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Quantitative determination of oxidative base damage in DNA by stable isotope-dilution mass spectrometry

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For understanding of the role of oxidative DNA damage in biological processes such as mutagenesis and carcinogenesis, it is essential to identify and quantify this type of DNA damage in cells. This can be achieved by gas chromatography/mass spectrometry. The present study describes the quantification of modified bases in DNA by isotope-dilution mass spectrometry with the use of stable isotope-labeled analogues as internal standards. A number of isotopically labeled DNA bases were synthesized. The mass spectra of their trimethylsilyl derivatives were recorded. Calibration plots were obtained for known quantities of modified bases and their isotope-labeled analogues. Quantification of various modified DNA bases by isotope-dilution mass spectrometry was demonstrated in isolated chromatin exposed to ionizing radiation. The results indicate that gas chromatography/stable isotope-dilution mass spectrometry is an ideally suited technique for selective and sensitive quantification of modified bases in DNA.

Free radical; Hydroxyl radical; Isotope-labeled analogue; Modified base; Oxidative DNA damage; Radiation damage; Selected-ion monitoring

1. INTRODUCTION

Oxidative DNA damage produced by free radicals or other DNA-damaging agents has been implicated in biological processes such as mutagenesis, carcinogenesis, reproductive cell death and aging (reviewed in [1]). Free radicals may be generated in vivo by endogenous and exogenous sources, and cause DNA damage by a variety of mechanisms (reviewed in [2,3]). Superoxide radical (O_2^-) and H_2O_2, which are generated in all aerobic cells [4], do not appear to cause any DNA damage under physiological conditions [5–7]. The toxicity of these species has been attributed to the highly reactive hydroxyl radical (•OH), which can be generated by metal ion catalyzed reactions of O_2^- and H_2O_2 [1]. Exogenous sources such as ionizing radiation can also produce •OH in cells and tissues [2]. Hydroxyl radical causes a unique pattern of chemical modifications in DNA and nucleoproteins, including modified bases and DNA-protein cross-links (reviewed in [2,3,8–10]). Some of these lesions are also produced by non-radical pathways (e.g., photosensitization, direct effect of ionizing radiation) [11–16]. Because of the importance of oxidative DNA damage in biological processes such as carcinogenesis, it is essential to chemically characterize and quantify DNA lesions resulting from this type of DNA damage. Identification and quantification of modified DNA bases can be achieved using a number of analytical techniques including immunochemical techniques, postlabeling assays and HPLC with absorbance, electrochemical and radioactivity measurements (reviewed in [3,10]). In recent years, we have described the use of the technique of gas chromatography/mass spectrometry (GC/MS) for chemical characterization and quantification of a variety of modified bases in DNA and chromatin (reviewed in [9,17]). Unlike the other techniques, GC/MS provides on-line structural evidence for the compounds analyzed and permits in a single analysis the measurement of a large number of pyrimidine- and purine-derived modified bases in the same sample of DNA or chromatin.

Using GC/MS, quantification of analytes is achieved by adding a suitable internal standard to DNA samples at an early stage of analysis such as prior to hydrolysis of DNA. In mass spectrometry, a stable isotope-labeled analog of an analyte can be used as an internal standard (reviewed in [18]). This procedure is often called isotope-dilution mass spectrometry. The use of stable isotope-labeled analogues permits compensation of possible losses of the analyte during the sample preparation and GC/MS-analysis because the analyte and its analog have essentially the same chemical and physical properties. With the use of the selected-ion monitoring (SIM) mode, an intense and characteristic ion of the analyte

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Abbreviations: O_2^-, superoxide radical; •OH, hydroxyl radical; BSTFA, bis(trimethylsilyl)trifluoroacetamide; GC/MS-SIM, gas chromatography/mass spectrometry with selected-ion monitoring; rel., relative; Gy, gray (J/kg); EI, electron-ionization; 8-OH-Ade, 8-hydroxyadenine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Gua, 8-hydroxyguanine.

and that of its analog are simultaneously monitored for quantification [18]. If stable isotope-labeled analogues are not available, structurally similar compounds may be used as internal standards [18]. Recently, we have reported the quantification of modified DNA bases with the use of structurally similar compounds as internal standards [19]. We have also studied the stability of modified DNA bases and their release from DNA under various acidic conditions, since these factors are important for accurate quantification [19,20]. Structurally similar compounds were used as internal standards because stable isotope-labeled analogues of modified DNA bases were not available commercially or were very expensive to synthesize and purify. Recently, a stable isotope-labeled analog has been used for quantification by GC/MS of 5-(hydroxymethyl)uracil in DNA [21].

