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Quantification of testosterone and metabolites released after alkaline treatment in human urine

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A rapid, accurate, and sensitive method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the quantification of the testosterone (T) fraction released under basic conditions and its metabolites 1,4-androstadien-3,17-dione (1,4-AD), 4,6-androstadien-3,17-dione (4,6-AD) and 4,6-androstadien-17β-ol-3-one (6-T) obtained in the same way has been developed and validated. Urine samples (5 ml) were alkalinized and the released analytes were extracted by liquid-liquid extraction. The chromatographic separation was performed in 8 min. MS/MS determination was performed under selected reaction monitoring mode using electrospray ionization in positive mode. The method was shown to be linear from 0.05 to 50 ng/ml for 1,4-AD, 6-T and T while for 4,6-AD the linearity from 0.5 to 500 ng/ml was demonstrated ($r > 0.99$). Limits of detection below 0.05 ng/ml were obtained for all analytes. Intra-assay precision and accuracies, evaluated at three concentrations levels (0.1, 1 and 20 ng/ml) were below 20%. The applicability of the analytical method was confirmed by analysis of several samples collected from healthy volunteers and samples collected after oral testosterone undecanoate administration. The analytical method was found to be able to quantify the metabolites either at the low concentrations levels present in the urine from untreated volunteers or at the high concentrations found after T administration. Short analysis time, simple sample preparation, and satisfactory quantitative parameters make this method potentially useful for anti-doping control purposes.

Keywords: testosterone; metabolites; mass spectrometry; doping analysis; quantitative method

Introduction

Anabolic androgenic steroids are the most commonly detected substances prohibited in human sports. Testosterone (T) is the principal androgenic anabolic steroid in humans and it is the most frequently reported adverse analytical finding among anti-doping laboratories.[1] T is available in a wide range of forms and dosages; for example, it is available as gels, as an ester in oil by intra-muscular injections, and as oral preparations.

T has been used as a performance-enhancing substance in sport for decades,[2] and the performance benefits produced by T have been clearly demonstrated.[3] However, due to its endogenous nature, the detection of T misuse is an analytical challenge for doping control laboratories. The testosterone/epitestosterone ratio (T/E) is the most well established reference marker for T misuse.[4] T/E ratios greater than 4 are considered indicative of T misuse and need further investigations.[5] The T/E ratio can be determined either by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS).[5,6,7] Despite this, the T/E ratio has several limitations: genetic aspects can have significant effects on urinary T/E excretion,[8] values lower than 4 can be found in samples with a low basal T/E even after T administration.[9,10] In addition, low retrospectivity in T misuse detection can be found depending on the route of administration (e.g. main changes in steroid profiles were reported during the 0–12 h after oral administration[11]). In spite of these drawbacks, the measurement of the T/E ratio by GC/MS is still the reference methodology for screening testosterone misuse in sports.

The detection of additional metabolites can improve the screening for anabolic steroids by increasing retrospectivity and sensitivity. This fact has been demonstrated in the screening of some exogenous steroids, such as methandienone,[12] stanozolol,[13] and methyltestosterone,[14] where the discovery of long-term metabolites allowed for an improvement in the detection of the misuse to these steroids.

Recently, four additional T metabolites (1,4-androstadien-3,17-dione, 4,6-androstadien-3,17-dione, 4,6-androstadien-17β-ol-3-one, and 15-androsten-3,17-dione) have been described in urine after the treatment with KOH and analysis by LC-MS/MS. The structure of three of them has been confirmed by analysis of reference compounds while 15-androsten-3,17-dione has been proposed as a feasible structure for the fourth metabolite.[15] As T metabolites, these compounds could help in the detection of T misuse in the same way that recently reported metabolites improved the detection of exogenous steroids.[12–14]

In the case of exogenous steroids, the sole identification of the metabolite is enough to declare an adverse analytical finding; qualitative methods are normally applied for this purpose.

