Comparison of Negative and Positive Ion Electrospray Tandem Mass Spectrometry for the Liquid Chromatography Tandem Mass Spectrometry Analysis of Oxidized Deoxynucleosides

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Oxidized deoxynucleosides are widely used as biomarkers for DNA oxidation and oxidative stress assessment. Although gas chromatography mass spectrometry is widely used for the measurement of multiple DNA lesions, this approach requires complex sample preparation contributing to possible artifactual oxidation. To address these issues, a high performance liquid chromatography (HPLC)-tandem mass spectrometric (LC-MS/MS) method was developed to measure 8-hydroxy-2'-deoxyguanosine (8-OH-dG), 8-hydroxy-2'-deoxyadenosine (8-OH-dA), 2-hydroxy-2'-deoxyadenosine (2-OH-dA), thymidine glycol (TG), and 5-hydroxymethyl-2'-deoxyuridine (HMDU) in DNA samples with fast sample preparation. In order to selectively monitor the product ions of these precursors with optimum sensitivity for use during quantitative LC-MS/MS analysis, unique and abundant fragment ions had to be identified during MS/MS with collision-induced dissociation (CID). Positive and negative ion electrospray tandem mass spectra with CID were compared for the analysis of these five oxidized deoxynucleosides. The most abundant fragment ions were usually formed by cleavage of the glycosidic bond in both positive and negative ion modes. However, in the negative ion electrospray tandem mass spectra of 8-OH-dG, 2-OH-dA, and 8-OH-dA, cleavage of two bonds within the sugar ring produced abundant S_1 type ions with loss of a neutral molecule weighing 90 u, $[M - H - 90]^-$. The signal-to-noise ratio was similar for negative and positive ion electrospray MS/MS except in the case of thymidine glycol where the signal-to-noise was 100 times greater in negative ionization mode. Therefore, negative ion electrospray tandem mass spectrometry with CID would be preferred to positive ion mode for the analysis of sets of oxidized deoxynucleosides that include thymidine glycol. Investigation of the fragmentation pathways indicated some new general rules for the fragmentation of negatively charged oxidized nucleosides. When purine nucleosides contain a hydroxyl group in the C_8 position, an S_1 type product ion will dominate the product ions due to a six-membered ring hydrogen transfer process. Finally, a new type of fragment ion formed by elimination of a neutral molecule weighing 48 (CO_2H_4) from the sugar moiety was observed for all three oxidized purine nucleosides. (J Am Soc Mass Spectrom 2000, 12, 80–87) © 2000 American Society for Mass Spectrometry

Oxidative damage of DNA bases contributes to cancer initiation, promotion, and progression as well as to the progression of degenerative diseases [1–4]. Reactive oxygen and nitrogen species are considered to be the main sources of this oxidative stress [1, 4–6]. Although not all mechanisms of how these base lesions lead to cancer and other diseases are understood, considerable circumstantial evidence has demonstrated a correlation between the incidence of DNA oxidation and various types of cancer and degenerative diseases [1, 4, 7, 8].

The standard methods for the analysis of DNA oxidation products are gas chromatography-mass spectrometry (GC-MS) [1, 9, 10] and high performance liquid chromatography (HPLC) with electrochemical detection (EC) [1, 11]. However, serious limitations or problems are associated with each of these methods [1, 12]. HPLC-EC methods can only be used to measure

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electroactive components, whereas GC-MS requires derivatization of free nucleic acid bases in order to increase their stability and volatility. HPLC coupled with electrospray ionization mass spectrometry-mass spectrometry (ESI-MS/MS) is emerging as an alternative approach for the analysis of a variety of modified nucleosides including covalent adducts [13, 14] and even 8-hydroxy-2'-deoxyguanosine (8-OH-dG) using positive ion electrospray [15, 16]. Here we report LC-MS/MS methods for the analysis of multiple DNA oxidation products using both positive and negative ion electrospray. We have chosen five important oxidized nucleosides for detailed study [17–19] out of the approximately 20 known DNA oxidation products [2]. These compounds include 8-OH-dG, 8-hydroxy-2'-deoxyadenosine (8-OH-dA), 2-hydroxy-2'-deoxyadenosine (2-OH-dA), thymidine glycol (TG), and 5-hydroxymethyl-2'-deoxyuridine (HMDU). Among these five oxidation products, 8-OH-dG has been the most widely studied biomarker of DNA damage [1]. From the comparison of positive and negative ion electrospray MS/MS, negative mode shows superior signal/noise levels. So we have developed a convenient HPLC-negative electrospray-MS/MS method for the simultaneous measurement of five oxidized deoxynucleosides from DNA samples.

