Anal Bioanal Chem (2014) 406:5293–5302
DOI 10.1007/s00216-014-7979-1

RESEARCH PAPER

New validated LC-MS/MS method for the determination of three alkylated adenines in human urine and its application to the monitoring of alkylating agents in cigarette smoke

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Received: 14 January 2014 / Revised: 8 June 2014 / Accepted: 16 June 2014 / Published online: 5 July 2014

Abstract A highly specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for simultaneous determination of urinary N^3-methyladenine (N^3-MeA), N^3-ethyladenine (N^3-EtA), and N^3-(2-hydroxyethyl)adenine (N^3-HOEtA). Chromatographic separation was achieved on a hydrophilic interaction liquid chromatography column, with a mobile phase gradient prepared from aqueous 10 mM ammonium formate–acetonitrile (5:95 v/v, pH 4.0). Quantification of the analytes was done by multiple reaction monitoring using a triple-quadrupole mass spectrometer in positive-ionization mode. The limits of quantification were 0.13, 0.02, and 0.03 ng/mL for N^3-MeA, N^3-EtA, and N^3-HOEtA, respectively. Intraday and interday variations (relative standard deviations) ranged from 0.6 to 1.3 % and from 3.7 to 7.5 %. The recovery ranges of N^3-MeA, N^3-EtA, and N^3-HOEtA in urine were 80.1–97.3 %, 83.3–90.0 %, and 100.0–110.0 %, respectively. The proposed method was successfully applied to urine samples from 251 volunteers including 193 regular smokers and 58 nonsmokers. The results showed that the levels of urinary N^3-MeA, N^3-EtA, and N^3-HOEtA in smokers were significantly higher than those in nonsmokers. Furthermore, the level of urinary N^3-MeA in smokers was found to be positively correlated with the level of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (r=0.48, P<0.001, N=192). This method is appropriate for routine analysis and accurate quantification of N^3-MeA, N^3-EtA, and N^3-HOEtA. It is also a useful tool for the surveillance of alkylating agent exposure.

Keywords N^3-Methyladenine · N^3-Ethyladenine · N^3-(2-Hydroxyethyl)adenine · Urine · Liquid chromatography–tandem mass spectrometry

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Introduction

Tobacco smoke contains more than 5,000 chemicals, including many carcinogenic agents (e.g., tobacco-specific nitrosamines), which are known to cause detrimental effects on human health, such as cancer, cardiovascular issues, and pulmonary diseases [1–3]. Human exposure to these carcinogenic agents can lead to formation of covalently bound adducts in DNA, including those methylated and ethylated 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 positions of thymine [4, 5]. As the N-3 position of adenine is the predominant reaction site, the glycosidic linkages of N^3-methyladenine (N^3-MeA), N^3-ethyladenine (N^3-EtA), and N^3-(2-hydroxyethyl)adenine (N^3-HOEtA) are removed from DNA either by spontaneous depurination or by the action of glycosylases to produce apurinic sites, followed by excretion in urine [6]. N^3-MeA is a cytotoxic and promutagenic lesion, whereas N^3-EtA and N^3-HOEtA are not considered to be promutagenic DNA lesions [7, 8]. However, if the apurinic sites are not efficiently repaired, they could lead to opening of the imidazole ring and DNA strand break. Thus, N^3-MeA, N^3-EtA, and N^3-HOEtA are potential biomarkers for DNA damage induced by carcinogenic agents (e.g., *N*-nitrosamines and ethylene oxide) in tobacco smoke [9, 10].

N-Nitrosamines are thought to be one of the primary causes of alkylated DNA adducts [11, 12]. 4-(Methylnitrosamino)-

1-(3-pyridyl)-1-butanone (NNK) is one of the tobacco-specific nitrosamines formed by nitrosation of nicotine, ranging from 3.22 to 246.67 ng per cigarette in mainstream smoke in Chinese commercial cigarettes [13]. NNK is a strong lung carcinogen in experimental animals [14]. In vivo, NNK is converted metabolically to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which is also a potent pulmonary carcinogen [15]. NNAL can be further detoxified by forming *N*-glucuronide NNAL and *O*-glucuronide NNAL, which are excreted in urine along with free NNAL [16]. The relationship between urinary total NNAL (free NNAL and its glucuronides) and N^3-MeA has been studied as a rationale for the mechanisms of carcinogenesis by NNK [17]. Previous research indicated that metabolic activation of NNK and NNAL in target tissues can result in the formation of methylated DNA adducts (Fig. 1) [15, 16].

DNA adduct level in urine is an indicator of the balance between adduct formation and the repair system in response to the chemical damage at a certain point in time. Certain DNA adducts have served as biomarkers for carcinogen exposure and evaluation of cancer risk [18]. Analysis of alkylated DNA adducts in urine requires a highly sensitive, specific, and quantitative method. A variety of analytical techniques have been applied for the quantification of alkylated DNA adducts in urine, including enzyme-linked immunosorbent assay [19], gas chromatography–mass spectrometry [20–22], and high-performance liquid chromatography [9]. Although these methods have been successful, they have drawbacks, such as requiring labor-extensive sample preparation, having low sensitivity, and/or requiring chemical derivatization.

