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Measurement of the Heterocyclic Amines 2-Amino-9H-pyrido[2,3-*b*]indole and 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in Urine: Effects of Cigarette Smoking

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Abstract

2-Amino-9H-pyrido[2,3-*b*]indole (AαC) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) are carcinogenic heterocyclic aromatic amines (HAAs) formed during the combustion of tobacco and during the high-temperature cooking of meats. Human enzymes biotransform AαC and PhIP into reactive metabolites, which can bind to DNA and lead to mutations. We sought to understand the relative contribution of smoking and diet to the exposure of AαC and PhIP, by determining levels of AαC, its ring-oxidized conjugate 2-amino-9H-pyrido[2,3-*b*]indole-3-yl sulfate (AαC-3-OSO₃H), and PhIP in urine of smokers on a free-choice diet before and after a six week tobacco smoking cessation study. AαC and AαC-3-OSO₃H were detected in more than 90% of the urine samples of all subjects during the smoking phase. The geometric mean levels of urinary AαC during the smoking and cessation phases were 24.3 pg/mg creatinine and 3.2 pg/mg creatinine, and the geometric mean levels of AαC-3-OSO₃H were 47.3 pg/mg creatinine and 3.7 pg/mg creatinine. These decreases in the mean levels of AαC and AαC-3-OSO₃H were, respectively, 87% and 92%, after the cessation of tobacco ($P < 0.0007$). However, PhIP was detected in < 10% of the urine samples, and the exposure to PhIP was not correlated to smoking. Epidemiological studies have reported that smoking is a risk factor for cancer of the liver and gastrointestinal tract. It is noteworthy that AαC is a hepatocellular carcinogen and induces aberrant crypt foci, early biomarkers of colon cancer, in rodents. Our urinary biomarker data demonstrate that tobacco smoking is a significant source of AαC exposure. Further studies are warranted to examine the potential role of AαC as a risk factor for hepatocellular and gastrointestinal cancer in smokers.

*Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: Figure S-1. Calibration curves of AαC, AαC-3-OSO₃H, and PhIP and Figure S-2 UPLC/MS² analysis of urinary PhIP during smoking and cessation of tobacco.

Introduction

Heterocyclic aromatic amines (HAAs) are formed during the grilling of meats, poultry, and fish.¹ Many HAAs are carcinogens in experimental animals and thus, may contribute to human cancers.^{1,2} While the principal source of exposure to HAAs is generally thought to occur through the diet,^{2,3} several HAAs are formed during the combustion of tobacco or occur in diesel exhaust.⁴⁻⁹ 2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) is by far the most abundant of the HAAs and structurally related aromatic amines formed in tobacco smoke.¹⁰ The levels of AαC reported in tobacco smoke range from 25 to 260 ng per cigarette.^{4,9,11,12} These amounts are 25–100 -fold higher than the levels of 4-aminobiphenyl (4-ABP) and the polycyclic aromatic hydrocarbon, benzo[*a*]pyrene, and comparable to the levels of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) that occur in tobacco smoke.¹³⁻¹⁵ 4-ABP, benzo[*a*]pyrene, and NNK are recognized as human carcinogens.^{16,17} There is one report on the identification of PhIP in tobacco smoke with levels ranging from 11 to 23 ng/cigarette.⁷ Furthermore, PhIP has been implicated as a major DNA-damaging agent in the urine of smokers, based on ³²P-postlabeling analysis of urinary mutagens.¹⁸

Epidemiological studies have consistently reported that smoking is a risk factor for cancer of gastrointestinal tract¹⁹ and hepatocellular carcinoma;^{20,21} however, the chemicals in tobacco smoke responsible for these cancers are unknown. AαC induces aberrant crypt foci, an early biomarker of neoplasia, in colon of mice,²² and AαC is a potent *lacI* transgene colon mutagen in mice.²³ Mice exposed to AαC also develop hepatocellular carcinoma.²⁴ PhIP is a pancreatic and colon carcinogen in rats.^{1,25} Human hepatocytes efficiently transform AαC and PhIP to their genotoxic *N*-hydroxylated metabolites, 2-hydroxamino-9*H*-pyrido[2,3-*b*]indole (HONH-AαC) and 2-hydroxamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HONH-PhIP), which bind to DNA and induce mutations.^{26,27} Thus, AαC and PhIP present in tobacco smoke could contribute to DNA damage and the risk of developing cancer of the liver and digestive tract in smokers.

Urine is a useful biological matrix for the screening of hazardous chemicals and their metabolites, since large quantities can be obtained noninvasively. Many biomarkers of tobacco-associated carcinogens have been measured in urine.¹⁶ We previously identified AαC in urine of adult men of the Shanghai Cohort study.²⁸ The findings showed that AαC represented a major HAA exposure and that tobacco smoke was an important point source of their AαC exposure. The number of cigarettes smoked per day was positively and significantly related to urinary levels of AαC in study subjects. The relative contribution of tobacco smoke and the diet as sources of exposure to AαC and PhIP in the United States is not known. In this study, we measured urinary levels of AαC, its ring-oxidized conjugate, 2-amino-9*H*-pyrido[2,3-*b*]indole-3-yl sulfate (AαC-3-OSO₃H), and PhIP in a group of smokers in the United States who underwent a tobacco cessation program.

