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Review

Formation and analysis of heterocyclic aromatic amine–DNA adducts in vitro and in vivo

Robert J. Turesky [a,*], Paul Vouros [b]

[a] *Division of Chemistry, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079, USA*
[b] *Department of Chemistry and Barnett Institute, Northeastern University, Boston, MA 02115, USA*

Abstract

The detection and quantification of heterocyclic aromatic amine (HAA)–DNA adducts, critical biomarkers in interspecies extrapolation of toxicity data for human risk assessment, remains a challenging analytical problem. The two main analytical methods currently in use to screen for HAA–DNA adducts are the ^{32}P-postlabeling assay and mass spectrometry, using either accelerated mass spectrometry (AMS) or liquid chromatography and electrospray ionization mass spectrometry (LC–ESI–MS). In this review, the principal methods to synthesize and characterize DNA adducts, and the methods applied to measure HAA–DNA adduct in vitro and vivo are discussed.

Keywords: Reviews; Heterocyclic aromatic amine–DNA adducts; DNA

Contents

1. Introduction

Heterocyclic aromatic amines (HAAs) induce cancer at multiple sites in experimental animals during long-term feeding studies [1]. Because of their wide spread occurrence in cooked meat products [2], HAAs may contribute to common forms of human cancers including colorectal, prostate, and breast that are associated with frequent consumption of diets high in meats and fat [3–5].

The adduction of genotoxic carcinogens such as HAAs to DNA is believed to be the first step in chemically induced carcinogenesis [6,7], and the identification of biomarkers representing genotoxic damage, such as DNA adducts, may aid in assessing human health risks [7,8]. Over the past several decades, different analytical methods have been established to detect and measure DNA adduct formation in experimental animals and humans. Early studies in animals used tritium- or radiocarbon-labeled aromatic amine carcinogens to assess DNA binding. Adduct identification was determined by high performance liquid chromatography (HPLC) with co-migration of non-radiolabeled adducts serving as UV markers [9]. A major limitation in these studies was the use of large amounts of radioactivity that precluded investigations in humans.

More recently Randerath et al. have developed the ^{32}P-postlabeling assay [10], which uses polynucleotide

* Corresponding author. Tel.: +1-870-543-7301; fax: +1-870-543-7686.
E-mail address: rturesky@nctr.fda.gov (R.J. Turesky).

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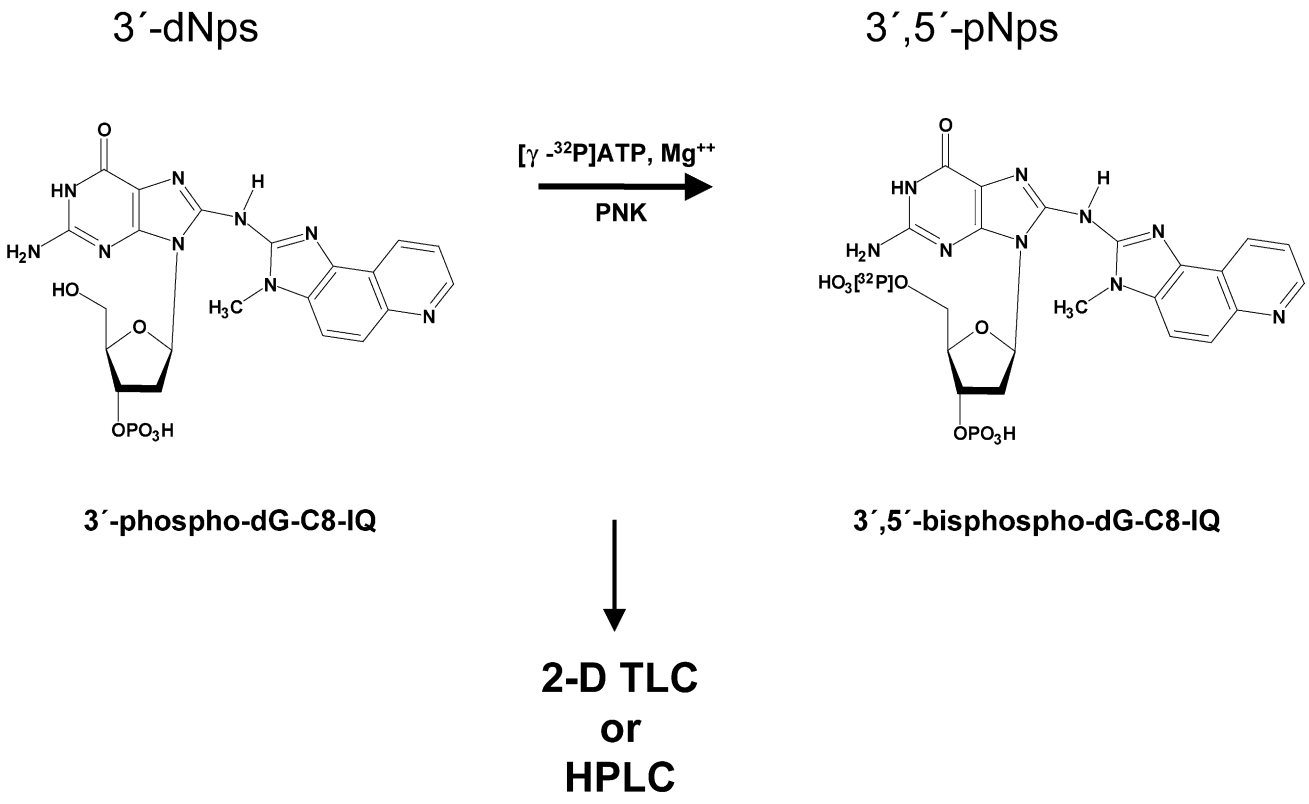

Fig. 1. The ^{32}P-postlabeling of 3′-phospho-dG-C8-IQ and non-modified nucleotides (3′,5′-bisdeoxynucleotides (pNps) by polynucleotide kinase followed by separation with TLC or HPLC).

kinase to enzymatically derivatize adducted 3′-nucleotides with [γ-^{32}P]ATP of very high specific activity to form the radiolabeled 3′,5′-bisnucleotide adducts. Thus, the need for radioactively labeled carcinogens was eliminated. The adducts are separated from non-modified 3′,5′-nucleotides (pNps) by two-dimensional TLC or HPLC (Fig. 1) [10]. The limits of detection of the ^{32}P-postlabeling assay can approach 1 adduct per 10^{10} non-modified DNA bases and the technique has been used to detect a wide variety of genotoxins in animals and humans [10,11]. The ^{32}P-postlabeling method is still a mainstay for human biomonitoring of DNA adducts because it is highly sensitive and the cost of establishing an analytical laboratory is relatively inexpensive. However, the method has several disadvantages that include: the requirement of significant amounts of radioactive phosphorous, a strong ß-emitter and potential health hazard; the lack of suitable internal standards to account for adduct recovery and labeling efficiency, which can vary by more than 100-fold [12]; and the lack of structural information of the lesion, which leaves the identity of the adduct ambiguous. DNA adduct identification and quantification by the ^{32}P-postlabeling method can be particularly challenging in humans where many lesions may be present at trace levels [13].

