Intestinal bacteria metabolize the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine following consumption of a single cooked chicken meal in humans

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Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a carcinogenic heterocyclic amine formed in meats during cooking. Although the formation of PhIP metabolites by mammalian enzymes has been extensively reported, the involvement of the intestinal bacteria remains unclear. This study examined the urinary and fecal excretion of a newly identified microbial PhIP metabolite 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2',4',5']imidazo[1,2-a]pyrimidin-5-ium chloride (PhIP-M1) in humans. The subjects were fed 150 g of cooked chicken containing 0.88–4.7 μg PhIP, and urine and feces collections were obtained during 72 h after the meal. PhIP-M1 and its trideuterated derivate were synthesized and a LC/MS/MS method was developed for their quantification. The mutagenic activity of PhIP-M1, as analyzed using the Salmonella strains TA98, TA100 and TA102, yielded no significant response. Of the ingested PhIP dose, volunteers excreted 12–21% as PhIP and 1.2–15% as PhIP-M1 in urine, and 26–42% as PhIP and 0.9–11% as PhIP-M1 in feces. The rate of PhIP-M1 excretion varied among the subjects. Yet, an increase in urinary excretion was observed for successive time increments, whereas for PhIP the majority was excreted in the first 24 h. These findings suggest that besides differences in digestion, metabolism and diet, the microbial composition of the gastrointestinal tract also strongly influences individual disposition and carcinogenic risk from PhIP.

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1. Introduction

Diet is a major risk factor in human cancer (Doll and Peto, 1981). Epidemiological studies indicate that the consumption of cooked meat and meat products predisposes individuals to neoplastic disease, particularly of the colon (Deverdier et al., 1991; Doll, 1992). Dietary factors which may be important in the etiology of human cancer include heterocyclic amines (Felton et al., 1986a). Of the 19 heterocyclic amines identified, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most mass abundant heterocyclic amine produced during the cooking of beef, pork and chicken (Felton et al., 1986b; Sinha et al., 1995; Wong et al., 2005). 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 (Sinha et al., 1995). 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 (Layton et al., 1995; Zimmerli et al., 2001). Experimentally, PhIP is a potent mutagen and genotoxin and has been shown to produce mammary gland, prostate and colon tumors in rats (Ito et al., 1991; Shirai et al., 1997; Sugimura, 2000). 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 well-done meat have an elevated risk of breast (Zheng et al., 1998) and colorectal (Gunter et al., 2005; Sinha, 1999) cancers. Not all studies have shown a positive correlation, however (Augustsson et al., 1999).

Until recently, studies of human PhIP metabolism mainly focused on the activation and detoxification of heterocyclic amines by mammalian enzymes. PhIP must first be metabolized via Phase I and Phase II enzymes to exert its mutagenic and carcinogenic effect. This involves an initial cytochrome P450A2 (CYP1A2) catalyzed N-hydroxylation step, to form N²-hydroxy-PhIP. N²-hydroxy-PhIP, which is mutagenic on its own, can be converted to a more biologically reactive form via Phase II metabolizing enzymes, to electrophilic O-sulfonfyl and O-acetyl esters which have the capacity to bind DNA and cellular proteins (Boobis et al., 1994; Buonarati et al., 1991; Edwards et al., 1994). Detoxification primarily involves glucuronidation. N²-hydroxy-PhIP can form stable glucuronide conjugates at the N² and N³ positions, which can be excreted or transported to extra-hepatic tissue for further metabolism (Alexander et al., 1991; Kaderlik et al., 1994). PhIP can also be hydroxylated at the 4' position. 4'-Hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted (Buonarati et al., 1992; Watkins et al., 1991). In addition, the parent compound can be directly glucuronidated at the N² and N³ positions. These glucuronides are not reactive and therefore considered as detoxification products (Kaderlik et al., 1994; Styczynski et al., 1993).

