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RECENT ADVANCES IN DOPING ANALYSIS (7)

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Over-the-Counter □5 Anabolic Steroids 5-Androsten-3,17-dione; 5-Androsten-3□,17□-diol; Dehydroepiandrosterone and 19-Nor-5-androsten-3,17-dione: Excretion Studies in Men

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Over-the-Counter Δ^5 Anabolic Steroids 5-Androsten-3,17- dione; 5-Androsten-3β,17β-diol; Dehydroepiandrosterone and 19-Nor-5-androsten- 3,17-dione: Excretion Studies in Men.

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Introduction

In 1997, Δ^4 precursors of testosterone and nandrolone were introduced on the nutritional supplement market as performance enhancing agents. Δ^5 Analogs have appeared now as well, commonly as components of multi-compound formulations. Their role as precursors to testosterone suggests their anabolic potency. They are banned in sports under the clause "related substances" by the definition of doping of IOC. Dehydroepiandrosterone (DHEA), androsten-3,17-dione, -diol and their 19-nor analogs are now specifically banned by the IOC and the major sports federations. On the other hand, they are legally sold without a prescription in the US.

We reported (1) excretion profiles of some Δ^4 over-the-counter (OTC) steroids. Δ^5 analogs 5-androsten-3,17-dione, 5-androsten-3β,17β-diol; DHEA; and 19-nor-5(10)-androsten-3,17-dione are the subject of the present study. None of them except DHEA (2-5) was investigated previously. DHEA has been available as an OTC supplement since 1994, longer than any other steroid discussed. Its commercial history, as well as being longer, has displayed a different emphasis. While the androstendiones and -diols are marketed through the bodybuilding media and sold through specialized outlets as anabolic agents, DHEA is more generally advertised, and commonly available in grocery stores. DHEA excretion studies (2-5) were especially concerned with the effect on urinary T/E ratio. Although some elevation of T/E was observed, no positive results were recorded after DHEA ingestion and its oral administration was concluded to be safe (3, 4). Recently, Bowers (5) has demonstrated T/E elevation above 6 after a dose of DHEA.

Experimental

Excretion studies and steroids

A single oral dose of each drug was administered to three male volunteers at different times over a 1-year period. All took 200 mg of DHEA (Marathon Nutrition capsules, Rolling Hills Estates, CA). Two received authentic standards of 5-androsten-3,17-dione and 19-nor-5-androsten-3,17-dione, 100 mg each (Steraloids, Inc., Newport, RI). One took 200 mg of 5-androsten-3β,17β-

diol standard (Steraloids). GC/MS analysis did not reveal impurities in these products. Urine specimens were collected before and after drug administration at sequential voids for 24 h, and then periodically for several days.

Reagents and materials

β-Glucuronidase/arylsulfatase crude solution from *Helix Pomatia* (Cat.# G-7017) and dithioerythritol were purchased from Sigma Chemical Co. N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was supplied by Campbell Science Corp. (Rockton, IL), ammonium iodide 99+% by Aldrich Chemical Co. (St. Louis, MO). C_{18} solid phase (200 mg) extraction cartridges were purchased from Varian (Harbor City, CA). Steroid internal standard mixture (ISTD) was prepared by adding 400 μL of 5β-androstan-17-one (1 mg/mL), 200 μL of 4-chlorotestosterone (1 mg/mL) and 9.4 mL methanol.

Urine sample preparation

To 4 mL urine in a glass tube, 1 mL of acetate buffer (pH 5.2)/ β-glucuronidase/ ISTD mixture (467:25:8 v/v/v) was added and incubated for 3 h at 52°C. After 3 h the tubes were centrifuged and the liquid was applied to C_{18} solid phase extraction columns prewashed with 3 mL methanol and 3 mL water. After urine passed through, the columns were washed with 2 mL of 30% (v/v) acetonitrile in water. Columns were dried under 350 mm vacuum for 15 min. Steroids were eluted with 3 mL methanol. The methanolic eluate was evaporated to dryness.

The dry residue was derivatized with 75 μL of MSTFA/NH_4I/Dithioerythritol (1000:2:3 v/w/w) for 15 min at 70°C. Samples were transferred into vials, 1 μL was injected into the GC/MS.

