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Determination of methyl-, 2-hydroxyethyl- and 2-cyanoethylmercapturic acids as biomarkers of exposure to alkylating agents in cigarette smoke☆

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ABSTRACT

Alkylating agents occur in the environment and are formed endogenously. Tobacco smoke contains a variety of alkylating agents or precursors including, among others, *N*-nitrosodimethylamine (NDMA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), acrylonitrile and ethylene oxide. We developed and validated a method for the simultaneous determination of methylmercapturic acid (MMA, biomarker for methylating agents such as NDMA and NNK), 2-hydroxyethylmercapturic acid (HEMA, biomarker for ethylene oxide) and 2-cyanoethylmercapturic acid (CEMA, biomarker for acrylonitrile) in human urine using deuterated internal standards of each compound. The method involves liquid/liquid extraction of the urine sample, solid phase extraction on anion exchange cartridges, derivatization with pentafluorobenzyl bromide (PFBBr), liquid/liquid extraction of the reaction mixture and LC–MS/MS analysis with positive electrospray ionization. The method was linear in the ranges of 5.00–600, 1.00–50.0 and 1.50–900 ng/ml for MMA, HEMA and CEMA, respectively. The method was applied to two clinical studies in adult smokers of conventional cigarettes who either continued smoking conventional cigarettes, were switched to test cigarettes consisting of either an electrically heated cigarette smoking system (EHCSS) or having a highly activated carbon granule filter that were shown to have reduced exposure to specific smoke constituents, or stopped smoking. Urinary excretion of MMA was found to be unaffected by switching to the test cigarettes or stop smoking. Urinary HEMA excretion decreased by 46 to 54% after switching to test cigarettes and by approximately 74% when stopping smoking. Urinary CEMA excretion decreased by 74–77% when switching to test cigarettes and by approximately 90% when stopping smoking. This validated method for urinary alkylmercapturic acids is suitable to distinguish differences in exposure not only between smokers and nonsmokers but also between smoking of conventional and the two test cigarettes investigated in this study.

1. Introduction

Alkylating agents such as *N*-nitrosamines, ethylene oxide and acrylonitrile are toxicologically important chemicals, because of their carcinogenic potential [1–4]. These agents can covalently bind to nucleophiles [5] which may account for their toxic, mutagenic and carcinogenic effects. Reaction products of alkylating agents with DNA, proteins and glutathione have been used as biomarkers of exposure to these chemicals [6–10]. With the advent of more sensitive and specific detection methods including mass spectrometry coupled with gas or liquid chromatography, the quantification of protein and DNA adducts as well as mercapturic acids related to individual alkylating agents became possible [9–11]. Assessing the exposure to these compounds by suitable biomonitoring methods, therefore, might be relevant for understanding the potential biological effect from these compounds, which would need further investigation with risk markers.

Tobacco smoke contains both direct alkylating agents (alkyl halides, acrolein, crotonaldehyde, ethylene oxide, propylene oxide, acrylonitrile and acrylamide) as well as indirect alkylating agents which require metabolic transformation to reactive species (tobacco-specific nitrosamines such as NNK, volatile dialkylnitrosamines such as NDMA, and ethylene) [12,13]. We have developed and validated a non-invasive method for measuring biomarkers of exposure to methylating (NDMA, NNK), hydroxyethylating (ethylene, ethylene oxide) and cyanoethy-

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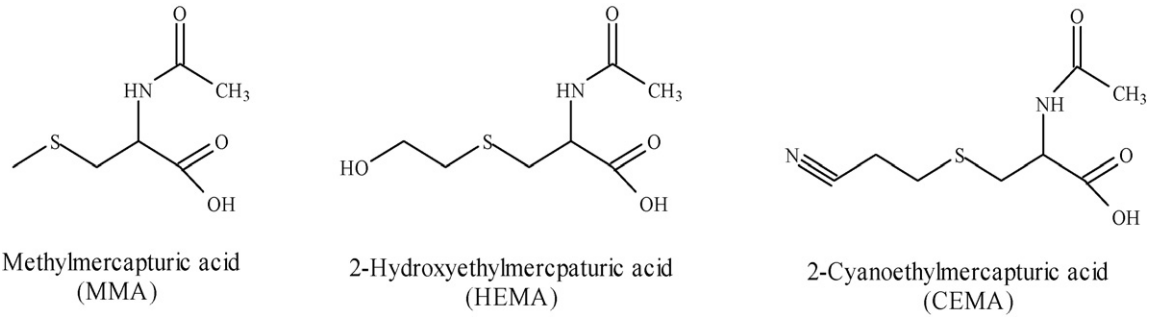

Fig. 1. Chemical structures of MMA, HEMA and CEMA.

lating (acrylonitrile) agents in tobacco smoke resulting from the conjugation of these compounds with glutathione and excretion as mercapturic acid metabolites in urine. For this purpose, methyl- (MMA), 2-hydroxyethyl- (HEMA) and 2-cyanoethylmercapturic acid (CEMA) were regarded to be most suitable. Fig. 1 shows the chemical structures of these three mercapturic acids.

Methylating chemicals in tobacco smoke comprise methyl halides (e.g., methyl chloride, mainstream smoke yield: 150–840 μg/cigarette [14], *N*-nitrosodimethylamine (NDMA, 0.1–180 ng/cigarette [12]) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 110–133 ng/cigarette [12]). NDMA and NNK are capable of methylation after metabolic activation. Ethylene oxide (7 μg/cigarette [12]) is a potential 2-hydroxyethylating agent in tobacco smoke. Ethylene (400–700 μg/cigarette [14]) is also a potential 2-hydroxyethylating agent because a small fraction of it can convert to ethylene oxide in the body. *N*-Nitrosodiethanolamine (NDELA, up to 36 ng/cigarette [12]) has also been shown to form 2-hydroxyethyl adducts [12]. Hydroxyethylation is also effected by vinyl chloride, ethylene dibromide and other electrophilic compounds with a two-carbon backbone [6,15]. Acrylonitrile is the major cyanoethylating agent. Its yields in mainstream smoke of cigarettes amount to 3–15 μg/cigarette [12].

