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A New LC-MS/MS Method for the Quantification of Endogenous and Vinyl Chloride-Induced 7-(2-Oxoethyl)Guanine in Sprague–Dawley Rats

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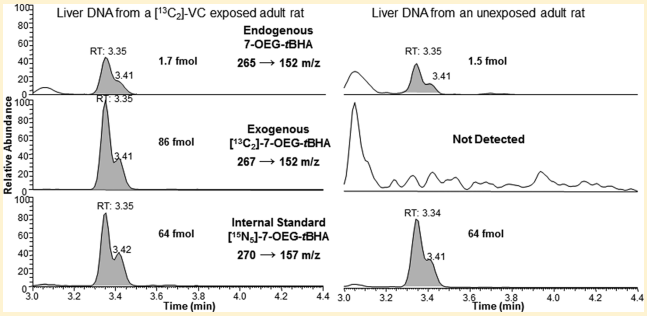
ABSTRACT: Vinyl chloride (VC) is an industrial chemical that is known to be carcinogenic to animals and humans. VC primarily induces hepatic angiosarcomas following high exposures (≥50 ppm). VC is also found in Superfund sites at ppb concentrations as a result of microbial metabolism of trichloroethylene and perchloroethylene. Here, we report a new sensitive LC-MS/MS method to analyze the major DNA adduct formed by VC, 7-(2-oxoethylguanine) (7-OEG). We used this method to analyze tissue DNA from both adult and weanling rats exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days. After neutral thermal hydrolysis, 7-OEG was derivatized with *O*-*t*-butyl hydroxylamine to an oxime adduct, followed by LC-MS/MS analysis. The limit of detection was 1 fmol, and the limit of quantitation was 1.5 fmol on the column. The use of stable isotope VC allowed us to demonstrate for the first time that endogenous 7-OEG was present in tissue DNA. We hypothesized that endogenous 7-OEG was formed from lipid peroxidation and demonstrated the formation of [$^{13}C_2$]-7-OEG from the reaction of calf thymus DNA with [$^{13}C_{18}$]-ethyl linoleate (EtLa) under peroxidizing conditions. The concentrations of endogenous 7-OEG in liver, lung, kidney, spleen, testis, and brain DNA from adult and weanling rats typically ranged from 1.0 to 10.0 adducts per 10^6 guanine. The exogenous 7-OEG in liver DNA from adult rats exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days was 104.0 ± 23.0 adducts per 10^6 guanine (n = 4), while concentrations in other tissues ranged from 1.0 to 39.0 adducts per 10^6 guanine (n = 4). Although endogenous concentrations of 7-OEG in tissues in weanling rats were similar to those of adult rats, exogenous [$^{13}C_2$]-7-OEG concentrations were higher in weanlings, averaging 300 adducts per 10^6 guanine in liver. Studies on the persistence of [$^{13}C_2$]-7-OEG in adult rats sacrificed 2, 4, and 8 weeks postexposure to [$^{13}C_2$]-VC demonstrated a half-life of 7-OEG of 4 days in both liver and lung.



INTRODUCTION

Vinyl chloride (VC) is an industrial chemical that is known to be a human and rodent carcinogen that also is found in over 100 Superfund sites as a result of microbial metabolism of trichloroethylene (TCE) and perchloroethylene (PCE). VC had been regarded as a relatively nontoxic industrial chemical until in 1974, the first epidemiology studies of occupationally exposed workers identified hepatic angiosarcomas, a rare neoplasm in humans.[1−3] VC is still widely used in industry for the preparation of polyvinyl chloride. While prolonged exposure to high concentrations of VC is known to cause liver angiosarcoma,[4] it remains unclear why carcinogenesis is primarily associated with high exposures (≥50 ppm VC). VC is metabolized by CYP450 2E1 to chloroethylene oxide (CEO).[5,6] CEO, the ultimate carcinogenic metabolite of VC,[7] covalently binds to DNA and induces four DNA adducts, whereas chloroacetaldehyde, a secondary metabolite, reacts primarily with proteins.[8] Although 7-(2-oxoethyl)guanine (7-OEG) is the major DNA adduct (~98%)[9−13] that arises from the reaction of CEO with DNA, it has been reported to be devoid of miscoding properties,[14] and it is lost primarily by chemical depurination. Conversely, exocyclic etheno adducts of VC, N^2,3-ethenoguanine (εG), 1,N^6-ethenodeoxyadenosine (εdA), and 3,N^4-ethenodeoxycytidine (εdC), display clear promutagenic activity during DNA synthesis.[15−17] Because promutagenic properties of etheno adducts are known, they have been studied in more detail than 7-OEG to further characterize their molecular dosimetry, promutagenic properties, and repair.[16−18] Low concentrations of endogenous etheno adducts have been detected in tissues[19−25] and are believed to result from lipid peroxidation and oxidative stress.[26−29] In contrast, 7-OEG

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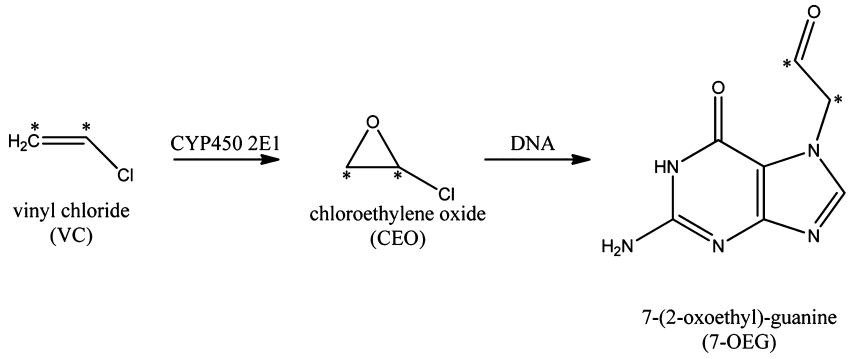
Scheme 1. VC-Induced Major DNA Adduct, 7-OEG[a]



[a]In the case of [$^{13}C_2$]-VC exposures, * indicates positions of labeled atoms.

