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Development and validation of a liquid chromatography–tandem mass spectrometry assay for the analysis of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and its metabolite 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) in plasma, urine, bile, intestinal contents, faeces and eight selected tissues from mice

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ABSTRACT

The development and validation of a bioanalytical assay is described for the simultaneous analysis of 2-amino-1-methyl-6-phenylimidazo[4-5-*b*]pyridine (PhIP) and its main metabolite 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) in plasma, urine, faeces, bile, liver, kidney, testis, spleen, brain, as well as colon-, cecum- and small intestinal tissue and contents from mice. The effect of the matrix on the accuracy of the method was extensively investigated. The bioanalytical assay is based on reversed phase liquid chromatography coupled with tandem mass spectrometry in the positive ion mode using multiple reaction monitoring for analyte quantification. The assay is validated from 1 to 250 ng/mL and the sample pretreatment consists of protein precipitation with acetonitrile using only 100 μL matrix (plasma, bile diluted in 4% (m/v) BSA, intestinal contents, faeces and tissue samples homogenized in 4% (m/v) BSA). The measured concentrations of PhIP and N-OH-PhIP in homogenates were expressed in ng/mL. Based on the weight of the isolated intestinal contents, faeces or tissue the amount of PhIP and N-OH-PhIP per mass unit intestinal content, faeces or tissue was calculated. The validated range for PhIP in urine is from 10 to 1000 ng/mL using 20 μL urine. For N-OH-PhIP quantification, mouse urine was diluted 100× in blank human urine to compensate for matrix effects. The developed method is simple, robust and reproducible. The applicability of the method was demonstrated and the assay could be successfully used to support *in vivo* toxicokinetics studies of PhIP and N-OH-PhIP in mice.

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1. Introduction

Heterocyclic amines are a large group of carcinogenic compounds found in proteinaceous foods such as cooked meats and fish, coffee, alcohol beverages, cigarette smoke, air, river and rainwater [1]. The most abundant of these heterocyclic amines, 2-amino-1-methyl-6-phenylimidazo[4-5-*b*]pyridine (PhIP), has been shown to specifically induce tumors of the colon, breast and prostate in rodents [2–4]. PhIP is formed as a by-product of the Maillard reaction during cooking or frying of protein-rich foods at high temperatures [5]. Shioya et al. suggested phenylalanine, creatinine and glucose to be the most probable precursors of PhIP [6]. For PhIP to exert its carcinogenic effect, a multiple metabolism pathway has been suggested [7–9], starting with N-hydroxylation at the amine group by CYP1A1 and CYP1A2 [10–12] resulting in 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP).

The hydroxyl group can be sulphated by SULT1A1 enzymes [13–17] or acetylated by NAT1 or NAT2 enzymes [13,17,18]. Heterolytic cleavage of this sulphate or acetate group results in a reactive radical cation which can form adducts with DNA purines [19,20]. PhIP and N-OH-PhIP are precursors for this multiple metabolism pathway which stresses the importance of the ability to quantify PhIP and N-OH-PhIP in biological samples in order

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to understand the distribution of PhIP and the formation and distribution of its carcinogenic metabolite N-OH-PhIP.

The enzymes that are mainly responsible for the metabolism of PhIP are found in different organs and therefore biotransformation of PhIP is not restricted to specific tissues such as the liver [10,21–26]. As it has been shown that PhIP induces tumors in numerous organs such as the colon, breast and prostate, the distribution profile of PhIP may be very relevant. This could provide further insight into the carcinogenic potential of PhIP.

A partially validated assay for the analysis of PhIP and N-OH-PhIP in human liver was published in 2001 by Prabhu et al. [27]. A fully validated bioanalytical liquid chromatography–tandem mass spectrometry method for the quantification of PhIP and its metabolite N-OH-PhIP in plasma, urine, bile, faeces, intestinal contents and eight selected tissues from mice has, to the best of our knowledge, not been published hitherto. We designed such an assay with the purpose to support preclinical toxicokinetic studies.

2. Experimental

2.1. Chemicals

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and its deuterated internal standard 2-amino-1-(trideuteromethyl)-6-phenylimidazo[4,5-*b*]pyridine (PhIP-D3) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository at the Midwest Research Institute (Kansas City, USA). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]-5-hydroxypyridine (5-OH-PhIP) was a kind gift from Henrik Frandsen of the National Food Institute, Technical University of Denmark. Methoxyflurane (Metofane) was obtained from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia). Methanol originated from Biosolve Ltd. (Amsterdam, The Netherlands). Ammonium formate was purchased from Acros Organics (Geel, Belgium). LiChrosolv water for chromatography, Bovine Serum Albumin (BSA), dimethylsulphoxide (DMSO) and formic acid were from Merck (Darmstadt, Germany).

2.2. Instrumentation

2.2.1. HPLC

HPLC experiments were performed on an Agilent 1100 series liquid chromatograph system (Agilent technologies, Palo Alto, CA, USA) consisting of a binary pump, an in-line degasser, autosampler and column oven. Mobile phase A was prepared by adjusting a 3.5 mM ammonium formate solution to pH 3.5 with a 98% formic acid solution. Mobile phase B consisted of methanol. Mobile phase A and B were pumped through a Synergi Hydro 110 Å column (150 mm × 2.0 mm I.D., 4 μm; Phenomenex, Torrance, CA, USA) at a flow rate of 0.2 mL/min using a step and a linear gradient as shown in Tables 1A and 1B. The step gradient was used for plasma, urine and bile analysis. The linear gradient was used for intestinal content, faeces and tissue analyses. The separation was performed at ambient temperature and 40 °C, respectively. Volumes of 20 μL were injected using an autosampler thermostated at 7 °C. The total run times were 13 and 19 min, respectively. The autosampler needle was rinsed with methanol before injection. During the first 1.5 and last 1.0 min the eluate was directed to waste using a divert valve to prevent the introduction of endogenous compounds into the mass spectrometer.

2.2.2. Mass spectrometry

The LC eluate was directed, via a divert valve, into an API 3000 triple quadrupole MS equipped with an electrospray ion source (Sciex, Thornhill, ON, Canada) operating in the positive ion mode. HPLC-MS/MS data were acquired and processed (integrated) using software application Analyst™ version 1.5 (Sciex, Canada). Calibration curves of analyte/internal standard peak area ratio versus respectively PhIP and N-OH-PhIP concentrations were constructed and weighted $1/x^2$ (the reciprocal of the squared concentration) linear regression was applied to the data. The ion spray voltage was set to 3.0 kV. The turbogas temperature was held constant at 300 °C. Nebulizer and heated turbo gas (both compressed air) were operated at 10 and 7 L/min, respectively. The curtain gas and collision gas (both nitrogen gas) were operated at 6 and 8 psi, respectively. Mass transitions and operating parameters are given in Table 2.

