Ultrasensitive Simultaneous Quantification of 1,N^2-Etheno-2′-deoxyguanosine and 1,N^2-Propano-2′-deoxyguanosine in DNA by an Online Liquid Chromatography−Electrospray Tandem Mass Spectrometry Assay

Camila C. M. Garcia, Florêncio P. Freitas, Paolo Di Mascio, and Marisa H. G. Medeiros*

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CEP 05508-000, São Paulo, Brazil

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Exocyclic DNA adducts produced by exogenous and endogenous compounds are emerging as potential tools to study a variety of human diseases and air pollution exposure. A highly sensitive method involving online reverse-phase high performance liquid chromatography with electrospray tandem mass spectrometry detection in the multiple reaction monitoring mode and employing stable isotope-labeled internal standards was developed for the simultaneous quantification of 1,N^2-etheno-2′-deoxyguanosine (1,N^2-εdGuo) and 1,N^2-propano-2′-deoxyguanosine (1,N^2-propanodGuo) in DNA. This methodology permits direct online quantification of 2′-deoxyguanosine and ca. 500 amol of adducts in 100 μg of hydrolyzed DNA in the same analysis. Using the newly developed technique, accurate determinations of 1,N^2-etheno-2′-deoxyguanosine and 1,N^2-propano-2′-deoxyguanosine levels in DNA extracts of human cultured cells (4.01 ± 0.32 1,N^2-εdGuo/10^8 dGuo and 3.43 ± 0.33 1,N^2-propanodGuo/10^8 dGuo) and rat tissue (liver, 2.47 ± 0.61 1,N^2-εdGuo/10^8 dGuo and 4.61 ± 0.69 1,N^2-propanodGuo/10^8 dGuo; brain, 2.96 ± 1.43 1,N^2-εdGuo/10^8 dGuo and 5.66 ± 3.70 1,N^2-propanodGuo/10^8 dGuo; and lung, 0.87 ± 0.34 1,N^2-εdGuo/10^8 dGuo and 2.25 ± 1.72 1,N^2-propanodGuo/10^8 dGuo) were performed. The method described herein can be used to study the biological significance of exocyclic DNA adducts through the quantification of different adducts in humans and experimental animals with pathological conditions and after air pollution exposure.

Introduction

Exocyclic DNA adducts arise from the reaction of DNA with lipid peroxidation products, such as malonaldehyde, 4-hydroxy-2(*E*)-nonenal, 4-oxo-2(*E*)-nonenal, 2,4-decadienal, acrolein, and crotonaldehyde (CRO[1]) (*1, 2*). The direct addition of α,β-unsaturated aldehydes to DNA bases yields cyclic substituted propano adducts, such as 1,N^2-propano-2′-deoxyguanosine (*3, 4*). Alternatively, α,β-unsaturated aldehydes can be oxidized to reactive epoxides, giving ethano or etheno derivatives upon reaction with DNA (*5, 6*). Some of these DNA lesions have been shown to be highly mutagenic (*7*−*9*) and are considered to be possible pathways leading to the carcinogenic effects involved in the lipid peroxidation process (*10*).

CRO is an important industrial chemical and an environmental pollutant formed by the combustion of plant materials, including tobacco, and mobile source emissions. Additionally, it is generated endogenously by the oxidative degradation of unsaturated lipids and as a metabolite of *N*-nitrosopyrrolidine (*11, 12*). CRO is mutagenic and carcinogenic (*13, 14*); its reaction with dGuo in DNA leads to the formation of the (6*S*,8*S*) and (6*R*,8*R*) diastereomers of 1,N^2-propano-2′-deoxyguanosine adducts (*15, 16*). These adducts can also be formed by the reaction of acetaldehyde (AA) with dGuo (*17, 18*). AA is present in tobacco smoke, vehicle exhaust due to alcohol fuel combustion, and some beverages and foods. It is endogenously formed by the metabolic oxidation of ethanol through hepatic NAD-dependent alcohol dehydrogenases in the liver and during threonine catabolism (*19*−*21*). The production of significant amounts of acetaldehyde in saliva was observed after the ingestion of moderate amounts of ethanol (*22*). AA reacts with dGuo in DNA, first forming N^2-ethylidene-dGuo (*18*). The subsequent reaction of another molecule of AA with N^2-ethylidene-dGuo gives rise to 1,N^2-propano-2′-deoxyguanosine (Scheme 1) in both the (6*S*,8*S*) and (6*R*,8*R*) configurations. The reaction between acetaldehyde and dGuo favors the formation of the (8*R*,6*R*) diastereomer, while the reaction involving CRO promotes (6*S*,8*S*) formation (*18*). In addition, AA-1,N^2-propano-2′-deoxyguanosine adduct formation is stimulated by polyamines and histones (*23, 24*). In DNA, the 1,N^2-propano-2′-deoxyguanosine adduct exists in an equilibrium between the closed and opened forms because the opened form is more favored in double-stranded DNA, and the closed-form is predominant in single-stranded DNA (*25*).

1,N^2-PropanodGuo promotes DNA miscoding in human cells, principally through G → T transversions, and can inhibit DNA synthesis (*8, 26*). The ring-opened form can lead to an interstrand cross-link when the adduct is formed in a 5′ CpG sequence (*27*). Cross-links involving DNA and proteins can also occur as shown by Kurtz and Lloyd after the reaction between

* To whon correspondence should be addressed. Tel: ++ (55) 11 30912153. Fax: ++ (55) 11 30912186. E-mail: mhgdmede@iq.usp.br.

[1] Abbreviations: CRO, crotonaldehyde; AA, acetaldehyde; 1,N^2-εdGuo, 1,N^2-etheno-2′-deoxyguanosine; 1,N^2-propanodGuo, 1,N^2-propano-2′-deoxyguanosine; dGuo, 2′-deoxyguanosine; HPLC/ESI/MS-MS, high performance liquid chromatography/electrospray ionization tandem mass spectrometry; MRM, multiple reaction monitoring.

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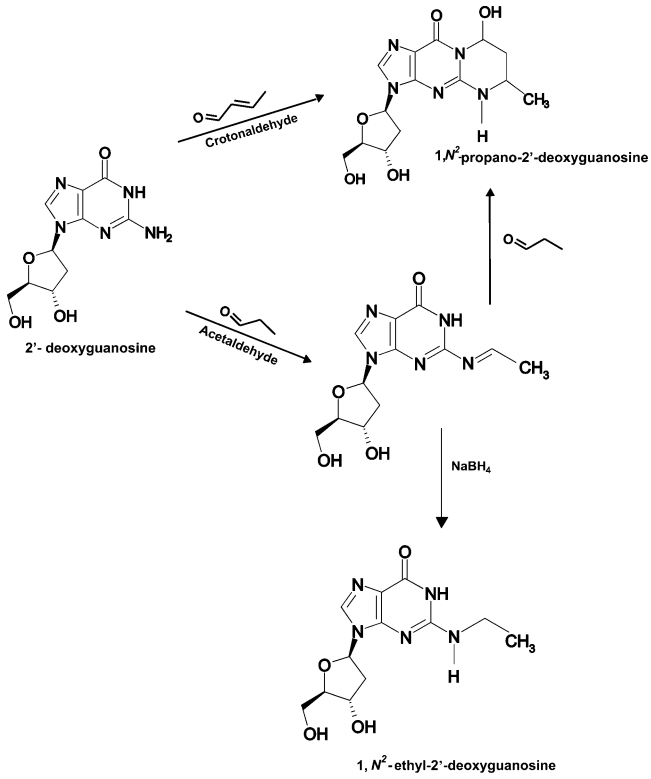
Scheme 1. Formation of 1,N^2-PropanodGuo in the Reaction of Acetaldehyde or Crotonaldehyde with dGuo



the free aldehyde group of the adduct ring-opened form and peptides (*28*).

