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Determination of styrene oxide adducts in DNA and DNA components

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Abstract

The reaction of styrene oxide with DNA components was studied using separation by capillary zone electrophoresis (CZE) and detection by negative-ion electrospray mass spectrometry (MS). The CZE–MS interface was built for a sector field mass spectrometer. The reaction of styrene oxide with mononucleotides (dGMP, dAMP) was used to optimize the relevant separation parameters and to gather the first information about the behaviour of the possible products. With these mixtures, sample stacking procedures were developed and the scope of collision-induced dissociations were studied. From the fragments recorded, information about the reaction sites in the nucleotides was obtained. Further, the reaction with intact calf thymus DNA was investigated. The DNA was digested into oligonucleotides using the previously described approach with Benzonase, an unspecific nuclease, and alkaline phosphatase. Styrene oxide mono-adducts in dinucleotides, trinucleotides and tetranucleotides were detected, whereas pentanucleotides exhibit mono- and discernible amounts of di-adducts. The hexanucleotides were generally modified twice. The alkylated species moved faster than the unmodified oligomers.

1. Introduction

Adduct formation of alkylating or oxidizing carcinogenic compounds with DNA has reportedly been a crucial step in the initiation of tumours [1]. Especially some synthetic chemicals are enzymatically oxidized, yielding very reactive electrophiles. One of these chemicals is styrene. Stryene is among the most important chemicals with its major use for the production of plastics and polymeric resins. Since the monomer can be liberated from such materials during industrial application of the polymers, occupational exposure of workers in such work places occurs. It finds its way into the body generally through inhalation. In the lung, it is easily absorbed in blood and metabolized primarily in the liver by the cytochrome P-450 pathway to styrene-7,8-oxide, an epoxide which is mutagenic in both procaryotic and eucaryotic test systems and carcinogenic in rodents. Workers occupationally exposed to styrene oxide has been found with increased chromosome aberration levels [2,3]. Hence styrene is considered a potential carcinogen and its allowed concentration in occupational situations is restricted.

With such a background, several in vitro studies addressing the genotoxic effects of styrene and particularly styrene oxide were performed using DNA or DNA components as models [4]. For the determination of the different reaction products, post-labelling and co-chro-

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matography with synthetic standards proved to be most successful [5]. The results indicate that alkylation through the epoxide takes place predominantly at the N-7-, N^2- and O^6-positions of guanine (Fig. 1).

Several attempts have been made to use mass spectrometric techniques also, since the identification of unknowns would be possible; the general problem is the lack of sufficient detection power. It could be demonstrated, however, that with appropriate derivatization the detection limits for modified nucleobases using GC–MS can be low enough to study biological samples [6,7].

Since the introduction of capillary zone electrophoresis (CZE) by Jorgenson and Lukacs [8], the technique has been used in high-resolution separation analyses for polar and particularly ionic substances at very low levels employing UV or fluorescence detection, the first studies in DNA adduct research have been described [9]. To increase the detection capabilities further, interfacing CZE with mass spectrometry (MS) was introduced using fast atom bombardment (FAB) [10] or electrospray ionization (ESI) [11]. Electrospray is the most often used means of detection since the detection capabilities of ESI are superior owing to much lower chemical backgrounds, but the greatest advantage is the ability to detect high molecular masses due to higher charge numbers per molecule giving access to mass spectra of large bioorganic compounds. For the detection of modifications in DNA, such a combination appeared most promising and we have constructed a capillary electrophoresis system interfaced to an electrospray ion source for a double-focusing sector field mass spectrometer built in this laboratory [12].

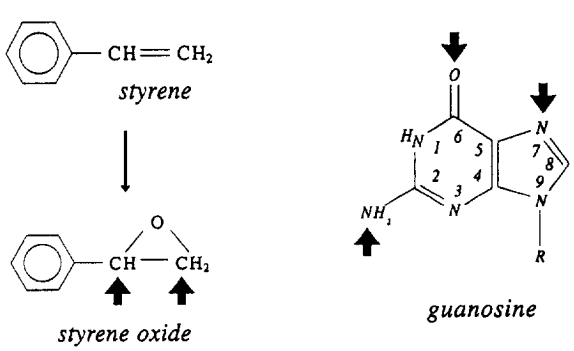

Fig. 1. Reaction sites of styrene oxide and deoxyguanosine.

