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Annales d'Endocrinologie 77 (2016) 43–48
Annales d'Endocrinologie
Annals of Endocrinology

Review

Role of mass spectrometry in steroid assays

Apport de la spectrométrie de masse au dosage des stéroïdes

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Abstract

In addition to protein hormones, steroids measurement constitutes the basis of modern endocrinology. Immunoassays have shown their limits in this field. In contrast, mass spectrometry shows an excellent sensitivity and specificity that make it the method of choice for steroids assays. The recent introduction of UHPLC-MS is a major advance which reinforces this position. In fact, mass spectrometry provides a lot of advantages such as determination of certain steroids in saliva, diagnosis of enzyme deficiencies, or measurement of molecules previously inaccessible like aldosterone. However, standardization is still needed to ensure good comparability of results between laboratories. In the future, mass spectrometry should not replace the immunoassays but rather complement it.
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Keywords: Mass spectrometry; Steroids; Estrogens; 17-hydroxyprogesterone; Testosterone

Résumé

À côté des hormones protéiques, le dosage des stéroïdes est à la base de l'endocrinologie moderne. Les techniques immunologiques ont montré leurs limites dans ce domaine. Au contraire, l'excellente sensibilité et spécificité de la spectrométrie de masse en font la méthode de choix et l'introduction récente de l'UHPLC-MS constitue une avancée majeure qui conforte cette position. En effet, la spectrométrie de masse apporte beaucoup d'avantages comme le dosage de certains stéroïdes dans la salive, le diagnostic des déficits génétiques en enzymes de la biosynthèse de ces hormones, ou encore le dosage de molécules autrefois difficilement accessibles comme l'aldostérone. Toutefois, la standardisation reste nécessaire pour permettre une bonne comparabilité des résultats entre les laboratoires. Dans l'avenir, la spectrométrie de masse ne devrait pas remplacer l'immunoanalyse, mais plutôt lui être complémentaire.
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Mots clés : Spectrométrie de masse ; Stéroïdes ; Estrogènes ; 17-hydroxyprogestérone ; Testostérone

1. Introduction

Chromatography coupled to mass spectrometry is a very useful method for the measurement of steroids. These methods are characterized by high sensitivity and selectivity, while using *samples with small volume* [1]. The most often, the developed methods associate the power of chromatographic separation with the sensitivity of mass spectrometry. Different mass analyzers can be used like *time* of *flight mass spectrometry* (TOF-MS), high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS), and *gas chromatography mass spectrometry* (GC-MS). Mass spectrometry has known in last few *years* a huge progress in medical biology. Thus, these techniques have been used in various applications such as the identification of germs in bacteriology, neonatal screening for inborn errors of metabolism, proteomics and hormonology. The aim of this article is to treat the recent advances regarding steroid assays using mass spectrometry.

2. Steroid assays history

In addition to protein hormones, the determination of steroids constitutes the basis of modern endocrinology. The use of mass

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http://dx.doi.org/10.1016/j.ando.2016.01.004
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spectrometry for steroids measurement started with analysis of these hormones in urine by GC-MS, that was described for the first time by Horning and Sweeley in 1960 [2]. Immunoassays have been a significant development in the 1960s. The first steroid measurement by radioimmunoassay (RIA) dates back to 1969 with the determination of estradiol (E2). Then, RIA has been quickly adapted to other steroids such as testosterone and progesterone. The sensitivity, accuracy and reproducibility of RIA made that it was the reference method whether for research or clinical practice. In the late 1970s, others immunoassays were developed based on enzymometry, fluorimetry or chemiluminescence. Like RIA, these methods can require pretreatment with a chemical extraction or chromatography, depending on antibody specificity. But, automation made them much faster and simple to use, which contributed to their generalization use in daily practice of clinical laboratories. In recent years, mass spectrometry has experienced major technological advance and has become the method of choice for steroids measurement [3].

