Published in final edited form as:

Anal Chem. 2011 February 1; 83(3): 1093–1101. doi:10.1021/ac102918b.

An Ultra Performance Liquid Chromatography – Tandem Mass Spectrometry Method for Biomonitoring Cooked Meat Carcinogens and Their Metabolites in Human Urine

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Abstract

The cooked meat carcinogens 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and their principal metabolites produced by cytochrome P450 and/or uridine diphosphate glucuronosyltransferases were simultaneously measured at the parts per trillion level in urine of omnivores, by ultra performance liquid chromatography (UPLC) with a Michrom Advance CaptiveSpray™ source and a triple stage quadrupole mass spectrometer. Quantitation was performed in the selected reaction monitoring mode. The UPLC method is much more rapid and sensitive than our earlier capillary HPLC method: the duty cycle of the UPLC method is 19 minutes compared to 57 minutes for capillary HPLC. The performance of the UPLC assay was evaluated with urine samples from three subjects over 4 different days. The intraday and interday precisions of the estimates of PhIP, MeIQx, and their metabolites, reported as the coefficients of variation, were ≤10%. The limit of quantification (LOQ) values for PhIP and MeIQx were about 5 pg/mL, whereas the LOQ values of their metabolites ranged from 10 to 40 pg/mL. Furthermore, the identities of the analytes were corroborated by acquisition of full scan product ion spectra, employing between 0.5 and 5 pg of analyte for assay.

INTRODUCTION

Heterocyclic aromatic amines (HAAs) are formed during the cooking of meats, fish, and poultry.¹ HAAs are experimental animal carcinogens and thought to contribute to human cancers, particularly for individuals who frequently consume well-done cooked meats that contain HAAs.² 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) are two of the most mass-abundant carcinogenic heterocyclic aromatic amines (HAAs) formed in cooked meats: concentrations can range from less than 1 part per billion (ppb) to ~15 ppb in meats prepared under common household cooking conditions.³ The chronic consumption of foods containing these HAAs constitutes a potential human health hazard: the Report on Carcinogens, Eleventh Edition, of the National Toxicology Program, concluded that several prevalent HAAs,

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including MeIQx and PhIP, are “reasonably anticipated” to be human carcinogens.⁴ There is a critical need to develop noninvasive and rapid methods to biomonitor HAAs for human risk assessment.⁵ Urine is a useful biological matrix for the assessment of recent exposures to carcinogens, since large quantities can be obtained noninvasively. Moreover, the characterization of the urinary metabolic profiles of the genotoxicants can provide an estimate of the relative extent of bioactivation, as opposed to detoxification, undergone by the chemicals in vivo.⁶

The analysis of unaltered MeIQx and PhIP, and their metabolites, in human urine is an analytical challenge, because usually only ~1 to several μg of each compound is ingested per day, for individuals eating well-done cooked meat.⁷ Thus, the concentrations of HAAs and their metabolites are often well below the part-per-billion (ppb) level in urine. The chemical structures of MeIQx and PhIP, and their principal metabolites are shown in Figure 1. The polar and ionic nature of the metabolites presents difficulties for their isolation along with the parent HAAs, from thousands of other components in the urine matrix.⁸ Various analytical approaches have been devised to isolate MeIQx or PhIP from human urine. The techniques have included solvent extraction,^{9,10} solid-phase extraction (SPE),¹¹ use of molecularly imprinted polymers,¹² and immunoaffinity methods,¹³ followed by quantification by gas chromatography and negative ion chemical ionization mass spectrometry (GC-NICI-MS),^{9,10,14} or liquid chromatography-electrospray ionization/mass spectrometry/tandem mass spectrometry (LC-ESI/MS/MS),^{11,12} or alternatively followed by fluorescence detection.¹⁵ Urinary metabolites have also been detected by LC-ESI/MS/MS,^{16,17} or indirectly, after chemical reduction or acid hydrolysis of HONH-PhIP conjugates, with detection by LC-ESI/MS/MS or GC-NICI-MS.^{18,19}

We recently reported a facile solid phase extraction (SPE) method, employing a mixed-mode reverse phase cation exchange resin enrichment procedure, to simultaneously isolate PhIP, MeIQx and their principal metabolites in urine of human volunteers who ate cooked meat.^{20,21} The unaltered urinary HAAs and their metabolites were quantified by capillary HPLC-ESI/MS/MS.^{20,21} The analytical method was robust. The interday and intraday coefficients of variation were <10%, at analyte concentrations above the limit of quantification (LOQ). However, the duty cycle time of the analysis was almost 1 hr per sample.²¹ For large scale population studies, much shorter LC/MS run times are required. In our original analytical methods validation study,^{17,21} the subjects drank water as the sole fluid with the meal. However, individuals may prefer to drink juices, or beverages, such as colas, wine, beer, tea, or coffee with their meal, instead of water. A number of constituents in these beverages could adversely affect the efficacy of our SPE method. Moreover, beverages may contain isobaric interferences of HAAs or their metabolites and restrict the utility of the LC-ESI/MS/MS method. In this article, we describe a rapid UPLC-ESI/MS/MS method to assay for MeIQx, PhIP, and their principal metabolites in urine of subjects who have eaten cooked meat along with different beverages, employing the Waters NanoAcquityTM UPLC with a Michrom Advance CaptiveSprayTM source and a triple stage quadrupole mass spectrometer.

