Chapter 18
Mass Spectrometric DNA Adduct Quantification by Multiple Reaction Monitoring and Its Future Use for the Molecular Epidemiology of Cancer

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Abstract The formation of DNA adducts is considered essential for tumor initiation. Quantification of DNA adducts may be achieved by various techniques of which LC-MS/MS-based multiple reaction monitoring has become the most prominent in the past decade. Adducts of single nucleosides are analyzed following enzymatic break-down of a DNA sample following adduct enrichment usually by solid-phase extraction. LC-MS/MS quantification is carried out using stable isotope-labeled internal reference substances. An upcoming challenge is the use of DNA adducts as biomarkers either for internal exposure to electrophilic genotoxins or for the approximation of cancer risk. Here we review recent studies in which DNA adducts were quantified by LC-MS/MS in DNA samples from human matrices. We outline a possible way for future research to aim at the development of an "adductome" approach for the characterization of DNA adduct spectra in human tissues. The DNA adduct spectrum reflects the inner exposure of an individual's tissue to electrophilic metabolites and, therefore, should replace the conventional and inaccurate external exposure in epidemiological studies in the future.

Abbreviations

2-AAF	2-Acetylaminofluorene
4-ABP	4-Aminobiphenyl
BaP	Benzo[*a*]pyrene
CYP	Cytochrome P450 monooxygenase

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dA	2′-Deoxyadenosine
dC	2′-Deoxycytidine
dG	2′-Deoxyguanosine
HAA	Heterocyclic aromatic amine
LC-MS/MS	Liquid chromatography—tandem mass spectrometry
MP	1-Methylpyrene
MRM	Multiple reaction monitoring
PAH	Polycyclic aromatic hydrocarbon
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
TLC	Thin-layer chromatography

18.1 Introduction

Chemical carcinogens cause different DNA lesions, such as adducts, strand breaks, and cross-linked DNA strands. If not fixed by DNA repair mechanisms the damage can lead to mutation events. If this occurs in genes involved in the regulation of the cell cycle, differentiation, or cell–cell interaction, autonomous growth and metastatic potential could be achieved [21]. The vast majority of chemical carcinogens, e.g., heterocyclic aromatic amines (HAA) [24], polycyclic aromatic hydrocarbons (PAHs) [25], *N*-nitrosamines [23], and halogenated hydrocarbons [19, 22] are relatively inert. Enzyme-catalyzed oxidation and conjugation reactions can convert procarcinogens into electrophilic metabolites that can react via nucleophilic substitutions with proteins, RNA or DNA. Cytochrome P450 (CYP) is primarily involved in oxidation reactions. For example, the epoxidation of aflatoxin B1 to aflatoxin B1 *exo*-8,9-oxide is catalyzed primarily by CYP3A4 [20]. CYP1A1 and 1B1 substantially contribute to the bioactivation of benzo[*a*]pyrene (BaP) generating two enantiomers of *trans*-7,8-diols that can be further epoxidized to the ultimate carcinogen BaP-7*R*,8*S*-diol-9*S*,10*R*-epoxide by CYP1A1, 1A2, 1B1, and 2C9 [26, 50]. But also conjugation reactions catalyzed by sulfotransferases, glutathione *S*-transferases, and *N*-acetyl transferases play critical roles in metabolic activation of procarcinogens resulting in covalent modifications of the DNA [48].

Formation of DNA adducts is considered the initial event in cancer development. Studies of animals exposed to common carcinogenic compounds showed that increasing concentrations of DNA adducts were usually associated with growing tumor numbers; however, the correlations were not necessarily linear [36, 42]. Also, DNA adducts can be found in organs that do not develop tumors indicating that other factors, e.g., the tissue-specific capacity of cell-proliferation, co-determine the risk for tumor induction. As tumors do not form in the absence of DNA adducts in animal models, DNA adduct formation is considered a “necessary but not sufficient” requirement for cancer development.

Concentrations of DNA adducts in a particular tissue of an animal treated with a test substance or of human origin may be quantified by different techniques, such as ^{32}P-postlabeling [46] or LC-MS/MS multiple reaction monitoring (MRM) [12, 27].

