Analysis of 3,N^4-Ethenocytosine in DNA and in Human Urine by Isotope Dilution Gas Chromatography/Negative Ion Chemical Ionization/Mass Spectrometry

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The promutagenic etheno DNA adducts have been detected in tissue DNA of rodents and humans from various exogenous and endogenous sources. While other etheno DNA adducts have been detected and quantified by isotope dilution gas chromatography/negative ion chemical ionization/mass spectrometry (GC/NICI/MS), similar analysis for 3,N^4-ethenocytosine (ϵCyt) has not been available. In this report, a GC/NICI/MS assay was developed for detection and quantification of ϵCyt in DNA and in human urine samples. The stable isotope of ϵCyt with 7 mass units higher than the normal ϵCyt 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 selective ion monitoring at $[M - 181]^-$ fragments of pentafluorobenzylated ϵCyt and its isotope analogue. One femtogram ($S/N > 40$) of pentafluorobenzylated ϵCyt was detected when injected on column with selective ion monitoring mode. The limit of quantification for the entire assay was 7.4 fmol of ϵCyt, which was approximately one thousand times lower than that of the HPLC/fluorescence assay for the nucleoside 3,N^4-etheno-2′-deoxycytidine in DNA. Analysis of chloroacetaldehyde-treated calf thymus DNA by both GC/NICI/MS and HPLC/fluorescence methods provided similar adduct levels and thus verified the assay. This GC/NICI/MS method was used for analysis of ϵCyt in two smokers' urine samples and the average level of ϵCyt was 101 ± 17 pg/mL/g of creatinine. Thus, quantification of ϵCyt in DNA and in urine by this highly specific and ultrasensitive isotope dilution GC/NICI/MS assay may facilitate research on the role of ϵCyt in carcinogenesis and in cancer development.

Introduction

Formation of etheno DNA adducts, 1,N^6-ethenoadenine (ϵAde),[1] 3,N^4-ethenocytosine (ϵCyt), N^2,3-ethenoguanine (ϵGua), and 1,N^2-ϵGua, has been shown to derive from exposure of chemicals such as the industrial chemical vinyl chloride (*1*, *2*), the ethyl carbamate (urethane) in bread and alcoholic beverages (*3*, *4*), mucochloric acid in drinking water (*5*), and their metabolites, epoxyethyl carbamate, 2-chloroethylene oxide, and 2-chloroacetaldehyde (CAA). (*6*). In addition to exogenous sources, endogenously generated DNA damage is recognized to play an important role in the multistep carcinogenesis process (*7*−*10*). The endogenous DNA damage could cause mismatched base pairing, leading to chromosomal instability and mutation and thus contribute to cancer development (*11*). *trans*-4-Hydroxy-2-nonenal is one of the major α,*β*-unsaturated aldehydic products of lipid peroxidation (*12*−*16*). Our previous study showed that 4-hydroxynonal can be epoxidized to 2,3-epoxy-4-hydroxynonanal by biological oxidants and, thus, could contribute to the endogenous formation of etheno DNA adducts (*17*). Background levels of etheno DNA adducts detected in rodents and human (*18*) may originate from lipid peroxidation (*12*) or from oxidation of the sugar backbone of DNA (*19*). Their levels appear to increase with oxidative stress and are implicated in cancer development (*20*−*22*). Etheno adducts are also formed in nitric oxide induced lipid peroxidation and are thus associated with DNA damages by chronic infections and inflammation (*23*). A recent study demonstrates that etheno adducts can derive from decomposition of lipid hydroperoxides by ascorbate (*24*).

The mutagenic properties of CAA-derived DNA adducts have been investigated both in bacteria and in mammalian cells. Transfection experiments in bacteria have shown that CAA treatment caused the most efficient mutations in cytosines, less efficiently at adenines, but not at guanines or thymines. Among mutations targeted to cytosine, 80% were C to T transitions and 20% were C to A transversions (*25*). There are data indicating that ϵCyt is highly mutagenic and may give noninstructional DNA lesion in vivo (*26*). In the bacteria system, ϵAde and ϵCyt induce low mutation frequency (*27*−*29*), but they are highly mutagenic in mammalian cells. The types of mutations for ϵCyt are mainly C to A transversions and C to T transitions (*29*, *30*).

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[1] Abbreviations: CAA, 2-chloroacetaldehyde; ϵ, etheno; ϵAde, 1,N^6-ethenoadenine; ϵCyd, 3,N^4-etheno-2′-deoxycytidine; ϵCyt, 3,N^4-ethenocytosine; ϵGua, N^2,3-ethenoguanine; ESI, electrospray ionization; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NICI, negative ion chemical ionization; PFB, pentafluorobenzyl; SIM, selective ion monitoring; SPE, solid-phase extraction.

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One of the responses of the living cells to such DNA adduction is to replace the abnormal adducts with the normal bases so that the damage is reversed. Etheno DNA adducts are repaired by separate repair enzymes and their repair efficiencies by human DNA glycosylases varies (reviewed in ref *31*). Among glycosylases capable of repairing the etheno DNA adducts, a mammalian m^3A-DNA glycosylase is reported to excise ϵAde and ϵGua, but not ϵCyt (*32*, *33*). Human mismatch-specific thymine-DNA glycosylase, a 55-kDa protein (*34*, *35*), and a 66-kDa DNA glycosylase from human He La cells (*36*) have been shown to possess high ϵCyt glycosylase activity. A homologous enzyme in *E. coli* is also capable of repairing ϵCyt in DNA (*35*). Sine two human ϵCyt glycosylases are identified, the presence of ϵCyt in human urine is investigated in this study. DNA adducts have been used not only as biomarkers for cancer risk assessment in molecular epidemiology studies, but also in understanding the mechanisms of carcinogenesis (*37*, *38*). Among DNA adducts, exocyclic etheno adducts are found to be useful biomarkers in cancer etiology and chemoprevention (*39*). The development of accurate and quantitative assay of DNA adducts is thus very important.

