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DEVELOPMENT AND APPLICATION OF AN LC-MS/MS METHOD FOR THE DETECTION OF THE VINYL CHLORIDE-INDUCED DNA ADDUCT *N*^{2},3-ETHENOGUANINE IN TISSUES OF ADULT AND WEANLING RATS FOLLOWING EXPOSURE TO [$^{13}C_2$]-VC

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Abstract

In the 1970s exposure to vinyl chloride (VC) was shown to cause liver angiosarcoma in VC workers. We have developed a new LC-MS/MS method for analyzing the promutagenic DNA adduct *N*^{2},3-ethenoguanine (εG) and have applied this to DNA from tissues of both adult and weanling rats exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days or 1100 ppm VC for 1 day. This assay utilizes neutral thermal hydrolysis and an HPLC clean-up prior to quantitation by LC-MS/MS. The number of endogenous and exogenous εG adducts in DNA from tissues of adult rats exposed to [$^{13}C_2$]-VC for 5 days was 4.1±2.8 adducts/10^8 guanine of endogenous and 19.0±4.9 adducts/10^8 guanine of exogenous εG in liver, 8.4±2.8 adducts/10^8 guanine of endogenous and 7.4±0.5 adducts/10^8 guanine of exogenous εG in lung and 5.9±3.3 adducts/10^8 guanine of endogenous and 5.7±2.1 adducts/10^8 guanine of exogenous εG in kidney (n=4). Additionally, the data from weanling rats demonstrated higher numbers of exogenous εG, with ~4 fold higher amounts in liver DNA of weanlings (75.9±17.9 adducts/10^8 guanine) in comparison to adult rats and ~2 fold higher amounts in lung (15.8±3.6 adducts/10^8 guanine) and kidney (12.9±0.4 adducts/10^8 guanine) (n=8). The use of stable isotope labeled VC permitted accurate estimates of the half life of εG for the first time by comparing [$^{13}C_2$]-εG in adult rats with identically exposed animals killed 2, 4 or 8 weeks later. The half life of εG was found to be 150 days in liver and lung and 75 days in kidney, suggesting little or no active repair of this promutagenic adduct.

Introduction

Vinyl chloride (VC) is widely used for the production of polyvinyl chloride polymers (PVC) and is also present in tobacco smoke, PVC pipes, packaging products, and ground water as a result of microbial degradation of trichloroethylene (TCE) and perchloroethylene (PCE) (1–5). Induction of hepatic angiosarcomas in VC workers was first recognized in the 1970s, resulting in the classification of VC as a known human carcinogen (6–11). Studies in VC-exposed rodents also showed cancer at multiple organ sites (12–15).

After exposure via inhalation or oral uptake, VC is metabolized by CYP2E1 to chloroethylene oxide (CEO), which rapidly rearranges to chloroacetaldehyde (CAA) (16–19). CEO and CAA are genotoxic metabolites known to alkylate nucleic acids and proteins (20–23) and have been shown to be mutagenic in bacteria, yeast, and mammalian cells

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(24;25). Several DNA adducts have been reported to form from VC exposure *in vivo* and *vitro*(26–30). Although 7-(2-oxoethyl)guanine (7-OEG) is the major DNA adduct of VC exposure, it does not cause mispairing during DNA replication. N^2,3-Ethenoguanine (εG) is mutagenic, resulting in G→A transitions (31), making it an important biomarker of VC. εG has also been detected endogenously in unexposed humans and animals with concentrations in rat liver appearing lower than those in human liver (32–34). It is believed that the major source of endogenous εG is lipid peroxidation (35–37). Therefore, to specifically determine exposure-related εG, it is critical to develop a specific method to measure both endogenous and exogenous εG.