GC/MS parameters

GC/MS was Hewlett-Packard 5890/5970 with HP 7673A autoinjector and UNIX-Target software. Column: HP-1 fused silica, crosslinked methylsilicon, 17m, 0.2 mm I.D., 0.11 μm film thickness. Carrier gas helium at linear velocity 40 cm/sec was used. Injection split ratio was 1:10. Oven temperature program: hold at 180°C for 0.3 min; raise at 3°C/min to 231°C; then 30°C/min to 310°C, hold for 1.07 min. Injector temperature was 270°C, transfer line 280°C.

Results and Discussion

Dehydroepiandrosterone. Volunteers in this excretion study comprised two distinct endogenous urine profiles at baseline. One volunteer is designated as high T/E (T/E > 4/1), and the other two as low T/E (T/E <0.5). Results are presented in fig. 1. Both androsterone and etiocholanolone rise sharply, immediately following DHEA ingestion. After androsterone peaks, in the first few hours, and begins a rapid return to baseline levels, etiocholanolone for two volunteers is seen to

remain elevated and even continue to rise, before gradually falling to the baseline. Etiocholanolone may remain elevated above baseline for 72 hours or longer, while testosterone returns to normal in 24 to 36 hours. The protracted elevation of etiocholanolone compared with androsterone is seen for the low T/E volunteers, although there is some individual variation in the actual rate of decay for both substances. Urinary DHEA (fig.1) and dehydroandrosterone (DHA, not shown in fig. 1) rise along with androsterone and etiocholanolone, peak coincidently with androsterone and, like it, rapidly return to baseline levels. Although DHEA and DHA concentrations show great individual variation, the qualitative nature of their appearance in urine closely resembles that of androsterone. The behavior of etiocholanolone, subsequent to the peaking of androsterone, DHEA and DHA reflects a continued more gradual rise to a peak occuring 2 to 3 times later than that for androsterone, DHEA and DHA, that is 16-24 hours vs. 8 hours, followed by gradual return to normal. The behavior of 5α- and 5β-androstan-3α,17β-diols (fig.1) reflects the relationship of androsterone and etiocholanolone respectively, i.e. 5β isomer reaches its maximum later and remains elevated for a longer time than the 5α isomer. These observations match those reported by Kazlauskas (2). One high T/E volunteer (top graphs in fig.1) did not show protracted elevation of etiocholanolone.

The T/E ratio is affected similarly for all volunteers. We see about a three fold increase in the T/E ratio as a result of DHEA ingestion under these circumstances. The high T/E individual displays a rise from 4.5 to 16. One of the low T/E persons rises from 0.3 to about 0.7, while the initial T/E of the third one was low enough to cast doubt on the total increase seen, although the level rises to T/E 0.6. For a normal T/E distribution, the minority of individuals whose baseline T/E exceeds 2/1 are at risk for a positive steroid test as a result of DHEA ingestion.

5-Androsten-3β,17β-diol. At early excretion hours, parent 5-androstendiol and especially DHEA are abundant (fig. 2) in urine of the low T/E volunteers. Two chromatograms indicate the change in the steroid profile with time, after ingestion. The fall in androsterone, DHEA and DHA relative to etiocholanolone are characteristic for the later collection. Androsterone, DHEA and DHA rise and fall in the same manner as when DHEA was consumed. Similarly, etiocholanolone remains elevated for a longer period of time. The time changes in relative steroid concentrations for this individual are consistent with those resulted from DHEA administration. Total effect of 5-androstendiol ingestion in this case is similar to that of DHEA.

5-Androsten-3,17-dione. A chromatogram of the steroid profile prior and 7 hours after administration of 100 mg to a low T/E volunteer (fig. 3) reveal some significant changes. The entire profile is amplified after ingestion. Etiocholanolone concentration rises relative to

androsterone, as was observed for the two previous substances. Particularly remarkable is the rise in DHA compared to DHEA. We see in addition, the presence of 5-androsten-3α,17β-diol, eluting between androsterone and etiocholanolone (fig. 3). This change is noticeable only after ingestion of 5-androstendione, it is not observed for DHEA or for 5-androsten-3β,17β-diol ingestion. A closer look in fig. 3 reveals that 7 hours after ingestion of 5-androstendione, DHEA has not changed, in marked contrast to DHA, androsterone and etiocholanolone.

