Quantification of Tamoxifen DNA Adducts Using On-Line Sample Preparation and HPLC-Electrospray Ionization Tandem Mass Spectrometry

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The nonsteroidal antiestrogen tamoxifen is used as an adjuvant chemotherapeutic agent for the treatment of all stages of hormone-dependent breast cancer and more recently as a chemopreventive agent in women with elevated risk of developing the disease. While clearly beneficial for the treatment of breast cancer, tamoxifen has been reported to increase the risk of endometrial cancer in women. Furthermore, it has been shown to be hepatocarcinogenic in rats. Tamoxifen is clearly genotoxic in rat liver, as indicated by the formation of DNA adducts; the occurrence of tamoxifen DNA adducts in human endometrial tissue is more controversial. The detection and quantitation of tamoxifen DNA adducts have relied primarily upon ^{32}P-postlabeling, with other techniques, such as immunoassays and accelerator mass spectrometry, being used to a much lesser extent. To expand the range of available analytical methodologies for quantifying tamoxifen DNA adducts, we have developed an assay using on-line sample preparation, coupled with HPLC and electrospray ionization tandem mass spectrometry (ES-MS/MS). α-Acetoxytamoxifen was reacted with salmon testis DNA at ratios between 0.1 ng and 1 mg α-acetoxytamoxifen per mg DNA. After enzymatic hydrolysis to nucleosides, the most highly modified DNA samples were analyzed by HPLC−UV, which indicated the presence of two adduct peaks in approximately a 1:4 ratio. The major adduct was isolated, rigorously characterized as (*E*)-α-(deoxyguanosin-N^2-yl)tamoxifen, and quantified on the basis of its molar extinction coefficient. A similar reaction was conducted with [$N(CD_3)_2$]-α-acetoxytamoxifen to prepare a deuterated adduct that could serve as an internal standard for ES-MS/MS. The limit of detection for the HPLC-ES-MS/MS method was approximately 5 adducts/10^9 nucleotides, with an intra- and interassay precision of 3% relative standard deviation. The method was validated over the range of 8−1 520 000 adducts/10^8 nucleotides using 100 μg samples of DNA modified in vitro. Analysis of liver DNA from female Sprague−Dawley rats treated by gavage with seven daily doses of 20 mg tamoxifen/kg body weight gave a value of 496 ± 16 adducts/10^8 nucleotides for (*E*)-α-(deoxyguanosin-N^2-yl)tamoxifen and 626 ± 18 adducts/10^8 nucleotides for (*E*)-α-(deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen. These data indicate that the HPLC-ES-MS/MS methodology has sufficient sensitivity and precision to be useful in the analysis of tamoxifen DNA adducts formed in vivo in experimental models and may be able to detect tamoxifen DNA adduct formation in human tissue samples.

Introduction

The nonsteroidal antiestrogen tamoxifen[1] (**1**, Figure 1) has been used for nearly three decades as an adjuvant chemotherapeutic agent for the treatment of all stages of hormone-dependent breast cancer (*1*). More recently, data from a large-scale clinical trial in the United States have shown that the administration of tamoxifen to women at high risk of developing the disease resulted in a 50% reduction in the occurrence of invasive breast cancer (*2*). This observation has prompted the U.S. Food and Drug Administration to approve the use of tamoxifen as a prophylactic agent against breast cancer in high-

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[1] Abbreviations: AlkOPh, alkoxyphenyl; AMS, accelerator mass spectrometry; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane; C_{quat}, quaternary carbon; CIA, chemiluminescence immunoassay; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; *N*-desmethyltamoxifen, (*Z*)-1-[4-(2-methylamino)ethoxy]phenyl-1,2-diphenyl-but-1-ene; dG, 2′-deoxyguanosine; dG-desMeTam, (*E*)-α-(deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen; dG-Tam, (*E*)-α-(deoxyguanosin-N^2-yl)tamoxifen; dG-Tam-d_6, (*E*)-α-(deoxyguanosin-N^2-yl)-*N*,*N*-bis(trideuteriomethyl)tamoxifen; dR, 2′-deoxyribosyl; EI, electron ionization; ES, electrospray ionization; FAB, fast atom bombardment; HRMS, high-resolution mass spectrometry; α-hydroxy-*N*-desmethyltamoxifen, (*E*)-4-[4-[2-(methylamino)ethoxy]phenyl]-3,4-diphenylbut-3-en-2-ol; α-hydroxytamoxifen, (*E*)-4-[4-[2-(dimethylamino)ethoxy]phenyl]-3,4-diphenylbut-3-en-2-ol; α-hydroxytamoxifen-d_6, (*E*)-4-[4-[2-(bis(trideuteriomethyl)amino)ethoxy]phenyl]-3,4-diphenyl-but-3-en-2-ol; LOD, limit of detection ($S/N = 3$); LOQ, limit of quantitation ($S/N = 10$); MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; RSD, relative standard deviation (SD/mean × 100); *S*/*N*, signal-to-noise ratio; tamoxifen, (*Z*)-1-[4-(2-dimethylamino)ethoxy]phenyl-1,2-diphenylbut-1-ene.

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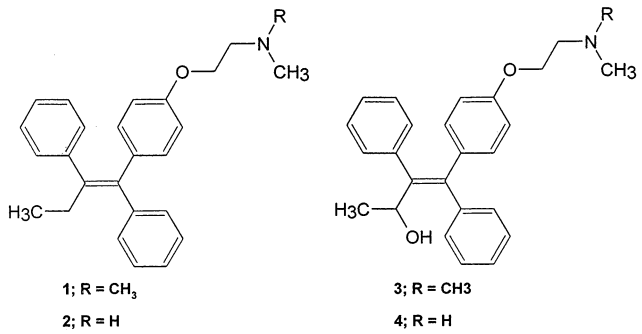

Figure 1. Structures of tamoxifen (**1**) and its metabolites *N*-desmethyltamoxifen (**2**), α-hydroxytamoxifen (**3**), and α-hydroxy-*N*-desmethyltamoxifen (**4**).

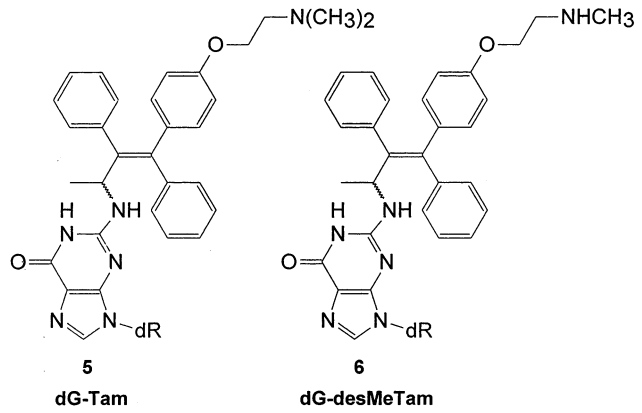

Figure 2. Structures of (*E*)-α-(deoxyguanosin-N^2-yl)tamoxifen (dG-Tam; **5**) and (*E*)-α-(deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen (dG-desMeTam; **6**), the major adducts formed in the livers of tamoxifen-treated rats.

risk women. Despite this beneficial effect regarding breast cancer, a major cause of concern is the increased incidence of endometrial cancer among women administered tamoxifen (*1*, *2*). In particular, suggestions that long-term tamoxifen users develop endometrial cancers with a worse prognosis than that of sporadic endometrial cancers (*3*, *4*) raise questions about the net benefit from the prophylactic use of the drug by healthy women. The mechanisms underlying the induction of endometrial tumors by tamoxifen have yet to be established, although both the genotoxic (reviewed in *5*) and the hormonal (*6*−*10*) properties of the drug have been implicated.