Previously, the formation of εG *in vivo* was determined by GC-NICI-MS and immunoaffinity coupled-GC/HRMS methods (27–30;38–41). Fedtke *et al*. demonstrated the formation and persistence of εG in rats exposed to 600 ppm VC for 5 days by GC-NICI-MS. The concentration of εG adducts was highest in liver, followed by kidney and lung, while no adducts were detected in brain or spleen. The adduct amounts determined from this study were later found to be derived from both endogenous and exogenous sources. This study was followed by the work of Morinello *et al.* (39–41) using an immunoaffinity coupled-GC/HRMS approach. εG was separated from DNA hydrolysates by immunoaffinity columns specific for N^2,3-εG adducts and quantified by high resolution GC-MS after derivatization with pentafluorobenzylbromide (38). This study was the first use of mass spectrometry to demonstrate the relationship between endogenous and exogenous DNA adducts in the same animals by exposing them to [$^{13}C_2$]-VC. The molecular dose of εG was determined in rats exposed to 0, 10, 100, and 1100 ppm VC for 1 or 4 weeks and showed a rapid increase in εG adducts in liver DNA between 10 and 100 ppm exposures, followed by a greatly decreased rate of formation at 1100 ppm.

The present investigation describes a specific and sensitive LC-MS/MS assay, which enabled us to detect εG DNA adducts in tissues from adult and weanling rats exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days (6hrs/day) or 1100 ppm VC for 1 day. In addition to quantifying endogenous and exogenous adducts after exposure to the stable isotope-labeled VC, the half life of [$^{13}C_2$]-εG in these target organs was determined.

Chemicals

VC is a known carcinogen and should be handled carefully with protective equipment (i.e., gloves and laboratory coat) in an operating fume hood. [$^{13}C_4{}^{15}N_2$]-N^2,3-εG was previously synthesized by Ham *et al.* (42). [$^{13}C_2$]-VC (98%+ chemical purity; 99% isotopic purity) was obtained from Cambridge Isotope Laboratories (Andover, MA). HPLC grade water, methanol, and acetic acid were purchased from Thermo Fisher Scientific. RNase A, calf thymus DNA, and dG were purchased from Sigma Aldrich (St. Louis, MO). Nucleic acid purification grade lysis buffer, protein precipitation solution and proteinase K were purchased from Qiagen (Valencia, CA).

Animals, Exposure and Tissue Collection

Exposures were conducted at the USEPA NHEERL facility in Research Triangle Park, NC. All procedures that involved the use of animals were approved by the Institutional Animal Care and Use Committee. For the adult study, 10 week old (~300 g) and for the weanling study, 21 day old (50–60 g) Sprague Dawley male 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 air exposed to 1100 ppm VC in a nose-only inhalation apparatus for 1 day [6 h/day, 1 day] or to 1100 ppm [$^{13}C_2$]-VC [6 h/day] for 5 days. The vinyl chloride vapor was generated by metering pure [$^{13}C_2$]-vinyl chloride vapor into a mixing chamber with medical grade air

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using mass flow controllers (Tylan Instruments, Torrance CA). Vinyl chloride was delivered to the inlet of a 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 vinyl chloride (Scott Gas, 99.96% purity). The 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. At the end of exposure, the rats were anesthetized with Euthasol® by intraperitoneal injection and killed by exsanguination via the vena cava. The liver, lung, kidney, spleen, and brain were removed, frozen on dry ice, and stored at −80 °C.

DNA Isolation

DNA isolation from tissues was performed as previously described (40) with minor modifications using the Gentra Systems DNA extraction kit. The homogenized tissues were lysed in lysis buffer with 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 was then resuspended in lysis buffer with TEMPO and incubated with RNase A at 37 °C for 30 min, 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 Hydrolysis

DNA solutions (250 μg) were spiked with the internal standard (200 fmol, [$^{13}C_4{}^{15}N_2$]-N^2,3-εG) and diluted to 500 μL with HPLC-grade water. Samples were incubated at 100°C for 40 min. Immediately after incubation, samples were cooled on ice. The DNA backbone was separated from solution by Microcon 10 filtration (11500 rpm, 4 °C, 40 min). After the filtrate was separated, an additional 400 μL HPLC-grade water was added to the retentate to wash the DNA backbone (11500 rpm, 4 °C, 40 min) and both filtrates were combined. Samples were enriched for εG using an Agilent 1200 HPLC system equipped with a Beckman Ultrasphere C18 column (250 × 4.6 mm, 5μm). Following the injection of samples, the column was eluted at a flow rate of 1 mL/min with a 7–80 % MeOH gradient in 10mM ammonium acetate buffer as follows: 7 %–80 % MeOH over 17 min; hold at 80 % for 6 min; reequilibrate at 7 % for 7 min. εG was isolated by collection of a fraction between 16.10 min and 19.10 min. The fractions were dried by vacuum evaporation in a SpeedVac concentrator for LC-MS/MS analysis. The dried fractions were transferred to LC-MS/MS vials by rehydration with HPLC-grade water (3 × 100 μL), dried under vacuum, then dissolved in 20 μL of water for LC-MS/MS analysis.