The most powerful procedures for the unequivocal chemical characterization and quantification is to use gas chromatography/negative ion chemical ionization/mass spectrometry (GC/NICI/MS) with selective ion monitoring (SIM) (*40*, *41*). Due to the fast development of instrumentation, liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) has become a very useful technique in measurement of DNA adducts, although the sensitivity is generally lower than GC/NICI/MS. The LC/ESI/MS/MS methods have been developed for ϵAde (*42*, *43*), ϵCyt (*43*), ϵGua (*44*), and 1,N^2-ϵGua (*45*) in DNA. Assays based on GC/NICI/MS have been developed for ϵAde (*46*), ϵGua (*47*, *48*), and 1,N^2-ϵGua (*49*), but a similar method for ϵCyt is not available. Unlike ϵAde and ϵGua possessing strong fluorescent properties, ϵCyt is merely fluorescent. Therefore, a sensitive assay for ϵCyt is demanded. In this report, a highly specific and ultrasensitive assay based on isotope dilution GC/NICI/MS with SIM was developed for detection and quantification of ϵCyt in DNA and in human urine. This assay should be useful in biological monitoring of ϵCyt from exogenous as well as endogenous sources.

Experimental Procedures

Caution: *CAA is a mutagen and tumorigen. Precautions should be taken in handling these compounds, e.g., wearing protective gloves, performing experiments under a fume hood, and disposing of waste according to appropriate safety guidelines.*

Materials. Calf thymus DNA and ϵCyt were from Sigma Chemical Co. (St. Louis, MO). [$^{13}C_9$,$^5N_3$]Cytidine ([$^{13}C_9$,$^5N_3$]Cyd) was purchased from Cambridge Isotope Laboratories (Andover, MA). 2,3,4,5,6-Pentafluorobenzyl bromide (PFB-Br), chloroacetaldehyde (CAA), diisopropylethylamine, anhydrous methanol, and anhydrous phosphorus pentoxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bond Elut C18, C18-OH, and Si solid-phase extraction (SPE) columns were from Varian (Harbor City, CA). 3,N^4-Etheno-2′-deoxycytidine (ϵdCyd) was synthesized from reaction of dCyd with CAA at pH 4.5 and collected by HPLC as described (*50*).

Instruments. NMR spectra were recorded on a Bruker DPX 400 MHz (Billerica, MA) instrument.

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 or a F-4500 fluorescence detector (Hitachi, Taiwan). *System 1*. A Prodigy ODS (3) 250 × 4.6 mm 5 μm column (Phenomenex, Torrance, CA) was eluted at a linear water and CH_3CN gradient: 0 to 5 min, 0% CH_3CN; 5 to 20 min, 0 to 53% CH_3CN at a flow rate of 1.0 mL/min. *System 2*. A Prodigy ODS (3) 250 × 4.6 mm 5 μm column was eluted with a linear gradient: 0 to 5 min: 0% B; 5 to 60 min: 5 to 50% B (solvent A, water; solvent B, 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 an isocratic condition of 2% B (solvent A, 10 mM ammonium formate, pH 3.0; solvent B, 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.

Synthesis of Isotope-Labeled ϵCyt Internal Standard. [$^{13}C_9$,$^{15}N_3$]Cytidine (1.0 mg/mL in 0.1 m phosphate buffer, pH 4.5) was added CAA (200 μM, final concentration), and the reaction mixture was stirred at 60 °C for 24 h, followed by evaporation to dryness. The residue was added 88% formic acid and heated at 120 °C for 6 h to obtain [$^{13}C_4$,$^{15}N_3$]Cyt. The reaction mixture was neutralized and purified by a C18 SPE (500 mg, 3 mL) precondition with 15 mL of methanol, followed by 15 mL of water. The SPE column was washed with 12 mL of water and the fraction containing [$^{13}C_4$,$^{15}N_3$]Cyt was eluted by 3 mL of 10% methanol. The eluant was dried and quantified based on the molar UV absorbance of ϵCyt using HPLC system 1, and its identity and purity was confirmed by GC/NICI/MS after pentafluorobenzylation.

Verification of the Structure of the PFB-ϵCyt Derivative. To a 20 mL vial containing dried ϵCyt (4.7 mg, 35 μmol) was added a solution of diisopropylethylamine (43 μL, 245 mmol) and PFB-Br (25 μL, 165 mmol) in 1.0 mL of anhydrous methanol and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was evaporated under vacuum and collected from HPLC system 2 to afford 9.5 mg of PFB-ϵCyt (86% yield) as a white solid. ^{1}H NMR (CD_3OD) δ 5.30 (s, 2H, benzyl), 6.65 (d, J = 7.9 Hz, 1H, H-5), 7.36 (d, J = 1.4 Hz, 1H, H-7), 7.56 (d, J = 7.9 Hz, 1H, H-6), 7.75 (d, J = 1.4 Hz, 1H, H-8). NICI-MS (assignment and relative abundance in parentheses) m/z 134 ([M-PFB]$^-$, 100%). Positive EI-MS (assignment and relative abundance in parentheses) 134 ([M-PFB]$^+$, 100%), 181 (PFB^+, 38%).

Modification of Calf Thymus DNA with CAA. Calf thymus DNA (1.0 mg/mL) was incubated with 50 μM CAA in 0.1 M phosphate buffer (pH 7.0) at 25 °C for 15 min. The reaction mixture was extracted with chloroform (2 × volume) after centrifugation at 15 000 rpm for 20 min. The chloroform layer was discarded to remove unreacted CAA.