Experimental

2'-Deoxyguanosine (dG), 2'-deoxyadenosine, thymidine, 8-OH-dG, and HMDU were purchased from Sigma Chemical (St. Louis, MO). 8-OH-dG [20], 2-OH-dA [21], and TG [22] were synthesized according to published methods, and their structures and purity were verified using electrospray mass spectrometry, proton-NMR and ^{13}C-NMR. [$^{13}C_{10}$, $^{15}N_5$]-8-Hydroxy-2'-deoxyguanosine (L-8-OH-dG) [23] and [$^{13}C_{10}$, $^{15}N_2$]-thymidine glycol [22] were synthesized from [$^{13}C_{10}$, $^{15}N_5$]-2'-deoxyguanosine and [$^{13}C_{10}$, $^{15}N_2$]-thymidine (Cambridge Isotope Laboratories, Andover, MA), respectively, for use as internal standards and to verify fragmentation pathways.

Salmon testes DNA samples were digested to nucleosides using enzymatic hydrolysis. For each 100 μg DNA, 33 μL water, 2 μL 10 mM desferol, and 50 μL buffer solution (50 mM ammonium acetate, 0.2 mM ZnCl$_2$, pH 5.3) were added to dissolve DNA. Then 10 μL of nuclease P1 (0.39 unit/μL) and 5 μL of alkaline phosphatase (1 unit/μL in 10 mM Tris pH 7.4) were added, and the solution was incubated at 37 °C for 30 min. A 60 μL aliquot of the DNA digest and 10 μL internal standard (L-8-OH-dG, 3.6 ppb in methanol) were combined in a 30,000 molecular weight cutoff ultracentrifuge tube (Amicon, Bedford, MA). The solution was centrifuged for 15 min at 14,000 rpm and 4 °C before LC-MS/MS analysis.

MS/MS studies were carried out by infusion of aqueous solutions of each oxidized nucleoside (~10 pM) using a syringe pump at 20 μL/min into a Micro-

mass (Manchester, UK) Quattro II triple quadrupole mass spectrometer equipped with an electrospray ionization source. Both positive and negative ion electrospray mass spectra and tandem mass spectra were recorded. Nitrogen was used as the drying and nebulizing gas at 8 and 0.8 L/min, respectively. Argon was used as the collision gas for CID at a pressure of 3×10^{-3} mtorr and a collision energy of 25 eV. The source temperature was maintained at 130 °C. Product ion scans were collected at 2 s/scan.

The HPLC system consisted of a Waters (Milford, MA) model 2690 pump, autoinjector, and model 2487 UV detector. A YMC (Wilmington, NC) AQ C$_{18}$ column (2.0 × 250 mm) and guard column (4.0 × 20 mm) were used at a flow rate of 200 μL/min. A gradient solvent system from water (solvent A) to methanol (solvent B) was used for the separation of oxidized and native nucleosides. The gradient conditions were: 0–14.5 min 6%–20% B, 14.5–28 min 20% B, and at 28.5 min 90% B. Absorbance detection at 260 nm was used for the on-line quantitation of the native nucleosides.

After the UV detector, the HPLC flow was directed to a VICI EHMA switch valve (Valco, Houston, TX), which diverted the solvent front eluting from the HPLC column to waste in order to minimize contamination of the electrospray ion source. The other outlet of the switch was connected to the electrospray source via a 40-cm capillary tube. The MS/MS conditions in the LC-MS/MS configuration are same as above except the drying and nebulizing gas flow rates were 12 and 1 L/min, and the electrospray source was maintained at 140 °C.

Results and Discussion

Comparison of Positive and Negative Product Ion Mass Spectra

Each of the five oxidized deoxynucleosides formed abundant protonated or deprotonated molecules during positive ion or negative ion electrospray, respectively. CID was used during MS/MS to fragment each protonated or deprotonated molecule, and then the product ion mass spectra were recorded. The positive and negative ion electrospray MS/MS product ion spectra for 8-OH-dG are shown in Figure 1. In positive mode, protonation of the purine ring of 8-OH-dG was probably followed by cleavage of the glycosidic bond with hydrogen transfer leading to loss of the sugar, [MH − 116]$^+$, and formation of an abundant fragment ion of m/z 168. An ion corresponding to the sugar was observed at m/z 117 but at low abundance.