During the past decade, mass spectrometry methods have been employed to measure DNA adducts. Accelerator mass spectrometry (AMS) has been used to measure radiocarbon isotope with attomole (10^{-18} mol) sensitivity [14]. Because of the extraordinary sensitivity of AMS, only trace levels of radioactivity are required. However, this technique requires that the isolated DNA is devoid of non-covalently bound radioactivity to assure accurate estimates of DNA adduct levels. Although structural information is not provided by this technique, the use of HPLC in combination with AMS provides a greater degree of confidence in analyte identity. Soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have emerged as techniques to detect non-volatile and thermally labile compounds [15,16]. The on-line coupling of HPLC combined with ESI-tandem mass spectrometry (LC–ESI–MS/MS) provides structural information on the adducts and the incorporation of stable, isotopically labeled internal standards into the assay assures precision and accurate quantification of the DNA adducts [17–20]. Although LC–ESI–MS/MS is not as sensitive as those of ^{32}P-postlabeling or AMS, HAA–DNA adduct detection limits have been reported to range from 1 adduct per 10^7 to 10^9 bases using 100–500 μg of DNA and may be amenable to biomonitoring studies in humans [21–24]. In this review, the biochemical and synthetic approaches to form HAA–DNA adducts and the analytical methods used to detect these adducts in experimental animals and humans are presented.

2. Genotoxic heterocyclic aromatic amine metabolites and DNA adduct formation

HAAs must be metabolically activated to bind covalently to DNA. Metabolic activation occurs primarily by hepatic cytochrome P450 1A2 (CYP1A2) and to a lesser extent by CYP1A1 and CYP1B1-mediated N-oxidation in extrahepatic tissues [25–29] to form the *N*-hydroxy-HAA derivatives. The *N*-hydroxy-HAA metabolites formed in liver may enter the general circulation to react with DNA in various target tissues or undergo phase II metabolism reactions to form highly reactive esters that bind to DNA [30].

The synthetic *N*-hydroxy-HAA derivatives are prepared through reduction of the nitro species. In the case of the amino-imidazo type HAAs, the nitro derivatives are prepared through a diazotization reaction in the presence of excess $NaNO_2$ where NO_2 displaces the diazonium ion in a Sandmeyer-type reaction to form the nitro HAAs [31]. The nitro derivatives of the pyrolysate mutagens, 2-amino-6-methyldipirido[1,2-*a*:3′,2′-d]imidazole (Glu-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), and 2-amino-1-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), are prepared by oxidation of the parent compounds with hydrogen peroxide in the presence of trifluoroacetic acid and catalytic amounts of molybdenum hexacarbonyl [32–34]. *N*-Hydroxy-HAAs are obtained by reduction of the nitro-HAAs with hydrazine using a palladium on carbon catalyst at −10 °C [35]. A facile and quantitative reduction of nitro-IQ and nitro-MeIQx to the *N*-hydroxy species also may be accomplished with ascorbic acid under alkaline pH conditions [36].

A number of the *N*-hydroxy-HAAs exhibit low reactivity with DNA under neutral pH, and in contrast to arylhydroxylamines, the reactivity is enhanced only modestly under acidic pH conditions [36,37]. However, DNA binding of the *N*-hydroxy compounds is greatly enhanced by the generation of reactive esters, such as the *N*-acetoxy derivatives, which undergo heterolytic cleavage to produce the reactive nitrenium ion-acetate anion pair (Fig. 2) [38,39]. *N*-Acetyltransferases (NAT), sulfotransferases (SULT), phosphorylases, and aminoacyl-tRNA syntheses contribute to the bioactivation of *N*-hydroxy-HAAs through formation of reactive esters [40,41]. The *N*-acetoxy derivatives of IQ and MeIQx are highly unstable with lifetimes of seconds or less in physiologic phosphate buffer [39], but *N*-acetoxy-PhIP is relatively more stable and has been characterized by mass spectrometry [42]. The *N*-acetoxy-HAAs are prepared in situ by reaction of the *N*-hydroxy-HAAs with acetic anhydride [38,39] or ketene gas [32] and form adducts in the presence of DNA or deoxynucleosides. HAA–DNA adduction products have been reported to form with 2'deoxyguanosine (dG) but not with other deoxynucleosides. HAA–DNA adducts have been synthesized with 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) [38,39], 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) [43], 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) [39,44], 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) [45], 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) [42,46], Glu-P-1, and Trp-P-2 [32], where the adduction occurs through the exocyclic amine group of the HAAs and the C8 atom of guanine (dG-C8-HAA adducts). All of these HAA–DNA adducts have been spectroscopically characterized by NMR and mass spectrometry and the chemical structures are presented in Fig. 3. dG-C8 adducts of MeAαC and AαC also have been prepared by reduction of the respective nitro derivatives in the presence of DNA or dG [33,34]. Under these reaction conditions, the yields of dG-C8 adducts are low and form at only about several percent of the starting HAA intermediate. The reaction yields of *N*-acetoxy-PhIP with oligonucleotides are also low [47,48]. The C8 guanyl base adducts of Glu-P-1, MeAαC, and AαC also have been prepared by reaction of the parent amines with guanine-*N*3-oxide with acetic anhydride [33,34].

In addition to these dG-C8 adducts, adducts are formed at the N^2 atom of guanine by reaction at the C5 positions of IQ and MeIQx, indicating nitrenium ion formation and charge delocalization at this site (Fig. 2) [39]. The dG-N^2 adducts of IQ and MeIQx are formed at 5–10-fold lower amounts than the respective dG-C8 adducts with both DNA and dG.

The photoactivated azides of IQ, MeIQx, and PhIP bind to DNA to form the same adducts as the *N*-acetoxy species, indicating that the nitrenium ion is a common intermediate for both reactive intermediates [49,50]. Recently, a non-biomimetic approach has been reported for the syntheses of dG-C8 adducts of arylamines and IQ through

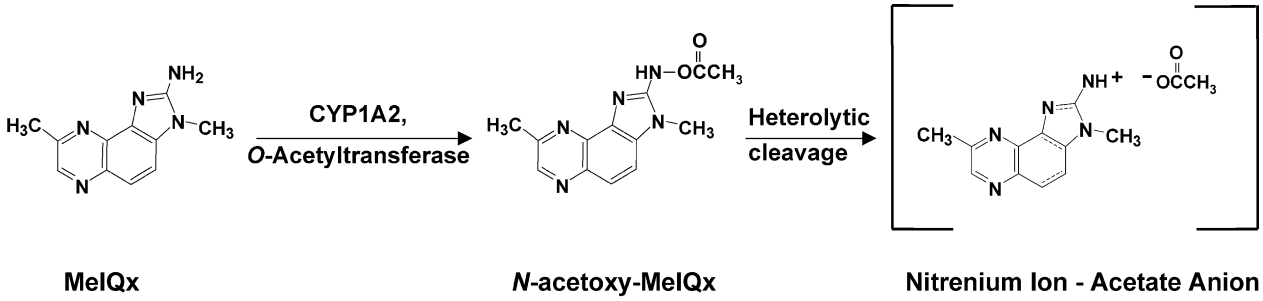

Fig. 2. Metabolic activation of MeIQx by CYP1A2 followed by NAT to form *N*-acetoxy-MeIQx, and generation of nitrenium ion-acetate anion pair with the positive charge delocalized about the exocyclic amino group and the C5 atom of MeIQx. The reactive nitrenium ion intermediate binds to DNA bases.

palladium-catalyzed N-arylation of a protected 8-bromo-dG derivatives with yields as high as 60% [51].

