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Quantitative determination of urinary N3-methyladenine by isotope-dilution LC–MS/MS with automated solid-phase extraction

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ABSTRACT

A rapid, highly specific and sensitive liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method was developed for measuring urinary N3-methyladenine (N3-MeA). With the use of an isotope internal standard (d_3-N3-MeA) and on-line solid-phase extraction (SPE), urine samples can be directly analyzed within 12 min without prior sample purification. The detection limit of this method was estimated as 0.035 ng/mL on-column (11.7 fmol). Intra- and inter-day imprecision (CV) were <7.4% and <10.8%, respectively. Mean recovery of N3-MeA in urine was 97–101%. This method was further applied to study the methylating agents exposure arising from cigarette smoke. Eighty-six volunteers were recruited including 41 regular smokers and 45 nonsmokers. The results showed that the urinary levels of N3-MeA observed in smokers (10.9 ± 13.2 ng/mg creatinine) were significantly higher than those in nonsmokers (4.1 ± 4.3 ng/mg creatinine; $P < 0.001$). It was further noted that the urinary level of N3-MeA was found to be highly associated with cotinine for smokers (Spearman correlation coefficients, $r = 0.69$, $P < 0.001$), implying that cigarette smoking resulted in increased DNA methylation, followed by depurination and excretion of N3-MeA in urine. As a result of the on-line SPE system, this method is capable of routine high-throughput analysis and accurate quantification of N3-MeA, and would be a useful tool for the surveillance of methylating agent exposure and its associated cancer risk.

1. Introduction

Human exposure to carcinogenic agents can lead to the formation of covalently bound adducts in DNA. The formation of such DNA adducts is believed to be the earliest indicator of biologically effective dose arising from carcinogen exposure. Exposure to methylating agents can result in the formation of a number of methylated base residues in DNA including those methylated at the O^6, N^2, N-7 and N-3 positions of guanine, the N-7 and N-3 positions of adenine, the O^2 position of cytosine and the O^2 and O^4 position of thymine [1]. As the N-7 position of guanine and N-3 position of adenine are the predominant reaction sites, N7-methylguanine (N7-MeG) and N3-methyladenine (N3-MeA) have been proposed as useful markers of exposure to endogenous and exogenous methylating agents such as *N*-nitrosamines [2]. 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 [3].

Substantial amounts of N7-MeG are present in urine since N7-MeG is derived in large part from tRNA turnover. Alternatively, urinary N3-MeA could be a more sensitive marker for the non-invasive evaluation of human exposure to methylating agents than N7-MeG, because background levels are low [4]. A variety of analytical techniques have been developed for quantification of urinary N3-MeA including high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [5], immunoassay [6], and gas chromatography–mass spectrometry (GC–MS) [7–9]. Although these methods have been successful in analysis of N3-MeA, they have drawbacks such as being labor intensive, requiring chemical derivatization, and low sensitivity or limited specificity due to possible interferences arising from the complex biological matrix (i.e., crude urine).

Recently, liquid chromatography with tandem mass spectrometry (LC–MS/MS) has become a powerful technology to overcome the sensitivity and specificity issues in analysis of DNA adducts [10]. Accurate quantification of adducted bases at extremely low concentrations has frequently relied on the use of stable isotope-labeled standards to compensate for the loss of analyte during

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sample preparation, which has been the most critical step in eliminating the matrix effect for analysis of modified bases by mass spectrometry [11]. Moreover, the on-line sample extraction using a column-switching device is an extremely useful technique to get the biological samples prepared automatically for LC–MS methods [12]. Its advantages include less ion suppression, relatively short run time as well as higher sensitivity and selectivity, especially for the biological samples containing a considerable amount of coeluting interferences.

