Published in final edited form as:

J Steroid Biochem Mol Biol. 2016 September ; 162: 70–79. doi:10.1016/j.jsbmb.2016.01.002.

Ultra-high sensitivity analysis of estrogens for special populations in serum and plasma by liquid chromatography-mass spectrometry: assay considerations and suggested practices

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Abstract

Estrogen measurements play an important role in the clinical evaluation of many endocrine disorders as well as in research on the role of hormones in human biology and disease. It remains an analytical challenge to quantify estrogens and their metabolites in specimens from special populations including older men, children, postmenopausal women and women receiving aromatase inhibitors. Historically, immunoassays have been used for measuring estrogens and their metabolites in biological samples for risk assessment. However, the lack of specificity and accuracy of immunoassay-based methods has caused significant problems when interpreting data generated from epidemiological studies and across different laboratories. Stable isotope dilution (SID) methodology coupled with liquid chromatography-selected reaction monitoring-mass spectrometry (LC-SRM/MS) is now accepted as the 'gold-standard' to quantify estrogens and their metabolites in serum and plasma due to improved specificity, high accuracy, and the ability to monitor multiple estrogens when compared with immunoassays. Ultra-high sensitivity can be obtained with pre-ionized derivatives when using triple quadrupole mass spectrometers in the selected reaction monitoring (SRM) mode coupled with nanoflow LC. In this review, we have examined the special issues related to utilizing ultra-high sensitivity SID LC-SRM/MS-based methodology to accurately quantify estrogens and their metabolites in the serum and plasma from populations with low estrogen levels. The major issues that are discussed include: sample

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preparation for both unconjugated and conjugated estrogens, derivatization, chromatographic separation, matrix effects, and assay validation.

Graphical Abstract

Keywords

Estrogens and their metabolites; Older men; Children; Postmenopausal women; Liquid chromatograph-tandem mass spectrometry; Stable isotope dilution methodology; Derivatization

Estrogen measurement, one subset of steroid analysis, plays an important role in the clinical evaluation of many endocrine disorders as well as in research on the role of hormones in human biology and disease [1, 2]. For almost 30 years the predominant methodologies used to quantify circulating levels of estrogens were conventional radioimmunoassays (RIAs) or direct enzyme immunoassays [3–5]. These assays afford good sensitivity, but often lack specificity due to the cross-reaction of the antibodies used in the assays with other steroids. The inter-laboratory variability of immunoassays has caused significant problems when interpreting epidemiologic studies [4, 6]. Another limitation of immunoassay-based methods is the reliance on single analyte-by-analyte measurement, thus multiple assays are required to measure all the metabolites from a single estrogen [7]. Over the last decade, significant advances in stable isotope dilution (SID) methodology coupled with liquid chromatography-selected reaction monitoring-mass spectrometry (LC-SRM/MS) have been made that can potentially offer a solution to these problems [4–9]. However, it is still challenging to quantify estrogens and their metabolites where ultra-high sensitivity is required, such as serum from older men [10], children [11–13], postmenopausal women and women receiving aromatase inhibitors for breast cancer treatment [4, 6]. The present review is focused on the issues and considerations when ultra-high sensitivity estrogen analysis is employed in serum and plasma. Some suggested practices for sample preparation, analyte derivatization, chromatographic separation, minimization of matrix effects, and assay validation for optimal use of SID LC-SRM/MS-based methodology have been discussed.

1. Estrogen metabolism

Several clinical and experimental studies showed that relatively high estrogen levels in the serum or plasma in women were associated with increased breast cancer risk [4, 6, 14, 15], endometrial cancer [16, 17], and ovarian cancer [18] although notably, these studies used RIA assays of questionable validity [5]. Increased circulating estrogens are also a potential risk factor for prostate cancer in men [10, 19]. In addition, diagnosis and treatment of disorders of puberty and sexual development have demonstrated the critical nature of quantitation of the levels of estradiol (E2) and testosterone (T) present in children [11, 12].