3. HAA–DNA adducts in experimental animal studies assayed by ^{32}P-postlabeling

A number of studies have reported the formation of HAA–DNA adducts in experimental animals including, rats, mice, and non-human primates using the ^{32}P-postlabeling technique, and the results are summarized in several review articles [30,37,52,53]. In order to obtain accurate and quantitative estimates of DNA adduct levels by ^{32}P-postlabeling, the modified DNA must be completely digested to recover the mononucleotide adducts, and the labeling efficiency of polynucleotide kinase must be determined with synthetic DNA adduct standards. In the case of dG-C8-IQ, enzymatic hydrolysis of IQ-modified calf thymus DNA with micrococ-

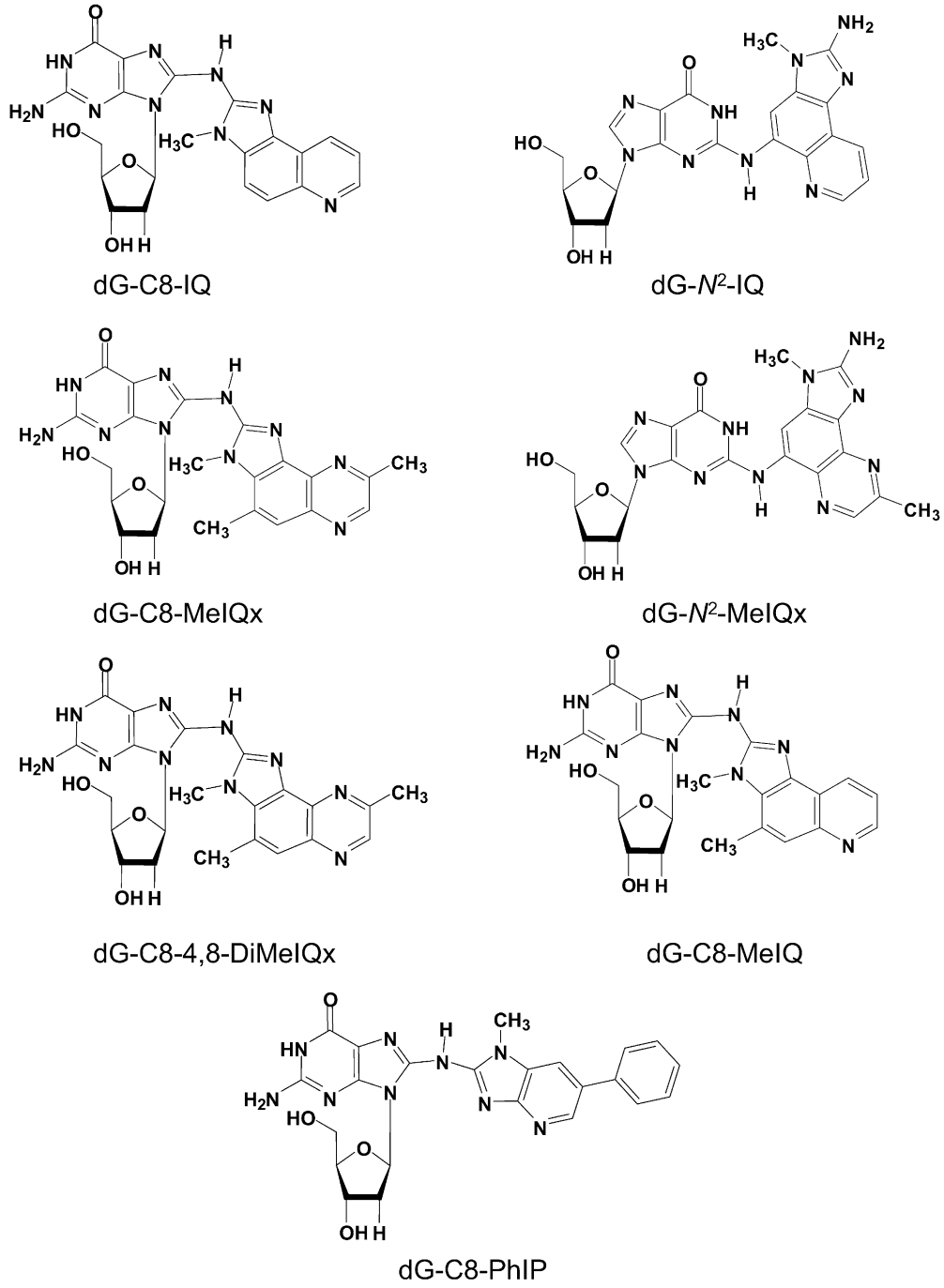

Fig. 3. Chemical structures of HAA–DNA adducts.

