolyzing d_3-5-medC in 1 N HCl at 95 °C for 60 min. The resulting hydrolysate was dried under vacuum in a SpeedVac. The d_3-5-meC was then purified using a semipreparative HPLC system, confirmed by MS analysis, and quantified using unlabeled 5-meC. The chemical structures of 5-meC, 5-medC, and their corresponding internal standards are also shown in Figure 1.

Participants and Urine Samples. This study was approved by the Institutional Review Board of Chung Shan Medical University Hospital. Spot urine samples were obtained from 376 healthy male subjects, including 160 smokers and 216 nonsmokers. A questionnaire was used to obtain data on subject age, body mass index (BMI), and the smoking status. Urine samples were kept at 4 °C during sampling and then stored at −20 °C before analysis.

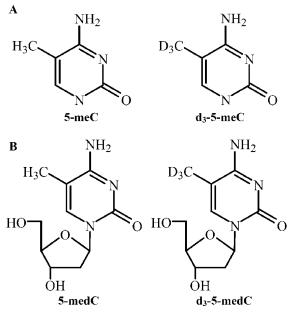

Figure 1. Chemical structures of (A) 5-meC or (B) 5-medC and their corresponding stable isotopes.

Simultaneous Analysis of Urinary 5-MeC and 5-MedC Using Online SPE LC-MS/MS. *Preparation of Urine Samples.* The urine samples were thawed, vortexed, and then heated to 37 °C for 10 min to release possible 5-meC and 5-medC from precipitate. After centrifugation at 5000*g* for 5 min, 12 μL of urine was diluted 10 times with a solution containing 0.9 ng of d_3-5-meC and 1.2 ng of d_3-5-medC as internal standards in 5% (v/v) methanol (MeOH)/3 mM ammonium acetate (AA).

Standard stock solutions of two analytes (1000 μg/mL) were prepared by individually dissolving the analyte in 5% (v/v) MeOH and then further diluting with 5% (v/v) MeOH/3 mM AA to a final concentration of 100 ng/mL. For establishing the linear calibration curve, the stock solutions of 5-meC and 5-medC (100 ng/mL) were mixed in equal volumes and then serially diluted 1:1 with 5% (v/v) MeOH/3 mM AA to yield aqueous standard solutions. Two linear ranges were determined for both 5-meC and 5-medC from 0.006 to 0.094 ng (low range: 0.006, 0.012, 0.023, 0.047, and 0.094 ng) and 0.094−1.5 ng (high range: 0.094, 0.188, 0.375, 0.75, and 1.5 ng); each calibrator contained 0.9 ng of d_3-5-meC and 1.2 ng of d_3-5-medC.

Automated Online SPE. The column-switching system used in this study was as described in detail elsewhere.[27] It consisted of a switching valve (2-position microelectric actuator from Valco) and a C18 trap column (75 mm × 2.1 mm i.d., 5 μm, ODS-3, Inertsil). The switching valve function was controlled by PE-SCIEX control software (Analyst; Applied Biosystems). The column-switching operation, including the LC gradients used during the online cleanup and the analytical procedures, is summarized in detail in Table 1. When the switching valve was at position A, 50 μL of prepared urine sample was loaded onto the trap column using an Agilent 1100 series autosampler (Agilent Technology), and a binary pump (Agilent 1100 series) delivered the 5% (v/v) MeOH/3 mM AA at a flow rate of 200 μL/min as the loading and washing buffer (solvent Ia). After the column was flushed with the loading buffer for 2.0 min, the valve switched to the injection position (position B) to inject the sample (enriched 5-meC) into the LC system. At 3.5 min after injection (Table 1), the valve was switched back to position A, and the trap column was washed with a mobile phase of 90% solvent Ia and 10% of 50% (v/v) MeOH containing 3 mM AA (solvent Ib) for 2 min, followed by valve switching to injection position B again to inject the sample (enriched 5-medC) into the LC system. At 7.5 min after injection, the valve was switched back to position A, and the column was washed using a mobile phase (eluent I) with a gradient from 100% solvent Ib to 100% solvent Ia for 4 min, followed by 100% solvent Ia for 5.5 min for equilibration of the column and preparation for the next analysis. The total run time was 17 min.

Liquid Chromatography. After automatic sample cleanup (see Table 1 at the 2.0 min time point), the sample was automatically transferred onto a C18 column (150 mm × 2.1 mm i.d., 5 μm, ODS-3, Inertsil). The column was washed with a gradient from 100% solvent IIa to 90% sovlent IIa for 1.5 min for eluting the 5-meC to the analytical column. At 5.5 min after injection, the same mobile phase was applied for 2 min to elute the 5-medC. The column was then washed using eluent II with a gradient from 90% solvent IIa to 0% solvent IIa and back to 100% solvent IIa.

