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ORIGINAL PAPER

Evaluation of urinary steroid profile after administration of pharmaceutical preparations

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Abstract

On World Anti-Doping Agency (WADA) initiative, in the near future, a passport will be developed for the athletes, comprising the main parameters of the steroid profile. The most characteristic endogenous steroids that define the steroid profile, one of the most versatile and informative screening tools for the detection of steroid abuse in sports drug testing, are testosterone, epitestosterone, androsterone, etiocholanolone, dehydroepiandrosterone, 5α-androstane-3α,17β-diol and 5β-androstane-3α,17β-diol. Knowledge of factors that influence the steroid profile pattern is of central importance. In addition to parameters such as specific gravity, pH-value, gender, sport discipline and time of sampling, other factors such as pharmaceutical, technical and biological issues need consideration when interpreting steroid profile. The purpose of this study was to monitor the values of steroid profile for two male volunteers after the administration of pharmaceutical preparations that contained human chorionic gonadotropin hormone. The analysis was performed by a selective, specific and sensitive gas chromatography/mass spectrometry method in selective ion monitoring (SIM) mode. The study showed that the concentrations of the main parameters of steroid profile increased, but the testosterone/epitestosterone ratio has not exceeded the WADA action limit for human doping control (ratio 4) after the intramuscular administration of one single dose (5000UI) of Pregnyl injection.

Keywords: excretion study, steroid profile, gas chromatography/mass spectrometry

Introduction

In the last years, the research in biomedical field registered considerable progresses related to the development of analytical methods for the detection and quantification of peptide hormones and endogenous steroids in urine samples. Such methods should be specific and should provide a high precision, sensitivity and accuracy without interference from unrelated matrix components. These methodological aspects are very important when the analyses are done in the framework of the fight against doping in sports.

Determination of steroid profile parameters is a major concern of specialists in the field of doping control, on the one hand because these parameters will be introduced at the request of World Anti-Doping Agency (WADA) in the Athlete Biological Passport (AP), and on the other hand to successfully meet the requirement of WADA for harmonization of detection and confirmation of endogenous anabolic androgenic steroids. The Athlete Biological Passport which was first proposed by the WADA in 2002, represents a complex analytical procedure which could harmonize the samples collected both in competition and out of competition, thus ensuring an increasingly efficient fight against doping at the most sophisticated level, with this systematic and robust monitoring program. The collection and

monitoring parameters corresponding to this program will constitute an individual and longitudinal profile which means that AP will become athlete's own reference.

The data shown in specific literature in the last years in doping control field, as well as the information provided by the specialists of doping control laboratories, revealed that monitoring the hormonal profile and identification of the substances used by the athletes aiming to stimulate the secretion of endogenous anabolic steroids is a major objective of the research.

Steroid profile is one of the most versatile and informative screening tools for the detection of steroid abuse in sports drug testing**.** The urinary steroid profile [1] is composed of concentrations and ratios of various endogenously hormones, including testosterone (T), epitestosterone (E), androsterone (Andro), etiocholanolone (Etio), dehydroepiandrosterone (DHEA), 5α-androstane-3α, 17β-diol (5α-diol) and 5β-androstane-3α,17β-diol (5β-diol). The alteration of one or more of the concentrations of these parameters interferes with the naturally well-balanced system and raises suspicion in routine doping controls either by increased or decreased concentrations and ratios. Steroid profiles, as determined routinely in doping control, provide essential information for several purposes as they are recorded, stored, and attributed to individuals incrementally with each collected sports drug testing sample. Several studies demonstrated only small intra individual variations of steroid profile parameters, especially within the ratios utilized for doping control purposes such as T/E, Andro/Etio, Andro/T and 5α-diol/5β-diol [2,3,4,5,6].

The interrelation of these substances is illustrated in Figure 1, whereas the exact pathway leading to the formation of E has not yet been elucidated.

