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Optimization of LC method for the determination of testosterone and epitestosterone in urine samples in view of biomedical studies and anti-doping research studies

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ABSTRACT

A sensitive and rapid liquid chromatographic (LC) method for the simultaneous determination of testosterone (T) and epitestosterone (E) in human urine samples has been developed and elaborated. The ratio of the both steroids (T/E) in human urine is a widely used as doping control indicator. A sample pretreatment by solid-phase extraction (SPE) after hydrolysis using 36% hydrochloric acid for determination of total level of T has been applied. Unconjugated (free) form of the both androgens were determined without hydrolysis steps, what makes novelty of the method, because simplifies the proposed procedure. In turn, the measurements of urinary free T and E provided the diagnostic information for excess adrenal production of steroids. The proposed LC assay was evaluated by analyzing a series of urine samples containing T, E and methyltestosterone (MT) as internal standard at the range of concentration 2–300 ng^{-1} mL of both analyzed hormones. The proposed method was fully validated for specificity, linearity, limits of detection and quantitation, precision and trueness according to the current requirements concerning analytical methods. Interestingly, the developed LC method allows to obtain a sensitive enhancement with respect to UV detection with the quantitation limit for T and E equaled 2 ng mL^{-1}. The method was selective and reliable for identity and enable to detect changes of endogenous levels of T and E in urine independently of fluctuations characteristic for both analyzed endogenous hormone level in plasma.

1. Introduction

Testosterone (T) is the major steroid secreted by Leydig cells and it is widely used in androgen replacement therapy for the treatment of male hypogonadism [1–4]. Analysis of T in urine as steroid hormone from the androgen group is essential for androgen-related disorders such as male-pattern alopecia in men [5], androgenic alopecia in women [6], risk of breast cancer [7,8], and effects of chrysin on T level [9]. In addition, T is important for health and well-being as well as in preventined osteoporosis. In men, T plays a key role as biomarker of prostate cancer [10] as well as promoting increased muscle and bone mass. Moreover, it was found that the measurement of T is important source of information as the amount of the clinical useful hormone produced over 24 h. Urinary total T level is commonly determined using different kinds of hydrolysis whereas the fraction of nonprotein bounded as free T is determined without hydrolysis step the biologically active fraction. Furthermore, the measurements of urinary non-conjugated T excretion in healthy subjects can be useful in the diagnosis of androgen excess or androgen deficiency in clinical conditions [8]. The normal amounts of total endogenous T and epitestosterone (E) practically measured in healthy male in urine are in the range 30–60 ng mL^{-1} [11]. T and E and their ratio T/E is stable in males, what was well established [12]. Since 1983, T was forbidden in sports by the International Olympic Committee (IOC). The detection of illicit use of T is currently carried out measuring the ratio between the concentration of T and its isomer E. A ratio of their concentrations (T/E ratio) higher than 4 is considered as potentially indicative of T administration. On the other hand, because the T/E ratio can be artificially modified by the administration of E, a urinary concentration of epitestosterone above 200 ng mL^{-1} has been established as indicative of its misuse as a masking agent [13]. The World Anti-Doping Agency (WADA) indicated that if the T/E ratio was equal or above 4, or concentration of E higher than 200 ng mL^{-1}, a confirmation procedure to prove doping would be necessary [14].

According to a knowledge described above, determination of non-conjugated form of T and E is clinically suitable with anabolic and androgenic effects, hence the sensitive, simple, fast and low-cost method to monitor both T and its epimer E is needed, which could be used in routine investigations.

Several immuno-based methods for the determination of T and E have been reported in the literature including radioimmunoas-

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say [15], enzyme-linked immunosorbent assay (ELISA) [16] and immunosensor [17]. In immunoassays, cross-reactions noted and the reagents are expensive and would be also inappropriate for the determination of the potential presence of degradation product. The immunoassay methods are rapid and simple, although cross-reactivities can give the false positive results as falsely increased estimations of both T and E together, thus specificity of these methods are not attractive. Further, most number of publication described immunoassay applications for urinary total T level and do not measure free T alone, but also possibilities of the interfering material with apparently T metabolites can occur. Thus, immunoassay methods are not good enough to quantity analysis because of cross-reactivity risk between different steroids. In summary, immunological methods have some disadvantages such as long wait times for results, high cost and high labour requirements. Additionally fact is that among immunoassays' techniques a different assay is needed for each steroid and also the low dynamic range.

Gas chromatography (GC) is another alternative for the analysis of T and E. This technique is fast, high power of resolution and sensitivity, but its performance requires considerable experience. Free T and E are not sufficiently volatile, they have to be converted into volatile derivatives to be determined, however derivatization procedure could be complicated and time-consuming [18]. GC coupled with mass spectrometry (MS) is method usually applied for the determination of anabolic steroid level ranging from micrograms to nanograms in biological samples [19]. Among liquid chromatographic (LC) methods coupled with MS, such as LC–MS and LC–MS/MS, were also used to evaluate of anabolic steroids in urine samples [20,21]. A powerful MS detector can improve detection sensitivity, however, it is not widely applicable and it could be expensive for a common laboratory. On the other hand, techniques based upon capillary electrophoresis (CE) [11,22,23] for the study of T and E total level in human biological samples were also applied. CE methods have advantages of low sample and reagent consumption, high efficiency and speed. The possibility of separation both neutral and ionized anabolic steroids are associated finally with the advantages of micellar electrokinetic chromatography (MEKC) [22,23].

A number of LC methods have been reported for the study of total level of T and E [14,24,25]. LC is a commonly used separation technique for the determination of T and E in body fluids because of its enough sensitivity, good resolution and respectively short analysis time. Moreover, practical aspect because of freely available LC apparatus, especially with UV detector is important, too. Many methods for determination of T and E in different kind of biological samples (plasma, serum, urine, saliva) have been described in recent years, unfortunately all of them also possess several limitations [26–28]. The measuring of T and E levels in human blood has many disadvantages because the sampling of blood may induce itself a stress, it represents invasive technique of taking biological samples before analytical investigations. For this reason, saliva could be considered as non-invasive, stress-free method, which additionally allows for frequent, rapid and easy sampling. However, salivary T levels are only 50–70% of serum free T levels due to the conversion of T to E by 11β-hydroxysteroid dehydrogenase type 2 activity in saliva [28]. Described LC methods with UV detection for quantification of androgens in biological fluids with the use of various kinds of online preconcentration were performed [14,24,25]. However, the method employed a labor intensive manual, time-consuming as well as no economical liquid/liquid extraction (LLE) procedure with methylene chloride and large volume of organic solvent used. Despite of the successful use of halogenated solvents, such as dichloromethane and chloroform, this solvent should be avoided when new methods are developed, since these solvents are banned from the use in a number of countries because of their negative effects on the environment. Moreover, nowadays the most widely used SPE has many advantages such as the possibility of isolation and concentration of volatile and non-volatile analytes, which has a positive effect on further analysis and allows the lower limit of detection and determination of compounds of interest. In addition, it allows the storage of test compounds adsorbed on the sorbent for a long time and eliminates the formation of emulsions that occur during the LLE extraction. An important advantage is the reduction in the use of organic solvents. Additionally, SPE can be easily incorporated into automated analytical procedures, which can lead to greater trueness and precision, as well as greater laboratory productivity. The application of LC to urine samples is potentially difficult due to the low sensitivity for absorbance detection. Moreover, urine is complex biological matrix and difficult to clean and prepare before proper analysis. However, through suitable pretreatment of samples the amount level of the analytes can be fortified sufficiently for clinical applications. Since its introduction in 1905, LC has gained wide popularity and it has been used for the separation of various hydrophobic compounds such as steroids. Different kinds of mobile phases were applied as well as various stationary phases were used. By the way, addition of the organic modifiers to mobile phase such as triethylamine [27] was found to be essential in achieving separation of steroids in a mixture because its influence on resolution. Special stationary phases [14,24] used in chromatographic systems allow to achieve baseline separation of T and E in less than 10 min with the detection limit of 1–5 $\mu g\,mL^{-1}$. Separation of T and E as hydrophobic steroids by LC with UV detection at 244 nm can even provide a detection limit of 5 $ng\,mL^{-1}$ for analyzed steroids in urine samples [24]. Qiu et al. [25] evaluated and optimized the buffers as component of mobile phase for the determination of E with LC. According to the authors the detection limit of free T and E in urine was 2 $ng\,mL^{-1}$ applying solid-phase extraction (SPE) compared to 100 $ng\,mL^{-1}$ without SPE.