Endogenous Δ^5 steroid summary. The diagram in fig. 4 summarizes findings for the metabolism of the endogenous Δ^5 steroids. Comparing observations for 5- and 4-androstendiones ingestion (1), we see that the increase in T/E ratio is comparable and that neither steroid results in a change in DHEA levels. Contrasting the results, we see that although the change in the T/E ratio is consistent both in time and degree for both -dions, the relative change in absolute amounts of testosterone, and ultimate urinary metabolites is not. Δ^4 causes more dramatic changes (1). In addition 5-androstendione results in a protracted elevation of etiocholanolone not seen for its 4-analog. The similarity in the initial rise of testosterone and its metabolites indicates that conversion to 4-androstendione occurs. The quantitative difference in the change in the metabolites indicates that the conversion occurs to only a limited extent. We suggest the following: the absence of additional DHEA and increase in DHA after ingestion of 5-androstendione indicates that reduction of the 3-keto function yields a 3α-hydroxy isomer only. Some DHA is excreted along with the 5-androsten-3α,17-diols.

When DHEA is ingested, we observe results similar to those following 5-androstendione ingestion, with the additions now of measurable changes of DHEA and the 5-androsten-3β,17-diols. This suggests that, in addition to the documented conversion of DHEA to its diols (6) and to 4-androstendione, DHEA also converts, via 5-androstendione, to DHA (fig. 4) and that long term elevation of etiocholanolone following DHEA ingestion is due to the mediation of 5-androstendione. Additional etiocholanolone at the later stages of excretion represents that portion of 5-androstendione that survived the first path metabolism and circulates in the bloodstream. Ingestion of 5-androsten-3β,17β-diol yields results essentially identical to those for DHEA. We suggest that administration of 5-androsten-3β,17β-diol is equivalent to DHEA administration, because these two steroids convert into each other, as shown on the diagram in fig. 4.

19-Nor-5-Androsten-3,17-dione. 19-Nor-5-androstendione differs from other Δ^5 steroids we are discussing also in the orientation of its double bond, which extends between carbons 5 and 10. Fig. 5 contrasts early (3 hours) with later (23 hours) excretions after ingestion of 50 mg.

Abundant 19-nor compliments to the endogenous steroids appear. Some of the analogs to the early 5-androstendione metabolites are 19-nor androsterone, etiocholanolone, and proposed nor-5-androstendiols, and nor-DHA. There is some lack of symmetry with 5-androstendione metabolism, however, as witness the presence of nor-DHEA (proposed structure).

Where early excretion is dominated by norandrosterone, nor-DHA, and nor-DHEA, later, it is nor-etiocholanolone that stands out, analogously to the observations for 5-androstendione. Predominance of noretiocholanolone over norandrosterone is an indication of 19-nor Δ^5 steroid administration. This ratio is reversed for 19-nor Δ^4 analogs (1, 7).

