ORIGINAL ARTICLE

NEW LC-MS/MS METHOD FOR TESTOSTERONE AND DIHYDROTESTOSTERONE ANALYSIS IN MALE RAT PLASMA

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Abstract

A new LC-MS/MS method for testosterone and dihydrotestosterone determination in male rat plasma is proposed. After a simple protein precipitation, the compounds were separated on a C18 column with 1 mM ammonium formate solution and methanol in gradient elution at 40°C and detected by an ion trap mass spectrometer after positive electrospray ionisation. The monitoring ions were m/z 97.2, 109.2, 253.2 for testosterone and m/z 255.2 for dihydrotestosterone. The linearity domain was established between 1.0 and 64 ng/mL for both compounds. The ranges of values for within- and inter-day accuracy and precision were 89 - 111% and 2 - 13%, respectively. The proposed method is suitable for research purpose such as studies regarding the influence of aromatase inhibitors on male rat plasma levels of testosterone and dihydrotestosterone.

Rezumat

Lucrarea propune o nouă metodă LC-MS/MS de determinare a testosteronei și dihidrotestosteronei în plasma de șobolan mascul. După precipitarea proteinelor, compuşii au fost separaţi pe o coloană C18 la 40°C prin eluţie cu gradient, folosind ca fază mobilă o soluţie formiat de amoniu 1 mM şi metanol, detecţia făcându-se prin spectrometrie de masă cu trapă ionică după ionizare pozitivă prin *electrospray*. Ionii monitorizaţi au fost m/z 97.2, 109.2, 253.2 pentru testosteronă şi m/z 255.2 pentru dihidrotestosteronă. Domeniul de liniaritate pentru ambii compuşi a fost stabilit între 1,0 şi 64,0 ng/mL. Precizia şi acurateţea în aceeaşi zi şi în zile diferite s-au încadrat în intervalele 89 - 111% şi, respectiv, 2 - 13%. Metoda propusă poate fi aplicată la studiul influenţei inhibitorilor de aromatază asupra nivelurilor de testosteronă şi dihidrotestosteronă în plasma de șobolan mascul.

Keywords: testosterone, dihydrotestosterone, rat, LC-MS/MS

Introduction

Steroids determination in humans is mainly related to the endocrine disorder investigation or doping assessments in sports. The sex hormone testosterone (TST) and its active metabolite dihydrotestosterone (DHT) are biochemical parameters which are used related to confirm TST's excess or deficiency, i.e. the diagnosis of hypogonadism, boys with delayed or precocious puberty, to monitor the testosterone replacement therapy, including supplements, or its nonmedical use (doping) [3, 5, 10, 17, 18].

The routine laboratory methods for TST and DHT determination are mainly immunological, but in the past 20 years the analysis of these hormones by chromatography coupled with mass spectrometry spread all over the world [1]. Testosterone immuno-assays require minimal human intervention, are automated and allow high-throughput analysis. However, there is increased interest for TST (and DHT, too) determination in serum with high accuracy and precision, these requirements being fulfilled by LC-MS/MS methods [6], together with other advantages: reduced matrix effect, no cross-reactivity with structurally related steroids and high sensitivity [19].

The LC-MS/MS capability in clinical laboratory was subjected to many discussions in review articles and there is no doubt about the demand for its routine use. Even today, however, there are still drawbacks to be overcome: no ready to use reagent kits (just for few biological relevant parameters); mass spectrometers should be chosen wisely in terms of desired applications due to their variation in terms of specificity, sensitivity, cost and ease of use; training and maintaining highly qualified analysts [1, 2, 6, 11].

