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Detection of DNA Damage Derived from a Direct Acting Ethylating Agent Present in Cigarette Smoke by Use of Liquid Chromatography−Tandem Mass Spectrometry

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Cigarette smoke contains a complex mixture of chemicals, including some that are genotoxic. A number of epidemiological and clinical studies have reported the association of increased DNA adduct levels with the development of lung cancer in smokers. The majority of chemicals present in cigarette smoke require cytochrome P450-mediated metabolic activation to form the ultimate reactive species that covalently binds with DNA. We have investigated the presence of a direct-acting ethylating agent present in cigarette smoke by studying the formation of N-7 ethylguanine (N-7EtG) following exposure of DNA to cigarette smoke in vitro. A sensitive liquid chromatography−tandem mass spectrometry (LC-MS/MS) method with multiple reaction monitoring (MRM) was developed for the detection of N-7EtG in DNA. DNA samples were subjected to thermal hydrolysis to selectively release the N-7EtG, which was then quantified by LC-MS/MS MRM using a stable isotope internal standard [$^{15}N_5$]N-7EtG. The limit of detection of the method for N-7EtG was 2.0 fmol injected on column with 100 μg of calf thymus DNA as the matrix (0.6 N-7EtG adducts per 10^8 nucleotides). A linear dose−response was observed for the formation of N-7EtG in calf thymus DNA treated with diethyl sulfate at concentrations ranging from 1 to 1000 μM. Calf thymus DNA treated with smoke generated from 1, 5, and 10 commercially available cigarettes resulted in the formation of 1.3, 3.6, and 8.4 N-7EtG adducts per 10^8 nucleotides, respectively. There was a positive correlation between the formation of N-7EtG and the number of cigarettes ($r = 0.9938$). These results confirm the presence of an as yet unidentified direct acting ethylating agent in cigarette smoke, which is present at levels that can produce DNA damage that could ultimately have adverse implications for human health, particularly in the case of the development of lung cancer.

Introduction

Smoking cigarettes is associated with the development of numerous human cancers in various tissues (including the lung, pharynx, and larynx as well as the bladder) and accounts for 30% of all deaths related to cancers in more developed countries (*1*). Lung cancer is the most prevalent cancer arising from cigarette smoking with approximately 87% of all lung cancer cases being caused by exposure to cigarette smoke (*2*, *3*).

Cigarette smoke consists of a complex mixture of chemicals that have the potential to react with DNA, resulting in the formation of DNA adducts which may result in mutations (*4*). If these mutations are not repaired and occur in critical genes that are involved in cellular regulation such as the *ras* gene, they may lead ultimately to the development of cancer (*5*, *6*). For example *p53* mutations in human lung cancers are more prevalent in smokers when compared to nonsmokers (*7*). The nature of chemicals found in cigarette smoke is diverse, ranging from the nonpolar polycyclic aromatic hydrocarbons and aromatic amines to more polar nitrosamines as well as free radical generating species and metals. In total cigarette smoke contains about 4000 chemicals, of which about 60 are classified carcinogens in humans and animals as assessed by the International Agency for Research on Cancer (*8*, *9*, *10*). Numerous studies have been conducted with samples from smokers with regard to the formation of cigarette smoke-related DNA adducts showing that they are involved in the mechanism of cigarette smoke-induced carcinogenesis (*11*, *12*).

The majority of chemicals present in cigarette smoke require cytochrome P450-dependent metabolism to form electrophilic species that covalently react with DNA to form adducts. For example the reactive species for benzo[*a*]pyrene is benzo[*a*]pyrene diol epoxide (*13*). Similarly alkylating agents such as *N*-nitrosodiethylamine and the tobacco-specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone are metabolized via an α-hydroxynitrosamine to an alkyldiazonium ion (*14*, *15*). There are a few chemicals, such as ethylene oxide and acetaldehyde, that do not require metabolism for reaction with the DNA. However evidence suggests that there is a direct acting ethylating agent present in cigarette smoke of unknown chemical identity. Two separate human studies showed that there were higher levels of N-3 ethyladenine in the urine of smokers compared to that of the nonsmokers (*16*, *17*). A further study assessed exposure of smokers to ethylating agents in cigarette smoke by determining the N-terminal *N*-ethylvaline in hemoglobin, which was shown to be significantly elevated compared to non

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smokers (*18*).

To investigate this direct-acting ethylating agent further, we studied the formation of N-7 ethylguanine (N-7EtG)[1] following exposure of DNA to cigarette smoke. Alkylation of the N-7 position of guanine in DNA represents a good biomarker for determining exposure to alkylating agents since it is the most nucleophilic site in DNA, hence highly reactive, and is slowly repaired by a base excision repair pathway involving DNA glycosylases. However, it is not regarded to be a promutagenic DNA adduct: even though depurination may occur, the resulting apurinic sites are repaired again by a base excision repair pathway (*19*, *20*). The ability to react with oxygen atoms influences the relative mutagenicity and/or carcinogenicity of different alkylating agents (*21*). The main mutagenic adducts formed are O^6-alkylguanine and O^4-alkylthymidine, the latter is considered most mutagenic due to being poorly repaired by O^6-alkylguanine DNA alkyltransferases (*20*, *22*, *23*). The distribution of the different DNA adducts formed is very much dependent on the nature of the alkylating agent with the N-7 position of guanine being modified preferentially by dialkyl sulfates and alkyl methanesulfonates compared with ethyl nitrosourea, which prefers exocyclic oxygen atoms in guanine and thymidine. Ethylating agents compared to analogous methylating agents are thought to be more potent carcinogens, even though the extent of ethylation is much less than that of methylation (*24*).

A number of methods are available for the detection of N-7 alkylguanine adducts including immunochemical (*25*), ^{32}P-postlabeling (*26*, *27*, *28*), HPLC-electrochemical (*29*, *30*) or fluorescence detection (*31*), and gas chromatography−mass spectrometry (*32*) as well as liquid chromatography−mass spectrometry (*33*).

