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Development of a Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry Method for Analysis of Stable 4-Hydroxyequilenin-DNA Adducts in Human Breast Cancer Cells

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Abstract

Estrogen-DNA adducts are potential biomarkers for assessing cancer risk and progression in estrogen-dependent cancer. 4-Hydroxyequilenin (4-OHEN), the major catechol metabolite of equine estrogens present in hormone replacement therapy formulations, autoxidizes to a reactive *o*-quinone that subsequently causes DNA damage. Formation of stable stereoisomeric cyclic 4-OHEN-DNA adducts has been reported *in vitro* and *in vivo*, but their removal by DNA repair processes in cells has not been determined. Such studies have been hampered by low yields of cyclic adducts and poor reproducibility when treating cells in culture with 4-OHEN. These problems are attributed in part to the instability of 4-OHEN in aerobic, aqueous media. We show herein that low yields and reproducibility can be overcome by 4-OHEN diacetate as a novel, cell-permeable 4-OHEN precursor, in combination with a sensitive LC-MS/MS method developed for detecting adducts in human breast cancer cells. This method involves isolation of cellular DNA, DNA digestion to deoxynucleosides, followed by the addition of an isotope-labeled internal standard (4-OHEN-$^{15}N_5$-dG adduct) prior to analysis by LC-MS/MS. A concentration-dependent increase in adduct levels was observed in MCF-7 cells after exposure to 4-OHEN diacetate. The chemical stabilities of the adducts were also investigated to confirm that adducts were stable under assay conditions. In conclusion, this newly developed LC-MS/MS method allows detection and relative quantification of 4-OHEN-DNA adducts in human breast cancer cells which could be adapted for adduct detection in human samples.

Keywords

LC-MS/MS; *o*-quinone; catechol estrogens; DNA adducts; hormone replacement therapy

Introduction

Most chemical carcinogens exhibit their genotoxic activities through metabolic activation to form reactive intermediates that cause a variety of DNA damage, including strand breaks, oxidized bases, apurinic sites, and covalent adducts. DNA adducts are considered to be

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important for mutagenicity and carcinogenicity, which can generate mutations in genomic DNA if not detected and repaired efficiently (1). Long-term exposure to estrogens is associated with an increased risk of developing estrogen-dependent cancers (2–4). The most widely prescribed hormone or estrogen replacement therapy (HRT/ERT) in the USA contains conjugated human estrogens and B-ring unsaturated conjugated equine estrogens (5). The equine estrogens have been shown to be potent estrogens, which increase breast cancer risk in postmenopausal women, with similar estrogen receptor (ER) binding affinity and cell proliferation potency as compared to 17β-estradiol (E_2) (6). In addition to these hormonal effects, the genotoxic effects of equine estrogen metabolites are considered to be important in cancer initiation (7–9). Equine estrogens (equilin, equilenin) are hydroxylated to form 4-hydroxyequilin (4-OHEQ) or 4-hydroxyequilenin (4-OHEN). 4-OHEQ is autoxidized to an *o*-quinone which isomerizes to 4-OHEN (10–12). Studies have shown that 4-OHEN can be readily autoxidized to genotoxic 4-OHEN-*o*-quinone, which induces a variety of different types of DNA damage (8), causing DNA mutations (13), apoptosis (14), and cellular transformation (15). In addition, 4-OHEN is considered to be more reactive, cytotoxic, and carcinogenic than the endogenous catechol estrogens (8,14,15).

It has been reported that 4-OHEN readily reacts with DNA in aqueous solutions and gives rise to unusual stable adducts via its *o*-quinone and semiquinone forms (Fig. 1) (16–18). Each of these adducts contain three chiral centers, and in theory eight stereoisomers should be observed. However, only four different stereoisomers were reported from 4-OHEN when reacted with 2'-deoxynucleosides (dNs) in aqueous solution (16). Characterization of these nucleoside adducts was established by LC-MS/MS and 2D-NMR experiments, and their stereochemical characteristics were further investigated using theoretical models (19–23). The miscoding properties of these adducts were determined by using site-specifically modified oligodeoxynucleotides, which contain a single diastereoisomeric form (24–26). For example, human DNA Polymerases η and κ can efficiently bypass 4-OHEN-dC (major adduct) by incorporating dAMP or dCMP opposite the lesion, which eventually leads to C → T transitions and C → G transversions (26). Mutagenic events were observed in a *supF* shuttle vector plasmid propagated in human cells (13). In an animal study, several stereoisomers of stable 4-OHEN-dA and -dG adducts were detected when 4-OHEN was directly injected into rat mammary tissue (27). 4-OHEN-derived DNA adducts were also detected in human breast tissues of patients, who have a known history of Premarin-based HRT (28). These observation suggested that formation of bulky adducts by 4-OHEN may cause mutagenesis *in vivo*.

The development of a sensitive method for detection of 4-OHEN-DNA adducts is a crucial task to quantify these adducts in human samples. High-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-MS/MS) has been widely employed to detect adducts in biological samples with high selectivity, precision, and sensitivity (29). In the present study, a sensitive LC-MS/MS method was developed for analyzing 4-OHEN-DNA adducts in breast cancer cells which could be adapted for adduct detection in human samples.

Experimental Procedures

Caution

The catechol estrogens are potentially hazardous materials. Protective clothing should be worn, and appropriate safety procedures should be followed.

Materials

All other chemicals were obtained from Sigma or Fisher Scientific unless stated otherwise. Alkaline phosphatase (ALP) and venom phosphodiesterase I (VPH) were purchased from Worthington (Lakewood, NJ). 4-OHEN was synthesized by treating equilin with Fremy's salt

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as described previously (30) with minor modifications (11). $^{15}N_5$ isotope labeled 4-OHEN-dG adduct as an internal standard was prepared by reaction of 4-OHEN with $^{15}N_5$-isotope labeled dG at 37 °C and separated from reaction mixture by HPLC. 4-OHEN diacetate was synthesized as follows. Acetic anhydride (134 μL, 1.4 mmol), pyridine (115 μL, 1.4 mmol) and 4-dimethylaminopyridine (0.035 mmol) were dissolved in anhydrous dichloromethane (DCM, 200 μL), and 4-OHEN (0.035 mmol) in DCM (200 μL) was added dropwise. The reaction mixture was stirred overnight at room temperature. Solvents were removed under reduced pressure and the residue was purified by column chromatography (hexanes/ethyl acetate, 3:2), and the product was obtained as slightly yellow power (13 mg). ^{1}H and ^{13}C - NMR spectra were obtained with Bruker Ultrashield 400 spectrometer. ^{1}H-NMR (acetone-d_6, 400 MHz): δ 7.95 (d, 1H, *J*=9.2 Hz), 7.83 (d, 1H, *J*=8.8 Hz), 7.43 (d, 1H, *J*=8.4 Hz), 7.42 (d, 1H, *J*=9.2 Hz), 3.32–3.36 (m, 2H), 3.21–3.25 (m, 1H), 2.81–2.84 (m, 1H), 2.56–2.67 (m, 2H), 2.46 (s, 3H), 2.36–2.40 (m, 1H), 2.32 (s, 3H), 2.11–2.17 (m, 1H), 1.67–1.89 (m, 1H), 0.76 (s, 3H); ^{13}C-NMR (Acetone-d_6, 100 MHz): δ 218.36, 168.88, 168.81, 139.87, 138.51, 136.30, 132.43, 131.67, 127.79, 125.99, 122.89, 122.72, 120.50, 47.77, 47.32, 36.77, 29.85, 24.70, 22.33, 20.60, 20.33, 13.32. ESI-MS: m/z 389.3 $[M + Na]^+$.

