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Impact of nonsteroidal aromatase inhibitors on steroid profile in a Chinese population

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Abstract

Steroid profiling was introduced to determine the endogenous steroid misuse in sports. Thus, screening for the exogenous use of these prohibited substances can be established by monitoring a range of endogenous steroids, which constitute the steroid profile and evaluate their concentrations and ratios against reference values. The steroid profiling is currently based on population statistics. As large interindividual variations exist, athlete biological passport (ABP) analysis is ongoing. This study aimed to identify new biomarker(s) for aromatase inhibitor detection in sports using statistical analysis and adapt the model into ABP analysis.

Forty-one Chinese nonathlete volunteers (21 males and 20 females) were administered 3 nonsteroidal aromatase inhibitors (aminoglutethimide, letrozole, and anastrozole) independently. Statistical analysis was performed on 16 steroid profile parameters.

After administration, the concentrations of endogenous androgen biomarkers including testosterone (T), epitestosterone, androsterone (AN), etiocholanolone (ETIO), 5α-diol, 5β-diol, and dehydroepiandrosterone were increased, while the level of estrogen was decreased. These biomarkers returned to the baselines levels within 1 month. In females, the concentrations of endogenous biomarkers were affected by nonsteroidal aromatase inhibitors, without a common trend. Three new endogenous biomarkers (AN/estrone, ETIO/estrone, and T/estrone) elevated significantly after treatment. The 3 new models were more sensitive than the World Anti-Doping Agency ratio biomarkers. They were also effective in exponentially weighted moving average chart analysis.

Verification experiment demonstrated that the biomarker T/estrone was valid in judging the steroidal aromatase inhibitor abuse. The screening of these new endogenous biomarkers can provide additional parameters to support ABP monitoring and specific information regarding the administered steroids.

Abbreviations: ABP = athlete biological passport, AN = androsterone, DHEA = dehydroepiandrosterone, DHT = dihydrotestosterone, ET = epitestosterone, ETIO = etiocholanolone, EWMA = exponentially weighted moving average, GC-MS = gas chromatography–mass spectrometry, IOC = International Olympic Committee, ISTD= Internal Standard, LOQ = limit of quantification, T = testosterone, TD = technical document, TMS = trimethylsilyl, WADA = World Anti-Doping Agency.

Keywords: doping, nonsteroidal aromatase inhibitors, steroid profiling

1. Introduction

Androgenic steroids are ergogenic drugs enhancing the muscular mass and strength in both male and female athletes.[1,2] Consequently, androgens are banned for usage in sports by the World Anti-Doping Agency (WADA).[2,3] To circumvent the imposed ban, several strategies have been developed; for instance, the use of aromatase inhibitors.[3] Aromatase inhibitors are widely used in clinical settings and are of 2 types: steroids and nonsteroids.[4] Exemestane is the representative of steroidal aromatase inhibitors in the clinic.[4] Aminoglutethimide, letrozole, and anastrozole are the most commonly available nonsteroid aromatase inhibitors.[4]

Aromatase (estrogen synthetase) is omnipresent in several human tissues, and it catalyzes the conversion of androgens to estrogens. In addition, it converts the adrenally generated androstenedione into estrone and testosterone into estradiol in peripheral tissues, as well as in tumors.[5] Aromatase inhibitors can suppress the aromatase activity by competitively binding to the heme of its cytochrome P450 subunit.[6,7] They are commonly employed for the treatment of postmenopausal women with hormone-sensitive breast cancer.[8] After oral administration, increased serum testosterone levels have been observed.[7] Hence, male athletes use aromatase inhibitors to improve athletic performance and treat the adverse effects of an extensive abuse of anabolic androgenic steroids (gynecomastia). Therefore, the usage of aromatase inhibitors was prohibited by the International Olympic Committee and WADA[1] for male and female athletes in September 2001 and January 2005, respectively.[9]

Although gas chromatography–mass spectrometry (GC-MS) with trimethylsilyl derivatization is traditionally used for the detection of aromatase inhibitors and related metabolites, this method is limited due to the detection window of the elimination

