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Review

Advantages and challenges of mass spectrometry assays for steroid hormones☆

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ABSTRACT

Although steroid hormones have been measured, primarily in urine, by gas chromatography–mass spectrometry (GC–MS) assays for many years, in the past decade both clinical and research laboratories have dramatically increased usage of liquid chromatography–tandem mass spectrometry (LC–MS/MS) assays for measuring circulating levels of steroid hormones. Because of their high validity and throughput, mass spectrometry (MS) assays have replaced conventional radioimmunoassays (RIAs) and direct immunoassays for steroid hormones in larger reference laboratories, and they are touted to become the "gold standard" for steroid hormone quantitation. These advances in MS assays present several major challenges, which include the affordability of smaller laboratories to purchase MS instruments and pay for related operating costs; improving assay sensitivity, especially for measuring low estradiol levels in postmenopausal women and women treated with aromatase inhibitors; developing assays for quantitating profiles of steroid hormone metabolites in serum and tissues; standardizing steroid MS assays; and obtaining reliable reference intervals. The present review discusses the advantages of MS assays over conventional RIAs and direct immunoassays in steroid hormone measurements, and points out some of the important challenges facing the rapid increase in usage of MS assays.

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1. Introduction

During a period of about 30 years the predominant methodologies used to measure circulating levels of steroid hormones were conventional radioimmunoassays (RIAs), which require preceding purification steps, and direct immunoassays on automated platforms. In the past decade this has changed due to significant advances in mass spectrometry (MS) technology that have facilitated routine analyses of steroid hormones in clinical and research laboratories. With these advances, clinical laboratories can achieve

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greater throughput of patient samples with high accuracy and precision. Also, the MS assay methodology is now sufficiently rapid and robust for quantitating steroid hormones in large epidemiologic studies. The purpose of the present review is to show the advantages of MS assays over conventional RIAs and direct immunoassays in measurements of circulating levels of steroid hormones, and to discuss important challenges facing the rapid increase in usage of MS assays.

2. Development of mass spectrometry assays and radioimmunoassays for steroid hormones

Practical MS of steroid hormones was the outcome of technological developments that began with the first successful analysis of steroid hormones using gas chromatography (GC) reported by Sweeley and Horning in 1960 [1]. Soon after that, introduction of derivatization techniques (TMSE and oximes) allowed more polar steroids to be protected and volatilized prior to GC. Also, an interface was developed at the Karolinska Institute that combined a mass spectrometer with a gas chromatograph. Sjovall's group set the standard for steroid hormone analysis by gas chromatography–mass spectrometry (GC–MS) in the 1960s [2]. As early as 1966, Horning et al. produced the first comprehensive urinary steroid profile by GC–MS [3]. Today's descendants of the same methodology produce comparable data.

A few years after urinary steroids began to be measured by GC–MS, the RIA method for steroid hormones was developed. In 1969, the first RIA for a steroid hormone in serum was developed by Abraham, and this was for estradiol (E_2) [4]. The E_2 RIA method involved separation of E_2 from interfering metabolites by organic solvent extraction and Celite or Sephadex column chromatography, prior to quantitation of the compound by RIA. The RIA included a specific antiserum against E_2 in conjunction with tritiated E_2, and separation of the antibody-bound and unbound E_2 fractions using charcoal. This method was shown to be sensitive, specific, precise and accurate, and soon afterwards was applied successfully to other sex steroid hormones, such as testosterone and progesterone. During the decade of the 1970s, RIAs were developed for a variety of natural and synthetic steroid hormones. A notable change in the RIA procedure during those years was replacement of tritium with iodine in the radioactive marker to improve assay sensitivity. Due to the reduced technical complexity and lower cost of RIAs compared to GC–MS assays for quantifying steroid hormones, RIAs became widely used in research and diagnostic laboratories.

3. Advantages and disadvantages of conventional radioimmunoassays

Steroid RIA methods with purification steps, which are often referred to as conventional RIAs, have the following advantages: first, steroid-binding proteins (e.g., SHBG) are denatured, thereby releasing the steroids (e.g., E_2 and testosterone) that they bind. Second, the purification steps remove numerous potentially interfering metabolites prior to RIA. Third, the RIAs are highly reliable when properly validated. Finally, multiple steroids (usually up to 5) can be measured in a single aliquot of serum.