Some studies also showed that higher levels of estrogens at earlier ages are correlated with the onset of breast development in girls, suggesting that hormones during development might modulate disease risk later in life [20].

Estrogens are thought to act through either estrogen receptor (ER)-dependent or ER-independent mechanisms [21, 22]. The mechanism mediated by ER results in direct stimulation of aberrant cell proliferation, a factor causally related to breast cancer development [23, 24]. The ER-independent carcinogenic effects are believed to occur through the actions of genotoxic estrogen metabolites [23–26]. Parent estrogens undergo oxidative metabolism by cytochrome P450 (CYP) 1B1 and CYP3A4 to form 2,3-catechol (2-OH-E1 and 2-OH-E2) and 3,4-catechol (4-OH-E1 and 4-OH-E2) estrogen metabolites. CYP1B1 has considerable regioselectivity (9:1) for formation of 4-OH-E1 and 4-OH-E2 [27]; it has been suggested that 4-OH-E1 and 4-OH-E2 are more genotoxic than the corresponding 2-OH-E1 and 2-OH-E2 metabolites [28, 29]. Furthermore, 2-OH-E2 is a potent inhibitor of aromatase [30] and 2-OH-E1 is associated with reduction of risk of ER+ breast cancer in postmenopausal women after adjustment for circulating estrone concentrations [31]. Both catechols are rapidly converted by catechol-O-methyl transferase (COMT) to methoxy (OMe) metabolites [32–34]. It is noteworthy that 2-OMe-E2 has significant anti-proliferative activity [35].

Estrogens and their hydroxylated metabolites are also readily modified by glucuronide, sulfate, or glutathione (GSH) moieties to give conjugated estrogen metabolites. The most abundant circulating estrogen conjugates are sulfates [36], which can serve as estrogen precursors in breast tissue through the action of steroid sulfatases [37, 38]. Circulating androgens can also serve as estrogen precursors in postmenopausal women (through the action of aromatase), which provides a fundamentally different source of estrogens when compared to the contributors to estrogen metabolites in premenopausal women [6, 31, 38]. Thus, considering their effects and metabolism, estrogens as well as their metabolites in the circulation are valuable biomarkers of tissue estrogen biosynthesis and metabolism [22, 39, 40].

2. Special considerations in ultra-high sensitivity required specimens

Typical serum concentrations in postmenopausal women and in older men for unconjugated E2 are often <5 pg/mL and for unconjugated E1 are 11.8–37.4 pg/mL [20, 41]. E2 levels in some pediatric endocrinology clinical studies were reported to be some 100-fold lower than those in adults, with the lowest levels in the pre-pubertal stages [42, 43]. These low pg/mL levels are very challenging for most LC-MS-based assays [6, 14, 41]. Furthermore, other unconjugated estrogen metabolites, such as MeO-estrogens or catechols, have even lower concentrations, with circulating levels generally below the lower limit of quantification (LLOQ) of most LC-MS assays [6, 41, 44].

The sensitivity challenge for LC-MS-based procedures is due primarily to the low ionization efficiencies of unconjugated estrogens and their metabolites. Therefore, many derivatization strategies have been developed in order to improve ionization and increase sensitivity [4, 6, 14]. This also often also helps with sample clean up. Choosing fit-for-purpose sample

preparation processes and establishing derivatization approaches are the first two considerations for ultra-high sensitivity analysis of estrogens in serum and plasma. It is less challenging to quantify total (unconjugated + conjugated) estrogens after hydrolysis or solvolysis because the total levels of estrogens are at least 2–3 fold higher than the unconjugated estrogens [6, 41, 44]. However, the evaluation of hydrolysis efficiency and uniformity across samples is still problematic and could give rise to erroneous results because some authentic standards and stable isotope standards are not available.

