Detection of 7-(2′-Carboxyethyl)guanine but Not 7-Carboxymethylguanine in Human Liver DNA

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7-Carboxymethylguanine (7-CMGua) and 7-(2′-carboxyethyl)guanine (7-CEGua) are DNA adducts that potentially could be formed upon the metabolism of the carcinogenic nitrosamines *N*-nitrososarcosine (NSAR) and 3-(methylnitrosamino)propionic acid (MNPA), respectively, or from other sources such as nitrosation of glycine (7-CMGua) or reaction of DNA with acrylic acid (7-CEGua). Since both NSAR and MNPA have been detected in human urine and there are plausible sources of exposure to other precursors to these adducts, we analyzed human liver DNA for 7-CMGua and 7-CEGua, using liquid chromatography−electrospray ionization−tandem mass spectrometry−selected reaction monitoring (LC-ESI-MS/MS-SRM). Human hepatic DNA was mixed with [$^{15}N_5$]7-CMGua and [$^{15}N_5$]7-CEGua as internal standards and enzymatically hydrolyzed. The hydrolysate was partially purified by solid-phase extraction, and the resulting fraction was treated with acetyl chloride in methanol to convert 7-CMGua and 7-CEGua to their methyl esters. After a second solid-phase extraction, LC-ESI-MS/MS-SRM analysis was carried out using the transitions *m/z* 224 $[M + H]^+ \rightarrow$ *m/z* 164 $[(M + H) - HCOOCH_3]^+$ and *m/z* 238 $[M + H]^+ \rightarrow$ *m/z* 152 $[BH]^+$ for the methyl esters of 7-CMGua and 7-CEGua, respectively. The method was sensitive, accurate, precise, and apparently free from artifact formation. 7-CEGua, as its methyl ester, was detected in all 24 human liver samples analyzed, mean ± SD, 373 ± 320 fmol/μmol Gua (74.6 adducts per 10^9 nucleotides), range 17−1189 fmol/μmol Gua, but the methyl ester of 7-CMGua was not detected in any sample. These results demonstrate the ubiquitous presence of 7-CEGua in human liver DNA. Acrylic acid may be a likely endogenous precursor to 7-CEGua.

Introduction

The ability of DNA adducts to induce mutations has been clearly demonstrated in multiple studies (*1*). When these mutations occur in critical regions of important genes such as *ras* and *p53*, the result can be loss of normal cellular growth control mechanisms, genomic instability, and cancer (*2*−*4*). By characterizing DNA adducts in human tissues and establishing their miscoding potential, new leads for understanding cancer etiology can potentially be obtained.

In this study, we focused on the analysis of 7-carboxymethylguanine (7-CMGua, **5**, Scheme 1) and 7-(2′-carboxyethyl)guanine (7-CEGua, **13**). These adducts could potentially be formed from the carcinogenic nitrosamines *N*-nitrososarcosine (NSAR, **1**) and 3-(methylnitrosamino)propionic acid (MNPA, **10**). NSAR causes esophageal tumors in rats, nasal tumors in Swiss mice, and liver tumors in newborn mice (*5*). The major sources of human exposure to NSAR are believed to be consumption of smoked and cured meats, and endogenous formation (*5, 6*). NSAR has been consistently detected in human urine (*6*). Levels of NSAR in the urine of healthy individuals averaged 0.42 and 0.94 μg/day in two studies in Germany, 0.36 μg/day in the United Kingdom, 0.1 μg/day in Poland, 3.4 μg/day in Egypt, and 0.1−1.5 μg/day in China. Increased levels were found in regions of high esophageal cancer incidence in China and high gastric cancer incidence in Poland (*6*). NSAR is "reasonably anticipated to be a human carcinogen" according to the U. S. Department of Health and Human Services (*7*).

Although 88% of NSAR is excreted unchanged in rats (*8*), its carcinogenicity strongly indicates that it is metabolized to some extent. Cytochrome P450 catalyzed metabolism of NSAR by hydroxylation of its methyl group, a well established and common metabolic pathway for *N*-methyl-*N*-nitroso compounds (*9*), is expected to yield the unstable intermediate α-hydroxymethyl NSAR (**2**). Intermediate **2** will spontaneously eject formaldehyde with the formation of the carboxymethyl diazonium ion **3**, which exists in equilibrium with diazotate **4**. Multiple studies have demonstrated that this intermediate, generated from various sources, is a carboxymethylating agent and reacts with DNA to produce 7-CMGua as well as O^6-CMGua (**6**). Thus, Zurlo et al. demonstrated that 7-CMGua was formed in pancreatic acinar cells exposed to azaserine (**7**) (*10*). The Shuker group carried out a series of studies investigating the formation of O^6-CMGua. They demonstrated that the nitrosated bile acid conjugate *N*-nitrosoglycocholic acid (**8**) and related *N*-nitroso-compounds produced 7-CMGua and O^6-CMGua (as well as O^6-methylGua) in vitro (*11, 12*). They also showed that O^6-CMGua could be formed upon the nitrosation of glycine (**9**) in vitro, presumably via intermediate **4**, and detected O^6-CMGua in three human blood samples using an immunoslot blot assay (*13*). Using immunohistochemistry techniques, they demonstrated positive staining of exfoliated colonic cells for O^6-CMGua, and this staining was significantly higher in cells from humans who consumed a high red meat diet (*14*). Further studies demonstrated that potassium diazotate, a stable precursor to **4**, produced mutations in the *p53* gene in vitro (*15*).