Materials and Methods

Caution

HAAs and derivatives are hazardous and should be handled with caution and with appropriate clothing.

Study subjects

Thirty compliant volunteers were selected from a completed smoking cessation conducted at the University of Minnesota Transdisciplinary Tobacco Use Research Center, Minneapolis, MN. The full details of the treatment and cessation program were reported.²⁹ The selected group was comprised of 13 females and 17 males. Three of the subjects were African American and the remaining volunteers were Caucasians. The volunteers smoked their own preferred brand of cigarettes *ad libitum* during the baseline 2 week smoking phase. The mean number of cigarettes smoked per day (CPD) was 23.8 ± 6.4 . First morning void urine samples were collected at the end of the two week baseline smoking phase. Thereafter, the subjects participated in a six week program, where either low yield nicotine cigarettes (0.3 mg or 0.05 mg nicotine per cig, Quest cigarettes, manufactured by Vector; Vector Tobacco Inc., Durham, NC) or nicotine lozenges (4 mg) were provided to relieve the acute effects of cigarette withdrawal. Thereafter, the usage of all treatment products ceased for a period of 6 weeks, and spot urine samples were obtained for chemical analysis the end of this cessation period. Urinary cotinine was measured to confirm that subjects had successfully refrained from smoking.²⁹ The goal of this study was to assess the level of exposure to AαC and PhIP during smoking and cessation phases. Since the combustion of low nicotine cigarettes are still expected produce AαC and PhIP, the measurement of these HAAs in urine of the volunteers was restricted to the baseline smoking phase and following the 6 week period where all treatment products had ceased.

Chemicals and reagents

ACS grade ethyl acetate and formic acid and reagent grade sodium hydroxide were purchased from Sigma Aldrich (St Louis, MO). LC/MS grade water, methanol, acetonitrile and formic acid and OPTIMA LCMS grade ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). AαC, PhIP, and 2-amino-1-trideuteromethyl-6-phenylimidazo[4,5-*b*]pyridine ($[^2\text{H}_3\text{C}]$ -PhIP) were purchased from Toronto Research Chemicals (Toronto, Canada). $[4\text{b},5,6,7,8,8\text{a}-^{13}\text{C}_6]$ -AαC was a kind gift of Dr. Daniel Doerge, National Center for Toxicological Research (Jefferson, AR). Liver microsomes from rats pretreated with polychlorinated biphenyls were purchased from Molecular Toxicology, Inc. (Boone, NC). SOLA SCX SPE cartridges (10 mg/mL) were purchased from Thermo Scientific (Bellefonte, PA). Oasis WAX SPE cartridge (30 mg/mL) were purchased from Waters (Milford, MA).

NMR Characterization of AαC Metabolites

^1H -NMR resonance assignments for the metabolites of AαC were conducted at 25 °C with a Bruker Avance III 600 MHz spectrometer equipped with a triple resonance cryoprobe (Bruker BioSpin Corp., Billerica, MA). The ^1H chemical shifts were referenced directly from the DMSO- d_6 multiplet at 2.50 ppm.

Biosynthesis of 2-Amino-3-Hydroxy-9H-pyrido[2,3-*b*]indole (AαC-3-OH)

AαC-3-OH was prepared enzymatically by incubation of liver microsomes (1 mg protein/mL) of rats pretreated with polychlorinated biphenyls with cofactors and AαC (200 μM) for 1 h at 37 °C as previously described.³⁰ The microsomal protein was precipitated

with 1 vol of CH₃OH, and the supernatant was diluted with 10 vol H₂O. 3-HO-A α C was enriched by solid phase extraction with a C18 resin, followed by purification with an HPLC Agilent 1260 Infinity HPLC (Santa Clara, CA) equipped with a UV photodiode array detector. A Thermo Aquasil C18 reversed-phase column (4.6 x 250 mm, 5 μ m particle size, Bellefonte, PA) was employed for isolation of the desired product. A linear gradient starting from 99% A (10 mM NH₄CH₃CO₂ in H₂O) and 1% B: (100% CH₃CN) and arriving at 100% B over 20 min at a flow rate of 1 mL/min.³¹ The desired metabolite was dried in vacuo. The proton assignments were: δ 10.92 (s, 1H, H-N9) 7.79 (d, 7.67 Hz, 1H, H-5); 7.53 (s, 1H, H-4); 7.34 (d, 7.97 Hz, 1H, H-8), 7.19 (dd, 7.55, 7.97 Hz, 1H, H-7), 7.06 (dd, 7.55, 7.67 Hz, 1H, H-6), 5.75 (s, 2H, NH₂). Full scan spectra under ESI/MS conditions with the TSQ showed the protonated molecule [M+H]⁺ at m/z 200.1.

