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Characterization of Thioether-Linked Protein Adducts of DNA Using a Raney-Ni Mediated Desulfurization Method and Liquid Chromatography-Electrospray-Tandem Mass Spectrometry

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Abstract

This unit contains a complete procedure for the detection and structural characterization of DNA protein crosslinks (DPCs). The procedure also describes an approach for the quantitation of the various structurally distinct DPCs. Although various methods have been described in the literature for labile DPCs, characterization of non-labile adducts remain a challenge. Here we present a novel approach for characterization of both labile and non-labile adducts by the use of a combination of chemical, enzymatic, and mass spectrometric approaches. A Raney Ni-catalyzed reductive desulfurization method was used for removal of the bulky peptide adducts, enzymatic digestion was used to digest the protein to smaller peptides and DNA to nucleosides, and finally LC-ESI-tandem mass spectrometry (MS) was utilized for detection and characterization of nucleoside adducts.

Keywords

DNA-protein crosslinks; desulfurization; structural characterization; Raney Ni; LC-MS

Introduction

DNA-protein crosslinks (DPCs) are common DNA lesions generated by various endogenous and exogenous agents and proteins. Proteins including AGT, topoisomerase I, topoisomerase II, and DNA polymerase β have been shown to form DPCs (Liu et al., 2002). DPCs are bulky lesions that can impart significant toxicity by blocking replication and transcription or even causing mutations. Similar to small molecule DNA adducts, DPCs can produce a variety of DNA lesions with structural diversity. In order to understand the mechanism of DPC formation and its physiological consequences, it is necessary to elucidate the structure of the DPCs.

Structural characterization of DPCs has been largely limited to labile adducts (*N*⁷-alkyl dGuo) owing to the ease with which the alkylated/adducted proteins can be separated from DNA. In contrast, structural characterization of non-labile covalent DPCs (e.g. *O*⁶-dGuo,

N^6 -dAdo, N^2 -dGuo) is a challenge. In order to characterize the structure of various thioether-linked DPCs (e.g. Cys adducts), we developed a novel method using a combination of chemical, enzymatic, and LC-ESI tandem mass spectrometric approaches (Chowdhury et al., 2013). We utilized a Raney Ni-mediated reductive desulfuration approach (Hecht et al., 1971) to convert the bulky DNA-ethylene-protein crosslinks into small ethyl adducts that can be readily digested to the nucleoside level and characterized by LC-ESI tandem MS and comparison against authentic standards. In this unit we present a detailed protocol of this method to characterize thioether-linked DPCs.

Strategic Planning

Authentic standards

For unambiguous structural characterization of the DPCs (Figure 1), it is necessary to have authentic standards of anticipated alkyl adducts of nucleoside that may result from Raney Ni-catalyzed reductive desulfurization and enzymatic digestion of the DPCs (Figure 2). These alkylated nucleosides can be obtained either from commercial sources or by synthesis (chemical or enzymatic means) and purification (Figure 3). Synthetic methods for small alkyl adducts (methyl, ethyl) are published, while for larger adducts synthetic procedures will need to be designed and developed.

Protease digestion of DPCs

DPCs are bulky lesions that presents significant inhibition to enzyme catalyzed digestion of both the protein and DNA due to steric factors. In the method presented here the protein part of the DPCs are digested first by either trypsin or proteinase K. Because proteinase K cleaves peptide bonds non-specifically and generates smaller peptides, it is the preferred enzyme, although other proteases can also be used. To prevent hydrolysis of labile DPCs, it is also recommended that the peptide digestion be done for about 3 h at 37 °C. To ensure maximum efficiency of the digestion, a high concentration of enzyme can be used.

Raney Ni-catalyzed desulfurization reaction

The Raney Ni-catalyzed reductive desulfurization reaction is the key step in this method for the detection and characterization of DPCs (Chowdhury et al., 2013). The yield of the desulfurization reaction is thus important for the success of this procedure. Accordingly, it is advisable to standardize the reaction conditions by performing the desulfurization reaction with analogous model compounds. The model compounds can be nucleosides or DNA adducted with the small molecule generating the DPCs and a small peptide or GSH (Figure 4). In the absence of suitable model compounds standard conditions reported here can be used. Alternatively, a methyl or ethyl GSH adduct of deoxyadenosine (dAdo) or deoxyguanosine (dGuo) can be used for standardization of reaction conditions.

