Current Trends of Liquid Chromatography Tandem Mass Spectroscopy Use in Clinical Gynecology

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In the present environment of staggering technical innovations and increasing expectations of quality healthcare it is evident that we need to fine tune our diagnostic abilities in order to fulfil patients' demands for more efficient therapies and augmented quality of life. We are looking for current trends in clinical gynecology that make use of Liquid chromatography tandem mass spectroscopy, technology not yet employed in Romanian laboratories for the clinical practice but that is rapidly becoming the worldwide method of choice for accurate characterization of the hormonal milieu essential for the requirements of women healthcare.

Keywords: LC-MS/MS, testosterone, estradiol, vitamin D, PCOS, menopause, breast cancer

The need for accurate diagnosis is as stringent as ever, but the last 100 years have seen a growing rate of knowledge accumulation. This trove of data is bringing new life in the medical debate and there are shifting definitions and new understanding of disease mechanisms. At the same time there are competing methods of achieving the same goal and a cacophony of supporters paralleled by the almost silence of the communication gap between diverse fields of use. There is an overlap of interest concerning the sex steroids and gonadal function in women that arouse the interest both of endocrinologists and gynecologists. To these two parties there are now rallying the medical oncologists and cancer surgeons and they, too, need accurate measurement of sex steroids in order to assess treatment efficacy and disease relapse.

The various steroid hormones including glucocorticoids, mineralocorticoids, progestins, androgens and estrogens are biosynthesized from cholesterol by the action of enzymes like cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs) and steroid reductases. Biologically active steroids can be synthesized in the adrenal gland, testis, ovary, brain, placenta and adipose tissue; but the adrenal gland, testis, and ovary specialize in de novo steroid production [1]. Steroids may be targeted in diverse biological materials and due to the metabolic versatility of the steroids, there are complex combinations of steroidal metabolism products to be analyzed and measurement frequently needs to be preceded by chromatographic procedures. The basic steroid molecular structure consists of four rings of carbon atoms, perhydro-1,2-cyclopentenophenanthrene with one or more methyl groups at carbon positions that are shared by two rings, and they may be categorized according to the numbers of carbon atoms gonane C17, estrane C18, androstane C19, pregnane C21, cholane C24 and cholestane C27. Many of these compounds are endogenous hormones or precursors, androgens, corticoids, estrogens, progestogens, other are bile acids, cholane, and vitamin D compounds (9,10 secosteroids) and cardiac steroids.

There is an ongoing discussion referring to methods of analyzing low levels of steroids and the best method to be employed. Steroid hormones have been measured, since 1930, primarily in urine, by GC-MS (gas chromatography – mass spectroscopy) and by RIA (radio-immunoassay) usually in serum and plasma and to these stable isotope dilution LC-SRM/MS was added. RIA methods have applications in the rapid determination of higher levels of the main estrogen metabolites. Accumulating data suggest that the RIAs are measuring cross-reacting material, which falsely elevates the estrogen levels [2]. A well-known limitation of GC-MS, long considered the gold standard in steroid analysis, is represented by the requirement of analytes volatilization raising the necessity of multiple extraction and purification steps along with a chemical derivatization to render the analytes suitable for analysis being, thus, tedious and time consuming. From around 1960, being faster and higher yielding methods, automated immunoassays have been promoted for use in routine clinical laboratories as they skip the separation and derivatization steps. Commercial immunoassays widely used in clinical labs have been repeatedly proved to be highly prone to interference as a result of their inherent speciûcity problems mostly in low molecular weight, low volume analytes [3-6]. The analytical specificity equates to the ability of a mass spectrometer to determine the mass-to-charge ratio (m/z) of the ions of interest.