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Table 1. Timetable for the Column-Switching Procedure

	eluent I (trap column)		eluent II (analytical column)				
time (min)	solvent Ia[a] (%)	solvent Ib[b] (%)	solvent IIa[a] (%)	solvent IIb[b] (%)	valve position	flow rate (μL/min)	remarks
0	100	0	100	0	A	200	injection and washing of sample
2.0	100	0	100	0	B	200	start of elution of 5-meC to the analytical column
3.5	90	10	90	10	A	200	end of elution, washing continued
5.5	90	10	90	10	B	200	start of elution of 5-medC to the analytical column
7.5	0	100	90	10	A	200	end of elution; trap column cleanup and reconditioning
11.4	0	100	90	10	A	200	
11.5	100	0	90	10	A	200	
14	100	0	0	100	A	200	
15	100	0	100	0	A	200	
17	100	0	100	0	A	200	

[a]5% (v/v) MeOH/3 mM AA. [b]50% (v/v) MeOH/3 mM AA.

Electrospray Ionization MS/MS. The sample eluting from the HPLC system was introduced into a TurboIonspray source installed on an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems), operating in the positive mode with a needle voltage of 5.0 kV, using nitrogen as the nebulizing gas and with the turbogas temperature set at 400 °C. Data acquisition and quantitative processing were accomplished with Analyst software, Ver. 1.4 (Applied Biosystems). For all of the samples, the $[M + H]^+$ ion was selected by the first mass filter. After collisional activation, two fragment ions were selected as follows: the most abundant fragment ion was used for quantification (quantifier ion), and the second most abundant ion was used for qualification (qualifier ion). Optimal multiple reaction monitoring (MRM) conditions were obtained for six channels: 5-meC (m/z 126 → 83 and 126 → 109), d_3-5-meC (m/z 129 → 112), 5-medC (m/z 242 → 126 and 242 → 109), and d_3-5-medC (m/z 245 → 129). The dwell times per channel were set at 200 ms both for the analytes and the internal standards. The optimized electrospray ionization (ESI) conditions were as follows: nebulizer gas flow, 8; curtain gas flow, 8; collision-assisted dissociation (CAD) gas flow, 4; turbo gas flow, 8; collision energy (CE), 25 V for both quantifier and qualifier ions of 5-meC, 30 V for d_3-5-meC, 15 V for quantifier ion, 45 V for qualifier ion of 5-medC, and 25 V for d_3-5-medC; declustering potential (DP) voltage, 40 V for 5-meC, 50 V for d_3-5-meC, and 20 V for both 5-medC and d_3-5-medC; focusing potential (FP) voltage, 200 V for 5-meC, 180 V for d_3-5-meC, 120 V for 5-medC, and 110 V for d_3-5-medC; and entrance potential (EP) voltage, 10 V. Peak full-width at half-maximum was set to 0.7 Th (Thomson = 1 amu per unit charge) for both Q1 and Q3.

Urinary Analysis of Methylated and Oxidized Lesions Using Online SPE LC-MS/MS. Urinary N7-meG and N3-meA concentrations were determined, respectively, using two previously described methods of online SPE LC-MS/MS.[25,26] The detection limits of methods were 8.0 (4.8 fmol) and 35 pg/mL (11.7 fmol) on-column for N7-meG and N3-meA, respectively.

Urinary concentrations of 8-oxoGua and 8-oxodG were measured using also a previously reported method of online SPE LC-MS/MS.[27] The detection limits of this method were 50 (30 fmol) and 10 pg/mL (3.5 fmol) on-column for 8-oxoGua and 8-oxodG, respectively.

Furthermore, urinary cotinine, a major metabolite of nicotine, has been widely used as a biomarker of tobacco exposure and was measured by an isotope-dilution LC-MS/MS method previously described by Hu et al.[28] The concentrations of modified DNA bases and nucleosides and cotinine were adjusted to the urinary concentration of creatinine (ng/mg creatinine) to control for variation in urinary output. Urinary creatinine was measured for each sample using a HPLC-UV method described by Yang.[29]

Statistical Methods. Means and SDs were used to describe the distributions of urinary-modified bases and nucleosides, cotinine, and the demographic data for study subjects. The data were analyzed using the SAS statistical package (SAS, version 9.1). The Mann−Whitney test was used to compare the continuous variables among groups. Spearman correlation coefficients were used to study the relationship of urinary 5-meC (or 5-medC) concentrations to various methylated or oxidized lesions concentrations. In multiple linear regression models, the relationships of urinary 5-meC (or 5-medC) concentrations to various methylated and oxidized lesions concentrations were investigated after adjusting for other variables (i.e., age, BMI and urinary cotinine). Meanwhile, these modified bases and nucleosides might also be affected by diet. However, because of a large sample size in our study, it was difficult to conduct a controlled diet experiment or dietary survey (if subjects on free-choice diets). The diet impact was therefore not included in the regression models.

