Simultaneous Quantitation of N^2,3-Ethenoguanine and 1,N^2-Ethenoguanine with an Immunoaffinity/Gas Chromatography/High-Resolution Mass Spectrometry Assay

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We have previously described an immunoaffinity/gas chromatography/electron capture negative chemical ionization high-resolution mass spectrometry (IA/GC/ECNCI-HRMS) assay for quantitation of the promutagenic DNA adduct N^2,3-ethenoguanine (N^2,3-ϵGua) in vivo. Here we present an expanded assay that allows simultaneous quantitation of its structural isomer, 1,N^2-ethenoguanine (1,N^2-ϵGua), in the same DNA sample. 1,N^2-ϵGua and N^2,3-ϵGua were purified together from hydrolyzed DNA using two immobilized polyclonal antibodies. GC/ECNCI-HRMS was used to quantitate the 3,5-bis(pentafluorobenzyl) (PFB) derivative of each adduct against an isotopically labeled analogue. Selected ion monitoring was used to detect the $[M - 181]^-$ fragments of 3,5-$(PFB)_2$-N^2,3-ϵGua and 3,5-$(PFB)_2$-[$^{13}C_4$,$^{15}N_2$]-N^2,3-ϵGua and the $[M - 201]^-$ fragments of 3,5-$(PFB)_2$-1,N^2-ϵGua and 3,5-$(PFB)_2$-[$^{13}C_3$]-1,N^2-ϵGua. The demonstrated limits of quantitation in hydrolyzed DNA were 7.6 fmol of N^2,3-ϵGua and 15 fmol of 1,N^2-ϵGua in ~250 μg of DNA, which corresponded to 5.0 N^2,3-ϵGua and 8.7 1,N^2-ϵGua adducts/10^8 unmodified Gua bases, respectively. 1,N^2-ϵGua was found to be the predominant ethenoguanine adduct formed in reactions of lipid peroxidation products with DNA. The respective ratios of 1,N^2-ϵGua to N^2,3-ϵGua were 5:1 and 38:1 when calf thymus DNA was treated with ethyl linoleate or 4-hydroxynonenal, respectively, under peroxidizing conditions. Only N^2,3-ϵGua was detected in DNA treated with the vinyl chloride (VC) metabolite 2-chloroethylene oxide and in hepatocyte DNA from rats exposed to 1100 ppm VC for 4 weeks (6 h/day for 5 days/week). These data suggest that 1,N^2-ϵGua plays a minor role relative to N^2,3-ϵGua in VC-induced carcinogenesis, but that 1,N^2-ϵGua may be formed to a larger extent from endogenous oxidative processes.

Introduction

The exocyclic DNA adducts N^2,3-ethenoguanine (N^2,3-ϵGua)[1] and 1,N^2-ethenoguanine (1,N^2-ϵGua) have been identified in reactions of the vinyl chloride (VC) metabolites 2-chloroacetaldehyde and 2-chloroethylene oxide (CEO) with DNA (*1*−*3*). The formation of N^2,3-ϵGua in vivo has been characterized in rats exposed to VC and other chemicals by several methods, including ultrasensitive, highly specific gas chromatography/electron capture negative chemical ionization high-resolution mass spectrometry (GC/ECNCI-HRMS) assays developed in this laboratory (*4*, *5*). The amount of 1,N^2-ϵGua formed from in vitro reactions with DNA has been measured using HPLC with UV or fluorescence detection (*2*, *3*), a competitive immunoassay (*6*), and LC/MS/MS (*7*−*9*). However, these methods were unable to detect the low concentrations of 1,N^2-ϵGua expected to be present in vivo. A sensitive ^{32}P-postlabeling assay used for the detection of 1,N^2-ϵGua standard added to DNA (*10*) has not been applied to the routine analysis of DNA.

Both 1,N^2-ϵGua and N^2,3-ϵGua have been shown to be highly promutagenic when tested with in vitro and in vivo systems (*11*−*16*). 1,N^2-ϵGua has been detected in reactions of dGuo with the lipid peroxidation products *trans*-4-hydroxy-2-nonenal (HNE) or 2,3-epoxy-4-hydroxy-2-nonanal (*17*, *18*), and both 1,N^2-ϵGua and N^2,3-ϵGua were demonstrated in reactions of dGuo with HNE or ethyl linoleate (EtLA) in the presence of *tert*-butylhydroperoxide (*t*-BuOOH) (*9*). Recently, 1,N^2-ϵdGuo was demonstrated from the reaction of calf thymus DNA with the linoleic acid peroxidation product *trans*,*trans*-2,4-decadienal in the presence of peroxides (*8*). Consequently, both etheno adducts are suspected to play significant roles as endogenous mutagens, especially under conditions of oxidative stress (*19*).

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[1] Abbreviations: N^2,3-ϵGua, N^2,3-ethenoguanine; 1,N^2-ϵGua, 1,N^2-ethenoguanine; VC, vinyl chloride; CEO, 2-chloroethylene oxide; ECNCI, electron capture negative chemical ionization; HRMS, high-resolution mass spectrometry; HNE, *trans*-4-hydroxy-2-nonenal; EtLA, ethyl linoleate; *t*-BuOOH, *tert*-butyl hydroperoxide; IA, immunoaffinity; KLH, keyhole limpet hemocyanin; PFB, pentafluorobenzyl; ESI, electrospray ionization; EI, electron impact; BHT, butylated hydroxytoluene.

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In this work, we describe the extension of the existing immunoaffinity (IA)/GC/ECNCI-HRMS assay for N^2,3-ϵGua to allow the simultaneous quantitation of 1,N^2-ϵGua in DNA. The formation of both adducts in DNA was examined to determine the relative role each may play in carcinogenesis under various conditions.

Experimental Procedures

Materials. Caution: *VC, CEO, 2-chloroacetaldehyde, and chloroform are known or probable carcinogens. Phenol is severely caustic. Pentafluorobenzyl bromide is a potent lachrymator. Each of these chemicals should be handled carefully with gloves in an operating fume hood*. VC (99% chemically pure) was purchased from Supelco (Bellefonte, PA). Solubilized keyhole limpet hemocyanin (KLH), calf thymus DNA, and RNAases A and T_1 were obtained from Sigma (St. Louis, MO). *Clostridium histolyticum* type 2 collagenase was obtained from Worthington Biochemical (Freehold, NJ). Nucleic acid purification grade lysis buffer, a phenol/chloroform/water mixture, and proteinase K were purchased from PE Biosystems (Foster City, CA). 1,N^2-ϵdGuo and [2,9,9a-$^{13}C_3$]-1,N^2-ϵdGuo were generous gifts from F. Peter Guengerich (Vanderbilt University, Nashville, TN) and were synthesized as described by Müller et al. (*7*). CEO was synthesized as previously described (*20*). 2-Chloroacetaldehyde and 2,3,4,5,6-pentafluorobenzyl bromide were purchased from Aldrich (Milwaukee, WI). Other materials were obtained as described by Ham et al. (*5*, *9*).

