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Methodological considerations in estrogen assays of breast fluid and breast tissue

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ABSTRACT

Estradiol (E2) in nipple aspirate fluid (NAF), ductal lavage fluid (DLF), and random fine needle aspirates (rFNA) are compared. Quantification was by immunoassay or tandem MS. The percent of women yielding NAF varied between 24% and 48% and for DLF was 86.3%. Variation between ducts within a breast was not less than variation between breasts within women but variation between breasts and within women over time was significantly less than variation between women. Serum E2 was highly significantly different among phases of the menstrual cycle but NAF E2 was not different. The correlation between serum and breast fluid E2 concentrations in premenopausal women had coefficients of determination of less than 15%. The correlation between serum and NAF in studies of postmenopausal women varied greatly and may depend on patient selection. The difference between NAF E2 between pre- and postmenopausal women was only 22%; for rFNA it was non-significantly 44% lower in a similar group of postmenopausal women. Progesterone was 96% and 98% lower in postmenopausal NAF and rFNA samples, respectively. Measurements of E2 in breast fluid or breast tissue appears to provide similar estimates of E2 exposure. E2 levels in breast fluid do not reflect the rapid changes that occur in serum and, thus, serum availability of E2 is only one factor determining its levels in the breast. The similarity of levels between breasts and between ducts suggests that estimates of estrogen exposure does not require multiple samples, however, unavailability of fluid may require rFNA in some cases.

1. Introduction

There are a number of reasons to suggest that serum concentrations of estrogens may not be representative of the concentrations of estrogens available to the breast parenchyma. Both sulfation and hydrolysis of estrogens sulfates occurs in the breast [1–3] and changes in metabolism may result in alterations in tissue concentrations. Uptake of sulfated estrogens may also be affected [4,5]. Earlier studies of nipple aspirate fluid indicated that the concentration of estradiol is higher in the breast than in serum, and the coefficients of determination (the percent of the variance in breast fluid or tissue that can be accounted for by variation in serum) were generally less than 0.5 [6–8]. Nevertheless, the concentrations remain sufficiently constant over time within individuals to be useful as indicators of exposure of individuals [9].

2. Experimental

2.1. Collection of NAF and DLF

The methods for collection of NAF and DLF have been described in detail in previous publications [9,10]. Briefly, after warming and massage of the breast, droplets of NAF are collected from the nipple in calibrated capillary tubes. The volume is measured, the sample is flushed out with 200 μL of phosphate-buffered saline, and the diluted sample is sealed and stored at −80 °C. Lavage for DLF was performed as described previously [10]. The breast was massaged, and the Cytyc aspirator (Cytyc Corp., Boxborough, MA) was used to elicit nipple aspirate fluid. Lavage of fluid-yielding ducts and visualized-non-fluid yielding ducts was performed through a microcatheter (Cytyc), using plasmalyte, an isotonic electrolyte solution (Baxter Healthcare Corp., Deerfield, IL). The

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DLF was collected in 20 of Cytolyte (Cytyc), a buffered solution containing 20% methanol. The samples were centrifuged and placed at −80 °C.

2.2. Collection of rFNA

Random fine needle aspirates of the breast were collected by the method of Fabian and colleagues [11] after NAF was expressed. The sample was rinsed into phosphate-buffered saline, 0.1% ascorbic acid was added, and the samples were frozen at −80 °C.

2.3. Analysis of NAF and DLF for estrogens

Methanol from the thawed sample was removed by a centrifugal evaporator and the aqueous remaining solution was reduced in a freezer dryer. The lipid residue from the NAF and DLF samples was extracted into ethyl acetate–hexane (3:2). The estrogens were either separated by the alkaline partition method [9] or by HPLC [12]. In the solvent partition method estradiol is extracted from the NAF or concentrated DLF samples into ethyl acetate–hexane (3:2) and, after evaporation of the solvent, the residue is partitioned between 0.4 N aq. NaOH and isooctane. The alkaline fraction is neutralized and re-extracted into ethyl acetate–hexane (3:2). The ethyl acetate–hexane solvent is evaporated and the residue is dissolved in phosphate buffer containing 0.1% gelatin for immunoassay. We used radioimmunoassay kits from DSL (Webster, TX) for estradiol and estrone sulfate.

Alternatively, the initial ethyl acetate extract is separated by HPLC on a reversed phase HPLC column and eluted with 58% 15 mmol/L phosphate buffer (pH 6) and 42% of a 50:50 mixture of acetonitrile and methanol. A gradient was started at 40 min to a final concentration of 71% of the acetonitrile-methanol solvent at 50 min [12]. In this case, the fractions containing estradiol, estrone, and other steroids are collected separately, the solvent is evaporated, and the residue is dissolved in the assay buffer for radioimmunoassay.