In this study we describe the development of a liquid chromatography−tandem mass spectrometry (LC-MS/MS) method for the sensitive and specific detection of N-7EtG in DNA samples using a stable isotope internal standard. The N-7EtG adduct was isolated from alkylated DNA by filtration following thermal hydrolysis. Using this method, we investigated the formation of N-7EtG in DNA that was exposed to cigarette smoke generated from commercially available cigarettes in vitro.

Experimental Procedures

Caution: *Diethyl sulfate and ethyl methanesulfonate are mutagens and carcinogens. Protective clothing should be worn and appropriate safety procedures followed when working with both compounds.*

Chemicals. N-7EtG was kindly provided by Professor M. Jarman (Institute of Cancer Research, Sutton, Surrey, U.K.). Diethyl sulfate (DES) and ethyl methanesulfonate were purchased from Sigma (Poole, U.K.). The cigarettes used for the experiments were a commercially available leading brand purchased from a local retail outlet. [$^{15}N_5$]-2′-deoxyguanosine ([$^{15}N_5$]dG) was purchased from Cambridge Isotope Laboratories Inc. (Cambridge, MA). All other reagents (analytical grade), HPLC (electrochemical) grade methanol, HPLC-grade acetic acid, and HPLC-grade 2-propanol were purchased from Fisher Scientific (Loughborough, U.K.). HPLC grade water, 18.2 MΩ output quality was obtained from Maxima purification equipment (Elga, High Wycombe, U.K.).

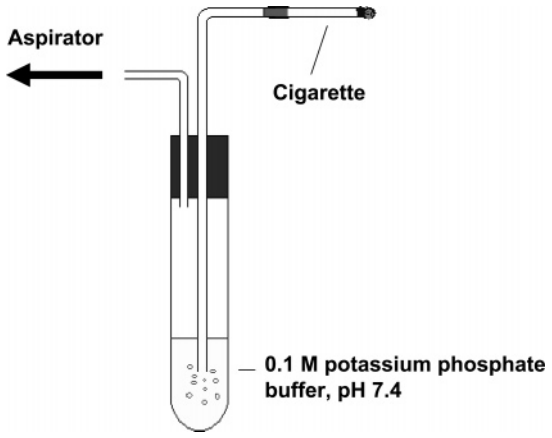


Figure 1. Diagram of the apparatus used to collect the cigarette smoke. The aspirator was attached to a water tap.

Preparation of N-7EtG Standard Stock Solution. The N-7EtG standard stock solution was prepared by dissolving N-7EtG in 0.1 M formic acid, pH 2.3. The concentration of this solution was calculated from the extinction coefficient for N-7EtG ($\epsilon = 7700\ M^{-1}\ cm^{-1}$) by determining the UV absorbance at 282 nm (U-3010 spectrophotometer, Hitachi, Tokyo, Japan) (*34*).

Synthesis of [$^{15}N_5$]N-7EtG Stable Isotope Internal Standard. To 0.5 mg of [$^{15}N_5$]dG was added 1 mL of 0.1 M ethyl methanesulfonate dissolved in 0.05 M sodium phosphate buffer, pH 7.2 (*35*). This reaction mixture was incubated at 37 °C for 16 h, followed by a further incubation at 70 °C for 1 h. The [$^{15}N_5$]N-7EtG was purified by injecting aliquots of the reaction mixture onto a Waters HPLC system consisting of Alliance 2690 separations module and 2487 UV detector connected to a Hypersil (ThermoQuest Hypersil, Runcorn, U.K.) C18 BDS (5 μm, 250 × 4.6 mm) column and a C18 BDS (5 μm, 10 × 4.6 mm) guard column. The column was eluted with a gradient at a flow rate of 1 mL/min with solvent A (0.05 M ammonium formate) and solvent B (methanol). The following gradient was used: 0 min, 0% B; 15 min, 20% B; 20 min, 0% B; 25 min, 0% B. The UV absorbance was monitored at 254 nm. The fraction corresponding to [$^{15}N_5$]N-7EtG was collected, pooled, and dried down on a centrifugal vacuum evaporator (Speedvac, Savant, Farmingdale, NY). The [$^{15}N_5$]N-7EtG fraction was further purified by reinjection onto the HPLC system described above. However, this time the column was eluted with a gradient at a flow rate of 1 mL/min with solvent A [HPLC-grade water/methanol (5:95 v/v)] and solvent B (methanol). The following gradient was used: 0 min, 0% B; 20 min, 20% B, 25 min, 0% B; and 30 min, 0% B. The UV absorbance was monitored at 254 nm. The [$^{15}N_5$]N-7EtG fraction was again dried down and dissolved in HPLC-grade water. The concentration of the solution was calculated from the extinction coefficient by determining the UV absorbance at 282 nm as described above for N-7EtG.

Treatment of Calf Thymus DNA with DES or Cigarette Smoke. To 2-mg aliquots of calf thymus DNA were added 1.0 mL of DES dissolved in 0.1 M potassium phosphate buffer, pH 7.4, at different concentrations (1, 10, 50, 100, 250, 500, and 1000 μM). For control calf thymus DNA, 1.0 mL of 0.1 M potassium phosphate buffer, pH 7.4, was added. Each calf thymus DNA/DES solution (and also the control) was left mixing on a rotating table for 8 h. The DES-treated and control DNA were precipitated by the addition of 800 μL of ice-cold 2-propanol. Following mixing by inversion, the solutions were centrifuged at 14 000 rpm for 5 min to pellet the DNA.