Exhibiting similar signal-to-noise to the positive ion analysis, the negative ion electrospray tandem mass spectrum of 8-OH-dG is shown in Figure 1B. Although the positive ion tandem mass spectrum of 8-OH-dG has been published previously [16], the negative ion tandem mass spectrum has not been reported elsewhere to the best of our knowledge. The most abundant frag-
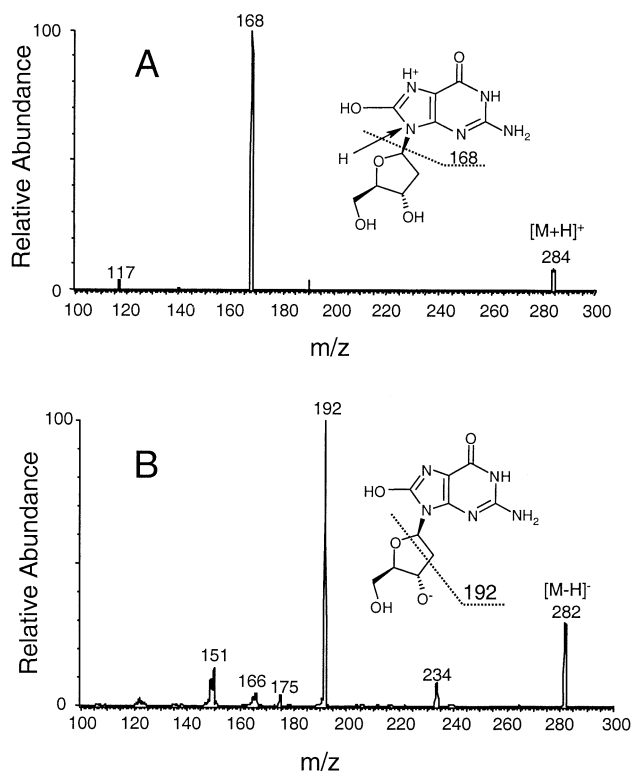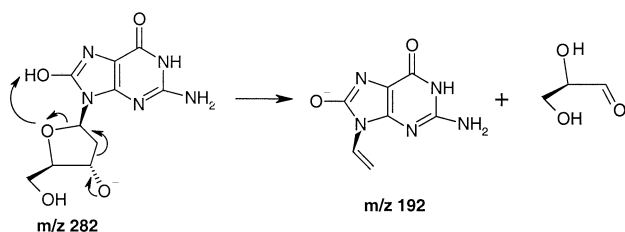
Figure 1. Comparison of (**A**) positive ion and (**B**) negative ion electrospray CID tandem mass spectra of 8-hydroxy-2'-deoxyguanosine.
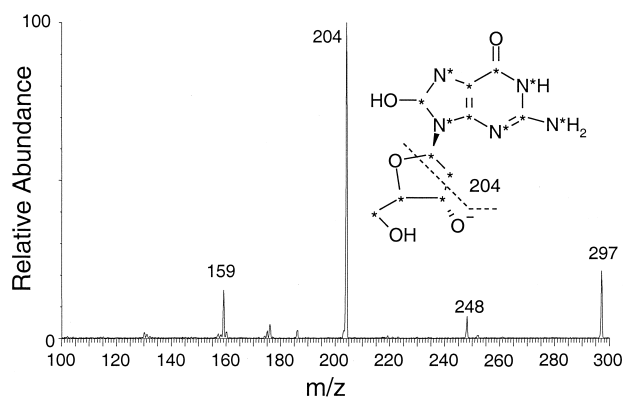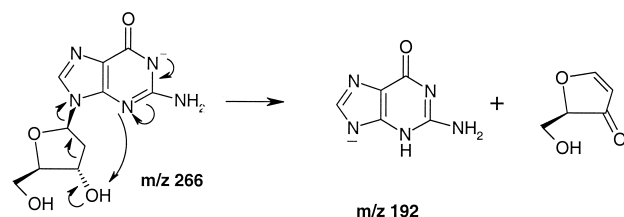

Figure 2. Negative ion electrospray CID tandem mass spectrum of $[^{13}C_{10}, {}^{15}N_5]$-8-hydroxy-2'-deoxyguanosine. Atoms labeled with heavy isotopes are marked with an asterisk.

ment ion was observed during CID resulting in loss of a group weighing 90 and formation of an ion of m/z 192. This ion was probably formed by the cleavage of C–C and C–O bonds within the sugar ring with the charge localized to the sugar as a deprotonated hydroxyl group. This pathway has been defined as S_1 type fragmentation of nucleosides and has been observed in both positive and negative modes [24, 25]. The detailed fragmentation mechanisms in positive mode were described by Wilson and McCloskey [24]. In Scheme **1**, we propose a fragmentation mechanism for nucleoside cleavage in negative mode. After deprotonation of the hydroxyl group at the C_3' position, the negative charge can migrate from the sugar to the base through an intermediate six-membered ring hydrogen transfer, which is conformationally and energetically favorable. This fragmentation mechanism was confirmed by MS/MS of the isotopically labeled compound L-8-OH-

dG, whose negative ion product ion mass spectrum of m/z 297 exhibited a base peak of m/z 204, resulting from loss of a neutral molecule containing three ^{13}C atoms from the sugar moiety (Figure 2). The fragmentation behavior of 8-OH-dG is distinctly different from unoxidized dG and many other modified purine nucleosides [25], which produced negative fast-atom bombardment (FAB) product ion mass spectra dominated by nucleic base ions (B$^-$) formed by the loss of sugar moiety. This difference may be rationalized by the lack of a hydroxyl group on the purine ring of dG which facilitated hydrogen transfer and charge migration in 8-OH-dG. Therefore in negative ion mode, dG is initially deprotonated on the purine ring at N_1 followed by hydrogen transfer without charge migration as shown in Scheme 2.