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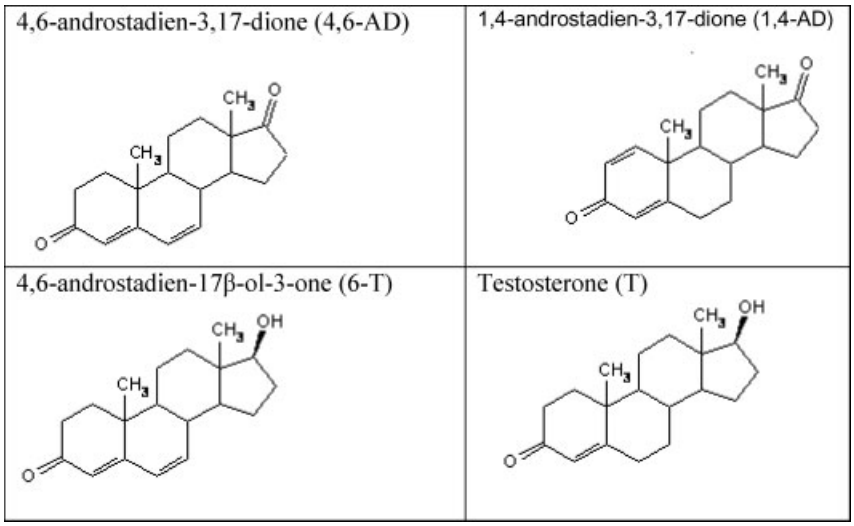

Figure 1. Structure for selected analytes.

Contrarily, due to their endogenous nature, T metabolites are present in every sample, and screening methods for detection of T misuse should differentiate between basal concentration levels and the concentrations found after T administration. Moreover, the tendency in the doping control field is to establish an individual biological passport where an accurate quantification is compulsory. Therefore, appropriate methods for the quantification of endogenous metabolites in urine are required for their application in the doping control field.

The aim of the present work study was to develop and validate a quantitative method for the direct determination of these new T metabolites based on LC-MS/MS. The application of the developed method was tested by quantifying these metabolites in samples collected from healthy people and samples collected after intake of a single oral dose of testosterone undecanoate

Experimental

Chemicals and reagents

4,6-androstadien-3,17-dione (4,6-AD) and 4,6-adrostadien-17β-ol-3-one (6-T) were obtained from Steraloids Inc. (Newport,RI, USA). 1,4-androstadien-3,17-dione (1,4-AD) was purchased from NMI (New South Wales Pymble, Australia). Methandienone, used as an internal standard (ISTD) and Testosterone (T) were obtained from Sigma-Aldrich (St Louis, MO, USA). For structures of the analytes see Figure 1.

Diagnostics GmbH (Mannheim, Germany). Citric acid, sodium bicarbonate, urea, calcium chloride, sodium chloride, iron II sulfate, magnesium sulfate, sodium sulphate, potassium dihydrogen phosphate, ammonium chloride, analytical grade potassium carbonate, potassium hydroxide pellets, di-sodium hydrogen phosphate, sodium hydrogen phosphate and *tert*-butyl-methyl ether were obtained from Merck (Darmstadt, Germany). Lactic acid, creatinine and urea were purchased from Sigma-Aldrich (St Louis, MO, USA).

Acetonitrile and methanol (LC gradient grade), formic acid (LC/MS grade) (Merck, Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). Detectabuse® columns were obtained from Biochemical Diagnostics Inc. (Edgewood, NY, USA).

Instrumentation

LC-MS/MS

Selected reaction monitoring (SRM) method was carried out using a triple quadrupole (Quattro Premier XE) mass spectrometer equipped with an orthogonal Z-spray-electrospray ionization source (ESI) (Waters Associates, Milford, MA, USA) interfaced to an UPLC system, Acquity (Waters Associates, Milford, MA, USA) for the chromatographic separation. Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h and the cone gas flow to 50 L/h. A cone voltage of 25 V, and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 450 °C and the source temperature to 120 °C.

The LC separation was performed using an Eclipse Plus C_{18} column (50 × 2.1 mm i.d., 1.8 µm) (Agilent, Palo Alto, CA, USA), at a flow rate of 300 µL/min. Water and methanol both with formic acid (0.01%) and ammonium formate (1 mM) were selected as mobile phase solvents. pH was not monitored, despite of this all steroids showed stable retention times. A gradient program was used; the percentage of organic solvent was linearly changed as follows: 0 min., 45%; 1 min., 45%; 3.5 min., 65%; 4.5 min., 95%; 5 min., 95%; 5.5 min., 45%; 8 min, 45%.