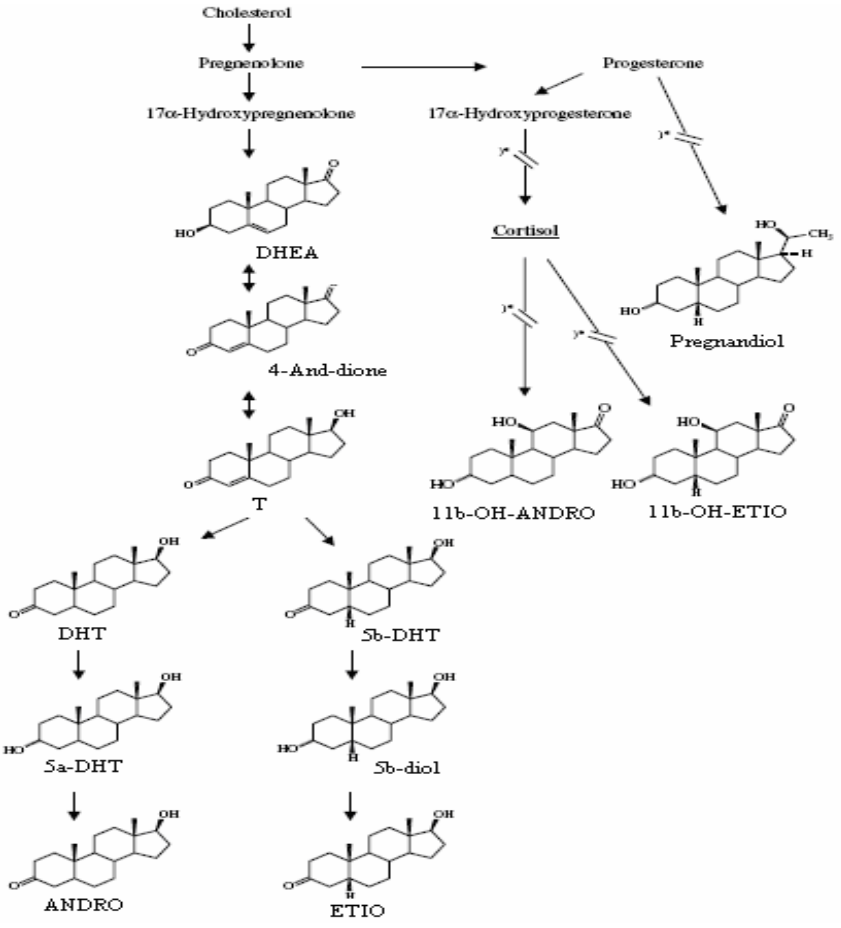

Figure 1. Biosynthesis of endogenous steroids

All steroid hormones, therefore endogenous hormones too, are biosynthesized in the body from cholesterol. Cholesterol – under the action of a mitochondrial enzymatic complex which includes cholesterol – desmolasis and cytochrome P_{450}, is broken in six carbon atoms which take the position 17β of pregnenolone, forming progesterone. One should notice that pregnenolone is a precursor of all steroid hormones and by retro-inhibition, regulator of steroid genesis from cholesterol. Once the pregnenolone is formed, there are two ways for the biosynthesis of androgynous hormones: Δ_4 and Δ_5. Studies using marked intermediates ^3H and ^{14}C showed that in adult individuals Δ_5 predominates, therefore T is synthesized easier from pregnenolone than from progesterone.

Endogenous anabolic steroids are extensively metabolized in the human body and only small amounts of the parent substances are excreted in the urine. The most important metabolic phase-I reactions include oxidation or reduction in position C-3 and C-17 [7].

The great importance of urinary steroid profiles and their utility in doping controls are manifold, and the information obtained is vital to the successful screening for steroid anabolic agents. It is known that the administration of testosterone or its precursors, other prohibited substances [8] or other substances involved in the regulation of endocrine system, such as human chorionic gonadotropin hormone (hCG) [9,10] and luteinizing hormone (LH) [11,12], alters one or more of the parameters of the urinary steroid profile. Thus, knowledge of factors that influence the steroid profile pattern is of central importance. In addition to parameters such as specific gravity, pH-value, gender, sport discipline and time of sampling, other factors such as pharmaceutical, technical and biological issues need consideration when interpreting steroid profile [13].

hCG is produced abundantly by the placental trophoblastic cells during pregnancy, stimulating steroid hormone production in the ovaries. Therefore, hCG is used as a marker for pregnancy tests [14]. However, due to its stimulation of endogenous steroid production, leading to increased testosterone levels without affecting the T/E ratio (used as criteria for detecting doping with exogenous testosterone), hCG has also been used for doping purposes [10,16]. Linked to this action, the side-effects associated with hCG misuse are similar to those induced by anabolic androgenic steroids.

