Detection and Quantification of 5-Chlorocytosine in DNA by Stable Isotope Dilution and Gas Chromatography/ Negative Ion Chemical Ionization/Mass Spectrometry

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Hypochlorous acid (HOCl) is generated from activated phagocytes during infections and inflammation. One of the major products of HOCl reaction with DNA was 5-chlorocytosine (5Cl-Cyt). In this report, a gas chromatography/negative ion chemical ionization/mass spectrometry (GC/NICI/MS) assay with stable isotope dilution was developed for detection and quantification of 5Cl-Cyt in DNA. During hydrolysis of DNA, 5Cl-Cyt undergoes spontaneous deamination quantitatively forming 5-chlorouracil (5Cl-Ura). The stable isotope of 5Cl-Ura with six mass units higher than the normal 5Cl-Ura was synthesized and used as internal standard of the assay. The adduct-enriched fraction of DNA hydrolysate was derivatized with pentafluorobenzyl bromide before GC/NICI/MS analysis with selected ion monitoring at $[M - 181]^-$ fragments of bispentafluorobenzylated 5Cl-Ura and its isotope analogue. The limit of detection was 20 amol ($S/N = 8$) of bispentafluorobenzylated 5Cl-Ura injected on column with selective ion monitoring mode and the limit of quantification for the entire assay was 14 fmol of 5Cl-Cyt. Analysis of hypochlorous acid-treated calf thymus DNA by both GC/NICI/MS and HPLC/UV detection provided similar adduct levels and thus verified this new GC/NICI/MS assay. Using this highly specific and ultrasensitive GC/NICI/MS method, the levels of 5Cl-Cyt in untreated calf thymus DNA and human placental DNA were determined as 0.6 and 6.6 adducts per 10^7 normal cytosine, respectively. Peroxynitrite also contributed to 5Cl-Cyt formation in DNA. Level of 5Cl-Cyt in DNA treated with peroxynitrite in the presence of chloride was higher than that without addition of chloride. Thus, quantification of 5Cl-Cyt in DNA by this isotope dilution GC/NICI/MS assay may facilitate research on the role of DNA chlorination in carcinogenesis and in cancer development.

Introduction

Upon stimulation, neutrophils and phagocytes excrete hypochlorous acid (HOCl)[1] as a defense mechanism for killing the invading microorganisms. Formation of HOCl at the sites of infection and inflammation is through oxidation of chloride (Cl^-) by hydrogen peroxide (H_2O_2) catalyzed by the heme enzyme myeloperoxidase (MPO) (*1*, *2*). Being a bactericidal agent, HOCl also damages the host cells by reaction with various biological molecules, including protein, DNA, carbohydrates, lipids, ascorbate, GSH, and NADH (*3*−*11*). It also leads to formation of cross-links between protein and DNA (*12*). The major reactions of DNA with HOCl include oxidation of pyrimidines, but not purines, and chlorination of cytosine. 5-Chlorocytosine (5Cl-Cyt) was referred as a "fingerprint" for damage of DNA by HOCl (*13*). Reaction of HOCl with nitrite, of which the concentration is elevated during inflammation (*14*, *15*), forms nitryl chloride (NO_2Cl). Nitryl chloride has been shown to form in activated human neutrophils (*16*), and it is capable of chlorination, nitration, and oxidation of DNA, protein, and low-density lipoproteins in vitro (*17*−*21*). The level of 5Cl-Ura in HOCl-treated DNA increased in the presence of nitrite compared to HOCl alone, indicating that NO_2Cl was a better chlorinating species than HOCl (*17*). Thus, 5Cl-Cyt is not a specific biomarker for HOCl, but it could be considered as a biomarker for "reactive chlorine species". Chlorination of adenine was also found in DNA exposed to HOCl (*21*), but the amount of 8-chloroadenine was much lower than 5Cl-Cyt. Furthermore, only 5Cl-Cyt, not 8-chloroadenine, formation was significantly increased when HOCl was added in the presence of nitrite (*17*).

Detection of 5Cl-Cyt in vivo was first reported in salmon sperm DNA (*22*). It was not clear whether 5Cl-Cyt was formed endogenously or from molecular chlorine (Cl_2) in processing of water (*23*). Exposure of human respiratory epithelial cells to both HOCl and nitrite significantly increased the yield of 5Cl-Cyt compared to HOCl treatment alone (*24*). Henderson et al. detected 5Cl-Cyt in RNA, but not DNA, of intact *Escherichia coli* exposed to the myeloperoxidase/H_2O_2/Cl^- system (*25*). However, they cannot conclude that the chlorinating agent did not reach the nucleus and react with DNA since the limit of quantification was approximately 1 in 10^4 nucleobases. Apparently, a highly sensitive and specific assay for 5Cl-Cyt in cellular or tissue DNA is demanded.

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[1] Abbreviations: TBDMS, *t*-butyldimethylsilyl; 5Cl-Cyt, 5-chlorocytosine; 5Cl-dCyd, 5-chloro-2′-deoxycytidine; 5Cl-dUrd, 5-chloro-2′-deoxycytidine; 5Cl-Ura, 5-chlorouracil; HOCl, hypochlorous acid; MPO, myeloperoxidase; NICI, negative ion chemical ionization; NO_2Cl, nitryl chloride; PFB, pentafluorobenzyl; SIM, selective ion monitoring; SPE, solid-phase extraction; TMS, trimethysilyl.

