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Quantitation of Acrolein-Derived 3-Hydroxypropylmercapturic Acid in Human Urine by Liquid Chromatography-Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry: Effects of Cigarette Smoking

Steven G. Carmella, Menglan Chen, Yan Zhang, Siyi Zhang, Dorothy K. Hatsukami, and Stephen S. Hecht*

The Cancer Center and Transdisciplinary Tobacco Use Research Center, University of Minnesota, Minneapolis, Minnesota 55455

Abstract

Recently published data suggest that acrolein (**1**), a toxic but weakly carcinogenic constituent of cigarette smoke, may be involved as a causative factor for the mutations frequently observed in the *p53* tumor suppressor gene in lung cancer in smokers. Biomarkers are needed to further assess the possible relationship between acrolein uptake and cancer. In this study, we analyzed 3-hydroxypropylmercapturic acid (3-HPMA, **2**) in human urine. 3-HPMA is a major metabolite of acrolein in laboratory animals. The method employs [¹³C₃]3-HPMA as internal standard, with analysis and quantitation by LC-APCI-MS/MS-SRM. Clean, readily quantifiable chromatograms were obtained. The method was accurate and precise and required only 0.1 mL of urine. Median levels of 3-HPMA were significantly higher (2900 pmol/mg creatinine, N = 35) in smokers than in non-smokers (683 pmol/mg creatinine, N = 21) (P = 0.0002). The effect of smoking was further assessed by determining levels of 3-HPMA before and after a 4 week smoking cessation period. There was a significant 78% decrease in median levels of urinary 3-HPMA after cessation (P < 0.0001). The relationship between levels of urinary 3-HPMA and those of acrolein-derived 1,N²-propanodeoxyguanosine (PdG) adducts in lung was investigated in 14 smokers. There was a significant inverse relationship between urinary 3-HPMA and α -hydroxy-PdG (**3**) but not γ -hydroxy-PdG (**4**) or total adduct levels. The results of this study clearly demonstrate that acrolein uptake in smokers is significantly higher than in non-smokers, and underline the need for further investigation of the possible relationship of acrolein uptake to lung cancer.

Keywords

acrolein; 3-hydroxypropylmercapturic acid; human urine

Introduction

While the toxic effects of acrolein (**1**) in cigarette smoke have been established for over 4 decades (1,2), only recently has it emerged as a possible participant in the genetic damage caused by cigarette smoking in humans (3,4). Feng et al demonstrated that acrolein produces a pattern of DNA damage in the *p53* tumor suppressor gene which is similar to the spectrum of mutations found in this commonly mutated gene in lung tumors from smokers (3). This

*To whom correspondence should be addressed: The Cancer Center, University of Minnesota, MMC 806, 420 Delaware St SE, Minneapolis, MN 55455, USA. ph: (612) 626-7604 fax: (612) 626-5135 e-mail: hecht002@umn.edu.

observation challenges the widely held view that polycyclic aromatic hydrocarbons (PAH), which produce a similar spectrum of DNA damage via their diol epoxide metabolites, are responsible for the observed *p53* mutations (5). The role of acrolein in producing this significant genetic damage is plausible because of its relatively high concentration in cigarette smoke – 18 – 98 μg per cigarette – far greater than that of PAH (6). On the other hand, the carcinogenic activity of acrolein in laboratory animals, in contrast to that of PAH, is weak or possibly non-existent, although it does produce genetic damage in bacteria and in some human cell assays (7). Furthermore, we have recently shown that acrolein-DNA adducts are present in human lung tissue (8). Collectively, these observations raise some intriguing questions about the possible role of acrolein in cancer induction by cigarette smoke.

