Quantification of Steroids in Human Plasma by Liquid Chromatography Coupled with the Affordable Q Exactive Focus HRMS

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Key Words
Steroids, LC-HRMS, plasma, Q Exactive Focus, quantification

Goal
To develop a robust liquid chromatography–high-resolution mass spectrometry (LC-HRMS) analysis for the routine quantification of steroids in plasma that fulfills clinical research demand for sensitivity and selectivity.

Introduction
Steroid hormones are endogenous metabolites that are synthesized by various enzymes from cholesterol. There are tens of these hormones with different effects even at very low concentrations (nM–pM levels).[1,2] Their determination can be relevant for clinical research. Steroid determinations by immunoassays are affected by interferences and are not fully appropriate for children and women.

The steroid backbone of cholesterol does not contain amino groups; therefore, various ionization sources have been employed. However, recent routine LC-MS research analyses of steroids (underivatized) use a 2.1 mm ID (inner diameter) analytical column coupled with electrospray ionization (ESI) and triple quadruople MS.

Indeed, LC-MS research methods almost exclusively use triple-quadrupole mass spectrometry. However, many articles have recently shown that LC-MS analysis using high resolution (HR) technology can perform robust, quantitative and sensitive analyses of drugs and peptides in routine research environments.[3] Interestingly, most LC-HRMS analyses are performed in HR full scan acquisition which allows an overview of all ions such as steroid metabolites.

The work presented here is to analyze quantitatively eight steroids using an affordable HRMS instrument, the Thermo Scientific™ Q Exactive™ Focus MS, for clinical research purposes.

Experimental
Chemicals
Cortisol, corticosterone, 11-deoxycortisol, deoxycorticosterone, androstenedione, 17-hydroxyprogesterone, and progesterone standards were purchased from Sigma-Aldrich chemie GmbH. Internal standards (IS), d_9-progesterone, d_4-cortisol, d_8-corticosterone, d_2-11-deoxycortisol, d_8-deoxycorticosterone, and d_8-17-hydroxyprogesterone were purchased from C/D/N Isotopes Inc. D_5-androstenedione and d_3-testosterone were purchased from Cambridge Isotope Laboratories, Inc. and Lipomed AG, respectively.

Sample Types
Human plasma samples were prepared by centrifugation of donor whole blood withdrawn in heparin tubes.

Plasma samples were stored at -80 °C. Two quality control samples (QCs), one as a pool of female plasma and one as a pool of male plasma samples, were prepared. Calibrators (Cs) were prepared from stripped fetal bovine serum and fortified with seven different concentrations (see Table 1).

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Sample Preparation

Plasma samples (100 µL) were diluted with 100 µL of 5% phosphoric acid (H_3PO_4) containing the internal standards (IS) in water. The solution was stirred for 10 minutes. A solid phase extraction (SPE) was performed using 96-well plates (mixed mode cation extraction 96-well plate, 10 mg of phase). The SPE plate was activated with 200 µL of methanol (MeOH) and 200 µL of water. The samples were loaded on the plate with positive pressure, washed with 200 µL of 5% ammonium hydroxide (NH_4OH) and 200 µL of 10% MeOH in water. Then, the steroids were eluted with 2 x 75 µL of isopropanol. The wells were dried under nitrogen flow and the dried residues were reconstituted in 100 µL of H_2O/MeOH (50:50) prior injection.

LC Conditions

The LC system consisted of a Rheos Allegro UHPLC pump (Flux Instruments, Basel, Switzerland) and a CTC PAL® autosampler (CTC Analytics, Switzerland). A Thermo Scientific™ Accucore™ C18 LC column (2.1 x 50 mm and 2.6 µm, P/N 17126-052130) was used and placed in an oven set at 60 °C. Mobile phase was composed of A) H_2O and B) MeOH with 50 µL/L of formic acid. The gradient was delivered as follows: at 0 min: 10% of B; at 15 min: 75% B; at 15.10 min: 100% of B kept to 17.90 min; 18 min: 10% of B and maintain isocratic to 20 min for column initial conditions. The flow rate was 600 µL/min. The injection volume was 40 µL.

MS Conditions

The LC system was connected to a Q Exactive Focus high-resolution mass spectrometer; the following MS conditions were used:

Source type	Heated-electrospray ionization (HESI II)
Ionization mode	Positive
Spray voltage	4000 V
Sheath gas, N2	60 AU
Sweep gas, N2	20 AU
S lens	70 V
Capillary temperature	380 °C
Auxiliary gas heater	Deactivated
Data acquisition mode	HR full scan
Scan range	*m/z* 200 to 400
Resolution	70,000 (FWHM) at *m/z* 200

A second acquisition was studied and consisted of an HR full scan (same range and resolution) and a targeted single ion monitoring (SIM) scan, which allowed the detection of both testosterone and d_3-testosterone in the same SIM scan:

SIM scan	Centered on *m/z* 290
Isolation window	8 *m/z*
C-trap capacity	5 x 10^5 charges
Maximum injection time	100 ms

Results and Discussion

The determined steroids are presented in Table 1 and were detected as singly charged molecular ions $[M+H]^+$. Extracted ion chromatograms (XIC) were constructed on the theoretical *m/z* (m/z_{theor}; Table 1) with a mass extraction window of ± 5 ppm.

