JOURNAL OF MASS SPECTROMETRY, VOL. 33, 363–376 (1998)

Quantitative Analysis of 1,3-Butadiene-induced DNA Adducts *In vivo* and *In vitro* using Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

Natalia Yu. Tretyakova, Su-Yin Chiang, Vernon E. Walker and James A. Swenberg*
Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC 27599-7400 and New York State Department of Health, Albany, NY 12201

1,3-Butadiene (BD) is a high volume industrial chemical which is known as a multi-site rodent carcinogen and is classified as a probable human carcinogen. Covalent interactions of the reactive epoxy metabolites of BD with DNA lead to the formation of DNA adducts which may cause mutations and tumor formation. In the present work, liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was employed for analyses of BD-induced DNA adducts *in vitro* and *in vivo*. Selected reaction monitoring (SRM) using the fragmentation of the $[M + H]^+$ ions of the adducts to the corresponding protonated nucleobases under collision-induced dissociation was performed. Quantitation was based on isotope dilution with ^{13}C- and ^{15}N-labeled internal standards. The methods were applied *in vitro* [calf thymus DNA and TK6 cell cultures treated with epoxy metabolites of BD, 3,4-epoxy-1-butene (EB) and diepoxybutane (DEB)] and *in vivo* [DNA isolated from tissues of BD-exposed laboratory animals]. Two regioisomers of *N-7*-EB-guanine adducts, *N-7*-(2-hydroxy-3-buten-1-yl)guanine (*N-7*-EB-Gua I) and *N-7*-(1-hydroxy-3-buten-2-yl)guanine (*N-7*-EB-Gua II) and two *N-3*-EB-adenine isomers, *N-3*-(2-hydroxy-3-buten-1-yl)adenine and *N-3*-(1-hydroxy-3-buten-2-yl)adenine (*N-3*-EB-Ade I and II), were found in EB-exposed samples. *N-7*-(2′,3′,4′-trihydroxybut-1′-yl)guanine (*N-7*-THB-Gua), N^6-(2′,3′,4′-trihydroxybut-1′-yl)adenine (N^6-THB-Ade), and *N-3*-(2′,3′,4′-trihydroxybut-1′-yl)adenine (*N-3*-THB-Ade) were detected in DEB-treated DNA. DNA isolated from liver and lung of rats and mice exposed to 1250 ppm BD for 2 weeks contained both regioisomers of *N-7*-EB-Gua and *N-3*-EB-Ade, as well as *N-7*-THB-Gua and N^6-THB-Ade. The methods developed in this work provide the means to study accumulation, repair and dose–response relationships of BD–DNA adducts *in vivo*. Although less sensitive than gas chromatography/electron capture negative ionization high-resolution mass spectrometry (GC/ECNI-HRMS), LC/ESI^+-MS/MS in the SRM mode is extremely useful for analysis of BD–DNA adducts, which are not amenable to GC and derivatization owing to the presence of several adjacent polar functional groups. Using LC/ESI-MS/MS and isotope dilution, multiple structurally diverse BD–DNA adducts can be analyzed simultaneously in the same sample with minimal sample preparation. © **1998 John Wiley & Sons, Ltd.**

J. Mass Spectrom. **33**, 363–376 (1998)

KEYWORDS: DNA adduct; 1,3-butadiene; liquid chromatography/electrospray ionization tandem mass spectrometry; alkylguanine; alkyladenine

INTRODUCTION

1,3-Butadiene (BD) is a major chemical in rubber and plastics manufacturing[1] and a common environmental contaminant.[2] Although it has been classified as a probable human carcinogen,[3] the mechanisms of BD-induced mutations and cancer are not well understood. BD requires metabolic activation to reactive epoxides for its activity. It undergoes cytochrome P450-mediated oxidation to the major metabolite, 3,4-epoxy-1-butene (EB).[4] EB participates in further oxidation to 1,2,3,4-diepoxybutane (DEB), hydrolysis to 1-buten-3,4-diol or glutathione conjugation (Fig. 1).[5,6] 3,4-Epoxy-1,2-butanediol (EBD) can be produced through partial hydrolysis of DEB and/or epoxidation of 1-butene-3,4-diol (Fig. 1). EB, DEB and EBD are direct mutagens in the Ames test and cause genotoxic effects in a number of *in vivo* and *in vitro* systems.[7,8] BD is a multi-site carcinogen in both rats and mice, but the tumorigenic response in mice is induced at concentrations of BD three orders of magnitude lower than in rats. The increased sensitivity of mice to BD has been attributed to the production of significantly higher amounts of DEB in this species.[6]

Although the exact mechanisms of BD-induced mutagenesis and carcinogenesis are unknown, chemical reactions of epoxy metabolites of BD with DNA resulting in covalently bound adducts are likely to be critical steps in multi-stage carcinogenesis. The chemistry of the interactions of EB and DEB with DNA nucleobases has been extensively studied in this and other laboratories

* Correspondence to: J. A. Swenberg, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, North Carolina 27599-7400, USA.
E-mail: james_swenberg@unc.edu.

CCC 1076–5174/98/040363–14 $17.50

Received 13 October 1997
Accepted 9th January 1998

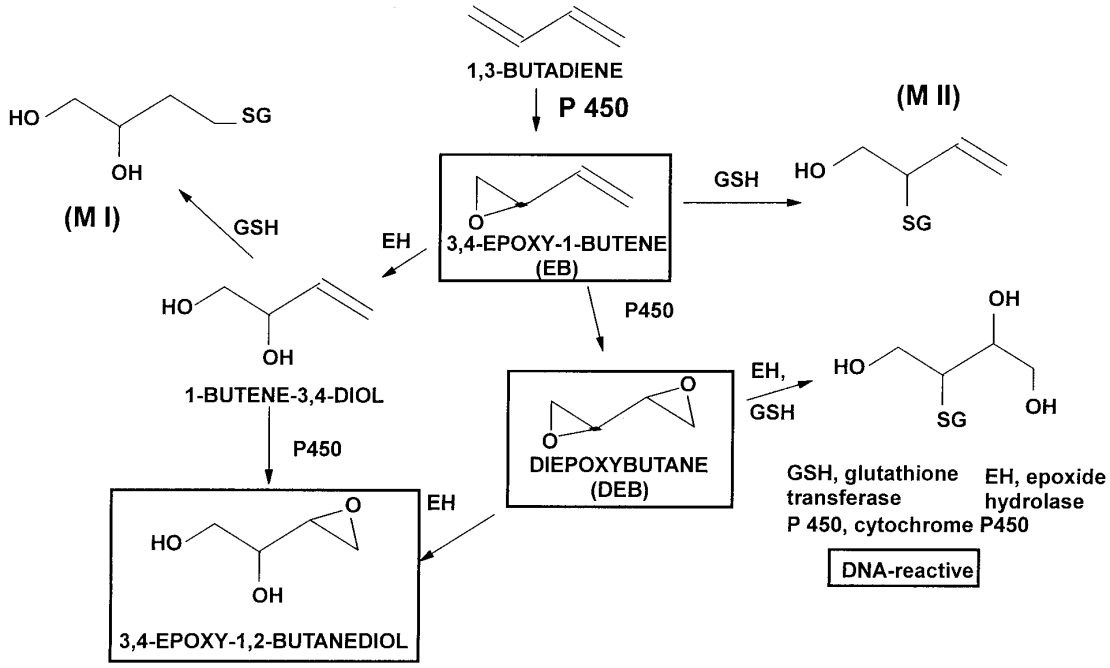

Figure 1. Metabolism of 1,3-butadiene.

and multiple DNA adducts have been identified (Fig. 2).[9–19] *N-7*, N^1- and N^2-guanine and *N-3*-, N^6-and *N-7*-adenine BD adducts have been isolated from reactions of DNA nucleobases and nucleosides with the epoxides of butadiene. Regioisomeric pairs of *N-7*-guanine and *N-3*-adenine adducts have been found in EB-treated calf thymus DNA (CT DNA).[9,14,15] CT DNA exposed to DEB contained *N-7*-THB-guanine and N^6-THB-adenine adducts.[18,19] The *N-7* position of guanine was demonstrated to be the most reactive position in DNA towards BD epoxides, followed by the *N-3* and the N^6 of adenine.[15,18,19] However, the information available to date on the formation of these adducts *in vivo* in DNA of BD-exposed laboratory animals and occupationally exposed humans is very limited. *N-7*-THB-Gua and *N-7*-EB-Gua II have been detected in liver DNA of BD-exposed mice but not rats,[10] but no quantitative analyses were performed. Very small amounts of *N-7*-EB-Gua adducts (2.4 per 10^7 normal nucleobases) were found in liver DNA of BD-exposed rats (200 ppm for 5 days) using ^{32}P-postlabeling.[17] The latter method is not very specific and is extremely laborious, and the low postlabeling efficiency of *N-7*-guanine and *N-3*-adenine adducts makes it difficult to perform accurate quantitation. No quantitative data for BD–DNA adducts in tissues of exposed mice are available in the literature.Quantitative analysis of BD–DNA adducts *in vivo* is required in order to improve our understanding of the mechanisms of BD-induced carcinogenesis and mutagenesis, determine the BD metabolite(s) causing DNA damage and the lesions responsible for the observed biological effects. The goal of the present work was to develop sensitive and specific mass spectral methods for identification and quantification of BD-induced DNA adducts. Using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI$^+$-MS/MS) in the selected reaction monitoring (SRM) mode, we analyzed a variety of BD-induced DNA adducts *in vitro* (CT DNA and TK6 cell cultures treated with EB and DEB) and *in vivo* (tissues of rats and mice exposed to 1250 ppm BD by inhalation). Highly efficient chromatographic separation combined with the excellent selectivity of tandem mass spectrometry allowed the sensitive and accurate quantitation of BD-induced DNA adducts in animal tissues.