Recently, we obtained custom-synthesized, stable isotope-labeled analogues of a number of modified bases which result from oxidative damage to pyrimidines and purines in DNA. In the present paper, we report on the use of these compounds for quantification by GC/MS-SIM of modified DNA bases.

2. MATERIALS AND METHODS

2.1. *Materials*

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

4,6-Diamino-5-formamidopyrimidine, 8-hydroxyadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 8-hydroxyguanine, dialuric acid, 4,6-diamino-5-formamidopyrimidine-1,3-$^{15}N_2$-2-^{13}C-(5-aminoformyl-^{15}N,^{2}H), 8-hydroxyadenine-1,3,7-$^{15}N_3$-2,8-$^{13}C_2$, 2,6-diamino-4-hydroxy-5-formamidopyrimidine-1,3-$^{15}N_2$-(5-amino-^{15}N)-2-^{13}C, 8-hydroxyguanine-1,3-$^{15}N_2$-(2-amino-^{15}N)-2-^{13}C and dialuric acid-1,3-$^{15}N_2$-2,4-$^{13}C_2$ were purchased from Program Resources, Inc., Frederick, MD. *cis*-Thymine glycol-$\alpha,\alpha,\alpha,6$-$^{2}H_4$ was obtained from Merck Inc./Isotopes, St. Louis, MO. All these compounds were custom-synthesized. *cis*-Thymine glycol was a gift from Dr. W.F. Blakely of the Armed Forces Radiobiology Research Institute, Bethesda, MD. Thymine-$\alpha,\alpha,\alpha,6$-$^{2}H_4$ was purchased from Merck Inc./Isotopes, St. Louis, MO. Xanthine, xanthine-1,3-$^{15}N_2$, and alloxan were purchased from Sigma. 5-Hydroxyhydantoin and 5-hydroxyhydantoin-1,3-$^{15}N_2$-2,4-$^{13}C_2$ were obtained by treatment of dialuric acid and dialuric acid-1,3-$^{15}N_2$-2,4-$^{13}C_2$, respectively, with 88% formic acid at 150°C for 1 h. Formic acid was purchased from Mallinckrodt. Acetonitrile and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylsilylchlorosilane were obtained from Pierce.

2.2. *Purity of compounds and preparation of samples*

The purity of modified DNA bases and their labeled analogues was assessed by GC/MS and found to be greater than 98%. The identity of each compound was confirmed on the basis of the known mass spectra of trimethylsilyl (Me_3Si) derivatives of the unlabeled compounds [22,23]. Stable isotope-labeled compounds had no measurable contamination by their respective unlabeled analogues. All compounds were dissolved in water at a concentration of 0.1 mM. 8-Hydroxyguanine and 8-hydroxyguanine-1,3-$^{15}N_2$(2-amino-^{15}N)-2-^{13}C were not completely soluble. However, a complete solubility was obtained by increasing the pH of the solutions to 9.5 with dilute NaOH and by subsequent stirring the solutions for several hours at room temperature. Aliquots of the solutions of modified bases and their stable isotope-labeled analogues were mixed in different ratios and then lyophilized.

2.3. *Isolation of chromatin and irradiations*

Isolation of chromatin from cultured K562 cells was performed as described previously [24]. Chromatin was obtained in 1 mM Tris buffer (pH 7.4) and then dialyzed against 1 mM phosphate buffer (pH 7.4). Chromatin suspensions (0.1 mg of DNA/ml) were saturated with N_2O and irradiated in a ^{60}Co-γ-source (dose rate, 85.3 Gy/min). To aliquots of chromatin samples containing 0.1 mg of DNA, aliquots of stable isotope-labeled compounds (0.5 nmol each) including thymine-$^{2}H_4$ (20 nmol) were added. Samples were then lyophilized.

