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Detection and quantitation of *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine adducts in DNA using online column-switching liquid chromatography tandem mass spectrometry

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Abbreviations

Heterocyclic aromatic amine, HAA; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; PhIP-C8-dG, *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; ESI, electrospray ionization; LC-MS/MS, liquid chromatography-tandem mass spectrometry (mass spectrometry/mass spectrometry); SRM, selected reaction monitoring; CID, collision induced dissociation

Abstract

The heterocyclic aromatic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is formed by the grilled cooking of certain foods such as meats, poultry and fish. PhIP has been shown to induce tumours in the colon, prostate and mammary glands of rats and is regarded as a potential human dietary carcinogen. PhIP is metabolically activated via cytochrome P450 mediated oxidation to an *N*-hydroxylamino-PhIP intermediate that is subsequently converted to an ester by *N*-acetyltransferases or sulfotransferases and undergoes heterolytic cleavage to produce a PhIP-nitrenium ion, which reacts with DNA to form the *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP-C8-dG) adduct. Thus far, the detection and quantification of PhIP-DNA adducts has relied to a large extent on ^{32}P-postlabelling methodologies. In order to expand the array of available techniques for the detection and improved quantification of PhIP-C8-dG adducts in DNA we have developed an online column-switching liquid chromatography (LC)-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) selected reaction monitoring (SRM) method incorporating an isotopically [$^{13}C_{10}$]-labelled PhIP-C8-dG internal standard for the analysis of DNA enzymatically hydrolysed to 2'-deoxynucleosides. A dose-dependent increase was observed for PhIP-C8-dG adducts when salmon testis DNA was reacted with *N*-acetoxy-PhIP. Analysis of DNA samples isolated from colon tissue of mice treated by oral gavage daily for 5 days with 50 mg/kg body weight of PhIP resulted in the detection of an average level of 14.8 ± 3.7 PhIP-C8-dG adducts per 10^6 2'-deoxynucleosides. The method required 50 µg of hydrolysed animal DNA on column and the limit of detection for PhIP-C8-dG was 2.5 fmol (1.5 PhIP-C8-dG adducts per 10^8 2′-deoxynucleosides). In summary, the LC-ESI-MS/MS SRM method provides for the rapid automation of the sample clean up and a reduction in matrix components that would otherwise

interfere with the mass spectrometric analysis, with sufficient sensitivity and precision to analyse DNA adducts in animals exposed to PhIP.

Introduction

The heterocyclic aromatic amine (HAA), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is formed following the pyrolysis of amino acids and reaction with sugars in the presence of creatine/creatinine during the grilled cooking of certain foods, such as meats, poultry and fish [1-5]. PhIP represents one of the most abundantly formed of more than 20 HAAs that have been shown to be mutagenic in bacterial assays as well as mammalian cells [3,5]. The carcinogenic properties of HAAs have been demonstrated in rodent bioassays following long-term feeding studies [6]. PhIP has been found to induce tumours in the colon, prostate and mammary glands of rats, which represent the principal sites that are associated with human dietary related cancer implying that it could be a human carcinogen [3,4,7-9]. The International Agency for Research on Cancer (IARC) has classified PhIP as a group 2B carcinogen (possibly carcinogenic to humans) [10].

The genotoxicity of PhIP is related to metabolic activation resulting in an electrophilic species that is capable of generating covalent DNA adducts [1]. PhIP undergoes an initial oxidation step to an *N*-hydroxylamino-PhIP intermediate which is mediated by cytochrome (CYP) P450 enzymes, primarily by CYP1A2 in humans [11-13]. Subsequent metabolism by *N*-acetyltransferases (NAT) or sulfotransferases (SULT) converts the *N*-hydroxylamino-PhIP into an ester capable of undergoing heterolytic cleavage to produce a PhIP-nitrenium ion, which is the ultimate reactive species that reacts with DNA (**Scheme 1**) [5,13,14]. The major covalent DNA adduct detected *in vivo* resulting from the exposure of experimental animals and humans to PhIP is *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP-C8-dG) [14,15].

Numerous methods have been developed for the detection of PhIP-derived DNA adducts, which include ^{32}P-Postlabelling in combination with thin layer chromatography (TLC) or high performance liquid chromatography [16-20], immunological methods [7,9], gas chromatography-mass spectrometry following alkaline hydrolysis of PhIP adducts to the parent amine [21,22] and accelerator mass spectrometry [23-25]. ^{32}P-Postlabelling has been the most widely used method for the detection of PhIP-DNA adducts in animal and human tissues offering the advantage that only small amounts of DNA are required for analysis. The development of electrospray ionization (ESI) has permitted the coupling of liquid chromatography (LC) to mass spectrometry (MS), which has been used for the detection of numerous DNA adducts [26]. LC-mass spectrometry has made sufficient gains in sensitivity for the detection of DNA adducts resulting in it being a viable alternative to other detection methods such as ^{32}P-postlabelling and immunoassays, to provide more accurate and precise results through the use of stable isotope internal standards [27,28]. Recently, LC-tandem mass spectrometry (MS/MS) methods using deuterated stable isotope internal standards and off-line pre-purification of the PhIP-C8-dG adduct using solid phase extraction or online column-switching LC have been described for the detection and quantitation of adducts in cells and DNA treated *in vitro* with PhIP, and also in the liver and colon of rats treated *in vivo* with PhIP [20,22,29-31]. Mass spectrometry based methods offer a number of advantages with respect to the identification and quantitation of DNA adducts, as they rely on the detection of adduct-specific ions and characteristic transitions of the ions being monitored, respectively, and because quantification is based upon the use of isotopically-labelled internal standards.

We describe the development of a method involving online column-switching LC-MS/MS using selected reaction monitoring (SRM) for the determination of PhIP-C8-dG in DNA

that has been enzymatically hydrolysed to 2'-deoxynucleosides. This newly developed method allows for the rapid automation of the sample clean up and a reduction in matrix components that would otherwise interfere with the mass spectrometric analysis. We applied this method to the detection of PhIP-C8-dG adducts in DNA samples from salmon testis DNA treated with different concentrations of *N*-acetoxy-PhIP *in vitro* and DNA obtained from mouse colon tissue following dosing with PhIP.