Synthesis of 2-Amino-9H-pyrido[2,3-b]indole-3-yl sulfate (A α C-3-OSO₃H)

A α C or [¹³C₆]-A α C (0.35 μ mol) in C₂H₅OH (100 μ L) was reacted with a 1.2-fold molar excess of potassium persulfate in 0.4N NaOH, (0.4 mL).³² The mixture was placed in a thermomixer at 37 °C and agitated at 650 rpm for two h before neutralization with 1N HCl. The products were purified via the Agilent 1260 Infinity HPLC system and chromatographic conditions as described above for 3-HO-A α C. The desired product was collected and vacuumed centrifuged to dryness. The A α C-3-OSO₃H was dissolved in CH₃OH, and the concentration was determined by UV spectroscopy with an Agilent 8453 UV spectrophotometer employing a molar extinction coefficient ϵ (M⁻¹ cm⁻¹) = 21,563 at λ_{335} nm. The yield in product ranged between 10 – 20%. The ¹H NMR spectrum of A α C-3-OSO₃H is in agreement to that of the previously biosynthesized product.³³ (¹H NMR (DMSO-*d*₆) δ 11.64 (s, 1H, H-N9) 8.28 (s, 1H, H-4); 8.00 (d, 7.67 Hz, 1H, H-5); 7.50 (d, 8.00 Hz, 1H, H-8); 7.35 (dd, 7.55, 8.00 Hz, 1H, H-7), 7.22 (dd, 7.55, 7.67 Hz, 1H, H-6). Due to rapid exchange with the sulfate group, the NH₂ signal was not detected. Full scan spectra with the TSQ showed the protonated molecule [M+H]⁺ at m/z 280.1 and in negative ion mode the deprotonated molecule [M-H]⁻ was observed at m/z 278.1.

Isolation of A α C and PhIP from Urine

The isolation procedure followed that of our previous method except that a fritless SOLA SCX SPE cartridge was employed for isolation of HAAs. Urine (0.5 mL) was spiked with [¹³C₆]-A α C and [²H₃C]-PhIP (25 pg) and made alkaline with 10N NaOH (0.055 mL). The samples were heated at 37 °C in a thermo-mixer at 450 rpm for 2 h to deconjugate metabolites.²⁸ After cooling, samples were extracted twice with two volumes of ethyl acetate. The organic fractions were acidified with HCO₂H (2% v/v), and applied to SOLA SCX SPE cartridges, which were pre-washed with CH₃OH/5% NH₄OH followed by H₂O/2% HCO₂H before loading samples. Cartridges were washed in series with 1 mL volumes of H₂O/2% HCO₂H twice, followed by CH₃OH/2% HCO₂H, then H₂O, and finally with 40% CH₃OH/5% NH₄OH. Thereafter, the analytes were eluted with CH₃OH/5% NH₄OH and collected into Eppendorf tubes and dried by vacuum centrifugation at 43 °C. The samples were reconstituted in H₂O/0.01% HCOOH (50 μ L) and centrifuged at 21,000 x g for three min before transferring to silylated glass conical capLC sample vials (Wheaton, Microliter vials, Millville, NJ).

Isolation of AαC-3-OSO₃H from Urine

[¹³C₆]-AαC-3-OSO₃H (500 pg) was added to urine (0.5 mL). The samples were acidified with CH₃CO₂H (2% v/v), followed by two volumes of CH₃OH to precipitate salt and protein. Samples were placed on ice for 30 min before centrifugation at 17,000 x g for 10 min. The supernatant was applied to an Oasis WAX SPE cartridge, which was pre-washed with CH₃OH/5% NH₄OH, followed by H₂O/2% HCO₂H before loading samples. Cartridges were washed in series with 1 mL of H₂O/2% HCO₂H (twice), followed by CH₃OH/2% HCO₂H, H₂O, and finally 40% CH₃OH/5% NH₄OH. AαC-3-OSO₃H and its internal standard were eluted with CH₃OH/5% NH₄OH and collected into Eppendorf tubes, followed by vacuum centrifugation to dryness as described above. The samples were reconstituted in 5 mM NH₄HCO₃ (pH 9.0) (50 μL), followed by addition of chloroform (5 μL). Samples were vortexed and then sonicated with a Branson CPX 3800 ultrasonic cleaner for 10 min, and then centrifuged at 21,000 x g for three min. The supernatant was transferred into a 300 μL polypropylene capLC vial (ChromTech, Apple Valley, MN).

