REVIEW

Analysis of DNA adducts in human samples: Acrolein-derived exocyclic DNA adducts as an example

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Acrolein is an environmental pollutant that is also derived endogenously through lipid peroxidation and protein degradation. The reaction of acrolein with 2′-deoxyguanosine produces exocyclic 1,N^2-propano-2′-deoxyguanosine (AdG) adducts, mutagenic lesions that play important roles in multistage carcinogenesis processes. Accurate quantification of acrolein-derived DNA adducts is a critical step toward elucidating the mode of action of acrolein carcinogenicity. Exposure of humans to acrolein can occur through the smoking of tobacco and the dietary consumption of oxidized polyunsaturated fatty acids. This review describes the use of ^{32}P-postlabeling- and MS-based methods for the analyses of acrolein-derived DNA adducts in humans as well as present trends toward improving the sensitivity, specificity, and accuracy of the quantification of trace amounts of DNA adducts in DNA of limited availability.

Keywords:
Acrolein / DNA adduct / Nanoflow LC / MS / ^{32}P-postlabeling

1 Introduction

Acrolein, a highly reactive α,β-unsaturated aldehyde, is a ubiquitous environmental pollutant to which humans are commonly exposed through the atmosphere, diesel exhaust, cooking fumes, and cigarette smoke [1, 2]. It is also produced in vivo through lipid peroxidation and polyamine catabolism [3, 4]. Stevens and Maier provided a comprehensive review of the exogenous and endogenous sources of acrolein and its metabolism [5]. The reactivity of acrolein is mainly attributed to the electrophilicity of its β-carbon atom toward electron-rich groups, such as thiols and the amino groups of biomolecules, forming Michael addition products. Being a bifunctional electrophile, acrolein also reacts at both its β-carbon atom and aldehyde moiety, producing interchain cross-links of double-stranded DNA [6] and DNA–protein cross-links [7]. The reaction of acrolein with cellular glutathione is considered an effective detoxification mechanism [8]. Nonetheless, those molecules that escape from the endogenous detoxification pathways might react with cellular proteins, leading to inactivation of protein activity or cell death. Adduction of DNA, if not repaired efficiently, can induce mutations in critical genes, subsequently leading to cancer development [9–13].

Acrolein reacts with the 2′-deoxyguanosine (dGuo) moieties of DNA to form exocyclic 1,N^2-propano-2′-deoxyguanosine adducts as two pairs of regioisomers: (6*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8-tetrahydro-6-hydroxypyrimido[1,2-*a*]purine-10(3*H*)one (α-AdG) and (8*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8-tetrahydro-8-hydroxypyrimido[1,2-*a*]purine-10(3*H*)one (γ-AdG) (Fig. 1) [14]. α-AdG is formed after initial Michael addition of the N-1 nitrogen atom of dGuo to the β-carbon atom of acrolein, followed by addition/cyclization of the N^2 amino group to the aldehyde; initial Michael addition of the N^2 amino group of dGuo to the β-carbon atom of acrolein, followed by addition/cyclization of the N-1 nitrogen atom of dGuo, gives γ-AdG. Each regioisomer is

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Abbreviations: **α-AdG,** (6*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8-tetrahydro-6-hydroxypyrimido[1,2-*a*]purine-10(3*H*)one; **γ-AdG** (8*R*/*S*)-3-(2′-deoxyribos-1′-yl)-5,6,7,8-tetrahydro-8-hydroxypyrimido[1,2-*a*]purine-10(3*H*)one; **AD,** Alzheimer's disease; **CdG,** crotonaldehyde-derived 1,N^2-propano-2′-deoxyguanosine adduct; **dGuo,** 2′-deoxyguanosine; **DHA,** docosahexaenoic acid; **HNE,** *trans*-4-hydroxynonenal; **3-HPMA,** (3-hydroxypropyl)mercapturic acid; **PhIP,** 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; **PUFAs,** polyunsaturated fatty acids; **SRM,** selected reaction monitoring

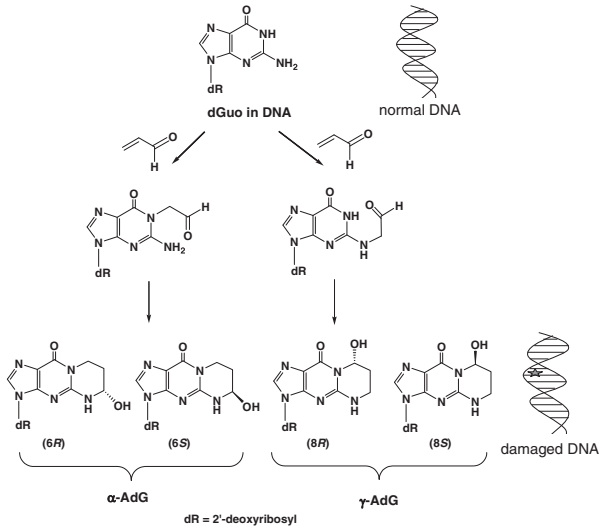

Figure 1. Formation of AdG from acrolein and 2′-deoxyguanosine in DNA.