To summarize LC methods described above and published during the last decade, to date, no real straightforward, simple and low-cost LC method for trace determination of T concentrations of free and total levels in urine samples has been developed. It should be emphasized that, although many systematic investigations have been carried out to establish new analytical methods for these compounds in urine there are still problems in simple, without hydrolysis step and routine analysis at the trace levels.

The aim of the present study is to evaluate the potential LC method for T and E and the T/E ratio monitoring. Moreover, the study was also intended to apply the proposed LC method for estimation the through concentrations of free and total levels of androgen not only in large group of healthy volunteers (116), but also including 10 athletes in goal of check possibility who among them was administered T dose for doping. The proposed LC method with SPE was then combined with the elution using methanol as sample pretreatment for free-level and total-level T and E determinations, respectively. The work proposes an easy and rapid method using LC with UV detector to determine T and E in human urine. Researchers applying UV detection have mostly used the method of pre-concentration of following volume of urine, namely 1 mL [14], 16 mL [25]. For this reason we decided to develop 6 mL volume sample, lower than [25]. It is well known that the concentration of T and E in biological fluids is at the level of $ng\,mL^{-1}$, but their unconjugated (free) form exemplifies only about 2% of total value. This manner allowed to us to determine this hormone in real samples. Moreover, to improve detection of T and E, suitable preconcentration of urine samples with hydrophilic–lipophilic balance (HLB) cartridges with high recovery has been employed. Finally, UV absorption at 240 nm should be easily to set with UV detector for analyzed androgens. Due to limited sensitivity of absorbance measurements, several attempts have been made to improve detection limits concerning optimization of chromatographic conditions such

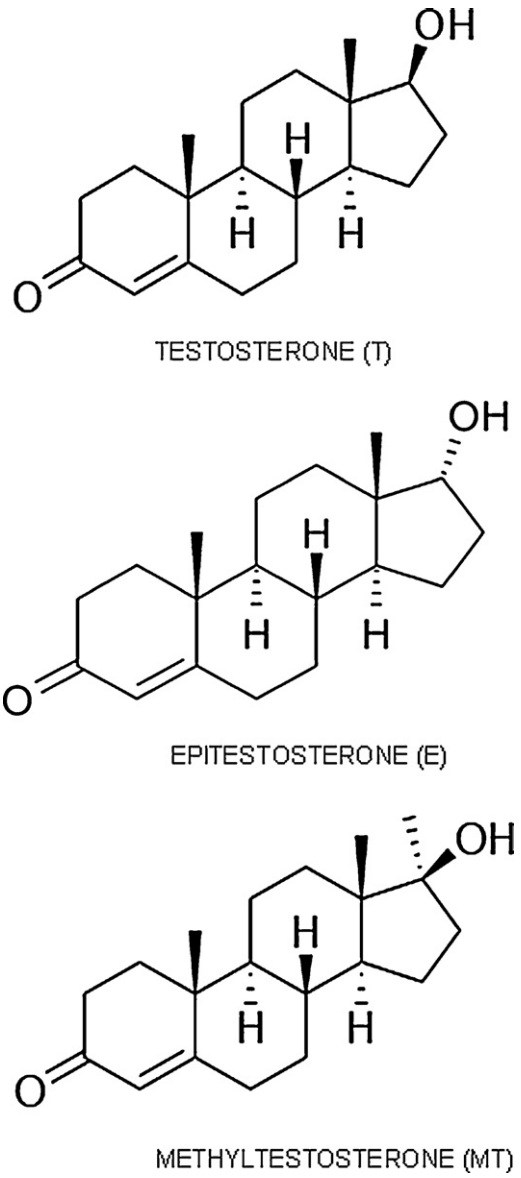

Fig. 1. Chemical structure of T, E and MT.

as mobile phase, stationary phase and parameters of sample extraction. Validation parameters such as linearity, specificity, precision, trueness and sensitivity were successfully assessed. Additionally, the application of LC method to screen for level of total as well as free T and E level in human urine samples was also investigated and applied in large group of healthy volunteers (116) as well as for 10 athletes.

2. Experimental

2.1. Reagents and chemicals

Testosterone (17β-hydroxyandrost-4-en-3-one) its epimer, epitestosterone (17-α-hydroxyandrost-4-en-3-one) and methyltestosterone as internal standard (17β-hydroxy-17α-androst-4-ene-3-one) (Fig. 1) were purchased from Sigma (St. Louis, MO, USA) and were of a minimum purity of 99%. Each steroid was accurately weighed, dissolved in methanol and diluted to an appropriate concentration. To avoid decomposition all solutions of analytes were stored at 4 °C until analysis. Hydrochloric acid was delivered by POCh (Gliwice, Poland).

Methanol, acetonitrile, dichloromethane, and acetone were obtained from Merck (Darmstadt, Germany). Highly pure water was obtained from Milli-Q equipment (Millipore, Bedford, MA, USA). Separation of compounds of interest before LC assay was preceded by sample preparation with solid-phase extraction (SPE) using hydrophilic–lipophilic balance (HLB) cartridges (200 mg, 6 mL), which were purchased from Supelco (Park Belefonte, PA, USA).

2.2. Instrumentation

The LC system consisted of a solvent pump (Mini-Star K-500), spectrophotometric detector with selectable fixed-wavelength operated at 240 nm, autosampler (K-3800) with a 20 μL sample loop volume. Experimental data were collected and processed using a computer data acquisition program (Eurochrom 2000). The separation was performed on a reversed phase C18 Nucleosil-100 column (125 × 4.6 mm I.D., 5 μm) from Knauer (Berlin, Germany). As the mobile phase a binary mixture of acetonitrile–water (48:52 *v/v*) was applied. Measurements were carried out at a flow rate of 1 mL min^{-1}. The chromatographic system was operated at 25 °C.

2.3. Sample preparation

The urine was obtained from adult volunteers and frozen at −20 °C. Before extraction samples were left to thaw and equilibrated to room temperature. T and E levels were expressed finally as analyzed steroid-to-creatinine ratio after standardizated with correction for creatinine excretion.

Creatinine in urine was investigated using a diagnostic kit for the determination of creatinine PZ Cormay (Lublin, Poland). The colorimetric method based on the reaction with picrinic acid was used, according to the methodology supplied.