Experimental Procedures

Chemicals and Reagents

PhIP was acquired from Toronto Research Chemicals (North York, Ontario, Canada), and [$^{13}C_{10}$]-2′-deoxyguanosine was purchased from Cambridge Isotope Laboratories (Andover, MA). All other reagents used in the preparation of the DNA adduct standards and *in vitro* modified DNA samples were of the highest available purity and purchased from Sigma-Aldrich (St. Louis, MO). **Caution:** *PhIP and its derivatives are mutagens and carcinogens and should be handled with care. Protective clothing should be worn and appropriate safety procedures followed when working with these compounds.* Salmon testis DNA, deoxyribonuclease I (from bovine pancreas), snake venom phosphodiesterase I (from *Crotalus adamanteus*), alkaline phosphatase (from *E. coli*) and mass spectrometry grade formic acid were purchased from Sigma (Poole, UK). HPLC grade water, 18.2 MΩcm output quality was obtained from Maxima purification equipment (Elga, High Wycombe, UK). All other reagents (analytical grade) and HPLC grade acetonitrile were purchased from Fisher Scientific (Loughborough, UK).

Synthesis of *N*-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-acetoxy-PhIP)

2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine (*N*-hydroxylamino-PhIP) was prepared as described previously, by diazotization of PhIP and reaction with sodium nitrite followed by catalytic reduction of the nitro derivative thus obtained with Pd/C to the *N*-hydroxylamine. [32] The hydroxylamine was acetylated as described previously to afford *N*-

acetoxy-PhIP. [14] Given the expected instability of *N*-acetoxy-PhIP, it was used without any further purification.

Preparation of unlabelled and isotopically labelled *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine adduct standards

The PhIP-C8-dG adduct standards were prepared as described previously [14], by reacting the crude acetylation mixtures containing *N*-acetoxy-PhIP with solutions of 2′-deoxyguanosine or [$^{13}C_{10}$]-2′-deoxyguanosine. Unlabelled and [$^{13}C_{10}$]-labelled PhIP-C8-dG thus obtained were purified by reverse-phase HPLC [14]. The identity and purity of the unlabelled PhIP-C8-dG was confirmed by ^{1}H NMR and ESI-MS/MS (data not shown). The adduct standard solutions were quantified by UV spectrophotometry, based upon an extinction coefficient of ε = 33,300 $M^{-1}cm^{-1}$ at λ_{max} 360 nm. [22]

Preparation of salmon testis DNA samples modified *in vitro* with *N*-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

A range of DNA samples with different levels of in-vitro modification were prepared by adding serial 10-fold dilutions of *N*-acetoxy-PhIP in THF to solutions of salmon testis DNA (1 mg/mL in 25 mM phosphate buffer, pH 7.0). The estimated concentrations of the *N*-acetoxy-PhIP in the reaction mixtures ranged from *ca.* 0.0001 mg/mL to 0.1 mg/mL. The reaction mixtures were incubated overnight at 37ºC, extracted with 1 volume of ethyl acetate then 1 volume of water-saturated *n*-butanol, and then DNA was precipitated by addition of 0.1 volume of 5 M NaCl and 2 volumes of ice-cold ethanol. DNA samples were centrifuged, the pellets thus obtained were washed twice with 5 mL of ice-cold ethanol/water (70:30, *v/v*) and the residues

finally obtained upon centrifugation were redissolved in 5 mM BisTris buffer, pH 7.1, 0.1 mM EDTA, allowing an overnight rehydration at 4ºC before freezing the samples at −80ºC until further use. The DNA concentrations in the final solutions were determined by UV spectrophotometry.

Enzymatic hydrolysis of *in vitro* modified salmon testis DNA for LC-ESI-MS/MS analysis

Each of the *in vitro* modified salmon testis DNA samples (400 µg) was evaporated to dryness using a centrifugal vacuum evaporator. The dried DNA samples were dissolved in 990 µL of 50 mM BisTris, 0.1 mM EDTA, pH 7.1 buffer plus 10 µL of 1 M $MgCl_2$ and incubated with 100 µL of deoxyribonuclease I (2 mg/mL dissolved in 0.15 M NaCl, 10 mM $MgCl_2$) at 37ºC for 6 h. The samples were further incubated with 80 µL of snake venom phosphodiesterase I from *Crotalus adamanteus* (0.001 U/µL dissolved in 0.11 M Tris-HCl, 0.11 M NaCl, 15 mM $MgCl_2$, pH 8.9) and 26.2 µL of alkaline phosphatase from *E. coli* (0.305 U/µL) at 37ºC for 15 h. The hydrolysed DNA samples were centrifuged at 14,000 rpm for 1 min and the supernatants transferred to new microfuge tubes for storage at −80ºC. Aliquots of the hydrolysed DNA samples were removed to which were added 1333.3 fmol of the [$^{13}C_{10}$]-labelled PhIP-C8-dG internal standard (100 fmol/µL) and evaporated to dryness using a centrifugal vacuum evaporator. The dried samples were dissolved in 20 µL of HPLC grade water/methanol (80:20, *v*/*v*), centrifuged at 14,000 rpm for 2 min and transferred to HPLC vials with low volume inserts. A 15 µL aliquot of each sample was injected on to the online column switching LC-ESI-MS/MS, equivalent to 5 or 10 µg of hydrolysed DNA depending on the extent of modification by PhIP with 1000 fmol of the [$^{13}C_{10}$]-labelled PhIP-C8-dG internal standard on column.