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half-time, the timing of sample collection, and sophistication of some doping regimens.[10] The detection of misuse of prohibited substances is a great challenge for doping control laboratories. Steroid profiling was adapted and introduced to determine endogenous steroid misuse in sports.[11,12] The urinary steroid profile is composed of concentrations and ratios of various endogenously produced steroidal hormones as well as their precursors and metabolites, including but not limited to testosterone (T), epitestosterone (ET), dihydrotestosterone (DHT), androsterone (AN), etiocholanolone (ETIO), dehydroepiandrosterone (DHEA), 5α-androstane-3α,17β-diol (5α-diol), 5β-androstane-3α,17β-diol (5β-diol), and estrone.[13–15] Moreover, the ratios of selected steroids such as T/ET, AN/T, 5α-diol/5β-diol, AN/ETIO, and 5α-diol/ET are considered valuable markers in an athlete biological passport (ABP), which could be used to indicate doping product abuse.[16,17] In early 2012, 25 antidoping organizations implemented the ABP biomarkers according to WADA requirements. Since then, ABP plays a major role in doping control and represents a highly promising complementary approach to traditional drug testing, as, by definition, doping aims to change the physiology of the athlete, and ABP precisely aims to detect these changes.[18] The abbreviation and ion traces of these substances are illustrated in Table 1. Steroid profiling is a well-known method of clinical endocrinology, used for the detection of enzyme deficiencies.[19,20] Several studies have shown that steroid profile parameters, especially the steroid ratios used for doping control purposes, are extremely stable.[21,22] The intake of exogenous substances which affect the metabolic pathways can alter the steroid profile.

With the aim to assess the impact of nonsteroidal aromatase inhibitor intake on steroid profile, human subjects administered the above-mentioned 3 drugs were evaluated. Interestingly, some new biomarkers for ABP evaluation were obtained due to the effects of exogenous substances on various metabolic pathways.

2. Materials and methods

2.1. Design of excretion study

This is a prospective, randomized, single-blinded, interventional study conducted between December 2014 and January 2016. Steroid administration was approved by the Ethics Committee of CHINADA. All volunteers were fully informed by seminars and written materials, and they provided signed consent forms.

Table 1

Abbreviation and ion traces (m/z) of endogenous steroids monitored in steroid profiling (as TMS derivatives).

Substance	Abbreviation	m/z
Androsterone, bis-TMS	AN	434
Etiocholanolone, bis-TMS	ETIO	434
5α-androstane-3α,17β-diol, bis-TMS	5α-diol	241
5β-androstane-3α,17β-diol, bis-TMS	5β-diol	241
Dehydroepiandrosterone, bis-TMS	DHEA	432
Epitestosterone, bis-TMS	ET	432
Testosterone, bis-TMS	T	432
Methyltestosterone, bis-TMS (ISTD)	MT	446
Estrone, bis-TMS	Estrone	414

TMS = trimethylsilyl.

In total, 48 nonathlete healthy volunteers (24 males and 24 females) at Beijing Sports University and Beijing University of Chinese Medicine were enrolled. Before inclusion, all the volunteers underwent a routine physical examination. Then, the volunteers were randomly divided into 3 groups; each group consisted of 8 male and 8 female subjects. A stratified-block randomized trial was applied for this study to ensure that the number and gender of subjects were similar in each group. During the excretion study, 7 individuals dropped out after randomization for personal reasons (Fig. 1). They were traveling for a business trip, and it was impossible for them to store the urine samples in the icebox and deposit them in time). Finally, 41 volunteers completed the excretion study, with each group comprising of at least 6 males and 6 females.

2.2. Interventions and sample collection

Finally, there were 12 (6 males and 6 females) subjects in Group A, 14 (8 males and 6 females) subjects in Group B, and 15 (7 males and 8 females) in Group C who completed the study. Subjects in Group A received 2 daily oral dosages of aminoglutethimide (250 mg, 100 tabs; Nantong General Pharmaceutical Factory, China) at 500 mg/day for 3 consecutive days. Subjects in Groups B and C were administered letrozole (2.5 mg/day) (2.5 mg, 10 tab; Hengshun General Pharmaceutical Factory, China) and anastrozole (1 mg/day) (1.0 mg, 14 tab; AstraZeneca Pharmaceuticals LP, USA) for 5 consecutive days, respectively. All drugs were administered at 9 am every day, while the other dose in Group A was taken at 9 pm daily. All subjects were blinded for their drugs. Before treatment, the volunteers produced 4 daily blank urine samples (morning, noon, afternoon, and evening) for 7 days. During treatment and 3 successive days, urine samples were also collected at the above-mentioned 4 time points. From the 4th to 28th day after treatment, the morning urine samples were collected. All urine samples were stored at –20°C until analysis.