3. Mass spectrometry

Mass spectrometry is an analysis method used to quantify, identify compounds, and elucidate the structure and chemical properties of different molecules (M). The process of analysis by mass spectrometry involves the ionization of molecules and conversion into a gas phase to allow analysis. This step can be performed with or without fragmentation. Thereafter, the separation is done as a function of mass to charge ratio (m/z). We are treating here only the main methodological aspects of mass spectrometry, essential to understand steroid assays. For readers interested in learning more, we recommend the review of Menet [4].

3.1. Ionization

3.1.1. Atmospheric pressure chemical ionization (APCI)

Liquid effluent is introduced directly into the ionization source through a probe. The sample solution undergoes a nebulization to form an aerosol spray of fine droplets and is rapidly heated in a stream of nitrogen gas. The mixture of the nebulizing gas and those from evaporation is driven to a corona discharge, where acid-base reactions occur. In general, proton transfer occurs in the positive or negative ion mode to yield $[M\text{-}H]^+$ or $[M\text{-}H]^-$ ions, respectively.

3.1.2. Atmospheric pressure photoionization (APPI)

APPI is the last arrival in the family of atmospheric pressure ionization methods to couple mass spectrometry to liquid-phase separation techniques [5]. This method is used for low to moderately polar compounds. The samples are ionized by using UV light; the molecules interact with photon of UV light and vapors of nebulizer liquid solution. The analyte molecules absorb a photon (hv) and become electronically excited molecules. If the ionization energy of molecules is lower than that of photons, molecules lose an electron to form cations.

3.1.3. Desorption electrospray ionization (DESI)

This method has high sensitivity, is virtually instantaneous in response time, and is applicable to small-molecule organic compounds as well as to biological macromolecules [6].

Ions are generated from the sample surface by way of bombardment with high velocity, charged micro-droplets through the atmosphere. The spray impact causes the formation of microscopic liquid layers on the sample surface in which the condensed-phase analyte dissolves. This process is followed by desorption via momentum transfer when additional droplets collide with the liquid layer forcing the dissolved analyte into the gas (atmospheric air) phase in the form of micron-sized droplets [7].

3.1.4. Atmospheric pressure photoionization (DAPPI)

DAPPI is an ambient ionization technique for mass spectrometry; it relies on a heated nebulizer microchip delivering a heated jet of vaporized solvent and a photoionization lamp emitting 10-eV photons. The solvent jet is directed toward sample spots on a surface, causing the desorption of analytes from the surface. The photons emitted by the lamp ionize the analytes, which are then directed into the mass spectrometer [8].

3.1.5. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)

They allowed analysis of non-volatile and thermolabile compounds under adequate experimental conditions, with minimal fragmentation. These two techniques can be easily coupled with liquid chromatography systems. ESI can proceed via different mechanisms. Low molecular weight analytes follow the ion evaporation model, whereas the charged residue model applies to large globular species [9].

In MALDI analyses, the sample must be mixed with matrix and spotted in a stainless steel plate prior the analysis in the mass spectrometer. The sample is co-crystallized with the matrix, which has an essential function in MALDI. The co-crystallized sample is ionized by short laser pulses [10].

3.2. Analyzers

The analyzers are usually composed of several quadrupoles (generally three). Each quadrupole includes four electrodes that apply two electric fields: constant and alternative. In the quadrupoles, the trajectory of an ion subjected to these electric fields depends on both its mass and its load.

In case of tandem experiments, parent ions are separated from most others of the mixture in the first quadrupole. Then, parent ions are fragmented in the second quadrupole and analyzed in the third one. The combination of two or more analyzers in the same mass spectrometer yielded the high performance and resolution of the nowadays equipments. In tandem mass spectrometry, simple reaction monitoring (SRM) and multiple reactions monitoring (MRM) are used in quantitative analysis. In SRM, one of the fragment ions is analyzed, whereas multiple fragments ions are monitored and quantified in MRM [11]. The SRM gives a more important signal than MRM, but the background noise of

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SRM is much higher. Thus, MRM offers better sensitivity than SRM, which significantly reduces the risk of false negatives.

