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Steroid analysis in clinical routine diagnostics – discussing crucial questions

Steroid-Analytik in der klinischen Routinediagnostik – die Gretchenfrage der Methodik

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Abstract: Quantitative steroid analysis via liquid chromatography-tandem mass spectrometry (LC-MS/MS) is applicable to clinical routine diagnostics by now, substituting immunoassays due to its superior selectivity and comparable sensitivity. Multiplexed assays covering a multitude of analytes represent the gold standard in this regard. There are commercially available kits which are easily adapted to individual LC-MS/MS systems required. Prior to and even after their appearance, in-house method development represented the flexible alternative in terms of solving specific analytical problems or focusing on a narrower steroid profile while maximizing sensitivity and high throughput applicability. In this work, commercial assays and in-house methods are discussed in relation to a benchmark LC-MS/MS method. Thereby, prerequisites and results are compared. Furthermore, the effect of concomitant medication on steroid assays was tested and requirements regarding quality assurance in routine steroid analysis are discussed. Most of the different commercially available or in-house LC-MS/MS methods for steroid analysis show a good or reasonable agreement of results. However, the harmonization in the methodology of mass spectrometric assays has to be improved to further reduce their variability. Such a procedure would facilitate the performance of diagnostic tests that involve the measurement of steroid hormones by the tremendous improvement of diagnostic sensitivity and specificity.

Keywords: clinical diagnostics; liquid chromatography; quality assurance; routine application; steroid quantitation; tandem mass spectrometry.

Zusammenfassung: Quantitative Steroidanalytik mittels LC-MS/MS ist in der klinischen Routinediagnostik angekommen und ersetzt immunologische Assays dank überlegener Selektivität bei vergleichbarer Sensitivität. Multiplex-Assays, die eine Vielzahl von Analyten abdecken, stellen in dieser Hinsicht den Goldstandard dar. Es gibt kommerziell verfügbare Kits, die problemlos an die individuellen LC-MS/MS-Systeme der Nutzer angepasst werden können. Vor deren Erscheinen und ebenso danach stellte die in-house Methodenentwicklung eine flexible Alternative dar, um spezifische analytische Fragestellungen zu bearbeiten. In einer Routineanwendung bietet sich beispielsweise die Fokussierung auf ein engeres Steroidprofil und gleichzeitig die Erhöhung der Sensitivität und eine Hochdurchsatzoptimierung an. Kommerzielle Assays und in-house Methoden werden in Bezug auf eine Benchmark-LC-MS/MS-Methode diskutiert. Dabei werden deren Voraussetzungen und Ergebnisse verglichen. Darüber hinaus wird in dieser Arbeit der Einfluss von Begleitmedikation auf Steroid-Assays getestet und Anforderungen an die Qualitätssicherung der Routineanalytik diskutiert. Die meisten der verschiedenen kommerziell verfügbaren oder in-house LC-MS/MS Methoden für Steroidanalytik zeigen eine gute oder angemessene Übereinstimmung der Ergebnisse. Allerdings muss die Harmonisierung massenspektrometrischer Assays verbessert werden, um ihre Variabilität weiter zu reduzieren. Dies könnte zu einer allgemeinen Verbesserung der Sensitivität und Spezifität führen, wodurch die Leistung diagnostischer Tests erhöht würde.

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Introduction

The use of commercially available assays utilizing liquid chromatography-tandem mass spectrometry (LC-MS/MS) like MassChrom® from Chromsystems Instruments & Chemicals GmbH (Munich, Germany) and AbsoluteIDQ Stero17 Kit from Biocrates Life Sciences AG (Innsbruck, Austria) can be advantageous since they provide a nearly ready to use system on delivery. A sophisticated sample preparation and an easily adaptable chromatographic setup featuring long chromatography offer high selectivity. Therefore, a relatively high number of 13 to 17 steroids can be measured by these kits. Yet, the advantages of commercial kits also come with their biggest shortcomings. Up to 500 µL serum and a processing time of more than 12 h are necessary to obtain results [1]. This may be acceptable if a comprehensive steroid profile is requested. In the majority of the daily routine analysis, however, the number of requested steroid hormones is from one to four. Hence, a high-throughput procedure (<5 min run time) that uses small sample volumes (20–100 µL) for the six to eight most frequently required steroid parameters might be more helpful in routine analysis. Fortunately, the appearance of commercially available reference material as well as samples of external proficiency enabled the elaborate validation of in-house assays covering endocrine issues from serum aldosterone to hair cortisol [2–7].

