Biomonitoring of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and Its Carcinogenic Metabolites in Urine

Jean-Marie Fede[1], **Anup P. Thakur**[1], **Nigel J. Gooderham**[2], and **Robert J. Turesky**[1,*]

[1]Division of Environmental Health Sciences, Wadsworth Center, New York State Department of Health, Albany, New York 12201

[2]Imperial College London, Sir Alexander Fleming Building, London SW7 2AZ

Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a carcinogenic heterocyclic aromatic amine that is produced in cooked meats. The simultaneous analysis of PhIP and its metabolites in human urine is a challenge, because these biomarkers only occur in urine at parts-per-billion or lower concentrations, and must be selectively purifed from thousands of other urinary constituents. We have developed a facile solid-phase extraction method, employing a mixed-mode reverse phase cation exchange resin, to simultaneously isolate PhIP, its glucuronide conjugates, and the glucuronide conjugates of the genotoxic metabolite 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine from urine of meat-eaters. PhIP and its metabolites were quantified by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS), using a triple stage quadrupole mass spectrometer in the selected reaction monitoring scan mode (SRM). The lower limit of quantification (LOQ) of PhIP is 5 parts-per-trillion (ppt), and the LOQ values for the glucuronide conjugates are 50 ppt, when 25 μL of urine are employed for assay. The extraction scheme is versatile and has been employed to isolate other ring-hydroxylated and glucuronidated metabolites of PhIP, for characterization by LC-ESI/MS/MS.

INTRODUCTION

Heterocyclic aromatic amines (HAA)[1] are a class of carcinogenic compounds that form in cooked meats (1). PhIP is one of the most mass-abundant HAAs formed in red meats and poultry cooked well-done: the concentrations of PhIP can range from several parts-per-billion (ppb) up to 500 ppb (2,3). Putative DNA adducts of PhIP have been detected in

[1]Abbreviations: PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; 5-HO-PhIP, 2-amino-1-methyl-6-(5-hydroxy)phenylimidazo[4,5-*b*]pyridine; 4′-HO-PhIP, 2-amino-1-methyl-6-(4′-hydroxy)-phenylimidazo[4,5-*b*]pyridine; HONH-PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; HON-PhIP-*N*[2]-G1, *N*[2]-β-D-glucosidurony1-2-(hydroxyamino)-1-methy1-6-phenylimidazo[4,5-*b*]pyridine; PhIP-*N*[2]-G1, *N*[2]-β-D-glucosidurony1-2-amino-1-methy1-6-phenylimidazo[4,5-*b*]pyridine; HON-PhIP-*N*3-G1, *N*3-β-D-glucosidurony1-2-(hydroxyamino)-1-methy1-6-phenylimidazo[4,5-*b*]pyridine; PhIP-*N*3-G1, *N*3-β-D-glucosidurony1-2-amino-1-methy1-6-phenylimidazo[4,5-*b*]pyridine; PhIP-4′-O-Gl; 4′-β-D-glucosiduronyloxy-2-amino-4′-hydroxy1-1-methy1-6-phenylimidazo[4,5-*b*]pyridine; CID, collision induced dissociation; HAA, heterocyclic aromatic amine; LC-ESI/MS/MS, liquid chromatography-electrospray ionization/ tandem mass spectrometry; LOQ, limit of quantification; MS, mass spectrometry; GC-NICIMS, negative ion chemical ionization mass spectrometry; ppb, parts-per-billion; ppt, parts-per-trillion; SPE, solid-phase extraction; SRM, selected reaction monitoring; TSQ, Finnigan TSQ Quantum Ultra triple stage quadrupole mass spectrometer; UDPGA; uridine-5′-diphosphoglucuronic acid; UGTs, diphosphate glucuronosyltransferases.

human tissues, even though the dietary concentrations of PhIP generally are low (4,5). Thus, PhIP is a health hazard and is reasonably anticipated to be a human carcinogen (6).

The metabolism of PhIP has been extensively studied in vitro with tissue fractions, purified and recombinant enzymes (7–10), and hepatocytes (11,12), and in vivo in experimental laboratory animals (11–15), and humans (16–22). Cytochrome P450s (P450s 1A1, 1A2 and 1B1) catalyze the oxidation of the exocyclic amine group of PhIP to form the genotoxic metabolite, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HONH-PhIP) (9,10,23). This metabolite can undergo conjugation by sulfotransferases (SULTs) (24,25) or *N*-acetyltransferases (NATs) (26), to produce highly reactive esters that bind to DNA (27), or undergo solvolysis to produce 2-amino-1-methyl-6-(5-hydroxy)phenylimidazo[4,5-*b*]pyridine (5-HO-PhIP) (Figure 1). These same P450s catalyze oxidation at the 4′ position of PhIP to form 2-amino-1-methyl-6-(4′-hydroxy)-phenylimidazo[4,5-*b*]pyridine (4′-HO-PhIP), a principal detoxication product of PhIP in rodents and non-human primates (11,14). However, human P450s primarily catalyze the formation of HONH-PhIP as the major oxidation product, the formation of 4′-HO-PhIP occurs at considerably lower levels (9,15,23).

PhIP undergoes extensive metabolism, by uridine diphosphate glucuronosyltransferases (UGTs). Both PhIP and HNOH-PhIP undergo conjugation by UGT1A1 isoforms to produce N^2- and $N3$-glucuronide conjugates (28–30). UGT activity toward PhIP and HONH-PhIP has been detected in human liver and colon microsome samples (28,31–34). The glucuronide conjugates of HONH-PhIP have been viewed as detoxication products (21), although bacterial glucuronidases can hydrolyze HON-PhIP-*N3*-Gl to liberate HONH-PhIP for futher metabolism and potential DNA adduct formation (35).