obtained as a pair of epimers/diastereoisomers as a result of the stereochemical outcome of the addition/cyclization step [14]. Possibly because of the higher nucleophilicity of the N^2 amino group relative to that of the N-1 nitrogen atom of dGuo, γ-AdG is formed predominantly over α-AdG after the incubation of acrolein with dGuo or DNA in vitro [15–17]. In all of the in vivo DNA samples that have been analyzed, γ-AdG prevails over α-AdG, except in human lung DNA [22]. For 1,N^2-propano-2′-deoxyguanosine adducts derived from α,β-unsaturated aldehydes featuring longer carbon atom chains (e.g. crotonaldehyde and *trans*-4-hydroxynonenal (HNE)), only the regioisomers analogous to γ-AdG are formed [18, 19].

α-AdG and γ-AdG are mutagenic lesions in bacterial and human cells, with the predominant mutations being G→T transversions [9–13]. Interestingly, these regioisomeric adducts are not equally mutagenic: α-AdG induces much higher mutagenicity than γ-AdG [12]. These adducts might also be involved in producing the characteristic mutational spectrum in the *p53* tumor suppressor gene [13] that has been observed in tumors of lung cancer patients who are also smokers [20]. The concentration of acrolein in cigarette smoke is approximately 1000 times greater than that of benzo[*a*]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) that is well established as a lung procarcinogen. Thus, acrolein is implicated as a key component contributing to smoking-induced lung cancer [21]. In addition, acrolein can induce bladder tumors in rodents [22].

Although AdG has been detected and quantified in various human tissues [15, 16, 23–27], clinical DNA samples are often limited in quantity, especially those obtained from biopsy. Thus, highly sensitive and accurate methods are required when investigating the roles of AdG in carcinogenesis. This review outlines the historical development of the analytical techniques used for the detection and quantification of AdG in humans. The degree of DNA damage from exposure of acrolein through diet, environment, and lifestyle (smoking) can be evaluated by measuring AdG, thereby shedding light into its role in cancer development and possible prevention.

2 Analytical approaches

Because of the low levels of adducts present in vivo and the limited availability of clinical tissues, highly specific, accurate, sensitive, and quantitative assays are required when investigating the roles of DNA adducts in tumorigenesis and in cancer risk assessment. Among the analytical methods used for quantification of AdG, ^{32}P-postlabeling and liquid chromatography–mass spectrometry (LC-MS) techniques are the most frequently used.

2.1 ^{32}P-postlabeling

The ^{32}P-postlabeling-based assay is a very sensitive method for analyzing DNA adducts. In the multi-step procedures for AdG measurement [17], the assay involves the use of micrococcal nuclease and spleen phosphodiesterase for the enzyme hydrolysis of tissue DNA, forming nucleotide 3′-monophosphates. A UV-detectable amount of standard AdG 3′-monophosphates is added to the DNA hydrolysate and the fraction corresponding to the AdG 3′-monophosphates is collected using HPLC-UV. Nuclease P1 treatment of the collected fraction dephosphorylates only the unmodified nucleotides, thereby enriching the adducted 3′-monophosphates. The enriched AdG 3′-monophosphates are labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase (T4 PNK) as a catalyst, to form AdG 3′,5′-bisphosphates. Any excess of [γ-^{32}P]ATP is subsequently removed by treatment with apyrase. The mixture is developed on a polyethyleneimine (PEI)/cellulose thin-layer chromatography (TLC) plate (at pH 3.5) and visualized using autoradiography. The spots corresponding to AdG 3′,5′-bisphosphates are excised, extracted with isopropanol/6 N ammonium hydroxide, spiked with a UV-detectable amount of standard AdG 3′,5′-bisphosphates, and purified through HPLC, monitoring the signals from the UV markers. The collected fraction is analyzed by HPLC with tandem UV and radioflow detectors (Fig. 2). Adduct quantification in DNA samples is based on the radioactivity of the AdG 3′,5′-bisphosphate peaks comigrating with the added UV standard AdG 3′,5′-bisphosphates, and corrected for the decay and recovery factors as well as the labeling efficiency [23–25].