LC-MS/MS Analysis

Quantitative LC-MS/MS data were obtained using a Waters Acquity UPLC coupled to a Thermo Finnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer. A heated electrospray ionization (HESI) interface was operated in positive ionization mode. Tandem MS/MS quantitation of the molecular ions was achieved by using a pseudo-selected reaction monitoring (pseudo-SRM) method and monitoring the signals *m/z* 176 → 176 for endogenous εG, *m/z* 178 → 178 for [$^{13}C_2$]-εG, and *m/z* 182 → 182 for [$^{13}C_4{}^{15}N_2$]-εG internal standard (IST). Explanation for this mass spectrometric technique is given later with Results and Discussion. Samples were kept at 4 °C during analysis, and the sample injection volume was 15 μL. Separation was performed on an Aquasil C18 column (150 × 1 mm, 3) with a flow rate of 0.05 mL/min using the following gradient (A) 0.1% acetic acid in water and (B) methanol; 0 min, 1% B; 0–10 min, 80% B; 10–14 min, 80 % B; 14–15 min, 1 % B; 15–20 min, 1 % B.

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Instrument conditions were optimized for maximum signal of εG by direct infusion of analyte and internal standard. MS settings were as follows: electrospray voltage (3000 V), ion transfer 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 of argon). Collision energy and collision gas pressure were chosen to achieve lowest background noise possible without appreciably degrading transmission of the molecular ions. Calibration curves using pure εG and [$^{13}C_4{}^{15}N_2$]-εG internal standard in water were generated with each sample set. Curves were calculated using the peak area ratio of εG to internal standard versus fmol εG injected. The amounts of both εG and exogenously derived [$^{13}C_2$]-εG were calculated against the εG calibration curves.

Results and Discussion

Etheno adducts are formed after exposure to VC and vinyl carbamate (urethane), as well as endogenously as a result of lipid peroxidation (35–37). The DNA adducts formed from VC exposure provide critical information for understanding the molecular dosimetry of VC. Although 7-OEG is the major DNA adduct of VC, it has a short half life and is rapidly lost from DNA by chemical depurination (29;30). In contrast, the etheno adduct, εG, is persistent in DNA, and little is known about its repair *in vivo*. εG is known to cause G→A transitions (31). While smaller amounts of εG adducts are formed in comparison to 7-OEG following VC exposure, the persistence of εG may play an important role in VC-induced carcinogenicity. The promutagenic properties and persistence of εG highlight the need for the development of a reliable and specific method for the detection and quantification of this biomarker following VC exposure.

In this study we developed a new sensitive LC-MS/MS analysis to determine the amount of endogenous and exogenous εG in liver, lung, kidney, spleen and brain DNA of rats exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days. Following neutral thermal hydrolysis, εG was separated from DNA hydrolysates by HPLC fraction collection and measured by LCMS/MS. In comparison with an established GC-MS assay (40), which used immunoaffinity enrichment and derivatization, the new LC-MS/MS assay eliminated these steps to become more time efficient. Our LC-MS/MS method was based on the previously published LC-ESI/MS method by Yen *et al.* (43). The present method provided increased sensitivity with a lower limit of detection (LOD): (3 fmol in pure standards) and allowed quantitation of endogenous levels of εG in 250 ug of DNA isolated from liver, lung, and kidney with a LOQ of 4–5 fmol. During method development, it was apparent that neutral thermal hydrolysis followed by HPLC fraction collection *vs*. acid hydrolysis followed by immunoaffinity enrichment or HPLC fraction collection resulted in cleaner samples and lower background for LC-MS/MS analysis. We added acetic acid to the mobile phase to enhance ionization in the presence of matrix instead of using the previous water/methanol mobile phase (43). This change resulted in linear standard curves over a wider dynamic range (Figure 1). The previous method measured εG in selected ion monitoring (SIM) mode with a LOD of 50 fmol per injection in samples. The fragmentation pattern of εG, whose molecular ion is *m/z* 176 for $[M+H]^+$, was previously investigated by Yen, *et al.*, and showed product ions at *m/z* 135 for $[(M+H)^+$-a-1] and 94 for $[(M+H)^+$-a-b] (42). Figure 2 shows the proposed fragments of εG and a product ion scan acquired during the present study. According to Yen, *et al.*, high collision energy was needed to determine the product ion spectrum, but this resulted in low ion abundances. The lack of fragmentation prompted Yen, *et al.* to use SIM to optimize instrument sensitivity. The present tandem MS/MS method actually takes advantage of εG's stability during collision-induced fragmentation by significantly reducing background noise, while leaving the abundance of the molecular ion virtually unchanged. The use of collision gas and moderate collision energy in the new instrument method eliminated much of the non-specific ion background at *m/z* 176, which allowed reproducible detection of 3 fmol εG per