Urine is a useful biological matrix for the measurement of PhIP and its metabolites, since large quantities can be obtained noninvasively. Although measurements of PhIP or its metabolites in urine do not shed light on DNA damage, they can reflect the capacity of an individual to bioactivate and detoxicate this procarcinogen (36). PhIP is rapidly absorbed from the gastrointestinal tract and is eliminated in urine as multiple metabolites within 24 hr of consumption of grilled meats (19,37,38). Approximately 70% of the ingested dose of PhIP has been reported to undergo metabolism by P450 1A2 in humans (38), while often < 1% of the ingested dose is eliminated in urine as the unaltered compound.

Several different analytical approaches have been devised to isolate PhIP from human urine: These techniques include solvent extraction (38,39), solid-phase extraction (SPE) (40), molecularly imprinted polymers (22), or immunoaffinity methods (17), followed by quantification by gas chromatography and negative ion chemical ionization mass spectrometry (GC-NICI-MS) (38,39,41), or LC-ESI/MS/MS (22,40), or followed by fluorescence detection (18). [^{14}C]PhIP and several radiolabeled metabolites have been identified in human urine by accelerator mass spectrometry (AMS) (21,42): urinary metabolites have also been detected by LC-ESI/MS/MS (19,20), or indirectly after chemical reduction or acid hydrolysis of HONH-PhIP conjugates, employing either LC-ESI/MS/MS or GC-NICI-MS (43,44).

To understand the interindividual variation in the activities of xenobiotic metabolism enzymes involved in PhIP metabolism, we require a robust, analytical method for simultaneous quantitation of unaltered PhIP and its principal metabolites in urine. To our knowledge, there is no report in the literature of a validated method to conduct measurements of these biomarkers simultaneously, and details about intra-day and inter-day peformance of the method (accuracy and precision) are lacking. Such an analysis is a challenge, because the concentrations of PhIP and its metabolites occur at or below the ppb

level in urine. Moreover, the polar and ionic nature of the PhIP metabolites presents difficulties for the selective isolation of them with PhIP, from thousands of other components in the urine matrix (45). In this paper, we describe a facile extraction scheme that employs a mixed-mode reverse phase cation exchange solid phase resin, to isolate PhIP and its glucuronide metabolites from urine of meat-eaters (21,42). Quantification of these biomarkers is achieved by LC-ESI/MS/MS, in the selected reaction monitoring (SRM) scan mode, using the stable isotope dilution method. Moreover, the sensitivity of the triple-stage quadrupole mass spectrometer has allowed us to corroborate the structures of these known PhIP metabolites; it has also enabled us to characterize three other unknown glucuronide conjugates of hydroxylated-PhIP metabolites, in the full product ion scan mode.

EXPERIMENTAL PROCEDURES

Caution: PhIP and several of its derivatives are potential human carcinogens and should be handled with caution in a well-ventilated fume hood with the appropriate protective clothing.

Materials and Methods

PhIP and 1-[^{2}H$_3$C]-PhIP (99% isotopic purity) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). NADPH, NADH, glucose-6-phosphate, uridine-5′-diphosphoglucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase, alamethicin, and NH$_4$OH solution (25%) were from Sigma (St. Louis, MO). All solvents used were high-purity B & J Brand® from Honeywell Burdick and Jackson (Muskegon, MI). ACS reagent grade HCO$_2$H (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). Male SD rat liver microsomes of animals pretreated with polychlorinated biphenyls (PCB, Aroclor-1254) were obtained from Moltox (Boone, NC). Human liver microsomal samples were from Tennessee Donor Services, Nashville, TN, and were kindly provided by Dr. F. P. Guengerich, Vanderbilt University. The P450 1A2 protein expression and metabolic activity were previously characterized (9). All other chemical reagents were ACS grade, and purchased from Sigma Aldrich.

Synthesis of HONH-PhIP and 5-HO-PhIP

Briefly, the nitro derivative of PhIP (46) was reduced to HONH-PhIP with hydrazine and Pd/C (46,47), followed by purification of HNOH-PhIP with a Baker C18 SPE resin. The product was eluted with DMSO:C$_2$H$_5$OH (3:1) and stored in liquid nitrogen prior to use. 5-HO-PhIP was prepared by reaction of HONH-PhIP with acetic anhydride in C$_2$H$_5$OH to produce the reactive *N*-acetoxy-PhIP intermediate, which decomposes to produce 5-HO-PhIP as one of several solvolysis products (48). The product was isolated and characterized by UV, ^{1}H NMR, and MS as previously described (32).

Biosynthesis of 4′HO-PhIP, and PhIP-4′-O-Gl, PhIP-*N*^{2}-Glu, PhIP-*N*3-Gl, HON-PhIP-*N*^{2}-Glu and HON-PhIP-*N*3-Glu conjugates

The biosyntheses of PhIP-*N*^{2}-Gl and PhIP-*N*3-Gl were done with human liver microsomal protein (2 mg/mL) in 100 mM Tris HCl buffer (pH 7.5), containing 10 mM MgC1$_2$, PhIP (0.5 mM) and UDPGA (5 mM). The mixture was preincubated with alamethicin (50 μg/mg protein) on ice for 15 min (28). Thereafter, the glucuronidation assay was conducted at 37 °C for 3 h. The reaction was terminated by the addition of 3 vol CH$_3$CN and placed on ice for 30 min, to precipitate proteins. The CH$_3$CN was evaporated under a stream of argon gas. The aqueous solution was diluted with 10 vol of H$_2$O, and metabolites were partially purified by SPE with a Baker C18 resin (500 mg), and then purified by HPLC (32). The

biosynthesis of HON-PhIP-N^2-Glu was done as described above except that HONH-PhIP (0.5 mM) was used instead of PhIP. The HON-PhIP-N3-Glu conjugate was prepared in a similar manner, except that microsomes (2 mg protein/mL) of rats pretreated with PCBs were employed. The corresponding isotopically labeled internal standards were prepared in the same manner, employing 1-[^{2}H$_3$C]-PhIP or 1-[^{2}H$_3$C]-HONH-PhIP. The concentrations of the glucuronide metabolites were determined from their UV absorption spectra at their maxima absorbance in CH$_3$OH (HON-PhIP-N^2-Glu, 318 nm; HON-PhIP-N3-Glu, 320 nm; PhIP-N^2-Gl 305 nm), using the molar extinction coefficient for PhIP (315 nm; ε (M^{-1}cm^{-1}) was determined as 22,220).

