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Quantitative determination of estrone by liquid chromatography–tandem mass spectrometry in subcutaneous adipose tissue from the breast in postmenopausal women

Veera Vihma[a,b,*], Feng Wang[a,b], Hanna Savolainen-Peltonen[a,c], Ursula Turpeinen[d], Esa Hämäläinen[d], Marjut Leidenius[e], Tomi S. Mikkola[a,c], Matti J. Tikkanen[a,b]

[a] Folkhälsan Research Center, 00290 Helsinki, Finland
[b] University of Helsinki and Helsinki University Central Hospital, Heart and Lung Center, 00290 Helsinki, Finland
[c] University of Helsinki and Helsinki University Central Hospital, Obstetrics and Gynecology, 00290 Helsinki, Finland
[d] Helsinki University Central Hospital, HUSLAB, 00290 Helsinki, Finland
[e] Helsinki University Central Hospital, Breast Surgery Unit, 00290 Helsinki, Finland

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ABSTRACT

Estrone is the most abundant estrogen after the menopause. We developed a liquid chromatography-tandem mass spectrometric method (LC–MS/MS) for determination of estrone in adipose tissue. Subcutaneous adipose tissue from the breast was collected during elective surgery in postmenopausal women undergoing mastectomy for treatment of breast cancer ($n = 13$) or reduction mammoplasty (controls, $n = 11$). Homogenized adipose tissue was extracted with organic solvents and the estrone fraction was purified by LH-20 column chromatography from the excess of lipids. The concentration of estrone was analyzed by LC–MS/MS. The method was accurate with an intra-assay variation of 8% and an interassay variation of 10%. The median concentration of estrone in subcutaneous adipose tissue from the breast did not differ between breast cancer and control women, 920 pmol/kg and 890 pmol/kg, respectively. In breast cancer patients but not in the controls, breast adipose tissue estrone levels correlated positively with the serum estrone concentration. In conclusion, the new method provides a reliable means to measure estrone concentrations in adipose tissue in postmenopausal women.
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1. Introduction

In postmenopausal women, adipose tissue is the principal source of estrogen [1]. The most abundant estrogen after the menopause is estrone which is formed in peripheral tissues primarily from androstenedione by aromatase, a cytochrome p450 enzyme [2]. Activity of aromatase in adipose tissue is positively related to weight and age in women [3,4]. Moreover, there are relatively high concentrations of estrone sulfate in the circulation which may be taken up and hydrolyzed to free estrone in tissues by steroid sulfatase (STS) [5]. Estrogens are thought to play an important role in the maintenance and growth of estrogen receptor positive breast cancers [6,7].

The concentration of estrone in adipose tissue as reported in postmenopausal women is several times higher than its circulating levels [8,9]. Breast adipose tissue estrone levels have been shown to correlate with body mass index (BMI) in postmenopausal women [10]. Estrone is a precursor of 17β-estradiol in the biosynthesis of estrogens in adipose tissue and, accordingly, concentration of estrone is higher than that of 17β-estradiol in breast adipose tissue as studied in breast cancer patients [8–12].

Estrone is more lipophilic compared to 17β-estradiol. The previous reports on estrone concentrations in adipose tissue from the breast have used radioimmunoassay (RIA) as the analytical method [8–15]. When quantified in adipose tissue, the estrone fraction needs to be separated from great amounts of free cholesterol, triglycerides and other lipids that might otherwise interfere with the analytical method for example by causing signal suppression. In the present study, we describe a new quantitative method to determine estrone in adipose tissue by liquid chromatography-tandem mass spectrometry (LC–MS/MS), and

Abbreviations: BMI, body mass index; GC–MS, gas chromatography-mass spectrometry; IS, internal standard; LC–MS/MS, liquid chromatography–tandem mass spectrometry; RIA, radioimmunoassay; STS, steroid sulfatase.

* Corresponding author at: Folkhälsan Research Center, Biomedicum Helsinki C315a, Haartmaninkatu 8, 00290 Helsinki, Finland. Tel.: +358 9 47171881; fax: +358 9 47171882.
E-mail address: veera.vihma@helsinki.fi (V. Vihma).

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applied the method for analyzing the estrone concentrations in subcutaneous adipose tissue from the breast in postmenopausal women with or without breast cancer.

2. Materials and methods

2.1. Subjects

Subcutaneous adipose tissue from the breast was obtained from postmenopausal women during mastectomy for treatment of breast cancer ($n = 13$) or reduction mammoplasty ($n = 11$) as described in [16]. Two distinct adipose tissue biopsies were taken from every subject. Blood samples were collected before the operation, and the tissue and blood samples were stored as described in [16]. The study was approved by the Ethics committee of Helsinki University Central Hospital and the subjects gave their written informed consent.

2.2. Estrogens and solvents

Estrone (vetranal) was purchased from Sigma–Aldrich, St. Louis, MO. [2,4,6,7-^{3}H(N)]Estrone (specific activity 94 Ci/mmol) was purchased from NEN, Boston, MA. Prior to use, [^{3}H]estrone was purified by Sephadex LH-20 column chromatography (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using 9% methanol in toluene as eluent [17]. $^{13}C_3$-estrone was purchased from Iso-Sciences, King of Prussia, PA.

Methanol, hexane, diethyl ether and ethyl acetate were HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland). Chloroform and toluene were purchased from Merck (Kenilworth, NJ).