EXPERIMENTAL SECTION

Caution

MeIQx, PhIP, and several of their derivatives are potential carcinogens and they should be handled in a well-ventilated fume hood with the appropriate protective clothing.

Chemicals

MeIQx, PhIP, and 2-amino-3-trideutromethyl-8-methylimidazo[4,5-*f*]quinoxaline ($[\text{}^2\text{H}_3\text{C}]$ -MeIQx) and 2-amino-1-trideutromethyl-6-phenylimidazo[4,5-*b*]pyridine ($[\text{}^2\text{H}_3\text{C}]$ -PhIP)

(both at 99% isotopic purity) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). 2-Amino-1,7-dimethylimidazo[4,5-*g*]quinoxaline (MeIQx) was synthesized as previously reported.²² The following metabolites and their trideuterated internal standards were prepared by chemical means²³ or biosynthetically as previously reported:²¹ 2-Amino-3-methylimidazo[4,5-*f*]quinoxaline-8-carboxylic acid (IQx-8-COOH); 2-amino-8-(hydroxymethyl)-3-methylimidazo[4,5-*f*]quinoxaline (8-CH₂OH-IQx); *N*²-(β-1-glucosiduronyl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx-*N*²-GI); *N*²-(β-1-glucosiduronyl)-2-(hydroxyamino)-3,8-dimethylimidazo[4,5-*f*]quinoxaline (HON-MeIQx-*N*²-GI), *N*²-(3,8-dimethylimidazo[4,5-*f*]quinoxalin-2-yl)-sulfamic acid (MeIQx-*N*²-SO₃H); *N*²-(β-1-glucosiduronyl)-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HON-PhIP-*N*²-GI); *N*3-(β-1-glucosiduronyl)-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HON-PhIP-*N*3-GI); *N*²-(β-1-glucosiduronyl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP-*N*²-GI); and *N*3-(β-1-glucosiduronyl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP-*N*3-GI). All solvents used were high-purity B & J Brand® from Honeywell Burdick and Jackson (Muskegon, MI). ACS reagent grade HCO₂H (88%) was purchased from J.T. Baker (Phillipsburg, NJ). Retain CX resins (30 mg) were purchased from ThermoFisher Scientific (Palm Beach, FL) and Baker C18 solid-phase extraction (SPE) resins (500 mg) were purchased through Krackeler Scientific Inc. (Albany, NY). All other chemical reagents were ACS grade, and purchased from Sigma Aldrich.

Human Subjects and Meat Consumption

Urine samples were obtained from volunteers who agreed to participate in a dietary intervention study. The study was approved by the Institutional Review Board at the University of Arkansas Medical Sciences and the Wadsworth Center. The volunteers consumed cooked chopped beef patties and either a fruit juice, vegetable juice or beverage. Subjects began at 8:00 a.m., continuing at 4-hour intervals until/including midnight, one of following dietary interventions: a) orange juice (8 fl. oz./serving); b) apiaceous vegetable juice (4 fl. oz./serving); c) muscadine grape juice (5 fl. oz./serving); d) Fresca (8 fl. oz./serving); e) black tea (5 fl. oz./serving; 2% hot effusion); and f) non-alcoholic beer (12 fl. oz./serving). An exception was made in the non-alcoholic beer regimen in which the non-alcoholic beer was given the day before Day 1 over four 4-hour doses beginning at 8:00 a.m., as the prenylflavonoids require metabolism by intestinal microflora in order to become bioactive.²⁴ At noon on Day 1, subjects consumed of a grilled beef mixture on a lettuce salad with Ranch dressing. The beef was prepared in a large batch on a high-temperature grill (275 °C) and contained about 7.5–12 ng/g of PhIP and 5–7 ng/g of MeIQx.²⁵ The volunteers consumed 200 g of this grilled beef at noon. Urine was collected in two lots from noon-midnight of Day 1 (0–12- hour); and morning-noon of Day 2 (12–24 hour). The urine samples were collected on ice and then stored at –80 °C prior to chemical analysis. This study design was based upon the known rapid absorption of PhIP and MeIQx from the diet, the excretion of the these HAAs and their urinary metabolites occurs during the 6–12 hour interval.^{16,17,26,27} The urine samples were collected on ice and then stored at –80 °C prior to chemical analysis.