2.4. *Hydrolysis, trimethylsilylation and gas chromatography/mass spectrometry*

Lyophilized chromatin samples were hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed tubes at 140°C for 30 min [20]. Samples were lyophilized and then trimethylsilylated with 0.1 ml of a BSTFA/acetonitrile (4/1; v/v) mixture in poly(tetrafluoroethylene)-capped hypovials under nitrogen at 130°C for 30 min. Lyophilized mixtures of modified bases and their stable isotope-labeled analogues were trimethylsilylated in the same manner. Analysis of derivatized samples by GC/MS-SIM was performed as described previously [20,24]. In addition, small quantities (≈0.2 mg) of individual modified bases and their stable isotope-labeled analogues were trimethylsilylated in separate vials. These samples were analyzed by GC/MS with total-ion monitoring to check the purity of the compounds and to obtain their full mass spectra. All mass spectral recordings were performed in the electron-ionization (EI) mode at 70 eV.

3. RESULTS AND DISCUSSION

Trimethylsilylated modified bases and their stable isotope-labeled analogues were analyzed separately by GC/MS with total-ion monitoring to obtain their full EI-mass spectra. Two examples of mass spectra are illustrated in Figs. 1 and 2. EI-mass spectra of Me_3Si derivatives of unlabeled modified bases have been published previously [22,23]. These mass spectra are dominated generally by an intense molecular ion ($M^{+\bullet}$ ion) and an intense $(M-15)^+$ ion, which results from loss of a methyl radical from $M^{+\bullet}$ ion. In some instances, an $(M-1)^+$ ion, which results from loss of an H atom from $M^{+\bullet}$ ion, is also present. In the mass spectrum of the Me_3Si derivative of 4,6-diamino-5-formamidopyrimidine [4,6-diamino-5-formamidopyrimidine-$(Me_3Si)_3$] (Fig. 1A), $M^{+\bullet}$, $(M-1)^+$ and $(M-15)^+$ ions appear at *m/z* 369, 368 and 354, respectively. The intense ion at *m/z* 280 results from loss of $^{\bullet}OSiMe_3$ (89 Da) from $M^{+\bullet}$ ion [23]. As expected, the masses of these ions are shifted by 5 Da to 374, 373, 359 and 285 Da, respectively, in the mass spectrum of 4,6-diamino-5-formamidopyrimidine-$^{15}N_3$,^{13}C,^{2}H-$(Me_3Si)_3$ (Fig. 1B). The ion at *m/z* 372 may be due to loss from $M^{+\bullet}$ ion of an ^{2}H atom located at the formyl group of the molecule. The absence of ion currents at *m/z* 280, 354, 368 and 369 in Fig. 1B indicates that the labeled analog was not contaminated by the unlabeled material. Ions at *m/z* 73 and 147 in Figs. 1A and B are common fragments of Me_3Si derivatives

