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Liquid chromatography–tandem mass spectrometry method of urine analysis for determining human variation in carcinogen metabolism

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Abstract

We developed a solid-phase extraction LC–MS–MS method for the analysis of the four major metabolites of PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) in human urine after a meal of well-done chicken. Ten volunteers each ate either 150 or 200 g of well-done chicken breast containing 9–21 μg of PhIP. Among the individual volunteers there is 8-fold variation in the total amount of metabolites and 20-fold variation in the relative amounts of individual metabolites, showing individual differences in carcinogen metabolism. PhIP metabolites were also detected in urine from a subject consuming chicken in a restaurant meal, demonstrating the method's sensitivity after real-life exposures. Published by Elsevier Science B.V.

Keywords: Amines, heterocyclic aromatic; Aminomethylphenylimidazo[4,5-*b*]pyridine; Pyridines; Glucuronides

1. Introduction

PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine) is a potent mutagen and rodent carcinogen formed in meats from natural precursors during the cooking process. PhIP is found at the highest levels in grilled or fried meats and is frequently the most mass abundant heterocyclic amine produced during the cooking of beef, pork, and chicken [1–5], and in meats cooked by professional chefs and purchased in restaurants [6,7]. 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]. Because humans are routinely exposed to varying amounts of these food-derived compounds there is a concern that they may play a role in human carcinogenesis.

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 to a hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-hydroxy-PhIP). *N*-hydroxy-PhIP is then converted to a more biologically reactive form via Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases. PhIP can also be hydroxylated at the 4′ position, forming 2-amino-1-methyl-6-(4′-hydroxy) phenylimidazo[4,5-*b*]pyridine (4′-hydroxy-PhIP). This hydroxylation does not produce an active intermediate. 4′-Hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted. Detoxification primarily involves glucuronidation. *N*-Hydroxy-PhIP can form stable glucuronide conjugates at either the N^2 or *N*3 positions. In addition, the parent compound can be directly glucuronidated at the N^2 and *N*3

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positions. These glucuronides are not reactive and are excreted in the urine.

There is conclusive evidence that PhIP, a genotoxic carcinogen, is involved in tumorigenesis in animals. In rats and mice, dose-dependent tumor formation has been consistently demonstrated after PhIP administration, and the most common tumor sites in the rat appear to be colon, prostate, and breast [9–14].

Less is known about the role of PhIP in human carcinogenesis. Until recently, studies of human PhIP metabolism have been limited to hepatic microsomes or cells in culture. Pioneering studies in in vivo human metabolism demonstrated the presence of PhIP and PhIP conjugates in human urine, but in these studies the urine was first treated with acid to hydrolyze the Phase II conjugates to the parent amine. These investigations proved that PhIP is bioavailable in humans, but did not give information about specific metabolic pathways [15,16]. Specific results about the identity of human PhIP metabolites were obtained in studies that investigated human PhIP metabolism following administration of ^{14}C-labeled PhIP to patients undergoing cancer surgery. We recently described human PhIP metabolism in cancer patients receiving a single dose of radiolabeled PhIP in a capsule. These studies identified four major human PhIP metabolites: N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, PhIP-4′-sulfate, and N^2-OH-PhIP-N3-glucuronide [17].

In the present study we describe our development of a solid-phase extraction LC–MS–MS method for quantifying the four most abundant PhIP metabolites in human urine, following a meal of well-cooked chicken. We applied this method to characterize PhIP metabolism in 10 healthy individuals receiving a known dose of naturally produced PhIP. We have also extended this method to monitor metabolite excretion in a subject consuming chicken as part of a restaurant meal, demonstrating that our method is sensitive enough to detect PhIP metabolites after common real-life exposures.