2.3.1. Sample preparation of free testosterone and epitestosterone

To 6 mL of human urine samples, 12 μL of internal standard solution at concentration of 100 μg mL^{-1} in methanol (MT, obtaining concentration 200 ng mL^{-1} in sample) was added. After shaking, the urine sample was divided into two parts, each of 3.0 mL, separately placed in a 7 mL tube, and 3 mL of triple distilled water were added. Resulting mixture was shaken mechanically for 10 min, centrifuged for 15 min at 8000 rpm, and the sample was transferred to the SPE cartridges. Next, sample was slowly passed through a HLB cartridge. Sorbent of column was conditioned with 3 mL of methanol and 3 mL of deionized water prior to the sample loading. After passing the respectively spiked urine samples, the sorbent was washed with a 3 mL of acetone/water mixture at the ratio of 25:75 (*v/v*). Next, the column was rinsed with 3 mL of triple distilled water and the final elution of steroids was achieved using 1.0 mL of methanol. Each two parts of eluate concerning the same initial sample of urine were connected and mixed. The organic solvent was transferred to conical tube and evaporated to dryness in a water bath at 45 °C under a stream of argon. Finally, the residue was reconstituted in 70 μL of mobile phase, centrifuged at 8000 rpm for 7 min, and a 20 μL aliquot of the sample was injected into chromatographic column. Standard samples were prepared by spiking blank urine samples with known amounts of T and E and used for construction of calibration curves.

As a control, unspiked blank urine sample was also carried through the extraction procedure. The blank urine samples were obtained in following step. Four grams of activated charcoal were added to 100 mL urine and mixed using a magnetic stirrer for 2 h. The mixture was then centrifuged for 2 h at 3000 rpm. After centrifugation the supernatant was filtered using a glass filter (grade 4).

2.3.2. Sample preparation of total testosterone and epitestosterone

Procedure of sample preparation was preceded by hydrolysis using 0.5 mL of 36% hydrochloric acid added to each 3.0 mL of urine sample at 95 °C for 1 h in a water bath. After hydrolysis urine samples were adjusted to room temperature and prepared by procedure described above.

3. Results and discussion

The aim of this study was to develop a LC as an alternative method during the routine determination of endogenous low-hydrophilic steroid hormone. To detect levels of T and E, preconcentration of urine samples was necessary. The study started by analyzing 1 mL of urine sample, and then 3 and 4 mL of it. However, in each case the extraction of the urine provided not satisfactory LOD and LOQ limits. It was not possible to detect both of the steroids in the full range of physiological concentrations. T at these physiological levels can be easily detected using 6 mL of urine sample to clean-up before injection to chromatographic system after preconcentration.

3.1. Optimization of sample extraction

Steroids possess the similar chemical structure (chemical difference from each other only in the configuration of the hydroxyl group on C-17 on the D ring) then they can be extracted in the same conditions. Several organic solvents (dichloromethane, ethyl acetate, acetonitrile, methanol) were used for the extraction of T and E as a hydrophobic reagents. The SPE was preferred by us because of high efficiency, environmental and health protection. During passing of the sample over the nonpolar C18 sorbents, some of sample analytes were retained, so samples were selectivity cleaned up. The results of extraction efficiency of analyzed steroids using different organic solvents for the elution were collected in Table 1.

To obtain the desired interaction between analytes and sorbent, analytes must be in unbound form. Low flow-rates and dilution of the sample are reported to increase the concentration of unbound drug available for sorbent interaction. Biological samples are generally viscous and dilution will also make easier their passage through the sorbent level. When a large volume of aqueous sample is loaded, the silica-based material may no longer be wetted and a reduction in recovery and poor reproducibility might be observed. In the SPE procedure non-endcapped C18 (Merck, Darmstadt, Germany) and HLB cartridges (Supelco, Park Belefonte, PA, USA) were employed. However, the recovery of the analytes was 25% higher in the case of HLB cartridges comparing to C18 (Table 1). As eluents for SPE column were applied different solvents, including methanol, dichloromethane and acetonitrile. Dichloromethane appeared to be better for clean-up of urine background, but lower recovery of analytes after using dichloromethane in compare to methanol was observed. The choice of methanol as the best eluent was dictated by the fact that the chromatograms were free of interfering peaks and recoveries for androgens showed the highest values.

Extraction efficiency was experimentally demonstrated by carrying out of all extraction procedure for concentration of analytes of 10, 50 and 100 ng mL^{-1}. Different sorbents and organic solvents were applied. The best results as higher efficiency was obtained after methanol was used as elate for elution of analytes of interest from C18 sorbents. The extraction efficiency after using methanol and HLB sorbents equaled 98.0% for 10 ng mL^{-1}, 100.4% for 50 ng mL^{-1} and 102.2% for 100 ng mL^{-1}. In conclusion, SPE with methanol for the elution of analytes causes cleaner background and higher recovery of steroids in comparison to LLE. Therefore SPE has been finally proposed to eliminate ballast substances from the biological matrix. Recoveries (mean ± SD) were also determined for the internal standard at concentration of 200 ng mL^{-1} after SPE equaled 85.9 ± 6.8%.

Table 1
Analytical extraction efficiency test of T after various extraction solvents.

Nominal concentration (ng mL^{-1})	Recovery (%)	RSD (%)
LLE (ethyl acetate)		
10	75.0	5.3
50	89.2	6.3
100	85.3	9.6
LLE (dichloromethane)		
10	81.0	2.5
50	96.2	4.4
100	92.1	4.6
SPE (dichloromethane) with HLB cartridge		
10	95.0	1.0
50	97.8	3.1
100	95.3	6.9
SPE (methanol) with HLB cartridge		
10	98.0	1.0
50	100.4	1.0
100	102.2	1.2
SPE (acetonitrile) with HLB cartridge		
10	97.0	3.1
50	90.4	3.3
100	85.0	6.5
SPE (dichloromethane) with C18 cartridge		
10	86.0	4.6
50	90.4	3.3
100	85.0	5.4
SPE (methanol) with C18 cartridge		
10	95.0	8.4
50	95.8	7.3
100	100.5	3.8
SPE (acetonitrile) with C18 cartridge		
10	76.0	1.3
50	90.4	3.3
100	85.0	6.6

Important parameter for determination of androgen steroids is also pH for SPE. Hence, the influence of pH values on the recoveries of these steroids has been investigated as a function of pH. Our experiments confirmed that the best pH value for SPE of T and E is slightly acidic or neutral (pH 7.0). This conclusion is in accordance with the results received by Fenske [29].

3.2. Optimization of LC parameters

The proposed LC method was optimized to provide a simple procedure for the determination of androgen steroids in human urine samples. First, the UV absorption spectra of T, E, and MT were carried out in the wavelength range between 190 and 300 nm. Due to the fact that all analyzed steroids are perfectly absorb UV at 240 nm, while methanol and acetonitrile exhibit maximum absorption at short wavelengths (205–210 nm), the methanol was used as a solvent for the analytes, and acetonitrile was the mobile phase component. Moreover, acetonitrile as mobile phase component improved shape of peaks of interest as well as their symmetry. Our experience has shown that steroids and the internal standard (MT) dissolved in mobile phase containing acetonitrile show maximum absorbance at a wavelength of 240 nm, hence this wavelength has been selected for further study as optimal.