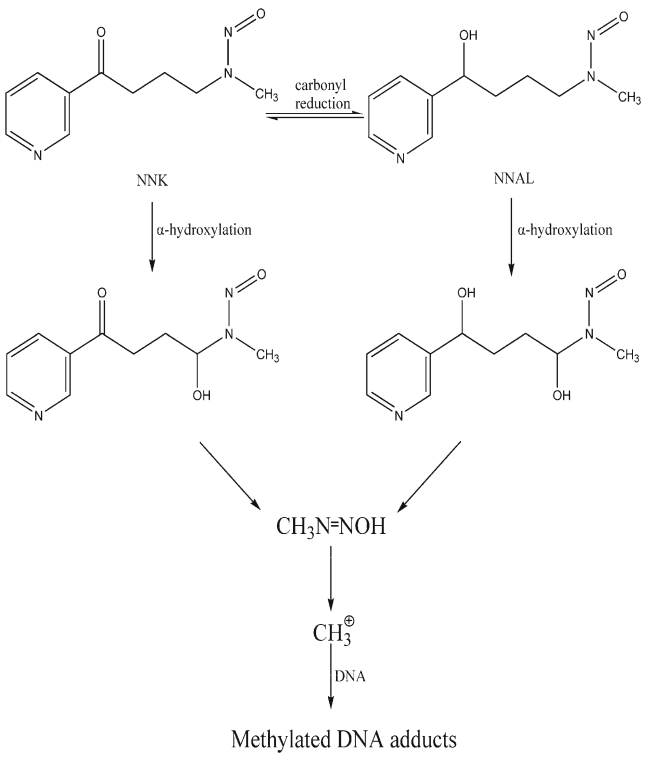

Fig. 1 Overview of the metabolic pathways of NNK and NNAL leading to methylated DNA adducts

Recently, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become a powerful technology to overcome the sensitivity and specificity issues in the quantitative analysis of DNA adducts [8, 23]. LC–MS/MS methods are thought to be especially suitable to detect low levels of alkylated DNA adducts [24]. A particular LC–MS/MS method was developed by Chao et al. [7]. They firstly applied an online sample extraction using a column-switching device to automatically prepare urinary samples, and used nonradioactive-isotope-labeled standards to compensate for the loss of analyte during sample preparation and to correct for the matrix effect of the urinary samples [25]. The application of LC–MS/MS methods has resulted in as series of series of achievements [8, 17, 24, 25], including the correlation of nicotine and N^3-MeA [8], and the correlation between NNAL and N^3-MeA [17]. To the best of our knowledge, an LC–MS/MS method for simultaneous determination of N^3-MeA, N^3-EtA, and N^3-HOEtA has not yet been reported in the literature.

The purpose of this study was to develop a specific and sensitive LC–MS/MS method for simultaneous determination of N^3-MeA, N^3-EtA, and N^3-HOEtA in human urine to monitor the urinary concentrations of N^3-MeA, N^3-EtA, and N^3-HOEtA in Chinese smokers and nonsmokers. In addition, the relationship between urinary NNAL and these alkylated DNA adducts will be discussed.

Materials and methods

Chemicals

Ammonium formate, methanol, ethyl acetate, ammonia, and acetonitrile were obtained from TEDIA (Fairfield, OH, USA). All solvents were high-performance liquid chromatography grade. N^3-MeA, N^3-EtA, N^3-HOEtA, N^3-MeA-d_3, and N^3-EtA-d_5 (Fig. 2) were purchased from Toronto Research Chemicals. (North York, ON, Canada).

Urine sample preparation

The sample preparation was performed according to a previous published method [23]. Ultrapure water (4 mL) was added to a 4-mL urine sample, then acetonitrile (100 μL) containing N^3-MeA-d_3 at 100 ng/mL and N^3-EtA-d_5 at 4 ng/mL was added to the mixture. The cartridge (Waters Oasis MCX, 500 mg, 6 mL) was preconditioned with 3 mL methanol and 6 mL water, and was then washed with 1 mL water/methanol (9:1 v/v) and 2 mL ethyl acetate, and the contents were eluted with 12 mL ethyl acetate/methanol/ammonia (47.5:47.5:5 v/v/

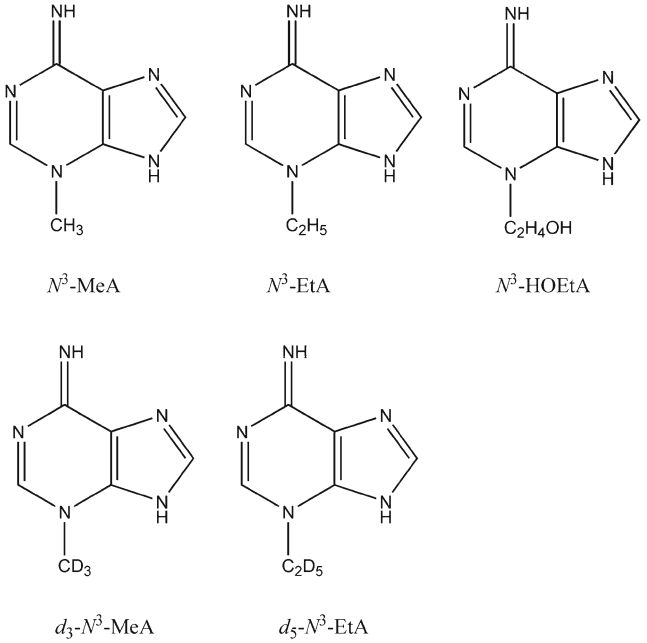

Fig. 2 Structures of the analytes (N^3-MeA, N^3-EtA, N^3-HOEtA) and their stable-isotope-labeled internal standards (N^3-MeA-d_3, N^3-EtA-d_5)

v). The extract was dried in a SpeedVac evaporator (Thermo Fisher, Dreieich, Germany) and dissolved in 100 μL ultrapure water.

