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Analysis of Tamoxifen–DNA Adducts by High-Performance Liquid Chromatography Using Postcolumn Online Photochemical Activation

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Tamoxifen, a widely used nonsteroidal antiestrogen in the treatment of breast cancer, forms several metabolites. 4-Hydroxytamoxifen (4-OHTam), a metabolite found in the bloodstream, has much higher affinity for the estrogen receptor than tamoxifen itself. Oxidative activation of 4-OHTam induces DNA damage. DNA isolated from HL-60 cells exposed to 10 μM 4-OHTam in the presence of 1 μM hydrogen peroxide was digested enzymatically to release both normal and modified nucleosides. The modified nucleosides were enriched by butanol extraction. Using UV detection, HPLC analysis of the butanol extract from 200 μg DNA digest detected ~4 4-OHTam-dG adducts per 10^7 nucleotides (n = 3). Online postcolumn UV irradiation in HPLC and fluorescence detection improved the detection sensitivity by 3×10^2 times. Using 4-OHTam as an example, this report demonstrated for the first time the power of the technique to assay tamoxifen-DNA adducts directly in the DNA digest without relying on postlabeling.

Online postcolumn UV irradiation in HPLC is used to convert non-fluorescent analytes to highly fluorescent species (1–3). Tamoxifen, a nonsteroidal antiestrogen, is widely used in the treatment of breast cancer. It was recently approved as a prophylactic agent for preventing breast cancer, with a standard therapy duration of ≥5 years (4). Tamoxifen, inherently a nonfluorescent agent, has complex pharmacological activity due to the metabolism of the parent drug to numerous compounds that are biologically active. The photochemical conversion of tamoxifen and its metabolites to highly fluorescent phenanthrenes decreased their HPLC limits of detection to sub-nanogram levels (5). However, when the photolysis was carried out off-line, broad, irregular peaks and irreproducible results caused by degradation of these derivatives affected the HPLC analysis. Brown *et al.* were the first to avoid these problems by using postcolumn online irradiation (6). Later work optimized postcolumn treatment by refining the design of the photoreactor unit. The photochemical reactor is easy to construct and a commercial version is also available (Aura Industries Inc.). Using commercially available components, fully automated method has been developed for the determination of tamoxifen and its major metabolites in plasma by this technique (7). The analyses of other anti-inflammatory agent, such as sulindac and its metabolites in human serum, and more recently tamoxifen and its metabolite in human liver microsomes following protein precipitation have also been reported using online postcolumn UV activation in HPLC with excellent reproducibly and precision (8, 9).

Tamoxifen treatment is reported to be associated with increased risk of endometrial cancer in human (10, 11). Long-term administration of tamoxifen to rats results in a dose-dependent increase in hepatic tumors (12, 13). In rodents, the weight of evidence suggests that tamoxifen may be carcinogenic, at least partially, through genotoxic mechanism (14, 15). α-Hydroxylation of tamoxifen is reported to be the key metabolic step involved in the formation DNA adducts in rat hepatocytes (16, 17). Whether this mechanism elucidated in rodent models is directly applicable to the human tissue is currently controversial (18–21). The possibility of involvement of more than one reactive intermediate cannot be overlooked (22). In breast cancer patients exposed to the standard chronic clinical dose of tamoxifen, steady-state plasma levels are reached in 3 to 5 weeks. Online postcolumn photochemical activation in HPLC analysis of tamoxifen-exposed

Abbreviations used: HPLC, high-performance liquid chromatography; FL, fluorescence; 4-OHTam, 4-hydroxytamoxifen; 4-OHTam-dG, 4-hydroxy-(deoxyguanosin-N^2-yl)-tamoxifen; H_2O_2, hydrogen peroxide; HL-60 cells, promyelocytic leukemia cells; UV, ultraviolet; nmr, nuclear magnetic resonance.

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plasma has detected 4-hydroxytamoxifen and tamoxifen-ol as metabolites of hydroxylation and two others *N*-desmethyltamxifen and *N*-desdimethyltamoxifen by N-demethylation (7). Although *N*-desmethyltamoxifen is the major species, 4-Hydroxytamoxifen (4-OHTam) is found in detectable levels in the blood stream and has been shown to have much higher affinity for the estrogen receptor than tamoxifen itself (23). Microsomal, chemical and *in vivo* oxidation of 4-OHTam produce DNA adducts (24–26). The structures of the major adducts induced by quinone methide intermediate of 4-OHTam were elucidated (27, 28).