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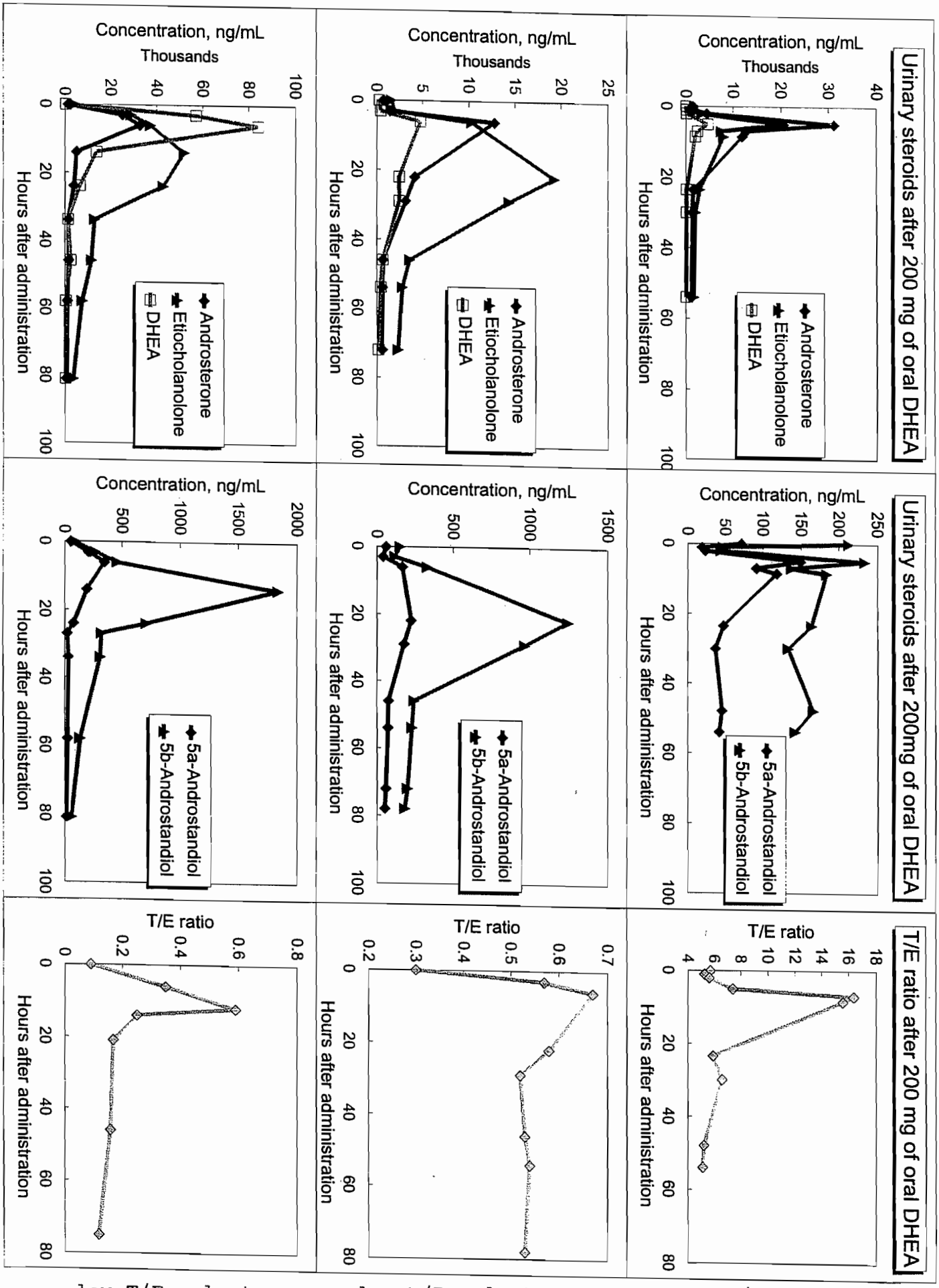
Urinary steroids after 200 mg of oral DHEA
Urinary steroids after 200mg of oral DHEA
T/E ratio after 200 mg of oral DHEA
Concentration, ng/mL
Thousands
Androsterone
Etiocholanolone
DHEA
5a-Androstandiol
5b-Androstandiol
T/E ratio
Hours after administration
high T/E volunteer
low T/E volunteer
low T/E volunteer

Figure 1.

