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Overall biological markers of oral testosterone undecanoate misuse

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Introduction

According to the regulation of international Olympic committee, a urinary T/E (testosterone glucuronide to epitestosterone glucuronide) ratio higher than 6 is suspicious for exogenous testosterone intake (1). However, this parameter is influenced by several factors, such as ethnic differences, inter- and intra- individual variations or administration of other substances (2). Thus, this criterion has limitations for urine samples corresponding to subjects with a naturally elevated T/E ratio or urine samples showing low T/E ratios even after administration of exogenous testosterone (3). Developing additional urinary markers for detecting testosterone intake is of interest, and some interesting progress having been made by isotope ratio mass spectrometry (IRMS) for differentiating the isotope compositions of carbon atoms between exogenous and endogenous testosterone in urine (4, 5). In recent years attention has been paid to plasma samples in addition to urine samples. In fact the detectability of minute amount of intact testosterone esters in plasma provide unequivocal proof of testosterone intake (6). Exogenous testosterone also suppresses pituitary secretion of luteinizing hormone (LH) and as a result, the production of testosterone precursor 17 α -hydroxyprogesterone (17 α OHP) is also inhibited. Plasma T/17 α OHP and T/LH (both in urine and plasma) are suggested as potential indirect indicators of testosterone abuse (7, 8). However, at present, plasma sampling is not a common practise yet in doping control. An alternative for plasma sample is 1~2 drops of whole blood, which can be punched from fingertips, spotted on filter paper and transferred easily from the field to the laboratories. This alternative has been discussed for determination of some steroids (9) and could be used for direct detection of testosterone and its esters by GC/MS with high sensitivity.

The goal of the present work has been to review the effects produced by the controlled administration of oral testosterone undecanoate on those already known direct and indirect markers available in urine and blood. One single dose of 120 mg testosterone undecanoate

was orally administered to six healthy volunteers. Those potential plasma and urinary markers studied are the following:

a) Urine

Steroid profile

Isotope ratio measurements of testosterone metabolites

b) Blood plasma

Direct detection of testosterone undecanoate

Steroid profile and gonadotrophins

Extent of glucuronidation

c) Dried blood spots of whole blood on filter paper

Direct detection of the ester

Extent of glucuronidation

Due to the work still undergoing, this presentation will summarise partial results of the study.

Experimentals

Clinical protocol and sample collection

Six spanish healthy volunteers (Mean \pm SD; 27.2 ± 2.1 years; 73.4 ± 4.0 kg; 1.75 ± 0.03 m) were administrated with one dose of 3 x 40 mg testosterone undecanoate (Androxon; Organon, Asker, Norway) orally.

Plasma A volume of 10 ml of venous blood from antecubital vein were collected, plasma was separated by centrifugation at 4°C and frozen at -20°C until analysis. Blood samples were collected - 72, -48, -24 hours before administration, -0.5, 0 (09:00 am), 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 hours on administration day (day 1), and at 0 h on days 2, 3, 4, 5, 7 after administration .

Dried blood spots on filter paper Before each whole blood sample was centrifuged , aliquots of 20 μ l of fresh blood were spotted on filter paper (Whatman 41#) with a pipette , dried in the air , sealed in plastic bags and stored at -20°C until analysis.

Urine The night urine was collected on the 3 days before administration. Urine samples were collected from 0~4, 4~8, 8~12, 12~24 hours on administration day (day 1), from 0~12, 12~24 hours the next day (day 2) and night urine on days 3, 4 and 6. The volume of each urine was measured and an aliquot of 50 ml was frozen at -20°C until analysis.

Sample preparation

Urine

Urine steroid profiles were analysed by GC/MS according to the routine screening procedure for antidoping testing (10). Briefly, 2.5 ml of urine were extracted in Detectabuse™ Column (Biochemical Diagnostics Inc., NY) previously washed with methanol and water. Steroids were eluted with methanol, then the solvent was evaporated and the hydrolysis was carried out with 5000 IU of β -glucuronidase from *E. coli* (k12) in 1 ml of phosphate buffer (0.2 M, pH=7) for 1 h at 55°C. After cooling, the pH was adjusted to 9-10 and steroids were extracted with 5 ml t-butylmethylether. The organic solvent was evaporated to dryness and the samples were kept in a desiccator for at least 30 min before derivatization. Methyltestosterone, [2, 2, 4, 4-²H₄]-etiocholanolone, [13,16,17-²H₃]-testosterone, [13,16,17-²H₃]-epitestosterone and [2, 2, 4, 4-²H₄]-11 β -hydroxyandrosterone were used as internal standards. In each analytical batch, a calibration solution of distilled water (including androsterone, etiocholanolone, testosterone, epitestosterone, DHT, estradiol, 3 α ,5 β -androstandiol, 3 α ,5 α -androstandiol, and other steroids) was treated, derivatised like urine samples and injected into GC/MS system to calculate response factor to quantify each substance (3).

Plasma

Method 1: For extraction of plasma samples, a method similar to others (11, 3) described in the literature was used with modification. Frozen plasma sample (1 ml) was allowed to stand at room temperature for thawing. Testosterone phenylpropionate (TPh) was added at a final concentration of 4 ng/ml as internal standard. Immediately after subsequent addition of 50 μ l of 3 M KOH, the samples were extracted twice with 4 ml of n-hexane: ethyl acetate (7: 3) mixture using a rocking mixer for 20 min. The organic phases were pipetted out in a tube, mixed and washed with 1 ml of 5% acetic acid and then with 1 ml of distilled water. After evaporating the solvent, the residue was kept in a desiccator at least 30 min before derivatization. Quantitative analysis of testosterone undecanoate (TU) were performed by GC/MS/MS using a calibration curve obtained by adding known amounts of TU to 1 ml of drug-free plasma at a final concentration of 0.5, 1, 5, 10, 50, 100 ng/ml.

Method 2: 1ml of plasma sample was diluted with 1.5 ml phosphate buffer (0.2 M, pH 7) and treated like the procedure for urine samples described above, with the modification that,

before hydrolysis, the free fraction of steroids in plasma was extracted with 5 ml t-butyl-methyl ether, evaporated with nitrogen and kept in a desiccator until derivatization. The aqueous phase was hydrolysed and extracted like the urine samples. Quantitation of steroids (including 17aOHP) in both fractions was performed by the response factor of a calibration sample similar to that for urine analysis.

