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GC/HRMS Determination of the Serum (Plasma) Testosterone / 17α-Hydroxyprogesterone Ratio and Its Significance

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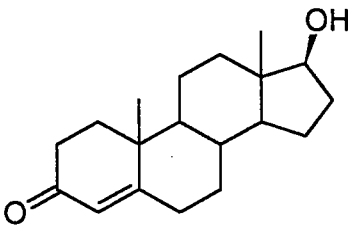
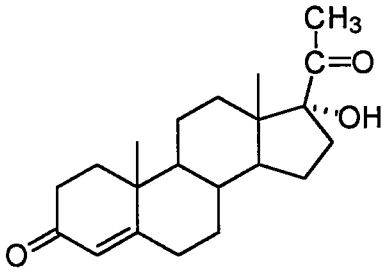
Introduction

This report is a follow up of the paper entitled "Steroid Profiling in Human and Horse Blood: Some Results" and discusses the determination of testosterone and one of its biosynthetic precursors, 17α-hydroxyprogesterone.



Testosterone



17a-hydroxyprogesterone

17α-hydroxyprogesterone is an intermediate in the bioconversion of C_{21} into C_{19} steroids and in males is largely produced by testicular secretion. In recent work by Carlström and coworkers [1,2] it was shown that the serum level ratio of testosterone to 17α-hydroxyprogesterone (T/17OHP) can be used as a marker for testosterone doping and/or doping with anabolic steroids. The serum T/17OHP ratio can also be used as an indicator for testosterone doping in cases where high urinary testosterone to epitestosterone (T/E) ratios are measured. This is especially useful in cases in which the high T/E ratio is due to an abnormally low epitestosterone concentration.

Here we report our first results for blood steroid profiles of 17α-hydroxyprogesterone and testosterone determined for top male and female athletes at IAAF meets in the summer of 1993. The GC/HRMS analytical procedure has been described in the above mentioned article, as has the use of radio immunoassay for the determination of endogenous serum steroids. In

addition to the results obtained for the IAAF meets, data gathered from volunteers in this laboratory and from endocrinology studies are also presented.

Experimental

Sample Preparation and Selection of Internal Standards

Testosterone and 17α-hydroxyprogesterone are largely unconjugated in serum and can easily be prepared for GC/HRMS analysis by extraction in ether followed by derivitization using MSTFA/TMIS. Quantification of testosterone is performed using deuterated D_3-testosterone as an internal standard. A similarly useful internal standard for the quantification of 17α-hydroxyprogesterone would be a deuterated analogue; however, synthesis of such a standard is not straight forward. Selection of an internal standard for GC/HRMS is based on the mass of the internal standard and the substance to be quantified as well as their elution times from the GC column. The mass should be in the same range as that of the species to be quantified for analysis via electric field scanning of the mass spectrometer. With respect to the elution time, the most reliable results are obtained when the internal standard and the species of interest are in the same ion group. Oxymesterone fulfils both of these criteria and is therefore used as the internal standard for the quantification of 17α-hydroxyprogesterone.

Internal Standard Mixture Added to 1 ml of Serum	**Amount**
D_3-Testosterone	2 ng
Oxymesterone	2 ng

Table 1. Internal standards added to 1 ml of serum for the analysis of testosterone and 17α-hydroxy-progesterone.

Gas Chromatography / Mass Spectrometry

GC/HRMS was performed using a Finnigan MAT 95 double focussing mass spectrometer interfaced with a Hewlett Packard 5890 gas chromatograph. The gas chromatograph and mass spectrometer operating parameters are given in the paper entitled "Steroid Profiling in Human and Horse Blood: Some Results". In Table 2 the exact masses of the ions which are used to monitor the derivatized steroids are listed together with their temperature programmed Kovat indices.

STEROID	MASS MONITORED	KOVAT INDEX
testosterone bis-TMS	432.2879 amu	2660
D_3-testosterone bis-TMS	435.3068	2658
oxymesterone tris-TMS	534.3381	2950
17α-hydroxyprogesterone tris-TMS	546.3380 531.3145	3007

Table 2. Endogenous steroids, internal standards and the exact ion masses of their per-TMS derivatives (molecular ion and abundant fragment ion) recorded in GC high-resolution selective ion monitoring (GC/HRSIM). Also included are temperature programmed Kovat indices (185° C for 0 min, then 5° C/min to 320 ° C).

For 17α-hydroxyprogesterone tris-TMS, the molecular ion and the $[M^{+\cdot} - 15]$ fragment ion, m/z 531.3145 amu, are monitored since in some cases a substance coelutes with the TMS derivative, influencing the response of the molecular ion. The ratio of the fragment ion to the molecular ion is used as a criteria to determine whether or not 17α-hydroxyprogesterone can be quantified in the sample.

