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^{32}P-postlabelling analysis of 1,3-butadiene-induced DNA adducts *in vivo* and *in vitro*

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Butadiene monoepoxide (BMO), epoxybutanediol (EBD) and diepoxybutane (DEB) are reactive metabolites of 1,3-butadiene (BD), an important industrial chemical classified as a probable human carcinogen. The covalent interactions of these metabolites with DNA lead to the formation of DNA adducts which may induce mutations or other types of DNA damage, resulting in tumour formation. In the present study, two pairs of diastereomeric *N*-1-BMO-adenine adducts were identified in the reaction of BMO with 2′-deoxyadenosine-5′-monophosphate (5′-dAMP). The major products formed by reacting EBD with 2′-deoxyguanosine-5′-monophosphate (5′-dGMP) were characterized as diastereomeric *N*-7-(2′,3′,4′-trihydroxybut-1′-yl)-5′-dGMP by UV and electrospray mass spectrometry. The formation of *N*-7-BMO-guanine adducts (1′-carbon, 60; 2′-carbon, 54/10^4 nucleotides) in BMO-treated DNA was about four times higher than that of *N*-1-BMO-adenine adducts (1′-carbon, 20; 2′-carbon, 8.7/10^4 nucleotides). However, the recovery of *N*-1-BMO-adenine adducts in DNA (45 ± 5%) was two times higher than that of *N*-7-guanine adducts (20 ± 4%) by ^{32}P-postlabelling analysis. Using the ^{32}P-postlabelling/HPLC assay, *N*-1-BMO-adenine, *N*-7-BMO-guanine and *N*-7-EBD-guanine adducts were detected in BMO- or DEB-treated DNA and in liver DNA of rats exposed to BD by inhalation. The amount of *N*-7-EBD-guanine adducts (11/10^8 nucleotides) in rat liver was about three-fold higher than *N*-7-BMO-guanine adducts (4.0/10^8 nucleotides). The novel finding of *N*-1-BMO-adenine adducts formed *in vivo* may contribute to the understanding of the mechanisms of BD carcinogenic action.

Keywords: 1,3-butadiene, butadiene monoepoxide, epoxybutanediol, diepoxybutane, ^{32}P-postlabelling, DNA adducts, HPLC.

Abbreviations: BD, 1,3-butadiene; BMO, butadiene monoepoxide; EBD, epoxybutanediol; DEB, diepoxybutane; HPLC, high performance liquid chromatography; ESI-MS, electrospray ionization-mass spectrometry; TLC, thin-layer chromatography.

Introduction

1,3-Butadiene (BD), an important industrial chemical, has been classified by IARC as a 'probable human carcinogen' (Fajen *et al*. 1993). Humans are exposed to BD from cigarette smoke, from automobile exhaust and in the workplace (Brunnemann *et al*. 1990, Pelz *et al*. 1990). BD is carcinogenic to mice and rats, mice being by far the more sensitive species (Huff *et al*. 1985, Melnick *et al*. 1990). In humans, epidemiological data showed an increase in the incidence of various lymphohaematopoietic cancers in industrial workers exposed to BD (Divine 1990, Matanoski *et al*. 1990, 1993, Santos-Burgoa *et al*. 1992). In addition, an increase in *hprt* mutant frequency in the peripheral lymphocytes of non-smoking workers exposed to BD over controls was reported (Ward *et al*. 1994).

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BD undergoes a complex pathway of metabolic transformation and forms several intermediates with potential reactivity towards DNA (figure 1). The alkylation of DNA is assumed to be the basis for its mutagenic action. The compound is metabolized by cytochrome P-450-dependent monooxygenases to the primary metabolite butadiene monoepoxide (BMO). BMO is subjected to further metabolism: oxidation to diepoxybutane (DEB), hydrolysis to butenediol and conjugation to glutathione. Epoxybutanediol (EBD) may be formed by hydrolysis of DEB or by oxidation of butenediol (Malvoisin *et al*. 1979, Malvoisin and Roberfroid 1982, Bolt *et al*. 1983, Csanady *et al*. 1992). BMO, DEB and EBD are mutagenic and genotoxic in different *in vivo* and *in vitro* systems (Xiao and Tates 1995, Anderson *et al*. 1997, Tates *et al*. 1998) and are thought therefore to be responsible for at least a proportion of the genotoxic activity seen after exposure to BD. DEB is a bifunctional alkylating agent and is a considerably more effective mutagen than BMO (Ehrenberg and Hussain 1981, Xiao and Tates 1995). The precise contribution of these metabolites to BD-related effects is not known as yet, because of limited understanding of BD metabolism, especially in humans.

The epoxide metabolites of BD are DNA alkylating agents and multiple DNA adducts have been identified. BMO has been shown to react with deoxyguanosine and DNA *in vitro* to form regioisomeric pairs at *N*-7-guanine, *N*-1- and *N*-3-adenine (Citti *et al*. 1984, Neagu *et al*. 1995, Kumar *et al*. 1996, Selzer and Elfarra 1996, Tretyakova *et al*. 1997). These result from the nucleophilic attack by *N*-7 of guanine, *N*-1 and *N*-3 of adenine at 1-carbon and 2-carbon of BMO. Other adducts of BMO include N^6-alkyladenine, which is formed by Dimroth rearrangement of the initial *N*-1-adenine adducts (Koivisto *et al*. 1995). DEB has also been shown to react with guanine and DNA to form two major adducts identified as *N*-7-(2′,3′,4′-trihydroxybut-1′-yl)guanine and N^6-(2′,3′,4′-trihydroxybut-1′-yl)adenine (Tretyakova *et al*. 1997a,b). The N^6-adenine adducts of DEB have been detected by ^{32}P-postlabelling/HPLC in DNA from cultured cells (Leuratti *et al*. 1994). So far, limited data are available concerning *in vivo* formation of DNA adducts of BD. Few studies have investigated the *N*-7-guanine adducts of BMO and DEB formed in the tissues of rats and mice which were inhalation-exposed to BD (Koivisto *et al*. 1996, 1997, 1998a,b, 1999, Tretyakova *et al*. 1998). The measurement of BD-induced DNA adducts *in vivo* will provide a basis for understanding the mechanisms of carcinogenic process due to BD. In the present study *N*-1-BMO-adenine, *N*-7-BMO-guanine and *N*-7-EBD-guanine adducts were analysed in BMO- and DEB-treated DNA *in vitro* and in liver DNA obtained from BD-exposed rats by using the ^{32}P-postlabelling/HPLC assay. The

