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Testing for natural and synthetic anabolic agents in human urine

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Abstract

This paper describes a comprehensive method for the detection of natural and synthetic anabolic agents, including some veterinary preparations such as trenbolone, zeranol (a non-steroidal agent) and clenbuterol (a β_2-agonist). For the natural steroids such as testosterone, the precise determination of urinary androgens during routine procedures allowed the description of statistical distribution of relevant parameters of the endogenous steroid profile amongst male athletes. The validity of the results is discussed, taking into account some factors that may cause the degradation of the specimen.

Keywords: Anabolic agents; Trenbolone; Zeranol; Clenbuterol; Steroids; Testosterone

1. Introduction

Since 1968, the Medical Commission of the International Olympic Committee and major international sports federations have been concerned by the reported abuse of doping agents in the athletic community. One of the first duties of these organizations was to establish a list of classes of substances and methods that athletes were forbidden to use during competition and training. The latest 1995 IOC list of banned and restricted classes of substances [1] includes the anabolic agents, covering the anabolic androgenic steroids and the β_2-agonists, such as those used as growth promoters in the cattle industry. The testing of anabolic agents in the urine of competing athletes was implemented on a large scale during the Montréal 1976 Olympic Games and was mainly based upon RIA techniques. In the last ten years, several studies were aimed at the identification and characterization of anabolic agents and their metabolites in human urine using gas chromatography–mass spectrometry (GC–MS). Nowadays, most of the laboratories involved in athletic drug testing programs are using procedures based upon the solid-phase extraction of the urine sample, enzymatic hydrolysis of the glucuroconjugates, followed by the liquid–liquid extraction at basic pH and the trimethylsilyl derivatization prior to the GC–MS analysis (selected ion monitoring) [2–4]. The confirmation of the identity of the anabolic agent or its metabolite requires the unequivocal correspondence of the GC and MS properties with those of the pure authentic standards or of a reference excretion study. For the natural anabolic androgenic steroids such as testosterone and 5α-dihydrotestosterone (DHT), strategies developed were taking into account the expected values of reference population-based parameters of the steroid profile [5–7].

This paper describes a comprehensive method for the detection and confirmation of some synthetic and natural anabolic agents and provides references to published studies, aimed at the identification and characterisation of urinary metabolites of anabolic agents. The statistical distribution of some key elements of the steroid profile is presented. In reference to the determination of testosterone abuse,

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data were obtained, over years, for individuals permitting the discrimination between normal and abnormal results. Finally, the consequences of improper storage and of potential microbial growth on the validity of the results are discussed.

2. Experimental

2.1. Standards

5α-Androstan-17-one (external standard) was obtained from Steraloids (Wilton, NH, USA), androsterone from UpJohn (Don Mills, Canada), androsterone glucuronide, epietiocholanolone (internal standard), epitestosterone, etiocholanolone, etiocholanolone glucuronide were purchased from Sigma (St. Louis, MO, USA), 17α-methyl-5α-androstan-3β,17β-diol (internal standard) was from Amersham/Searle (Oakville, Canada) and testosterone, NF reference standard, from U.S.P.C. (Rockville, MD, USA). Authentic anabolic agents and pharmaceutical preparations were supplied by the pharmaceutical manufacturers. Metabolites of synthetic anabolic androgenic steroids not commercially available were synthetised and kindly distributed to IOC accredited laboratories by W. Schänzer [4].

2.2. Chemicals, solvents, reagents

Acetic acid, anhydrous dibasic sodium phosphate, monobasic sodium phosphate, potassium carbonate, sodium acetate, sodium bicarbonate, sodium carbonate and sulfuric acid were of analytical reagent grade and were obtained from either J.T. Baker (Phillipsburg, NJ, USA), Fisher Scientific (Montreal, Canada), BDH (Ville St-Laurent, Canada) or Malinckrodt (Paris, KY, USA). Acetonitrile, 1-chlorobutane, dichloromethane, diethyl ether, hexane, methanol and ethylacetate, of glass-distilled or HPLC grades, were purchased from Caledon (Montreal, Canada). Deionized water was obtained by reverse osmolysis and filtered over a Milli-Q water purification system (Millipore, Ville St-Laurent, Canada). Nitrogen, zero grade, was obtained from Air Liquide Canada (Montreal, Canada).

2.3. Enzymatic preparations

β-Glucuronidase from *Escherichia coli*, type IX-A, lyophilised powder, containing 1 500 000–2 000 000 U/g of β-glucuronidase activity was obtained from Sigma.

2.4. Trimethylsilyl derivatizations

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), trimethyliodosilane (TMIS) and ethanethiol were obtained from Aldrich (Milwaukee, WI, USA). Pyridine, silylation grade, was purchased from Regis (Morton Grove, IL, USA) and trimethylchlorosilane (TMCS) was purchased from Pierce (Rockford, IL, USA). The TMS ether, TMS enol derivatization [8] was carried out by the addition of 50 μl of a mixture of 3.0 ml of MSTFA, 60 μl of ethanethiol and 60 μl of a 0.1 *M* trimethyliodosilane solution. The reaction mixture was heated in a stoppered vial under nitrogen at 70°C for 30 min. The TMIS solution was prepared by adding 70 μl of TMIS, 1 μl of triethylamine and 430 μl of dichloromethane, in a stoppered vial under a nitrogen atmosphere. The reaction mixture was analysed as such. The TMS ethers were obtained by reaction with MSTFA (10 μl), TMCS (1 μl) in pyridine (90 μl) at 70°C for 20 min. The reactives were evaporated under nitrogen and the residue was dissolved in hexane prior to the GC–MS analysis.

2.5. Preparation of calibration standards

2.5.1. Testosterone and epitestosterone

Eight calibration standards containing testosterone and epitestosterone in ratios ranging from 0.54 to 10.73 were prepared by adding the authentic standards to child urine. The final concentrations of the urinary standards varied from 1.8 to 352 ng/ml for testosterone and from 1.6 to 328 ng/ml for epitestosterone.

2.5.2. Androsterone and etiocholanolone

The urinary standards prepared as described in Section 2.5.1 also contained androsterone and etiocholanolone glucuronides in concentrations equivalent to 332–2657 ng/ml of free androsterone and to 320–2561 ng/ml of free etiocholanolone. In these

calibration standards, androsterone and etiocholanolone were present in ratios varying from 0.13 to 8.30. Three quality control urinary standards contained testosterone and epitestosterone in the ratios 0.05, 6.6, 10.73 and androsterone and etiocholanolone in the ratios 0.80, 1.43 and 4.01.

2.6. Isolation of the free and glucuroconjugated substances

To aliquots of 1.5 to 8 ml (depending upon the specific gravity of the urine sample) that were accurately measured, were added 2.5 μg of epietiocholanolone and 250 ng of 17α-methyl-5α-androstan-3β,17β-diol. An equivalent volume of acetate buffer (pH 5.2) was added and the sample was passed through a Sep-Pak Plus C_{18} cartridge (Millipore) that had been previously washed with 5 ml of methanol and 5 ml of water. The cartridge was washed with 5 ml of water, 3 ml of hexane and elution was carried out with 5 ml of methanol. The solvent was evaporated to dryness under a stream of nitrogen at 50°C. The enzymatic hydrolysis was carried out after the addition of 1 ml of phosphate buffer (pH 6.9) and of a freshly prepared solution containing 1 mg of the lyophilised powder of β-glucuronidase from *E. coli* type IX-A in 50 μl of water. The hydrolysis was completed in 1 h at 50°C or in 16 h at 37°C. After cooling at room temperature, around 50 mg of a 10:1 (w/w) mixture of sodium bicarbonate–sodium carbonate were added (pH 8.5) and the extraction was done with 5 ml of diethyl ether. The organic layer was evaporated to dryness under a nitrogen stream. The residue was dissolved in methanol, transferred to a conical vial containing 250 ng of 17α-androstan-3-one (external standard) and submitted to derivatization as described in Section 2.4.

2.7. Isolation of the sulfoconjugated substances

After the extraction with diethyl ether, the residue of the enzymatic hydrolysis was brought to pH 5 and 1 ml of acetate buffer (pH 5.2) and the internal standards were added. The solution was passed again through a prepared Sep-Pak Plus C_{18} cartridge: washing and elution was performed as described previously. After the evaporation of the solvent, the residue was dissolved in 1 ml of ethyl acetate and 2 μl of an aqueous solution of 0.4 *M* sulfuric acid were added. The reaction mixture was heated at 40°C for 1 h. Then, 4 ml of ethyl acetate and 1 ml of an aqueous solution of sodium bicarbonate 5% (v/v) were added. The organic layer was washed with a saturated sodium chloride aqueous solution and evaporated.

2.8. GC and GC–MS

GC–MS analyses were carried out on a Hewlett-Packard 5970 mass-selective detector (Hewlett-Packard, Mississauga, Canada), with direct coupling to a Model 5890 (Series II) gas chromatograph equipped with a Model 7673 autosampler. Instrumentation control and data handling were performed by Hewlett-Packard Vectra VL2 computers, running under Hewlett-Packard MS ChemStations (DOS series) software (version C.02.03). The separation was achieved on Hewlett-Packard HP-5 capillary columns (19091J-102) of 5% phenyl polymethyl siloxane (25 m×0.25 mm I.D., 0.33 μm film thickness).

A Hewlett-Packard 5890 gas chromatograph (Series II) with an autosampler and one flame ionization detector operating under HP 3365 Series II Chemstation (DOS series) software (version B.02.04) and with the same injection port was used for GC experimentation. The separation was achieved on a J&W DB-1 capillary column (Chromatographic Specialties, Brockville, Canada) of methylsilicone (15 m×0.25 mm I.D., 0.25 μm film thickness). All computers were linked to a file server operating under Novell 3.2 network software.

