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Abstract: The population based Steroid Profile (SP) ratio of testosterone
(T) and epitestosterone (E) has been considered as a biomarker approach
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Laboratories apply the World Antidoping Agency (WADA) Technical Document
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of SP during their screening. The SP Athlete Biological Passport (ABP)
adaptive model uses the concentrations of the total of free and
glucuronide conjugated forms of six EAASs concentrations and ratios
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antidoping male and female urine samples during screening.
Concentrations, statistical parameters and selected ratios for the 6
EAAS, the 6 sulfate EAAS and 29 proposed ratios of concentrations from
both EAAS and sulfate EAAS, which potentially used as SP ABP biomarkers,
population reference limits and distributions have been estimated after
the GC/MSMS analysis for EAAS and LC/Orbitrap/MS analysis for sulfate
EAAS.

Detailed Response to Reviewers

No.	pages	Reviewers Comments	Reply to reviewer's comments for the last submission cycle.
14		Comment: The argument of the author to this question relies upon the presented works of others. The selected steroid ratios in this paper demonstrate little critical reflection of relevant biomarkers; the reviewer believes that at least some of the 37 ratios presented ratios in table 2 are irrelevant for doping analysis. In addition, statistics of other markers present very high variation due to the use of T-S or E-S in the denominator, two compounds with high incidence of below LOQ concentrations. This drastically increases the variation of the presented ratios and the relevance of its statistics, particularly when these concentrations are even more suppressed, which is the reason for putting them in the denominator. If the author does not want to cut in the presented markers, at least the high variance due to analytical limitations should be discussed.	Accepted and corrected according to reviewer's comment . The following text was added to the Results and Discussion section, page 13. Correction: -The T-S and E-S concentrations and ratios biomarkers showed high variation, due to the existence of significant percentages of values below LOQ and LOD (Table 1). In relation to analytical variation based on LOQ, the reduction of variation in E-S and T-S determinations can be achieved in lowering the validated LOQ [32]. The variation of T-S and E-S biomarkers due to samples without measurable MS signals, will be more difficult to be reduced, since the current used MS technology is of high mass accuracy and sensitivity. The intra-individual athlete baseline or after doping application variation of EAAS-S has to be defined in clinical studies. The inter-athlete population variation will play an important role and it has to be determined and considered in a potential incorporation of EAAS-S biomarkers in the ABP steroidal module.
15		Comment: The author prefers to leave the data with a small database, thereby referring to the use of established software and other papers with similar database sizes e.g. P10L52. Instead of this reference, true sample size should be calculated using the formula in paragraph 3.5 of Solberg et al [1]. The correct sample size applied for most of the used markers. If the author does not want to increase the database size, this shortage should be discussed for ratios with T_S in denominator for women. 1.Solberg, H.E., RefVal: A program implementing the recommendations of the International Federation of Clinical Chemistry on the statistical treatment of reference values. Comput. Meth. Programs Biomed.,	Accepted and corrected according to reviewer's comment. The following text was added to the Results and Discussion section, page 10 and 12. Correction: -In another criterion, the RefVal software generates error messages if the number of population samples is below the specification of International Federation of Clinical Chemistry (IFCC) [36, 37] related to the selected confidence interval of the study. In the data processing of the current study by RefVal, no such error messages were generated, consequently the numbers of female and male samples included in the current population are in compliance with both specifications. -The RefVal estimations of Tables 4, 5 and 6 were provided following compliance with the RefVal criterion of $1 / N < \alpha$, where N is the values in the male and female populations [41, 42].

		1995. 48(3): p. 247-256.	-Addition of two references "41.H.E. Solberg, The IFCC recommendation on estimation of reference intervals. The RefVal Program. Clin. Chem. Lab Med (2004)710- 4. https://doi.org/10.1515 /CCLM .2004.121. 42. H.E. Solberg, Approved recommendation (1987) on the theory of reference values 5. Statistical treatment of collected reference values-determination of reference limits. Clin. Chim. Acta (1987) S13–32.https://doi.org/10.1016/0009-8981(87)90151-3."

- Quantitation of intact endogenous sulfate steroids in sports antidoping screening.
- LC/MS sulfates endogenous steroids for WADA Athlete Biological Passport.
- Ratios from sulfate and free-glucuronide concentrations of athlete population.
- Distribution and reference limits for male and female athlete population.

Population Reference Ranges of Urinary Endogenous Sulfate Steroids Concentrations and Ratios as Complement to the Steroid Profile in Sports Antidoping

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Abstract

The population based Steroid Profile (SP) ratio of testosterone (T) and epitestosterone (E) has been considered as a biomarker approach to detect testosterone abuse in '80s. The contemporary Antidoping Laboratories apply the World Antidoping Agency (WADA) Technical Document (TD) for Endogenous Androgenic Anabolic Steroids (EAAS) in the analysis of SP during their screening. The SP Athlete Biological Passport (ABP) adaptive model uses the concentrations of the total of free and glucuronide conjugated forms of six EAASs concentrations and ratios measured by GC/MS. In the Antidoping Lab Qatar (ADLQ), the routine LC/MS screening method was used to quantitatively estimate the sulfate conjugated EAAS in the same analytical run as for the rest qualitative analytes. Seven sulfate EAAS were quantified for a number of routine antidoping male and female urine samples during screening. Concentrations, statistical parameters and selected ratios for the 6 EAAS, the 6 sulfate EAAS and 29 proposed ratios of concentrations from both EAAS and sulfate EAAS, which potentially used as SP ABP biomarkers, population reference limits and distributions have been estimated after the GC/MSMS analysis for EAAS and LC/Orbitrap/MS analysis for sulfate EAAS.

1. Introduction

The sports anti-doping movement is led worldwide by the World Anti-Doping Agency (WADA). WADA publishes every year the Prohibited List Substance, which is the definition of doping [1]. The Prohibited List incorporates several pharmacological classes and examples of drugs as endogenous or synthetic substances. Anabolic Androgenic Steroids (AAS) are the most frequently reported prohibited substances according to the several years' statistics that can be retrieved in the official WADA site report [2]. Externally administrated testosterone (T) and its metabolically related substances, the synthetic forms of the Endogenous AAS (EAAS), are often abused by the athletes in order to avoid urinary detection, because conventional mass spectrometry (MS) the mass spectra of the endogenously produced and the exogenous introduced T are the same and cannot be distinguished, unless Isotope Ratio Mass Spectrometry (IRMS) is used [3,4]. In 1982, the implementation of a population based steroid profile (SP) ratio for T and its epimer epitestosterone (E) has been considered as a biomarker to detect testosterone abuse [5]. After 1982, the SP was enriched by adding more T metabolites, and a plethora of studies have been published to describe the stability of SP, the population reference ranges and conditions that SP changes [3,4]. The anti-doping laboratories accredited by WADA apply the Technical Document for EAAS (TD2018EAAS) [6] in the analysis and evaluation of the SP during their Initial Testing Procedure (ITP). The current SP evaluation is based on individual athlete and athletes' population reference ranges using the Bayesian statistical model developed by Sottas [7-9], which is based on the model used for the blood Athlete Biological Passport (ABP) [10]. The steroidal module of the ABP uses the concentrations of the following EAAS, which are extracted from urine and include total of the free and glucuronide conjugated forms: androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol), T, E and ratios between them T/E, A/T, A/Etio, 5αAdiol/5βAdiol. All EAAS are analyzed by gas chromatography/mass spectrometry (GC/MS) [6, 11-13].

