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^{32}P-postlabelling/HPLC analysis of various styrene-induced DNA adducts in mice

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Styrene oxide (SO), a reactive metabolite of styrene, modifies DNA at several nucleophilic sites. In the present work we have determined the SO–DNA adducts *in vitro* and *in vivo* by two different versions of ^{32}P-postlabelling/HPLC assays. When anion-exchange cartridges were used for adduct enrichment the β-isomer of 7-substituted guanines was detected in *in vitro* SO-treated DNA as well as in mice lungs exposed to styrene at 750 and 1500 mg m^{-3} for 21 days (6 h day^{-1}, 7 days week^{-1}). In the lungs, the adduct levels were 6.5 and 23 per 10^8 nucleotides for the two doses, respectively. When the nuclease P1 resistant adducts were studied by the ^{32}P-postlabelling/HPLC assay involving nuclease P1/prostatic acid phosphatase hydrolysis, the main adducts in *in vitro*-treated DNA were the α-isomer of N^2-substituted guanine, β-isomers of 1-substituted adenine and 3-substituted uracil. β1-SO–adenine adduct was detected in the mice lung tissues after conversion of the 1-substituted adduct to the βN^6-SO–adenine adduct by the Dimroth rearrangement. The 1-adenine adduct levels for the two doses were found to be 0.17 and 0.51 per 10^8 nucleotides. The current results show the potential of using the 7-guanine and 1-adenine adducts as biomarkers in biomonitoring of styrene exposure.

Keywords: DNA adducts, ^{32}P-postlabelling, styrene, styrene 7,8-oxide.

Abbreviations: dAMP, 2′-deoxyadenosine 5′-monophosphate; dCMP, 2′-deoxycytidine 5′-monophosphate; dGMP, 2′-deoxyguanosine 5′-monophosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; HPLC, high-performance liquid chromatography; MN, micrococcal nuclease; NucP1, nuclease P1; PAP, prostatic acid phosphatase; SO, styrene 7,8-oxide; SPD, spleen phosphodiesterase; SVPD, snake venom phosphodiesterase; TLC, thin-layer chromatography.

Introduction

Exposure to a chemical carcinogen can produce a continuum of events from absorption, DNA binding and mutations resulting, in the worst case, to the development of disease (Perera and Weinstein 2000). Several biomarkers can be used to follow these events to elucidate the mechanisms of the carcinogenic process as well as the individual response to the carcinogens. Among the biomarkers for genotoxic compounds, DNA adducts represent an important integral measure of

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DNA damage, which relates the individual exposure, metabolic capacity and DNA repair capacity (Hemminki 1997, Collins 1998, Perera and Weinstein 2000). Styrene, an important industrial chemical, is classified by IARC as a possible human carcinogen (group 2B, IARC 1994a) and its major metabolite, styrene oxide (SO) as a probable human carcinogen (Group 2A, IARC 1994b). The possible carcinogenecity of styrene is believed to be related to the genotoxic effects of SO, especially because SO has been shown to react at several sites with the DNA constituents *in vitro* and *in vivo* (Hemminki and Vodicka 1995, Rappaport *et al.* 1996, Vodicka *et al.* 1999). Therefore, the DNA adducts represent an important biomarker in biomonitoring of styrene exposure, and sensitive analytical methods are needed to detect these endpoints in the continuum from the exposure to the biological effects.

The ^{32}P-postlabelling technique represents the method with the sensitivity required for the determination of low levels of the SO-induced DNA-adduct *in vivo*, without the need of giving radiolabelled styrene or SO. Earlier the technique has suffered from to the poor separation capacity and reproducibility of the thin-layer chromatography (TLC) and from difficulties for the quantitation of the adducts. However, the recent advances in the high-performance liquid chromatography (HPLC) techniques with on-line ^{32}P-detection for the separation of the adducts have markedly improved the strength of the ^{32}P-postlabelling assay when regarding the identification and quantitation of the adducts, especially when properly characterized standards are available (see for example, Koskinen *et al.* 1997).

Only a few animal experiments on DNA adducts have been carried out so far on styrene exposure. Pauwels *et al.* (1996), by using the ^{32}P-postlabelling method, reported 7-and O^6-guanine adducts in various mice tissues after a single i.p. administration of styrene. For doses of 0–4.35 mmol kg^{-1} bw adduct levels up to 6.3 of 7-guanine adducts per 10^7 nucleotides and 3.8 of O^6-guanine adducts per 10^7 nucleotides were detected, with a clear dose–response relationship. Later, Ottender *et al.* (1999) questioned the stability of O^6-guanine adducts in the ^{32}P-postlabelling method used. By using a modified method they obtained a liver adduct level of seven O^6-guanine adducts per 10^7 nucleotides in rats exposed for 2 years for 1000 ppm styrene.

Recently we have revised the spectrum of the SO-induced DNA adducts *in vitro* (Koskinen *et al.* 2000a, b). A vast majority of the alkylation takes place in the 7-guanine position, the β-isomer being formed to a slight excess over the α-isomer. Therefore the 7-substituted guanines should be one of the main targets of the SO-alkylation when the *in vivo* exposure of styrene is considered. Another main alkylation site is the 3-position of adenine, but its suitability for a ^{32}P-postlabelling analysis is doubtful due to the instability of the adduct as well as to the impossibility to prepare nucleotide standards (Koskinen *et al.* 2000b). Other adducts that have been identified in double-stranded DNA, that should be considered in the *in vivo* biomonitoring, include N^2- and O^6-substituted guanines, 1- and N^6-substituted adenines as well as the deamination products due to the 1-substituted adenine, i.e. the 1-substituted hypoxanthines. In addition, we have recently identified in double-stranded DNA alkylation at the 3-cytosine (that deaminates to the corresponding uracil), as well as the stable N^4-substituted cytosine (Koskinen *et al.* 2000a).

