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Quantitation of Steroid Hormones in Thin Fresh Frozen Tissue Sections

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Abstract

As analytical technologies in proteomics and metabolomics continue to mature, there is an increasing need to apply these to clinically relevant biologic samples. In this study, a liquid chromatography-tandem mass spectrometry method that utilizes selected reaction monitoring was used to measure the absolute quantity of estrogens and estrogen metabolites and testosterone in 8- μ m tissue sections obtained from a fresh frozen lymph node tumor infiltrated by metastatic breast carcinoma. Total (conjugated plus unconjugated) and unconjugated levels of these steroid hormones were measured using two cohorts, each containing five adjacent serial sections cut from this tumor. The results were highly reproducible across replicate samples, showing that typical histological tissue sections represent an important sample type for the measurement of these specific metabolites.

As analytical technologies continue to increase in their sensitivity and specificity, there is a surge to apply these methods to clinically relevant samples such as biofluids and tissues. There is great hope that mass spectrometry (MS) represents the next generation of technologies that will greatly improve physicians', and especially the pathologists', ability to diagnose and classify diseases using biologic samples.^{1,2} While MS has existed for decades, it has seen an incredible resurgence in the past 12–15 years primarily due to improvements in technology that have enabled an increasing number of biomolecule classes to be measured with high sensitivity and specificity. These improvements have made MS the key technology in proteomics and metabolomics, in which the aim is to characterize as many proteins and metabolites in complex mixtures as possible in a single experiment. Mass spectrometry is playing an ever-increasing role in clinical-based studies, as many efforts have demonstrated its ability to comprehensively analyze the proteomes and metabolomes of biofluids such as

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urine and serum/plasma.^{1,3} While measuring proteins and metabolites in biofluids is important for early diagnosis and monitoring therapeutic interventions, the opportunity to analyze tissues may allow the mechanistic roles that specific analytes play in tumor initiation and progression to be elucidated. Unfortunately, obtaining fresh human tissue samples in sufficient amounts to perform proteomic or metabolomic studies is difficult. Typically, such samples are processed into fresh frozen or formalin-fixed tissue blocks that are archived and used for established technologies, such as standard histopathology, immunohistochemistry (IHC), fluorescence in situ hybridization, loss of heterozygosity, and nucleic acid sequencing.^{4,5}

This present situation suggests the importance of developing technologies that can be adapted to readily available samples (e.g., fresh frozen tissue sections). Fortunately, many in the proteomics community have recognized this situation and have developed methods to characterize proteomes of thin sections obtained from both fresh frozen and formalin-fixed, paraffin-embedded tissues.⁶⁻⁹ Although heavily relying on MS as well, metabolomics has not yet shown the promise of proteomics for the analysis of tissue sections. Recent advances in imaging using matrix-assisted laser desorption/ionization MS have made the detection of specific metabolites in tissue sections possible; however, this method does not provide the quantitative power required for most clinical studies and is extremely labor- and time-intensive.¹⁰

Steroid hormones represent an important class of molecules that are valuable to measure in clinical tissue sections. There is considerable evidence linking steroid hormones to breast cancer risk.¹¹⁻¹³ Understanding the roles that hormones play in certain human cancers has a relatively long and interesting history. Approximately 100 years ago, a Scottish physician named George Beatson controlled the growth of many breast tumors by removing the patients' ovaries.¹⁴ Retrospectively, this study provided evidence that some breast cancers are hormonally driven. Charles Huggins was a Canadian-born American physician/scientist who first discovered that prostate tumors in male dogs could be shrunk by castration or via estrogen infusion.¹⁵ Hormone manipulation by this method led to the control of prostate cancer in many male patients. Dr. Huggins then developed a Sprague-Dawley rat model for breast cancer that was hormone-dependent and widely used in breast cancer research for two decades. Using this model, he demonstrated that some breast cancers are hormone dependent, and hormone deprivation could be used as a cancer treatment.¹⁶ The Nobel Prize for Physiology or Medicine was awarded to Dr. Huggins in 1966 for his discovery of the hormone dependence of cancer cells in experimental animals and his contributions to both treatment and prevention of cancers in humans.

