THE ALTERATION OF THE URINARY STEROID PROFILE UNDER THE STRESS

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ABSTRACT: In the second part of twentieth century anabolic-androgenic steroids were introduced into doping practice and received continuously increasing significance. In order to prove the usage of doping substances, the determination of steroid profile in the urine came into practice. Several factors may be responsible for alterations in the normal steroid profile for example age, sex and diet. The aim of this study was to find out, whether the psychological stress may cause modifications in the steroid profile and T/Et ratio. The effect of physical activity was also considered. The steroid profile was determined in the group of 34 students being in non-stress conditions and under stress immediately before an important university exam. The intensity of stress was rated by self-reported questionnaire. The GC/MS method was applied to determine the steroid profile in the urine samples. The results of the experiment have shown that psychological stress may cause significant changes in the steroid profile, especially in females. Physical activity, independently of stress significantly modified the steroid profile. In summary, observed changes in steroid profile suggest, that major fluctuations of T/Et and A/E ratios under the influence of stressogenic factors and physical activity are unlikely.

KEY WORDS: doping, steroid profile, stress, physical activity

INTRODUCTION

There are several factors that could potentially alter the normal man steroid profile for example an age, sex and diet. From doping control view-point, for the adequate interpretation of the analytical results it is necessary to possess an ability of selecting adaptive changes in the body that response to physiological impulses having nothing in common with intended doping. Among the best recognized factors influencing quantitatively the androgenic profile are training loads forcing high physical activity, restricted energy intake and training specificity of a given sport discipline and interethnic variation [3,4,10,16].

Although numerous studies were conducted to evaluate the effect of various stimuli, including pre-competition stress in competing athletes, on the behavior of endogenous blood androgens in humans, the effect of stress on urinary androgen excretion is still pure recognized. It should be noticed that under stress condition or following an exertion, the level of an androgen in urine does not always closely reflects that in blood because of strong effect of mentioned stimuli on renal function. For that reason the behavior proof urinary T/Et ratio being a proof of testosterone abuse by athletes may fluctuate dependently on external conditions. This problem is an important one, when anti-doping controls are carried out among competitors exposed to stressful psychological and physical challenges, which might influence on the rate of excretion urinary steroids and affect the doping markers. It is worth to note, that stress effecting on doping markers may give positive false result, as was reported by Armanini et al. The authors described a history of female athlete, Italian Olympic gold medalist, who was accused of growth hormone abuse based on very high the hormone levels in blood samples prior to competition. However, further investigations showed huge variation of endogenous growth hormone in this subject exposed to psychological stress. [1]. As to urinary T/Et status under competition stress condition the only study was carried out [6]. It was found, that pistol shooting resulted in rise of blood testosterone, whereas T/Et in urine was unchanged. Assuming that anticipatory stress in students before exams is similar to that in competitors prior to competition,the purpose of the study was to investigate the effect of highly stressogenic situation of waiting for exam on the steroid profile in the urine of students.

MATERIALS AND METHODS

The steroid profile was determined in the group of 14 female (average age 24.3 yrs) and 20 male (average age 25.2 yrs) students of Pharmaceutical Faculty of Medical University of Warsaw. The urine samples were collected twice: firstly 30 minutes before highly stressogenic exam and secondly one month later providing the tested persons were refreshed and relaxed. Each time the self-reported questionnaire was completed. The inquiry comprised questions on physical activity, the degree of stress (4 point scale) during sample collection, the quality of sleep during preceding night, consumption of drugs and beverages, about possible casual events that could influence the results of the investigation.

The samples were analysed to procedure for anabolic agents (procedure based on procedure described by Geyer et al [5] with separation on GC/MS 6890/5973N).

The GC/MS method was applied to determine the steroid profile in the urine samples.

Instrument GC/MS Agilent Technologies 6890A/Agilent Technologies 5973N

GC column:

HP-1 (25 m length, 0.2 mm ID, 0.11 μm film thickness)

flow parameters

carrier gas: helium

flow 0.6 ml/min at 120 ºC

head pressure constant pressure mode, 14.80 psi

injector parameters

injection mode:	split 10:1
injection volume:	2 μl
injector temperature:	280 ºC

oven temperature program

initial temperature:	120 ºC
initial time:	1.11 min
rate 1:	50 ºC/min (up to 200 ºC)
rate 2:	2 ºC/min (up to 245 ºC)
rate 3:	25 ºC/min
final temperature	300 ºC
final time:	5 min