Method 3: Follicle stimulating hormone (FSH), luteinizing hormone (LH) were analysed by MEIA (Abbott, Co.) directly in plasma following manufacturer's instructions, and sex hormone-binding globulin (SHBG) was measured by immunoflorescence assay.

Dried blood spots on filter paper

Two spots of dry blood on filter paper were cut into small pieces to a tube. 1 ml of phosphate buffer (0.2 M, pH 7) was added. Then a similar procedure for the extraction of plasma samples described above (**Method 1** for plasma sample) was used for the extraction of testosterone undecanoate and free fraction of steroids from blood spots on filter paper, with the modification of using $^2\text{H}_3$ -testosterone (T-D₃) and $^2\text{H}_4$ -etiocholanolone (Et-D₄) as additional internal standards with final concentrations of 0.9 ng/ml and 5 ng/ml in buffer, respectively. Then the aqueous phase was incubated with 30 μl of β -glucuronidase at 37°C overnight after adding internal standards, and extracted with t-butylmethyl ether for those conjugated fraction of steroids.

Endogenous steroids (testosterone, androsterone and etiocholanolone) both in free and conjugated fractions from whole blood were quantified by a response factor calculated from an extracted sample of distilled water containing 1.2 ng/ml testosterone, 20 ng/ml androsterone and 20 ng/ml etiocholanolone.

Derivatization

The trimethylsilylation of steroids in extracts of urine, plasma and whole blood spots on filter paper was carried out by dissolving in 50 μl of MSTFA/ NH_4I / 2-mercaptoethanol (1000: 2: 6) and heating at 60°C for 30 min. 1 μl ~2 μl of each solution were directly analysed by GC/MS or GC/MS/MS.

Instrumentation

GC/MS

For analysis of endogenous steroid profile in urine, plasma, and whole blood, a Hewlett-Packard 5890 II GC model fitted with a HP 7673A autosampler was connected to a mass selective detector HP 5970. The separation was carried out by a methylsilicone fused silica capillary column (HP Ultra-1, length 17 m, ID 0.2 mm, film thickness 0.11 μm) with an oven temperature programme: From an initial temperature of 180°C to 230°C with a rate of 3.0 °C/min, then to 310°C with a rate of 40 °C/min and kept for 3 min. Helium was used as carrier gas with a flow rate of 0.8 ml/min. The injector (operated in split mode 10:1) and the interface were maintained both at 280°C. The mass spectrometer was operated in selected ion monitoring (SIM) acquisition mode with one or more ions selected for each substance.

GC/MS/MS

For the detection of testosterone undecanoate from plasma and whole blood spots, a Finnigan GCQ ion trap mass spectrometer was connected with a GCQ gas chromatograph equipped with a model A200S autosampler. A HP Ultra1 capillary column (19 m, 0.2 mm, 0.11 μm) was used for the separation with an oven temperature programme: from 180°C (initial temperature, kept for 0.5 min) to 210°C with a rate of 30 °C/min, then to 230°C with a rate of 4 °C/min, and finally to 315°C with a rate of 40 °C/min (kept for 7 min). The injector (in split mode 15:1) and the transferline were maintained at 290°C, 300°C respectively. Helium was used as carrier gas with a flowrate of 0.8 ml/min at 180°C. Ion trap detector was operated in selected reaction monitoring (SRM) mode with a ion source temperature of 200°C. Molecular ions of testosterone phenylpropionate (ISTD, m/z 492) and testosterone undecanoate (m/z 528) were chosen as parent ions and a collision energy of 1.3 volts was used for both ions. Product ions were 209, 464 ($\text{M}^+ - 28$), 477 ($\text{M}^+ - 15$) for TPh and 209, 500 ($\text{M}^+ - 28$), 513 ($\text{M}^+ - 15$) for TU, respectively.

GC/C/IRMS measurements

Urinary samples for gas chromatography / combustion / isotope ratio mass spectrometry measurements are prepared by a procedure similar to the screening of urinary steroid profile by GC / MS, with the modifications that n-pentane was used as extracting instead of t-butylmethyl ether, acetylation was carried out instead of trimethylsilylation, then the

derivatization mixture was taken to dryness and reconstituted in cyclohexane for analysis. A Supelco SPB 50 capillary column (50% phenylmethylsilicone, 30 m, 0.25 mm, 0.25 μ m) was installed in a Hewlett-Packard 6890 GC, which was connected to a isotope ratio mass spectrometer (Finnigan Delta^{plus}) through a combustion interface (Finnigan GC combustion III). The oven temperature programme: initial temperature 150°C (kept for 1 min), 20°C/min up to 260°C, 1.5 °C/min to 280°C, then 2 °C/min to 290°C (kept for 4.5 min), 5 °C/min to 300°C. The injector was maintained at 280°C and helium was used as carrier gas at a flow rate of 1.5 ml/min. The oxidation and reduction reactors were maintained at 940°C and 600°C, respectively. δ values of urinary endogenous steroids, such as pregnandiol (P2), 5 β -androstan-3 α ,17 β -diol (5 β Diol), and so on were obtained.

Results

Urine

Steroid profile

The steroid profile of T, E, androsterone (A), etiocholanolone (Et), 11OH-androsterone (11OHA), 11OH-etiocholanolone (11OHEt), DHT, 5 β -androstan-3 α ,17 β -diol (5 β Diol) and 5 α -androstan-3 α ,17 β -diol (5 α Diol) were obtained from the urine samples of the six volunteers after the oral administration of testosterone undecanoate. All the monitored steroids showed a fluctuating increase in 0~4 h urine for five volunteers. In 8~12 h urine all of them returned to basal levels. Urinary ratios T/E, A/E, Et/E showed remarkable increase during 6 h after administration. Ratios of 5 α / 5 β metabolites of testosterone: A/Et, 11OHA/11OHEt and 5 α Diol/5 β Diol were decreased to some extent after exogenous testosterone intake.