The current SP ABP system has been originated from various studies made in the past. In 1976, Harris *et al.* proposed models of time series data obtained from an individual for clinical purposes [14]. The basis of the time series data evaluation is that each individual has a biological average about which the parameter concentration varies as a function of time. In the anti-doping literature, several reference ranges and SP population statistics have been published, e.g. [15-17] and a thorough review has been published by Ayotte *et al.* [4]. Factors that affect the SP divided into endogenous and exogenous factors and these factors have been reviewed and identified such as, confounding factors as alcohol, masking agents, exogenous steroids [3, 6, 18], genetic polymorphisms [19] or urine dilution [20]. In order to enhance the detectability of T and its prohormones abuse, SP improvement with additional biomarkers has been proposed [17]. The application of ultra-high performance liquid chromatography (UHPLC) on the measurements of blood concentrations of T, its major metabolites, and precursors could be a complementary information of SP for the EAAS [21]. The detection of four T metabolites extracted after urine alkaline treatment has been developed after a single dose administration of T in a gel formulation and prohormone forms [22].

The complementary SP of T and sulfate metabolites has been the subject of several studies. In 1996, Dehennin *et al.* [23] proposed the ratio of T glucuronide to the total E fractions (free,

sulfate, glucuronide). Bowers *et al.* developed a method for detecting directly the sulfate and glucuronide conjugates of urine steroid metabolites by using HPLC/MS/MS [24]. A different approach from Schänzer and coworkers has developed by analyzing urine using a sequential extraction of free with glucuronide and sulfate conjugates in two steps and the examination of population distribution [25]. A steroidomic approach for biomarkers discovery has been developed with the participation of the WADA accredited laboratory of Lausanne (Switzerland), which concluded the monitoring of both glucuronide and sulfate steroid conjugates as biomarkers and included them in the SP ABP [26]. Another study from the same group described an investigation of the steroid metabolism after transdermal and oral T administration and concluded that sulfo-conjugated steroids might provide helpful screening information for individuals with homozygous UGT2B17 deletion, while the hydroxy-glucuroconjugated EAAS could enhance the detection window of oral T undecanoate abuse [27]. The use of the sulfate fraction biomarkers has been also supported by other studies. Schulze and coworkers found that after a dose of T enanthate, the T sulfate (T-S) levels were markedly decreased to negligible levels because exogenous T is not sulfated at all [28]. In another study, also Schulze and coworkers concluded that after administration of exogenous T (T enanthate), the urinary excretion rate of dehydroepiandrosterone sulfate (DHEA-S) and T-S decreased approximately by 50% and 80% respectively [29]. Finally, a study conducted in the WADA accredited laboratory in Cologne (Germany), found that the retrospective detection of T misuse can be performed by monitoring the epiandrosterone sulfate (epiA-S) [30]. In a recent study, 14 endogenous steroid sulfates were measured to improve the detection capabilities of oral T administration [31].

WADA accredited laboratories estimate quantitatively the SP of EAAS and their ratios results are reported electronically during routine ITP [6]. The analysis is conducted either by GC/MS or gas chromatography/tandem mass spectrometry (GC/MSMS) in an extracted and hydrolyzed urine to cleavage only the glucuronidate conjugations moiety, while in this condition the Phase II conjugated EAAS-S remain intact and are not measured. A recent study by ADLQ group has shown the capability of the routine LC/MS ITP method to quantitatively estimate the EAAS-S in the same analytical run as for the rest qualitative analytes [32]. In the current study, seven EAAS-S related to T metabolism, selected according to the existing literature, were quantified in a set of routine anti-doping samples. Female and male elite athlete population statistical parameters, reference limits, distributions of nominal concentrations and ratios have been estimated by combining GC/MSMS [11] analysis for the EAAS and LC/MS analysis [32] for the EAAS-S. The EAAS and EAAS-S concentrations and the ratios between them were considered as potential biomarkers to enhance specificity and sensitivity of the urinary SP applied in sports antidoping through the WADA ABP system. For this purpose, athletes' samples with EAAS and EAAS-S concentrations measured below the established from analytical method validation limit of quantitation (LOQ) were not excluded from the current database, as this would create biased and unrealistic population statistics.

2. Materials and Methods

2.1. Samples

Routine Samples received at ADLQ within a period of eight months has been selected and analyzed by GC/MS and LC/MS as part of ITP, as described in sections 2.3 and 2.4. No additional analysis was conducted for the current study other than the mandatory ITP. There is an extensive variability of the ethnicities of the athletes, whose samples were analyzed, because of the geographical diversity of the testing authorities collaborating with ADLQ. As all anti-doping samples are always received as anonymized, the athletes' gender was the only information available for the samples used in this study. These anti-doping samples were classified as non-research samples according to the paragraph 6.3 of the World Anti-Doping Code [33]. In order to create population statistics originated from samples negative for doping substances, the following exclusion criteria were used: samples officially reported to contain any kind of substances and confounding factors and samples with bacteria urine degradation signs according to TD2018EAAS[6] were not included in the study. Moreover, after the statistical analysis described in section 2.6. herein, samples characterized as outliers were also removed before applying the population statistics.

All samples had been stored frozen in $-20^{o}C$ prior analysis. The sample preparation had been performed one or two days after the delivery of the samples at ADLQ. The specific gravity and pH measurements were conducted before ITP analysis by using a digital refractometer Atago 3464 and a Thermo Scientific Orion Star A111 pH-meter respectively.

2.2 Materials and reagents

2.2.1. Chemicals

The following chemicals were purchased from Sigma Aldrich (Darmstadt, Germany): sodium hydrogen bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), di-potassium hydrogen phosphate trihydrate ($K_2HPO_4 \times 3H_2O$), potassium dihydrogen phosphate (KH_2PO_4), acetonitrile and methanol (HPLC grade). β-Glucuronidase from *Escherichia Coli* (*E. coli*) was purchased from Roche Diagnostics (Mannheim, Germany). Ethyl acetate, diethyl ether, N-methyl-N-trimethylsily-trifluoroacetamide (MSTFA) and 2-Propanethiol were supplied from Merck (Darmstadt, Germany). Formic acid (HCOOH) and 5 M ammonium formate ($HCOONH_4$) were obtained from Agilent Technology (Santa Clara, United States). Ammonium Iodide (NH_4I) was purchased from Acros Organics (Geel, Belgium).

2.2.2. Reference standards

The following reference standards and deuterated internal standards were used for LC/MS analysis: androsterone sulfate (A-S), etiocholanolone sulfate (Etio-S), T-S, epitestosterone sulfate (E-S), DHEA-S, 5α-Dihydrotestosterone Sulfate (5α-DHT-S), androsterone sulfate-d_4 (AS-d_4), 5α-dihydrotestosterone sulfate-d_3 (5α-DHTS-d_3), testosterone sulfate-d_3 (TS-d_3) were

purchased from National Measurement Institute (NMI, Pymble, Australia). epiA-S and epiandrosterone sulfate-d5 (epiAS-d5) were obtained from Toronto Research Chemicals (TRC, Toronto, Canada). Dehydroepiandrosterone-d_5-3-sulfate (DHEAS-d_5) was obtained from Cerilliant (Texas, United States). The deuterated internal standards for qualitative screening were mefruside-d_3, morphine 3-β-D-glycuronide-d_3 and phendimetrazine-d_3 and acquired from TRC (Toronto, Canada).

The following reference standards and deuterated internal standards were used for GC/MS analysis: A, Etio, T, E, 5αAdiol, 5βAdiol, etiocholanolone-d_5 (Etio-d_5), androsterone-β-glucuronic acid-d_4 (AGlu-d_4), 5β-androstane-3α,17β-diol-d_5 (5βAdiol- d_5), testosterone-d_3 (T-d_3) , epitestosterone -d_3 (E- d_3) were purchased from National Measurement Institute (NMI, Pymble, Australia).