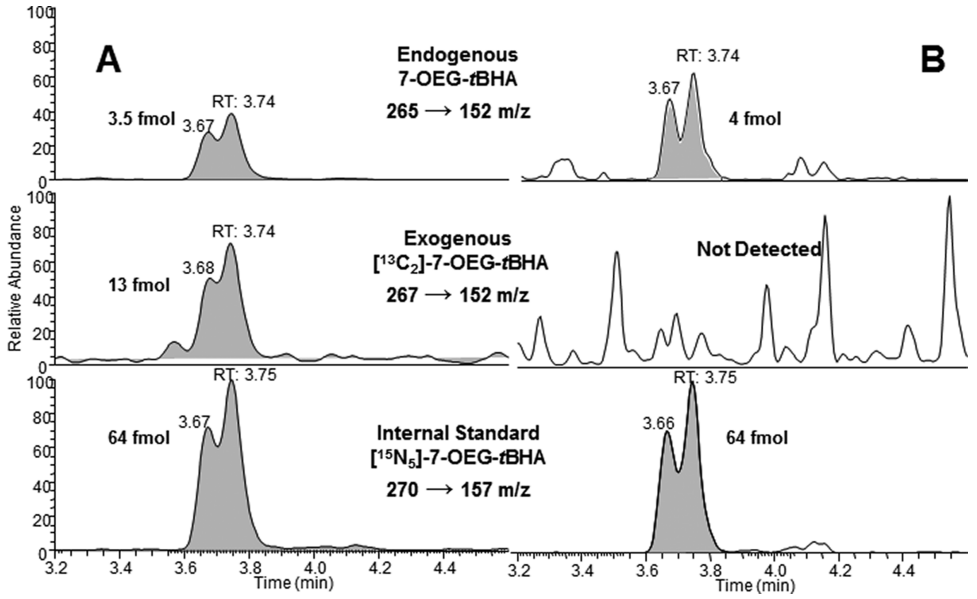


Figure 1. Chromatograms of (A) brain DNA from an adult rat exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days, 6 h per day, and (B) brain DNA from an unexposed adult rat.

adducts were only known to result from the reaction of CEO (Scheme 1) or the epoxide of urethane with the nucleophilic N7 position on dG.

Reactive oxygen species (ROS), such as super oxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxy radical (·OH), are generated during oxidative stress by biochemical reactions and cellular functions.[30] They are highly reactive with DNA, protein, lipids, and glycation products and are well-known to be cytotoxic.[31] While ROS are produced as a product of normal cellular function (i.e., mitochondrial metabolism), oxidative stress occurs when an imbalance arises between ROS production and antioxidant capacity, either through excess ROS production and/or antioxidant depletion. Oxidative stress can induce oxidative damage in cellular DNA, which if left unrepaired may induce mutations and, thus, play an important role in multistage carcinogenesis and progression of cancer.[32] Excess free radical formation and oxidative stress are associated with various human diseases including a variety of cancers, neurodegenerative diseases, aging, and cardiovascular diseases.[33−35]

Oxidative degeneration of lipids, also known as "lipid peroxidation" (LPO), consists of three major steps: initiation, propagation, and termination. LPO is first initiated by abstraction of hydrogen from unsaturated fatty acids to generate a fatty acid radical. This is followed by a reaction with O_2 to give a peroxy radical (propagation), which continues a chain reaction until production is stopped by the formation of a nonradical species. Following oxidation, α,β-unsaturated aldehydes, such as 4-oxo-2-nonenal (ONE), 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA), and acrolein, form by the decomposition of hydroperoxides.[36,37] Such aldehydes react with DNA and proteins to induce cell proliferation and apoptosis.[36−38] Some of the LPO-induced DNA adducts are εdA, εG, and pyrimido-[1,2-*a*]-purin-10(3*H*)-one (M_1G).[39,40]

Previously, VC has been shown to be more carcinogenic in young animals.[41−44] Maltoni et al. showed that 1 day old Sprague−Dawley rats exposed to 6000 or 12000 ppm VC (4 h/day, 5 day/week, 5 weeks) had about a 50% incidence of angiosarcoma, whereas 13 week old rats exhibited less than a 10% incidence.[41] Drew et al. also reported that rats, mice, and hamsters exposed to 50−200 ppm VC by inhalation (6 h/day, 5 days/week), regardless of duration of exposure, had a higher incidence of neoplasms when exposures are started early in life.[42] It was also shown that rat fetuses and neonates have higher susceptibility to angiosarcomas and hepatocellular carcinomas.[44,45]

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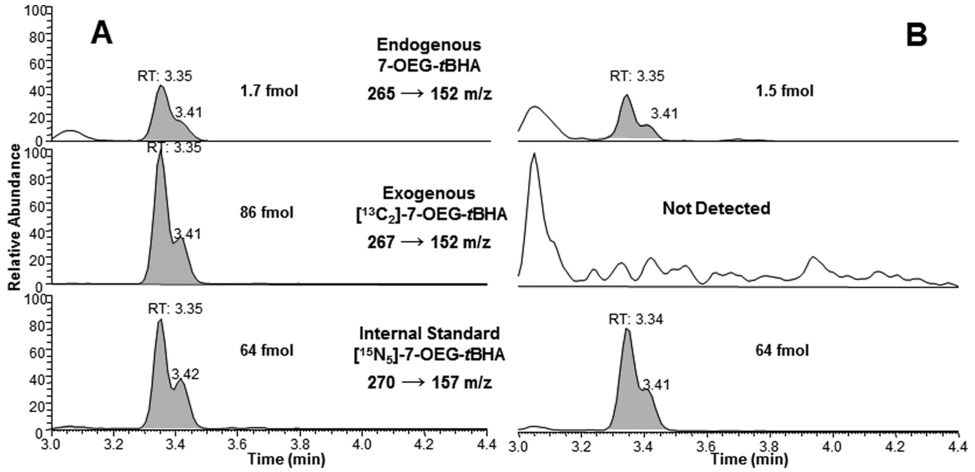

Figure 2. Chromatograms of (A) liver DNA from an adult rat exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days, 6 h per day, and (B) liver DNA from an unexposed adult rat.