MSD parameters

ionisation mode:	EI, 70 eV
acquisition mode:	SIM
interface temperature:	280 ºC
multiplier voltage:	according to tune
Ions used to calculations [m/z]:	
11beta-OH-Androsterone	522
11beta-OH-Etiocholanolone	522
Androsterone	434
Etiocholanolone	434
D4-Etiocholanolone	438
5beta-Androstane-3beta,17beta-diol	241
5alfa- Androstane-3alfa,17beta-diol	241
5beta-Androstane-3alfa,17beta-diol	241
5alfa-Androstane-3beta,17beta-diol	241
Testosterone	432
Epitestosterone	432
D3-Testosterone	435
D3-Epitestosterone	435
Dihydrotestosterone	434
Dehydroepiandrosterone	432
Methylotestosterone	446

The results were analyzed statistically, separately for men and women. Test t for dependent variables was used to study the effect of stress before exam and test t for independent variables in studying the effects of intensity of stress and physical activity.

Over the research period the analytical procedure was unchanged and the relative analytical uncertainties were 7.30, 7.20 and 12.60% for T, Et and T/Et respectively

TABLE I. THE EFFECT OF STRESS ON STEROID PROFILE IN FEMALES (N=14)

Steroid (ng/ml)/ Steroid Ratio	Relax	Stress	p value
11beta-OH-Androsterone	913.46±477.08	1357.83±697.29	**0.004**
11beta-OH-Etiocholanolone	670.47±413.44	881.04±530.60	**0.041**
Androsterone	2996.08±2004.98	4235.76±2912.11	**0.018**
Etiocholanolone	3434.78±2218.01	4545.04±2784.81	**0.047**
5beta-Androstane-3beta,17beta-diol	43.14±18.98	55.71±26.13	**0.020**
5alfa-Androstane-3alfa,17beta-diol	53.38±22.43	61.65±24.54	0.120
5beta-Androstane-3alfa,17beta-diol	54.20±35.02	60.49±47.04	0.275
5alfa-Androstane-3beta,17beta-diol	128.79±80.24	109.17±58.70	0.327
Testosterone	16.13±11.40	18.55±15.59	0.326
Epitestosterone	18.41±14.17	21.44±17.40	0.570
Dihydrotestosterone	8.25±5.89	10.22±7.12	**0.047**
Dehydroepiandrosterone	75.91±40.05	104.33±59.13	**0.015**
T/Et	1.18±0.76	1.38±1.36	0.569
A/E	0.88±0.22	0.94±0.32	0.327

RESULTS

20 male and 12 female students participated in the trial. The study has shown that women responded the most intensively to stressogenic situation. Among twelve investigated hormones and their metabolites, as many as seven have shown statistically significant increase in the urine, and further four exhibited growth tendency (Table 1). In men statistically significant increase of urine concentration was observed for two hormones: 11β-OH-androsterone and androsterone, and three others exhibited growth tendency (Table 2). This not significant increase was observed with etiocholanolone, its metabolite 11β-OH- etiocholanolone and epitestosterone.

In women there were statistically significant increases in dihydrotestosterone, 3β-,5β-,17β-androstantriole and dehydroepiandrosterone. These compounds in the majority are metabolites of testosterone, but urine concentration of testosterone and epitestosterone did not differ significantly. Only in the urine of women, the testosterone concentration exhibited growth tendency under the stress influence.

In the questionnaire filled by the trial participants it was attempted to describe the degree of intensity of stress in the four point scale (1-2-3-4). The results of inquiry enabled to separate for statistical estimation only two groups with stress level 2 and 3, both for men and women. In women, the increasing feeling of stress (groups 2-3) did not change significantly urinary steroid profile except for the weak growth tendency in testosterone level (Table 3). In men, increased stress feeling was accompanied by the increasing tendency in 11β-OH-etiocholanolone, 3α-,5α-,17β-androstantriole and 3β-,5β-,17β-androstantriole (Table 4). The declining tendency for testosterone and increasing tendency for epitestosterone also appeared. These two opposed trends resulted in significant decrease of the value of T/Et factor proportionally to the intensification of stress.

