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CHARACTERIZATION OF FREE RADICAL-INDUCED DAMAGE TO DNA BY THE COMBINED USE OF ENZYMATIC HYDROLYSIS AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The use of gas chromatography–mass spectrometry (GC–MS) for characterization of free radical-induced base damage to DNA is presented. Damage introduced to DNA by reactive oxygen species such as hydroxyl radicals appears to play an important role in mutagenesis, carcinogenesis and aging. Elucidation of the chemical nature of such DNA lesions is necessary for the assessment of their biological consequences and enzymatic repair. DNA exposed to radiation-generated hydroxyl radicals in aqueous solution was hydrolyzed to 2'-deoxyribonucleosides with a mixture of DNase I, venom and spleen exonucleases and alkaline phosphatase. The hydrolysate was subsequently trimethylsilylated and analyzed by GC–MS. A large number of DNA lesions were separated and identified. Mass spectra obtained were interpreted on the basis of the typical fragmentation pathways of trimethylsilylated nucleosides. The use of GC–MS with selected-ion monitoring facilitated the detection of these lesions at the very low quantities and radiation doses (below 10 Gray) that might be relevant to those in biological systems.

INTRODUCTION

Evidence indicates that damage to deoxyribonucleic acid (DNA) induced by oxygen radicals plays an important role in mutagenesis, carcinogenesis, and aging[1]. Hydroxyl radicals are the most reactive among active oxygen species, which are generated in living cells by cellular metabolism or by the interaction of external agents such as ionizing radiations with cellular water[2]. Reactions of these radicals with DNA create a number of lesions including modified bases, base-free sites, and single and double strand breaks, as revealed by the study of DNA and its components in aqueous solution[3]. Chemical characterization of such lesions is necessary for an understanding of their biological consequences and enzymatic repairability.

In the past, various analytical techniques have been applied to characterization of free radical-induced products of DNA. For instance, thin-layer chromatography, high-performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy and mass spectrometry have been used for identification of base products

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of DNA[4,5] and capillary gas chromatography–mass spectrometry (GC–MS) has been applied to characterization of products of the sugar moiety[6,7].

Recent results from this laboratory have shown that the GC–MS technique is also very useful for characterization of free radical-induced base damage in DNA[8–11]. Moreover, the application of GC–MS with selected-ion monitoring (GC–SIM-MS) facilitated the detection of base products released by acidic hydrolysis from damaged DNA at very low concentrations that might be relevant to those in cellular systems[12]. This paper presents the application of the GC–MS technique with the use of enzymatic hydrolysis to detection of DNA damage induced in aqueous solution by radiation-generated hydroxyl radicals.

EXPERIMENTAL*

Materials

Calf thymus DNA, bis(trimethylsilyl)trifluoroacetamide (BSTFA), and DNA nucleosides were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile was obtained from Pierce (Rockford, IL, U.S.A.). All enzymes used were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Thymidine glycol was obtained by OsO_4-oxidation of thymidine, and purified by HPLC. 2-Amino-4-hydroxy-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine was kindly provided by Dr. J. Cadet of the Centre d'Etudes Nucleaires, Grenoble, France.

Irradiations

Aqueous solutions of DNA (0.25 mg/ml) were saturated with N_2O for 30 min and irradiated with γ-rays in a ^{60}Co-γ-source (dose rate, 1 Gray/min or 80 Gray/min) (1 Gray = 100 rad = 1 J/kg). The samples were then lyophilized. Control samples were treated in the same manner except for irradiation.

Enzymatic hydrolysis and trimethylsilylation

One milligram of DNA was dissolved in 0.5 ml of 10 m*M* Tris–HCl buffer (pH 8.5) containing 2 m*M* magnesium chloride. This solution was incubated with DNase I (100 units), spleen exonuclease (0.01 unit), snake venom exonuclease (0.5 unit), and alkaline phosphatase (10 units) for 24 h at 37°C. The sample was lyophilized and then trimethylsilylated in polytetrafluoroethylene-capped hypovials (Pierce) with 0.2 ml of a mixture of BSTFA and acetonitrile (1:1) by heating for 30 min at 130°C. Unirradiated DNA samples were hydrolyzed and derivatized in the same manner.