MNPA is a relatively weak inducer of lung adenomas in A/J mice in the one reported study of its carcinogenicity (*16*). The greatest known source of human exposure to MNPA is

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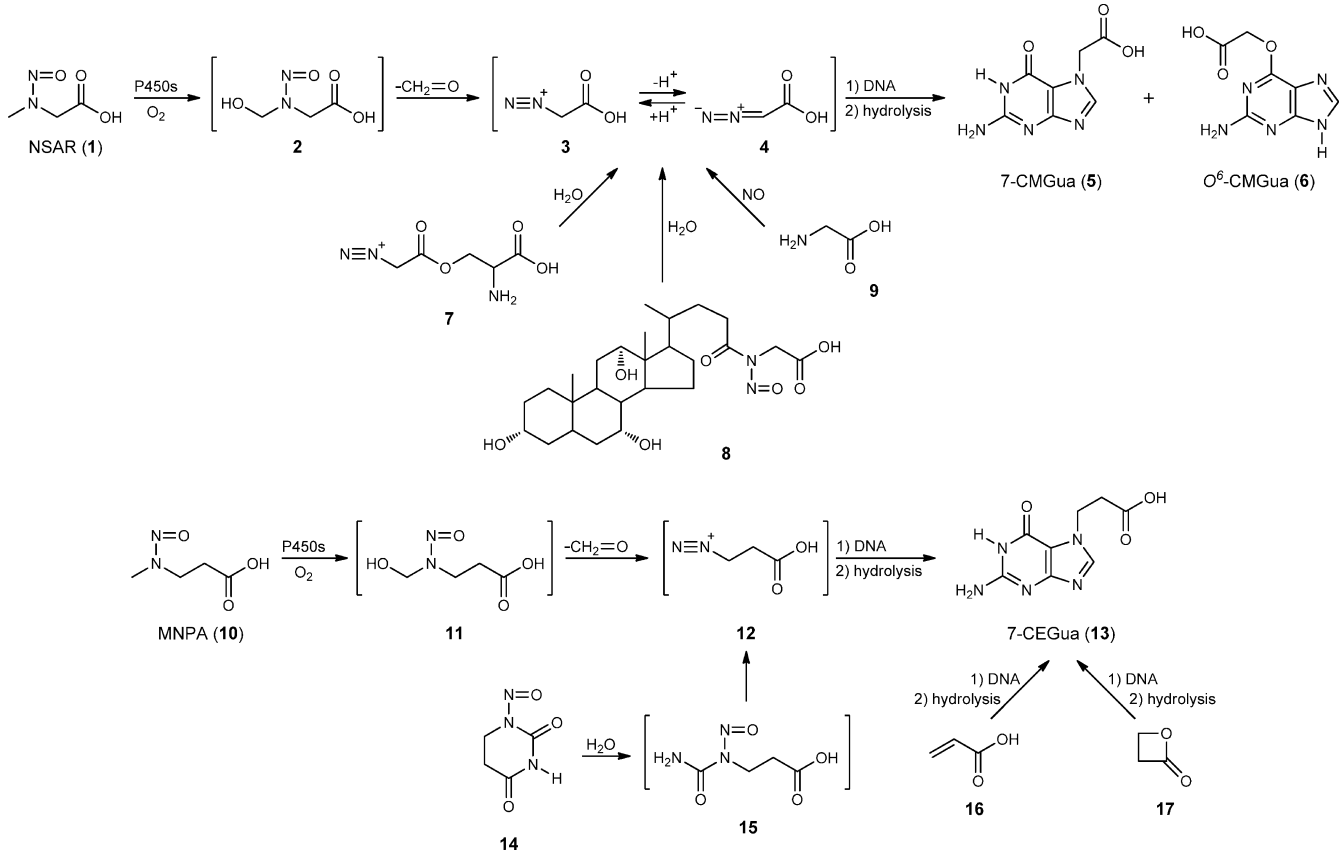
Scheme 1. Reactions Which Can Produce 7-CMGua (5) and 7-CEGua (13)[a]



[a] Carboxylic acids are shown in protonated form for consistency with adduct structures **5** and **13** which are detected in this form.

smokeless tobacco which contains substantial amounts of this nitrosamine (*17*). MNPA has been reported in human urine at levels of 0.1−0.4 μg/day in studies of healthy individuals on normal diets (*18*). Its levels were higher in areas of Poland where inhabitants have a relatively high risk for stomach cancer compared to low risk areas (*18, 19*). It is not clear whether the occurrence of MNPA in human urine is related to tobacco use. α-Methyl hydroxylation of MNPA would produce intermediate **11**, which upon loss of formaldehyde yields the carboxyethyl diazonium ion **12**. This intermediate would be expected to react with DNA to produce 7-CEGua (**13**). 7-CEGua has been previously detected in liver DNA of rats treated with the hepatocarcinogen 1-nitroso-5,6-dihydrouracil (**14**) (*20*). 7-CEGua is also formed in the reactions of acrylic acid (**16**) and β-propiolactone (**17**) with DNA (*21*).

Collectively, these data provided a strong rationale for investigating the presence of 7-CMGua and 7-CEGua in human DNA. Therefore, we used liquid chromatography−electrospray ionization−tandem mass spectrometry−selected reaction monitoring (LC-ESI-MS/MS-SRM) to analyze human liver DNA for these adducts.

Experimental Procedures

HPLC-UV Analysis. This was carried out using Waters Associates (Milford, MA) instruments equipped with a model 996 Photodiode array detector for system 1 and a Shimadzu SPD-10A UV detector (Shimadzu Scientific Instruments, Columbia, MD) for system 2. The UV detector was operated at 254 nm. System 1 used a 10 mm ×25 cm 5 μm Luna C18 column (Phenomenex, Torrance, CA) with a gradient from 5% CH_3OH in 0.35% aq formic acid to 40% CH_3OH over the course of 35 min at a flow rate of 3.3 mL/min. This system was used for collecting 7-CMGua, [$^{15}N_5$]7-CMGua, 7-CEGua, and [$^{15}N_5$]7-CEG. System 2 used a 4.6 mm × 25 cm Luna 5 μm C18 column with a gradient from 5 to 40% CH_3OH in H_2O over the course of 35 min at a flow rate of 0.7 mL/min. This system was used for the quantitation of dGuo or Gua in DNA samples. Results from the two methods agreed within 5%. The Gua values reported here were calculated from measured dGuo.