HPLC. Individual systems and conditions are described in detail below. Reverse-phase chromatography was performed with either Beckman Ultrasphere semipreparative (250 mm × 10 mm × 5 μm) or analytical (250 mm × 4.6 mm × 5 μm) octadecyl silane columns. Strong cation-exchange chromatography was performed as described by Ham et al. (*5*). The eluent was monitored with either a HP 1040A diode array UV detector or an Applied Biosystems 757 single-wavelength UV detector. Injections were made manually with a Rheodyne injector, or automatically with a Waters 712 WISP autosampler. In each case, Waters 510 pumps were used.

Synthesis of 1,N^2-ϵGuo. 1,N^2-ϵGuo was synthesized by the reaction of 2-chloroacetaldehyde (45% aqueous solution) with Guo as described by Sattsangi et al. (*21*). The product was purified by semipreparative reverse-phase HPLC, and A_{285} of the eluent was monitored. Waters Baseline software was used for data acquisition and to control a Pharmacia FRAC-200 fraction collector. The methanol concentration was 20% from 0 to 16 min, was linearly increased to 50% at 19 min, and was maintained at 50% until 35 min. The 1,N^2-ϵGuo fractions (15.7 min) were collected, pooled, and lyophilized. 1,N^2-ϵGuo was purified a second time using the same system with an isocratic 20% methanol mobile phase. The flow rate was 2 mL/min in each case.

Production of the KLH−1,N^2-ϵGuo Conjugate. 1,N^2-ϵGuo was conjugated to KLH by a modified periodate oxidation procedure (*22*). 1,N^2-ϵGuo (5 mg) was dissolved in 1 mL of 100 mM sodium acetate buffer (pH 5.5). Sodium periodate was added to a concentration of 30 mM and incubated for 15 min at room temperature before the reaction was stopped by addition of 50 μL of 1 M ethylene glycol. After 5 min at 4 °C, the product was purified in a single 1 mL injection using a Pharmacia FPLC system fitted with a HR10/10 gel filtration column. The mobile phase was 100 mM sodium bicarbonate (pH 9.5) used at a flow rate of 3 mL/min. The A_{254} and A_{280} of the eluent were monitored with a Pharmacia UV-2 detector coupled to Waters Baseline software for data acquisition. The eluent corresponding to the low-molecular weight peak was collected (3.5−7.0 min), and 10 mL of KLH (57 mg) was added. The solution was incubated for 24 h at 4 °C, and the product was reduced with 190 μL of 100 mM sodium borohydride. After 30 min at 4 °C, the KLH−1,N^2-ϵGuo conjugate was purified with multiple 1 mL injections using the same system with water as the mobile phase at a rate of 4 mL/min. The high-molecular weight product (0.6−1.2 min) was collected, pooled, and lyophilized.

Immunizations. Two New Zealand white rabbits (2−3 kg) were purchased from Robinson Services (Clemmons, NC). The KLH−1,N^2-ϵGuo conjugate (3.0 mg) was added to 1.5 mL of sterile 10 mM phosphate buffer and 0.85% saline (pH 7.5) (PBS), and the suspension was emulsified in 1.5 mL of Freund's complete adjuvant. Immunizations (1.0 mL total) were made at four sc and six id sites on the back and hind of each rabbit. The rabbits were boosted in the same manner at 2, 4, and 6 weeks with the conjugate similarly prepared in Freund's incomplete adjuvant. A test bleed from the ear vein was conducted at 7 weeks. Blood was harvested 3 days later by exsanguination from the abdominal aorta after each rabbit was anesthetized with acepromazine and ketamine by a member of the veterinary staff. Plasma was obtained by centrifuging the blood from each rabbit at 1000*g* for 15 min at 4 °C and collecting the supernatant. The plasma was stored at −80 °C until it was used.

Preparation of Immunoaffinity Columns. Immunoaffinity columns were prepared as described by Ham et al. with modifications (*5*). To prepare hybrid columns, 4 mL of anti-1,N^2-ϵGuo plasma was added to 2.5 mL of protein A−Sepharose, and 1.5 mL of anti-N^2,3-ϵGuo plasma was added to 1.0 mL of protein A−Sepharose. Following the coupling procedures, the resins were pooled and used to construct 20 columns. To prepare 10 columns for the purification of 1,N^2-ϵGua only, 2 mL of anti-1,N^2-ϵGuo plasma was added to 1.25 mL of protein A−Sepharose. The columns were washed sequentially with 5 mL of PBS containing 0.02% sodium azide (w/v), 5 mL of water, 20 mL of methanol, 5 mL of water, 20 mL of 0.1 M formic acid, 10 mL of water, and 10 mL of PBS before use.

