Published in final edited form as:

Chem Res Toxicol. 2008 August ; 21(8): 1509–1513. doi:10.1021/tx8001737.

Estrogen Carcinogenesis:

Specific Identification of Estrogen-modified Nucleobase in Breast Tissue from Women

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Abstract

Prolonged exposure to estrogens correlates with increased risk for breast cancer. One explanation is that estrogen metabolites cause mutations by reacting with DNA, leading to depurination. We describe an extraction procedure and a liquid chromatographic tandem mass spectrometric (LC/MS/MS) assay to detect estrone-metabolite-modified adenine (Ade) in 100-200 mg samples of human breast tissue. To insure reliable analyses, we used a synthetic estrone-metabolite-modified, U-¹⁵N labeled Ade as an internal standard (IS). Appropriate high pressure liquid chromatography gives sharp (~ 5 s at half height) with identical retention times for the analyte and the IS. In breast tissue from women with and without cancer, we found a co-eluting material with similar MS/MS fragmentation as the IS, providing high specificity in the identification of the modified Ade; the recovery was approximately 50%. For women with and without breast cancer, the levels of the modified Ade are in the range of 20-70 fmol/g of breast tissue from five women and not detectable in tissue from another women. The sample size and detection limits are not yet sufficient to permit distinctions between cancer and noncancer patients.

Introduction

The correlation between risk of breast cancer and cumulative exposure to estrogens is well known. Several mechanisms explain estrogen's role. The better-known hypothesis is that the estrogen receptor (ER) mediates cell proliferation, increasing errors in DNA replication (1-3). Another explanation is that estrogen metabolites react covalently with DNA bases by either redox cycling or forming an abasic site. Subsequent error-prone repair of the modified DNA generates oncogenic mutations that initiate cancer(4-8). The two mechanisms may also act in concert.

According to the second mechanism, P450 metabolism of estrone (E₁) and estradiol (E₂) gives catechol estrogens (CEs), 2-hydroxyestrogen (2-OH-E) and 4-hydroxyestrogen (4-OH-E). Further oxidation leads to 2,3-catechol-estrogen quinone (E-2,3-Q) and 3,4-catechol-estrogen quinone (E-3,4-Q), respectively, which react directly with DNA via a Michael addition or indirectly via generation of reactive oxygen species (ROS). Methylation of CEs by catechol-*O*-methyltransferase, conjugation of the catechol estrogen quinones (CEQs) with glutathione, and enzymatic reduction to reform CEs are processes that prevent accumulation of the reactive metabolites. If these latter protective processes are insufficient, the CEQs accumulate and damage DNA by either oxidation or depurination with release of CE-modified purines.

In the depurination process, the E-3,4-Q isomer is more reactive with DNA (9), possibly because its longer half life allows more reaction time with DNA.(10) The subsequent

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Although evidence from cell-culture and animal studies support the estrogen adduct-mediated pathway, data from humans have

ion-trap mass spectrometry. We also tested the method on a small number of samples of normal breast tissue obtained from both cancer containing and non-cancer containing breasts.

Experimental

Materials

Oxone, 2-iodobenzoic acid (IBA), dimethyl formamide (DMF), chloroform (CHCl_3), methanol (MeOH, Omnisolv grade), acetonitrile (ACN, Omnisolv grade), formic acid (FA, LC/MS grade) double-distilled acetic acid (AcOH, Teflon grade, 99.5%) and $\text{NH}_3\cdot\text{H}_2\text{O}$, LC/MS grade, 25% in H_2O) were from Sigma-Aldrich (St. Louis, MO). E_1 was from Steraloids (Newport, RI). Isotope-labeled ($\text{U-}^{15}\text{N}$) adenine 5'-monophosphate (AMP), was from Cambridge Isotope Laboratory (Andover, MA). Milli-Q water was used for semi-prep HPLC separation and LC/MS analysis. The 2-iodoxybenzoic acid (IBX), was synthesized from IBA and oxone (18).

