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Review

Measurement of endogenous estrogens: analytical challenges and recent advances

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Abstract

Recent developments in the analysis of endogenous estrogens (including both free and conjugated estrogens) are reviewed. Largely due to urging by some cancer researchers, new demands are now being placed on such measurements in terms of sensitivity, throughput, multi-analyte detection and accuracy. Especially high sensitivity is required for detecting estrogens in serum from postmenopausal women, children and men, where concentrations at the low pg/ml level are encountered, and one would prefer to test much less than 1 ml of serum. Aside from throughput, meeting all of these demands may be beyond the reach of immunoassay, the method that has created and continues to dominate this field. Both HPLC and GC versions of mass spectrometry are emerging that have some potential to improve the testing of physiological samples for endogenous estrogens. The following topics are covered in this review: related analyses (e.g. detection of estrogens in environmental samples such as water, where 1-l samples can be collected to provide ng amounts of estrogens); structure and metabolism of estrogens; biological actions (with an emphasis on their role in cancer); immunoassays; HPLC with electrochemical detection; GC-ECD; and various forms of mass spectrometry.

Keywords: Reviews; Estrogens

Contents

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1. Introduction

In spite of 100 years of chromatography during which thousands of articles have been published on steroid analysis including estrogens, further advances are still needed in the analysis of endogenous estrogens in physiological samples. Largely this demand comes from concern about the role of estrogens especially in breast and ovarian cancer. More sensitive, comprehensive, faster and accurate assays of these analytes are needed.

This review will first discuss the clinical aspects of endogenous estrogens that are relevant to estrogen analysis, and then consider the potential for current and emerging methodology to address the above analytical challenges. Ongoing and recent methods for measurement of endogenous estrogens will be evaluated relative to these criteria, even though not every estrogen analysis needs to be conducted with high sensitivity or throughput.

2. Related analysis

Although this review focuses on endogenous estrogens, there is much information that can help in their analysis from studies of related analytes or of estrogens in other matrices. Recent reviews or selected articles along these lines are as follows, where A designates an article and R designates a review: steroid analysis including estrogens in physiological samples (R [1–3]); estradiol and its conjugates in meat (A [4]); estrogens in environmental (generally water) samples (A [5–11]); synthetic estrogens (A [12,13]); neuroactive steroids (R [14]); endocrine disrupting chemicals which includes natural and synthetic estrogens (R [15]); and phytoestrogens (plant products such as isoflavones and ligands which have estrogenic activity) (R [16]).

Croley et al. [6], in their work on the analysis of estrogens in the environment, compared gas chromatography–tandem mass spectrometry, liquid chromatography–mass spectrometry with selected ion monitoring, and liquid chromatography–tandem mass spectrometry for this analysis, and discussed the advantages and disadvantages of each technique. The LC–MS–MS method was reported to be the most promising, reaching a detection limit of 5 μg/l at $S/N \geq 10$ for spiked 1-l environmental samples, and also for a 2-ml serum sample spiked with 10 ng. Estradiol, estriol, estrone, and ethynylestradiol were tested. Mass chromatograms for these detection limits in real samples were not shown. Van Poucke and Van Peteghem [17] used liquid chromatography–tandem mass spectrometry to detect synthetic, anabolic steroids in animal urine, reaching levels below 1 ng/ml.

3. Structural and metabolism of endogenous estrogens

We will use the term ‘‘estrogens’’ in this review to refer to both the free and conjugated forms (e.g. glucuronide- or sulphate-conjugates) of endogenous estrogens. The structures and metabolism of the major free estrogens, including catecholestrogens (e.g. 4HE2), are shown in Fig. 1. The structures of representative estrogen quinones, that arise from oxidation of catecholestrogens, are shown in Fig. 2. The estrogens have a complicated physiology in that their bioactivity may be beneficial (e.g. estradiol as a hormone drives the development of female sex organs), harmful (e.g. some estrogens are now classified as carcinogens; estrogen quinones form DNA adducts), or neutral (e.g. inactive estrogen metabolites en route to excretion). At least several of the estrogens and estrogen quinones are of analytical

