Formation, Persistence, and Repair of 1,2,3,4-Diepoxybutane-Induced Bifunctional DNA Adducts in Tissues of Rodents Exposed to 1,3-Butadiene by Inhalation

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Abstract

1, 3-butadiene (BD) is an industrial chemical and environmental pollutant. It is also classified as a probable human carcinogen based on animal tumorigenesis studies and human epidemiologic evidence. BD is metabolized to three reactive epoxides, of which 1,2,3,4-diepoxybutane (DEB) is the most mutagenic, likely due to its bifunctional electrophilic structure that enables it to form DNA-DNA cross-links, 1,4-\textit{bis}-(guan-7-yl)-2,3-butanediol (\textit{bis}-N7G-BD) and 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD), and 1,N\textsubscript{6}-(2-hydroxy-3-hydroxymethyl-1,3-propanediyl)-dA (1,N\textsubscript{6}-HMHP-dA) exocyclic DNA adducts. The purpose of this research is to identify mechanism-based biomarkers of exposure to BD. Our laboratory has identified DNA-DNA cross-links and exocyclic deoxyadenosine adducts formed from DEB that could potentially be used as biomarkers of exposure. Highly specific and sensitive isotope dilution HPLC-ESI\textsuperscript{+}-MS/MS methods have been developed to analyze DEB-induced DNA-DNA cross-links and exocyclic DEB-dA adducts in DNA extracted from laboratory animals exposed to BD by inhalation. \textit{bis}-N7G-BD was the most abundant adduct, N7G-N1A-BD was \~10 times less abundant than \textit{bis}-N7G-BD, and 1,N\textsubscript{6}-HMHP-dA was the least abundant (\~4 fold lower than N7G-N1A-BD). The quantitative isotope dilution mass spectrometry methods developed in this work were employed to investigate the dose dependent formation, persistence, and repair of the BD-induced DNA adducts in laboratory rodents. Species, gender, and tissue differences in adduct levels were observed. Mouse DNA contained a greater number of DEB-specific adducts than rat DNA at identical exposures. Adduct levels were also higher in female as compared to male rodents, and in liver DNA (compared to lung, brain, kidney, and thymus).

Although \textit{bis}-N7G-BD was most abundant DEB-DNA adduct immediately following exposure, it did not persist in mouse or rat liver while the other adducts did. We did not observe repair of the bifunctional lesions by BER or NER, however, \textit{in vitro} studies suggest that 1,N\textsubscript{6}-HMHP-dA is repaired by AlkB. The research presented in this thesis is consistent with animal inhalation studies where mice were more susceptible to tumor formation. The data also suggests that these differences are due to species differences
in the extent of BD metabolism to DEB. Two of the adducts, N7G-N1A-BD and 1,N6-HMHP-dA were persistent in DNA and may be responsible for the mutagenicity of BD.
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LIST OF ABBREVIATIONS

1,N²-ε-dGuo  1,N²-etheno-2'-deoxyguanosine
4-ABP        4-aminobiphenyl
A            adenine
AA           acrylamide
BD           1,3-butadiene
bis-N7G-BD   1,4-bis-(guan-7-yl)-2,3-butanediol
BP-6-N7Gua   N7-(benzo[a]pyrene-6-yl)guanine
BPDE         trans-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-
tetrahydrobenzo[a]pyrene
C            Cytosine
C8(1-HE)g    C8-(1-hydroxyethyl)guanine
C8-dG-MeIQx  N-(deoxyguanosine-8-yl) MeIQx; N²-dG-MeIQx, 5-
              (deoxyguanosin-N²-yl) MeIQx
capLC-nanoESI capillary HPLC-nanoelectrospray ionization-tandem mass
              spectrometry
CedG         N2-(1-carboxyethyl)-2'-deoxyguanosine
CNL          constant neutral loss
CP-d(GpG)    cisplatin 1,2-guanine-guanine intrastrand cross-link
DEB          1,2,3,4-diepoxybutane
dG-BPDE      10-(deoxyguanosin-N²-yl)-7,8,9-trihydroxy-7,8,9,10-
tetrahydrobenzo[a]pyrene
dG-C8-4-ABP  N-(deoxyguanosin-8-yl)-4-ABP
dG-C8-IQ     N-(deoxyguanosine-8-yl)-2-amino-3-methylimidazo [4,5-f]
              quinoline
dG-desMeTam  (E)-α-(deoxyguanosin-N²-yl)-N-desmethyl tamoxifen
dG-Tam       (E)-α-(deoxyguanosin-N²-yl) tamoxifen
EB           3,4-epoxy-1-butene
EBD          3,4-epoxy-1,2-butanediol
EH           epoxide hydrolase
G
GA
gdG
HEB
His
HPLC-ESI\(^{+}\)-MS/MS
IDMS
IQ
IS
LOD
LOQ
MelQx
mel-dGuo
MP-dAdo
MP-dGuo
N1-THBA
N7G-N1A-BD
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methylpyrene deoxyguanosine
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1-(guan-7-yl)-4-(hypoxanth-1-yl)-2,3-butanediol
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N7-(2,3,4-trihydroxybut-1-yl)-guanine
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cytochrome P450 monooxygenase
N,N-(2,3-dihydroxy-1,4-butanediyl)-valine
reactive oxygen species
serine
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<td>SRM</td>
<td>selected reaction monitoring</td>
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<tr>
<td>T</td>
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<td>THB</td>
<td>trihydroxybutyl</td>
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<td>THB-Val</td>
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I. LITERATURE REVIEW

1.1 Isotope Dilution Mass Spectrometry for Quantitative Analysis of DNA Adducts

1.1.1 DNA adducts and their role in chemical carcinogenesis

The formation of covalent DNA adducts is a key event in the development of cancer (1,2). If not repaired, DNA lesions can be misread by DNA polymerases, leading to heritable mutations (1). DNA adducts can be formed by endogenous agents (i.e. lipid peroxidation products, reactive oxygen species) and also by exogenous chemical agents. Many carcinogens must be metabolically activated to electrophilic species such as epoxides, diazonium ions, or nitrenium ions which are capable of forming covalent bonds with nucleophilic sites in DNA (3). Other chemicals, such as α, β-unsaturated carbonyl compounds and aldehydes are inherently reactive and do not require metabolic activation (3).

Chemical stability of DNA adducts varies significantly. For example, adducts to the N7 position of guanine, the N7 or the N3 of adenine, and the O2 of cytosine destabilize the glycosidic bond, leading to spontaneous depurination of the structurally altered nucleobases and the formation of abasic sites (4). The latter can be repaired via base excision repair mechanisms, or may cause point mutations and DNA strand breaks (5). DNA lesions also differ in their ability to mispair during DNA synthesis. For example, \( O^6 \)-alkylguanines preferentially mispair with thymine instead of the normal partner, cytosine, leading to \( G \rightarrow T \) transversions (6). Exocyclic adducts, such as \( 1,N^6 \)-etheno-2'-deoxyadenosine (εA), are strongly promutagenic because the formation of fused ring adducts alters the molecular shape and the base pairing characteristics of the parent nucleobase (7).
Living organisms have evolved to protect themselves against DNA damage induced by endogenous and exogenous electrophiles. Every living cell contains thousands of genes responsible for the repair of DNA adducts, including direct repair, base excision repair (BER), nucleotide repair (NER), and homologous recombination repair. Simple alkylated and oxidized lesions are primarily repaired by BER, while bulky adducts that distort DNA structure are excised by NER, and DNA-protein cross-links are repaired by homologous recombination pathways (8). Furthermore, specialized repair proteins such as 6-alkylguanine DNA alkyltransferase and AlkB restore the parent nucleobases, preventing mutagenesis (9-11). However, some lesions such as interstrand DNA-DNA cross-links are difficult to repair because they affect both strands of DNA, preventing accurate repair synthesis; these adducts are often cytotoxic and cause large deletions (12).

Because DNA adducts play a central role in carcinogenesis, they can be used as biomarkers of exposure to carcinogens and as indicators of the formation of reactive intermediates available for binding to DNA (13). Sensitive, accurate, and specific methods have been developed for quantifying DNA adducts in biological samples (14-16). Information about DNA adduct formation can be used to identify populations at risk for cancer development, and also for setting human exposure limits for industrial chemicals (13). Typical levels of DNA adducts present in tissues as a result of industrial or environmental chemical exposures are in the range of 0.1-1 adduct per 10^8 normal nucleotides (17). Therefore, methods used for their analysis must be ultra sensitive and capable of accurate quantification of target adducts in the presence of a high molar excess of normal nucleosides. Isotope dilution mass spectrometry (IDMS)
is an ideal method for quantification of DNA adducts because it uses isotopically labeled internal standards that are chemically and chromatographically identical to the analyte, allowing for reproducible, precise, and specific quantitation (15).

**Sources of DNA for analyses of DNA adducts.** Typical sources of DNA for adduct analysis include organ tissues, blood, and urine. Larger amounts of DNA can be isolated from abundant tissues such as liver; however, blood samples are easier to obtain, and may be the only feasible choice in the case of human analyses. Another non-invasive source of DNA adducts that can be used for human biomonitoring is urine that contains depurinated or repaired free base and nucleoside adducts (7;18;19). Similar to blood samples, urine is easy to obtain, however, it is a complex matrix containing high amounts of salts and other components that must be removed prior to MS analysis.

**Sample preparation.** Most biological samples (e.g. DNA extracted from tissues or blood, or urine specimens) require extensive sample clean-up steps prior to MS analysis to remove normal nucleosides, proteins, salts, and other components of biological fluids that interfere with MS analysis (15;16). Examples of sample preparation methods include size exclusion chromatography, liquid/liquid extraction, solid phase extraction, and offline HPLC purification. The simplest form of sample clean-up is size exclusion filtration to remove large molecular weight components (i.e. proteins, DNA backbone). For liquid/liquid extraction, hydrophobic DNA adducts are extracted with organic solvents, while other components of the biologic matrix remain in an aqueous phase. Solid phase extraction (SPE) employs a small disposable column of chromatographic packing materials capable of binding the adducts. In SPE based on
reversed phase separation, samples are loaded in water or buffer, and matrix components are washed away with water, buffer, or other weak solvents. Finally, the adducts of interest are eluted with a stronger solvent such as methanol or acetonitrile. Immunoaffinity purification employs a similar principle, with the exception that the sample enrichment is achieved \textit{via} analyte binding to a monoclonal or polyclonal antibody attached to a solid support. Offline HPLC is the use of chromatographic separation to isolate DNA adducts and to remove the bulk of the biological matrix prior to MS analysis. HPLC fractions containing the analyte are concentrated under vacuum and injected onto an HPLC column for HPLC-MS analysis. While off-line HPLC achieves optimal analyte purification/enrichment, it can be very time consuming and is not practical for studies employing large numbers of samples.

1.1.2. Instrumentation for HPLC-ESI\(^+\)-MS/MS

Several types of mass spectrometry instruments are available commercially, including single and triple quadrupoles, ion traps, orbitraps, and time of flight (TOF) mass spectrometers (20). Different types of MS instruments vary in their resolution power, duty cycle, dynamic range, and MS/MS capabilities, as well as their cost and their ease of use. In this section, we will describe the main types of MS instruments and their advantages and disadvantages in quantifying DNA adducts. We will also discuss MS scanning methods used to increase selectivity and sensitivity of MS/MS analyses of DNA adducts.

Several scanning methods can be used to increase the selectivity of mass analyzers for a specific analyte, including selected ion monitoring (SIM), selected
reaction monitoring (SRM, also MRM), constant neutral loss (CNL), and enhanced product ion scan (EPI). SIM is simplest of methods for instruments unable to perform tandem mass spectrometry experiments. SIM adds selectivity by only monitoring ions with a narrow \( m/z \) range, increasing sensitivity 100-fold due to an improved duty cycle. Many of the most sensitive scanning methods require the use of instruments capable of performing MS/MS experiments, such as triple quadropoles (QqQ) and QqQtrap. In the SRM mode, \( Q_1 \) scans all ions and selects only ions of a user-specified \( m/z \) to pass to \( q_2 \) where the ions are fragmented by CID. The fragmented ions (product ions) enter \( Q_3 \), where they are filtered by another user-specified \( m/z \) and only ions of the specified \( m/z \) enter the mass detector. This adds selectivity, as it is unlikely that matrix components will have the same precursor and fragment masses (\( m/z \)). CNL mode adds selectivity by allowing the user to select a mass difference between precursor and product ions. This method is typically used when analytes with similar structures are being analyzed that all lose a similar fragment (e.g. a deoxyribose or phosphate group) (21). In CNL, \( Q_1 \) scans all \( m/z \) ions, which are then dissociated in \( q_2 \) before passing to \( Q_3 \) where the product ions are scanned, and only ions with user-specified difference between \( Q_1 \) and \( Q_3 \) are transmitted to the mass detector. Lastly, EPI can be performed with a hybrid QqQtrap analyzer. This method is similar to SRM, with the major difference being the trapping of fragmented precursor ions between \( q_2-Q_3 \) prior to mass filtering in \( Q_3 \).

**Quadropoles**

A quadropole is a mass filter (Q) that forms an electromagnetic field in which only ions falling within a narrow range of mass to charge ratio (\( m/z \)) values have a
stable trajectory (15;20). Triple quadropoles (Q_1q_2Q_3) contain two quadropole mass filters (Q_1 and Q_3) and a collision cell/ion guide (q_2). Triple quadropole mass analyzers can perform tandem mass spectrometry in the precursor ion mode, selected reaction monitoring mode (SRM), product ion mode, or constant neutral loss (CNL) mode. For example, in the SRM mode, parameters for Q_1 are set to isolate ions of a specific m/z of a precursor ion, those ions are fragmented in the second quadropole (q_2) by collision induced dissociation (CID) and are directed to Q_3 where product ions of a specified m/z are selected and enter the mass detector. Triple quadropoles are the main type of mass analyzer used for trace quantitative analysis due to their high duty cycle in selected ion monitoring (SIM) and SRM modes, high resolution power 100-1000, relatively low cost, and the ease of operation.

**Ion traps**

Ion trap mass spectrometers store ions in time rather than in space; the ions can be trapped and accumulated to increase signal to noise ratios (22). Ion traps are typically not used for trace quantitative analysis due to their low duty cycle, which limits their sensitivity in quantitative analyses (20). Ion traps can operate in the MS^n mode where n = 1-10, and have a m/z limit of ~5000 Da. Therefore, ion traps are popular for structural analysis. Recently, a hybrid Q_1q_2/TQ_3/T instrument that can be used as triple quadropole linear ion trap has been used for quantitation of DNA adducts (23;24). The QqQtrap can be operated in the enhanced product ion (EPI) mode, in which precursor ions pass through Q_1 to q_2 where they are fragmented by CID, as in QqQ. The ions are then trapped between q_2 and Q_3 prior to mass filtering in Q_3 and ion
detection. Although some methods report similar results and sensitivity for QqQ MRM and QqQtrap EPI modes (20), triple quadrupoles are more commonly used.

**Time of Flight Mass Analyzers**

Time of flight (TOF) mass analyzers are extended m/z range mass analyzers with a resolving power of 10,000 or better. TOF mass analyzers are often coupled with MALDI for use in peptide sequencing (20). The SIM mode is not possible for trace quantitative analysis; therefore TOF LOD values are typically 10 fold lower than SIM/SRM QqQ methods (25). Another hybrid mass analyzer couples quadrupoles and TOF in the QqTOF (25). The QqTOF is similar to QqQ; however, the third Q is replaced by TOF analyzer. The QqTOF can be operated in a SIM-like mode similar to SIM/SRM of QqQ except with higher resolution of the fragment ions (25). QqTOF has received some attention for trace analysis, however, it is mostly used for sequencing peptides/proteins (26).

**Magnetic sector analyzers, FTICR and Orbitrap**

Magnetic sector analyzers have a high resolving power (10,000), and can be readily coupled with GC (20). These analyzers are typically used in the SIM mode for trace analysis of environmental analytes, such as chlorinated dibenzodioxins (27). Disadvantages of magnetic sector analyzers are difficulty in coupling with HPLC-ESI/API. Furthermore, they are expensive to install and maintain.

In ion cyclotron resonance (ICR), molecule ionization, mass analysis, and detection all occur in a single cell (22). Fourier Transform Ion Cyclotron Resonance
(FTICR), instruments that employ fourier transform pulse techniques have become an important tool in MS-based proteomics (28). FTICR has a high resolving power and high sensitivity, however it has a low duty cycle, and a high cost. Therefore, FTICR is not typically used for quantification of trace analytes (20).

Orbitrap is a modified ion trap in which ions of a specific mass to charge ratio oscillate around the central electrode in rings. The Orbitrap is a relatively new invention (29). The use of Orbitraps for trace quantitative analysis is rare (20). While Orbitraps have high resolving power (150,000) and mass accuracy, a recent review of the Orbitrap mass analyzer, does not report any references of employing this analyzer for trace quantitative analyses, likely due to its high cost and recent availability (30).

**MS method validation**

DNA adducts present in biological samples often go through several processing/enrichment steps (e.g. solid phase extraction) prior to MS analysis. Therefore, a certain amount of analyte is lost during processing, and recovery can vary from sample to sample. To increase the reproducibility and accuracy of quantitative analyses, isotope dilution methodology (IDMS) can be used. Isotope dilution-MS/MS analysis is the use of stable isotope labeled analogs of analyte as internal standards. Increased reproducibility and accuracy are achieved when a stable isotope labeled internal standard is spiked into samples early in the analysis to account for any loss of the analyte, and matrix effects/suppression during MS analysis. Quantitative analysis is achieved by comparing HPLC-ESI-MS peak areas in ion channel corresponding to the analyte and the internal standard.
Quantitative HPLC-MS/MS methods must be validated to determine their accuracy and precision by spiking sample matrix with known amounts of analyte and internal standard, processing the spiked samples by the same enrichment methods and HPLC-MS/MS methods as actual samples to determine if the measured level of analyte matches the level that was spiked into the matrix. Similar spiking experiments can be used to determine the limit of detection (LOD) (S/N ≥ 3) and limit of quantitation (LOQ) (S/N ≥ 10) for the analyte in a given matrix.

The role of IDMS for in vivo quantitative analysis of DNA adducts, focusing on HPLC-ESI-MS/MS, and recent advances in on-line sample processing, nanospray-MS, and chip-based LC-MS methods are discussed below. This methodology has been used to quantify a wide range of DNA adducts, including adducts formed from natural products/drugs, aromatic amine adducts, nitrosamine adducts, etheno adducts, polyaromatic hydrocarbon adducts, etc. Several examples for each group are described in more detail below, and typical limits of detection (LOD) are summarized in Table 1.1.

1.1.3. Examples of HPLC-ESI-MS/MS analyses of DNA adducts

Capillary HPLC-ESI-MS/MS conducted on a triple quadropole mass spectrometer using isotopically-labeled internal standards is most commonly used to quantify many DNA adducts in biological samples due to its high selectivity, sensitivity, and accuracy. IDMS has been used for DNA adducts formed from drugs/natural products, including melphalan and cisplatin, chemotherapeutic agents used in the treatment of multiple myeloma and ovarian cancers (31). Isotope dilution
mass spectrometry has also been used to quantify DNA adducts formed from exposure to carcinogens present in air, food, drinks, cigarettes, and occupational settings. For example, 4-aminobiphenyl (4-ABP), a carcinogen present in cigarette smoke, can induce N-(deoxyguanosine-8-yl)-4-ABP (dG-C8-4-ABP) adducts in DNA (32). 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) is a heterocyclic aromatic amine found in cigarette smoke and also present in cooked meat and fish. Following bioactivation to IQ, N-(deoxyguanosine-8-yl)-2-amino-3-methylimidazo [4,5-f] quinoline adducts (C8-dG-IQ) form (33). The tobacco specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent lung carcinogen in rodents, and possibly in human smokers (2). The Hecht laboratory has developed IDMS methods to quantify four POB adducts of NNK, 7-POB-Gua, O6-POB-dGuo, O2-POB-dThd, and O2-POB-Cyt, in rodents exposed to NNK (34,35). Exocyclic etheno DNA adducts arise from exposure to exogenous chemicals such as vinyl chloride, and from endogenous lipid peroxidation products. Etheno DNA adducts are promutagenic and may be involved in cancer initiation. Therefore, several etheno-DNA adducts, 1,N2-ε-dGuo (36), ε-dA (37) have been quantified by IDMS for risk assessment. An increased occurrence of colon cancer has been associated with alcohol consumption, attributed to the formation of acetaldehyde, reactive oxygen species (ROS), and 1-hydroxyethyl radical following metabolic processing of ethanol. Wang and colleagues developed an HPLC-ESI-MS/MS method for quantitation of an acetaldehyde-dG adduct in human liver DNA (38). Hundreds of PAHs have been identified in automobile exhaust, cigarette smoke, and coal tar; many of these are carcinogenic to animals and humans. One PAH extensively studied is benzopyrene (BP), which reacts with guanine to form N7-
(benzo[a]pyrene-6-yl)-guanine (BP-6-N7Gua). These adducts can be depurinated and excreted in urine; therefore, IDMS methods for quantification of BP-6-N7-Gua in human urine have been developed for risk assessment (39). 1-Methylpyrene (1-MP) is an alkylated PAH that requires metabolic activation to its reactive form, 1-sulfooxymethylpyrene (1-SMP). Monien et al. compared IDMS and $^{32}$P-postlabeling for quantitation of 1-Methylpyrene adducts to adenine and guanine in rats treated with 1-SMP (40). Interestingly, they observed 3.4-fold more adducts by the IDMS methods than from $^{32}$P-postlabeling, due to more accurate quantitation by IDMS. While $^{32}$P-postlabeling has low limits of detection, it is not as accurate and specific a method as IDMS. Several improvements to mass spectrometry instruments and methods, discussed in the following section, have lead to detection limits approaching those of $^{32}$P-postlabeling.
Table 1.1 Summary of IDMS methods for several DNA adducts.

<table>
<thead>
<tr>
<th>Natural Products/Drug adducts</th>
<th>Adduct</th>
<th>Method</th>
<th>LOD</th>
<th>Sample Prep.</th>
<th>Species</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary 4-OHE2-N7G</td>
<td>Mel-dGuo</td>
<td>IDMS w/ column switching</td>
<td>900 fg</td>
<td>SPE, offline HPLC</td>
<td>Rat</td>
<td>liver, mamm</td>
<td>(41)</td>
</tr>
<tr>
<td></td>
<td>dG-Tam</td>
<td>IDMS w/ column switching</td>
<td>1 adduct/10^8 nts</td>
<td>SPE, offline HPLC</td>
<td>Rat</td>
<td>liver, uteri</td>
<td>(42)</td>
</tr>
<tr>
<td></td>
<td>AFB1-N7Gua</td>
<td>IDMS</td>
<td>0.02 pg</td>
<td>SPE, immunoaffinity column</td>
<td>Human</td>
<td>urine</td>
<td>(19)</td>
</tr>
<tr>
<td></td>
<td>4-OH-E1-N3Ade</td>
<td>capLC-nanoESI</td>
<td>5 fmol/g tissue</td>
<td>SPE, offline HPLC</td>
<td>Human</td>
<td>breast</td>
<td>(43)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aromatic amino adducts</th>
<th>Adduct</th>
<th>Method</th>
<th>LOD</th>
<th>Sample Prep.</th>
<th>Species</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IQ-dG</td>
<td>IDMS</td>
<td>6 fmol</td>
<td>SPE</td>
<td>Rat</td>
<td>liver</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td>dG-C8-ABP</td>
<td>IDMS</td>
<td>5 fmol/300 µg DNA</td>
<td>SPE</td>
<td>Human</td>
<td>pancreas</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td>dG-C8-MeIQx</td>
<td>IDMS</td>
<td>500 fg</td>
<td>SPE</td>
<td>Rat</td>
<td>liver</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td>dG-N2-MeIQx</td>
<td>IDMS</td>
<td>750 fg</td>
<td>SPE</td>
<td>Rat</td>
<td>liver</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td>N8-Ade-benzidine</td>
<td>IDMS</td>
<td>22 pg</td>
<td>SPE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N8-Ade-2-</td>
<td>IDMS</td>
<td>51 pg</td>
<td>SPE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>aminofluorene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrosamine adducts</td>
<td>Method</td>
<td>LOD</td>
<td>Sample Prep.</td>
<td>Species</td>
<td>Source</td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
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<td>---------</td>
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<td>------</td>
<td></td>
</tr>
<tr>
<td>Hydroxyethyl-dG</td>
<td>IDMS</td>
<td>100-150 fmol</td>
<td>filter</td>
<td>rat</td>
<td>liver</td>
<td>(46)</td>
<td></td>
</tr>
<tr>
<td>O&lt;sup&gt;6&lt;/sup&gt;-POBdG</td>
<td>IDMS</td>
<td>50 fmol</td>
<td>SPE</td>
<td>mouse</td>
<td>liver</td>
<td>(47)</td>
<td></td>
</tr>
<tr>
<td>POB adducts</td>
<td>IDMS</td>
<td>3 fmol G, 1 fmol dGuo, 100 amol Thd, 2 fmol Cyt</td>
<td>SPE</td>
<td>Rat</td>
<td>liver/lung</td>
<td>(34)</td>
<td></td>
</tr>
<tr>
<td>7-POB-Gua</td>
<td>IDMS</td>
<td>SPE</td>
<td>Rat</td>
<td>liver/lung</td>
<td>(34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O&lt;sup&gt;6&lt;/sup&gt;-mdGuo</td>
<td>IDMS w/column switching</td>
<td>24 fmol O&lt;sup&gt;6&lt;/sup&gt;-mdGuo</td>
<td>SPE</td>
<td>rat</td>
<td>liver</td>
<td>(48)</td>
<td></td>
</tr>
<tr>
<td>O&lt;sup&gt;6&lt;/sup&gt;-methyl/O&lt;sup&gt;6&lt;/sup&gt;-ethyl-dG</td>
<td>IDMS w/column switching</td>
<td>0.03 O&lt;sup&gt;6&lt;/sup&gt;-Me-dG/10&lt;sup&gt;8&lt;/sup&gt; nts</td>
<td>Centrifugation</td>
<td>mouse</td>
<td>liver</td>
<td>(49)</td>
<td></td>
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<table>
<thead>
<tr>
<th>Exocyclic adducts</th>
<th>Adduct</th>
<th>Method</th>
<th>LOD</th>
<th>Sample Prep.</th>
<th>Species</th>
<th>Source</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td>Etheno guanine</td>
<td>IDMS</td>
<td>1 pg</td>
<td>SPE</td>
<td>human</td>
<td>urine</td>
<td>(51)</td>
<td></td>
</tr>
<tr>
<td>εA</td>
<td>IDMS</td>
<td>2-3 pg</td>
<td>SPE</td>
<td>human</td>
<td>urine</td>
<td>(52)</td>
<td></td>
</tr>
<tr>
<td>ε,N&lt;sup&gt;2&lt;/sup&gt;-propano-dG</td>
<td>IDMS</td>
<td>0.015 fmol/µg DNA</td>
<td>Filtration, SPE</td>
<td>rat</td>
<td>liver</td>
<td>(54)</td>
<td></td>
</tr>
<tr>
<td>Propane-dG</td>
<td>IDMS</td>
<td>4 adducts/10&lt;sup&gt;9&lt;/sup&gt; nts</td>
<td>SPE</td>
<td>human</td>
<td>lung</td>
<td>(55)</td>
<td></td>
</tr>
<tr>
<td>Cro-dGuo</td>
<td>IDMS</td>
<td>0.2 fmol</td>
<td>SPE</td>
<td>human</td>
<td>liver/lung</td>
<td>(56)</td>
<td></td>
</tr>
<tr>
<td>PdG, Et-dG</td>
<td>IDMS</td>
<td>250 fg</td>
<td>Liquid extraction</td>
<td>human</td>
<td>blood</td>
<td>(57)</td>
<td></td>
</tr>
<tr>
<td>1,N&lt;sup&gt;2&lt;/sup&gt;-εGuo</td>
<td>IDMS</td>
<td>20 fmol</td>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt; extraction</td>
<td>rat</td>
<td>liver</td>
<td>(36)</td>
<td></td>
</tr>
<tr>
<td>εC εdA εAde</td>
<td>Column switching</td>
<td>70 pM dAde, 100 pM dC, 17 pM dA</td>
<td>SPE</td>
<td>human</td>
<td>urine</td>
<td>(58)</td>
<td></td>
</tr>
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</table>
Table 1.1 (continued from pp. 11-12)
Ethanol/acetaldehyde induced DNA adducts

<table>
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<th>Adduct</th>
<th>Method</th>
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<th>Sample Prep.</th>
<th>Species</th>
<th>Source</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td>N7-Ethyl-dG</td>
<td>IDMS</td>
<td>0.25 fmol</td>
<td>SPE</td>
<td>human</td>
<td>liver</td>
<td>(59)</td>
</tr>
<tr>
<td>N7-Ethyl-dG</td>
<td>column switching IDMS</td>
<td>0.59 pg/mL urine</td>
<td>SPE</td>
<td>human</td>
<td>urine</td>
<td>(60)</td>
</tr>
<tr>
<td>N2-ethyl-dGuo</td>
<td>IDMS</td>
<td>0.4 fmol</td>
<td>SPE</td>
<td>human</td>
<td>liver</td>
<td>(38)</td>
</tr>
<tr>
<td>N2-ethyl-Gua</td>
<td>IDMS</td>
<td>0.05 fmol on column, 3 adducts/10^9 nts</td>
<td>SPE</td>
<td>human</td>
<td>leukocytes</td>
<td>(61)</td>
</tr>
<tr>
<td>C8-HEG</td>
<td>IDMS</td>
<td>50 fmol</td>
<td>HPLC</td>
<td>rat</td>
<td>liver</td>
<td>(62)</td>
</tr>
</tbody>
</table>

Polyaromatic hydrocarbon DNA adducts

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Method</th>
<th>LOD</th>
<th>Sample Prep.</th>
<th>Species</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-dGuo, MP-dAdo</td>
<td>IDMS</td>
<td>10 fmol/2 fmol in 100 µg DNA</td>
<td>SPE</td>
<td>rat</td>
<td>liver</td>
<td>(40)</td>
</tr>
<tr>
<td>dG-N²-BPDE</td>
<td>IDMS</td>
<td>NA</td>
<td>NA</td>
<td>mouse</td>
<td>liver, lung, kidney</td>
<td>(63)</td>
</tr>
<tr>
<td>dG-N²-BPDE</td>
<td>IDMS column switching</td>
<td>1/10^8 nts</td>
<td>SPE</td>
<td>mouse</td>
<td>liver</td>
<td>(64)</td>
</tr>
<tr>
<td>dG-BPDE</td>
<td>IDMS column switching</td>
<td>1/10^9 nts</td>
<td>SPE</td>
<td>mouse</td>
<td>liver</td>
<td>(65)</td>
</tr>
<tr>
<td>BP-6-N7Gu</td>
<td>IDMS w/ column switching</td>
<td>2.5 fmol/1 mL urine</td>
<td>SPE</td>
<td>human</td>
<td>urine</td>
<td>(39)</td>
</tr>
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</table>
Table 1.1 (continued from pp. 11-13)

1,3-butadiene/epoxide

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Method</th>
<th>LOD</th>
<th>Sample Prep.</th>
<th>Species</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>THBG</td>
<td>IDMS</td>
<td>3.5 fmol</td>
<td>filtration</td>
<td>mouse/rat</td>
<td>liver/lung</td>
<td>(66)</td>
</tr>
<tr>
<td>HMVK-dGuo</td>
<td>IDMS</td>
<td>5 fmol/200 µg DNA</td>
<td>filtration, HPLC</td>
<td>rat</td>
<td>liver</td>
<td>(67)</td>
</tr>
<tr>
<td>N7-GA-Gua,</td>
<td>IDMS</td>
<td>1.5-2 adducts/10⁸ nts</td>
<td>filtration</td>
<td>mouse</td>
<td>liver/lung/kidney</td>
<td>(68)</td>
</tr>
<tr>
<td>N3-GA-Ade</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bis-N7G-BD</td>
<td>IDMS</td>
<td>6 adducts/10⁹ nts</td>
<td>filtration, offline HPLC</td>
<td>mouse/rat</td>
<td>liver, lung, kidney, brain, thymus</td>
<td>(69)</td>
</tr>
<tr>
<td>N7G-N6A-BD</td>
<td>nanoLC-nanoESI-MS/MS</td>
<td>3 adducts/10⁹ nts</td>
<td>Filtration, SPE</td>
<td>mouse/rat</td>
<td>liver</td>
<td>(70;71)</td>
</tr>
<tr>
<td>N7-HEG</td>
<td>IDMS column switching</td>
<td>0.25 ng/ mL urine</td>
<td>filtration</td>
<td>human</td>
<td>urine</td>
<td>(72)</td>
</tr>
<tr>
<td>N7-HEG</td>
<td>IDMS</td>
<td>0.1 fmol</td>
<td>filtration</td>
<td>rat</td>
<td>liver, heart, spleen, kidney, colon, stomach, lung</td>
<td>(73)</td>
</tr>
<tr>
<td>N7-HEG, N1-HedA</td>
<td>IDMS</td>
<td>0.5 fmol ea.</td>
<td>filtration, HPLC</td>
<td>rat</td>
<td>liver</td>
<td>(74)</td>
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Oxidative stress induced DNA adducts

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<tr>
<th>Adduct</th>
<th>Method</th>
<th>LOD</th>
<th>Sample Prep.</th>
<th>Species</th>
<th>Source</th>
<th>Ref.</th>
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<tr>
<td>8-oxo-dG</td>
<td>IDMS</td>
<td>2 fmol</td>
<td>Immunoaffinity column purification</td>
<td>mouse</td>
<td>prostate</td>
<td>(75)</td>
</tr>
<tr>
<td>8-oxo-dG/A</td>
<td>IDMS</td>
<td>10 fmol 8-oxodA</td>
<td>SPE</td>
<td>human</td>
<td>urine</td>
<td>(76)</td>
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<tr>
<td>8-OH-dG</td>
<td>IDMS</td>
<td>0.024 ng/mL</td>
<td>SPE</td>
<td></td>
<td></td>
<td>(77)</td>
</tr>
<tr>
<td>CEdG</td>
<td>IDMS</td>
<td>0.2 pg</td>
<td>SPE</td>
<td>rat</td>
<td>urine</td>
<td>(78)</td>
</tr>
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1.1.4. New approaches to improve analytical methods for DNA adduct analysis

Several novel approaches in mass spectrometry have recently been developed for quantitation of DNA adducts in vivo. In this section we will discuss three new approaches: nanospray ionization, column switching, and Chip-MS. Nanospray MS improves sensitivity of HPLC-MS methods by lowering HPLC flow rates (100 – 500 nL/min), leading to a decreased chemical noise, a greater ionization efficiency, and a more complete sample transfer from HPLC to the ion source. Column switching methods couple sample clean-up with HPLC separation and MS analysis on-line, potentially eliminates offline cleanup steps, saves time, and prevents sample loss. Lastly, we will discuss chip-MS. Chip-MS is the use of a microchip with electrospray needles or nano columns embedded on it. This method also increases sample throughput as it eliminates the HPLC component of MS, and samples are infused directly through a nanospray needle into the mass spectrometer (79).