Chemicals, Enzymes, and Chromatography Supplies. [$^{15}N_5$]dGuo was purchased from Spectra Stable Isotopes (Columbia, MD). Ethanol was obtained from AAPER Alcohol and Chemical Co. (Shelbyville, KY). 2-Propanol was procured from Acros Organics (Morris Plains, NJ). Puregene DNA purification solution was obtained from Qiagen (Morris Plains, NJ). Oasis MCX 3 cc, 60 mg LP Extraction cartridges were obtained from Waters (Milford, MA). Strata-X cartridges (33 μm, 30 mg/1 mL) were obtained from Phenomenex. Alkaline phosphatase was purchased from Roche Diagnostic Corp. (Indianapolis, IN). N^2-(1′-Carboxyethyl)deoxyguanosine was prepared essentially as described (*22*). All other chemicals and enzymes were obtained from Sigma-Aldrich (St. Louis, MO).

7-Carboxymethylguanine (7-CMGua, 5) and [$^{15}N_5$]7-CMGua. 7-CMGua was prepared as described by Zurlo et al (*10*) with minor modifications. Iodoacetic acid (238.6 mg, 1.3 mmol) and Guo (92.2 mg, 0.33 mmol) were dissolved in 600 μL of H_2O containing 16.3 mg of LiOH. The mixture was heated at 100 °C for 70 min. The color of the solution turned to brownish red. The product was purified using HPLC system 1. ^{1}H NMR: δ8.25 (bs, 1H, N1H), 7.66 (s, 1H, C8H), 6.00 (s, 2H, NH_2), 4.46 (s, 2H, CH_2). Positive ESI-MS: *m/z* 210 $[M + H]^+$, MS/MS of *m/z* 210, *m/z* 152 $[BH]^+$; UV λ_{max} 252 nm, λ_{min} 230 nm.

[$^{15}N_5$]7 -CMGua was prepared from [$^{15}N_5$]dGuo using the same procedure. Positive ESI-MS: *m/z* 215 $[M + H]^+$, MS/MS of *m/z* 215, *m/z* 157 $[BH]^+$.

The concentration of 7-CMGua in standard solutions was determined by ^{1}H NMR using toluene as internal standard, as

previously described (*23*). The concentration of [$^{15}N_5$]7-CMG was determined by UV at 254 nm.

7-(2′-Carboxyethyl)guanine (7-CEGua, 13) and [$^{15}N_5$]7-CEGua. 7-CEGua was prepared essentially as described above. 3-Iodopropionic acid (240 mg, 1.2 mmol) and dGuo (80 mg, 0.3 mmol) were dissolved in 1 mL of dimethylacetamide, and the mixture was stirred at room temperature overnight, then heated at 100 °C for 5 h. The product was purified by HPLC using system 1. ^{1}H NMR δ 8.23(bs, 1H, N1H), 7.79 (s, 1H, C8H), 6.11 (s, 2H, NH_2), 4.28 (t, 2H, CH_2CH_2COOH), 2.74 (t, 2H, CH_2CH_2COOH). Positive ESI-MS: *m/z* 224 $[M + H]^+$, MS/MS of *m/z* of 224, *m/z* 152 $[BH]^+$; UV λ_{max} 250 nm, λ_{min} 233 nm. [$^{15}N_5$]7-CEG was prepared from [$^{15}N_5$]dGuo using the same procedure. Positive ESI-MS: *m/z* 229 $[M + H]^+$, MS/MS of *m/z* of 229, *m/z* 157 $[BH]^+$.

The concentrations of 7-CEGua and [$^{15}N_5$]7-CEGua in standard solutions were determined by ^{1}H NMR and UV at 254 nm, respectively.

7-(1′-Carboxyethyl)guanine (18). This was prepared as described for 7-CEGua from 2-bromopropionic acid and dGuo followed by heating at 100 °C for 5 h. Positive ESI-MS: *m/z* 224 $[M + H]^+$, MS/MS of *m/z* of 224, *m/z* 152 $[BH]^+$; UV λ_{max} 250 nm, λ_{min} 233 nm.

Human Liver Samples and DNA Isolation. This study was approved by the University of Minnesota Research Subjects' Protection Program Institutional Review Board Human Subjects Committee. Twenty-four de-identified liver samples were obtained through The Masonic Cancer Center Tissue Procurement Facility. The samples were removed during surgery, immediately frozen in liquid N_2, confirmed histopathologically as normal, and stored at −80 °C.

DNA isolation was performed as previously described with minor modifications (*24*). Briefly, the liver samples (0.5 g) were homogenized with 15 mL of cell lysis solution. After the samples were treated with RNase A and protein was precipitated, DNA was precipitated with 2-propanol. The DNA was dissolved in 4 mL of 10 mM Tris-HCl/1 mM EDTA buffer (pH 7), and the mixture was extracted twice with $CHCl_3$ containing 4% isoamyl alcohol. The DNA was precipitated by the addition of 5 M NaCl and ice-cold ethanol, and washed with 70% and 100% ethanol.

For experiments using *N*-acetylcysteine to investigate artifactual formation of 7-CEGua, 10 mM *N*-acetylcysteine was added to the cell lysis solution before the tissue was homogenized. The DNA was isolated as described above.