In time of flight analyzers, the ionization source is the most often MALDI source. Accelerated ions enter a tube containing no electric fields. The time it takes for a ion to traverse the tube and reach the detector is directly related to the m/z ratio and thus enables to differentiate it.

4. Limits of immunoassays and advantages of mass spectrometry

In order to automate immunoassays, the first steps of extraction by solvents or chromatography have been removed for getting results quickly (10–15 minutes) at the detriment of specificity [12]. Immunoassays also exhibit a number of other limitations such as cross-reactivity of human heterophile antibodies directed against animal antibodies contained in reagents, and the possible presence of autoantibodies that can lead to defaults or excess values and therefore false the results. In addition, matrix differences exist between patient's sera (hemolysis, icterus and lipemia) and calibrators used for the preparation of calibration curves. Furthermore, immunoassays often do not have a removal step of hormone transport proteins like the sex hormone binding globulin (SHBG) which transports 60 to 70% of circulating testosterone.

Mass spectrometry does not appeal to antibodies and thus overcomes these interferences. In addition, LC-MS/MS allows measurement of a panel of steroids like the androgen panel (testosterone, androstenedione, dehydroepiandrosterone and/or dehydroepiandrosterone sulfate). The automation or semi-automation of these methods is a real technological revolution for clinical diagnosis.

5. Applications of mass spectrometry in steroid assays

5.1. Corticosteroids

Isotope dilution high performance liquid chromatography/tandem mass spectrometry (ID-LC-MS/MS) with *online solid phase extraction* is an automatable method that presents good accuracy, high precision and no interference with structural analogs, making it the reference method for the determination of cortisol and their precursors [13]. Moreover, the majority of cortisol assays measure total cortisol, whereas it is the free fraction that is only active. Mass spectrometry showed robustness and sufficient reproducibility for a routinely determination of the free fraction in serum [14]. A major progress in this domain is the possibility of measuring free cortisol in other body fluids such as saliva and urine by LC-MS/MS with ESI in positive and negative ion mode [15–17]. The measurement of cortisol in saliva was impossible for many years because cortisone is in much important concentrations due to conversion of cortisol by an 11β-hydroxysteroid dehydrogenase type 2 and immunoassays are not sufficiently discriminative to differentiate these two hormones [18]. Mass spectrometry is also very useful for screening inborn errors of steroids metabolism. Indeed, it is possible to determine simultaneously various precursors and metabolites such as corticosterone, androstenedione, deoxycorticosterone, testosterone, dehydroepiandrosterone (DHEA) and progesterone [19]. The determination of certain of these metabolites such as 11-deoxycortisol, 21-deoxycortisol, 17-hydroxypregnenolone and pregnenolone was exclusively reserved to specialized laboratories.

Congenital adrenal hyperplasia (CAH) describes a group of autosomal recessive disorders of cortisol biosynthesis [20]. The most frequent form is due to a mutation in the 21-hydroxylase gene which results in the increase in 17-hydroxyprogesterone production. Accordingly, measurement of 17-hydroxyprogesterone in blood collected in filter paper is the basis of neonatal screening for CAH. But, many false positives have been reported, particularly in premature infants. The simultaneous determination of metabolites by mass spectrometry avoids these false positives [21].

Aldosterone is the main mineralocorticoid, it is secreted by the zona glomerulosa of adrenal cortex. Since 1970, this steroid is essentially measured by RIA after extraction. Currently, aldosterone assay may be performed by ID-HPLC-MS/MS or ultra high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) [22,23]. Aldosterone is not well ionized by ESI, whereas APPI gives a better sensitivity than APCI, but it is not compatible with the determination of a number of other steroids.