Here we demonstrate the application of the technique to the analysis of styrene oxide adducts obtained in in vitro reactions with mononucleotides and calf thymus DNA.

2. Experimental

2.1. Materials

Chemicals were purchased from the following suppliers: NH_4HCO_3, Sigma (Deisenhofen, Germany); alkaline phosphatase [1500 U suspension in $(NH_4)_2SO_2$], nucleotides (Böhringer, Mannheim, Germany); calf thymus DNA (lyophilized, research grade), Serva (Heidelberg, Germany); $Mg(OAc)_2$ (analytical-reagent grade), Benzonase (250 U/μl, purity I, 100 000 E per vessel), epoxystyrene (styrene-7,8-oxide), diethyl ether, 2-propanol, NaOH (analytical-reagent grade), Merck (Darmstadt, Germany); methanol (HPLC grade), Promochem (Wesel, Germany).

2.2. Capillary electrophoresis–mass spectrometry

A schematic diagram of the CZE–MS system is given in Fig. 2. A laboratory-constructed CE apparatus is interfaced with an electrospray ion source designed for a Finnigan MAT (Bremen, Germany) Model 90 double-focusing sector field mass spectrometer. The CE system is equipped with a pneumatically actuated sample tray providing space for eight sample reservoirs. Injection can be made electrokinetically or with pressure. The instrument is controlled by means of a single-board computer (Intel 8052). The CE potential is applied at the injection port via a gold electrode inserted in the buffer solution and the other end of the capillary is at electrospray potential. The use of titanium reservoirs [12] was discontinued since gas bubbles evolving during the separations occasionally caused breakdowns of the current. With PTFE cups this problem was solved. The CE apparatus is equipped with a 30

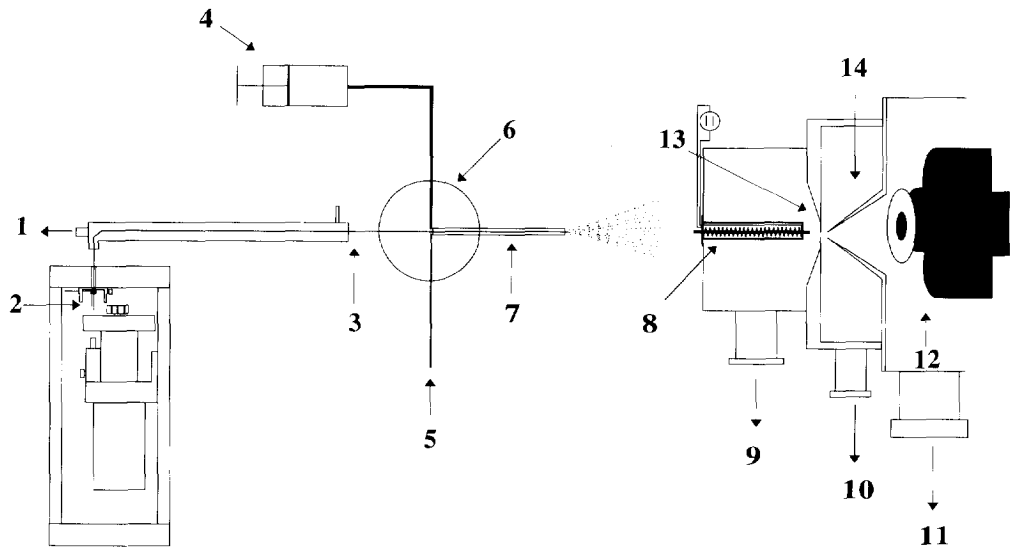

Fig. 2. Schematic diagram of the CZE–MS interface. 1 = air cooling using membrane pump; 2 = high-voltage CZE; 3 = CZE capillary; 4 = liquid sheath flow; 5 = high-voltage ESI; 6 = four-way junction at ESI potential (−8.3 kV); 7 = stainless-steel capillary (sprayer); 8 = indirectly heated capillary; 9 = first pumping stage; 10 = second pumping stage; 11 = source vacuum; 12 = ion optics with draw lens (−2.8 kV) and static quadrupoles; 13 = sampling orifice (0.6 mm); 14 = skimmer (0.8 mm).

kV power supply (Heinzinger, Rosenheim, Germany), which can be floated up to 5 kV.