Notably, the evaluation of matrix effects is more complicated for analysis of estrogens when a suitable blank matrix is not available. The general approach is to prepare calibration standards and quality control (QC) samples in surrogate matrix, such as charcoal stripped serum [41, 45, 46]. However, analysts should be aware that calibration standards might not be truly representative of the unknown samples because components of real samples that cause matrix effect may be removed during the charcoal stripping process. Thus, spiking in isotope labeled internal standards (INSTDs) at the beginning of sample preparation as well as improving chromatographic separation of the isomers with longer LC gradients are important to ensure accurate absolute quantification. In addition, thorough method validation should be conducted to confirm the assay sensitivity, specificity, and reproducibility prior to clinical samples analysis [5, 47].

SID LC-SRM/MS methods are now accepted as the current ‘gold-standard’ to for quantification estrogen and their metabolites in serum or plasma of postmenopausal women [14]. For example, a high sensitivity SID-LC-SRM/MS method for analysis of E1 and its metabolites was reported in 2011, which employed the Girard P (GP) pre-ionized derivative that had been employed previously to analyze to keto steroids [48] (Fig. 1 and Fig. 2). The high sensitivity allowed the use of only 0.5 mL of serum. The LLOQ for each estrogen was 0.156 pg/mL (15.6 fg on column). In 2015, a similar pre-ionization concept was implemented for E2 and its metabolites using the N-methyl pyridinium-3-sulfonyl (NMPS) derivative [41] (Fig. 3 and Fig. 4). This method required only 0.1 mL of serum, and yet was capable of reaching 1.0 fg on column sensitivity for five estrogens and their metabolites.

3. LC-tandem MS (MS/MS)-based assays for estrogen analysis

3.1. Sample preparation

Sample preparation for analysis of serum and plasma estrogen analysis by LC-MS/MS-based methods such as LC-SRM/MS typically involves extraction, clean up, and concentration. For the conjugated estrogens, hydrolysis can be performed before analysis, or they can be quantified as intact conjugates. The hydrolysis step converts the conjugates into unconjugated forms, hence increasing their concentration in the sample. Subsequent liquid-liquid extraction (LLE) or solid phase extraction (SPE) can be employed to efficiently extract estrogens from serum and plasma [2, 49].

LLE is inexpensive, fast, and often the primary extraction for estrogen analysis. Some drawbacks of LLE are that it is labor-intensive, time-consuming and harder to automate than SPE. The solvents most commonly used are methyl *tert*-butyl ether (MTBE), diethyl ether, dichloromethane or mixtures of organic solvents. MTBE can be used to extract both

unconjugated estrogen from serum samples and estrogen derivatives from derivatization buffer [41, 48]. A thorough investigation of extraction efficiency for MTBE, diethyl ether, hexane and 2-methylbutane from serum samples was reported by Keski-Rahkonen *et al.* [50]. This study showed that LLE with MTBE fully recovered the tested steroid hormones in contrast to the other solvents.

Off-line or on-line SPE coupled with LC-MS is a very promising technique for semi-automated sample analysis. Advantages of on-line SPE include shorter analysis time, more concentrated chromatographic band and greatly reducing of contaminations. One study by Zhao *et al.* reported a LC-MS method for determination of 12 unconjugated estrogens and their intact conjugates in blood and urine [51]. This method used only one SPE step for both unconjugated estrogens and their conjugates. After loading samples on an Oasis HLB (hydrophilic-lipophilic balance) cartridge (Waters, Milford, MA), unconjugated estrogens were first eluted with ethyl acetate, and conjugates were then eluted with methanol containing 0.1% ammonium hydroxide. A more recent study reported an automated on-line trap-and-elute sample treatment process by using a weak cation exchange (WCX) restricted access material (RAM) trap column (Waters) and on-line dilution [52]. This approach has potential application to older men, children, and postmenopausal women samples since the streamlined procedure requires only 100 μ L of serum and an LLOQ of 3 pg/mL with excellent accuracy and precision.

