Identification of urine metabolites of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine following consumption of a single cooked chicken meal in humans

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Many studies suggest that mutagenic/carcinogenic chemicals in the diet, like 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), may play a role in human cancer initiation. We have developed a method to quantify PhIP metabolites in human urine and have applied it to samples from female volunteers who had eaten a meal of cooked chicken. For this analysis, urine samples (5 ml) were spiked with a deuterium-labeled internal standard, adsorbed to a macroporous polymeric column and then eluted with methanol. After a solvent exchange to 0.01 M HCl, the urine extracts were passed through a filter, applied to a benzenesulfonic acid column, washed with methanol/acid and eluted with ammonium acetate and concentrated on a C18 column. The metabolites were eluted from the C18 column and quantified by LC/MS/MS. In our studies of human PhIP metabolism, eight volunteers were fed 200 g of cooked chicken containing a total of 27 µg PhIP. Urine samples were collected for 24 h after the meal, in 6 h aliquots. Although no metabolites could be found in urine collected from volunteers before eating the chicken, four major human PhIP metabolites, N2-OH-PhIP, N2-glucuronide, PhIP-N2-glucuronide, 4′-PhIP-sulfate and N2-OH-PhIP-N3-glucuronide, were found in the urine after the chicken meal. The volunteers in the study excreted 4–53% of the ingested PhIP dose in the urine. The rate of metabolite excretion varied among the subjects, however, in all of the subjects the majority of the metabolites were excreted in the first 12 h. Very little metabolite was detected in the urine after 18 h. In humans, N2-OH-PhIP-N2-glucuronide is the most abundant urinary metabolite, followed by PhIP-N2-glucuronide. The variation seen in the total amount, excretion time and metabolite ratios with our method suggests that individual digestion, metabolism and/or other components of the diet may influence the absorption and amounts of metabolic products produced from PhIP.

Introduction

Cooked muscle meats, major components of the Western diet, contain potent mutagens and rodent carcinogens belonging to the heterocyclic amine class of chemical compounds. Humans are routinely exposed to varying amounts of these food-derived compounds and there is a concern that they may play a role in human carcinogenesis. Of the 19 heterocyclic amines identified, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is frequently the most abundant heterocyclic amine produced during the cooking of beef, pork and chicken (1–5) and in meats purchased in restaurants (6,7).

PhIP is naturally formed in meats during the cooking process, at least in part due to a heat-dependent condensation of creatine and phenylalanine, two natural components of muscle meats. The highest levels of PhIP can be found in grilled or fried meats. In very well-done flame-grilled chicken PhIP can be found at levels up to 400 ng/g (2). The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day, depending on individual dietary and cooking preferences (8).

The role of PhIP in cancer initiation has been well established in animals. PhIP has been shown to cause DNA strand breaks, sister chromatid exchanges and form DNA adducts both in vitro and in vivo (9–14). Short-term exposure to PhIP produces mutations in the large and small intestine of mice (15,16). In rats and mice dose-dependent tumor formation has been consistently demonstrated after PhIP administration and the most common tumor sites appear to be colon, prostate and breast (17–22). Other studies have confirmed the carcinogenicity of PhIP in rodent breast and prostate gland (23,24). In the CDF1 mouse PhIP induces lymphomas, while in the newborn B6C3F1 mouse model it induces adenocarcinomas of the liver (25,26). PhIP exposure can also occur via breast milk: DNA adducts were found in pups that received breast milk from PhIP-exposed lactating rats (27) and increased intestinal tumors were shown in multiple intestinal neoplasia (Min) mice exposed to PhIP via breast milk (28).

