Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (3)

M. Donike
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1996

D. Goudreault, C. Ayotte:
Investigation of Mesterolone Urinary Metabolites by GC/MS
In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (3). Sport und Buch Strauß, Köln, (1996) 55-80

D. Goudreault and C. Ayotte

Investigation of Mesterolone urinary metabolites by gc/ms[1]

Institut national de la recherche scientifique, INRS-Santé, Montréal, Québec

Mesterolone (17β-hydroxy-1α-methyl-5α-androstan-3-one) is mostly an androgenic agent. It is however frequently abused in some sports. In 1993, the IOC laboratories reported 940 findings of anabolic agents, 31 of which were due to mesterolone. Our results show that mesterolone was found in the last years in five urine samples, most of the time along with several other anabolic agents. These samples were obtained from Bodybuilders (4) and Powerlifter (1).

Our research activities have mainly been directed towards the identification of the excreted metabolites of anabolic agents taking into account their structural characteristics. We previously reported[2] the results of the investigations made on two steroids : methenolone and stenbolone. Both possess a 1-en methylated A-ring and showed metabolites arising from oxydoreduction and hydroxylation pathways. Mesterolone retains the 1α-methyl group of methenolone but lacks the double bond. In this study, we have identified new interesting metabolites permitting a better knowledge of the overall biotransformation of mesterolone in man.

The urine samples were collected after the oral administration of 50 mg of Proviron®. The urinary metabolites were isolated by solid phase and liquid-liquid extractions. The free fraction was separated and the enzymatic hydrolysis by β-glucuronidase (*E. coli* type IX-A) was followed by the chemical solvolysis (H_2SO_4 in ethylacetate). This last step was preferred over the uncomplete enzymatic hydrolysis of the sulfoesters by the *H. pomatia* mixtures. The identification was done by gc/ms on several derivatives mainly : the TMS and methoxime derivatives. The 3α configuration of the reduced metabolites was proved by the selective

[1] This study was undertaken with the financial support of the Canadian Centre for Drug-free Sport.
[2] a) D. Goudreault and R. Massé : J. Steroid Biochem. Molec. Biol., 37, 137 (1990) b) idem, 38, 639 (1991)

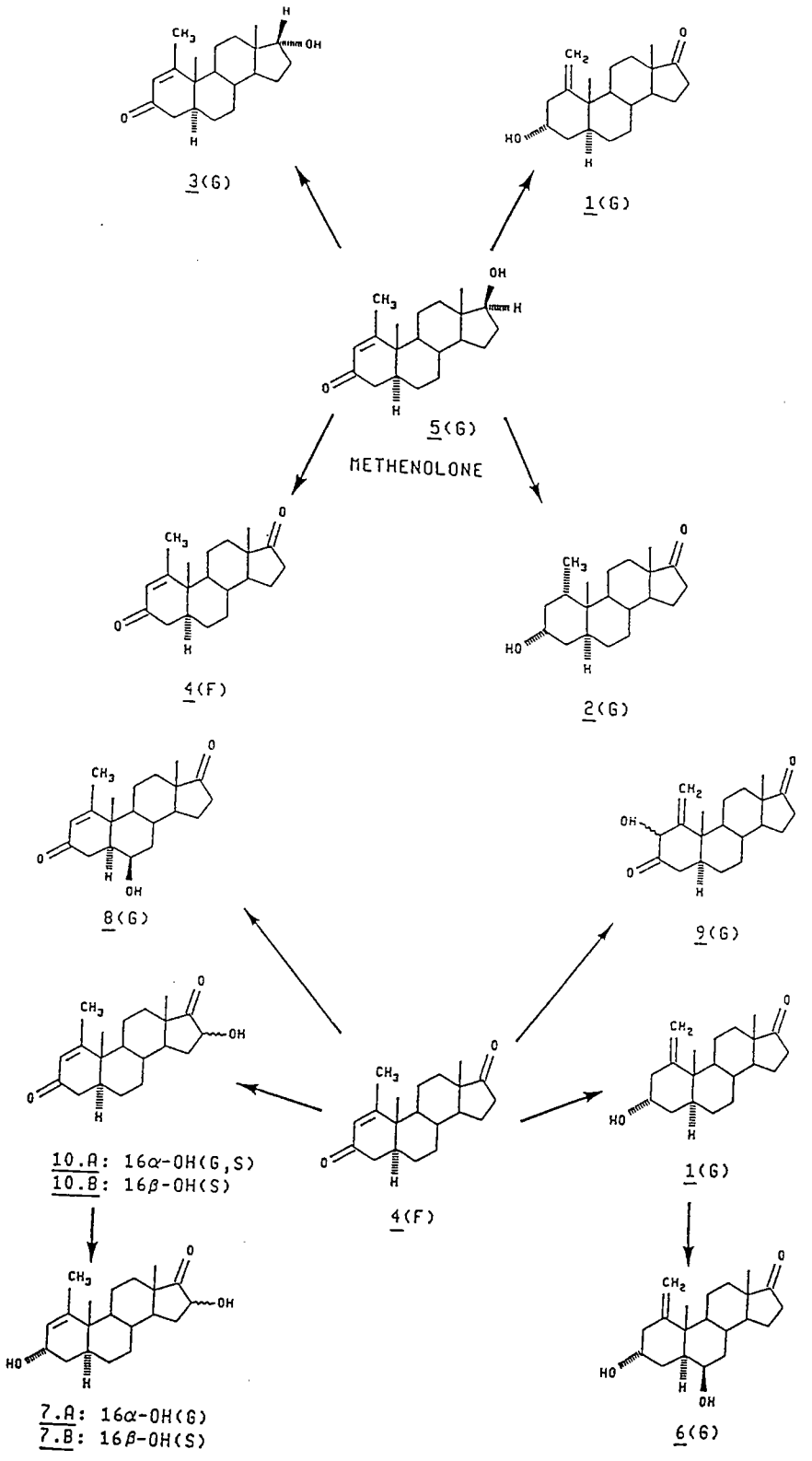
enzymatic oxidation with 3α-hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas testosteroni* (E.C.1.1.1.50)[3].

Mesterolone is mostly excreted as its 3-ol-17-one metabolite as previously reported[4]. Three isomeric 3,17-diols we also identified along with the 3,17-dione and 16-hydroxylated compounds. The presence of urinary mesterolone was observed in all cases. As reported for methenolone and stenbolone, mass spectral evidences were found supporting the identification of some 18-hydroxylated metabolites[5].

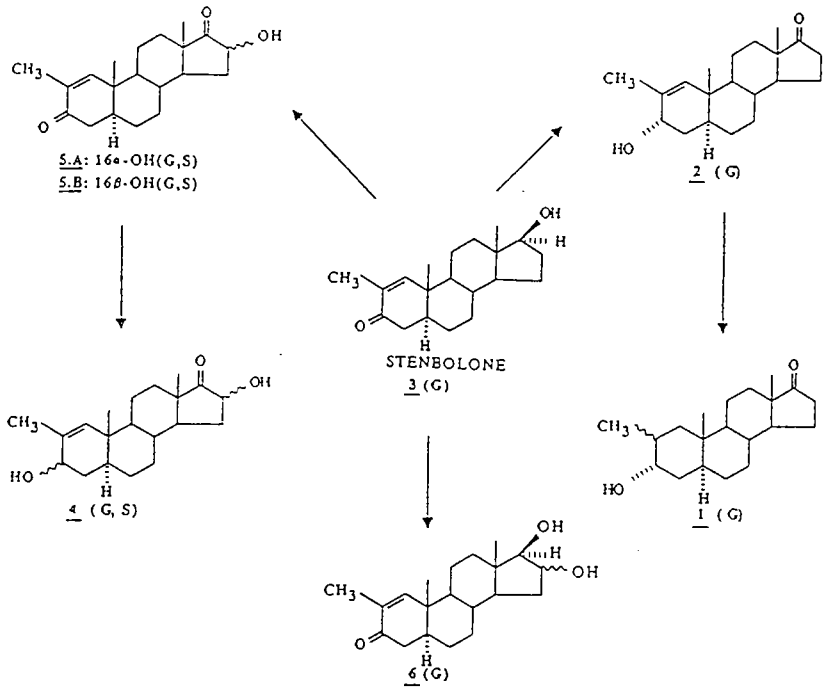
[3] a) K. Kudo et al.: Biochimica Biophys. Acta, 1046, 12 (1990) b) J.W. Ricigliano et al.: Biochem. J., 262, 139 (1989)

[4] a) D. De Boer et al.: J. Steroid Biochem. Molec. Biol., 42, 411 (1992) b) W. Schanzer et al.: J. Steroid Biochem. Molec. Biol., 38, 441 (1991) c) B. Chung et al.: J. Anal. Toxicol., 14, 91 (1990) d) G. P. Cartoni et al. : J. Chromatogr., 279, 515 (1983)

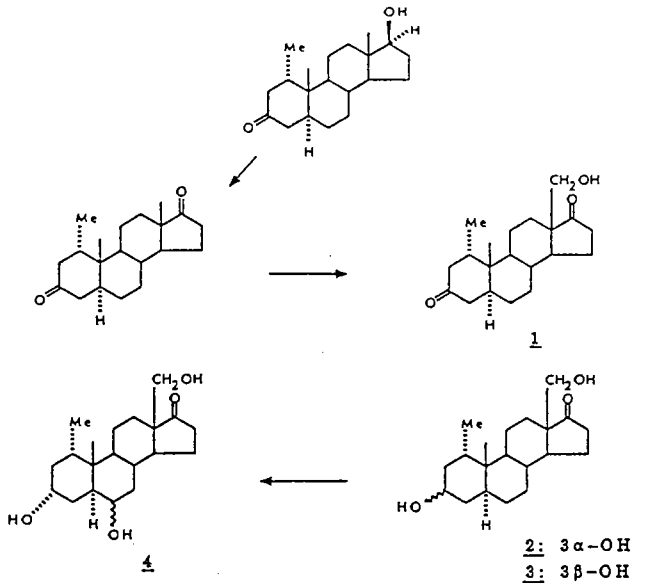
[5] R. Massé and D. Goudreault : J. Steroid Biochem. Molec. Biol., 42, 399 (1992)



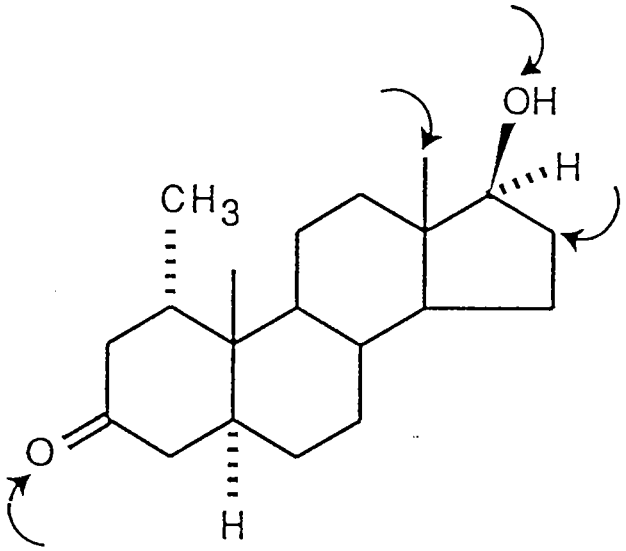
Proposed oxido-reduction and hydroxylation pathways of methenolone metabolism in human.