The major weaknesses of the ^{32}P-postlabeling-based assay are its laborious multi-step procedure (including purification through three HPLC-UV separations and extraction from one-dimensional TLC), poor labeling efficiency and recovery, and large assay variation. When this assay is applied for the detection of AdG in tissue DNA from

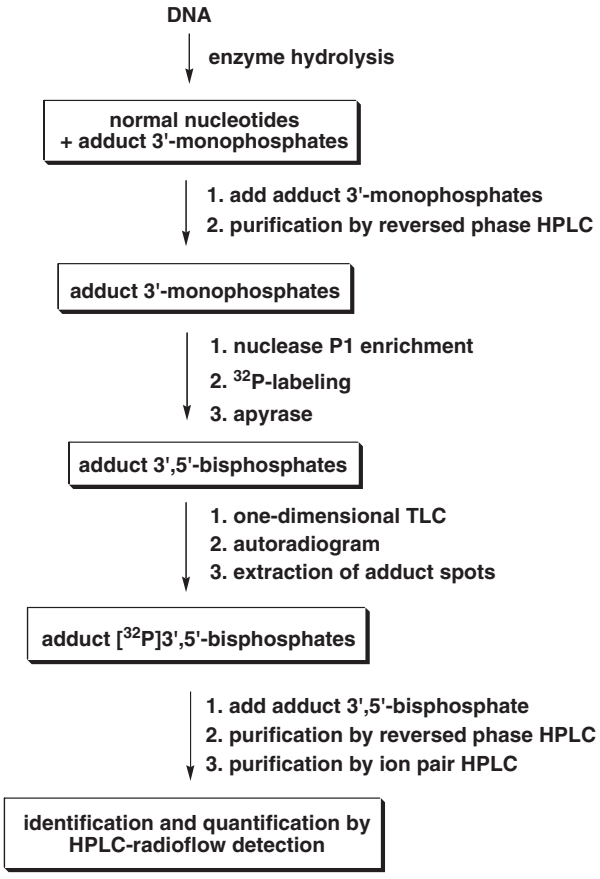

Figure 2. Procedures for analyzing AdG and CdG using the ^{32}P-postlabeling method [24, 25].

rodents (liver, lung, kidney, brain, breast, prostate, colon, skin, leukocytes, mammary glands) and humans (liver, mammary glands, leukocytes, gingiva), α-AdG is found to be present in much lower quantities (sometimes not at all) than those of γ-AdG [23–25]. Although the levels of AdG in human gingival DNA are significantly higher ($p = 0.003$) in smokers than those in nonsmokers, they do not correlate with the number of cigarettes smoked per day [25]. Table 1 summarizes the levels of AdG in human tissues.

Chung and co-workers improved the assay for the analysis of AdG along with other 1,N^2-propanodeoxyguanosine adducts derived from crotonaldehyde, pentenal, heptenal, and HNE in the same DNA sample. The labeling efficiency increases after replacing the PEI/cellulose TLC plate with a reversed-phase solid-phase extraction (SPE) column for the separation of the adducted nucleotide bisphosphates from the unmodified nucleotides, which interfere with the labeling efficiency [28]. When the purification of the AdG nucleotides is achieved through the sequential use of two SPE columns, a reversed-phase HPLC system, and an anion-exchange HPLC system, the overall recovery for the AdG analysis increases to an average of 14% – three times greater than the previous method – with the intra-assay variation of $<10\%$ [29]. In the latter study, contamination of acrolein in water and in commercial nucleotides was observed; it could be trapped through the addition of glutathione [29]. This finding confirms that acrolein is a ubiquitous contaminant in the environment; therefore, caution should be taken, through appropriate control experiments, when measuring AdG.

2.2 Mass spectrometry

2.2.1 For measurement of DNA adducts in general

^{32}P-Postlabeling-based analytical methods cannot provide structural information regarding the analytes and they lack suitable internal standards for reliable quantification. In contrast, MS does allow structural characterization of the analytes. Although accelerator MS is the most sensitive analytical method developed to date for detecting DNA adducts, with a limit of detection (LOD) as low as 1 adduct per 10^{12} unmodified DNA bases [30], the need to incorporate ^{14}C or ^{3}H radioisotopes into the DNA hinders its application.

The use of stable isotopomers as internal standards for MS-based assays can provide accurate quantification of the analytes because the internal standards are structurally identical to the analytes – that is, they have identical chemical and physical properties, except for the molecular weight. Therefore, errors due to recovery in each step of the assay procedure and due to the matrix effect can be corrected by using isotope-labeled internal standards. Most importantly, stable-isotope internal standards can serve as carriers for the ultra-trace amounts of the analytes present in the biological samples – such small amounts are easily lost during the multi-step assay procedures. Thus, isotopic dilution mass spectrometry (IDMS) has been widely applied for the specific detection and accurate quantification of low-abundance DNA adducts in vivo [31].

Electrospray ionization (ESI), which results in negligible fragmentation of molecular ions, is used more frequently than atmospheric pressure chemical ionization (APCI) for DNA adduct analysis. The latter has been applied for the analyses of less polar adducts, such as Schiff bases, malondialdehyde-dGuo, and substituted and unsubstituted etheno adducts [32–36].