Capillary HPLC-nanospray ionization mass spectrometry (capLC-nanoESI) involves using a capillary HPLC column (0.3-0.8 mm I.D.) and flow rates of 5-10 µL/min with a flow splitter and a nanoelectrospray ion source. Liu et al. have recently developed capLC-nanoESI methods for quantitation of two different DNA adducts (80;81). The first method was employed for monitoring acrolein-dG (Acro-dG) adducts in DNA from human brain tissue. Acro-dG is formed from reaction of a lipid peroxidation product, hydroxyl-2-nonenal (80). Lipid peroxidation products have been found in increased amounts in tissues of subjects with Alzheimer’s disease (80). Therefore, DNA from brain tissue of Alzheimer’s patients and matched controls were compared for Acro-dG levels. An UltiMate capillary/nano LC system was interfaced
with a Bruker Daltonics high capacity ion trap (Bruker nanospray source, 50 µm i.d. tapered stainless steel emitter). Using these methods, the LOD for Acro-dG was 31 amol, which enabled them to observe a difference in adduct levels in brain of Alzheimer Disease (AD) patients and the corresponding controls (5150 ± 640 as compared to 2800 ± 460 Acr-dG/10^9 nts) (80). The second method was used to measure HNE-derived cyclic 1, N^2-propanodeoxyguanosine adducts (HNE-dG) formed from the lipid peroxidation product trans-4-hydroxy-2-nonenal (HNE) in AD brain tissue. The same capLC-nanoMS system described above was used. The LOD was 24 amol for pure HNE-dG standard, or 40 adducts/10^9 nts in matrix (81). It had previously been shown that HNE levels were elevated in brains of AD patients, thus the authors compared HNE-dG levels in control and AD brain samples. No statistically significant differences were observed; however, thus HNE-dG may not be a useful biomarker of oxidative stress in AD.

A capLC-nanoESI-IDMS/MS method has also been developed for quantitation of estrogen-modified adenine adducts in human breast tissue (43). Cytochrome P450 metabolism of estrone leads to the formation of 3,4-catechol-estrogen quinone, which can react directly with DNA to form 4-hydroxyestrogen-2-N7-guanine (4-OHE2-N7Gua) and 4-hydroxyestrogen-1-N3-adenine (4-OH-E1-N3-Ade) adducts. The authors (43) employed an LCQ Deca quadropole ion-trap mass spectrometer (ThermoFisher) interfaced to a capillary HPLC (Waters Corp.). A custom packed Luna column (3 µm, 120 mm x 75 µm) with a 15 µm PicoFrit nano tip was used for analyses. The initial flow rate (8 µL/min) was split to achieve a flow rate of 270 nL/min at the tip. The LOD of the method was 5 fmol 4-OH-E1-N3-Ade/g tissue. DNA was extracted from tissue,
and the enzymatic digest was enriched by SPE and offline HPLC prior to capLC-nanoESI-MS/MS analysis. Breast tissues from 6 patients (3 controls, 3 breast cancer patients) were analyzed. 4-OH-E₁-N₃-Ade was detected in all 6 samples; however, the study size was not large enough to determine whether 4-OH-E₁-N₃-Ade levels are greater in cancer patients than in controls.

Nanoflow HPLC-nanospray ionization mass spectrometry (nanoLC-nanoESI) uses nanobore HPLC columns (0.025 – 0.1 mm I.D. eluted at flow rates of 100-500 nL/min) with nanoelectrospray source. Currently, nanoLC-nanoESI is used mainly for proteomics (82). However, Embrechts et al. have used nanoLC-nanoESI-MS/MS for analysis of estrogen-DNA adducts, 17α-ethynylestradiol-2′-deoxyadenosine, estrone-2′-deoxyguanosine, equilenin-2′-deoxyguanosine, and estradiol-2′-deoxyguanosine, in human breast tumor tissue. The LOD was 200 fg; however, they did not use stable isotope internal standards for quantitation (83).

Our laboratory has recently developed quantitative nanoLC-nanoESI methods for the analysis of 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD) a DNA cross-link of 1,3-butadiene in mouse liver DNA (Chapter III, (71)). A 10-fold increase in sensitivity was observed with nanoESI over capLC-ESI-MS/MS methods (3 adducts/10⁹ nts compared to 30 adducts/10⁹ nts). Using this methodology, we were able to detect N7G-N1A-BD in DNA from mice exposed to 62.5 ppm BD (71).

Quantification of DNA adducts in biological samples can be difficult due to MS signal suppression by sample matrix (salts, proteins, unmodified nucleosides). Therefore, extensive sample processing is required prior to injection on an analytical column for LC-MS analysis. Examples of sample clean-up include size exclusion
filtration, liquid/liquid extraction, solid phase extraction, and offline HPLC. This extra processing of samples can lead to significant sample loss and can be very time consuming. Column switching methods can avoid sample loss and increase sample throughput.

Column switching involves loading of the sample onto a trap column and washing away salts/contaminants. Following valve switching, the sample is back-flushed onto an analytical column for separation and MS analysis. Column switching has been successfully used by several laboratories to quantify various DNA adducts (48;53;84-86). Our laboratory has also recently developed a column switching HPLC-ESI-MS/MS method for quantification of an exocyclic DEB-dA adduct in mice exposed to 1,3-butadiene (Chapter IV).

In a recent review by Singh and Farmer, they state that development of chip-MS is the future of DNA adduct quantification because it uses small volumes and requires little sample processing (87). Chip-MS utilizes a silicon chip containing an array of ESI nozzles that allows for the direct infusion of a sample without an HPLC column. Advantages of this method are that it uses small volumes of sample, thereby decreasing the levels of matrix entering the mass spectrometer. A new ESI needle is also used for each sample, eliminating problems with carryover from high level analyte samples to low level samples. A major disadvantage is the absence of chromatography, which means that the analyte enters the MS system at the same time as the matrix, and matrix effects such as ion suppression and interference may occur. In some cases, simple liquid/liquid extraction of samples prior to chip-MS/MS can greatly reduce matrix effects (79).
Currently the majority of applications of chip-MS have been for the analysis of peptides, drugs, or drug metabolites (79;88). To our knowledge only the Vorous group at Northeastern University has used chip-MS analysis for quantitation of DNA adducts. The Vorous group first reported a Chip-based nano-LC/MS ion trap method for the quantitation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) induced DNA adducts (89). The same laboratory has recently used a small molecule chip for quantitative analysis of 4-ABP-dG adducts in DNA extracted from bladder tissue of rats exposed to 4-ABP (90). Their methods employ an Agilent LC/MSD XCT Ultra Ion Trap and Agilent small molecule Chip Zorbax 80SB-C18, 5 µm particle size. The Agilent Chip contains an enrichment column (40 nL) and analytical column (75 µm i.d., 43 µm in length). Two ions are isolated and fragmented ($m/z$ 435.4 $\rightarrow$ 319 and $m/z$ 444 $\rightarrow$ 328) corresponding to dG-ABP and dG-ABP-$d_9$. The limit of detection for this method is 2 dG-ABP/10$^8$ nts, requiring only 1.25 µg DNA. The method was used to quantify dG-ABP in bladder DNA from rats dosed with ABP by IP injection, dG-ABP was detected at levels starting at 80 adducts/10$^8$ nts in these samples.

1.1.5. Conclusions and relevance of IDMS to this thesis

IDMS-MS is a sensitive and specific method for quantification of DNA adducts. It is superior to previous methods such as $^{32}$P-post-labelling and fluorescence because it provides structural information about the analyte. Furthermore, the use of stable isotope labeled internal standard makes the method specific, accurate, and reproducible. Recent improvements in electrospray mass spectrometry (i.e. the development of nano columns/needles, chip-cubes) have increased the sensitivity of MS methods to levels
approaching the sensitivity of $^{32}$P-postlabeling methods. Advancements in column switching have led to cleaner samples, resulting in less suppression, low quantitation limits, and high throughput. The increased sensitivity observed with nanospray methods suggests the use of nanospray for quantitation of DNA adducts shows great promise for quantitation of low abundance adducts and human samples. Furthermore, the use of Chip-MS, which combines column switching and nanospray MS, reduces time spent per sample, minimizes sample loss, increasing sensitivity. While we have presented many methods in this review that are able to measure DNA adducts \textit{in vivo}, mainly in animal studies, future advancements in instrumentation (nanospray, Chip-MS) will surely lead to increased sensitivity of current methods for quantification of DNA adducts for human risk assessment.

1.2 1,3-Butadiene Overview

1,3-butadiene (BD) is an important industrial chemical used in the production of plastics and rubber, with a global demand of 9 million metric tons in 2004 (91). BD is also an environmental pollutant present in automobile exhaust and cigarette smoke (20 – 75 µg/cigarette) (2;92). It is classified as a carcinogen based on animal studies in which laboratory rodents exposed to BD by inhalation develop tumors (93;94), and epidemiologic evidence suggesting that human BD industry workers have an increased incidence of leukemia (95). For nearly forty years research has been conducted to discover the mechanisms of toxicity and tumorigenicity of BD.

1.2.1 Metabolism of 1,3-Butadiene
BD is metabolically activated by cytochrome P450 monooxygenases (P450) to several reactive electrophiles. As shown in Scheme 1.1, BD is first oxidized by P450 to 3,4-epoxybutene (EB), which can either be detoxified by way of epoxide hydrolase (EH) to 1-butene-3,4-diol or further oxidized to 1,2,3,4-diepoxybutane (DEB). DEB can also be hydrolyzed, forming 3,4-epoxy-1,2-butanediol (EBD). All three epoxide-containing metabolites of BD (EB, DEB, and EBD) can react with nucleophilic sites in biomolecules, including DNA and proteins, forming covalent adducts. DEB, while not the major metabolite formed, is considered the ultimate carcinogenic species of BD as it is by far the most mutagenic and genotoxic (96;97). DEB is 50 times more mutagenic than EB and 100 times more mutagenic than EBD (96;97). The mutational spectra of human TK6 cells exposed to DEB reveal a high occurrence of A → T transversion mutations, as well as large deletions (98). In contrast, EB induces primarily GC → AT and AT → TA point mutations and requires exposure concentrations 100 fold greater concentration than that of DEB (98). Bone marrow of Laci mice exposed to 625 – 1250 ppm BD exhibited primarily AT → GC or AT → TA point mutations (98;99). The similarities of mutational spectra of DEB and BD provide further evidence that DEB is the ultimate carcinogen of BD capable of inducing mutagenic and genotoxic events associated with BD exposure (98). Similar results were observed at the hprt locus of T-lymphocytes of mice and rats exposed to BD metabolites. DEB-exposed animals and BD-exposed animals had similar levels of mutation frequencies, while EB-exposed animals had lower levels of mutation frequencies (100).

The potent toxicity and mutagenicity of DEB have been attributed to the presence of two epoxide rings in its structure, leading to the formation of DNA-DNA
and DNA-protein cross-links (Scheme 1.2). Upon initial reaction of DEB with DNA, one of the epoxide rings alkylates the N7 position of guanine nucleobases, forming 2-hydroxy-3,4-epoxybut-1-yl (HEB) monoadducts. HEB monoadducts can be either hydrolyzed to trihydroxy-butyl (THB) monoadducts, or can react with another nucleophilic site in DNA to form interstrand and intrastrand cross-links, and exocyclic nucleobase adducts (Scheme 1.2). DNA-DNA cross-links and exocyclic lesions of DEB are hypothesized to be the cytotoxic and pro-mutagenic adducts responsible for the adverse biological effects of BD exposure.
Scheme 1.1 Metabolism of 1,3-butadiene to DNA reactive electrophiles.
Scheme 1.2 DEB reactions with DNA to yield bifunctional DNA adducts.
1.2.2 Effects of stereochemistry on genotoxicity of 1,2,3,4-diepoxybutane.

All three possible stereoisomers of DEB (S,S; R,R; and meso) are formed metabolically (Chart 1.1). Interestingly, these optical isomers have varying toxicity in animals and in human cell lines. For example, in mouse skin painting studies S,S + R,R DEB was more carcinogenic than meso DEB (101). Also, in the supF gene (S,S)-DEB induced the greatest level of mutations (9.8 fold increase over controls), compared to 4x and 5-fold increases by (R,R) and meso DEB, respectively (102). The majority of supF mutations observed were single base substitutions, including A:T \rightarrow T:A transversion mutations (102), consistent with the mutation spectra of human lymphoblast TK6 cells treated with DEB and laci bone marrow mutation spectra of mice exposed to BD (98).

Based on DNA renaturation studies of DNA individually treated with the three isomers of DEB, (S,S)-DEB formed the greatest level of interstrand DNA cross-links, followed by (R,R)-DEB and meso DEB (103). Similar results were observed in DEB-treated T7 coliphage (104), and in restriction fragments (105). Treatment with (S,S)-DEB inactivated phages more efficiently than (R,R) or meso DEB, and induced the greatest number of interstrand cross-links (104). Interstrand DNA-DNA cross-links are thought to be more cytotoxic than the corresponding intrastrand lesions as the former affect both strands of DNA preventing DNA transcription, replication, and repair. Therefore, the observation of more efficient interstrand cross-linking by S,S-DEB (106) is likely a reason for its increased toxicity. Interestingly, S,S-DEB is considered the active metabolite of the chemotherapeutic agent Treosulfan used to treat ovarian cancer (107).
Chart 1.1 Stereoisomers of 1,2,3,4-diepoxybutane.
1.2.3 Species and Gender Differences in Metabolism and Toxicity of BD

Animal studies have shown striking differences in species susceptibility to BD-induced tumor formation. For example, in chronic BD exposure studies, mice developed tumors at 6.25 ppm BD, while rats did not develop tumors until exposures reached 1000 ppm BD (93;94). These remarkable interspecies differences in susceptibility to BD-induced cancer are believed to be due to differences in the extent of formation of DEB in rat and mouse tissues. Microsome studies looking at metabolism of BD by mouse, rat, and human microsomes have shown that mice indeed form greater levels of DEB than both rat and human microsomes (108). This is due to a greater rate of oxidation of BD to EB and EB to DEB in mouse tissues, as well as a slower rate of hydrolysis of EB and DEB in mice as compared to rat and human microsomes (108). The rate of P450 oxidation of BD to EB and EB to DEB is roughly 3.5-fold faster in mice as compared to rats (108). Furthermore, the rate of epoxide hydrolase hydrolysis of EB to 1-butene-3,4-diol is 2.7-fold faster in rats as compared to mice (108).

There is also evidence that rat cytochrome P450 2E1 monooxygenase, the major isoform of mixed function monooxygenase responsible for BD metabolism to EB and DEB, can be inactivated by phosphorylation of Ser residues by PKA/cAMP (109) or by covalent binding of BD metabolites, specifically EB, to Tyr and His residues within its active site (110). If this inactivation is less common in the mouse, it could be another explanation for why BD-exposed rats form less DEB than mice, and are less susceptible to BD induced cancer.
BD inhalation studies have also shown gender differences in BD metabolism and carcinogenicity, although those were not as pronounced as interspecies differences. Female mice developed lung tumors after exposure to 6.25 ppm BD, whereas male mice did not form tumors until exposures to 62.5 ppm BD or higher (111). In another study, female rats exposed to a single 6 hour exposure of 62.5 ppm BD had 4.75-fold higher blood concentrations of DEB than did male rats that received the same dose (112). Similarly, female mice had greater DEB concentrations in blood, femur, lung and fat tissue as compared to male mice receiving the same BD exposure (112).

A possible cause of the observed gender differences in BD metabolism is differences in hormone levels between the genders. The enzymes involved in the metabolism of BD, P450 monooxygenases and epoxide hydrolase (EH) (Scheme 1.1) are regulated by endogenous hormone levels. P450s are responsible for the production of DEB as it oxidizes BD to EB, and EB to DEB (Scheme 1.1). There is evidence that P450s are regulated by growth hormone levels. Growth hormone levels vary between the sexes. While females maintain a constant level of growth hormone, males release bursts of growth hormone that drop to zero between bursts (113). For this reason, some isoforms of cytochrome P450 (CYP2C12) are expressed only in females only, while others (CYP2C6 and CYP2A1) are expressed in both sexes, but are more abundant in females (113). An increased level of expression of BD-oxidizing enzymes in females as compared to males may be responsible for the observed differences in toxicity of BD between the sexes (113).

Another key enzyme involved in the metabolism of BD is epoxide hydrolase (EH), which is involved in detoxification of BD epoxides to the corresponding diols.
Testosterone has been observed to stimulate cytosolic EH expression \( (114;115) \), which could lead to more efficient detoxification of DEB in male animals. In general, there is greater activity of Cytochrome P450 monooxygenases in female mice, and a greater activity of epoxide hydrolase in male mice. The end result is an increased formation and decreased hydrolysis of reactive BD metabolites in female mice, leading to greater overall levels of DEB greater in females than in males. Similar results were observed for rats, where following BD exposure, blood of female animals contained higher concentrations of DEB as compared to males \( (116) \). While no differences in \( hprt \) gene mutations have been observed between occupationally exposed female and male butadiene workers \( (117) \), future studies are needed to see whether gender differences in biomarker levels are observed in humans.

### 1.3 Previous Biomarkers of 1,3-Butadiene Exposure and Metabolism

Over the past 10 years, several DNA and protein adducts of BD metabolites have been developed as biomarkers of BD exposure and metabolic activation to reactive epoxides. Quantification of biomarkers has been performed by radioactive scintillation counting, \( ^{32}P \)-postlabelling DNA, and isotope dilution mass spectrometry. These studies are briefly reviewed below.

#### 1.3.1 DNA-BD monoadducts

Several N7-guanine DNA monoadducts of BD, N7-(2,3,4-trihydroxybut-1-yl)guanine, N7(1-hydroxymethyl-2,3-dihydroxypropyl)guanine, and N7-(2-hydroxy-3-buten-1-yl)guanine (see Chart 1.2) have been quantified in laboratory animals exposed to \( ^{14}C \)-BD and \( ^{14}C \)-EB \( (118;119) \). Although somewhat greater adduct levels were
observed in mice compared to rats exposed to the same concentrations of BD, these differences cannot explain the ~100-fold greater sensitivity of mice to BD-induced cancer. Furthermore, because in these studies adduct concentrations were determined by measuring radioactivity of DNA and DNA hydrolysates, no structural information of the adducts was obtained.

Koivisto et al. employed $^{32}$P-postlabeling to quantify N7-G and, N1-A adducts (Chart 1.2) in laboratory animals exposed to 1,3-butadiene (120-124). N7-guanine adduct levels were ~100 fold higher than the concentrations of N1-adenine adducts. Yet Zhao et al. were still able to detect N1-(2,3,4-trihydroxybut-1-yl)adenine (N1-THBA) adducts in human lymphocytes obtained from 14 butadiene industry workers (124). 4.5 ± 7.7 adducts/10$^9$ nucleotides were observed in BD-exposed workers, while only 0.8 ± 1.2 adducts/10$^9$ nucleotides were measured in non-BD exposed individuals (124). The problem with using radiolabeled carcinogens and $^{32}$P-postlabeling analysis of DNA adducts is that these methodologies do not yield any structural information. Adducts are identified by HPLC peak retention times as compared to those of authentic standards. The absence of an internal standard limits the methods accuracy and precision.

N7-(2’,3’,4’-trihydroxybut-1’-yl)guanine (THBG) (Chart 1.2) adducts of BD have been quantified in rodents exposed to BD using isotope dilution mass spectrometry (125-127). These adducts can result either from guanine reactions with DEB, followed by hydrolysis of the second epoxide, or directly by guanine alkylation by EBD. EBD is much more abundant than DEB, and 98% of THBG observed in vivo is produced from the reaction of EBD with DNA (125). Furthermore, only a 2-fold difference in THBG
levels was observed between mice and rats (125), which is inconsistent with the 100 fold difference in mutagenicity among the two species and suggests that DNA adducts other than THBG monoadducts are responsible for the genotoxic effects of BD. Since DEB is the most mutagenic metabolite of BD, there is an urgent need for DEB-specific biomarkers to be used in risk assessment (128).
Chart 1.2  Structures of BD-DNA monoadducts
1.3.2 Protein Biomarkers

Several adducts to the N-terminal valine of hemoglobin have been developed as biomarkers of exposure to BD. For example, 1,2,3-trihydroxybutyl-valine (THB-Val) and 2-hydroxy-3-butenyl-valine (HB-Val) adducts (Chart 1.3) have been quantified in both laboratory animals and humans exposed to BD in an occupational setting. THB-Val and HB-Val were quantified by GC-MS with $^{13}$C$_5$-labeled THB-Val and $^{13}$C$_5$-HB-Val. The alkylated N-terminal valine of hemoglobin is selectively cleaved by Edman degradation, internal standards were added, and the acetylated pentafluorophenyl thiohydantoin derivatives were analyzed by GC-MS. A 4 fold greater level of THB-Val was observed in rats compared to mice exposed to DEB (300 µmol/kg for 24 h) (129). In contrast, similar levels of THB-Val were observed in both rats and mice exposed to EBD (129). In a separate study, rodents were exposed to butenediolic, and 2 fold greater levels of THB-Val were observed in mice as compared to rats (67). THB-Val has also been monitored in BD workers in China, however there was no significant difference in adduct concentrations between BD exposed (2-21 ppm BD) and control subjects (117).

Recently, a DEB-specific valine adduct, N,N-(2,3-dihydroxy-1,4-butadiyl)-valine (pyr-Val), has been developed as a biomarker (130). Unlike THB-Val/HB-Val, pyr-Val adduct is quantified as a peptide by HPLC-ESI-MS/MS. For these methods hemoglobin is digested with trypsin to release the N-terminal peptide (1-11), which is then spiked with a synthetic deuterated pyr-Val peptide (1-11) as an internal standard. The pyr-Val peptides are purified from the tryptic digest with immunoaffinity columns prior to HPLC-MS/MS analysis. Pyr-Val has been monitored in laboratory animals exposed to BD, and in blood from occupationally exposed workers (131). A 4-10 fold
greater concentration of pyr-Val was observed in mice as compared to rats at the same BD exposure \((130;132)\), which is consistent with tumorigenesis studies. However, the amounts of pyr-Val were below the LOQ (50 fmol on column) in globin obtained from BD-exposed workers \((0.397 – 0.808 \text{ mg/m}^3 \text{ BD}) (131)\). A benefit of using globin adducts as biomarkers is that it is easy to obtain blood samples for analysis. Because protein adducts are not repaired, they accumulate over the lifetime of red blood cells, allowing their ready detection. However, globin adducts are not involved in the mechanisms of mutagenesis and carcinogenesis and cannot be used to look at the extent of metabolite formation in target tissues. Furthermore, while protein adducts can be used as biomarkers of BD metabolism to DEB, they cannot be used to determine what DNA adducts are responsible for the mutagenicity of BD, or to investigate potential repair pathways for such adducts. To accomplish these goals, DEB-specific DNA biomarkers are required.
Chart 1.3 Structures of EB, EBD, and DEB adducts to the N-terminal valine of hemoglobin.
1.4 1,2,3,4-Diepoxybutane Specific DNA Adducts – *in vitro* studies.

1.4.1 Guanine-guanine-butanediol cross-links

As early as 1967 there was evidence that DEB formed DNA-DNA interstrand cross-links. Lawley and Brookes studied renaturation of thermally denatured DNA and found that DEB-treated DNA went back to a duplex structure faster than control DNA, suggesting that interstrand DNA-DNA cross-links were present. These authors further hypothesized that DEB formed di-(guanin-7-yl) adducts based on a decrease in renaturation rates with extended heating, which they attributed to depurination of the N7-guanine adducts (103). Over 30 years later, our laboratory was the first to structurally characterize the major DEB-induced DNA cross-link using modern NMR and mass spectrometry methods (133).

The major cross-linked lesions formed upon incubation of calf thymus DNA with DEB were identified as 1,4-*bis*-(guan-7-yl)-2,3-butanediol (bis-N7G-BD) (Chart 1.4). Structural conformation was made by NMR, UV spectroscopy, and mass spectrometry of authentic standards prepared synthetically (133). Capillary HPLC-ESI-MS/MS analysis of neutral thermal hydrolysates of DEB-treated DNA revealed a major peak that co-eluted with synthetic *bis*-N7G-BD and had the same UV and MS/MS spectra as the authentic standard. As mentioned earlier, the metabolic formation of DEB from BD is not stereospecific; three isomers, *R,R*; *S,S*; and *meso* DEB are formed (Chart 1.1), thus there are three isomers of *bis*-N7G-BD that can be formed (Chart 1.4). *In vitro* work shows that the stereochemistry of DEB determines which type of DNA-DNA cross-link will form (interstrand vs. intrastrand) (106). *S,S*-DEB primarily
induces interstrand cross-links; $R,R$-DEB forms significantly more interstrand than intrastrand cross-links, and $meso$-DEB forms equal numbers of interstrand/intrastrand bis-N7G-BD lesions \((106)\). The different types of cross-links also have varying stability. The half-life of interstrand bis-N7G-BD cross-links in DNA under physiological conditions is 147 h, while that of intrastrand cross-links is only 35 h due to spontaneous depurination of N7-alkylated guanines (Scheme 1.3) \((106)\). Interstrand cross-links are more toxic because they affect both DNA strands, preventing DNA transcription, replication, and repair. As seen in Scheme 1.3, the formation of an N7-guanine adduct creates a positive charge at the N7 of guanine, which destabilizes the glycosidic bond, leading to spontaneous depurination of the adduct \((4)\).
Chart 1.4 Three possible stereoisomers of bis-N7G-BD cross-links formed from the reactions of DEB isomers with DNA.
Scheme 1.3 Formation and spontaneous depurination of *bis*-N7G-BD.
1.4.2 Guanine-adenine-butanediol cross-links

Four regioisomers of guanine-adenine cross-links of DEB (G-A cross-links) have been identified from the reactions of DEB with DNA in vitro (134). These isomers contain a 2,3-butanediol bridge between the N7 position of guanine and different sites within adenine heterocycle (N1, N7, N3, or N6): 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD), 1-(guan-7-yl)-4-(aden-7-yl)-2,3-butanediol (N7G-N7A-BD), 1-(guan-7-yl)-4-(aden-3-yl)-2,3-butanediol (N7G-N3A-BD), and 1-(guan-7-yl)-4-(aden-6-yl)-2,3-butanediol (N7G-N6A-BD) (Chart 1.5). As with bis-N7G-BD, authentic standards were synthesized independently, and their structures were confirmed by NMR, UV, and MS. These authentic standards were used to identify G-A nucleobase adducts present in acid hydrolysates of DEB-treated DNA. N7G-N1A-BD was the most abundant G-A cross-link of DEB, followed by N7G-N3A-BD, N7G-N7A-BD, and N7G-N6A-BD (134). The relative ratios of N1A:N3A:N7A:N6A were 1:0.2:0.6:0.1 (134). Similar to the GG cross-links, adducts to the N3 and N7 of adenine carry a positive charge, destabilizing the glycosidic bond (4). Therefore, the N7G-N3A-BD and N7G-N7A-BD cross-links are not hydrolytically stable, with half-lives under physiological conditions of 31 and 17 h, respectively (134). While the mutagenicity of these adducts is unknown, it is likely that the hydrolytically stable adducts, N7G-N1A-BD and N7G-N6A-BD, are mispairing if not repaired.
Chart 1.5 Structures of the regioisomeric N7-guanine-adenine cross-links of DEB.
1.4.3 Guanine-Hypoxanthine-BD Cross-links

HPLC-ESI$^+$/MS/MS analysis of acid hydrolysates of DEB-treated DNA revealed additional lesions with a MW of 373 g/mol and MS/MS transitions at $m/z$ 374 $\rightarrow$ 223, 137, 152, consistent with the presence of hypoxanthine-guanine butanediol conjugates. N1-adenine adducts can undergo deamination to yield N1-hypoxanthine adducts (Scheme 1.4), thus we hypothesized that these were deamination products of N7G-N1A-BD, 1-(hypoxanth-1-yl)-4-(guan-7-yl)-2,3-butanediol (N7G-N1HX-BD). An authentic standard of N7G-N1HX-BD was prepared and characterized by NMR, UV and mass spectrometry (135). The authentic standard had identical HPLC retention time and MS/MS fragmentation as the nucleobase adduct observed in acid hydrolysates of DEB-treated calf thymus DNA, supporting adduct identity as N7G-N1HX-BD (135).
Scheme 1.4 Possible conversion pathways of N7G-N1A-BD to N7G-N^{6}A-BD by base-induced Dimroth rearrangement, and to N7G-N1HX-BD by deamination.
1.4.4 Exocyclic nucleoside adducts

Exocyclic dG adducts, 7-hydroxy-3-(2-deoxy-\(\beta\)-D-\textit{erythro}\-pentofuranosyl)-6-hydroxymethyl-5,6,7,8-tetrahydro-10\(H\)-pyrimido [1,2-\(\alpha\)]-purin-10-one (P4-1) and 7,8-dihydroxy-3-(2-deoxy-\(\beta\)-D-\textit{erythro}\-pentofuranosyl)-3,5,6,7,8,9-hexahydro-11\(H\)\-[1,3]diazepino-[1,2-\(\alpha\)]-purin-11-one (P6) (Chart 1.6), have been observed when dG is reacted with DEB \textit{in vitro} (136). Adducts at the N1 and the N\(^2\) of guanine should be hydrolytically stable in DNA, and are likely to interfere with normal Watson-Crick base pairing. Therefore, these adducts are potentially mutagenic if formed \textit{in vivo}.