Proposed structures of stenbolone metabolites resulting from oxidation of the 17-hydroxyl group and reduction of A-ring substituents, with or without concomitant hydroxylation at the C_{16} position.



Proposed pathways for the formation of 18-hydroxylated metabolites of mesterolone.



MESTEROLONE

Literature:

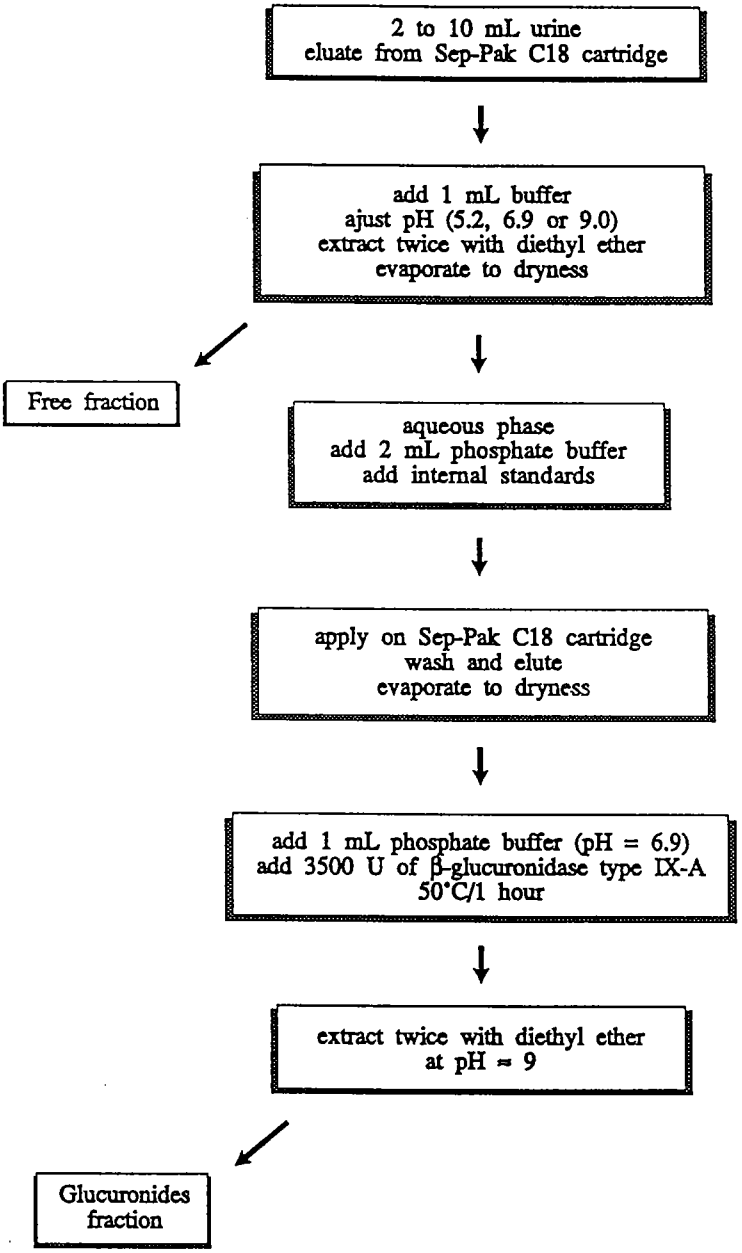
Proposed metabolites:

mesterolone (parent compound)
3α-hydroxy-1α-methyl-5α–androstan-17-one (major, confirmed)
3ß-hydroxy-1α-methyl-5α–androstan-17-one
3α,17ß-dihydroxy-1α-methyl-5α–androstane
3β,17ß-dihydroxy-1α-methyl-5α–androstane
3α– and 3β,18-dihydroxy-1α-methyl-5α–androstan-17-one
18-hydroxy-1α-methyl-5α–androstan-3,17-dione
All these metabolites: conjugated to glucuronic acid.

PROCEDURE IV

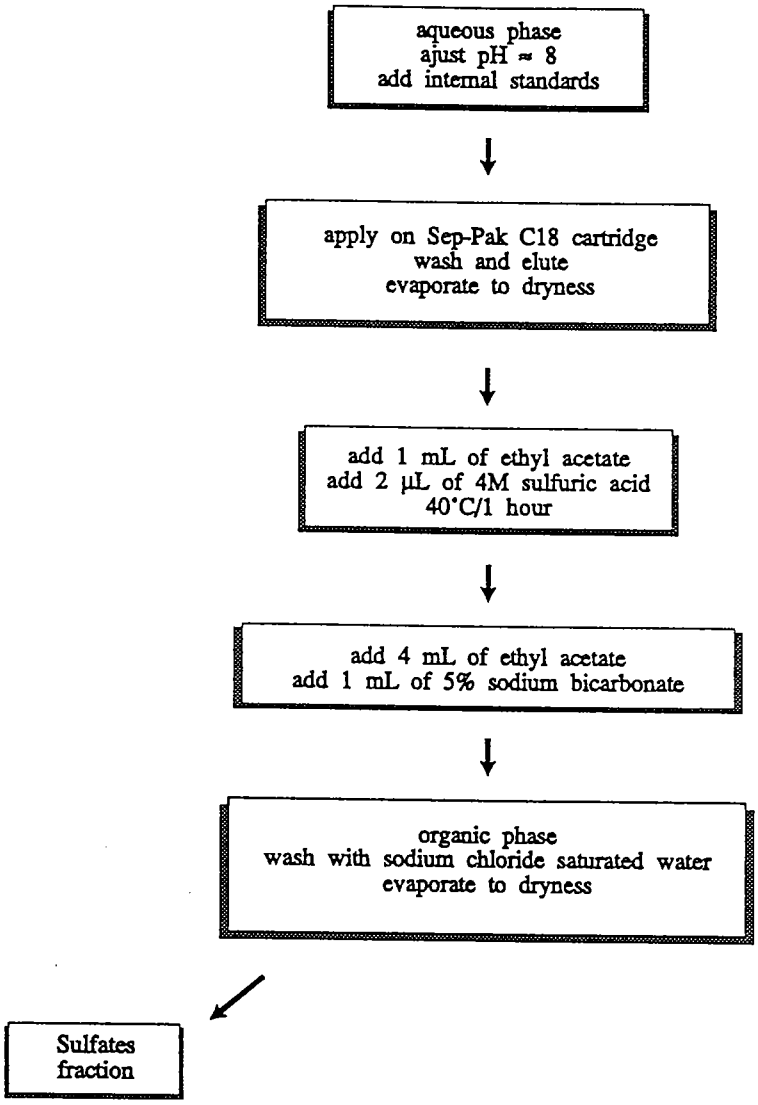
CONFIRMATION

(Free, glucuronides and sulfates fractions)



PROCEDURE IV

CONFIRMATION
(Free, glucuronides and sulfates fractions)
(continued)



Instrumental details

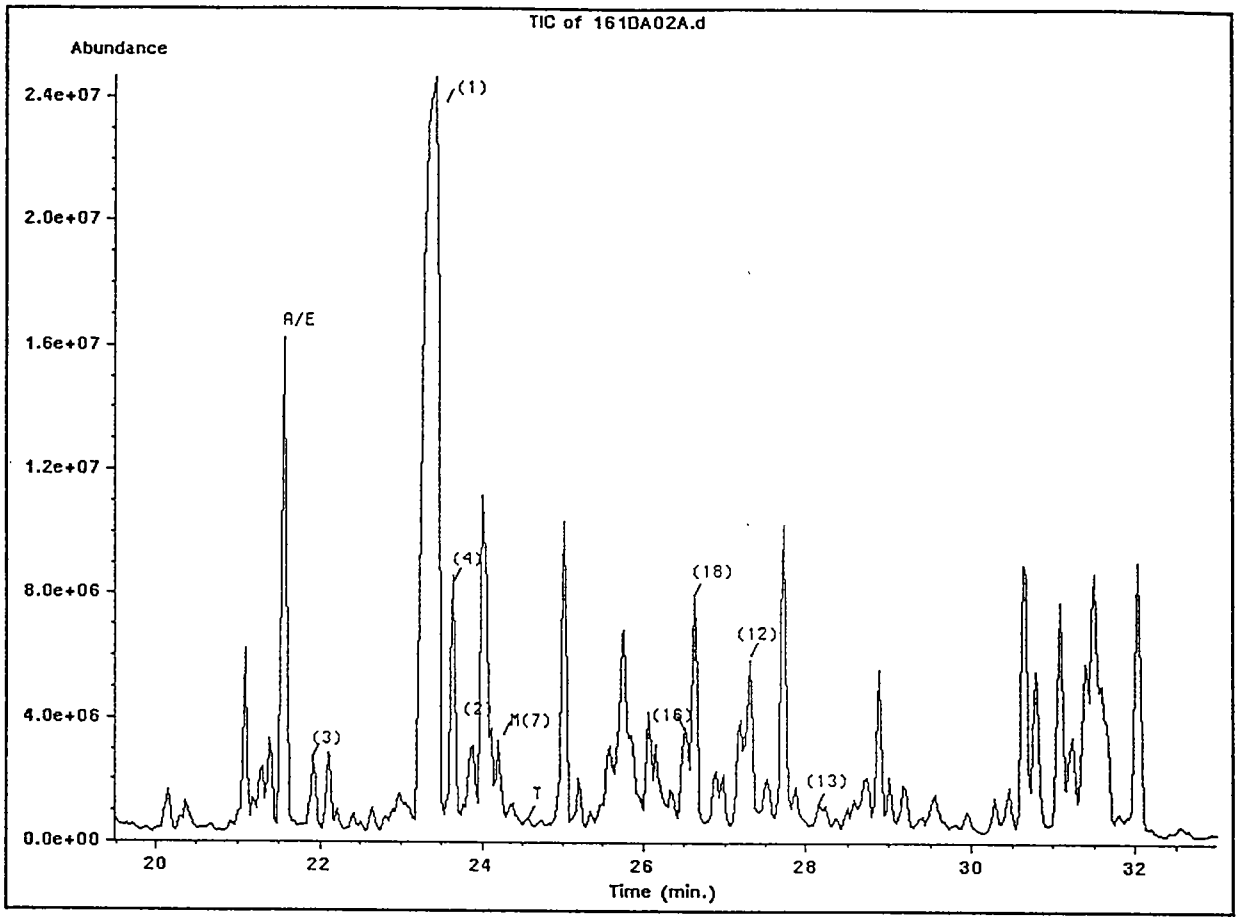
GC/MS analyses in the scan mode are carried out with Hewlett Packard HP5890 gas chromatograph with direct coupling to HP-MSD 5970.