One volunteer (vol 23) showed T/E lower than six even after the dosing. However, the A/E and Et/E were found increased stronger than in other volunteers. The average recovery of testosterone undecanoate from 0~12 h urine of the six volunteers was (40.4 \pm 7.0)%. The urinary T/E ratios were shown in Figure 1.

IRMS measurements

The IRMS data obtained from one volunteer (vol 24, Figure 2) showed testosterone intake can be detected until at least 24 h according the reference cut-off value (5 β Diol/P2 >1.10) suggested by literature (12), which agree with the result obtained from the urine after an oral administration of a single dose of 40 mg testosterone undecanoate (13).

Plasma

Testosterone undecanoate

The analysis of plasma samples of four out of six volunteers has already been carried out. All the four volunteers showed very fast change of TU concentrations during 6 hours after administration. The highest plasma TU concentration with a mean value of 88 ng/ml for three volunteers were found 3 h after administration. Testosterone undecanoate can be detected from 1~1.5h to 4~6h after administration. For vol 23 (urinary T/E < 2 during the controlled study), the detectable point began at 1 h and the highest concentration of TU was reached (about 11 ng/ml) at 2 h, one hour early than others. Direct detection of testosterone undecanoate in these four volunteers provide unequivocal indication of exogenous intake and the detectability time is up to 6 h after dosing (Figure 3).

Steroid profile (free and glucuronide fractions)

Usually the basal level of testosterone glucuronide (TG) in plasma is much lower compared with free testosterone. Free testosterone measured in plasma after oral administration of T undecanoate does not show significant changes (Figure 4a). Instead, testosterone glucuronide measured in plasma suffered remarkable increase until 8 h after administration. Similar results for the respective glucuronides occurred for other important metabolites of testosterone like A, Et, 5 α Diol, 5 β Diol, DHT and E₂. Thus hydrolysis of plasma sample played an important role in the interpretation of the plasma steroid profile. Increased conjugated steroids appear to be a manifest proof of exogenous testosterone intake. TG/T (Figure 4b, obtained from vol 22 for the ratio between glucuronide fraction and free fraction), AG/A and EtG/Et (data not shown) also keep elevated up to 6~8 h after administration.

LH, SHBG, FSH and 17 α OHP

Plasma SHBG, FSH and LH were measured. No changes in FSH and SHBG were observed. LH (Figure 5a, for vol 22), with high fluctuation, was slightly inhibited after TU administration. 17 α OHP was also decreased after exogenous T intake (Figure 6a). Here again, TG/LH and TG/17OHP were found significantly increased up to 8 h and 12 h after administration, respectively, but not T/LH nor T/17 α OHP (Figure 5b and Figure 6b).

Dried blood spots on filter paper

Several samples of blood spots on filter paper from one volunteer have been analysed. The preliminary results showed that with only 40 μ l of whole blood, testosterone undecanoate can be detected by GC/MS/MS method. The highest concentration of TU was found at 3 h after the administration in whole blood just as found in plasma. Figure 7 shows the chromatograms obtained from one sample of dried blood spots.

Conjugated and free fractions of testosterone have been measured for those dried blood spots from two volunteers (Figure 8a). As obtained in plasma, testosterone glucuronide increased significantly after oral intake of testosterone undecanoate. TG/T (shown for two volunteers in Figure 8b) remained increased until 8 h after administration.

Discussion

From the result obtained, the steroid profile either in urine or in plasma suffered fluctuating changes after oral administration of testosterone undecanoate, which agree with results obtained by a long-term study of oral testosterone undecanoate administration (14). Among the six subjects, three of them showed T/E higher than 6 until 4~8h urine, two of them only in 0~4h urine, and one subject never reached the cut-off value. As a marker for doping control, T/E ratio was a sensitive but short-lasting parameter, showing inter-individual differences.

On the other hand, the presence of testosterone undecanoate in plasma for the four subjects analysed can be detected from 1~1.5h to 4~8h, including even the subject never reaching high urinary T/E ratio. Detectability of esters in plasma offered unequivocal demonstration of testosterone intake. Regarding the presence of testosterone glucuronide, plasma TG/T in the only subject studied remained elevated until 8 h, as observed in a study of oral administration of both testosterone undecanoate and epitestosterone undecanoate (15). In order to compare the oral route with the non-oral route of administration, the plasma samples from the same volunteer after intramuscular injection with a single dose of 25 mg TP+110 mg TE have been analysed for TG and T concentrations. Free testosterone increased until 10 days while no significant change was observed in the fraction of conjugated testosterone. It is suggested that TG/T in plasma may be a selective marker for oral testosterone administration.

The changes of hypothalamic-hypophysary-gonadal axis were not remarkable as judged by LH level after oral administration of testosterone undecanoate. However, TG /LH was

elevated until 8 h after administration and TG/17aOHP was increased even more significantly.

The use of small amount of whole blood dry spots on filter paper has the advantage of being less invasive and could be a good alternative for plasma. It is more convenient for sampling, transporting and storage. In the present study testosterone undecanoate and increased TG/T can be detected in 40 µl of whole blood spotted on filter paper. However for the use of this technique as a real sampling procedure (e.g. fingerprick or earprick), the usefulness of capillary blood, as compared with venous blood (studied in the present work) should be verified.

The sample preparation procedure for IRMS measurement in the present study is practical regarding the low volume of urine needed and δ values of many endogenous steroids obtained at the same time in one extract.

Conclusion

Among those parameters evaluated in our study after oral administration of testosterone undecanoate, the ratio of δ values of urinary 5 β Diol/P2 by IRMS and detection of testosterone ester in plasma or whole blood provide direct proof of testosterone intake. IRMS measurement prolonged the detectability.

Plasma TG/T, TG/LH and TG/17aOHP are good, and probably selective, markers of oral administration of testosterone undecanoate.

Whole blood spotted on filter paper (simple way of sampling, low volume, easy-transporting and less invasive) can be a promising alternative to blood plasma for detecting unchanged testosterone ester or for the ratio of testosterone glucuronide to unconjugated testosterone after oral testosterone administration.

