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Mass Spectrometric Characterization of an Acid-Labile Adduct Formed with 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and Albumin in Humans

Yi Wang^{†,||}, Peter W. Villalta[†], Lijuan Peng[‡], Karen Dingley[§], Michael A. Malfatti[§], K.W. Turteltaub[§], and Robert J. Turesky^{*,†,||}

[†]Masonic Cancer Center, University of Minnesota, 2231 6th Street, Minneapolis, MN 55455, USA

^{||}Department of Medicinal Chemistry, Cancer and Cardiovascular Research Building, University of Minnesota, 2231 6th Street, Minneapolis, MN 55455, USA

[‡]School of Food Science and Engineering, Wuhan Polytechnic University, ChangQing Garden, Hankou, Wuhan 430023, P.R. China

[§]Biosciences and Biotechnology Division, Center for Accelerator Mass Spectrometry Lawrence Livermore National Laboratory, Livermore 94550, CA

Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a carcinogenic heterocyclic aromatic amine formed during the high temperature cooking of meats. The cytochrome P450-mediated *N*-hydroxylation of the exocyclic amine group of PhIP produces 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HONH-PhIP), an electrophilic metabolite which forms adducts with DNA and proteins. Previous studies conducted by our laboratory showed that the reaction of N-oxidized-PhIP metabolites with human albumin *in vitro* occurs primarily at the Cys³⁴ residue, to produce an acid-labile linked sulfenamide adduct. Based on these findings, we developed a sensitive ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) method to measure acid-labile albumin-PhIP adducts in humans volunteers administered a dietary-relevant dose of ¹⁴C-labelled-PhIP (Dingley *et al.*, Cancer Epidemiol. Biomarkers Prev. 8:507–512). Mild acid-treatment of albumin (0.1N HCl, 37 °C for 1 h) or proteolytic digestion with pronase (50 mM ammonium bicarbonate buffer pH 8.5 at 37 °C for 18 h) released similar amounts of covalently bound PhIP, which was characterized by multistage scanning (MS³) and quantified by Orbitrap mass spectrometry. The amount of ¹⁴C-PhIP recovered by acid-treatment of albumin 24 hour following dosing accounted for 7.2 to 21.3% of the ¹⁴C-PhIP bound to albumin based on accelerator mass spectrometry measurements. 2-Amino-1-methyl-6-(5-hydroxy)-phenylimidazo[4,5-*b*]pyridine (5-HO-PhIP), a hydrolysis product of the Cys³⁴ S–N linked sulfenamide adduct of PhIP, was not detected in either acid-treated or protease treated samples.

***Corresponding Author:** Robert J. Turesky, Masonic Cancer Center and Department of Medicinal Chemistry, Cancer and Cardiology Research Building, University of Minnesota, 2231 6th Street, Minneapolis, MN 55455, USA. Tel.: +1 612-626-0141; fax: +1 612-624-3869.

Supporting Information

Reconstructed ion chromatograms of PhIP, [²H₃C]-PhIP, and [¹⁴C]-PhIP standards. Product ion spectra of [²H₃C]-PhIP. Calibration curve of PhIP in plasma extract. Product ion spectra of [¹⁴C]-PhIP and [²H₃C]-PhIP recovered from human plasma. This material is available free of charge via the Internet at <http://pubs.acs.org>.

These findings suggest that a portion of the PhIP bound to albumin *in vivo* probably occurs as an acid labile-sulfonamide adduct formed at the Cys³⁴ residue.

TOC image

Keywords

Carcinogens; heterocyclic aromatic amines; serum albumin adducts; protein adducts

INTRODUCTION

Heterocyclic aromatic amines (HAA) are produced during the high-temperature cooking of meats, poultry, and fish, and some HAA are formed during the combustion of tobacco.^{1,2} 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass-abundant carcinogenic HAA formed in well done cooked red meats.^{1,3-5} PhIP undergoes metabolic activation by cytochrome P450 (P450) to form 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HONH-PhIP), a genotoxic metabolite capable of reacting with DNA and proteins.⁶ The Working Group of the International Agency for Research on Cancer (IARC) recently classified red meat as probably carcinogenic to humans (Class 2A). The classification was based on a large body of epidemiological observations and mechanistic studies in humans and animal studies for the induction of colorectal cancer.^{6,7} However, imprecise estimates of exposure and the lack of physicochemical evidence that mutagens formed in cooked meat induce DNA damage and mutations in target organs are critical missing links in the published literature and vital to performing a complete evaluation of the consumption of cooked meat and the risk of developing cancer.⁸

The biomonitoring of DNA adducts represents the most definitive biomarker to assess the biologically effective dose and genotoxic potential of a chemical.^{8,9} However, the measurement of DNA adducts is often limited because of the lack of tissue samples. Moreover, the repair of DNA adducts can diminish the levels of DNA adducts, and the measurement of DNA adducts remains a challenging endeavor even by the most sensitive of mass spectrometry (MS) instruments.⁶ The use of blood protein-carcinogen adducts is an alternative approach to biomonitor exposures to hazardous chemicals.¹⁰⁻¹² Stable blood protein adducts do not undergo repair *in vivo*, and can accumulate during chronic exposure, and facilitate detection. In the case of PhIP, the same genotoxic metabolites that adduct to albumin are involved in DNA adduct formation.⁶ The albumin adduct levels are thus not only a measure of exposure but also of bioactivation of PhIP.

Human albumin contains one reduced cysteine residue – the Cys³⁴; a strong nucleophile at physiological pH ($pK_a = \sim 6.5$) and scavenger of a variety of electrophiles.^{12,13} Early studies reported that the Cys³⁴ residues of mouse or rat albumins reacted with the N-oxidized metabolites of several HAA, including 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ),¹⁴ 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline,¹⁵ and PhIP.¹⁶ In the case of IQ, the sulfenamide adduct formed with albumin in the rat, was isolated, digested with pronase and characterized by mass spectrometry. The ¹H-NMR spectrum of the corresponding adduct formed *in vitro* with rat albumin was identified as a tripeptide C*PY containing N²-cysteinylsulfinyl-IQ.¹⁴ A model peptide containing sequence homology surrounding Cys³⁴ of rat albumin was shown to react with the N-acetoxy derivative of PhIP to form a sulfur-nitrogen linked sulfenamide adduct, when characterized by ion trap MS.¹⁷ We employed ion trap MS to characterize human albumin adducts formed with N-oxidized metabolites of PhIP *in vitro*, followed by digestion of the modified protein with a mixture of trypsin and chymotrypsin.^{18–21} The primary adduct formed occurred at Cys³⁴ and the peptides were identified as ³¹LQQC*PF³⁶ and the single missed-cleaved ³¹LQQC*PFEDHVK⁴¹ sulfenamide (C-[SO]-PhIP); the ³¹LQQC*PFEDHVK⁴¹ sulfonamide (C-[SO₂]-PhIP) was identified as a minor adduct.^{18,20} The hydrolysis of the albumin sulfenamide adduct of PhIP occurs under conditions sufficiently mild that the albumin is not degraded,^{14,20} and the released PhIP can be readily separated from the macromolecular protein and assayed by sensitive MS based methods. The hydrolysis of albumin modified with N-oxidized metabolites of PhIP, by pronase digestion or mild acid treatment, resulted in the recovery of PhIP, which was principally derived from the sulfenamide (C-[SO]-PhIP) linked adduct.^{19,20} The ring-oxidized product 2-amino-1-methyl-6-(5-hydroxy)-phenylimidazo[4,5-*b*]pyridine (5-HO-PhIP) was also identified in the proteolytic digest of N-(acetoxy)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-acetoxy-PhIP) adducted albumin, suggesting a sulfenamide adduct of PhIP had formed at the Cys³⁴ of albumin (Scheme 1).^{19,21,22}