Although much less abundant, loss of 48 was observed in the negative ion product ion tandem mass spectrum of 8-OH-dG (Figure 1B) corresponding to elimination of CH_4O_2 from the sugar moiety. The ion of $[M - H - 48]^-$ was probably formed by bond cleavage between C_4' and C_5' with elimination of formaldehyde and water. This interpretation is supported by the observation of loss of 49 from L-8-OH-dG which is consistent with loss of $^{13}CH_4O_2$. Loss of 48, $[M - H - 48]^-$, was observed also in the negative ion tandem product ion mass spectra of 8-OH-dA (Figure 3) and 2-OH-dA (Figure 4). Observed using electrospray ionization, this fragmentation pathway is particularly in-

Scheme 1. Proposed fragmentation pathway of 8-OH-dG during negative ion electrospray tandem mass spectrometry with CID.
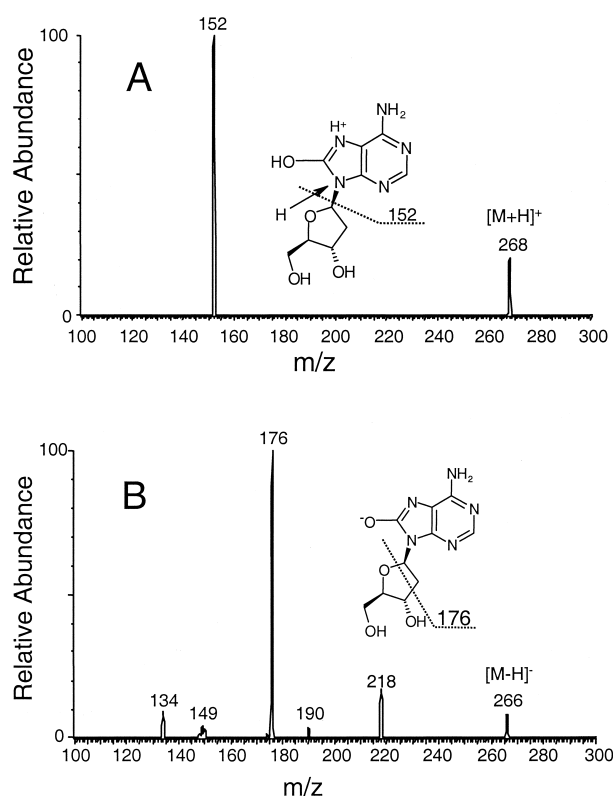

Scheme 2. Proposed fragmentation pathway of dG during negative ion electrospray tandem mass spectrometry with CID.

Figure 3. Comparison of (**A**) positive ion and (**B**) negative ion electrospray CID tandem mass spectra of 8-hydroxy-2'-deoxyadenosine.

Figure 4. (**A**) Positive ion and (**B**) negative ion electrospray CID tandem mass spectra of 2-hydroxy-2'-deoxyadenosine.

teresting because similar ions were not reported for the negative FAB tandem mass spectra of nucleosides [25].

The positive and negative ion electrospray MS/MS product ion spectra for 8-OH-dA are shown in Figure 3. The fragmentation pattern is similar to 8-OH-dG in that loss of 116 and 90 are the dominant pathways during CID after positive and negative electrospray ionization, respectively. This similarity correlates well with the structure similarity between 8-OH-dG and 8-OH-dA. Like 8-OH-dG, loss of 48 was observed during negative ion electrospray with CID and verifies that this fragmentation originates in the sugar moiety. The signal-to-noise ratio of product ions was similar in both ionization modes. Therefore, either positive or negative electrospray ionization may be utilized with equal efficiency for a quantitative MS/MS assay of 8-OH-dG and 8-OH-dA.