The presence of high hCG levels in a male urine are highly suspicious for doping, except for the situation when the concentration was due to a physiological or pathological condition. In females, establishing the origin of these peptide hormones (exogenous or naturally produced) constitutes a challenging task [16].

This paper work shows the evolution of urinary steroid profile after intramuscular administration of hCG, peptide hormone that may be used by the athletes to stimulate the testosterone and epitestosterone production, aiming to obtain effects in increasing the muscular mass and to mask these effects by maintaining the T/E report within physiological limits.

Materials and Methods

Chemicals and reagents

The reference substances for testosterone (17β-hydroxyandrost-4-en-3-one), epitestosterone (17α-hydroxyandrost-4-en-3-one), androsterone (3α-hydroxy-5α-androstan-17-one), etiocholanolone (3α-hydroxy-5β-androstan-17-one), 5α-diol (5α-Androstan-3α,17α-diol), 5β-diol (5β-Androstan-3α,17α-diol) and methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one, internal standard) were purchased from National Measurement Institute (Germany). Derivatization agent N-methyl-N-trimethylsilyltrifluoroacetamide MSTFA (for gas chromatography) was produced by Alfa Aesar GmbH&CohG, and the

enzyme β-Glucuronidase from E.coli was from Roche Diagnostics Manheim. All the other reagents and solvents were of analytical and chromatographic grade and were purchased from Sigma and Merck.

Urine samples

An excretion study was performed on two male volunteers V1 and V2 (28 and 34 years). Pregnyl (1mL containing 5.000UI of hCG) has been injected intramuscularly on the male volunteers in accordance with the rules of the local ethical commission. Urine samples were collected before administration and every morning for 9 days after administration. The samples have been stored by freezing and their pH and specific gravity have been measured before analysis.

Urine sample preparation

Urine samples were prepared for regular doping control analysis adopted after Schänzer and Dönike [16] and implemented in Romanian Doping Control Laboratory, according to the internal laboratory standard operating procedures, in order to detect the anabolic agents.

For quantification of steroid profile, internal standard (methyltestosterone, 500ng/mL), 1mL phosphate buffer (pH 7) and 25µL of β-glucuronidase from E. coli solution were added to 2mL of urine. The samples were hydrolyzed during 90 minutes at 50°C. After adjusting the pH to approximately 9 with potassium carbonate, the analytes were extracted with tert-butyl-methyl-ether. The samples were shaken mechanically for 15 minutes (100 oscillations/ min) and were centrifuged for 15 minutes at 2500 rpm and the organic phase was separated and evaporated to dryness under nitrogen stream at 40^0C. The samples were dried in a desiccator for 60 minutes and for GC/MS analysis, the dry residues were derivatized using 100µL MSTFA/NH4I/ethanethiol (1000/2/3; v/w/v) at 60°C for 30 minutes and 3µL were injected into the gas chromatography/mass spectrometry (GC/MS) system.

GC/MS parameters

The identification of the parameters of steroid profile was performed in selective ion monitoring (SIM) mode. GC/MS method was run on an Agilent 6890 gas chromatograph directly coupled to an Agilent 5973 mass spectrometer. The GC column was an UTRA 1 J&W Scientific (cross-linked dimetil polisiloxan, length 17m, i.d. 0.2mm, film thickness 0.11µm). Helium was used as carrier gas in the constant flow mode (0.8mL/min). A total of 3µL was injected split 1:10 on the chromatographic system. The oven temperature program was as follows: 160°C (2 min), 5°C/min→ 255°C (0 min), 30°C/min → 285°C (5 min) and 60°C/min →300°C (3.75 min). The electron energy was set at 70eV and the ion source temperature was set at 230°C.

Results and Discussions

By developing this research we intended to establish the changes in the concentrations of endogenous androgynous hormones and the reports between them after administration of one single dose (5000UI of hCG) of Pregnyl.

In Table 1 are presented the concentrations of β-hCG for the both volunteers. The values show that physiological excretion of β-hCG is similar for the both subjects. The highest levels of β-hCG concentrations, 216 and 147.2mUI/mL, are observed in the first day after administration of a single dose of Pregnyl. In the next days it can be noticed a slow decrease, but the β-hCG concentrations stay above the upper reference limit for 6-8 days after administration.