DNA Hydrolysis and Adduct Enrichment by C18 SPE Chromatography. DNA sample (10 μg) was added [$^{13}C_4$, $^{15}N_3$]ϵCyt (1.0 ng) and hydrolyzed in 1 N HCl at 100 °C for 2 h. The hydrolysate were neutralized (pH 7.0) with sodium hydroxide solution. The DNA hydrolysate was applied to a C18 SPE column preconditioned with 15 mL of MeOH, followed by 15 mL of water. After the reaction mixture was loaded, the column was washed with 12 mL of water and ϵCyt was eluted with 3 mL of 10% methanol in water. The eluant was collected in a silanized glass vial, evaporated by the centrifuge concentrator (Pan-Chum, Taiwan), and dried over phosphorus pentoxide under vacuum overnight.

Urine Pretreatment and Enrichment by C18-OH SPE Chromatography. Urine samples (3 mL) stored at −80 °C freezer were defrosted in an ice−water bath, [$^{13}C_4$,$^{15}N_3$]ϵCyt (3.6 ng) was added, and the solution was centrifuged at 15000*g* for 10 min at 4 °C. The supernatant was removed for workup, and the precipitate was discarded. The supernatant of pretreated urine sample was loaded on a C18-OH SPE column preconditioned with 15 mL of MeOH, followed by 15 mL of water. After the volume of the sample was eluted, to the column was added 9 mL of water. The fraction containing ϵCyt was eluted with 6 mL of 7% methanol in water, collected in a silanized glass vial, and evaporated under vacuum.

GC/NICI/MS Assay Calibration. The stock solutions of ϵCyt and [$^{13}C_4$,$^{15}N_3$]ϵCyt (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. [$^{13}C_4$,$^{15}N_3$]ϵCyt (1.0 ng) was added to each sample as internal standard. Various amounts of ϵCyt ranging from 0, 1.0, 1.5, 3, 6, 15, 50, 100, and 300 pg were added. The samples were processed through the same procedures as DNA samples, i.e., acid hydrolysis, C18 SPE enrichment, pentafluorobenzylation, Si SPE purification, and GC/NICI/MS analysis.

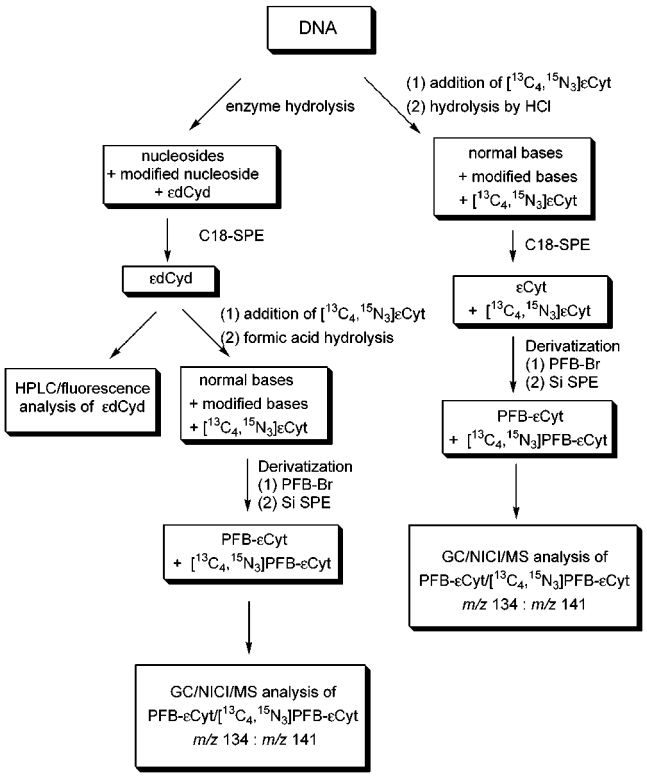
GC/NICI/MS Analysis of ϵCyt in Calf Thymus DNA and in Human Urine. The vial containing ϵCyt and [$^{13}C_4$,$^{15}N_3$]ϵCyt was dried over phosphorus pentoxide, followed by addition of PFB-Br (5 μL) and diisopropylethylamine (7 μL) in 0.1 mL of anhydrous methanol under argon atmosphere and incubation at 40 °C for 2 h. After the reaction mixture was evaporated to dryness in a vacuum, it was dissolved in dichloromethane (0.2 mL), and the pentafluorobenzylated adduct was purified by a Si SPE column preconditioned with 15 mL of dichloromethane. The column was washed with 3 mL of 1% methanol in dichloromethane (v/v) and 1 mL of 5% methanol in dichloromethane (v/v), and PFB-ϵCyt was eluted with 2 mL of 5% methanol in dichloromethane (v/v). The eluant was evaporated, transferred to an insert, and evaporated to dryness. For DNA samples, the residue was dissolved in 10 μL of acetone and 1 μL aliquot was analyzed by GC/NICI/MS with SIM at *m/z* 134 and 141 for PFB-ϵCyt and [$^{13}C_4$,$^{15}N_3$]PFB-ϵCyt, respectively. Urine samples were reconstituted in 100 μL of acetone and a 1 μL aliquot was analyzed by GC/NICI/MS. The quantification of ϵCyt in the sample was based on intrapolation of the ratio of the peak area of the pentafluorobenzylated adduct versus that of the isotope and the calibration curve.

GC/NICI/MS experiments were conducted using a Hewlett-Packard 6890 GC with 5973 MSD mass detector with the negative ion chemical ionization source (Agilent, Taiwan). 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 an 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 reagent 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 (SIM) at *m/z* 134 and 141 was used to detect and quantify the $[M - 181]^-$ fragment ions of PFB-ϵCyt and [$^{13}C_4$,$^{15}N_3$]PFB-ϵCyt, respectively. The quantification was based on intrapolation of the ratio of the peak area of PFB-ϵCyt versus [$^{13}C_4$,$^{15}N_3$]PFB-ϵCyt and the calibration curve.