Table 1. Steroids determined, chemical composition, *m/z* used for XIC construct, retention time, and the lower and upper calibrator levels (Cs).

Name	Formula	m/z theor $[M+H]^+$	R_T [min]	Lower Cs [nM]	Upper Cs [nM]
Cortisol	$C_{21}H_{30}O_5$	363.21660	8.5	2.19	1400
Corticosterone	$C_{21}H_{30}O_4$	347.22169	9.8	0.47	60
11-Deoxycortisol	$C_{21}H_{30}O_4$	347.22169	10.1	0.16	20
Deoxycorticosterone	$C_{21}H_{30}O_3$	331.22677	11.8	0.16	20
Androstenedione	$C_{19}H_{26}O_2$	287.20056	10.7	0.31	40
Testosterone	$C_{19}H_{28}O_2$	289.21621	11.3	0.13	80
17-hydroxyprogesterone	$C_{21}H_{30}O_3$	331.22677	11.2	0.16	20
Progesterone	$C_{21}H_{30}O_2$	315.23186	13.3	0.50	160

Typical XIC traces constructed from HR full scan acquisition are presented in Figure 1 and show the resolution of many steroid isomers in donor plasma in less than 14 minutes. Similar chromatograms presenting various isomers have been shown with SRM acquisitions[4-6] revealing that an efficient chromatographic resolution, typically using UHPLC or core shell columns, is mandatory.