Recent research has shown that the amount of PhIP metabolites excreted in the 0–24 h urine represented 17 ± 10% of the ingested PhIP in a meat matrix (Kulp et al., 2004). In an earlier study with patients given PhIP in a capsule, 90% of the ingested dose was recovered in the urine (Malfatti et al., 1999). This indicates that PhIP provided in capsule form is more bioavailable than PhIP ingested from meat. The non-bioavailable fraction reaches the colon in an intact form to come into contact with the resident microbiota. Direct binding of heterocyclic amines to the cell walls of intestinal bacteria has been reported and is currently considered as a detoxification mechanism since it prevents absorption of heterocyclic amines through the intestinal mucosa (Bolognani et al., 1997; Turbic et al., 2002). However, little has been done to characterize PhIP metabolism by the human intestinal microbiota, although

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**Fig. 1.** Metabolite of PhIP formed by the human intestinal microbiota: 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-a]pyrimidin-5-ium chloride.

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2. Materials and methods

2.1. Chemicals

PhIP and its trideuterated analogue [2-amino-1-(trideuteromethyl)-6-phenyl-imidazo[4,5-b]pyridine; [2H₃] PhIP] were purchased from Toronto Research Chemicals (Ontario, Canada). The constituents of the culture media, namely tryptone and yeast extract were obtained from AppliChem (Darmstadt, Germany), L-cystein was purchased from Sigma–Aldrich (Bornem, Belgium). Extrelut-20 extraction cartridges were provided by Merck (Darmstadt, Germany), diatomaceous earth refill material was obtained from Sigma–Aldrich NV/SA (Bornem, Belgium). Oasis MCX (30 and 60 mg) cartridges were from Waters (Milford, MA, USA).
The solvents for LC/MS/MS analysis were of gradient grade and purchased from Acros Organics (Geel, Belgium).

2.2. Synthesis of 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydroxyprido[3′,2′:4,5]imidazo[1,2-a]pyrimidin-5-imium chloride and its trideuterated derivative

7-Hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydroxyprido[3′,2′:4,5]imidazo[1,2-a]pyrimidin-5-imium chloride (PhIP-M1) and its trideuterated derivative [2H3] PhIP-M1 were synthesized using procedures modified from previous studies (Vanhaecke et al., 2006). Briefly, incubation mixtures consisted of 0.1 mM PhIP or 0.02 mM [2H3] PhIP in TY broth (tryptone 30 g/L, yeast extract 20 g/L, cysteine 0.5 g/L, pH 7.0) supplemented with 10% (v/v) fecal inoculum in phosphate buffered saline (0.1 M, pH 7) in a final volume of 50 mL. Each sample was sealed with a butyl rubber top and anaerobiosis was obtained by flushing the flasks with N2 during 15 min. Each sample was processed using a previously published liquid/liquid extraction procedure (Vanhaecke et al., 2006). The yield of PhIP-M1 from PhIP and [2H3] PhIP-M1 from [2H3] PhIP was ~90%. Purification was obtained by preparative high performance liquid chromatography on Gilson preparative HPLC system (Gilson International B.V., Middleton, United States) comprising a H322 pump system and a 206 fraction collector, coupled to a model 156 UV/vis detector. Chromatographic separation was achieved using a 10 μm 21.4 × 250 mm Omnisphere C18 column obtained from Varian (St.-Ketelijne-Waver, Belgium). Compounds were eluted by an isocratic solvent mixture containing 85% water with 0.05% formic acid and 15% acetonitrile with 0.05% formic acid, at a flow rate of 20 mL/min. Absorbance was monitored at 307 nm. The identities of the microbial PhIP metabolites were confirmed by their LC/MS/MS fragmentation pattern (see below). The peaks corresponding to PhIP-M1 and [2H3] PhIP-M1 were collected and evaporated to dryness under nitrogen gas. Purity of the PhIP-M1 and its deuterated derivative was 97 ± 0.8% as determined by LC/MS/MS. Isotopic purity of [2H3] PhIP-M1 was 99%.