Table 1A
HPLC step gradient parameters used for the separation of PhIP and N-OH-PhIP in plasma, urine and bile on a Phenomenex Synergi Hydro 110 Å column (150 mm × 2.0 mm I.D., 4 μm) at ambient temperature.

Time (min)	Flow rate (mL/min)	Mobile phase A[a] (%)	Mobile phase B[b] (%)
0.0	0.2	70	30
4.0	0.2	70	30
4.1	0.2	20	80
8.0	0.2	20	80
8.1	0.2	70	30
13.0	0.2	70	30

[a] Mobile phase A: 3.5 mM ammonium formate buffer pH 3.5.
[b] Mobile phase B: Methanol.

Table 1B
HPLC linear gradient parameters used for the separation of PhIP and N-OH-PhIP in intestinal content, faeces and tissue on a Phenomenex Synergi Hydro 110 Å column (150 mm × 2.0 mm I.D., 4 μm) at 40 °C.

Time (min)	Flow rate (mL/min)	Mobile phase A[a] (%)	Mobile phase B[b] (%)
0.0	0.2	80	20
13.0	0.2	20	80
16.0	0.2	20	80
16.1	0.2	80	20
19.0	0.2	80	20

[a] Mobile phase A: 3.5 mM ammonium formate buffer pH 3.5.
[b] Mobile phase B: Methanol.

2.3. Preparation of calibration standards, quality controls and internal standard solutions

Two separate stock solutions were prepared for PhIP and N-OH-PhIP by dissolving accurately weighed approximately 1 mg of these analytes in 1.0 mL of methanol and DMSO, respectively. An internal standard stock solution was prepared by dissolving accurately weighed approximately 1 mg of PhIP-D3 in 1.0 mL of methanol.

One stock solution was used for the preparation of calibration standards and the other stock solution was used for the preparation of quality control standards. The preparation of the two stock

Table 2
Mass transitions and operating parameters for PhIP, N-OH-PhIP and PhIP-D3 on an API 3000 triple quadrupole MS equipped with an electrospray ion source operating in the positive ion mode.

Parameters	PhIP	PhIP-D3	N-OH-PhIP
Parent mass (*m*/*z*)	225	228	241
Product mass (*m*/*z*)	210	210	223
Focusing potential (V)	340	340	270
Declustering potential (V)	76	76	61
Entrance potential (V)	10	10	10
Collision energy (V)	43	43	37
Collision exit potential (V)	14	14	22
Dwell time (ms)	80	80	80
Polarity	Positive	Positive	Positive
Retention time (min) block gradient	8.8	8.8	9.1
Retention time (min) linear gradient	9.4	9.4	11.5

solutions for each compound was checked and in all cases deviations were less than ±5%. 1.0 mL of a 25 μg/mL working solution containing PhIP and N-OH-PhIP was prepared in plasma. Plasma and 4% (m/v) BSA calibration standards at nominal concentrations of 1, 5, 10, 25, 75, 100, 175 and 250 ng/mL were prepared from the plasma working solution.

Plasma and 4% (m/v) BSA calibration standard solutions were aliquoted in 100 μL portions and stored at nominally −20 °C. 2.0 mL of a 50 μg/mL working solution containing PhIP and N-OH-PhIP was prepared in urine. Calibration standards were prepared from the urine working solution by spiking blank urine, acidified with formic acid to pH 3.5, at nominal concentrations of 10, 25, 75, 100, 175, 250, 500 and 1000 ng/mL. Urine calibration standard solutions were aliquoted in 20 μL portions and stored at nominally −20 °C.

Two internal standard working solutions with PhIP-D3 were prepared: (i) in acetonitrile with a target concentration of 75 ng/mL and (ii) in methanol–ammonium formate buffer pH 3.5 (30:70, v/v) with a target concentration of 175 ng/mL. Due to limited volumes of murine blank plasma and urine, human plasma and urine from healthy volunteers was used for the preparation of calibration standards and quality control samples.

2.4. Sample preparation

2.4.1. Plasma

Directly after sampling, plasma was processed from whole blood, subsequently snap frozen on dry-ice and stored at −70 °C. After thawing plasma samples were processed immediately by addition of 300 μL internal standard working solution (75 ng/mL) in acetonitrile to each 100 μL plasma aliquot. The sample was vortex mixed for 10 s and centrifuged for 10 min at 11,300 × *g*. The clear supernatant was diluted 1:1 (v/v) with 3.5 mM ammonium formate buffer pH 3.5, vortex mixed for 10 s and subsequently transferred to an autosampler vial. The final extracts were stored at 2–8 °C until analysis.

2.4.2. Urine

After sampling, urine was snap frozen on dry-ice and stored at −70 °C. After thawing, 180 μL internal standard working solution (175 ng/mL) in methanol–ammonium formate buffer pH 3.5 (30:70, v/v) was added to a 20 μL urine sample aliquot. The 10× diluted urine was subsequently transferred to an autosampler vial and stored at 2–8 °C until analysis.

2.4.3. Bile

After sampling, bile was snap frozen on dry-ice and stored at −70 °C. A volume of 10 μL bile was diluted in 400 μL 4% (m/v) BSA solution. A 100 μL aliquot of diluted bile was subsequently processed by addition of 300 μL internal standard working solution (75 ng/mL) in acetonitrile. The sample was vortex mixed for 10 s and centrifuged for 10 min at 11,300 × *g*. The clear supernatant was diluted 1:1 (v/v) with 3.5 mM ammonium formate buffer pH 3.5, vortex mixed for 10 s and subsequently transferred to an autosampler vial. The final extracts were stored at 2–8 °C until analysis.

2.4.4. Tissue, intestinal contents and faeces

After sampling and weighing, tissues, intestinal contents and faeces were snap frozen on dry-ice and stored at −70 °C. After thawing, the complete organ or the total volume of sampled intestinal contents and faeces was homogenized in different volumes of a 4% (m/v) BSA solution using a blender. 1 mL was used for spleen, colon and cecum tissue, 2 mL for kidney, brain, testis, colon content and small intestinal tissue, 3 mL for cecum and small intestinal content, 5 mL for liver and 1 mL 4% (m/v) BSA solution was added per 100 mg faeces.

