Molecular and Cellular Endocrinology 466 (2018) 81–85

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Mass spectrometry-based metabolic signatures of sex steroids in breast cancer

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ARTICLE INFO

Article history:
Received 17 April 2017
Received in revised form
15 September 2017
Accepted 15 September 2017
Available online 18 September 2017

Keywords:
Sex steroid
Mass spectrometry
Chromatography
Metabolite profiling
Breast cancer

ABSTRACT

Owing to controversy over the effects of steroids on breast cancer pathophysiology, comprehensive quantification of steroid hormones has been extensively considered in both clinical practice and biomarker discovery studies. In contrast to the traditional immunoaffinity-based assays, which show cross-reactivity and have poor validity at low levels of sex steroids, mass spectrometry is becoming a promising tool for measuring steroid levels in complex biological specimens. The Endocrine Society has announced and continuously updated on technical advances to apply high-quality breakthroughs in the clinical sciences. To avoid incorrect estimation of the steroids of interest, however, further emphasis should be made on the efficient separation by chromatography, such as gas and liquid chromatography, prior to mass spectrometric (MS) detection. Recent advances in MS-based analysis of sex steroids associated with breast cancer enable accurate quantification of circulating as well as localized steroids from frozen tissue slices, allowing these assays to be more powerful in clinical practice.

Contents

1. Introduction

Breast cancer (BCa) risk is associated with reproductive events and lifestyle parameters that lead to altered sex steroid levels. Estrogens play a primary biochemical role in the development and growth of human BCa, which is based on estrogen receptor (ER)-mediated mitogenic and genotoxic effects (Preston-Martin et al., 1990). The levels of endogenous estrogens have a strong relationship with BCa risk (Colditz, 1998) and they are converted into active derivatives catalyzed by hydroxylases and catechol-*O*-methyltransferase, for independent expression in target tissues (Fig. 1).

Hydroxylated estrogens may have an important biochemical role in the onset and clinical progression of BCa. Increased intratumor 16α-hydroxyestrone level was associated with prolonged survival of patients with BCa (Castagnetta et al., 2002), while no association with 2-hydroxyestrone was observed in ER (+)/progesterone receptor (PR) (+) tumors, but a good correlation was observed in ER (−)/PR (−) tumors (Eliassen et al., 2008). In contrast, no significant correlation between BCa risk and serum levels of 2-

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http://dx.doi.org/10.1016/j.mce.2017.09.023
0303-7207/

Abbreviations

BCa	breast cancer
ER	estrogen receptor
DHT	5α-dihydrotestosterone
DHEA	dehydroepiandrosterone
LC-MS	liquid chromatography-mass spectrometry
GC-MS	gas chromatography-mass spectrometry
CRM	certified reference material
ESI	electrospray ionization
MS/MS	tandem mass spectrometry

hydroxyestrone, 16α-hydroxyestrone, and their ratio was reported in a case-control study on premenopausal women (Arslan et al., 2009).

High aromatase activity in BCa results in increased levels of intratumor estrone and 17β-estradiol, which are metabolized from the two androgens, androstenedione and testosterone, respectively (Fig. 1). Therefore, testosterone levels should be monitored together as a part of the prognostic follow-up because increased plasma testosterone may be a good predictor of poorer prognosis in postmenopausal women with BCa (Micheli et al., 2007; Secreto et al., 2009). Serum testosterone levels were also increased in the presence of a metabolic syndrome, which was closely related to BCa progression (Pasanisi et al., 2006). In addition, testosterone rather than 17β-estradiol might be more strongly associated with BCa risk (Cummings et al., 2005).

The metabolic balance between the catechol and methoxylated estrogens as well as between major estrogens and androgens, may affect the BCa risk. Thus, an overview of the sex steroid profile, which provides the metabolic signatures (Ha et al., 2009; Baglietto et al., 2010), is necessary and helps understand the pathophysiological actions of sex steroids in BCa development and treatment. Here, the recent advances in chromatographic separation coupled to mass spectrometric detection of sex steroids over the traditional immunoaffinity-based assays are discussed.