Capillaries with a thinner silica wall (75 μm I.D., 190 μm O.D.) (SGE, Weiterstadt, Germany) caused difficulties owing to very small hair leaks resulting in gas bubbles inside the capillary. Similar effects have been reported by Moseley et al. [13] and termed electrodrilling. It could be reduced by using capillaries with thicker capillary walls (360 μm O.D., 75, 50 and 25 μm I.D.) (Analyt, Müllheim, Germany). However, even then keeping a constant CZE current for prolonged periods is one of the limiting problems in this interface and the reasons are not yet understood. As a liquid sheath we used 2-propanol and methanol at flow-rates of 5–15 μl/min. The separation capillary ended in a stainless-steel capillary (gauge 22 = 410 μm I.D.) (Hamilton, Darmstadt, Germany), which both ended in a four-way HPLC tee that was used as ESI sprayer. The capillary for liquid sheath was connected to a syringe pump (TSE Systems, Bad Homburg, Germany) and the fourth inlet was used as an electrode connection to the ESI potential.

In our set-up, it seems to be necessary to adjust the separation (inner) capillary so that the end is in line with the outer stainless-steel capillary; otherwise the spray may not be stable. A sheath flow of typically 5 μl also appeared essential to stabilize both the end potential of the CE voltage and the spray, because fluctuations in the eluting salt concentrations may destabilize the performance of the ESI spray, lowering the ion yield. Generally, support by additional air flow using nitrogen was not required.

For desolvation we replaced the earlier used resistively heated capillary with an indirectly heated capillary, providing a uniform temperature profile. The nozzle–skimmer system of the ESI ion source [14] was redesigned using SIMION calculations [15]. The "nozzle" is now conical towards the source with a hole of 600 μm, while the skimmer has an 800 μm entrance at a distance of 4 mm from the nozzle. The distance from the end of the desolvation capillary to the nozzle is 10 mm. Recently, the nozzle was redesigned to a dish-shaped form with a flat base of 2 mm and a hole of 600 μm to avoid contamination reducing ion transmission.

The ion optics design employing two sets of static quadrupoles has been reported for thermo-

spray [16], fast atom bombardment [17] and inductively coupled plasma MS [18] ion sources; with electrospray it gives much better ion transmission when the acceleration potential of the draw lens was held at 2.8 kV, allowing the ions to accelerate in the high-pressure region more moderately.

2.3. Sample preparation

One milligram each of 2′-deoxyadenosine-5′-monophosphate (dAMP), 2′-deoxyguanosine-5′-monophosphate (dGMP) and calf thymus DNA were dissolved in 1 ml of doubly distilled, deionized water and 10 μl of styrene-7,8-oxide were added. The reaction mixture was incubated at 37°C for 48 h. The DNA mixtures were extracted twice with 300 μl of diethyl ether to remove unreacted styrene oxide. The DNA mixtures were digested enzymatically using Benzonase and alkaline phosphatase. The solutions were used without further treatment for electrophoresis using 30 m*M* NH_4HCO_3 buffer, (pH 6.5–7.0).

2.4. Hydrolysis of styrene oxide in water

A 10-μl volume of styrene oxide was dissolved in 1 ml of water and kept for 48 h at 37°C. The solution was subjected directly to Raman Spectroscopy (Raman 2000 R spectrometer; Perkin-Elmer, Überlingen, Germany).

2.5. Sample stacking in the CE capillary

For this procedure the capillary was filled completely with the sample and by inverting the injection port potential of the CZE from positive to negative voltage, the sample migrated from the end of the capillary to the front, increasing the sample concentration. A precondition for this method is that the buffers at both ends have a higher ionic strength than the sample at the beginning. Usually, with CZE–MS there is no buffer reservoir at the sprayer end of the capillary. Therefore, an Eppendorf vessel filled with buffer solution was held in place over the sprayer needle until the preconcentration was finished. The zones were compressed and concentrated, which was indicated by reaching 95% of the normal CZE current. Then the potential was reversed from negative to positive and the CZE–MS run was started.

