Steroids 76 (2011) 1400–1406

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Short communication

Relevance of the selective oestrogen receptor modulators tamoxifen, toremifene and clomiphene in doping field: Endogenous steroids urinary profile after multiple oral doses

Monica Mazzarino [a], Maria Cristina Braganò [a], Xavier de la Torre [a], Francesco Molaioni [a], Francesco Botrè [a,b,*]

[a] Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Largo Giulio Onesti, 1, 00197 Rome, Italy
[b] Dipartimento di Management, "Sapienza" Università di Roma, Via del Castro Laurenziano, 9, 00161 Rome, Italy

ARTICLE INFO

Article history:
Received 16 November 2010
Received in revised form 13 June 2011
Accepted 15 June 2011
Available online 30 June 2011

Keywords:
Anti-doping analysis
Selective oestrogen receptor modulators

ABSTRACT

The present study was performed to investigate the influence of the intake of selective oestrogen receptor modulators on the urinary endogenous steroids profile. For this purpose the circadian variability of luteinizing hormone, follicle-stimulating hormone, testosterone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol, epitestosterone, 4-androstenedione, androsterone and etiocholanolone were measured on eight subjects (four males and four females) by gas chromatography–mass spectrometry and chemiluminescent immunometric assay techniques before and after oral administration of multiple doses of either tamoxifen (80 mg for 2 days) or toremifene (120 mg for 2 days) or clomiphene (100 mg for 2 days). The individual baseline variability of the steroids studied was set up by collecting the urine samples every 3 h, for 3 days prior to the treatment; whereas the evaluation of the effects of the oral administration of multiple doses of selective oestrogen receptor modulators on the steroid urinary profile was assessed by collecting urine samples every three hours for at least five days from the first administration.

The results of our measurements showed that, only in male subjects, the relative urinary concentrations of testosterone, epitestosterone and 4-androstenedione were significantly altered generally after the second day of drug administration. While no significant effects were recorded in both sexes on the luteinizing hormone, follicle-stimulating hormone, androsterone, etiocholanolone, 5α-androstan-3α,17β-diol and 5β-androstan-3α,17β-diol urinary levels and on testosterone/epitestosterone, 5α-androstan-3α,17β-diol/5β-androstan-3α,17β-diol and androsterone/etiocholanolone ratios.

1. Introduction

According to the annual statistic data of the World Anti-Doping Agency (WADA) based on the accredited laboratories reporting [1], anabolic androgenic steroids are still the most frequently abused drugs used in sports. Nevertheless, the evolution of the analytical technologies has led the athletes to use a variety of indirect strategies to circumvent the ban of direct androgen doping, such as, for example, the indirect stimulation of hypothalamic gonadotropin-releasing-hormone (GnRH) secretion by increasing endogenous luteinizing hormone [2–4]. One of the factors known to enhance endogenous hypothalamic GnRH secretion includes the use of selective oestrogen receptor modulators (SERMs) that binding competitively to oestrogens hypothalamic and pituitary receptors block sex steroid negative feedback. Previous researchers demonstrated that the administration of these agents increases the blood testosterone levels in men [3,4]. The same finding was not observed in women, because in contrast to men in whom blood testosterone concentrations are strictly controlled by negative feedback regulation of the hypothalamic–pituitary–gonad axis, in women blood testosterone is derived mainly from extra-gonad sources that are not involved in any homoeostatic feedback regulation [2–4].

Since 2005, SERMs are included, with other similar drugs, in the Section 4 "agents with anti-oestrogenic activity" of the WADA list of prohibited substances and methods [5]. The athletes could illicitly use SERMs for two main reasons: (i) to increase endogenous testosterone levels, with the aim to by-pass the specific testing regimens for known synthetic androgens including exogenous testosterone, and (ii) to balance the adverse effects of an extensive abuse of anabolic androgenic steroids [3,4]. Little is known about the effect of SERMs administration on the sport performance and on their possible interaction with the normal circadian fluctuation of the endogenous hormonal parameters that are presently selected as markers of an atypical urinary steroid profile in the doping field [6]. The purpose of this study was to verify whether the oral administration of multiple doses of SERMs (tamoxifen, toremifene

* Corresponding author at: Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Largo Giulio Onesti, 1, 00197 Rome, Italy. Tel.: +39 06 36859600; fax: +39 06 8078971.
E-mail address: francesco.botre@uniroma1.it (F. Botrè).

