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Formation of a Mutagenic Heterocyclic Aromatic Amine from Creatinine in Urine of Meat Eaters and Vegetarians

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Liquid chromatography electrospray ionization mass spectrometry (MS) with a triple quadrupole MS was used to identify known and novel heterocyclic aromatic amines (HAAs) in human urine. The identities of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (8-MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) were confirmed by their product ion spectra. The constant neutral loss scan mode was employed to probe for other analytes in urine that display the transition $[M + H]^+ \rightarrow [M + H - CH_3^{\bullet}]^{+\bullet}$, which is common to HAAs containing an *N*-methylimidazo moiety, and led to the detection of a previously unreported isomer of 8-MeIQx [Holland, R., et al. (2004) *Chem. Res. Toxicol. 17*, 1121−1136]. We now report the identification of another novel HAA, 2-amino-1-methylimidazo[4,5-*b*]quinoline (IQ[4,5-*b*]), an isomer of the powerful animal carcinogen 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQ). The amounts of IQ[4,5-*b*] measured in the urine of human volunteers who consumed grilled beef ranged from 15 to 135% of the ingested dose, while the amounts of 8-MeIQx and PhIP excreted in urine were on average <2% of the ingested dose. Base treatment of urine at 70 °C increased the concentrations of 8-MeIQx and PhIP by as much as 6-fold, indicating the presence of phase II conjugates; however, the amount of IQ[4,5-*b*] increased by more than 100-fold. IQ[4,5-*b*] was also detected in the urine of vegetarians following base hydrolysis. The formation of IQ[4,5-*b*], but not IQ, 8-MeIQx, or PhIP, also occurred in urine incubated at 37 °C. Creatinine and 2-aminobenzaldehyde are likely precursors of IQ[4,5-*b*]. The detection of IQ[4,5-*b*] in the urine of both meat eaters and vegetarians suggests that this HAA may be present in nonmeat staples or that IQ[4,5-*b*] formation may occur endogenously within the urinary bladder or other biological fluids.

Introduction

Urine is a useful biological fluid to assess human exposure and health risk of dietary and environmental toxins. Because large quantities of urine can be obtained noninvasively, chemical analyses of urine may be more amenable than for blood samples. The measurement of urinary carcinogens can be used to determine exposure and bioavailability, while the measurement of carcinogenic metabolites may be used to assess the capacity of an individual to bioactivate or detoxicate carcinogens. A wide range of tobacco, food, and environmental carcinogens and their metabolites have been measured in urine (*1*). Excised DNA adducts of several different classes of genotoxic carcinogens and oxidized DNA lesions also have been identified in urine (*1*−*4*), and these biomarkers represent indices of genetic damage and oxidative status, respectively. Some urinary biomarkers may serve to facilitate the extrapolation of experimental animal toxicity data for human risk assessment, which requires consideration of species differences in the catalytic activities and expression of xenobiotic metabolism enzymes that modulate the toxicity of genotoxins and health risk in exposed populations.

Heterocyclic aromatic amines (HAAs)[1,2] are one class of ubiquitous mutagens found in cooked meats, poultry, fish, and tobacco smoke condensate (*5*). A number of HAAs have been reported to induce tumors at multiple sites in experimental animals (*5*−*8*). Several HAAs have been identified in human urine following the consumption of cooked meats, indicating that HAAs are readily absorbed from the gastrointestinal tract (*9*−*11*). Human tissues metabolically activate HAAs to genotoxins (*12*,

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[1] Abbreviations: 2-ABA, 2-aminobenzaldehyde; APNH, 9-(4′-aminophenyl)-9*H*-pyrido[4,3-*b*]indole; AA, anthranilic acid; 8-MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; IQ[*iso-f*], 2-amino-3-methylimidazo[4,5-*f*]isoquinoline; IQ[*iso-h*], 2-amino-3-methylimidazo-[4,5-*h*]isoquinoline; 1-CH_3-IQ, 2-amino-1-methylimidazo[4,5-*f*]quinoline; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; IQ[*h*], 2-amino-3-methylimidazo[4,5-*h*]quinoline; IQx, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; CID, collision-induced dissociation conditions; CNL, constant neutral loss; ESI-MS/MS, electrospray ionization tandem mass spectrometry; HAAs, heterocyclic aromatic amines; LOQ, limit of quantitation; MCX, mixed mode cation resin/C18 reversed phase resin; SIM, selected ion monitoring; SRM, selected reaction monitoring.

[2] Disclaimer: The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.

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13), and some epidemiological investigations have linked the frequent consumption of foods such as grilled meats containing HAAs with an elevated risk of colon and breast cancer (*14*−*16*). Therefore, HAAs may contribute to the etiology of some common forms of human cancers.

Our research has focused on the development of analytical methods to measure urinary biomarkers of HAAs in experimental animals and humans to understand interspecies differences in HAA metabolism, which may influence toxicity and health risk of these genotoxins (*17*−*19*). We recently developed a rapid tandem solvent−solid phase extraction method to isolate a variety of known HAAs from human urine, which also led to the detection of a novel isomer of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (8-MeIQx) designated as Iso-MeIQx (*20*). This HAA was subsequently detected in grilled meats (*20*, *21*), and the structure remains to be elucidated through chemical synthesis. By employing the different scan modes of the triple quadrupole MS, we detected another analyte in urine that we speculated was an HAA based upon its product ion mass spectrum with characteristic fragment ions attributed to losses of $[M + H - CH_3^{\bullet}]^{+\bullet}$ and $[M + H - CH_3^{\bullet} - HCN]^{+\bullet}$ (*22*, *23*). In this current study, we have identified this second novel HAA in human urine as 2-amino-1-methylimidazo[4,5-*b*]quinoline (IQ[4,5-*b*]), an isomer of the powerful animal carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (*5*, *24*). The presence of IQ[4,5-*b*] in the urine of both meat eaters and vegetarians following base treatment suggests that this HAA may be present in nonmeat staples or that IQ[4,5-*b*] formation may occur within the body.

Experimental Procedures

Caution: *HAA derivatives are hazardous and should be handled with caution*.

Chemicals and Reagents. IQ, IQ[4,5-*b*], 8-MeIQx, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-1-methylimidazo[4,5-*f*]quinoline (1-CH_3-IQ), 2-amino-3-methylimidazo[4,5-*f*]isoquinoline (IQ[*iso-f*]), 2-amino-3-methylimidazo-[4,5-*h*]quinoline (IQ[*h*]), 2-amino-3-methylimidazo[4,5-*h*]isoquinoline (IQ[*iso-h*]), 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 3-[^2H_3C]IQ, 3-[^2H_3C]-8-MeIQx, 1-[^2H_3C]PhIP (isotopic purities >98%) were purchased from Toronto Research Chemicals (Downsview, Ontario, Canada). Anthranilic acid (AA) was purchased from Aldrich (Milwaukee, WI). 1-[^2H_3C]creatinine (isotopic purity >99.7%), 2-aminobenzaldehyde (2-ABA), and *o*-toluidine were purchased from Sigma (St. Louis, MO). MicroLC vials, C_{18} sep-pak, and mixed mode cation resin/C18 reversed phase resin (MCX) cartridges (30 and 500 mg) were obtained from Waters Corp. (Milford, MA). Extrelut 20 resins were obtained from EMD Chemical (Gibbs Town, NJ).

Synthesis of 1-[^2H_3C]IQ[4,5-*b*]. The synthesis of this HAA was performed by reaction of 1-[^2H_3C]creatinine with 2-ABA as previously described with minor modifications (*25*). 1-[^2H_3C]-creatinine (40 mg) was dissolved in diethylene glycol (1 mL) in a glass vial (10 mL), followed by the addition of 2-ABA (20 mg), and the reaction was placed on a heating block set at 155 °C for 8 h. The reaction mixture was allowed to cool to room temperature and then was diluted with 0.05 N NaOH (5 mL). The solution was applied to a C_{18} sep-pak cartridge, which had been preconditioned with CH_3OH, followed by 0.05 N NaOH. After application of the sample, the cartridge was washed with 5% CH_3OH, followed by 25% CH_3OH. The desired product was eluted with CH_3OH. The chemical (>98%) and isotopic purity of the product (>99.6%) were determined by UV and LC/MS measurements and based on comparison to IQ[4,5-*b*].

Human Subjects and Meat Consumption. The analysis of HAAs was conducted on the urine of male volunteers from a previous investigation, and full details were reported previously (*26*). The human volunteer study was conducted under the written unanimous approval of the Carshalton Medical Research Ethics Committee. In brief, subjects consumed 275 g of cooked minced beef patties that had been fried without added oil or fat for approximately 6 min on each side using a hot metal griddle at ∼300 °C until the meat was well-browned. A 10 h urine collection was then commenced, and urine samples were stored at −80 °C prior to analysis. As a negative control, each subject provided a spot urine sample (50−100 mL) following a 24 h time period where the subjects had refrained from consuming grilled foods known to contain HAAs. During that time, the volunteers consumed cheese, boiled eggs, tomato soup, bread with butter, cucumbers, tomatoes, lettuce, yogurt, fruit cake, cheddar cheese sauce, potatoes, and fruit salad. A subset of these urine samples that had been stored for 3 years at −80 °C was sent blind-coded on dry ice to NCTR for further analysis. Another set of urine samples was collected over ice for up to 24 h from four other volunteers following consumption of well-done cooked beef (100−170 g) that was prepared either by pan-frying or by barbeque over a charcoal or gas flame. These subjects also provided a 24 h urine sample collection after refraining from meat consumption for >24 h. Two vegetarians also provided urine samples over a 24 h time period. All urine samples were blind coded, and the identities of the volunteers were anonymous. The amounts of HAA ingested from meat were estimated by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI/MS) following solid phase extraction as previously reported (*20*, *21*).

Urinary Creatinine Measurements. The creatinine content in urine was based upon the colorimetric method of Jaffee and performed following the instructions provided with the kit purchased from Pointe Scientific (Lincoln Park, MI).