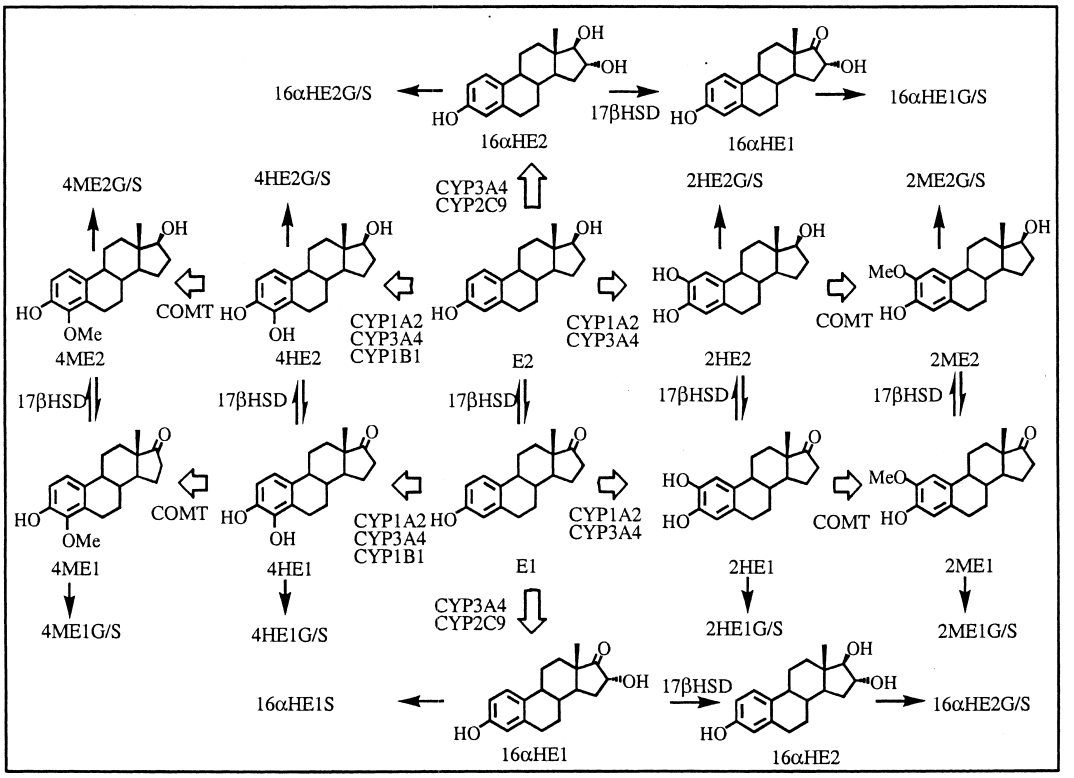

Fig. 1. Structures and some of the metabolism of endogenous estrogens. COMT, catechol *O*-methyl transferase; CYP, cytochrome P450; E1, estrone; E2, estradiol; G, glucuronide; H, hydroxy; HSD, hydroxysteroid dehydrogenase; M, methoxy; S, sulfate; SULT, sulfotransferase; UGT, uridine diphosphate glucuronosyltransferase. This figure was provided by Ian Blair.

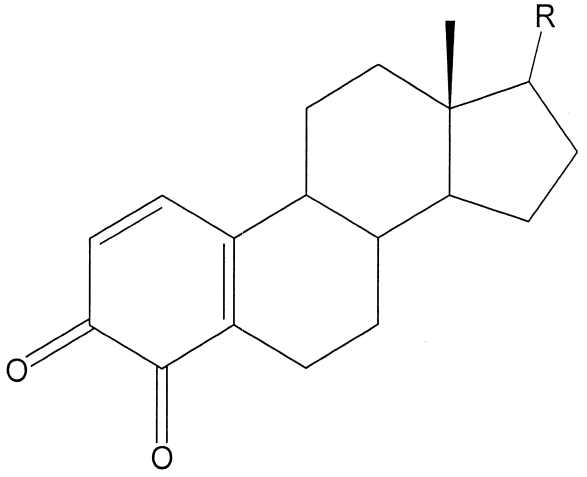

Fig. 2. Structure of estradiol-3,4-quinone (R=–OH) and estrone-3,4-quinone (R==O).

interest because of incomplete knowledge concerning their exact role and associated metabolism in health and disease. Their analysis can be challenging, e.g. the concentration of estradiol in postmenopausal women is usually <20 pg/ml (<73 pmol/l), and concentrations <5 pg/ml are common [18]. Estradiol in men is <8 pg/ml, and in children <5 to 45 pg/ml [19]. One would actually prefer to analyze volumes of physiological samples much less than 1 ml.

4. Biological actions of endogenous estrogens

Estrogens are well known as stimulants of secondary sexual characteristics along with some systemic effects such as the growth and maturation of long bones. What has more recently amplified an interest in their clinical measurement is primarily a

concern about their role in breast, ovarian and possibly prostate cancer. Interest in the role of estrogens in ovarian cancer intensified recently because epidemiology studies linked use of postmenopausal estrogens to this disease [20,21].

There is a close relationship between exposure to estrogens and breast cancer risk, as has been reviewed [22]. Evidence for this comes from numerous studies involving cell culture, animal and human systems. For example, 17β-estradiol and the carcinogen, benzo[a]pyrene, induce similar transformation phenotypes from human breast epithelial cells [23]. While the general importance of estrogen exposure as a risk factor for breast cancer is clear, the details are not, and more study is needed towards goals of improving prevention, early detection, and treatment of this disease.