Stock standard solutions were prepared in methanol for each of the examined analytes. The working standard solutions was prepared in methanol by appropriate dilutions of the respective stock solutions. All stock and working standard solutions were stored at -20 ° C in dark vials. The steroid profile for EAAS and EAAS-S were prepared in a different working solutions.

Seven-points calibration curves were prepared for each of EAAS-S steroid spiked in a pool clean-up female child urine: T-S, E-S, 5α-DHT-S: 0.5-100 ng/mL, epiA-S: 2.5-500 ng/mL, A-S, Etio-S, and DHEA-S: 10-2000 ng/mL. The internal standard (ISTD) mixture solution of 5α-DHTS-d_3/AS-d_4/ TS-d_3/ epiAS-d_5/ DHEAS-d_5 was prepared at the concentrations of 1.0/20.0/5.0/10.0/10.0 μg/mL, respectively.

The steroid profile calibration curves of EAAS were prepared using the same pool of female child urine at different concentration levels, where the exact concentrations depended on each steroid: T, E: 1-200 ng/mL, 5αAdiol, 5βAdiol: 4-800 ng/mL, A, and Etio: 100-8000 ng/mL. The ISTD mixture solution of Etio-d_5/ AGlu-d_4/ T-d_3/ E-d_3/ 5βAdiol- d_5 was prepared at the concentrations of 50.0/50.2/1.60/0.40/2.0 μg/mL, respectively.

2.3. GC/MS analysis

2.3.1. Sample preparation

A 2.5 mL urine sample were added 25 μL of ISTD followed by enzymatic hydrolysis using 1 mL of phosphate buffer (pH=7) and 50 μL of β-glucuronidase enzyme from *E. coli* for 1.5 h at 50° C. After hydrolysis, the pH of urine sample was adjusted to 9-10 using solid mixture of sodium hydrogen bicarbonate and sodium carbonate (10:1) (w/w) followed by liquid-liquid extraction (LLE) using 5 mL diethyl ether. Then, anhydrous sodium sulfate was added to all samples and shaken for 20 min. After centrifugation, the organic layer was separated from aqueous phase by freezing the samples at –80° C then evaporated under nitrogen flow at 50° C. The residues were derivatized with 50 μL of MSTFA/NH_4I/2-Propanthiol (1000:4:8) and incubated in 100° C for 60 min. Finally, the samples were transferred to the relevant vials and a 2 μL was injected into the instrument.

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2.3.2. Instrumentation

GC/MS analysis was achieved by using an Agilent GC 7890 coupled with an Agilent 7000 QQQ MS equipped with an Agilent 7693 auto sampler (Agilent Technologies, Santa Clara, CA, USA). The GC separation was performed in a SGE BPX5 column (30 m length, 0.250 mm i.d., 0.1 µm film thickness, Trajan Scientific Ringwood, Australia). GC oven temperature program was the following: an initial oven temperature was 160° C, ramped at 10° C /min to 200° C, then ramped at 2° C /min to 220° C, ramped at 6 ° C /min to 292° C, 50° C /min increased to 310° C and held for 3 min before cooling-down to initial conditions. The injection volume was 2 µL in a split ratio of 1:10. The total analysis time was 28.4 min with a solvent delay of 2.60 min. The collision cell parameter for helium quench gas flow was 2.25 mL/min and the nitrogen gas flow was 1.5 mL/min. Additionally, helium carrier gas was set at flow of 1.1 mL/min. The front inlet and the interface transfer line heater temperatures were maintained at 280° C. The mass spectrometer was operated with the electron impact (EI) mode under the following setting: ion source temperature was 230° C, electron energy was 70 eV and quadrupole temperature was set to 150° C. The data acquisition was performed in multiple reaction monitoring (MRM) mode with the collision energy optimized from 5 eV and 35 eV depending on the analyzed compounds. The transitions used for quantification were m/z 432.3→209 for T and E, m/z 434.3→ 239.1 for A and Etio, and m/z 256.2→ 185.2 for 5αAdiol and 5βAdiol. MassHunter Software (Agilent, Santa Clara, CA, USA) was used for acquisition and data analysis.

2.4. LC/MS analysis

2.4.1. Sample preparation

The ISTD mixture and calibration curves in this study were adjusted by including epiA-S calibrartion, epiAS -d_5 and DHEAS-d_5 ISTDs and altering the calibration ranges of T-S, E-S and 5α-DHT-S. 5 ml of urine aliquots were added 50 µL of ISTDs. The enzymatic hydrolysis were applied for all samples by adding 100 µL of β -Glucuronidase from *E. coli*, 1 mL phosphate buffer at pH 7 (Na_2HPO_4 0.8 M and NaH_2PO_4 0.4 M) and incubated at 50° C for 1.5 hour. After cooling the samples, the pH was adjusted to 9–10 by addition of solid mixture $NaHCO_3$:Na_2CO_3 (10:1) (w/w). LLE was performed by the addition of 5 mL ethyl acetate and anhydrous sodium sulfate. The samples were shaken for 20 min and centrifuged at 3000 rpm for 12 min. The organic layer was separated from the aqueous phase by frozen samples using ethanol at -80° C. Subsequently, the organic phase of each sample was transferred to the relevant conical tube and 200 µL of 3 M acetic acid in ethyl acetate were added. The organic layer was evaporated up to dry under stream of nitrogen at 40° C. The residue was dissolved in 200 µL of reconstitution solvent (mobile phase A/B 80:20; v/v). Finally, the reconstituted extract were mixed with 20 µL of the non-extracted original urine and 5 µL of the mixture were analyzed by LC/MS.

2.4.2. Instrumentation

The LC/MS analysis [32] was performed using a Dionex UHPLC system (Thermo Scientific, Bremen, Germany) equipped with QExactive bench top Orbitrap based mass spectrometer

(Thermo Scientific, Bremen, Germany). The chromatographic separation was performed using a Zorbax Eclipse Plus C18 column (100 × 2.1 mm i.d., 1.8 μm particle size; Agilent Technologies, Santa Clara, CA, USA). The column oven and the auto-sampler temperatures were set at 30° C and 7° C, respectively. Water containing 5 mM $HCOONH_4$ and 0.02% (v/v) HCOOH (solvent A) and mixture of acetonitrile/water (90:10 v/v) containing 5 mM $HCOONH_4$ and 0.02% formic acid (solvent B) were used as mobile phase solvents. A gradient elution program was set at a constant flow rate of 0.2 mL/min. The gradient organic solvent (B) started with 5% for 1 min, changed to 32% in 2.5 min, then remained isocratic for 13 min, and changed to 100% within 8 min, held for 2.5 min before returning to the initial 5% within 1 min. The analysis run time was 28 min and the post-run equilibrium time was 4 min. The injection volume was 5 μL.