Instrumentation

All samples were analyzed using a model 1200 rapid resolution liquid chromatograph from Agilent Technologies (Wilmington, NC, USA) coupled with an API 5500 triple-quadrupole mass spectrometer equipped with a TurboIonSpray™ source from Applied Biosystems (Foster City, CA, USA). A hydrophilic interaction liquid chromatography column (2.1 mm × 150 mm, 2.5 μm) from Waters (Philadelphia, PA, USA) was used for liquid-chromatographic separation.

Analytical conditions

The liquid chromatography conditions were as follows: column temperature, 25 °C; mobile phase solvent A, 10 mM ammonium formate (pH 4.0), and mobile phase solvent B, acetonitrile (pH 4.0) (5:95 v/v); flow rate, 250 μL/min; injected volume, 3 μL.

The mass specrometry conditions were as follows: nebulizer gas, N_2 (50 psi); TurboIonSpray voltage, 5,500 V; TurboIonSpray temperature, 400 °; ionization mode, positive ion. Optimization results for each analyte in multiple reaction monitoring scan mode are given in Table 1. N^3-MeA, N^3-EtA, N^3-HOEtA, N^3-MeA-d_3, and N^3-EtA-d_5 were assayed by quantifying the multiple reaction monitoring transition of the $[M + H]^+$ ion of N^3-MeA at *m/z* 150.0 → 123.0, N^3-MeA-d_3 at *m/z* 153.1 → 126.1, N^3-EtA at *m/z* 164.2 → 136.1, N^3-EtA-d_5 at *m/z* 168.9 → 137.1, and N^3-HOEtA at *m/z* 180.2 → 136.1 (Table 1).

Method validation

The method was validated according to the US Food and Drug Administration guidelines for bioanalytical methods [26]. Recoveries were determined by comparing the analyte concentrations at low, middle, and high levels (with the background subtracted). As a criterion, the accuracy at the average concentration tested in these three matrices should be in the range of 80–110 %. The intraday precision was determined by analyzing seven times human urine samples containing N^3-MeA, N^3-EtA, and N^3-HOEtA at concentrations of 10.0, 1.5, and 1.0 ng/mL, respectively. For intraday precision, each sample was analyzed seven times. For interday precision, human urine samples containing N^3-MeA, N^3-EtA, and N^3-HOEtA at concentrations of 30.0, 2.5, and 1.7 ng/mL, respectively, were analyzed once on five separate days within 2 weeks. Acceptance criteria for precision should not exceed 15 % and 20 % of the coefficient of variation. The limit of detection (LOD) and the limit of quantification (LOQ) were estimated with the signal-to-noise method using the integrated function of Analyst. Matrix effects were determined by calculating the peak area ratios of the analyte at certain concentrations when added to urine and the same amounts of analyte in solvent (water).

Urine samples

The studies were approved by Zhengzhou University Ethics Committee, and 251 healthy volunteers were recruited, including 58 nonsmokers and 193 smokers smoking 15 Chinese cigarettes per day with 8 mg (N=63), 10 mg (N=64), and 13 mg (N=66) of tar. For all the subjects, 24-h urine samples were obtained at the baseline from ongoing studies (urinary biomarkers related to smoke exposure) in the Institute of Clinical Pharmacology of Zhengzhou University. Informed consent was obtained from each of the subjects.

Data analysis

All chromatographic peaks were reviewed, and integration correction was made manually, if necessary. Calibration curves were prepared using a linear regression with $1/x$ weighting. All standard and sample concentrations were determined using analyte area versus internal standard area. The data were analyzed using Analyst 1.5.1, SPSS 17.0, and Microsoft Office Excel 2010. The Spearman correlation coefficient was used to study the relationship among the urinary N^3-MeA, N^3-EtA, N^3-HOEtA, and NNAL. All statistical tests of significance were performed as two-tailed tests with

Table 1 Tandem mass spectrometry parameters for alkylated purines

Compound	Q1 mass (amu)	Q3 mass (amu)	Dwell time (ms)	DP (V)	CE (V)
N^3-MeA	150.0	123.0[a]	40	100	30
	150.0	108.0	40	100	30
N^3-MeA-d_3	153.1	126.1	40	100	30
N^3-EtA	164.2	136.1[a]	40	85	26
	164.2	119.1	40	85	26
N^3-EtA-d_5	168.9	137.1	40	120	26
N^3-HOEtA	180.2	136.1[a]	40	100	25
	180.2	119.1	40	100	25

See Fig. 2 for the structures of the analytes

CE collision energy, *DP* declustering potential, *Q1* quadrupole 1, *Q3* quadrupole 3

[a] Quantifier transition

$P<0.001$. The differences between smokers and nonsmokers were evaluated using one-way ANOVA.