However, the use of column-switching (on-line solid-phase extraction, SPE) with LC–MS to investigate alkylated DNA adducts has been limited. To our knowledge, no on-line extraction chromatographic method has been presented to date that enables direct monitoring of trace levels of N3-MeA in urine. In this study, an isotope-dilution LC–MS/MS method coupled with on-line SPE system was developed for direct and sensitive analysis of urinary N3-MeA. This method was then applied to investigate the urinary concentrations of N3-MeA in smokers and nonsmokers, and their association with cotinine concentrations.

2. Experimental

2.1. Chemicals

Solvents and salts were of analytical grade. Reagents were purchased from the indicated sources: N3-methyladenine (see Fig. 1) and cotinine (Sigma–Aldrich, St Louis, MO); d_3-N3-methyladenine (d_3-N3-MeA, Fig. 1) and d_3-cotinine (Cambridge Isotope Laboratories, Andover, MA).

2.2. Urine samples

This study was approved by the Institutional Review Board of Chung Shan Medical University Hospital. Urine samples were obtained from 86 apparently healthy males (41 regular smokers and 45 nonsmokers). A questionnaire was used to obtain data on subject age, body mass index (BMI), and the smoking status (self-reported daily cigarette consumption). Urine samples were kept at 4 °C during sampling, and stored at –20 °C prior to analysis. Urinary creatinine was also measured for each sample using a HPLC-UV method described by Yang [13].

For urinary N3-MeA analysis, the urine samples were thawed, vortexed and then heated to 37 °C for 10 min to release possible N3-MeA from precipitate. After centrifugation at 5000 g for 5 min, 20 μL of urine was diluted 10 times with 3% (v/v) methanol (MeOH)/0.1% (v/v) trifluoroacetic acid (TFA). A 100 μL aliquot of diluted urine was spiked with 20 μL of d_3-N3-MeA solution (20 ng/mL in 3% MeOH/0.1% TFA) as internal standard, and then mixed by vortex for approximately 5 s. The N3-MeA standard stock solution was prepared by dissolving N3-MeA in 10% (v/v) MeOH; it was then freshly diluted in 3% (v/v) MeOH/0.1% (v/v) TFA to yield aqueous solutions for establishing the calibration curve. Because N3-MeA is usually present in urine, there was no blank matrix available for matrix-matched calibration in this study. However, the use of an isotope-labeled coeluting internal standard should compensate for the effects of electrospray ionization (ESI) suppression by other matrix components.

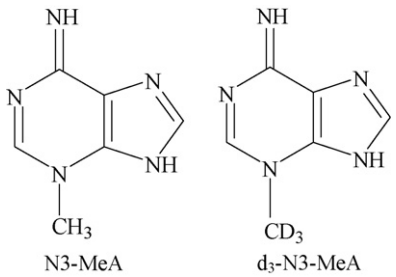

Fig. 1. Chemical structure of N3-MeA and d_3-N3-MeA.

2.3. Automated on-line extraction system and liquid chromatography

The column-switching system was described in detail in a previous publication [12]. It consisted of a switching valve (two-position microelectric actuator, Valco) and a C18 trap column (33 mm × 2.1 mm i.d., 5 μm, Inertsil, ODS-3). The switching valve function was controlled using PE-SCIEX control software (Analyst; Applied Biosystems). The detailed column-switching operation sequence is summarized 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 3% (v/v) MeOH/0.1% (v/v) TFA at a flow rate of 0.2 mL/min as the loading and washing buffer (Solvent Ia). The trap column was flushed with the loading buffer for 1.5 min, followed by valve switching to injection position B to inject the sample (enriched N3-MeA) into the LC system. At 3 min after injection, the valve was switched back to the loading position A, and the trap column was washed using a mobile phase (Eluent I) with a gradient from 100% of 75% (v/v) MeOH/0.1% (v/v) TFA (Solvent Ib) to 100% Solvent Ia for 5 min, followed by 100% Solvent Ia for 4 min for equilibration of the column and preparation for the next analysis. The total run time was 12 min.