3. Results

3.1. Mononucleotides

In first experiments, the reaction of styrene oxide with mononucleotides was studied. On that basis a typical reaction product pattern could be established and the electrophoretic separation parameters were optimized. The electrophoretic separation of reaction products of dAMP incubated with styrene oxide shows three peaks in the reconstructed ion mass electropherogram (Fig. 3, bottom).The corresponding mass electropherograms of *m/z* 330 (Fig. 3, top) identifies peak 3 as unreacted dAMP, while peaks 1 and 2 both contain two different species of styrene oxide adducts to dAMP (*m/z* 450; Fig. 3, middle).

Similar results were obtained by the reaction between the other mononucleotides and styrene oxide, with the exception that dGMP shows two additional compounds at *m/z* 586, probably doubly alkylated dGMP. Both bi-adducts migrate slightly faster than the corresponding mono-adducts, while unmodified dGMP migrates last. In Fig. 4, the separation of the products is shown using sample stacking. Since the absolute amount of sample is always of concern in CZE–MS, sample stacking [19] (on-column preconcentration) techniques were applied. The gain in detection power is approximately one order of magnitude, but there is obviously a loss of resolution, particularly for the main components; the reason for this is that the stacking procedure was terminated before the optimum current was reached to avoid sample loss. Without stacking the di-adducts were not detectable since their concentration is lower by at least two orders of magnitude compared with the starting material,

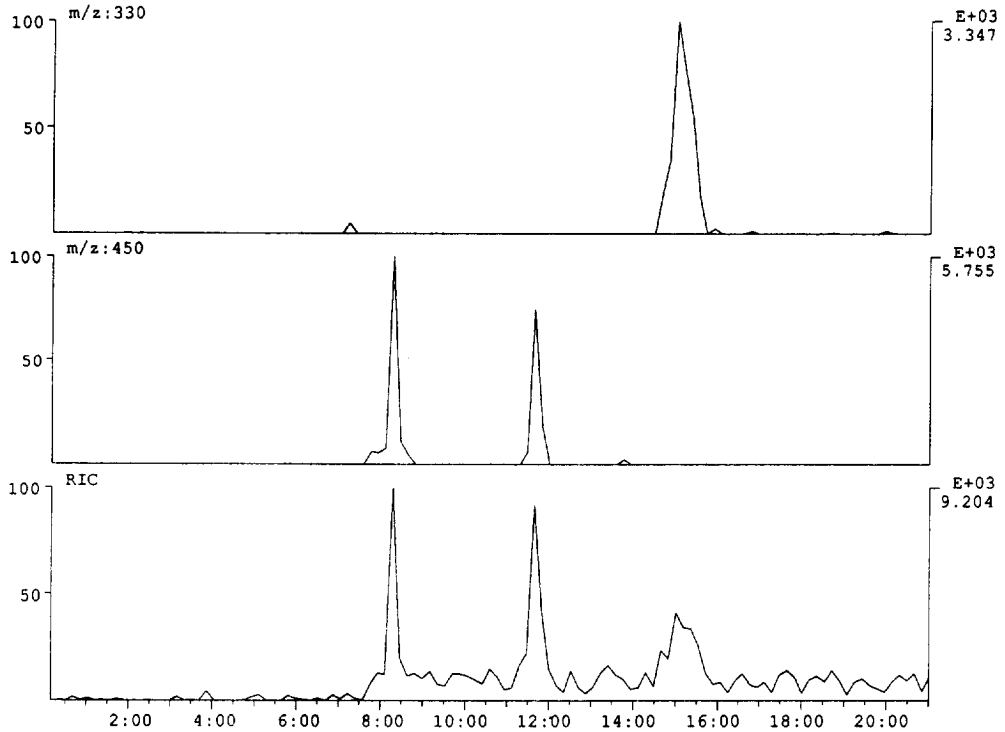

Fig. 3. CZE–MS of dAMP after reaction with styrene oxide. Top trace, electropherogram, m/z 330 (dAMP); middle trace, m/z 450 (mono-adducts dAMP–stryrene oxide); bottom trace, RIC. Separation conditions: capillary, 82 cm × 75 μm I.D.; buffer, 30 m*M* NH_4HCO_3 (pH 6.8); 280 V/cm.

as can be seen from the absolute intensities of the signals.