Analysis of DNA for 7-CMGua and 7-CEGua. The procedure for enzymatic hydrolysis of DNA was previously described (*25*). Briefly, DNA (1 mg) was dissolved in 1 mL of 10 mM Tris-HCl/5 mM $MgCl_2$ buffer (pH 7.0) containing [$^{15}N_5$]7-CMGua (1920 fmol) and [$^{15}N_5$]7-CEGua (1040 fmol). The DNA was enzymatically hydrolyzed for 70 min at 37 °C with 1060 units of DNase I (type II, bovine pancreas), 0.05 units of phosphodiesterase I (type II, *Crotalus adamanteus* venom), and 300 units of alkaline phosphatase (calf intestine). The hydrolysate, after the removal of a 10 μL aliquot for dGuo quantitation, was purified using a mixed mode cation exchange (MCX Vac RC, 60 mg, Waters) cartridge. The cartridge was conditioned with 2 mL of CH_3OH and 2 mL of 2% H_3PO_4. The sample, which had been acidified with 10 μL of 86% H_3PO_4, was loaded, and the column was washed with 2 mL of 0.1% H_3PO_4 and 2 mL of CH_3OH, which were discarded. It was then washed with 2 mL of 3% NH_4OH in CH_3OH. This fraction was collected and evaporated to dryness on a SpeedVac. To the vial was added 1 mL of freshly prepared 10% CH_3COCl in CH_3OH, and the mixture was heated for 1 h at 50 °C, then concentrated to dryness. The residue was dissolved in 1 mL of 15 mM NH_4OAc buffer (pH 6.6) and purified using a Strata-X (33 μm, 30 mg/1 mL, Phenomenex) solid-phase extraction cartridge. The cartridge was conditioned with 1 mL of CH_3OH, 1 mL of H_2O, and 1 mL of 15 mM NH_4OAc buffer (pH 6.6). The sample was loaded on the cartridge, which was washed with 1 mL of 15 mM NH_4OAc buffer (pH 6.6), 1 mL of H_2O, and 1 mL of 2% aq CH_3OH. It was then washed with 1 mL of 80% CH_3OH, and this eluant was collected and evaporated to dryness. The residue was dissolved in 40 μL of 15 mM NH_4OAc buffer (pH 6.6), and 8 μL aliquots were analyzed by LC-ESI-MS/MS-SRM.

The analysis was carried out with either a Quantum Ultra AM (with Ion Max ESI source) or a Discovery Max triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) interfaced with an Agilent Technologies (Palo Alto, CA) 1100 capillary flow HPLC equipped with a 150 mm × 0.5 mm Zorbax SB C18 column (Agilent). The column was operated at 50 °C and eluted with a gradient from 5% to 25% CH_3CN in 15 mM NH_4OAc buffer (pH 6.6) for 10 min, then a gradient from 25−80% CH_3CN in 2 min, then held at 80% for 6 min, and finally returning to 5% CH_3CN in 2 min, at a flow rate of 15 μL/min. The first 2 min of eluant was directed to waste, and the 2−15 min fractions were diverted to the ESI source, then the remaining 5 min eluant to waste.

A second HPLC system was used for the confirmation of identity. A 150 mm × 0.5 mm, 4 μm, Synergi Polar-RP column (Phenomenex) was eluted with a gradient from 5 to 35% CH_3OH in 15 mM NH_4OAc buffer (pH 6.6) in 35 min at a flow rate of 10 μL/min at 50 °C. The first 15 min of eluant was directed to waste, and the 15 to 35 min fraction was diverted to the ESI source.

The MS parameters were set as follows: spray voltage, 4 kV; sheath gas pressure, 30; capillary temperature, 250 °C; collision energy, 22 V; scan width, 0.1 amu; scan time, 0.4 s; Q1 peak width, 0.7; Q3 peak width, 0.7; Q2 pressure, 1.0 mTorr; source CID, 8 V; and tube lens offset, 95 V. Transitions monitored were as follows, for the methyl esters of 7-CMGua, [$^{15}N_5$]7-CMGua, 7-CEGua, and [$^{15}N_5$]7-CEGua, respectively: *m/z* 224 $[M + H]^+ \rightarrow$ *m/z* 164 $[(M + H) - HCOOCH_3]^+$; *m/z* 229 $[M + H]^+ \rightarrow$ *m/z* 169 $[(M + H) - HCOOCH_3]^+$; *m/z* 238 $[M + H]^+ \rightarrow$ *m/z* 152 $[BH]^+$; and *m/z* 243 $[M + H]^+ \rightarrow$ *m/z* 157 $[BH]^+$.

Calibration curves were constructed before each analysis using standard solutions of 7-CEGua and [$^{15}N_5$]7-CEGua. A constant amount of [$^{15}N_5$]7-CEGua (1040 fmol) was mixed with differing amounts of 7-CEGua (20, 40, 80, 160, 320, and 640 fmol), and these were esterified with CH_3COCl and CH_3OH and analyzed by LC-ESI-MS/MS-SRM. Each set of human liver samples contained negative (buffer blanks) and positive (calf thymus DNA samples) controls.

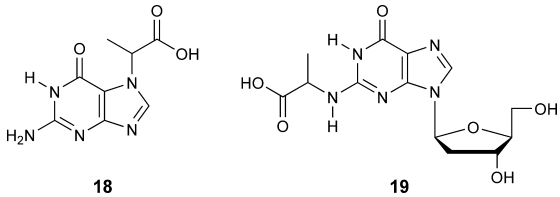
Accuracy and precision were determined by adding 20, 40, 80, 160, or 320 fmol of 7-CEGua in triplicate to 0.5 mg aliquots of calf thymus DNA and analyzing each sample in duplicate. The amount of 7-CEGua which was present in calf thymus DNA (52 fmol/0.5 mg) was subtracted from the amount detected. Recovery was determined by adding 1040 fmol of [$^{15}N_5$]7-CEGua to 0.5 mg of calf thymus DNA and processing as described above. The results were compared to the same analysis without solid-phase extraction.