Results

LC–MS/MS characteristics of alkylated adenines

Typical LC–MS/MS chromatograms of the alkylated adenines and their isotopically labeled internal standards are shown in Fig. 3. The retention time of N^3-EtA was 10.8 min. The positive electrospray ionization mass spectrum of N^3-EtA contained an $[M + H]^+$ precursor ion at *m/z* 164 and product ions at *m/z* 136 (quantifier ion, Fig. 3, chromatogram a) and *m/z* 119 (qualifier ion, Fig. 3, chromatogram b) due to loss of C_2H_4 or $C_2H_4NH_3$; a precursor ion at *m/z* 169 and a product ion at *m/z* 137 characterized N^3-EtA-d_5 (Fig. 3, chromatogram c). For N^3-MeA, the retention time was 12.4 min. The $[M + H]^+$ precursor ion of N^3-MeA was at *m/z* 150 and the product ions appeared at *m/z* 123 (quantifier ion, Fig. 3, chromatogram d) and *m/z* 108 (qualifier ion, Fig. 3, chromatogram e), resulting from the loss of HCN or CH_3HCN; a precursor ion at *m/z* 153 and a product ion at *m/z* 126 characterized N^3-MeA-d_3 (Fig. 3, chromatogram f). The retention time of N^3-HOEtA was 14.5 min. Its $[M + H]^+$ precursor ion was at *m/z* 180 and the product ions appeared at *m/z* 136 (quantifier ion, Fig. 3, chromatogram g) and *m/z* 119 (qualifier ion, Fig. 3, chromatogram h), resulting from the loss of C_2H_4O or $NH_3C_2H_4O$.

LOQ and LOD

The LOQ were 0.130, 0.020, and 0.030 ng/mL for N^3-MeA, N^3-EtA, and N^3-HOEtA, respectively. The LOD were 0.043, 0.006, and 0.010 ng/mL for N^3-MeA, N^3-EtA, and N^3-HOEtA, respectively.

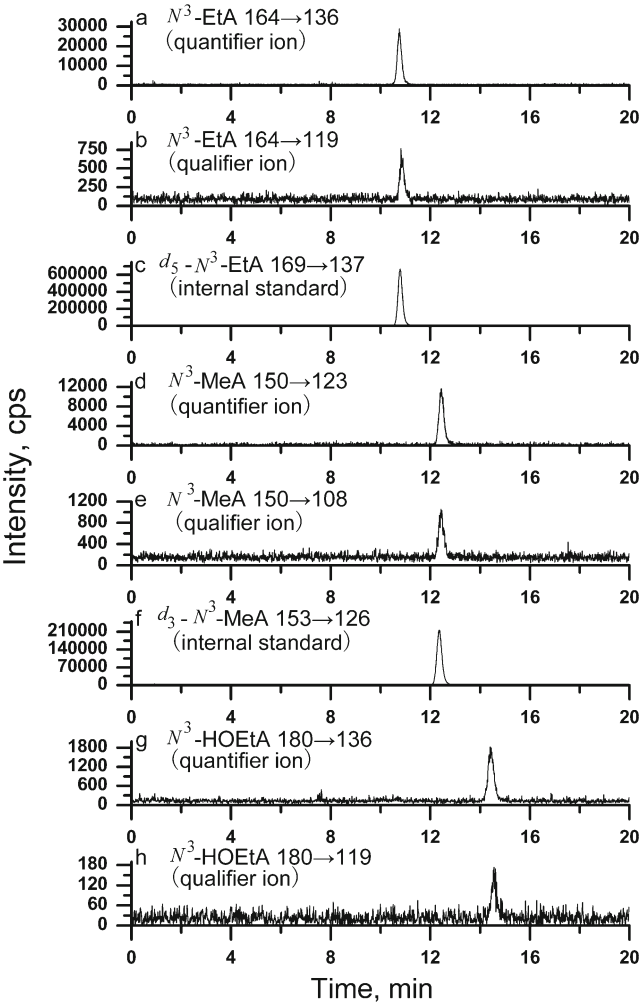

Fig. 3 Chromatograms of the ion transitions for N^3-EtA (0.1 ng/mL) monitored at *m/z* 164→136 (**a**) and *m/z* 164→119(**b**), the internal standard N^3-EtA-d_5 (4 ng/mL) monitored at *m/z* 169→137 (**c**), N^3-MeA (2.0 ng/mL) monitored at *m/z* 150→123 (**d**) and *m/z* 150→108 (**e**), the internal standard N^3-MeA-d_3 (100 ng/mL) monitored at *m/z* 153→126 (**f**), and N^3-HOEtA (0.1 ng/mL) monitored at *m/z* 180→136 (**g**) and *m/z* 180→119 (**h**)

Recovery

The recovery ranges were 80.0–97.3 %, 83.3–90.0 %, and 100.0–110.0 % for N^3-MeA, N^3-EtA, and N^3-HOEtA, respectively (Table 2). The results indicate that the recoveries conform to Food and Drug Administration regulations.

Linearity and precision

The method was calibrated with 0.1, 0.2, 0.5, 1, 1.5, 2, 2.5, 3, and 5 ng/mL for N^3-EtA (Fig. 4a), 10, 20, 40, 60, 100, 120, 150, and 180 ng/mL for N^3-MeA (Fig. 4b), and 0.1, 0.2, 0.5, 1, 1.5, 2, 2.5, and 3 ng/mL for N^3-HOEtA (Fig. 4c). Each calibrator was analyzed twice. The means of the analyte area to internal standard area ratio were used to calculate the regression functions, and the linear regressions, which were calculated with non-zero-forced. The correlation coefficients (r) obtained were higher than 0.999 in all cases.