EXPERIMENTAL

Materials

HPLC-grade water and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and Mallinckrodt (Paris, KY, USA), respectively. EB and DEB were obtained from Aldrich (Milwaukee, WI, USA). Calf thymus DNA, nucleobases, nucleosides, Ribonuclease T1, Ribonuclease A and Proteinase K were acquired from Sigma Chemical (St Louis, MO, USA). Solid-phase extraction columns (ODS-AQ, 500 mg, 3 ml) were obtained from YMC (Wilmington, NC, USA). Other chemicals were purchased from Fisher Scientific. Guanine and adenine adducts of EB and DEB were prepared by the reactions of purine nucleobases or nucleosides with EB or DEB as described elsewhere[14,15,18,19] (Fig. 2). Isotopically labeled analogs of the adducts for use as internal standards in mass spectral studies were prepared analogously starting with [$^{13}C_4$]-guanosine, [$^{15}N_5$]-adenine or [$^{15}N_5$]-adenosine (Fig. 3). [$^{13}C_4$]-Guanosine was kindly provided by Dr R. Sangaiah at the Department of Environmental Sciences and Engineering of the University of North Carolina at Chapel Hill. [$^{15}N_5$]-Adenosine was obtained from Cambridge Isotope Laboratories (Cambridge, MA, USA). [$^{15}N_5$]-Adenine was prepared from [$^{15}N_5$]-adenosine by heating in 0.2 M HCl for 5 h. We determined that the residues of unlabeled adducts in

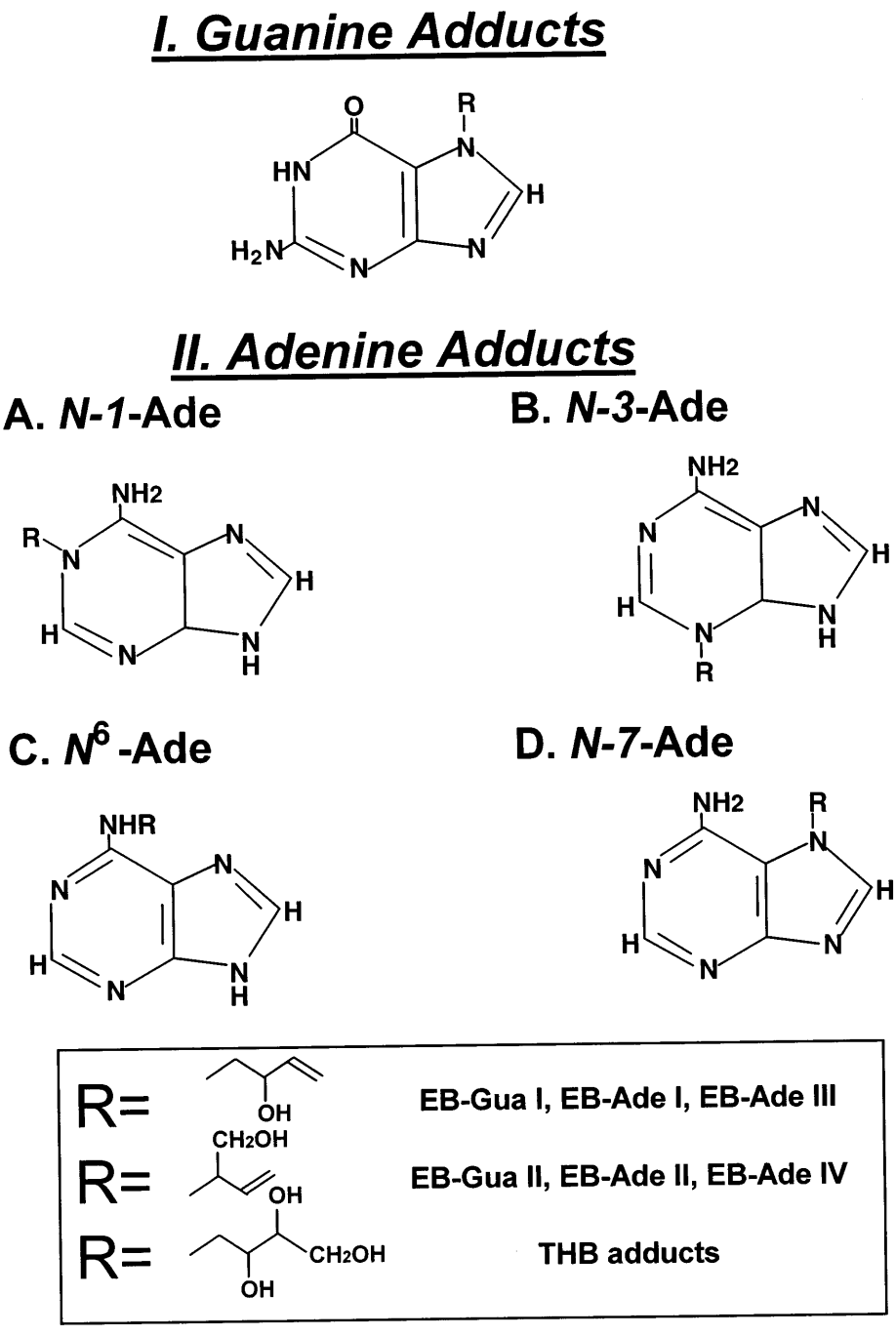

Figure 2. DNA adducts induced by 1,3-butadiene and its metabolites.[14–17]

standard solutions of [$^{13}C_4$]- and [$^{15}N_5$]-labeled adducts were <0.5% by conducting LC/ESI$^+$-MS/MS analysis in which both compounds were monitored.

Stock solutions of analytes and internal standards (20–50 μM) were prepared in water and stored at −20 °C. The concentrations were periodically checked by UV spectrophotometry. Standard solutions were prepared from stock solutions by serial dilutions with water and stored at −20 °C.

Reactions of EB and DEB with calf thymus DNA (CT DNA)

CT DNA solutions (6 mg in 2 ml of 5 mM Tris buffer, pH 7) were treated with 38 μl EB or 36 μl DEB (neat) at 37 °C for 18 h (environmental shaker). At the end of the incubations, the mixtures were extracted with 3 × 2 ml of diethyl ether to remove the unreacted epoxides. Aliquots of 100 μl of the resulting solution were spiked with 8–10 pmol of internal standards.

CT DNA hydrolysis and purification of adducts by solid-phase extraction

The DNA samples were hydrolyzed at 95 °C for 30 min in water (neutral thermal hydrolysis) or at 75 °C for 30 min in 0.1 M HCl (mild acid hydrolysis) with mixing. The acid hydrolyzates were neutralized with KOH and all hydrolyzates were filtered through Centricon-3 filters (Amicon, Lexington, MA, USA) and desalted using

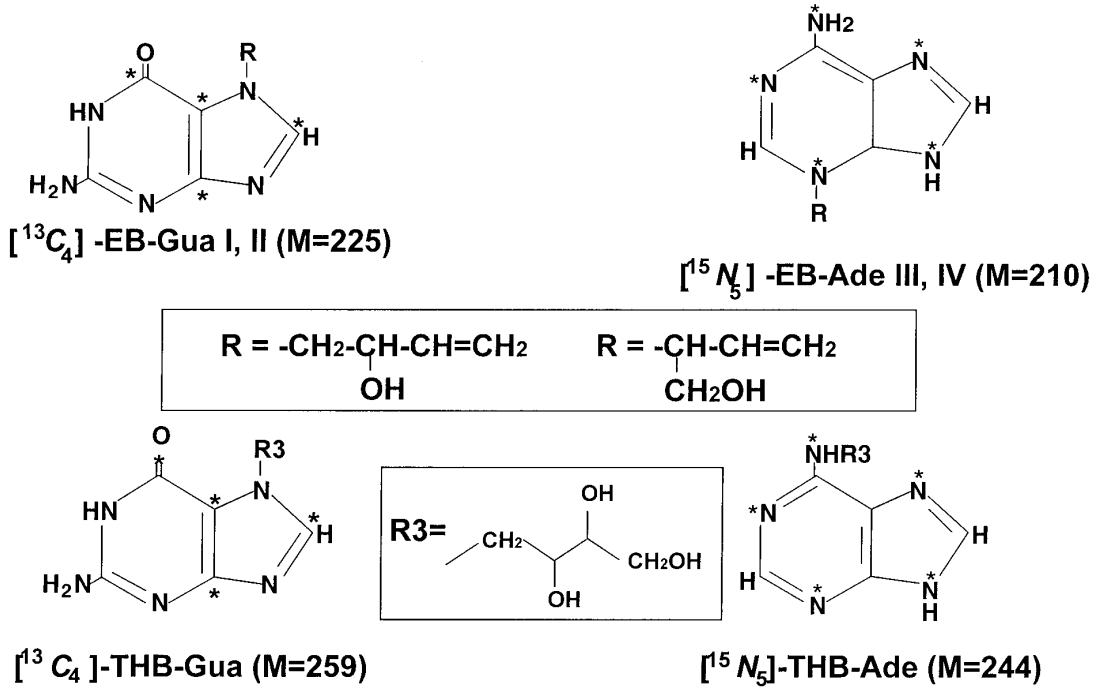

Figure 3. Internal standards for quantitation of BD-induced DNA adducts.

ODS-AQ solid-phase extraction columns (500 mg, 3 ml, YMC). The cartridges were primed with 6 ml of methanol and 10 ml of water and the samples were loaded using gravity. The columns were washed with either 4 ml of water (neutral thermal hydrolyzates) or 8 ml of water (mild acid hydrolyzates) and the samples were eluted with 6 ml of methanol. The resulting solutions were evaporated under reduced pressure and redissolved in 50 µl of buffer. A 1–2 µl volume of this solution was injected into the LC system for LC/ESI+-MS/MS analyses (Fig. 4).