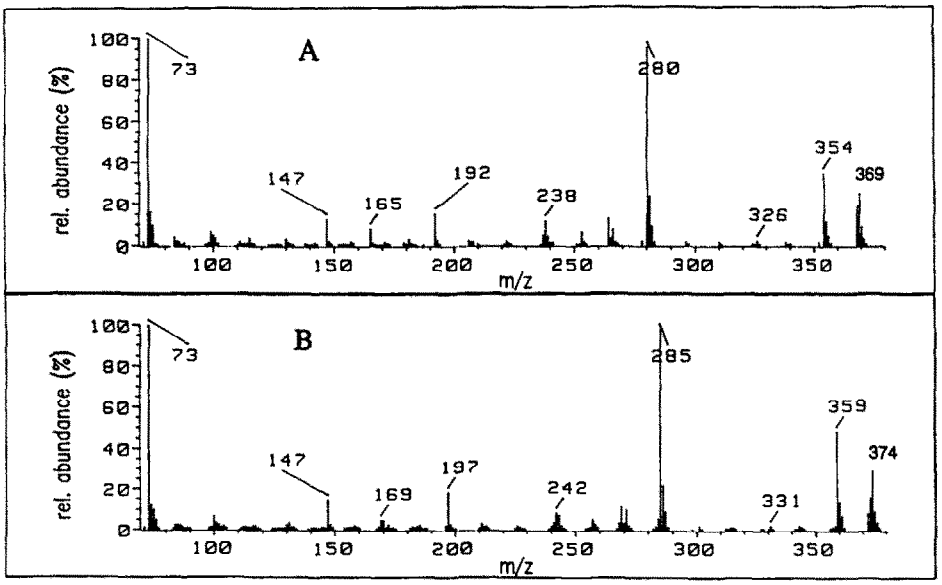

Fig. 1. (A) EI-mass spectrum of 4,6-diamino-5-formamidopyrimidine-$(Me_3Si)_3$; (B) EI-mass spectrum of 4,6-diamino-5-formamidopyrimidine-$^{15}N_3$,^{13}C,^{2}H-$(Me_3Si)_3$.

and serve no diagnostic purpose [25]. In the mass spectrum of thymine glycol-$(Me_3Si)_4$ (Fig. 2A), $M^{+\bullet}$ and $(M—15)^+$ ions appear at *m/z* 448 and 433, respectively. These ions are shifted by 4 Da to 452 and 437, respectively, in the mass spectrum of thymine glycol-$^2H_4$-$(Me_3Si)_4$ (Fig. 2B). The mass of *m/z* 259 is shifted to 262 Da. The difference of 3 Da between the masses of these ions indicates that the carbon-6 of thymine may not be contained in these fragment ions, since three of the four ^2H atoms of the labeled material are part of the methyl group and one is at carbon-6.

In the case of 8-hydroxyadenine-$(Me_3Si)_3$, 2,6-diamino-4-hydroxy-5-formamidopyrimidine-$(Me_3Si)_4$ and 8-hydroxyguanine-$(Me_3Si)_4$, the masses of characteristic ions were also shifted in the mass spectra of their labeled analogues according to the extent of labeling (data not shown). When dialuric acid and dialuric acid-$^{15}N_2$,$^{13}C_2$ were trimethylsilylated directly without dissolving them in water first, they gave single gas chromatographic peaks and were detected by mass spectrometry as the Me_3Si derivatives of their enol forms, i.e. 5,6-dihydroxyuracil-$(Me_3Si)_4$ and 5,6-dihydroxyuracil-$^{15}N_2$, $^{13}C_2$-$(Me_3Si)_4$, respectively. The mass spectrum of 5,6-dihydroxyuracil-$(Me_3Si)_4$ has been published previously

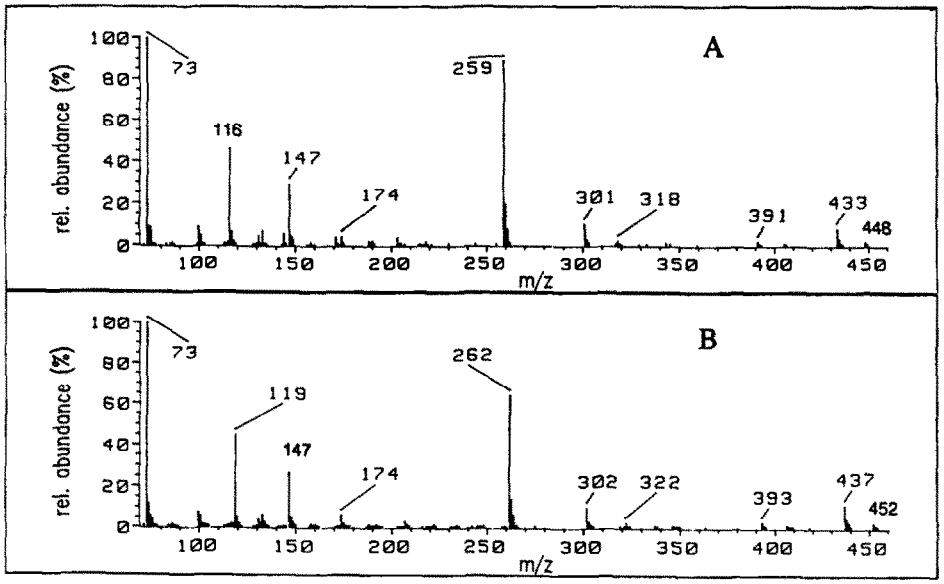

Fig. 2. (A) EI-mass spectrum of thymine glycol-$(Me_3Si)_4$; (B) EI-mass spectrum of thymine glycol-$^2H_4$-$(Me_3Si)_4$.