For the GC–MS analysis of anabolic agents, 1 μl of the TMS ether, TMS enol derivatization mixture was injected in the splitless mode (30 s) into a split–splitless injection port with an inner glass liner containing silanized glass wool. Helium was used as the carrier gas and the oven temperature program was as follows: 100°C (1 min) to 220°C at a rate of 16°C/min, to 301°C at a rate of 3.8°C/min and finally to 320°C at a rate of 20°C/min. The final temperature was held for 5.5 min. The temperature of the injection port was kept at 270°C and that of the transfer line at 315°C. The electron multiplier

Table 1
GC–MS analysis (SIM mode) of anabolic agents in urine samples

Anabolic agent	Urinary metabolite	t^{a}_{RR}	Ions	Other metabolites and remarks
Androstanolone	Androsterone/etiocholanolone (G)			Elevated DHT concentration and elevated values of the
(5α-DHT)	5α-Dihydrotestosterone (G)	0.95	405.4, 143.1	5α/5β metabolites [6,7,58]
Bolasterone	Bolasterone (G) (7α,17α-dimethyl-4-androsten-17-ol-3-one)	1.041	445.4, 315.3	7α,17α-Dimethyl-5α,5β-androstan-3,17-diol (G)
Boldenone	Boldenone (G) (1,4-androstadien-17β-ol-3-one)	0.961	430.4, 206.2	Several other metabolites (G,S) [9]
	5β-Androst-1-en-17β-ol-3-one (G)	0.809	432.4, 194.2	
Calusterone	Calusterone (G) (7β,17α-imethyl-4-androsten-17-ol-3-one)	1.048	445.4, 315.3	7β,17α-Dimethyl-5α,5β-androstan-3,17-diol (G)
4-Chlortestosterone	4-Chloro-4-androsten-3a-ol-17-one (G)	0.990	466.4, 451.4, 431.4	4-Chloro-5α,5β-androstan-3-ol-17-one (G,S)
4-Chloro-methandienone	4-Chloro-17α-methyl-1,4-androstadien-3,12,17-triol (F) (proposed structure)	1.187	170.1, 143.1	TMS derivatives are more stable,
	4-Chloro-17α-methyl-1,4-androstadien-6β,12, 17-triol-3-one (F)	1.316	315.3, 243.2, 170.1, 143.1	4-Chloro-methandienone and the 17β-epimer (F),
				6β-hydroxy-4-chloro-methandienone and epimeric (F), several other metabolites (F) [4,21,22]
Clenbuterol	Clenbuterol (F)	0.565	335.3, 300.3, 86.1	
Drostanolone	Drostanolone (G) (2α-methyl-5α-androstan-17β-ol-3-one)	0.974	448.4, 433.4, 141.1	2α-Methyl-5α-androstan-3-ol-17-one (G)
Epitestosterone	Epitestosterone (G)	0.953	432.4, 417.4	
Fluoxymesterone	Fluoxymesterone (F, G) (9α-fluoro-17α-methyl-4-androsten-11β,17β-diol-3-one)	1.155	552.5, 462.4, 407.4	Several other metabolites (F, G) [4]
	9α-Fluoro-17α-methyl-4-androsten-3α,6β,11β, 17β-tetrol (F,G)	1.060	642.6, 552.5, 462.4, 143.1	
Formyldienone	17α-Methyl-4-androsten-11α,17β-diol-3-one (G)	1.081	534.5, 444.4, 389.3	Several other metabolites (F,G) [17,18]
Furazabol	Furazabol (G)	1.194	402.4, 387.3, 143.1	
	16-Hydroxyfurazabol (G)	1.393	490.4, 231.2, 218.2	
Internal standard	17α-Methyl-5α-androstan-3β,17β-diol	1.000	435.4, 143.1	
Mestanolone	17α-Methyl-5α-androstan-3α,17β diol (G)	0.925	450.4, 435.4, 143.1	Metabolites present as glucuro- and sulfo- conjugates,
				17α-methyl-5α-androstan-3α,16,17-triol (S)
Mesterolone	Mesterolone (G) (1α-methyl-5α-androstan-17β-ol-3-one)	0.956,0.960	448.4, 433.4	18-Hydroxylated metabolites observed [13],
	1α-Methyl-5α-androstan-3α-ol-17-one (G)	0.925	448.4, 433.4	16-hydroxylated metabolites (S) persistent

Methandienone	17α-Methyl-5β, (α)-androstan-3α,17β-diol (G)	0.925	450.4, 435.4, 143.1	Several metabolites (F,G,S) [15,16],
	17α-Methyl-5β-androst-1-en-3α,17β-diol (F, G)	0.921	448.4, 433.4, 143.1	methandienone (G) was found in some cases,
	17α-Methyl-1,4-androstadien-6β,17β-diol-3-one (F)	1.117	532.5, 517.5	17β-methyl-5β-androst-1-en-3α,17β-diol (G) persistent
Methandriol	17α-Methyl-5β-androstan-3α,17β-diol (G)	0.925	450.4, 435.4, 143.1	Methandriol (G),
				16-hydroxymethandriol metabolites (G,S)
Methenolone	Methenolone (G)	0.993	446.4, 431.4, 195.1	18-Hydroxylated and several other metabolites observed [13],
	1-Methylen-5α-androstan-3α-ol-17-one (G)	0.905	446.4, 431.4	methenolone and 17α-methenolone were found in some cases,
				1-methyl-5α-androst-1-en-3,17-dione-16α-ol (S) persistent
17α-Methyltestosterone	17α-Methyl-5β, (α)-androstan-3α,17β-diol (G)	0.925	450.4, 435.4, 143.1	Metabolites present as glucuro- and sulfo- conjugates,
				17α-methyl-5β,(α)-androstan-3α,16,17β-diol (S,G)
Mibolerone	Mibolerone (G) (7α,17α-dimethyl-4-estren-17β-ol-3-one)	1.012	446.4, 431.4, 301.3	7α,17α-Dimethyl-5-estran-3,17-diol (G),
				TMS ether derivatives useful for confirmation
Norethandrolone	17α-(2-Hydroxyethyl)-5α-estran-3α,17β-diol (G) (proposed structure)	1.078	538.5, 421.4, 254.2	Several other metabolites [4]
19-Nortestosterone	Norandrosterone (G) (5α-estran-3α-ol-17-one)	0.797	420.4, 405.4, 315.3	Metabolites in the sulfo- and glucuroconjugated form
	Noretiocholanolone (G) (5β-estran-3α-ol-17-one)	0.830	420.4, 405.4, 315.3	
Oxandrolone	Oxandrolone (F)	1.111	378.3, 363.3, 143.1	17-Epimer (F); extraction at pH 5
Oxymesterone	Oxymesterone (G) (17α-methyl-4-androsten-4,17β-diol-3-one)	1.169	534.5, 519.5, 389.3	17α-Methyl-5-androstan-3,4,17-triol isomers (S,G)
Oxymetholone	17α-Methyl-5α-androstan-3α,17β-diol (G)	0.925	450.4, 435.4, 143.1	Several metabolites (G,S),
	2-Hydroxymethyl-17α-methyl-5-androstan-3,17-diol (G) (proposed structure)	1.106	552.5, 462.4, 143.1	acidic metabolites were identified [19,20]
	2-Hydroxymethyl-17α-methyl-5-androstan-3,6,17-triol (G) (proposed structure)	1.180	640.5, 550.5, 460.4, 143.1	
Stanozolol	Stanozolol (G)	1.310	472.4, 457.4, 143.1	Several other metabolites (G) [23,24],
	3′-Hydroxystanozolol (G)	1.380	560.5, 545.5, 254.2, 143.1	16-hydroxylated metabolites are persistent
	4β-Hydroxystanozolol (G)	1.393	560.5, 545.5, 254.2, 143.1	
Steranabol	5-Androstan-3,4-diol-17-one (G) (proposed structure)	0.887	508.5, 493.4	Metabolites found either as sulfo- or glucuroconjugates
Testosterone	Testosterone (G)	0.970	432.4, 417.4	
Trenbolone	17α-Trenbolone (G)	0.943	412.4, 307.3	TMS derivatives are useful
Zeranol	Zeranol (G)	1.083	538.5	

[a] t_{RR} given to the internal standard: 17α-methyl-5α-androstan-3β,17β-diol.

was fixed during the analysis at 200 eV higher than the voltage obtained from the automatic tuning of the mass-selective detector with electron impact ionisation at 70 eV. The analysis was carried out in the SIM mode acquiring 110 ions (50 ms/ion) separated in eleven groups. Mass spectra were obtained by scanning from m/z 50 to 660.

Under standard operating protocols, each sequence of samples analysed included the analysis of a standard mixture in order to verify the analytical performance of the GC–MS. This mixture contained clenbuterol, salbutamol, epitrenbolone (1 ng/μl injected), epitestosterone, testosterone, 6β-hydroxymethandienone, stanozolol (2 ng/μl injected), 5α-androstan-17-one (external standard) and 17α-methyl-5α-androstan-3β,17β-diol (internal standard) (5 ng/μl injected). To assess the efficiency of the whole procedure, a control urine sample, prepared from excretion studies and addition of authentic standards containing clenbuterol, epitrenbolone, oxandrolone, 6β-hydroxymethandienone, stanozolol and its metabolites, and a quality control urine sample (testosterone, epitestosterone) were analysed before the samples.

The quantitation of androsterone and etiocholanolone was performed by GC–FID on a shorter column to prevent its saturation. All samples analysed in GC–MS were reanalysed in GC–FID.

2.9. Luteinizing hormone (LH) measurements

Urinary LH was measured using the Serono MAIAClone IRMA test, purchased from Starplex (Mississauga, Canada). The Lyphochek immunoassay controls were purchased from Bio-Rad (Mississauga, Canada).

3. Results and discussion

3.1. GC–MS detection and identification of anabolic agents

The anabolic androgenic steroids are either excreted unchanged in the urine or, more often, as metabolites (mostly glucuronides or sulfates). Several papers have been published (especially in the last ten years) on the identification and characterization of urinary metabolites of anabolic agents such as boldenone [9], methenolone [10], mesterolone and drostanolone [11–13], oxandrolone [14], methandienone [15,16], mestanolone [15], formyldienone [17,18], oxymetholone [2,19,20], 4-chloro-methandienone [21,22], stanozolol [23,24] and mibolerone [25]. The epimerisation and degradation of the 17β-sulfate-17α-methyl anabolic steroids in urinary matrix were studied in detail and permitted the characterization of several metabolites [26,27]. Schänzer and Donike [4] published the results of a very comprehensive study on the synthesis and use of reference substances in the identification of synthetic steroid excretion.

With the knowledge gained from these studies and our own research work, a GC–MS method was developed [2] which has been constantly improved, and now permits the detection of twenty-five anabolic agents (Table 1).