Analytes were determined by an SRM method including two transitions for each compound (Table 1). The most abundant and specific was selected for quantitative purposes.

Sample preparation

After addition of the ISTD (methandienone 1 µg/ml, 50 µl), urine samples (5 ml) were directly alkalinized by addition of 300 µl of KOH 6M (no previous enzymatic hydrolysis was performed). Incubation was performed fo 15 min at 60 °C. After cooling to room temperature, a liquid-liquid extraction was performed by addition of 6 ml *tert*-butylmethylether. The sample was centrifuged and the organic layer separated and evaporated.

The residue was dissolved into 150 µl of a mixture of water : acetonitrile (50 : 50, *v*/*v*) and 10 µL were directly injected into the LC-MS/MS system.

Table 1. MS/MS conditions for selected analytes

Analyte	Precursor ion m/z		Product ion specie	Product ion Collision energy (eV)
1,4-AD	285	$[M+H]^+$	121[a]	20
			147	15
4,6-AD	285	$[M+H]^+$	149[a]	25
			97	35
6-T	287	$[M+H]^+$	151[a]	20
			133	25
T	289	$[M+H]^+$	97[a]	30
			109	20
Methandienone	301	$[M+H]^+$	121	20

[a] Ion selected for quantitative purposes.

Artificial urine preparation

The artificial urine was prepared based on a protocol described elsewhere.[16] 0.1g of lactic acid, 0.4g of citric acid, 2.1g of sodium bicarbonate, 10g of urea, 0.07g of uric acid, 0.8g of creatinine, 0.37g of calcium chloride*$2H_2O$, 5.2g of sodium chloride, 0.0012g of iron II sulfate*$7H_2O$, 0.49g of magnesium sulfate*$7H_2O$, 3.2g of sodium sulfate*$10H_2O$, 0.95g of potassium dihyrogen phosphate, 1.2g of di-potassium hydrogen phosphate, and 1.3g of ammonium chloride were dissolved in one liter of ultrapure water. The artificial urine presented a pH of 6.8 and a specific gravity of 1.0126

Method validation

Linearity

Calibration standards at ten concentration levels (0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 ng/ml for 1,4-AD, 6-T and T and at 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/ml for 4,6-AD) were prepared in ultrapure water and analyzed. Calibration curves were calculated by least-squares linear regression. Method was considered linear if a correlation coefficient (r) higher than 0.99 was obtained.

Intra-assay accuracy and precision

Quality control samples were prepared to check for precision and accuracy of the method. Since T and its metabolites are endogenous compounds ubiquitously present in human urine, quality control samples were prepared by spiking appropriate amounts of methanolic solutions of each compound to artificial urine. Three concentration levels were chosen along the calibration ranges. For 1,4-AD, 6-T and T, control samples were prepared at 0.1, 1, and 20 ng/ml. For 4,6-AD the control samples were prepared at 1, 10, and 200 ng/ml.

Intra-assay precision of the method is expressed as the relative standard deviation (%) of the estimated concentrations obtained for four replicates of the quality control samples analyzed in one assay, at each concentration level. Intra-assay accuracy is expressed as the relative error (%) in the estimation of each concentration for the quality control samples.

Inter-assay accuracy and precision

Inter-assay precision is given as the relative standard deviation (%) of the estimated concentrations obtained for all replicates analyzed along the three validation assays and by two different operators (n = 10, at each concentration level). Inter-assay accuracy is obtained as described for intra-assay accuracy (n = 10, at each concentration level).

Limit of detection (LOD)

The LOD, defined as the lowest concentrations with a value of the signal/noise ratio (S/N) of 3, was calculated by estimating the S/N from the chromatogram at the lowest concentration assayed.

Limit of quantification (LOQ)

The LOQ was defined as the lowest concentration assayed, which gave acceptable recoveries and precision.

Stability

Freeze/thaw stability was studied during a week by performing three cycles of freezing at −20 °C and thawing at room temperature. Concentrations calculated after these cycles were compared with those obtained in the analysis of the fresh urine.