Synthesis and Characterization of Bis(pentafluorobenzyl)-1,N^2-ϵGua Derivatives. 1,N^2-ϵGuo (3.0 mg) was dissolved in 2 mL of 1 N hydrochloric acid and hydrolyzed to 1,N^2-ϵGua by incubation at 80 °C for 8 h. The solution was neutralized with 1 N sodium hydroxide, and 1,N^2-ϵGua was purified by reverse-phase semipreparative HPLC. The mobile phase was 20% methanol at 2 mL/min, and A_{285} was monitored with a diode array detector. 1,N^2-ϵGua was collected (10.9 min) and lyophilized. The pentafluorobenzyl (PFB) derivatives were synthesized as described in the next section, except that 26 μL of undiluted pentafluorobenzyl bromide was used. A portion of the product was purified by reverse-phase semipreparative HPLC. The mobile phase was 50% methanol from 0 to 15 min, and the methanol concentration was linearly increased to 90% at 45 min with a flow rate of 2 mL/min. Full scan mass spectra of the crude product and each major HPLC peak were obtained by GC/MS in the ECNCI and electron impact (EI) modes at a mass resolving power of 1×10^3 as described below. Electrospray ionization (ESI) mass spectra of the analytical standards were obtained by infusion with a Finnigan (San Jose, CA) TSQ7000 instrument. Each HPLC peak was analyzed by ESI-MS by infusion with a Finnigan LCQ^{DECA} instrument. ^{1}H NMR spectra were recorded at 500 MHz on a Varian (Palo Alto, CA) Inova500 spectrometer at the University of North Carolina School of Medicine facility. 1,N^2-ϵGua: ESI-MS (assignments in parentheses) m/z 176 (MH^+); ^{1}H NMR (DMSO-d_6) δ 12.52 (br s, 1H, N1 or N3-H), 12.22 (br s, 1H, N1 or N3-H), 8.18 and 8.12 (2 s, 1H, N5), 7.82 (s, 1H, H-2), 7.56 (d, 1H, J = 2.4 Hz, H-7), 7.37 (d, 1H, J = 2.4 Hz, H-6); UV (20% methanol/H_2O) λ_{max} = 223, 292 nm. [$^{13}C_3$]-1,N^2-ϵGua: ESI-MS m/z 179 (MH^+)], [3,5-$(PFB)_2$-1,N^2-ϵGua: ESI-MS (assignments and relative abundances in parentheses) m/z 535 (M^+, 100), 516 (M − F^+, 18), 354 (M − PFB^+, 37); ECNCI-MS (assignments and relative abundances in parentheses) m/z 354 (M − PFB^-, 83), 334 (M − PFB − HF^-, 100); ^{1}H NMR ($CDCl_3$) δ 7.79 (s, 1H, H-2), 7.68 (d, 1H, J = 1.9 Hz, H-7), 7.35 (d, 1H, J = 1.9 Hz, H-6), 6.15 (s, 2H, CH_2-N3), 5.74 (s, 2H, CH_2-N5); UV (77% methanol/H_2O) λ_{max} = 226, 253, 308 nm. 1,5-$(PFB)_2$-1,N^2-ϵGua: EI-MS (assignments and relative abundances in parentheses) m/z 535 (M^+, 100), 516 (M − F^+, 15), 354 (M − PFB^+, 34); ECNCI-MS (assignments and

relative abundances in parentheses) *m*/*z* 354 (M − PFB$^-$, 100), 181 (PFB$^-$, 7); ^{1}H NMR ($CDCl_3$) δ 8.88 (br s, 1H, H-2), 7.58 (d, 1H, J = 2.5 Hz, H-7), 6.97 (d, 1H, J = 2.5 Hz, H-6), 5.90 (s, 2H, CH_2-N1), 5.40 (s, 2H, CH_2-N5); UV (67% methanol/H_2O) λ_{max} = 227, 308 nm. O^9,5-(PFB)$_2$-1,N^2-ϵGua: ESI-MS (assignments in parentheses) *m*/*z* 536 (MH^+); ^{1}H NMR ($CDCl_3$) δ 7.69 (br s, 1H, H-2), 7.62 (d, 1H, J = 1.5 Hz, H-7), 6.92 (d, 1H, J = 1.5 Hz, H-6), 5.34 (s, 2H, OCH_2), 5.31 (s, 2H, CH_2-N5).

Animal Exposures and Tissue Collection. Exposures were conducted at Huntington Life Sciences (East Millstone, NJ). Eleven-week-old male Sprague-Dawley rats (450−550 g) were purchased from Charles River Laboratories (Portage, MI) and housed in stainless steel cages with a 12 h light/dark cycle. Certified Rodent Diet No. 5002 (PMI Feeds, St. Louis, MO) was provided ad libitum during nonexposure periods, and water was provided without restriction at all times. Ten animals per group were acclimated for 1 week and then exposed to nominal concentrations of 0 or 1100 ppm VC in a whole-body inhalation apparatus for 1 or 4 weeks (6 h/day for 5 days/week). The animals were anesthetized ip with pentobarbital, iv heparinized, and killed by incision of the abdominal aorta. The livers were perfused in situ with collagenase via the portal vein (*23*). After ∼5 min, the livers were removed and sieved through 149 and 105 μm polypropylene meshes to obtain mixed cell suspensions. Hepatocytes were isolated by centrifugation at 50*g* for 3 min (*24*), frozen on dry ice, and stored at −80 °C.

DNA Extraction. Cellular DNA was isolated using a modified phenol/chloroform extraction procedure with 2% butylated hydroxytoluene (BHT) in 2-propanol (w/v) added to reagents and samples as an antioxidant. The hepatocyte pellet was suspended in 5 mL of lysis buffer containing 0.04% BHT (w/v), and an additional 25 μL of 2% BHT solution was added. RNAases A (5 units) and T_1 (270 units) were added, and the samples were incubated for 2 h at 37 °C. Next, proteinase K (45 units) was added, and the samples were incubated for an additional 2 h at 37 °C. The samples were extracted twice with 5 mL of a phenol/chloroform/water mixture containing 0.04% BHT (w/v) and once with 5 mL of alumina-purified chloroform containing 0.04% BHT (w/v). DNA was precipitated by addition of 0.5 mL of 3 M sodium acetate and 10 mL of cold 95% ethanol. The pellet was washed with cold 70% ethanol and redissolved overnight in water at 4 °C. The concentration of each sample was estimated by A_{260}, and the DNA was stored at −80 °C until analysis was carried out.

Standards. N^2,3-ϵGua and [$^{13}C_4$,$^{15}N_2$]-N^2,3-ϵGua standards were prepared and characterized as described by Ham et al. (*5*). 1,N^2-ϵdGuo and [$^{13}C_3$]-1,N^2-ϵdGuo were hydrolyzed to the corresponding nucleobases in 2.5% formic acid at 60 °C for 4 h. The bases were purified by reverse-phase analytical HPLC. The methanol concentration was increased from 5 to 18% from 0 to 10 min, and was then increased to 45% from 10 to 15 min. The pooled nucleobase fractions (8.1 min) were dried in a rotary evaporator, and the concentration of each standard was determined by UV absorbance in 0.1 N HCl (*21*). GC/ECNCI-HRMS was used to calibrate the internal standard by comparison to the better-characterized analyte standard, and the calculated concentration was within 1% of that determined by UV. No contamination of the internal standard with unlabeled 1,N^2-ϵGua was detected by GC/ECNCI-HRMS (data not shown).

DNA Reactions. The reaction of HNE and EtLA with calf thymus DNA was performed as described by Ham et al. (*9*). For CEO reactions, calf thymus DNA (3 mg/mL) was dissolved in 100 mM potassium phosphate buffer (pH 7.4) and sheared to homogeneity. Three 1 mL aliquots were equilibrated at 4, 23, and 37 °C for 30 min. Next, 15 μL of CEO (53% pure as determined by GC; 0.1 mmol) was added, and each sample was incubated at the appropriate temperature for 30 min before being stored at −80 °C.

Assay Procedures. Samples were prepared using the mild acid hydrolysis method described by Ham et al. (*5*) with minor modifications as described below.