A volume of 300 μL internal standard working solution (75 ng/mL) in acetonitrile was added to 100 μL tissue or intestinal content homogenate. For faeces analysis, the internal standard working solution was added to 100 μL clear supernatant which was obtained when the faeces homogenate was centrifuged for 10 min at 11,300 × *g*. The sample was vortex mixed for 10 s and centrifuged for 10 min at 11,300 × *g*. The clear supernatant was diluted 1:1 (v/v) with 3.5 mM ammonium formate buffer pH 3.5, vortex mixed for 10 s and subsequently transferred to an autosampler vial. The final extracts were stored at 2–8 °C until analysis.

The measured concentrations of PhIP and N-OH-PhIP were expressed in ng/mL homogenate. Based on the weight of the isolated intestinal contents, faeces or tissue the amount of PhIP and N-OH-PhIP per mass unit intestinal content, faeces or tissue was calculated.

2.5. Animals

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were male wild-type mice of an FVB genetic background, between 9 and 14 weeks of age. Animals were kept in a temperature-controlled environment with a 12 h light/12 h dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

2.6. PhIP administration and sampling

For i.v. administration, 5 μL/g body weight of a 0.2 mg/mL PhIP solution in DMSO–0.9% NaCl solution (20:80, v/v) was injected into the tail vein of male mice. After 30 min, animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia and plasma, urine and organs were collected. Intestinal contents and tissue were separated. Bile was obtained in a separate experiment by gall bladder cannulation as described previously [28], followed by i.v. administration of 1 mg/kg PhIP. Faeces were collected for 24 h after i.v. administration of 1 mg/kg PhIP in a metabolic cage experiment, as described [28]. Blank plasma, urine, bile, liver, kidney, testis, spleen, brain, colon-, cecum- and small intestinal tissue and contents were collected from untreated mice.

3. Validation procedures

3.1. Linearity

Eight non-zero calibration standards in plasma, urine and 4% (m/v) BSA were prepared in duplicate for each run and analyzed in three independent runs. Calibration curves (area ratio with the internal standard versus nominal concentration) were fitted by least-squares linear regression using the reciprocal of the squared concentration ($1/x^2$) as a weighting factor. To assess linearity, deviations from the mean calculated concentrations over three runs should be within ±15% of nominal concentrations. At the lower limit of quantification level (LLQ) a deviation of ±20% was permitted and the response of the analyte should be at least five times higher than the response of a blank sample.

3.2. Accuracy and precision in plasma, urine and 4% (m/v) BSA

Intra- and inter-assay accuracies and precisions of the method were determined by assaying five replicates of each of the quality control (QC) samples at the LLQ, low, mid and high concentration level in three separate runs. The concentration of each QC sample was calculated using the calibration standards that were analyzed

in duplicate in the same run. The differences between the nominal and the measured concentrations were used to calculate the accuracies. The accuracy should be within 85–115% except at the LLQ, where an accuracy of 80–120% is allowed. The precision, expressed as the coefficient of variation, should not exceed 15%, except for the LLQ, where it should not exceed 20% [29]. The ability to dilute samples originally above the upper limit of quantification (ULQ) was demonstrated in 4% (m/v) BSA by analyzing quality control samples containing 10 times the concentration of the high quality control sample. These samples were prepared in 5-fold and analyzed after a 10-fold dilution in 4% (m/v) BSA.

3.3. Accuracy and precision in matrices isolated from mice

To check whether calibration standards prepared in control human plasma, human urine or 4% (m/v) BSA could be adequately used for the quantification of PhIP and N-OH-PhIP in matrices from mice, QC samples were prepared in control matrices obtained from male mice and accuracies and precisions were determined.

3.4. Carry-over

Carry-over was determined by injecting a blank processed sample after an ULQ sample. Carry-over was determined in plasma, urine and 4% (m/v) BSA.

Areas of peaks in the blank processed sample should be less than 20% of the peak area of the LLQ sample [29].

3.5. Stability

The stability of the analytes was investigated in the stock solutions at −20 °C. Furthermore, stability was tested in mouse plasma, mouse urine and 4% (m/v) BSA for 1 h at room temperature and after 10 months of storage at −20 °C. Stability of homogenized tissue and intestinal contents was tested after storage at −70 °C. Due to limited amounts of available blank tissue homogenate each tissue stability experiment was performed only once, without replication. Stability was tested in mouse brain, small intestine and testis for 1 h at room temperature (22 °C). Stability in other used tissues was assumed to be equal to brain, small intestine and testis. Final extract stability was investigated during 12.5 h at 2–8 °C. Analytes were considered stable in stock solutions when 95–105% of the original concentration was found. Analytes were considered stable in the biological matrix or extract when 80–120% of the initial concentration was recovered. Stability of the deuterated internal standard was assumed to be equal to the corresponding undeuterated analyte. Isotopic purity of the deuterated analyte (i.e. internal standard) was investigated during each analytical run by spiking blank plasma, urine or 4% (m/v) BSA with an internal standard working solution. The peak area in the undeuterated analyte window should be less than 20% of the peak area of the analyte at its LLQ level [29].

4. Results and discussion

4.1. HPLC-MS/MS

To obtain high sensitivity, a triple quadrupole mass spectrometer was chosen as a detector. An analytical column was selected with a stationary phase endcapped with polar groups to provide retention of both hydrophobic as well as hydrophilic compounds.