Since its introduction in 1981 ^{32}P-postlabeling was the gold standard of DNA adduct quantification at the end of the last century due to its superior sensitivity. The applicability of the method resulted in a multitude of studies intending to establish correlations between concentrations of DNA adducts in particular tissues of tumor patients vs. control subjects. Various reviews summarize exemplary data in the field [1, 15, 39, 56]. However, many attempts to correlate DNA adducts and tumor incidence were inconclusive and the value of the data recorded by ^{32}P-postlabeling is questioned today due to the insufficient specificity of the method. Technical details are explained in the next paragraph. In the last decade LC-MS/MS-based techniques were used increasingly for the highly sensitive and specific quantification of DNA adducts via monitoring of analyte-specific molecular fragmentation reactions [27]. We present the results of several recent studies describing the application of LC-MS/MS analytical techniques for the quantification of DNA adducts from human biological matrices. Future research will be directed towards monitoring of multiple DNA adducts in order to characterize the inner exposure of the human genome to electrophilic substances. This "adductome" approach may greatly improve the interpretation of epidemiological data related to cancer development.

18.2 Quantification of DNA Adducts: Technical Details

A variety of analytical methods for the quantification of DNA adducts are available. Until 1981, radioactively labeled carcinogens were used to calculate DNA adduct levels. Later, alternative methods have been used, such as ^{32}P-postlabeling [46], immunofluorescent detection [43], a competitive radioimmunoassay [54], and LC-MS/MS MRM. Table 18.1 provides a brief overview of the techniques. The choice of methods depends on various factors such as the amount of DNA available, the chemical nature of DNA adducts (hydrophobicity), and the scientific question (for example, the search for genotoxins of yet unknown identity in a complex mixture of compounds or quantification of well-defined DNA adducts). ^{32}P-postlabeling was introduced in 1981 and is still attractive because of the sensitivity in the detection of adducts formed from large hydrophobic substances such as PAHs. The method combines the insertion of a radioactive [^{32}P]phosphoryl group at the 5′-hydroxy position of the 3′-mononucleotide adducts after DNA cleavage with subsequent separation of labeled adducts by thin-layer chromatography (TLC). Sensitive autoradiography is used to visualize the chromatographic pattern of DNA adducts on the TLC plate. ^{32}P-postlabeling is still a valuable tool in DNA adduct analyses, especially when molecular adduct structures are unidentified. Thus, ^{32}P-postlabeling is advantageous for the detection of DNA adducts of genotoxic substances in mixtures of environmental xenobiotics of unknown composition, for example, in food plants [2]. The sensitive technique enables the detection of adduct levels in the range of 1 adduct/10^{10} nucleotides using only 10 micrograms DNA [11]. However, the pattern on the TLC plate often shows a number of spots of unknown origin, whose identities can only be conjectured by co-chromatography of standard substances.

Table 18.1 Common techniques for the quantification of DNA adducts

Method	Procedure	Advantages	Drawbacks
^{32}P-postlabeling	– Enzymatic hydrolysis of DNA into 3′-mononucleotides – Nucleotide adducts are labeled with ^{32}P-phosphate at the 5′-end – Separation of adducts over TLC and detection by scintillation counting	– Requires only 1–10 µg DNA – Sensitive – Knowledge of DNA-adduct structures is not required	– Radioactive labeling – Unspecific detection – Underestimation of adduct levels
LC-MS/MS	– Enzymatic hydrolysis of DNA into nucleosides – Enrichment of adducts by extraction methods – LC-MS/MS	– Specific detection – Sensitive quantification	– Requires >10 µg DNA – Isotope-labeled reference substances are desirable
Immunoassays	– Use of antiserum for a DNA adduct in competitive immunoassay, endpoints, e.g., radioactivity or fluorescence	– Inexpensive – Immunohisto-chemistry allows studying localization of adducts	– Unspecific detection (crossreactions) – Requires 50–100 µg DNA – Overestimation of adduct levels