The detection of some steroids requires stringent protocols due to their instability or their sensitivity towards active sites during GC. For this reason, the glass liner and the glass wool in the injection port must be silanised [28] and replaced every thirty injections. Furthermore, careful removal of moisture in the helium gas line with an efficient trap is mandatory and will lenghten the lifetime of the capillary column. The lactone ring of oxandrolone, excreted unchanged along with its 17-epimer, is unstable under alkaline conditions [14] and it must be extracted at pH 5 for optimal recovery. For some specific purposes, such as the confirmation of the presence of oxandrolone, the free fraction of steroids was obtained by the extraction of the buffered eluent prior to the enzymatic hydrolysis. Poor chromatographic conditions also caused the retention of oxandrolone and of stanozolol (pyrazole ring) and its metabolites. Carefully controlled inert chromatographic conditions permitted the detection of trenbolone and its 17α-epimer by this method and we were successful in identifying trenbolone in athletes' samples [29]. As depicted in Fig. 1, the mass spectra of 17α-trenbolone TMS enol, TMS ether and dehydro derivatives were characterised by the presence of molecular ions at m/z 414 and 412, respectively, and of characteristic ions at m/z 309 and 307 (–$CH_3^{\cdot}$, –TMSOH), 298 and 296 (–HC(OTMS)CH_2), 283 and 281 (–HC(OTMS)CH_2, –$CH_3^{\cdot}$). However, due

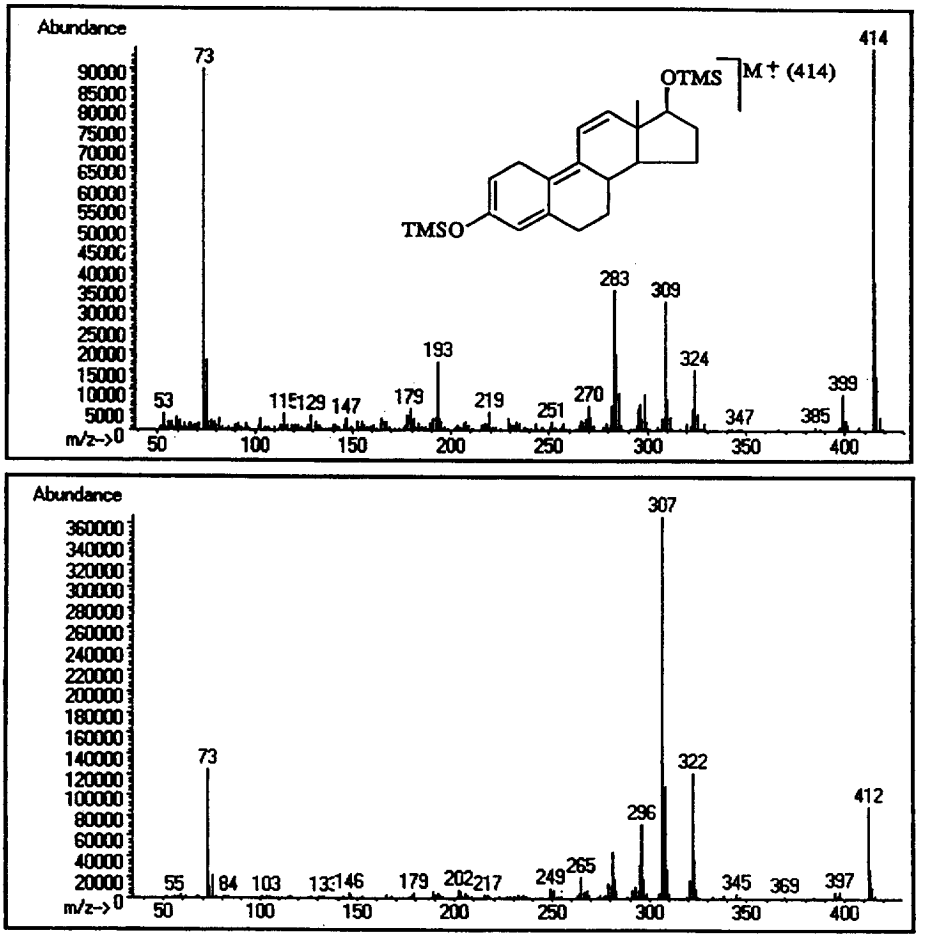

Fig. 1. Mass spectrum of the TMS ether, TMS enol derivatives of 17α-trenbolone (upper panel) and 17α-dehydrotrenbolone (lower panel) obtained from the GC–MS analysis of the authentic standard (20 ng injected).

to the instability of trenbolone in the derivatization mixture which gave rise to the formation of other species with time [30], sensitivity was improved by carrying out the derivatization in the injection port of the chromatograph [31]. For this purpose, the extracted steroids, dissolved in 50 μl of ethyl acetate (trenbolone and its epimer are excreted as glucuroconjugates) were injected along with the TMS enol, TMS ether derivatization mixture, on a clean silanized glass liner. Accordingly, the TMS ether derivatives were obtained in the same way [30,31].

Zeranol is a non-steroidal agent used as a growth promoter and available as implants. Zeranol's main metabolites in man [32] were reported to be zeranol and zearalanolone (oxidation of the hydroxyl group at position 7). We have identified on one occasion, zeranol as its glucuroconjugate in a urine sample provided by a competing athlete. The mass spectrum of the tri-TMS derivative of zeranol is shown in Fig. 2 and is characterised by the presence of the molecular ion at m/z 538 ($M^{+\cdot}$) and by extensive fragmentation producing ions at m/z 523 ($-CH_3^{\cdot}$), 453 (–TMSOH), 433 ($-CH_3^{\cdot}$ –TMSOH) and others.

In 1992, we reported [33] the detection of clenbuterol, a β_2-agonist agent, in several urine samples collected from competing athletes. The identification of clenbuterol was performed by the analysis of the free fraction extracted at pH 11 and the mass spectrum of its di-TMS derivative [33] is shown in

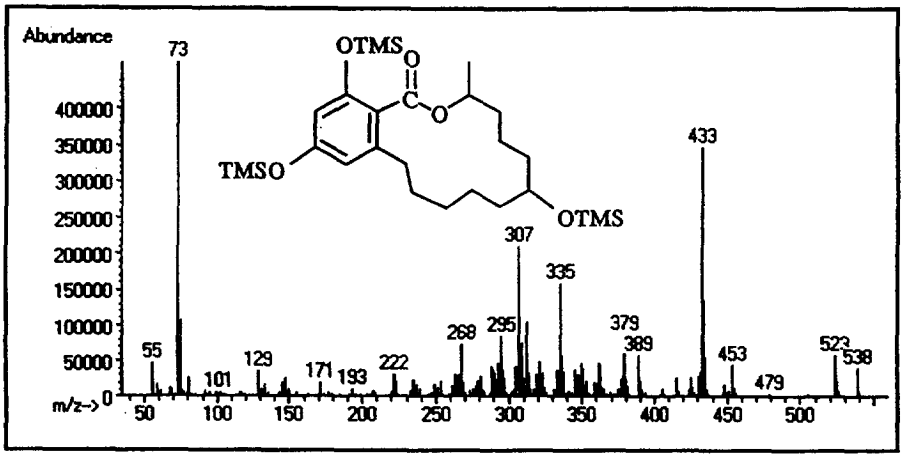

Fig. 2. Mass spectrum of zeranol tri-TMS obtained from the GC–MS analysis of the authentic standard (20 ng injected).

Fig. 3. 1-Chlorobutane was used for the extraction of low concentrations of clenbuterol, when the background of the urinary matrix was important.

The identification of the synthetic anabolic agent was made by comparison of the retention times and of the mass spectra. When the urinary concentration prevented the obtention of the mass spectrum, the comparison of characteristic ion ratios was made. Other conclusive evidence was gained by the identification of other metabolites or by the analysis of the free or the sulfoconjugated fractions (Table 1). The chemical hydrolysis was preferred in several cases to the enzymatic hydrolysis by *Helix pomatia* mixtures, since some sulfates of steroids were resistant under these conditions [34–36].

For the purpose of confirmation and quantitation of testosterone and epitestosterone, three other aliquots of the specimen were prepared and analysed in duplicate by GC–MS. The urinary testosterone and epitestosterone standards were analysed accordingly. In one aliquot, the free fraction was extracted prior to the enzymatic hydrolysis and analysed to

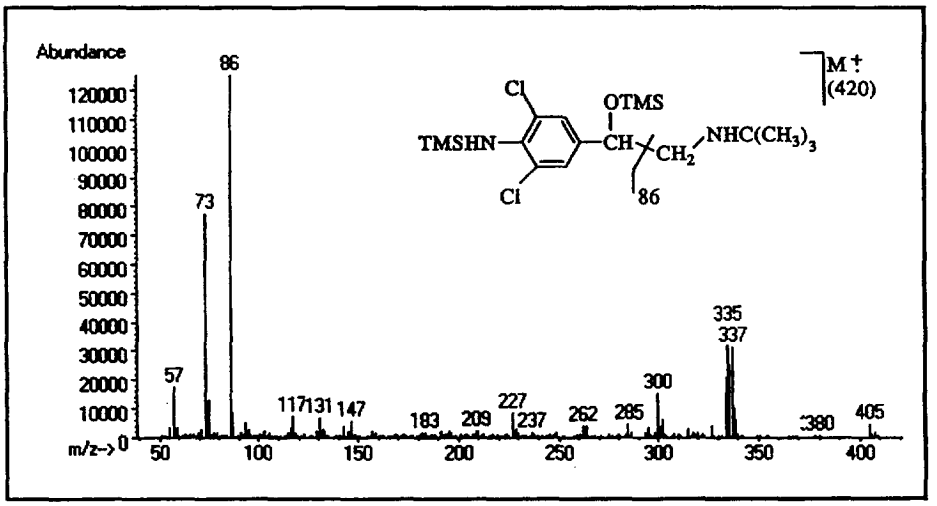

Fig. 3. Mass spectrum of clenbuterol di-TMS obtained from the GC–MS analysis of the control urine sample (20 ng injected).

Table 2
Reference ranges of the T/E, A/Etio, A/T, T/LH values and of the concentration of testosterone and epitestosterone glucuronides measured in samples collected from male athletes

Ratio	Population type	*n*	95% Percentile cut-off level		99% Percentile cut-off level	
			Minimum	Maximum	Minimum	Maximum
T/E	Lognormal (2)	3667	0.11	3.71	0.05	5.25
A/Etio	Extreme value	2237	0.72	2.89	0.43	4.14
A/T	Lognormal	2077	27	614	17	1375
T/LH	Gamma (nmole/UI)	2313	2.5	73	1.0	110
Testosterone glucuronide			maximum: 180 ng/ml		maximum: 225 ng/ml	
Epitestosterone glucuronide			maximum: 150 ng/ml		maximum: 180 ng/ml	