Kimble/Kontes all-glass Potter-Elvehjem 1 mL tissue grinders and non-coated 10 mL borosilicate glass centrifuge tubes were purchased from VWR (West Chester, PA). Solid-phase extraction (SPE) cartridges (Focus, 50 mg sorbent mass, 6 mL capacity) were from Varian Inc (Palo Alto, CA). Semi-prep C_{18} column (10×150 mm, 3 μm) and Guard columns (C_8 , 9.7×10 mm, 5 μm) were from P.J. Cobert (St. Louis, MO). Eppendorf 1.5 mL microcentrifuge tubes were from ThermoFisher (San Jose, CA).

Synthesis of $\text{U-}^{15}\text{N}$ -labeled 4-OH- E_1 -1-N3Ade Adduct

The synthesis and purification of the reference materials and the $\text{U-}^{15}\text{N}$ -labeled IS were reported recently (16).

Extraction of Human Breast Tissue

Normal breast tissues were removed in surgery by RLA from women with or without detectable breast carcinoma. In women with a diagnosis of breast cancer, normal tissue was collected from the quadrant farthest from the cancer. Three of the patients were premenopausal (samples 1, 5, 6, Table 1) and three were postmenopausal (2,3,4). None were using hormone replacement therapy at the time of collection. Specimens were stored at $-80\text{ }^\circ\text{C}$, and thawed in dry ice before homogenization. The Institution Review Board of Washington University in St. Louis approved the study, allowing the collection of breast tissue, and all patients gave written informed consent before enrollment.

A small sample (~ 200 mg) of breast tissue was homogenized in 1.25 mL 3% AcOH and transferred to a glass centrifuge tube (10 mL) with an additional 1.25 mL 3% AcOH, 3.75 mL MeOH/ CHCl_3 (2/1, v/v), and 1.25 mL CHCl_3 . The IS (1 pmol) was added to the homogenate, and the suspension was vortexed three times (10 s duration). The homogenate was then sonicated (Sonics, 500-watt Ultrasonic, Fisher-Thermo, San Jose, CA) three times for 20 s each and incubated overnight with agitation. The suspension was centrifuged (ThermoFisher/IEC Central CL3R) at 4000 rpm for 10 min. After discarding the lower lipid layer and the middle protein disc, the upper non-lipid layer was transferred to a new uncoated glass centrifuge tube, and 600 μL of $\text{NH}_3\cdot\text{H}_2\text{O}$ (1/10 dilution of 25% NH_3) was added to adjust the pH to 6-7. The suspension was further centrifuged to fractionate additionally precipitated proteins and to assure separation of the MeOH/ CHCl_3 layer (~ 50 μL). The clear upper layer was taken for SPE cleanup.

A Focus SPE cartridge was conditioned with 5 mL MeOH, followed by 5 mL H_2O and 5 mL 10% ACN. The analyte was eluted with 5 mL MeOH/ACN/ H_2O /TFA (60/30/10/0.25) to a standard Eppendorf centrifuge tube, the solvent removed under reduced pressure, and the residue reconstituted in 150 μL H_2O /ACN/FA (3/97/0.1) for injection into to a guard column

for filtration followed by a semi-preparative LC column. New injection needle, injection valve, guard column, semi-prep column, UV flow cell, and tubing were used to avoid contamination from the synthetic standard. The flow rate was 5 mL/min, and the mobile phases were A (H₂O with 0.1% FA) and B (ACN with 0.1% FA). The gradient profile was started with pure A and ramped to 5% B in 5 min, then ramped to 100% B in 20 min, and held at 100% B for 5 min. Control experiments showed that 4-OH-E₁-1-N³Ade eluted at ~ 10.9 and 11.3 min. Fractions were collected at 0.2 min intervals from 10-12 min to ensure minimal losses, dried, reconstituted in 10 µL of H₂O/ACN/FA (93.5/6.5/0.1), and analyzed by nano LC/MS/MS.