Treatment of animals

$Por^{lox/lox}/Cre^{CYP1A1}$ mice on a C57BL/6 background (Reductase Inducible Cre, RIC) were bred in-house at the Biomedical Research Institute, Dundee [33]. In these mice the cytochrome P450 oxidoreductase (Por) gene can be deleted tissue-specifically after the administration of chemical inducers (e.g. 3-methylcholanthrene) of CYP1A1. However, in the absence of the chemical inducer (as in the present study), these mice are phenotypically normal and indistinguishable from wild-type animals. Adult female RIC mice, > 3 weeks old, were dosed by oral gavage daily for 5 days with 50 mg/kg body weight of PhIP dissolved in corn oil. Control animals were dosed with corn oil alone. Animals were sacrificed and the colon tissue was removed. DNA isolation was performed by using standard phenol/chloroform extraction. All animal experiments were carried out under license in accordance with the law, and following local ethical review.

Enzymatic hydrolysis of animal DNA for LC-ESI-MS/MS analysis

Prior to enzymatic digestion 1333.3 fmol of the [$^{13}C_{10}$]PhIP-C8-dG internal standard (100 fmol/μL) was added to 66.7 μg of the mouse DNA samples and evaporated to dryness using a centrifugal vacuum evaporator. The dried DNA samples were dissolved in 99 μL of 50 mM BisTris, 0.1 mM EDTA, pH 7.1 buffer plus 1.0 μL of 1 M $MgCl_2$ and incubated with 10 μL of deoxyribonuclease I (2 mg/mL dissolved in 0.15 M NaCl, 10 mM $MgCl_2$) at 37ºC for 6 h. The samples were further incubated with 6.0 μL of snake venom phosphodiesterase I from *Crotalus adamanteus* (0.001 U/μL dissolved in 0.11 M Tris-HCl, 0.11 M NaCl, 15 mM $MgCl_2$, pH 8.9) and 3.18 μL of alkaline phosphatase from *E. coli* (0.315 U/μL) at 37ºC for 15 h. The hydrolysed DNA samples were centrifuged at 14,000 rpm for 2 min and the supernatant transferred to a new

eppendorf tube and then evaporated to dryness using a centrifugal vacuum evaporator. The dried samples were dissolved in 20 µL of HPLC grade water/methanol (80:20, *v*/*v*) and transferred to HPLC vials with low volume inserts. A 15 µL aliquot of each sample, equivalent to 50 µg of hydrolysed DNA and 1000 fmol of the [$^{13}C_{10}$]PhIP-C8-dG internal standard on column was injected on to the online column switching LC-ESI-MS/MS.

Online column-switching LC-ESI-MS/MS analysis

The online column switching valve system consisted of an automated switching valve (motorized two-position six-port valve, Waters Ltd., Hertfordshire, UK) connected to pump A incorporating an autosampler (Waters Alliance 2695 separations module with a 100 µL injection loop) and an isocratic pump B (Gynotek GmbH, Germering, Germany). Pump A was connected via the switching valve to the trap column, a Synergi Fusion-RP 80A C_{18}, 4 µm, 30 × 2.0 mm column attached to a KrudKatcher disposable pre-column (0.5 µm) filter (Phenomenex, Macclesfield, UK) and Pump B was connected via the switching valve to the analytical column, a Synergi Fusion-RP 80A C_{18} (4 µm, 250 × 2.0 mm) column attached to a Synergi Fusion-RP 80A C_{18} (4 µm, 4.0 × 2.0 mm) guard column and KrudKatcher disposable pre-column (0.5 µm) filter. The outlet of the analytical column was directly connected to the mass spectrometer. The configuration of the online column switching valve system is summarised in **Figure 1**.

Sample loading: A 15-µL aliquot of the *in vitro* modified (equivalent to 5 or 10 µg of hydrolysed DNA) or animal (equivalent to 50 µg of hydrolysed DNA) DNA sample containing 1000 fmol of [$^{13}C_{10}$]PhIP-C8-dG internal standard was injected onto the trap column using pump A with the switching valve in position 1. The impurities on the trap column were eluted to waste using a gradient with solvent A, 0.1% formic acid/HPLC grade water (0.1:99.9, *v*/*v*) and solvent

B, 0.1% formic acid/acetonitrile (0.1:99.9, v/v) at a flow rate of 120 µL/min. The following gradient was used: 0 min–15%B, 5 min–15%B, 15 min–65%B, 24 min–65%B, 24.1 min–15%B, 40 min–15%B. Concurrently isocratic flow at 120 µL/min via the analytical column, which was eluted with 0.1% formic acid/acetonitrile (0.1% formic acid) (75:25, *v*/*v*), was maintained to the mass spectrometer by means of pump B.

Sample elution: At 12.0 min the switching valve was switched to position 2 allowing the purified PhIP-C8-dG adduct to be back-flushed from the trap column onto the analytical column and then subsequent elution into the mass spectrometer. The isocratic flow was maintained by pump B at flow rate of 120 µL/min with 0.1% formic acid/ acetonitrile (0.1% formic acid) (75:25, *v*/*v*) for 12.0 min. Concurrently the flow from pump A bypassed the trap column and was diverted directly to waste. At 24.0 min the switching valve was switched back to position 1 and the configuration of the online column switching system reverted back to initial conditions as described for the sample loading above. The total run time was 40 min.

Mass spectrometry: A Waters Micromass Quattro Ultima Pt (Micromass, Waters Ltd., Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface was used. The temperature of the electrospray source was maintained at 120ºC and the desolvation temperature at 350ºC. Nitrogen gas was used as the desolvation gas (650 L/h) and the cone gas (25 L/h). The capillary voltage was set at 3.20V. The cone and RF1 lens voltages were 42 V and 15 V, respectively. The collision gas was argon (indicated cell pressure 2.0×10^{-3} mbar) and the collision energy set at 12 eV. The dwell time was set to 200 ms and the resolution was two *m/z* units at peak base. The mass spectrometer was tuned by using a standard solution of PhIP-C8-dG (10 pmol/µL) in 0.1% formic acid/acetonitrile (0.1% formic acid) (75:25, *v*/*v*) introduced by continuous infusion at a flow rate of 10 µL/min with a Harvard model 22 syringe pump (Havard

Apparatus Ltd., Edenbridge, UK). The hydrolysed DNA samples were analysed in positive ESI MS/MS selected reaction monitoring (SRM) mode for the $[M+H]^+$ ion to base $[B+H_2]^+$ transitions of *m/z* 490 to 374 for PhIP-C8-dG and *m/z* 500 to 379 for $[^{13}C_{10}]$PhIP-C8-dG. The level of the adduct in the DNA samples was determined from the ratio of peak area of the $[^{13}C_{10}]$PhIP-C8-dG internal standard.