The injection sample volume (5–20 μL) was tested to achieve the required sensitivity for the separation of T, E and MT. It was checked that 20 μL was enough to allow suitable limit of detec-

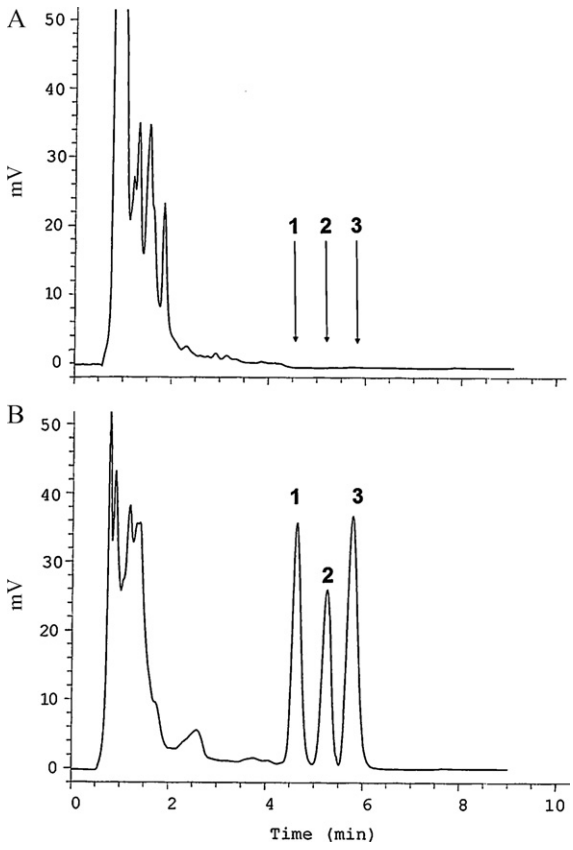

Fig. 2. Representative chromatogram of blank human urine and urine samples spiked with T (1) 150 ng mL^{-1}, E (2) 150 ng mL^{-1} and MT (3; I.S.) 200 ng mL^{-1}.

tion, good resolution for the analytes studied and maximum peak enhancement. Although, larger volume injection than 5 μL caused that peak signal was increased at the same time to 20 μL, but above 20 μL no good shape of peak was observed.

Temperatures of 18, 19, 20, 21, 22, 23, 24, 25, 26 and 27 °C were tested. An increased temperature from 18 to 25 °C caused the decreasing retention times, hence optimal temperature 25 °C was chosen. Further increasing of temperature caused worse resolution.

3.3. Validation of the method

The chromatographic method was further validated for linearity, specificity, sensitivity, precision and accuracy according to ICH guidelines.

3.3.1. Specificity

Specificity of the method was determined by a comparative analysis of six different blank urine extracts with extract sample spiked with steroids. Specificity was demonstrated by the absence of any endogenous interference at retention times of peaks of interest as evaluated by chromatograms of blank human urine against those spiked with steroids (Fig. 2). Endogenous steroids and internal standard were well separated and identified by their relative retention time e.g. 4.7 ± 0.2, 5.3 ± 0.2, and 5.9 ± 0.2 min, respectively. No interferences were observed during the chromatographic run of the urine samples in the area where steroid or internal standard peaks appear. Additionally, the specificity of method has been confirmed by the identification of steroid peaks and internal standard in term of the retention time and UV spectrum.

Table 2
Results of regression model for total T and total E.

LOF	Mandel's test value	QC %	*p*
Linear regression model for total T			
3.08	6.46	1.93	0.01
4.42	4.84	2.23	0.00
2.13	5.29	1.67	0.02
1.99	1.73	1.79	0.03
2.43	3.21	2.03	0.09
3.91	3.02	1.53	0.02
Linear regression model for total E			
4.08	7.54	2.93	0.05
4.82	5.66	3.52	0.04
3.23	4.18	2.76	0.03
2.98	2.13	2.44	0.00
3.46	2.33	3.42	0.09
4.33	2.19	3.13	0.01

3.3.2. Linearity

Method linearities ($n = 6$) for T and E were determined by the addition of T to charcool-stripped urine over the range of 2–300 ng mL^{-1}. Each sample was analyzed according to the extraction procedure and the concentrations were calculated from a calibration curve. The linear calibration curve was constructed by plotting the analyte/I.S. peak high ratio (y) against analyte concentrations (x), was as follows: $y = 0.0074$ (±0.00003)x − 0.0061 (±0.0033) with correlation coefficient $r = 0.9998$ and the $r^2 = 0.9999$ for total T and $y = 0.006$ (±0.00036)$x + 0.0253$ (±0.045) with coefficient $r = 0.9994$ and the $r^2 = 0.9996$ for total E, suggested that the developed method has good linearity. Regression equations for free T and free E were also estimated by least squares method, $y = 0.0066$ (±0.00001)$x + 0.0125$ (±0.0095) with correlation coefficient $r = 0.9996$ and the $r^2 = 0.9998$ and $y = 0.0058$ (±0.00004)$x + 0.0051$ (±0.0062) with coefficient $r = 0.9994$ and the $r^2 = 0.9996$, respectively.

However it can be noted that different statistical tests, like *e.g.*, such as the *F*-value of the Lack-of-fit (LOF) test and Mandel's fitting test have been recently recommended also to evaluate the goodness of fit of the calibration curves because of the disputable or even controversial position of the correlation coefficient r as linearity indicator [30,31]. Therefore, results for Lack-of-fit (LOF) test and Mandel's fitting test are additionally presented in Table 2 and confirming good linearity of the method. For this reason the proposed method could be linear in the range of concentration from 2 to 300 ng mL^{-1}. The *F*-value of the Lack-of-fit (LOF) test were compared with the quality coefficient and the correlation coefficient for several linear calibration lines for T. The significant values at the 95% confidence level are underlined. The results showed for the Lack-of-fit test as linear reference model LRM must systematically be accepted at the 95% confidence level, and for Mandel's fitting test even accepted at the same confidence of level. Thus, despite the fact that r and QC are greater than 0.997 and lower than 5%, respectively, the linearity of the calibration lines were rejected on the basis of the before mentioned *F*-test. The results indicate that r should be used with care when evaluating the linearity of calibration lines according to rules of Analytical Methods Committee. However, in this case, linear regression model was chosen $y = a + bx$. The test for Lack-of-fit reveals that this LRM adequately fits the calibration data at the 95% confidence level in all cases. The *p*-value was systematically smaller than 1%. Therefore, the linear reference model was chosen as the reference model.

3.3.3. Limit of detection (LOD) and limit of quantification (LOQ)

The detection limit (LOD) was defined as the lowest concentration level resulting in a peak height of three times the baseline noise. The ratio of signal size to that of noise was calculated and found to be 0.5 ng mL^{-1}.

Table 3
Assay validation results obtained from repeatability and reproducibility experiments on analysis of total T and E by LC method.

Nominal concentration (ng mL^{-1})	Mean concentration (ng mL^{-1}) $n=6$	Precision, as RSD (%)	Accuracy as recovery (%)	Mean recovery (%)
T				
Intra-day reproducibility				
20	19.5 ± 0.8	4.1	97.5	96.7 ± 3.2
50	51.2 ± 2.5	4.9	102.4	
100	98.6 ± 3.8	3.8	98.6	
Day-to-day reproducibility				
20	20.9 ± 2.3	11.0	104.5	
50	53.5 ± 4.3	8.0	107.0	
100	100.6 ± 4.8	4.8	100.6	
E				
Intra-day reproducibility				
20	19.1 ± 0.8	4.2	95.5	95.9 ± 5.6
30	31.2 ± 1.5	4.8	104.0	
50	52.2 ± 1.8	3.4	104.4	
Day-to-day reproducibility				
20	20.3 ± 2.3	11.3	101.5	
30	32.6 ± 2.5	7.7	108.7	
50	50.2 ± 4.9	9.8	100.4	

The limit of quantification (LOQ) was determined as the lowest concentration on the standard calibration curve that provided a peak height with a signal-to-noise ratio higher than 10 with a precision below 15% and accuracy of 80–120% of its nominal value. The LOQs obtained for each analyzed steroid by the method was 2 ng mL^{-1}.

3.3.4. Precision and accuracy

The accuracy and precision of the method were evaluated with quality control (QC) samples at concentrations of 20, 50 and 100 ng mL^{-1} for T and 20, 30, and 50 ng mL^{-1} for E. The intra- and day-to-day accuracy and precision values of the assay method are shown in Table 3. The precision of the method was calculated as the relative standard deviation (RSD) of the concentrations determined in all replicates. The intra-day reproducibility for steroids was below 4.9%. All values of intra-day reproducibility were below 11.3%. The accuracies were determined by comparing the mean calculated concentration to the spiked target concentration of the QC samples. The intra- and day-to-day accuracies for analyzed steroids were found to be within 95.5–104.4% and 100.4–108.7%, respectively, of the target values.