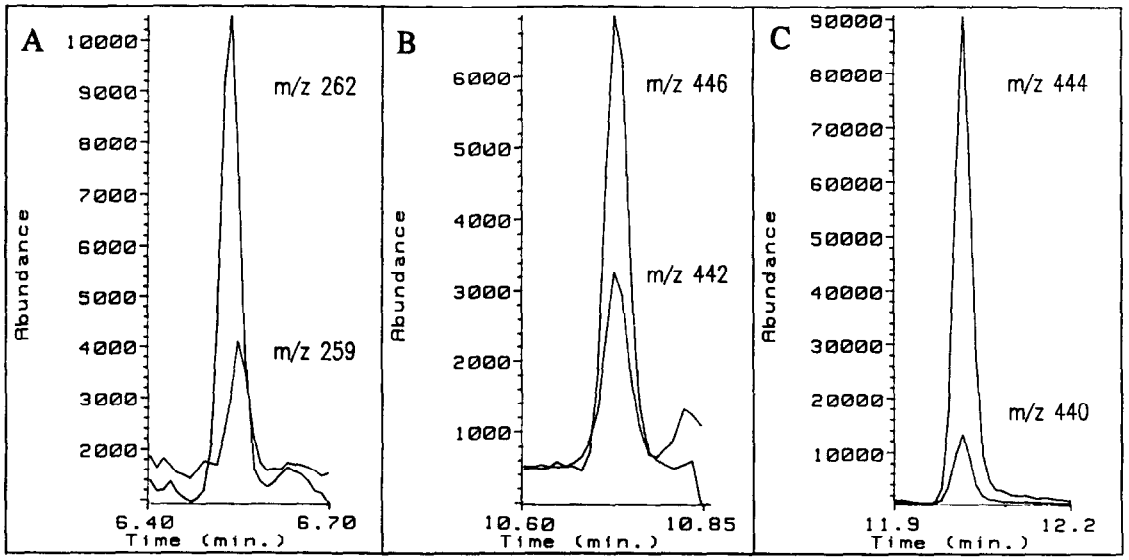

Fig. 3 Superimposed selected ion-current profiles, (A): at *m/z* 259 [thymine glycol-$(Me_3Si)_4$] and 262 [thymine glycol-$^2H_4$-$(Me_3Si)_4$]; (B). at *m/z* 442 [2,6-diamino-4-hydroxy-5-formamidopyrimidine-$(Me_3Si)_4$] and 446 [2,6-diamino-4-hydroxy-5-formamidopyrimidine-$^{15}N_3$,^{13}C-$(Me_3Si)_4$; (C): at *m/z* 440 [8-hydroxyguanine-$(Me_3Si)_4$] and 444 [8-hydroxyguanine-$^{15}N_3$,^{13}C-$(Me_3Si)_4$]. Profiles were obtained during the GC/MS-SIM analysis of a trimethylsilylated hydrolysate of irradiated chromatin.