Since so far only the molecular ions $[M - H]^-$ were observed without relevant fragments, preliminary experiments using collisionally induced dissociation (CID) were made. A mixture of incubated dGMP was continuously infused at a flow-rate of 5 μl/min. The fragmentation was induced by a potential of −40 V between the nozzle and skimmer in the first pumping stage. Fig. 5 shows a mass spectrum of 20 accumulated scans from an experiment with incubated dGMP. At m/z 150 the negative ion of guanine appears, while m/z 195 indicates the loss of guanine from dGMP [M − guanine]. Two peaks in the spectrum are significant for two different types of adducts: the signal at m/z 270 belongs to a guanine–styrene oxide adduct, whereas that at m/z 216 is styrene oxide phosphate. This means that one of the adducts is a phosphate derivative, an artifact not common in DNA. From the two adducts in the electropherogram (Fig. 4, middle trace) the first is the phosphate adduct, since it is less polar, thus eluting first.

3.2. DNA digestion

The attempt to identify modifications of DNA at the oligonucleotide level was made by means of digested calf thymus DNA after styrene oxide incubation using two enzymes. The unspecific endonuclease Benzonase cleaves the DNA into oligonucleotides while the 5′-phosphates were removed with alkaline phosphatase. This digestion steps produce oligonucleotides of the type n-nucleotide–$(n-1)$-phosphate with chain lengths of 2–8 nucleotides, irrespective of modification. Under the separation conditions used here, the oligonucleotides were separated into groups according to the same chain length.

