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Philippe Liere & Michael Schumacher

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Mass spectrometric analysis of steroids: all that glitters is not gold

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Philippe Liere

Neuroregenerative and Remyelinating Small Molecules, U1195 Inserm and University Paris-Sud, 94276 Kremlin-Bicêtre, France

Michael Schumacher

Author for correspondence: Neuroprotective, Neuroregenerative and Remyelinating Small Molecules, U1195 Inserm and University Paris-Sud, 80 rue du Général Leclerc, 94276 Kremlin-Bicêtre, France
Tel.: +33 149 591 895
michael.schumacher@inserm.fr

Steroid hormones are small molecules (MW around 300 Da) characterized by a large range of polarity and their analysis has always presented a serious challenge. Persistent problems with the specificity of conventional immunological methods are the cause of inconsistent results in the literature, a particularly problematic situation for healthcare decisions. At present, mass spectrometric methods have become the gold standard for accurate steroid profiling, and their advent will require the re-analysis of previously published data. However, it is a common misconception to consider the use of theses sophisticated technologies as a guarantee for accurate measures. Steroid analysis, especially in nervous tissues, indeed requires well-validated purification and separation steps before mass spectrometry, only then will mass spectrometric analysis be the absolute reference methodology.

The analysis of steroids in biological samples relied on bioassays before the advent of radioimmunoassays (RIA) in 1969 [1]. Both steroid hormone RIA and ELISA operate on the basis of competition between the hormone to be measured in a sample and a fixed amount of labeled or enzyme-conjugated hormone for a limiting amount of antibody. Concentrations are then determined against a standard curve established with known hormone concentrations [2]. It is obvious that such methods present serious limitations with regard to specificity, and that they need careful purification of samples and chromatographic pre-separation of steroids before the final analysis step. Direct RIA or ELISA indeed do not allow accurate measures of steroid hormones. It is thus surprising that even reference journals continue to publish steroid measures in biological fluids based on poorly validated RIA or ELISA 'according to the instructions provided by the manufacturer'.

The combination of gas chromatography (GC) or liquid chromatography (LC) with mass spectrometry (MS) or tandem MS (MS/MS) has provided reference methods for steroid hormone analysis. It is interesting to note that the analysis of steroids by GC-MS started developing in the early sixties, before the report of the first steroid RIA [3]. It indeed took time before the GC-MS technology became sufficiently robust for the analysis of steroids in biological samples [4]. The advent of MS has allowed the standardization of steroid determinations for diagnostic purposes, especially in fluids, such as human urine or saliva [5,6]. However, it has become a dangerous trend to consider the use of GC-MS(/MS) or LC-MS(/MS) as a guarantee of accuracy for steroid analysis. Indeed, steroid analysis in complex matrices, such as blood plasma or tissue relies on careful sample preparation, involving multiple steps prone to errors before mass spectrometric analysis. Challenges facing the increasing use of MS for the assay of steroids in biological samples have been recently discussed [7]. Here, we wish to insist on the absolute requirement for carefully validating sample workup, including essential purification and fractionation steps, without which even mass spectrometric methods may generate erroneous measures.

EXPERT REVIEWS

KEYWORDS: gas chromatography • liquid chromatography • mass spectrometry • neurosteroids • steroid hormones

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A key step in sample workup is to remove cholesterol as quickly as possible. Cholesterol can indeed be oxidized during the entire sample preparation before mass spectrometric analysis, both in solution and dry condition, generating steroid hormone precursors, such as pregnenolone, dehydroepiandrosterone (DHEA), and androstenediol [8]. This is a major problem for tissues rich in cholesterol, such as nervous tissues. Cholesterol represents indeed >20% of dry brain weight and can easily become a major source for the artifactual formation of oxysterols, but also of steroid hormone precursors [8,9]. Thus, removing cholesterol and lipids rapidly from samples on reverse-phase cartridges by a fractionation process, that is, steroid isolation by groups according to their polarity, named solid phase extraction, is an absolute requirement. Most important, tissue or plasma extracts have to be resuspended in suitable solvents, such as methanol or ethanol before solid phase extraction for allowing their complete solubilization. Lipids, such as cholesterol, and also mid-polar and polar steroids, including steroid sulfates, are efficiently solubilized in alcohol solvents. It is thus a big mistake to dissolve tissue extracts in an aqueous medium, such as methanol/water, a very commonly used method that has even been recommended [10], because the hydrophobic compounds cannot be fully solubilized under such conditions. In fact, the presence of water reduces the solubilization of cholesterol and other lipids and results in their ineffective adsorption by the reverse-phase cartridge designed to retain hydrophobic compounds. As a consequence, cholesterol is eluated and contaminates the steroid fraction to be analyzed, leading to the artifactual formation of significant amounts of pregnenolone, DHEA or androstenediol [8]. This may explain why DHEA continues to be detected in the rat or mouse brain by LC-MS/MS [11], although this steroid is normally not present in adult rodents [8].