Conventional steroid RIAs also have disadvantages. They are cumbersome, time-consuming, costly, and require relatively large sample volumes, especially when the steroid is present in low concentrations. Also, although multiple steroids can be measured in a single aliquot of serum, the measurements have to be done very carefully and are especially time-consuming. In addition, as with all antibody-based assays, since the measurement of the analyte is a surrogate approach (i.e., measurement of radioactivity rather than the actual analyte itself), there is always the possibility of antibody cross-reactivity giving an erroneous result. Furthermore, the presence of auto-antibodies within patients can further affect an assay, leading to falsely high or low values depending on the type of antibody interaction that occurs. Despite these concerns, a well-validated RIA preceded by organic solvent extraction and chromatography steps in a good laboratory is typically a very accurate and precise assay for the majority of applications.

4. Impact of the conventional RIA method

The immediate impact of the conventional RIA method was that it allowed measurement of an immensely wide range of compounds of clinical and biological importance, and it opened new horizons in endocrinology. The long-term impact of the RIA method was that its use in numerous studies enriched the field of endocrinology with new knowledge, and its use in diagnostic testing provided physicians with valuable information for diagnosing and treating countless patients. The RIA methodology also allowed substantial research into the physiologic and pathophysiologic roles of steroid hormones in applications such as sexual differentiation, puberty, neuroendocrinology, the menstrual cycle, pregnancy, menopause, and male endocrinology. In addition, the RIA method opened the door for epidemiologic studies that permitted us to better understand the role of steroid hormones in the etiology of numerous diseases, notably the hormone-dependent breast and prostate cancers.

5. Direct immunoassays and their advantages and disadvantages

Due to the time-consuming limitations of conventional RIAs, in the late 1970s, the radioligands in RIAs were replaced with non-radioactive ligands (chemiluminescent, enzymatic or fluorescent) and the organic solvent extraction and chromatography steps used prior to RIA were eliminated, allowing direct immunoassays to be performed on an automated platform; this resulted in rapid measurements of steroid hormones. Conventional RIAs continued to be used, but their use was overwhelmingly surpassed by direct immunoassays, particularly in diagnostic clinical laboratories.

The development of automated platforms gave direct assays, such as chemiluminescent immunoassays, the advantages of being convenient, simple, rapid, and relatively inexpensive, and requiring a lower sample volume (usually 0.1 ml). However, these assays also have serious disadvantages. They often overestimate the measurements due to lack of specificity of the antibody, especially in samples obtained from women treated with exogenous steroid hormones [5]. Also, matrix differences may exist between serum samples (particularly hemolyzed and lipemic samples) and solutions of the standard used to prepare the standard curve in the assay. In addition, steroids such as testosterone and E_2 may not be released efficiently from proteins such as sex hormone-binding globulin to which they bind with high affinity in blood. Furthermore, direct immunoassays generally lack the sensitivity to measure low levels of certain steroid hormones such as E_2 with accuracy and reliability [5]. Finally, direct immunoassays can only measure one analyte at a time. Limitations of direct immunoassays for quantifying E_2 in postmenopausal women and testosterone in both premenopausal and postmenopausal women are now well documented in the literature [6–11].

6. Advances in mass spectrometry assays

Following their introduction in 1980, solid phase extraction columns transformed biological sample work-up procedures [12]. Subsequently, marketing of the HP MSD instrument with high res-

olution fused silica columns led to routine use of GC–MS in the mid 1980s, and the descendant of this instrument remains in use today. Introduction of fast atom bombardment (FAB) and related techniques led to the development of the high performance liquid chromatography (HPLC)-MS instrument in 1987. In the past decade, HPLC coupled with tandem mass spectrometry (LC–MS/MS) has revolutionized measurement of steroid hormones. In addition, invention of an electrospray source by Nobel laureate John B. Fenn, Ph.D., in 1990, and the subsequent development of atmospheric chemical ionization have facilitated routine analysis of steroids in clinical laboratories [13]. This technology facilitates ionization of the analytes present in liquid droplets and sprays the molecules directly into the mass spectrometer from the HPLC. These advancements allowed for simple coupling of the liquid chromatography (LC) eluent with the mass spectrometer and often negated the need for derivatization of the steroid, which reduced the complexity of the assay and shortened the assay run time dramatically. These factors greatly increased the throughput of patient samples, while still providing highly accurate and precise results.

