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ARTICLES

Carbon Isotope Mass Spectrometry in Doping Control

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Abstract—A procedure that makes it possible to establish the origin of endogenous steroids present in human urine by the determination of the $^{13}C/^{12}C$ carbon isotope ratio is described. A combination of solid-phase and liquid–liquid extraction, as well as semipreparative liquid chromatography, was used to separate fractions containing testosterone, 5α- and 5β-androstane-3α, 17β-diols, androsterone, ethiocholanolone, 5β-pregnane-3α, 20*S*-diol, and 16(5α)-androstene-3α-ol. More than 100 samples were analyzed by gas chromatography coupled with isotope mass spectrometry. The resulting values of $^{13}C/^{12}C$ were statistically processed, and the ranges required for the interpretation of the results were found. The procedure was certified and accredited in accordance with ISO 17025 requirements.

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The use of doping substances that are close analogs of hormones produced in the human body or their metabolic precursors (testosterone, dehydroepiandrosterone, androstenedione, androstenediols, etc.) should be detected in the current practice of doping control. This cannot be made based on the results of classical techniques—gas chromatography coupled with mass spectrometry, and only so-called atypical changes in the hormonal profile of a athlete can be detected.

The use of synthetic testosterone in sport was prohibited more than 25 years ago. Previously, the determination of the concentration ratio of testosterone (**T**) and its inactive isomer epitestosterone (**E**) in urine was the only way for detecting the use of this substance. However, this approach has a number of important limitations because the T/E ratio in a population varies over a wide range (from 0.1 to 4.0 or higher) [1]. Moreover, prohormones (substances that are testosterone precursors or metabolites, which cannot be determined by traditional doping control methods) became widespread recently in the sports nutrition market. Because the vast majority of synthetic steroids are prepared from plant raw materials with a carbon isotope composition of about –30‰ or lower (with reference to the belemnite international standard), the use of these substances can be detected with the use of isotope mass spectrometry. The method is based on the measurement of the $^{13}C/^{12}C$ isotope ratio of endogenous steroids in the human body; this ratio lies in a range from –17 to –26‰, depending on the residence and diet of the human being, and it is never lower than –27‰ [2–4].

In 1994, Becchi et al. [5] were the first to propose a procedure for the selective extraction of testosterone metabolites from urine followed by analysis using gas chromatography–combustion–isotope ratio mass spectrometry (**GC–C–IRMS**); however, this procedure required 50 mL of a sample.

In 2004, the World Anti-Doping Agency (**WADA**) published a technical document [6] to regulate the use of GC–C–IRMS; however, currently, there is no universal approach for the analysis procedure in antidoping laboratories, because this analysis is complicated and labor-intensive. Multistep solid-phase extraction (**SPE**) [7–10] and SPE in combination with semipreparative liquid chromatography [11] are most frequently used.

The aim of this work was to develop a procedure for the determination of the $^{13}C/^{12}C$ isotope ratios in steroids, separated from human urine using gas chromatography in combination with isotope ratio mass spectrometry, and to determine intralaboratory values for the evaluation of the results of analysis.

EXPERIMENTAL

Instrumentation. The experiments were performed on a Delta V Advantage isotope mass spectrometer (Thermo, Bremen) coupled to a TRACE GC gas chromatograph (Thermo, Italy) through a Combustion III interface. A DSQ II mass spectrometer (Thermo, United States) coupled to a second gas chromatograph was used for the identification of analytes. A TriPlusAS autosampler (Thermo, Italy) was used for the injection of liquid samples; it allowed us to alternately introduce the samples into the injectors of two gas chromatographs.