[23]. Upon dissolving in water, however, dialuric acid and dialuric acid-$^{15}N_2$,$^{13}C_2$ were readily oxidized to alloxan [2,4,5,6(1H,3H)-pyrimidinetetrone] and alloxan-$^{15}N_2$,$^{13}C_2$, respectively [26]. Authentic alloxan was available commercially. The mass spectrum of alloxan-$(Me_3Si)_4$ is characterized by an $M^{+\bullet}$ ion of low intensity (*m/z* 448, 1% relative intensity), an $(M-15)^+$ ion (*m/z* 433, 10%) and an intense ion at *m/z* 331 (100%), which most likely results from loss of $^{\bullet}CO_2Me_3Si$ (117 Da) from $M^{+\bullet}$ ion (mass spectrum not shown). The mass of the $M^{+\bullet}$ ion and the occurrence of the intense *m/z* 331 ion indicate that alloxan assumes its free acid form either in solution or upon trimethylsilylation. In the mass spectrum of alloxan-$^{15}N_2$,$^{13}C_2$-$(Me_3Si)_4$, these masses were shifted by 4 Da to 452, 437 and 335 Da, respectively. Acidic treatment quantitatively converted alloxan and alloxan-$^{15}N_2$,$^{13}C_2$ into 5-hydroxyhydantoin and 5-hydroxyhydantoin-$^{15}N_2$,$^{13}C_2$, respectively. In the past, 5-hydroxyhydantoin has been identified as an $^{\bullet}OH$-induced product of cytosine (reviewed in [9,27]). The fragmentation patterns of 5-hydroxyhydantoin-$(Me_3Si)_3$ were very similar to those of 5-hydroxy-5-methylhydantoin-$(Me_3Si)_3$, the mass spectrum of which has been published previously [17]. $M^{+\bullet}$, $(M-1)^+$ and $(M-15)^+$ ions were observed at *m/z* 332 (1% rel. intensity), 331 (1%) and 317 (60%), respectively (mass spectrum not shown). An intense ion at *m/z* 202 (42%), which results from loss of Me_3SiOCN (115 Da) from $(M-15)^+$ ion [17], was also observed. The common fragment ions *m/z* 73 and 147 appeared as intense peaks with 55% and 100% relative intensities, respectively. The masses of $M^{+\bullet}$, $(M-1)^+$ and $(M-15)^+$ ions were shifted by 4 Da to 336, 335 and 321 Da in the mass spectrum of 5-hydroxyhydantoin-$^{15}N_2$,$^{13}C_2$-$(Me_3Si)_3$ (mass spectrum not shown). The mass of the *m/z* 202 ion was shifted by 2 Da to 204 Da because of loss of 1-^{15}N and 2-^{13}C or 3-^{15}N and 4-^{13}C in the form of Me_3SiOCN as indicated above.

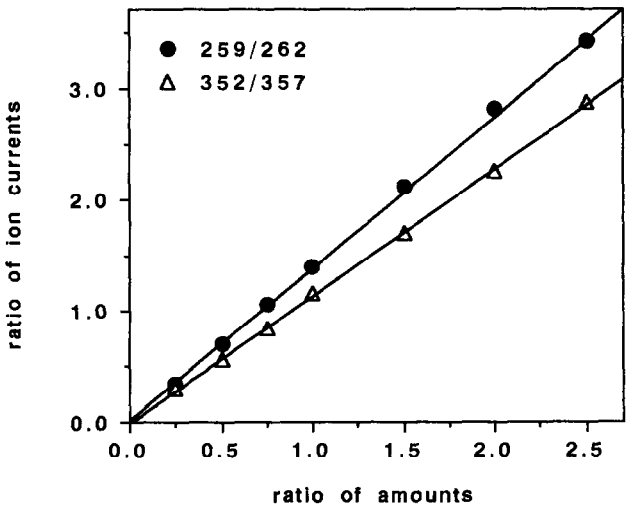

Fig. 4. Calibration plots for ratios of ion currents at indicated masses versus ratios of molar amounts of thymine glycol-$(Me_3Si)_4$ and its labeled analog (259/262), and 8-hydroxyadenine-$(Me_3Si)_3$ and its labeled analog (352/357).

The mass spectrum of xanthine-$(Me_3Si)_3$ was similar to that of guanine-$(Me_3Si)_3$ [25], except for the difference of 1 Da in the masses of typical ions because of the difference in the molecular masses by 1 Da. Masses of typical ions of xanthine-$(Me_3Si)_3$ were shifted by 2 Da in the mass spectrum of its labeled analog xanthine-$^{15}N_2$-$(Me_3Si)_3$. Xanthine has recently been detected in