References

1. Donike M, Barwald KR, Klostermann K, Schänzer W, Zimmermann J, Nachweis von exogenen Testosteron. In Sport: Leistung und Gesundheit, ed. Heck H, Hollmann W, Liesen H, Rost R. Köln: Deutscher Arzte Verlag, 1983, 293
2. Geyer H, Schänzer W, Mareck-Engelke U, Donike M, Factors influenceing steroid profile. In Recent Advances in Doping Analysis (3), ed. Donike M, Geyer H, Gotzmann A, Mreck-Engelke U. Köln: Sport und Buch Strauß, 1996

3. De la Torre X, Segura J, Yang ZY, Ye L, Wu M. Testosterone detection in different ethnic group. In: Recent advances in doping analysis (4). Schänzer W, Geyer H, Gotzman A, Mareck-Engelke U (Eds.) Sport un Buch Strau ß Köln, P71-89, 1997
4. Southan G, Mallet A, Jumeau J, Craig S , Poojara N, mitchell D, Wheeler M, Brooks RV. Programme and Abstracts of the Second international Symposium on Applied Mass Sepectrometry in Health Sciences, Barcelona. P306, 1990
5. Becchi M, Aguilera R, Farizon Y, Flament MM, Casabianca H, James P. Gas Chromatography / combustion / isotope ratio mass spectrometry analysis of urinary steroids to detect misuse of testosetrone in sport. Rapid Comm Mass Spectrom, 8: 304-308, 1994
6. De la Torre X, Segura J, Poletini A, and Montagna M. Detection of testosterone esters in human plasma. J. Mass Spectrometry, 30: 1393-1404, 1995
7. Kickman AT, Brooks RV, Collyer SC, Cowan DA, Nanjee NN, Southan GJ, Wheeler MJ. Criteria to indicate testosterone administration. Br. J. Sports Med. 24: 253-264,1990
8. Carlström K, Palonek E, Garle M, Oftebro H, Stanghelle J And Bjökhem I. Detection of testosterone administration by increased ratio between serum concentrations of testosterone and 17 a-hydroxy progesterone. Clin. Chem. 38 (9): 1779-1784,1992
9. Howe CJ and Handelsman DJ. Use of filter paper for sample collection and transport in steroid Pharmacology. Clin. Chem.43 (8): 1408-1415, 1997
10. Segura J, Pascual JA, Ventura R, Ustaran JI, Cuevas A, Gonzalez R. Clin Chem. 39: 836-845 (1993)
11. Shinohara y, Fujioka M and Baba S. Pharmacokinetic studies of testosterone propionate using gas chromatography/mass spectrometry/ selected ion monitoring. Biomed,. Envir. Mass Spectrom., 16: 241-244, 1988
12. Shackleton CHL, Phillips A, Chang T, Li Y. Confirming testosterone administration by isotopic ratio mass spectrometric analysis of urinary androstenediol, Steroids, 62: 379-387,1997
13. Horning S, Geyer H, Machnik M, Schäzer W, Hilkert A, Oeßelmann J, Detection of exogenous testosterone by $^{13}\text{C}/^{12}\text{C}$ analysis. In: Recent advances in doping analysis (4). Schänzer W, Geyer H, Gotzman A, Mareck-Engelke U (Eds.) Sport un Buch Strau ß Köln, P275-283, 1997
14. Wright F, Lafarge JP, Antréassian J, Lagoguey M and Péres G. Long term study of steroid and peptidic hormones in the plasma of healthy young men under controlled testosterone

undecanoate therapy. In: blood samples for doping control. Peter Hemmersbach and Kare I. Birkeland (Eds.) , Pensumtjeneste, Norway, 65-74, 1994

15. Dehennin L and Pérès G . Plasma and urinary markers of oral testosterone misuse by healthy men in presence of masking epitestosterone administration. *Int J. Sports Med* , 17: 315-319, 1996

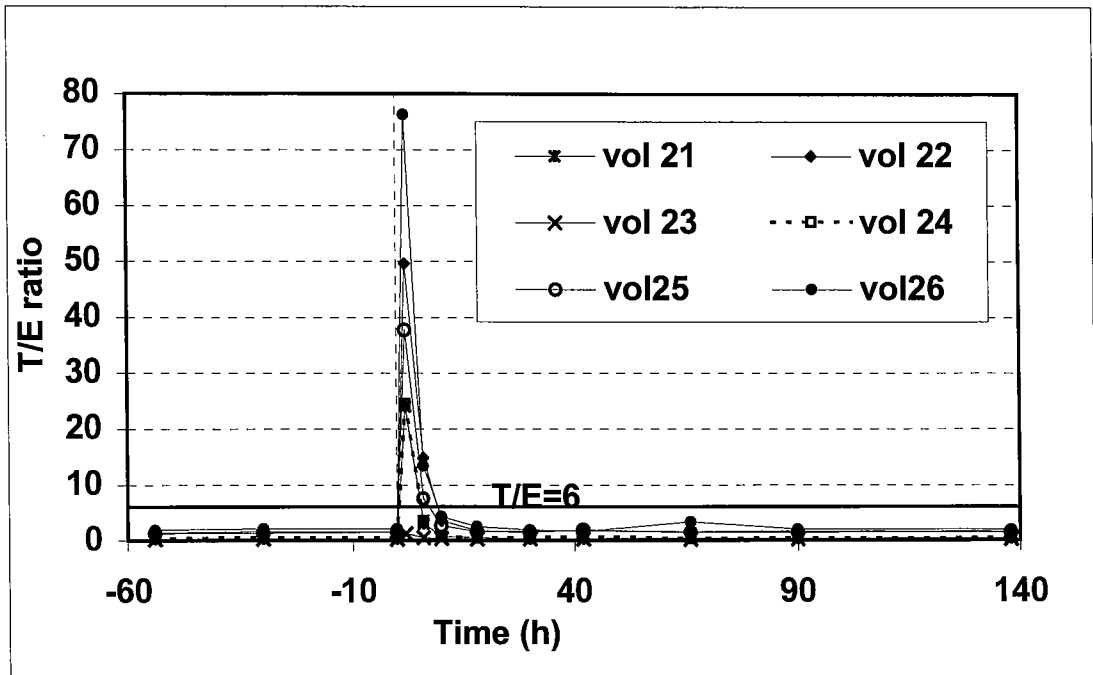


Figure 1: Urinary T/E ratios of six volunteers after oral administration of 120 mg testosterone undecanoate