Methodology

The LC-MS/MS method representing our benchmark was published in 2016 featuring a multi-matrix approach [2]. In brief, 100 µL of saliva, serum, plasma, urine dilution and hair extracts were treated with a precipitating agent including the internal standards, thoroughly mixed and centrifuged. Prior to this dilution process, urine had to be acidified using hydrochloric acid and hair samples underwent methanolic extraction. A Prominence UFLC system from Shimadzu (Duisburg, Germany) was coupled to a QTRAP® 6500 from SCIEX (Framingham, MA, USA). Sample purification via online solid phase extraction (SPE) and reverse phase chromatographic separation were achieved by applying an automated column switching strategy with a total run time of 4 min. Electrospray ionization (ESI) was applied in positive and negative modes and detection was carried out using multiple reaction monitoring in MS^2 as well as MS^3. Calibration and quality control were performed using a 6PLUS1® multilevel serum calibrator and MassCheck® steroid serum control from Chromsystems Instruments & Chemicals GmbH (Munich, Germany) as well as in-house produced calibration and stock solutions. Between-day precision ranges were 2.9%–10.6% for plasma, 2.6%–11.5% for saliva, 3.4%–19.0% for urine and 15%–19% for hair. Accuracy ranged from 90%–107% for plasma. Recovery ranges were 97%–115% for saliva, 93%–102% for urine and 82%–112% for hair. LLOQ ranges were 0.02–3.1 nmol/L for plasma, 0.1–0.28 nmol/L for saliva, 0.07–0.1 nmol/L for urine and 0.8–1.6 pg/mg for hair (normalized to 10 mg of hair). For comparison purposes the sensitivity for serum estradiol is arbitrarily set as a measure for the quality of a steroid assay. In that regard, it could be expected that commercial kits with their broadband adaptability approach fall behind more specialized in-house developments and that is in fact the case for the MassChrom® kit with rather unsatisfactory LLOQs of 220 pmol/L and 239 pmol/L using a 4500 Triple Quad and a QTRAP® 5500 from SCIEX, respectively. The AbsoluteIDQ kit, on the other hand, reaches 73 pmol/L using a QTRAP® 4000 [1]. Both are using electrospray ionization over atmospheric pressure chemical ionization. Of course the actual LLOQ that can be reached strongly depends on the used mass spectrometer. A previously developed in-house method using a QTRAP® 4000 reached a LLOQ for estradiol of 220 pmol/L [8]. Substituting the mass spectrometer with a QTRAP® 6500 and adapting LC conditions to recent findings regarding mobile phase modification led to an improvement of the LLOQ to 37 pmol/L which is suitable for the challenging estradiol diagnostic [2, 9]. In our experience, this is as good as it gets considering the simple sample preparation and rapid liquid chromatography. For breaching into lower concentration areas, the sample volume has to be increased or derivatization has to be included in the sample preparation for enhancing the ionization. Such an approach can even go as low as 1.8 pmol/L [10], whereas the high throughput applicability might be questioned due to the additional derivatization step. Keeping the hands-on time low reduces variability of the results and increases the robustness of the method. Fortunately, steroid analysis lends itself nicely to sample preparation via protein precipitation, especially when combined with online SPE. This dilute-and-shoot approach is applicable to serum, saliva and urine, whereas the latter has to be acidified prior to precipitation for rendering aldosterone available to analysis. Furthermore, proteinuric samples do not pose a clogging threat to the LC-MS/MS system compared to a direct injection of unprocessed urine as previously suggested [11].

Routine analysis in saliva

Analysis of steroid hormones in saliva is an attractive option for physicians and researchers with its non-invasive sample collection method and the implication that the salivary hormones reflect the bioactive free hormones in the blood. Salivary neuroendocrine bioindicators, such as cortisol, testosterone and aldosterone, significantly correlate with blood in healthy adults when stringent methodologic controls are used [12]. Saliva collection can be performed using the Salivette® from Sarstedt (Nürnbrecht, Germany). It consists of a polypropylene tube and a perforated inlay containing an absorbent wad produced in three different versions,

cotton or polyethylene [13]. Mucin, which increases the viscosity of saliva, can be filtered out through the wad. Salivary viscosity can be reduced further through freeze-and-thaw cycles [14]. According to Gaudl et al. [2], the specially developed Salivette® for cortisol produced a generally lower overall level of background noise in mass spectrometric analysis. Furthermore, it showed good recovery of salivary steroids [13]. Steroid hormones in collected saliva samples have a high long-term stability. For example, salivary cortisol remained stable for 3 years at −80 °C [15]. Using LC-MS/MS is beneficial over using immunoassays in saliva analysis because of its higher selectivity. Immunoassays are more susceptible to cross-reactivity or matrix interference, especially in lower concentration ranges, than LC-MS/MS. This is of clinical relevance in saliva diagnostics, particularly with cut-offs in low concentration ranges. For example, the cut-off for midnight salivary cortisol in diagnosis of Cushing's syndrome ranges from 2.2 to 12 nmol/L depending on the immunoassay kits [2, 16, 17].