The positive and negative electrospray MS/MS product ion spectra of 2-OH-dA are shown in Figure 4. The signal-to-noise ratios for positive and negative ionization were similar. Like 8-OH-dA, fragmentation in positive ion electrospray (Figure 3A) with CID occurred by cleavage of the glycosidic bond and loss of 116 yielding a product ion of m/z 152. However, the peak at m/z 152 was significantly less intense for the 2-OH-dA isomer relative to 8-OH-dA. Additionally, the protonated sugar of m/z 117, which was absent in the MS/MS spectrum of 8-OH-dA, was observed at higher
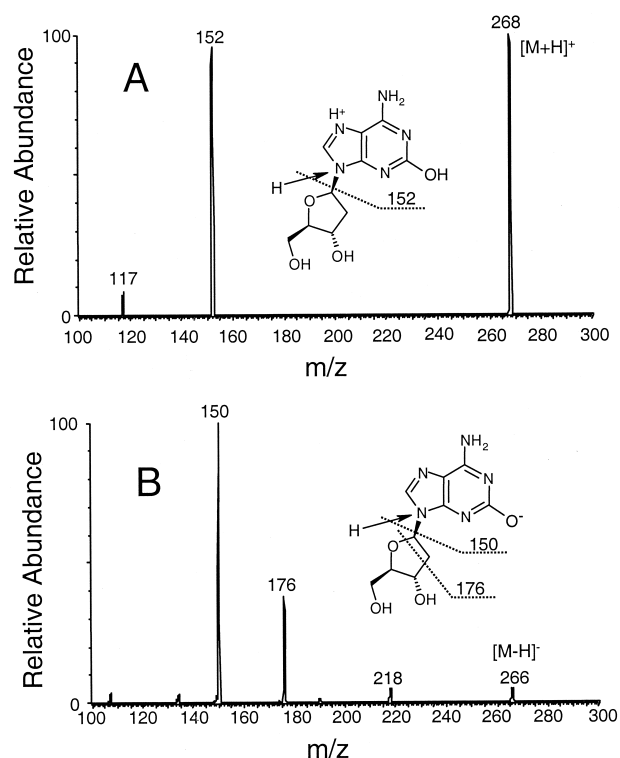
abundance than in the tandem mass spectrum of 8-OH-dG. In the negative ion mode (Figure 4B) loss of either 116 or 90 was observed forming ions of m/z 150 or 176, respectively, corresponding to cleavage of the glycosidic bond or the cleavage of C–C and C–O bonds within the sugar ring. These competing fragmentation pathways are probably similar to the pathways shown in Schemes **2** and **1**. However, the conformation of 2-OH-dA will not allow a six-member-ring hydrogen transfer as in 8-OH-dG (Scheme **1**). Instead an eight-membered ring hydrogen transfer may be possible as shown in Scheme **3** to form a product ion of m/z 176. Because this process is not as favorable as the six-membered intermediate of Scheme **1**, the base peak of m/z 150 is formed by the competing pathway. By comparing the abundances of the product ions, the free energy of each scheme may be ranked as Scheme **1** > Scheme **2** > Scheme **3**. Like 8-OH-dG and 8-OH-dA, the ion of m/z 218 corresponds to elimination of a neutral weighing 48 u.
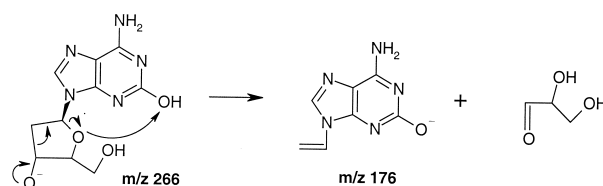
Scheme 3. Proposed fragmentation pathway of 2-OH-dA to give m/z 176 during negative ion electrospray MS/MS with CID.
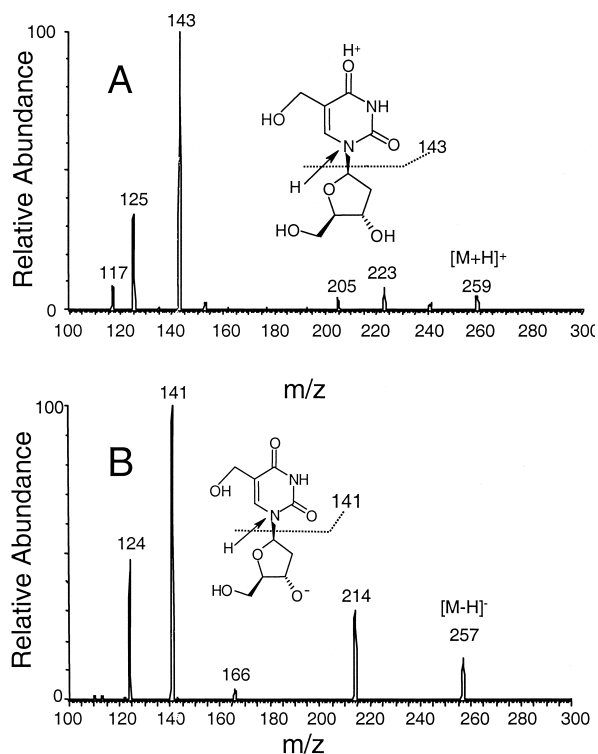
Figure 5. (**A**) Positive ion and (**B**) negative ion electrospray CID tandem mass spectra of 5-hydroxymethyl-2'-deoxyuridine.

Figure 6. Comparison of (**A**) positive ion and (**B**) negative ion electrospray CID tandem mass spectra of thymidine glycol.