Two studies used accelerator mass spectrometry (AMS) to measure ¹⁴C-PhIP blood protein adduct formation in humans.^{23,24} Both studies reported that PhIP bound poorly to hemoglobin, but levels approaching up to ~ one percent of the dose bound to albumin. However, the structures of the adducts were not determined. The Airoldi laboratory developed gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods to measure acid-labile albumin adducts of PhIP in a cohort from Italy.²⁵ PhIP was detected in approximately 30 percent of the subjects. The mean level of albumin adducts of PhIP was significantly higher in meat consumers than in vegetarians (6.7 vs. 0.7 fmol/mg albumin). A more recent study conducted with a cohort from the United Kingdom did not reproduce the findings obtained from the Italian study. The authors concluded that acid-labile albumin adducts of PhIP or other HAA were not formed at levels sufficient to be detected by LC-MS/MS, and the proposed albumin sulfenamide adducts of HAA were unlikely to be viable biomarkers of HAA exposure.²⁶

Based on our understanding of the chemistry of reactivity of N-oxidized metabolites of PhIP with human albumin *in vitro*,^{18–21} we sought to determine if acid-labile albumin adducts of PhIP are formed in humans. We used our characterized ³¹LQQC*PFEDHVK⁴¹ sulfenamide (C-[SO]-PhIP) as a reference standard, and developed a sensitive UPLC-MSⁿ method,

employing high resolution accurate mass spectrometric measurement with Orbitrap MS, to characterize and measure the PhIP released from albumin purified from humans, following mild acid hydrolysis or proteolytic digestion. The samples were drawn from the Dingley study, where human volunteers were administered a single dose of [^{14}C]-PhIP.²⁴

MATERIALS AND METHODS

Caution

PhIP is a carcinogen and should be handled in a well-ventilated fume hood with the appropriate protective clothing.

Chemicals and Materials

PhIP, 2-amino-1- $^{2}\text{H}_3\text{C}$ -methyl-6-phenylimidazo[4,5-*b*]pyridine ($^{2}\text{H}_3\text{C}$ -PhIP, 99% isotopic purity), and ^{14}C -PhIP (10 mCi/mmol) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Human serum albumin, pronase E, *meta*-chloroperoxybenzoic acid (*m*-CPBA), hydrochloric acid (HCl), and LC-MS grade formic acid were purchased from Sigma-Aldrich (St. Louis, MO). LC-MS grade solvents were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were ACS grade, and purchased from Sigma-Aldrich unless stated. Isolute C18 solid-phase extraction (SPE) columns (25 mg) were from Biotage (Charlotte, NC). SOLA HRP solid-phase extraction (SPE) columns (10 mg) were from Thermo Fisher Scientific (Rockford, IL). Amicon Ultra centrifugal filter units (10,000 mw cutoff) were from Millipore (Billerica, MA). Human plasma was purchased from Bioreclamation LLC (Hicksville, NY). Reference peptide LQQCPFEDHVK was purchased from New England Peptide (Gardner, MA). N-Oxidized derivatives of PhIP were synthesized as previously reported.^{20,21,27}

Human Protocol

The details of this study were previously reported.²⁴ Four volunteers were administered a dietary-equivalent of [^{14}C]-PhIP (70 – 84 μg , at a specific activity of 42 or 56 mCi/mmol) before colon cancer surgery. At various times up to 24 h after dosing, blood samples (30 mL) were collected and separated into plasma, RBCs, and buffy coat by centrifugation, and immediately stored at -80°C . Plasma samples were stored on dry-ice and transported to the University of Minnesota for chemical analyses. For reference, the dose of PhIP that the subjects in this study received was approximately equivalent to eating 175 g of very well-done chicken,⁵ and the radioactive dose was <1% of an individual's yearly exposure to background sources of radiation. The protocol for this study was approved by the human subject review boards at Lawrence Livermore National Laboratory, the University of Arkansas Medical School Hospital, and the J. L. McClellan Memorial Veterans Administration Medical Center in Little Rock, AR.

AMS Analysis

The carbon-14:carbon-13 ratios of albumin samples were assayed previously as described.²⁴ For subjects 1 and 2, AMS analyses were performed and reported as the mean $\pm$ standard deviation of four separate analyses. The AMS analyses for subjects 7 and 10 were derived from single values ($\pm$ standard deviation of the AMS measurement).

Syntheses of the PhIP Sulfonamide and Sulfonamide Adducts of LQQC*PFEDHVK

The protocol for the synthesis of sulfonamide and sulfonamide adducts was described previously.²⁸ Briefly, LQQCPFEDHVK (20 nmol, 250 μ L in 10 mM potassium phosphate buffer pH 8.5) was modified with 2-nitroso-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (NO-PhIP) (60 nmol, 50 μ L in MeOH) at 5 °C for 1 h. LQQC*PFEDHVK sulfonamide (C-[SO]-PhIP) was formed by adding 250 μ L 50 mM potassium phosphate buffer pH 3.6 to the reaction mixture, which agitated at 37 °C for 1 h. LQQC*PFEDHVK sulfonamide (C-[SO₂]-PhIP) was formed by oxidation of LQQC*PFEDHVK sulfonamide (C-[SO]-PhIP) by *m*-CPBA at a molar ratio of 1:1 at 37 °C for 1 h. LQQC*PFEDHVK sulfonamide (C-[SO]-PhIP) and sulfonamide (C-[SO₂]-PhIP) were purified by HPLC (Acquasil C18, 5 μ m, 4.6 $\times$ 250 mm, Thermo Fisher) with a 25 min gradient from 100% A (5% ACN and 95% H₂O) to 100% B (100% ACN), at a flow rate of 1 mL/min. The concentrations of LQQC*PFEDHVK sulfonamide and sulfonamide were estimated by the UV absorbance of the PhIP chromophore at 315 nm (molar extinction coefficient, 22,220 M⁻¹ cm⁻¹).²⁹ Structures of LQQC*PFEDHVK sulfonamide and sulfonamide were confirmed by sequencing and multistage scanning (MSⁿ).^{18,20}

Modification of Commercial Human Albumin with HONH-PhIP in vitro

Mixed disulfides formed at Cys³⁴ of albumin were reduced by treatment with β -mercaptoethanol.¹⁴ Albumin was modified with HONH-PhIP at a molar ratio of 1:3, at 37 °C for 18 h. Unbound HONH-PhIP and its decomposition products were removed by liquid extraction with 2 $\times$ volume of ethyl acetate, twice. To fully convert the LQQC*PFEDHVK sulfonamide (C-[SO]-PhIP) to the LQQC*PFEDHVK sulfonamide (C-[SO₂]-PhIP), the modified human albumin was oxidized by *m*-CPBA at a molar ratio of 1:1, at 37 °C for 5 h.²¹ The solution was subjected to a buffer exchange (450 μ L of 100 mM potassium phosphate buffer, pH 7.4, 2-times) using centrifugal filters (10,000 mw cutoff).