The target compounds (as acetates) were separated on identical RTX-35MS capillary columns (30 m × 0.25 mm × 0.25 μm) with an integrated precolumn 5-m long (Restek, United States) under the same tem-

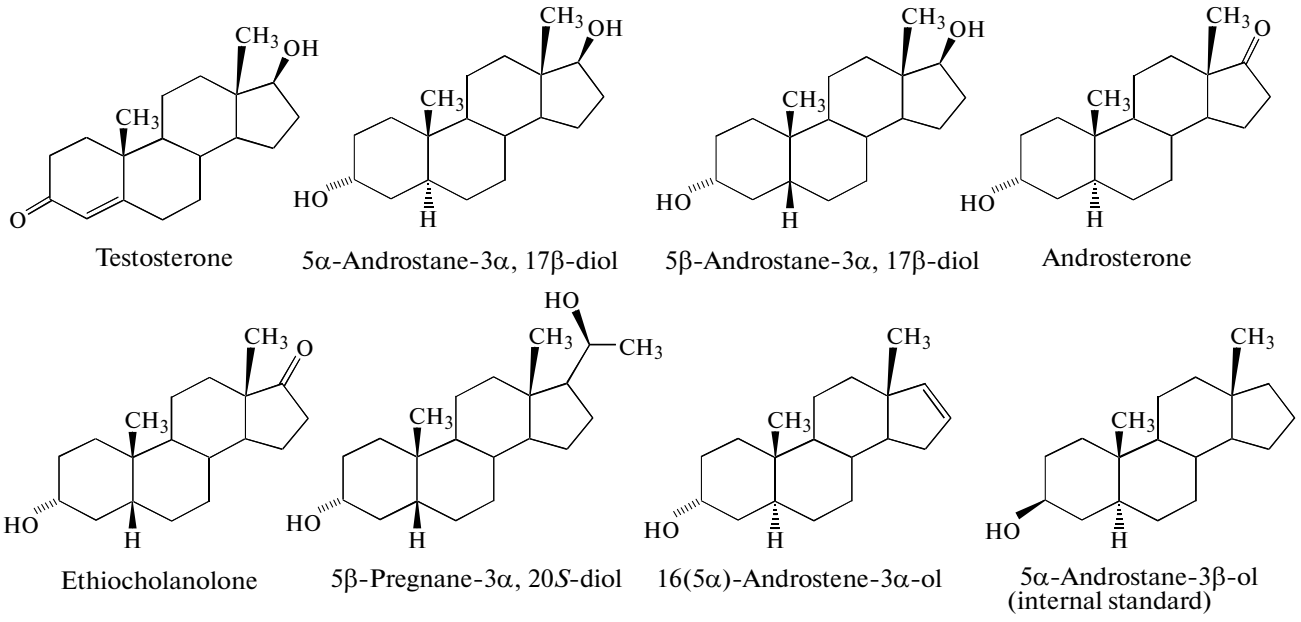

Fig. 1. Structural formulas of the test compounds.

perature programming conditions: 120°C (2 min); heating to 260°C at a rate of 30 K/min; heating to 276°C at a rate of 1 K/min; and heating to 310°C at a rate of 30 K/min (2 min). The sample was injected in the splitless mode at 250°C. The flow rate of a carrier gas (99.9999% helium) was 1.7 mL/min in the case of GC–MS or 2 mL/min in the case of GC–C–IRMS. The carrier gas for GC–C–IRMS was additionally purified using a thermocatalytic converter (Supelco, United States). In GC–C–IRMS, CO_2 of 99.995% purity (reference gas) and O_2 of 99.999% purity were also used. The oxidation reactor temperature in the combustion interface was 940°C, and the reduction reactor temperature was 600°C. The oxidation reactor was regenerated by saturation with oxygen for 15 min at the working temperature several hours before each series of analyses. In the case of GC–MS, data were measured in the scanning mode from 50 to 410 amu under electron-impact ionization at 70 eV. The injected sample volume was 2 or 0.5 μL for GC–C–IRMS or GC–MS, respectively.