Method Validation and Calibration Curves

The performance of the method was determined by inter-day and intra-day estimates of the levels of urinary biomarkers. The samples were assayed in quadruplicate on four different days. The limit of detection (LOD), limit of quantification (LOQ), the within-day and between-day reproducibility were performed by spiking pooled urine samples from four non-smokers who had refrained from eating well-done cooked meat.³⁴ AαC or PhIP was spiked into urine at levels of 10 or 30 pg/mL, and AαC-3-OSO₃H was spiked at a level of 100 or 500 pg/mL. Based upon recommended guidelines for data acquisition and quality evaluation in environmental chemistry, the values of the LOD and LOQ for analytes were set at 3σ and 10σ SD units above the background level signal of an uncontaminated matrix.³⁵

A combined calibration curve was constructed for AαC and PhIP, and an independent curve was constructed for AαC-3-OSO₃H. The same urine samples pooled from four non-smoking volunteers who had refrained from eating well-done cooked meat were used for method validation and constructing the calibration curves.³⁴ The matrix was verified to be free of HAA analytes by LC/MS (data not shown). Data were fitted to a straight line (area of response of analyte/internal standard versus the amount of analyte/internal standard) using ordinary least-squares with equal weightings. A seven-point calibration curve was established containing HAA biomarkers at concentrations ranging from 0, 2.5 to 50 pg/mL urine for AαC and PhIP, and a 9-point calibration curve ranging from 0, 20 to 1000 pg/mL urine was created for AαC-3-OSO₃H. Analytes were added to urine extracts following SPE. All calibration points were done in triplicate.

Ultraperformance Liquid Chromatography/Tandem Mass Spectrometry (UPLC/MS²)

The chromatographic separation of AαC and PhIP was performed with a Waters NanoAcquity UPLC system with a Magic C18AQ reversed-phase column (0.3 x 150 mm, 3 μm particle size, 100 Å pore size) (Michrom Corp., Auburn, CA) and heated to 50 °C. The flow rate was set to 6 μL/min. A linear gradient 10 min was employed, starting at 90% A (H₂O/0.01% HCO₂H) and 10% B solvent (CH₃CN/0.01% HCO₂H) and ended at 100% B.

The same UPLC system was employed for AαC-3-OSO₃H, but used a BEH130 C18 reversed-phase column (0.3 x 100 mm, 1.7 μm particle size, 130 Å pore size) (Waters Corp) heated to 50 °C. The flow rate was set at 5 μL/min. A 10 min linear gradient was employed and started at 90% A (5 mM NH₄HCO₃, pH 9.0) and 10% B (CH₃CN) and ended at 100% B.

The mass spectral data were acquired with TSQ Quantiva triple stage quadrupole (TSQ) mass spectrometer (Thermo Scientific, San Jose, CA) employing the HESI II source in the positive ionization mode. The instrument tune parameters for AαC and PhIP were as follows: 3.3 kV source spray voltage, 70 °C vaporizer temperature, sheath gas setting of 3 (arbitrary units), auxiliary gas 1 (arbitrary units), and no sweep gas. The ion transfer tube temperature was 400 °C. The tune method for AαC-3-OSO₃H used a 3.2 kV source spray voltage; the other parameters were identical to those employed for AαC and PhIP.

The selected reaction monitoring (SRM) mode was used to measure HAA biomarkers. Each SRM transition was independently optimized, with collision energies ranging from 29 – 48 V. The dwell time was 100 ms for each transition of AαC and PhIP. The resolution of Q1 and Q3 was set at 0.7 resolution (FWHM). The RF lens offset was 95 V for AαC and 120 V for PhIP. There was no in-source CID offset voltage. The quantification of AαC ([M + H]⁺ at *m/z* 184) was done using the transition 184.1 → 140.1 ([M + H]⁺ → [M + H – NH₃ – HCN]⁺). Two additional qualifier transitions were used to corroborate the purity of AαC at *m/z* 167.1 ([M + H]⁺ → [M + H – NH₃]⁺) and *m/z* 113.1 ([M + H]⁺ → [M + H – NH₃ – 2HCN]⁺). PhIP ([M + H]⁺ at *m/z* 225.1) was measured by the transition 225.1 → 210.1 [M + H]⁺ → [M + H – CH₃]⁺, and the transition 225.1 → 140.1 [M + H]⁺ → [M + H – C₃H₇N₃]⁺ was used as a qualifier ion. For AαC-3-OSO₃H ([M + H]⁺ at *m/z* 280.1), the RF lens offset was set at 107 V and an elevated in-source CID voltage offset of 60 V was employed to cause the loss of SO₃ moiety (–80 Da) in the source. The transition of the resultant AαC-3-OH ([M + H – SO₃]⁺ at *m/z* 200.1) was subjected to CID, and the transition 200.1 → 155.1 ([M + H – SO₃]⁺ → [M + H – SO₃ – CO – NH₃]⁺) was used for quantitation. The qualifier transitions 200.1 → 128.1 ([M + H – SO₃]⁺ → [M + H – SO₃ – CO – NH₃ – HCN]⁺) and 200.1 → 101.1 ([M + H – SO₃]⁺ → [M + H – SO₃ – CO – NH₃ – 2HCN]⁺) were used to confirm the analyte purity. Full scan product ion spectra were acquired on AαC employing a collision energy ramp of 20 – 55 V and for AαC-3-OH spectra were acquired at 40 V collision energy. The scanning for both molecules was from *m/z* 100 – 300.

