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Development and validation of a testosterone assay using liquid chromatography tandem mass spectrometry without derivatization

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Analysis of testosterone is helpful for investigation of several conditions such as hypogonadism or limited testis function in man, hirsutism hyperandrogenism or polycystic ovarian syndrome in women, and early or late onset of puberty in boys. Unfortunately the analytical performance of the commonly used testosterone immunoassays is limited in terms of sensitivity and specificity for analysis of low testosterone concentrations that are normally found in females and children. Since improved sensitivity and specificity in the low testosterone concentration range has been reported for testosterone assays using liquid chromatography tandem mass spectrometry (LC-MS/MS), we intended to set-up such an assay (1-5). Our goal was to develop a method applicable for routine testing, and although methods that utilize testosterone derivatization generally result in even higher testosterone assay sensitivity, we choose to avoid derivatization in order to simplify sample preparation.

Methods

Sample preparation

Patient samples were obtained by venous phlebotomy using serum BD vacutainer coagulation tubes and serum was obtained after centrifugation.

50 µL of internal standard solution (2000 ng/dL d3-testosterone in methanol; Sigma-Aldrich) was added to 200 µL of quality control, calibrator or patient sample and incubated for 20 minutes at room temperature (RT). Next, samples were extracted for 30 minutes at RT using 1 mL of methyl t-butyl ether. After transferring the organic phase to new vials and evaporation of the solvent, the residue was reconstituted in 150 µL water-methanol solution (1:1 v/v).

LC-MS/MS

LC was run on a Shimazu HPLC system consisting of two pumps, auto-sampler and column oven. 30 µL of sample was injected and separation was performed using a Kinetex reverse phase C18 column (2.6 µm, 100 x 3 mm, Phenomenex) kept at 40˚C. The flow-rate was kept constant at 0.45 mL/min and 30% mobile phase A (0.1% formic acid in water) and 70% mobile phase B (0.1% formic acid in methanol) was used as starting liquid phase condition. After 1 minute, mobile phase B was increased linearly to 95% in 2 minutes and left at 95% for another 1.5 minutes. Thereafter the system was reset at starting condition and allowed to equilibrate for 2 minutes. The total run time was 5.5 minutes.

MS/MS analyses were performed on an API 5000 (AB Sciex). Positive mode electrospray ionization (ESI, Turbospray) was applied. The ion-source settings were: Curtain gas 40, CAD 9, GS1 50, GS2 50, Temperature 650˚C and ion source 3500 V.

Sample analysis was performed using multiple reaction monitoring (MRM) with a dwell time of 50 ms. The 289.4/97.1 and 289.4/109.1 transitions were used to monitor testosterone and the 292.4/97.0 and 292.4/109.2 transitions for d3-testosterone. The first was used as IS for all testosterone concentration calculations. N_2 was used as collision gas and declustering potential, entrance potential, cell entrance potential and collision cell exit potential settings were optimized for each transition.

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Quantification
Quantification was performed using a calibration curve containing 0, 0.175, 0.7, 1.75, 7.0 and 35 nmol/L testosterone standards (Cerilliant). Standards were prepared by addition of 20 µL testosterone standard solution prepared in methanol, to 180 µL of double charcoal stripped serum.
Peak area of both testosterone and d3-testosterone was used for quantification and (linear) calibration curves were generated using a $1/x^2$ weighing to ensure accuracy at lower concentrations. Quantification was performed for both testosterone transitions in order to exclude assay interference.

Assay validation
For initial analytical validation of the testosterone assay, the CLSI EP10 protocol was performed. Linearity was investigated using the CLSI EP6 protocol. The lower limit of quantification (LLOQ) was performed by repeated inter-assay measures of low samples (n=5) and defined as the lowest concentration resulting in CV<20%.
For investigation of specificity, the zero calibrator, 0.82 nmol/L (23.5 ng/dL) and 5.85 nmol/L (167 ng/dL) testosterone standards were spiked with several drugs and structurally related compounds.

