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Plasma and urinary markers of oral testosterone undecanoate misuse

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Abstract

Orally administered testosterone undecanoate (TU), an anabolic, androgenic steroid, can potentially be abused by athletes. Indirect evidence for detecting oral TU intake could be deduced from the changes in steroid profile post-administration. Direct evidence could be obtained by detection of unchanged TU in plasma. To this end, both urinary and plasma steroid profiles of six healthy male subjects given a single oral dose of 120 mg of TU were studied by gas chromatography/mass spectrometry (GC/MS) and gas chromatography/tandem mass spectrometry (GC/MS/MS). The increased concentration of glucuronidated testosterone in plasma appears to be the most characteristic sign of oral TU intake. The testosterone glucuronide (TG)/nonconjugated testosterone (T) ratio, TG/17-hydroxyprogesterone (17OHP) ratio, and TG/luteinizing hormone (LH) ratio were observed to be significantly elevated above their basal levels for 10 h, 10 h, and 6 h, respectively. Urinary ratios of TG/epitestosterone glucuronide (EG) were found to be higher than the cut-off value of 6 for the period 4∼8 h post-administration, but only in three subjects. One subject failed to respond with respect to all of the above-mentioned indirect markers, as TG was not significantly increased in either plasma or urine. Unchanged TU was directly detected in plasma of all six subjects from 1∼1.5 h to 4∼6 h after oral TU intake by GC/MS/MS, providing unequivocal proof of exogenous testosterone intake. Distinct and complementary markers for detecting oral TU intake could be obtained from plasma and urine, respectively.

Keywords: Oral testosterone undecanoate; Doping; Plasma; Urine; Gas chromatography/mass spectrometry

1. Introduction

Discriminating the intake of exogenous testosterone (T) from endogenous testosterone still remains a challenge in sports drug testing. Until now, the urinary concentration ratio of testosterone glucuronide to epitestosterone glucuronide (TG/EG), with a cut-off value of six, has been established and commonly accepted by the International Olympic Committee as a general marker for indicating suspicious cases of exogenous testosterone intake in athletes [1]. However, it is already known that a potentially positive urinary sample judged by this criterion needs elaborate follow-up studies for a final decision due to the fact that some individuals have a physiologically or pathologically elevated urinary TG/EG ratio higher than six [2–4]. In contrast, some individuals will not show a urinary TG/EG ratio higher than six even after exogenous testosterone administration. This occurs, for example, in people of Asian origin, who have low basal TG/EG ratios [5]. Efforts have been made to develop complementary direct and indirect markers for detecting testosterone administration. On one hand, the unambiguous detection of minute intact testosterone esters in plasma or hair by tandem mass spectrometry (MS/MS) or high resolution mass spectrometry (HRMS) has been proposed [6,7]. Also, the direct differentiation of $^{13}C/^{12}C$ isotope compositions between endogenous and exogenous testosterone or between their relative metabolites in urine by isotope ratio mass spectrometry (IRMS) [8] can provide direct proof of testosterone intake [9–12]. On the other hand, the urinary TG to urinary luteinizing hormone (LH) ratio [13], blood plasma testosterone to plasma LH ratio, and blood plasma T to 17-hydroxyprogesterone (17OHP) ratio have been proposed as potential indirect markers for testosterone intake [14,15].

Among the pharmaceutical testosterone preparations commercially available, testosterone undecanoate (TU), which can be self-administered orally, can potentially be abused by athletes. It is known that oral TU produces a very

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brief change in the urinary TG/EG ratio [16]. Thus, oral TU intake could be difficult to detect in sports drug testing. Complementary plasma markers for detecting oral TU intake would be useful. After oral TU intake, it was found that plasma TG significantly increased instead of T [17]. Thus, the use of plasma TG/T and TG/17OHP ratios (instead of the T/17OHP ratio) has been proposed [18] for the detection of oral TU intake. The detectability of intact TU in plasma has been proved in one subject after multiple oral dosing of TU by both GC/MS and GC/MS/MS [6,19].

The aims of the present work were to study in detail the changes in steroid profile induced by a single oral administration of TU and to review its effects on known indirect markers of steroids in both urine and plasma. Also, the unequivocal detection of unchanged TU in plasma after oral administration was confirmed by the GC/tandem MS technique.

2. Experimental

2.1. Clinical protocol and sample collection

Six healthy Spanish male volunteers (subject #1–6; age, 27.2 ± 2.1 years; weight, 73.4 ± 4.0 kg; height, 1.75 ± 0.03 m; mean ± SD) were given one oral dose of 120 mg of TU (Androxon™, three 40-mg capsules; Organon). Ethical approval for the study had been 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.

2.1.1. Plasma

Venous blood (10 ml) was collected in heparin-containing tubes at −0.5 h before administration of TU, at 0 h (09:00 in the morning), and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 h after oral TU administration. Plasma was separated by centrifugation at 4°C immediately after the collection of blood and frozen at –20°C until analysis.

2.1.2. Urine

Overnight urine samples were collected on the day of TU administration (day 1). Urine samples were collected at 0∼4, 4∼8, 8∼12, and 12∼24 h on day 1 and 0∼12 and 12∼24 h on the next day (day 2). Overnight urine samples were collected on days 3, 4, and 6. The volume of each collected urine sample was measured, and an aliquot of 50 ml was frozen at –20°C until analysis.

2.2. Chemicals

17β-Hydroxyandrost-4-en-3-one (testosterone, T), 17α-hydroxyandrost-4-en-3-one (epitestosterone, E), 3α-hydroxy-5α-androstan-17-one (androsterone, A), 3α-hydroxy-5β-androstan-17-one (etiocholanolone, Et), 17α-hydroxypregn-4-ene-3,20-dione (17-hydroxyprogesterone, 17OHP), 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone, DHT), estra-1,3,5 [10]-triene-3,17β-diol (estradiol, E_2), 5β-androstane-3α,17β-diol (5β3αDiol), 5α-androstane-3α,17β-diol (5α3αDiol), testosterone glucuronide (TG), androsterone glucuronide (AG), etiocholanolone glucuronide (EtG), and 17α-methyl-testosterone (methyltestosterone) were purchased from Sigma (St Louis, MO, USA). 17β-Hydroxy-[16,16,17,$^2H_3$]androst-4-en-3-one (testosterone-D_3, T-D_3), 17α-hydroxy-[16,16,17,$^2H_3$]-androst-4-en-3-one (epitestosterone-D_3, E-D_3), and 3α-hydroxy-5β-[2,2,4,4,$^2H_4$]-androstan-17-one (etiocholanolone-D_4, Et-D_4) were purchased from Institut für Biochemie, Deutsche Sporthochschule Köln (Cologne, Germany). *Escherichia coli* β-glucuronidase was purchased from Boehringer Mannheim (Mannheim, Germany). Testosterone 17β-undecanoate (TU) was purchased from Research Plus (Bayonne, New Jersey, USA), and testosterone 17β-phenylpropionate (TPh) was kindly provided by Laboratorios Leo (Madrid, Spain).