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In this study, an isotope dilution GC negative ion chemical ionization (NICI) mass spectroscopic assay is developed for quantification of 5Cl-Cyt in DNA. With incorporation of stable isotope of the analyte as internal standard and labeling the analyte with electrophore, followed by GC/NICI/MS analysis, this assay offers high sensitivity and specificity. The level of 5Cl-Cyt in untreated calf thymus DNA is accurately quantified. Peroxynitrite is another strong oxidant released in inflamed tissues (*26*, *27*), and 5Cl-Cyt is first found in peroxynitrite-treated DNA in the presence of Cl^-. The present work provides a useful tool in investigating the role of cytosine chlorination in DNA in in vivo experiments.

Experimental Procedures

Materials. Calf thymus DNA, human placental DNA, 5-chlorouracil (5Cl-Ura), cytosine, 2′-deoxycytidine, and methionine were from Sigma Chemical Co. (St. Louis, MO). [$^{13}C_4$,$^5N_2$]Uracil (U-$^{13}C_4$, 99%, U-$^5N_2$, 98%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Diisopropylethylamine, 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br), anhydrous methanol, and anhydrous phosphorus pentoxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bond Elut C18-OH and Si solid-phase extraction (SPE) columns were from Varian (Harbor City, CA). The concentration of NaOCl was determined by the absorbance at 292 nm (pH 12, $\epsilon = 350\ M^{-1}\ cm^{-1}$) (*28*). Peroxynitrite was synthesized according to the previously described procedures using isoamylnitrite and hydrogen peroxide (*29*) and was stored at −80 °C. The concentration of peroxynitrite was determined by the absorbance at 302 nm in 1 N NaOH ($\epsilon = 1670\ M^{-1}\ cm^{-1}$) (*30*).

Instruments. NMR spectra were recorded on a Bruker DPX 400 MHz (Billerica, MA) instrument. GC/NICI/MS experiments were conducted using a Hewlett-Packard 6890 GC with 5973 MSD mass detector (Agilent, Taiwan).

Liquid Chromatography. HPLC system was equipped with Hitachi L-7000 pump system with D-7000 interface, a Rheodyne injector, and L-7450A photodiode array detector (Hitachi, Taiwan). *System 1.* A Prodigy ODS (3) 250 × 4.6 mm 5 μm column was eluted with a linear gradient of solvents A and B: 0−20 min, 0% B; 20−40 min, 0 to 100% B (solvent A, 50 mM ammonium formate, pH 4.0; solvent B, methanol) at a flow rate of 1.0 mL/min. *System 2.* A Prodigy ODS (3) 250 × 4.6 mm 5 μm column (Phenomenex, Torrance, CA) was eluted at a linear gradient of water and methanol: 0−10 min, 5% methanol; 10−20 min, 5 to 25% methanol; 20−30 min, 25 to 100% methanol at a flow rate of 1.0 mL/min. *System 3.* A Prodigy ODS (3) 250 × 4.6 mm 5 μm column was eluted with a linear gradient of water and methanol: 0−35 min: 0 to 100% methanol at a flow rate of 1.0 mL/min. *System 4.* A Prodigy ODS (3) 250 × 4.6 mm 5 μm column was eluted with 50 mM ammonium formate (pH 4.0) at a flow rate of 1.0 mL/min.

Formation of 5Cl-Cyt. To a solution containing Cyt (1.0 mM) in potassium phosphate buffer (0.2 M) was added NaOCl (1.0 mM) or peroxynitrite (20 mM) in the presence or absence of NaCl (100 mM) with a final pH of 7.4. The reaction mixture was incubated at 37 °C for 20 min, followed by immediate analysis by HPLC using system 1 monitoring at 270 and 280 nm. The reaction of Cyt with H_2O_2 was performed under similar conditions except that the concentration of H_2O_2 was 100 mM and the incubation time was 1 day.

Synthesis of 5Cl-Cyt and 5Cl-dCyd. To a solution containing Cyt or dCyd (1.0 mM) and NaCl (100 mM) at pH 2.0 was added NaOCl (1.5 mM), and the pH was adjusted to 2.0. The reaction was incubated at 37 °C for 1 h in a shaking water bath and quenched with methionine (1.5 mM). The reaction mixture was collected by HPLC using system 2 monitoring at 280 nm. 5-Chlorocytosine and 5Cl-dCyd was collected and evaporated to dryness.

Synthesis of Isotope-Labeled 5Cl-Ura Internal Standard. To a solution containing [$^{13}C_4$,$^{15}N_2$]Ura (1.3 mM) was added NaCl (200 mM) and NaOCl (2.1 mM, final concentration). The reaction mixture was adjusted to pH 2.0, shaked at 37 °C for 1 h, and quenched with methionine (2.1 mM). The reaction mixture was purified by a C18-OH SPE (500 mg, 3 mL) precondition with 13.5 mL of methanol, followed by 13.5 mL of water. The SPE column was washed with 2.7 mL of water and the fraction containing [$^{13}C_4$,$^{15}N_2$]5Cl-Ura was eluted with 2.7 mL of water. The eluant was dried and analyzed by HPLC using system 1 for its chemical purity. The yield (59%) was quantified based on the molar UV absorbance of 5Cl-Ura at 280 nm. The isotopic purity was confirmed by GC/NICI/MS after pentafluorobenzylation of 1.0 ng of [$^{13}C_4$,$^{15}N_2$]5Cl-Ura. No peak corresponding to the pentafluorobenzylated 5Cl-Ura at *m*/*z* 325 was detected.