Animal studies also indicated that young animals are more susceptible than adults to the formation of DNA adducts by VC exposures.[46−48] It was previously reported that following VC exposure via inhalation (600 ppm, 4 h/day, 5 days), 7-OEG and εG concentrations in 10 day old rats were ∼4-fold higher than lactating rats.[46] Age-dependent differences in the formation of εG by VC exposure were also studied by Morinello et al.[47] The concentration of εG in hepatocytes from weanling rats exposed to 1100 ppm VC (6 h/day, 5 days/week, 1 week) was reported to be ∼1.8-fold greater than that found in adult rats.[47]

Several methods were developed to measure 7-OEG induced by VC in mice and rats.[12,13,49,50] Fedtke et al. used HPLC to determine the formation and persistence of 7-OEG in female rats exposed to 600 ppm VC.[9,10,51] The half-life of 7-OEG in these animals was reported as 62 h, which was shorter than found for the etheno adducts. The concentration of 7-OEG was also measured by LC-MS/MS after the reduction of 7-OEG to 7-(2-hydroxyethyl)guanine (7-HEG) with $NaCNBH_3$.[47] In that study, 7-OEG was detectable in hepatocytes but not adjoining nonparenchymal cells.

In the present study, we developed a new highly sensitive and specific LC-MS/MS method to analyze 7-OEG in tissues from both adult and weanling male rats exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days and 1100 ppm VC for 1 day. Because of the increased sensitivity of the new assay and the use of stable isotope-labeled VC, we were able to detect and quantify endogenous 7-OEG in vivo in tissues of control rats and [$^{13}C_2$]-VC-exposed rats (Figures 1 and 2). We hypothesized that the source leading to the formation of 7-OEG was LPO and present data to support our hypothesis. The reaction of calf thymus DNA (CtDNA) with [$^{13}C_{18}$]-ethyl linoleate (EtLa) under peroxidizing conditions resulted in the formation of [$^{13}C_2$]-7-OEG,[52] which could be quantified by LC/MS-MS (Figure 3). In addition to quantitating endogenous and exogenous 7-OEG after [$^{13}C_2$]-VC exposures, the half-life of [$^{13}C_2$]-7-OEG in liver and lung was determined.

MATERIALS AND METHODS

Caution: *VC is a known carcinogen and should be handled carefully in an operating fume hood with protective equipment (i.e., gloves and laboratory coat).*

Chemicals. [$^{13}C_2$]-VC (≥98% chemical purity; 99% isotopic purity) and [$^{15}N_5$]-dG (98% isotopic purity) were obtained from Cambridge Isotope Laboratories (Andover, MA). HPLC grade water, methanol, and acetic acid were purchased from Thermo Fisher Scientific (Raleigh, NC), ethylene oxide, *O*-*t*-butyl hydroxylamine, RNase A, CtDNA, and dG were purchased from Sigma Aldrich (St. Louis, MO), and *t*-butyl hypochlorite was purchased from TCI America (Portland, OR). Nucleic acid purification grade lysis buffer, protein precipitation solution, and proteinase K were purchased from Qiagen (Valencia, CA).

Synthesis of 7-OEG Analyte and Internal Standards. 7-OEG was synthesized from the reaction of CEO with dG. CEO was synthesized as previously described by Holt et al.[53] by photochlorination of ethylene oxide with *t*-butyl hypochlorite. After distillation of the crude product, the exact composition of the collected product was verified by ^{1}H NMR. A solution of dG (20 mM) in water was reacted with excess CEO in a sealed tube at room temperature for 15 min and then at 37 °C for an additional 15 min. After hydrolysis with 0.1 N HCl at 70 °C for 40 min, 7-OEG standard was purified by HPLC. Chromatography was performed on a RP-18 (15 mm × 3.2 mm) precolumn (Brownlee Laboratories) attached to RP-SCX column (ES Industries, Chromega column, 250 mm × 4.6 mm, 5 μm) with a flow rate of 1.5 mL/min using a linear gradient program from 20 to 100% B in 20 min [A, 75 mM ammonium formate/10% acetonitrile (ACN), pH 2.8, and B, 250 mM ammonium formate/10% ACN, pH 2.8).[49] 7-OEG analyte standard (AST) was collected at ∼7 min and quantified by fluorescence measurement with excitation wavelength of 255 nm and a 340 nm emission cutoff filter. Isotopically labeled 7-OEG internal standard (IST) also was synthesized from the reaction of [$^{15}N_5$]-dG with CEO as described and purified by the same procedure as for AST.

Animals, Exposure, and Tissue Collection. Inhalation exposures were conducted at the U.S. EPA NHEERL facility in Research Triangle Park, NC. All procedures that involved the use of animals were approved by the U.S. EPA Institutional Animal Care and Use Committee. Ten week old (300−325 g) adult and 21 day old (50−60 g) weanling male Sprague−Dawley rats were purchased from Charles River Laboratories (Raleigh, NC). The rats were acclimated for 1 week and housed in stainless steel cages with a 12 h light/dark cycle. Four adult and eight weanling animals per group were exposed to 1100 ppm VC in a nose-only inhalation apparatus for 1 day (6 h/day) or to 1100 ppm [$^{13}C_2$]-VC (6 h/day) for 5 days. The VC vapor was generated by metering pure VC or [$^{13}C_2$]-VC vapor into a mixing chamber with medical grade air using mass flow controllers (Tylan Instruments, Torrance, CA). VC vapor was delivered to the inlet of the nose-only chamber (CH Technologies, Westwood, NJ) after the concentration was determined using a Miran 1A infrared gas analyzer (Foxboro, MA), which was calibrated with VC (Scott Gas, 99.96% purity). Temperature and relative humidity were monitored during the exposures using an Omega model RH411 Thermo-Hygrograph (Stamford, CT) and were within the acceptable limits for nose-only exposures.