3.3.5. Stability

The stability of the steroids in urine samples at room temperature (24 °C) and at −20 °C was assessed by analyzing of samples after three repeated freeze-thaw cycles was determined to assess the integrity of the analytes. Control samples were analyzed without thawing as initial, and compared to the same samples that were frozen and thawed three times. Table 4 summarizes results for processed urine sample freeze–thaw cycles at three various concentrations of steroids of 10, 30 and 50 ng mL^{-1} during three months. It can be seen that three cycles of freezing and thawing had minimal effect on the quantitation of samples at the three concentrations for these analytes, which showed that the T and E had a good stability within three months.

Table 4
Results for processed urine sample freeze–thaw cycles during three months ($n=6$).

Urine total concentration (ng mL^{-1})		
Spiked	Initial	After three freeze-thaw cycles
T		
10	12.4 ± 0.7	10.7 ± 1.8
30	33.5 ± 1.3	29.1 ± 1.3
50	53.3 ± 3.9	53.8 ± 4.3
E		
10	10.4 ± 0.8	10.9 ± 1.9
30	32.5 ± 1.5	29.3 ± 1.4
50	57.1 ± 4.2	54.8 ± 4.5

3.4. Application to the real urine sample

The amount of T in the urine reflected the average T concentration in the blood at the time that the urine was formed. Moreover, evaluated T level in urine is stable and credible, especially in urine collected during all over the clock while plasma level of T could be incidentally changed. On the other hand, this hormone measurement in urine collected disposable at the morning is depending on the density of the urine. Due to the fact that creatinine is a product of muscle metabolism and it is normally lost in the urine at a relatively steady rate, the ratio of T and E to creatinine in the urine can be used to account for the effect of urine concentration. Simple stress may cause a mild increase in urinary T/E ratio. The presence of other illnesses may also result in increased T production by the adrenal glands and therefore increase this ratio. The level of creatinine in urine samples of subjects was studied, because the increased value indicates of renal failure and/or a reduced glomerular filtration. It may cause an increase (or decrease) of the contents of analyzed substances in urine.

3.4.1. Subjects

Ethical approval was obtained for this part of study and 116 healthy female and male volunteers and 10 athletes were used. All subjects participated in experiment previously had been signed a written consent with approbate of rules concerning questionnaire with personal details, especially diet and no taking drugs and alcoholic drinks during two weeks before experiment. As an application, real urine samples from 37 males and 79 females aged 20–57 years were collected and investigated by the method presented here. Their average age was 28.81 ± 10.23 years, body weight of 70.16 ± 15.37 kg and height of 171.09 ± 8.85 cm (average ± standard deviation). Urinary creatinine level from 116 healthy volunteers was measured in all samples using a diagnostic kit for the determination of creatinine from PZ Cormay (Lublin, Poland). The obtained results indicated that the concentrations of creatinine in urine samples were between 0.57 and 3.85 mg/dL. It confirmed that no volunteer possessed a dysfunction of kidney. All samples were collected in polyethylene urine containers and then frozen at −20 °C. Urinary average total and free T lev-

Table 5
Average urinary T and E levels in human healthy volunteers and athletes.

Number of volunteers	Sex of volunteers	Measured total concentration of total T ($ng\,mL^{-1}$)	Measured free concentration of T ($ng\,mL^{-1}$)	Measured total concentration of E ($ng\,mL^{-1}$)	Measured free concentration of E ($ng\,mL^{-1}$)
116	All healthy volunteers both sexes	44 ± 62	5.7 ± 4	33 ± 47	4.5 ± 3
79	Female	18 ± 14	Below limit of quantitate	13 ± 10	Below limit of quantitate
37	Male	99 ± 86	5.7 ± 4	74 ± 65	4.5 ± 3
10	Athletes (male)	170 ± 254	22 ± 6	79 ± 56	18 ± 5

els found in human volunteers and measured by LC method was expressed as T-to-creatinine ratio and is presented in Table 5. Practical application of the method for determination of investigated endogenous steroids in each healthy volunteers participated in the experiment was presented in Table 6. Concentrations of total T in urine of most volunteers fit within the range practically evaluated in healthy subjects, namely 30–60 $ng\,mL^{-1}$ [11]. The average concentration equaled $44 \pm 62\,ng\,mL^{-1}$, free T level was evaluated on $5.7 \pm 4\,ng\,mL^{-1}$ (both are presented in Table 5). The results for E were as follows: $33.00 \pm 47\,ng\,mL^{-1}$ and $4.5 \pm 3\,ng\,mL^{-1}$, respectively. Female have significantly lower T level comparing to male, hence, average concentration of investigated steroids for all volunteers reflects concentration in male. Few volunteers indicated concentrations exceeding the established standards of T and could be testified to the increased susceptibility to stress, diseases and efforts. However, the final diagnosis required additional research. The chromatograms of real urine samples from healthy volunteers (Fig. 3) were also presented.

The results of LC method established in this paper could be successfully applied to the determination of the doping biomarker T in the urine samples. This method is especially convenient for the analysis of the number of samples. Chromatograms obtained from athletes participated in the investigation can be seen in Fig. 4 and quantity level of urinary T and E were summarized in Tables 5 and 6. Higher average T and E level in urine samples in the case of athletes comparing to healthy volunteers was noticed. These results are in agreement with knowledge that the hormone levels increase after physical effort. Moreover, doping control using T in one competitor was confirmed and established in one athlete (competitor No. 3), who represents high T level as well as E level above $200\,ng\,mL^{-1}$ and T/E ratio > 4. No doping using T in the other athletes was observed.

3.4.2. Chemometric considerations

All calculations, mean values with their standard deviation (mean ± SD), regression model and multivariate tests were presented using computer program *Statistica* 8,0 (StatSoft, Tulsa, OK, USA) software.

The data on 116 healthy volunteers and 10 athletes were evaluated. Among all healthy volunteers data of 37 males were retrospectively evaluated using chemometric analysis including physical data, T and E level (Table 6) by modern data processing methods, namely principal component analysis (PCA). The general idea of PCA is to reduce the dimensionality of the original multivariable data set by a finding linear combination of those variables that explains most the variability within the set of data considered. The variables positioned in the space determined by the first principal components produced a plot of principal component "loadings". The principal components most significant for separation of subjects ("object scores") are PC1 and PC2. In this study, the variance of the data analyzed accounted by PC1 and PC2 cumulatively of 75.11 (t1) and 24.89% (t2), respectively. The projection of points assigned to individual subjects as principal component "scores", in the space determined by the first two principal components axes,