2. Analytical issues about steroids

The immunoaffinity-based biochemical assays for analysis of sex steroids, such as testosterone and 17β-estradiol, are simple to perform and have good detection sensitivity (Sikaris et al., 2005; Handelsman et al., 2014). Although they are very useful as a screening method in clinical practice, drawbacks of immunoassays, such as cross reactivity caused by structural similarity and conjugation diversity of steroid backbone, have been well described (Taylor et al., 2015). Testosterone detection using immunoassays is often hampered due to background interference by dehydroepiandrosterone (DHEA) sulfate, which is an abundant steroid in the human serum (Middle, 2007), similar to cortisol precursors obtained from patients administered with metyrapone that interfere in serum cortisol quantification (Monaghan et al., 2011). Immunoaffinity-based assays provide valuable results in primitive experimental conditions without any special requirements, but their variability and specificity should be considered if steroids are present at low levels with matrix interference. As an alternative assay, liquid chromatographic purification prior to radioimmunoassay using estradiol-6-(*O*-carboxymethyl)-oximino-2-(2 [^{125}I]-iodo-histamine) as a ligand was firstly introduced to overcome the methodological problems in the low range of estrogens. The method successfully reduced possible cross reactivity by other compounds in breast tissues obtained from patients with malignant (Geisler et al., 2000) and undergoing aromatase inhibitor

