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RECENT ADVANCES IN DOPING ANALYSIS (6)

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V.P. URALETS, P.A. GILLETTE, R.K. LATVEN:
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Over-the-Counter Anabolic Steroids 4-Androsten-3,17-dione; 4-Androsten-3β,17β-diol, and 19-Nor-4-androsten-3,17-dione: Excretion Studies in Men.

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Introduction

4-Androsten-3,17-dione (androstendione), 4-androsten-3β,17β-diol (androstendiol), and 19-nor-4-androsten-3,17-dione (norandrostendione) are at this time available over-the-counter in the United States as nutritional supplements for the enhancement of physical performance. Their structures are shown in figure 1. Chemically and pharmacologically, these substances belong to the class of androgenic anabolic steroids. According to general rules of human steroid metabolism (1-6), they should rapidly convert in the body to the potent anabolics testosterone or nandrolone, which are controlled substances. Consequently, they are banned by the International Olympic Committee (IOC) and major sports federations, which define them as performance enhancing anabolic agents.

Androstendione was first advertised in early 1997, in bodybuilding magazines, as a natural muscle building supplement. Since that time, we have seen evidence of its use by athletes engaged in "power" sports. Androstendione, as an anabolic and endurance promoting agent, became an attractive, legal alternative to testosterone for athletes, and an athletic enhancer for the sporting public. It was not specifically listed as a controlled substance, because androstendione had no pharmacological use at that time. Two newer " supplements", androstendiol and norandrostendione are now also in use, as we have observed by a characteristic deterioration of endogenous steroid profiles in routine testing.

Detection of banned synthetic anabolic steroids, their metabolites, and testosterone in athletes' urine is well developed (7, 8). It has become an efficient tool to deter the abuse of regular anabolic

steroids in sports. As a result, abuse of some unusual endogenous and designer steroids has become more frequent. These are difficult to detect, and are not specifically listed as controlled substances in the United States.

We performed human excretion studies with the three over-the-counter steroids under investigation here, to observe their effects on steroid profiles, in order to explore the possibility of detection. The detection of the abuse of endogenous steroids like testosterone, 5α-dihydrotestosterone, and dehydroepiandrosterone has been an analytical challenge in doping control. Their detection is currently based on the changes in relative ratios of some urinary steroids (9-14), or more recently, on the difference in carbon isotope ratio of endogenous from exogenous steroids, which are synthesized from vegetable material (15-20).

Experimental

Over-the-counter steroids

4-Androsten-3,17-dione (*Androsten)* 50 mg capsules and 4-androsten-3β,17β-diol (*Androdiol*) 100 mg capsules were purchased from OSMO™, San Francisco, California. 19-Nor-4-androsten-3,17-dione (*Norandro)* 50 mg capsules were purchased from So Cal Sports Supplements, La Jolla, California.

Excretion studies

Five healthy male subjects volunteered for this study. During the study, they were engaged in normal physical activity. Oral administration of each of the three drugs was performed once for each individual. Drug administration was separated in time by a period of from several weeks, to months. Urine specimens were collected before and after drug administration at each urine void for 24 hours, and in larger time intervals after that. Norandrostendione excretion specimens were collected for up to 14 days. Upon collection, sodium azide was added to urine as a preservative. Urine specimens were stored in a refregirator at 4°C prior to extraction.

Reference steroids

Major endogenous reference steroids were obtained from Sigma and Research Plus, Inc. Reference

4-androsten-3,17-dione was from Sigma; 4-androsten-3β,17β-diol from Steraloids, Inc.; 4-estren-3,17-dione from Research Plus. 4-Androsten-3β-ol-17-one was from Research Plus, 4-androsten-3α-ol-17-one from Steraloids. Five different available hydroxy-4-androsten-3,17-diones were tested: 11α-ol and 11β-ol from Research Plus, and 6β-ol, 16α-ol, and 19-ol from Steraloids.

Urine sample preparation

To 4 mL urine in a glass tube, 1 mL of acetate buffer (pH 5.2)/ β-glucuronidase from *Helix Pomatia* / ISTD mixture is added and incubated for 3 hours at 52°C. After hydrolysis the tubes are centrifuged and the liquid is applied to C_{18} solid phase extraction columns (200 mg, Varian) prewashed with 3 mL methanol and 3 mL water. After urine passes through, the column is washed with 2 mL of 30% acetonitrile in water. Columns are dryed under vacuum. Steroids are eluted with 3 mL methanol. The methanolic eluate is evaporated to dryness.

The dry residue is derivatized with 75 µL of MSTFA/NH_4I/Dithioerythritol 1000:2:3 for 15 minutes at 70°C. Samples are vialed, 1 µL is injected into the GC/MS.

GC/MS parameters

GC/MS was HP 5890/5970. Column: HP-1 fused silica, crosslinked methylsilicon, 17m, 0.2 mm I.D., 0.11µ film thickness. Temperature programm: 180°C(0.3 min); 3°/min - 231°C; 30°C/min - 310°C (1.07 min).

Results and Discussion

Androstendione

Metabolism of androgenic anabolic steroids (21) in its final stages in man is shown in figure 2. According to the scheme, ingestion of androstendione will increase urinary concentrations of testosterone and its ultimate metabolites: androsterone, etiocholanolone, 5α- and 5β-androstandiols. Indeed, in our excretion study all male participants had approximately 100 times higher concentrations of androsterone and etiocholanolone in their urine shortly after oral administration of a 50 mg androstendione pill. Figure 3 compares two chromatograms of urinary steroids before

and after androstendione administration. Elevated steroid concentrations return to normal in less than 24 hours.

Concentrations of both testosterone and epitestosterone increase briefly (figure 4) for a typical man with initially normal testosterone/ epitestosterone urinary ratio (T/E). Testosterone rises faster than epi, which causes T/E to rise (figure 5) above the positive cutoff value of 6. In this way, androstendione may result in a positive anabolic steroid test. Most men respond to androstendione administration by increasing uranary testosterone excretion relative to epitestosterone, and, consequently, display an elevated T/E ratio.

Ethnic variability in normal excretion patterns of testosterone and epitestosterone is well known (22). Many Asian men have very low T/E ratios (0.1), with normal excretion of epitestosterone and below normal testosterone. On the other extreme, there are very few men with unusually high T/E. We tested the response of both to androstendione administration.

Figure 6 summarizes our data on the urinary excretion of major steroids affected by androstendione for three types: high T/E~4 (upper graphs), normal T/E~1.2 (middle), and low (Asian) T/E~0.1 (bottom). Concentrations of androsterone and etiocholanolone increase dramatically, shortly after androstendione administration for all three types of men, reaching their maximum after 3-6 hours, as shown in the left column of figure 6. Concentrations of testosterone rise from five to ten times for normal and high T/E persons (middle column, upper and middle graphs of figure 6) with epitestosterone rising at a slower rate. The T/E ratio increases to 6 for the normal man and to a much higher value (above 20) for the high T/E person (upper right graph of figure 6). For the low T/E person, however, the concentration of testosterone does not change significantly, which causes T/E ratio even to drop below its already low value (lower right graph, figure 6).

Significant increase in concentrations of other urinary steroids has been observed, including 11-hydroxy-androsterone and -etiocholanolone. Dehydroepiandrosterone, pregnandiol and corticosteroid levels remain unchanged. The increase of parent androstendione concentration in urine is not remarkably high.

A specific hydroxy metabolite of androstendione was found. Its exact structure has not been elucidated, mass spectrum is shown in figure 7. It does not match any of five hydroxyandrostendione reference standards, listed in "Experimental". This is a minor endogenous compound, the concentration of which rises sharply after androstendione administration.