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doi:10.1016/j.steroids.2011.06.005

or clomiphene) leads to significant variations on testosterone, epitestosterone, 4-androstenedione, androsterone, etiocholanolone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol, luteinizing hormone and follicle-stimulating hormone urinary concentration. For this purpose the steroids urinary levels measured before the oral administration of multiple doses of tamoxifen, toremifene or clomiphene were compared with those obtained after administration of the drug(s) using the Student *t*-test ($p < 0.05$). The data obtained were also evaluated taking into account the procedure currently adopted by the WADA to give an atypical or a positive result in case of longitudinal studies evaluation (CV% $\geqslant$30 for males and CV% $\geqslant$60 for females). Specifically for each subject the coefficient of variation was calculated considering all the measurements of the samples obtained before and after SERMs administration. In addition the mean concentrations value obtained before drug administration (individual reference baseline value) were compared with every single value obtained after the administration [6].

2. Materials and methods

2.1. Standards, chemicals and reagents

Tamoxifen (Nolvadex®) was purchased from AstraZeneca (Milan, Italy). Toremifene (Fareston®) was purchased from Orion Pharma (Milan, Italy). Clomiphene (Clomid®) was purchased from Bruno Farmaceutici S.p.A. (Rome, Italy).

Androsterone and etiocholanolone were supplied by Sigma–Aldrich (Milan, Italy).

Testosterone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol, epitestosterone, 4-androstenedione, deuterated testosterone, deuterated epitestosterone, deuterated androsterone and deuterated etiocholanolone (testosterone d3, epitestosterone d3, androsterone d4, etiocholanolone d5 used as internal standards) were purchased from NMI (National Measurement Institute, Pymble, Australia).

The luteinizing hormone and the follicle-stimulating hormone were purchased from NIBSC (National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK).

All chemicals (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate, *tert*-butylmethylether) were from Carlo Erba (Milan, Italy). The enzyme β-glucuronidase from *Escherichia coli*, needed for the enzymatic hydrolysis of glucuro-conjugates, was purchased from Roche (Monza, Italy).

The derivatizing agent was a mixture of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide/ammonium iodide/dithierythritol (1000:2:4:v/w/w) stored in screwed cap vials at 4 °C for maximum 2 weeks. *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was supplied by Alfathech (Genova, Italy). Ammonium iodide (NH_4I) and dithierythritol (DTE) were supplied from Sigma–Aldrich (Milan, Italy).

The artificial urine was prepared following the protocol described by Leinonen et al. [7].

2.2. Administration study and data analysis

Excretion studies were performed on four male and four female subjects (age: 25–43 years; weight: 50–85 kg). Written consents were obtained from patients allowing the use of urine samples for research purposes (observational study).

To obtain the individual baseline urinary variability of endogenous androgen steroids, urine samples were collected for 3 days before oral administration of multiple doses of SERMs (control samples). Whereas after treatment with tamoxifen (Nolvadex®; 80 mg for 2 days; administered at 8:00 a.m. by male subjects 1 and 2 and female subjects 3 and 4), toremifene (Fareston®; 120 mg for 2 days; administered at 8:00 a.m. by male subjects 1 and 2 and female subjects 3 and 4) or clomiphene (Clomid®; 100 mg for 2 days; administered at 8:00 a.m. by male subjects 5 and 6 and female subjects 7 and 8) separately, the urine samples were collected for at least 5 days. Before and after the treatment, the urine samples were collected every three hours, starting with the morning urine (usually around 8:00 a.m.) and finishing with the last urine in the evening (usually around 8:00 p.m.).

The urine samples were stabilized with sodium azide (1 mg/mL) and stored at −20 °C to prevent changes in the concentrations of steroids caused by bacterial contamination or thermal degradation [8–12]. All concentration values were adjusted for a value of the specific gravity of 1.020, following the guidelines reported in the WADA Technical Document-TD2004 EAAS "Reporting and evaluation guidance for testosterone, epitestosterone, T/E ratio and other endogenous steroids" [6].

The following endogenous compounds were considered: 4-androstenedione (4-AD), testosterone (T), epitestosterone (E), androsterone (Andro), etiocholanolone (Etio), 5α-androstan-3α, 17β-diol (5a-diol), 5β-androstan-3α,17β-diol (5b-diol), luteinizing hormone (LH) and the follicle-stimulating hormone (FSH).

The urinary concentrations of each compound measured before and after oral administration of multiple doses of SERMs were plotted versus the urine collection time. Specifically for each collection the urinary concentration mean value of the two male or two female subjects taking the same drug was plotted versus the correspondent collection time. Data were then evaluated using the Student *t*-test ($p < 0.05$).