HPLC/Fluorescence Analysis of ϵdCyd in Calf Thymus DNA. Briefly, DNA (200 μg) was treated with hydrolytic enzymes as described by Crane (*51*). The hydrolysate was filtered through a 0.22 μm nylon filter, and the filtrate was loaded on a C18 SPE column. After the volume of the hydrolysate was eluted, the column was washed with 12 mL of water followed by 6 mL of 10% methanol, and the ϵdCyd-containing fraction was eluted with 3 mL of 30% methanol. These fractions were combined, evaporated to dryness, reconstituted in 100 μL of water, and analyzed by HPLC with fluorescence detector using system 3 with excitation wavelength at 290 nm and emission wavelength at 340 nm. The quantification was based on the calibration curve constructed from solutions containing 0, 1.5, 2.0, 4.0, 10, 50, 100, and 300 ng of ϵdCyd. Duplicated experiments were performed. The samples were hydrolyzed and analyzed by procedures described above and plotted as mol % ϵdCyd recovered from the amount of ϵdCyd started. The equation of the calibration curve was obtained by linear regression.

Determination of Cytosine in Calf Thymus DNA. The cytosine content of DNA samples was determined by hydrolysis of DNA with 88% formic acid at 120 °C for 2 h, followed by evaporation and analysis by HPLC using system 4 for guanine and adenine. The content of cytosine was quantified as the same as guanine since cytosine underwent partial deamination under the hydrolysis condition.

Scheme 1. Procedures Involved in the Assays of ϵCyt in DNA and in Urine.

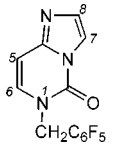


Determination of Creatinine in Human Urine. Urine was pretreated as described above, and its creatinine content was analyzed with a modified method (*52*) by HPLC equipped with Hitachi L-7000 pump system with D-7000 interface, a Rheodyne injector, and L-7450A photodiode array (PDA) detector (Hitachi, Taiwan). Fifty microliters of the sample was diluted 50 times with 10 mM potassium phosphate buffer (pH 3.0), and 1 mL of the aliquot was passed through a 13 mm disposable syringe filter containing a 0.45 μm Nylon membrane. Twenty microliters of the filtrate was injected into a Prodigy ODS (3) 250 × 4.6 mm 5 μm (Phenomenex, Torrance, CA) column eluting with 50 mM ammonium formate buffer (pH 5.5) at a flow rate of 1.0 mL/min and the column effluent was monitored at 216 nm.

Results

The procedures of the new GC/NICI/MS assay for ϵCyt involve addition of internal standard, hydrolysis/pretreatment and adduct enrichment, pentafluorobenzylation, postderivatization cleanup, and GC/NICI/MS analysis as depicted in Scheme 1.

Synthesis of Isotope-Labeled ϵCyt Internal Standard. The isotope analogue being 7 mass units higher than the analyte [$^{13}C_4$,$^{15}N_3$]ϵCyt was added to the DNA samples as internal standard to monitor loss of analyte during the workup procedures. The use of this isotope standard allows unequivocal detection and accurate quantification of the adduct in the complex mixture of DNA hydrolysate. The isotope standard [$^{13}C_4$,$^{15}N_3$]ϵCyt used in this assay was synthesized from [$^{13}C_9$,$^{15}N_3$]-cytidine, which was allowed to react with CAA at elevated temperature under acidic pH to form [$^{13}C_4$,$^{15}N_3$]3,N^4-ethenocytidine, which was monitored by reversed-phase HPLC for optimum yield. The etheno nucleoside was



PFB-ϵCyt

Figure 1. Structure of PFB-ϵCyt.

hydrolyzed to release [$^{13}C_4$,$^{15}N_3$]ϵCyt, which was purified by a disposable C18 solid-phase extraction (SPE) column. This procedure gave [$^{13}C_4$,$^{15}N_3$]ϵCyt in 20% overall yield with high chemical purity as determined by reversed-phase HPLC with photodiode array detection. The isotopic purity of [$^{13}C_4$,$^{15}N_3$]ϵCyt used in the assay was determined by derivatization with pentafluorobenzyl bromide (PFB-Br) followed by analysis using GC/NICI/MS at *m*/*z* 134 and 141 for unlabeled ϵCyt and for [$^{13}C_4$,$^{15}N_3$]ϵCyt, respectively. The ratio of *m*/*z* 134 versus 141 was 0.0013.

The yield of [$^{13}C_4$,$^{15}N_3$]ϵCyt was lower when [$^{13}C_9$,$^{15}N_3$]-cytidine was first hydrolyzed to [$^{13}C_4$,$^{15}N_3$]cytosine and followed by CAA treatment. It was due to the fact that hydrolysis of cytidine resulted in substantial deamination product, uridine. Purification of the reaction mixture by a brand new reversed-phase HPLC column gave [$^{13}C_4$,$^{15}N_3$]ϵCyt with contamination as determined by GC/NICI/MS analysis, even when the injector of HPLC was thoroughly cleaned (*53*).