Steroids have an important role throughout pregnancy. In a recent study, urinary metabolites of steroids of pregnant women were measured by mass spectrometry using two methods: DESI or DAPPI, for screening biomarkers during pregnancy. In DESI, signals of eleven steroid metabolite ions were found to increase as the pregnancy proceeded, and in DAPPI ten steroid ions showed at least an order of magnitude increase during pregnancy. These two different profiles can be complementary, because they do not evaluate the same steroids C18 and C21 in DESI, and C19 and C21 in DAPPI. Analyzes are also conducted with various modalities negative mode in the first and positive mode in the other [24].

5.2. Estrogens

Estrogen assay is indicated to evaluate ovarian follicular maturation, as part of medically assisted procreation for the diagnosis of ovarian or pituitary insufficiency, to look for delayed puberty, and also for menopause diagnosis. There is a wide variability in results of immunoassays compared to GC-MS [25]. In addition, recent studies report that analytical performances of LC-MS/MS without derivatization are greater than those of immunoassays for the measurement of E2 and oestrone (E1) in low concentrations, which is particularly useful for man, child, women after menopause and patients treated with anti-aromatase [26–28]. The measurement of free E2 provides a better representation of active fraction. It is carried out by equilibrium dialysis-liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS) in positive ionization mode with MRM [29]. Moreover, estrogens are known to have neuroprotective effects. Nguyen et al. developed a LC-MS/MS method

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that allows simultaneous detection of four estrogens in cerebrospinal fluid of ischemic trauma patients. Ischemic trauma patients, namely estriol (E3), E1, 17β-estradiol (17β-E2) and 17α-estradiol (17α-E2). The sensitivity and accuracy were greater than 86% for the two E2 compounds and 76% for E1 and E3 while the extraction recovery ranged from 91% to 104% [30].

5.3. Androgens

Immunoassays have been criticized for a long time for their low sensitivity. It was suggested that these methods give bad results for low values of testosterone. This poses problem when investigating a polycystic ovarian syndrome (PCOS) or primary ovarian failure in women, or for exploring the clinical stages of puberty in children and adolescents, as well as for men treated with antiandrogens. Whereas, recent studies report that LC-MS/MS offers good sensitivity, precision and accuracy that are comparable to those of RIA in testosterone measurement and therefore provide the same clinical information [31,32].

A steroidomic approach consisting on determining a steroid profile allows the differential diagnosis of diseases caused by different enzyme deficiencies. For instance, the differential diagnosis of CAH, Cushing's disease and POCS can only be obtained by the measurement of a number of steroids [33].

A study conducted on 196 sera of patients with prostate cancer suggests that low levels of DHEA examined by UHPLC-MS MS are in favor of a poor prognosis [34]. Furthermore, measurement of *unconjugated* and *conjugated estrogens*, and androgens by LC-MS/MS in serum and plasma of postmenopausal women allows assessing the risk for breast cancers [35]. This is now achieved by advanced technology of polarity switching which allowed detection of both positive (testosterone and progesterone) and negative (estrogens) ions in the same analytical experiment [36].

5.4. Vitamin D

In addition to conventional effects of vitamin D on bone which are well known. Vitamin D deficiency has been associated with many diseases such as diabetes mellitus and cancers. The vitamin D status is evaluated by measurement of 25(OH)-D. In 2004, Vogeser et al. proposed a reference method using an LC-MS/MS [37]. Then several variants have been proposed and two methods were selected by the joint committee for traceability in laboratory medicine (JCTLM) and the international federation of clinical chemistry and laboratory medicine (IFCC-LM). These two methods differ essentially by ionization mode: APCI or ESI; and derivatization procedure [38].