Finally our measurements were evaluated taking into account the procedure currently used by the WADA to establish an atypical or a positive result in case of longitudinal studies evaluation (CV% $\geqslant$30 for males and CV% $\geqslant$60 for females) [6]. Specifically for each subject the coefficient of variation was calculated considering all the measurements of the samples obtained before and after SERMs administration. In addition the mean concentrations values obtained before drug administration (individual reference baseline value) were compared with every single value obtained after the administration.

2.3. Sample pre-treatment

Both control samples (used to set up the endogenous steroids individual baseline variability) and excretion study samples (used to study the effect of SERMs on the endogenous steroids) were pre-treated according to a specific procedure accredited according to ISO17025 and presently followed by the WADA-accredited anti-doping laboratory of Rome [12,13]. Briefly, to 3 mL of urine, 1 mL of phosphate buffer (1 M, pH 7.4), 50 μL of β-glucuronidase from *E. coli* and 10 μL of the internal standards solution (17α-methyltestosterone 12 μg/mL, deuterated androsterone 120 μg/mL, deuterated etiocholanolone 120 μg/mL, deuterated testosterone 4 μg/mL and deuterated epitestosterone 1 μg/mL) were added and incubated for 1 h at 50 °C. After hydrolysis, 1 mL of carbonate/bicarbonate buffer (0.8 M, pH 9) was added to alkalinize the sample and the extraction was carried out with 10 mL of *tert*-butylmethylether for 6 min on a mechanical shaker. After centrifugation, the organic layer was transferred to a 10 mL tube and evaporated to dryness. The residue was then derivatized, at 70 °C for 20 min, using 50 μL of a mixture of MSTFA/NH_4I/DTE (1000:2:4:v/w/w) and 1 μL of the derivatized extract was injected directly into the GC–MS.

2.4. GC–MS procedure

Quantitative analysis by GC–MS of excreted steroids was performed on an Agilent Technologies 6890 series gas chromatographer

coupled to a 5973 MSD quadrupole mass spectrometer (Hewlett–Packard, Cernusco sul Naviglio, Milano, Italy), in electron impact ionization (70 eV), using a 17 m fused silica capillary column cross-linked methyl silicone (HP1), ID 0.20 mm, film thickness 0.11 μm. The carrier gas was helium (flow rate: 1 mL/min, split ratio 1:10), and the temperature program was as follows: 180 °C (hold 4.5 min), 3 °C/min to 230 °C, 20 °C/min to 290 °C, 30 °C/min to 320 °C; the transfer line temperature was set at 280 °C. Acquisition was carried out in selected ion monitoring (SIM). The diagnostic ion *m*/*z* 432 was monitored for the quantitative analysis of both testosterone and epitestosterone. The diagnostic ion *m*/*z* 434 was monitored for the quantitative analysis of both androsterone and etiocholanolone. The diagnostic ion *m*/*z* 241 was monitored for the quantitative analysis of both 5α-androstan-3α,17β-diol and 5β-androstan-3α,17β-diol. Whereas the diagnostic ion *m*/*z* 430 was monitored for the quantitative analysis of 4-androstenedione.

The values of the urinary concentration of testosterone and epitestosterone were calculated by the peak areas of the detected signals relative to the internal standards deuterated testosterone and epitestosterone (*m*/*z* 435). The values of the urinary concentration of androsterone and etiocholanolone were calculated by the peak areas of the detected signals relative to the internal standards deuterated androsterone (*m*/*z* 438) and etiocholanolone (*m*/*z* 439), respectively. Whereas the values of the urinary concentration of 4-androstenedione, 5α-androstan-3α,17β-diol and 5β-androstan-3α, 17β-diol were calculated by the peak areas of the detected signal relative to the internal standard 17α-methyltestosterone (*m*/*z* 301).

2.5. Calibration curve and calibration sample

The calibration curve was obtained fortifying aliquots of 3 mL of the artificial urine, with 4-androstenedione, androsterone, etiocholanolone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol, testosterone and epitestosterone at 10 concentration levels. Specifically, the concentrations ranged from 2 to 200 ng/mL for 4-androstenedione, 5α-androstan-3α,17β-diol, 5β-androstan-3α, 17β-diol, testosterone and epitestosterone whereas for androsterone and etiocholanolone the concentrations ranged from 100 to 10,000 ng/mL.