The smoke generated from 1, 5, and 10 cigarettes was bubbled through 10.0 mL of 0.1 M potassium phosphate buffer, pH 7.4, at room temperature by use of an apparatus similar to that used by Leanderson and Tagesson shown in Figure 1 (*36*). The

[1] Abbreviations: N-7EtG, *N*-7 ethylguanine, [$^{15}N_5$]N-7EtG, ^{15}N stable isotope labeled N-7 ethylguanine, dG, 2′-deoxyguanosine, [$^{15}N_5$]-dG, ^{15}N stable isotope labeled 2′-deoxyguanosine, DES, diethyl sulfate, LC-MS/MS, liquid chromatography−tandem mass spectrometry (mass spectrometry/mass spectrometry); MRM, multiple reaction monitoring.

solutions were used immediately or stored at −20 °C. To calf thymus DNA aliquots (0.5 mg/mL) dissolved in 0.1 M potassium phosphate buffer, pH 7.4 (6.0 mL) were added 4.0 mL of the 1, 5 and 10 cigarette smoke-exposed solutions and incubated at 37 °C for 48 h. The control DNA was incubated with 0.1 M potassium phosphate buffer, pH 7.4. Following the incubation, the DNA solutions were centrifuged at 4000 rpm (Beckman, Allegra 6R centrifuge) for 30 min. For each sample the supernatant was removed, to which was added 8.0 mL of ice-cold 2-propanol to precipitate the DNA followed by centrifugation at 4000 rpm to pellet the DNA.

The DNA pellets for DES or cigarette smoke-treated calf thymus DNA were washed with 1.0 mL of ethanol, centrifuged at 14 000 rpm for 10 min, then washed again with 1.0 mL ethanol/water (70:30 v/v) and centrifuged at 14 000 rpm for 10 min. The pellets were air-dried and dissolved in 1.0 mL of HPLC-grade water. The concentration of each DNA sample was calculated by determining the absorbance at 254 nm (GeneQuant spectrophotometer, Biochrom, Cambridge, U.K.), with the assumption that one absorbance unit equals 50 μg/mL for double-stranded DNA. The samples were stored at −20 °C.

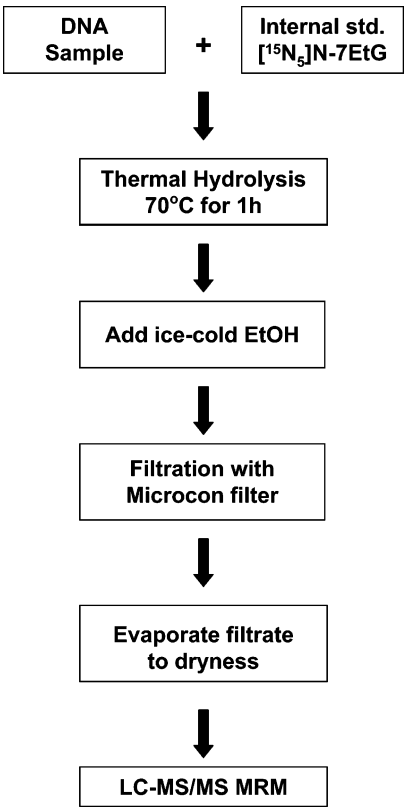
Determination of N-7EtG in DNA Samples. To a volume of the DES or cigarette smoke-treated calf thymus DNA sample giving 133.3 μg was added 666.7 fmol of the [$^{15}N_5$]N-7EtG internal standard (50 fmol/μL) and the mixture was then dried down by use of a centrifugal vacuum evaporator. The dried DNA sample was dissolved in 100 μL of HPLC-grade water and incubated at 70 °C for 1 h in a heating block (Stuart Scientific, Staffordshire, U.K.). Following the addition of 80 μL of ice-cold ethanol, the DNA solution was transferred to a 10 000 MWCO Microcon centrifugal filter device (Millipore, Massachusetts, US) and centrifuged at 14 000 rpm for 1h. The filtrate was dried down by use of a centrifugal vacuum evaporator and redissolved in 20 μL of 0.1% acetic acid for analysis by LC-MS/MS.

The LC-MS/MS consisted of a Waters Alliance 2695 separations module with a 100 μL injection loop connected to a Micromass Quattro Ultima Pt. (Micromass, Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface. The temperature of the electrospray source was maintained at 110 °C and the desolvation temperature at 350 °C. Nitrogen gas was used as the desolvation gas (650 L/h) and the cone gas (25 L/h). The capillary voltage was set at 3.20 kV. The cone and RF1 lens voltages were 42 and 30 V, respectively. The photomultiplier was set at 850 V. The mass spectrometer was tuned by using a N-7EtG (1 pmol/μL) standard solution dissolved in 0.1% acetic acid/methanol (90:10 v/v) introduced by continuous infusion at a flow rate of 10 μL/min with a Harvard model 22 syringe pump (Havard Apparatus Ltd., Edenbridge, U.K.).

A 15 μL aliquot of the DNA sample (equivalent to 100 μg of thermally hydrolyzed DNA containing 500 fmol of [$^{15}N_5$]N-7EtG) was injected onto a HyPurity (ThermoQuest Hypersil, Runcorn, U.K.), C18 (3 μm, 150 × 1.0 mm) column connected to a Uniguard C18 (3 μm, 10 × 1.0 mm) guard cartridge attached to KrudKatcher disposable precolumn (0.5 μm) filter. The column was eluted isocratically with 0.1% acetic acid/methanol (90:10 v/v) at a flow rate of 50 μL/min. The collision gas was argon (indicated cell pressure 4.0×10^{-3} mbar) and the collision energy was set at 15 eV. The dwell time was set to 200 ms and the resolution was two *m/z* units at peak base. The samples were analyzed in positive-ionization MS/MS multiple reaction monitoring (MRM) mode for the $(M + H)^+$ ion to guanine base $(B + H)^+$ transitions of N-7EtG (*m/z* 180 to 152) and [$^{15}N_5$]N-7EtG (*m/z* 185 to 157). The procedure has been summarized in Scheme 1.