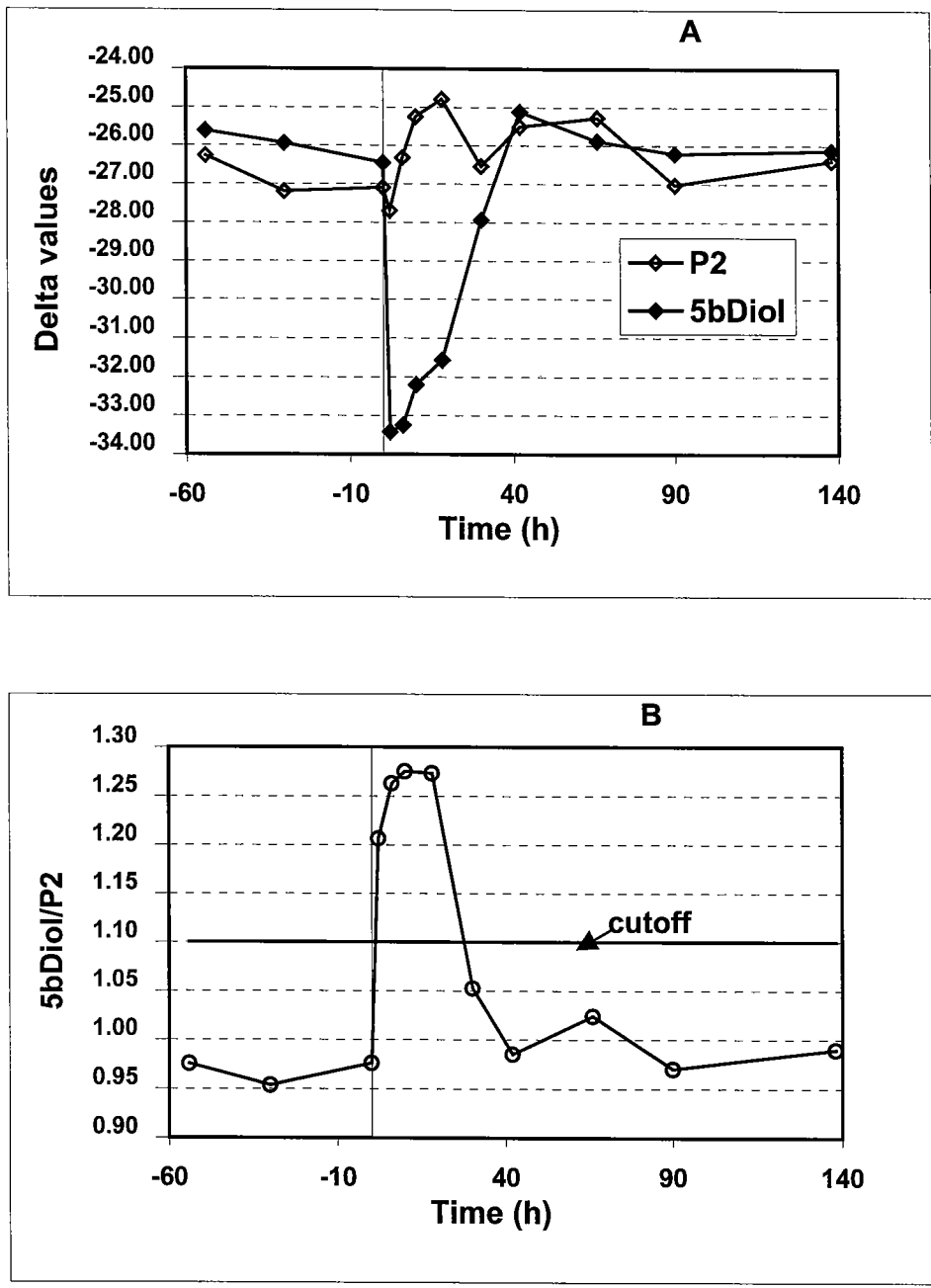


Figure 2: $\delta^{13}\text{C}$ ‰ values (A) and their ratios(B) of 5b-androstandiol (5bdiol) and pregnandiol (P2) obtained from urine samples of one volunteer (volunteer 24) after oral administration of 120 mg testosterone undecanoate

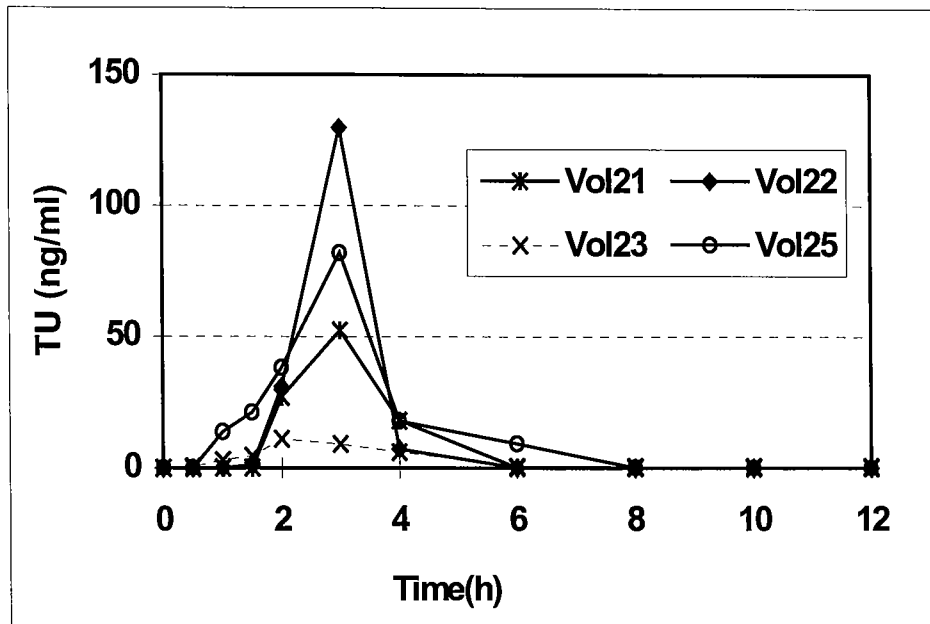


Figure 3: Time course of testosterone undecanoate (TU) in plasma after oral administration of 120 mg testosterone undecanoate