Mass spectrometry vs. immunoassay: influence of concomitant medication

Concomitant medications used in the patients with endocrine disorders have similar chemical structures to endogenous steroid hormones. Thus, structurally related compounds can cross-react with the antibodies used in steroid hormone immunoassays [18]. Cross-reactivity with a variety of endogenous and synthetic hormones is reported in the assay package inserts by the manufacturers. However, there is no clear standard in testing and calculating the magnitude of cross-reactivity in immunoassays [19]. If the cross-reactivity is calculated based on the measurement of the amount of the synthetic compound that was required to generate the same signal for the analyte, the cross-reactivity could be negligible although the recovery due to the cross-reactivity with the synthetic compound has clinical significance. Furthermore, the degree of cross-reactivity depends on the concentration of the analyte in a competitive immunoassay, which tends to higher cross-reactivity in lower concentration ranges [15]. Such limitations of immunoassays can be overcome by adopting LC-MS/MS for the measurement of steroid hormones.

Cross-reactivity with concomitant medication in immunoassays can mislead the interpretation in monitoring the treatment with Fulvestrant in postmenopausal women with hormone receptor positive metastatic breast cancer. Fulvestrant is a selective estrogen receptor degrader (SERD) and works by binding to the estrogen receptor and destabilizing it, causing the cell's normal protein degradation processes to destroy it. It has a similar chemical structure to estradiol and may cross-react with the antibodies used in immunoassays leading to falsely elevated estradiol results. Several manufacturers of estradiol immunoassay, such as Siemens, Roche and Abbott, issued an urgent field safety notice saying that their immunoassay kits are not suitable for the measurement of estradiol in serum samples from patients treated with Fulvestrant and the results could lead to misinterpretation of the menopausal status of these women. The impact of Fulvestrant in estradiol analysis was tested comparing the results of the same samples measured with immunoassay as well as LC-MS/MS. While estradiol results of the sample from the postmenopausal woman treated with a Fulvestrant analogue was unexplainably high in the immunoassay, the estradiol level measured by LC-MS/MS was below the lower limit of quantification. Our method comparison showed that data were not in a linear relationship in the lower concentration range (Figure 1). As this example shows, it is important to measure low estradiol levels using an accurate and reliable assay method with high sensitivity and specificity such as gas chromatography-tandem mass spectroscopy (GS-MS/MS) or LC-MS/MS in order to assess the treatment efficacy correctly.

Quality assurance considerations for LC-MS/MS analysis of steroids in clinical diagnostics

LC-MS/MS assays for steroid hormones can be used in patient routine analysis if appropriate formal and analytical preconditions are fulfilled. Thus, the performance of quality assurance (QA) of the analytical method has to be in agreement with the recommendations of the RiliBÄK and in correspondence with clinical background that is associated with the individual steroid analyte. The guidelines of the BÄK imply internal as well as external quality assurance recommendations for the steroids cortisol, estradiol, testosterone, and progesterone [20]. QA criteria for steroids that are not covered by these recommendations can be provided by manufacturers of quality control (QC) material.