The level of N-7EtG in each DNA sample was determined from the ratio of the peak area of N-7EtG to that of the internal standard [$^{15}N_5$]N-7EtG from a calibration line. The calibration line was constructed by the addition of different amounts of the N-7EtG standard (ranging from 2.0 to 5000 fmol) and 500 fmol of the [$^{15}N_5$]N-7EtG internal standard to 100 μg of calf thymus DNA for the final amount on column injected onto the LC-MS/MS. The standards were subjected to the entire thermal hydrolysis procedure before analysis by LC-MS/MS (Scheme 1).

Scheme 1. Summary of the Procedure for Determination of N-7EtG in DNA Samples



Results

LC-MS/MS Determination of N-7EtG. The stable isotope internal standard for N-7EtG was prepared by the reaction of ethyl methanesulfonate with [$^{15}N_5$]dG followed by thermal hydrolysis, resulting in depurination of the ethylated base from the deoxynucleoside. The [$^{15}N_5$]N-7EtG product was separated from unreacted [$^{15}N_5$]guanine, which was also produced by depurination, via HPLC as shown in Figure 2. Confirmation of the identity of the [$^{15}N_5$]guanine peak was obtained by tandem mass spectrometry with the $(M + H)^+$ ion at *m/z* 157 for [$^{15}N_5$]guanine giving rise to product ions at *m/z* 139 and 113. The identity of the unreacted [$^{15}N_5$]dG peak was also confirmed by tandem mass spectrometry with the $(M + H)^+$ ion at *m/z* 273 for [$^{15}N_5$]dG, giving rise to product ions at *m/z* 157 and 117 corresponding to [$^{15}N_5$]-guanine and deoxyribose, respectively. The tandem mass spectrum of N-7EtG is shown in Figure 3A with four product ions being observed following fragmentation of the $(M + H)^+$ ion at *m/z* 180 for N-7EtG. The identity of the [$^{15}N_5$]N-7EtG peak was confirmed by tandem mass spectrometry as shown in Figure 3B. The fragmentation pattern of N-7EtG was similar to that observed by Liao et al. for N-7(2-hydroxy)ethylguanine for which they postulated the structural identity of the various product ions formed (*37*). The products ions at *m/z* 152, 135, and 110 are thought to be formed by loss of the ethyl group, but the product ion at *m/z* 163 has the ethyl group still attached and is formed by the loss of the -NH_2 group. The tandem mass spectrum of [$^{15}N_5$]N-7EtG confirmed the identity of these product ions as predicted by Liao et

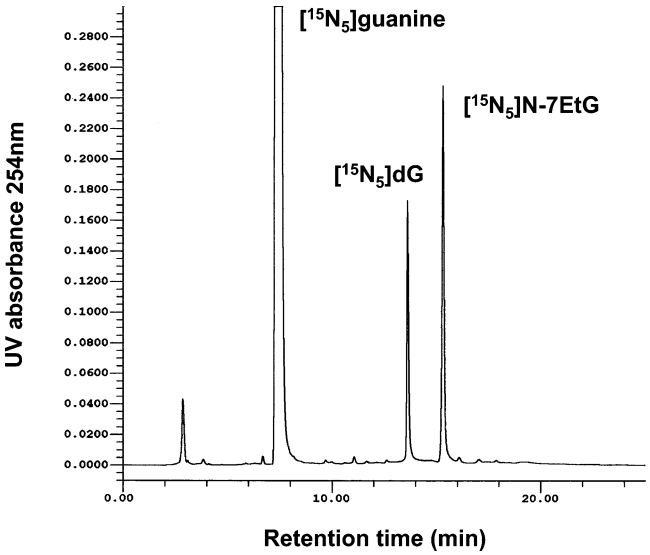


Figure 2. HPLC−UV chromatogram for the separation of $[^{15}N_5]$N-7EtG formed following the incubation of $[^{15}N_5]$dG with ethyl methanesulfonate at 37 °C for 16 h and then 70 °C for 1 h. The column was eluted by use of a gradient (0 min, 0% B; 15 min, 20% B; 20 min, 0% B; and 25 min, 0% B) at a flow rate of 1 mL/min with solvent A (0.05 M ammonium formate) and solvent B (methanol).

al. due to the presence of ^{15}N atoms in the structure and resultant change in *m/z* for each product ion. The transition of the $(M + H)^+$ precursor ethylated guanine ion to guanine (*m/z* 152 or 157) resulted in the product ion with highest intensity. Therefore, for the MRM analysis, the transition of *m/z* 180 to 152 was determined for N-7EtG and the corresponding transition of *m/z* 185 to 157 for $[^{15}N_5]$N-7EtG.

A calibration line was constructed by preparing a series of standards each containing varying amounts of N-7EtG and a constant amount of the stable isotope internal standard $[^{15}N_5]$N-7EtG (500 fmol) added to 100 μg of calf thymus DNA to account for any matrix effects. Each of the standards were then subjected to the entire analysis procedure (Scheme 1). The response was linear over a range of 2.0 up to 5000 fmol of N-7EtG added to 100 μg of calf thymus DNA. Varying the amount of the calf thymus DNA added from 10 up to 125 μg had no effect on the LC-MS/MS signal observed for both N-7EtG and $[^{15}N_5]$N-7EtG, implying that the depurinated DNA was adequately removed by the Microcon filter (data not shown). The calibration line for levels of N-7EtG ranging from 0 to 100 fmol is shown in Figure 4. The calibration line was used to determine the level of N-7EtG in the calf thymus DNA samples treated with DES or cigarette smoke containing 500 fmol of the stable isotope internal standard $[^{15}N_5]$N-7EtG. The limit of detection on column for N-7EtG was 2.0 fmol (with a signal-to-noise ratio of 3.0), which is equivalent to 0.6 N-7EtG adducts per 10^8 nucleotides for 100 μg of calf thymus DNA (assuming that 1 μg of DNA is equal to 3240 pmol of nucleotides (*38*)). In contrast, the detection limit on column following injection of the N-7EtG standard directly onto the LC-MS/MS was 1 fmol with a signal-to-noise ratio of 5.0.