The hypothesis that localized production and metabolism of steroid hormones in the breast is believed to play a major role in the development of hormone-dependent breast carcinomas has led many studies to investigate enzymes such as aromatase, steroid sulfatase, and 17 β -hydroxysteroid dehydrogenase, which are involved in the synthesis and metabolism of estrogens.^{13,17,18} While characterization of enzymes responsible for estrogen biosynthesis and metabolism and their receptors is critical, it would be valuable to be able to measure the absolute levels of individual steroid hormones directly within tissue sections. In this study, we describe a liquid chromatography-tandem MS (MS²) method^{19,20} to measure various

estrogens and estrogen metabolites (EM) and testosterone in 8- μ m-thick human tissue sections. If MS is to influence disease diagnostics and therapeutics, it is important to develop methods that apply this technology to routinely collected clinical samples.

MATERIALS AND METHODS

Reagents and Materials.

Testosterone and 15 estrogens and estrogen metabolites (EM) including estrone (E_1), 17 β -estradiol (E_2), estriol (E_3), 2-hydroxyestrone (2-OHE₁), 2-methoxyestrone (2-MeOE₁), 2-hydroxy-17 β -estradiol (2-OHE₂), 2-methoxy-17 β -estradiol (2-MeOE₂), 2-hydroxyestrone-3-methyl ether (3-MeOE₁), 4-hydroxyestrone (4-OHE₁), 4-methoxyestrone (4-MeOE₁), 4-methoxy-17 β -estradiol (4-MeOE₂), 16R-hydroxyestrone (16R-OHE₁), 17-epiestriol (17-epiE₃), 16-keto-17 β -estradiol (16-ketoE₂), and 16-epiestriol (16-epiE₃) were obtained from Steraloids, Inc. (Newport, RI). Four deuterium-labeled estrogens and estrogen metabolites (d-EM) including 17 β -estradiol-2,4,16,16- d_4 (d_4 -E₂), estriol-2,4,17- d_3 (d_3 -E₃), 2-hydroxy-17 β -estradiol-1,4,16,16,17- d_5 (d_5 -2-OHE₂), and 2-methoxy-17 β -estradiol-1,4,16,16,17- d_5 (d_5 -2-MeOE₂) were purchased from C/D/N Isotopes, Inc. (Pointe-Claire, QB, Canada). A fifth d-EM, 16-epiestriol-2,4,16- d_3 (d_3 -16-epiE₃), was obtained from Medical Isotopes, Inc. (Pelham, NH). All EM and d-EM and testosterone have reported chemical and isotopic purity of 98% and were used without further purification. Dichloromethane, methanol, and formic acid were obtained from EM Science (Gibbstown, NJ). Glacial acetic acid, sodium bicarbonate, and L-ascorbic acid were purchased from J. T. Baker (Phillipsburg, NJ), and sodium hydroxide and sodium acetate were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium bicarbonate and β -glucuronidase/sulfatase (*Helix pomatia*, type HP-2, 500 units of β -glucuronidase and 37.5 units of sulfatase activity) was obtained from Sigma Chemical Co. (St. Louis, MO). Dansyl chloride and acetone were purchased from Aldrich Chemical Co. (Milwaukee, WI). All chemicals and solvents used in this study were HPLC or reagent grade unless otherwise noted.

Fresh Frozen Tissue Section Sample Collection.

Ten 8- μ m-thick adjacent tissue sections were cut serially from a fresh frozen lymph node tissue infiltrated by metastatic breast carcinoma. Five sections were used for measurement of total (conjugated plus unconjugated) EM and testosterone levels, while the remaining five were used to measure free (unconjugated only) EM and testosterone levels. The tissue collection was approved by the NCI/NIH Institutional Review Board.

Preparation of Stock and Working Standard Solutions.

Stock solutions of testosterone, EM, and d-EM were each prepared at 80 μ g/mL by dissolving 2 mg of each steroid powder in methanol containing 0.1% (w/v) L-ascorbic acid to a final volume of 25 mL in a volumetric flask. Stock solutions were monitored by measuring the absolute peak height of each analyte using LC-MS² to verify that no time-dependent degradation of the testosterone, EM, and d-EM standards had occurred. The stock solutions were stable for at least two months while stored at -20 °C. Working standard solutions of testosterone and each EM or d-EM at 8 ng/mL were prepared by dilutions of the stock solutions with methanol containing 0.1% (w/v) L-ascorbic acid.