Since there are two common chromatographic methods by which to separate the analyte from its matrix and introduce it into the MS, it is worthwhile to briefly examine their relative strengths and weaknesses. GC is a very highly resolving technique allowing baseline resolution of minor structural differences between analytes (e.g., isomers). Furthermore, the sample volume injected is very small (1–5 μl typically). However, runtimes tend to be very long (30 min or more is not unusual), non-volatile compounds such as steroids need to be chemically derivatized, and the small sample injection volume requires intense analyte cleanup and concentration prior to analysis to provide sufficient analyte intensity for detection. In contrast, LC typically does not provide analyte resolution as high as that of GC and can have difficulty separating analytes with very closely related structures, although it is possible. However, since the sample is already in liquid form there is no need to derivatize the analyte to make it volatile. Furthermore, the LC allows rapid analysis times but does require a high specificity detector to make up for the lack of baseline resolution provided by the separation technique.

While the advent of LC–MS/MS in the past decade has resulted in dramatic improvements in the sensitivity, specificity, and automation of serum steroid hormone measurements, there are still situations where a GC–MS or GC–MS/MS assay provides higher chromatographic resolution and even sensitivity. A particular strength of GC–MS and GC–MS/MS is their high applicability to measurement of large numbers of structurally similar analytes. They remain the most powerful discovery tool for defining steroid disorder metabolomes. For example, since the 1930s, almost all inborn errors in steroidogenesis have been first defined through the study of urinary steroid excretion, and in the last 30 years this has been carried out exclusively by GC–MS.

Both GC- and LC-based mass spectrometry has been utilized with great success in the identification and quantitation of novel steroidal compounds in various body fluids. The strength of tandem MS is that one does not need to know the structure of an analyte to detect it. Rather, some very specialized operational modes can be used to identify compounds that have "family resemblance" to other well understood compounds in a homologous series of compounds. A classic example of this is in metabolite identification of new therapeutic entities after their administration to laboratory animals or humans [14]. Such approaches have been utilized for identification of urinary steroid metabolites by GC–MS of circulating steroid compounds. Since MS is capable of quantitating multiple analyses in a single run, ratios have been developed to help define and diagnose certain very important endocrine conditions.

Because of the high validity and throughput of MS assays, there is a rapidly growing use of this methodology for quantitating steroid hormones in both clinical and research laboratories. In larger reference laboratories, these assays have replaced conventional RIAs, which are cumbersome and time-consuming, and direct immunoassays, which lack specificity and/or sensitivity. This technology has been implemented successfully for routine analysis of steroids in major laboratories at the Mayo Clinic, Quest Diagnostics, and Esoterix in the United States. Although the high cost of MS instrumentation, related operating costs, and requirement for high technical expertise have prohibited smaller laboratories from using this instrumentation for high-throughput routine testing of steroid hormones, this situation is changing and MS assays are becoming much more widely used. Furthermore, once the MS instrument has been purchased the components for the assay to be run are "off-the-shelf" from multiple sources rather than a vendor's kit. This can rapidly reduce the cost per test to the same amount or less than a traditional kit and removes the possibility of reagent rentals, which typically tie the laboratory to the vendor. All of these factors provide a higher degree of freedom for price negotiations and give the laboratory more autonomy.

7. Challenges in mass spectrometry assays

Although there seems to be general agreement that MS assays will become the gold standard for steroid hormone measurements, there are many challenges to be overcome before this occurs. Conventional RIAs and direct immunoassays have been carried out for a substantial number of years and have provided a considerable amount of data on the role of steroid hormones in endocrinology, through studies as well as clinical testing. How will MS assays improve on this? There are several ways, and they pertain to clinical diagnostic testing, assay sensitivity, metabolomics, and assay standardization.

7.1. Clinical diagnostic testing

MS assays have made an important contribution in endocrine testing [15,16]. A major advantage of using MS assays for quantitating steroid hormones in clinical diagnostic laboratories is that accurate and reliable testing of these hormones can be carried out with high throughput [17]. Although conventional RIAs can also produce accurate results, they are time-consuming, especially when Celite column partition chromatography is used to separate interfering steroids from the steroid being measured. While the columns provide reasonably good resolution, they have to be packed prior to each assay. Using LC–MS/MS assay methodology, large clinical diagnostic laboratories can now perform several million endocrine tests per year. The challenge is to have smaller clinical diagnostic laboratories carry out steroid hormone measurements using MS assays. This will be achieved gradually as the cost and complexity of instrumentation decreases, allowing diagnostic laboratories to generate operating budgets that will cover both the costs associated with instrumentation and the higher salaries required for specialized personnel.