However, to our knowledge P4-1 and P6 have not been observed in DNA treated with DEB, or in DNA from animals exposed to BD.

Because of the large numbers of mutations observed at A:T base pairs following exposure to DEB, our laboratory proposed that DEB induces strongly mispairing lesions on adenine nucleobases. Postoligomerization synthesis methods were utilized to prepare oligonucleotides containing site specific \(N^6\)-(2-hydroxy-3,4-epoxybut-1-yl)-2’-deoxyadenosine (\(N^6\)-HEB-dA) adducts (137). Upon purification of the \(N^6\)-HEB-dA containing oligomers, additional peaks were observed. These were subsequently characterized as exocyclic DEB-dA products, \(\bar{N}^6,N^6\)-(2,3-dihydroxy-1,4-butadiyl)-2’-deoxyadenosine (\(N^6,N^6\)-DHB-dA); \(1,N^6\)-(2-hydroxy-3-hydroxymethyl-1,3-propanodiy)-2’-deoxyadenosine (\(1,N^6\)-\(\gamma\)-HMHP-dA); and \(1,N^6\)-(1-hydroxymethyl-2-hydroxy-1,3-propanodiyl)-2’-deoxyadenosine (\(1,N^6\)-\(\alpha\)-HMHP-dA) produced by spontaneous cyclization of \(N^6\)-HEB-dA (Chart 1.6) (Seneviratne et al. submitted for publication). The \(1,N^6\)-HMHP-dA regioisomers were also observed in DEB-treated
DNA, while $N^\delta,N^\delta$-DHB-dA adducts were not. The mutagenicity of the exocyclic DEB-dA adducts remains to be established; however, other exocyclic $1,N^\delta$-dA adducts are highly mutagenic since the presence of the $1,N^\delta$ exocycle blocks normal Watson-Crick base pairing. For example, $1,N^\delta$-ethenoadenine ($\varepsilon$A) and $1,N^\delta$-ethanoadenine (EA) (Chart 1.7) are highly blocking to DNA polymerases, and induce $A \rightarrow T$ transversion mutations in human cells (7;11). Therefore, due to structural similarities, it is hypothesized that if formed *in vivo* $1,N^\delta$-HMHP-dA adducts are likely culprits of BD mutagenicity.
Chart 1.6 Structures of exocyclic nucleoside adducts of DEB, P4-1 and P6 (136), 1, $N^\alpha$-HMHP-dA and $N^\alpha$, $N^\alpha$-DHB-dA adducts (Seneviratne et al. submitted for publication).
Chart 1.7 Structures of exocyclic adenine adducts, 1,6-ethanoadenine and 1,6-ethenoadenine.
1.4.5 Mutagenesis of DNA adducts

The mutagenicity of \textit{bis-N7G-BD} adducts has not been investigated because of their rapid hydrolytic degradation and technical difficulties associated with preparing site-specifically modified DNA substrates. In contrast, hydrolytically stable intrastrand 1,4-\textit{bis-N2}-guanine-2,3-butanediol cross-links (N\textsuperscript{2}G-N\textsuperscript{2}G, Chart 1.8) have been incorporated into DNA substrates and replicated \textit{in vivo} (138). These site-specific mutagenesis studies have shown that the \textit{S,S N2G-N2G} cross-link is more mutagenic than the \textit{R,R} cross-link, inducing mostly G \rightarrow A transitions, but also G \rightarrow T and G \rightarrow C transversion mutations (138). While N\textsuperscript{2}G-N\textsuperscript{2}G intrastrand lesions of DEB are mutagenic, they block DNA polymerases, but cannot explain A \rightarrow T transversion mutations, the major mutation induced in human lymphoblast exposed to DEB. Unlike \textit{bis-N7G-BD}, N\textsuperscript{2}G-N\textsuperscript{2}G cross-links of DEB have not been detected \textit{in vivo}, or in DEB-treated DNA.

Site specific N\textsuperscript{6}-N\textsuperscript{6}-adenine intrastrand cross-links of DEB (1,4-\textit{bis-N6}-adenine-2,3-butanediol) (Chart 1.8) have also been incorporated into synthetic oligonucleotides (139). For these adducts, the \textit{R,R} isomer was more mutagenic than the \textit{S,S} isomer (139), which is inconsistent with other mutagenicity studies of DEB isomers. The N\textsuperscript{6}-N\textsuperscript{6}-adenine cross-links of DEB induced mainly A \rightarrow G mutations (40\%) and a small number of A \rightarrow C (9\%) and A \rightarrow T (5\%) mutations (139). Similar to the N\textsuperscript{2}G-N\textsuperscript{2}G intrastrand cross-links of DEB, the N\textsuperscript{6}-N\textsuperscript{6}-adenine intrastrand lesions have not been observed in DEB-treated DNA, and there is no experimental evidence for their presence in tissues of animals exposed to BD.
**Chart 1.8** Structures of synthetic DNA-DNA cross-links of DEB

1,4-*bis*-(\(\hat{N}^2\)guanine)-2,3-butanediol

1,4-*bis*-(\(\hat{N}^6\)adenine)-2,3-butanediol
Several DNA-BD monoadducts have been incorporated into oligomers for site specific mutagenesis studies (Table 1.2). $N^2$-(2,3,4-trihydroxybut-1-yl)-guanine adducts formed from $R,R$ or $S,S$-EBD blocked $E.coli$ polymerases, and were weakly mutagenic (only 1%) inducing $G \rightarrow T > G \rightarrow A > G \rightarrow C$ mutations (140). This data is inconsistent with BD/DEB mutation spectra. Similarly, an $N^6$-(2,3,4-trihydroxybut-1-yl) adenine adduct was shown to mispair with dG, leading to $A \rightarrow C$ mutations (141;142), also inconsistent with BD mutation spectra. An N1-inosine adduct of EB, N1-(1-hydroxy-3-buten-2-yl)-inosine was shown to be highly mutagenic, inducing $A \rightarrow G$ (80%), $A \rightarrow C$ (10 %), and $A \rightarrow T$ (8 %) mutations (139). A uridine, N3-(2-hydroxy-3-buten-1-yl)-deoxyuridine adduct of EB was shown to by highly mutagenic and blocking. Point mutations induced were $C \rightarrow T > C \rightarrow A > C \rightarrow G$, also inconsistent with BD mutation spectra (143).

In summary, studies to date have failed to uncover the mechanisms of DEB-induced mutagenesis, in particular, the structural basis for the observed $A \rightarrow T$ transversions.
### Table 1.2 Mutagenicity of site specific BD-DNA adducts

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Mutations</th>
<th>Blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-\textit{bis}-(N^2\text{-guanine})-2,3-butanediol</td>
<td>G $\rightarrow$ A (41 %), deletions</td>
<td>Yes</td>
</tr>
<tr>
<td>1,4-\textit{bis}-(N^6\text{-adenine})-2,3-butanediol</td>
<td>A $\rightarrow$ G (40 %)</td>
<td>No</td>
</tr>
<tr>
<td>N^2-(2,3,4-trihydroxybut-1-yl)-guanine</td>
<td>Weakly (1%) G $\rightarrow$ T</td>
<td>Yes</td>
</tr>
<tr>
<td>N^6-(2,3,4-trihydroxybut-1-yl)-adenine</td>
<td>A $\rightarrow$ C (0.25%)</td>
<td>No</td>
</tr>
<tr>
<td>N1-(1-hydroxy-3-buten-2-yl)-inosine</td>
<td>A $\rightarrow$ G (80%)</td>
<td>No</td>
</tr>
<tr>
<td>N3-(2-hydroxy-3-buten-1-yl)-dU</td>
<td>Highly C $\rightarrow$ T (53.4 %)</td>
<td>Yes</td>
</tr>
</tbody>
</table>
1.5 Summary and Thesis goals

In summary, BD is an important industrial chemical which is also present in urban air, leading to a widespread exposure of human populations. BD is classified as a known human carcinogen based on the results of epidemiological studies and animal inhalation data. Species and gender differences observed in susceptibility to BD tumorigenesis are believed to be due to differences in BD metabolism to its ultimate carcinogenic metabolite, DEB. Several DNA mono-adducts have been quantified in tissues of laboratory animals exposed to BD, however, they are not specifically formed by DEB, and do not correlate with cancer incidence. More recently a DEB-specific globin biomarker, N,N-(2,3-dihydroxy-1,4-butadiyl)valine, has been developed and employed in animal and human studies as a measure of DEB formation. However, hemoglobin adducts cannot provide information about tissue distribution of reactive metabolites. Furthermore, they do not directly quantify promutagenic DNA adducts. Our laboratory has identified several DEB-specific DNA-DNA cross-links and exocyclic deoxyadenosine adducts from in vitro reactions of DEB with deoxynucleosides and DNA. The goals of this thesis work were to develop quantitative HPLC-ESI\(^+\)-MS/MS methods for the quantitation of these adducts in DNA extracted from tissues of laboratory animals exposed to BD, and to investigate interspecies differences in the formation of DEB-specific adducts, their persistence, tissue distribution, and possible repair mechanisms. Taken together, our results provide important information about the mechanisms of BD-mediated genotoxicity in an animal model to facilitate human risk assessment for occupational and environmental exposure to BD.
II. QUANTITATIVE HPLC-ESI-MS/MS ANALYSIS OF N7-GUANINE-N7-GUANINE DNA CROSS-LINKS IN TISSUES OF MICE EXPOSED TO 1,3-BUTADIENE BY INHALATION


2.1 Introduction

1,2,3,4-diepoxybutane (DEB) (Scheme 1.1) is an important metabolite of 1,3-butadiene (BD), an industrial chemical and an environmental pollutant found in automobile exhaust and in cigarette smoke (92;144). BD is classified as “reasonably anticipated to be a human carcinogen” (U.S. Department of Health and Human Services) based on its multi-site carcinogenicity in laboratory animals and its pronounced genotoxic effects, including the induction of point mutations, large deletions, and chromosomal aberrations (93;95;145-148). In chronic inhalation experiments with B6C3F1 mice and Sprague-Dawley rats, BD was carcinogenic in both species (93;94). However, while mice developed tumors after chronic exposure to as little as 6.25 ppm BD (93), much higher concentrations (1,000 ppm) were required to induce tumors in rats (94). These interspecies differences in sensitivity have been attributed to differences in BD metabolism in mice and rats, in particular, the increased formation of DEB and other DNA-reactive metabolites in the mouse (149).

BD requires metabolic activation before it can exert its biological effects (150). *In vivo* metabolism of BD to DEB is catalyzed by cytochrome P450 2E1 and P450 2A6 monooxygenases (108;151;152). The first epoxidation step yields (R)- and (S)-3,4-epoxy-1-butene (EB) (151;152) (Scheme 1.1). EB can then be hydrolyzed to 1-butene-3,4-diol or can undergo a second oxidation to yield R,R; S,S; and meso-DEB (108).
(Scheme 1.1, Chart 1.1). Although DEB is a relatively minor metabolite of BD, experimental evidence suggests that it is responsible for many of the adverse effects of BD. DEB is 50-100 fold more genotoxic and mutagenic in human cells \textit{in vitro} than its monoepoxide analogues, EB and 3,4-epoxy-1,2-butanediol \textit{(96;153)}. Efficient metabolism of BD to DEB in target tissues of laboratory mice is thought to cause the increased susceptibility of this species to BD carcinogenesis \textit{(149)}. The widespread human exposure to BD, as well as its potent carcinogenic effects, warrant further investigation of the origins of interspecies differences in susceptibility to BD carcinogenesis.

While all three DEB stereoisomers are generated metabolically \textit{(108)}, biological studies reveal significant differences between the ability of \textit{R,R; S,S; and meso-DEB} to inactivate T7 coliphage \textit{(104)}, induce chromosomal aberrations \textit{(154)}, and cause mutagenesis in maize \textit{(155)}. Among the three stereoisomers, \textit{S,S} DEB exhibits the most potent genotoxicity and cytotoxicity, followed by \textit{R,R} and then \textit{meso} DEB \textit{(104;154;155)}. Furthermore, DEB stereoisomers induce different spectra of mutations induced in the \textit{supF} gene, suggesting stereospecific differences between the formation and/or repair of DNA adducts induced by the optical isomers of DEB \textit{(102)}.

The genotoxicity and cytotoxicity of DEB are thought to result from its ability to form bifunctional DNA adducts by sequentially alkylating two nucleophilic sites within the DNA duplex. DNA-DNA cross-linking by DEB was first discovered by Lawley and Brookes in the 1960s. These authors isolated \textit{N7}-guanine – \textit{N7}-guanine adducts from DEB-treated salmon sperm DNA \textit{(103)}. More recently, our laboratory employed modern spectroscopic methods to determine the chemical structure of these lesions,
which were identified as 1,4-\textit{bis}-(guan-7-yl)-2,3-butanediol (\textit{bis}-N7G-BD) (Chart 1.4) (133). Lawley and Brooks noted that DEB-treated DNA renatured more rapidly than control DNA, suggesting that interstrand DNA-DNA cross-links of DEB were formed (103). This was further confirmed by gel electrophoresis experiments of Millard and White (156). Interestingly, a strong sequence preference for interstrand DNA cross-linking between the N-7 positions of distal guanine nucleobases within 5’-GNC context was observed both in synthetic oligomers and in chromatinized restriction fragments (105;106;157;158). More recently, stable isotope labeling was employed to demonstrate that DEB produced both inter- and intrastrand cross-links (106). While S,S isomer specifically gave rise to interstrand lesions, \textit{meso} DEB induced equal numbers of intrastrand and interstrand cross-links (106). These findings are consistent with a greater cytotoxic effect of S,S DEB (104;154;155). In addition to guanine-guanine lesions, DEB can form adenine-guanine conjugates, e.g. 1-(aden-1-yl)-4-(guan-7-yl)-2,3-butanediol, 1-(aden-3-yl)-4-(guan-7-yl)-2,3-butanediol, 1-(aden-7-yl)-4-(guan-7-yl)-2,3-butanediol, and 1-(aden-N\textsuperscript{6}-yl)-4-(guan-7-yl)-2,3-butanediol (Chart 1.5) (134), exocyclic DNA lesions (159), and N7-2, 3, 4-trihydroxy-1-y1 adducts that result from hydrolysis of one of the two epoxy groups of DEB. The \textit{bis}-N7G-BD cross-links appear to be the most abundant bifunctional DNA lesions of DEB (133;134).

Because of the proposed key role of DNA-DNA cross-links of DEB in mutagenesis and carcinogenesis of BD, there is an urgent need for a specific biomarker of their formation and repair (160). Previous studies have analyzed N7-(2, 3, 4-trihydroxy-1-y1)guanine (THBG) adducts in DNA of BD-treated laboratory animals (125-127). However, THBG lesions cannot be used as a specific biomarker of BD
metabolism to DEB, because they are also formed by another, more prevalent metabolite of BD, 3,4-epoxy-1,2-butanediol (125;126). In the present work, capillary HPLC-ESI\textsuperscript{+}-MS/MS methods for quantitative analyses of bis-N7G-BD were developed and validated. These methods were used to analyze DEB-induced DNA-DNA cross-links in tissues of laboratory mice exposed to BD by inhalation. Our methods provide the first DEB-specific DNA biomarker available for studies of bifunctional DNA adduct formation and repair in vivo.

2.2 Experimental

**Note: DEB is a suspected human carcinogen and must be handled with adequate safety precautions.**

**Materials.** All solvents and chemicals were obtained from Sigma-Aldrich (Milwaukee, WI) unless specified otherwise. Bis-N7G-BD stereoisomers and their \textsuperscript{15}N\textsubscript{10} analogs were prepared at our laboratory as described previously (106;133). HPLC-ESI-MS/MS confirmed that the residues of unlabeled adduct in standard solutions were < 0.2%. Stock solutions of racemic and meso bis-N7G-BD and their internal standards were prepared in 0.1 mol/L HCl (20-30 µM) and stored at -20°C. The concentrations were periodically checked by UV spectrophotometry ($\varepsilon_{252} = 15 700$ at pH 1) and HPLC-ESI-MS/MS. Standard solutions were prepared from stock solutions by serial dilutions with 0.1 mol/L HCl and stored at -20°C.

**Animals and treatment.** C57BL/6 mice were exposed to 625 ppm BD by inhalation for five days (7 hours/day) at the Department of Preventive Medicine and Community Health at the University of Texas Medical Branch. All procedures were carried in accordance with our ACUC protocol 880202401. Mice were exposed in
Hinners-type stainless steel chambers (0.85 l/m3) to approximately 625 ppm BD for 7 hours per day, for 5 days (30 air volume changes/hour). Temperature and humidity were maintained at 24±1°C and 50±5% respectively. During exposures, animals were housed in individual stainless steel wire mesh cages within the chambers to ensure uniform delivery of the gas phase BD. BD, from a 99% pure source (Scott Specialty Gases; Pasadena, TX), was metered through a mass flow controller to achieve the desired concentration. Chamber concentrations were continuously monitored using gas chromatography (GC) and photoionization detection. Chamber atmospheres were continuously drawn through the GC sample loop and periodically introduced into the flow of N2 carrier gas using a computer-controlled, air-actuated valve. The concentration of BD was calculated by integrating the peak areas. The GC was calibrated at the beginning of each day using a 60 ppm BD certified standard (Scott Specialty Gases). Air controls were maintained in identical chambers. Animals were euthanized immediately following inhalation exposure period. Tissues including liver and lung were removed and flash frozen in liquid nitrogen. Frozen tissues were then transferred to a -80°C freezer prior to overnight shipment on dry ice to the University of Minnesota.

**DNA isolation.** The tissue was weighed (0.1 – 0.5 g) and homogenized in 10 mL of cold Tris-EDTA buffer. The nuclei were isolated by centrifugation at 2700 rpm for 15 min and re-suspended in lysis buffer. Following addition of RNase T1 and RNase A, the lysates were incubated at 37°C for 1 h. Proteinase K was added, and the resulting mixture was incubated for an additional 4 h at 37°C. DNA was extracted using phenol/chloroform extraction and ethanol precipitation according to previously
reported methodology (161). The isolated DNA was hydrated overnight, and the solutions were homogenized by shearing through a 22 gauge needle. The amounts and purity of DNA were determined by UV spectrophotometry (20 $A_{260} = 1$ mg /ml DNA). The $A_{260}/A_{280}$ ratios were found to be between 1.7 and 1.9, ensuring minimal protein contamination. Any samples exhibiting lower $A_{260}/A_{280}$ ratio were re-extracted by the same procedure.

**dG quantitation.** DNA was quantified by dG analysis in enzymatic hydrolysates. Approximately 10 µg of each DNA sample was dissolved in 70 µL of 10 mM ammonium acetate/1 mM ZnCl$_2$, pH 5.3 and digested with nuclease P1 (2.3 U) and alkaline phosphatase (10 U) at 37 ºC for 30 min. dG was quantified by HPLC-UV analysis on an Agilent Technologies model 1100 HPLC system (Wilmington, DE) incorporating a diode array detector and an autosampler. A Zorbax Eclipse XDB-C8 (4.6 x 150 mm, 5 µm) column (Agilent Technologies, Palo Alto, CA) was eluted with a gradient of 150 mM ammonium acetate (A) and acetonitrile (B). The solvent composition was kept at 100% A for 2.5 min and then linearly changed to 4.5 % B in 19 min, further to 30 % B in 3 min, and then maintained at 30 % B for 5.5 min. UV signal was monitored at 260 nm. Calibration curves were constructed by injecting known amounts of dG standard.

**DNA hydrolysis and sample preparation.** DNA samples (100 µg) were spiked with a mixture of racemic and meso $^{15}$N$_{10}$-bis-N7G-BD internal standards (500 fmol each) and subjected to neutral thermal hydrolysis (1 h at 70 ºC) to release bis-N7G-BD adducts (Scheme 2.1). The partially depurinated DNA was removed by Centricon YM-10 filtration (Millipore Corp., Billerica, MA). Bis-N7G-BD adducts were isolated by solid
phase extraction (SPE). Oasis MAX SPE cartridges (500 mg, 6 mL, Waters Corp., Milford, MA) were employed. Cartridges were prepared by washing with methanol (6 mL) and 0.2 N NaOH (6 mL) prior to loading samples in 0.1 M NaOH. Samples were washed with 0.01 mol/L NaOH (6 mL), 0.01 mol/L KOH in methanol (6 mL), water (2 mL), 1 mol/L ammonium acetate pH 6.8 (2 x 4 mL), water (2 mL), and 5% methanol (6 mL) prior to elution of bis-N7G-BD and its internal standard with 80% methanol/water (6 mL). SPE fractions containing bis-N7G-BD adducts were dried under nitrogen and dissolved in buffer A (25 µL) prior to analysis by capillary HPLC- ESI+-MS/MS. The injection volume was 8 µL.

**Capillary-HPLC-ESI+-MS/MS method.** An Agilent 1100 capillary HPLC system (Wilmington, DE) interfaced to a Thermo-Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific Corp., Waltham, MA) was used in all analyses. In our first set of experiments chromatographic separation was achieved with a Zorbax Extend C18 column (3.5 µm, 150 x 0.5 mm) eluted isocratically with 5.2% acetonitrile in 15 mM ammonium acetate, pH 5.5. The HPLC flow rate was 12 µL/min. The injection volume was typically 8 µL. With this solvent system, the retention time of racemic bis-N7G-BD was 5.9 min, while meso bis-N7G-BD eluted at 7.9 min (Figure 2.1). The mass spectrometer was operated in the positive ion mode, with nitrogen used as a sheath gas (5 L/min). Electrospray ionization was achieved at a spray voltage of 4.0 kV and a capillary temperature of 270°C. The mass spectrometer parameters were optimized for maximum response during infusion of standard solutions of bis-N7G-BD.
Scheme 2.1 Sample preparation for bis-N7G-BD analysis in DNA.
Bis-N7G-BD lesions were quantified by isotope dilution with racemic and meso $^{15}$N$_{10}$-bis-N7G-BD internal standards. Quantitative analyses were performed in the selected reaction monitoring (SRM) mode using HPLC-ESI$^+$-MS/MS peak areas corresponding to the loss of guanine from protonated molecules of bis-N7G-BD ($m/z$ 389.1 [M + H]$^+$ → 238.0 [M + H-Gua]$^+$ and $m/z$ 389.1 [M + H]$^+$ → 152.0 [Gua + H]$^+$) (Figure 2.1). Meso and racemic $^{15}$N$_{10}$- bis-N7G-BD internal standards were analyzed analogously using the transitions $m/z$ 399.1 [M + H]$^+$ → 243.0 [M + H-$^{15}$N$_5$-Gua]$^+$ and 399.1 [M + H]$^+$ → 157.0 [$^{15}$N$_5$-Gua + H]$^+$. Quantitative analyses were based on the area under the peak in the selected ion chromatogram corresponding to the analyte to the peak area of the internal standard (relative response ratios). Standard curves were constructed by analyzing solutions containing 0 – 1000 fmol racemic and meso bis-N7G-BD and 500 fmol racemic and meso $^{15}$N$_{10}$- bis-N7G-BD, followed by regression analysis of the relative response ratios calculated from HPLC-ESI$^+$-MS/MS peak area ratios corresponding to analytes and their internal standards (Figure 2.2).

**HPLC-ESI$^+$-MS/MS method validation.** For our original capillary HPLC-MS/MS method, the lower limit of quantitation for bis-N7G-BD diastereomers was determined to be 5 fmol racemic or meso bis-N7G-BD for pure standards and 10 fmol for spiked DNA (100 µg), determined as the lowest levels of bis-N7G-BD detectable with a signal-to-noise ratio of 3. A method calibration curve for the HPLC-MS/MS method was obtained by spiking mouse liver DNA (100 µg) with racemic and meso bis-N7G-BD standards (0-500 fmol) and the corresponding $^{15}$N$_{10}$-internal standards (500 fmol) (Figure 2.3). Analysis of three replicates at 10 fmol and 50 fmol was used to determine the accuracy and precision of the method (Table 2.1). Method accuracy was
calculated from the equation: \(\frac{100 \times C_m}{C_a}\) where \(C_m\) is the mean concentration measured and \(C_a\) is the actual concentration added. The coefficient of variation was calculated from the equation: \(\frac{100 \times SD}{C_m}\) where \(SD\) is the standard deviation.

**Method Optimizations.** Following analysis of mouse DNA samples with a LOD of 10 fmol/100 µg DNA, we tried to optimize methods for the future analysis of lower level exposures and rat DNA, which were expected to contain lower levels of bis-N7G-BD. We developed an offline HPLC method to purify bis-N7G-BD in DNA neutral thermal hydrolysates. The method employs a Zorbax Extend C18 column (4.6 x 150 mm, Agilent) eluted with a gradient of 0.4% formic acid (A) and acetonitrile (B) at 1 mL/min. The solvent composition was held at 0% B for 3 min, followed by a linear increase to 3% B in 10 min, and further to 40% B in 5 min. The column was equilibrated for 15 min before injections. dT and dA standards (0.5 µg each) are spiked into samples as retention time markers. The retention time of bis-N7G-BD determined with \(^{15}\text{N}_10\)-bis-N7G-BD (2 nmol) standards was 12.4 min, while dT eluted at 9.8 min, and dA eluted at 16.6 min. Three fractions (10.5 – 12 min, 12 – 13 min, and 13 - 14.5 min) were collected between dT and dA, with bis-N7G-BD eluting in fraction 2. The fractions were dried under vacuum, and dissolved in 0.05% acetic acid for HPLC-ESI-MS/MS analysis.

Improvements to the capLC-MS/MS method were also made. The same Zorbax Extend-C18 column was employed, however, 0.05% acetic acid (A) and methanol (B) were used as HPLC solvents. The optimized method employed a gradient from 0% B to 10% in 5 minutes, further to 50% B in 15 minutes, followed by returning to 0% B and equilibrating for 10 min before samples. Using these optimized methods, the LOQ was
1 fmol and 2 fmol *bis*-N7G-BD for pure standards and spiked DNA, respectively. Method validation was performed as described above for these methods, except that the control DNA (100 µg) was spiked with lower levels of *bis*-N7G-BD (5 fmol).
Figure 2.1  HPLC-ESI⁺-MS/MS analysis of racemic and meso bis-N7G-BD authentic standards (A) and ¹⁵N-labelled internal standards (B).
Figure 2.2  Calibration curves for racemic (A) and meso (B) bis-N7G-BD.
2.3 Results

2.3.1 Capillary HPLC-MS/MS method development.

We have developed HPLC-ESI$^+$-MS/MS methodologies to quantify bis-N7G-BD cross-links in DNA isolated from BD-exposed laboratory animals. In our approach, DNA is spiked with $^{15}$N-labeled racemic and meso bis-N7G-BD (internal standards for mass spectrometry) and subjected to neutral thermal hydrolysis to release N7-alkylguanine adducts (Scheme 2.1). Following Centricon ultrafiltration to remove partially depurinated DNA, bis-N7G-BD conjugates and their internal standards are purified via solid phase extraction on Oasis mixed mode cartridges, or by offline HPLC. Quantitative analysis of bis-N7G-BD diastereomers is then performed by capillary HPLC-ESI$^+$-MS/MS (Figure 2.1). The mass spectrometer is operated in selected reaction monitoring mode by following transitions corresponding to the loss of guanine base from protonated molecules of the adduct, $m/z$ 389.1 [M + H]$^+$ → $m/z$ 238.1 [M + H – Gua]$^+$, and the formation of protonated guanine, $m/z$ 389.1 [M + H]$^+$ → $m/z$ 152.1 [Gua + H]$^+$ (Figure 2.1). $^{15}$N$_{10}$-labeled internal standards are analyzed analogously using the transitions $m/z$ 399.1 → $m/z$ 243.1 and $m/z$ 399.1 → $m/z$ 157.1 [M + H – Gua]$^+$. While $S,S$ and $R,R$ bis-N7G-BD lesions (Chart 1.4) are enantiomeric and thus have identical HPLC retention times (5.9 min at our conditions), the meso adducts elute slightly later from the HPLC column ($t_R$, 7.9 min) (Figure 2.1). The HPLC-ESI$^+$-MS/MS responses were linear between 10 and 1000 fmol of each adduct, with $R^2$ values $> 0.9995$ (Figure 2.2). The detection limits for bis-N7G-BD diastereomers (5 fmol for standards, and 10 fmol for spiked DNA) are not as low as those for other DNA adducts analyzed in our laboratory (sub-fmol). This difference in sensitivity may be attributed
to the use of free nucleobases for bis-N7G-BD adduct analyses. Under ESI\(^+\)-MS/MS conditions, nucleoside adducts fragment much more readily than free nucleobases because of the facile cleavage of the glycosidic bond and the formation of protonated nucleobases. Unfortunately, bis-N7G-BD nucleosides are not amenable to quantitative analysis because of the intrinsic destabilization of the glycosidic bond when the N-7 position of guanine is alkylated, which leads to partial loss of deoxyribose during sample processing (Scheme 1.3).