In mass spectrometers featuring a single quadrupole, selective ion monitoring or single ion monitoring (SIM) is performed by selecting the most abundant or characteristic ion of the analyte. Coupling of the LC system with a triple quadrupole mass spectrometer operated in the selected reaction monitoring (SRM) mode – sometimes referred to as multiple reaction monitoring (MRM) – is now the most commonly used LC/MS method for quantification of small molecules because the highest sensitivity is achieved when the mass filters at both quadrupole 1 (Q1) and quadrupole 3 (Q3) are operated in the static mode, selecting specific

Table 1. Levels of AdG in human tissues

Tissue	Adduct level or range	Amount of DNA used/analyzed	Method[a)]	Reference
Liver	0.03–0.74/10^6 guanine ($n=5$)	–/–	^{32}P-postlabeling	[23]
Mammary	0.010–0.660/10^6 guanine ($n=3$)	50 μg	^{32}P-postlabeling	[24]
Leukocyte	0.003–0.025/10^6 guanine ($n=3$)			
Gingiva	1.36 ± 0.90/10^6 guanine (S) ($n=11$)* 0.46 ± 0.26/10^6 guanine (NS) ($n=12$)	23–122 μg/–	^{32}P-postlabeling	[25]
Brain	5150 ± 640/10^9 nucleosides (range: 3360–7990) (AD) ($n=8$)* 2800 ± 460/10^9 nucleosides (range: 1060–3720) (control) ($n=5$)	10 μg/1–2 μg	nanoLC-NSI/MS/MS (500 nL/min)	[15]
Lung	16–209/10^9 nucleosides ($n=30$)	0.03–1.25 mg	capLC-ESI/MS/MS (10 μL/min)	[26]
Placenta	108/10^8 nucleosides ($n=1$)	20 μg/4 μg	nanoLC-NSI/MS/MS (600 nL/min)	[16]
Leukocyte	78 ± 23/10^8 nucleosides (range: 43–109) ($n=9$)	30 μg/6 μg		
Buccal cells	>5/10^7 nucleosides (S) ($n=6$)	100 μg	capLC-QIT/MS^3 (6 μL/min)	[27]

a) Flow rate of the LC system presented in parentheses. *Abbreviations*: AD, Alzheimer disease; NS, nonsmokers; S, smokers.
* Statistically significant; $p<0.05$.

values of m/z for the precursor and daughter ions, respectively. Alternatively, the constant neutral loss (CNL) occurs when both Q1 and Q3 are operated in the scanning mode. In a comprehensive approach toward analysis of the adductome map, the loss of a 2′-deoxyribose moiety from the protonated molecular ion $[M+H]^+$ to the fragmented aglycone ion $[M+H-116]^+$ was monitored; through comparison with synthetic adduct standards, the presence of seven adducts, including AdG, was confirmed in human lung and esophagus DNA [37].

An assay based on hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometry (Q-TOF-MS), with mass resolution of approximately 8000, has been developed to characterize selected PAH-derived DNA adducts in terms of their fragmentation patterns, using a column having an inner diameter (id) of 75-μm and eluting at a flow rate of 140–150 nL/min [38, 39]. Quantification was, however, not attempted.

Although the sensitivity of an ion trap mass spectrometer is generally lower than that of a triple quadrupole mass spectrometer employing MRM, the former provides the ability to perform multi-stage tandem mass spectrometry (MS^n) for structural identification and to increase specificity in samples experiencing interference or large matrix effects. Although the increase in specificity employing MS^n is accompanied by a sacrifice in sensitivity, the rapid scanning rate and accumulation of trapped ions can contribute to its enhanced sensitivity by increasing the signal-to-noise (S/N) ratio of a measurement. Turesky and co-workers were the first to apply MS^n techniques for DNA adduct quantification. They quantified adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-dGuo using two-dimensional linear quadrupole ion trap (LTQ) mass spectrometry and both SRM and MS^3 scanning modes. Starting from 27 μg of DNA, the MS^3 and MS^4 scanning modes were applied for adduct characterization from in vivo samples at a level of three adducts per 10^8 normal nucleosides [40].

Analysis of DNA adducts using gas chromatography/mass spectrometry (GC-MS) offers ultra-high assay sensitivity with structural information of the analytes, especially when the use of an electrophore as the derivatizing reagent is coupled with an electron capture negative chemical ionization source for detection [41]. The GC-MS approach is hampered, however, by the need to hydrolyze DNA to afford the adducted nucleic acid bases (often requiring strong or mildly acidic conditions at elevated temperature) and the need to derivatize the polar nonvolatile analytes for GC analysis. Nevertheless, the GC-MS methodology is a good choice for analyzing DNA base adducts released in biological fluids (e.g. urine) that would cause serious matrix effects if analyzed directly through LC-MS. In this instance, electrophore-labeling of the adducted DNA bases followed by post-derivatization clean-up can provide clean chromatographs for adduct quantification with high assay sensitivity. We have applied this approach successfully to the analyses of exocyclic 1,N^6-ethenoadenine (εAde) and 3,N^4-ethenocytosine (εCyt) in human urine [42–45]. The on-column LODs for pentafluorobenzylated εAde and εCyt were 5.9 and 3.2 amol, respectively; the limits of quantification (LOQs) for the entire assays of εAde and εCyt were 3.1 and 1.8 fmol, respectively – that is, 31 and 18 pM, respectively, when starting from 0.1 mL of human urine [43, 44].