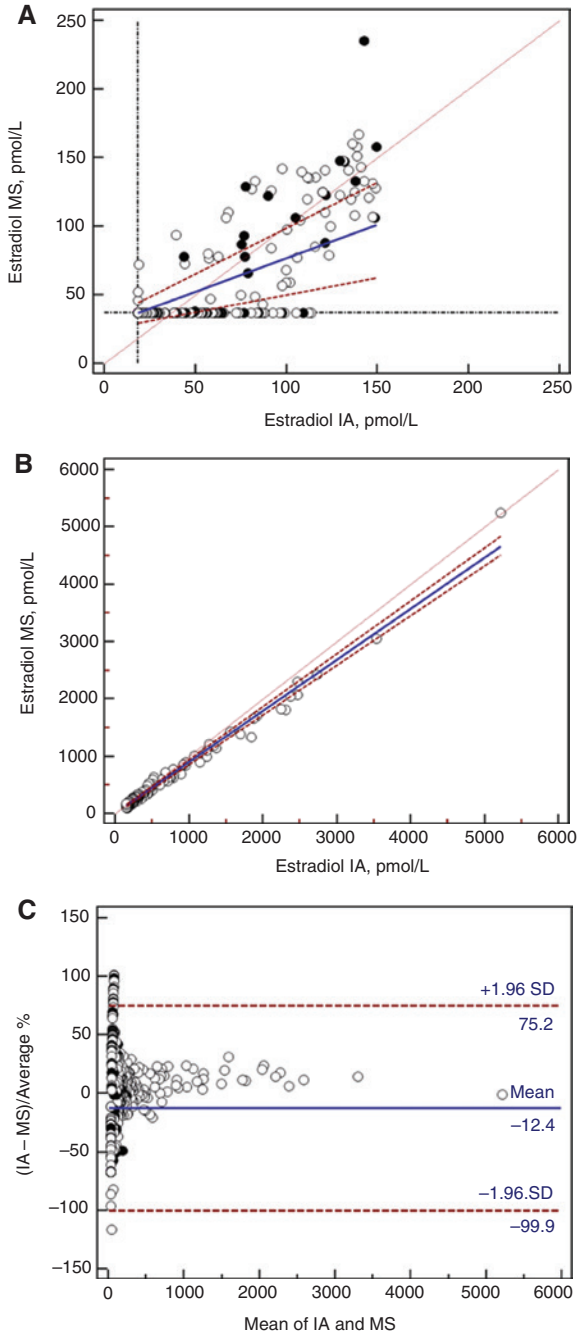

Figure 1: Method comparison between LC-MS/MS and immunoassay for the measurement of estradiol in serum depending on the concentration range.
(A) Estradiol IA <150 pmol/L, $R^2 = 0.54$, n = 243, non-linear relationship. (B) Estradiol MS >150 pmol/L MS = 0.89*IA + 9.38, $R^2 = 0.96$, n = 122, linear relationship. (C) Bland-Altman plot (open circle: female sample, closed circle: male sample).

Internal QA

The permissible relative deviation of a single result from the target value is dependent on the respective hormone level in the range between 16% and 22% as shown by the RiliBÄK Table B1A [20]. As the preparation of QC samples according to the routine-conform CE-guidelines [21], needed for quality assurance, is a very complex process, control samples can rather be purchased by manufacturers of LC-MS/MS kits or QC material. The package insert of these QC samples contains the above-mentioned manufacturer-dependent applicable acceptance ranges for RiliBÄK and non-Rili-BÄK steroid parameters.

External QA

According to the RiliBÄK recommendations, the permissible relative deviation in external QA trials was determined parameter dependent between 30% and 35% [20]. This range is comparable with the established range for non-RiliBäk steroid parameters by German external QA providers, such as RfB (Reference Institute for Bioanalytics, Bonn, Germany) and Instand (Düsseldorf, Germany). However, the maximum deviation range for aldosterone (±44% RfB) and 17-hydroxyprogesterone (17-OHP) (±60% for levels ≥5 nmol/L, RfB) are much wider, reflecting a generally higher variance in the measurement of both parameters. Interestingly, the number of laboratories participating in the external QA trial of the RfB for steroid measurements by mass spectrometry ranged from 5 (for estradiol) to 28 (for 17-OHP) at the end of year 2016 [22]. In relative numbers, 0.7% to 17.2% of all participating labs used a mass spectrometric method for steroid measurements. Despite a minor degree of outliers, the respective data were largely between the 16th and 84th percentile of the acceptance range and demonstrated a high accuracy and comparability of results. External quality assurance trials may also give insight into the method-specific variance, whether the range between the 16th–84th percentile is related to the median of submitted results [22]. As shown in Table 1, this indicator of variance was lower or equal if mass spectrometric methods, which should be comprised in their majority by LC-MS/MS methods, were compared with fully automated immunoassays based on luminometric detection. This means that the variance of both assay systems is very similar. In the case of aldosterone, testosterone and DHEA-S the variance of the mass spectrometric methods was even lower. In contrast, the variance of mass spectrometric methods compared to immunoassays based on photometric detection was distinctly lower. As this variance is determined by sensitivity, reproducibility, accuracy and specificity of the respective analytical method, mass spectrometric assays appear to be demonstrating tremendous advantages regarding the quality criteria compared to the variety of immunometric assays.

Table 1: Comparison of variances in method-specific steroid results from external quality assurance trial 3/2016 (RfB) as indicated by the percentage concentration difference of 84th and the 16th percentile from the whole number of submitted data adjusted for the value of the median concentration (50th percentile).

