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Analysis of Estrogens in Serum and Plasma from Postmenopausal Women: Past Present, and Future

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Abstract

Previous studies have shown that the selection of women who are at high breast cancer risk for treatment with chemoprevention agents leads to an enhanced benefit/risk ratio. However, further efforts to implement this strategy will require the development of new models to predict the breast cancer risk of particular individuals. Postmenopausal women with elevated plasma or serum estrogens are at increased risk for breast cancer. Therefore, the roles of various enzymes involved in the biosynthesis of estrogens in postmenopausal women have been reviewed in detail. In addition, the potential genotoxic and/or proliferative effects of the different estrogen metabolites as risk factors in the etiology of breast cancer have been examined. Unfortunately, much of the current bioanalytical methodology employed for the analysis of plasma and serum estrogens has proved to be problematic. Major advances in risk assessment would be possible if reliable methodology were available to quantify estradiol and its major metabolites in the plasma or serum of postmenopausal women. High performance liquid chromatography (HPLC) coupled with radioimmunoassay (RIA) currently provides the most sensitive and best validated immunoassay method for the analysis for estrone and estradiol in serum samples from postmenopausal women. However, inter-individual differences in specificity observed with many other immunoassays have caused significant problems when interpreting epidemiologic studies of breast cancer. It is almost impossible to overcome the inherent assay problems involved in using RIA-based methodology, particularly for multiple estrogens. For reliable measurements of multiple estrogens in plasma or serum, it will be necessary to employ stable isotope dilution methodology in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Extremely high sensitivity can be obtained with pre-ionized estrogen derivatives when employed in combination with a modern triple quadrupole mass spectrometer and nanoflow LC. Using [$^{13}\text{C}_6$]-estrone as the internal standard it has proved possible to analyze estrone as its pre-ionized Girard T (GT) derivative in sub-fg (low amol) amounts on column. This suggests that in the future it will be possible to routinely conduct LC-MS assays of multiple estrogen metabolites in serum and plasma at even lower concentrations than the current lower limit of quantitation of 0.4 pg/mL (1.6 pmol/L). The ease with which the pre-ionization derivatization strategy can be implemented will make it possible to readily introduce high sensitivity stable isotope dilution methodology in laboratories that are currently employing LC-MS/MS methodology. This will help conserve important plasma and serum samples as it will be possible to conduct high sensitivity analyses using low sample volumes.

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Keywords

estrogens; catechol estrogens; radioimmunoassay; electrochemiluminescence immunoassay; stable isotope dilution; gas chromatography/mass spectrometry liquid chromatography/mass spectrometry

1. Introduction

17 β -estradiol (estradiol) induces tumors in animal models and in humans and elevated estrogen levels in postmenopausal women are associated with increased breast cancer risk [1]. This is thought to arise from a dual mechanism in which estradiol can act either as a hormone to stimulate aberrant cell proliferation or as the precursor to the formation of genotoxic metabolites [2]. Estrogen biosynthesis which occurs in the breast tissue of postmenopausal women is fundamentally different from that which occurs in the ovaries of premenopausal women. Unlike the ovaries, breast tissue lacks the ability to synthesize androgen precursors. Hence, estrogen production is dependent upon the availability of circulating C-19 androgen precursors and local conversion to estrogens in target tissues such as the breast. The estrogens can then be released into the circulation, which provides biomarkers of tissue estrogen biosynthesis.

Investigative studies and new therapies have significantly improved the recurrence-free and overall survival rates in breast cancer patients [3]. However, the ability to prevent breast cancer in the first place is a much more desirable goal, particularly as the world population is aging and age is an important determinant of breast cancer risk [4,5]. This requires the selection of women who are at higher breast cancer risk for treatment with chemoprevention agents because previous studies have shown that this approach leads to an enhanced benefit/risk ratio [6,7]. Further implementation of this strategy will require the development of new models to predict breast cancer risk of particular individuals [8]. Postmenopausal women with elevated plasma or serum estrogens are at increased risk for breast cancer [4,9-13]. Unfortunately, much of the current bioanalytical methodology employed for the analysis of plasma or serum estrogens has proved to be problematic [14,15]. Major advances in risk assessment would be possible if more reliable methodology were readily available to quantify estradiol and its major metabolites in the plasma or serum of postmenopausal women [4]. These measurements could then be coupled with other risk factors such as mammographic density [16], bone density [17], BMI [18], and single-nucleotide polymorphisms associated with breast cancer [19] to provide an improved model of breast cancer risk [6]. In addition, the availability of sensitive and specific plasma or serum estrogen assays would make it possible to more reliably assess the effects of aromatase inhibitors in postmenopausal women [20]. The present review will focus on the enzymes involved in estrogen biosynthesis, the biological effects of different estrogen metabolites, and the analysis of total (free and non-covalently bound) forms of estradiol and its metabolites in plasma and serum.