The intraday precision of the three alkylated adenines in spiked human urine samples was 0.6–3.0 % and the interday precision was 3.5–7.5 % (Table 3).

Matrix effect

The matrix effects of the three alkylated adenines were 21 %, 41 %, and 34 % for N^3-MeA, N^3-EtA, and N^3-HOEtA, respectively. This indicates a lack of significant matrix effects, which is mainly due to cleanup of the aqueous urine samples by solid-phase extraction (SPE). The matrix effects were adjusted by addition of the labeled internal standards.

N^3-MeA, N^3-EtA, and N^3-HOEtA in human urine

The method was applied to 58 nonsmokers and 193 smokers (63 smokers with 8-mg tar yields, 64 smokers with 10-mg tar yields, and 66 smokers with 13-mg tar yields) (Figs. 5 and 6). The concentrations of total urinary N^3-MeA for nonsmokers, smokers with 8 mg tar, smokers with 10 mg tar, and smokers with 13 mg tar were 5.78–82.72 μg in 24 h, 8.96–91.71 μg in 24 h, 5.72–83.38 μg in 24 h, and 10.46–164.97 μg in 24 h, respectively (Fig. 7). Urinary N^3-EtA for nonsmokers,

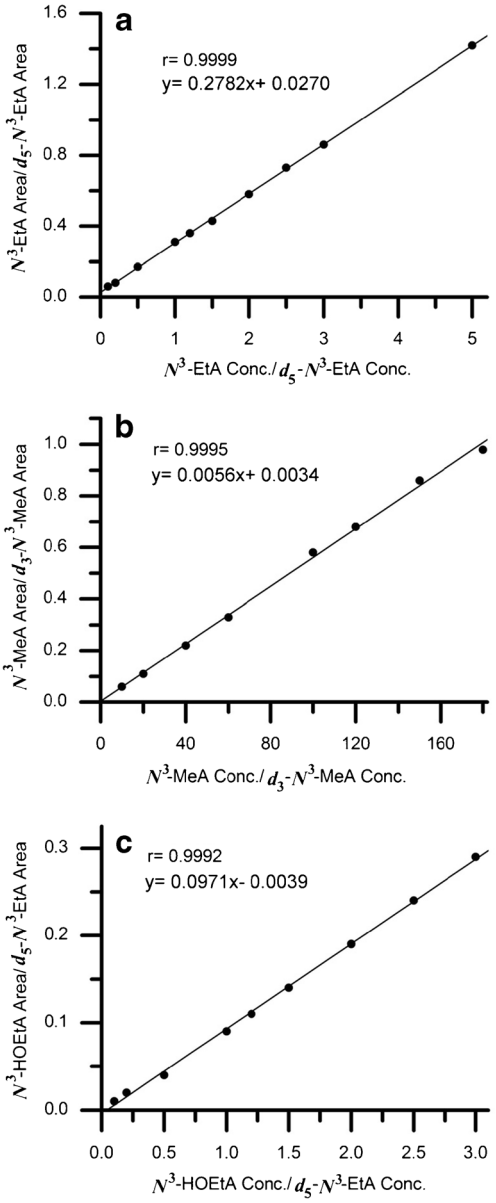

Fig. 4 Calibration curves of the three alkylated DNA adducts with an internal standard

Table 2 Recovery

	Background (ng/mL)	Spiked amount (ng/mL)	Concentration (ng/mL)	Recovery (N=5) (%)
N^3-MeA	0.9	10.0	9.6	87.0
		30.0	30.1	97.3
		80.0	65.0	80.1
N^3-EtA	0.0	1.0	0.9	90.0
		3.0	2.5	83.3
		5.0	4.2	84.0
N^3-HOEtA	0.5	0.8	1.3	100.0
		1.0	1.6	110.0
		1.5	2.1	106.6

Table 3 Intraday and interday precision

	Concentration (ng/mL)	Intraday precision (N=7) (%)	Concentration (ng/mL)	Interday precision (N=5) (%)
N^3-MeA	9.8	0.6	30.4	3.7
N^3-EtA	1.0	3.0	2.5	3.5
N^3-HOEtA	1.5	1.3	1.7	7.5