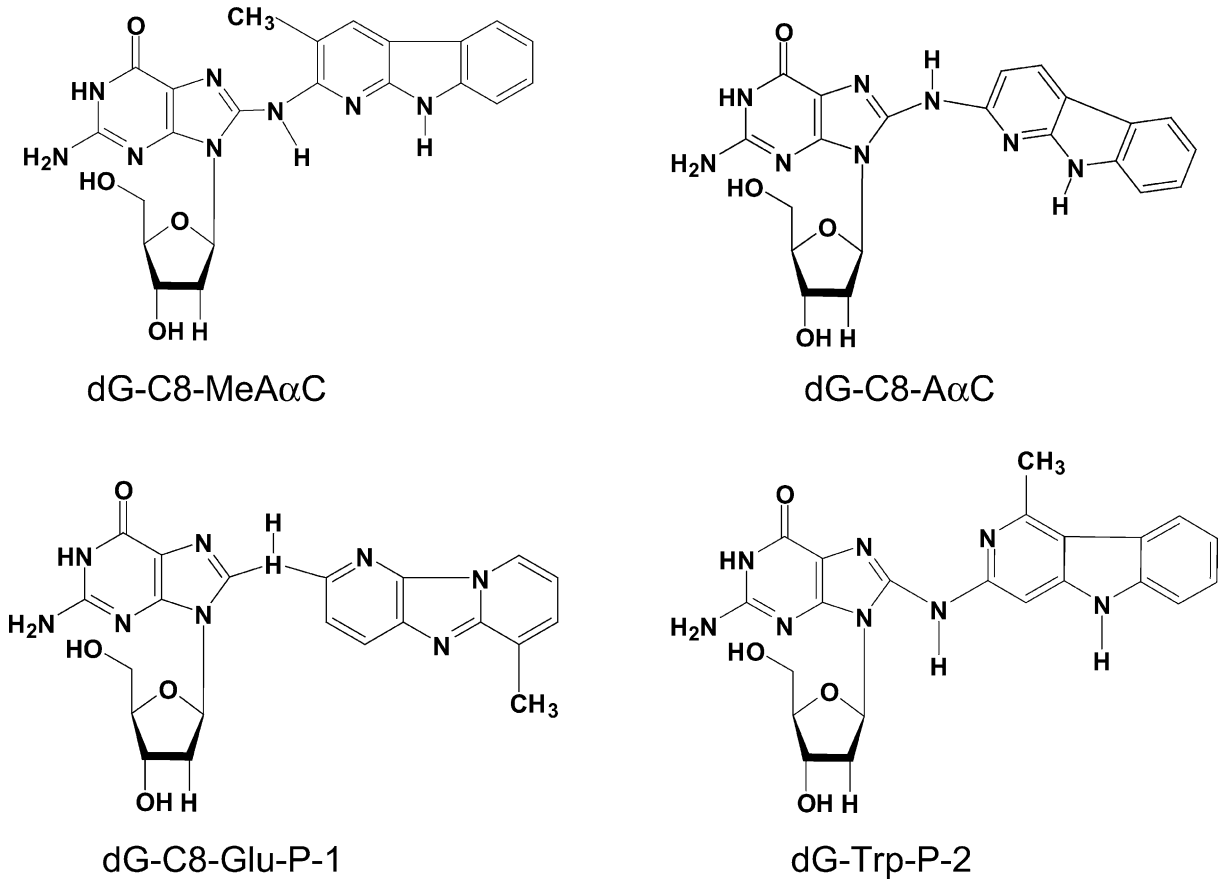

Fig. 3. (*Continued*).

cal nuclease and spleen phosphodiesterase required 24 h for completion, while the release of the isomeric dG-N^2 adduct was complete within 4 h [54]. Some of the early investigations on HAA–DNA adduct formation reported the presence of numerous adducts when assayed by ^{32}P-postlabeling. Many of the lesions were possibly dG-C8-HAA adducted dimers or oligonucleotides that were resistant to hydrolysis by micrococcal nuclease and spleen phosphodiesterase [55]. These lesions were susceptible to hydrolysis by nuclease P1, resulting in an adduct profile that was simplified to just one or two major spots [55]. In an extension of this approach, nuclease P1 and phosphodiesterase I were employed to convert IQ-adducted oligonucleotides to the ^{32}P-labeled-mononucleotide adduct 5′-phosphate forms for in vivo studies and this method may be applicable to assay other HAA–DNA adducts [44,56].

Several variations in the ^{32}P-postlabeling conditions also have been used to detect DNA adducts. In the initial studies, excess [γ-^{32}P]ATP was used to assure complete labeling of both non-modified and adducted nucleotides [10,57]. Subsequently, it was observed that limiting amounts of [γ-^{32}P]ATP in the postlabeling assay could enhance the limits of detection of some bulky carcinogen adducts, including dG-C8-HAA–DNA adducts, because of their preferential labeling over non-modified nucleotides [58]. It is noteworthy that dG-N^2-IQ is a relatively poor substrate for polynucleotide kinase and not readily detected unless labeled with excess [γ-^{32}P]ATP [54]. Another approach used solid phase extraction for removal of non-modified nucleotides and enrichment of DNA adducts prior to postlabeling [54]. As depicted in Fig. 4, both the duration of enzyme hydrolysis and utilization of excess ATP (or solid phase adduct enrichment to remove non-modified nucleotides) were critical for the detection of dG-C8-IQ and dG-N^2-IQ [54]. The conditions employed for enzymatic digestion and postlabeling of modified DNA are critical for the successful detection of DNA adducts. There is a need for standardization and interlaboratory validation of the postlabeling method(s) for HAA–DNA adducts [59,60].

In most experimental animal studies using ^{32}P-postlabeling, the doses of HAAs employed were very large (10–50 mg/kg body weight), exceeding daily human exposure by more than a million-fold. At these dose levels, the dG-C8 adducts of IQ [54,56,58,61], MeIQ [43], MeIQx [44,61], 4,8-DiMeIQx [45], PhIP [46,61–63], MeAαC [34], and AαC [33] were the most prominent lesions. For most HAAs, DNA adduct formation is greatest in the liver, which may be attributed to the high levels of CYP1A2 expression [64]. However, adducts have been detected in all tissues investigated [30,53]. In contrast to other HAAs, the levels of adducts

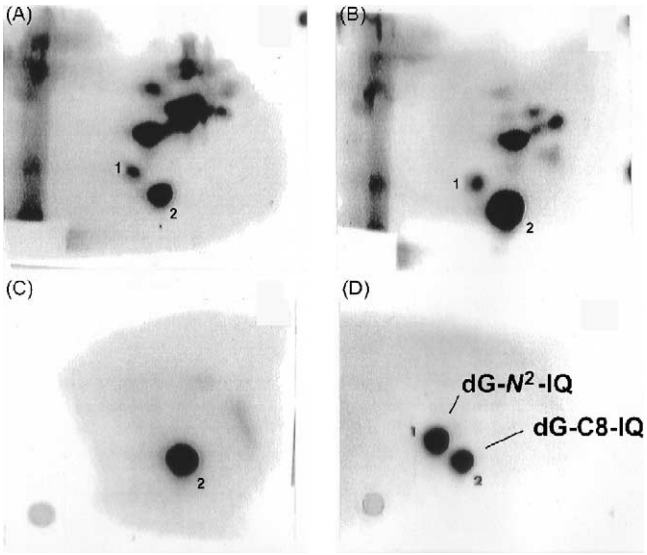

Fig. 4. The effect of enzyme hydrolysis time (micrococcal nuclease and spleen phosphodiesterase) and [γ-^{32}P]ATP concentration on recovery of dG-C8-IQ and dG-N^2-IQ from IQ-modified calf thymus DNA by ^{32}P-postlabeling and analysis by 2-D TLC. (A) 1 h digestion time and (B) 24 h digestion time with excess [γ-^{32}P]ATP, (C) 24 h digestion time using limiting amounts of [γ-^{32}P]ATP, (D) Synthetic dG-C8-IQ and dG-N^2-IQ adducts (100 fmol). Adapted from reference [54].

formed with PhIP are low in liver relative to extrahepatic tissues; adduct levels are particularly elevated in colon, pancreas, prostate, and the mammary gland of female rodents [63,65–67]. Glutathione *S*-transferase-mediated detoxication of reactive PhIP metabolites has been shown to be high in liver and may explain the relatively lower level of PhIP–DNA adduct formation in this tissue [68]. Significant levels of MeAαC–DNA adducts have also been detected in pancreas of rats [69]. Since HAA–DNA adducts are formed in non-target tissues for carcinogenesis, HAA–DNA adduct formation alone is not sufficient for tumorigenesis and other factors influence the susceptibility of tissues to the carcinogenic effects of HAAs.