N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was provided by Macherey-Nagel (Düren, Germany). Ammonium iodide and 2-mercaptoethanol were obtained from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

2.3. Sample preparation

2.3.1. Urine

Urinary steroid profiles were analyzed by GC/MS according to a routine screening procedure for anti-doping test described previously [5,20]. Briefly, to 2.5 ml of urine, methyltestosterone (500 ng/ml), Et-D_4 (500 ng/ml), T-D_3 (90 ng/ml), and E-D_3 (15 ng/ml) were added as internal standards [21]. The sample was then loaded onto Detectabuse™ Column (Biochemical Diagnostics Inc., NY) pretreated with methanol and water. The on-column sample was washed with water, and steroids were eluted with methanol. After the solvent was evaporated, the residue was redissolved in 1-ml sodium phosphate buffer (0.2 mol/l, pH 7), hydrolyzed with 6 international units of β-glucuronidase for 1 h at 55°C, and then extracted with 5-ml t-butylmethylether after adjusting the pH to 9∼10 with 50 g/l potassium carbonate. The organic phase was evaporated, and the residue was kept in a desiccator maintained at 60°C and 60 kPa (phosphorous pentoxide was used as desiccant and was changed when the formation of phosphoric acid became notable) for at least 30 min before derivatization (described below). In each analytical batch, a calibration sample including 2000 ng/ml A, 2000 ng/ml Et, 120 ng/ml T, 20 ng/ml E, 20 ng/ml DHT, 50 ng/ml E_2, 120 ng/ml 5β3αDiol, 80 ng/ml 5α3αDiol, and other steroids in distilled water were treated, derivatized like the urine samples, and injected into the GC/MS system to calculate response factors to quantify each substance [5].

Urinary luteinizing hormone (LH) was measured by a

heterogeneous sandwich magnetic separation assay (MSA; Bayer Corporation, USA) following the manufacturer's instructions.

2.3.2. Plasma

2.3.2.1. Nonconjugated and glucuroconjugated steroids. A 1-ml aliquot of each plasma sample was diluted by adding 1.5-ml sodium phosphate buffer (0.2 mol/l, pH 7). Methyltestosterone (50 ng/ml), T-D_3 (9 ng/ml), and Et-D_4 (50 ng/ml) were then added as internal standards. The sample was loaded onto a Detectabuse™ column pre-treated with methanol and water, then washed with water, and eluted with methanol. After evaporating the organic solvent, the residue was reconstituted in 1-ml sodium phosphate buffer (0.2 mol/l, pH 7). Nonconjugated steroids (containing T and 17OHP) were extracted with 5-ml t-butylmethylether, evaporated with nitrogen, and kept in a desiccator until derivatization. Glucuroconjugated steroids in the remaining aqueous phase were hydrolyzed after a second addition of the same amounts of the same internal standards (methyltestosterone, T-D_3, and Et-D_4) and extracted as described above for the urine samples. Quantitation of steroids in both fractions of plasma was performed by calculating their relative response factors to internal standards obtained from a calibration sample of distilled water containing 5 ng/ml 17OHP, 12 ng/ml T, 200 ng/ml A, 200 ng/ml Et, 8 ng/ml 5α3αDiol, 12 ng/ml 5β3αDiol, 2 ng/ml DHT, and 5 ng/ml E_2. Pooled plasma samples of non-treated subjects either with or without spiked T, TG, AG, and 17OHP were extracted in each analytical batch as quality control samples (see details below).

2.3.2.2. Testosterone undecanoate. For extraction of TU from plasma, a method similar to those previously described [6,22] was used, with some modifications. Testosterone 17β-phenylpropionate (TPh) was added as an internal standard to 1-ml of plasma to give a final concentration of 4 ng/ml. Immediately after the subsequent addition of 40 μl of 3 mol/l potassium hydroxide, the sample was extracted twice with 4 ml of a n-hexane:ethyl acetate (7:3 v/v) mixture using a rocking mixer for 20 min. The organic phase was pipetted out in a tube, mixed, and washed with 1 ml of 50 g/l acetic acid and with 1 ml of distilled water. After evaporating the organic solvents, the residue was kept in a desiccator for at least 30 min before the derivatization (described below). A calibration curve of plasma samples from non-treated subjects spiked with 0, 0.25, 0.5, 1.0, 5, 25, 50, 75, and 150 ng/ml of TU was constructed for plasma TU quantitation.

2.3.2.3. FSH, LH and SHBG. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) were analyzed by a heterogeneous sandwich magnetic separation assay (MSA; Bayer Corporation, USA) directly on plasma following the manufacturer's instructions. Sex hormone-binding globulin (SHBG) in plasma was measured with a two-site chemiluminescent immunometric assay (IMMULITE® SHBG; EURO/Diagnostic products Corporation, UK).

2.4. Derivatization

Trimethylsilylation (TMS) of steroids (including TU) in all extracts from urine and plasma was carried out by adding 50 μl of MSTFA/ammonium iodide/2-mercaptoethanol (1000:2:6 v/w/v) and heating at 60°C for 30 min. 1 ~ 2 μl of each solution were directly analyzed by GC/MS or GC/MS/MS.

2.5. Instrumentation

2.5.1. GC/MS

A Hewlett-Packard 6890 II GC model fitted with a HP 7673A autosampler was connected to a HP 5973 mass selective detector. The separation was carried out by a methylsilicone fused-silica capillary column (HP Ultra-1; 17 m × 0.2 mm (i.d.), film thickness 0.11 μm) with the following oven temperature program: an initial temperature of 181°C increased to 230°C at a rate of 3.0°C/min, then to 310°C at a rate of 40°C/min and held for 3 min. Helium was used as carrier gas with a flow rate of 0.8 ml/min (measured at 180°C). The injector (operated in 10:1 split mode) and the interface were both maintained at 280°C. The mass spectrometer was operated in selected ion monitoring acquisition mode with one or more ions selected for the TMS derivative of each substance (Table 1).