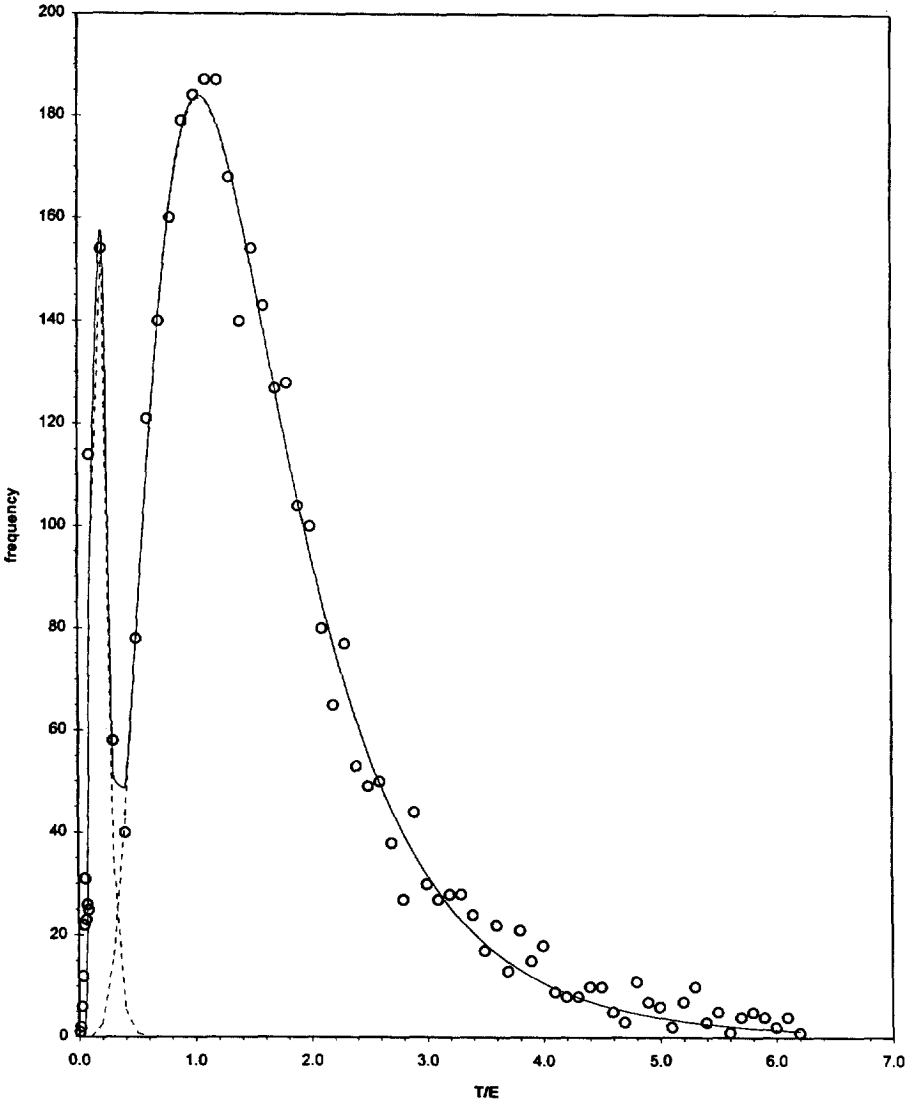

Fig. 4. Lognormal distribution of 3667 urinary T/E values collected during routine controls from male athletes (see Section 3.2 for description).

verify the absence of deconjugated steroids. The T/E value (area ratio) and the concentration of testosterone and epitestosterone was determined. The lower limit of quantitation was 50 pg injected (equivalent to 1.6 ng/ml) and the limit of detection was 0.5 ng/ml.

This procedure enhabled the detection of other doping agents as their TMS-derivatives, such as morphine, pemoline [37], buprenorphine, cannabis metabolite and of some β-blocking agents.

3.2. Detection of testosterone administration and determination of the steroid profile

In 1982, Donike et al. [5] reported a method for the detection of the administration of testosterone in urine samples that was based upon the measurable increase of the urinary concentration of testosterone glucuronide in comparison to the 17α-epimer, epitestosterone, normally present and not increased after testosterone administration. The International Olympic Committee adopted the measurement of a ratio of the urinary glucuronides of testosterone and epitestosterone exceeding 6:1 as evidence of the administration of testosterone. However, some cases were reported of athletes normally producing urine specimens in which the T/E values were consistently greater than 6:1 [38–41]. On the other hand, some recent work has shown that for some individuals, the administration of testosterone will not produce a T/E value higher than 6:1 [42,43]. Donike et al. [44,45,47] and Mareck-Engelke et al. [46] also

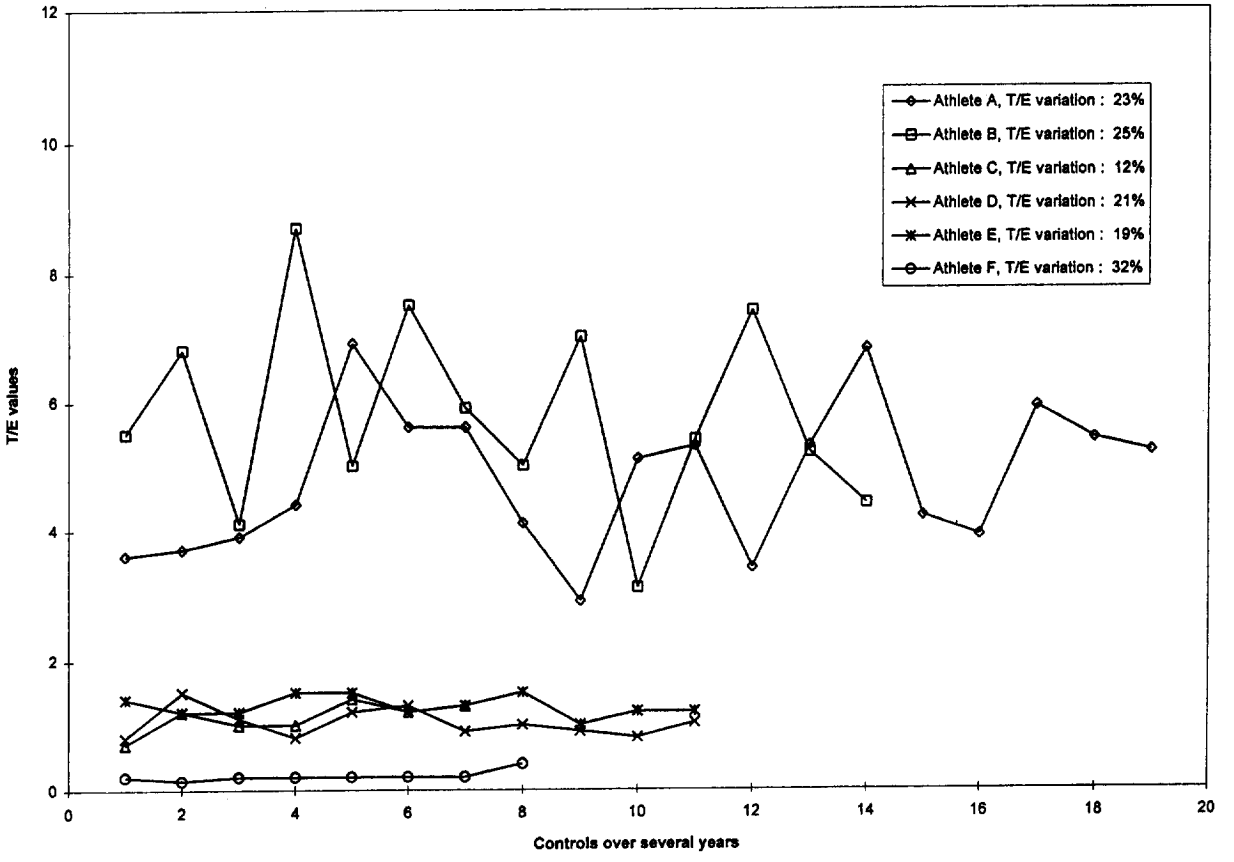

Fig. 5. Distribution of the urinary T/E values of four male athletes over time. Athlete A, mean value of 1.11 (4 years); athlete B, mean value of 1.03 (8 years); athlete C, mean value of 1.30 (8 years); athlete D, mean value of 0.22 (6 years); athlete E, mean value of 4.8 (9 years) and athlete F, mean value of 5.79 (6 years).

described some reference ranges for urinary concentrations of androsterone (A), etiocholanolone (Etio), testosterone (T) and epitestosterone (E) and respective ratios. The administration of testosterone and possibily of other anabolic agents was expected to produce deviation from the reference values. The comparison of previous and subsequent tests, referred to as the "longitudinal studies", may represent a useful discriminating tool [47–49]. Different approaches to prevent false positives or negatives were proposed, such as the use of secondary markers to the T/E values based upon LH [50,51] and epitestosterone sulfate measurements [42,52]. The use of inhibitors of testosterone biosynthesis such as ketoconazole [53] was also suggested. In blood, it was demonstrated that 17α-hydroxyprogesterone and its ratio to testosterone were good markers of the administration of testosterone [54] and other researchers determined reference ranges of some serum hormones like LH, steroid hormone binding globulin (SHBG), estradiol and others [55].

The T/E values and the urinary concentration of testosterone, epitestosterone, androsterone and etiocholanolone were quantitated precisely during screening procedures. The results obtained from quality control samples were compared, to assess reproducibility. Under routine operations, the quantitative measurements made in the quality control samples varied within 10%.

We have established the distribution, in a popula-

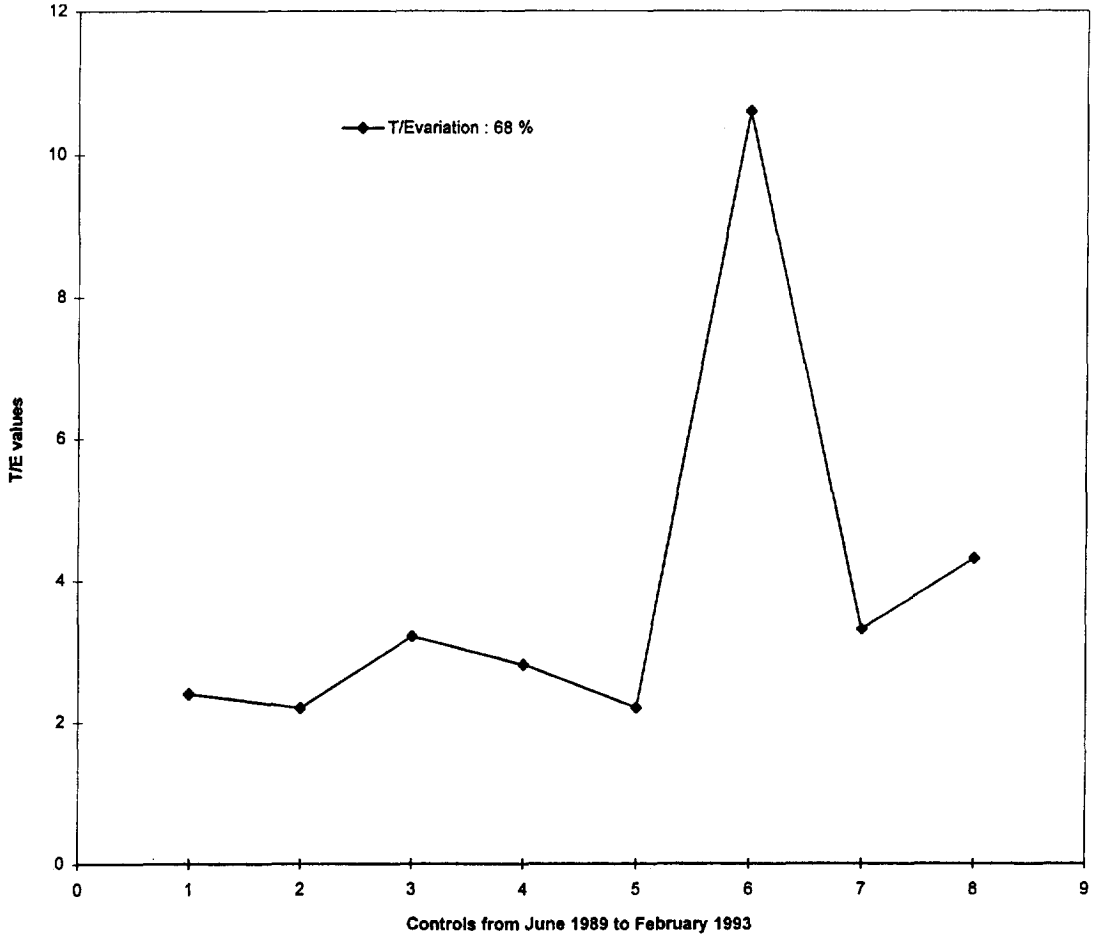

Fig. 6. Distribution of the urinary T/E values of one male athlete over 4 years. Test 6 afforded a positive T/E result of 10.6.

tion of several thousands male athletes, of the T/E, A/Etio, A/T and T/LH values (Table 2). The positive cases were not included.

