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Mass spectrometry techniques in the survey of steroid metabolites as potential disease biomarkers: A review

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ABSTRACT

Mass spectrometric approaches have been fundamental to the identification of metabolites associated with steroid hormones, yet this topic has not been reviewed in depth in recent years. To this end, and given the increasing relevance of liquid chromatography–mass spectrometry (LC–MS) studies on steroid hormones and their metabolites, the present review addresses this subject. This review provides a timely summary of the use of various mass spectrometry-based analytical techniques during the evaluation of steroidal biomarkers in a range of human disease settings. The sensitivity and specificity of these technologies are clearly providing valuable new insights into breast cancer and cardiovascular disease.

We aim to contribute to an enhanced understanding of steroid metabolism and how it can be profiled by LC–MS techniques.

1. Mass spectrometry overview

Mass spectrometry has an important history in the identification of drug metabolites and has recently emerged as the foremost technology in endogenous metabolite research [1], given its proven success in drug metabolite analysis and pharmacokinetic studies [2–12]. In fact, the recent rise of the «metabolomics era» stems from the enhanced ability to

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Abbreviations: APCI–MS, Atmospheric-pressure chemical ionization mass spectrometry; APPI–MS, Atmospheric-pressure photoionization mass spectrometry; CA, Cholic acid; CAT, 1,2-Dihydroxybenzene (benzene catechol); CAT-Q, 1,2-Dihydroxybenzene-Quinone; CID, Collision-induced dissociation; COMT, Catechol-O-methyltransferase; DCA, Deoxycholic acid; ESI–MS, Electrospray ionization; FIA, Flow injection analysis; FTMS, Fourier transform mass spectrometry; GC–MS, Gas chromatography–mass spectrometry; GP, Girard P; HPLC–EDC, High performance liquid chromatography–electro-chemical detection; LC–MS, Liquid chromatography–mass spectrometry; MALDI-TOF, Matrix-assisted laser desorption/ionization-time-of-flight; *N*-AcCys, *N*-acetylcysteine; NADA, *N*-acetyldopamine; NADA-Q, *N*-acetyldopamine-quinone; NQO-2, NRH quinone oxidoreductase 2; Resv, Resveratrol; SPE, Solid phase extration; SLOS, Smith–Lemli–Opitz syndrome; SRM, Selected reaction monitoring; TOF, Time-of-flight; UPLC–MS/MS, Ultra-performance liquid chromatography-tandem mass spectrometry.

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perform faster, more accurate and comprehensive metabolite analyses, along with the need to understand intracellular biochemical events towards identification of both disease and pharmaceutical biomarkers [1].

Metabolite analyses have been typically carried out by means of liquid or gas chromatography with mass spectrometry (LC–MS or GC–MS, respectively), or inclusively high field proton nuclear magnetic resonance (NMR). The primary advantage of NMR in metabolite analysis is its ability to measure analytes in biofluids quickly and accurately, without the need of initial processing or separation [13–15]. Over recent years, improvements have included higher spectral resolution, lower instrument cost, and the addition of stop-flow chromatography on fractions of samples. Yet, the major weakness of NMR is that it has a poor dynamic range (100–1000) that results in only the major components being observed [1]. High-resolution capillary GC–MS has also been a landmark in metabolite research and disease diagnosis, as it enables identification of key small molecules, such as fatty acids, amino acids and organic acids, in biofluids, particularly in urine and blood [16–18]. This technique has been influential in providing diagnostic information for many inherited diseases, such as numerous metabolic disorders, disorders of the metabolism of amino acids [19–22], bile acids [23,24] and steroids [25–27]. Nevertheless, GC–MS techniques have limited applicability to metabolite profiling, as they usually require (i) convoluted sample preparation including metabolite extraction and subsequent derivatization to volatile adducts, (ii) long analysis times, and (iii) ideal size and type of molecules to be analyzable; in other words, non-volatile, highly polar and/or large molecules cannot be studied by GC–MS [1]. In this context, LC–MS techniques present several advantages over NMR or GC–MS techniques in metabolite profiling, namely greater sensitivity and dynamic range. Therefore, LC–MS techniques will be overviewed in greater detail.

2. Competing MS technologies

LC–MS with an electrospray ionization interface (LC–ESI–MS) has become a popular choice for metabolite analysis and studies for new biomarkers [18,28]. This technique is advantageous over GC–MS in that sample preparation and analysis are relatively simple, providing access to metabolites of higher structural diversity. ESI offers many advantages over other ionization techniques, for example, the ability to analyze low- and high-molecular weight compounds, excellent quantitative capabilities and reproducibility, high sensitivity, simple sample preparation, amenability to automation, soft ionization and absence of matrix [29]. The utility of ESI lies in its ability to generate gas-phase ions directly from the liquid phase, which establishes the technique as a convenient mass-analysis platform for both LC and direct flow injection analysis (FIA), especially when combined with tandem mass spectrometry (MS/MS) [1]. While previous LC separation of the diverse molecules present in biofluids can reduce ESI ion suppression [30–32], making LC–MS especially attractive in the initial stages of metabolite research, it also delays data acquisition and analysis. Therefore, for ESI–MS quantitation of a known biomarker, extraction combined with flow injection analysis (FIA) is the method of choice, as the extracted sample is directly injected into the mass spectrometer, without prior chromatographic separation [33]. Altogether, ESI–MS techniques result in a selective approach that allows for both qualitative and quantitative metabolite analysis, while sensitivities in the pg/mL range can be readily achieved [34]. Still, a challenge in metabolite profiling is that potential biomarkers may be present in the biofluid in even lower abundances, thus requiring especially sensitive techniques, like nano-LC–ESI–MS; this technique is performed at flow rates ($\sim 200\ \text{nL} \cdot \text{min}^{-1}$) much lower than those in standard LC–ESI–MS ($\sim 300\ \mu\text{L} \cdot \text{min}^{-1}$), which produces ions with less evaporation, thus enabling detection of highly diluted species. This improves the sensitivity and ultimately offers a greater dynamic range in metabolite discovery [30–36].

Finally, though atmospheric-pressure chemical or photo-ionization mass spectrometry (APCI–MS or APPI–MS, respectively) is not widely used in metabolite-profiling studies, these techniques have been employed in the analysis of more easily ionizable molecules, such as phospholipids, to produce molecular and fragment ions complementary to those obtained by ESI with collision-induced dissociation (CID). APCI–MS provides a dynamic range higher than ESI–MS and is considered robust, easy to operate and relatively tolerant to higher buffer concentrations. Yet, it is a mass-sensitive rather than concentration-sensitive technique, so no sensitivity gain can be reached with smaller columns or lower flow rates.

A summary of the main characteristics of the techniques outlined above is provided in Table 1.