2.5.2. GC/MS/MS

For the detection of TU in plasma, a Finnigan GCQ ion trap mass spectrometer was connected with a GCQ gas chromatograph equipped with a model A200S autosampler. A HP Ultra-1 capillary column (19 m × 0.2 mm (i.d.), film thickness 0.11 μm) was used for the separation with the following oven temperature program: an initial temperature of 180°C (maintained for 0.5 min) increased to 210°C at a rate of 30°C/min, then to 230°C at a rate of 4.0°C/min, and finally to 315°C at a rate of 40°C/min and held for 7 min. The injector (in 15:1 split mode) and the interface were maintained at 290°C and 300°C, respectively. Helium was used as carrier gas with a flow rate of 0.8 ml/min (measured at 180°C). The ion source temperature was maintained at 200°C. The EI mass spectra of TU and TPh by GCQ system are shown in Fig. 1(A) and 1(C), respectively. The molecular ions of TPh (m/z 492) and TU (m/z 528) were chosen as parent ions. The mass spectrometry of daughter ions m/z 492 and m/z 528 are presented in Fig. 1(B) and 1(D), respectively. Main product ions were 209, 464 ($M^{+\cdot}$-28), and 477 ($M^{+\cdot}$-15) for TPh and 209, 500 ($M^{+\cdot}$-28), and 513 ($M^{+\cdot}$-15) for TU, and these were used as identification ions (Table 1). The collision energy for MS/MS fragmentation of TU and TPh was optimized for enhancement of sensitivity (data not shown). A collision energy of 1.3 V was used for

Table 1
Characteristic ions selected for TMS derivatives of steroids in GC/MS or GC/MS/MS analysis

GC/MS

Compound	RT (min)	Selected ions (m/z)	Compound	RT (min)	Selected ions (m/z)
A	10.50	434	DHT	12.56	434, 143
Et-D_4	10.65	438	E_2	12.97	416
Et	10.69	434	T-D_3	13.21	435
5α3αDiol	10.89	241	T	13.24	432
5β3αDiol	11.06	241	MT (ISTD)	15.02	446, 301
E	12.37	432	17OHP	18.16	546
E-D_3	12.37	435			

GC/MS/MS

Compound	RT (min)	Parent ions (m/z)	c.e. (Volts)	Daughter ions (m/z)
TPh	9.68	492	1.3	477, 464, 209
TU	10.00	528	1.3	513, 500, 209

c.e. = collision energy.

both parent ions. After the optimization of MS/MS fragmentation, the ion trap detector was operated in selected reaction monitoring mode for analysis of TU in plasma samples. Ions m/z 477 (for TPh) and 513 (for TU), produced by fragmentation from $M^{+\cdot}$ to $M^{+\cdot}$-15, were used as quantitation ions.

2.6. Statistical parameters

Pooled plasma samples, non-spiked and spiked with T (0.8 ng/ml and 5 ng/ml), TG (15 ng/ml and 75 ng/ml), AG (50 ng/ml) and 17OHP (0.5 ng/ml and 1 ng/ml), were included in each analytical batch as control samples in the analysis of the plasma steroid profile. Control samples were stored at – 20°C as those plasma samples collected after TU administration. The stability of steroids in plasma was not evaluated under the conditions of storage. Intra- and inter-assay variabilities of the quantitation with single-point calibrations as described above were evaluated (Table 2) with coefficients of variation (CVs) in the range of 2.7%–15% for T, TG, AG, EtG, and 17OHP. Relative accuracy errors for measuring T, TG, AG, and 17OHP in spiked plasma samples ranged from −5.4% to −17.9%. The average recovery of spiked T, TG, AG, and 17OHP in pooled plasma were 79.7%, 86.3%, 81.6%, and 84%, respectively.

The calibration curves for TU were linear over the concentration range of 0.25 ng/ml (0.55 nmol/l)–150 ng/ml (330 nmol/L), and the mean correlation coefficients for the calibration regression lines were around 0.996.

Both urinary and plasma steroid concentrations are presented in nmol/l; accordingly, excretion rates and concentration ratios are presented in nmol/h and nmol/l to nmol/l, respectively. Excretion rates, concentrations, and log-transformed concentration ratios either in urine or in plasma after administration of TU were compared with their basal values by ANOVA for significant differences.

3. Results

3.1. Urine steroid profile

The urinary excretion rates of the measured steroids (TG, AG, EtG, EG, DHT glucuronide (DHTG), E_2 glucuronide (E_2G), 5β3αDiol glucuronide (5β3αDiolG), and 5α3αDiol glucuronide (5α3αDiolG)) significantly increased 0∼4 h, 4∼8 h, and 8∼12 h (except for EG) post-TU administration in five of six subjects (Table 3). However, by 12∼24 h, most excretion rates returned nearly to their basal values, except for those of 5α3αDiolG, E_2G, and 5β3αDiolG, which remained clearly elevated over their basal values until 12∼24 h, 24∼36 h, and 24∼36 h, respectively. In the five subjects mentioned above, the mean urinary concentration (nmol/l) ratios of TG/EG and AG/EG showed remarkable increases until 4∼8 h post-administration (Fig. 2), while those ratios corresponding to 5α-/5β-metabolites of testosterone (AG/EtG and 5α3αDiol/5β3αDiol) only showed slight or no changes after oral TU intake.

One subject (subject # 3), whose basal urinary excretion rates of steroids were always much lower (Table 3), did not show a TG/EG ratio higher than six even after TU intake (negative for T intake, as judged by the official criterion). However, this subject showed significantly elevated excretion rates of AG, EtG in the 0∼4 h period, which were comparable to those of the other five subjects. Subject #3 also showed greater increases in the ratios of AG/TG and AG/EG, which suggested a faster conversion of testosterone to androsterone post-TU administration.

The 0∼24 h urinary recovery of metabolites, expressed as TU administered, was calculated (Table 4). The TU recovery (30%) from subject # 3 was found to be not far below the TU recovery range (from 33% for subject # 4 to 53% for subject # 6) of the other five subjects. This may

