What are the main considerations for bioanalysis of estrogens and androgens in plasma and serum samples from postmenopausal women?

“LC–SRM/MS-based methods allow the simultaneous determination of multiple estrogens in a single analysis.”

Keywords: androgens • breast cancer biomarkers • endometrial cancer biomarkers estrogens
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In postmenopausal women, circulating androgens from the adrenals serve as precursors for the formation of unconjugated estrogens in tissues. Estrogens are synthesized in breast tissue and endometrium as well as in other tissues from androgen precursors by the enzyme aromatase, which is expressed by the cytochrome P-450 (CYP) 19 gene. This pathway of biosynthesis provides the major source of circulating unconjugated estrogens in postmenopausal women. In contrast, much higher concentrations of circulating estrogens are released primarily from the ovaries of premenopausal women. Other circulating forms of estrogens in postmenopausal women include methyl ether metabolites as well as β -glucuronide and sulfate conjugates. However, only the sulfate conjugates can serve as precursors (through the action of sulfatases) of the corresponding unconjugated estrogens in tissues [1]. Estradiol (E2) is one of the essential sex hormones that are required for reproduction in premenopausal women. E2 is thought to promote breast and endometrial cancer in postmenopausal women through its ability to enhance abnormal cell proliferation as well as by the biotransformation of estrone (E1) and E2 into genotoxic catechol metabolites [2]. Different CYP isoforms can oxidize E2 and E1 to the corresponding 2,3- and 3,4-catechols. The catechols can subsequently be detoxified by catechol O-methyltransferases, uridine diphosphate-glucuronosyltransferases or sulfotransferases. If these pathways are overwhelmed, the catechols will form highly reactive quinones

that can modify DNA directly or induce the formation of reactive oxygen species resulting in oxidative DNA-adduct formation. It has been proposed that 2,3-quinones form stable DNA adducts, whereas the 3,4-quinones form unstable depurinating DNA-adducts that result in abasic sites in the DNA [3,4].

Elevated unconjugated plasma or serum estrogens in postmenopausal women are thought to be associated with an increased risk for breast and endometrial cancer. The levels of unconjugated estrogens in serum and plasma are extremely low (low pg/ml range, even fg/ml for some metabolites) and their accurate quantification is particularly challenging. Over the years three major analytical approaches have been implemented: the radioimmunoassay (RIA) coupled with chromatography, stable isotope dilution GC–SRM/MS and stable isotope dilution LC–SRM/MS. All three methods are still used today. RIA methods have applications in the rapid determination of higher levels of the main estrogen metabolites. LC–SRM/MS methods are becoming the most used for the extremely low abundance metabolites in postmenopausal women. One of the advantages of the LC–SRM/MS methods is the simultaneous determination of multiple estrogens in a single analysis [5] making the most efficient method for large population studies, where a panel of estrogen metabolites can be quantified. More relevant conclusions about the cancer risk can be made based on correlation between levels of different estrogens or androgen precursors.

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Reported values for circulating E2 have a mean value of 7.3 pg/ml [1,6]. E1 measurements are not particularly challenging since its concentration is higher, but all the other metabolites are at the same levels as E2 or lower. Many of the available methods using GC–SRM/MS and LC–SRM/MS have a LOQ higher than the mean concentration of the estrogens of interest.

To improve the sensitivity for estrogen analysis by LC–SRM/MS, three different derivatization approaches have been used. The first and the most widely employed method uses dansyl chloride [7] to derivatize the phenolic hydroxyl group coupled with ESI/MS. The second approach involves derivatization of the phenolic hydroxyl group with an electron-capturing group such as pentafluorobenzyl [8] followed by electron capture atmospheric pressure chemical ionization. Electron capture atmospheric pressure chemical ionization is known to be less susceptible to suppression of ionization than ESI and LODs in the low pg/ml range for most estrogens can be attained [9]. The third approach involves the use of a pre-ionized derivatives, so the analyte does not have to undergo protonation from the mobile phase before entering the ESI source [10]. When pre-ionized derivatives are coupled with nanoflow LC [11], the LOD is lower than for the previous methods allowing analysis in the fg/ml range.