In the past decade, LC-MS/MS techniques have become more important for the quantification of DNA adducts, even though standard substances are indispensable. The LC-MS/MS techniques are characterized by several advantages, including high specificity for the detected DNA adducts, straightforward quantification by isotope-labeled internal reference compounds, and high-throughput capability. The specific detection of nucleoside adducts is based on collision-induced fragmentation of recurring structural motifs. Usually, the adducts are formed by nucleophilic substitution of reactive metabolites and atoms of 2′-deoxyadenosine (dA), 2′-deoxyguanosine (dG), and 2′-deoxycytidine (dC). Figure 18.1a shows the dG adduct of 1-methylpyrene (MP), N^2-((pyren-1-yl)methyl)-dG (N^2-MP-dG). MP is a common carcinogenic food contaminant [33]. It can be bioactivated by CYP-catalyzed hydroxylation at the exocyclic methyl group and subsequent sulfo conjugation resulting in a highly reactive sulfate ester, which undergoes nucleophilic substitutions with exocyclic nitrogens of dA, dG, or dC [33].

Figure 18.1b shows the dG adduct of 2-amino-1-methyl-6- phenylimidazo[4,5-*b*] pyridine (PhIP), a highly carcinogenic HAA isolated from well-done meat [13]. Similar to MP, PhIP is bioactivated by CYP-catalyzed hydroxylation at the exocyclic nitrogen and subsequent sulfo conjugation [14]. The sulfate ester of N^2-hydroxy-PhIP causes the formation of an adduct of dG on *C*8, *C*8-(2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-N^2-yl)-dG (*C*8-PhIP-dG) (Fig. 18.1b).

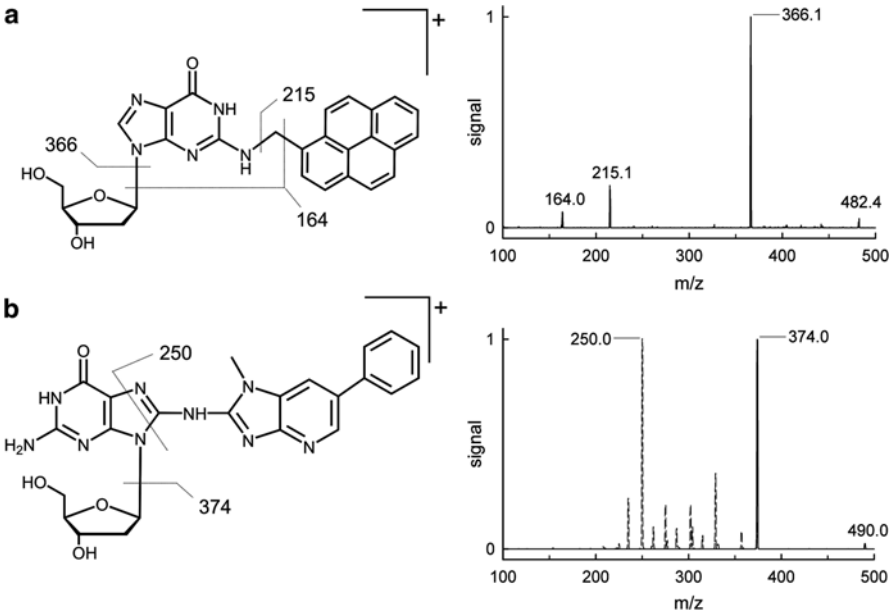

Fig. 18.1 Molecular fragmentation of DNA adducts in the collision cell of a triple quadrupole mass spectrometer. Fragmentation patterns of (**a**) N^2-((pyren-1-yl)methyl)-dG (N^2-MP-dG) and (**b**) *C*8-(2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-N^2-yl)-dG (*C*8-PhIP-dG). The fragment spectrum of N^2-MP-dG (**a**) recorded by collision-induced dissociation showed ions at $m/z = 366.1$ (the aglycone of N^2-MP-dG), $m/z = 215.1$ (the MP-cation), and $m/z = 164.0$ (protonated N^2-methylguanine). The collision-induced dissociation of *C*8-PhIP-dG (**b**) generated fragments of $m/z = 374.1$ (the aglycone of *C*8-PhIP-dG) with a collision energy of 20 eV (*solid line* in the mass spectrum) and a group of fragments at a collision energy of 50 eV (*dashed line* in the mass spectrum) with a dominating signal at $m/z = 250.0$