2. Enzymology of estrogen biosynthesis in postmenopausal women

The adrenal cortex is the principal source of the C19 androgens, dehydroepiandrosterone (DHEA) and androstenedione (Figure 1). Cholesterol is first metabolized to pregnenolone by cytochrome P-450 (CYP) 11A1, the side-chain cleavage enzyme [21]. Pregnenolone is then metabolized to progesterone through the action of 3 β -hydroxysteroid dehydrogenase (HSD) or to 17 α -hydroxy-pregnenolone by CYP17A. 17 α -hydroxy-pregnenolone is further metabolized to 17 α -hydroxy-progesterone by 3 β -HSD. 17 α -hydroxy-progesterone can also arise from CYP17A1-mediated hydroxylation of progesterone. Progesterone serves as the precursor to the formation of corticosterone and aldosterone, whereas 17 α -hydroxy-progesterone is the precursor to the formation of cortisol (Figure 1). DHEA arises from

CYP17A1-mediated metabolism of 17α -hydroxy-pregnenolone. It is converted to androstenedione by 3β -HSD-2 [22]. Androstenedione can also arise from CYP17A1-mediated oxidation of 17α -hydroxy-progesterone [21].

Circulating DHEA and androstenedione derived from the adrenal cortex in postmenopausal women can undergo further metabolism to androgens and estrogens in the breast (Figure 1). DHEA is metabolized to androstenediol by 17β -HSDs [21,23] and to androstenedione by 3β -HSD-1 [22]. Androstenediol is converted to testosterone by 3β -HSD-1 [22]. Testosterone can also arise from 17β -HSD-3-mediated reduction of androstenedione in testicular Leydig cells [24] and so this pathway is not significant in premenopausal women. 17β -HSD-1, 5, 7, and 12; the individual isoforms that can metabolize androstenediol to androstenedione, are expressed in epithelial cells of acini and/or ducts as well as in the stromal cells in the breast [25]. Human 17β -HSD-5 belongs to the aldo-keto reductase (AKR) superfamily [26] is identical to AKR1C3 [27]. It is widely expressed in human tissues including the prostate, endometrium, and mammary gland [28]. AKR1C3 as well as 17β -HSD-1, 7, and 12 are all expressed in breast tumor tissue [26,29]. Aromatase (CYP19), which converts androstenedione to estrone and testosterone to estradiol, is expressed in stromal and carcinoma or parenchymal components of breast cancer tissue [30]. Estrone is converted to estradiol in breast tissue by AKR1C3 together with 17β -HSD-1, 7, and 12 (Figures 1 and 2) [23,27,31,32]. In contrast, 17β -HSD-2, converts estradiol back to estrone using NAD^+ as a co-factor [31], in a similar mechanism to that observed with hydroxyprostaglandin dehydrogenase [33]. It is expressed in normal epithelium of the breast and is thought to modulate the exposure of breast tissue to estradiol. In addition to its ability to metabolize estradiol, 17β -HSD-2 can convert testosterone back to androstenedione [34]. The more recently discovered 17β -HSD-14, is also involved in NAD^+ -mediated conversion of estrone to estradiol and so it could also be involved in increasing the exposure of breast tissue to estradiol [35].