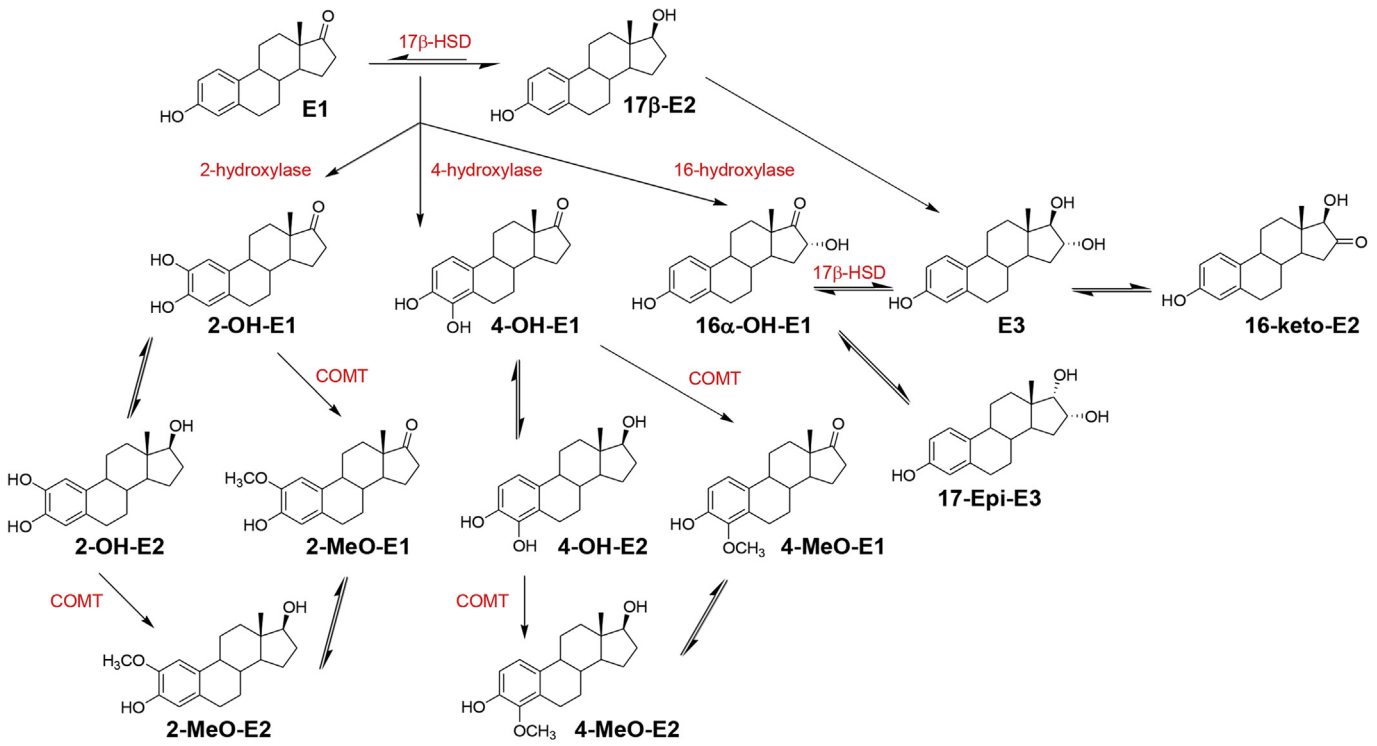

Fig. 1. Representative metabolic pathways of estrogens. Major estrogens, estrone and 17β-estradiol, which are synthesized from androstenedione and testosterone, respectively, are hydroxylated at the 2, 4, and 16 carbon positions, to yield 2/4-OH-estrogens and 16α-OH-estrone. Further metabolisms are also catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) and catechol-O-methyltransferase. E1: estrone; E2: estradiol; E3: estriol.

treatment (Geisler et al., 2008b). It showed the classical immunoassays are still applicable enough in clinical studies. In recent, mass spectrometry-based methods have led to the development of high-throughput profiling techniques enabling more comprehensive clinical diagnostics with less labor intensive processes to evaluate the altered steroid metabolism (Handelsman et al., 2014).

Mass spectrometry is not used alone, but is coupled to chromatographic separation, which increases analytical selectivity and makes more feasible in quantification from complex biological specimens. Metabolomic platform coupled to gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) can help in understanding the pathophysiological changes and investigating the predictable biomarkers of biological variations (Shackleton, 2010; Choi and Chung, 2015). Non-targeted metabolite profiling gives a broad spectrum of potent biomarkers from energy metabolism and lipid profiles (Rijk et al., 2012). However, steroid hormones, including sex steroids, have not been mainly measured to reveal metabolic signatures due to lack of the structural discrimination and biological concentration even in hormone-dependent cancers (Lloyd et al., 2015). Alternatively, the data-dependent non-targeted metabolite profiling focused on steroid molecules may be applicable to discover potent biomarkers of steroid metabolism (Jung et al., 2010). Metabolite profiling of several steroid molecules at a time enables individual quantification as well as calculation of metabolic ratios, which may represent enzyme activities of interest (Moon et al., 2009; 2013; Ha et al., 2009). Evaluation of endogenous metabolic ratios associated with specific enzyme may be the useful tool as the multiplexed enzyme assay, and its pharmacometabolomic approach could be applicable to predict drug clearance and further insights of drug metabolism (Shin et al., 2013).

Nonetheless, the clinical mass spectrometric assay may have some drawbacks to be indicated as the “gold standard”. Accurate quantification of sex steroids could be done by a standardized method conducted using either certified reference materials (CRMs) or quality-controlled calibration samples combined with isotope-labeled internal standards. Sampling steps, including collection, delivery, and storage, are very important to obtain comprehensive results, and they have been well documented and applicable in many clinical laboratories. For calibration sets, steroid-free biological specimens are carefully prepared as the negative control samples. Solid-phase or liquid-liquid extraction techniques for isolation of sex steroids from pooled specimens (Moon et al., 2011a; Jung et al., 2011) are commonly used relative to the commercially available materials (Ha et al., 2009), but most of them failed to meet the real negative specimens and the commercial products often needed for further purification (Lee et al., 2016b). Although the calibration protocols of some methods have clearly improved using the CRM in sex steroid analysis (Thienpont and De Leenheer, 1998; Botelho et al., 2013), these methods are still necessary the progress the harmonization of MS-based clinical laboratory methods.