Tandem Solvent Solid Phase Extraction of HAAs from Urine. Urine, stored at −80 °C, was allowed to thaw to room temperature. Isotopically labeled HAAs as internal standards (50 pg, 10 μL) were added to urine (1 mL) in polypropylene tubes (15 mL), and the solutions were diluted with 1 M KH_2PO_4 buffer (pH 7.5) (2 mL). The HAAs were then extracted from the urine by mixing thoroughly with ethyl acetate (5 mL). The solution was centrifuged for 1 min at 3000*g*. The ethyl acetate fraction was retrieved, and the extraction procedure was repeated. The combined ethyl acetate fractions were acidified with glacial acetic acid (CH_3CO_2H) (20 μL) and applied to an MCX cartridge (30 mg) prewashed with 5% aqueous NH_4OH in CH_3OH, followed by 2% CH_3CO_2H in CH_3OH and by CH_3OH. The urine extract was passed through the MCX resin under a gentle vacuum at a flow rate of 3−5 mL/min. Then, the resin was washed successively with 0.04 N HCl in CH_3OH (40% v/v) (1 mL), followed by CH_3OH (1 mL), and last 2% NH_4OH containing 15% CH_3OH (1 mL). Then, the HAAs were eluted from the resin with 5% NH_4OH in CH_3OH (855 μL), collected into microLC vials containing 10% diethylene glycol in CH_3OH (10 μL), and concentrated to dryness by vacuum centrifugation at 43 °C at a pressure of 40 mbar.

IQ[4,5-*b*] was isolated from urine (50 mL) of a meat eater that had been spiked with 2-ABA (1 μg/mL). The urine was treated with base (1 N NaOH, 70 °C for 3 h) in order to obtain sufficient material for an online UV spectrum by HPLC. The isolation of IQ[4,5-*b*] was done as described above except the volume of ethyl acetate used for extraction was increased to 50 mL. The pooled organic extracts were applied to an MCX cartridge (500 mg), and the solvents used to wash the resin and elute IQ[4,5-*b*] were increased to 5 mL. The extract was evaporated to dryness in vacuo and subjected to HPLC using an Agilent 1100 system (Palo Alto, CA), which consisted of a quaternary pump and a UV diode array detector. The separation was done with an Aquasil C_{18} reversed phase column (4.6 mm × 250 mm, 5 μm particle size) from Thermo Electron (Bellafonte, PA) using a linear gradient from 5 mM $NH_4CH_3CO_2$ (pH 6.8) containing 10% CH_3CN to 100% CH_3CN over 40 min at a flow

rate of 1.0 mL/min. The extract was monitored at 260 and 330 nm.

Tandem Solid Phase Extraction of HAAs from Cooked Meat. Grilled meat samples (4 g) were spiked with isotopically labeled HAAs (1 ng/g), thoroughly homogenized, and then applied to an Extrelut 20 resin connected in series with an Oasis MCX LP extraction column (500 mg). The MCX cartridge was used instead of tandem C18 and propylsulfonic acid resins to allow the recovery of all of the HAAs in one fraction (*20*, *27*). The HAAs adsorbed on to the MCX cartridge were washed and eluted with solvents (5 mL) as described above. Samples were concentrated to dryness by vacuum centrifugation at 43 °C at a pressure of 40 mbar.

Kinetics of HAA Hydrolysis in Urine under Base Treatment. Urine samples (1 mL) from individuals were fortified with isotopically labeled internal standards (50 pg) and hydrolyzed under alkaline conditions (1 N NaOH; final concentration made by addition of 110 μL of 10 N NaOH). The samples were heated at 70 °C for 0, 1, 3, and 5 h. After the samples were cooled, the HAAs were subjected to the tandem solid phase extraction procedure described above.

Identification of Precursors of IQ[4,5-*b*] in Urine. Urine (1 mL) was spiked with either *o*-toluidine (100 μg), 2-aminobenzyl alcohol (100 μg), AA (100 μg), or 2-ABA(1 μg), with 2.5 ng of trideuterated labeled IQ, MeIQx, and PhIP and 1.6 ng of IQ[4,5-*b*] added as internal standards. The urine samples were incubated at 37 °C for up to 5 h. The reaction was terminated by the addition of 1 M K_2HPO_4 buffer (pH 7.5) (2 mL), and the HAAs were extracted by mixing with ethyl acetate (5 mL), followed by centrifugation for 1 min at 3000*g*. The aqueous phase was extracted a second time with ethyl acetate, and the organic extracts were combined. A small amount of anhydrous Na_2SO_4 was added to the ethyl acetate extracts. A portion of the organic extract (855 μL) was placed into an LC sample vial containing 10% diethylene glycol in CH_3OH (10 μL), and the samples were taken to dryness with a vacuum centrifugation system set at 43 °C at a pressure of 40 mbar.

Determination of Artifactual Formation of IQ[4,5-*b*] during Workup of Urine. Urine (1 mL) from a subject pre- and postconsumption of grilled beef was spiked with 1-[$^2H_3$C]-creatinine (3 mg) and 50 pg of isotopically labeled MeIQx and PhIP. The urine was immediately neutralized with 1 M K_2HPO_4 buffer (pH 7.5) (2 mL), and the HAAs were extracted as described above. Urine samples (1 mL) were also spiked with 1-[$^2H_3$C]creatinine (3 mg) and 50 pg of isotopically labeled MeIQx and PhIP, followed by addition of 110 μL of 10 N NaOH and heated for 3 h at 70 °C, and then extracted as above.

HAA Analysis in Urine and Grilled Meat by HPLC/ESI-MS/MS. The chromatography of the HAAs from urine extracts was conducted with a capillary HPLC 1100 and autosampler systems from Agilent. An Aquasil C_{18} Pioneer reversed phase column (1 mm × 250 mm, 3.5 μm particle size) (Thermo Electron Corp.) with a Javelin C_{18} precolumn (1 mm × 5 mm) (Thermo Electron) was used for separation of the HAAs. The chromatography was done with a linear gradient starting from 0.1% HCO_2H and 0.5% CH_3CN in H_2O to 90% CH_3CN containing 0.1% HCO_2H and H_2O over 20 min, and then held for 3 min at this final solvent composition at a flow rate of 50 μL/min. For some analyses on the characterization of the IQ[4,5-*b*] isomer, HCO_2H was replaced with $NH_4CH_3CO_2$ (5 mM, pH 7.5) as a buffer, which resulted in a shift of the t_R of this HAA by more than 2 min.

HAA detection and quantification were done by ESI/MS/MS with a Finnigan Quantum Ultra triple quadruople MS (San Jose, CA). Quantitative analysis was done in positive ionization mode using the selected reaction monitoring (SRM) transitions $[M + H]^+ \rightarrow [M + H - 15]^{+\bullet}$ for the HAAs (loss of $CH_3^\bullet$) and $[M + H]^+ \rightarrow [M + H - 18]^{+\bullet}$ for the isotopically labeled internal standards (loss of $CD_3^\bullet$). Secondary fragment ions were monitored at *m/z* 142 and 157 to corroborate the identity of IQ[4,5-*b*]. The capillary spray voltage was optimized for IQ[4,5-*b*] and set at 1000 V, the in source CID was 18 V, the capillary temperature was set at 350 °C, and the sheath gas was maintained at 80 units. The scan width was set at 1 mass unit, and the resolution, peak width at half-height, was set at 0.7 for both Q1 and Q3. The dwell time for each transition was set at 0.1 s. Argon was the collision gas and set at a pressure of 1.5 mTorr. The collision energy was set at 34 eV for PhIP and at 31 eV for all other HAAs. Product ion and constant neutral loss (CNL) scan modes ($[M + H]^+ \rightarrow [M + H - CH_3^\bullet]^{+\bullet}$) were obtained on the protonated molecules $[M + H]^+$ scanning from *m/z* 100 to 250 at a scan speed of 150 amu/s using the same acquisition parameters.

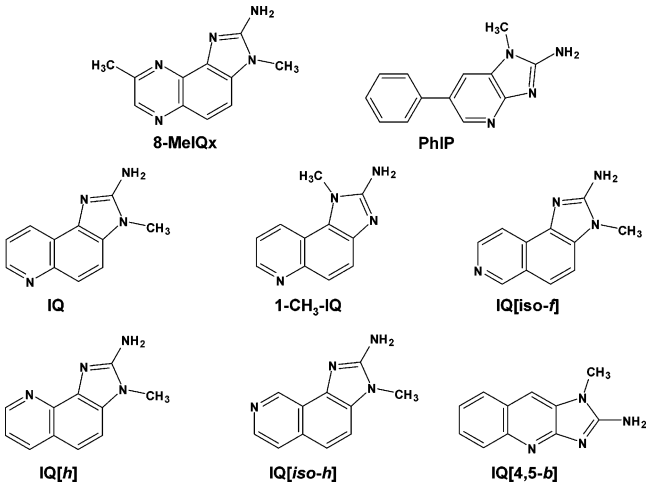


Figure 1. Chemical structures of 8-MeIQx, PhIP, and IQ isomers investigated in this study.

Results

The chemical structures of HAAs investigated in this study are presented in Figure 1. 8-MeIQx and PhIP are two of the most abundant HAAs found in cooked meats, and they have been identified in the urine of subjects following the consumption of grilled meats (*9*, *10*, *20*, *28*). IQ, angular tricyclic ring isomers of IQ, and the linear tricyclic ring isomer IQ[4,5-*b*] are also presented in Figure 1. The structure of IQ[4,5-*b*] is also similar to that of PhIP, except that PhIP contains a freely rotating phenyl group attached to the C6 atom of 2-amino-1-methylimidazo[4,5-*b*]pyridine moiety that is common to both HAAs. To date, only IQ from this series of isomers has been reported to form in foods that include cooked beef extract paste (*29*−*32*) and well-done grilled fish (*33*). However, IQ has rarely been detected at concentrations exceeding 0.1 ppb in meats, fish, or poultry prepared under common household cooking conditions (*34*−*36*) and has not been detected in human urine following consumption of cooked meats (*9*, *20*). IQ[4,5-*b*] has not been identified in cooked foods prior to this study. In this investigation, we report the presence of IQ[4,5-*b*] in the urine of meat eaters as well as its formation in urine of vegetarians and presence in cooked meat. Identification of this novel HAA in human urine and grilled meats was based upon UV spectroscopy, ESI-MS/MS spectrometry techniques, and by cochromatography with the synthetic IQ[4,5-*b*] derivative.