An important approach to determining individual and collective risk from exposure to tobacco products is assessment of tobacco carcinogen biomarkers (9,10). Urinary metabolites have emerged as highly practical biomarkers for determining uptake of specific carcinogens and toxicants in tobacco smoke, and ultimately are likely to have more utility in predicting tobacco-associated harm than machine measurements of smoke constituents. Studies in laboratory animals have demonstrated that 3-hydroxypropylmercapturic acid (3-HPMA) (**2**) is a major metabolite of acrolein, formed by glutathione conjugation followed by reduction and normal metabolic processing to a mercapturic acid (7,11). The identification of 3-HPMA in the urine of cigarette smokers has been reported (12). In this study, we developed a practical LC-ESIMS/MS method for quantitation of 3-HPMA in human urine. The method was applied to determine the relationship of 3-HPMA levels to smoking by analyzing urine from smokers and non-smokers, and by assessing levels in smokers who stopped smoking for 4 weeks. We also investigated the relationship of 3-HPMA levels to those of acrolein-DNA adducts in lung.

Experimental Section

Chemicals

3-HPMA and *N*-acetyl- $[\text{}^2\text{H}_3]$ -*S*-(4-hydroxybut-2-yl)-*L*-cysteine were purchased from Toronto Research Chemicals, Toronto, Ontario, Canada. All other chemicals were obtained from Aldrich Chemical Co., Milwaukee, WI

3-Hydroxy $[\text{}^{13}\text{C}_3]$ propylmercapturic acid $[\text{}^{13}\text{C}_3]$ 3-HPMA

To 0.5 mL of $[\text{}^{13}\text{C}_3]$ glycerol in a 25 mL 2-neck flask was added 100 mg NaHSO_4 . The mixture was heated to 175 $^\circ\text{C}$ for 1h and the evolved acrolein was led into a 10 mL flask by purging with a stream of N_2 . The 10 mL flask contained 80 mg *N*-acetylcysteine in 2 mL H_2O at room temperature and was stirred for 2h. The pH was adjusted to 7 with ammonium bicarbonate and 100 mg of NaBH_3CN was added. The mixture was stirred at room temperature overnight. The product was collected by reverse-phase HPLC and identified and quantified by LC-APCI-MS/MS comparison to 3-HPMA.

Analysis of 3-HPMA in Urine

Internal standard, $[\text{}^{13}\text{C}_3]$ 3-HPMA (50 ng) in H_2O , was added to urine (0.2 mL). A 500 mg Oasis MAX (Waters Associates, Milford, MA) solid phase extraction cartridge was conditioned with MeOH (6 mL) and then 2% aq NH_4OH (6 mL). The urine was applied to the cartridge and it was washed with 2% NH_4OH (6 mL) and then methanol (6 mL). The cartridge was then dried briefly under vacuum and further blown dry with N_2 . It was then washed with

2% aq formic acid (6 mL) and then the fraction containing 3-HPMA was collected with 30% MeOH/2% aq formic acid in a 15 mL glass disposable centrifuge tube. The solvent was removed under vacuum, overnight, using a Speed-Vac. The residue was transferred to a 0.25 mL autosampler vial with $2 \times 80 \mu\text{L}$ of 80:20 acetonitrile: MeOH, and the solvents were removed on the Speed-Vac. The samples were stored at -20°C until analysis by LC-APCI-MS/MS-SRM. Prior to MS analysis, the samples were redissolved in $50 \mu\text{L}$ of 93% 15 mM NH_4OAc / 7% MeOH containing 50 ng of the injection standard, *N*-acetyl- $[\text{}^2\text{H}_3]$ -*S*-(3-hydroxypropyl-1-methyl)-*L*-cysteine. Ten μL were injected for analysis.

LC-APCI-MS/MS-SRM analysis was carried out with an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) equipped with a $4.6 \text{ mm} \times 25 \text{ cm } 4\mu$ Synergi Max-RP C12 column (Phenomenex, Torrance, CA) and a Discovery Max (Thermoelectron, San Jose, CA) triple quadrupole mass spectrometer operated in the negative ion APCI mode. Solvent A was 15 mM NH_4OAc and solvent B was MeOH. The solvent elution program was as follows: time (%A, %B) 0–10 min (93, 7); 10–15 min (60, 40); 15–31 min (93, 7) at 0.8 mL/min. The MS source CID collision energy was 10v and the Q2 collision gas pressure was 1 mTorr.