Calibration curve for PhIP-C8-dG adducts with DNA matrix

The calibration curvess were constructed by the addition of different amounts of the unlabelled PhIP-C8-dG standards (ranging from 2.0 to 4000 fmol) plus 1000 fmol of the $[^{13}C_{10}]$PhIP-C8-dG internal standard to 50 μg of enzymatically hydrolysed calf thymus DNA as the matrix. The standards were subjected to the online column switching LC-ESI-MS/MS analysis procedure as described above.

Results

Validation of the online column switching LC-ESI-MS/MS analysis

Accurate DNA adduct quantitation by mass spectrometry is best achieved by coupling selected reaction monitoring (SRM) with the use of stable isotope-labelled internal standards. Mass spectrometric analysis of the [$^{13}C_{10}$]PhIP-C8-dG internal standard was performed in positive mode LC-ESI-MS/MS using the trap column only and resulted in a typical product ion spectrum that is normally observed for adducted 2′-deoxynucleosides following the neutral loss (121 u) of the [$^{13}C_5$]-labelled 2′-deoxyribose moiety. The ^{13}C-labelled atoms were equally distributed between the adducted base and 2′-deoxyribose moiety of the molecule as shown in **Figure 2**. The collision induced dissociation (CID) product ion mass spectrum consisted of the adducted base as being the most abundant product ion formed from the $[M+H]^+$ ion at *m/z* 500. The adducted base product ion $[B+H_2]^+$ at *m/z* 379 was formed by the cleavage of the glycosidic bond and the accompanied proton transfer from the 2′-deoxyribose moiety to the adducted base (**Figure 2**). A similar fragmentation pattern with the *m/z* decreased by 5 for the adducted base product ion $[B+H_2]^+$ was observed for the corresponding unlabelled PhIP-C8-dG standard $[M+H]^+$ ion. [$^{13}C_{10}$]PhIP-C8-dG was chemically pure as determined by HPLC-UV and no unlabelled adduct was detectable in the isotopic standard solutions as determined by LC-ESI-MS/MS SRM.

Initial experiments involved optimisation of the mobile phase conditions for the separation of the PhIP-C8-dG adduct on the trap column from unmodified 2'-deoxynucleosides and matrix impurities. A gradient mobile phase system consisting of 0.1% formic acid and acetonitrile (0.1% formic acid) was found to give the optimal separation on the trap column with a retention time of 14.16 min for PhIP-C8-dG. The online column switching valve system was

configured as shown in **Figure 1** and the switching valve was switched to position 2 at 12.0 min following injection of the hydrolysed DNA sample. This allowed for unmodified 2′-deoxynucleosides plus any other matrix impurities that may have been present to be eluted to waste while the PhIP-C8-dG adduct was back-flushed onto the analytical column for analysis by the mass spectrometer.

A typical online column-switching LC-MS/MS calibration curve for the detection of PhIP-C8-dG is shown in **Figure 3**. The calibration curve was obtained by adding different amounts of the PhIP-C8-dG standard with 1000 fmol of the [$^{13}C_{10}$]PhIP-C8-dG internal standard to 50 μg of enzymatically hydrolysed calf thymus DNA to account for any matrix effects.. A linear response over the range from 2.5 to 4000 fmol, corresponding to 1.5 to 2,469.1 adducts per 10^8 2′-deoxynucleosides (**Figure 3**), was obtained for the detection of PhIP-C8-dG with a correlation coefficient (r) of 0.9998 ($y = 0.001x$-0.0008). The limit of detection on column of the method was 2.5 fmol (S/N = 3) corresponding to 1.5 PhIP-C8-dG adducts per 10^8 2′-deoxynucleosides and the lower limit of quantitation was 10.0 fmol (S/N = 8) obtained by spiking the PhIP-C8-dG adduct and the labelled internal standard into calf thymus DNA (50 μg) enzymatic hydrolysate. The intra- and inter-assay coefficients of variation of the online column-switching LC-ESI-MS/MS method, which were obtained following the analysis of calf thymus spiked with 25, 100 and 1000 fmol of the PhIP-C8-dG standard are shown in **Table 1**. The average accuracy of the method, determined by spiking known amounts (2.5 to 4000 fmol) of the PhIP-C8-dG adduct and 1000 fmol of the [$^{13}C_{10}$]PhIP-C8-dG internal standard in calf thymus DNA enzymatic hydrolysate was 100.2%.

Online column switching LC-ESI-MS/MS analysis of *in vitro* modified and animal DNA samples

Salmon testis DNA was incubated at 37 °C overnight with solutions of *N*-acetoxy-PhIP. The typical online column-switching LC-MS/MS SRM ion chromatograms obtained for the determination of PhIP-C8-dG adducts in control (untreated) DNA and salmon testis DNA treated with 0.001mg/mL of *N*-acetoxy-PhIP are shown in **Figure 4A and B**, respectively. A linear dose-dependent increase was observed for the levels of PhIP-C8-dG adducts detected in salmon testis DNA following exposure to levels of *N*-acetoxy-PhIP ranging from 0.0001 to 0.1 mg/mL (**Figure 5**). The levels of PhIP-C8-dG adducts observed for the different DNA samples and the intra- and inter-assay coefficients of variation for the analyses are shown in **Table 2**. The typical online column-switching LC-MS/MS ion chromatograms obtained for a mouse colon DNA sample is shown in **Figure 4C** for a control animal that was treated with the vehicle (corn oil) alone and **Figure 4D** shows the typical ion chromatogram obtained following the daily oral dosing of a mouse with 50 mg/kg body weight of PhIP for 5 days. The average level of PhIP-C8-dG adducts detected in colon DNA from three animals treated under these conditions was 14.8 ± 3.7 PhIP-C8-dG adducts per 10^6 2'-deoxynucleosides.