Table 1. The concentrations of the β-hCG

Time (days)	hCG (mUI/mL)	hCG (mUI/mL)
	V1	V2
0	0.10	1.3
1	216	147.2
2	126.4	96.4
3	112.9	60.7
4	77	32.5
5	45.7	15.4
6	17.3	6.2
7	13.9	0.9
8	6.2	0.7
9	4.4	0.4
10	3.2	0.4

For the purposed aim, a set of urinary concentrations of several endogenous steroids was determined by the gas chromatography/mass spectrometry (GC/MS) method used for the detection of steroid abuse.

Aiming to obtain the most concrete values of steroid profile parameters concentrations, three replicates were prepared from each urine sample, each replicate was injected twice in GC/MS system and the values showed in Table 2 are the average of all concentrations of each hormone in a urine sample. The quantification of each hormone was made by one calibration point which contains 2000ng/mL Andro and Etio, 80ng/mL 5α-diol, 180ng/mL 5β-diol, 15ng/mL E and 60ng/mL T.

The evolution of steroid profile parameters was followed over a period of 9 days after intramuscular administration of hCG. Some parameters were determined in volunteers before administration and the resulted values were considered as witness values for determination of the parameters reported for other times of investigation.

Before starting the experiment the 2 volunteer subjects had normal concentrations of main steroid profile parameters (T, E, Andro, Etio, 5α-diol, 5β-diol).

Table 2. The concentrations of the main steroid profile parameters

Time (days)	ANDRO (ng/mL)		ETIO (ng/mL)		5α-diol (ng/mL)		5β-diol (ng/mL)		E (ng/mL)		T (ng/mL)		T/E	
	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2
0	276.8	5470.3	221.6	6298.9	6.0	150.7	21.2	889.2	9,1	81.3	5.5	185.4	0.60	2.26
1	2020.2	5846.7	1631.6	5995.3	58.0	150.6	152.2	766.4	98.3	164.7	66.7	289.2	0,67	1.74
2	1241.8	5100.6	696.9	4449.0	88.5	149.5	90.6	557.9	52.8	161.2	37.6	285.2	0.71	1.75
3	1917.6	7488.6	1246.5	6288.7	103.4	195.0	133.0	796.4	78.8	180.7	68.8	397.9	0.87	2.18
4	1829.7	9239.9	1434.7	7419.3	61.6	224.3	108.8	776.3	61.7	209.7	63.6	574.9	1.02	2.72
5	2815.3	4483.6	2202.0	3666.0	92.9	119.7	177.4	499.1	95.6	80.7	91.9	277.5	0.95	3.41
6	856.6	3917.3	677.1	3708.0	22.5	128.1	64.4	552.5	18.5	72.1	20,6	246.9	1.10	3.40
7	1049.3	3267.1	806.6	3014.9	33.5	291.7	94.1	794.4	21.7	50.6	17.3	292.0	0.79	3.72
8	298.6	2105.4	236.6	2223.0	7.5	74.7	21.3	386.3	9.0	23.4	6.6	87.7	0.73	3.73
9	1478.2	2328.3	1259.6	2451.3	45.7	76.4	142.2	347.3	58.9	19.1	36.7	56.6	0.62	2.94
10	1095.4	5339.3	891.2	4957.7	26.9	138.3	81.7	541.0	41.6	64.0	31.3	209.9	0.75	3.26

When studying the values of steroid profile parameters concentrations, one may notice that volunteer 1 showed a trend of increase in the concentrations of androgenous hormones after hCG administration, with a maximum of excretion in day 5, except for 5α-diol, which

shows a maximum concentration in day 3. As for the second volunteer, the maximum values of androgenous hormones concentrations can be seen in different days, which reveal different metabolizing behaviors of the two subjects.

Testosterone and epitestosterone production are both increased by hCG [17]. hCG is administered intramuscularly by athletes in order to reduce hypogonadotrophic hypogonadism after anabolic steroid use [18]. Another interesting aspect of hCG is that it decreases a high T/E due to T administration [19,20]. Because the T/E ratio can be decreased to a value far below 4 in this way, hCG can be regarded as a masking agent for T.