For the daily calibration of the GC–MS instrument, a calibration sample (CS), obtained adding to 3 mL of the artificial urine all the compounds considered in this study (50 ng/mL final concentration for 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol and

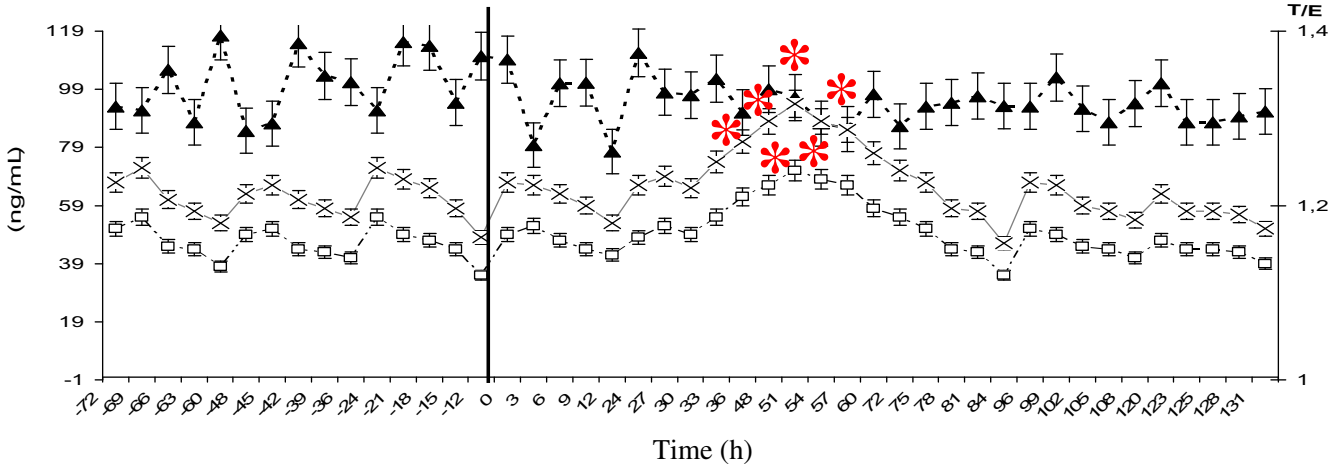

Fig. 1A. Testosterone (X, mean ± SD), epitestosterone (□, mean ± SD) and T/E ratio (▲, mean ± SD) urinary profiles before and after the oral administration of multiple doses (at $t = 0$ and $t = 24$) of tamoxifen in two males (subjects 1 and 2). Statistical significance of the effect ($p < 0.05$) is indicated by the asterisks.

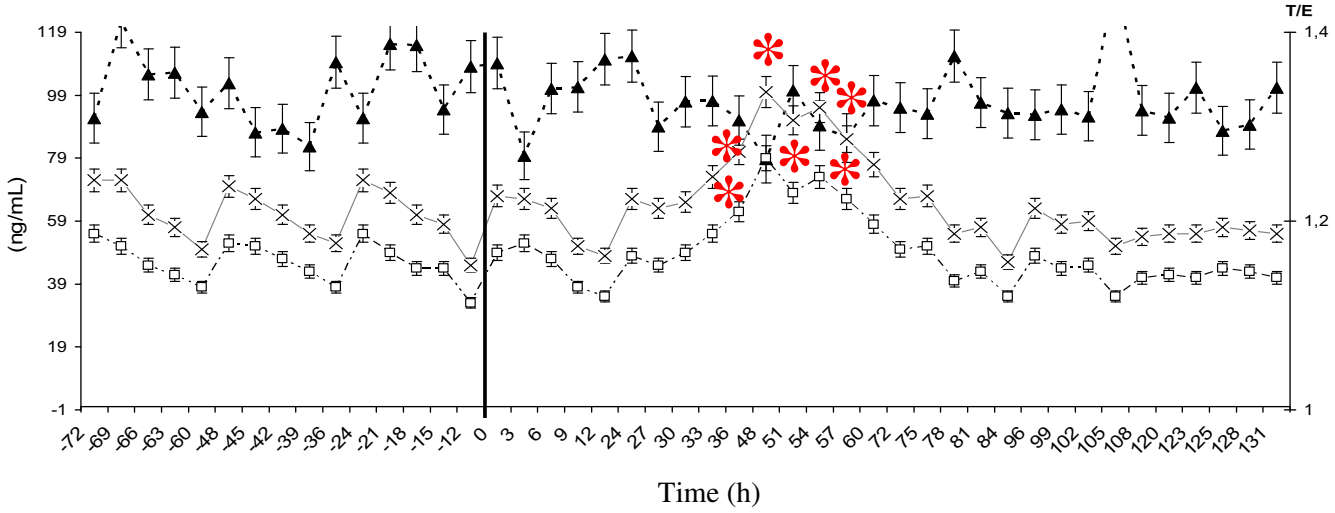

Fig. 1B. Testosterone (X, mean ± SD), epitestosterone (□, mean ± SD) and T/E ratio (▲, mean ± SD) urinary profiles before and after the oral administration of multiple doses (at $t = 0$ and $t = 24$) of toremifene in two males (subjects 1 and 2). Statistical significance of the effect ($p < 0.05$) is indicated by the asterisks.

testosterone; 10 ng/mL final concentration for epitestosterone and 4-androstenedione; 2000 ng/mL final concentration for androsterone and etiocholanolone).