Tamoxifen is a strong hepatocarcinogen in rats (*11*−*13*) and has been shown to cause endometrial tumors in rats and mice after transplacental, neonatal, or chronic exposure (*14*−*18*). Substantial evidence for a genotoxic mechanism in rat and mouse liver stems from the detection of hepatic DNA adducts following dosing regimens with tamoxifen (*12*, *19*−*23*) and some of its metabolites, such as *N*-desmethyltamoxifen, α-hydroxytamoxifen, and α-hydroxy-*N*-desmethyltamoxifen (**2**−**4**, Figure 1) (*24*−*30*). A considerable body of evidence has indicated that metabolic activation to DNA binding electrophiles in rat liver occurs primarily through cytochrome P450-catalyzed hydroxylation at the allylic (α) carbon of tamoxifen (and presumably **2**) (*31*), followed by *O*-esterification, mainly through sulfotransferase-catalyzed sulfation (*32*−*35*). The major DNA adducts arising from these metabolic pathways have been identified as dG-Tam (**5**, Figure 2) and dG-desMeTam (**6**, Figure 2) by using spectroscopic techniques and through comparison with fully characterized synthetic standards (*28*, *36*−*43*). Some additional observations consistent with tamoxifen genotoxicity include the induction of aneuploidy and chromosomal aberrations in rat liver and of micronuclei in metabolically proficient human cells (reviewed in *5*). Furthermore, tamoxifen has been associated with mutations in the *p53* tumor supressor gene of rat hepatocarcinomas (*44*), and both tamoxifen and α-hydroxytamoxifen have been shown to cause mutations in the liver *lacI* and *cII* genes of Big Blue transgenic rats (*45*−*50*). Similarly to what has been shown in vitro for all of the dG-Tam diastereomers (*51*), these mutations were predominantly G → T transversions.

With few exceptions (*52*, *53*), most studies have failed to detect DNA adducts in the uterus and other extrahepatic tissues from rats administered tamoxifen or tamoxifen derivatives (*18*, *23*, *24*, *29*, *54*). These results, combined with the lack of detectable mutations in extrahepatic tissues (*29*, *46*, *47*, *49*), have suggested that tamoxifen-induced endometrial tumors in the rat do not arise from a genotoxic pathway. Although very few studies have been conducted in other animal models, recent preliminary reports of the detection of tamoxifen DNA adducts in various tissues of female cynomologous monkeys, including the uterus (*55*, *56*), support the existence of interspecies variations in tamoxifen activation.

The evidence for uterine DNA damage in women on tamoxifen therapy has been the focus of intense debate. Using the ^{32}P-postlabeling methodology, Hemminki et al. (*57*) were the first to report the detection of DNA adducts in endometrial samples from women administered tamoxifen, at levels of 2.7 adducts/10^9 nucleotides, but their study has been criticized, due to high background levels and the lack of adduct standards (*58*). Using an improved ^{32}P-postlabeling procedure, coupled with HPLC detection, Shibutani and colleagues corroborated the detection of tamoxifen DNA adducts in the endometrial tissue of patients administered tamoxifen (*59*, *60*). The adducts were identified on the basis of co-chromatography with synthetic standards and were estimated to be in the range of 0.2−18 adducts/10^8 nucleotides. However, using a similar approach, Carmichael, Phillips, and co-workers found no evidence for tamoxifen binding to human endometrial DNA (*61*, *62*). In a preliminary study, Martin et al. used the extremely sensitive technique of AMS to assay uterine DNA following a single dose of [^{14}C]-tamoxifen to women prior to surgery (*63*). Radiolabeled carbon was detected in the uterus of the treated patients, but the evidence for DNA damage was not conclusive.

As indicated above, the ^{32}P-postlabeling technique has been, in most instances, the method of choice for the detection and quantitation of tamoxifen DNA adducts formed in vitro and in vivo, with a few studies reporting the use of AMS (*53*, *63*) or immunoassays (*55*, *64*). Despite their sensitivity, each of these techniques has a range of different limitations, particularly concerning the unequivocal characterization of the adducts (reviewed in *65*). In view of the controversy surrounding the detection of tamoxifen DNA adducts in human endometrial samples, the availability of analytical methodologies capable of measuring, with high chemical specificity, small amounts of tamoxifen-derived adducts in target tissues is clearly required. In the present study, we describe the development and validation of an on-line method that involves HPLC separation and ES-MS/MS detection, using a deuterated adduct as internal standard and MRM. The

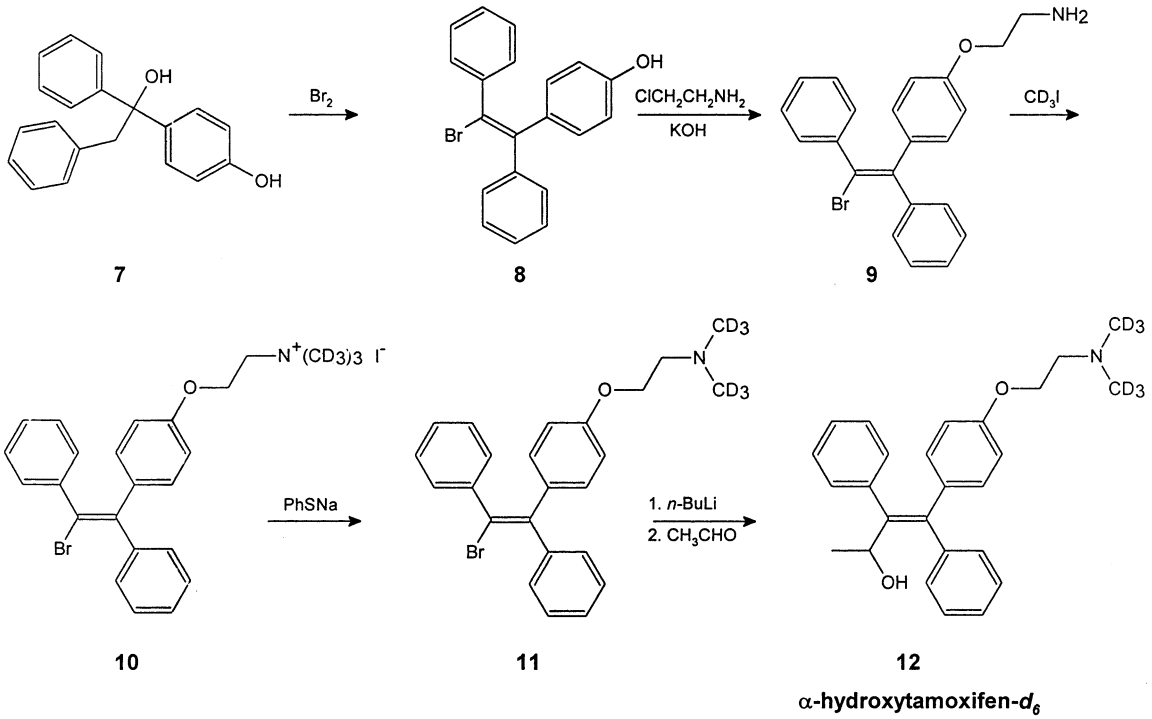

Figure 3. Synthesis of deuterium-labeled α-hydroxytamoxifen (**12**). Compounds **8**−**11** were obtained as mixtures of the *E* and *Z* isomers; compound **12** was separated from the *Z* isomer by TLC.

method is applied to DNA modified in vitro with α-acetoxytamoxifen and from tissues of experimental animals administered tamoxifen.

Materials and Methods

Caution: *Tamoxifen and its derivatives are potentially genotoxic and should be handled with proper care.*

Chemicals. Tamoxifen, salmon testis DNA, Bis-Tris, trioctanoin, and the enzymes used in DNA hydrolysis were purchased from Sigma Chemical Co. (St. Louis, MO). All other commercially available reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI) or Sigma-Aldrich Química, S. A. (Madrid, Spain) and were used as received. Whenever necessary, solvents were purified by standard procedures (*66*).