7.2. Assay sensitivity

A second challenge for MS is to enhance assay sensitivity for measuring certain steroids in serum, particularly estrogens. For example, ultrasensitive measurements of low levels of E_2 may be important for prediction of risk of fractures, and for monitoring the extent of E_2 suppression in women receiving aromatase inhibitor therapy and the response to anti-estrogens for prevention of breast cancer. In addition, evaluating the degree of suppression of gonadal steroids in the treatment of precocious puberty is another application that will benefit from enhanced assay sensitivity.

In vitro recombinant DNA bioassays for E_2 have been reported to have sensitivities ranging from 0.02 to 1 pg/ml [18]. However, it is not known whether these bioassays more truly reflect blood E_2 levels or detect lower E_2 levels due to artefactual influences. Regardless, these ultrasensitive bioassays are too cumbersome and time-consuming for routine use and are realistically only of use in their smaller-scale application to research projects.

Conventional E_2 RIAs generally have a sensitivity of 2–3 pg/ml, whereas the sensitivity of MS assays for E_2 appears to be 2 pg/ml or less. One GC–MS/MS assay reports a sensitivity of 0.6 pg/ml for E_2. No reliable studies have yet been carried out comparing the sensitivities of GC–MS/MS versus LC–MS/MS assays.

Ultrasensitive assays are also needed for certain studies. For example, there is presently a great interest in the role of catechol and 16α-hydroxylated estrogens in the etiology of breast cancer. However, these estrogen metabolites are present in very low concentrations in serum and breast tissue. There is a major need to obtain valid ultrasensitive assays for the unconjugated forms of these estrogens.

7.3. Metabolomics

Presently, there are two main approaches in metabolomics: (a) a targeted approach in which a chosen set of metabolites is quantified; and (b) a non-targeted approach in which there is a search for potential biomarkers. Both approaches have the potential to provide highly valuable information in diagnosing patients and in a variety of studies, particularly epidemiologic studies.

A serious limitation of conventional RIAs and direct immunoassays of steroid hormones is their inability to measure multiple steroids in a single aliquot of serum or urine. A good example of the enormous progress made in developing MS assays for quantifying patterns of steroid hormone metabolites can be seen in the methodology established recently by Xu et al. [19,20] at the National Cancer Institute in the United States. This group has developed a stable isotope dilution LC–electrospray ionization-MS/MS assay that can measure concurrently a total of 15 estrogens in 0.5 ml of serum or urine with high validity. The estrogens include the unconjugated and conjugated (sulfates and glucuronides) forms of E_2 and estrone, and their metabolites. The assay method is sufficiently rapid and robust for large epidemiologic studies.

Using the LC–MS/MS assay methods developed by Xu et al. [19,20], epidemiologic studies can assess total estrogen exposure, concentrations of specific estrogen metabolites, and profiles of estrogen metabolites. In addition, various hypotheses pertaining to mutagenic and genotoxic effects of certain estrogen metabolites can be tested.

Using MS, the Metabolomic Platform (MetaP) of the Helmholtz Zentrum Munchen in Munich, Germany, is making important contributions through studies elucidating metabolomic effects in health and disease. MetaP is designed to mediate progress in science through development of new metabolomic methods. In research related to targeted metabolomics at MetaP, over 150 endogenous metabolites of lipids, amino acids, acylcarnitines, carbohydrates, and other compounds have been quantified reliably in only 10 μl of plasma. The effects of these metabolites in complex diseases are being studied. There are many challenges that will be encountered in the rapidly growing research field of metabolomics, but the results are likely to provide a valuable contribution to understanding different diseases.

7.4. Standardization of MS assays

Probably the most difficult challenge of MS assays is standardization of their measurements, which, because of their high specificity and sensitivity, are frequently referred to as the "gold standard". However, it is important to realize that MS technology faces variability issues similar to those of conventional RIAs and immunoassays that need to be addressed. One study, investigating the performance of MS assays for serum total testosterone in eight different laboratories against an MS reference method (NIST standard), showed mean biases ranging between −14.1% and 19.2% at levels >100 ng/dl; at levels <100 ng/dl, the biases were as high as 25.3% [21]. In the same study, the coefficients of variation measured for two samples with testosterone levels of 296 ng/dl and 8.47 ng/dl ranged between 2.2% and 11.4%, and 2.7% and 25.6%, respectively. The MS assays also differed in their detection limits and reportable ranges. The results of this study showed clearly that a heterogeneous group of assay methods was being used in the different laboratories with the same measurement principles but with major differences in assay performances.