TK6 cell cultures

TK6 human lymphoblastoid cell cultures were grown in cytidine–hypoxanthine–aminopterin–thymidine medium for 3 days prior to treatment in order to reduce the level

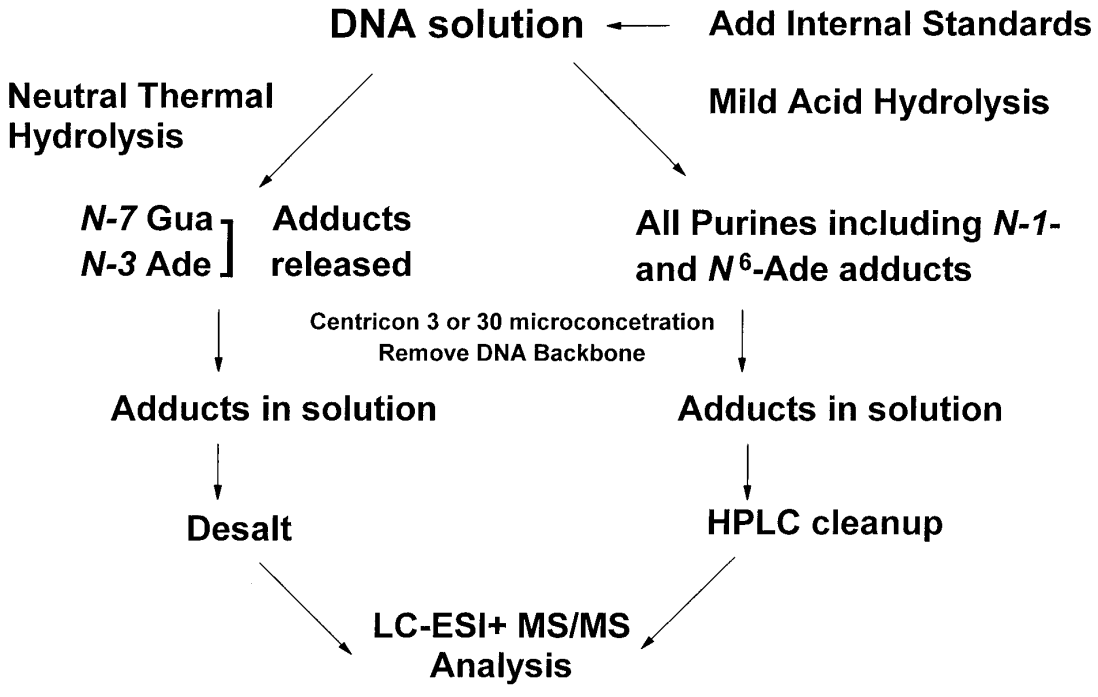

Figure 4. Experimental scheme for analysis of BD–DNA adducts by LC/ESI+-MS/MS.

of spontaneous mutants. Cultures (180 ml) at a density of 4×10^5 cells ml^{-1} (three replicates) were exposed to 5–25 μM DEB or 5–500 μM EB administered as aqueous solutions for 24 h. At the end of treatment, cells were collected for DNA adduct studies or re-suspended in fresh medium and grown for a period of 8 days. The cells were then plated in microwell plates in the media containing 2 μg ml^{-1} trifluorothymidine (TFT) and incubated for 10–11 days prior to scoring fast-growing *tk* colonies. The plates were re-fed with TFT and scored for slow-growing *tk* mutants after incubating for a further 10–11 days as described by Cochrane and Skopek.[8]

Animal exposures

B6C3F1 mice and F344 rats were subjected to whole-body exposure to 1250 ppm of 1,3-butadiene for 2 weeks (6 h per day, 5 days per week) as described previously.[18] Control animals were exposed to chamber supply air alone. After the exposure period, rats and mice were immediately killed by exsanguination. Organs were collected, packed in foil, frozen and stored at −70 °C until analysis. See Ref. 18 for further details.

DNA isolation from animal tissues and TK6 cell cultures

DNA was isolated from tissues either manually or using a Model 340 Nucleic Acid Extractor (Applied Biosystems, Foster City, CA, USA). An amount of 1–2 g of tissue was homogenized in 10 ml of PBS solution and the resulting suspensions were centrifuged to separate nuclei. The nuclear pellets were suspended in lysis buffer and incubated with Ribonucleases A and T1 at 37 °C for 1–1.5 h. The samples were treated with Proteinase K for 4 h followed by an automated phenol–chloroform extraction. The DNA was rehydrated in 3 ml of water and frozen at −70 °C until needed. The amounts and purity of DNA in the extracts were determined by UV spectrophotometry (20 $A_{260} = 1$ mg ml^{-1} DNA). The A_{260}/A_{280} ratios were found to be between 1.6 and 1.9, ensuring minimal protein contamination. Guanine and adenine contents were determined by HPLC analyses. The HPLC system employed consisted of a Rheodyne injector (Baxter, Charlotte, NC, USA) equipped with a 2 ml injection loop, two Waters Model 510 HPLC pumps (Waters, Milford, MA, USA) and an Applied Biosystems (Ramsey, NJ, USA) Model 757 absorbance detector. A Partisil 10 SCX strong cation-exchange column (250 × 4.6 mm i.d., Whatman, Hillsboro, OR, USA) was eluted isocratically with 100 mM ammonium formate, pH 2.8, containing 10% methanol, at a flow rate of 1.8 ml min^{-1}. Guanine and adenine concentrations were determined by measuring the UV absorbance at 254 nm and comparing the peak areas with calibration graphs constructed with standard compounds. DNA concentrations were calculated from the guanine content. The DNA from TK6 cells was isolated and quantitated in the same way but omitting the homogenization step.

TK6 and rodent DNA hydrolysis and sample clean-up

For the analyses of *N-3*-Ade and *N-7*-Gua adducts in DNA isolated from cell cultures and animal tissues, 0.2–1 mg of DNA was spiked with 1.73 pmol of [$^{13}C_4$]-*N-7*-EB-Gua I, 1.67 pmol of [$^{13}C_4$]-*N-7*-EB-Gua II, 0.15 pmol of [$^{15}N_5$]-*N-3*-EB-Ade I, 0.16 pmol of [$^{15}N_5$]-*N-3*-EB-Ade II, and 1.75 pmol of [$^{13}C_4$]-*N-7*-THB-Gua. The samples were diluted to 1 ml with purified water and subjected to neutral thermal hydrolysis, Centricon-30 filtration and desalting on solid-phase extraction columns as described above for CT DNA. For the determination of N^6-THB-Ade, partially depurinated DNA remaining after neutral thermal hydrolysis was dissolved in 1 ml of 0.1 M HCl, spiked with 2.26 pmol of [$^{15}N_5$]-N^6-THB-Ade standard, and subjected to mild acid conditions filtered and desalted as described above for CT DNA. The desalted samples were evaporated under vacuum and the residues were dissolved in 50 μl of buffer. Volumes of 10–15 μl of these solutions were injected into the LC system for LC/ESI$^+$-MS/MS analysis (Fig. 4).

LC/ESI$^+$-MS/MS analyses

LC/ESI$^+$-MS/MS studies were conducted using a VG Quatro II LC/ESI$^+$-MS/MS triple quadrupole system interfaced with a Hewlett-Packard Model 1050 liquid chromatograph. For analyses of EB adducts, a narrow-bore BDS Hypersil C_8 reversed-phase column (50 × 2.1 mm i.d., 3 μm; Phenomenex, Torrance, CA, USA) was eluted at a flow rate of 150 μl min^{-1}. The solvent system was 25% acetonitrile–5 mM ammonium formate, pH 3.5 (A) and 5 mM ammonium formate, pH 3.5 (B), with a linear gradient from 20 to 60% A in 10 min. For THB adduct analysis, a BDS Hypersil C_8 reversed-phase column (100 × 2.1 mm i.d., 3 μm; Phenomenex) was eluted isocratically with 25% acetonitrile–5 mM ammonium formate, pH 3.5, at a flow rate of 100 μl min^{-1}. Nitrogen (90–95 psi) was used as a nebulizing and drying gas and the ion source temperature was held at 120 °C. The mass spectrometer was operated in the positive ion mode with a potential of 2.5–2.84 kV applied to the ESI needle. Selected reaction monitoring (SRM) scanning was performed by setting the parameters in the first quadrupole (Q1) to select the protonated molecules of the adducts and the internal standards. Collision-induced dissociation (CID) was performed using argon at 2×10^{-3}–5×10^{-3} mbar in the second quadrupole (Q2). The third quadrupole (Q3) was set to the masses of protonated adenine for Ade adducts and protonated guanine for Gua adducts or the corresponding fragment ions of the internal standards. The cone energy and collision offset energy were optimized to allow maximum sensitivity for each adduct (see Table 2). Quantitative analyses were based on the ratio of the area under the peak in the selected ion chromatogram corresponding to the analyte to the peak area of the internal standard (relative response factors). The linearity was checked using calibration graphs constructed by spiking internal standards with known amounts of analytes (e.g. see Fig. 5). The mass spectrometer parameters were optimized for maximum

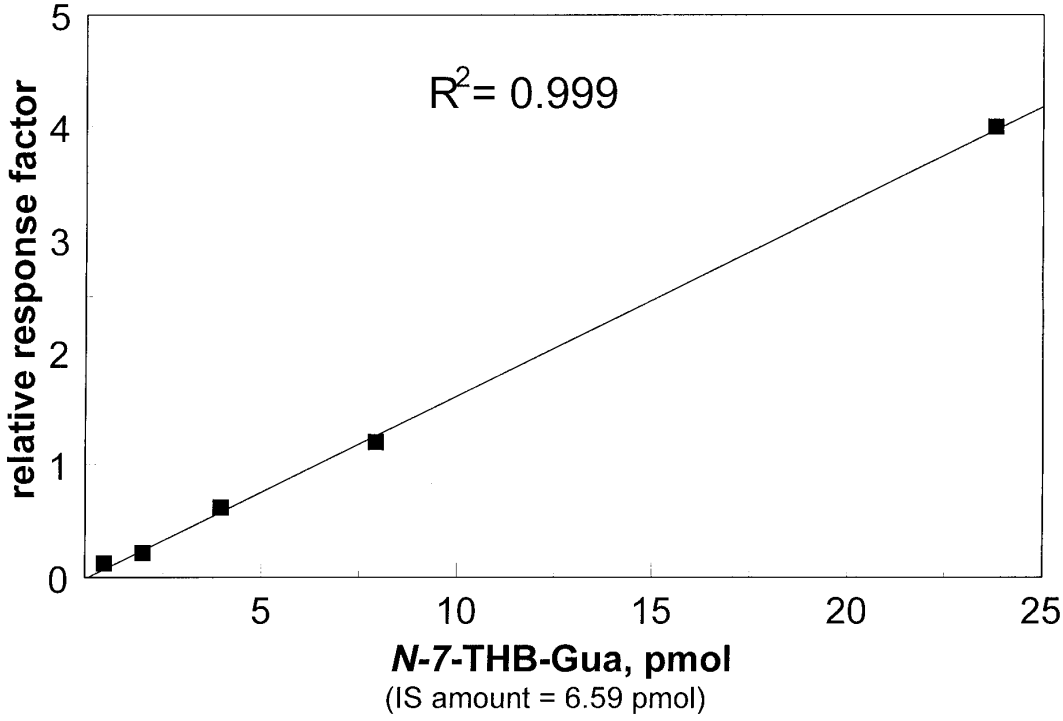

Figure 5. Calibration curve for LC/ESI+-MS/MS analysis of *N*-7-THB-Gua.

response during infusion of standard solutions of the adducts.