Besides, more than 50 metabolites of vitamin D were reported. Among them the C3-epimer of 25 (OH)-D is especially present in children and contributes to the overestimation of 25(OH)-D concentration. The LC-MS/MS enables a specific determination of a profile of vitamin D metabolites in a single experiment setting [39]. Similarly to cortisol, measurement of vitamin D in saliva is now possible [40]. Moreover, vitamin D-binding protein (DBP) is involved in the transport of more than 99% of vitamin D. In case of nephrotic syndrome, urinary excretion of this protein is increased, leading to a hypovitaminosis D. A vitamin D assay in urine using LC-MS/MS has been described. This simple method has a better sensitivity than RIA, does not require derivatization and allows measurement of both 25(OH)-D forms with an imprecision (CVs) below 7% [41].

6. Disadvantages of mass spectrometry

The high instrumental cost and the need for highly *qualified personnel* have for a long time stopped the expansion of mass spectrometers in clinical laboratories. Lower costs and especially benefits of these techniques make them progressively more and more used. Mass spectrometry is often considered as the reference method with high precision and accuracy characteristics. However, extraction of steroids is mandatory before LC-MS/MS to avoid some interference such as matrix effects or the risk of signal inhibition, and limits the application of this technique to a large volume of samples. In addition, the results of external quality controls showed significant differences between the results obtained by different laboratories. In a study conducted by eight laboratories, determination of testosterone by different methods LC-MS/MS was performed. The results were comparable to those obtained by immunoassay with however a difference between average value and reference value given by the national institute of standards and technology (NIST) about 11% [42]. The variability of the results obtained by immunoassay is lower than that obtained by mass spectrometry. The multiplicity of extraction methods can explain this variability, but it can also be linked to the lack of standardization. This implies the need to standardize assays of testosterone and other steroids by mass spectrometry. Standardization should also touch the pre- and post-analytical phases. A large majority of reference intervals are established by RIA methods during the last fifty years of studies, so it seems essential to determine new intervals specific to mass spectrometry.

7. What to choose: LC-MS/MS or GC-MS?

Derivatization is essential for separation of hormones by gas chromatography, which significantly increase time of analysis; it takes at least thirty minutes to perform the assay [43]. The resolution of GC-MS is excellent, allowing separation of very similar structure molecules. Moreover, this resolution quality is widely exploited in the field of research for identifying new therapeutics metabolites.

LC-MS/MS is simpler to use. Derivatization is not necessary; the analysis time is shorter, about ten minutes. Overall, the GC-MS is useful for unidentified molecules and non-conjugated steroids with low molecular weight (molecular weight < 500 Da), while LC-MS/MS is useful for determination of a large number of conjugated and unconjugated steroids [44] (Table 1).

Recent introduction of UHPLC/TOF-MS allows identification of many hormones, when accurate mass databases are available [45]. The coupling UHPLC to mass spectrometry is a major technological advance which improves robustness, sensitivity and resolution, extends the column lifetime. Furthermore,

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Table 1
Comparison of LC-MS/MS and GC-MS for steroid assays.

Characteristics	LC-MS/MS	GC-MS
Derivatization	No	Required when the compound is not volatile
Time analysis (speed)	Tens of minutes	Thirty minutes
Adaptation to large series	Yes	No
Resolution	Excellent	Good
Applications	Research and clinical research	Research

measuring range is larger and the interactive software is more attractive [46].

8. The future of steroids measurement

In future, immunoassays should keep their place in measuring steroids for which the specificity and sensitivity are satisfactory like progesterone and cortisol. While the LC-MS/MS would be reserved for others steroids for which immunoassays have shown their limits such as the 17-hydroxyprogesterone, and testosterone [47]. The future of steroids measurement resides therefore in the complementarity between these two groups of analytical methods, that allow better respond to the needs of clinical practice [48].

9. Conclusion

Mass spectrometry and in particular LC-MS/MS has contributed much to the development of endocrinology both in clinical practice and research. Thus, LC-MS/MS brings many applications such as determining a panel of steroids for the detection of inborn errors of steroids metabolism and the measurement of steroids in others biological fluids than serum.

Disclosure of interest

The authors declare that they have no competing interest.

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