In humans, less is known about the potential role of PhIP and related heterocyclic amines in tumor development. Several studies have shown that individuals who eat their meat well done have an elevated risk of cancers at various sites. Zheng et al. showed a significant dose–response relation between doneness levels of meat and breast cancer risk, reporting that women who preferred well-done hamburger, steak and bacon have a 4.62-fold greater risk of breast cancer than did women who preferred meats cooked ‘rare’ or ‘medium’ (29). Other studies showed increased risk of colorectal adenomas with increased well-done meat consumption (30,31). Lung cancer has also been related to the consumption of fried, well-done meat (32). Other studies, however, have shown either negative or equivocal associations with well-done meat and cancers of the breast (33,34), colon or rectum (35) or prostate gland (1). In all of these studies PhIP and related heterocyclic amine exposure levels are based upon answers to dietary questionnaires. However, the formation of heterocyclic amines is variable and the levels of these compounds found in foods depends on many cooking variables. Dietary surveys have several flaws, including bias, inconsistent reporting and, most importantly, the difficulty in quantifying cooking doneness.

Abbreviations: 4′-hydroxy-PhIP, 2-amino-1-methyl-6-(4′-hydroxy)phenylimidazo[4,5-b]pyridine; N-hydroxy-PhIP, 2-hydroxymino-1-methyl-6-phenylimidazo[4,5-b]pyridine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.
PhIP must first be metabolized via Phase I and Phase II enzymes to exert its mutagenic and carcinogenic effect. During Phase I metabolism PhIP is oxidized via cytochrome P4501A2 (CYP1A2) enzymes to a hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N\(^{-}\)hydroxy-PhIP). N\(^{-}\)hydroxy-PhIP, which is itself mutagenic, can be converted to a more biologically reactive form via Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases. This esterification generates electrophilic O-sulfonylelimines and O-acetyl esters, which have the capacity to bind DNA and cellular proteins (10,36–38). Detoxification primarily involves glucuronidation. N\(^{-}\)hydroxy-PhIP can form stable glucuronide conjugates at the N\(^{2}\) and N3 positions which can be excreted or transported to extrahepatic tissue for further metabolism (39,40). PhIP can also be hydroxylated at the 4\(^{\prime}\) position, forming 2-amino-1-methyl-6-(4\(^{\prime}\)-hydroxy)phenylimidazo[4,5-b]pyridine (4\(^{-}\)hydroxy-PhIP). 4\(^{-}\)hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted (41,42). In addition, the parent compound can be directly glucuronidated at the N\(^{2}\) and N3 positions. These glucuronides are not reactive and this is believed to be a detoxification pathway (40,43). The structure of these metabolites and probable pathways of formation are shown in Figure 1.

Fig. 1. Major metabolites of PhIP found in human urine.
The individuals participating were recruited from the local workforce, were all female, in good health, non-smokers and of normal weight.

**Meat preparation and controlled dietary period**

Boneless, skinless chicken breasts were cut into ~2.5 cm pieces and fried in a non-stick coated pan, sprayed with a vegetable-based non-stick cooking spray, for 25–35 min. Pan temperature was recorded every 5 min, averaging 186°C for the cooking period. At the end of the cooking time the chicken was white with some browning. A representative chicken sample was removed for heterocyclic amine analysis using previously published methods (63). The first two study subjects were provided with 200 g chicken containing 105 p.p.b. PhIP along with other non-meat foods and beverages. The total PhIP dose was 21 µg. The remaining six subjects were given chicken containing 94 p.p.b. PhIP, for a total dose of 18.8 µg.

Subjects were asked to abstain from meat consumption for 24 h prior to eating the well-done chicken breast. There were no other dietary restrictions. Control urine was collected before eating the chicken and for 24 h after in 6 h increments. Samples were refrigerated until analysis. Repeated analysis of these samples over prolonged periods of time (>1 year) have shown no noticeable change in metabolite levels.