Estrogens synthesized in the breast from androgens in postmenopausal women are probably only biologically active at a local tissue level in a paracrine [21] or intracrine fashion [36]. Therefore, the total amount of estrogens synthesized in the breast tissue is quite low but the local concentrations are sufficient to exert significant biological activity. In postmenopausal women mesenchymal cells of the adipose tissue become an important source of estrogens [21]. As a result, the extent of estrogen biosynthesis in postmenopausal women is determined to a significant extent by the amount of adipose tissue that is present. This is of clinical importance because there is a decreased risk of osteoporosis in overweight postmenopausal women, which is thought to arise from increased estrogen biosynthesis [6]. For example, women with a body mass index (BMI) $< 18.5 \text{ kg/m}^2$ (underweight) nearly tripled the fracture risk compared with a BMI $> 25 \text{ kg/m}^2$ (overweight) [37]. In contrast, obesity is positively correlated with an increase in breast cancer risk, which is thought to arise from increased estrogen biosynthesis in the breast [6,38]. Obese subjects have an approximately 1.5-3.5-fold increased risk of developing breast cancer compared with normal-weight subjects, and between 15 and 45% of these cancers have been attributed to being overweight (BMI, 25.0-29.9 kg/m^2) [39]. Interestingly, recent studies have shown that exercise-induced fat loss can lead to a decrease in serum levels of estradiol and estrone [40,41].

3. Estradiol metabolism

Estradiol is metabolized to a 3-glucuronide by UGT1A1 [42] and to a 17β -glucuronide by UGT1A3 and 2B7 (Figure 2) [43,44]. It is metabolized to estradiol-3-sulfate by SULT1A1 [45,46], SULT1E1 [46,47], and SULT2A1 [48]. Estradiol- 17β -sulfate was also observed as a minor metabolite in SULT2A1-mediated estradiol metabolism (Figure 2). Tissue steroid sulfatase can convert estradiol-3-sulfate back to estradiol [49]. Therefore, in menopausal women, circulating estradiol-3-sulfate can serve as a precursor to estradiol formation in breast

tissue. Estradiol also undergoes extrahepatic oxidation by CYP1A1 and hepatic oxidation by CYP1A2 and CYP3A to give the catechol derivatives, 2-hydroxy-17 β -estradiol (2-hydroxy-estradiol) and 4-hydroxy-17 β -estradiol (4-hydroxy-estradiol) (Figure 2) [50]. Estradiol-17 β -glucuronide is a substrate for hepatic CYP2C8-mediated oxidation to the corresponding 2-hydroxy-catechol metabolite [51]. There is also evidence that estradiol-17 β -sulfate can be metabolized to 2- and 4-hydroxylated metabolites [52]. 2-hydroxy-estradiol undergoes COMT-mediated metabolism to 2-methoxy-3-hydroxy-17 β -estradiol (2-methoxy-estradiol) and with lower catalytic efficiency *in vitro* it also forms 2-hydroxy-3-methoxy-17 β -estradiol [53]. It also undergoes UGT1A8- and UGT1B7-mediated metabolism to glucuronide conjugates [44], and SULT1E1-mediated metabolism to sulfate conjugates [54]. Similarly, 2-methoxy-estradiol is converted to glucuronide conjugates by UGT1A1, 1A3, and 1A8 [44], and to sulfate conjugates by SULT1E1 [55].