2. Material and methods

2.1. Synthesis of N^2-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide internal standard

The biological synthesis of deuterium labeled *N*-OH-PhIP-N^2-glucuronide was carried out in two steps as described previously [18]. Briefly, pentadeutero PhIP was reacted with baculovirus infected insect cell microsomes expressing human cytochrome P4501A2 (Gentest, Woburn, MA, USA) to produce the *N*-OH-[$^2H_5$-phenyl]PhIP intermediate. The reaction products were concentrated under N_2 and then isolated by HPLC using a Waters Alliance HPLC system equipped with a 5 μm, 220×4.6 mm TSK-Gel ODS-80 TM column (TosoHaas, Montgomeryville, PA, USA). Metabolites were detected using a Waters 990 photodiode array detector. The *N*-OH-[$^2H_5$-phenyl]PhIP was eluted at 1.0 ml/min using a gradient starting at 30% aqueous methanol, 0.1% triethylamine, pH 6, to 55% aqueous methanol, 0.1% triethylamine, pH 6, at 8 min. The methanol concentration was maintained at 55% from 8 to 20 min. After evaporation of the mobile phase, the yield of *N*-OH-[$^2H_5$-phenyl]PhIP from [$^2H_5$-phenyl]PhIP was approximately 40%.

Purified *N*-OH-[$^2H_5$-phenyl]PhIP was reacted with microsomes derived from the AHH-1 TK+/-human lymphoblastoid cell line which expresses human UDP-glucuronosyltransferase 1A1 (Gentest). The *N*-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide was isolated and purified by HPLC using the conditions described above to give a 15% yield from *N*-OH-[$^2H_5$-phenyl]PhIP.

2.2. Study design

The study protocol was reviewed and approved by the Institutional Review Board for Human Research at Lawrence Livermore National Laboratory. Informed consent was obtained from each subject prior to beginning the study. The individuals participating were recruited from the local workforce, were males and females aged 22–45 years, in good health, nonsmokers, and of normal weight.

2.3. Meat preparation and controlled dietary period

Boneless, skinless chicken breasts were cut into approximately 2.5 cm pieces and fried for 25 to 35 min in a non-stick coated pan sprayed with a vegetable-based cooking spray. Pan temperature averaged 186°C for the cooking period. At the end of the cooking time the chicken was white with some

browning. PhIP analysis was performed according to previously published methods [19].

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. The first two study subjects were provided with 200 g chicken containing 105 ng/g PhIP. The total PhIP dose was 21 μg. Subjects three to eight were given 200 g of chicken containing 94 ng/g PhIP, for a total dose of 18.8 μg. The remaining two subjects were given 150 g of chicken containing 62 ng/g PhIP, for a total dose of 9.2 μg. All subjects were provided with other non-meat foods and beverages with the cooked chicken.

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 (greater than 1 year) have shown no noticeable change in metabolite levels.

2.4. PhIP metabolite analysis after a restaurant meal

To test the sensitivity of detection of this method, one subject ordered and consumed chicken that was prepared as ''chicken mango'' at a local restaurant. The subject ate approximately 80 g of grilled chicken containing 33 ng/g of PhIP (a portion of the entrée was reserved and later analyzed using previously published methods [19]). Urine was collected for approximately 4 h, 4–8 h after eating the meal.

2.5. 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, USA). Metabolites were eluted with 5 ml methanol. The elution aliquot was evaporated to dryness under nitrogen and the metabolites were re-dissolved in 2.5 ml 0.01 *M* HCl. Proteins and high-molecular-mass contaminants were removed by filtering the solution through a Centricon YM-3 centrifugal filter (Millipore, Bedford, MA, USA). The samples were centrifuged in the filter at 3000 *g*, overnight. The filtrate was applied to a pre-conditioned benzenesulfonic acid column (SCX, 500 mg, Varian, Harbor City, CA, USA) and the column washed with 6 ml of 10% (v/v) methanol in 0.01 *M* aqueous HCl. The metabolites were eluted onto a coupled C_{18} column (Baker-bond spe, 1000 mg, J.T. Baker, Phillipsburg, NJ, USA) with 0.05 *M* ammonium acetate, pH 8. The C_{18} column was washed with 3 ml of methanol–water (5:95, v/v) and eluted from the C_{18} column with methanol–water (50:50, v/v). The metabolites were dried under nitrogen and 1 ml urine equivalent was injected into the LC–MS–MS in a volume of 20 μl.