In the course of sample preparation, an Agilent Series 1100 high-performance liquid chromatograph (Germany) equipped with a binary pump, an autosampler, a diode-array detector, and a G1364B preparative fraction collector was used. The separation was performed on a SunFire C18 column (Waters, United States) of size 250 mm × 4.6 mm with a sorbent grain size of 5 μm. A precolumn (20 mm × 4.0 mm) with an analogous sorbent was used to protect the analytical column. The conditions of gradient elution were the following: (1) *A* (water) from 70 to 0% for 20 min; 10 min at 100% *B* (acetonitrile); then, to 70% *A* for 5 min and 5 min at 70% *A* or (2) from 30 to 0% *A* for 33 min; 5 min at 100% *B*; then, to 30% *A* for 5 min and 5 min at 30% *A*. The eluant flow rate through the column was 1 mL/min; the column was thermostated at 35°C. Detection was performed at 197 and 240 nm.

Reagents and materials. Figure 1 shows the structural formulas of the test steroids. The certified reference materials of testosterone (**T**), 5α- and 5β-androstane-3α, 17β-diols (**5α**, **5β**), and 5β-pregnane-3α, 20*S*-diol (**PD**) were obtained from LGC Standards (Germany). Androsterone (**A**), ethiocholanolone (**E**), 16(5α)-androstene-3α-ol (**16en**), 5α-androstane-3β-ol, dehydropregnenolone acetate, testosterone acetate, pyridine, acetic anhydride, heptane, diethyl ether, and salts for the preparation of buffer solutions were obtained from Sigma-Aldrich (United States). β-Glucuronidase from *E. coli* K12 was obtained from Roche (Germany). High-purity gases were manufactured at NII KM (Russia).

Deionized water (18.2 MΩ) (Milli-Q Synthesis, Millipore, United Kingdom) and acetonitrile of far-UV grade (JT Baker, Netherlands) were used as eluants. Bond Elut LRC C18 10-mL cartridges (Varian, United States) containing 500 mg of a sorbent were used for SPE.

Sample preparation. A urine sample of 10 mL (in some cases, 20 or 40 mL) was passed through a preconditioned cartridge for SPE; then, it was washed with water and eluted with methanol (four times with 1-mL portions). The eluate was evaporated; the residue was dissolved in 1 mL of a 0.8 M phosphate buffer solution (pH 6.3) and extracted with 5 mL of diethyl ether. The organic layer was rejected; thereafter, 100 μL of β-glucuronidase was added to the aqueous layer and the mixture was incubated at 57°C for 1 h.

After cooling, a 3 M carbonate buffer solution (pH 10) and 5 mL of diethyl ether were added and extraction on an automated shaker was performed for 10 min. After centrifugation and freezing of the aqueous layer at –30°C, the ether extract was transferred to a new test tube and evaporated at 60°C. The dry residue was dissolved in 60 μL of methanol containing 50 ng/μL of dehydropregnenolone acetate (an internal standard for retention time monitoring); 40 μL of water were added, and the contents were transferred to a vial with a small-volume insert.

Then, 90 μL of the resulting sample was injected into a liquid chromatograph under conditions ***1*** (wavelength of 197 nm). Fractions with retention times corresponding to testosterone (I), 5α- and 5β-androstane-3α, 17β-diols (II), androsterone and ethiocholanolone (III), pregnanediol (IV), and androstenol (V) were collected. A solution of 5α-androstane-3β-ol (300 ng/μL) in an amount of 1/10 of the final fraction volume (see below) was added to fractions III–V; the contents were evaporated to dryness and acetylated with 100 μL of a mixture (50 : 50) of pyridine and acetic anhydride at 80°C for 2 h. The reaction mixtures were evaporated, and fractions I and II were dissolved in 60 μL of methanol containing 50 ng/μL of dehydropregnenolone acetate. Water (40 μL) was added, and the contents were transferred to a vial; 90 μL of the mixture was injected into a liquid chromatograph. A fraction corresponding to testosterone acetate was collected under conditions ***1*** at a wavelength of 240 nm, and a fraction containing 5α- and 5β-androstane-3α, 17β-diol acetates was collected under conditions ***2*** at a wavelength of 197 nm.