Stability Studies of 4-OHEN-DNA Adducts

Potential differences in the stabilities of the cyclic 4-OHEN-dA, -dC, and -dG adducts were investigated to determine if the decomposition of the adducts could play a role in the above-mentioned lack of reproducibility of DNA adduct levels in cells treated with 4-OHEN. Adduct solutions were prepared as previously described (16). Briefly, 4-OHEN (1 mM) was incubated with dNs (5 mM) in 10 mM potassium phosphate buffer (PPB, pH 7.4) at 37 °C for 1 h, 1 mL total volume, in amber glass vials in the dark. The adducts were then separated from 4-OHEN and dNs by HPLC and dried under vacuum. The following stability studies were performed and analyzed by LC-MS/MS: *i) Photosensitivity*. Adduct solutions in PPB were exposed to fluorescent light, and then collected in amber vials at different time intervals for 24 h. *ii) pH stability*. The stability of these adducts were tested under acidic (pH 5.3), neutral (pH 7.4), and basic (pH 9.8) conditions. The samples were then collected at different time intervals for 24 h. *iii) Thermal stability*. Adduct solutions were incubated in amber vials at 4 °C, 37 °C, and 55 °C in the dark and collected into new vials at different time intervals for 24 h. *iv) Redox cycling conditions*. Adduct solutions with ascorbic acid (1 mM) were treated with/without catalytic amount of $FeCl_3$ (10 μM). Samples were then collected at different time intervals for 24 h. The results were expressed as percentage of adducts remaining (% = integrated peak area/initial peak area × 100%).

Hydrolysis of 4-OHEN Diacetate with Pig Liver Esterase

The hydrolysis of 4-OHEN diacetate was determined in the presence of pig liver esterase (PLE). A solution containing 4-OHEN diacetate (30 μM), 4-OHEN diacetate with PLE (0.1 mg/mL), or 4-OHEN diacetate with PLE, GSH (1 mM) in 100 mM PPB (pH 7.4) in a total volume of 1 mL was incubated for 20 min at 37 °C. The reactions were terminated by chilling in an ice bath followed by the addition of perchloric acid (50 μL/mL). The reaction mixtures were centrifuged at 18,000 g for 10 min; aliquots (90 μL) of supernatant were analyzed using an Agilent 6310 ion trap mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a model 1100 HPLC system and electrospray ionization source. 4-OHEN, 4-OHEN-*o*-quinone and 4-OHEN di-GSH conjugate were identified by LC-electrospray ionization (ESI) MS in positive ion mode as $[M + H]^+$ m/z 283.1, 281.1, and 893.4 respectively. HPLC was performed using an Agilent Zorbax Bonus reverse phase C_{18} column (3.0 × 150 mm, 3.5 μm) with UV absorbance detection at 280 nm. The composition of HPLC mobile phase was the mixture of water with 10% methanol, 0.1% formic acid (solution A) and acetonitrile with 0.1% formic acid (solution B). The mobile phase was initially composed of solvent A/solvent B (90:10), held for 3 min, and then a linear gradient of solution B from 10 – 30% over 20 min,

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10 min gradient of solution B from 30 – 70%, and at finally 70 – 90% of solution B over 1 min at a flow rate of 0.5 mL/min. The hydrolysis of 4-OHEN diacetate (150 μM) in 100 mM PPB (pH 7.4, containing 5% MeOH) catalyzed by PLE (0.1 mg/mL) was also monitored on a Hewlett-Packard 8452A photodiode array UV-Vis spectrophotometer. Scans were performed every 30 s.

Cell Culture Conditions

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless stated otherwise. The MCF-7 WS8 human breast cancer cells (a kind of gift form Dr. V.C. Jordan, Fox Chase Cancer Center, Philadelphia, PA) were maintained in RPMI 1640 containing 10% fetal bovine serum (Atlanta Laboratory, Atlanta, GA), 2 mM Lglutamine, 0.1 mM nonessential amino acids, and 6 μg/ mL bovine insulin (Sigma). Estrogen-free media were prepared from phenol-red-free RPMI1640 media supplied with 10% charcoal-dextran-treated fetal bovine whereas other components remained the same. The cells (1.5×10^6cells/plate) were placed in 10-cm dishes for 24 h and changed to phenol red-free media 72 h prior to treatment. Compounds were freshly dissolved in DMSO with 0.1% final concentration in media. After 20 min incubation, the cells were washed with cold phosphate-buffered saline (pH 7.4), collected by scraping, and then harvested by centrifugation at 450 g for 5 min. The cell pellets were collected for DNA isolation.

Extraction and Hydrolysis of Cellular DNA

DNA extraction from cells was performed as previously published (27,31) with minor modification. The cell pellets were homogenized in lysis buffer (320 mM sucrose, 10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 10 mM Triton X-100, and 50 mM mannitol). After centrifugation, the nuclei pellets were treated with RNase T1 and RNase A in solution buffer (1% SDS, 1 mM EDTA, 10 mM Tris, pH 7.4, and 0.45 M NaCl) and further incubated with proteinase K at 37 °C. NaCl and Tris were added to achieve final concentrations of 0.62 M and 20 mM, respectively. An equal volume of n-butanol was added, the samples were thoroughly mixed and centrifuged, and the bottom aqueous layer was isolated. After isopropanol precipitation, the DNA was washed twice with 70% ethanol. The DNA was dissolved in 25 mM ammonium acetate buffer (pH 5.3) containing 0.1 mM $ZnCl_2$. The DNA concentrations were calculated by measuring the absorbance at 260 nm and assuming that one absorbance unit equals 50 μg/ mL of DNA. DNA solutions were hydrolyzed by incubation with nuclease P1 (NP1, from *Penicillium citrinum*, 20 U) for 4 h at 55 °C, and then further incubated with ALP (from calf intestine, 30 U) and VPH (1 U) for 4 h at 37 °C in a reaction buffer containing 0.5 mM $MgCl_2$, with 0.1 M diethanolamine to achieve final pH ~9.8. After digestion, an aliquot (790 fmol) of $^{15}N_5$ isotope labeled 4-OHEN-dG adduct was added into the digests as an internal standard. The mixtures were then centrifuged for 25 min at 13,000 g using an YM-10 ultrafiltration centrifuge tube (Millipore), and the elutes were concentrated by centrifugation under vacuum in the dark and then re-dissolved in methanol. An aliquot (5 μL) was immediately analyzed by LC-MS/MS.

Instrumentation

All LC-MS/MS measurements were carried out using positive mode electrospray ionization method on API 3000 (Applied Biosystem, Foster City, CA, USA) triple quadrupole mass spectrometer attached to Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA). Ionization voltage was 3.5 kV, focusing potential (230 V), declustering potential (51 V), source temperature 400 °C, nubulizer gas (10 instrument units) curtain gas (7 instrument units), collision gas (5 instrument units), and specified collision energy (4-OHEN-dA: 35 V, -dC:25 V, -dG: 23 V). Multiple Reaction Monitoring (MRM) channels of 548 → 432, 524 → 408, 564 → 448 and 569 → 453 were set to detect 4-OHEN-dA, -dC, -dG and -$^{15}N_5$-labled-dG

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adducts, respectively. Fine tuning of each parameter using standards for each isomer was found to be critical for optimum results. HPLC was performed on an Xbridge Phenyl (3.5 μm; 2.1 mm × 150 mm) column (Waters Corporation, Milford, MA) using methanol (B)-water (10 mM ammonium acetate, pH 4.8) (A) as a mobile phase, and flow rate was 0.2 mL/min with a 24 min linear gradient from 25% B to 50% then to 95% in 28 min and then holding at final conditions for an additional 5 min. The area of these peaks was measured to identify the subsequent levels of DNA adducts and normalized to internal standard and to 1 μg of DNA.