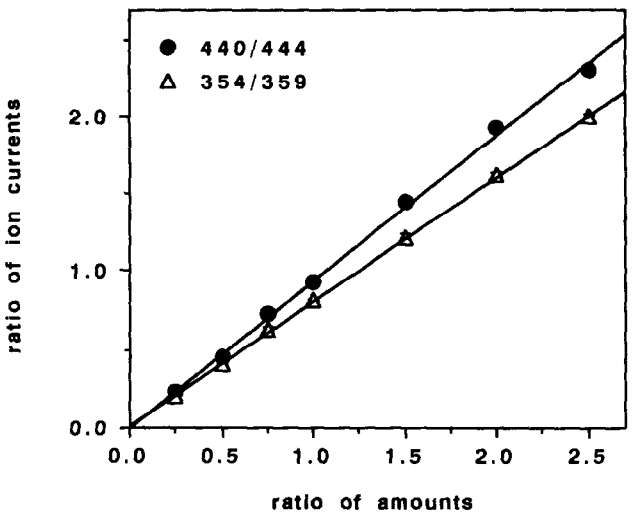

Fig. 5. Calibration plots for ratios of ion currents at indicated masses versus ratios of molar amounts of 8-hydroxyguanine-$(Me_3Si)_4$ and its labeled analog (440/444), and 4,6-diamino-5-formamidopyrimidine-$(Me_3Si)_3$ and its labeled analog (354/359).

cancerous human tissues in elevated amounts when compared with non-cancerous surrounding tissues [28]. Xanthine may result from attack of $^{\bullet}OH$ at the carbon-2 of guanine in DNA and thus it is of interest in studies of oxidative DNA damage.

Gas chromatographic retention times of the Me_3Si derivatives of modified bases were the same as those of their stable isotope-labeled analogues with the exception of thymine glycol. Co-elution of unlabeled compounds with their labeled analogues indicates no isotope effect on elution behaviors of corresponding compounds. Three examples of selected-ion current profiles are illustrated in Fig. 3. A slight resolution of thymine glycol-$(Me_3Si)_4$ from its ^2H-labeled analog by 0.02 min was observed (Fig. 3A), indicating a well known isotope effect on the elution behavior of ^2H-containing compounds.

For quantification, calibration plots must first be obtained for the response of the mass spectrometer to known quantities of both a modified base and its stable isotope-labeled analog as the internal standard [18]. For this purpose, mixtures containing known quantities of modified bases and their analogues were analyzed by GC/MS-SIM and their $M^{+\bullet}$ and $(M-15)^+$ ions were monitored. In the case of thymine glycols, *m/z* 259 and 262 ions were also recorded. The ratios of ion currents at selected masses were plotted as a function of the ratios of the molar amounts of an analyte and its analog. The ratio of molar amounts was varied up to 10-fold. Examples of calibration plots are illustrated in Figs. 4 and 5. Linear relationships of the ratio of ion currents to the ratio of quantities were obtained. Each of the data points on these plots was obtained by three independent measurements and contain standard deviations. The error bars are not discernable because standards deviations were equal to, or less than 1%. This clearly indicates the precision of these measurements by GC/MS-SIM.

The stable-isotope labeled analogues were used to demonstrate the quantification of modified DNA bases, which were formed in isolated chromatin in aqueous suspension upon exposure to ionizing radiation. Radiation doses from 7.5 to 72 Gy were used to vary the amounts of modified bases. Trimethylsilylated hydrolysates of chromatin samples were analyzed by GC/MS-SIM. Typical ions of Me_3Si derivatives of modified bases and their stable isotope-labeled analogues were recorded simultaneously during their respective time periods of elution. Quantities of the modified bases were calculated using the areas of the ion-current profiles of the monitored ions and the corresponding calibration plots. To verify the amount of DNA in each chromatin sample, thymine was also quantified in the same manner by the use of thymine-$^2H_4$ as the internal standard [21]. As examples, the dose–yield plots of some of the modified bases are illustrated in Figs. 6 and 7. Linear dose–yield relationships were obtained. At the lowest level of radiation dose (7.5 Gy), which was available in the radiation source used, significant elevations in the quantities of modified bases over control levels were observed. Dose–yield plots of modified bases were similar to those obtained under similar experimental conditions by using 6-azathymine and 8-azaadenine as internal standards [24].