Fractions I and II were evaporated to dryness; thereafter, a solution of pentacosane in heptane (100 ng/μL) in an amount of 1/10 of the final fraction volume and pure heptane in an amount of 9/10 of the volume were added to fractions I–V. The resulting samples of fractions I–V were analyzed by GC–MS and GC–C–IRMS.

RESULTS AND DISCUSSION

The main problem in the determination of the isotope composition of endogenous steroids in urine is that this matrix is complex and the concentrations of target compounds vary over a wide range from tens of nanograms (testosterone) to ten or more micrograms (androsterone and ethiocholanolone). Moreover, the linear range of an isotope mass spectrometer under continuous flow conditions is restricted, and the analytical signal should vary in a narrow range corresponding to 30–100 ng of steroid injected into the chromatographic column in order to obtain accurate results. Because of this, the sample should be fractionated.

To improve the chromatographic properties of steroids, derivatives that are more highly volatile should often be obtained. Silylation, which belongs to the simplest derivatization procedures, is unsuitable for GC–C–IRMS because silicon dioxide is formed upon the oxidation of silyl derivatives, and the depositions of silicon dioxide affect the functionality of the oxidation reactor with time. Fluorine-containing reagents (trifluoroacetic anhydride) poison the oxidation reactor because of the formation of stable copper and nickel fluorides, resulting in the impossibility of its regeneration. On the other hand, the use of acetic anhydride as a derivatizing agent does not adversely affect the system, and the reaction products exhibit good chromatographic properties. The change in the isotope composition of steroids is calculated based on experimental data [12].

Note that chromatographic analysis in the GC–C–IRMS system has a few special features related to the design of the interface between a gas chromatograph and an isotope mass spectrometer. First, a great number of joints and long capillaries broaden the chromatographic zones, including the peak of the CO_2 formed upon the combustion of sample components. To minimize this spreading, we modified the interface of the GC–C–IRMS system by replacing a metal T-piece in the GC oven by a glass connector (press fit) and passing a fused-silica capillary (through which the sample arrived at the oxidation reactor) directly into the reactor (in the original design, the joint was made through a metal connector). Even after these changes, the average peak width in GC–C–IRMS was 25–40 s, depending on the substance and its amount; this imposed additional requirements on the efficiency of the separation of neighboring peaks.

Second, almost 80% of a sample injected into the chromatograph was lost because the joint of the mass spectrometer and the interface is an open flow splitter. Because of this, a greater sample volume should be injected into the GC–C–IRMS system, as compared with GC–MS; in a number of cases, this overloads the chromatographic column. This can be avoided by using a column with a greater inner diameter (0.32 mm) or increasing the film thickness of a stationary phase. However, the efficiency of separation of structurally similar test compounds (5α- and 5β-androstanediols, androsterone, and ethiocholanolone) can decrease in the former case, whereas the analysis time can considerably increase in the latter case. In this context, we considered that a capillary column of 30 m × 0.25 mm × 0.25 μm is a reasonable compromise for solving the problem.

We found that the operation of the standard injector of the TRACE GC chromatograph was inadequately efficient in the splitless mode because noticeable analyte losses occurred in the injector. To minimize the losses of target compounds in the course of sample injection, the injector pressure was increased by a factor of 2.5 for 1 min after injection. This was made only in the GC–C–IRMS system. As the injector pressure was increased, the chromatographic peak

areas of steroid acetates increased by a factor of about 2, whereas we found in preliminary experiments that the peak areas of native steroids increased by almost one order of magnitude. Such losses were unacceptable, taking into consideration the low concentrations of the target compounds in urine. Moreover, substance losses facilitated isotope fractionation [13] and the detection of wrong $\delta^{13}C/^{12}C$ ratios, which is also unacceptable.