We describe here an LC–MS/MS method for the simultaneous determination of MMA, HEMA and CEMA in human urine. This method was applied to urine samples from two clinical studies.

2. Experimental

2.1. Standards and chemicals

N-Acetyl-*S*-methyl-L-cysteine (methylmercapturic acid, MMA), [D_3]-*N*-acetyl-*S*-methyl-L-cysteine (MMA-D_3), *N*-acetyl-*S*-(2-hydroxyethyl)-L-cysteine (2-hydroxyethylmercapturic acid, HEMA), *N*-acetyl-*S*-([D_4]-2-hydroxyethyl)-L-cysteine (HEMA-D_4), *N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine (2-cyanoethylmercapturic acid, CEMA), [D_3]-*N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine (CEMA-D_3) were purchased from Toronto Research Chemicals, North York, Canada. The supplier stated a purity of 98% or greater for all reference compounds. Results of this study were not corrected for purity

Acetonitrile, ammonium acetate and formic acid were purchased from Merck KGaA, Darmstadt, Germany. Sodium hydroxide, ammonium hydroxide, N,N-diisopropylethylamine and pentafluorobenzyl bromide (PFBBr) were obtained from Sigma–Aldrich GmbH, Taufkirchen, Germany. Acetone, methanol (both HPLC grade) and ethyl acetate were purchased from Promochem, Wesel, Germany. Hydrochloric acid (32%) was obtained from Carl Roth GmbH, Karlsruhe, Germany. Deionized water was prepared with Seradest equipment (Munich, Germany). All chemicals were of analytical grade or higher.

2.2. Urine clean-up

Six (6) ml urine were adjusted to pH 1.5 with first 4N and subsequently, for fine adjustment, with 1N hydrochloric acid and 50 μl internal standard (IS) solution in methanol containing 2 ng/μl of each MMA-D_3, HEMA-D_4 and CEMA-D_3 was added. The mixture was extracted twice with 4 ml ethyl acetate by shaking. The combined organic phases were evaporated to dryness by means of a SpeedVac evaporator (Thermo Fisher Scientific GmbH, Dreieich, Germany). The residue was re-dissolved in 4 ml ammonium hydroxide (5%) and applied to an anion exchange cartridge (MAX 6 cc, 500 mg, Waters GmbH, Darmstadt, Germany) conditioned with 6 ml methanol and 6 ml water (pH 8–9, adjusted with 5% ammonium hydroxide). The cartridge was washed consecutively with 5 ml 5% ammonium hydroxide, 4 ml water (pH 8–9), 3 ml acetonitrile, 3 ml methanol and 4 ml acetone. The cartridge was then dried under reduced pressure (600–700 mbar, 5 min) and eluted with 4 ml water:methanol:formic acid, 50:40:10, vol:vol:vol). The eluate was evaporated to dryness in a SpeedVac evaporator.

2.3. Derivatization of the urine extract

The residue from the clean-up procedure was re-dissolved in 100 μl methanol containing 10% *N,N*-diisopropylethylamine and derivatized with 100 μl pentafluorobenzyl bromide (PFBBr) (10% in methanol) at 50 °C for 1 h. The mixture was evaporated to dryness (SpeedVac evaporator), re-dissolved in 0.5 ml water and extracted twice with 2 ml ethyl acetate. The extract was evaporated (SpeedVac evaporator) and the residue re-dissolved in 200 μl acetonitrile:water (3:1, vol:vol).

2.4. LC–MS/MS analysis

Ten (10) μl of the final extract was injected into an LC–MS/MS system consisting of a temperature controlled autosampler HTC PAL (Axel Semrau, Sprockhoevel, Germany), an HPLC Model 1200 device (Agilent Technology, Waldbronn, Germany) with a binary pump, a column oven and a degasser connected to an API 5000, triple quadrupole mass spectrometer with atmospheric pressure ionization inlet (Applied Biosystems, Darmstadt, Germany). Chromatography was performed on Luna C18(2) analytical column (150 mm × 2 mm, 3 μm particle size, 100 Å pore size; Phenomenex, Aschaffenburg, Germany) at 50 °C and a flow rate of 0.3 ml/min. A gradient was applied with 1% formic acid (A) and acetonitrile with 0.1% formic acid (B): 0 min: 70% A, 10 min: 5% A, 12.5 min: 5% A, 12.6 min: 70% A, 15 min: 70% A. The MS/MS system was operated in positive electrospray ionization (ESI+) mode. The nebulizer heater was maintained at 600 °C. This instrumental setting was found to

Table 1
Retention times (RT) and mass transitions for analytes and internal standards.

	RT (min)	Quantifier (*m*/*z*)	Qualifier (*m*/*z*)
MMA	7.62	358 → 132	358 → 130
MMA-D_3	7.62	361 → 135	–
HEMA	5.85	388 → 130	388 → 310
HEMA-D_4	5.83	392 → 130	–
CEMA	7.22	397 → 130	397 → 355
CEMA-D_3	7.22	400 → 133	–

be the optimum when increasing the setting from 400 to 700 °C in 50 °C steps. Nitrogen was used as nebulizing, auxiliary, and curtain gas at 50, 40, and 30 psi, respectively. Parent ions were filtered in the first quadrupole and dissociated in the collision cell. Ion transitions were recorded in multiple reaction monitoring (MRM) mode. Retention times, mass transitions for quantifier and qualifier ions of the analytes and the deuterated internal standards are shown in Table 1.