HPLC/ESI-MS/MS and UV Spectroscopic Analyses of IQ[4,5-*b*] in Human Urine. HAAs containing the *N*-methylimidazole moiety undergo facile cleavage of the *N*-methyl group under collision-induced dissociation conditions (CID) with a triple quadrupole mass spectrometer (*22*, *23*, *37*). Therefore, MS/MS was employed in the CNL

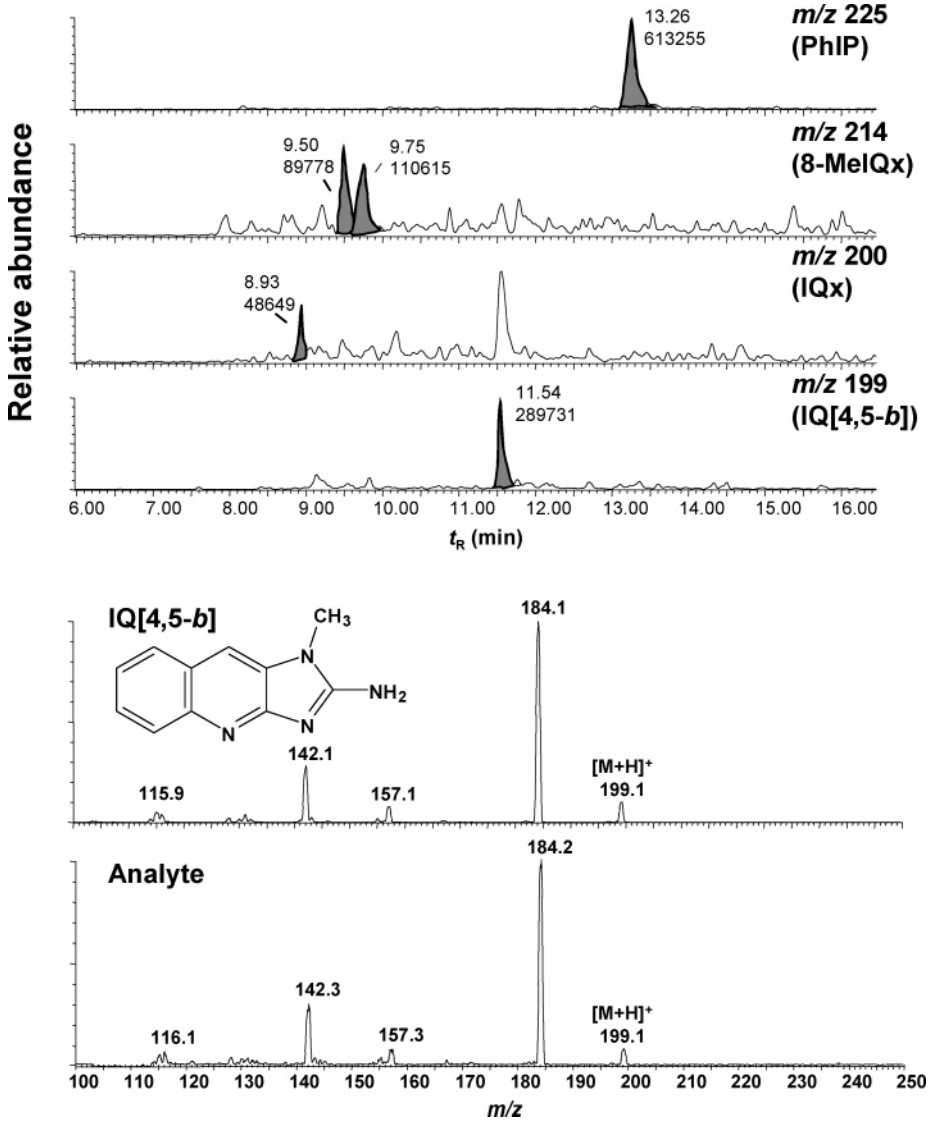


Figure 2. HPLC/ESI-MS/MS extracted ion chromatogram monitoring the CNL transition $[M + H]^+ \rightarrow [M + H - CH_3^{\bullet}]^{+\bullet}$, which is common to HAAs containing an *N*-methylimidazo moiety. The t_R (min) and area of known HAAs are presented, and peaks are shaded. The full scan product ion spectra of the analyte at $t_R = 11.54$ min and IQ[4,5-*b*] are presented. The product ion spectra of 8-MeIQx, PhIP, and IQx were previously reported (*20*).

scan mode monitoring the transition $[M + H]^+ \rightarrow [M + H - CH_3^{\bullet}]^{+\bullet}$ to probe for HAAs containing an *N*-methylimidazole moiety. With the CNL scan mode, we detected 8-MeIQx, a previously unknown isomer of 8-MeIQx (Iso-MeIQx), PhIP, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), and IQx (*20*). The identities of these HAAs were confirmed by their product ion spectra (*20*). Using the CNL scan mode, we also detected a prominent peak at *m/z* 199, which corresponds to the nominal mass of the protonated ion $[M + H]^+$ of IQ (Figure 2). However, the t_R of the analyte was about 2 min or longer than that of IQ, the 1-CH_3-IQ isomer, and other angular tricyclic ring IQ isomers. In contrast to these isomers, the linear tricyclic ring isomer IQ[4,5-*b*] was found to coelute with the analyte in urine under both acidic (0.1% HCO_2H) and neutral pH conditions (5 mM $NH_4CH_3CO_2$, pH 7.5), where the t_R values of the analyte and all IQ compounds were shifted by more than 2 min.

The product ion spectrum of the analyte in urine is indistinguishable from that of IQ[4,5-*b*] (Figure 2) but differs from those of IQ and other angular ring IQ isomers (Figure 3). Both IQ[4,5-*b*] and angular ring IQ isomers are relatively stable molecules, and only a few fragment ions are formed under these CID conditions. Many of the same principal fragment ions are observed in the spectra of all IQ isomers but with different relative abundances (Figure 3). The most prominent fragment ion occurs through cleavage of the *N*-methylimidazole moiety of these molecules to produce the radical species $[M + H - CH_3^{\bullet}]^{+\bullet}$ at *m/z* 184. Two other common fragment ions are present at *m/z* 157 $[M + H - CH_3^{\bullet} - HCN]^{+\bullet}$ and 142 $[M + H - CH_3^{\bullet} - NCNH_2]^{+\bullet}$. The latter fragment ion is more predominant in the spectrum of IQ[4,5-*b*] than in the spectra of any of the other IQ isomers. The product ion spectra of the trideuterated homologues 1-[$^2H_3$C]IQ[4,5-*b*] and 3-[$^2H_3$C]IQ also contain the same fragment ions (data not shown), demonstrating that the initial fragmentation of these IQ molecules occurs through cleavage of the *N*-CH_3 bond of the aminoimidazole moiety, followed by further fragmentation of the protonated radical species (*22*, *23*).

The UV spectra of synthetic IQ[4,5-*b*] and the analyte purified from urine spiked with 2-ABA (vide infra) were acquired online by HPLC with a UV diode array detector.

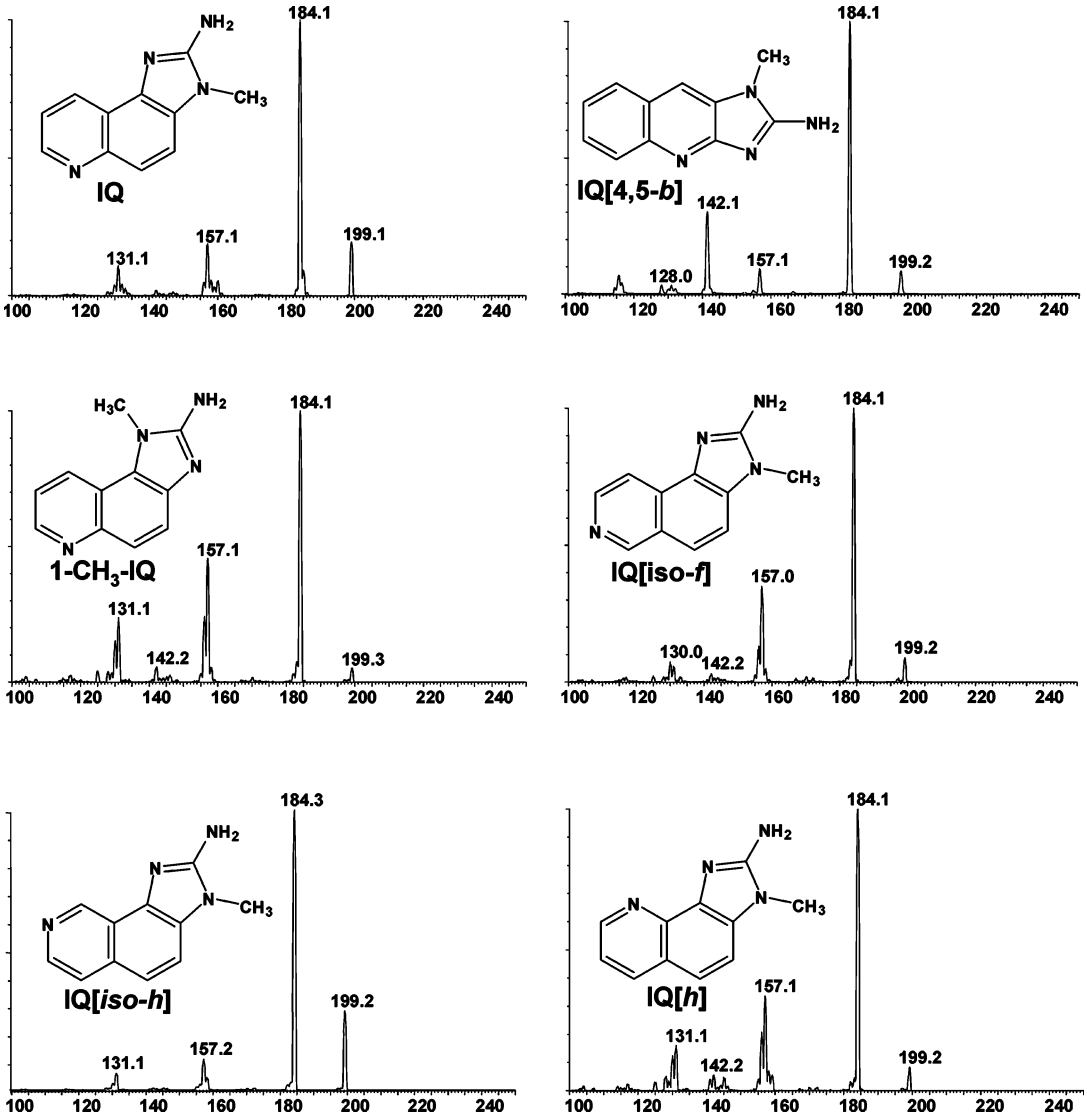


Figure 3. LC/ESI-MS product ion spectra of synthetic IQ[4,5-*b*], IQ, and angular tricyclic ring isomers of IQ.