Systems are equipped with automatic samplers 7673A. Instrumentation control and data handling are performed by a Hewlett Packard Vectra
VL2 4/50 data system running under HP DMS 5970 operating software.

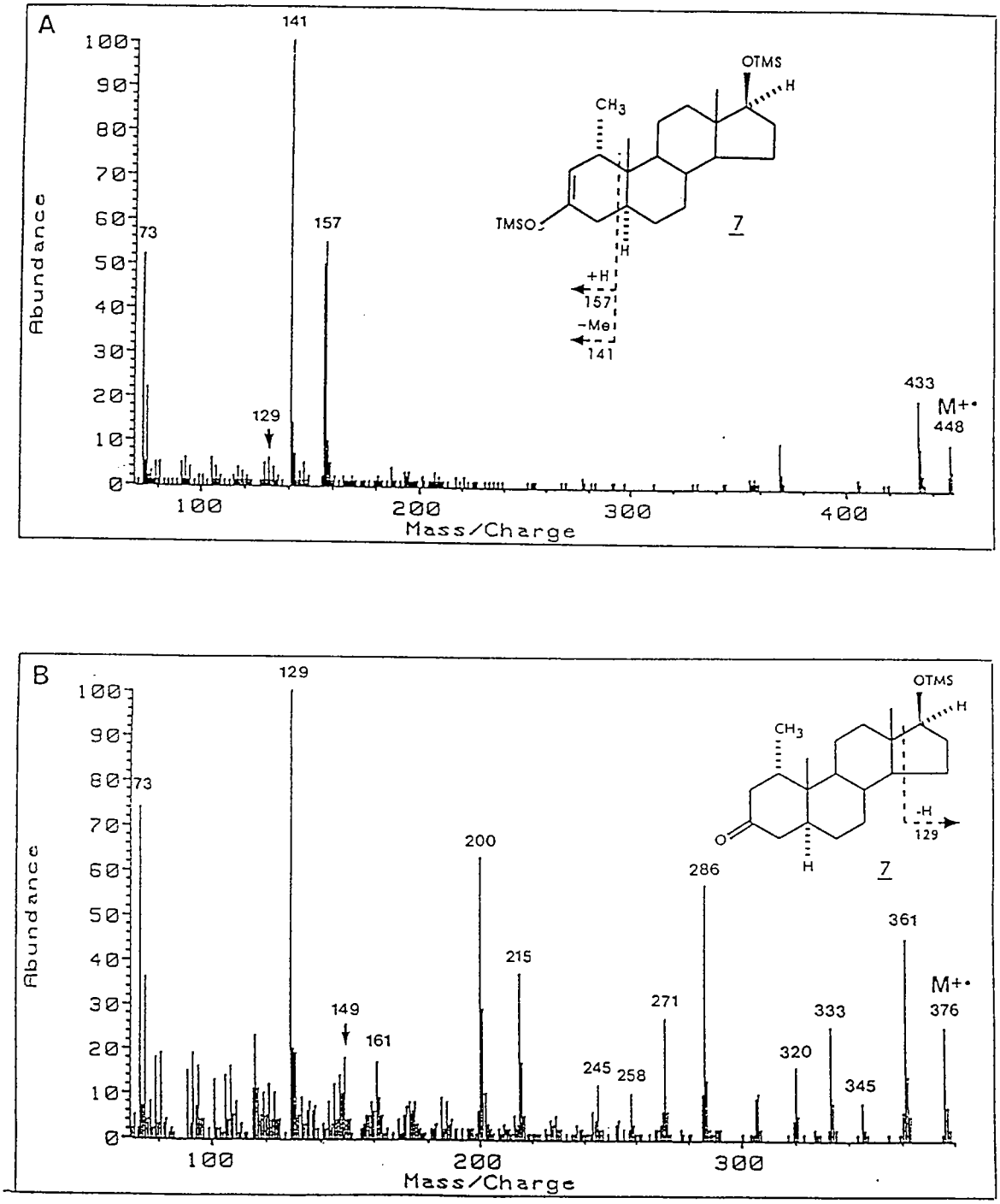
The injections are carried out in the splitless mode (1μL) and the separation is achived on HP-5 capillary columns (5% phenyl methylsilicone phase, 25m X 0.25mm, film thickness:0.33μm).

Chromatographic parameters

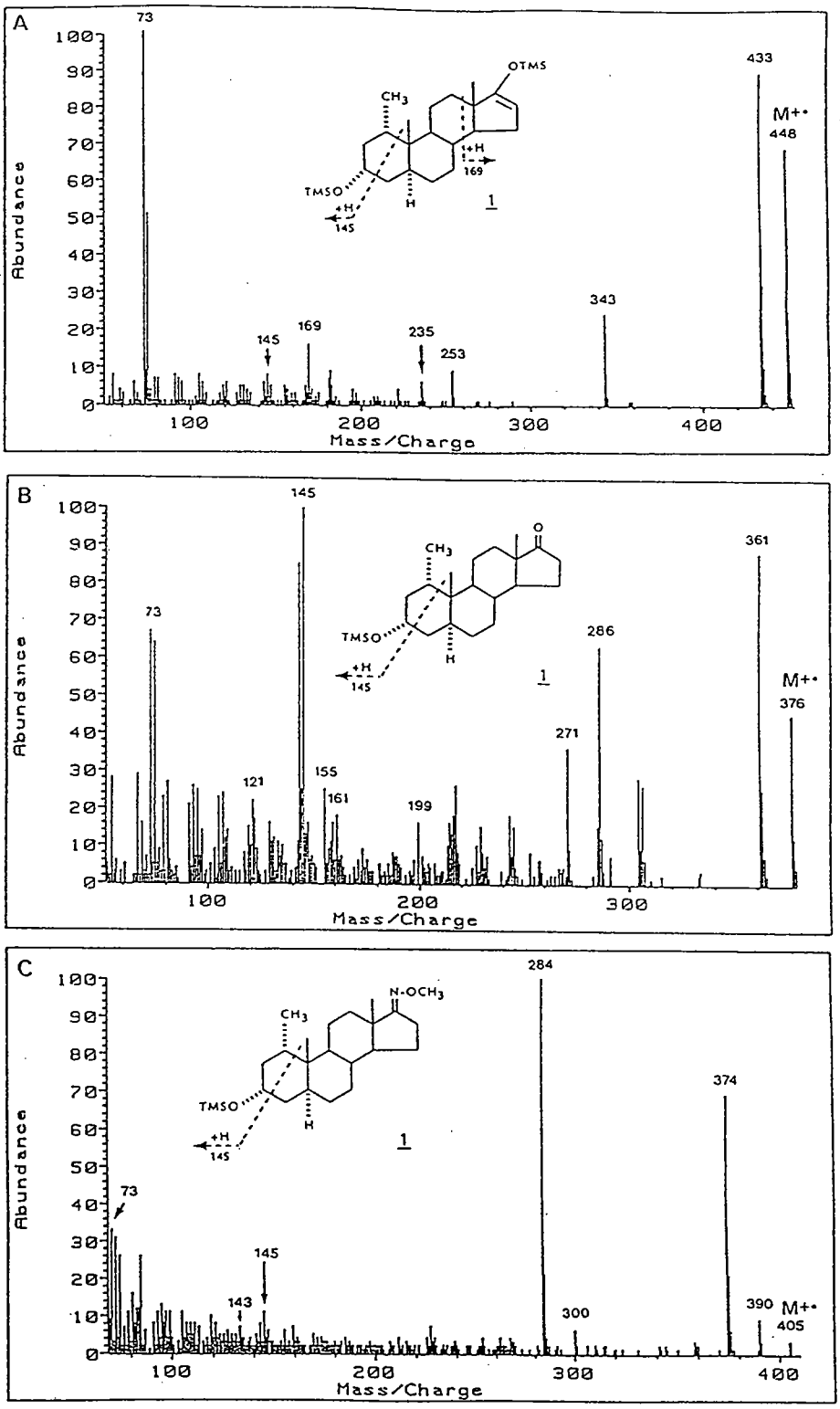
carrier gas: He
injector port: 270°C
transfert line: 310°C
injection mode: splitless 30 sec
initial temperatureof the oven: 100°C(1 min.)
initial rate: 16°C/min.
first temperature: 220°C
final rate: 3.8°C/min.
final temperature: 300°C(10 min.)



GC/MS analysis (full scan mode) of the mesterolone reference urine (glucuronide fraction, TMS-enol, TMS-ether derivative) 6h post administration.



Mass spectra of urinary mesterolone 7: A)TMS-enol, TMS-ether and B) TMS-ether derivatives.



Mass spectra of urinary 3α-hydroxy-1α-methyl-5α-androstan-17-one 1: A) di-TMS, B) mono-TMS and C) TMS, methoxime derivatives.