Effects of *N*-Acetylcysteine on the Formation of 7-CEGua in Reactions of Acrylic Acid with dGuo and Calf Thymus DNA. A mixture of dGuo (30 mg, 110 μmol), acrylic acid (0.32 mg, 4.5 μmol), and *N*-acetylcysteine (final concentration 0, 1, 2, 5, or 10 mM) in 15 mL of 0.1 M phosphate buffer, pH 7, was incubated at 37 °C for 24 h in a shaking water bath. The resulting mixture was analyzed as described above for 7-CEGua. Reactions with calf thymus DNA (30 mg) were carried out and analyzed in a similar fashion.

Effects of *N*-Acetylcysteine on Levels of 7-CEGua in Human Liver DNA. *N*-Acetylcysteine (final concentration, 10 mM) was added to the cell lysis solution before homogenizing the tissue, and DNA was isolated and analyzed as described above.

Results

The internal standards, [$^{15}N_5$]7-CMGua and [$^{15}N_5$]7-CEGua, were prepared by reactions of iodoacetic acid and 3-iodopropionic acid, respectively, with [$^{15}N_5$]dGuo, followed by neutral thermal hydrolysis. We also prepared adducts **18** and **19**, which could have interfered with the analysis of 7-CMGua and 7-CEGua. Adduct **18** was prepared by the reaction of dGuo with 2-bromopropionic acid followed by neutral thermal hydrolysis and adduct **19** by reaction of methylglyoxal with dGuo (*22*).



Since 7-CMGua and 7-CEGua would be released from DNA by neutral thermal hydrolysis due to the lability of the Gua N^9-deoxyribose 1′ bond, our initial plan was to use neutral thermal hydrolysis followed by solid-phase extraction to enrich the adducts. This was not successful because neutral thermal hydrolysis produced a relatively high background in the LC-ESI-MS/MS-SRM analysis of these adducts. Experimentation with calf thymus DNA (which contains 7-CEGua) showed that enzyme hydrolysis was superior to neutral thermal hydrolysis or the combination of both methods of hydrolysis in the release of 7-CEGua from DNA because it produced the highest signal-to-noise ratio in the LC-ESI-MS/MS-SRM analysis.

Solid-phase extraction of 7-CMGua and 7-CEGua using mixed mode cation exchange cartridges was successful, but we were unable to achieve good results with Strata-X reverse-phase cartridges. Since both enrichment steps were necessary and the high polarity of the adducts was suspected to be a reason for their poor retention on Strata-X columns, we converted 7-CMGua and 7-CEGua to their methyl esters by treatment with CH_3COCl and MeOH.

The analytical method is summarized in Scheme 2. Human hepatic DNA and the internal standards were subjected to enzyme hydrolysis followed by solid-phase extraction using mixed mode cation exchange cartridges. The adducts were converted to their methyl esters **20** and **21**, which proceeded in 90% yield, and the resulting mixture was purified by solid-phase extraction on Strata-X cartridges. The appropriate fraction was collected and analyzed by LC-ESI-MS/MS-SRM at *m/z* 224 $[M + H]^+ \rightarrow$ *m/z* 164 $[M - HCOOCH_3]^+$ for **20** and *m/z* 238 $[M + H]^+ \rightarrow$ *m/z* 152 $[BH]^+$ for **21**.

A typical chromatogram obtained upon LC-ESI-MS/MS-SRM analysis using this method is shown in Figure 1. A clear peak for 7-CEGua methyl ester **21** was observed in the *m/z* 238 → *m/z* 152 channel, and this peak coeluted with the corresponding internal standard [$^{15}N_5$]7-CEGua methyl ester, monitored at *m/z* 243 → *m/z* 157. In contrast, no peak was observed for 7-CMGua methyl ester **20**, in the *m/z* 224 → *m/z* 164 channel. Using a different LC column (Synergi Polar-RP) to confirm the presence of 7-CEGua methyl ester **21**, a sharp peak corresponding to **21** was observed, and it coeluted with the internal standard (Figure 2). The related standards **18** and **19**, as their methyl esters, eluted at retention times different from those of **21** under the conditions used in Figure 1: 0.1 min later for **18** and 3.6 min later for **19**.