Solid-phase Extraction (SPE) of MeIQx, PhIP and their Metabolites from Urine

Urine samples (1.0 mL) were spiked with isotopically labeled internal standards 100 pg of [²H₃C]-MeIQx and [²H₃C]-PhIP; 300 pg of [²H₃C]-8-CH₂OH-IQx and IQx-8-COOH; and 1000 pg of each [²H₃C]-PhIP-*N*²-GI, [²H₃C]-PhIP-*N*3-GI, [²H₃C]-HON-PhIP-*N*²-GI, and [²H₃C]-HON-PhIP-*N*3-GI. The samples were acidified with HCO₂H (88% v/v, 20 μL) and then placed on ice for 30 min, before being centrifuged at 15,000 g for 2 min to remove particulates. The supernatants were applied to ThermoFisher HyperSep Retain CX (30 mg resin) cartridges that had been prewashed with CH₃OH containing 5% NH₄OH (1 mL),

followed by 2% HCO₂H in H₂O (1 mL). The resins were attached to a vacuum manifold, under slight pressure (~5 inches of Hg), to achieve a flow rate of the eluent of approximately 1 mL/min. After application of the samples, the cartridges were washed with 2% HCO₂H in H₂O (1 mL), followed by 2% HCO₂H in CH₃OH (1 mL), H₂O (1 mL) and 5% NH₄OH (1 mL). The resin was allowed to run to dryness. Then, the analytes were eluted from the resin with CH₃OH containing 1% NH₄OH (1.5 mL). The extract was collected into Eppendorf tubes (2.0 mL), and placed in a ventilated hood for 5 min to allow the NH₃ to evaporate, and then concentrated to approximately 0.1 mL, by vacuum centrifugation. Then, the samples were transferred into silylated glass conical μ Liter vials and evaporated to dryness by vacuum centrifugation. The samples were resuspended in H₂O (40 μ L).

LC-ESI/MS/MS Analyse

Chromatography was performed with a NanoAcquity™ UPLC system (Waters Corporation, Milford, MA) equipped with a Michrom C18 AQ column (0.3 $\times$ 150 mm, 3 μ m particle size, Michrom Bioresources Inc., Auburn, CA). Analytes were separated by a gradient. The A solvent was 0.01% HCO₂H in H₂O, and the B solvent contained 0.01% HCO₂H and 5% H₂O in CH₃CN. The flow rate was set at 6 μ L/min, starting at 100% A increased by a linear gradient to 65% B in 13 min, and then to 100% B at 14 min holding for 1 min. The gradient was reversed to the 100% A over 1 min and at the same time the flow rate was increased from 6 μ L/min to 10 μ L/min. A post-run time of 3 min was required for re-equilibration. The back pressure of the column was 3,700 psi at the initial solvent conditions. The sample injection volume was 0.4 μ L. The manipulation of UPLC system was done by MassLynx software (Waters Corp., Milford, MA). Alternatively, chromatography was performed with an Agilent 1100 series capillary LC system (Agilent Technologies, Palo Alto, CA) equipped with an Agilent Zorbax-SB-C18 column (0.3 $\times$ 250 mm; 5 μ m particle size). The solvents were the same as those described above. The flow rate was set at 6 μ L/min, starting at 100% A and holding for 1 min, followed by a linear gradient to 60% B at 35 min, and then to 100% B at 36 min, and holding for 4 min. The gradient was reversed to the starting conditions over 1 min, and a post-run time of 16 min was required for re-equilibration.

The mass-spectral data were acquired on a Finnigan™ Quantum Ultra Triple Stage Quadrupole MS (Thermo Fisher, San Jose, CA) and processed with Xcalibur version 2.07 software. Analyses were conducted in the positive ionization mode and employed an Advance CaptiveSpray™ source from Michrom Bioresources Inc. (Auburn, CA). The spray voltage was set at 1400 V; the in-source fragmentation was -5 V; and the capillary temperature was 200 °C. There was no sheath or auxiliary gas. The peak widths (in Q1 and Q3) were set at 0.7 Da. The measurement of analytes was done by selected reaction monitoring (SRM). The following transitions and collision energies were used for the quantification of MeIQx, PhIP, and metabolites: MeIQx and [²H₃C]-MeIQx: 214.1 $\rightarrow$ 199.1 and 217.1 $\rightarrow$ 199.1, at 30 eV; 8-CH₂OH-IQx and [²H₃C]-8-CH₂OH-IQx: 230.1 $\rightarrow$ 197.1 and 233.1 $\rightarrow$ 197.1, at 35 eV; IQx-8-COOH and [²H₃C]-IQx-8-COOH: 244.1 $\rightarrow$ 183.1 and 247.1 $\rightarrow$ 183.1, at 38 eV; PhIP and [²H₃C]-PhIP: 225.1 $\rightarrow$ 210.1 and 228.1 $\rightarrow$ 210.1 at 33 eV; PhIP-*N*²-Gl, PhIP-*N*³-Gl, and [²H₃C]-PhIP-*N*²-Gl and [²H₃C]-PhIP-*N*³-Gl: 401.1 $\rightarrow$ 210.1 and 404.1 $\rightarrow$ 210.1 at 55 eV; HON-PhIP-*N*²-Gl, and [²H₃C]-HON-PhIP-*N*²-Gl: 417.1 $\rightarrow$ 225.1 and 223.1 and 420.1 $\rightarrow$ 228.1 and 225.1 @ 34 eV; HON-PhIP-*N*³-Gl and [²H₃C]-HON-PhIP-*N*³-Gl: 417.1 $\rightarrow$ 225.1 and 224.1 and 420.1 $\rightarrow$ 228.1 and 227.1 @ 34 eV. The dwell time for each transition was 10 ms. Argon was used as the collision gas and was set at 1.5 mTorr. Product ion spectra were acquired on the protonated molecules [M + H]⁺, scanning from *m/z* 50 to 250 or 500 at a scan speed of 500 amu/s using the same acquisition parameters as above.