**Extraction of PhIP metabolites**

Urine samples (5 ml) were spiked with internal standard (4.2 ng in 5 µl water) and applied to a pre-conditioned 60 mg Oasis SPE macroporous polymeric column (Waters, Milford, MA). Metabolites were eluted with 5 ml of methanol. The elution aliquot was evaporated to dryness under nitrogen and the metabolites were redissolved in 2.5 ml of 0.01 M HCl. Proteins and high molecular weight contaminants were removed by filtering the solution through a Centricyn YM-3 centrifugal filter (Millipore, Bedford, MA). The filtrate was applied to a preconditioned benzenesulfonic acid column (500 mg) (Varian Sample Preparation Products, Harbor City, CA) and the column washed with 6 ml of 10% (v/v) methanol/0.01 M HCl. The metabolites were eluted onto a coupled C₁₈ column (1000 mg) (Bakerbond spe; J.T. Baker, Phillipsburg, NJ) with 0.5 M ammonium acetate, pH 8. The C₁₈ column was washed with 3 ml of 5% (v/v) methanol/H₂O and eluted from the C₁₈ column with 50% (v/v) methanol/H₂O. The metabolites were dried under nitrogen and 1 ml of urine equivalent was injected into the LC/MS in a volume of 20 µl.

Chromatography was done on an Ultra-Plus HPLC system (Microtech, Sunnyvale, CA) equipped with a YMC basic column (3.0 x 250 mm). Metabolites were eluted at a flow rate of 200 µl/min using a mobile phase of A (water/methanol/acetic acid, 97:2:1) and 5% B (methanol/water/acetic acid, 95:4:1) for 1 min, to 25% B at 5 min, a linear gradient to 100% B at 30 min and held for 5 min.

Urine samples were detected with a mass spectrometer (model LCQ; Finnigan, San Jose, CA) in the MS/MS positive ion mode using an electrospray interface. A capillary temperature of 240°C, a source voltage of 4.5 kV and sheath gas of 70 units was used. An ion trap injection time of 1000 ms and one microscan were used. Alternating scans were used to isolate [M+H]⁺ ions at masses 417, 401 and 321 for the pentadeuterio-labeled internal standard and 2-glucuronide; range of peaks seen in this study; least squares regression demonstrated no significant correlation to data.

**Recovery studies and precision of the assay**

The overall recovery of the metabolites was determined by spiking each urine sample with known amounts of N- OH-[H₃]PhIP-N₂-glucuronide. Final metabolite amounts were adjusted based on recovery of the internal standard. The effect of the urine matrix on the recovery of metabolites was determined by spiking increasing amounts of the internal standard in 5 ml of water and comparing these recoveries to recovery of the internal standard in 5 ml urine. Ion suppression in the mass spectrometer by co-eluting interference was investigated by spiking human urine extracts with mouse urine containing high levels of the metabolites.

Replicate analyses of different urine samples were made during the course of the study to determine the precision of the assay.

**Results**

**Method development and urine analysis**

The goal of this study was to develop a method that reliably quantifies PhIP metabolites and could be applied to large numbers of urine samples. The initial step of the method (Figure 2) utilized non-specific absorption to remove all the metabolites from the urine, extracting them from the water and salts. Urinary proteins and larger molecular contaminants were removed by centrifuging the extracts through a filter with a molecular weight cut-off of 3000 kDa. Protein determinations of the urine samples before and after filtering demonstrated that 60–80% of the color-reacting material could be removed from the sample during the filtering step (data not shown). This greatly improved HPLC column lifetime. After the initial purification, secondary purifications were designed to exploit the protonation of the heterocyclic nitrogen atoms common to the metabolites in an ion exchange adsorption step removing uncharged interference. Finally, the urine extract was concentrated and washed on reversed phase silica.

Because of the complexity of the urine extracts and the low amounts of metabolite present, metabolites could not be seen by UV or fluorescence detection. Due to co-elution of hundreds of compounds into the mass spectrometer, no signal can be seen above the background with single ion monitoring MS for the parent masses (data not shown). MS/MS detection is necessary for these analyses. Urine samples from rodents receiving high doses of PhIP were used to optimize the HPLC separation and fragmentation of the metabolites. Metabolites in rodent urine were used to determine the linear range of the instrument. The LC/MS/MS peak areas were linear over the range of peaks seen in this study; least squares fits to data gave r² values ranging from 0.984 to 0.999.