Purification of Albumin from Human Plasma

Albumin was purified from human plasma samples using the Pierce Albumin Depletion Kit. Briefly, human plasma samples (200 μ L) were desalted using centrifugal filters (10,000 mw cutoff) with 25 mM potassium phosphate buffer pH 7.4, twice. Desalted human plasma was applied to affinity resins (500 μ L vol), which were equilibrated in 25 mM potassium phosphate buffer pH 7.4. The resins bound with albumin were rinsed with 500 μ L 25 mM potassium phosphate buffer, pH 7.4 (three times), and eluted with 1 mL of 25 mM potassium phosphate buffer, pH 7.4 containing 1.5 M potassium chloride. The elutes were desalted using centrifugal filters (10,000 mw cutoff).

Acid Hydrolysis of Peptide Adducts of PhIP or Albumin-PhIP Adducts from Human Plasma

The PhIP products unbound to albumin were removed by solvent extraction with 2 $\times$ volume of ethyl acetate, twice. Thereafter, the synthetic peptides LQQC*PFEDHVK sulfonamide (C-[SO]-PhIP) or sulfonamide (C-[SO₂]-PhIP) (200 ng in 90 μ L H₂O), or albumin purified from human plasma containing [¹⁴C]-PhIP (10 mg albumin in 90 μ L 25 mM potassium phosphate buffer, pH 7.4) was acidified with 10 μ L 1 N HCl. [²H₃C]-PhIP was spiked into the mixture as an internal standard at level of 500 fg [²H₃C]-PhIP/mg albumin. The mixture was agitated

at 37 °C for 1 h. Then, the mixture was made alkaline with 1N NaOH (15 μ L), and the PhIP released from albumin was extracted by CH₃OH or ethyl acetate. For the CH₃OH extraction method, pre-chilled CH₃OH (900 μ L, –80 °C) was added to the mixture to precipitate the proteins at –20 °C for 2 h. The solution was centrifuged at 18,000 g for 20 min, and the supernatant was transferred to a 2 mL Eppendorf tube, vacuum centrifuged for 30 min to remove CH₃OH, and resuspended in H₂O (1 mL). For the ethyl acetate extraction method, ethyl acetate (1 mL) was added to the mixture. After vortexing, the upper layer containing the organic fraction was transferred to an Eppendorf tube, vacuum centrifuged to dryness, and resuspended in H₂O (1 mL). The mixture was applied to a SOLA HRP cartridge (10 mg). The cartridge was rinsed with 10% CH₃OH (2 mL), and PhIP was eluted with CH₃OH (1 mL). The elute was vacuum centrifuged to dryness. The levels of adducts measured were the same for both clean-up methods (data not shown); however, the extraction with ethyl acetate provided cleaner chromatograms and was used for all in vivo studies.

Proteolytic Digestion with Pronase E

Peptide or protein adducts were digested with pronase E (1:2 w/w) in 50 mM ammonium bicarbonate buffer pH 8.5 at 37 °C for 18 h. The mixture was applied to an Isolute C18 SPE column. Polar peptides or amino acids were removed with 10% CH₃OH (2 mL) and adducts were eluted with CH₃OH (1 mL), followed by concentration to dryness by vacuum centrifugation.

Stability of PhIP-Albumin Adducts in Human Plasma *in vitro*

Human plasma containing [¹⁴C]-PhIP (150 μ L) was diluted with an equal volume of 100 mM potassium phosphate buffer (pH 7.4) and incubated at 37 °C for 0 h, 6 h, 1 d, 3 d or 7 d. HONH-PhIP modified commercial human albumin (150 pmol) was added to 100 mM potassium phosphate buffer (pH 7.4, 1 mL) or commercial human plasma (1 mL) and also incubated overtime. Procedures for albumin purification and ensuing acid hydrolysis of PhIP adducts are the same as those conditions reported above. The PhIP products unbound or released from albumin were removed by solvent extraction with 2× volume of ethyl acetate, twice, prior to acid hydrolysis of the remaining PhIP bound to albumin, followed by isolation of the released PhIP using the sample clean-up by SPE as described above.

Measurement of PhIP and 5-HO-PhIP by Ultraperformance Liquid Chromatography-Electrospray Ionization-Multistage Mass Spectrometry (UPLC-ESI/MSⁿ)

The acid hydrolysis products of albumin PhIP adducts were assayed by UPLC-ESI/MSⁿ employing a Dionex Ultimate 3000 LC and an Orbitrap Elite™ Hybrid Ion Trap-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Following SPE, the extracts were resuspended in 10% DMSO:90% H₂O (25 μ L), and 5 μ L was loaded on to a trapping column (Thermo Fisher Scientific Acclaim PepMap 100 C18, 100 Å, particle size 5 μ m, 300 μ m × 5 mm) with 99% A (1% ACN, 0.01% HCO₂H) at a flow rate of 5 μ L/min for 6 min. The products were back flushed onto a Michrom C18 column (0.1 × 150 mm, 3 μ m particle), resolved with a 25 min linear gradient from 99% A (1% CH₃CN, 0.01% HCO₂H) to 99% B (95% CH₃CN, 0.01% HCO₂H) at a flow rate of 1 μ L/min. Chromeleon 7.2 Chromatography Data System was used for the UPLC management. The Advance CaptiveSpray (Michrom Bioresource, Auburn, CA) was employed as the ion source and MS

parameters were set as follows: capillary temperature, 270 °C; ionization voltage, 2 kV at positive ion mode; 1 μ scan; maximum injection time, 100 ms for MS and 200 ms for MSⁿ; MS fragmentation used a normalized collision energy of 45%; no auxiliary and sheath gases were used. Resolution was set as 60,000 at m/z 400 for selected ion monitoring (SIM), and 15,000 at m/z 400 for MS² and MS³. The isolation width was set at m/z 1 for MS² and MS³ scan modes, and the activation Q was set at 0.3. AGC (automated gain control) was set at 50,000 for Orbitrap (FT) MS² and MS³. The Orbitrap was routinely calibrated in positive ion mode using Pierce LTQ Velos ESI Positive Ion Calibration Solution (2 μ g/mL caffeine, 1 μ g/mL MRFA, 0.001% Ultramark 1621 and 0.00005% *n*-butylamine).