### 2.3.2 Optimization of sample preparation.

Our original methodology employed C18 SPE cartridges; however, harsh elution conditions (0.1 mol/L HCl) were required to elute bis-N7G-BD from C18 packing. Therefore, we decided to use mixed phase SPE cartridges that combine reversed phase and anion-exchange chromatography separation (Oasis MAX). We found that large cartridges (500 mg capacity) were required for purification of mouse liver DNA hydrolysates (100 µg), since smaller cartridges (50 mg) did not have sufficient capacity for this sample size. Bis-N7G-BD cross-links are readily eluted from Oasis MAX columns with 80% methanol. Using the optimized SPE methodology, we consistently obtain 80-90% recovery of racemic and meso bis-N7G-BD standards spiked into DNA. Further modifications were made to sample processing to improve sensitivity; bis-N7G-BD was purified by offline HPLC clean-up, instead of SPE. HPLC-ESI-MS/MS solvents were changed to 0.05% acetic acid/methanol, instead of ammonium acetate/ACN to increase sensitivity. With this new method, we can detect 2 fmol bis-N7G-BD/100 µg DNA (6 adducts/10\(^9\) nts).
2.3.3 Method validation.

Control mouse liver DNA (0.1 mg) was spiked with 0-500 fmol bis-N7G-BD isomers and 500 fmol of the corresponding $^{15}$N$_{10}$-labelled internal standards, followed by the sample processing and HPLC-MS/MS analysis as employed for real samples (Scheme 2.1). We found that method detection limit was 15 fmol/0.1 mg DNA for racemic and meso bis-N7G-BD adducts (S/N ratio of 3 or better), or 2 fmol/0.1 mg DNA for samples processed by off-line clean-up prior to LC-MS/MS analysis. A good correlation was observed between the expected and measured levels of bis-N7G-BD in mouse liver DNA (Figure 2.3). The accuracy and precision determined for 50 fmol bis-N7G-BD were 110 ± 5% (racemic) and 83 ± 7% (meso) using the original methods (SPE and ammonium acetate capillary LC-MS/MS) (Table 2.1). For 5 fmol bis-N7G-BD analyzed by the optimized methods, we observed 87 ± 3% (racemic) and 74 ± 3% (meso) (Table 2.1).

2.3.4 In vivo analysis of bis-N7G-BD.

The original quantitative HPLC-MS/MS method was employed to analyze the formation of bis-N7G-BD cross-links in DNA extracted from liver and lung tissues of C57BL/6 mice exposed to 0 or 625 ppm BD for 5 days by inhalation. We found that liver DNA of treated mice contained 3.17 ± 0.35 racemic adducts per $10^6$ guanines, while DNA of control animals did not contain any bis-N7G-BD lesions (Figure 2.4). Lung DNA isolated from treated animals contained slightly lower amounts of racemic bis-N7G-BD (1.79 ± 0.54 per $10^6$ guanines). No meso bis-N7G-BD lesions were detected in either tissue (Table 2.2). Representative HPLC-ESI$^+$-MS/MS analysis chromatograms of a BD-exposed and control mouse DNA are shown in Figure 2.4. The
optimized methods have been used to quantify bis-N7G-BD lesions in rodents exposed to 0, 6.25, 62.5, 200, and 625 ppm BD for 10 days to obtain dose response curves (see Chapter V). With the optimized methods, we observe meso bis-N7G-BD in mice at exposures as low as 62.5 ppm BD (see Chapter V).
Figure 2.3  Method calibration curves for racemic (A) and meso bis-N7G-BD spiked into control DNA (100 µg) (B).
Table 2.1  Accuracy and precision of capillary HPC-ESI$^+$-MS/MS methods for \textit{bis-}N7G-BD spiked into control mouse liver DNA.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Racemic \textit{bis}-N7G-BD (%)</th>
<th>\textit{Meso} \textit{bis}-N7G-BD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 fmol (n = 4)</td>
<td>97.1 ± 20.8</td>
<td>92.1 ± 20.4</td>
</tr>
<tr>
<td>50 fmol (n = 3)</td>
<td>110.1 ± 5.3</td>
<td>82.6 ± 6.5</td>
</tr>
<tr>
<td>5 fmol (n = 4)</td>
<td>87.0 ± 3.3</td>
<td>74.3 ± 2.7</td>
</tr>
</tbody>
</table>
Figure 2.4 HPLC-ESI$^+$-MS/MS analysis of bis-N7G-BD in liver DNA from a female mouse control (A) and a mouse exposed to 625 ppm BD for 1 week (B).
Table 2.2 Levels of bis-N7G-BD levels in liver and lung DNA of mice exposed to 625 ppm BD for 1 week.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Racemic bis-N7G-BD /10⁶ Guanines</th>
<th>Meso bis-N7G-BD /10⁶ Guanines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Liver Control (N=5)</td>
<td>&lt; 0.05</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Mouse Liver 625 ppm BD (N=4)</td>
<td>3.17 ± 0.35</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Mouse Lung Control (N=4)</td>
<td>&lt; 0.05</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Mouse Lung 625 ppm BD (N=4)</td>
<td>1.79 ± 0.54</td>
<td>&lt; 0.1</td>
</tr>
</tbody>
</table>
2.4 Discussion

Specific biomarkers of BD exposure and metabolic formation of DEB are needed to help identify the origins of interspecies differences in the sensitivity to BD carcinogenesis and to evaluate human risk associated with BD exposure. Previous \textit{in vivo} analyses of DEB-DNA adducts have been limited to N7-(2,3,4-trihydroxybut-1-yl) guanine (N7-THBG) (125-127), N1-THB-Ade (162), N3-THB-Ade (127), and N6-THB-Ade (123). However, more recent studies revealed that the vast majority of N7-THBG lesions (~98%) are derived from another metabolite of BD, EBD (125), suggesting that THB monoadducts cannot be used as biomarkers of DEB (125;126;160). In contrast, \textit{bis}-N7G-BD cross-links (Chart 1.4) can only be derived from DEB and therefore can be used as specific biomarkers of metabolic activation of BD to DEB. However, because the amounts of cross-linked DNA lesions are low compared with the corresponding monoadducts, they are more difficult to detect \textit{in vivo}.

We have developed and validated sensitive and specific methods for the quantitative analysis of \textit{bis}-N7G-BD based on isotope dilution - capillary HPLC-ESI$^+$-MS/MS. Using our methods, the amounts of \textit{bis}-N7G-BD lesions were determined in liver and lung DNA of laboratory mice exposed to 625 ppm BD for a relatively short period of time (5 days, 7 h/day). Interestingly, higher levels of \textit{bis}-N7G-BD were detected in the mouse liver (3.17 adducts per $10^6$ guanines) DNA than in the lung (1.79 adducts per $10^6$ guanines). Our observation of DEB-induced DNA cross-links in the lung is not unexpected because mice were exposed to BD by inhalation, and BD may be metabolized in the lung tissue. Higher levels of P450s responsible for the metabolism of BD to DEB are present in the liver, explaining the higher levels of \textit{bis}-N7G-BD cross-
links in this tissue. Because DEB formed in the liver enters systemic circulation, further research is needed to determine the relative degree to which formation of this DNA-reactive intermediate in the liver versus the lung contribute to the total amount of DNA alkylation occurring in the lung. For example, Koc et al. observed similar levels of dG adducts induced by monoepoxide metabolites of BD in liver, lung, and kidney DNA of exposed mice (125). Our observation of DEB-induced DNA cross-links in mouse lung is significant because this is one of the major target tissues for BD carcinogenesis in this species (146).

The amounts of racemic bis-N7G-BD G-G cross-links found in liver DNA of BD-exposed mice are 4-5 fold lower than those previously reported for THBG monoadducts (126). This is consistent with the in vitro analyses of DEB-treated calf thymus DNA, which detected higher amounts of dG monoadducts as compared with bis-N7G-BD cross-links (133). Although mice form more DEB upon BD metabolism than rats do, DEB is a minor metabolite of BD in both species as compared with much more prevalent EBD (130;163). Therefore, our observation of significant amounts of bis-N7G-BD in liver DNA of exposed mice suggests that G-G cross-links are more stable in vivo than the corresponding monoadducts. Later chapters will examine the persistence of bis-N7G-BD in animal tissues (see Chapter VI).

Several explanations can be proposed for the apparent absence of meso bis-N7G-BD cross-links in mouse DNA. Because our HPLC-ESI-MS/MS method is less sensitive for meso adducts compared with racemic bis-N7G-BD, the meso adducts may have escaped analysis, if formed in low amounts (<5% of the total bis-N7G-BD adducts). Meso bis-N7G-BD has been detected using the optimized methods (offline
HPLC and water/methanol solvents), levels were ~10% of racemic bis-N7G-BD (Chapter V). One possibility is that BD metabolism in the mouse produces greater amounts of (S,S)- and (R,R)-diepoxides as compared with meso DEB. For example, studies using cDNA-expressed human P450 monooxygenases have shown that individual P450s can generate meso and racemic forms of DEB in different molar ratios (164). In particular, P450 2A6 and 2E1 favor meso over racemic (2:1), whereas 2C9 forms equal levels of each (164). Alternatively, mice may detoxify meso bis-N7G-BD at a faster rate than racemic bis-N7G-BD. For example, epoxide hydrolase (EH), an enzyme that detoxifies DEB by hydrolyzing the epoxide ring to a diol (Scheme 1.1), has been reported to hydrolyze meso DEB at a faster rate than racemic DEB (164), potentially leading to smaller amounts of meso DEB available to cross-link DNA. In addition, diastereomeric bis-N7G-BD lesions may have different stability in DNA. Previous studies have shown that meso DEB forms both interstrand and intrastrand bis-N7G-BD, whereas (S,S)-DEB forms specifically interstrand bis-N7G-BD (106). The half-life for spontaneous depurination of intrastrand bis-N7G-BD (35 h) is much shorter than that for interstrand bis-N7G-BD (147 h) (106). Furthermore, depending on their type, bis-N7G-BD may be processed differently by DNA repair systems. In general, interstrand lesions are expected to be more difficult to repair because they affect both strands of the DNA duplex.

To our knowledge, this method represents the first DNA-based biomarker of DEB formation upon exposure to BD. Quantitative methods for DEB-specific hemoglobin adducts, N,N-(2,3-dihydroxy-1,4-butadiyl)-valine, have been reported (130). Although these protein adducts are specific to DEB exposure and can be used as
a biomarker of BD metabolism to DEB, hemoglobin adduct levels cannot predict the amounts of biologically relevant bifunctional DNA adducts of DEB that are formed and persist in target tissues.

The quantitative HPLC-MS/MS methods provided in this work provide the means to study accumulation, repair, and dose-response relationships of bifunctional DEB-DNA adducts in vivo. Previous investigators have noted the differences between BD metabolism by human, mouse, and rat microsomes (164;165). In particular, mice form DEB from butadiene monoepoxide at much faster rate than rats do (164), potentially leading to higher levels of bis-N7G-BD cross-links in the mouse. Furthermore, due to interspecies differences, there may be variations in the total amounts and the stereochemical identities of DEB generated in different species, the rates of DEB detoxification by epoxide hydrolase, and repair of bis-N7G-BD cross-links. The availability of specific biomarker of DEB available for binding to cellular DNA has made it possible to conduct studies analyzing interspecies variations in the metabolism of BD to DEB and the repair of bis-N7G-BD cross-links (see Chapters V and VI).
III. QUANTITATIVE HPLC-ESI-MS/MS METHODS FOR THE ANALYSIS OF GUANINE-ADENINE CROSS-LINKS IN MICE EXPOSED TO BUTADIENE


3.1 Introduction

1,3-Butadiene (BD) is a large volume industrial chemical commonly used in the rubber and plastics industries, for example, the manufacturing of styrene-butadiene rubber and tires (91). The worldwide demand for BD in 2004 was approximately 9 million metric tons (91). BD is also an environmental toxin present in automobile exhaust and in cigarette smoke (20-75 µg per cigarette in mainstream smoke, 205-360 µg in sidestream smoke) (2;92). According to recent toxicological risk analyses, BD has the highest cancer risk index among all tobacco constituents (2). BD is classified as a human carcinogen based on “sufficient evidence” in humans of an increased risk for leukemia, its multi-site carcinogenicity in laboratory animals, and its pronounced genotoxic effects, including the induction of point mutations, large deletions, and chromosomal aberrations (93;95;145-148). Because of the widespread exposure from the environment or occupational exposure, there is a need for biomarkers to assess human risk associated with exposure.

Chemical modification of genomic DNA by the epoxide metabolites of BD is considered an early critical event in its carcinogenic mechanisms (150). Upon metabolic activation, BD is first oxidized to 3,4-epoxy-1-butene (EB), which can be further oxidized to 1,2,3,4-diepoxybutane (DEB) or can be metabolized by epoxide hydrolase to form 1-butene-3,4-diol (108;151;152;163) (Scheme 1.1). DEB can also be
hydrolyzed to 3,4-epoxy-1,2-butanediol (EBD) (108) or further to the corresponding tetrol. The three epoxide metabolites of BD (EB, DEB, and EBD) are reactive electrophiles capable of binding to nucleophilic sites within biomolecules to form covalent adducts (160;166).

Although DEB is a relatively minor metabolite of BD, it is considerably more genotoxic and mutagenic than its monoepoxide analogues, EB and EBD (128;167). DEB is 50x more effective in inducing sister chromatid exchanges and chromosomal aberrations in human lymphocytes than EB (97;168;169) and is two orders of magnitude more mutagenic than EB in TK6 lymphoblasts (96). The types of mutations induced by the two epoxides are distinct. While EB exposure results in base substitutions at GC basepairs (98), DEB induces deletions and point mutations at both AT and GC basepairs (145;170-172).

Animal studies revealed pronounced interspecies difference in sensitivity towards BD-induced cancer. In chronic inhalation experiments with B6C3F1 mice and Sprague-Dawley rats, BD was carcinogenic in both species (93;94). However, B6C3F1 mice developed tumors at BD exposure concentrations three orders of magnitude lower than those that cause cancer in Sprague-Dawley rats (93;94;149;173). Furthermore, the mutational spectrum found in BD-induced tumors of mice was different from that in rats (174). The increased susceptibility of mice to carcinogenicity of BD may result from a more efficient metabolic activation of BD to DEB in this species (149;167;175). Indeed, several studies have found that target tissues of BD-exposed mice, especially lung, contain significant levels of DEB (173). DEB specific N,N-(2,3-dihydroxy-1,4-butanediyl)-valine (pyr-Val) globin adducts were detected in mice treated with as little
as 3 ppm BD by inhalation (130). In contrast, formation of DEB in rat tissues is negligible, providing a possible explanation for the weak tumorigenic response to BD in this species (130;149;163;173;176). Consistent with this model, all three species are equally sensitive to the genotoxic effects of DEB when it is introduced directly into isolated rat, mouse, or human lymphocytes (168;169).

The ability of DEB to induce characteristic mutations and chromosomal aberrations has been attributed to its bifunctional nature. Because of the presence of two electrophilic epoxide groups in its structure, DEB can cross-link cellular biomolecules and form exocyclic DNA lesions. We have previously identified 1,4-\textit{bis}-(guan-7-yl)-2,3-butanediol (\textit{bis}-N7G-BD) as the major DEB-induced DNA-DNA cross-link and quantified this lesion in liver and lung DNA of mice that had been exposed to 625 ppm BD for 5 days (69). However, guanine-guanine cross-linking by DEB cannot explain the induction of AT base pair mutations following exposure to BD and DEB (98;102;177).

In addition to guanine-guanine butanediol conjugates, DEB has been shown to form four regioisomeric adenine-guanine cross-links, e.g. 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD), 1-(guan-7-yl)-4-(aden-3-yl)-2,3-butanediol (N7G-N3A-BD), 1-(guan-7-yl)-4-(aden-7-yl)-2,3-butanediol (N7G-N7A-BD), and 1-(guan-7-yl)-4-(aden-6-yl)-2,3-butanediol (N7G-N^6A-BD) (Chart 1.5) (134). Among these, N7G-N3A-BD and N7G-N7A-BD are thermally labile and can be spontaneously released from the DNA backbone under physiological conditions, while N7G-N1A-BD and N7G-N^6A-BD require acid hydrolysis to undergo depurination (134). In the present study, we report an HPLC-ESI+ MS/MS method for sensitive and accurate quantitation
of adenine-guanine cross-links of DEB in vitro and in vivo and its application to analyses of guanine-adenine (G-A) BD cross-links in liver DNA of BD-exposed and control female B6C3F1 mice. We also introduce a nanoflow-HPLC-nanoelectrospray ionization-MS/MS method for accurate and sensitive quantitation of low levels of G-A cross-links in vivo.

3.2 Experimental

Note: DEB is a known carcinogen and must be handled with adequate safety precautions.

Materials. Calf thymus DNA and racemic DEB were obtained from Sigma-Aldrich (St. Louis, MO). Meso-DEB, unlabeled N7G-N1A-BD, N7G-N3A-BD, N7G-N7A-BD, and N7G-N^6A-BD were synthesized as previously reported (134). \(^{15}\)N\(_3\), \(^{13}\)C\(_1\)Guanine was a gift from Roger Jones (Rutgers University). Stock solutions of N7G-N1A-BD, N7G-N3A-BD, N7G-N7A-BD, and N7G-N^6A-BD were prepared in 0.1 M HCl and stored at -20ºC.

Synthesis of \(^{15}\)N\(_3\), \(^{13}\)C\(_1\)-N7G-N1A-BD and \(^{15}\)N\(_3\), \(^{13}\)C\(_1\)-N7G-N3A-BD (internal standards for mass spectrometry). 2’-Deoxyadenosine (49.7 mg), \(^{15}\)N\(_3\), \(^{13}\)C\(_1\)-guanine (8.8 mg), and glacial acetic acid (1 mL) were combined in a microcentrifuge tube and heated to 80 ºC. Racemic DEB (3 µL) was added and the mixture heated at 80 ºC for 1 hour. The solution was cooled to 37 ºC and 5 volumes of ether:acetone (4:1) were added. The resulting white precipitate was brought up in 0.1 M HCl and hydrolyzed at 80 ºC for 1 hour. The products were purified by HPLC using a Luna C18 (4.6 x 150 mm) column. The cross-links were separated using a gradient from 0 to 6% acetonitrile
(B) in 20 mM ammonium acetate, pH 4.9 (A) in 6 minutes, and further to 10% B in 14 minutes. Under these conditions, $^{15}$N$_3, ^{13}$C$_1$-N7G-N1A-BD eluted at 9.8 minutes and $^{15}$N$_3, ^{13}$C$_1$-N7G-N3A-BD eluted at 10.5 minutes. Internal standard stock solution concentrations were determined by comparing LC-MS/MS peak areas with peak areas of analyte of known concentration.

**Animals and treatment.** B6C3F1 mice were purchased from Charles River Breeding Labs (Raleigh, NC, Portage, MI, or Hollister, CA) and were acclimated for about 10 days before initiation of chemical exposures. Animals were randomly separated into air-control and exposure groups by weight and were housed individually in hanging wire stainless steel cages according to NIH guidelines (NIH Publication 86-23, 1985). All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee.

Experimental animals were exposed using multi-tiered whole-body exposure chambers (H-2000, Lab Products, Aberdeen, MD). Rodents in one chamber received filtered air only as a control group, and rodents in the other chamber received nominal 625 ppm BD for 2 weeks (6 h/day, 5 days/week). Animals were housed within exposure chambers throughout the experiment, and had free access to food and water except for removal of food during the 6-h exposure periods. Within 2 hours after cessation of the final day of exposure, animals were euthanized via cardiac puncture, and tissues were harvested and snap-frozen for storage at −80°C.

**DNA isolation.** Liver tissue (0.1 – 0.5 g) was homogenized in Tris-EDTA buffer (10 mL), and DNA was extracted by reported methods (69). DNA purity and amounts
were determined by UV spectrophotometry. Typical \( A_{260}/A_{280} \) ratios were between 1.7 and 1.9, ensuring minimal protein contamination.

**DNA hydrolysis and sample preparation.** DNA samples (100 \( \mu \)g) were spiked with \(^{15}\text{N}_3, ^{13}\text{C}_1\)-N7G-N3A-BD (300 fmol) and subjected to neutral thermal hydrolysis (70 °C for 1 hour) to release thermally labile adducts (N7G-N7A-BD and N7G-N3A-BD) (Scheme 3.1). The partially depurinated DNA was removed by Centricon YM-10 filtration (Millipore Corp., Billerica, MA), while the filtrates containing N7G-N7A-BD, N7G-N3A-BD, and \(^{15}\text{N}_3, ^{13}\text{C}_1\)-N7G-N3A-BD were stored at –20 °C until further analysis. Partially depurinated DNA was recovered by reversing the filters and washing with water into new collection tubes. The partially depurinated DNA was spiked with \(^{15}\text{N}_3, ^{13}\text{C}_1\)-N7G-N1A-BD internal standard (300 fmol), and subjected to mild acid hydrolysis (0.1 M HCl at 70 °C for 30 min) to release all purine bases including N7G-N1A-BD and N7G-N\(^6\)A-BD. Following Centricon YM-10 filtration to remove the DNA backbone, pH was neutralized with NaHCO\(_3\) for solid phase extraction. Sep Pak C18 cartridges (100 mg, 1 mL, Waters Corp., Millford, MA) were equilibrated with methanol (1 mL) and water (3 x 1 mL) prior to loading samples in 0.05 M NaHCO\(_3\) (1 mL). The hydrolysates were washed with water (2 x 1 mL), and eluted with 50 % methanol (1 mL). The eluates were concentrated to 500 \( \mu \)L under vacuum and pH adjusted to 12 with 1 M NH\(_4\)OH (500 \( \mu \)L). The solutions were incubated at 70° C overnight to induce Dimroth rearrangement of N7G-N1A-BD adducts to the corresponding N7G-N\(^6\)A-BD species (Scheme 1.3). The NH\(_4\)OH was removed under vacuum and samples were dissolved in 0.05% acetic acid (25 \( \mu \)L) for HPLC- ESI\(^+\)-MS/MS analysis (injection volume, 8 \( \mu \)L).
Scheme 3.1  DNA hydrolysis and sample preparation for HPLC-ESI<sup>+</sup>-MS/MS analysis of guanine-adenine cross-links of DEB.
**HPLC-ESI⁺-MS/MS.** An Agilent 1100 capillary HPLC system (Wilmington, DE) interfaced to a Finnigan TSQ Quantum triple quadrupole mass spectrometer was used in all analyses. Chromatographic separation was achieved with a Zorbax SB-C18 column (150 x 0.5 mm, 5 µm) eluted at a flow rate of 15 µL/min. The solvent system consisted of 0.05% acetic acid (A) and methanol (B). A linear gradient from 3 to 9% B in 5 minutes and further to 24% B in 3 minutes was employed.

The regioisomeric G-A cross-links of DEB were quantified by isotope dilution with $^{15}$N$_3$, $^{13}$C$_1$-labeled internal standards. $^{15}$N$_3$, $^{13}$C$_1$-N7G-N3A-BD was used as an internal standard for quantitation of both N7G-N3A-BD and N7G-N7A-BD as they are both released from DNA by neutral thermal hydrolysis. $^{15}$N$_3$, $^{13}$C$_1$-N7G-N1A-BD was used as an internal standard for quantitation of both N7G-N1A-BD and N7G-N$^6$A-BD as these cross-links are analyzed in acid hydrolysates.

Quantitative analyses of DEB-induced G-A cross-links were performed using HPLC-ESI⁺-MS/MS peak areas corresponding to the loss of guanine ($m/z$ 373.1 [M + H]$^+$ → $m/z$ 222.1 [M + H – Gua]$^+$) or the loss of adenine and water ($m/z$ 373.1 [M+H]$^+$ → 220.0 [M+H-Ade-H$_2$O]$^+$) from protonated molecules of the adducts (M = 372). $^{15}$N$_3$, $^{13}$C$_1$-N7G-Ade-BD internal standards (M = 376) were analyzed analogously by following the transitions $m/z$ 377.1 [M + H]$^+$ → $m/z$ 222.0 [M+H-$^{15}$N$_3$, $^{13}$C$_1$-Gua]$^+$ and $m/z$ 377.1 [M+H]$^+$ → $m/z$ 224.0 [M+H-Ade-H$_2$O]$^+$ (Figure 3.1). Calibration curves were constructed by analyzing solutions containing known amounts of N7G-Ade-BD standards and $^{15}$N$_3$, $^{13}$C$_1$-GA internal standards (Figure 3.2).

**HPLC-ESI⁺-MS/MS method validation.** A method calibration curve was constructed by spiking control mouse liver DNA (100 µg) with known amounts of
N7G-Ade-BD cross-links (0 to 500 fmol) and isotopically-labeled internal standard (500 fmol). Three replicates at 5 fmol/100 µg DNA were also prepared to determine accuracy and precision of the capillary methods. Validation samples were processed and analyzed by HPLC-ESI$^+$-MS/MS in the same manner as actual samples on three separate days.
Figure 3.1  HPLC-ESI\(^+$\)-MS/MS analysis of thermally labile (A) and thermally stable G-A cross-links (B) and the corresponding isotopically labeled internal standards.
Figure 3.2 Calibration curves for thermally labile (A) and thermally stable G-A cross-links (B).
Calf thymus DNA treatment with DEB and sample processing. Calf thymus DNA (500 µg) in 10mM Tris-HCl, pH 7.2 was incubated with varying concentrations of racemic or meso DEB (0.05 – 1.0 mM in Tris-HCl, pH 7.2) in triplicate at 37 ºC for 24 hours. The reaction mixtures were extracted with diethyl ether (2 x 400 µL) to remove unreacted DEB. Internal standard, $^{15}$N$_3$, $^{13}$C$_1$-N7G-N3A-BD (25 pmol) was added, and samples were subjected to neutral thermal hydrolysis (70 ºC for 1 hour) to release N7G-N7A-BD and N7G-N3A-BD adducts. Partially depurinated DNA was removed by Centricon YM-30 ultrafiltration, and the filtrates containing N7G-N7A-BD and N7G-N3A-BD were concentrated to 100 µL under vacuum prior to HPLC-ESI$^+$-MS/MS analysis. The DNA was recovered from the filter, spiked with $^{15}$N$_3$, $^{13}$C$_1$-N7G-N1A-BD internal standard (25 pmol), and subjected to mild acid hydrolysis to release N7G-N1A-BD and N7G-N$_6$A-BD (0.1 M HCl at 70 ºC for 30 minutes). Samples were filtered through Centricon YM-30 filters, concentrated to 100 µL under vacuum, and analyzed by capillary HPLC-ESI$^+$-MS/MS.

N7G-N1A-BD stability studies. Authentic N7G-N1A-BD standard was dissolved in Tris-HCl buffer, pH 7.4 and incubated at 37 ºC for up to 72 hours. Aliquots were removed at various time points and immediately frozen. Samples were analyzed by HPLC-ESI$^+$-MS/MS on an Agilent 1100 Ion Trap (Agilent Technologies) using the same HPLC conditions as above. The MS was operated in MS$^2$ mode monitoring for m/z 373.1 [M + H]$^+$ for N7G-N1A-BD and N7G-N$_6$A-BD, and 374.1 [M + H]$^+$ for N7G-N1HX-BD. MS peak areas of N7G-N1A-BD, N7G-N$_6$A-BD, and N7G-
N1HX-BD were plotted versus time to determine stability of N7G-N1A-BD at physiological conditions (Figure 3.7).

**Nanoflow HPLC-nanoelectrospray ionization-MS/MS analysis of N7G-N6A-BD.** A Waters nanoAquity UPLC system (Waters Corp., Millford, MA) interfaced to a Thermo-Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific Corp., Waltham, MA) was used for these analyses. HPLC solvents were 0.01% acetic acid in water (A) and 1:1 LC-MS grade methanol/acetonitrile (B). Samples (4-8 µL) were loaded on a Symmetry C18 nanoAcquity trapping column (0.18 x 20 mm, Waters Corp.) for 1 min at 0% B. Chromatographic separation was achieved using an Atlantis C18 (75 x 100 µm, Waters Corp.) column eluted at a flow rate of 0.350 µL/min. A linear gradient program was employed from 0 to 12 % B in 2 minutes, further to 25% B over 7 minutes, and finally to 45% B over 15 minutes. The column was equilibrated at 0% B for at least 12 minutes before each run. Under these conditions, both racemic and meso N7G-N6A-BD eluted at 16.9 minutes (Figure 3.3). The mass spectrometer was operated in the selected reaction monitoring (SRM) mode by following mass transitions corresponding to the neutral loss of guanine from protonated molecules of the adduct:

\[ m/z \text{ 373.1} \to m/z \text{ 222.1} \ [M + H - \text{Gua}]^+ \ (\text{N7G-N6A-BD}) \] and

\[ m/z \text{ 377.1} \to m/z \text{ 222.1} \ [M + H - [^{13}\text{C}_1,^{15}\text{N}_3]\text{Gua}]^+ \ (^{13}\text{C}_1,^{15}\text{N}_3-\text{N7G-N6A-BD}) \].