After automatic sample cleanup (see Table 1 at the 1.5 min time point), the sample was automatically transferred onto a C18 column (150 mm × 2.1 mm i.d., 5 μm, Inertsil, ODS-3). The mobile phase was 3% (v/v) MeOH/0.1% (v/v) formic acid (FA) (Solvent IIa) and delivered at a flow rate of 0.2 mL/min for 1.5 min for eluting the N3-MeA to the analytical column. At 6.0 min after injection, the mobile phase was varied to 90% Solvent IIa and 10% of 75% (v/v) MeOH/0.1% (v/v) FA (Solvent IIb). The column was then washed using an Eluent II with a gradient from 90% Solvent IIa to 0% Solvent IIa and rapidly back to 100% Solvent IIa.

2.4. Electrospray ionization tandem mass spectrometry (ESI-MS/MS)

The sample eluted from the HPLC system was introduced into a Turbolonspray source installed on an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems) operating in the positive mode with a needle voltage of 5.5 kV, using nitrogen as the nebulizing gas and with the turbogas temperature set at 400 °C. Data acquisition and quantitative processing were accomplished using Analyst software, version 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: 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 three channels: m/z 150 → 123 (quantifier ion) and 150 → 108 (qualifier ion) for N3-MeA, and m/z 153 → 126 for d_3-N3-MeA. The dwell times per channel were set at 100 ms for both the analyte and internal standard. The optimized ESI conditions were as follows: nebulizer gas flow, 8; curtain gas flow, 8; collision-induced dissociation (CID) gas flow, 12; turbo gas flow, 8; collision energy (CE), 30 V for quantifier ion and 40 V for qualifier ion with nitrogen as the collision gas; declustering potential (DP) voltage, 50 V; focusing potential (FP) voltage, 200 V; entrance potential (EP) voltage, 10 V. Peak full-width at half-maximum was set to 0.7 Th (Thompson = 1 amu per unit charge) for both Q1 and Q3.

Table 1
Timetable for the column-switching procedure.

Time (min)	Eluent I (trap column)		Eluent II (analytical column)		Valve position	Flow rate (mL/min)	Remarks
	Solvent Ia[a] (%)	Solvent Ib[b] (%)	Solvent IIa[c] (%)	Solvent IIb[d] (%)			
0	100	0	100	0	A	0.2	Injection and washing of sample
1.5	100	0	100	0	B	0.2	Start of elution to the analytical column
2	100	0	100	0	B	0.2	
3	0	100	100	0	A	0.2	End of elution; trap column cleanup and reconditioning
4	0	100	100	0	A	0.2	
6	0	100	90	10	A	0.2	
7	0	100	0	100	A	0.2	
8	100	0	0	100	A	0.2	
10	100	0	0	100	A	0.2	
10.1	100	0	100	0	A	0.2	
12	100	0	100	0	A	0.2	

[a] 3% (v/v) MeOH/0.1% (v/v) TFA.
[b] 75% (v/v) MeOH/0.1% (v/v) TFA.
[c] 3% (v/v) MeOH/0.1% (v/v) FA.
[d] 75% (v/v) MeOH/0.1% (v/v) FA.

2.5. Determination of urinary cotinine

Urinary cotinine, a major metabolite of nicotine, was measured by an isotope-dilution LC–MS/MS method following a liquid–liquid extraction pretreatment previously described by Chao et al. [14].

2.6. Statistical methods

The data were analyzed using a SAS statistical software (SAS, version 9.1). Mann–Whitney *U*-test was used to compare urinary concentrations of N3-MeA and cotinine between nonsmokers and smokers. Spearman correlation coefficients were used to study the relationship of urinary N3-MeA concentrations to cotinine concentrations or self-reported daily cigarette consumption. Multiple linear regression models were used to investigate the relationship of urinary N3-MeA concentrations to cotinine concentrations or daily cigarette consumption after adjusting for other variables (i.e., age and BMI).