Calibration Curve and Quantitation of PhIP

Seven calibrant points (0, 50, 100, 250, 500, 1000, 1250 fg PhIP or 5-HO-PhIP/mg albumin) were constructed using unlabeled PhIP and 5-HO-PhIP, which were added to the extract post-cleanup of acid-treated commercial albumin purified from plasma. [²H₃C]-PhIP and 5-HO-[²H₃C]-PhIP were added internal standards at a level of 500 fg/mg albumin. PhIP and [²H₃C]-PhIP were measured at the MS³ scan stage using product ions at m/z 225.1 or 228.1 > 210.1 > 168.0682 and 183.0791 for PhIP, and [¹⁴C]-PhIP was measured at MS³ scan stage using product ions at m/z 227.1 > 212.1 > 168.0682, 183.0791 and 185.0823. 5-HO-PhIP, 5-HO-[²H₃C]-PhIP and 5-HO-[¹⁴C]-PhIP were measured at MS³ scan stage using product ions at m/z 241.1 > 223.1 > 196.0867, 208.0742; m/z 244. 1 > 226.1 > 198.0902, 210.0776; and m/z 243.1 > 225.1 > 199.1058, 208.0742, respectively. The calibration curves were generated with unlabeled PhIP and 5-HO-PhIP because the [¹⁴C]-PhIP used for the human studies was largely carrier free (42 or 56 mCi/mmol; 67 or 90% labeled, respectively) and no longer available. Given that the signals of response of PhIP and [¹⁴C]-PhIP (10 mCi/mmol) are comparable, when the specific activity of the radioisotope is accounted for (Supporting information, Figs S-1A – S-1D), with little background noise in the MS³ scan stage chromatograms, we used PhIP as a substitute calibrant to estimate the acid-labile [¹⁴C]-PhIP recovered from the albumin *in vivo*. This also minimized the contamination of radioactive PhIP in the mass spectrometer.

Method Validation

The assay was validated by the accuracy and within-day and between-day reproducibility using human albumin modified with HONH-PhIP at a known level of acid-labile adduct and diluted with commercial human plasma to arrive at a level of adduction of 675 fg PhIP/mg albumin. This level of PhIP adduction to albumin was close to the average level [¹⁴C]-PhIP bound to albumin reported in the AMS study of Dingley.²⁴ The reproducibility studies were based on four independent measurements conducted on three different days. The accuracy and percent coefficient of variation (%CV) were used as criteria for the performance and reproducibility of the method.

RESULTS

The AMS studies conducted by Dingley reported that up to ~0.2 percent of the dose of [¹⁴C]-PhIP administered to volunteers had bound to albumin; however, the structure(s) of the adduct(s) and stability towards acid (or base) were not reported.^{23,24} The levels of [¹⁴C]-

PhIP bound to albumin were estimated, by AMS, at ~ 500 pg per g albumin (~1.5 PhIP molecules bound per 10⁷ molecules of albumin),²⁴ a level of modification far too low to directly measure the tryptic or chymotryptic peptide adduct. We sought to quantify the amount of [¹⁴C]-PhIP bound to albumin in humans that could be recovered as [¹⁴C]-PhIP or 5-HO-[¹⁴C]-PhIP, following mild acid treatment, as would be expected for the PhIP sulfinamide or sulfenamide linkages (Scheme 1). We examined the stability of S-N linked adducts of the sulfinamide and sulfenamide adducts of with LQQC*PFEDHVK formed with HONH-PhIP following treatment with acid or pronase; however, the S-N linked adduct of the sulfenamide LQQC*PFEDHVK formed with *N*-acetoxy-PhIP was too unstable to isolate and characterize. Thereafter, we examined the recovery of acid-labile adducts of albumin modified *in vitro* with HONH-PhIP. Subsequently, we measured for [¹⁴C]-PhIP and 5-HO-[¹⁴C]-PhIP hydrolysis product(s) from *in vivo* samples of purified albumin of volunteers of the Dingley study.²⁴

Mass Spectra of PhIP, [²H₃C]-PhIP and [¹⁴C]-PhIP Standards

The product ion spectra of the synthetic PhIP analogues were acquired online by UPLC/MSⁿ. The ion chromatograms of protonated PhIP ([M+H]⁺ at *m/z* 225.1135) and [¹⁴C]-PhIP ([M+H]⁺ at *m/z* 227.1167) are shown in Fig. S-1. The ratios of peak areas of [¹⁴C]-PhIP to PhIP by SIM and MS² scan stage were 21.2 ± 0.2% and 20.8 ± 0.1%, respectively (Figs S-1A – S-1D). Since the maximum specific activity available for carrier free molecule containing a single ¹⁴C label is 62.4 mCi, high resolution accurate mass measurements of the [¹⁴C]-PhIP/[¹²C]-PhIP ratio correspond to a specific activity of 13.2 and 13.0 mCi/mmol in SIM and MS², respectively. The ratio of [¹⁴C]-PhIP to PhIP at the MS³ scan stage was 19.1 ± 0.4%, or 12.1 mCi/mmol (Fig. S-1E and S-1F), which is consistent with the specific activity of [¹⁴C]-PhIP reported by the vendor (10 mCi/mmol). There was no overlap of signals of the reconstructed ion chromatograms of [²H₃C]-PhIP with either PhIP or [¹⁴C]-PhIP at the MS² and MS³ scan stages (Supporting information, Fig. S-2), when employing an isolation width of 1 *m/z* for MS² and MS³, ensuring specificity of the transitions of PhIP analogues used for assay.

The product ion spectra of PhIP and [²H₃C]-PhIP obtained by collision induced dissociation (CID), (Fig 1) show a major product ion at *m/z* 210.0897 (observed vs. calculated 210.0900, 1.4 ppm) as the base peak, and the [¹⁴C]-PhIP undergoes CID to form a major fragment ion at *m/z* 212.0928 (observed vs. calculated 212.0932, 1.9 ppm). These ions are attributed to the loss of CH₃[•] for PhIP and [¹⁴C]-PhIP, and the loss of [²H₃C][•] from the trideuterated internal standard (Figs 2A–2C), an observation in agreement with the literature.³⁰ A minor product ion at *m/z* 208.0874 (observed vs. calculated 208.0875, 0.5 ppm) for PhIP, *m/z* 211.1065 (observed vs. calculated 211.1063, 1.0 ppm) for [²H₃C]-PhIP, or *m/z* 210.0906 (observed vs. 210.0907, 1.5 ppm) for [¹⁴C]-PhIP results from the loss of NH₃ (Figs 2A–2C). Consecutive reaction monitoring of the radical cation at *m/z* 210.09 for PhIP and [²H₃C]-PhIP at the MS³ scan stage produces an ion at *m/z* 183.0787 (observed vs. calculated 183.0791, 2.2 ppm, loss of HCN), *m/z* 168.0680 (observed vs. calculated 168.0682, 1.1 ppm, loss of H₂CN₂), and *m/z* 167.0601 (observed vs. calculated 167.0604, 1.8 ppm, loss of H₃CN₂[•]) (Figs. 2D and 2E). Similarly, the product ion spectrum acquired for the *m/z* 212.0932 of [¹⁴C]-PhIP includes ions at *m/z* 168.0680, *m/z* 167.0600, *m/z* 183.0786

attributed to the loss of H^{14}CN , and an ion at m/z 185.0823 (observed vs. calculated 185.0823, 0 ppm, loss of HCN).