For volunteer V1, after hCG stimulation, the testosterone concentration increased, maximal increases being observed 5 days after administration. In volunteer V2 there was a significant increase of renal excretion of testosterone concentration in the early days, the maximum value being observed on the 4^{th} day after administration, then the testosterone concentration decreased gradually.

At 9 days after administration, for volunteer V1 testosterone concentration (31.3ng/mL) remains much higher than the baseline value (5.5ng/mL) and for volunteer V2 testosterone concentration tends to return to baseline value. A most suggestive representation is shown in Figure 2, which shows the evolution of testosterone concentration throughout the study.

The ratio between testosterone and epitestosterone (T/E), both excreted as glucuronides, introduced for first time into routine doping control by Donike et al. [21], is the most frequently used parameter for detecting the misuse of testosterone or testosterone prohormones by athletes. False-negative results can also be produced by stimulation of testicular steroid genesis by administering human chorionic gonadotropin, which would result in a concomitant increase in the urinary excretion rate of T and E, but with no significant change in the T/E ratio [11].

The increase of the T/E ratio in urine may be attributed to an increased concentration of testosterone or a decreased concentration of epitestosterone, or both. In our study, although T and E values increased very much compared to the blank sample, the T/E ratio has not exceeded the WADA action limit for human doping control (T/E ratio=4) after hCG administration. The ratios rose from basal values of 0.6 and 2.26 to maximum values on day 6 and 8, respectively. After hCG stimulation, the T/E ratios rose for each subject, the maximum values of 1.10 and 3.73 being observed for subject 1 on day 6 and for subject 2 on day 8. Changes in T/E ratios are displayed in Figure 3.

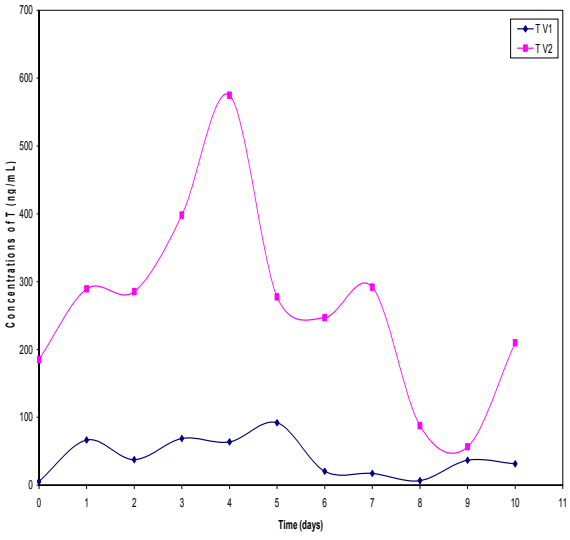

Figure 2. Excretion curves of testosterone

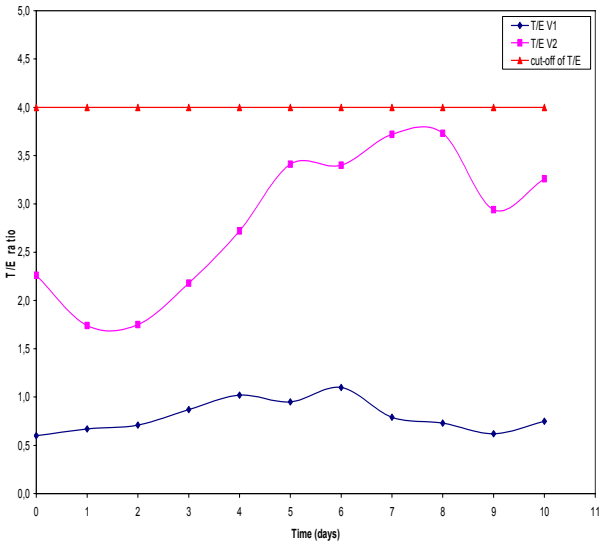

Figure 3. Evolution of T/E ratio

Androsterone (Andro) and Etiocholanolone (Etio), the final products of metabolism of testosterone, are considered some of the most stable parameters of steroid profile [2]. Concentrations of Andro and Etio for the two subjects are presented in Table 2 and Figure 4, illustrating the evolution of concentration for these two parameters. The maximum values were reached in day 5 for first volunteer and day 4 for the second volunteer. Different values for Andro and Etio in the two subjects are due to metabolic particularities of each individual. As with T and E, administration of hCG induces an increase in concentration of Andro and Etio.