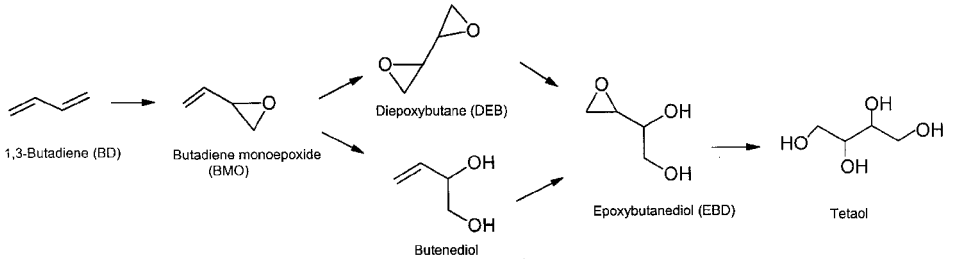

Figure 1. The metabolism of 1,3-butadiene.

novel aspect of the study was that we analysed *N*-1-adenine adducts of BMO *in vivo* by using a revised HPLC-based method of high specificity and sensitivity. As a novel technical approach for the adenine adducts, Dimroth rearrangement of the postlabelled *N*-1-adenine adduct fractions was utilized to convert these to the corresponding N^6-adenine adducts which were reanalysed by HPLC.

Materials and methods

Chemicals

Chemicals were used as purchased from the manufacturer. 2′-Deoxyguanosine-5′-monophosphate (5′-dGMP), 2′-deoxyadenosines-5′-monophosphate (5′-dAMP), salmon testis DNA (sodium salt) and micrococcal nuclease were from Sigma Chemical Co. (St Louis, MO). BMO and DEB were from Aldrich Chemical Co. (Milwaukee, WI), spleen phosphodiesterase and nuclease P1 from Boehringer Mannheim (Mannheim, Germany), [γ-^{32}P]ATP and T4 polynucleotide kinase from Amersham (Little Chalfont, UK). The polyethyleneimine cellulose TLC plates were purchased from Macherey-Nagel (Düren, Germany). Methanol was gradient grade from Merck (Darmstadt, Germany). All other chemicals were either from Sigma or Merck.

Reaction of BMO with 5′-dAMP and 5′-dGMP

5′-dAMP (2 mg ml^{-1}) was incubated at 37 °C for 24 h with 100 mM BMO in 50 mM Tris–HCl (pH 7.4), followed by extraction with ethylacetate. The charged adducts were enriched on a 1 ml Bakerbond anion exchange cartridge (J. T. Baker) and eluted with 5 mM ammonium formate (AF) buffer, pH 5.2. The eluents were injected into a Beckman HPLC system consisting of a 116 dual head pump and a 168 diode array UV detector. UV spectra were recorded by a Beckman DU-640 spectrophotometer. For purification of *N*-1-BMO-5′-dAMP, a C-18 (4.6 × 250 mm) reversed phase column was used. It was run with a linear gradient of 98% of 50 mM AF, pH 4.6, to 20% of methanol for 80 min. Flow-rate was 0.7 ml min^{-1} and the adducts were detected by UV-absorbance at 260 nm. N^6-BMO-5′-dAMP was prepared by the Dimroth rearrangement of *N*-1-BMO-5′-dAMP at pH 13 (80 °C, 30 min).

N-7-BMO-5′-dGMP was a gift from Dr Kumar, and it had been synthesized and characterized as described in Kumar *et al*. (1996). *N*-7-BMO-guanine was prepared by depurinating the nucleotide adduct at neutral pH, 100 °C for 30 min and separating the products by HPLC.

Reaction of EBD with 5′-dGMP

EBD was prepared by hydrolysis of DEB (200 µl, 2.6 mmol) in water (1.5 ml) for 3 days at 37 °C, as described by Licea Pérez *et al*. (1997). The remaining DEB was extracted with toluene. Assuming a half-life of ~100 h (Ehrenberg and Hussein 1981) it was estimated that the EBD concentration obtained was 670 mM. 5′-dGMP (2 mg ml^{-1}) was treated with 67 mM EBD in 50 mM Tris–HCl, pH 7.4 at 37 °C for 24 h. The samples were analysed by HPLC with a gradient starting isocratically from 100% of 50 mM AF, pH 4.6 for 15 min, followed by a linear gradient to 40% methanol for 60 min.

Reaction with DNA

Salmon testis DNA (2 mg ml^{-1} 50 mM Tris–HCl, pH 7.4) was treated with 100 mM BMO or 100 mM DEB at 37 °C for 24 h. At the end of the incubation, the mixtures were extracted with ethylacetate followed by ethanol precipitation of DNA. The purified DNA was depurinated at pH 1 (0.1 M HCl, 70 °C, 30 min) and the released DNA bases were separated by HPLC coupled with a diode array detector. A C-18 (4.6 × 250 mm) reversed phase column was used and it was run isocratically with 100% of 50 mM AF, pH 4.6, for 20 min, followed by a linear gradient of 100% of 50 mM AF, pH 4.6, to 20% methanol for 60 min. Flow-rate was 0.7 ml min^{-1}. Adduct levels of *N*-1-BMO-adenine and *N*-7-BMO-guanine were calculated from standard curves obtained by injecting samples with known concentrations of *N*-7-BMO-guanine, *N*-1-BMO-adenine. Concentrations of standards were determined by UV absorption using published molar extinction coefficients for corresponding methylation products (*Handbook of Biochemistry* 1970).