CYP1B1, which is primarily expressed in extrahepatic tissues such as the breast and lung, converts estradiol to 4-hydroxy-estradiol with relatively high regioselectivity [56]. CYPs 1A1, 2C8, 3A4, 3A5, and 3A7 can also metabolize estradiol to 16 α -hydroxy-estradiol [57]. 4-hydroxy-estradiol was found to be mutagenic in the I-select cII assay in BB rat2 cells [58]. Under similar conditions, 2-hydroxy-estradiol was inactive [58]. The mutational spectrum obtained after treatment of the BB rat 2 cells with 4-hydroxy-estradiol contained a considerable proportion of mutations at A:T base pairs, suggesting that 2'-deoxyadenosine was a major target of the reactive metabolite derived from the catechol estrogen. In addition, 4-hydroxy estradiol but not 2-hydroxy estradiol induced the expression of hypoxia-inducible factor 1 α and vascular endothelial growth factor A in human ovarian carcinoma cells [59]. Furthermore, 4-hydroxy-estradiol can readily redox cycle with the corresponding semiquinones and quinones causing the generation of reactive oxygen species together with the formation of quinone-derived DNA adducts [60,61]. A potential route for quinone detoxification is through the formation of glutathione (GSH)-adducts [62]. However, the quinone derivative from 4-hydroxy-estradiol was found to covalently modify and inactivate the glutathione-S-transferases involved in GSH-adduct formation, suggesting that alternative detoxification pathways are more important [63]. Detoxification of 4-hydroxy-estradiol occurs by COMT-mediated metabolism with high catalytic efficiency *in vitro* to give 3-hydroxy-4-methoxy-17 β -estradiol (4-methoxy-estradiol) [53] as well as by UGT2B7-mediated metabolism to glucuronide conjugates [44,64] and SULT1E1-mediated metabolism to sulfate conjugates [47]. Similarly 4-methoxy-estradiol undergoes UGT1A8-mediated metabolism to a glucuronide conjugate [44] and SULT1A1-mediated conversion to a sulfate conjugate [55]. It has been suggested that CYP1B1-mediated 4-hydroxy-estradiol formation in breast tissue is involved in the initiation of carcinogenesis [65-69]. Conversely, COMT-mediated conversion of 2-hydroxy-estradiol to its 2-methoxy metabolite potentially inhibits the proliferation of breast cancer cells *in vitro* [70,71]. Therefore, 2-methoxy-estradiol could be a chemopreventive agent. Mechanistic studies indicate that the actions of 2-methoxy-estradiol are mediated through inhibition of the pro-angiogenic transcription factor hypoxia-inducible factor 1 α , c-Jun NH2-terminal kinase signaling, and the generation of reactive oxygen species [72]. 2-methoxy-estradiol also efficiently induces mitotic arrest, apoptosis, and autophagic cell death in glioma cells *in vitro*, which has stimulated the search for new drugs to treat gliomas based on the structure of 2-methoxy-estradiol [73].

4. Estrone metabolism

Estrone is metabolized to a 3-glucuronide by UGT1A8 [44] and to a 3-sulfate at high concentrations by SULT1A1 and at low concentrations by SULT1E1 (Figure 3) [74]. Estrone also undergoes extrahepatic oxidation by CYP1A1 and hepatic oxidation by CYP1A2 and CYP3A in a similar manner to estradiol to give the catechol derivatives, 2-hydroxy-estrone and 4-hydroxy-estrone (Figure 3) [50]. In contrast, CYP1B1, metabolizes estrone to 4-hydroxy-estrone with relatively high regioselectivity [56]. 4-hydroxy-estrone stimulated the

growth of MCF-7 breast cancer cells *in vitro* and had carcinogenic effects in animal models [71]. Significantly, 2-hydroxy-estrone was unable to induce tumors in the same animal models [71]. 2-hydroxy-estrone undergoes COMT-mediated metabolism to 2-methoxy-estrone and with low catalytic efficiency to 2-hydroxy-3-methoxy-estrone, whereas 4-hydroxy-estrone is primarily metabolized to 4-methoxy-estrone [53]. 2-hydroxy-estrone is converted to a 3-glucuronide conjugate by UGT1A1, whereas 4-hydroxy-estrone is converted to the 3-glucuronide conjugate by UGT1A8 [44]. 4-hydroxy-estrone is also converted to a 4-glucuronide conjugate by UGT1A9 [44] and there is evidence that glucuronide conjugation is mediated through the action of UGT2B7 [75].

4-hydroxy-estrone can form depurinating DNA-adducts [2] and unlike 2-hydroxy-estrone, it possesses partial estrogenic activity [76]. 2-methoxy-estrone does not appear to have antitumor activity in its own right in contrast to the corresponding 2-methoxy-estradiol metabolite [70]. It has been suggested that reductive metabolism by 17 β -HSD type 1 in the breast could convert 2-methoxy-estrone to the active 2-methoxy-estradiol [70]. However, oxidative metabolism of the resulting 2-methoxy-estradiol by 17 β -HSD type 2 could convert it back to 2-methoxy-estrone (Figure 3) [77]. Therefore, the net anti-tumor activity would depend upon the relative amounts of reducing and oxidizing HSDs that are present in a particular target tissue.