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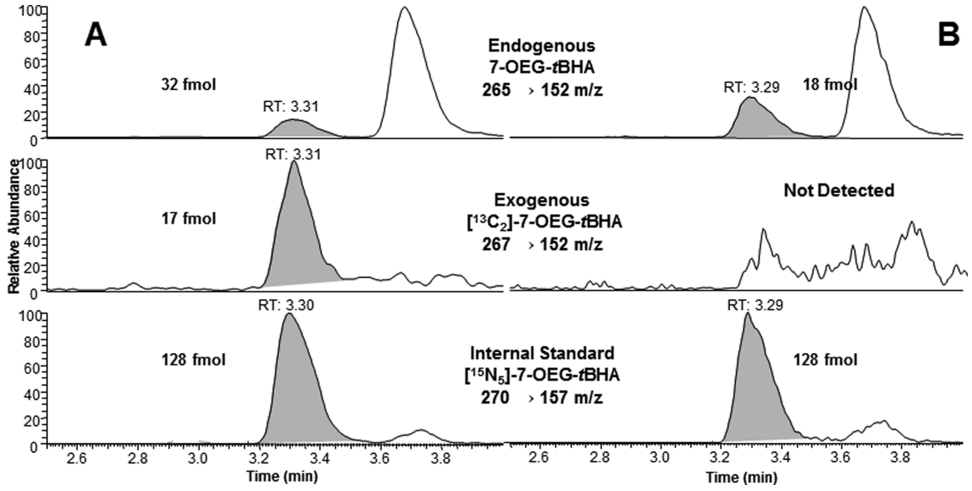

Figure 3. Chromatograms of (A) CtDNA reacted with [$^{13}C_{18}$]-EtLa for 89 h and (B) control CtDNA.

At the end of exposure, the rats were anesthetized with Euthasol by intraperitonial injection and euthanized by exsanguination via the vena cava. The liver, lung, kidney, spleen, testis, and brain were removed, frozen on dry ice, and stored at −80 °C. Following 5 days of [$^{13}C_2$]-VC exposure, four adults and all eight weanling rats were euthanized within 2 h of the completion of exposure. Three additional groups of four adults were euthanized 2, 4, or 8 weeks later to determine the half-life of [$^{13}C_2$]-VC DNA adducts.

DNA Isolation. DNA isolation from tissues was performed as previously described[22] with minor modifications using the Gentra Systems DNA extraction kit. The homogenized tissues were incubated in lysis buffer with the addition of 20 mM 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), followed by protein precipitation solution. After protein precipitation, the DNA/RNA mixture was precipitated by isopropanol. The DNA/RNA pellet then was resuspended in lysis buffer containing TEMPO and incubated with RNase A at 37 °C for 30 min, which was then followed by protein and DNA precipitation. The DNA pellet was resuspended in distilled water containing 1 mM TEMPO and stored at −80 °C until analysis. DNA was quantified by a Biomate UV spectrophotometer (Thermo Scientific) at A_{260} and calculated as 1 AU = 50 μg/mL.

DNA Hydrolysis. DNA solutions (50 μg) were spiked with the IST (640 fmol, [$^{15}N_5$]-7-OEG) and diluted to 500 μL with HPLC water. Samples were incubated at 100 °C for 30 min. Immediately after incubation, samples were cooled on ice. The DNA backbone was separated by Microcon 10 filtration (11500 rpm, 4 °C, 40 min), after which the filtrate was placed in a separate tube, and 400 μL of HPLC water was added to the retentate to wash the DNA backbone (11500 rpm, 4 °C, 40 min), followed by combining both filtrates. Four mM *O*-*t*-butyl hydroxylamine (*O*-*t*BHA) (50 μL) and methanol (100 μL) were added to each DNA filtrate, incubated at 45 °C for 30 min, and dried by vacuum evaporation in a SpeedVac concentrator. The dried fractions were transferred to glass autosampler vials by rehydration with HPLC water (3 × 100 μL), dried under vacuum, and then diluted in 50 μL of HPLC water to be analyzed by LC-MS/MS. Positive CtDNA controls (with and without *O*-*t*BHA) and negative reagent-only controls (with and without *O*-*t*BHA) were also prepared as described above. These samples proved no artificial contribution to the exogenous mass transitions, and endogenous 7-OEG was present only in samples containing DNA.

LC-MS/MS Analysis. Quantitative LC-MS/MS data were obtained using a Thermo Finnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer equipped with Waters Acquity UPLC. A heated electrospray ionization (HESI) interface was operated in positive selected reaction monitoring (SRM) mode. The transitions monitored were *m*/*z* 265 → 152 for 7-OEG-*t*BHA (Figure 4), *m*/*z* 267 → 152 for $^{13}C_2$-7-OEG-*t*BHA, and *m*/*z* 270 → 157 for [$^{15}N_5$]-7-OEG-*t*BHA. The method gave a detection limit of 1 fmol on the column using an injection volume of 5 μL. Samples were kept at 4 °C in the autosampler during analysis. Separation was performed on a Thermo Scientific Aquasil C18 column (150 mm × 1 mm, 5 μ) with a flow rate of 0.120 mL/min using the following gradient: (A) 0.1% acetic acid in water and (B) acetonitrile; 0−1.5 min, 5% B; 1.5−3 min, 30% B; 3−6 min, 90% B; 6−7 min, 5% B; and 7−12 min, 5% B.

