BACKGROUND: New high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS) methods are among the most successful approaches to improve specificity problems inherent in many immunoassays.

CONTENT: We emphasize problems with immunoassays for the measurement of steroids and review the emerging role of LC-MS/MS in the measurement of clinically relevant steroids. The latest generation of tandem mass spectrometers has superior limits of quantification, permitting omission of previously employed derivatization steps. The measurement of steroid profiles in the diagnosis and treatment of congenital adrenal hyperplasia, adrenal insufficiency, chronic pelvic pain and prostatitis, oncology (breast cancer), and athletes has important new applications.

CONCLUSIONS: LC-MS/MS now affords the specificity, imprecision, and limits of quantification necessary for the reliable measurement of steroids in human fluids, enhancing diagnostic capabilities, particularly when steroid profiles are available.

Steroid hormones are synthesized from cholesterol, and many are of great clinical importance (1). Synthesis occurs in the mitochondria and smooth endoplasmic reticulum of cells in the adrenal cortex, the gonads, and the placenta (Fig. 1). The adrenal gland is composed of the adrenal medulla and cortex. The latter is divided into 3 anatomic zones: the zona glomerulosa, which produces the mineralocorticoids such as corticosterone and aldosterone, and the zone fasciculata and reticularis, which together produce the glucocorticoids (11-deoxycortisol, cortisol) and the adrenal androgens [dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEAS)], which in the adrenals are produced in far higher amounts than androstenedione and testosterone. Aromatase [cytochrome P (CYP)-450 19, CYP19A1] adds 2 double bonds to ring A of testosterone, yielding estradiol with an aromatic ring A, and it also aromatizes ring A of androstenedione to form estrone (E1).

One of the goals in the treatment of some breast cancers is reduction of estrogen concentrations. This can be achieved through the use of aromatase inhibitors (AIs), which block the conversion of androgens to estrogens (2). AIs do not sufficiently block estrogen synthesis by the ovaries, however, but do block other tissues from converting androgens to estrogens. For this reason, AIs are used mostly in women who have reached menopause, when the ovaries no longer synthesize steroids. E1 synthesized mainly by aromatase conversion of androstenedione is metabolized to several steroid metabolites, including CYP1A2 conversion to 2-hydroxyestrone (2-OHE1) and CYP3A4 and CYP1A2 to 16α-hydroxyestrone (16-OHE1). The former is weakly estrogenic and inhibits breast cell proliferation (3-6), whereas the latter is carcinogenic and genotoxic, enhances breast cell growth, and increases DNA synthesis and oncogene expression (3). Attempts to modify the 2-OHE1:16-OHE1 ratio in young women using the antidepressant fluoxetine are currently being explored (6). Fluoxetine is a known inhibitor of multiple P450 isoenzymes, including 3A4, 2C9, and 2D6, and known to affect estrogen concentrations.

Methods of Measurement

Endogenous steroids have been measured by immunoassay (IA) (7–12), GC-MS (13–15), and high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) (16–21). An evaluation of the results for steroid measurement in the College of American Pathologists (CAP) proficiency testing (PT)

5 Nonstandard abbreviations: DHEA, dehydroepiandrosterone; DHEAS, DHEA-sulfate; CYP, cytochrome P; E1, estrone; AI, aromatase inhibitor; 2-OHE1, 2-hydroxyestrone; 16-OHE1, 16α-hydroxyestrone; IA, immunoassay; LC-MS/MS, liquid chromatography–tandem mass spectrometry; CAP, College of American Pathologists; PT, proficiency testing; ESI, electrospray ionization; LOD, limit of detection; APPI, atmospheric pressure photoionization; APCI, atmospheric pressure chemical ionization; CAH, congenital adrenal hyperplasia; ACTH, adrenocorticotropic hormone.
program is very informative. The majority of laboratories participating in this program employ IA methods, each using a different antibody. For each method, CAP calculates the method mean, so it is possible to divide the mean value of the method giving the highest mean by the mean value of the method giving the lowest mean. Table 1 summarizes the IA results for one of the CAP challenges for 2008. For this particular challenge, laboratories using the method giving the highest results differed from those using the procedure giving the lowest results by a factor of 2.8, 9.0, and 3.3 for testosterone, estradiol, and progesterone, respectively.