The positive and negative ion electrospray tandem mass spectra of HMDU are shown in Figure 5. The most abundant fragment ions were formed by cleavage of the glycosidic bond with the positive or negative charge located on the base. Loss of the deoxyribose sugar with the transfer of a hydrogen atom to the base yielded m/z 143 and 141 in positive and negative modes, respectively. The abundance of these base ions may be attributed to resonance stability [25]. The ion of m/z 214 observed in negative mode may result from loss of O=C=NH from the deprotonated molecule. An ion corresponding to deoxyribose (m/z 117) was observed in positive mode but was absent in the negative ion tandem mass spectrum. Three sequential losses of water were observed in the positive ion tandem mass spectrum, m/z 259 → m/z 241 → m/z 223 → m/z 205, but not in negative mode. The signal-to-noise ratio of product ions was similar in both ionization modes. Therefore, either positive or negative electrospray ionization may be utilized with equal efficiency for a quantitative MS/MS assay of HMDU.

The positive and negative ion electrospray MS/MS spectra of TG are shown in Figure 6. In both cases, cleavage occurred at the glycosidic bond with the positive or negative charge residing on the sugar yielding the abundant product ions of m/z 117 and 116, respectively, in contrast to HMDU in which the base carried the charge. Also, elimination of a molecule of water was observed from the protonated or deprotonated molecule. In negative ion mode, the deprotonated
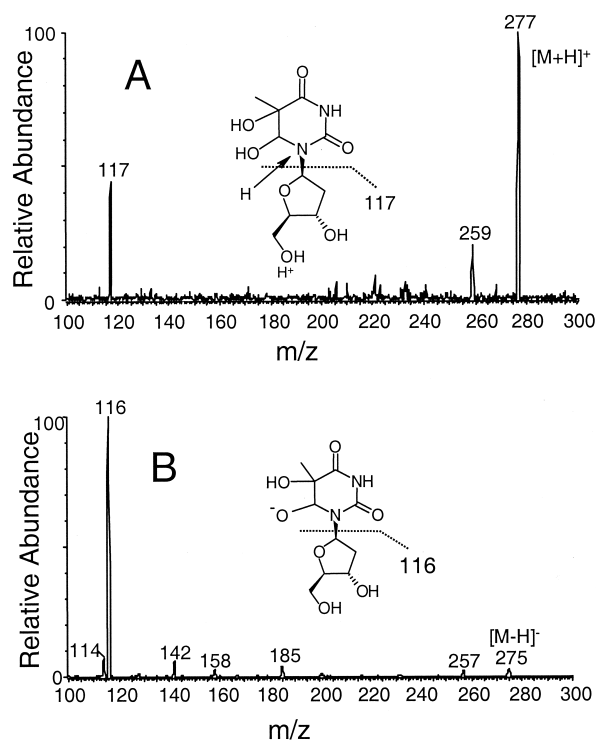
base was observed at m/z 158, however, its relative abundance was 30 times less than the ion corresponding to the deprotonated sugar. Cleavage across the sugar similar to 8-OH-dG and 8-OH-dA with the corresponding loss of 90 was also observed in negative ion mode to form an ion of m/z 185. Overall, the signal-to-noise ratio was approximately 100 times greater in the negative ion tandem mass spectrum of TG than in the positive ion spectrum. The absence of highly abundant product ions of TG in the positive ion mode is possibly due to the lack of a double bond in the TG ring, which apparently facilitated the glycosidic bond cleavage in unoxidized thymidine and HMDU. Increasing the CID energy did not improve the signal-to-noise ratio in the positive ion tandem mass spectrum. Therefore, negative ion electrospray MS/MS with CID would be preferred for the quantitative analysis of TG.

LC-UV-MS/MS Nucleoside Analysis in DNA Samples

In order to measure five different oxidized nucleosides including TG during the same LC-MS/MS analysis, negative electrospray is preferred to achieve the highest overall sensitivities. The LC-UV-MS/MS chromatograms for a standard solution containing four native deoxynucleosides and five oxidized deoxynucleosides are shown in Figure 7. All nine components were separated within 30 min.