Limit of Detection

Limit of detection for LC-MS/MS was calculated using a calibration curve for the range of adduct concentrations from 10 pM to 100 nM with $^{15}N_5$-labeled 4-OHEN-dG as an internal standard (Fig. S1). The volume of each injection to the column was 5 μL. After the peak areas were normalized to the internal standard, the detection limits were determined by seven replicate injections of calibration standards and calculated by the following equation: MDL = $t_{99\%} \times S_{(n=7),}$ where t is student's t at 99% confidence intervals ($t_{99\%, n=7} = 3.143$) and S is the standard deviation of seven samples. The detection limits for 4-OHEN-dA, -dC, and -dG adducts were approximately 274 fg, 264 fg, 28.2 pg, respectively. A possible reason for the difference in the detection limits of 4-OHEN-dG adducts could be that oxidation or fragmentation of 4-OHEN-dG adduct in the electrospray source might occur since dG has a lower oxidation potential and higher photosensitivity (32). This detection limit is similar to that of 8-oxo-dG using this instrument.

Results

Stability of 4-OHEN-DNA Adducts in Aqueous Solutions

Previous studies have shown that stable 4-OHEN-dC and 4-OHEN-dG adducts were formed in calf thymus DNA exposed to 4-OHEN using similar LC-MS/MS method (16). In contrast, Shibutani's group have found that only 4-OHEN-dA and 4-OHEN-dC, but no 4-OHEN-dG adducts were observed in calf thymus DNA treated with 4-OHEN using a ^{32}P-postlabeling method (33). This result was consistent with the findings from Geacintov's group (22). Although different methods were used in these experiments, the degradation of stable 4-OHEN adducts might occur during DNA preparation and analysis processes, and consequently only some DNA adducts may be detectable. Therefore it was important to study and validate the analytical method taking into account possible optical, thermal, and redox cycling stabilities of these adducts.

Optical stability experiments were conducted by exposure of adducts to fluorescent light at room temperature; control experiments were performed in the dark. Data indicated that all 4-OHEN-dA, -dC, and -dG adducts were sensitive to light (Fig. 2). The degradation rate of 4-OHEN-dA and -dC adducts was essentially the same, while 4-OHEN-dG adducts were degraded 2-fold faster; after 24 h exposure to light, the 4-OHEN-dG was almost undetectable. In control experiments, no degradation was observed within 24 h in the dark (data not shown). These data suggested that the lack of detection of 4-OHEN-dG adducts in calf thymus DNA by ^{32}P-postlabeling methods might be due to its higher photosensitivity as well as their low yield. Exposure to light should be avoided during the entire experimental process.

The stability of these adducts was also examined as functions of temperature, pH, and redox conditions (Table 1). These experiments were performed in dark and the pH values and temperatures were chosen to match the conditions used in the experimental processes. No significant degradation was detected by comparing the adduct peak area with initial control samples, indicating these adducts were not sensitive to these temperatures and pH values. Furthermore, these adducts were relatively tolerant to redox cycling conditions in the dark. In

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the absence of metal ions, all of the adducts were stable in aqueous solution with ascorbic acid (1 mM). After addition of the metal ion Fe^{3+}, 4-OHEN-dA and -dC adducts showed a very slow degradation rate, while 4-OHEN-dG adducts were oxidized much faster; ~ 60% of the adducts were degraded after 48 h (Table 1).

Optimization of DNA Hydrolysis Procedure

Although we have reported a DNA hydrolysis method for detecting 4-OHEN-DNA adducts *in vivo* (27), the hydrolysis efficiency was limited by the short incubation times. Since complete hydrolysis is critical for quantitative analysis of adducts in cells, the DNA hydrolysis procedure was optimized in the present study. Based on a series of control experiment, the following concentrations of enzymes and experimental reaction conditions were identified as most efficient for generating cyclic 4-OHEN-DNA adducts; 10 U of NP1, 15 U of ALP, and 0.5 U of VPH were used for 100 μg of DNA. In Method I (original), as previously described (27), both NP1 and ALP were added to DNA solutions in ammonium acetate (pH 5.3) for 45 min at 37 °C. In Method II (optimized), since the alkaline pH is optimum for ALP, NP1 and ALP were added separately, and ALP coupled with VPH was added after the NP1 incubation (Fig. 3). Control experiments were conducted using different incubations times and the digests were monitored by HPLC-MS to determine hydrolysis efficiency. For example, increasing the incubation time of NP1 from 45 min to 4 h, dramatically increased the quantity of adducts relative to the quantities obtained by Method I. Further increases in incubation time did not significantly increase the yields of adducts (data not shown). Also, the 4 h time period was chosen for ALP and VPH enzyme incubations after pH adjustment to 9.8 using diethanolamine (0.1 M). The inclusion of VPH was necessary for full digestion. As shown in Fig. 3B, the adduct yields were greatly enhanced (~ 20-fold) using the optimized method instead of Method I.

Hydrolysis of 4-OHEN Diacetate by Pig Liver Esterase

Since 4-OHEN can be autoxidized to a reactive *o*-quinone in aqueous solution, it might be readily trapped by nucleophiles before entering into cells, giving lower DNA adduct yields. To minimize this effect, 4-OHEN diacetate was synthesized to block autoxidation of 4-OHEN until it enters the cytosol where it is hydrolyzed by esterases generating 4-OHEN (Fig 1). The hydrolysis of 4-OHEN diacetate was modeled using PLE *in vitro*. As shown in Fig. 4, 4-OHEN diacetate is stable in 100 mM PPB (pH 7.4). After adding PLE (0.1 mg/mL), 4-OHEN diacetate was rapidly hydrolyzed to 4-OHEN, which autoxidized to 4-OHEN-*o*-quinone. Once the *o*-quinone was formed, it was readily trapped by excess amount of GSH, and the major product detected was 4-OHEN di-GSH conjugate (Fig. 4A). The rate of *o*-quinone formation was monitored by measuring the increase in absorbance 390 nm at room temperature, and the maximum absorbance was reached within 400 s, demonstrating that rapid hydrolysis of 4-OHEN diacetate (Fig. 4B).

Detection of Adducts in MCF-7 cells

The amount of DNA adducts produced by 4-OHEN and 4-OHEN diacetate was compared in MCF-7 cells. Representative LC-MS/MS chromatograms of stable 4-OHEN-dA, -dC, and -dG adducts formed by incubation of 4-OHEN diacetate in MCF-7 cells are shown in Fig. 5. Each of 4-OHEN-dA, -dC and -dG adducts formed four stereoisomers in MCF-7 cells. The rank order of adduct level was 4-OHEN-dC > 4-OHEN-dA >> 4-OHEN-dG adducts. Treatment with 4-OHEN diacetate gives a ~4-fold higher yield of adducts in cells than 4-OHEN-treated cells under the same conditions (Fig. 6). Furthermore, 4-OHEN diacetate seems to yield more reproducible levels of adducts. These data suggested that use of 4-OHEN diacetate improves the yield of 4-OHEN-DNA adducts in cells and also enhances the reproducibility of the data.

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Since 4-OHEN diacetate was found to be superior to 4-OHEN in terms of adduct formation, further experiments were conducted with 4-OHEN diacetate. When MCF-7 cells were incubated with 1–10 μM 4-OHEN diacetate, a dose-dependent increase in adduct formation was observed (Fig. 7). We chose the four major stereoisomeric 4-OHEN-dA3, -dA4, -dC3, and -dC4 adducts for the relative quantification of adduct levels. The total level of 4-OHEN-dC adduct was higher than of 4-OHEN-dA adducts. These data are consistent with previous results using 4-OHEN-treated calf thymus DNA where the major products were dC adducts (22,33). The levels of 4-OHEN-dC3 isomers were slightly higher than of the 4-OHEN-dC4 adducts, and the levels of 4-OHEN-dA3 and 4-OHEN-dA4 were essentially the same (Fig. 7).