^{32}P-postlabelling of N-1-adenine adducts

The BMO modified salmon testis DNA (5 µg) and rat liver DNA (10 µg) were analysed by postlabelling procedure as described by Randerath *et al*. (1989). DNA was hydrolysed with a mixture of nuclease P1 (20 mU µg^{-1} DNA) and prostatic acid phosphatase (0.2 µg µg^{-1} DNA) at pH 5. After incubation at 37 °C for 45 min, the reaction was terminated by adding 100 µl cold ethanol. Proteins were

precipitated for 20 min at –20 °C. After centrifugation the supernatant was evaporated to dryness. Labelling of adducted dinucleotides was carried out in a mixture (2 μl) containing 2.4 U T4 polynucleotide kinase and 2.3 pmol [γ-^{32}P]ATP (specific activity > 5000 Ci per mmol). The reaction was carried out at pH 9.6, followed by adding snake venom phosphodiesterase (0.5 mU μg^{-1} DNA). The postlabelled sample mixed with the synthesized UV-marker, *N*-1-BMO-5′-dAMP, was injected into a Beckman HPLC coupled with a UV and a Beckman 171 Radioisotope detector. HPLC fractions containing postlabelled *N*-1-BMO-adenine adducts were collected and treated with 0.1 M NaOH at pH 13 (80 °C, 30 min), representing conditions under which *N*-1-adenine is completely converted to N^6-adenine adducts. The corresponding N^6-adenine adducts formed by the Dimroth rearrangement were further analysed by HPLC.

For collection of *N*-1-BMO-adenine adducts, the column was run isocratically with 100% of 0.2 M AF containing 20 mM phosphoric acid, pH 4.6, for 15 min, followed by a linear gradient of 100% of 50 mM AF, pH 4.6, to 5% methanol for 65 min. For separation of N^6-BMO-adenine adducts, the column was run isocratically with 99% of 0.2 M AF containing 20 mM phosphoric acid, pH 4.6, for 20 min, followed by a linear gradient of 99% of 0.2 M AF containing 20 mM phosphoric acid, pH 4.6, to 20% methanol for 35 min and then maintained for 20 min at which time the N^6-BMO-adenine adducts eluted. Flow rate was 0.2 ml min^{-1}.

^{32}P-postlabelling of N-7-guanine adducts

The BMO or DEB modified salmon testis DNA (5 μg) and rat liver DNA (10 μg) were analysed by the postlabelling assay as described previously (Zhao *et al*. 1997). Briefly, DNA samples were incubated at 37°C with micrococcal nuclease (80 mU μg^{-1} DNA, 2 μl) in 3 mM Bicine(0.5 μl), pH 9.0 and 0.2 mM $CaCl_2$ (0.5 μl) for 2 h followed by addition of spleen phosphodiesterase (1.6 mU μg^{-1} DNA, 2.0 μl) and 20 mM ammonium acetate (1.0 μl), pH 5.0. The incubation was continued for further 2 h. The adduct enrichment was carried out by applying DNA digests to anion exchange cartridges. ^{32}P-postlabelling was carried out as described above without SVPD treatment. Instead the labelling mixtures were treated with nuclease P1 (2.5 μg, 0.5 μl) for 15 min. The postlabelled sample mixed with the synthesized UV-marker, *N*-7-BMO-5′-dGMP or *N*-7-EBD-5′-dGMP, was injected into the HPLC. HPLC fractions containing postlabelled *N*-7-BMO-guanine or *N*-7-EBD-guanine adducts were collected and applied to 10×20 cm PEI TLC plates (pre-washed with water) and developed with 0.1 M AF, pH 5.2 in the first dimension (D1) and 0.6 M AF, pH 5.2, mixed with 40% *n*-propanol in the second dimension (D2). Adducts were visualized by autoradiography, and radioactivity of the adduct spots measured by Cerenkov counting.

For collection of *N*-7-BMO-guanine adducts, the column was run isocratically with 98% of 0.2 M AF containing 20 mM phosphoric acid, pH 4.6, for 20 min, followed by a linear gradient of 98% 50 mM AF, pH 4.6, to 5% methanol for 60 min. For collection of *N*-7-EBD-guanine adducts, the column was run isocratically with 99% of 50 mM AF, pH 4.6, for 30 min. Flow rate was 0.2 ml min^{-1}.

Mass spectrometry

Electrospray ionization-mass spectra (ESI-MS) were obtained using a Finnigan LCQ LC/MS^n system (equipped with an ion trap mass analyser), connected to a Waters 2690 separation module. The samples were analysed in negative ion mode with a spray voltage of 5.0 kV. Capillary temperature was maintained at 250 °C. The samples (~10–100 ng) were dissolved in 10 μl of the eluent (water/acetonitrile/NH_3, 49/50/1 %) and injected via loop into the running solvent, at a flow-rate 0.2 ml min^{-1}. The full scan data was acquired for 70–1000 *m/z* and for MS/MS data 70–500 *m/z*.

Animal exposure

Male Sprague–Dawley rats were acclimatized for 5 days before the start of exposure. Food and water were given *ad libitum* except during exposure. Animals were exposed to BD for 5 days (6 h per day) in a 1 m^3 steel chamber with glass doors, essentially as described by Koivisto *et al*. (1997). The mean concentration of BD for 5 days was 300 ppm.