Materials

Calf thymus DNA

Crosslinking agent (in this case ethylene dibromide (DBE))

Crosslinking protein (in this case *O*⁶-alkylguanine DNA alkyltransferase (AGT))
Potassium phosphate buffer, pH 7.7 and 7.4
Ethylenediaminetetraacetic acid (EDTA)
Tris.HCl (Trizma HCl)
Sodium dodecyl sulfate (SDS)
Proteinase K
Calcium chloride
Ammonium bicarbonate buffer
Dithiothreitol (DTT)
Ethanol (100%, absolute)
DNase free water
Sodium acetate
Raney Ni
DNase I
Phosphodiesterase I
Nuclease P1
Alkaline phosphatase
Sonicator
Table top centrifuge
Agilent C18 (octadecylsilane) SPE HPLC column
Acquity ultra performance liquid chromatography (UPLC) system (Waters Associates)
LTQ Orbitrap mass spectrometer (Thermo Fisher)
Acquity BEH octadecylsilane (C18) UPLC column (2.1 mm × 100 mm, 1.7 μm)
Octadecylsilane (C18) semi-prep HPLC column

Preparation of sonicated calf thymus DNA

- 1 Prepare a 5 ml solution of calf thymus DNA (2 mg/ml) in DNase-free water by sonicating with a probe in intervals of 5 s for 1 minute. Store the DNA solution at −20 °C for future use.

Synthesis of authentic standards

- 2 Obtain authentic standards from commercial sources or by synthesis. In the case of 1,2-dibromoethane and AGT, the anticipated ethyl nucleoside adduct *N*²-ethyl-dGuo was obtained from Sigma, St. Louis, MO. The other anticipated ethyl nucleoside adducts, *O*⁶-ethyl dGuo, *N*¹-ethyl dGuo and *N*⁶-ethyl dAdo

were synthesized and purified following literature procedures (Figure 2 and 3) (Chowdhury et al., 2013).

- 3 Briefly, treat the respective deoxynucleosides with 1.5 equivalent of ethyl iodide in the presence of excess potassium carbonate (~6 equivalents) in DMF at 25 °C. Follow the reaction by TLC for disappearance of the starting material. When the reaction has gone to completion or near completion, separate and purify the various ethyl adducts using preparative HPLC by monitoring the UV absorbance at 260 nm.

The flow rate and gradient conditions (water and acetonitrile) depend on the dimensions of the column used. For adduct identification, use the published characteristic UV-visible spectrum of the respective ethyl adduct and HR-MS (Figure 4).

Standardization of Raney Ni-catalyzed desulfurization reaction

- 4 Prepare *S*-(2-chloroethyl)GSH as described (Humphreys et al., 1990), and incubate 10 equiv with (2'-) dGuo or -dAdo in H₂O/(CH₃)₂SO (1:1, v/v) for 2 h at 37 °C. Subsequently heat the reactions for 30 min at 90 °C under neutral conditions to release some of the modified bases (i.e., *N*⁷-alkyl dGuo). Separate and purify the products by HPLC as previously described and identify *S*-[2-(*N*⁷-guanyl)ethyl]GSH, *S*-[2-(*N*¹-deoxyadenosyl)ethyl]GSH, and *S*-[2-(*N*⁶-deoxyadenosyl)ethyl]GSH adducts (Cmarik et al., 1992) by UV spectroscopy and LC-MS/MS (Figure 5). Treat the purified ethylene-GSH adducts with Raney Ni to perform desulfurization and analyze the corresponding *N*⁷-Et dGuo, *N*¹-Et dAdo, and *N*⁶-Et dAdo adducts using [¹⁸O]-*N*⁷-dGuo-(OH)₃ butane (Cho and Guengerich, 2012) as an internal standard (ISTD) for LC-MS/MS.

Various amounts of Raney Ni should be used, and the amount needs to be adjusted based on the amount of the thioether reactants and the final yield of the desulfurized products (60 °C for 30 min with vigorous stirring in small glass test tubes or vials).

Formation of DPCs

- 5 Mix 50 mg of sonicated calf thymus DNA from the prepared solution (*vide supra*) with 50 mM potassium phosphate buffer (pH 7.7) containing 5 mM EDTA, 10 nmol of AGT, and 10 mM DBE (in case of ¹⁴C-DBE, the specific radioactivity was 6.5 μCi/μmol).

The use of ¹⁴C-DBE is necessary if quantitation of the various DPCs formed is needed or required.