Instrument conditions were optimized for maximum signal of 7-OEG by direct infusion of AST and IST. MS settings were as follows: electrospray voltage, 3000 V; capillary temperature, 285 °C; HESI temperature, 150 °C; sheath and auxiliary gas pressures, 30 and 20 arbitrary units; collision energy, 20 V; and Q2 collision gas pressure, 1.5 mTorr. Calibration curves using *O*-*t*BHA derivatized 7-OEG AST and [$^{15}N_5$]-7-OEG IST in HPLC water were generated by diluting from derivatized stock standards with each sample set. Standard curves were calculated using the peak area ratio of 7-OEG-*t*BHA to IST versus fmol of 7-OEG-*t*BHA injected (Figure 5). The amounts of both 7-OEG and exogenously derived [$^{13}C_2$]-7-OEG in each sample were calculated from the ratio of the AST and IST peak areas.

Injection of a high amount of [$^{15}N_5$]-7-OEG-*t*BHA IST (>3 pmol) showed no contribution to either the endogenous or the exogenous mass transitions. The assay performance was evaluated by adding known amounts of 7-OEG to freshly isolated CtDNA. DNA was quantified by UV before spiking 50 μg aliquots with 3.2, 32, and 64 fmol of 7-OEG and 640 fmol of IST. Sample sets were assayed on two different days by separate analysts, and results are summarized in Table 1. The endogenous 7-OEG concentration found in CtDNA was determined to be 2.4 ± 0.8 fmol (n = 5) per 50 μg of DNA.

Reaction of [$^{13}C_{18}$]-EtLa with CtDNA. The reaction of [$^{13}C_{18}$]-EtLa with CtDNA was carried out according to a previously published method[29] with minor modifications. Ten milligrams of [$^{13}C_{18}$]-EtLa (100 μL, 100 mg/mL in methanol) was added to 300 μg of CtDNA in 1 mL of 50 mM sodium phosphate buffer (pH 7.4). After the addition of 10 μL of *t*-butylhydroperoxide (70% solution, 70 μmol), the mixture was incubated at 37 °C for 24 h. After the addition of 1 mL of water, 10 μL of 2% butylated hydroxytoluene in isopropanol (w/v), and 0.5 mL of methanol to the reaction mixture, the sample was extracted two times with 2 mL of chloroform containing 0.5% *t*-butylhydroperoxide (w/v); the aqueous layer was dried under vacuum, and the sample was resuspended in 1 mL of water. Samples were left at 4 °C overnight to rehydrate and stored at −80 °C until analysis.

RESULTS AND DISCUSSION

Although 7-OEG lacks miscoding properties, it is the major DNA adduct formed by VC and is therefore an important biomarker for VC exposure. This paper details a new approach to determine the formation and persistence of 7-OEG. We developed a sensitive LC-MS/MS analysis to determine 7-OEG

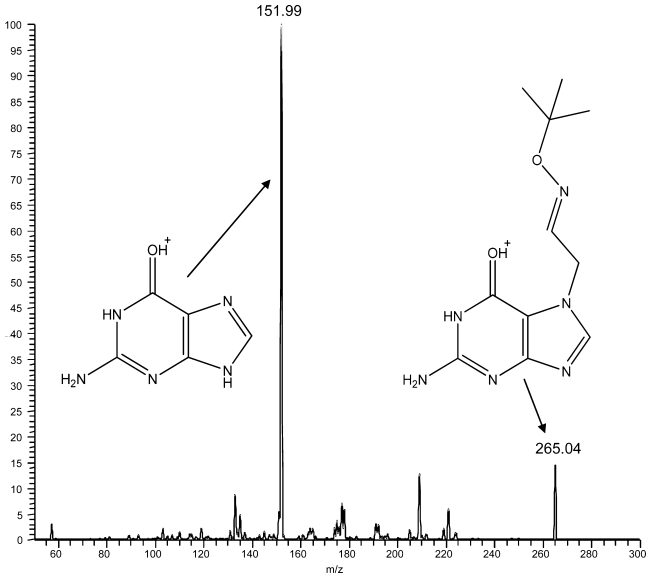

Figure 4. Product ion spectrum of 7-OEG-*t*BHA (precursor ion *m*/*z* 265). The spectrum was obtained by injecting 6.4 pmol of 7-OEG-*t*BHA on the LC system, selecting *m*/*z* 265 in Q1, and scanning Q3 from *m*/*z* 50 to 300. The argon collision gas pressure was 1.5 mTorr, and the collision energy was 20 eV.

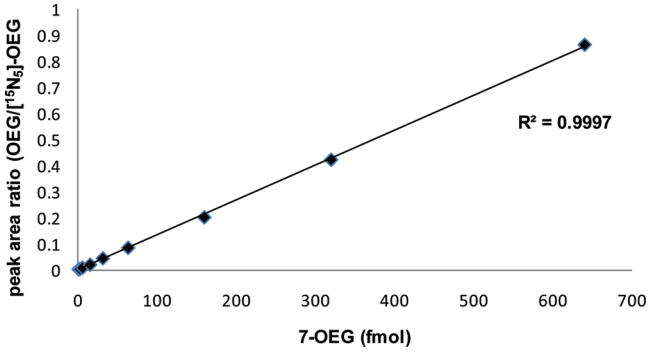

Figure 5. Calibration curve for 7-OEG-*t*BHA obtained under SRM conditions using the present LC-MS/MS method. The plot shows the peak area ratio of 7-OEG-*t*BHA to IST vs increasing fmol of 7-OEG-*t*BHA. Data points correspond to standard mixtures that give 1.6, 3.2, 6.4, 16, 32, 64, 160, 320, and 640 fmol of 7-OEG-*t*BHA and 640 fmol of [$^{15}N_5$]-7-OEG-*t*BHA on column.

concentrations in tissue DNA of male Sprague−Dawley rats exposed to 1100 ppm VC for 1 day or [$^{13}C_2$]-VC for 5 days, including postexposure periods of 2, 4, or 8 weeks. The new 7-OEG assay utilized an oximation reaction with O-*t*BHA, which allowed us to measure 7-OEG as a more stable oxime adduct. Using this new highly sensitive assay, we determined that 7-OEG concentrations were ∼200-fold higher than εG in liver of adult rats similarly exposed to VC.[48] Laib et al. reported similar results for adduct formation in liver of rats exposed to VC (1:100 ratio).[12]