The mass spectrometer was operated in positive–negative polarity switching mode and equipped with a heated electrospray ionization (HESI) source with the following settings: capillary temperature 300°C, the heater temperature 30°C, S-lens radio frequency (RF) level 55, and sheath gas, ion sweep gas, and auxiliary gas flow rates set at 40, 10, and 1 arbitrary units, respectively. Nitrogen was used as sheath, ion sweep and auxiliary gas. The ion spray voltage was set to 4000 kV for the positive ionization and 3800 kV for the negative ionization. All EAAS-S were detected in full scan (FS) negative ionization mode. The settings of the FS acquisition mode were as follow: scan range m/z from 100-1000 at 17500 resolving power, automatic gain control (AGC) target was set at 10^6 and duty cycle was 100 ms. The deprotonated molecules $[M-H]^-$ were used for the quantification of the EAAS-S: m/z 369.1585 for T-S, E-S, DHEA-S, m/z 369.1741 for 5α-DHT-S, epiA-S, A-S and Etio-S, m/z 373.1992 for AS-d_4, m/z 370.1773 for TS-d_3, m/z 372.1899 for DHEAS-d_5, m/z 374.2055 for epiAS-d_5 and m/z 372.1929 for 5α-DHTS-d_3. Thermo Xcalibur and LCquan^{TM} version 3.0 (Thermo Scientific, Bremen, Germany) were used for acquisition and data analysis, respectively.

2.5. Data Processing

Identification and integration of the analytes obtained by standard integrated software were checked manually, as routine ITP process implemented at ADLQ. ITP quality control (QC) samples spiked with EAAS and EAAS-S from reference solutions were included in the study. Samples with concentrations above the highest concentration level of the calibrations' curves, were re-analyzed with an appropriate dilution. Samples with concentrations below LOQ and limit of detection (LOD) were included in the study for the sake of the statistical processing [34] and to create the real and unbiased image of the athlete population for all those new SP markers. For the latter samples, the steroid concentration considered for the study was the one calculated from the instrument's calibration curve.

The quantitative analysis of EAAS was performed using calibration curves of seven concentration levels which included one spiked QC sample and two low and high steroid profile for quality control purposes and acceptance criteria. The concentration of EAAS were calculated based on the peak height ratios of compounds versus corresponding deuterated internal standards. The correlation coefficient of each calibration curves were assessed for each steroid to be greater than 0.990. The calibration curves of EAAS-S comprised seven concentration levels and were checked by two QC samples for each steroid spiked in blank urine. The concentration

of EAAS-S were built based on the peak area ratios of T-S and E-S to T-S d_3, A-S and Etio-S to A-S d_4, DHEA-S to DHEAS-d_5, α5-DHT-S to α5-DHTS-d_3 and epiA-S to epiAS-d_5. Instrument tuning and calibration and analytes' method calibration curves were performed at every analytical batch.

The SP concentrations of EAAS and EAAS-S where normalized by the application of the SG-adjustment method used by WADA based on the Levine-Fahy equation [6, 20]:

$$C_{SG} \left(\frac{ng}{mL}\right) = C \left(\frac{ng}{mL}\right) \cdot \left(\frac{1.020 - 1}{SG_{sample} - 1}\right)$$

The instrument measured concentration C in ng/mL and was adjusted to a population reference SG value of 1.020 [5].

2.6. Statistical analysis

Data analysis was performed by SPSS version 25.0 (IBM SPSS Statistics for Windows, Version 25.0, IBM Corporation, Armonk, NY, USA) software package. Data distribution was tested using the Kolmogorov-Smirnov (K-S) normality test, applied for all steroids and corresponding ratios. The same K-S test was applied after logarithmic transformation of the data. Significance was set at $p < 0.05$ level and all tests were 2-tailed with 95% confidence intervals (CI). Outlier detection occurred with the Horn`s algorithm applying a Tukey Fence Factor of 2.1 [35] using RefVaL program [36, 37]. The outliers were removed from the statistics. Determination of upper reference limits (RL) was performed using RefVaL including 97.5%, 99% and 99.5% fractiles each calculated within a 95% CI. Furthermore, the non-parametric approach of applying twice 'far outside' value, defined as '2 × [75th percentile + (3 × interquartile range)]', was adopted to determine a threshold value [38, 39]. The minimum number of population samples of the study were in compliance with RefVal specifications. Pearson correlation coefficients (r_p) were calculated to determine the strength of the relationship between the calculated ratios by SPSS.

3. Results and Discussion

Two separate databases, 780 males' samples and 373 females' samples, were created with EAAS and EAAS-S concentrations (ng/mL) and selected concentration ratios. The GC/MS EAAS [11] and LC/MS EAAS-S [32] analytical batches quality control comprised criteria in compliance with the specifications of WADA Technical Document [6] for EAAS. More specifically, the hydrolysis efficiency was controlled to all samples by isotopically labeled Phase II glucuronide conjugated ISTD for EAAS. The deuterated morphine glucuronide was used as an ISTD in EAAS-S aliquots for the non-sulfate conjugates glucuronidated analytes. The derivatization efficiency was checked in EAAS analysis in all samples by monitoring the mono-trimethylsilyl derivative of A. The extraction efficiency and matrix effects were checked by the deuterated ISTD in both EAAS and EAAS-S. The microbial degradation signs were monitored in EAAS aliquots by the urine degradation products [6]. Finally, the confounding factors that may affect the SP in EAAS analysis were also monitored according to TD2018EAAS [6].

The EAAS concentrations of the databases in this study were those officially reported for the WADA ABP, except for the concentrations that were estimated below LOQ. The specification of WADA [6] requests that the concentrations of EAAS below LOQ to be reported as "-1" and below LOD as "-2". This specification has been introduced in order to harmonize the reporting concentrations below LOQ among all WADA Accredited laboratories. The low concentration SP reporting harmonization is mandatory for the longitudinal intra-athlete of ABP module evaluation. The current study presents the unbiased population data as a monitoring concept rather than data to be introduced for ABP module evaluation according to the official WADA reporting specifications. Consequently, the "-1" and "-2" annotations of the officially reported concentrations were replaced by the concentrations calculated by the instrument's calibration curves per steroid, despite the fact that those concentrations were lower than the lowest concentration level of the calibration curve. The original EAAS officially reported parameters did not comprise any "-2"; i.e. all EAAS, in all male and female samples of this study, the MS signal was always monitored and converted to a concentration level. The same practice applied for EAAS was followed to the concentrations of EAAS-S. However, 6.9% (54 out of 780) of samples for males and 21% (79 out of 373) of samples for females had no T-S MS signal, as they contained no measurable signal of T-S. Similarly, for E-S, one sample for males and three samples for females contained no measurable signal. The zero numerical values of those samples with no MS signal for T-S and E-S were replaced with 0.005 ng/mL for the sake of statistical processing, as referenced in a previous anti-doping population study [39]. Those values were not adjusted for SG, as for the rest of the concentrations. A minimum number of 300 samples is required to create meaningful results, as it has been considered in the study of the Cologne Laboratory [15]. In another criterion, the RefVal software generates error messages if the number of population samples is below the specification of International Federation of Clinical Chemistry (IFCC) [36, 37] related to the selected confidence interval of the study. In the data processing of the current study by RefVal, no such error messages were generated, consequently the numbers of female and male samples included in the current population are in compliance

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with both specifications. All data transformations described in this paragraph were applied before any statistical processing.

The K-S test results revealed that none of the parameters set in both populations could be considered as normal (Gaussian) distributed, not even after logarithmic transformation. 5α-DHT-S was removed from the database, since its signal was not detected in the vast majority of male and female samples.

In Table 1, EAAS and EAAS-S statistics in male (n=780) and female (n=373) populations, including the inter-quartile range (IQ1–IQ3), median, mean, minimum, maximum concentration, the percentage of measurements below LOD and below LOQ and the number of outliers, are presented. Four steroids, A, Etio, T, E were included in both populations, for EAAS and for EAAS-S. For all four steroids in both males and females, lower mean EAAS-S concentrations were measured compared to mean EAAS. The 5αAdiol and 5βAdiol of EAAS were not included in EAAS-S, because of the lack of reference materials and the existence of two hydroxyl-groups in each molecule, which creates multiple Phase II conjugates. On the other hand, in EAAS-S populations, DHEA-S [29] and epiA-S [30] were included, since the former is suppressed, and the latter is increased with T administration. The concentrations of the EAAS are higher in males compared to females, as already reported in previous studies. The mean concentrations of EAAS are similar to the previous studies [15, 16] in both the male and female populations. The mean concentrations of EAAS-S in males are more than 50 % higher than in females, except the Etio-S. The median values of T-S, A-S and DHEA-S are higher than the previous study done by Kuk *et al.* [24]. In the referred article, the EAAS-S population concentrations after ITP analysis were not published and therefore cannot be compared with our findings.