2.6. Chemiluminescent immunometric assay

The determinations of LH and FSH were obtained by an Immulite 2000 (DPC instrument), using Siemens FSH and LH chemiluminescent immunoassay kits (Siemens Medical Solutions Diagnostic, Los Angeles, USA). The detection was made by means of LUMIGEN® PPD [4-metoxy-(3-phosphatephenyl)-spiro-(1,2-dioxethane-3,2′-adamantane)] substrate. The calibration of the immunoanalyzer was performed using the calibrators provided by the manufacturer

3. Results

3.1. Individual baseline

Figs. 1A–C and 2A–C show that in both sexes the excretion of testosterone and epitestosterone was maximal in the morning and decreased along the day reaching a minimum value in the evening. The same circadian trend was registered for 4-androstenedione, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol, androsterone and etiocholanolone (data not shown). The variation in T/E ratio along the day was less than 20% in both males and females (see Figs. 1A–C and 2A–C). Whereas the Andro/Etio and the 5a-diol/5b-diol ratios were the most stable parameters (circadian variability less than 10%, data not shown).

No significant differences were recorded on the urinary levels of the 4-androstenedione, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol, testosterone, epitestosterone, androsterone and etiocholanolone (in males and females) for samples collected from the same subject at the same time in different days; on the contrary in both sexes, marked inter-individual differences were measured for the endogenous steroids considered, confirming the data obtained by previous studies [13–16].

Finally the urinary luteinizing hormone and follicle-stimulating hormone showed a fluctuation along the day higher than 30% for both sexes (data not shown). High variability was registered, also, for samples collected in different days at the same time and to the same subject.

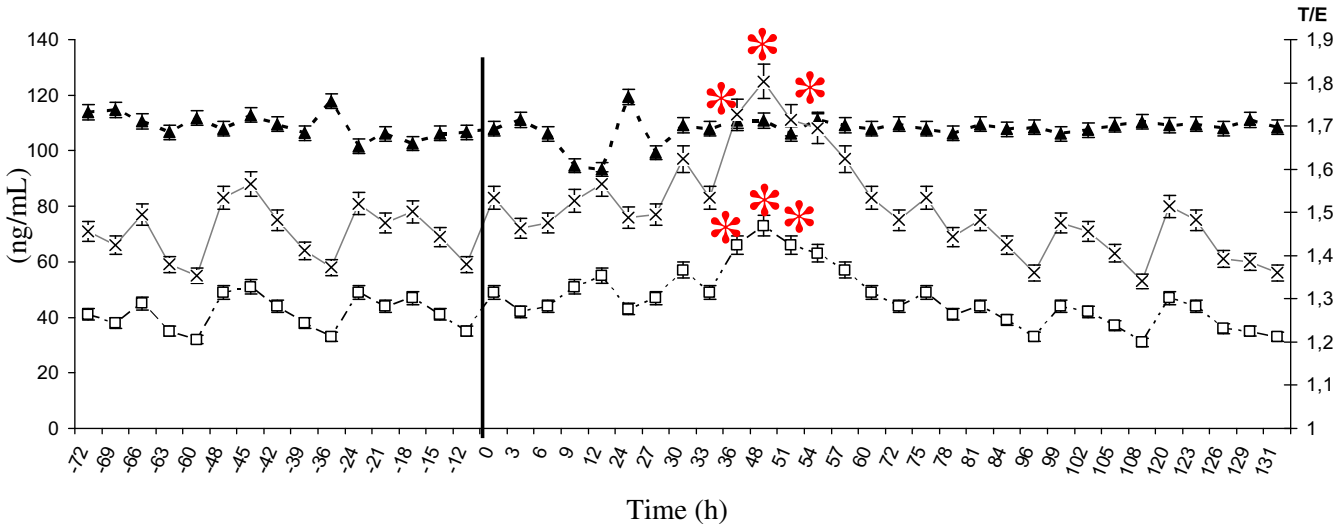

Fig. 1C. Testosterone (X, mean ± SD), epitestosterone (□, mean ± SD) and T/E ratio (▲, mean ± SD) urinary profiles before and after the oral administration of multiple doses (at $t = 0$ and $t = 24$) of clomiphene in two males (subjects 5 and 6). Statistical significance of the effect ($p < 0.05$) is indicated by the asterisks.