Discussion

In the present study we have described the development of an online column-switching LC-ESI-MS/MS SRM method that is suitable for the routine detection and quantification of PhIP-derived DNA adducts formed *in vivo*. Mass spectrometry offers great potential when applied to the detection and quantification of DNA adducts due to its very high sensitivity and chemical specificity, which allows the identification of specific DNA adducts in complex biological matrices. The advantage of using online column-switching LC is that an enzymatically hydrolysed DNA sample can be analysed directly without the requirement for off-line pre-purification such as solid phase extraction of the DNA adduct, from the vast excess of unmodified 2′-deoxynucleosides that could potentially interfere with the analysis. The PhIP-C8-dG adduct is retained on a trap (enrichment) column while unmodified 2′-deoxynucleosides are eluted to waste. Following elution of the unmodified 2′-deoxynucleosides as well as any matrix impurities that may cause concomitant ionisation suppression, the switching valve diverts the flow from the trap column into the analytical column, which is connected to the mass spectrometer. We have previously applied a similar approach for the determination of DNA adducts derived from reactive oxygen species, acetaldehyde and 3-nitrobenzanthrone [28,34,35]. Interferences from the matrix may result in poor sensitivity by competition for charge, or increased droplet viscosity and surface tension from high concentrations of co-eluting interfering compounds for the formation of gas phase ions in the electrospray source [36]. The addition of [$^{13}C_{10}$]PhIP-C8-dG internal standard prior to enzymatic hydrolysis of the DNA samples allowed for the accurate quantification of the adduct and also provided confirmation of the identity of the analyte peak since it eluted at the identical retention time as the unlabelled PhIP-C8-dG. The ^{13}C-labelled stable isotope internal standard affords the advantage, when compared to deuterium

labelled internal standards, that it is not subject to isotope (eg. deuterium-hydrogen) exchange. Furthermore the fragmentation properties of deuterated compounds may be different from the corresponding natural isotope abundance containing compounds due to the more stable nature of the C-D covalent bond in relation to the C-H bond. Therefore, the accurate quantitation of the analyte may require the determination of a response factor to correct for this difference in fragmentation [37]. For both the PhIP-C8-dG adduct and the corresponding stable isotope internal standard SRM transitions were used for the $[M+H]^+$ ion to adducted base product ion $[B+H_2]^+$ formed by the cleavage of the glycosidic bond. SRM offers the greatest sensitivity due to decreased solvent and matrix interferences, and increased specificity due to the monitoring of a characteristic fragmentation following CID of the compound being investigated. Initial method validation was performed by the construction of calibration curves following the spiking of PhIP-C8-dG into calf thymus DNA within a range of 2.5 to 4000 fmol. The method presented good statistical performance with average intra- and inter-assay coefficients of variation of 4.9 and 5.6%, respectively. The estimated LOD and LOQ was 2.5 and 10 fmol on column for PhIP-C8-dG, respectively. These values correspond to a LOD and LOQ of 1.5 and 6.2 PhIP-C8-dG adducts per 10^8 deoxynucleosides in a 50 µg DNA sample, respectively.

In order to develop the online column-switching LC-ESI-MS/MS method for the detection and quantification of PhIP derived DNA adducts formed *in vivo*, salmon testis DNA samples were treated with different concentrations of the reactive metabolite *N*-acetoxy-PhIP ranging from 0.0001 to 0.1 mg/mL. A dose-dependent increase was observed for PhIP-C8-dG adducts following reaction of salmon testis DNA with *N*-acetoxy-PhIP. The levels ranged from 5.5 to 2728.2 PhIP-C8-dG adducts per 10^6 2′-deoxynucleosides. The average intra- and inter-assay coefficients of variation for the analysis of these samples were 3.3 and 5.3%, respectively.

Analysis of DNA samples isolated from colon tissue of mice treated by oral gavage daily for 5 days with 50mg/kg body weight of PhIP resulted in the detection of an average level of 14.8 ± 3.7 PhIP-C8-dG adducts per 10^6 2'-deoxynucleosides. No PhIP-C8-dG adducts were detected in control animals dosed with corn oil alone. Previous studies have shown higher levels of PhIP-C8-dG adducts are detected in colon tissue when compared to the liver, which is consistent with the colon being the target tissue for tumour development [29,30].

In the past detection and quantification of PhIP DNA adducts has relied to a large extent on the ^{32}P-postlabelling technique [16-18]. The limitations of ^{32}P-postlabelling are that it does not provide structural confirmation of the adduct being detected and lacks the incorporation of an internal standard to account for adduct recovery and labelling efficiencies. Furthermore ^{32}P-postlabelling of HAA-DNA adducts has resulted in considerable inter-laboratory variation in the patterns of adduct spots detected following TLC separation making the interpretation of the results very difficult. It has been suggested that the majority of the additional spots observed correspond to adducted oligonucleotides as consequence of partial enzymatic hydrolysis of the DNA [16]. The online column-switching LC-ESI-MS/MS SRM method described here would require higher amounts of DNA for the analysis of human samples exposed to dietary levels of PhIP and is generally less sensitive when compared to ^{32}P-postlabelling methods. However, it presents the clear advantages of providing accurate quantitation with information on the chemical identity of the adduct and constitutes a more automated and faster analytical method. Presently accelerator mass spectrometry represents the most sensitive method for the detection of DNA adducts at levels normally encountered in human biomonitoring studies and allows dosing with HAAs at dose levels comparable to those found in the diet [23-25]. However the technique is disadvantaged by the requirement of dosing with ^{3}H- or ^{14}C-labelled carcinogens which is not

amenable for general human population based studies and does not provide any structural information [23-25]. The use of capillary- or nano-LC coupled to micro- or nanoelectrospray ionisation mass spectrometry in theory should provide for improvements in sensitivity and allow the detection of PhIP-C8-dG adducts in animal and human samples following dietary levels of exposure to PhIP.