Results

Reaction of BMO with 5′-dAMP

The reaction of BMO with 5′-dAMP, followed by adduct enrichment on anion exchange cartridges, resulted in four major products (figure 2). The UV spectra of all these fractions were identical to each other, with λ_{max} at 260 nm (pH 1), 260 nm (pH 7) and 262 nm (pH 13), being similar to those of other reported *N*-1-adenosine

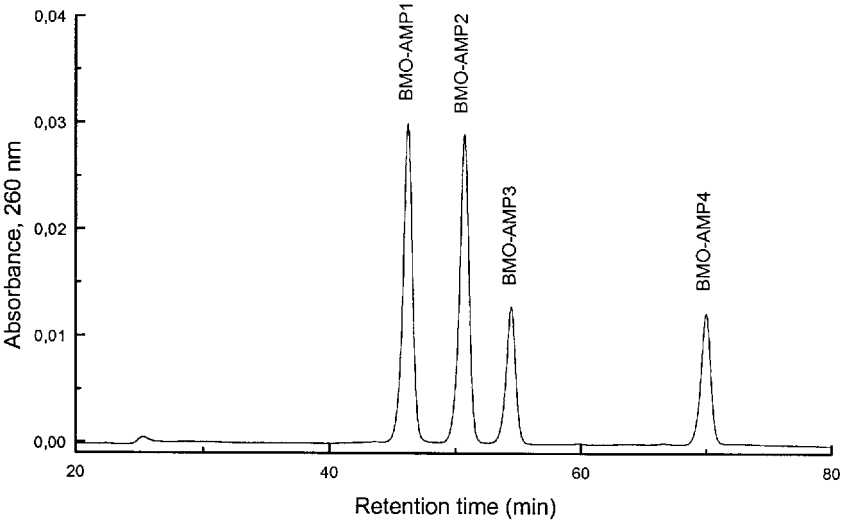

Figure 2. HPLC separation of products formed in a reaction of 5′-dAMP with BMO. Products BMO-AMP1 and BMO-AMP2 correspond to *N*-1-(2′-hydroxy-3′-buten-1′-yl)-5′-dAMP, BMO-AMP3 and BMO-AMP4 to *N*-1-(1′-hydroxy-3′-buten-2′-yl)-5′-dAMP.

adducts. After treatment with 0.1 M NaOH (pH 13, 80 °C, 30 min) the adduct fractions were converted to new products, which were assigned as N^6-BMO-5′-dAMP. Thus, the new adducts had λ_{max} at different pH values, identical to those of authentic N^6-methyladenosine, and MS analysis on the adducted base after acidic depurination showed $[M–H]^-$ of *m/z* 204.5, corresponding to a mono-alkylated BMO-adenine. After depurination of the *N*-1-dAMP adducts at pH 1, the fractions BMO-AMP1 and BMO-AMP2 and the following BMO-AMP3 and BMO-AMP4 eluted as a single peak, respectively, indicating the pairs to be different diastereomeric forms. The depurination products exhibited the characteristic UV spectra at different pH values for *N*-1-adenine adducts. MS analyses gave $[M–H]^-$ of *m/z* 204.5 corresponding to a *N*-1-BMO-adenine in the imino form. The earlier eluting depurination product can be assumed as a *N*-1-adenine adduct reacted through 1-carbon of the BMO, and the later eluting as an adduct reacted through 2-carbon, on the basis of what has been reported on the relative proportions of the adducts formed (Tretyakova *et al*. 1997). Therefore it can be assumed that the adducts BMO-AMP1 and BMO-AMP2 were a diastereomeric pair of *N*-1-(2′-hydroxy-3′-buten-1′-yl)-dAMP, and BMO-AMP3 and BMO-AMP4 were a diastereomeric pair of *N*-1-(1′-hydroxy-3′-buten-2′-yl)-dAMP.

Reaction of EBD with 5′-dGMP

In the reaction of EBD with 5′-dGMP two major products were detected (EBD-GMP1 and EBD-GMP2 in figure 3). The two adducts had identical UV spectra and λ_{max} typical of other known *N*-7-alkylguanosine adducts. The molecular weights of these adducts were confirmed by ESI-MS analysis. Thus, both of the nucleotide adducts gave a deprotonated molecular ion $[M–H]^-$ at *m/z* 450.6. By MS/MS analysis the $[M–H]^-$ was cleaved to a fragment of *m/z* 254.1

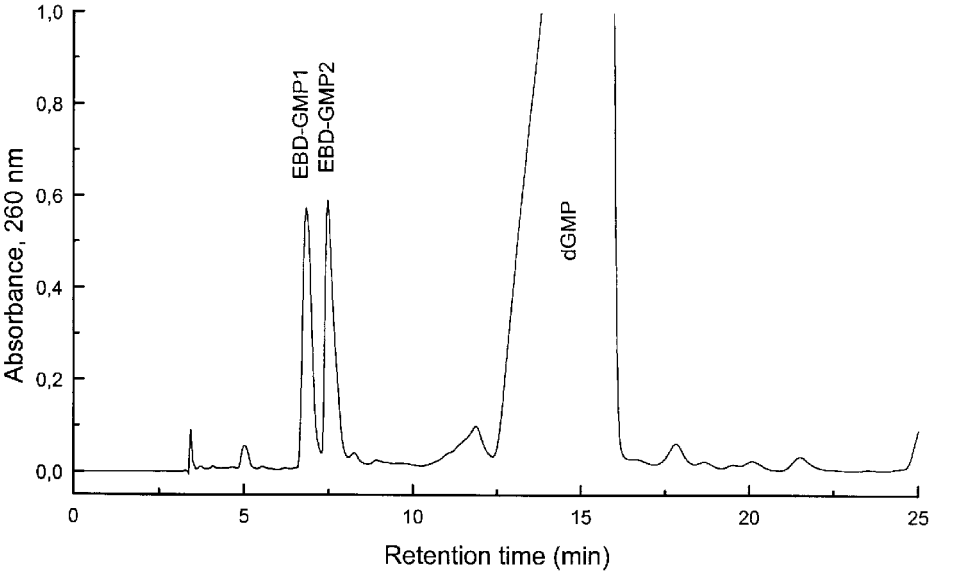

Figure 3. HPLC separation of products formed in a reaction of 5′-dGMP with EBD. Products EBD-GMP1 and EBD-GMP2 correspond to *N*-7-(2′,3′,4′-trihydroxybut-1′-yl)-5′-dGMP.