Another critical parameter in MS-based metabolite studies is the mass analyzer, a central piece in the performance of any mass spectrometer. Among the most commonly used are the quadrupole, the quadrupole ion trap, the time-of-flight (TOF) reflectron, and the Fourier transform ion cyclotron resonance (FTMS) analyzer.

Quadrupole is presently the most common type of mass analyzers; quadrupoles tolerate relatively high pressures, have the capability of analyzing up to an m/z of 4000 and are relatively low cost instruments. Yet, a triple-quadrupole is required if tandem mass analysis is to be performed; the three quadrupoles are placed in series, and each of them has a separate function: the first (Q1) is used to scan across the full m/z range and select an ion of interest; the second (Q2), also known as the collision cell, focuses and transmits the ions while introducing a collision gas (argon or helium) into the flight path of the selected ion; the third (Q3) serves to analyze the fragment ions generated in the collision cell (Q2) [29].

Quadrupole ion trap analyzers are also useful in tandem MS analysis, as a single ion species can be isolated by ejecting all others from the trap, enabling the isolated species to be further fragmented by collisional activation (CID); a key advantage of quadrupole ion traps is that multiple CID experiments can be performed quickly without requiring multiple analyzers. Other advantages include their ability to trap and accumulate ions to provide a better signal-to-noise ratio and their mass range up to ~4000 m/z. Yet, quadrupole ion traps are unable to perform high-sensitivity triple quadrupole-type precursor-ion scanning and neutral loss scanning experiments. Also, the upper limit on the ratio between precursor m/z and the lowest trapped fragment ion is

Table 1 – Summary of the major characteristics of LC–MS, FIA, GC–MS and NMR techniques used in metabolite-profiling studies.

Technique Characteristics	LC–MS	FIA	GC–MS	NMR
Sample preparation	extraction	extraction	Extraction and chemical modification	Typically none
Chromatographic Separation	Medium-resolution separation	No separation	High-resolution separation	No separation
Sensitivity	Millimoles per liter to nanomoles per liter	Millimoles per liter to micromoles per liter	Millimoles per liter to nanomoles per liter	Millimoles per liter to high micromoles per liter
Dynamic range	10^6	10^4	10^6	10^3
Speed	Slow (5–90 min)	Rapid (1 to 5 min)	Slow (~30 min)	Rapid (1 to 5 min)
Quantitative accuracy	±10%	±10%	±10%	±10%
Significant advantages	Soft ionization Large mass range	Data in one spectrum fast	High resolution ESI–MS library available	No sample preparation
Significant disadvantages	Speed of analysis	Signal suppression from multiple components	Significant sample preparation with chemical modification; Slow analysis; Harsh ionization; Limited number of molecules can be analyzed	Poor sensitivity and dynamic range Some chemical classes are not detected

about 0.3 (also known as the “one-third rule”) and their dynamic range is limited, as when too many ions are in the trap, space charge effects diminish the performance of the ion-trap analyzer [29].

The linear time-of-flight (TOF) is the simplest mass analyzer with a virtually unlimited mass range. It has gained wide use due to its fast scanning capabilities (milliseconds), good mass range (up to m/z ~10,000), and an accuracy in the order of 5 ppm. Quadrupole-TOF mass analyzers combine the stability of a quadrupole analyzer with high efficiency, sensitivity, and accuracy of time-of-flight reflectron mass analyzer and are typically coupled to ESI sources. The quadrupole can act as a simple quadrupole analyzer to scan across a specified m/z range. Quadrupole-TOF exploits the quadrupole’s ability to select a particular ion and the ability of TOF-MS to achieve simultaneous and accurate measurements of ions across the full mass range. They offer significantly higher sensitivity and accuracy of tandem quadrupole instruments when acquiring full-fragment mass spectra.

FTMS offers high resolution, the ability to perform multiple collision experiments (MSn), and high-accuracy fragment masses (often at the part-per-million level). It is now becoming more common to couple ultra high resolution (> 105) FTMS to a wide variety of ionization sources, including MALDI, ESI, APCI and EI (electron impact ionization). Quadrupole-FTMS and quadrupole linear ion-trap FTMS mass analyzers that have recently been introduced are typically coupled to ESI sources. The quadrupole FTMS combines the stability of a quadrupole analyzer with high accuracy of FTMS. A specified m/z range can be scanned by using the quadrupole, which can also be used to selectively isolate a precursor ion. This ion can be directed into the collision cell or the FTMS, and the resultant precursor and fragment ions can then be analyzed by the FTMS [29].

MS instrumentation information outlined above will be important to understand some conclusions in the next MS biological studies; and hence we hope our review of capacities of these instruments allows the reader clearer insights concerning the biological findings involving MS that we review below.

2.1. *MS studies on estradiol metabolites as potential cancer biomarkers*

Metabolism of estrogens is characterized by two major pathways, respectively started by hydroxylation in ring A of molecule (Fig. 1) to produce the 2- and 4-catechol estrogens, and hydroxylation at the 16α position (Fig. 1). In the catechol pathway, the metabolism involves further oxidation to semiquinones and quinones, including formation of the catechol estrogen-3,4-quinones (E-3,4-Q), the major carcinogenic metabolites of estrogens. These electrophilic compounds react with DNA to form the depurinating adducts 4-hydroxyestrone(estradiol)-1-N3Adenine (4-OHE$_1$(E$_2$)-1-N3Ade) and 4-hydroxyestrone- (estradiol)-1-N7Guanine (4-OHE$_1$(E$_2$)-1-N7Gua) [1]. Oxidation of catechol estrogens to their quinones is homeostatically controlled which minimizes formation of the quinones and their apurinic adducts. When the homeostasis is disrupted, excessive amounts of catechol estrogen quinones are formed and the resulting increase in depurinating DNA adducts can lead to initiation of carcinogenesis [37].

Cavalieri and co-workers conducted several studies using ESI–MS and LC–MS/MS techniques to prove the formation of the aforementioned DNA adducts and their relevance in the initiation of carcinogenesis. Moreover, they used those techniques to study the effect of specific antioxidants, such as *N*-acetylcysteine and resveratrol (Resv), as well as of different enzymes, on estrogen metabolism [38].