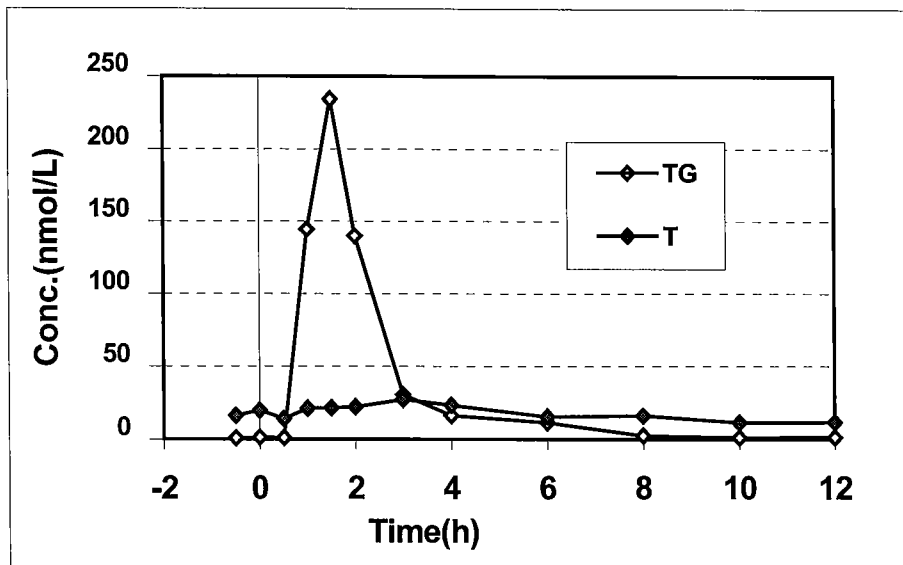


Figure 4a : Testosterone glucuronide(TG) and free testosterone (T) in plasma of volunteer 22 after oral administration of 120 mg testosterone undecanoate

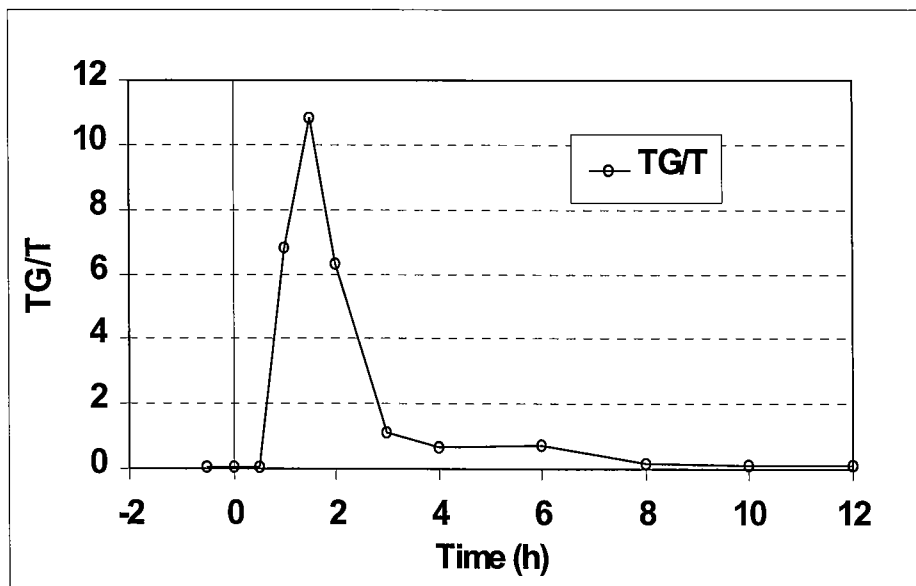


Figure 4b: Plasma TG/T of volunteer 22 after oral administration 120 mg of testosterone undecanoate

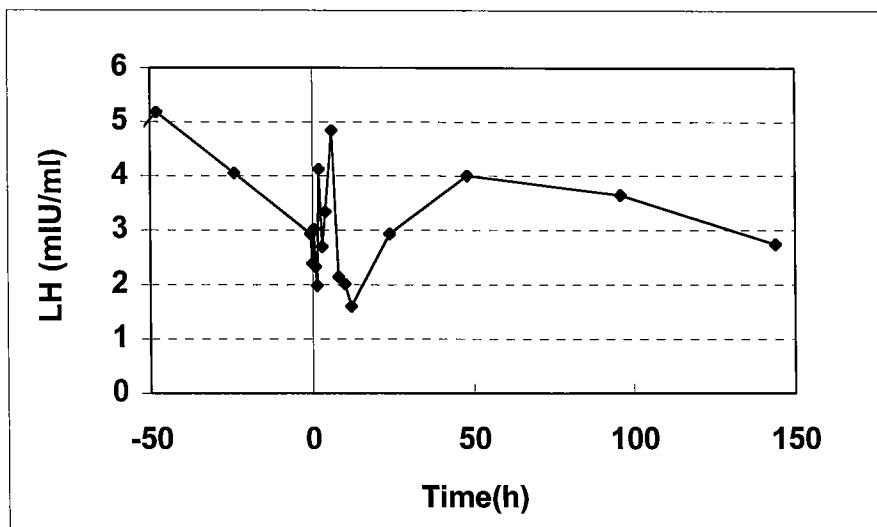


Figure 5 a : Plasma LH concentrations of volunteer 22 after oral administration of 120 mg testosterone undecanoate