Matrix effect

Due to the unavailability of truly blank urine samples, the matrix effect was evaluated by standard additions. In a first step, the concentration levels of the analytes in different urine samples (n = 6) were calculated by mean of a external calibration. Once the external calibration concentrations were established, standard additions were carried out in every sample by the addition of four concentration levels (0.5, 1, 2, and 3 times the external calibration concentration). All samples were then analyzed and the areas obtained were plotted against de added concentration The standard addition concentration value was calculated for each sample using a least squares linear regression by extrapolation to the x axis.

The matrix effect was calculated for each analyte as the difference between the external calibration concentration and standard addition concentration.

Application to real samples

In order to evaluate the applicability of the developed method, 16 samples collected from healthy volunteers (males, 20–35 years) were analyzed. Furthermore, post-administration samples collected from four volunteers after oral administration (from 0 to 4 h) of a single dose of 120 mg of testosterone undecanoate (TU) (Androxon™, three 40-mg capsules; Organon) were analyzed. These samples were stored at −20 °C until analysis. Ethical approval for the study was granted by Comité Ètic d'Investigació Clínica of our institute (CEIC-IMAS No. 94/467) and the Spanish Health Ministry (DGFPS No. 95/75). All of the subjects participating in the study gave their written informed consent.

Results and Discussion

Method optimization

The 1,4-AD, 4,6-AD, 6-T were recently reported as T metabolites released after alkalinization of the sample.[15] Although the origin of these analytes is still unknown, they are probably coming from the release of a conjugate formed in phase II metabolism.

The qualitative approach proposed in the previous study[15] for the extraction of the metabolites presented several steps. Briefly, urine were pre-concentrated in a Detectabuse® column, then, after enzymatic hydrolysis, the glucuronides and the free steroids were removed. Finally, a basic treatment (100 µl of KOH 6M, 15 min to 60 °C) was performed in order to extract the above mentioned metabolites. Although this method was found to be useful for qualitative purposes, some of the steps performed in the method exhibited uncontrolled losses of analytes which would hamper its quantitative application. For this reason, the method was revised in order to avoid these drawbacks.

First, the efficacy of the column (a XAD-2 Detectabuse®) for the retention of the conjugated form of these metabolites was questioned. For this purpose, four urine samples were passed through the columns, previously conditioned with methanol and water.[15] The column was then washed with water and both the fraction corresponding to the loading of the sample and the water-washing step were mixed and analyzed as non-retained fraction. Finally, the column was eluted with methanol. This eluate was analyzed as retained fraction.

The results for both fractions were compared. A significant percentage of the metabolites was detected in the unretained fraction indicating loses during this step which can affect the quantitative parameters of the method. For 1,4-AD, 4,6-AD and 6-T the unretained fraction ranged from 8% to 71% while T was the highest retained compound and almost quantitative retention was achieved in most of samples. The percentage of analyte lost in the pre-concentration step was not constant, showing that the retention efficiency of these metabolites may depend in part of the urine matrix or the variability within the columns hampering the quantification of the analytes. For this reason, the pre-concentration step was eliminated.

A complete release of the analytes is crucial for the quantitative determination of the metabolites. In order to optimize the incubation step, different concentrations of KOH and different times of incubation were tested using blank samples from healthy volunteers. The addition of different volumes of KOH 6 M was studied. Adding 300 µl of KOH 6 M allowed for the complete release of the selected metabolites.

The incubation time was also studied. To optimize this step, three samples were analyzed using different times (5 min, 15 min, 30 min, 1 h and overnight) with 300 µl of KOH 6 M at 60 °C.

Table 2. Concentrations obtained for each metabolite and the parent compound as a function of the incubation time in a sample from a healthy volunteer: 5 minutes (5′), 15 minutes (15′), 30 minutes (30′), 1 hour (1 h) and overnight

	Concentration (ng/ml)				
Analyte	5′	15′	30′	1 h	Overnight
1,4-AD	0.5	0.5	0.6	0.6	0.5
4,6-AD	25.7	25.9	28.3	25.2	27.9
6-T	2.8	2.5	2.9	2.6	3.2
T	0.8	0.8	0.9	0.8	0.5

The concentration for each sample was calculated (**Table 2**). The concentrations did not change between 5 min to overnight. Only T showed variations in the concentration obtained after overnight incubation, probably due to the fact that higher matrix effects are obtained when increasing the incubation time; 15 min was selected to assure the complete release of the metabolites.