Etheno adducts have been used as biomarkers for DNA damage resulting from the reactions of endogenous lipid peroxidation end products (*29*). 1,N^2-Etheno-2′-deoxyguanosine (1,N^2-εdGuo) can be formed by the reaction between 2′-deoxyguanosine and reactive aldehydes, such as chloroacetaldehyde, 2,3-epoxyaldehydes and vinyl chloride, that are derived from oxidized lipids, sugars, or amino acids (*6, 30, 31*).The biological relevance of the occurrence of 1,N^2-εdGuo has been revealed by studies showing the mutagenicity of this etheno adduct both in *Escherichia coli* and in human cells (*7, 9, 32, 33*). The presence of 1,N^2-εdGuo leads to misincorporation and mutations in *in vitro* systems involving DNA polymerases in bacteria and mammalian cells (*7, 32, 33*). Recently, it was reported that the human DNA polymerase κ produces frame shift deletions when bypassing a 1,N^2-εdGuo adduct in DNA (*9*).

Today, exocyclic DNA adducts are emerging as new tools in the study of oxidative stress-related diseases and cancer etiology. Several methods, including ^{32}P-postlabeling, competitive immunoassay, gas chromatography/electron capture negative chemical ionization high-resolution mass spectrometry (GC/ECNCI-HRMS), and high performance liquid chromatography/tandem mass spectrometry (HPLC/ESI/MS-MS), have been employed to quantify DNA adducts and have been previously reviewed (*1, 2, 10*).

This work describes the development and validation of a sensitive method for the simultaneous quantitative determination of 1,N^2-εdGuo and 1,N^2-propanodGuo from *in vitro* and *in vivo* samples using online reverse-phase HPLC separation with tandem mass spectrometry detection by multiple reaction monitoring (MRM). In addition, the technique permits the detection and quantification of dGuo from crude or extracted DNA in the same analysis. This methodology enabled us to determine the basal levels of 1,N^2-εdGuo and, 1,N^2-propanodGuo in cultured human lung fibroblasts (IMR-90 cells) and in liver, lung, and brain tissues from male rats.

Experimental Procedures

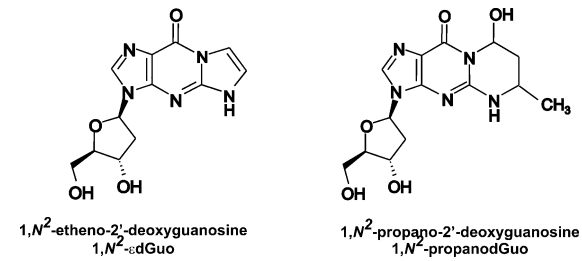
Chemicals. All of the chemicals employed were of the highest purity grade commercially available. [$^{15}N_5$]-2′-Deoxyguanosine was provided by Cambridge Isotope Laboratories (Andover, MA). Chromatography grade acetonitrile, 2-propanol, and ethanol were obtained from Merck (Darmstadt, Germany). Fetal calf serum was acquired from Atena Biotecnologia (Campinas, SP, Brazil). 2′-Deoxyguanosine (dGuo), formic acid, sodium hydroxide, potassium phosphate, magnesium sulfate, saccharose, magnesium chloride, acetaldehyde, 2-chloroacetaldehyde, and all the other chemicals used were from Sigma (St. Louis, MO). Water was purified using a Milli-Q system (Millipore, Bedford, MA).

DNA Modified *in Vivo*. Twenty-week-old untreated male Wistar rats ($n = 21$) were sacrificed by using a guillotine. Their livers, lungs, and brains were removed, snap-frozen in liquid nitrogen, and stored at −80 °C.

Cell Culture. Human lung fibroblasts (line IMR-90) were routinely grown in Dulbecco's modified Eagle's medium (DMEM, pH 7.4) supplemented with 10% (v/v) fetal calf serum, 3.7 g/L sodium bicarbonate, 0.04 g/L penicillin, and 0.1 g/L streptomycin sulfate. The cells were incubated in a CO_2/air atmosphere (1:19) at 37 °C and were allowed to grow until confluence. DNA was extracted, hydrolyzed, and analyzed as described below.

DNA Extraction. DNA was isolated by the modified chaotropic NaI method, as previously described (*34*). Briefly, the tissues (500 mg) or the cellular pellets (3×10^8 cells) were homogenized in 10 mL of a lysis solution (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, 0.1 mM desferroxamine, and 1% (v/v) Triton X-100 at pH 7.5). After centrifugation at 1500*g* for 10 min, the pellets were resuspended in 10 mL of the lysis solution and centrifuged one more time at 1500*g* for 10 min. The pellets were then suspended in 6 mL of 10 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, 0.15 mM desferroxamine, and 350 μL of 10% SDS. The enzymes RNase A (30 μL, 10 mg/mL) and RNase T1 (4 μL, 20 U/μL) in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 2.5 mM desferroxamine were added, and the reaction mixture was incubated at 37 °C. After 1 h, 300 μL of proteinase K (20 mg/mL) was added, and the reaction was incubated at 37 °C for 1 h. After centrifugation at 5000*g* for 15 min, the liquid phase was collected, 1 mL of a solution containing 7.6 M NaI, 40 mM Tris-HCl (pH 8), 20 mM EDTA, and 0.3 mM desferroxamine was added, and then 5 mL of isopropanol was added. The contents of the tube were mixed well by inversion until a white precipitate appeared. The precipitate was collected by centrifugation at 5000*g* for 15 min, washed with 5 mL of 60% isopropanol, centrifuged at 5000*g* for 15 min, washed with an additional 5 mL of 70% ethanol, and centrifuged at 5000*g* for 15 min. The DNA pellet was solubilized in 500 μL of desferroxamine (0.1 mM). The DNA concentration was measured spectrophotometrically at 260 nm.