Characterization of the Hydrolysis Products of LQQC*PFEDHVK Sulfinamide and Sulfonamide Adducts of PhIP

The LQQC*PFEDHVK sulfinamide (C-[SO]-PhIP) undergoes hydrolysis under mild acidic pH conditions to produce PhIP and the cysteine sulfinic acid containing peptide.^{19,20} The LQQC*PFEDHVK sulfinamide (100 pg = 63 fmol) was subjected to acid hydrolysis in 0.1N HCl for 1 h or pronase E digestion for 18 h. [$^2\text{H}_3\text{C}$]-PhIP (200 fg) was spiked as an internal standard prior to hydrolysis. PhIP was quantitatively recovered after acid hydrolysis, whereas 51 fmol of PhIP was recovered from the LQQC*PFEDHVK sulfinamide following digestion with pronase E (Table 1). The lower recovery of PhIP by digestion of the modified albumin with pronase E was because the enzyme also produced some C* and C*PF sulfinamide adducts.^{18,21}

The LQQC*PFEDHVK sulfinamide (C-[SO]-PhIP) linkage can be oxidized to the acid-stable sulfonamide linkage (C-[SO₂]-PhIP), by treatment of the adducted peptide with *m*-CPBA.²⁰ We examined the stability of the sulfonamide linkage towards the same acid hydrolysis or proteolytic digestion conditions described above. PhIP was not recovered by either the acid or pronase treatment of LQQC*PFEDHVK sulfonamide (C-[SO₂]-PhIP). The digestion with pronase produced the tripeptide adduct C*PF sulfonamide (C-[SO₂]-PhIP) (Table 1). These data demonstrate that the S-N linkage of the LQQC*PFEDHVK sulfinamide (C-[SO]-PhIP) is labile, and the adduct can be quantitatively hydrolyzed to PhIP by acid treatment or by pronase E digestion. In contrast, S-N linkage of the LQQC*PFEDHVK sulfonamide (C-[SO₂]-PhIP) is stable and does not undergo hydrolysis to form PhIP.

Acid Hydrolysis and Proteolytic Digestion of Commercial Human Albumin Modified with HONH-PhIP with or without Oxidation of *m*-CPBA

LQQC*PF and LQQC*PFEDHVK sulfinamide (C-[SO]-PhIP) are major adducts identified in HONH-PhIP modified albumin following digestion with trypsin/chymotrypsin.²⁸ *m*-CPBA oxidizes the sulfenamide and sulfonamide-PhIP linked adducts to the stable sulfonamide (C-[SO₂]-PhIP).²⁸ Here we measured the recovery of PhIP from HONH-PhIP-modified human albumin without or with *m*-CPBA-pretreatment, followed by acid hydrolysis or proteolytic digestion with pronase E.

To ensure that the PhIP recovered by hydrolysis was attributed to covalently bound PhIP-albumin adducts, a liquid extraction was performed with 2× volume of ethyl acetate followed by sample cleanup with filtration, which effectively removes all unbound PhIP spiked into a solution of albumin.²¹ In the HONH-PhIP-modified albumin, 88 ± 0.8 and 64 ± 14.6 (fmol PhIP/pmol albumin) PhIP was recovered after acid hydrolysis and proteolytic digestion with pronase E, respectively (Table 2). By comparison, only 1.3 ± 0.1 and 3.3 ± 0.3 (fmol PhIP/pmol albumin) was recovered from the *m*-CPBA oxidized albumin modified with HONH-PhIP (Table 2). These data recapitulate the findings observed with LQQC*PFEDHVK (C-[SO]-PhIP and C-[SO₂]-PhIP) peptides: the sulfinamide adduct of

PhIP in albumin is labile under acidic pH conditions, while sulfonamide adduct of PhIP is stable towards acid hydrolysis.

Validation of the Method

The levels of PhIP used for the calibration curve were chosen based on the level of [^{14}C]-PhIP bound to albumin *in vivo* by AMS measurements.²⁴ Seven calibrant points, ranging from 0, 50 up to 1250 fg PhIP/mg albumin, were constructed for the calibration curve. [$^2\text{H}_3\text{C}$]-PhIP was used as the internal standard and spiked at level of 500 fg/mg albumin. The linearity of calibration curve was confirmed by the slope ($s = 1.03 \pm 0.0072$) and linear regression value ($r^2 = 0.9997$) (Supporting information, Fig. S-3). The [^{14}C]-PhIP of high specific activity used for the human study was no longer available, therefore, the calibration curves, limit of detection (LOD), and limit of quantification (LOQ) values were determined with unlabeled PhIP. The ions at m/z 168.0682 and 183.0791 (and the ion at m/z 185.0823 for [^{14}C]-PhIP) were used to determine the LOD and LOQ. Since no measurable ions m/z 168.0682, 183.0791, and 185.0823 were observed in the unspiked sample at the MS³ scan stage using high-resolution Orbitrap (5 ppm mass tolerance), the LOD and LOQ were estimated by the formula $3.3\sigma/s$ and $10\sigma/s$, respectively (σ is the standard deviation of the slope (s) of calibration curve).^{31,32} The LOD was estimated at 11 fg PhIP/mg albumin, and the LOQ was estimated at 33 fg PhIP/mg albumin. Approximately 12–13 scans were acquired across the full width of the peak of the calibrant spiked with the lowest amount of PhIP (50 fg PhIP/mg albumin). The spiking of commercial albumin extracts with [^{14}C]-PhIP (10 mCi/mmol) provided similar responses at the LOD and LOQ values (data not shown).

The amount of plasma available from treated volunteers was limited, thus we conducted the validation of the analytical method with commercial human plasma spiked with *in vitro* HONH-PhIP modified human albumin, which was diluted with commercial plasma containing non-detectable levels of PhIP modified albumin. The level of modification was 675 fg PhIP/mg albumin, a level that is close to those levels of [^{14}C]-PhIP bound to albumin *in vivo* by AMS measurement.²⁴ The method of validation was done with four independent measurements of HONH-PhIP modified albumin on three different days, following acid hydrolysis (Table 3). The accuracy was $94.7 \pm 2.5\%$. The SD values for the within-day and between-day precision were 15% and 14%, respectively. The reproducibility studies were further conducted with samples of albumin obtained from two volunteers dosed with [^{14}C]-PhIP and based on three independent measurements on two different days (Table 4). The values of within-day and between-day precision (%CV) were 3.9 and 6.9% for volunteer 2, and 6.6 and 7.2% for volunteer 10, respectively.