The negative ion CI spectrum of 3-HPMA had a base peak of m/z 220 $[\text{M} - \text{H}]^-$. MS/MS of m/z 220 gave a base peak of m/z 91 $[\text{HO}(\text{CH}_2)_3\text{S}]^-$. The transitions monitored were m/z 220 – m/z 91 for 3-HPMA, m/z 223 – m/z 94 for $[\text{}^{13}\text{C}_3]$ 3-HPMA, and m/z 237 – m/z 105 for the injection standard.

Calibration curves were constructed using 1 ng/ μL $[\text{}^{13}\text{C}_3]$ 3-HPMA, 1 – 5 ng/ μL 3-HPMA, and 1 ng of *N*-acetyl- $[\text{}^2\text{H}_3]$ -*S*-(3-hydroxypropyl-1-methyl)-*L*-cysteine. Accuracy was determined by adding 25 – 300 ng 3-HPMA to a urine sample from a non-smoker, and analyzing each sample in duplicate. Precision was determined by analyzing 6 aliquots of a non-smoker's urine.

Urine Samples

The studies were approved by the University of Minnesota Research Subject's Protection Programs Institutional Review Board Human Subjects Committee. Urine samples from 35 smokers and 21 non-smokers were obtained at baseline from ongoing studies in the University of Minnesota Transdisciplinary Tobacco Use Research Center. Urine samples from smokers who stopped were obtained as described in a study of the effects of smoking cessation on acetaldehyde-DNA adducts in leukocytes (13). Briefly, subjects were included in this study if they were: 1) smoking at least 10 cigarettes per day; 2) motivated to quit; 3) drinking alcohol only occasionally or not at all (e.g., drinking less than or equal to 6 alcoholic drinks per month and willing to abstain from alcohol during the study); 4) not using marijuana on a regular basis (greater than once a month); 5) not diagnosed with a history of alcohol dependence; 6) in stable and good physical and mental health; and 7) not pregnant. The protocol called for a six week study with two weeks of baseline smoking data collection and four weeks of data collection during abstinence from all tobacco products. After two weeks of baseline smoking, subjects were asked to quit smoking after the second clinic visit. In order to facilitate smoking abstinence, subjects were provided brief behavioral counseling and the nicotine patch and/or the nicotine lozenge. First void morning urine samples were assessed for 3-HPMA. Levels of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (total NNAL) were determined and reported previously (13). One baseline sample and one sample after 4 weeks of abstinence were analyzed. Twenty-five subjects of the 46 eligible completed the study with biochemically confirmed abstinence, as previously described (13).

Urine samples were also obtained through The Cancer Center Tissue Procurement Facility from some subjects just prior to surgery for lung cancer (8). Tissue samples were histopathologically confirmed as normal and were taken during surgery. Levels of adducts 3 and 4 were determined and reported previously (8).

Statistical Analysis

The descriptive statistics of 3-HPMA and acrolein-DNA adduct levels were calculated. The distribution and variation of the data were examined. Because the biomarkers did not follow a normal distribution, hypothesis testing was conducted using nonparametric methods. 3-HPMA levels between smokers and non-smokers were compared using Wilcoxon's rank sum test. Changes in 3-HPMA levels after 4 weeks of abstinence were evaluated using Wilcoxon's signed-rank test. The relationship between 3-HPMA and NNAL in urine was evaluated with a non-parametric Spearman's correlation coefficient. Correlations between 3-HPMA and acrolein-DNA adducts were evaluated based on Spearman's rank-order correlation. The analyses were conducted using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). All tests were two-sided with a significance level of 0.05.

Results

The method is straightforward, involving addition of internal standard [$^{13}\text{C}_3$]3-HPMA to urine, solid phase extraction on a mixed anion exchange-reverse phase cartridge, and analysis by negative ion LC-APCI-MS/MS-SRM. The transitions analyzed were m/z 220 - m/z 91 for 3-HPMA, m/z 223 - m/z 94 for the internal standard, and m/z 237 - m/z 105 for the injection standard. A typical chromatogram from a smokers' urine is illustrated in Figure 1. Clean symmetric co-eluting peaks were observed for the analyte and internal standard. Similar traces were observed upon analysis of non-smokers' urine. These results confirm the presence of 3-HPMA in human urine.

