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Liquid chromatography tandem mass spectrometry determination of free and conjugated estrogens in breast cancer patients before and after exemestane treatment

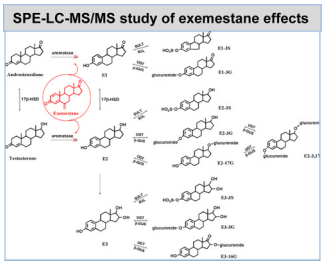
Yuli Zhao [a], Jessica M. Boyd [a], Michael B. Sawyer [b], Xing-Fang Li [a,*]

[a] Division of Analytical and Environmental Toxicology, Department of Laboratory Medicine and Pathology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada T6G 2G3
[b] Division of Medical Oncology, Department of Oncology, Faculty of Medicine and Dentistry, University of Alberta, Cross Cancer Institute, Edmonton, AB, Canada T6G 1Z2

HIGHLIGHTS

- New SPE fractionation with LC–MS/MS determination.
- Determination of intact estrogen conjugates.
- Quantitative profiling of twelve estrogens.
- Unique findings of exemestane effects on estrogens in breast cancer patients.
- Evidence of exemestane inhibition of aromatase activity.

GRAPHICAL ABSTRACT



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ABSTRACT

We report liquid chromatographic separation with tandem mass spectrometry determination of 12 endogenous estrogens and their intact conjugates in blood and urine and its application to study effects of exemestane treatment on estrogen generation and metabolism in postmenopausal women with estrogen-dependent breast cancer. A 0.5 mL aliquot of each urine or serum sample is fractionated with solid phase extraction to a fraction of free estrogen and another fraction of their conjugates. The reversed phase LC/MS/MS determines dansylated estrogens with positive ionization and intact conjugates with negative ionization. The method provides reproducible separation and limit of detection as low as 1 pg mL^{-1} for free estrogens and 0.03 ng mg^{-1} creatinine for the conjugates in serum and urine samples. The method enabled us to acquire unique concentration profiles of 12 endogenous estrogens and their intact conjugates in 30 breast cancer patients before and after one month of exemestane treatment. Exemestane suppressed total serum and urinary estrogens by 11–97% ($P < 0.0001$) and 8.7–91% ($P < 0.0001$), respectively. Specifically, these data show that exemestane preferentially suppressed E1, E1-3S, E1-3G, and E2-17G more than other estrogens. Linear regression analysis of estrogen concentrations before and after treatment showed correlation coefficients of 0.8385 ($n = 289$, $P < 0.0001$) and 0.8863 ($n = 360$, $P < 0.0001$). This study provides urinary and blood estrogen concentration profiles in breast cancer patients to demonstrate the effect of exemestane on estrogen generation, supporting inhibition of aromatase activity.

1. Introduction

Endogenous estrogen levels have been positively correlated with an increased risk of breast cancer, particularly in postmenopausal women [1]. The metabolic pathway of estrogen is

* Corresponding author. Tel.: +780 492 5094; fax: +780 492 7800.
E-mail address: xingfang.li@ualberta.ca (X.-F. Li).

