A19

Steroids panel by LC-MS/MS: analytical validation and reference ranges establishment

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Short abstract:

We validated a quantification method of 13 steroid hormones to switch from the immunoassays or radiommunoassays to Liquid Chromatography triple quadrupole mass spectrometry. Finally we have established new references range values for these steroids . The method showed good precision, accuracy, detection limits, and robustness. However the sensitivity of DHT and oestradiol is still too low.

Introduction:

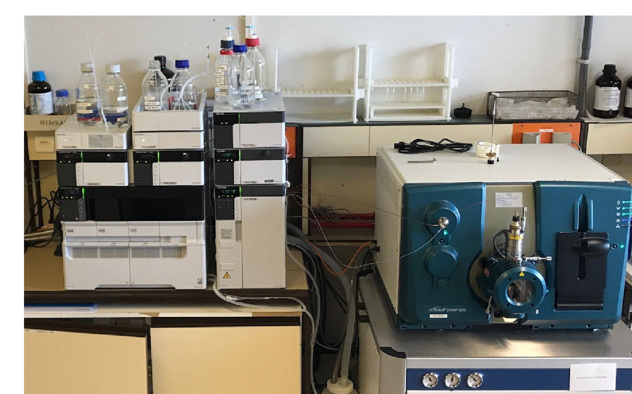
Steroid hormones play a crucial role in controlling metabolism, immune functions and inflammation. Modifications in steroid profiling reflect disease status and help research into a wide number of health disorders, including congenital adrenal hyperplasia, Cushing's disease and polycystic ovarian disease (1). However, the quantification of steroids by immunoassays may underestimate some of them. Prior to the introduction of mass spectrometry (MS) in clinical laboratories, the main techniques for measurement of steroids were immunoassays (IA) and radioimmunoassays (RIA)(2). Antibody–based methods can have good sensitivity but they usually lack specificity, particularly in the competitive formats required for small molecule analysis (3–6). To overcome these limitations most clinicians are switching to Liquid Chromatography (LC) coupled to Tandem Mass Spectrometry (MS/MS) in order to obtain an accurate and precise dosage of steroid hormones (7–9). This technique has become an essential tool for small molecule quantification due to its high sensitivity, specificity, excellent reproducibility and the ability to perform simultaneous analysis to overcome the limitations of immunoassays.

The reliable and simultaneous analysis of a panel of steroid hormones is a powerful tool for investigation of hormone status, which is relevant for a variety of clinical questions and diagnoses like adrenal insufficiency in congenital adrenal hyperplasia (CAH)(10).

Aim of the Study:

The aim of our work was the evaluation and validation of the MassChrom® for Steroids in Serum/Plasma kit by LC-MS/MS (Chromsystems) as well as the establishment of the new reference values. Indeed, as we change the methods, new reference values must be determined. This method is able to detect and quantify aldosterone(ALDO), cortisol(COR), cortisone, cortsicosterone, 11-deoxycortisol(S), androstenedione (AND), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate(DHEAS), dihydrotestosterone(DHT), estradiol (E2), 17 α-hydroxyprogesterone (HYP), progesterone and testosterone(TST) as total steroids.

Figure 1: Qtrap 6500 (Sciex)



Materials and Methods:

The analysis was performed with the MassChrom® kit from Chromsystems (Heimburgstrasse, Munich, Germany) using:

→ panel 1 for corticosterone, cortisone, cortisol (COR), aldosterone (ALDO) and 11-desoxycortisol(S)
→ panel 2 for AND, DHEA, DHEAS, DHT, E2, HYP, progesterone and TST

Liquid chromatography and electrospray tandem mass spectrometry

- Analytical evaluation of the new methodology has been carried out using a Sciex 6500 Qtrap MS/MS (Framingham, MA, USA) equipped with LC-30A Nexera UHPLC system (Shimadzu Co., Kyoto, Japan) (Figure 1).
- Injection volume was 30µL for prepared samples.
- The flow rate varied from 0.8 to 1 ml/min.
- The run time was 10.5 min and 12.5 min for the panel 1 and 2 respectively.
- A gradient was used for the mobile phases.
- Electrospray ionization in negative mode has been used for aldosterone while positive mode has been used for the rest of compounds.
- Two MRM transitions (one as quantifier and another one as qualifier) were used to monitor each analyte, and one MRM transition was used to monitor each deuterated internal standard.
- Quadrupoles Q1 and Q3 were tuned to unit resolution and the MS parameters optimized for maximum signal intensity for each mass transition.
- Data acquisition and processing was carried out using Analyst 1.6.2 and calibration curves were prepared using $1/x^2$.