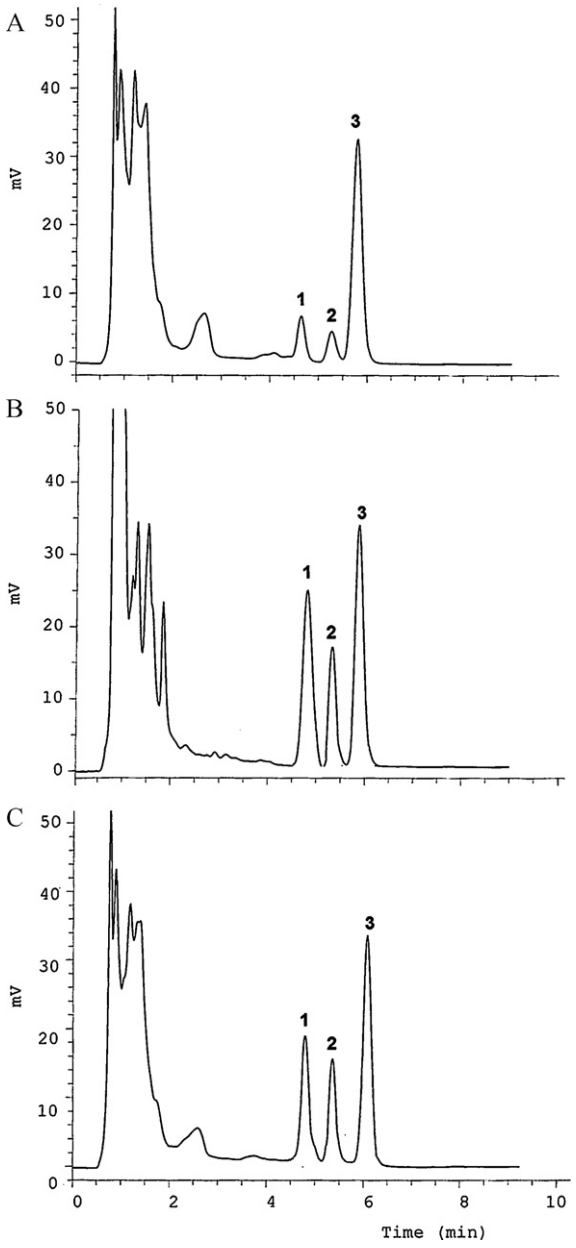

Fig. 3. Chromatograms of the urine sample obtained from healthy volunteer No. 32, 82, 38, respectively; (A) 29.02 $ng\,mL^{-1}$ of total T (1) and 21.76 $ng\,mL^{-1}$ of total E (2), (B) 122.05 $ng\,mL^{-1}$ of total T (1) and 91.54 $ng\,mL^{-1}$ of total E (2), (C) 89.96 $ng\,mL^{-1}$ of total T (1) and 67.47 $ng\,mL^{-1}$ of total E (2). All samples were spiked with 200 $ng\,mL^{-1}$ of MT (3; I.S.)

Table 6
Results of the urinary total T and E levels in healthy volunteers using the LC method.

Volunteer No	Sex	Age	Height (cm)	Body mass (kg)	Smoker or nonsmoker or passive smoker	Creatinine level (mg/dL)	Total T level (ng mL^{-1})	Total E level (ng mL^{-1})
1	F	20	180	83	Passive sm	3.85	3.42	2.56
2	F	21	168	55	Nonsmoker	0.67	15.67	11.28
3	F	21	170	68	Passive SM	2.64	6.82	5.16
4	F	21	182	67	Passive SM	1.32	6.92	5.19
5	F	21	161	53	Nonsmoker	1.90	42.2	31.65
6	F	25	168	70	Nonsmoker	2.25	12.64	9.48
7	F	30	164	60	Smoker	1.87	2.86	2.14
8	F	37	166	56	Nonsmoker	1.79	24.22	18.17
9	M	39	186	80	Passive SM	1.5	86.81	65.98
10	M	57	187	106	Smoker	1.86	96.88	72.66
11	M	28	187	76	Smoker	0.57	161.84	121.38
12	F	54	167	74	Smoker	1.12	2.95	2.2
13	F	27	165	52	Passive SM	3.26	8.64	6.57
14	F	24	165	57	Passive SM	1.15	11.5	8.64
15	F	34	164	65	Nonsmoker	1.06	9.92	7.54
16	M	21	181	100	Smoker	1.07	39.04	27.33
17	F	24	158	69	Nonsmoker	3.55	21.2	16.11
18	F	21	152	55	Passive SM	1.75	51.6	37.15
19	F	23	167	63	Smoker	1.09	39.52	29.79
20	F	21	164	59	Nonsmoker	1.20	18.32	13.19
21	M	20	173	64	Nonsmoker	3.30	132.44	100.65
22	F	27	176	74	Nonsmoker	0.78	7.66	5.75
23	M	33	194	96	Passive SM	1.67	124.04	94.27
24	M	43	173	96	Nonsmoker	2.15	325.21	247.16
25	F	34	176	60	Smoker	1.86	8.64	6.48
26	F	44	168	75	Passive SM	1.37	22.34	16.76
27	M	47	182	92	Smoker	1.53	34.56	25.92
28	F	26	169	56	Passive SM	1.75	20.62	15.46
29	F	48	164	75	Nonsmoker	1.43	12.22	9.17
30	F	49	164	60	Nonsmoker	2.25	21.14	15.86
31	M	26	194	107	Passive SM	1.82	94.84	72.08
32	M	23	176	85	Smoker	1.14	29.02	21.76
33	F	28	179	73	Nonsmoker	1.42	12.92	9.69
34	F	24	170	62	Passive SM	2.27	7.63	5.72
35	F	32	168	65	Passive SM	2.88	39.43	29.57
36	M	37	180	105	Smoker	2.12	81.62	61.22
37	F	31	166	58	Smoker	1.69	10.68	8.01
38	M	52	175	86	Nonsmoker	1.69	89.96	67.47
39	F	19	168	60	Nonsmoker	2.51	18.42	13.82
40	M	27	187	86	Nonsmoker	1.90	169.24	126.93
41	F	31	159	48	Nonsmoker	1.56	9.06	6.8
42	F	24	158	42	Smoker	0.97	7.58	5.69
43	F	37	164	81	Nonsmoker	1.98	24.32	18.24
44	M	31	176	86	Nonsmoker	1.75	145.75	109.31
45	F	21	166	60	Nonsmoker	0.99	35.25	26.44
46	M	50	176	85	Nonsmoker	1.91	332.88	252.99
47	F	39	158	60	Smoker	0.8	Nd	Nd
48	M	40	181	85	Smoker	1.79	25.8	19.35
49	M	37	175	79	Nonsmoker	2.15	184.96	138.72
50	M	40	170	68	Passive SM	2.68	69.88	53.11
51	F	48	167	50	Smoker	2.08	2.18	Nd
52	M	50	180	76	Smoker	1.18	41.04	30.78
53	F	32	173	73	Smoker	1.13	17.09	12.82
54	M	32	175	115	Passive SM	2.93	50.96	38.22
55	F	27	162	76	Passive SM	1.39	40.42	30.31
56	M	55	176	90	Passive SM	1.78	43.44	32.58
57	M	26	174	89	Nonsmoker	0.75	54.64	40.98
58	F	53	159	65	Nonsmoker	0.83	18.13	13.61
59	F	21	172	59	Nonsmoker	2.85	22.44	16.83
60	M	28	184	92	Nonsmoker	2.16	25.11	18.83
61	F	20	171	58	Passive SM	1.70	43.22	32.42
62	M	21	178	73	Smoker	0.82	28.45	21.34
63	F	21	164	55	Passive SM	1.89	24.86	18.64
64	M	21	185	57	Nonsmoker	2.42	48.86	36.65
65	M	21	184	115	Nonsmoker	2.16	39.96	29.97
66	M	32	190	87	Smoker	2.62	41.48	31.11
67	F	29	169	80	Passive SM	2.54	24.8	18.6
68	F	41	158	65	Passive SM	2.36	19.02	14.27
69	M	34	164	65	Smoker	1.45	19.44	14.58
70	F	21	164	58	Passive SM	1.54	1.32	0.99
71	F	21	174	65	Nonsmoker	1.99	10.22	7.67
72	F	22	168	63	Nonsmoker	1.17	22.44	16.83
73	F	21	174	79	Nonsmoker	2.50	2.44	1.83
74	F	21	170	59	Nonsmoker	1.63	18.99	14.24
75	F	21	165	53	Nonsmoker	1.26	6.62	4.97

Table 6 (*Continued*)