TABLE 2. THE EFFECT OF STRESS ON STEROID PROFILE IN MALES (N=20)

Steroid (ng/ml)/ Steroid Ratio	Relax	Stress	p value
11beta-OH-Androsterone	917.96±561.66	1391.20±622.39	**0.007**
11beta-OH-Etiocholanolone	410.88±334.81	477.13±243.64	0.305
Androsterone	2640.40±1932.20	4209.90±3054.10	**0.011**
Etiocholanolone	2673.40±1869.60	3270.7±1738.00	0.176
5beta-Androstane-3beta,17beta-diol	54.74±42.45	50.65±20.31	0.606
5alfa-Androstane-3alfa,17beta-diol	55.90±48.55	47.40±17.29	0.361
5beta-Androstane-3alfa,17beta-diol	105.48±84.16	105.21±63.52	0.988
5alfa-Androstane-3beta,17beta-diol	241.45±260.81	211.30±184.62	0.394
Testosterone	40.52±36.69	37.02±25.43	0.472
Epitestosterone	35.19±22.82	39.93±20.94	0.246
Dihydrotestosterone	78.87±42.92	83.68±44.98	0.653
Dehydroepiandrosterone	8.98±4.76	8.84±3.70	0.891
T/Et	1.67±2.36	1.10±0.73	0.319
A/E	1.13±0.52	1.31±0.43	**0.038**

TABLE 3. THE EFFECT OF INTENSITY OF STRESS (DEGREE 2 VS DEGREE 3) ON STEROID PROFILE IN FEMALES (N=6 VS N=8, RESPECTIVELY)

Steroid (ng/ml)/ Steroid Ratio	2nd degree	3rd degree	p value
11beta-OH-Androsterone	1633.17±528.73	1151.32±767.83	0.213
11beta-OH-Etiocholanolone	917.75±483.67	853.51±594.75	0.833
Androsterone	4759.80±2521.65	3842.73±3285.64	0.581
Etiocholanolone	4588.01±2602.80	4512.82±3092.04	0.962
5beta-Androstane-3beta,17beta-diol	60.71±27.93	51.96±25.96	0.557
5alfa-Androstane-3alfa,17beta-diol	60.25±26.51	62.69±24.76	0.862
5beta-Androstane-3alfa,17beta-diol	62.24±40.48	59.18±54.18	0.910
5alfa-Androstane-3beta,17beta-diol	97.56±76.80	117.89±44.54	0.543
Testosterone	13.02±5.48	22.70±19.60	0.267
Epitestosterone	23.15±14.14	20.16±20.37	0.765
Dihydrotestosterone	10.17±8.68	10.27±6.36	0.980
Dehydroepiandrosterone	96.92±52.49	109.90±66.65	0.701
T/Et	1.05±1.41	1.62±1.36	0.456
A/E	1.09±0.27	0.83±0.33	0.143

TABLE 4. THE EFFECT OF INTENSITY OF STRESS (DEGREE 2 VS DEGREE 3) ON STEROID PROFILE IN MALES (N=13 VS N=5, RESPECTIVELY)

Steroid (ng/ml)/ Steroid Ratio	2nd degree	3rd degree	p value
11beta-OH-Androsterone	1417.69±612.84	1277.69±751.85	0.688
11beta-OH-Etiocholanolone	462.77±208.01	631.59±275.48	0.176
Androsterone	4222.98±3485.16	4371.54±2597.44	0.933
Etiocholanolone	3321.06±1871.76	3575.98±1662.50	0.794
5beta-Androstane-3beta,17beta-diol	47.75±22.89	59.95±11.32	0.277
5alfa-Androstane-3alfa,17beta-diol	44.28±17.17	60.75±13.71	0.074
5beta-Androstane-3alfa,17beta-diol	114.00±71.71	86.91±47.47	0.450
5alfa-Androstane-3beta,17beta-diol	254.38±213.51	152.50±75.63	0.320
Testosterone	43.61±28.24	24.18±15.71	0.170
Epitestosterone	37.03±22.32	51.98±14.61	0.188
Dihydrotestosterone	9.31±4.307	8.18±1.74	0.581
Dehydroepiandrosterone	81.20±47.12	86.38±31.88	0.825
T/Et	1.28±0.69	0.53±0.370	**0.037**
A/E	1.29±0.49	1.20±0.28	0.687

TABLE 5. THE EFFECT OF PHYSICAL ACTIVITY (YES/NO) IN RELAX STATE ON STEROID PROFILE IN FEMALES (N=5/N=9, RESPECTIVELY)

Steroid (ng/ml)/ Steroid Ratio	No	Yes	p value
11beta-OH-Androsterone	1118.38±393.45	544.59±406.86	**0.024**
11beta-OH-Etiocholanolone	875.02±323.95	302.26±286.39	**0.006**
Androsterone	3513.13±2171.71	2065.38±1395.69	0.207
Etiocholanolone	4085.76±2361.48	2263.02±1471.64	0.147
5beta-Androstane-3beta,17beta-diol	47.24±19.18	35.77±18.15	0.297
5alfa-Androstane-3alfa,17beta-diol	57.67±27.16	45.67±6.67	0.358
5beta-Androstane-3alfa,17beta-diol	64.98±38.09	34.81±18.77	0.126
5alfa-Androstane-3beta,17beta-diol	159.95±79.02	72.70±48.16	0.046
Testosterone	20.42±12.00	8.41±4.31	0.055
Epitestosterone	20.68±16.27	14.31±9.54	0.442
Dihydrotestosterone	10.40±6.11	4.39±3.00	0.064
Dehydroepiandrosterone	86.78±45.21	56.34±19.47	0.188
T/Et	1.22±0.56	1.11±1.13	0.804
A/E	0.85±0.19	0.92±0.29	0.592