Scheme 2. Structure of DNA Adducts, 1,N^2-PropanodGuo and 1,N^2-εdGuo



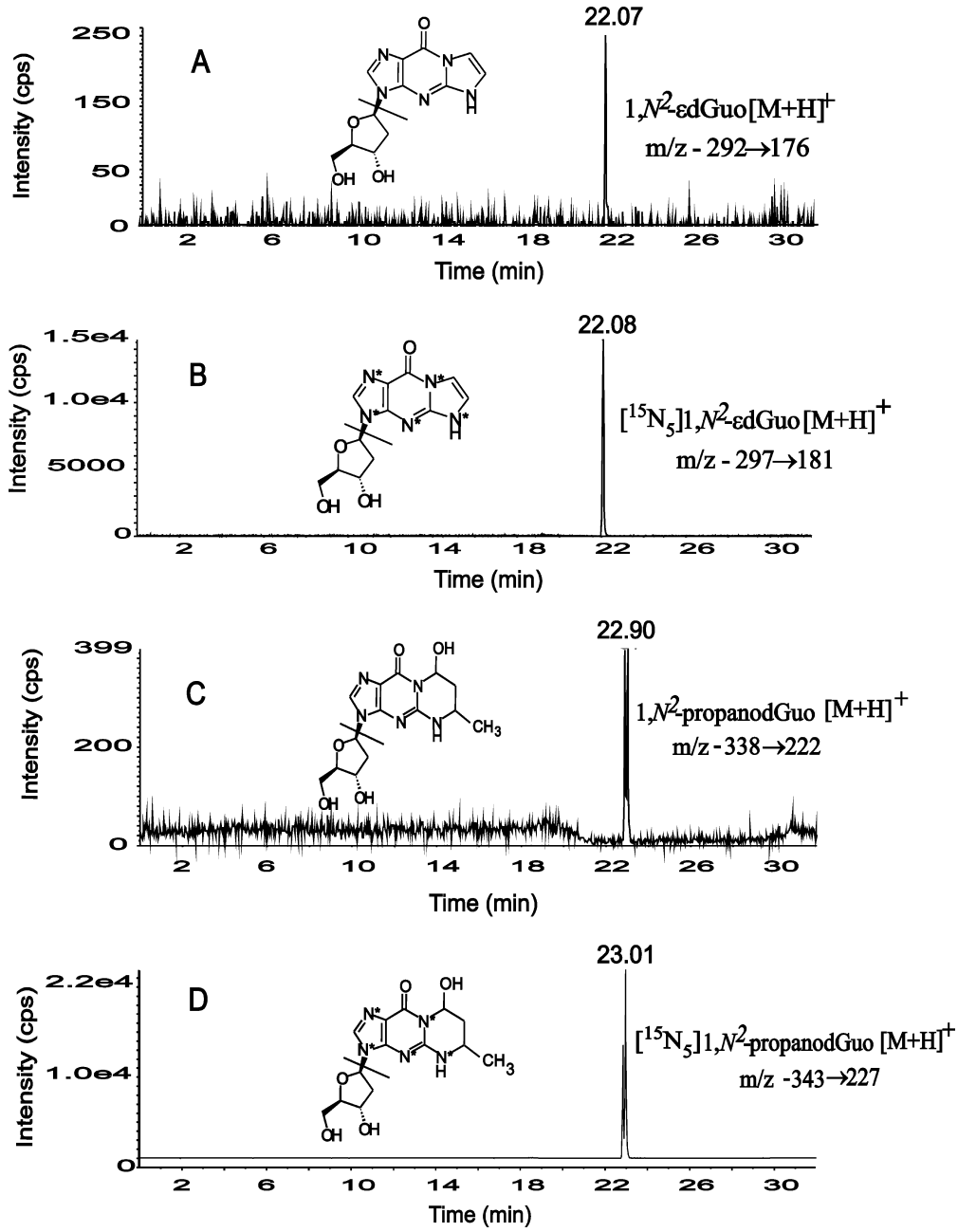


Figure 1. Detection of a 500 amol of DNA adducts standard by HPLC/ESI/MS-MS analysis in the MRM mode. (A) 1,N^2-εdGuo: *m/z* 292 → 176. (B) [$^{15}N_5$]-1,N^2-εdGuo: *m/z* 297 → 181. (C) 1,N^2-propanodGuo: *m/z* 338 → 222. (D) [$^{15}N_5$]-1,N^2-propanodGuo: *m/z* 343 → 227.

Enzymatic Hydrolysis of DNA. Sodium acetate buffer (1 M, pH 5, 4 μL) and 33 fmol of [$^{15}N_5$]-1,N^2-εdGuo were added to an aliquot of a 0.1 mM desferroxamine solution containing 200 μg of DNA. The DNA was then digested with 2 units of nuclease P1 at 37 °C for 30 min. Tris-HCl buffer (1 M, pH 7.4, 8 μL), 8 μL of phosphatase buffer, and 6 units of alkaline phosphatase were then added for an additional 1 h of incubation at 37 °C. The final volume of the solution was adjusted to 200 μL with water before the second incubation. The enzymes were precipitated by centrifugation at 5000*g* for 3 min, and the resulting aqueous layer was subjected to HPLC/ESI/MS-MS analysis (100 μL of the DNA solution/injection). The amounts of the reagents and labeled internal standards were proportionally adjusted for hydrolysis and analysis of other DNA quantities.

Synthesis of the 1,N^2-Etheno-2′-deoxyguanosine Unlabeled Standard. The 1,N^2-εdGuo unlabeled standard was obtained by reacting dGuo with chloroacetaldehyde with subsequent purification by HPLC, as described by Loureiro et al. *(35)*. The identity of the compound was confirmed by ESI/MS and ^{1}H NMR.

Synthesis of the [$^{15}N_5$]-1,N^2-Etheno-2′-deoxyguanosine Internal Standard. [$^{15}N_5$]-1,N^2-εdGuo was obtained by reacting [$^{15}N_5$]-dGuo with chloroacetaldehyde with subsequent purification by HPLC, as described by Loureiro et al. *(36)*. The identity of the [$^{15}N_5$]-adduct was confirmed by mass spectrometry analysis.

Synthesis of the 1,N^2-Propano-2′-deoxyguanosine (6*R*,8*R*) and (6*S*,8*S*) Unlabeled Standards. dGuo (25 μmol) was dissolved in 2 mL of phosphate buffer (50 mM, pH 7.5) containing 1 mmol of AA and 0.05 mmol of lysine (as a catalyst for adduct formation). The solution was mixed at 500 rpm at 37 °C for 12 h. The adducts were purified by HPLC (Shimadzu, Kyoto, Japan) with a Luna C18(2) analytical column (250 mm ×4.6 mm i.d., 5 μm, Phenomenex, Torrance, CA). The following water/acetonitrile gradient method was used: from 0 to 30 min, 0 to 8% acetonitrile and 0.8 to 0.5 mL/min; from 30 to 50 min, 8 to 15% acetonitrile and 0.5 to 0.6 mL/min; from 50 to 60 min, 15 to 50% acetonitrile, 0.6 to 0.8 mL/min; from 60 to 65 min, 50 to 0% acetonitrile and 0.8 mL/min; and from 65 to 70 min, 0% acetonitrile and 0.8 mL/min.

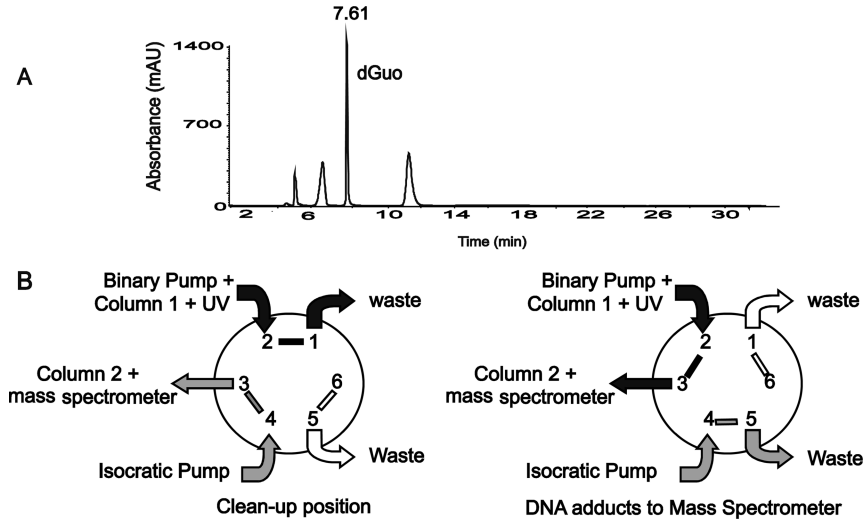


Figure 2. (A) Enzymatically hydrolyzed DNA; $\lambda = 260$ nm. Conditions were as described in the Experimental Procedures section. (B) Positions of the automated switching valve during the HPLC/ESI/MS-MS analysis, cleaning and mass spectrometer positions.