3. Results

3.1. LC–MS/MS characteristics of N3-MeA

Product ion spectra of N3-MeA and its isotope internal standard are shown in Fig. 2. The spectra are recorded by selecting the protonated ion ($[M+H]^+$) in the first quadrupole (Q1). After collision activation of the selected ions in the collision cell, the product ion spectra are recorded by scanning the last quadrupole (Q3). The transition of the $[M+H]^+$ (m/z 150) precursor ion of N3-MeA to $[MH-HCN]^+$ (m/z 123) resulted in the product ion with highest intensity, while the second most intense product ion was observed at $[MH-CH_3HCN]^+$ (m/z 108). In this study, the most abundant fragment ion (quantifier ion) was used for quantification and the second abundant fragment ion (qualifier ion) for confirmation of the identity of analyte. For the isotope internal standard only one fragment ion was selected. Therefore, the samples were analyzed in positive-ion MRM mode and the transitions of the precursors to the product ions were as follows: m/z 150 → 123 (quantifier) and 150 → 108 (qualifier) for N3-MeA, and m/z 153 → 126 for the internal standard d_3-N3-MeA.

Fig. 3 shows a typical on-line SPE LC–MS/MS chromatogram for N3-MeA and d_3-N3-MeA in the urine of a smoker. The retention time was 5.3 min for N3-MeA, with a total analysis time of 12 min per sample.

3.2. Method validation

The limit of quantification (LOQ) was defined as the lowest concentration of N3-MeA that could be reliably and reproducibly measured with values for accuracy and intra- and inter-day imprecision [CV (coefficient of variation)]<20%. Using the present method, the LOQ was determined to be 0.1 ng/mL on-column (33.6 fmol in a 50 μL injection volume), based on direct measurement of diluted calibration solutions. Meanwhile, the limit of detection (LOD), defined as the lowest concentration that gave a signal-to-noise ratio of at least 3, was 0.035 ng/mL on-column (11.7 fmol).

Two linear calibration curves ranging from 0.0625 to 1 ng/mL (low range: 0.0625, 0.125, 0.25, 0.5 and 1 ng/mL) and 1–16 ng/mL (high range: 1, 2, 4, 8 and 16 ng/mL) were obtained by serial dilution of aqueous calibrator solutions. Each calibration solution (100 μL)

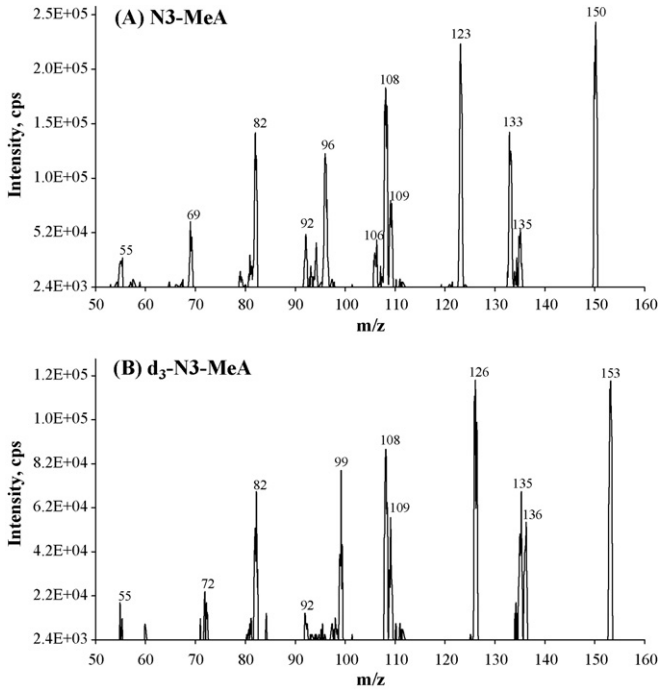

Fig. 2. Positive-ion electrospray MS/MS spectra for the $[M+H]^+$ ion (A) at m/z 150 for N3-MeA standard and (B) at m/z 153 for the stable isotope internal standard d_3-N3-MeA.