To minimize the effects of various factors (as a rule, the fluctuations of state in the GC–C–IRMS system) on the results of isotope measurements, it is the practice of doping control to use the difference ($\Delta\delta$) between the values obtained for testosterone metabolites and steroids that do not participate in the metabolism of androgens, the isotope composition of which does not depend on the administration of exogenous substances (so-called endogenous markers) rather than the absolute values of the isotope composition of steroids ($\delta^{13}C/^{12}C$). Pregnanediol and 11-ketoethiocholanolone are most frequently used as these markers; however, other compounds, such as androstenol, can also be used [14]. In accordance with WADA criteria, the test is considered negative if this difference is no higher than 3‰. However, Piper et al. [11] found that it is more preferable to use so-called reference ranges (individual threshold values for each particular steroid) determined within the laboratory upon the validation of the test procedure. This is due to the fact that the values of $\delta^{13}C/^{12}C$ for individual steroids can be biased because of the systematic error caused by isotope fractionation upon sample preparation and analysis to cause false-positive results.

We determined the isotope composition of the above steroids in more than 100 urine samples from athletes and volunteers by GC–C–IRMS analysis. This was necessary for formulating statistically substantiated values to assess the results of the analysis, that is, to determine reference ranges.

In the development of the procedure, it was necessary to obtain the values of $\delta^{13}C/^{12}C$ for steroids that occur in urine in both minor and very large amounts. For example, the concentrations of steroids in urine are usually the following: testosterone, 20–70 ng/mL (in males); 5α- and 5β-androstanediols, 70–250 ng/mL; androsterone and ethiocholanolone, 1500–5000 ng/mL; pregnanediol, 200–1000 ng/mL; and androstenol, 500–2000 ng/mL (in males). To obtain correct results, it is necessary to previously estimate the concentrations of target steroids in the test urine sample in order that the analytical signal on an isotope mass spectrometer occurred within the linearity range. We determined the concentrations of the steroids by the preliminary analysis of a sample in accordance with the current doping-control procedure [15]; thereafter, we calculated the volume in which a particular fraction should be dissolved to obtain a steroid concentration of 20–50 ng/μL in the final extract. If the calculated fraction volume was smaller than 50 μL, the dry residue was dissolved in 50 μL of heptane; thereafter, the solution was evaporated to the specified volume in a flow of nitrogen. Because of the use of glass microinserts in vials, the minimum fraction volume in this work was as low as 10 μL.

The following two compounds were used as internal standards: pentacosane and androstanol. Androstanol was added to fractions III–V before acetylation in order to control isotope fractionation upon derivatization. In the case of fractions I and II, which were repeatedly separated by HPLC, androstanol was not added because an additional fraction would be collected in this case.

The average value of $\delta^{13}C/^{12}C$ for pentacosane (-30.0 ± 0.8‰), determined from more than 300 analyses, served as a characteristic of the state of the oxidation reactor and the system as a whole.

Table 1 summarizes the retention times of the test compounds in the GC–C–IRMS system. As an example, Fig. 2 shows the chromatograms of a test mixture of steroids and individual urine fractions; they indicate that the use of semipreparative HPLC allowed us to selectively separate the test steroids and to minimize the effect of interfering matrix components.

Because this procedure involves a long-term and labor-intensive sample preparation process (the preparation of a series of six samples took three days), in consideration of the complexity of the GC–C–IRMS system, each test sample (series of samples) should be accompanied by two quality control samples: knowingly positive and knowingly negative. The knowingly positive sample was obtained from a volunteer who took two capsules of the drug Andriol (testosterone undecanoate, 40 mg), whereas the knowingly negative sample was obtained from a volunteer who did not take steroid preparations. Both of the volunteers collected urine for two days; thereafter, the urine was stabilized with sodium azide (1 g/L), filtered, poured into 10-mL vials, and frozen; the concentrations of the target steroids were determined in advance. Then, each of these samples was repeatedly analyzed by GC–C–IRMS to obtain information on the values of $\delta^{13}C/^{12}C$ for the target steroids and the intermediate reproducibility of the method. We found that the relative standard deviation (**RSD**) under reproducibility conditions was 0.4–0.8‰, depending on the compound. Table 2 summarizes the data on the isotope compositions of the knowingly positive and knowingly negative samples obtained in the course of the approval of the procedure.