Results and Discussion

Serum Steroid Profiles in Humans

Serum concentrations of unconjugated testosterone and 17α-hydroxyprogesterone have been determined for male and female volunteers from this laboratory and some of the results are listed in Table 3. In males, the serum testosterone concentration lies around 4 ng/ml and the 17α-hydroxyprogesterone levels range from 1 ng/ml to 2 ng/ml. For females the testosterone levels are much lower than for males, about 0.5 ng/ml; however, the 17α-hydroxyprogesterone levels in females are similar to those found in males. The serum ratio of testosterone to 17α-hydroxyprogesterone (T/17αP) is consistently smaller for females than for males and range from values of 0.24 to 4.75. For the males the T/17αP ratio is about 3.

Analyses were also performed on serum samples taken from top male and female athletes during four IAAF meets in Europe in the summer of 1993. The samples were also analyzed using radio immunoassay in the Doping Laboratory at the Aker Hospital, Oslo, Norway. The GC/HRMS serum testosterone and 17α-hydroxyprogesterone concentrations are summarized in Tables 4 and 5.

TESTOSTERONE AND 17α-HYDROXYPROGESTERONE SERUM CONCENTRATIONS			
GENDER	**T**	**17αP**	**T / 17αP**
F	0.53	2.22	0.24
F	0.44	0.39	1.13
F	0.64	1.46	0.44
F	0.75	2.27	0.33
M	3.16	2.13	1.49
M	3.77	0.95	3.95
M	3.73	1.47	2.54
M	5.86	1.23	4.75

Table 3. Serum concentrations of unconjugated testosterone and 17α-hydroxyprogesterone [ng/ml] in male and female volunteers from this laboratory determined using GC/HRMS. Testosterone (T), 17α-hydroxyprogesterone (17αP), T/17αP ratio of testosterone to 17α-hydroxyprogesterone.

The unconjugated testosterone and 17α-hydroxyprogesterone serum levels in the female athletes, see Table 4, were near the values determined for the female volunteers from this laboratory. The range of testosterone levels in the female athletes is quite large (0.2 ng/ml to 2.1 ng/ml) compared to the female volunteers (0.44 ng/ml to 0.75 ng/ml). The 17α-hydroxyprogesterone serum levels in the female volunteers was consistently higher than the levels determined for the female athletes. The ratio T/17αP for both groups of females was quite similar and varied over a wide range (0.2 to 1.8). One female athlete had a high T/17αP ratio value of 4, which was due to a very low serum concentration of 17α-hydroxyprogesterone.

TESTOSTERONE AND 17α-HYDROXYPROGESTERONE SERUM CONCENTRATIONS IN FEMALE ATHLETES AT IAAF MEETS IN 1993				
EVENT	**Nr.**	**T**	**17αP**	**T / 17αP**
Brussels	A75	0.20	0.19	1.1
Zurich	23	0.28	0.33	0.85
Brussels	A81	0.36	0.09	4.0
Brussels	A80	0.37	0.23	1.6
Brussels	A87	0.39	0.41	0.95
Zurich	14	0.40	1.27	0.31
Berlin	33	0.41	0.38	1.1
Berlin	31	0.43	1.11	0.39
Berlin	38	0.56	0.63	0.89
Oslo	48	0.60		
Zurich	15	0.63	0.95	0.66
Berlin	32	0.72	1.92	0.38
Oslo	42	0.85		
Oslo	30	0.90		
Oslo	36	1.11		
Berlin	28	1.23	0.67	1.8
Oslo	31	1.34		
Brussels	A84	1.57		
Oslo	28	2.10		

Table 4. Serum levels of unconjugated testosterone and 17α-hydroxyprogesterone [ng/ml] in female athletes determined using GC/HRMS. Testosterone (T), 17α-hydroxyprogesterone (17αP), T/17αP ratio of testosterone to 17α-hydroxyprogesterone. Note that at the Oslo event, 17α-hydroxyprogesterone was not determined by GC/HRMS.

The unconjugated testosterone serum levels in the male athletes, see Table 5, were spread over a wide range (0.5 ng/ml to 12.5 ng/ml), whereas the values determined for male volunteers from this laboratory varied only over a small range (3.2 ng/ml to 5.9 ng/ml). Interestingly, the 17α-hydroxyprogesterone levels in the male volunteers were consistently higher than for the male athletes. As a result of the low 17α-hydroxyprogesterone levels, the male athletes often have very high T/17αP ratios. Approximately half the male athletes have a T/17αP ratio between 5 and 10, more than one third have a T/17αP ratio of 20 or larger, and only a very small number of male athletes have a T/17αP ratio lower than 5.