These methods were validated as described above for capillary HPLC-MS/MS methods, except that replicates of 1 fmol/100 µg DNA were used for accuracy and precision, instead of 5 fmol.
Figure 3.3 NanoLC-nanoESI$^+$-MS/MS analysis of N7G-N\textsuperscript{6}A-BD and $^{15}\text{N}_3, ^{13}\text{C}_1$-N7G-N\textsuperscript{6}A-BD internal standard in liver DNA of a B6C3F1 mouse exposed to 625 ppm BD.
3.3 Results

3.3.1 Development of capillary HPLC-ESI\(^+\)-MS/MS methods for quantification of G-A DEB adducts. To enable sensitive and specific detection of guanine-adenine cross-links of DEB (Chart 1.5) in biological samples, isotope dilution HPLC-ESI\(^+\)-MS/MS methodology was developed. In our approach, G-A cross-links are quantified as nucleobase conjugates following their release from the DNA backbone by neutral thermal hydrolysis (N7G-N3A-BD, N7G-N7A-BD) or acid hydrolysis (N7G-N1A-BD and N7G-N6A-BD). Early in the analysis, DNA is spiked with \(^{15}\text{N}_3\), \(^{13}\text{C}_1\)-N7G-N3A-BD internal standard, followed by neutral thermal hydrolysis to release N7G-N7A-BD and N7G-N3A-BD adducts (Scheme 3.1). Centricon ultrafiltration is used to remove the partially depurinated DNA, which is then spiked with \(^{15}\text{N}_3\), \(^{13}\text{C}_1\)-N7G-N1A-BD internal standard and subjected to mild acid hydrolysis to release N7G-N1A-BD and N7G-N6A-BD. G-A BD lesions and their internal standards. The hydrolysates are purified by ultrafiltration and solid phase extraction on SepPak C18 cartridges. Quantitative analysis of the G-A BD regioisomers is performed by capillary HPLC-ESI\(^+\)-MS/MS. The mass spectrometer is operated in the selected reaction monitoring mode by following the transitions corresponding to the neutral loss of adenine base and water or guanine base from the protonated molecules of the adducts, \(m/z\) 373.1 [M + H]\(^+\) \(\rightarrow\) \(m/z\) 220.1 [M + H – Ade – H\(_2\)O]\(^+\) and \(m/z\) 373.1 [M + H]\(^+\) \(\rightarrow\) \(m/z\) 222.1 [M + H – Gua]\(^+\). \(^{15}\text{N}_3\), \(^{13}\text{C}_1\)-labeled internal standards are analyzed analogously using the transitions \(m/z\) 377.1 \(\rightarrow\) \(m/z\) 224.1 and \(m/z\) 222.1. \(^{15}\text{N}_3\), \(^{13}\text{C}_1\)-N7G-N3A-BD internal standard was used to quantify the depurinating G-A cross-links (N7G-N7A-BD and N7G-N3A-BD).
(Figure 3.1A), while $^{15}\text{N}_3$, $^{13}\text{C}_1$-N7G-N1A-BD internal standard was used to quantify the thermally stable G-A adducts (N7G-N1A-BD and N7G-N$^6$A-BD) (Figure 3.1B). Based on calibration curves, N7G-N7A-BD adducts had approximately 2-fold greater HPLC-ESI$^+$-MS/MS response as compared with N7G-N3A-BD (Figure 3.2A), while N7G-N1A-BD had a 3-fold higher HPLC-ESI$^+$-MS/MS response than N7G-N$^6$A-BD (Figure 3.2B).

3.3.2 Concentration dependence curves in DEB-treated DNA.

To quantify the formation of regioisomeric G-A DEB cross-links in vitro, calf thymus DNA was treated with racemic or meso DEB (0 – 1000 µM), followed by HPLC-ESI$^+$-MS/MS analysis of the resulting G-A cross-links with the capillary HPLC-MS/MS method. Adduct amounts increased linearly with increased DEB concentration (Figure 3.4). We found that the major G-A BD cross-link formed was N7G-N1A-BD, followed by N7G-N7A-BD, N7G-N3A-BD, and N7G-N$^6$A-BD. N7G-N$^6$A-BD amounts were below the detection limit in samples treated with low DEB concentrations (< 500 µM). Consistent with our previous findings (134), N7G-N1A-BD was the most abundant N7G-Ade-BD cross-link formed in vitro. No stereospecific differences in adduct amounts were observed in DNA treated with racemic or meso DEB (Figure 3.4).
Figure 3.4 Dose dependent formation of G-A cross-links in calf thymus DNA treated with increasing amounts of DEB.
3.3.3 Method validation.

Method calibration curves were obtained by spiking control mouse liver DNA with known amounts of G-A BD cross-links and the corresponding $^{15}$N$_3$, $^{13}$C$_1$-internal standards, followed by HPLC-ESI$^+$-MS/MS analysis by the same methods as used for real samples. Forced Dimroth rearrangement was employed to convert N7G-N1A-BD to N7G-N$^6$A-BD because of the interfering signal originating from liver DNA hydrolysates that co-eluted with N7G-N1A-BD. A good correlation was observed between the expected and measured levels of N7G-Ade-BD spiked in mouse liver DNA (Figure 3.5). Accuracy and precision were obtained for the three regioisomers of Gua-Ade-BD cross-links at 10 fmol per 100 µg mouse liver DNA. For 5 replicate samples analyzed on 3 separate days, the calculated amounts of N7G-N3A-BD, N7G-N7A-BD, and N7G-N$^6$A-BD were 105 ± 17%, 102 ± 25%, and 79 ± 11% of the theoretical value, respectively (Table 3.1). The limits of quantitation (S/N > 10) for N7G-N3A-BD, N7G-N7A-BD, and N7G-N$^6$A-BD spiked into 100 µg of mouse DNA were 1.5 - 3 adducts per 10$^8$ nucleotides. The limits of detection (S/N > 3) were calculated as 0.6 - 1.5 adducts per 10$^8$ nucleotides for the capillary HPLC-ESI-MS/MS method, and 3 N7G-N1A-BD/10$^9$ nucleotides for nanoLC-nanoESI-MS/MS methods. The limit of detection was 1 fmol N7G-N1A-BD for nanoLC-nanoESI-MS/MS methods.
Figure 3.5  Method calibration curves of thermally labile G-A cross-links (A) and thermally stable G-A cross-links (B) spiked into control mouse liver DNA.
Table 3.1  Validation results for capillaryHPLC-ESI\(^+\)-MS/MS analysis of N7G-N3A-BD, N7G-N7A-BD, and N7G-N\(^6\)A-BD (10 fmol) spiked into mouse liver DNA (0.1 mg) and for nanoHPLC-ESI\(^+\)-MS/MS analysis of N7G-N\(^6\)A-BD (1 fmol) spiked into mouse liver DNA (0.1 mg).

<table>
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3.3.4 *In vivo* analysis of N7G-Ade-BD.

DNA extracted from liver tissue of female B6C3F1 mice exposed to 625 ppm BD for 10 days by inhalation and the corresponding air-only controls was analyzed using the new quantitative capillary HPLC-MS/MS method. We found that DNA isolated from liver of BD-exposed mouse contained 3.1 ± 0.58 N7G-N1A-BD adducts per $10^8$ nucleotides (N = 5, quantified as N7G-N$^6$A-BD following forced Dimroth rearrangement), while control DNA did not contain any N7G-N1A-BD lesions. The amounts of N7G-N3A-BD and N7G-N7A-BD in the same samples were below the limits of detection of our current methods (1.5 adducts per $10^8$ nucleotides).

Representative HPLC-ESI$^+$-MS/MS chromatograms of a control and BD-exposed mouse DNA are shown in Figure 3.6.

3.4 Discussion

BD is an important industrial chemical classified as a probable human carcinogen based on laboratory animal studies and human epidemiology data. However, human risk assessment from exposure to BD is complicated because of the documented interspecies differences in metabolism and carcinogenic response (128;169). Because of its central role in BD-mediated genotoxicity, DEB-specific biomarkers of BD exposure are needed to identify adducts responsible for BD-associated mutagenesis, and to determine which animal model will be most suitable in evaluating human risks associated with BD exposure.
Figure 3.6 HPLC-ESI²-MS/MS analysis of N7G-N²A-BD in liver DNA from mouse exposed to 0 ppm BD (A) and 625 ppm BD (B).
DEB causes a large number of A to T transversion mutations (98;102); yet, previous studies have failed to identify DEB-DNA adducts responsible for these genetic changes. Polymerase bypass of synthetic DNA templates containing N6-(2,3,4-trihydroxybut-1-yl)-adenine monoadducts of (R,R)- and (S,S)-DEB is mostly error-free, leading to low levels of A → G and A → C base substitutions (< 0.3%) (138). N1-(2,3,4-trihydroxybut-1-yl)-dI lesions originating by deamination of the corresponding N1-dA adducts can induce high levels of A → G transitions (139), but these lesions have not been detected in vivo. None of the DEB monoadducts tested induced A→T transversions, limiting our understanding of the structural origins of BD-mediated mutagenesis.

Earlier in vivo analyses of DEB-DNA adducts have been limited to DEB-induced trihydroxybutyl monoadducts, e.g. N7-(2,3,4-trihydroxybut-1-yl) guanine (N7-THBG) and N6-(2,3,4-trihydroxybut-1-yl)-Ade (127). These monoadducts are not DEB-specific because they can be formed by another, more abundant BD metabolite, EBD (Scheme 1.1). Recently, our laboratory has developed sensitive and specific methods for the quantification of guanine-guanine cross-links of DEB (bis-N7G-BD). We found that mouse liver DNA from animals exposed to 625 ppm BD for 1 week contained 3.2 bis-N7G-BD adducts per 10^6 normal guanines (69). Unlike trihydroxybutyl monoadducts, bis-N7G-BD lesions can only be formed by DEB and represent a specific biomarker of exposure to DEB.

In the present study, we developed and validated sensitive and specific isotope dilution capillary HPLC-ESI+−MS/MS and nanoLC-nanoESI-MS/MS methods for the quantitative analysis of G-A cross-links of DEB. Using the capillary methods, N7G-
N⁶A-BD was detected in DNA extracted from liver tissue of mice exposed to BD (625 ppm for 10 days). As expected from *in vitro* studies, levels of N7G-N⁶A-BD (3.08 ± 0.58/10⁸ nucleotides) were an order of magnitude lower than the amounts of *bis*-N7G-BD adducts in the same animals (3.9 adducts/10⁷ nucleotides, see Chapter V). Neither N7G-N3A-BD nor N7G-N7A-BD were detected *in vivo*, which may be explained by their rapid spontaneous depurination at physiological conditions (t½ = 35 and 17 h, respectively (134)).

Although in the present study, a sum of N7G-N1A-BD and N7G-N⁶A-BD adducts was quantified following forced Dimroth rearrangement of N7G-N1A-BD to N7G-N⁶A-BD (Scheme 1.3), we hypothesize that N7G-N1A-BD is the dominant G-A cross-link *in vivo* based on the following evidence. N7G-N1A-BD is by far the most abundant G-A conjugate following *in vitro* treatment of double stranded DNA with DEB (Figure 3.4) and is very stable at physiological conditions, undergoing only minimal rearrangement to N7G-N⁶A-BD (< 16 % at 72 h) (Figure 3.7). Hydrolytic deamination of N7G-N1A-BD to the corresponding hypoxanthine-guanine cross-link (N1HX-N7G-BD) (135) is even slower (1.4 % following 72 h incubation) and may not be relevant in cells.
**Figure 3.7** Stability of N7G-N1A-BD at physiological conditions.
The biological significance of the formation of G-A DEB conjugates in BD-exposed animals remains to be established. Although their concentrations are only 1/10 of the amounts of the dominant G-G DNA cross-links of DEB (*bis*-N7G-BD), N7G-N1A-BD and N7G-N⁶A-BD conjugates are more hydrolytically stable and may accumulate in target tissues over time (4). For example, Koivisto et al. observed higher levels of N7-THBG lesions than N⁶-THBA *in vivo* immediately following BD exposure, however, 21 days post exposure no N7-THBG was detected while N⁶-THBA levels remained the same (121). Spontaneous hydrolysis of the N7-substituted guanine moiety within N7G-N1A-BD and N7G-N⁶A-BD under physiological conditions would lead to the formation of a bulky adduct opposite an abasic site (Scheme 3.2). Repair of such a lesion is likely to be hindered or promutagenic because of the presence of DNA damage in both strands of DNA.
**Scheme 3.2** Hydrolytic stability of the four G-A cross-links, N7G-N1A-BD, N7G-N6A-BD, N7G-N3A-BD, and N7G-N7A-BD.
Interspecies differences in metabolism of BD have been observed in studies with human, rat and mouse microsomes (178). Mice metabolize BD to DEB at faster rates than rats and humans (179), which suggests that greater amounts of DEB-specific lesions such as N7G-N^6A-BD and bis-N7G-BD may be formed in the mouse. Furthermore, there may be interspecies differences in repair of N7G-N1A-BD cross-links. Due to lower abundance of N7G-N1A-BD cross-links as compared to bis-N7G-BD, we have developed nanospray methods to increase sensitivity for N7G-N1A-BD. The quantitative nanoHPLC-nanoESI^+^MS/MS methods presented in this chapter have allowed us to conduct the analyses of interspecies BD dose-response relationships, repair, and accumulation of DEB-specific G-A cross-links *in vivo* (see chapters V and VI).
IV. DEVELOPMENT AND VALIDATION OF COLUMN SWITCHING HPLC-ESI*-MS/MS METHODS FOR THE QUANTITATION OF EXOCYCLIC-dA ADDUCTS IN MICE EXPOSED TO 1,3-BUTADIENE

4.1 Introduction

1,2,3,4-diepoxybutane (DEB) is the proposed ultimate carcinogenic form of 1,3-butadiene (BD) (96;111;133;136), a chemical used commonly in the rubber and plastic industries, and classified as a human carcinogen (91). Although DEB is a relatively minor metabolite of BD, it is by far the most mutagenic, inducing large numbers of base substitutions, sister chromatid exchanges, and chromosomal aberrations (93;95;147;148). The mutational spectra of DEB in human lymphocytes show a high occurrence of point mutations at A:T base pairs, specifically A → T transversion mutations (98). However, DNA adducts responsible for these genetic changes remain to be identified, limiting our understanding of the mechanism of DEB-mediated mutagenesis.

We hypothesized that DEB forms highly mispairing exocyclic adducts at adenine nucleobases. This hypothesis was based on our studies of N6-(2-hydroxy-3,4-epoxybut-1-yl)-dA (N6-HEB-dA) adducts which spontaneously cyclized under physiological conditions to form exocyclic dA adducts, 1,N6-(2-hydroxymethyl-1-hydroxypropanodiyl)-dA (1,N6-γ-HMHP-dA) and 1,N6-(1-hydroxymethyl-2-hydroxypropanodiyl)-dA (1,N6-α-HMHP-dA) (Senevirante and Tretyakova, submitted for publication). We hypothesized that DEB reacts first with the N1 of adenine to form, N1-(2-hydroxy-3,4-epoxybut-1-yl)-dA (N1-HEB-dA), followed by cyclization to 1,N6-
α-HMHP-dA (Scheme 4.1). 1,N⁶-α-HMHP-dA undergoes a slow Dimroth rearrangement to 1,N⁶-γ-HMHP-dA (Scheme 4.1)

Based on the mutagenicity of structurally analogous 1,N⁶-exocyclic-dA adducts we hypothesized that 1,N⁶-HMHP-dA adducts may be responsible for the mutagenicity of DEB. For example, 1,N⁶-ethenoadenine and 1,N⁶-ethanoadenine are both mutagenic in human cells because the 1,N⁶-exocyte of dA prevents normal Watson-Crick base pairing (10;11). In the present work, we have developed quantitative column switching capillary HPLC-ESI⁺-MS/MS methods for the quantification of 1,N⁶-HMHP-dA in liver DNA of laboratory mice exposed to 1,3-butadiene by inhalation.
**Scheme 4.1** Formation of $1,N^6$-α-HMHP-dA and $1,N^6$-γ-HMHP-dA from the reaction of DEB with DNA.
4.2 Experimental

Materials. Authentic 1,N<sup>6</sup>-HMHP-dA and 15N<sub>4</sub>-1,N<sup>6</sup>-HMHP-dA (internal standard for quantitation) were prepared as previously reported (Seneviratne et al. submitted for publication). Phosphodiesterase I, Phosphodiesterase II, and DNAase I were obtained from Worthington Biochemical Corp. (Freehold, NJ). All other reagents were from Sigma (Milwaukee, WI) unless otherwise noted.

Methods.

Animals and DNA extraction. Female B6C3F1 mice were exposed to 625 ppm 1,3-butadiene by inhalation for 2 weeks at the Lovelace Respiratory Research Institute (Albuquerque, NM) as described in Chapters II and III. Following BD exposure, animals were sacrificed via cardiac puncture (70). All animal procedures were approved by the Institutional Animal Care and Use Committee. Tissues were flash frozen and shipped to the UMN on dry ice. DNA was extracted from liver tissue (200 – 400 mg) using NucleoBond-AXG500 extraction kits (Macherey-Nagel, Bethlehem, PA) following manufacturers protocol. DNA amounts and purity were determined by UV spectrometry.

DNA Hydrolysis and sample preparation. Partially depurinated DNA was obtained by neutral thermal hydrolysis of 100 µg of DNA (60 min at 70 ºC), followed by ultrafiltration using YM-10 Centricon filters (Millipore Corp., Billerica, MA). DNA was spiked with 15N<sub>4</sub>-1,N<sup>6</sup>-HMHP-dA (27.5 fmol) and digested with phosphodiesterase I (1.3 mU/µg DNA), phosphodiesterase II (1.4 mU/µg DNA), DNAse (0.67 U/µg DNA), and alkaline phosphatase (0.26 U/µg DNA) in 20 mM Tris-HCl/30 mM MgCl<sub>2</sub> at 37 ºC overnight. Following DNA digestion, samples were filtered using YM-10
Centricon filters and purified by solid phase extraction. Solid phase extraction was performed using Extract Clean Carbo cartridges (150 mg) from Grace Davidson Discovery Science (Deerfield, IL). Cartridges were prepared with methanol (2 x 3 mL) and water (2 x 3 mL) prior to loading samples in water (1 mL) by gravity. Samples were washed with water (3 mL), 5 % methanol (3 mL), and eluted in 30 % methanol (3 mL). The SPE fractions containing \(1,N^6\)-HMHP-dA were dried under vacuum and dissolved in 0.05% acetic acid (25 µL) prior to HPLC-ESI\(^+-\)MS/MS analysis.

**In vitro DEB treatment of calf thymus DNA.** Calf thymus DNA (500 µg aliquots, in 500 µL water) was treated with increasing amounts of \(d\), \(l\), or meso DEB (0-1 mM) at 37 °C for 24 hours. DNA was precipitated with cold ethanol and re-suspended in 500 µL of water. Following spiking with \(^{15}\text{N}_4\-1,N^6\)-HMHP-dA (500 fmol), DNA was digested to deoxynucleosides in the presence of Nuclease P1 (10 U) and alkaline phosphatase (60 U, 37 °C for 1 hour). Samples were filtered through YM-30 Centricon filters to remove proteins, concentrated under vacuum, and re-dissolved in 25 µL of water. About 8 µL was injected onto a capillary HPLC column for HPLC-ESI\(^+-\)MS/MS analysis.

**HPLC-ESI-MS/MS analysis of DEB-treated DNA.** \(1,N^6\)-HMHP-dA and its internal standard were analyzed with an Agilent 1100 capillary HPLC system (Wilmington, DE) interfaced to a Thermo-Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific Corp., Waltham, MA). A Phenomenex Synergy (250 x 0.5 mm, 0.4 µm) column was eluted with a gradient of 10 mM ammonium formate, pH 4.2 (A) and methanol (B). The solvent composition was kept at 100 % A for 5 min, linearly increased to 6 % B over 1 min, and finally to 30 % B over 10 min.
Under these conditions, $1,N^6$-HMHP-dA eluted at 14.5 min. The mass spectrometer was operated in the positive ion mode, with nitrogen used as a sheath gas (5 L/min). Electrospray ionization was achieved at a spray voltage of 4.0 kV and the capillary temperature of 250 °C. Collision Induced Dissociation (CID) was achieved with Ar as a collision gas (1 mTorr) and a collision energy of 20 V. The mass spectrometer parameters were optimized for maximum response during infusion of standard solutions. Both exocyclic DEB-dA lesions were quantified by isotope dilution with the corresponding $^{15}N_4 - 1,N^6-\alpha$-HMHP-dA and $^{15}N_4 - 1,N^6-\gamma$-HMHP-dA internal standards. Quantitative analyses were performed in the selected reaction monitoring (SRM) mode using HPLC-ESI$^+$-MS/MS peak areas corresponding to the neutral loss of deoxyribose ($m/z$ 338.1 [M + H]$^+$ → 222.0 [M + 2H-dR]$^+$ and $m/z$ 342.1 [M + H]$^+$ → 226.0 [M + 2H-dR]$^+$ for analytes and their internal standards, respectively.

**Column switching HPLC-ESI$^+$-MS/MS Method.** Samples containing $1,N^6$-HMHP-dA were loaded onto an SCX trap column (300Å 5µm, nanoAcquity Waters Corp., Millford, MA) using an auxiliary Agilent 1100 pump delivering 0.5 % methanol in water isocratically at 15 µL/min for 10 min. During this time, the divert valve was in position A (Figure 4.1). After 10 min loading time, the divert valve was switched to position B, and the adducts were released from the trap column and transferred to the analytical Synergi Hydro-RP (250 x 0.5 mm, Phenomenex) by backflushing for five minutes with a gradient of 0.05% acetic acid (A) and MeOH (B) delivered by a Waters Acquity pump (Waters Corp.). The solvent composition was maintained at 0.5% B for 11 minutes, linearly increased to 5% B in 2 minutes, maintained at 5% B for 4 minutes, and further increased to 20% B over 9 minutes and then returned to 0.5% in 1 minute.
The divert valve was returned to position A at 15 minutes (Figure 4.1). Under these conditions \(1,N^6\)-HMHP-dA eluted as a sharp peak at 14.5 min (Figure 4.2). A Thermo Finnigan Ultra TSQ mass spectrometer was operated in the selected reaction monitoring mode by following the mass transition \(m/z\) 338.1 \([M + H]^+\) \(\rightarrow\) 222.1 \([M + 2H – dR]^+\), and the corresponding transition for the \([^{15}N_4]-1,N^6\)-HMHP-dA internal standard \((m/z - 342.1 \rightarrow 226.1)\). Quantitative analyses were based on the ratios of areas under the peak in the selected ion chromatogram corresponding to the analyte and the internal standard (relative response ratios). Standard curves were constructed by analyzing the solutions containing \(1,N^6\)-HMHP-dA (0 – 5000 amol) and \(^{15}N_4\)-\(1,N^6\)-HMHP-dA (2550 amol), followed by regression analysis of the relative response ratios calculated from HPLC-ESI\(^+\)-MS/MS peak area ratios corresponding to \(1,N^6\)-HMHP-dA isomers and their internal standards (Figure 4.2).
Figure 4.1 Diagram of column switching valve positions. In position A, samples are loaded onto the trap column and impurities are washed to waste. In position B, the trap column is backflushed with acid to load the analyte onto the analytical column for HPLC-MS/MS analysis.
Figure 4.2 Column switching HPLC-ESI+MS/MS analysis of 1,\(N^6\)-HMHP-dA authentic standards (A) and 15\(N_4\)-1,\(N^6\)-HMHP-dA internal standard (B).
**Method validation.** Control DNA was extracted from human cervical carcinoma (HeLa) cell cultures using NucleoBond AXG kits (Macherey-Nagel, Bethlehem, PA). DNA (0.1 mg) was spiked with \(1,N^6\)-HMHP-dA (0-5 fmol) and \(^{15}\text{N}_4\)-\(1,N^6\)-HMHP-dA (5.5 fmol). DNA was enzymatically digested, filtered, purified by SPE, and analyzed by column switching HPLC-ESI-MS/MS as described above. Three replicates spiked with 0.5 fmol \(1,N^6\)-HMHP-dA were also processed as above and analyzed on three separate days to determine accuracy and precision of the method (Table 4.1).

4.3 Results

4.3.1 Formation of \(1,N^6\)-HMHP-dA in DEB-treated DNA.

Aliquots of calf thymus DNA (500 µg) were treated with increasing amounts of \(S,S\), \(R,R\), or \(meso\) DEB. All three isomers of DEB (\(S,S\), \(R,R\), and \(meso\)) were employed in order to investigate potential effects of diepoxide chirality on the formation of exocyclic DEB-dA adducts. The resulting alkylated DNA was enzymatically digested to deoxynucleosides, spiked with \(^{15}\text{N}_4\)-\(1,N^6\)-HMHP-dA internal standard and the amounts of \(1,N^6\)-HMHP-dA adducts formed were determined by column switching HPLC-MS/MS. We found that the concentrations of \(1,N^6\)-DEB-dA in DNA increased linearly as the concentrations of DEB were gradually raised from 50 µM to 1000 µM (Figure 4.3). Similar numbers of exocyclic DEB-dA adducts were observed in DNA treated with \(S,S\), \(R,R\) or \(meso\) DEB, suggesting that the three DEB stereoisomers are equally capable of inducing exocyclic \(1,N^6\)-HMHP-dA lesions in DNA.
4.3.2 Development of column switching HPLC-ESI$^+$-MS/MS methods for $1,N^\delta$-HMHP-dA adducts in DNA.

Since the $1,N^\delta$-HMHP-dA adducts have a positive charge at the N1 of dA at physiological pH (Scheme 4.1), they are not well retained on reversed phase HPLC columns, eluting with the solvent front (results not shown) and suffering from signal suppression. Although somewhat better retention was obtained on a Synergi Hydro-RP C18 column, this column could not resolve $1,N^\delta$-HMHP-dA from dC present in high levels in DNA digests. To overcome this problem we have developed column switching methods to further purify $1,N^\delta$-HMHP-dA from DNA digests with little sample loss. We took advantage of the positive charge on $1,N^\delta$-HMHP-dA to capture the adducts and their internal standards on a SCX trap column. Following loading, the analytes are backflushed with 0.05% acetic acid to enter an analytical Synergi Hydro-RP C18 column for capillary HPLC-ESI$^+$-MS/MS analysis (Figure 4.1). We determined that the HPLC-ESI$^+$-MS/MS responses for $1,N^\delta$-DEB-dA were linear between 10 and 1000 fmol of each adduct, with $R^2$ values $> 0.997$ (Figure 4.4). The detection limits for $\alpha$ and $\gamma$ $1,N^\delta$-HMHP-dA were 125 amol for pure standards, and 300 amol for standards spiked into blank DNA.
Figure 4.3 Concentration dependent formation of $1,N^6$-HMHP-dA in calf thymus DNA treated with $S,S$; $R,R$; and meso DEB.
Figure 4.4 Calibration curve for authentic $I,N^6$-HMHP-dA standard and $^{15}N_4-I,N^6$-HMHP-dA internal standard.
4.3.3 Method validation.

Aliquots of control DNA from human cervical carcinoma (HeLa) cells (0.1 mg) were spiked with 0-5000 amol \(1,N^6\)-HMHP-dA and 5000 amol of the corresponding \(\text{^{15}N}_4\)-labelled internal standards, followed by the sample processing and column switching HPLC-MS/MS analysis as employed for actual samples. We found that method quantitation limit was 500 amol for \(1,N^6\)-HMHP-dA adducts (S/N ratio of 10 or better). A good correlation was observed between the expected and measured levels of \(1,N^6\)-HMHP-dA in HeLa DNA (Figure 4.5). Method accuracy and precision determined for 500 amol \(1,N^6\)-HMHP-dA was 87.0 ± 15.2 % (Table 4.1).
Figure 4.5 Method calibration curve for $1,N^6$-HMHP-dA spiked HeLa DNA (0.1 mg)

\[
y = 0.8426x + 0.062
\]

\[
R^2 = 0.997
\]
Table 4.1  Accuracy and precision for $1,N^6$-HMHP-dA (500 amol) spiked into HeLa DNA (100 µg) analyzed on three separate days.

<table>
<thead>
<tr>
<th></th>
<th>$1,N^6$-HMHP-dA</th>
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</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.4708</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>14.3</td>
</tr>
<tr>
<td>accuracy (%)</td>
<td>94.2</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
</tr>
<tr>
<td><strong>Day 2</strong></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.3716</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>11.8</td>
</tr>
<tr>
<td>accuracy (%)</td>
<td>74.3</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
</tr>
<tr>
<td><strong>Day 3</strong></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.4621</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>9.45</td>
</tr>
<tr>
<td>accuracy (%)</td>
<td>94.2</td>
</tr>
<tr>
<td>n</td>
<td>3</td>
</tr>
<tr>
<td><strong>Interday</strong></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.4348</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>15.2</td>
</tr>
<tr>
<td>accuracy (%)</td>
<td>87</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
</tr>
</tbody>
</table>
4.3.4 *In vivo* analysis of $1,N^6$-HMHP-dA.

The new quantitative column switching HPLC-MS/MS method was employed to analyze the formation of $1,N^6$-HMHP-dA in liver DNA of B6C3F1 mice which were exposed to 0 or 625 ppm BD for 10 days by inhalation. We found that DNA extracted from tissues of BD-treated mice contained $0.44 \pm 0.08$ $1,N^6$-HMHP-dA adducts per $10^8$ nucleotides, while DNA of control animals did not contain detectable amounts of $1,N^6$-HMHP-dA lesions ($N = 5$) (limit of detection, $0.015$ $1,N^6$-HMHP-dA per $10^8$ normal nucleotides. Representative extracted ion chromatograms for HPLC-ESI$^+$-MS/MS analysis of $1,N^6$-HMHP-dA in samples from BD-exposed and control mouse DNA are shown in Figure 4.6.
Figure 4.6 Column switching HPLC-ESI$^+$-MS/MS analysis of $1,N^6$-HMHP-dA in liver DNA of control (A) and 625 ppm BD-exposed (B) mice.
4.4 Discussion

While it is known that DEB is the most mutagenic metabolite of BD (96;111;133;159), specific DNA adducts responsible for its mutagenicity remain to be identified. In particular, a large number of mutations are observed at adenine bases, which suggests that DEB forms strongly mispairing deoxyadenosine adducts (98). We have recently identified two types of exocyclic DEB-dA adducts, \(1,N^\delta-(2\text{-hydroxy}-3\text{-hydroxymethylpropan-1,3-diyl})-2\text{'-deoxyadenosine}\) \((1,N^\delta-\gamma\text{-HMHP-dA})\) and \(1,N^\delta-(1\text{-hydroxymethyl-2-hydroxypropan-1,3-diyl})-2\text{'-deoxyadenosine}\) \((1,N^\delta-\alpha\text{-HMHP-dA})\). Both lesions were observed \textit{in vitro} in DEB-treated DNA (Senevirante and Tretyakova, submitted for publication). The objective of the present work was to develop quantitative HPLC-ESI-MS/MS methods to analyze the novel exocyclic DEB-dA adducts \textit{in vitro} and \textit{in vivo}.

Our method is based on column switching HPLC-ESI-MS/MS, where an additional sample enrichment step is achieved by column switching prior to analysis. In the column switching step, the analyte is retained on a trap column while impurities are washed away; ideally the trap column will specifically retain only the analyte. The analyte is then released from the trap column onto an analytical column for HPLC-MS/MS analysis. In our approach, \(1,N^\delta\text{-HMHP-dA}\) is selectively retained by a strong cation exchange (SCX) trap column due to the positive charge on N1 (Scheme 4.1), while the bulk of natural nucleosides present in DNA hydrolysates are washed away. This makes our method selective for \(1,N^\delta\text{-HMHP-dA}\) and also sensitive due to the extremely low levels of background, which is necessary for this low abundance lesion.
The method is accurate and reproducible due to use of isotopically labeled internal standards (Figure 4.2).

The new methodology was employed to quantify $1,N^6$-HMHP-dA adducts in DEB-treated DNA and liver DNA extracted from B6C3F1 mice exposed to 625 ppm BD for two weeks by inhalation. There is evidence that the different isomers of DEB ($S,S; R,R; \text{ and } meso$) have varying genotoxicities, with $S,S$ being the most toxic (90-92). Therefore, we investigated the formation of $1,N^6$-HMHP-dA in DNA treated with DEB isomers. A dose dependent formation of the $1,N^6$-HMHP-dA adducts was observed at similar rates for each of the DEB stereoisomers (Figure 4.3), suggesting that each isomer is capable of forming the $1,N^6$-HMHP-dA lesions. Therefore, any variation in toxicity of the DEB stereoisomers must be from varying levels of $S,S; R,R; \text{ and } meso$ DEB present in vivo, or different stability/repair of $1,N^6$-HMHP-dA isomers in vivo.