Based on the statistical processing of data, the values of $\delta^{13}C/^{12}C$ for steroids were obtained (Table 3). The normalcy was tested visually by constructing quantile–quantile plots with the use of the SPSS version 17 software for statistical data manipulation. Figure 3 shows bar diagrams for each particular steroid,

Table 1. Retention times of the test compounds (steroids as acetates; IS refers to an internal standard)

Compound	t_R, s	Compound	t_R, s
Pentacosane (IS)	548.0	5β-Androstanediol	970.8
Androstenol	606.1	5α-Androstanediol	995.5
5α-Androstane-3β-ol (IS)	657.7	Testosterone	1127.1
Ethiocholanolone	893.5	Pregnanediol	1223.9
Androsterone	910.4		

Table 2. Values of $\delta^{13}C/\delta^{12}C$ for target steroids in knowingly positive and knowingly negative urine samples ($n = 20$)

Compound	Positive sample		Negative sample	
	average, ‰	RSD	average, ‰	RSD
Testosterone	−28.2	0.8	−22.6	0.8
5α-Androstanediol	−29.4	0.7	−22.1	0.8
5β-Androstanediol	−27.4	0.5	−21.9	0.4
Androsterone	−27.5	0.5	−22.8	0.5
Ethiocholanolone	−27.3	0.4	−22.9	0.4
Pregnanediol	−22.2	0.5	−22.4	0.6
Androstenol	−22.5	0.4	−21.6	0.6

Table 3. Values of $\delta^{13}C/\delta^{12}C$ for target steroids found in this work

Compound	Average, ‰	Range, ‰	RSD	Number of measurements
Testosterone	−23.4	−(21.2–25.5)	0.93	83
5α-Androstanediol	−23.8	−(20.8–25.7)	0.91	105
5β-Androstanediol	−22.0	−(19.5–25.2)	0.94	127
Androsterone	−22.9	−(20.7–24.9)	0.78	131
Ethiocholanolone	−22.8	−(20.8–24.6)	0.71	128
Pregnanediol	−21.9	−(19.9–24.1)	0.73	131
Androstenol	−22.6	−(21.2–24.1)	0.65	130