- 6 Prepare appropriate control reaction mixtures where either the DPC forming agent 1,2-dibromoethane or the protein AGT is omitted. Incubate all the reaction mixtures simultaneously for 3 h at 37 °C.

Note: turbidity may be observed in the DNA+DBE+AGT samples due to precipitation of alkylated AGT.

- 7 Following incubation, ethanol precipitate the DNA along with the DPCs using 70% cold ethanol (v/v) and 0.3 M sodium acetate (final concentration). Centrifuge at $10,000 \times g$ for 10–15 min and discard the supernatant. Wash the pellet three times with cold 80% ethanol (v/v) to remove any unreacted excess DBE.
- 8 Redissolve the DNA and DPC pellets in 50 mM Tris buffer (pH 7.4, prepare by adding appropriate amount of Trizma HCl in water) containing 1 mM CaCl_2 and incubate with proteinase K for 3 h at 37 °C (to avoid depurination of the labile adducts avoid prolonging the incubation or increase the temperature) to digest the proteins. In case of the radioactive samples, transfer 1% of the reaction mixture to a scintillation vial (containing 4–10 mL of scintillation cocktail) and count using a scintillation counter.
- 9 Ethanol precipitate the DNA, along with the adducts (*vide supra*), to remove digested peptides. Centrifuge at $10,000 \times g$ for 10–15 min and discard the supernatant. Wash the pellet three times with cold 80% ethanol (v-v).
- 10 Dissolve the DNA precipitate in 50 mM potassium phosphate buffer (pH 7.4) and heat at 90 °C for 30 min in a heating block to depurinate of any labile adducts.
- 11 Again precipitate the DNA using 70% cold ethanol (v/v) and 0.3 M sodium acetate. Centrifuge at $10,000 \times g$ for 10–15 min to form a DNA pellet. DO NOT discard the supernatant. It contain the labile adducts (crosslinks).
- 12 Transfer the supernatant into a vial for the desulfurization reaction. In the case of radioactive samples, transfer 1% of the this solution to a scintillation vial containing 4–10 ml of scintillation cocktail and count using a scintillation counter to quantify the amount of labile adducts.

Conversion of DPCs to alkylated nucleosides

- 13 Dissolve the pellet in 50 mM potassium phosphate buffer (200 μL total volume). For radioactive samples analyze 1% (2 μL) by scintillation counting to quantify the yield of non-labile adducts.
- 14 Dry the supernatant from step 12, (containing the labile crosslinks) under a stream of N_2 and redissolve it in 50 mM potassium phosphate buffer (pH 7.4, 200 μL total volume).
- 15 Treat both the solutions (labile and nonlabile) separately with Raney Ni (30 mg were used, however standardized conditions should be used and the amount needs to be adjusted based on the yield of the DPCs) at 60 °C for 30 min with vigorous stirring. Use a stir bar with a plastic extension (Figure 6) and put it in the vial containing the reaction mixture in a way such that the magnetic bar is not immersed in the solution. This will prevent binding of the Ni on the magnetic bar.

- 16 Let the reaction mixtures cool, centrifuge to remove the Ni (at $3,000 \times g$ for 10–15 min), and purify the DNA or the adducted bases using a C18 SPE column (Agilent, 3 mL).
- 17 For the radioactive labile crosslinks, analyze the reaction mixtures using an on-line HPLC-flow counter and for non-radioactive labile crosslinks by LC-ESI-tandem MS.
- 18 For the non-labile DPCs, digest the SPE-purified products with nuclease, phosphodiesterase, and phosphatase following standard literature procedures (Taghizadeh, et. al. 2008). After digestion, pass the reaction mixture through an Amicon filter (M_r 3,000 cutoff) to remove the proteins. Store the eluent at -20 or -80 °C for LC-MS analysis.

LC-ESI MS analysis of non-radioactive samples

Prepare the instruments and column for LC-MS analysis

- 19 For LC-ESI-tandem analysis of the digested products (nucleosides), an Acquity UPLC system connected to a Thermo Fisher LTQ orbitrap mass spectrometer has been used. Although it is advisable to use the above mentioned instrument, HPLC systems connected to a low-resolution ion trap or a QToF MS instrument can also be used. Operate the MS in the electrospray ionization (ESI) positive ion mode.