Acid Hydrolysis of Albumin and Measurement of [^{14}C]-PhIP Bound Albumin in Human Volunteers

We employed the UPLC-ESI/MS³ method to measure [^{14}C]-PhIP recovered from hydrolysis of albumin purified from plasma of four volunteers. The specific activity of [^{14}C]-PhIP in capsules taken by human volunteers was 42 or 56 mCi/mmol (67% and 90% labeled, respectively).²⁴ We monitored [^{14}C]-PhIP by SIM (m/z 220 – 230) following SPE of the acid hydrolyzed extract of albumin (0.1 N HCl, 37 °C for 1 h) (Supporting information Fig. S4). The mass chromatograms at the SIM stage of (Fig. S4A, C, E) [^{14}C]-PhIP ($[\text{M}+\text{H}]^+$ m/z

227.1167), and (S4B, D, F) [$^2\text{H}_3\text{C}$]-PhIP ($[\text{M}+\text{H}]^+$ m/z 228.1323 with mass tolerances set at 2, 5 or 10 ppm (Fig. S4) revealed high levels of isobaric interferences, particularly in the m/z window for [^{14}C]-PhIP, even at 60,000 mass resolution (Supporting information Fig. S4A, C, E). Thus, multistage scanning (MS^2 and MS^3) was used to characterize and identify [^{14}C]-PhIP recovered from albumin. The reconstructed ion chromatograms of [^{14}C]-PhIP and [$^2\text{H}_3\text{C}$]-PhIP at MS^2 and MS^3 stages from acid hydrolyzed albumin (0.1 N HCl, 37 °C for 1 h) obtained from commercial plasma and plasma of a volunteer who was administered with [^{14}C]-PhIP are shown in Fig. 2. Since the [^{14}C]-PhIP used in the study was largely carrier free, unlabeled PhIP was not monitored. The product ions of [^{14}C]-PhIP were not detected in the extract of commercial human plasma (Figs 2A and 2E), but the ions of [^{14}C]-PhIP were readily detected in plasma from subjects at 24 h following dosing (Figs 2B and 2F). Some isobaric interferences are seen in the reconstructed ion chromatogram of [^{14}C]-PhIP at the MS^2 scan stage (scan resolution 15,000) even when the mass tolerance is set at 2 ppm (Fig. 2B). The isobaric interference is greatly reduced in the reconstructed ion chromatogram at the MS^3 scan stage (Fig. 2F). The mass spectra of [^{14}C]-PhIP and its internal standard [$^2\text{H}_3\text{C}$]-PhIP in human plasma are shown in Supporting information, Fig. S5. The product ion spectra at the MS^2 and MS^3 scan stages are in excellent agreement to the mass spectra of the reference standards (Fig. 2).

Kinetics and Recovery of [^{14}C]-PhIP Bound to Albumin in Human Volunteers

A kinetics experiment was conducted *in vitro* to determine the effect of temperature and duration of acid hydrolysis on the recovery of [^{14}C]-PhIP. In a plasma sample of a volunteer, which was collected 24 h after dosing, [^{14}C]-PhIP was below the LOD prior to treatment of albumin with acid or pronase E digestion (Fig. 3). The amount of acid-labile [^{14}C]-PhIP released from albumin was estimated at a level of 16 fg/mg albumin following acid hydrolysis (0.1 N HCl) at 37 °C for 30 min, and the amount of the released [^{14}C]-PhIP peaked at ~54 fg/mg albumin at 60 min (Fig. 3). The level of [^{14}C]-PhIP was not significantly different in the samples after hydrolysis at 37 °C for 60 and 180 min (Fig. 3), or following acid hydrolysis at 65 °C for 180 min (data not shown, $p > 0.05$).

Levels of [^{14}C]-PhIP Adduct Formation with Albumin in Humans

The levels of [^{14}C]-PhIP in albumin purified from the plasma of four volunteers following acid hydrolysis or proteolytic digestion with pronase E is reported in Table 5. The amount of acid labile [^{14}C]-PhIP bound to albumin ranged from 28 to 57 fg/mg albumin collected 24 h after dosing (T_{24}). These estimates were between 7.2 and 21.3% of the amount of [^{14}C]-PhIP bound to albumin based on AMS measurements performed when the samples were originally collected. We also examined for 5-HO- ^{14}C -PhIP following acid or pronase treatment of albumin, using 5-HO- $^2\text{H}_3\text{C}$ -PhIP as an internal standard. Since the same amounts of [$^2\text{H}_3\text{C}$]-PhIP and 5-HO- $^2\text{H}_3\text{C}$ -PhIP injected for UPLC/MS analysis yield similar ion counts of peak areas at MS^3 scan stage (with no isobaric interferences), we estimate the values of LOD and LOQ values of 5-HO- ^{14}C -PhIP to be close to that of PhIP and [^{14}C]-PhIP. 5-HO- ^{14}C -PhIP was below the LOD in human plasma of these subject (< 11 fg/mg albumin) (Fig. S-6).

Stability of [^{14}C]-PhIP-Acid Labile Albumin Adducts

The stability of the presumed [^{14}C]-PhIP-sulfonamide adduct formed *in vivo* was examined over time *in vitro* at 37 °C, following a 2-fold dilution with commercial human plasma and measurement, by UPLC-ESI/MS³, of the remaining acid-labile [^{14}C]-PhIP bound to albumin over seven days. We acknowledge that these samples were stored for nearly 20 years at -80 °C, and the stability of acid-labile adducts over this prolonged period of storage has not been determined. The level of acid-labile [^{14}C]-PhIP bound to albumin decreased by 17% and 39% after human plasma was incubated for 6 h and 24 h, and then by 80% after 7 days, under neutral pH conditions (Fig. 4). The curve approximates a first-order rate decay, and the half-life of labile [^{14}C]-PhIP bound to albumin is estimated at ~50 h. However, the inclusion of the last time point at 7 days may indicate that the rate of decay is biphasic. Additional time points would be required to fully characterize the decay curve of the labile [^{14}C]-PhIP albumin adduct. We also incubated HONH-PhIP modified with albumin *in vitro* and diluted it in human plasma; the level of PhIP bound to albumin (primarily as the sulfonamide) also decreased at a similar rate to the adduct formed *in vivo* (data not shown). These data suggest that the acid-labile albumin adducts of PhIP may have a shorter half-life *in vivo* than albumin, which is approximately 20 days.³³

DISCUSSION

The Cys³⁴ of rodent and human albumin reacts with N-oxidized metabolites of PhIP,^{16,20,21} and many other electrophiles to form adducts.¹² UPLC-MSⁿ characterization of proteolytic digest of human albumin modified *in vitro* with N-oxidized metabolites of PhIP revealed that the Cys³⁴ residue is the major site of adduction and forms the sulfonamide adduct with PhIP (Scheme 1).^{20,21} We measured the presumed Cys containing sulfonamide (C-[SO]-[^{14}C]-PhIP) formed *in vivo* indirectly, following mild acid treatment or pronase digestion of the purified albumin. Acid or pronase treatment results in hydrolysis of the sulfonamide linkage and regenerates PhIP. The C* sulfonamide (C-[SO₂]-PhIP) adduct is stable toward acid or protease treatment, and does not produce PhIP. The proposed the S-N linked adduct of the PhIP sulfenamide undergoes hydrolysis by mild acid treatment or proteolysis to produce both PhIP and 5-HO-[^{14}C]-PhIP (Scheme 1). However, we did not detect 5-HO-[^{14}C]-PhIP in the human samples. In contrast, 5-HO-PhIP was identified as a major product recovered in pronase hydrolysates of rat albumin reacted with *N*-acetoxy-PhIP.¹⁶ Our laboratory also identified 5-HO-PhIP in the pronase digest of albumin recovered from human hepatocytes treated with PhIP,²¹ and from human albumin treated with *N*-acetoxy-PhIP *in vitro*, but not with albumin modified with HONH-PhIP.^{18,21} Thus, we deduce that a large portion of the acid labile [^{14}C]-PhIP bound to albumin in humans is likely present as the sulfonamide linked adduct, which is formed by reaction of nitroso-PhIP with Cys³⁴ of albumin (Scheme 1).^{18,21} However, these plasma samples were stored at -80°C ever since the study was conducted in 1997 – 1998,²⁴ a labile [^{14}C]-PhIP sulfenamide may have undergone hydrolysis over this long time period.