Steroid ratios are applied metric in anti-doping screening of athletes [3, 6, 17]. Based on the databases (Table 1), a number of ratios between EAAS and EAAS-S concentrations were created and presented in Tables 2 and 3 for males and females respectively, together with the inter-quartile range (IQ1–IQ3), median, mean, minimum, maximum concentration and the number of outliers per ratio. The WADA Technical Document [6], except the SP concentration markers presented also in Table 1, incorporates additional markers as EAAS ratios, which are referred herein as ratios 1-6 in Table 2. The ratio 1 has been calculated following the specifications of TD2018EAAS [6]; i.e. EAAS T/E ratios per sample were determined from the ratios of the chromatographic peak heights and the respective response factors, as determined by MassHunter software. However, all other ratios in the current study, were determined as the concentration ratios of EAAS and EAAS-S. Consequently, for consistency between T/E and the rest determined ratios in this study, the T/E ratio 2 was based on concentrations. The numerical values of T/E ratios 1 and 2 per sample were similar with a Pearson Correlation equal to 0.996. In the current study, mean and median of T/E in male population were calculated 1.2 and 0.96 respectively (Table 2), while previous researchers [15, 16, 17] calculated the same statistics as 1.6 and 1.4 respectively. The slightly lower values calculated in the current study could be explained by the fact that ADLQ analyzed samples mainly originated from Asian populations, where the homozygous UGT2B17 deletion genotype [28] is more common compared to the Caucasian population. For female population, the mean of T/E is lower than male population as expected and lower than the ratios described in the previous study [16]. No outliers were statistically detected for T/E in both populations. For A/T in both males and females, the mean is

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higher than in earlier studies [16]. For females, the mean A/T is four times higher than for males, due to the lower concentration of T in females. Mean values for A/Etio in male population is similar to previous studies [15, 16, 17, 23] after the removal of three outliers. For female population, the mean A/Etio is similar to the value found in pervious study [16] and one outlier was removed. For both populations, similar mean values for ratios 5αAdiol/5βAdiol and 5αAdiol/E were found between the present study and previous ones [16, 17].

The ratios 7-37, either they have been proposed from literature, like ratio 27 [23], or they have been presented herein, following the concept that after T or prohormones administration the numerator is increased, and the denominator remains either unaffected or even suppressed, because the exogenous applied steroid is not converted to sulfate EAAS-S. The only exception to this concept is the ratio 26 for T-S/E-S, which was included in the database as comparable biomarker to the classical ratio T/E [5]. More specifically, the ratios 8 to 16 were introduced with T-S in denominator, following data from previous studies. This data shows that exogenous T probably is not sulfated, 95% of TS is originated from testis and it is decreased after T administration due to suppression of the luteinizing hormone (LH) secretion [28]. Additional data from T administration studies, comprising different subject genotypes, need to be created for the ratios 8 to 16. Population data for ratios 8-16 did not incorporate the samples with TS concentrations replaced by 0.005 ng/mL. Similarly, the ratios 17-25 and 29-37, having E-S and DHEA-S as denominators, would be expected to increase after T abuse. In a previous study [23], E-S was introduced in the denominator of the T/E (ratios 1, 2 herein), also included in the current database (ratio 27 herein), in order to improve marker discrimination between physiologically or pharmacologically abnormal T/E. The same conclusion has also been created from another recent study [31] for E-S. The suppression of E and LH, most probably of E-S as well, is not systematic. Depended on the individual metabolism, the E-S is potentially a valid SP parameter, especially in contributing in ratios in denominator position. Moreover, E-S measured above LOD except in a very few samples. Similarly, the DHEA-S is suppressed following T administration [28]. The ratio 27 has been included in our study as it has been proposed in a previous study [23] based only on GC/MS analysis. The ratios 7 and 28 have been included following the SP ABP for E, where E is used as denominator in ratios. In general, very few outliers are listed in Tables 2 and 3 and the samples corresponding to the outliers were removed from the ratio calculation.

The ratios of male samples 1, 2, 17 and 27 composed by T as numerator showed bimodal distributions [3], similarly to T concentrations of the current study. The male ratio 29, T/DHEA-S did not show a bimodal distribution, probably due to the fact that the numerical values are distributed in the area of 0.2 (mean). T/DHEA-S is probably a sensitive biomarker of the T abuse.

In Table 4, the 97.5% (α=2.5%), 99% (α=1%), 99.5% (α=0.5%) reference limits (RL) and the respective 95% confidence intervals (CI) of all compounds' concentrations for male and female populations are presented. In Tables 5 and 6, the same statistical parameters listed in Table 4 have been evaluated for the ratios presented in Tables 2 and 3, for males and females respectively. The data in Table 4 showed similar 97.5% reference limits for EAAS in both male and female populations, to those reported in a previous study [17]. Similarly 97.5% RL values for of T/E , A/Etio and 5αAdiol/5βAdiol in both populations were found comparable with the previous study [17]. In the WADA International Standard for Testing and Investigations (ISTI),

Annex L [40], the 99% reference range, from a lower limit of 0.5% to an upper limit of 99.5%, for the normal physiological biomarkers, is applied for the ABP. The RefVal estimations of Tables 4, 5 and 6 were provided following compliance with the RefVal criterion of $1 / N < \alpha$, where N is the values in the male and female populations [41, 42].

In Table 7, the calculated tentative upper limit of the monitored steroids concentrations and ratios in male and female populations are presented. Moreover, for each parameter of Table 7, the number of samples exceeding the double of far outside value per figure, is presented. The ratios 8-16 statistics provided large numerical values due to the fact that T-S concentrations in the denominators of the ratios were low in a significant part of the athlete population. This condition resulted in high 'far outside' x 2 numerical figures and additionally, a large number of samples with ratios exceeding that upper limit. It can be concluded that the ratios 8-16 could be applied only to athletes with higher T-S concentrations after collecting data from T administration studies.

The T-S and E-S concentrations and ratios biomarkers showed high variation, due to the existence of significant percentages of values below LOQ and LOD (Table 1). In relation to analytical variation based on LOQ, the reduction of variation in E-S and T-S determinations can be achieved in lowering the validated LOQ [32]. The variation of T-S and E-S biomarkers due to samples without measurable MS signals, will be more difficult to be reduced, since the current used MS technology is of high mass accuracy and sensitivity. The intra-individual athlete baseline or after doping application variation of EAAS-S has to be defined in clinical studies. The inter-athlete population variation will play an important role and it has to be determined and considered in a potential incorporation of EAAS-S biomarkers in the ABP steroidal module.