General Instrumentation. Melting temperatures were measured with a Leica Galen III hot-stage apparatus and are uncorrected. HPLC analyses of tamoxifen derivatives and their synthetic precursors were conducted with a μBondapak C_{18} column (0.39 cm × 30 cm; Waters Associates, Milford, MA), using either a Varian system consisting of a Star 9012 ternary gradient pump and a Polychrom 9065 diode array spectrophotometric detector (Varian, Inc., Palo Alto, CA), equipped with a Rheodyne model 7125 injector (Rheodyne, Cotati, CA), or a Waters Associates system consisting of two model 510 pumps and a model 660 automated gradient controller, equipped with a Rheodyne model 7125 injector and a Hewlett-Packard 1050 diode array spectrophotometric detector (Hewlett-Packard Co., Palo Alto, CA). The peaks were monitored at 254 or 280 nm. UV spectra were recorded with either a Beckman DU-40 UV/vis (Beckman Coulter, Fullerton, CA) or a Shimadzu 1202 UV/vis (Shimadzu Europe, Duisburg, Germany) spectrophotometer.

^{1}H NMR spectra were obtained either on a Varian Unity 300 spectrometer (Varian Deutschland Gmbh, Darmstadt, Germany), operating at 300 MHz, or a Bruker AM500 spectrometer (Bruker Instruments, Inc., Billerica, MA), operating at 500 MHz. ^{13}C NMR spectra were recorded on the Varian Unity 300 instrument, operating at 75.4 MHz. Chemical shifts are reported in ppm downfield from tetramethylsilane, and coupling constants are reported in Hz.

Mass spectral characterization of synthetic intermediates was obtained on either a VG Trio 2000 spectrometer (Thermo Onix, Winsford, U.K.), operated in the FAB mode, with the sample dispersed in a matrix of 3-nitrobenzyl alcohol, a Finnigan TSQ-700 GS/MS system (Thermo Finnigan, San Jose, CA), operated in the EI mode, with the sample being introduced via a direct exposure probe, or a Finnigan TSQ-7000 LC/MS system, operated in the ES mode. For ES spectral measurements, 50% methanol containing 0.1% ammonium formate (pH 3.5) was used at a flow rate of 0.2 mL/min. HRMS was performed at the Instituto Tecnológico e Nuclear, Sacavém, Portugal. Elemental analyses were performed at the M-H-W Laboratories (Phoenix, AZ) or at the Analytical Laboratory, Instituto Superior Técnico, Lisboa, Portugal.

Syntheses. α-Hydroxytamoxifen (**3**) was synthesized by the method of Foster et al. (*67*) and converted to α-acetoxytamoxifen as described by Osborne et al. (*36*). α-Hydroxy-*N*-desmethyltamoxifen (**4**) was synthesized and converted into its sulfate ester derivative as described in Gamboa da Costa et al. (*28*). α-Hydroxytamoxifen-d_6 (**12**, Figure 3) was synthesized by the series of reactions outlined below.

(±)-[1-(4-Hydroxyphenyl)]-1,2-diphenylethanol (7). A solution of benzyl bromide (26 mL, 214 mmol) in dry THF (50 mL) was added dropwise, over a period of 1 h, to a refluxing suspension of magnesium powder (11 g, 452 mmol) in dry THF (100 mL). The mixture was further refluxed for 20 min and then allowed to reach room temperature. 4-Hydroxybenzophenone (10 g, 49.4 mmol) in THF (20 mL) was subsequently added over 5 min, and the mixture was stirred for 1 h. The dark green solution was then quickly filtered to remove excess magnesium, the magnesium was washed with THF (20 mL), and the combined THF solutions were poured into a saturated ammonium chloride solution (800 mL). The product was extracted with methylene chloride (3 × 1 vol), and the organic extracts were combined and dried with anhydrous sodium sulfate. The solvent was evaporated under vacuum, and the crude residue was washed repeatedly with *n*-hexane to remove 1,2-diphenylethane. The carbinol (12.23 g, 85%) was obtained upon recrystallization from toluene; mp 135−37 °C. ^{1}H NMR (acetone-d_6): δ 3.59 (2H, s, PhC**H**$_2$), 4.28 (1H, s, CH_2CO**H**), 6.71 (2H, d, J = 8.7, Ar**H**), 6.98 (2H, dd, J_o = 6.6, J_m = 2.4, Ar**H**), 7.04−7.07 (3H, m, Ar**H**), 7.13 (1H, t, J = 7.2, Ar**H**), 7.22 (2H, t, J = 7.2, Ar**H**), 7.28 (2H, d, J = 8.7, Ar**H**), 7.42 (2H, d, J = 7.2, Ar**H**), 8.21 (1H, s, ArO**H**). ^{13}C NMR (acetone-d_6): δ 48.52 (**C**H$_2$), 78.18

(CH_2**C**OH), 115.14 (Ar**C**H), 126.49 (Ar**C**H), 126.85 (Ar**C**H), 127.23 (Ar**C**H), 127.90 (Ar**C**H), 128.25 (Ar**C**H), 128.47 (Ar**C**H), 131.92 (Ar**C**H), 138.47 ($\mathbf{C}_{quat}$), 139.88 ($\mathbf{C}_{quat}$), 149.06 ($\mathbf{C}_{quat}$), 156.70 ($\mathbf{C}_{quat}$). MS (FAB): *m/z* 290 (M^+, 4%), 289 [$(M - H)^+$, 11%], 273 [$(MH - H_2O)^+$, 100%], 199 [$(MH - C_7H_8)^+$, 60%]. Anal. calcd for $C_{20}H_{18}O_2$: C, 82.73%; H, 6.25%. Found: C, 82.83%; H, 6.31%.

(*E*,*Z*)-1-Bromo-2-[(4-hydroxy)phenyl]-1,2-diphenylethene (8). A solution of bromine (2.35 mL, 45.6 mmol) in carbon tetrachloride (15 mL) was added to a solution of **7** (13 g, 44.8 mmol) in the same solvent (100 mL), and the mixture was stirred at room temperature. The reaction product started to precipitate after approximately 5 min. The mixture was stirred for an additional hour and then concentrated to approximately 50% of the original volume. Methylene chloride (150 mL) was added to dissolve the precipitate, and the solution was washed sequentially with 1 vol of saturated $NaHCO_3$ and 1 vol of water. The organic extract was dried with anhydrous sodium sulfate and evaporated. The bromoalkene **8** (15.22 g, 97%), obtained as a mixture of the *E* (75%) and *Z* (25%) isomers, was recrystallized from toluene/*n*-hexane (3/7). ^{1}H NMR (acetone-d_6): δ 6.57 (2H, d, J = 8.7, alkOPh**H**, *E* isomer), 6.80 (2H, d, J = 8.7, alkOPh**H**, *E* isomer), 6.86 (2H, d, J = 8.4, alkOPh**H**, *Z* isomer), 6.96−7.42 (10H, *E* isomer + 12H, *Z* isomer, m, Ar**H**), 8.42 (1H, s, ArO**H**, *E* isomer), 8.56 (1H, s, ArO**H**, *Z* isomer). ^{13}C NMR (acetone-d_6): δ 115.48 (Ar**C**H), 115.57 (Ar**C**H), 121.08 ($\mathbf{C}_{quat}$), 122.71 (Ar**C**H), 128.20 (Ar**C**H), 128.60 (Ar**C**H), 128.84 (Ar**C**H), 128.95 (Ar**C**H), 130.10 (Ar**C**H), 131.02 (Ar**C**H), 131.62 (Ar**C**H), 132.32 (Ar**C**H), 132.97 ($\mathbf{C}_{quat}$), 142.38 ($\mathbf{C}_{quat}$), 144.64 ($\mathbf{C}_{quat}$), 145.02 ($\mathbf{C}_{quat}$), 157.25 ($\mathbf{C}_{quat}$), 157.38 ($\mathbf{C}_{quat}$). MS (EI): *m/z* 352 (M^+, 43%), 350 (M^+, 41%), 271 [$(M - Br)^+$, 100%]. Anal. calcd for $C_{20}H_{15}BrO$: C, 68.39%; H, 4.30%. Found: C, 68.27%; H, 4.31%.