Statistical Analyses

The paired t-test or linear regression was performed on tobacco-associated urinary biomarkers using GraphPad Prism (v. 6) for Windows, GraphPad Prism Software (San Diego, CA). A P value <0.05 was considered statistically significant.

Results

We employed our previously established a tandem solvent/SPE method to isolate AαC and PhIP from human urine; the LOQ values are 2.5 pg/mL for both HAAs.³⁶ In this study, we also developed a method to monitor AαC-3-SO₃H, a sulfate conjugate of 3-HO-AαC, which is one of the major metabolites of AαC formed by human liver microsomes.³⁷ A mixed-

mode weak anion exchange resin was successfully employed to isolate AαC-3-SO₃H from urine. The LOD and LOQ values for AαC-3-SO₃H, following in-source fragmentation of the sulfate bond to form AαC-3-OH, were 9.0 and 23.3 pg/mL urine. These values were set at 3σ and 10σ SD units above the signals of unspiked urine samples obtained from volunteers who did not smoke and had refrained from eating well-done cooked beef.^{34,35} The data on the precision and reproducibility of the urinary measurements of AαC and AαC-3-SO₃H are summarized in Table 1. The validation of the method was previously reported for PhIP.³⁶ The calibration curves are provided in Supporting Information, Figure S-1.

Identification of Urinary Biomarkers of HAAs During Baseline Smoking Phase and Six Weeks Following Tobacco Cessation

Representative UPLC/MS² chromatograms of AαC present in urine during the smoking phase and 6 weeks after cessation of tobacco usage are shown in Figure 2. The purity of the peak attributed to AαC was shown by the qualifier ions at m/z 167.1 and 113.1, attributed to $[M + H - NH_3]^+$ and $[M + H - NH_3 - 2HCN]^+$, which were within 20% of the relative abundance of ions observed for the pure standard.³⁸ The full scan product ion spectrum, which was in excellent agreement to the spectrum obtained for synthetic AαC, confirmed the identity of the analyte (Figure 2 right panel).

The analysis of AαC-3-OSO₃H in urine, by tandem MS with the TSQ, was analytically challenging because of isobaric interferences. Initially, AαC-3-OSO₃ (m/z 278.1) was assayed in the negative ionization mode, by monitoring the transition 278.1 → 198.1, attributed to the loss of SO₃.³⁹ However, isobaric interferences were extensive and precluded measurement with this transition (Figure 3A). Surprisingly, the signal for protonated AαC-3-OSO₃H (m/z 280.1) in positive ionization mode was relatively strong; however, the transition 280.0 → 200.1, also due to loss of SO₃, lacked specificity and could not be employed for measurement (Figure 3B). Subsequently, a “pseudo MS³” scan in the positive ionization mode was conducted, by applying an elevated voltage to the skimmer that resulted in the in-source fragmentation of the sulfate bond of AαC-3-OSO₃H, to produce protonated AαC-3-OH ($[M + H]^+$ at m/z 200.1) (Figure 3C). The “pseudo MS³” scan method was selected to measure AαC-3-OSO₃H, by the monitoring the transition of AαC-3-OH (200.1 → 155.1, the loss of CO + NH₃). Representative UPLC-ESI/MS² chromatograms depicting AαC-3-OSO₃H in a urine of a smoker during the smoking phase and 6 weeks after cessation of tobacco are shown in Figure 4. The product ion spectrum confirmed the structure of AαC-3-OH: prominent ions observed at m/z 155.1 ($[M + H - NH_3 - CO]^+$), m/z 128.1 ($[M + H - NH_3 - CO - HCN]^+$), and m/z 101.1 ($[M + H - NH_3 - CO - 2HCN]^+$). The spectrum was identical to that of the biosynthesized AαC-3-OH (data not shown).

Urinary Levels of AαC, AαC-3-OSO₃H, and PhIP in Volunteers of the Tobacco Cessation Study

AαC and AαC-3-OSO₃H were successfully measured in urine of twenty-eight out of the 30 subjects. Isobaric interferences in urine precluded measurements in two subjects during both smoking and cessation phases. Creatinine was not measured in urine of a third subject,

hence the data on urinary levels of AαC, when normalized to pg/mg creatinine, are reported on 27 subjects. The linear regression curve showing the correlation between the levels of AαC and AαC-3-OSO₃H in urine during the smoking phase is shown in Figure 5. The urinary levels of AαC and AαC-3-OSO₃H were highly correlated during the smoking phase ($p < 0.0007$, correlation coefficient $r = 0.64$, 95% CI of slope: 0.4247, 1.371). The slope of the curve reveals that the amount of AαC, which is a mixture of unmetabolized AαC and its hydrolyzed conjugates, is comparable to the amount of AαC-3-OSO₃H in urine of smokers.