2.3. Study design

The study protocol was reviewed and approved by the Ethics Committee of the Ghent University Hospital (EC UZG 2005/404). Informed consent was obtained from each subject prior to beginning the study. The six individuals participating were recruited from the local workforce, were all male, between 20 and 30 years old, in good health, non-smokers and of normal weight. None had a history of digestive pathology nor had received antibiotics during 3 months prior to the study.

2.4. 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 non-stick cooking spray, for 25–35 min. Pan temperature was recorded every 5 min, averaging 180 °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 (Kuize et al., 1995). Total PhIP dose depended on the exact cooking time and was different for each of the three batches of chicken cooked. The PhIP content in the various batches ranged from 4.4 to 39 ng/g. Two first study subjects (A, B) were provided chicken containing 39 ng/g PhIP along with other non-meat foods and beverages. The total PhIP dose was 4.7 μg PhIP. The next two study subjects (C, D) were given chicken containing 4.4 ng/g, for a total dose of 0.88 μg. The remaining two subjects (E, F) received chicken containing 18 ng/g PhIP, for a total dose of 2.7 μg. The subjects were all provided with 150 g of chicken.

Subjects were asked to abstain from meat consumption for 3 days prior and 3 days after eating the well-done chicken breast. There were no other dietary restrictions. Control urine and feces samples were received before eating the chicken and all urine and feces was collected for 3 days afterwards, in 8 h increments for urine and 24 h increments for feces. Fecal slurries of 20% (w/v) fresh fecal inocula were prepared by homogenizing the feces with phosphate buffered saline (0.1 M, pH 7). Samples were coded, the volume recorded and stored frozen at −20 °C until analysis.

2.5. Analysis of PhIP and PhIP-M1 in human feces and urine

Urine samples (5 mL) and fecal slurries (5 mL) were spiked with 100 μL internal standard containing 125 ng/μL [2H3] PhIP and [2H3] PhIP-M1 in dimethylsulfoxide (DMSO), added with 0.5 mL of 6 M NaOH and mixed with 5 g of diatomaceous earth. The mixture was placed into an empty Exetrelut-20 cartridge and extracted with 30 and 60 mL of dichloromethane for the urine and fecal samples, respectively. The eluate was directly passed through an Oasis MCX (30 and 60 mg) cartridge, preconditioned with either 1 or 2 mL of dichloromethane. After washing the cartridges with 1 mL of 0.1 M HCl and 1 mL of acetonitrile, heterocyclic amines were eluted with 6 or 12 mL of 10% NH4 in acetonitrile for the urine and fecal samples, respectively. Finally, the extracts were evaporated to dryness under a stream of nitrogen, redissolved in 100 μL of acetonitrile-5 mM formic acid (75:25) and injected into the LC/MS/MS in a volume of 20 μL.

Acid hydrolysis of urine was carried out by adding 0.5 mL of 1 M HCl to 5 mL of urine and heating at 90 °C for 1 h. For fecal samples 0.5 mL of 6 M HCl was used. After hydrolysis was completed, 0.5 mL of 1 and 6 M sodium hydroxide was added to the urine and feces, respectively, to obtain a basic medium. Subsequently, the samples were processed using the optimized clean-up procedure mentioned above.

Chromatography was carried out on a Thermo Finnigan HPLC system (San Jose, CA, USA) comprising a P4000 quaternary pump and an AS3000 autosampler, equipped with a 5 μm, 21.4 × 150 mm Symmetry C18 column obtained from Waters (Milford, MA, USA). Metabolites were eluted at a flow rate of 300 μL/min using a mobile phase of 98% A (0.01% aqueous formic acid) and 2% B (acetonitrile) for 2 min, increasing linearly to 100% B at 22 min, maintaining 100% B for 8 min, and finally increasing to 100% B in the minute.

Analytes were detected with a LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) in the MS/MS positive ion mode using an Electrospray Ionisation (ESI) interface. A capillary temperature of 240 °C, a source voltage of 4.5 kV and sheath gas of 70 units with no auxiliary gas were used.