Mass Spectrometry Analysis

Nano LC-ESI-MS/MS was with a LCQ Deca quadrupole ion-trap mass spectrometer (ThermoFisher, San Jose, CA) coupled to a capillary LC (Waters Corp., Milford, MA). An uncoated 360/75 µm OD/ID fused-silica capillary column with 15 µm PicoFrit™ nano tip (New Objective Inc., Woburn, MA) was custom-packed with C₁₈(2) particles (Luna 3 µm 100 Å, Phenomenex, Torrance, CA) to afford a column that was ~ 12 cm in length. The sample solution (5 µL) was loaded and delivered with 97% solvent A (H₂O/ACN/FA, 97/3/0.1) and 3% B (H₂O/ACN/FA, 3/97/0.1). The flow was started at 8 µL/min, decreased to 1 µL/min in 5 min, and held at 1 µL/min for 10 min without splitting. The LC analysis started with 97% A for 8 min and then a 37 min linear gradient to 100% B. The eluent (total flow of 8 µL/min) was split before the column to achieve 270 nL/min at the tip.

The mass spectrometer was operated in the positive-ion mode with a spray voltage of 1.8-2.5 kV, and no nitrogen sheath gas. The maximum time for ion injection into the trap was 300 ms to allow one “microscan.” The scan range was *m/z* 200-430. For MS² experiments, the collision energy was optimized at 40% of the maximum energy available (~ 5 eV, laboratory frame).

Results and Discussion

Sample Extraction

Successful extraction of breast tissue is achievable by balancing compromises in specimen processing and avoiding pitfalls. Silanized glass tubes decreased the recovery of 4-OH-E₁-1-N³(U-¹⁵N)Ade from these tubes to approximately 25%. Using a lower percentage of AcOH led to insufficient precipitation of proteins in the tissue homogenate, whereas higher percentages of AcOH gave lower recovery of the IS. We chose 3% to achieve acceptable protein precipitation and analyte recovery. With glass containers and the use of TFA or TCA, nearly complete loss of analyte occurred. Protein precipitation must also be carefully done. After adding MeOH and CHCl₃, the upper-layer solvent became ~ 50% MeOH with some CHCl₃ at an apparent pH ≈ 3. With centrifugation, precipitated proteins sometimes do not accumulate completely at the protein disc formed between the upper and lower layers, resulting in a blurry upper layer. A small percentage of residual proteins in this layer can lead to complete loss of the IS. This is avoidable by clarifying the cloudy upper layer by adding 2.5% NH₃·H₂O to adjust the pH to 6-7 and centrifuging. The two-step protein precipitation removes the majority of proteins from tissue extracts, leading to a clear, non-viscous upper layer that is ready for SPE cleanup.

We chose a hybrid Focus cartridge for SPE. This cartridge retains the analyte by both hydrophobic and electrostatic interactions. From the tissue extract, we obtained an upper layer containing ~ 50% MeOH. To apply SPE, it is critical to adjust the extracting solvent to neutral pH to retain the analyte by the electrostatic interactions. We used TFA (0.25%) in the elution step to disrupt the electrostatic interaction and then collected in normal Eppendorf centrifuge tubes, avoiding both “low-bind” tubes, which add interferences with 0.1% or higher TFA, and

glass containers, as discussed above. The percent TFA is a compromise; lower concentration gives a larger volume of eluent while higher concentrations generate interfering species.

After semi-preparatory HPLC fractionation and solvent removal, we reconstituted the analyte in 10 μ L H₂O/ACN/FA (93.5/6.5/0.1%). The presence of organic solvent and the addition of acid assisted the solvation of the analyte; however, higher organic content leads to peak broadening in the LC analysis. Typical recovery was $\sim$ 50%, and detections are achievable with 100 mg of tissue (wet weight).