TESTOSTERONE AND 17α-HYDROXYPROGESTERONE SERUM CONCENTRATIONS IN MALE ATHLETES AT IAAF MEETS IN 1993				
EVENT	**Nr.**	**T**	**17αP**	**T/17αP**
Zurich	13	0.53	0.95	0.56
Brussels	A73	0.55	0.13	4.23
Zurich	22	1.12	0.20	5.6
Zurich	20	1.36	0.29	4.7
Brussels	A85	1.48	0.03	49.3
Zurich	16	1.50	0.20	7.5
Brussels	A78	1.59	0.04	39.8
Zurich	27	1.69	0.34	5.0
Brussels	A92	1.74	0.28	6.2
Oslo	49	2.31		
Zurich	17	2.52		
Zurich	26	2.72		
Berlin	39	2.77	0.82	3.4
Oslo	40	2.91		
Zurich	21	2.99		
Berlin	35	3.00	0.51	5.9
Berlin	34	3.32	0.76	4.4
Brussels	A91	3.34	0.22	15.2
Oslo	34	3.35		
Berlin	29	3.42	0.60	5.7
Berlin	37	3.66	0.80	4.6
Berlin	36	3.98	0.64	6.2
Zurich	24	4.02		
Berlin	30	4.34	0.47	9.4
Oslo	46	4.44		
Zurich	19	4.49	0.22	20.4
Oslo	44	4.59		
Brussels	A72	6.42	0.16	40.1
Zurich	18	6.79	0.38	17.8
Oslo	32	8.74		
Zurich	25	12.49	0.66	18.9

Table 5. Serum levels of unconjugated testosterone and 17α-hydroxyprogesterone [ng/ml] in female athletes determined using GC/HRMS. Testosterone (T), 17α-hydroxyprogesterone (17αP), T/17αP ratio of testosterone to 17α-hydroxyprogesterone. Note that at the Oslo event, 17α-hydroxyprogesterone was not determined by GC/HRMS.

Steroid Profiles in Human Serum - An Endocrinology Study

Serum testosterone and 17α-hydroxyprogesterone levels were also determined for male individuals as a part of an endocrinological study. One sample was submitted in a forensic investigation. As seen in Table 6, this individual had an extremely high blood testosterone level, 28.2 ng/ml, which is more than 6 times the normal value. The remaining candidates had consistently high urinary T/E ratios, assumed to result from low epitestosterone excretion. One individual also participated in a test using ketoconazole, a cytochrome P_{450} inhibitor which suppresses testicular testosterone production [3,4]. All of the test candidates had normal testosterone serum levels and the ratio T/17αP was within the normal range. The testosterone serum level following application of ketoconazole was strongly suppressed. Interestingly in this individual the 17α-hydroxyprogesterone level was also strongly suppressed following ketoconazole administration.

TESTOSTERONE AND 17α-HYDROXYPROGESTERONE SERUM LEVELS IN MALES - AN ENDOCRINOLOGICAL STUDY			
SAMPLE	**T**	**17αP**	**T/17αP**
Forensic Study	28.2	0.25	113
Low Urinary T/E	4.44	1.76	2.52
Low Urinary T/E	5.04	14.47	0.35
Low Urinary T/E	2.14	0.67	3.21
Subject Prior to Ketoconazole Application	5.37	16.50	0.33
Subject Following Ketoconazole Application	2.34	2.13	1.10

Table 6. Serum levels of unconjugated testosterone and 17α-hydroxyprogesterone [ng/ml] in males determined using GC/HRMS. Testosterone (T), 17α-hydroxyprogesterone (17αP), T/17αP ratio of testosterone to 17α-hydroxyprogesterone.

Conclusion

Using GC/HRMS it is possible to determine serum testosterone and 17α-hydroxy-progesterone levels. This preliminary study has investigated serum levels of these steroids in male and female volunteers from this laboratory and from top male and female athletes participating in IAAF meets in the summer of 1993. In the female groups there was little difference between serum testosterone levels. The serum 17α-hydroxyprogesterone levels, however, were somewhat higher for the female volunteer than for the female athletes. The T/17αP ratios were similar in both groups and ranged between 0.2 and 1.8. Testosterone serum levels in the male athletes were spread over a wide range (0.5 ng/ml to 12.5 ng/ml), in contrast to the values determined for male volunteers in this laboratory which varied over a narrow range (3.2 ng/ml to 5.9 ng/ml). The T/17αP ratios were widely distributed for the male athletes. More than more than one third of the male athletes had a T/17αP ratio of 20 or larger, half of the male athletes had a T/17αP ratio between 5 and 10, and only a few male athletes have a T/17αP ratio lower than 5.

A large number of endogenous steroids can be determined in human blood. Further work, which is in progress, includes a wider spectrum of steroids, which in turn provides a more complete steroid profile. In addition, extensions of these studies are being made towards the detection of steroids in horse blood.

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