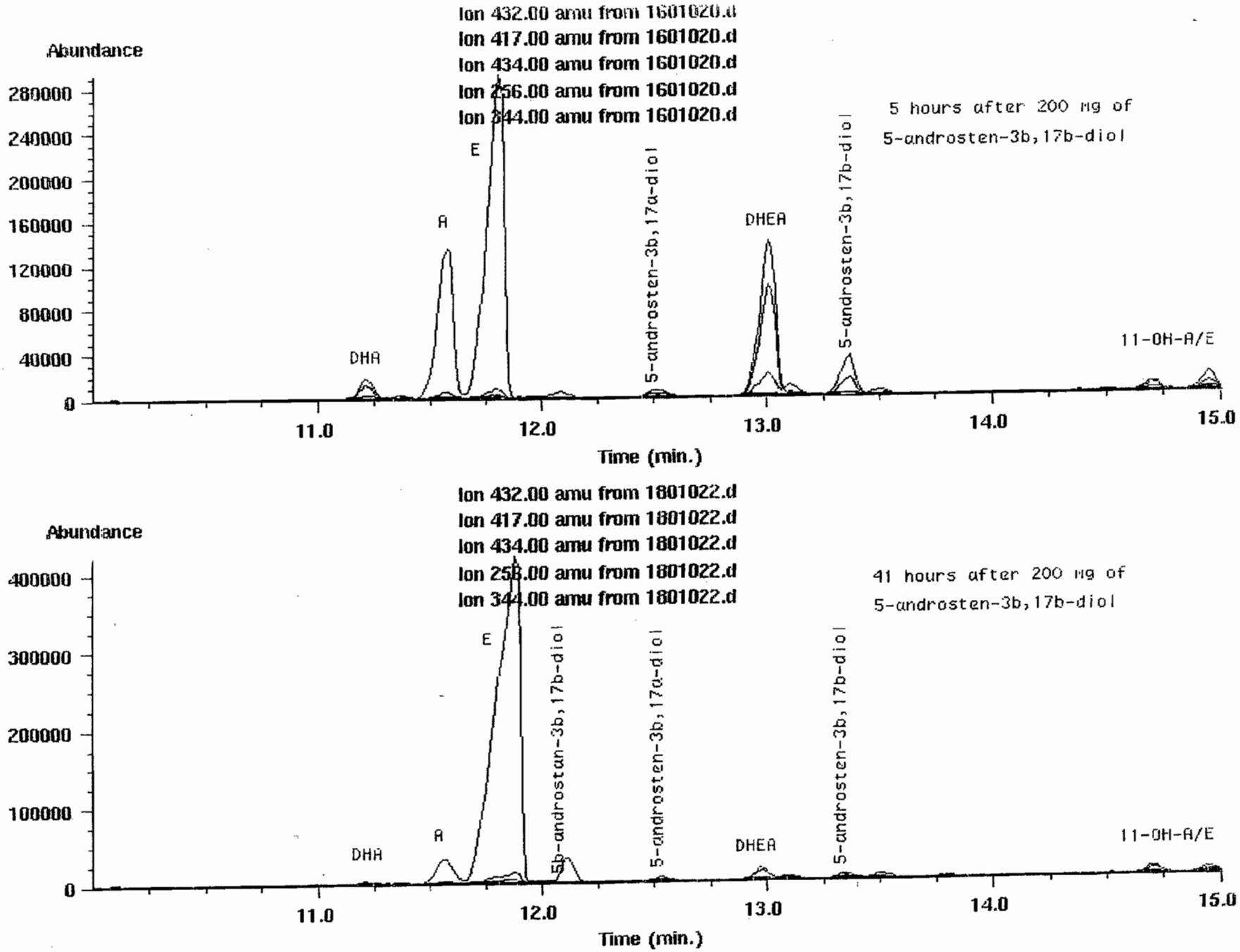
Abundance
Ion 432.00 amu from 1601020.d
Ion 417.00 amu from 1601020.d
Ion 434.00 amu from 1601020.d
Ion 256.00 amu from 1601020.d
Ion 344.00 amu from 1601020.d
5 hours after 200 mg of
5-androsten-3b,17b-diol
280000
240000
200000
160000
120000
80000
40000
0
DHA
A
E
5-androsten-3b,17a-diol
DHEA
5-androsten-3b,17b-diol
11-OH-A/E
11.0
12.0
13.0
14.0
15.0
Time (min.)
Abundance
Ion 432.00 amu from 1801022.d
Ion 417.00 amu from 1801022.d
Ion 434.00 amu from 1801022.d
Ion 256.00 amu from 1801022.d
Ion 344.00 amu from 1801022.d
41 hours after 200 mg of
5-androsten-3b,17b-diol
400000
300000
200000
100000
0
DHA
A
E
5b-androstan-3b,17b-diol
5-androsten-3b,17a-diol
DHEA
5-androsten-3b,17b-diol
11-OH-A/E
11.0
12.0
13.0
14.0
15.0
Time (min.)

Figure 2.