Historically, the extensive sample preparation schemes required for GC-MS analysis limited the widespread clinical application of mass spectroscopy (MS) because of low throughput and high cost. Atmospheric pressure ionization techniques such as electrospray ionization (ESI) combined with high performance and ultra-high-performance LC-MS/MS enabled MS to become an affordable platform for routine clinical laboratories. Semi-automated oû-line solid phase extraction has been implemented in many laboratories in order to achieve higher throughput, being used for sample preparation prior to ultra-performance LC-MS/MS analysis using electrospray ionization with detection by LC-MS/MS measurement of serum steroids. LC-MS/MS machines have slowly permeated the clinical laboratory practice, finding its niche besides high-throughput but interference susceptible immunoassays and the highly speciûc and laborious GC-MS. In the last twenty years LC-MS/MS has

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been pushed to the forefront, catching-up with immunoassays for high-throughput, offering speciûc measurement of serum steroids and becoming standard in many clinical reference laboratories.

LC-MS/MS measurement of serum steroids provides accurate insights for the clinical investigation of conditions such as polycystic ovarian syndrome (PCOS) and infertility and have, also, been proved useful in monitoring treatment in patients with antihormonal therapies. The majority of steroids are measured in positive ionization mode, but use of negative ionization mode is common for such compounds as aldosterone and dehydroepiandrosterone sulphate (DHEAS) [7]. In order to obtain an accurate LC-MS/MS quantitation, the technique requires a stable isotope internal standard (IS) and is added to all samples, calibrators and quality controls being performed, prior to extraction and LC-MS/MS analysis. The eûects of the matrix upon signal intensity due to ion suppression or enhancement are corrected by determining the ratios of analyte to IS signals. Some LC-MS/MS methods employ IS with two or more hydrogens replaced by deuterium atoms but other methods make use of C13.

The IS has a diûerent mass and ion transition when compared to the analyte, but have identical chemical and physical properties exhibiting the same behavior as the analyte through the procedure. Carbon-13 labelled IS are becoming more available and increasingly being used as they co-elute at a higher percentage with the non-labelled analyte and seem to be more eûective at compensating for matrix efects than deuterium labelling [8]. A potential problem in steroid LC-MS/MS assays is that of speciûcity, due to the close structural similarities of the intermediate metabolites in the steroidogenic pathway, some having identical molecular weight and being isobaric. It is essential that these are resolved by measures that ensure selectivity of the mobile phase such as reverse phase chromatography to undergo the same ion transitions in the mass spectrometer or, else, cause positive bias by cumulation.

Testosterone testing

In the clinical laboratory, testosterone is one of most the frequently serum steroid measured by LC-MS/MS analysis [9]. In an audit of over 5000 female samples in which testosterone was measured with over 800 samples referred for conûrmation of elevated levels, 23% were found to have LC-MS/MS results within the reference range [10]. It is considered that one of the most common causes of positive interference in immunoassays for testosterone in female samples may be norethisterone, a synthetic form of progesterone used in hormonal contraceptives [11].

Polycystic ovarian syndrome (PCOS) is the most frequent endocrinological disorder in women as it may affect up to 15-20% of women of reproductive age, depending on the diagnostic criteria [12,13] and the condition is characterized by increased levels of testosterone. PCOS is a known cause of anovulation and infertility through impaired oocyte-follicle maturation [14]. It may be difficult to quantify the costs of medical assistance employed in reducing the stress burden of affected women and the economic impact PCOS is important since almost 4 billion dollars are being spent in United States every year to test for the disease and treat its conditions and the Australian Health System reportedly allocates 800 million dollars per year [15]. On the cost side we should also address the fact that being cheap, testosterone and other hormones levels are repeatedly measured by inadequate methods with no better results. Beyond the fact that health boards all over the world struggle to find unifying lines for PCOS it is evident that accurate and precocious diagnosis of PCOS is stringent for future health comorbidities prevention and, less important, also to reduce costs and increase efficiency.