For some incubations, NADPH and NADH (1 mM), glucose-6-phopshate (5 mM), and glucose-6-phosphate dehydrogenase (1 unit) were added to the microsomal samples in 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM MgCl$_2$, to catalyze the formation of HONH-PhIP and 4′-HO-PhIP and their glucuronide conjugates *in situ*. After completion of the reaction, the supernatants were either assayed directly by LC-ESI-MS/MS (*vide infra*) or purified by solid phase extraction (SPE), followed by HPLC (32). The products were characterized by UV spectroscopy, LC-ESI/MS/MS, and ^{1}H NMR (32).

Human Subjects and Meat Consumption

The analyses of PhIP metabolites were conducted with urine samples from male volunteers who participated in a previous investigation; and full details were reported previously (20,49). In brief, each subject consumed 275 g of cooked minced beef patties that had been fried without added oil or fat for 6 min on each side, using a hot metal griddle at 300 °C, until the meat was well-browned. The average amount of PhIP ingested was estimated to be 4,900 ng (49). A 10-h urine collection was then commenced, and urine samples were stored at −80 °C. A subset of samples were sent blindcoded on dry-ice to the Wadsworth Center for further analyses. This study was approved by the Institutional Review Board at the Wadsworth Center.

Solid-phase Extraction (SPE) of PhIP and its Metabolites from Urine

Urine samples (0.5 mL) were added to chilled CH$_3$OH/Acetone (1:1) (1.5 mL) in Eppendorf tubes. The samples were placed on ice for 15 min, and the precipitated protein and salts were removed by centrifugation at 15,000*g* for 5 min at 4 °C. The supernatants were transferred into clean Eppendorf tubes, and the organic solvent was removed by vacuum centrifugation at room temperature. The remaining aqueous fraction (~ 0.5 mL) was acidified with HCO$_2$H (10 μL). Then, the urine samples were applied to ThermoFisher HyperSep Retain CX (30 mg resin) cartridges that had been prewashed with CH$_3$OH containing 5% NH$_4$OH (1 mL), followed by 2% HCO$_2$H in H$_2$O (1 mL). The resins were attached to a vacuum manifold, under slight pressure (~5 mm 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% HCO$_2$H in H$_2$O (1 mL), followed by 2% HCO$_2$H in CH$_3$OH (1 mL), H$_2$O (1 mL), 5% NH$_4$OH (2 × 1 mL), and H$_2$O (1 mL). PhIP and its metabolites were eluted from the resin with CH$_3$OH containing 1% NH$_4$OH (1 mL), and were collected in total recovery cap LC vials (Waters, New Milford, MA). The samples were placed in a ventilated hood for 15 min to allow the NH$_3$ to evaporate. Then, the samples were evaporated to dryness by vacuum centrifugation and resuspended in 1:1 CH$_3$OH:H$_2$O (20 μL).

LC-ESI/MS/MS Analyses

Chromatography was performed with an Agilent 1100 series capillary LC system (Agilent Technologies, Palo Alto, CA) equipped with an Agilent Zorbax-XDB-C18 column (0.3 × 250 mm; 5 μm particle size). Analytes were separated by a gradient. The A solvent contained 0.01% HCO$_2$H and 5% CH$_3$CN in H$_2$O, and the B solvent contained 0.01%

HCO_2H and 5% H_2O in CH_3CN. The flow rate was set at 6 μL/min, starting at 100% A and holding for 1 min, followed by a linear gradient to 60% B at 25 min, and then to 100% B at 26 min. The gradient was reversed to the starting conditions over 1 min, and a post-run time of 15 min was required for re-equilibration. The mass-spectral data were acquired on a Finnigan™ Quantum Ultra Triple Stage Quadrupole MS (TSQ/MS) (Thermo Fisher, San Jose, CA), data manipulations were done with Xcalibur version 2.07 software. Analyses were conducted in the positive ionization mode and employed an ADVANCE nanospray source from Michrom Bioresources Inc. (Auburn, CA). The spray voltage was set at 1500 V; the in-source fragmentation was −10 V; and the capillary temperature was 250 °C. There was no sheath or auxiliary gas. The peak and scan widths (in Q1 and Q3) were set at 0.7 Da. The following transitions and collision energies were used for the quantification of PhIP and its metabolites: PhIP and $[^2H_3C]$-PhIP: 225.1→ 210.1 and 228.1→ 210.1 @ 33 eV; PhIP-N^2-Gl and $[^2H_3C]$-PhIP-N^2-Gl: 401.1→ 210.1 and 404.1→ 210.1 @ 55 eV; HON-PhIP-N^2-Gl, HON-PhIP-N3-Gl, and $[^2H_3C]$-HON-PhIP-N^2-Gl and $[^2H_3C]$-HON-PhIP-N3-Gl: 417.1→ 225.1 and 224.1 and 420.1→ 228.1 and 227.1 @ 32 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 100 to 500 at a scan speed of 500 amu/s using the same acquisition parameters.

Calibration Curves

Calibration curves were produced in quadruplicate by the addition of a fixed amount of $[^2H_3C]$-PhIP (50 pg) and 0, 2.5, 5, 10, 15, or 20 pg of PhIP per 0.5 mL urine from a volunteer who had not consumed cooked meat for at least 24 h. The calibration curves of PhIP metabolites were also constructed in quadruplicate with $[^2H_3C]$-PhIP-N^2-Gl, $[^2H_3C]$-HON-PhIP-N^2-Gl, and $[^2H_3C]$-HON-PhIP-N3-Gl added at a fixed concentration of 500 pg; the unlabeled analytes were each added at concentrations of 0, 50, 100, 250, 500 or 1000 pg per 0.5 mL urine. The calibration data were fitted to a straight line using the ordinary least-squares method with equal weightings. Each urine sample was subjected to the organic solvent and SPE processing conditions described above. The within- and between-day precisions for PhIP and its metabolites were calculated in quadruplicate as described (50), with urine samples from three different subjects collected over 10 hr, following consumption of cooked meat (20). The measurements were done on four different days over a time period of 2 months.