The exposure of adult and weanling rats to 1100 ppm [$^{13}C_2$]-VC for 1 week induced [$^{13}C_2$]-7-OEG in liver, lung, kidney, spleen, brain, and testis as summarized in Table 2. Stable isotope-labeled VC exposures allowed us to distinguish 7-OEG from [$^{13}C_2$]-7-OEG and demonstrated that 7-OEG is formed endogenously. This is clearly shown in Figures 1 and 2, which contrast chromatograms from an unexposed control animal to one subjected to 5 days of [$^{13}C_2$]-VC exposure. The greatest

Table 1. Intraday and Interday Assay Variability Summarized for 50 μg Aliquots of CtDNA Spiked with Increasing Amounts of 7-OEG[a]

fmol of 7-OEG spiked	fmol of 7-OEG found day 1 (n = 5)	fmol of 7-OEG found day 2 (n = 5)	combined interday results (n = 10)
3.2	7.9 ± 3.3	7.4 ± 3.3	7.6 ± 3.1
32	34.8 ± 7.3	38.3 ± 7.7	36.6 ± 7.1
64	70.4 ± 10.3	69.0 ± 9.2	70.0 ± 9.2

[a]Data are presented as the mean fmol found per sample ± standard deviation. Amounts measured in this standard deviation are the combination of the endogenous concentration and spiked concentration 7-OEG.

concentration of [$^{13}C_2$]-7-OEG detected in DNA from adult rats exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days was found in liver, with 104.0 ± 23.0 adducts per 10^6 guanine. Lung, kidney, and spleen had the next highest concentrations of [$^{13}C_2$]-7-OEG with 39.0 ± 2.0, 28.0 ± 7.0, and 10.0 ± 2.0 adducts per 10^6 guanine, respectively. Brain (3.0 ± 0.3) and testis (1.0 ± 0.1) had the lowest number of [$^{13}C_2$]-7-OEG adducts per 10^6 guanine.

We found that the concentrations of exogenous 7-OEG adducts were ∼50-fold higher than endogenous 7-OEG in adult livers and ∼300-fold higher in weanling rat livers. The endogenous 7-OEG adduct concentrations in target tissues in adult and weanling rats showed no significant difference. Therefore, we conclude that there is no effect of age on the endogenous 7-OEG formation. The ratio of exogenous/endogenous 7-OEG in adult rats was ∼200-fold in lung, ∼40-fold in kidney, ∼4-fold in brain, and ∼1.7-fold higher in spleen, while in testis it was ∼0.1-fold.

The formation of [$^{13}C_2$]-7-OEG in liver of weanling rats was ∼3-fold greater than adult rats, with 299.0 ± 129.0 adducts per 10^6 guanine. This is thought to be due to age-related differences in metabolism due to greater CYP2E1 activity in weanling rats. A recent study has shown up to 2-fold higher CYP2E1 activity in 3 week old rat liver.[54] In comparison to the adult rats, weanling rats had [$^{13}C_2$]-7-OEG concentrations in lung, kidney, and spleen that were approximately 2-fold higher than adults (Tables 2 and 3). Three-fold higher [$^{13}C_2$]-7-OEG was also detected in brains of weanling rats exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days, as compared to the exogenous concentrations found in similarly exposed adult rats, but was still 30 times lower than weanling liver. The lowest exogenous 7-OEG was detected in spleen for both weanling and adult rats, which might suggest that P450 metabolism of VC in this tissue is minimal. In contrast, endogenous 7-OEG formation was highest in spleen and testis for both adult and weanling rats as compared to other tissues.

The persistence of 7-OEG was also determined in adults rats euthanized 2, 4, and 8 weeks after exposure to [$^{13}C_2$]-VC for 5 days (Table 2). The half-life of [$^{13}C_2$]-7-OEG was calculated in liver and lung DNA by using the plot of log (adducts) versus days (Figure 6). The half-life of [$^{13}C_2$]-7-OEG in liver and lung was ∼4 days, which is consistent with other studies on N7-guanine adducts. This is thought to primarily represent chemical depurination.

Previously, 7-OEG formation was determined by Fedtke et al. in rats exposed to 600 ppm VC (1 week).[10] DNA (1−2 mg) was depurinated by acid hydrolysis and analyzed by HPLC using fluorescence detection with a limit of detection 10 pmol 7-OEG per μmol guanine. 7-OEG was shown to be 144 times higher than εG. While the data obtained from these studies

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Table 2. 7-OEG (Endogenous) and [$^{13}C_2$]-7-OEG (Exogenous) Adduct Concentrations Determined from Adult (n = 4) Rats Exposed to 1100 ppm [$^{13}C_2$]-VC for 5 Days and Cage Controls

	adult control		adult postexposure time							
			2 h		2 weeks		4 weeks		8 weeks	
	7-OEG	$^{13}C_2$-7-OEG	7-OEG	$^{13}C_2$-7-OEG	7-OEG	$^{13}C_2$-7-OEG	7-OEG	$^{13}C_2$-7-OEG	7-OEG	$^{13}C_2$-7-OEG
liver (add/10^6 gua)	1.3 ± 0.5	–	2.0 ± 1.0	104.0 ± 23.0	1.0 ± 0.3	4.0 ± 3.0	1.0 ± 0.4	1.0 ± 0.6	1.0 ± 0.7	ND
lung (add/10^6 gua)	0.6 ± 0.1	–	0.2 ± 0.1	39.0 ± 2.0	0.4 ± 0.1	2.0 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.8 ± 0.4	ND
kidney (add/10^6 gua)	1.2 ± 0.4	–	0.7 ± 0.4	28.0 ± 7.0	0.4 ± 0.2	1.0 ± 0.6	0.2 ± 0.1	ND	1.5 ± 0.6	ND
brain (add/10^6 gua)	3.0 ± 2.0	–	0.8 ± 0.5	3.0 ± 0.3	NA	NA	NA	NA	NA	NA
spleen (add/10^6 gua)	3.2 ± 1.5	–	6.0 ± 4.0	10.0 ± 2.0	NA	NA	NA	NA	NA	NA
testis (add/10^6 gua)	2.3 ± 1.5	–	9.0 ± 4.0	1.0 ± 0.1	NA	NA	NA	NA	NA	NA