Volunteer No	Sex	Age	Height (cm)	Body mass (kg)	Smoker or nonsmoker or passive smoker	Creatinine level (mg/dL)	Total T level (ng mL^{-1})	Total E level (ng mL^{-1})
76	F	21	169	60	Nonsmoker	2.74	24.62	18.47
77	F	20	165	68	Smoker	2.51	4.48	3.36
78	F	20	170	54	Nonsmoker	0.34	8.02	6.02
79	F	22	168	71	Smoker	1.13	Nd	Nd
80	F	21	167	55	Passive SM	2.32	20.13	15.1
81	M	22	166	57	Nonsmoker	2.89	99	74.25
82	M	35	188	87	Nonsmoker	3.18	122.05	91.54
83	F	21	180	64	Passive SM	2.07	35.22	26.41
84	F	21	168	56	Smoker	2.07	9.37	7.03
85	F	22	172	72	Smoker	2.08	4.56	3.42
86	M	24	180	80	Passive SM	2.08	81.88	61.41
87	F	25	168	57	Nonsmoker	2.09	16.55	12.41
88	M	26	178	75	Nonsmoker	2.87	97.241	72.93
89	F	22	160	63	Nonsmoker	2.15	15	11.25
90	F	22	172	48	Nonsmoker	1.37	10.12	7.59
91	F	20	176	65	Nonsmoker	2.88	38.58	28.94
92	F	21	167	56	Nonsmoker	1.40	11.12	8.34
93	F	46	162	55	Smoker	1.79	26.35	19.76
94	M	41	178	90	Passive SM	1.29	147.96	110.97
95	F	23	167	63	Passive SM	1.81	14.44	10.83
96	F	22	180	84	Passive SM	2.97	48.86	50.67
97	F	21	165	69	Passive SM	1.15	20.02	15.02
98	F	21	170	67	Nonsmoker	1.70	8.02	6.02
99	M	22	176	94	Passive SM	1.42	62.6	46.95
100	F	21	165	55	Passive SM	1.59	14.02	10.52
101	F	21	162	52	Passive SM	1.49	61.42	46.07
102	F	21	165	60	Passive SM	1.70	52.3	39.23
103	F	22	160	62.8	Nonsmoker	2.95	27.22	20.42
104	F	46	160	99	Passive SM	1.83	3.71	2.78
105	M	47	182	84	Nonsmoker	2.70	359.18	269.39
106	F	21	170	70	Passive SM	1.90	7.31	5.48
107	M	22	174	68	Smoker	2.43	29.92	22.44
108	F	21	160	56	Nonsmoker	1.13	27.71	20.78
109	F	21	165	57	Smoker	1.64	19.93	14.95
110	F	20	163	54	Smoker	1.44	4.26	3.19
111	F	22	159	72	Nonsmoker	1.45	22.29	17.72
112	F	21	171	55	Nonsmoker	0.54	Nd	Nd
113	F	22	174	70	Passive SM	3.92	9.11	6.83
114	M	23	179	75	Nonsmoker	1.32	45.61	34.21
115	F	23	165	55	Passive SM	1.26	7.81	5.86
116	M	23	190	80	Passive SM	2.49	29.77	22.33
Average		28.81	171.09	70.16		1.85	43.91	33.00
SD		10.22	8.85	15.37		0.72	62.08	46.88
Athletes								
1	M	24	192	86	Nonsmoker	2.68	175.36	115.94
2	M	23	188	92	Nonsmoker	1.76	78.66	57.2
3	M	25	194	84	Nonsmoker	2.11	876.46	205.26
4	M	24	190	88	Nonsmoker	1.98	182.78	123.93
5	M	23	186	88	Nonsmoker	2.33	24.36	22.19
6	M	24	188	90	Nonsmoker	1.54	36.89	27.67
7	M	24	190	90	Nonsmoker	2.32	126.69	78.99
8	M	23	186	92	Nonsmoker	0.99	64.76	48.86
9	M	25	188	88	Nonsmoker	1.56	48.66	41.16
10	M	24	190	92	Nonsmoker	1.34	89.96	68.93
Average		23.90	189.20			1.86	170.46	79.01
SD		0.74	2.53			0.52	254.00	55.88

Nd – not detected

PC1 and PC2, is depicted in Fig. 5 as two-dimensional plot and in Fig. 6 as three-dimensional plot. An apparent clustering of the subjects and variables was observed – these have been interpreted in terms of their similarities and dissimilarities.

Lower values of PC1 for subjects suggest no differences among these healthy volunteers (left top corner in Fig. 5). On the other hand, as it can be seen in two-dimensional plot of loadings corresponding with eigeinvalues attributed to subjects (male No. 24, 46 and 105) attracts attention because of highest eigeinvalues (above 3) as well man No. 32 (above 0.5). To our knowledge, concentration of T is strongly associated with age, however, sometimes is typically not age-adjusted, but based on an average of a test group which includes elderly males with low T levels. Therefore, a value of 3 ng mL^{-1} might be normal for a 65-year-old male, but not normal for a 30-year-old. Identification of inadequate T in an aging male by symptoms alone can be difficult. Volunteer No. 32 was young man (23-years old) with low concentration of T, whereas subjects No. 24, 105 and 46 were 43-, 47- and 50-years old, respectively and they had high level of urinary T. Further, body mass is the second factor which affects on concentration of T and E. Volunteer No. 16 possessed obesity, probably it causes that the volunteer is in the same cluster as subjects 50, 52, 56 and 62. Next cluster can be seen in the middle of the plot containing male No. 23, 49, 82 and 94 with average aging and weight. Studies show there is no total agree-

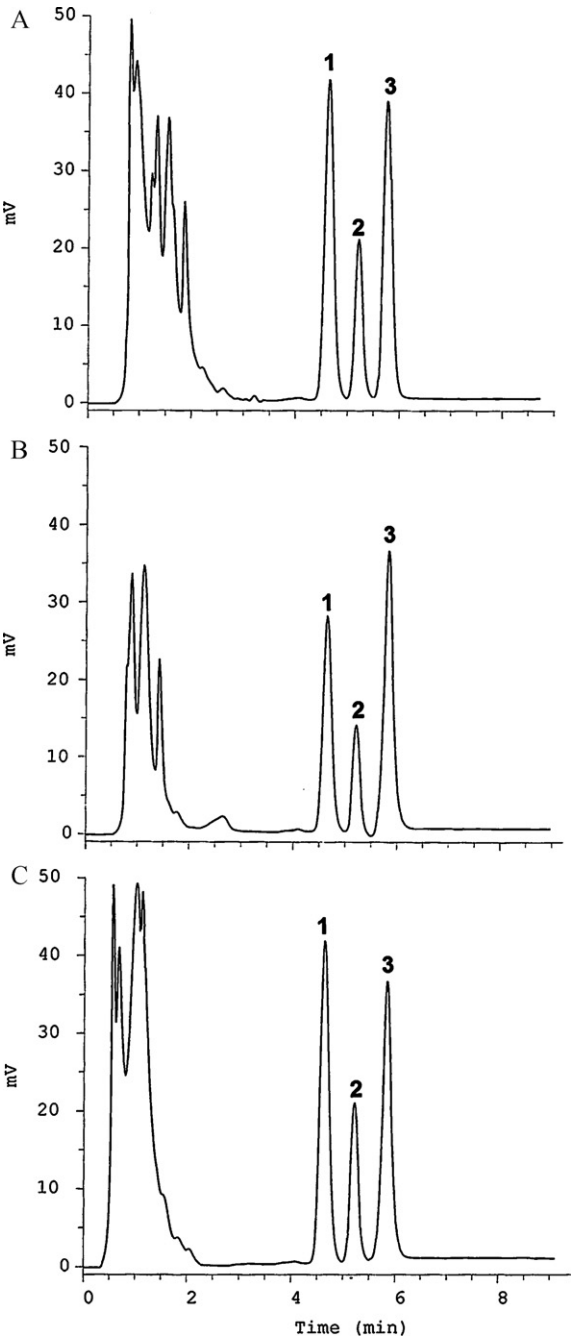

Fig. 4. Chromatograms of the urine sample obtained from athletes No. 4, 7, 1, respectively; (A) 182.78 ng mL^{-1} of total T (1) and 123.93 ng mL^{-1} of total E (2), (B) 126.69 ng mL^{-1} of total T (1) and 78.99 ng mL^{-1} of total E (2), (C) 175.36 ng mL^{-1} of total T (1) and 115.94 ng mL^{-1} of total E (2). All samples were spiked with 200 ng mL^{-1} of MT (3; I.S.)