RESULTS

LC-ESI/MS Characterization of PhIP, HONH-PhIP, 4′-HO-PhIP, 5-HO-PhIP, and their Glucuronide Metabolites Produced by Rat or Human Liver Microsomes

Rat liver (pretreated with PCBs) and human liver microsomal samples containing high levels of PhIP N-oxidation acitivity (rat liver microsomes: 11.3 nmol HONH-PhIP/min/mg protein; human liver microsomes: 2.1 nmol HONH-PhIP/min/mg protein) were used to produce PhIP metabolites (Figure 2). Interspecies differences in regioselectivity of P450-catalyzed oxidation and UGT-catalyzed glucuronidation of PhIP were observed. Both rat and human microsomal preparations produced large quantities of HONH-PhIP. The rat liver microsomes also produced large amounts of 4′-HO-PhIP and its glucuronide conjugate 4′-β-glucosiduronyloxy-2-amino-1-methyl-6-(4′-hydroxy)-phenylimidazo[4,5-*b*]pyridine (PhIP-4′-O-Gl); however, these PhIP metabolites were produced in low quantities by human liver microsomes. The levels of 5-HO-PhIP and its glucuronide conjugates, if these were formed in either type of microsomal sample, were negligible. The rat liver microsomes catalyzed low levels of glucuronidation of PhIP at both the endocyclic N3 and exocyclic N^2 atoms of PhIP. The N3 atom of HONH-PhIP was the predominant position of glucuronidation by rat liver microsomes, whereas the exocyclic N^2 atom was the principal

site of glucuronidation of PhIP and HONH-PhIP by human liver microsomes. These interspecies differences in regioselectivity of PhIP metabolism have previously been observed for liver microsomes, purified and recombinant P450s, recombinant UGTs, and human and rodent hepatocytes (9,23,28–33).

The ESI product ion spectra of the protonated molecules PhIP and its metabolites are shown in Figure 3A, B. The principal fragment ion of PhIP occurs at m/z 210.0 $[M + H - 15]^{+\bullet}$ and is attributed to cleavage of the $N1$-CH_3 bond. The HONH-PhIP ($[M + H]^+$ at m/z 241.1) undergoes fragmentation to form ions at m/z 224.2 $[M + H - 17]^{+\bullet}$ and m/z 223.0 $[M + H - 18]^+$, respectively due to the loss of $OH^\bullet$ and of H_2O The $[M + H - 17]^{+\bullet}$ is a characteristic fragment ion for N-hydroxy-HAAs, but not for ring-hydroxylated HAAs, under these LC-ESI/MS/MS conditions (32,51). The product ion spectrum of 1-$[^2H_3C]$-HONH-PhIP ($[M + H]^+$ at m/z 244.1) displays prominent ions at m/z 227.2 $[M + H - 17]^{+\bullet}$ and 225.1 $[M + H - 19]^{+\bullet}$, due to losses of $OH^\bullet$ and HOD. However, the ion at m/z 226.1 $[M + H - 18]^+$, due to the loss of H_2O, is minor. Therefore, the principal mechanism of dehydration of HONH-PhIP occurs by homolytic cleavages at a deuterium atom bonded to the C1 atom and at the HO-N^2 bond of 1-$[^2H_3C]$-HONH-PhIP, to produce HOD: The loss of 18 (H_2O), by solvolysis of HONH-PhIP, is a minor pathway (Scheme 1). The isomeric 4′-HO-PhIP metabolite ($[M + H]^+$ at m/z 241.1) undergoes collision-induced dissociation (CID) to form product ions at m/z 226.1 $[M + H]^+$ and m/z 213.0 $[M + H - 28]^+$, due to losses of $CH_3^\bullet$ and CO. The isomeric, synthetic 5-HO-PhIP undergoes fragmentation to produce prominent product ions at m/z 225.9, 223.1, 208.1, and 195.9, respectively, attributed to losses of $CH_3^\bullet$, H_2O, $CH_3^\bullet$ and H_2O, and H_2O and HCN.

Both PhIP-N^2-Gl and PhIP-$N3$-Gl conjugates ($[M + H]+$ at m/z 401.1) undergo CID to form ions at m/z 225.1 $[M+H–176]^+$, due to the loss of the glucuronic acid moiety; a secondary fragmentation of the $N1$-CH_3 bond of PhIP occurs to produce the ion at m/z 210.1 $[M+H–191]^{+\bullet}$ Two fragment ions are observed at m/z 237.2 and 267.2 in the product ion spectrum of PhIP-$N3$-Gl, but are not seen in the spectrum of PhIP-N^2-Gl. These ions are proposed to occur via ring opening of the glucuronide, followed by cleavage at the C1–C2 bond (m/z 237.2) or C2–C3 bond (m/z 267.2) of the glucuronide moiety (Scheme 2). The corresponding fragment ions are observed at m/z 240.2 and m/z 270.2, in the product ion spectrum of 1-$[^2H_3C]$-PhIP-$N3$-Gl (data not shown).

Both HON-PhIP-N-Gl conjugates ($[M+H]^+$ at m/z 417.1) undergo fragmentation of the glycosidic linkage $[M+H –176]^+$, to produce HONH-PhIP at m/z 240.9, as well as other product ions at m/z 225.0, 223.9, and 223.0. The product ion spectra of 1-$[^2H_3C]$-HON-PhIP-N-Gl conjugates ($[M+H]^+$ at m/z 420.1) display ions at m/z 244.1 $[M+H–176]^+$ (the formation of 1-$[^2H_3C]$-HONH-PhIP), and ions at m/z 228.1, 227.1, 226.1, and 225.1. The HONH-PhIP, m/z 240.9, (or 1-$[^2H_3C]$-HONH-PhIP, m/z 244.1), undergoes fragmentation to lose $OH^\bullet$ and H_2O (or $OH^\bullet$, H_2O, HOD), to form the ions at m/z 224.1 and 223.1 (or m/z 227.1, 226.1, and 225.1). The precursor ion scan mode revealed that the ions at m/z 225.1 and m/z 228.3 in the product ion spectra of the unlabeled and labeled Gl conjugates, respectively, were derived from the parent molecules, indicative or rearrangement of the hydroxyl group of HONH-PhIP to the glucuronide moiety. Subsequent cleavage of the glycosidic linkage produces protonated PhIP (m/z 225.1) (or protonated 1-$[^2H_3C]$-PhIP m/z 228.1) (Scheme 3).