Two approaches have been employed for the analysis of conjugated estrogens. The first approach involves hydrolysis of β -glucuronide and sulfate conjugates before extraction and derivatization steps. The most often used enzyme is β -glucuronidase/arylsulfatase purified from the snail, *Helix pomatia* [46, 53–55]. The enzyme from *Helix pomatia* naturally contains β -glucuronidase and sulfatase activities in almost equal amounts. In contrast, the enzyme from *E. coli* contains only β -glucuronidase and is essentially free of sulfatase activity. However, some evidences showed that *Helix pomatia* extract is contaminated with 3β -hydroxysteroid dehydrogenase (HSD) or cholesterol oxidase activity and could confound studies in which the analytes of interest are 3β -HSD substrates [56]. This is a very important issue if androgens are being analyzed in the same sample. Experiments performed with synthesized estrogen sulfate conjugates showed that only the 3-sulfate is cleavage by enzymatic hydrolysis, whereas the 17-sulfate group is resistant to the enzymatic hydrolysis [57]. A promising method for overcoming this problem involves solvolysis of the conjugates with anhydrous methanolic hydrogen chloride an approach that was first published by Tang and Crone in 1989 [58]. Several groups have used this approach subsequently [57, 59]. Surprisingly, it does not appear to have been employed in studies conducted with serum and plasma samples from older men, children, and postmenopausal women.

The second approach involves analysis of the intact conjugate by MS in negative ion mode without enzyme hydrolysis or derivatization. Recent studies observed that total E1 concentration in postmenopausal women is in the range of 61.3 to 442.1 pg/mL including E1 sulfate at mean concentration of 244.8 pg/mL [6, 44]. These higher levels of E1 glucuronide or E1 sulfate could easily be quantified by an LC-MS-based method. E1 sulfate in serum samples can be efficiently extracted using Oasis HLB [60, 61] or weak anion exchange (WAX) cartridge (Waters, Milford, MA) [62] and eluted with ammonium acetate or

ammonium hydroxide. The use of intact conjugates is very promising but is hampered by the lack of authentic estrogen conjugate standards and heavy stable isotope analogs for use as internal standards. For example, only 17 β -E2-2,4,6-[^2H]₄-3-sulfate is currently available among five possible E2 sulfates (3-sulfate, 17-sulfate, 3-sulfate 17-glucuronide, 3-glucuronide 17-sulfate, 3-,17-*bis*-sulfate).

3.2. Derivatization

Due to the extremely low concentrations of unconjugated estrogens in serum or plasma in older men, children, and postmenopausal women, it is currently necessary to employ derivatization approaches for reliable measurement. A number of chemical derivatization reagents have been used with the purpose of improving the estrogen ionization efficiencies. The derivatization of the phenolic hydroxyl group of estrogen by pentafluorobenzyl (PFB) [63], pyridyl-3-sulfonyl (PS) [64], dansyl (D) [65–67], 2-picolinoyl (P) [68], N-methyl-2-pyridinyl (NMP) [69], N-methyl-nicotinoyl (NMN) [70], 1-(2,4-dinitro-5-fluorophenyl)-4,4-dimethylpiperazinyl (MPPZ) [71], 3-pentafluorobenzyl-17 β -pyridinium (PFBPY) [72] and 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS) [73] derivatives have all been reported in the recent literature. Most of the derivatives (PFB, PNB, PS, D, P-derivatives) do not carry a charge on the molecule, which limits their ability to reach low pg/mL detection limits. Furthermore, due to the chemical similarity of estrogens, the derivatives tend to provide nonspecific fragmentation patterns, which causes poor analytical specificity. The MP, NMN, MPPZ, PFBPY and DMIS derivatives carry a permanent charge making them more amenable to reaching pg/mL sensitivity range. Recently, we developed a new pre-ionized derivatization procedure, which formed E2 and its metabolites as pre-ionized N-methyl pyridinium-3-sulfonyl (NMPS) derivatives. The LLOQ of 1.0 fg on column with 1 μL injection volume makes it very powerful to absolutely quantify unconjugated estrogens in serum samples from postmenopausal women and older men [41]. The derivatization occurs at the phenolic hydroxyl group making the method generalizable to the all of the estrogens. As with any high sensitivity analysis, caution should be taken since small amounts of contamination or carryover from a previous injection, or background from processing may affect the quality of the data. It is particularly important to utilize and appropriate blank matrix to check system background including tubes, extract solvent, derivatization buffer and derivatization solvent used in the sample preparation, and the instrument itself. Therefore tubes, reagents, and enzymes should be pre-tested to make sure they will not bring any contamination or interference for determination of estrogen [14]. For example, we unexpectedly found that some glass tubes cause interference into the E2 signal (equivalent to approximately 10 pg/mL in serum) during enzymatic hydrolysis.