2.5. Method calibration

The method was calibrated by spiking a nonsmoker pool urine with 5, 10, 25, 50, 100, 150, 300 and 600 ng/ml MMA, 1, 2, 5, 10, 25 and 50 ng/ml HEMA and 1.5, 5, 25, 50, 100, 150, 300, 900 ng/ml CEMA. The background peak area ratios (area analyte/area IS) in unspiked urine were subtracted from each calibrator to account for endogenous levels. Each calibration level was analyzed in duplicate. The means of the analyte/IS peak area ratios were used to calculate the regression function. The calibration functions were linear for the calibration range of all analytes. Deviations from the nominal value were <15% (<20% at the lowest level) for all calibrators.

2.6. Method validation

Method validation was performed according to the FDA guidelines for bioanalytical methods [16]. Specificity, sensitivity, precision, accuracy, recovery, matrix effects, carryover, and stability of the analytes in human urine at ambient temperature, frozen at −20 °C, following three cycles of freezing and thawing and in reconstituted sample extracts were assessed during the validation exercise. The recovery was determined by comparing the MS/MS response obtained when spiking the analyte before the derivatisation step (=100%) and with that obtained when spiking the analyte at the start of the urine clean-up procedure. The matrix effect (ion suppression) was investigated by spiking pool urine extracts (prior to derivatization) with low and high levels of the analytes. A nonsmoker pool and a smoker pool urine were used for the low and high spike, respectively. The peak areas resulting from these samples (after subtracting the areas originating from the background analyte levels) were compared with those of the corresponding standards. Carry-over effects were checked by injecting 5 times a worked-up sample followed by a blank and repeating this procedure for 3 times.

2.7. Human studies

The study design for these investigations was a randomized (randomly assigned), controlled (housed in a clinic under a controlled environment), forced-switching (subjects randomly assigned to a smoking group), open label (due to the uniqueness of the test product, subjects were not blinded), parallel-group (all the group measurements occurred in parallel), single center design. After screening, 100 adult smokers of conventional cigarettes were admitted to the research clinic for a 10-day confinement period. In both studies, 24-h urine (approximately 07:00–07:00) was collected from Baseline through Day 8. Aliquots were obtained and stored frozen at −20 °C until analysis. The samples collected at Baseline and Day 8 were analyzed and compared by group. The protocol and informed consent form were reviewed and approved by the MDS Pharma Services Institutional Review Board. All subjects provided written consent before enrolling into the study, were paid for participating, and were free to discontinue the study at any time for any reason.

2.7.1. Study 1

Subjects were randomized into one of three study groups for a period of 8 days: Group 1 (n = 20) continued to smoke conventional cigarettes (CC1, 11 mg tar and 0.8 mg nicotine delivery according to the Federal Trade Commission (FTC) smoking regimen), Group 2 (n = 60) was switched to a test cigarette (TC1), which is an electrically heated smoking system (EHCSS), and Group 3 (n = 20) stopped smoking for the remainder of the study (SS1). The EHCSS has a puff-activated lighter/heater as described previously [13]. Its overwrap paper is designed to decrease generation of formaldehyde in the mainstream smoke. The EHCSS delivers a maximum of eight puffs per cigarette, one from each of the eight tobacco-heating blades of the puff-activated lighter. Tar and nicotine yield according to FTC amount to 5 and 0.4 mg/cigarette, respectively. As tobacco is only heated during a puff, the EHCSS does not generate side-stream smoke. Details of the study design, subject characteristics and cigarette characteristics have been reported [17].

2.7.2. Study 2

In this study 60 adult smokers of conventional cigarettes were randomized into one of three study groups after screening and Baseline investigations: Group 1 (n = 20) continued smoking conventional cigarettes (11 mg FTC tar and 0.8 mg FTC nicotine delivery) (CC2), Group 2 (n = 20) was switched to a test cigarette consisting of a conventional lit-end cigarette having 11 mg FTC tar and 0.8 mg FTC nicotine as well as a highly activated carbon granule filter (TC2). Group 3 (n = 20) stopped smoking for the remainder of the study (SS2). Details of this study design, subject characteristics and cigarette characteristics have been reported [18].

2.8. Statistical methods and data analysis

SAS® for Windows (SAS Institute, Cary, NC, release 9.1) was used for conducting the statistical analyses. A linear mixed model for repeated measures analysis of variance was used to test for differences in daily biomarker urinary excretion levels between the study groups (CC, TC1, TC2, SS) and time points. The terms in the model were study group, time point and their interactions. SAS® Proc Mixed was used for this analysis. The relationship between the daily biomarker urinary excretion levels and nicotine equivalents urinary excretion levels were examined using Pearson product–moment correlation analysis. For non-normal distribution of biomarker variables natural logarithm transformation was applied. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Chromatography and mass-selective detection

Product ion mass spectra of the PFBBr derivates of MMA, HEMA, and CEMA and of the deuterated internal standards together with the suggested structures of the ion fragments are shown in Fig. 2. The $[M+H]^+$ ion of MMA (m/z 358), MMA-D_3 (m/z 361), HEMA (m/z 388), HEMA-D_4 (m/z 392), CEMA (m/z 397) and CEMA-D_3 (m/z 400) are formed from all compounds. The mercapturic acid-specific ion