Differences in accuracy among MS assay methods appear to be attributable to calibration, but differences in assay precision seem to be explained, in part, by variations in sample preparation. Although reasons for differences in assay imprecision are not fully understood, it has been suggested that the differences could be due to ion suppression effects by compounds such as salts, ion-pairing agents, drugs, and proteins [22]. These compounds may bias the amount of analyte that can become charged in the gas phase and thus ultimately reach the detector in the mass spectrometer, resulting in lower values of the analytes. It should be noted that ion suppression is a factor in all MS analyses, whether the separation technique be GC or LC. One way around this problem is to add an isotope-labeled version of the analyte being measured (known as the internal standard or IS) to all samples, calibrators, and controls. This is known as isotope-dilution MS and is the standard approach in quantitative analysis. The presence of an isotope within the molecule (most commonly deuterium replacing one or more hydrogens) causes the IS to have a different mass compared to the analyte being measured, while retaining all of the chemical and physical properties of the analyte. Dividing the analyte measurement by the IS measurement value in each sample, calibrator, and control provides automatic normalization of the results. Because of the large variability in steroid hormone measurements among different assay methods, and the lack of valid reference intervals and cutoffs for clinical treatment and epidemiologic studies, the US Centers for Disease Control and Prevention (CDC) started a standardization project to overcome deficiencies in testosterone testing [23]. The need for standardization of testosterone measurements was established by researchers and professional organizations because these measurements are widely used for diagnosing diseases and disorders and for monitoring treatments, and are also used in numerous studies. The testosterone standardization efforts are focusing not only on the analytic measurement process, but also on pre-analytical and post-analytical issues such as test selection and reference intervals. This approach to testosterone assay standardization is based on similar CDC standardization programs that have been successful, which include those for cholesterol and other blood lipids and glycated hemoglobin A1c.

The aim of the analytical component of the assay standardization process at CDC is to assure that measurement results from a sample are the same, independent of the methodology or technology used in a laboratory. This can be achieved by adding a certified reference material (e.g., a pure testosterone primary standard) to a matrix-based material such as serum, and assigning values to this material to establish matrix-based calibrators. The CDC is using sets of single-donor sera for calibrating clinical and research assays because the matrix-based calibrators would be as similar to a patient's sample as possible. Due to the limited volumes of sera available from individual donors, many serum samples will be needed over time, which will require frequent changes in reference values assigned to calibrators. However, this approach to

assay standardization should provide highly accurate and precise measurements of steroid hormones. The CDC is collaborating with other organizations, such as the Clinical and Laboratory Standards Institute (CLSI), National Institutes of Health (NIH), and College of American Pathologists (CAP), to assess assay calibration.

8. Reference intervals

Yet another important challenge for MS measurements is the requirement for reference intervals derived from well-characterized, adequate-sized populations using standardized procedures such as those formulated by the CLSI. Frequently, only limited information is available about subjects used to establish reference intervals. In a study using a reference panel of sera from healthy eugonadal young men with verified normal reproductive function, testosterone levels and reference intervals were compared between the reference method, GC–MS, and direct immunoassays carried out on different commercial automated platforms [10]. The results show not only significant differences in testosterone levels between the direct assays but also substantial discrepancies between reference intervals.

An often neglected aspect in establishing reference intervals for steroid hormone measurements is biologically influencing factors that may affect these measurements. The major factors include sex, age, body mass index, pubertal stage, menopausal status, phase of menstrual cycle, pregnancy and diurnal rhythm. These factors are essential to consider in establishing valid reference intervals for MS assays that are presently being used and developed.

It will be especially important to compare reference intervals for steroid hormones measured by MS assays to those obtained by conventional RIAs, since much of our knowledge about the role of steroid hormones in normal women and men, as well as in different diseases, is based on the data obtained by the latter methodology. Presently, it appears that there will not be significant differences in reference intervals between the two methods for most of the commonly measured steroid hormones, with the possible exception of serum E_2 levels in postmenopausal women, men, and prepubertal children, and testosterone levels in women and prepubertal children.

9. Conclusions

It is obvious in this review that there are many challenges that lie ahead for MS assay methodology and technology. Nevertheless, in a relatively short period of time enormous advances have been made in quantitating analytes by MS. Of particular note, major advances have been made in clinical diagnostic testing, assay sensitivity, and metabolomics. In addition, a great start has also been made by the CDC in standardizing steroid hormone assays. It is evident from previous efforts at the CDC that vast improvements in measurement performance can be achieved through assay standardization. However, based on their experience it could take years to accomplish such achievements. Continuing efforts and support by different organizations involved in laboratory testing are essential to achieve these goals.

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