Calibration Standards.

A normal breast cell line grown in steroid-free medium, and having no detectable levels of testosterone or EM, was used as the matrix for preparation of calibration standards. MCF-10A cells were grown in complete growth medium (ATCC, Manassas, VA) with 10% charcoal-stripped fetal bovine serum (Invitrogen, Carlsbad, CA). When cells reached 80% confluency, they were washed three times with PBS and dislodged from cell culture dishes following incubation with 5 mM EDTA in PBS for 5 min. The cells were pelleted by centrifugation at 500g for 10 min at 4 °C. The cell lysate was prepared as described below. Calibration standards were prepared by adding 5 μ L of the d-EM working internal standard solution (40 pg of each d-EM) to various volumes of the EM and testosterone working standard solution, which contained 0.4–320 pg of testosterone and each EM and were assayed in duplicate. Each calibration standard contained lysate from ~50 000 MCF-10A cells.

Sample Preparation Procedure.

Each entire tissue section was quantitatively transferred into a 1.5-mL Eppendorf tube containing 200 μ L of 12.5 mM ammonium bicarbonate by scrapping the section off of the glass slide using a fresh razor blade. The sample was sonicated in a water bath at ambient temperature for 20 min, followed by vortexing at high speed for 10 min and an additional 10-min sonication in an ambient-temperature water bath. Each sample was transferred to a clean screw-capped glass tube.

The sample preparation procedures were designed to quantitatively measure the following: (1) unconjugated testosterone and EM and (2) total (conjugated plus unconjugated) testosterone and EM. For total tissue testosterone and EM, 5 μ L of the d-EM working standard solution (40 pg of each d-EM) was added to the tissue cell lysate, followed by the addition of 0.5 mL of freshly prepared enzymatic hydrolysis buffer containing 2 mg of L-ascorbic acid, 5 μ L of β -glucuronidase/sulfatase, and 0.5 mL of 0.15 M sodium acetate buffer (pH 4.1) as previously described.^{19,20} Samples were incubated for 20 h at 37 °C. After hydrolysis with β -glucuronidase and sulfatase, each sample underwent slow inverse extraction at 8 rpm (RKVSD, ATR, Inc., Laurel, MD) with 6 mL of dichloromethane for 30 min. After extraction, the aqueous layer was discarded. The organic solvent portion was transferred into a clean glass tube and evaporated to dryness at 60 °C under nitrogen gas (Reacti-Vap III, Pierce, Rockford, IL). For measuring unconjugated testosterone and EM, the same sample preparation was used except that a 0.5-mL aliquot of hydrolysis buffer, without β -glucuronidase/sulfatase, was added.

To each dried sample, 40 μ L of 0.1 M sodium bicarbonate buffer (pH at 9.0) and 40 μ L of dansyl chloride solution (1 mg/mL in acetone) were added. After vortexing, samples were heated at 60 °C (Reacti-Therm III Heating Module, Pierce) for 5 min to form the EM and d-EM dansyl derivatives (EM-dansyl and d-EM-dansyl, respectively).^{19,20} The dansyl derivatization method modifies the phenolic hydroxyl group of EM and will not react with testosterone.^{19,20} For measuring unconjugated testosterone and EM, the same sample preparation was used except that a 0.5-mL aliquot of hydrolysis buffer, without β -glucuronidase/sulfatase, was added. Calibration standards were hydrolyzed, extracted, and

derivatized following the same procedure as that used for the unknown tissue samples. After derivatization, all samples were analyzed by the capillary LC-MS².

Capillary Liquid Chromatography–Tandem Mass Spectrometry Analysis.