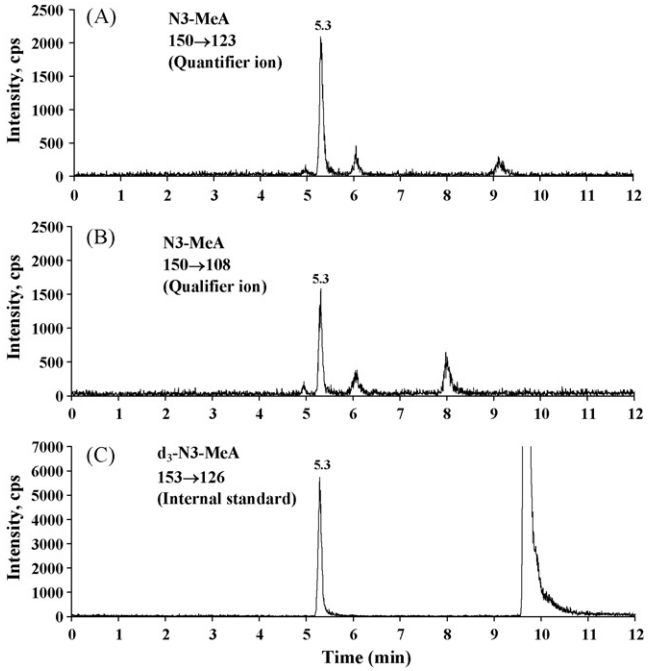

Fig. 3. Chromatograms of N3-MeA in human urine, as measured by LC–MS/MS coupled with on-line SPE. N3-MeA was monitored at (A) m/z 150 → 123 and (B) m/z 150 → 108, and the internal standard d_3-N3-MeA was monitored at (C) m/z 153 → 126.

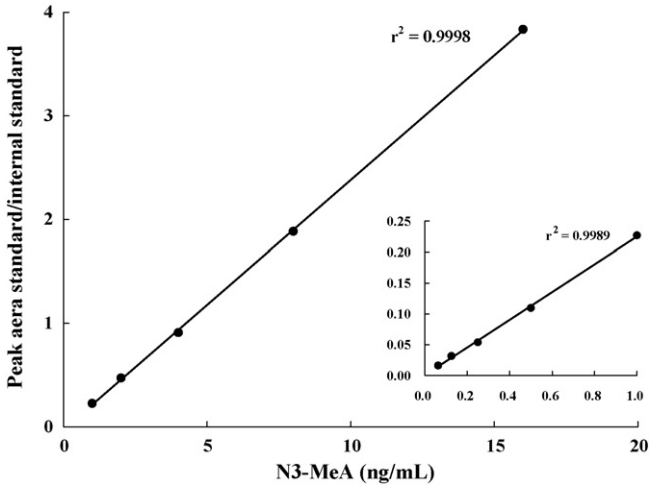

Fig. 4. Calibration curve of N3-MeA with internal standard.

was spiked with 20 μL of 20 ng/mL d_3-N3-MeA. Linear regression was calculated with non-weighting and non-zero-forced, and 2 linear equations were obtained: $y = 0.2242x + 0.0007$ ($r^2 = 0.9989$) for the low range and $y = 0.2407x - 0.0263$ ($r^2 = 0.9998$) for the high range (see Fig. 4). Over the entire concentration range of the calibration curves, the mean observed percentage deviation of back-calculated concentrations was between −5.0% and +10.5%, with an imprecision (CV) < 10%.