In B6C3F1 mice exposed to 625 ppm BD by inhalation for 2 weeks, $0.44 \pm 0.08$ $1,N^6$-HMHP-dA adducts per $10^8$ nucleotides were observed, while no $1,N^6$-HMHP-dA lesions were detected in the corresponding air controls. Although $1,N^6$-HMHP-dA is the least abundant of the three bifunctional DEB-DNA adducts quantified (Table 4.2), we hypothesize it will be a highly mutagenic adduct based on the mutagenicity of other known exocyclic dA lesions (10;11). Exocyclic adducts are typically highly mutagenic because of their effects on the molecular shape and hydrogen bonding properties of the parent nucleobase (180-183). For example, $1,N^6$-ethenoadenine ($\varepsilon$A) and $1,N^6$-ethanoadenine (EA) (Chapter I, Chart 1.7) are highly blocking during DNA polymerization, and induce $A \rightarrow T$ transversion mutations in human cells (10;11).
Similar to $1,N^6$-HMHP-dA, these adducts are present in low levels, $0.12 – 0.45 \varepsilon A/10^7$ nucleotides in control mice (184).

In the mutation spectra of BD/DEB exposed mice and human T6K cells, there is a high occurrence of mutations at A:T base pairs (A $\rightarrow$ T transversion mutations) (98). If the $1,N^6$-HMHP-dA adducts present in vivo are not repaired, the normal A:T Watson-Crick base pairing is not possible because of the steric hindrance induced by the substituted propane group of the adduct. However, $1,N^6$-HMHP-dA could be forced to the syn conformation about the glycosidic bond similar to $\varepsilon A$ (182). In the syn conformation, $1,N^6$-HMHP-dA could form a Hoogsteen base pair with a protonated dA (Figure 4.7), which would lead to A $\rightarrow$ T transversion mutations. While the mutagenicity of $1,N^6$-HMHP-dA adducts is currently unknown, experiments in our laboratory are currently underway to synthesize oligonucleotides containing the $1,N^6$-HMHP-dA to determine its base pairing characteristics in site-specific mutagenesis experiments.
Table 4.2  Summary of bifunctional DEB-DNA adduct levels in liver DNA of female B6C3F1 mice exposed to 625 ppm BD for 2 weeks by inhalation.

<table>
<thead>
<tr>
<th>Adduct Type</th>
<th># adducts/10⁷ nts</th>
</tr>
</thead>
<tbody>
<tr>
<td>bis-N7G-BD</td>
<td>3.95 ± 0.89</td>
</tr>
<tr>
<td>N7G-N1A-BD</td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td>1,N⁶-HMHP-dA</td>
<td>0.044 ± 0.008</td>
</tr>
</tbody>
</table>
Figure 4.7 Proposed Hoogsteen base pair of $1,N^6$-HMHP-dA with protonated dA that could lead to A $\rightarrow$ T transversion mutations.
V. MOLECULAR DOSIMETRY OF BIFUNCTIONAL DNA ADDUCTS IN B6C3F1 MICE AND F344 RATS EXPOSED TO BUTADIENE BY INHALATION


5.1 Introduction

1,3-Butadiene (BD) is a high volume industrial chemical used in the production of plastics and rubber (91). It is also an environmental toxin present in automobile exhaust and in cigarette smoke (2;92). BD is classified as a human carcinogen based on laboratory animal data linking it to the formation of tumors, human epidemiology data revealing increased incidence of leukemia and lymphohematopoietic cancers in occupationally exposed workers, and genotoxicity data demonstrating the induction of point mutations, large deletions, and chromosomal aberrations following exposure to BD (93;95;147;148). Due to the widespread human exposure to BD, there is a pressing need to identify biomarkers of BD exposure for use in quantitative risk assessment. In particular, specific biomarkers of the metabolic activation of BD to DNA-reactive intermediates are required.

BD is metabolized by cytochrome P450 monooxygenases to generate three reactive epoxides, e.g. 3,4-epoxybutene (EB), 3,4-epoxy-1,2-butanediol (EBD), and 1,2,3,4-diepoxybutane (DEB) (Scheme 5.1) (108;151;152). Although all three epoxide metabolites of BD can react with DNA, DEB is considered the ultimate carcinogenic form of BD because of its potent genotoxicity and its ability to form bifunctional DNA adducts such as DNA-DNA cross-links and exocyclic DNA lesions (133;159). Studies in human cell culture reveal that DEB is 100-200-fold more mutagenic than BD-derived
epoxides that possess a single epoxide functionality (111). DEB preferentially alkylates the N7 position of guanine bases in DNA to form N7-(2'-hydroxy-3', 4'-epoxybut-1'-yl)-guanine (N7-HEB-dG) adducts (185). The epoxide group of N7-HEB-dG can then be hydrolyzed to N7-(2’, 3’, 4’-trihydroxybut-1’-yl)-guanine (THBG), or, less frequently, can react with another site in DNA, such as the N7 of another guanine or the N1 of an adenine. The latter reaction forms 1,4-bis-(guan-7-yl)-2,3-butanediol (bis-N7G-BD) and 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD) cross-links (Scheme 5.1) (133;134). Alkali-catalyzed Dimroth rearrangement of N7G-N1A-BD leads to the corresponding N7G-N6A-BD adducts (Scheme 5.1) (70). While less reactive, the N1 of adenine can also react with DEB to form N1-(2’-hydroxy-3’,4’-epoxybut-1’-yl)-deoxyadenosine (N1-HEB-dA) adducts that spontaneously to form 1,N6-(1-hydroxymethyl-2-hydroxypropanodiyl)-dA (1,N6-HMHP-dA, Scheme 5.1) (Seneviratne et al. submitted for publication).
**Scheme 5.1** Metabolic activation of 1,3-butadiene to reactive electrophiles and reaction with DNA.
Chronic BD inhalation studies in mice and rats revealed that it was carcinogenic in both species, but with a striking difference in sensitivity and tissue specificity. Mice were far more sensitive to BD than rats, forming tumors in lung, heart, and hematopoietic system at 6.25 ppm BD exposure (93), while rats did not develop tumors until 1000 ppm BD exposures (94). In rats tumors were observed in thyroid, pancreas, testis, uterus (different target tissues than for mice). The increased susceptibility of mice to the carcinogenicity of BD may result from a more efficient metabolic activation of BD to DEB in this species (149;175;179).

Indeed, several studies have found that target tissues of BD-exposed mice, especially lung, contain significant levels of DEB (173). DEB-specific N,N-(2,3-dihydroxy-1,4-butanediyl)-valine (Pyr-Val) globin adducts were detected in mice treated with as little as 3 ppm BD by inhalation (130). In contrast, the formation of DEB in rat tissues is negligible, providing a possible explanation for the weak tumorigenic response to BD in this species (130;163;173). Consistent with this model, rats, mice, and humans are equally sensitive to the genotoxic effects of DEB when it is introduced directly into isolated lymphocytes (168;169). However, the formation of DEB-specific DNA-DNA adducts in tissues of laboratory mice and rats exposed to BD have not been previously investigated.

Earlier studies have analyzed DEB monoadducts in rodent tissues in an attempt to explain the observed interspecies differences in carcinogenic response. Koc et al. examined the formation of THBG adducts (Scheme 5.1) in liver DNA of B6C3F1 mice and F344 rats treated with 62.5-625 ppm of BD for 4 weeks (125). THBG levels were only 2-fold higher in mice than in rats (125) and thus could not account for the 1000-
fold greater sensitivity of mice to BD-mediated cancer. The study concluded that over 95% of the THBG adducts were formed not from DEB, but from another, more prevalent metabolite of BD, 3,4-epoxy-1,2-butanediol (EBD) (Chapter I, Scheme 1.1). These results emphasized the need for a unique biomarker for DEB to use in predicting the carcinogenic risk of BD (125;128).

We have recently developed quantitative HPLC-ESI-MS/MS methods for three types of DEB-specific bifunctional DNA adducts, 1,4-\textit{bis}-(guan-7-yl)-2,3-butanediol (\textit{bis}-N7G-BD), 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD), and 1,N\textsuperscript{6}-(1-hydroxymethyl-2-hydroxy-1,3-propanediyl)-2’-deoxyadenosine (1,N\textsuperscript{6}-HMHP-dA) (69;70) (Chapters II, III, and IV). In our approach, the bifunctional DNA adducts are released from the DNA backbone by neutral thermal hydrolysis, mild acid hydrolysis, or enzymatic digestion, and enriched prior to analysis by HPLC-ESI-MS/MS using the corresponding $^{15}$N-labeled adducts as internal standards (69;70). The N7G-N1A-BD adducts are converted to the corresponding 1-(guan-7-yl)-4-(aden-6-yl)-2,3-butanediol (N7G-N\textsuperscript{6}A-BD) lesions \textit{via} forced Dimroth rearrangement prior to analysis in order to improve the sensitivity of the method and to eliminate adduct decomposition during sample processing (70). We found that liver DNA of female C57BL/6 and B6C3F1 mice exposed to 625 ppm BD by inhalation contained \textit{bis}-N7G-BD adducts in ten fold greater amounts than N7G-N1A-BD, and 90 fold greater amounts than 1,N\textsuperscript{6}-HMHP-dA (Table 4.2) (69;70). However, unlike \textit{bis}-N7G-BD cross-links which are hydrolytically labile and are spontaneously released from DNA by depurination ($t_{1/2}$, 81.5 h) (133), N7G-N1A-BD and 1,N\textsuperscript{6}-HMHP-dA lesions may persist in DNA and accumulate in tissues over time.
The objective of this work was to quantify DEB-specific DNA adducts in tissues of laboratory rats and mice exposed to BD by inhalation in an attempt to establish the structural basis of species differences in sensitivity to BD-mediated carcinogenesis and mutagenesis. We obtained dose response relationships for DEB-induced DNA-DNA cross-links in tissues of B6C3F1 mice and F344 rats exposed to increasing concentrations of BD (6.25, 62.5, 200, and 625 ppm for 2 weeks). Unlike previous results for THBG adducts (125), the amounts of DEB-specific bifunctional DNA adducts exhibited important interspecies and gender differences which correlate with previously observed differences in tumorigenic susceptibility, supporting the idea that DEB plays an important role in BD-mediated cancer in mice.

5.2 Experimental

*Note: DEB is a known carcinogen and must be handled with adequate safety precautions.*

**Materials.** All chemicals and solvents were obtained from Sigma-Aldrich (Milwaukee, WI), unless stated otherwise. Racemic and meso bis-N7G-BD, [\(^{15}\text{N}_{10}\)]-bis-N7G-BD, N7G-N1A-BD, [\(^{15}\text{N}_{3},^{13}\text{C}_{1}\)]-N7G-N1A-BD, 1,\(^{N}\)-HMHP-dA, and [\(^{15}\text{N}_{4}\)]-1,\(^{N}\)-HMHP-dA were prepared in our laboratory as described elsewhere (70;106).

**Animals and treatment.** Animals (B6C3F1 mice and F344 rats) were randomly separated into air-control and exposure groups by weight and were housed individually in hanging wire stainless steel cages according to NIH guidelines (NIH Publication 86-23, 1985). All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee. Experimental animals were exposed
using multitiered whole body exposure chambers (H-2000, Laboratory Products, Aberdeen, MD). Rodents in one chamber received filtered air only as a control group, and rodents in the other chamber received nominal 6.25, 62.5, 200, or 625 ppm BD for 2 weeks (6 h/day, 5 days/week). Animals were housed within exposure chambers throughout the experiment and had free access to food and water except for removal of food during the 6 h exposure periods. Within 2 h after cessation of the final day of exposure, animals were euthanized via cardiac puncture, and tissues were harvested and snap-frozen for storage at -80 °C. Tissues were shipped on dry ice to the University of Minnesota where they were stored at -80 °C until DNA extraction.

**DNA isolation.** DNA was isolated using NucleoBond AXG500 anion exchange cartridges (Macherey-Nagel Bethlehem, PA) according to manufacturer’s procedures. In brief, tissues (0.1 – 0.4 g) were homogenized and incubated with RNAse and proteinase K at 50 °C for 2-4 hours. Samples were loaded on NucleoBond AXG cartridges, which were prepared and washed following the kit instructions. Following elution, DNA was precipitated by the addition of isopropanol. The DNA was spooled, washed 3 times with cold 70% ethanol, dried, and dissolved in Milli-Q water (500 µL). DNA purity and amounts were determined by UV spectro-photometry. Typical A$_{260}$/A$_{280}$ ratios were between 1.7 and 1.9, ensuring minimal protein contamination.

**Bis-N7G-BD sample preparation.** DNA (100 µg) was dissolved in 200 µL of water, spiked with racemic and meso $^{15}$N$_{10}$-bis-N7G-BD internal standards (300 fmol each) and heated at 70 °C for 1 hour to release N7-alkylguanine adducts. Following thermal hydrolysis, the partially depurinated DNA backbone was removed by ultrafiltration with Centricon YM-10 filters (Millipore Corp., Billerica, MA). The
filtrates containing bis-N7G-BD were further purified by offline HPLC as described below, while partially depurinated DNA was recovered for future analysis of N7G-N1A-BD or 1,6-HMHP-dA (see below). Offline HPLC purification of bis-N7G-BD was performed using a Zorbax Eclipse XDB-C18 (4.6 x 150 mm, 5 µm) column. Bis-N7G-BD was eluted with a gradient consisting of 0.4% formic acid in H2O (A) and acetonitrile (B). The column was maintained at 0% B for 5 minutes, followed by a linear increase to 3% B in 10 minutes, and then further to 40% B over 5 minutes. The system was equilibrated for 15 minutes between runs. The retention time of bis-N7G-BD (12.4 minutes) was determined with 15N10-labeled internal standard to prevent carryover contamination. Samples were spiked with 2'-deoxythymidine (retention time 9.8 min) and 2'-deoxyadenosine (retention time 16.6 min) (0.5 µg each) as HPLC retention time markers. HPLC fractions containing bis-N7G-BD were dried under vacuum and reconstituted in 0.05 % acetic acid (25 µL) for HPLC-MS/MS analysis.

**N7G-N1A-BD sample preparation.** Partially depurinated DNA backbone recovered from neutral thermal hydrolysis was spiked with 13C1, 15N3-N7G-N1A-BD (300 fmol, internal standard for mass spectrometry) and hydrolyzed in the presence of 0.1 M HCl to release all purine bases, including N7G-N1A-BD. The hydrolysates were filtered by YM-10 filters and purified by solid phase extraction on C18 cartridges as previously reported (Chapter III, (70)). Samples were heated in base (1.0 M NH4OH at 70 °C for 16 h) to force Dimroth rearrangement of N7G-N1A-BD to N7G-N6A-BD. The ammonium hydroxide was removed under vacuum, and samples were dissolved in 0.05% acetic acid (25 µL) prior to HPLC-MS/MS analysis.
**1,N⁶-HMHP-dA hydrolysis and sample preparation.** Partially depurinated DNA backbone (recovered from 100 µg DNA after neutral thermal hydrolysis) was spiked with \(^{15}N_4\)-1,N⁶-HMHP-dA (27.5 fmol), enzymatically digested with PDE I, PDE II, DNase, and alkaline phosphatase at 37 °C, and filtered using YM-10 size exclusion filters as described in Chapter IV. 1,N⁶-HMHP-dA and its internal standard were purified by Extract Clean Carbo solid phase extraction prior to column switching HPLC-ESI\(^+\)-MS/MS analysis as described in Chapter IV.

**HPLC-µESI\(^+\)-MS/MS of bis-N7G-BD.** MS instrumentation and chromatographic separation of bis-N7G-BD was achieved using previously described methods (see Chapter II of this thesis). Our optimized method utilizes 0.05% acetic acid (A) and methanol (B) as HPLC solvents. A Zorbax Extend C18 column (3.5 µm, 150 x 0.5 mm) was eluted with a gradient of 0 to 10 % B in 5 minutes, further from 10% to 60% over 10 minutes, and ramped back to 0% B over 3 minutes. The HPLC flow rate was 10 µL/min, the column temperature was maintained at 40 °C, and the injection volume was typically 8 µL. With this solvent system, the retention time of racemic bis-N7G-BD was 9.9 min, while meso bis-N7G-BD eluted at 10.9 min. The mass spectrometer was operated in SRM mode monitoring \(m/z\) 389.1 \(→\) 238.1, 152.1, and the corresponding transitions for IS \(m/z\) 399.1 \(→\) 243.1, 157.1.

**nanoHPLC-nanoESI\(^+\)-MS/MS of N7G-N1A-BD.** MS instrumentation and chromatographic separation of N7G-N1A-BD were as described in Chapter III. Briefly, a Waters nanoAquity UPLC system interfaced to a Thermo-Finnigan TSQ Quantum Ultra mass spectrometer were used for these analyses. Samples (4-8 µL) were loaded on a Symmetry C18 nanoAcquity trapping column (0.18 x 20 mm) for 1 min and
separated on an Atlantis C18 (75 x 100 µm) column eluted at a flow rate of 0.350 µL/min. N7G-N1A-BD/N7G-N6A-BD elutes at 16.9 minutes.

**Column switching capillary HPLC-ESI+MS/MS of 1,N6-HMHP-dA.** MS instrumentation and chromatographic separation of 1,N6-HMHP-dA were as described in Chapter IV. Briefly, an Agilent 1100 capillary HPLC system was employed to load and trap 1,N6-HMHP-dA onto an SCX trap column for 10 min prior to backflushing with a Waters Acquity pump and loading of 1,N6-HMHP-dA on to a Synergi Hydro-RP (250 x 0.5 mm, 0.4 µm, Phenomenex) column for separation and elution into a Thermo-Finnigan TSQ Quantum Ultra mass spectrometer for analysis.

**Statistical analysis.** Statistical analyses were performed using Microsoft Excel spreadsheet analysis tools. A student’s t-test (2 sample assuming unequal variances) was used to determine p-values for female/male differences in adduct levels.

**5.3 Results**

Because all three stereoisomers of DEB are formed metabolically (R,R; S,S; and meso DEB), three stereoisomers of the DEB-DNA cross-links can potentially be formed (Scheme 5.1). Our current methods analyze R,R and S,S bis-N7G-BD isomers as a racemic mixture separate from meso bis-N7G-BD. N7G-N1A-BD adducts are converted to the corresponding N7G-N6A-BD adducts by forced Dimroth rearrangement (Scheme 5.1), because our previous studies demonstrated that this results in a greater sensitivity for *in vivo* sample analyses. Based on our *in vitro* studies, N7G-N1A-BD is the most abundant Gua-Ade cross-link of DEB, while little N7G-N6A-BD is formed at concentrations below 500 µM DEB. Therefore, the majority of N7G-N6A-BD adducts
being measured by our methods represent N7G-N1A-BD lesions formed \textit{in vivo}. \(1,N^6\)-HMHP-dA isomers are not separated by our current column switching HPLC methods.

5.3.1 Dose Response Curves.

The optimized HPLC-ESI\(^+\)-MS/MS methods described above were used to quantify \textit{bis}-N7G-BD cross-links and N7G-N1A-BD/N7G-N^6A-BD cross-links, and \(1,N^6\)-HMHP-dA adducts in liver DNA of B6C3F1 mice and F344 rats exposed to a range of BD concentrations (0 to 625 ppm) by inhalation for 10 days (Figures 5.1- 5.3). We found that \(S,S + R,R\) (racemic) \textit{bis}-N7G-BD was the most abundant bifunctional DEB-DNA adduct formed upon exposure to BD in both species, followed by \textit{meso} \textit{bis}-N7G-BD, N7G-N1A-BD/N7G-N^6A-BD, and \(1,N^6\)-HMHP-dA. The molar ratio of G-G DEB cross-link, G-A DEB cross-link, and \(1,N^6\)-HMHP-dA observed in current work (~10:1:0.14) is consistent with a lower reactivity of adenine nucleobases towards epoxide electrophiles and is similar to the molar ratios of N7-(2’,3’,4’-trihydroxybut-1’-yl)guanine (N7-THBG) and N^6-(2’,3’,4’-trihydroxybut-1’-yl)adenine (N^6-THBA) monoadducts reported previously. The ratios of the three bifunctional DEB-DNA adducts in DEB-treated DNA are ~12:1:0.35, suggesting there may be repair of \(1,N^6\)-HMHP-dA \textit{in vivo}.

Significant interspecies differences were observed between DEB-DNA adduct levels in mouse and rat DNA (Figures 5.1 and 5.2). Concentrations of racemic \textit{bis}-N7G-BD were 4-10 fold higher in mice than in rats at all exposure levels. While the shape of the \textit{bis}-N7G-BD dose response curve for mice appears curvilinear, bending downward slightly between 6.25 and 625 ppm BD, rat adduct levels reached a plateau at 62.5 ppm, suggesting that the metabolic activation of EB to DEB is saturated in this species.
(Figure 5.1). The only DEB-DNA crosslink observed at the lowest BD exposure (6.25 ppm) in mice was \( S,S^+ R,R \text{bis}-N7G-BD \) (0.32 ± 0.09 adducts/\( 10^7 \) nts). The lowest exposure at which measurable levels of \( \text{bis}-N7G-BD \) were observed in rats was 62.5 ppm, although we did not have access to tissues of animals exposed to BD amounts between 6.25 and 62.5 ppm.

A similar trend was observed for N7G-N1A-BD/N7G-N6A-BD, which was significantly more abundant in liver DNA of mice as compared to the corresponding rat tissue (Figure 5.2). The dose response curve for N7G-N1A-BD/N7G-N6A-BD is much steeper in the mouse than in the rat, which exhibits signs of metabolic saturation at exposures above 62.5 ppm BD (Figure 5.2). These results are fully consistent with the data for \( \text{bis}-N7G-BD \) (Figure 5.1). While similar curvilinear dose response curve was obtained for \( 1,N^6\text{-HMHP-dA} \) in BD-treated mice, adduct levels in rat DNA were below the detection limits of our method (0.015 \( 1,N^6\text{-HMHP-dA}/10^8 \) nts) at all exposures (Figure 5.3).
Figure 5.1  Dose response curves for racemic and meso bis-N7G-BD in liver DNA of female B6C3F1 mice and F344 rats exposed to 1,3-butadiene (0-625 ppm) for 2 weeks. ML = mouse liver DNA, RL = rat liver DNA
Figure 5.2 Dose response curves of N7G-N^6A-BD in liver DNA of female B6C3F1 mice and F344 rats exposed to 1,3-butadiene (0-625 ppm) for 2 weeks.
Figure 5.3 Dose response curves for the formation of $1,N^6$-HMHP-dA in liver DNA of female B6C3F1 mice exposed to 1,3-butadiene (0-625 ppm) for 2 weeks. The concentrations of $1,N^6$-HMHP-dA adduct in rat tissues was below our limit of detection.
5.3.2 Tissue distribution of bis-N7G-BD.

To analyze the tissue distribution of DEB-DNA adducts following exposure of laboratory mice and rats to BD by inhalation, bis-N7G-BD adduct levels were determined in liver, lung, kidney, brain, and thymus tissues. In both species, bis-N7G-BD levels were highest in the liver (Table 5.1). For BD-exposed mice, liver adduct levels (3.95 S,S + R,R bis-N7G-BD/10^7 nts) were about 3 fold higher than in the other tissues (0.38 – 1.35 S,S + R,R bis-N7G-BD/10^7 nts) (Table 5.1). In BD-exposed rats, liver adduct levels (0.36 S,S + R,R bis-N7G-BD/10^7 nts) were ~1.5 fold higher than in the other tissues (0.21 – 0.30 S,S + R,R bis-N7G-BD/10^7 nts) (Table 5.1).

5.3.3 Gender differences in DEB-DNA adduct formation.

Female mice develop tumors at lower BD exposure than males, indicating gender differences in susceptibility (111). It has been previously reported that when exposed to 62.5 ppm BD for 6 h, the concentration of DEB in the blood of female rats is 6 fold greater than in males (149). Similarly, the formation of DEB-specific globin adducts (N,N-(2,3-dihydroxy-1,4-butanediyl)-valine, pyr-Val) is 2-4 fold higher in female than in male rats exposed to 1,000 ppm BD (130). Therefore, we investigated potential gender differences in the formation of bis-N7G-BD adducts in the liver DNA of female and male mice and rats exposed to 625 ppm BD (Figure 5.4). We found that in both species, the amounts of S,S, R,R bis-N7G-BD were 2 - 2.5 fold higher in female animals as compared to males subjected to the same exposure conditions. Furthermore, the amounts of meso bis-N7G-BD adducts were 3-fold higher in female mouse liver DNA than the corresponding amounts in male mouse liver DNA (data not shown). Tissues were not available for full dose response analyses in male animals; however,
similar gender differences were observed in animals exposed to 200 ppm BD (Figure 5.4).

5.4 Discussion

Although BD is a known mutagen and carcinogen present ubiquitously in urban air, the exact mechanisms of its mutagenic and carcinogenic activity remain to be established. Furthermore, human BD exposure risk assessment is complicated by large interspecies differences in sensitivity to BD-induced cancer. BD is a potent carcinogen in mice, but is only a weak carcinogen in rats. B6C3F1 mice develop tumors at BD exposure concentrations three orders of magnitude lower than those that cause cancer in Sprague-Dawley rats (93;151;173). It has been proposed that the carcinogenic potency of BD in a given organism can be predicted from the relative amounts of the diepoxide metabolite, DEB, generated upon metabolic activation. However, in vivo formation and repair of bifunctional DEB-DNA adducts in laboratory rats and mice exposed to BD have not been previously investigated.

We have developed sensitive and specific HPLC-ESI+ -MS/MS methods which, for the first time, quantify the formation of DEB-specific DNA adducts in vivo following inhalation exposure to BD. Our results for dose-dependent formation of bis-N7G-BD and N7G-N1A-BD/N7G-N6A-BD in tissues of laboratory mice and rats exposed to 0-625 ppm BD by inhalation (Figures 5.1 - 5.2) reveal remarkable interspecies differences between adduct levels. For example, the concentrations of bis-N7G-BD adducts in mouse liver following 10 day exposure to 625 ppm BD were 10-
fold greater than in rats exposed at the same conditions (Figure 5.1). $1,N^6$-HMHP-dA adduct levels in rat DNA were below our detection limits.
Table 5.1  Tissue differences in the formation of racemic \textit{bis}-N7G-BD in female B6C3F1 mice and female F344 rats exposed to 625 ppm BD by inhalation for 2 weeks.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Racemic \textit{bis}-N7G-BD per $10^7$ nts in mouse</th>
<th>Racemic \textit{bis}-N7G-BD per $10^7$ nts in rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (N = 4)</td>
<td>3.95 ± 0.89</td>
<td>0.36 ± 0.23</td>
</tr>
<tr>
<td>Lung (N = 4)</td>
<td>1.35 ± 0.12</td>
<td>0.30 ± 0.01</td>
</tr>
<tr>
<td>Kidney (N = 4)</td>
<td>1.10 ± 0.13</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>Brain (N = 4)</td>
<td>0.38 ± 0.15</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>Thymus (N = 4)</td>
<td>1.15 ± 0.26</td>
<td>0.21 ± 0.06</td>
</tr>
</tbody>
</table>
Figure 5.4  Gender differences in the formation of racemic bis-N7G-BD in B6C3F1 mice and F344 rats exposed to 625 ppm BD, and mice exposed to 200 ppm BD by inhalation for 2 weeks. *Differences between female and male mice at 625 ppm and 200 ppm BD is statistically significant, p-values = 0.014 and 0.006. Difference between female and male rats is not statistically significant, p-value = 0.19.
Both \textit{bis}-N7G-BD and N7G-N1A-BD/N7G-N^6A-BD reach a plateau in rat after inhalation exposures above 62.5 ppm, possibly a result of P450 2E1 inactivation through phosphorylation or suicide inhibition of the protein by covalent binding of epoxide products to the active site. These results are consistent with a greater sensitivity of laboratory mice to BD-mediated carcinogenesis, suggesting that DEB-induced bifunctional DNA lesions play an important role in BD-mediated cancer.

Our results are in accord with previously published dose response data for DEB-specific hemoglobin adducts (pyr-Val), which revealed that DEB adduct levels in mice were 4-10x greater than levels in rats exposed to the same conditions. The dose response curves for both DEB-induced hemoglobin adducts and bifunctional DNA adducts (Figures 5.1 and 5.2) in the rat are supralinear, providing evidence for the saturation of metabolic activation pathways in this species following exposure to 62.5 ppm BD. In contrast, the dose response curves for the formation of DEB-specific DNA adducts in the mouse are curvilinear between 6.25 to 625 ppm BD, and do not show any signs of metabolic saturation (Figures 5.1 - 5.3).

Taken together, these results suggest that the interspecies differences in the carcinogenic potency of BD in rats and mice are related to metabolic differences. Mice form 5-fold more DEB than rats per unit of BD exposure, which is reflected in a higher efficiency for the formation of DEB-globin adducts and DEB-DNA adducts in this species (Table 5.2). As demonstrated in Table 5.2, the number of \textit{bis}-N7G-BD adducts per BD exposure level in mice is greatest following a low BD exposure, but exceeds the efficiency of DEB-mediated DNA cross-linking in the rat at all BD exposures examined.
Table 5.2. *Bis*-N7G-BD adduct levels in mouse liver DNA per unit dose of 1,3-butadiene. ND, not detected.

<table>
<thead>
<tr>
<th>BD exposure (ppm)</th>
<th>Racemic <em>bis</em>-N7G-BD adducts /10^6 nts/ppm BD in mouse liver DNA</th>
<th>Racemic <em>bis</em>-N7G-BD adducts /10^6 nts/ppm BD in rat liver DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6.25</td>
<td>0.51</td>
<td>ND</td>
</tr>
<tr>
<td>62.5</td>
<td>0.13</td>
<td>0.031</td>
</tr>
<tr>
<td>200</td>
<td>0.098</td>
<td>0.010</td>
</tr>
<tr>
<td>625</td>
<td>0.063</td>
<td>0.006</td>
</tr>
</tbody>
</table>
In addition to significant differences in carcinogenic sensitivity, rats and mice also differ in tissue specificity of BD-mediated tumor formation. In long-term inhalation studies, mice developed tumors of the lung, lymphatic system, liver, forestomach, and heart, while rats exhibited tumors in different tissues, including pancreas, testis, mammary gland, and thyroid gland. Our results presented in Table 5.1 indicate that these differences are not a result of tissue-dependent generation/accumulation of DEB.

In both species, DEB-DNA adduct levels were highest in the liver (Table 5.1), consistent with the high activity of cytochrome P450 enzymes which activate BD to DEB in this tissue. Bis-N7G-BD concentrations in extrahepatic tissues (lung, kidney, brain, and thymus) were 1.5-3-fold lower than those in liver DNA, but similar to one another. These results are consistent with a model in which DEB is formed primarily in the liver and is transported throughout the body to reach other tissues.