(*E*,*Z*)-1-Bromo-2-[4-(2-aminoethoxy)phenyl]-1,2-diphenylethene (9). To a solution of **8** (8 g, 22.8 mmol) in dioxane/toluene (1/3, 150 mL) was added a 10-fold molar excess of powdered potassium hydroxide. The mixture was then taken to reflux, and an excess of solid 2-chloroethylamine hydrochloride (ca. 5 equiv) was added portionwise until TLC (silica gel, 15% methanol in methylene chloride) indicated no further change. The mixture was then concentrated to approximately 50% of the original volume, water (400 mL) was added, and the organic materials were extracted with methylene chloride (2 × 0.5 vol). The organic extract was washed sequentially with 0.5 M sodium hydroxide (1 vol) and water (2 × 1 vol), dried with anhydrous sodium sulfate, and evaporated. The residue was purified by flash chromatography on silica gel H (Type 60, E. Merck, Darmstadt, Germany) by eluting with a stepwise gradient of 0−10% methanol in methylene chloride. The alkylated product **9** (6.26 g, 70%) was obtained as a mixture of the *E* (33%) and *Z* (66%) isomers. ^{1}H NMR (acetone-d_6): δ 3.47 (2H, t, J = 6.0, C**H**$_2$N, *E* isomer), 3.57 (2H, t, J = 6.0, C**H**$_2$N, *Z* isomer), 4.06 (2H, t, J = 6.0, C**H**$_2$O, *E* isomer), 4.21 (2H, t, J = 6.0, C**H**$_2$O, *Z* isomer), 6.66 (2H, d, J = 8.7, alkOPh**H**, *E* isomer), 6.89 (2H, d, J = 8.7, alkOPh**H**, *E* isomer), 6.95−7.40 (10H, *E* isomer + 14H, *Z* isomer, m, Ar**H**). ^{13}C NMR (acetone-d_6): δ 51.08 (**C**H$_2$N), 51.20 (**C**H$_2$N), 68.86 (**C**H$_2$O), 69.02 (**C**H$_2$O), 114.58 (Ar**C**H), 114.76 (Ar**C**H), 121.41 ($\mathbf{C}_{quat}$), 121.88 ($\mathbf{C}_{quat}$), 127.72 (Ar**C**H), 128.21 (Ar**C**H), 128.60 (Ar**C**H), 128.73 (Ar**C**H), 128.83 ($\mathbf{C}_{quat}$), 128.94 (Ar**C**H), 130.05 (Ar**C**H), 130.95 (Ar**C**H), 131.47 (Ar**C**H), 132.16 (Ar**C**H), 133.89 ($\mathbf{C}_{quat}$), 136.66 ($\mathbf{C}_{quat}$), 142.18 ($\mathbf{C}_{quat}$), 144.40 ($\mathbf{C}_{quat}$), 144.84 ($\mathbf{C}_{quat}$), 158.91 ($\mathbf{C}_{quat}$), 159.36 ($\mathbf{C}_{quat}$), 168.52 ($\mathbf{C}_{quat}$). MS (EI): *m/z* 395 (M^+, 4%), 393 (M^+, 4%), 352 [$(M - CH_2CHNH_2)^+$, 7%], 350 [$(M - CH_2CHNH_2)^+$, 6%], 315 [$(M - Br + 1)^+$, 35%], 272 [$(M - CH_2CHNH_2 - Br + 1)^+$, 100%], 178 {$[(C_6H_5)_2C_2]^+$, 21%}, 44 [$(CH_2CH_2NH_2)^+$, 14%]. Anal. calcd for $C_{22}H_{20}BrNO$: C, 67.01%; H, 5.11%; N, 3.55%. Found: C, 66.82%; H, 5.14%; N, 3.41%.

(*E*,*Z*)-1-Bromo-2-[4-[2-(tris(trideuteriomethyl)ammonio)ethoxy]phenyl]-1,2-diphenylethene iodide (10). Potassium carbonate (6.25 g, 45.3 mmol) and iodomethane-d_3 (1.4 mL, 22.4 mmol) were added to a solution of **9** (500 mg, 1.3 mmol) in methanol (10 mL), and the mixture was stirred at room temperature for 48 h. Following evaporation of the methanol, the residue was resuspended in methylene chloride (200 mL) and washed with water (3 × 0.5 vol). The organic extract was dried with anhydrous sodium sulfate, filtered, evaporated, washed three times with *n*-hexane, and finally dried under vacuum to yield **10** (644 mg, 86%) as mixture of the *E* (33%) and *Z* (66%) isomers. ^{1}H NMR (DMSO-d_6): δ 3.70 (2H, t, J = 3.9, C**H**$_2$N, *E* isomer), 3.80 (2H, t, J = 3.3, C**H**$_2$N, *Z* isomer), 4.33 (2H, bs, C**H**$_2$O, *E* isomer), 4.48 (2H, bs, C**H**$_2$O, *Z* isomer), 6.74 (2H, d, J = 8.7, alkOPh**H**, *E* isomer), 6.89 (2H, d, J = 8.7, alkOPh**H**, *E* isomer), 6.93−7.40 (10H, *E* isomer + 14H, *Z* isomer, m, Ar**H**). ^{13}C NMR (DMSO-d_6): δ 52.09 (m, **C**D$_3$N), 61.43 (**C**H$_2$N), 61.57 (**C**H$_2$N), 63.89 (**C**H$_2$O), 63.90 (**C**H$_2$O), 114.16 (Ar**C**H), 114.40 (Ar**C**H), 120.73 ($\mathbf{C}_{quat}$), 121.11 ($\mathbf{C}_{quat}$), 127.08 (Ar**C**H), 127.56 (Ar**C**H), 127.98 (Ar**C**H), 128.11 (Ar**C**H), 128.24 (Ar**C**H), 128.35 (Ar**C**H), 128.87 (Ar**C**H), 129.72 (Ar**C**H), 129.84 (Ar**C**H), 130.42 (Ar**C**H), 131.05 (Ar**C**H), 133.51 ($\mathbf{C}_{quat}$), 136.30 ($\mathbf{C}_{quat}$), 140.65 ($\mathbf{C}_{quat}$), 140.81 ($\mathbf{C}_{quat}$), 143.05 ($\mathbf{C}_{quat}$), 143.50 ($\mathbf{C}_{quat}$), 156.26 ($\mathbf{C}_{quat}$), 156.68 ($\mathbf{C}_{quat}$). MS (ES): *m/z* 447 (M^+, 100%), 445 (M^+, 91%), 367 [$(MH - Br)^+$, 81%].

(*E*,*Z*)-1-Bromo-2-[4-[2-(bis(trideuteriomethyl)amino)-ethoxy]phenyl]-1,2-diphenylethene (11). The iodide salt **10** (400 mg, 0.70 mmol) was dissolved in 2-butanone (15 mL) that had been dried over anhydrous sodium sulfate. Sodium benzenethiolate (470 mg, 3.4 mmol) was then added, and the mixture was refluxed for 1 h. After the solvent was evaporated, the residue was resuspended in methylene chloride (100 mL) and washed sequentially with 0.5 M sodium hydroxide (2 × 1 vol) and water (2 × 1 vol). The organic extract was concentrated, and the product was purified by flash chromatography. Elution was started with methylene chloride to remove traces of thiophenol, and then the product (285 mg, 95%) was recovered with 10% methanol in methylene chloride as a mixture of the *E* (26%) and *Z* (74%) isomers. ^{1}H NMR (acetone-d_6): δ 2.58 (2H, t, J = 5.9, C**H**$_2$N, *E* isomer), 2.68 (2H, t, J = 5.9, C**H**$_2$N, *Z* isomer), 3.95 (2H, t, J = 5.9, C**H**$_2$O, *E* isomer), 4.10 (2H, t, J = 5.9, C**H**$_2$O, *Z* isomer), 6.65 (2H, d, J = 8.7, alkOPh**H**, *E* isomer), 6.89 (2H, d, J = 8.7, alkOPh**H**, *E* isomer), 6.94−7.39 (10H, *E* isomer + 14H, *Z* isomer, m, Ar**H**). ^{13}C NMR (acetone-d_6): δ 45.13 (m, **C**D$_3$N), 58.68 (**C**H$_2$N), 58.72 (**C**H$_2$N), 66.96 (**C**H$_2$O), 67.10 (**C**H$_2$O), 114.59 (Ar**C**H), 114.77 (Ar**C**H), 121.47 ($\mathbf{C}_{quat}$), 121.91 ($\mathbf{C}_{quat}$), 127.73 (Ar**C**H), 128.22 (Ar**C**H), 128.62 (Ar**C**H), 128.75 (Ar**C**H), 128.84 ($\mathbf{C}_{quat}$), 128.95 (Ar**C**H), 130.06 (Ar**C**H), 130.96 (Ar**C**H), 131.49 (Ar**C**H), 132.18 (Ar**C**H), 133.97 ($\mathbf{C}_{quat}$), 136.75 ($\mathbf{C}_{quat}$), 142.21 ($\mathbf{C}_{quat}$), 144.42 ($\mathbf{C}_{quat}$), 144.88 ($\mathbf{C}_{quat}$), 158.81 ($\mathbf{C}_{quat}$), 159.25 ($\mathbf{C}_{quat}$). MS (ES): *m/z* 430 (MH^+, 89%), 428 (MH^+, 100%), 350 [$(MH_2 - Br)^+$, 77%]. HRMS calcd for $C_{24}H_{18}D_6BrNO$, 427.1412; found, 427.1412.