RESULTS

Simultaneous Determination of 5-MeC and 5-MedC in Human Urine Using Online SPE LC-MS/MS. *Chromatography and Mass Spectra.* A typical online SPE LC-MS/MS chromatogram for 5-meC and 5-medC in the urine of a nonsmoker is shown in Figure 2. Detailed product ion spectra of 5-meC, 5-medC, and their isotope internal standards are also given in Figure S1 in the Supporting Information. The positive ESI mass spectrum of 5-meC contained a $[M + H]^+$ precursor ion at m/z 126 and product ions at m/z 83 (quantifier ion, Figure 2A) and m/z 109 (qualifier ion, Figure 2B) due to loss of NH_3 and CN groups or NH_3 alone; a precursor ion at m/z 129 and product ion at m/z 112 characterized the d_3-5-meC (Figure 2C). Because the coeluting interference was occasionally observed at the first most abundant fragment ion (m/z 109), a second abundant fragment ion (m/z 83) was used as quantifier ion for 5-meC quantification. Meanwhile, the $[M + H]^+$ precursor ion of 5-medC was at m/z 242, and product ions appeared at m/z 126 (quantifier ion, Figure 2D) and m/z 109 (qualifier ion, Figure 2E), resulting from the loss of the neutral 2′-deoxyribose moiety or together with NH_3; a precursor ion at m/z 245 and product ion at m/z 129 characterized the d_3-5-medC (Figure 2F). The retention times were 7.2 and 13.6 min for 5-meC and 5-medC, respectively. However, it was found that the deuterated internal standards eluted slightly earlier than the analytes, and this could be attributed to the altered hydrophilic nature of the internal standards labeled with three deuterium atoms, which was known as "deuterium isotope effect" during reversed phase LC separation.[30] Interestingly, it was noted that the transitions of m/z 126 → 83 and 126 → 109 (as well as the d_3-5-meC internal standard, m/z 129 → 112) were also detected at the retention time (13.6 min), corresponding to 5-medC (Figure 2), suggesting that an

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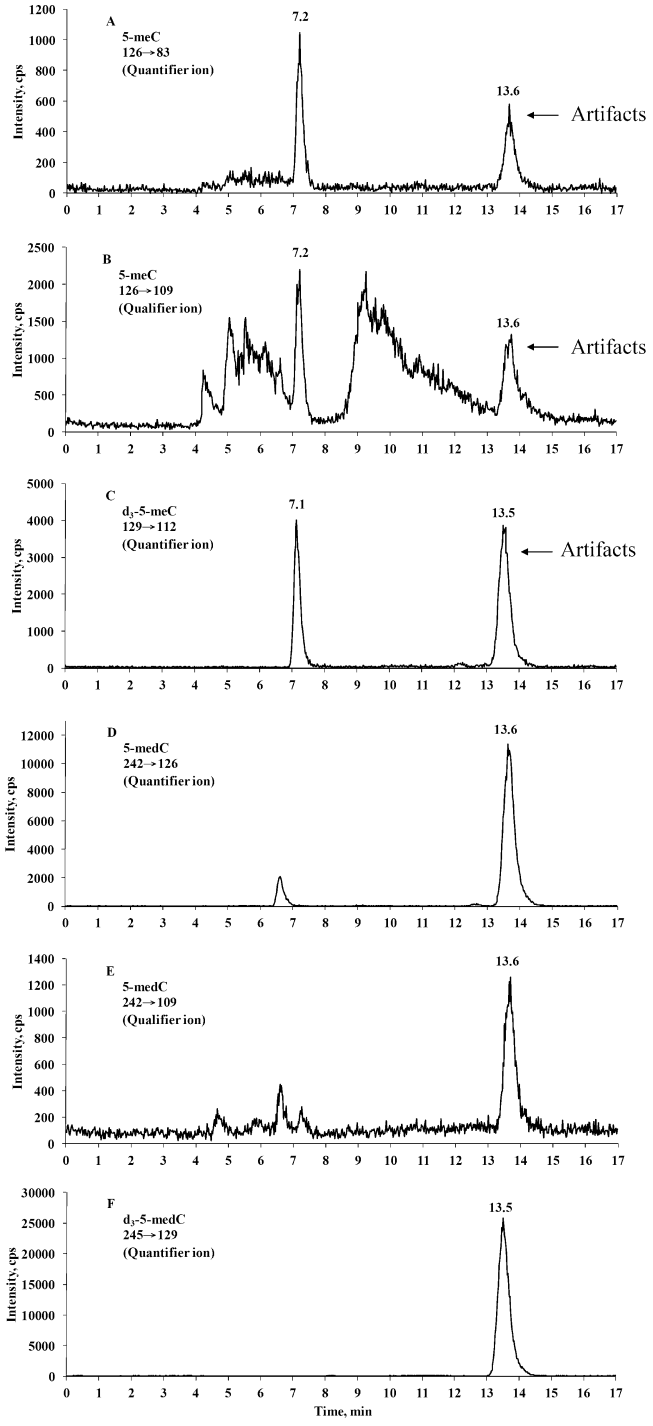