Interestingly, the T/E values of specimens from male athletes and shown in Fig. 4, were distributed in two different lognormal populations. Within the 99% percentile cut-off level, the T/E values were found to be between 0.05 and 5.25. The first minor population was characterised by a mode of 0.16 and within the 95% percentile cut-off level, values were found between 0.07 and 0.25, and from 0.35 within the 99% percentile cut-off level. The mode of the second population was 1.0, 95% of values being distributed between 0.5 and 3.5. The observation of two discrete populations of specimens, one showing T/E values lower than 1, can be attributed to the multi-ethnic origins of the Americans [56,57]. Concerning the 6:1 threshold for laboratory positive findings, these results supported this limit as being realistic. However, it does not rule out the possibility of false positive or false negative values. For individuals producing specimens of the first, low T/E population-type, the administration of testosterone may produce a lower than 6:1 value, however, for an individual of the second type, a result exceeding the cut-off level will be reached.

Some cases were selected to show the stability of individual T/E values (Fig. 5). For some athletes, data over several years were available and variations of less than 20 to 30% were recorded. The usefulness of such "longitudinal studies" was proven on several occasions, two cases being depicted in Fig. 6 and Fig. 7. After the obtention of positive laboratory findings, of around ten, other specimens were col-

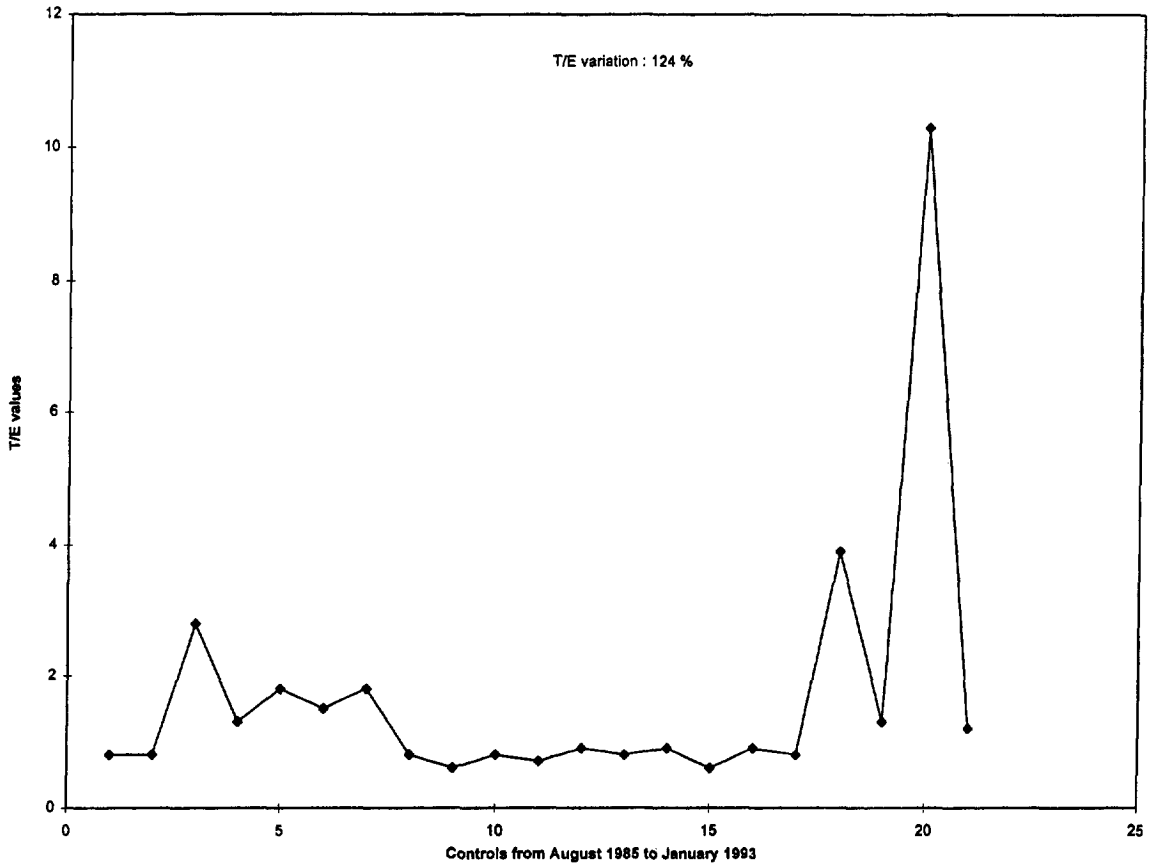

Fig. 7. Distribution of the urinary T/E values of one male athlete over 8 years. Test 20 afforded a positive T/E result of 10.3.

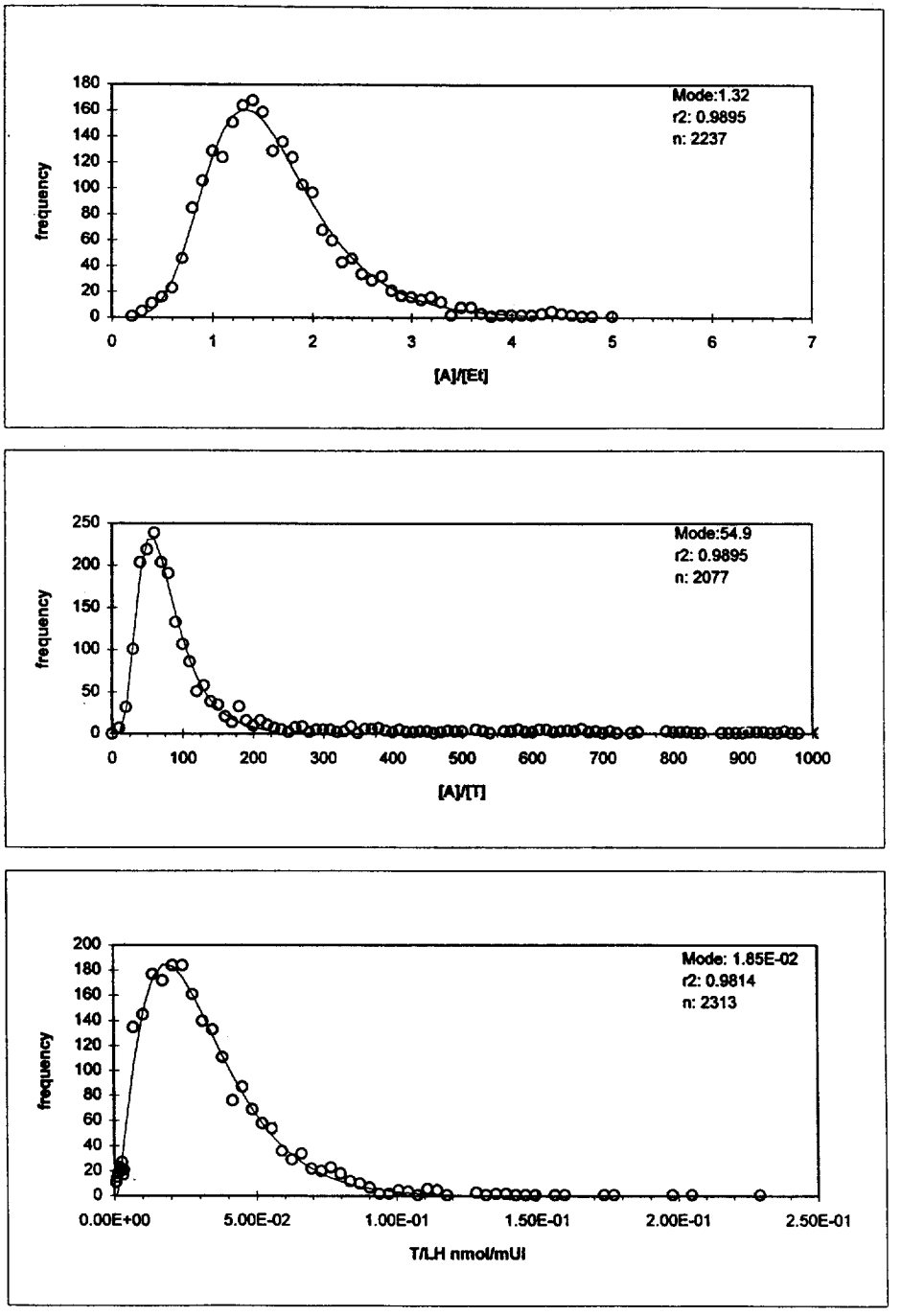

Fig. 8. Distribution of the urinary A/Etio, A/T and T/LH values among male athletes.

lected and results of the previous and subsequent tests were compared. Eight test results over four years and twenty one over eight years were studied, respectively. Clearly, in both cases, the two values of ten could not be related to a systematically elevated excretion of T/E from such variations of 68% and 124% from the mean values, respectively.

The A/Etio, A/T and T/LH distributions amongst male athletes are presented in Fig. 8 and are described in Table 2. Their significance as secondary markers of testosterone administration was deduced from observations made on testosterone-positive samples and seemed mainly to be useful for intra-individual comparison. We have observed that a clear deviation from the normal reference ranges was not systematic and that all these parameters were not influenced. However, when results of previous and subsequent tests were available, the comparison of A/T and T/LH was informative although the variations were less pronounced that those recorded for the T/E values. Some examples are shown in Fig. 9 and Fig. 10. The first case illustrates the stability of individual measurements gathered from three different laboratories in the world. The data were collected over 4 months and the results showed a narrow variation of 20% of T/E values from a mean vlaue of 6.3 (Fig. 9). The complete suppression of the LH excretion as measured by our procedure, was not systematic, but in some cases an increase of the T/LH was observed (Fig. 10).