2.3. Sample analysis

Urine analysis method was described by Liu et al.[23] This quantitative GC-MS method measures the free and glucuronidated steroid concentrations in agreement with WADA requirements on endogenous steroids. Briefly, the hydrolysis with β-glucuronidase from *Escherichia coli* was performed for 3 hours followed by diethyl ether extraction and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide/ammonium iodide/ethanethiol derivatization. Seven endogenous androgens were monitored: T, ET, AN, ETIO, 5α-diol, 5β-diol, and DHEA. One endogenous estrogen (estrone) was added to the protocol. Cross-contamination was avoided by the injection of the derivatization mixture before and after the quality control of the sample.

2.4. Data analysis

The accurate integration of every GC-peak area for the monitored steroids was checked manually. All values below the limit of quantification were excluded from the statistical analysis to ensure analytical validity. Samples with steroid concentrations exceeding the highest calibrator were diluted and reanalyzed.

To compare the measured concentrations between different samples, the intersample variability in urine due to dilution was reduced by adjusting the measured values according to the

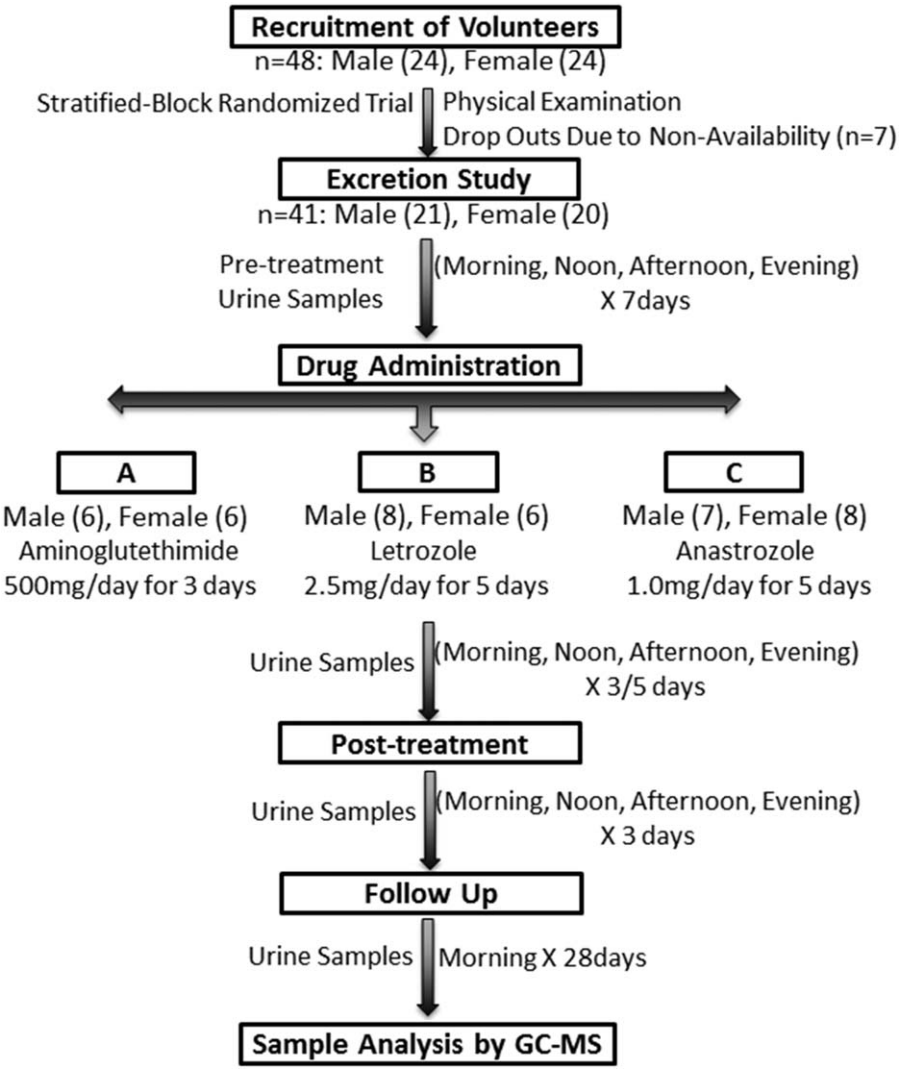

Figure 1. Flowchart of the study.

specific gravity to a value of 1.020 as recommended in the technical document of WADA on endogenous steroids.[1]

$$-C_{1.020} = \frac{(1.020-1)}{\text{Specific gravity}_{\text{sample}} - 1} * C_{\text{sample}}$$

Samples with specific gravities <1.004 were discarded to avoid substantial correction factors for highly diluted urine samples.