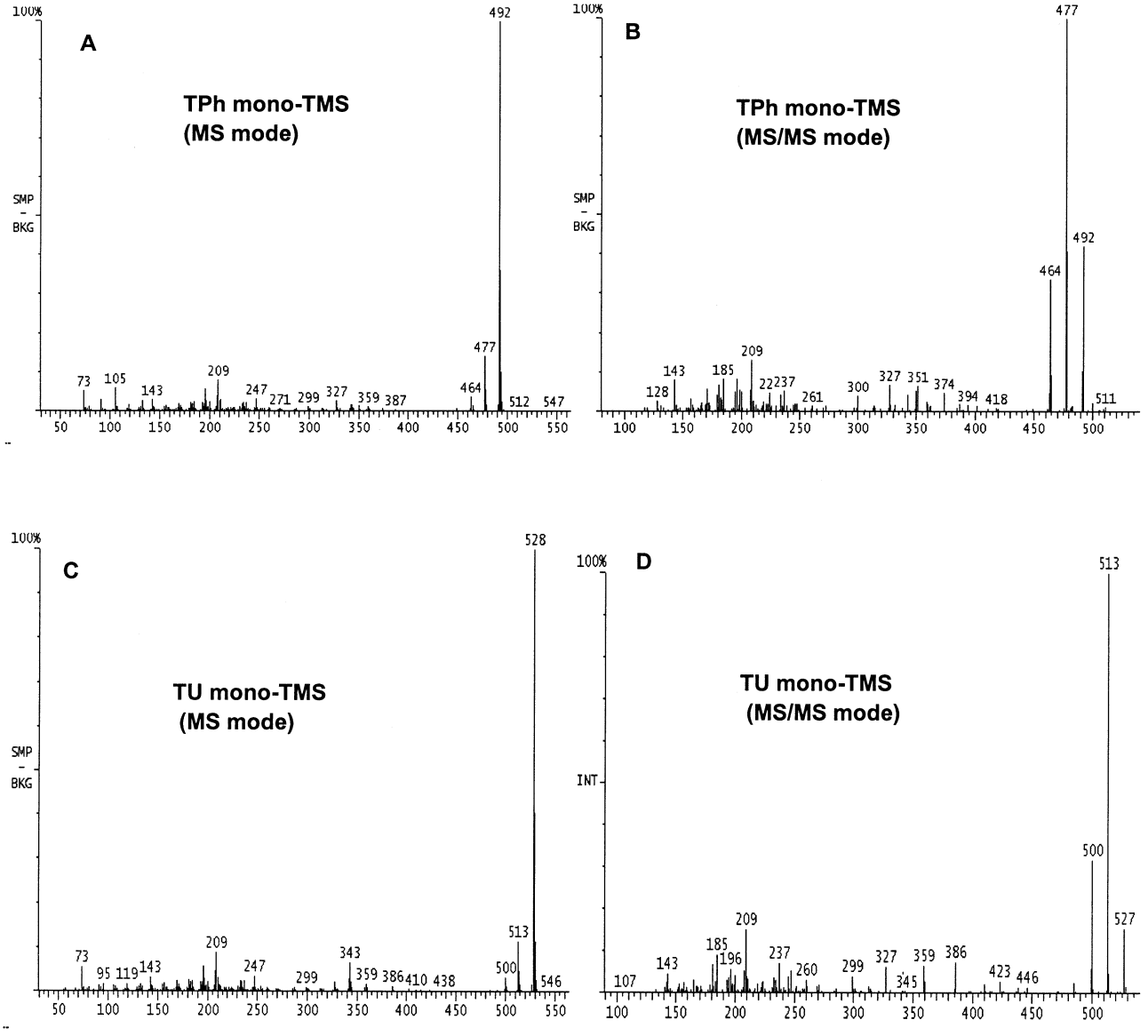

Fig. 1. Ion trap full scan mass spectra of mono-O-TMS dervatives of testosterone phenylpropionate (TPh mono-TMS; A) and testosterone undecanoate (TU mono-TMS; C); Ion trap full scan tandem mass spectra from the fragmentation of the molecular ions of mono-O-TMS derivatives of testosterone phenylpropionate (TPh mono-TMS; B) and testosterone undecanoate (TU mono-TMS; D).

suggest that there was no significant difference in the absorption of TU between subject # 3 and the other five subjects.

No significant changes in urinary LH concentrations (data not shown) were observed.

3.2. Plasma steroid profile

Basal concentrations of plasma TG were much lower in all subjects, compared with basal nonconjugated T. However, after oral administration of TU, plasma T did not show significant increases (Table 5), which is in opposition to what is usually observed after intramuscular (i.m.) administration of T esters [5,14,15]. Instead, TG showed a remarkable increase until 6 h post-administration in five of the six subjects. Similar results occurred for other important metabolites of testosterone, like A, Et, DHT, E_2, 5β3αDiol, and 5α3αDiol, which also showed significant increases of their glucuronide conjugates in plasma. Increases in glucuronidated steroids appear to be clear proof of oral TU intake.

On the other hand, subject #3 did not show a significant increase of TG. Further, neither DHTG nor E_2G were detectable in his plasma, both before and after oral TU administration, with the sensitivity achieved by the present bench-top GC/MS system (about 0.1 ng/ml). However, plasma AG, EtG, 5β3αDiol, and 5α3αDiol in this subject did show increases similar to those observed in the other five subjects.

Table 2
Precision and accuracy of GC/MS analysis of nonconjugated and glucuronidated steroids in control and steroid-spiked pooled plasma

		T (ng/ml)	TG (ng/ml)	AG (ng/ml)	EtG (ng/ml)	17OHP (ng/ml)
Non-spiked	Intra-assay precision (n = 6)					
	Mean ± SD	3.66 ± 0.10	0.41 ± 0.04	12.19 ± 0.87	13.53 ± 0.82	0.36 ± 0.03
	CV (%)	2.7	10.4	7.2	6.1	9.4
	Inter-assay precision (n = 12)					
	Mean ± SD	3.70 ± 0.21	0.38 ± 0.06	12.85 ± 1.17	13.79 ± 0.70	0.39 ± 0.05
	CV (%)	5.8	15.1	9.1	5.1	13.1
	Low concentration control					
	Added	0.80	15.00	50.00	0*	0.50
	Expected	4.50	15.38	62.85	13.79	0.89
	Measured (n = 6)	4.25 ± 0.17	12.62 ± 1.81	53.92 ± 6.42	13.01 ± 0.89	0.80 ± 0.09
	CV (%)	4.0	14.4	11.9	6.8	11.7
	Relative error (%)	−5.5	−17.9	−14.2	−5.6	−10.1
Spiked						
	High concentration control					
	Added	5.00	75.00	50.00	0*	1.00
	Expected	8.70	75.38	62.85	13.79	1.39
	Measured (n = 6)	8.23 ± 0.29	68.66 ± 9.24	53.35 ± 7.36	13.67 ± 1.13	1.25 ± 0.12
	CV (%)	3.5	13.4	13.8	8.2	11.0
	Relative error (%)	−5.4	−8.6	−15.1	−0.8	−10.1

T, testosterone; TG, testosterone glucuronide; AG, androsterone glucuronide; EtG, etiocholanolone glucuronide, * standard not available; 17OHP, 17α-hydroxyprogesterone.

3.3. Changes in the hypothalamic-hypophysial-gonadal (HHG) axis

The possible inhibitory effect of oral TU administration on the hypothalamic-hypophysial-gonadal (HHG) axis was studied by monitoring FSH, LH, and 17OHP in plasma. No significant changes in FSH was observed (data not shown), while LH, with high fluctuations on the administration day (Table 5), slightly decreased for 1∼2 days after TU administration (data not shown). Production of plasma 17OHP (precursor of endogenous T) also slightly decreased from 4 h to 12 h post-administration. Plasma concentrations of LH and 17OHP from subject #3 showed some slight fluctuations. Thus, a single oral dose of TU administration seems unlikely to produce an inhibitory effect on the HHG axis.