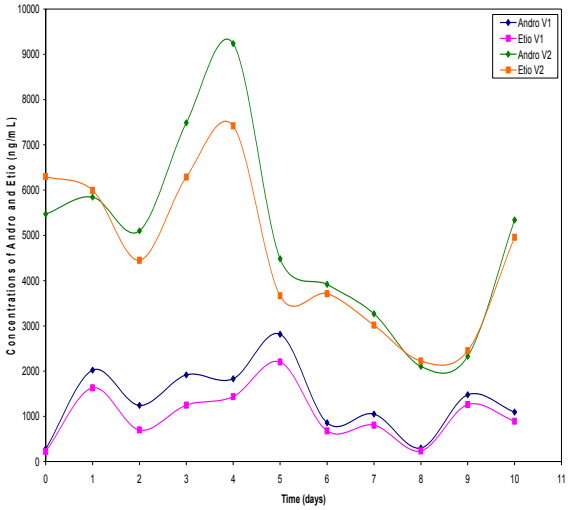

Figure 4. Excretion curves of Andro and Etio

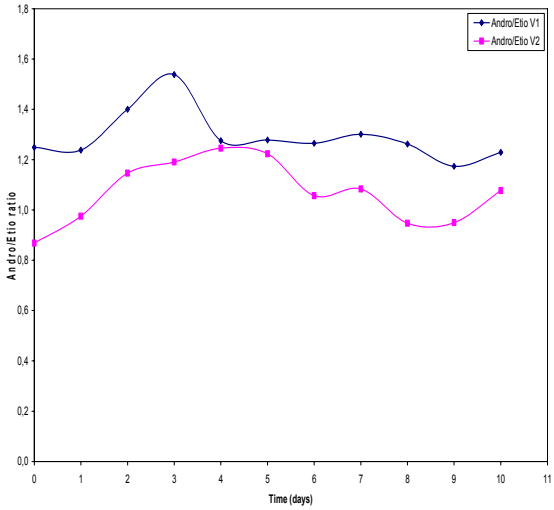

Figure 5. Evolution of Andro/Etio ratio

The increase of Andro and Etio concentrations did not cause a significant change to Andro/Etio ratio (Figure 5), which were also determined on selected days. During the 9 days of study, the Andro/Etio ratio showed similar values for both volunteers.

Andro/T ratio is a significant steroid profile parameter used to indicate particularly exogenous testosterone abuse. Experts have shown that if exists a contribution of exogenous testosterone, Andro/T ratio decreases as a result of increased concentration of testosterone renal excretion. Thus, for the two subjects that are included in the study it was noted a decrease in the values of this parameter until day 5 and then can be observed an increase of Andro/T ratio (Figure 6).

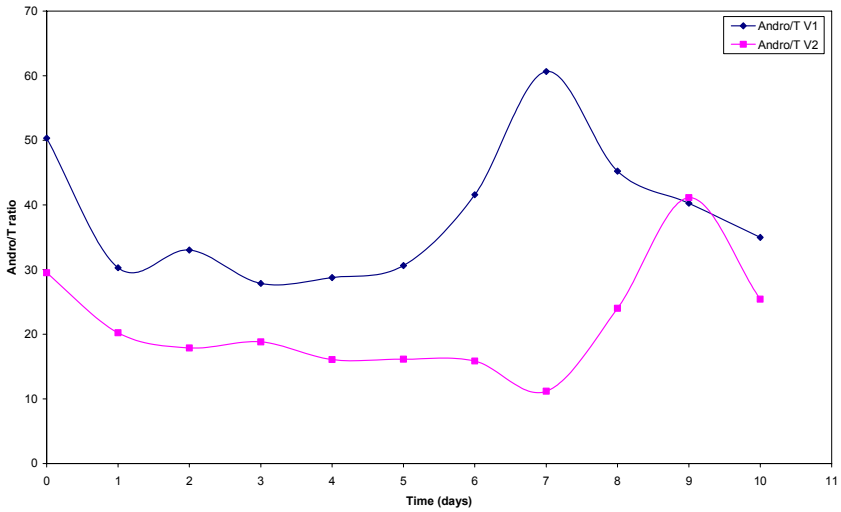

Figure 6. Evolution Andro/T ratio

5α and 5β-diols resulted by degradation of testosterone and DHT, direct metabolite of testosterone by "5α-reduction way", by enzymatic reduction of the double connection between C_4 and C_5.