The importance of both precise analytical procedures and careful sample workup for the accurate measure of steroids is particularly well documented by the artifactual detection, over decades, of pregnenolone sulfate (PREGS) and dehydroepiandrosterone sulfate (DHEAS) in rodent plasma and brain by RIA and even inferred by GC/MS [12–14]. The reality of the presence of these steroid sulfates in rodents was then challenged by direct LC-MS/MS methods, which failed to detect even trace amounts [15,16]. In fact, conjugated steroids can only be measured by LC, as they are pyrolized by the high temperatures required for GC. They are also not recognized by antibodies used in RIA. For this reason, GC/MS and RIA analysis indirectly estimate levels of PREGS and DHEAS by measuring free pregnenolone or DHEA released by the derivatization and solvolysis/hydrolysis of the sulfated conjugates. The problem at the origin of the erroneous detection of PREGS and DHEAS was twofold: the inadequate solubilization of the biological extract, and the contamination of the steroid sulfate fraction by endogenous cholesterol and the subsequent artifactual formation of steroid hormone precursors [8]. Then, no steroid sulfate was detected in rodent brain and plasma using validated solid phase extraction fractionation methods before GC-MS analysis [17]. Nevertheless, the presence of PREGS and DHEAS has been verified in human brain [8].

However, when combined with careful sample workup, mass spectrometric methods offer an unequalled tool for the analysis of steroids. In addition to their specificity and sensitivity, they allow establishing extended steroid profiles (steroid metabolomes) thanks to the coupling with high-resolution GC or LC in nervous tissues [13,18] or in the circulation [19]. In contrast to the conventional immunological methods, mass spectrometric analysis indeed allows measuring a large number of steroids, including a large range of stereoisomers, even within small tissue samples.

There are two major methodologies for the chromatographic separation of steroids before MS or MS/MS: GC and LC. Advantages of LC are, no absolute need for steroid derivatization (the conversion of steroids to larger, less polar and more volatile compounds – a step required before GC) and, as pointed out above, the possibility to directly measure conjugated steroids, such as sulfates. On the other hand, although LC and GC have comparable runtimes [20], GC provides higher chromatographic resolution than LC for unconjugated steroids and offers, in general, a more powerful tool for extensive steroid profiling, allowing analyzing a large number of structurally similar steroids, and especially fully reduced steroid isomers, in a single fluid or tissue sample [7]. However, for both GC-MS and LC-MS, assay sensitivity, reliability, and absolute specificity remain major challenges. They have been improved by adding a second analyzer together with a collision cell (MS/MS). The strategy consists of obtaining additional structural information by fragmenting within a collision cell specific ions isolated by the first MS. The second MS then analyses the generated product ions. Tandem MS markedly reduces matrix interferences and background noise, and significantly improves assay selectivity, sensitivity and precision [21]. GC-MS/MS and LC-MS/MS are today the reference technologies for the quantification of steroids in a biological matrix. It is thus difficult to understand that, at the same time that such sophisticated mass spectrometric methods for the analysis of steroids are developed, and mass spectrometers are even assembled in tandem to achieve higher selectivity and sensitivity, the cellular distribution and regulation of neuroactive steroids, such as allopregnanolone and estradiol, are analyzed by immunohistochemistry on brain sections [22–24]. Most disturbing is the validation of the specificity of the affinity-purified antibodies by RIA before immunohistochemical steroid analysis.

The analysis of steroids in biological fluids and tissues covers a period of nearly five decades for immunological and of two decades for mass spectrometric methods. During these periods, both technologies have evolved, and it is not always easy to appreciate the validity of reported steroid levels. When interpreting results, it is important to be aware of methodological limitations, even for the mass spectrometric technologies. Thus, GC-MS(/MS) and LC-MS(/MS), and even the new generation of high-resolution mass spectrometers, such as quadrupole time-of-flight or Orbitrap, have their limits and may generate erroneous data when combined with inadequate sample workup. An analytical chemistry expertise in the steroidomic field is absolutely required to overcome these limitations.

Although mass spectrometric methods for the analysis of steroids are associated with high equipment and operating costs and require technical expertise, their deployment is becoming more widespread in research laboratories. They are even making its way into clinical testing, and their use requires specific recommendations [25–27].

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