LC-ESI/MS/MS Analysis of PhIP and its Metabolites in Human Urine

The analysis of urine from a subject before and after consumption of grilled meat is shown in Figure 4. Trace levels of PhIP at (t_R 16.3 min, <3 pg/mL) are detected in urine of the subject prior to meat consumption; none of the other metabolites are detected. The LC-ESI/MS/MS chromatogram of metabolites from urine collected over 10 h after meat

consumption displays signals for PhIP (t_R 16.3 min, 20 pg/mL), HON-PhIP-N^2-Gl (t_R 16.7 min 1680 pg/mL), and HON-PhIP-N3-Gl (t_R 20.1 min 276 pg/mL) (Figure 4). The PhIP-N^2-Gl (t_R 15.4 min) was difficult to discern when the transition $[M+H]^+ \rightarrow [M+H-176]^+$ (401→225) was employed for monitoring and many isobaric interferences were observed (data not shown). Increasing the CID voltage from 32 to 55 eV resulted in secondary fragmentation of PhIP-N^2-Gl $[M+H]^+ \rightarrow [M + H-191]^{+\bullet}$ (401→ 210), attributed to the loss of the glucuronide, followed by loss of the $CH_3^{\bullet}$ group of PhIP. This transition greatly improved the signal to noise for monitoring the PhIP-N^2-Gl analyte in urine. However, the amount of PhIP-N^2-Gl was below 50 pg/mL (the LOQ). There were small quantities of 4′-HO-PhIP (t_R 11.5 min). On the basis of ionization efficiency and response similar to those of PhIP, we deduce that 4′-HO-PhIP was present in urine samples at levels of about 16 pg/mL. Trace levels of the PhIP-4′-O-Gl (t_R 5.2 min) were also detected.

The quantities of HON-PhIP-N^2-Gl and HON-PhIP-N3-Gl were sufficient that product ion spectra could be acquired for corroboration of their identities. The product ion spectra are in excellent agreement with the reference standards (Supporting Information, Figure S-1, S-2). There was evidence for three other glucuronide conjugates of hydroxylated PhIP metabolites $[M+H]^+$ at m/z 417.1, (t_R 13.3–14.5 min), when the transition $[M+H]^+ \rightarrow [M+H-194]^+$ (417.1→ 223.1), due to loss of glucuronide and H_2O. These analytes were not seen in urine samples of subjects prior to consumption of cooked meat (Figure 5). The product ion spectra of these metabolites differ from those spectra of the glucuronide conjugates of HONH-PhIP and 4′-HO-PhIP. Prominent fragment ions are observed at m/z 241.1, 223.1, and 167.1 (Supporting Information, Figure S-3).

Performance of the Analytical Method

The recoveries of each internal standard $[^2H_3C]$-PhIP (100 pg/mL), or $[^2H_3C]$-PhIP-N^2-Gl, $[^2H_3C]$-HON-PhIP-N^2-Gl and $[^2H_3C]$-HON-PhIP-N3-Gl (each at 1000 pg/mL), added to urine prior to sample processing were consistently between 50 and 80%, based on the response of the signals to those of pure standards measured by LC-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 (40). The calibration curves for PhIP, PhIP-N^2-Gl, HON-PhIP-N^2-Gl and HON-PhIP-N3-Gl (4 independent replicates per calibrant level), constructed in urine from a subject who refrained from eating cooked meat for 48 h, are depicted in Figure 6. The LOQ for PhIP was estimated at approximately 5 pg/ml, and the LOQs for the glucuronide conjugates of PhIP and HONH-PhIP were estimated at ~50 pg/mL.

The isomeric glucuronide conjugates of HONH-PhIP and their isotopically labelled internal standards were monitored by use of the transitions $[M+H]^+ \rightarrow [M+H-192]^+$ and $[M+H-193]^+$, which are attributed to the loss of the glucuronide moiety and an oxygen atom or loss of the glucuronide moiety and the hydroxyl group. These ions were chosen because isobaric interferences were observed in the most abundant transition for the internal standard ($[M+H]^+ \rightarrow [M+H-194]^+$ (loss of glucuronide and H_2O), resulting in less reliable estimates of the urinary analytes (data not shown). The precision (CV(%)) of the estimates in the calibration curve at the lowest calibrant levels of PhIP (5 pg/mL) was ≤20%, and the precisions (CV(%)) for PhIP-N^2-Gl, HONH-PhIP-N^2-Gl, and HONH-PhIP-$N3$-Gl metabolites at the lowest calibrant levels (100 pg/mL) were ≤11%; these precisions improved at the higher calibrant levels.

The peformance of the method was assessed by the within-day and between-day estimates and precision of measurements of PhIP and the glucuronide conjugates of NHOH-PhIP, in urine of three volunteers determined over 4 days ($n = 4$ independent measurements per day), within a time period of two months. The results are summarized in Table 1. The within-day

and between-day precision values (CV(%)) in estimates of PhIP were <8.8% and 14.0%, respectively. The within-day and between-day precision values (CV(%)) in estimates of the $N3$- and N^2-glucuronide metabolites of HONH-PhIP were <10.2% and 10.8%, respectively. Thus, the analytical method is precise, and inter-day estimates for the quantification of PhIP and its metabolites are highly reproducible.