DNA Hydrolysis. One hundred microliters of 1 N HCl, 30 μL of the [$^{13}C_4$,$^{15}N_2$]-N^2,3-ϵGua standard (66.3 fmol), and 50 μL of the [$^{13}C_3$]-1,N^2-ϵGua standard (177 fmol) were added to 5−800 μg of aqueous DNA. Water was added to bring the total volume of each sample to 1000 μL, and the tubes were incubated at 70 °C for 60 min. The samples were cooled, transferred to Centricon-10 concentrators (Millipore, Bedford, MA), and centrifuged at 5000*g* (60 min at 4 °C). In the case of DNA samples, a 50−250 μL aliquot of the filtrate was retained for Gua quantitation (see a section below). Each tube was rinsed with 1 mL of 500 mM sodium phosphate buffer (pH 7.2), which was transferred to the Centricon retentate vial. An additional 1 mL of buffer was added to each retentate, and the samples were similarly centrifuged for 90 min.

Immunoaffinity Chromatography. All steps were performed at 4 °C unless otherwise noted. The IA columns were preconditioned by the sequential addition of 5 mL of water, 10 mL of methanol, 10 mL of water, and 10 mL of PBS/sodium azide. Each Centricon filtrate was diluted with 2 mL of water and transferred to an IA column at room temperature. The filtrate vials were rinsed with 5 mL of PBS/sodium azide, which was also transferred to the columns. The samples were washed with 5 mL of water and 10 mL of 5% methanol. The adducts were eluted with 5 mL of methanol into silanized test tubes and were dried in a rotary evaporator. The columns were reconditioned with 5 mL of methanol, 5 mL of water, 10 mL of 0.1 M formic acid, 10 mL of water, and 10 mL of PBS/sodium azide.

Derivatization. To each sample were added ∼25 mg of oven-dried, powdered potassium carbonate and 500 μL of acetone. Next, 35 μL of a 10% pentafluorobenzyl bromide/acetone mixture (v/v) was added. The tubes were capped and mixed vigorously at 50 °C for 70 min. The samples were dried in a rotary evaporator and were then extracted and transferred in two 100 μL portions of dichloromethane to disposable silica gel columns prepared as previously described (*5*). The samples were washed with 4 mL of hexane and 6 mL of a 5% ethyl acetate/hexane mixture (v/v). The derivatives were eluted in 3 mL of ethyl acetate and dried again. The samples were redissolved in 15 μL of toluene and transferred to silanized GC vial inserts for analysis.

GC/ECNCI-HRMS. The instruments and conditions described by Ham et al. (*5*) were used for the GC/ECNCI-HRMS analyses. The mass resolving power was 7−10 × 10^3. A 2 μL volume of each sample was injected. Baseline resolution of the derivatized adducts was achieved without modification of the GC parameters. Selected ion monitoring at *m*/*z* 334.0351 and 337.0453 was used to monitor the [M − 201]$^-$ fragments of 3,5-(PFB)$_2$-1,N^2-ϵGua and 3,5-(PFB)$_2$-[$^{13}C_3$]-1,N^2-ϵGua, respectively. The [M − 181]$^-$ fragments of 3,5-(PFB)$_2$-N^2,3-ϵGua and 3,5-(PFB)$_2$-[$^{13}C_4$,$^{15}N_2$]-N^2,3-ϵGua were monitored at *m*/*z* 354.0414 and 360.0489, respectively. The absolute retention times of the peaks decreased over time since the GC column was shortened by ∼30 cm before each reinstallation.

Guanine Quantitation. The Gua content of each sample was determined with HPLC system 2 described by Ham et al. (*5*). In the case of in vitro DNA reactions using less than 50 μg of DNA, each 200−250 μL aliquot was analyzed undiluted. Otherwise, 50 μL aliquots were diluted to 200 μL with water and analyzed. The Gua content of each sample was determined by comparison to an external standard curve prepared with each experiment.

Basic pH Incubations. Calf thymus DNA (200 μg) was hydrolyzed, and the first centrifugation was performed as described above. To stop hydrolysis of the DNA backbone, 2 mL of 500 mM sodium phosphate buffer (pH 7.2) was added to the retentate vial. A 50 μL Gua aliquot was removed from the filtrate vial, and the filtrates were treated with 1 mL of 50 mM sodium bicarbonate buffer (pH 10.5) and 55 μL of 1 N sodium hydroxide. The pH of each sample was measured at pH 10.5−10.6 with a micro Ag/AgCl electrode. The samples were incubated at 4 °C for 1 or 12 h before the filtrates were neutralized

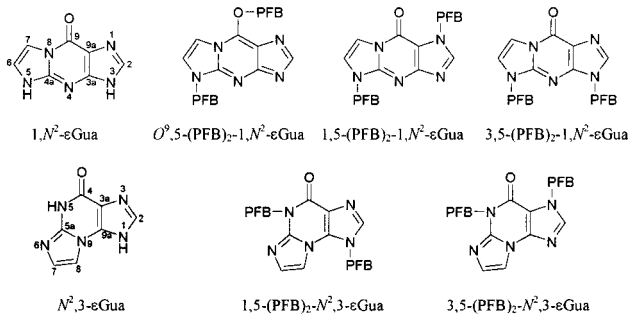

Figure 1. Structures of 1,N^2-ϵGua, N^2,3-ϵGua, and their bispentafluorobenzyl derivatives.

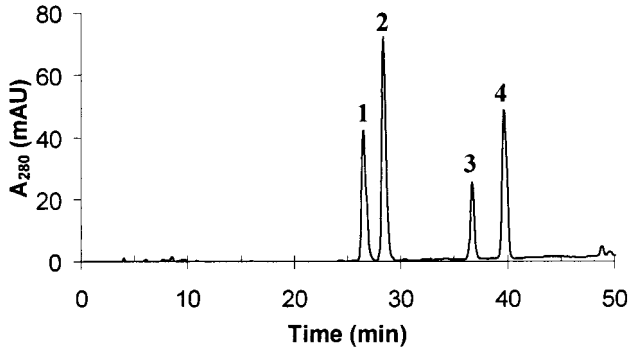

Figure 2. Reverse-phase HPLC profile of 1,N^2-ϵGua derivatization products. Peak 1 was identified as O^9,5-(PFB)$_2$-1,N^2-ϵGua. Peaks 2 and 3 correspond to 1,5-(PFB)$_2$-1,N^2-ϵGua and 3,5-(PFB)$_2$-1,N^2-ϵGua, respectively. Peak 4 was due to a trisubstituted derivative of HO-ethanoGua that contaminated the 1,N^2-ϵGua synthesis product.