Two types of general mechanisms are usually considered for how estrogens contribute to breast cancer. The first is that elevated levels of some of these substances increase cell proliferation, and the second is that some of the estrogens (especially the quinone metabolites) are directly or indirectly genotoxic. (A direct genotoxin is a substance that directly damages DNA; an indirect genotoxin stimulates DNA damage by other substances, as by increasing reactive oxygen species such as hydroxyl radicals.) In turn, those who study the metabolites, whether as mitogens or as DNA-damaging agents, tend to fall into two camps [24,25]. One camp considers especially the 4-hydroxy metabolites (apparently their daughter semiquinone or quinone metabolites) as most toxic, while others suspect the 16α-hydroxy products. Quinone metabolites of the estrogens form DNA adducts [26]. Since catecholestrogens are substrates for human sulfotransferases, a class of polymorphic enzymes, then individual variation in the isoforms of these enzymes might influence risk for breast cancer [27]. Those who argue against a significant genotoxic role for estrogen metabolites in breast cancer consider that the amounts of these metabolites are relatively small. Overall the field is characterized by much complexity and many contradictions both in the observations and their interpretation, aside from complete agreement that estrogen exposure is an important risk factor for breast cancer [22].

Estrogens may also play a role in prostate cancer, although their role here is even less clear than for breast cancer. Earlier observations (prior to 1997) linking estrogens to prostate cancer have been reviewed [28], and some observations reported in this latter review are as follows: (i) serum estrone and estradiol levels are elevated in healthy young African American males, who have the highest rate of prostate cancer in the world, while middle-aged Japanese males, who have a low incidence of this disease, have low serum estradiol levels relative to age-matched Caucasians; (ii) in the stromal compartment of the aged prostate, estradiol is elevated along with estrogen receptor; and (iii) a low dose of androgen plus estrogen induces a proliferative response in the prostate glands of several mammalian species, including full-blown prostate carcinoma of the Noble rat after prolonged dosing. Indeed, estrogens alone can induce growth and differentiation of epithelial cells of the prostate [29].

More recent observations also seem to connect variations in estrogens (or at least their receptors) to prostate cancer. For example, estrogen receptor-β, which is thought to be an inhibitor of prostate growth, is frequently lost in primary human prostate cancer [30,31], while it is the predominant receptor subtype in most metastases of this disease [31].

5. Analytical challenges

Several forces are at play that are reducing the amounts of physiological samples available for clinical diagnostic testing, which in turn creates a need for more sensitive methods including those for estrogens. The first one is that the number of available tests are increasing, so only a smaller portion of the sample may be available for a given test. Second, tumors are being diagnosed earlier, making smaller tissue samples available. Third are the increasing demands being placed on clinical research studies by human subject review committees. Fourth is the increasing accumulation from epidemiology studies of precious case control samples, or very precious cohort samples, which preferably are ‘‘never’’ used up.

Three other, more specific challenges face the testing of endogenous estrogens. The first one is the interest in measuring several to many of them at the

same time. Figs. 1 and 2 only reveal part of the diversity of estrogens: there are additional quinone metabolites, and also conjugated metabolites. Second is the large number of samples that one needs to test for potentially meaningful epidemiology studies, demanding a high throughput. Third is that the concentrations of the estrogens can be very low in physiological samples, as pointed out earlier. Thus, the estrogens are challenging analytes given the problems in some cases of small samples, estrogen multiplicity, large numbers of samples, and low concentrations.

6. Recent analytical advances

While we are mainly concerned in this review about meeting the four challenges of sensitivity, analyte diversity, throughput and accuracy for estrogen analysis, it must be appreciated that other considerations, along with the usual trade-offs in satisfying different analytical criteria simultaneously, leave room for many kinds of methods to contribute to estrogen analysis. For example, some clinical applications may only require the measurement of a single estrogen, high throughput is not always essential, and cost is always important. Thus, every valid analytical method for estrogens deserves both some encouragement and some criticism. Nevertheless, the four challenges that we have highlighted for estrogen analysis remain as the primary focus of this review, and our discussion of the analytical methodology for measuring estrogens will proceed accordingly.

6.1. Immunoassays

Immunoassays, such as radioimmunoassay [32], first must be praised for their important and ongoing contribution to estrogen analysis, contributing thereby to our understanding of estrogen biochemistry and physiology. These assays readily provide a high throughput, but the challenges of assay accuracy and estrogen diversity are severe, and, when it comes to some estrogen testing, every assay can be criticized for its limited sensitivity. Two general classes of immunoassays for estrogens are those that subject the initial sample to extraction or chromatography before quantitation and those that do not. Historically, immunoassays for estrogens and other steroids were preceded by tedious bioassays, and engineered versions of estrogen bioassays (e.g. use of recombinant estradiol receptor) are of ongoing interest.

In a study of laboratory reproducibility between four well-established endocrine laboratories in the United States, the laboratory error for the measurement of estrogen and estradiol in plasma samples of postmenopausal women by radioimmunoassay was often large ($>25\%$), and no single laboratory was found that could measure all of the seven steroid hormones of interest in this study acceptably [33]. Ziegler and coworkers [34] observed a correlation of only 0.6 for the measurement of 2-hydroxyestrone in urine samples from postmenopausal women by enzyme immunoassay and mass spectrometry, and the correlation was only ≥ 0.9 even for samples from premenopausal women where levels are higher. Currently 0.4 ml or more of plasma is needed to test just one postmenopausal estrogen by immunoassay in general. Recently an ultrasensitive non-extraction chemiluminescent assay was reported for estradiol in which the calibration curve reached 0.48 pg/ml (1.8 pmol/l) [18]. This enables duplicate or triplicate aliquots of a 0.4-ml plasma sample to be tested (as usual) even when a low (less than 20-pg/ml) concentration of estradiol is present. Comparisons continue to be made between immunoassay and mass spectrometry for conventional estrogen analysis [35,36].