Our studies on the characterization of the reactivity of the N-oxidized metabolites of PhIP with albumin *in vitro* revealed that >60% of the PhIP bound to albumin was acid-labile and recovered as PhIP (Scheme 1).²⁰ A very high percentage of this acid labile PhIP bound to

albumin occurs at Cys³⁴ since pretreatment of albumin with 4-chloromercuribenzoic acid, a selective agent that binds to Cys³⁴ of albumin,³⁴ abolished most of the PhIP species bound as an acid labile albumin adduct. The percent of [¹⁴C]-PhIP bound to albumin in humans and recovered as PhIP, following protein purification and acid treatment, is considerably lower (7.2 – 21.3%) than those levels observed with albumin modified *in vitro* with N-oxidized PhIP metabolites. We note that the stability of the sulfinamide C-[SO]-[¹⁴C]-PhIP has not been assessed under prolonged storage conditions and some portion of the adduct may have undergone hydrolysis. A portion of the sulfinamide C-[SO]-[¹⁴C]-PhIP also could have undergone oxidation to form the acid-stable sulfonamide C-[SO₂]-[¹⁴C]-PhIP during prolonged storage (Scheme 1).¹⁹ Other sites of PhIP adduct formation with albumin also occur. *N*-sulfooxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-sulfooxy-PhIP), a likely genotoxic metabolite of PhIP formed in humans,³⁵ reacts *in vitro* with the sole tryptophan (Trp²¹⁴) residue of human albumin to form a minor adduct that is recovered by trypsin digestion as AW*[PhIP]AVAR.²¹ Another adduction product was detected at His residue(s) following pronase digestion of albumin modified with *N*-sulfooxy-PhIP.²¹ The proposed structures of these adducts are depicted in Figure 5. Neither adduct undergoes proteolysis to regenerate PhIP, although the chemical stability of the adducts towards acid treatment was not investigated.²¹ The amounts of AW*[PhIP]AVA and His albumin adducts formed *in vivo* may be larger than those which occur *in vitro*. Because of the very large amounts of non-modified peptides in the proteolytic digest of *in vivo* samples, we do not yet have selective enrichment methods to isolate Trp and His adducts of PhIP for measurement by UPLC-ESI/MSⁿ.

An LC-MS/MS method with a triple quadrupole MS was recently developed to quantify the level of HAAs released from acid treatment of albumin in humans: the LOD and LOQ values reported for PhIP were 125 and 375 fg PhIP/mg albumin, respectively (the albumin content in human plasma ~40 mg/mL).²⁶ The authors concluded that the formation of acid-labile albumin HAA adducts was very low and would not be viable biomarkers to biomonitor HAA exposure in humans. However, the volunteers were on a free-choice diet, and the amount of PhIP ingested was not known. Our sensitive UPLC-MSⁿ method employing the high resolution accurate mass measurement of the Orbitrap at the MS³ scan stage has superior sensitivity to the triple quadrupole MS method; the LOD and LOQ values are 11 and 33 fg PhIP/mg albumin, respectively. Our kinetic studies *in vitro* with [¹⁴C]-PhIP bound to albumin in isolated plasma reveal the adduct has a half-life of about 50 h, which is considerably less than the ~20 day half-life of albumin in humans.³³ A kinetics study measuring PhIP albumin adduct formation and decay over time with healthy subjects on a diet containing known amounts of PhIP is required to determine if the acid labile PhIP albumin adduct can be implemented in human biomonitoring studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AMS	accelerator mass spectrometry
βME	β -mercaptoethanol
IQ	2-amino-3-methylimidazo[4,5- <i>f</i>]quinoline PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
5-HO-PhIP	2-amino-1-methyl-6-(5-hydroxy)-phenylimidazo[4,5- <i>b</i>]pyridine
N-acetoxy-PhIP	<i>N</i> -(acetyloxy)-2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
HONH-PhIP	2-hydroxyamino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
NO-PhIP	2-nitroso-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
N-sulfooxy-PhIP	<i>N</i> -sulfooxy-2-amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine
Hb	hemoglobin
HAA	heterocyclic aromatic amines
NATs	<i>N</i> -acetyltransferases
MS	mass spectrometry
<i>m</i>-CPBA	<i>meta</i> -chloroperoxybenzoic acid
SIM	selected ion monitoring
SPE	solid phase extraction
SULTs	sulfotransferases
UPLC-MSⁿ	ultraperformance liquid chromatography-multistage scan mass spectrometry

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Figure 1.

Product ion spectra of (A) PhIP ($[M+H]^+$ m/z 225.1 >), (B) $[^2H_3C]$ -PhIP ($[M+H]^+$ m/z 228.1 >), and (C) $[^{14}C]$ -PhIP ($[M+H]^+$ m/z 227.1 >) of $[^{14}C]$ -PhIP and $[^2H_3C]$ -PhIP reference standards at the MS² scan stage. Product ion spectra of (D) PhIP ($[M+H]^+$ m/z 225.1 > 210.1 >), (E) $[^2H_3C]$ -PhIP ($[M+H]^+$ m/z 228.1 > 210.1 >), and (F) $[^{14}C]$ -PhIP ($[M+H]^+$ m/z 227.1 > 212.1 >) of $[^{14}C]$ -PhIP and $[^2H_3C]$ -PhIP standards at MS³ scan stage. The m/z values shown in the structure scheme are the calculated values. The m/z values shown in the mass spectra are the observed values.

Figure 2.

Reconstructed ion chromatograms at MS² stage of (A, B) $[^{14}\text{C}]\text{-PhIP}$ ($[\text{M}+\text{H}]^+$ m/z 227.1 > 212.0932) recovered from human plasma of volunteer 2 given a capsule containing 56 mCi/mmol $[^{14}\text{C}]\text{-PhIP}$. Reconstructed ion chromatograms at MS³ stage of (E, F) $[^{14}\text{C}]\text{-PhIP}$ ($[\text{M}+\text{H}]^+$ m/z 227.1 > 212.0932 > 168.0682, 183.0791, 185.0823) recovered from *in vivo* human plasma. (C, D, G, H) $[^2\text{H}_3\text{C}]\text{-PhIP}$ were spiked to the samples as internal standards. The mass extraction window was ± 5 ppm.