In addition to CYP3A4-mediated metabolism of estrone to the corresponding 2- and 4-hydroxy catechols [78], estrone also undergoes metabolism to 16 α -hydroxy-estrone [57,79]. In addition, CYPs 1A1, 2C8, 3A5, and 3A7 can also metabolize estrone to a 16 α -hydroxy-estrone, which could also potentially arise from spontaneous rearrangement of 3,16 α -dihydroxy-17 β -estradiol, (16 α -hydroxy-estradiol) [80]. 16 α -hydroxy-estrone has been identified as a circulating metabolite [50,81], which is present at higher concentrations in breast cancer tissue when compared with normal tissue [82]. In addition, 16 α -hydroxy-estrone caused genotoxic damage and aberrant proliferation in mouse mammary epithelial cells [83]. 16-hydroxy-estrone was also shown to increase cyclin D1 protein levels in MCF7 breast cancer cells by almost fourfold compared with control un-treated cells [84]. This suggests that 16 α -hydroxy-estrone is involved in tumor initiation through its genotoxic effects [85,86] and in tumor promotion and progression through its ability to induce cellular proliferation [80]. It has been proposed that a shift toward 2-hydroxy-estrone from the 16 α -hydroxy-estrone metabolic pathway, as indexed by the 2-hydroxy-estrone to 16 α -hydroxy-estrone ratio, will be inversely associated with breast cancer risk [87]. In fact, several recent epidemiological studies have found that women with a high ratio of serum 2-hydroxy-estrone metabolites to 16 α -hydroxy-estrone metabolites are at a decreased risk for breast cancer [12,88].

5. Quantitative Analysis of estrogens in the plasma and serum of postmenopausal women

Estradiol and its metabolites are present in plasma and serum in the free unbound form, non-covalently bound to steroid binding proteins, and as glucuronide and sulfate conjugates. Concentrations of the free (unbound) forms of plasma and serum estrogens in postmenopausal women are in the fg/ml range, which puts them below the limit of quantitation (LOQ) of routine assays [89,90]. The LOQ is defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy [91]. A minimum requirement is that replicate determinations (n=5) can be conducted with a precision of better than 20 % and an accuracy of between 80 % and 120 %. Therefore, estrogens are quantified as a combination of free and non-covalently bound (total) forms with typical serum estradiol concentrations of 2-21 pg/mL in postmenopausal women as determined using specific MS-based methodology [92]. The concentrations of free unbound forms are then determined by analyzing the amount of plasma steroid binding protein [93] and subtracting the amount of each individual estrogen calculated to non-covalently bind to this protein [94,95]. In contrast,

glucuronide and sulfate estrogen conjugates re present in much higher concentrations can be readily quantified after hydrolysis of the plasma or serum with arylsulfatase/ β -glucuronidase and methanolysis with acetyl chloride in methanol [96]. The minor amounts of non-conjugated forms can then be subtracted to determine the concentrations of conjugated estrogens that are present in the plasma or serum sample.

High-quality estrogen assays with high sensitivity, specificity, and reproducibility are essential for conducting sophisticated epidemiologic studies [97]. There are three major bioanalytical methods used currently, immunoassay [90], GC-MS/MS [98], (LC)-MS/MS [92]. RIAs and electrochemiluminescence immunoassays (ECLIA) are by far the easiest to implement and the most widely used. However, they are fraught with numerous problems it difficult to provide accurate concentrations for the low level samples in postmenopausal women [89,99-101]. For example, conjugated estrogens are present in plasma and serum at 2-3 orders of magnitude higher in concentration than the corresponding un-conjugated forms [102,103]. Therefore, even if cross-reactivity is in only in the 1 % range, they can contribute to the antigen-antibody interaction and provide falsely elevated values. In addition, un-conjugated steroids such as estriol that are present in plasma can lead to elevated values [104]. When prior chromatographic separations are performed to remove interfering cross-reacting substances, corrections for recovery can be made using radiolabeled analogs as internal standards [103]. Unfortunately, there is no way to readily determine whether the radioactive analogs have decomposed during the analytical procedure. In addition, the trace amounts of radioactivity that are used cannot act as carriers through the assay. Therefore, if selective binding to active sites on glassware or other surfaces occurs during extraction and chromatography, significant losses of the estrogen analytes can occur. Poor recoveries can significantly impact on assay precision and recovery. Furthermore, it is possible that there are endogenous substances present in an individual plasma or serum sample that can modulate the antibody/antigen interaction, which would lead to erroneous values. In spite of these potential problems, HPLC coupled RIA currently provides the most sensitive and best validated immunoassay method for the analysis for estrone and estradiol in serum samples obtained from postmenopausal women [105,106]. This contrasts with the inter-individual differences in specificity observed with many other immunoassays [100,101,104,107], which have caused significant problems when interpreting epidemiologic studies of breast cancer risk in menopausal women [15].