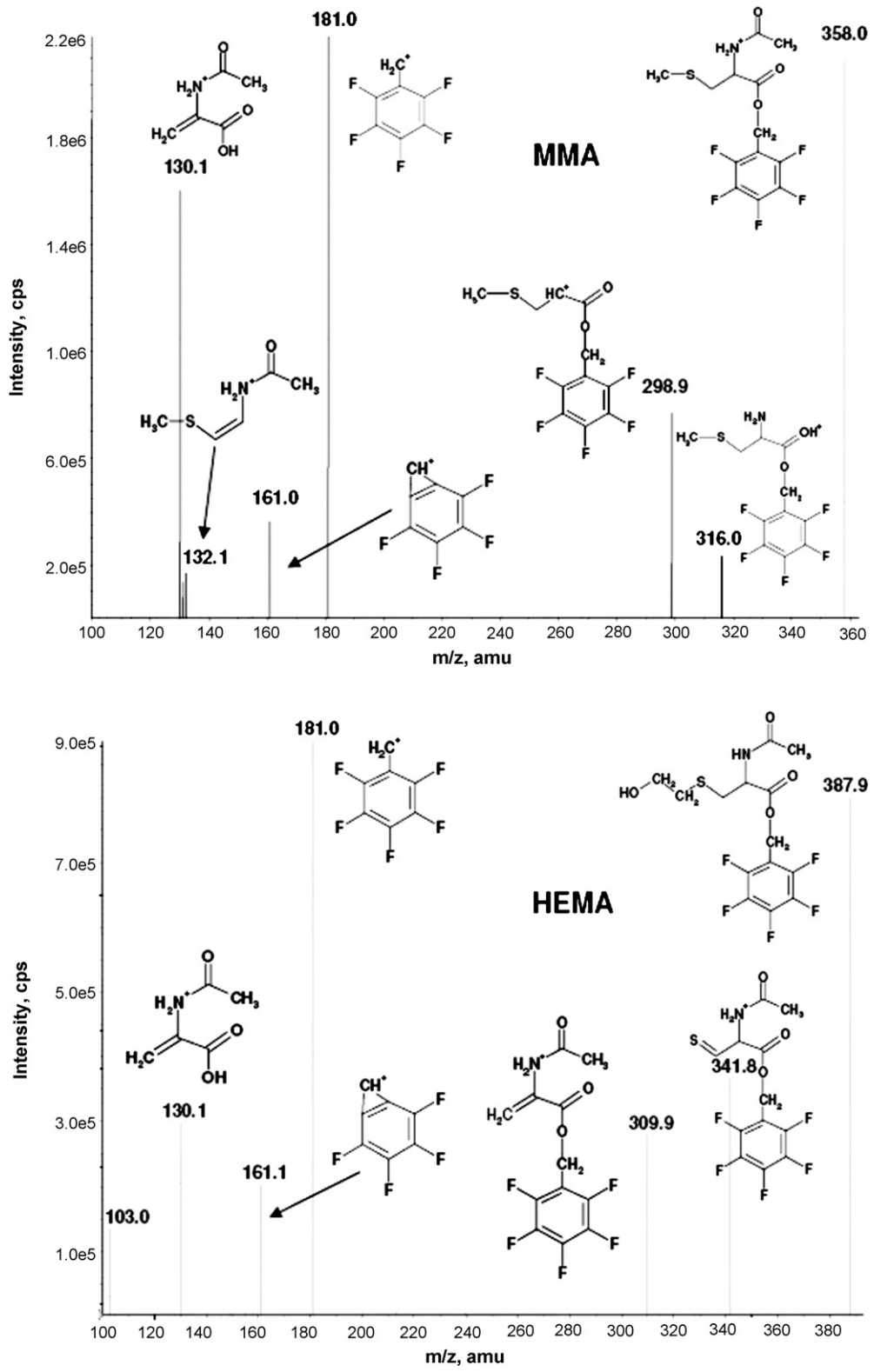

Fig. 2. Product ion mass spectra of the PFBBr derivatives of MMA, HEMA and CEMA.

fragment of *m*/*z* 130 (133 for CEMA-D_3) is formed from each analyte and internal standard derivative. The same is true for the fragment ions *m*/*z* 181 and 161, which are formed from the pentafluorobenzyl (PFB) residue of the derivatives. The most intensive ion fragment *m*/*z* 181, which is due to the PFB residue in the molecule, could not be used because of high background levels of this mass transition. It is notable that the ion fragments corresponding to the parent mercapturic acids ($MH^+ - 181$) do not occur in the ion mass spectra under these conditions.

Typical chromatograms of derivatives of the analytes and internal standards obtained with extracts from a conventional cigarette smoker are shown in Fig. 3.