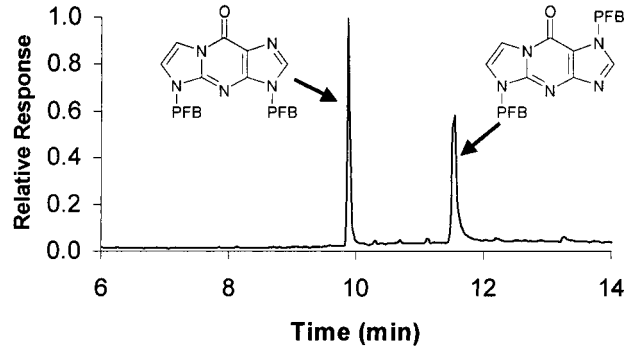

Figure 3. GC/ECNCI-MS total ion chromatogram of the crude 1,N^2-ϵGua derivatization product. The first peak corresponds to 3,5-(PFB)$_2$-1,N^2-ϵGua, and the second corresponds to 1,5-(PFB)$_2$-1,N^2-ϵGua.

by centrifugation at 5000*g* for 90 min. The samples were purified with IA chromatography and analyzed as described above.

Statistical Analysis. All results are reported as the means ± SD. Statistical analyses were completed with the aid of InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA). Two-tailed *p* values were used in each case.

Results

Characterization of Pentafluorobenzyl Derivatives of 1,N^2-ϵGua. Four products of the 1,N^2-ϵGua derivatization were detected with HPLC (Figure 2). When analyzed with ESI-MS, HPLC peaks 1−3 demonstrated molecular ions that corresponded to (PFB)$_2$-1,N^2-ϵGua derivatives. Two of these derivatives, corresponding to HPLC peaks 2 and 3, were eluted from the GC column when the crude product was analyzed by EI-MS (Figure 3). Each of the isolated HPLC peaks was analyzed by GC/MS in the EI and ECNCI modes (Figure 4A,B). The identification of the isomers was made with NMR in analogy to the pentafluorobenzyl derivatives of N^2,3-ϵGua (*4*), and in accordance with the work of Kjellberg and Johansson (*25*). The downfield shift of the H-2 proton in HPLC peak 3 relative to that in HPLC peak 2 indicates that the former corresponds to the 3,5-(PFB)$_2$-1,N^2-ϵGua isomer, while the latter corresponds to the 1,5-(PFB)$_2$-1,N^2-ϵGua isomer (Figure 5). The 3,5-(PFB)$_2$-1,N^2-ϵGua isomer was monitored in subsequent quantitative experiments since it produced the strongest signal in the ECNCI mode (Figure 4B), and displayed superior chromatographic properties. In the ECNCI mode, there was significant fragmentation of 3,5-(PFB)$_2$-1,N^2-ϵGua into both [M − 201]$^-$ (*m/z* 334) and [M − 181]$^-$ (*m/z* 354) ions. The major (66%) [M − 201]$^-$ fragment was chosen for quantitative analyses. The minor fragment of 3,5-(PFB)$_2$-1,N^2-ϵGua did not interfere with quantitation of the major fragment of 3,5-(PFB)$_2$-N^2,3-ϵGua (and vice versa) since the two isomers were resolved by GC.

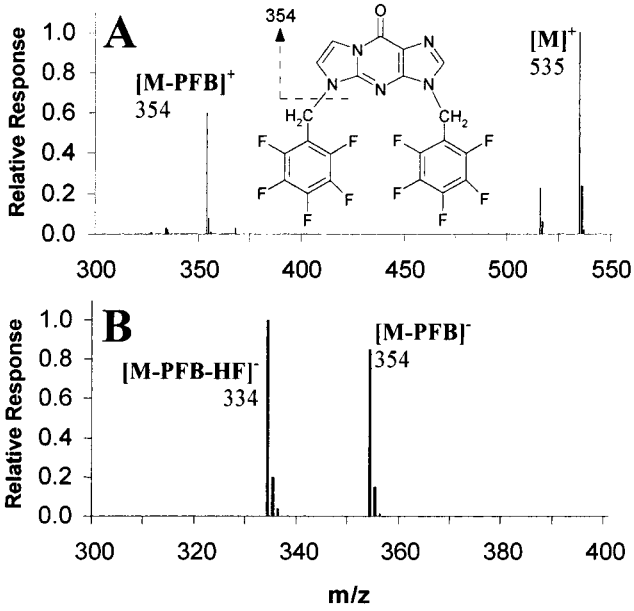

Figure 4. Full scan EI (A) and ECNCI (B) mass spectra of 3,5-(PFB)$_2$-1,N^2-ϵGua. The spectra of 1,5-(PFB)$_2$-1,N^2-ϵGua are similar.

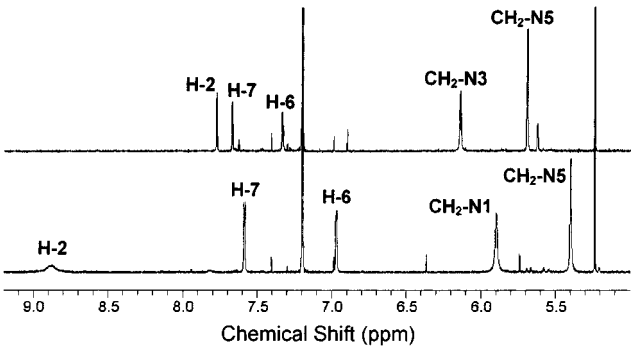

Figure 5. ^{1}H NMR spectra (500 MHz) of 1,5-(PFB)$_2$-1,N^2-ϵGua (top) and 3,5-(PFB)$_2$-1,N^2-ϵGua (bottom) in $CDCl_3$ (δ 7.24 ppm). Chemical shifts are reported relative to TMS. The assignment of the fragmented group is arbitrary.

The NMR and ESI-MS spectra of HPLC peak 1 were consistent with a novel O^9,5-(PFB)$_2$-1,N^2-ϵGua derivative. This derivative was identified as an O^9-substituted isomer on the basis of the large upfield shift of the PFB

Table 1. Recovery of Adduct Standards

		% recovered (mean ± SD)	
	n	1,N^2-ϵGua	N^2,3-ϵGua
hydrolysis + IA[a]	5	84.4 ± 2.2	71.4 ± 2.6
IA only[b]	5	85.3 ± 2.7	84.3 ± 2.1

[a] Recovery was determined by processing 177 fmol of 1,N^2-ϵGua and 50.0 fmol of N^2,3-ϵGua standards through the entire method and adding both internal standards to the immunoaffinity eluent. [b] Recovery was determined by applying 177 fmol of 1,N^2-ϵGua and 50.0 fmol of N^2,3-ϵGua standards directly to the columns and adding both internal standards to the immunoaffinity eluent.