The histograms depicting the concentrations of AαC and AαC-3-OSO₃H in urine of all 28 subjects during the smoking and cessation phases are shown in Figure 6, and the scatter dot plots of the urinary levels of cotinine and AαC during the smoking and cessation phases are summarized in Figure 7. The geometric mean level of cotinine was 4684 ng/mg creatinine (95% CI: 3644, 5723) during the baseline smoking phase and decreased by >98% to a mean of 74 ng/mg creatinine (95% CI: 15, 134) six weeks following cessation of smoking (Figure 7). These differences in urinary mean levels of cotinine are highly significant (paired t-test, $p < 0.0001$), and confirm that the subjects had abstained from smoking during the cessation period. AαC was above the LOQ in twenty-eight of the subjects during the smoking phase (Figures 6 and 7): the geometric mean concentration of AαC in urine was 24.3 pg/mg creatinine (95% CI: 19, 35), and it decreased to a level of 3.2 pg/mg creatinine (95% CI: 1.8, 5.5), following the six week cessation period. The difference in geometric mean levels of urinary AαC between the smoking and cessation phases was highly significant (paired t-test, $p < 0.0001$). The levels of AαC in urine were still above the LOQ in fifteen out of the 28 subjects 6 weeks following abstinence from smoking, and two of the subjects had relatively high levels of AαC (32 and 118 pg/mg creatinine) in urine. These data demonstrate that smoking is a major source of exposure to AαC for the majority of volunteers.

The geometric mean urinary level of AαC-3-OSO₃H during the smoking phase was 47.3 pg/mg creatinine (95% CI: 36 – 63) and decreased to a level of 3.7 pg/mg creatinine (95% CI: 2.1, 6.5) 6 weeks after cessation of tobacco usage. The difference in the geometric mean levels of AαC-3-OSO₃H between the smoking and cessation phases was highly significant (paired t-test, $p < 0.0007$). AαC-3-OSO₃H was above the LOD in four subjects and above the LOQ only in two subjects 6 weeks after cessation of tobacco. The two subjects (Subjects 3 and 8) harboring very high levels of AαC-3-OSO₃H (~200 pg/mg creatinine) were the same subjects who had high amounts of AαC in their urine postcessation of tobacco. The infrequent detection of AαC-3-OSO₃H in many of these urine samples postcessation of tobacco usage may be attributed to the 9-fold higher LOQ value of AαC-3-OSO₃H than the value of AαC.

PhIP, in contrast to AαC, was detected in urine of only four subjects during the smoking phase (3.4, 8.1, 13.3, and 18.0 pg/mg creatinine); these subjects were not positive for PhIP after cessation (Supporting Information, Figure S-2). Two other subjects harbored PhIP at levels above the LOQ (3.0 and 7.1 pg/mg creatinine) after cessation of tobacco, but neither subject contained detectable levels of PhIP during the smoking phase.

Discussion

There are several reports on the assessment of exposures to aromatic amines, including *o*-toluidine, 4-ABP, and 2-naphthylamine, by measurement of these carcinogens in urine of nonsmokers, smokers, or occupationally exposed workers.^{40–44} The estimates of the arylamines in urine vary widely among the different laboratories. The levels of *o*-toluidine in urine of smokers and nonsmokers are reported to be as high as 6300 ng/24 h urine collection.⁴⁰ The mean levels of 4-ABP in urine of nonsmokers and smokers ranged, respectively, between 5.6 ng/24 h and 17.3 ng/24 h urine collection,⁴³ a second study reported means levels of 4-ABP at 9.6 and 15.3 ng/24 h urine collection for nonsmokers and smokers;⁴² a third study reported 4-ABP at levels ranging from 68 ng/24 h and 79 ng/24 h, respectively, in nonsmokers and smokers.⁴¹ 2-Naphthylamine was reported at mean levels as high as 2100 ng/L and 3900 ng/L of urine of nonsmokers and smokers.⁴⁴ Some of these studies employed HPLC with electrochemical detection or gas chromatography with electron capture detector for detection, and employed surrogate external standards for quantification. These methods are less precise than MS-based methods which employ stable, isotopically labeled internal standards for identification and quantification. Moreover, the peak purity and identity of the analytes are equivocal in the earlier studies that did not employ MS, and the estimates of aromatic amine concentrations in urine may be inaccurate. Many of these analyses were performed following acid or base treatment, or by enzyme treatment of urine with β -glucuronidase/arylsulfatase to hydrolyze metabolic conjugates to the parent amines. These different types of treatments may have selectively hydrolyzed different types of conjugates and recovered different amounts of the parent amines.

The data reported in the literature on urinary biomarkers of tobacco-associated HAAs are limited to two reports.^{28,45} We previously identified AaC in urine of adult men of the Shanghai Cohort study.²⁸ The number of cigarettes smoked per day was positively and significantly related to urinary levels of AaC in the study subjects: the mean level of AaC in non-hydrolyzed urine of subjects who smoked greater than 20 cig/day was 11.9 pg/mg creatinine (9.2 pg/mL urine), and the mean level in nonsmokers urine was 2.54 pg/mg creatinine (2.2 pg/mL urine).²⁸ AaC was detected in 51% of the smokers and 19% of the non-smokers. Smoking and not cooked meat was the major source of AaC exposure.