In the present study, we demonstrate the spectrum of SO-induced DNA

adducts *in vitro* by ^{32}P-postlabelling using two different enrichment methods. In particular, 7- and N^2-substituted 2′-deoxyguanosine 5′-monophosphates (7-SO–dGMP and N^2-SO–dGMP, respectively) and 1- and N^6-substituted 2′-deoxyadenosine 5′-monophosphates (1-SO–dAMP and N^6-SO–dAMP) are considered as well as the 3-substituted 2′-deoxycytidine 5′-monophosphate (3-SO–dCMP) and the corresponding deaminated 2′-deoxyuridine 5′-monophosphate (3-SO–dUMP) (see figure 1 for structures). These adducts were also studied *in vivo* in mice exposed to styrene by inhalation.

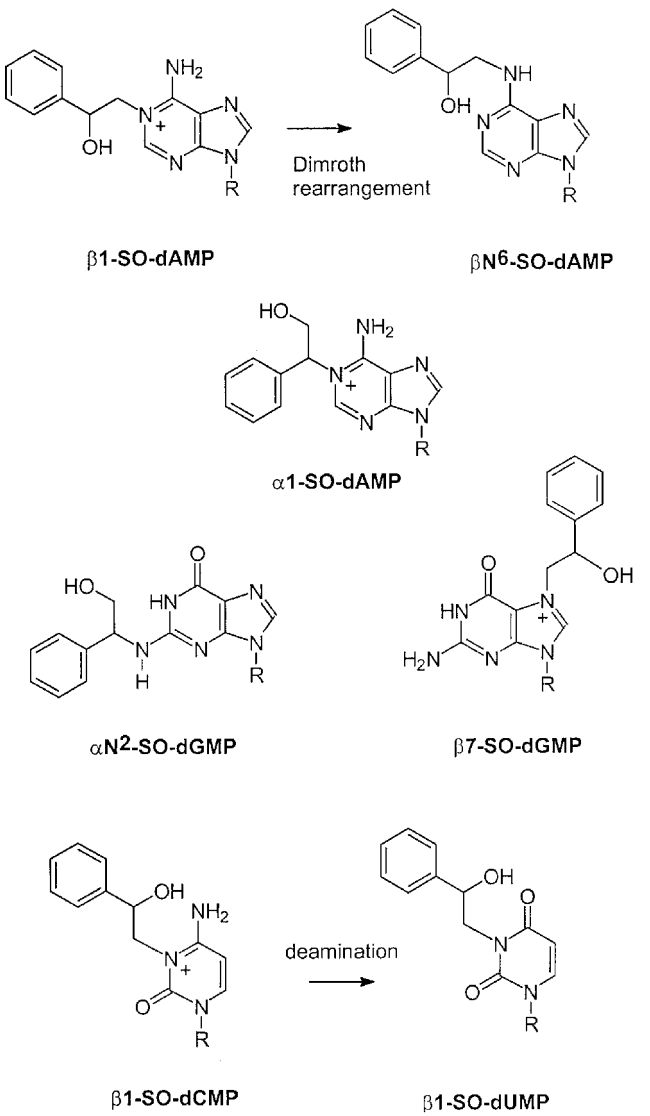

Figure 1. Structural formulae and the interconversions of compounds studied.

Materials and methods

Chemicals

Chemicals were used as purchased from the manufacturers. Salmon testis DNA (sodium salt), micrococcal nuclease (MN) and prostatic acid phosphatase (PAP) were from Sigma Chemical Co. (St Louis, MO). Racemic SO (>97% pure) was from Fluka Chemie AG (Buchs, Germany), spleen phosphodiesterase (SPD) and nuclease P1 (NucP1) from Boehringer Mannheim (Mannheim, Germany), [γ-^{32}P]ATP and T4 polynucleotide kinase from Amersham (Little Chalfont, UK). Methanol was gradient grade from Merck (Darmstadt, Germany). All other chemicals were either from Sigma or Merck.

Standards

Preparation of SO substituted dGMPs and dGpdT dinucleotides has been described in Koskinen *et al*. (1999) and the preparation of dAMP standards has been described in Koskinen *et al*. (2000b). β3-SO–dCMP and β3-SO–dUMP were prepared, separated and identified as the SO-alkylated deoxycytidines described in Koskinen *et al*. (2000a). The 5′-nucleotide standards were used as UV-markers in the HPLC separations of the adducts.

In vitro *reaction with DNA*

Salmon testis DNA was dissolved in 10 mM Tris–HCl, 0.15 M NaCl or 50 mM Tris–HCl, both pH 7.4. SO was added in an amount corresponding to 200 mM solution. SO was not completely dissolved but remained as droplets in the mixture. DNA was incubated at 37°C for 32 h. The reaction was stopped by extraction of the unreacted SO twice by 1 vol. ethyl acetate. The SO-modified DNA was precipitated by 4 M sodium acetate (0.1 vol.) and absolute ethanol (1.5 vol.), and the pellet was washed three times with 70 % ethanol. The SO–DNA was dissolved in water. The 7-guanine alkylation level was determined by neutral thermal hydrolysis and the other adducts by enzymatic hydrolysis as described in Koskinen *et al*. (2000a). The SO–DNA obtained was used as standard in the determination of recoveries in the ^{32}P-postlabelling analyses. The standard DNA for 7-subsituted guanines was prepared in 50 mM Tris–HCl and was diluted with mouse lung DNA, 1:1000. For the other adducts the standard DNA was prepared in 10 mM Tris–HCl, 0.15 M NaCl, and was used without further dilutions. Table 1 shows the alkylation levels for the latter standard DNA.