Figure 2. Chromatograms of 5-meC and 5-medC in human urine, as measured by LC-MS/MS coupled with online SPE. Multiple reaction monitoring transitions of m/z 126 → 83 (A) and m/z 126 → 109 (B) for 5-meC, m/z 129 → 112 (C) for d_3-5-meC, m/z 242 → 126 (D) and m/z 242 → 109 (E) for 5-medC, and m/z 245 → 129 (F) for d_3-5-medC. cps, counts per second.

artifactual 5-meC was generated during the ionization process. The false response of 5-meC was confirmed by direct injection of a 5-medC standard solution only (see Figure S2 in the Supporting Information), showing that the transitions of 5-meC (m/z 126 → 83 and 126 → 109) eluted at the time corresponding to 5-medC. This is because 5-medC is a fragile molecule that tends to lose its 2-deoxyribose moiety to become 5-meC during ion transfer from the ion source to Q0, probably due to in-source thermolysis and/or collisionally induced dissociation. With our online SPE LC-MS/MS method, the artifactual formation of 5-meC from 5-medC during ion transfer from the ion source to Q0 was estimated to be around 0.53−0.74 pmol generated per picomole of 5-medC analyzed. A similar phenomenon was also previously reported by Hu et al.[27]

Limit of Quantification and Limit of Detection. The limit of quantification (LOQ) was defined as the lowest concentration that could be reliably and reproducibly measured with values for accuracy and intra- and interday imprecision [CV (coefficient of variation) < 20%]. Using this method, the LOQs of 5-meC and 5-medC were determined to be 0.075 ng/mL on column (3.8 pg in a 50 μL injection volume) and 0.02 ng/mL on column (1 pg), respectively, based on direct measurement of diluted calibration solutions. The limits of detection (LODs), defined as the lowest concentration that gave a signal-to-noise ratio of at least 3, were 0.024 (1.2 pg) and 0.006 ng/mL on column (0.3 pg) for 5-meC and 5-medC, respectively.

Linearity, Precision, and Recovery. Two linear calibration curves covering the low concentration range (0.006−0.094 ng) and the high concentration range (0.094−1.5 ng) were obtained by dilution of aqueous standard solutions. Each calibrator contained 0.9 ng of d_3-5-meC and 1.2 ng of d_3-5-medC. Linear regression was calculated with nonweighting and nonzero-forced, and the linear equations were obtained: low range, $y = 0.4760x + 0.0018$ ($r^2 = 0.9996$), and high range, $y = 0.4675x + 0.0057$ ($r^2 = 0.9999$) for 5-meC, and low range, $y = 1.608x - 0.0001$ ($r^2 = 1$), and high range, $y = 1.5242x + 0.0111$ ($r^2 = 0.9999$) for 5-medC. Over the entire concentration range of the calibration curves, the mean observed percentage deviation of back-calculated concentrations was between −4.3 and 1.4% for 5-meC and −5.6 and 5.9% for 5-medC with an imprecision (CV) < 10%. For 5-meC and 5-medC in urine, the peak identity was also confirmed by comparing the peak area ratios (quantifier/qualifier) with those of the calibrators. As an acceptance criterion, ratios in urine samples should not deviate by more ±25% from the mean ratios in the calibrators.

The precision of this method was evaluated by performing replicate determinations of 5-meC and 5-medC in three different urine samples (see Table 2). The intraday imprecision was 2.4−3.8% for 5-meC and 0.5−2.9% for 5-medC. The interday test was carried out by assaying the same sample on 5 different days over a period of 50 days. The interday imprecision of 5-meC and 5-medC was determined to be 3.3−5.4 and 5.3−8.3%, respectively.