In routine testing, androstendione affected profiles are easy to recognize by abnormally high concentrations of androsterone and etiocholanolone. High testosterone and elevated epitestosterone often make such urine specimens positive for testosterone (T/E>6), epitestosterone (above cutoff, 200 ng/mL), or both.

Because of the form (pill or capsule) in which androstendione is commonly consumed in its current popular form, there is some uncertainty as to its efficiency. Androstendione and its active metabolites are mostly destroyed in their initial pass through the liver, prior to distribution to the bloodstream and transfer to tissues, where they have their anabolic effect. This necessity, for the liver to metabolize supraphysiological doses prior to its effective absorbtion, may constitute some risk of liver damage.

Androstendiol

4-Androstendiol capsules (*Androdiol*), which we were able to puchase for this excretion study, contained mostly 4-androsten-3β,17β-diol with minor mixture of α- and β- isomers. Figure 8 shows typical steroid profiles for the normal T/E man before and after oral administration of androstendiol. The effect on steroid profile seems to be greater than that for androstendione. The proposed pathway of androstendiol is presented in figure 9. The major qualitative difference from androstendione is formation of two isomeric 3α- and 3β-hydroxy-4-androsten-17-ones, which have similar mass spectra shown in figure 10. These are intermediate products in the conversion of androstendiol into androstendione, testosterone and its ultimate urinary metabolites. This intermediate step slows down first pass metabolism, allowing a more efficient release of testosterone into the bloodstream. Participants in the excretion study reported a higher perceived anabolic effect than that associated with ingestion of androstendione.

Indeed, concentrations of testosterone in urine after administration of androstendiol are ten times higher than after androstendione. They are peaking at unbelievable 3000-5000 ng/mL levels, as can be seen in figure 11 (central column graphs), where concentratios of urinary steroids are presented before and after administration of a 100 mg androstendiol pill. Concentrations of ultimate metabolites androsterone and etiocholanolone (left column, figure 11) are of the same magnitude as was observed previously for androstendione (compare corresponding data in figure 6). Epitestosterone concentrations in the case of androstendiol administration (figure 11) reach above 1000 ng/mL, much higher than was observed for androstendione (fugure 6), and higher than the IOC positive cutoff value of 200 ng/mL. Relatively higher epitestosterone may be explained by a substancial presence of the 17α-ol isomer in the androstendiol pill. Consequently, in spite of higher release of testosterone after androstendiol ingestion, the rise in T/E is not as sharp as for androstendione. T/E for the normal man (middle right graph in figure 11) rises to only 2.5. The high T/E person, however, easily goes beyond (upper right graph) the T/E cutoff of 6. The asian man demonstrates a little increase in urinary testosterone (middle, bottom, figure 11), but the T/E time profile (bottom right) has a characteristic dive where androstendiol has its maximum effect on urinary steroids.

Signs of androstendiol administration mostly disappear after 20 hours. Some secondary metabolites may, however, remain elevated. Figure 12 shows a full scan urinary steroid profile 22 hours after androstendiol administration, with unusually high 5β-androstan-3β,17β-diol, comparable in abundance with androsterone and etiocholanolone.

Oral androstendiol is a potent anabolic agent, because it is an efficient precursor of testosterone. Androstendiol reveals itself in urine in a manner similar to androstendione, and can be distinguished from it by abundant characteristic metabolites: 3α- and 3β-hydroxy-4-androsten-17-ones.

Norandrostendione

19-Norandrostendione, as may be expected, follows a metabolic pathway similar to that already discussed for androstendione, except that niether it nor its metabolites are endogenous steroids. Two chromatograms of urinary steroids after oral administration of norandrostendione are shown in figure

13. The main excretion products are norandrosterone and noretiocholanolone, the same as those observed for common nandrolone (nortestosterone, controlled substance), because norandrostendione quickly converts to nortestosterone in the body. Concentrations of major metabolites in the first urine voids are unusually high (100,000 ng/mL). Such concentrations of urinary metabolites have never been seen for injectable oil based nandrolone esters, which by design release nandrolone slowly to create a strong and steady therapeutic effect for a long period of time. Norandrostendione impact is immediate and short. First pass metabolism deactivates most of the dosage before it reaches the bloodstream and target tissues, similar to androstendione. Norandrosterone and noretiocholanolone are detectable in urine 7-10 days after a single 50 mg oral dose of norandrostendione. Metabolites are virtually indistinguishable from nandrolone preparations, except for the first urine voids, when elevated metabolic concentrations reveal norandrostendione administration.

Minor metabolites, including nortestosterone and parent norandrostendione are well detectable during the first few hours after administration. Most are tentatively identified in figure 13 as norepitestosterone, norandrostandione, hydroxynorandrostendiones, hydroxynorandrosterone and hydroxynoretiocholanolone. Interestingly, dehydrogenated (-2H) norandrosterone and noretiocholanolone are found, which we previously detected (23) as nandrolone metabolites. One metabolite we tentatively identified as norandrostadiendione. Dehydrogenated nortestosterone is perhaps present as well. Dehydrogenation is most likely in position 1 of the steroid structure, which possibly is facilitated by the absense of the C_{19} methyl group.

The difference in excretion patterns of norepitestosterone and nortestosterone for low (upper chromatogram, figure 13) and normal (lower chromatogram) T/E persons is consistent with what we observed for androstendione. Nortestosterone is more abundant for the normal T/E person, epi - for the low T/E person.

Ethnic variability

Low concentrations of testosterone or nortestosterone in Asian male urine is evidently a result of rapid and complete conversion of these substances (but not epi isomers) to ultimate urinary

metabolites, due to higher specific enzymatic activity towards testosterone. Ethnic differences in enzyme activities, such as alcohol dehydrogenase for instance, are well known. Asian and Caucasian men have, in urine, comparable concentrations of androsterone and etiocholanolone, ultimate metabolites of testosterone, which allows the conclusion that the rates of testosterone production in these two groups are comparable. Additional testosterone from an external source such as testosterone enanthate intramuscular injection (22), or oral androstendione or androstendiol, is more rapidly eliminated by Asian men by means of complete conversion of testosterone prior to its release to urine. Time excretion curves of major steroids are noticeably narrower for Asian men, as shown by the bottom graphs in figures 6 and 11. A similar variation between Caucasian and Asian men was observed by De la Torre et al. (22) for plasma testosterone concentrations.

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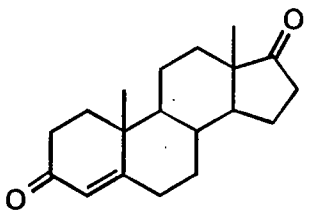
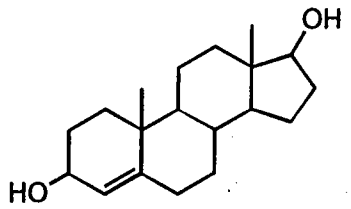


4-ANDROSTEN-3,17-DIONE



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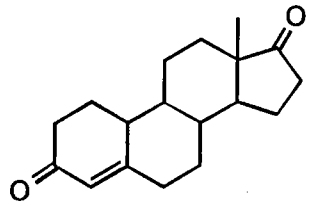


19-NOR-4-ANDROSTEN-3,17-DIONE

Figure 1.