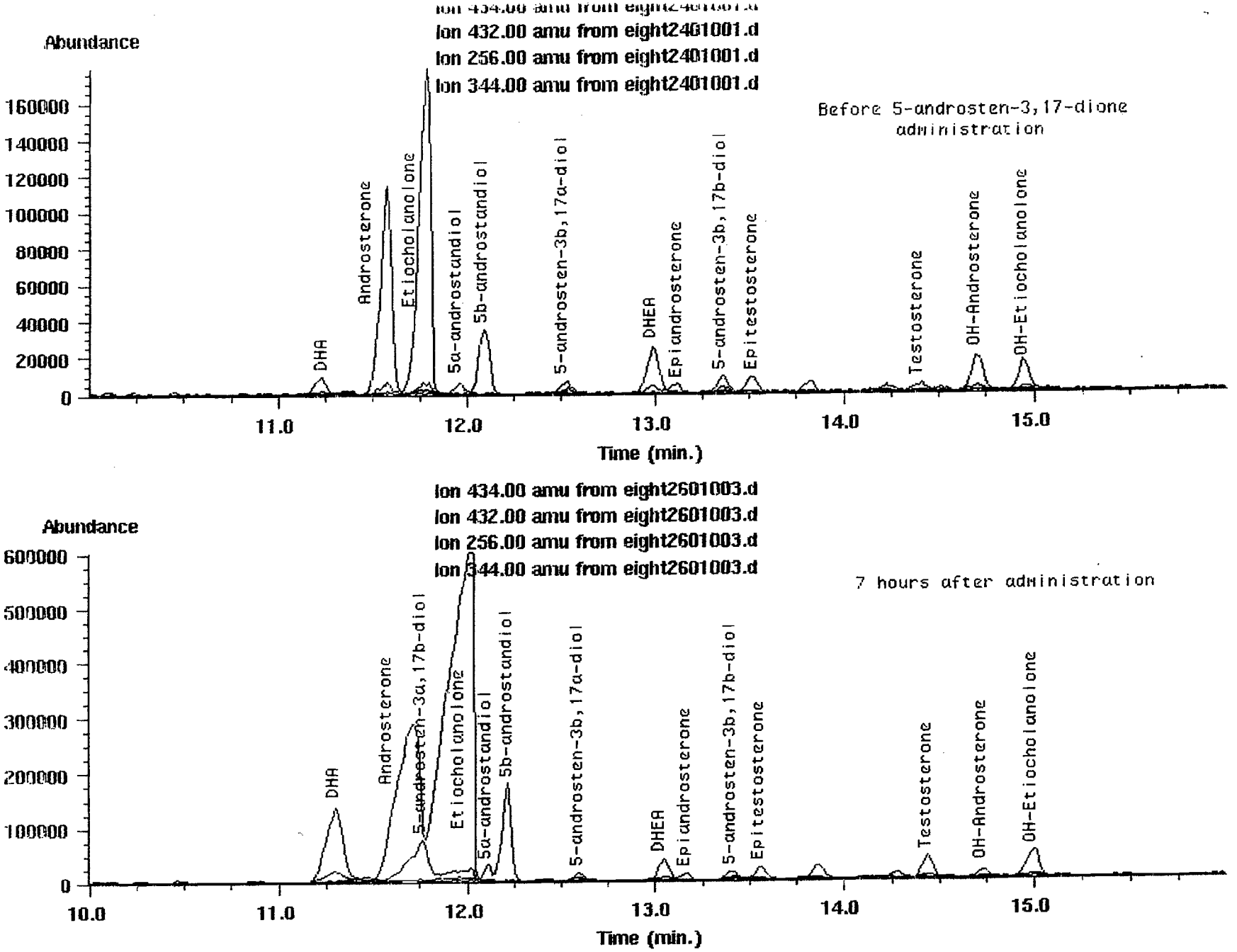
Abundance
Ion 432.00 amu from eight2401001.d
Ion 256.00 amu from eight2401001.d
Ion 344.00 amu from eight2401001.d
Before 5-androsten-3,17-dione administration
DHA
Androsterone
Etiocholanolone
5a-androstandiol
5b-androstandiol
5-androsten-3b,17a-diol
DHEA
Epiandrosterone
5-androsten-3b,17b-diol
Epitestosterone
Testosterone
OH-Androsterone
OH-Etiocholanolone
Time (min.)
Abundance
Ion 434.00 amu from eight2601003.d
Ion 432.00 amu from eight2601003.d
Ion 256.00 amu from eight2601003.d
Ion 344.00 amu from eight2601003.d
7 hours after administration
5-androsten-3a,17b-diol
Time (min.)

Figure 3.