The molecular structures of the adducts determine the fragmentation reactions in the collision cell of the mass spectrometer, which allows their specific detection and quantification. The breakage of the glycosidic bond in the protonated nucleoside adduct $[M+H]^+$ leads to the neutral loss of 2′-deoxyribose with a mass of 116 Da and the formation of the base adduct $[B+H]^+$ (Fig. 18.1). Another route of collision-induced dissociation of the precursor ion $[M+H]^+$ leads to the release of a positively charged fragment of adduct molecules, which can be observed for numerous different N^6-adducts of dA and N^2-adducts of dG [30–32, 44]. However, the adduct *C*8-PhIP-dG breaks at higher collision energies in many different fragments that cannot be assigned unambiguously to particular molecular structures (see mass spectrum in Fig. 18.1b). Figure 18.2 shows four chromatograms resulting from the collision-induced fragmentation analysis of another common exemplary DNA adduct formed after the uptake of the rodent carcinogen furfuryl alcohol which is a food contaminant present at high levels in the human diet [31]. The upper panel shows the neutral loss of the 2′-deoxyribose ($332.1 \rightarrow 216.1$), which is used as a *quantifier signal*. The chromatogram of the second panel results from the cleavage of the positively

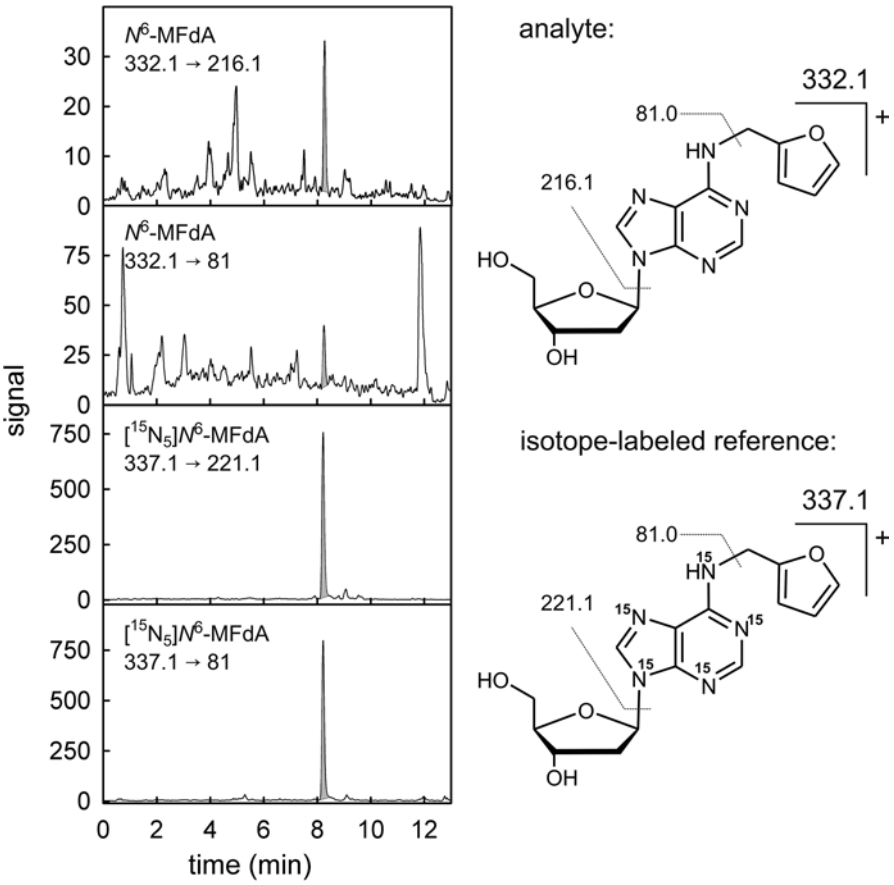

Fig. 18.2 LC-MS/MS analytical quantification of N^6-((furan-2-yl)methyl)-2′-deoxyadenosine (N^6-MFdA) formed by the rodent carcinogen furfuryl alcohol. The chromatograms are LC-MS/MS MRM traces of N^6-MFdA in a digest of DNA isolated from the liver of furfuryl alcohol-treated mice. The fragmentations 332.1 → 216.1 (*first panel*) and 332.1 → 81 (*second panel*) allowed detecting N^6-MFdA and were monitored together with the transitions 337.1 → 221.1 (*third panel*) and 337.1 → 81 (*fourth panel*) of the internal isotope-labeled standard [$^{15}N_5$]N^6-MFdA (38.0 fmol/injection). The ratio of peak areas for the transition 332.1 → 216.1 (N^6-MFdA) and for the transition 337.1 → 221.1 ([$^{15}N_5$]N^6-MFdA) was used to calculate the N^6-MFdA content of the DNA. Details of the method are outlined in [31]