Oxidation with 3α-hydroxysteroid dehydrogenase (3α-HSD)

3α-HSD (E.C. 1.1.1.50) from Pseudomonas Testosteroni

EXPERIMENTALS

A) Specificity of 3α-HSD

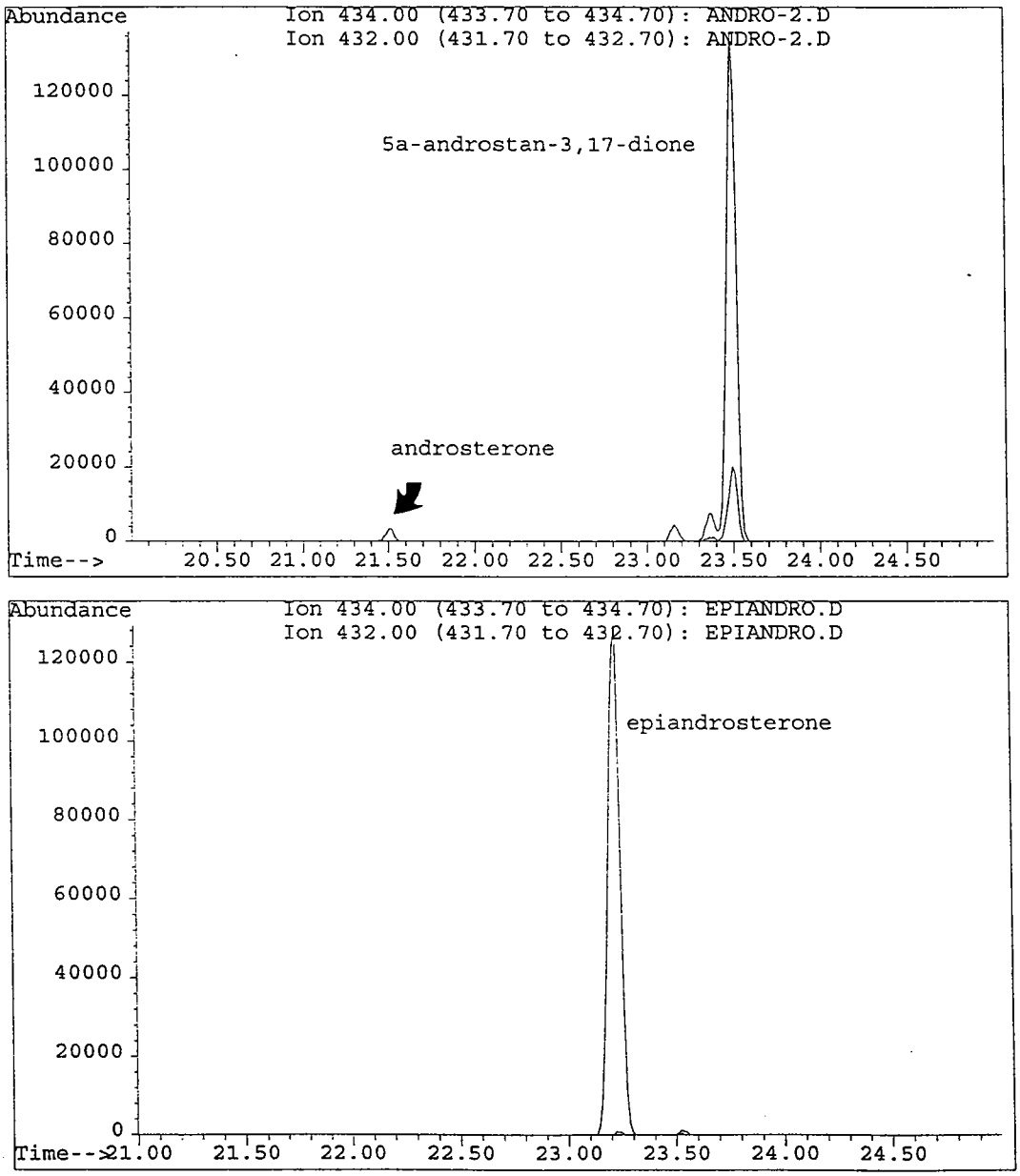
2µg androsterone and epiandrosterone
150µL 3α-HSD (0.1U/mL)
140µL NAD^+ 4mM
1mL phosphate buffer 0.1M pH 8.5

Incubation 1h/37°C
Extraction with 5mL of diethylether
TMS enol-TMS ether derivatization

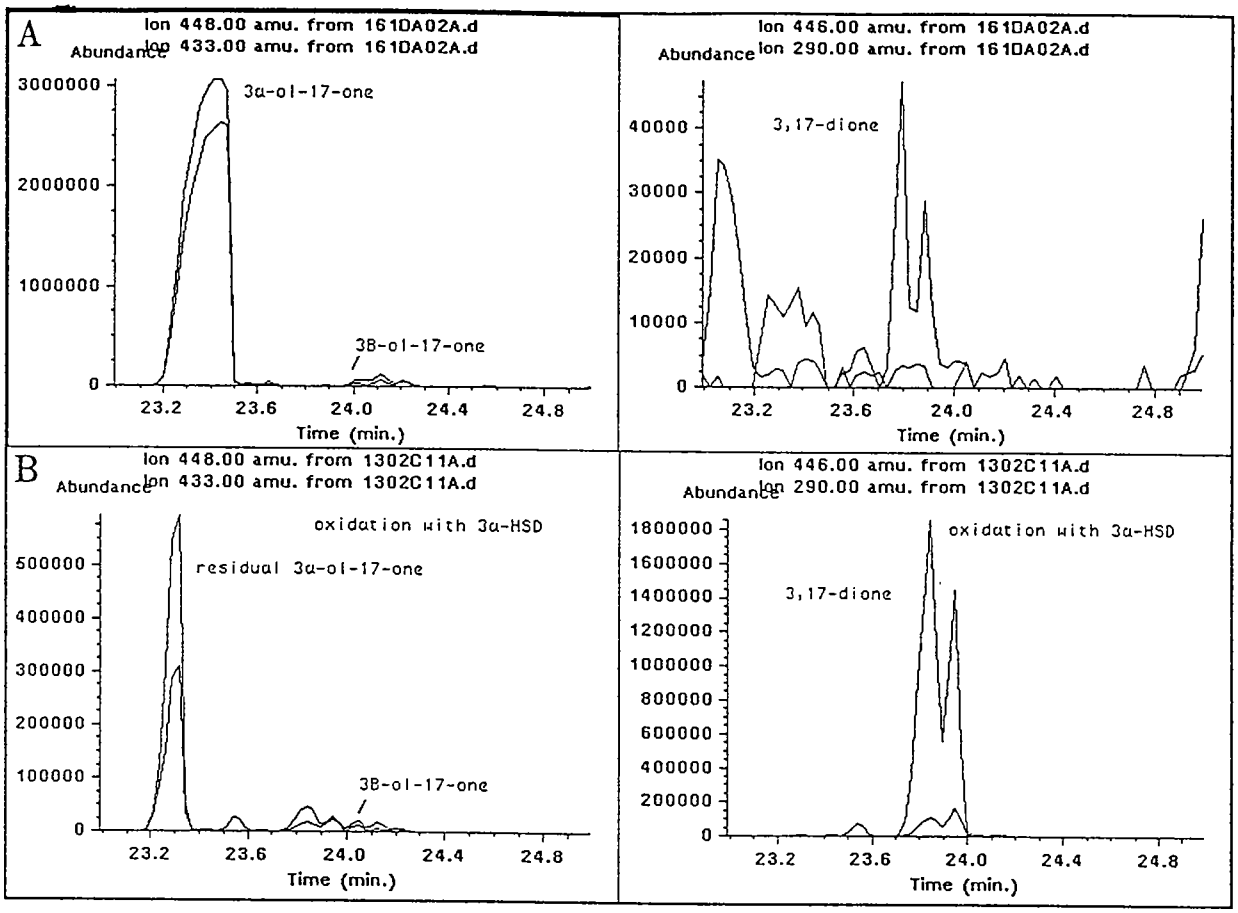
B) Oxidation of urinary fractions (according to Kudo 1990)

urinary fractions (100µL in methanol)
1mL 3α-HSD (2U/mL)
250µL NAD^+ 12mM
2mL phosphate buffer 0.1M pH 8.5

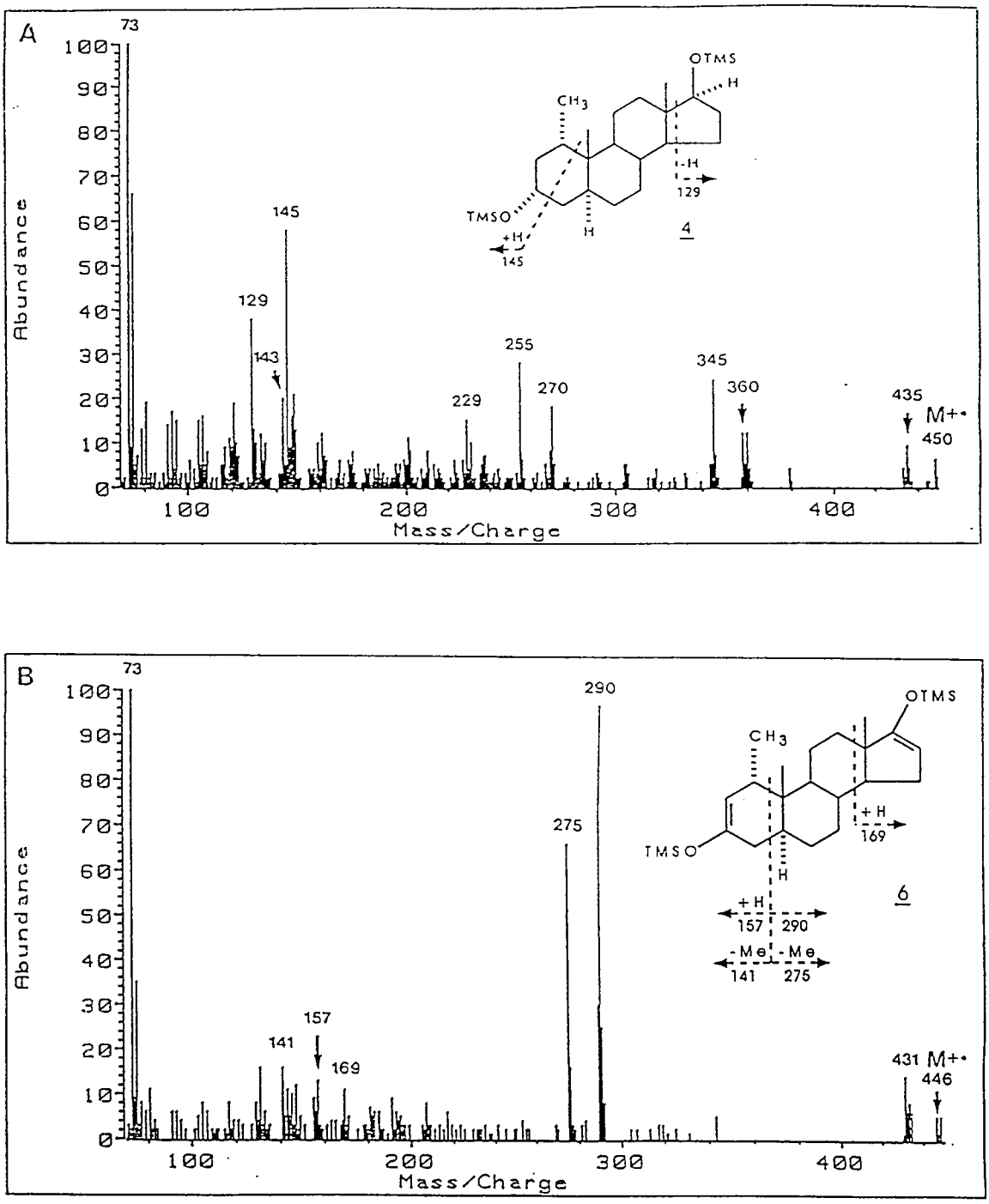
Incubation 3h/37°C
Extraction with 5mL of diethylether
Derivatization