This example illustrates vividly the magnitude of the IA problem, due to many factors, including lack of specificity of antibodies purported to measure a particular steroid. This poor IA performance for steroid measurement encompasses more than just the 3 steroids shown in Table 1 and is true for all steroids measured in the CAP PT program. This contrasts with the good IA performance for the measurement of drugs such as phenytoin, phenobarbital, carbamazepine, etc., where the mean values for drugs in the CAP PT program for

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Lowest mean (L)</th>
<th>Highest mean (H)</th>
<th>Factor H/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone, ng/dL</td>
<td>52.6</td>
<td>148.7</td>
<td>2.8</td>
</tr>
<tr>
<td>Estradiol, pg/mL</td>
<td>25.4</td>
<td>229.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Progesterone, ng/mL</td>
<td>0.83</td>
<td>2.72</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table 1. Lack of specificity of steroid hormone immunoassays; data from CAP PT Program Y-A Survey 2008.
MEASUREMENT OF STEROIDS

FACTORS MERITING CONSIDERATION FOR MS/MS
MEASUREMENT OF STEROIDS

Derivatization vs nonderivatization. This is currently an important issue, with advocates on both sides. Derivatization proponents claim that both enhanced limit of quantification and specificity can be achieved by adopting this approach. This is questionable, however; derivatization has its disadvantages, which include decreased precision due to the added derivatization steps (extraction, derivatization at an extreme of pH). Accuracy could be compromised as well through the possible hydrolysis of conjugates, which would clearly affect the accuracy of the assay by giving falsely increased results. An example of such a problem could occur with estradiol conjugates, which on hydrolysis would yield estradiol. While serving on the CAP PT committee, we compared the values obtained for testosterone, progesterone, and estradiol in 3 leading laboratories using tandem mass spectrometry. Although excellent agreement was found for testosterone and progesterone, the laboratories using derivatization at an extreme of pH for estradiol measurement had results approximately 10%–20% higher than the laboratory that avoided derivatization. Derivatization methods are also lengthy and therefore more time-consuming. Over the past 15 years, the detection limits with modern mass spectrometers have improved greatly and have made the derivatization of analytes unnecessary. For this reason, we have been able to avoid derivatization approaches for the measurement of steroids (17–19).

Type of ionization. Electrospray ionization (ESI) in the negative mode yields the best results for the estrogens (estradiol, estriol, E1, and 16-OHE1) (18). The method used, which avoids derivatization, has a lower limit of detection (LOD) of 1–2 pg/mL for all 4 estrogens when run on the API-5000 tandem mass spectrometer (Applied Biosystems). Total sample requirement is only 0.2 mL, and total chromatography time for each estrogen profile is 8 min. The use of C-8 analytical columns (Supelco LC-8-DB; 3.3 by 3.0 mm, 3 μm particle size) is preferred over C-18 columns, as they markedly reduce retention times of the analytes in question. Our experience, and that of others (14, 18, 22), has shown that IAs for estrogens have problems at low estrogen concentrations (<80 pg/mL), frequently reporting falsely increased results.

For DHEA, DHEAS, androstenedione, testosterone, progesterone, cortisol, 11-deoxycortisol, corticosterone, and aldosterone, we have found that atmospheric pressure photoionization (APPI) has potential advantages over ESI or APCl (atmospheric pressure chemical ionization) in that it is a soft ionization source that effectively ionizes these steroids fairly selectively, leading to cleaner chromatograms. Alary (23) compared APPI-MS/MS with APCl for the measurement of steroids in biological matrices and reported that the signal obtained using the APPI source was 3–10 times more intense than that obtained employing the APCl source. In our APPI method (19), the sample size is 0.2 mL serum or plasma. After protein precipitation with acetonitrile containing the deuterated internal standards, the solution is vortex-mixed and centrifuged, and the supernatant is injected directly onto a C-8 column, as with the estrogen profile assay. The column is run on the API-5000 tandem mass spectrometer. Although excellent agreement was found for testosterone and progesterone, the laboratories using derivatization at an extreme of pH for estradiol measurement had results approximately 10%–20% higher than the laboratory that avoided derivatization. Derivatization methods are also lengthy and therefore more time-consuming. Over the past 15 years, the detection limits with modern mass spectrometers have improved greatly and have made the derivatization of analytes unnecessary. For this reason, we have been able to avoid derivatization approaches for the measurement of steroids (17–19).