There are many LC-MS/MS published methods for TST and DHT determination in different biological human samples. Many articles reviewed the laboratory methodologies for sex hormones assays, the overall conclusions being almost the same, LC-MS/MS is a powerful technique and it is necessary to become a routine one [1, 9, 12, 13]. In 2016, eight LC–MS/MS methods for TST determination in human serum were compared, proving the continuous

interest and progress on this topic [4]. Some methods published in literature proposed a variety of chemical derivatization procedures to achieve a higher sensitivity of the measurements, i.e. reaction schemes to introduce a proton affinitive, but this procedure enhances the complexity and duration of analysis [9]. Besides these methods, there are other dedicated to the determination of TST in rat plasma for various research purposes [7, 8, 16, 22].

Regarding the advantages of mass spectrometers, ion trap analysers have the ability to "fragment and isolate ions several times in succession before the final mass spectrum is obtained, resulting in so-called MS^n capabilities" [17]. Due to this ability, ion trap analysers proved to be suitable for analysing compounds with cyclopentanoperhydro-phenanthrene nucleus i.e. steroidal structures like spironolactone, canrenone (the active metabolite of spironolactone) or progesterone [14, 21].

The aim of the present study was to propose a new, simple and fast LC-ion trap mass spectrometric assay of testosterone and dihydrotestosterone in male rat plasma after simple protein precipitation which minimizes the complexity and facilitates the automation.

Materials and Methods

Chemicals and biological matrix

Testosterone and dihydrotestosterone solution 1 mg/mL were the reference standards obtained from Sigma-Aldrich. Methanol, acetonitrile, formic acid, and ammonium formate were Sigma-Aldrich and Merck products. Pure water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) system. The blank plasma of male rats was supplied by the Experimental Department on Animal of the University of Medicine and Pharmacy from Târgu Mureş, Romania. The study was approved by the research ethics committee of the same institution.

Standard solutions

A quantity of 3.3 mg TST, accurately weighted on an Analytical Plus balance (Ohaus Corporation), were dissolved and adjusted to 10 mL with methanol. The already prepared standard solution of 1 mg/mL DHT was used as initial solution. After further dilutions, two working solutions of 201 ng/mL TST and 200 ng/mL DHT were obtained in order to be used for the preparation of calibration and quality control (QC) standards. Series of seven calibration standards, ranging from 1.0 to 64.3 ng/mL, and four QC solutions, with the concentrations 1.0 ng/mL, 2.0 ng/mL, 8.0 ng/mL and 32.2 ng/mL, respectively, for both analytes, were obtained.

HPLC and mass spectrometry conditions

The chromatographic system consisted of an 1100 series model HPLC equipment (Agilent Technologies, USA) with binary pump, solvent degasser, autosampler with controlled temperature, column thermostat, and an Ion Trap VL mass spectrometer detector (Brucker Daltonics GmbH, Germany). Data acquisition and processing were performed using QuantAnalysis software. The detection of the analyte was in MS2 mode after positive electrospray ionisation. The ionisation source parameters were: capillary voltage 4500 V, nebulizer 60 psi, dry gas 12 L/min, dry temperature 350°C. The mass spectrometer parameters: skimmer 55 V, capillary exit 75 V, Oct 1 DC 6.4 V, Oct 2 DC 1.5 V, trap drive 35. The analytes were detected by monitoring the sum of ions from the following transitions: TST m/z 97.2, 109.2, 253.2 from m/z 289.2; DHT m/z 255.2 from m/z 291.2.

Chromatographic separation was performed at 40°C on a Zorbax SB-C18 100 x 3 mm, 3.5 μm column (Agilent Technologies), protected by an in-line filter. The mobile phase consisted of a mixture of ammonium formate 1 mM (phase A) and methanol (phase B) and it was delivered with the flow rate of 0.9 mL/min. A gradient elution was used: 0 - 4 min 44% A → 32% A; 4 - 4.6 min 32% A; 4.6 - 4.61 min 32% A → 44% A; 4.61 - 5.5 min 44% A.

Sample preparation

The plasma samples were prepared as follows: a volume of 0.3 mL plasma was precipitated with 0.1 mL solution 7% $HClO_4$. The mixture was vortex-mixed for 10 seconds, then centrifuged for 5 minutes at 12,000 rpm. A volume of 80 μL was injected into the HPLC system.