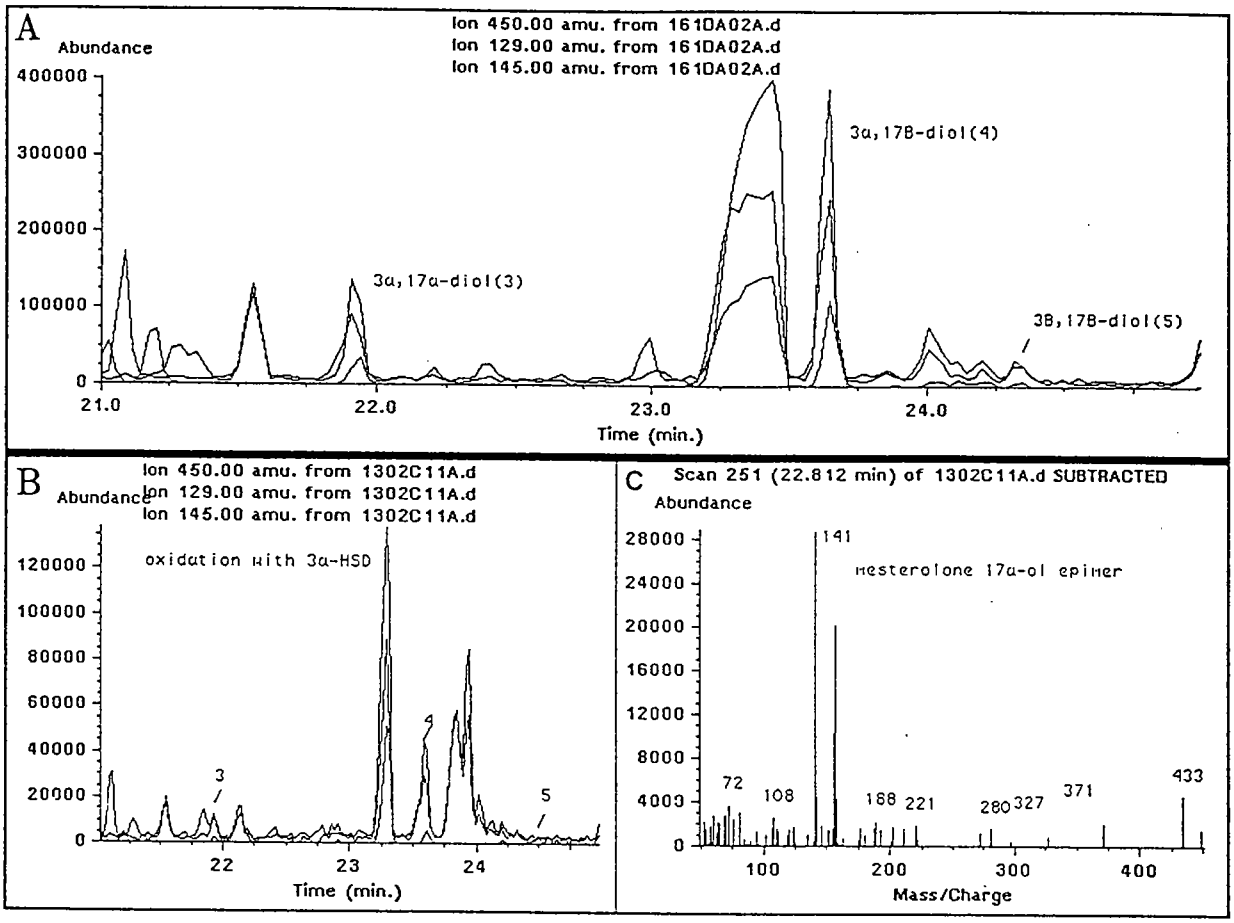
GC/MS analyses (full scan mode): A) androsterone oxidized to 5α-androstan-3,17-dione and B) epiandrosterone unchanged following incubation with 3α-HSD.



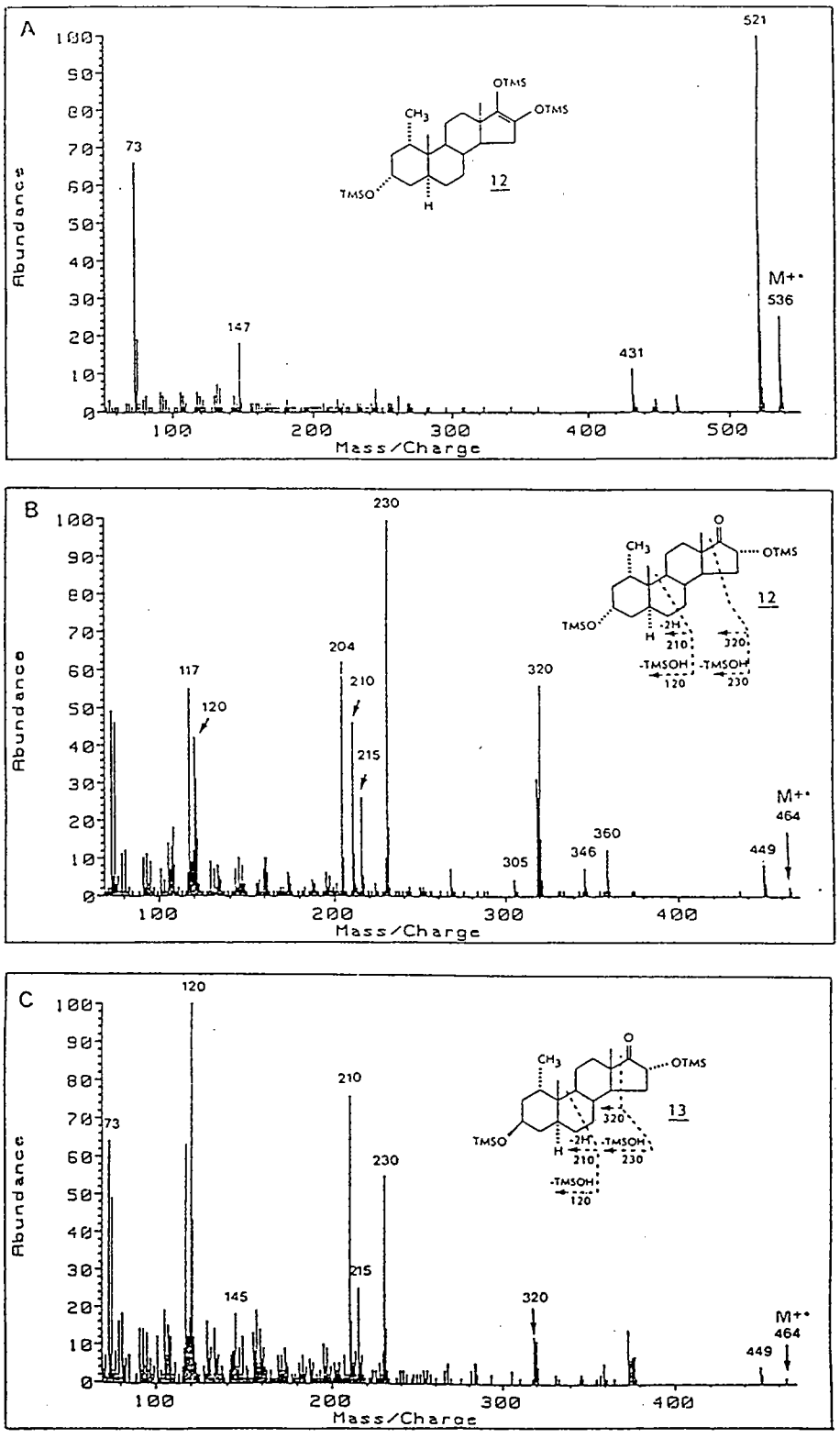
GC/MS analyses (full scan mode) showing mesterolone metabolites 3α- and 3ß-hydroxy-1α-methyl-5α-androstan-17-one 1 and 2 A)without and B) following incubation with 3α-HSD. (glucuronide fraction, TMS-enol, TMS-ether derivative)