Discussion

The formation of stable covalent DNA adducts is an important step in the initiation of carcinogenesis. Many epidemiology studies have already employed DNA adducts as biomarkers of carcinogen exposure (34). 4-OHEN can be readily autoxidized to 4-OHEN-*o*-quinone, which reacts with nucleophilic DNA bases to form unusual cyclic 4-OHEN-dA, -dC, and -dG stable adducts (8). Methods for the detection of 4-OHEN adducts by ^{32}P-postlabeling and LC-MS/MS have been reported (16,22,33). The ^{32}P-postlabeling assay has been widely used for the detection of stable DNA adducts. It is considered to be a sensitive method for detecting adducts with a limit of detection as low as 1 adduct in 10^{10} nucleotides (35). This assay uses γ-^{32}P-labeled ATP to incorporate a ^{32}P-containing phosphate group into nucleotides derived from DNA hydrolysis. It has been successfully applied to detect 4-OHEN adducts in oligomers and calf thymus DNA (13,22,33). However, the disadvantages of this technology are it does not provide structural characterization of adducts and, without internal standards for identifying the products, has poor selectivity (34). In addition, the requirement for radioactive γ-^{32}P-labeled ATP raises safety concerns. In contrast, the advantages of LC-MS/MS for the determination of DNA adducts are it provides improved information about the structural identity of these adducts, thus confirming that the correct adduct is being studied. Considering the complexicity of the biological matrix, selective identification and quantification is vital to explain the biological effects of these adducts. Using stable isotope labeled adducts as internal standards achieves accurate quantification using the LC-MS/MS techniques (29).

Questions about the stability of 4-OHEN-DNA adducts were examined under different conditions. Our results indicate that the light source is critical for adduct analysis; all 4-OHEN-DNA adducts are sensitive to light exposure. The 4-OHEN-dG adduct ($t_{1/2}$ ~ 3.5 h) decomposed at twice the rate of the 4-OHEN-dA and -dC adducts, which might explain the failure to observe 4-OHEN-dG adducts in experiments with calf thymus DNA treated with 4-OHEN by ^{32}P-postlabeling methods (22,33). Avoiding exposure to light is crucial for detection of 4-OHEN-DNA adducts in cells. By using this improved LC-MS/MS method, we detected all four stereoisomeric forms of 4-OHEN-dA, -dC, and -dG adducts in calf thymus DNA (data not shown).

To achieve a sufficient sensitivity for LC-MS/MS detection, isolation of high amounts of cellular DNA from treated cells, followed by the optimized DNA hydrolysis method for DNA adduct enrichment were performed prior to analysis. In contrast to the ^{32}P-postlabeling method that uses small amounts of DNA (~ 10 μg) for analysis, the current LC-MS/MS method usually requires more than 200 μg of cellular DNA to achieve sufficient sensitivity, thereby isolation of such significant amounts of DNA from treated cells (10 million cells per dish, 3 dishes) is important for successfully detecting 4-OHEN-DNA adducts. In addition, the hydrolysis procedure was modified to generate fully hydrolyzed DNA samples for increased detection sensitivity. Although 4-OHEN-DNA adducts were detectable in rat mammary tissue (27), the previous hydrolysis method only gave ~ 10% hydrolysis efficiency with a large amount of

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incompletely hydrolyzed DNA fragments (data not shown). To achieve maximum hydrolysis, control experiments were conducted using different concentrations of enzymes and incubation times, exploring the optimal pH and temperature for each enzyme used in this procedure. The optimized conditions are described in detail in the Experimental Procedures.

The use of 4-OHEN diacetate improved the yield of adducts formed in MCF-7 cells and minimized the variations in the adduct levels compared to incubations with 4-OHEN. The latter is rapidly autoxidized to reactive *o*-quinone electrophiles which are readily trapped by nucleophiles prior to entering the nucleus. 4-OHEN diacetate was synthesized to block the autoxidation allowing release of 4-OHEN after hydrolysis in the cytoplasm. As expected, the use of 4-OHEN diacetate achieved ~ 3 to 4-fold higher yields of adducts as compared to 4-OHEN-treated cells, and the formation of DNA adducts was found to depend on the 4-OHEN diacetate concentration. All four diastereoisomeric forms of 4-OHEN-dA, -dC, and -dG adducts were detected in MCF-7 cells. Therefore, acylation of the reactive hydroxyl groups of 4-OHEN provided an improved approach, enhancing DNA adduct formation, and may be of application to studies *in vivo*.

In conclusion, we have developed a sensitive LC-MS/MS method for detecting 4-OHEN-DNA adducts in MCF-7 human breast cancer cells. A series of control experiments were conducted to increase the sensitivity of detection. To the best of our knowledge, this is the first report of 4-OHEN-DNA adducts in human cells. The biological significance of these adducts as well as the repair efficiency of the different adducts will be the subject of future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ALP, alkaline phosphatase
DCM, dichloromethane
2D-NMR, two dimensional nuclear magnetic resonance
dA, deoxyadenosine
dC, deoxycytidine
dG, deoxyguanosine
dNs, 2’-deoxyribonucleosides
E_2, 17β-estradiol
EN, equilenin
ER, estrogen receptor
HRT, hormone replacement therapy
LC-MS/MS, liquid chromatography-tandem mass spectroscopy
NP1, nuclease P1
4-OHEN, 4-hydroxyequilenin
4-OHEQ, 4-hydroxyequilin
8-oxo-dG, 8-oxo-7,8-dihydro-2'-deoxyguanosine
PLE, pig liver esterase
PPB, potassium phosphate buffer
ROS, reactive oxygen species
VPH, venom phosphodiesterase I