Recovery was evaluated by adding unlabeled 5-meC (0, 1.13, 2.25, 4.5, and 9 ng) and 5-medC (0, 1.2, 2.4, 4.8, and 9.6 ng for 5-medC) at five amounts to a crude urine sample. As shown in Table 2, the recovery of this method, as calculated from the slope of the regression, was 113% for 5-meC and 116% for 5-medC ($r^2 > 0.99$), and the mean recovery was 108 and 112% for 5-meC and 5-medC, respectively, as estimated from the increase in the measured amounts after addition of 5-meC (or 5-medC) divided by the amounts added.

Matrix Effects. Matrix effects were calculated from the peak areas of the internal standard added to the calibration solutions and compared with the peak areas of the internal standard that was added to each urinary sample. The relative change in peak area of the internal standard was attributed to matrix effects, which reflect online extraction losses and ion suppression due to the urinary matrix. In this study, the matrix effects were less

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Table 2. Precision and Recovery of Isotope Dilution LC-MS/MS with Online SPE for Urinary 5-MeC and 5-MedC Analysis

	5-meC			5-medC		
	urine 1	urine 2	urine 3	urine 1	urine 2	urine 3
			precision[a]			
intraday variation (mean ± SD, ng/mL) (CV, %)	15.2 ± 0.5 (3.3)	24.0 ± 0.9 (3.8)	54.4 ± 1.3 (2.4)	3.5 ± 0.1 (2.9)	10.3 ± 0.1 (1.0)	19.6 ± 0.1 (0.5)
interday variation (mean ± SD, ng/mL) (CV, %)	15.0 ± 0.8 (5.3)	24.1 ± 1.8 (3.3)	54.0 ± 2.9 (5.4)	3.7 ± 0.3 (8.1)	10.8 ± 0.9 (8.3)	20.9 ± 1.1 (5.3)
			recovery[b]			
slope of regression	c	1.13			1.16	
mean (SD) recovery (%)		108 (3.2)			112 (7.5)	

[a]Each urine analysis was repeated five times for the intraday and interday tests; the interday test was carried out over a period of 50 days. [b]Recovery of 5-meC and 5-medC in urine was estimated by the addition of 5-meC and 5-medC standard mixture in five different amounts (0, 1.13, 2.25, 4.5, and 9 ng for 5-meC; 0, 1.2, 2.4, 4.8, and 9.6 ng for 5-medC) to a crude urine sample. The recovery was estimated from (a) the slope of the regression of measured 5-meC (or 5-medC) vs added 5-meC (or 5-medC) and (b) the increase in measured amounts after addition of 5-meC (or 5-medC) divided by the amounts that were added. [c]Not measured.

than 50% for 5-meC and 5-medC in all urine samples. Although the use of stable isotope-labeled internal standard could have compensated for different matrix effects, a low matrix effect achieved in this study ensures a high sensitivity of the method.[31]

Urinary Excretion of 5-MeC, 5-MedC, N7-MeG, N3-MeA, 8-OxoGua, and 8-OxodG in Healthy Male Subjects. The characteristics of 376 participants and the modified bases and nucleosides concentrations are summarized in Table 3.

Table 3. Overall Characteristics of Study Subjects

variable	smokers (n = 160)	nonsmokers (n = 216)	P value[b]
age, years	33.4 ± 6.6[a]	31.9 ± 6.4	0.01
BMI (kg/m^2)	23.5 ± 4.0	24.0 ± 4.0	0.23
cotinine (ng/mg creatinine)	992 ± 867	0.43 ± 2.31	<0.001
	(1238 ± 789)[c]	(0.90 ± 3.41)	
	(636 ± 557)[d]	(0.27 ± 1.47)	
5-meC (ng/mg creatinine)	28.7 ± 14.5	28.3 ± 14.2	0.70
	(46.2 ± 35.1)	(42.9 ± 31.4)	
	(25.9 ± 13.1)	(25.6 ± 12.8)	
5-medC (ng/mg creatinine)	7.04 ± 8.16	7.04 ± 6.47	0.17
	(11.4 ± 13.3)	(11.3 ± 13.2)	
	(3.30 ± 3.8)	(3.30 ± 3.0)	
N7-meG (ng/mg creatinine)	3644 ± 969	3598 ± 865	0.56
	(6172 ± 3924)	(5842 ± 3745)	
	(2495 ± 663)	(2464 ± 592)	
N3-meA (ng/mg creatinine)	5.60 ± 5.7	4.14 ± 7.0	<0.001
	(8.43 ± 11.2)	(5.44 ± 7.7)	
	(4.25 ± 4.4)	(3.14 ± 5.3)	
8-oxoGua (ng/mg creatinine)	10.3 ± 7.0	12.0 ± 6.0	<0.001
	(17.9 ± 18.8)	(19.0 ± 14.2)	
	(6.93 ± 4.8)	(7.78 ± 4.3)	
8-oxodG (ng/mg creatinine)	3.72 ± 1.7	3.30 ± 1.3	0.014
	(6.1 ± 4.1)	(5.4 ± 3.7)	
	(1.48 ± 0.7)	(1.32 ± 0.5)	