For N3-MeA in urine, the peak identity was also confirmed by comparing the peak area ratios (quantifier ion/qualifier ion) with those of the calibrator solutions. As an acceptance criterion, ratios in urine matrix should not deviate by more ±25% from the mean ratios in the calibrator solution. The precision of the present method was evaluated by performing replicate determinations of N3-MeA in 3 different urine samples (see Table 2). The intra-day precision was 2.3–7.4%. The inter-day test was carried out by assaying the same sample on 5 different days over a period of 60 days. The inter-day precision was determined to be 5.2–10.8%. Recovery was evaluated by adding unlabeled N3-MeA at 5 concentrations to crude urine samples. As shown in Table 2, the recovery of the present method, as calculated from the slope of the regression, was 98–103% ($r^2 > 0.99$), and the mean recovery was 97–101%, as estimated from the increase in the measured concentration after addition of N3-MeA divided by the concentration added.

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 samples. The relative change in peak area of the internal standard was attributed to matrix effects, which reflect both on-line extraction losses and ion suppression due to the urinary matrix. In the present study, the matrix effects were found to be less than 30% for N3-MeA 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 [15].

3.3. Urinary excretion of N3-MeA and cotinine in smokers and nonsmokers

The characteristics of the participants and the urinary N3-MeA and cotinine concentrations are summarized in Table 3. It was found that smokers and nonsmokers were similar in age and BMI. Smokers had a mean age of 42.2 and a mean BMI value of 23.6 while nonsmokers had a mean age of 40.5 and a mean BMI value of 24.7. As for the urinary N3-MeA adjusted by urinary creatinine, smokers had a mean urinary N3-MeA concentration of 10.9 ± 13.2 ng/mg creatinine and nonsmokers had a mean concentration of 4.1 ± 4.3 ng/mg creatinine. Smokers had significantly higher urinary N3-MeA concentrations than did nonsmokers ($P < 0.001$). Moreover, mean urinary cotinine concentrations in smokers (946 ± 672 ng/mg creatinine) were significantly higher than in nonsmokers (3.8 ± 3.9 ng/mg creatinine; $P < 0.001$).

Table 2
Precision and recovery of isotope-dilution LC–MS/MS with on-line SPE for urinary N3-MeA analysis.

	Urine 1	Urine 2	Urine 3
Precision[a]			
Intra-day variation (mean ± SD, ng/mL) (CV, %)	4.2 ± 0.3 (7.4)	18.6 ± 0.4 (2.3)	86.5 ± 2.5 (2.9)
Inter-day variation (mean ± SD, ng/mL) (CV, %)	4.3 ± 0.5 (10.8)	17.9 ± 0.9 (5.2)	88.7 ± 6.8 (7.7)
Recovery[b]			
Slope of regression	1.03	0.98	0.98
Mean (SD) recovery (%)	101 (9.6)	97 (3.8)	99 (8.5)

[a] Each urine analysis was repeated 5 times for the intra-day and inter-day tests; inter-day test was carried out over a period of 60 days.
[b] Recovery of N3-MeA in urine was estimated by the addition of N3-MeA at 5 different concentrations to crude urine samples (1–20 ng/mL for urine 1, 5–100 ng/mL for urine 2 and 20–400 ng/mL for urine 3). The recovery was estimated from (a) the slope of the regression of measured N3-MeA vs. added N3-MeA and (b) the increase in measured concentration after addition of N3-MeA divided by the concentration that was added.

Table 3
Overall characteristics of the study subjects.

Variables	Smokers	Nonsmokers	*P*-Value[a]
Numbers	41	45	
Age (years)	42.2 ± 10.4[b]	40.5 ± 10.0	0.46
BMI (kg/m^2)	23.6 ± 3.3	24.7 ± 3.1	0.11
Cigarettes/day	20.4 ± 10.0	0	
Cotinine (ng/mg creatinine)	946 ± 672	3.8 ± 3.9	<0.001
N3-MeA (ng/mg creatinine)	10.9 ± 13.2	4.1 ± 4.3	<0.001
	(15.5 ± 20.3)[c]	(5.7 ± 7.3)	<0.001

[a] Comparison by Mann–Whitney *U*-test.
[b] Data are mean ± SD.
[c] Urinary concentration of N3-MeA as expressed in ng/mL.