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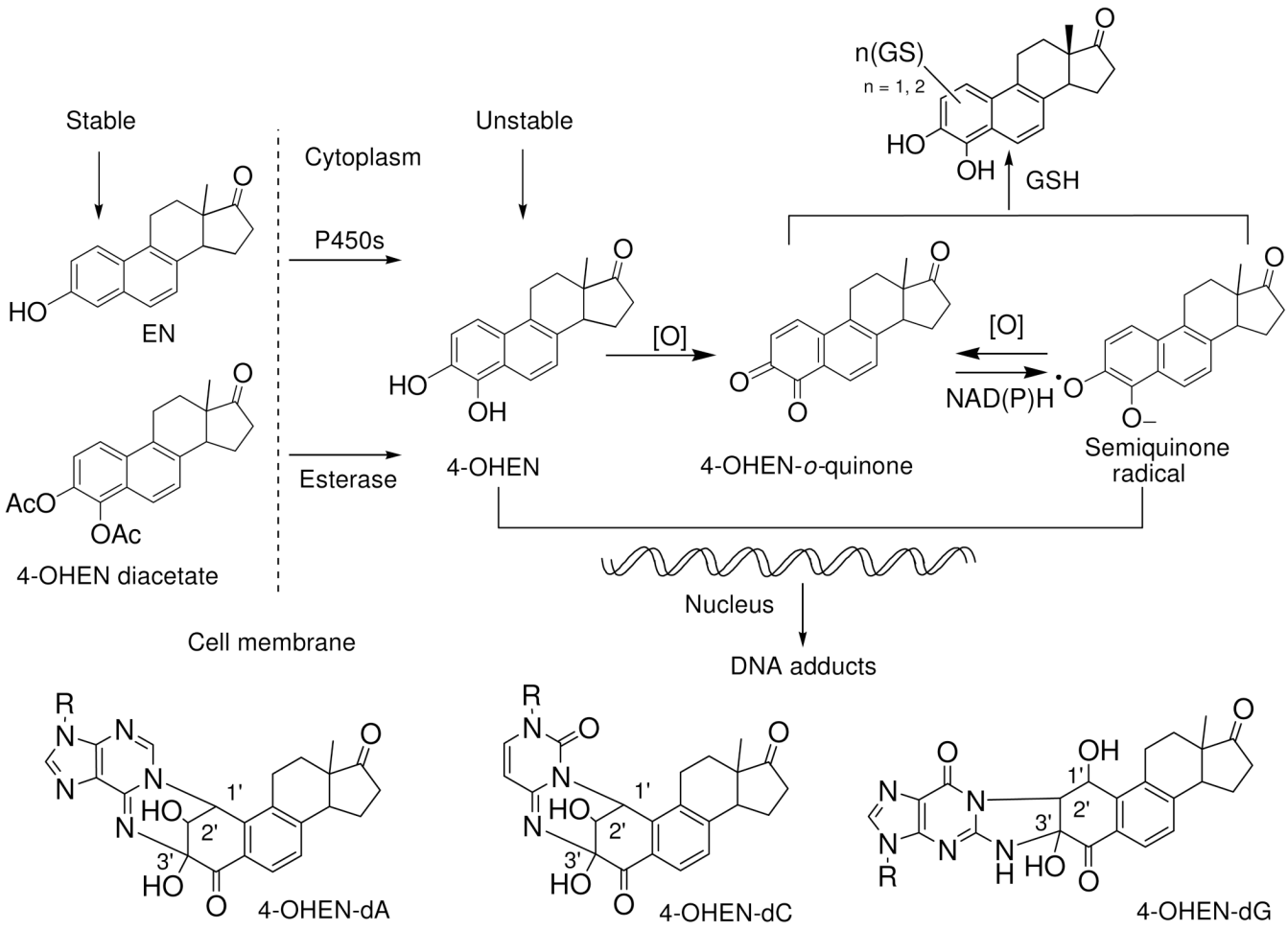

Fig. 1.

A proposed pathway for 4-OHEN diacetate entering into cytoplasm, generating 4-OHEN by esterases to form cyclic bulky DNA adducts. The three chiral centers (1', 2', and 3') in 4-OHEN-DNA adducts are labeled.

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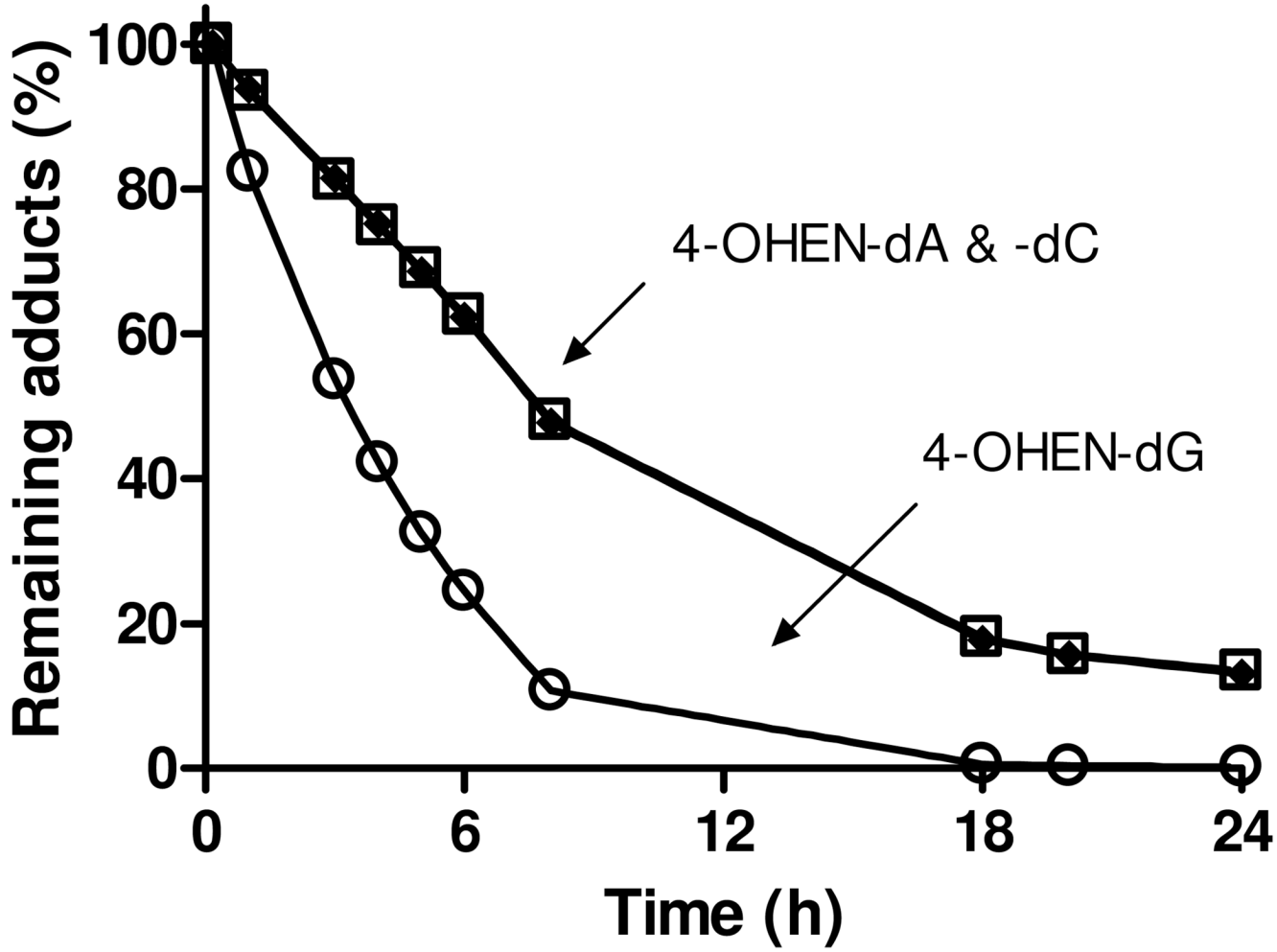

Fig. 2.

Time course on the effect of ambient light exposure on the stability of 4-OHEN-dA, -dC, and -dG adducts. 4-OHEN-DNA adducts in 10 mM phosphate buffer (pH 7.4) were exposed to light at room temperature; control experiment was performed in the dark. Aliquots were analyzed by LC-MS/MS at various time points. Opened squares, 4-OHEN-dA adduct; closed diamonds, 4-OHEN-dC adduct; opened circles, 4-OHEN-dG adduct. Data were expressed as the mean of percentage of adducts remaining (% = integrated peak area/initial peak area × 100%) (n = 3).