2.5. Data evaluation

Intraindividual variations of all biomarkers made it reasonable for the data to be hypothesized as log-normally distributed rather than abnormally distributed. R-studio version 0.99.467 was used for statistical analyses. Results are presented as scatter plots, index ratio from 95% or 99% percentile, and exponentially weighted moving average chart. For statistics, the correlation analysis and rank-sum test were used. The normality of the associations of different markers evaluated in this study for the doping control field (T, ET, AN, ETIO, 5α-diol, 5β-diol, estrone, T/ET, AN/T, 5α-diol/5β-diol, AN/ETIO, 5α-diol/ET, AN/estrone, ETIO/estrone, and T/estrone) was carried out using the Wilcoxon rank-sum test. $P < 0.05$ was considered statistically significant. Moving average charts of the 3 novel markers (AN/estrone, ETIO/estrone, and T/estrone) were generated for each. Control limits (upper control limit and lower control limit) were used to determine when the marker is out of control.

3. Results

3.1. Physical examination of volunteers

For the 41 subjects, the mean age was 22.5 ± 3.7 years, and the average body weight was 64 ± 15 kg. None of them presented endocrine disorder, chronic disease history, and combined drug use. Moreover, they were not registered players. For the physical examination, there were no significant differences in the subjects' kidney, liver, and other functions among the groups.

3.2. Concentrations of endogenous biomarkers

For males, the concentrations of endogenous biomarkers (T, ET, AN, ETIO, 5α-diol, 5β-diol, DHEA, and estrone) fluctuated within a small range before treatment. After treatment, the