Table 3. 7-OEG (Endogenous) and [$^{13}C_2$]-7-OEG (Exogenous) Adduct Concentrations Determined from Weanling (n = 8) Rats Exposed to 1100 ppm [$^{13}C_2$]-VC for 5 Days and Cage Controls

	weanling control		weanling 2 h postexposure	
	7-OEG	$^{13}C_2$-7-OEG	7-OEG	$^{13}C_2$-7-OEG
liver (add/10^6 gua)	0.8 ± 0.1	–	1.0 ± 0.5	299.0 ± 129.0
lung (add/10^6 gua)	0.5 ± 0.1	–	0.4 ± 0.1	83.0 ± 19.0
kidney (add/10^6 gua)	2.7 ± 1.0	–	0.8 ± 0.3	66.0 ± 12.0
brain (add/10^6 gua)	5.0 ± 2.0	–	1.0 ± 0.3	10.0 ± 0.8
spleen (add/10^6 gua)	3.8 ± 1.1	–	7.0 ± 5.0	16.0 ± 2.0
testis (add/10^6 gua)	1.0 ± 0.6	–	6.0 ± 0.2	1.2 ± 0.1

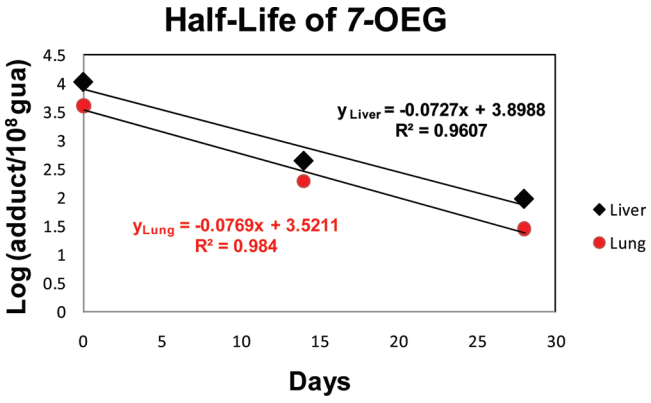

Figure 6. Plot of log (7-OEG/10^8 gua) vs days was used to determine the half-life of 7-OEG in the liver and lung of adult rats exposed to [$^{13}C_2$]-VC (1100 ppm, 5 days, 6 h per day) and euthanized at the end of exposure or 2, 4, or 8 weeks later. The half-life of 7-OEG in liver and lung is 4 days on the basis of the equations.

gave important information on DNA adduct formation from VC exposures, it lacked the sensitivity of the present LC-MS/MS assay. Using our mass spectrometry method, we were able to quantify DNA adducts by using a stable isotope-labeled 7-OEG IST, while in the previous HPLC method, adducts were quantified by external calibration using CtDNA spiked with 7-OEG. In the previous studies, neither endogenous 7-OEG in control samples, nor exogenous 7-OEG in brain and spleen of exposed weanling rats could be detected. In comparison, the adduct ratio of 7-OEG was 1:1 in lung of adult and weanling rats, whereas in liver it was 4-fold higher in weanlings. The half-life of 7-OEG was also calculated as 63 h, while in the present study, it was ~96 h.[10]

Morinello et al. also examined 7-OEG concentrations in hepatocyte and brain DNA of rats exposed to 1100 ppm VC for 1 week (6 h/day, 5 days/week) and demonstrated that 7-OEG was only detectable in hepatocyte DNA at 4 ± 0.9 adducts per 10^6 guanine.[47] 7-OEG was measured as 7-HEG after Na-CNBH$_3$ reduction and analyzed by LC-MS/MS with a limit of detection 0.3 adducts per 10^6 guanine. Because only DNA of unlabeled VC exposed rats was analyzed, no distinction was made between endogenous and exogenous 7-OEG formation. 7-OEG concentrations reported by Morinello et al.[47] were ~25-fold lower than exogenous 7-OEG concentrations that we report in this study (Table 2). Because the sufficiency of NaCNBH$_3$ reduction of 7-OEG is not known, we suggest that incompletion of the reduction of 7-OEG in DNA might be an important factor for the lower 7-OEG concentration found in that study. For comparison reasons, we also analyzed lung, kidney, and brain DNA of rats exposed to [$^{13}C_2$]-VC from Morinello et al. study.[47] Endogenous and exogenous 7-OEG concentrations in those samples were very similar to the concentrations reported in this study (Table 4).

Table 4. 7-OEG (Endogenous) and [$^{13}C_2$]-7-OEG (Exogenous) Adduct Concentrations Determined from Adult Rats Exposed to 1100 ppm [$^{13}C_2$]-VC for 5 Days[a]

	adult control		adult 2 h postexposure	
	7-OEG	$^{13}C_2$-7-OEG	7-OEG	$^{13}C_2$-7-OEG
lung (n = 8) (add/10^6 gua)	NA	–	0.6 ± 0.2	31.0 ± 11.0
kidney (n = 7) (add/10^6 gua)	NA	–	0.8 ± 0.4	22.0 ± 3.0
brain (n = 3, 1)* (add/10^6 gua)	5.0 ± 1.0	–	1.0	2.0

[a]Tissues were analyzed from Morinello et al.[47] *Brain control, n = 3; brain exposed, n = 1.