The identities of the two diastereomeric products were confirmed by the following spectroscopic features: UV λ_{max} 260 nm, $\varepsilon = 15600$ M^{-1} cm^{-1} (6*S*,8*S*) and $\varepsilon = 15700$ M^{-1} cm^{-1} (6*R*,8*R*) (SPD-E10A/VP Shimadzu). ESI/MS: *m*/*z* 338 ([M + H − 2-D-*erythro*-pentose]$^+$), 222 ([M + H]$^+$). ^{1}H, ^{1}H-^{1}H COSY NMR (D_2O) (Bruker DRX 500 MHz). Circular dichroism was recorded in water on a JASCO J-720 spectropolarimeter (Figures 1 and 2, Supporting Information).

Synthesis of the [$^{15}N_5$]-1,N^2-Propano-2′-deoxyguanosine Internal Standard. [$^{15}N_5$]-1,N^2-propanodGuo was obtained by reacting [$^{15}N_5$]-dGuo with AA with subsequent purification by HPLC, as described above. The identity of the [$^{15}N_5$]-adduct was confirmed by mass spectrometry analysis. ESI/MS for [$^{15}N_5$]-1,N^2-propanodGuo: *m*/*z* 343 ([M + H − 2-D-*erythro*-pentose]$^+$), 227 ([M + H]$^+$).

High-Performance Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry (HPLC/ESI/MS-MS). Online HPLC/ESI/MS-MS analyses were carried out in the positive mode using an API-4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA). The 1,N^2-εdGuo and 1,N^2-propanodGuo adducts in the DNA samples were detected by multiple reaction monitoring (MRM). An Agilent HPLC system (Kyoto, Japan) consisting of an autosampler (1200 High performance), a column oven at 18 °C (1200 G1216B), an automated switching valve, a 1200 Binary Pump SL, a 1200 Isocratic Pump SL, and a UV detector (1200 DAD G1315C) were used for sample injection and cleanup of the analytical column (Luna C18(2), 250 mm ×4.6 mm i.d., 5 μm, Phenomenex, Torrance, CA). The adduct was eluted from this column with a gradient of water and acetonitrile containing 0.1% formic acid with the following method: from 0 to 10 min, 10 to 40% acetonitrile and 0.65 to 0.2 mL/min; from 10 to 16 min, 40 to 30% acetonitrile and 0.2 mL/min; from 16 to 20 min, 30 to 60% acetonitrile and 0.2 mL/min; from 20 to 21 min, 60 to 40% acetonitrile and 0.2 mL/min; from 21 to 22 min, 40 to 90% acetonitrile and 0.2 to 0.65 mL/min; from 22 to 26 min, 90% acetonitrile and 0.65 mL/min; from 26 to 27 min, 90 to 10% acetonitrile and 0.65 mL/min; from 27 to 32 min, 10% acetonitrile and 0.65 mL/min.

An isocratic pump was used to simultaneously load a second column (Eclipse XDB-C18, 150 mm × 4.6 mm i.d., 5 μm, Agilent) at 0.2 mL/min with a solution of 60:40 water/acetonitrile with 0.1% formic acid and maintained a constant flow of the mobile phase to the mass spectrometer during the analysis. The position of the switching valve was changed twice: at 13 min to allow the eluent from the first column to enter the second column and at 25 min to permit the first column to be washed while the adduct was eluted through the second column to the mass spectrometer. The total time spent on this analysis was 32 min.

The DNA hydrolysates containing 33 fmol of the [$^{15}N_5$]-1,N^2-εdGuo and [$^{15}N_5$]-1,N^2-propanodGuo internal standards were injected into the system described above. The [M + H]$^+$ ions corresponding to the *m*/*z* values 292/176 (1,N^2-εdGuo), 297/181 ([$^{15}N_5$]-1,N^2-εdGuo) and 338/222 (1,N^2-propanodGuo), 343/227([$^{15}N_5$]-1,N^2-propanodGuo) were monitored with a dwell time of 200 ms.

All of the parameters of the mass spectrometer were adjusted for acquisition of the best [M + H]$^+$/[M + H − 2-D-*erythro*-pentose]$^+$ transition. The curtain gas was adjusted to 25 psi, the source temperature was held at 450 °C, the nebulizer and auxiliary gas were maintained at 60 psi, the Turbo Ion Spray voltage was 5500 V, the collision gas was set on high, the interface heater was held at 100 °C, and the entrance potential was set to 10 V. For the 338/222 and 343/227 transitions, the following were selected: collision energy, 19 V; collision cell exit, 20 V; and declastering potential, 51 V. For the 292/176 and 297/181 transitions were selected 17 V of collision energy, 16 V of collision cell exit, and 41 V of declastering potential. The data were processed using Analyst 1.4.2 software.

Results

The online HPLC/ESI/MS-MS methodology reported here permits direct quantification of two DNA adducts, 1,N^2-propanodGuo and 1,N^2-εdGuo (Scheme 2), formed from the reaction of dGuo with exogenous or endogenous reactive aldehydes. Figure 1 shows a representative chromatogram of 1,N^2-εdGuo and 1,N^2-propanodGuo (Figure 1A and C). The use of the stable isotopic internal standards [$^{15}N_5$]-1,N^2-εdGuo and [$^{15}N_5$]-1,N^2-propanodGuo, five units of mass larger than the native adducts, ensures accurate quantification of the adducts (Figure 1B and D). The internal standards were synthesized as described in the Experimental Procedures section, characterized by UV and ESI-MS/MS and quantified with the UV extinction coefficients of the unlabeled adducts. The stable isotopic internal standards (33 fmol) were added to the DNA samples prior to enzymatic hydrolysis. The HPLC program developed herein allowed for adequate separation of the adducts from the last eluted nucleoside (dThd), as shown in Figure 2A. The method also allowed the simultaneous quantification of dGuo from hydrolyzed DNA samples