corresponding to the trihydroxybutylguanine. After heating an aliquot of these adduct fractions, only one peak was observed in the HPLC separation. This product had a UV spectrum typical of other known *N*-7-alkylguanine adducts. The MS analysis of the depurination product gave [M-H$^-$] at *m/z* 254.7, which was selected for further analysis. Thus, by MS/MS, product ions of *m/z* 236.1 and 150.2 were observed, corresponding to cleavage of a hydroxyl group and the trihydroxybutyl moiety, respectively (figure 4). In addition to these, a product with *m/z* 164.3 was observed which was interpreted as guanine with a CH_2-group. This indicates that the trihydroxybutyl is bound to guanine at the terminal 1′-carbon. Such a product cannot be formed if one of the central 2′- or 3′-carbons were reacted with guanine. Therefore, products EBD-GMP1 and EBD-GMP2 were assigned as a diastereomeric pair of *N*-7-(2′,3′,4′-trihydroxybut-1′-yl)-dGMP.

Reaction of BMO with DNA

After hydrolysis at pH 1, the HPLC analysis of the BMO treated salmon testis DNA showed four products, not present in untreated DNA (figure 5). The products BMO-Ade1 and BMO-Ade2 had retention times and UV spectra, obtained by diode array detection, identical to those of *N*-1-BMO-adenine obtained from corresponding depurinated nucleotide adducts. Fractions BMO-Gua1 and BMO-Gua2 corresponded to *N*-7-BMO-guanine, verified by retention times and UV-spectral properties of corresponding depurinated nucleotide adducts. Neutral depurination of DNA (30 min, 100 °C, pH 7.0) did not change the yield of N7-guanine adducts (compared with depurination at pH 1), but no *N*-1-adenine adducts were detected. The levels of these adducts are shown in table 1. Formation of *N*-7-guanine adducts was predominant and a similar ratio of the two isomers was obtained. The level of *N*-1-adenine adducts was about four times

Table 1. *N*-7-BMO-guanine and *N*-1-BMO-adenine adducts in salmon testis DNA exposed to BMO (adducts/10^4 nucleotides)

N-7-Gua (1′-C)	*N*-7-Gua (2′-C)	*N*-1-ade (1′-C)	*N*-1-ade (2′-C)
60	54	20	8.7

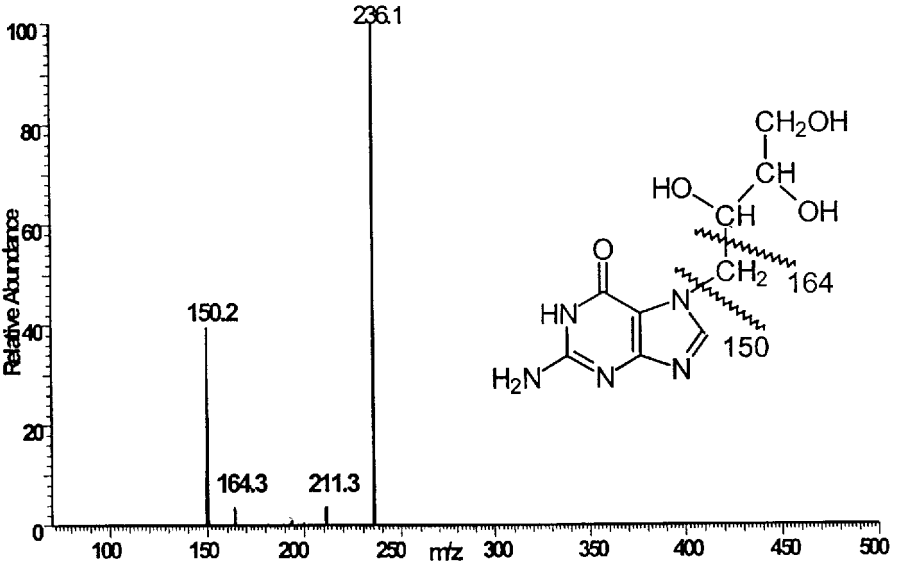

Figure 4. ESI-MS/MS spectrum obtained with the $[M–H]^-$ = 254.7 for 7-alkylguanine adduct of EBD as precurser ion.

lower than *N*-7-guanine adducts and the products of nucleophilic attack at the 1-carbon were about two times more abundant than those resulting from attack at the 2-carbon.

Postlabelling of BMO-treated DNA or DEB-treated DNA

For *N*-7-guanine adduct analysis the BMO-treated DNA or DEB-treated DNA was enzymatically hydrolysed and passed through an anion-exchange cartridge for adduct enrichment. Postlabelled *N*-7-guanine adducts were analysed by HPLC. The adduct peaks in BMO-treated DNA corresponding to diastereomers of the 1′- and 2′-carbon adducts were identified by their co-migration with corresponding *N*-7-BMO-5′-dGMP used as UV markers (figure 6(a)). In DEB-treated DNA the two adduct peaks co-migrated with the *N*-7-EBD-5′-dGMP UV markers (figure 6(b)). These products were not detected when the enriched adduct fractions were heated prior to postlabelling, thus further confirming their identities. Based on known adduct levels in BMO-treated DNA, the recovery of *N*-7-BMO-guanine