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injection. Higher background signal in SIM mode prevented quantitation of low amounts of endogenous εG, whereas the lower limit of detection allowed by MS/MS analysis could readily detect this. Consequently, we measured εG as *m/z* 176 $[M+H]^+ \rightarrow 176$ $[M+H]^+$, exogenous $[^{13}C_2]$-εG as *m/z* 178→178, and $[^{13}C_4{}^{15}N_2]$-N^2,3-εG internal standard as *m/z* 182→182 under pseudo-SRM conditions. Figure 3 compares detection of 3 fmol εG in SIM and pseudo-SRM and shows the significant improvement in detection limit using the new pseudo-SRM instrument parameters.

Assay performance was tested by adding known amounts of εG to freshly isolated calf thymus DNA. DNA was quantitated by UV before spiking 250 μg aliquots with 5, 20, or 50 fmol εG and 200 fmol internal standard. The low spike level of 5 fmol was chosen to show assay performance close to LOQ. Sample sets were assayed on two different days by separate analysts, and the results are summarized in Table 1.

The results for liver, lung and kidney from adult and weanling exposures to $[^{13}C_2]$-VC are shown in Table 2. The data demonstrated that after the exposure to 1100 ppm $[^{13}C_2]$-VC, exogenous εG adducts were formed in liver, lung and kidney. No $[^{13}C_2]$-εG adducts were detected in adult and weanling brain or spleen DNA. The amount of endogenous εG in liver of adult rats was similar in amount to that measured previously in our lab (40). One of the advantages of $[^{13}C_2]$-VC exposures was that one can differentiate adducts derived from endogenous sources (lipid peroxidation) and exogenous sources in the same sample. This is clearly shown in Figure 4, which contrasts chromatograms from an unexposed control animal with 5 day $[^{13}C_2]$-VC exposure. In this study it was found that the amounts of exogenous εG adducts in adult rat livers were 4.6-fold higher than endogenous εG and ~50-fold higher in weanling rat livers. The endogenous εG amounts in lung and kidney were measured in adult and weanling rats for the first time, which enabled us to compare the effect of age on the endogenous formation of εG. In lung and kidney the ratio of exogenous:endogenous εG was ~1:1 in adult tissues and ~3:1 in weanlings. There was no significant difference in the number of endogenous εG adducts in lung and kidney for adults *vs* weanling animals, but endogenous εG in liver was 2.6 fold higher in adult rats than in weanling rats. Similar findings were also reported previously by Morinello *et al.*(40). Furthermore, the amount of endogenous εG was not affected by VC exposure. It is not clear how much of the difference in the amount of endogenous εG in liver of weanling versus adult rats is due to formation versus dilution due to rapid cell replication in weanling rats.

εG was readily induced in target organs for VC carcinogenesis after 1100 ppm $[^{13}C_2]$-VC exposures in both adult and weanling rats. The concentration of $[^{13}C_2]$-εG adducts was greatest in liver as expected, followed by lung and kidney in both adult and weanling rats. The amount of exogenous εG in weanling rat liver was 4-fold greater than that found in adult rats, which is thought to be due to age related differences in metabolism. Exposure to 1100 ppm $[^{13}C_2]$-VC did not induce detectable amounts of εG in brains of either adult or weanling rats, while the endogenous εG adducts in adults were 2.18±0.31 adducts/10^8 guanine, which is consistent with the previous study by Morinello *et al*(34;40). No endogenous or exogenous εG was detected in splenic DNA of rats exposed to $[^{13}C_2]$-VC, which is also similar to previous findings (28). It is not clear why endogenous εG has not been detected in spleen, but the data suggest that lipid peroxidation is minimal in this tissue.