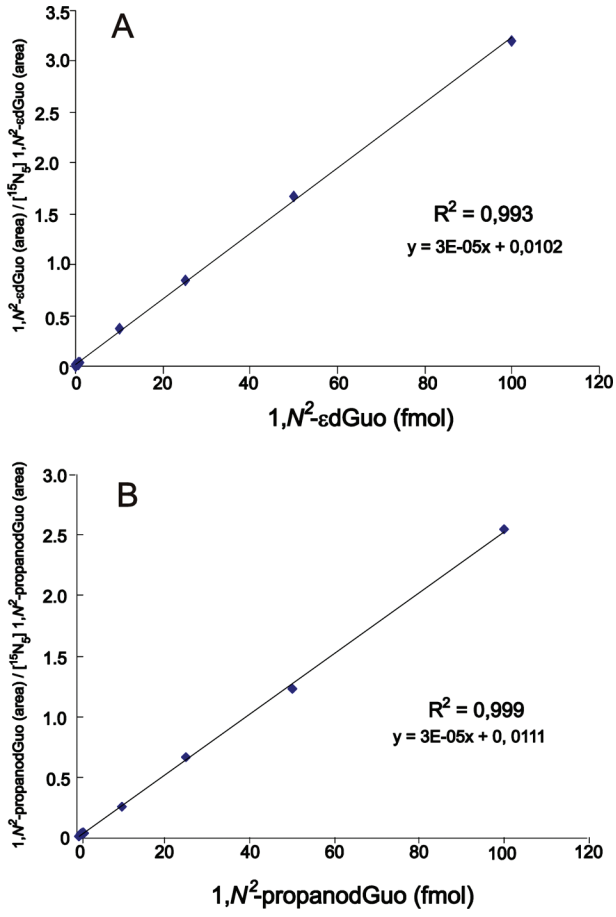


Figure 3. HPLC/ESI/MS-MS calibration curve for $1,N^2$-etheno-2′-deoxyguanosine (A) and $1,N^2$-propano-2′-deoxyguanosine (B), obtained by plotting the relative area ratios of unlabeled adducts to the isotopically $^{15}N_5$-labeled adduct vs increasing amounts of $1,N^2$-etheno-2′-deoxyguanosine and $1,N^2$-propano-2′-deoxyguanosine. Conditions were as described in the Experimental Procedures section.

through absorbance measurements (λ = 260 nm) in the UV detector (Figure 2A). An automated switching valve (Figure 2B) was programmed to change its position in the interval between the end of the elution of the last DNA base and the beginning of the elution of the first DNA adduct ($1,N^2$-εdGuo). The automated switching valve directed the unmodified nucleotides, after elution through column 1 and UV detection, to the waste (cleaning time 13 min), preventing the loss of mass spectrometer sensitivity. The adducts were then eluted through a second narrow bore column at a low flow rate to concentrate the adduct into a sharp peak, increasing the sensitivity of mass spectrometer detection in MRM mode. The *m*/*z* transition chosen for the MRM detection experiments corresponds to the [M + H − 2-D-*erythro*-pentose]$^+$ ion of the adducts, a predominant fragmentation product characterized by the loss of 116 mass units from MH$^+$. Representative ion chromatograms obtained by MRM detection using the *m*/*z* transitions from 292 to 176 ($1,N^2$-εdGuo), from 297 to 181 ([$^{15}N_5$]-$1,N^2$-εdGuo), from 338 to 222 ($1,N^2$-propanodGuo), and from 343 to 227 ([$^{15}N_5$]-$1,N^2$-propanodGuo) with samples containing 500 amol of the unlabeled adducts and 33 fmol of the stable isotopic internal standards are shown in Figure 1A−D.

The calibration curves shown in Figure 3 were obtained from 14 different concentration points. The chromatographic peak area ratios of $1,N^2$-εdGuo to [$^{15}N_5$]-$1,N^2$-εdGuo (Figure 3A, r^2 = 0.993) and $1,N^2$-propanodGuo to [$^{15}N_5$]-$1,N^2$-propanodGuo (Figure 3B, r^2 = 0.999) from each injection were plotted against the amount (in fmol) of the unlabeled adduct injected (0.01−100 fmol/injection). The amount of the [$^{15}N_5$]-labeled internal standard that was injected was kept constant at 33 fmol. Blank injections (water) under the same conditions revealed that no carryover of adducts from previous analyses occurred (data not shown).

The limit of detection and the possible suppressor effect of the excess amount of normal DNA bases on the response of the adduct were evaluated using hydrolyzed calf thymus DNA spiked with different quantities (0 to 100 fmol) of the unlabeled DNA adduct standards and 33 fmol of the [$^{15}N_5$] DNA adducts. All of the standards were added prior to DNA hydrolysis. The limit of detection of both adducts (S/N = 3 ± 0.2) was 150 amol on the column, and the limit of quantification (S/N = 10 ± 0.3) was 500 amol on the column, corresponding to approximately 9.5 adducts/10^9 dGuo in 100 μg of hydrolyzed DNA. The basal levels present in the calf thymus DNA were minimized from all DNA samples before quantification and validation. Figure 4 shows a representative chromatogram of calf thymus DNA contaminated with 500 amol of adduct standards, corresponding to an S/N value of approximately 10, the limit of quantification for each adduct.

The accuracy and precision of the method were determined by analysis of nine different amounts of hydrolyzed calf thymus DNA contaminated with 1 fmol of $1,N^2$-εdGuo, 1 fmol of each diastereomer of the $1,N^2$-propanodGuo standards, and 33 fmol of each internal standard. These injections were performed on three consecutive days with three injections per day. Table 1 summarizes the quantification of the adducts and the coefficients of variance of the analysis within the same day and on subsequent days. The values accurately reflect the known additional amounts of adduct. On the first occasion, the $1,N^2$-εdGuo and $1,N^2$-propanodGuo levels were found to be 0.95 ± 0.07 fmol or 0.52 ± 0.03 adducts/10^8 dGuo and 2.06 ± 0.10 fmol or 1.14 ± 0.05 adducts/10^8 dGuo, respectively. On the second occasion, the measured levels were 0.99 ± 0.04 fmol or 0.52 ± 0.04 adducts/10^8 dGuo and 2.07 ± 0.11 fmol or 1.08 ± 0.07 adducts/10^8 dGuo, respectively, and on the third occasion, they were 1.04 ± 0.02 fmol or 0.58 ± 0.01 adducts/10^8 dGuo and 2.08 ± 0.1 fmol or 1.14 ± 0.03 adducts/10^8 dGuo, respectively (Table 1). The chromatograms (Figure 5) show one of the DNA samples that contained 1 fmol of unlabeled adduct. The S/N ratio of the unlabeled adducts shown in Figure 5 is approximately two times higher than the S/N ratio obtained with 500 amol of the unlabeled standards (Figure 4), confirming the linearity in adduct quantification and the fact that larger hydrolyzed DNA samples did not cause suppression of the adduct response.

The newly developed methodology was applied to the quantification of the basal levels of $1,N^2$-εdGuo and $1,N^2$-propanodGuo in cultured IMR-90 cells and in the liver, brain, and lung tissues from untreated male Wistar rats. Analysis of DNA extracted from the cultured cells (n = 7 trials) confirmed the presence of basal levels of 4.01 ± 0.32 $1,N^2$-εdGuo/10^8 dGuo and 3.43 ± 0.33 $1,N^2$-propanodGuo/10^8 dGuo (data not shown).