^{32}P-postlabelling using NucP1/PAP hydrolysis: the HPLC separation conditions

The 'dinucleotide' version of the ^{32}P-postlabelling analysis of SO–DNA was based on the method by Randerath *et al*. (1989). A 10 µg sample of DNA was hydrolysed to dinucleotides with a mixture of

Table 1. Alkylation levels detected in the standard DNA treated for 32 h with SO in 10 mM Tris–HCl, 0.15 M NaCl, pH 7.4, determined as described by Koskinen *et al*. (2000a)[a].

Adduct	Alkylation level (adducts per 10^4 nucleotides)	Proportion of the identified alkylation products (%)
β7-SO–guanine[b]	52.6	44.3
α7-SO–guanine[b]	34.0	28.6
α3-SO–adenine[b]	6.7	5.6
β3-SO–adenine[b]	2.0	1.7
β1-SO–deoxyadenosine[c]	2.8	2.3
αN^6-SO–deoxyadenosine	7.6	6.4
βN^6-SO–deoxyadenosine[c]	1.6	1.4
αN^2-SO–deoxyguanosine	3.9	3.3
β3-SO–deoxyuridine	4.1	3.5
αN^4-SO–deoxycytosine	1.6	1.4
α-1SO–deoxyinosine[3]	1.8	1.6

[a]7-Guanine and 3-adenine adducts were determined by neutral thermal hydrolysis, the others were determined by enzymatic hydrolysis.

[b]The alkylation level contains all 7-guanine and 3-adenine adducts formed, i.e. both spontaneously depurinated adducts as well as those in DNA.

[c]The adducts were not described in Koskinen *et al*. (2000a). They were identified by UV-spectroscopy at different pH values. β1-SO–deoxyadenosine and βN^6-SO–deoxyadenosine were also verified by interconversion due to the Dimroth rearrangement.

The data are the means of two values.

NucP1 (0.2 μg μg^{-1} DNA) and PAP (20 mU μg^{-1} DNA) in 20 mM sodium acetate buffer, pH 5. After incubation at 37 °C for 45 min 100 μl of cold ethanol was added to precipitate proteins (30 min, –20 °C). After centrifugation the supernatant was evaporated to dryness and the dinucleotides were ^{32}P-labelled in a mixture (2 μl) containing 2.4 U T4 polynucleotide kinase and 2.3 pmol [γ-^{32}P]ATP (specific activity > 5000 Ci $mmol^{-1}$). The pH of the labelling mixture was adjusted with a buffer containing 20 mM CHES, 10 mM $MgCl_2$, 10 mM dithiotreitol, 1 mM spermidine, pH 9.6. The mixture was incubated at 37 °C for 50 min after which the dinucleotides were cleaved to 5′-mononucleotides by snake venom phosphodiesterase (SVPD, 0.8mU μg^{-1} DNA, 30 min at 37 °C).

The ^{32}P-labelled components in the labelling mixture were separated, together with the UV-marker compounds, by injecting the whole mixture to HPLC and were detected with sequential on-line radioisotope and UV detection. The separation system consisted of an Isco 2360 Gradient programmer and an Isco Model 2350 pump with a Packard Radiomatic 500TR Flow Scintillation analyser and Isco CV^4 capillary absorbance detector. The separations were carried out with a C-18 column (Prodigy ODS 250 × 2.0 mm, 5 μm, Phenomenex) using a binary gradient consisting of 0.2 M ammonium formate, 20 mM H_3PO_4, pH 4.6, and methanol, with a flow-rate of 0.2 ml min^{-1}. Initial elution was with 5% methanol in the buffer for 10 min, followed by an increase of the proportion of methanol to 40% in 60 min and further to 100% in 10 min; 100% methanol was maintained for 10 min and decreased to 10% in 10 min.

A 5μ Kromasil 2.0 × 50 mm C-18 precolumn was installed in the front of the analytical column. Inorganic phosphate, residual ^{32}P-ATP and the residual normal nucleotides were separated from the adducts on the precolumn by diverting the first 10 min after injection of the sample to waste using a four-port switching valve.

Analysis of 1-substituted dAMP adducts

β1-SO–dAMP was analysed by converting it to βN^6-SO–dAMP by the Dimroth rearrangement. Thus, the DNA hydrolysis and labelling were performed as described in the above section. The β1-SO–dAMP fraction was collected using a similar HPLC gradient to the one described in the previous section except after 10 min from the sample injection the buffer was changed to 50 mM ammonium formate pH 4.6 to avoid high salt concentration in the collected fraction. The collection was started when the UV-absorbance for the β1-SO–dAMP marker started increasing and was continued for 5 min after the top of the peak. The fraction was frozen and lyophilized. The rearrangement of 1-substituted adenine to the N^6-substituted one was performed by dissolving the sample in 12 μl of 0.3 M NaOH and by incubating at 80 °C for 30 min. The mixture was neutralized with 12 μl of 1 M ammonium formate, pH 5, and was injected into the HPLC. For the separation of the rearranged product the gradient started with 10% of methanol in 0.2 M ammonium formate, 20 mM H_3PO_4, pH 4.6, for 5 min, followed by an increase to 45% of methanol in 20 min and a slower increase to 50% of methanol in 20 min during which time the βN^6-SO–dAMP was detected. The washing step included an increase of methanol to 100%, which was maintained for 10 min followed to return to initial conditions in 10 min.