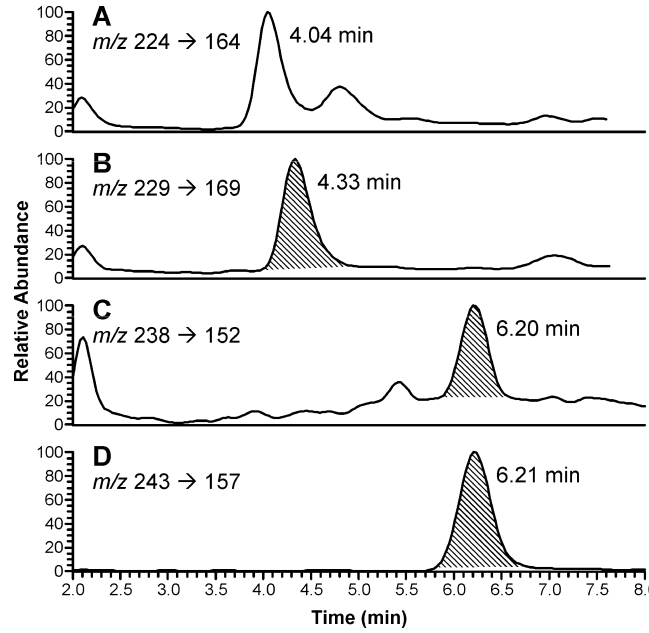
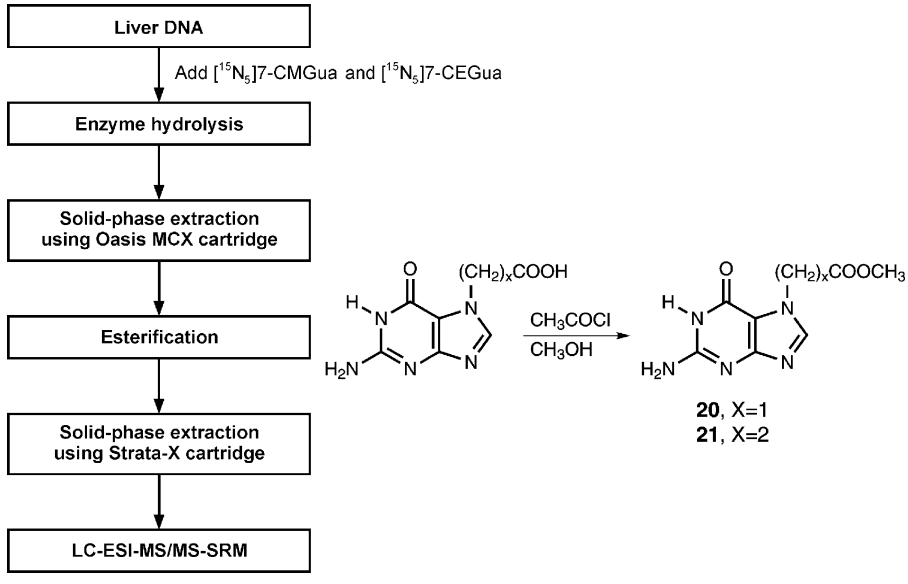


Figure 1. Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis of a hydrolysate of human liver DNA for the methyl esters of 7-CMGua and 7-CEGua. A Zorbax SB C18 column was used for the analysis. Shaded peaks correspond to the methyl esters of [$^{15}N_5$]7-CMGua internal standard (panel B); 7-CEGua (panel C); and [$^{15}N_5$]7-CEGua internal standard (panel D). No peak was observed corresponding to the methyl ester of 7-CMGua (panel A).

A calibration curve for methyl ester **21** was linear in the range measured (20−640 fmol); $R^2 = 1$. The accuracy and precision of the method were tested by adding various amounts of 7-CEGua to calf thymus DNA and carrying out the analysis of three aliquots at each added concentration. The results which are summarized in Table 1 demonstrate outstanding accuracy

Scheme 2. Method of Analysis of Human Liver DNA for 7-CMGua and 7-CEGua



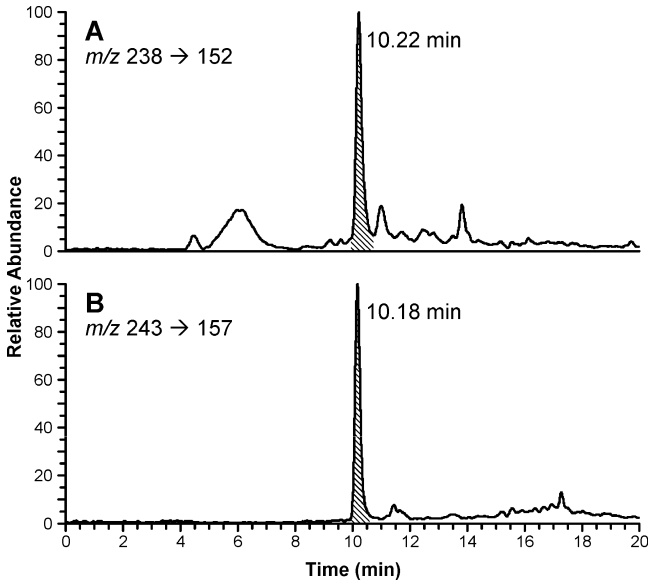


Figure 2. Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis of a hydrolysate of human liver DNA for the methyl ester of 7-CEGua. A Synergi Polar-RP column was used for the analysis. Shaded peaks correspond to the methyl esters of (A) 7-CEGua and (B) [$^{15}N_5$]7-CEGua internal standard.

Table 1. Relationship between Added and Measured Amounts of 7-CEGua in Calf Thymus DNA

added[a]	detected[b,c]	accuracy (%)	precision (CV, %)
20	22.3 ± 0.4	111	1.2
40	40.3 ± 3.5	100	8.7
80	81.0 ± 5.0	100	6.2
160	169 ± 3.6	106	2.1
320	328 ± 4.9	103	1.5

[a] Added to 0.5 mg calf thymus DNA. [b] Amount present in calf thymus DNA was subtracted from each value. 7-CEGua was detected as its methyl ester **21**. [c] Mean ± SD, $N = 3$.

and precision. The on column detection limits for esters **20** and **21** were 10 fmol and 5 fmol, respectively. Recovery was about 50%. The detection limit for **21** in DNA, starting with 1 mg, was approximately 8 fmol.