Table 2 reports the quantification and SD of basal levels of $1,N^2$-εdGuo and $1,N^2$-propanodGuo in the liver (2.47 ± 0.61 $1,N^2$-εdGuo/10^8 dGuo and 4.61 ± 0.69 $1,N^2$-propanodGuo/10^8 dGuo), brain (2.96 ± 1.43 $1,N^2$-εdGuo/10^8 dGuo

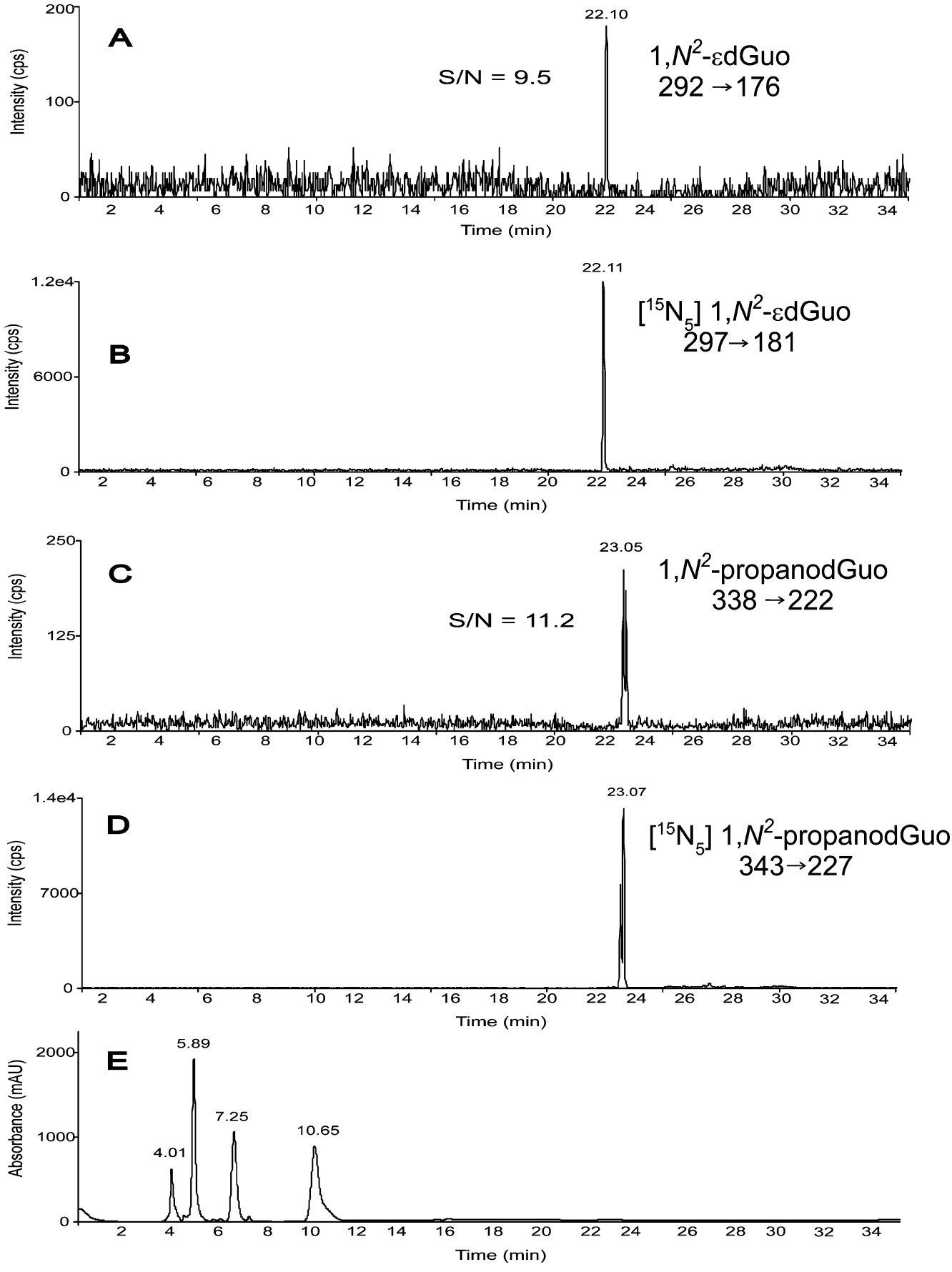


Figure 4. HPLC/ESI/MS-MS detection of 500 amol of DNA adducts standard contaminated in 100 μg of calf thymus DNA. (A) 1,N^2-εdGuo: *m/z* 292 → 176. (B) [$^{15}N_5$]-1,N^2-εdGuo: *m/z* 297 → 181. (C) 1,N^2-propanodGuo: *m/z* 338 → 222. (D) [$^{15}N_5$]-1,N^2-propanodGuo: *m/z* 343 → 227. (E) Enzymatically hydrolyzed DNA at 260 nm. Conditions were as described in the Experimental Procedures section.

Table 1. Levels of of 1,N^2-PropanodGuo and 1,N^2-εdGuo in Hydrolyzed DNA Calf Thymus Spiked with 1 fmol of Each Adduct[a]

		1,N^2-εdGuo-fmol	1,N^2-εdGuo/10^8dGuo	1,N^2-propanodGuo-fmol	1,N^2-propanodGuo/10^8dGuo
day 1	DNA A	0.93	0.49	2.16	1.20
	DNA B	1.02	0.56	1.98	1.10
	DNA C	0.94	0.52	2.04	1.13
	mean	*0.95*	*0.52*	*2.06*	*1.14*
	SD	*0.07*	*0.03*	*0.1*	*0.05*
day 2	DNA A	1.01	0.56	2.17	1.01
	DNA B	1.03	0.48	1.95	1.08
	DNA C	0.95	0.53	2.09	1.16
	mean	*0.10*	*0.52*	*2.07*	*1.08*
	SD	*0.04*	*0.04*	*0.11*	*0.07*
day 3	DNA A	1.02	0.58	2.20	1.18
	DNA B	1.07	0.59	2.02	1.12
	DNA C	1.05	0.58	2.03	1.13
	mean	*1.04*	*0.58*	*2.08*	*1.14*
	SD	*0.02*	*0.01*	*0.10*	*0.03*
DNA A	*mean*	*0.96*	*0.54*	*2.17*	*1.13*
	SD	*0.05*	*0.05*	*0.02*	*0.10*
DNA B	*mean*	*1.04*	*0.54*	*1.98*	*1.10*
	SD	*0.03*	*0.07*	*0.03*	*0.02*
DNA C	*mean*	*0.98*	*0.54*	*2.05*	*1.14*
	SD	*0.06*	*0.03*	*0.03*	*0.02*
total	***mean***	***1.00***	***0.55***	***2.11***	***1.15***
	SD	***0.06***	***0.05***	***0.17***	***0.11***

[a] The analysis were performed in 3 subsequent days by HPLC-MS/MS ($n = 3$).

and 5.66 ± 3.70 1,N^2-propanodGuo/10^8 dGuo), and lung (0.87 ± 0.34 1,N^2-εdGuo/10^8 dGuo and 2.25 ± 1.72 1,N^2-propanodGuo/10^8 dGuo) samples from 21 untreated male Wistar rats. A representative chromatogram of one of these samples is shown in Figure 6.

Discussion

The ultrasensitive methodology described herein permits us to evaluate and compare the deleterious effects of reactive aldehydes, such as CRO and AA, from endogenous or exogenous exposure through the direct and simultaneous quantification of two resulting DNA adducts, 1,N^2-propanodGuo and 1,N^2-εdGuo. Advantages of the method include the online separation of adducts from normal nucleosides coupled with the accuracy provided by tandem mass spectrometry detection. The methodology also excludes the necessity for a second method for the quantification of unmodified dGuo, thereby increasing precision and efficiency. The addition of an isotopically labeled adduct prior to DNA hydrolysis improves the specificity of the method on adduct quantification because it allows for the correction of any possible loss of adducts during the procedure.