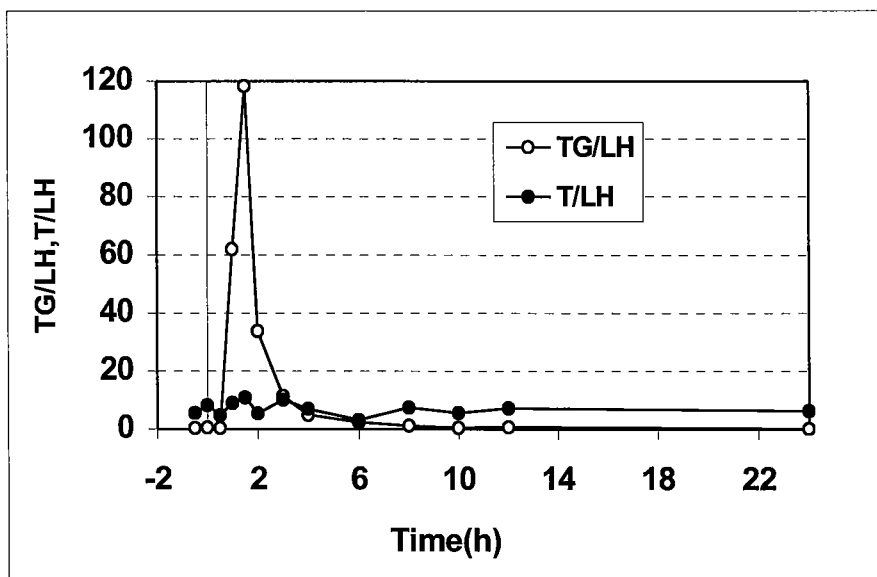


Figure 5b : Plasma TG/LH and T/LH ratios of volunteer 22 after oral administration of 120 mg testosterone undecanoate

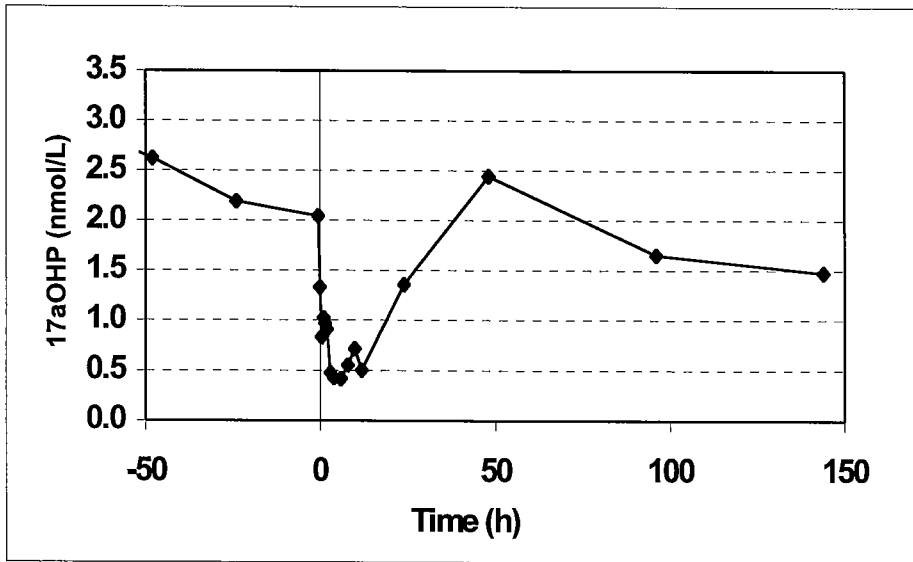


Figure 6a : Plasma 17a-hydroxyprogesterone concentrations of volunteer 22 after oral administration of 120 mg testosterone undecanoate

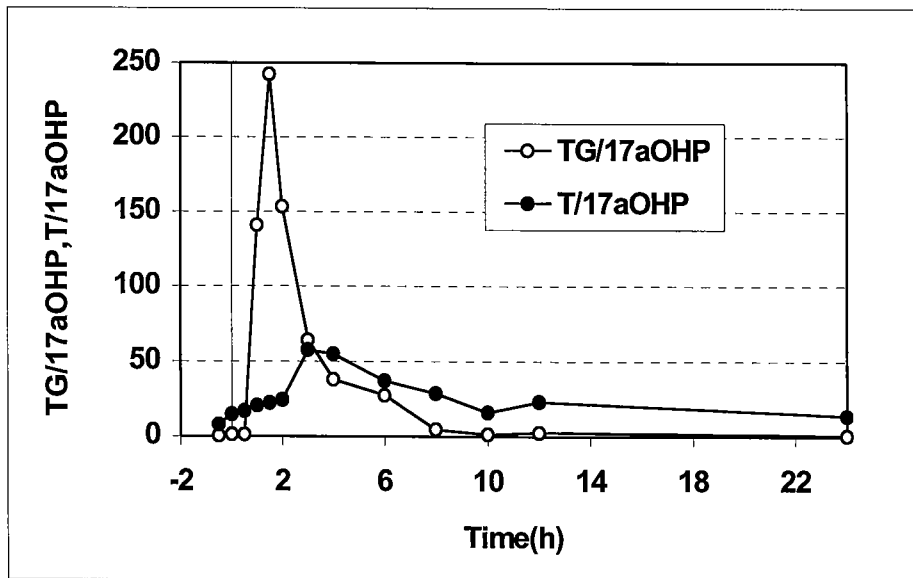


Figure 6b. Plasma TG/17aOHP and T/17aOHP of volunteer 22 after oral administration of 120 mg testosterone undecanoate