Synthesis of N^1,N^3-Bis(pentafluorobenzyl)-5-chlorouracil Derivative. To a 20 mL vial containing dried 5Cl-Ura (3.0 mg, 21 μmol) was added a solution of diisopropylethylamine (280 μL, 1.6 mol) and PFB-Br (50 μL, 310 mmol) in 0.6 mL anhydrous methanol and the reaction mixture was incubated at 42 °C for 2 h. The reaction mixture was evaporated under vacuum and collected using HPLC system 3 monitoring at 270 nm to afford 2.7 mg of $(PFB)_2$-5Cl-Ura (77% yield) as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.01 (s, 2H, benzyl), 5.23 (s, 2H, benzyl), 7.49 (s, 1H, C6−H). NICI-MS (assignment and relative abundance in parentheses) 291 ([MH − PFB − Cl]$^-$, 56%), 325 ([M − PFB]$^-$, 100%), 327 (39%), 486 ([M − HF]$^-$, 5.6%), 506 (M$^-$, 2.3%).

Modification of Calf Thymus DNA with HOCl. Two conditions were performed to obtain 5Cl-Cyt-containing calf thymus DNA. *Condition A.* Calf thymus DNA (2.48 mg/mL) in potassium phosphate buffer (0.2 M, pH 7.4) was incubated with NaOCl (1.74 mM) and NaCl (100 mM) at 37 °C for 20 min, followed by addition of methionine (17.4 mM) and stand at room temperature for 30 min to quench the reaction. *Condition B.* Calf thymus DNA (0.5 mg/mL) in potassium phosphate buffer (0.2 M, pH 7.4) was incubated with HOCl (0.35 mM) and NaCl (100 mM) at 37 °C for 60 min, followed by addition of methionine (3.5 mM).

Peroxynitrite-Treated DNA. Calf thymus DNA (0.5 mg/mL) in potassium phosphate buffer (0.2 M, pH 7.25) with or without NaCl (100 mM) was added peroxynitrite (final concentration 0.35 mM, final pH 7.4) and incubated at 37 °C for 60 min, followed by addition of methionine (3.5 mM) and stand at room temperature for 30 min.

DNA Hydrolysis and Adduct Enrichment by C18-OH SPE. DNA sample was evaporated, added [$^{13}C_4$,$^{15}N_2$]5Cl-Ura (1.0 ng) and 60% formic acid (0.5 mL) and heated at 150 °C for 45 min. The hydrolysate was evaporated to dryness, reconstituted in a 1.0 mL aqueous solution (pH 4.0), and enriched by a C18-OH SPE column preconditioned with 13.5 mL of methanol, followed by 13.5 mL of ammonium formate (50 mM, pH 4.0) solution. The column was washed with 2.7 mL of ammonium formate (50 mM, pH 4.0) and collected the second 2.7 mL of ammonium formate solution.

Pentafluorobenzylation and Si SPE Column. The eluant was evaporated and dried over phosphorus pentoxide, followed by addition of an anhydrous methanolic solution (0.3 mL) containing pentafluorobenzyl bromide (40 μL) and diisopropylethylamine (57 μL) under argon atmosphere. The reaction mixture was incubated at 42 °C for 2 h, followed by evaporation to dryness. The residue was added dichloromethane (0.4 mL) and purified by Si SPE column preconditioned with 15 mL of dichloromethane. The column was washed with 1 mL of dichloromethane and eluted with 2 mL of dichloromethane. The eluant was evaporated, transferred to an insert, and evaporated to dryness. The residue was dissolved in 10 μL of acetone and 1 μL aliquot was analyzed by GC/NICI/MS with selective ion monitoring (SIM) at *m*/*z* 325 and 331 for PFB_2-5Cl-Ura and [$^{13}C_4$,$^{15}N_2$]PFB_2-5Cl-Ura, respectively.

GC/NICI/MS Analysis of $(PFB)_2$-5Cl-Ura. GC/NICI/MS studies used a Hewlett-Packard 6890 GC with 5973 MSD mass

detector with the negative ion chemical ionization source. In the SIM mode, the filament was operated at 120 eV with the ion source at 150 °C. The analyses were carried out with a cool-on-column inlet, a precolumn (J&W, 1.0 m, 0.53 mm, deactivated silica), and a HP-5MS capillary column (Hewlett-Packard, 30 m × 0.25 mm, 0.25 μm film thickness) inserted into the ion source. Methane (99.999% pure) was the moderating gas with a flow rate of 2.0 mL/min and the pressure at the ion gauge was 2.2×10^{-4} Torr. Helium was used as the carrier gas (99.999% pure) at a flow rate of 1.2 mL/min. The oven temperature was held at 50 °C for 2 min and then raised to 300 °C at 10 °C/min. Selective ion monitoring at *m/z* 325 and 331 was used to detect and quantify the $[M - 181]^-$ fragment ions of PFB_2-5Cl-Ura and $[^{13}C_4,^{15}N_2]PFB_2$-5Cl-Ura, respectively. The quantification was based on intrapolation of the ratio of the peak area of PFB_2-5Cl-Ura versus $[^{13}C_4,^{15}N_2]PFB_2$-5Cl-Ura and the calibration curve. The level of 5Cl-Cyt in DNA was obtained from dividing the amount of 5Cl-Cyt by the amount of cytosine in the DNA sample (*31*).

Assay Calibration. The stock solutions of 5Cl-Cyt and $[^{13}C_4,^{15}N_2]$5Cl-Ura (1.0 mg/mL) in water were stored at −80 °C. Sample solutions for calibration were freshly prepared by diluting the stock solutions in H_2O for each analysis. The internal standard $[^{13}C_4,^{15}N_2]$5Cl-Ura (1.0 ng) was added to samples containing various amounts of 5Cl-Cyt ranging from 0, 1, 2, 5, 10, 20, 50, 100, and 150 pg. The samples were processed through the entire procedures, i.e., formic acid hydrolysis, C18-OH SPE enrichment, pentafluorobenzylation, Si SPE purification, and GC/NICI/MS analysis.