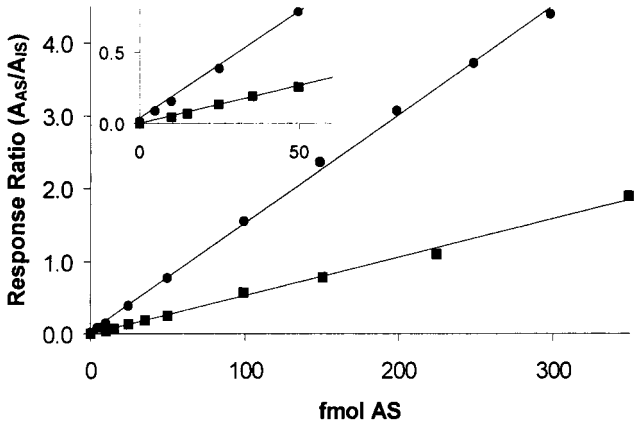

Figure 6. Calibration curves for N^2,3-ϵGua (●) and 1,N^2-ϵGua (■) standards processed through the entire method (r^2 > 0.995 in each case). The ratio of each analyte standard (AS) to the appropriate internal standard (IS) was calculated.

methylene protons relative to those in the 1,5- and 3,5-$(PFB)_2$ isomers. This substitution was unexpected since no analogous product was reported from the pentafluorobenzylation of N^2,3-ϵGua (*4*). The ESI mass spectrum of HPLC peak 4 was consistent with a trisubstituted PFB derivative of HO-ethanoGua (*3*). Although this related adduct contaminated the product of the large-scale synthesis, its presence did not affect the quantitation of 1,N^2-ϵGua since the analytical standards were obtained from a different source, and both were demonstrated to be free of HO-ethanoGua with ESI-MS.

Immunoaffinity Chromatography. The recovery of 1,N^2-ϵGua and N^2,3-ϵGua standards from the IA columns was evaluated by GC/ECNCI-HRMS (Table 1). No carryover of either adduct from previous samples was observed, and no decrease in the recovery of either adduct was apparent after the columns were each used seven times over a 3 month period.

Method Performance. The incubation time for the 0.1 N HCl DNA hydrolysis was increased from 30 to 60 min to account for the relatively strong glycosidic bond associated with 1,N^2-ϵdGuo (*2*). 1,N^2-ϵdGuo was quantitatively converted to 1,N^2-ϵGua under these conditions (data not shown). 1,N^2-ϵGua and N^2,3-ϵGua standards were processed together through the entire method to generate the standard curves shown in Figure 6. When the detector was operated at maximum sensitivity, its response was linear when analyzing samples containing 5−300 fmol of the N^2,3-ϵGua standard and 10−350 fmol of the 1,N^2-ϵGua standard. If the derivatization efficiency and recovery of 1,N^2-ϵGua are assumed to be equal to those of N^2,3-ϵGua at 30% (*4*), these values correspond to quantitation limits of ∼200 amol of N^2,3-ϵGua and ∼400 amol of 1,N^2-ϵGua per injection.

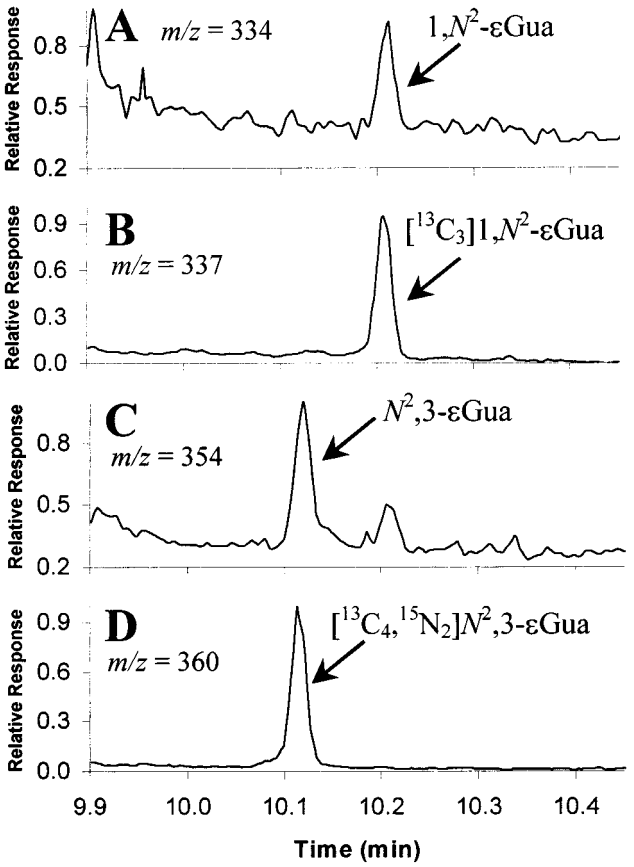

Figure 7. Detection of 15 fmol of the spiked 1,N^2-ϵGua standard (A) and 5 fmol of endogenous N^2,3-ϵGua (C) in 250 μg of calf thymus DNA. The chromatograms corresponding to the respective internal standards are shown in panels B and D.

The accuracy of adduct measurements in samples of hydrolyzed DNA was evaluated by spiking 200 μg of calf thymus DNA with 177 fmol of the 1,N^2-ϵGua standard and 50.0 fmol of the N^2,3-ϵGua standard. The amount of 1,N^2-ϵGua detected in four samples was 177 ± 6 fmol. The N^2,3-ϵGua determinations were complicated by the endogenous presence of this adduct in calf thymus DNA. When the amount of N^2,3-ϵGua measured in an equivalent amount of control calf thymus DNA was subtracted from the amount of N^2,3-ϵGua detected in the spiked DNA, the result was 56.4 ± 8.0 fmol of N^2,3-ϵGua.

Detection Limits in DNA. The limits of quantitation for 1,N^2-ϵGua and N^2,3-ϵGua processed through the entire method were determined in samples containing calf thymus DNA. Known amounts of the 1,N^2-ϵGua standard were added to the DNA prior to hydrolysis. Since N^2,3-ϵGua is present as an endogenous lesion in DNA (*5*, *26*), it was unnecessary to add any standard to the samples. The demonstrated limits of quantitation for N^2,3-ϵGua and 1,N^2-ϵGua in these samples were 7.6 and 15 fmol of adduct/sample, respectively (Figure 7). In the ∼250 μg DNA samples, these values corresponded to 5.0 N^2,3-ϵGua and 8.7 1,N^2-ϵGua adducts/10^8 unmodified Gua bases. The assay is inherently less sensitive toward 1,N^2-ϵGua than N^2,3-ϵGua since only 24% of the N^2,3-ϵGua derivative is present as the minor isomer, and since 3,5-$(PFB)_2$-N^2,3-ϵGua fragments almost exclusively to the $[M - 181]^-$ ion (*4*).