Determination of N-7EtG in DNA Samples. Calf thymus DNA was incubated with different concentrations of DES for 8 h at room temperature. No N-7EtG was detectable in control calf thymus DNA treated with phosphate buffer (Figure 5A). Typical LC-MS/MS MRM ion chromatograms are shown in Figure 5B,C for 1 and 10 μM DES-treated calf thymus DNA, respectively. A linear correlation was observed for the amount of N-7EtG formed and the concentration of DES from 1 to 1000 μM used to treat calf thymus DNA. The levels of N-7EtG formed were 3.3 ± 1.4, 42.6 ± 3.7, 206.8 ± 7.4, 445.2 ±

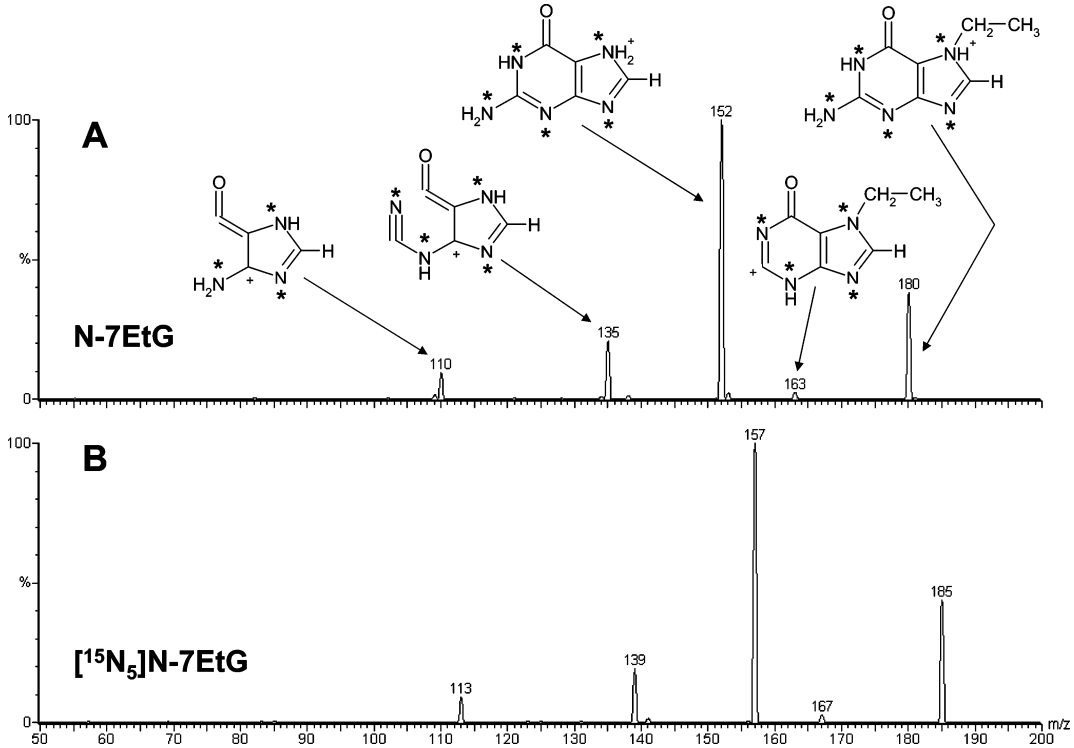


Figure 3. Positive-ion electrospray MS/MS spectra for the $(M + H)^+$ ion (A) at *m/z* 180 for N-7EtG standard and (B) at *m/z* 185 for the synthesized stable isotope internal standard $[^{15}N_5]$N-7EtG. Each spectrum was obtained by continuous infusion of a 1.0 pmol/μL solution into the mass spectrometer at 10 μL/min. The structures corresponding to the product ions are shown (the asterisks indicate the ^{15}N-labeled sites in the internal standard).

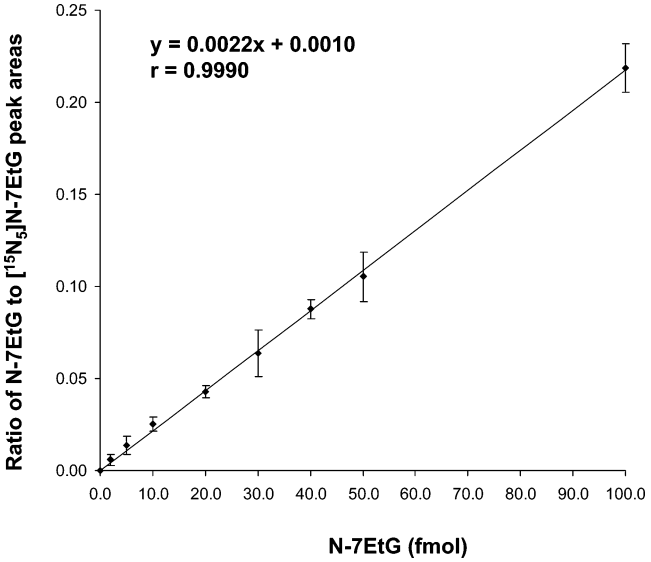


Figure 4. Calibration line for the determination of N-7EtG with [$^{15}N_5$]N-7EtG as the internal standard and 100 μg of calf thymus DNA matrix by LC-MS/MS (MRM transitions *m/z* 180 to 152 and *m/z* 185 to 157 for N-7EtG and [$^{15}N_5$]N-7EtG, respectively). The ratio of the peak area of N-7EtG to 500 fmol of [$^{15}N_5$]N-7EtG was plotted against the amount of N-7EtG (femtomoles). The error bars represent the standard deviation of the mean ($n = 5$).