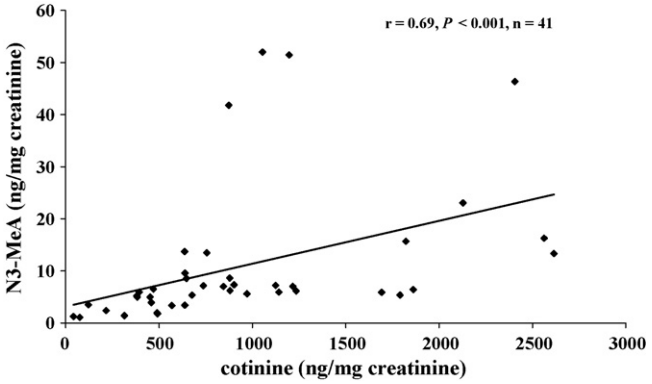

Fig. 5. Correlation between urinary N3-MeA and cotinine in smokers as estimated by Spearman correlation.

3.4. Correlation between urinary N3-MeA and cotinine for smokers

The correlation between urinary N3-MeA and cotinine in smokers is shown in Fig. 5. Urinary N3-MeA concentrations were found to be associated with urinary cotinine concentrations (Spearman correlation coefficients, $r = 0.69$, $P < 0.001$). No significant correlation was observed between urinary N3-MeA and self-reported daily cigarette consumption (Spearman correlation coefficients, $r = 0.20$, $P = 0.21$). In multiple linear regressions, the correlation between urinary N3-MeA and cotinine was not confounded by other variables, including age and BMI ($P < 0.001$). There was no correlation between urinary N3-MeA and self-reported daily cigarette consumption after adjustment for age and BMI ($P = 0.37$).

4. Discussion

In this study, we have developed a rapid, specific, and sensitive isotope-dilution LC–MS/MS method incorporating column-switching (on-line SPE) and an isotopic internal standard that can detect urinary N3-MeA with a low LOD of 0.035 ng/mL on-column (11.7 fmol) and a total analysis time per sample as short as 12 min.

In previous studies, several attempts were made to monitor the urinary excretion of N3-MeA. However, to achieve satisfactory specificity and sensitivity for analysis of urine, most developed methods have been combined with tedious manual (off-line) sample cleanup procedures. Prevost et al. [6] developed a method combined with immunoaffinity column cleanup and enzyme-linked immunosorbent assay (ELISA) for determination of N3-MeA in urine that resulted in a LOD of 600 fmol. Urinary N3-MeA has been also determined by competitive radioimmunoassay after separation by HPLC, with a LOD of 160 fmol [16]. The GC-MS method described by Prevost et al. [9] involved 2-step off-line SPE cleanup and derivatization, and had a LOD of 1000 fmol. Interestingly, there has not been much effort devoted to the development of quantitative assays using LC–MS for this important methylated DNA adduct. The use of LC–MS has been recently proposed for the analysis of N3-MeA in DNA standard by Chadt et al. [17], who reported a LOD of 33.6 fmol. Feng et al. [18] established a LC–MS/MS method following an off-line SPE cleanup for detection of urinary N3-MeA and reported a LOD of 167 fmol. Apparently, our newly developed on-line sample enrichment coupled to isotope-dilution LC–MS/MS method has a lower LOD (11.7 fmol) than these previously reported methods and provides relatively simple and rapid determination of urinary N3-MeA. Furthermore, this method requires only 20 μL of urine for analysis compared with previous studies, which required ~2–50 mL of urine [6,9,16,18,19]. The requirement of a very small sample volume could allow repeated measurements if a second sample is not available and could reduce the required storage space for samples.