After derivatization, all samples were analyzed using LC-MS². Capillary LC-MS² analysis was performed using an Agilent 1200 series nanoflow LC system (Agilent Technologies, Palo Alto, CA) coupled to a TSQ Quantum Ultra triple-quadrupole mass spectrometer (Thermo Electron, San Jose, CA). The LC separation was carried out on a 150 mm long $\times$ 300 μ m i.d. column packed with 4- μ m Synergi Hydro-RP particles (Phenomenex, Torrance, CA) and maintained at 40 °C. A total of 8 μ L of each sample was injected onto the column. The mobile phase, operating at a flow rate of 4 μ L/min, consisted of methanol as solvent A and 0.1% (v/v) formic acid in water as solvent B. A linear gradient increasing from 72 to 85% solvent A in 75 min was employed for the separation. The MS conditions were as follows: source, ESI; ion polarity, positive; spray voltage, 3500 V; sheath and auxiliary gas, nitrogen; sheath gas pressure, 7 arbitrary units; ion-transfer capillary temperature, 270 °C; scan type, selected reaction monitoring (SRM); collision gas, argon; collision gas pressure, 1.5 mTorr; scan width, 0.7 u; scan time, 0.50 s; Q1 peak width, 0.70 u full width half-maximum (fwhm); Q3 peak width, 0.70 u fwhm. The optimized SRM conditions for the protonated molecules [MH]⁺ of EM-dansyl and d-EM-dansyl were similar to those previously described.^{19,20} In addition, the specific SRM transitions of protonated testosterone m/z 289 $\rightarrow$ 97 and 109 were used for measuring tissue testosterone.

Identification and Quantitation of Tissue Section Testosterone and EM.

The identification and quantitation of testosterone and EM were carried out using Xcalibur (Thermo Electron) similar to the method previously described.^{16,17} The testosterone and EM in each tissue section were identified by comparing the chromatographic retention time and mass spectral profile of the specific steroids with those from the reference chemical standards. For quantitation, calibration curves for each EM and testosterone were constructed by plotting for the calibration standards the peak area ratios for each analyte and its assigned stable isotope internal standard versus the amount of each analyte that was injected on the column and fitting these data using linear regression with 1/ X weighting. The amounts of testosterone and each EM in the tissue sections were then interpolated using this linear function. Based on similarity of structures and retention times, d₄-E₂ was used as the internal standard for E₂ and E₁; d₃-E₃ for E₃; d₅-2-MeOE₂ for 2-MeOE₁ and 2-MeOE₂; and d₅-2-OHE₂ for 2-OHE₁ and 2-OHE₂.

RESULTS

We acquired 8- μ m-thick, serial sections from a fresh frozen lymph node tissue infiltrated by metastatic breast carcinoma. The tissue sections were assigned to two groups comprising five adjacent serial sections each. Total (conjugated plus unconjugated) levels of various EM and testosterone were measured in each of the individual tissue sections in one group. Unconjugated levels of EM and testosterone were measured in the other group of tissue sections.

After extraction and derivatization of the sample, 8 μL (1/10) of the extract's final volume was injected onto the reversed-phase column and analyzed using capillary LC-MS² operating in SRM mode. The LC-MS² SRM profiles of the unconjugated EM levels observed in a single tissue section are shown in Figure 1A. In this chromatogram, seven EM were observed: E₁, E₂, E₃, 2-MeOE₁, 2-MeOE₂, 2-OHE₁, and 2-OHE₂. Total levels of testosterone and EM were also measured in serial sections taken from the same metastatic lymph node tissue. To measure total levels of these steroid hormones (compared to unconjugated levels), a hydrolysis step with β -glucuronidase/sulfatase is employed prior to the extraction and dansylation. The action of the enzymes removes glucuronidate and sulfate moieties that may exist on a subpopulation of the steroid hormones. The chromatogram of the total EM is shown in Figure 1B. The same seven EM observed in Figure 1A could be observed; however, the peaks corresponding to many of these hormones had obviously higher intensity and greater signal-to-noise ratios. The chromatographic profiles of unconjugated and total testosterone levels extracted from the same tissue section are shown in Figure 2. As observed for the EM, the testosterone signal observed from the sample that had undergone hydrolysis was more intense and exhibited a much higher signal-to-noise ratio.

To ensure that the correct steroid hormones were being assigned to each peak, the data were acquired in a SRM mode, in which a transition ion specific to each of the targeted steroid hormones was measured. This acquisition mode, coupled with the chromatographic behavior of the steroid hormones of interest, ensures reasonable confidence that the correct metabolite is being identified and quantitated at specific points in the chromatogram. The chromatographic retention time and fragment ion spectra for two of the EM detected in the tissue sections (E₁ and E₂) were compared to standards of each compound in Figure 3A and B. In both cases, the EM detected in the tissue sections showed the same chromatographic retention time and fragmentation characteristics as the reference standards. The same comparison was conducted using a testosterone standard and testosterone extracted from the tissue section. As with the EM, the chromatographic retention time and fragment ion spectrum of the reference standard matched exactly to that of the testosterone extracted from the tissue section (Figure 3C).