^{32}P-postlabelling using anion-exchange enrichment

The analysis of 7-substituted guanines was based on an earlier method described by Kumar *et al.* (1997) with some modifications. A 10 μg sample of DNA was incubated at 37 °C in a mixture (6 μl) containing 0.5 mM Bicine (pH 9.0), 3 μM $CaCl_2$ and 400 mU micrococcal nuclease for 2 h. Two μl of 20 mM ammonium acetate and 48 mU spleen phosphodiesterase in 6 μl were added to the mixture and incubation was continued for 40 min to obtain 3′-nucleotides. The hydrolysate was frozen, evaporated to dryness and dissolved in 150 μl of 5 mM ammonium formate, pH 5.1. The adducts were separated from the normal nucleotides by applying the mixture to 1 ml Bakerbond anion-exchange cartridges (JT Baker, Deventer, The Netherlands), and then manual pressure was applied. One ml of 5 mM ammonium formate buffer was added to the cartridge and was pressed through the column. The passed fraction was collected to a tube on an ice-bath and was repassed through the cartridge a total of four times. Multiple passes were performed to better retain the normal nucleotides in the resin. The adducts were then collected by a total of 7 ml of 5 mM ammonium formate buffer (pH 5.1) using a centrifuge for elution. The collected fraction was frozen, lyophilized and dissolved in 80 μl of water, frozen again and evaporated to dryness in a vacuum centrifuge.

The ^{32}P-labelling was carried as described above, except after the incubation with ^{32}P-ATP and T4-kinase, 1 μl of 1 M ammonium formate and 2.5 μg of Nuclease P1 were added and were incubated for 15 min at 37 °C to dephosphorylate the bisphosphates to corresponding 5′-nucleotides. HPLC separation conditions were the same as described in the section '^{32}P-Postlabelling using NucP1/PAP hydrolysis'.

Animal samples

Male NMRI mice (2 months old, 25 g) were exposed to styrene in a dynamic inhalation chamber for 21 days, 6 h day^{-1} and 7 days $week^{-1}$, with concentrations of 0, 750 and 1500 mg m^{-3}. DNA was isolated

from lungs by RNase A, RNase T1 and subsequent proteinase K treatements, followed by phenol and chloroform/isoamylalcohol extraction. The DNA was precipitated overnight in freezer by addition of 0.1 vol. 4 M sodium acetate and 1.5 vol. absolute ethanol. The DNA pellet was washed three times by 70 % ethanol and was dissolved in water.

For the analysis of β1-SO–dAMP 30 μg of DNA was hydrolysed and labelled in 10 μg portions which were combined prior to HPLC separation, and for the analysis of 7-substituted guanines 10 μg of DNA was used.

Results

^{32}P-postlabelling with NucP1/PAP hydrolysis

The SO-modified DNA was hydrolysed enzymatically to dinucleotides, ^{32}P-labelled and after final hydrolysis by SVPD to obtain adducted 5′-nucleotides, the products were separated by HPLC with on-line radioisotope detection. The main SO adducts detected were those of the two diastereomers of αN^2-SO–dGMP, β1-SO–dAMP and β3-SO–dUMP (figure 2). In addition, the α-isomers of 1-SO–dAMP was detected as well as the minor adducts αN^6-SO–dAMP and β3-SO–dCMP.

The αN^2-SO–dGMP adducts were assigned by co-elution of the corresponding synthetic 5′-nucleotide UV-markers and by addition of the ^{32}P-labelled and SVPD-treated dinucleotide standards. The β1-SO–dAMP product was identified, in addition to co-elution of the UV-standard, by Dimroth rearrangement, i.e. by isolating the adduct fraction and treating it in sodium hydroxide. Re-running the fraction gave a new product which co-eluted with the βN^6-SO–dAMP UV-marker. Further, the β1-SO–dAMP peak was sensitive to heating under neutral pH (30 min at 90 °C) in which Dimroth rearrangement and deamination are taking place (Qian and Dipple 1995). Moreover, when the whole ^{32}P-labelling mixture, after SVPD incubation, was treated with 0.4 M NaOH for 2 h at 37 °C, the β1-SO–dAMP product had almost completely disappeared in the subsequent HPLC analysis, as

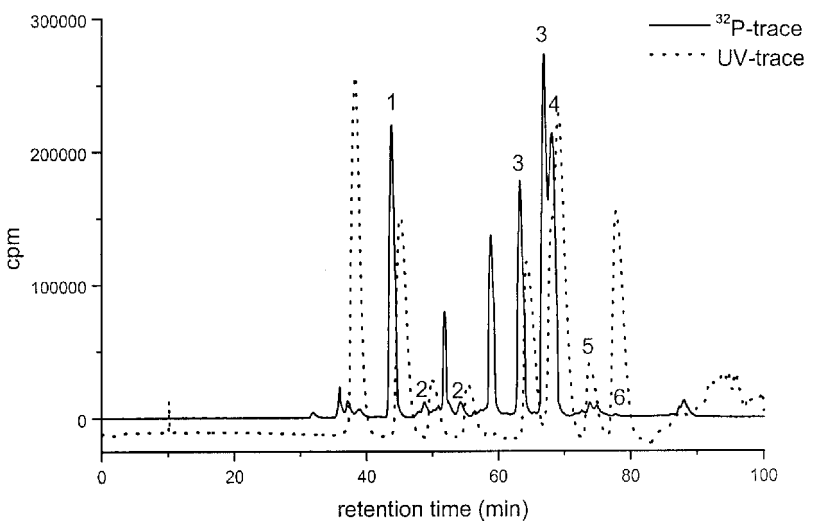

Figure 2. An HPLC separation of the ^{32}P-postlabelled NucP1/PAP hydrolysed SO–DNA. Peaks: 1, β1-SO–dAMP; 2, α1-SO–dAMP; 3, αN^2-SO–dGMP; 4, β3-SO–dUMP; 5, βN^6-SO–dAMP; 6, αN^6-SO–dAMP.

had the two peaks that co-eluted with the α1-SO–dAMP standards. At the same time, the peaks corresponding the α- and β-isomers of N^6-substituted dAMP adducts were increased, the latter increasing more. In the SO-treated DNA prepared in 50 mM Tris–HCl, pH 7.4, αN^6-SO–dAMP was detected as a minor product but no βN^6-substituted adenines were directly detected. The αN^2-SO–dGMP peaks were resistant to alkaline treatment. No separate peaks were detected for deaminated 1-SO–dAMP. These deamination products were tested with the UV-markers, which eluted at approximately the same retention time as the first diastereomer of αN^2-dGMP adduct.