The LC-MS-based methodology does have the advantage of allowing direct analyses of polar DNA adducts without derivatization; great advancements in LC interfaces and MS ionization technologies have been achieved in recent years to enhance the sensitivity [46, 47]. To further increase the sensitivity of LC-MS analysis, the coupling of capillary or nanoflow LC with micro- or nanoelectrospray (NSI) ionization MS has shown promise for DNA adduct analysis [48].

In 1997, Vanhoutte and co-workers reported the first application of nanoflow LC/MS for DNA adduct analysis. Relative to conventional LC/MS (flow rate: 0.8 mL/min), they observed an improvement of 3300-fold in mass sensitivity for the 2′-deoxyguanosine 5′-monophosphate adduct of bisphenol A diglycidyl ether when using the nanoflow LC/MS system (flow rate: 200 nL/min). This enhancement in mass sensitivity was mainly due to an increase in the peak concentration of the analyte delivered to the mass spectrometer. This method was applied to the detection of the adduct in a calf thymus DNA sample treated with the carcinogen in vitro, but the adduct was not quantified [49].

2.2.2 For AdG measurement

In 2005, Lynn and co-workers analyzed AdG in the hippocampus/parahippocampal gyrus region of postmortem brains using a nanoflow LC system coupled with a high-capacity ion trap mass spectrometer operated in the MS/MS mode [15]. They used a nanobore capillary column of (id: 100 μm) packed in-house with a commercially available material polystyrene–divinylbezene (particle size: 3 μm). The column was eluted at a flow rate of 500 nL/min and coupled to a NSI (or nanoESI) source for MS analysis, achieving an on-column detection limit of 31 amol. Notably, however, the isomers of AdG were not resolved in the capillary LC-MS/MS system; instead, they were quantified as total AdG. The assay achieved an LOQ of 1.5 fmol in 10 μg of DNA or 50 adducts per 10^9 normal nucleosides. The levels of AdG in Alzheimer's disease (AD) patients (5150 ± 640 per 10^9 normal nucleosides (mean $\pm$ SD)) were significantly higher ($p = 0.025$) than those in age-matched healthy subjects (2800 ± 460 per 10^9 normal nucleosides (mean $\pm$ SD)). It is not known whether such high adduct levels are due to the fact that the brain tissue samples were obtained from autopsy approximately 3 h after death, during which time the degree of lipid peroxidation could increase in this fatty tissue. With such high adduct levels, only 1–2 μg of DNA digest was required for analysis. Nonetheless, the significantly higher AdG levels in AD patients are consistent with the higher acrolein concentrations in two regions of the brains of AD patients, as determined through HPLC/fluorescence detection after derivatization [50].

In 2007, Hecht and co-workers analyzed AdG in human lung DNA [26]. They used a micropore (id: 500 μm) capillary LC column containing C18 packing material (particle size: 1.8 μm) eluting at a flow rate of 10 μL/min. This LC system allowed separation of AdG isomers into three peaks: the two α-AdG epimers and γ-AdG. Contrary to the case in other human tissues [16, 23–25], α-AdG was the major regioisomer in the majority of the human lung DNA samples. The possibility of acrolein contamination was excluded by adding $NaBH_3CN$ to the solutions used for DNA isolation to chemically reduce acrolein in the solution; no difference in adduct levels was found in the presence or absence of the reducing agent. The LOQ in DNA samples was 4 adducts/10^9 nucleotides, starting from 0.5 mg of DNA. The levels of α-AdG and γ-AdG in human lung DNA were 40 ± 38 adducts per 10^9 nucleotides (mean $\pm$ S.D.) and 29 ± 31 adducts per 10^9 nucleotides (mean $\pm$ SD), respectively. There was no difference, however, in the adduct levels between smokers and nonsmokers, and the adduct levels were not correlated with urinary nicotine and cotinine, the self-reported time since cessation of smoking, gender, or age [26].