Five separate tissue sections were analyzed separately to measure the absolute quantity of total testosterone and total EM. Seven EM, shown in Figure 1, were detected. The amounts of each steroid hormone are shown in Table 1. The amounts of EM found in the tissue sections ranged from a high of 44.40 pg/tissue section for E₂ to a low of 1.31 pg/tissue section for 2-MeOE₁. The coefficients of variation (CVs) for the five analyses of total steroid hormone levels ranged from 2.4 (E₁) to 12.4% (2-OHE₂). The quantitative results for unconjugated testosterone and unconjugated EM measured within five additional serial sections from the same metastatic lymph node tissue are shown in Table 2. As expected, the levels of unconjugated hormones were consistently less than the total amounts shown in Table 1. For E₁, E₃, 2-MeOE₁, 2-MeOE₂, 2-OHE₁, 2-OHE₂, and testosterone, the levels of unconjugated hormone were 41–66% that of the total levels. The level of unconjugated E₂ that exists in the tissue section, however, was only 14% of the total amount of E₂. The CVs for the five analyses of free steroid hormone levels ranged from 6.3 (E₁) to 13.9% (2-OHE₁ and 2-MeOE).

The described assay was applied to measure total EM levels in serial sections cut from brain schwannoma, ovarian cancer (two different grades, enumerated 1 and 2), uterine cancer, pancreatic islet cell tumor, and pituitary adenoma fresh frozen tissue blocks. The results showing the levels of the various EM detected in a triplicate analysis of adjacent sections of each of these various tissue types are shown in Table 3. Various levels of the targeted EM were seen in the different tissue sections showing that different tumor types have unique EM profiles. Triplicate analysis showed the results to be highly reproducible. The chromatographic profiles of total levels for seven of the EM extracted from the ovarian cancer 2 tissue sections are shown in Figure 4. Two catechol EM, specifically 4-OHE₁ and 2-OHE₂, were the most abundant of the seven EM detected in this tissue. In fact, these two species accounted for more than 65% of all unconjugated EM found in this ovarian cancer tissue section. Our findings suggested that most of the parent estrogens were metabolized to catechol estrogens at the local level of this ovarian cancer tissue. In contrast, the levels of methylated catechol estrogens, an indication of localized catechol-*O*-methyl transferase activity, were low. Given the potential carcinogenicity of catechol estrogens, our findings of an imbalance between catechol estrogen formation and inactivation at local ovarian cancer tissue level certainly warrant further investigation.

DISCUSSION

Medical science is currently striving toward personalized medicine. This trend is especially true in oncology given the significant toxicities associated with many therapies, high rates of partial or no response to certain treatments, and limited information for determining appropriate treatments. Advanced molecular diagnostics are required to better enable a rational assignment of therapy based on the molecular portrait and analysis of an individual's tumor. To this end, tumor tissue EM profiling may offer a new dimension to further the understanding of estrogen receptor (ER)-positive breast cancers. Finally, given the relatively new evidence for cardiac dysfunction associated with long-standing testosterone suppression, especially in the setting of hormone refractory prostate cancer, measuring tumor tissue testosterone may provide scientific insights to modulate therapy and thus avoid cardiac toxicity.

Today, therapy for a newly diagnosed breast cancer is determined by analysis of several parameters, including tumor size, number of disease positive/infiltrated lymph nodes (LN), evidence of disease outside of the breast, and local axillary LN, ER status, and HER2neu receptor status. Considerable emphasis is placed on ER status given the limited toxicity of hormonal treatment approaches. However, a significant degree of molecular and prognostic heterogeneity exists among ER-positive breast cancers.