23.9, 1068.5 ± 78.9, 2350.7 ± 216.0, and 4840.4 ± 353.0 adducts per 10^8 nucleotides (±standard deviation) for 1, 10, 50, 100, 250, 500, and 1000 μM DES-treated calf thymus DNA, respectively. The dose−response curve obtained for 1−250 μM DES-treated calf thymus DNA is shown in Figure 6.

Calf thymus DNA was incubated with the smoke generated from 1, 5, and 10 cigarettes for 48 h at 37 °C. There was no N-7EtG detected in control calf thymus DNA incubated with phosphate buffer alone (Figure 7A). Typical LC-MS/MS MRM ion chromatograms are shown in Figure 7B,C for calf thymus DNA treated with the smoke generated from 1 and 10 cigarettes, respectively. The level of N-7EtG determined following exposure of calf thymus DNA to the smoke generated from 1, 5, and 10 cigarettes was 1.3 ± 0.17, 3.6 ± 0.23 and 8.4 ± 0.51 adducts per 10^8 nucleotides (± standard error), respectively, from 100 μg of DNA analyzed (Figure 8). The results shown in Figure 8 represent the average levels of N-7EtG obtained following the incubation of cigarette smoke from 1 and 10 cigarettes with calf thymus DNA for three separate experiments and in the case of 5 cigarettes for two separate experiments.

Discussion

A LC-MS/MS method has been developed allowing the detection of N-7EtG in DNA samples that have been exposed to ethylating agents. The method allows for the sensitive and accurate detection of N-7EtG with the presence of the stable isotope internal standard [$^{15}N_5$]N-7EtG. The N-7EtG was selectively released from the DNA by thermal hydrolysis since this adduct is extremely labile at increased temperatures. Interference from other modified bases (for example, O^4- and O^2-ethylthymidine) and unmodified bases was not encountered since they require acid hydrolysis and high temperature to be released from the DNA (*39*). Although N-3 and N-7 ethyladenine are also released by thermal hydrolysis, they did not interfere with the method due to the

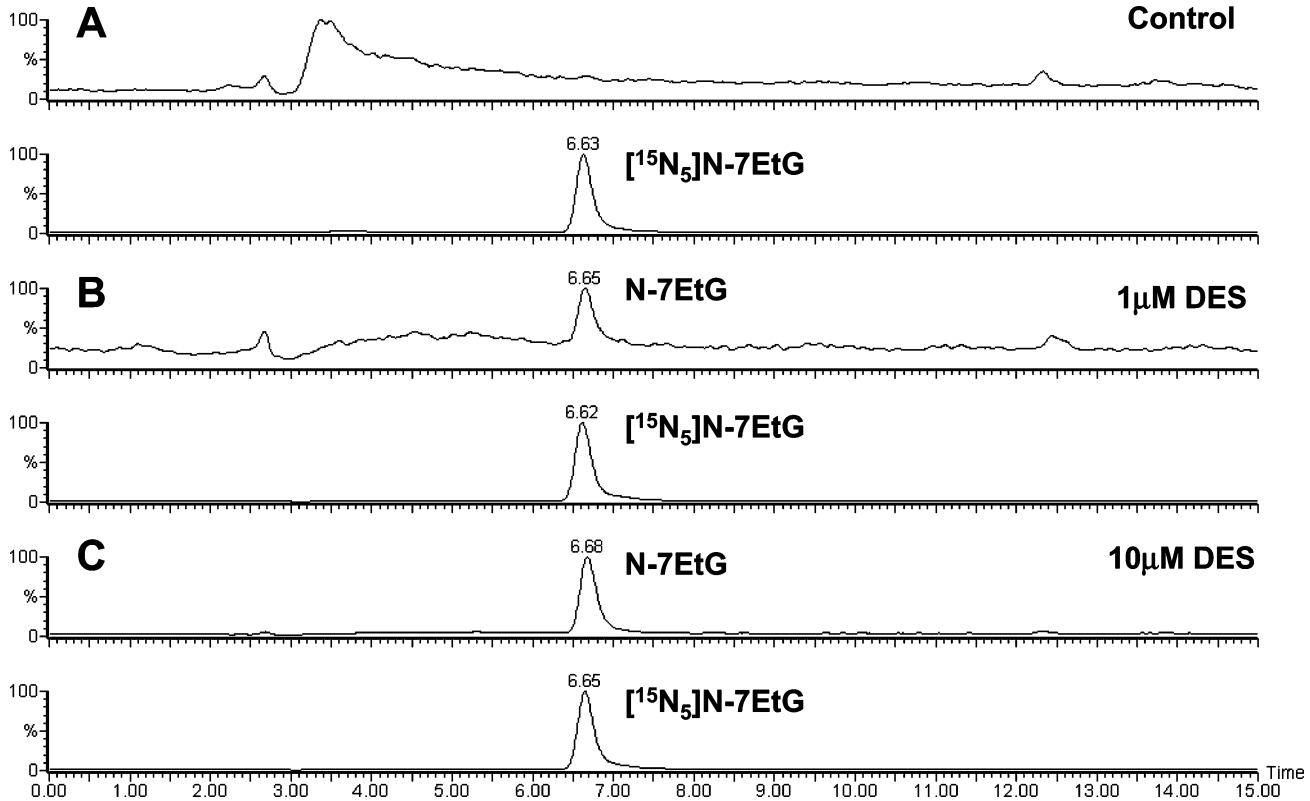


Figure 5. Determination of N-7EtG in DNA samples treated with DES by LC-MS/MS by use of a C18 microbore column eluted isocratically with 0.1% acetic acid/methanol (90:10 v/v) at a flow rate of 50 μL/min. Typical LC-MS/MS MRM ion chromatograms for (A) control DNA, (B) 1 μM DES-treated calf thymus DNA, and (C) 10 μM DES-treated calf thymus DNA (MRM transitions *m/z* 180 to 152 and *m/z* 185 to 157 for N-7EtG and 500 fmol of the internal standard [$^{15}N_5$]N-7EtG, respectively).