While immunoassays may continue to dominate the field of estrogen analysis for quite some time, their shortcomings, especially for a class of structurally similar, trace analytes such as the endogenous estrogens, spur on efforts to create a replacement method. Ideally sample volumes much less than 1 ml could be analyzed for multiple estrogens, including the ones at a very low concentration.

6.2. HPLC with electrochemical detection

This technique utilizes a relatively inexpensive detector but has provided only moderate sensitivity to date for estrogens, and does not utilize stable isotope internal standards to control accuracy. Nakagomi and Suzuki [37] detected 1–15 ng of catechol estrogens and their *N*-acetylcysteine conjugates with this method in urine (0.75–7.5 ml) of rats

and hamsters after these animals were treated with estradiol or 4-hydroxyestradiol. The chromatograms showed a considerable background response and the accuracy of the method was not established. Devanesan et al. [38] detected catechol estrogens in hamsters (1-g tissue samples) treated with 4-hydroxyestradiol by HPLC–electrochemical detection (and also by HPLC–MS). Detection limits by neither technique were evaluated, but HPLC–electrochemical detection was used routinely to detect 1 pmol (~300 pg) of estrogen injected on-column. Yamada and coworkers detected estradiol in a 100-μl sample of rat plasma that contained 1 ng of spiked-in estradiol [39].

6.3. Mass spectrometry

Various forms of mass spectrometry (MS) need to be considered for estrogen analysis, since MS is a family of techniques. High accuracy is a well known attribute of MS because it is a very specific technique, and because stable isotope internal standards can be used. Nevertheless, MS is not always accurate even when such standards are employed [40]. The high specificity overall in an MS-based method is achieved in one of three ways: (i) include a chromatographic, electrophoretic, immunoextraction or another resolving technique prior to MS detection; (ii) use a high resolution form of MS such as a dual-sector, time-of-flight or ion cyclotron resonance instrument; or (iii) rely on one of several forms of tandem MS. These high specificity options can be combined, and may need to be, for ultrasensitive estrogen detection. Overall, as we will shortly see, MS shows some promise but has yet to meet the current challenges of some clinical estrogen testing. Recently advances in mass spectrometry for the measurement of estrogens and other endocrine disrupting compounds in aquatic environmental samples have been reviewed [15]. Trialkylsilyl derivatization of estrogens was studied recently, enabling the detection of 5–10 ng of spiked estrogens in 1-l of water by GC–electron impact-ion trap MS [41].

6.3.1. Electrospray mass spectrometry (ESI-MS)

For estrogen analysis according to the criteria of this review, ESI-MS encounters three general problems. The first one, which is a major shortcoming for trace ESI-MS in general, is that response is often analyte-dependent (see below). The second problem is related to the first: the conditions in the overall system for highest sensitivity of each analyte may be different. However, this problem can be minimized by instantly changing some of the conditions during the analysis. Third, response can be very dependent on analyte purity (e.g. as an HPLC peak), which gets worse with high throughput (fast HPLC). This is due to increased matrix effects that suppress or enhance analyte signal. This general problem for ESI-MS has been studied in more detail recently for drug analysis, and it was found that atmospheric pressure chemical ionization also can have this problem [42].

Zhang and Henion [43] reported the analysis in urine of three endogenous estrogen sulfates, E_1-3S, E_3-3S, and E_2-3S, along with two synthetic estrogens and a stable isotope internal standard, by LC–ESI-MS–MS. The structures of the compounds are shown in Fig. 3. In spite of the structural similarity of these analytes including a permanent charge, which would seem to make them ideal for detection by ESI-MS–MS, their trace analysis turned out to be very challenging, especially when under-

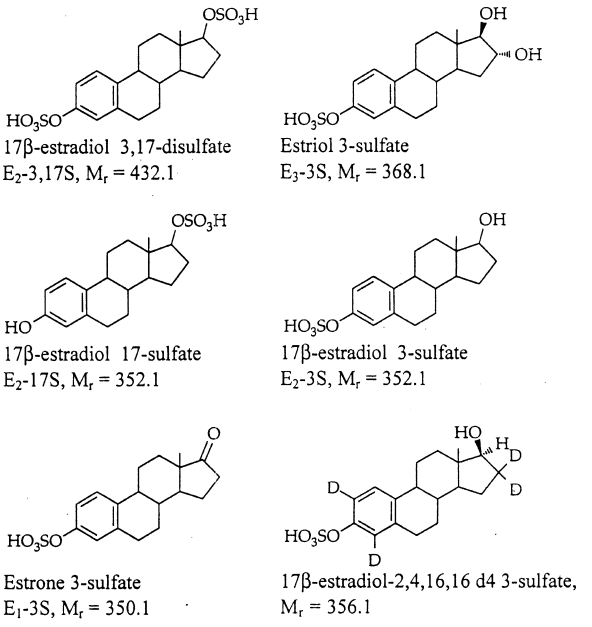

Fig. 3. Structures and masses of some estrogen sulfates. Reprinted with permission from Ref. [43].