Specificity, linearity, accuracy and precision

In order to verify the specificity of TST and DHT signals against the other endogenous plasma compounds, blank plasma from rats after castration was used.

The external standard calibration method was applied using singlicate calibration standard and the calibration curve model was determined by the least squares analysis. A linear calibration function was applied: $y = ax + b$, $1/y^2$ weight, where y – peak area and x – concentration. The calibration model was accepted if the following cumulative conditions were accomplished: random distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration); at least 2/3 of the standards, including the highest and lowest calibration levels, had the residuals within ± 20% interval for the lowest level and within ± 15% for the other concentrations, the conditions being compulsory for both analytes. The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%.

The intra- and inter-day precision (expressed as coefficient of variation, CV%) and accuracy (the ratio between obtained and theoretical concentrations, Re%) of the assay procedure were determined by

analysis on the same day of three replicates at each of the lowest (1 ng/mL), lower (2 ng/mL), medium (8 ng/mL), and higher (32 ng/mL) levels of TST and DHT concentrations and one replicate on three different occasions, respectively.

Results and Discussion

The aim of the study was to propose a fast and reliable method for TST and DHT determination in rat plasma by using LC-MS/MS analysis with ion trap detection. Taking in account the limitation in terms of sensitivity of the ion trap mass spectrometer in comparison with other more powerful mass spectrometers such as triple quadrupole or hybrid triple quadrupole time of flight, two ionisation sources were tested and maximum fragmentation signals were assigned and used for acquisition. The simple protein precipitation was chosen to acquire a high-speed and low cost sample preparation.

Selecting the ionisation source

Two ionisation sources, electrospray ionisation ESI and atmospheric chemical ionisation APCI, were tested in terms of sensitivity. As it is well known, ESI has a large applicability, including molecules which cannot be ionized by other techniques, but signal suppression due to matrix interferences is more common in this case. APCI is not so sensitive to matrix effects as ESI and it is used to analyse smaller, thermally stable polar and non-polar compounds. The tests were conducted in positive mode, on the same column, at the same temperature and by changing the type of mobile phase. The composition of the aqueous and organic mobile phase was adjusted in order to have the same retention time of the analytes for a better comparison. In each case, the source and mass spectrometer parameters were optimised.

As it can be seen in Table I, DHT is almost undetectable using the APCI source. In TST molecule, the double bond carbon-carbon is separated from the carbonyl group by one simple carbon-carbon bond, thus its influence on its electron distribution is obvious. The common fragmentation behaviour of TST includes a positive charge at the oxo group followed by fragmentation to m/z 97 and 109 [15]. The presence of the carbonyl group in testosterone structure is related to its biological activity and the 3-keto group is responsible for the interaction with the receptor through hydrogen bonds [20]. A possible explanation regarding the low ionisation behaviour of DHT could be related to the absence of the double carbon-carbon bond in α-β position from carbonyl group.

The best detection for both compounds was obtained using the ESI source, by using ammonium formate 1 mM and methanol as mobile phase constituents.

Table I

Comparison of the ionisation sources for TST detection in terms of signal heights, 10^5 units (tested solution 100 ng/mL)

Aqueous phase	Organic phase	ESI		APCI	
		TST	DHT	TST	DHT
Formic acid 0.1%	Methanol	1.20	0.62	1.5	0.11
Formic acid 0.1%	Acetonitrile	0.92	0.39	1	0
Ammonium formate 1 mM	Methanol	2.23	0.83	1.25	0.16
Ammonium formate 1 mM	Acetonitrile	0.92	0.68	0.8	0

Under the final chromatographic and mass spectrometry conditions, previously presented, the retention times of TST and DHT were 4.0 min and 5.0 min, respectively, with a total runtime of 5.5 min.

Specificity, linearity, accuracy and precision

The method proved to be specific as it can be seen in Figures 1, 2 and 3. No interference from rat plasma after castration was detected at the retention times of TST and DHT.