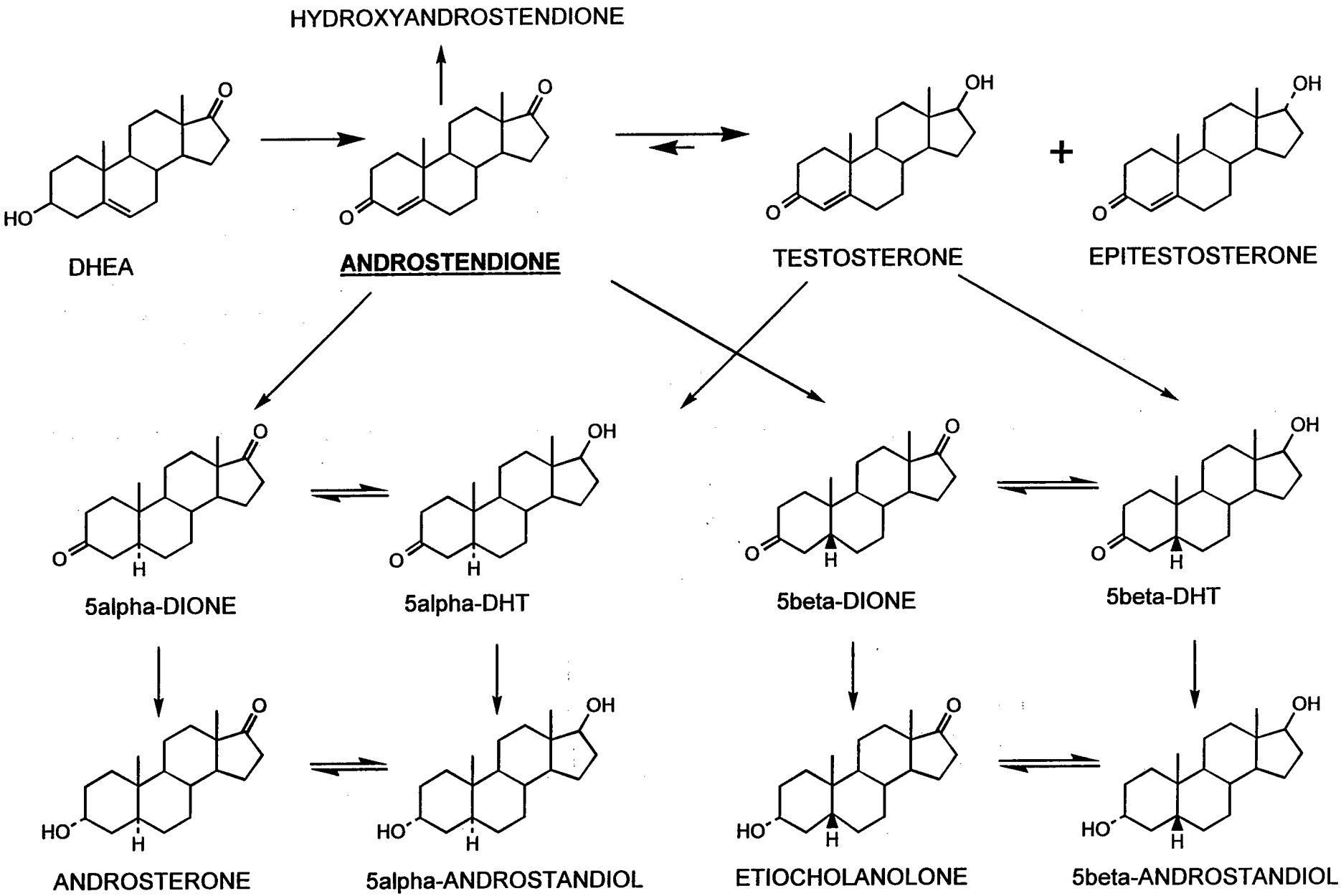


Figure 2. Metabolism of androgens (21).

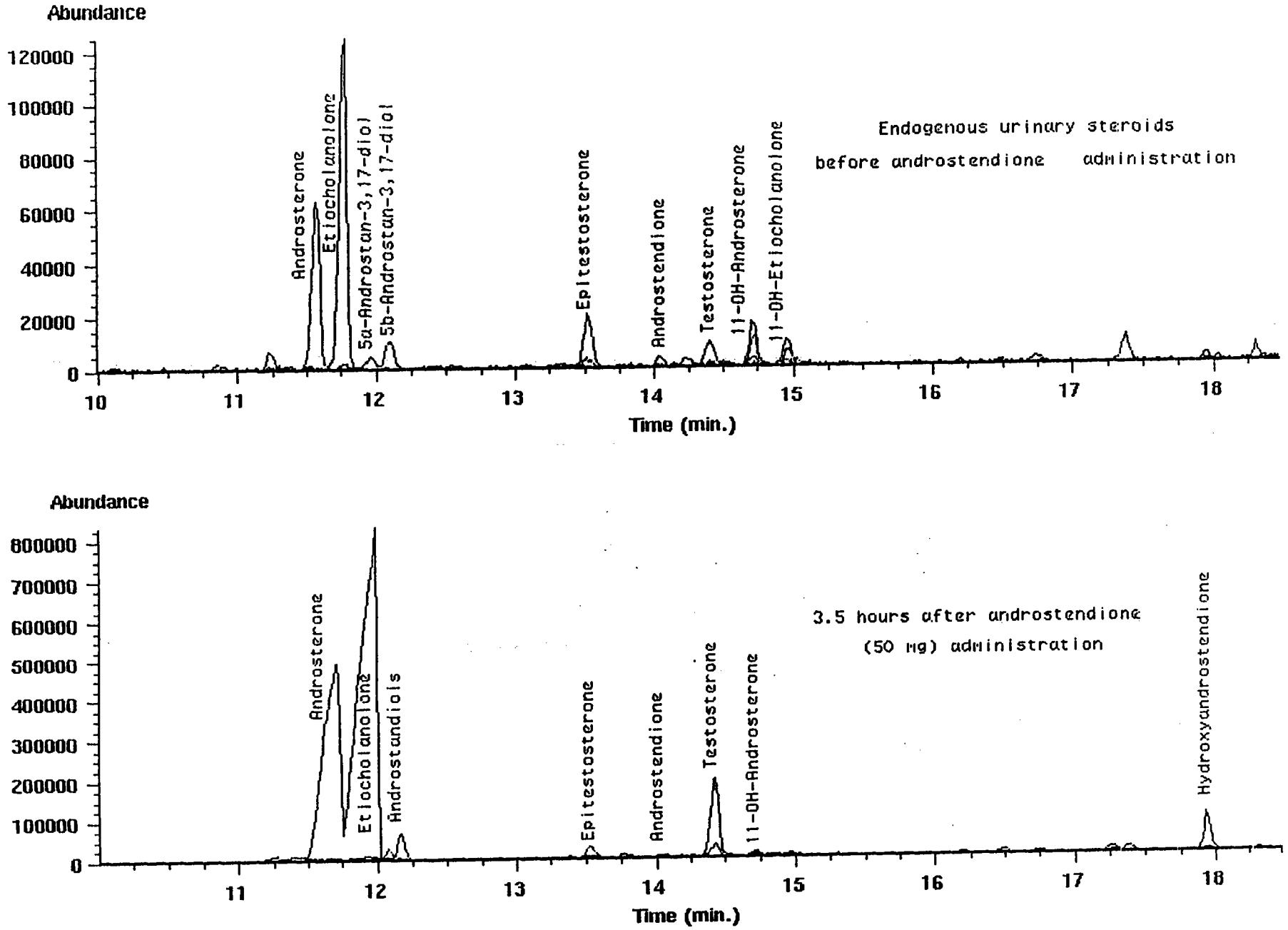


Figure 3. Urinary steroids before (top) and after (bottom) androstendione (50mg) administration.