Gas chromatography–mass spectrometry

A Hewlett-Packard Model 5880A microprocessor-controlled gas chromatograph interfaced to a Hewlett-Packard Model 5970A mass selective detector was used. The injection port and GC–MS interface were both maintained at 250°C and the ion source at *ca.* 220°C. Separations were carried out using a fused-silica capillary

* Certain commercial equipment or materials are identified in this paper in order to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified is necessarily the best available for the purpose.

column (12 m × 0.2 mm I.D.) coated with crosslinked SE-54 (5% phenylmethyl silicone; film thickness, 0.11 μm) (Hewlett-Packard, Avondale, PA, U.S.A.). Helium was used as the carrier gas at an inlet pressure of 100 kPa. The split ratio was 20:1. Mass spectra were obtained at 70 eV.

RESULTS AND DISCUSSION

Enzymatic hydrolysis

When DNA is exposed to radiation-generated hydroxyl radicals in aqueous solution, sugar products and intact bases are released due to the sugar damage[6,7], whereas the modified bases generally remain attached to the polynucleotide chain[4]. Therefore, the removal of the modified bases from the sugar-phosphate backbone of DNA prior to their chemical analysis is necessary, and this was accomplished in the past by the use of acidic hydrolysis[4,9]. Enzymatic hydrolysis by a mixture of endo- and exonucleases and a phosphatase, which degrades DNA to mononucleosides[13], has also been utilized to release several base products as nucleosides from DNA before their identification by various analytical techniques[10,11,14]. The use of nuclease P_1 for the same purpose has also been reported[15,16]. On the other hand, a study previous to those mentioned above had shown that mixtures of endo- and exonucleases only partially hydrolyzed DNA damaged by hydroxyl radicals, and some oligonucleotides resistant to hydrolysis remained[17]. Similar results have been obtained when nuclease P_1 was used[15]. Lesions causing this resistance, however, have not been elucidated. The present paper demonstrates the usefulness of the GC–MS technique for a simultaneous identification of a large number of base products released as nucleosides by a mixture of DNase I, venom and spleen exonucleases and alkaline phosphatase from DNA damaged by radiation-generated hydroxyl radicals. The hydrolysis procedure used in this work was similar to that applied in ref. 17. DNA lesions resistant to hydrolysis may not have been observed here.

Gas chromatography–mass spectrometry

Fig. 1. shows a total ion chromatogram obtained from a trimethylsilylated enzymatic hydrolysate of DNA exposed to hydroxyl radicals. Peaks I, II and III represent the trimethylsilyl (Me_3Si) derivatives of 2′-deoxyinosine, 2′-deoxyadenosine, and 2′-deoxyguanosine [2′-deoxyinosine-$(Me_3Si)_3$, 2′-deoxyadenosine-$(Me_3Si)_3$, 2′-deoxyguanosine-$(Me_3Si)_4$], respectively, as confirmed by the use of authentic materials. 2′-Deoxyinosine was also observed in unirradiated samples and was probably formed to a large extent by deamination of 2′-deoxyadenosine under the hydrolysis conditions used here. Peak IIIa also corresponds to a Me_3Si derivative of 2′-deoxyguanosine; however, with one Me_3Si group missing from the amino group. No peak was observed for 2′-deoxycytidine-$(Me_3Si)_3$, and peak for thymidine-$(Me_3Si)_2$ was much smaller than expected.

Peaks 1 and 2 represent the two stereoisomers of thymidine glycol-$(Me_3Si)_5$. Mass spectra taken from these peaks were identical to each other, and to the mass spectrum of chemically synthesized authentic material. One of them is shown in Fig. 2. This and other mass spectra presented in this work could be interpreted on the basis of the well-known fragmentation patterns of nucleoside Me_3Si derivatives[18,19]. Thymidine glycol and other free radical-induced products of DNA nucleosides dis-