We recently developed an assay (Fig. 3) based on isotope dilution nanoflow LC with nanospray ionization tandem mass spectrometry (nanoLC-NSI/MS/MS) for the simultaneous detection and quantification of the isomers of AdG and the crotonaldehyde-derived 1,N^2-propano-2′-deoxyguanosine adduct (CdG) [16]. The LC system featured a commercially available capillary column (id: 100 μm) and C18 packing material (particle size: 3.5 μm), with elution at a flow rate of 600 nL/min to separate the AdG isomers into two peaks; the later-eluting α-AdG regioisomer was not separable from γ-AdG. The on-column LOD of AdG was 15 amol; the LOQ for the assay was 619 amol in 20 μg of DNA, corresponding to 9.8 AdG in 10^9 normal nucleotides. We determined the level of AdG in a commercially available human placental DNA (20 μg) to be 108 AdG in 10^8 normal nucleotides, while the AdG levels in nine human leukocyte

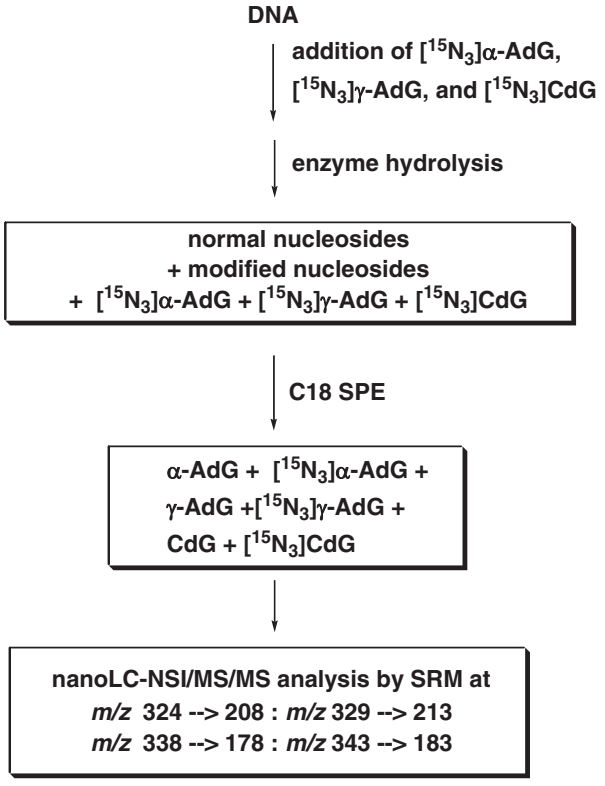

Figure 3. Procedures for measuring AdG and CdG using nanoLC-NSI/MS/MS [16].

DNA samples were 78 ± 23 AdG (mean ± SD) in 10^8 normal nucleotides, starting from 30 μg of DNA. In addition, AdG was not detectable in untreated calf thymus DNA. Only 4–6 μg of DNA was required for analysis using this nanoLC-NSI/MS/MS system; accordingly, only 1–1.5 mL of blood, providing 20–30 μg of DNA, was necessary when measuring both the AdG and CdG levels in human leukocyte DNA. Figure 4 displays representative chromatograms of a human leukocyte DNA sample under the SRM transitions for AdG and the isotope-labeled internal standard [$^{15}N_5$]AdG. In this LC system, the later-eluting α-AdG was not separated from γ-AdG. In all the samples analyzed, γ-AdG was detected and was the predominant species; the mutagenic α-AdG was the minor product [16].

To obtain more structural information during DNA adduct analysis, an ion trap mass spectrometer can record full product ion spectra time-dependently, while allowing multi-stage tandem mass spectrometry (MS^n) to be performed. Turesky and co-workers reported the simultaneous screening for multiple DNA adducts from seven classes of carcinogens using two-dimensional quadrupole linear ion trap (QIT) mass spectrometer with data-dependent CNL scanning followed by MS^3; they quantified AdG at levels of greater than 5 adducts per 10^7 normal nucleotides in buccal cell DNA from smokers [27].

Overall, strategies for improving the sensitivity and specificity of these assays include miniaturization of the LC system, performing multi-stage tandem mass spectrometry (MS^n), and increasing the mass resolution and accuracy.

2.3 Enzyme hydrolysis

The enzymes used for hydrolysis in ^{32}P-postlabeling methods are universal: micrococcal nuclease and spleen phosphodiesterase convert DNA into the nucleotide 3′-monophosphates. On the other hand, various enzyme hydrolysis conditions, in terms of the types and amounts of the nucleases as well as the incubation time, have been used to release DNA adducts as nucleosides from DNA for LC/MS analyses. In many studies, the same amounts of nucleases were used for hydrolysis of a wide range of amounts of DNA.

We have found [51] that different enzyme hydrolysis conditions can give very different levels of the three glyoxal-induced DNA cross-linked products (Fig. 5). In the highly cross-linked glyoxal-treated calf thymus DNA, adduct levels obtained under two sets of enzyme hydrolysis conditions varied from 270- to 1500-fold for the three cross-linked adducts. At low adduct levels, such as those found in human placental DNA, the cross-link levels differed by 2.8- to 4.1-fold. These results clearly demonstrate the substrate specificity of the hydrolytic enzymes in recognizing the adducts [51]. For quantification of AdG and CdG, we compared four different enzyme hydrolysis conditions using human placental DNA as a model DNA to optimize the enzyme hydrolysis conditions, observing up to a two-fold difference for AdG. Because 20–30 μg of DNA was typically used, we also examined the effect of reducing the amounts of the hydrolytic enzymes, finding that it had a great effect on the release of the adducts from the DNA (Table 2) [16]. Thus, as part of the method development to assure the accuracy of adduct quantification, caution must be exercised when optimizing the enzyme hydrolysis conditions for the adduct of interest.