Figure 4. Urinary concentrations of testosterone and epitestosterone after 50 mg of androstendione

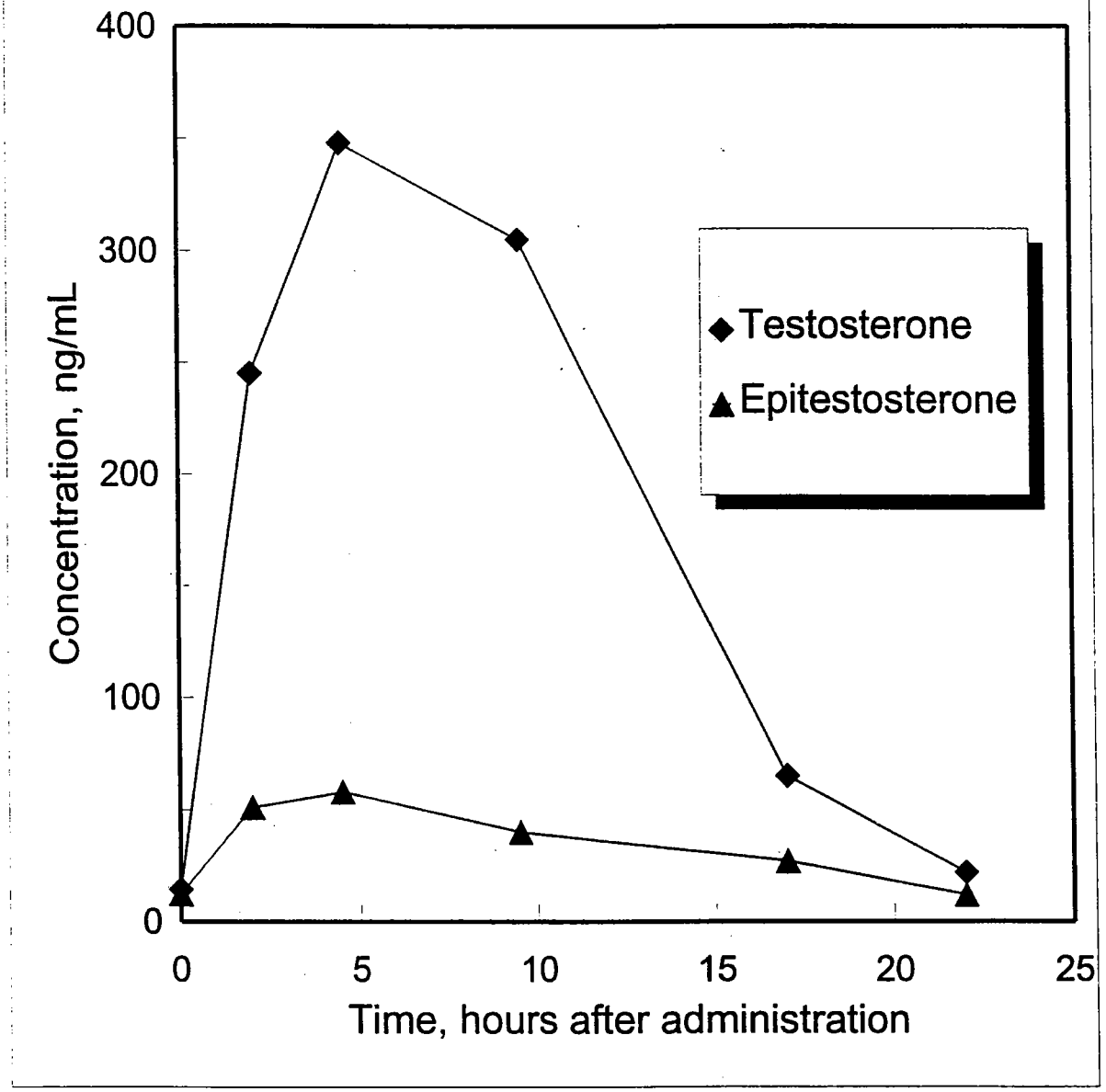
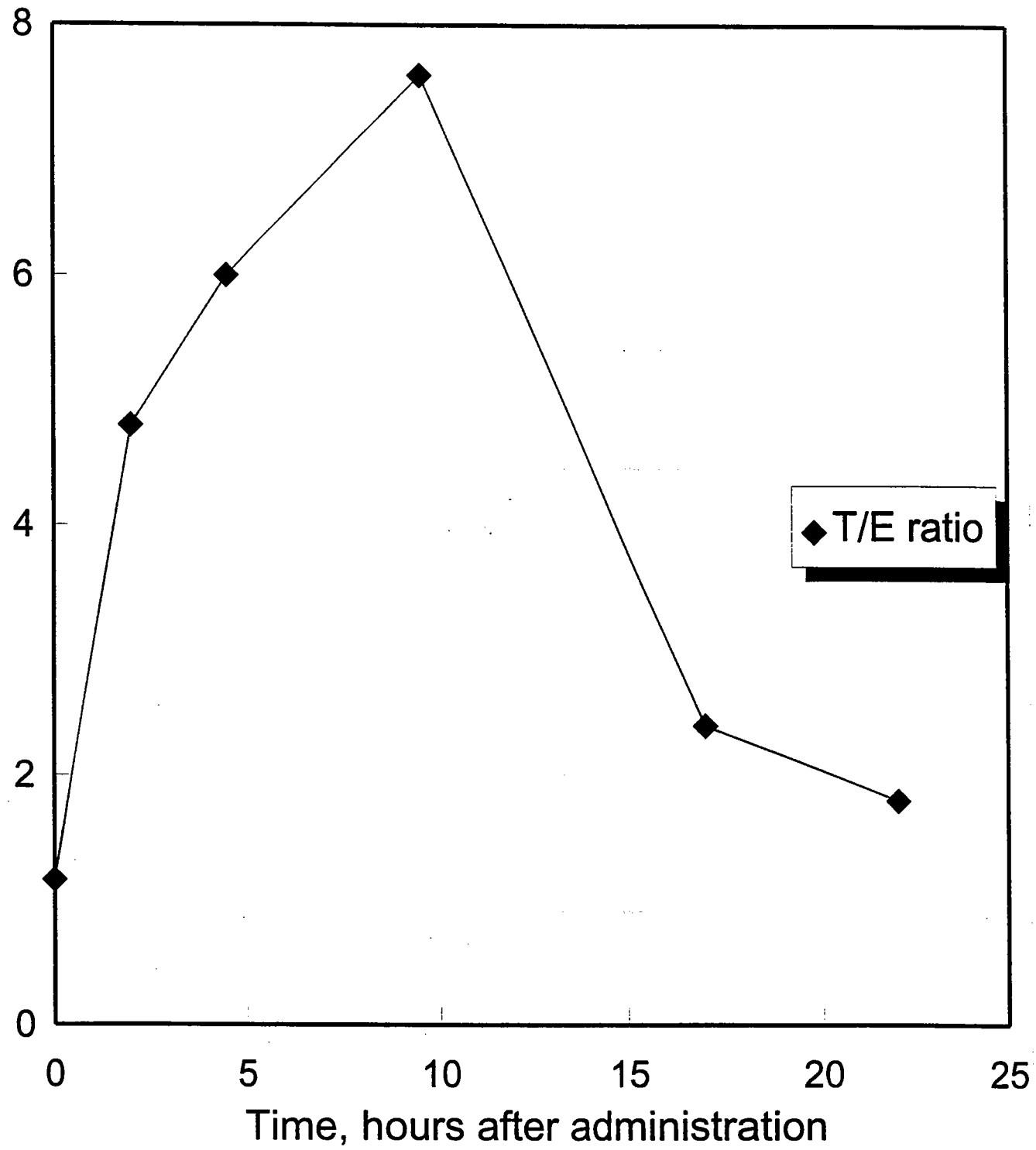