(*E*)-4-[4-[2-(Bis(trideuteriomethyl)amino)ethoxy]phenyl]-3,4-diphenylbut-3-en-2-ol (12). The synthetic procedure was based on the method of Foster et al. (*67*). Briefly, a 120 mM solution of the (*E*,*Z*)-bromoalkene **11** in freshly distilled dry THF was cooled to −110 °C and kept under argon. A 3-fold molar excess of 1.6 M *n*-butyllithium was then added, while keeping the temperature below −80 °C. The dark brown vinyllithium intermediate was then quenched by dropwise addition of an excess of dry acetaldehyde until the mixture became pale yellow. The excess of acetaldehyde was quickly removed by evaporation, and saturated aqueous ammonium chloride was added to the mixture. Following extraction with methylene chloride, **12** was separated from the *Z* isomer by TLC on silica gel (Merck) by eluting twice with methylene chloride/methanol (9/1) and recovered in 34% yield by precipitation from diethyl ether; mp 121−122 °C. ^{1}H NMR ($CDCl_3$): 1.20 (3H, d, J 6.6, C**H**$_3$CH), 2.66 (2H, t, J = 5.9, C**H**$_2$N), 3.92 (2H, t, J = 5.9, C**H**$_2$O), 4.83 (1H, q, J = 6.6, CH$_3$C**H**), 6.55 (2H, d, J = 8.7, alkOPh**H**), 6.80 (2H, d, J = 8.7, alkOPh**H**), 7.17−7.40 (10H, m, Ph**H**). ^{13}C NMR ($CDCl_3$): 22.46 (**C**H$_3$CH), 42.69 (m, **C**D$_3$N), 58.00 (**C**H$_2$N), 65.49 (**C**H$_2$O), 68.12 (CH$_3$**C**H), 113.35 (Ar**C**H), 126.55 (Ar**C**H), 126.99 (Ar**C**H), 127.75 (Ar**C**H), 128.26 (Ar**C**H), 129.51 (Ar**C**H), 131.06 (Ar**C**H), 131.37 (Ar**C**H), 134.54 ($\mathbf{C}_{quat}$), 138.34 ($\mathbf{C}_{quat}$), 140.60

($\mathbf{C}_{quat}$), 141.50 ($\mathbf{C}_{quat}$), 141.94 ($\mathbf{C}_{quat}$), 156.93 ($\mathbf{C}_{quat}$). MS (EI): *m/z* 393 (M^+), 78 $[CH_2CH_2N(CD_3)_2]^+$, 64 $[CH_2N(CD_3)_2]^+$. HRMS calcd for $C_{26}H_{23}D_6NO_2$, 393.2569; found, 393.2575.

DNA Adduct Standards. α-Acetoxytamoxifen was reacted with DNA using the method of Osborne et al. (*36*), as modified by Gamboa da Costa et al. (*29*). Following sequential extraction of the unbound materials with diethyl ether and *n*-butanol, both of which had been presaturated with 5 mM Bis-Tris and 0.1 mM EDTA (pH 7.1), the DNA was precipitated with sodium chloride and ethanol and redissolved in 5 mM Bis-Tris and 0.1 mM EDTA (pH 7.1) at a concentration of ∼1 mg/mL. After enzymatic hydrolysis to nucleosides (*68*), the adducts were partitioned into *n*-butanol, which had been presaturated with 5 mM Bis-Tris and 0.1 mM EDTA (pH 7.1), the *n*-butanol was evaporated, and the residue was redissolved in methanol. The major adduct, dG-Tam (*36*), was then isolated by HPLC, at a flow rate of 2 mL/min, using a 17 min linear gradient of 0−60% acetonitrile in 100 mM ammonium acetate (pH 5.7), followed by a 3 min linear gradient to 100% acetonitrile and a 5 min isocratic elution with acetonitrile. The adduct was thoroughly dried under vacuum and quantified on the basis of its molar extinction coefficients (ϵ_{250} = 16 800 M^{-1} cm^{-1}; ϵ_{275} = 13 200 M^{-1} cm^{-1}) as described in Gamboa da Costa et al. (*29*).

dG-desMeTam, the major adduct from reaction of α-sulfoxy-*N*-desmethyltamoxifen with DNA, was prepared and purified as detailed in Gamboa da Costa et al. (*28*). The deuterated adduct standard, dG-Tam-d_6, was synthesized from **12**, isolated as described above for dG-Tam (*29*, *36*), and characterized by MS/MS. MS (ES): *m/z* 643 (MH^+). MS/MS (643): *m/z* 527 $[(MH_2 - dR)^+]$, 376 $[(M - dG)^+]$, 350 $[(MH_2 - dG - CH_3CH)^+]$, 178 $[(Gua + CH_3CH)^+]$, 78 $[CH_2CH_2N(CD_3)_2]^+$.

For quantification purposes, the molar extinction coefficients of both dG-desMeTam and dG-Tam-d_6 were assumed to be identical to those determined for dG-Tam, since the minor structural differences between the three adducts were not expected to affect the corresponding chromophores. Stock solutions containing known concentrations of dG-Tam, dG-desMeTam, or dG-Tam-d_6 in methanol were prepared and kept at −20 °C in vials sealed with Teflon septa to prevent evaporation of the solvent.

In Vitro Modified DNA Samples. DNA samples with different extents of modification were prepared by conducting a series of overnight incubations at 37 °C in which α-acetoxytamoxifen was reacted with salmon testis DNA at ratios varying by 10-fold increments, from 0.1 ng to 1 mg α-acetoxytamoxifen/mg DNA. After it was purified, as described above, the DNA was redissolved in 5 mM Bis-Tris and 0.1 mM EDTA (pH 7.1) at a concentration of ∼1.7 mg/mL. For comparative purposes, a control incubation was conducted without α-acetoxytamoxifen. Aliquots of each solution, containing 100 μg of DNA, were subsequently hydrolyzed to nucleosides (*68*) and analyzed directly by either HPLC−UV or HPLC-ES-MS/MS without prior partition of the adducts into *n*-butanol.

Treatment of Animals. Four female Sprague−Dawley rats (8 weeks old, obtained from the breeding colony at the National Center for Toxicological Research) were treated by gavage (*29*) with seven daily doses of tamoxifen (20 mg/kg, 54 μmol/kg, dissolved in 200 μL of trioctanoin). Four additional control rats were treated in the same manner with 200 μL of trioctanoin alone. Twenty-four hours after the last treatment, the animals were killed by exposure to carbon dioxide and the livers and uteri were quickly excised. Hepatic nuclei were isolated by the method of Basler et al. (*69*), and DNA was prepared from the liver nuclei and uteri by slight modifications of the procedure described in Beland et al. (*70*).