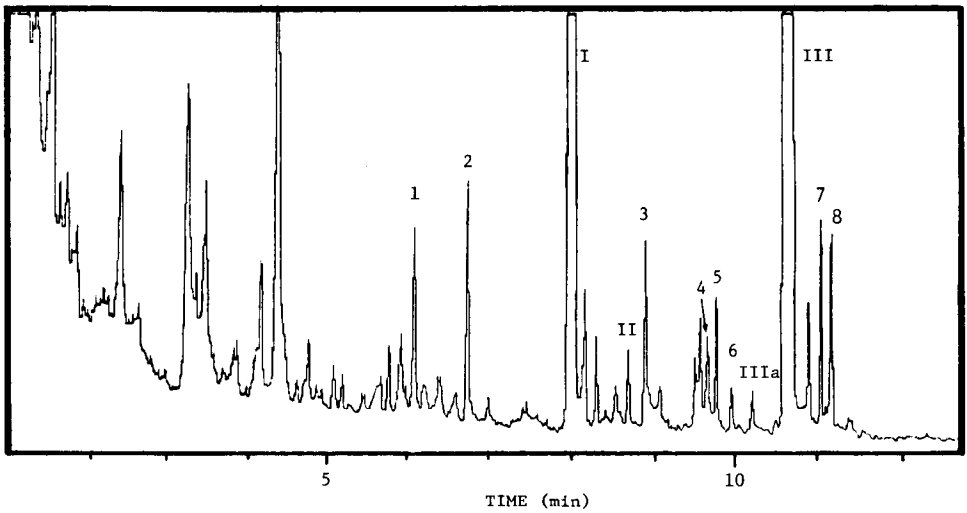
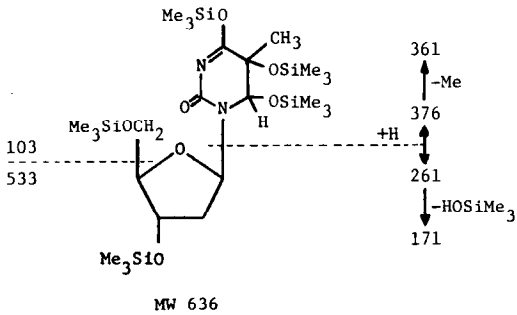


Fig. 1. Total ion chromatogram obtained from a trimethylsilylated enzymatic hydrolysate of DNA exposed to radiation-generated hydroxyl radicals in aqueous solution (radiation dose, 400 Gray). Column, fused-silica capillary coated with SE-54 (12 m × 0.2 mm I.D.), programmed from 190 to 250°C at 7°C/min after 3 min at 190°C. Peak assignment: 1 and 2, two diastereomers of thymidine glycol-$(Me_3Si)_5$; I, 2′-deoxyinosine-$(Me_3Si)_3$; II, 2′-deoxyadenosine-$(Me_3Si)_3$; 3, 8-hydroxy-2′-deoxyinosine-$(Me_3Si)_4$; 4, 4-amino-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine-$(Me_3Si)_5$; 5 and 6, two diastereomers of 8,5′-cyclo-2′-deoxyguanosine-$(Me_3Si)_4$; IIIa, 2′-deoxyguanosine-$(Me_3Si)_3$; III, 2′-deoxyguanosine-$(Me_3Si)_4$; 7, 8-hydroxy-2′-deoxyguanosine-$(Me_3Si)_5$; 8, 2-amino-4-hydroxy-5-formylamino-6-(2′-deoxyribosyl)amino-pyrimidine-$(Me_3Si)_6$.

cussed below were not observed in unirradited DNA samples. A molecular ion (M) and a characteristic M − Me ion were observed at *m/z* 636 and 621 in the mass spectrum of thymidine glycol-$(Me_3Si)_5$. The ion at *m/z* 546 corresponds to the loss of trimethylsilanol ($HOSiMe_3$) from M, which is common to all nucleoside Me_3Si derivatives[18,19]. A further elimination of $HOSiMe_3$ from *m/z* 546 accounts for the ion at *m/z* 456. The fragmentation pathways of thymidine glycol-$(Me_3Si)_5$ leading to the characteristic ions indicated in Fig. 2 are shown below:



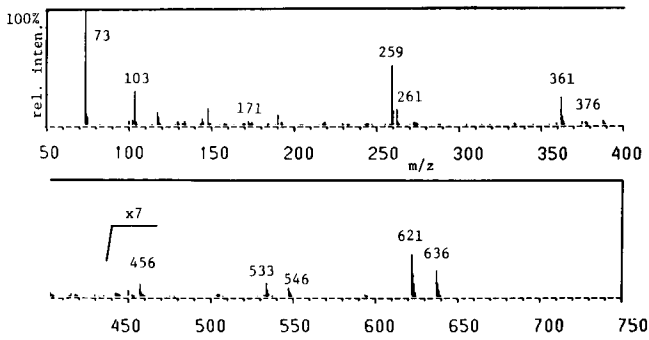
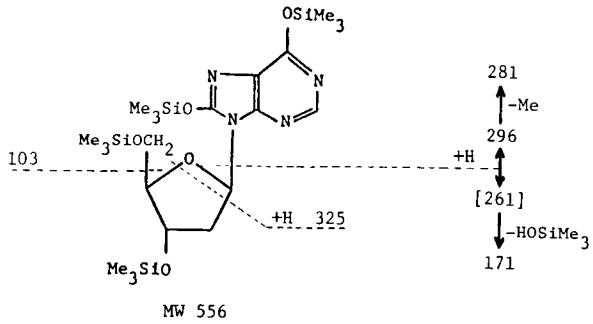


Fig. 2. One of the identical mass spectra taken from peaks 1 and 2 in Fig. 1.

Cleavage of the C–N glycosidic bond accompanied by transfer of an H atom[18,19] leads to the ion at *m/z* 376 [base + H (B + 1) ion]. The B ion is also found at *m/z* 375. The low abundances of the B and B + 1 ions are in agreement with previous observations for pyrimidine nucleosides[19]. The intense ion at *m/z* 361 corresponds to the "base + H − Me" (B − 14) ion[19]. The sugar fragment is represented by the *m/z* 261 ion. Elimination of the $HOSiMe_3$ from this ion accounts for *m/z* 171. The abundant ion at *m/z* 103 extensively occurs in the mass spectra of Me_3Si nucleosides and was assigned mostly to the carbon-5′ silyloxy group[19]. The same fragmentation with the charge remaining on the larger part of the molecule leads to the ion at *m/z* 533. The second most intense ion at *m/z* 259 could not be explained; however, it is believed to contain carbons-4, -5, and -6, and nitrogen-2. This is supported by the fact that this ion also occurs as an intense ion in the mass spectrum of thymine glycol-$(Me_3Si)_4$[20]. The *m/z* 73 and 147 ions are commonly observed with Me_3Si derivatives, and serve no diagnostic purpose.

The mass spectrum taken from peak 3 is illustrated in Fig. 3. It was attributed to 8-hydroxy-2′-deoxyinosine-$(Me_3Si)_4$. This compound may have been formed during the incubation by deamination of 8-hydroxy-2′-deoxyadenosine, which is a known hydroxyl radical-induced product of DNA[21]. M, M − Me, and M − Me − $HOSiMe_3$ ions are found at *m/z* 556, 541, and 451, respectively. The most intense ion at *m/z* 296 and the ion at *m/z* 281 correspond to the B + 1 and B − 14 ions, respectively. The ion of low intensity at *m/z* 325 represent the base plus a portion of the sugar moiety as shown below:



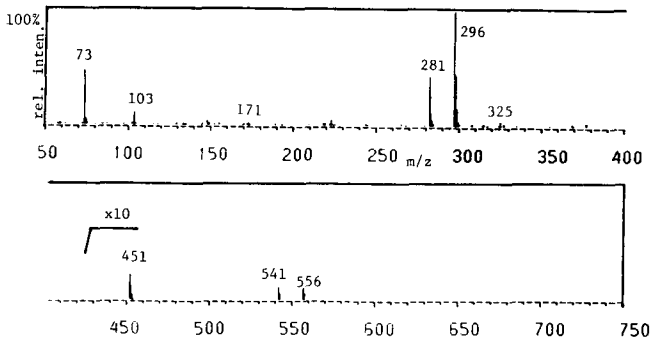
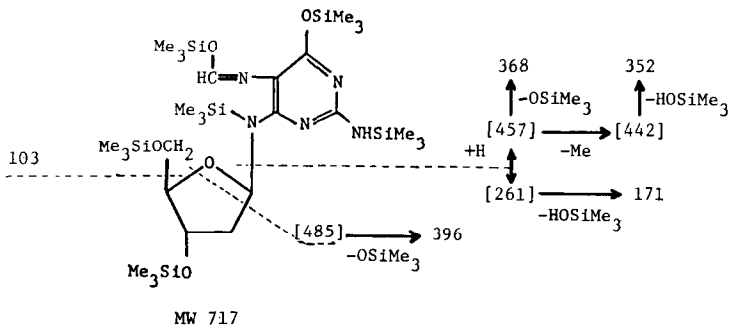


Fig. 3. Mass spectrum taken from peak 3 in Fig. 1.