The use of varying PCOS diagnostic criteria highlighted issues of finding common grounds for PCOS research worldwide, which then translated in confused clinical practice and a much-delayed progress in understanding the syndrome. The International evidence-based guideline for the assessment and management of polycystic ovary syndrome aims to promote PCOS recognition, whilst avoiding over diagnosis, especially in adolescents, endorsing the recommendation of the National Institutes of Health (NIH) evidence-based methodology workshop of PCOS 2012. Among the diagnostic criteria the guideline counts calculated free testosterone, free androgen index or calculated bioavailable testosterone that should be used to assess biochemical hyperandrogenism in the diagnosis of PCOS. Also, it is recommended that high quality assays such as LC-MS/MS and extraction/chromatography immunoassays, should be used for the most accurate assessment of total or free testosterone in PCOS. It indicates that assessment of biochemical hyperandrogenism in PCOS by direct free testosterone assays, such as radiometric or enzyme-linked assays, should not be used due to low sensitivity, accuracy and precision [16,17]. O'Reilly et al. study indicate that high androstenedione levels are a sensitive indicator of PCOS-related androgen excess and it is suggested that concomitant measurement of serum testosterone and androstenedione is useful for predicting metabolic risk in PCOS [18]. Increased use of LC-MS/MS assays into the clinical laboratory and the measurement of testosterone and androstenedione in the same run underline androstenedione as an important cause of hyperandrogenism in a subgroup of patients with PCOS [19]. These cases would have been, otherwise, undiagnosed by testosterone measurement alone. Are obvious the benefits of measuring two or more steroids simultaneously, representing an advantage over radio-immunoassays or routine automated immunoassays.

Williams, E. describes a semi-automated solid phase extraction LC-MS/MS method for the simultaneous measurement of androstenedione, testosterone and 17-OH progesterone with excellent recovery and minimal ion suppression/enhancement, seemingly comparing favorably with stand-alone LC-MS/MS methods [10]. As a poster of the method's high yield and ease of use, the assay is reportedly routinely done several times a week with 80 patient sample plates, standards and controls, automated sample extraction being completed in around 90 minutes and 5-minute LC-MS/MS sample to sample injection time. There are numerous published studies on LC-MS/MS methods for testosterone quantitation that highlight the multiplex capabilities of the technique and mainstream becoming [20-23].

Estrogen testing

Breast cancer is the most frequent neoplasm in women [24] and endometrial carcinoma is the 4th most frequent [25,26] and are both supposed to be dependent on the estrogen levels after menopause. Management and therapeutic options for these conditions may employ Aromatase Inhibitors agents that require accurate estrogen levels monitoring. The post-menopausal blood levels of unconjugated estrogens are in the extremely low ranges (low pg/mL, even fg/mL for some metabolites) and their accurate measurement is distinctly challenging. LC–MS/

MS methods are becoming the most used for the extremely low level metabolites in postmenopausal women [27]. Reliable estrogen levels determination is necessary in cancer prevention for menopausal women for whom it has been demonstrated that high levels of estrogen may increase the risk for both breast and endometrial cancers [28-30]. Lukanova et al. showed that postmenopausal women in the highest quartiles of plasmatic E2 and E1 levels have their risk of endometrial cancer increased by 4.1- and 3.7-fold, respectively [31]. Endometrial cancer mainly occurs in postmenopausal women, implying that in absence of ovarian estrogen synthesis, the contribution of extraovarian tissues to circulating estrogens levels becomes essential.

Data from a large prospective European study reported by Kaaks and colaborators provide strong evidence for the independent roles played by androgens and estrogens levels in predicting breast cancer risk [32]. Another study, conducted by Key et al. collected data from nine studies of similar type and came to similar conclusions [33]. The conversion of androgen-estrogen adrenal precursors, dehydroepiandrosterone and androstenedione, to E2, E1, and E1-sulfate (E1-S) takes place in peripheral tissues and relies on the actions of an enzymatic complex, but estrogen can also be formed from sulphated estrogens such as E1-S and sulfatase produces E1 from the E1-S metabolite, having an important contribution to estrogen synthesis in malignant endometrium [34], facts that highlight the potential use of multiple reaction monitoring LC-MS/MS that can quantify these metabolites and contributing to risk assessment. In the continuous stride to develop methods for LC MS-MS that are both reliable, reproducible and fast, Wang et al. described an ultrasensitive stable isotope dilution liquid chromatography-tandem mass spectrometry method (LC-MS/MS) for multiplexed quantitative analysis of six unconjugated and conjugated estrogens in human serum [35].