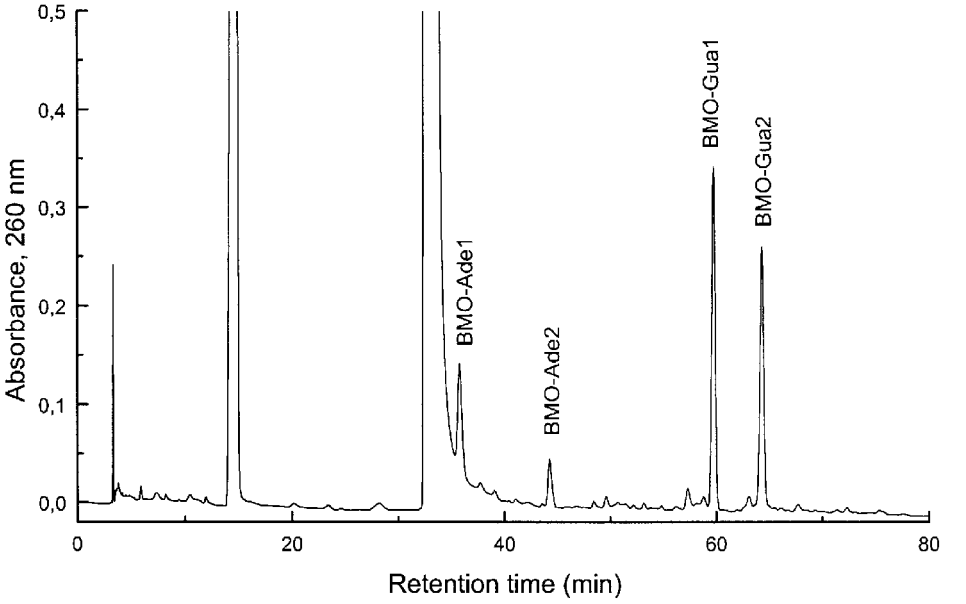

Figure 5. HPLC separation of depurination (pH 1) products from DNA treated with BMO. BMO-Ade1 and BMO-Ade2 correspond to adducts reacted at *N*-1 adenine through 1′ and 2′ -carbons of BMO, respectively. BMO-Gua1 and BMO-Gua2 correspond to adducts reacted at *N*-7 guanine through 1′ and 2′ -carbons of BMO, respectively.

adducts from DNA hydrolysis through all steps of the postlabelling procedure was 20 ± 4 %. Two isomers (1′ and 2′-carbon) showed identical recoveries.

The *N*-1-adenine adducts in BMO-treated DNA were enriched by nuclease P1 digestion. HPLC analysis of the postlabelled sample showed four adduct peaks which were assigned to diastereomeric pairs of adducts reacted through 1- and 2-carbon of BMO, identified by their co-migration with corresponding *N*-1-BMO-5′-dAMP UV markers (figure 7(A)). Upon treatment of collected *N*-1-BMO-adenine adducts with base they were completely converted to two N^6-BMO-adenine adducts (diastereomers co-migrated), which were identified by their co-migration with synthesized N^6-BMO-5′-dAMP adducts (figure 7(B)). Based on known adduct levels in BMO-treated DNA, the recovery of *N*-1-adenine adducts from DNA hydrolysis through all steps of the postlabelling procedure was 45 ± 5 % and the recovery of rearrangement of *N*-1-adenine to N^6-adenine adducts was close to 100 %. The two isomers (1′- and 2′-carbon) had similar recovery. The detection limit of the method was estimated to be 1 adduct/10^{10} using 20 μg DNA.

Analysis of BD-induced DNA adducts in rat liver

The BMO-treated DNA sample was used as an external standard in each individual experiment. The total recovery of *N*-7-guanine adducts and *N*-1-adenine adducts was used to correct the adduct levels determined in rat livers.

The *N*-7-BMO-guanine and *N*-7-EBD-guanine adducts formed in rat liver were analysed by HPLC as described above for the *in vitro*-treated DNA. However,