The protonated molecular ions ($[M+H]^+$) were used as precursor ions to generate product-ion spectra (Fig. 1A and B). The most intense product ions were optimized and used as MRM transitions

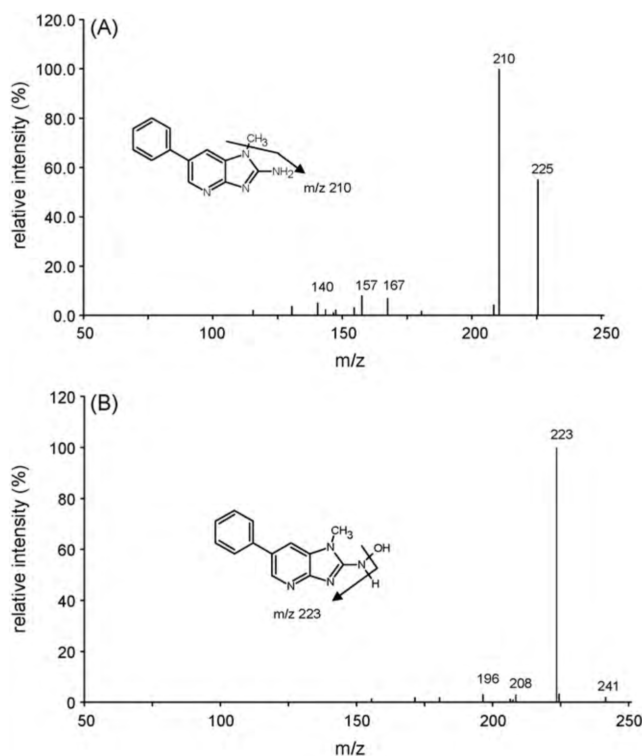

Fig. 1. (A) Product Ion Scan of PhIP, measured on an API 3000 triple quadrupole MS equipped with an electrospray ion source operating in the positive ion mode. Instrument settings are summarized in Table 2. (B) Product Ion Scan of N-OH-PhIP, measured on an API 3000 triple quadrupole MS equipped with an electrospray ion source operating in the positive ion mode. Instrument settings are summarized in Table 2.

to ensure high sensitivity and selectivity. Ionization and fragmentation parameters for PhIP and N-OH-PhIP were optimized by continuous infusion of a standard solution in methanol:ammonium formate buffer pH 3.5 (30:70, v/v) into the mass spectrometer. The most dominant and reproducible fragment of PhIP was the loss of the methyl group from the imidazole moiety. The loss of a fragment with an m/z of 18 from N-OH-PhIP indicated the loss of a water molecule. The transition appeared to be stable and reproducible during the validation and was selected to obtain maximum sensitivity. Fig. 2A and B shows representative chromatograms of PhIP and N-OH-PhIP in a processed calibration standard at LLQ level in 4% (m/v) BSA.

4.2. Linearity

For all the calibration curves, in plasma, urine and 4% (m/v) BSA, linear regression was applied using a weighting factor of $1/x^2$. For every calibration curve the concentrations were back-calculated from the responses. The deviation from the nominal concentration was in all cases less than 15%. PhIP-D3 was used as an internal standard for both PhIP and N-OH-PhIP.

4.3. Accuracy and precision in plasma, urine and 4% (m/v) BSA

Three full validation runs were performed in 4% (m/v) BSA to determine inter- and intra-assay accuracy and precision. A partial validation (i.e. one run) was performed in both plasma and urine to determine intra-assay accuracy and precision. Assay performance data of PhIP and N-OH-PhIP in 4% (m/v) BSA, plasma and urine are presented in Table 3. Four concentration levels per range were measured. In all matrices the accuracy was within 85–115% and the precision (expressed as the coefficient of vari-

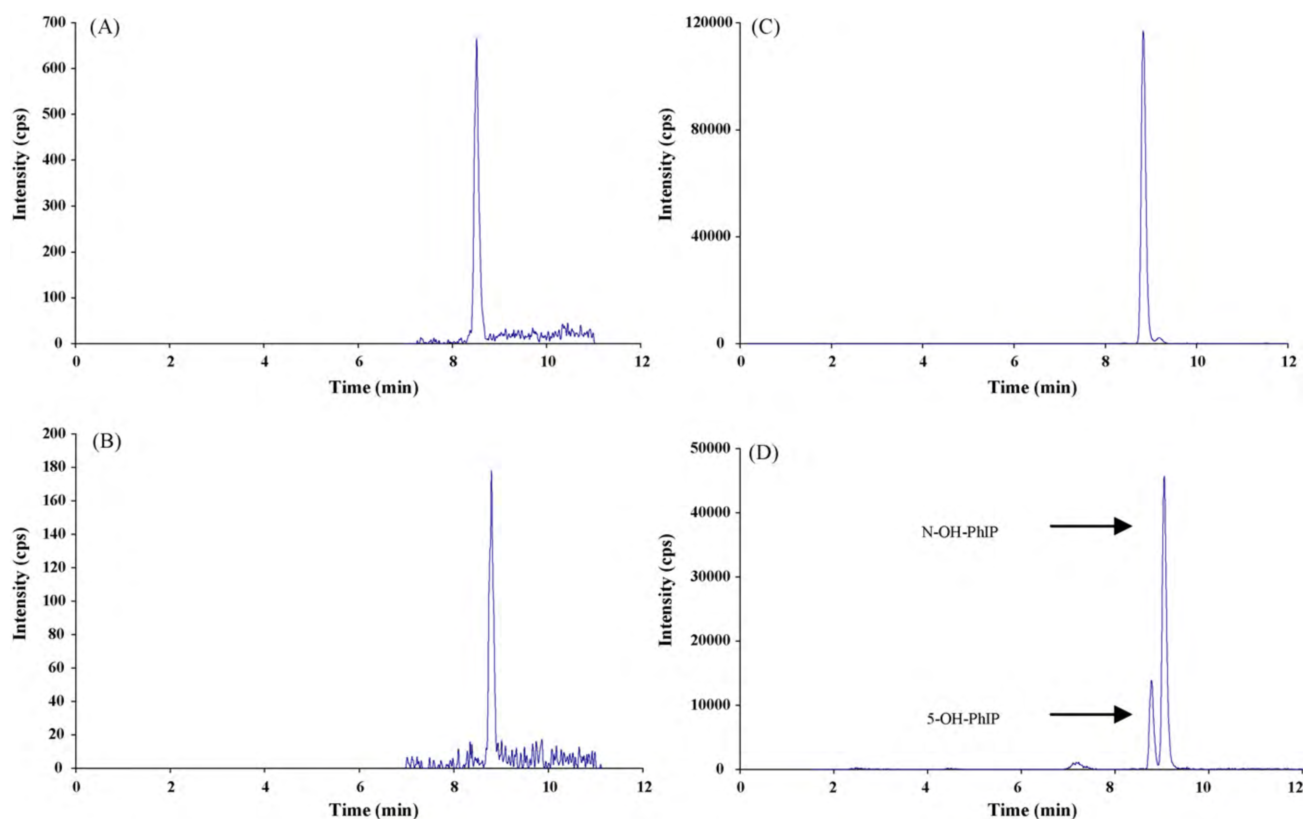

Fig. 2. Representative HPLC-MS/MS chromatograms from: (A) PhIP (LLQ: 0.978 ng/mL) in 4% (m/v) BSA, (B) N-OH-PhIP (LLQ: 1.11 ng/mL) in 4% (m/v) BSA, (C) PhIP in kidney homogenate (in 4% (m/v) BSA) 0.5 h after 1 mg/kg i.v. PhIP administration, (D) N-OH-PhIP and 5-OH-PhIP in mouse urine 0.5 h after 1 mg/kg i.v. PhIP administration.

ation) was less than 15% for both compounds. This also accounts for the 4% (m/v) BSA samples containing high concentrations of the analytes (i.e. >ULQ) that were diluted 10 times using blank 4% (m/v) BSA.