The linearity domain was established between 1.0 and 64.3 ng/mL for TST and between 1.0 and 64 ng/mL for DHT, with the LLOQ of 1 ng/mL for both compounds. The average calibration curves were: a) TST $y = 84373(\pm 5135)X + 26302(\pm 13626)$, $R^2 > 0.994$; b) DHT $y = 8178(\pm 1094)X - 1420.6(\pm 3616)$, $R^2 > 0.996$, for N = 7 calibration levels, n = 3 replicates in different days, weighting $1/Y^2$. All the residuals were within 85-115% limits, except one replicate for TST.

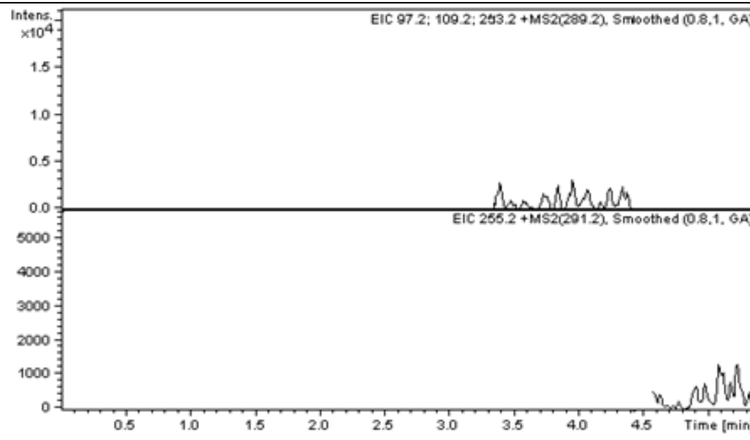

Figure 1.
Chromatogram of male rat plasma after castration
(upper image – TST chromatogram, lower image, DHT chromatogram)

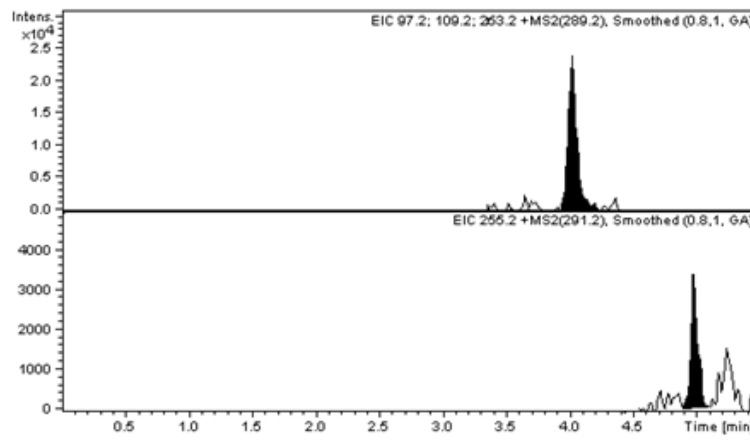

Figure 2.
Chromatogram of a standard solution
(upper image – TST chromatogram, lower image, DHT chromatogram)

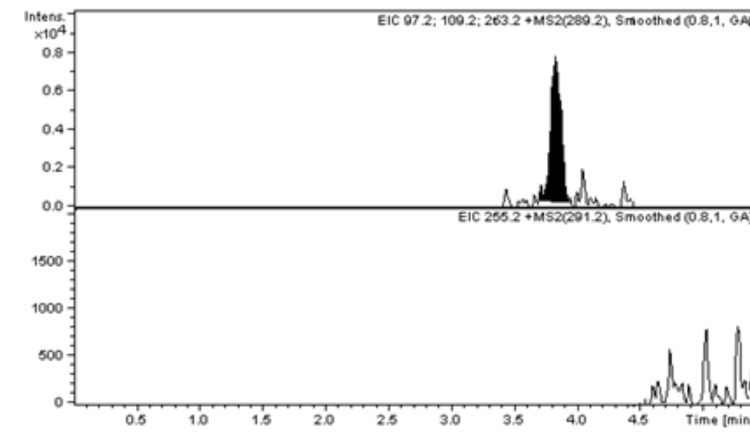

Figure 3.
Chromatogram of normal male rat plasma
(upper image – TST chromatogram, lower image, DHT chromatogram)