Evidence suggests that incomplete hydrolysis occurs when using micrococcal nuclease and spleen phosphodiesterase to convert the adducts induced by the dietary carcinogen PhIP to the 3′-monophosphates required for ^{32}P-postlabeling analysis [52, 53]. Comparison of PhIP-derived adducts in the same DNA sample has revealed that, relative to LC-MS/MS analysis, the adduct levels were 15- to 20-fold lower when as determined using ^{32}P-postlabeling [40]. Similarly, a five-fold underestimation of the adducts of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) has been reported [54]. Taken together, incomplete enzyme hydrolysis, uncertain labeling efficiencies, and low assay recoveries (typically 5–10% [55]) can all lead to underestimated DNA adduct quantification when using the ^{32}P-postlabeling technique.

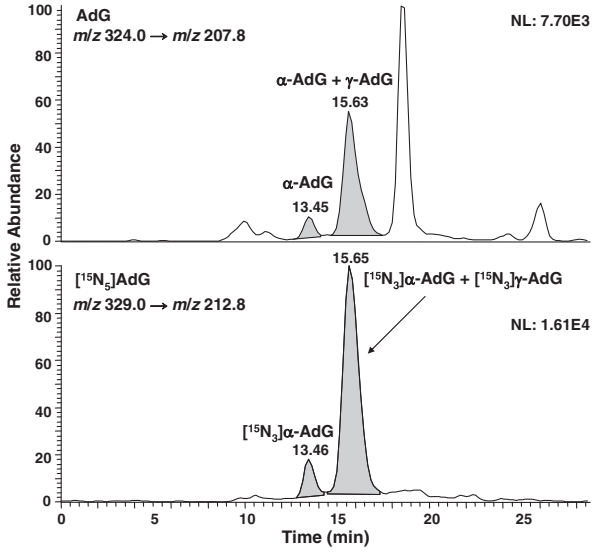

Figure 4. Chromatographs of α-AdG and γ-AdG in human leukocyte DNA, analyzed using nanoLC-NSI/MS/MS.

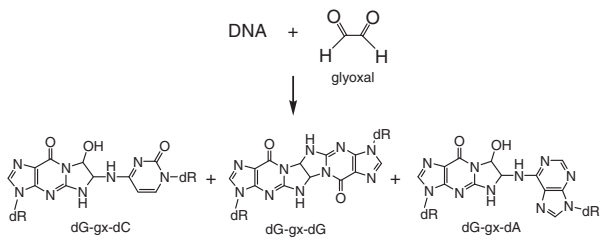

Figure 5. Glyoxal-induced cross-linked adducts of DNA [51].

Table 2. Effects of various hydrolysis methods on the levels of AdG and CdG in human placental DNA [16]

	Adduct level (adducts/10^8 nucleotides)[a)b)]	
	AdG	CdG
Method A [72]	67±8	22±0.2
Method B [19]	69±2	30±0.2
Method C [15]	77±2	32±0.4
Method D [26]	111±2	26±0.8
Method D1[c)]	108±0.7	26±0.1
Method D2[d)]	82±0.8	21±0.8

a) Each experiment started with 20 μg of human placental DNA; an equivalent of 4 μg of DNA hydrolysate was subjected to the LC-NSI/MS/MS analysis.
b) Adduct levels are presented as mean±standard deviations (SDs) from triplicate experiments.
c) The amounts of all enzymes used were 20% of those used in method D.
d) The amounts of all enzymes used were 10% of those used in method D.

3 Biological implications

3.1 Smoking as the predominant source of acrolein

Hecht and co-workers reported that there was no difference in the AdG levels in lung tissue or leukocyte DNA between smokers and nonsmokers [26, 56]. Nevertheless, a five-fold increase in the level of an acrolein-derived mercapturic acid metabolite, (3-hydroxypropyl)mercapturic acid (3-HPMA), has been found in the urine of smokers, relative to nonsmokers [8], suggesting that conjugation of glutathione to acrolein is effective at removing acrolein produced by cigarette smoking, thereby protecting cellular DNA from damage. After smoking cessation for 4 wk, the 78% decrease in the median level of urinary 3-HPMA demonstrated that smoking is the predominant source of acrolein in humans. There was a significant inverse relationship between urinary 3-HPMA and α-AdG, but not the γ-AdG or total adduct levels [8]. The possible relationship between acrolein uptake and lung cancer warrants further investigation.