Sample preparation

- For sample preparation, to 500 µl serum/QC/calibrator both Internal standard Mix and Extraction buffer were added before performing an extraction in a 96-well solid phase extraction(SPE) plate.

LC–MS/MS analytical evaluation

- The procedure was validated by testing 3 levels of concentration in triplicate during 3 different days. Statistical analysis was performed using the Enoval validation software (Arlenda, Liège, Belgium). Statistical analysis was performed using Medcalc version 12.7.7.0 (Ostende, Belgium) software.
- A calibration curve was prepared using 6 points and responses were determined by calculating the integrated peak area ratio between endogenous steroids to deuterated steroids. According to these results the precision (intra-assay and inter-assay) and an accuracy profile were established. In this profile, we set the acceptance limits at ±15% for serum for each level tested. We settled at each level, β-expectations with a probability of 95%. These β-expectations allow calculation of, with a probability of 95%, the variation at each tested point. To be considered as valid, the β-expectations of the method should be within the pre-defined tolerance limits.
- Linearity of the calibration curves for serum was assessed by performing linear regression.
- Recovery was calculated with the expected values of the internal control.
- The limit of detection (LOD) and limit of quantification (LOQ) were calculated with the lowest concentration that we tested. LOD and LOQ were respectively defined as 3:1 and 10:1 signal/noise ratio respectively.

Reference values establishment

- For the serum reference interval study, recruited participants were normotensive (clinical blood pressure <140/90 mmHg), without antihypertensive or corticosteroid treatment, non-smokers and did not take any oral contraception. All participants gave informed consent and were fasting.

- We enrolled 514 healthy Caucasian volunteers (144 F: mean age 55 ± 15 years old (yo), 117 M: mean age 37 ± 13 yo and children: n=253: mean age 8.4±4.4 yo).

- Blood samples were centrifuged immediately after the draw (morning) for 10 minutes at 2500 g, aliquoted and stored frozen at -80° C before further analysis. Study was approved by ethic committee of the university in Liege. The 95% percentile was calculated with the CLSI C28-A3 recommendation.

Results:

Analytical evaluation

The evaluation of the mentioned kit was carried out at the following concentrations (µg/L) for each steroid. Panel 1: ALDO (0.025-3.08), COR (10.2-288), cortisone (1.03-38.9), corticosterone (0.52-48.2) and S (0.09-13.9). Panel 2: AND (0.18-14), DHEA (0.97-55.9), DHEAS (105-5975), DHT (0.06-1.34), E2 (0.04-4.94), HYP (0.1-15.1), progesterone (0.17-25.6) and TST (0.05-11.8).
The mean recoveries values did not differ significantly from 100% while the precision, as CV%, was below 10% for both the intra-assay and inter-assay variability except for corticosterone (11%).
The developed method was shown to be linear (R^2>0.99) for all steroids in serum.
The limit of detection (LOD) and limit of quantification (LOQ) were calculated with the lowest concentration tested.
For the panel 1, LOQ's(LOD's) were 0.0014(0.004) µg/L for S, 0.025(0.007) µg/L for ALDO,0.67(0.2) nmol/L for COR,0.057(0.017) µg/L for cortisone, 0.051(0.015) µg/L for corticosterone. For the panel 2, 0.07(0.0021) µg/L for AND, 0.236(0.167) µg/L for DHEA, 0.020(0.006) µmol/L for DHEAS, 0.079(0.024) µg/L for DHT, 0.037(0.011) ng/L for E2, 0.052(0.016) µg/L for HYP, 0.024(0.005) µg/L for progesterone and 0.043(0.013) nmol/L for TST.

Reference values establishment

Difference for the gender was observed so different reference values were calculated according to age, Tanner Stage and gender. The reference values were obtained with a robust and validated method and are presented in the table 1.