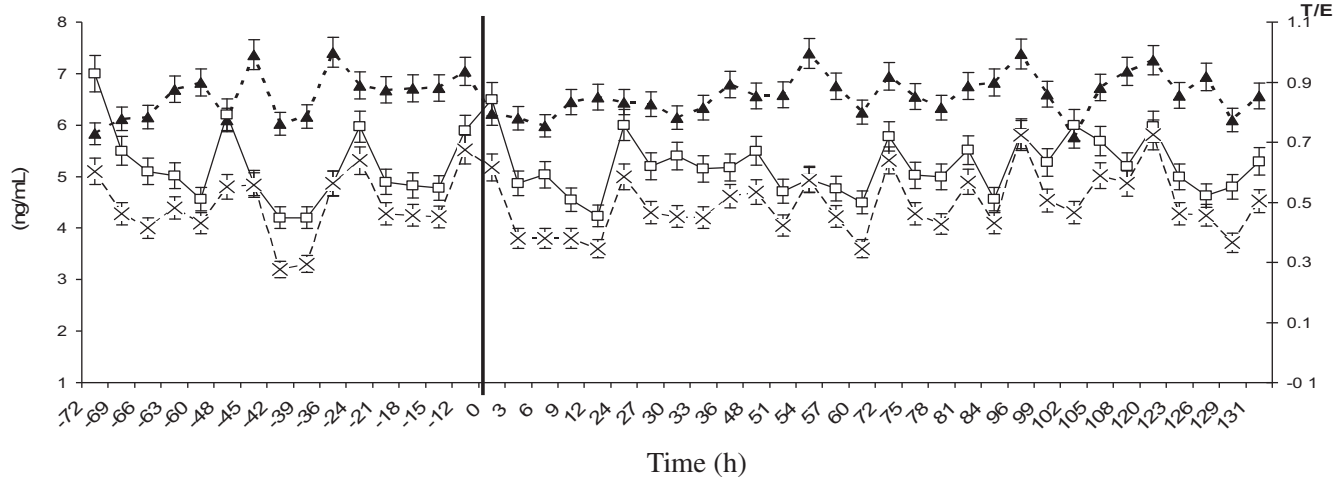

Fig. 2A. Testosterone (X, mean ± SD), epitestosterone (□, mean ± SD) and T/E ratio (▲, mean ± SD) urinary profiles before and after the oral administration of multiple doses (at $t = 0$ and $t = 24$) of tamoxifen in two females (subjects 3 and 4).

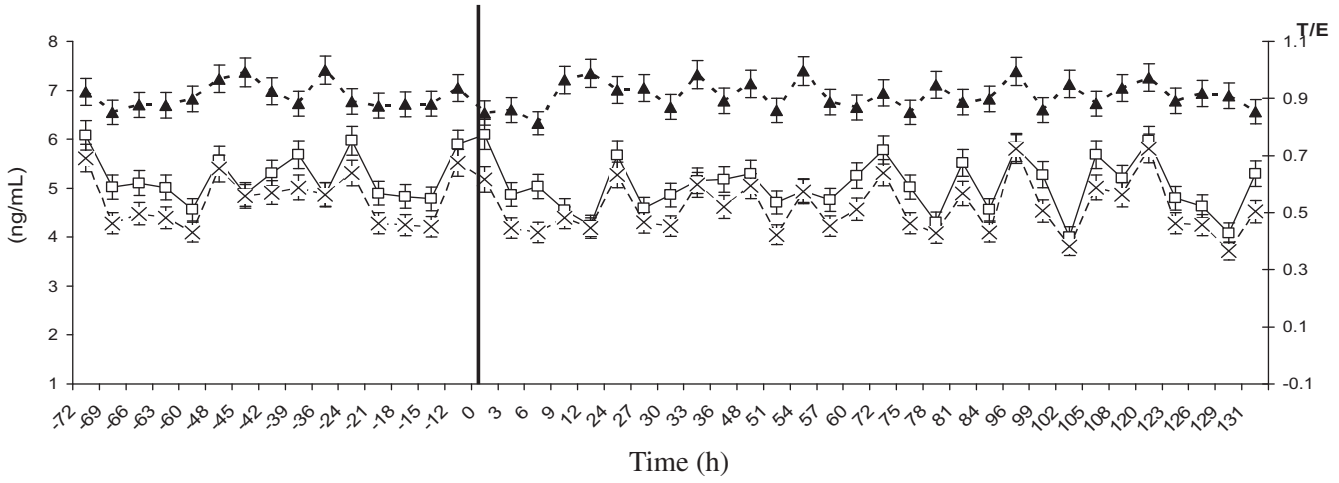

Fig. 2B. Testosterone (X, mean ± SD), epitestosterone (□, mean ± SD) and T/E ratio (▲, mean ± SD) urinary profiles before and after the oral administration of multiple doses (at $t = 0$ and $t = 24$) of toremifene in two females (subjects 3 and 4).