β3-SO–dCMP was assigned as a minor product in DNA. The identification was based on the deamination reaction to the corresponding uracil product. Thus, when the isolated β3-SO–dCMP was treated in water at 100 °C for 30 min, a new product was detected in a subsequent HPLC analysis at slightly longer retention time as the later eluting αN^2-SO–dGMP diastereomer. The new product co-eluted with the β3-SO–dUMP UV-marker, confirming deamination of the 3-cytosine adduct. The deaminated product was seen as a major peak in the chromatogram obtained from SO-treated DNA prepared in 10 mM Tris–HCl, 0.15 M NaCl, indicating that the deamination of cytosine adducts was readily taking place. Further evidence for the *N*-3 dCMP adduct was that the peak was found to be relatively resistant to acid treatment (0.1 M HCl, 30 min at 90 °C) in which all the purine adducts were depurinated.

When the adduct level obtained by the ^{32}P-postlabelling method was compared with the adduct level found in the standard DNA by UV-detection (diluted 1:1000 by mouse lung DNA), 33 % of the αN^2-SO–dGMP was recovered. The labelling efficiencies of the two diastereomers of αN^2-dinucleotide standards were found to be 41 % and 43 %, respectively. The recoveries of 36 % and 15 % were determined for β1-SO–dAMP and β3-SO–dUMP, respectively. The lower recovery of β3-SO–dUMP, as compared with other adducts, was explained because the determination of the alkylation of standard DNA involved a heating step that deaminated all the 3-SO–dCMP, while by using the ^{32}P-postlabelling method, some original products were still detectable.

Analysis of 1-substituted dAMP adducts

When the β1-SO–dAMP product was isolated with the aid of the corresponding UV-marker and was treated with sodium hydroxide, the 1-substituted product was converted to the βN^6-SO–dAMP by the Dimroth rearrangement. The N^6-product eluted in the subsequent HPLC analysis in a region where no background products were detected. No original β1-SO–dAMP products were detected after the alkali treatment, as tested with the corresponding ^{32}P-labelled 3′-nucleotide standard and the UV-marker. The adducts were, however, not completely stable in the alkali treatment since the rearranged product gave roughly 75% of the radioactivity of the labelled original β1-SO–dAMP standard. The total recovery by the method involving the Dimroth rearrangement for β1-SO–dAMP was 20 %.

Analysis of 7-substituted dGMP adducts

For the analysis of 7-substituted guanines the SO-treated DNA was hydrolysed by MN and SPD to 3′-nucleotide monophosphates and the adducts were enriched

by applying the mixture to an anion exchange cartridge. By this method only the β7-SO–dGMP was quantitatively analysed because α7-SO–dGMP, even though constituting a high proportion of the adducts in the SO-treated DNA, was poorly labelled.

In the optimal anion exchange enrichment the 7-substituted 3′dGMPs and 1-substituted 3′dAMPs were not retained in the column at pH 5.1, while the normal nucleotides were retained. We found, however, that the SO-substituted 3′dGMP had interactions with the anion-exchange material, leading to tailing of the adducts while eluting them from the cartridge. Therefore, a total of 7 ml of the buffer was needed to elute the main fraction of adducts from the cartridge. In addition, we found that the normal nucleotides tended to elute with the adducts to such a high levels that they may consume the main proportions of the ^{32}P-ATP in the labelling step. A repassing of the applied hydrolysis mixture in 1 ml portions through the cartridge for four times before the final elution with 7 ml of the buffer reduced the normal nucleotides to an acceptable level. Thus, by the repassing the normal nucleotides had more time to interact with the resin.

By the treatment of DNA with SO, it was expected that both diastereomers for each isomer were formed to an equal extent. The postlabelling analysis gave, however, higher response for the first eluting diastereomer (see figure 3(A)). Thus, the recoveries varied for β7-SO–dGMP diastereomers, being 19 – 18 % for the first and 6 – 3 % (n = 10) for the second diastereomer.

Analysis of SO-induced DNA adducts in mouse lungs

The above described methods were used to characterize the adduct formation *in vivo* using mouse lung as the model system. The adducts formed to the highest levels were those of the β7-SO–dGMP (table 2, figure 3(B)). They were identified by co-elution with the UV-markers and they were also found to be sensitive to heat treatment at neutral pH during which the 7-substituted dGMPs are depurinated. The detection limit for the β7-SO–dGMP in the lung samples was *ca* 1 adduct per 10^9 nucleotides using 10 μg DNA. The different diastereomers showed different rates of formation, the first eluting one accounting for 64 – 6 % of the total β7-SO–dGMP. The anion-exchange enrichment allowed also the detection of the β1-SO–dAMP adducts. However, the quantitation was done using the method involving the Dimroth rearrangement.