No changes in the SHBG were observed, in accordance with little changes observed in nonconjugated T.

3.4. Identification of TU in plasma

Unchanged TU was detected by GC/MS/MS in plasma of all of the six subjects (Fig. 3) from 0.5∼1 h to 4∼6 h post-administration. Remarkable inter-individual variations of plasma TU concentrations were observed, with maximal concentrations ranging from 20 nmol/l to 285 nmol/l. The time to reach maximal concentrations ranged from 1.5 h to 3 h post-administration. However, the time-windows for the detection of TU were quite similar in all six subjects.

3.5. Plasma markers as compared to urinary markers

As observed above, after oral TU intake, plasma TG increased significantly and appeared to be the most specific plasma indicator for oral TU administration, while nonconjugated T, 17OHP, and LH were observed to slightly decrease in plasma, probably only due to their diurnal variations. Consequently, TG/T, TG/17OHP, and TG/LH, but not T/17OHP and T/LH, could act as potential plasma markers for indicating oral testosterone intake (Fig. 4). Plasma TG/T, TG/LH, and TG/17OHP were indicative of testosterone intake in five of the six subjects for 10, 10, and 6 h post-intake, respectively [23,24]. However, the identification of intact TU provide unequivocal evidence for testosterone intake in all subjects. Table 6 shows the comparison of detection windows between plasma markers and the urinary TG/EG ratio (The mean basal TG/T ratio of the five subjects was about 0.1, so a cut-off value of 0.6 was tentatively used).

One subject (subject #3) failed to respond post-TU administration with respect to all of the above-discussed indirect markers in both urine and plasma. This appeared to be due to a lack of change in his TG levels in both plasma and urine. Unchanged TU detected in this subject's plasma was the only effective indicator of TU administration.

4. Discussion

After a single oral administration of TU, the main changes in steroid profiles in both urine and plasma were

Table 3
Urinary excretion rates (nmol/h) of steroids from six healthy subjects after a single oral dosing of 120 mg of testosterone undecanoate

Interval	TG	EG	AG	EtG	DHTG	E_2G	5α3αDIOLG	5β3αDIOLG
				Mean ± SE (n = 5, subjects # 1, 2, 4, 5, 6)				
Basal	6.7 ± 1.8	6.0 ± 0.6	671 ± 89	513 ± 120	1.6 ± 0.4	1.1 ± 0.3	13.2 ± 2.2	52.2 ± 17.2
0–4 h	896[a] ± 196	21.4[a] ± 1.9	13 087[a] ± 1200	8948[a] ± 1481	46.1[a] ± 9.0	198[a] ± 36	148[a] ± 50	435[a] ± 80
4–8 h	71.6[a] ± 19.6	8.9[a] ± 0.6	2627[a] ± 235	1799[a] ± 308	11.8[a] ± 2.1	23.5[a] ± 4.3	50.6[a] ± 6.2	197[a] ± 30
8–12 h	13.7[a] ± 4.3	6.1 ± 1.0	1052[a] ± 140	1137[a] ± 244	4.9[a] ± 1.3	6.9[a] ± 2.2	31.2[a] ± 5.7	158[a] ± 43
12–24 h	7.1 ± 2.2	4.9 ± 0.5	611 ± 96	659 ± 175	2.4 ± 0.8	2.3[a] ± 1.0	20.5[a] ± 5.0	93.2[a] ± 39.1
24–36 h	5.9 ± 1.6	5.3 ± 0.5	747 ± 85	652 ± 160	2.2 ± 0.5	1.8[a] ± 0.7	13.3 ± 2.8	83.0[a] ± 27.2
36–48 h	7.6 ± 1.7	6.8 ± 1.2	783 ± 53	593 ± 103	1.9 ± 0.2	1.3 ± 0.5	17.9 ± 3.0	69.8 ± 18.2
60–72 h	6.1 ± 1.4	5.8 ± 1.1	591 ± 115	386 ± 80	1.5 ± 0.2	0.44 ± 0.15	10.5 ± 3.5	41.1 ± 14.4
84–96 h	7.0 ± 2.3	5.6 ± 0.8	618 ± 112	459 ± 122	1.5 ± 0.4	0.37 ± 0.11	12.4 ± 3.1	52.7 ± 20.4
132–144 h	5.5 ± 1.6	4.4 ± 0.6	527 ± 84	419 ± 117	1.6 ± 0.3	0.29 ± 0.11	9.3 ± 1.9	39.6 ± 12.4
				Subject # 3				
Basal	0.61	2.32	356	234	1.06	0.26	4.8	4.5
0–4 h	9.61	8.08	10 241	7068	3.94	6.99	52.0	47.7
4–8 h	1.60	2.86	1554	998	0.87	1.36	15.8	12.2
8–12 h	1.13	3.04	1388	888	0.99	0.63	14.0	12.3
12–24 h	0.82	2.84	547	409	0.72	0.29	8.1	6.9
24–36 h	0.61	2.08	428	268	1.38	0.29	4.3	3.4
36–48 h	0.58	2.02	318	213	0.63	0.18	4.3	4.3
60–72 h	0.57	2.11	384	247	0.81	0.15	4.9	4.7
84–96 h	0.37	1.35	246	148	0.58	0.07	2.9	2.7
132–144 h	0.54	2.09	348	228	0.40	0.22	4.2	3.9

[a] Significant increase above basal levels ($P < 0.05$).