taken in a high throughput mode as was done here. Several trade-offs in conditions were required, for example: (i) optimum reversed-phase solid-phase extraction conditions giving high recovery were different for polar E_3-3S versus less polar E_2-3S and E_1-3S; (ii) ammonium acetate in the reversed-phase HPLC mobile phase was necessary for analyte retention but lowered MS sensitivity; (iii) the best pH for HPLC peak shapes (5.4) was different from the best pH (5.8) for MS sensitivity; and (iv) use of acetonitrile rather than methanol in the mobile phase greatly improved the resolution of E_1-3S and E_2-3S, but drastically reduced sensitivity. This latter trade-off was problematic due to the similar precursor ion/product ion transitions for E_2-3S (m/z 351/271) versus E_1-3S (m/z 349/269), creating an opportunity for signal spillover.

Further, superimposed on these trade-offs were the following difficulties: (i) some polypropylene tips used for sample preparation yielded an ion that interfered due to tailing with the ion from E_2-3,17S; and (ii) fine tuning of the source declustering potential was necessary for E_2-3,17S due to the structural fragility of this compound. Different LC–MS–MS tuning parameters for the ionization and collision-induced dissociation stages were employed for each analyte except E_2-3S and E_1-3S, which essentially co-eluted from the HPLC column in the methanolic mobile phase. A robotic sample preparation scheme was set up involving sequential use of 96-well liquid–liquid extraction and solid-phase extraction plates for high throughput. Time of evaporation after solid-phase extraction (SPE) was minimized by using SPE extraction discs which could be eluted with a small solvent volume since the discs incorporated a small mass of extraction particles in a thin bed volume. The method achieved the detection of 20 pg of the estrogen sulfates in a 100-μl volume of urine, and enabled detection of these compounds even in a non-gravid urine sample, as demonstrated by the data shown in Fig. 4. An impressive throughput equivalent to 192 samples per day was reported.

Xu et al. [44] recently reported the detection of 2- and 4-hydroxyestrones in human urine by derivatization–LC–ESI-ion trap-MS. The lower limit of quantitation (1 ng per 10-ml urine sample) was sufficient to reach the levels of these analytes in urine from postmenopausal women. The on-column

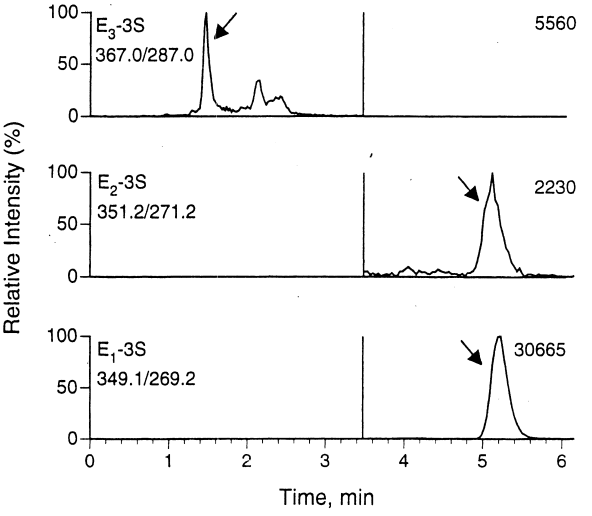

Fig. 4. Selected reaction monitoring LC–MS chromatograms for an extract of non-gravid female human urine. Column, C_{18} Betasil (2 mm×100 mm, i.d. 5 μm); mobile phase, 50:50 methanol/5 m*M* ammonium acetate, pH 5.4; flow rate, 200 μl/min. Reprinted with permission from Ref. [43].

detection limit for derivatized standards was 50 pg ($S/N \geq 15$). In this procedure, hydrazone derivatives of the estrones (which possess a ketone group) were formed by reaction with toluensulfonhydrazide. This enhanced the sensitivity, at least in part because these derivatives could be eluted from the LC column at a higher percentage of methanol. The authors also attributed the improved sensitivity to the ease with which the derivatives formed stable, protonated molecules in the electrospray process, which were used for quantitation (with inclusion of stable isotope internal standards). Interestingly, higher resolution was observed by reversed-phase HPLC for the derivatives rather than the starting estrones. One can speculate that masking the polar carbonyl group as a non-polar hydrazone then directed the polar selectivity to the dihydroxyaryl moiety where their structures differed. This method has little scope for general estrogen analysis since it is directed only towards estrones.