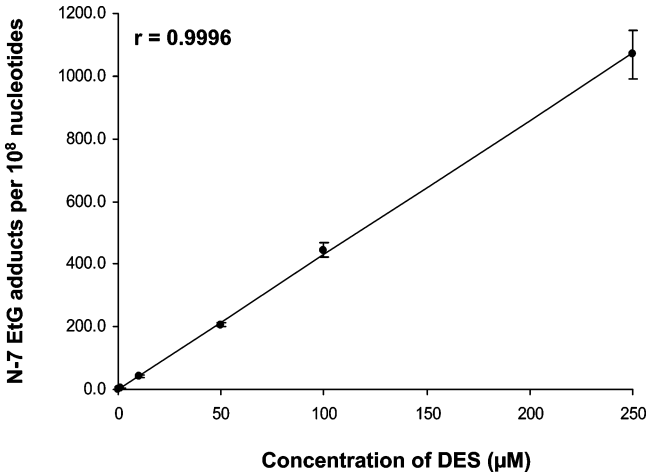


Figure 6. Formation of N-7EtG following the treatment of calf thymus DNA with different concentrations of DES. The error bars represent the standard deviation of the mean ($n = 5$).

selectivity of the LC-MS/MS. This method offers numerous advantages over the ^{32}P-postlabeling assays used for the detection of N-7 alkylguanines, which encounter problems with low labeling efficiencies due to depurination and also require adduct enrichment to remove the unmodified nucleotides that interfere with the assay. The LC-MS/MS method described here with a limit of detection of 0.6 N-7EtG adduct/10^8 nucleotides has comparable sensitivity to ^{32}P-postlabeling methods for determining N-7 alkylguanine adducts in terms of the amount of DNA required for analysis, typically ranging from 10 to 100 μg. Shields et al. (*27*) quoted a detection limit of 2.5 N-7 methylguanine adducts per 10^8 nucleotides for 100 μg of DNA analyzed by a combined HPLC and ^{32}P-postlabeling method. Using a similar approach but using a two-step HPLC purification step, Haque et al. (*40*) reported a detection limit of 4.0 N-7 methylguanine adducts per 10^8 nucleotides with only 10 μg of DNA analyzed. A two-step HPLC purification step was also used by Kato et al. (*41*), and they reported a detection limit of 0.25 N-7 alkylguanine adduct/10^8 nucleotides with 100 μg of DNA analyzed.

The LC-MS/MS method was validated by using calf thymus DNA that had been treated with DES at concentrations ranging from 1 to 1000 μM and allowed the detection of alkyl DNA damage at low micromolar DES concentrations. The level of N-7EtG formed was linear with respect to the dose of DES used for treatment of calf thymus DNA. DES is a direct acting alkylating agent and may form DNA adducts via a bimolecular nucleophilic substitution (S_N2) mechanism predominantly forming N-7EtG adducts and is less reactive in forming oxygen adducts as compared to ethyl nitrosourea, which forms adducts via a unimolecular nucleophilic substitution (S_N1) mechanism. For ethyl nitrosourea, the ultimate reactive species is an ethyl diazonium ion (*24*, *42*).

The presence has been previously reported of an unknown ethylating agent derived from exposure to cigarette smoke, by the formation of protein adducts of N-terminal *N*-ethylvaline in hemoglobin as well as DNA adducts by the detection of N-3 ethyladenine in urine from smokers (*17*−*19*). N-3-Ethyladenine has also been detected in DNA exposed to cigarette smoke in vitro (17). Using the LC-MS/MS method developed here, we can confirm the presence of a direct acting ethylating agent present in the smoke generated from commercially avail-