Recently, new commercial multigene assays aimed at assessing prognosis and potential treatments have been being routinely used in the United States and Europe. The early success of OncoType Dx (a 21-gene assay based on reverse transcriptase-polymerase chain reaction by Genomic Health) has resulted in a recent enhancement of this assay. The new aim is to better quantify the ER receptor status of a breast tumor, given the problems of determining ER status by IHC. Heterogeneity in ER positive breast tumors and the resultant variable responses to hormone therapy require new scientific approaches.

Improved understanding of hormonally driven malignancies is needed. Central to this need are assays with advanced quantitative abilities. In this report, we have shown the utility of a targeted metabolomics approach to measure the absolute quantity of testosterone and a panel of EM in a single histological section obtained from a fresh frozen metastatic lymph node infiltrated with breast carcinoma and ovarian cancer tissues. This method allows differentiation and quantitation of testosterone and EM including conjugated and unconjugated forms for each hormone. The overall CVs (relative standard deviation) for the measurements ranged between 2.4 and 12.4% RSD for total steroid levels and between 6.3 and 13.9% RSD for unconjugated steroid levels.

Mass spectrometry is arguably the most promising technology for improving the accuracy and precision of quantitative measurements that support clinical diagnostic and therapeutic monitoring. As important as the improved ability to accurately and precisely quantitate specific molecules is the enhanced sensitivity. Advancements made over the past decade have increased the sensitivity, resolution, and mass accuracy of MS and enabled the analysis of entire proteomes and metabolomes. Historically, one of the major stumbling blocks in bringing MS to bear on clinical problems has been a disconnect between the types of samples collected by clinicians and those that are routinely analyzed by mass spectrometrists. The last five years, however, have seen a huge breakthrough in the application of MS for the characterization of proteomes extracted from a variety of biofluids¹ and tissue sections, including both fresh frozen and formalin-fixed.^{7,8} On the metabolomics side, MS² has been used for analyzing dried blood spots obtained from newborns to screen for over 30 metabolic disorders, including those associated with amino acids (e.g., phenylketonuria) and fatty acids.²¹ Increasing the uses of MS for analyzing routinely collected, as well as archived, clinical samples will require continued method developments with the aim of generating routine tests that can be used for both diagnostic and therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Liquid chromatography-tandem mass spectrometry SRM chromatographic profiles of (A) unconjugated and (B) total (conjugated + unconjugated) estrogens and estrogen metabolites in an 8- μ m tissue section obtained from a metastatic lymph node infiltrated with metastatic breast carcinoma. The *X*-axis is retention time and the *Y*-axis is relative intensity.

Abbreviations: estrone (E_1), 17 β -estradiol (E_2), estriol (E_3), 2-methoxyestrone (2-MeOE₁), 2-methoxy-17 β -estradiol (2-MeOE₂), 2-hydroxyestrone (2-OHE₁), 2-hydroxy-17 β -estradiol (2-OHE₂).

Figure 2. Liquid chromatography-tandem mass spectrometry SRM chromatographic profiles of (A) unconjugated and (B) total (conjugated + unconjugated) testosterone in an 8- μ m tissue section obtained from a metastatic lymph node infiltrated with metastatic breast cancer. The *X*-axis is retention time and the *Y*-axis is relative intensity.

Figure 3. Tandem mass spectrometry profiles of (A) dansylated estrone (E₁), (B) dansylated 17 β -estradiol (E₂), and (C) testosterone observed for the reference standards for each steroid (top) and for each steroid measured within a single thin tissue section (bottom).

Figure 4.

Liquid chromatography--tandem mass spectrometry SRM chromatographic profiles of seven of the unjugated estrogens and estrogen metabolites extracted from an 8- μ m ovarian cancer 2 tissue section. For each individual estrogen, the mean (pg/tissue section) and standard deviations for three replicate analyses are indicated. The *X*-axis is retention time and the *Y*-axis is relative intensity.