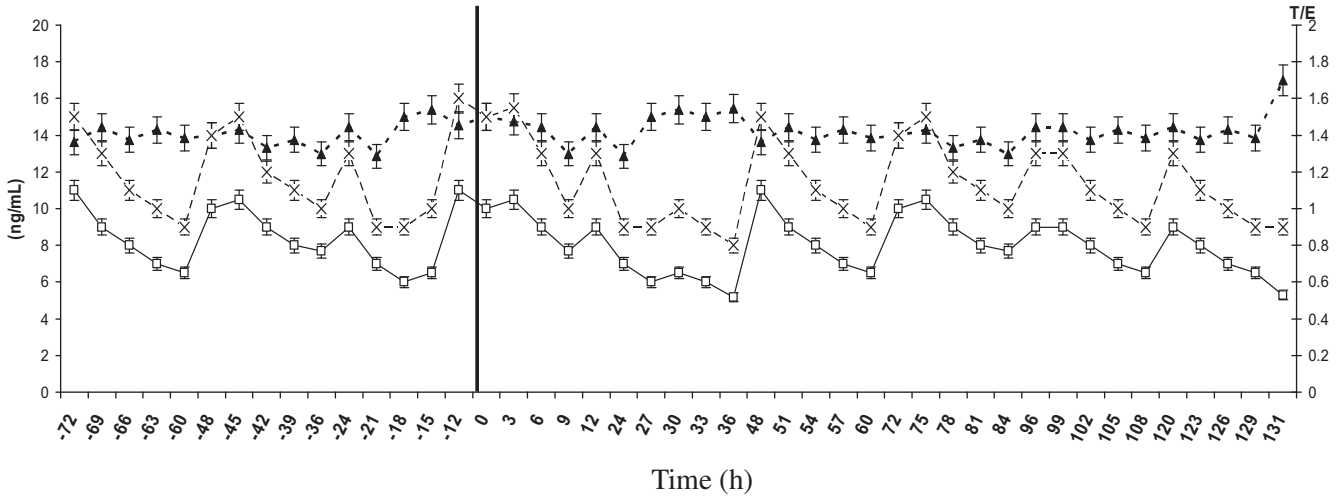

Fig. 2C. Testosterone (X, mean ± SD), epitestosterone (□, mean ± SD) and T/E ratio (▲, mean ± SD) urinary profiles before and after the oral administration of multiple doses (at $t = 0$ and $t = 24$) of clomiphene in two females (subjects 7 and 8).

Table 1
Percentage of variation of testosterone urinary levels measured 24 h before and after the oral administration of multiple doses of tamoxifen, toremifene or clomiphene from the testosterone mean value obtained in the individual baseline experiments for the male subjects 1, 2 and 5.

	Tamoxifen (subject 1)					Toremifene (subject 2)					Clomiphene (subject 5)				
	t_0					t_0					t_0				
	−24 h	−21 h	−18 h	−15 h	−12 h	−24 h	−21 h	−18 h	−15 h	−12 h	−24 h	−21 h	−18 h	−15 h	−12 h
% from the mean of individual baseline	−2	7	5	4	−3	−3	3	5	−2	−4	−2	5	−9	−12	−5
	t_1 (80 mg at 8:00 a.m) 1st administration					t_1 (80 mg at 8:00 a.m) 1st administration					t_1 (80 mg at 8:00 a.m) 1st administration				
	0 h	3 h	6 h	9 h	12 h	0 h	3 h	6 h	9 h	12 h	0 h	3 h	6 h	9 h	12 h
% from the mean of individual baseline	−3	6	5	5	−4	−4	5	4	4	−2	−4	5	4	4	−2
	t_2 (80 mg at 8:00 a.m) 2nd administration					t_2 (80 mg at 8:00 a.m) 2nd administration					t_2 (80 mg at 8:00 a.m) 2nd administration				
	24 h	27 h	30 h	33 h	36 h	24 h	27 h	30 h	33 h	36 h	24 h	27 h	30 h	33 h	36h[a]
% from the mean of individual baseline	−2	7	1	13	25	2	5	3	10	21	2	5	7	15	31
	t_3					t_3					t_3				
	48h[a]	51h[a]	54h[a]	57h[a]	60 h	48h[a]	51h[a]	54h[a]	57h[a]	60 h	48h[a]	51h[a]	54h[a]	57 h	60 h
% from the mean of individual baseline	35	46	40	35	20	44	53	48	36	25	51	45	48	22	18
	t_4					t_4					t_4				
	72 h	75 h	78 h	81 h	84 h	72 h	75 h	78 h	81 h	84 h	72 h	75 h	78 h	81 h	84 h
% from the mean of individual baseline	13	9	5	−5	−7	15	4	5	−7	−9	10	4	5	−7	−9

[a] CV% >30.