Although only a single, non-endogenous estrogen, ethinylestradiol, was detected using derivatization–ESI-MS–MS by Anari et al. [12], remarkable sensitivity was reported. In this procedure, as little as 250 fg of ethinylestradiol in 50-μl of plasma was

detected at a $S/N>100$, even with injection of only 10-μl from a final sample volume of >100 μl into the instrument (a 200-μl sample of 50% acetone was evaporated at 60 °C for ~3 min prior to injection). For this measurement the sensitivity of this compound was enhanced by derivatizing it with dansyl chloride as part of sample preparation. The authors commented that even a lower detection limit might be possible if carryover in the HPLC could be overcome. While the HPLC separation and the parent ion selection in the triple quadrupole mass spectrometer contributed specificity, a daughter ion characteristic of the dansyl group rather than the estrogen was used for quantitation.

Limitations of current assays for non-estrogenic steroids by ESI-MS are also informative. Ma and Kim [45] evaluated liquid chromatography–mass spectrometry for the detection of a variety of non-estrogenic steroids as standards in an effort to establish a method that would overcome the inaccuracy of immunoassays. Unfortunately, detection limits for steroids (non-derivatized) varied from 5 pg to several ng by ESI-MS. Liu et al. [46] resorted to conversion of oxosteroids to oximes in order to improve their detection by ESI-MS, and reported ~20-fold improvement for three of them. With nanospray, detection limits for oxime standards of progesterone, pregnenolone and dehydroepiandrosterone were 2.5, 5 and 25 pg/μl. However, the amounts actually derivatized were 10 μg, and no real samples were tested.

6.3.2. Atmospheric pressure chemical ionization mass spectrometry (APCI-MS)

Conventional APCI-MS apparently faces the same limitations that have been encountered by ESI-MS for estrogen analysis. This is implied from the study of non-estrogenic steroids by Ma and Kim [45] already discussed above, that explored APCI-MS along with ESI-MS. The detection limits for standards by LC–APCI-MS in this study varied from 50 pg to 8 ng. Fredline et al. [47] detected as little as 124 pg/ml of aldosterone in 2 ml of serum using HPLC–APCI-MS–MS. However, aside from the internal standard, only a single steroid was detected, and this steroid was a favorable analyte in terms of its response.

6.3.3. Liquid chromatography electron capture atmospheric pressure chemical ionization tandem mass spectrometry (LC–EC–APCI-MS–MS)

Blair and coworkers [48] recently introduced a new form of electron capture mass spectrometry in which the low energy electrons for capture are produced in an APCI source by using nitrogen as the sheath gas. A conventional APCI source can be used: the corona discharge needle initiates ionization of nitrogen leading to the production of electrons. Several estrogens including estrone (E1) and 2-methoxyestrone (2-ME1) were tested as *O*-pentafluorobenzyl derivatives. For derivatives of these two estrogens, detection limits were evaluated for diluted standards (prepared at the 100-ng level) and found to be 740 and 170 amol (333- and 82-fg), respectively. An HPLC–triple quadrupole mass spectrometer was used, in which the parent anion (from loss of a pentafluorobenzyl radical) was subjected to collisionally induced dissociation (CID) to form a daughter anion for detection. The one formed from estrone is shown in Fig. 5. In Fig. 6 is shown the detection of the four estrogens tested, along with two stable isotope internal standards, after a 13-min reversed-phase separation. As seen, different CID conditions were needed to optimize the sensitivity for each estrogen, and even slightly different CID conditions were used for the non-isotopic and isotopic forms of a given estrogen. Essentially identical detection limits were observed when the estrogen derivatives underwent a normal-phase separation prior to detection by EC–APCI-MS–MS. It will be interesting to follow the further development of this important new technique as it seeks to mature even further in terms of sensitivity, analyte diversity and throughput for estrogen analysis.

6.3.4. Liquid chromatography atmospheric pressure photoionization tandem mass spectrometry (LC–APPI-MS–MS)

Recently Robb et al. [49] introduced LC–APPI-MS–MS, in which a dopant such as toluene or acetone is introduced as a vapor into basically a conventional atmospheric pressure chemical ionization source, but ionization is initiated with 10-eV photons from a krypton lamp rather than by corona discharge. Analytes are ionized secondarily via direct or indirect interactions with the cation radicals

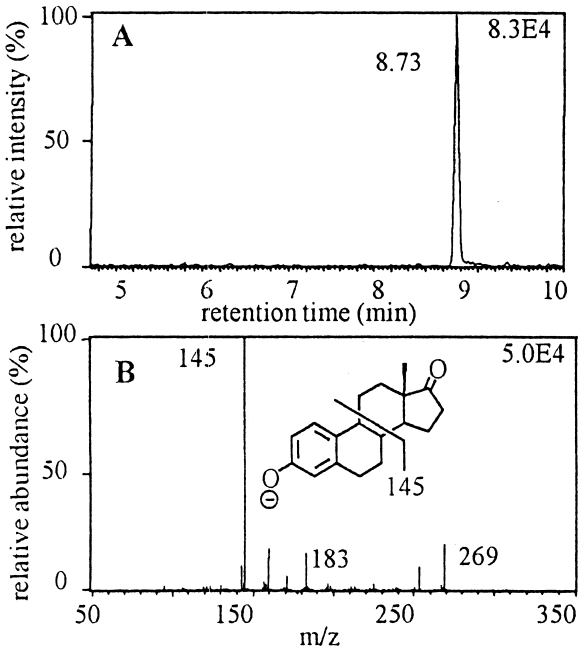

Fig. 5. (A) LC–EC–APCI-MS chromatogram of 10 pg of derivatized estrone on column with a retention time of 8.73 min. (B) Product ion spectra from CID of $[M-PFB]^-$ ion m/z 269 at a collision energy of 44 eV. Reprinted with permission from Ref. [48].