Several recent studies suggested that there may be gender differences in cancer susceptibility and BD metabolism in laboratory animals. In carcinogenesis studies on BD, female mice develop tumors at lower BD concentrations than males (111). Furthermore, female rats contained higher blood DEB concentrations than male rats following a single 6 h exposure to 62.5 ppm BD. Female rats also had 3-4 fold greater levels of pyr-Val hemoglobin adducts than male rats following a 90 day exposure to 1000 ppm BD. Our results presented here are consistent with these earlier findings.

Bis-N7G-BD adduct amounts in female rats and mice exposed to 625 ppm BD are 2-2.5-fold higher than in males (Figure 5.4). A similar trend is observed following lower BD exposure (200 ppm, Figure 5.4). In contrast, human epidemiology studies comparing male and female industry workers exposed to BD observed no significant
difference in gene mutations (e.g., *HPRT* mutant frequencies) or sister chromatid exchanges between male and female workers. Future studies of DNA and protein adducts in occupationally exposed humans are needed to determine whether gender differences in biomarker levels of BD exposure and metabolism are observed in humans.

The possible roles of *bis*-N7G-BD, N7G-N1A-BD/N7G-N^6^-A-BD, and *1,N^6^-HMHP-dA lesions investigated in the present work in the genotoxicity of DEB remain to be established. Several laboratories, including our group, have shown that *bis*-N7G-BD cross-links are formed preferentially within the 5’-GNC sequence context (105;156;186), with nucleosome structure having little effect on sequence selectivity of DEB-DNA cross-link formation. Interestingly, while the *S,S* stereoisomer of DEB produces a greater number of interstrand cross-links, all three DEB stereoisomers target 5’-GNC trinucleotides. Large local distortions of the DNA helix are required to accommodate 1,3-interstrand *bis*-N7G-BD lesions because the four-carbon tether length (6 Å) is much shorter than the spacing between the distal N7-dG atoms in 5’-GNC sequences of canonical B-DNA (8.9 Å). In addition, *meso* DEB is capable of inducing 1,2-intrastrand *bis*-N7G-BD lesions. Molecular dynamics simulations predict that the base stacking and hydrogen bonding interactions in the vicinity of 1,3-interstrand and 1,2-intrastrand *bis*-N7G-BD cross-links are disrupted as a result of twisting of the cross-linked residues with respect to the base-pairing plane. 3’-Exonuclease activity of *E. coli* Polymerase I is blocked one nucleotide ahead of the interstrand *bis*-N7G-BD lesions, consistent with an induced structural change in the vicinity of the cross-link. These
structural changes may be important for the recognition of \textit{bis}-N7G-BD adducts by DNA repair enzymes.

Another factor that must be considered when evaluating possible contributions of \textit{bis}-N7G-BD, N7G-N1A-BD, and \textit{1,N}^6-\text{HMHP-dA} to BD-mediated mutagenesis and cancer is differences in their hydrolytic stability. All N7-alkylguanine adducts are hydrolytically labile because of the intrinsic destabilization of the glycosidic bond when the N7 position of guanine is alkylated. Both glycosidic bonds of \textit{bis}-N7G-BD cross-links can be hydrolyzed, with a half-life in double stranded DNA of 147 h (interstrand) and 35 h (intrastrand). In contrast, spontaneous depurination of the N-7-guanine portion of N7G-N1A and N7G-N^6A DEB cross-links (Scheme 5.1) results in hydrolytically stable adenine adducts containing a butanediol cross-link to free guanine in one DNA strand and an abasic site (Ab) in the other (Scheme 5.2). If produced \textit{in vivo}, semi-depurinated interstrand cross-links may be important to DEB mutagenesis, because repair synthesis on either strand must proceed past the damaged nucleobase in the opposite strand. The \textit{1,N}^6-\text{HMHP-dA} adducts are hydrolytically stable.
Scheme 5.2 Hydrolytic stability of bifunctional DNA adducts.

bis-N7G-BD

Two abasic sites and release of bis-N7G-BD cross-link

N1A-N7G-BD or N6A-N7G-BD

Abasic site and bulky lesion at A

DNA stable
In summary, our results presented herein provide additional support to the hypothesis that DEB is the key metabolite largely responsible for the interspecies differences in sensitivity to BD-induced cancer. Our study provides a greater molecular detail of the consequences of interspecies metabolic differences by quantifying DNA lesions specific for DEB. This work also has implications for human risk assessment. The current industry BD exposure limits are set based on a mouse model because mice are the more sensitive species to BD-induced cancers. However, studies in liver microsomes suggest that mice form DEB at faster rates than both rats and humans. Human microsomes also have higher epoxide hydrolase activity than rats and mice, and therefore hydrolyze DEB more efficiently than both rat and mouse microsomes. Therefore, if DEB is responsible for BD-induced cancer in the mouse, a rat model of sensitivity may be more appropriate for setting the limits of human exposure.
VI. PERSISTENCE AND REPAIR OF BIFUNCTIONAL DNA ADDUCTS IN RODENTS EXPOSED TO 1,3-BUTADIENE BY INHALATION

6.1 Introduction

1,3-Butadiene (BD) is an important industrial chemical used widely in the production of plastics and rubber (91). It is also an environmental toxin present in automobile exhaust and cigarette smoke, leading to widespread exposure of human populations (2;92). BD is classified as a human carcinogen based on the results of laboratory animal exposure studies and human epidemiology studies in occupationally exposed workers (93;95). BD undergoes metabolic activation by cytochrome P450 monooxygenases to form reactive electrophiles, 3,4-epoxybutene (EB), 3,4-epoxy-1,2-butandiol (EBD), and 1,2,3,4-diepoxybutane (DEB) (Scheme 1.1). Although it is a relatively minor metabolite of BD, DEB is 50 – 100 fold more mutagenic than metabolites containing only one epoxide functionality (e.g. EB and EBD) and is therefore considered the ultimate carcinogenic species of BD (97;99). DEB is a bifunctional electrophile capable of forming DNA-DNA cross-links and exocyclic deoxyadenosine adducts (133;134;159). We have previously quantified two types of DNA-DNA cross-links, 1,4-bis-(guan-7-yl)-2,3-butanediol (bis-N7G-BD) and 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD), and an exocyclic adenine adduct, 1,N^6-(1-hydroxymethyl-2-hydroxy-1,3-propanediyl)-2’-deoxyadenosine (1,N^6-α-HMHP-dA), in tissues of animals exposed to BD by inhalation (Chapters II, III, and IV (69;70)). The goal of the present study was to investigate the persistence and repair of DEB-induced DNA-DNA cross-links and exocyclic adducts in vivo.
Persistence of DNA adducts \textit{in vivo} is a combination of their hydrolytic stability in DNA, and a lack of active repair. The majority of N7-guanine adducts, including \textit{bis}-N7G-BD, are hydrolytically unstable, as alkylation on the N7 of guanine forms a positive charge and destabilizes the \(\beta\)-glycosidic bond, leading to their spontaneous depurination and the formation of abasic sites (4,69). The \textit{in vitro} half-life of racemic \textit{bis}-N7G-BD adduct in calf thymus DNA is 81.5 h (133). In contrast, DEB-induced G-A cross-links, N7G-N1A-BD, are expected to be more stable as they are only partially hydrolyzed (at the N7G), leaving an abasic site opposite a bulky lesion to the N1 of adenine (Scheme 5.2) (134). Therefore, unless enzymatic repair mechanisms exist to remove N7G-N1A-BD \textit{in vivo}, they should persist and are likely to lead to mutations. The exocyclic DEB-dA adduct, \(1,N^6\)-\(\alpha\)-HMHP-dA, is also expected to be stable \textit{in vivo}, and lead to mutations if not repaired.

Several DNA damage repair pathways have evolved in living cells to help protect against alkylating agents. In base excision repair (BER), a glycosylase enzyme specifically removes damaged bases, leaving abasic sites (187). The abasic sites are cleaved on the 5' side by AP endonucleases and on the 3' side by deoxyribophosphodiesterases (187). The resulting gap is filled by DNA polymerases using the undamaged DNA strand as a template, and sealed by DNA ligase to yield undamaged DNA (187). An example of BER repair protein is methyl purine glycosylase (MPG), which specifically excises alkylated purines, including 3-methyladenine, 3-methylguanine, 7-methylguanine, hypoxanthine, and etheno-dA (Scheme 6.1) (10,187-189). There is evidence that this protein plays a role in the repair of N7-guanine nitrogen mustard monoadducts (190); therefore, we investigated the
Scheme 6.1 Structures of DNA lesions repaired (A) and not repaired by mpg (B).

A

\[
\begin{align*}
\text{m7G} & : \quad \begin{array}{c}
\text{H}_3\text{C} \\
\begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{O} \\
\text{N} \\
\text{H} \\
\text{N} \\
\text{N} \\
\end{array}
\end{array} \\
\text{m1G} & : \quad \begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{O} \\
\text{N} \\
\text{H} \\
\text{N} \\
\text{N} \\
\end{array} \\
\varepsilon\text{A} & : \quad \begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{H} \\
\text{N} \\
\text{N} \\
\end{array} \\
\text{EA} & : \quad \begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\end{array} \\
1,N^2-\varepsilon\text{G} & : \quad \begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{O} \\
\text{N} \\
\text{H} \\
\text{N} \\
\end{array} \\
\text{Hx} & : \quad \begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{OH} \\
\text{H} \\
\text{N} \\
\end{array}
\end{align*}
\]

B

\[
\begin{align*}
\text{M1G} & : \quad \begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{O} \\
\text{N} \\
\text{H} \\
\text{N} \\
\end{array} \\
\text{m1A} & : \quad \begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{NH}_2 \\
\text{N} \\
\text{H} \\
\text{N} \\
\end{array} \\
\end{align*}
\]
possibility that MPG recognizes DEB-induced N7-guanine cross-links. There is also evidence that MPG repairs $1,N^6$-ethenoadenine adducts (188;189); therefore, due to structural similarity of the $1,N^6$-α-HMHP-dA adduct, it may also be a substrate for repair by MPG. If $1,N^6$-HMHP-dA lesions, or the preceding N1-adenine monoadducts are substrates for MPG, higher adduct levels should be observed in animals deficient in this repair pathway.

Repair of interstrand DNA-DNA cross-links, such as bis-N7G-BD is difficult because they affect both strands of DNA, preventing DNA unwinding and strand separation (3). Furthermore, since both strands are damaged, neither one can be employed as a template for repair synthesis (3). There is some evidence that interstrand DNA-DNA cross-links can be repaired by nucleotide excision repair (NER), homologous recombination repair, and mismatch repair (187;191;192).

Nucleotide excision repair pathway recognizes large, bulky DNA adducts and lesions that distort DNA secondary structure (187). Repair endonucleases make incisions several nucleotides 5’ and 3’ of the damaged site, releasing a short oligonucleotide, and the resulting gap is filled by repair polymerases (187). Xeroderma Pigmentosum (XP) is a condition in which patients have a NER defect and are extremely sensitive to UV radiation due to the inability to repair UV-induced dT dimers (193). This condition can be caused by deficiencies in XPA, XPB, XPC, XPD, XPF, or XPG genes (193). The XPA gene codes for a protein involved in the recognition of local distortions in the DNA helix associated with adduct formation and signaling to other proteins to complete the excision (193). Since the formation of G-G and G-A cross-links by DEB is associated with DNA helix distortion, they are likely substrates
for NER \((106;134)\). Furthermore, NER may recognize partially depurinated G-A lesions generated from the spontaneous hydrolysis of N7-alkylated guanine (Scheme 1.3). This results in a bulky adenine lesion linked to free guanine base that may also be a substrate for NER (Scheme 3.2).

Direct repair protein AlkB is a non-heme iron dependent protein that oxidatively removes the side chains of DNA adducts such as N1-methyl-adenine (m1A), 3-methylcytosine (3MeC), 3-methyl-thymine (3MeT), and 1-methyl-guanine (1MeG), restoring the natural base \((194)\). It also recognizes exocyclic DNA lesions such as etheno-dA and ethano-dA, generating less mutagenic ring opened adducts \((9;195)\). Due to their structural similarity to known AlkB substrates, \(1,N^6\)-etheno-dA and \(1,N^6\)-ethano-dA, we hypothesized that \(1,N^6\)-HMHP-dA may be repaired by AlkB.

Here we present a study of the persistence and repair of three types of bifunctional DEB-DNA adducts, \(bis\)-N7G-BD, N7G-N1A-BD, and \(1,N^6\)-a-HMHP-dA (Scheme 5.1), in laboratory animals exposed to BD by inhalation. We also investigated a possible role of AlkB in the repair of \(1,N^6\)-a-HMHP-dA adducts \textit{in vitro}.

6.2 Experimental

Isotopically labeled internal standards, \(^{15}N_{10\text{-bis}}\)-N7G-BD, \(^{15}N1, {^{15}N2, {^{15}N3,}
^{13}C2\)-N7G-N1A-BD, and \(^{15}N1, {^{15}N3, {^{15}N7, {^{15}N9 -1,N^6\text{-HMHP-dA were prepared as previously described in Chapters II, III, and IV. All reagents were from Sigma (Milwaukee, WI) unless otherwise noted. PDE I, PDE II, DNAase were from Worthington Biochemical Corp. (Freehold, NJ), and the \textit{E.coli} AlkB protein was received from Dr. Chuan He (University of Chicago, Chicago, IL).}
Animals and treatment. Persistence studies- BD exposures were performed as previously reported (Chapter III, (70)) at the Lovelace Respiratory Research Institute (Albuquerque, NM). Briefly, B6C3F1 mice, or F344 rats (5 per group) were exposed to 625 or 1250 ppm BD, respectively, by inhalation for ten days (7 hours/day). Animals were euthanized either immediately at the end of the exposure period, or at 1 day, 3 days, 6 days, or 10 days post exposure. Liver tissues were collected, flash frozen, shipped to University of MN on dry ice, and stored at -80º C until DNA extraction.

Repair studies- BD exposures of mpg deficient animals and the corresponding controls were performed at the University of Texas-Medical Branch (Galveston, TX) as described above (Chapter II). In brief, C57BL/6 mice were exposed to 62.5 ppm BD for 10 days and Xpa deficient mice and matched controls were exposed to 625 ppm BD for 10 days at Lovelace Respiratory Research Institute (Albuquerque, NM).

DNA isolation. DNA was isolated using NucleoBond AXG500 anion exchange cartridges (Macherey-Nagel, Bethlehem, PA) according to manufacturer’s procedures. In brief, tissues (0.1 – 0.4 g) were homogenized in G2 buffer (20 mL) and incubated with RNAse and proteinase K at 50 ºC for 2 - 4 hours. Samples were loaded on NucleoBond AXG cartridges, washed, and eluted according to kit directions. DNA was precipitated by the addition of isopropanol. The DNA was spooled, washed 3 times with cold 70% ethanol, dried, and dissolved in Milli-Q water (500 µL). DNA purity and amounts were determined by UV spectrophotometry. Typical $A_{260}/A_{280}$ ratios were between 1.7 and 1.8, ensuring minimal protein contamination.

Sample hydrolysis and preparation of bis-N7G-BD. Bis-N7G-BD samples were prepared as described in Chapter II. In brief, DNA (100 µg) was spiked with
racemic and *meso* $^{15}$N$_{10}$-labelled internal standards (300 fmol each) and incubated at 70 °C for 1 hour. Following thermal hydrolysis, partially depurinated DNA was removed by ultrafiltration with Centricon YM-10 filters (Millipore Corp., Billerica, MA). The filtrates containing *bis*-N7G-BD and its internal standard were further purified by offline HPLC. A Zorbax Eclipse XDB-C18 (4.6 x 150 mm, 5 µm) column was eluted with a gradient of 0.4% formic acid in water (A) and acetonitrile (B). HPLC fractions containing *bis*-N7G-BD (12 – 13 min) were collected, dried under vacuum, and dissolved in 0.05% acetic acid (25 µL) for HPLC-MS/MS analysis.

**HPLC-ESI$^+$-MS/MS analysis of *bis*-N7G-BD.** A Zorbax Extend C18 column (3.5 µm, 150 x 0.5 mm) was eluted with a gradient of 0.05% acetic acid (A) and methanol (B). The gradient program was 0 to 10% B in 5 minutes, further to 60% over 10 minutes, and back to 0% B over 3 minutes. The HPLC flow rate was 10 µL/min, the column temperature was maintained at 40 °C, and the typical injection volume was 8 µL. With this solvent system, the retention time of racemic *bis*-N7G-BD was 9.9 min, while *meso* *bis*-N7G-BD eluted at 10.9 min.

**Sample hydrolysis and preparation of N7G-N1A-BD.** N7G-N1A-BD samples were prepared as described in Chapter III. Briefly, partially depurinated DNA backbone (from 100 µg DNA, see above) was recovered from YM-10 filters following neutral thermal hydrolysis, spiked with $^{13}$C$_1$,$^{15}$N$_3$-N7G-N1A-BD internal standard (300 fmol), and hydrolyzed in 0.1 M HCl (70 °C, 30 min). The hydrolysates were filtered through YM-10 filters, and N7G-N1A-BD (and its internal standard) was isolated by solid phase extraction as described in Chapter III. Samples were heated in base (1.0 M NH$_4$OH at 70 °C for 16 h) to force Dimroth rearrangement of N7G-N1A-BD to N7G-
N^6-A-BD (Chapter III, Figure 3.). Ammonium hydroxide was removed under vacuum, and samples were dissolved in 0.05% acetic acid (25 µL) prior to HPLC-MS/MS analysis.

**nanoLC-nanoESI^+**-MS/MS analysis of N7G-N1A-BD. Waters nanoAquatix UPLC system (Waters Corp., Millford, MA) interfaced to a Thermo-Finnigan TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific Corp., Waltham, MA) was used in all analyses. HPLC solvents were 0.01% acetic acid (Fluka, LC-MS grade) in water (A) and a 1:1 mix of LC-MS grade methanol and acetonitrile (Sigma, Milwaukee, WI) (B). Samples (4-8 µL) were loaded on a trapping column (Symmetry C18 nanoAcquity, 0.18 x 20 mm) for 1 min at 0% B. Chromatographic separation was achieved using an Atlantis dC18 (75 µm x 100 mm) column eluted at a flow rate of 0.35 µL/min. The gradient program went from 0 to 12% B in 2 minutes, then to 25% B over 7 minutes, and further to 45% B over 15 minutes. The column was equilibrated at 0% B for at least 12 minutes before each run. Under these conditions, N7G-N^6-A-BD isomers eluted as a single peak at 16.9 minutes. The mass spectrometer was operated in selected reaction monitoring mode of transitions corresponding to loss of guanine from protonated molecules of the adduct: m/z 373.1 [M + H]^+ \rightarrow m/z 222.1 [M + H - Gua]^+ and m/z 377.1 [^{13}C_1,^{15}N_3-M + H]^+ \rightarrow m/z 222.1 [M + H - [^{13}C_1,^{15}N_3]Gua]^+.

**Sample hydrolysis and purification of 1,N^6-α-HMHP-dA.** Liver DNA (100 µg) was spiked with ^15N_4-1, N^6-HMHP-dA internal standard (27.5 fmol). DNA was digested with DNase I (35 U/100 µg DNA), PDE I (70 mU/100 µg DNA), PDE II (80 mU/100 µg DNA), and alkaline phosphatase (14.6 U/100 µg DNA) in 10 mM Tris-HCl/15 mM MgCl₂ at 37 °C for 18 h. The DNA hydrolysates were purified by...
Centricon ultrafiltration (YM-10) and solid phase extraction on Extract Clean Carbo (Grace Davidson, Deerfield, IL) cartridges (3 mL). SPE cartridges were prepared by washing with methanol (2 x 3 mL) and water (2 x 3 mL) prior to loading samples in water (1 mL). Samples were washed with water (3 mL) and 5 % methanol (3 mL) prior to elution with 30 % methanol (3mL). The 30 % methanol elution was dried under vacuum, resuspended in 25 µL 0.05% acetic acid, and 8 µL was injected for LC-MS/MS analysis.

**Column Switching HPLC-ESI+−MS/MS.** Column switching methods were as reported in Chapter IV. In brief, trapping was achieved with water and an SCX (Waters Corp., Milford, MA) trap column (300 Å) that was backflushed with 0.05% acetic acid to elute 1,N^6-α-HMHP-dA onto the analytical column, a Synergi Hyrdo-RP (250 x 0.5 mm, Phenomenex). 1,N^6-HMHP-dA was eluted with a solvent system of 0.05% acetic acid (A) and methanol (B) at 10 µL/min. The gradient was increased linearly from 0.5 % B to 5% B in 5 min, and further to 20% in 10 min. The mass spectrometer was operated in selected reaction monitoring mode of the neutral loss of deoxyribose from protonated molecules of 1,N^6-HMHP-dA (m/z 338.1 → 222.1, and m/z 342.1 → 226.1 for internal standard).

**AlkB repair of 1,N^6-HMHP-dA in vitro.** Calf thymus DNA (500 µg/500 µL in water) was treated with racemic DEB (1 mM) at 37 ºC for 24 h. The unreacted DEB was extracted with diethyl ether (5 x 400 µL). To the DEB-treated DNA (500 µg) was added HEPES-KOH (50 mM, pH 8), Fe(NH₄)(SO₄)₆H₂O (75 µM), α-ketoglutarate (2 mM), BSA (35 µg), ascorbate (2 mM), and *E. coli* AlkB (1400 pmol). The AlkB repair reaction was conducted at 37 ºC. Aliquots (90 µL) were removed at 0, 2, 5, 8, 15, 30,
45, and 90 min. The reactions were quenched with EDTA (11 mM), and samples were immediately frozen at -20 ºC. Each sample was spiked with internal standard (55 fmol), enzymatically digested, filtered through YM-10 membranes, and purified by SPE on Extract Clean Carbo cartridges as described above for the *in vivo* samples. Repair samples were analyzed by capillary HPLC-ESI-MS/MS method described below.

**Hypercarb HPLC-MS/MS Analysis of AlkB repair samples.** A Hypercarb column (100 x 0.5 mm, Thermo) was eluted with a gradient of 0.05% acetic acid (A) and ACN (B). A linear gradient of 0 to 20% B in 30 minutes was employed at a flow rate of 14 µL/min at 25 ºC. Under these conditions 1,N$^6$-α-HMHP-dA eluted at 21.5 min and 1,N$^6$-γ-HMHP-dA eluted at 26 min. The mass spectrometer was operated in the SRM mode as described above for the *in vivo* samples. Data was analyzed by plotting percent 1,N$^6$-HMHP-dA remaining in DNA versus time.

### 6.3 Results

#### 6.3.1 Persistence of bifunctional BD DNA adducts *in vivo*.

As expected, *bis*-N7G-BD adducts did not persist in DNA due to their spontaneous depurination. By 6-10 days post BD exposure, the amounts of *bis*-N7G-BD were 10 fold lower than those immediately after exposure (Figure 6.1). The half-life of racemic *bis*-N7G-BD in mouse and rat liver DNA was 48 and 50 h, respectively. The half-life of *meso* *bis*-N7G-BD in mice was slightly lower (38 h), probably because *meso* DEB forms more intrastrand cross-links, which are less hydrolytically stable than the corresponding interstrand cross-links formed from *S,S* and *R,R* DEB (106). The *in vivo*
*bis*-N7G-BD half-lives are slightly shorter than the half-life observed *in vitro* (81.5 h) (133), suggesting that there may be some active repair mechanisms *in vivo*.

Unlike *bis*-N7G-BD, N7G-N1A-BD levels did not decrease in mice or rats post exposure, but remained constant over the time analyzed (up to 240 and 144 h, respectively) (Figure 6.1). 1,N⁶-HMHP-dA also appears to persist in mouse liver DNA (Figure 6.1a), while its levels in rat liver DNA were below our current LOQ (0.01 1,N⁶-HMHP-dA/10⁷ nts).
Figure 6.1 Persistence of bifunctional DEB-DNA adducts in mouse and rat liver DNA. (A) Female B6C3F1 mice were exposed to 625 ppm BD for 2 weeks and tissues were collected 2, 72, or 240 h post exposure. (B) Female F344 rats were exposed to 1250 ppm BD for 2 weeks, and tissues were collected 2, 24, 72, or 144 h post exposure.
6.3.2 Effect of mpg on BD bifunctional DNA adduct levels.

We hypothesized that a base excision repair mechanism such as mpg may play a role in the repair of bis-N7G-BD and N7G-N1A-BD due to similarities in the structures of N7-HEB-dG (precursor to bis-N7G-BD and N7G-N1A-BD, Scheme 6.2) and the structure of N7-methyl-guanine (m7G Scheme 6.1), a known substrate for mpg. However, our results indicate that there was no significant difference between mpg deficient and proficient animals in bis-N7G-BD, N7G-N1A-BD cross-link levels, suggesting that these adducts/adduct precursor monoadducts are not repaired by mpg. Perhaps the HEB sidechain (butenyl as compared to methyl) is too large for mpg protein active site.

Similarly, we hypothesized that 1,N⁶-HMHP-dA would be a substrate for mpg repair, due to similarity in its structure to other 1,N⁶-exocyclic-dA adducts, EA and εA (Scheme 6.1) that are repaired by mpg (10). Again however, there was no significant difference in 1,N⁶-HMHP-dA adduct levels in mpg deficient and proficient animals. This may be due to the larger size of the 1,N⁶-HMHP-dA six-membered exocyclic ring as compared to the five-membered rings of EA and εA. In fact, a similar observation was made for exocyclic-dG lesions. 1,N²-εG (Scheme 6.1A), containing a five-membered exocyclic ring is repaired by mpg, while pyrimido[1,2-α]purin-10(3H)-one (M1G) (Scheme 6.1B) containing a six-membered exocyclic ring is not (10). Another important difference between structures of 1,N⁶-HMHP-dA and EA/εA is the presence of a positive charge on the N1 of adenine in 1,N⁶-HMHP-dA. 1-methyladenine (m1A, Scheme 6.1B) lesions also have a positive charge on the N1 of adenine, and this adduct
is also not repaired by mpg; therefore, positive charge at this position may not be favorable for mpg protein active site (189).
Scheme 6.2 Summary of bifunctional DEB-DNA adduct repair by mpg, xpa, and AlkB.
Table 6.1 Concentrations of BD-induced bifunctional DNA adduct levels in liver DNA from *mpg* proficient and deficient animals exposed to 62.5 ppm BD for 2 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Racemic bis-N7G-BD/10^7 nts*</th>
<th>Meso bis-N7G-BD/10^7 nts</th>
<th>N7G-N1A-BD/10^7 nts</th>
<th>1,N^6-HMHP-dA/10^7 nts</th>
</tr>
</thead>
<tbody>
<tr>
<td>mpg -/- 62.5 ppm (n = 4)</td>
<td>1.13 ± 0.35</td>
<td>0.12 ± 0.033</td>
<td>0.086 ± 0.012</td>
<td>0.007 ± 0.009</td>
</tr>
<tr>
<td>mpg +/- 62.5 ppm (n = 4)</td>
<td>1.13 ± 0.23</td>
<td>0.096 ± 0.019</td>
<td>0.084 ± 0.011</td>
<td>0.011 ± 0.006</td>
</tr>
</tbody>
</table>

*adduct levels in *mpg/-/* animals were not significantly different from *mpg +/-/* animals. Racemic *bis*-N7G-BD (p-value = 0.87), *meso* *bis*-N7G-BD (p-value = 0.40), N7G-N1A-BD (p-value = 0.18), or *1,N^6*-HMHP-dA (p-value = 0.72).
6.3.3 Effect of XPA on BD bifunctional DNA adduct levels.

We hypothesized that NER plays a role in repair of bifunctional DEB-DNA adducts due to structural similarities to other substrates of NER. For example, cyclobutane thymine dimers, cisplatin 1,2-d(GpG) [CP-d(GpG)] cross-links, and bulky α-(N²-deoxyguanosiny)tamoxifen (Scheme 6.3) are substrates for NER (10). Several exocyclic DNA adducts, 1,N²-propanoguanine (PdG) and M1G (Scheme 6.3) are also substrates for NER, suggesting the 1,N⁶-HMHP-dA may also be a substrate (10). However, there was no significant difference between bis-N7G-BD and N7G-N1A-BD cross-link concentrations in XPA deficient and proficient animals (Table 6.2). This data suggests that these adducts are not repaired by NER, or that the XPA protein does not play an important role in excision of these adducts.

6.3.4 Repair of 1,N⁶-α-HMHP-dA and 1,N⁶-γ-HMHP-dA by AlkB.

We hypothesized that 1,N⁶-HMHP-dA lesions may be recognized and repaired by AlkB protein because of their similary to known AlkB substrates, 1,N⁶-ethano-dA and 1,N⁶-etheno-dA. Indeed, HPLC-MS/MS analysis of DEB-treated calf thymus DNA revealed that the levels of 1,N⁶-HMHP-dA adducts decreased rapidly upon incubation with purified AlkB protein. The α and γ isomers of 1,N⁶-HMHP-dA were quantified separately, and a faster repair rate was observed for 1,N⁶-α-HMHP-dA (Figure 6.2). Nearly 80% of 1,N⁶-α-HMHP-dA adducts were repaired within 60 minutes, while only 30 - 40% of 1,N⁶-γ-HMHP-dA was repaired in the same time frame (Figure 6.2). This difference is likely due to a greater steric bulk at the γ-carbon in 1,N⁶-γ-HMHP-dA, which is the target site for AlkB-mediated oxidation (Scheme 4.1).
Scheme 6.3 Structures of DNA adducts repaired by nucleotide excision repair.
Table 6.2  Concentrations of BD- induced bifunctional DNA adducts in liver DNA of *xpa* proficient and deficient mice exposed to 62.5 ppm BD for 2 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Racemic bis-N7G-BD/10^7 nts*</th>
<th>Meso bis-N7G-BD/10^7 nts</th>
<th>N7G-N1A-BD/10^7 nts</th>
<th>1,N^6-HMHP-dA/10^7 nts</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPA +/- 625 ppm (n = 4)</td>
<td>1.19 ± 0.51</td>
<td>0.055 ± 0.022</td>
<td>0.14 ± 0.04</td>
<td>0.036 ± 0.017</td>
</tr>
<tr>
<td>XPA -/- 625 ppm (n = 4)</td>
<td>1.14 ± 0.53</td>
<td>0.094 ± 0.02</td>
<td>0.16 ± 0.02</td>
<td>0.041 ± 0.018</td>
</tr>
</tbody>
</table>

* The concentrations of racemic bis-N7G-BD (p-value = 0.63), meso bis-N7G-BD (p-value = 0.20), N7G-N1A-BD (p-value = 0.44), and 1,N^6-HMHP-dA (p-value = 0.67) in XPA-/- animals were not significantly different from XPA+/+ animals.
Figure 6.2 Time course for the *in vitro* repair of $1,N^6$-HMHP-dA adducts present in DEB-treated calf thymus DNA by purified *E. coli* AlkB repair protein.
Discussion

1,3-Butadiene is a high volume industrial chemical used to synthesize plastics and rubber, leading to occupational exposure of workers in the US and worldwide (91). It is also an environmental pollutant present in urban air (2; 92). DEB is the most mutagenic metabolite of BD, hypothesized to be the ultimate carcinogenic species of BD based on its potent genotoxicity and the ability to cause bifunctional DNA adducts (97; 148; 153; 196). Our laboratory has developed quantitative methods for three DEB-specific DNA adducts: bis-N7G-BD, N7G-N1A-BD, and 1,N6-HMHP-dA, as biomarkers of BD metabolism to DEB (Chapters II, III, and IV) (69; 70). We found that immediately after BD exposure, bis-N7G-BD is the most abundant adduct, followed by N7G-N1A-BD, and 1,N6-HMHP-dA (Table 4.2). Bifunctional DEB-DNA adducts were predicted to have different stabilities in DNA because alkylation of the N7 of guanine yields unstable adducts that spontaneously depurinate, while alkylation of the N1 and N6 of adenine form hydrolytically stable adducts (4). In the present work, we investigated the persistence of DEB-induced adducts in DNA extracted from animals exposed to BD and possible repair mechanisms for the removal of the adducts from DNA.