Because of the interfering background products that eluted with the 1-adenine adduct when using the NucP1/PAP hydrolysis, the β1-SO–adenine adduct was converted to the N^6-substituted adenine, as described for the *in vitro*-treated DNA (figure 4). In the subsequent HPLC analysis, the βN^6-SO–dAMP eluted later without interfering radioactivity. Thus, the β1-adenine adducts were quantitated as the N^6-adenine adduct, the detection limit being *ca* 1 adduct per 10^9 nucleotides for 30 μg of DNA.

Even though αN^2-SO–dGMP showed good response by using the NucP1/PAP–method in the case of the *in vitro* SO-treated DNA, no clearly evident N^2-substituted products were observed in the mouse lung. The background products around the expected peaks were quite prominent decreasing the detection limit to, roughly estimated, one adduct per 10^8 nucleotides. In some samples of the higher dose there were some minor radioactive products co-eluting with the first diastereomer of αN^2-SO–dGMP. These may be related also to the spontaneously deaminated

Table 2. Adduct levels in mice lung tissues after the exposure to styrene by inhalation for 21 days, 6 h per day and 7 days per week (expressed as adducts per 10^8 normal nucleotides)[a].

	0 mg m^{-3}	750 mg m^{-3}	1500 mg m^{-3}
β7-SO–dGMP	n.d.[b]	6.5; $n = 2$	23.0 – 11.9; $n = 6$
β1-SO–dAMP	n.d.	0.17 – 0.05; $n = 3$	0.51 – 0.22; $n = 8$

[a] Adduct levels are corrected for recoveries. In the case of β7-SO–dGMP recoveries used in calculations were 19% and 6% for the first and second eluting diastereomers, respectively, and 20% for β1-SO–dAMP.

[b] No adducts detected.

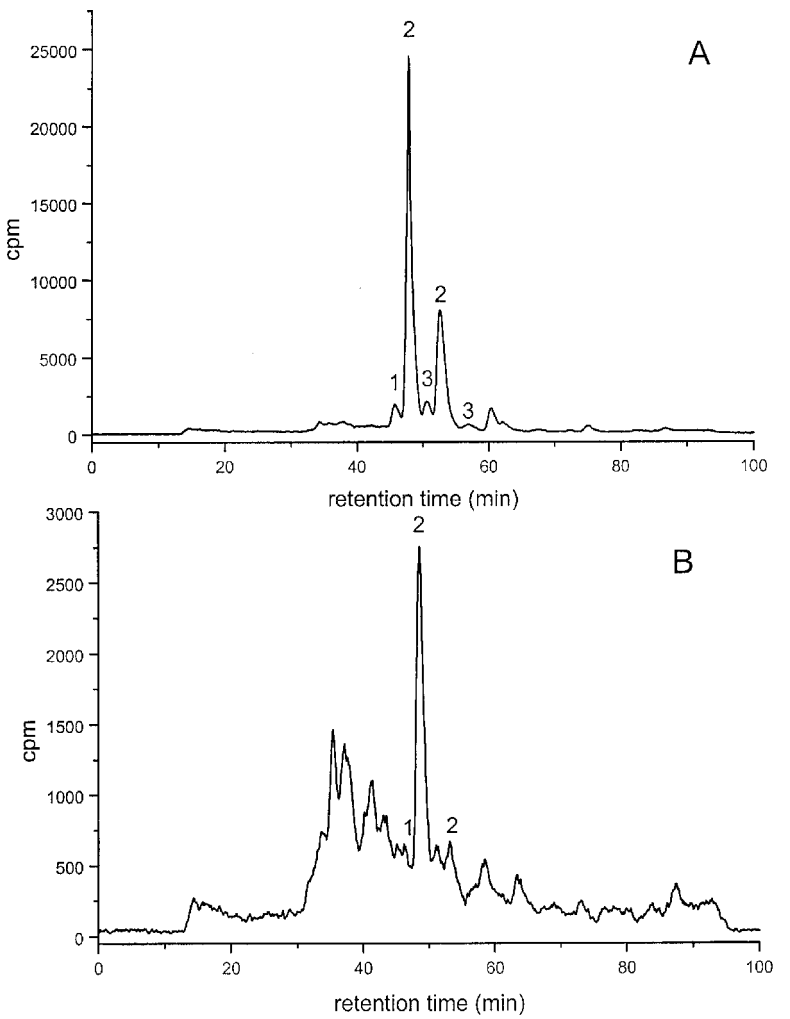

Figure 3. HPLC separations of anion exchanged enriched adducts after ^{32}P-postlabelling. A: *In vitro*-treated SO–DNA, diluted with mouse lung DNA 1:1000. Peaks: 1, β1-SO–dAMP; 2, β7-SO–dGMP; 3, α7-SO–dGMP. B: Mouse lung DNA, exposed to 1500 mg m^{-3} of styrene. Peaks: 1, β1-SO–dAMP; 2, β7-SO–dGMP.