3.2. Steroid profile after administration of multiple doses of SERMs

The results obtained comparing the steroids urinary levels measured before and after the oral administration of multiple doses of SERMs (tamoxifen, toremifene or clomiphene) showed that significant variations from the individual baseline values, generally after second day of drug administration, were recorded for 4-androstenedione, testosterone and epitestosterone only in male subjects. Not significant differences from individual baseline values were instead registered for luteinizing hormone, follicle-stimulating hormone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol, androsterone and etiocholanolone urinary levels and for 5α-androstan-3α,17β-diol/5β-androstan-3α,17β-diol, testosterone/epitestosterone and androsterone/etiocholanolone ratios. More specifically Fig. 1A–C show the urinary profile of testosterone, epitestosterone and T/E ratio (mean value ± standard deviation) in male subjects before and after the oral administration of multiple doses of SERMs. We can notice that after second day of drug administration significant variations from the baseline values were recorded for testosterone and epitestosterone; whereas no significant differences were measured for T/E ratio. Fig. 2A–C show the urinary profiles of testosterone, epitestosterone and the T/E ratio (mean value ± standard deviation) in female subjects before and after oral administration of multiple doses of SERMs. We can notice that in this case after the oral administration of multiple doses of SERMs no significant variations from the baseline values were registered for testosterone, epitestosterone and T/E ratio.

4. Discussion and conclusions

At present, and to the best of our knowledge, little is known about the effect of the administration of SERMs on the normal circadian fluctuation of the endogenous hormonal parameters that are presently selected as markers of an atypical urinary steroid profile in doping field [6]. Data collected in this study have shown that in male subjects the relative urinary concentrations values of testosterone were significantly altered generally the second day of oral administration of multiple doses of tamoxifen, toremifene or clomiphene. The same finding was not observed in women, because in this case the blood testosterone is derived mainly from extra-gonad sources (adrenal and extra-glandular) that are not directly involved in any homeostatic feedback regulation and thus not influenced by SERMs action.

In summary, after oral administration of multiple doses of tamoxifen, toremifene or clomiphene:

(i) The 4-AD, T and E urinary concentrations increased significantly only in males. This might be due to the blockage of the negative feedback mechanism on the hypothalamus–pituitary–gonads axis.
(ii) No significant variations were measured for the urinary concentrations of androsterone, etiocholanolone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol and Andro/Etio and 5a-diol/5b-diol ratios in both males and females.
(iii) No variations were recorded for the urinary T/E ratio values. The blockage of the feedback mechanism on the hypothalamus-pituitary-gonads axis, seems not to modify the production of T and E from their precursor 4-AD.
(iv) In male subjects, after second day from oral administration of multiple doses of SERMs, the calculated coefficient of variation from the baseline value was significantly higher than 30% for testosterone (see Table 1) and epitestosterone, whereas for androsterone, etiocholanolone, 5α-androstan-3α,17β-diol, 5β-androstan-3α,17β-diol and the Andro/Etio, 5a-diol/5b-diol and T/E ratios the calculated coefficient of variation from the baseline values was lower than 30%.
(v) In female subjects, after oral administration of multiple doses of SERMs, the calculated variation from the baseline values was for all parameters studied lower than the coefficient of variation (CV% 60) currently employed by WADA to give an atypical or a positive result in case of longitudinal studies evaluation.
(vi) In male subjects the oral administration of multiple doses of SERMs induced a transient increase of the urinary concentration of both LH and FSH after 24 h. Nonetheless this variation was not statistically significant, maybe due to their high intra and inter-days variability.

In conclusion, we have shown that the oral administration of multiple doses of SERMs produced significant alterations in the urinary endogenous steroids profile, and specifically a significant increase in 4-androstenedione, testosterone and epitestosterone urinary concentrations only in males, confirming the fact that in men estradiol (derived from aromatization of testosterone) plays a significant role in negative feedback regulation.

Acknowledgement

This work has been supported in part by a Research Grant of the Italian Department of Health (“Ministero della Salute, Commissione per la vigilanza sul doping e sulla tutela sanitaria delle attività sportive”).

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