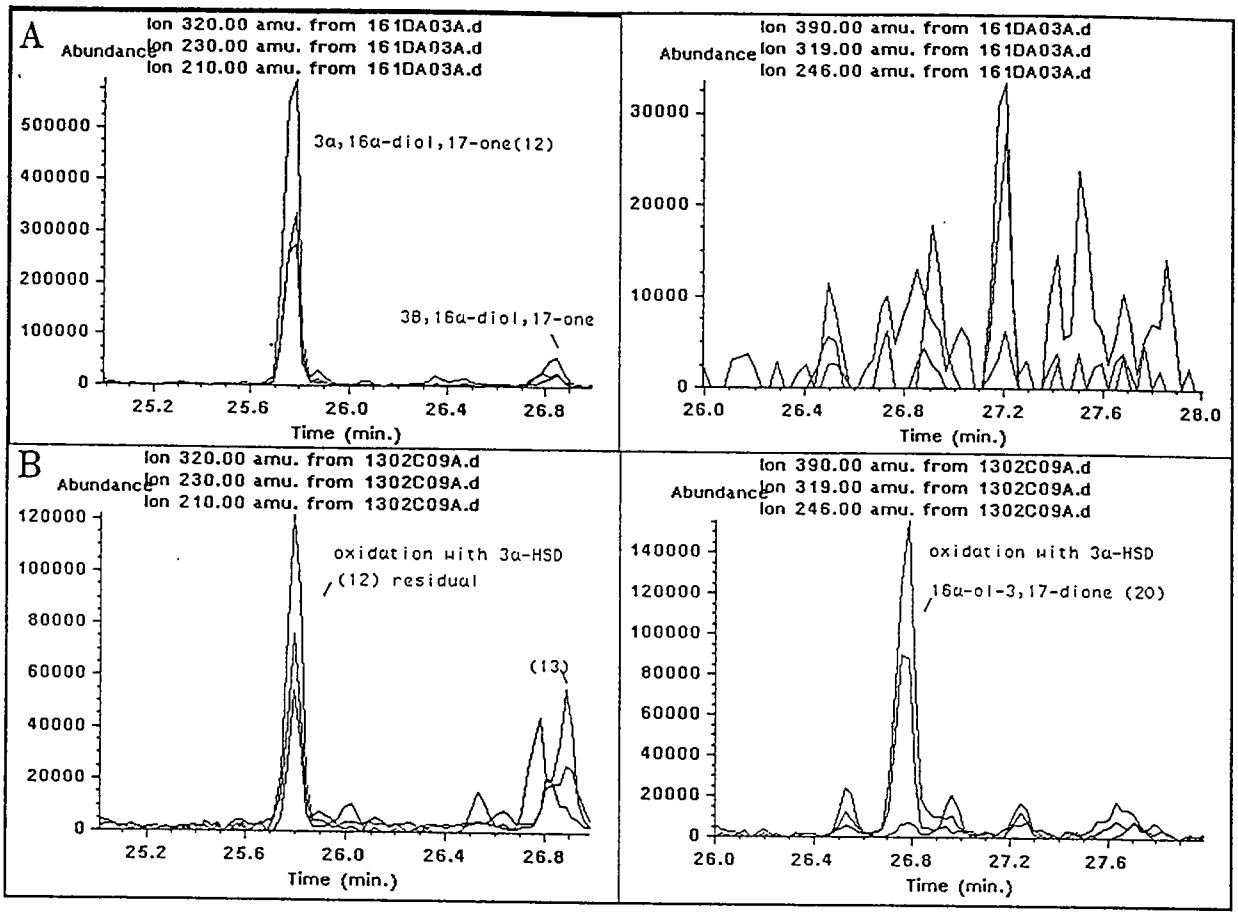
Mass spectra of urinary mesterolone metabolites A) 3α,17ß-dihydroxy-1α-methyl-5α-androstane 4 as TMS-ether derivative and B) 1α-methyl-5α-androstan-3,17-dione 6 as TMS-enol, TMS-ether derivative.



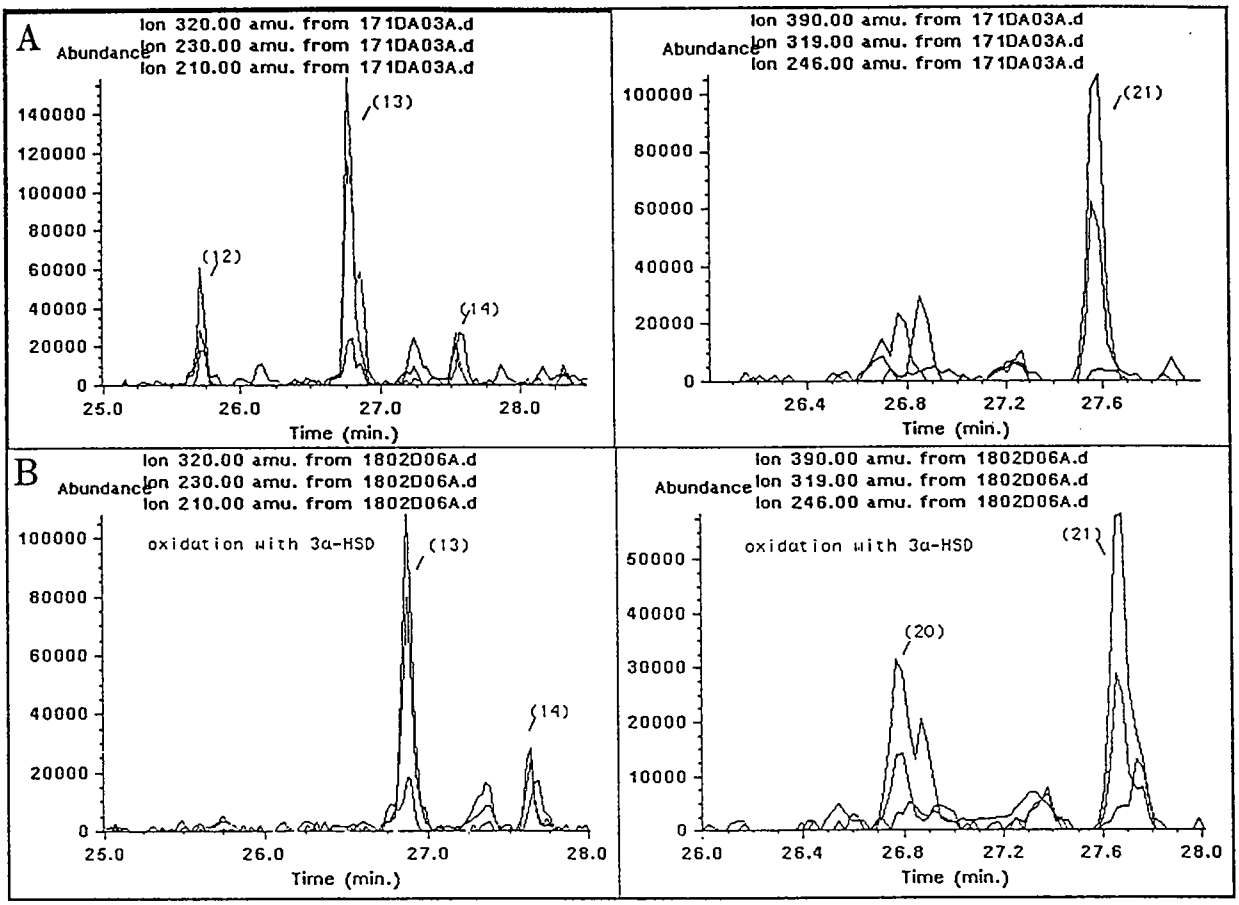
GC/MS analyses (full scan mode) showing urinary 3,17-dihydroxy-1α-methyl-5α-androstane A) without and B) following incubation with 3α-HSD. (glucuronide fraction, TMS-ether derivatives, proposed structures)



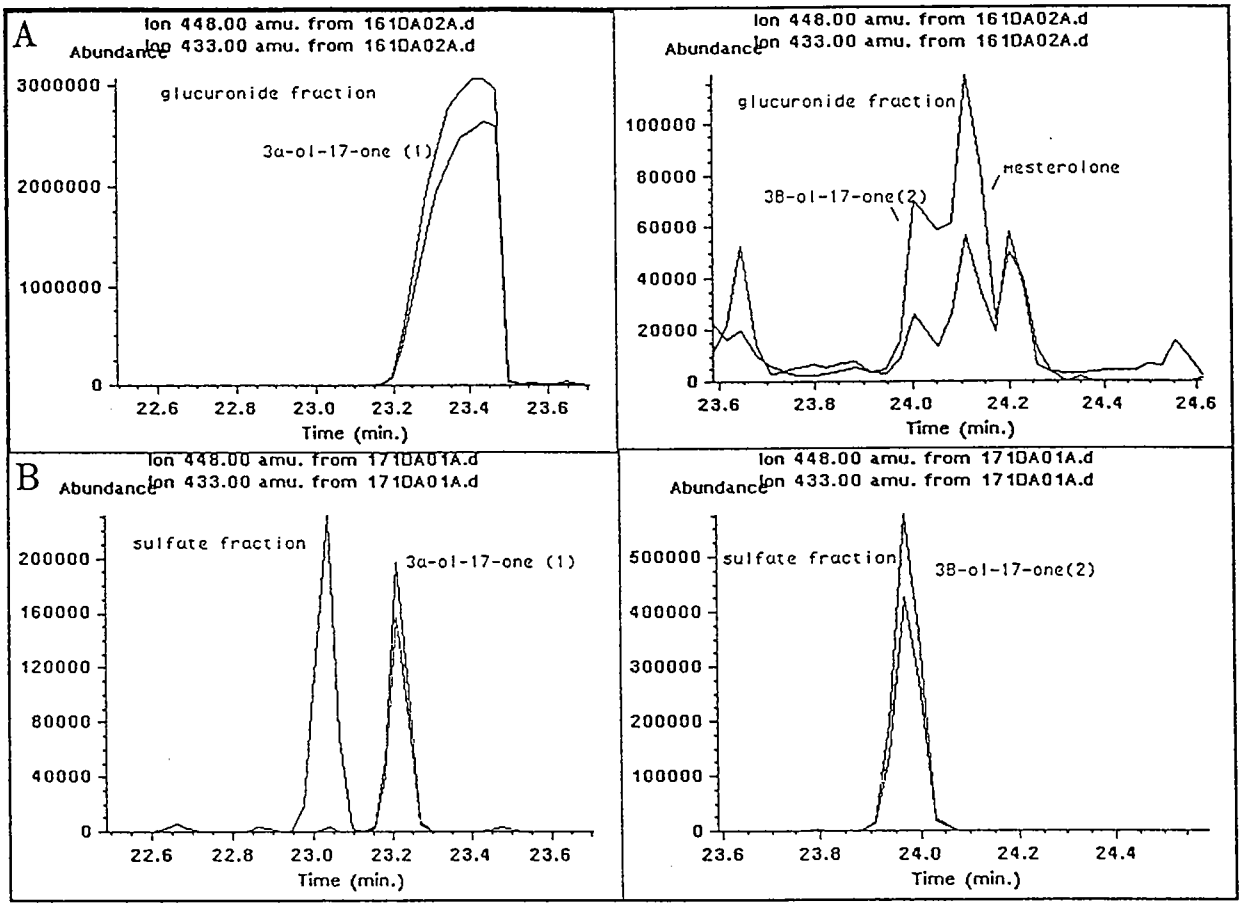
Mass spectra of urinary 3α,16α-dihydroxy-1α-methyl-5α-androstan-17-one 12 as A) TMS-enol,TMS-ether, B) TMS-ether derivatives and C)3ß,16α-dihydroxy-1α-methyl-5α-androstan-17-one 13 as TMS-ether derivative. (proposed structures)