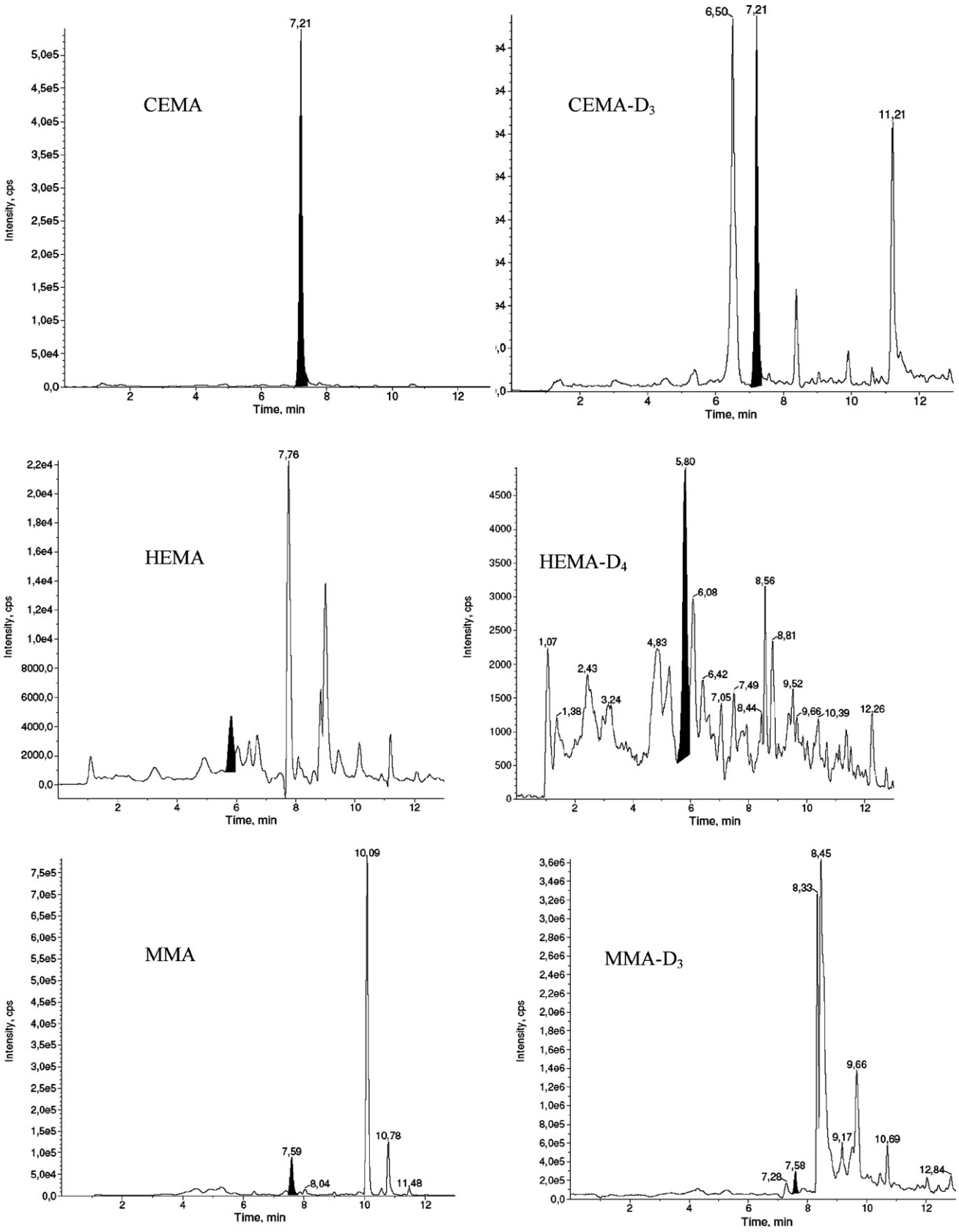

Fig. 3. Ion chromatograms of a urine sample from a subject (# 88) smoking a conventional cigarette: ion transitions for CEMA, HEMA and MMA derivatives are shown on the left panel, the ion transitions for the corresponding deuterated internal standards are shown on the right panel. The analytes were present in the following concentrations: CEMA: 240.8 ng/ml, HEMA: 17.0 ng/ml, MMA: 19.5 ng/ml.

Table 2
Performance data of the analytical method for MMA, HEMA and CEMA in urine.

	MMA	HEMA	CEMA
Specificity			
Deviations quantifier/qualifier in 4 different urine matrices	358 → 132/358 → 130 < 33%	388 → 130/388 → 310 < 7%	397 → 130/397 → 355 < 5%
Accuracies in 6 different urine matrices:	163 ng/ml: 95–103%	9.4 ng/ml: 96–104%	102 ng/ml: 97–103%
Matrix effect	5.0 ng/ml: 107.9%	0.5 ng/ml: 41.9%	2.0 ng/ml: 21.3%
Peak area ratios between analyte in matrix (urine) and solvent (methanol). Mean of $n = 3$ measurements	50.0 ng/ml: 34.8%	10.0 ng/ml: 37.6%	130.0 ng/ml: 36.7%
Precision			
Intra-day ($n = 5$)	12.5 ng/ml: 0.9% 23.2 ng/ml: 4.9% 28.7 ng/ml: 2.2%	5.5 ng/ml: 5.0% 13.8 ng/ml: 1.2% 46.2 ng/ml: 4.8%	1.6 ng/ml: 5.7% 167 ng/ml: 4.9% 305 ng/ml: 2.7%
Inter-day (6 days)	12.6 ng/ml: 7.9% 23.2 ng/ml: 3.2% 28.7 ng/ml: 2.5%	5.5 ng/ml: 8.3% 13.8 ng/ml: 6.5% 46.2 ng/ml: 4.4%	1.6 ng/ml: 13.9% 167 ng/ml: 4.2% 305 ng/ml: 2.2%
Accuracy ($n = 5$)	5.0 ng/ml: 80.6% 10.0 ng/ml: 99.9% 50.0 ng/ml: 96.3%	3.0 ng/ml: 100.2% 15.0 ng/ml: 103.3% 30.0 ng/ml: 97.6%	2.0 ng/ml: 116.1% 50.0 ng/ml: 101.4% 130 ng/ml: 102.4%
Recovery (whole analytical procedure without derivatization)	5.0 ng/ml: 89.0% 10.0 ng/ml: 87.5% 50.0 ng/ml: 76.5%	2.0 ng/ml: 20.9% 15.0 ng/ml: 11.4% 30.0 ng/ml: 16.6%	2.0 ng/ml: 24.5% 50.0 ng/ml: 20.1% 130 ng/ml: 37.5%
LOD	~1.4 ng/ml	~0.3 ng/ml	~0.2 ng/ml
LOQ[a]	5.0 ng/ml (CV: 9.5%)	1.0 ng/ml (CV: 8.3%)	1.5 ng/ml (CV: 2.2%)
Linearity	0–600 ng/ml $y = 0.02331\,x$ ($R^2 = 0.9996$)	0–50 ng/ml $y = 0.05379\,x$ ($R^2 = 0.99998$)	0–900 ng/ml $y = 0.04881\,x$ ($R^2 = 0.99999$)
Stability ($n = 3$)	23.2 ng/ml: 92.7%	5.5 ng/ml: 103.5%	1.6 ng/ml: 94.1%
Short-term (20 h, 21 °C)	28.7 ng/ml: 108.8%	45.2 ng/ml: 101.3%	305 ng/ml: 100.0%
Long-term (9 weeks, −20 °C)	12.6 ng/ml: 90.4% 28.7 ng/ml: 94.4%	5.5 ng/ml: 112.6% 46.2 ng/ml: 105.4%	1.6 ng/ml: 102.6% 305 ng/ml: 105.5%
Freeze/thaw (3 cycles)	23.2 ng/ml: 92.6% 28.7 ng/ml: 90.6%	5.5 ng/ml: 110.3% 45.5 ng/ml: 107.4%	1.6 ng/ml: 99.9% 305 ng/ml: 101.6%
Autosampler (~65 h, 10 °C)	12.6 ng/ml: 100.5% 28.7 ng/ml: 95.8%	5.5 ng/ml: 100.9% 46.2 ng/ml: 102.4%	1.6 ng/ml: 97.1% 305 ng/ml: 96.8%