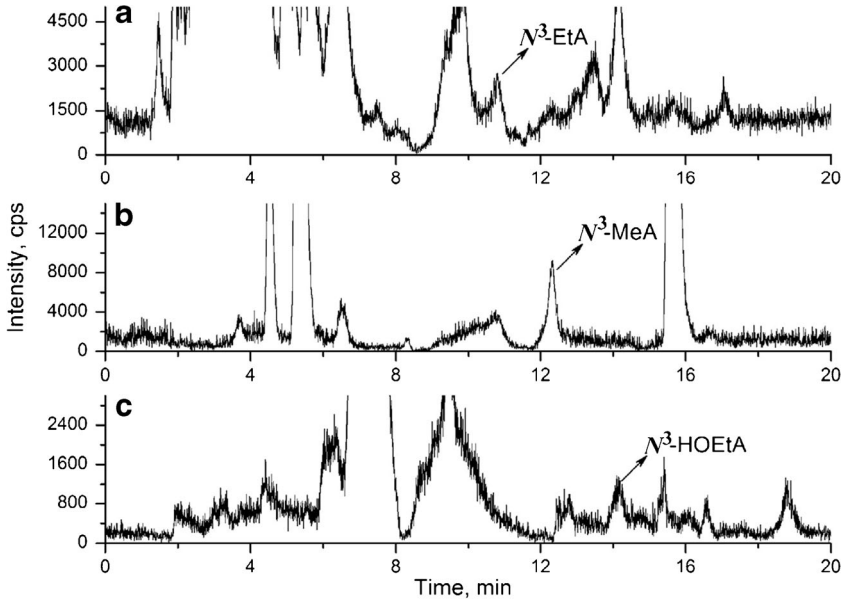

Fig. 5 Liquid chromatography–tandem mass spectrometry (LC–MS/MS) chromatograms of nonsmoker's urine: N^3-EtA (**a**), N^3-MeA (**b**), and N^3-HOEtA (**c**)

smokers with 8 mg tar, smokers with 10 mg tar, and smokers with 13 mg tar ranged from 0 to 2,241.18 ng in 24 h, from 0 to 3,234.53 ng in 24 h, from 0 to 3,661.88 ng in 24 h, and from 125.66 to 3,580.50 ng in 24 h (Fig. 8), Urinary N^3-HOEtA ranged from 0 to 605.00 ng in 24 h, from 0 to 1,581.15 ng in 24 h, from 0 to 1,395.00 ng in 24 h, and from 0 to 1,386.00 ng in 24 h (Fig. 9).

The correlation between urinary NNAL and urinary N^3-MeA, N^3-EtA, and N^3-HOEtA

Urinary NNAL was determined by LC–MS/MS by adapting a previously published method [27]. A positive correlation between urinary levels of N^3-MeA and NNAL for smokers was found (r=0.48, N=192) and was not confounded by other variables, including age and gender (Fig. 10). However, there was not the same trend between urinary NNAL and urinary N^3-EtA (r=0.09, N=192) and between urinary NNAL and urinary N^3-HOEtA (r=0.04, N=192).

Discussion

In this study, a specific and sensitive LC–MS/MS method with SPE and isotopically labeled internal standards has been

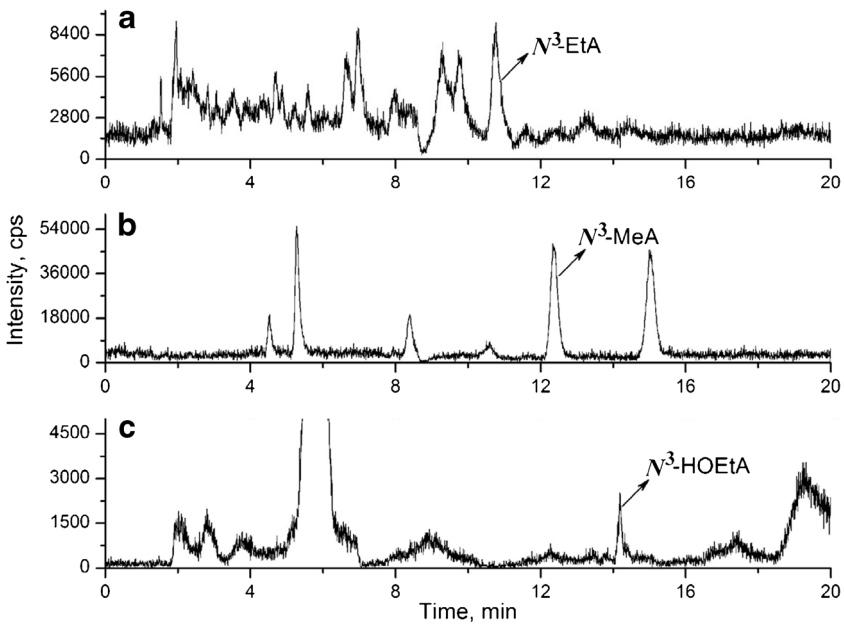

Fig. 6 LC–MS/MS chromatograms of smoker's urine: N^3-EtA (**a**), N^3-MeA (**b**), and N^3-HOEtA (**c**)

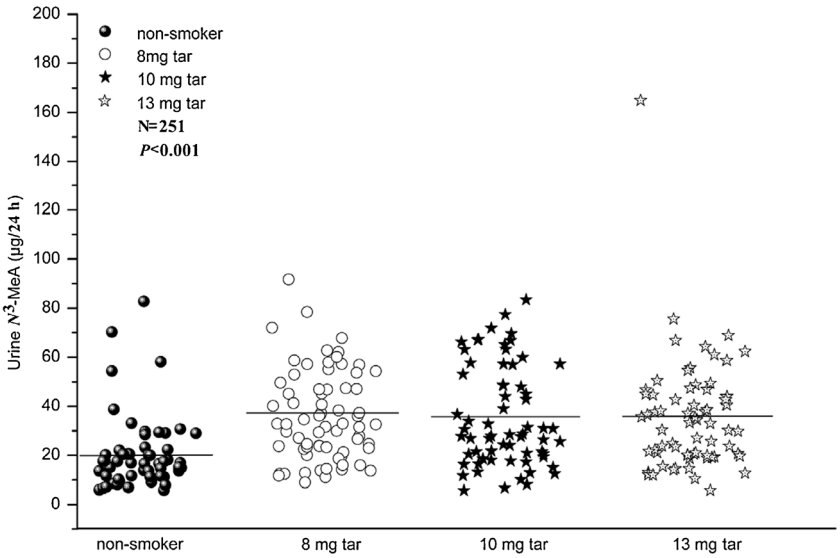

Fig. 7 Concentration of N^3-MeA in urine of nonsmokers and smokers of cigarettes with different tar yields. *Horizontal lines* show the means ($P<0.001$ refers to statistical comparison of the nonsmokers' group with each of the three smokers' groups)