The A/Etio value is influenced by the administration of 5α-DHT; as shown from the latest observations made on DHT-positive test samples and from excretion studies [6], the concentration of the DHT metabolite, androsterone, increased, therefore producing a noticeable elevation of the A/Etio values [58]. The need for descriptive reference ranges within several ethnic populations is obvious. We observed that in the American male athletic population tested, the ratios were distributed in an

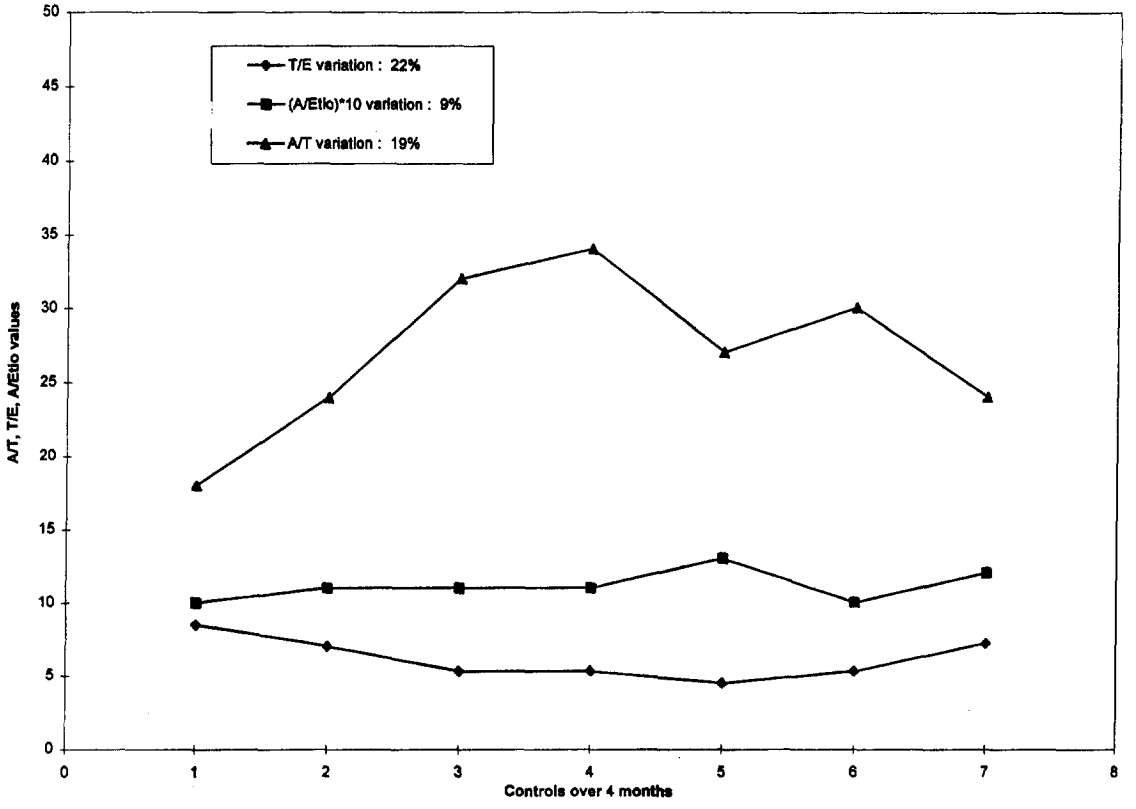

Fig. 9. Distribution of the urinary T/E, A/Etio, A/T of one male athlete. Data were collected from three different laboratories over 4 months.

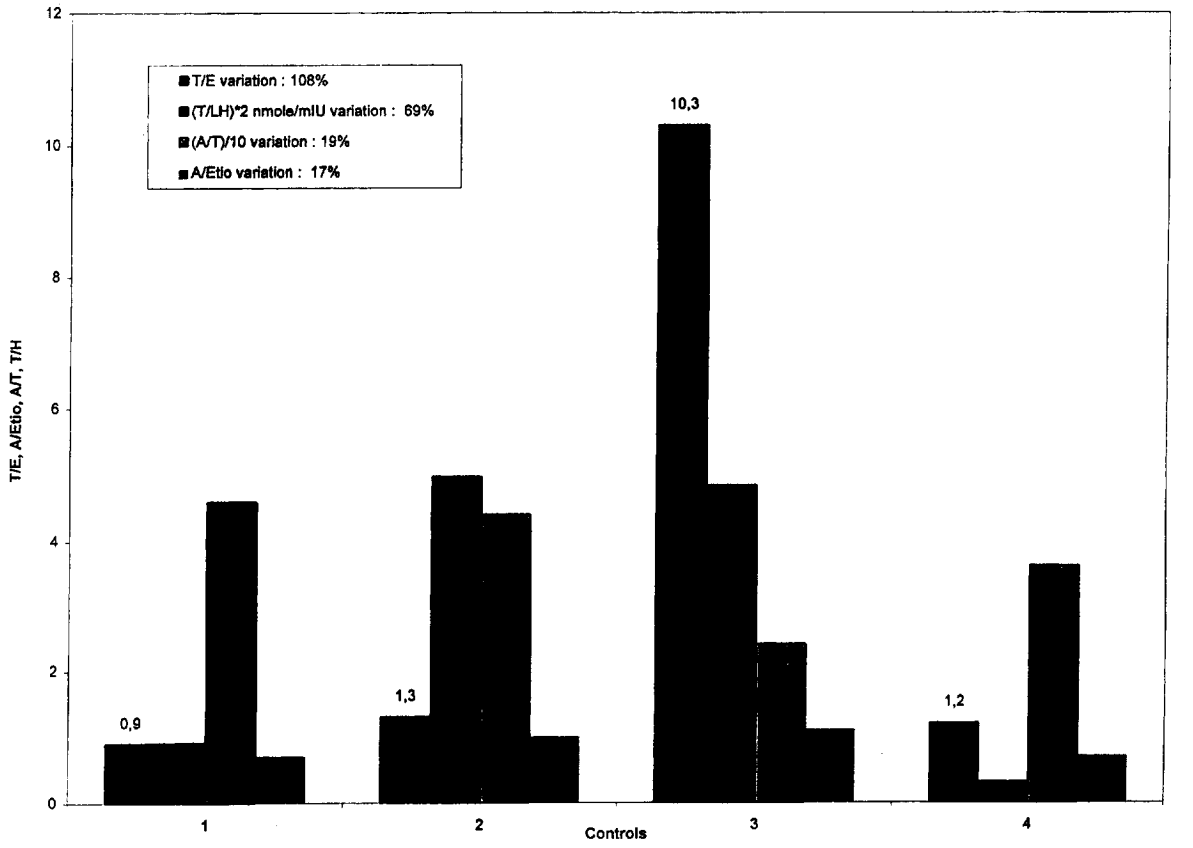

Fig. 10. Distribution of the urinary T/E, A/Etio, A/T and T/LH of one male individual. Test 3 afforded a positive result.

extreme value-type population and that 99% of values were found between 0.43 and 4.14 (Fig. 8 and Table 2).

We also studied the distribution of the urinary concentration of testosterone and epitestosterone glucuronides, taking into account the dilution of the specimen and using the specific gravity for that purpose. Even if such relations are usually made using creatinine as the reference, the measurement of specific gravity is routinely made in Doping Control Laboratories and therefore represents a more useful tool.

As shown in Fig. 11 and Fig. 12, as obviously expected, the concentration of urinary testosterone and epitestosterone glucuronides was related to the dilution of the specimen and a range of concentrations were expected to be found in a sample of a given specific gravity. Therefore, since the administration of testosterone and epitestosterone resulted in an increase of the respective glucuronide concentration, these normal reference ranges can be helpful in assessing their administration. In the IOC latest list of banned classes of substances [1], epitestosterone was described as a masking agent and laboratories were to report urinary concentrations exceeding 200 ng/ml. This definition of a positive finding would benefit from a correlation with the dilution of the sample.

The urinary testosterone glucuronide concentrations were distributed within two populations (Fig. 11), indicating that lower T/E values were due to low testosterone glucuronide excretion and supporting the observations made on some individuals. A distinct population of low epitestosterone glucuronide concentrations was not observed.

3.3. Degradation of urinary samples

We have studied the stability of some urinary steroids when urine specimens were submitted, dur-

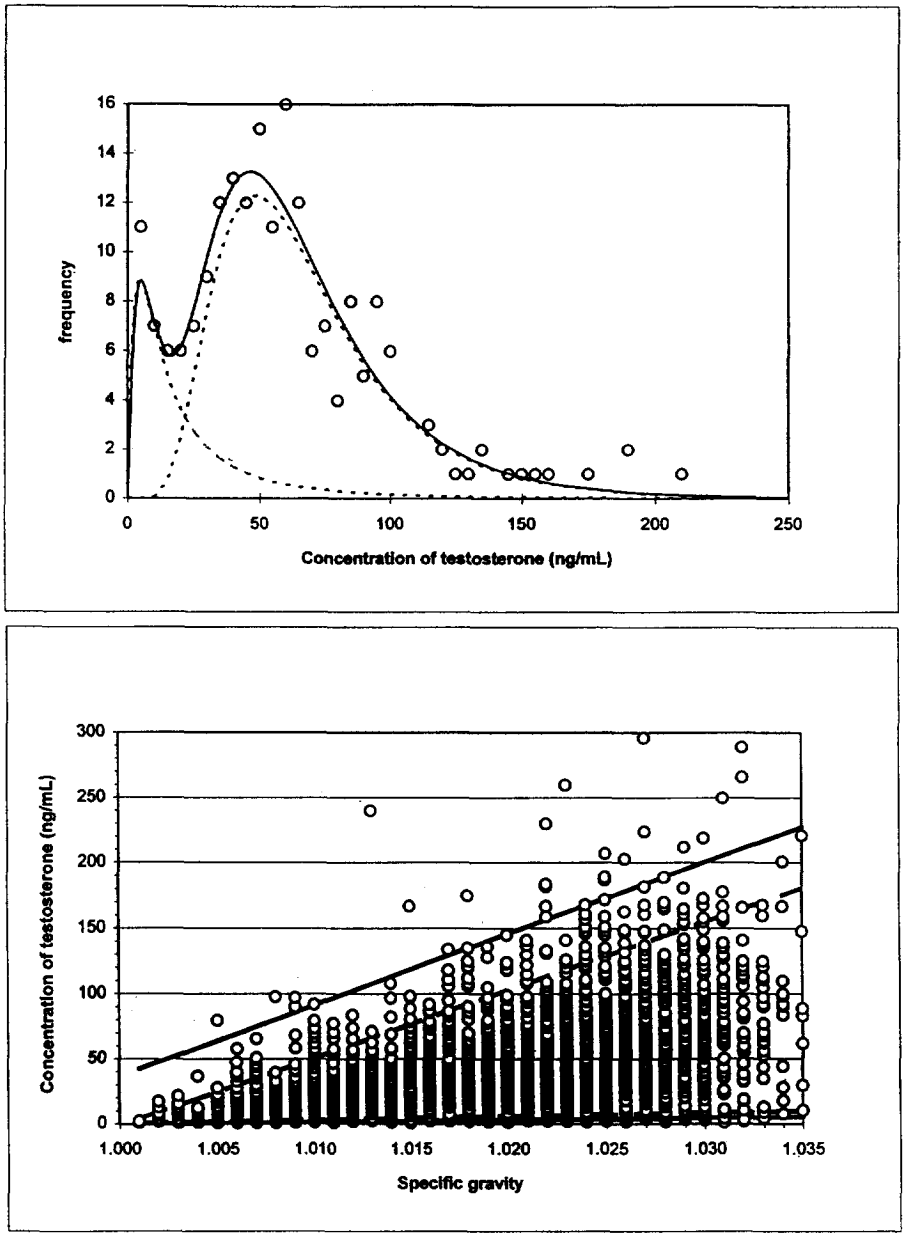

Fig. 11. Distribution of testosterone glucuronide concentrations according to the specific gravity of the urine samples. Values measured at 1.025 (upper panel) and all values (lower panel). The 99% and 95% limits are indicated by lines.