[a] Derived from the lowest calibrator levels not exceeding 20% of deviation from the nominal level. CVs were derived from the duplicate analysis of the lowest calibrator.

3.2. Performance of the method

Performance data of the method for MMA, HEMA and CEMA are summarized in Table 2.

Specificity of the method was demonstrated for all three analytes. The quantifier/qualifier ratio for MMA deviated by 33% in one of the six urine sources, although the deviation was less than 7% for the other matrix sources. *Intra*- and *inter*-day precision of the method did not exceed 15%. Accuracies for all analytes at all levels were within 81–116%. Extraction efficiency was >75% for MMA, and approximately 20% for HEMA and CEMA. LODs for MMA, HEMA and CEMA were 1.4, 0.3 and 0.2 ng/ml, respectively. The corresponding LOQs amounted to 5.0, 1.0 and 1.5 ng/ml, respectively. For all three analytes (except for MMA at low level in a nonsmoker pool urine) significant matrix effects were observed (suppression by 80–60%, Table 2). No losses of the analytes were observed under short-term storage conditions at 21 °C for 20 h and long-term storage conditions at −20 °C for 9 weeks. Stability following three cycles of freezing and thawing as well as post-preparative stability testing showed no analyte losses. No carry-over of the IS to the blank samples was observed.

3.3. Study 1

In this study the group of 20 subjects that continued smoking conventional cigarettes (CC1) smoked on average, 17 cigarettes/day (cig/day) at Baseline (Day-1) and 18.6 cig/day at Day 8. The group of 60 subjects that was switched to TC 1 (EHCSS) smoked 17.5 cig/day at Baseline and 17.7 cig/day at Day 8. The group of 20 subjects that stopped smoking, smoked 16.0 cig/day at Baseline. Table 3 shows the daily urinary excretion levels of each biomarker at Baseline and Day 8 for each of the three groups. For all three urinary biomarkers, on Day 8 in the CC1 group, no statistically significant ($p > 0.05$) difference was found compared to Baseline. There was a statistically significant decrease ($p < 0.0001$) in the excretion levels for CEMA and HEMA for the other two groups (TC1 and SS1) on Day 8. MMA, however, did not show a statistically significant difference between Day 8 and Baseline.

The Pearson product–moment correlation coefficients showed significant correlations between CEMA and HEMA and nicotine equivalents in all smokers at Baseline ($r = 0.71$ and 0.50 respectively, $p < 0.0001$), when all subjects smoked CC1. MMA had no significant correlation with nicotine equivalents ($r = 0.06$). The Pearson product–moment correlation coefficients showed significant correlations between CEMA and HEMA and nicotine equivalents in all smokers at Day 8 ($r = 0.68$ and 0.72 respectively, $p < 0.0001$). MMA had no significant correlation with nicotine equivalents ($r = 0.11$).

3.4. Study 2

In this study the group of 20 subjects that continued smoking conventional cigarettes (CC2) smoked on average, 18.5 cig/day at Baseline and 18.6 cig/day at Day 8. The group of 20 subjects that was

Table 3
Daily urinary biomarker excretion levels [mean[a] ± SD (range)] from Study 1 and Study 2.