Vitamin D testing

The Endocrine society has successively stated calls and issued recommendations for clear protocols for testosterone and estradiol testing, promoting LC-MS/MS essays and encouraging similar attitude towards vitamin D measuring. [36-38]. Vitamin D is a secosteroid with endocrine activity, with synthesis pathways in the human body taking place in skin, liver and kidneys and presence of vitamin D receptors in many tissues [39,40]. The primary circulating metabolite, 25-hydroxyvitamin D_3 ($25(OH)D_3$), requires at first a hydroxylation step in the liver microsomes and the active form, 1á,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), occurs after a second hydroxylation step, mainly in the kidney. Through upregulation of a number of diverse genes, $1,25(OH)_2D_3$ produces the majority of the actions of the biological spectrum of vitamin D_3. It is usually known for its controlling calcium and phosphorus homeostasis role, being considered essential in absorption and metabolism of calcium and phosphate, most evident in bones health [41]. It influences a large array of processes such as immune function, cell proliferation, differentiation, and apoptosis [42] and during pregnancy [43], has an important role in fetal growth, development of the nervous system, lung maturation and fetal immune system function.

Vitamin D insufficiency has been related to the development of diabetes, pre-eclampsia and fetal neurological disorders [44] and is correlated with reduced female fertility [45], with complex underlying mechanisms, diminished levels of ovarian anti-Müllerian hormone (AMH) representing an alleged factor [46]. Vitamin D receptor is expressed in the ovary, endometrium, and myometrium and there are data indicating possible correlation between vitamin levels and receptor expression in endometrium or endometrial pathologies and, also, variations of receptor expression during the normal menstrual cycle.

The potential anti-proliferative and anti-inflammatory effects of vitamin D_3 for the treatment of endometriosis have been evaluated and the effect upon ectopic endometrial cells could demonstrate reduced cytokine-mediated inflammatory responses [47]. Similar, an alteration of vitamin D metabolism in terms of increased 24-hydroxylase mRNA and protein expression has been investigated in endometrial cancer, and the effect of D_3 as an anti-proliferative, pro-apoptotic, anti-inflammatory and differentiation-inducing agent has been demonstrated in various endometrial cancer cell lines [48]. Normal range for the circulating blood levels of $1,25(OH)_2D_3$ are very low, in the low picograms per milliliter spectrum [49] and low concentrations and interference from other vitamin D_3 metabolites make precise evaluation of $1,25(OH)_2D_3$ relatively difficult.

Progress has been made to increase the sensitivity of LC-MS-MS assays by either using a more sensitive derivatization reagent [50] or by using microflow LC–MS-MS concept to improve the instrument sensitivity [51]. Inconveniences associated with those methods, including cost, availability, the stability of the agents used, and the complexity of the system involved have led to new and effective methods to being continually developed [52,53].

The active form of vitamin D circulates in much lower levels [54], thus, 25-OH-D is in the ng/mL range, $1,25-(OH)_2-D_2$ and $-D_3$ are in the low pg/mL range, and testing for it requires RIA or LC/MS/MS methods that present higher sensitivities. Conventional methods lack sufficient accuracy, specificity, sensitivity and repeatability and these traits have made LC-MS/MS become the method of choice for $1,25-(OH)_2-D_2$ and $-D_3$ analysis [55]. RIA perform accurate measurements, but they necessitate extensive preparation due to antibody cross-reactivity with vitamin D metabolites, and these methods do not separate the two isoforms of $1,25(OH)_2$-D without chromatography. Although the LC/MS/MS methods are considered the *gold standard* for measuring active forms of vitamin D, their use remains a challenge due to the poor ionization efficiency caused by a lack of ionizable polar groups [55]. Derivatizing techniques have been developed to enhance the detection response of the poorly ionizable compounds [56]. Though several derivatizing agents have been reported, only 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD is readily available commercially for laboratory use and has demonstrated the ability to allow analysis of $1,25-(OH)_2-D_2$ and $-D_3$. The addition of PTAD derivatization to multiple solid phase extraction techniques has improved sensitivity and decreased sample size [57].