DISCUSSION

A robust analytical method has been established to simultaneously quantitate PhIP, PhIP-N^2-Gl, and the isomeric N^2 and $N3$-glucuronide conjugates of HONH-PhIP. The extraction method is facile: It entails treatment of the urine with an organic solvent, to promote precipitation of proteins and salts, followed by SPE of the supernatant with a mixed-mode reverse phase cation exchange resin. The LC-ESI/MS/MS analyses of glucuronide conjugates of HONH-PhIP in human urine have been previously reported, in two different studies. The first study employed a labor-intensive extraction scheme prior to LC-ESI/MS/MS: the analysis was done with a Finnigan™ LCQ (Thermo Electron), a 3-dimensional ion trap mass spectrometer (19). A second study measured these urinary metabolites by triple stage quadrupole MS, employing a Quattro LC (Micromass Ltd.). In the latter study, urine was assayed without any prior purification of the metabolites (20). In our current study, we detected numerous isobaric interferences in urine samples without prior purification of PhIP and its metabolites, when monitored with the Finnigan™ Quantum TSQ/MS. Apparently, the different designs of the ion sources of MS instruments can influence the degree of analyte purification that is required prior to MS analysis. Moreover, we observed that the performance of the analytical LC column deteriorated rapidly, when unprocessed urine was assayed. Our estimates of PhIP and its N^2- and $N3$-glucuronide metabolites of HONH-PhIP are in excellent agreement with the values previously determined on the same urine samples that were purified and measured by different techniques (20,49): The inter-laboratory estimates of PhIP and these metabolites are within 25%.

The prominent role of UGTs in the metabolism of HONH-PhIP by humans is well-known (20,21,32,37,44). Together, the amount of the HON-PhIP-N^2-Gl and HON-PhIP-$N3$-Gl conjugates have been reported to account for 50 to 70%, and the levels of PhIP-N^2-Gl ranged between 4 and 11% of the ingested dose that were excreted in urine within 24 h, when assayed by AMS (21). The other major urinary metabolite detected by AMS was the 4′-sulfate conjugate of 4′-HO-PhIP, which accounted for as much as 10% of the ingested dose that was excreted in urine (21). We did not detect either the N^2 or the $N3$-Gl conjugate of PhIP (<50 pg/mL), by LC-ESI/MS/MS, in urine of subjects from our study. Glucuronidation has been reported to be less important to the metabolism of PhIP than is the glucuronidation of HONH-PhIP in vivo (21), as well as in human hepatocytes (32). Possibly, the amounts of the PhIP-N-Gl isomers present in these urine specimens were just below the LOQ in our analysis. We did detect trace levels of 4′-HO-PhIP; however, the 4′-sulfate conjugate of 4′HO-PhIP was not found (data not shown). This sulfate metabolite exists as a zwitterion under our LC solvent conditions, and may have escaped detection. Increased coverage of this PhIP metabolite can be achieved by pre-treatment of urine with arylsulfatase, to cleave this sulfate conjugate to form 4′-HO-PhIP (22).

Three other minor glucuronide conjugates of hydroxylated PhIP metabolites were detected; their structures are unknown. Frandsen (22) tentatively identified three glucuronide conjugates of 5-HO-PhIP, a solvolysis product of the reactive N-acetoxy-PhIP intermediate. We have seen that a prominent fragment ion occurs at m/z 223.1 in the product ion spectra of all three of these glucuronide metabolites (Supporting Information, Figure S-3), and is also prominent in the product ion spectrum of 5-HO-PhIP (Figure 3A). Further studies are

required to determine whether these three novel glucuronide metabolites are derived from 5-HO-PhIP or other uncharacterized hydroxylated PhIP metabolites.

In summary, our new method for the measurement of PhIP and its urinary metabolites is an effective, non-invasive approach, by which to determine exposure to this carcinogen during the previous 24 h (21). Although the measurements of PhIP urinary metabolites do not shed light on DNA damage, which occurs at a distant target tissues, such as the colon (21), urinary metabolite analyses can aid in identifying genetic polymorphisms in xenobiotic metabolism enzymes that influence the genotoxic potency of this procarcinogen, and they can be used to assess chemoprotective agents that modulate the metabolism and the potential deleterious effects of PhIP (20).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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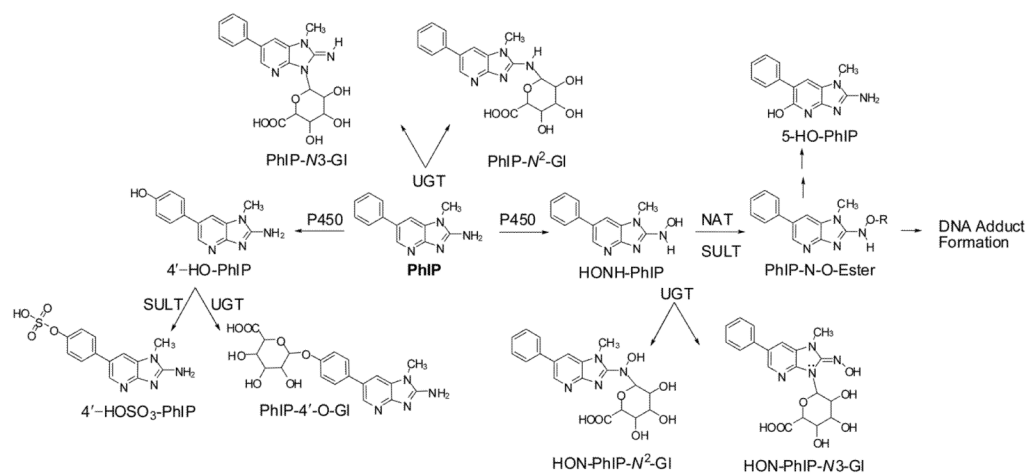
SYNOPSIS
Biomonitoring of PhIP and metabolites in urine.

Figure 1.
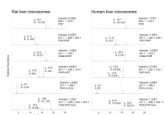Major pathways of metabolism of PhIP in experimental laboratory animals and humans.