Alternating scans were used to isolate [M+H]+ ions at masses 225 and 281 for PhIP and PhIP-M1 and 228 and 284 for the deuterated internal standards. The precursor isolation width was set to 2 Da, the activation Q to 0.35 and the collision energy to 45%. Daughter ions were detected at appropriate masses: 210 [M+H–CH3]+ from 225 for PhIP, 263 [M+H–OH]+ and 225 [M+H–tetrahydroxypridine–OH]+ from 281 for PhIP-M1, 210 [M+H–CD3]+ from 228 for [2H3] PhIP, 266 [M+H–OH]+ and 228 [M+H–tetrahydroxypridine–OH]+ from 284 for [2H3] PhIP-M1. The overall recovery of PhIP and PhIP-M1 was determined by spiking each urine and feces sample with known amounts of their deuterated analogues. Final PhIP and PhIP-M1 concentrations were adjusted based on recovery of the internal standard. The effect of the urine or fecal matrix on the overall recovery of PhIP and PhIP-M1 was determined by spiking increasing amounts of the internal standard in 5 mL of water and comparing these recoveries to the recovery of the internal standard in 5 mL urine or fecal slurry. Replicate analyses of several different urine and fecal samples were made during the course of the study to determine the precision of the assay.

2.6. Salmonella mutagenicity assay

The mutagenic activity of the purified extract of PhIP-M1 in DMSO (100 ng/μL for TA98 and 2 μg/μL for TA100 and TA102) was determined using the standard plate incorporation assay described by Ames et al. (1973), with S. typhimurium strains TA98, TA100 and TA102 (gifts of Professor Bruce Ames, University of California, Berkeley) and tested in 5,
Aroclor-induced rat liver S9 protein (2 mg per plate) was used for metabolic activation. As a positive control, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) was used. DMSO was the negative control (spontaneous revertant counts). Dose–response curves of the mutagenic activity were calculated using the method of Moore and Felton (1983). A minimum of four dose points from duplicate platings was used, and the linear portion of the curve was used to calculate the number of revertants per µg of PhIP-M1 extract.

3. Results

3.1. Method development, urine and feces analysis

The goal of this study was to develop and apply a method that reliably quantifies PhIP and its newly identified microbial metabolite PhIP-M1 in urine and feces samples of healthy individuals administered a known dose of PhIP. The initial step of the method utilized an acid hydrolysis to release Phase II conjugates. Strickland et al. found that the optimal conditions for releasing PhIP from urine conjugates was incubation at 90°C for 60 min at a final HCl concentration of 0.05–0.1 N (Strickland et al., 2001). For fecal samples a final HCl concentration of 0.5–0.6 N is required for optimal hydrolysis (unpublished data). During the next step of the method liquid/liquid extraction was applied to eliminate macromolecules from the urine and fecal matrices. In order to avoid problems due to emulsions and manipulation of the sample, the contact was increased between both liquids by the addition of a solid support of diatomaceous earth. After this initial purification, secondary purifications were designed to exploit the protonation of the heterocyclic nitrogen atoms common to PhIP and PhIP-M1 in an Oasis MCX cartridge, combining reversed-phase silica and cation-exchange mechanisms. During this final step, the removal of uncharged interference and concentration of the compounds was achieved.

Because of the complexity of the urine and fecal extracts and the overlapping retention times of the analytes and the internal standards, UV or fluorescence detection could not be used. Due to co-elution of hundreds of compounds into the mass spectrometer, detection of a signal above the background with single ion monitoring MS for the parent masses was only possible for a limited amount of samples (data not shown). Therefore, multiple MS detection was necessary for these analyses. An authentic standard of PhIP and a synthesized standard of PhIP-M1 were used to optimise the HPLC separation and fragmentation. The LC/MS/MS peak areas were linear over the range 0.25–100 µg/L with R² values of 0.999 and 0.997 for PhIP and PhIP-M1.