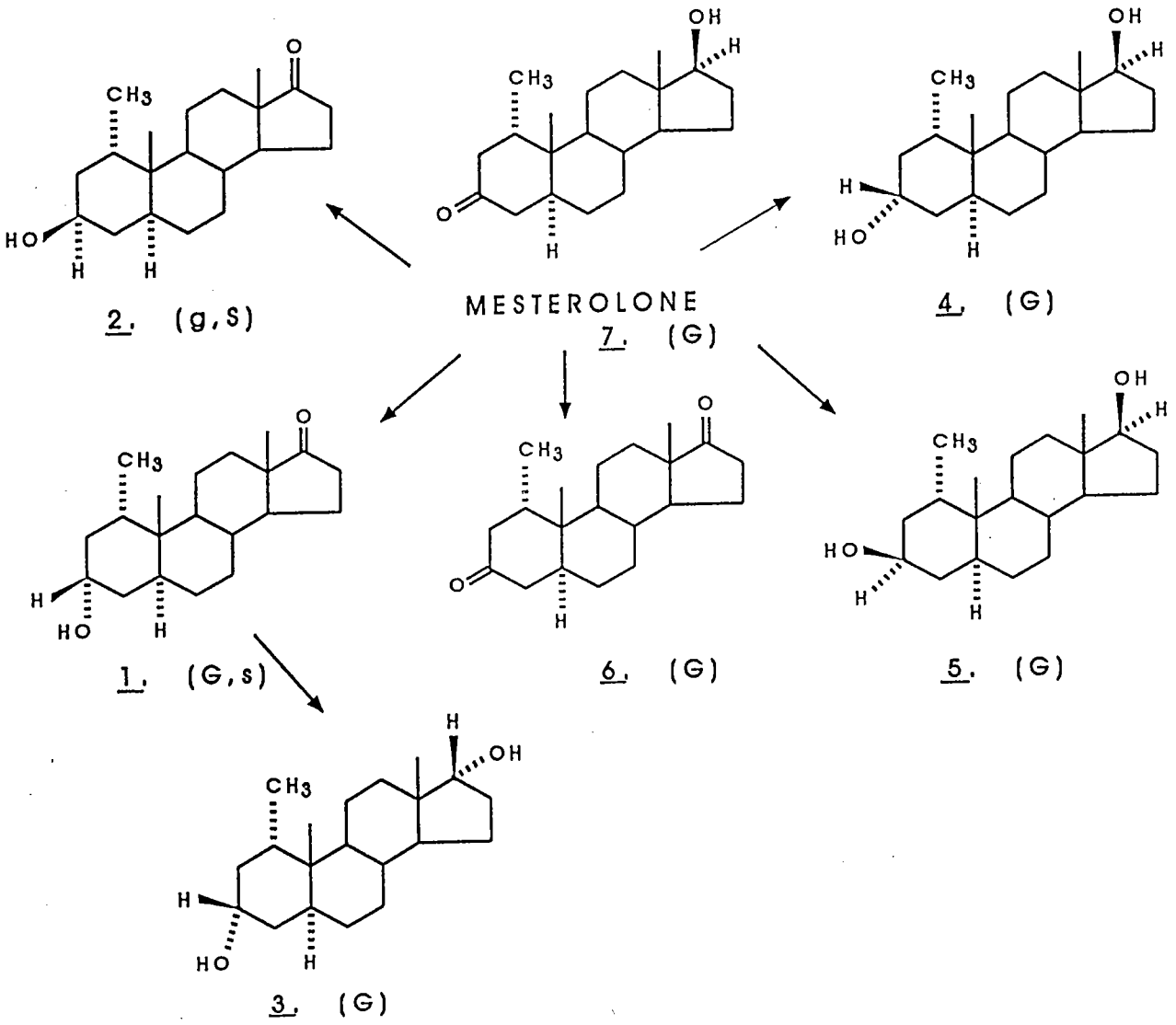
GC/MS analyses (full scan mode) showing urinary 3,16-dihydroxy-1α-methyl-5α-androstan-17one 12 and 13 A) without and B) following incubation with 3α-HSD. (glucuronide fraction, TMS-ether derivatives)



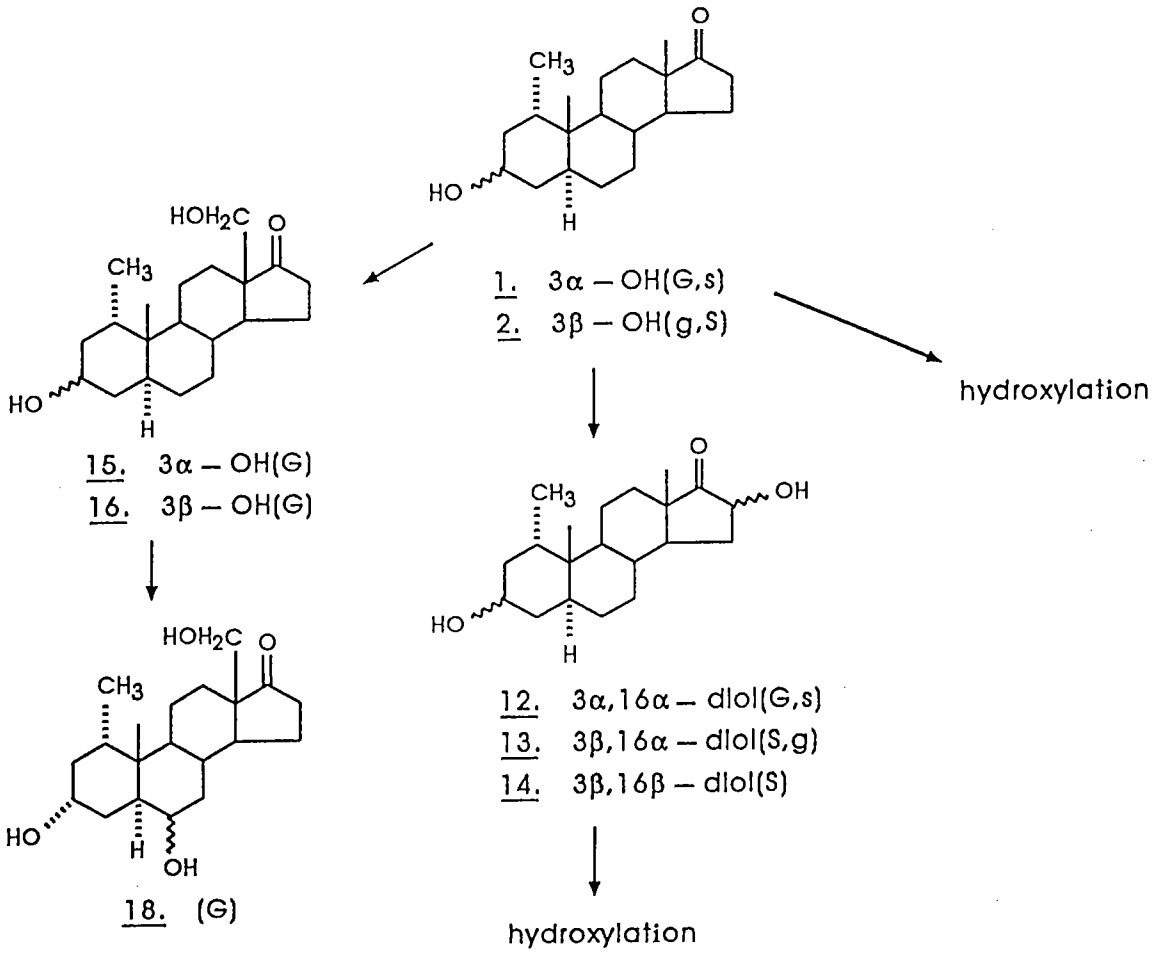
GC/MS analyses (full scan mode) showing urinary 3,16-dihydroxy-1α-methyl-5α-androstan-17-one 12, 13 and 14 A) without and B) following incubation with 3α-HSD. (sulfate fraction, TMS-ether derivatives)



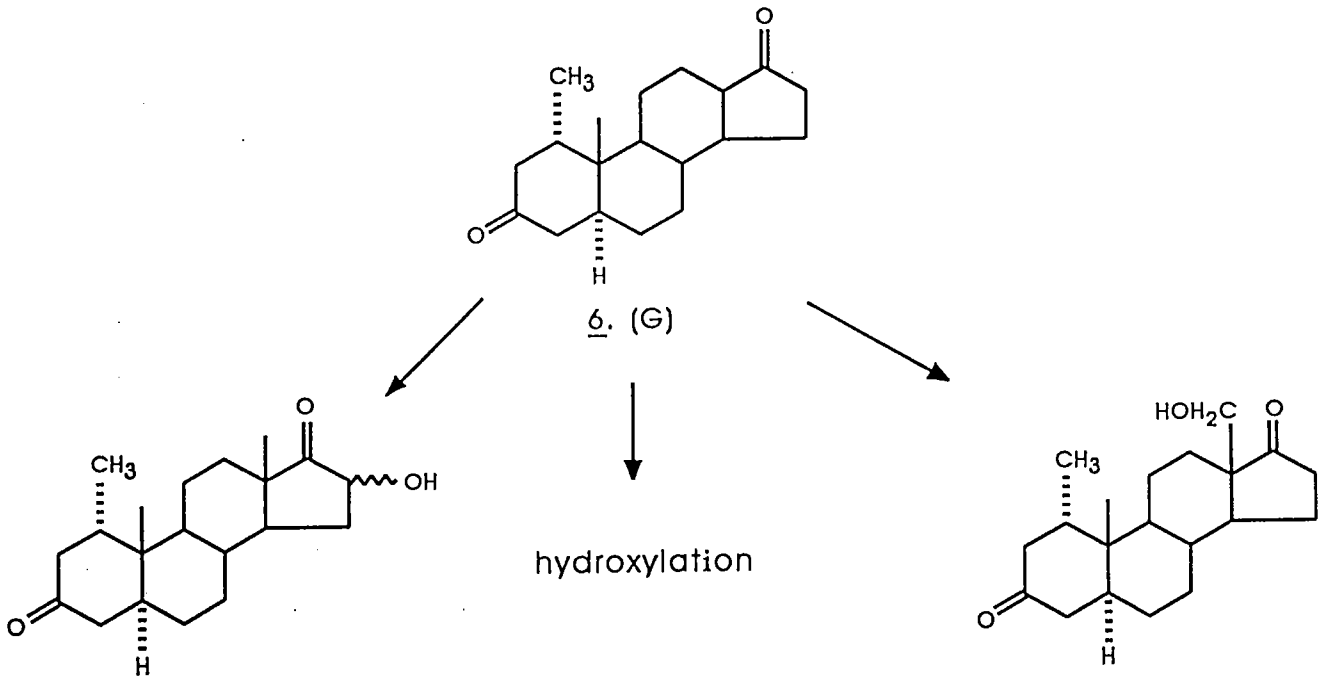
GC/MS analyses (full scan mode) of urinary 3α- and 3ß-hydroxy-1α-methyl-5α-androstan-17-one 1 and 2 as TMS-enol, TMS-ether derivatives of A) glucuronide and B) sulfate fractions.