Chromatography was done on a Microtech Ultra-Plus HPLC system (Sunnyvale, CA, USA) equipped with a YMC ODS-A column (250×3.0 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, and a linear gradient to 100% B at 30 min and held for 5 min.

Analytes were detected with an ion trap mass spectrometer (model LCQ, Finnigan, San Jose, CA, USA) in the MS–MS positive ion mode using an electrospray interface. The capillary temperature was 240°C and the spray voltage was 4.5 kV. The sheath gas was set at 70 units and no auxiliary gases were used. The ion trap injection time was 1000 ms and a setting of one microscan was used.

Alternating scans were used to isolate $[M+H]^+$ ions at mass 417, 401, and 321 for natural PhIP metabolites, and 422, for the pentadeutero-labeled internal standard metabolite. Collision energy was 25%. Daughter ions were detected at appropriate masses: 241 [M+H-glucuronic acid]$^+$ and 225 [M+H-glucuronic acid-OH]$^+$ from 417 for the *N*-hydroxy-N^2 and *N*3 glucuronide, respectively, 225 [M+H-glucuronic acid]$^+$ from 401 for the PhIP N^2 glucuronide, 241 $[M+H\text{-}SO_3]^+$ from 321 for PhIP-4′-sulfate, and 246 [M+H-glucuronic acid]$^+$ and 230 [M+H-glucuronic acid-OH]$^+$ from 422 for the internal standard, *N*-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide. An external standard of naringenin was used in later samples, its $[M+H]^+$ ion isolated at mass 273 with protonated fragments detected at mass 147, 153, and 185.

2.6. 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-[$^2H_5$]PhIP-N^2-glucuronide. Final metabolite amounts were adjusted based on the recovery of the internal standard. The effect of the urine matrix on the recovery of the metabolites 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.

Ion suppression in the mass spectrometer by co-eluting interferences was investigated by spiking human urine extracts with mouse urine containing high levels of metabolites. In our method, the *N*-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide is used as a surrogate standard for all of the metabolites because of the structural similarity of the metabolites and our belief that it is representative of the other metabolites, within the precision of other aspects of our assay. An external standard of naringenin added to later samples shows that ion suppression is consistent and suppresses the signal by 65% compared to the external standard injected alone.

Replicate analyses of several different urine samples were made during the course of the study to determine the precision of the assay. The coefficient of variation was approximately 28% for urine extractions and LC–MS–MS, with much of the variation occurring in the LC–MS–MS instrument. Consequently, samples were injected three times and the results averaged.

3. Results and discussion

3.1. Method development and urine analysis

The goal of this work 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 utilizes non-specific adsorption to remove all the metabolites from the water and salts in the urine. Other materials were tried in preliminary work, such as C_4, C_8, and C_{18} solid-phase extraction materials and styrenedivinylbenzene medium packed into columns, but none recovered all four metabolites as well as the polymeric material in the Oasis columns.

Our initial attempts at sample clean-up resulted in samples that did not chromatograph well. Poor HPLC column lifetime, peak broadening, and increasing retention time for two of the metabolites, N^2-OH-PhIP-N^2-glucuronide and PhIP-N^2-glucuronide were the symptoms of this problem. Suspecting that urinary proteins and larger molecule contaminants were the cause of some of these symptoms, they were removed by centrifuging the extracts through a filter with a molecular mass cut-off of 3×10^6. 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 improved HPLC column lifetimes somewhat. After the centrifugation step, further purifications exploited the protonation of the heterocyclic nitrogen atoms that are common to the all the metabolites. This ion-exchange adsorption step was designed to remove uncharged interferences. Finally, the urine extract was concentrated and washed on reversed-phase silica.