The method developed in this study using LC/MS/MS detects peaks for PhIP, the microbial metabolite PhIP-M1 and the deuterated internal standards in a single chromatographic run (Fig. 2) and has been successfully applied for urine as well as feces. Since other ion peaks are sometimes present in the chromatograms that are not PhIP or PhIP-M1 (Fig. 2), expected peak retention times were compared with the internal standards and calibration standards to identify PhIP and PhIP-M1. PhIP typically exhibits a sharp peak and a good signal-to-noise ratio (Fig. 2a). The internal standard [2H₃] PhIP elutes at the same time as the non-labeled product (Fig. 2b). PhIP-M1 is separated in time from PhIP and fragments into two daughter ions with masses 225 and 263. The sum of those two peaks is used for quantitation (Fig. 2c). The internal standard [2H₃] PhIP-M1 shows a similar profile as the natural product (Fig. 2d).

Fig. 2. Ion plots of PhIP, the microbial metabolite PhIP-M1 and the deuterated internal standards from hydrolyzed urine of subject B 8–16 h after consuming the well-done chicken. (a) Mass 210 peak plot after fragmenting mass 225, representing PhIP. (b) Mass 210 peak plot after fragmenting mass 228, representing the internal standard [2H₃] PhIP. (c) Sum of masses 225 and 263 after fragmenting mass 281, representing PhIP-M1. (d) Sum of masses 228 and 266 after fragmenting mass 284, representing the internal standard [2H₃] PhIP-M1.
3.2. Recovery and reproducibility

Spiking human urine and feces samples with increasing concentrations of $[^{2}H_{3}]$ PhIP and $[^{2}H_{3}]$ PhIP-M1 allowed us to determine the recovery of the compounds while optimizing the extraction protocol. Typical recoveries ranged from 74% to 83% for $[^{2}H_{3}]$ PhIP and 51% to 86% for $[^{2}H_{3}]$ PhIP-M1 in urine samples and from 51% to 59% for $[^{2}H_{3}]$ PhIP and 24% to 31% for $[^{2}H_{3}]$ PhIP-M1 in fecal samples (Table 1). Recovery of the internal standards was obviously better in water (81–100%) compared to urine and feces, indicating that the complexity of the urine and fecal matrices interferes with the efficiency of the solid phase extraction columns or lowers the sensitivity of the mass spectrometer through ion suppression.

Recovery using the optimized method for the kinetic samples was quantified by spiking each urine or fecal sample with the deuterium-labeled internal standards $[^{2}H_{3}]$ PhIP and $[^{2}H_{3}]$ PhIP-M1. Final PhIP and PhIP-M1 concentrations in each sample were adjusted based upon recovery of the internal standards in that sample. Because of the small peak sizes in our assay, there is variation inherent in the mass spectrometry detection. To account for this variation, each extract was injected three times and the peak areas averaged.

3.3. Microbial PhIP metabolite quantitation

Control urine and feces samples were collected from each of the six volunteers the day before the consumption of the well-done chicken, during the period that they abstained from eating cooked meat. PhIP was detectable in one of six control urine samples (72 ng/L) and in all six control feces samples (593 ± 342 ng/L). PhIP-M1 was detectable in two of six control urine samples (18 ± 14 ng/L) and in four of six control fecal samples (28 ± 9.7 ng/L). Because of the low concentrations detected in the control urine samples compared to the urine after chicken consumption, these background concentrations were not taken into account for quantification. The fecal pre-feeding concentrations were however a factor 10 higher and therefore a correction were made by subtracting the volume corrected pre-feeding amounts. Total urine and feces excreted after chicken consumption were collected for 72 h in 8 h increments for urine and 24 h increments for feces. Values shown are corrected for the total volumes of urine and feces. Fig. 3 shows the absolute dose percentages of PhIP and the microbial metabolite PhIP-M1 recovered in urine and feces for the six subjects. These varied from 12% to 21% for PhIP and 1.2% to 15% for PhIP-M1 in urine, and from 26% to 42% for PhIP and 0.9% to 11% PhIP-M1 in feces. No significant differences in absolute PhIP or PhIP-M1 dose percentage excreted could be observed for the different PhIP doses administered. Fig. 4 shows the rate of excretion of PhIP and the microbial PhIP metabolite for the respective time periods collected. Our results demonstrate that excretion rates for PhIP and PhIP-M1 vary among volunteers, but that most urinary PhIP (Fig. 4a) was excreted during the first 24 h, while for the microbial metabolite (Fig. 4c) the urinary excretion increased throughout time with a maximum between 48 and 72 h. Subject A however excreted only 1.2% of PhIP-M1 in urine. Fecal PhIP excretion (Fig. 4b) was the highest during the 24–48 h period for subjects A and E, whereas subjects C and F excreted most in the 48–72 h period. Subject’s B fecal PhIP excretion was almost equal all three days. Subject D excreted most PhIP during the first 24 h. Fecal PhIP-M1 excretion (Fig. 4d) was the highest during the 24–48 h period for subjects A, C and E; subject B excreted most during the first 24 h; subject F excreted most during the 48–72 h period. Subject D excreted almost equally all 3 days.