Method validation

Calibration curves were produced in triplicate by the addition of a fixed amount of [$^2\text{H}_3\text{C}$]-PhIP and [$^2\text{H}_3\text{C}$]-MeIQx (100 pg) and 0, 6, 12, 16, 20, 40, 60 or 100 pg of the unlabeled standards per 1.0 mL urine from a volunteer who had not consumed cooked meat for at least 48 h. [$^2\text{H}_3\text{C}$]-8-CH₂OH-IQx and [$^2\text{H}_3\text{C}$]-IQx-8-COOH were added at a level of 300 pg/mL of urine. The unlabeled 8-CH₂OH-IQx standard was added at 0, 6, 12, 16, 20, 40, 60 or 100 pg, whereas the unlabeled IQx-8-COOH standard was added at 0, 30, 60, 80, 100, 200, 300 or 500 pg/mL of urine. The calibration curves of PhIP metabolites were constructed with [$^2\text{H}_3\text{C}$]-PhIP-*N*²-GI, [$^2\text{H}_3\text{C}$]-PhIP-*N*³-GI, [$^2\text{H}_3\text{C}$]-HON-PhIP-*N*²-GI, and [$^2\text{H}_3\text{C}$]-HON-PhIP-*N*³-GI added at a fixed concentration of 1000 pg/mL of urine, and each unlabeled analyte was added at concentrations of 0, 60, 120, 160, 200, 400, 600 or 1000 pg/mL of urine. The urine samples were processed by SPE (*vide supra*). The calibration curves for each urine sample were run twice on two different days, to have six data points per calibrant level. The calibration data were fitted to a straight line using the ordinary least-squares method with equal weightings.

The performance of the analytical method was conducted on urine samples from three different subjects collected over 12 hrs, following consumption of cooked meat. The subjects drank orange juice as the beverage. Urine (1 mL) underwent the SPE processing conditions described above. The within- and between-day precisions for MeIQx, PhIP and their metabolites were calculated in quadruplicate as described,²⁸ with urine samples. The sample preparation and data analyses were done on four different days over a time period of 1 month.

RESULTS AND DISCUSSION

UPLC-ESI/MS/MS Analysis of of MeIQx, 8-CH₂OH-IQx, IQx-8-COOH, PhIP, PhIP-*N*²-GI, PhIP-*N*³-GI, HON-PhIP-*N*²-GI, and HON-PhIP-*N*³-GI in the Urine of Omnivores

The chemical structures of PhIP, MeIQx, and their metabolites are shown in Figure 1. A mixed-mode reverse phase cation exchange resin enrichment procedure, developed in our lab,²¹ is a rapid and high throughput method for the simultaneous isolation of PhIP, MeIQx, and six of their metabolites from urine. The metabolism of both HAAs was extensive: Less than 9% of the dose was eliminated in urine as unaltered MeIQx and <1% was eliminated as unaltered PhIP.²¹ We estimated that 60 to 85% of the ingested dose of MeIQx was eliminated in urine of subjects, as a combination of unaltered MeIQx and cytochrome P450 1A2 derived metabolites, 8-CH₂OH-IQx and IQx-8-COOH (Figure 1), within 12 h of consumption of cooked beef. The rate of elimination of PhIP and its *N*-glucuronidated metabolites was slower than that for MeIQx and its metabolites, and up to 35% of the ingested dose of PhIP was eliminated within 12 h of consumption of cooked beef;²¹ PhIP and its *N*-glucuronidated metabolites account for 60–82% of the ingested dose that was eliminated in urine within 24 h.²⁹

The capillary HPLC-ESI/MS/MS analytical method was sensitive. The LOQ values of the unaltered HAAs and metabolites, were 50 parts per trillion (ppt) or less, when 100 μL equivalent of urine was assayed on column.²¹ However, the duty cycle time was protracted at 57 min: this duty cycle included a 41 min gradient for analyte separation, two min column washing cycle, and a 14 min equilibration time (Supporting Information, Figure S-1). We sought to determine if the duty cycle time could be significantly shortened by UPLC without sacrificing resolution, diminished sensitivity, or reproducibility in the quantification of the urinary analytes. The duty cycle time of the UPLC-ESI/MS/MS assay could be decreased to 19 min; the duty cycle includes a 13 min gradient for separation and analysis of the analytes, a 2 min column wash at 100% B, followed by a 4 min equilibration time.