It is almost impossible to overcome the inherent assay problems involved in using RIA-based methodology, particularly for multiple estrogens. For reliable measurements of multiple estrogens in plasma or serum, it is necessary to employ stable isotope dilution methodology in combination with LC-MS/MS or GC-MS/MS. These technologies represent the “gold standard” for the analysis of multiple estrogens when they are used under rigorously validated conditions. Losses during the extraction and chromatographic analysis are inherently taken into account by the use of an internal standard for each estrogen analyte that has identical physical properties but differs only in mass. Stable isotope analogs also act as a carriers to prevent non-selective losses of trace analytes through binding to active surfaces during extraction and analyses [108]. Until recently, this ideal condition was not possible for estrogens because only deuterated analogs were available for use as internal standards. There is a small but significant separation of the deuterium analog internal standards and their corresponding endogenous protium forms during chromatography. Therefore, differential suppression or enhancement of ionization could still affect the quality of the analytical data. The recent availability of [$^{13}\text{C}_6$]-estrogen analogs from Cambridge Isotope Laboratories (Andover, MA) means that going forward it will be possible to use internal standards that have identical chromatographic retention times but a mass difference of 6-Da. The specificity of GC-MS/MS and LC-MS/MS methodology arises from three analytical parameters. The estrogen must have an identical relative retention time to the heavy isotope analog internal standard determined during assay validation, an identical parent ion on MS analysis and an identical product ion on

MS/MS analysis. Immunoaffinity purification can improve potentially improve specificity still further as we found with the difficult amyloid β -peptides [108]. Unfortunately, the sensitivity of conventional immunoaffinity purification/MS-based procedures is inadequate for the analysis of plasma and serum samples from postmenopausal women [109].

Stable isotope dilution methodology coupled with GC-MS or LC-MS can in principle provide the optimal specificity for estrogen analysis because (as noted above) internal standards with identical physicochemical properties to the relevant analytes are carried through the entire analysis procedure. GC-MS/MS when used in the electron ionization mode does not have the sensitivity for the analysis of estrogens in postmenopausal serum and plasma samples. However, pentafluorobenzyl (PFB) and trimethylsilyl (TMS) derivatization of the 2- and 17-hydroxyl groups, respectively coupled with electron capture negative chemical ionization (ECNCI)/MS/MS provides outstanding sensitivity with a LOQ for serum estradiol of 0.6 pg/mL (2.3 pmol/L) [98] (Table 1). Elaboration of this methodology to multiple estrogens on a routine basis will be very challenging and limited to selected laboratories. LC-MS/MS methodology is not so challenging but unfortunately, endogenous estrogens are not effectively ionized using conventional electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) methodology. This has restricted the use of these ionization techniques to samples with higher concentrations of plasma and serum estrogens [92,101,109-111]. It is necessary enhance the ionization characteristics of estrogens by first converting them to suitable derivatives in order to conduct analyses in plasma or serum samples from postmenopausal women.