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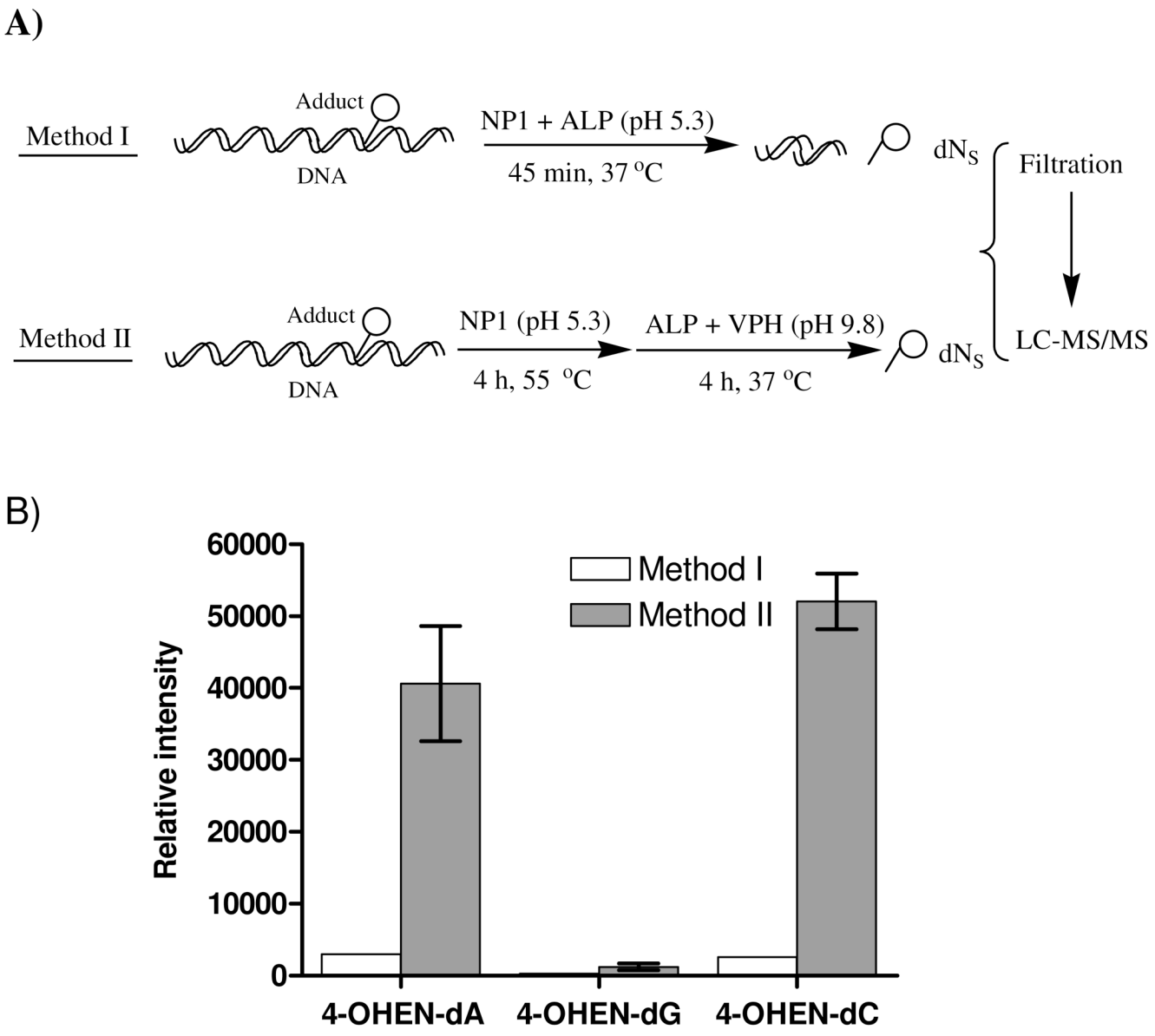

Fig. 3.

Optimization of hydrolysis conditions for detecting stable 4-OHEN-DNA adducts. A) Method I, incubation with NP1 and ALP together in 25 mM ammonium acetate (pH 5.3) for 45 min at 37 °C. Method II, the DNA solutions were incubated with NP1 for 4 h in ammonium acetate at 55 °C, and then ALP and VPH were added to mixture for 4 h at 37 °C after pH adjustment to 9.8 by 0.1 M diethanolamine. B) Equal amounts of calf thymus DNA were hydrolyzed using Method I and Method II. The hydrolysates were analyzed by LC-MS/MS and compared by total relative intensity of each adduct peak.

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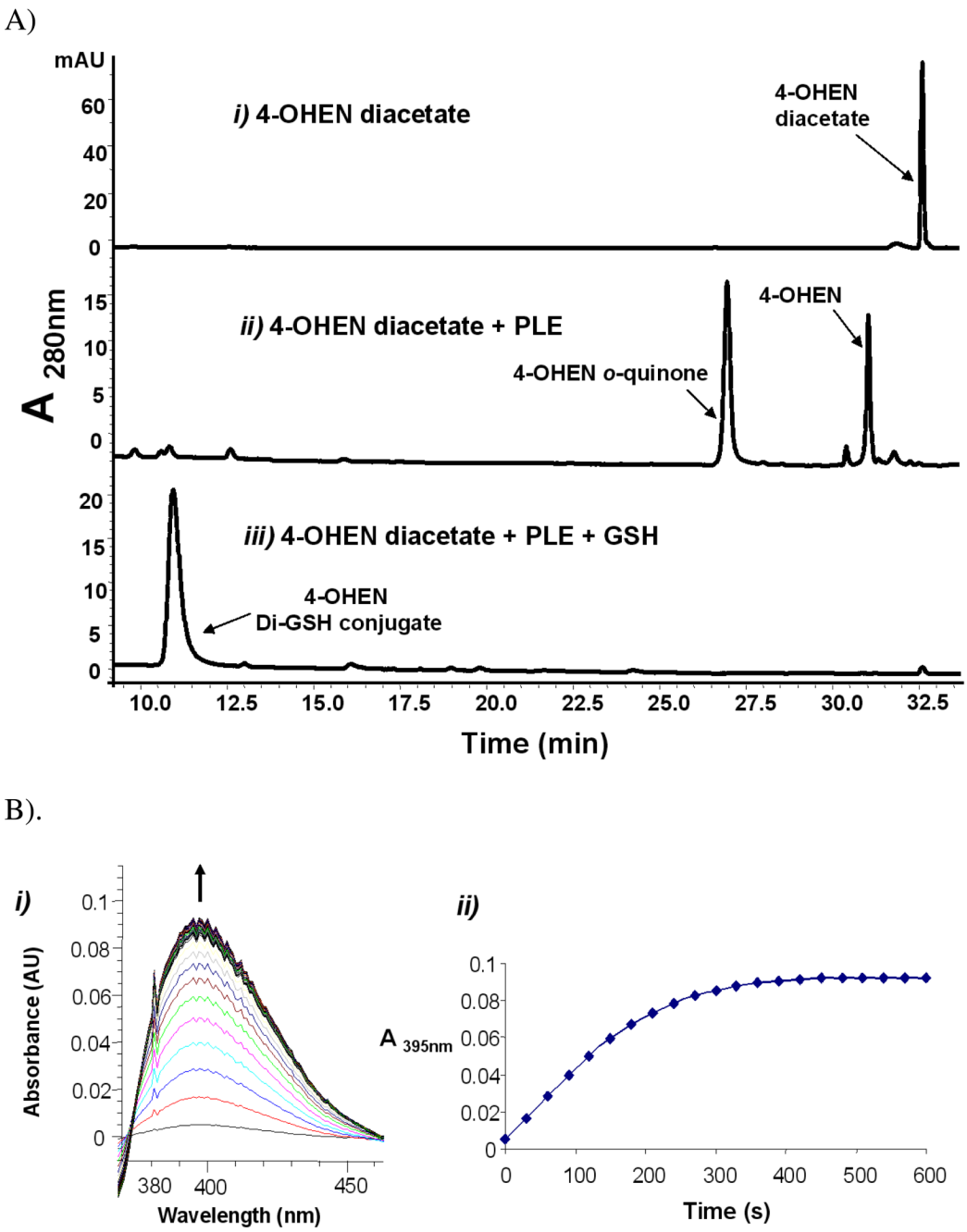

Fig. 4.