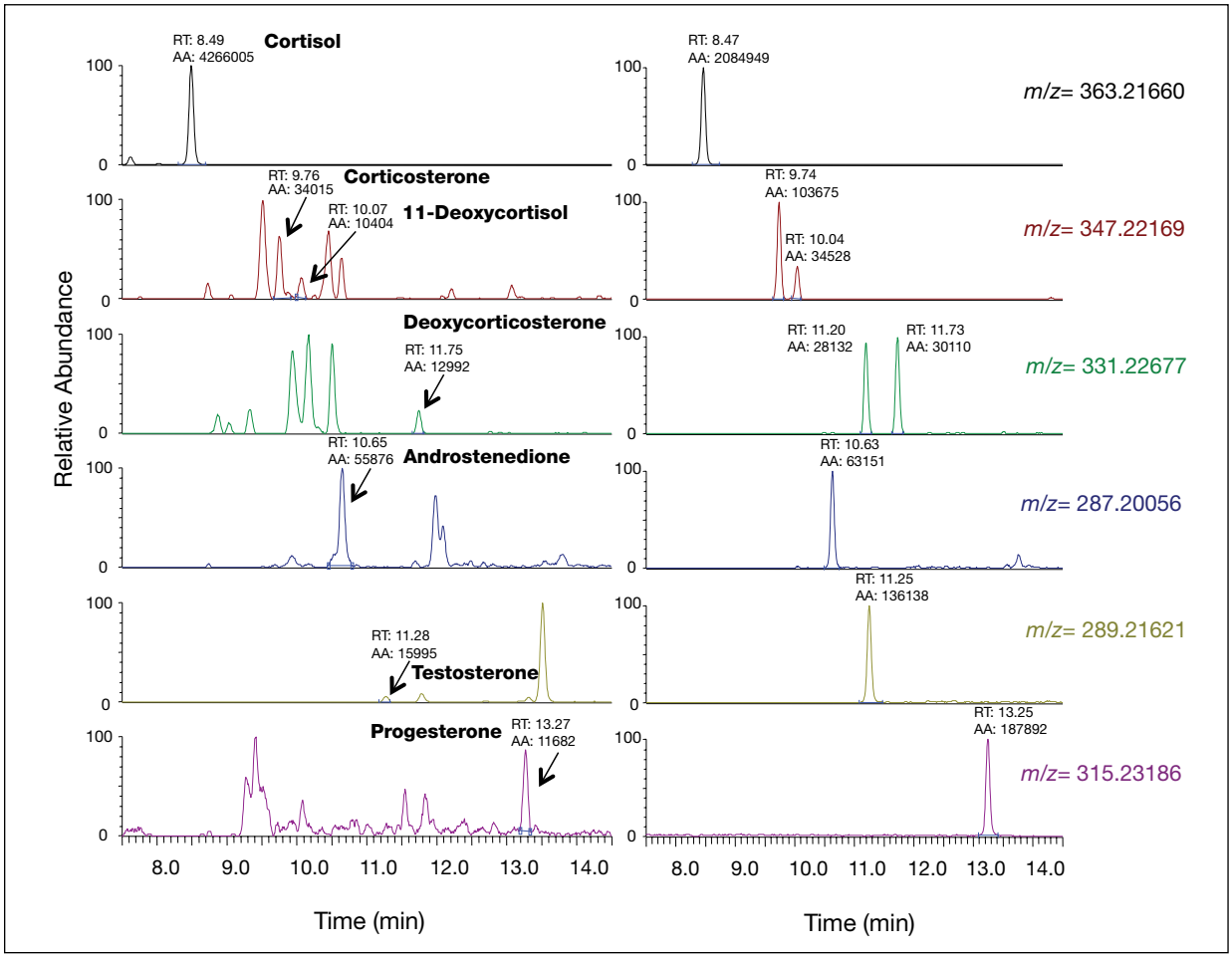

Figure 1. Example of XIC chromatograms extracted from HR full scan acquisition. Left: a male donor plasma sample. Right: a calibrator sample (stripped plasma fortified). Extracted *m/z* is listed on the right.

Calibration curves and equations were generated using the internal standard methodology. An example of a calibration curve is depicted in Figure 2. Analyses were validated according to international guidelines. Accuracy of calibrators, QCs, and lower limit of quantification (LLOQ) levels were ≤15% and ≤20%, respectively.

To determine the LLOQ for testosterone, HR full-scan and tSIM mode data were acquired successively. Various Cs at expected LLOQ values (N = 5 extracts) included in a full calibration (with the upper limit of calibrations = 80 nM) were injected. Generic ESI source parameters were employed (no specific adjustments of the ESI source parameters were made for testosterone or any steroids tested).