Conditions for LC

- 20 Use an Acquity BEH octadecylsilane (C18) UPLC column (2.1 mm $\times$ 100 mm, 1.7 μ m particle size) for liquid chromatography (LC).
- 21 Mobile phase for LC:
Buffer A: 0.1% HCO₂H in 5% CH₃CN and 95% H₂O (v/v)
Buffer B: 0.1% HCO₂H in 95% CH₃CN and 5% H₂O (v/v)
- 22 Prepare the gradient program for LC according to Table 1.
- 23 Maintain the column temperature at 40 °C.
- 24 Tune the mass spectrometer using an authentic nucleoside adduct (in this case N²-Et dGuo was used).

Detect and identify the nucleoside adduct/s (DPCs)

- 25 Equilibrate the C18 column by running 100% buffer A at a flow rate of 300 μ L/min for 30 min.
- 26 Set up a method in the mass spectrometer involving two scan events, one LC-MS² and another LC-MS³ using m/z values of the expected nucleoside adducts. Alternatively, set up a data-dependent method with an inclusion list comprising of the m/z values of the expected nucleoside adducts. If there are difficulty in detecting adducts, perform a neutral loss experiment (neutral loss of 116 amu,

corresponding to the deoxyribose sugar) to detect all possible nucleosides including the unmodified ones. The peaks corresponding to unmodified nucleosides can be easily identified using authentic standards.

- 27 Infuse 10–20 μ l of the samples using an autosampler system.
- 28 Acquire ion spectra over a range that is suitable for the m/z values of the expected nucleoside adducts (Figures 7 and 8).
- 29 Analyze the collected LC-MS² and LC-MS³ data and compare it with authentic standards of the expected nucleoside adducts to characterize the various DPCs. In the event of unavailability of the authentic standards, potential structures of the detected adducts can be proposed based on fragmentation analysis.

Detect and identify the nucleoside adduct/s (DPCs) for radioactive samples

- 30 For radioactive samples with enough counts, inject 50–100 μ l (determine the amount depending on the specific activity of the sample) of the sample into a HPLC system connected on-line to an UV-vis detector and flow counter. Compare the retention time (t_R) of the detected radioactive peaks with the t_R of the UV peaks (use a wavelength of 260 nm) of authentic standards for identification (Figure 9). Alternatively, if the counts are low for flow counter detection, spike the samples with cold authentic standards and collect fractions using the UV signal of the authentic standards. Add scintillation liquid (4–8 ml depending on the volume of the fractions) and count them in a scintillation counter to detect, identify, and quantify the presence of the various adducts (DPCs).

For unambiguous structure elucidation of the adducts and DPCs, the availability of authentic standards of potential nucleoside adducts is necessary.

Background Information

DNA-protein crosslinks (DPCs) are common DNA lesions generated by various endogenous and exogenous agents, including aldehyde metabolites, physical damage such as ionizing radiation and UV light, chemical agents including formaldehyde, 1,3-butadiene, 1,2-dibromoethane, transition metals, and bifunctional chemotherapeutic drugs such as nitrogen mustards and platinum compounds. Proteins including AGT, topoisomerase I, topoisomerase II, and DNA polymerase β have been shown to form DPCs (Liu et al., 2002). DPCs are bulky lesions that can disrupt normal physiological processes including replication, transcription, DNA repair, and chromatin remodeling. Genetic and biochemical studies have shown that both nucleotide excision repair (NER) and homologous recombination (HR) could remove DPCs and both pathways play different roles (Reardon and Sancar, 2006a; 2006b). In bacteria, NER repair DPCs that contain proteins that are less than 15 kDa, whereas oversized DPCs are processed by HR. In mammalian cells, NER does not contribute to the repair of DPCs unless the proteins are <10 kDa whereas HR plays a pivotal role to process DPCs. However, previous model systems cannot be used to study the heterogeneity of DNA repair of DPCs at the DNA base level and the precise effect of DPCs

in vivo. DPC-inducing agents can produce a variety of DNA lesions with structural diversity. In order to understand the mechanism of DPC formation and its physiological consequences it is necessary to elucidate the structure of the DPCs.