Analyses of EB-Gua adducts by gas chromatography/electron capture negative chemical ionization mass spectrometry

DNA (50–100 μg) was spiked with 1.7 and 3.1 pmol of [$^{13}C_4$]-EB-Gua I and II, respectively, and subjected to neutral thermal hydrolysis as described above. The DNA backbone was removed by cold acid precipitation [21] and the adducts were derivatized with pentafluorobenzyl bromide (PFBBr) using the methods described by Fedtke *et al.*[22] and Rios-Blanco *et al.*[23]. GC/HRMS was carried out on an HP 5890 gas chromatograph interfaced to a VG 70 250SEQ mass spectrometer (VG, Manchester, UK) operated in the negative ion chemical ionization mode. The mass resolving power was 10K. Direct injections using a press-fit liner were made on a DB-5MS (J&W Scientific, Folsom, CA, USA) fused-silica capillary column (15 m × 0.32 mm i.d.) and the temperature gradient was as follows: 70 °C (1 min), 20 °C min^{-1} to 290 °C (1 min), 15 °C min^{-1} to 300 °C (3 min). The helium head pressure was 10 psi and the injector temperature was 290 °C. The GC retention times of derivatized EB-Gua I and II at these conditions were 10.38 and 10.42 min, respectively. The ion source temperature was 250 °C. Methane (3×10^{-5} mbar) was used as a reagent gas. The electron emission current was 0.5 mA. Quantitation of *N*-7-EB-Gua was performed by isotope dilution mass spectrometry. Selected ion monitoring of the $[M - PFB]^-$ fragment of EB-Gua (*m/z* 581.0671) and the corresponding fragment of [$^{13}C_4$]-*N*-7-EB-Gua internal standard (*m/z* 585.0807) were performed and the amounts were calculated from the peak area ratios using calibration curves. The calibration curves were linear with $r^2 = 0.999$ from 0.2 to 10 pmol of *N*-7-EB-Gua I and II.

RESULTS

LC/ESI+-MS/MS method development

Early in these studies we attempted to develop a method based on GC/ECNI-HRMS for detecting BD-induced adducts after electrophore labeling with pentafluorobenzyl bromide (PFBBr). This technique has been widely applied in our laboratory for analyses of other *N*-7-alkylguanines, such as *N*-7-(2-hydroxyethyl) guanine[22] and *N*-7-(2-hydroxypropyl)guanine.[23] Although *N*-7-EB-Gua adducts were successfully derivatized with PFBBr using the same methods,[22–23] major difficulties were encountered in the case of THB adducts. The latter compounds contain a trihydroxybutyl group (Fig. 2), of which at least two of the hydroxyls have to be modified in order to make them amenable to separation by GC. Although a PFB derivative was obtained for *N*-7-THB-Gua, the derivatization yields were extremely low, probably owing to the steric hindrance created by the first PFB group that enters the molecule. Moreover, the molecular mass of the derivative with two PFB groups on the side-chain of *N*-7-THB-Gua was 976 Da (the major MS peak under ECNCI conditions was at *m/z* 955), making GC separation and mass calibration of the instrument challenging. Similar difficulties were expected for THB-Ade adducts. Therefore, it was decided to analyze the BD-induced DNA adducts directly, without derivatization, using liquid chromatography combined with electrospray ion-

ization mass spectrometry (LC/ESI$^+$-MS). Tandem mass spectrometry is a particularly useful method for the analysis of complex mixtures of compounds in biological matrices which has been applied previously to alkylated nucleobases and nucleosides in urine[24] and animal tissues.[25–28]

Up-front CID mass spectra of EB-and THB-induced adducts with adenine and guanine are summarized in Table 1. The ESI$^+$ CID mass spectra of all BD adducts were dominated by two major peaks. The peak at a higher mass corresponded to $[M + H]^+$ ions of each adduct (*m/z* 226 for *N-7*-EB-Gua, *m/z* 256 for *N-7*-THB-Gua, *m/z* 206 for *N-3*-EB-Ade and *m/z* 240 for N^6-THB-Ade, Table 1). The second most abundant peak in each case was a fragment peak due to the characteristic loss of the alkyl side-chain to give the ions of protonated nucleobases (*m/z* 136 for adenine adducts and *m/z* 152 for guanine adducts, Table 1). N^6-THB-Ade was an exception since it had a significant peak at *m/z* 148, corresponding to the cleavage of the C-1′—C-2′ bond of the substituent (Table 1). These differences are probably due to the fact that N^6-THB-Ade is the only adduct in this set which is substituted at an exocyclic nitrogen. McLafferty-type rearrangement of the $[M + H]^+$ ions of N^6-THB-Ade leads to aromatic ions at *m/z* 148, whereas the $(Ade + H)^+$ ions at *m/z* 136 are non-aromatic (Fig. 6(a)). The situation is the opposite for the adducts substituted at endocyclic nitrogens, which produce abundant $[Ade + H]^+$ ions at *m/z* 136 and do not have significant peaks at *m/z* 148 (Fig. 6(b) and Table 1). The same mass spectra, with a +4 mass shift for [$^{13}C_4$] and a +5 mass shift for [$^{15}N_5$], were observed for the stable isotope-labeled standards (Table 1).

We developed SRM methods for analyzing BD adducts on the triple quadrupole system, taking advantage of the fact that one major fragmentation process was observed for all the adducts under CID conditions (Table 2). Protonated molecules of the adducts were selected and focused on the first quadrupole, Q1, their fragmentation was achieved by CID in Q2 and the selected product ions were detected on Q3. The collision energy and cone energy were optimized individually for each adduct to obtain the highest response and the instrument was tuned using standard solutions of the analytes introduced by infusion. We found that the sensitivity of this method for *N-3*-adenine adducts was generally higher than for the *N-7*-guanine adducts. This difference could be due to more efficient protonation of the *N-3*-adenine adducts in solution, since they have higher p*K* values than *N-7*-alkylguanines. *N-3*-Alkyladenines have been reported previously to give a higher response than *N-7*-substituted guanines under ESI$^+$ conditions.[29]

The LC conditions significantly affected our ability to detect and separate cleanly the regioisomers of the EB-

Table 1. ESI$^+$ up-front CID spectra of BD-induced DNA adducts

Adduct	Major peaks (% of the main peak)
EB-Ade I	206 (100), 136 (70), 149 (5)
EB-Ade II	206 (100), 136 (90), 228 (5)
EB-Gua I	222 (100), 152 (40), 175 (5)
EB-Gua II	222 (100), 152 (25), 202 (5)
N^6-THB-Ade	240 (100), 148 (50), 136 (50), 162 (30)
N-7-THB-Gua	256 (100), 152 (60), 278 (15)
[$^{15}N_5$]-EB-Ade I	211 (100), 141 (65)
[$^{15}N_5$]-EB-Ade II	211 (100), 141 (65)
[$^{13}C_4$]-EB-Gua I	226 (100), 156 (45)
[$^{13}C_4$]-EB-Gua II	226 (100), 156 (45)
[$^{13}C_4$]-THB-Gua	260 (100), 156 (50)
[$^{15}N_5$]-THB-Ade	245 (100), 141 (45)

Table 2. Selected reaction monitoring of BD-induced DNA adducts and internal standards

Adduct	SRM transition	Cone energy (V)	Collision energy (eV)
N-3-EB-Ade	*m/z* 206 $[M + H]^+ \rightarrow$ *m/z* 136 $[Ade + H]^+$	25	20
[$^{15}N_5$]-*N-3*-EB-Ade	*m/z* 211 $[M + H]^+ \rightarrow$ *m/z* 141 [$^{15}N_5$-Ade + H]$^+$	25	20
N-7-EB-Gua	*m/z* 222 $[M + H]^+ \rightarrow$ *m/z* 152 $[Gua + H]^+$	16	20
[$^{13}C_4$]-*N-7*-EB-Gua	*m/z* 226 $[M + H]^+ \rightarrow$ *m/z* 156 [$^{13}C_4$-Gua + H]$^+$	16	20
N^6-THB-Ade	*m/z* 240 $[M + H]^+ \rightarrow$ *m/z* 136 $[Ade + H]^+$	23	25
[$^{15}N_5$]-N^6-THB-Ade	*m/z* 245 $[M + H]^+ \rightarrow$ *m/z* 141 [$^{15}N_5$-Ade + H]$^+$	23	25
N-7-THB-Gua	*m/z* 256 $[M + H]^+ \rightarrow$ *m/z* 152 $[Gua + H]^+$	16	20
[$^{13}C_4$]-*N-7*-THB-Gua	*m/z* 260 $[M + H]^+ \rightarrow$ *m/z* 156 [$^{13}C_4$-Gua + H]$^+$	16	20