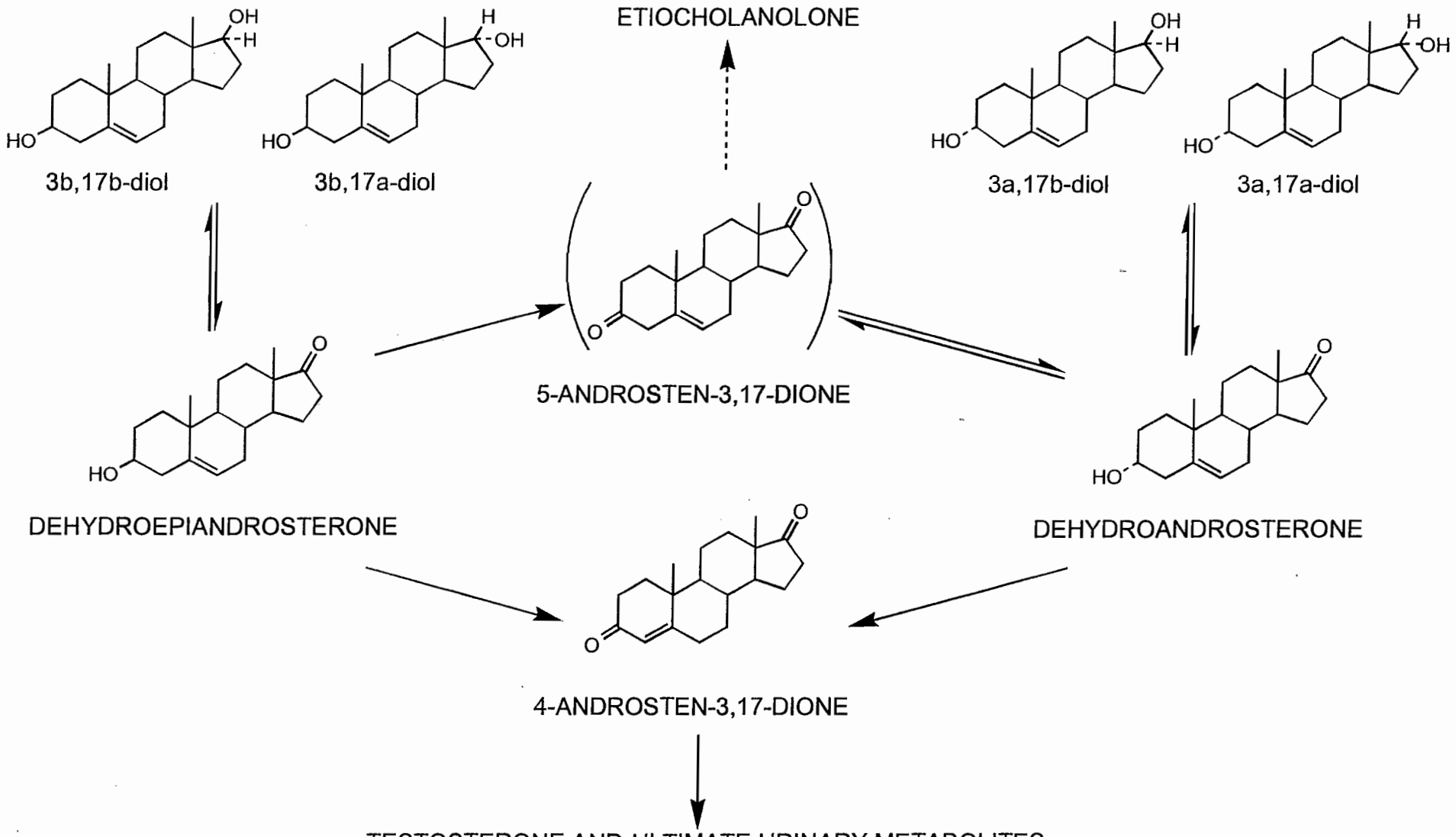

Figure 4. Proposed metabolism of endogenous delta 5 steroids.