The ^{32}P-postlabeling assay is currently in widespread use for measuring Tam-DNA adduct (15, 18–21, 24, 25, 29, 30). Martin *et al.* (31) reported by accelerated mass spectrometry irreversible ^{14}C-radiolabeled tamoxifen binding to DNA in extra hepatic organs at levels below detection using ^{32}P-postlabeling. Recently, in response to the need for a specific Tam-DNA adduct assay, specific immuno assays have been developed (32). Although highly sensitive, the various assays reported for analysis of Tam-DNA adducts are either laborious and/or involve handling and disposal of radioactive wastes. We have explored the potential of online postcolumn UV irradiation in HPLC and fluorescence detection to assay DNA adducts induced by tamoxifen. This report describes analysis of DNA adducts in cells (HL-60) exposed to 4-OHTam.

MATERIALS AND METHODS

Materials. 4-Hydroxytamoxifen, salmon testes DNA, bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane, bovine pancreas DNAse I, phosphodiesterase I from crotalus adamanteus venom, and bacterial alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade solvents and ammonium acetate were obtained from Fisher Scientific (Bedford, MA).

Methods. Quinone derivative of 4-OHTam was prepared following reported procedure with minor modification (28). Treatment of salmon testes DNA with quinone methide followed by DNA isolation and enzymatic digestion of DNA to excise the modified nucleosides were followed as reported by Marques *et al.* (27). The major peaks isolated from 30 mg DNA were combined, lyophilized and after desalting. We reported earlier the identification of the lyophilized product by nmr and mass spectroscopic analyses (28). In agreement with the reported study (27), the major adducts were identified by nmr as isomers of 4-OHTam-dG (28).

HL-60 cells, obtained from American Type Culture Collection (CCL 240), were cultured and exposed to 4-OHTam following previously reported procedure (28). DNA was isolated from the cell pellets using a Mannheim Boehringer DNA isolation kit following manufacturer's protocol. The isolated DNA was digested enzymatically to nucleosides as described earlier (28).

HPLC apparatus and chromatographic conditions. The HPLC system consisted of a fully programmable binary pump system and an injection valve with variable loops (20–200 μl) from Rainin Instruments Co., Inc., a Radial-Pak 8MBC18 LC cartridge (10 μm, 8 mm i.d., 10 cm) with a compatible 2-cm guard column from Waters, and a postcolumn photochemical reactor from Aura Industries Inc. containing a 0.25 mm i.d., 5-m PTEF knitted reactor coil and a 254-nm UV lamp which converted the compounds of interest to fluorophore. The effluent from the photochemical converter was connected in succession to a Hatachi variable wavelength UV detector with setting at 254-nm and a Shimadzu 530 RF fluorescence detector operating at an excitation and emission wavelengths 260 and 375 nm, respectively. The detector signal was integrated by Shimadzu Integrator CR501.

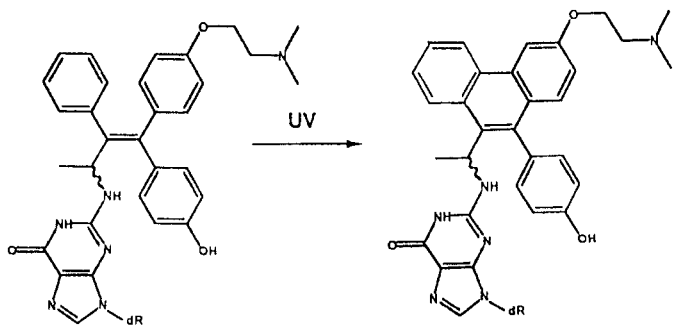

FIG. 1. Structures of 4-OHTam-dG adduct and its fluorescent phenanthrene derivative (R, ribose).