3.3 Chromatographic separation

In LC-SRM/MS-based estrogen quantification, interference arising from isobaric exogenous or endogenous estrogens and other steroids can be a critical factor [2, 74]. Unfortunately, estrogens and their metabolites tend to form similar product ions on collision-induced dissociation (CID) in analysis by tandem MS (MS/MS) [65–67]. For example, the product ion m/z 171 is commonly selected as a quantifier or qualifier of all estrogens and their metabolites, which originated from dansyl group when dansyl-derivatives are analyzed. Therefore, if E1 and its metabolites are not chromatographically separated from E2 and its

metabolites, overestimation of unconjugated E2 may occur since unconjugated E1 is usually 2–3 folds higher. It is more challenging to accurately quantify 2- and 4-OH-E1 and 2- and 4-OH-E2 and their corresponding methoxy-metabolites because the individual isomers must be chromatographically separated from each other. In this regard, increasing peak capacity and optimization of gradient elution are helpful strategies. Furthermore, particle size of the stationary phase can have a profound effect on peak performance and increasingly sub 2 μm particles are used to improve chromatographic capacity as well as sensitivity and speed of analysis [75, 76]. For example, in our recent study, 12 estrogen metabolites can be successfully separated on Waters BEH130 C18 column (150 $\mu\text{m} \times 100 \text{ mm}$, 1.7 μm , 130 Å) within 45 min following pyridinium sulfonyl derivatization including four catechol estrogens (4-OHE1, 2-OHE1, 4-OHE2, 2-OHE2) and four MeO-estrogens (4-MeOE1, 2-MeOE1, 4-MeOE2, 2-MeOE2) (Fig. 5).

3.4 Matrix effects

Serum or plasma contains components such as phospholipids and salts which may enhance or suppress the ionization efficiency of estrogen. Furthermore, Keski-Rahkonen *et al.* encountered matrix effects when LC-MS-based assay was performed in test tubes or well plates made of plastic [50]. Matrix effects have been designated as the potential “Achilles heel” of LC-MS/MS-based analyses of biological samples [77]. In LC-MS and LC-SRM/MS especially, it is important to be aware that the co-eluting compounds will not be always seen in the monitored ranges or transitions, as part of a phenomenon termed “ghost peaks”.

For estrogens analysis, positive mode electrospray ionization (ESI) is the most widely used ionization type after conducting derivatization, but it has been argued to be more susceptible to ion suppression than atmospheric pressure chemical ionization (APCI) [44, 78]. However, it was shown that underivatized E2 had at least a 10-fold increase in sensitivity in the ESI negative mode when compared with atmospheric pressure photoionization (APPI) in the positive mode and did not suffer from interference by co-eluting isobaric compounds [79]. To assess the matrix effects there are three general strategies: (1) post-column infusion, (2) post-extraction addition and (3) a comparison of the slopes of calibration curve. The details of these assessment approaches were discussed in several reviews [80, 81]. FDA guidance from 2013 suggests that appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, especially if the matrix used for production batches is different from the matrix used during method validation. Also, the acceptance criteria are that the measured value should be within the 15% bias range of the nominal value while the CV should be less than 15% [82]. For estrogens quantification, reducing matrix effects has been suggested by improving the sample preparation procedure [83], optimizing chromatographic separation [74], or employing stable isotope labeled internal standards [4]. Caution with [^2H]-analog internal standards should be taken, as a shift in retention time between unlabeled and [^2H]-labeled compounds can be analytically significant. This can lead to differential suppression of the signals from the analyte and internal standard [84]. Ideally, [^{13}C]-containing analogs of the target analytes should be used as internal standards since they exactly co-elute under all chromatographic conditions [84]. Furthermore, they eliminate the possibility of potential deuterium exchange in protic