This study was particularly designed to determine the half life of εG in tissues of adult rats exposed to $[^{13}C_2]$-VC. The number of endogenous and exogenous adducts in lung and kidney were similar. The half life of εG was calculated in liver, lung, and kidney DNA in adult rats sacrificed 2, 4, and 8 wks after exposure to $[^{13}C_2]$-VC by using the plot of log (adducts) versus days. The half life of εG in liver and lung was ~150 days, while in kidney it was ~75 days (Figure 5). Previous analysis of εG in Sprague-Dawley rats exposed to 600

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ppm VC (5 days, 4h/day) and 3, 7 and 14 days post exposures demonstrated the half life of εG to be 30 days (28), but that study could not differentiate between exogenous and endogenous εG. Thus, the present data are the only information that accurately determines the half life of εG. The extremely long half lives of εG in liver, lung and kidney suggest that εG is not actively repaired. It is more likely that the small decrease in εG over 8 weeks post exposure is the result of cell replication and cell death. In related studies on 7OEG and 1,N^6-ethenodeoxyadenosine (εdA) on these tissues, rapid repair of εdA ($T_{1/2}$ ~ 24 hours) and loss of 7-OEG ($T_{1/2}$ ~4 days) demonstrate major differences between these three adducts (unpublished data).

We also determined the number of εG adducts in liver and lung DNA of rats exposed to 1100 ppm VC for 1 day (Table 3). In adult and weanling rats exposed to unlabeled VC, the amount of εG in liver was 5.85±0.84 and 11.16±2.86 adducts/10^8 guanine, respectively, and in lung, 7.63±4.80 and 3.78±0.66 adducts/10^8 guanine. These animals were exposed to VC to test the nose-only inhalation apparatus before starting [$^{13}C_2$]-VC exposures, but provided additional information on tissue and age differences in εG. εG adduct concentrations in weanling rat liver were ~2 fold higher than in adult rats, whereas it was slightly less in weanling rat lung that in adult rat lung. The decreased differences between the data from these animals, in contrast to the [$^{13}C_2$]-VC exposed rats, are most likely due to the combined measurement of endogenous and exogenous εG and the decreased number of exposure days, which blunt the actual effect of exposure.

As anticipated, the data for εG adducts from exposure to [$^{13}C_2$]-VC for 5 days demonstrated that liver, lung and kidney are subjected to genotoxic damage by exposure to VC. The data presented in this study are also consistent with the previous hepatic and brain findings by Morinello *et al.* (39;40).

In this study we were able to differentiate endogenous εG from exogenous εG formation in multiple target organs in adult and weanling rats. A higher amount of εG adduct in liver was expected since P450 plays a critical role for VC metabolism. Since chloroethylene oxide (CEO) is known to be highly reactive and its half life very short, we hypothesized that VC itself is metabolized in distant tissues rather than via CEO circulation in the body. The negative spleen and brain data support this. Young animals are more susceptible to the induction of neoplasia by VC. This highly sensitive period corresponds to greater metabolism of VC to CEO, resulting in greater amounts of VC-induced DNA adducts during a period of rapid cell proliferation when compared to that of adults.

Maltoni *et al*. reported that high VC exposures (>2500 ppm) in rats induced brain tumors, however, it was later determined that these neoplasms originated in the nasal olfactory epithelium and invaded into the brain (11;44). The absence of detectable exogenous εG adducts in brain of adult and weanling rats following exposure to 1100 ppm [$^{13}C_2$]-VC suggests that exogenous εG is not formed in brain by VC in either adult or weanling rats. Small amounts of endogenous εG were detectable in some of the animals.