The methodology enables us to determine the basal levels of 1,N^2-εdGuo (4.01 ± 0.32 adducts/10^8 dGuo) and 1,N^2-propanodGuo (3.43 ± 0.33 adducts/10^8 dGuo) in the DNA of human lung fibroblasts (IMR-90). Our group had previously reported basal levels of 1,N^2-εdGuo in the DNA of green monkey kidney fibroblasts (CV1-P cells) (4.5 ± 0.4 adducts/10^7 dGuo) (*35*).

Additionally, we measured here the adduct levels in different Wistar rat tissues (liver, 2.47 ± 0.61 1,N^2-εdGuo/10^8 dGuo and 4.61 ± 0.69 1,N^2-propanodGuo/10^8 dGuo; brain, 2.96 ± 1.43 1,N^2-εdGuo/10^8 dGuo and 5.66 ± 3.70 1,N^2-propanodGuo/10^8 dGuo; and lung, 0.87 ± 0.34 1,N^2-εdGuo/10^8 dGuo and 2.25 ± 1.72 1,N^2-propanodGuo/10^8 dGuo).

Table 2. Levels of 1,N^2-Propano-2′-deoxyguanosine and 1,N^2- Etheno-2′-deoxyguanosine in DNA from Wistar Rat Tissues (Lung, Liver, and Brain) ($n = 21$)

	1,N^2-εdGuo/10^8 dGuo			
	min. value	max. value	mean	SD
liver	1.77	3.41	2.47	0.61
lung	0.39	2.14	0.87	0.34
brain	1.83	4.19	2.96	1.43
	1,N^2-propanodGuo/10^8 dGuo			
	min. value	max. value	mean	SD
liver	3.02	6.93	4.61	0.69
lung	1.47	3.42	2.25	1.72
brain	1.86	10.19	5.66	3.70

The endogenous formation of 1,N^2-propanodGuo and its quantification in animals models are still controversial in the literature. Previously, Nath and Chung found 0.18−0.94 adducts/10^6 dGuo in the liver DNA of Fisher 344 rats using a ^{32}P-postlabeling methodology (*37*). In another study, they analyzed DNA from the brain (0.397−0.691 adducts/10^6 dGuo), lung (0.117−0.469 adducts/10^6 dGuo), and other tissues from the same rat model (*38*). However, Schuler and Eder did not detect basal levels of 1,N^2-propanodGuo in the liver DNA of Fisher 344 rats using ^{32}P-postlabeling coupled with TLC (*39, 40*). Later, Pan et al., using a methodology based on solid-phase extraction/high-performance liquid chromatography and ^{32}P-postlabeling, reported basal levels of 1,N^2-propanodGuo (25.9 ± 7.8 adducts/10^9 dGuo) in the liver DNA of Long-Evans rats (*41*). This level is comparable with that in liver samples of Wistar rats reported here (4.61 ± 0.69 1,N^2-propanodGuo/10^8 dGuo).

The presence of 1,N^2-propanodGuo has previously been reported in human tissue (liver and lung) and blood using two different methodologies, ^{32}P-postlabeling combined with HPLC (*37*) and HPLC/MS/MS analysis (*42*). Different quantities and frequencies of the adducts were reported, probably due to differences in the methodologies and in the levels of aldehyde exposure and individual repair efficiencies, as discussed before (*38, 42, 43*).

Additionally, etheno adducts, such as 1,N^6-etheno-2′-deoxyadenosine (1,N^6-εdAdo), 3,N^4-etheno-2′-deoxycytidine (3,N^4-εdCyd), 3,N^2-etheno-2′-deoxyguanosine (3,N^2-εdGuo), and 1,N^2-etheno-2′-deoxyguanosine (1,N^2-εdGuo), are well-validated biomarkers for DNA damage arising from the reaction of endogenous lipid peroxidation end products (*29*). The *in vivo* basal levels of 1,N^2-εdGuo was first reported by our group using a methodology based on HPLC/MS-MS. This adduct was quantified in the liver DNA of untreated female Wistar rats (5.22 ± 1.37 adducts/10^7 dGuo) (*36*). These levels are 10 times higher than those reported here. This difference can be attributed to improvement in the present methodology, simultaneous dGuo quantification, and rat gender. More recently, 1,N^2-εdGuo was quantified by Pang et al. (*44*) in tissue DNA of SJL mice. The basal levels reported in the liver (39 ± 38/10^9 dGuo), kidney (20 ± 7/10^9 dGuo), and spleen (24 ± 9/10^9 dGuo) are comparable with those reported here.

The quantification of the basal levels of DNA adducts in different tissues and animals is extremely important for the unequivocal use of these adducts as biomarkers of disease development and environmental exposure. Additionally, the accurate measurement of adducts can be useful for mechanistic elucidation in pathological situations associated with oxidative stress.