ESI-MS/MS Analysis

The catechol-estrone-modified Ade is readily ionized in electrospray and forms a protonated species, [4-OH-E₁-1-N3Ade + H]⁺ of m/z 420. To characterize specifically the presence of 4-OH-E₁-1-N3Ade from breast-tissue extract, the analyses were carried out with an ion-trap instrument to monitor alternatively the product ions generated from precursor ions of m/z 420 (native analyte) and 425 ([4-OH-E₁-1-N3(U-¹⁵N)Ade + H]⁺). We chose a narrow mass spectral m/z scan from 200 to 430, resulting in a duty cycle of 50% for the trap. A maximum injection time of 300 ms and a single “microscan” gave $\sim$ 15 points to define the LC peak for quantitative analysis. The MS analysis achieved a limit of detection (LOD) of 150 attomol of 4-OH-E₁-1-N3Ade with signal-to-noise ratio (S/N) $\geq$ 3 in control experiments. Matrix effects from the complex tissue sample increased the LOD to 1 fmol/200 mg tissue (5 fmol/g) on average with S/N $\geq$ 3.

For native 4-OH-E₁-1-N3Ade, we selected product ions of m/z 256, 257, 296, 385, 391 (MS/MS) and the precursor ion of m/z 420 to reconstruct the ion chromatogram. The ions of m/z 256 and 257 come from the cleavage of estrone ring B, that of m/z 385 from consecutive losses of NH₃ + H₂O, and that of m/z 391 from loss of CH₂NH. We chose these ions because they are abundant and characteristic of the modified Ade. For example, the fragmentation 420 $\rightarrow$ 392 occurs in many interfering species whereas that of 420 $\rightarrow$ 391 is characteristic of a modified Ade. (We described the fragmentation in a recent paper (16).) For U-¹⁵N labeled IS, the product ions of m/z 261, 262, 301, 389 and 395 and precursor ion of m/z 425 correspond to those of the unlabeled analyte, and they were the basis of the reconstructed ion chromatogram for the IS.

Identification and quantification of 4-OH-E₁-1-N3Ade

Incorporation of 1 pmole of the IS in the sample before the extraction and analysis permits good detection (Figure 1) and allows the coelution of the native and labeled species to be established. The product-ion spectra of the m/z 425 species (IS) eluting at 19.2 min, shows that the characteristic products of m/z 407, 395, 389, 377, 345, 301, 262 and 261 (Figure 2, top). The product-ion spectrum of the co-eluting native species of m/z 420 shows the expected product ions of m/z 403, 402, 391, 385, 373, 345, 296, 257 and 256 (Figure 2, bottom). The ions of m/z 403 and 402, generated from NH₃ and H₂O losses from the analyte merge into a single ion of m/z 407 for U-¹⁵N labeled IS. The good agreement in these spectra and the sharp coelution of the native and IS provide high specificity in the identification.

Adding 1 pmol of IS to an adjoining semi-prep HPLC fraction that does not contain native material provides a solution with a similar matrix as that of the sample. Comparing that signal with one from the extract gave for the six samples an average recovery of 50%. The TIC of 4-OH-E₁-1-N3Ade has a S/N of 17 (Figure S1 in supplementary information); this can be improved by reconstructing the TIC by including signals from the characteristic product ions of precursors of m/z 425 and m/z 420 (Figure 1). After the reconstruction, the S/N increases to 2000 for the IS and 36 for 4-OH-E₁-1-N3Ade, respectively.

Levels in Breast Tissue

We used this procedure to quantify the levels of 4-OH-E₁-1-*N*3Ade in six breast-tissue samples, two from controls and four normal tissue samples taken from cancerous breasts. Owing to the difference in recovery, the limit of detection varies from 5-20 fmole/gram. The results are an average of two replicates. The precision (Table 1) is not sufficiently high to permit conclusion about whether the levels of the modified Ade are elevated in the cancer patients. The levels of 4-OH-E₁-1-*N*3Ade are approximately two orders lower than previously reported in breast tissue from a cancer patient.(11)

We also investigated the range over which quantitative measurement are possible. Control experiments show that the ion-trap responds linearly over the range of 1-500 fmol of native material relative to 500 fmol of 4-OH-E₁-1-*N*3(U-¹⁵N)Ade internal standard (Figure S2 in supplementary information).