In conclusion, we have developed an online column-switching LC-ESI-MS/MS SRM method with sufficient sensitivity and precision for the analysis of PhIP-C8-dG adducts formed in *in-vitro* experiments and *in-vivo* animal studies.

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Legends

Scheme 1 Bioactivation of PhIP and DNA adduct formation.

Figure 1 Schematic representation of the online column-switching system.

Figure 2 Positive LC-ESI-MS/MS CID product ion spectrum of the [$^{13}C_{10}$]PhIP-C8-dG internal standard. The spectrum was acquired following gradient elution of the trap column with solvent A, 0.1% formic acid and solvent B, acetonitrile (0.1% formic acid) at a flow rate of 120 μL/min (the asterisks indicate the ^{13}C labelled sites)

Figure 3 Online column-switching LC-MS/MS SRM calibration curve for the detection of PhIP-C8-dG (error bars represent the standard deviation, $n = 3$). The ratio of the peak area of the PhIP-C8-dG standard to that of the [$^{13}C_{10}$]PhIP-C8-dG internal standard spiked into 50 μg on column of calf thymus DNA subjected to enzymatic hydrolysis was plotted against the amount of the PhIP-C8-dG (fmol). The inset shows the expanded view of the calibration curve at the lower levels of PhIP-C8-dG.

Figure 4 Typical online column-switching LC-MS/MS SRM ion chromatograms for the determination of PhIP-C8-dG in (A) control calf thymus DNA (B) following the treatment of salmon testis DNA with 0.001 mg/mL of *N*-acetoxy-PhIP (C) colon DNA from mouse dosed with corn oil and (D) colon DNA from mouse treated with 50 mg/kg body weight of PhIP (50 μg of hydrolysed DNA was analysed (for (B) 10μg) and 1000 fmol of [$^{13}C_{10}$]PhIP-C8-dG internal

standard on column). The SRM transitions monitored were *m/z m/z* 490 to 374 for PhIP-C8-dG and *m/z* 500 to 379 for [$^{13}C_{10}$]PhIP-C8-dG. The trap column was eluted using a gradient with solvent A, 0.1% formic acid and solvent B, acetonitrile (0.1% formic acid) at a flow rate of 120 μL/min. The analytical column was eluted isocratically with 0.1% formic acid/acetonitrile (0.1% formic acid) (75:25, *v/v*) at a flow rate of 120 μL/min.

Figure 5 Plot for the formation of PhIP-C8-dG adducts following the treatment of salmon testis DNA with *N*-acetoxy-PhIP concentrations ranging from 0.0001 to 0.1 mg/mL as determined by online column-switching LC-MS/MS SRM analysis.

Table Legends

Table 1 Intra- and inter-assay precision for the determination of PhIP-C8-dG spiked into calf thymus DNA (50 μg of enzymatically hydrolysed DNA on column)

[a and b] $n = 4$ and 4, respectively

Table 2 Determination of PhIP-C8-dG adducts in salmon testis DNA treated with PhIP using online column-switching LC-MS/MS SRM analysis.

[a and b] 10 and 5 μg of enzymatically hydrolysed DNA analysed on column, respectively

[c] the ± error represents the standard deviation ($n = 3$)

[d] $n = 3$

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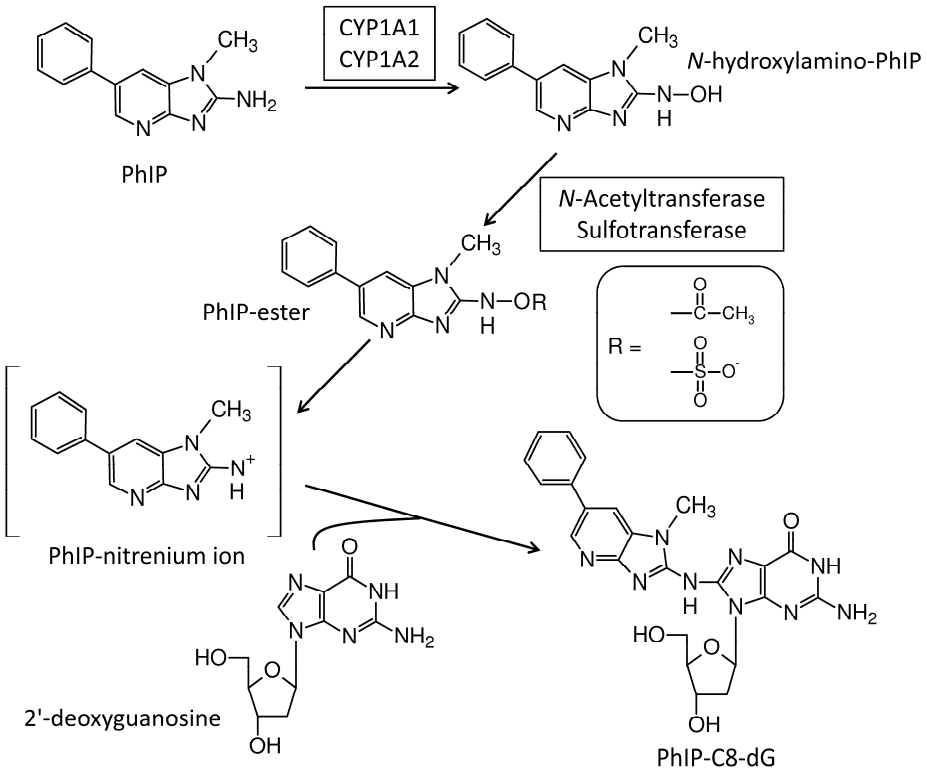
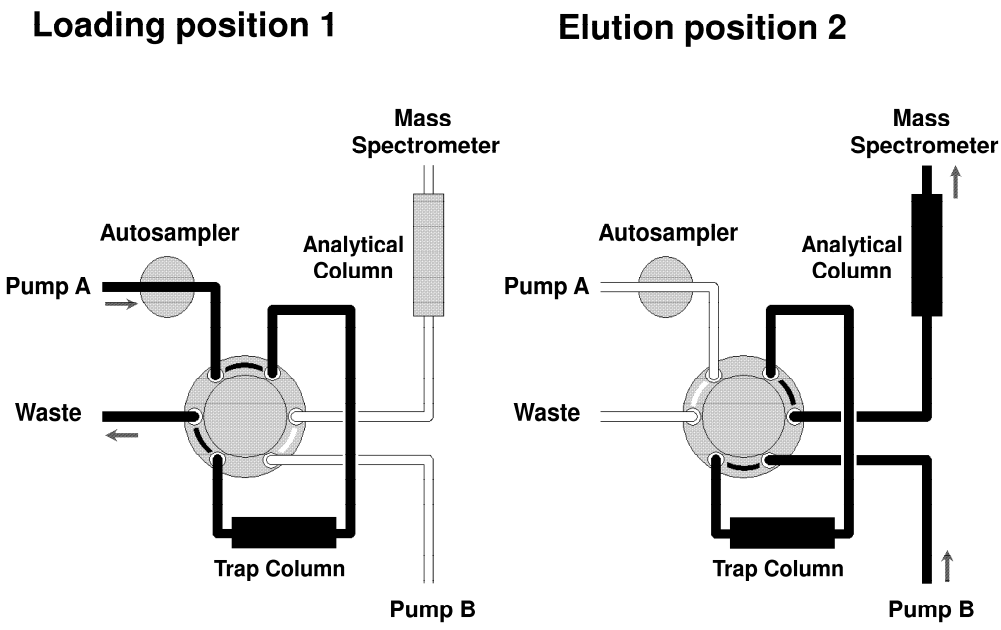
Figures