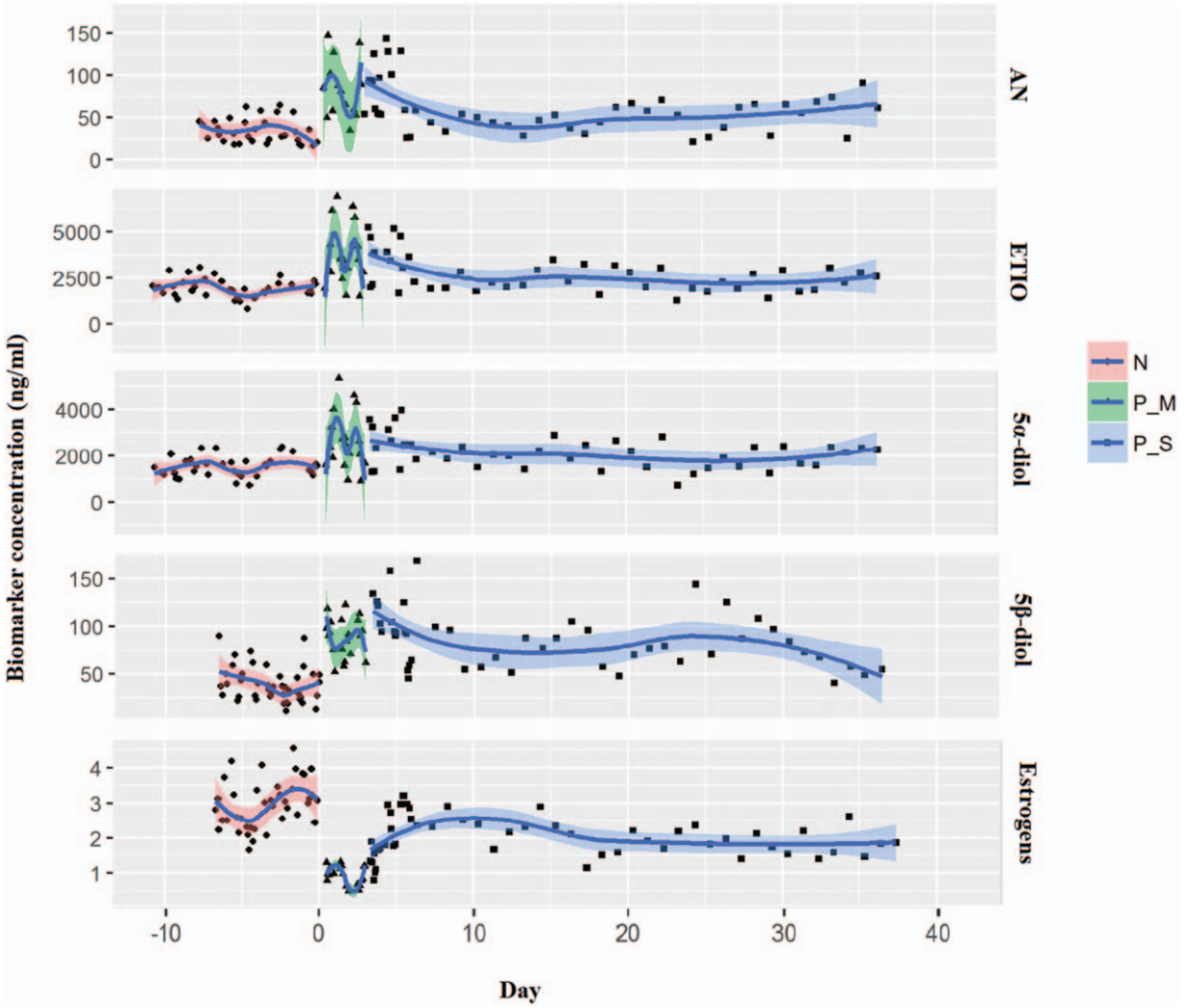

Figure 2. Trends of AN, ETIO, 5α-diol, 5β-diol, and estrogens concentration changes in male subjects after drug administration. AN = androsterone, ETIO = etiocholanolone.

concentrations of endogenous androgen biomarkers such as AN, ETIO, 5α-diol, 5β-diol, and DHEA increased to some extent, while the levels of estrogens (such as estrone) decreased notably. The concentration changes of T, ET, and DHEA did not show a general trend. All values returned to normal level within 1 month. The typical trends of AN, ETIO, 5α-diol, 5β-diol, and estrogens concentration changes in male subjects is shown in Fig. 2. In the case of females, the concentrations of endogenous biomarkers were also influenced by nonsteroidal aromatase inhibitors. However, no general trend was found among them. Hence, the variations were less regular in females than males.

3.3. Diagnostic ratio biomarkers

In this study, WADA TD ratios were applied to analyze the data after administration. The scatter plots revealed that T/ET trend began to rise soon after treatment among B and C groups (Appendix 1, http://links.lww.com/MD/B784). The trend also can be observed in 5α-diol/5β-diol of group A (Appendix 2, http://links.lww.com/MD/B784). As for other ratios including AN/T, AN/ETIO, and 5α-diol/ET, they slightly increased but returned to normal level within 5 days after treatment. No common trend can be observed in the established WADA ratio biomarkers. However, the present study found that the ratio of the 3 new biomarkers (T/estrone, AN/estrone, and ETIO/estrone) increased sharply after the drug administration and the trend lasted about 15 to 20 days. Moreover, the trend was similar in both male and female (Fig. 3) subjects.

3.4. Percentile estimation and detection rates

Percentiles play a critical role in descriptive statistics of continuous data, and their use is recommended for estimating suspicious samples. In accordance with the WADA Technical Document-TD2016EAAS, if the ratio biomarkers exceed the limits, further confirmation procedures should be applied. In this study, 3 new ratio biomarkers (T/estrone, AN/estrone, and ETIO/estrone) were proposed and 5 WADA ratio biomarkers (T/ET, AN/T, 5α-diol/5β-diol, AN/ETIO, and 5α-diol/ET) were evaluated and compared, and the percentiles were calculated for these biomarkers by the blank urine samples from the male and female subjects, respectively. The percentiles calculated at the 95% and 99% levels of confidence intervals (CI) are presented in Tables 2 and 3, respectively. By comparing 2 different evaluation