The spectra of the analyte and IQ[4,5-*b*] are in excellent agreement and distinct from the spectra of IQ and other angular tricyclic ring isomers (Figure 4). A prominent absorbance maximum is situated at 335 nm for IQ[4,5-*b*], while the principal maximum for IQ (263 nm) and other angular tricyclic IQ isomers (data not shown) occurs between 265 and 275 nm (spectra acquired in 5 mM NH_4-CH_3CO_2/CH_3CN, pH 6.8). The indistinguishable UV and ESI mass spectral data of IQ[4,5-*b*] from the analyte in urine, combined with the coelution of both compounds under different solvent conditions, collectively support the structure of the urinary analyte as IQ[4,5-*b*]. The isomer 2-amino-3-methylimidazo[4,5-*b*]quinoline can be excluded as a plausible structure because its formation occurs through condensation of 2-ABA with the *N*3-CH_3 isomer of creatinine.

HPLC/ESI-MS/MS Analyses and Kinetics of IQ[4,5-*b*], 8-MeIQx, and PhIP Formation in Urine of Meat Eaters and Vegetarians Treated with Base. The HPLC/ESI-MS/MS traces of HAAs in urine samples in the SRM scan mode are presented in Figure 5. The isotopically labeled internal standards of IQ, IQ[4,5-*b*], 8-MeIQx, and PhIP spiked in urine at 50 pg/mL were readily detected in all urine samples.

IQ, MeIQx, and PhIP (<1 pg/mL) were not detected in urine samples collected over 24 h from 10 carnivores who had refrained from meat consumption for 24 h prior to collection of the urine, which is consistent with previous investigations (*10*, *20*, *26*). However, IQ[4,5-*b*] was detected in urine samples of four of those subjects who abstained from neat consumption (Figure 5A, left panel). After eating cooked meat, 8-MeIQx, Iso-MeIQx (*20*), PhIP, IQ[4,5-*b*], but not IQ, were readily detected in the urine of volunteers and well above the LOQ (~2 pg/mL) (Figure 5A, right panel). The base treatment of urine of meat eaters was found to increase the concentrations of 8-MeIQx and PhIP as previously reported (*20*) (data not shown); however, much higher concentrations of IQ[4,5-*b*] were found in these urine samples, both pre- and post-meat consumption, following base treatment (vide infra). The HPLC/ESI-MS/MS traces of HAAs in a urine sample of a vegetarian (subject 11) are presented in Figure 5B before (left panel) and after base hydrolysis (right panel). None of the HAAs are present in untreated urine, yet IQ[4,5-*b*] was formed in abundant amounts (>100 pg/mL) following base treatment.

Glucuronidation and sulfamation are important phase II conjugation pathways of metabolism of 8-MeIQx and PhIP and lead to the formation of stable phase II

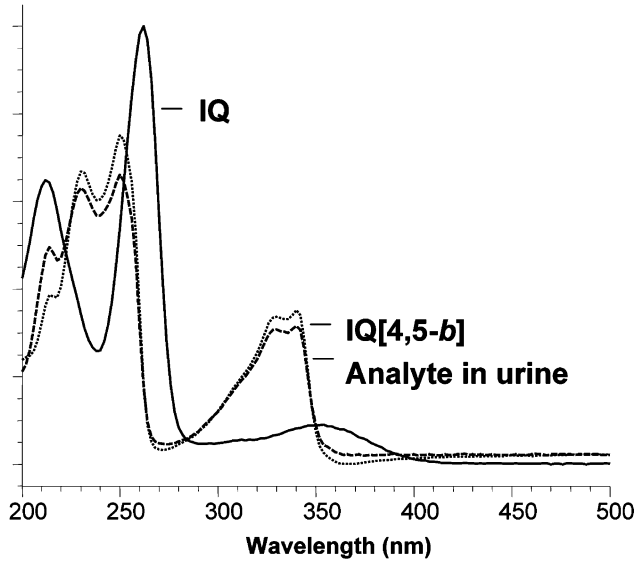


Figure 4. Online HPLC-UV spectra of analyte isolated from human urine spiked with 2-ABA (1 μg/mL) and treated with 1 N NaOH for 3 h at 70 °C prior to isolation. The UV spectra of synthetic IQ[4,5-*b*] and IQ are also presented.

conjugates that are excreted in urine (*17*, *18*, *38*). Acid or base treatment results in the hydrolysis of these phase II conjugates and regeneration of the parent amines (*28*, *39*, *40*). The 6-fold increase in concentrations of 8-MeIQx and PhIP in base-treated urine of subjects who consumed cooked beef (1 N NaOH, 70 °C for 5 h) is indicative of phase II conjugates. Under the same hydrolysis conditions, the concentration of IQ[4,5-*b*] increased by more than 100-fold. The kinetics of HAA formation in base-treated urine of a carnivore (subject 2) postmeat consumption (Figure 6a), after refraining from meat for >24 h (Figure 6b), and in base-treated urine of a vegetarian (subject 11) (Figure 6c) reveal that much higher amounts of IQ[4,5-*b*] than 8-MeIQx or PhIP are present in human urine. Similar findings were obtained from urine samples of two other carnivores and a second vegetarian (data not shown). These results suggest that IQ[4,5-*b*] may be present in foods other than cooked meats and that this HAA undergoes more extensive metabolism through phase II conjugation reactions than either 8-MeIQx or PhIP, which leads to recovery of high levels of IQ[4,5-*b*] following base treatment of urine. Alternatively, the formation of IQ[4,5-*b*] in urine may occur through the chemical reaction of an identified precursor with creatinine.

Mechanism of Formation of IQ[4,5-*b*] in Urine. We explored the possibility that the high levels of IQ[4,5-*b*] found in urine of meat eaters and vegetarians may be

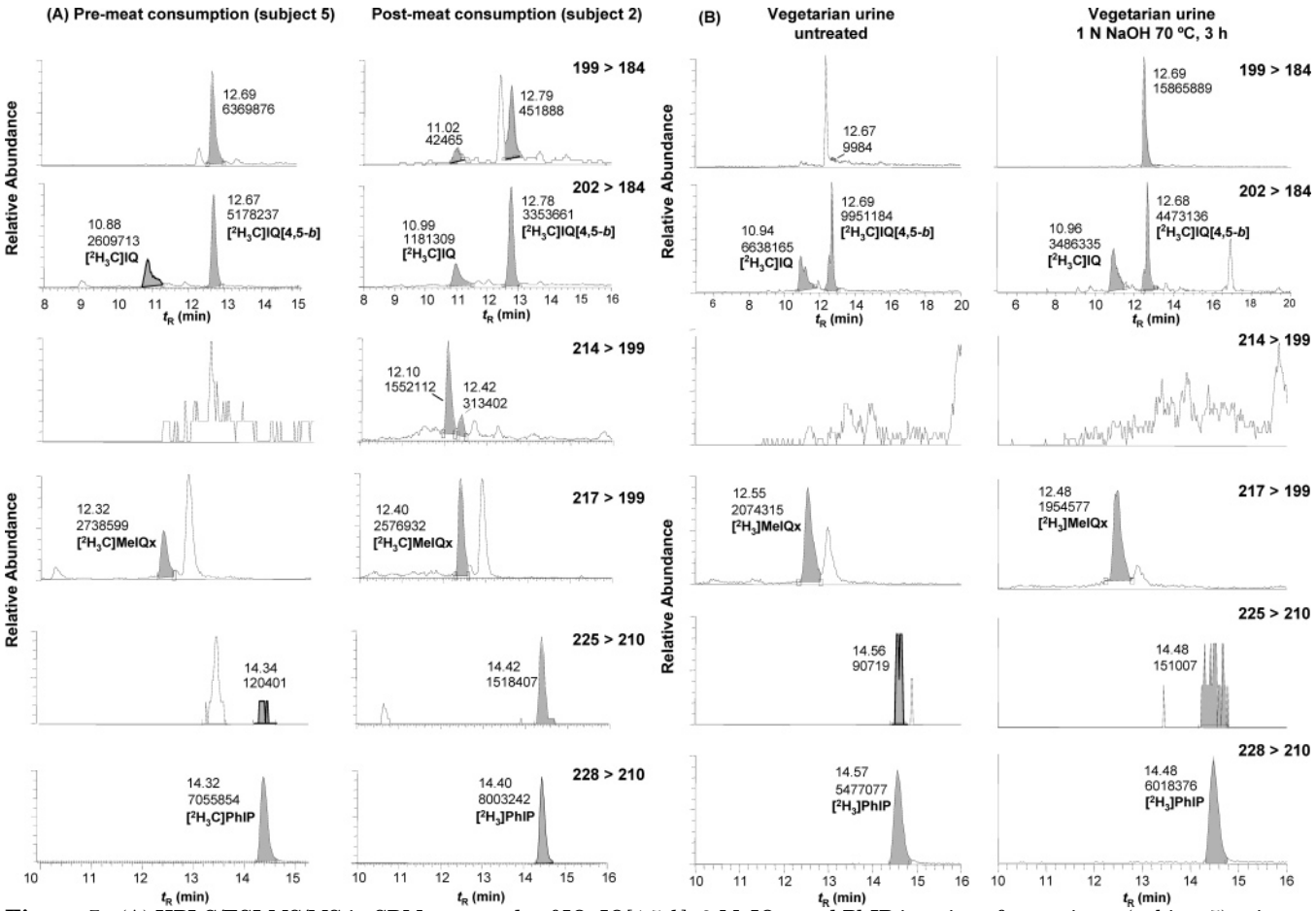


Figure 5. (A) HPLC/ESI-MS/MS in SRM scan mode of IQ, IQ[4,5-*b*], 8-MeIQx, and PhIP in urine of a carnivore (subject 5) prior to meat consumption (left panel), which shows the presence of IQ[4,5-*b*] but not 8-MeIQx or PhIP. Urine of a carnivore (subject 2) post-meat consumption (right panel) where a novel isomer of 8-MeIQx (t_R 12.10 min), 8-MeIQx (t_R 12.42 min), and PhIP (t_R 14.42 min) are detected. (B) Urine of a vegetarian (subject 11) either untreated (left panel) or treated with 1 N NaOH (right panel) at 70 °C for 3 h. The SRM transitions employed for detection and quantification were: 202 > 184 for [^2H_3C]IQ and [^2H_3C]IQ[4,5-*b*]; 199 > 184 for unlabeled IQ and IQ[4,5-*b*]; 217 > 199 for [^2H_3C]MeIQx and 214 > 199 for unlabeled 8-MeIQx; and 228 > 210 for [^2H_3C]-PhIP and 225 > 210 for unlabeled PhIP. The t_R (min) and area of known HAAs and internal standards are presented and peaks are shaded.