CYP1A2 is a principal enzyme involved in the bioactivation of HAAs [26,29,70] but other enzymes may contribute to their bioactivation. The role of CYP1A2 in the formation of DNA adducts of IQ and PhIP was examined in CYP1A2-null and wild-type mice [71]. The IQ–DNA adduct levels in liver and kidney of CYP1A2-null mice were 20–30% of the levels present in wild type mice, while the adduct levels were about three-fold less in the colon of CYP1A2-null mice. The contribution of CYP1A2 to PhIP–DNA adduct formation was even more striking; adduct levels in liver and kidney of wild type mice were at least 100-fold higher than in the CYP1A2-null mice and adduct levels in mammary gland and colon of wild type mice were 10-fold greater than in the CYP1A2-null mice. Therefore, CYP1A2 strongly contributes to HAA–DNA adduct formation but other cytochromes P450 and pathways of bioactivation make a contribution. Given the high doses of HAAs used in this study (25–150 mg/kg), some enzymes may have contributed to IQ and PhIP bioactivation that would not be involved in metabolism under the low exposure conditions of HAAs that occur in the human diet [2,72].

Several studies have been reported on the biochemical fate of HAA–DNA adducts. The formation and removal of the isomeric dG-C8-IQ and dG-N^2-IQ adducts were examined in rats and monkeys by ^{32}P-postlabeling following DNA adduct enrichment by solid phase extraction [73,74]. In both species, dG-C8-IQ, the principal adduct formed following a single acute dose (10 or 20 mg/kg), was removed more rapidly than dG-N^2-IQ in slowly dividing tissues such as liver, kidney, pancreas, and heart. In contrast to these tissues, both adducts were removed at comparable rates in the colon, where the epithelial cells turn over rapidly. In non-human primates, the level of dG-N^2-IQ in the liver of chronically treated animals (20 mg/kg, 9 years) was approximately three to five-fold greater than dG-C8-IQ. Moreover, the levels of dG-N^2-IQ increased by more than 100-fold over a single acute dose in slowly dividing extrahepatic tissues [75]. The differences in the kinetics of removal of these isomeric dG-IQ adducts may be attributed to differences in adduct conformation of the glycosidic linkage of the IQ adducted DNA. Proton NMR studies have shown that the dG-C8-IQ adduct exists preferentially in the *syn* form and may induce a greater distortion of the DNA helix at the site of adduction than the dG-N^2-IQ adduct, which preferentially exists in the normally occurring *anti* form [39]. Therefore, repair enzymes may more readily recognize and remove the dG-C8-IQ adduct [76]. In two other studies [77,78], no differences between the rates of IQ–DNA adduct removal in various tissues of rats were reported, and there was no evidence for the accumulation of any specific IQ adduct. However, the limiting amounts of ATP used for postlabeling in these studies would have precluded detection of dG-N^2-IQ. In a fourth study, significant levels of dG-N^2-IQ formation were reported in liver of rats chronically treated with IQ when DNA was labeled with excess ATP [56]. Thus, the relative contribution of dG-N^2-IQ to DNA adduct formation in vivo is far greater than that which would be expected based upon reactions of *N*-acetoxy-IQ with calf thymus DNA in vitro, where dG-N^2-IQ accounts for only a minor percentage of the total adducts [39].

The removal of MeIQx–DNA adducts in liver of rats was reported to occur in a biphasic manner [79]. Unfortunately, dG-C8-MeIQx and dG-N^2-MeIQx were not used as reference adducts in the investigation to determine if there was preferential removal of either adduct. Rates of total DNA adduct removal of PhIP in male Fischer-344 rats (50 mg/kg) were similar in colon, spleen, cecum, liver, lungs, stomach and small intestine, with levels at day twenty declining to <16% of those amounts found 1 day post-treatment [63]. There was no evidence for preferential removal of any specific adducts.

4. HAA–DNA adducts analyses in vitro and in experimental animal studies using mass spectrometry

During the past decade, HAA–DNA adduct formation has been characterized by various mass spectrometry techniques. Because of the extraordinary sensitivity of AMS, animals may be dosed with radiolabeled carcinogens at dose exposure levels present in the human diet and interspecies comparisons can be made on DNA adduct formation and biologically effective doses [14]. AMS was used to measure MeIQx–DNA adduct formation in rats at doses ranging from 0.1 to 500 ng/kg body weight and radioisotope doses of less than 10 nCi/kg [80]. A linear dose response in MeIQx–DNA adduct formation in rats was observed at these dose ranges in liver tissue, indicating that a constant proportion of MeIQx is bioactivated to the carcinogenic species even at doses approaching human exposure levels. The dose–response curves for hepatic [^{14}C]-MeIQx–DNA under chronic dosing regimens reached steady-state levels between 4 and 12 weeks depending upon the dose, and the adducts increased as a power function over a dose range spanning four orders of magnitude [14].

The dG-C8-HAA adducts may be measured indirectly by gas chromatography-mass spectrometry. This technique, which has been used to measure dG-C8 adduct of 4-aminobiphenyl [81], is based upon alkaline hydrolysis of dG-C8-HAA adducts with formation of the parent HAA compounds. Following chemical derivatization with trifluoromethylbenzylbromide, the di(3,5-bistrifluoromethylbenzyl)HAA derivative is measured by gas chromatography-electron-capture detection or gas chromatography-negative chemical ionization mass spectrometry [82]. This technique is applicable to dG-C8-HAA adducts but has not been validated for dG-N^2-HAA adducts, which are important contributors to the total adducts of IQ [75] and MeIQx [24]. All non-covalently bound carcinogen must be removed from the DNA prior to base hydrolysis for accurate quantification of the adducts. The alkaline GC-electron capture and ^{32}P-postlabeling methods were used to assess DNA adducts in tissues of male Fischer-344 rats treated daily with PhIP at doses ranging from 1 to 0.0001 mg/kg for 23 days [83]. PhIP–DNA adducts could be detected only in animals receiving 1 or 0.1 mg/kg per day, with highest adduct levels found in the pancreas, heart, and kidneys. There was a good correlation between the ^{32}P-postlabeling and GC-electron capature analyses with average adduct levels determined by ^{32}P-postlabeling approximately 1.4 times higher than those determined by alkaline GC electron capture method. In another study, the alkaline GC-electron capture method was compared to LC–ESI–MS analysis of dG-C8-PhIP in the selected ion monitoring mode (SIM) using deuterated dG-C8-PhIP as an internal standard for both assays [23]. Both methods provided comparable data with a limit of quantification of 200 pg adduct per 500 μg DNA.