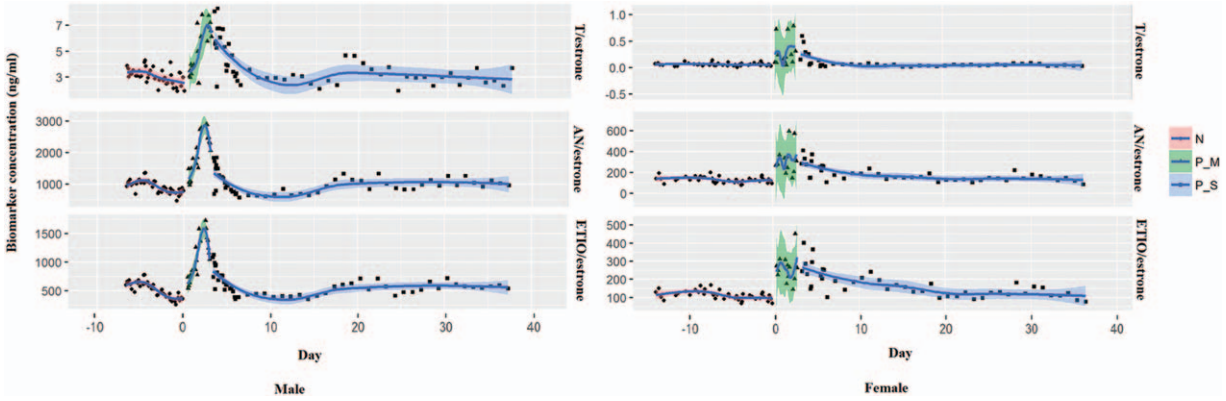

Figure 3. Trends of T/estrone, AN/estrone, and ETIO/estrone changes in male and female subjects after drug administration. AN = androsterone, ETIO = etiocholanolone, T = testosterone.

approaches (3 new models and 5 WADA ratio biomarkers), the average detection rates were increased sharply in 3 new models as follows: T/estrone (35.76%), ETIO/estrone (54.59%), and AN/estrone (52.45%), at the level of 95% CI; T/estrone (24.39%), ETIO/estrone (42.68%), and AN/estrone (40.56%), at the level of 99% CI. By comparison, using WADA EAAS TD, the average detection rates were T/ET (5.53%), 5α-diol/5β-diol (5.45%), AN/T (9.37%), AN/ETIO (4.90%), and 5α-diol/ET (4.14%) at the level of 95% CI; the rates were T/ET (3.25%), 5α-diol/5β-diol (0.29%), AN/T (6.00%), AN/ETIO (2.11%), and 5α-diol/ET (1.16%) at the level of 99% CI. The higher the detection rate was, the longer the detection window limits were. Comparisons of detection rates in different biomarker ratios in 3 study groups are shown in Fig. 4.

3.5. Exponentially weighted moving average control charts for doping control analysis

Exponentially weighted moving average (EWMA) control is a mature theory applied in ABP analysis. In this study, a moving average chart was plotted for the 3 new ratio biomarkers for ABP purpose. In the EWMA chart, the control limits were determined by the negative urine data of the participants. Any out data was considered a suspicious sample. In Group A, all male volunteers at least had at least 2 groups out data after drug administration. For females, half of the samples (AF02, AF04, and AF06) were judged as suspicious. The new ratio biomarkers fluctuated but not exceeding the reference limits in the remaining 3 female volunteers (Appendix 3, http://links.lww.com/MD/B784). In Group B, the data from all volunteers were assessed by the EWMA chart analysis. In addition, the detected window was relatively long in this group (Appendix 4, http://links.lww.com/MD/B784). These ratio biomarkers were effective in analyzing Group C data. Twelve of 14 subjects (except CF06) were considered to be suspicious by this method (Appendix 5, http://links.lww.com/MD/B784). Therefore, the new biomarkers could be a supplement to ABP evaluation due to their impact on steroid profile.

3.6. Verification experiment

To verify the validity of the model, another experiment was designed. Exemestane (25 mg, 30 tabs; Pfizer Italia s.r.l, USA) was administered at a single dose of 25 mg to 1 male volunteer. After

Table 2

Percentiles evaluated by the blank urine samples at a 95% confidence interval level and detection rates of different ratio biomarkers.

Ratio biomarkers	T/Estrone		ETIO/Estrone		AN/Estrone		T/ET		5α-diol/5β-diol		AN/T		5α-diol/ET		AN/ETIO	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Percentile	18.08	1.45	738.85	395.11	1403.44	483.19	1.92	1.00	2.27	1.78	635.73	7021.85	4.15	4.04	3.38	2.51
Group A detection rate, %	7.26	75.00	11.73	85.26	7.82	100.00	11.73	0.00	2.79	4.49	27.93	0.00	3.35	10.30	8.48	8.94
Group B detection rate, %	19.46	38.85	52.40	69.43	40.12	68.79	0.00	0.00	11.98	0.00	1.80	22.29	4.49	0.00	9.05	0.00
Group C detection rate, %	58.02	15.97	84.36	24.37	77.37	20.59	20.58	0.84	0.41	13.03	0.41	3.78	0.00	6.70	0.00	2.94
Average detection rate, %	35.76		54.59		52.45		5.53		5.45		9.37		4.14		4.90	

AN = androsterone, ET = epitestosterone, ETIO = etiocholanolone, T = testosterone.