observed during the 0∼12 h period post-administration in six healthy Caucasian subjects studied. The most relevant feature was the detection of exogenous testosterone and/or its metabolites in plasma in their glucuronidated form. Although measurements of the increases in the glucuroconjugates of testosterone, DHT, and 5α3αDiol in plasma after oral TU intake either by RIA [17] or by GC/MS [18] have been reported before, a more comprehensive steroid profile is presented here. In plasma, T, A, Et, 3α5αDiol, 3α5βDiol, DHT, and E_2 were mainly in their glucuroconjugated derivatives after oral TU administration in five of the six subjects studied. It had been previously shown that AG and EtG clearly increased in plasma from the same six subjects after a single i.m. injection of Testoviron Depot 100 (combined 25-mg testosterone propionate (TP) and 110-mg testosterone enanthate (TE)) [24]. In contrast, in plasma testosterone was mainly detected, nonconjugated, instead of glucuroconjugated, after i.m. administration of TP plus TE, just as was observed after i.m. TE administration [5,14,15]. Thus, the increase in TG appears to be characteristic of oral TU intake, as compared with i.m. administration of T esters. The increase of glucuronidated testosterone after oral TU intake was similar to the behavior of orally administered, non-esterified testosterone, as it is known that orally administered testosterone is mainly deactivated into TG due to the first-pass effect [25]. However, in case of orally administered TU, the glucuronidation of the hydrolysed testosterone may occur noticeably in tissues or splachnic organs other than the liver. It's usually considered that the esterification of testosterone with undecanoic acid shifts the route of absorption of orally given TU from the portal vein to the lymph system [26] and that TU enters the blood via the thoracic duct. However, in the present study, plasma TG sharply increased post-TU administration, with the mean peak concentration being reached 1 h after the dosing, whereas unchanged TU was found in plasma of all the six subjects receiving oral TU, with the mean peak concentration appearing at 3 h after the dosing. This observation suggests that still, a substantial percentage of TU may lose the undecanoate group in gastrointestinal track and be absorbed via portal vein after being glucuronidated in liver or in gastrointestinal track (the presence of several UDP-glucuronosyltransferase enzymes in the gastrointestinal tract was recently reported [27]). On the other hand, the remaining nonmetabolised TU could be absorbed via the lymphatic system, enter the blood, be hydrolyzed into testosterone, then be glucuronidated in liver or other tissues. The above hypothesis about the possible absorption routes of oral TU administration is in accordance with the oral TU absorption behavior suggested by Coert et al. [28] in a controlled study of rats after oral TU administration. Partial discussion concerning the oral TU absorption has been presented in our former publication [24]. In summary, clear increases in glucuronidated testosterone and its metabolites in plasma directly reflect the oral intake of exogenous testosterone.

Whether TG or T should be used in plasma parameters for indicating T intake is worth discussing. In the past, when the plasma T/17OHP ratio was suggested as a complementary marker to test for doping [14,15], plasma testosterone (T) was usually measured by RIA and was supposed to

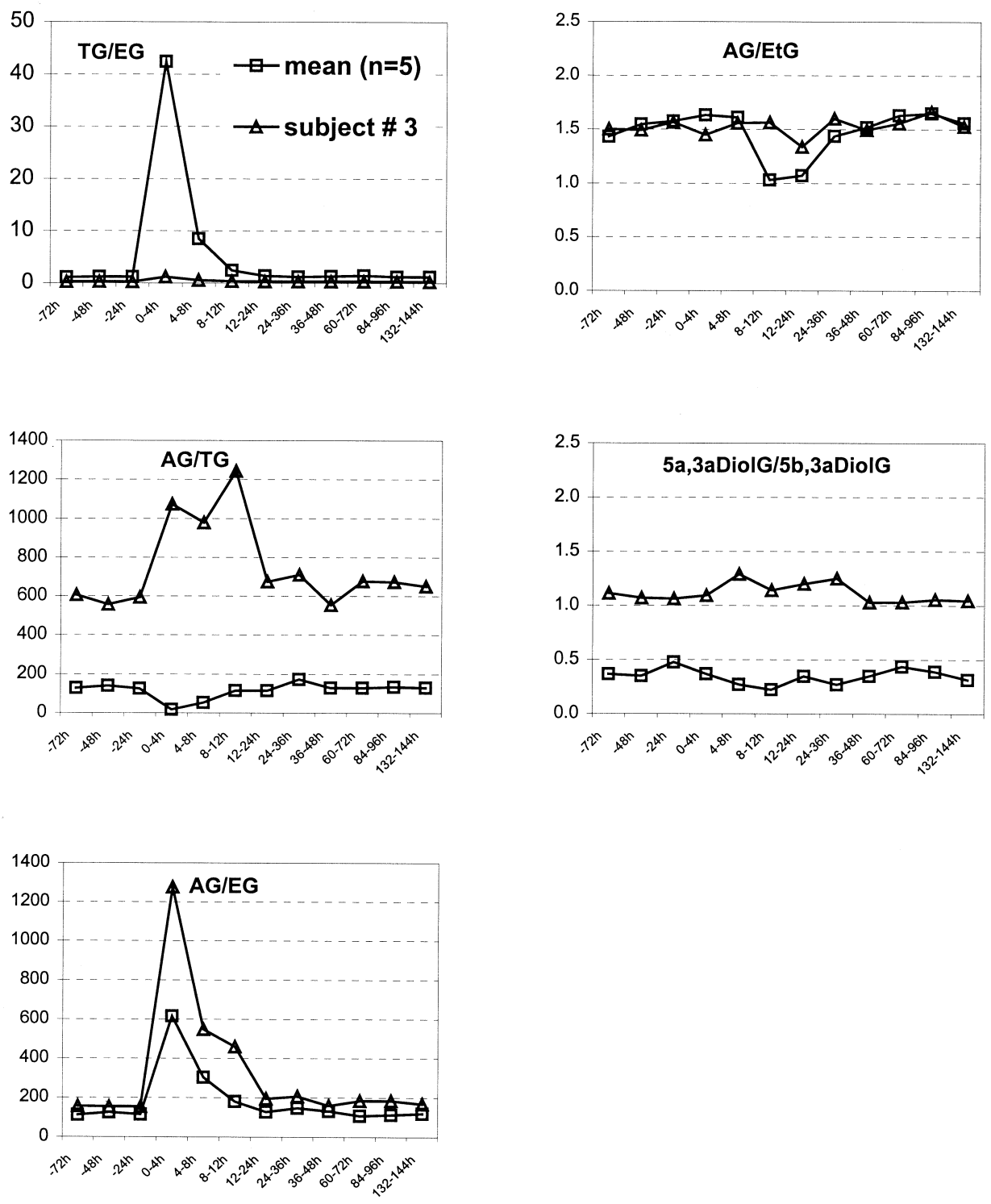

Fig. 2. Profiles of urinary steroid molar concentration ratios for six subjects after a single oral dose of 120 mg of testosterone undecanoate (□ for mean, n = 5, subjects # 1, 2, 4, 5, 6; Δ, subject # 3).

represent the nonbound (or nonconjugated) fraction of total plasma testosterone. More recently, the GC/MS technique was used for specifically measuring nonconjugated T in plasma and for calculating the T/17OHP ratio [5] after i.m. administration of TE. An increase in the plasma T/17OHP ratio, demonstrated by both RIA and GC/MS, appeared to be a sensitive marker for indicating exogenous testosterone after i.m. administration of T esters [14,15]. However, after oral TU administration, the absence of a clear increase in plasma nonconjugated T concentrations obtained by

Table 4
TU recovery (% of dose administered) in 0~24 h urine of six healthy subjects after a single oral administration of 120 mg of TU

	% (Mean ± SE, $n = 5$) Subjects # 1, 2, 4, 5, 6	% Subject # 3
TG	1.46 ± 0.32	0.02
EG	0.03 ± 0.00	0.01
AG	23.78 ± 1.57	17.85
EtG	16.26 ± 2.56	12.21
DHTG	0.10 ± 0.02	0.01
5β,3αDiolG	2.00 ± 0.51	0.16
5α,3αDiolG	0.35 ± 0.08	0.12
Total recovery	43.98 ± 3.34%	30.38%