Hydrolysis of 4-OHEN diacetate with/without esterase. A) HPLC chromatogram of the hydrolysis of 4-OHEN diacetate (30 μM) with/without PLE (0.1 mg/mL). Incubations were conducted in 100 mM phosphate buffer (pH 7.4) at 37 °C for 20 min and quenched by the addition of perchloric acid (50 μL/mL). *i)* 4-OHEN diacetate; *ii)* 4-OHEN diacetate and PLE; *iii)* 4-OHEN diacetate and PLE in the presence of GSH (1 mM). B) The hydrolysis of 4-OHEN diacetate (150 μM) in 100 mM phosphate buffer (pH 7.4, 5% MeOH) catalyzed by PLE (0.1 mg/mL) was monitored by UV spectrometer. *i)* Characteristic absorbance of 4-OHEN-*o*-quinone around 395 nm; *ii)* Time-dependent increase in absorbance at 395 nm.

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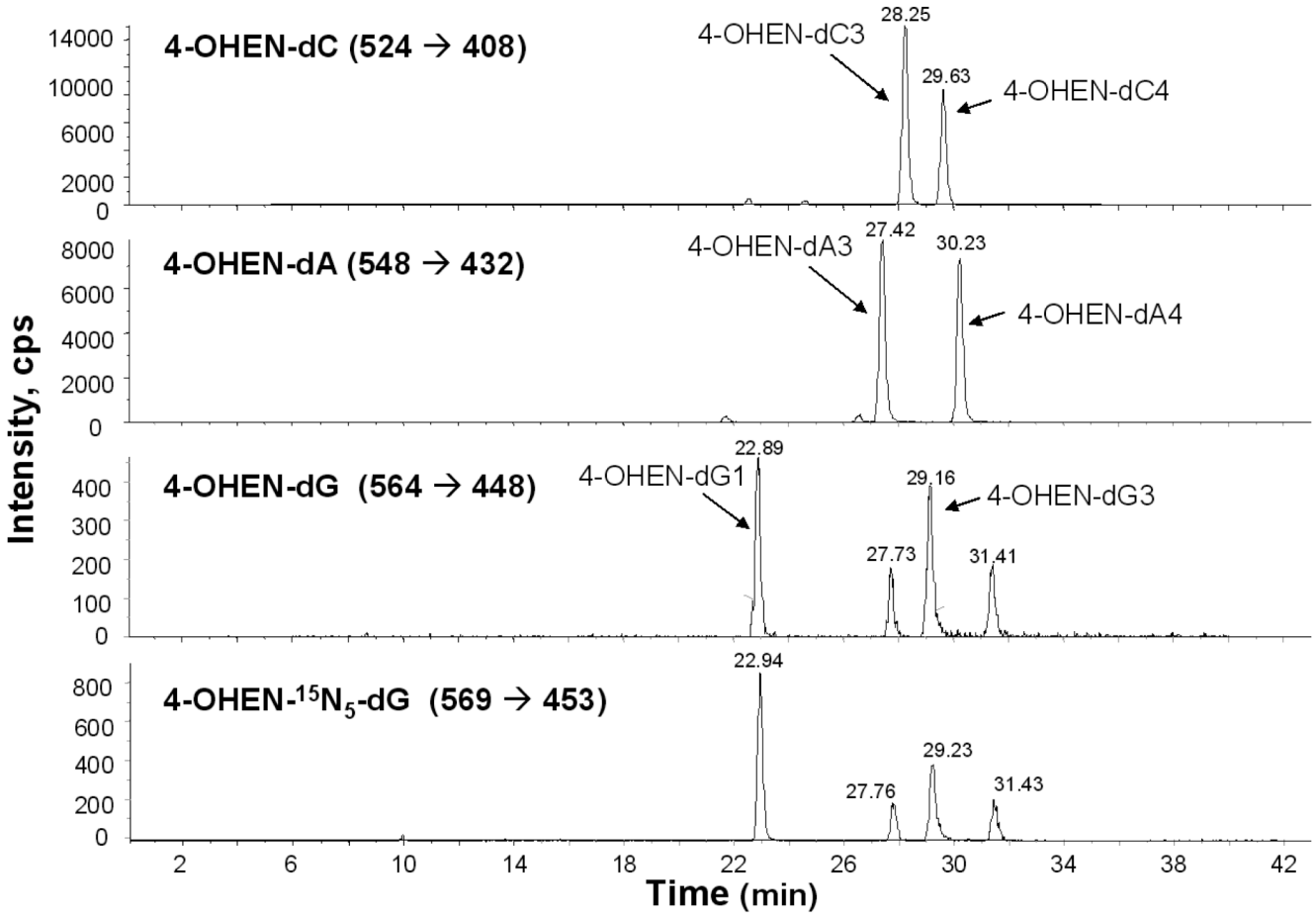

Fig. 5.

Representative LC-MS/MS chromatograms of stable 4-OHEN-DNA adducts. MCF-7 cells were incubated with 4-OHEN diacetate (10 μM) for 20 min. Cellular DNA was extracted and hydrolyzed for LC-MS/MS analysis. MRM channels of 524 → 408, 548 → 432, 564 → 448, and 569 → 453 were set to detect 4-OHEN-dC, -dA, -dG, and - $^{15}N_5$-labeled-dG adducts respectively.

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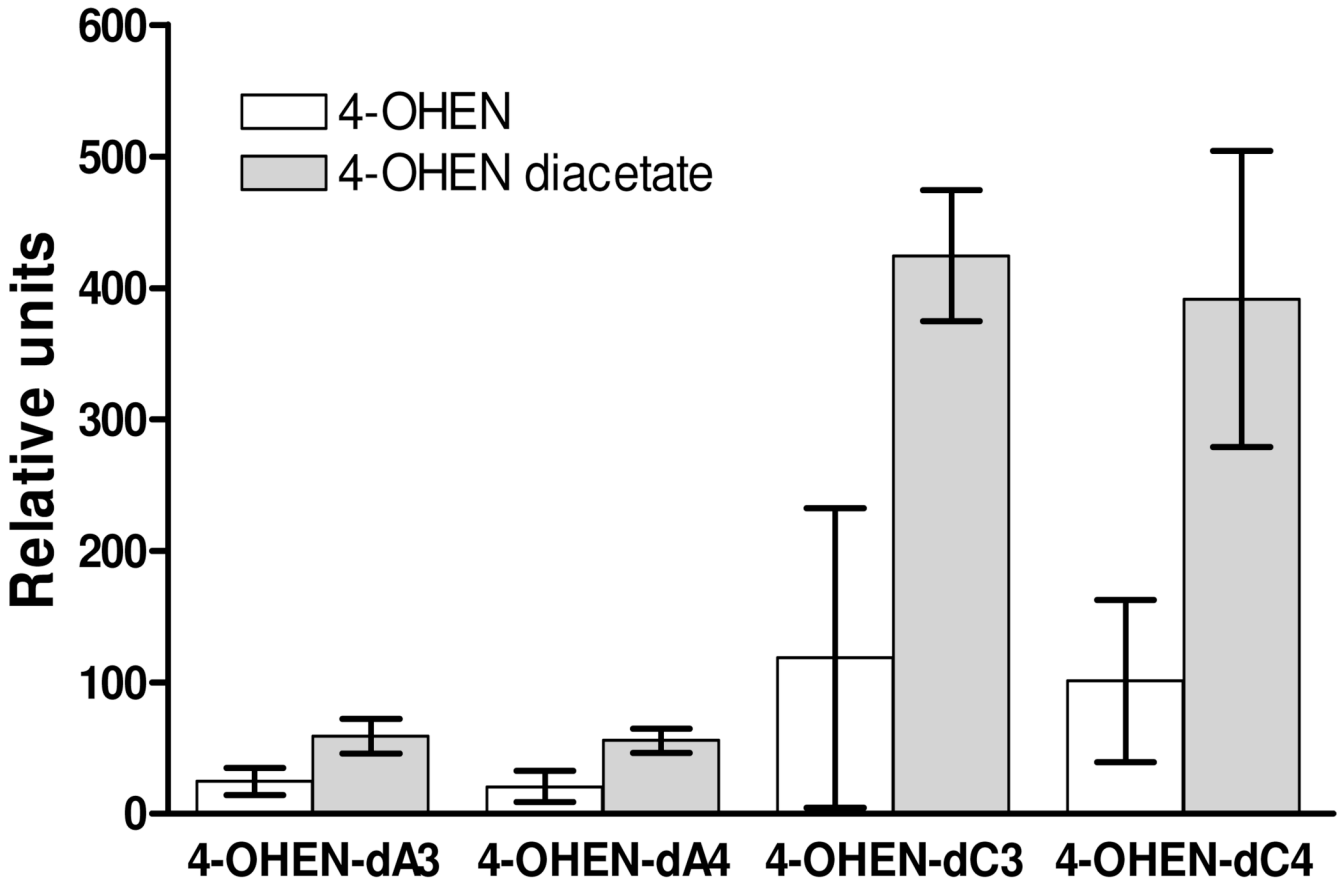