Verification of the Structure of the PFB-ϵCyt Derivative. Pentafluorobenzylated ϵCyt (PFB-ϵCyt) was synthesized in high yield (86%) under the same conditions as for PFB-ϵAde (*46*). The pentafluorobenzyl moiety should attach at N1 of cytosine since it is the only position containing active proton (Figure 1). The ^{1}H NMR measured in methanol-d_4 showed the two benzyl protons as a singlet with a chemical shift of 5.30 ppm. Two pairs of protons were assigned based on the coupling constants. The coupling constant for the pair of protons (C-5 and C-6) on the pyrimidine ring (J = 7.9 Hz) was larger than the one (C7 and C8) on the imidazole rings (J = 1.4 Hz). The small coupling constant for the exocyclic protons was also observed for other etheno adducts (*43*, *44*). The proton on C-5 is more upfield than the C-6 proton as it is the case for cytosine. The C-7 proton geminal to the N(C═O) group is assigned to the proton at 7.75 ppm. The identity of the product was also confirmed by the full scan mass spectrum in both NICI and positive electron impact modes. The NICI mass spectrum shows no molecular ion peak and the [M-181]$^-$ ion at *m*/*z* 134 was the major fragment ion, indicating that the compound lost the pentafluorobenzyl moiety easily. The *m*/*z* 134 ion was used for monitoring PFB-ϵCyt in the GC/NICI/MS assay. The positive electron impact spectrum of PFB-ϵCyt showed both [M − PFB]$^+$ and PFB$^+$ ions at *m*/*z* 134 and 181, respectively. Although no molecular ion peak was detected, the presence of these two fragments suggested the identity of this compound.

DNA Hydrolysis and Adduct Enrichment by C18 SPE Chromatography. Acid hydrolysis of DNA released normal and adducted bases, which were separated by a C18 solid-phase extraction (SPE) column to enrich ϵCyt. The isotope standard [$^{13}C_4$,$^{15}N_3$]ϵCyt was added before acid hydrolysis. Unlike ϵAde that tends to decompose during acid hydrolysis (*42*), ϵCyt is stable under acidic conditions. Hydrolysis of 3,N^4-etheno-2′-deoxycytidine (ϵdCyd) with 1 N HCl at 100 °C for 2 h or 88% formic acid at 120 °C for 1.5 h completely released ϵCyt with almost quantitative yield as determined by reversed-phase HPLC with photodiode array detection (data not shown). After hydrolysis, ϵCyt in the DNA hydrolysate was enriched by a C18 SPE column to remove most of the normal bases. With the optimized C18 SPE condition in which ϵCyt was eluted with 10% methanol in aqueous solution, ca. 3% of adenine was collected with ϵCyt. As compared to the adduct present in DNA, the amount of adenine was tremendous. Although pentafluorobenzylated Ade and ϵCyt cannot be distinguished by mass spectrometry since they have the same molecular weight, they can be separated by gas chromatography.

Urine Pretreatment and Enrichment by C18-OH SPE Chromatography. Urine samples were added isotope standard (3.6 ng) and centrifuged to remove proteins. Enrichment of ϵCyt was performed by a C18-OH SPE column, which provided a cleaner sample than using the C18 SPE column. The rest of the assay procedures were the same for both DNA and urine samples, and the recoveries of the two procedures were similar.

GC/NICI/MS Analysis of PFB-ϵCyt. High sensitivity of PFB-ϵCyt on the electron capture GC/NICI/MS was observed. One femtogram (3.2 amol) of PFB-ϵCyt was detected at 20.8 min with a S/N = 41 when injected on column with selective ion monitoring (SIM) mode at *m*/*z* 134 (Figure 2). The GC condition was virtually identical to that for PFB-ϵAde, and the intrinsic sensitivity of PFB-ϵCyt was higher than that for PFB-ϵAde (10 fg, 30 amol) (*46*).

The adduct-enriched fraction of DNA hydrolysate was dried and derivatized under mild conditions (42 °C, 2 h) with PFB−Br in the presence of a tertiary amine, diisopropylethylamine. The derivatization mixture was evaporated to dryness so that the excess reagents are removed. The pentafluorobenzylated analyte was purified from the mixture by a Si SPE column as postderivatization sample cleanup before GC/NICI/MS analysis. To minimize the unwanted compounds collected during the assay procedures, each milliliter of the eluant was analyzed. We found that the first milliliter elution of 5% methanol in dichloromethane did not contain the analyte, and it contained a peak interfering with the analysis. Thus, collecting the second and third milliliters of the eluant was the optimized cleanup condition. The purified sample was analyzed by GC/MS at *m*/*z* 134 and 141 for the [M − 181]$^-$ ions for PFB-ϵCyt and [$^{13}C_4$,$^{15}N_3$]PFB-ϵCyt, respectively.

GC/NICI/MS Assay Calibration. Calibration of the assay was performed by addition of a fixed amount of [$^{13}C_4$,$^{15}N_3$]ϵCyt with various quantities of ϵCyt ranging from 0 to 300 pg, the lowest amount detected with quantitative linearity was 1.0 pg with a recovery of 30 ± 2% and a correlation coefficient (γ^2) of 0.9999 (Figure 3). However, this calibration curve did not pass the origin. Background peaks corresponding to the peak area ratio of 0.005 was observed, which was not entirely due to the contamination of the normal ϵCyt during synthesis of the isotope standard. Derivatization of the isotope standard alone followed by Si SPE column cleanup gave a peak area ratio of 0.001. Thus, the background level might come from procedures before derivatization, i.e., hydrolysis, C18 SPE, and evaporation by centrifuge concentrator. The last procedure might be the main source of contamination since high levels of standards such as those in the