Table 2. MS/MS data of steroid hormones; CAP PT Program Y-A Survey 2008.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Lowest value (L)</th>
<th>Highest value (H)</th>
<th>Factor H/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone, ng/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone no. 1</td>
<td>52</td>
<td>72</td>
<td>1.4</td>
</tr>
<tr>
<td>Testosterone no. 2</td>
<td>182</td>
<td>225</td>
<td>1.2</td>
</tr>
<tr>
<td>Estradiol, pg/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol no. 1</td>
<td>109</td>
<td>109</td>
<td>1.0</td>
</tr>
<tr>
<td>Estradiol no. 2</td>
<td>628</td>
<td>630</td>
<td>1.0</td>
</tr>
<tr>
<td>Progesterone, ng/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone no. 1</td>
<td>0.7</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Progesterone no. 2</td>
<td>8.1</td>
<td>8.6</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 2. MS/MS data of steroid hormones; CAP PT Program Y-A Survey 2008.
occurrence with the method described. Use of deuterated internal standards and an online sample wash step are partly responsible for the good performance.

Profile vs single steroid testing. Large commercial reference laboratories have high daily volumes for many of the clinically important steroids discussed above. To accommodate this volume and meet the need for rapid turnaround time with short chromatography time, these laboratories have adopted a 1 tandem/1 steroid philosophy, with a different tandem mass spectrometer used for each steroid. Smaller teaching hospital laboratories with a less pressing workload can, on the other hand, evaluate the potential role of steroid profile testing. As LC-MS/MS allows the simultaneous measurement of several steroids, sample sizes can be reduced compared to IA platforms that require an additional sample for each steroid measured; this is particularly germane in the evaluation of infants, where specimen size is limited.