4. Conclusions

The technological progress on MS instruments during the last decade and their uses for ITP analysis, in particular the high-resolution LC/MS, it has improved the sensitivity and specificity of the analytes detection. The improvement in the sensitivity resulted in the detection of the analytes in full scan MS acquisition mode and, subsequently, resulted in the increase of the detected analytes in a single run for both positive and negative ionizations. The latter proved the feasibility of measurement of a number of intact sulfate Phase II EAAS-S. The present study used the recent MS instrumental improvements to create males and females athlete population reference ranges for EAAS and EAAS-S concentrations. Several ratios from the WADA GC/MS and LCMS ITP have also been presented. The study created unbiased and realistic statistics for the population. To support the unbiased statistics, samples with detected EAAS and EAAS-S below LOQ were not excluded from the studied population. On the contrary, samples were excluded from the population if one or more of the following criteria was met: samples reported as non-negative and samples characterized as not valid according to the WADA Technical Document [6] for EAAS analysis related to detection of confounding factors or microbial degradation. The new SP biomarkers, in the context of individual analyte concentrations or ratios, were described through their population statistics, like mean, median, confidence interval, reference limits, outliers. Those additional biomarkers may be useful to improve the detectability of the SP ABP [40] after the incorporation of data from administration studies.

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Table 1. Statistics of the monitored steroids in a male (n=780) and a female (n=373) population including the inter-quartile range (IQ1–IQ3), the percentage of measurements below LOD and below LOQ, median, mean, minimum, maximum concentration and the number of outliers.

Compounds			Male								# Outliers
	LOD (ng/mL)	LOQ (ng/mL)	% < LOD	% < LOQ	1Q1 (ng/mL)	Median (ng/mL)	Mean (ng/mL)	IQ3 (ng/mL)	Min (ng/mL)	Max (ng/mL)	
EAAS											
T	0.33	1.0	0.13	0.13	8.4	28	34	46	0.10	190	0
E	0.33	1.0	-	-	18	31	38	49	3.6	199	0
A	6.7	20	-	-	2124	3039	3374	4267	523	12868	1
Etio	6.7	20	-	-	1198	1772	1967	2454	217	6755	0
5αAdiol	0.33	1.0	-	-	30	48	59	77	5.4	543	2
5βAdiol	0.33	1.0	-	-	55	100	153	201	7.3	1258	0
EAAS-S											
T-S	0.15	0.50	9.8	10	0.65	1.7	3.8	4.2	0.005	54	0
E-S	0.15	0.50	-	-	7.8	12	13.3	16.8	1.2	49	2
A-S	3.0	10	-	0.27	295	539	736	1011	5.2	5566	0
Etio-S	3.0	10	-	0.90	71	190	276	403	6.4	2349	0
DHEA-S	3.0	10	-	0.13	141	395	1235	1186	9.7	39612	0
epiA-S	0.76	2.5	-	0.26	49	106	161	211	1.5	2255	0

Table 1. (Continued)

Compounds	LOD (ng/mL)	LOQ (ng/mL)	Female % < LOD	% < LOQ	1Q1 (ng/mL)	Median (ng/mL)	Mean (ng/mL)	IQ3 (ng/mL)	Min (ng/mL)	Max (ng/mL)	# Outliers
EAAS											
T	0.33	1.0	3.2	14	1.7	4.6	6.9	9.6	0.05	78	0
E	0.33	1.0	-	0.27	5.7	9.8	13	16	0.50	104	0
A	6.7	20	-	-	1056	1682	2030	2609	74	10275	0
Etio	6.7	20	-	-	889	1391	1654	2043	171	8982	0
5αAdiol	0.33	1.0	-	-	10	16	20	24	1.0	120	1
5βAdiol	0.33	1.0	-	-	15	33	62	79	3.6	999	0
EAAS-S											
T-S	0.15	0.50	32	24	0.08	0.39	0.81	0.87	0.005	36.5	0
E-S	0.15	0.50	1.6	4.3	1.4	2.6	4.5	4.4	0.005	364	0
A-S	3.0	10	-	0.27	138	249	378	512	4.57	2208	0
Etio-S	3.0	10	-	-	109	249	312	425	10.16	2067	0
DHEA-S	3.0	10	0.54	2.1	66	151	533	536	0.03	8730	0
epiA-S	0.76	2.5	0.27	0.80	17	35	63	84	0.52	556	0

Table 2. Statistics of the monitored steroid ratios in male population including the inter-quartile range (IQ1–IQ3), median, mean, minimum, maximum concentration and the number of outliers.

No.	Ratios	Male						
		IQ1	Median	Mean	IQ3	Min	Max	#Outliers
1	T/E_TD	0.36	0.96	1.2	1.6	0.04	9.9	0
2	T/E_Conc.	0.34	0.95	1.1	1.6	0.01	9.2	0
3	A/T	66	107	268	321	16	14200	0
4	A/Etio	1.3	1.8	1.9	2.3	0.39	5.6	3
5	5αAdiol/5βAdiol	0.27	0.48	0.61	0.82	0.05	2.8	1
6	5αAdiol/E	0.38	0.65	0.81	1.0	0.03	5.7	1
7	A/E	62	98	128	163	13	864	0
8	T/T-S	3.3	13	39	38	0.04	1892	0
9	E/T-S	5.0	16	50	45	0.18	3603	0
10	A/T-S	575	1501	4011	3791	54	120395	0
11	Etio/T-S	366	884	2116	2204	24	61359	0
12	5αAdiol/T-S	9.3	25	63	63	0.41	1959	0
13	5βAdiol/T-S	19	56	155	147	0.91	6064	0
14	A-S/T-S	118	268	611	648	2.2	24441	0
15	Etio-S/T-S	31	91	304	253	0.67	10540	0
16	epiA-S/T-S	26	58	103	121	0.79	1744	0
17	T/E-S	0.99	2.4	3.1	4.1	0.02	19	0
18	E/E-S	1.5	2.7	3.5	4.2	0.38	35	0
19	A/E-S	171	256	318	384	41	1853	1
20	Etio/E-S	99	146	185	230	27	1297	2

Table 2. (Continued)

No.	Ratios	Male						
		IQ1	Median	Mean	IQ3	Min	Max	#Outliers
21	5αAdiol/E-S	2.5	4.1	5.5	7.0	0.38	45	0
22	5βAdiol/E-S	4.6	9.5	14	18	0.59	107	0
23	epiA-S/E-S	5.1	9.5	13	16	0.19	117	1
24	A-S/E-S	25	49	62	82	0.779	446	0
25	Etio-S/E-S	5.9	16	26	36	0.498	299	0
26	T-S/E-S	0.09	0.16	0.27	0.34	0.003	2.5	0
27	T/(E+E-S)	0.22	0.69	0.73	1.0	0.006	4.5	0
28	epiA-S/E	1.5	3.7	6.6	8.0	0.03	191	0
29	T/DHEA-S	0.01	0.05	0.21	0.21	0.0001	3.9	0
30	E/DHEA-S	0.02	0.07	0.24	0.25	0.0003	5.2	0
31	A/DHEA-S	2.6	7.5	19	22	0.16	297	0
32	Etio/DHEA-S	1.5	4.7	11	12	0.08	143	0
33	5αAdiol/DHEA-S	0.04	0.12	0.32	0.35	0.002	6.26	0
34	5βAdiol/DHEA-S	0.07	0.27	0.81	0.83	0.003	15	0
35	epiA-S/DHEA-S	0.13	0.24	0.33	0.45	0.02	2.4	0
36	A-S/DHEA-S	0.57	1.2	2.1	2.6	0.08	29	0
37	Etio-S/DHEA-S	0.14	0.43	1.2	1.3	0.001	18	0

Table 3. Statistics of the monitored steroid ratios in female population including the inter-quartile range (IQ1–IQ3), median, mean, minimum, maximum concentration and the number of outliers.