TABLE 6. THE EFFECT OF PHYSICAL ACTIVITY (YES/NO) IN STRESS STATE ON STEROID PROFILE IN FEMALES (N=4/N=10, RESPECTIVELY)

Steroid (ng/ml)/ Steroid Ratio	No	Yes	p value
11beta-OH-Androsterone	1541.38±736.12	898.97±297.06	**0.123**
11beta-OH-Etiocholanolone	1035.54±535.58	494.79±284.54	**0.084**
Androsterone	4812.34±3218.05	2794.31±1342.49	0.257
Etiocholanolone	4764.63±3028.34	3996.06±2351.74	0.659
5beta-Androstane-3beta,17beta-diol	63.12±27.34	37.18±8.70	0.094
5alfa-Androstane-3alfa,17beta-diol	65.20±26.02	52.77±20.74	0.414
5beta-Androstane-3alfa,17beta-diol	72.86±50.93	29.56±4.83	0.123
5alfa-Androstane-3beta,17beta-diol	120.40±56.80	81.10±61.48	0.274
Testosterone	22.02±17.16	9.88±5.42	0.199
Epitestosterone	23.93±20.21	15.23±3.88	0.420
Dihydrotestosterone	12.36±7.24	4.90±3.11	0.075
Dehydroepiandrosterone	116.85±63.43	73.06±35.40	0.224
T/Et	1.66±1.52	0.68±0.41	0.235
A/E	1.01±0.33	0.76±0.28	0.207

The stressogenic state before exam caused also statistically significant increase in the value of A/E factor in men (Table 2). It can be explained by the higher intensification of metabolic pathway testosterone – androsterone than testosterone – etiocholanolone.

One of the questionnaire questions concerned the quality of sleep. This factor, both in stressogenic and relaxed situations did not correlate with the changes in steroid profile, therefore we did not include that results.

Meaningful question directed to participants of the trial referred to physical activity. 5/12 women and 12/20 men declared physical activity during both phases of experiment. Physical activity significantly influenced urinary steroid profile; speaking generally its effect was opposite to the effect of stress.

Comparing groups of physically active and inactive women under relax conditions it can be noticed that physical activity decreased the concentration of every measured hormones, and for 11β-OH-androsterone, 11β-OH-etiocholanolone, 5α-,3β-,17β-androstantriole and testosterone the decrease was statistically significant (Table 5). Also dihydrotestosterone concentration tended to decline. Under stress conditions the declining tendencies did not reach statistical significance ($p<0.05$), possibly as the result of stress effect on hormone concentrations increase and limited size of experimental groups (Table 6).

In men, also downward tendency in the concentrations of hormones of steroid profile was dominating, but to less extent than in women (Tables 7 and 8).

DISCUSSION

Anti-doping control and the requirement for exact interpretation of analytical results were stimulus for a number of researches on steroid profile in human blood and urine. The variability of steroid profile was fulfilled by the effect of internal factors having source in

TABLE 7. THE EFFECT OF PHYSICAL ACTIVITY (YES/NO) IN RELAX STATE ON STEROID PROFILE IN MALES (N=13/N=7, RESPECTIVELY)

Steroid (ng/ml)/ Steroid Ratio	No	Yes	p value
11beta-OH-Androsterone	959.00±598.69	895.86±564.58	0.818
11beta-OH-Etiocholanolone	433.22±443.22	398.85±280.75	0.833
Androsterone	3057.14±2156.83	2416.08±1851.84	0.494
Etiocholanolone	2718.27±1997.22	2649.26±1881.02	0.940
5beta-Androstane-3beta,17beta-diol	77.63±60.73	42.41±23.19	0.076
5alfa-Androstane-3alfa,17beta-diol	77.44±75.87	44.31±20.93	0.150
5beta-Androstane-3alfa,17beta-diol	140.76±126.26	86.48±46.13	0.175
5alfa-Androstane-3beta,17beta-diol	306.24±377.31	206.57±180.99	0.430
Testosterone	38.21±32.97	41.77±39.79	0.842
Epitestosterone	27.07±20.26	39.56±23.66	0.253
Dihydrotestosterone	9.53±2.08	8.69±5.79	0.719
Dehydroepiandrosterone	95.77±56.91	69.78±32.26	0.204
T/Et	2.68±3.83	1.12±0.74	0.163
A/E	1.31±0.53	1.03±0.50	0.244