Multiple circumstances in which steroid profiles have been employed.
1. Congenital adrenal hyperplasia (CAH) is an inborn error of steroid biosynthesis. CAH is a group of inherited diseases caused by defective activity of 1 of 5 enzymes in the adrenal cortex. The defective enzyme leads to decreased production of cortisol (causing an increased corticotropin) and excess production of hormones proximal to the defect. The 2 most common forms of CAH are caused by either 21-hydroxylase deficiency (defect in the P450c21 enzyme) or 11-β-hydroxylase deficiency (defect in P450c11 enzyme). Individuals with CAH due to 11- or 21-hydroxylase enzyme deficiency cannot produce adequate amounts of cortisol and, in some cases, are also aldosterone deficient. These hormones are essential in glucose metabolism and sodium reabsorption. Untreated CAH can lead to sudden adrenal insufficiency, with dehydration, shock, and even death. Steroids that have been recommended for the assessment of CAH are cortisol, androstenedione, and 17α-hydroxyprogesterone to 11-deoxycorticosterone and 17α-hydroxyprogesterone to 11-deoxycorticisol (31) (Fig. 1).
2. The evaluation of adrenal insufficiency is historically recommended by measuring cortisol at 0, 30, and 60 min after an adrenocorticotropic hormone (ACTH) stimulation test (27–29). We have improved the diagnostic reliability of this approach by measuring the 3 steroids aldosterone, cortisol, and most importantly, 11-deoxycortisol at 0, 30, and 60 min. Including aldosterone in the profile allows the differentiation of primary from secondary adrenal insufficiency. In primary adrenal insufficiency, no aldosterone response is observed, whereas an adequate response is found in secondarily adrenal insufficiency (30). The concentration of 11-deoxycortisol increases 15- to 20-fold in controls after an ACTH stimulation test, which compares to an approximately 3-fold increase for the more traditionally measured analyte cortisol. Our study demonstrated greater diagnostic accuracy if these 3 steroids were measured instead of measuring only cortisol (30).
3. We have also assessed the role of steroid profiles in patients with chronic prostatitis/chronic pelvic pain syndrome. Our results suggest reduced activity of CYP21A2 (P450c21), which is the 21-hydroxylase enzyme that converts progesterone to 11-deoxycorticotocosterone and 17α-hydroxyprogesterone to 11-deoxycorticisol (31) (Fig. 1).
4. We assessed whether steroid profiles provided insight into the reasons for premature adrenarche and infants with genital hair. In both these groups, the concentrations of testosterone, androstenedione, DHEA, and DHEAS were somewhat higher than inagematched controls (32). We have also assessed the reference intervals for these steroids during pregnancy and 1 year postpartum using isotope dilution tandem mass spectrometry (33).
5. Sera from active smokers, passive smokers, and nonsmokers have been analyzed for 15 steroid hormones and thyroid hormones to examine the associations between smoke exposure and hormone concentrations (34, 35). Although we do not know whether the blood concentrations of the hormones reflect changes that parallel physiological variation in steroid hormone concentrations, the assumption is that differences reflect associations with tobacco smoke exposure.
6. LC-MS/MS after solid-phase extraction has been used in lipidomic profiling of some female steroid hormones in human urine, and can be potentially applied clinically and to metabolomic research (36).
7. Diabetes strongly affects neuroactive steroids in the nervous system. LC-MS/MS assessment of the concentrations of neuroactive steroids provides a basis for new therapeutic tools based on neuroactive steroids aimed at countering diabetic neuropathy (37).
8. Even in trace amounts, estrogens such as E2, E1, estriol, and 17α-ethinyl estradiol may have adverse effects on humans and the aquatic ecosystem. It is there-
fore essential to be able to reliably determine trace amounts (at environmentally relevant concentrations) of steroid estrogens in water. Using LC-MS/MS, it is now possible to detect these chemicals in small samples of water at concentrations as low as 0.04 ng/L. 

9. Finally, steroid profiling has been used to assess changes in adrenal steroids before and after a 56-km ultramarathon race (42). Concentrations of the mineralocorticoids corticosterone and aldosterone increased significantly, as did concentrations of the glucocorticoids cortisol, 11-deoxycorticisol, and 17-OH progesterone and the adrenal steroids DHEA, DHEAS, and androstenedione (P < 0.0001 for all).

Some future areas for research include the assessment of the role of neoestrogens in epilepsy, particularly in pubertal girls in whom a marked increase in seizure activity has been found (43–45), and analysis of androgens in males, patients with benign prostatic hyperplasia, and prostate cancer (46, 47).

In conclusion, LC-MS/MS now affords the specificity, imprecision, and levels of quantification necessary for the reliable measurement of steroids in human fluids, thereby enhancing diagnostic capabilities, particularly when steroid profiles are available. Major advantages of tandem mass spectrometry include small sample size, the simultaneous measurement of many analytes, and enhanced specificity compared to IA methods. Mass spectrometric methods are still fairly labor intensive, and certainly require a higher level of laboratory expertise than do IA platforms. Occasional interferences when using mass spectrometric methods have been described, such as prednisolone/prednisone metabolite interference in urinary free cortisol measurements (48). It should be noted that currently reimbursement for steroid profile testing is not yet approved by Medicare (with the exception of the CAH steroid profile), nor are steroid profiles ordered frequently by clinicians. This could well change as steroid hormone profiling becomes more appreciated in the years ahead. Although mass spectrometric assays are not always more precise than IAs, they are more specific for measuring the analyte of interest. By omitting extraction and derivatization steps, the steroid tandem mass spectrometric procedures described here have good intrarun and intrarun imprecision (18, 19). Drug interference has been tested and found not to be a problem for the steroid and estrogen profiles reported in this review (18, 19). These two methods are also relatively free of ion suppression.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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Mini-Review


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