charged methylfuran fragment (332.1 → 81). These traces are monitored together with two additional MRM signals from the transitions 337.1 → 221.1 (third panel) and 337.1 → 81 (fourth panel) of the internal isotope-labeled standard [$^{15}N_5$]N^6-MFdA. The ratio of peak areas for the transition 332.1 → 216.1 (N^6-MFdA) and for the transition 337.1 → 221.1 ([$^{15}N_5$]N^6-MFdA) is used for the quantification of the N^6-MFdA in the sample.

The main advantage of this procedure is the specificity of detection. The identity of a particular nucleoside adduct is confirmed by a specific retention time of the chromatography and the MS/MS-monitoring of several selected fragmentation reactions.

In return, the scientist accepts that other possible adducts remain unobserved. Further, the sensitivity of LC-MS/MS detection with detection limits in the range 1–10 adducts/10^9 nucleotides using 100 μg DNA is somewhat lower compared to that of ^{32}P-postlabeling [12, 27]. However, the quantification of the adducts by LC-MS/MS provides more accurate results in comparison with ^{32}P-postlabeling and immunoassays: Beland et al. determined adduct levels by LC-MS/MS, ^{32}P-postlabeling, and a fluoroimmunoassay in liver DNA of mice treated with [2,2′-^{3}H]-4-aminobiphenyl (4-ABP) and compared the results with the quantification by the ^{3}H-labeled DNA. The result of the LC-MS/MS method agreed best to the adduct concentration determined by scintillation counting, while ^{32}P-postlabeling and the fluoroimmunoassay grossly under- or overestimated the correct adduct concentration [4]. This is consistent with other comparative studies. For a methodological comparison between LC-MS/MS and ^{32}P-postlabeling, we determined the adduct concentrations in hepatic DNA of rats treated with the active metabolite of MP. The concentrations of N^2-MP-dG as determined by LC-MS/MS were on average 3.4 times higher compared to the amounts determined by ^{32}P-postlabeling. Also in the case of adducts of BaP [51] and PhIP [16] LC-MS/MS methods reported 3.7- and 20-fold higher adduct levels compared to ^{32}P-postlabeling, respectively. Factors that may contribute to the underestimation of DNA adduct concentration by ^{32}P-postlabeling are the incomplete digestion of the sample DNA as well as a partial phosphorylation of the modified nucleotides [4, 40, 47]. We showed that an over-digestion of the MP adducts leading to an unintentional loss of the 3′-phosphate also contributes to the adduct loss. The N^2-MP-dG-3′-phosphate proved not to be entirely resistant to dephosphorylation in the presence of micrococcus nuclease, spleen phosphodiesterase, and nuclease P1. About 20 % of the N^2-MP-dG-3′-phosphate was hydrolyzed to N^2-MP-dG and was thus lost to the ^{32}P-postlabeling by T4 polynucleotide kinase [33].

In summary, the advantages of the LC-MS/MS MRM prevail. There are no difficulties resulting from nonspecific enzymatic reactions as in ^{32}P-postlabeling because the DNA samples are digested completely to nucleosides in preparation for LC-MS/MS analysis. An effective solid-phase extraction for enrichment of the adducts allows for almost total isolation of modified nucleosides. The use of stable isotope-labeled standard substances ensures compensation of the analyte losses during the workup for a highly specific detection and ultimately for a convenient quantification of the analytes. In addition, the use of radioactivity is avoided and the time for sample preparation is shorter compared to ^{32}P-postlabeling, which allows the daily processing of 100 samples and the future application of LC-MS/MS techniques in routine analyses of DNA adducts.