[a]Mean ± SD. [b]The comparisons were made among the groups by Mann–Whitney test. [c]Concentration expressed in ng/mL. [d]Concentration expressed in nmol/mmol.

Smokers were slightly order than nonsmokers (P = 0.01), but they were similar in BMI (P = 0.23). As compared with nonsmokers, smokers had similar urinary concentrations for 5-meC, 5-medC, and N7-meG but had significantly higher urinary concentrations for cotinine, N3-meA, and 8-oxodG (P < 0.05). Meanwhile, urinary concentrations of N3-meA and 8-oxodG were found to be positively associated with urinary cotinine concentrations (Spearman correlation coefficients, r = 0.35, P < 0.001, for N3-meA and r = 0.15, P = 0.003, for 8-oxodG). No significant correlations were observed between urinary N7-meA, 5-meC, 5-medC, and urinary cotinine. However, in multiple linear regressions, urinary N3-meA, 8-oxodG, and N7-meG were all found to be highly associated with urinary cotinine concentrations after adjustment for age and BMI (n = 376, P = 0.02 for N7-meG, P < 0.001 for N3-meA, and P < 0.001 for 8-oxodG). There was no significant correlation between urinary 5-meC (or 5-medC) and urinary cotinine after adjustment for age and BMI.

Correlation between 5-MeC (or 5-MedC) and Methylated or Oxidized Lesions. The association among 5-meC, 5-medC, N7-meG, N3-meA, 8-oxoGua, and 8-oxodG was analyzed using Spearman correlation coefficients. The urinary concentrations of 5-medC, N7-meG, N3-meA, 8-oxoGua, and 8-oxodG were found to be individually positively associated with the urinary 5-meC (n = 376, r = 0.15, P = 0.0043 for 5-medC; r = 0.16, P = 0.0015 for N7-meG; r = 0.49, P < 0.001 for N3-meA; r = 0.19, P < 0.001 for 8-oxoGua; r = 0.14, P = 0.0082 for 8-oxodG). In multiple linear regressions (see Table 4), the correlations between urinary 5-meC concentrations and the concentrations of modified bases and nucleosides (i.e., 5-medC, N7-meG, N3-meA, and 8-oxodG) were not confounded by other variables, including age, BMI, and cotinine concentrations (P < 0.05). Interestingly, multiple linear regression analysis revealed no significant correlations between urinary 5-medC and any methylated or oxidized lesions measured after adjustment for age, BMI, and cotinine.

DISCUSSION

In this study, we reported a sensitive and reliable LC-MS/MS method for the direct and simultaneous determination of 5-meC and 5-medC in urine. With the use of isotope internal standards and online SPE, this method exhibited low LODs of 1.2 and 0.3 pg on column for 5-meC and 5-medC, respectively.

5-MedC was first detected in urine using an inhibition ELISA method;[12] however, because of the fact that ELISA may suffer from poor selectivity and sensitivity, later urinary measurements of 5-medC were developed mainly based on the chromatographic techniques. Zambonin et al. developed the HPLC-UV[13] and LC-ion trap MS/MS[32] methods following off-line SPE cleanup and reported LODs of 4000 and 250 pg, respectively.

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Table 4. Multivariate Regression Analysis for Urinary 5-MeC

variables	b(SE)[a]	P	b(SE)[b]	P	b(SE)[c]	P	b(SE)[d]	P
age	0.132 (0.113)	0.246	0.127 (0.113)	0.260	0.160 (0.100)	0.109	0.102 (0.114)	0.373
BMI	0.190 (0.186)	0.307	0.178 (0.185)	0.338	0.130 (0.165)	0.427	0.179 (0.186)	0.336
cotinine	0.001 (0.001)	0.229	0.001 (0.001)	0.355	−0.001 (0.001)	0.399	0.001 (0.001)	0.458
5-medC	0.217 (0.100)	0.030						
N7-meG			1.845 (0.806)	0.023				
N3-meA					1.040 (0.100)	<0.001		
8-oxodG							1.029 (0.513)	0.046

[a]Regression model 1 (variables: age, BMI, cotinine, and 5-medC). [b]Regression model 2 (variables: age, BMI, cotinine, and N7-meG). [c]Regression model 3 (variables: age, BMI, cotinine, and N3-meA). [d]Regression model 4 (variables: age, BMI, cotinine, and 8-oxodG).