solvents. With the better chromatographic resolution provided by the sub 2 μm particles, larger separation between analytes and their corresponding [^2H]-analogs could occur, which would further reduce the benefit of internal standard. For example, Berg and Strand investigated the use of [^2H]- and [^{13}C]-analog internal standards using an ultraperformance LC-MS/MS-based method and observed an improved ability to compensate for ion suppression effects when [^{13}C]-analog were used [85]. Fortunately, most of the [^{13}C]-analog internal standards are available for estrogens and their metabolites except for 2-OH-E2.

3.4 Assay validation

Prior to implementing a clinical study, any developed LC-MS method must be rigorously validated to ensure assay reliability [82]. Fundamental parameters for this validation include accuracy, precision, selectivity, sensitivity, reproducibility and stability. It is particularly important to ensure that the assay is accurate because it is relatively easy to ensure LC-MS assays are precise. This means that the authentic standards must be pure and that all standard solutions are monitored by HPLC-UV and are calculated by their extinction coefficient for any decomposition during storage.

Typically, accuracy and precision are evaluated by intra-batch or inter-batch spike-in QC samples. Recovery and matrix effects are then determined by comparing the accuracy and precision data from both surrogate matrix and the actual serum matrix. The spike-in QC samples usually are prepared in a matrix, which lacks the endogenous estrogen and analyzed in replicates of three to five over several days. However, the use of endogenous QCs as well as spike-in QCs can be extremely helpful for monitoring assay performance [45]. The endogenous QC samples are prepared in a pooled serum of unstripped serum, which contains estrogens and their metabolites. LQC, MQC and HQC serum samples are then prepared by spiking at known concentration with an appropriate QC working solution. Caution should be taken when evaluating matrix effect using surrogate matrices (e.g. charcoal stripped serum) since some components of real serum or plasma samples might be removed during their preparation [41, 45]. It is noteworthy that other approaches have been used to evaluate assays without the use of surrogate matrices, such as generating calibration curves by stable isotope labeled analog internal standards coupled with standard addition [45, 75].

Another technique that is often used to confirm assay accuracy is to conduct a standard curve in water or buffer. The regression line should be parallel to that observed in serum or plasma and the intercept on the x-axis of the serum or plasma regression lines should correspond to the negative value for the endogenous analyte concentration. Before implementing a validated LC-SRM/MS-based assay on a large scale of real samples, it is valuable to compare the initial data with other available assays by analyzing a small set of patient samples. Data agreement between two methods should be assessed with a Deming regression [86] and by a Bland Altman comparison [87]. This will help to identify potential problems with the assay, especially those related to endogenous metabolites and matrix effects. It is of particular concern to minimize suppression of ionization from the matrix when using ESI/MS-based methods because this can affect the accuracy of analyses close to the LLOQ where, unfortunately, many samples from postmenopausal women tend to lie.