Three approaches to enhancing sensitivity of estrogen analysis through derivatization have been reported. The first approach, which we have pioneered, involves the preparation of an electron capturing PFB derivative of the estrogen 2-hydroxyl groups coupled with the use of electron capture atmospheric pressure chemical ionization (ECAPCI)/MS (Figure 4) [112]. The Higashi group has also explored the utility of ECAPCI/MS for estrogen analysis by using different electron capturing derivatives [113]. We showed that it was possible to quantify estrogens in the low pg/mL range in plasma using LC-ECAPCI/MS {Penning, 2010 1972 /id} (Table 1). The second approach uses conventional derivatization coupled with LC-ESI/MS. This approach is exemplified by studies of the Ziegler [115], Tai [111], and Kushnir [92] groups using the dansyl (D) derivative and studies by the Yamashita [116], and Spink [117] groups in which a picolinoyl (P), or pyridyl-3-sulfonyl (PS) derivatives were employed (Figure 4). The third approach involves the preparation of pre-ionized (quaternized) derivatives, so that ionization is not required in the ESI source of the mass spectrometer. This approach is exemplified by studies of the Chen [118], Higashi [119], and Adamec [120] groups in which N-methyl-2-pyridyl (NMP), 1-(2,4-dinitro-5-fluorophenyl)-4,4-dimethylpiperazine (MPPZ), or N-methyl-nicotinyl (NMN) groups are attached to the 3-hydroxy moiety of the estrogen moiety (Figure 4).

6. Future Directions

The three derivatization strategies described above make it possible to quantify plasma and serum estrogens with LOQs in the low pg/mL (pmol/L) range (Table 1) {Santen, 2007 1941 /id; Penning, 2010 1972 /id; Xu, 2005 1860 /id; Yamashita, 2007 1858 /id; Yang, 2008 1855 /id}. The availability of methodology based on these novel ionization techniques coupled with GC-MS/MS or LC-MS/MS will greatly facilitate future studies to rigorously establish the precise levels of individual estrogens that are present in the plasma of postmenopausal women.

The ESI process requires ionization to occur in solution followed by desolvation of resulting protonated molecules in the source of the mass spectrometer. Therefore, it is difficult to achieve complete ionization of all analyte molecules. This contrasts with pre-ionized derivatives, which

are already completely ionized (Figure 4). The Adamec group has employed the NMN pre-ionized derivative for the high sensitivity analysis of plasma and serum estrogens [120] (Table 1). Our laboratory has also explored this approach using the GT derivative, which has been employed previously to analyze keto steroids [122,123]. Extremely high sensitivity can be obtained with the pre-ionized estrone GT derivative (Figure 4) when employed in combination with a modern triple quadrupole mass spectrometer coupled with nanoflow LC. For example, using [$^{13}\text{C}_6$]-estrone as the internal standard, we have demonstrated linear standard curves in the range of 0.625 fg to 10.00 fg (2.2 amol to 37.0 amol) of estrone on column as its GT derivative (Figure 5). This suggests that in the future it will be possible to conduct LC-MS/MS assays on multiple estrogen metabolites in serum and plasma at an order of magnitude lower than the impressive sensitivity that can be obtained with currently available GC-ECN/MS, LC-ECAPCI/MS and LC-ESI/MS methodology (Table 1). The ease with which the pre-ionized derivatization strategy can be implemented will make it possible to readily introduce high sensitivity methodology in laboratories that are currently employing LC-MS/MS methodology. We anticipate that the use of pre-ionized estrogen derivatives will also help conserve important plasma and serum samples as it will be possible to conduct high sensitivity analyses using low sample volumes. This will make it possible to use existing banked plasma and serum samples without significantly depleting the amounts that are available. This will permit other studies to be conducted on the same samples in order to help understand the factors that cause an increase in breast cancer risk.

It is possible that the ratio of 4-methoxy-estrogens to 2-methoxy-estrogens might provide an indirect measurement of the catechol estrogens 4-hydroxy-estradiol/2-hydroxy-estradiol and 2-hydroxy-estrone/4-hydroxy-estrone [124]. This is potentially important since the hydroxylated catechols are considered to be genotoxic estrogens while the 2-hydroxy catechols are metabolized to 2-methoxy-estrogens, which are considered to be anti-proliferative and protective against mammary carcinogenesis [12,13]. In contrast, 4-methoxy-estrogens do not appear to exert anti-proliferative effects. The ability to routinely conduct very high sensitivity analyses of 4-methoxy-estrogens to 2-methoxy-estrogens together with estrone, 16 α -hydroxy-estrone, and estradiol will make it possible to develop and evaluate new and improved models of breast cancer risk [6,97]. The development of such models would impact significantly on the implementation of chemoprevention strategies for women newly identified to be in a high breast cancer risk category. Previous studies have shown that this would significantly improve breast cancer prevention [6,7]. Therefore, the ability to routinely analyze plasma and serum estrogens with very high sensitivity could potentially save a large number of women from this devastating disease [97].