Gaikwad and colleagues studied the evidence for reduction of the carcinogenic *ortho*-quinones mediated by NRH quinone oxidoreductase 2 (NQO2). These authors showed for the first time that NQO2 catalyzes the reduction of electrophilic estrogen quinones and thereby may act as a detoxification enzyme. Binding of E$_2$-3,4-Q with NQO2 was confirmed by ESI–MS, and further corroborated by analyzing the NQO2-E$_2$-3,4-Q

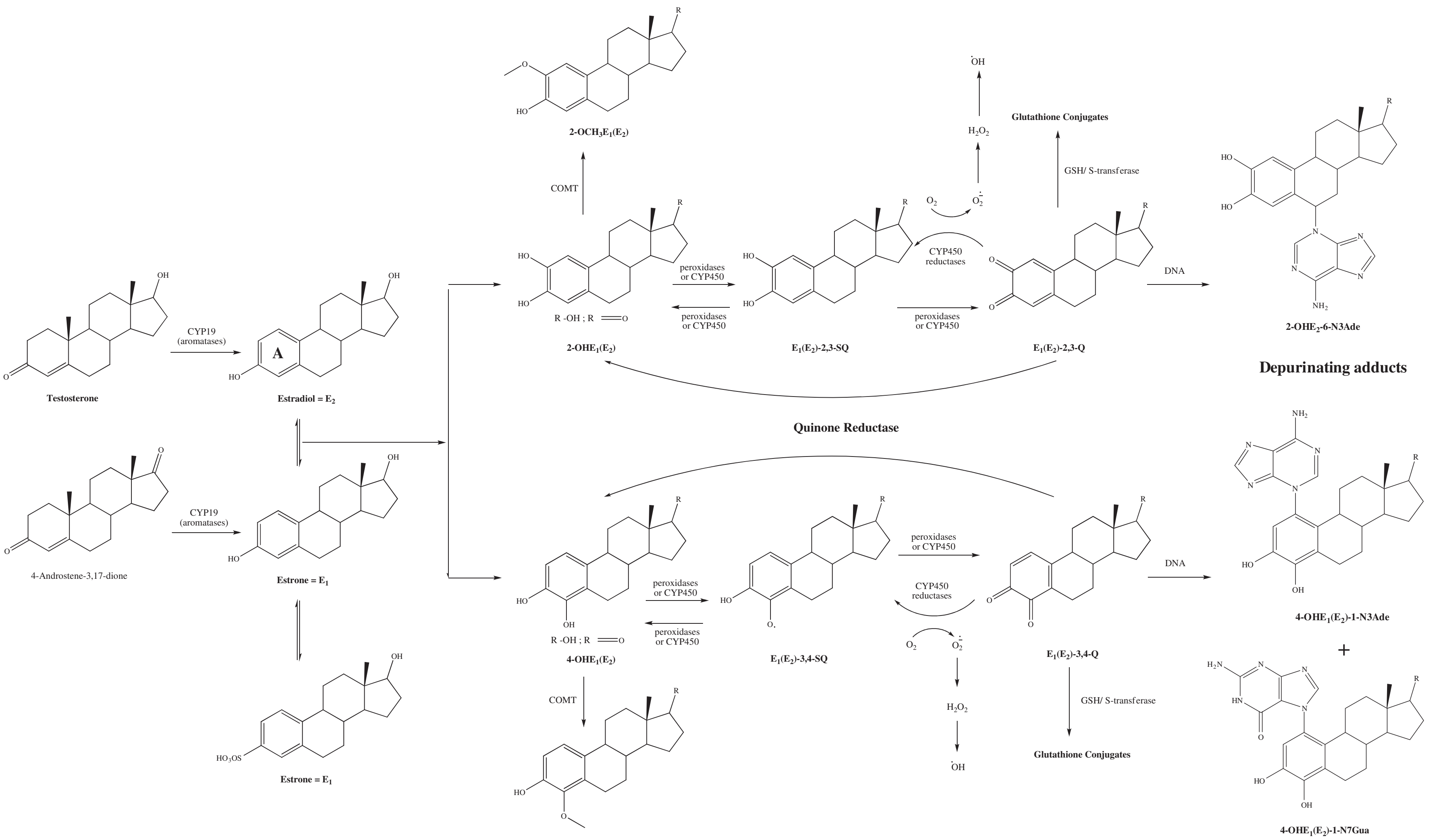

Fig. 1 – Biosynthesis and metabolic activation of estrogens E_1 and E_2. One of the major pathways of E1 and E2 leads to 2- and 4-catechol derivatives, which are further oxidized to yield the corresponding reactive quinones; these can react with DNA to form depurinating DNA adducts. In the deactivation pathway, which operates in parallel, the catechol derivatives are methylated to form methoxy catechol estrogens. In addition, the quinones are reduced by quinine reductase, as well as conjugated to GSH and thus rendered harmless. A shift in the apparent balance between activating and deactivating pathways towards formation of depurinating DNA adducts could lead to initiation of breast cancer (adapted from [38]) with permissions of authors. 2-OHE$_1$(E$_2$) **— 2-hydroxyestrone(estradiol);** 4-OHE$_1$(E$_2$) — **4-hydroxyestrone(estradiol);** 2-OCH$_3$E$_1$(E$_2$) — **2-methoxyestrone(estradiol);** 4-OCH$_3$E$_1$(E$_2$)- **4 —methoxyestrone(estradiol);** E$_1$(E$_2$)-2,3-SQ — **Estrone(estradiol)-2,3-semiquinone;** E$_1$(E$_2$)-2,3-Q — **Estrone(estradiol)-2,3-quinone;** E$_1$(E$_2$)-3,4-SQ — **Estrone(estradiol)-3,4-semiquinone;** E$_1$(E$_2$)-3,4-Q — **Estrone(estradiol)-3,4-quinones;** 2-OHE$_2$-6-N3Ade — **2-hydroxyestradiol-6-N3Adenine;** 4-OHE$_1$(E$_2$)-1-N3Ade — **4-hydroxyestrone(estradiol)-1-N3Adenine;** 4-OHE$_1$(E$_2$)-1-N7Gua — **4-hydroxyestrone(estradiol)-1-N7Guanine.**

complex by matrix-assisted laser desorption/ionization mass spectrometry with a TOF analyzer (MALDI-TOF). Both UV and LC–MS/MS assays unequivocally corroborate the reduction of estrogen ortho-quinones by NQO2, indicating that this could be a novel target for prevention of breast cancer initiation [39].

In a separate report, Gaikwad et al. analyzed urine samples from 46 healthy control women, 17 women with breast cancer and 12 women in high risk of acquiring breast cancer. After partial purification of urine samples by solid phase extraction (SPE), 40 estrogen-related compounds were identified and quantitated by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Each metabolite was detected and identified based on unique parameters including mass retention time and ionization mode (positive or negative). The authors proposed that levels of depurinating DNA adducts to their respective estrogen metabolites and conjugates could provide an invaluable biomarker allowing differentiation between women at high risk of developing breast cancer, women with breast cancer and healthy women [40].