Figure 5. T/E ratio after 50 mg androstendione



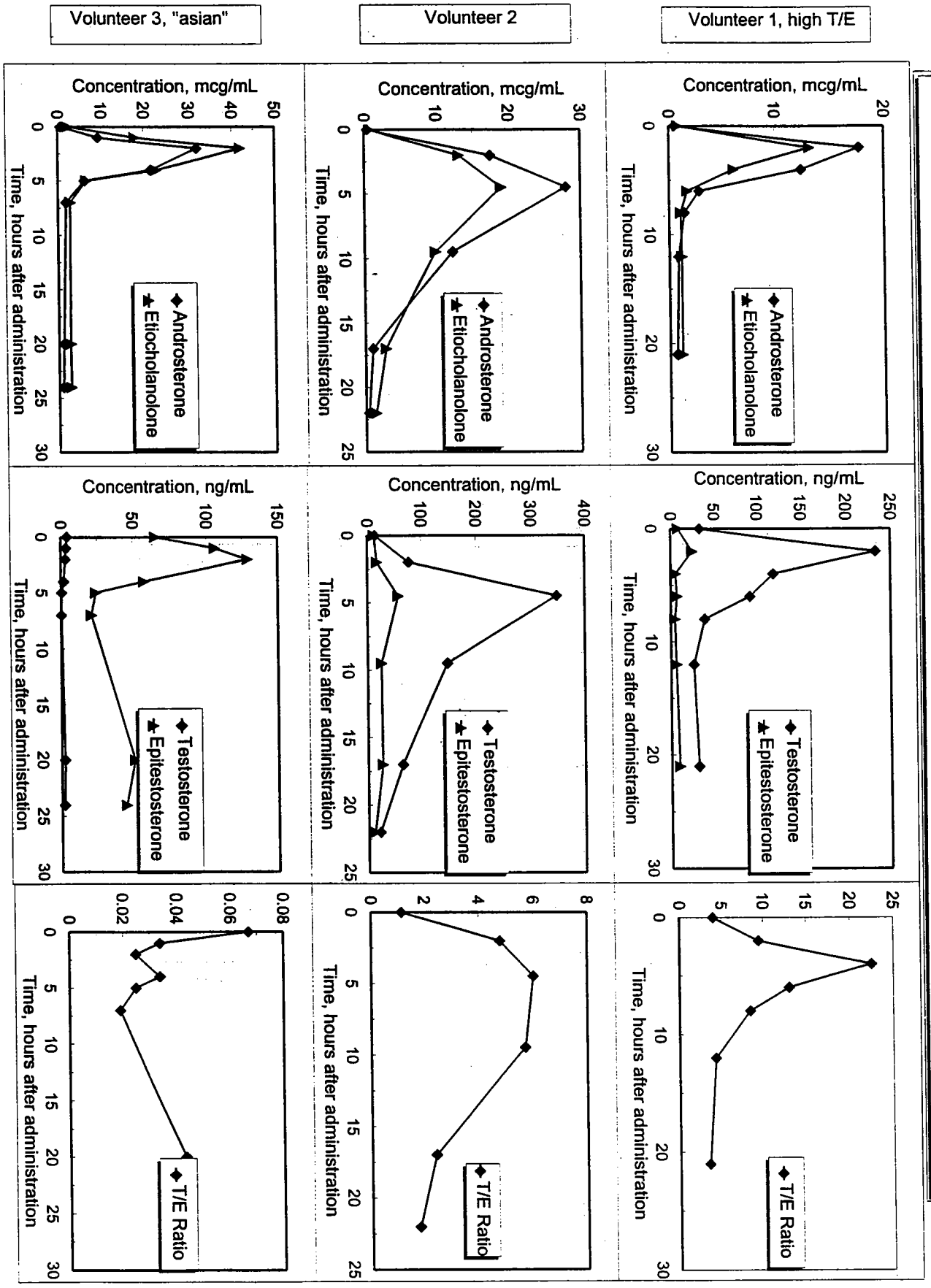


Figure 6. Urinary steroids after administration of 50 mg androstendione

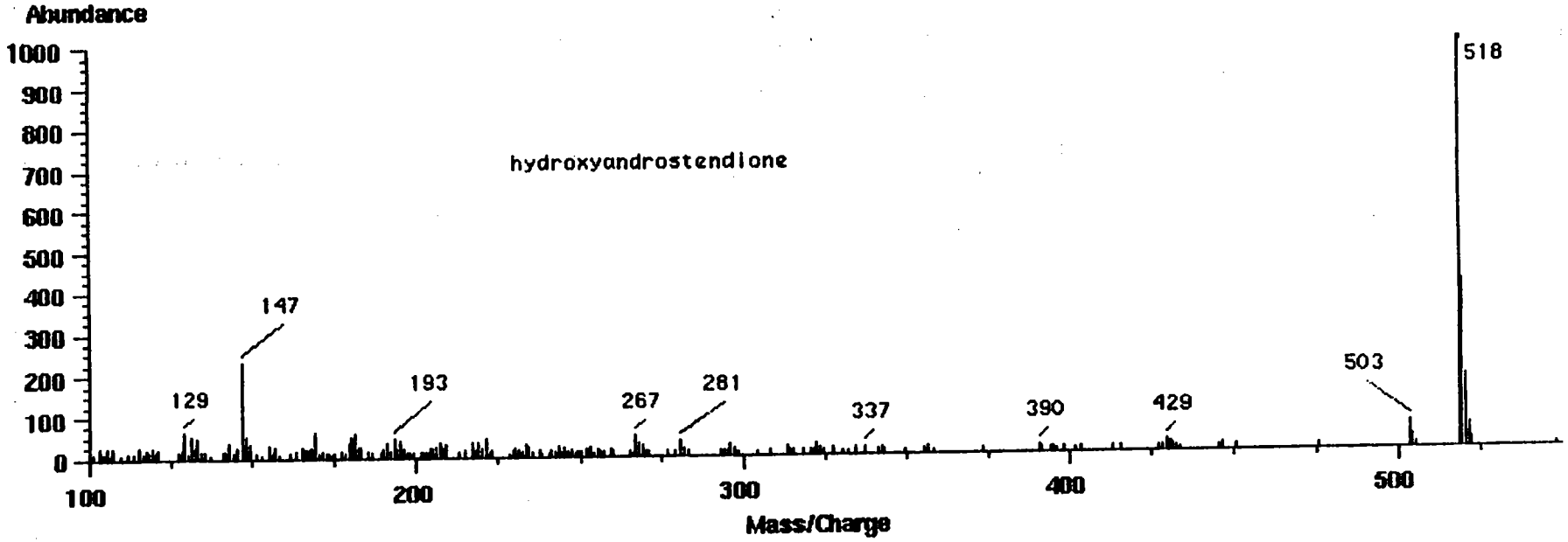


Figure 7. Androstendione hydroxy metabolite, enol-TMS, m.w. 518, EI mass spectrum.