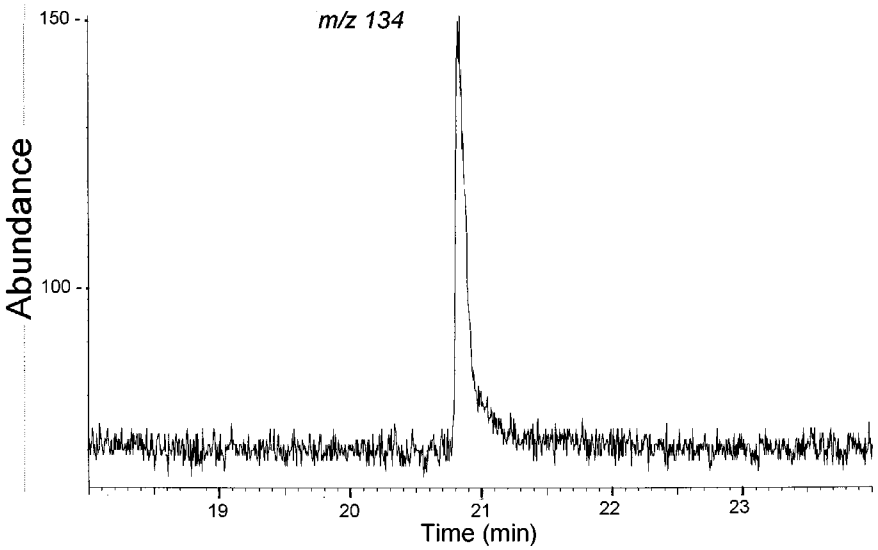


Figure 2. Detection of 1.0 fg (3.2 amol) of PFB-ϵCyt by GC/NICI/MS with selective ion monitoring at *m*/*z* 134.

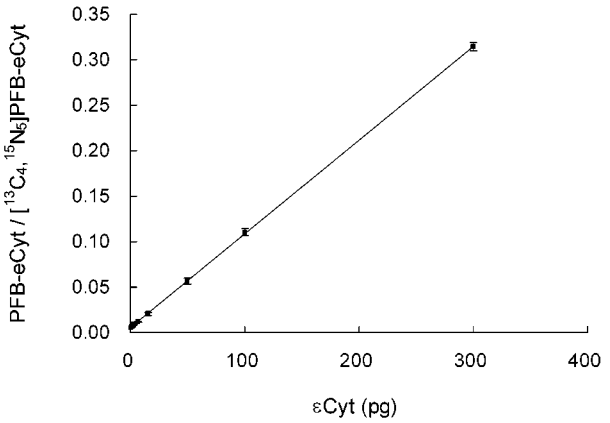


Figure 3. Calibration curve for the GC/NICI/MS analysis for PFB-ϵCyt at *m*/*z* 134 and [$^{13}C_4$,$^{15}N_3$]PFB-ϵCyt at *m*/*z* 141. Samples containing various amounts (0−300 pg) of ϵCyt was added a fix amount of [$^{13}C_4$,$^{15}N_3$]ϵCyt (1.0 ng) and subjected to the assay procedures described in Experimental Procedures. 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.

standard curve were also evaporated in the same concentrator. The peak area ratio of 0.005 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.

GC/NICI/MS Analysis of ϵCyt in Calf Thymus DNA. The assay with acid hydrolysis/C18 SPE procedure was used to analyze ϵCyt in CAA-treated calf thymus DNA. The chromatogram showed coelution of the peak of *m*/*z* 134 and 141 at 19.45 min and confirmed the identity of the analyte using as little as 10 μg of DNA (Figure 4). The huge peak at 20.4 min was the pentafluorobenzylated adenine. Fortunately, it did not interfere with the analysis. The level of ϵCyt in CAA-treated calf thymus DNA was 1.41 ± 0.11 adducts/10^4 Cyt as determined from duplicated experiments. This level was further verified by reversed-phase HPLC with fluorescence detection of ϵdCyd in the same DNA (Table 1).

GC/NICI/MS Analysis of ϵCyt in Human Urine. Three milliliters of the 24-h-urine samples from two smokers were analyzed after the assay procedures. Since

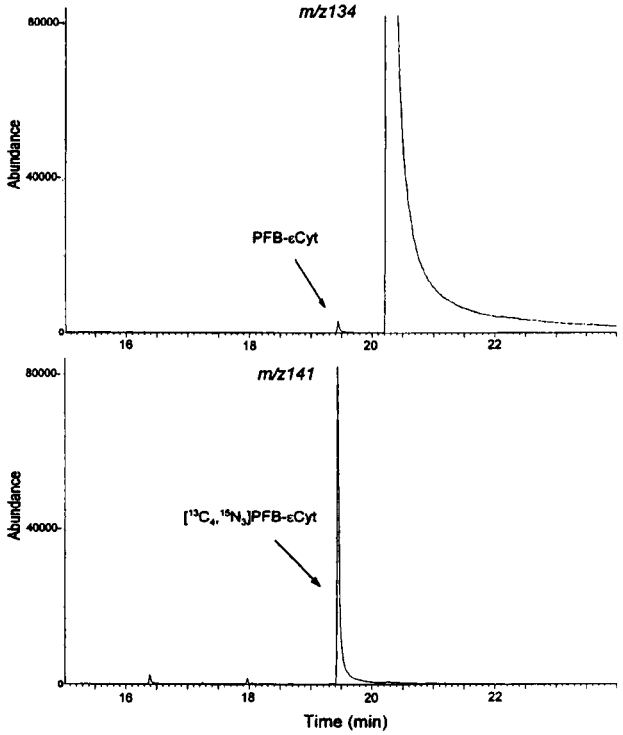


Figure 4. GC/NICI/MS SIM chromatogram of PFB-ϵCyt in CAA-treated DNA. Calf thymus DNA (10 μg) was treated with CAA (50 μM) at 25 °C for 15 min at pH 7.0, followed by workup and assay procedures described in Experimental Procedures (r^2 = 0.9999). The peak at 19.44 min represents 2.3 pmol (725 pg) of PFB-ϵCyt.