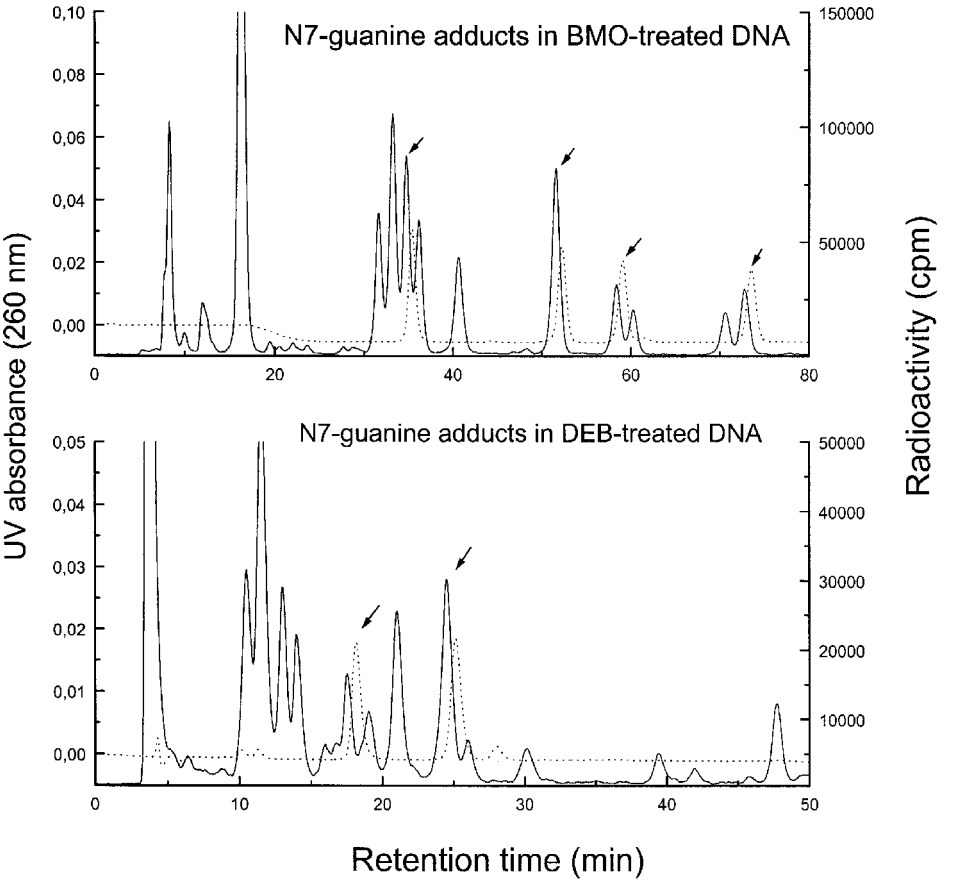

Figure 6. HPLC chromatograms of postlabelled BMO-treated DNA (a) and DEB-treated DNA (b) analysed with radioisotope and UV detectors. The positions of the adducts are indicated with arrows. —, radioactivity; ..., UV. Note that the UV detector is installed 0.6 min after the radioactivity detector.

since the background products interfered with the detection of the adducts, a subsequent TLC was combined to separate these adducts. The typical TLC separation of *N*-7-BMO-guanine adducts is shown in figure 8.

The *N*-1-BMO-adenine adducts in rat liver DNA were analysed by the same method as described for *in vitro* treated DNA. The interference of background peaks close to *N*-1-adenine adducts makes it difficult to detect directly low levels of adducts *in vivo*. The sensitivity of the assay could be considerably increased when the adduct fraction was rearranged to the N^6 position, the rearranged adduct eluted much later and was free of background radioactivity. Consequently, the quantification of *N*-1-BMO-adenine adducts in rat liver DNA was based on the level of the rearranged N^6-adenine adducts. The HPLC separation of corresponding N^6-BMO-adenine adducts from liver DNA in an exposed rat and a control rat are shown in figure 7 (C and D). The adduct levels in rat livers are presented in table 2. The amounts of *N*-7-BMO-guanine adducts were higher than

Table 2. BD-induced DNA adducts in livers of rats exposed to 300 ppm BD by inhalation (adducts/10^8 nucleotides)

N-7-BMO-guanine	*N*-1-BMO-adenine	*N*-7-EBD-guanine
4.0	1.6	11

corresponding *N*-1-adenine adducts. EBD-induced *N*-7-guanine adducts were more abundant than the corresponding BMO-induced adducts.

Discussion

In a previous study we described the formation of N^6-trihydroxybutyl-adenine adducts in rats exposed to BD (Zhao *et al*. 1998). In the present study, we extended the analysis of BD induced DNA-adducts with methodological improvements and described analysis of *N*-1-adenine and *N*-7-guanine adducts in rat liver resulted from exposure to BD.

In the reaction of EBD with 5′-dGMP the formation of 2′3′4′-trihydroxybut-1′-yl adducts at *N*-7 position of guanine was observed. We showed that the isomerism of the adduct can be determined by ESI-MS. The identification of the MS/MS product with a CH_2-group at the *N*-7 position excludes the formation of the other theoretically possible isomers of the trihydroxybutyl moiety, i.e. *N*-7 adduct through the 2′- or 3′-carbons. This is in agreement with the NMR-studies performed on the trihydroxybutyl substituted guanine (Tretyakova *et al*. 1997). In the case of the reaction products formed between BMO and dAMP, the current MS method did not allow the identification of different isomers, neither as *N*-1 nor N^6-adducts. However, the previous findings on adenine adducts (Tretyakova *et al*. 1997) allowed the assessment of the isomerism of two diastereomeric pairs of dAMP adducts. The products of nucleophilic attack at 1-carbon of BMO were twice as abundant as those resulting from attack at 2-carbon. This is in agreement with Tretyakova *et al*. (1997) who obtained a similar ratio of the two *N*-1 alkylated isomers and with Koivisto *et al*. (1995) who reported similar ratios to N^6-alkylated adducts which they interpreted to be from *N*-1 alkylation followed by the Dimroth rearrangement. In BMO-treated DNA at physiological pH, the *N*-7 alkylated guanine was the major binding product, comprising 80 % of the alkylation observed in this study.

The ^{32}P-postlabelling/HPLC method was used to analyse BD-induced adducts in liver DNA of rats exposed to 300 ppm BD by inhalation. The results show that the level of *N*-7-BMO-guanine adducts was higher than that of corresponding *N*-1-adenine adducts. Comparing with our previous study (Zhao *et al*. 1998), the amount of *N*-1-EBD-adenine adducts was ~2-fold higher than *N*-1-BMO-adenine adducts in livers of rats exposed under the same conditions. This is comparable with other studies which found more abundant *N*-7-EBD-guanine and EBD-haemoglobin adducts than the corresponding BMO-induced adducts (Licea Pérez *et al*. 1997, Tretyakova *et al*. 1998). A major improvement in the analysis of *N*-7-guanine adducts was the combination of separations involving HPLC and TLC, which removed the interfering background radioactivity. The analysis of *N*-1-adenine adducts has also been difficult due to the background products. However,