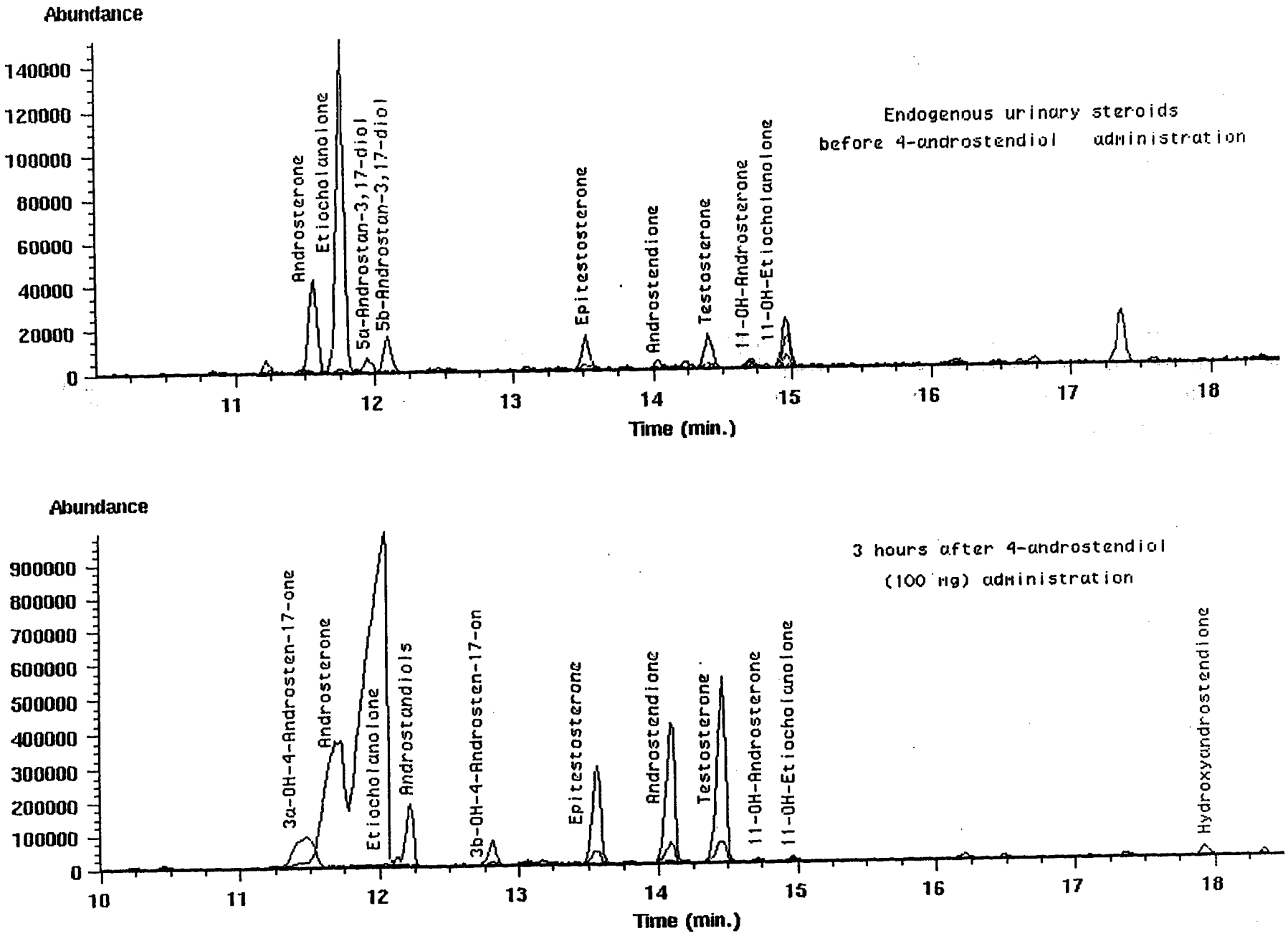


Figure 8. Urinary steroids before (top) and after (bottom) androstendiol (100mg) administration.