Our method using LC/MS/MS detects peaks for the four identified human PhIP metabolites and the deuterated internal standard in a single chromatographic run (Figure 3). For increased sensitivity, data acquisition was over three segments, isolating mass 321 for 22 min, masses 417, 401 and 422 for 7 min and mass 417 only for the final 5.5 min. Since other ion peaks are often present in the chromatograms that are not one of the four identified PhIP metabolites (Figure 3), expected peak retention times were compared with the internal standard and reference samples to identify PhIP metabolites. N₂-OH-PhIP-N₂-glucuronide is typically a broader HPLC peak that fragments into daughter ions with masses of 225 and 241. The sum of these two peak areas is used for quantitation (Figure 3A). The N₂-OH-PhIP-N₃-glucuronide is separated in time from the N₂-OH-PhIP-N₂-glucuronide and fragments to mass 225 only (Figure 3A). The internal standard, N₂-OH-[H₅]-phenyl[PhIP-N₂-glucuronide, elutes slightly before the non-labeled natural product (Figure 3B). The PhIP-N₂-glucuronide
Fig. 4. Percent of the dose of PhIP excreted as urinary metabolites for eight volunteers over a 24 h period. The recovery-corrected sum of the amount of $N^2$-OH-PhIP-$N^2$-glucuronide, PhIP-$N^2$-glucuronide, 4'-PhIP-sulfate and $N^2$-OH-PhIP-$N^3$-glucuronide detected is shown.

Table I. Percent recovery of $N^2$-OH-$N^2$-[2H5-phenyl]PhIP-glucuronide spiked into water or urine (numbers in parentheses represent peak area standard deviations of at least two injections of two different samples)

<table>
<thead>
<tr>
<th>Spike (ng)</th>
<th>Water</th>
<th>Urine</th>
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<tbody>
<tr>
<td>0.5</td>
<td>45.6 ± 8.0</td>
<td>39.8 ± 13.8</td>
</tr>
<tr>
<td>1.5</td>
<td>47.3 ± 7.4</td>
<td>40.6 ± 12.3</td>
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<tr>
<td>2.5</td>
<td>52.4 ± 26.4</td>
<td>39.7 ± 5.9</td>
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Fig. 3. Ion plots of PhIP metabolites and the internal standard from urine of subject H 0–6 h after consuming well-done chicken. A 1 ml equivalent of urine was used. See Materials and methods for LC/MS/MS conditions. (A) Sum of masses 225 and 241 after fragmenting mass 417. These peaks represent $N^2$-OH-PhIP-$N^2$-glucuronide and $N^2$-OH-PhIP-$N^3$-glucuronide. (B) Sum of mass 230 and 246 ions fragmented from mass 422. The indicated peak represents the internal standard. (C) Mass 225 peak plot after fragmenting mass 401, representing PhIP-$N^2$-glucuronide. (D) Mass 241 ion plot from mass 321. This peak represents PhIP-4'-sulfate.

Recovery and reproducibility

Spiking human samples with animal urine containing high levels of metabolites allowed us to determine the recovery of the metabolites while optimizing the extraction process. This was necessary to ensure that each of the four metabolites was recovered at each step in the clean-up process. Recovery through the final method for each sample was quantified by spiking each urine sample with a deuterium-labeled internal standard, $N$-OH-[2H5]PhIP-$N^2$-glucuronide. Typical recoveries ranged from 37 to 40% (Table I), although final metabolite amounts in each sample were adjusted based upon recovery of the internal standard in that sample. Recovery of the internal standard is slightly better in water (45–52%), indicating that the complexity of the urine matrix either interferes with the efficiency of the solid phase extraction columns or lowers the sensitivity of the mass spectrometer through ion suppression. Several of the samples were repeatedly analyzed over the course of the study to determine the reproducibility of the assay. Because of the small peak sizes in our assay, there is variation inherent in the mass spectrometry detection. To account for this variation, each urine extract was injected three times and the peak areas averaged. Variation within a sample ranged from 20 to 30%.