3. Mass spectrometry in sex steroid analysis

Due to increasing questions about the validity of immunoassays, as most of the estrogens, especially 17β-estradiol, are present at extremely low levels in men, pre-pubertal children, and postmenopausal women (Lee et al., 2014; Faqehi et al., 2016). Increased sensitivity with a good reproducibility of the quantitative analysis of estrogens is therefore required over the conventional detectability at sub-ng/mL concentrations (Jaque et al., 2013). The levels of estrogens in BCa tissue on an average exceed plasma as well as benign breast tissue levels, while plasma concentrations of 17β-estradiol are commonly <184 pmol/L in men and 7–77 pmol/L in postmenopausal women (Faqehi et al., 2016). The plasma levels of estrone and 17β-estradiol during therapy with an aromatase inhibitor are 70–80 pmol/L and 15–20 pmol/L, respectively (Lønning et al., 2009). The optimized immunoassay could detect down to 1.14 pmol/L for estrone and 0.67 pmol/L for 17β-estradiol (Geisler et al., 2008a).

The classical techniques, such as chemical derivatization, coupled with GC-MS for analyzing sex steroids from human biological fluids have been continuously utilized (Krone et al., 2010; Choi and Chung, 2015). Compared to traditional GC-MS steroid profiling with trimethylsilyl derivatization (Moon et al., 2009; Hoffmann et al., 2010), alternative chemical reactions have been studied for improved analytical sensitivity of active androgen and estrogen metabolites, which could successfully quantify sex steroids at concentrations as low as sub-ng/mL (Moon et al., 2011a; 2011b; Lee et al., 2012). Despite recent advances in GC-MS, the GC-MS-based methods have been hampered due to inadequate sensitivity for screening of sex steroids from limited amounts of clinical specimens. In addition, sample size is often limited with dried blood spots and hair as the non-invasive samples (Dietzen et al., 2016; Son et al., 2016), especially in clinical practice and diagnostic development (Blonder et al., 2008). The GC-MS/MS method has been introduced in steroid analysis and it provides improved analytical efficiencies in terms of sensitivity and selectivity (Caron et al., 2015; Lundell et al., 2017). The exact quantification of steroids with an acceptable reproducibility at concentrations much lower than ng/mL (sub-nmol/L) derived from small amounts of clinical samples should be conducted with clinical evaluation and biomarker discovery studies. However, GC-MS is still compromised due to better chromatographic resolution of epimeric steroids, which is particularly useful in screening metabolic disorders, and it allows the database-dependent non-targeted metabolite profiling as a discovery tool of novel biomarkers (Krone et al., 2010; Jung et al., 2010; Asiago et al., 2010).

LC-MS, particularly electrospray ionization coupled to tandem mass spectrometry (ESI-MS/MS), has been replacing classical GC-MS methods for targeted steroid analysis (Rijk et al., 2012). Both chromatographic resolution and analytical sensitivity in LC-MS-based methods have been recently improved by high-pressure column techniques derived from small particle LC column materials (<2.0 μm) and/or high flow-rate against column dimension (Cho et al., 2009; Ke et al., 2014; Lee et al., 2016b). As compared to GC-MS, LC-MS analysis of steroids does not require hydrolysis and chemical derivatization steps during sample preparation in the presence of a good sensitivity (Schofield et al., 2017). However, the advances in ultra-sensitive LC-MS-based analysis of sex steroids over that of immunoassays have been achieved with chemical derivatization (Faqehi et al., 2016; Kim et al., 2016), and the steroids could be detectable to as low as 0.5 fg/mL (0.036 pmol/L) levels from 0.1 mL human serum (Keski-Rahkonen et al., 2015; Wang et al., 2015). The LC-MS-based ultra-sensitive steroid profiling techniques for screening and diagnostic steps in clinical practice will be continuously developed and may be applicable for ensuring the quality of pathophysiological sciences as the routine clinical mass spectrometry.