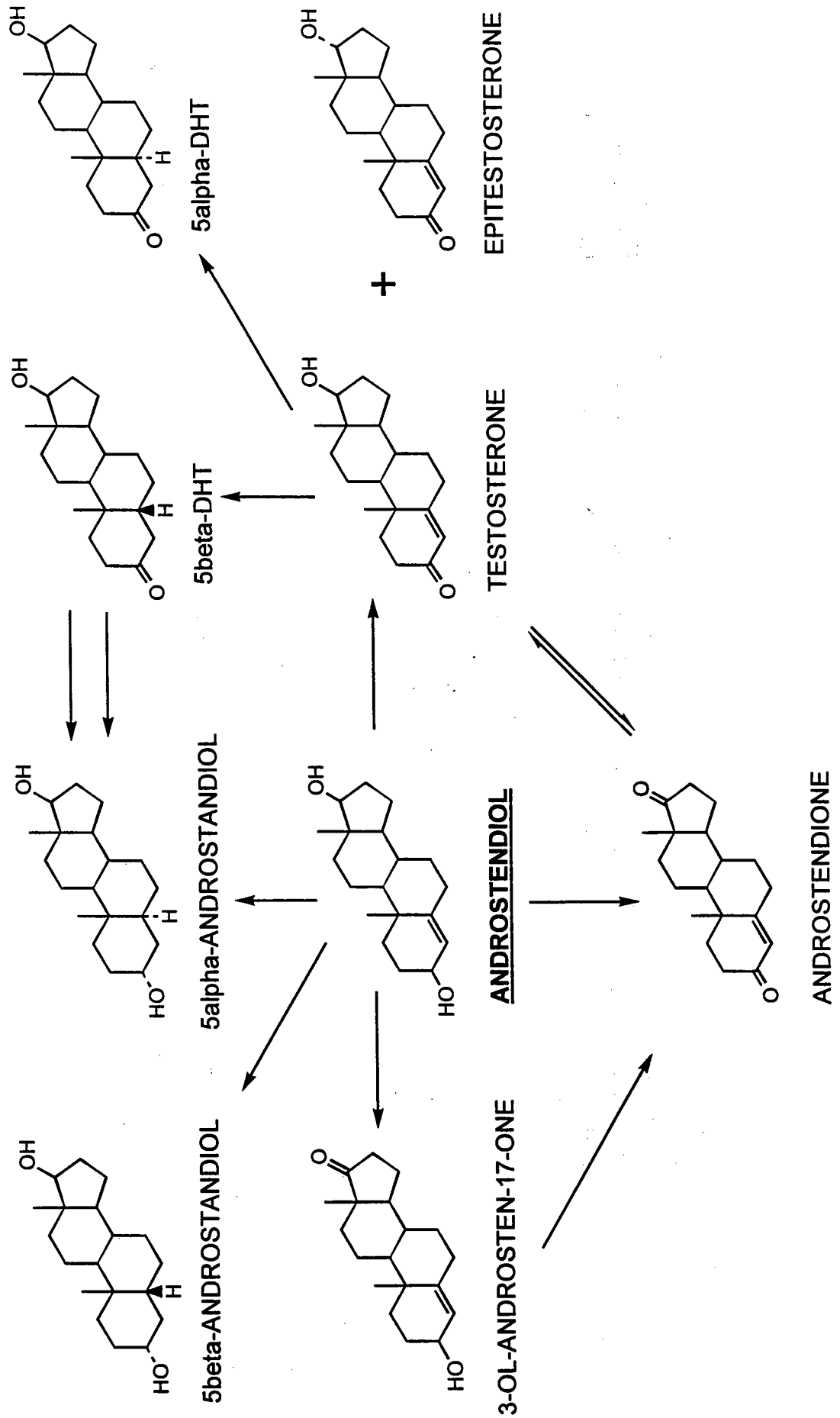


Figure 9. Metabolism of Androstendiol

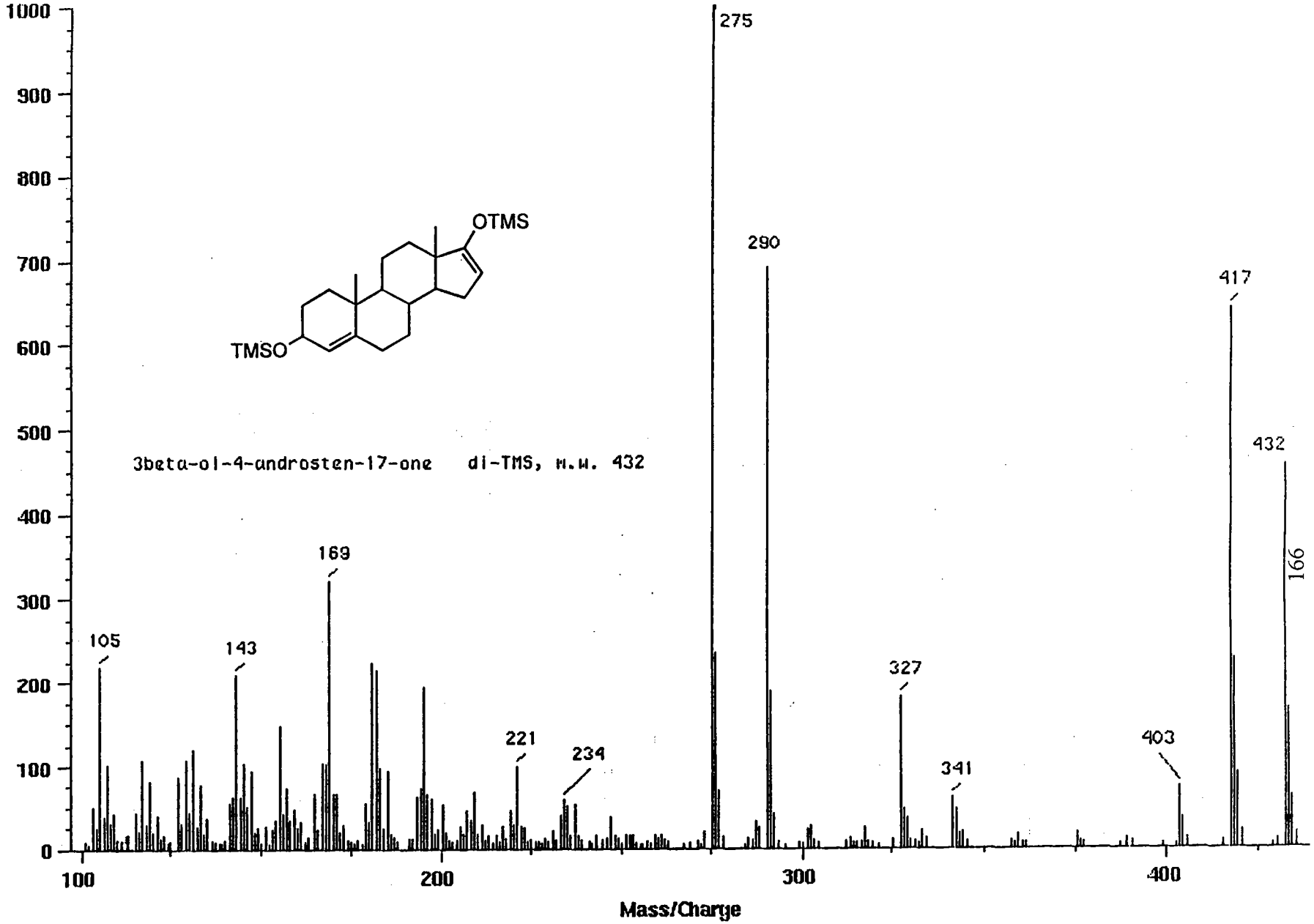
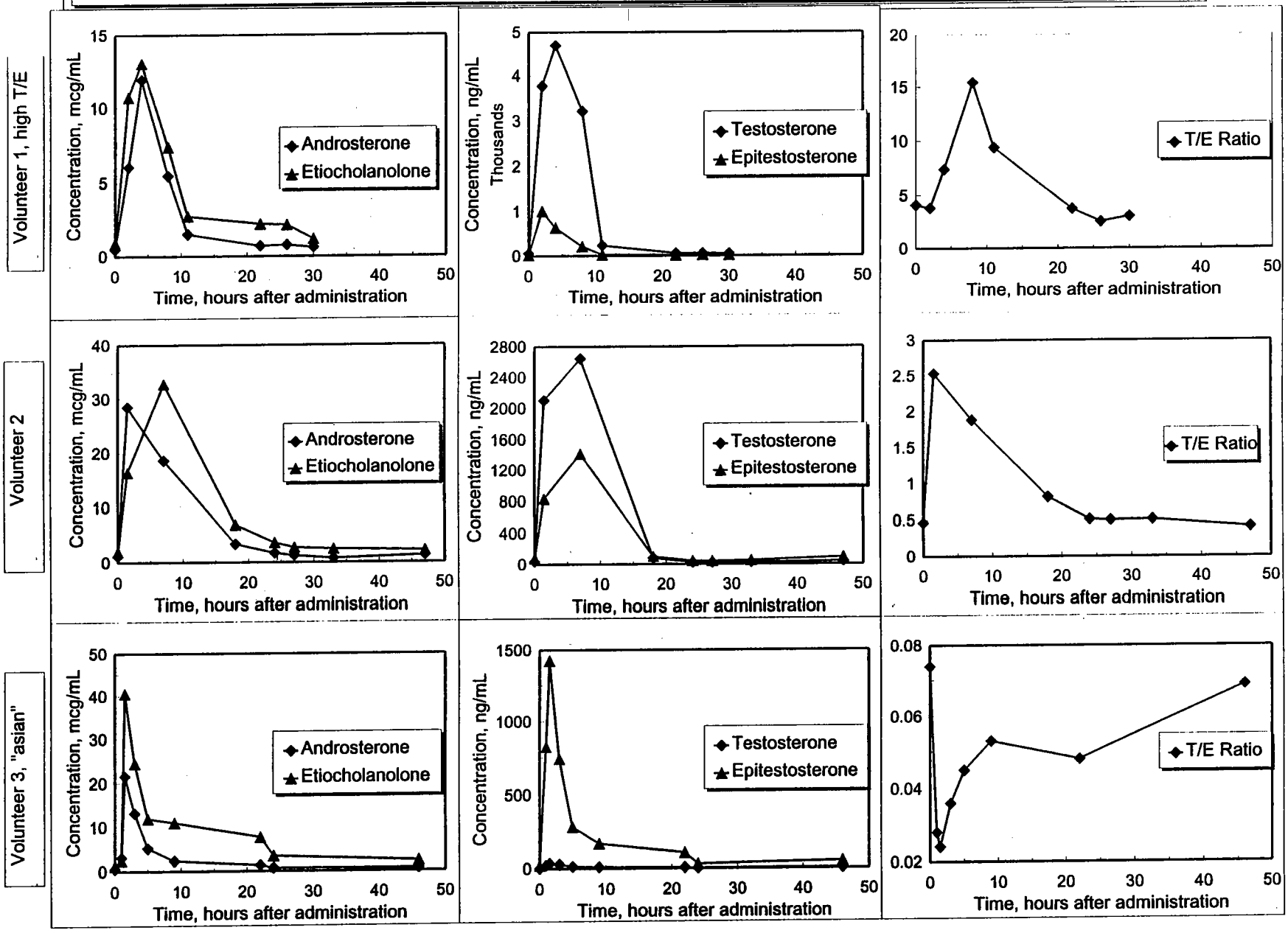


Figure 10. EI Mass spectrum of 3β-hydroxy-4-androsten-17-one, di-TMS.