ment on the thresholds of T value below which a man would be considered hypogonadal disease. T can be measured as "free" (that is, bioavailable and unbound) or more commonly, "total" (including the percentage which is chemically bound and unavailable). In the United States, male total T levels below 3 ng mL^{-1} from a morning serum sample are generally considered low. The signs and symptoms are non-specific, and might be confused with normal aging characteristics, such as loss of muscle mass and bone density as well as decreased physical endurance. Recent studies suggest that T level plays a major role in a risk taking during financial decisions. Fatherhood also decreases T levels in males, suggesting that the resulting emotional and behavioral changes promote paternal

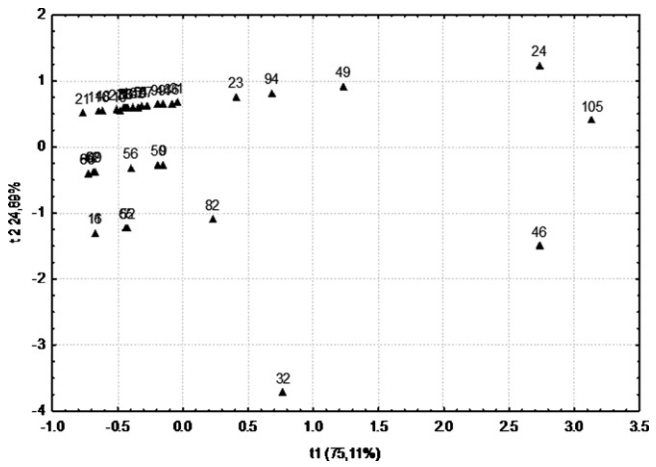

Fig. 5. Two-dimensional PCA plot based on urinary total T, total E levels and data of volunteers for investigated group of healthy males (37).

care and loss of libido, that falling in love decreases men's T levels while increasing women's T levels. It is confirmed by male No. 60. It is speculated that these changes in T result in the temporary reduction of differences in behavior between the sexes. In the research performed, influence of age on analyzed hormones level was noticed mainly. However, our results show that the best meaning seems to have influence of smoking. Smokers in spite of young age represent significantly lower T level (like No. 62) than older nonsmokers like males No. 105 and 46 (Fig. 5).

It should be emphasized that plasma T level can be misleading due to the circadian rhythm variation [4] while total urinary T, first of all glucuronide, levels are independent of short-term changes in blood and because of that they can be used as an index of androgen production [2]. Moreover, urine is biological matrix readily obtainable through noninvasive collection procedures. Taking of blood is stressful situation for patients and could evoke of fluctuation of T level in volunteers. The level of steroid hormone in urine after correction to creatinine ratio is stable, independently from con-

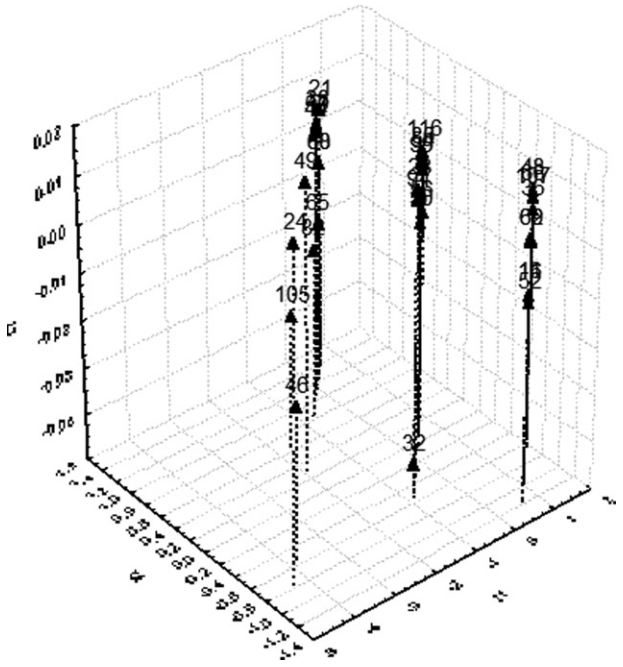

Fig. 6. Three-dimensional PCA plot of subjects based on urinary total T, total E levels and data of volunteers for investigated group of healthy males (37).

centration of urine, which should be different in various time for night or day.

It is known that T is primarily excreted by glucuronide, but only in small amounts as sulphate, therefore determination of total T level is usually made after hydrolysis of the conjugate. For this reason acid hydrolysis both glucuronide and sulphate conjugates has been used so far to free the analyzed substance from its conjugate [24]. In order to carry out acid hydrolysis of the steroid high temperature is necessity. However, acid hydrolysis causes that analyst has difficulty to continue of analysis because of intensive dark pigment formed in the extract. Nevertheless, a good resolution of this problem was proposed by washing the extract on sorbent column with mixture of acetone and water (25:75 v/v). Acetone is the best solvent which allows eliminating the pigment. Futhermore, structure of the steroid can alter. Hence our colleague decided to determine urinary non-conjugated T level carried out without hydrolysis because of urinary nonconjugated T values could be useful in clinical conditions of androgen excess or deficiency. We also know that non-conjugated T is the biologically active form of the hormone, hence the idea of its measuring. It was also interesting to work out the determination method of total urinary T including conjugated and non-conjugated excretion using hydrolysis as well as separately for free T and comparative evaluation to assess correlation between both forms of T. Novelty is showing that the information generated in this investigation on human nonconjugated T level in urine and its comparison to conjugated form level, may be used in studies where functional properties need to be determined for prediction of different disease androgen hormone origin. Additionally comparative studies between urinary male and female T excretion makes our experience more realistic and practically usefulness.

4. Conclusions

In summary, the proposed LC method for quantification of T and E in urine samples showed that the analytical procedure developed in this work is fast, specific, precise, no expensive with acceptable accuracy. Moreover, the described method is sufficiently sensitive to determine free- and total-level T for monitoring the concentrations of T in healthy volunteers, in patients with androgen related disorders as well as in athletes to detect doping with androgens. By applying this method a good selectivity was achieved with quite satisfactory distinguished peaks of T, E, and MT (as I.S) in human urine samples. Further, LC method produces less environmental pollution through replacement of dichloromethane for methanol, gives high efficiency, reproducibility and causes with a low cost using simple and commonly available UV detection. The implementation of LC method would be facilitated because it uses a simple UV detector, which is standard instrumentation in each laboratory and low demands in terms of maintenance. The extraction of the free form of analytes is based on a simple SPE step without hydrolysis, it significantly shortened time need to prepare urine samples before chromatographic analysis. Finally, the present analysis method could be also useful for clinical application to check androgen excretion level, improvement of prognosis of cancer treatment as well as doping control in athletes. Good correlation was observed between free and total steroids level in urine samples for all subjects participated in described research.

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