Figure 2.
LC-ESI/MS/MS chromatograms of PhIP metabolites produced with (A) Rat liver microsomes and (B) Human liver microsomes. Both microsomal samples were fortified with UDPGA and cofactors for P450 catalysis (respective t_R: PhIP-N3-Gl 13.6 min; PhIP-N^2-Gl 15.5 min; HON-PhIP-N^2-Gl 14.9 min; HON-PhIP-N3-Gl 18.6 min). The ion intensity and area counts $\times 10^{-3}$ are reported.

Figure 3A
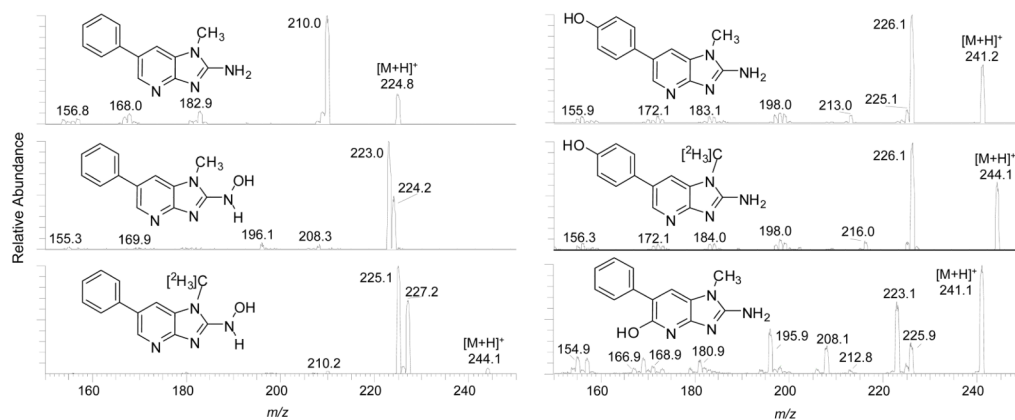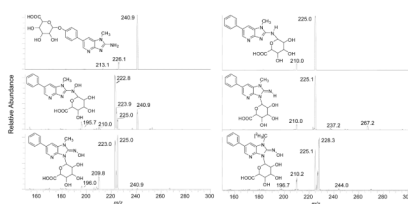
Figure 3.
LC-ESI/MS/MS product ion spectra of PhIP and its metabolites. (A) PhIP, 4′-HO-PhIP and 5-HO-PhIP @ a CID of 32 eV, and HONH-PhIP and 1-[²H₃C]-HONH-PhIP @ a CID of 20 eV. (B) Isomeric PhIP-*N*-Gl and HONH-PhIP-*N*-Gl conjugates and 1-[²H₃C]-HONH-PhIP-*N*3-Gl @ 32 eV. The ion intensity and area counts $\times 10^{-3}$ are reported.
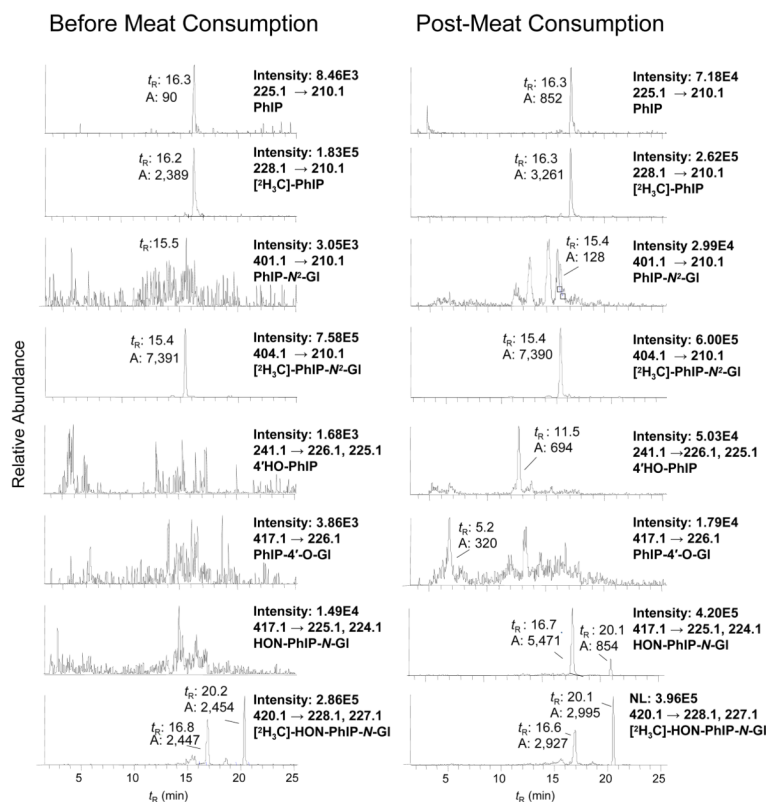
Figure 4.
SRM traces of PhIP and its glucuronide metabolites in human urine before and after
consumption of cooked beef. Note, 0.01% HCO_2H replaced the 0.1% HCO_2H that was
employed in the analysis of microsomal metabolites presented in Figure 2. The change in
acid concentration resulted in shifts of t_R values of metabolites: PhIP-N^2-Gl 15.2 min; HON-
PhIP-N^2-Gl 16.7 min; and HON-PhIP-N3-Gl 20.1 min). The ion intensity and area counts $\times$
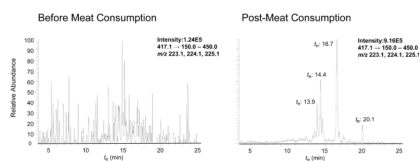10^{-3} are reported.