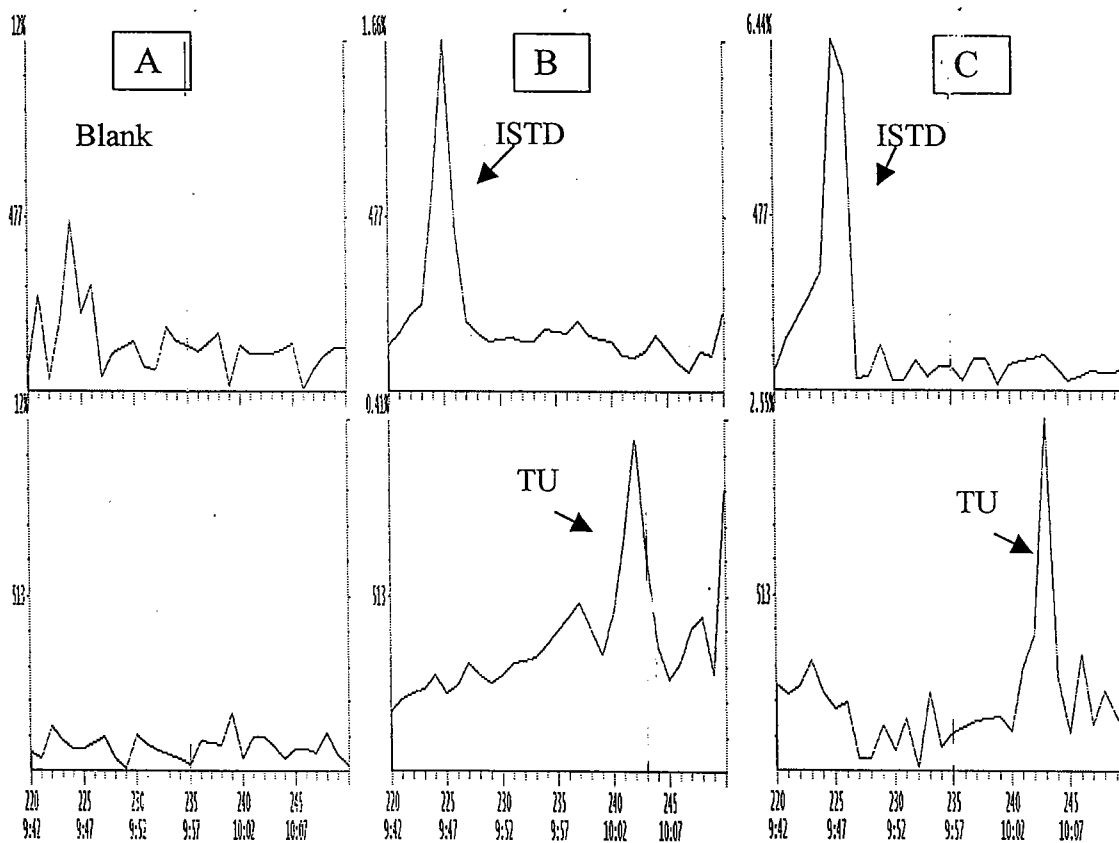


Figure 7 : Chromatographic profiles obtained by GC/MS/MS analysis of blood spot samples (ion chromatograms for TPh-TMS at m/z 477 and for TU-TMS at m/z 513) (A) Blank blood spots; (B) Blood spots spiked with TPh(ISTD) and TU; (C) Blood spots collected 2h after oral administration of testosterone undecanoate from volunteer 21.

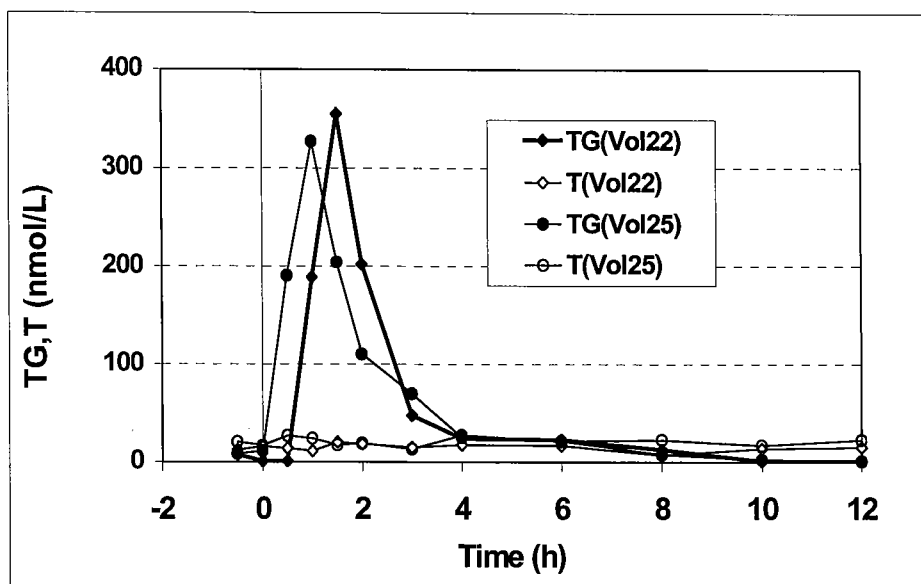


Figure 8a : Concentrations of testosterone glucuronide and free testosterone in dried blood spots from two volunteers after oral administration of 120 mg testosterone undecanoate

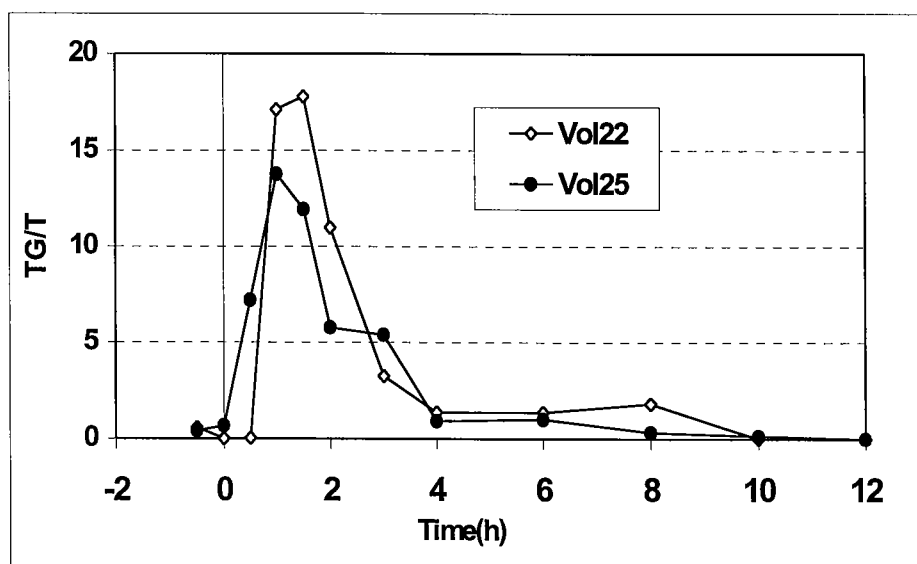


Figure 8b: Time course of TG/T obtained from dried blood spots of two volunteers after oral administration of 120 mg testosterone undecanoate.