HPLC−UV Analyses. HPLC−UV quantification of in vitro modified DNA samples was conducted by direct injection of the DNA hydrolysates. The adducts were separated at a flow rate of 2 mL/min, using a 20-min linear gradient of 20−60% acetonitrile in 100 mM ammonium acetate (pH 5.7), followed by a 5-min isocratic elution with 60% acetonitrile in the same buffer, and a 10-min linear gradient of 60−100% acetonitrile. The adduct levels in each sample were determined by comparison of the adduct peak areas to those obtained from injection of known amounts of the dG-Tam adduct standard.

HPLC-ES-MS/MS Analyses. (1) LC. The liquid handling system consisted of an Alliance 2790 pump (Waters Associates), a Dionex GP40 quaternary gradient pump (Dionex, Sunnyvale, CA), and two automated switching valves (TPMV, Rheodyne, Cotati, CA). Valve 1 allowed the pump eluent to either load a sample onto the trap column and then wash it or to bypass the trap column and clean the analytical column. Valve 2 was used to divert the trap column effluent to either waste or the analytical column. The Alliance 2790 pump was used for sample injection and cleanup and regeneration of the trap and analytical columns; the Dionex pump, containing 73% of 0.1% (v/v) aqueous formic acid and 27% of acetonitrile, was used to backflush the trap column to the analytical column during analysis and to keep a constant flow of mobile phase going into the mass spectrometer during the sample loading and preparation periods.

Each sample was loaded onto a reversed phase trap column [Luna C18(2), 2 mm × 30 mm, 3 μm, Phenomenex, Torrance, CA] and washed to waste for 4.5 min with 95% of 0.1% formic acid and 5% of acetonitrile at a flow rate of 0.75 mL/min. After valve 2 was switched, the concentrated sample zone was backflushed from the trap column onto the analytical column [Luna C18(2), 2 mm × 150 mm, 3 μm, Phenomenex] with 73% of 0.1% formic acid and 27% of acetonitrile at 0.2 mL/min, and the sample components were eluted into the mass spectrometer. Following an 8-min run, valve 2 was switched and the trap column was cleaned to waste with 95% of acetonitrile and 5% of 0.1% formic acid for 2 min, at a flow rate of 0.75 mL/min. Next, valve 1 was switched and the analytical column was cleaned for 2 min with the same eluent, at a flow rate of 0.2 mL/min. Both valves were then switched to their initial positions, to equilibrate both the trap and the analytical column at the starting mobile phase compositions, and the process was repeated.

(2) MS. A Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, U.K.), equipped with an ES interface, was used with a source block of 150 °C and a desolvation temperature of 450 °C. Nitrogen was used as the desolvation (750 L/h) and nebulizing (90 L/h) gas. Argon was used as the collision gas, at a collision cell pressure of 1.5 × 10^{-3} mBar. Positive ions were acquired in the MRM mode (dwell time of 0.3 s, span of 0.02 Da, and interchannel delay of 0.03 s) for the transitions from the doubly charged protonated nucleoside molecule $[(M + 2H)^{2+}]$ to the doubly charged protonated purine base $[(BH + 2H)^{2+}]$ for both dG-Tam (*m/z* 319 → 261) and dG-desMeTam (*m/z* 312 → 254), and the internal standard, dG-Tam-d_6 (*m/z* 322 → 264). The cone voltage was 15 V, and the collision energy was 9 eV for all three transitions.

Results

Synthesis of the Deuterated Adduct Standard, dG-Tam-d_6. Adduct quantitation by mass spectrometry is best achieved by coupling selected ion recording or MRM with the use of a stable isotope-labeled internal standard (*65*). Therefore, a simple synthetic strategy was devised to obtain a deuterated analogue of dG-Tam. Deuteration of the two methyl groups attached to the alkylamino substituent was judged appropriate to provide sufficient *m/z* separation from dG-Tam, since no lability of these groups was expected during the synthetic or analytical procedures. The approach to obtain the adduct precursor, **12**, is outlined in Figure 3. The preparation of the bromoalkene **9** followed standard procedures, involving bromination of the benzylic carbon of **7**, with concomitant dehydration to **8**, and then alkylation with 2-chloroethylamine hydrochloride in alkali to yield **9**. The problem of controlled trideuteriomethylation to a tertiary

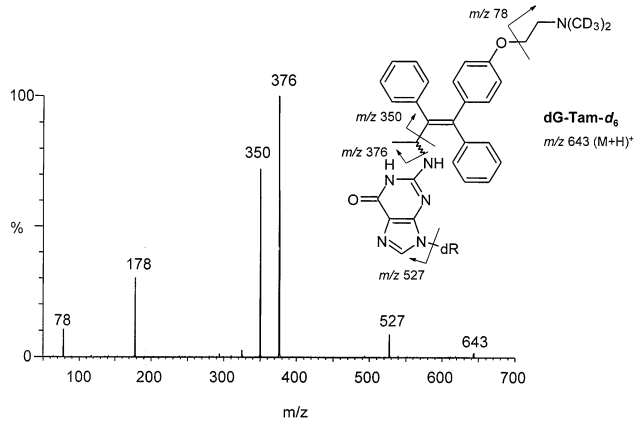

Figure 4. MS/MS characterization of the deuterated adduct standard, dG-Tam-d_6, using ES ionization. The major fragments derived from the protonated molecule $(M + H)^+$ are outlined in the structure.

amine was circumvented by full alkylation with CD_3I to the ammonium salt **10**, followed by removal of one trideuteriomethyl group through nucleophilic displacement with benzenethiolate (*71*). The bromoalkene **11** was then converted to **12** through lithiation and subsequent addition of acetaldehyde, essentially as described for α-hydroxytamoxifen (*67*). Spectroscopic data for **12** (^{1}H and ^{13}C NMR, standard EI-MS, and HRMS; cf. Materials and Methods) were in full agreement with the assigned structure.

Synthesis of dG-Tam-d_6 from **12** was performed as described for dG-Tam (*29*, *36*). The adduct had an HPLC retention time and UV spectrum similar to those of its nondeuterated analogue (not shown). MS, with ES ionization, indicated a protonated molecule (*m*/*z* 643) consistent with the expected structure. A product ion analysis of the protonated molecule in the ES-MS/MS mode further confirmed the characterization of the isolated adduct as dG-Tam-d_6 (Figure 4). Thus, the fragment ions detected at *m*/*z* 527 and 376 corresponded to loss of deoxyribose and deoxyguanosine, respectively, while a small fragment ion at *m*/*z* 78, consistent with *N*,*N*-bis-(trideuteriomethyl)aziridinium $[CH_2CH_2N(CD_3)_2^+]$, confirmed the incorporation of the trideuteriomethyl groups in the aminoethoxy substituent. Additional fragment ions at *m*/*z* 350 $[(MH_2 - dG - CH_3CH)^+]$ and *m*/*z* 178 $[(Gua + CH_3CH)^+]$ were fully compatible with attachment of deoxyguanosine to the allylic carbon of the deuterated tamoxifen segment. This fragmentation pattern was virtually identical to that obtained for dG-Tam (not shown).

In Vitro Modification of Salmon Testis DNA with α-Acetoxytamoxifen. α-Acetoxytamoxifen was prepared by acetylation of α-hydroxytamoxifen with acetic anhydride and reacted with salmon testis DNA (*29*, *36*) at different ratios, varying by 10-fold increments from 0.1 ng to 1 mg α-acetoxytamoxifen/mg DNA. After the DNA was purified by solvent extractions and precipitation, aliquots of the highest modified samples, containing 100 μg of DNA, were hydrolyzed enzymatically to nucleosides and the hydrolysates were analyzed directly by HPLC with UV detection. Figure 5 depicts representative chromatograms obtained with nonmodifed DNA (panel A) and with DNA modified with 1 mg of α-acetoxytamoxifen/mg DNA (panel B). With the modified DNA, two peaks were present at a ratio of approximately 4:1. The major peak co-chromatographed with the synthetic standard, dG-Tam, whereas the minor peak corresponded to a diastereomer or a mixture of diastereomers of the same adduct (*37*, *38*). The total extent of modification for the sample represented in Figure 5B was estimated to be 207 adducts/10^4 nucleotides (Table 1). This estimate was based upon the molar extinction coefficient determined for the major adduct, dG-Tam (*29*), and on the assumption of a similar value for the adduct(s) in the minor peak, since both epimerization at the α carbon of the tamoxifen segment and *E*−*Z* isomerization of the olefinic double bond were not expected to introduce significant alterations in the chromophore. A similar adduct pattern was observed with DNA samples modified with 0.1 and 0.01 mg of α-acetoxytamoxifen/mg DNA, with the adduct levels being estimated to be 58 and 3.9 adducts/10^4 nucleotides, respectively (Table 1). The adduct levels in the samples modified with lower ratios of α-acetoxytamoxifen/DNA were too low to be detected by HPLC−UV when using 100 μg of DNA.