In conclusion, the results obtained in the present study indicate that, as with any other class of organic compounds, isotope-dilution mass spectrometry is an ideally suited technique for selective and sensitive quantification of modified bases that result from oxidative

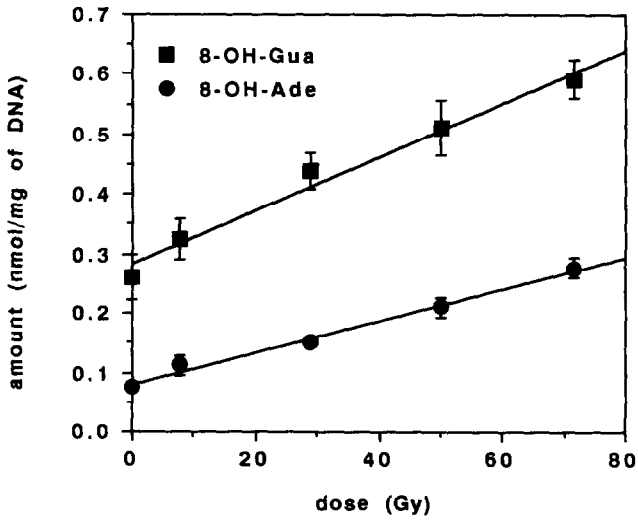

Fig. 6. Radiation dose–yield plots of 8-hydroxyadenine and 8-hydroxyguanine formed in chromatin exposed to ionizing radiation. Each data point represents the mean ± S.D. from three independent experiments.

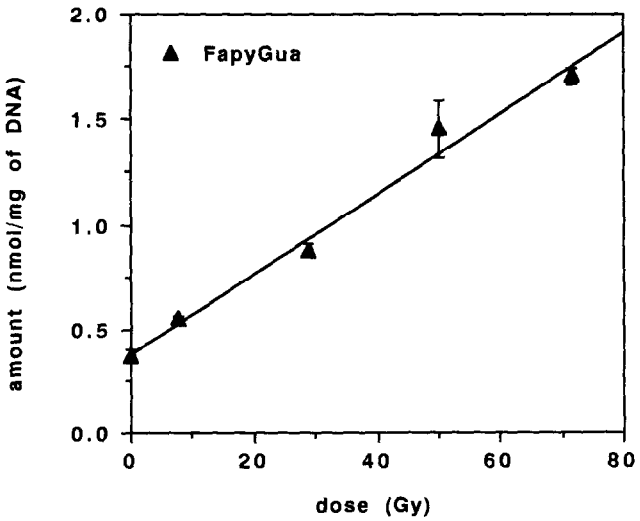

Fig. 7. Radiation dose–yield plot of 2,6-diamino-4-hydroxy-5-formamidopyrimidine formed in chromatin exposed to ionizing radiation.

damage to DNA. We have recently investigated the stability of various pyrimidine-derived and purine-derived modified bases and their release from DNA under acidic conditions of DNA hydrolysis [20]. Structurally similar compounds have been used as internal standards for this purpose. We have found that most of the bases are stable under these conditions, and only a few undergo partial destruction, the extent of which depends on the concentration of formic acid. However, reducing the acid concentration (e.g. to 60%) minimizes their destruction. Compensation can be achieved for losses even at high acid concentrations by the use of the relative molar response factors determined under the same acidic conditions [20]. The use of labeled analogues as internal standards will further improve quantification of those modified bases that undergo partial destruction under acidic conditions.

As was suggested by us previously [23,29], the GC/MS technique should be useful in chemical characterization and quantification of numerous modified DNA bases in biological fluids. The use of isotope-dilution mass spectrometry will permit highly accurate quantification of modified bases in biological fluids, since stable isotope-labeled analogues can be added to fluid samples at an early stage of sample preparation, which may involve a number of steps. Labeled analogues are expected to suffer the same type of losses during sample preparation as modified bases that may result from DNA repair in vivo and be excreted into biological fluids. Thus, possible losses not only during GC/MS analysis but also during sample preparations will be compensated resulting in accurate quantitative measurements of modified DNA bases in biological fluids.

Although the present study reports on the use of seven labeled compounds, stable isotope-labeled analogues of other modified bases such as 5-hydroxy-5-methylhydantoin, 5-hydroxyuracil (isobarbituric acid), 5-hydroxycytosine, isodialuric acid (5,6-dihydroxyuracil) and 2-hydroxyadenine, the identification of which can also be achieved by GC/MS [9,17], will be available in the near future. The quantification of those modified bases in DNA by isotope-dilution mass spectrometry will be similar to the procedures described in this study.

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