DNA Reactions. Both adducts were formed in large amounts in DNA treated with EtLA in the presence of *t*-BuOOH (Figure 8). When 8 μg of DNA was analyzed

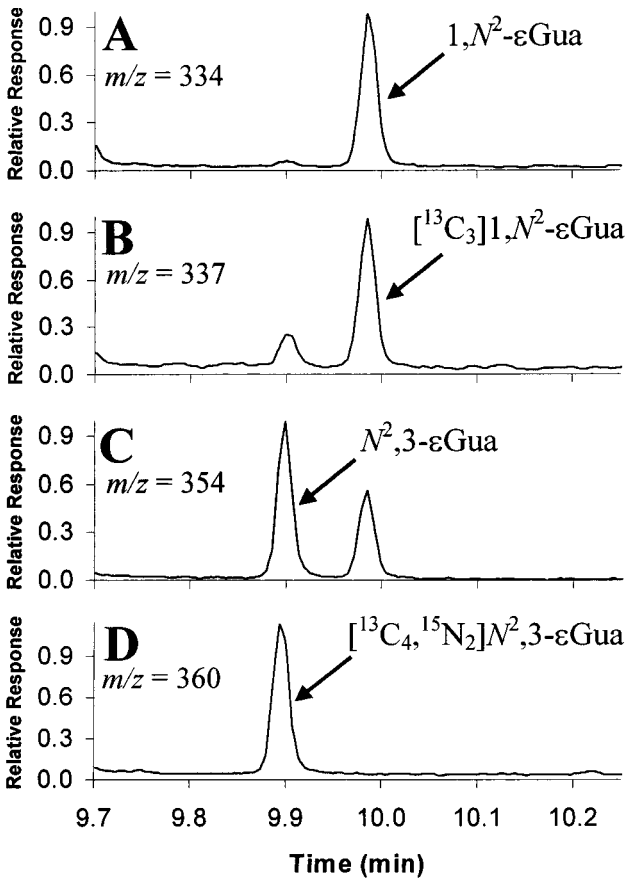

Figure 8. Formation of 1,N^2-ϵGua (A) and N^2,3-ϵGua (C) in 200 μg of DNA treated with EtLA in the presence of *t*-BuOOH. The chromatograms corresponding to the respective internal standards are shown in panels B and D. The smaller peak in panels B is due to the minor fragment of 3,5-$(PFB)_2$-1,N^2-ϵGua at *m*/*z* 354.

Table 2. Formation of 1,N^2-ϵGua and N^2,3-ϵGua in DNA Reacted with EtLA or HNE

		mol of ϵGua/10^6 mol of Gua	
treatment	*n*	1,N^2-ϵGua	N^2,3-ϵGua
t-BuOOH (EtLA control)	3	ND[a]	0.14 ± 0.03
t-BuOOH + EtLA[d]	3	28.1 ± 3.5[b]	5.28 ± 0.61[c]
t-BuOOH + EtLA[e]	3	28.3 ± 5.2[b]	5.22 ± 0.96[c]
t-BuOOH (HNE control)	3	ND	0.20 ± 0.02
t-BuOOH + HNE	3	146 ± 66	3.87 ± 1.33[c]

[a] ND, not detectable (see the text). [b] Significantly greater than the amount of N^2,3-ϵGua in the same samples using the paired *t* test ($p < 0.02$). [c] Significantly greater than the matched control ($p < 0.05$) using the unpaired *t* test with a Welch correction. [d] Eight micrograms of DNA analyzed. [e] Sixteen micrograms of DNA analyzed.

from each of triplicate reaction mixtures, the concentrations were measured at 28.1 ± 3.5 1,N^2-ϵGua and 5.28 ± 0.61 N^2,3-ϵGua adducts/10^6 unmodified Gua bases (Table 2). The linearity of the assay was demonstrated when similar adduct concentrations were measured after the amount of DNA analyzed from each reaction was doubled (Table 2). The mean 1,N^2-ϵGua:N^2,3-ϵGua ratio in these six samples was 5.4:1. The precision of the assay was demonstrated when 23.8 ± 1.5 1,N^2-ϵGua and 4.57 ± 0.21 N^2,3-ϵGua adducts/10^6 unmodified Gua bases were found in quadruplicate samples of 8 μg of DNA from a single EtLA−DNA reaction. When the same sample was analyzed on three separate occasions over the course of 5 months, the adduct concentrations were determined to be 26.1 ± 3.3 1,N^2-ϵGua and 4.75 ± 0.38 N^2,3-ϵGua adducts/10^6 unmodified Gua bases.

1,N^2-ϵGua was also the predominant ethenoGua adduct formed in reactions of DNA with HNE in the presence of *t*-BuOOH (Table 2). When 5 μg of DNA from each of triplicate reaction mixtures was analyzed, the mean 1,N^2-ϵGua:N^2,3-ϵGua ratio was 38:1.

When calf thymus DNA was reacted with the VC metabolite CEO at several temperatures, only N^2,3-ϵGua was detected (Table 3). When 100 μg of CEO-treated calf thymus DNA (reacted at 23 °C) was analyzed using IA columns constructed using only 1,N^2-ϵGua antibodies (to prevent suppression due to the very large amounts of N^2,3-ϵGua present in this DNA), the amount of 1,N^2-ϵGua was still below the limit of quantitation (data not shown). Thus, the N^2,3-ϵGua:1,N^2-ϵGua ratio observed in this experiment is clearly much greater than that previously reported using HPLC/fluorescence (*3*) and LC/MS/MS (*7*) assays.

VC-Treated Rats. Only N^2,3-ϵGua, at a concentration of 8.06 ± 2.58 N^2,3-ϵGua adducts/10^7 unmodified Gua bases, was detected in hepatocyte DNA from three rats exposed to 1100 ppm VC for 4 weeks (5 days/week) by inhalation (Table 3). On the basis of the demonstrated detection limit of 15 fmol of 1,N^2-ϵGua/DNA sample, and a mean sample size of 150 μg of DNA, the upper limit for 1,N^2-ϵGua in these samples was estimated to be 1.5 1,N^2-ϵGua adducts/10^7 unmodified Gua bases. 1,N^2-ϵGua was also not detected in brain and whole liver DNA from similarly exposed rats (data not shown).