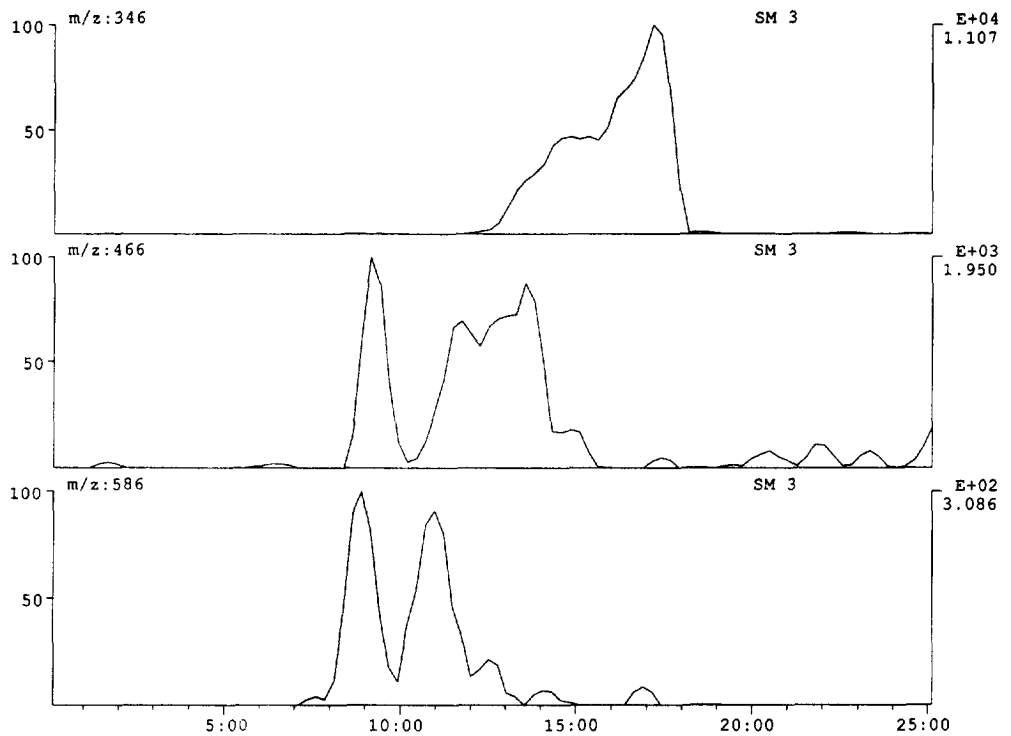

Fig. 4. CZE–MS of dGMP reacted with styrene oxide; sample stacking (capillary was filled completely). Top trace, *m/z* 346 (dGMP); middle trace, *m/z* 466 (two mono-adducts); bottom trace, *m/z* 586 (two di-adducts). Separation conditions: capillary, 82 cm × 75 μm I.D.; buffer, 30 m*M* NH_4HCO_3 (pH 6.8); 280 V/cm.

Fig. 6 shows a total ion electropherogram indicating four peaks. The first peak contains all dinucleotide monophosphates, the second the trinucleotide diphosphates and so on up to pentanucleotide tetraphosphate in this particular electropherogram; in other digestion experiments we have found the oligomers up to octanucleotides.

In Fig. 7 a CZE–MS separation is shown as an "eagle's view" [20], which allows the facile recognition of modified and unmodified oligomers by their separation in time and mass. The modified oligomers migrate slightly faster than the unmodified counterparts; hence it is difficult to separate them in different scans. It should be noted, however, that it is not possible on the basis of these data to distinguish between the 3′- and 5′-isomers of the same sequence, since they are not separated electrophoretically. From Fig. 7 it is evident that styrene oxide produces a variety of adducts under these in vitro conditions. All the oligomers with the exception only of thymine-containing sequences were found to be modified at least once. Di-, tri- and tetranucleotides were found to be modified once, while pentanucleotides showed mono- and di-adducts. Hexanucleotides were found only in doubly modified form and no unmodified oligomers remained. Since the reaction is possible at each nucleobase in the polymer, the probability of modification increases with increase in chain length.

To verify that the modification reaction has occurred before digestion of DNA and not with the oligonucleotides, the reaction was stopped with extraction of styrene oxide using diethyl