Scheme 1.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Pump A
Waste
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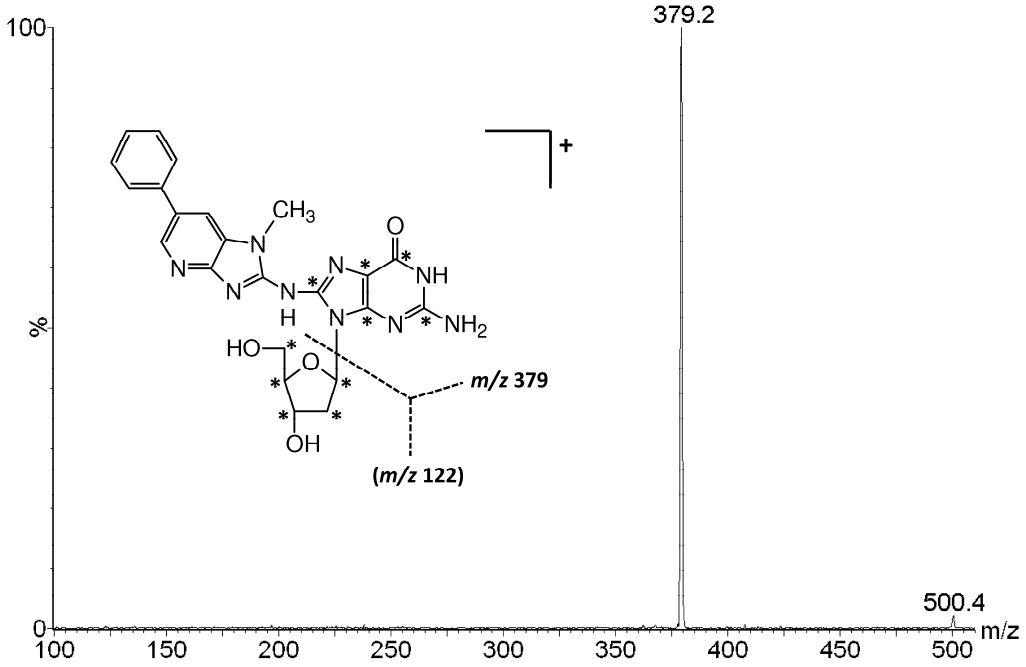
Analytical Column
Pump A
Waste
Trap Column
Pump B