HPLC/UV Analysis of 5Cl-Cyt in DNA. DNA (0.52 mg) treated with HOCl under condition A was hydrolyzed with 60% formic acid (0.5 mL) at 150 °C for 45 min. The hydrolysate was evaporated to dryness, reconstituted in water (0.2 mL), and analyzed by HPLC using system 3 monitoring at 280 nm.

Results and Discussion

Formation and Stability of 5Cl-Cyt. Reaction of Cyt with equal molar of HOCl under physiological temperature and pH led to formation of 5Cl-Cyt in 6.7% yield, respectively, as determined by HPLC analysis with photodiode array detection. The reaction was quenched with a scavenger methionine, the fastest HOCl-reacting amino acid residue of protein (*32*). Since HOCl is generated via oxidation of Cl^- by H_2O_2, we examine if other physiologically relevant oxidants are also capable of Cl^- oxidation. Like HOCl, peroxynitrite is also found in inflamed tissues. It is formed by the rapid reaction of superoxide anion with nitric oxide, both of which are released from macrophages and neutrophils (*26, 27*). Peroxynitrite plays an important role in carcinogenesis related to chronic infections and inflammation (*33*). In the presence of plasma concentration of Cl^- (100 mM), small amount of 5Cl-Cyt (0.015%) was detected in the incubation mixture of Cyt with excess peroxynitrite. Under similar conditions, no 5Cl-Cyt was detected with peroxynitrite alone. (Table 1). These results show that peroxynitrite is capable of oxidizing Cl^- forming an intermediate that is a much weaker chlorinating agent than HOCl. The identity of this intermediate will be investigated, but it is not the focus of this study. In contrast, large excess of H_2O_2 did not oxidize Cl^- during prolonged incubation in the absence of a peroxidase, such as myeloperoxidase or eosinophil peroxidase (*25*), indicating the importance of peroxidases in generation of HOCl in vivo.

5-Chlorocytosine and its nucleoside 5Cl-dCyd are not stable adducts. After collection from the reaction mixture by reversed-phase HPLC followed by evaporation, the dry solid samples of 5Cl-Cyt and 5Cl-dCyd decomposed during storage at room temperature for 1 week without protection from the air or light. The decomposed products were their deaminated analogues, 5Cl-Ura and 5Cl-2′-deoxyuridine. Under the conditions described above, 23% of 5-chlorouracil (5Cl-Ura) derived from solid 5Cl-Cyt and 9% of 5Cl-dUrd derived from solid 5Cl-dCyd as determined by HPLC analysis with photodiode array detection (Figure 1). Incubation of purified 5Cl-Cyt at 37 °C for 20 min led to formation of 35% of 5Cl-Ura, whereas 5Cl-Ura was stable under similar conditions.

Table 1. Formation of 5Cl-Cyt from Reaction of Cyt with Oxidants

reaction and time	yield of 5Cl-Cyt (%)	yield of 5Cl-Ura (%)
Cyt + HOCl (1:1), 20 min[a]	6.74 ± 0.08	ND[c]
Cyt + $ONOO^-$ (1:20), 20 min[a]	ND[d]	ND
Cyt + $ONOO^-$ (1:20) + Cl^-, 20 min[b]	ND	ND
Cyt + H_2O_2 (1:100) + Cl^-, 1 day[b]	ND	ND

[a] The incubation was performed with Cyt (1.0 mM) with HOCl or peroxynitrite in potassium phosphate buffer (0.2 M, final pH 7.4) at 37 °C, followed by quench with methionine and HPLC analysis using system 1. The percentage yields are presented as mean ± standard deviation from at least duplicated experiments. [b] The incubation was performed in the presence of 100 mM of Cl^-. [c] Not detectable. The limit of detection for 5Cl-Ura is ca. 1.5 ng. [d] Not detectable. The limit of detection for 5Cl-Cyt is ca. 1.5 ng.