The UPLC-ESI/MS/MS chromatograms of MeIQx, PhIP and their metabolites in urine of a subject, collected before and after consumption of meat, are depicted in Figure 2. The subject drank black tea with the meal. The SRM traces of parent compounds, all of the analytes and the internal standards in the urine extract are well resolved from isobaric interferences. A steeper gradient could not be employed to further reduce the time of analysis, because of an isobaric interferent detected at t_R 7.9 min monitored with the same transition as MeIQx (214.1 $\rightarrow$ 199.1, t_R = 8.0 min). This compound was identified as 2-amino-3,7-dimethylimidazo[4,5-g]quinoxaline (7-MeIQx, t_R = 7.9 min), a recently discovered linear tricyclic ring isomer of MeIQx²² (Supporting Information, Figure S-2). The base-line resolution of these isomers is lost, when a steeper gradient is employed. The isomeric *N*²-glucuronide and *N*³-glucuronide conjugates of PhIP also coalesce (Figure 3), when a steeper gradient is employed for chromatography.

We compared several columns designed for UPLC (1.7 μ m particle size) from different vendors; however, the Michrom C18 MAGIC AQ column (0.3 $\times$ 150 mm; 3 μ m particle size) provided the highest level of resolution for all of the analytes. The peak widths of the analytes were about 6–10 s, when separated by UPLC, as opposed to peak widths of ~45 s, when assayed by capillary HPLC (Supporting Information, Figure S-1). The superior chromatography and narrower peak shape obtained by the UPLC system resulted in an improved signal over the background noise and enhanced the sensitivity of analyte detection. With the UPLC-ESI/MS/MS method, only 10 μ L equivalent of urine was injected on the column. More than 700 urine samples have been analyzed by UPLC without a decline in performance of the column, demonstrating that our SPE clean-up procedure is highly effective in column preservation.

Product Ion Spectra of PhIP, MeIQx and their Metabolites

The high level of sensitivity provided by the UPLC system interfaced with the Michrom Advance CaptiveSprayTM source and the MS allowed us to acquire product ion spectra of PhIP, MeIQx and their metabolites with only 12 μ L equivalent of urine. Several of the product ion spectra are shown in Figure 3. The proposed mechanisms of fragmentation of these compounds have been previously described.^{20,21,30} Remarkably, high quality product ion scan spectra were acquired on less than 1 pg (< 5 fmol equivalent of HAA) for PhIP, MeIQx (not shown), and several of the metabolites. The product ion spectra data confirm the identities of the compounds and prove that the peaks attributed to these urinary analytes are rather pure.

The original validation of the analytical method to measure PhIP, MeIQx, and their metabolites, by capillary HPLC-ESI/MS/MS,^{20,21} was conducted with urine samples from subjects who ate meat cooked well-done and who restricted their consumption of fluids to water. However, individuals on a free-choice diet may drink beverages, such as colas, juices, wine, beer, tea, or coffee with their meal, instead of water. Many of these beverages could contain isobaric interferences of the HAAs or their metabolites and adversely affect the usefulness of the HPLC-ESI/MS/MS method. Representative chromatograms of a subject who drank either black tea (Figure 2), or Fresca, grape juice or vegetable juice with cooked meat reveal that the unaltered compounds and their metabolites are well resolved from isobaric interferences, when assayed by UPLC-ESI/MS/MS (Supporting Information, Figure S-3–S-5).

Performance of the Analytical Method

The performance of the method was assessed by the within-day and between-day estimates and the precision of measurements of PhIP, PhIP-*N*²-GI, PhIP-*N*³-GI, HON-PhIP-*N*²-GI, HON-PhIP-*N*³-GI, MeIQx, 8-CH₂OH-IQx and IQx-8-COOH, in urine samples from three

subjects assayed in quadruplicate over 4 different days. The subjects ate 250 grams of well-done cooked beef and drank orange juice as their beverage with the meal. These independent measurements were conducted during a time period of 1 month. The recoveries of each internal standard [$^2\text{H}_3\text{C}$]-MeIQx and [$^2\text{H}_3\text{C}$]-PhIP (100 pg/mL); [$^2\text{H}_3\text{C}$]-8-CH₂OH-IQx and [$^2\text{H}_3\text{C}$]-IQx-8-COOH (each at 300 pg/mL); PhIP-*N*²-G1, PhIP-*N*³-G1, HON-PhIP-*N*²-G1 and HON-PhIP-*N*³-G1 (each at 1000 pg/mL), added to urine prior to sample processing, were consistently between 40 and 80%, based on the response of the signals to those of pure standards measured by UPLC-ESI/MS/MS. The response of the signals of the processed internal standards is a function of the recoveries of the compounds and the potential ion suppression effects of the urine matrix.¹¹