Accuracy was determined by adding various amounts of 3-HPMA to the urine of a non-smoker. As shown in Figure 2, there was excellent agreement between the added and quantified amounts, and the y-intercept, corresponding to 440 ng/mL, agreed with the amount determined in the urine of this non-smoker, 422 ng/mL. Precision was determined by analyzing 6 aliquots of a non-smoker's urine. The results were 425 ± 6.7 ng/mL, RSD = 1.57%. Recoveries were approximately 40%. The limit of detection of 3-HPMA injected on the column was 11 pg/injection (S/N = 3). The estimated limit of quantitation of 3-HPMA in urine was 0.9 ng/mL.

Levels of 3-HPMA in the urine of 35 smokers were (mean $\pm$ S.D.) 3950 ± 2950 pmol/mg creatinine and in 21 non-smokers were 1900 ± 3000 pmol/mg creatinine. The median levels were 2900 pmol/mg creatinine in smokers and 683 pmol/mg creatinine in non-smokers ($P=0.0002$) (Figure 3). For the cessation study, we used urine which had been collected in a previous study of the effect of smoking cessation on levels of acetaldehyde-DNA adducts (13). Levels of 3-HPMA decreased significantly upon cessation of smoking, median decrease 78% ($P < 0.0001$) (Table 1 and Figure 4). Levels of 3-HPMA correlated with those of total NNAL, previously measured in these samples, ($R = 0.55$, $P = 0.0009$), when both were expressed as pmol/mg creatinine. Levels of 3-HPMA at baseline did not correlate with cigarettes smoked per day.

In a recent study, we quantified levels of acrolein-DNA adducts **3** and **4** in human lung (8). Urine samples were available from 14 of these subjects, and levels of 3-HPMA were determined. There was a significant inverse correlation between levels of 3-HPMA in urine and those of adduct **3** in lung (Spearman correlation coefficient, -0.58 , $P = 0.03$), but no relationship between amounts of 3-HPMA and adduct **4**, or the sum of adducts **3** and **4** ($P > 0.05$).

Discussion

The results of this study clearly demonstrate that levels of 3-HPMA are higher in smokers than non-smokers and that they decrease significantly on abstinence from smoking. These results show therefore that urinary 3-HPMA, formed from the toxic cigarette smoke constituent acrolein, is a smoking-related biomarker.

Our results are consistent with previous studies and with the amount of acrolein in mainstream cigarette smoke. Mascher et al reported a method similar to ours and found levels of acrolein in smokers and non-smokers similar to those reported here (12). In a recent study, Scherer et al found that when smokers switched from cigarettes containing cellulose acetate filters to charcoal filters, there was a small reduction of 3-HPMA, of borderline significance (14). The levels of 3-HPMA in urine are quite consistent with the measured levels in cigarette smoke. If the average mainstream delivery of acrolein were 50 μg per cigarette (6), then a smoker of 1 pack per day would excrete a maximum of about 4 mg per day of 3-HPMA. The average amount of 3-HPMA in our smokers was 1095 ng/ml, which amounts to about 1.7 - 2 mg per day (assuming urine volume of ~1.6 L - 2.0 L per day), consistent with the assumption that 3-HPMA is a major metabolite of acrolein, as it is in the rat (11).

All subjects in our study had 3-HPMA in their urine, independent of smoking status. Acrolein is a common product of combustion and air pollutant, occurring in gasoline and diesel engine exhaust, aircraft emissions, and industrial emissions (7). It is formed in high temperature cooking and may contribute to lung cancer in women who perform wok cooking (15). It is also found in a wide variety of foods (7), and occurs endogenously as a lipid peroxidation product (16). However, it is clear from the cessation study that cigarette smoking represents a major source of acrolein exposure and 3-HPMA in urine.