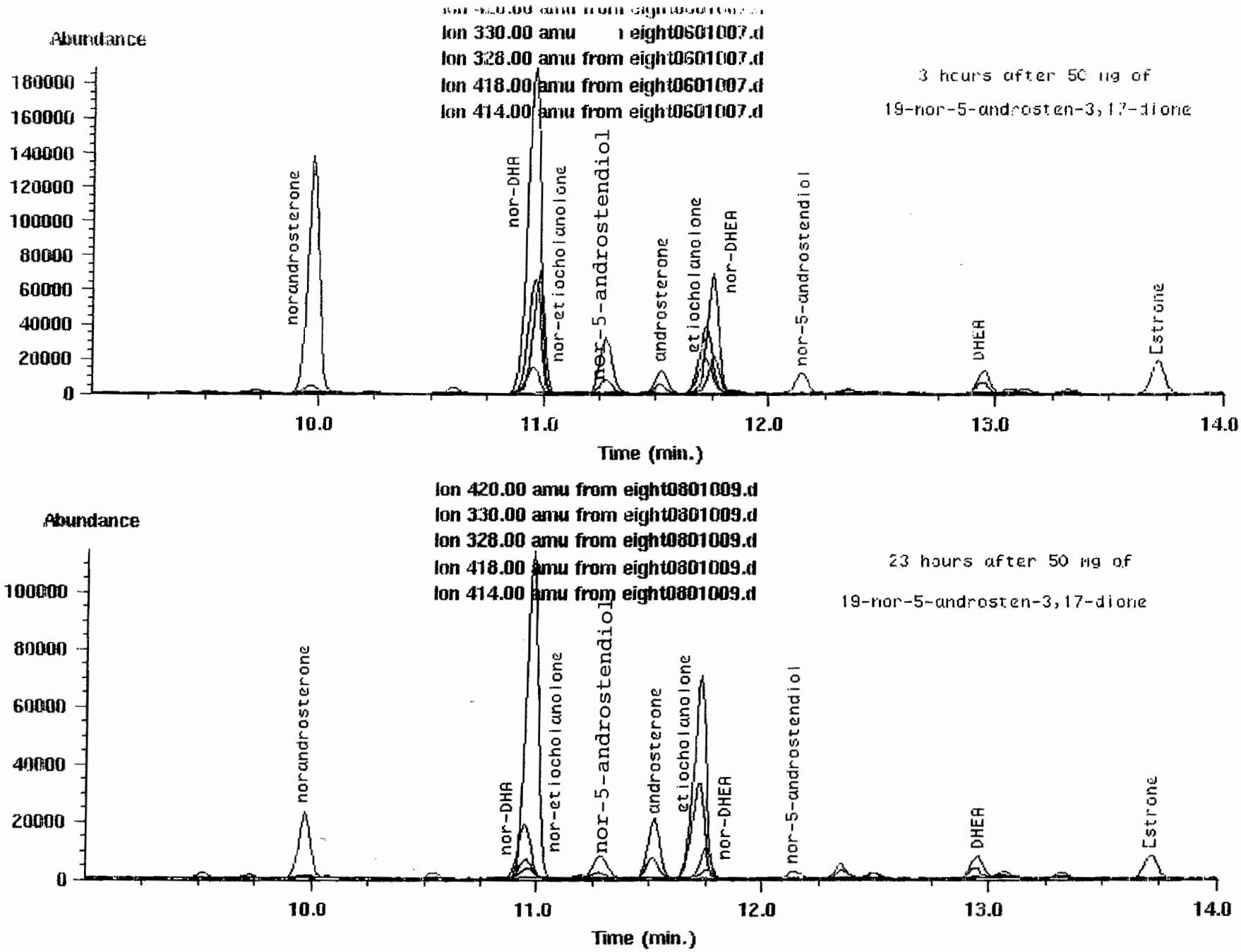
Abundance
Ion 330.00 amu eight0601007.d
Ion 328.00 amu from eight0601007.d
Ion 418.00 amu from eight0601007.d
Ion 414.00 amu from eight0601007.d
3 hours after 50 mg of
19-nor-5-androsten-3,17-dione
norandrosterone
nor-DHA
nor-etiocholanolone
nor-5-androstendiol
androsterone
etiocholanolone
nor-DHEA
nor-5-androstendiol
DHEA
Estrone
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
10.0
11.0
12.0
13.0
14.0
Time (min.)
Ion 420.00 amu from eight0801009.d
Ion 330.00 amu from eight0801009.d
Ion 328.00 amu from eight0801009.d
Ion 418.00 amu from eight0801009.d
Ion 414.00 amu from eight0801009.d
Abundance
23 hours after 50 mg of
19-nor-5-androsten-3,17-dione
norandrosterone
nor-DHA
nor-etiocholanolone
nor-5-androstendiol
androsterone
etiocholanolone
nor-DHEA
nor-5-androstendiol
DHEA
Estrone
100000
80000
60000
40000
20000
0
10.0
11.0
12.0
13.0
14.0
Time (min.)

Figure 5.