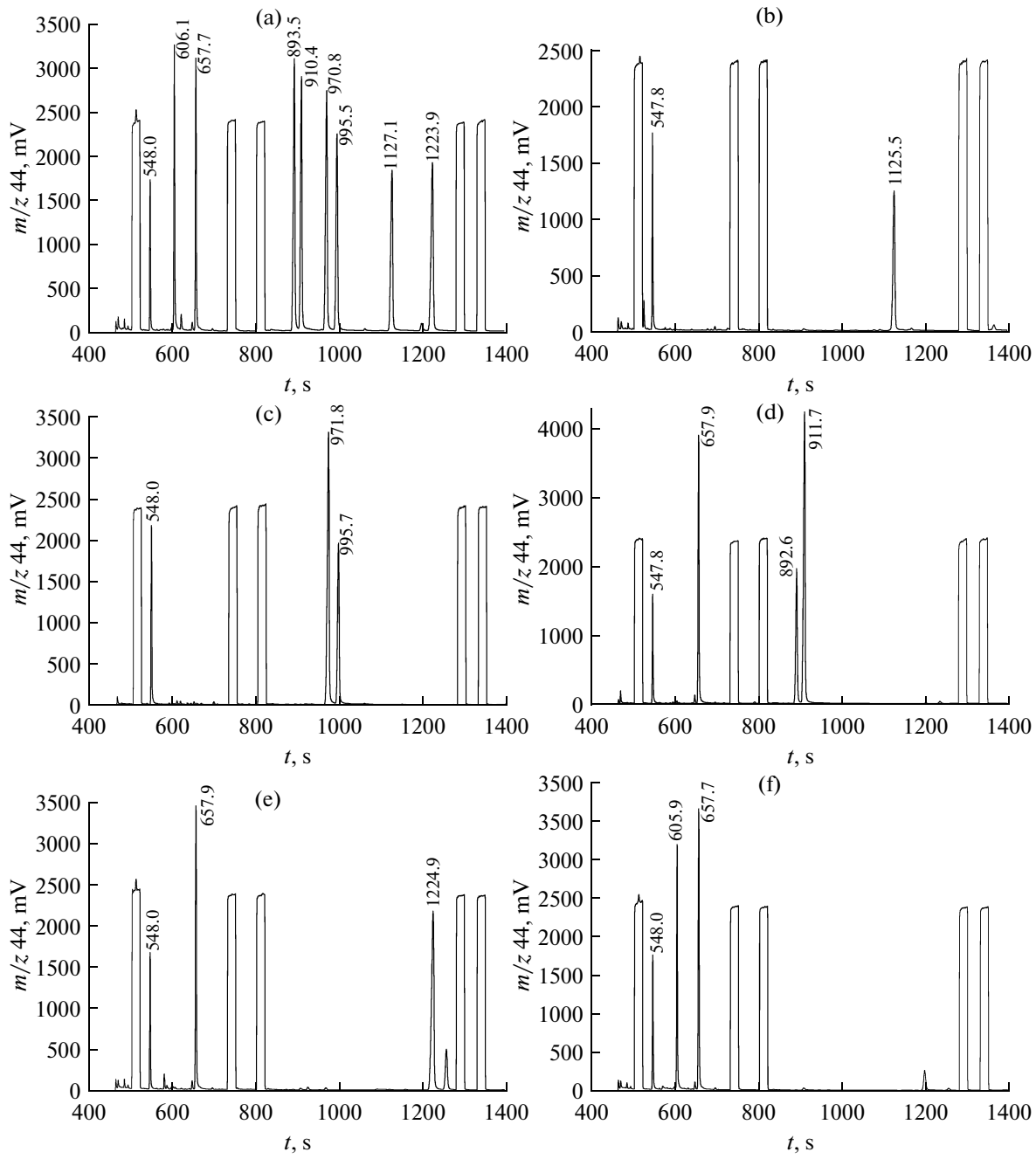

Fig. 2. Chromatograms of a test mixture of steroids and individual fractions of an urine sample. Rectangular peaks are CO_2 pulses with known isotope composition: (a) test mixture of steroids used for checking the state of the system and identifying the components (Table 1 specifies the names and retention times); (b) fraction I (testosterone); (c) fraction II (androstanediols); (d) fraction III (androsterone and ethiocholanolone); (e) fraction IV (pregnanediol); and (f) fraction V (androstenol).

which characterize the probability density of the corresponding values of $\delta^{13}C/^{12}C$.

Table 4 summarizes the reference ranges calculated for two endogenous markers (pregnanediol and androstenol) and five target steroids (testosterone, 5α-androstanediol, 5β-androstanediol, androsterone, and ethiocholanolone). The reference ranges are the steroid – endogenous marker differences ($\Delta\delta$) averaged over all of the samples analyzed in the laboratory. We obtained critical values for each pair of steroids by adding the triple standard deviation to this value, which corresponds to a confidence probability of 99.7% (in the calculations, we rounded the results to