The calibration curves for MeIQx, PhIP and their metabolites, constructed from urine samples from a subject who had refrained from eating cooked meat for 48 h, displayed good linearity ($R^2 > 0.997$) (Supporting Information, Figure S-6). The LOQ values were derived based on a threshold of 10 σ SD units above the background signal levels,³¹ in the urine samples from three volunteers, during the pre-exposure phase of the study. The background signals are attributed to endogenous levels of the target metabolites, isobaric interferences, or chemical noise that serve as the basis for the establishment of the limit of detection (LOD) and LOQ levels. The LOQ values were <5 pg/mL for MeIQx and PhIP; the LOQ value for 8-CH₂OH-IQx was 7 pg/mL; the LOQ value for IQx-8-COOH was 20 pg/mL; and the LOQ values for the glucuronide conjugates of PhIP and HON-PhIP ranged between 20 to 40 pg/mL.

The results of the within-day and between-day estimates and the precisions [CV(%)] of measurements by UPLC-ESI/MS/MS are summarized in Table 1 and Table 2. MeIQx, 8-CH₂OH-IQx and IQx-8-COOH, were present at concentrations above the LOQ values for all three subjects; the [CV (%)] of the measurements ranged from 3.7 to 10.2%. PhIP was above the LOQ value in only one subject, and the concentration was measured at 9.7 pg/mL. The within-day and between-day precisions [CV(%)] were <10.1%. The urinary concentrations of PhIP were above the LOD in the other two subjects, and the within-day and between-day precisions [CV(%)] were <18.5%. The isomeric PhIP-*N*²-G1 and PhIP-*N*³-G1 conjugates were also present as minor components in urine. PhIP-*N*²-G1 occurred at levels above the LOQ value in only one subject and the within-day and between-day precision [CV(%)] of the measurements were <8.5%. For the other two subjects, the concentrations of PhIP-*N*²-G1 and PhIP-*N*³-G1, were above the LOD value: the within-day and between-day precisions [CV(%)] were $\leq 18.9\%$. The HON-PhIP-*N*²-G1, HON-PhIP-*N*³-G1 concentrations were above the LOQ values for all three subjects: the within-day and between-day precision [CV(%)] of the measurements of HON-PhIP-*N*²-G1, HON-PhIP-*N*³-G1 ranged between 3.7 and 7.7 %.

CONCLUSIONS

To our knowledge, no report in the literature has described the simultaneous analysis of MeIQx and PhIP and their principal metabolites in human urine, other than our recent study.²¹ Previous studies either investigated the individual unaltered HAAs or focused on measuring one or two metabolites. The concurrent analysis of both MeIQx, PhIP and their respective urinary metabolites is important since the urinary excretion levels of either MeIQx or PhIP can only serve as an approximate measure for one another, in assessment of exposures in humans consuming unrestricted diets.³² Our facile mix-mode reverse phase cation exchange resin enrichment procedure can be employed to isolate MeIQx, PhIP, and their principal phase I and phase II metabolites in urine; its use is followed by quantitative measurements by LC-ESI/MS/MS.²¹ The simultaneous isolation and separation of these analytes from thousands of other constituents in urine⁸ is challenging, and prolonged

gradients and run times are required by capillary HPLC.²¹ The use of UPLC has greatly decreased the duty cycle over the capillary HPLC method.²¹ Several of the oxidized metabolites of MeIQx and PhIP are produced by cytochrome P450 1A2, an enzyme implicated in the metabolic activation of these procarcinogens (Figure 1).³³ Our immediate goal is to determine if naturally occurring inhibitors of cytochrome P450 1A2 that are present in vegetables or beverages^{34,35} can modulate the metabolism and genotoxic potential of MeIQx and PhIP, by measuring the changes in the urinary metabolic profiles of these carcinogens with this UPLC-ESI/MS/MS method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The project was supported by grant R01CA-122320 from the National Cancer Institute (D.G. and RJT), and by grant number 2007/58 funded by the World Cancer Research Fund International (D.G., R.J.T., M.M.R., and F.F.K.).