The method was accurate and precise (Tables II and III). Within-day accuracy and precision ranges were 92 - 110.97% and 2.27 - 11.69%, respectively. For inter-day accuracy and precision the limits were 89.26 - 107.29% and 2.98 - 13.28%, respectively.

Table II

Within-day accuracy and precision, n = 3

TST				DHT			
C_t, ng/mL	Mean C_f, ng/mL	Re%	CV%	C_t, ng/mL	Mean C_f, ng/mL	Re%	CV%
1.01	0.93	92.00	7.35	1	0.93	92.55	8.00
2.01	1.97	98.24	9.77	2	2.03	101.34	3.11
8.04	7.92	98.51	9.86	8	7.80	97.44	11.69
32.16	35.69	110.97	2.27	32	30.30	94.70	7.08

Table III

Inter-day accuracy and precision, n = 3

TST				DHT			
C_t, ng/mL	Mean C_f, ng/mL	Re%	CV%	C_t, ng/mL	Mean C_f, ng/mL	Re%	CV%
1.01	1.01	100.34	4.45	1	0.92	92.14	2.98
2.01	2.16	107.29	3.05	2	1.86	92.81	5.74
8.04	8.38	104.19	13.28	8	7.14	89.26	5.26
32.16	33.65	104.64	9.59	32	31.08	97.13	4.37

Drawbacks

The high injection volume of acidic sample, necessary for a reasonable sensitivity, influences the column stability. Therefore, the column should be washed more often. On the other hand, the proposed method has a limit of quantification which makes it unsuitable for plasma levels below 1 ng/mL, i.e. female rat plasma.

Conclusions

The proposed method allows the determination of testosterone and dihydrotestosterone in male rat plasma after simple protein precipitation. The optimised LC-MS/MS conditions are suitable for research purpose analysis such as the study regarding the influence of aromatase inhibitors on TST and DHT plasma levels.

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References

1. Adaway J.E., Keevil B.G., Owen L.J., Liquid chromatography tandem mass spectrometry in the clinical laboratory. *Ann. Clin. Biochem.*, 2015; 52(1): 18-38.
2. Burcea Dragomiroiu G.T.A., Cimpoieşu A., Ginghină O., Baloescu C., Bârcă M., Popa D. E., Ciobanu A.M., Anuţa V., The development and validation of a rapid HPLC method for determination of piroxicam. *Farmacia*, 2015; 63(1): 123-131.
3. Boccard J., Badoud F., Grata E., Ouertani S., Hanafi M., Mazerolles G., Lantéri P., Veuthey J.L., Saugy M., Rudaz S., A steroidomic approach for biomarkers discovery in doping control. *Forensic Sci. Int.*, 2011; 213: 85-94.
4. Büttler R.M., Martens F., Ackermans M.T., Davison A.S., van Herwaarden A.E., Kortz L., Krabbe J.G., Lentjes E.G.W., Comparison of eight routine unpublished LC–MS/MS methods for the simultaneous measurement of testosterone and androstenedione in serum. *Clin. Chim. Acta*, 2016; 454: 112-118.
5. Gosetti F., Mazzucco E., Gennaro M.C., Marengo E., Ultra high performance liquid chromatography tandem mass spectrometry determination and profiling of prohibited steroids in human biological matrices. A review. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 2013; 927: 22-36.
6. Grebe S.K.G., Singh R.J., LC-MS/MS in the Clinical Laboratory–Where to From Here?. *Clin. Biochem. Rev.*, 2011; 32: 5-31.
7. Han J., Zhang S., Liu W., Leng G., Sun K., Li Y., Di X., An analytical strategy to characterize the pharmacokinetics and pharmacodynamics of triptorelin in rats based on simultaneous LC-MS/MS analysis of triptorelin and endogenous testosterone in rat plasma. *Anal. Bioanal. Chem.*, 2014; 406: 2457-2465.
8. Higashi T., Ninomiya Y., Iwaki N., Yamauchi A., Takayama N., Shimada K., Studies on neurosteroids XVIII: LC–MS analysis of changes in rat brain and serum testosterone levels induced by immobilization stress and ethanol administration. *Steroids*, 2006; 71: 609-617.
9. Higashi T., Ogawa S., Chemical derivatization for enhancing sensitivity during LC/ESI-MS/MS quantification of steroids in biological samples: a review. *J. Steroid Biochem. Mol. Biol.*, 2016; 162: 57-69.
10. Iliescu R., Hăncianu M., Reckelhoff J., Testosterone supplements accelerate progression of kidney injury in a rat model of reduced renal mass. *Farmacia*, 2014; 62: 119-128.
11. Keevil B.G., LC-MS/MS analysis of steroids in the clinical laboratory. *Clin. Biochem.*, 2016, 49(13-14): 989-997.
12. Ketha H., Kaur S., Grebe S.K., Singh R.J., Clinical applications of LC-MS sex steroid assays: evolution of methodologies in the 21st century. *Curr. Opin. Endocrinol. Diabetes Obes.*, 2014; 21: 217-226.