Human PhIP metabolite quantitation

Control urine samples were collected before consuming the well-done chicken, during the period that the volunteers abstained from eating cooked meat. No PhIP metabolite peaks were seen in the control samples from the eight individuals (data not shown). Total urine excreted after chicken consumption was collected for 24 h in 6 h increments. Values shown are corrected for the total volume of urine. Figure 4 shows the percentage of the total dose recovered in urine for the eight subjects. These varied 13-fold, despite the fact that all urine was collected and that each volunteer ate the same amount of chicken.

Figure 5 shows the rate of excretion of the PhIP metabolites in time periods of 0–6, 6–12 and 12–24 h. Three of the individuals (C–E) did not void during the 12–18 h period, thus the data for the 12–18 and 18–24 h periods were combined for the other five subjects. In all of the subjects the majority of the metabolites were excreted in the first 12 h (62–85%). The individuals showed variation in the time of metabolite excretion. Subjects B and H excreted most of the metabolite in the 0–6 h time period (55 and 45% of total metabolites, respectively), whereas subjects C and D excreted the majority in the 6–12 h time period (73 and 69% of total metabolites, respectively).

The total amounts of each of the four individual metabolites

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Urine metabolites of PhIP

Most common cancer sites in humans: the breast, colon, and prostate gland. In addition, exposure to PhIP need not be ubiquitous, but can be determined and modified through intervention, making PhIP-induced tumor formation preventable.

The metabolism of PhIP has been well characterized in animals, however, little is known about PhIP metabolism in humans. To take advantage of the opportunity to compare animals with humans and humans with each other and see the influence of diet on carcinogen absorption and metabolism, we developed a method for quantifying PhIP metabolites in human urine. This study reports the variation in PhIP metabolism between eight healthy human subjects.

Optimizing a solid phase extraction method was surprisingly difficult due to the polarity of the metabolites. The Oasis brand polymeric absorbant used proved superior to the various brands of C8 and C18 supports. Because of the wide range in polarity among the metabolites, analyte retention was problematical. To retain as much analyte as possible, washing steps were minimized. Due to the complexity of the urine matrix, there were still co-extracted impurities present in the final sample, even after our extensive clean-up. Our initial attempts at metabolite quantification showed good peak signals and metabolite recoveries, but very poor HPLC column life. Many experiments were done to produce even cleaner samples while maintaining good recoveries. The PhIP-4-H11032-sulfate metabolite was especially problematical because it is more polar than the glucuronide metabolites and thus easily lost during purification. Finally, a satisfactory procedure was devised that gives an acceptable column life of at least 100 injections/column, meeting our goal of devising a method to quantify PhIP metabolites in large numbers of samples.

Well-done chicken is the best source of PhIP exposure because at high temperatures and long cooking times chicken breast preferentially forms more PhIP and less of the related heterocyclic amines as compared with beef. Formation of PhIP seems to be favored by the higher amounts of the amino acids phenylalanine, isoleucine, leucine and tyrosine and lower amounts of glucose that are present in chicken (64). Both the amounts of chicken consumed by our volunteers and the PhIP levels were comparable with consumption levels possible in households or restaurants (7).

The percentage of the dose accounted for in the urine varied among individuals from 4 to 53% and was lower than previous studies of human subjects given radiolabeled PhIP. In the earlier study (60) the subjects were hospitalized elderly cancer patients who were given PhIP in a gelatin capsule. This route of administration resulted in recovery of 90% of the ingested dose in the urine for two of the three subjects. Our study consisted of younger women on their normal diet, which was unrestricted except for refraining from meat consumption for the 24 h prior to dosing. It is probable that the PhIP, when formed in the meat matrix, is not as bioavailable as PhIP in capsule form. In addition, the presence of additional foods in the gastrointestinal tract may influence the absorption of PhIP. We are exploring possible interventions that may reduce PhIP absorption, thereby reducing the biologically available dose.