Quantity (pg/Tissue Section) of Total (Conjugated plus Unconjugated) Estrogens and Estrogen Metabolites and Testosterone in an 8- μ m Tissue Section Taken from Fresh Frozen Metastatic Lymph Node Infiltrated with Metastatic Breast Carcinoma

Table 1.

slide	E ₁	E ₂	E ₃	2-MeOE ₁	2-MeOE ₂	2-OHE ₁	2-OHE ₂	T	total
1	3.796	40.538	2.634	1.275	2.629	3.115	6.941	4.504	65.4
2	3.798	45.579	2.114	1.513	2.329	2.960	7.428	4.306	70.0
3	3.835	45.811	2.703	1.274	2.812	3.143	7.398	3.881	70.9
4	3.941	45.419	2.883	1.124	2.667	3.096	8.944	3.511	71.6
5	4.007	44.656	2.863	1.350	3.021	3.629	9.104	3.648	72.3
mean	3.875	44.401	2.639	1.307	2.692	3.188	7.963	3.971	70.0
SD	0.094	2.202	0.425	0.141	0.254	0.256	0.989	0.425	2.71
CV (%)	2.4	5.0	10.7	10.8	9.5	8.0	12.4	10.7	3.9

Quantity (pg/Tissue Section) of Unconjugated Estrogen and Estrogen Metabolites and Testosterone in an 8- μ m Tissue Section Taken from a Fresh Frozen Metastatic Lymph Node Infiltrated with Metastatic Breast Carcinoma

Table 2.

slide	E ₁	E ₂	E ₃	2-MeOE ₁	2-MeOE ₂	2-OHE ₁	2-OHE ₂	T	total
1	1.795	6.862	1.483	0.893	1.815	1.654	3.143	1.601	19.2
2	1.734	5.852	1.457	0.745	1.530	1.283	3.814	2.147	18.6
3	1.513	5.644	1.207	0.833	1.246	1.391	3.396	2.195	17.4
4	1.673	6.345	1.444	0.909	1.486	1.826	2.907	2.023	18.6
5	1.719	6.653	1.512	0.928	1.684	1.556	3.057	1.989	19.1
mean	1.687	6.271	1.421	0.861	1.552	1.542	3.263	1.992	18.6
SD	0.106	0.517	0.122	0.074	0.214	0.214	0.355	0.234	0.715
CV (%)	6.3	8.2	8.6	8.6	13.9	13.9	10.9	11.7	3.9

Concentrations (pg/Tissue Section) of Unconjugated Estrogen and Estrogen Metabolites in 8- μ m Tissue Sections Obtained from Six Different Tumor Tissues^a

	brain schwannoma	ovarian cancer 1	ovarian cancer 2	uterine cancer	pancreatic islet cell tumor	pituitary adenoma
E ₁	0.295 ± 0.005	0.495 ± 0.012	0.518 ± 0.009	0.530 ± 0.009	0.189 ± 0.007	0.208 ± 0.013
E ₂	0.555 ± 0.010	1.428 ± 0.081	1.185 ± 0.068	1.083 ± 0.049	0.361 ± 0.020	0.412 ± 0.017
E ₃	0.429 ± 0.017	0.269 ± 0.031	0.197 ± 0.009	0.270 ± 0.019	nf ^b	nf
16-ketoE ₂	0.204 ± 0.002	0.261 ± 0.004	0.865 ± 0.008	0.126 ± 0.002	0.151 ± 0.001	0.112 ± 0.001
16 α -OHE ₁	0.192 ± 0.001	0.416 ± 0.006	1.118 ± 0.010	0.239 ± 0.003	0.297 ± 0.001	0.196 ± 0.001
3-MeOE ₁	0.241 ± 0.009	nf	0.198 ± 0.035	nf	nf	nf
2-MeOE ₁	nf	nf	0.639 ± 0.031	0.557 ± 0.022	nf	nf
4-MeOE ₁	0.202 ± 0.007	0.122 ± 0.001	0.177 ± 0.029	nf	nf	nf
2-MeOE ₂	0.217 ± 0.007	0.185 ± 0.008	0.240 ± 0.019	0.490 ± 0.017	0.105 ± 0.005	0.058 ± 0.003
2-OHE ₂	0.762 ± 0.028	2.323 ± 0.103	3.974 ± 0.051	1.561 ± 0.068	0.053 ± 0.016	0.129 ± 0.013
4-OHE ₁	nf	nf	5.884 ± 0.146	nf	0.245 ± 0.038	3.715 ± 0.062
total	3.097 ± 0.063	5.501 ± 0.010	14.99 ± 0.367	4.856 ± 0.134	1.401 ± 0.048	4.831 ± 0.068

^aData are expressed in mean ± standard deviation of triplicate analyses.

^bnf, not found.