LC–ESI–MS/MS with triple quadrupole mass spectrometers has been used successfully for the detection of IQ and MeIQx adduct formation in vitro, as well as in tissues of rodents and non-human primates [21,24,84]. DNA adducts of PhIP-modified calf thymus DNA and adducts in various cell lines also have been characterized [23,85]. For trace analysis, the tandem MS system is operated in the single (or multiple) reaction monitoring (SRM or MRM) mode. In this mode, the protonated adduct ions $[M+H]^+$ are transmitted by the first mass analyzer (Q_1) and are subjected to collision induced dissociation (typically with argon gas) in the second quadrupole (Q_2). These collision conditions result in the facile loss of deoxyribose (dR) to form the protonated base adduct $[BH_2]^+$, which is selectively transmitted through the third quadrupole (Q_3). Significantly, this fragmentation is common to the deoxynucleoside adducts of all HAAs reported thus far. Higher collision energy conditions result in more extensive fragmentation of the guanyl adducts, providing extensive structural information about the compounds. For example, isomeric dG-C8 and dG-N^2 adducts of IQ and MeIQx display characteristic fragmentation of the guanyl moiety, where the dG-N^2 adducts preferentially lose NH_3 (17 Da) from the guanine base $[BH_2–NH_3]^+$, while there is a preferential loss of 45 Da $[BH_2–CONH_3]^+$ from the guanine moiety of the dG-C8 adducts (Fig. 5) [24,84]. These differences in fragmentation have been previously reported for dG-C8- and dG-N^2-substituted adducts of the aromatic amine 2-aminofluorene and may be used to distinguish between C8 and N^2-substituted derivatives of dG [86].

In view of this selective and universal fragmentation of the dR (116 Da) in the class of HAA–DNA adducts, an alternative tandem MS mode known as constant neutral loss (CNL) has proved to be extremely useful to search for unknown, novel adducts. When used in combination with separation methods, the CNL mode can target adducts in complex mixtures and establish the molecular weights of these compounds. This scan mode is particularly useful for examining carcinogen-modified DNA in vitro, where adduct levels may be on the order of 1–10 adducts per 10^5 bases or greater. With CNL scanning mode, the isomeric dG-C8 and dG-N^2 adducts of MeIQx and IQ were readily detected in vitro following reaction of calf thymus DNA with the *N*-acetoxy-HAAs with no evidence for other DNA adducts [24,84]. LC–ESI–MS/MS characterization of PhIP-modified calf thymus DNA also revealed that dG-C8-PhIP was the principal lesion but analysis in CNL scan mode revealed the presence of at least two other isomeric dG adducts [85]. These results were in qualitative agreement to those obtained by ^{32}P-postlabeling where one major lesion (dG-C8-PhIP) and two minor spots were observed (Fig. 6) [85]. dG-C8-PhIP was reported to be the major adduct following the reaction of *N*-acetoxy-PhIP with an 11-mer oligodeoxyribonucleotide, but at least eight minor polar adducts were also formed [48]. MALDI-time of flight mass spectrometry (MALDI Tof-MS) characterization of three of these adducts revealed products consistent with a

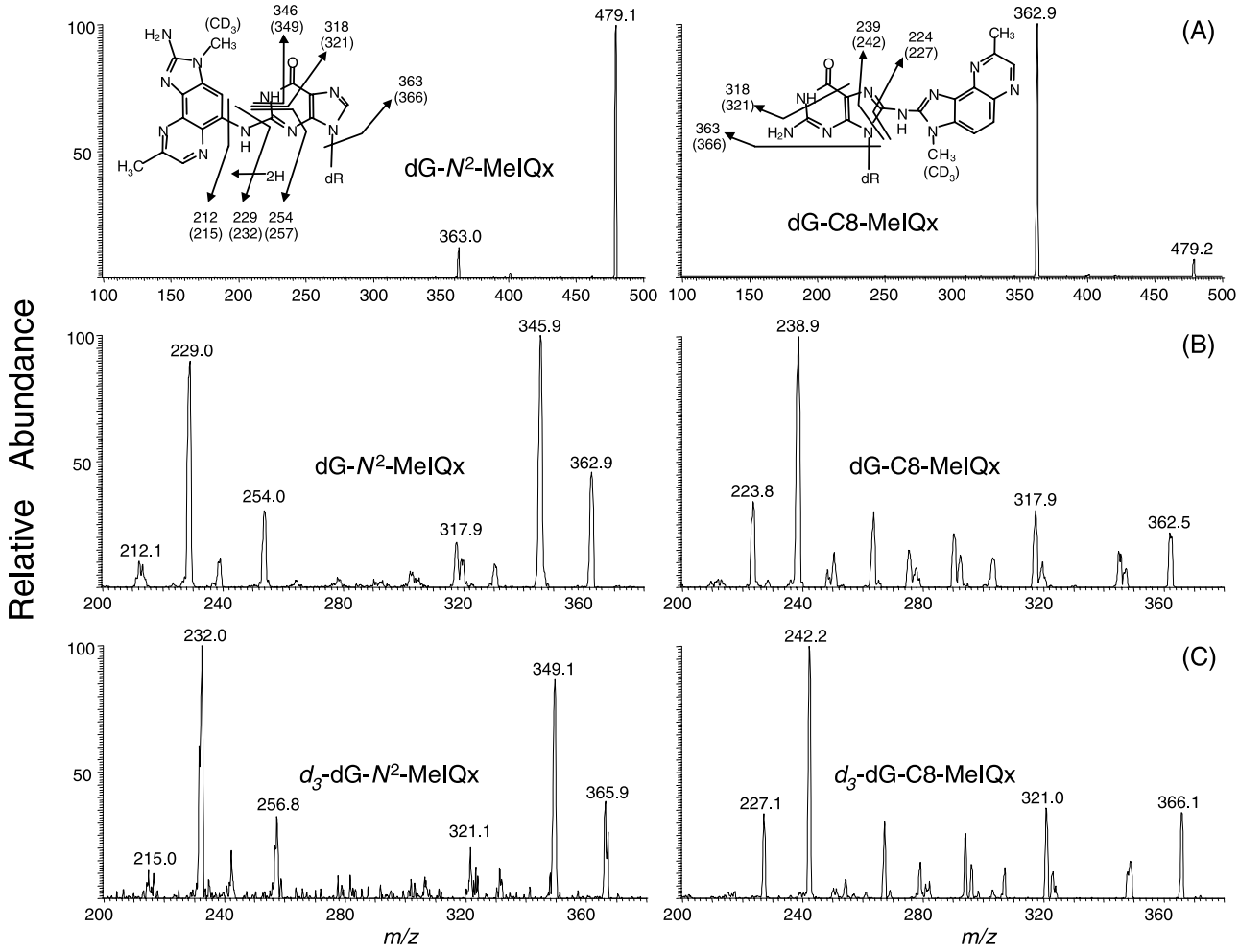

Fig. 5. Product ion spectra and proposed fragmentation of dG-C8-MeIQx and dG-N^2-MeIQx at $m/z = 479$ (m/z 484 for CD_3 analogue) corresponding to $[M + H]^+$. (A) Fragmentation of $[M + H]^+$ under low collision energy conditions, (B) product ion spectra of $m/z = 363$, corresponding to $[BH_2]^+$, (C) product ion spectra of $m/z = 366$, corresponding to $[BH_2]^+$ of the d_3-labeled internal standards. Adapted from reference [24].

spirobisguanidino-PhIP derivative and a ring opened adduct of dG. The third adduct had the same mass as dG-C8-PhIP and may be an adduct of PhIP bound to the N^2 atom of guanine.