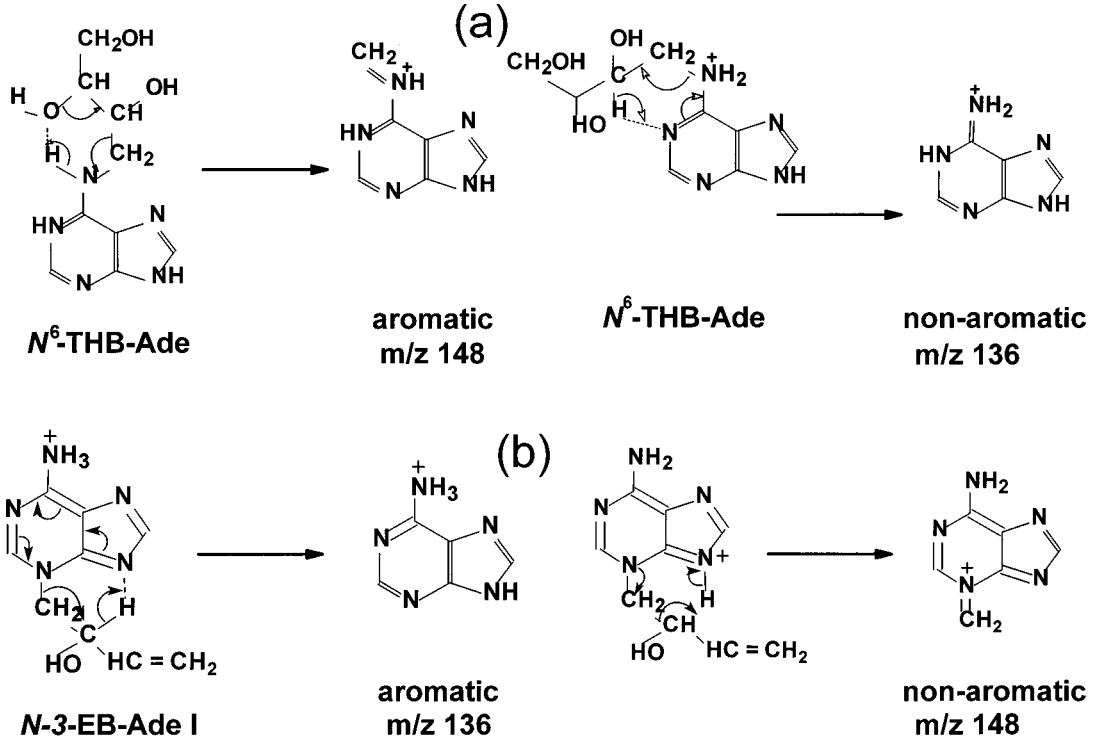

Figure 6. Fragmentation of (a) N^6-THB-Ade and (b) *N-3*-EB-Ade under CID.

induced adducts. We found that *N-3*-Ade adducts were best analyzed using C_8 columns. Application of C_{18} columns caused a major deterioration of the peak shape. The buffer pH and the nature of the organic solvent were also important owing to their effect on LC separation and the electrospray response of the modified nucleobases. It has been observed previously[29] that acidification of the solvent with weak acid increased the ESI^+ ion signal of certain DNA adducts owing to increased protonation. The pK_a values for the DNA adducts used in this study fall into the range 3.7–6.5 and the sensitivity increased when the pH of the buffer was decreased to 4 or below. However, the retention times of the adducts became very short when the pH was below 3.5. Hence the pH range 3.5–4.5 was optimal for the LC separation of the BD-induced DNA adducts.

Calibration curves for each of the adducts were constructed using the described SRM parameters. Fixed amounts of internal standards were spiked with various amounts of the corresponding analytes and the relative response of the analyte to the internal standard was plotted against the concentration of the analyte (for an example, see Fig. 5). The responses of BD adducts to the corresponding $^{13}C_4$ or $^{15}N_5$ isotopically labeled internal standards were linear ($r^2 = 0.995$–0.999) and reproducible from 0.1 to 10 pmol injected on-column. The limits of quantitation for the BD adducts were 20–100 fmol depending on the nature of the adduct, with *N-3*-Ade being most sensitive.

The quantitation was performed using stable isotope analogs of the adducts as internal standards, which were added to samples early in the analyses to account for losses during sample work-up (Fig. 4). During sample analyses, the analytes and the corresponding internal standards were monitored simultaneously by switching between the *m/z* ratios characteristic for each compound during the MS/MS scan. This allowed specific detection of the analytes of interest based on the correct molecular mass, fragmentation to the parent nucleobases and co-elution with the corresponding internal standards.

CT DNA reactions with epoxybutene

A representative LC/ESI^+-MS/MS ion chromatogram of a neutral thermal hydrolyzate of EB-exposed CT DNA is shown in Fig. 7. Two major peaks of equal size at 3.6 and 5.1 min were seen in the ion chromatogram corresponding to EB-Gua adducts (*m/z* 222 → *m/z* 152). These two compounds co-eluted with the $^{13}C_4$-labeled internal standards for *N-7*-(2-hydroxy-3-buten-1-yl) guanine (*N-7*-EB-Gua I) and *N-7*-(1-hydroxy-3-buten-2-yl)guanine (*N-7*-EB-Gua II), which allowed their identification as *N-7*-EB-Gua I and II (Fig. 2). In addition, two minor EB-Gua adducts with shorter retention times were observed, but their identities have not been established owing to the lack of the corresponding standard compounds. MS/MS selected ion chromatograms for EB-Ade adducts (*m/z* 206 → *m/z* 136) contained four peaks at 2.23, 3.06, 3.68 and 4.09 min (Fig. 7). Since the compounds at 3.06 and 4.09 min co-eluted with the $^{15}N_5$-labeled standards for *N-3*-(2-hydroxy-3-buten-1-yl)adenine (*N-3*-EB-Ade I) and *N-3*-(1-hydroxy-3-buten-2-yl)adenine (*N-3*-EB-Ade II), they were identified as the two regioisomers of *N-3*-EB-Ade adducts I and II (Fig. 2). These assignments were further confirmed by comparison of the full spectra of the adducts with those of the standard compounds. The amounts of *N-3*-EB-Ade adducts in EB-exposed CT DNA (2.1 and 7.6 nmol μmol Ade for I and II, respectively, Table 3) were ~1/7 of the EB-Gua adducts (26.9 and 36.2 nmol μmol Gua). The formation of regioisomeric pairs of EB adducts in

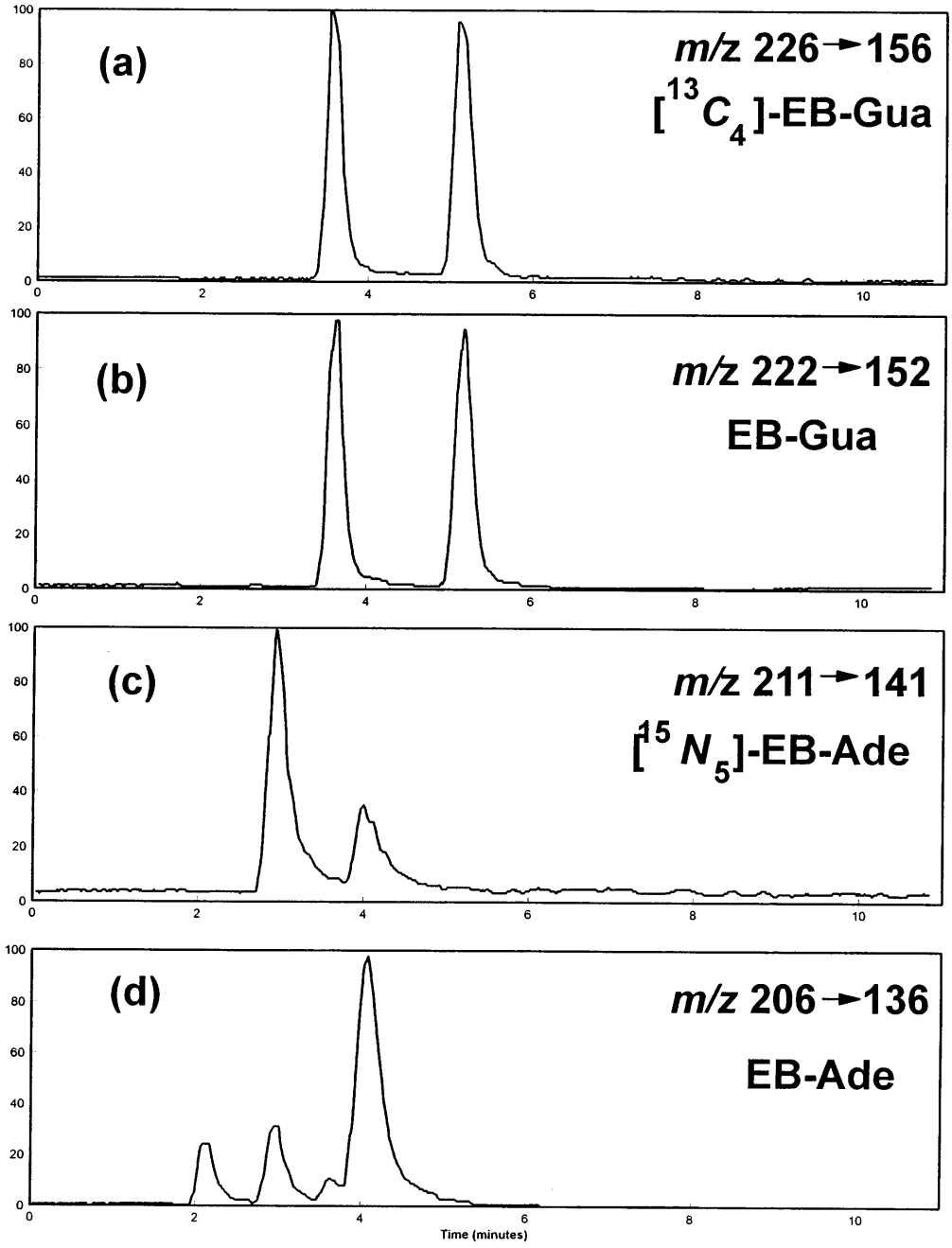

Figure 7. LC/ESI$^+$-MS/MS analysis of a neutral thermal hydrolyzate of calf thymus DNA treated with 3,4-epoxy-1-butene.