In summary, we developed a new sensitive, selective and more efficient LC-MS/MS method for quantifying the amount of εG in DNA. [$^{13}C_2$]-εG and endogenous εG were present in liver, lung and kidney DNA of adult and weanling rats exposed to VC. The use of stable isotope-labeled VC permitted accurate determinations of the half-lives of εG to be calculated and demonstrated that this adduct has little or no active DNA repair. This is the only DNA adduct of VC that is not lost due to chemical depurination or active DNA repair. In contrast, [$^{13}C_2$]-εG was not detectable in spleen or brain, and endogenous εG was only present in brain in small amounts and was not detectable in spleen. These data support a genotoxic

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mode of action for VC in those tissues that metabolize it to CEO, but do not support CEO being a circulating metabolite.

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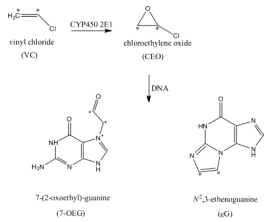

Scheme 1.

Formation of vinyl chloride induced DNA adducts, 7-OEG and εG. In the case of [$^{13}C_2$]-VC exposures, * indicates positions of labeled atoms.

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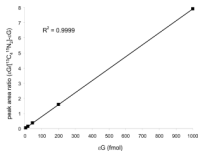

Figure 1.

Calibration curve for εG obtained under pseudo-SRM conditions using the present LC-MS/MS method. The plot shows the peak area ratio of εG to internal standard versus increasing fmol of εG. Data points correspond to standard mixtures that give 4.6, 18.5, 46, 200, and 1000 fmol εG and 144 fmol [$^{13}C_4$ $^{15}N_2$]-εG per injection.

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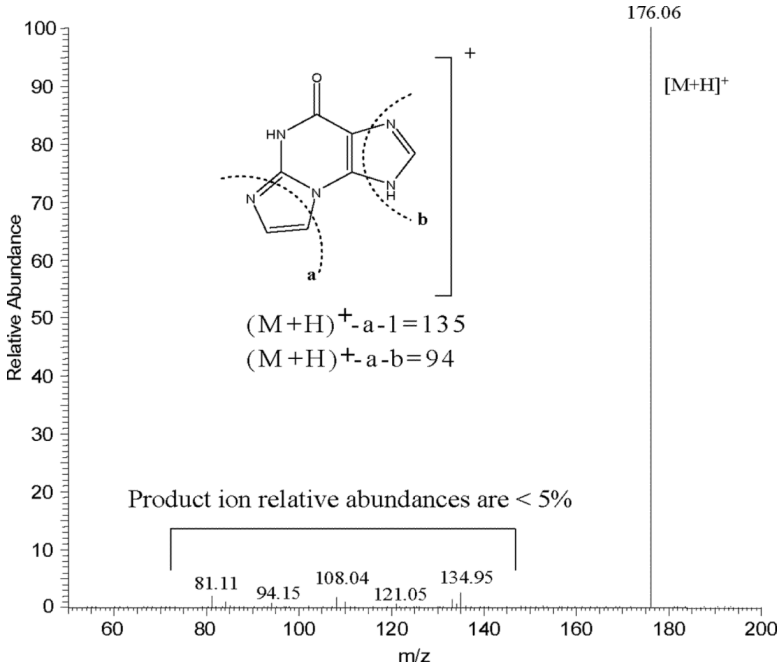

Figure 2.

The product ion spectrum of εG (precursor ion *m/z* 176) shows low abundances of major products. The spectrum was obtained by injecting 200 fmol of εG on the LC system as described in the text, selecting *m/z* 176 in Q1, and scanning Q3 from *m/z* 50 to 200. Argon collision gas pressure was 1.5 mTorr, and collision energy was 20 eV.

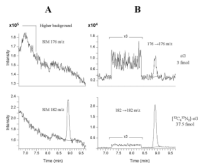

Figure 3.

These chromatograms prove the utility of the pseudo-SRM method for quantitation of low amounts of εG. A standard mixture giving 3 fmol εG and 37.5 fmol [$^{13}C_4$ $^{15}N_2$]-εG on column was analyzed using SIM and pseudo-SRM. Chromatograms are plotted as absolute ion intensity versus time. Panel A) SIM results show only internal standard is visible above baseline. Panel B) Pseudo-SRM decreases background noise to reveal the low-level εG peak. A portion of the baseline is magnified 3-fold to illustrate improved S/N over SIM. Note the y axis scale and baseline level in Panel A are about 10 times higher than in Panel B for each ion monitored.