ABBREVIATIONS

MeIQx	2-amino-3,8-dimethylimidazo[4,5- <i>f</i>]quinoxaline
MeIgQx	2-amino-1,7-dimethylimidazo[4,5- <i>g</i>]quinoxaline
HONH-MeIQx	<i>N</i> -hydroxy-2-amino-3,8-dimethylimidazo[4,5- <i>f</i>]quinoxaline
8-CH₂OH-IQx	2-amino-8-(hydroxymethyl)-3-methylimidazo[4,5- <i>f</i>]quinoxaline
IQx-8-COOH	2-amino-3-methylimidazo[4,5- <i>f</i>]quinoxaline-8-carboxylic acid
MeIQx-<i>N</i>²-GI	<i>N</i> ² -(β-1-glucosiduronyl)-2-amino-3,8-dimethylimidazo[4,5- <i>f</i>]quinoxaline
HON-MeIQx-<i>N</i>²-GI	<i>N</i> ² -(β-1-glucosiduronyl)-2-(hydroxyamino)-3,8-dimethylimidazo[4,5- <i>f</i>]quinoxaline
MeIQx-<i>N</i>²-SO₃H	<i>N</i> ² -(3,8-dimethylimidazo[4,5- <i>f</i>]quinoxalin-2-yl)sulfamic acid
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
HONH-PhIP	<i>N</i> -hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
4'-HO-PhIP	2-amino-4'-hydroxy-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
HON-PhIP-<i>N</i>²-GI	<i>N</i> ² -(β-1-glucosiduronyl)-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
HON-PhIP-<i>N</i>3-GI	<i>N</i> 3-(β-1-glucosiduronyl)-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5- <i>b</i>]
pyridine PhIP-<i>N</i>²-GI	<i>N</i> ² -(β-1-glucosiduronyl)-2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
PhIP-<i>N</i>3-GI	<i>N</i> 3-(β-1-glucosiduronyl)-2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
HAAs	heterocyclic aromatic amines
LC-ESI/MS/MS	liquid chromatography-electrospray ionization/mass spectrometry/tandem mass spectrometry

LOD	limit of detection
LOQ	limit of quantification
ppb	parts per billion
GC-NICI-MS	negative ion chemical ionization mass spectrometry
SPE	solid phase extraction
SRM	selected reaction monitoring
UGTs	uridine diphosphate glucuronosyltransferases
UPLC	ultra performance liquid chromatography

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Figure 1.

Chemical structures of MeIQx, PhIP, and their metabolites. The HONH-MeIQx and HONH-PhIP are unstable and not assayed. The MeIQx- N^2 SO₃H, MeIQx- N^2 -Gl and HON-MeIQx- N^2 -Gl are not recovered from urine, by this SPE method.

Figure 2.

SRM traces for MeIQx, PhIP, and their metabolites in a urine sample collected before and after meat consumption. The retention time (t_R), area, and ion intensity are reported. The large peak eluting at t_R 7.9 min and monitored with the transition 214.1 > 199.1 is 7-MeIQx, an isomer of MeIQx, which elutes at t_R 8.0 min. The t_R of PhIP- N^2 -Gl and PhIP- N^3 -Gl are 9.6 and 10.0 min, respectively, and the t_R of HON-PhIP- N^2 -Gl and HON-PhIP- N^3 -Gl are 10.6 and 12.4 min, respectively. The subject drank black tea with the cooked beef.

Figure 3.

Product ion spectra of selected metabolites of MeIQx and PhIP (the amount of urinary analyte used for acquiring product ion spectra are reported). Product ion spectra of the urinary metabolites (A) 8-CH₂OH-IQx (0.5 pg) and synthetic standard, (B) IQx-8-COOH (2.8 pg) and synthetic standard, (C) PhIP-N²-GI (0.6 pg) and synthetic standard, and (D) HON-PhIP-N²-GI (4.9 pg) and synthetic standard.

Table 1

The intraday and interday measurements of MeIQx, 8-CH₂OH-IQx, IQx-8-COOH

Subject	Metabolite	Amount (pg/mL)							CV(%) within-day	CV(%) between-day
		Day 1	Day 2	Day 3	Day 4	Overall				
1	IQx-8-COOH	Mean	169	164	174	142	162			
		SD	5.3	4.7	5.3	7.8	13.5			
		RSD(%)	3.1	2.9	3.0	5.5	8.3	3.6		9.0
	8-CH ₂ OH-IQx	Mean	82	88	85	74	82			
		SD	5.9	5.8	8.3	7.9	8.3			
		RSD(%)	7.1	6.6	9.8	10.7	10.1	8.4		10.2
	MeIQx	Mean	32	37	36	36	35			
		SD	3.0	3.7	1.3	4.2	3.5			
		RSD(%)	9.3	10.0	3.7	11.5	9.8	9.1		9.9
2	IQx-8-COOH	Mean	162	176	151	161	163			
		SD	7.0	5.7	5.2	5.8	10.8			
		RSD(%)	4.3	3.2	3.4	3.6	6.6	3.7		7.3
	8-CH ₂ OH-IQx	Mean	27	27	28	25	27			
		SD	2.2	2.4	3.0	1.8	2.5			
		RSD(%)	8.0	8.8	10.6	7.2	9.2	9.0		9.4
	MeIQx	Mean	17	16	16	15	16			
		SD	0.9	1.4	1.2	1.1	1.3			
		RSD(%)	5.5	8.6	7.7	7.5	7.8	7.4		8.0
3	IQx-8-COOH	Mean	117	112	129	111	117			
		SD	7.7	6.6	4.0	6.1	9.3			
		RSD(%)	6.6	5.9	3.1	5.5	8.0	5.1		8.4
	8-CH ₂ OH-IQx	Mean	34	30	34	34	33			
		SD	2.3	1.4	2.7	0.8	2.4			
		RSD(%)	6.7	4.6	8.1	2.3	7.3	4.2		6.7
	MeIQx	Mean	17	16	17	16	16			
		SD	1.1	0.4	1.6	1.4	1.1			