Structural characterization of DPCs has been largely limited to labile adducts (*N*⁷-alkyl dGuo) owing to the ease with which the alkylated/adducted proteins can be separated from DNA. In contrast, structural characterization of non-labile covalent DPCs (e.g. *O*⁶-dGuo, *N*⁶-dAdo, *N*²-dGuo) is a challenge. The available methods for the characterization of DNA modifications involve digestion of the DNA to nucleosides and the protein to peptides or amino acid level followed by LC-MSⁿ analysis and comparison with authentic standards. In the case of DNA-ethylene-AGT crosslinks (Figure 10), digestion of the DNA and protein to nucleosides and amino acids is inhibited due to steric hindrance for the hydrolases. Chemical methods of digestion of complexes are generally too harsh to preserve the linkages for structural characterization. Moreover, MS methods of detection are optimized either for nucleic acids or protein/peptides (negative vs positive electrospray ionization (ESI)) and, therefore, in the case of DPCs the presence of one negates the other. Thus, structure elucidation of non-labile DPCs is a challenge.

In order to characterize the structure of various thioether-linked DPCs (e.g. Cys adducts), we developed a novel method using a combination of chemical, enzymatic, and LC-ESI tandem mass spectrometric approaches (Figure 11). Raney Ni reduces thioether functional groups to the corresponding sulfides and alkyl groups. Hecht and coworkers have successfully used this approach on tRNA (Hecht et al., 1971), and we previously applied a similar approach with nickel borate (Ozawa and Guengerich, 1983). Accordingly, we utilized Raney Ni-mediated reductive desulfuration approach on DPCs containing thioether linkages. A Raney Ni-mediated reductive desulfuration method was utilized to convert the bulky DNA-ethylene-AGT crosslinks into small ethyl adducts that can be easily digested to the nucleoside level by standard enzymatic digestion methods. The generated nucleosides and ethyl adducted nucleosides were detected and characterized by LC-ESI tandem MS and comparison against authentic standards. Using this approach, one labile and four non-labile covalent DPCs at guanine and adenine bases (the *N*², *O*⁶, and *N*¹ atoms of guanine and *N*⁶ of adenine, Structures 2–5) have been systematically identified in case of DNA-ethylene-AGT crosslinks.(Chowdhury et al., 2013)

LC-ESI tandem MS analysis of the final products

Following enzymatic digestion and cleanup, the DNA samples are analyzed by LC-ESI tandem MS to detect and characterize the presence of the alkylated nucleoside adducts. LC is performed on an end-capped C18 column using a shallow gradient of water and acetonitrile (each containing 0.1% formic acid, v-v). MS will be performed in the positive ESI mode and the potential adducts will be detected either by a neutral (116 amu) loss experiment or using the anticipated mass of the potential adducts. Nucleosides are known to fragment along the glycosidic bond resulting in the neutral loss of the deoxyribose sugar. Once the various alkylated nucleoside adducts are detected, structural characterization are performed by further fragmenting the deglycosylated adducted bases (LC-MS³) and

comparing the retention times and fragmentation patterns with anticipated synthetic authentic standards.

Critical Parameters and Troubleshooting

The success of the experiment depends primarily on the yield of the DPCs and recovery of adducts from various precipitation steps during the workup procedure (Figure 11). Generally, more than one type of non-labile DPCs is formed and the corresponding yield of the various adducts may be low and variable. The peptide digestion step (i.e., with proteases) may be an issue owing to steric hindrance and needs to be optimized for maximum yield. For success with the Raney Ni-catalyzed desulfurization step, it is necessary that the alkyl thiols used in the previous steps (for example peptide digestion) are completely removed. Using a tripeptide adduct of nucleosides and bases, the yield in the overall Ni reaction was found to be high and did not vary much (89–91%). (Chowdhury et al., 2013) Finally, it is very important that control reactions (where either the DPC forming agent or the protein is omitted) are performed, because even DNA from sources not exposed to known alkylating agents may contain low levels of endogenous alkyl adducts.

Anticipated Results

Generally the anticipated results are unmodified nucleosides (dAdo, dThd, dGuo, dCyd) and alkylated (in this case ethylated) bases and nucleosides depending on the structure of the DPCs. For example, in case of labile DPCs (DPC formed on the N7 atom), alkylated bases will be obtained, while for non-labile DPCs, alkylated nucleosides will be obtained. The number of alkylated nucleosides will depend on the structures of DPCs formed.

Time Consideration

The time required for the generation of DPCs will depend on the nature of the reagents (agent and protein used to generate the DPC) and the chemistry associated with it. However the structural characterization of the DPC using the approach discussed above will require 2–3 days, depending on the bulk and nature of the DPC. This time consideration is based on the assumption that the potential alkyl adducts (obtained after Raney Ni reaction) are commercially available. If the adducts are not readily available, they will have to be separately synthesized, purified, and characterized, which will require additional time.