Table 1. Comparison of GC/NICI/MS and HPLC/ Fluorescence Assays for ϵCyt in DNA

assay	LOD	LOQ	recovery
GC/NICI/MS	1.0 fg (3.2 amol, S/N = 41)	1.0 pg (7.4 fmol)	30%
HPLC/fluorescence	0.6 ng (2.4 pmol, S/N = 3)	1.5 ng (5.9 pmol)	76%

only one hundredth of the urine sample was analyzed, 3.6 ng (instead of 1.0 ng used in DNA samples) of the isotope standard was added to clearly locate the [$^{13}C_4$, $^{15}N_3$]PFB-ϵCyt peak among other peaks at *m*/*z* 141 by GC/NICI/MS analysis. The GC/NICI/MS chromatogram of

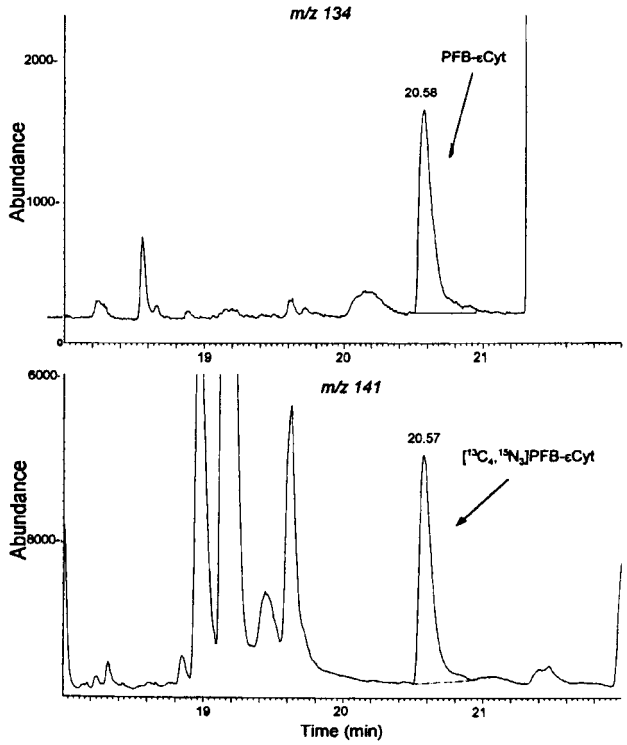


Figure 5. GC/NICI/MS analysis with selective ion monitoring chromatogram of PFB-ϵCyt in human urine. Urine (3 mL) was added [$^{13}C_4$,$^{15}N_3$]ϵCyt (3.6 ng) and processed as described in Experimental Procedures. The peak at 20.56 min represents 24 fmol (7.5 pg) of PFB-ϵCyt.

one of the urine samples showed coelution of the peak at 20.58 min at *m*/*z* 134 with the one at 20.57 min at *m*/*z* 141 (Figure 5). The levels of ϵCyt in these two samples were determined to be 105 and 104 pg/mL from duplicated experiments after subtracting the levels in the control samples containing isotope standard only. The precision of the assay was 4%. The averaged ϵCyt levels of these two smokers after adjusting for creatinine, which were measured by a modified HPLC method (*52*), were 101 ± 17 pg/mL/mg of creatinine (Table 2).

HPLC/Fluorescence Analysis of ϵdCyd in Calf Thymus DNA. The analysis of ϵdCyd in DNA hydrolysate was performed with 2% methanol in ammonium formate buffer at the optimum pH with the excitation wavelength at 290 nm and the emission one at 340 nm. The limit of detection (LOD) was 0.6 ng, which was about 10 times lower than the reported value (*45*). The CAA-treated DNA (200 μg) was hydrolyzed to normal nucleosides and the adducted nucleosides, including ϵdCyd, by enzymes using the method by Crane (*51*). The enzyme hydrolysate was purified by a C18 SPE to enrich ϵdCyd using an optimized elution condition. Analysis by HPLC/fluorescence showed the peak of ϵdCyd eluted at 32 min (Figure 6). Quantification of the assay was calculated from the standard curve with ϵdCyd ranging from 0 to 300 ng (Figure 7). The recovery of the assay was 76%, obtained from linear regression of the calibration curve, and the correlation coefficient (γ^2) was 0.9997. The limit of quantification of the entire assay was 1.5 ng of ϵdCyd. The level of ϵdCyd in CAA-treated DNA analyzed by this assay was 1.79 ± 0.09 adducts/10^4 Cyt after intrapolation of the calibration curve and adjustment for recovery. The sensitivity of two assays was compared in Table 1.

Table 2. Levels of ϵCyt in Human Urine

sample[a]	ϵCyt (pg/mL)[b]	creatinine (mg/mL)[c]	ϵCyt/creatinine[d]
1	105	0.89	118
2	104	1.24	84
mean ± SD			101 ± 17

[a] Urine (3 mL) was added isotope standard, followed by C18-OH SPE enrichment, pentafluorobenzylation, Si SPE purification, and one hundredth of the residue was analyzed by GC/NICI/MS as described in the Experimental Procedures. [b] Average from at least duplicated experiments. [c] Determined by HPLC with photodiode array detection as described in the Experimental Procedures. [d] Expressed as pg ϵCyt per mL per mg creatinine.

Discussion

Our results demonstrate that this new GC/NICI/MS assay is of practical use in quantification of ϵCyt in DNA and in urine samples. In this GC/NICI/MS assay, the detection limit of the analyte PFB-ϵCyt was at the subattomole level. This intrinsically high sensitivity indicates that this assay can be very sensitive once the