The kinetics of PhIP metabolite excretion in our study are similar to those seen previously for humans in a study that detected excretion of the parent compound (55). It is also in agreement with our previous study of the excretion of radiolabeled metabolites (60). Our results demonstrate that excretion times vary among the volunteers but that most of

Discussion

The opportunity to study a genotoxic dietary carcinogen at realistic levels in humans is rare. PhIP is of special interest because it causes tumors in animals and DNA damage in vitro in human tissues. The sites of PhIP damage are among the most common cancer sites in humans: the breast, colon and prostate gland. In addition, exposure to PhIP need not be ubiquitous, but can be determined and modified through intervention, making PhIP-induced tumor formation preventable.

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the dose is excreted in the first 18 h. This suggests that these metabolites are suitable for investigating individual variation in rates and ratios of PhIP metabolism. Further, these metabolite measurements may be used as biomarkers of recent exposure, but are not suitable for long-term exposure estimates.

The detection of individual metabolites confirms our earlier findings in cancer patients administered 14C-labeled PhIP. The major human PhIP metabolites are N2-OH-PhIP-N2-glucuronide, PhIP-N2-glucuronide, PhIP-4′-sulfate and N2-OH-PhIP-N3-glucuronide. As previously reported, human metabolism differs from that seen in rodents. In rodents PhIP-4′-sulfate and 4′-OH-PhIP are the major metabolites, whereas in humans glucuronidation, either directly to PhIP or after N2-hydroxylation, appears to be a major pathway for urinary excretion. With our assay PhIP activation by cytochrome P450 enzyme-mediated N2-hydroxylation may be determined, at least in part, by the sum of N2-OH-PhIP-N2-glucuronide and N2-OH-PhIP-N3-glucuronide metabolites.

The ratio of the individual metabolites varied among our eight individuals. The enzymes known to be involved in the metabolism of PhIP are found at varying levels and activities within the human population (65). Experiments in animals, in animal tissues grown in vitro, in cells grown in culture and in bacteria show that the expression of specific activating enzymes greatly affects the genotoxic response of PhIP. We believe that the N2-OH-PhIP-N2-glucuronide and N2-OH-PhIP-N3-glucuronide metabolites represent activation pathways, whereas the PhIP-N2-glucuronide and PhIP-4′-sulfate represent detoxification pathways. The variation that we can detect in these metabolites suggests that both activation by P450 enzymes and detoxification by UDP-glucuronosyltransferases is variable among individual volunteers and may be an indication of potential susceptibility to DNA damage, mutation and cancer.

Future studies will focus on improving the method by increasing the sensitivity of metabolite detection, allowing us to lower the amount of PhIP-containing meat given to the volunteers. Reducing the analysis time and variation for the LC/MS/MS analysis are also needed. Greater control of the individual’s diet before the dosing period may reduce the variation present among the individuals. Both the amount of the metabolite formed and the rate of metabolite excretion can be used to develop a PhIP metabolite phenotype. Repeated analysis of PhIP metabolism in the same individuals over time will help determine the consistency of PhIP metabolism, allowing us to correlate the PhIP metabolite phenotype with genotype.

Altering the metabolism of PhIP to prevent formation of biologically active species may reduce individual susceptibility and prevent the occurrence of cancers in target tissues. Of the metabolites we detected, two appear to be part of the activation pathway for PhIP, N2-OH-PhIP-N2-glucuronide and N2-OH-PhIP-N3-glucuronide. It is likely that interventions that reduce N2-hydroxylation or increase direct glucuronidation of PhIP are desirable, as are genotypes favoring these products. The method described here should make studies of individual susceptibility and dietary interventions possible in the future.

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