The need of the hydrolysis step was also evaluated. Avoiding the enzymatic hydrolysis reduces the total analytical time but the partial degradation of phase II metabolites after basic treatment can affect the final concentration of the analytes. In order to check that common phase II metabolites were not degraded under basic conditions, testosterone glucuronide and sulfate standards were treated as described above. Neither testosterone nor other analytes were found in the extract confirming the stability of common phase II metabolites under the studied conditions.

Therefore, 5 ml of urine, 300 µl of KOH 6 M and 15 min at 60 °C were used for the quantification of the metabolites released after basic treatment.

Method validation

The assay was shown to be linear over the range 0.05–50 ng/ml for 1,4-AD, 6-T and T, and over the range 0.5–500 ng/ml for 4,6-AD. A reproducible linear relationship between concentration and response was found over the measured concentrations range. The correlation coefficients for all metabolites were higher than 0.99 (**Table 3).**

Results for accuracy and precision are summarized in **Table 3**. Both, intra-assay RSD and inter-assay RSD were normally below

Table 3. Linearity, precision, accuracy and LOD for each metabolite and the parent compound

Analyte	Range (ng/ml)	Corr. coeff. (r)	Spiked conc. (ng/ml)	Error (%)	RSD intra-assay (%) (n = 4)	RSD inter-assay (%) (n = 10)	LOD (ng/ml)
1,4-AD	0.05–50	0.993	0.1	1.3%	16	11	0.006
			1	1.6%	7	10	
			20	−2.6%	5	11	
4,6-AD	0.5–500	0.992	1	−10%	18	19	0.001
			10	9.8%	4	10	
			200	−12.9%	6	8	
6-T	0.05–50	0.997	0.1	0%	10	12	0.047
			1	−4.2%	7	11	
			20	−11.7%	2	12	
T	0.05–500	0.994	0.1	−1.9%	23	28	0.006
			1	−1.7%	9	7	
			20	−13.4%	9	10	

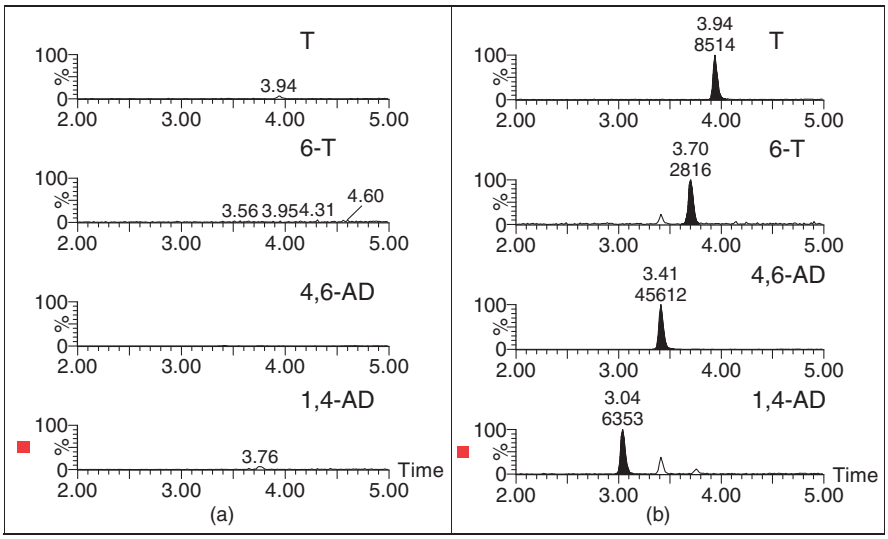

Figure 2. Chromatograms of (a) artificial urine and (b) artificial urine spiked to 0,1 ng/ml of T and metabolites.