The lack of correlation between AdG levels and smoking status might be related to individual differences in detoxification and the repair capability of these adducts. Although evidence suggests that AdG should be repaired primarily by the nucleotide excision repair (NER) system [57], the possibility of the involvement of base excision repair (BER) pathways [58] cannot be excluded because the structurally analogous five-membered exocyclic 1,N^2-ethenodeoxyguanosine can be repaired by human alkylpurine-DNA-*N*-glycosylase [59] and the base adduct 1,N^2-ethenoguanine has been detected and quantified in human urine [60]. To date, however, there have been no reports of AdG nucleoside or its base adduct released in human urine or other biological fluids. The inability to detect AdG in urine might be due to the inhibitory effect of acrolein on NER enzymes [13].

In all analyses of AdG together with its analogous adduct(s) derived from crotonaldehyde and/or HNE in the same tissue, the levels of 1,N^2-propano-2′-deoxyguanosine adducts have been consistent with their decreasing order of reactivity and in vivo concentration: acrolein > crotonaldehyde > HNE [16, 18, 19, 23–25].

3.2 Dietary factors

It has been documented that ω-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid, protect against colon cancer. On the other hand, ω-6 PUFAs, including linoleic acid and arachidonic acid, promote colon carcinogenesis [61–63]. Pan and Chung have demonstrated that AdG is derived predominantly from ω-3 PUFA, and to a lesser extent from ω-6 PUFA, under oxidative conditions (in the presence of ferrous sulfate) [64]. Measurement of AdG formation from various PUFAs has revealed that the degree of AdG formation is proportional to the number of double bonds in the PUFAs. In a recent study, correlations were established between AdG formation and DHA-induced apoptotic response, by measuring caspase-3 activity and poly(ADP-ribose) polymerase cleavage, as well as cell cycle arrest in human colon cancer cells. These results suggest an association between AdG adduct formation and the protective effect of DHA against colon tumorigenesis [65]. The potential role of AdG as the ''good'' adduct is intriguing.

In an in vitro incubation system, only the less-mutagenic γ-AdG regioisomer was formed in the presence of PUFA/Fe^{+2} [64]. When γ-AdG is placed complementary to 2′-deoxycytidine in double-stranded DNA, it undergoes ring-opening and rearranges to the N^2-(3-oxopropyl)-dG aldehyde, which then forms interstrand N^2-dG:N^2-dG cross-links in the 5′-CpG-3′ sequence, as evidenced using nuclear magnetic resonance (NMR) spectroscopy [66, 67]. Because the γ-hydroxytrimethylene linkage is located in the minor groove of the DNA, this rearrangement and cross-link formation is stereospecific for the (*R*) epimer of γ-AdG [67]. The stereospecificity cannot be verified in vivo, however, because the (*R*) and (*S*) epimers of γ-AdG cannot be separated using any the LC systems developed for biological samples. Nonetheless, DNA interstrand cross-link formation from AdG might represent a plausible mechanism for initiation of apoptotic response in colon tumor cells. Investigation of the dietary factors affecting AdG levels in vivo should provide important information relating to caner prevention.

4 Concluding remarks and future perspectives

We and others have demonstrated that decreasing the inner diameter of the column and, correspondingly, reducing the flow rate of the LC system to the range of several hundred

nanoliters per minute lead to increases in the analytical sensitivity of the DNA adducts of interest, by increasing the concentrations of the analytes and ensuring more-efficient ionization in the mass spectrometer [15, 25, 49, 51, 68, 69]. Decreasing the column diameter to $<75\,\mu m$ (id) can, however, result in high column backpressure and make the system prone to column plugging, thereby decreasing the robustness of the assay.

An ultraperformance LC (UPLC) system, which can handle high column backpressures, with sub-2-μm particles in the capillary column (id: $\leq 75\,\mu m$), coupled with an NSI/MS/MS system can provide high column resolution with high assay sensitivity. It also reduces the column duty cycle, making it feasible for use in the high-throughput analyses required for epidemiological studies.

High-resolution accurate mass measurement with various mass spectrometers, such as LTQ/Orbitrap or Fourier transform ion cyclotron resonance (FT-ICR) systems, has been used in metabolomic studies because it greatly reduces the number of potential elemental composition candidates in mono-isotopic mass and MS/MS searches [70, 71]. Despite the high prices of these instruments, increasing the mass accuracy results in significant increases in analytical specificity. In the future, accurate mass measurement should play an important role in the field of DNA adduct quantification and adductome map searching in vivo.

In summary, we cannot overemphasize the requirements of high sensitivity, specificity, and accuracy for analytical techniques applied to the measurement of trace amounts of DNA adducts available in limited quantities of DNA from human samples. The relationship between the levels of DNA adducts in biological fluids, such as urine and blood, and target tissues (with tumors) must be established if nontarget site adducts are to be used as non- or low-invasive biomarkers for epidemiological studies.

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