Figure 11. Urinary steroids after administration of 100 mg androstendiol



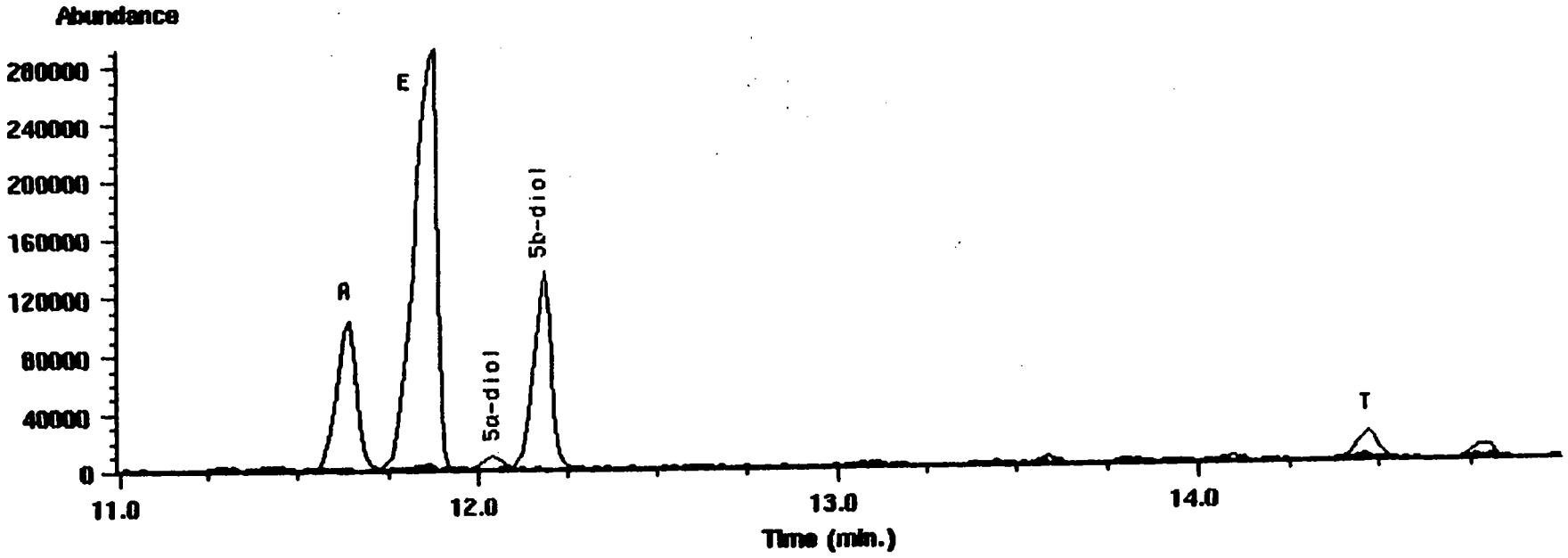


Figure 12. Urinary steroid profile 22 hours after androstendiol administration: A - androsterone; E - etiocholanolone; 5a-diol - 5α-androstan-3α,17β-diol; 5β-diol - 5β-androstan-3α,17β-diol; T - testosterone.

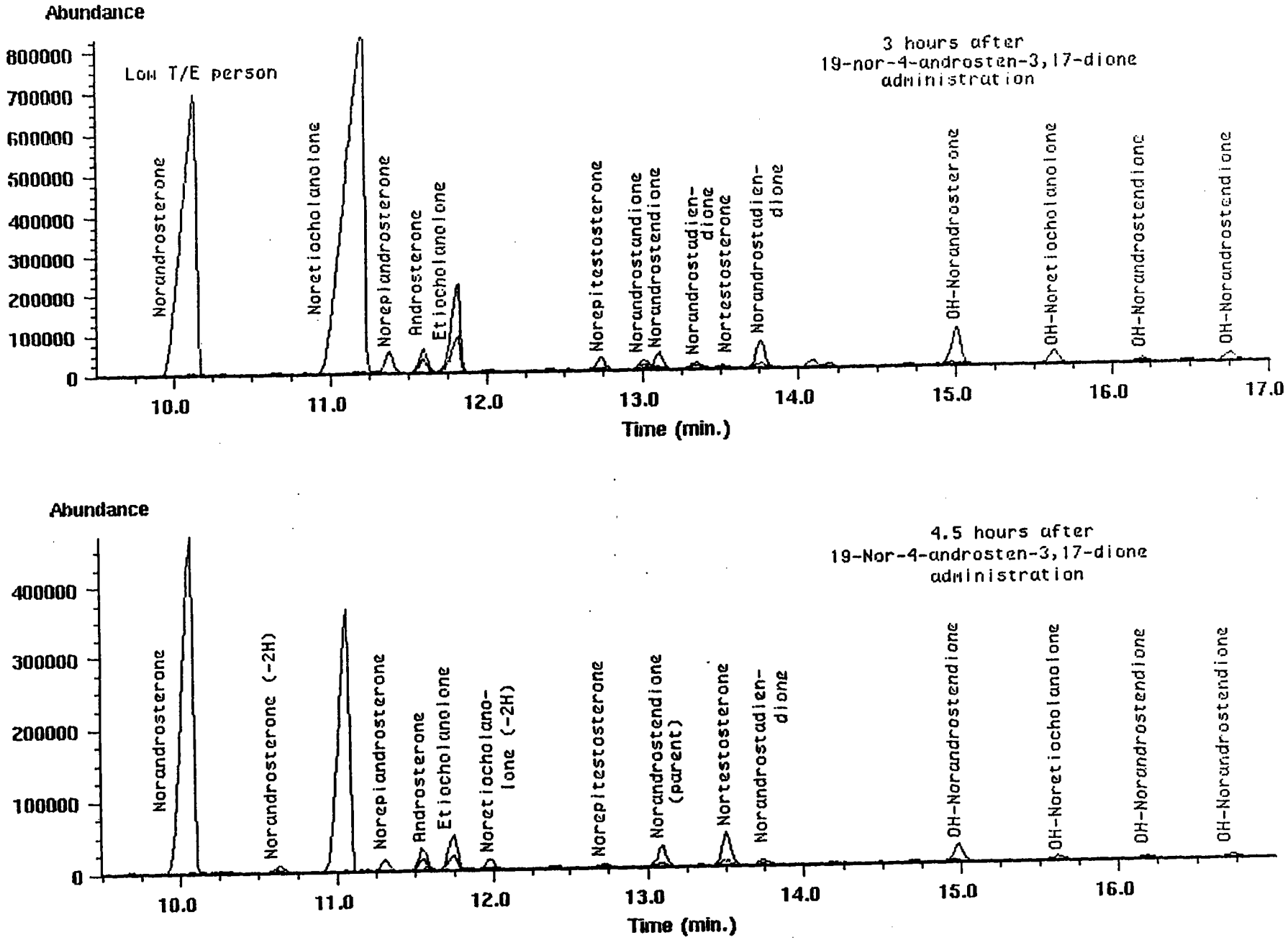


Figure 13. Metabolites of norandrostendione for low (top) and normal (bottom) T/E persons.