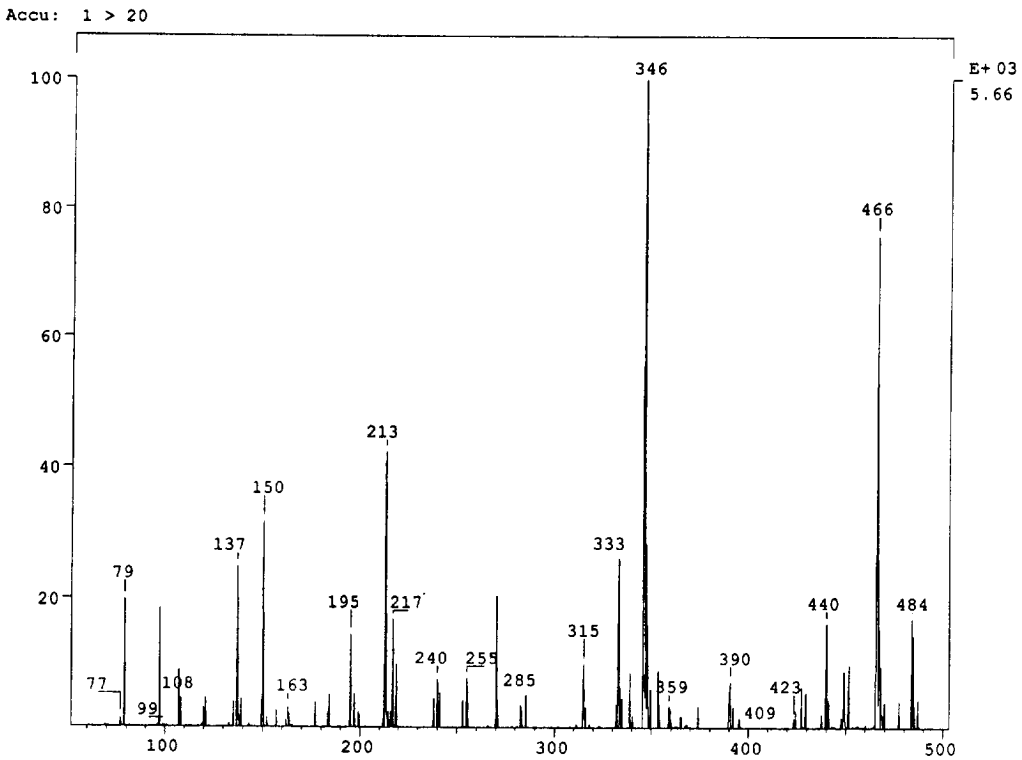

Fig. 5. CID in the skimmer region of reacted dGMP. Mass spectrum, 20 scans accumulated; nozzle voltage, −40 V.

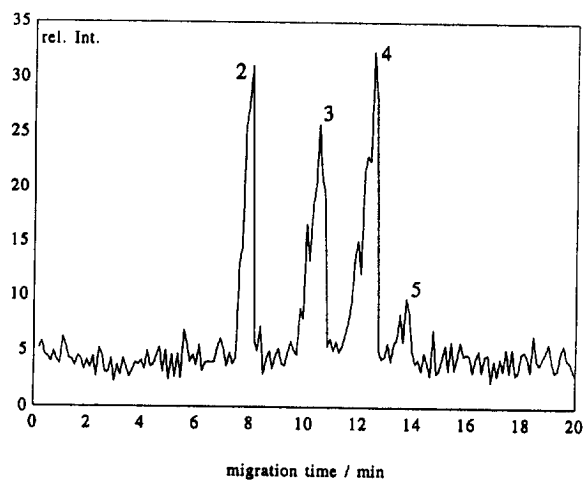

Fig. 6. CZE–MS of oligonucleotides: total ion electropherogram of oligonucleotides from enzymatically digested DNA. Separation conditions as in Fig. 3.

ether, but no differences to the reaction scheme without extraction were observed. Further, we have shown by Raman spectroscopy that after 48 h the epoxide is completely hydrolysed.

4. Discussion

The results shown here indicate that this approach to detecting modifications in DNA may be used to determine the frequency at which a particular reaction occurs in DNA and additionally any sequence specificity. The styrene oxide model seems not to exhibit sequence specificity, at least not at the high concentration level used for the orienting experiments. The observation that thymine does not react with styrene oxide shows the potential to detect such reactions in appropriate model systems. Additionally, at the