Table 3

Percentiles evaluated by the blank urine samples at a 99% confidence interval level and detection rates of different ratio biomarkers.

Ratio biomarkers	T/Estrone		AN/Estrone		ETIO/Estrone		T/ET		5α-diol/5β-diol		AN/T		5α-diol/ET		AN/ETIO	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Percentile	22.49	2.02	1838.86	658.49	853.21	482.03	2.20	1.14	2.60	3.26	753.37	9861.66	5.44	5.46	4.05	2.80
Group A detection rate, %	3.91	37.18	2.23	92.95	6.70	59.62	8.38	0.00	0.00	0.00	26.26	0.00	1.12	3.85	0.89	0.00
Group B detection rate, %	14.67	32.48	27.84	53.50	42.81	58.60	0.00	0.00	0.90	0.00	0.60	8.28	0.30	0.00	7.33	0.00
Group C detection rate, %	55.56	2.52	57.61	9.24	74.90	13.45	10.29	0.84	0.00	0.84	0.00	0.84	0.00	1.68	0.00	0.00
Average	24.39		40.56		42.68		3.25		0.29		6.00%		1.16%		2.11	

AN = androsterone, ET = epitestosterone, ETIO = etiocholanolone, T = testosterone.

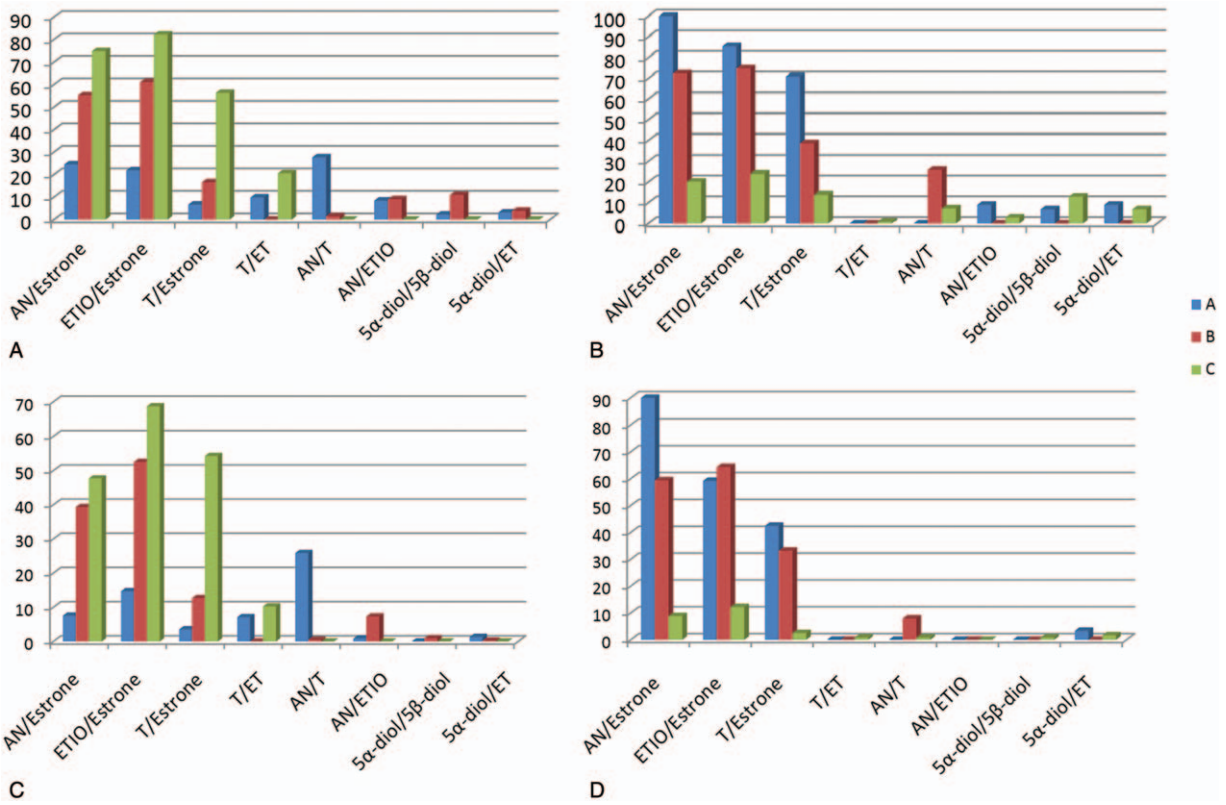

Figure 4. Comparisons of detection rates in 8 different ratio biomarker in 3 study groups with the percentiles calculated by blank urine samples at (A) 95%CI in male subjects; (B) 95%CI in female subjects; (C) 99%CI in male subjects; and (D) 99%CI in female subjects. CI = confidence interval.

administration, all the urine was collected for 3 days. From 4th to 15th day, the morning urine samples were collected. Exemestane was previously monitored by its major metabolite for doping control purpose.[24] Using the current method, the drug could not be detected after 25.5 hours (Appendix 6, http://links.lww.com/MD/B784). However, there was overt change in the EWMA of T/estrone, with detection window increasing from 1 to 10 days (Appendix 7, http://links.lww.com/MD/B784).

4. Discussion

The present study identified 3 new endogenous biomarkers (AN/estrone, ETIO/estrone, and T/estrone) that can be potentially applied to steroid profiling in the determination of steroid misuse in sports. Furthermore, we also demonstrated that biomarker T/estrone was significantly ($P < .05$) sensitive in the determination of steroidal aromatase inhibitor misuse.

Several therapeutic dose administration studies were carried out with a comprehensive steroid profiling method, evaluating population-based reference ranges for 16 parameters. However, the concentration range of the urinary anabolic steroids is highly variable depending on the origin of the athlete.[25,26] Therefore, the estimation of the ratio with endogenous steroids can be valuable for antidoping purpose.[27] To eliminate the influence of gravity in different urine samples of various geographical origin, the ratio biomarkers have been established to detect steroid misuse in sports.