4.4. Accuracy and precision in matrices isolated from mice

It is pivotal to check whether control human plasma and urine used for the preparation of calibration standards are adequate surrogate matrices. Therefore, QC samples were prepared in six different batches of control plasma and urine obtained from male mice and accuracies and precisions were determined based on calibration standards prepared in control human plasma and urine. Table 4 shows the results of these experiments.

The accuracies related to PhIP concentrations are excellent in both plasma and urine due to the presence of a deuterated internal standard. Accuracies and precisions are within the 15% criteria for all tested concentrations in six batches. For N-OH-PhIP accuracies are between 71.6 and 106% for plasma and between 174 and 238% for urine. Precisions are within 15%. Interferences of endogenous compounds in mouse plasma and urine and the lack of a suitable deuterated internal standard for N-OH-PhIP were hypothesized to be causes of the poor accuracies. To test this hypothesis, QC samples in mouse plasma and urine were diluted 10 and 100 times in control human plasma and urine, respectively. In plasma, accuracies were within 85–115% when samples were diluted 10 times. In urine, accuracies were significantly improved after 10 times dilution and were within 80–120% after 100 times dilution to a concentration at LLQ level. These results clearly show that the matrix effect of mouse plasma and urine on the MS signal of N-OH-PhIP is different in comparison to human plasma and urine.

The above-mentioned hypothesis was alternatively tested by adjusting the gradient (Table 1B) to obtain more selectivity for N-OH-PhIP. Undiluted samples from two batches were re-assayed using this linear gradient (Table 5). The accuracies related to PhIP concentrations were again excellent in both plasma and urine. The accuracies related to N-OH-PhIP concentrations in plasma were within the 85–115% criteria without the need to dilute samples in blank human plasma. However, N-OH-PhIP accuracies were between 167 and 198% in urine. The linear gradient appeared to have little effect on the accuracy of N-OH-PhIP in urine. Therefore, as shown in Table 4 urine had to be diluted 100× in blank human urine to ensure accuracies within 85–115%.

To check the influence of mouse tissues and intestinal contents on the accuracy and precision of PhIP and N-OH-PhIP when these samples are quantified on calibration standards prepared in 4% (m/v) BSA, QC samples in mouse tissues and intestinal contents were spiked, processed and analyzed. Results are presented in Table 6. For PhIP accuracies and precisions were excellent, but again the quantification of N-OH-PhIP was biased. To investigate this matrix effect, matrix effect profiles were recorded by infusing a PhIP and N-OH-PhIP working solution at a concentration of 1 μg/mL post-column while simultaneously a processed tissue or intestinal content extract was injected onto the analytical column via the autosampler. In Fig. 3A an overlay is presented of a chromatogram of PhIP and N-OH-PhIP and the recorded matrix effect profile. Using a step gradient (Table 1A), N-OH-PhIP elutes at a point where there is a steep slope of ion enhancement and suppression. Since no suitable deuterated internal standard was available for N-OH-PhIP to correct for matrix effects, a linear gradient (Table 1B) was used instead to obtain more selectivity from endogenous compounds. An overlay of chromatograms of PhIP and N-OH-PhIP and the recorded matrix effect profile recorded using the new linear gradient is presented in Fig. 3B. Assay performance data of PhIP and N-OH-PhIP were

Table 3
Assay performance data for the analysis of PhIP and N-OH-PhIP in 4% (m/v) BSA, human plasma and human urine. Analyses were performed using a step gradient (Table 1A).

Analyte (linear range)	Matrix	Nominal concentration (ng/mL)	n	Intra-assay accuracy (%)	Intra-assay precision (% CV)	Inter-assay accuracy (%)	Inter-assay precision (% CV)
PhIP (0.978–245 ng/mL)	4% (m/v) BSA	0.978	5	86.1	8.00	–	–
			5	96.9	4.77	–	–
			5	93.4	4.95	–	–
			15	–	–	89.7	6.38
		2.93	5	102	2.26	–	–
			5	101	2.26	–	–
			5	104	3.17	–	–
			15	–	–	103	2.64
		97.8	5	99.1	1.23	–	–
			5	102	1.23	–	–
			5	98.3	1.13	–	–
			15	–	–	98.7	1.20
		196	5	101	1.84	–	–
			5	101	1.93	–	–
			5	107	9.16	–	–
			15	–	–	104	5.65
		1220	5	−14.3	1.09	–	–
N-OH-PhIP (1.11–278 ng/mL)	4% (m/v) BSA	1.11	5	111	3.69	–	–
			5	91.9	4.10	–	–
			5	109	11.9	–	–
			15	–	–	104	11.4
		3.34	5	93.5	7.42	–	–
			5	91.8	8.11	–	–
			5	93.4	8.29	–	–
			15	–	–	92.9	7.37
		111	5	94.1	1.45	–	–
			5	101	6.47	–	–
			5	100	3.93	–	–
			15	–	–	98.2	5.06
		223	5	96.5	3.14	–	–
			5	106	3.79	–	–
			5	109	11.2	–	–
			15	–	–	104	8.61
		1380	5	6.09	4.28	–	–
PhIP (0.978–245 ng/mL)	Plasma	0.978	5	110	9.16	–	–
		2.93	5	108	3.34	–	–
		97.8	5	103	1.62	–	–
		196	5	104	1.59	–	–
N-OH-PhIP (1.11–278 ng/mL)	Plasma	1.11	5	107	9.19	–	–
		3.34	5	103	6.13	–	–
		111	5	96.8	5.13	–	–
		223	5	96.1	3.68	–	–
PhIP (9.78–978 ng/mL)	Urine	9.78	5	91.2	5.27	–	–
		29.3	5	92.8	1.46	–	–
		293	5	103	1.60	–	–
		782	5	104	5.90	–	–
N-OH-PhIP (11.1–1112 ng/mL)	Urine	11.1	5	93.2	1.62	–	–
		33.4	5	93.5	2.56	–	–
		334	5	105	3.46	–	–
		890	5	104	5.70	–	–

CV: coefficient of variation.

again investigated using the linear gradient. Accuracies appeared to be within 85–115% for both PhIP and N-OH-PhIP with the exception of small intestinal contents (Table 6). The recovered concentration of N-OH-PhIP in this matrix was low using both the linear and the step gradient. Most probably N-OH-PhIP is not fully recovered from small intestine content after addition of acetonitrile.