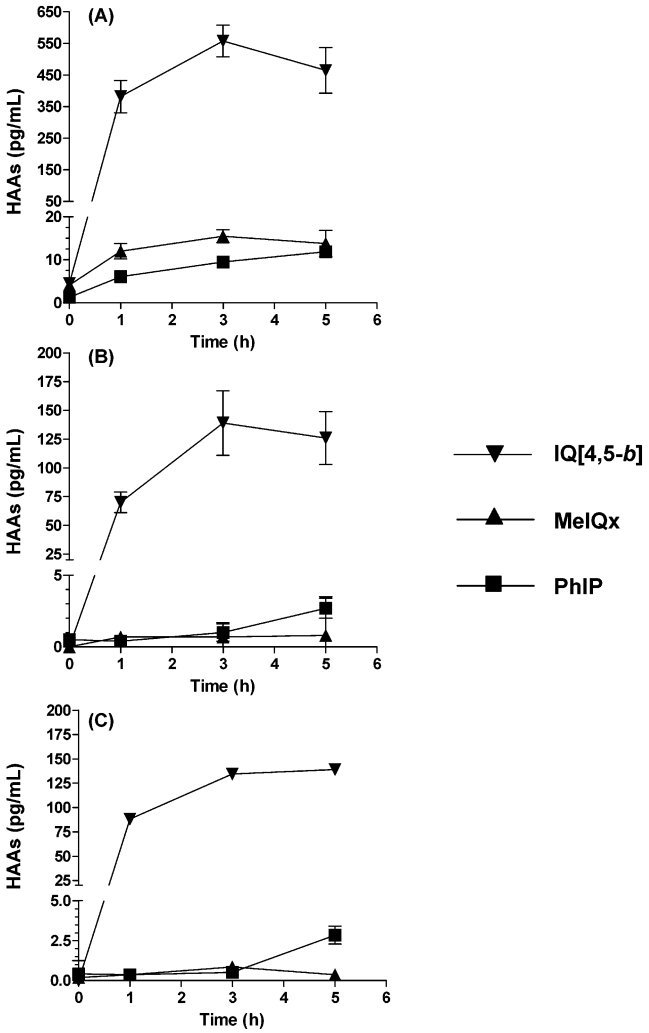
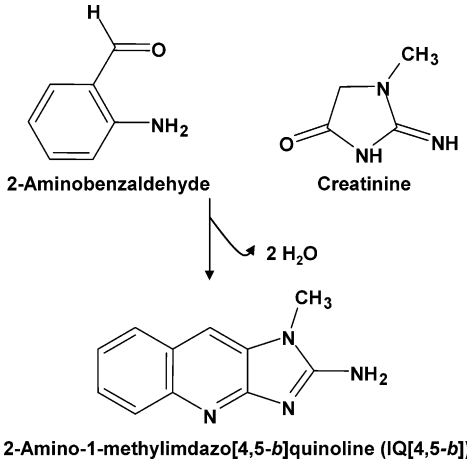


Figure 6. Kinetics of IQ[4,5-*b*], 8-MeIQx, and PhIP formation in urine treated with 1 N NaOH at 70 °C. (A) Urine from a carnivore (subject 2) collected for 24 h after meat consumption. (B) Urine from subject 2 prior to meat consumption. (C) Urine from a vegetarian (subject 11).

Scheme 1. Formation of IQ[4,5-*b*]



attributed to the biosynthesis of this HAA within urine. One plausible pathway of IQ[4,5-*b*] formation in vivo is through the condensation of creatinine with 2-ABA (Scheme 1), a product of tryptophan and indole metabolism (*41*−*44*). The reaction of creatinine with several metabolites derived from tryptophan and the environmental contaminant *o*-toluidine (*45*, *46*) that included

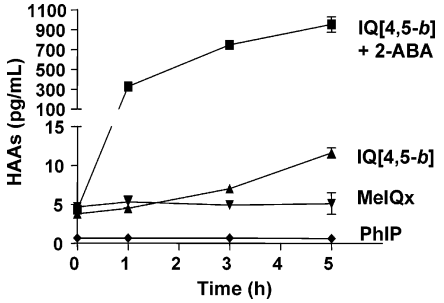


Figure 7. Kinetics of formation of IQ[4,5-*b*] in urine of a carnivore (subject 2) who had consumed meat within the past 24 h in the presence of 2-ABA (1 μg/mL urine) or solely in urine incubated at 37 °C. Other IQ isomers were not detected. The concentrations of 8-MeIQx or PhIP remained unchanged, irrespective of the addition of 2-ABA.

2-aminobenzyl alcohol, AA, as well as 2-ABA, and *o*-toluidine, were investigated as potential precursors of IQ[4,5-*b*]. The urine of a carnivore (subject 2) was incubated with these various constituents at 37 °C over time, and IQ[4,5-*b*] was quantitated by LC/ESI-MS. An increase in the content of IQ[4,5-*b*] was observed only in urine samples treated with 2-ABA. Approximately 60% of the 2-ABA was converted to IQ[4,5-*b*], which led to a 250-fold increase of this HAA (Figure 7). Moreover, the formation of IQ[4,5-*b*] also increased by 3-fold over time in unspiked urine while other isomers of IQ were not detected (<1 pg/mL). The concentrations of 8-MeIQx and PhIP also remained unchanged in urine in the presence or absence of 2-ABA. Therefore, constituents in urine are able to react with creatinine under physiological conditions to produce IQ[4,5-*b*], a genotoxic HAA (*47*).

Potential Artifactual Formation of IQ[4,5-*b*] in Urine during Isolation. The potential for artifactual formation of IQ[4,5-*b*] was investigated in a urine sample from the same individual pre- and postmeat consumption that was used for kinetics experiments described above. The urine samples were spiked with isotopic 1-[^2H_3C]-creatinine (3 mg/mL), and the HAAs were isolated as described in the Experimental Procedures. The procedure used to isolate HAAs from urine in this study deviates from our published method where the urine was made strongly alkaline (1 N NaOH) in order to deprotonate the HAAs and quantitatively extract the compounds into ethyl acetate (*20*). In this study, the pH of urine samples was carefully controlled by neutralization with 1 M KH_2PO_4 buffer (pH 7.5) prior to solvent extraction. The employment of a less alkaline pH resulted in lower recoveries of IQ[4,5-*b*], as well as for 8-MeIQx and PhIP from urine [recoveries ranging from 25 to 50% vs the previously reported recoveries of 70−80% (*20*)] but avoided potential artifactual, base-catalyzed formation of IQ[4,5-*b*] during the extraction procedure. The concentration of the isotopic 1-[^2H_3C]IQ[4,5-*b*] in urine samples of this subject pre- and postmeat consumption was below the LOD (∼1 pg/mL), but following base treatment for 3 h at 70 °C, the amount of 1-[^2H_3C]IQ-[4,5-*b*] exceeded 200 pg/mL. The concentration of unlabeled IQ[4,5-*b*] was also <1 pg/mL in urine collected over 24 h premeat consumption but present at a concentration of 5.4 pg/mL in urine collected for 24 h after meat consumption. Thus, the extraction procedure under neutral pH conditions does not catalyze the formation of IQ-[4,5-*b*] in urine extracts at quantifiable concentrations. The formation of 1-[^2H_3C]IQ[4,5-*b*] under alkaline condi-

Table 1. HAA Content in Grilled Meats (pg/g) Consumed by Volunteers[a]

meat sample	8-MeIQx	PhIP	IQ[4,5-*b*]	comments, cooking temperature
1	2570 ± 830	16360 ± 5240	230 ± 73	subject 1, barbequed meat over charcoal, 300 °C
2	1200 ± 100	664 ± 93	30 ± 4	subject 2, pan-fried meat 175 °C
3	3790 ± 175	450 ± 10	30 ± 7	subject 3, pan-fried meat, 200 °C
4	1290 ± 175	660 ± 40	132 ± 15	subject 4, barbequed meat over gas flame, 180 °C
5	5310 ± 720	15170 ± 2900	210 ± 48	subjects 5−10, pan-fried, 300 °C

[a] Average + SD ($N = 3$ or 6 independent extractions and measurements).

tions reinforces the observation that endogenous constituents of urine are able to react with creatinine to form this HAA.

8-MeIQx, PhIP, and IQ[4,5-*b*] Content in Cooked Meats. The concentrations of HAAs in grilled meat ingested in this study were determined by LC/ESI-MS/MS following tandem solid phase extraction of grilled meats as previously reported (*20*, *27*), and the data are summarized in Table 1. Relative to 8-MeIQx and PhIP, the concentrations of IQ[4,5-*b*] in cooked beef were low in all meat samples (<1 ppb), with the highest amounts formed in beef that was cooked well-done at 300 °C. The identity of IQ[4,5-*b*] in cooked beef was confirmed by its product ion spectrum and by coelution with a synthetic standard under acidic and neutral pH conditions and monitoring by LC/ESI/MS-MS (*21*).

IQ[4,5-*b*] Content in Urine following Consumption of Cooked Meat. The amounts of 8-MeIQx, PhIP, and IQ[4,5-*b*] quantified in urine of individuals following consumption of cooked beef and the percent of the ingested dose excreted as the parent compounds are reported in Table 2. As previously reported, the percent of the dose in urine recovered as unmetabolized 8-MeIQx and PhIP is low and on average corresponds to <2% of the ingested HAA 10 h following consumption of cooked meats (*10*, *17*, *48*, *49*). In contrast to 8-MeIQx and PhIP, the amount of unmetabolized IQ[4,5-*b*] recovered in urine ranged from 15 to 130% of the estimated intake. The absorption of 8-MeIQx and PhIP from the gastrointestinal tract is rapid. More than 80% of the ingested dose that is eliminated as the parent compounds occurs within 10 h, and >90% is eliminated within 24 h of consumption of grilled meats (*10*, *48*). The rate of elimination of IQ[4,5-*b*] in urine of humans is not known but may be slower that of 8-MeIQx or PhIP since a higher percentage of the unmetabolized IQ[4,5-*b*] ingested is recovered in urine of meat eaters that had been collected over 24 h than collected over shorter time periods (10−16 h). IQ[4,5-*b*] was also detected in spot urine collections of three out of eight individuals that had refrained from meat consumption for >24 h, suggesting that IQ[4,5-*b*] may be present in foods other than cooked meats.