The potential function of various cytochrome CYP450 enzymes in oxidizing catechol estrogens to quinones was identified by Zhang et al. These investigators used different human CYP isoforms with the aim of oxidizing the catechol estrogens 2-OHE$_2$ and 4-OHE$_2$ to their respective estrogen quinones, which then react with DNA to form depurinating adducts in vitro. The reaction products were analyzed by UPLC–MS/MS. These experiments demonstrated that CYP isoforms are able to oxidize catechol estrogens to their respective quinones, which can further react with proteins, GSH and DNA, the latter resulting in depurinating adducts that can lead to mutagenesis [41].

Pruthi et al. conducted a study to determine whether the ratio of estrogen DNA adducts to their metabolites and conjugates in serum differed between women with early-onset breast cancer and those with average or high risk of developing breast cancer (serum samples from women at average risk (n = 63) or high risk (n = 80) using Gail model). The goal of this study was to investigate the imbalance of estrogen metabolism in serum expressed as estrogen-DNA adduct ratio to examine its potential as a biomarker for increased breast cancer risk [42]. Serum samples from women diagnosed with early breast cancer were analyzed by UPLC–MS/MS, allowing observation that levels of depurinating estrogen-DNA adducts were significantly higher in women at high risk for developing breast cancer than in women at average risk. These findings suggest that serum estrogen-DNA adducts are potential biomarkers not only for determining the risk for developing breast cancer, but also for monitoring the effects of therapy. Yet, these findings are quite recent and require further studies towards unequivocal validation [42].

Zahid et al. reported a further study of benzene catechol (1,2-dihydroxybenzene, CAT) and *N*-acetyldopamine (NADA), which is itself a catechol. Benzene is metabolized to phenol in the liver by cytochrome CYP2E1. Other metabolites include CAT and hydroxyquinone (1,4-dihydroxybenzene). Oxidation of CAT and hydroxyquinone is catalyzed by peroxidases, forming quinones that can exert myelotoxic effects, and produce stable and depurinating DNA adducts. In this study, those authors analyzed reactions of catechol quinones such as the leukemogenic benzene quinone (CAT-Q) and *N*-acetyldopamine quinone (NADA-Q) with dG or DNA, using ESI–MS and UPLC–MS. They concluded that catechol quinones of natural and synthetic estrogens, benzene, naphthalene and dopamine react with DNA through an 1,4-Michael addition to form predominantly depurinating N3Ade and N7Gua adducts. With all of these compounds, the N3Ade adduct depurinates instantaneously from DNA, whereas the N7Gua adduct depurinates slowly, with a half-time of a few hours. For that reason, Zahid et al. proposed that common features may lead to the initiation of cancer and neurodegenerative diseases [43].

The effects of *N*-acetylcysteine N-AcCys on the metabolism of two cell lines MCF-10F (a normal human breast epithelial cell line) and E6 (a normal mouse breast epithelial cell line) were studied by Zahid et al. [43]. The cells were treated with 4-OHE$_2$ or E$_2$-3,4-Q, after which analysis using HPLC–EDC (electro-chemical detection) and UPLC–MS/MS demonstrated that N-AcCys inhibits the formation of depurinating adducts in an apparently similar way for both cell lines, despite originating from different mammal species. Therefore, it seems that, by blocking formation of estrogen-DNA adducts, N-AcCys could prevent the initiation of cancer by estrogens [44].

The same authors conducted another study using MCF-10F cells to evaluate the prevention of estrogen-DNA adduct formation by resveratrol (Resv), as Resv acts as both an antioxidant and an inducer of the phase II enzyme NQO1. The effects of Resv on the metabolism of estrogens were assessed by treatment of MCF-10F cells with 4-OHE$_2$ or E$_2$-3,4-Q, where UPLC–MS/MS was employed to analyze the estrogen-DNA adducts formed and determine the activity of NQO1 and catechol-*O*-methyl transferase (COMT). It was found that Resv decreased the formation of depurinating estrogen-DNA adducts from 4-OHE$_2$ or E$_2$-3,4-Q and increased formation of methoxycatechol estrogens; DNA adducts were not detectable, which indicates that Resv may be effective in preventing estrogen-mediated carcinogenesis by estrogen by blocking multiple sites in the estrogen genotoxicity pathway, as shown in Fig. 2 [45].

Gaikwad and colleagues demonstrated the possibility of circumventing the problem of nonenzymatic reduction of estrogen quinone by NAD(P)H, given that NQO1 catalyzes the reduction of estrogen quinones. They performed mass spectrometric binding studies involving E$_2$-3,4-Q or menadione with NQO1 which support the hypothesis of formation of an enzyme–substrate complex. Two different strategies were employed to ascertain the NQO1 activity in estrogen quinone reduction: first, the ping–pong mechanism of NQO1 catalysis was utilized to overcome the problem of nonenzymatic reduction of the substrate by NAD(P)H; second, tetrahydrophilic acid, which has a reducing potential lower than NAD(P)H was used as an alternative cofactor. Both strategies confirmed the reduction of E$_2$-3,4-Q by NQO1, according to UV or LC/MS–MS analysis of the assay mixtures [10]. In view of this, the authors concluded that E$_2$-3,4-Q is a substrate for NQO1, and NQO1 has a significant role in deactivation of estrogen *ortho*-quinone that might lead to initiation of cancer after formation of depurinating DNA adducts. These conclusions have broad implications for development of potential