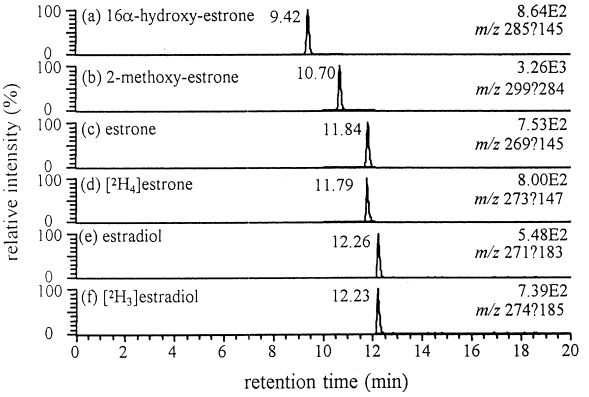

Fig. 6. Reversed-phase separation of PFB derivatives of estradiol (50 pg) and its metabolites (50 pg of each) by LC–EC–APCI-SRM-MS. (a) 16*R*-hydroxyestrone-PFB, retention time 9.42 min, monitoring the transition m/z 285 to 145 at a collision energy of 45 eV; (b) 2-methoxyestrone-PFB, retention time 10.70 min, monitoring the transition m/z 299 to 284 at a collision energy of 24 eV; (c) estrone-PFB, retention time 11.84 min, monitoring the transition m/z 269 to 145 at a collision energy of 40 eV; (d) $[^2H_4]$estrone-PFB, retention time 11.79 min, monitoring the transition m/z 273 to 147 at a collision energy of 41 eV; (e) estradiol-PFB, retention time 12.26 min, monitoring the transition m/z 271 to 183 at a collision energy of 52 eV; and (f) $[^2H_3]$estradiol-PFB, retention time 12.23 min, monitoring the transition m/z 274 to 185 at a collision energy of 48 eV. Reprinted with permission from Ref. [48].

formed by primary photoionization of the dopant. Depending on the analyte, $(M+H)^+$, $(M-H)^-$, M^+, or a subsequent fragment ion may predominate for detection. Alvary [50] subsequently used the technique with toluene as the dopant to detect 200 pg of standard ethynyl estradiol injected on-column and reported an extrapolated detection limit from this analysis of 1 pg under reversed-phase LC conditions. Three, non-estrogenic steroids were also tested, giving responses which ranged from 0.08 to 5.7 relative to that of ethynyl estradiol under these conditions. Relative to LC–APCI-MS–MS, the response for ethynyl estradiol was 8.1 times higher by LC–APPI-MS–MS. Similar detection limits were observed after a normal-phase separation, but in this case the dopant was not needed.

6.3.5. Gas chromatography with electron capture detection (GC–ECD) and gas chromatography electron capture mass spectrometry (GC–EC-MS)

With derivatization, non-conjugated estrogens fall into the volatility range of gas chromatography. For highest sensitivity, electron capture derivatives must be prepared. One of the general advantages of GC for multi-analyte testing is high-resolution separation. For example, even the isomers of hydroxyestrone and also of hydroxyestradiol can be separated by GC [51,52]. The detection of steroids in general by GC techniques including MS was reviewed in 1999 [3].

In conventional gas chromatography electron capture mass spectrometry (GC–EC-MS), an ion source is set up to establish low energy, gas-phase, secondary electrons by bleeding in a gas such as methane at a low pressure (e.g. 1 Torr), and bombarding this gas with high energy electrons (e.g. 150 eV) emitted from a hot filament [53,54]. Recent advances in EC-MS have been reviewed [55]. Compounds with a high electron affinity in the gas phase ionize by capturing a secondary electron, yielding an anion radical, which may dissociate into separate anion and radical products. What is detected then

may be an anion radical or an anion. Gas chromatography with electron capture detection (GC–ECD) is a related technique, but electron capture takes place at atmospheric pressure (the secondary electrons are formed from the eluent GC gas; the primary electrons are provided typically by a radioactive foil such as ^{63}Ni), and what is measured is the reduction in gas phase current [56]. Thus GC–ECD is much less specific than GC–EC-MS. Estrone after derivatization with heptafluorobutyric anhydride has been measured as a standard by GC–ECD [57]. Recently the catecholestrogens 2-HE2 and 4-HE2 were determined in rat tissue at the ng level by derivatization with heptafluorobutyric anhydride followed by gas chromatography with electron capture detection [58].