Discussion

Both GC/MS and LC/MS are sensitive techniques to identify genotoxic HAAs and their metabolites in human urine (*10*, *11*, *17*, *38*). While negative ion chemical ionization mass spectrometry in the selected ion monitoring (SIM) scan mode is a highly selective method to measure HAAs in cooked foods or urine following chemical derivatization (*10*, *50*, *51*), the different scan modes provided by the triple quadrupole MS have allowed us to corroborate the identities of known HAAs and probe for previously unreported HAAs in human urine and cooked meats by tandem LC/ESI-MS techniques (*20*, *21*). The SRM scan mode was used to quantitate known HAAs in human urine at levels approaching 1 pg/mL urine, and the full product ion scan mode was used to confirm the identities of these analytes (*20*). The usage of the CNL scan mode monitoring the transition $[M + H]^+ \rightarrow [M + H - CH_3^{\bullet}]^{+\bullet}$ that is common to 8-MeIQx, PhIP, and other HAAs containing the *N*-CH_3-aminoimidazole moiety (*22*, *23*, *37*) resulted in the detection of IQx and the discovery of a novel isomer of 8-MeIQx in human urine (*20*). As an extension of that investigation, we have identified IQ[4,5-*b*], an isomer of the powerful animal carcinogen IQ (*5*, *24*), in human urine by LC/ESI-MS/MS and LC/UV spectroscopy. Prior to this investigation, IQ[4,5-*b*] has not been identified in cooked meats, fish, or poultry, although its chemical synthesis has been reported (*25*).

In agreement with previous investigations (*10*, *26*), 8-MeIQx and PhIP were identified in the urine of meat eaters but not the urine of subjects who refrained from meat consumption for >24 h or in urine of vegetarians. In contrast to 8-MeIQx and PhIP, IQ[4,5-*b*] was detected in the urine of four of the eight subjects who had refrained from meat consumption for >24 h and was also found in the urine of two vegetarians following base hydrolysis, which increased the concentration of IQ[4,5-*b*] by more than 100-fold. The four subjects that had quantifiable amounts of IQ[4,5-*b*] in their urine after refraining from meat consumption had each eaten cheese and/or boiled eggs as part of their diet while abstaining from cooked meats. IQ and several other HAAs have been reported in fried eggs (*52*, *53*). It is plausible that IQ[4,5-*b*], which forms at temperatures well below 100 °C, may be present in boiled eggs or possibly other foods containing creatinine, such as cheese.

The amount of IQ[4,5-*b*] recovered in some urine samples and in urine samples of all of the subjects following base treatment exceeded the amount of IQ[4,5-*b*] ingested in grilled meats by more than 100-fold, which suggests that IQ[4,5-*b*] may be formed in the urinary bladder or other biological fluids rather than being derived exclusively from the diet. The constituents in urine that are responsible for the formation of IQ[4,5-*b*] are most likely creatinine, which is present at 40−100 mg/dL urine and 2-ABA. The source of 2-ABA in the urinary bladder is unknown. To the best of our knowledge, 2-ABA has not been reported as a byproduct of tryptophan metabolism in humans (*41*, *54*). However, 2-ABA is biosynthesized by bacteria from indole (*42*, *44*), and the bacterial flora of the gut may contribute to the formation of 2-ABA in vivo. The direct reduction of AA and other benzoic acids to the corresponding aldehydes occurs with high efficiency in plants and fungi (*43*, *55*), and biochemical reduction of AA in humans may contribute to 2-ABA formation. 2-ABA is also a metabolite of the environmental carcinogen *o*-toluidine, which has been identified in rat and human urine (*45*, *46*). Given the efficient reaction of 2-ABA with urinary creatinine, only low concentrations (<10 ng/mL urine) of this alde-

Table 2. Estimates of HAA Excretion in Urine before and after Meat Consumption[a]

				IQ[4,5-b]			MeIQx			PhIP			
subject	pH	creatinine (mg/dL)	urine (g)	ingested (ng total)	pg/mL urine	% dose in urine	ingested (ng total)	pg/mL urine	% dose in urine	ingested (ng total)	pg/mL urine	% dose in urine	comment
1	6.73	81.8 ± 3.8	1784	27.0 ± 8.5	16.9 ± 3.4	113 ± 23	301 ± 97	9.8 ± 2.9	5.8 ± 1.7	1780 ± 820	10.2 ± 1.7	1.0 ± 0.2	24 h urine collect, postmeat
	7.34	41.0 ± 2.0	1700		ND			ND			ND		24 h urine collect, premeat
2	5.79	188 ± 3.4	1062	4.2 ± 0.5	5.4 ± 1.3	136 ± 33	168 ± 14	3.6 ± 0.8	2.3 ± 0.5	93 ± 13	1.1 ± 0.2	1.3 ± 0.3	24 h urine collect, postmeat
	6.80	69.2 ± 4.2	100		ND			ND			ND		spot urine collect, premeat
3	5.90	46.4 ± 2.6	1250	2.9 ± 0.8	2.5 ± 0.7	108 ± 30	433 ± 20	3.4 ± 0.9	1.0 ± 0.3	51 ± 1	0.6 ± 0.1	1.5 ± 0.1	24 h urine collect, postmeat
	6.10	46.4 ± 0.8	1460		ND			ND			ND		24 h urine collect, premeat
4	6.87	139 ± 13	1120	22.0 ± 2.5	7.2 ± 0.7	37.0 ± 3.4	216 ± 29	5.0 ± 0.87	2.6 ± 0.5	110 ± 7	3.2 ± 0.6	3.2 ± 0.6	24 h urine collect, postmeat
	6.24	127 ± 2.0	1206		2.8 ± 0.5			ND			ND		16 h urine collect, premeat
5	7.56	37.2 ± 1.0	1857	36.0 ± 6.4	3.8 ± 0.1	20.0 ± 0.6	918 ± 124	4.3 ± 0.3	0.9 ± 0.1	2590 ± 531	9.0 ± 0.4	0.7 ± 0.0	10 h urine collect, postmeat
	5.90	90.2 ± 2.0	421		40 ± 0.6			ND			ND		spot urine collect, premeat
6	7.49	73.0 ± 6.0	1589	57 ± 10	5.6 ± 1.2	16.0 ± 3.3	1460 ± 197	10.2 ± 1.0	1.1 ± 0.1	4120 ± 840	16.0 ± 0.3	0.6 ± 0.0	10 h urine collect, postmeat
	7.27	75.6 ± 1.0	80		ND			ND			ND		spot urine collect, premeat
7	7.21	64.6 ± 1.8	1919	57 ± 10	4.6 ± 1.9	15.0 ± 6.3	1460 ± 197	9.4 ± 0.6	1.3 ± 0.1	4120 ± 840	14.0 ± 1.1	0.7 ± 0.1	10 h urine collect, postmeat
	7.82	111 ± 4.2	86		ND			ND			ND		spot urine collect, premeat
8	7.46	92.8 ± 3.4	1396	57 ± 10	10.5 ± 1.9	26.0 ± 4.6	1460 ± 197	26.0 ± 4.0	2.5 ± 0.4	4120 ± 840	22.0 ± 1.5	0.8 ± 0.1	10 h urine collect, postmeat
	7.77	92.8 ± 5.6	124		11.0 ± 1.9			ND			ND		spot urine collect, premeat
9	6.89	100 ± 4.4	1181	57 ± 10	11.3 ± 1.3	24.0 ± 2.7	1460 ± 197	21.0 ± 7.2	1.8 ± 0.6	4120 ± 840	28.0 ± 3.1	0.8 ± 0.1	10 h urine collect, postmeat
	6.46	99.6 ± 4.4	74		2.7 ± 0.4			ND			ND		spot urine collect, premeat
10	7.06	76.6 ± 0.6	1723	57 ± 10	9.0 ± 0.5	28.0 ± 1.5	1460 ± 197	16.0 ± 2.4	1.9 ± 0.3	4120 ± 840	19.0 ± 0.3	0.8 ± 0.0	10 h urine collect, postmeat
	5.94	67.2 ± 2.4	264		ND			ND			ND		spot urine collect, premeat
11	7.07	41.0 ± 2.8	960		ND			ND			ND		24 h urine collect, vegetarian
12	7.03	23.0 ± 0.8	1590		ND			ND			ND		24 h urine collect, vegetarian

[a] Percent dose is the amount found in the total volume of urine collected divided by meat value multiplied by 100%. Mean = SD ($N = 3$ or 6 independent urine extractions and measurements). ND, not detected; <1 pg/mL.

hyde are required to produce the amounts of IQ[4,5-*b*] observed in urine samples.

More than 20 HAAs have been reported in cooked meats, fish, and poultry prepared under common household cooking conditions (*5*, *36*, *56*). Investigations with model systems have shown that HAAs containing the aminoimidazole moiety are formed through reactions of sugars, amino acids, and creatinine as precursor molecules (*56*, *57*). All of these model reactions were conducted at temperatures that exceeded 100 °C, and the formation of HAAs at lower temperatures was reported to be negligible (*56*), although trace amounts of PhIP were reported to form from a mixture of creatinine, phenylalanine, and D-ribose incubated at 37 °C (*58*). In contrast to PhIP and other known HAAs, the formation of IQ[4,5-*b*] occurs in appreciable amounts by incubation of creatinine with 2-ABA in aqueous solutions at 37 °C. However, the amount of IQ[4,5-*b*] formed in cooked meats (<1 ppb) is much lower than the amounts of PhIP or 8-MeIQx, indicating that 2-ABA or other critical precursors of IQ[4,5-*b*] are present at limiting quantities in meat.