Fig. 6.

Comparison of 4-OHEN and 4-OHEN diacetate on formation of stable DNA adducts. MCF-7 cells were incubated with 4-OHEN or 4-OHEN diacetate (10 μM) for 20 min. Cellular DNA was extracted and hydrolyzed for LC-MS/MS analysis. The areas of these peaks were measured to identify the subsequent levels of DNA adducts and normalized to internal standard and to 1 μg of DNA. Data represent as the average ± S.D. (n = 3).

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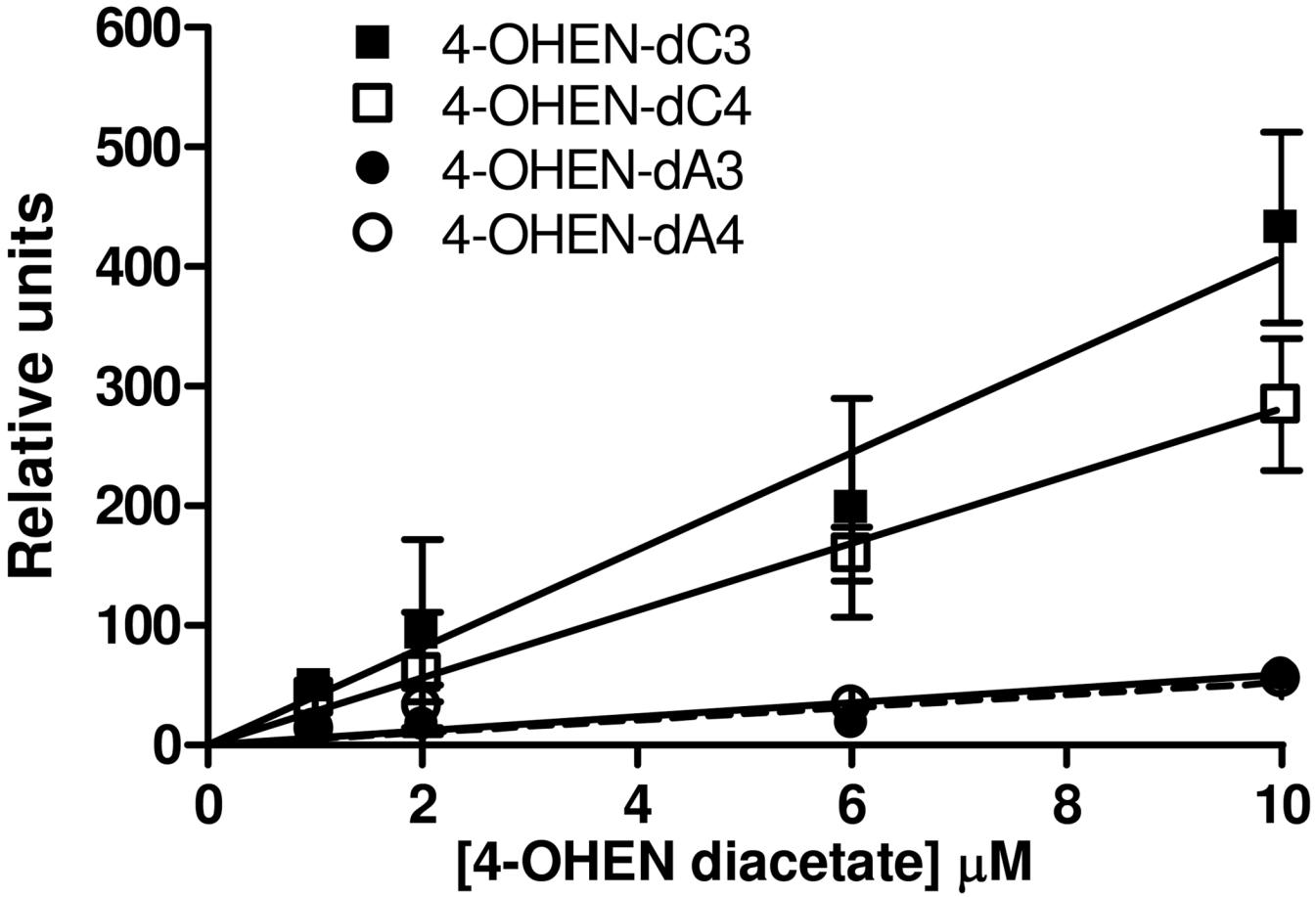

Fig. 7.

Concentration-dependent formation of DNA adducts. MCF-7 cells were incubated with various concentrations of 4-OHEN diacetate for 20 min. Cellular DNA was extracted and hydrolyzed for LC-MS/MS analysis. The area of these peaks was measured to identify the subsequent levels of DNA adducts and normalized to internal standard and to 1 μg of DNA. Closed squares, 4-OHEN-dC3 adduct; opened squares, 4-OHEN-dC4 adduct; Closed circles (dashed line), 4-OHEN-dA3 adduct; opened circles, 4-OHEN-dA4 adduct. Data represent as the average ± S.D. (n = 3).

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Table 1

Stability of 4-OHEN-dA, -dC, and -dG adducts to temperature, pH, and redox conditions.[a]

Reaction Conditions		Time Points (h)	Adducts (% remaining)		
			4-OHEN-dA	4-OHEN-dC	4-OHEN-dG
Temperature (°C)	4	4	100	99	99
		24	99	96	100
	37	4	99	99	102
		24	96	94	99
	55	4	100	99	94
		24	96	94	98
pH	5.3	4	99	105	102
		24	100	112	105
	7.4	4	105	95	110
		24	120	96	116
	9.8	4	97	98	107
		24	89	100	96
Redox Conditions	ascorbic acid	4	99	89	95
		24	100	97	98
		48	100	100	100
	ascorbic acid/Fe^{3+}	4	82	104	82
		24	90	98	76
		48	86	90	39

[a] Adduct solutions were prepared as follow. 4-OHEN (1 mM) was incubated with dNs (5 mM) in 10 mM phosphate buffer (pH 7.4) at 37 °C for 1 h, 1 mL total volume, in amber glass vials in the dark. The adducts were then separated from 4-OHEN and dNs mixture by HPLC and dried under vacuum. Stability studies were performed under different conditions and aliquots were collected in amber vials at different time intervals as indicated and then analyzed by LC-MS/MS. Experimental details are described under Experimental Procedures. Data were expressed as the mean of percentage of adducts remaining (n = 2).