To monitor the recovery of the metabolites through the method, a deuterium-labeled internal standard is added to the urine before extraction. Typical recoveries range from 37 to 40%. Final metabolite levels for each sample were adjusted based upon the recovery of the internal standard in that sample. Because of the small peak sizes in the 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 samples ranged from 20 to 30%.

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. Mass spectrometry must be employed.

Urine samples from rodents receiving high doses of PhIP were used to optimize the HPLC separation and the 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, which is approximately 20-fold higher than the limit of detection. Internal calibration curves

were calculated for each metabolite based upon rodent urine spiked into a human urine matrix. R^2 values were: N^2-OH-PhIP-N^2-glucuronide, 0.9703, PhIP-N^2-glucuronide, 0.978, PhIP-4′-sulfate, 0.999, and N^2-OH-PhIP-N^3-glucuronide, 0.9954.

Further, because of the 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 (Fig. 1A). MS–MS detection is necessary for these analyses. Fig. 1B shows a human urine sample analyzed by LC–MS–MS, showing peaks for the fragments of four metabolites after the isolation of the parent masses.

Volunteers are asked to refrain from eating meat for 24 h before eating the cooked chicken, and a control urine sample is collected at the end of the meat-free period. A chromatogram that represents a typical sample of control urine is provided in Fig. 2A. No metabolite peaks are seen at the retention times of PhIP metabolites. Fig. 2B represents urine from the same individual, collected during the first 6 h after consuming the chicken. Peaks are clearly seen for each of the four PhIP metabolites.

Fig. 3 shows the percentage that each individual metabolite represents of the total of all metabolites excreted over 24 h for 10 individuals. The N^2-OH-PhIP-N^2-glucuronide was the major metabolite in all cases. PhIP-N^2-glucuronide is the second most abundant, but the ratio of these two metabolites varies from almost equal amounts for subject 2 to 9-fold more N^2-OH-PhIP-N^2-glucuronide in subject 6. With the exception of subject number 10, N^2-OH-PhIP-N^2-glucuronide and PhIP-N^2-glucuronide together account for 90% or greater of the total metabolite excreted. Subject 10 excreted a much higher proportional amount of N^2-OH-PhIP-N3-glucuronide (22%) in contrast to the other individuals, in whom N^2-OH-PhIP-N3 glucuronide accounted for 7% or less of the total metabolite excreted. The time of excretion of metabolites also varies (data not shown), with some individuals excreting most of the metabolites in the 0–6 h time period and some later, in the 6–12 h time period. Little or no metabolite is detected in the 18–24 h time period.

To extend our method to real-life exposures, we collected urine from an individual who had consumed chicken as part of a restaurant meal. Fig. 4 shows the LC–MS–MS chromatogram of a urine extract collected 4–8 h after consuming the meal. Peaks for all four metabolites and the deuterium-labeled internal standard can be detected.

Our method provides an opportunity to study a genotoxic dietary carcinogen at realistic levels in humans. PhIP is of special interest because it causes tumors in animals that 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.

Several different types of studies can be supported by this analysis method. Relative amounts of PhIP metabolites can be used to determine individual metabolic phenotype. The effect of diet on carcinogen metabolism can be determined by controlled feeding studies that analyze the changes in the relative amounts and time of excretion of metabolites. Urine metabolites can also be quantified for individuals on a normal diet, to monitor for exposure levels.

The enzymes known to be involved in the metabolism of PhIP are found at varying levels and activities within the human population [20]. The expression of specific activating enzymes has a great affect on the biological reactivity of PhIP. We believe that the N^2-OH-PhIP-N^2-glucuronide and N^2-OH-PhIP-N3-glucuronide metabolites represent the metabolic products of activation pathways, whereas the PhIP-N^2-glucuronide and 4′-PhIP-sulfate represent detoxification pathways. The variation that we detect in these metabolites suggests that the levels of both activation and detoxification enzymes varies among individual volunteers and may be a way to quantify individual phenotype or genotype. Using our method to generate a metabolic profile could provide an indication of potential susceptibility to DNA damage, mutation, and cancer.