[28] WADA Technical Document-TD2016EAAS chooses ratios such as T/ET, AN/T, 5α-diol/5β-diol, AN/ETIO, and 5α-diol/ET as a basis for defining suspicious samples.[1] These ratio biomarkers are used in ABP analysis. ABP is an individual electronic document that stores any information valuable for the interpretation of indirect evidence of doping.[29] However, these biomarkers, which are included in ABP currently, are concerned with the ratio of androgen only.

In this study, we compared every 2-concentration biomarkers (T, ET, AN, ETIO, DHEA, 5α-diol, 5β-diol, and estrone) and selected the most sensitive ratio (AN/estrone, ETIO/estrone, and T/estrone) as the new biomarkers. In our analysis, no common trend was observed among the established WADA ratio biomarkers. Hence, these ratio biomarkers included in WADA TD could not provide any information to judge suspicious samples after administration of nonsteroidal aromatase inhibitors. The ratios of T/estrone, AN/estrone, and ETIO/estrone were significantly affected by aromatase inhibitor intake. We show that these 3 new biomarkers increased sharply after the administration and the trend persisted for 15 to 20 days. Moreover, the trend was similar in both male and female volunteers. After determining the percentile, the EWMA model, to estimate the abuse of aromatase inhibitors, was established. Compared to the ABP biomarkers, these 3 new biomarkers take estrogen into account simultaneously, which makes the trend easier to be observed. Females displayed a larger intraindividual variation in estrone levels as compared to males; however, the new biomarkers were still effective in most women. The minimal detection time for nonsteroidal aromatase inhibitors was 10 days; for a maximal detection time of more than 30 days, the sensitive new biomarkers were utilized. The prolonged detection time for steroidal aromatase inhibitors could play a great supplementary role for metabolite monitoring in doping control routine work. The new biomarkers can also be used for ABP purpose.

Overall, this study indicated that use of nonsteroidal aromatase inhibitors affects both ovarian and adrenal androgens. However, the decision limits of WADA TD do not always show sufficient accuracy to detect substance misuse unambiguously. A combination of the 3 new biomarkers with appropriate reference levels can increase the detection accuracy, and the inclusion of estrogens (such as estrone) in steroid profiling can improve the sensitivity of the currently used biomarkers.

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