In our current tobacco cessation study conducted in the United States, the subjects smoked an average 24 ± 6 CPD. During the baseline smoking phase, the mean urinary level of AaC was 35 ± 29 pg/mg creatinine (46 ± 41 pg/mL urine), and the mean level dropped to 8 ± 23 pg/mg creatinine (9 ± 18 pg/mL) six weeks following the cessation of tobacco usage. We treated urine specimens with base, which increased the amounts of AaC recovered from urine by about 3-fold.²⁸ The increase in AaC content may be attributed to the hydrolysis of the N-acetyl metabolite of AaC³³ which would explain the higher mean levels of AaC observed in the study subjects of the United States compared to the subjects of the Shanghai cohort.²⁸ However, the levels of AaC present in different brands of cigarettes can vary by 10-fold and impact the levels of AaC in urine.^{4,9,11,12} Acid treatment of urine (2N HCl at 70 °C for 6 h) recovers up to 10-fold higher amounts of AaC compared to non-hydrolyzed urine;²⁸ these acidic conditions hydrolyze the *N*²-glucuronide conjugate of AaC,³¹ but interfering isobaric components are formed during the hydrolysis and impede reliable

measurement of AαC in many acid-treated urine samples by TSQ/MS (unpublished observations, R. Turesky). Thus, the total amount of AαC and its conjugates in urine is probably several fold higher than the actual levels measured in our study.

The mean level of urinary cotinine decreased by more than 98% following 6 weeks of abstinence from smoking, yet AαC was still detected above the LOQ value in 50% of the subjects. We cannot exclude the possibility that the subjects were exposed to low levels of second-hand smoke. However, AαC occurs an atmospheric pollutant,⁶ and it also forms in well-done cooked meats and charred vegetables,^{5,46} which may have contributed to the non-tobacco associated exposure to AαC.

A recently published study confirmed our previous findings that AαC is present urine of smokers and nonsmokers in China,²⁸ with approximately a 2.5 fold higher level of AαC present in urine of smokers than nonsmokers (20 vs 8 pg/mg creatinine).⁴⁵ The authors also reported that 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), an HAA with a similar heterocyclic structure to PhIP, was detected at ~2-fold higher levels in urine of smokers than nonsmokers. In contrast, the amounts and frequency of PhIP detected in urine of smokers were comparable to nonsmokers, and urinary concentrations of PhIP hovered just above the LOQ value (4.3 pg/mL). Creatinine, which is an abundant amino acid present in animal proteins and fish muscle,⁴⁷ is thought to be a critical precursor in the formation of DMIP and PhIP during the high-temperature cooking of meats and poultry.⁴⁸ However, these nitrogenous chemicals occur at low levels in soils and plants.⁴⁹ To our knowledge, the amounts of creatine/creatinine have not been reported in tobacco plants. Thus, it is surprising to find PhIP and possibly DMIP are present in cigarette smoke condensate.¹¹ PhIP was infrequently detected in urine specimens of smokers from the greater Minneapolis metropolis even though the LOQ value for PhIP is very low (2.5 pg/mL urine). PhIP is extensively metabolized in humans, and on average, less than 1% of the ingested dose of PhIP is eliminated in urine of omnivores, and PhIP is often undetectable in urine.^{34,50} Thus, urinary PhIP is not a sensitive biomarker, and several of its urinary metabolites³⁴ may be superior biomarkers to assess exposure to this procarcinogen from tobacco smoke.

The ring-oxidized sulfate conjugate, AαC-3-OSO₃H, was also frequently detected in urine of subjects during the smoking phase. Human P450s catalyze the oxidation of AαC to form AαC-3-OH and the HONH-AαC.³⁷ P450-mediated ring-oxidation is viewed as a pathway of detoxication of HAAs, whereas P450-mediated N-oxidation of the exocyclic amine group to form HONH-AαC is considered the pathway of bioactivation.³⁷ The AαC-3-OSO₃H in urine may represent a pathway of detoxication, but the metabolite also may form as a rearrangement product of the reactive *N*-sulfooxy-2-amino-9*H*-pyrido[2,3-*b*]indole (*N*-sulfooxy-AαC) intermediate, a penultimate metabolite of AαC that reacts with DNA (Scheme 1).^{51,52} AαC-3-OSO₃H is prepared synthetically by the Boyland-Sims persulfate oxidation of AαC under alkaline pH conditions.³² Based on studies with arylamines, the Boyland-Sims reaction occurs by a nucleophilic displacement by the arylamine nitrogen on the peroxide oxygen to form an intermediate arylhydroxylamine-O-sulfonate, which rearranges to form the *o*-arylaminesulfate.^{32,53} The synthesis of AαC-3-OSO₃H likely occurs through the *N*-sulfooxy-AαC intermediate, with subsequent rearrangement to AαC-3-

OSO₃H (Scheme 1). Future studies are required to determine if AαC-3-OSO₃H formed in vivo represents a biomarker of bioactivation or detoxication of AαC.