3.4. Salmonella mutagenicity data

As a positive control, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) gave 800–1130 revertants per 5 ng dose for TA98, 1300–1400 revertants per 0.2 µg dose for TA100 and 500–600 revertants per 1 µg dose for TA102. DMSO gave TA98 values of 20–40 revertant colonies per

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Table 1

Percent recovery of $[^{2}H_{3}]$ PhIP and $[^{2}H_{3}]$ PhIP-M1 spiked into water, urine or feces

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spike (ng)</th>
<th>Water</th>
<th>Urine</th>
<th>Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{2}H_{3}]$ PhIP</td>
<td>0.5</td>
<td>95.7 ± 3.1</td>
<td>77.7 ± 5.1</td>
<td>50.7 ± 15.6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>91.8 ± 12.8</td>
<td>74.0 ± 2.1</td>
<td>59.2 ± 29.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>99.4 ± 3.6</td>
<td>82.8 ± 8.9</td>
<td>52.0 ± 0.9</td>
</tr>
<tr>
<td>$[^{2}H_{3}]$ PhIP-M1</td>
<td>0.5</td>
<td>97.0 ± 1.2</td>
<td>85.9 ± 3.4</td>
<td>24.0 ± 12.0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>80.9 ± 12.5</td>
<td>51.1 ± 2.6</td>
<td>30.9 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>93.6 ± 3.3</td>
<td>57.8 ± 9.7</td>
<td>29.7 ± 3.1</td>
</tr>
</tbody>
</table>

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Fig. 3. Total 72 h excretion of urinary and fecal PhIP and PhIP-M1 for six individuals after ingesting a well-done chicken meal. The recovery-corrected sum of the amount of PhIP and PhIP-M1 (mean ± SD) detected in hydrolyzed fecal and urine samples are shown ($n = 3$).
plate, TA100 values of 140–170 revertant colonies per plate and TA102 values of 260–300 revertant colonies per plate.

Analysis of the mutagenic activity of PhIP-M1 using the Ames test with strains TA98, TA100 and TA102 without metabolic activation gave no positive result. S9-mediated analysis, gave a positive response (a positive slope for the dose–response curve) for strain TA98 and strain TA100 (Table 2). For each strain the revertant colonies per Petri plate were plotted against the mass equivalents of PhIP-M1 extract. The slope of this line was used to determine the mutagenic response (Table 2). Yet, a mutagenic potency for the microbial metabolite was measured of about 2–4% of that of PhIP (Table 2).

4. Discussion

The metabolism of PhIP has been well characterized in animal species (Buonarati et al., 1992; Davis et al., 1994) and several studies have been undertaken to examine the disposition of PhIP in humans (Kulp et al., 2000, 2004; Malfatti et al., 1999). Yet, little is known about the contribution of the intestinal microbiota to the overall metabolism of PhIP. The present study is the first to detect the excretion of a microbial PhIP metabolite in human urine and feces. The variation in microbial PhIP metabolism between six healthy human subjects, the kinetics of PhIP microbial metabolite excretion and the mutagenic activity of this newly identified microbial PhIP metabolite are reported.