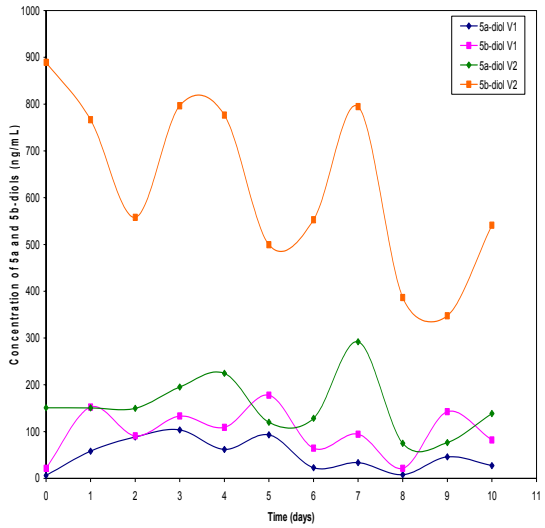

Figure 7. Excretion curves of 5α and 5β-diols

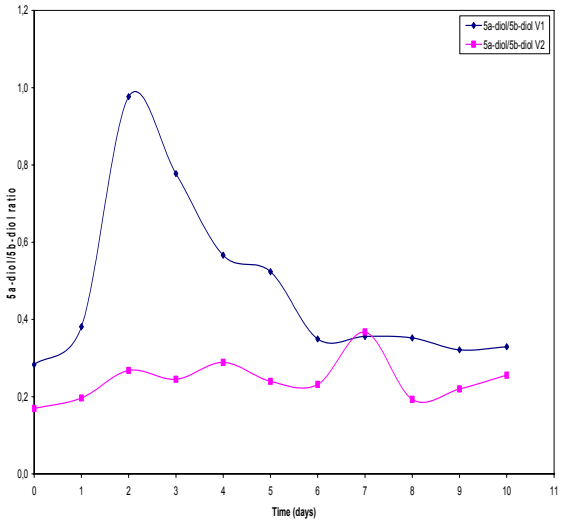

Figure 8. Evolution of 5α-diol/5β-diol ratio

As for 5α and 5β-diols excretion (Figure 7), volunteer 1 shows an emphasized increase of 5α-diol with a maximum concentration of 103.4ng/mL in day 3, while for 5β-diol maximum excretion takes place in day 5. Volunteer 2 shows a different behavior in the excretion of the two hormones. Thus, for 5α-diol maximum excretion is noticed in day 8, with a far less increase of the concentration in comparison with the initial moment, if compared with volunteer 1, while the concentration of 5β-diol decreases slowly in the first days but the values are closed in the first seven days and a small increase is seen in the last days.

Administration of hCG induces less changes in 5α-diol/5β-diol ratio as compared with the other hormonal ratios discussed. The review of 5α-diol/5β-diol (Figure 8) ratio value evolution for the 2 studied subjects shows an increase of this parameter on value 1 (V1), respectively 0.4 (V2) as compared with the witness samples of 0.3 (V1) and 0.2 (V2).

Conclusions

In the present study, a set of urinary concentrations of several endogenous steroids (testosterone, epitestosterone, androsterone, etiocholanolone, 5α and 5β-idol) was determined by the gas chromatography/mass spectrometry method used for the detection of steroid abuse.

The aim of this work was to improve the doping control activity and to conduct more efficient and rigorous doping controls to elite athletes, thus contributing to the fight against doping in sport. In this respect, the values of steroid profile for two male volunteers were monitored, after the administration of pharmaceutical preparations that contained human chorionic gonadotropin hormone.

The study showed that intramuscular administration of one single dose (5000UI) of Pregnyl increases the concentrations of the main parameters of steroid profile. hCG stimulation leads to a simultaneous increase in the urinary excretion rate of both testosterone and epitestosterone, but the testosterone/epitestosterone ratio has not exceeded the WADA action limit for human doping control (T/E ratio=4).

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