4. Future perspectives

It is clear that the analysis of estrogens as biomarkers for many endocrine disorders will continue to be an important area for research and there may continue to be a role for RIA-based procedures as long as specific antibodies reagents employed, and assay robustness carefully monitored [47]. SID LC-SRM/MS procedures can potentially provide a gold standard for assessing such procedures [14]. However, as assay sensitivity has increased such as by the use of pre-ionized derivative [41], the potential for interfering substances that elute with similar retention times to the estrogens of interest has also increased. This poses a great dilemma for the analyst particularly with serum or plasma samples that are close to the LLOQ. Often the use of alternative LC stationary phases or changes in extraction procedure cannot resolve the problem and so samples have to be classified as below the detection limit. Fortunately, the availability of instrumentation with higher resolution could potentially provide a solution to this dilemma. For triple quadrupole instruments, the high sensitivity Triple TOF mass spectrometer (Sciex, Redwood City, CA) can operate at a resolution of 30,000. It will be interesting to determine whether this substantial increase in resolution over conventional triple quadrupole instruments (typically 1,000) can improve selectivity. Alternatively, the new hybrid quadrupole/Orbitrap high resolution Q-Exactive HF instruments (Thermo Scientific, San Jose, CA), can routinely offer resolutions exceeding 150,000 with chromatographically compatible scan speeds [88] and provide an alternative approach. It is noteworthy that one study using a previous generation of Q-Exactive, when coupled with the use of 1-methylimidazole-2-sulfonyl estrogen derivatives, provided substantial improvements to sensitivity and specificity as compared with current triple quadrupole-based methodology [89].

The wider availability of higher resolution instruments coupled with their improved sensitivity suggests that the analysis of intact estrogen conjugates will be introduced as a more specific approach compared with the enzymatic hydrolysis procedures that currently predominate. Two major problems that have to be overcome are the lack of appropriate standards and the potential segregation of the LC-MS signal for a specific estrogen into numerous glucuronide and sulfate conjugates. The ultra-high sensitivity and resolution of instruments such as the Thermo Q-Exactive HF Orbitrap should overcome the problem of analyzing numerous conjugates. However, there is a real need for synthetic approaches to build upon the excellent studies of Caron *et al.* [1] to make available appropriate labeled and unlabeled standards. As the importance of estrogen biomarkers for breast cancer risk is more widely recognized, it seems realistic to assume that these standards will become available in the not too distant future. Finally, there is a real need to implement routine specific assays for urinary estrogen DNA-adducts [90] in order to more fully test the hypothesis that estrogens could induce cancer through a genotoxic mechanism [91].

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Highlights

- It remains an analytical challenge to quantify estrogens and their metabolites in specimens from special populations.
- Estrogen levels are at pg/mL in serum or plasma samples from older men, children, postmenopausal women and women receiving aromatase inhibitors for breast cancer treatment.
- Stable isotope dilution LC-SRM/MS assays provide high specificity and accuracy.
- Estrogen derivatives facilitate ultra-high sensitivity LC-SRM/MS-based analysis.
- Suggested practices for ultra-high sensitivity LC-SRM/MS-based methodology are reviewed
- Future perspectives on the use of high-resolution mass spectrometry are discussed.

Figure 1.
Girard P (GP) derivatives of estrone (E1) and its metabolites.

Figure 2.

LC-SRM/MS chromatograms for analysis of E1 and its metabolites extracted from double charcoal-stripped human serum as GP derivatives. A. LLOQ sample (0.156 pg/mL). B. HQC sample (16.0 pg/mL). E1, estrone, 16α-OH-E1, 16α-hydroxyestrone, 4-ME1, 4-methoxyestrone, 2-ME1, 2-methoxyestrone. Reprinted with permission from Rangiah *et al.* [35].

Figure 3.
N-methyl pyridine-3-sulfonyl (NMPS) derivatives of estradiol (E2) and its metabolites.

Figure 4.

LC-SRM/MS chromatograms for analysis of E2 and its metabolites extracted from double charcoal-stripped human serum as NMPS derivatives. A. LOQ sample (1.5 pg/mL). B. HQC sample (175.0 pg/mL). E2, estradiol, 16α-OH-E2, 16α-hydroxyestradiol, 4-MeO-E2, 4-methoxyestradiol, 2-MeO-E2, 2-methoxyestradiol, 4-OH-E2, 4-hydroxyestradiol, 2-OH-E2, 2-hydroxyestradiol. Adapted with permission from Wang *et al.* [31].

Figure 5.

LC-SRM/MS chromatograms for analysis of estrogens and their metabolites extracted from double charcoal-stripped human serum as pyridinium sulfonyl (PS) derivatives.