We also measured the recoveries of 4-OH-E₁-1-*N*3Ade and 4-OH-E₁-1-*N*3(U-¹⁵N)Ade from extraction in the absence of breast tissue. A constant amount of 4-OH-E₁-1-*N*3(U-¹⁵N)Ade (100 fmole) is combined with various amount of 4-OH-E₁-1-*N*3Ade. The control experiments show that the procedure has a linear recovery from 3 to 500 fmol (Figure S3 in supplementary information). Owing, however, to the low abundance of naturally occurred 4-OH-E₁-1-*N*3Ade and matrix effect in tissue samples, the responses are in the lower end of the standard curve and would probably account for the low precision in quantification.

Conclusions

Four conclusions arise from our results. First, an extraction and ion-trap MS-based analysis for 4-OH-E₁-1-*N*3Ade in human breast tissue is successful. We recommend three criteria for reliable identification: 1) sharp chromatographic peak (half peak width of 5 s), 2) co-elution with U-¹⁵N labeled internal standard, and 3) match of product-ion spectrum with that of the coeluting standard. This approach may be made more reliable by including detection by accurate mass, now possible with quadrupole time-of-flight and Fourier transform instruments.

Second, the levels 20-70 fmol/g of tissue of the analyte in breast tissue are close to the detection limits of the instrument. Third, the precision for analyses thus far is not high ($\pm 50\%$), which may hinder, at this stage, establishing a distinction between normal breast tissue and breast tissue from cancer-containing breasts. To optimize the results and answer the question of whether the modified nucleobase is a biomarker, either improved instrumentation or a larger tissue specimen must be used for analysis. Triple quadrupoles or newer 2D ion trap technology, for example, are reputed to be more sensitive than the 3D ion trap.

Fourth, the levels are consistent with estimates of the yield expected for the in-vivo conversion of estrogen into a modified nucleobase. The interplay of protection/detoxification mechanism complemented by results from appropriate in-vitro and in-vivo studies suggest that the conversion of CE_s to catechol-estrogen-modified nucleobases is on the order of 1% of the amount of estrogen present in tissue.(19,20) Accepting that the level in breast tissue of premenopausal and postmenopausal women is ~ 1 pmole/gram of breast tissue and that E and CE in breast tissue are comparable (21), we calculated that the yields of catechol-estrogen-modified nucleobases needed to give 20-70 fmol/g are 2-7%. These yields are reasonable, whereas the 8400 fmol/g detection, reported elsewhere (11), is considerably more than would be accommodated by the amount of estrogens in breast tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

The work was supported by the National Centers for Research Resources of the NIH (Grant No. 2P41RR000954). We thank Dr. Kevin Yarasheski and Ms. Amanda Becker for advice and help in the preparation and extraction of tissue samples.

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Figure 1.
Reconstructed ion chromatograms for the improved identification of 4-OH-E₁-1-N₃(U-¹⁵N) Ade (top panel) and 4-OH-E₁-1-N₃Ade (bottom panel).

Figure 2. Product-ion mass spectra of 4-OH-E₁-1-N₃(U-¹⁵N)Ade recovered (top panel) and 4-OH-E₁-1-N₃Ade identified from breast tissue extract (bottom). Peaks with red dots are characteristic product ions for IS and native material, respectively.

Table 1Amounts of 4-OH-E₁-1-N3Ade in six breast-tissue specimens

Patient	Type of Tissue	Amount of 4-OH-E ₁ -1-N3Ade (fmol/g tissue)
1	Non-cancer	nd. (< 5)
2	Non-cancer	26±10
3	Cancer	70±20
4	Cancer	16±10
5	Cancer	19±10
6	Cancer	40±20