13. Leung K.S.Y., Fong B.M.W., LC–MS/MS in the routine clinical laboratory: has its time come?. *Anal. Bioanal. Chem.*, 2014; 406: 2289-2301.
14. Mihu D., Vlase L., Imre S., Mihu C.M., Achim M., Muntean D.L., New LC/MS method for determination of progesterone in human plasma for therapeutic drug monitoring in pregnancy and gynecological disorders. *Stud. U. Babes. Bol. Che.*, 2009; 54(3): 151-160.
15. Nielena M.W.G., Lasaromsa J.J.P., Muldera P.P.K., Van Hendeb J., van Rhijn J.H.A., Groota M.J., Multi residue screening of intact testosterone esters and boldenone undecylate in bovine hair using liquid chromatography electrospray tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 2006; 830: 126-134.
16. Niwa M., Watanabe N., Ochiai H., Yamashita K., Determination of testosterone concentrations in rat plasma using liquid chromatography-atmospheric pressure chemical ionization mass spectrometry combined with ethyl oxime and acetyl ester derivatization. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 2005; 824: 258-266.
17. Pitt J.J., Principles and applications of liquid chromatography mass spectrometry in Clinical Biochemistry. *Clin. Biochem. Rev.*, 2009; 30: 19-34.
18. Pop A., Lupu D., Cherfan J., Kiss B., Loghin F., Evaluation of the androgenic/antiandrogenic activity of selective serotonin reuptake inhibitors. *Farmacia*, 2015; 63(3): 343-348.
19. Settlage J., Oglesby T., Rajasekaran A., Williard C., Scott G., The importance of chromatographic resolution when analyzing steroid biomarkers. *Steroids*, 2015; 99: 45-48.
20. Takahata Y., Vendram R., Ionization energies, electron affinities and excitation energies of some steroid hormones calculated with the semiempirical HAM/3 method. *J. Braz. Chem. Soc.*, 2001; 12: 165-172.
21. Vlase L., Imre S., Muntean D., Achim M., Muntean D.L., Determination of spironolactone and canrenone in human plasma by high-performance liquid chromatography with mass spectrometry detection. *Croat. Chem. Acta*, 2011; 84(3): 361-366.
22. Zhang S., Han J., Leng G., Di X., Sha C., Zhang X., Li Y., Liu W., An LC-MS/MS method for the simultaneous determination of goserelin and testosterone in rat plasma for pharmacokinetic and pharmacodynamic studies. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 2014; 965: 183-189.