LC–ESI–MS/MS in SRM mode was used to quantify dG-C8-IQ and dG-N^2-IQ adducts in tissues of non-human primates and liver of rats where the estimates showed good correlation to data obtained by ^{32}P-postlabeling at dose levels ranging from 0.05 to 10 mg of IQ/kg with adduct levels ranging from 3 to 40 adducts per 10^8 bases (Fig. 7) [21]. Both dG-C8-MeIQx and dG-N^2-MeIQx adducts were also measured by LC–ESI–MS/MS in SRM mode in rat liver tissue 24 h following treatment of MeIQx (0.5 and 10 mg/kg) [24]. Moreover, full product ion spectra were acquired on both adducts for unambiguous identification; the first time such spectral data has been acquired on HAA–DNA adducts in vivo. At the 10 mg/kg dose, dG-C8-MeIQx and dG-N^2-MeIQx adducts were estimated at 3.07 ± 0.84 and 0.45 ± 0.27 adducts per 10^7 DNA bases, respectively. However, at the lower dose, the dG-N^2-MeIQx predominated (0.40 ± 0.26 adducts per 10^7 bases) and was present at 10-fold greater amounts than dG-C8-MeIQx. The reason(s) for this inversion of the ratio of dG-C8- and dG-N^2-MeIQx adduct formation as a function of dose is not known but could be attributed to differences in adduct stability or repair [24]. The lower limits of detection of MeIQx- and IQ–DNA adducts in these studies were reported to approach 1–2 adducts per 10^8 DNA bases using 100 μg of DNA.

5. HAA–DNA adduct formation in human tissues

Several HAAs induce cancer of the colon, prostate, and mammary glands of experimental animals, and there has been considerable interest in the role that HAAs may have in etiology of these types of cancer in humans [3–5]. Human liver containing CYP1A2 is the most active tissue in metabolism of HAAs to the *N*-hydroxy species [41] and inter-individual expression of CYP1A2 may vary by more than 50-fold [26,29,87]. Moreover, the CYP1A2 content of

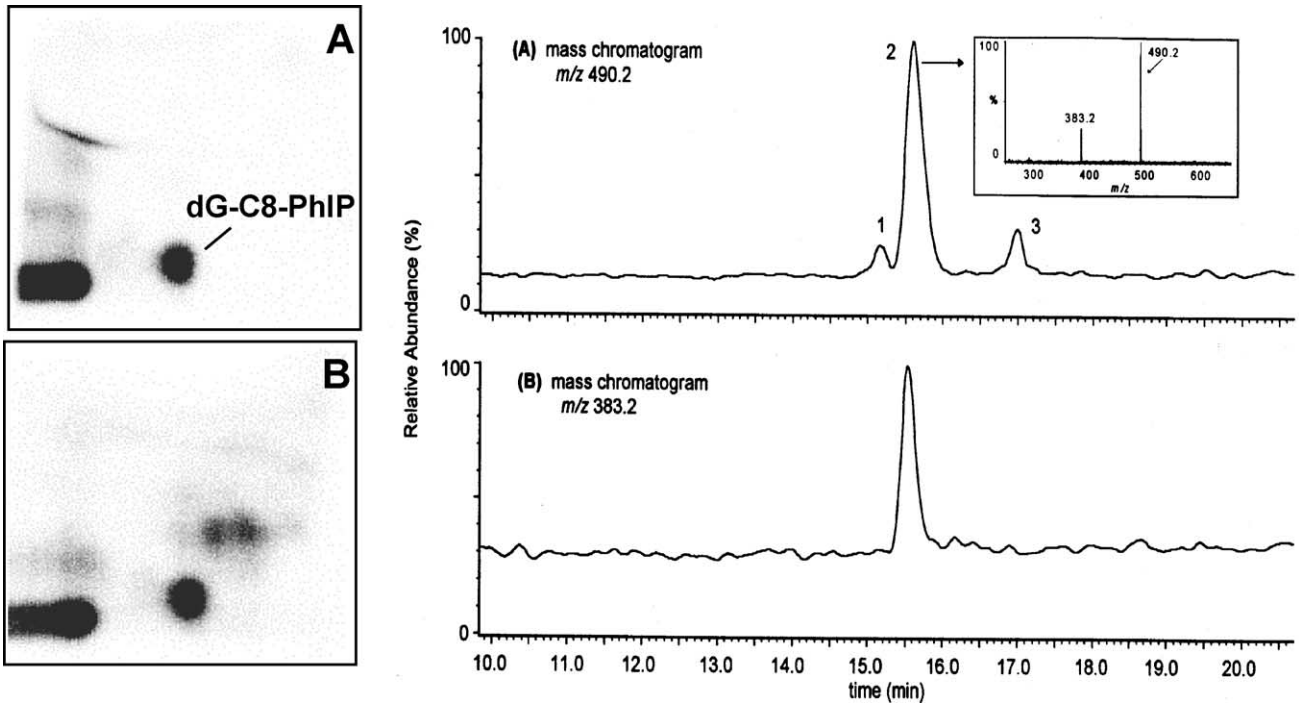

Fig. 6. ^{32}P-postlabeling 2-D TLC profiles of (A) 3′,5′-bisphospho-dG-C8-PhIP standard and (B) in vitro reaction of calf thymus DNA with *N*-acetoxy-PhIP (left panel). Mass chromatograms obtained from the CNL scanning mode (loss of 116 Da) (A) *m*/*z* 490.5 attributable to dG-C8-PhIP and isomers and (B) *m*/*z* 383.6 attributable to a ring-opened dG-C8-PhIP adduct (right panel). Adapted from reference [85].

many human liver samples greatly exceeds that amount expressed in liver of rodents used for carinogen bioassays [29]. Because of the higher CYP1A2 content in human liver, combined with the superior catalytic efficiency of human CYP1A2 in bioactivation of some HAAs, the experimental animal models used in toxicity studies may underestimate the health risk of HAAs to humans [29,70,87,88].

Although the liver is the most active tissue in metabolism of HAAs, extrahepatic tissues can convert HAAs to genotoxins capable of binding to DNA. Human mammary epithelial