Automatization

The direction of development for LC-MS/MS is toward full automatization for use in the clinical lab. There are still employed methods that are too labor intensive and are attended by doctoral level scientists, facts that impede reaching cost efficiencies of hospital or clinical level. A routine task in a research lab such as reconfiguring the LC-MS/MS system for a new assay, issues like calibration standards, HPLC columns, solvents and buffers, and sample-specific handling procedures arise may prove very difficult even for highly trained clinical laboratory technician. In the background of rising demand for steroid

and vitamin D levels measurement there surges a need for less demanding LC-MS/MS machines that can, for example, assess both vitamin D2 and D3 levels simultaneously in a single assay or a multiplexed panel of sex steroids defining in one stroke the hormonal picture needed for the complete diagnostic conclusion.

LC-MS/MS is better suited to measure multiple molecules in a single run and automated, integrated LC-MS/MS systems should provide around the clock LCMS/MS testing, attended by technicians with basic lab tech skills. Until now automation meant bringing automated sample handling capabilities to preexistent LC-MS/MS machines, thus replacing manual processes, combining mass spectrometry and automated handling system to an evident improvement and creating manually interconnected areas of automation, furthering the potential for error that automation is expected to lower. When systems do not produce the expected accuracy, the complexity of tracing the issue is multiplied by the number of diverse components. For the LC-MS/MS systems to become practical for hospitals and clinics, it would have to encompass all HPLC components including pumps, columns, solvents, buffers, mass spectrometer calibrants, sample calibrators, internal and calibration standards, sample handling robotics like centrifuges and cool storage, waste disposal, etc. in an integrated unit with software that further integration within and with other lab or hospital services. As major companies that supply LC-MS/MS machines get closer to achieving the goal of completing fully automated analyzers (ThermoFisher Scientific just launched one and is on course to certify different analytes), thus lowering the need for extremely high qualified lab staff, we expect costs to lower and the presence of this technology in clinical labs to enhance.

Conclusions

We have no knowledge of LC-MS/MS employed in clinical lab testing in Romania as clinical laboratories use immunoassay-based testing. As numerous studies highlighted the inappropriateness of immunoassays in evaluating sex steroid due to cross reactivity and to the high number of closely related metabolites, the MS technology has come of age, the slow progress being related to the complex processes employed in this sophisticated technique. There are now more and more fields in which mass spectrometry is involved and the growing numbers of machines used will hopefully lead to lowering prices and to an increasing ease of use.

Advances in LC-MS/MS technology have enabled the development of high-throughput, sensitive and precise methods for steroid measurement, LC-MS/MS assays have, by now, been published for all of the steroids in the steroidogenesis and metabolism pathways, using a variety of approaches for sample preparation prior to analysis, like protein precipitation, liquid-liquid extraction, solid phase extraction and supported liquid extraction. LC-MS/MS steroid panels may become the choice for female hyperandrogenism, postmenopausal sex hormones evaluation, infertility investigation and steroidogenic defects testing.

The more frequent use of semi-automated, high-throughput LC-MS/MS assays for multiplexed steroid measurement provides a window of opportunity for a neater image of female steroidome. There are serious technical hurdles to pass ensuring good sensitivity and specificity and there are described methods that both simplify and retain good accuracy. Besides numerous uses and methods employed in diverse labs from environmental studies to doping control, metabolomics and cancer research, there is a real need for precise determination of steroids in low levels cases.

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