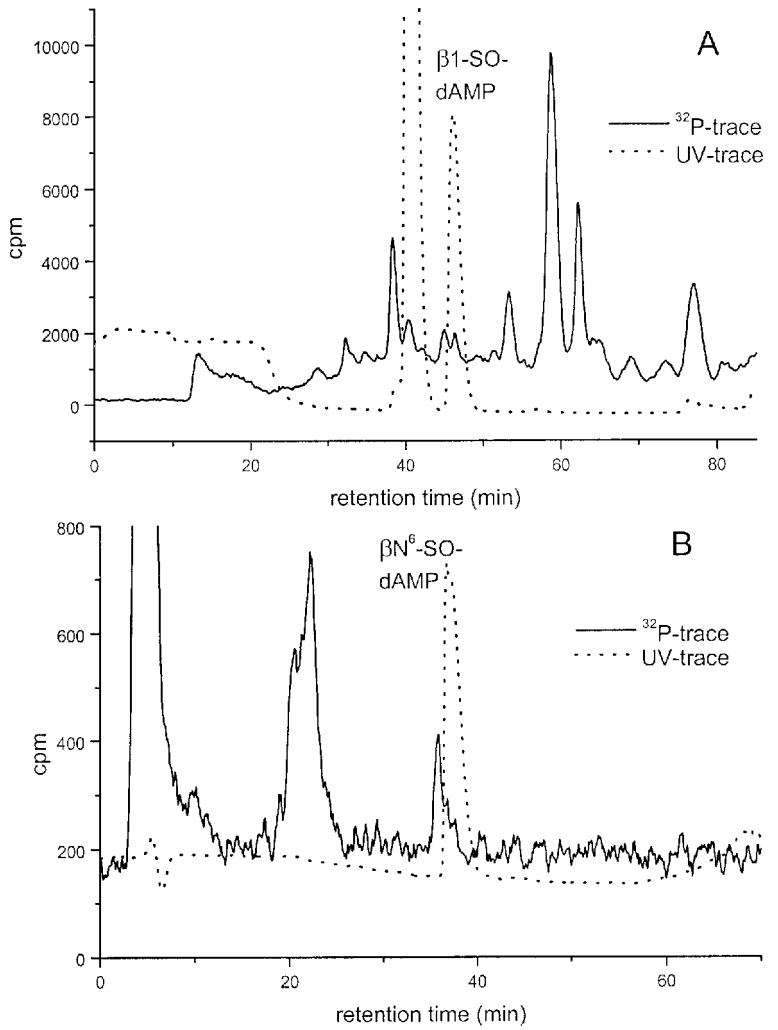

Figure 4. A: An HPLC separation of ^{32}P-postlabelled mouse lung DNA hydrolysed by NucP1/PAP. β1-SO–dAMP fraction collected for NaOH treatment. B: Dimroth rearranged β1-SO–dAMP fraction.

1-SO–dAMP adducts. The interfering products made an unambiguous assignment impossible. Changes in the HPLC gradient did not lead to better separation. The alkylation level of the product would be <1 adduct per 10^8 nucleotides. Also some other peaks were detected in the exposed animals whose identity was not revealed by the currently used UV-markers. Further studies are needed in these respects.

Discussion

SO-adduct analysis in vitro

^{32}P-postlabelling assay has been applied in several studies involving DNA alkylation by SO *in vitro* (Liu *et al*. 1988, Pongracz *et al*. 1989, 1992, Kaur *et al*. 1993, Vodicka *et al*. 1996, Ottender *et al*. 1999). In all of these studies the

^{32}P-postlabelling procedure has involved MN/SPD hydrolysis. Furthermore, these studies were mainly based on TLC separations of relatively low resolution. In the present work we have studied the possibilities of determining the SO-adducts using a method involving NucP1/PAP hydrolysis, combined with HPLC separations giving a superior resolution, and reliable identification and quantitation of the adducts. The method allowed a detection of a wide variety of nuclease P1 resistant adducts in SO-treated DNA, including αN^2-SO–dGMP, β1-SO–dAMP and β3-SO–dUMP. The recoveries of these adducts by ^{32}P-postlabelling was good considering the possible use of these adducts for *in vivo* biomonitoring.

Even though αN^6-SO–dAMP was one of the main adducts in the *in vitro*-treated DNA it was detected only as a minor alkylation product by the ^{32}P-postlabelling method. This is because the adduct was sensitive towards NucP1. The N^6-adenine adduct identified in the *in vitro*-treated DNA possibly arose out of the Dimroth rearrangement from the corresponding 1-substituted dAMP. No products were identified as O^6-substituted dGMPs, which are also partially sensitive towards NucP1. Previously, we have analysed DNA alkylation products in humans that were then identified as O^6-substituted dGMPs (Vodicka *et al*. 1993. 1994, 1999). The assignment was based on co-elution of the synthetic standards on TLC plates and on an observation that O^6-SO–dGMP was resistant to NucP1 (Vodicka and Hemminki 1993). Recently, Ottender *et al*. (1999) reported the instability of the O^6-guanine adducts towards NucP1, and suggested an alternative enrichment for these adducts combining micropreparative HPLC followed by a mild NucP1 treatment. Therefore, obviously the O^6-SO–guanine is not completely sensitive towards NucP1, and it might be that there have been some minor differences in the enzyme activities between the previous and current studies that have a major effect in the sensitivity of O^6-substituted products towards NucP1. Further, we noticed that treatment of O^6-SO–dGMP with NucP1 resulted in a new product eluting at a longer retention time (data not shown). Therefore, further studies on the properties of O^6-substituted guanines are needed, especially to verify our previous results whether the adducts analysed were really O^6-SO–dGMPs, some new products originating from them or some other nuclease P1 resistant products related to styrene exposure that had the same chromatographic properties on the TLC separation system used.