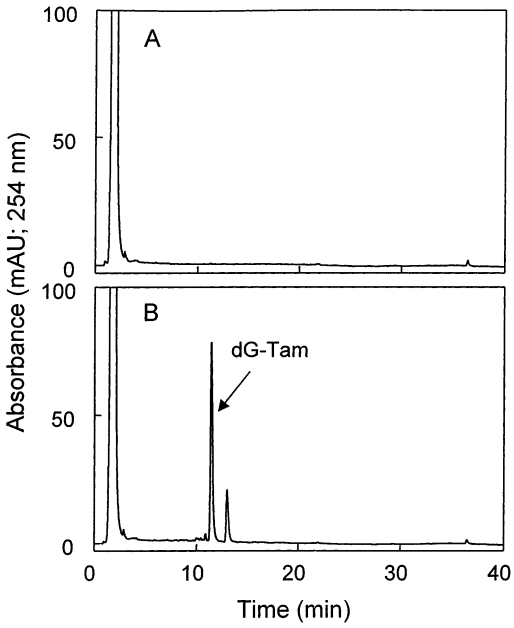

Figure 5. HPLC−UV analysis of a hydrolyzed DNA sample from a modification conducted in vitro with 1 mg of α-acetoxytamoxifen/mg DNA (panel B). The absorbance profile obtained from nonmodified DNA (panel A) is shown for comparison. The elution conditions are outlined in Materials and Methods. Determination of the total extent of modification was based upon the molar extinction coefficient of dG-Tam, as described in Materials and Methods.

Table 1. Adduct Levels (Expressed as dG-Tam/10^8 Nucleotides), as Determined by HPLC/UV and HPLC-ES-MS/MS, in DNA Samples Modified In Vitro with α-Acetoxytamoxifen

reaction ratio[a]	HPLC/UV[b]	HPLC-ES-MS/MS[c]
1	2 070 000	1 520 000
10^{-1}	580 000	540 000
10^{-2}	39 000	48 000
10^{-3}	NA[d]	5500
10^{-4}	NA	560
10^{-5}	NA	61
10^{-6}	NA	20
10^{-7}	NA	8

[a] Expressed as mg of α-acetoxytamoxifen/mg DNA. [b] The amount of dG-Tam as determined by comparison of the HPLC peak area with those of injected dG-Tam standards of known concentration. [c] The amount of dG-Tam as determined by HPLC-ES-MS/MS by quantifying against a dG-Tam-d_6 internal standard. [d] NA, not analyzed.

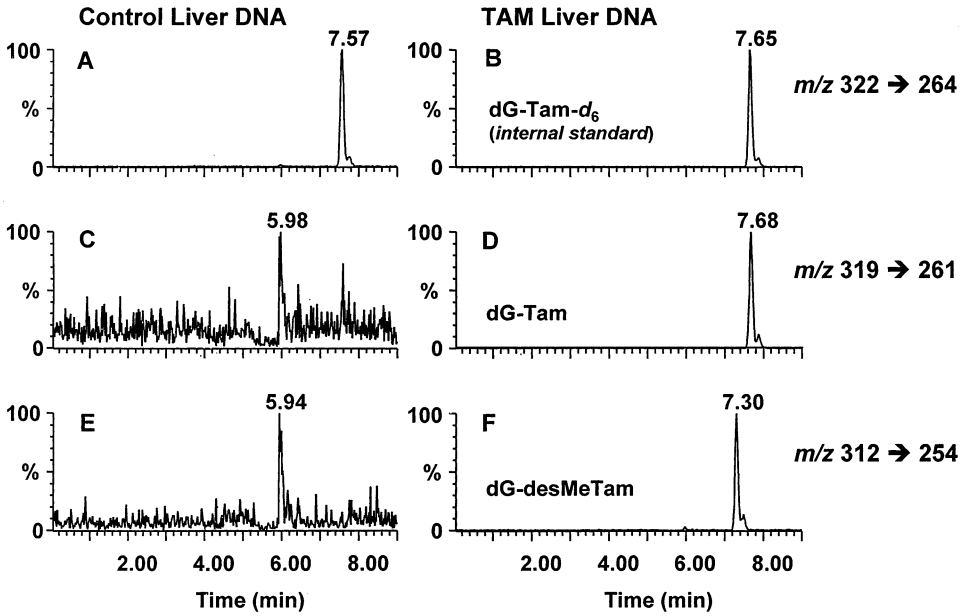

Figure 6. Representative HPLC-ES-MS/MS analyses of tamoxifen DNA adducts in liver DNA from control (panels A, C, and E) and tamoxifen-treated female Sprague−Dawley rats (panels B, D, and F). The rats were treated by gavage with seven daily doses of 20 mg tamoxifen/kg body weight and killed 24 h after the last dose, as outlined in Materials and Methods. Panels A and B: MRM (relative signal intensity vs time) for the transition from the doubly charged protonated nucleoside molecule $[(M + 2H)^{2+}]$ to the doubly charged protonated purine base $[(BH + 2H)^{2+}]$ for 100 pg of the internal standard, dG-Tam-d_6 (m/z 322 → 264). Panels C and D: MRM for the transition from $[(M + 2H)^{2+}]$ to $[(BH + 2H)^{2+}]$ for dG-Tam (m/z 319 → 261). Panels E and F: MRM for the transition from $[(M + 2H)^{2+}]$ to $[(BH + 2H)^{2+}]$ for dG-desMeTam (m/z 312 → 254). The retention time for the most prominent peak in each chromatogram is indicated. The elution conditions are outlined in Materials and Methods.

HPLC-ES-MS/MS Analyses of DNA Samples Modified In Vitro with α-Acetoxytamoxifen. The HPLC-ES-MS/MS method was validated with respect to intra- and interassay precision and accuracy by analyzing, on different days, an unmodified salmon testis DNA hydrolysate (100 μg) to which dG-Tam (10 pg) was added. On day 1, the dG-Tam level measured in the sample was 0.103 ± 0.003 pg/μg DNA (2.9% RSD, $n = 4$). On day 2, the dG-Tam level was found to be 0.105 ± 0.003 (3.1% RSD, $n = 4$). This corresponds to an adduct level of 5.2 dG-Tam adducts in 10^8 normal nucleotides. No observable responses for either dG-Tam or dG-desMeTam were detected in the control DNA in the absence of added adduct standards. The LOD ($S/N = 3$) was approximately 1 pg on-column, which is equivalent to 5 dG-Tam adducts in 10^9 normal nucleotides, and the LOQ ($S/N = 10$) was approximately 1 dG-Tam adduct in 10^8 normal nucleotides. In the presence of 25 pg of the deuterium-labeled internal standard (dG-Tam-d_6), a plot of the response ratio vs the concentration ratio (not shown) was found to be linear from the LOD up to 250 pg of dG-Tam (correlation coefficient > 0.99; slope, ∼1). Similarly, the response for dG-desMeTam (not shown) was also determined to be linear (correlation coefficient > 0.99; slope, 0.085). The method was validated within the range of 8−1 520 000 adducts/10^8 nucleotides, using 100 μg of the various DNA samples modified in vitro to different extents with α-acetoxytamoxifen. Although the process produced no observable analyte carryover to the subsequent injection, injections of reagent blanks, control DNA hydrolysates, and adduct standards were regularly incorporated into analytical runs for confirmation.