We found that among the three DEB-DNA lesions, bis-N7G-BD was the least persistent adduct (Figure 6.1). This was expected because of the hydrolytic instability of the glycosidic bond of N7-guanine adducts (4; 106; 133). The half-life of bis-N7G-BD in vivo (48 h), however, was shorter than its half-life observed in vitro (81 h) (4; 133), suggesting that there may be active repair mechanisms for bis-N7G-BD in vivo.

In contrast, the levels of N7G-N1A-BD and 1,N6-HMHP-dA in mouse liver DNA
remained steady up to 10 days post exposure, suggesting that these adducts persist in tissues and could be responsible for the mutagenicity/cytotoxicity of BD.

In the second part of this study, we tested the ability of three DNA repair mechanisms, BER, NER, and oxidative demethylation (AlkB) to recognize and repair DEB-DNA adducts. Methyl-purine glycosylase repairs 7-methyl guanine adducts (Scheme 6.1) (4;188;189), we therefore hypothesized that it may be able to remove N7-(2-hydroxy-3,4-epoxy-butyl)guanine monoadduct, the precursor to both bis-N7G-BD and N7G-N1A-BD (Scheme 6.2), reducing the concentration of bifunctional DEB adducts in vivo. However, the same concentrations of bis-N7G-BD and N7G-N1A-BD were observed in mpg deficient and proficient mice exposed to BD (Table 6.1). MPG has also been shown to repair 1,N6-etheno-dA adducts (Scheme 6.1) (4;188;189), thus we hypothesized that it may also repair the 1,N6-HMHP-dA adducts of DEB (Scheme 6.2). However, little difference between 1,N6-HMHP-dA concentrations was observed in mpg deficient and proficient mice (Table 6.1). Lee et al. recently reported that N1-methyl-dA is not a substrate for MPG, perhaps due to its positive charge at the N1 position (Scheme 6.1) (4;189). 1,N6-α-HMHP-dA similarly contains a positive charge at N1, and this may be responsible for the lack of its repair by MPG. These results are consistent with data from Wickliffe in which no increase in hprt mutation frequencies of the same animals exposed to EB was observed (unpublished data).

NER is responsible for removing bulky and DNA distorting adducts, such as cyclobutane thymine dimer, cisplatin 1,2-d(GpG) intrastrand cross-link, 1,N2-propanoguanine (PdG), pyrimido[1,2-α]purin-10(3H)-one (M1G), and α-(N2-deoxyguanosinyl)tamoxifen (Scheme 6.3) (10). Both interstrand and intrastrand bis-
N7G-BD cross-links of DEB are predicted to distort the DNA helix (106). 1,N\(^6\)-HMHP-dA is a bulky adduct containing a substituted propanoexocycle, thus we hypothesized that it may also be repaired by NER (10). However, we did not see any effect of XPA on the concentrations of DEB-induced cross-links and exocycles (Table 6.2).

A possible explanation for these results is that XPA may be required to recognize intrastrand cross-links, but it may not be able to recognize DNA-DNA interstrand cross-links such as bis-N7G-BD and N7G-N1A-BD. Studies comparing normal human fibroblasts to those deficient in XPA or XPF have shown that XPA deficient cells are more sensitive to UV-induced intrastrand cross-links, while XPF deficient cells were more sensitive to cross-links formed from the nitrogen mustard mechlorethamine (197). There is evidence that mechlorethamine induces mainly 1,3-interstrand guanine-guanine and guanine-adenine cross-links (198), similar to those induced by DEB (134). Another possibility is that DEB adducts are repaired by NER, but do not require XPA protein to be recognized. For example, Wickliffe et al. have observed increased mutation frequencies in mice deficient in XPC treated with EB (the metabolic precursor to DEB, Scheme 1.1) as compared to wild-type mice at the same exposure (199). This data suggests that promutagenic adducts of DEB are repaired by NER, requiring XPC. Further studies are required to determine the role of NER in the repair of bifunctional DEB-DNA adducts.

AlkB protein is known to oxidatively dealkylate several methylated DNA adducts, 1-MeA, 3-Me-C, 3-Me-T, and 1-Me-G, restoring normal nucleobases (194). It also oxidatively repairs 1,N\(^6\)-etheno-dA adducts, therefore we suspected that it may repair 1,N\(^6\)-HMHP-dA as it is structurally similar to etheno-dA (Scheme 6.1) (9).
$1,N^6$-HMHP-dA–containing calf thymus DNA was incubated with AlkB, we observed reduced adduct levels over time, suggesting that $1,N^6$-HMHP-dA is repaired by AlkB (Figure 6.2). Interestingly, $1,N^6$-$\alpha$-HMHP-dA isomer was repaired more efficiently than $1,N^6$-$\gamma$-HMHP-dA. We hypothesize this is due to a greater steric bulk at the $\gamma$ position of $1,N^6$-$\gamma$-HMHP-dA as compared to the $\alpha$ isomer (Scheme 4.1). Oxidation of the $\gamma$ position of $1,N^6$-HMHP-dA may lead to ring opening, producing a less mutagenic $N^6$-adenine lesion (Scheme 6.1). While the rapid repair we observed by AlkB in vitro is inconsistent with our in vivo persistence data, the in vitro experiments were performed with E.coli AlkB protein, which is known to be more promiscuous than the human homolog ABH2 (194).

In summary, we have identified two DEB-specific DNA adducts, N7G-N1A-BD and $1,N^6$-HMHP-dA, formed in vivo following BD exposure that persist in DNA over time. Thus these adducts, N7G-N1A-BD and $1,N^6$-HMHP-dA, may be involved in the mutagenicity of BD. Further experiments are in progress in our laboratory to prepare oligonucleotides with site-specific adducts to investigate their ability to be bypassed by DNA polymerases and induce mutations.
VII. SUMMARY AND CONCLUSIONS

1,3-Butadiene is a known animal and human carcinogen (95; 146-148). However, it is also an important industrial chemical used extensively in the rubber and plastic industries, and is present in urban air as a combustion product from automobile exhaust and cigarette smoke (2; 91; 92). Striking interspecies differences have been observed in animal BD-inhalation studies where mice were much more susceptible to tumor formation than rats, complicating human risk assessment (93; 94). Mice developed tumors following exposure to 6.25 ppm BD, while rats did not show signs of tumorigenesis until 1000 ppm BD exposures (93; 94). The observed interspecies differences in tumorigenicity of BD are hypothesized to be due to differences in metabolic activation of BD to reactive electrophilic metabolites (151).

BD is metabolized by cytochrome P450 monooxygenases to three epoxide metabolites (108; 151; 163). The first epoxidation of BD yields 3,4-epoxy-1-butene (EB), which can be further oxidized to 1,2,3,4-diepoxybutane (DEB). EB and DEB can be detoxified by epoxide hydrolase, to 1-butene-3,4-diol and 3,4-epoxy1,2-butanediol (EBD), respectively (Scheme 1.1) (108; 151; 163). All three epoxide metabolites of BD (EB, DEB, and EBD) can react with nucleophilic sites in biomolecules including DNA and proteins to form covalent adducts (160). DEB is, however, the most mutagenic metabolite. DEB induces sister chromatid exchanges, chromosomal aberrations, and has a mutation spectra similar to that of BD (97; 98; 153). BD and DEB both induce point mutations at A:T base pairs, while this is not the case for the other BD metabolites, EB and EBD (98). Therefore, DEB is considered the ultimate carcinogenic metabolite of BD primarily responsible for its deleterious biological effects.
Due to widespread human exposure to BD, from both occupational sources and environmental exposures from urban air, there is a great need for mechanism-based biomarkers of BD. While DEB is known to be mutagenic, specific DNA adducts responsible for the mutagenicity have not been identified. Several groups have studied BD-DNA adducts; however, most of the previous studies have focused on DNA monoadducts such as THBG (Chart 1.2). DNA monoadducts are formed mainly from EB and EBD, which are significantly less mutagenic than DEB. These studies revealed that the amount of DNA monoadducts, THBG and EB-Gua, in BD-exposed laboratory animals do not exhibit large interspecies differences like those observed in tumor studies, suggesting that BD metabolites other than EB and EBD are responsible for the carcinogenicity of BD. Based on its potent genotoxicity, DEB is a likely metabolite involved in the carcinogenicity of BD. Therefore, there is a great need for DEB-specific biomarkers in vivo.

The Swenberg laboratory at UNC-Chapel Hill has recently developed a DEB-specific hemoglobin biomarker, N,N-(2,3-dihydroxy-1,4-butadiyl)-valine (pyr-Val). While protein adducts can be used to measure DEB levels in blood following BD exposures, they are not involved in the mechanism of BD-induced cancer and cannot be used to examine tissue distribution of DEB. Our laboratory has discovered three DEB-specific bifunctional DNA adducts, bis-N7G-BD, N7G-N1A-BD, and $N^6$-HMHP-dA (Scheme 5.1). While the mutagenicity of these adducts remains to be established, they can be used to investigate interspecies differences in DEB concentrations available for binding to DNA (Chapter V), as well as persistence and repair DEB-DNA adducts (Chapter VI). Taken together, these analyses should gain
insight into the identities of DEB-DNA adducts leading to the observed mutations and other adverse biological effects of BD.

Stable isotope dilution mass spectrometry methodology was selected for adduct quantitation. As described in Chapter 1.5, IDMS is the most selective, sensitive, and accurate method available for quantitative analysis of DNA adducts in vivo (16). We observed significant interspecies differences in the concentrations of bis-N7G-BD and N7G-N1A-BD cross-links in tissues of mice and rats exposed to BD by inhalation (Chapter V) (74). The levels of bis-N7G-BD and G-A DEB cross-links in the liver were 4-10 fold greater in mice than in rats exposed to the same concentrations of DEB. 1,N\textsuperscript{6}-HMHP-dA lesions were observed only in mice as their amounts in rats were below the detection levels of our current methods. Our molecular dosimetry studies (Figure 5.1-5.2) revealed a saturation of DEB adduct formation in rats following exposure to 62.5 ppm BD. This metabolic saturation of DEB formation in rats may explain the observed interspecies differences in tumorigenesis (130). In both species, bis-N7G-BD was by far the most abundant bifunctional adduct observed (93% of total DEB adducts in mice and 84% in rats), followed by N7G-N1A-BD (6% in mice, 16% in rats) and 1,N\textsuperscript{6}-HMHP-dA (1%, observed only in mice). As mentioned earlier (Chapter I) three isomers of DEB (R,R; S,S; and meso) are formed upon metabolism of BD by P450 monooxygenases, therefore, there are potentially three stereoisomers of each bifunctional DEB-DNA adduct. We analyzed bis-N7G-BD and 1,N\textsuperscript{6}-HMHP-dA adducts formed from R,R and S,S DEB as a racemic mixture separate from the corresponding adducts formed by meso DEB. For both types of adducts, the in vivo concentrations of the isomers formed from racemic DEB were more abundant than
those from meso DEB (~10:1 for bis-N7G-BD and ~3:1 for 1,N^6-HMHP-dA) (Figure 2.4). In contrast, no stereoselective adduct formation was observed in vitro where DNA was exposed to the three DEB isomers individually (Figures 3.4 and 4.3), ruling out any differences in reactivity of DEB isomers towards DNA nucleobases. Therefore, the observed sterochemical composition of DEB-DNA adducts is likely due to differences in DEB isomer concentrations, or differences in stability of the stereoisomers of bifunctional DEB-DNA adducts.

Besides large interspecies differences in BD exposure required for tumor formation, there are also species differences in tumor site formation (93). Mice develop tumors of the lung, heart, and hematopoietic systems, whereas rats develop cancer of the pancreas, uteri, and testes (93). Therefore, we examined tissue distribution of the DEB-DNA adducts. DNA was extracted from liver, lung, kidney, thymus, and brain tissue of rats and mice exposed to 625 ppm BD for 2 weeks. In both species, the highest concentration of bis-N7G-BD adducts (3.95 adducts /10^7 nts) was in the liver, consistent with high activity of P450 monooxygenase P450 activity in this tissue (Table 5.1). All other tissues examined similar adduct levels, suggesting that DEB is formed in the liver and is transported to the other tissues by blood.

Although bis-N7G-BD is the most abundant bifunctional adduct of BD, it does not persist in DNA due to its spontaneous depurination, making it an unlikely adduct to be responsible for BD mutagenicity. Furthermore, there is an increase in A \rightarrow T transversion mutation in human lymphoblasts exposed to DEB, suggesting that adenine adducts play a key role (98). In our persistence studies described in Chapter VI, the half-life of bis-N7G-BD cross-links was \sim 48$ h, while N7G-N1A-BD and 1,N^6-HMHP-
dA were persistent for up to 240 h. Since the N7G-N1A-BD and \(1,N^6\)-HMHP-dA adducts involve adenine nucleobases, they have a potential to induce A \(\rightarrow\) T transversions if not repaired. In Chapter VI, we investigated possible repair pathways for the removal of bifunctional DEB-DNA adducts using transgenic animals deficient in specific DNA repair pathways and purified repair proteins.

Initially we tested a base excision repair protein, N-methyl purine glycosylase (MPG). While MPG repairs N7-methyl-adenine, N7-methylguanine, and εdA adducts, it has also been shown to be involved in the repair of N7-guanine nitrogen mustard monoadducts (190). Therefore, we hypothesized N7-HEB-guanine monoadducts (precursors to both \(bis\)-N7G-BD and N7G-N1A-BD, Scheme 5.1) would also be substrates for MPG. Due to its structural similarity to εdA, \(1,N^6\)-HMHP-dA was also a likely substrate for repair by MPG. To examine the potential role of MPG, bifunctional DEB-DNA adduct levels were determined in \(mpg\) deficient and proficient mice exposed to 1,3-butadiene. No differences were observed (Table 6.1, Chapter VI), however, suggesting the adducts are not repaired by MPG. Similar results were obtained for animals deficient in nucleotide excision repair pathways. The concentrations of bifunctional DEB-DNA adducts were similar in DNA from \(xpa\) deficient and proficient mice (Table 6.2, Chapter VI).

The last DNA repair pathway studied was AlkB, an oxidative demethylation repair protein shown to repair εdA adducts (9). We hypothesized that AlkB would also repair exocyclic DEB-dA adducts based on their structural similarity to εdA. While knockout animals for this gene are not available, we did have access to purified \(E.coli\) AlkB repair protein. DEB-treated DNA was incubated with the AlkB protein, in the
presence of α-ketoglutarate, Fe(NH₄)(SO₄)6H₂O, and ascorbate; 1,N⁶-HMHP-dA adduct levels were determined. We did observe repair of 1,N⁶-HMHP-dA by AlkB (Figure 6.2), however, this is not completely consistent with our persistence data showing that 1,N⁶-HMHP-dA adducts persist in mouse liver up to 10 days post 625 ppm BD exposure (Figure 6.1). It is important to note that these repair experiments were performed with the E.coli protein, and it remains to be determined whether the human analog of AlkB, ABH2 (194), can repair 1,N⁶-HMHP-dA.

In summary, our studies have established three DEB specific DNA biomarkers that will be useful in BD risk assessment. Quantitative HPLC-ESI-MS/MS methods were developed for bis-N7G-BD, N7G-N1A-BD, and 1,N⁶-HMHP-dA (Chapters I, II, and III, Scheme 5.1). We have used our methods to analyze the molecular dosimetry of DEB-DNA adducts in mice and rats exposed to BD by inhalation (Chapter V), identify the stability of the adducts in vivo (Chapter VI), and investigate their possible repair mechanisms (Chapter VI). This work represents the first DEB-specific DNA biomarkers of BD metabolism to DEB. Our results suggest that interspecies differences in BD-mediated mutagenicity and carcinogenicity arise from differences in the extent of metabolism of BD to DEB. Furthermore, because of their persistence in vivo and the inability of BER and NER to repair N7G-N1A-BD and 1,N⁶-HMHP-dA adducts, they are likely to be involved in the mutagenicity of DEB and BD.
VIII. FUTURE WORK

8.1 Quantification of depurinated DNA-DEB adducts in smoker’s urine.

Our previous studies have focused on the quantitative analysis of bifunctional DNA adducts of BD in animal tissues. However, BD metabolism and DNA adduct formation in humans remains to be elucidated. Because human tissues are not readily available, we are interested in analyzing BD adducts in urine. As mentioned earlier, N7-guanine adducts spontaneously depurinate and are excreted as free base conjugates. Due to the presence of large amounts of BD in cigarette smoke (25 – 70 µg/cigarette) (2), smokers are exposed to BD on a daily basis. Therefore, we will quantify depurinated N7-guanine adducts of BD (bis-N7G-BD and THBG) in smoker’s urine.

Many components of tobacco products have been studied and found to be carcinogenic (2). It is well documented that tobacco use increases risk for cancer, especially lung cancer (200). However, only one out of ten smokers will develop lung cancer (201). Ethnic/racial differences have been observed in smoking-induced cancer susceptibility, with African American and Pacific American smokers exhibiting a higher incidence of lung cancer than European and Asian Americans (202). We hypothesize that the observed differences in lung cancer incidence is a result of racial/ethnic differences in the rates of formation and detoxification of the reactive metabolites, such as DEB. In our preliminary studies of bis-N7G-BD in human urine, we discovered that our quantitative methods used for the quantitation of bis-N7G-BD in DNA from animal tissues needed to be modified due to the complexity of urine specimens. Our current methodology for bis-N7G-BD in smoker’s urine employs two solid phase extraction methods (C18 and covalent chromatography on phenyl boronic acid packings),
followed by off-line HPLC prior to HPLC-ESI⁺-MS/MS analysis. Using this methodology, we have quantified bis-N7G-BD in urine from a small set of Caucasian and African American smokers (Table 8.2).

Levels of bis-N7G-BD in smoker’s urine were low (0.14 – 0.24 pg bis-N7G-BD/mg creatinine) starting with 1 mL urine (Figure 8.1). We did however see higher adduct concentrations in African American smokers as compared to Caucasian smokers (Table 8.2). Standard deviations were very large, and adducts were not quantifiable in some samples due to a low signal/noise ratio. Because our current methods involve multiple drying and transferring steps, they are very time consuming, and also lead to sample loss. Therefore, for future analysis of bis-N7G-BD in urine specimens, a column switching HPLC-ESI-MS/MS method may be required to help eliminate some clean-up steps and prevent sample loss. The bis-N7G-BD is retained relatively well on C18 reversed-phase columns, thus a dC18 Atlantis trap column (Waters Corp.) could be employed for trapping bis-N7G-BD while salts and polar components of the urine matrix can be washed away with water (and likely up to 20 % methanol). Bis-N7G-BD can then be backflushed from the trap column with 0.05% acetic acid and higher organic concentrations for HPLC-MS analysis using the same Extend C18 analytical column currently employed for analyses of bis-N7G-BD.
Table 8.1 Levels of bis-N7G-BD in urine of African American (AA) and Caucasian (C) smokers, normalized to creatinine and the number of cigarettes smoked per day.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Race</th>
<th>pg bis-N7G-BD/mg</th>
<th>Sample #</th>
<th>Race</th>
<th>pg bis-N7G-BD/mg</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Std Dev</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>Creatinine/# cig</td>
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<td>216-0025</td>
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<td>216-0032</td>
<td>C</td>
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<tr>
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<td>216-0100</td>
<td>C</td>
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</tr>
<tr>
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<td>216-0101</td>
<td>C</td>
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</tr>
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<td>C</td>
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</tr>
<tr>
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<tr>
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<tr>
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<td>C</td>
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<tr>
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<td>AA</td>
<td>0.05</td>
<td>216-0220</td>
<td>C</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Average= $0.24 \pm 0.28$  
Average= $0.14 \pm 0.20$
Figure 8.1 HPLC-ESI+-MS/MS analysis of bis-N7G-BD in smoker’s urine (1 mL) spiked with $^{15}\text{N}_{10}$-bis-N7G-BD internal standard.
We have also developed methods for quantifying the monoalkylated guanine, N7-(2,3,4-trihydroxybut-1-yl)guanine (THBG) (Scheme 5.1), formed mainly from the EBD metabolite of BD. THBG is expected to be a more abundant adduct and may be easier to quantify in urine. Our current methodology employs two solid phase extraction steps (mixed mode anion exchange/C18 and CarboClean) followed by HPLC-ESI-MS/MS on a Hypercarb column. Although THBG is a more abundant adduct, it is also much more polar because of the presence of three hydroxyl groups and lack of second guanine moiety, so it does not retain well on most reversed phase HPLC columns, co-eluting with other polar components of urine specimens. Therefore, it is very difficult to quantify peaks from the high background (Figure 8.2). Future directions for this project include the development of a column switching HPLC-ESI-MS/MS method for THBG, however, it may be difficult to find a trapping column that retains THBG long enough to wash away other components of urine. A new packing Scherzo SM-C18 (Chromtech) has recently been developed for polar compounds; it is a mixed mode packing- C18 plus cation and anion exchange. We have previously determined that mixed mode solid phase extraction cartridges, Waters Oasis-MAX, retain THBG in basic conditions. Assuming the Scherzo column will have similar anion exchange properties, we will use a Scherzo trap column to retain THGB and wash away other urinary components prior to backflushing THBG onto a Hypercarb column for HPLC-MS/MS analysis. Alternatively, we can perform off-line HPLC with a Hypercarb column (which is known to retain THBG) prior to HPLC-ESI-MS/MS analysis.
Figure 8.2 HPLC-ESI+ MS/MS analysis of THBG standard (2.6 pg) (A) and smoker’s urine spiked with \textsuperscript{15}N\textsubscript{5}-THBG (128 pg) (B).
8.2 Improve method sensitivity for bis-N7G-BD. To enable the application of our quantitative methods for BD-DNA adducts to human tissues, they need to be ultra sensitive to detect low levels of bifunctional lesions. This is because humans are exposed to 2.5 – 1600 ppb in an occupational setting, and 0.1 – 10 ppb in urban air (203). The lowest animal exposure we have analyzed so far was 6.25 ppm BD for 2 weeks. At this exposure, we were able to quantify bis-N7G-BD in mouse liver DNA using our capillary HPLC-ESI-MS/MS methods. We now propose to employ nanospray ionization methods to detect bifunctional BD adducts in animals exposed to 1 ppm BD and lower. We recently obtained a 5 fold increase in sensitivity of the GA cross-link when switched from capillary to nanospray (71). Preliminary studies using nanospray ionization of bis-N7G-BD suggest that we will also see a 5 fold increase in sensitivity for this adduct. Therefore, we intend to employ this new method to analyze bis-N7G-BD in DNA of mice exposed to lower levels of BD to see if we can detect bis-N7G-BD in tissues of animals exposed to BD concentrations approaching the human industry exposure limit (1 ppm). Our collaborators at the University of North Carolina, Professor James Swenberg, recently sent us tissues from B6C3F1 mice exposed to 0.5, 1.0, and 1.5 ppm BD for 2 weeks. In the future, it would also be interesting to look at long-term exposures at low levels that would be more accurate of occupational exposures (e.g. 1 ppm for 50 weeks).
8.3 Quantify bifunctional DNA adducts in DNA from cell-lines deficient in various DNA repair genes. As described in Chapter VI, we did not see repair of bifunctional DEB-DNA adducts by MPG or NER. In these studies, however, we only looked at XPA, which is one of six XP cofactors required for NER. To examine the role of other cofactors in NER of the DEB-DNA cross-links, we propose to use commercial cell lines that are deficient in various DNA repair pathways. Our current results for MPG and XPA suggest these proteins are not involved in the repair of bis-N7G-BD, N7G-N1A-BD, or 1,N^6-HMHP-dA (Tables 6.1 and 6.2). There is some evidence that XPF is the major NER gene required for repair of damaged caused by cross-linking agents (197). There is also evidence that XPC/- mice exposed to EB, the precursor of DEB, have increased mutation frequencies as compared to wild-type mice (199).

While XPF and XPC deficient cell lines are not currently commercially available, there are several other NER deficient cell lines that are available for investigation (Table 8.1). V79 and CHO-9 cell lines are parental cell lines obtained from Chinese hamster lung and ovary tissues, respectively, which repair deficient cell lines were derived from (Coriell Institute, Camden, NJ). Therefore, V79 and CHO-9 cells will be used as controls for comparison to repair deficient cells. Several cell lines with NER defects are available, including XPG and XPD deficient cell lines, UV-135 and V-H1 (Table 8.1). Several cell lines containing defects in double strand break and recombination repair (XRCC4 and complementation group 7 deficient) are also available, V-B11, XR-1, and XR-C1 (Table 8.1). Since the repair of interstrand DNA-DNA cross-links leads to double strand breaks (187), it will be interesting to see whether bis-N7G-BD/N7G-N1A-BD levels increase in these deficient cells. Lastly,
there are BM-C11 cells that are deficient in the repair of single strand breaks, EM-C11, which may be important for N7G-N1A-BD lesions that partially depurinate leaving abasic sites.

The experimental design for these experiments will be to plate cells (deficient in various repair genes and their parental controls, Table 8.1), treat them with DEB, starting with 17-247 pmol/g, concentrations similar to those observed in rats/mice exposed to 62.5 ppm BD for 6 h (176), and DEB-containing media will be exchanged with fresh media. Cells will be harvested for DNA extraction at various times following exposure to determine repair rates of the three bifunctional DNA adducts using our validated quantitative HPLC-ESI+-MS/MS methods. If either of the repair mechanisms is involved in removing the bifunctional DNA adducts of DEB, we should see increased persistence of adducts in deficient cells as they will be unable to repair the adduct.
Table 8.1 Repair deficient cell lines available from Coriell Institute (Camden, NJ) for investigation of bifunctional DEB-DNA lesion repair.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Derived from</th>
<th>Repair defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79 (GM16136)</td>
<td>Parental line</td>
<td>Control</td>
</tr>
<tr>
<td>V-B11 (GM16137)</td>
<td>V79</td>
<td>NER complementation group 7</td>
</tr>
<tr>
<td>V-H4 (GM 16142)</td>
<td>V79</td>
<td>Hypersensitive to cross-linking agents, resembles Fanconi anemia</td>
</tr>
<tr>
<td>V-H1 (GM16141)</td>
<td>V79</td>
<td>NER (XPD/ERCC2)</td>
</tr>
<tr>
<td>CHO-9 (GM16132)</td>
<td>Parental line</td>
<td>Control</td>
</tr>
<tr>
<td>EM-C11 (GM16133)</td>
<td>CHO-9</td>
<td>BER (XRCC1)</td>
</tr>
<tr>
<td>XR-C1 (GM16135)</td>
<td>CHO-9</td>
<td>Complementation group 7 (double strand break repair/V(D)J recombination)</td>
</tr>
<tr>
<td>UV-135 (GM10908)</td>
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<td>NER (ERCC5/XPG)</td>
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<tr>
<td>XR-1 (GM16147)</td>
<td>4364</td>
<td>NER (XRCC4), double strand break repair</td>
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</tbody>
</table>
8.4 Synthesize oligonucleotides containing site specific N7G-N\textsuperscript{6}A-BD cross-links for mutagenesis studies. As mentioned earlier, DNA adduct/adducts responsible for the ability of BD and DEB to induce A → T transversion mutations have not been identified. Studies are currently under way in our laboratory to synthesize oligonucleotides containing site-specific and stereospecific 1, N\textsuperscript{6}-HMHP-dA lesions to investigate the mutagenesis of this adduct using site-specific mutagenesis (Senevirante and Tretyakova, unpublished data). The N7G-N1A-BD/N7G-N\textsuperscript{6}A-BD lesions of DEB (Scheme 5.1) also persist in DNA, so it would be interesting to also design oligonucleotides containing site specific N7G-N1A-BD/N7G-N\textsuperscript{6}A-BD adducts for similar mutagenicity/polymerase bypass assays.

Our laboratory has previously synthesized oligomers containing a site specific N\textsuperscript{6}-(2-hydroxy-3,4-epoxybut-1-yl)-adenine adduct 4 (Scheme 8.1) (137). In our approach, a 6-chloropurine was incorporated into synthetic oligodeoxynucleotides by solid phase DNA synthesis using the corresponding phosphoramidite (1). The 6-chloropurine containing oligomer (2) was reacted with 1-amino-3,4-epoxybutan-1-ol (3) to form the corresponding N\textsuperscript{6}-(2-hydroxy-3,4-epoxybut-1-yl)-dA (N\textsuperscript{6}-HEB-dA) oligonucleotide (4). We initially attempted to use N\textsuperscript{6}-HEB-dA containing DNA to form a G-A butanediol cross-link to the complementary strand (6) (Scheme 8.1), however, N\textsuperscript{6}-HEB-dA spontaneously cyclized to form 1, N\textsuperscript{6}-(1-hydroxymethyl-2-hydroxypropandiy1)-dA (1, N\textsuperscript{6}-HMHP-dA) (5). Even in the presence of a large excess of 2’-deoxyguanosine (2’dG), cyclization was favored, and no N7G-N\textsuperscript{6}A-BD cross-links were observed (Scheme 8.1). Therefore, perhaps coupling of 2’-dG with the amino-epoxide (3) prior to reaction with the 6-chloropurine oligo (2) is needed to form
the site-specific N7G-N⁶A-BD oligonucleotide (Scheme 8.2). In the new proposed synthetic scheme, compound 3 will be incubated with 2’-dG to yield N7-(1-amino-2,3-dihydroxybut-1-yl)-dG (7) which should depurinate to N7-(1-amino-2,3-dihydroxybut-1-yl)-guanine (8). Compound 8 will be dissolved in DMSO in the presence of DIPEA to deprotect the Fmoc-protected amino, and will be reacted with oligo 2 to yield the cross-linked oligo 6.

The N7G-N⁶A-BD containing oligo will be used for polymerase and mutagenicity assays to determine whether it is blocking to DNA polymerases, and what mutations it induces (e.g. which bases are placed opposite the cross-link), similar to studies of monoadducts (139-141).
Scheme 8.1 Previous synthetic scheme for N7G-N^6A-BD containing oligonucleotide.
**Scheme 8.2** Proposed new synthetic scheme for N7G-N\(^6\)A-BD containing oligonucleotide.
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