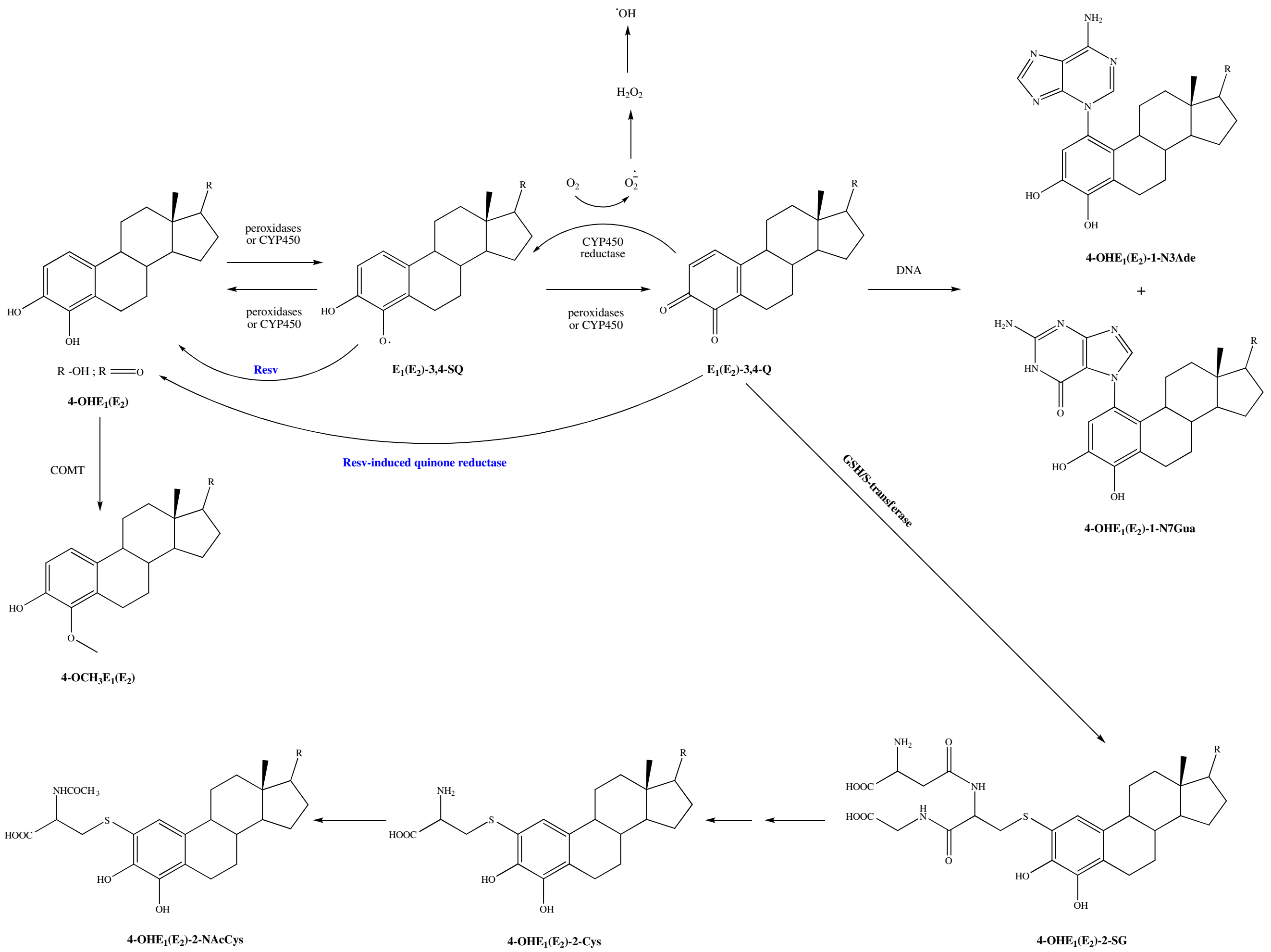

Fig. 2 – Mechanism by which Resv is proposed to prevent estrogen-initiated breast cancer (reproduced from [45]) with permission of authors.

inducers of NQO1 that could ultimately prevent estrogen-initiated carcinogenesis [46].

In another study by Zahid et al., natural antioxidants, such as N-AcCys, melatonin, reduced lipoic acid and Resv, were investigated for their ability to prevent the reaction of E_2-3,4-Q with DNA [43]. DNA was incubated with E_2-3,4-Q or lactoperoxidase-activated 4-OHE$_2$ in the presence of the antioxidants. After precipitation of DNA and centrifugation, supernatants were analyzed by LC–MS/MS and it was found that Resv and melatonin did not affect the formation of depurinating adducts when E_2-3,4-Q was reacted with DNA [11]. On the other hand, N-AcCys and lipoic acid showed a significant inhibition of the formation of depurinating adducts by E_2-3.4-Q. In the case of lactoperoxidase-activated 4-OHE$_2$ reaction with DNA, Resv achieved the highest level of inhibition, N-AcCys and reduced lipoic acid produced moderate inhibition, whereas melatonin had the least inhibitory capability. Therefore, this LC–MS/MS study provided demonstration that all four antioxidants inhibited formation of adducts involved in malignant transformation of mammary epithelial cells, identifying them as potential chemo-preventing agents of cancer initiation, particularly breast and prostate cancer [47].

Gaikwad et al. also reported a study where the aim was to investigate urine biomarkers of risk in the molecular etiology of breast cancer. To this end, urine samples from 40 healthy control women, 40 high risk women (Gail Model scores were 1.67%–11.7%) and 40 women newly diagnosed with breast cancer were analyzed by UPLC–MS/MS for quantitation of estrogen metabolites such as conjugates and depurinating DNA adducts [38]. At the outset, they confirmed that relatively high levels of depurinating estrogen-DNA adducts were present in women at high risk for breast cancer or diagnosed as already diseased. Estrogen metabolism was shifted from protective methoxylation and conjugation pathways in the healthy individuals towards activating pathways leading to formation of depurinating DNA adducts in women at high risk or with breast cancer. These results support the hypothesis that breast cancer is initiated by mutations derived from depurination of estrogen-DNA adducts. Therefore, relative levels of depurinating estrogen–DNA adducts could become biomarkers for early detection of breast cancer risk and aid in prevention [38].

A recent analysis of urine samples from premenopausal women during the luteal phase showed that nine of the steroidal estrogens (E_1, E_2, 16α-OHE$_1$, E_3, 16-ketoE$_2$, 2-OHE$_1$, 2-OHE$_2$, 2-MeOE$_1$, 4-OHE$_1$) represent more than 90% of the measured urinary estrogen metabolites [48]. This was corroborated by an analogous study involving 10 premenopausal women also during luteal phase, where the same nine compounds represented 89% of all measured urinary estrogen metabolites [49]. At a much larger scale, Franke et al. analyzed steroids in 232 urine samples from 78 premenopausal women, using a benchtop orbitrap LC–MS and a single quadrupole GC–MS; while GC–MS allows the measurement of a wide range of steroids, including non-polar analytes that elude detection by LC–MS, orbitrap-based LC–MS is more sensitive, faster, cheaper and allows post-data acquisition reinterrogation of analytes not targeted a priori [50]. In this combined LC–MS/GC–MS study, sixteen steroidal estrogens, including oxidized metabolites, could be detected; the LC–MS/GC–MS Spearman rank correlation coefficients (ρ) for the relative concentrations of major estrogens E_1, E_2, E_3, 16α-OHE$_1$ and 2-OHE$_1$ were very high (ρ ~ 0.72 to 0.91), and absolute concentrations as determined by both techniques were also in agreement (below 5% difference for E_1, E_2, E_3, 16α-OHE$_1$). LC–MS allowed reinterrogation of the acquired data due to the orbitrap technology, which permitted post-analysis quantitation of progesterone, cortisol, and cortisone with an LC–MS/GC–MS ρ between 0.80 and 0.84, and differences between absolute concentrations below 7% (n = 13).