Creatine is consumed in numerous food products and also produced in vivo through the reaction of arginine and glycine by A:G amidinotransferase (*59*, *60*). Dietary supplementation of creatine by sports enthusiasts has become ever increasingly popular and believed to be efficacious for maintaining higher energy phosphates during vigorous exercise and for developing increased muscle mass during training. Dietary supplementation of creatine is viewed as safe when consumed in moderation; however, the safety of consumption of large amounts of creatine (*59*−*61*), where as much as 20−30 g of the supplement may be consumed per day by endurance athletes (*62*), has been questioned in several recent review articles. The review articles raised the possibility that high consumption of creatine could result in formation of genotoxic HAAs in the body (*59*−*61*). Because of the elevated temperatures required for the formation of known HAAs (*56*, *63*), it seems unlikely that molecules such as 8-MeIQx or PhIP would form under physiological conditions. However, the formation of IQ[4,5-*b*] does occur in urine incubated under physiological conditions, and the potential for the endogenous formation of this HAA within the urinary bladder or other biological fluids cannot be excluded, particularly in individuals who consume large quantities of creatine. Recently, several novel HAAs that include 9-(4′-aminophenyl)-9*H*-pyrido-[4,3-*b*]indole (APNH) and 9-(4′-amino-3′-methylphenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole were reported to occur in vitro through reaction of norharman with a P450-mediated hydroxylated metabolite of aniline or *o*-toluidine, respectively (*64*−*66*). APNH was also detected in the urine of rats following simultaneous administration of norharman and aniline by gavage. Therefore, APNH is likely to be also produced from norharman and a metabolite of aniline in the human body and may be considered as a novel class of endogenous mutagens and carcinogens (*65*, *67*). IQ[4,5-*b*] may be another class of HAAs that are formed endogenously.

There is little data on the toxicological properties of IQ[4,5-*b*], and the potential risk of this HAA for human health is unknown. IQ[4,5-*b*] is mutagenic in the Ames reversion assay in strain TA98 in the presence of liver S9 obtained from polychlorinated biphenyl pretreated rats and a more potent mutagen in tester strain YG1024 (*47*). This tester strain contains elevated *O*-acetyltransferase, suggesting that IQ[4,5-*b*] undergoes bioactivation by P450-catalyzed *N*-oxidation followed by *O*-acetylation to produce the reactive *N*-acetoxy intermediate as reported for other HAAs (*68*, *69*). Fortunately, the mutagenic potency of IQ[4,5-*b*] in the Ames reversion is more than 1000-fold weaker than that of its isomer IQ (*47*), a powerful carcinogen in rodents and nonhuman primates (*5*, *24*). However, the carcinogenic potency of many HAAs in experimental animals is within 10-fold even though the mutagenic potencies of these compounds in bacterial assays span over 5 orders of magnitude (*5*, *70*). Because of the potential for IQ[4,5-*b*] to form in vivo, further studies on biomonitoring of this compound in human biological fluids and assessment of the toxicological properties of this HAA in mammalian cells and in vivo may be warranted.

References

(1) Hecht, S. S. (2002) Human urinary carcinogen metabolites: Biomarkers for investigating tobacco and cancer. *Carcinogenesis 23*, 907−922.

(2) Wogan, G. N., Hecht, S. S., Felton, J. S., Conney, A. H., and Loeb, L. A. (2004) Environmental and chemical carcinogenesis. *Semin. Cancer Biol. 14*, 473−486.

(3) Chen, H. J., Wu, C. F., Hong, C. L., and Chang, C. M. (2004) Urinary excretion of 3,N4-etheno-2′-deoxycytidine in humans as a biomarker of oxidative stress: Association with cigarette smoking. *Chem. Res. Toxicol. 17*, 896−903.

(4) Weimann, A., Belling, D., and Poulsen, H. E. (2002) Measurement of 8-oxo-2′-deoxyguanosine and 8-oxo-2′-deoxyadenosine in DNA and human urine by hig performance liquid chromatography-electrospray tandem mass spectrometry. *Free Radical Biol. Med. 30*, 757−764.

(5) Sugimura, T., Wakabayashi, K., Nakagama, H., and Nagao, M. (2004) Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. *Cancer Sci. 95*, 290−299.

(6) Ohgaki, H., Takayama, S., and Sugimura, T. (1991) Carcinogenicities of heterocyclic amines in cooked food. *Mutat. Res. 259*, 399−410.

(7) Ito, N., Hasegawa, R., Sano, M., Tamano, S., Esumi, H., Takayma, S., and Sugimura, T. (1991) A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PHIP). *Carcinogenesis 12*, 1503−1506.

(8) Nagao, M., and Sugimura, T. (1993) Carcinogenic factors in food with relevance to colon cancer development. *Mutat. Res. 290*, 43−51.

(9) Ushiyama, H., Wakabayashi, K., Hirose, M., Ito, N., Sugimura, T., and Nagao, M. (1991) Presence of carcinogenic heterocyclic amines in urine of healthy volunteers eating normal diet, but not of inpatients receiving parenteral alimentation. *Carcinogenesis 12*, 1417−1422.

(10) Lynch, A. M., Knize, M. G., Boobis, A. R., Gooderham, N., Davies, D. S., and Murray, S. (1992) Intra- and interindividual variability in systemic exposure in humans to 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine, carcinogens present in food. *Cancer Res. 52*, 6216−6223.

(11) Stillwell, W. G., Turesky, R. J., Sinha, R., Skipper, P. L., and Tannenbaum, S. R. (1999) Biomonitoring of heterocyclic aromatic amine metabolites in human urine. *Cancer Lett. 143*, 145−148.

(12) Rich, K. J., Murray, B. P., Lewis, I., Rendell, N. B., Davies, D. S., Gooderham, N. J., and Boobis, A. R. (1992) *N*-Hydroxy-MeIQx is the major microsomal oxidation product of the dietary carcinogen MeIQx with human liver. *Carcinogenesis 13*, 2221−2226.

(13) Turesky, R. J., Constable, A., Richoz, J., Varga, N., Markovic, J., Martin, M. V., and Guengerich, F. P. (1998) Activation of heterocyclic aromatic amines by rat and human liver microsomes and by purified rat and human cytochrome P450 1A2. *Chem. Res. Toxicol. 11*, 925−936.

(14) Lang, N. P., Butler, M. A., Massengill, J. P., Lawson, M., Stotts, R. C., Hauer-Jensen, M., and Kadlubar, F. F. (1994) Rapid metabolic phenotypes for acetyltransferase and cytochrome P4501A2 and putative exposure to food-borne heterocyclic amines increase the risk for colorectal cancer or polyps. *Cancer Epidemiol. Biomarkers Prev. 3*, 675−682.

(15) Le Marchand, L., Hankin, J. H., Wilkens, L. R., Pierce, L. M., Franke, A., Kolonel, L. N., Seifried, A., Custer, L. J., Chang, W.,

Lum-Jones, A., and Donlon, T. (2001) Combined effects of well-done red meat, smoking, and rapid *N*-acetyltransferase 2 and CYP1A2 phenotypes in increasing colorectal cancer risk. *Cancer Epidemiol. Biomarkers Prev. 10*, 1259−1266.

(16) Zheng, W., Gustafson, D. R., Sinha, R., Cerhan, J. R., Moore, D., Hong, C. P., Anderson, K. E., Kushi, L. H., Sellers, T. A., and Folsom, A. R. (1998) Well-done meat intake and the risk of breast cancer. *J. Natl. Cancer Inst. 90*, 1724−1729.

(17) Turesky, R. J., Garner, R. C., Welti, D. H., Richoz, J., Leveson, S. H., Dingley, K. H., Turteltaub, K. W., and Fay, L. B. (1998) Metabolism of the food-borne mutagen 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline in humans. *Chem. Res. Toxicol. 11*, 217−225.

(18) King, R. S., Kadlubar, F. F., and Turesky, R. J. (2000) In vivo Metabolism of Heterocyclic Amines. In *Food Borne Carcinogens: Heterocyclic Amines* (Nagao, M., and Sugimura, T., Eds.) pp 90−111, John Wiley & Sons Ltd., Chichester, England.

(19) Turesky, R. J., and Vouros, P. (2004) Formation and analysis of heterocyclic aromatic amine-DNA adducts in vitro and in vivo. *J. Chromatogr. B 802*, 155−166.

(20) Holland, R. D., Taylor, J., Schoenbachler, L., Jones, R. C., Freeman, J. P., Miller, D. W., Lake, B. G., Gooderham, N. J., and Turesky, R. J. (2004) Rapid biomonitoring of heterocyclic aromatic amines in human urine by tandem solvent solid phase extraction liquid chromatography electrospray ionization mass spectrometry. *Chem. Res. Toxicol. 17*, 1121−1136.

(21) Turesky, R. J., Taylor, J., Schoenbachler, L., Freeman, J. P., and Holland, R. D. (2004) Quantification of carcinogenic heterocyclic aromatic amines and detection of novel heterocyclic aromatic amines in cooked meats and grill scrapings by HPLC/electrospray-mass spectrometry. *J. Agric. Food Chem*. In press.

(22) Guy, P. A., Gremaud, E., Richoz, J., and Turesky, R. J. (2000) Quantitative analysis of mutagenic heterocyclic aromatic amines in cooked meat using liquid chromatography-atmospheric pressure chemical ionisation tandem mass spectrometry. *J. Chromatogr. A 883*, 89−102.

(23) Toribio, F., Moyano, E., Puignou, L., and Galceran, M. T. (2002) Multistep mass spectrometry of heterocyclic amines in a quadrupole ion trap mass analyser. *J. Mass Spectrom. 37*, 812−828.

(24) Adamson, R. H., Thorgeirsson, U. P., Snyderwine, E. G., Reeves, J., Dalgard, D. W., Takayama, S., and Sugimura, T. (1990) Carcinogenicity of 2-amino-3-methylimidazo[4,5-*f*]quinoline in nonhuman primates: Induction of tumors in three macaques. *Jpn. J. Cancer Res. 81*, 10−14.

(25) Ronne, E., Olsson, K., and Grivas, S. (1994) One-step synthesis of 2-amino-1-methylimidazo[4,5-*b*]quinoline. *Synth. Commun. 24*, 1363−1366.

(26) Murray, S., Lake, B. G., Gray, S., Edwards, A. J., Springall, C., Bowey, E. A., Williamson, G., Boobis, A. R., and Gooderham, N. J. (2001) Effect of cruciferous vegetable consumption on heterocyclic aromatic amine metabolism in man. *Carcinogenesis 22*, 1413−1420.

(27) Gross, G. A., and Gruter, A. (1992) Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food products. *J. Chromatogr. 592*, 271−278.

(28) Stillwell, W. G., Turesky, R. J., Gross, G. A., Skipper, P. L., and Tannenbaum, S. R. (1994) Human urinary excretion of sulfamate and glucuronde conjugates of 2-amino-3,8-dimethylimdazo[4,5-*f*]-quinoxaline (MeIQx). *Cancer Epidemiol. Biomarkers Prev. 3*, 399−405.

(29) Spingarn, N. E., Kasai, H., Vuolo, L. L., Nishimura, S., Yamaizumi, Z., Sugimura, T., Matsushima, T., and Weisburger, J. H. (1980) Formation of mutagens in cooked foods. III. Isolation of a potent mutagen from beef. *Cancer Lett. 9*, 177−183.