Optimizing a solid phase extraction procedure for PhIP and its microbial metabolite encountered some difficulties due to the complexity of the urine and fecal matrices.
The Oasis MCX brand was selected because of its dual nature in retaining heterocyclic amines and was found superior in recovery compared to the various brands of C18 and cation exchange supports. Diatomaceous earth extract proved a suitable substrate for eliminating emulsion and manipulation problems and increasing contact between analytes and solvent (Galconan et al., 1996). Subsequent liquid/liquid extraction with dichloromethane achieved a significant decrease in matrix interferences without completely ruling out co-extracted impurities in the final sample. To retain as much analyte as possible, further washing steps were minimized and a satisfactory procedure was devised meeting our goal to quantify PhIP and PhIP-M1 in both urine and fecal samples. Urine and fecal samples were heated with acid prior to analysis in order to hydrolyze Phase II conjugates (Reistad et al., 1997; Stillwell et al., 1997). A large increase (7–10-fold) in the amount of PhIP detected following this acid treatment has been reported for urine (Lynch et al., 1992; Stillwell et al., 1997; Strickland et al., 2001) and indicates that acid-labile PhIP metabolites represent a major proportion of the PhIP in human urine. This has been confirmed in recent studies on the metabolism of ingested PhIP indicating that PhIP-N2-glucuronide, N2-OH-PhIP-N2-glucuronide and N2-OH-PhIP-N2-glucuronide are common metabolites in human urine (Kulp et al., 2004). Overall, the acid treatment enhances the amount of free PhIP and should provide an estimate of total mammalian PhIP metabolites excreted, without having to analyze each liver metabolite separately. Analysis of urine and feces samples as such have shown that acid hydrolysis does not affect the recovery of PhIP-M1 (data not shown), implying that PhIP-M1 is not conjugated by mammalian enzymes.

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 aromatic amines as compared with beef. Formation of PhIP seems to be favored by higher amounts of the amino acids phenylalanine, isoleucine, leucine and tyrosine and lower amounts of glucose that are present in chicken (Pais et al., 1999). Both the amounts of chicken consumed by our volunteers and the PhIP levels were comparable with consumption levels measured in households or restaurants.

It is unlikely that PhIP-M1 was formed de novo during hydrolysis in the urine or feces from PhIP. We spiked PhIP and PhIP-M1 in baseline urine and fecal samples and no production of PhIP-M1, respectively, PhIP was measured. Numerous publications describe the incubation of PhIP with liver hepatocytes or enzymes and none of them report the detection of a metabolite resembling PhIP-M1 (Zhao et al., 1994; Crofts et al., 1998; Turesky et al., 2002), whereas incubation of PhIP with specific intestinal bacterial species in the presence of glycerol and a protein rich feed source, does give rise to the formation of this metabolite (Vanhaecke et al., submitted for publication). Therefore our results confirm that the intestinal microbiota contribute to the overall metabolism and disposition of PhIP in vivo, although a high degree of interindividual variation in the urinary and fecal excretion exists. The percentage of the PhIP dose excreted in the 0–72 h hydrolyzed urine varied from 12% to 21% with an average of 15 ± 3.9% for PhIP and from 1.2% to 15% with an average of 5.7 ± 5.1% for its microbial metabolite PhIP-M1. Our findings for PhIP are comparable with data previously obtained by Strickland et al., where the average 24 h urinary excretion of PhIP (unchanged plus acid-labile conjugates) from individuals fed a uniform diet containing high-temperature cooked meat, amounted 17 ± 7.4% (Strickland et al., 2001). The percentages of the total PhIP dose excreted in this study as PhIP (26–42%) and PhIP-M1 (0.9–11%) in feces were surprisingly high and could explain the relatively low PhIP dose percentages measured in urine in previous metabolism studies of human subjects given PhIP in a meat matrix (Kulp et al., 2004; Strickland et al., 2001). The total percentage of the PhIP dose accounted for in the 72 h urine and feces as PhIP and PhIP-M1 varied among individuals from 49% to 71% with an average of 51 ± 8.8%. When N-OH-PhIP-N2-glucuronide, the major human N-oxidation metabolite of PhIP is hydrolyzed under acidic conditions, the deaminated product 2-OH-PhIP is formed. This derivate was not quantified during this study, but Stillwell et al. measured 2-OH-PhIP in urine collected from 66 subjects after ingestion of a meat-based meal and reported that 25 ± 8.4% of the ingested PhIP dose was excreted as 2-OH-PhIP in the 0–24 h urine (Stillwell et al., 2002). The formation of this hydroxylated derivate might explain the deficit in dose percentage encountered in this study. The variability in PhIP-M1 excretion can be explained by the interindividual variability in microbial community composition and activity between test subjects (Eckburg et al., 2005). In vitro incubation of PhIP with intestinal bacteria derived from stools freshly collected from healthy volunteers confirms these results, measuring PhIP transformation efficiencies from 37% to 90% within the first 24 h of incubation (Vanhaecke et al., 2006). Interindividual differences in microbial metabolic activities are not uncommon. A striking example is the microbial conversion of the dietary phytoestrogen daidzein (Decroos et al., 2005).