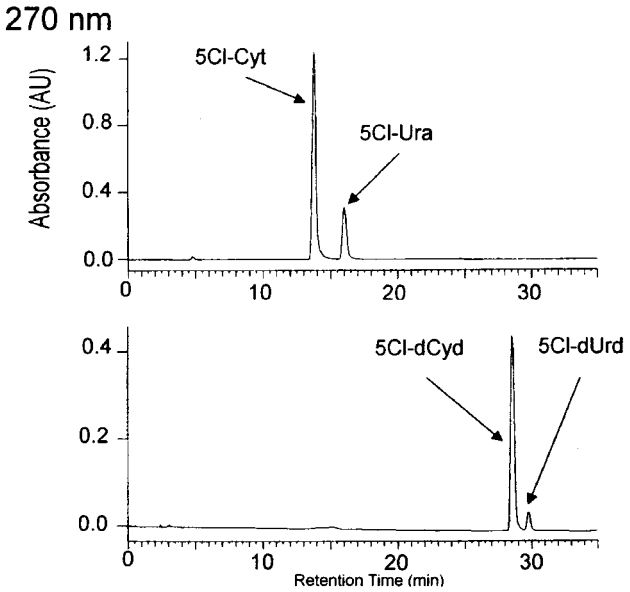
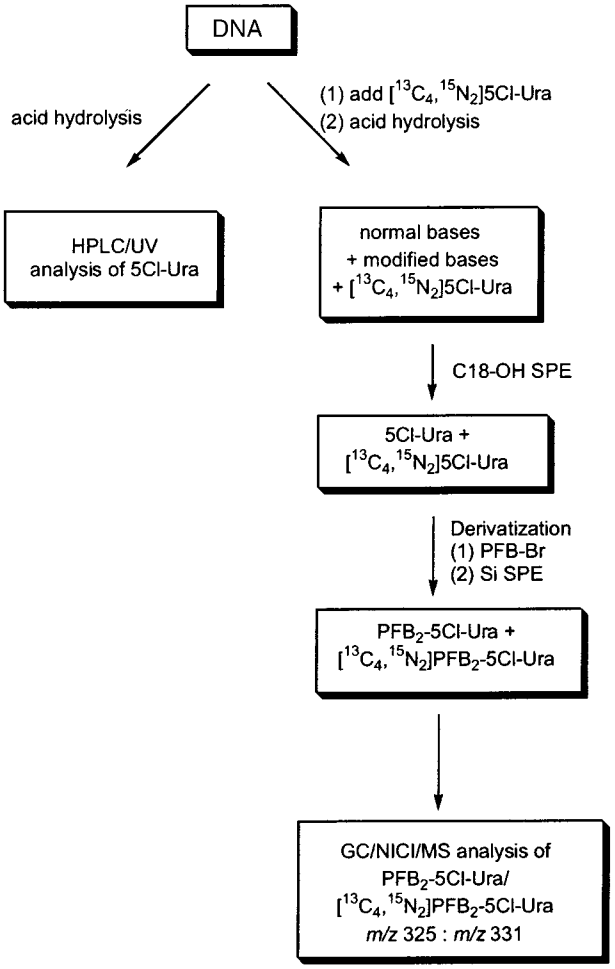


Figure 1. Decomposition of (a) 5Cl-Cyt to 5Cl-Ura and (b) 5Cl-dCyd to 5Cl-dUrd upon storage at room temperature for 1 week.

GC/NICI/MS Analysis of 5Cl-Cyt in DNA. The procedures of this new GC/NICI/MS assay for 5Cl-Cyt in DNA are modified from the isotope dilution GC/NICI/MS methods developed in this laboratory for 3,N^4-ethenocytosine and 1,N^6-ethenoadenine in DNA (*31, 34, 35*). The entire assay involves addition of a stable isotope labeled internal standard, acid hydrolysis, adduct enrichment, derivatization with an electrophore, postderivatization cleanup, and GC/NICI/MS analysis as illustrated in Scheme 1.

Under the hydrolysis condition of DNA with 60% formic acid at 150 °C for 90 min, 5Cl-dCyd converted to 5Cl-Ura quantitatively, while 98% formic acid at 150 °C or 1 N HCl at 100 °C for 2 h led to incomplete deamination (data not shown). Thus, hydrolysis of DNA with 60% formic acid was used and 5Cl-Ura was measured as the

Scheme 1. Procedures Involved in the Assays of 5Cl-Cyt in DNA



end product for quantification of 5Cl-Cyt in DNA hydrolysate (*13*). The isotope analogue having six mass units higher than the analyte, $[^{13}C_4,^{15}N_2]$5Cl-Ura, was added to the DNA samples as internal standard. The isotope standard $[^{13}C_4,^{15}N_2]$5Cl-Ura used in this assay was synthesized from $[^{13}C_4,^{15}N_2]$uracil reacting with HOCl in the presence of Cl^- under acidic pH to form $[^{13}C_4,^{15}N_2]$5Cl-Ura in optimum yield (*25*). The isotope standard was obtained in good yield (59%) after purification by a disposable C18-OH solid-phase extraction (SPE) column, which was sufficient to provide high chemical purity, determined by reversed-phase HPLC with photodiode array detection. The isotopic purity of $[^{13}C_4,^{15}N_2]$-5Cl-Ura used in the assay was confirmed by derivatization with pentafluorobenzyl bromide (PFB-Br) followed by analysis using GC/NICI/MS at *m/z* 325 and 331 for pentafluorobenzylated 5Cl-Ura and $[^{13}C_4,^{15}N_2]$5Cl-Ura, respectively, and no peak corresponding to the pentafluorobenzylated 5Cl-Ura at *m/z* 325 was detected. The purity of $[^{13}C_4,^{15}N_2]$Ura purchased was U-$^{13}C_4$, 99%, U-$^{15}N_2$, 98%. Assuming there is no contamination during synthesis of $[^{13}C_4,^{15}N_2]$5Cl-Ura, the content of $[^{12}C_4,^{14}N_2]$5Cl-Ura should be $(0.01)^4 \times (0.02)^2 = 4 \times 10^{-10}$ % or 0.026 zmol in 1.0 ng of $[^{13}C_4,^{15}N_2]$5Cl-Ura, which is far below the detection limit of $(PFB)_2$-5Cl-Ura (20 amol). Thus, the contribution from natural abundance of this isotope standard can be neglected in sample quantification.