Similarly, Lee et al.[33] described also a LC-ion trap MS/MS method based on direct urine injection and had a LOD of 2400 pg. Apparently, our newly developed online sample purification/enrichment system coupled with isotope dilution LC-triple quadrupole MS/MS has a better sensitivity than previously reported methods. More importantly, this is the first chromatography method developed to directly and simultaneously determine both 5-medC and 5-meC in urine, which could be helpful to understand aberrant global DNA methylation in disease scenarios.

Interestingly, there is a discrepancy in the findings of urinary 5-medC levels in the literature. Itoh et al.[12] reported that the mean urinary levels of 5-medC were 1900 and 3600 ng/mg creatinine for healthy individuals and leukemia patients, respectively, using the ELISA assay. Lee et al.[14] also observed the mean urinary concentrations of ∼559 ng/mg creatinine for both healthy subjects and patients with Alzheimer's disease, using LC-ion trap MS/MS method. Contrary to these findings, Zambonin et al. used the LC-UV[13] or LC-ion trap MS/MS[32] methods and found that no 5-medC could be detected in the urines of both healthy individuals and leukemia patients. The authors therefore concluded that if 5-medC really is present in urine, its level must be below 50 ng/mL (roughly below 28 ng/mg creatinine after adjustment by a baseline creatinine level of 1.8 mg/mL) of LOD even in leukemia patients. In the present study, the 5-medC was detectable in all urine samples, and its mean level was 7.04 ng/mg creatinine (0.13−76 ng/mL) for healthy subjects, which was in accordance with previous speculation described by Zambonin et al.[32] Furthermore, the methylated base of cytosine, 5-meC, was for the first time identified in human urine in this study (Figure 2 and Table 3), existing in relatively high concentrations (3.8−197 ng/mL). Our finding may support the hypothesis that 5-meC in DNA could be removed by BER pathway initiated by DNA glycosylase[4,5,8] and further excreted in the urine.

Tobacco smoke is causally associated with cancers of the upper aerodigestive tract, lung, and bladder.[34,35] Tobacco-specific nitrosamines (TSNAs) are a group of carcinogens present in tobacco and tobacco smoke that are formed from nicotine and related tobacco alkaloids. The metabolic activation of TSNAs (e.g., 4-methylnitrosamino-1-3-pyridyl-1-butanone, NNK) and other tobacco *N*-nitrosamines in target tissues can result in the formation of methylated DNA.[36] As the N-7 position of guanine and N-3 position of adenine are the predominant reaction sites, both N7-meG and N3-meA have been proposed as useful markers of exposure to endogenous and exogenous methylating agents.[37] Although N7-meG and N3-meA are not considered to be promutagenic, they are removed from DNA either by spontaneous depurination or by the action of glycosylases to produce apurinic sites, followed by excretion in urine. If not repaired, apurinic sites can potentially cause mutations in mammalian cells.[38] Furthermore, urinary N7-meG could derive substantially from tRNA turnover,[39] while urinary N3-meA may also originate from the diet.[40] In the present study, both urinary N7-meG and N3-meA were positively associated with urinary cotinine although the urinary N3-meA has shown even better correlation with cotinine than that of N7-meG, suggesting that N3-meA was a more sensitive marker for the noninvasive evaluation of human exposure to methylating agents than N7-meG because of the low background level of N3-meA.[41,42] Meanwhile, tobacco smoke also contains various ROS and compounds (e.g., polycyclic aromatic hydrocarbons) that can generate oxygen free radicals during metabolic activation[34] and cause oxidatively damaged DNA. This study measured both urinary 8-oxoGua and 8-oxodG that represent the repair products of oxidatively damaged DNA in vivo, presumably through the BER and NER pathway, respectively, although urinary 8-oxodG may possibly also originate from enzymatic hydrolysis (i.e., MTH1 protein) of oxidized guanine nucleoside 5′-phosphates in the nucleotide pool.[43] Unlike urinary N3-meA, urinary 8-oxoGua and 8-oxodG levels do not depend on diet.[44] This study found that urinary 8-oxodG was positively correlated with urinary cotinine, which is in line with our previous finding.[45] It was however noted that urinary 8-oxoGua representing the results of the major pathway for repairing oxidatively damaged DNA[46] was not correlated to urinary cotinine in this study. This could have reflected the previous finding showing that both smokers and nonsmokers had a similar OGG activity for the BER pathway.[47] Furthermore, no significant correlation between urinary 8-oxoGua and 8-oxodG was observed in the present study that was different from a previous study reported by Foksinski et al.[48]