Epidemiological studies have reported that smoking is a risk factor for cancer of the liver and gastrointestinal tract, but the causative agents are uncertain.^{19,21} Apart from the endocyclic nitrogen atoms, AαC has the same chemical structure as 2-aminofluorene, one of the most well-studied carcinogenic aromatic amines over the past 60 years.⁵⁴ AαC induces aberrant crypt foci, early biomarkers of colon cancer,²² induces *lacI* transgene mutations in colon of mice,²³ and induces hepatocellular carcinomas in rats.¹ Moreover, AαC undergoes extensive bioactivation of by human hepatocytes to form persistent DNA adducts,^{26,55} suggesting that AαC can contribute to DNA damage liver and gastrointestinal tract of smokers. Given the relatively high amounts of AαC present in tobacco smoke, further studies on the potential role of AαC in tobacco-associated cancers are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

B[a]P	benzo[a]pyrene
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNAL	4AαC, 2-amino-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
AαC-3-OH	2-amino-3-hydroxy-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
AαC-3-OSO₃H	2-amino-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole-3-yl sulfate
HONH-AαC	2-hydroxyamino-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
N-sulfooxy-AαC	<i>N</i> -sulfooxy-2-amino-9 <i>H</i> -pyrido[2,3- <i>b</i>]indole
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
HONH-PhIP	2-hydroxyamino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
CID	collision-induced dissociation
CPD	cigarettes smoked per day
ESI	electrospray ionization
HAA	heterocyclic aromatic amine
HPLC	high pressure liquid chromatography

LOD	limit of detection
LOQ	limit of quantification
SPE	solid phase extraction
SRM	selected reaction monitoring
SCX	strong cation exchange
TSQ	triple stage quadrupole
UPLC/MS²	ultraperformance liquid chromatograph/tandem mass spectrometry
WAX	weak anion exchange

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Figure 1.
Chemical structures of AαC, AαC-3-OSO₃H, and PhIP.

Figure 2. UPLC/MS² analysis of AαC during the baseline smoking phase and 6 weeks after cessation of tobacco and product ion spectra of analyte and synthetic AαC standard.

Figure 3. UPLC/MS² analysis of AαC-3-SO₃H in urine of a subject who ate well-done cooked meat. (A) negative ion mode (278.1 → 198.1), (B) positive ion mode (280.1 → 200.1), and (C) positive ion mode, following in-source CID to fragment AαC-3-SO₃H to AαC-3-OH, and monitoring the transition 200.1 → 155.1, attributed to the loss of CO and NH₃ from protonated AαC-3-OH.

Figure 4.

UPLC/MS² analysis of AαC-3-SO₃H during the baseline smoking phase and 6 weeks after cessation of tobacco, and the product ion spectra of the analyte and synthetic AαC-3-OSO₃H standard, following in-source CID to fragment AαC-3-OSO₃H to AαC-3-OH

Figure 5.
Linear regression curve (dashed lines 95% CI) of the levels of AαC and AαC-3-OSO₃H during the baseline smoking phase.

Figure 6. Urinary levels of AαC and AαC-3-SO₃H during the baseline smoking phase and 6 weeks after cessation of tobacco.

Figure 7. Scatter dot plots of the levels of cotinine and AαC (showing the geometric mean and 95% CI) during the baseline smoking phase and 6 weeks after cessation of tobacco.

Scheme 1.

Mechanism of AαC-3-OSO₃H formation through sulfation of 3-HO-AαC or by rearrangement of *N*-sulfooxy-AαC.

Table 1
Within-day and between-day estimates of AaC and AaC-3-OSO₃H (pg/mL urine) spiked in urine.^a

	AaC (pg/mL urine)	Day 1	Day 2	Day 3	Day 4	CV (%)within- day	CV (%)between- day
Mean	10.0	13.4	11.7	13.3	12.2	10.7	11.6
SD		2.9	1.9	1.4	0.64		
RSD (%)		21.3	16.7	10.9	5.5		
Mean	30.0	34.9	32.2	36.23	31.3	7.0	9.9
SD		0.8	1.1	1.8	4.1		
RSD (%)		2.3	3.4	4.8	12.1		

	AaC-3- OSO ₃ H (pg/mL urine)	Day 1	Day 2	Day 3	Day 4	CV (%)within- day	CV (%)between- day
Mean	100.0	97.9	84.7	104.7	101.4	8.7	12.7
SD		12.0	12.0	2.1	3.0		
RSD (%)		12.2	14.1	2.0	3.0		
Mean	500.0	512	609	514	493	6.4	12.5
SD		26.4	21.0	15.1	51.7		
RSD (%)		5.2	3.4	2.9	10.5		

^aThe HAA biomarkers were isolated from urine on 4 different days (n=4 samples per day) and assayed by UPLC/MS².