We considered the possibility that 7-CEGua could be formed as an artifact by reaction of acrylic acid, a ubiquitous compound, with DNA during isolation and analysis. We investigated this problem by first determining the effects of *N*-acetylcysteine on the reaction of acrylic acid with dGuo or DNA. As shown in Table 2, *N*-acetylcysteine inhibited this reaction, presumably by scavenging acrylic acid by Michael addition. Maximum inhibition of about 80% was obtained with 10 mM *N*-acetylcysteine. We then analyzed three human liver DNA samples in the absence and presence of 10 mM *N*-acetylcysteine, added at the DNA isolation step. The amounts of **21** detected in these analyses, with or without *N*-acetylcysteine addition, were sample 1, 419 and 414 fmol/μmol Gua; sample 2, 1673 and 1491 fmol/μmol Gua; and sample 3, 177 and 180 fmol/μmol Gua. These results demonstrate that artifact formation was not occurring during the isolation of DNA and analysis for 7-CEGua.

The results of the analyses of 24 human liver DNA samples are presented in Table 3. 7-CEGua, as its methyl ester **21**, was detected in all of the samples; mean ± SD, 373 ± 320 fmol/μmol Gua (74.6 adducts per 10^9 nucleotides) and range 17−1189 fmol/μmol Gua. 7-CMGua, as its methyl ester **20**, was not detected in any sample. There was no relationship of 7-CEGua levels to age and gender. No information on tobacco use was available.

Table 2. Effects of *N*-Acetylcysteine on the Formation of 7-CEGua in Reactions of Acrylic Acid (16) with dGuo or DNA[a]

(A)

reaction of **16** with	*N*-acetylcysteine (mM)	7-CEGua (fmol/μmol Gua)[b]	% reduction
dGuo	0	5600	
	1	4030	28
	2	3640	35
	5	1810	68
	10	1110	80

(B)

	N-acetylcysteine (mM)	7-CEGua (fmol/μmol Gua)[c]	% reduction
DNA	0	1370	
	5	308	77
	10	168	88
	20	305	77

[a] Reactions were carried out with 0.3 mM **16** and dGuo (30 mg) or calf thymus DNA (30 mg) in 15 mL of 0.1 M phosphate buffer (pH 7) at 37 °C for 24 h. [b] dGuo contained 7-CEGua, 947 fmol/μmol Gua. This amount was subtracted from each value. [c] Calf thymus DNA contained 7-CEGua, 185 fmol/μmol Gua. This amount was subtracted from each value.

Table 3. 7-(2′-Carboxyethyl)guanine in Human Liver DNA[a]

sample	gender	age	DNA analyzed (mg)	7-CEGua (fmol/μmol Gua)
1	F	31	0.18	112
2	F	21	0.19	196
3	F	61	0.84	246
4	M	60	0.56	278
5	M	54	0.58	268
6	M	54	0.16	296
7	F	34	0.54	267
8	F	83	0.26	160
9	F	30	0.12	177
10	F	58	0.38	190
11	F	45	0.13	704
12	M	18	0.11	1120
13	F	51	0.17	769
14	F	50	0.20	326
15	F	51	0.95	17
16	F	38	0.12	1190
17	M	64	0.69	39
18	F	43	0.06	842
19	F	37	0.45	211
20	M	48	0.53	128
21	M	64	0.42	206
22	M	59	0.20	490
23	M	77	0.64	311
24	M	38	0.22	402

[a] 7-(2′-Carboxyethyl)guanine (7-CEGua) was determined as its methyl ester **21**. Negative controls did not have detectable **21**. Positive control values were 172 ± 42 fmol/μmol Gua in calf thymus DNA samples run with each set.

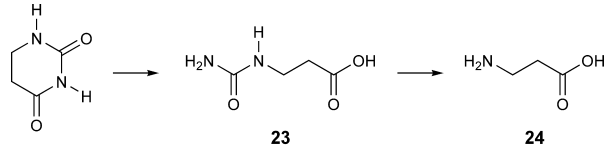
Discussion

We present convincing evidence for the presence of 7-CEGua in human liver DNA. We monitored the transition *m/z* 238 $[M + H]^+ \rightarrow$ *m/z* 152 $[BH]^+$, which is characteristic of our analyte **21**, the methyl ester of 7-CEGua. Our LC-ESI-MS/MS-SRM chromatograms showed clear peaks which coeluted with the internal standard [$^{15}N_5$]7-CEGua, as its methyl ester, in two different LC systems (Figures 1 and 2). We found no evidence however for the presence of 7-CMGua in these liver DNA

samples, as there were no clear peaks eluting at the retention time of its methyl ester **20**.

As indicated in Scheme 1, there are several possible sources of 7-CEGua (**13**) in human liver DNA. One of the most likely may be the reaction of acrylic acid (**16**) with DNA. Acrylic acid is a metabolite of acrolein in the rat, on the basis of in vitro studies with rat liver preparations and on in vivo studies in which the excretion of the mercapturic acid *N*-acetyl-*S*-(2-carboxyethyl)cysteine was observed in the urine of rats treated with either acrolein or acrylic acid (*26*−*29*). Acrolein is a commonly observed lipid peroxidation product and ubiquitous environmental pollutant, occurring in various types of engine exhaust and industrial emissions (*30*). It is formed in high temperature cooking and is found in various foods (*30, 31*). Acrolein forms exocyclic adducts with dGuo in DNA, and these have been detected in some human tissues (*30, 32*−*39*). Acrylic acid is widely employed in the production of acrylates which are used in the manufacturing of polymers, leading potentially to human exposure (*40*). Mercapturic acid metabolites of acrolein and acrylic acid, *N*-acetyl-*S*-(3-hydroxypropyl)cysteine and *N*-acetyl-*S*-(2-carboxyethyl)cysteine, are routinely detected in the urine of nonsmokers (*41, 42*). Acrylic acid reacts with DNA in vitro giving 7-CEGua as a product, although the reaction is slow and has not been reported in vivo (*21*). Nevertheless, these data collectively provide a plausible mechanism for our finding of 7-CEGua in human hepatic DNA.