	MMA (μg/24 h)	CEMA (μg/24 h)	HEMA (μg/24 h)
Study 1			
CC1 (n = 20)			
Baseline	25.4 ± 20.8 (8.37–82.6)	246.7 ± 82.8 (107.1–353.3)	26.9 ± 8.7 (11.2–40.3)
Day 8	27.2 ± 20.9 (9.84–84.7)	272.8 ± 76.5 (172.8–399.0)	28.8 ± 11.0 (13.5–49.9)
TC1 (EHCSS) (n = 60)			
Baseline	20.4 ± 12.2 (6.97–70.6)	264.1 ± 123.2 (47.6–597.1)	28.7 ± 12.0 (8.56–60.1)
Day 8	24.5 ± 18.0 (3.34–93.0)	69.0 ± 43.9[b,c] (4.93–181.0)	13.3 ± 9.53[b,c] (2.28–67.6)
SS1 (n = 20)			
Baseline	24.0 ± 21.8 (5.07–82.0)	259.2 ± 117.6 (128.2–608.0)	30.4 ± 14.7 (10.8–65.5)
Day 8	26.1 ± 31.6 (4.75–148.2)	25.7 ± 13.4[b,c](14.9–75.5)	7.61 ± 3.56[b,c] (3.92–19.2)
Study 2			
CC2 (n = 20)			
Baseline	19.6 ± 12.7 (8.97–63.2)	274.6 ± 157.9 (60.4–697.8)	24.0 ± 11.9 (6.10–54.0)
Day 8	19.5 ± 14.3 (6.60–72.2)	220.5 ± 99.9 (53.4–487.8)	23.3 ± 16.2 (6.82–76.6)
TC2 (n = 20)			
Baseline	26.4 ± 21.3 (4.05–100.3)	272.6 ± 115.8 (24.3–448.0)	30.2 ± 19.9 (9.32–96.0)
Day 8	26.8 ± 18.4 (7.22–70.2)	62.5 ± 34.7[b,c] (13.3–171.8)	16.4 ± 9.23[b,c] (0.760–46.2)
SS2 (n = 20)			
Baseline	21.0 ± 10.8 (6.60–42.1)	274.6 ± 108.1 (53.2–494.9)	25.8 ± 8.8 (9.88–41.4)
Day 8	19.1 ± 11.7 (5.87–52.0)	29.1 ± 17.9[b,c] (6.30–75.1)	6.79 ± 3.05[b,c] (0.905–13.0)

[a] Arithmetic means.
[b] Statistical significantly different between baseline level and Day 8 level. p-values for comparisons with the baseline values (reference) were obtained from the ANOVA model after the natural log transformation of the biomarker excretion levels, p-values were 0.0001.
[c] Statistical significantly different between the reference group (CC1or CC2) at Day 8 level and the other groups at Day 8 level. p-Values for comparisons with the reference group at baseline or Day 8 (reference) were also obtained from the ANOVA model after the natural log-transformation of the biomarker excretion levels, except for one case all of the p-values were <0.0001.

switched to TC2, smoked 17.9 cig/day at Baseline and 18.6 cig/day at Day 8. The group of 20 subjects that stopped smoking (SS2) smoked 18.5 cig/day at Baseline. The daily urinary excretion of each biomarker in the second study at Baseline and Day 8 for each of the three groups is shown in the lower half of Table 3.

There was no statistically significant difference ($p > 0.05$) between Baseline and Day 8 for any of the three urinary biomarkers in the CC2 group. There was a statistically significant decrease ($p < 0.0001$) in the excretion levels for CEMA and HEMA for the other two groups (TC2 and SS2) on Day 8. MMA, however, did not show a statistically significant difference between Day 8 and Baseline.

In a similar manner to Study 1, the Pearson product–moment correlation coefficients showed significant correlations between CEMA and HEMA and nicotine equivalents in all smokers at Baseline ($r = 0.91$ and 0.52, respectively, $p < 0.0001$), when all subjects smoked CC2. MMA had no significant correlation with nicotine equivalents. Correlations between CEMA and HEMA and nicotine equivalents were also significant in the remaining smokers who smoked at Day 8 ($r = 0.68$ and 0.72 respectively, $p < 0.0001$). MMA again had no significant correlation with nicotine equivalents ($r = 0.28$).

4. Discussion

We have developed and validated a LC–MS/MS method for the simultaneous determination of three major urinary metabolites of these compounds, namely, MMA, HEMA and CEMA. Orginally, ethylmercapturic acid (EMA, a potential biomarker of exposure to ethylating agents) was also included in this method. The method comprises liquid/liquid extraction of the urine sample, solid phase extraction on anion exchange cartridges, derivatization with PFBBr, liquid/liquid extraction of the reaction mixture and LC–MS/MS analysis with positive electrospray ionization. The elaborate procedure was necessary in order to achieve the required specificity and sensitivity. These features were particularly important for EMA, levels of which were about 50-fold lower than those of MMA and HEMA and >100-fold lower than that of CEMA. The derivatization step led to a significant improvement in selectivity and sensitivity, allowing the detection of EMA in all and the quantitation in about 50% of the samples from the studies presented. Since the latter fact was regarded as insufficient for evaluation, EMA was not included in this investigation. The recovery for the analytical procedure (without the derivatization step) was >75% for MMA, but only about 20% for HEMA and CEMA. The extraction efficiency for HEMA is in line with the ethyl acetate extraction efficiency of 15% reported by Barr et al. [19]. These authors, therefore, used anion exchange extraction of urine, yielding a recovery of 76% for HEMA. The lower extraction efficiencies for HEMA and CEMA compared to that for MMA is probably a result of the higher polarity of the former compounds which leads to their less efficient extraction from urine with ethyl acetate. The low extraction efficiencies should not affect quantitation because of the use of deuterated internal standards. For the same reason, the observed matrix effects should also not impair the quantification ability of the method, however, LOD and LOQ may be increased in some samples. In the two presented studies, we observed 1 (of 239) sample at the LOD and 50 samples (21%) between LOD and LOQ for MMA. No samples had levels of HEMA and CEMA below the LOD.