It should be noted that dG has the same molecular weight as 8-OH-dA and 2-OH-dA (267 u) and under-
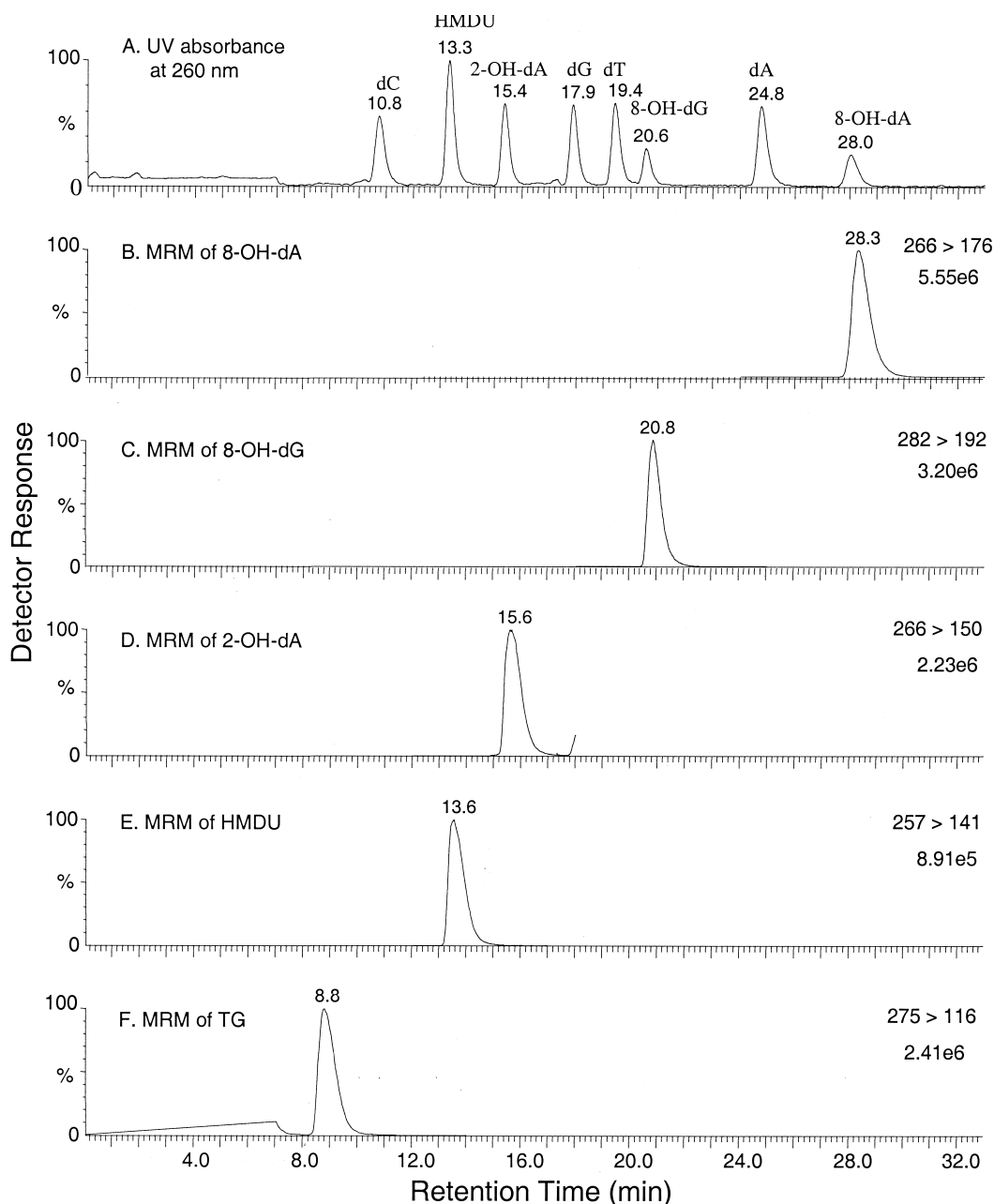
Figure 7. LC-UV-MS/MS of four native and five oxidized deoxynucleosides. The concentration of each of the nine deoxynucleosides TG, dC, HMDU, 2-OH-dA, dG, dT, 8-OH-dG, dA, and 8-OH-dA was 4.5, 6.4, 6.0, 4.1, 3.6, 4.5, 3.6, 3.5, and 3.6 μL/mL, respectively. An aliquot of 20 μL was injected onto the HPLC column.

goes similar fragmentation to 2-OH-dA, namely loss of 116 in positive mode and loss of 90 in negative mode. Fortunately, these three nucleosides are easily separated using our reversed phase chromatography conditions with 2-OH-dA eluting at 15.4 min, dG at 17.9 min, and 8-OH-dA eluting much later at 28.0 min. The extremely different chromatographic retention times for 8-OH-dA compared with 2-OH-dA and dG may be explained by differences in intramolecular hydrogen bonding. During reversed phase HPLC, 2-OH-dA elutes shortly before dG because it has one extra hy-

droxyl group making it slightly more polar than dG (Figure 7). However, isomeric 8-OH-dA elutes more than 12 min after 2-OH-dA probably because intramolecular hydrogen bonding between the 8-hydroxy group and an oxygen on the sugar mask some of its polar groups.