Figure 5.
SRM traces of novel glucuronides of hydroxylated PhIP metabolites in human urine before and after consumption of cooked beef. The novel glucuronide metabolites elute at t_R 13.9 – 14.6 min, the t_R of HON-PhIP-$N2$-Gl is 16.7 min; and t_R of HON-PhIP-$N3$-Gl is 20.1 min.
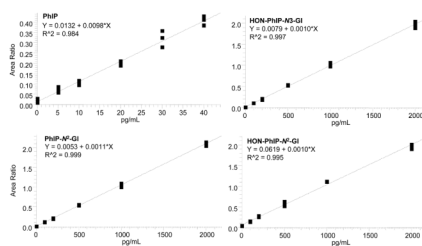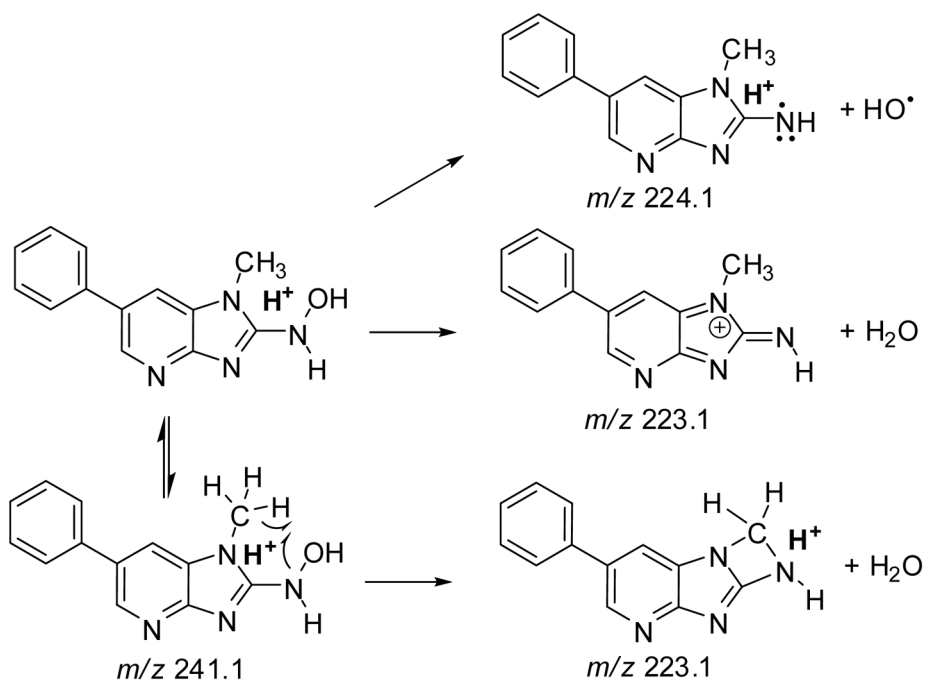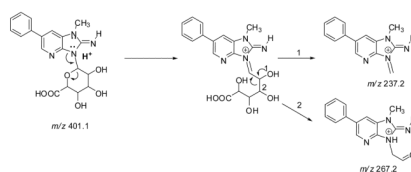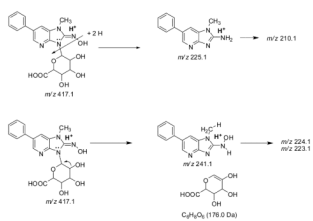
Figure 6.
Calibration curves for PhIP, PhIP-N^2-Gl, HONH-PhIP-N^2-Gl, and HONH-PhIP-N3-Gl.

Scheme 1.
Proposed pathways of fragmentation of HOHN-PhIP [M+H]$^+$ at *m/z* 241.1, To form the product ions at *m/z* 223.1 and 224.1.

Scheme 2.
Proposed pathways of fragmentation of PhIP-*N*3-Gl To produce the fragment ions at *m/z* 267.2 and 237.2.

Scheme 3.
Proposed pathways of fragmentation of HON-PhIP-*N*3-PhIP [M+H]$^+$ at *m/z* 417.1 To produce the product ions at *m/z* 241.1 and 225.1, and secondary fragmentations to form the product ions at *m/z* 223.1 and 224.1 (from HONH-PhIP) and *m/z* 210.1 (from PhIP).

Table 1

Performance of the Analytical Method to Measure PhIP and Its Metabolites in Urine

Subject	Metabolite		Amount (pg/mL)					CV(%) within-day	CV(%) between-day
			Day 1	Day 2	Day 3	Day 4	Overall		
1	HON-PhIP-N^2-Gl	Mean	970	1040	1080	1110	1050		
		SD	63	177	24.5	105	112		
		RSD(%)	6.5	17.1	2.3	9.4	10.6	10.2	10.6
	HON-PhIP-$N3$-Gl	Mean	145	135	149	136	142		
		SD	15.8	12.4	16.2	10.2	13.9		
		RSD(%)	10.9	9.2	10.8	7.5	9.9	10.2	10.2
	PhIP	Mean	27	25	28	24	26		
		SD	3.6	1.3	0.6	0.8	2.3		
		RSD(%)	13.2	5.0	2.1	3.4	8.7	8.8	9.3
2	HON-PhIP-N^2-Gl	Mean	990	950	1150	1120	1054		
		SD	27	24	96	96	105		
		RSD(%)	2.7	2.5	8.4	8.6	9.9	6.7	10.8
	HON-PhIP-$N3$-Gl	Mean	168	154	163	159	161		
		SD	6.5	18	16	5.0	12.8		
		RSD(%)	3.9	11.9	10.1	3.1	7.9	8.0	7.8
	PhIP	Mean	14	18	17	15	16		
		SD	1.0	2.3	0.5	0.4	2.0		
		RSD(%)	6.7	12.7	2.9	2.7	12.6	8.2	14.0
3	HON-PhIP-N^2-Gl	Mean	1610	1450	1450	1490	1490		
		SD	87	105	62	89	103		
		RSD(%)	5.4	7.2	4.3	6.0	6.9	5.5	7.0
	HON-PhIP-$N3$-Gl	Mean	258	227	231	222	235		
		SD	22	15	17	22	21		
		RSD(%)	8.5	6.6	7.4	6.3	8.9	6.5	8.8
	PhIP	Mean	18	19	23	19	20		
		SD	1.0	0.4	1.5	0.4	1.9		
		RSD(%)	5.5	1.8	6.5	2.1	9.7	2.8	10.3

$n = 4$ replicates per day