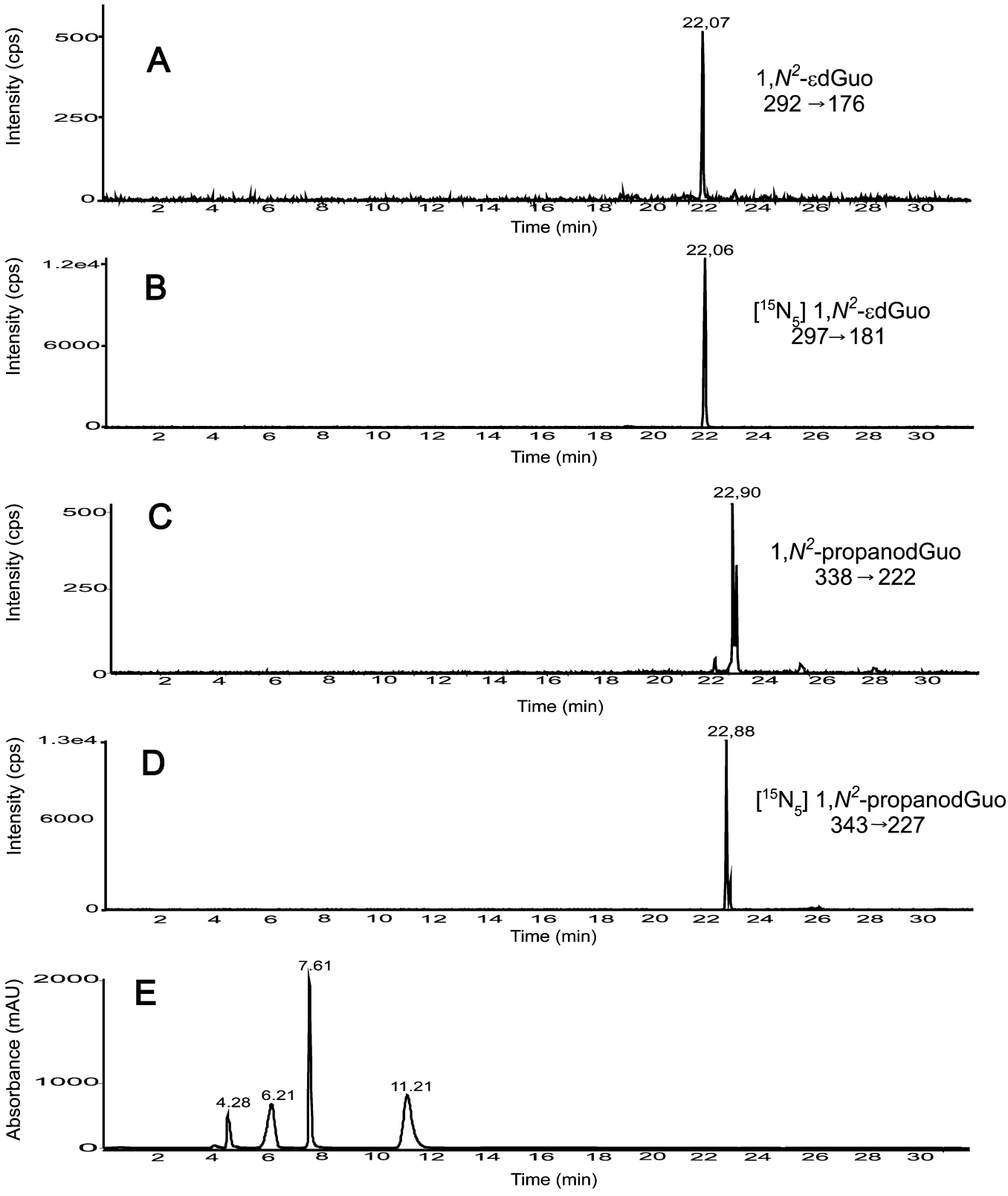


Figure 5. HPLC/ESI/MS-MS detection of DNA adducts in 100 μg of calf thymus DNA. (A) 1 fmol of 1,N^2-εdGuo: *m/z* 292 → 176. (B) [$^{15}N_5$]-1,N^2-εdGuo: *m/z* 297 → 181. (C) 1 fmol of each isomer of 1,N^2-propanodGuo: *m/z* 338 → 222. (D) [$^{15}N_5$]-1,N^2-propanodGuo: *m/z* 343 → 227. (E) Enzymatically hydrolyzed DNA at 260 nm. Conditions were as described in the Experimental Procedures section.

The methodology described herein provides a highly precise and specific approach to quantify 1,N^2-εdGuo and 1,N^2-propanodGuo adducts in DNA and allows for the future evaluation of their levels when tissues are subjected to factors that promote or decrease the lipid peroxidation process. In addition, this article demonstrates the unequivocal quantification of the basal levels of the two DNA adducts in the lung, liver, and brain tissue of male Wistar rats and in live cells (IMR-90). This quantification is fundamental for the establishment of these molecules as biomarkers involving redox processes.

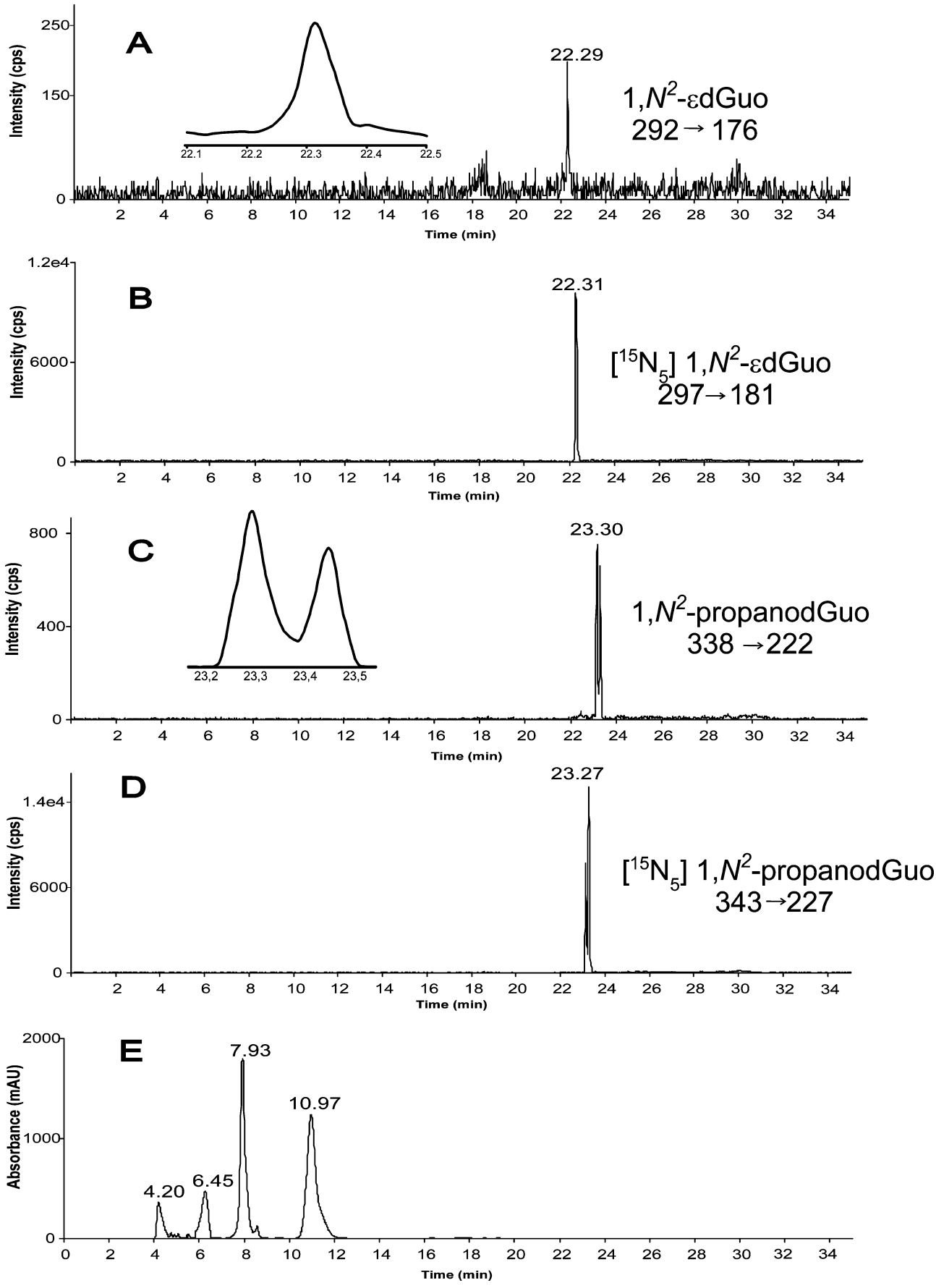


Figure 6. HPLC/ESI/MS-MS detection of DNA adducts in 100 μg of DNA extracted from the liver of an untreated male Wistar rat. The MRM mode was used. (A) 1,N^2-εdGuo: *m/z* 292 → 176. (B) [$^{15}N_5$]-1,N^2-εdGuo: *m/z* 297 → 181. (C) 1,N^2-propanodGuo: *m/z* 338 → 222. (D) [$^{15}N_5$]-1,N^2-propanodGuo: *m/z* 343 → 227. (E) Enzymatically hydrolyzed DNA at 260 nm. Conditions were as described in the Experimental Procedures section.

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Supporting Information Available: ^{1}H NMR spectrum and circular dichroism spectra of (6*R*,8*R*) and (6*S*,8*S*) 1,N^2-propanodGuo. This material is available free of charge via the Internet at http://pubs.acs.org.

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