The high abundance of the B + 1 ion is indicative of an electron-donating substituent at the carbon-8 of the purine ring[19]. The B + 1 ion also appears as the most intense ion (*m/z* 383) in the mass spectrum of 8-hydroxy-2′-deoxyguanosine-$(Me_3Si)_5$ published previously[10]. This compound is represented by peak 7 in Fig. 1.

Mass spectrum taken from peak 8 is illustrated in Fig. 4. It was assigned to the Me_3Si derivative of 2-amino-4-hydroxy-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine, which is a known hydroxyl radical-induced product of 2′-deoxyguanosine moiety of DNA[4]. Authentic material for this compound was available and gave a mass spectrum identical to that in Fig. 4. M, M − Me, M − $HOSiMe_3$, M − Me − $HOSiMe_3$ ions were observed at *m/z* 717, 702, 627, and 612, respectively. This compound is different from other nucleosides in terms of the position of the C–N glycosidic bond. The B + 1 and B − 14 ions (*m/z* 457 and 442, respectively) typically observed in the mass spectra of Me_3Si nucleosides are not found in Fig. 4. Ions at *m/z* 368 and 352 appear to be formed by the loss of $OSiMe_3$ from the B + 1 ion and of $HOSiMe_3$ from the B − 14 ion, respectively. The second most intense ion at *m/z* 396 was probably formed by loss of $OSiMe_3$ from *m/z* 485 representing the base plus a portion of the sugar moiety. The latter ion was not observed. The structure of this compound along with the suggested fragmentation pathways is shown below:



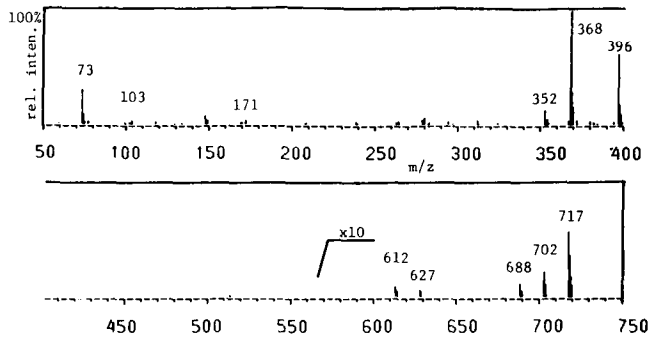


Fig. 4. Mass spectrum taken from peak 8 in Fig. 1.

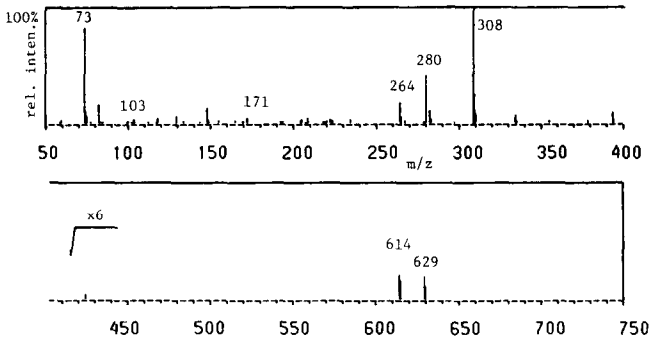
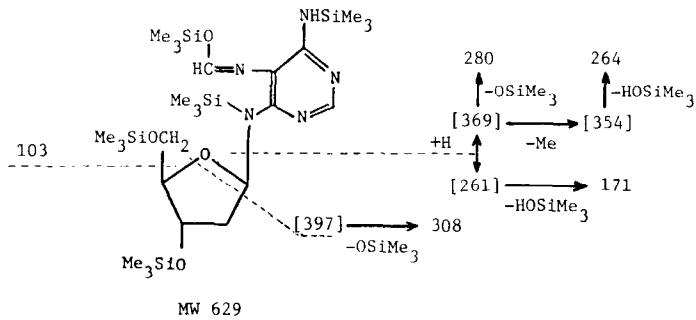


Fig. 5. Mass spectrum taken from peak 4 in Fig. 1.