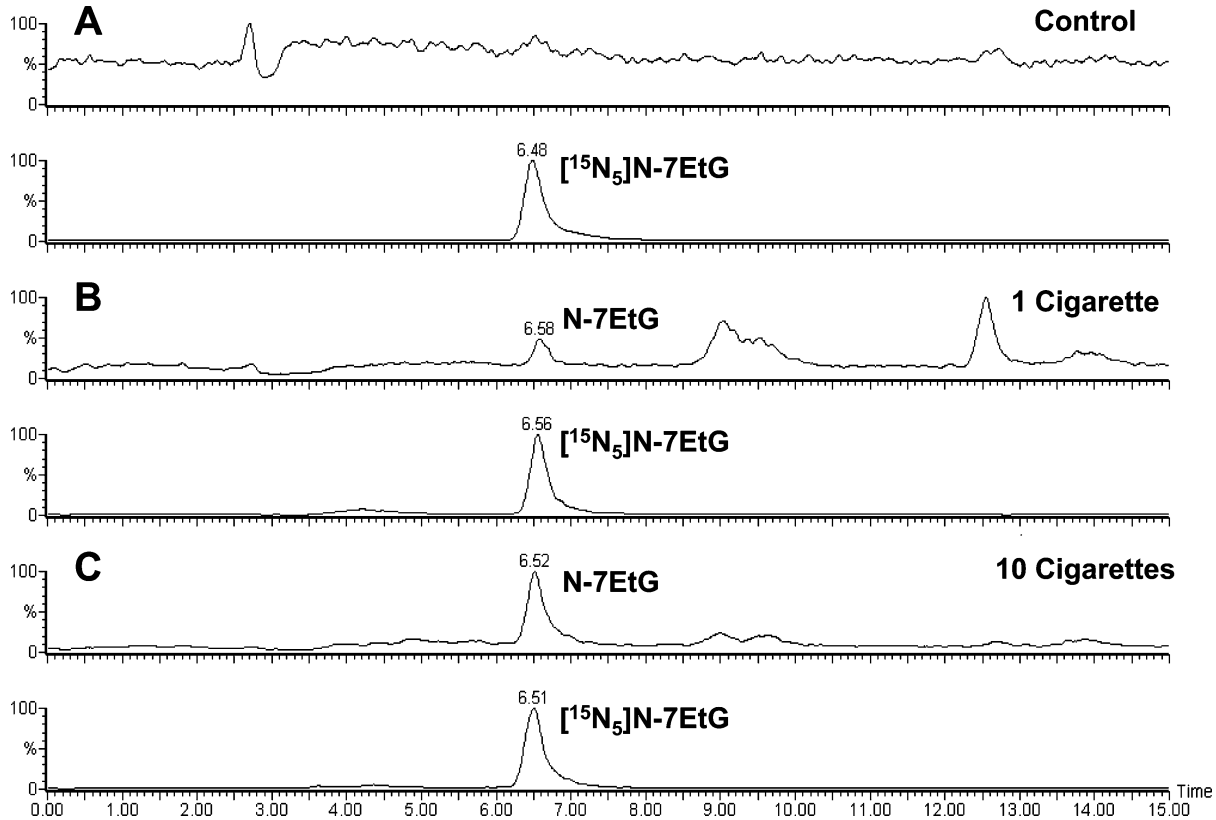


Figure 7. Determination of N-7EtG in DNA samples treated with cigarette smoke by LC-MS/MS by use of a C18 microbore column eluted isocratically with 0.1% acetic acid/methanol (90:10 v/v) at a flow rate of 50 μL/min. Typical LC-MS/MS MRM ion chromatograms for (A) control DNA, (B) 1 cigarette-treated calf thymus DNA, and (C) 10 cigarette-treated calf thymus DNA (MRM transitions *m/z* 180 to 152 and *m/z* 185 to 157 for N-7EtG and 500 fmol of the internal standard [$^{15}N_5$]N-7EtG, respectively).

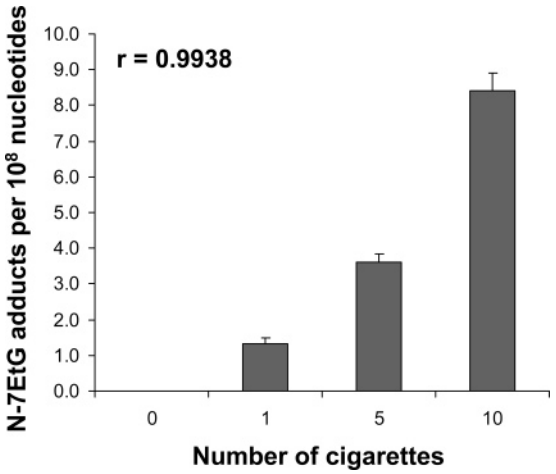


Figure 8. Formation of N-7EtG following the treatment of calf thymus DNA with smoke generated from different numbers of cigarettes. The error bars represent the ± standard error.

able cigarettes, which reacts with DNA resulting in the formation of N-7EtG. The level of N-7EtG formed was directly proportional to the number of cigarettes. The levels of N-7EtG adducts formed from the cigarette smoke are equivalent to the lowest dose of DES exposure to calf thymus DNA; however, these levels were formed after 8 h exposure at room temperature compared to 48 h at 37 °C for the cigarette smoke exposed DNA. Blomeke et al. have reported the detection of an average level of 4.0 N-7EtG adducts per 10^8 nucleotides in human lung tissue using ^{32}P-postlabeling (*43*). The levels of *N*-nitrosamines in cigarette smoke such as *N*-nitrosodiethylamine range from not detectable to 2.8 ng/cigarette (*2*). At these levels they could not account for the formation of alkyl protein and DNA adducts observed in smokers. Godschalk et al. have shown that levels of O^4-ethylthymidine are increased in lung tissue from cancer patients who are smokers compared to nonsmokers and concluded that cigarette smoke was the main source of exposure to an ethylating agent (*44*). The identity of the direct acting ethylating agent remains unknown. Potentially the ethylating agent could be generated by interactions between the diverse array of chemicals that are present in cigarette smoke. Carmella et al. (*18*) have postulated that it could be formed from the nitrosation of ethylamine, which is present at levels of approximately 1 μg/cigarette.

The majority of carcinogens present in cigarette smoke require metabolism to form the reactive species that can covalently bond to DNA forming adducts. It has been shown in the case of nitrosamine metabolism in humans that there is a high variability between individuals, hence affecting their risk to developing cancer from cigarette smoke (*45*). The results we have obtained showing the presence of a direct acting ethylating agent in cigarette smoke imply that smoking individuals are exposing themselves to an alkylating agent regardless of their capacity for metabolic activation. Hence the ability of an individual to reduce the harmful affects following exposure to this ethylating agent is dependent on their ability to detoxify the reactive metabolite or repair the DNA damage, which will be ultimately affected by the number of cigarettes and the length of time the individual smokes. The importance of DNA repair is highlighted by evidence that low activity of the repair enzyme 8-oxoguanine DNA *N*-glycosylase (OGG) is associated with an increased risk of lung cancer in smokers (*46*). Furthermore recent findings suggest that genetic polymorphisms in *CYP1A1*, *GSTM1*, *GSTP1*, *GSTT1*, and *NQO1* may not influence lung cancer risk in smokers, implying the role for a direct acting genotoxic agent in smoking-associated lung cancer (*47*, *48*).

In conclusion, we have developed a sensitive LC-MS/MS method for the detection and accurate quantitation of N-7EtG, using the stable isotope internal standard, in DNA that has been exposed to cigarette smoke in vitro. The results confirm the presence of a direct-acting ethylating agent present in cigarette smoke that increases with the number of cigarettes smoked, resulting in the corresponding increase of N-7EtG. The identity of the direct acting ethylating agent remains unknown, but these results show that it is present at levels that produce DNA damage, which could potentially have adverse implications to human health, particularly in the case of the development of lung cancer.

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