Table 4. Matrix effect calculated with standard addition and external calibration for each metabolite and the parent compound

	1,4-AD			4,6-AD			6-T			T		
Concentration (ng/ml)	Std. Add[a]	Ext. cal[b]	Matrix effect (%)	Std. Add[a]	Ext. cal[b]	Matrix effect (%)	Std. Add[a]	Ext. cal[b]	Matrix effect (%)	Std. Add[a]	Ext. cal[b]	Matrix effect (%)
Vol.1	0.31	0.48	53%	35.1	34.7	−1%	3.36	3.37	0%	0.72	0.43	−40%
Vol.2	0.15	0.20	31%	18.9	16.9	−11%	3.04	2.91	4%	0.05	0.06	8%
Vol.3	0.23	0.31	35%	24.1	25.1	4%	2.19	2.11	4%	0.14	0.20	43%
Vol.4	0.10	0.14	34%	24.2	22.3	−8%	1.71	1.50	−13%	0.02	0.02	−8%
Vol.5	0.47	0.72	53%	44.1	41.4	−6%	3.52.	3.80	8%	0.14	0.17	15%
Vol.6	0.55	0.71	30%	96.0	70.3	−27%	6.43	5.98	7%	0.09	0.10	11%
Matrix effect[c] (mean)			+39%			−7%			−2%			+5%
RSD			8%			12%			7%			26%

[a] Concentration (ng/ml) obtained using standard addition approach.
[b] Concentration (ng/ml) obtained using a external calibration.

20%. These data indicate that the method provides adequate accuracy and precision for the detection of these three metabolites in urine samples. Only for T, RSD values exceeding 20% (23% for intra-assay and 28% for inter-assay) were obtained at the lowest assayed concentration.

The established LOQ for both 1,4-AD and 6-T was 0,05 ng/ml, whereas for 4,6-AD a LOQ of 0,5 ng/ml was achieved. For T, the LOQ was estimated at 1 ng/ml due to the poor precision obtained at 0.1 ng/ml. On the other hand, the estimated LOD ranged from about 1 pg/ml for 4,6-AD to 47 pg/ml for 6-T (Table 3). Typical chromatograms obtained for a blank artificial urine sample and an artificial urine sample spiked at 0.1 ng/ml are presented in Figure 2 showing the sensitivity of the method.

Urine samples were found to be stable during a week at −20 °C and after three freeze/thaw cycles. Concentrations with deviations lower than 12% with respect to initial concentrations were found after these storage conditions.

Ion suppression or enhancement can affect the urinary quantification of anabolic steroids.[17,18] The common approach to quantifying this matrix effect is the comparison between a blank sample spiked at a known concentration after extraction and a solvent spiked at the same concentration. Due to the endogenous nature of these compounds, the use of this approach was not possible. Isotope-labelled compounds can be used to minimize this limitation.[6] However, isotope labelled analogues for most of the selected compounds were not commercially available. Therefore, the matrix effect was evaluated by standard additions (Table 4). The results for 4,6-AD, 6-T, and T showed no differences between the concentrations obtained by external calibration and by standard additions indicating that no matrix effect was observed for these compounds. In the case of the earliest eluting compound (1,4-AD), an enhancement of around 40% was observed probably due to some components of the matrix. The RSD of the six urine tested was also evaluated in order to check if the matrix effect was dependent on the sample. For 1,4-AD, 4,6-AD and 6-T low RSD were obtained indicating that the matrix effect did not depend on the sample. The higher RSD obtained for T (26%) could be associated either with a higher variability of the matrix effect depending on the sample or with the poor precision obtained for T (Table 3).

Method application

The developed method was applied to quantify 1,4-AD, 4,6-AD, 6-T and T in urine samples from volunteers who have declared not