Acknowledgments

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Figure 1.
Structures of DNA-ethylene-protein crosslinks (Reproduced with permission from Chowdhury et al., 2013)

Figure 2.

Structures of ethylene adducts of DNA formed after Raney Ni treatment (Reproduced with permission from Chowdhury et al., 2013)

Figure 3.

LC-MS/MS and LC-MS³ chromatograms of synthesized authentic Et dGuo adducts. a) Extracted ion chromatogram (EIC) using the m/z 296→180 transition. b) LC-MS³ chromatogram using CID of m/z 296@30, 180@30. Fragmentation of the daughter ion m/z 180 gave two major fragments at m/z 163 (N^2 -Et dGuo) and m/z 152 (N^1 -Et dGuo and O^6 -Et dGup adducts). Based on the UV-vis spectra, the peak at t_R ~3.2 min (with a major ion at m/z 152) was assigned to the N^1 -Et dGuo and the peak at t_R ~4.0 min (with a major ion at m/z 152) was assigned to the O^6 -Et dGuo adduct (Reproduced with permission from Chowdhury et al., 2013).

Figure 4. UV-vis spectra of *N*¹- and *O*⁶-Et dGuo adducts (Reproduced with permission from Chowdhury et al., 2013)

Figure 5.

Structures of *S*-[2-(*N*⁷-guanylyl)ethyl]GSH, *S*-[2-(*N*¹-deoxyadenosyl)ethyl]GSH, and *S*-[2-(*N*⁶-deoxyadenosyl)ethyl]GSH adducts (Reproduced with permission from Chowdhury et al., 2013)

Figure 6.

(A) A cartoonic representation of the Raney Ni reaction setup. (B) Stir bars that can be used for Raney Ni reactions.

Figure 7.

LC-MS/MS and LC-MS³ chromatograms and spectra showing the presence of *N*⁶-Et dAdo adducts from calf thymus DNA. a) LC-MS/MS chromatogram using the m/z 280→164 transition. b) LC-MS³ chromatogram using the m/z 280→164→136 transition. c) LC-MS/MS spectra of the region of the chromatogram at t_R ~3.6 min in a showing presence of the m/z 164 ion, which indicates presence of the ethyl dAdo adduct. d) LC-MS³ spectra of the region of the chromatogram at t_R ~3.6 min in Part b showing the presence of the m/z 136 ion, again indicating the presence of the Et dAdo adduct (Reproduced with permission from Chowdhury et al., 2013).

Figure 8.

LC-MS/MS and LC-MS³ chromatograms and spectra showing the presence of Et dGuo adducts in calf thymus DNA. a) LC-MS/MS chromatogram using the m/z 296→180 transition. b) LC-MS³ chromatogram using the m/z 296→180→163 transition (for N^2 -Et dGuo adducts). c) LC-MS³ chromatogram using the m/z 296→180→152 transition (for N^1 -Et dGuo and O^6 -Et dGuo adducts). d) LC-MS³ spectra of the region of the chromatogram at t_R ~3.6 min in b) showing the presence of the m/z 163 ion, which indicates the presence of the N^2 -Et dGuo adduct. e) LC-MS³ spectra of the region of the chromatogram at t_R ~4.1 min

in c) showing presence of the m/z 152 ion, which indicates the presence of the O^6 -Et dGuo adduct (Reproduced with permission from Chowdhury et al., 2013).

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Figure 9. HPLC chromatograms showing the presence of labile adducts (N^7 -Et dGuo) in calf thymus DNA. The ethyl adducts were analyzed by HPLC-flow counting (flow counter coupled in-line to an HPLC). a) Control (DNA+ ^{14}C -1,2-dibromoethane (DBE)). b) Sample (DNA + ^{14}C -DBE + AGT) (Reproduced with permission from Chowdhury et al., 2013).

Figure 10.
Mechanism of DBE derived crosslink formation by AGT.

Figure 11.

Flow chart for the detection and quantitation of various DPCs and DNA adducts

(Reproduced with permission from Chowdhury et al., 2013)

Table 1

UPLC gradient

Time (min)	% A	% B	Flow rate (μl/min)
0	100	0	300
5	75	25	300
5.5	0	100	300
6.5	0	100	300
7.0	100	0	300
10	100	0	300