Method comparison
25 patient samples, male and female, collected in proper collection tubes were used. All these patients were on mycophenolic acid therapy. The LC-MS/MS-assay was compared to Centaur testosterone immunoassay (Siemens, n=25).

Results
ESI was chosen over APCI as ionization source since the testosterone baseline for a blank sample was found to be the clearest. When various collection tubes were tested, severe interference was observed for some of them (6).
Total assay imprecision obtained using the CLSI EP10 protocol for the three different levels were; 5% at 0.47 nmol/L, 3.6% at 3.75 nmol/L and 3% at 7.0 nmol/L. The LLOQ was found to be 70 pmol/L (2 ng/dL) and linearity was confirmed (r^2=0.999). Samples were found to be stable for at least two weeks when kept at 4˚C (n=3) and prepared sample extracts were stable for at least nine days when kept at 4˚C.

Table 1. Assay interference tested for selected drugs, endogenous compounds and steroids

Compound tested	Concentration tested (ng/dL)	Interference	Peak at other RT	Effect on (testosterone)
Amoxicillin	750	No	No	None
Ascorbic acid	2500	No	No	None
Caffeine	2500	No	No	None
Chloroamphenicol	2500	No	No	None
Cyclosporin A	2500	No	No	None
Dexamethason	2500	No	No	None
Digoxin	1041	No	No	None
Esomeprazole	2500	No	No	None
Furosemide	2500	No	No	None
Ibuprofen	2500	No	No	None
Predisone	2082,5	No	No	None
Predisolone	2082,5	No	No	None
Spironalactone	2082,5	No	No	None
Creatinine	2082,5	No	No	None
Uric acid	2500	No	No	None
11-deoxycortisol	250	No	No	None
17OH-Pregnenolone	2500	No	No	None
17OH-Progesterone	25000	**Yes**	Co-eluting	+175 pmol/L (5 ng/dL)
17OH-Progesterone	6250	**Yes**	Co-eluting	+35 pmol/L (1 ng/dL)
17OH-Progesterone	375	No	No	None
17OH-Progesterone	37,5	No	No	None
21-OH-Progesterone	2500	No	No	None
Aldosterone	2500	No	No	None
Androstenedione	250	No	**Yes**	None
Corticosterone	2500	No	No	None
DHEA	2500	No	**Yes**	None
DHEAS	2500	No	No	None
Dihydrotestosterone	250	No	No	None
Epi-testosterone	250	No	**Yes**	None
Estradiol (E2)	2082,5	No	No	None
Estriol (E3)	2500	No	No	None
Estrone (E1)	1250	No	No	None
Progesterone	2500	No	No	None