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Abbreviations used

2-hydroxy-estradiol	2,3-dihydroxy-17 β -estradiol
2-methoxy-estradiol	2-methoxy-3-hydroxy-17 β -estradiol
4-methoxy-estradiol	3-hydroxy-4-methoxy-17 β -estradiol
4-hydroxy-estradiol	3,4-dihydroxy-17 β -estradiol
16 α -hydroxy-estradiol	3,16 α -dihydroxy-17 β -estradiol
estradiol	17 β -estradiol
AKR	aldo-keto reductase
APCI	atmospheric pressure chemical ionization
BMI	body mass index
COMT	catechol <i>O</i> -methyl transferase
CYP	cytochrome P-450
D	dansyl
DHEA	dehydroepiandrosterone
ECLIA	electrochemiluminescence immunoassay
ECAPCI	electron capture atmospheric pressure chemical ionization
ECNCI	electron capture negative chemical ionization
ESI	electrospray ionization
GSH	glutathione
GT	Girard T
LC	liquid chromatography
HSD	hydroxysteroid dehydrogenase
LOQ	limit of quantitation
MPPZ	1-(2,4-dinitro-5-fluorophenyl)-4,4,-dimethylpiperazine
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NMN	N-methyl-nicotinyl
NMP	N-methyl-2-pyridyl
P	picolinoyl

PFB	pentafluorobenzyl
PS	pyridyl-3-sulfonyl
RIA	radioimmunoassay
SULT	sulfotransferase
UGT	uridine diphosphate glucuronosyltransferases
TMS	trimethylsilyl

Figure 1.
Biosynthesis of estrogens from circulating C-19 androgens arising from cholesterol metabolism in the adrenal cortex of menopausal women.

Figure 2.
Enzymes involved in estradiol metabolism.

Figure 3.
Enzymes involved in estrone metabolism

Figure 4. Derivatives used to enhance the ionization efficiency of estrogens in order to improve sensitivity for GC-MS/MS and LC-MS/MS analysis.

Figure 5.

Analysis of estrone in the range 0.625 fg to 10.00 fg (2.3 amol to 37.0 amol) on columns as the GT derivative using LC-multiple reaction monitoring (MRM)/MS. Chromatography was performed using a Halo C18 column (150 × 0.1 mm × id, 2.7 μm, 90 Å; Advanced Materials Technology, Wilmington, DE) using a linear gradient of water/acetonitrile at a flow rate of 1,000 nL/min. MS was conducted using a Thermo Analytical Vantage triple stage quadrupole mass spectrometer. MRM/MS was conducted on the following ions m/z 384 (M^+ , estrone) → m/z 157 and m/z 390 (M^+ , [¹³C₆]-estrone) → m/z 157. Data points represent the means ± SEM (n=3).

Table 1

Limits of quantitation (LOQ) for analysis of serum and plasma estradiol and estrone by LC-MS/MS after derivatization to improve ionization efficiency. The LOQ is defined in section 5. Abbreviations: APCI atmospheric pressure chemical ionization; ECAPCI, electron capture atmospheric pressure chemical ionization; ECNCl, electron capture negative chemical ionization; ESI, electrospray ionization; LC, liquid chromatography; NMN, N-methyl-nicotinyl; P, picolinoyl; PFB, pentafluorobenzyl; PS, pyridyl-3-sulfonyl; TMS trimethylsilyl.

Chromatography	Ionization	Derivative	LOQ (pg/mL)			LOQ (pmol/L)			Reference
			Estradiol	Estrone		Estradiol	Estrone		
GC	ECNCl	PFB/TMS	0.6	ND		2.3	ND		97
LC	ECAPCI	PFB	4.0	4.0		14.7	14.8		114
LC	ESI	D	8.0	8.0		29.4	29.6		115
LC	ESI	P	0.5	1.0		1.8	3.6		116
LC	ESI	PS	10.0	ND		36.7	ND		117
LC	ESI	NMN	0.4	0.4		1.6	1.3		120

ND = not determined