Met is an essential amino acid in protein biosynthesis and is also the precursor for the synthesis of SAM catalyzed by methionine adenosyltransferase. Met residues of proteins, however, are susceptible to oxidation by almost forms of ROS that resulted in the formation of MetO,[17] and the MetO/Met ratio in proteins was also found to be increased with age, inflammation, and smoking.[17,49] Meanwhile, it has been recently shown that in the presence of MetO, the increase in hydroxyl radicals production could mediate the formation of methyl radicals, leading to potential chemical DNA hypermethylation.[18] Although it was reported that 2′-deoxyguanosine is alkylated by carbon-centered radicals (methyl radicals) mostly to C8-methyl-2′-deoxyguanosine,[19] a recent study has shown that the methylation of cytosine at the C5 position was even higher than that of the C8 position of guanine in DNA probably due to a steric effect.[18] On the other hand, SAM, the major methyl donor for enzymatic DNA methylation, is also

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able to methylate DNA nonenzymatically to produce the methylated DNA lesions (e.g., N7-meG, N3-meA, or O^6-meG). On the basis of the literatures and our findings that significant positive associations between urinary 5-meC, N7-meG, N3-meA, and 8-oxodG, a hypothetical mechanism was therefore proposed as provided in Figure S3 in the Supporting Information.

When humans are exposed to tobacco smoke that contains oxygen free radicals and TSNAs, it could induce oxidized (e.g., 8-oxodG) and methylated DNA lesions (e.g., N7-meG and N3-meA), and in the meantime, oxygen free radicals (i.e., hydroxyl radicals) may also trigger the formation of methyl radicals and further nonenzymatical DNA methylation (5-meC). Although previous studies have shown that smoking was associated with aberrant DNA methyation either global hypomethylation in whole blood[50] or site-specific DNA hypermethylation of tumor suppressor genes,[51] no significant association between urinary 5-meC (or 5-medC) and urinary cotinine was observed in this study. It could be because that measurement of urinary 5-meC and 5-medC reflected the results of cellular DNA excision repair process in the "whole body" and may not be interpreted as epigenetic changes within regional or individual gene.

In conclusion, this study describes a simple, rapid, and reliable LC-MS/MS method for direct and simultaneous determination of urinary 5-meC and 5-medC. With the use of isotopic internal standards and online SPE, this method could allow for high-throughput analysis of urinary 5-meC and 5-medC without compromising quality and validation criteria. By using this method, 5-meC was first identified in human urine, and its concentration was four times higher than that of 5-medC. There, however, could be dietary and other potential endogenous sources (e.g., 5-meC from RNA,[52] 5-medC from MutT-type enzymatic hydrolysis of 5-methyl-dCTP in the nucleotide pool[53]) contributing to 5-meC or 5-medC in urine that need to be investigated. Urinary 5-meC was found to be highly associated with urinary methylated (i.e., N7-meG and N3-meA) and oxidized DNA lesions (i.e., 8-oxodG) after adjustment for age, BMI, and cotinine. Good correlations observed may imply a possible association between genetic (DNA lesions) and epigenetic (DNA methylation) alterations derived from the exposure to environmental carcinogens and/or from endogenous cellular processes in human. However, because these modified bases and nucleosides measured in this study could be also affected by diet or other endogenous sources, such a hypothesis should be regarded as tentative and warrants further investigation. Taken together, the measurement of 5-meC and 5-medC in urine may help to assess changes of DNA methylation status in the whole body and to investigate the DNA demethylation mechanism in vivo.

■ ASSOCIATED CONTENT

Supporting Information

Figures of product ion spectra, chromatograms, and hypothetical association between DNA methylation and DNA lesions. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mrchao@csmu.edu.tw.

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Notes

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■ ABBREVIATIONS

5-hmeC, 5-hydroxymethylcytosine; 5-meC, 5-methylcytosine; 5-medC, 5-methyl-2′-deoxycytidine; 8-meG, C8-methylguanine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; BER, base excision repair; ESI, electrospray ionization; LC-MS/MS, liquid chromatography−tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; Met, methionine; MetO, methionine sulfoxide; N3-meA, N3-methyladenine; N7-meG, N7-methylguanine; NER, nucleotide excision repair; ROS, reactive oxygen species; SAM, *S*-adenosylmethionine; SPE, solid-phase extraction; TSNAs, tobacco-specific nitrosamines

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