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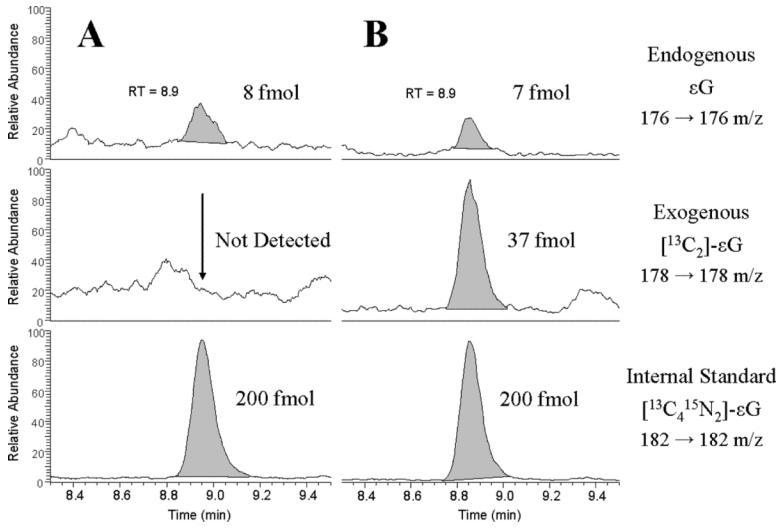

Figure 4.

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

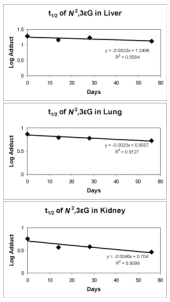

Figure 5.

The plot of log (adducts) versus days was used to determine the half life of N^2,3εG in liver, lung and kidney of adult rats exposed to [$^{13}C_2$]-VC (1100 ppm, 5 days, 6 hours per day) and killed at the end of exposure, or 2, 4 or 8 weeks later. The half life of N^2,3εG in liver and lung is 150 days and in kidney is 75 days based on the equations.

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Table 1

Intraday and interday assay variability are summarized for 250 ug aliquots of calf thymus DNA, which was spiked with increasing amounts of εG. Data are presented as mean fmol found per sample ± standard deviation with number of replicates in parentheses.

fmol εG Spiked	fmol εG Found Day 1	fmol εG Found Day 2	Combined Interday Results
5	6.7 ± 0.91 (5)	6.0 ± 0.24 (5)	6.3 ± 0.71 (10)
20	23 ± 1.5 (5)	23 ± 1.2 (4)	23 ± 1.3 (9)
50	55 ± 2.3 (5)	55 ± 8.2 (5)	55 ± 5.7 (10)

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Table 2

εG (endogenous) and [$^{13}C_2$]-εG (exogenous) adduct concentrations determined from weanling (n=8) and adult (n=4) rats exposed to 1100 ppm [$^{13}C_2$]-VC for 5 days.

	Liver (add/10^8 Gua)		Lung (add/10^8 Gua)		Kidney (add/10^8 Gua)	
	N^2,3-εG	$^{13}C_2$-N^2,3-εG	N^2,3-εG	$^{13}C_2$-N^2,3-εG	N^2,3-εG	$^{13}C_2$-N^2,3-εG
Weanling 2 hr post exposure	1.59±0.62	75.97±17.98	5.26±2.44	15.79±3.55	4.14±0.90	12.93±0.39
Adult 2 hr post exposure	4.14±2.77	18.96±4.93	8.37±2.77	7.39±0.49	5.93±3.26	5.71±2.14
2wks post exposure	3.68±3.10	14.24±4.19	5.96±0.06	6.24±0.99	5.06±3.86	3.68±1.17
4wks post exposure	3.07±1.00	16.91±1.57	2.98±0.90	6.08±0.55	9.34±7.49	3.81±0.34
8wks post exposure	3.73±1.48	13.21±2.53	2.73±1.36	5.33±0.23	10.68±2.02	2.90±0.06

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Table 3

εG (endogenous + exogenous) adduct concentrations determined from weanling and adult rats exposed to 1100 ppm VC for 1 day.

	Liver (N^2,3εG add/10^8 Gua)	**Lung (N^2,3εG add/10^8 Gua)**
Pup (2 hr post)	11.16±2.86	3.78±0.66
Adult (2 hr post)	5.85±0.84	7.63±4.80