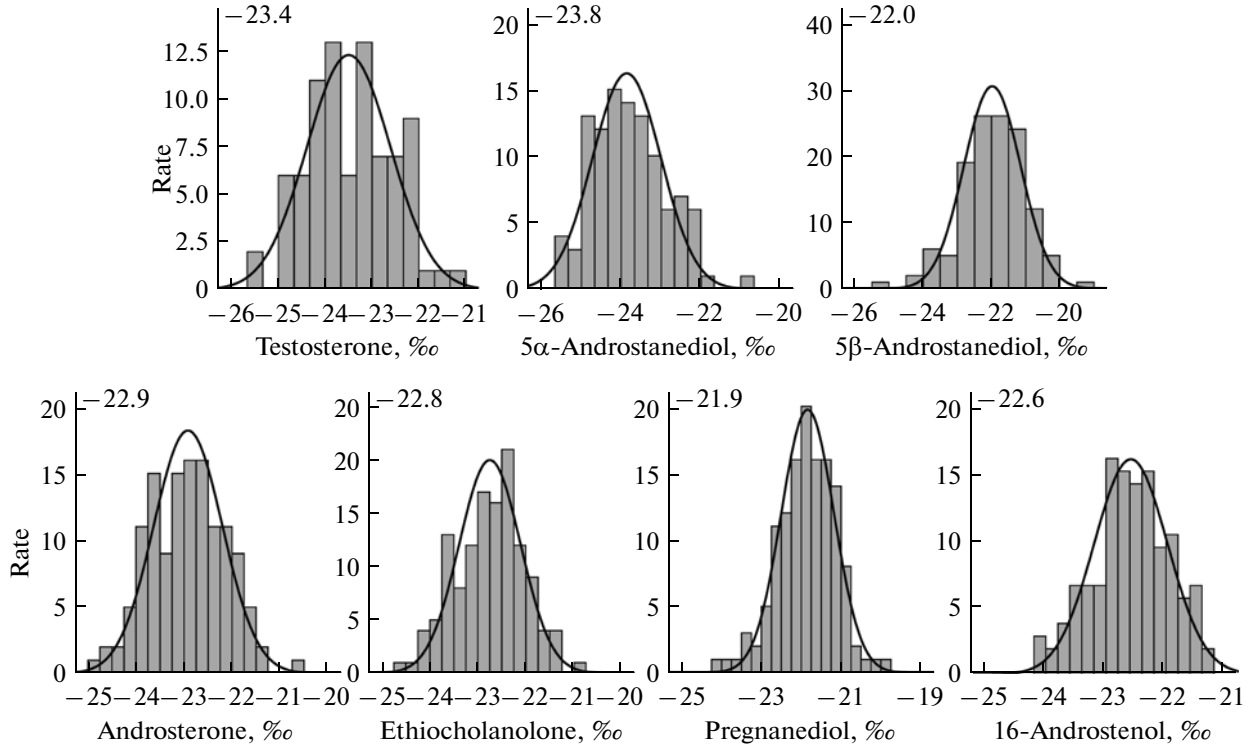

Fig. 3. Probability density plots for the values of $\delta^{13}C/^{12}C$ for the target steroids.

two decimal digits). Analogously, by constructing the quantile–quantile plots, we demonstrated that the values of Δδ were normally distributed.

Thus, our results demonstrate that, in the interpretation of experimental results, it is reasonable to use intralaboratory values rather than a single threshold of 3‰, which was established by WADA, because, for example, these values were 4.8 and 5.1‰ for testosterone and 5α-androstanediol, respectively (with reference to pregnanediol). If the value found in the laboratory is lower than 3‰, a threshold of 3‰ should be used in order to be consistent with the WADA document.

Table 4. Reference ranges for the test steroids

Pair difference	Average ± RSD	Critical value
Δδ PD – T	1.5 ± 1.1	4.8
Δδ PD – 5α	1.9 ± 1.1	5.1
Δδ PD – 5β	0.1 ± 0.6	2.0
Δδ PD – A	1.1 ± 0.8	3.4
Δδ PD – E	0.9 ± 0.6	2.5
Δδ 16en – T	0.9 ± 0.8	3.3
Δδ 16en – 5α	1.2 ± 0.8	3.8
Δδ 16en – 5β	–0.6 ± 1.0	2.3
Δδ 16en – A	0.4 ± 0.6	2.0
Δδ 16en – E	0.2 ± 0.7	2.3

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