The data obtained from CtDNA, control, and [$^{13}C_2$]-VC-exposed animals demonstrated that 7-OEG is formed endogenously in relatively high concentrations in comparison to other endogenous N7-guanine adducts. While endogenous concentrations of 7-HEG in tissues from control rats and mice were reported to range from 1 to 4 adducts per 10^8 nucleotides,[55,56] endogenous N7-methylguanine ranged from 2 to 2.5 adducts per 10^7 nucleotides.[57]

On the basis of previous data showing the formation of etheno DNA adducts by LPO, we hypothesized that endogenous 7-OEG was also formed as a result of LPO.[52] Previously, the key LPO intermediates that have been identified for the formation of DNA adducts[19,58−60] are the result of reactions of HNE, ONE, and MDA with dG and are associated with DNA adducts formed by VC exposure.[58]

Previously, the lack of evidence for endogenous 7-OEG formation did not suggest a rationale for studying its formation

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Table 5. 7-OEG (Endogenous + Exogenous) Adduct Concentrations Determined from Weanling (n = 8) and Adult (n = 4) Rats Exposed to 1100 ppm VC for 1 Day

	7-OEG			
	liver (add/10^6 Gua)	lung (add/10^6 Gua)	kidney (add/10^6 Gua)	brain (add/10^6 Gua)
weanling 1 day exposure	84.0 ± 47.0	37.0 ± 11.0	28.0 ± 12.0	1.5 ± 0.6
adult 1 day exposure	64.0 ± 49.0	28.0 ± 6.0	19.0 ± 3.0	1.6 ± 0.5

in vitro and/or in vivo. The detection of endogenous 7-OEG in the present study (Figures 1 and 2) prompted us to examine its route of formation. We tested the reaction of CtDNA with [$^{13}C_{18}$]-EtLa under peroxidizing conditions.[52] The formation of 7-OEG by direct alkylation was evaluated by monitoring the incorporation of the ^{13}C-labeled stable isotope from EtLa into 7-OEG (Figure 3). Previously, Chung et al.[61] and Ham et al.[52] had demonstrated the formation of etheno adducts 1,N^2-ethenoguanine, 1,N^6-ethenodeoxyadenine, and εG as a result of the reaction between dG with HNE, CtDNA with HNE and EtLa under peroxidation conditions.

7-OEG formation in adult and weanling rats exposed to 1100 ppm VC for 1 day was also determined (Table 5). In adult and weanling rats exposed to unlabeled VC, the concentration of 7-OEG adduct was 64.0 ± 49.0 and 84.0 ± 47.0 in liver, 28.0 ± 6.0 and 37.0 ± 11.0 in lung, 19.0 ± 3.0 and 28.0 ± 12.0 in kidney, 1.6 ± 0.5 and 1.5 ± 0.6 adducts per 10^6 guanine in brain, respectively. These animals were exposed to VC to test the nose-only inhalation apparatus before starting [$^{13}C_2$]-VC exposures but provided additional information about the effect of age, tissue, and duration of exposure on 7-OEG formation. The age-dependent formation of 7-OEG in liver from weanling rats exposed to 1100 ppm VC (1 day) was ∼1.3-fold greater than the concentrations in liver of adult rats exposed to 1100 ppm VC (1 day). The concentration of 7-OEG in lung, kidney, and brain from weanling rats was similar to adult rats exposed for the same duration. While 7-OEG adducts in liver, lung, and kidney were higher than control animals for both adult and weanling rats, in brain, it was similar to that of control animals. These data, however, reflect a mixture of endogenous and exogenous adducts, as no distinction between the two could be made.

In this study, we determined the concentration of 7-OEG in liver, lung, kidney, spleen, testis, and brain DNA from animals exposed to VC and [$^{13}C_2$]-VC and demonstrated the presence of endogenous 7-OEG in brain, liver, lung, kidney, spleen, and testis. The persistence of 7-OEG in liver, lung, kidney, and spleen was also demonstrated in this study. The molecular dosimetry data suggest that not only liver but also other organs such as lung and kidney might be considered target organs for VC-induced carcinogenesis following inhalation exposure. Because of the reactivity and short half-life of CEO, 1.6 min in aqueous solution at neutral pH,[62] we hypothesize that VC is metabolized in each organ by P450 rather than arising via the circulation of CEO in the body. The lower exogenous adduct level in brain, spleen, and testis supports this.

The data described in this study and its companion studies will help to understand the relative formation of endogenous and exogenous DNA adducts of VC. The identification of 7-OEG and the etheno DNA adducts as endogenous DNA lesions has important implications for risk assessment since background amounts will always be present. The new data support the carcinogenesis and epidemiology findings that VC is causally associated with cancer following relatively high exposures. In contrast, the number of endogenous adducts is greater than identical exogenous adducts formed at low exposures.

In summary, we developed a new sensitive, selective, and efficient LC-MS/MS method for quantification of 7-OEG in DNA. For the first time, endogenous 7-OEG was detected in control rats and [$^{13}C_2$]-VC exposed rats, and its endogenous formation from LPO was confirmed in vitro in CtDNA. The half-life of 7-OEG in liver and lung was determined to be ∼4 days.

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ABBREVIATIONS

VC, vinyl chloride; CEO, chloroethylene oxide; 7-OEG, 7-(2-oxoethyl)guanine; εG, N^2,3-ethenoguanine; εdA, 1,N^6-ethenodeoxyadenosine; εdC, 3,N^4-ethenodeoxycytidine; ROS, reactive oxygen species; LPO, lipid peroxidation; ONE, 4-oxo-2-nonenal; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; M_1G, pyrimido[1,2-a]-purin-10(3H)-one; 7-HEG, 7-(2-hydroxyethyl)-guanine; CtDNA, calf thymus DNA; EtLa, ethyl linoleate; AST, analyte standard; IST, internal standard; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; *O*-*t*BHA, *O*-*t*-butyl hydroxylamine; HESI, heated electrospray ionization; SRM, selected reaction monitoring

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