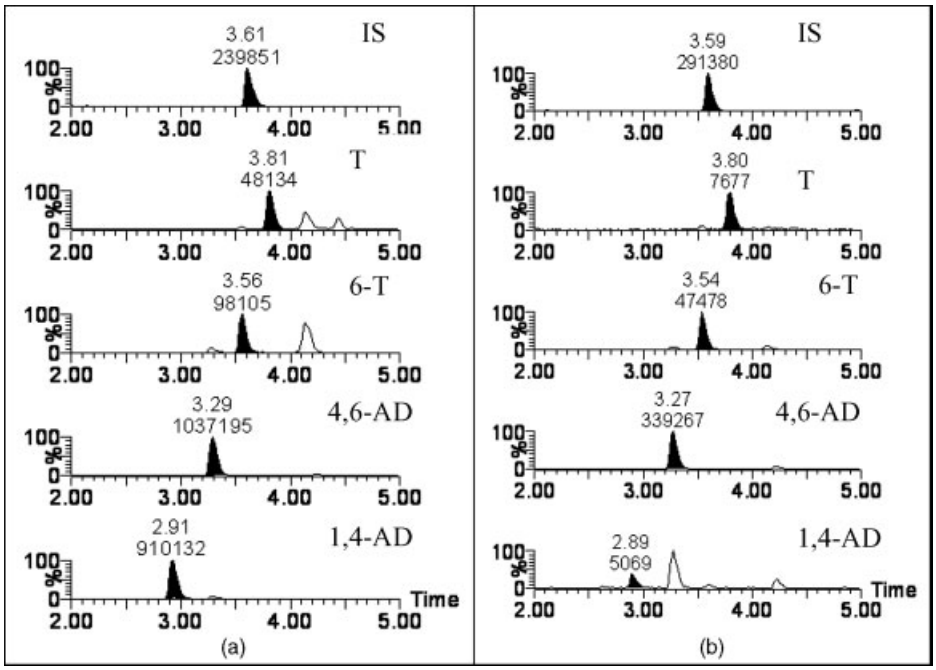

Figure 3. Chromatograms of actual urine samples: a) sample collected from 0 to 4h after TU intake (1,4-AD: 43.7 ng/ml, 4,6-AD: 104.7 ng/ml, 6-T: 16.6 ng/ml, T: 4.5 ng/ml) and b) sample from a untreated volunteer (1,4-AD: 0.14 ng/ml, 4,6-AD: 14.5 ng/ml, 6-T: 1.9 ng/ml, T: 0.17 ng/ml).

taking any forbidden substance (n = 16) and samples collected after the intake of a single oral dose of TU (n = 4). These metabolites were detected in all samples. For samples of untreated volunteers, the concentrations ranged from 0.06 to 0.5 for 1,4-AD, from 5.3 to 52.6 ng/ml for 4,6-AD and from 0,6 to 7.9 ng/ml for 6-T. In the case of T, concentrations between 0.02 and 0.4 ng/ml were obtained. However, since these values are below the LOQ established during validation, they should be taken as estimative.

For the samples collected between 0 and 4 h after TU intake, higher concentrations were obtained for all T metabolites. Concentrations from 27.7 to 69.9 ng/ml for 1,4-AD, from 73.5 to 195.4 ng/ml for 4,6-AD, from 2.92 to 22.5 ng/ml for 6-T and from 1.1 to 3.5 ng/ml for T were observed.

Therefore, the validated method is able to quantify both low concentrations obtained in non-treated volunteers and high concentrations obtained after oral testosterone administration. A moderate increase was observed for T, 4,6-AD and 6-T concentrations after T administration. However, in the case of 1,4-AD, the concentrations after T administrations were more than 50 times higher than the obtained in basal conditions. This fact seems to indicate that 1,4-AD can be an adequate marker for the detection of T misuse. The analysis of samples collected for longer periods after T administration would be necessary in order to confirm this suitability.

Representative chromatograms of a urine sample with a low concentration (untreated subject) and a urine sample with high concentration (sample collected after TU intake) are shown in Figure 3. As can be seen, the sensitivity of the method allowed for the correct determination of the metabolites at the low basal concentrations present in urine.

Conclusions

A rapid, sensitive, accurate, and precise quantitative method based on LC-MS/MS has been developed and validated for the determination of 1,4-AD, 4,6-AD, 6-T and T in urine after basic treatment. To our knowledge, it is the first method for the urinary quantification of these metabolites.

The method allowed for the quantification of these analytes at both the concentrations found in untreated samples and the concentrations observed after T intake. Only in the case of T did the poor precision obtained hamper the quantification at the basal concentration levels. However, the developed method allowed for the correct quantification of T at the levels detected in samples collected after T administration.

The satisfactory quantitative data obtained combined with the reduced analysis time resulting from a simple sample preparation and fast instrumental analysis makes this method appropriate for doping control analyses. The short overall analysis time is of special interest in the doping testing field, where a large number of samples have to be analyzed in a short period of time.

The application of this method to samples collected before and after T administration would be useful to evaluate the usefulness of these metabolites in the detection of T misuse mainly in those cases in which the measurement of T/E shows several limitations.

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