Standard pentafluorobenzylated 5Cl-Ura was synthesized under mild derivatization conditions (*31*). The

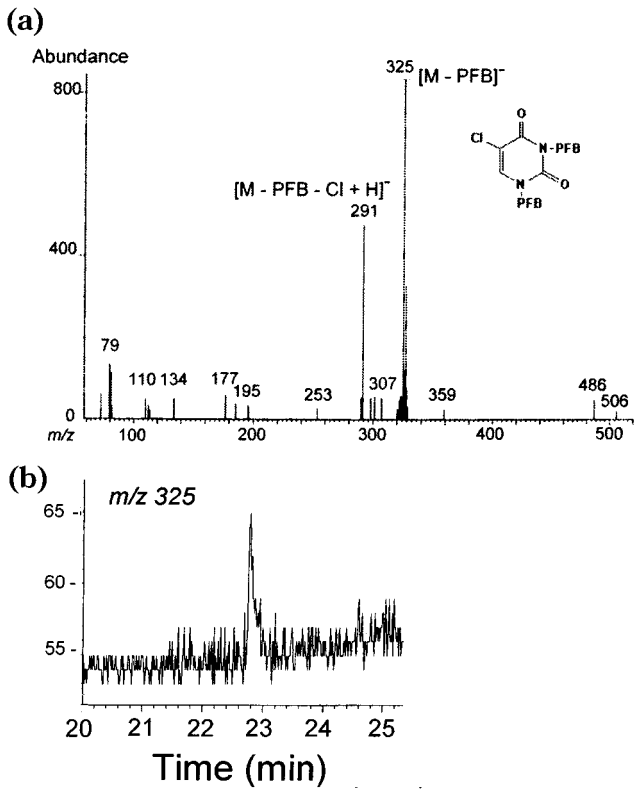


Figure 2. (a) Full scan NICI/MS spectrum of PFB_2-5Cl-Ura. (b) Detection of 10 fg (20 amol) of PFB_2-5Cl-Ura by GC/NICI/MS with selective ion monitoring at *m/z* 325.

pentafluorobenzyl group attached at both N1 and N3 of 5Cl-Ura, yielding N^1,N^3-bis(pentafluorobenzyl)-5-chlorouracil (PFB_2-5Cl-Ura), analogous to other pentafluorobenzyl derivatives of uracil (*36*). The ^{1}H NMR measured in $CDCl_3$ showed two sets of benzyl protons as two singlets with chemical shifts at 5.01 and 5.23 ppm in addition to C6-H at 7.49 ppm. The identity of the derivative was also confirmed by the full scan mass spectrum in NICI mode. The NICI mass spectrum showed a small molecular ion peak of *m/z* 506. The $[M - 181]^-$ ion of 325 was the base peak, indicating that the compound tends to lose one, but not two, pentafluorobenzyl moiety. The relative abundance of the ion at *m/z* 327 supported that the compound contained a chlorine atom (Figure 2a). The major fragment ion at *m/z* 325 was used for monitoring PFB_2-5Cl-Ura in the GC/NICI/MS assay. The limit of detection for PFB_2-5Cl-Ura by GC/NICI/MS was 10 fg (20 amol) with a $S/N = 8$ injected on column with selective ion monitoring (SIM) mode at *m/z* 325 (Figure 2b).

The isotope standard $[^{13}C_4,^{15}N_2]$5Cl-Ura was stable under the hydrolysis condition, and it was added before formic acid hydrolysis. The C18−OH SPE condition was optimized using a buffer of pH 4 to enrich 5Cl-Ura from the mixture of normal and adducted bases released by acid hydrolysis of DNA. Elution of 5Cl-Ura is not affected by pH ranging from 3 to 7 since it does not contain a free amino group. The optimum condition for its enrichment from the DNA hydrolysate was for removing maximum amount of the normal bases. Under this condition, 5Cl-Ura was collected with 85% of thymine, 49% of guanine, and 4% of cytosine. To ensure complete derivatization of 5Cl-Ura in the presence of large excess of normal bases, copious amount of derivatization agent

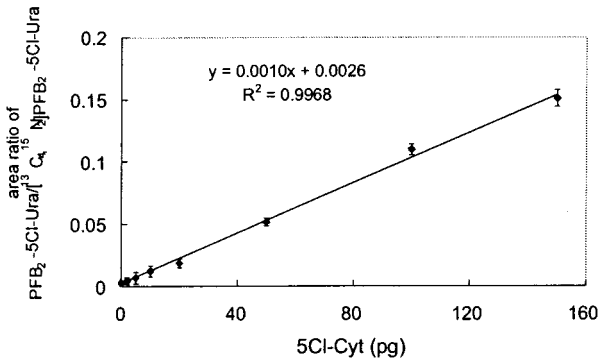


Figure 3. Calibration curve for the GC/NICI/MS analysis for PFB_2-5Cl-Ura at *m/z* 325 and $[^{13}C_4,^{15}N_2]PFB_2$-5Cl-Ura at *m/z* 331. Samples containing various amounts (0−150 pg) of 5Cl-Cyt was added a fix amount of $[^{13}C_4,^{15}N_2]$5Cl-Ura (1.0 ng) and subjected to the assay procedures described in Experimental Procedures (r^2= 0.9968). The data are combined from at least separate experiments in duplicates. The ratio of each analyte to the internal standard was calculated based on the peak areas.

was used. After derivatization, the pentafluorobenzylated normal bases were mostly removed by Si SPE chromatography.

GC/NICI/MS Assay Calibration. Calibration of the assay was performed by addition of a fixed amount of $[^{13}C_4,^{15}N_2]$5Cl-Ura (1.0 ng) with various quantities of 5Cl-Cyt ranging from 0 to 150 pg and processed through the assay procedures. The lowest amount detected with quantitative linearity, i.e., the limit of quantification (LOQ), was 2.0 pg (14 fmol) with a correlation coefficient (γ^2) of 0.9968 (Figure 3). The recovery of the entire assay was 44 ± 3%. Since only one-tenth of the processed sample was injected on GC/MS and the recovery of 44%, a 23-fold higher LOQ compared to LOD is apparent. This calibration curve did not pass the origin. Background peaks corresponding to the peak area ratio of 0.0026 was observed, which might come from evaporation by centrifuge concentrator since high levels of standards such as those in the standard curve were also evaporated in the same concentrator. The peak area ratio of 0.0026 was constantly obtained even after extensive cleaning of the concentrator. Since the calibration curve was linear and the correlation coefficient was high, this background level was subtracted. Once this control ratio is decreased, the limit of quantification can be lowered. It is possible, but remains to be proven in the future, that tandem mass spectrometry with selective reaction monitoring of fragmentation of $[M - PFB]^-$ ion at *m/z* 325 to $[M - PFB - Cl + H]^-$ ion at *m/z* 291 could achieve a lower LOQ than using GC/MS with SIM.