Stable isotope dilution LC–SRM/MS-based methodology is now accepted as the ‘gold-standard’ to quantify the low femtomolar amounts that are present in serum and plasma of postmenopausal women. There is still a need for highly sensitive methods that are able to utilize low serum volumes (most methods use 500 µl) and be able to quantify estrogens and androgens simultaneously. We have reported a method for the analysis E1 metabolites that can be extended to include all androgens [11] containing a keto group. This method, which employs a pre-ionized derivative coupled with nanoflow LC, allows the use of only 100 µl serum. We will outline the necessary steps for using this methodology to accurately quantify unconjugated estrogens and androgens in plasma and serum samples from postmenopausal women. Along with the preparation of calibration curves in the matrix of interest and quality control samples that need to be run with every batch of samples, we will emphasize several other aspects that require special attention when conducting estrogen analyses.

Sample clean-up

This is one of the most critical steps since interference coming from matrix and other isomers present in the biological fluids is a major challenge in estrogen analysis. Most protocols used for sample clean-up SPE [12] or liquid–liquid extraction [13]. The availability of the [¹³C]-labeled internal standards for all the estrogen

metabolites makes it possible to identify background contamination coming from extraction steps. It is particularly important to have blank samples with just the internal standard mix added from the beginning that are then carried through the entire workflow, including the hydrolysis step, for the determination of the conjugated estrogens and androgens. The β-glucuronidase/arylsulfatase enzymes used for the hydrolysis of conjugates, as well as the glassware used, can increase the background levels, which could interfere with the analyte signals, especially for the low abundant metabolites. In addition, it is advisable to test each internal standard individually and check for each endogenous estrogen levels, since we encountered cases when one internal standard was contaminated with a different unlabeled estrogen.

Derivatization

The same cautionary steps need to be taken for the derivatization step, as with the clean-up. We had a batch of derivatization reagent that was contaminated with E2, so this must be checked as well.

Chromatographic resolution

LC separations represent one of the most time-consuming aspects of estrogen analysis. Reducing the LC run time can have a profound impact on the number of assays that can be performed in a day. It is important that isomeric estrogens are separated from each other. Metabolites of E2 and E1 have only a 2 Da difference in mass and the E1 metabolites are often present at much higher levels. When using low resolution triple quadrupole instruments coupled with LC–SRM/MS a spillover of the E1 protonated molecule (MH⁺) arising from the endogenous isotopes such as ¹³C, and ¹⁸O (M + 2) can often be observed in the E2 metabolite channels. This requires adequate chromatographic resolution between all the estrogen metabolites, which leads to longer chromatographic runs. This must be balanced against the accuracy that arises from the clear separation of all the isomers.

Hydrolysis

The analysis of the total (unconjugated + conjugated) estrogens after the hydrolysis by β-glucuronidase/arylsulfatase enzymes is less challenging than the unconjugated estrogens since the levels of the released estrogens is higher. Caution should be taken to check for interferences from the enzymes. Since the conjugates could be glucuronide or sulfates, either an enzyme that has both of these activities can be used (such as *Helix pomatia*, which has both β-glucuronidase and arylsulfatase activity) or a β-glucuronidase (from *Escherichia coli*) followed by a β-glucuronidase + arylsulfatase. The difference between the treatments provides the sulfate levels [14].

Analysis of androgens by LC–SRM/MS is less technically challenging, since the androgens are more abundant than the estrogens in postmenopausal serum. However, the same care with appropriate controls needs to be taken as indicated for the estrogens. Particular attention should be taken in choosing the enzyme for deconjugation. Tamae *et al.* [14] showed that the *Helix pomatia* enzyme, which has both a β -glucuronidase + arylsulfatase activity, and is used quite extensively for the androgen analysis, is contaminated with 3β -hydroxysteroid dehydrogenase activity which can transform dehydroepiandrosterone into 5α -androstene-3,17-dione (AD). Considering that the levels of dehydroepiandrosterone are in the low ng/ml range [1] and the AD levels are about two orders of magnitude lower [1], even low 3β -hydroxysteroid dehydrogenase activity could lead to elevated levels of AD.

In summary, there are many pitfalls that can be encountered in the analysis of serum estrogens and androgens particularly when quantifying conjugated forms. One way to avoid the drawbacks associated with

the hydrolysis would be to analyze the intact conjugates directly as recently described by Zhao *et al.* [12]. In this way would be easier to get a complete picture of the different conjugates. The sulfates are probably the most relevant conjugates, since the circulating sulfates can be hydrolyzed by the tissue sulfatase to release the bioactive unconjugated estrogens [15]. Finally, there is increasing interest in the use of high-resolution mass spectrometers for estrogen analysis. This will almost certainly contribute substantially to the improvement of the existing assays by improving specificity and sensitivity [16].

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