GC/MS in the present study and others [18] does not agree well with the interpretation of results previously reported by RIA [29–31] after oral TU intake, where nonconjugated testosterone increased in serum, even though there were large inter-individual variations. This apparent disagreement between the results for T obtained in the past by RIA (increase) and now by GC/MS (no increase) after oral TU administration might be due to the possible cross-reactivity of the RIA anti-testosterone serum with either TG or TU, to large inter-individual variations of the steroid profile after oral TU intake, or to different target populations (healthy subjects this time vs. patients in previous studies). In our hands, the plasma TG/T, TG/17OHP, and TG/LH ratios, but not the T/17OHP or T/LH ratios, have been found to be sensitive markers for oral TU intake. In summary, as various pharmaceutical preparations of testosterone esters may be used by athletes via different administration routes (i.m. or oral), it is important to measure both the glucuroconjugated and nonconjugated testosterone in plasma during doping tests for exogenous T intake.

In urine, the mean dose recovery of TU was 44%, which agrees with other studies in which 40% radioactivity was recovered from urine samples of healthy subjects after oral administration of [^{3}H]-TU [26]. Testosterone and most of its metabolites (A, Et, 5β3αDiol, 5α3αDiol, DHT, and E_2) are almost completely glucuroconjugated, with the urinary TG/EG ratio being the most sensitive urinary marker (up to 40 times higher than basal values) for oral TU intake. However, in spite of urinary TG/EG or plasma TG/T, TG/17OHP, and TG/LH ratios, all being derived from changes in the steroid profile post-administration, the unequivocal identification of intact TU in plasma by tandem MS technique provided the stronger evidence of an exogenous TU intake.

None of the above-mentioned plasma and urine markers lasted longer than 10 h after oral TU intake. The relatively longer-lasting parameter was the urinary excretion rate of 5β3αDiolG, which lasted until the 24~36 h period in urine. This has been proven by directly differentiating this compound's exogenous origin from its endogenous origin by assessing the $^{13}C/^{12}C$ composition difference with IRMS [23]. Thus, information obtainable from both urine and plasma, and by different techniques, could be complementary to each other in sports drug testing.

As described above, one subject showed different changes in his steroid profile after oral TU administration. This finding was similar to the observation in one out of seven subjects in a previous controlled study of one oral administration of TU plus epitestosterone undecanoate who showed no significant changes in his urinary and plasma TG levels [18]. These particular subjects seem to represent the behavior of a small subgroup of the Caucasian population. Yet, this behavior could be more prevalent in individuals of other ethnic groups [5]. In fact, it has been reported that in four healthy Chinese subjects orally administered TU, none of them showed urinary TG/EG > 6 post-administration either with a single oral dose or with multiple oral doses of TU [32].

Table 5
Time courses of plasma concentrations (nmol/l) of T, TG, AG, EtG, DHTG, E_2G, 5β3αDiolG, 5α3αDiolG, 17OHP, and LH (in IU/l) in five subjects after an oral administration of 120 mg of TU

Time	T	TG	AG	EtG	DHTG	E_2G	5β3αDiolG	5α3αDiolG	17OHP	LH
					Mean ± SE, $n = 5$ (subjects # 1, 2, 4, 5, 6)					
−0.5	13.0 ± 3.2	1.7 ± 0.4	73.0 ± 26.8	48.5 ± 12.2	0.99 ± 0.36	0.44 ± 0.19	15.2 ± 5.0	3.7 ± 1.1	3.13 ± 0.74	3.74 ± 0.64
0	12.8 ± 3.3	1.6 ± 0.3	71.7 ± 19.7	51.2 ± 12.8	1.0 ± 0.3	0.55 ± 0.19	16.8 ± 4.7	3.4 ± 1.0	2.41 ± 0.63	3.72 ± 0.67
0.5	15.0 ± 3.6	84.8 ± 24.1[a]	334 ± 135[a]	314 ± 93[a]	6.7 ± 2.2[a]	2.5 ± 0.8[a]	33.6 ± 6.5[a]	7.2 ± 2.0[a]	2.66 ± 0.98	2.94 ± 0.32
1	14.7 ± 3.2	157 ± 50[a]	1943 ± 565[a]	1612 ± 485[a]	14.4 ± 3.5[a]	19.2 ± 5.6[a]	197 ± 68[a]	32.3 ± 11.9[a]	2.00 ± 0.74	3.47 ± 0.97
1.5	13.9 ± 3.1	97.6 ± 34.4[a]	1926 ± 534[a]	1061 ± 284[a]	17.6 ± 3.8[a]	16.2 ± 4.3[a]	268 ± 80[a]	45.2 ± 16.6[a]	2.62 ± 0.66	3.35 ± 0.67
2	14.1 ± 3.4	43.0 ± 12.2[a]	1285 ± 311[a]	635 ± 168[a]	10.7 ± 2.0[a]	7.7 ± 1.6[a]	192 ± 49[a]	30.2 ± 8.0[a]	2.02 ± 0.71	4.15 ± 0.40
3	12.4 ± 2.6	16.8 ± 5.9[a]	751 ± 202[a]	317 ± 79[a]	5.2 ± 1.6[a]	2.1 ± 0.5[a]	127 ± 31[a]	24.7 ± 6.6[a]	2.39 ± 0.81	3.92 ± 0.49
4	11.0 ± 2.9	6.3 ± 1.2[a]	378 ± 120[a]	158 ± 42[a]	5.7 ± 2.6[a]	1.2 ± 0.3[a]	75.1 ± 9.6[a]	17.3 ± 3.8[a]	1.49 ± 0.67[a]	3.14 ± 0.51
6	10.3 ± 3.1	3.1 ± 0.6[a]	142 ± 32[a]	86.9 ± 23.6[a]	2.8 ± 1.1[a]	0.96 ± 0.15[a]	41.3 ± 11.2[a]	9.4 ± 3.5[a]	1.93 ± 0.60	4.26 ± 0.65
8	8.2 ± 2.4	1.8 ± 0.3	100 ± 25	114 ± 33[a]	1.4 ± 0.2	0.73 ± 0.14	55.0 ± 17.3[a]	7.8 ± 2.3[a]	1.43 ± 0.64[a]	2.77 ± 0.51
10	6.1 ± 2.0	1.5 ± 0.3	61.8 ± 15.3	73.8 ± 21.2	1.8 ± 0.4[a]	1.00 ± 0.27[a]	45.6 ± 15.2[a]	7.7 ± 3.1[a]	1.25 ± 0.58[a]	3.59 ± 0.45
12	6.7 ± 1.7	1.1 ± 0.2	61.2 ± 15.8	74.7 ± 24.0	1.2 ± 0.3	0.47 ± 0.12	43.8 ± 14.2[a]	7.5 ± 2.3[a]	1.08 ± 0.31[a]	2.83 ± 0.43
24	11.2 ± 2.9	1.4 ± 0.3	80.6 ± 24.1	72.2 ± 19.5	0.99 ± 0.36	0.48 ± 0.10	34.9 ± 9.0[a]	5.3 ± 1.4[a]	2.14 ± 0.63	3.39 ± 0.49

[a] $P < 0.05$ compared to basal levels; n.d = not detectable.