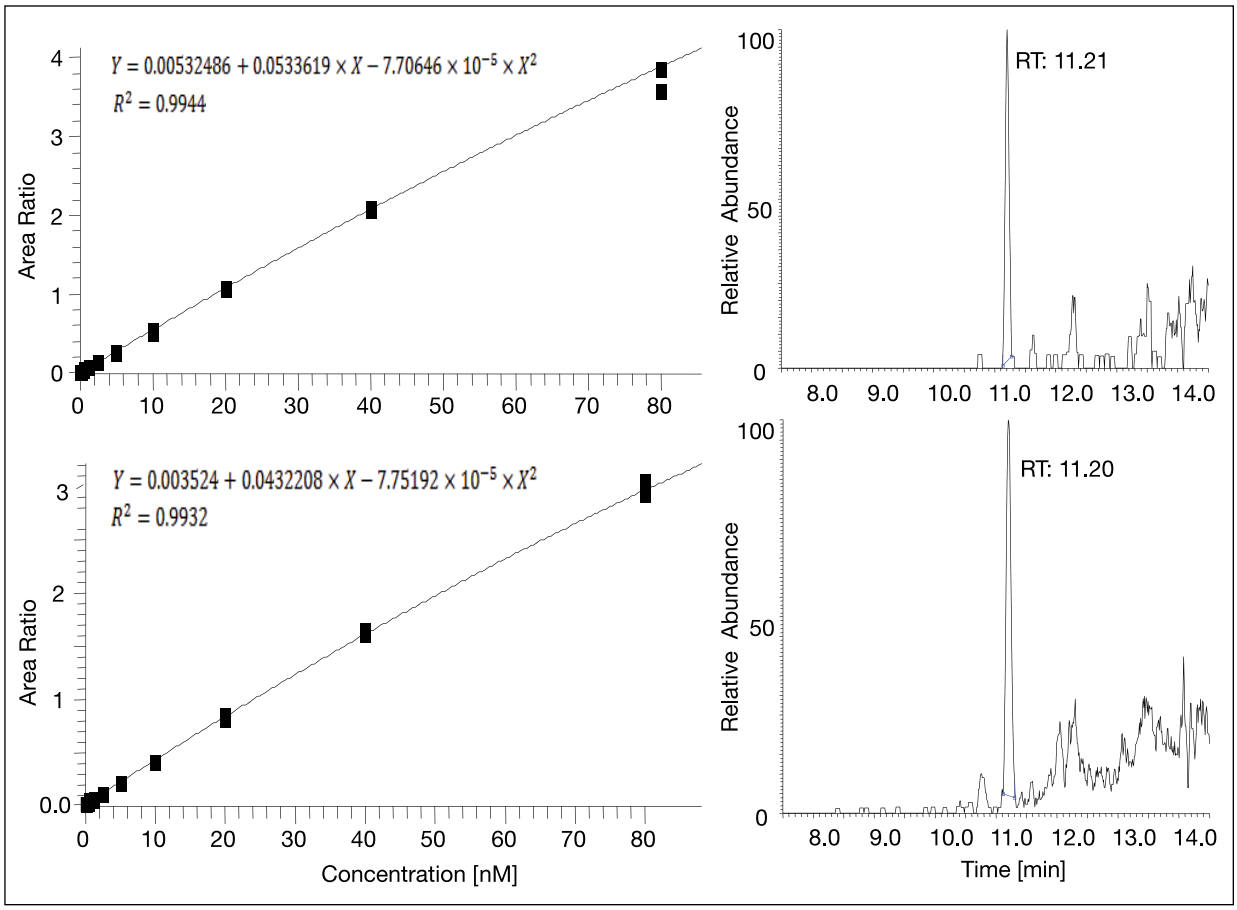

Figure 2. Left: Calibration curves of testosterone in fortified stripped plasma. Right: Corresponding XIC at the LLOQ level in plasma for testosterone (0.125 nM). Top and bottom: Data according to HR full-scan or targeted SIM acquisitions, respectively.

In HR full-scan acquisition on the Q Exactive Focus MS system, the LLOQ value for testosterone in plasma samples (volume = 100 µL) was 0.125 nM with a precision and accuracy of 19% and 8% (N = 4), respectively; whereas, in SIM mode, precision and accuracy were 21% and 7%, respectively. SIM acquisition did not significantly increase the LLOQ level (still at 0.125 nM). However, the limit of detection (LOD) for testosterone was increased by about five times in the SIM mode (not shown).

As an illustrative comparison only, the LLOQ levels (nM) and amounts (pg) on column at the LLOQ levels of steriods by ESI-MS in eleven published articles are presented in Table 2. Mean ±SD of the amounts on column at the LLOQ values is 1.77 ± 1.22 pg. Generic ESI parameters were used. Our LLOQ levels could probably be improved by adjusting ESI parameters for testosterone (e.g. heated gas temperature, etc.) or by extracting a bigger plasma volume (e.g. 200–400 µL instead of 100 µL). Nevertheless, even without tuning of parameters for sensitivity, the results show that the Q Exactive Focus MS has a comparable sensitivity to triple quadrupole instruments (Table 2). The amount on column at the LLOQ value was 1.45 pg. The obtained sensitivity allowed performing steroid determination in all donor samples.

Conclusion

A robust and rapid LC-MS method for the determination of steroids in plasma samples using an affordable HRMS instrument has been presented and is suitable for routine research analyses. Results acquired using this method demonstrate that the Q Exactive Focus MS exhibits accuracy, precision, and sensitivity that is comparable to triple quadrupole MS instruments. Various steroid isomers can be detected in full scan but also in MS/MS acquisition showing that there is a need for an efficient chromatography. In addition, with sensitive and selective full scan data, the Q Exactive Focus HRMS can be used for more in-depth investigations when requested by researchers or biochemists.

Table 2. LLOQ levels in plasma/serum matrices and amounts (pg) on column for testosterone in routine analyses in research found in the literature. HR-MS full scan (#7) and SRM (#1 to #6 and #8 to #13) acquisitions on various MS platforms can be compared but are illustrative only. The comparison should be taken with care because different LLOQ determinations, extraction procedures and yield, columns, ion sources, LC-MS conditions, S/N ratios, etc. have been used.

Reference Ordered by pg on column	Instrument	pg on column (*)	LLOQ [nM] (**)
1. Salameh et al., 2010[7]	TSQ Ultra, Thermo Scientific	**0.45**	0.01
2. Han et al., 2014[8]	QTRAP 5500, AB Sciex	**0.50**	0.13
3. Rhea et al., 2013[9]	QTRAP 5000, AB Sciex	**0.60**	0.03
4. Wang et al., 2014[10]	API 5500, AB Sciex	**0.75**	0.03
5. Søeborg et al., 2013[6]	TSQ Vantage, Thermo Scientific	**1.10**	0.10
6. Büttler et al., 2015[11]	Xevo TQS, Waters	**1.16**	0.10
7. Presented data, 2015	**Q Exactive Focus MS, Thermo Scientific**	**1.45**	0.13
8. Rhea et al., 2013[9]	QTRAP 4000, AB Sciex	**1.60**	0.06
9. Koal et al., 2012[12]	QTRAP 4000, AB Sciex	**2.00**	0.03
10. Keski-Rahkonen et al., 2011[13]	Agilent 6410, Agilent	**2.60**	0.08
11. Kyriakopoulou et al., 2013[14]	QTRAP 4000, AB Sciex	**3.00**	0.08
12. Ke et al., 2014[4]	QTRAP 6500, AB Sciex	**3.75**	0.17
13. Koren et al., 2012[5]	QTRAP 5500, AB Sciex	**4.00**	0.35

(*): according to a 100% extraction yield
(**): lower limit of quantification

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