Subject	Metabolite	Amount (pg/mL)					Overall	CV(%) within-day	CV(%) between-day
		Day 1	Day 2	Day 3	Day 4	Day 5			
		6.4	2.3	9.4	8.9	6.9	6.9	5.6	5.5

n = 4 replicates per day

Table 2

The intraday and interday measurements of PhIP, and *N*-glucuronide conjugates of PhIP and HONH-PhIP.

Subject	Metabolite	Amount (pg/mL)							CV(%) within-day	CV(%) between-day
		Day 1	Day 2	Day 3	Day 4	Overall				
1	HON-PhIP- <i>N</i> ² -Gl	Mean	698	764	691	710	716			
		SD	28.3	16.6	11.2	21.1	34.6			
		RSD(%)	4.1	2.2	1.6	3	4.8	2.9	5.3	
	HON-PhIP- <i>N</i> ³ -Gl	Mean	133	148	141	137	140			
		SD	7.5	5.2	5.3	5.8	7.7			
		RSD(%)	5.6	3.5	3.8	4.2	5.5	4.3	5.7	
	PhIP- <i>N</i> ² -Gl	Mean	77	90	86	91	86			
		SD	2.0	3.1	4.2	5.6	6.9			
		RSD(%)	2.6	3.4	4.9	6.1	8.0	4.5	8.5	
	PhIP- <i>N</i> ³ -Gl	Mean	14	17	15	19	16			
		SD	0.5	1.4	1.8	0.5	2.2			
		RSD(%)	3.4	8.4	12.0	2.8	13.1	7.4	14.4	
	PhIP	Mean	9.4	9.5	10.4	9.4	9.7			
		SD	0.6	1.2	1.0	0.8	0.9			
		RSD(%)	6.3	12.5	9.7	8.7	9.7	9.9	10.1	
2	HON-PhIP- <i>N</i> ² -Gl	Mean	700	723	765	767	739			
		SD	16.4	18.2	22.6	22.5	34			
		RSD(%)	2.3	2.5	3.0	2.9	4.7	2.7	5.1	
	HON-PhIP- <i>N</i> ³ -Gl	Mean	43	41	41	40	41			
		SD	3.7	4.4	3.2	1.6	3.2			
		RSD(%)	8.5	10.7	7.8	3.9	7.8	8.4	7.9	
	PhIP- <i>N</i> ² -Gl	Mean	22	32	27	30	28			
		SD	2.7	1.8	1.2	4.1	4.8			
		RSD(%)	12.4	5.4	4.5	13.8	17.2	9.8	18.9	
	PhIP- <i>N</i> ³ -Gl	Mean	10	11	11	9	10			
		SD	0.5	2.0	0.9	0.7	1.5			
		RSD(%)	5.2	17.7	8.0	7.8	14.2	12.1	15.5	

Subject	Metabolite	Amount (pg/mL)										CV(%) within-day	CV(%) between-day
		Day 1	Day 2	Day 3	Day 4	Overall	Day 1	Day 2	Day 3	Day 4	Overall		
3	PhIP	Mean	3	3	4	3	3	3	3	3	3		
		SD	0.4	0.3	0.7	0.4	0.4	0.4	0.4	0.4	0.4		
		RSD(%)	11.1	8.8	18.8	12.0	12.2	13.2	11.7				
	HON-PhIP-N ² -GI	Mean	494	522	495	554	516						
		SD	18.5	26.0	17.8	21.6	31.7						
		RSD(%)	3.7	5.0	3.6	3.9	6.1	3.7	6.4				
	HON-PhIP-N ³ -GI	Mean	56	53	58	53	55						
		SD	1.7	3.0	5.3	2.6	3.8						
		RSD(%)	3.1	5.6	9.1	5.0	6.8	4.0	5.6				
	PhIP-N ² -GI	Mean	19	15	15	15	16						
		SD	1.9	2.8	2.3	1.5	2.7						
		RSD(%)	9.7	18.6	14.9	9.9	16.6	11.3	16.1				
	PhIP-N ³ -GI	Mean	8	8	7	9	8						
		SD	1.0	0.9	1.2	0.8	13.9						
		RSD(%)	12.3	11.3	17.9	9.1	15.6	9.9	14.9				
	PhIP	Mean	3	4	4	3	4						
		SD	0.5	0.5	0.6	0.2	0.6						
		RSD(%)	15.8	15.1	13.2	6.9	17.9	11.4	18.5				

n = 4 replicates per day