No.	Ratios	female						
		IQ1	Median	Mean	IQ3	Min	Max	#Outliers
1	T/E_TD	0.18	0.53	0.76	1.1	0.02	8.5	0
2	T/E_Conc.	0.16	0.50	0.75	1.1	0.005	8.6	0
3	A/T	192	322	1064	804	27	16457	0
4	A/Etio	0.90	1.2	1.4	1.6	0.39	5.0	1
5	5αAdiol/5βAdiol	0.25	0.46	0.58	0.81	0.01	2.2	0
6	5αAdiol/E	0.34	0.61	0.87	1.1	0.05	4.6	0
7	A/E	104	177	230	306	14	1256	0
8	T/T-S	2.5	7.0	20	20	0.08	319	0
9	E/T-S	7.9	17	42	47	0.61	737	0
10	A/T-S	1274	2899	7021	7419	49	68264	0
11	Etio/T-S	1189	2391	5572	6102	25	73591	0
12	5αAdiol/T-S	13	28	64	71	0.79	1095	0
13	5βAdiol/T-S	27	67	158	152	1.5	2667	0
14	A-S/T-S	230	507	1252	1348	16	21992	0
15	Etio-S/T-S	147	448	1289	1106	3.1	27715	0
16	epiA-S/T-S	32	76	170	201	1.4	2343	0
17	T/E-S	0.63	2.0	3.2	3.5	0.01	91	0
18	E/E-S	2.1	3.6	6.0	7.3	0.08	67	0
19	A/E-S	376	667	1067	1087	1.1	15538	0
20	Etio/E-S	335	536	843	860	35	11378	1

Table 3. (Continued)

No.	Ratios	Female						
		IQ1	Median	Mean	IQ3	Min	Max	#Outliers
21	5αAdiol/E-S	3.4	5.7	11	11	0.02	222	0
22	5βAdiol/E-S	6.2	14	29	33	0.07	466	0
23	epiA-S/E-S	7.6	14	25	30	0.03	208	0
24	A-S/E-S	53	105	174	208	0.414	1887	0
25	Etio-S/E-S	40	90	170	182	0.072	4375	0
26	T-S/E-S	0.09	0.20	0.44	0.44	0.01	14	0
27	T/(E+E-S)	0.12	0.39	0.52	0.75	0.004	5.3	0
28	epiA-S/E	1.7	3.7	7.3	9.2	0.12	78	0
29	T/DHEA-S	0.01	0.02	0.16	0.08	0.00004	26	0
30	E/DHEA-S	0.02	0.06	0.56	0.16	0.001	143	0
31	A/DHEA-S	3.4	10	32	27	0.20	2807	0
32	Etio/DHEA-S	2.6	8.1	37	23	0.20	6356	0
33	5αAdiol/DHEA-S	0.03	0.09	0.62	0.26	0.001	127	0
34	5βAdiol/DHEA-S	0.07	0.20	1.9	0.60	0.002	471	0
35	epiA-S/DHEA-S	0.11	0.21	0.62	0.37	0.02	121	0
36	A-S/DHEA-S	0.64	1.4	7.3	3.1	0.08	1748	0
37	Etio-S/DHEA-S	0.39	1.3	13	3.8	0.01	3186	0

Table 4. The 97.5%, 99%, 99.5% reference limits (RL) and the respective 95% confidence intervals (CI) of all compounds for male population and female population.

Compounds	Male					
	97.5% RL	95%CI	99 % RL	95% CI	99.5% RL	95%CI
EAAS						
T	113	102-128	138	123-152	152	135-190
E	115	105-129	151	123-159	158	138-181
A	7408	7077-8106	8378	7747-9567	9554	8242-12868
Etio	4616	4212-5160	5503	4882-5824	5780	5319-6254
5αAdiol	162	145-187	214	176-270	270	206-543
5βAdiol	584	507-672	747	644-911	880	737-1135
EAAS-S						
T-S	19	17-23	26	23-34	33	25-43
E-S	34	31-38	41	35-44	44	41-49
A-S	2528	2166-2703	3166	2691-3562	3558	2865-4085
Etio-S	1037	904-1128	1213	1051-1530	1435	1136-2067
DHEA-S	8706	6606-10462	11588	9223-15396	15314	11244-18166
epiA-S	595	537-654	781	633-916	897	733-1356
Compounds	Female					
	97.5% RL	95%CI	99 % RL	95% CI	99.5% RL	95%CI
EAAS						
T	29	21-39	39	29-52	48	37-78
E	38	34-73	79	45-97	95	67-104
A	5904	4707-6432	7321	6367-9458	9171	6916-10275
Etio	4940	3767-5998	6350	5117-8685	8633	5998-8982
5αAdiol	70	61-80	81	72-97	91	78-120
5βAdiol	272	203-401	463	298-646	584	395-999
EAAS-S						
T-S	4.3	2.8-7.6	8.50	5.0-19	16	6.2-37
E-S	15	9.5-17	29.0	16 -124	74	19-364
A-S	1443	1222-1862	1885	1490-2024	1974	1862-2208
Etio-S	1040	905-1128	1213	1051-1530	1435	1131-2067
DHEA-S	3562	2628-5386	6610	3834-7709	7529	5181-8730
epiA-S	280	240-355	372	282-556	441	299-556

Table 5. The 97.5%, 99%, 99.5% reference limits (RL) and the respective 95% confidence intervals (CI) of all steroid ratios for male population.

No.	Ratio	Male					
		97.5% RL	95%CI	99 % RL	95% CI	99.5% RL	95%CI
1	T/E_TD	3.5	3.2-3.7	3.9	3.5-4.8	4.6	3.8-6.7
2	T/E_Conc.	3.4	3.1-3.65	3.8	3.5-4.7	4.5	3.7-6.7
3	A/T	1093	968-1229	1380	1207-1816	1829	1316-3281
4	A/Etio	3.8	3.5-4.3	4.8	4.2-5.3	5.3	4.7-5.4
5	5αAdiol/5βAdiol	1.7	1.6-2.0	2.1	1.9-2.2	2.2	2.0-2.8
6	5αAdiol/E	2.6	2.2-3.0	3.3	2.7-4.5	4.6	3.2-5.7
7	A/E	400	341-447	543	418-630	630	526-863
8	T/T-S	245	191-344	410	299-686	653	375-1383
9	E/T-S	308	248-374	500	338-720	698	479-1940
10	A/T-S	21778	18903-27211	39992	24747-83654	69791	36744-108539
11	Etio/T-S	10643	8701-14978	17093	13547-35349	32879	16784-59931
12	5αAdiol/T-S	359	284-426	542	383-880	778	520-1922
13	5βAdiol/T-S	853	642-1264	1862	1054-3314	2687	1527-5407
14	A-S/T-S	2972	2426-3588	5321	3231-7157	7183	5096-22449
15	Etio-S/T-S	2224	1527-2753	3253	2315-4881	4918	3163-9347
16	epiA-S/T-S	462	406-542	780	530-1239	1233	606-1713
17	T/E-S	11	9.3-12	13	12-16	16	13-18
18	E/E-S	12	10-13	16	12-19	19	14-21
19	A/E-S	1006	859-1143	1188	1075-1455	1459	1162-1615
20	Etio/E-S	560	504-592	640	575-715	712	616-934

Table 5.(Continued)