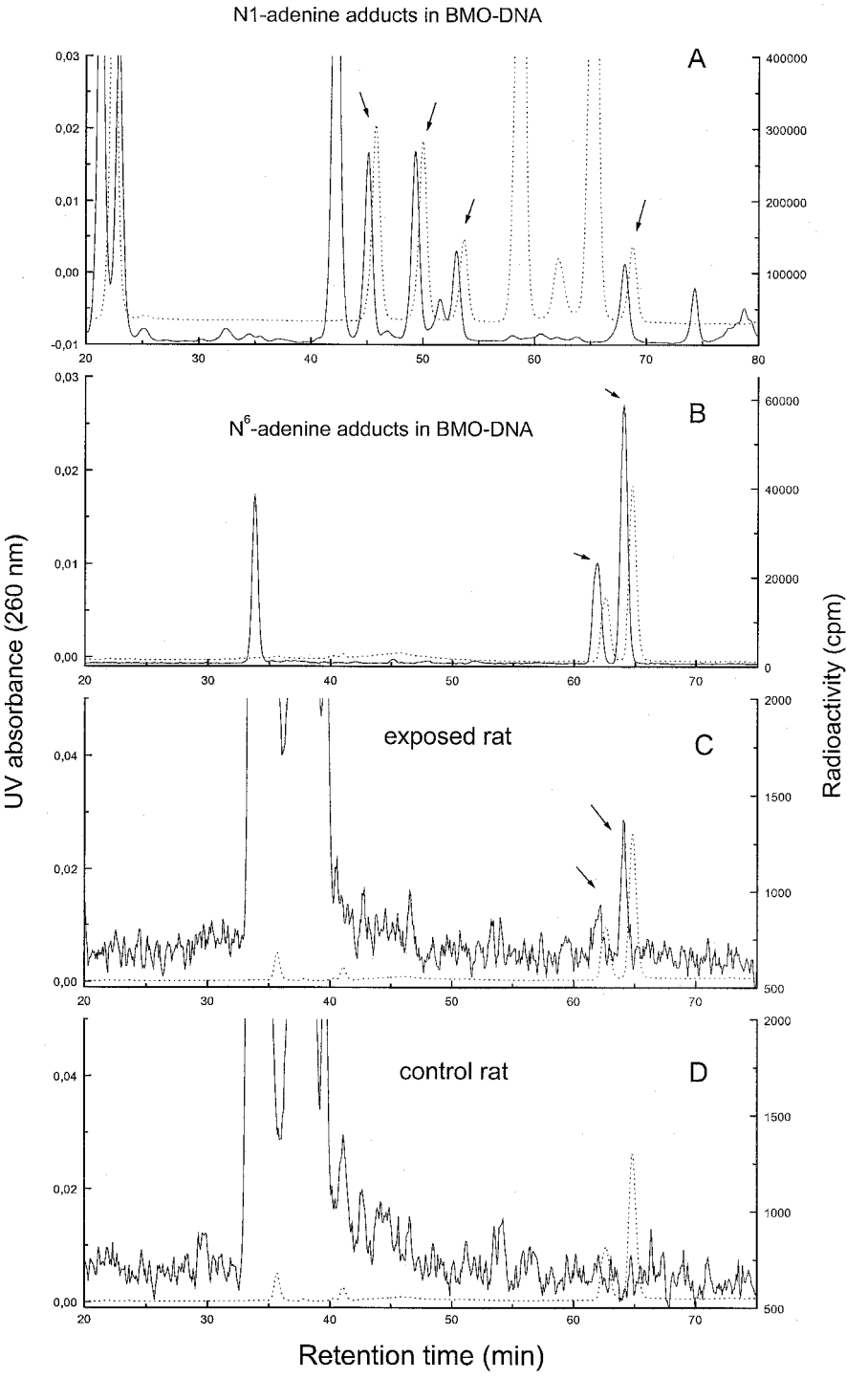

Figure 7. HPLC chromatograms of ^{32}P-postlabelled BMO treated DNA and rat liver DNA analysed with radioisotope and UV detectors. (A) *N*-1-adenine adducts in BMO treated DNA, (B) N^6 adenine adducts in BMO treated DNA, (C) an exposed rat liver sample, (D) a control rat liver sample. The positions of the adducts are indicated with arrows. —, radioactivity; ....., UV. Note that the UV detector is installed 0.6 min after radioactivity detector.

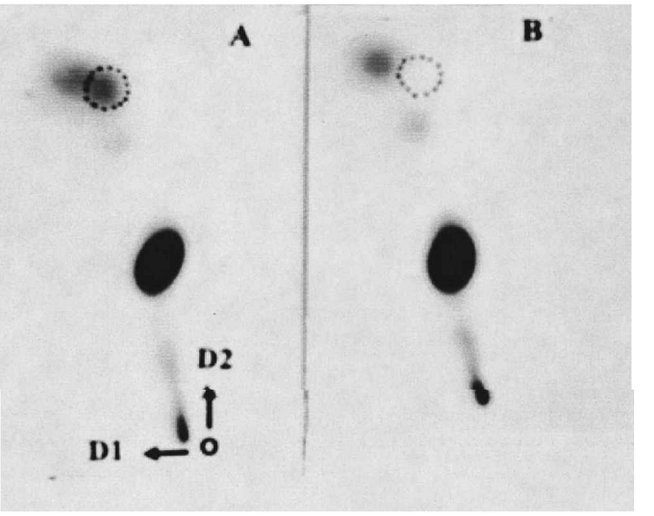

Figure 8. TLC analysis of *N*-7-BMO-guanine adducts in (A) an exposed rat liver sample, (B) a control rat liver sample. *N*-7-BMO-guanine adducts are indicated with circles. D1 and D2 are the directions of chromatography and 'O' is the origin.

when the *N*-1-adenine adducts were analysed by collecting the radioactive peaks and converted to corresponding N^6-adenine adducts by Dimroth rearrangement the products were well separated. Since much less radioactivity was injected into HPLC the sensitivity was increased. The sensitivity of the method can be increased by collecting the adduct peaks from several parallel DNA samples, which makes the *N*-1-adenine adducts a promising target for human biomonitoring. *In vivo* formation of N^6-BMO adducts originating probably from the *N*-1-adenine adducts was identified by Koivisto *et al*. (1995). Moreover, the higher frequency of point mutations at the A:T base pairs in the bone marrow of transgenic mice suggested that *N*-1-adenine adducts may be promutagenic lesions (Recio and Meyer 1995).

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