CT DNA is a result of a nucleophilic attack of the base at either the C-4 terminal carbon of EB (*N*-3-EB-Ade I and *N*-7-EB-Gua I) or at the C-3 of EB (*N*-3-EB-Ade II and *N*-7-EB-Gua II).[9,15] Interestingly, whereas the amounts of *N*-7-EB-Gua I and II were similar, EB-Ade II was more abundant than EB-Ade I, in accordance with our earlier findings from HPLC/UV analysis of EB-exposed CT DNA.[15]

Table 3. Guanine and adenine adducts in calf thymus DNA exposed to equimolar amounts of 3,4-epoxy-1-butene or diepoxybutane (nmol μmol^{-1} normal nucleobase)

	N-7-EB-Gua I	*N*-7-EB-Gua II	*N*-3-EB-Ade I	*N*-3-EB-Ade II	*N*-7-THB-Gua	*N*-3-THB-Ade
Exp. 1	23.2	35.0	2.1	7.9	57.4	6.2
Exp. 2	24.8	38.7	1.8	7.3	59.4	6.0
Exp. 3	32.8	35.1	2.5	7.5	59.2	6.2
Mean ± SD	26.9 ± 5.4	36.2 ± 2.1	2.1 ± 0.3	7.6 ± 0.3	58.7 ± 1.1	6.2 ± 0.2

CT DNA reactions with diepoxybutane

LC/ESI+-MS/MS analysis of the hydrolyzates of DEB-exposed CT DNA showed one major peak in the MS/MS ion chromatogram for THB-Gua adducts (*m/z* 256 → *m/z* 152, Fig. 8). This peak co-eluted with the internal standard signal in [$^{13}C_4$]-*N*-7-(2′,3′,4′-trihydroxybut-1′-yl)guanine ion channel, supporting its identification as *N*-7-THB-Gua. Adenine and guanine have been reported to react at the terminal carbon of DEB producing single regioisomers of the adducts.[10,18,19] The amount of *N*-7-THB-Gua in exposed DNA was 58.7 ± 1.1 adducts per 10^3 normal Gua (Table 3). This quantity is comparable to the sum of the amounts of the two regioisomers for *N*-7-EB-Gua in DNA exposed to the same molar amounts of EB (63.7 per 10^3 Gua, Table 3), suggesting that EB and DEB are equally reactive towards the *N*-7 position of guanine in DNA under these conditions.

THB-Ade MS/MS ion chromatogram (*m/z* 240 → *m/z* 136) contained a major peak at 6.0 min which was identified as N^6-THB-Ade since it co-eluted with the [$^{15}N_5$]-labeled internal standard (Fig. 8). The amount of this adduct (6.02 ± 0.2 per 10^3 normal Ade) was about 1/10 of the *N*-7-THB-Gua adduct, suggesting that the N^6-adenine position is significantly less reactive towards DEB than the *N*-7-guanine in DNA. The minor peaks at 2.03 and 3.46 min were tentatively iden-

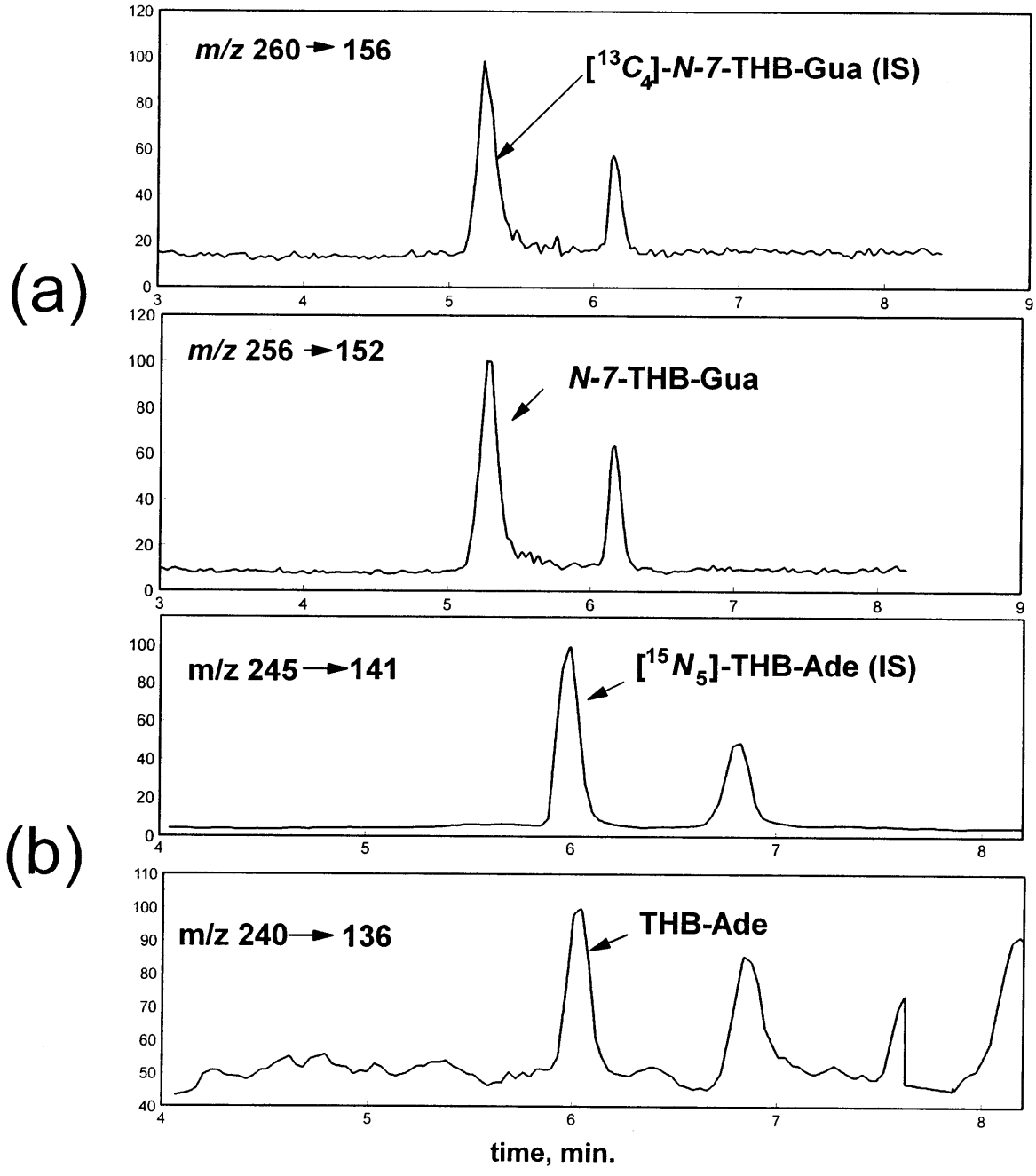

Figure 8. LC/ESI+-MS/MS analysis of trihydroxybutyl adducts of (a) guanine and (b) adenine in an acid hydrolyzate of CT DNA treated with diepoxybutane.

Table 4. DNA adducts in TK6 cell cultures exposed to the mono- and diepoxide of 1,3-butadiene

Exposure	Mutational frequency[a]	Amounts of adduct per 10^6 normal nucleobases					
		N-7-EB-Gua I	*N-7*-EB-Gua II	*N-3*-EB-Ade III	*N-3*-EB-Ade IV	*N-7*-THB-Gua	*N-3*-THB-Ade
5 μM EB	8.5	0.53	0.48	0.19	ND[c]	–[d]	–
100 μM EB	11.3	3.8	3.0	ND	ND	–	–
500 μM EB	38.3	14.0	10.1	1.2	0.5	–	–
5 μM DEB	43.3	–	–	–	–	<0.1	ND
25 μM DEB[b]	–	–	–	–	–	4.0	<1
Control	7.9	ND[e]	ND	ND	ND	ND	ND

[a] Per 10^6 surviving cells.
[b] The mutation frequency for these cells could not be measured owing to excessive cytotoxicity.
[c] ND = not detected.
[d] – = Not determined.

tified as *N-3*-THB-Ade and *N-7*-THB-Ade (results not shown), based on their retention times and fragmentation under CID, which were compared with those of the authentic standards.

DNA adducts of EB-and DEB-exposed TK6 cell cultures

DNA isolated from EB- and DEB-treated TK6 cell culture contained the same types of adducts as observed in exposed CT DNA (Table 4). The amounts of EB-guanine adducts increased proportionally with an increase of exposure concentration. The extent of alkylation at the *N-3* of adenine was significantly lower those at the *N-7*-guanine of DNA. LC/ESI$^+$-MS/MS analyses of DNA from cell cultures exposed to DEB indicated the presence of *N-7*-guanine and N^6-adenine adducts, although the latter was below the limit of quantitation of this method (Table 4).

In order to validate the LC/ESI$^+$-MS/MS method, parallel analyses of the DNA samples isolated from TK6 cells exposed to 500 μM EB was performed by LC/ESI$^+$-MS/MS and GC/ECNCI-HRMS. The latter method required derivatization of the adducts with PFBBr in order to increase their volatility and to increase the response under NCI conditions. The major peak in the NCI mass spectrum of the PFB derivative of EB-Gua (*m/z* 581) corresponded to the loss of one PFB group from the molecular ion (Fig. 9). Selected ion

Table 5. Comparison of GC/MS and LC/ESI$^+$-MS/MS for quantitation of DNA adducts with BD[a]

Analyte	Amount of adducts per 10^6 normal bases	
	GC/NICI-HRMS	LC/ESI$^+$-MS/MS
EB-Gua I	29.7	27.8
EB-Gua II	63.1	32.6

[a] Sample:DNA from EB-exposed TK6 cell culture (500 μM, 24 h).

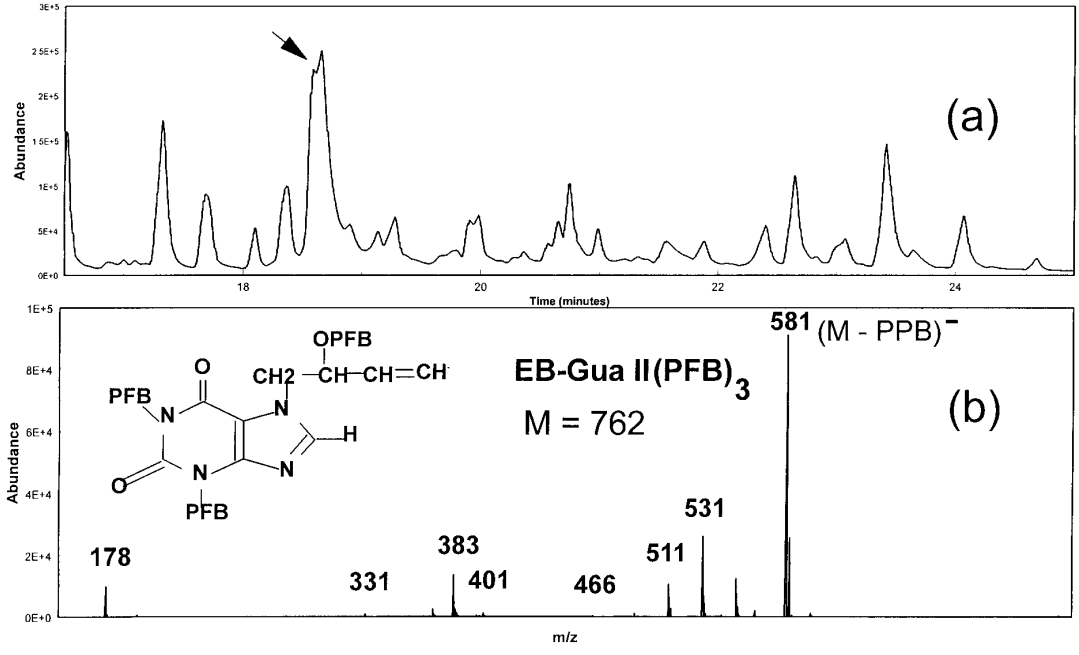

Figure 9. (a) GC/ECNI total ion current mass chromatogram of PFBBR-derivatized *N-7*-EB-guanine and (b) full mass spectrum of the major derivative.