4.5. Carry-over

Carry-over was determined in plasma, urine and 4% (m/v) BSA by injecting a blank processed sample after an ULQ sample. Areas of peaks in the blank processed sample were for PhIP: 1.27, 1.08, and 1.02% and for N-OH-PhIP: 2.10, 2.67 and 5.75% of the peak area in an LLQ sample in plasma, urine and 4% (m/v) BSA, respectively, and were found to be acceptable.

4.6. Stability

An overview of the stability experiments that were performed and the obtained results is presented in Table 7. A 1 mg/mL stock solution of PhIP in methanol was stable at −20 °C for at least 8 months whereas a 1 mg/mL stock solution of N-OH-PhIP in DMSO was stable at −20 °C for at least 10 months. A 1 mg/mL PhIP-D3 solution in methanol at −20 °C did not show significant degradation (<5%) over a period of 10 months (data not shown).

Both PhIP and N-OH-PhIP in either plasma, acidified urine (pH 3.5) or 4% (m/v) BSA were stable at −20 °C for up to 10 months.

Table 4
Assay performance data for the analysis of PhIP and N-OH-PhIP in mice plasma and urine ($n = 2$). Analyses were performed using a step gradient (Table 1A).

Analyte (linear range)	Matrix	Batch	Dilution factor[a]	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Accuracy (%)	Precision (% CV)
PhIP (1.02–102 ng/mL)	Plasma	1	1×	102	110	108	1.31
		2	1×		111	109	0.652
		3	1×		109	107	1.99
		4	1×		103	101	1.40
		5	1×		107	105	0.00
		6	1×		109	107	1.99
N-OH-PhIP (1.06–106 ng/mL)	Plasma	1	1×	106	112	106	6.67
		2	1×		113	106	8.91
		3	1×		92.0	86.8	5.21
		4	1×		94.5	89.1	9.52
		5	1×		80.4	75.8	5.97
		6	1×		75.8	71.6	8.99
	Plasma	1	10×	10.6	10.2	96.5	6.59
		2	10×		10.4	98.3	3.96
		3	10×		10.6	100	3.66
		4	10×		12.2	115	4.92
		5	10×		10.3	97.6	6.45
		6	10×		9.81	92.7	6.49
PhIP (10.2–1020 ng/mL)	Urine	1	1×	1060	1840	174	3.67
		2	1×		1900	179	7.11
		3	1×		2270	214	5.29
		4	1×		2320	219	3.87
		5	1×		2520	238	3.57
		6	1×		2520	238	2.68
N-OH-PhIP (10.6–1060 ng/mL)	Urine	1	10×	106	140	132	0.00
		2	10×		147	139	2.55
		3	10×		143	135	0.526
		4	10×		134	126	1.12
		5	10×		138	130	4.35
		6	10×		121	114	1.24
	Urine	1	100×	10.6	11.5	109	9.78
		2	100×		11.2	105	4.04
		3	100×		11.8	111	1.27
		4	100×		11.3	106	2.67
		5	100×		10.3	97.5	1.23
		6	100×		10.5	99.1	1.36

CV: coefficient of variation.
[a] Dilution in control human matrix before analysis.

Acidifying urine with formic acid to pH 3.5 is of vital importance to guarantee the stability of N-OH-PhIP [27]. A spiked sample of N-OH-PhIP in urine which was not acidified degraded at −20 °C to half its original concentration over a period of 10 months (data not shown). N-OH-PhIP appeared to be relatively unstable at room temperature (22 °C) in both plasma, acidified urine and 4% (m/v) BSA, but stability of both PhIP and N-OH-PhIP is guaranteed for at least 1 h at room temperature.

After sampling, tissue, intestinal contents and faeces were snap frozen and subsequently homogenized immediately after thawing. The homogenates were snap frozen and stored at −70 °C until analysis. Tissue homogenates were subsequently thawed at room temperature and processed immediately after thawing. Stability of tissue homogenates was demonstrated for 1 h at 22 °C using brain, small intestine and testis as model tissues. One hour is sufficient time for thawing and preparation of tissue homogenate, plasma, urine and 4% (m/v) BSA samples.

Stability of the final extract, when kept at 7 °C in the autosampler until analysis, was guaranteed for at least 12.5 h which is the total analysis time of one run containing calibration standards and 30 samples. Stability of PhIP and N-OH-PhIP in tissue and intestinal content homogenates during storage at −70 °C appeared to be highly dependent on the type of tissue and intestinal content homogenate. Therefore, the stability of PhIP and N-OH-PhIP is presented in Table 7 at variable storage durations. To keep the number of freeze/thaw cycles to a minimum, calibration standards were

Table 5
Assay performance data for the analysis of PhIP and N-OH-PhIP in mice plasma and urine ($n = 2$). Analyses were performed using a linear gradient (Table 1B).

Analyte	Matrix from mice (batch)	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Accuracy (%)	Precision (% CV)
PhIP	Plasma (1)	50.9	52.2	103	2.07
	Plasma (2)	50.9	52.2	103	2.07
	Urine (1)	102	100	98.4	6.69
	Urine (2)	102	113	111	0.640
N-OH-PhIP	Plasma (1)	52.9	56.1	106	0.00
	Plasma (2)	52.9	50.1	94.7	2.02
	Urine (1)	106	177	167	7.22
	Urine (2)	106	210	198	5.00

CV: coefficient of variation.

Table 6
Assay performance data for the analysis of PhIP and N-OH-PhIP in tissues isolated from mice. Analyses were performed using a step gradient (Table 1A) and a linear gradient (Table 1B).