Mass spectrometry techniques have also been used in the survey of potential biomarkers of bladder cancer caused by the parasitic flatworm *Schistosoma haematobium*; this eukaryotic pathogen infects millions of people mostly in the rural regions of sub-Saharan Africa, and is associated with high incidence of bladder cancer, although why this happens remains uncertain [51]. Since it has long been known that schistosomes have estradiol receptors, Botelho et al. conducted a series of studies on sera from *S. haematobium* infected persons, as potentially carcinogenic antigens/components from these parasites may be useful to decipher schistosome-associated oncogenesis [52]. These investigators readily identified and quantitated the sex hormones estradiol, testosterone and luteinizing stimulating hormone (LH) and found that, in all cases, serum levels of estradiol were remarkably high as compared to those in sera from non-infected persons; the other hormones were not dissimilar from normal levels [52]. In view of this, the authors hypothesized that the levels of estradiol observed in infected patients were of schistosome origin, and therefore analyzed the estradiol content in extracts of worms, finding significant expression of estradiol-related molecules [52,53]. The same investigators employed LC–ESI–MS to investigate the possible presence of additional undisclosed estrogenic molecules in worm extracts and sera of schistosome-infected persons [53,54]; novel estrogen-related molecules were identified in both worm tissues and sera of infected individuals, but not in the plasma of a healthy donor, and their structures suggest that they were formed upon reaction of estrogen quinone with DNA (Fig. 1). Therefore, given the aforementioned carcinogenic potential of this estrogen adduct, the estrogen-related molecules found in extracts of *S. haematobium* may be the link between schistosomiasis and squamous cell carcinoma of bladder [54].

2.2. MS methods in the analysis of cholesterol, bile acids and related metabolites

Estrogens have not been the only steroids targeted by MS-based metabolomics studies: androgens, corticoids and other steroids such as bile acids, vitamin D, cardiac steroids and cholesterol itself exhibit physiologically relevant activities, and therefore are frequently monitored in diverse biological samples [55]. Due to the metabolic versatility of steroid molecules, extremely complex mixtures are often encountered, making their analysis quite demanding and requiring chromatographic separation prior to MS analysis [52]. Both GC–MS and LC–MS have been used in the study of steroids and their metabolites, but LC–MS is considered as the most promising analytical method for determination of steroids and steroid conjugates, due to its sensitivity, specificity and versatility [55,56]. Cholesterol is undeniably the most emblematic of all steroids; it is a constituent of cell membranes and a bioprecursor of bile acids and steroid hormones. Cholesterol is also one of the major risk factors for arteriosclerotic diseases, such as hypertension and cerebrovascular disease. Thus, the remainder of this review will focus on informative reports that present MS-based studies on cholesterol, bile acids and derivatives.

Kock et al. developed an LC-isotope dilution-MS procedure to quantify total cholesterol in serum, without the need of an in-line derivatization method, using [25,26,27-^{13}C] cholesterol as the internal standard [56]. A particle-beam interface was used for coupling the LC and the MS [20], whereas alkaline hydrolysis followed by extraction with cyclohexane was employed for sample preparation; results obtained correlated well with those produced by the stable isotope dilution GC–MS method, but some interference by other steroids in the cholesterol quantitation by the LC–MS approach was observed [55,56].

Griffiths et al. developed prototypic LC–MS/MS methods for prenatal diagnosis of Smith–Lemli–Opitz syndrome (SLOS), a severe disorder in cholesterol synthesis that is classically diagnosed prenatally by GC–MS analysis of sterol in the amniotic fluids [57]. The 3β-hydroxysterols from amniotic fluids were oxidized with cholesterol oxidase to their corresponding 3-ketones, which were then derivatized with Girard P (GP) hydrazine in a “one-pot” reaction; the GP-hydrazones formed were analyzed by LC–ESI–MS/MS, and results provided proof of concept of the potential application of this approach, once optimized, in the prenatal diagnosis of SLOS [57].

Marbel et al. developed a novel LC–MS/MS method for quantitative determination of 4β-hydroxycholesterol after analyte extraction from plasma with hexane and extract purification by normal-phase SPE followed by 4β-hydroxycholesterol isolation from cholesterol and endogenous isobaric plasma oxysterols by reversed-phase HPLC; detection was achieved by APPI–MS/MS in the positive mode, using toluene as dopant [58]. This LC–APPI–MS/MS method allowed quantitation of 4B-hydroxycholesterol in human plasma at relevant physiological levels (10.0–250 nmol/L), which makes it highly suited for the clinics, given the method’s high sensitivity, selectivity, accuracy and relatively short analysis time [58].

Oxysterols, which are mostly oxygenated forms of cholesterol in mammals (Fig. 3) recently found to be biologically active *per se* [59], have also been the focus of methodological studies based on MS techniques. For example, Honda and co-authors [60] described a highly sensitive and specific method, based on a stable isotope dilution LC–MS/MS technique, for the quantitation of the oxysterol 7α-hydroxy-4-cholesten-3-one (C4), which has been used as a biomarker for bile acid biosynthesis [24]. C4 was extracted from human serum by a salting-out procedure, derivatized into its picolinyl ester and then isolated by SPE [60] for subsequent quantitation by LC–ESI–MS/MS; the method provided highly reliable and reproducible results for quantiation of C4 in sera, especially from small volumes of blood samples [60]. Application of this method was expanded by these workers for the quantitation, in serum or rat liver microsomes, of numerous key regulatory oxysterols [61], and also for the analysis of serum sterol profiles [62]. These studies provided confirmation that derivatization of (i) dihydroxy- and epoxysterols [61], and (ii) neutral sterols [62] into the respective picolinyl esters enabled fast LC–ESI–MS/MS quantitation with high sensitivity and reproducibility. Also, it allowed to identify, in addition to cholesterol, 19 cholesterol precursors, cholestanol, campesterol, sitosterol, and sitostanol [62]. Overall, this method seems to be potentially useful in lipid metabolism studies or in the clinic for (i) diagnosis of cholesterol/oxysterol metabolism-related disorders, or (ii) quantitation of serum biomarkers for the synthesis/absorption of cholesterol.

An alternative method for the quantitation of C4 in serum, without the need for chemical derivatization, was described by Lövgren-Sandblom [63]; this method was used successfully with sera from healthy fasting volunteers, and the findings obtained were consistent with the possibility that part of 7α-hydroxy-3-oxo-4-cholestenoic acid in blood originates from extrahepatic C4. The investigators hypothesized that the rate of production of C4 in liver is directly reflected by levels of α-hydroxy-3-oxo-4-cholestenoic acid in blood, which is consistent with use of the latter as a marker for cholesterol 7α-hydroxylase activity [63].