Table 6. BD adducts in tissues of rats and mice exposed to 1250 ppm BD for 10 days by inhalation

		Amount of adduct per 10^6 normal bases (mean ± SD)			
Animal	Tissue	EB-Gua I	EB-Gua II	EB-Ade I	THB-Gua
Control rat	Liver	ND[6]	ND	ND	ND
Exposed rat	Liver	1.4 ± 0.11	1.4 ± 0.02	ND	4.1 ± 1.5
Control mouse		ND	ND	ND	ND
Exposed mouse	Liver	3.0 ± 0.05	2.7 ± 0.1	0.07	7.6 ± 1.5
Exposed mouse	Lung	2.4	2.1	0.06	–[b]

[a] – = Not determined.
[b] ND = not detected.

monitoring of the $(M - PFB)^-$ ions of the derivatized adducts and the corresponding internal standards was performed at 10K resolution. The other half of the DNA hydrolyzate was analyzed by LC/ESI$^+$-MS/MS as described above. The results of both analyses are presented in Table 5. Excellent agreement between the results of the two methods was observed for EB-Gua I. The amounts of EB-Gua II from GC/ECNI-HRMS appeared higher than that from LC/ESI-MS/MS. The reasons for this difference are not clear but could reflect an impurity co-eluting either with the analyte in selected ion monitoring GC/HRMS or with the internal standard in LC/ESI$^+$-MS/MS analysis.

BD-induced DNA adducts in tissues of rats and mice exposed to 1250 ppm BD

Since the LC/ESI$^+$-MS/MS methods developed in this work were successfully applied to analyses of BD-induced adducts *in vitro* in samples exposed to the reactive epoxides of BD, the work was extended to an evaluation of the *in vivo* formation of these adducts in tissues of animals exposed to BD itself. The results of LC/ESI$^+$-MS/MS analyses of the DNA samples from tissues of several rats and mice exposed to 1250 ppm BD for 2 weeks by inhalation are presented in Table 6 and typical ion chromatograms obtained from ~1 mg of mouse liver DNA are given in Figs 10 and 11. Similarly to the results of *in vitro* experiments (see above), the amounts of the two regioisomers of *N*-7-EB-Gua were comparable (see Table 6 and Fig. 10(a)). No EB-Gua peaks were observed in control liver (Fig. 10(b)). The amounts of *N*-7-EB-Gua adducts in livers of BD-exposed mice (5.7 per 10^6 Gua) were ~2-fold higher than in livers of rats exposed under the same conditions (2.8 per 10^6 Gua).

LC/ESI$^+$-MS/MS using selected reaction monitoring of the transitions corresponding to THB adducts indicated the presence of *N*-7-THB-Gua and N^6-THB-Ade

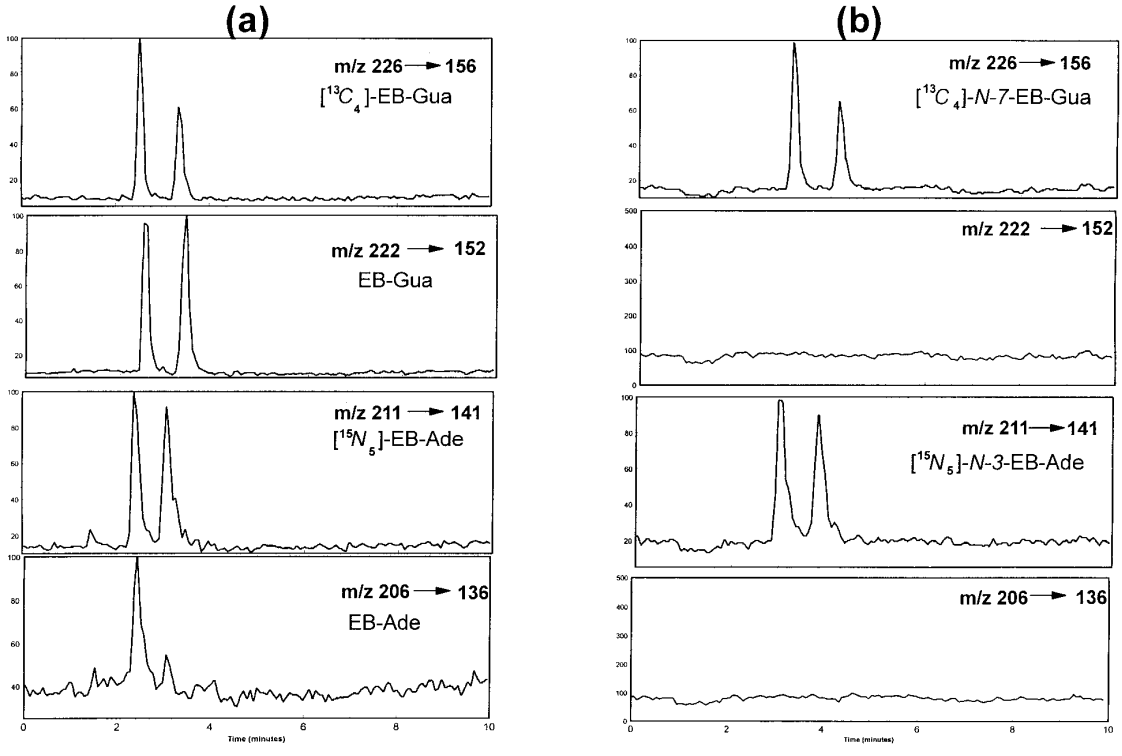

Figure 10. LC/ESI$^+$-MS/MS analysis of EB–DNA adducts in neutral thermal hydrolyzates of liver DNA from (a) a mouse exposed to 1250 ppm BD for 2 weeks and (b) a control mouse.

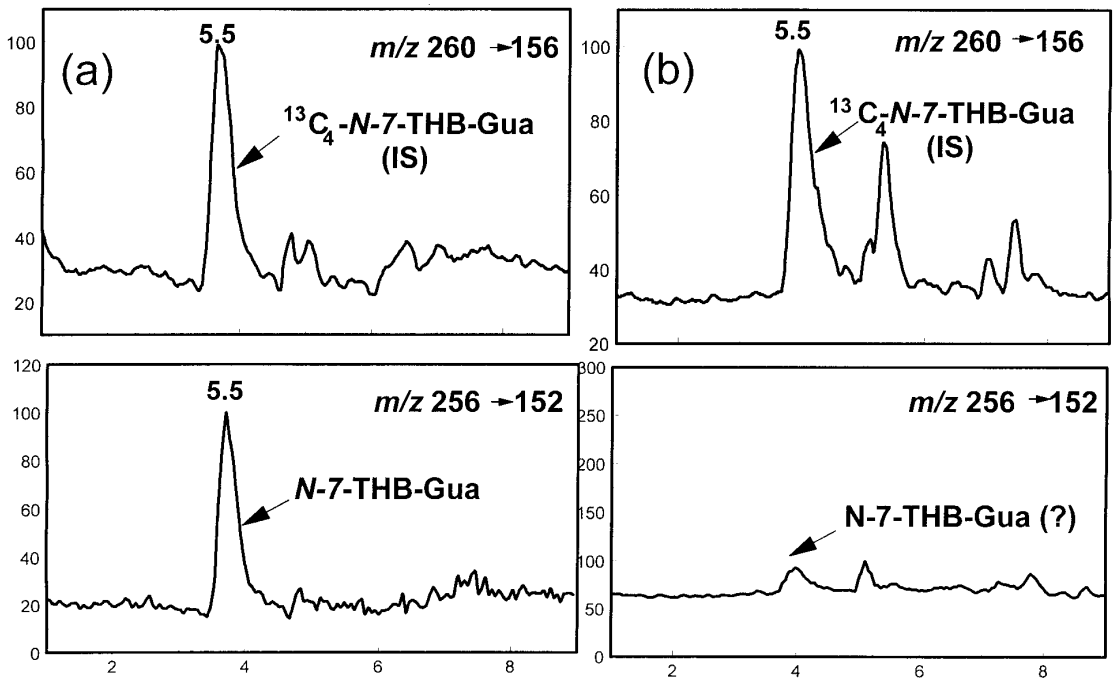

Figure 11. LC/ESI$^+$-MS/MS analysis of *N*-7-THB-Gua in neutral thermal hydrolyzates of liver DNA from (a) a mouse exposed to 1250 ppm BD for 2 weeks and (b) a control mouse.

in DNA of exposed rodents (Fig. 11). *N*-7-THB-Gua was somewhat more abundant than the corresponding EB-induced lesions (Table 6). Although the peak area ratios suggested that the amounts of N^6-THB-Ade in these samples were comparable to those of *N*-7-EB-Gua adducts (results not shown), the presence of an interferent co-eluting with the analyte peak prevented us from quantitating this adduct *in vivo*. Interestingly, control animals exposed to air only contained small peaks that may be THB adducts, but no similar peaks for EB adducts (e.g. Figs 10(b) and 11(b)), suggesting that THB adducts may be produced endogenously.