Table 1:Steroids reference values established by LC-MS/MS

Steroid name	Age(sexe , n)	Reference values
Aldosteron	>18 yo (F,n=119)	162 ng/L
	>18 yo (M, n=113)	128 ng/L
Cortisol(morning)	> 18yo (F+M,n=250)	3.7-22.7 µg/L
Cortisone	>18yo (F, n=141)	12.3-30.41 µg/L
	>18 yo (M, n=114)	16.1-29 µg/L
Corticosterone	<18 yo (M+F, n=227)	1671 ng/dL
	>18 yo (F, n=129)	<677 ng/dL
	> 18 yo (M, n=111)	<1085ng/dL
11- Desoxycortisol	<18 yo (M+F, n=240)	<211 ng/dL
	>18 yo (F, n=129)	<46.4 ng/dL
	>18 yo (M, n=106)	<69.5 ng/dL
Androstenedione	< 9 yo(Stade I)(F,n=20)	<30.35 ng/dL
	9.2-13.7yo (Stade II)(F,n=43)	<82.15 ng/dL
	10.0-14.4yo (Stade III)(F,n=26)	<112.5 ng/dL
	10.7-15.6 yo (Stade IV)(F,n=23)	<117.4 ng/dL
	11.8-18.6 yo (Stade V)(F,n=12)	<26.5 ng/dL
	>18yo (F, n=135)	>118 ng/dL
	< 9 yo (Stade I)(M, n=22)	<28.34 ng/dL
	9.8-14.5 yo (Stade II)(M, n=20)	<52.87 ng/dL
	10.7-15.4 yo (Stade III)(M, n=20)	<51.64 ng/dL
	11.8-17.3 yo (Stade IV and V)(M, n=23)	<62.31 ng/dL
	> 18 yo (M, n=57)	35-151 ng/dL
DHEA	6-10 yo ((F+M), n=30))	<1.3 ng/mL
	11-14 yo ((F+M),n=29))	<4 ng/mL
	19-30 yo ((F+M),n=41))	<13.5 ng/mL
	31-50yo ((F+M),n=66))	<7 ng/mL
	51-60 yo ((F+M),n=30))	<3.6 ng/mL
	>61 yo ((F+M),n=60))	<4.3 ng/mL
DHEAS	30-39 yo(F, n=19)	<205 µg/dL
	40-49 yo (F, n=23)	<232 µg/dL
	50-59 yo (F, n=33)	<147 µg/dL
	>60 yo (F, n=53)	<113 µg/dL
DHT	<7.1 yo (Stade I) (M, n=55)	< 50 pg/mL
	<12.1yo (Stade II) (M, n=32)	37-227 pg/mL
	> 19 yo (M, n=62)	<613 pg/mL
	<7.1 yo (Stade I) (F, n=55)	< 50 pg/mL
	<11.6 yo (Stade II +III+IV+V) (F, n=89)	<300 pg/mL
	20-55 yo (F, n=38)	<300 pg/mL
	>55yo(F, n=14)	<128 pg/mL
Progesterone	2-9 yo (M, n=33)	<0.15 ng/mL
	> 18 yo (M, n=50)	0.2-1.4 ng/mL
Testosterone	> 19 yo (M, n=61)	324-840 ng/dL

Conclusions:

The method based on LC-MS/MS using the MassChrom® Kit has been satisfactory validated and meets the requirements to be applied in daily routine with exception of oestradiol and DHT. Unfortunately at this moment the LOQ for these two analytes is still too high for the expected low references values. It has to be taken into account that some compounds are present at very low concentrations but their determination can be relevant for clinical practice.
However the use of mass spectrometry for the steroids analyses leads to a gain in specificity compared with IA's which are affected by interferences and are not fully appropriate for children and women.

With the actual analysis we have established new reference intervals in LC-MS/MS for 12 steroids in serum, despite of the low number of volunteers which have limited the validity of our study results. Consequently the recruitment is going to continue to obtain a larger number of reference values to be in line with CLSI recommendations.
As can be observed some age range is lacking or did not contain enough subjects. The lacking or incomplete reference values are going to be improved in the future. Indeed, it is difficult to recruit subjects according to the different period phases for women (luteal, follicular, ovulation) and for all the Tanner stages for the children…

Finally, we urge the Clinical Chemistry community to develop both an international standard reference material for steroids and a candidate reference method for LC-MS/MS. Our future works will deal with the development of a homemade method for the most challenging analytes.