Oxysterols occur in mammalian brain at ng/g–μg/g levels, while cholesterol is present at mg/g levels, which makes oxysterol analysis in brain rather challenging. Karu et al. developed a nano-LC–MS/MS method for the analysis of oxysterols in brain, based on SPE of the oxysterol fraction followed by an oxidation derivatization protocol and then nano-flow-LC–MS^n analysis [64]. According to these authors, while the oxidation derivatization method improved detection limits, nano-LC–MS^n provided separation of isomers and allowed for accurate oxysterol quantification [28]; in fact, they were able to identify 13 discrete oxysterols in rat brain, including 24S-hydroxycholesterol, 24S-25-epoxycholesterol and 7α,26-dihydroxycholest-4-en-3-one [64]. The same research group developed a novel LC–MS^n methodology for the identification of cholesterol metabolites in rat brain with high

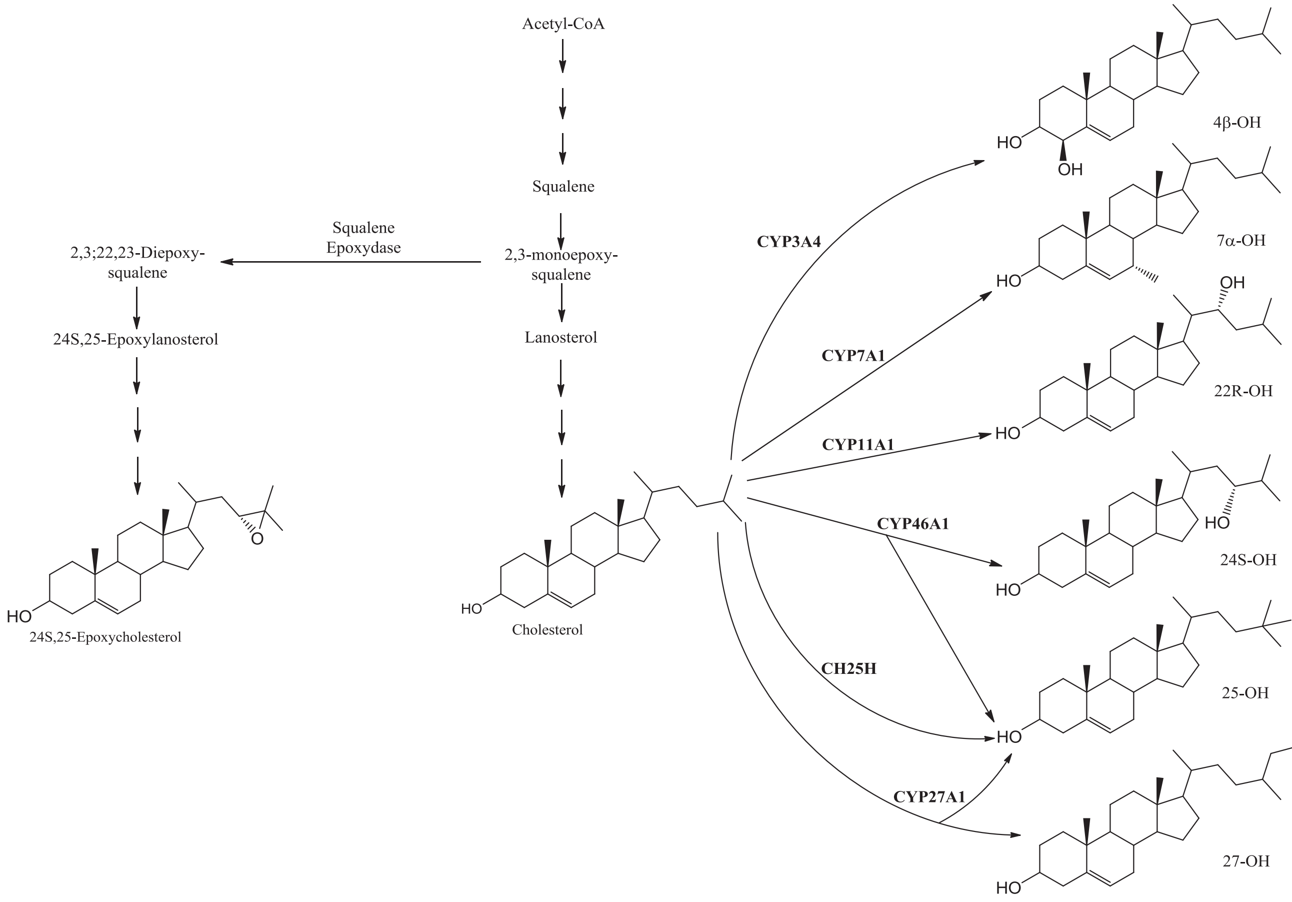

Fig. 3 – Biosynthetic pathways for key regulatory oxysterols. Hydroxycholesterols are synthesized from cholesterol, whereas 24S,25-epoxycholesterol is derived from a shunt in the cholesterol biosynthetic pathway. CH25H, cholesterol 25-hydroxylase; 4β-OH, 4β-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 22R-OH, 22R-hydroxycholesterol; 24S-OH, 24S-hydroxycholesterol; 25-OH, 25-hydroxycholesterol and 27-OH, 27-hydroxycholesterol (reproduced from [61].)

sensitivity, i.e., at the low pg level [65]. The method includes derivatization to enhance ionization, exact mass analysis at high resolution to identify potential metabolites, and MS^n ($n = 3$) to allow their structural characterization; this provided confirmation of 24S-hydroxycholesterol as a major oxysterol in rat brain, and identification of other formerly undisclosed oxysterols in brain, such as 24,25-, 24,27-, 25,27-, 6,24-, 7α-25-, and 7α-27-dihydroxycholesterols. Additionally, two molecules linked to protein amyloidogenesis, 3β-hydroxy-5-oxo-5,6-*sec*cholestan-6-al and its aldol, were identified in the same study [65].

An LC–MS/MS approach to determine 24S-hydroxycholesterol (24SOHChol) separately from 25-hydroxycholesterol in plasma was described by DeBarber and colleagues, as blood levels of 24SOHChol are a practical measure of cholesterol efflux from human brain; the method was found to be accurate and free of interference by endogenous 25-hydroxycholesterol interference, with the advantage of involving simplified sample work-up and analysis [66]. Development of highly sensitive and accurate methods for the analysis of 24SOHChol is extremely relevant also due to the fact that this oxysterol, as well as 27-hydroxycholesterol, is under investigation as a potential biomarker associated with neurodegenerative disorders such as Alzheimer's disease and multiple sclerosis [67]. In this context, Griffiths et al. developed a new LC–MS approach using charge-tagging and high resolution MS providing identification in plasma of several oxysterols and downstream metabolites such as, 7α-, 24S-, and 27-hydroxycholesterol, the cholestenetriol 7α-27-dihydroxycholesterol, and 3β-hydroxycholest-5-en-27-oic acid and its metabolite 3β-7α-dihydroxycholest-5-en-27-oic acid [67].