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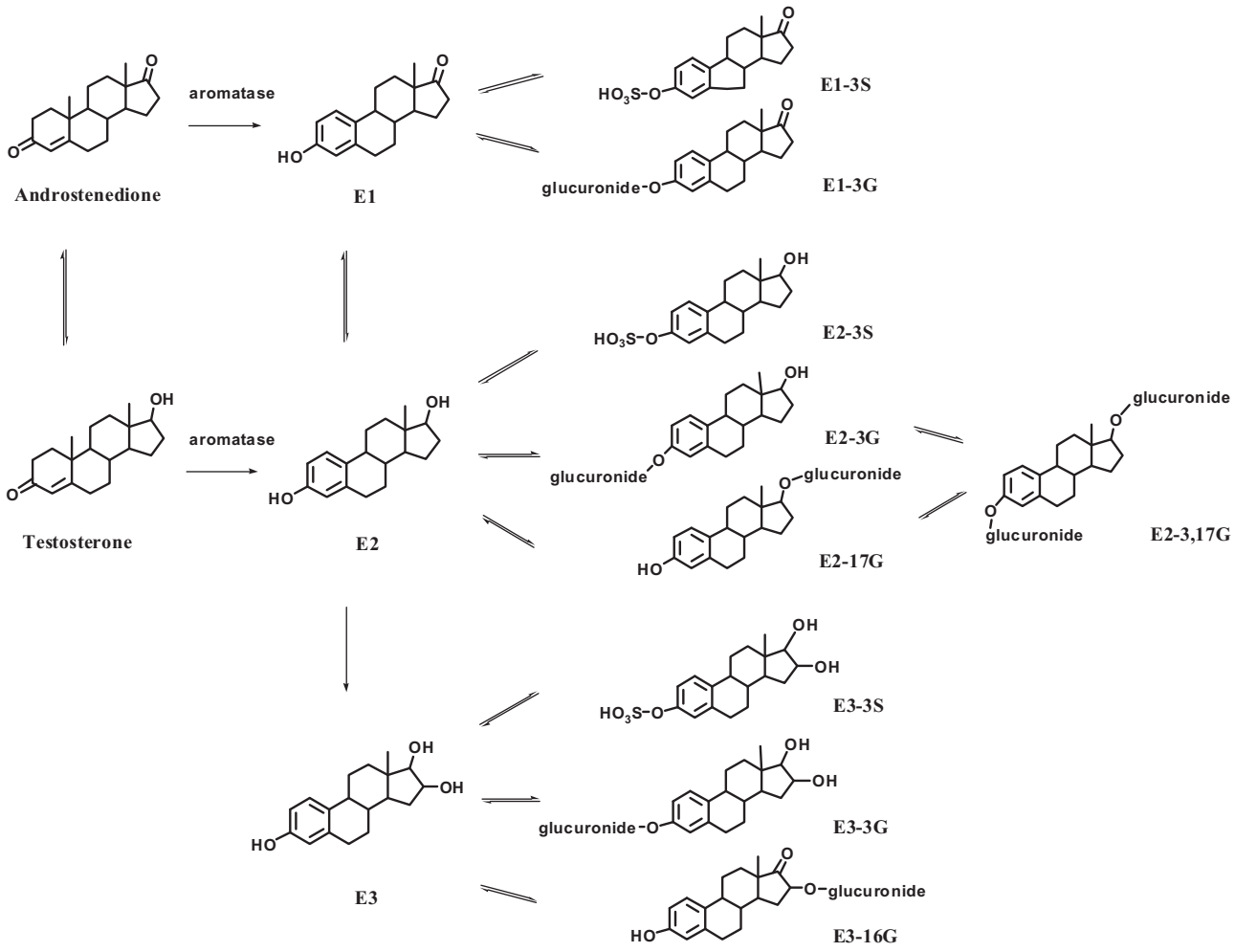

Fig. 1. Biosynthesis and metabolism pattern of estrogens.

complex, consisting of free estrogens and a number of metabolites, including glucuronidated and sulfonated conjugates [2] (Fig. 1). While free estrone (E1), estradiol (E2), and estriol (E3) are bioactive, their sulfonated and glucuronidated conjugates are not, or are less so [3,4]; however, conjugation affects the distribution and excretion of estrogens. Sulfonated estrogens have an increased half life in blood and are the most abundant estrogen conjugates present in serum. It is hypothesized that this increased half life may allow them to be transported to specific tissues where the sulfonate conjugate can be removed and the free estrogen can act upon cells [5]. Glucuronidated estrogens are more readily excreted in the urine than sulfonated conjugates, but can also be hydrolyzed back to bioactive free estrogens [6]. Due to these complex biosynthetic and metabolic processes, estrogens and estrogen conjugates coexist in human fluids in a dynamic equilibrium. Therefore, information on estrogen profiles, besides on just free estrogen levels, in circulating serum and urinary excretion may have clinical significance related to breast cancer [7]. However, little information is available, particularly in patients undergoing anti-estrogen chemotherapy treatments for breast cancer.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) based techniques have become popular for analysis of estrogens in clinical samples. Several methods have been published for analysis of estrogens in urine [8–14] or serum [15–18]. However, these methods must overcome several analytical challenges including limited sample volumes, differing ionization characteristics between estrogens and conjugates, and potentially laborious sample preparation steps. Methods capable of measuring both estrogens and their conjugates in one sample should also significantly reduce the serum/urine sampling volume. One method published analyzes for free estrogens and hydroxyl metabolites as well as their glucuronide and sulfate conjugates in both urine and serum [8,17,19]. The method measures the dansylated form of the free estrogens and metabolites. Conjugates are not directly detected, but rather are hydrolyzed and then derivatized like the free estrogens. The direct determination of estrogen conjugates is useful in order to avoid hydrolysis and derivatization steps. In addition, the conjugate concentrations are clinically relevant, particularly in response to breast cancer treatment.

To address these analytical issues, we developed a new reversed phase-LC–MS/MS method using electrospray ionization (ESI) and multiple reaction monitoring (MRM) to quantitatively determine three free estrogens (E1, E2 and E3) and nine estrogen conjugates [estradiol-3-sulfate (E2-3S), estrone-3-sulfate (E1-3S), estriol-3-sulfate (E3-3S), estrone-3-glucuronide (E1-3G), estradiol-3-glucuronide (E2-3G), estradiol-17-glucuronide (E2-17G), estradiol-3,17-glucuronide (E2-3,17G), estriol-3-glucuronide (E3-3G) and estriol-16-glucuronide (E3-16G)] in both urine and serum samples. The method involves using the same SPE step for both estrogens and their conjugates, after which the estrogen conjugates can be analyzed directly using negative ESI–MS. The free estrogens undergo a further dansyl chloride derivatization step after which they are analyzed by positive ESI–MS. The developed