Basic pH Incubations. As shown in Figure 9, both 1,N^2-ϵGua and N^2,3-ϵGua were readily induced in calf thymus DNA incubated under basic conditions. Although undetectable in control DNA, 1,N^2-ϵGua was apparent after a 1 h incubation at pH 10.5. The concentration of N^2,3-ϵGua detected following the 1 h incubation was similar to the endogenous concentration measured in untreated DNA. The concentrations of both adducts were increased significantly following a 12 h incubation under the same conditions ($p < 0.05$).

Discussion

This IA/GC/ECNCI-HRMS assay is at least as sensitive as the published LC/MS/MS methods (*7*, *8*) for the quantitation of 1,N^2-ϵGua in DNA, and offers the advantage of simultaneous N^2,3-ϵGua measurements. It is comparable in sensitivity, but is much more specific and quantitative than the existing ^{32}P-postlabeling method (*10*). Furthermore, the potential for contamination or artifactual adduct formation during sample preparation is minimized by the mild and rapid immunoaffinity enrichment procedure.

An unexpected finding was the apparent absence of 1,N^2-ϵGua in reactions of CEO with calf thymus DNA. The discrepancy between this and previous reports (*3*, *7*) may be due to minor differences between the reaction conditions, the higher specificity of this method for 1,N^2-ϵGua, or the complete avoidance of basic conditions. Here, it was demonstrated that both ethenoguanine adducts were readily formed by incubations of otherwise untreated DNA at pH 10.5. Although the sources of these adducts were not identified in this study, it has been reported that intermediates formed from the reaction of HNE or its epoxide with dGuo can be converted to 1,N^2-ϵGua at this pH (*17*, *18*). Thus, there appears to be a

Table 3. Quantitation of ϵGua Adducts in Calf Thymus and Rat Hepatocyte DNA

DNA sample	*n*	DNA (μg)	mol of ϵGua/10^6 mol of Gua	
			1,N^2-ϵGua	N^2,3-ϵGua
untreated calf thymus DNA	8	300	ND[a,b]	0.08 ± 0.03
CEO-treated calf thymus DNA (4 °C)	3[c]	5	ND	1330 ± 20
CEO-treated calf thymus DNA (25 °C)	3[c]	5	ND	1510 ± 50
CEO-treated calf thymus DNA (37 °C)	3[c]	5	ND	1730 ± 50
VC-exposed rat hepatocytes	3	150	ND	0.81 ± 0.26

[a] $n = 3$ since the 1,N^2-ϵGua standard was added to five other samples for detection limit determinations. [b] Not detectable (see the text). [c] Samples prepared from a single DNA reaction.

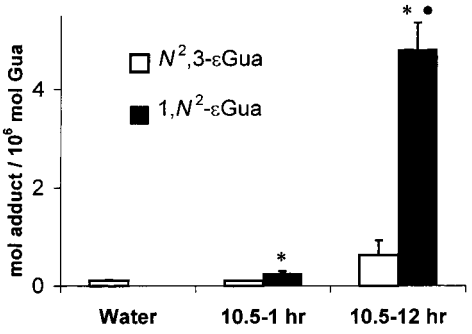

Figure 9. Formation of 1,N^2-ϵGua and N^2,3-ϵGua in untreated DNA under basic conditions. The results that are shown are the means ± SD of three reactions. Calf thymus DNA (200 μg) was incubated in pH 10.5 buffer for the indicated times as described in detail in Experimental Procedures. 1,N^2-ϵGua was not detected in the control DNA [an asterisk denotes that the level is significantly greater than that of N^2,3-ϵGua in the same samples by a paired *t* test ($p < 0.05$); the dot denotes that the level is significantly increased compared to the value at 1 h with ANOVA followed by the Tukey−Kramer multiple-comparisons test ($p < 0.01$)].

significant potential to form ethenoguanine adducts during sample preparation and analysis, and these results strongly suggest that basic conditions should be avoided at all times.

In lieu of positive results from the CEO−DNA samples, the reactions of EtLA and HNE with DNA served as positive controls during method development. As reported by Ham et al. using LC/MS/MS (*9*), both 1,N^2-ϵGua and N^2,3-ϵGua were formed from the reaction of either EtLA or HNE with DNA in the presence of *t*-BuOOH. Although 1,N^2-ϵGua was the predominant ethenoGua adduct in each case, the ratio of 1,N^2-ϵGua:N^2,3-ϵGua formed from the reaction of DNA with EtLA was lower than that formed from the reaction with its product, HNE. This suggests that products other than HNE are involved in the formation of N^2,3-ϵGua when DNA is subjected to peroxidizing conditions.

The results of 1,N^2-ϵGua and N^2,3-ϵGua measurements in hepatocyte DNA from rats exposed to VC mirrored the findings from the CEO−DNA reactions. While N^2,3-ϵGua was formed in relatively large amounts following exposure to VC, no 1,N^2-ϵGua was detected in these samples. Although it may be possible to detect small amounts of 1,N^2-ϵGua by analyzing greater amounts of DNA, these data suggest that 1,N^2-ϵGua plays a minor role in VC-induced carcinogenesis relative to N^2,3-ϵGua in this model.

The level of 1,N^2-ϵdGuo was recently determined with LC/MS/MS in calf thymus DNA to be 2.79 adducts/10^6 unmodified dGuo bases (*8*), a concentration that would be easily detected by the GC/MS method. However, the DNA in that experiment was apparently dissolved at pH 9.4, a condition that seems likely to generate 1,N^2-ϵGua artifactually. Since 1,N^2-ϵGua was not detected with the GC/ECNCI-HRMS method in untreated calf thymus DNA at neutral pH, its endogenous concentration appears to be very low under normal physiological conditions. Thus, despite the additional sensitivity offered by both MS-based methods, the existence of 1,N^2-ϵGua in vivo remains unproven. However, the results of the EtLA and HNE experiments suggest that 1,N^2-ϵGua may play a significant role when tissues are subjected to oxidative stress. This assay will be used in the future to determine if significant amounts of endogenous 1,N^2-ϵGua are present in animal models, disease states, or dietary conditions that promote lipid peroxidation.

The combination of IA chromatography and MS has had a significant impact on DNA adduct research in recent years. These techniques, coupled with the use of isotopically labeled internal standards, allow highly specific and precise measurements of adducts that are present in parts per million concentrations or lower relative to unmodified bases. To the best of our knowledge, this is the first example of the use of hybrid IA columns to purify multiple adducts from the same sample of hydrolyzed DNA. In this case, the similar chemical properties of these structural isomers allowed knowledge gained from the study of N^2,3-ϵGua to be applied to the quantitation of 1,N^2-ϵGua. The capability to measure levels of both ethenoGua adducts with one procedure will greatly aid in assessing the potential role of each in carcinogenesis under various conditions.

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