No.	Ratio	Male					
		97.5% RL	95%CI	99 % RL	95% CI	99.5% RL	95%CI
21	5αAdiol/E-S	18	17-23	25	21-40	30	24-34
22	5βAdiol/E-S	50	43-56	61	54-71	70	58-107
23	epiA-S/E-S	44	39-51	55	51-67	67	55-83
24	A_S/E_S	193	179-203	236	202-320	320	228-421
25	Etio_S/E_S	95	86-119	141	115-170	169	138-299
26	T-S/E-S	1.2	0.97-1.5	1.6	1.3-2.1	2.1	1.5-2.4
27	T/(E+E-S)	2.0	1.8-2.2	2.3	2.2-2.5	2.5	2.3-4.5
28	epiA-S/E	31	26-36	45	35-54	54	45-83
29	T/DHEA-S	1.4	1.1-1.8	1.8	1.6-2.3	2.3	1.8-3.7
30	E/DHEA-S	1.5	1.2-1.9	2.3	1.8-3.4	3.2	2.2-5.0
31	A/DHEA-S	112	90-125	148	119-190	189	143-254
32	Etio/DHEA-S	60	51-69	80	64-119	119	76-136
33	5αAdiol/DHEA-S	1.8	1.6-2.2	2.7	2.1-3.9	3.9	2.4-5.6
34	5βAdiol/DHEA-S	5.1	4.2-7.0	7.7	5.8-9.7	9.5	7.5-12.9
35	epiA-S/DHEA-S	1.1	1.0-1.3	1.4	1.2-1.7	1.7	1.4-2.0
36	A-S/DHEA-S	8.8	7.5-10	12	10-15	16	12-23
37	Etio-S/DHEA-S	7.6	6.4-9.1	12	8.5-14.3	14	12-16

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Table 6. The 97.5%, 99%, 99.5% reference limits (RL) and the respective 95% confidence intervals (CI) of all steroid ratios for female population.

No.	Ratio	Female					
		97.5% RL	95%CI	99 % RL	95% CI	99.5% RL	95%CI
1	T/E_TD	2.9	2.3-3.1	3.2	2.9-5.6	4.6	3.1-8.5
2	T/E_Conc.	2.7	2.4-3.2	3.7	2.8-5.5	5.0	3.2-8.6
3	A/T	6867	5646-8783	12920	6998-15453	15100	8545-16457
4	A/Etio	3.1	2.7-3.9	4.1	3.2-5.0	4.7	3.6-5.0
5	5αAdiol/5βAdiol	1.7	1.5-1.8	1.9	1.7-2.1	2.1	1.8-2.2
6	5αAdiol/E	3.1	2.7-3.7	3.9	3.2-4.3	4.2	3.5-4.6
7	A/E	740	614-830	887	766-1244	1242	828-1256
8	T/T-S	125	90 -200	213	130-319	318	158-319
9	E/T-S	245	160-301	367	255-737	557	285-737
10	A/T-S	43487	28512-60237	62180	43713-68264	65373	52725-68264
11	Etio/T-S	37733	24307-46500	46882	36562-73591	61700	44250-73591
12	5αAdiol/T-S	357	259-584	589	360-1095	852	409-1095
13	5βAdiol/T-S	1070	721-1655	1926	1137-2667	2315	1191-2667
14	A-S/T-S	8169	4815-11740	13697	7753-21992	18052	9895-21992
15	Etio-S/T-S	9124	5896-14274	15286	8661-27715	21811	12545-27715
16	epiA-S/T-S	971	650-1523	1659	943-2343	2105	1253-2343
17	T/E-S	15	12-20	24	16-50	34	20-91
18	E/E-S	27	18-29	45	27-64	63	31-67
19	A/E-S	4215	3709-6400	10595	4284-14482	14267	6161-15538
20	Etio/E-S	3666	2565-6178	7051	3607-10027	9449	4514-11378

Table 6.**(Continued)**

No.	Ratio	Female					
		97.5% RL	95%CI	99 % RL	95% CI	99.5% RL	95%CI
21	5αAdiol/E-S	53	43-74	79	53-134	100	77-222
22	5βAdiol/E-S	158	104-220	278	165-466	454	207-466
23	epiA-S/E-S	120	96-148	163	123-182	175	146-208
24	A_S/E_S	829	560-958	1452	874-1809	1778	1022-1887
25	Etio_S/E_S	804	523-1044	1402	853-3266	2791	1040-4375
26	T-S/E-S	2.6	1.6-4.0	4.3	2.6-14	9.3	3.1-14
27	T/(E+E-S)	1.7	1.4-2.2	2.4	1.8-3.6	3.2	2.2-5.3
28	epiA-S/E	33	26-44	49	33-78	62	41-78
29	T/DHEA-S	0.59	0.33-0.84	1.5	0.63-11	8.4	0.87-26
30	E/DHEA-S	1.2	0.87-2.3	3.3	1.3-41	23	2.4-143
31	A/DHEA-S	177	98-261	292	196-1087	785	247-2807
32	Etio/DHEA-S	122	81-193	266	136-6356	1174	162-6356
33	5αAdiol/DHEA-S	2.1	0.94-4.3	5.0	2.2-40	25	3.6-127
34	5βAdiol/DHEA-S	3.6	2.4-9.0	9.1	4.0-148	69	7.3-471
35	epiA-S/DHEA-S	1.0	0.80-1.7	1.9	1.2-36	17.8	1.7-121
36	A-S/DHEA-S	12	8.6-25	28	13-513	259	22-1748
37	Etio-S/DHEA-S	26	15-57	64	29-938	488	41-3186

Table 7. A calculated tentative upper limit of monitored steroids concentrations and ratios in a male and a female population

Parameter	Male population		Female population	
	(Far outside)x2	# Samples above upper limit	(Far outside)x2	# Samples above upper limit
T	320	0	66	1
E	283	0	96	1
A	21391	0	14537	0
Etio	12443	0	11009	0
5αAdiol	435	1	135	0
5βAdiol	1277	0	539	1
T-S	30	6	6.5	4
E-S	88	0	27	4
A-S	6320	0	3264	0
Etio-S	2805	0	2747	0
DHEA-S	8646	17	3891	7
epiA-S	1394	1	568	0
T/E_TD	11	0	7.8	1
T/E_Conc.	11	0	8.2	1
A/T	2174	1	5284	17
A/Etio	11	0	7.8	0
5αAdiol/5βAdiol	5.0	0	5.0	0
5αAdiol/E	5.9	0	6.7	0
A/E	928	0	1819	0
T/T-S	285	15	146	4
E/T-S	333	14	330	3
A/T-S	26880	11	51709	4
Etio/T-S	15433	10	41684	5
5αAdiol/T-S	448	10	486	4
5βAdiol/T-S	1064	14	1056	4
A-S/T-S	4474	9	9405	5
Etio-S/T-S	1835	25	7966	8
epiA-S/T-S	807	7	1419	5
T/E-S	27	0	24	3
E/E-S	25	1	46	3
A/E-S	2050	0	6439	4
Etio/E-S	1247	1	4872	4

Table 7. (Continued)

Parameter	Male population		Female population	
	(Far outside)x2	# Samples above upper limit	(Far outside)x2	# Samples above upper limit
5αAdiol/E-S	40	1	70	5
5βAdiol/E-S	114	0	229	4
epiA-S/E-S	99	1	197	1
A-S/E-S	506	0	1346	4
Etio-S/E-S	253	1	1216	3
T-S/E-S	2.2	2	3.0	4
T/(E+E-S)	7.0	0	5.3	0
epiA-S/E	55	3	64	1
T/DHEA-S	1.6	14	0.57	9
E/DHEA-S	1.8	14	1.2	10
A/DHEA-S	159	6	197	8
Etio/DHEA-S	88	7	169	5
5αAdiol/DHEA-S	2.5	8	1.9	9
5βAdiol/DHEA-S	6.2	14	4.4	7
epiA-S/DHEA-S	2.8	0	2.3	2
A-S/DHEA-S	17	2	21	5
Etio-S/DHEA-S	10.0	11	28	8

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