Xiao and McCalley [59] observed that estradiol, estrone, 2-hydroxyestrone, ethynylestradiol, 4-hydroxyestrone and 2-hydroxyestradiol can be derivatized efficiently with pentafluorobenzoyl chloride to yield products which give characteristic molecular ions by GC–EC-MS. The detection limit for diluted standards of these derivatives was ~100 fg, and the procedural recovery for spiked urine samples (10-ml volumes spiked at 1–5 ng/ml) was in the range of 84–101%. Corresponding trifluoroacetyl, heptafluorobutyryl, pentadecafluorooctanoyl and perfluorotolyl derivatives were also prepared and found to be inferior. The pentafluorobenzoyl derivatives apparently were superior largely because of their relatively high physicochemical stability, and relatively low volatility. Because of this latter feature, they eluted in a relatively clean region of the GC–EC-MS chromatogram. In the overall method of Xian and McCalley, human urine samples were subjected to metabolite hydrolysis with β-glucuronidase-arylsulfatase, extracted with ether, derivatized, evaporated and subjected to GC–EC-MS. The extrapolated detection limit of the method was 0.1 μg/l for a 10-ml urine sample, which corresponds to 1 ng.

GC–EC-MS detection of a pentafluorobenzyl/pivalyl derivative of estradiol at the low fg level is shown in Fig. 7 [60]. However, the derivative was prepared at the μg level and what was detected is a diluted standard, so much work remains to apply this technique to real samples. The corresponding deriva-

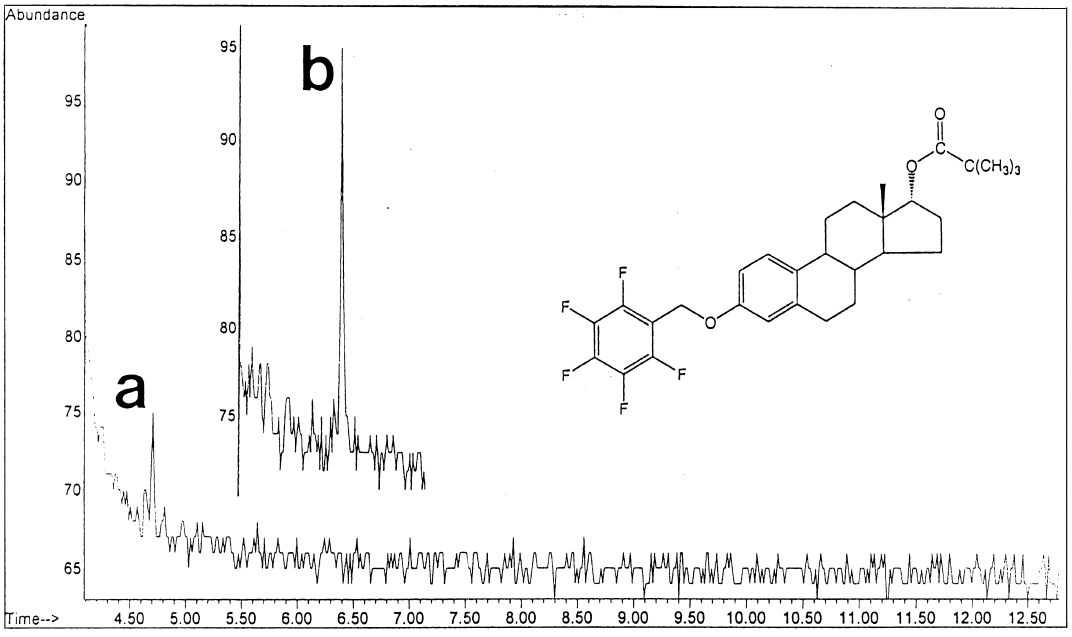

Fig. 7. Detection of electrophore-derivatized estradiol by GC–EC-MS: (a) 3.5 fg (71 amol); (b) 94 fg.

tive lacking the pivalyl group was detected at the pmol level ($S/N=50$) by laser-induced EC-MS [61].

Potentially, GC–EC-MS can become useful for the trace analysis of endogenous estrogens, if the challenges of derivatization at the trace level along with pre-and post-sample clean-up at this level can be overcome for real samples.

7. Conclusion and future

The measurement of endogenous estrogens in physiological samples with high sensitivity, throughput, multi-analyte detection and accuracy stands as a current challenge. Evidence and questions about the role of estrogens especially in breast, ovarian and possibly prostate cancer motivates such analytical goals. While immunoassays are used currently for clinical estrogen analysis and readily provide high throughput, there are fundamental limits to their sensitivity and accuracy for detection of multiple estrogens. It is fascinating that mass spectrometry methods now may be emerging that might become the major technique rather than immunoassay for estrogen testing in the future. However, not every estrogen in every circumstance needs to be detected according to these analytical criteria, aside from accuracy, especially if the cost of such analysis is high. We can anticipate that the future of estrogen testing will be rich in its diversity both in terms of the analytes and the kinds of methods, with special attention given to mass spectrometry techniques.

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