Analyte	Sample type	Nominal concentration (ng/mL)	Accuracy (%) (block gradient)	Accuracy (%) (linear gradient)
PhIP	Brain	17.8	98.3	92.5
	Kidney	17.8	98.1	91.7
	Liver	17.8	107	105
	Small intestinal content	17.8	104	106
	Small intestinal tissue	17.8	101	102
	Spleen	17.8	107	107
	Testis	17.8	93.6	95.2
N-OH-PhIP	Brain	18.5	142	105
	Kidney	18.5	112	111
	Liver	18.5	156	99.5
	Small intestinal content	18.5	19.8	59.9
	Small intestinal tissue	18.5	156	111
	Spleen	18.5	140	108
	Testis	18.5	114	119

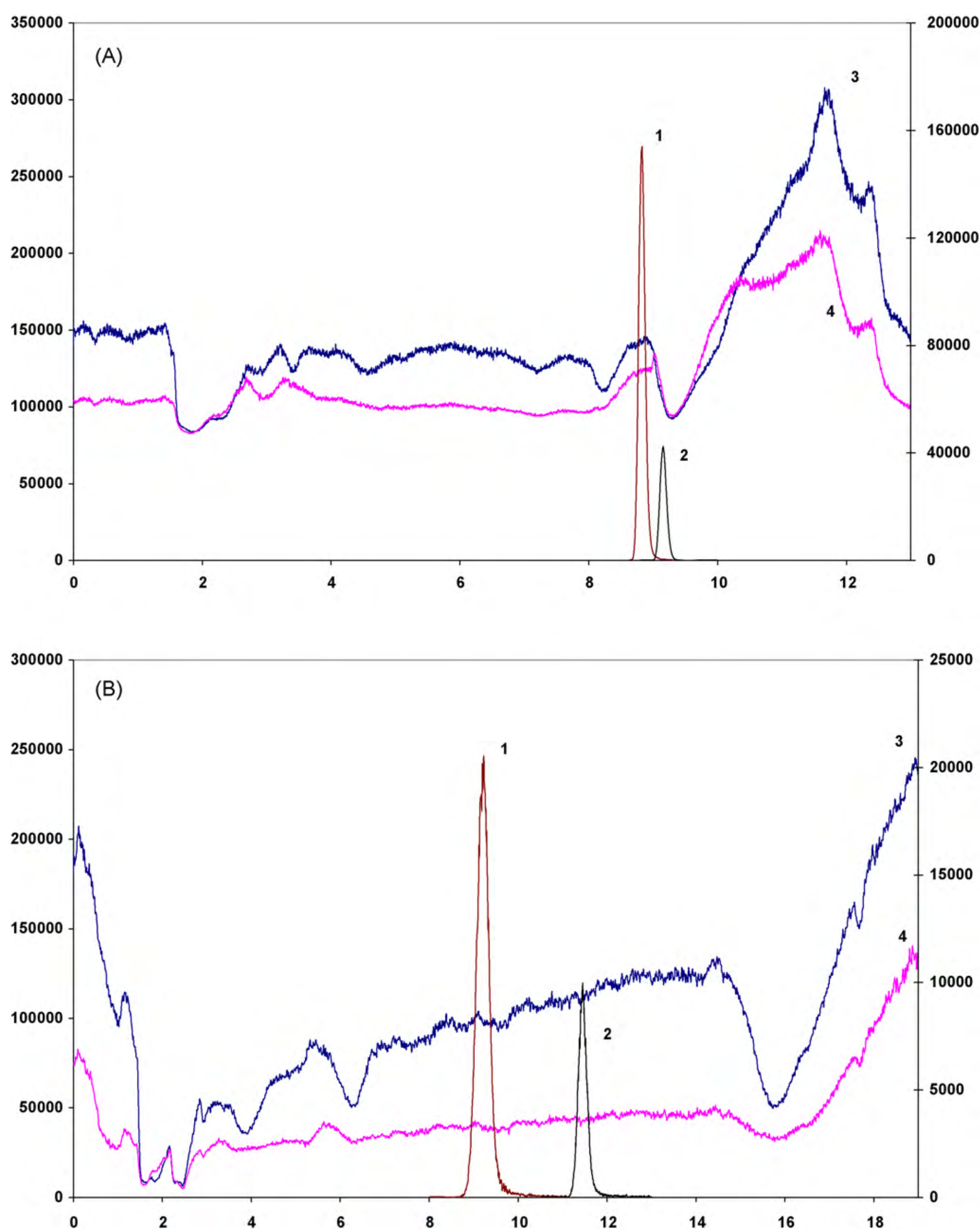

Fig. 3. Post-column infusion matrix effect profiles and representative HPLC-MS/MS chromatograms using: (A) a step and (B) a linear gradient. (1) Chromatogram of PhIP, (2) chromatogram of N-OH-PhIP, (3) matrix effect of liver on MS response of PhIP, (4) matrix effect of liver on MS response of N-OH-PhIP.

Table 7
Stability experiments.