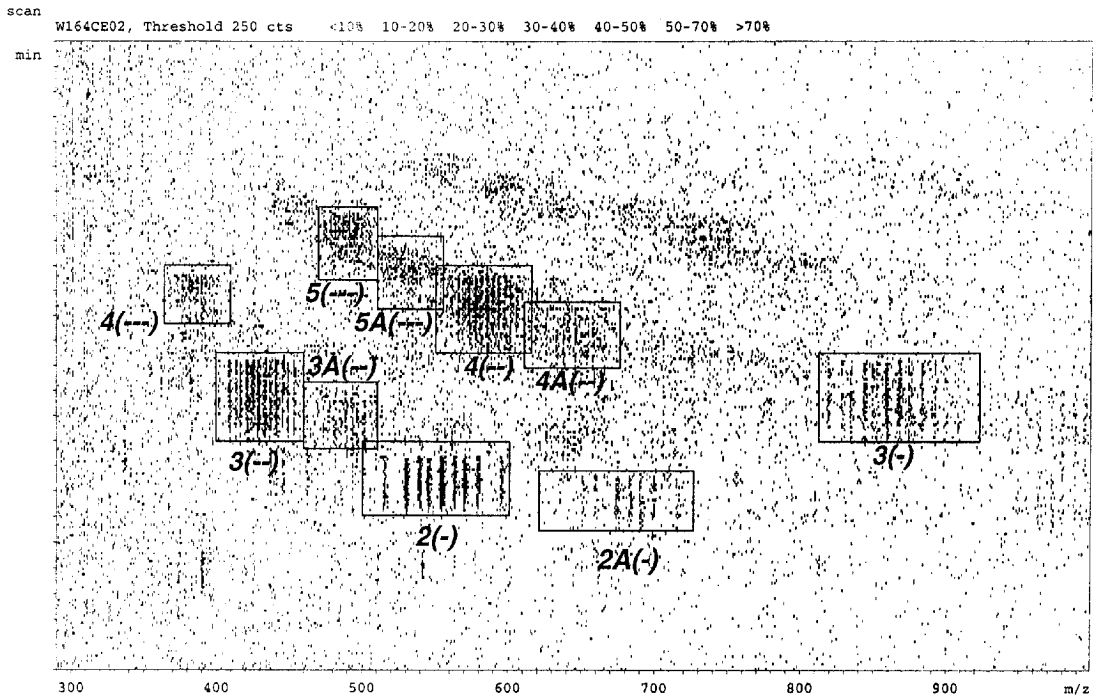

Fig. 7. "Eagle's view" of CZE–MS of oligonucleotides from calf thymus DNA incubated with styrene oxide. Experimental conditions: scan range, 300–1000, 15 s per decade; U(nozzle) = −70 V; T(capillary) = 120°C; separation capillary length, 82 cm; U(CZE) = 15 kV; U(ESI) = −8 kV; U(separation) = 23 kV (280 V/cm). The chain length and the charge state are given; A indicates the styrene oxide adducts.

dinucleotide level the adduct of d(CpC)[1] appears just above the background noise and the intensity of d(GpG) is only slightly higher. All other adducts of dinucleotides are readily detected.

At the trinucleotide level again only d(TpTpT) remains unmodified, whereas the adducts of d(TpTpC), d(ApApT) and d(ApApA) are barely visible. All other possible adducts of the trinucleotides appear with adequate intensities.

Two constraints need further attention. One is the insufficient separation of oligonucleotides of the same chain length, but preliminary results with different buffer systems are very promising. It seems possible to separate at least the different species [21], if not the isomers within the groups. Second, the level of detailed information about the reaction sites at the nucleobases could be optimized using CID either by means of skimmer CID or with MS–MS experiments. The preliminary results discussed here are promising. The adducts formed at N-7 of the guanine yielding a cation should cleave the glycosidic bond anyhow, releasing the modified nucleobase, and we found indications that this indeed happens. This means, on the other hand, that the guanine adducts in the oligomers are adducts to the exocyclic N^2 or O^6, which are chemically stable. This is further indicated by the low intensity of d(GpG) adduct found in the spectra.

Quantitative considerations are impossible as yet owing to the lack of any standard. This makes even simple estimations of the detection power of the technique nearly impossible. With simple dinucleotides such as TpT it is possible to detect nucleotides in the femtomole range. The development of standards and comparison or the combination with other, known methods for the determination of modifications, e.g., GC–MS and post-labeling, are prerequisites for future work.

Considering the toxicological relevance, we

[1] Abbreviations: d = deoxy; A = adenosine; G = guanosine; C = cytidine; T = thymidine; p = phosphate.

have already started to isolate DNA from various sources, particularly from liver homogenates and cell cultures, and have successfully applied the same analytical protocol.

5. Conclusion

We have been able to separate the reaction products of styrene oxide with DNA constituents by means of CZE and to detect the molecular ions $[M-H]^-$ by ESI–MS in a laboratory-built CZE–MS instrument. With CID, fragments were obtained giving some indications about the reaction sites in the molecules, but this approach is in a very preliminary stage.

The same reaction was studied with calf thymus DNA. The DNA was subsequently digested into a mixture of oligonucleotides, which could then be separated into groups of the same chain lengths, but modified species were clearly separated due to different electrophoretic mobility and mass. It seems that the procedure has the potential to detect the preferred reaction sites in DNA and even sequence specificity should be instantly recognized. Since the overall scheme is rapid and effective without the need for derivatization, it can be envisaged that this strategy could provide a valuable tool in the study of toxicologically relevant reactions and in the exploration of genotoxic effects of drugs and xenobiotics. With improved instrumentation, namely CZE injection schemes, stacking procedures and with mass spectrometers providing superior detection power, even biomonitoring could be accomplished.

Acknowledgements

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