The formation of bile acids and bile alcohols is of major importance for the maintenance of cholesterol homeostasis, as besides their functions in lipid absorption, bile acids/alcohols are regulatory molecules for a number of metabolic processes. Their effects are structure-dependent, and numerous metabolic conversions result in a complex mixture of biologically active and inactive forms. MS is the basic detection technique for the analysis of bile acids/alcohols in biological media, usually after at least an LC separation step. Capillary LC–ESI–MS normally provides the highest sensitivity, but depending on the nature of the bile acid/alcohol mixture and the range of concentrations, discrete sample preparation sequences, ranging from simple extractions to complex group separations and derivatizations, are applicable [68].

Ikegawa et al. developed an LC–ESI–MS method for simultaneous individual determination of different bile acid 3-sulfates in human urine. Urine was subjected to SPE followed by ion-exchange chromatography on a lipophilic gel, and then submitted to LC–ESI–MS analysis; the 3-sulfates were characterized by an abundant pseudo-molecular ion $[M\text{-}H]^-$ along with a doubly charged ion $[M\text{-}2H]^{2-}$, whose ratio was markedly influenced by an acidic component added to the LC mobile phase [69]. The application of this method to the analysis of the urine from a healthy volunteer allowed detection of chenodeoxycholic acid, deoxycholic acid and lithocholic acid 3-sulfate as glycine conjugates, with very small amounts of unconjugated and taurine-conjugated bile acid-3-sulfates [34]. In turn, analysis of urine from patients with obstructive jaundice led to identification of the glycine conjugates of chenodeoxycholic acid and cholic acid 3-sulfates, but not of lithocholic acid 3-sulfate [70]. Ikegawa's group also developed a method for separation and determination of bile acid 24-glucuronides in urine using LC–ESI–MS, to provide more information about the metabolic profile of bile acids and potentially serve as diagnosis tool for hepatobiliary diseases. The extracted glucoronides were subjected to LC–ESI–MS analysis employing an ^{18}O-labeled internal standard, and detected as intense peaks due to the deprotonated molecule $[M\text{-}H]^-$ and a fragment ion $[M\text{-}H\text{-}176]^-$ [69].

LC–ESI–MS studies by Goto et al. provided unprecedented identification of bile acid acyl galactosides in urine from healthy donors; urine specimens were subjected to SPE followed by LC separation and alkalyne hydrolysis, after which cholic acid (CA) and deoxycholic acid (DCA) were identified as liberated bile acids, detected along with other unknown components [71,72]. To identify the latter, a portion of the alkaline hydrolysate was reacted with 1-phenyl-3-methyl-5-pyrazolone, which enabled detection of galactose derivatives by LC–ESI–MS; further analyses, using adequate controls, provided confirmation of the derivatives structures as being CA 24-galactoside and DCA 24-galactoside, biosynthesis of which in the human body was thus confirmed.

Goto et al. have also developed a highly sensitive LC/ESI–MS/MS method, using selected reaction monitoring (SRM) analysis, for quantitation of bile acid derivatives in human urine. This provided simultaneous analysis of bile acid 3-sulfates, including nonamidated glycine-, and taurine-conjugated bile acid, cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid and lithocholic acid, and first-time identification of 3β,12α-dihydroxy-5β-cholanoic acid 3-sulfate in human urine [72].

Last, but not least, Muto et al. recently described a new, simple and sensitive LC–ESI–MS/MS method for the identification and characterization of 39 conjugated and unconjugated bile acids, including Δ^4-3-oxo- and $\Delta^{4,6}$-3-oxo-bile acids (markers for Δ^4-3-oxo-steroid-5β-reductase deficiency) [73]. In this method, a concentrated desalted urine sample was diluted in ethanol and directly injected into the LC–ESI–MS/MS, with detection in the negative ion mode and quantitation by SRM. The remarkable performance of this new approach was confirmed by comparison with a previously validated GC–MS analysis method, using urine from patients with genetically confirmed Δ^4-3-oxo-steroid-5β-reductase deficiency and from a patient with abnormally high levels of conjugated and unconjugated Δ^4-3-oxo-bile acids [73].

3. Mass spectrometry to assess androgen status

The highly sensitive and specific LC–MS/MS methods have been shown to be superior to conventional immunoassays to assess low sex hormone concentrations. Haring et al. [74] determined age-specific reference ranges for LC–MS/MS-measured total testosterone and prohormone androstenedione (is the most common sex hormone precursor in both sexes and plays a crucial role in the biosynthesis of testosterone) as well as calculated free testosterone, in a large population-based sample of women aged 20–80 years.

This LC–MS/MS method allows rapid, sensitive, and specific determination of serum sex hormone concentrations in women and is therefore suitable for routine clinical practice and research. Thus, the presented age-specific reference limits are particularly valuable to translate testosterone concentrations outside the reference range into clinical treatment and to establish appropriate cutoffs for clinical guidelines and epidemiological studies [74].

Reference ranges are essential for partitioning testosterone levels into low or normal and making the diagnosis of androgen deficiency. Bhasin et al. established reference ranges for total testosterone and free testosterone in a community-based sample of men, using liquid chromatography tandem mass spectrometry [75]. Reference ranges generated in a community-based sample of men provide a rational basis for categorizing testosterone levels as low or normal. Men with low total testosterone or free testosterone by these criteria had higher prevalence of physical dysfunction, sexual dysfunction, and diabetes [75].

The association between aging-related testosterone deficiency and late-onset hypogonadism in men remains a controversial concept. Mass spectrometry was used to develop criteria for identifying late-onset hypogonadism in the general population on the basis of an association between symptoms and a low testosterone level [76]. Late-onset hypogonadism can be defined by the presence of at least three sexual symptoms associated with a total testosterone level of less than 11 nmol/L (3.2 ng/mL) and a free testosterone level of less than 220 pmol/L (64 pg/mL) [76].

Recently, testosterone was measured for the investigation of female hyperandrogenism and male hypogonadism [77]. LC–MS/MS is becoming the method of choice but comprehensive reference ranges are lacking. Testosterone was measured by tandem MS on 90 healthy women, 67 young healthy men and pregnant women (59 first trimester and 60 second trimester). The male, male calculated free, first trimester and second trimester testosterone reference ranges (derived using the antilog of mean ± 1.96 SD of log transformed data) were 10.6–31.9, 0.23–0.63, 0.6–4.9 and 0.9–4.9 nmol/L, respectively. The female testosterone upper reference range limit, derived non-parametrically from the 97.5th centile, was < 1.7 nmol/L [77].

4. Conclusion

The introduction of soft ionization methods in MS and the evolution of highly sensitive and versatile LC–MS/MS techniques have made possible the analysis of steroid hormones with small sample requirements, and simplified sample preparation. The latest reports in this field, as those reviewed here, demonstrate that MS-based techniques have a central role in steroid metabolomics, contributing not only to a deeper understanding of steroid metabolism, but also to the identification of clinically relevant disease biomarkers.

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