18.3 The Scope of DNA Adducts as Human Biomarkers

DNA adducts are considered a prerequisite for the development of tumors. Otteneder et al. established a tentative correlation of hepatic DNA adduct concentrations and the incidence of liver tumors after chronic exposure of common carcinogens in mice and rats [36]. The calculated adduct concentrations at the TD_{50} (dose that caused a

50 % increase of tumors in the treated animals over the controls) ranged from 53 adducts/10^8 nucleosides for aflatoxin B1 to 2,083 adducts/10^8 nucleosides for *N*-nitrosodimethylamine in rats. In mice, the adduct concentrations at the TD_{50} ranged from 812 adducts/10^8 nucleosides for ethylene up to 5,543 adducts/10^8 nucleosides for 2-AAF. This vague correlations between adduct levels and tumorigenic effect of the substances pointed out that the carcinogenic potency of individual DNA adducts may differ greatly [36]. Nevertheless, due to the application of LC-MS/MS for the specific quantification of single DNA adducts it should be theoretically possible to predict the hepatic cancer risk. However, there are several reasons arguing against the prediction of tumor incidences from DNA adduct levels [37]: (1) Usually, the carcinogenicity of a particular genotoxin does not increase in a linear fashion with increasing DNA adduct levels but depends on the species, the gender, and the tissue [42]. This is in part due to tissue-specific differences in DNA repair and cell proliferation, both of which influence the effects of DNA adducts [37]. For example, male mice are twice as sensitive with respect to the hepatocarcinogenic effect of the adducts of *N*-nitrosodimethylamine compared to male rats [36]. (2) Tissue samples from healthy persons are usually not accessible. Non-invasive studies are restricted to the adduct analysis of DNA samples from leukocytes, cells of the sputum, breast milk, and from urine. This restricts the prospect of the possible future DNA adduct analyses for the prediction of a tissue-specific cancer risk in humans.

Although DNA adducts may not be used as biomarkers of effect, they may offer a superior tool for the characterization of the inner exposure to electrophilic compounds. Epidemiological studies investigating the association between exposure to complex mixtures of compounds, e.g., food, and tumor incidences, are greatly hampered by the modeling of the exposure. The subject's exposure, for example, food uptake, is usually deduced from questionnaires which are recognized as a source of inaccuracy ("recall bias"). Moreover, the "external" exposure of a subject to a compound does not necessarily reflect the internal effect of a bioactive metabolite to the individual's genome. The sequence of events between uptake of genotoxic carcinogens and a mutation includes the following steps: (1) absorption and bioactivation of the genotoxin, (2) possible detoxification of the reactive metabolite, (3) reaction with proteins, RNA or DNA (only the latter case is of importance for tumor initiation), (4) persistence of the DNA adduct or removal by repair mechanisms, and (5) proliferation of the cell containing the DNA adduct. This sequence of steps varies greatly between individuals. Further, factors of life style, e.g., alcohol consumption and permanent drug medication, were shown to exert considerable effects on tissue-specific concentrations of DNA adducts [28, 32]. And finally, there are carcinogens in complex mixtures for which the actual exposure is very difficult to calculate because accurate concentrations of the compounds cannot be determined. For example, the accurate intake of methyleugenol remains elusive due to variations of its content in food plants and spices [53]. Therefore, characterization of the "adductome," the pattern of an individual's inner exposure towards electrophilic compounds, would incorporate all interindividual differences in absorption, bioactivation, detoxification, and other parameters, such as life style and medication, that would influence the formation of DNA adducts. The replacement of the external exposure

with the characterization of the adduct-load of human DNA samples (the "adductome") may increase the scope of interpretation in future epidemiological studies targeted at the origins of tumor development.