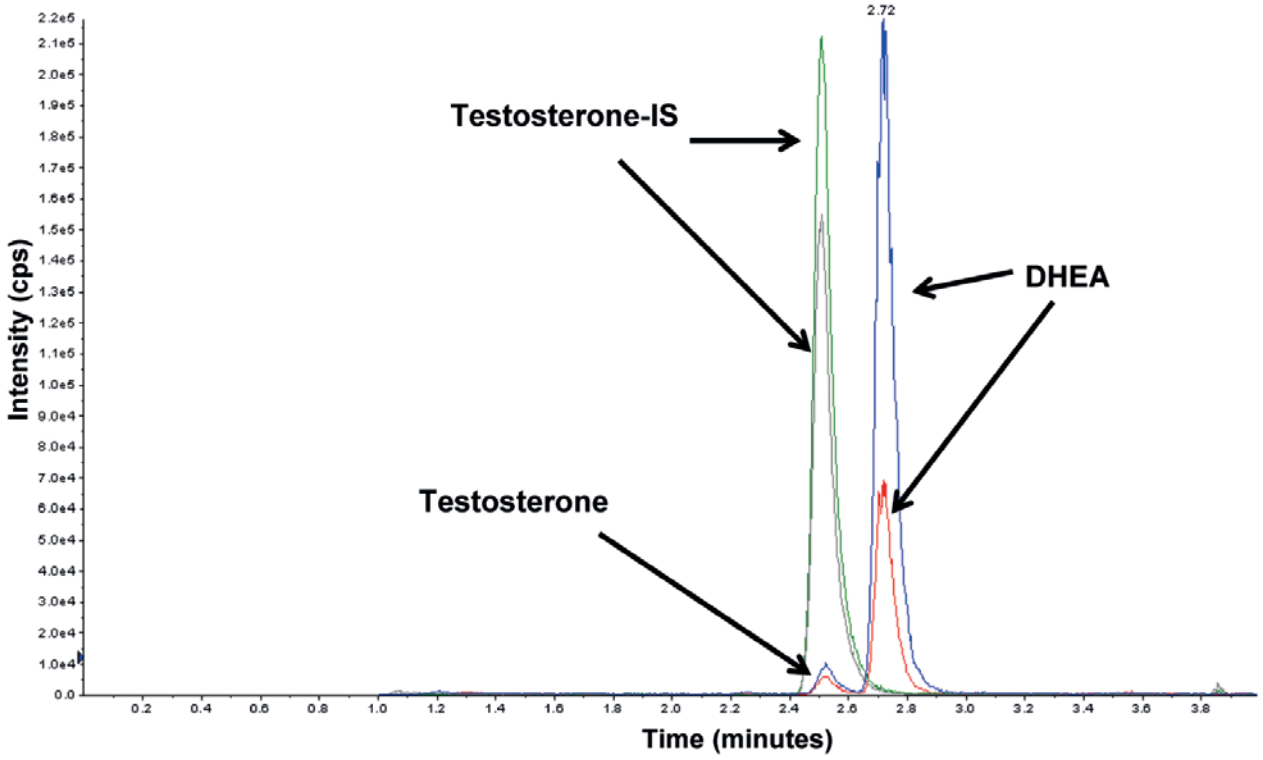

Figure 1. Chromatogram of a patient sample containing 0.82 nmol/L testosterone and spiked with 87 nmol/L (2500 ng/dL) DHEA (dehydroepiandrosterone). Blue: testosterone 289.4->97.1 transition, red: testosterone 289.4->109.1 transition, green: testosterone-d3 292.4->97.0 transition, grey: d3-testosterone 292.4->109.2 transition.

For the correlation experiment, eight female samples were below the analytical measurement range of the immunoassay, but could be quantified using our LC-MS/MS testosterone assay. An acceptable correlation with immunoassay was observed (r^2=0.94, slope=0.75). Eight female samples measurable by LC-MS/MS but with immunoassay results below the lower limit of detection were excluded from the correlation.

Specificity

An overview of compounds tested for interference is shown in the table.

17-OH-progesterone concentrations ≥190 nmol/L, did interfere with the testosterone quantification. These high concentrations are well above the upper limit of normal and could be recognized by peak broadening of the testosterone peak. We have not seen this interference in any of the patients tested thus far.

Conclusion

Measurement of low testosterone concentrations in pediatric and female samples is analytically challenging. LC-MS/MS has proven to allow superior sensitivity and specificity compared to the automated immunoassay platforms (1). In this respect a LC-MS/MS based testosterone assay was developed that does not require analyte derivatization. Attention should be paid on the use of correct collection tubes, sample handling vials and the potential interference of highly elevated 17-OH-progesterone concentrations (6). The method developed proved to be precise, linear and was not prone to interference by the drugs tested or physiological concentrations of endogenous compounds.

In conclusion, a rapid testosterone assay was developed that has proven to be superior to the Centaur immunoassay for the quantification of female testosterone concentrations and is useful for the measurement of low testosterone concentrations.

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