ing improper storage and transportation conditions, to elevated temperatures (37°C). We have observed frequently, and especially in the summertime, the degradation of the urinary steroids in the samples received. Recently, the possible bacterial contamination of the specimen was invoked to invalidate positive tests results [59]. The deconjugation of several steroid glucuronides and sulfates was observed at 37°C and after several days, most of the conjugated urinary steroids were isolated in the free

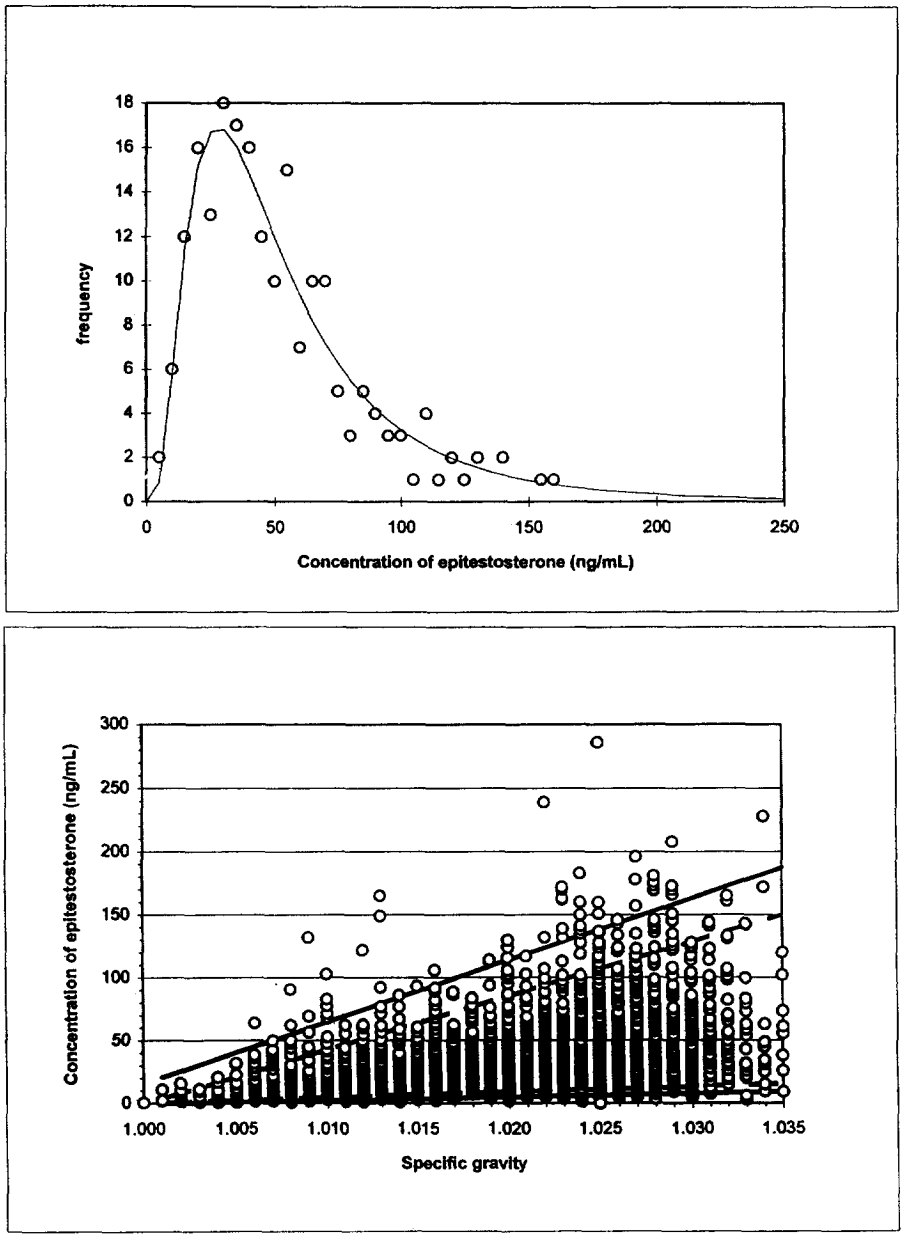

Fig. 12. Distribution of epitestosterone glucuronide concentrations according to the specific gravity of the urine samples. Values measured at 1.025 (upper panel) and all values (lower panel). The 99% and 95% limits are indicated by lines.

fraction (Fig. 13). The increase of free testosterone in urine samples was also reported by Kjeld et al. [60]. Bacteria such as *Enterococcus* and *Staphylococcus* are expected to be found in urine samples that are not collected under sterile conditions; however, several specimens containing such microbes did not show signs of degradation. Another source of bacterial contamination may be the laboratory itself

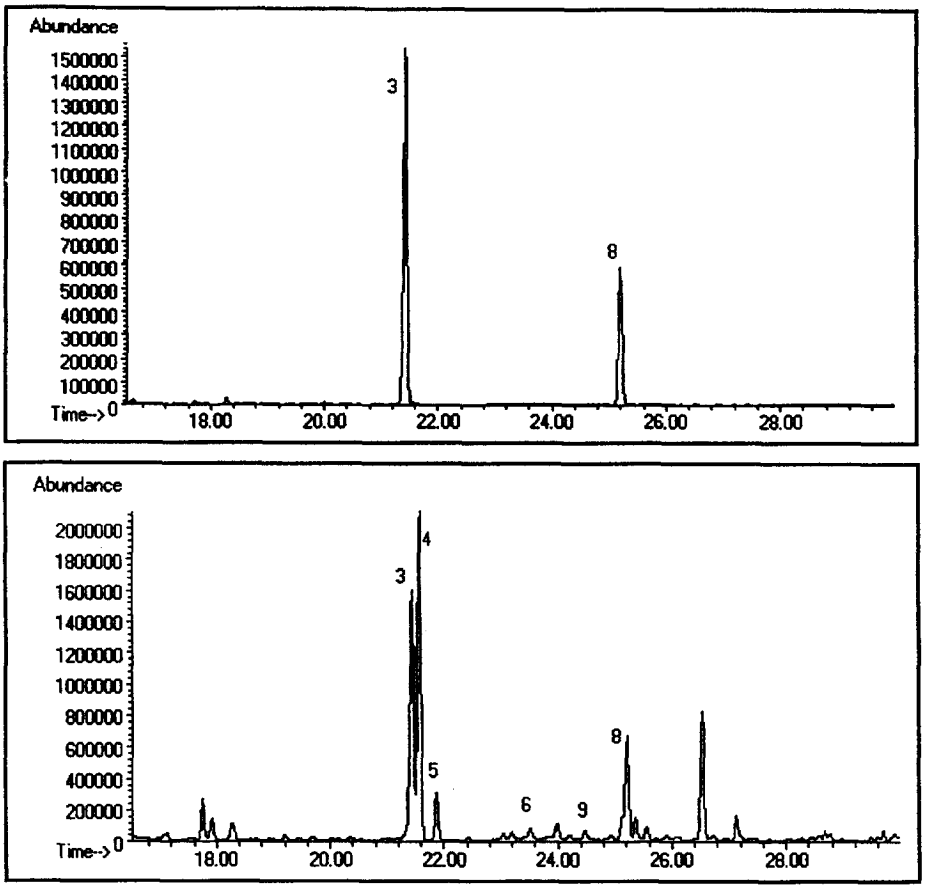

Fig. 13. GC–MS analysis (total ion chromatogram) of the extracted free fraction of a urine sample heated at 37°C for 24 h (upper panel) and 120 h (lower panel). Peaks: 3=epietiocholanolone (internal standard); 4=androsterone and etiocholanolone; 5=5α,β-androstan-3α,17β-diol; 6=epitestosterone; 8=17α-methyl-5α-androstan-3β,17β-diol (internal standard).

and care should be made to ensure proper operations. For example, water must be boiled before preparing the buffers used for the enzymatic hydrolysis. When urine samples were inoculated with urine specimens containing bacteria (1 ml in 50 ml) and incubated at 37°C for several days, the microbial growth resulted in the accumulation in the specimen of 5α- and 5β-androstan-3,17-dione, arising mainly from the deconjugation and the oxidation of androsterone, etiocholanolone and epiandrosterone (Fig. 14). Androstandiols were oxidised to the respective 5α- and 5β-androstanolones which were further oxidised to the androstandiones. The mass spectrum of the TMS-enol derivative of 5α-androstan-3,17-dione is shown in Fig. 15. This compound coeluted with epitestosterone and possessed the same molecular ion at m/z 432. Considering the degradation of testosterone and epitestosterone and the formation of androstandiones, the result of the degradation process on the T/E determination was an apparent reduction of the value, as shown in Fig. 16. Under such conditions, the analysis of natural steroids such as testosterone, epitestosterone and DHT is invalid.