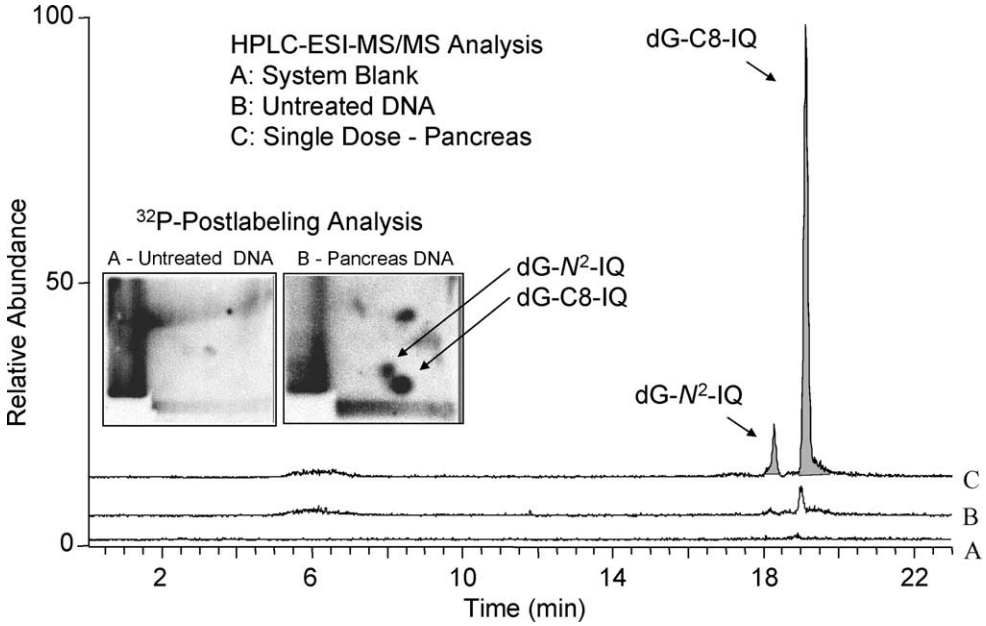

Fig. 7. HPLC–ESI–MS/MS and ^{32}P-postlabeling analyses of dG-C8-IQ and dG-N^2-IQ adducts in pancreas of non-human primates given a single dose of IQ (20 mg/kg) and analyzed 24 h post-treatment. The levels of dG-N^2-IQ and dG-C8-IQ were estimated at 5 and 9 adducts per 10^9 bases, respectively, by ^{32}P-postlabeling. Adapted from reference [22].

cells have been reported to activate IQ, MeIQ, and PhIP at variable levels to metabolites that bind to DNA, although activity is considerably lower than in liver [89,90]. CYP1A1 and peroxidases within breast tissue contribute to the bioactivation [91,92]. The *N*-hydroxy HAAs are substrates for NAT and SULT enzymes expressed in mammary tissue and convert *N*-hydroxy-IQ and *N*-hydroxy-PhIP to reactive esters that bind to DNA [66,93]. Human prostate epithelial cells also have been reported to metabolize Glu-P-2, MeIQx, and PhIP to genotoxins that result in DNA damage and mutagenicity [94,95]. Thus, extrahepatic human tissues are able to convert HAAs to genotoxins.

There have been several reports on HAA–DNA adduct formation in vivo in humans. Using the ^{32}P-postlabeling assay combined with HPLC, dG-C8-MeIQx was identified in 3 of 38 DNA samples of 13 individuals in colon, rectum, and kidney based upon thin layer chromatography and HPLC methods [13]. The adduct levels were reported to range from 2 to 20 adducts per 10^{10} DNA bases. In 24 individual tissue samples, including pancreas, colon mucosa, and urinary bladder epithelium, dG-C8-PhIP was detected in two of six colon samples at levels of 2.9 ± 0.5 adducts per 10^8 nucleotides, but adducts were not detected in human pancreas or urinary bladder by ^{32}P-postlabeling [82]. The identity of dG-C8-PhIP was further substantiated by the alkaline hydrolysis-GC electron capture detection method.

AMS has been employed to measure MeIQx–DNA adduct formation in colon of human subjects and rats [14]. The DNA adduct levels were approximately 10 times greater in human colon than rodent colon after treatment with the same dose of MeIQx (304 ng/kg body weight) and time point following exposure (24 h). In human colon, the mean adduct level was estimated at 26 ± 4 adducts per 10^{12} DNA bases. Approximately 90% of the MeIQx–DNA adducts, in both rodent and human colon, was identified as dG-C8-MeIQx based upon HPLC analysis and off-line AMS detection. Thus, MeIQx is readily bioavailable for both humans and rats. The higher levels of MeIQx–DNA adduct formation in human colon suggests that a larger portion of MeIQx is converted to the genotoxic species in humans and consistent with the superior catalytic activity of human CYP1A2 over the rat enzyme in N-oxidation of MeIQx [14,70,87,88].

AMS studies were also reported on five human subjects administered ^{14}C-labeled PhIP (70–84 μg/subject) 48–72 h before surgery for removal of colon tumors [96]. DNA adduct levels in the normal tissue of colon were estimated at 35–135 adducts per 10^{12} nucleotides. In another AMS study, female patients undergoing breast surgery were administered ^{14}C-labeled PhIP (20 μg/subject) and DNA adduct levels were reported to range from 26 to 480 adducts per 10^{12} DNA bases 24 h following treatment [97]. HPLC with off-line AMS detection was not performed in either study to determine the nature of the adducts. Thus, the accuracy in the amount of DNA adduct formation may be uncertain. Assuming that all of the radioactive material is bound covalently to DNA, the data indicate that PhIP is bioavailable to human colon and breast tissues to form DNA adducts following exposure to PhIP at dietary-relevant doses.

6. Relevance of HAA–DNA adduct measurements and future directions

Carcinogen–DNA adducts are considered as biomarkers of potential mutagenic events and cancer risk [7,8], and HAAs have been shown to induce mutations in oncogenes and tumor suppressor genes in experimental animals [98]. Therefore, measurement of HAA–DNA adducts in human tissues would provide an excellent means to assess genotoxic damage of these dietary carcinogens. A major limitation is the requirement of DNA from biopsy samples; an invasive procedure that may preclude the participation of healthy subjects. An alternative, non-invasive source of tissue that accurately reflects DNA adduct formation in target tissues is desirable. Buccal mucosa tissue has been successfully used to measure DNA adducts of smokers [99] and investigations on HAA–DNA adduct formation in buccal mucosa cells merits investigation. White blood cells are another alternative tissue source; however, the DNA adducts in white blood cells of rats or humans exposed to PhIP declines rapidly and does not appear to reflect PhIP–DNA adduct formation in colon or breast [83,96]. Thus, at least in the case of PhIP, white blood cells do not appear to be a promising surrogate tissue for biomonitoring DNA adducts.

Robust analytical methods that can be used routinely to measure HAA–DNA adducts in large studies still need to be developed. The use of ^{32}P-postlabeling is limited because it is labor intensive and requires large amounts of radioactivity. AMS applications in human studies also may be restrictive because of the requirement of radiolabeled isotopes, although a recent approach based upon administration of non-radioactive chemicals followed by postlabeling of DNA adducts by acetylation with ^{14}C-acetic anhydride shows promise [100]. LC–ESI–MS/MS has emerged as a major advancement in the analytical tools used in the field of DNA adduct analyses and continued improvements in LC–MS source interfaces to optimize analyte transmission may further decrease the limits of adduct detection and facilitate studies in humans [22,101]. Immunoaffinity techniques, which have been used for detection and purification of several different classes of DNA adducts [60], may further simplify HAA–DNA adduct isolation and detection methods for human studies. More investigations on HAA–DNA adduct formation in human populations using LC–ESI–MS/MS is warranted to corroborate the observations of previous studies that used less specific methods of detection.

The incorporation of HAA–DNA adducts as biomarkers in conjunction with enzyme polymorphisms that are involved in HAA bioactivation [102] combined with HAA exposure estimates may provide further insight into the role of HAA–DNA adducts in carcinogenesis in humans.

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