Figure 1

Figure 2


379.2
100
%
0
500.4
m/z
100
150
200
250
300
350
400
450
500
+
CH3
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N
H
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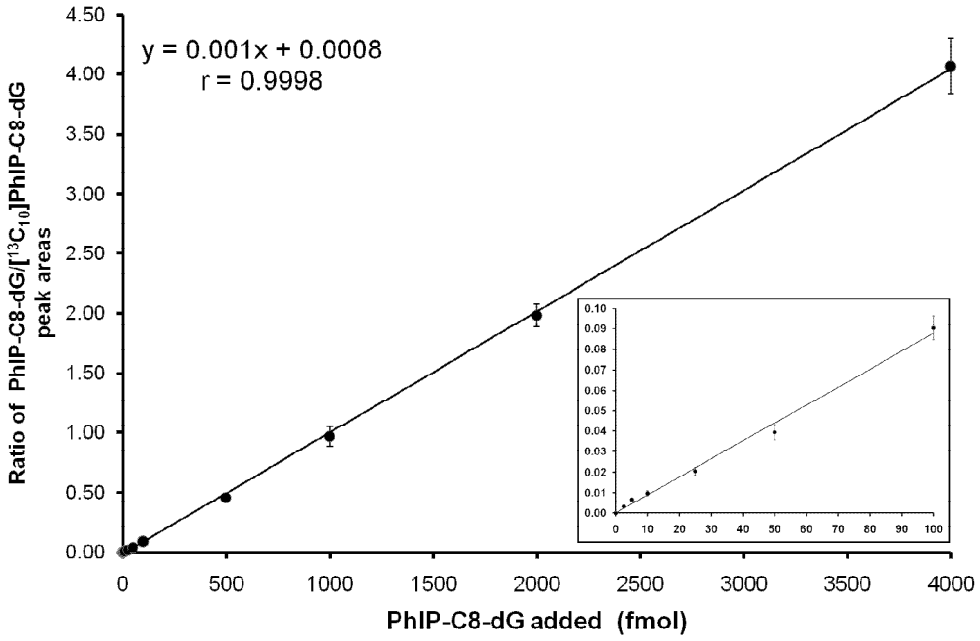
N
NH2
HO
O
OH
m/z 379
(m/z 122)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r = 0.9998
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PhIP-C8-dG added (fmol)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
500
1000
1500
2000
2500
3000
3500
4000
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
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0.00
0
10
20
30
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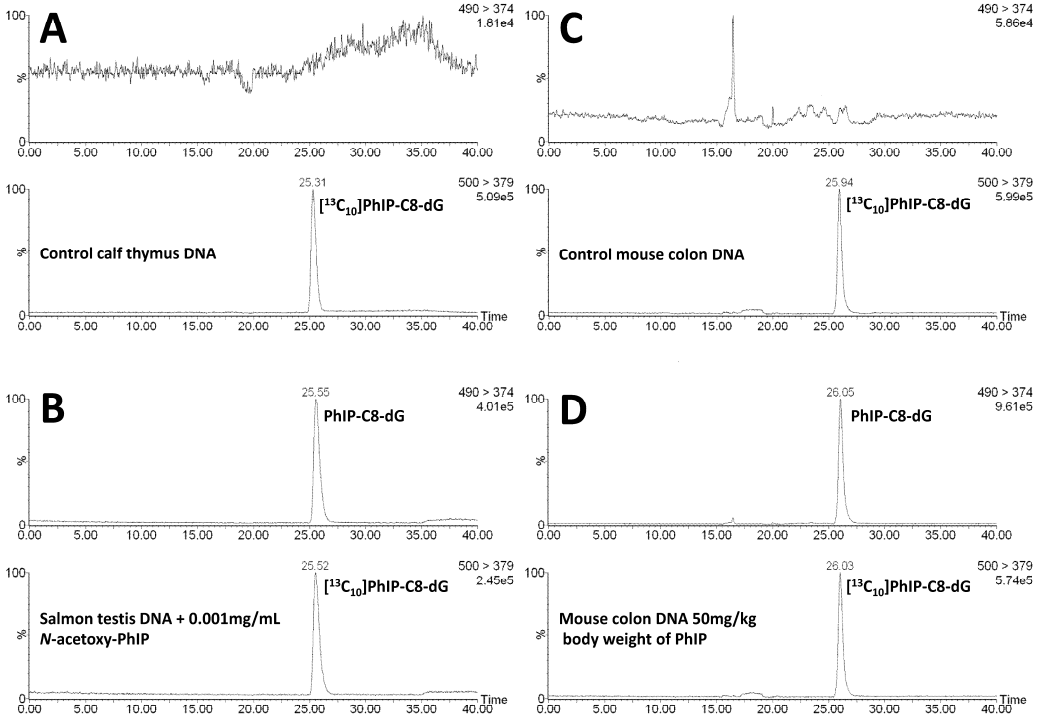
50
60
70
80
90
100

Figure 3.

Figure 4


A
490 > 374
1.81e4
C
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25.31
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Control calf thymus DNA
25.94
500 > 379
5.99e5
[13C10]PhIP-C8-dG
Control mouse colon DNA
B
25.55
490 > 374
4.01e5
PhIP-C8-dG
D
26.05
490 > 374
9.61e5
PhIP-C8-dG
25.52
500 > 379
2.45e5
[13C10]PhIP-C8-dG
Salmon testis DNA + 0.001mg/mL
N-acetoxy-PhIP
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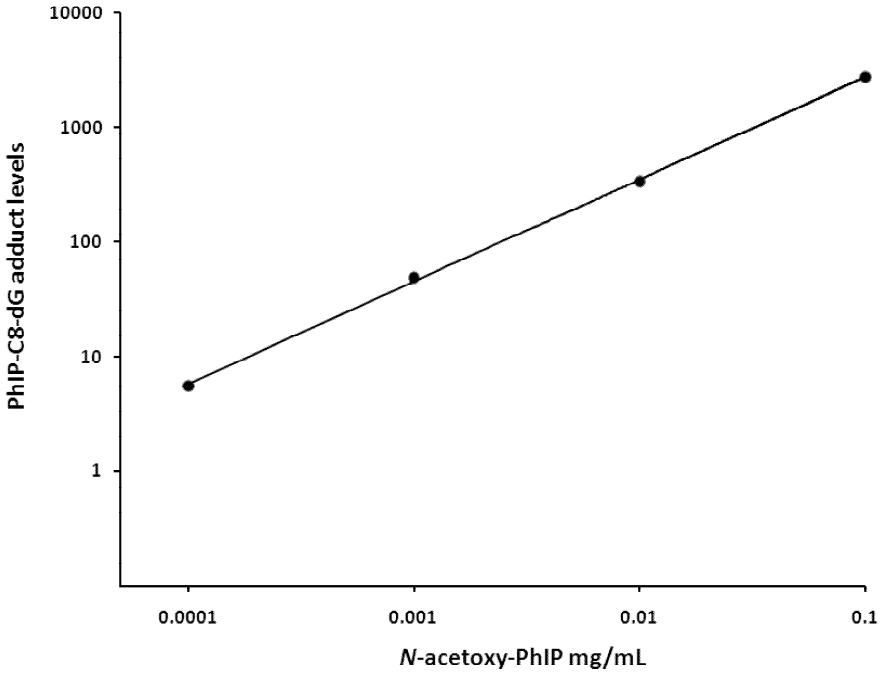
Mouse colon DNA 50mg/kg
body weight of PhIP
Time

Figure 5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100
10
1
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0.01
0.1
N-acetoxy-PhIP mg/mL