(30) Takahashi, M., Wakabayashi, K., Nagao, M., Yamamoto, M., Masui, T., Goto, T., Kinae, N., Tomita, I., and Sugimura, T. (1985) Quantification of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in beef extracts by liquid chromatography with electrochemical detection (LCEC). *Carcinogenesis 6*, 1195−1199.

(31) Turesky, R. J., Wishnok, J. S., Tannenbaum, S. R., Pfund, R. A., and Buchi, G. H. (1983) Qualitative and quantitative characterization of mutagens in commercial beef extract. *Carcinogenesis 4*, 863−866.

(32) Hargraves, W. A., and Pariza, M. W. (1983) Purification and mass spectral characterization of bacterial mutagens from commercial beef extract. *Cancer Res. 43*, 1467−1472.

(33) Yamaizumi, Z., Kasai, H., Nishimura, S., Edmonds, C. G., and McCloskey, J. A. (1986) Stable isotope dilution quantification of mutagens in cooked foods by combined liquid chromatography-thermospray mass spectrometry. *Mutat. Res. 173*, 1−7.

(34) Knize, M. G., Dolbeare, F. A., Carroll, K. L., Moore, D. H., and Felton, J. S. (1994) Effect of cooking time and temperature on the heterocyclic amine content of fried beef patties. *Food Chem. Toxicol. 32*, 595−603.

(35) Skog, K., Steineck, G., Augustsson, K., and Jagerstad, M. (1995) Effect of cooking temperature on the formation of heterocyclic amines in fried meat products and pan residues. *Carcinogenesis 16*, 861−867.

(36) Felton, J. S., Jagerstad, M., Knize, M. G., Skog, K., and Wakabayashi, K. (2000) Contents in Foods, Beverages and Tobacco. In *Food Borne Carcinogens Heterocyclic Amines* (Nagao, M., and Sugimura, T., Eds.) pp 31−71, John Wiley & Sons Ltd., Chichester, England.

(37) Holder, C. L., Preece, S. W., Conway, S. C., Pu, Y. M., and Doerge, D. R. (1997) Quantification of heterocyclic amine carcinogens in cooked meats using isotope dilution liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom. 11*, 1667−1672.

(38) Kulp, K. S., Knize, M. G., Fowler, N. D., Salmon, C. P., and Felton, J. S. (2004) PhIP metabolites in human urine after consumption of well-cooked chicken. *J. Chromatogr. B 802*, 143−153.

(39) Friesen, M. D., Rothman, N., and Strickland, P. T. (2001) Concentration of 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) in urine and alkali-hydrolyzed urine after consumption of charbroiled beef. *Cancer Lett. 173*, 43−51.

(40) Strickland, P. T., Qian, Z., Friesen, M. D., Rothman, N., and Sinha, R. (2002) Metabolites of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in human urine after consumption of charbroiled or fried beef. *Mutat. Res. 506*−*507*, 163−173.

(41) Wolf, H. (1974) Studies on tryptophan metabolism in man. *Scand. J. clin. Lab. Invest. 33* (Suppl. 136), 1−176.

(42) Spiteller, P., and Steglich, W. (2001) Biosynthesis of 2-aminobenzaldehyde in flowers of Robinia pseudoacacia and Philadelphus coronarius. *Phytochemistry 57*, 361−363.

(43) von Nussbaum, Spahl, W., and Steglich, W. (1997) Reduction of anthranilic acid and related amino acids in fruit-bodies of *Hebeloma Saccharioleus*. *Phytochemistry 46*, 261−264.

(44) Uchida, M., Sakamoto, Y., and Ichiara, K. (1953) The bacterial decomposition of indole. *Med. J. Osaka Univ. 3*, 487−495.

(45) Son, O. S., Everett, D. W., and Fiala, E. S. (1980) Metabolism of *o*-[*methyl*-^{14}C]toluidine in the F344 rats. *Xenobiotica 10*, 457−468.

(46) el-Bayoumy, K., Donahue, J. M., Hecht, S. S., and Hoffmann, D. (1986) Identification and quantitative determination of aniline and toluidines in human urine. *Cancer Res. 46*, 6064−6067.

(47) Vikse, R., Hatch, F. T., Winter, N. W., Knize, M. G., Grivas, S., and Felton, J. S. (1995) Structure-mutagenicity relationships of four amino-imidazonaphthyridines and imidazoquinolines. *Environ. Mol. Mutagen. 26*, 79−85.

(48) Boobis, A. R., Lynch, A. M., Murray, S., de la Torre, R., Solans, A., Farré, M., Segura, J., Gooderham, N. J., and Davies, D. S. (1994) CYP1A2-catalyzed conversion of dietary heterocyclic amines to their proximate carcinogens is their major route of metabolism in humans. *Cancer Res. 54*, 89−94.

(49) Stillwell, W. G., Turesky, R. J., Sinha, R., and Tannenbaum, S. R. (1999) N-oxidative metabolism of 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx) in humans: Excretion of the N^2-glucuronide conjugate of 2-hydroxyamino-MeIQx in urine. *Cancer Res. 59*, 5154−5159.

(50) Murray, S., Gooderham, N. J., Boobis, A. R., and Davies, D. S. (1988) Measurement of MeIQx and DiMeIQx in fried beef by capillary column gas chromatography electron capture negative ion chemical ionisation mass spectrometry. *Carcinogenesis 9*, 321−325.

(51) Murray, S., Lynch, A. M., Knize, M. G., and Gooderham, M. J. (1993) Quantification of the carcinogens 2-amino-3,8-dimethyl- and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in food using a combined assay based on gas chromatography-negative ion mass spectrometry. *J. Chromatogr. 616*, 211−219.

(52) Grose, K. R., Grant, J. L., Bjeldanes, L. F., Andresen, B. D., Healy, S. K., Lewis, P. R., Felton, J. S., and Hatch, F. T. (1986) Isolation of the carcinogen IQ from fried egg patties. *J. Agric. Food Chem. 34*, 201−202.

(53) Kato, T., Kikugawa, K., Asanoma, M., and Sakabe, Y. (1990) Occurrence of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and other heterocyclic amine mutagens in oil of charred egg yolk (ranyu). *Mutat. Res. 240*, 259−266.

(54) Kegg tryptophan metabolism reference pathway (2004) http://www.genome.jp/kegg/pathway/map/map00380.html

(55) Gross, G. G., and Zenk, M. H. (1969) [Reduction of aromatic acids to aldehydes and alcohols in the cell-free system. 1. Purification and properties of aryl-aldehyde: NADP-oxidoreductase from Neurospora crassa. *Eur. J. Biochem. 8*, 413−419.

(56) Skog, K. I., Johansson, M. A., and Jagerstad, M. I. (1998) Carcinogenic heterocyclic amines in model systems and cooked foods: A review on formation, occurrence and intake. *Food Chem. Toxicol. 36*, 879−896.

(57) Felton, J. S., Knize, M. G., Dolbeare, F. A., and Wu, R. (1994) Mutagenic activity of heterocyclic amines in cooked foods. *Environ. Health Perspect. 102* (Suppl. 6), 201−204.

(58) Manabe, S., Kurihara, N., Wada, O., Tohyama, K., and Aramaki, T. (1992) Formation of PhIP in a mixture of creatinine, phenylalanine and sugar or aldehyde by aqueous heating. *Carcinogenesis 13*, 827−830.

(59) Wyss, M., and Kaddurah-Daouk, R. (2000) Creatine and creatinine metabolism. *Physiol. Rev. 80*, 1107−1213.

(60) Brudnak, M. A. (2004) Creatine: Are the benefits worth the risk? *Toxicol. Lett. 150*, 123−130.

(61) Agence Française de sécurité sanitaire des aliments. Opinion of the French Agency for Food Safety and report on the assessment of the risks of creatine on the consumer and of the veracity of the claims relating ot sports performance and the increase of muscle mass (2001) www.afssa.fr/Ftp/Afssa/25500−25501.pdf.

(62) Poortmans, J. R., and Francaux, M. (2000) Adverse effects of creatine supplementation: fact or fiction? *Sports Med. 30*, 155−170.

(63) Sinha, R., Rothman, N., Salmon, C. P., Knize, M. G., Brown, E. D., Swanson, C. A., Rhodes, D., Rossi, S., Felton, J. S., and Levander, O. A. (1998) Heterocyclic amine content in beef cooked by different methods to varying degrees of doneness and gravy made from meat drippings. *Food Chem. Toxicol. 36*, 279−287.

(64) Totsuka, Y., Takamura-Enya, T., Nishigaki, R., Sugimura, T., and Wakabayashi, K. (2004) Mutagens formed from β-carbolines with aromatic amines. *J. Chromatogr B 802*, 135−141.

(65) Totsuka, Y., Kataoka, H., Takamura-Enya, T., Sugimura, T., and Wakabayashi, K. (2002) In vitro and in vivo formation of aminophenylnorharman from norharman and aniline. *Mutat. Res. 506−507*, 49−54.

(66) Hada, N., Totsuka, Y., Enya, T., Tsurumaki, K., Nakazawa, M., Kawahara, N., Murakami, Y., Yokoyama, Y., Sugimura, T., and Wakabayashi, K. (2001) Structures of mutagens produced by the co-mutagen norharman with o- and m-toluidine isomers. *Mutat. Res. 493*, 115−126.

(67) Kawamori, T., Totsuka, Y., Uchiya, N., Kitamura, T., Shibata, H., Sugimura, T., and Wakabayashi, K. (2004) Carcinogenicity of aminophenylnorharman, a possible novel endogenous mutagen, formed from norharman and aniline, in F344 rats. *Carcinogenesis 25*, 1967−672.

(68) Turesky, R. J. (1994) DNA adducts of heterocyclic aromatic amines, aryl azides and 4-nitroquinoline 1-oxide. In *DNA Adducts: Identification and Biological Significance* (Hemminki, K., Dipple, A., Shuker, D. E. G., Kadlubar, F. F., Segerbäck, D., and Bartsch, H., Eds.) pp 217−228, International Agency for Research on Cancer, Lyon.

(69) Schut, H. A., and Snyderwine, E. G. (1999) DNA adducts of heterocyclic amine food mutagens: Implications for mutagenesis and carcinogenesis. *Carcinogenesis 20*, 353−368.

(70) Sugimura, T. (1997) Overview of carcinogenic heterocyclic amines. *Mutat. Res. 376*, 211−219.

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