Method calibrations were performed in authentic matrix (human urine), in order to simulate any interferences form the matrix. Urine samples with especially low analyte levels were selected for this purpose. In principle, high analyte background levels in the unspiked calibrators could impair the LOQ of the method, which in our study was the lowest calibrator. Background levels were 2.0, 1.5 and 1.0 ng/ml for MMA, HEMA and CEMA, respectively. Since in this investigation <3, 1 and 0% of samples measured for MMA, HEMA and CEMA, respectively, were below these levels, we believe that this has no impact on the results.

To our knowledge, our method is the first, which allows the simultaneous determination of MMA, HEMA and CEMA at non-

occupational levels in human urine. Mean urinary excretion of MMA in adult smokers was found to be about 20 μg/24 h. We observed no influence of stopping smoking or switching to test cigarettes with lower yields of methylating smoke constituents on the MMA level in urine. In both studies, the percent change from baseline for MMA was <10% in all groups. The most intensively investigated biomarker for smoking-related methylation, methylvaline hemoglobin adducts, shows 20–30% higher levels in smokers compared to nonsmokers [20–23]. The 8-day without smoking or switching to test cigarettes (8 days) should be sufficient to show an effect on the urinary MMA excretion, because urinary mercapturic acids typically have half-lives in the range of 6–24 h [6]. In our view, it is more probable that other sources for urinary MMA, including endogenous methylation [24], have overwhelmed the potential effect of smoking. Our results show that urinary MMA is not a suitable biomarker for measuring the smoking-related exposure to methylating agents.

Smokers of conventional cigarettes excreted about 25 μg/24 h of HEMA. In both studies, after 8 days of stopping smoking, the HEMA level significantly decreased to 7–8 μg/24 h. This was an approximate 75% reduction from baseline in HEMA levels. Switching for 8 days to TC1 (EHCSS) or TC2 cigarettes significantly reduced the urinary HEMA excretion to approximately 13 and 17 μg/24 h, respectively which corresponded to an approximate 50% reduction from baseline levels. Calafat et al. [25] reported geometric means for HEMA in urine of smokers and nonsmokers of 2.6 and 1.1 ng/ml, respectively. Schettgen et al. [26] reported urinary median HEMA levels of 2.0 (range: 0.7–4.7) and 5.3 (0.8 7.5) ng/ml for nonsmokers and smokers, respectively. Ding et al. [27] found urinary HEMA ranges of <0.03–1.44 ng/ml for nonsmokers and <0.03–20.8 ng/ml for smokers. These levels appear lower than those observed in our study. On the other hand, Popp et al. [28] reported average urinary HEMA excretions of 558 μg/24 h (372 ng/ml) and 33 μg/24 h (22 ng/ml) for 10 subjects exposed to ethylene oxide and 10 unexposed controls, respectively. Both groups comprise smokers and nonsmokers, however, the distribution was not reported. The HEMA levels in controls are in close agreement with the levels we observed. Recently, Carmella et al. [29] reported that HEMA in urine of cigarette smokers decreased from 102 to 24.0 nmol/24 h three days after smoking cessation. These levels correspond to urinary excretion rates of 21.1 and 5.0 μg/24 h, respectively, and are thus in good agreement with our findings (Table 3). Haufroid et al. [30] investigated urinary HEMA in hospital workers temporarily exposed to ethylene oxide. At baseline (no exposure for at least 2 days), urinary HEMA levels amounted to 1.42 and 3.33 μg/g creatinine for nonsmokers and smokers, respectively. In all studies mentioned, except that of Popp et al. [28], urinary HEMA was measured by means of LC–MS/MS. Popp et al. [28] applied an amino acid analyzer after conversion of HEMA to hydroxyethylcysteine. Since urinary HEMA levels responded markedly to both stopping smoking and switching to the test cigarettes having lower levels of hydroxyethylating agents, our findings suggest that urinary HEMA is a suitable short-term biomarker for assessing the smoking-related exposure to hydroxyethylating agents.

Smokers of conventional cigarettes in our study excreted about 270 μg/24 h of CEMA. After 8 days of stopping smoking, the CEMA level significantly decreased to 30 μg/24 h, corresponding to an approximate 90% reduction from baseline levels for this biomarker. Switching for 8 days to EHCSS or TC2 cigarettes significantly reduced the urinary CEMA excretion to about 70 μg/24 h. This was an approximate 75% reduction from baseline levels. Norpoth and Mueller [31], using an amino acid analyzer after conversion of CEMA to cyanoethylcysteine, found CEMA concentrations of 127 and 252 ng/ml in urine of unexposed and exposed persons, respectively. Smokers and nonsmokers in the unexposed group were not differentiated. However, the CEMA levels would be in line with our results, if we assume that the group consists of both smokers and nonsmokers. The authors mentioned that smoking significantly influences the CEMA excretion, without giving details [31]. Recently, Schettgen et al. [32] found that urinary CEMA levels increased in nonsmokers with the extent of exposure to environmental tobacco smoke (ETS). The authors reported median CEMA levels of 2.0, 3.2 and 6.6 ng/ml for nonsmokers not exposed, slightly exposed or highly exposed to ETS, respectively. Median CEMA concentrations in smokers were 240 ng/ml. The latter finding is in line with our CEMA levels smokers of CC. Taken together, our results and those from the literature suggest that urinary CEMA is a suitable short-term biomarker for assessing the smoking-related exposure to cyanoethylating agents.

In conclusion, the newly developed LC–MS/MS method for the determination of urinary alkymercapturic acids meets commonly used validation criteria. The application of this method to two clinical studies in adult smokers revealed that HEMA and CEMA, but not MMA, are significantly associated with tobacco smoke exposure. HEMA and CEMA are suitable, non-invasive, short-term biomarkers which allow us to distinguish differences in exposure not only between smokers and nonsmokers but also between smokers of conventional and the two test cigarettes.

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