A second possible source is 1-nitroso-5,6-dihydrouracil (**14**). Favoring this hypothesis is the fact that 7-CEGua has been detected in the hepatic DNA of rats treated with **14**. Therefore, if there is human exposure to **14**, its metabolism to 7-CEGua in the liver is likely. Human exposure to **14** could potentially occur by endogenous nitrosation of 5,6-dihydrouracil (**22**). This is formed in the metabolism of uracil, catalyzed by dihydropyrimidine dehydrogenase. Subsequent steps in the metabolism of uracil lead to β-ureidopropionic acid (**23**) and finally to β-alanine (**24**). Levels of **22** in the urine of healthy volunteers averaged 1.41 μmol/mmol creatinine (about 1 mg per day), while those of **23** and **24** were 2.09 and 0.69 μmol/mmol creatinine, respectively (*43*). Levels of **22** in the plasma of healthy subjects averaged 36 ng/mL plasma (*44*). Endogenous nitrosation of **22** could potentially occur in situations of high nitrite exposure or the generation of endogenous NO, but this requires further investigation (*6, 45*−*47*). Nitrosation of **23** and **24** could also lead to carboxyethyl diazonium ion **12**.



22 **23** **24**

Our original hypothesis for this study pertained to the nitrosamines NSAR and MNPA. Although MNPA has not been reported frequently as a constituent of human urine, the levels reported in the one study carried out in Poland were similar to those of NSAR and indicate an environmental or endogenous source of exposure to this nitrosamine in humans (*6, 18*). α-Methyl hydroxylation of MNPA, while apparently not reported in the literature, is certainly a likely route of metabolism of this nitrosamine. Thus, the generation of carboxyethyldiazonium ion **12** in this way is completely reasonable and could be the source of 7-CEGua in hepatic DNA. Arguing against this hypothesis, however, is the lack of detection of 7-CMGua in our samples. Since, in most studies carried out in Western countries, levels of NSAR reported in human urine are similar to those of MNPA (*6*), we might have expected to detect both 7-CMGua and 7-CEGua if these nitrosamines were their sources. It is possible, however, that NSAR is metabolized to a lesser extent than MNPA by α-methyl hydroxylation. This requires further study.

In view of the extensive studies on O^6-CMGua formation, we were somewhat surprised to find no evidence for the presence of 7-CMGua in human liver DNA. In vitro reactions of *N*-nitrosoglycocholic acid (**8**) with DNA produced 7-CMGua and O^6-CMGua in an approximate 10:1 ratio, consistent with data on other alkylating agents (*11, 48*). Other studies by Shuker et al. provide convincing evidence for a number of *N*-nitroso compound precursors to O^6-CMGua in DNA via diazoacetate (**4**), a reaction which also produces O^6-methylGua via the decarboxylation of **4** (*12, 13*). They also reported the presence of this adduct in exfoliated colonic cells obtained from volunteers participating in a diet study to investigate the potential genotoxic effects of *N*-nitroso compounds arising from red meat consumption, an area of research that has been extensively and convincingly documented by the Bingham group (*14, 49, 50*). There are no reports in the literature of O^6-CMGua in hepatic DNA. It is possible that the formation of this adduct is specific to favorable conditions which exist in the colon. Decarboxylation of **4** to O^6-methylGua might also partially limit the observed levels of 7-CMGua.

The levels of 7-CEGua found in this study (373 fmol/μmol Gua) are quite comparable to those of the acetaldehyde DNA adduct N^2-ethylidene-dGuo (534 fmol/μmol dGuo), which we have previously quantified in human hepatic DNA by LC-ESI-MS/MS-SRM (*51*). 7-CEGua levels were substantially higher than those of 7-ethylGua (42 fmol/μmol Gua) (*24*), N^2-ethyl-dGuo (12 fmol/μmol dGuo) (*51*), and the 1,N^2-propano-dGuo adduct of crotonaldehyde and acetaldehyde (ND-15 fmol/μmol dGuo) (*52*), all quantified in human liver DNA by LC-ESI-MS/MS-SRM. The levels reported here are also higher than those of several endogenous DNA adducts reported in human hepatic DNA analyzed by various methods (*53*). These results suggest that there is a commonly occurring and rich source of 7-CEGua adducts in hepatic DNA.

There are no reports on the mutagenic properties of 7-CEGua. In general, 7-alkylGua derivatives are not considered to be mutagenic per se. However, they are prone to depurination, which can lead to abasic sites in DNA (*54*). If these are not repaired, the result can be G → T mutations and other changes (*54, 55*).

Our finding of 7-CEGua in human hepatic DNA raises significant questions regarding its source and the potential role of its precursors in human carcinogenesis. It will be important to determine whether acrylic acid, endogenous nitrosation of 5,6-dihydrouracil or glycine, or another mechanism is its main source. Another consideration, not addressed in this study, is tobacco use. These questions cannot be answered by assaying for this adduct in hepatic DNA because liver tissue is not routinely available. Therefore, we are investigating the detection of this adduct in human leukocyte DNA or urine.

A limitation of this study is that we do not know to what extent depurination of 7-CMGua or 7-CEGua may take place during DNA isolation, before the addition of the internal standards. If depurination does occur during DNA isolation, the values reported here would be lower than those that actually exist in hepatic DNA. A solution to this would be the synthesis of a DNA strand containing [^{15}N]7-CEdGuo, which could be added at the beginning of the DNA isolation procedure.

In summary, we report the detection of substantial amounts of 7-CEGua in human hepatic DNA. The related adduct, 7-CMGua, was not detected. These results provide some new insights on possible mechanisms of DNA damage in humans. While our original hypothesis involved the possible production of these adducts from *N*-nitroso compounds, our results suggest that acrylic acid may be a more likely precursor.

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