HPLC grade solvents and analytical grade reagents were used to prepare the solvent system. All solvents were filtered through a Nylon-66 filter (0.2 μm). A high pressure inline filter (SSI, 0.5 μm) was used as a further safe guard between each pump and the injector. The adducts were analyzed using a 20-min linear gradient of 20–60% acetonitrile in 100 mM ammonium acetate, pH 5, followed by 5 min at 60% acetonitrile, and finally a 10-min linear gradient of 60–100% acetonitrile. The flow rate was 2 ml/min unless stated otherwise.

RESULTS AND DISCUSSION

Our laboratory has developed fluorescence postlabeling assay for DNA damage by combining the basic idea of DNA digestion with fluorescence postlabeling (33–36). To assay tamoxifen-DNA adducts by this technique, salmon testes DNA exposed to activated 4-OHTam was digested enzymatically at the nucleotide levels and labeled with dansyl chloride following procedures reported earlier (33, 34). Because of the excess labeling reagents relative to the analyte, it is crucial in any postlabeling assay that the reagent peaks resolve clearly from the analyte of interest. Otherwise the analyte would be obscured. Dansyl sulfonic acid generated from hydrolysis of excess dansyl chloride is usually eluted early in reversed-phase HPLC whereas the dansylated derivatives of excess ethylenediamine used as a linker in the labeling procedure are retained much longer (33). Tamoxifen-DNA adducts are hydrophobic in nature and further labeling with dansyl chloride increases their hydrophobicity. Although HPLC conditions were developed in the laboratory to assay tamoxifen-DNA adducts by fluorescence postlabeling technique, the proximity of other hydrophobic reagent peaks could be critical to allow low level detection of tamoxifen-adducts, especially in biological samples (results not shown).

Figure 1 shows the structures of 4-OHTam-dG adduct and its photochemically-converted fluorescent de-

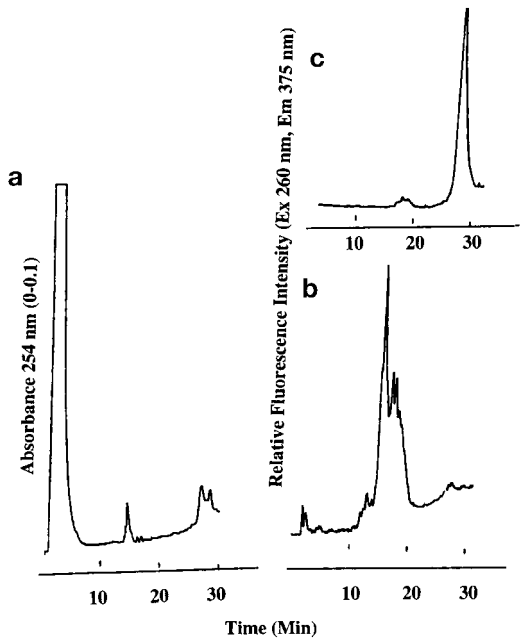

FIG. 2. HPLC analyses of butanol-extracts of DNA digest in HL-60 cells exposed to 4-OHTam (10 μM) + H_2O_2 (1 μM) (a) using UV detection; (b) using online photochemical activation and fluorescence detection; (c) reagent control. The conditions for analysis are described under Materials and Methods.

rivative. We and others have observed that the structures of the major DNA adducts induced by quinone methide intermediate of 4-OHTam are nearly identical to that of α-hydroxytamoxifen interacted major DNA adducts except for a phenolic hydroxyl function (27, 28). Recently, induction of DNA adducts by tamoxifen metabolites N-desmethyltamoxifen, *N,N*-didesmethyltamoxifen *in vivo* and tamoxifen *N*-oxide *in vitro* have also been reported (37, 38). Based on the reported observations (6, 7) of photochemical conversion of tamoxifen and its various metabolites to highly fluorescent phenanthrenes, DNA adducts induced by these metabolites are also expected to yield fluorescent phenanthrene derivatives upon irradiation by UV at 254 nm. With this end in view, DNA adducts induced by an activated tamoxifen metabolite, 4-OHTam were analyzed by HPLC using online postcolumn photochemical activation and fluorescence detection.