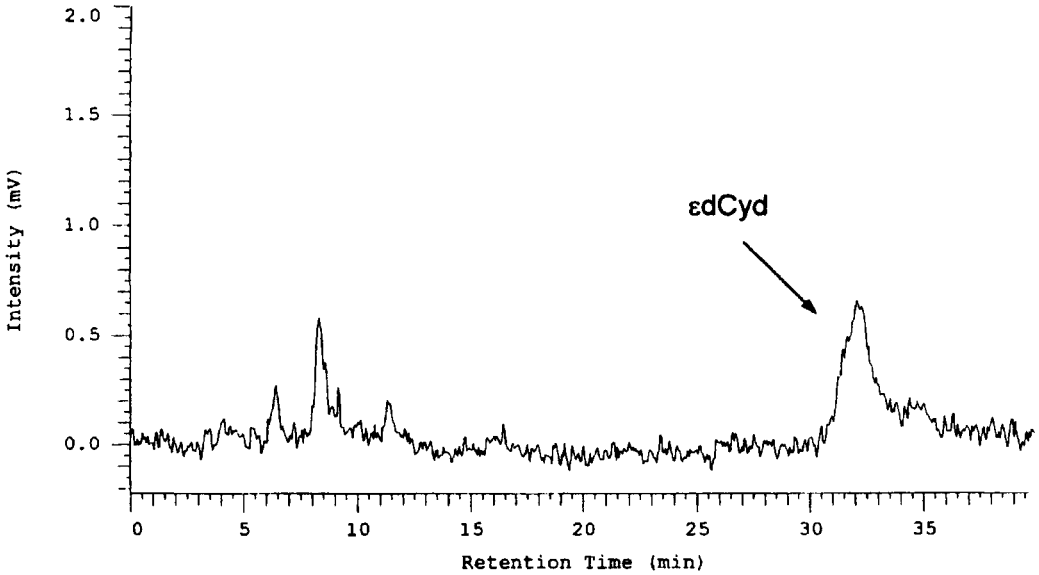


Figure 6. HPLC/fluorescence detection of ϵdCyd in the enzyme hydrolysate of CAA-treated calf thymus DNA (200 μg). The peak at 32.2 min represents 6.7 ng (27 pmol) of ϵdCyd. The excitation and emission wavelength was 290 and 340 nm, respectively.

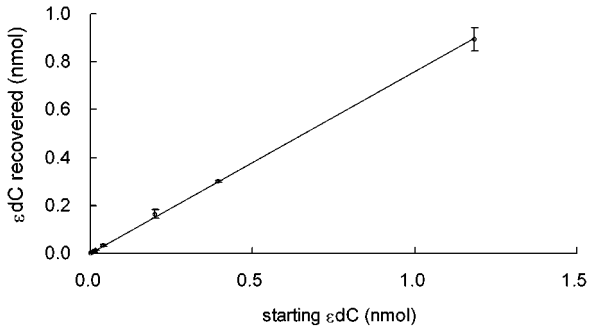


Figure 7. Calibration curve for HPLC/fluorescence assay of ϵdCyd in DNA. Samples containing various amounts (0−300 ng) of ϵdCyd was subjected to the assay procedures described in Experimental Procedures ($r^2 = 0.9997$). The data are combined from at least separate experiments in duplicates.

background contamination is reduced. Nonetheless, the limit of quantification (LOQ) of this GC/NICI/MS assay for ϵCyt in DNA is approximately 1000 times lower than that for ϵdCyd by our improved HPLC/fluorescence method. Although the background level limited the assay of being supersensitive, there is still plenty of room for future improvement. Once the background level in the control sample is reduced, so is the limit of the quantification of this assay. At this stage, this GC/NICI/MS assay is useful in analyzing samples containing picogram levels of ϵCyt, such as in human urine samples.

Accurate quantification of DNA adduct levels is very important in correlating adduct levels to cancer risk or cancer development. Discrepancy in measuring levels of DNA adduct using different methodologies has been reported and discussed (*8*, *41*). The reproducibility in isotope dilution mass spectrometric methods is much higher as compared to other assays. Mass spectrometry also provides the chemical identity of the analytes and offers advantage in characterization of DNA adducts that may be present in the complex mixture of DNA hydrolysate. Although high-resolution mass spectrometry offers very high specificity, the instrument is very expensive and thus is not affordable for general users or for routine measurements. Without the use of affinity chromatography, which might have the problem of batch-to-batch variation and aging of the column, our GC/NICI/MS assay uses a benchtop instrument that is relatively inexpensive and yet provides high sensitivity. It can be applied for routine analysis of large amount of samples in epidemiological studies.

3,N^4-Ethenocytosine was shown to exist in higher levels than ϵAde in vinyl chloride-treated rats (*54*, *55*). Like ϵAde, ϵCyt has also been detected in the tissue DNA of untreated rodents and humans by the immunoaffinity/^{32}P-postlabeling method (*18*). Being the most sensitive technique for DNA adduct measurement, ^{32}P-postlabeling was not able to analyze adducts as free bases or as nucleosides due to the nature of the assay. The endogenous ϵCyt has been postulated to derive from the epoxides of lipid peroxidation-generated enals (*12*, *16*−*18*). Levels of DNA adducts associated with oxidative stress have been compared in human pancreatic DNA and levels of ϵAde and ϵCyt in pancreatic DNA between smokers and nonsmokers were not statistically significant (*56*). Since levels of DNA adducts represent a balance between adduct formation and the repair, it is possible that different levels of ϵAde and ϵCyt will be found between smokers and nonsmokers. With this method in hand, the possibility can be examined. In addition, modification of this GC/NICI/MS assay for simultaneous detection of ϵAde and ϵCyt in the same sample can be achieved since the GC/NICI/MS conditions are the same.

In conclusion, this study describes the first GC/NICI/MS assay for analysis of ϵCyt in DNA and in human urine. Although the sample numbers are limited, ϵCyt in human urine was detected and its levels were determined for the first time using this highly specific and sensitive assay. This GC/NICI/MS assay of ϵCyt should provide a feasible tool in investigation of the correlation between levels of ϵCyt and disease states due to exogenous as well as endogenous sources.

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Note Added after ASAP Posting

This article was inadvertently released ASAP on 11/30/01 before final corrections were made. Figures 4 and 5 were exchanged. The correct version was posted 12/4/01.

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