18.4 DNA Adduct Analyses in Human Biological Matrices: Current Research

Numerous studies were published recently, in which LC-MS/MS MRM was used for the analysis of DNA adducts from different environmental carcinogens in human samples, e.g., from 4-ABP in pancreas tissue from smokers [49], from acrolein [57] or acetaldehyde [8] in leukocytes of smokers, from estrogen in breast tumor tissue [10], from PhIP and 4-ABP in saliva samples [6], or from tamoxifen in colon tissue [7]. All reports describe pilot studies with less than 50 persons using different kinds of biological matrices including saliva, pancreatic tissue, leukocytes, or breast tissue. The outcomes of the studies varied. Chen et al. showed that acetaldehyde adducts in DNA of leukocytes of 25 smokers decreased within several weeks of smoking abstinence [8]. The concentration of *C*8-PhIP-dG in the DNA of epithelial buccal cells did not correlate to consumption of grilled meat or smoking in 37 persons [6], and there was also no association between the 4-ABP adduct of dG in pancreatic tissue samples and smoker status of twelve participants [49]. The emphases of these works were on the description of the LC-MS/MS techniques demonstrating the feasibility of the studies [6–8, 10, 49, 57].

More recently, levels of *C*8-PhIP-dG were determined in adjacent tissue of mammary tumors from 70 patients using a sensitive LC-MS/MS method. The adduct was detectable in merely one sample, at a level of three molecules *C*8-PhIP-dG/10^9 nucleotides [18]. This result is in conflict with previous studies using immunohistochemistry and ^{32}P-postlabeling analytical methods. Zhu et al. reported elevated dG-PhIP adduct concentrations in normal breast tissue of 87 from 106 mammary tumor patients using immunohistochemistry (limit of detection ~1 adduct/10^7 nucleotides) [58]. Gorlewska-Roberts described the detection of dG-PhIP in 30 DNA samples from exfoliated ductal epithelial cells isolated from milk samples of 64 lactating women (mean value 4.7 adducts/10^7 nucleotides, no limit of detection reported) [17]. The discrepancies between results from studies using either highly specific LC-MS/MS MRM or the less selective immunohistochemistry and ^{32}P-postlabeling suggest critical revisions of many older biomarker studies that found correlations between tumor incidence and occurrence of DNA adducts [38, 39, 55]. The elaborate method of accelerator mass spectrometry was used by Brown et al. to detect dG-N^2-tamoxifen in colon DNA of women who were treated with a single dose of 20 mg [^{14}C]-labeled tamoxifen [7]. This supported the hypothesis of a causal relationship between tamoxifen therapy and the increased risk for the incidence of colorectal tumors in tamoxifen-treated women [35]. Taken together, these studies show that progressing development of LC-MS/MS technical equipment allows adduct analyses at sensitivities that were reported previously only for ^{32}P-postlabeling.

There are only few studies focusing on the correlation of increased DNA adduct levels and cancer risk in which specific LC-MS/MS was used to assess DNA damage. A Chinese cohort of 18,000 men were enrolled in a study in order to clarify the consequences of aflatoxin B1 intake and hepatitis B viral infection on the development of hepatic cancer. Samples of urine were analyzed for the adduct aflatoxin B1-N^7-guanine, which originates from hydrolysis of the *N*-glycosidic bond in the DNA adduct aflatoxin B1-N^7-dG. Men without hepatitis B infection but with measurable urine concentrations of aflatoxin B1-N^7-guanine faced a three-fold higher risk for the development of hepatic tumors compared to subjects of the control group. The relative risk was even increased in individuals infected with hepatitis (RR = 59.4, CI = 16.6, 212.0) [45].

Various studies presented correlations between concentrations of DNA adducts from the lipid peroxidation products malon dialdehyde and 4-hydroxy-2-nonenal as biomarkers for oxidative stress. The latter was shown to generate etheno-adducts, e.g., 1,N^6-etheno-dA (εdA) and N^2,3-etheno-dG (εdG). Increases of hepatic etheno-adducts were found in patients with either Wilson's disease or primary hemochromatosis, both of which induce hepatic oxidative stress [34]. Elevated urinary εdA concentrations were found in patients with alcoholic liver disease, chronic hepatitis, and liver cirrhosis, all of which are precancerous illnesses [3]. Bartsch et al. suggested that etheno-adducts in urine and needle liver biopsies may be explored as putative risk markers and to evaluate chemopreventive and therapeutic intervention strategies. However, laborious validation is required for the application of DNA adducts as biomarkers of cancer risk, in case a specific cancer incidence can be attributed to a particular DNA adduct. The validation of a tumor-DNA adduct correlation requires a prospective nested case-control study, in which a large group of participants have to be monitored over many years until cancer develops [41].