The relationship between levels of 3-HPMA in urine to adducts **3** and **4** in lung could be complex. At a given level of exposure to acrolein, individuals with more efficient glutathione detoxification capacity would be expected to have higher levels of urinary 3-HPMA, and this could contribute to lower adduct levels by scavenging acrolein. However, adduct levels could also be affected by repair and cell turnover. Furthermore, 3-HPMA may reflect predominantly hepatic detoxification of acrolein while our adduct measurements were in lung. Nevertheless, we did observe a negative correlation between urinary 3-HPMA and adduct **3**, but no relationship with adduct **4** or total adduct levels. These results were based on only 14 subjects for whom we had both urine and lung tissue and require further investigation.

The potential role of acrolein as a cause of lung cancer in smokers needs further research. The data reported here, together with the results of Feng et al demonstrating that it causes DNA damage at mutational hot spots in the *p53* gene (3), suggest involvement. Furthermore, adduct **3** has been shown to be mutagenic in human cells, causing G to T transversions (17), and we have shown that this adduct is present in human lung (8). However, levels of adducts **3** and **4** in human lung were unrelated to smoking status in our study, although the number of subjects

was small. Considering the results of the present study, the relationship of levels of adducts **3** and **4** in human lung to acrolein exposure from cigarette smoking requires further investigation. Arguing against a role of acrolein in smoking induced lung cancer is its weak carcinogenic activity (7). A working group of the International Agency for Research on Cancer concluded that there is inadequate evidence in experimental animals for the carcinogenicity of acrolein (7). Acrolein did not induce tumors in several studies in mice and rats and, when administered by inhalation to hamsters, did not enhance the carcinogenicity of either benzo [*a*]pyrene or *N*-nitrosodiethylamine (7). It did however produce bladder tumors in rats when given as a tumor initiator, followed by promotion with dietary uracil (18). The potential role of acrolein in cigarette smoke induced lung cancer requires further research.

In summary, the results of this study demonstrate that levels of 3-HPMA in human urine are related to cigarette smoking. The source of the higher levels in smokers is acrolein in cigarette smoke. While this paper was under review, two studies were published, one in which 3-HPMA levels were compared in smokers and non-smokers (19), and a second in which smokers abstained (20). The results were similar to those reported here. As acrolein is a highly toxic compound that could also be involved in tobacco smoke carcinogenesis, 3-HPMA should be incorporated as a standard urinary biomarker in investigations of cigarette smoke induced disease.

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Figure 1.

Chromatograms obtained upon LC-APCI-MS/MS-SRM analysis of a smoker's urine for 3-HPMA. Top panel, the peak for 3-HPMA elutes at 8.89 min. Bottom panel, the peak for the internal standard, $[^{13}\text{C}_3]$ 3-HPMA also elutes at 8.89 min.

Figure 2.
Relationship between added and measured 3-HPMA in urine. 3-HPMA was added to a non-smoker's urine and it was analyzed by the method described in the text.

Figure 3.

Levels of 3-HPMA in the urine of 35 smokers and 21 non-smokers. Boxes represent 25th and 75th percentile, lines represent 10th and 90th percentile, and dots represent outliers. The difference between the median levels in smokers and non-smokers was significant ($P = 0.0002$)

Figure 4.

Levels of 3-HPMA in the urine of smokers who abstained from smoking for 4 weeks. Solid bars represent levels in subjects at baseline, while still smoking. Open bars represent levels after these smokers had abstained.

Table 1

Sample Name	Baseline	Abstained 4 weeks	% Decrease
	3-HPMA pmol/mg creatinine	3-HPMA pmol/mg creatinine	
1	4600	1030	78
2	1850	954	48
3	5840	651	89
4	1330	296	78
5	8750	553	94
6	2670	491	82
7	2280	534	77
8	6370	368	94
9	4100	509	88
10	1180	842	29
11	7650	584	92
12	1660	680	59
13	3130	1300	58
14	2900	1010	65
15	10100	879	91
16	2820	867	69
17	3220	993	69
18	1660	346	79
19	1530	828	46
20	2350	452	81
21	1500	1130	24
22	5020	482	90
23	2760	718	74
24	5070	990	80
25	2790	489	82