2.4. Analysis of rFNA for estrogens

The samples required a different purification procedure to eliminate the bulk of triglycerides in the sample. Sample aliquots were spiked with 10 μL of acetonitrile (ACN) containing the internal standards {ISTD; E1-d4, E2-d3, (TRC, Toronto, Canada)} and extracted with twice its volume of methyl tert-butyl ether (MTBE). After centrifugation, the organic solution was evaporated in a tared tube and the residue was suspended in 90% aq. methanol. Triglycerides were precipitated at −70 °C, the solvent was evaporated, and the precipitate was weighed. The resulting purified lipid residue was derivatized by adding 0.1 mL of dansyl chloride solution (1 mg/mL in ACN) and 0.1 mL of sodium bicarbonate buffer (0.1 M, pH 9.5), and incubating at 60 °C for 20 min. The tube was then shaken and decanted into an auto-sampler vial for instrumental analysis.

Freshly prepared target analytes standard curves were analyzed along with study rFNA specimens. Instrument calibrators and quality control (QC) samples were prepared by adding 10 μL of stock standard solutions in ACN to PBS buffer (calibrators) and rFNA matrix (QA samples). Calibrator concentrations were prepared in the range of 5–200 pg (QC samples at 10, 40 and 100 pg) for E1 and E2 and 25–1000 pg. Calibrator and QC samples were processed for analysis following the procedure described above. Concentrations of target analytes in the study specimens were determined from each sample's peak area using the linear regression parameters derived for the calibration curves.

Instrumental analysis was performed using a tandem mass spectrometer (4000 Q-TRAP; AB SCIEX, Foster City, CA) equipped with a high performance liquid chromatography (Agilent 1200; Agilent Technologies, Wilmington, DE). Separation of target analytes from matrix components was achieved with a Luna PFP [2], 3μ column, 100 × 2.1 mm (Phenomenex, Torrance, CA). The column temperature was maintained at 25 °C, and a flow rate of 0.3 mL/min was used. The mobile phase consisted of Solvent A: 0.1% formic acid in water (v/v) and Solvent B: 0.1% formic acid in acetonitrile (v/v). The mobile phase gradient was as follows: after injection, initial conditions with Solvent A at 40% were held for 0.5 min, decreased to 5% in 5.5 min and held constant for 4 min, returning to initial conditions for another 4.5 min of re-equilibration time. Total run time was 15 min.

A heated nebulizer was used as the ion source (atmospheric pressure chemical ionization) operating in positive mode. Acquisition was performed in multiple reaction monitoring mode (MRM) using m/z 504.3 → 171.2, 508.3 → 171.2, 755.3 → 170.3 and 760.3 → 170.3 at low resolution for E1, E1-d4, E2, E2-d3, respectively. Ion source temperature was 325 °C, the declustering potential was 130 V and the collision gas was nitrogen with collision energy of 50 V.

3. Method performance

The accuracy (% of true value) of individual calibrators used to determine the calibration curves ranged from 90% to 114% for all target analytes. For the low, mid and high QC levels, precision ranged from 2% to 8% and 2–7% for E1 and E2, respectively. Accuracy ranged from 95% to 100% and 96–114% for E1 and E2, respectively. The mean recoveries of the internal standards with their standard errors were 72 ± 15% and 62 ± 15%.

4. Data analysis

The values from the immunoassays or mass spectrometer measurements were corrected for blank values and recovery of the internal standards. The distribution of the data for statistical analysis must be transformed. Natural log transformation normalizes the data (see Fig. 1). The data are given as log values and geometric means are presented. Subjects were designated as postmenopausal if they had not menstruated within the last 12 months or more or had had an oophorectomy. Hormonally, they had serum estradiol (E2) of ⩽30 pg/mL and progesterone (P4) of ⩽3 ng/mL. Subjects were considered premenopausal if they had regular menstrual cycles of between 24 and 33 days. The cycle was divided into three phases counting days back from the first day of the next menstrual cycle; follicular was days −20 to the first day of the previous menstrual cycle; mid-cycle was days −12 through −19; luteal was days 0 through −11. In addition, follicular phase had E2 ⩽ 30 pg/mL and P4 ⩽ 3 ng/mL; mid-cycle had E2 ⩾ 60 pg/mL and P4 ⩽ 3 ng/mL; luteal had E2 ⩾ 30 pg/mL and P4 ⩾ 3 ng/mL [13].

5. Results and discussion

5.1. NAF yielders

Adequate samples of nipple aspirate fluid (NAF) cannot be collected from all women. In earlier studies of pre- and postmenopausal women taking oral contraceptives or hormone replacement therapy, we found that only 26% of women taking oral contraceptives gave an adequate volume of NAF for hormone measurements; 30% of postmenopausal women not taking HRT, and 41% of postmenopausal women on HRT yielded sufficient NAF [6]. The proportion of yielders depends on what is considered an adequate volume; in this case we required 5 μL. More recently we have accepted 2 μL for some hormones and results from a