Conditions	Matrix	Analyte	Nominal concentration (ng/mL)	Dev. (%)	CV (%)	No. of replicates
Stock stability, −20 °C, 8 months	Methanol	PhIP	50.0	−3.46	0.343	3
Stock stability, −20 °C, 10 months	DMSO	N-OH-PhIP	50.0	−1.12	2.78	3
Sample stability, −20 °C, 10 months	Plasma	PhIP	25.0	−4.00	1.53	3
		N-OH-PhIP	25.0	−19.0	2.65	3
	Acidified urine (pH 3.5)	PhIP	50.0	−12.5	0.922	3
		N-OH-PhIP	50.0	−11.6	1.29	3
	4% (m/v) BSA	PhIP	25.0	−10.1	0.840	3
		N-OH-PhIP	25.0	−3.05	11.4	3
Sample stability, 22 °C, 1 h	Plasma	PhIP	76.3	10.0	0.626	3
		N-OH-PhIP	79.4	4.00	0.553	3
	Acidified urine (pH 3.5)	PhIP	76.3	1.00	0.990	3
		N-OH-PhIP	79.4	−2.70	0.371	3
	4% (m/v) BSA	PhIP	25.4	−0.800	0.993	3
		N-OH-PhIP	26.5	−14.2	7.14	3
Sample stability, 22 °C, 1 h,	Brain	PhIP	97.8	2.97	–	1
		N-OH-PhIP	111	8.99	–	1
	Small intestinal tissue	PhIP	97.8	6.45	–	1
		N-OH-PhIP	111	−14.2	–	1
	Testis	PhIP	97.8	2.80	–	1
		N-OH-PhIP	111	1.64	–	1
Final extract stability, 2–8 °C, 12.5 h	Final extract, plasma	PhIP	188	0.575	1.59	8
		N-OH-PhIP	188	−10.7	4.21	8
	Final extract, urine	PhIP	188	2.68	1.27	8
		N-OH-PhIP	188	−4.85	4.37	8
	Final extract, 4% (m/v)	PhIP	188	−0.897	0.701	8
	BSA	N-OH-PhIP	188	−1.75	7.71	8
Sample stability, −70 °C, homogenates in 4% (m/v) BSA						
4 days	Colon content	PhIP	17.8	3.53	–	1
12 days	Cecum content	PhIP	17.8	−12.6	–	1
12 days	Cecum tissue	PhIP	17.8	−14.1	–	1
12 days	Colon tissue	PhIP	17.8	−13.1	–	1
12 days	Kidney	PhIP	17.8	−8.67	–	1
12 days	Liver	PhIP	17.8	−13.5	–	1
12 days	Small intestinal tissue	PhIP	17.8	4.10	–	1
12 days	Testis	PhIP	17.8	−7.14	–	1
26 days	Brain	PhIP	17.8	−1.85	–	1
26 days	Spleen	PhIP	17.8	−2.63	–	1
4 days	Cecum content	N-OH-PhIP	18.5	−6.79	–	1
4 days	Cecum tissue	N-OH-PhIP	18.5	−3.40	–	1
4 days	Colon content	N-OH-PhIP	18.5	3.47	–	1
4 days	Colon tissue	N-OH-PhIP	18.5	11.2	–	1
4 days	Small intestinal tissue	N-OH-PhIP	18.5	5.26	–	1
4 days	Spleen	N-OH-PhIP	18.5	11.3	–	1
4 days	Testis	N-OH-PhIP	18.5	7.87	–	1
26 days	Brain	N-OH-PhIP	18.5	−12.3	–	1
26 days	Kidney	N-OH-PhIP	18.5	−5.60	–	1
26 days	Liver	N-OH-PhIP	18.5	−7.39	–	1

Dev: deviation from nominal. CV: coefficient of variation.
Plasma, urine and 4% (m/v) BSA analyses are performed with a step gradient (Table 1A).
Tissues are homogenized in 4% (m/v) BSA and analyzed using a linear gradient (Table 1B).
Analyses are performed with a linear gradient (Table 1B).

aliquoted immediately after preparation. Depending on the total analysis time, one or two sets of calibration samples were thawed before each analytical run.

The results of the stability experiments in tissue, intestinal contents and faeces homogenates stresses the importance for homogenates to be frozen after collection and homogenized immediately after thawing, while keeping the time span between sample collection and analysis as short as possible to prevent significant degradation of PhIP and N-OH-PhIP.

5. Toxicokinetic study

The applicability of the assay was demonstrated by i.v. administration of 1 mg/kg PhIP into the tail vein of a male mouse. After 0.5 h plasma, urine, intestinal contents and tissues were collected and analyzed. Bile was obtained in a separate experiment where gall bladder cannulation in a male mouse was performed. After cannulation, 1 mg/kg PhIP was administered i.v. and bile was collected in 15 min fractions. Faeces were collected for 24 h in a metabolic cage after i.v. administration of 1 mg/kg PhIP into the tail vein of a male mouse. Concentrations in plasma, urine, bile, intestinal contents, faeces and tissues are presented in Tables 8A and 8B. The concentrations in bile represent the fraction collected between 30 and 45 min after PhIP administration. The concentration of PhIP in urine is was above the highest calibration standard and was diluted 100× in control human urine before analysis. Fig. 2C and D shows chromatograms of PhIP and N-OH-PhIP in processed kidney homogenate and urine, respectively, both from mice 30 min

Table 8A
Concentration of PhIP and N-OH-PhIP in tissue, intestinal contents and faeces after iv administration of 1 mg/kg PhIP to male mice.

Matrix	PhIP (pg/mg)	N-OH-PhIP (pg/mg)
Brain	24.9	27.4
Kidney	1161	<LLQ
Liver	337	2.33
Spleen	143	12.0
Testis	51.5	<LLQ
Cecum content	1428	49.7
Colon content	1565	39.1
Small intestine content	2450	65.1
Cecum tissue	400	22.3
Colon tissue	257	15.7
Small intestine tissue	560	12.3
Faeces	706	38.4

Table 8B
Concentration of PhIP and N-OH-PhIP in plasma, urine and bile after iv administration of 1 mg/kg PhIP to male mice.

Matrix	PhIP (ng/mL)	N-OH-PhIP (ng/mL)
Plasma	25.8	12.0
Urine	5930	126
Bile	241	<LLQ

after i.v. administration of 1 mg/kg PhIP. Fig. 2D also clearly shows the presence of an additional compound in the N-OH-PhIP (m/z 241/223) window. This compound was hypothesized to be 5-OH-PhIP after comparison with a reference 5-OH-PhIP sample [8].

6. Conclusion

We have developed an assay for the sensitive analysis of PhIP and N-OH-PhIP in murine plasma, urine, bile, intestinal contents, faeces and tissue homogenates. The influence of matrix effects on the quantification of analytes is described. Two LC-gradients were developed: a step gradient and a linear gradient.

For the analysis of PhIP, a stable isotope labelled internal standard was available to compensate for matrix effects. Both gradients can be used for the quantitative analysis of this analyte. For N-OH-PhIP, deuterated PhIP was used as an internal standard. The step gradient was used for the analysis of N-OH-PhIP in plasma, urine and bile. However, the analysis in intestinal contents, faeces and tissue homogenates was biased due to matrix effects. By applying the linear gradient, matrix effects were reduced and accuracies were within ±15%, even when human matrices were used as calibration standards. For the quantification of N-OH-PhIP in mouse urine, samples had to be diluted 100× in human urine before analysis to ensure accuracies within 85–115%.

The assay was shown to be reproducible, accurate, and precise. The validated range for both PhIP and N-OH-PhIP is from 1 to 250 ng/mL in plasma and 4% (m/v) BSA, and the extended range in 4% (m/v) BSA is up to 1000 ng/mL. The dynamic range of both PhIP and N-OH-PhIP in urine is 10–1000 ng/mL.

The applicability of the method has been demonstrated and optimized for the above described matrices and it has been successfully used to study *in vivo* toxicokinetics of PhIP and N-OH-PhIP in mice.

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