The kinetics of PhIP excretion in our study are similar to those previously observed for humans on a meat based diet (Stillwell et al., 1997; Strickland et al., 2001). Our results demonstrate that excretion times vary among the volunteers, but that 72 ± 27% of total PhIP excretion takes place in the first 24 h. Malfatti et al. is to our knowledge the only paper in which the kinetics of PhIP, in this particular case [14C]PhIP, were examined over a period of 72 h (Malfatti et al., 1999). In the latter study the subjects were hospitalized elderly cancer patients who were given PhIP in a gelatine capsule. This route of administration resulted in a recovery of 90% of the ingested dose in the urine and in all subjects the majority of the dose was excreted in the first 12 h. Our study consisted of younger men on their normal
diet, which was unrestricted except for refraining from meat consumption for the 72 h prior to dosing and during the course of the study. It is probable that the PhIP when formed in a meat matrix, is not as bioavailable as PhIP in capsule form. In addition, the interaction with additional foods and the resident microbiota in the gastrointestinal tract influences the absorption, distribution and as demonstrated here, the metabolism and excretion as well. The kinetics of microbial PhIP metabolite excretion showed a significant interindividual variability as well. Compared to PhIP, the microbial metabolite excretion was shifted in time, 35 ± 18% was excreted in the first 24 h, 33 ± 19% during the 24–48 h period and 32 ± 18% during the 48–72 h period. Microbial metabolites have indeed the tendency to appear later in excretion profiles of plasma and urine (Li et al., 2006; Watanabe et al., 1998).

In a final part of this study, we assessed the microbial genotoxicity of the newly identified PhIP metabolite. A weak activity was measured upon S9 activation amounting up to 2.7 ± 0.2% of the original PhIP mutagenic potency for TA98 and 4.5 ± 0.6% for TA100. As the PhIP-M1 extract was, despite of the preparative separation, not entirely pure (97 ± 0.8%), a residual fraction of PhIP in this extract might explain the weak mutagenic activity measured after S9 activation. Based on these results, the microbial transformation of PhIP may be considered as a detoxification. Further studies will focus on determining the in vitro and in vivo mammalian toxicology of this microbial PhIP derivate.

In summary, we have developed a method for quantifying PhIP and its newly identified microbial metabolite PhIP-M1 in urine and feces utilizing solid phase extraction and LC/MS/MS. This method allowed to detect PhIP and PhIP-M1 in urine and fecal samples collected from six volunteers following ingestion of a natural dose of PhIP. These findings suggest that besides individual differences in digestion, metabolism and diet, the microbial composition of the gastrointestinal tract also strongly influences individual disposition and carcinogenic risk from PhIP.

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