This method has been successfully applied to analyze five oxidized nucleosides in salmon testes DNA samples. The LC-UV-MS/MS chromatograms are shown in Figure 8, and the concentration of 8-OH-dG was determined to be 0.93 ppb by comparison with
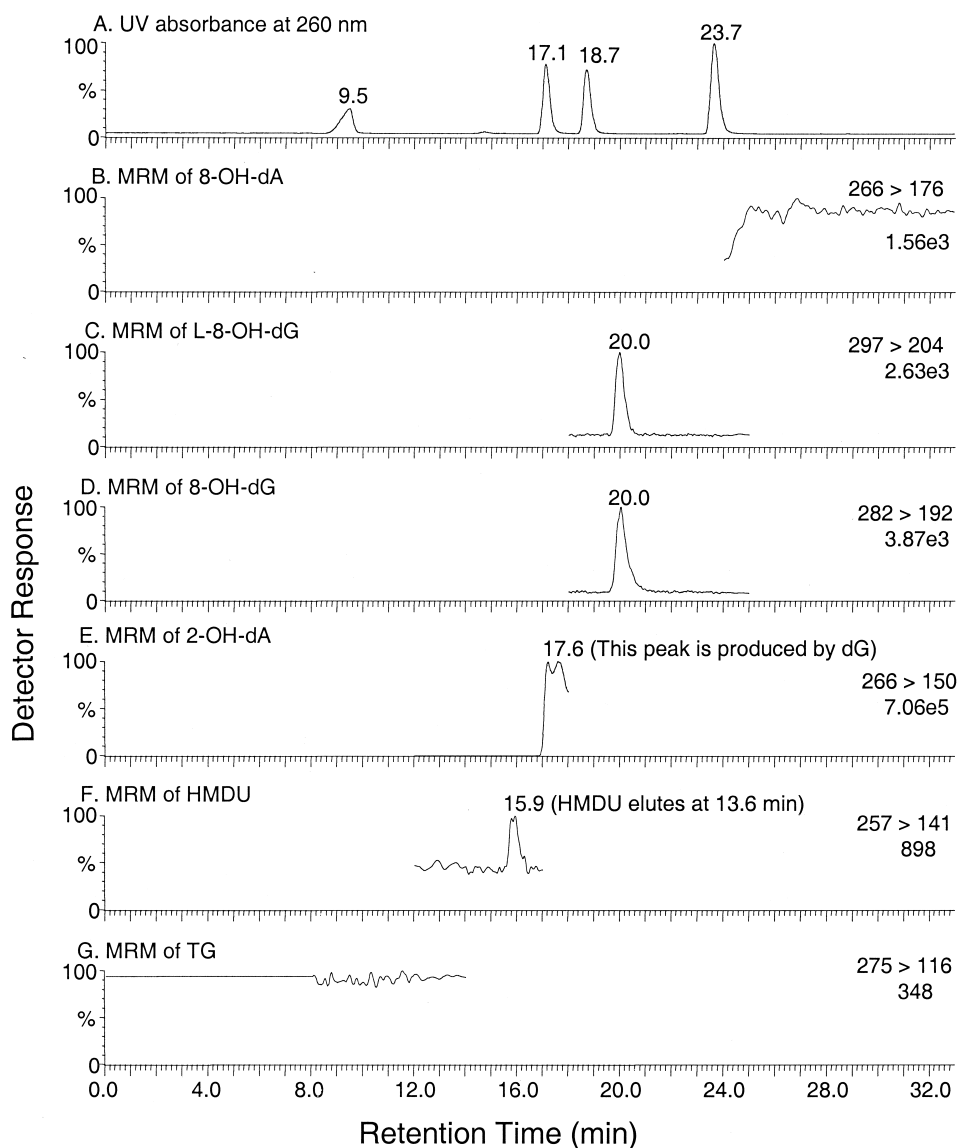
Figure 8. LC-UV-MS/MS of an enzymatically digested solution of 100 μg salmon testes DNA. An aliquot of 20 μL of 70 μL was analyzed.

labeled 8-OH-dG as internal standard using the calibration equation (Area$_{8\text{-OH-dG}}$/Area$_{L\text{-8-OH-dG}}$ = 1.79 $C_{8\text{-OH-dG}}$ − 0.01) for accurate quantitation. The degree of oxidation evaluated by 8-OH-dG/dG is calculated to be 0.89 × 10^{-5}, where the dG concentration (105 ppm) was measured using on-line UV absorbance using the calibration equation (Peak Area = 10.88C_{dG} − 102.8). Although the other four oxidized deoxynucleosides were monitored, their signals were below the detection limits of the triple quadrupole mass spectrometer used in this investigation.

Conclusions

This study describes a method using LC-UV-MS/MS for the simultaneous measurement of five DNA oxidation products. Investigation of the fragmentation mech-

anisms indicated some new general rules for the fragmentation of oxidized deoxynucleosides in negative ion mode. First, when deoxynucleosides contain purine bases with a hydroxyl group in the C$_8$ position, an S_1 type product ion will dominate the product ions due to a six-membered ring hydrogen transfer process. Second, a new type of fragment ion corresponding to loss of CO$_2$H$_4$, [M − H − 48]$^-$, from the deoxyribose moiety was observed for nucleosides containing hydroxylated purines. Finally, a comparison of negative ion electrospray with positive mode showed similar signal-to-noise for most of the compounds, but TG showed 100-fold higher signal-to-noise in negative mode. These fragmentation patterns may be used to detect and identify modified nucleoside unknowns, as well as be utilized during the development of a quantitative assay for oxidized deoxynucleosides.

Acknowledgments

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