N3-MeA can be formed in DNA by methylating agents as well as non-enzymatically by intracellular SAM [3]. Although there are sources of pre-formed N3-MeA in the diet [9,20], urinary N3-MeA has been proposed as a useful biomarker of exposure to methylating agents [16,21]. The basal level of urinary N3-MeA observed in this study was ~4.1 ng/mg creatinine (Table 3), which is in good agreement with several previous measurements using GC–MS in subjects without dietary control (~5–10 μg/day; ~3.3–6.6 ng/mg creatinine after adjustment by a typical daily creatinine excretion rate of 1.5 g/day) [9,16,22]. However, very high basal urinary N3-MeA values (1133–2863 ng/mg creatinine) were reported using HPLC-UV [5].

Tobacco smoke is causally related to the occurrence of a number of human cancers at different sites including lung, oral, bladder, oropharyngeal, hypopharyngeal, laryngeal and pancreatic cancers [23]. Tobacco-specific nitrosamines (TSNAs) are a group of carcinogens that are present in tobacco and tobacco smoke. They 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 adducts [24,25]. In the present study, smokers (10.9 ng/mg creatinine) had about 2.7 times higher level of N3-MeA than nonsmokers (4.1 ng/mg creatinine; Table 3), suggesting that the increased urinary N3-MeA was formed from cigarette smoke as the main exposure source. Our data are similar to previously reported results from 24 h urine collections showing that smokers excreted 1.5–3 times more N3-MeA in urine than did nonsmokers [16,22]. Prevost and Shuker [21] also collected 24 h urine samples of smokers and found a urinary level of N3-MeA ~11 μg/day on nonsmoking days (roughly equal to 7.3 ng/mg creatinine) and ~18 μg/day on smoking days (roughly equal to 12 ng/mg creatinine).

Cotinine is one of the major metabolites of nicotine. Since cotinine has a longer elimination half-life (about 20 h compared to 2 h for nicotine), the measurement of cotinine in biological fluids has been widely used as a reliable biomarker to estimate active smoking behavior. Previous studies reported that cotinine levels in nonsmokers were below 30 ng/mg creatinine while levels for passive/light smokers (<5 cigarettes/day) and regular smokers were 30–100 and 100–7000 ng/mg creatinine, respectively [26,27]. In this study, the smokers and nonsmokers had mean urinary levels of cotinine of 946 ng/mg creatinine (range from 42 to 2128) and 3.8 ng/mg creatinine (range from 0 to 18.8), respectively, which is consistent with these previously reported ranges. A positive correlation between urinary level of N3-MeA and cotinine for smokers was found in this study ($r = 0.69$, $P < 0.001$, $n = 41$) and was not confounded by other variables including age and BMI. Our literature review suggests this could be the first work to demonstrate a significant dose-dependent relationship between urinary excretion of N3-MeA and nicotine intake.

We found no significant correlation between self-reported daily cigarette consumption and the urinary N3-MeA levels with or without adjustment for age and BMI. One possible explanation for the lack of such a correlation is the inadequacy of self-reported data on smoking status because of recall bias, unwillingness to disclose smoking habits, invalid reported numbers of cigarettes consumed, and the use of various cigarette brands containing methylating agents at different concentrations [28].

5. Conclusions

This study describes a simple, rapid, and reliable LC–MS/MS method for direct determination of urinary N3-MeA. When combined with column-switching (on-line SPE) and isotope-dilution, this method could allow for high-throughput analysis of urinary N3-MeA without compromising quality and validation criteria. This method was demonstrated to exhibit a lower detection limit (11.7 fmol) than previously reported GC-MS, LC–MS and immunological methods, and might be also suitable for analysis of N3-MeA in human DNA. This study found that urinary N3-MeA was significantly correlated with urinary cotinine in smokers, suggesting that cigarette smoke is highly responsible for the increased urinary excretion of N3-MeA. It is believed that such non-invasive measurement of urinary N3-MeA could serve as a useful biomarker for assessing methylating agent exposure and its associated cancer risk.

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