The 7-substituted dGMPs are known to be sensitive toward the 3′-phosphatase activity of NucP1. Therefore, other enrichment methods have been applied for their analysis. An enrichment procedure using anion exchange cartridges has been introduced for the analysis of 7-SO–dGMPs (Kumar *et al*. 1997). In particular, analysis of the β-isomer of the 7-substituted product by this method showed promise. However, analysis of the α-isomer was not encouraging mainly because it has been shown to be only a poor substrate towards the T4-kinase mediated phosphorylation (Vodicka and Hemminki 1991). Therefore, no attempts were made to analyse α7-SO–guanines in this study. Even though the current modified version of the anion-exchange enrichment allowed the detection of the β7-SO–guanine at the levels of 1 adduct per 10^9 nucleotides with relatively good recovery of 20%, we have not been able to overcome the problem of the poor reproducibility of the method. The enrichment method has been successfully used for various alkylepoxides with relatively good reproducibility (Segerbäck *et al*. 1998, Plna *et al*. 2000, Zhao *et al*. 2000a). The difference between SO and the aliphatic alkyl epoxides seems to be the interactions of the SO-adduct with the

resin causing severe tailing during the elution of the adducts. In our laboratory, it has been found that for the enrichment of 7-alkyl-dGMPs of propylene oxide, butadiene monoxide and epichlorohydrin, the negatively charged normal nucleotides remain in the anion exchange cartridge while the uncharged adducts are completely eluted by using 4 ml of 5 mM ammonium formate buffer at pH 5.1. For SO adducts 7 ml of the buffer are needed to elute the main adduct fraction. Experiments involving changes in the buffer concentration, small changes in pH and addition of methanol to the eluent did not improve the separation of the adducts from the normal nucleotides, neither improved the reproducibility.

SO-adduct analysis in vivo

This is the first study reporting adenine adducts of SO *in vivo*. The alkaline treatment of the 1-substituted adenines converting them to the N^6-substituted adducts by the Dimroth rearrangement, allowed the separation and quantitation of the β1-SO–adenine adduct from otherwise interfering radioactive background products. The same approach has been recently applied for determination of 1-adenine adducts of butadiene monoxide and propylene oxide in animal samples (Plna *et al*. 1999, Zhao *et al*. 2000a) and in humans (Zhao *et al*. 2000b).

There are no previous data available in the literature on the 7-guanine adduct levels after inhalation of styrene. Butadiene monoepoxide adducts have been studied in mouse lungs, and they were found at levels of 34 adducts per 10^8 nucleotides after exposure to butadiene corresponding to 1100 mg m^{-3} for 5 days (Koivisto *et al*. 1998). Therefore, 7-guanine alkylation seems to be lower for styrene than butadiene exposure, at least through the β-carbon of the epoxides. The two diastereomers of β7-SO–guanines were found to be formed at different levels. Enantioselective adduct formation has also been observed in the case of the 7-guanine adducts of butadiene monoepoxide in mice lungs (Koivisto *et al*. 1998). Because the adduct formation itself is unlikely to be stereospecific, the formation of SO by the cytochrome P450 systems from styrene or the detoxification by epoxy hydrolases is therefore more likely to be enantiospecific. Indeed, different yields of the diastereomeric forms of SO has been shown to been formed in mice, the (*S*)-enantiomer being formed to a moderate excess (Linhart *et al*. 2000).

Recently, we reported the 7-guanine and 1-adenine adducts of butadiene monoepoxide in mice liver after a 660 mg m^{-3} exposure of butadiene for 5 days (Zhao *et al*. 2000a). The 7-guanine levels ($4/10^8$) were comparable to our current results. However, the 1-adenine adduct levels were clearly higher for butadiene exposure, being 40 % of the 7-alkylation. In the present study, the β1-SO–adenine adducts levels were *ca* 3 % of the level of β7-SO–guanine adducts. When the proportion of β1-SO–adenine formed in double-stranded DNA *in vitro* was determined by enzymatic hydrolysis the proportions of β1-SO–adenine and β7-SO–guanine were found to be 2.3 % and 44.3 %, respectively (table 1). Since the proportion of β7-SO–guanine includes also the spontaneously depurinated fraction, the true proportion of β1-SO–adenines as compared with that of β7-SO–guanine is obviously > 5.2 % in *in vitro*-treated DNA. Therefore, it seems that by this rather long exposure with high doses of styrene, β7-SO–guanines are formed to some excess over the β1-SO–adenines as compared with the *in vitro*-treated DNA. This may be due to the tendency of 1-substituted adenines to undergo spontaneous deamination or Dimroth rearrangement (Barlow *et al*. 1998).

SO-treatment has been found to increase the frequency of hypoxanthine guanine phosphoribosyl transferase (*hprt*) mutations in human T-cells *in vitro* (Bastlová *et al*. 1995). In the same experiment, elevated O^6-guanine adduct levels were also detected that correlated strongly with the single-strand breaks in DNA. However, somewhat puzzling was the finding that no correlation was observed between the O^6-guanine and *hprt* mutation frequencies (Bastlová *et al*. 1995). In a subsequent study, the molecular analysis of *hprt* mutations revealed the dominating type of mutation being AT→GC (Bastlová and Podlutsky 1996). This transition is most likely due to the 1-substitution of adenine or the corresponding hypoxanthine deamination product. Another candidate for these mutations could be the N^6-substituted adenine, but that is however less likely, since it has been found to be only a weakly mutagenic lesion (Latham *et al*. 1993). Therefore, it might be that the β1-SO–adenine adduct, identified *in vivo* in this study, is the main mutagenic lesions due to styrene.

With regard to human occupational biomonitoring, the present data show a methodology for analysing 7-guanine and 1-adenine adducts. To verify whether these adducts can really be detected in humans remains to be studied. Earlier, in humans exposed to styrene, N^2-guanine (Horvath *et al*. 1994) and O^6-guanine (Vodicka *et al*. 1993) were detected at mean levels of 16 and 5.4 adducts per 10^8 nucleotides, respectively. Our current animal study does not support either of these studies. The adduct levels now observed in mice were relatively low for the high doses of styrene. But on the other hand, also the exposure time was short as compared with chronic human exposure. A low-dose long-term animal study would be needed to better characterize the accumulation of the adducts and thus to predict the adducts to be detected in humans.

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