Figure 2 shows HPLC analysis of DNA isolated from HL-60 cells exposed to 10 μM 4-OHTam in culture in the presence of 1 μM H_2O_2. Enzymatic digestion of the isolated DNA excised both normal and modified nucleosides. The digested DNA was extracted with normal butanol to enrich the modified nucleosides. The butanol extract was evaporated under vacuum and the residue was reconstituted in methanol prior to analysis by HPLC. Previously we reported (28) the isolation and characterization of the major DNA adducts in salmon testes DNA exposed to activated 4-OHTam following Marques *et al.* procedure (27). The two closely eluting peaks at 14.2 and 14.6 min shown in the profile 2a from 4-OHTam-exposed cellular DNA were identified by cochromatography with the authentic standards as isomers of 4-OHTam-dG adducts. These peaks were not present in DNA isolated from untreated cells (28). The profile 2a, monitored by UV detection, represents analysis of 200 μg size DNA sample. The early big peak in this profile was due to some of the normal nucleosides present in the butanol extracted sample. Figure 2b shows a typical HPLC profile of the same sample (20 μg) using online postcolumn photochemical activation and fluorescence detection. The profile 2c shows analysis of butanol extract of an activated 4-OHTam sample in the absence of DNA. Thus, postcolumn online photochemical activation did not change the adduct pattern (see profiles 2a and 2b). Fluorescence detection improved the adduct signal (integrated peak area) by two orders of magnitude compared to UV detection.

Figure 3 shows HPLC analyses of digested cellular DNA. The cells (HL-60) were exposed in culture to 4-OHTam and DNA isolated following the same procedures discussed earlier. The isolated DNA was digested enzymatically as described before except for the butanol extraction step which was omitted. The objective was to determine whether 4-OHTam-DNA adducts could be detected in the DNA digest without enriching the modified nucleosides. The major peaks in the profile 3a were identified as 4-OHTam-dG adducts by cochromatography with the authentic standards (see

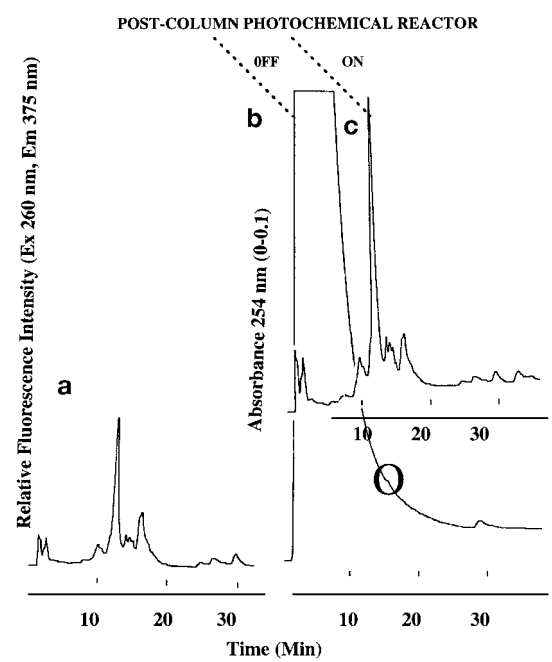

FIG. 3. HPLC analyses of DNA digest in HL-60 cells exposed to 4-OHTam (10 μM) + H_2O_2 (1 μM) (a and c) using online postcolumn photochemical activation and fluorescence detection; (b) using UV detection; (b and c) cochromatography of DNA digest with authentic marker. The conditions for analysis are same as in Fig. 2.