On possible mechanism for the protective effects of fruits and vegetables seen in human cancer studies is the influence of natural compounds on both primary and secondary metabolism. This suggests that the metabolism of carcinogens, including PhIP, can be modified by the addition of protective foods to the diet. Our method provides an invaluable tool for monitoring the effect dietary interactions on PhIP

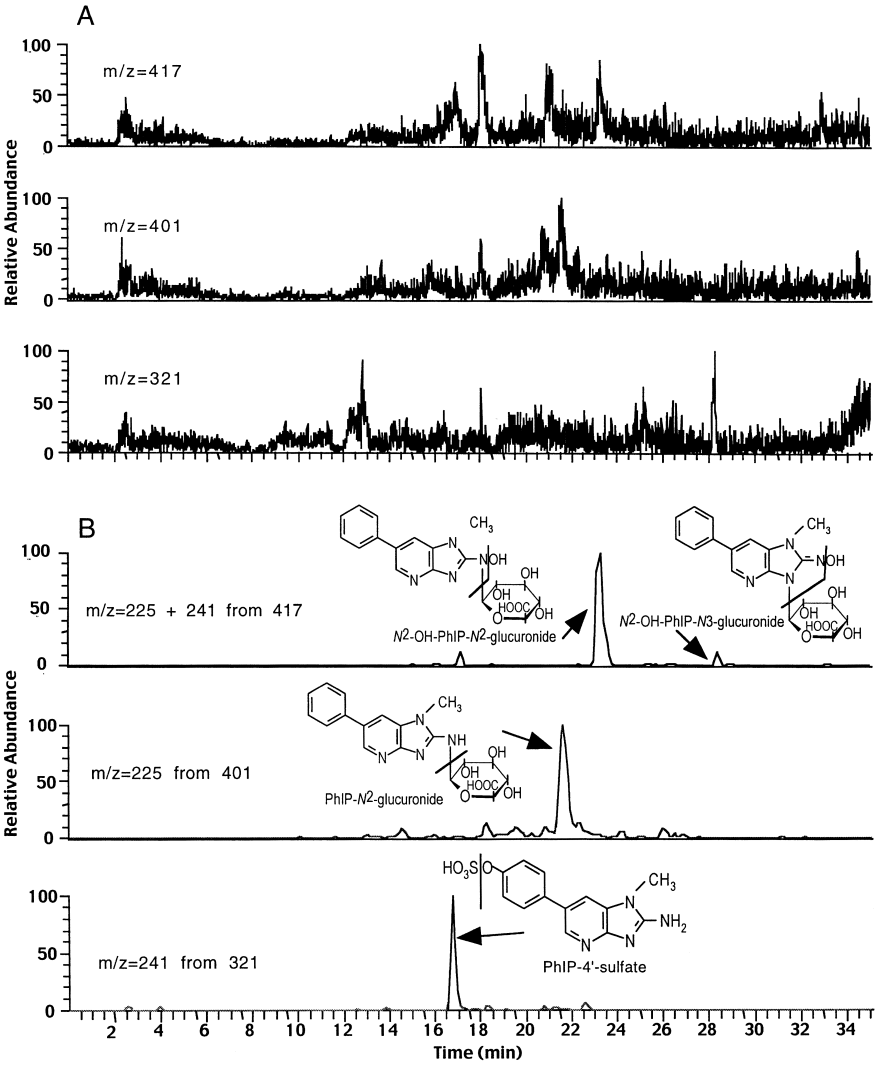

Fig. 1. Reversed-phase HPLC mass chromatograms of urine extract. (A) shows full scan plot of the m/z 417, 401 and 321 corresponding to PhIP metabolites. (B) MS–MS chromatograms of the human urine sample with masses isolated as indicated. Peaks are clearly seen for four metabolites indicated by arrows. Chemical structures and a line indicating the site of fragmentation for each structure are shown.

metabolism. These effects on metabolism can be quantified in humans at normal dietary levels using our method.