Table 1 outlines the adduct levels determined by HPLC-ES-MS/MS for the DNA samples modified in vitro at ratios of $1-10^{-7}$ mg of α-acetoxytamoxifen/mg DNA. The table also presents the values determined by HPLC−UV for the samples modified at ratios of $1-10^{-2}$ mg of α-acetoxytamoxifen/mg DNA. The correspondence between the results from the two methods is very good; in addition, the regular variation in adduct levels obtained for the different samples is consistent with what would be expected from their corresponding reaction ratios.

HPLC-ES-MS/MS Analyses of DNA Modified In Vivo with Tamoxifen. Female Sprague−Dawley rats were treated by gavage (*29*) with seven daily doses of tamoxifen (20 mg/kg). Hepatic and uterine DNA was isolated, and the extent of binding was assessed by HPLC-ES-MS/MS using conditions identical to those described for analysis of the in vitro modified DNA. Figure 6 illustrates representative HPLC-ES-MS/MS analyses of liver DNA from a control rat (panels A, C, and E) and from a rat administered tamoxifen (panels B, D, and F). The traces corresponding to MRM of the transitions from the doubly charged protonated nucleoside molecule $[(M + 2H)^{2+}]$ to the doubly charged protonated purine base $[(BH + 2H)^{2+}]$ for the internal standard, dG-Tam-d_6 (m/z 322 → 264), are presented in panels A and B. The corresponding MRM traces for dG-Tam (m/z 319 → 261) and dG-desMeTam (m/z 312→ 254) are presented in panels C and D and panels E and F, respectively. As expected, the signal from the deuterated internal standard is clearly present in both samples, whereas the signals ascribed to dG-Tam and dG-desMeTam were readily seen in the livers from the tamoxifen-treated animals but not in the hydrolysates from control livers. The identity of the signals at 5.98 and 5.94 min, which were detected at high expansion in the m/z 319 → 261 and 312→ 254 MRM traces of the control liver DNA samples (Figure 6), is not known. The dG-Tam and dG-desMeTam adduct levels determined by HPLC-ES-MS/MS in the liver DNA from tamoxifen-treated rats were 496 ± 16 and 626 ± 18 adducts/10^8 nucleotides, respectively. Neither dG-Tam nor dG-desMeTam was detected in the uterus DNA, with the LOD being 5

adducts/10^9 nucleotides for dG-Tam and 5 adducts/10^8 nucleotides for dG-desMeTam.

Discussion

Tamoxifen has been associated with an increased incidence of endometrial cancer, when used both as a chemotherapeutic or as a chemopreventive agent against breast cancer (*1*, *2*). Although the mechanism for the induction of these tumors is not known, conflicting evidence regarding the detection of DNA adducts in endometrial samples from women treated with tamoxifen (*57*−*63*) has led to suggestions that the estrogen agonist effect of tamoxifen, promoting uterine cell proliferation, may be involved (*72*). Tamoxifen and some of its metabolites, such as *N*-desmethyltamoxifen (**2**), *N*,*N*-didesmethyltamoxifen, and 4-hydroxytamoxifen, have been detected in endometrial samples from women on tamoxifen therapy at estimated levels of approximately 2−4 μg/g of tissue (*73*). In addition, cytochrome P450 enzymes, including 3A isoforms that have been implicated in the activation of tamoxifen to α-hydroxytamoxifen (*31*), appear to be expressed in the human endometrium (*74*). Therefore, the possibility of tamoxifen being activated in the human uterus by α-hydroxylation seems plausible, and even in the absence of significant sulfotransferase activity to ensure further activation (*5*), the intrinsic reactivity of α-hydroxytamoxifen (and presumably α-hydroxy-*N*-desmethyltamoxifen (**4**)) toward DNA at physiological pH (*36*) could conceivably generate uterine DNA adducts. Thus, to establish whether tamoxifen acts as a genotoxic agent in the human endometrium, the use of reliable adduct detection methodologies to search for putative tamoxifen DNA adducts is of critical importance, not only to establish if adducts are present but also to establish if the detected adduct levels are biologically relevant.

^{32}P-postlabeling has been the most widely used methodology for the assessment of tamoxifen DNA adducts, formed both in vitro and in vivo, with LODs reported in the ranges of 1 adduct/10^9 nucleotides (*62*) to approximately 2.5 adducts/10^{10} nucleotides (*59*, *60*). More recently, competitive DELFIA and CIA have been used to quantify tamoxifen DNA adducts (*64*), with the latter technique having a LOD estimated at 3 adducts/10^9 nucleotides. Despite the high sensitivity achieved in adduct detection by both the ^{32}P-postlabeling and immunoassay methodologies, none of these techniques can provide a chemical characterization of the adducts (*65*). Furthermore, quantitation by ^{32}P-postlabeling can suffer from a series of limitations including incomplete DNA digestion, inefficiency of adduct labeling by T4 polynucleotide kinase, and loss of adducts during the enrichment process (*65*), whereas immunoassay results may, for instance, be limited by crossreactivity of the antisera.

MS is a technique of great potential when applied to DNA adduct detection, due to its high chemical specificity, which permits the identification of particular DNA adducts even in the presence of other species, including chemically unrelated adducts. We have previously used HPLC-ES-MS in the analysis of DNA samples modified in vitro and in vivo with the arylamine carcinogen 4-aminobiphenyl (*75*, *76*). More recently, HPLC-ES-MS/MS, using MRM, was applied with good results to the routine quantification of etheno DNA adducts formed in vitro, and also in animal tissues and human placenta (*77*), at levels of 1 adduct in 10^8 nucleotides. The intrinsic advantage of ES-MS was further enhanced by the use of MS/MS, which can provide information on specific fragmentation patterns, and MRM, which results in very low analytical backgrounds (*65*).

In the present study, we have explored the HPLC-ES-MS/MS methodology as a suitable routine analytical tool for the detection and quantification of tamoxifen DNA adducts formed in vivo. The method involved on-line sample preparation and cleanup and MRM of the transitions from the doubly charged protonated nucleoside molecule $[(M + 2H)^{2+}]$ to the doubly charged protonated purine base $[(BH + 2H)^{2+}]$ for dG-Tam, dG-desMeTam, and an internal standard, dG-Tam-d_6. By quantifying against the deuterated internal standard, the method was validated within the range of 8−1 520 000 adducts/10^8 nucleotides using 100 μg of hydrolyzed DNA that had been modified in vitro with α-acetoxytamoxifen. The LOD and LOQ levels for dG-Tam were estimated at 5 adducts/10^9 nucleotides and 1 adduct/10^8 nucleotides, respectively, and good statistical performance (RSD ∼3%) was obtained. In addition, dG-Tam and dG-desMeTam were readily detected in rat liver DNA of female Sprague−Dawley rats administered tamoxifen under a typical dosing regimen (*29*). DNA adducts were not detected in uterus DNA, which confirms results previously obtained by ^{32}P-postlabeling analyses (*29*).

In conclusion, we have shown that HPLC-ES-MS/MS has sufficient sensitivity and precision to be useful in the routine analysis of tamoxifen DNA adducts formed in vivo in experimental animals. Despite being somewhat less sensitive than the ^{32}P-postlabeling or immunoassay methodologies, it has the clear advantage of providing information on the chemical identities of the adducts. Some investigators have reported tamoxifen DNA adducts to be present at 0.2−18 adducts/10^8 nucleotides in endometrial samples from women undergoing tamoxifen therapy (*59*, *60*). In preliminary studies, we have used HPLC-ES-MS/MS to assess the presence of dG-Tam in endometrial DNA from women treated daily with 20 mg of tamoxifen for up to 5 years. We did not detect dG-Tam in any of these samples (*56* and unpublished data), which is similar to what has been reported by other investigators using ^{32}P-postlabeling techniques (*61*, *62*). Additional studies are clearly necessary to resolve this controversy; nonetheless, the HPLC-ES-MS/MS methodology described in this paper should help clarify the genotoxic potential of tamoxifen in the human endometrium.

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