GC/NICI/MS Analysis of 5Cl-Cyt in Calf Thymus DNA. This assay was used to analyze 5Cl-Cyt in calf thymus DNA treated with equimolar HOCl (relative to cytosine in DNA) in the presence of plasma concentration of Cl^- (100 mM). In the GC/NICI/MS chromatogram, the peaks of *m/z* 325 and 331 coeluted (not shown) and thus confirmed the identity of the analyte using as little as 0.1 μg of DNA. The level of 5Cl-Cyt in HOCl/Cl^--treated calf thymus DNA was 1.2 adducts/10^2 Cyt from duplicated experiments after interpolation of the calibration curve. This level was further verified by reversed-phase HPLC analysis with photodiode array detection of 5Cl-Ura in hydrolysate of 520 μg of the same DNA (Table 2). Another preparation of HOCl/Cl^--treated calf thymus DNA was analyzed to examine the effect of Cl^- on 5Cl-Cyt formation. The yield of 5Cl-Cyt in DNA treated with HOCl in the presence of 100 mM of Cl^- was only 11% more than that with HOCl alone. In the latter sample, incubation was carried out in phosphate buffer dissolved in deionized water without special treatment to remove Cl^-. Contamination of Cl^- from the buffer was as little as 20 μM. This result indicates that HOCl plays a major role in 5Cl-Cyt formation in DNA under physiological pH, and the contribution of Cl^- in forming molecular chlorine is minor. Thus, 5Cl-Cyt formation in DNA is likely to occur at a low concentration of Cl^-, such as the intracellular concentration of 5−15 mM. The concentration of HOCl was reported to be approximately 100 μM in the vicinity of activated neutrophils (*37*). Furthermore, HOCl is a neutral and stable molecule, and it should have long enough half-life to reach and react with cellular DNA.

Table 2. Levels of 5Cl-Cyt in DNA

DNA sample (amount)	5Cl-Cyt/Cyt[a]	method
treated calf thymus DNA		
HOCl/Cl^- (A) (520 μg)[b]	1.1 ± 0.1/10^2	HPLC/UV[c]
HOCl/Cl^- (A) (0.1 μg)[b]	1.2 ± 0.1/10^2	GC/NICI/MS[d]
HOCl/Cl^- (B) (0.1 μg)[e]	8.9 ± 0.4/10^3	GC/NICI/MS
HOCl (B) (0.1 μg)[e]	7.9 ± 0.2/10^3	GC/NICI/MS
ONO_2^-/Cl^- (500 μg)[e]	2.2 ± 0.1/10^6	GC/NICI/MS
ONO_2^- (500 μg)[e]	5.3 ± 0.4/10^7	GC/NICI/MS
untreated calf thymus DNA (500 μg)	6.0 ± 0.4/10^8	GC/NICI/MS
human placental DNA (500 μg)	6.6 ± 0.4/10^7	GC/NICI/MS

[a] Values are presented as mean ± standard deviation from at least duplicated experiments. [b] A solution containing calf thymus DNA (2.48 mg/mL) in potassium phosphate buffer (0.2 M, final pH 7.4) and Cl^- (100 mM) was added HOCl (1.74 mM) and incubated at 37 °C for 20 min. The reaction was quenched with methionine (17.4 mM). [c] Reversed phase HPLC analysis with UV detection at 280 nm using system 1. [d] Described in Materials and Methods. [e] Calf thymus DNA (0.5 mg/mL) in potassium phosphate buffer (0.2 M) and in the presence or absence of Cl^- (100 mM) was added HOCl (0.35 mM) or peroxynitrite (0.35 mM) and incubated at 37 °C for 60 min, followed by addition of methionine (3.5 mM). The final pH of the reaction was 7.4.

This new GC/NICI/MS assay is sensitive enough to accurately quantify background levels of 5Cl-Cyt in untreated calf thymus DNA and in human placental DNA. In 500 μg each of commercially available calf thymus DNA and in human placental DNA, the levels of 5Cl-Cyt were determined to be 0.6 and 6.6/10^7 cytosine, respectively. The GC/NICI/MS chromatogram of untreated calf thymus DNA showed coelution of the peak of 23.48 min at *m/z* 325 and at 331 (Figure 4) injecting one-tenth of the sample. The peak of 23.48 min at *m/z* 325 represented 3.7 fmol of PFB_2-5Cl-Ura, which was about 2.6 times of the limit of quantification (14 fmol in the entire sample). This level of 5Cl-Cyt in untreated calf thymus DNA was much lower than what was reported in the control DNA samples using the TMS derivatization method in in vitro experiments of calf thymus DNA treated with HOCl (*13*, *17*). It is possible that derivatization by the TMS reagent at elevated temperature could give increased level of 5Cl-Ura, as that was the case for 8-hydroxyguanine (*38*, *39*), due to artifact formation. Although the involvement of chlorinating agents from drinking water cannot be excluded, the presence of 5Cl-Cyt in untreated calf thymus DNA suggests that chlorination of DNA might be a biological process.