18.5 "Adductomics": Monitoring of Multiple DNA Adducts

Recently, many reports were published about DNA adduct quantification using LC-MS/MS-based techniques. Usually, the scientists focused on DNA adducts derived from single carcinogens. However, the association between the exposure to a single carcinogen and the development of a specific tumor as observed for aflatoxin B1 and hepatic cancer is not a common observation. Humans are exposed to complex mixtures of carcinogens. For example, food uptake confronts the organism with a plethora of mutagenic and carcinogenic compounds including mycotoxins, HAA, PAH, heavy metals, *N*-nitrosoalkylamines, substituted furans, etc. Most reports presented in the preceding paragraph were proof-of-concept studies showing the applicability of a novel analytical method for the quantification of DNA adducts of single genotoxins. However, single genotoxins only contribute to the overall cancer risk, which should be better described by the sum of all DNA lesions. Apart from the efforts to further increase the sensitivities of LC-MS/MS-based methods future research will be aimed at simultaneous analyses of different DNA

adducts reflecting the exposure to many of carcinogens. Singh et al. described a method for the quantification of multiple DNA adducts by LC-MS/MS neutral loss of 2′-deoxyribose in a digest of calf thymus DNA incubated with a mixture of dihydrodiol-epoxides of different PAHs [52]. More recently, human autopsy tissue samples were analyzed for 16 different DNA adducts originating from lipid peroxidation demonstrating that the "adductomic" strategy is transferable to studies with human samples for the assessment of internal exposure to electrophilic substances [9]. This approach was further used to analyze samples of gastric mucosa from Japanese and Chinese cancer patients who underwent gastrectomy [29]. These studies were initial steps on the way to develop techniques for the quantification of multiple DNA adducts in human biomatrices. Two further hurdles should be mentioned that determine the progress in this field. Since tissue samples of living human subjects are usually not available (except from cancer patients undergoing surgery), the future use of the methods requires adaption to the analysis of (small amounts of) DNA samples obtained from noninvasive procedures, e.g., from leukocytes. Thus, the progress depends in part on the continuous instrumental advance yielding optimized chromatographic and mass spectrometric equipment. Second, future prediction of cancer risk from assessment of multiple DNA adduct levels, *i.e.*, the application of the DNA adduct spectrum as human biomarker, requires a validation in prospective molecular epidemiology studies. This will be a time-consuming endeavor. However, future analyses of DNA adduct spectra may greatly amplify the significance of human biomonitoring and may extend the scope of interpretations in epidemiological studies.

18.6 Conclusions

The number of reports on sensitive LC-MS/MS analytical techniques for quantification of adducts in DNA samples of human origin is constantly increasing. This is, in part, due to the continuous instrumental advance yielding optimized chromatographic and mass spectrometric equipment, which allows compensating the sensitivity advantage of ^{32}P-postlabeling. As a result, LC-MS/MS MRM is currently the method of choice for DNA adduct analyses. ^{32}P-postlabeling is still attractive for screening purposes if target DNA adducts are not yet characterized or if facilities for organic synthesis of standard substances are lacking.

The assessment of DNA adducts as biomarkers for the definition of internal exposure to environmental carcinogens has several advantages over traditional exposure estimation. Most importantly, DNA adducts account for interindividual differences in uptake, elimination, distribution, metabolism, and repair among exposed individuals as well as for different uptake routes. Consequently, DNA adducts may be helpful tools for the establishment of biologically plausible associations between exposure and disease in epidemiological studies. For example, application of DNA adducts as biomarkers of exposure, e.g., to reactive metabolites in common food carcinogens, such as furfuryl alcohol or methyleugenol, could be

very helpful because the external exposure of these substances resulting from many sources is very difficult to determine [5, 31]. Further, DNA adducts may serve as valuable endpoints in intervention studies. However, the association between tissue concentrations of single adducts and the outcome of a cancer study does not seem plausible. We believe that future analyses of multiple DNA adducts providing an overview of genomic damage due to reactive electrophiles of exogenous or endogenous origin will serve as a valid parameter for molecular epidemiology of cancer.

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