Percent	Photometry			Luminescence			Mass spectrometry		
	16th–84th	50th	(16th–84th)/50th (%)	16th–84th	50th	(16th–84th)/50th (%)	16th–84th	50th	(16th–84th)/50th (%)
	n = 14			n = 139			n = 16		
Aldst. A	0.75–1.56	1.2	67.75	1.28–1.61	1.41	23.40	1.52–1.87	1.73	20.23
Aldst. B	0.25-0.39	0.30	48.31	0.29–0.41	0.36	31.13	0.40–0.48	0.45	18.34
	n = 14			n = 457			n = 15		
Cortisol A	907–1532	1057	59.13	867–1042	945	18.52	887–1081	1005	19.30
Cortisol B	232–377	257	56.42	226–276	243	20.58	227–294	251	26.69
	n = 48			n = 497			n = 12		
Progest. A	6.36–8.05	6.58	25.68	5.91–7.24	6.55	20.31	5.94–7.64	6.8	25.00
Progest. B	38.2–41.9	39.4	9.39	32.4–45.1	38.9	32.65	36.2–44.6	40	21.00
	n = 53			n = 603			n = 22		
Testost. A	5.20–5.84	5.55	11.53	4.72–6.01	5.36	24.07	5.24–5.90	5.54	11.91
Testost. B	20.8–22.6	21.2	8.49	18.5–23.9	21.9	24.66	19.2–22.2	21.2	14.15
	n = 16			n = 407			n = 12		
DHEA-S A	2.90–4.36	3.4	42.94	3.55–5.22	4.23	39.48	3.36–4.07	3.7	19.19
DHEA-S B	4.40–6.92	5.15	48.93	5.43–7.76	6.43	36.24	4.04–5.87	5.4	33.89
	n = 78			n = 6			n = 28		
17-OHP A	5.76–7.07	6.53	20.06	n/a	n/a	n/a	5.82–7.54	6.47	26.58
17-OHP B	8.62–10.9	9.86	23.12	n/a	n/a	n/a	8.48–11.2	9.63	28.25

"n" reflects parameter-dependent total number of results sent by the labs. A and B represent the identification label of test sample.

Result comparability of different mass spectrometric methods

Despite the previously-mentioned low variance in quality assurance of mass spectrometric results, it is important to acquire knowledge about the direct comparability of individual mass spectrometric methods in clinically routine analysis. Recently, seven LC-MS/MS and one GC-MS/MS method were compared for the measurement of testosterone in female and male sera [23]. Within-run variability (n = 5) at 10.3 nmol/L and 0.29 nmol/L of all methods ranged from 1.40% to 11.36% and from 2.52% to 25.58%, respectively. In most cases, however, this variability was lower than for immunoassays [24]. The absolute values of the percent differences between assays and the reference method again showed a wide range between 2.1% and 19.2%. The slopes of the Deming regression were between 0.903 and 1.138 and significantly different from 1 in six assays indicating a relative bias. The intercepts were significantly different from 0 in four assays indicating a constant bias. The correlation coefficients of >0.996 indicated a good qualitative agreement. Additionally, this paper suggests a higher variability of data for the low range of female testosterone concentrations. The comparison of the individual methods to the reference assay revealed significant mean differences of 10% or less for the most methods. That difference is smaller than those between immmunoassays and MS assays [25, 26]. The significant differences between the mentioned methods could be provoked by different calibrators, different internal standards, and cross-reactivity or matrix interferences due to different sample preparations. Thus, despite a generally better applicability of MS assays than of immunoassays for clinical analysis the quality of the individual assay in the measurement of clinical challenges is strongly dependent on the assay methodology and assay optimization. This suggestion was supported by a recent article: 60 random serum samples from males and females were analyzed by eight routine LC-MS/MS methods for testosterone and androstenedione [27]. Intra-assay variation of various methods was very obviously different ranging from 3.7% to 16.0% for testosterone in females, from 0.9% to 5.2% for testosterone in males, and from 1.2% to 9.5% for androstenedione. The slopes for the regression lines were distinctly different as well. Inter-method coefficients of variation were 24%, 14%, and 29% for female testosterone, male testosterone and androstenedione, respectively. Despite these relatively high coefficients of variation the data appear to be lower than in immunoassays [28]. Again, differences in calibrators and internal standards as well as cross-reactivity or matrix interferences were assumed as reasons for the contrasting data.

We conclude that most of the different commercially available or in-house LC-MS/MS methods for steroids show a good or reasonable agreement of results. However, the harmonization in the methodology of mass spectrometric assays has to be improved to further reduce their variability. This is the most important precondition for the establishment of largely uniform reference ranges for mass spectrometry-based methods. Such a procedure would facilitate the performance of diagnostic tests that involve the measurement of steroid hormones by the tremendous improvement of diagnostic sensitivity and specificity.

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