developed for simultaneous determination of urinary N^3-MeA, N^3-EtA, and N^3-HOEtA, with LOD as low as 0.13, 0.02, and 0.03 ng/mL (0.9, 0.12, and 0.17 pmol/mL). With this method, smokers and nonsmokers can be easily and accurately distinguished by N^3-MeA, N^3-EtA, and N^3-HOEtA (as shown in Figs. 7, 8, 9). Feng et al. [23] described an LC–MS/MS method with SPE for analysis of urinary N^3-MeA and N^3-EtA. However, it required 50 mL urine, which may increase the consumption of solvent and interference by the matrix effect. Using this method allows simultaneous determination of three alkylated DNA adducts, requiring only 4 mL of urine and a three-step sample SPE cleanup. The requirement of a small sample volume not only allows repeated measurements, but also reduces the required storage space for samples. This is the first LC–MS/MS method that makes possible simultaneous determination of N^3-MeA, N^3-EtA, and N^3-HOEtA using a hydrophilic interaction liquid chromatography analytical column, which would be a useful tool in research and clinical practice for evaluation of DNA damage by alkylation.

Consistent with published results, the urinary levels of N^3-MeA, N^3-EtA, and N^3-HOEtA in smokers were significantly higher than those in nonsmokers (see Figs. 7, 8, 9), which indicates that cigarette smoking primarily increased the urinary excretion of N^3-EtA, N^3-MeA, and N^3-HOEtA. In previous studies, smokers were reported to have 5.0 times higher levels of N^3-EtA than nonsmokers [8], 2.7 times higher levels of N^3-MeA than nonsmokers [9], and 2.0–4.0 times higher levels of N^3-HOEtA than nonsmokers [6]. However, in this study, the level of urinary N^3-EtA in smokers was only 2.2 times higher than that in nonsmokers (Fig. 6a), the level of urinary N^3-MeA was only 1.8 times higher (Fig. 6b), and the level of N^3-HOEtA was only 2.3 times higher (Fig. 6c). These variations may be explained by differences in dietary habits,

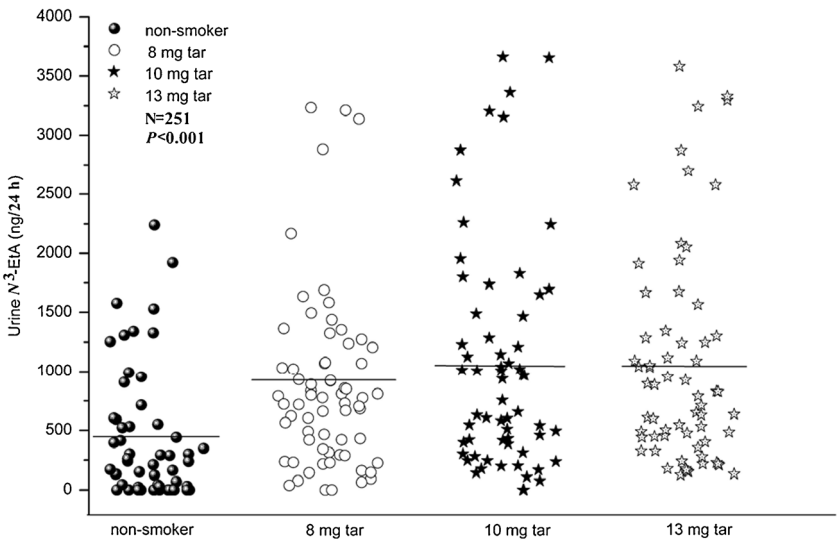

Fig. 8 Concentration of N^3-EtA in urine of nonsmokers and smokers of cigarettes with different tar yields. *Horizontal lines* show the means ($P<0.001$ refers to statistical comparison of the nonsmokers' group with each of the three smokers' groups)