The analogous product from the 2′-deoxyadenosine moiety of DNA[20], *i.e.*, 4-amino-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine, is represented by peak 4 in Fig. 1 as its Me_3Si derivative. The mass spectrum taken from peak 4 contains M and M − Me ions at *m/z* 629 and 614, respectively (Fig. 5). Abundant ions at *m/z* 264, 280, and 308 can be explained by fragmentations analogous to those discussed above for 2-amino-4-hydroxy-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine-$(Me_3Si)_6$:



One hydroxyl radical-induced reaction unique to purine nucleosides and nucleotides is the intramolecular cyclization between the carbon-5′ of the sugar moiety and the carbon-8 of the purine ring. This reaction has been observed in adenosine-5′-monophosphate and 2′-deoxyadenosine and the resultant products have been identified as 8,5′-cyclo-adenosine-5′-monophosphate and 8,5′-cyclo-2′-deoxyadenosine, respectively[22,23]. Recently, the formation of an analogous 8,5′-cyclo-2′-deoxyguanosine moiety in DNA upon hydroxyl radical attack has been reported from this laboratory[11]. This product is represented by peaks 5 and 6 in Fig. 1 as Me_3Si derivatives of its two diastereomers. The mass spectrum of 8,5′-cyclo-2′-deoxyguanosine-$(Me_3Si)_4$ has been published previously[11].

Selected-ion monitoring

Gas chromatography–mass spectrometry with selected-ion monitoring (GC–SIM-MS) permits identification of organic compounds at very low concentrations in complex mixtures. In the SIM mode, only ions that are characteristic of a particular compound are recorded. As a result, a mass spectrometer in this mode becomes far

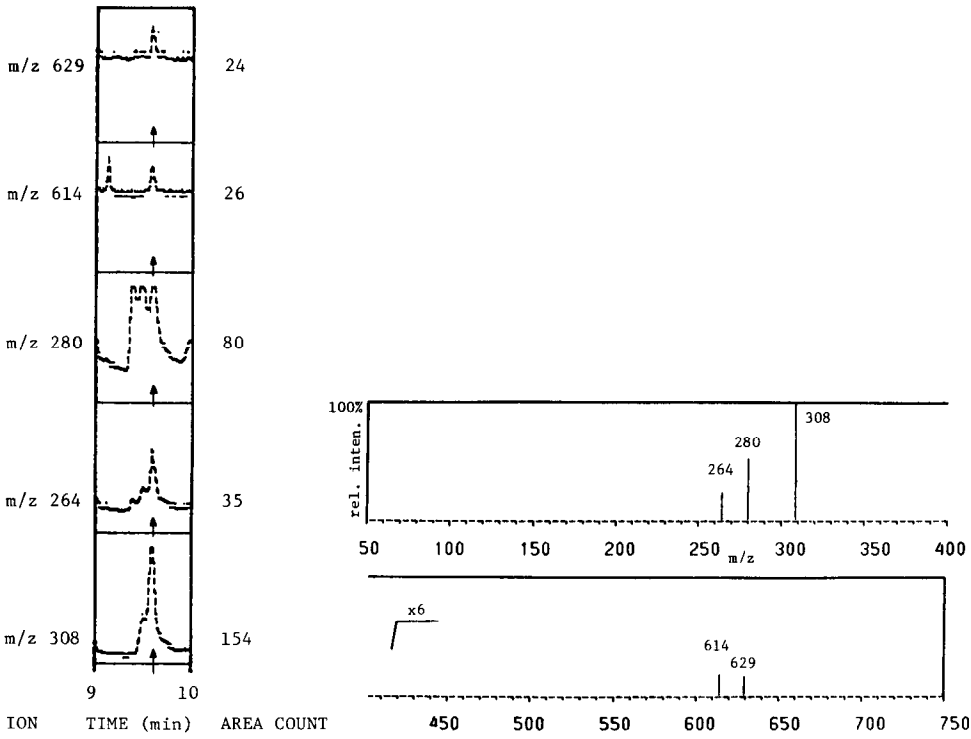


Fig. 6. SIM plots of some characteristic ions of 4-amino-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine-$(Me_3Si)_5$ obtained from a sample of DNA irradiated at a radiation dose of 5 Gray. (The full scales of the *m/z* 614 and 629 ions are 14 times and the full scale of the *m/z* 264 ion is twice higher than those of the 308 and 280 ions; area counts given here are arbitrary numbers calculated by the intergrator of the instrument).