2.3. Sample preparation

Control serum pools containing two concentrations of exogenous estrone (180 and 540 pmol/l) were prepared from pooled human male sera (Sigma–Aldrich). The control sera were divided into 1 ml fractions and stored at −20 °C. In addition, subcutaneous adipose tissue from the breasts of 24 women (13 reduction plasty and 11 cancer patients) was homogenized as described below, pooled and used as control.

The flow chart of the method is shown in Fig. 1. Weighed adipose tissue (~200 mg) was homogenized in 1 ml of distilled water. Tissue and control serum samples (1 ml) were pipetted into disposable borosilicate extraction tubes. [^{3}H]Estrone (~49,000 dpm in 10 μl of ethanol) was added to adipose tissue samples obtained from the control pool and used as an internal standard to measure recovery. The samples were extracted four times with 3.5 volumes of diethyl ether–ethyl acetate (1:1 by volume). The combined organic phases were evaporated to dryness and weighed. A mean of 78% of the original tissue sample's ($n = 56$) weight was recovered in the organic phase extract, calculated as [the weight of fat extracted from adipose tissue (mg)/the weight of the original adipose tissue sample (mg)] × 100%. After extraction, the samples were subjected to hydrophobic chromatography on Sephadex LH-20 in hexane–chloroform (2:1 by volume) (chromatography I). The samples were applied to the columns in two 0.3-ml aliquots of hexane–chloroform (2:1 by volume). The interfering fatty substances were eluted in the first lipoidal fraction with 10 ml of the same solvent and discarded. The estrone fraction was eluted with 5 ml of methanol and evaporated to dryness. To further purify the samples, a second Sephadex LH-20 column chromatography (chromatography II) was carried out in 9% methanol in toluene as described in [17]. The sample was applied to the column in two 0.1 ml aliquots of 9% methanol in toluene and estrone was eluted from the column in the first fraction in 2.4 ml of the same solvent. The estrone fraction was evaporated and dissolved in 1 ml of methanol. An aliquot of 50 μl was taken from the control samples for liquid scintillation counting to determine the recovery of the [^{3}H]estrone internal standard (Rack-beta, Wallac Oy, Turku, Finland). An aliquot of 250 μl was taken for analysis by LC–MS/MS as described below.

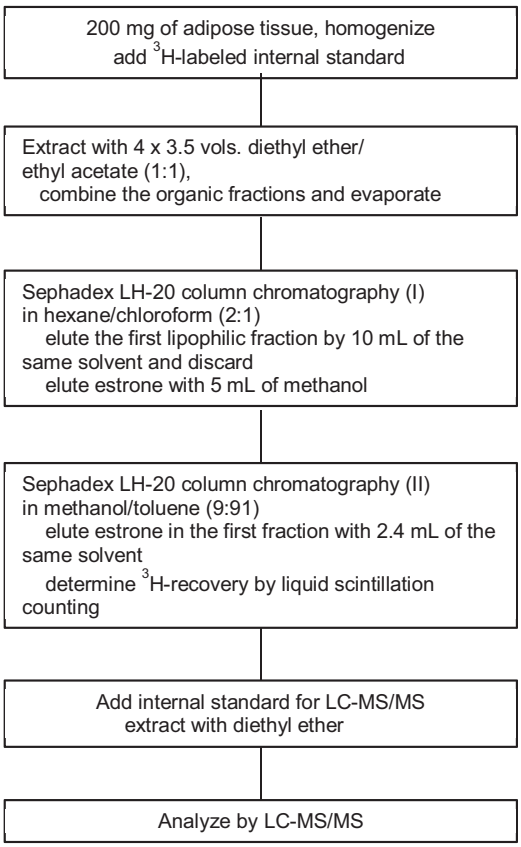

Fig. 1. Outline of the method. Flowchart for quantitative determination of estrone in adipose tissue by LC–MS/MS.

2.4. LC–MS/MS method for determining estrone concentration in adipose tissue

After evaporation to dryness, 30 μl of $^{13}C_3$-estrone (internal standard (IS), 2.5 nM) was added followed by 250 μl of water and 500 μl of 50 mM ammonium acetate/NH_3 (pH 9). The samples were extracted with 2 ml of diethyl ether. After mixing for 3 min, the organic layer was collected and evaporated to dryness under nitrogen. The residue was dissolved in 125 μl of 50% methanol. Calibrators containing 25–1000 pmol/l of estrone were prepared in 50% methanol. 25 μl was injected on an LC–MS/MS system equipped with an API 4000 triple quadrupole mass spectrometer (AB Sciex, Concord, Canada). Peripherals included an Agilent series 1200 HPLC system with a binary pump (Waldbronn, Germany). Separation was performed on a SunFire C18 column (2.1 × 100 mm; Waters, Milford, MA). The mobile phase was a linear gradient consisting of methanol (B) and water (A), at a flow rate of 300 μl/min. The gradient was: 0 min, 50% B; between 0 and 5 min linearly increased to 100% B; 5–8.5 min 100% B; between 8.5–9 min linearly decreased to 50% B; and 9–15 min 50% B. The column was directly connected to the electrospray ionisation probe. Estrone was detected with the following transitions: m/z 269.1 to m/z 145.0 (quantification) and m/z 269.1 to m/z 142.9 (confirmation) and IS, m/z 272.1 to m/z 148.0. Data were acquired and processed with the Analyst Software (version 1.4.2; Sciex). All results were generated in negative-ion mode: with the entrance potential at −8 V, the declustering potential at −100 V, and the collision cell exit

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