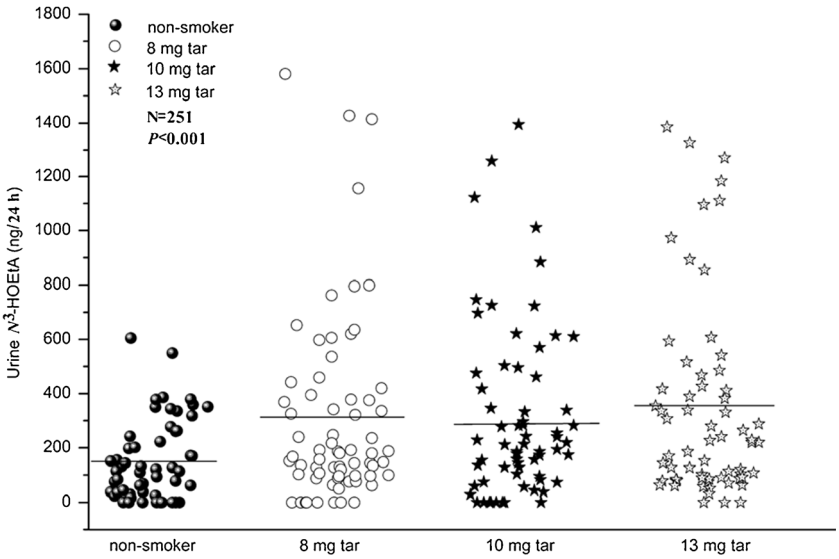

Fig. 9 Concentration of N^3-HOEtA in urine of nonsmokers and smokers of cigarettes with different tar yields. *Horizontal lines* show the means ($P<0.001$ refers to statistical comparison of the nonsmokers' group with each of the three smokers' groups)

the type of cigarette smoke (Chinese Virginia cigarette VS blended type cigarette), and smoking behaviors.

Compared with the results obtained by Feng et al. [23], higher background levels of urinary N^3-MeA and N^3-EtA were found in our study, which may be explained by the differences in diet and ethnicity (Chinese versus Caucasian). Fay et al. [14] in their study on urinary excretion of N^3-MeA after consumption of fish containing high levels of dimethylamine found that diet was a significant source of N^3-MeA in human urine. The levels of alkylated DNA adducts in smokers' urine were higher than those reported by Prevost et al. [20] (16.69–35.46 μgN^3-MeA in 24 h, 16.69–195.60 ngN^3-EtA in 24 h, and 60.86–1,664.70 ngN^3-HOEtA in 24 h) and Feng et al. [23] (7.14–19.68 μgN^3-MeA in 24 h and 124.64–210.53 ngN^3-EtA in 24 h). This difference may be attributed to the different races and smoking behaviors.

The positive association of NNAL and N^3-MeA in urine of smokers in the present study is consistent with the findings of Hu et al. [17], who found a significant correlation of NNAL with nicotine, cotinine, N^3-MeA, and N^7-methylguanine. Their results indicated urinary concentrations of NNAL were significantly correlated with urinary concentrations of nicotine, cotinine, N^3-MeA, and N^7-MeG in smokers ($P<0.001$). Their study first demonstrated in humans that in urine the levels of a urinary tobacco-specific nitrosamine metabolite (NNAL) are highly correlated with the levels of the

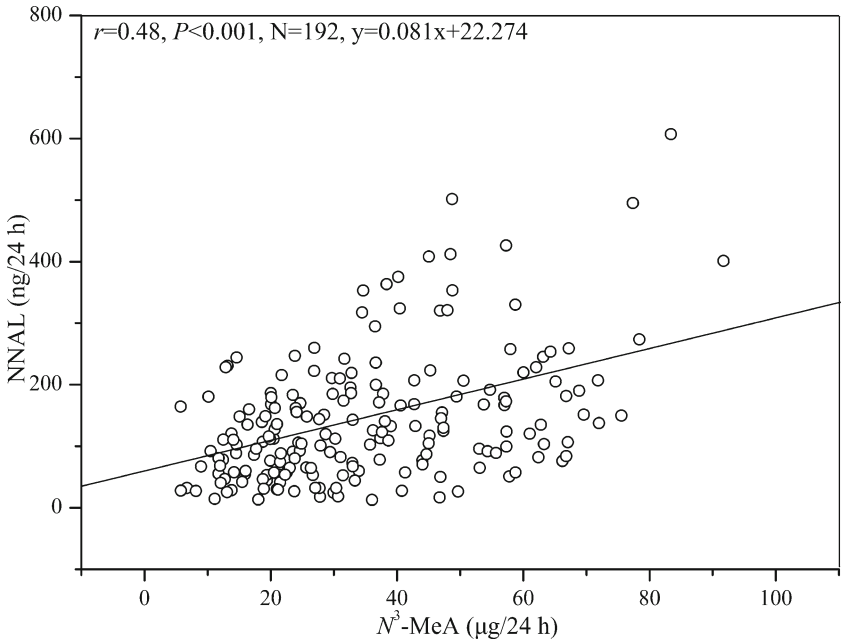

Fig. 10 Correlation between urinary N^3-MeA and NNAL in smokers

methylated DNA lesions resulting from exposure to cigarette smoke, which may help to substantiate an increased cancer risk associated with tobacco smoke exposure.

Conclusion

In conclusion, an analytical method using SPE coupled with LC–MS/MS for determination of N^3-EtA, N^3-MeA, and N^3-HOEtA in human urine has been developed and validated. The method was successfully applied to urine samples from 58 nonsmokers and 193 smokers. The levels of N^3-EtA, N^3-MeA, and N^3-HOEtA were found to be significantly higher in urine of smokers compared with nonsmokers. There was a significant association between the total carcinogen NNAL and urinary N^3-MeA in smokers. Total urinary N^3-MeA, N^3-EtA, and N^3-HOEtA may be valuable biomarkers for monitoring exposure to carcinogenic alkylating agents.

Acknowledgments This work was supported by the National Natural Science Foundation of China (No. 21277174, 21347002). The authors thank Gao Na from the Institute of Clinical Pharmacology (School of Medicine, Zhengzhou University, China) for providing 24-h urine samples of smokers and blank urine samples of nonsmokers.

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