4. Recent advances in analysis of steroids from breast tissue

In general, the levels of localized sex steroids are measured from tissue samples (Blankenstein et al., 1999; Bélanger et al., 2006; Stanczyk et al., 2015). Due to *in situ* production of both androgens and estrogens from circulating precursors in BCa, the levels of steroids in the breast tissue were evaluated to monitor the effects of estrogen therapy (Eliassen et al., 2006). The results of immunohistochemistry indicate that the epithelial cells contain the

mediating receptor of 17β-estradiol in the breast tissues and the luminal epithelial cells are responsible for most of the breast tumors, which can be confirmed with biochemical comparisons (Anderson et al., 1998). In general, estrogen levels are not exactly matched with the ER status in the breast tissues, but the intratissue estrogen levels are still comparable to provide better understanding of the pathophysiological events. For example, significantly increased levels of both 2- and 4-hydroxyestradiol, along with a marked increase in 16α-hydroxyestrone, were firstly observed in cancerous tissues as compared to the normal breast tissues (Castagnetta et al., 2002).

Based on analytical feasibility, the levels of epithelial and stromal cells, and fats, which are derived from neoplastic, glandular, and adipose tissues, in the breast tissue differ from those in the peripheral blood. The comprehensive analytical protocols are therefore challengeable (Falk et al., 2008). Comprehensive analytical methods with an acceptable sensitivity to investigate the localized steroid actions from practical biopsy samples would be essential features for routine clinical assays, especially, from the samples obtained from postmenopausal women as well as patients who are in treatment with aromatase inhibitors (Geisler and Lønning, 2005; Geisler et al., 2010). Highly sensitive estrogen analysis was successfully conducted with immune-affinity based detection (Lønning et al., 2009). This method describes that both estrone and estrone sulfate were converted into 17β-estradiol to allow final detection with a highly specific antibody. However, the indirect method for the evaluation of estrogen sulfates after the hydrolysis may be associated with incomplete deconjugation mainly caused by sample matrix effect and different hydrolytic activity. Therefore, immunoassay-based analysis of estrogens at the extremely low concentration may need a confirmatory analysis with better sensitivity and specificity, such as liquid chromatography-mass spectrometry (Jaque et al., 2013). A novel LC-MS/MS method showed excellent sensitivity and chromatographic resolution for 7 estrogens and testosterone in 8-μm tissue sections obtained from a frozen breast carcinoma sample (Blonder et al., 2008), which may be one of the cutting edge techniques in steroid analysis. Considering both merit and limitation which of different techniques, immune-affinity and MS-based analyses, the sensitive immunoassay detection coupled to the LC fractionation (Geisler et al., 2000) could be an alternative option when small numbers of targeted analytes and samples are conducted as the confirmatory analysis.

5. Understanding MS detection and conclusions

The importance of accurate measurement of steroids using clinical mass spectrometry cannot be over-emphasized and there is no argument recently that immunoassays of steroids are methodologically inadequate in clinical researches. The Endocrine Society has been continuously updated on the requirement for mass spectrometric sex steroid assays since it was specially featured in 2013 (Handelsman and Wartofsky, 2013). However, chromatographic separation, which can reduce sample complexity as well as increase analytical sensitivity, prior to mass spectrometric detection, should not be overlooked by clinical scientists. Although the advanced mass spectrometry provides better chances to identify unknown molecules with a good accuracy, quantification is achieved based on characteristic ions detected in the selected-ion monitoring and collision-induced dissociation scan modes, which means that the background noise may be present resulting in a systematic error for exact quantification.

To be the “gold standard” in both clinical practice and high-quality steroid research, the comprehensive calibration procedures based on CRM are standardized and harmonized in mass spectrometry-based sex steroid assays combined with the well-defined chromatographic selectivity, which should be extensively monitored matrix interferences against individual steroids, such as isobaric and metabolic effects (Middle, 2007; Lee et al., 2016a). In addition to the requirement in sex steroid analysis, it can be expected that mass spectrometry-based assays will extend to corticosteroids and sterols, which may be the risk factors for BCa although their pathophysiological mechanisms have not been well understood (Sørensen et al., 2012; Nelson et al., 2014).

Competing interests

The author has no conflicts of interest to declare. The funding sources had no any part in the writing of the manuscript or decision to submit for publication.

Acknowledgements

This study was supported by a grant from the Korea Institute of Science and Technology Institutional Program (Project No. 2E26990) and the Bio & Medical Technology Development Programs (NRF-2016M3A9B6902060) through the Ministry of Science and ICT.

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