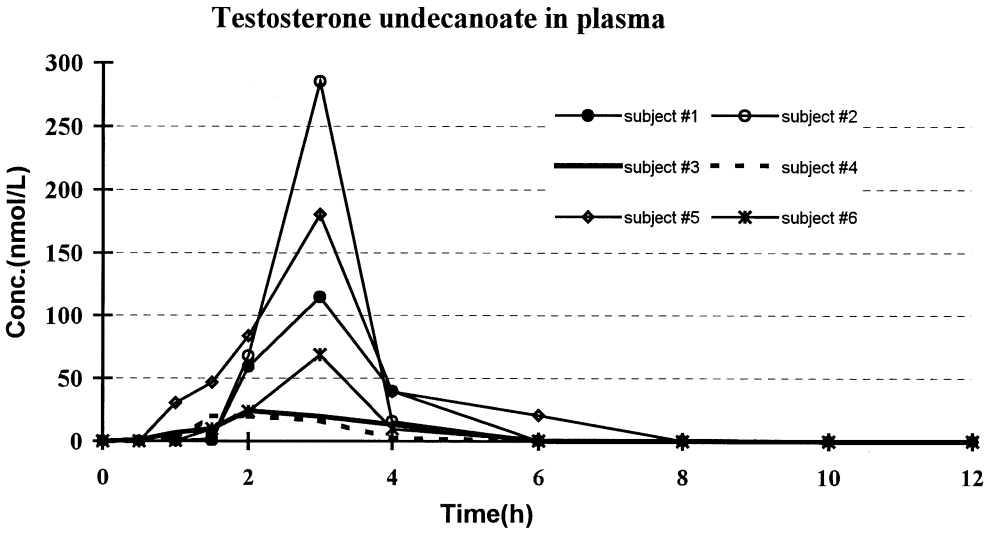

Fig. 3. Concentration versus time profiles of unchanged TU in blood plasma in six subjects after an oral administration of 120 mg of testosterone undecanoate.

One of the probable explanations for the above described intra-ethnic and inter-ethnic differences about testosterone glucuronidation after oral TU administration may be the polymorphism of UDP-glucuronosyltransferase isoforms which conjugate androgens [33,34].

In general, important information for detecting exogenous testosterone intake can be obtained from measuring changes in urinary and plasma steroid profiles. Hydrolysis of plasma samples in order to differentiate TG from T is relevant. Increased TG in plasma after exogenous administration is characteristic of oral TU intake. However, clearly increased concentrations of glucuronidated metabolites of testosterone (such as A, Et, DHT, E_2, 5β3αDiol, 5α3αDiol, and so on) in plasma appear to be common after both oral and i.m. administration of testosterone esters. The plasma TG/T, TG/17OHP, and TG/LH ratios, but not T/17OHP and T/LH ratios, appear to be sensitive markers of oral testosterone abuse. However, for application of all of the studied indirect markers in doping tests, both relevant population reference values and intra-individual variations should be studied and taken into consideration. Direct identification of testosterone undecanoate provided unambiguous proof of exogenous testosterone intake in all of the subjects studied. Blood plasma, as a complementary material to urine, could be used for detecting exogenous T intake in sports drug testing.

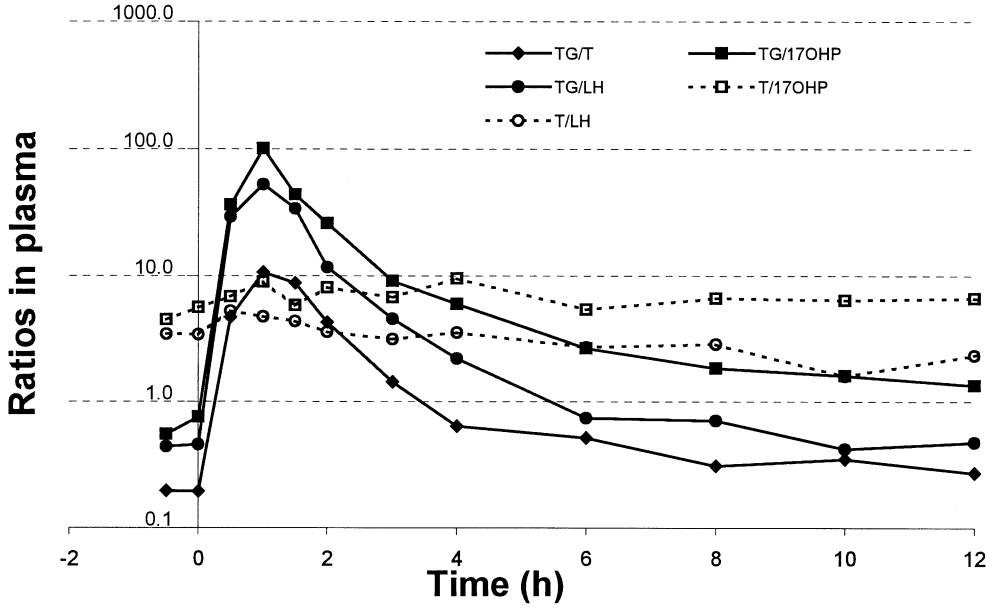

Fig. 4. Time course of plasma concentration ratios (mean, $n = 5$) in five subjects after a single oral dose of 120 mg of TU. Concentrations of TG, T, and 17OHP are in nmol/l; LH concentration is in IU/l.

Table 6
Comparison of individual detection windows as judged by plasma TG/T, plasma TU, and urinary TG/EG after a single oral administration of 120 mg of TU

Subjects	Plasma TG/T (>0.6[a])	Plasma TU	Urinary TG/EG (>6)
# 1	>4 h	2~4 h	0~4 h
# 2	>12 h	2~4 h	4~8 h
# 3	failed	0.5~4 h	failed
# 4	>3 h	1~4 h	0~4 h
# 5	>3 h	1~6 h	4~8 h
# 6	>4 h	1~4 h	4~8 h

[a] A tentative threshold value of 0.6 for TG (nmol/l)/T (nmol/l).

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