On the other hand, the level of 5Cl-Cyt in DNA treated with equimolar peroxynitrite (relative to cytosine in DNA) in the presence of 100 mM of Cl^- was determined

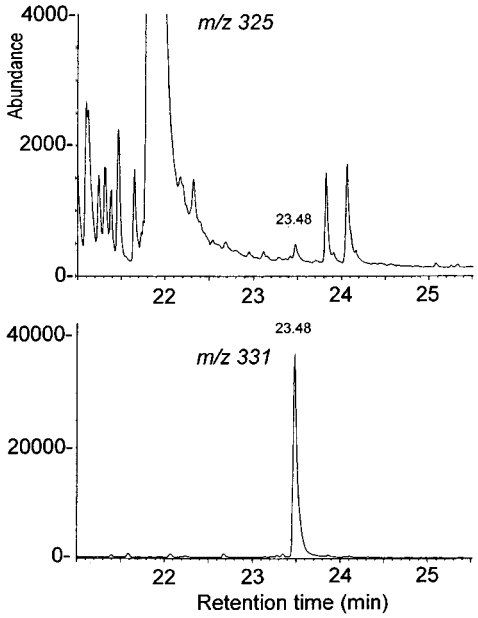


Figure 4. GC/NICI/MS SIM chromatogram of PFB_2-5Cl-Ura in untreated calf thymus DNA. Calf thymus DNA (500 μg) was hydrolyzed and subjected to the assay procedures described in Experimental Procedures. The peak of 23.48 min at *m*/*z* 325 represents 3.7 fmol (1.9 pg) of PFB_2-5Cl-Ura.

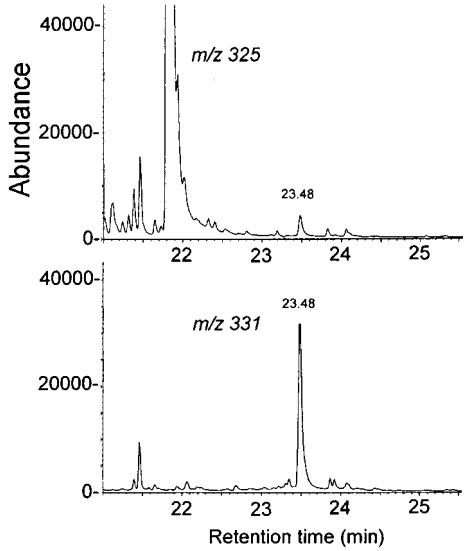


Figure 5. GC/NICI/MS analysis with selective ion monitoring chromatogram of PFB_2-5Cl-Cyt in peroxynitrite/chloride-treated calf thymus DNA (500 μg). The peak of 23.48 min at *m*/*z* 325 represents 79 fmol (40 pg) of PFB_2-5Cl-Ura.

to be $2.2/10^6$ cytosine, more than 4 times higher than that without addition of Cl^-. The peak of 23.48 min at *m*/*z* 325 represents 79 fmol of PFB_2-5Cl-Ura (Figure 5). The level of 5Cl-Cyt in DNA treated with peroxynitrite in the presence of 20 μM of Cl^- was $5.3/10^7$ cytosine. The accuracy of the assay was 5% in average ranging from 2 to 9% depending on the adduct levels (Table 2). To the best of our knowledge, this is the first report of 5Cl-Cyt formation in DNA by peroxynitrite through oxidation of Cl^-.

Isotope dilution mass spectrometry has several advantages over mass spectrometric methods without using the isotope as internal standard. Since the isotope has the same chemical properties as the analyte, it can monitor the recovery during each step of the assay and ensure accurate quantification of the analyte. Addition of nanogram quantity of the isotope standard also serves as carrier for trace amount of analyte in the sample, which might have poor recovery. In addition, the presence of the isotope standard assists in locating small amount of the analyte peak in the complicated chromatogram since the peak of the derivatized standard has virtually the same retention time (0−0.02 min deviation) as that for the analyte monitored at different *m*/*z* channels. Since quantification of the analyte is calculated from the ratio of the peak areas obtained from the GC/NICI/MS analysis, variation in sample recovery can be adjusted. Collectively, the use of this isotope standard allows unequivocal detection and accurate quantification of the analyte in the complex mixture of DNA hydrolysate. Electrophore labeling coupled with NICI/MS analysis offers high assay sensitivity. Our results demonstrate that this new isotope dilution GC/NICI/MS assay should be useful for 5Cl-Cyt analysis in DNA in in vivo studies since less amount of DNA sample is needed due to increase in sensitivity.

Little is known about the mutagenicity of 5Cl-Cyt and its repair mechanism. It was reported that tumor cells exposed to 5-chloro-2′-deoxycytidine (5Cl-dCyd), a radiosensitizer, incorporate 5-chloro-2′-deoxycytidine (5Cl-dUrd) into their DNA (*40*) and delayed tumor growth (*41*). The mechanism might be that 5Cl-dCyd was converted to the proximate radiosensitizer 5Cl-dUrd by dCMP deaminase and thus disrupted pyrimidine metabolism and DNA synthesis (*40*). It is highly possible that similar mechanisms apply to the bactericidal action of HOCl. Since drinking water contains Cl_2 that is in equilibrium with HOCl, humans are constantly exposed to exogenous chlorinating agents in addition to those formed endogenously. Obviously, more study is needed in order to understand the biological significance of 5Cl-Cyt. With this sensitive and specific assay of 5Cl-Cyt in hand, it is now feasible to perform in vivo investigations, such as the correlation between cytosine chlorination of DNA and carcinogenesis associated with chronic infections and inflammation, or the effect of chlorine processing in drinking water to human health.

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