Figure 3.

Kinetics study on acid hydrolysis of [¹⁴C]-PhIP-albumin obtained following purification of albumin from plasma of volunteer 10 given a capsule containing 42 mCi/mmol [¹⁴C]-PhIP. Each time point was measured in triplicate and reported as the mean $\pm$ SD. Transitions of [¹⁴C]-PhIP were monitored ($[M+H]^+$ m/z 227.1 > 212.0932 > 168.0682, 183.0791, 185.0823). The mass extraction window was $\pm$ 5 ppm.

Figure 4.

The level of labile [^{14}C]-PhIP remaining bound to albumin of volunteer 7 given a capsule containing 42 mCi/mmol [^{14}C]-PhIP as a function of time when the human plasma was incubated at 37 °C in vitro, following dilution with commercial plasma. Values represent the mean $\pm$ SD ($n=3$).

PhIP-Trp **PhIP-His****Figure 5.**

Proposed structures of PhIP adduct formed at Trp²¹⁴ and His residues of albumin. Mass spectral data support structures formed between the C-2 indole atom of Trp and the C-2 imidazole atom of histidine with the N-oxidized NH₂-group of PhIP.²¹

Scheme 1.

Formation of albumin (Alb) sulphenamide, sulfinamide and sulfonamide adducts formed with N-oxidized metabolites of PhIP, and proposed mechanism of hydrolysis of the linkages to produce PhIP and 5-HO-PhIP. NATs, N-acetyltransferases; SULTs, sulfotransferases.

Table 1

Amounts of PhIP recovered from LQQC*PFEDHVK sulfinamide and sulfonamide peptides after acid hydrolysis or digestion with pronase E.

	No treatment	Acid Hydrolysis	Pronase E Digestion
	fmol	fmol	fmol
LQQC*PFEDHVK sulfinamide	63.8 ±15.3	ND	ND
PhIP	3.7 ±0.3	64.3 ±0.2	50.6 ±0.2
	fmol	fmol	fmol
LQQC*PFEDHVK Sulfonamide	38.1 ±0.1	31.3 ±0.1	ND
PhIP	ND	ND	ND
C* sulfonamide	ND	ND	19.5±0.1

^aPhIP and peptides were determined by UPLC-MS² analysis of LQQC*PFEDHVK sulfinamide ([M+3H]³⁺ *m/z* 527.9 > 225.1, 679.1), PhIP ([M+H]⁺ *m/z* 225.1 > 210.1), and C*PF sulfonamide ([M+H]⁺ *m/z* 620.1 > 225.1, 602.1), [²H₃C]-PhIP ([M+H]⁺ *m/z* 228.1 > 210.1) was used the internal standard. Values are reported as the mean ± SD (*n*=3). ND, not detected, below the limit of detection. The amount of C*sulfonamide is an estimate based on the response relative to that of [²H₃C]-PhIP.

Table 2

The measurement of PhIP recovered from *in vitro* HONH-PhIP (45 pmol) modified commercial human albumin (15 pmol), subsequently treated with or without *m*-CPBA (3× molar excess of albumin).^a

	HONH-PhIP modified human albumin Without oxidization by <i>m</i> -CPBA		HONH-PhIP modified human albumin With oxidation by <i>m</i> - CPBA	
	Acid hydrolysis (fmol PhIP/ pmol albumin)	Pronase E digestion (fmol PhIP/pmol albumin)	Acid hydrolysis (fmol PhIP/ pmol albumin)	Pronase E digestion (fmol PhIP/pmol albumin)
PhIP	88±0.8	64±14.6	1.3±0.1	3.3±0.3

^aThe labile adduct of PhIP recovered was measured after acid hydrolysis (0.1N HCl, 37°C, 60 min) or digestion with pronase E for 18 h. Values are reported as the mean ± SD (*n*=3).

Table 3

Within-day and between-day measures of acid-labile PhIP (fg/mg albumin) recovered from spiked HONH-PhIP modified albumin diluted into commercial plasma.

	Spiked PhIP level (fg PhIP/mg albumin)	Day 1	Day 2	Day 3	CV(%) within-day ^a	CV(%) between-day ^a
Mean	675	618(92%) ^b	642(95%) ^b	658(97%) ^b		
SD		127	64	83		
RSD		21	9	13	15	14

^a Within-day and between-day estimates were conducted with four independent measurements on three different days.

^b Values in parentheses represent accuracy of the method.

Table 4

Within-day and between-day measures of [¹⁴C]-PhIP recovered from plasma of subjects 2 and 10 after acid hydrolysis.

Subject	[¹⁴ C]-PhIP (fg/mg albumin)				CV (%) within-day ^a	CV (%) between-day ^a
	Day 1	Day 2	Mean	SD		
2	50	47	Mean			
	2	2	SD			
	5	5	RSD		4.6	7.2
10	52	49	Mean			
	2	5	SD			
	5	11	RSD		7.9	8.4

^a Within-day and between-day measures were conducted with three independent measurements on two different days.

Table 5

An estimate of [^{14}C]-PhIP-albumin adduct level from subjects administered [^{14}C]-PhIP in vivo^{a,b}

Subject	Treatment	UPLC/MS ³	AMS	
		Acid Labile ([^{14}C]-PhIP fg/mg albumin)	([^{14}C]-PhIP bound fg/mg albumin)	% [^{14}C]-PhIP bound recovered as [^{14}C]-PhIP
1, T ₀	Acid hydrolysis or pronase E digestion	ND		
1, T ₂₄	Acid hydrolysis	57 ± 2	392 ± 4	14.5
1, T ₂₄	Pronase E digestion	44 ± 3		
2, T ₀	Acid hydrolysis & pronase E digestion	ND		
2, T ₂₄	Acid hydrolysis	49 ± 3	681 ± 10	7.2
2, T ₂₄	Pronase E digestion	38 ± 3		
7, T ₀	Acid hydrolysis or pronase E digestion	ND		
7, T ₂₄	Acid hydrolysis	28 ± 2	244 ± 3	11.5
10, T ₀	Acid hydrolysis	ND		
10, T ₂₄	Acid hydrolysis	51 ± 4	240 ± 4	21.3

^a Albumin was purified from plasma at T₀ or T₂₄ h and treated with 0.1 N HCl or pronase E digestion.

Values of acid-labile [^{14}C]-PhIP measured by UPLC/MS³ are reported as the mean ± SD ($n=3$). For subjects 1 and 2, the AMS measurement values are the mean ± SD of four independent analyses. The AMS analyses for subjects 7 and 10 are from single analyses (± SD of the AMS measurement). ND, not detected, below the LOD.

^b Albumin adduct data obtained by AMS represents the [^{14}C]-PhIP component of adduct levels and not unlabeled adducts. In the Dingley paper,²⁴ the total adduct levels (labeled and unlabeled PhIP) were reported.