Fig. 7. Mass spectrum generated on the basis of the ions and their area counts at the elution position indicated with the arrow in Fig. 6.

more sensitive than in the total ion monitoring mode. In the present work, the use of the GC–SIM-MS technique permitted the detection of the products discussed above in enzymatic hydrolysates of DNA exposed to radiation doses as low as 1 Gray. As an example of the application of this technique, Fig. 6 shows the SIM plots of some five characteristic ions of 4-amino-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine-$(Me_3Si)_5$ obtained from a sample of DNA exposed to 5 Gray. The presence of the recorded ions is clearly seen in Fig. 6 at the GC-retention time of this compound indicated with the arrow (for comparison, see also peak 4 in Fig. 1). No signals for these ions were observed in the SIM plots obtained from unirradiated DNA (data not shown here). Since not only the presence of the characteristic ions of a compound are important but also their intensities relative to one another for a positive identification, the area counts of the recorded ions were calculated by the integrator of the instrument. The acquired data were used subsequently to generate a mass spectrum by setting the area count of the most abundant ion equal to 100% of relative intensity. The partial mass spectrum obtained from the signals indicated with the arrow in Fig. 6 is illustrated in Fig. 7. This partial spectrum was directly correlatable to that of 4-amino-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine-$(Me_3Si)_5$ shown in Fig. 5.

Recently, the use of the GC–SIM-MS technique also facilitated the detection of (5′*R*)- and (5′*S*)-8,5′-cyclo-2′-deoxyadenosine moieties in DNA exposed to radiation-generated hydroxyl radicals[24]. The (5′*R*)- and (5′*S*)-epimers of 8,5′-cyclo-2′-deoxyadenosine-$(Me_3Si)_3$, although well separated from each other, coelute with 2′-deoxyinosine-$(Me_3Si)_3$ under the GC conditions used in Fig. 1. Thus, they could not be detected in the hydrolysate of damaged DNA by the use of the total-ion monitoring mode.

CONCLUSIONS

The results presented here show that the combined use of enzymatic hydrolysis and the GC–MS technique is well suited for the simultaneous identification of various hydroxyl radical-induced DNA lesions. The detection of such lesions at very low quantities and radiation doses (below 10 Gray) can be achieved by the use of the selected-ion monitoring technique. An appropriate data acquisition system can monitor simultaneously the necessary characteristic ions of all the products reported here in a single run. This means that the sample amount and the time required can be kept at a minimum level by the use of the GC–SIM-MS technique. Because of the simple hydrolysis conditions and the simple preparation of the Me_3Si derivatives, DNA can be hydrolyzed and derivatized in a single vial, without any further sample manipulation. It should be pointed out that a method involving GC is limited, of course, to products that can be successfully derivatized (if derivatization is needed) and passed through a GC column. In the present work the limitation was that the products of the cytosine moiety of DNA could not be analyzed as their nucleosides, and only one product of the thymine moiety, *i.e.*, thymidine glycol, was observed. The detection of cytosine products and other thymine products by GC–MS and GC–SIM-MS after acidic hydrolysis of DNA has been recently demonstrated[9,12]. On the other hand, the enzymatic hydrolysis of DNA was shown here to permit the detection by GC–MS of all the known hydroxyl radical-induced products of purine

nucleosides, especially that of 8,5′-cyclopurine nucleosides, which are not released from DNA by acidic hydrolysis. The conclusion can be drawn that these two modes of DNA hydrolysis are complementary and both should be used for an optimal assessment by GC–MS of hydroxyl radical-induced DNA damage. On the basis of the results obtained here and elsewhere[9,12], the GC–MS technique, especially with the use of SIM, appears to be an ideal analytical tool for characterization of hydroxyl radical-induced (or radiation-induced) damage to cellular DNA. Prior to the application of this technique, however, isolation of cellular DNA from intact cells is required.

ACKNOWLEDGEMENTS

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