Determining the dietary dose of PhIP is important for epidemiology studies and risk determination. Typically, exposure estimations are made through dietary questionnaires. However, the formation of PhIP is variable, and the amount in foods depends on the cooking methods. Dietary surveys have several flaws, including bias, inconsistent reporting, and most importantly, the difficulty in quantifying cooking doneness via questionnaire. As a result, dietary surveys give varying estimates of PhIP amounts that may or may not reflect actual exposures. PhIP metabolite detection in the urine of the subject who ate chicken prepared at a restaurant demonstrates that our method is sensitive enough to monitor PhIP exposure of individuals in real-life situations.

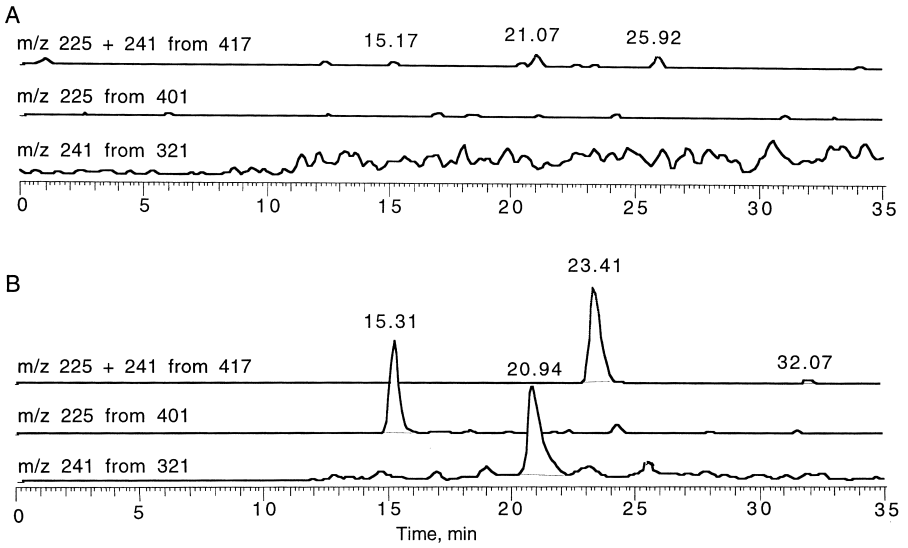

Fig. 2. LC–MS–MS chromatograms of urine from a subject abstaining from well-done meat for 24 h (A), and urine collected 0–6 h after consumption of well-done chicken (B).