As shown in Fig. 17, we have observed that although the measurement of elevated pH values of 8.0 and more may indicate deterioration, it was not always the case and half of the samples showing pH values between 8 and 9.2 did not show signs of degradation. Therefore, the rejection of a sample on the basis of its elevated pH value is not necessary,

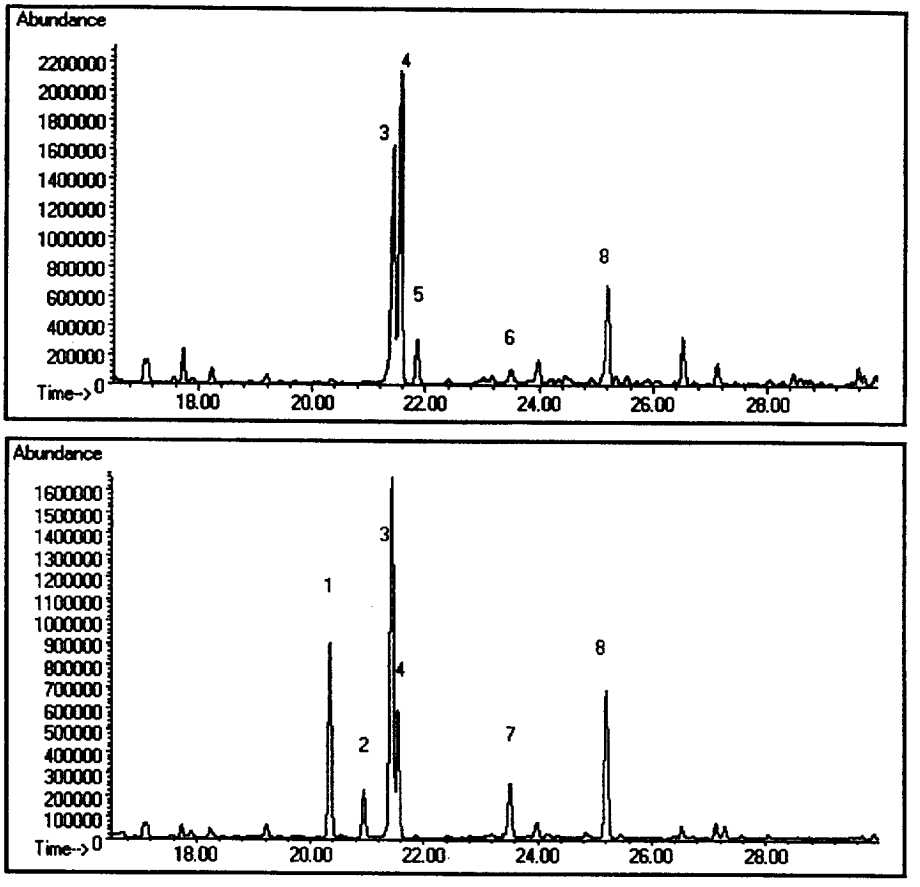

Fig. 14. GC–MS analysis (total ion chromatogram) of the free and glucuroconjugated fractions of a urine sample contaminated by bacteria, 6 h (upper panel) and 120 h after inoculation (lower panel). Peaks: 1 and 2=5β-androstan-3,17-dione (m/z 432.4); 3=epietiocholanolone (internal standard); 4=androsterone and etiocholanolone; 5=5α,β-androstan-3α,17β-diol; 6=epitestosterone; 7=5α-androstan-3,17-dione; 8=17α-methyl-5α-androstan-3β,17β-diol (internal standard).

but the absence of signs of degradation must be shown.

Similar effects can be observed during the enzymatic hydrolysis. Some crude mixtures of *Helix pomatia* juices are known to possess some unwanted enzymatic activities that cause the degradation of the urinary steroids [2,61].

4. Conclusion

The precise quantitative measurements obtained during analysis has enabled the determination of population-based reference values that are useful to the evaluation of test results. The distribution of the T/E values amongst male athletes confirmed that the probability of naturally occurring elevated values exceeding the threshold of 6:1 was low. Furthermore, as reported by several groups [5,52,54,62], the T/E value is the most sensitive probe of testosterone administration. This study has demonstrated the usefulness of studies of subsequent and previous test results in assessing testosterone administration. Other parameters of the steroid profile can be used in support of the findings.

Studies have shown [6] that the administration of DHT was detected by the comparison of population-based reference ranges. Moreover, the evaluation of individual longitudinal studies provided unequivocal proof. Athletes are competing around the world and

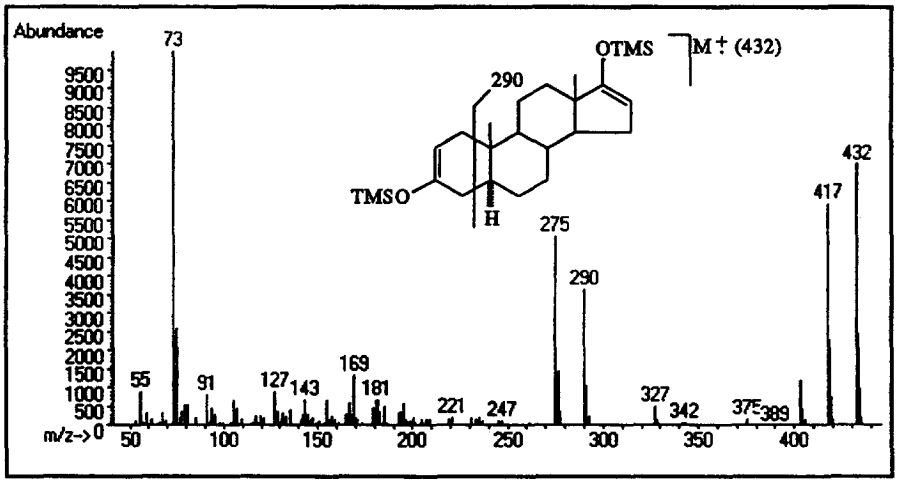

Fig. 15. Mass spectrum of the TMS enol derivative of 5α-androstan-3,17-dione obtained from the GC–MS analysis of the authentic standard (20 ng injected).

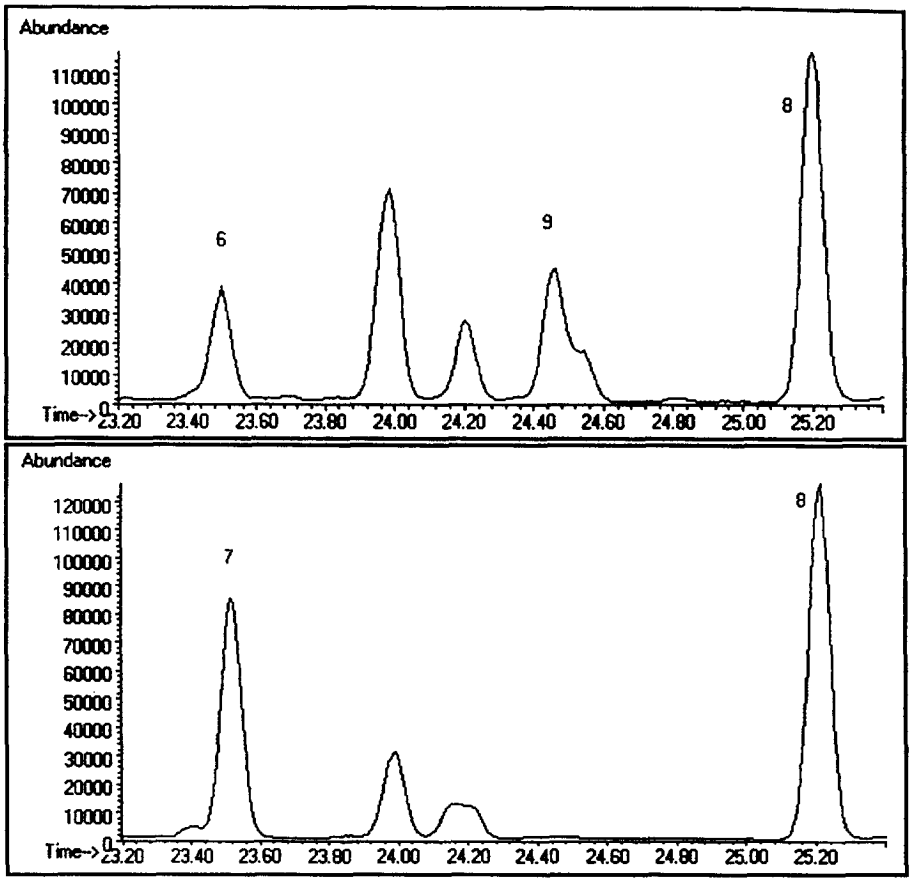

Fig. 16. GC–MS analysis (432.4 and 435.4 ion chromatograms) of the free and glucuroconjugated fractions of a urine sample contaminated by bacteria, 6 h (upper panel) and 120 h after inoculation (lower panel). Peaks: 6=epitestosterone; 7=5α-androstan-3,17-dione; 8=17α-methyl-5α-androstan-3β,17β-diol (internal standard); 9=testosterone.

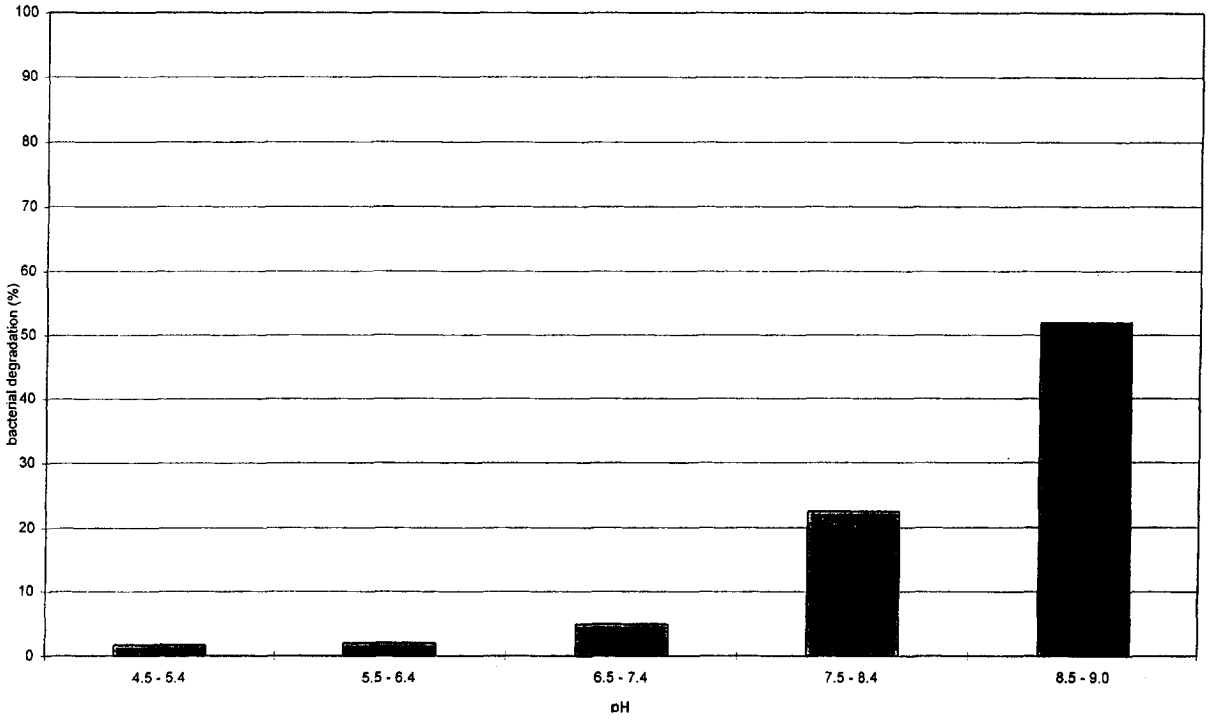

Fig. 17. Percentage of samples showing signs of bacterial degradation in relation to the pH values of the specimens received from doping control programs.

the results needed for the longitudinal studies are gathered from several laboratories. The quantitative measurements carried out on a routine basis must be precise, in order to ensure that a proper inter- and intra-laboratory comparison can be carried out. The increase in unannounced controls on short, or no notice, will permit a more representative collection of specimens. For example, by taking into account the rapid excretion of testosterone when it is administered orally [62] and that T/E values return to normal within 24 h, some athletes may use oral preparations and never be found positive.

Acknowledgments

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