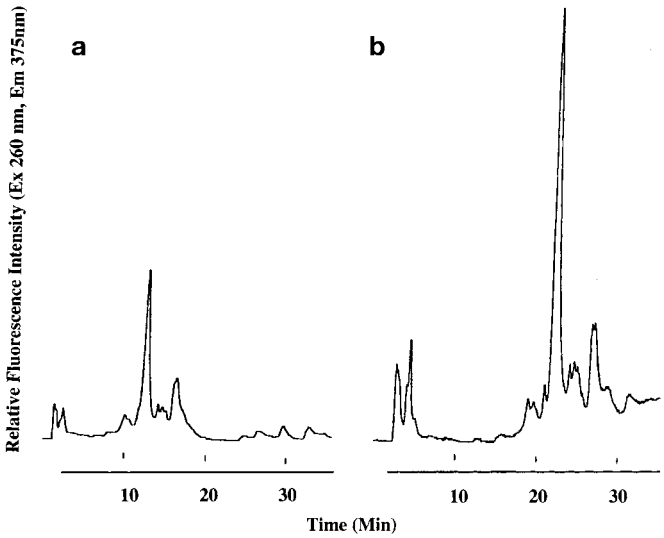

FIG. 4. HPLC analyses of DNA digest in HL-60 cells exposed to 4-OHTam (10 μM) + H_2O_2 (1 μM) using online photochemical activation and fluorescence detection (a) flow rate 2 ml/min; (b) flow rate 1 ml/min. The conditions for analysis are described in the text.

profile 3c). Several small peaks were also resolved in the profile 3a which were not identified. When the online photochemical reactor was turned off (Fig. 3b), HPLC analysis of the same sample shown in Fig. 3c detected the authentic marker only as a minute shoulder shown by the circle on the profile 3b. The large peak from the normal nucleosides released during the enzymatic digestion of DNA (20 μg) in this profile overlapped with the retention times of most of the adduct peaks detected by online photochemical activation (see profiles 3a and 3c). On the other hand, when the reactor was turned on, the normal nucleosides were resistant to photochemical conversion to fluorescent derivatives and did not interfere with the analysis of the 4-OHTam-DNA adducts. As a result, the direct detection of the 4-OHTam-DNA adducts was possible by this technique even in the presence of large excess of the normal nucleosides in the digested DNA (20 μg) sample. Further manipulation of the DNA digest, as shown in Fig. 2b, was unnecessary in order to detect the modified nucleotides.

Figure 4 shows the effect of dwell-time of the analyte in the photochemical reactor on the fluorescence signal as a function of flow rate in real sample analysis. Each profile in Fig. 4 represented direct analysis of digested DNA (20 μg) isolated from 4-OHTam-exposed HL-60 cells. The analyses were performed using a 20-min linear gradient of 20–60% acetonitrile in 0.1 M ammonium acetate, pH 5, followed by an isocratic elution with 60% acetonitrile in the same buffer. The flow rate was reduced to 1 ml/min in the profile b. The overall peak profiles did not change with flow rate. The retention time of the peaks was longer in profile b with better peak resolution, as expected. The fluorescence signal of the major adduct (the mean integrated peak area, $n = 4$) in the profile 4b was also three times higher than that in the profile 4a with the standard flow rate (2 ml/min). The change in the flow to 0.5 ml/min enhanced the fluorescence signal even further. However, there was no practical gain due to the large peak broadening effect and very long retention time (results not shown).

The minimum detectable quantity (M.D.Q.) for absorbance detection of 4-OHTam-dG adduct was 200 fmol which allowed the detection of a relative adduct level of ~4 adduct per 10^7 normal nucleotide in a 200-μg DNA sample (28). Online, postcolumn UV irradiation in HPLC using Shimadzu RF-530 fluorescence detector lowered the M.D.Q. by 3×10^2 times. Additional improvements in sensitivity were possible using McPherson FL-750/HSA (high sensitive accessory) fluorescence detector. Previously we observed that compared to conventional HPLC-FL detection, the use of a He-Cd laser as a radiation source improved the detection sensitivity of fluorescence postlabeling assay for DNA damage significantly (3×10^4 times) (34). Other ultraviolet-laser (257-nm emission from a frequency-doubled argon ion laser as a radiation source) induced fluorescence with liquid chromatography is also reported to offer highly impressive detection limit of some pharmaceutical compounds and substituted anthracenes in comparison with conventional fluorescence detection (39, 40).

In summary, this report demonstrated for the first time that HPLC analysis using postcolumn online photochemical activation is a powerful tool for assaying DNA-adducts induced by an important tamoxifen metabolite. The method is reproducible and offers sensitive detection without relying on fluorescence or radiochemical postlabeling. Since online photochemical activation is known to convert both tamoxifen and its various metabolites to highly fluorescent phenanthrene derivatives, it is reasonable to expect that this technique has potential to detect directly in DNA digest any adduct induced by tamoxifen and its metabolites which can be resolved by HPLC.

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