TABLE 8. THE EFFECT OF PHYSICAL ACTIVITY (YES/NO) IN STRESS STATE ON STEROID PROFILE IN MALES (N=12/N=8, RESPECTIVELY)

Steroid (ng/ml)/ Steroid Ratio	No	Yes	p value
11beta-OH-Androsterone	1623.91±335.41	1236.09±729.30	0.179
11beta-OH-Etiocholanolone	389.98±126.68	535.23±288.29	0.199
Androsterone	5190.56±3835.81	3556.11±2362.75	0.252
Etiocholanolone	3770.92±1842.41	2937.22±1659.50	0.306
5beta-Androstane-3beta,17beta-diol	55.94±18.95	47.12±21.22	0.356
5alfa-Androstane-3alfa,17beta-diol	50.35±18.60	45.43±16.90	0.548
5beta-Androstane-3alfa,17beta-diol	137.18±76.05	83.91±45.28	0.064
5alfa-Androstane-3beta,17beta-diol	258.67±242.71	179.72±136.63	0.363
Testosterone	45.04±25.42	31.67±25.05	0.260
Epitestosterone	40.19±12.76	39.76±25.56	0.966
Dihydrotestosterone	10.47±2.65	7.75±4.00	0.109
Dehydroepiandrosterone	98.69±52.34	73.68±38.44	0.233
T/Et	1.10±0.48	1.10±0.88	0.982
A/E	1.40±0.52	1.25±0.37	0.459

genetic status and biological rhythms [11,14], as well as by the external factors comprehensive of seasons of the year, physical activity, style of life, diet and various stress conditions [7,8,9,12,15,18]. Students constitute a group of young people, many times undergoing strong stresses similar in character to stress experienced by sport competitors immediately before start. We had an opportunity to compare urinary steroid profiles in the same probants at relax and stress conditions, that significantly reduced the deviation of concentrations of estimated hormones, and allowed us to gain statistically significant results in spite of limited size of experimental groups including 14 women and 20 men.

As expected, relaxed and stressed females demonstrated markedly lower urinary testosterone levels as compared to those recorded in males. That sex-related differences confirmed well known sexual dimorphism regarding androgenic status in blood, since females testosterone is on average 10-fold lower than that among healthy males. Another difference between sexes were contradictor direction of testosterone changes (baseline vs. stress) in response to psychological arousal, i.e. rise in female (by 14.9%) and drop in males (by 8.6%). Although these changes were not statistically significant, that phenomenon confirms that in females stress brought about secretion of adrenal androgens, androstendione and DHEA-S that are metabolized into testosterone in target tissues. Among males the same stressful event may bring about two various reactions, secretion of testosterone due to acute rapid rise of catecholamines, or inhibition its biosynthesis in testis by huge cortisolism, as was revealed in male junior judo players directly prior to a high rank competition [14].

Presented results indicate that both physical exertion and pre-examination stress influence much more seriously on steroid profile in women. Nevertheless, the values of T/Et and A/E ratios in women did not show statistically significant changes. The only tendency observed was to decrease the values of both factors under physical activity, that was seen both in relax and stress states. Differently in men, the stress produced small but statistically significant increase in A/E value and the value of T/Et significantly decreased with stress intensification.

Physical activity caused a decrease in urine concentrations of every hormone in women steroid profile. Bricout et al. [2] confirmed the effect of physical activity on the androgens levels in the urine with simultaneous lack of changes in the value of T/Et ratio. T/Et values did not change, also at different phases of menstruation cycle. Our results suggest a tendency to decrease T/Et and A/E values in physically active women exposed to psychological stress.

Similar tendency of decreasing the urine concentrations of every hormone appeared in physically active men. In active man in relax state, the exception was regular testosterone concentration and increasing tendency in epitestosterone concentration. The ratios T/Et and A/E tended to decrease. In men practicing endurance sports it was proved that androgens concentration decrease during training and increase during competition, however, the value of T/Et ratio remains unchangeable [13,14].

CONCLUSIONS

In summary, observed changes in steroid profile suggest, that major fluctuations of T/Et and A/E ratios under the influence of stressogenic factors and physical activity are unlikely. Even more, these values have shown declining tendency, so the risk of surpassing the limit value (limit is more restricted and amounts 4 - WADA The Prohibited List 2009 [17]) for T/Et is diminishing.

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