DISCUSSION

Covalent DNA modification with carcinogens or their metabolites can be the initial step in chemical carcinogenesis. If not repaired before DNA replication, DNA adducts can cause mispairing resulting in mutations and chromosomal alterations.[30] DNA adducts thus provide a 'biological dose' of a chemical which integrates its distribution and biotransformation and is related to the biological effects that follow. Analysis of DNA adducts *in vivo* is a challenge since the specificity and sensitivity of the method must be sufficient to allow identification and quantitation of modified nucleobases in the presence of a 10^6–10^7 excess of normal nucleobases. ^{32}P-postlabeling is currently the most popular method of analysis of DNA adducts.[11–13,17,31] ^{32}P-postlabeling is sensitive enough to detect one adduct per 10^8 normal bases and lower. Unfortunately, ^{32}P-postlabeling provides no structural information on the analytes which are detected as 'spots' on a TLC plate and quantitated from radioactive counts. Furthermore, some of the the most common types of DNA adducts (*N*-7-alkylguanine, *N*-3-alkyladenine) have poor labeling yields owing to the facile cleavage of their glycosyl bonds.[31] Methods based on gas chromatography/mass spectrometry are more specific than ^{32}P-postlabeling, since the adducts are identified by their molecular masses and GC retention times. Since DNA nucleobases are polar, some kind of chemical derivatization is usually required. This step represents a challenge for certain types of DNA adducts due to poor derivatization yields (e.g. THB adducts), production of artifacts during the derivatization step and sample to sample variability.[28] In addition, derivatization methods are usually analyte-specific and cannot be applied to analyze simultaneously chemically diverse compounds in the same sample.

LC/ESI$^+$-MS makes it possible to analyze DNA adducts directly, without derivatization, since volatility is not necessary. Sample preparation is based on the separation of the adducts from normal nucleobases and desalting, since the presence of large amounts of electrolytes can result in ion suppression and loss of sensitivity.[29,32] Tandem mass spectrometry adds further specificity to the detection of DNA adducts in complex biological mixtures since adduct identification is based on three parameters: LC retention time, molecular mass and characteristic MS fragmentation of the analyte. Several different analytes can be analyzed in the same sample. Unfortunately, the sensitivity of LC-ESI$^+$ MS methods is typically 10-fold lower than that of GC/ECNI-HRMS,[26] which is in turn less sensitive than ^{32}P-postlabeling.

In the present work, LC/ESI$^+$-MS/MS was used to analyze multiple BD-induced adducts in DNA hydrolyzates. The developed methods were applied to samples of treated CT DNA, TK6 cell cultures and tissues of laboratory animals exposed to BD by inhalation. The methods were validated by comparing the results of LC/ESI$^+$-MS/MS analyses with the data obtained independently by GC/NCI-HRMS. Whereas *N*-7-Gua adducts

could be accurately quantitated in 0.3–1 mg DNA samples extracted from animal tissues, larger DNA samples ($\geqslant$2 mg) are needed for *in vivo* quantitation of the less abundant *N*-3-EB-Ade and N^6-THB-Ade adducts. Although the amount of DNA alkylation was higher in mice than in rats ($\sim$2-fold, Table 6), this difference is not sufficient to explain the markedly increased sensitivity of mice to BD carcinogenesis. The overall level of DNA adduction in BD-exposed animals ($<$10 per 10^6 normal nucleobase) appears to be low compared with other simple alkylating agents (ethylene oxide),[22] suggesting that mechanisms other than point mutations resulting from mono-adducts may be involved in BD carcinogenesis. For example, DNA–DNA and/or DNA–protein cross-linking by DEB may play a role.[33] Interestingly, the trihydroxybutyl adducts were observed in both species in amounts comparable to or higher than that of the EB adducts (Table 6). Since trihydroxybutyl adducts can result from reactions of either DEB or EBD with DNA and rats do not produce DEB in any significant amount, EBD may be an important metabolite in this species.[34] A study is under way to determine the reactivity and adduct types from EBD reactions with DNA nucleobases.

The presence of small amounts of THB but not EB adducts in tissues of control animals is consistent with the presence of THB hemoglobin adducts in red blood cells of unexposed rodents and humans obtained in this laboratory.[35] Further studies are required to confirm the presence of THB–DNA adducts and to establish the source of these endogenous adducts. The methods described in this paper provide a foundation for future studies of dose–response relationships, accumulation and repair of various BD-induced DNA adducts. Although less sensitive than GC/ECNI-HRMS, LC/ESI$^+$-MS/MS in selected reaction monitoring mode is a useful method in analyses of BD–DNA adducts which are not amenable to GC and derivatization owing to the presence of several adjacent polar functional groups in the molecule.

Acknowledgements

This work would have been impossible without the generous gift of instrument time from Triangle Laboratories (Durham, NC, USA). We appreciate the assistance of Mike Allen (Triangle Laboratories) and Asoka Ranasinghe (UNC) in performing mass spectral analyses. We thank Dr J. Ronald Hass for suggesting the use of LC/ESI$^+$-MS and helpful discussions along the way, Dr Ramiah Sangaiah for the synthesis of [$^{13}C_4$]-dGuo and Dr T.-Y. Yen and Patricia Upton for critical reading of the manuscript. This work was sponsored in part by grants from the Chemical Manufacturers Association and the Health Effects Institute (agreement 94-6).

REFERENCES

1. N. L. Morrow, *Environ. Health Perspect.* 86, 7 (1990).
2. N. Pelz, A. M. Dempster and P. R. Shore, *J. Chromatogr. Sci.* **28**, 230 (1990).
3. International Agency for Research on Cancer (IARC), *IARC Monogr Eval Carcinogen Risk Chem Hum*, Vol. 54, p. 237. IARC, Lyon (1992).
4. H. M. Bolt, G. Schmiedel, J. G. Filser, H. P. Rolzhauser, K. Lieser, D. Wistuba and V. Schurig, *J. Cancer Res. Clin. Oncol.* **106**, 112 (1983).
5. E. Malvoisin and M. Roberfroid, *Xenobiotica* **12**, 137 (1982).
6. M. W. Himmelstein, J. F. Acquavella, L. Recio, M. A. Medinsky and J. A. Bond, *Crit. Rev. Toxicol.* **27**, 1 (1997).
7. C. de Meester, *Mutat. Res.* **195**, 273 (1988).
8. J. E. Cochrane and T. R. Skopek, *Carcinogenesis* **15**, 713 (1994).
9. L. Citti, G. Gervasi, G. Turchi, G. Bellucci and R. Bianchini, *Carcinogenesis* **5**, 47 (1984).
10. B. Jellito, R. R. Vangala and R. J. Laib, *Arch. Toxicol.* **13**, Suppl., 246 (1989).
11. P. Koivisto, R. Kostiainen, I. Kilpeläinen, K. Steinby and K. Peltonen, *Carcinogenesis* **16**, 2999 (1995).
12. C. Leuratti, N. J. Jones, E. Marafante, R. Kostiainen, K. Peltonen and R. Waters, *Carcinogenesis* **15**, 1903 (1994).
13. M. Sorsa, K. Peltonen, D. Anderson, N. A. Demopoulos, H-G. Neumann and S. Osterman-Golkar, *Mutagenesis* **11**, 9 (1996).
14. N. Yu. Tretyakova, Y-P. Lin, P. B. Upton, R. Sangaiah and J. A. Swenberg, *Toxicology* **113**, 70 (1996).
15. N. Yu. Tretyakova, Y-P. Lin, R. Sangaiah, P. B. Upton and J. A. Swenberg, *Carcinogenesis* **18**, 137 (1997).
16. R. R. Selzer and A. A. Elfarra, *Chem. Res. Toxicol.* **9**, 126 (1996).
17. P. Koivisto, M. Sorsa, F. Paccierotti and K. Peltonen, *Carcinogenesis***18**, 439 (1997).
18. N. Yu. Tretyakova, R. Sangaiah, T.-Y. Yen and J. A. Swenberg, *Chem. Res. Toxicol.* **10**, 779 (1997).
19. N. Yu. Tretyakova, R. Sangaiah, T.-Y. Yen and J. A. Swenberg, *Chem. Res. Toxicol.***10**, 1171 (1997).
20. Q. Meng, L. Recio, A. A. Reilly, B. A. Wong, M. Bauer and V. E. Walker, *Carcinogenesis* submitted for publication.
21. R. A. Becker, L. R. Barrows and R. C. Shank, *Carcinogenesis* **2**, 1181 (1981).
22. N. Fedtke, J. A. Boucheron, M. J. Turner and J. A. Swenberg, *Carcinogenesis* **11**, 1279 (1990).
23. M. Rios-Blanco, K. Plna, T. Saller, W. Kessler, K. Hakasson, P. E. Kreuzer, A. Ranasinghe, D. Segerbäck and J. A. Swenberg, *Mutat. Res.* **380**, 179 (1997).
24. D. E. G. Shuker and P. B. Farmer, *Chem. Res. Toxicol.* **5**, 450 (1992).
25. M. Muller, F. J. Belas, I. A. Blair and F. P. Guengerich, *Chem. Res. Toxicol.* **10**, 242 (1997).
26. A. K. Chaudhary, M. Nokubo, T. D. Oglesby, L. J. Marnett and I. A. Blair, *J. Mass Spectrom.* **30**, 1157 (1995). 27. Z. Liu, R. Young-Sciame and S. S. Hecht, *Chem. Res. Toxicol.* **9**, 774 (1996).
28. T.-Y. Yen, N. I. Christova-Gueorgieva, S. Holt, J. A. Swenberg and M. J. Charles, *J. Mass Spectrom.* **31**, 1271 (1996).
29. T.-Y. Yen, R. D. Voyksner and M. J. Charles, in *Proceedings of the 43rd ASMS Conference on Mass Spectrometry and Allied Topics*, Atlanta, GA, 1995, p. 247.
30. B. Singer and D. Grunberger, *Molecular Biology of Mutagens and Carcinogens.* Plenum Press, New York (1983).
31. R. Kumar, P. Vodicka, P. Koivisto, K. Peltonen and K. Hemminki, *Carcinogenesis* **17**, 1297 (1996).
32. A. P. Snuder (Ed.), *Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry*, ACS Symposium Series, No. 619 American Chemical Society, Washington, DC (1995).
33. P. Brookes and P. D. Lawley, *Biochem. J.El*] **80**, 496 (1961).
34. H. L. Perez, J. Lähdetie, H. H. Landin, I. Kilpeläinen, P. Koivisto, K. Peltonen and S. Osterman-Golkar, *Chem.-Biol. Interact.* **105**, 181 (1997).
35. A. Ranasighe, N. Christova-Georgieva and J. A. Swenberg, paper presented at the 45th ASMS Conference on Mass Spectrometry and Allied Topics. San Diego, CA (1997).