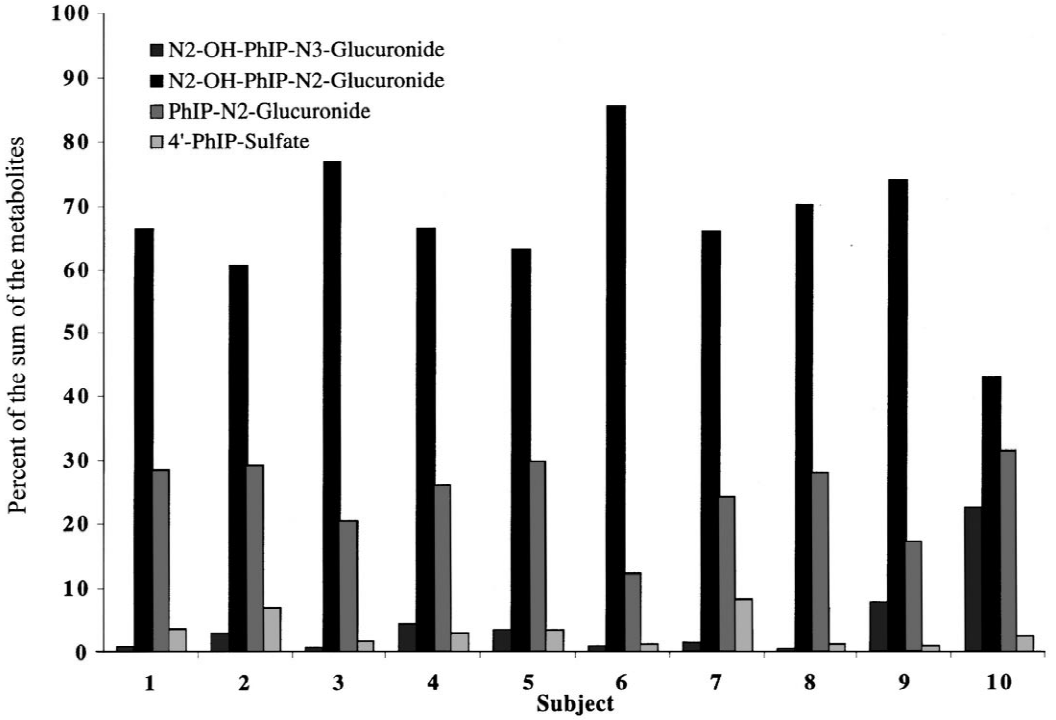

Fig. 3. Graph of individual PhIP metabolites excreted over 24 h from 10 individuals eating a single meal of well-done chicken.

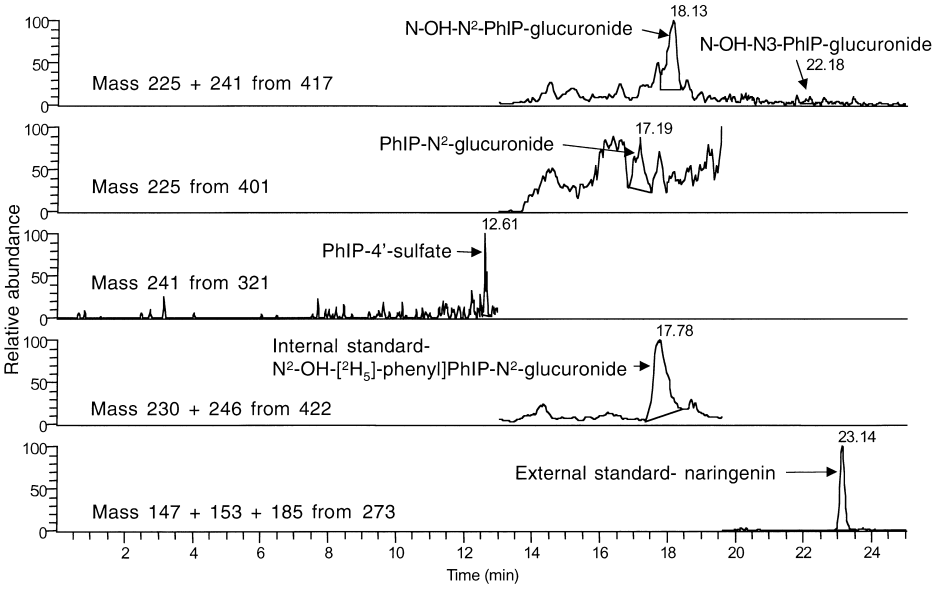

Fig. 4. LC–MS–MS mass chromatograms of urine collected after consumption of a restaurant meal of grilled chicken. Peaks identified are at the retention time of metabolites or the added internal or external standard. The equivalent of 2 ml of urine and 5 ng of internal standard were injected.

Future studies will focus on improving the method by increasing the sensitivity of metabolite detection, allowing us to lower the amount of food containing PhIP given to the volunteers. Reducing the analysis time and variation for the LC–MS–MS analysis are also needed. 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. The method described here should make studies of individual susceptibility and dietary interventions possible in the future.

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