Proposed pathways of mesterolone metabolism in human.

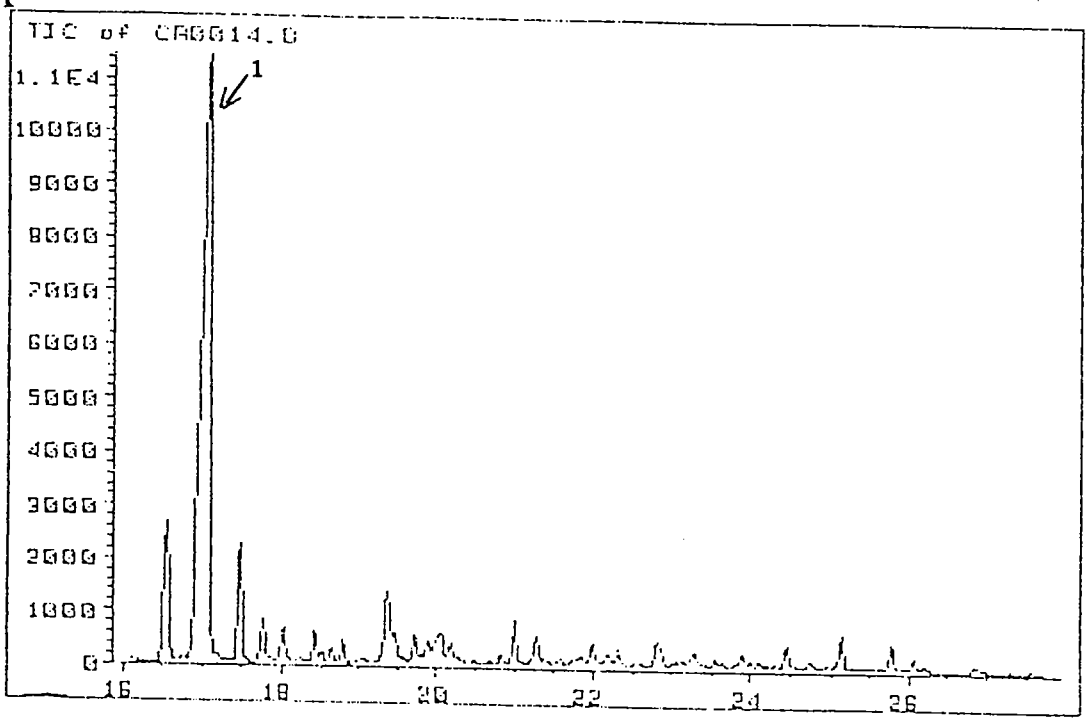


Proposed pathways of mesterolone metabolism in human.

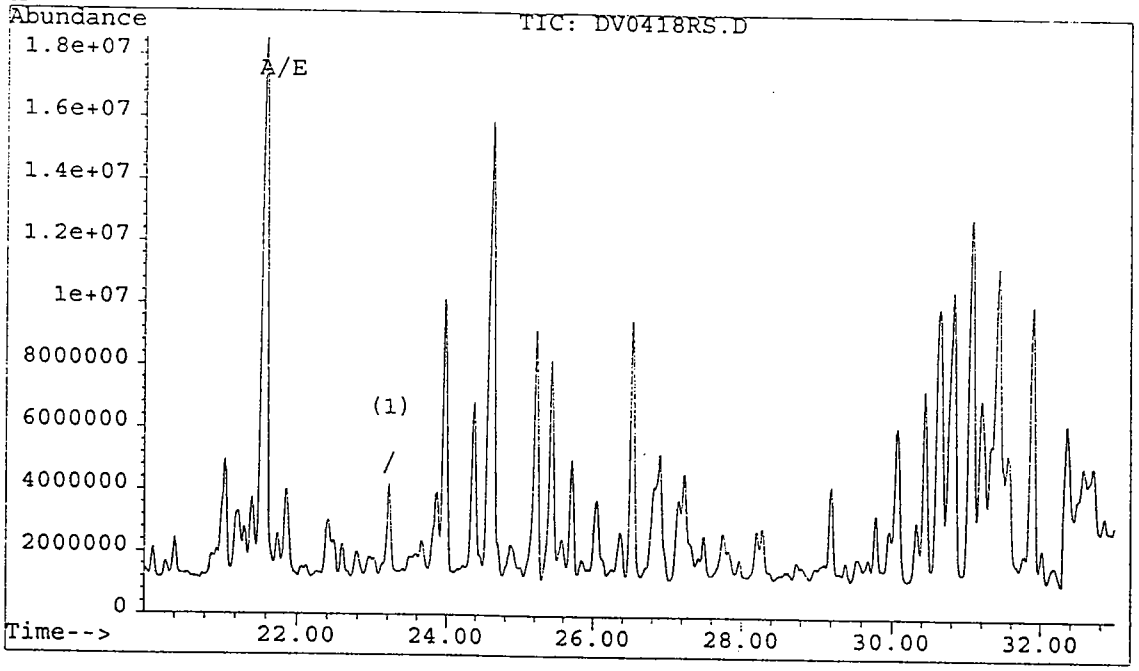


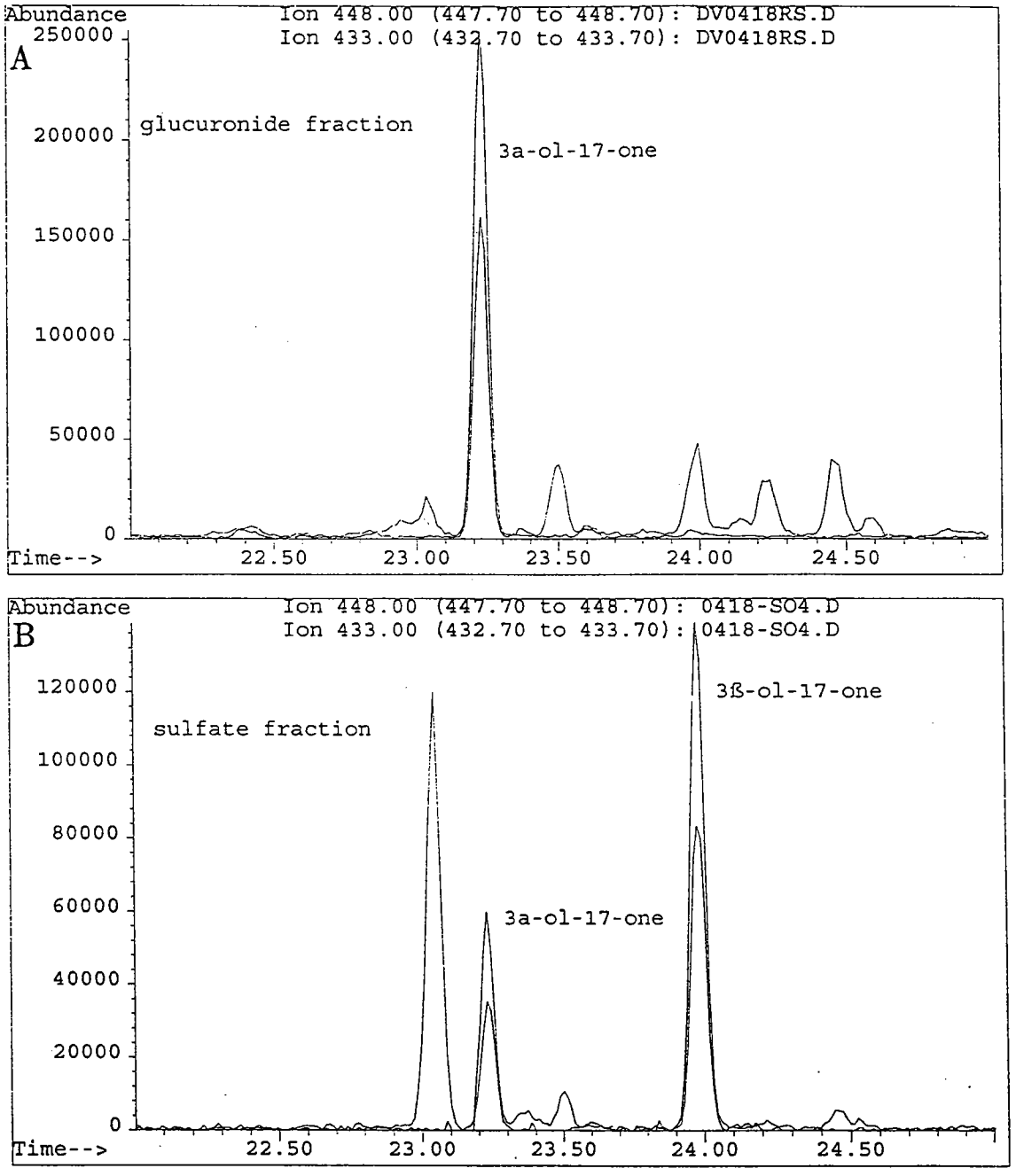
Proposed pathways of mesterolone metabolism in human.

A

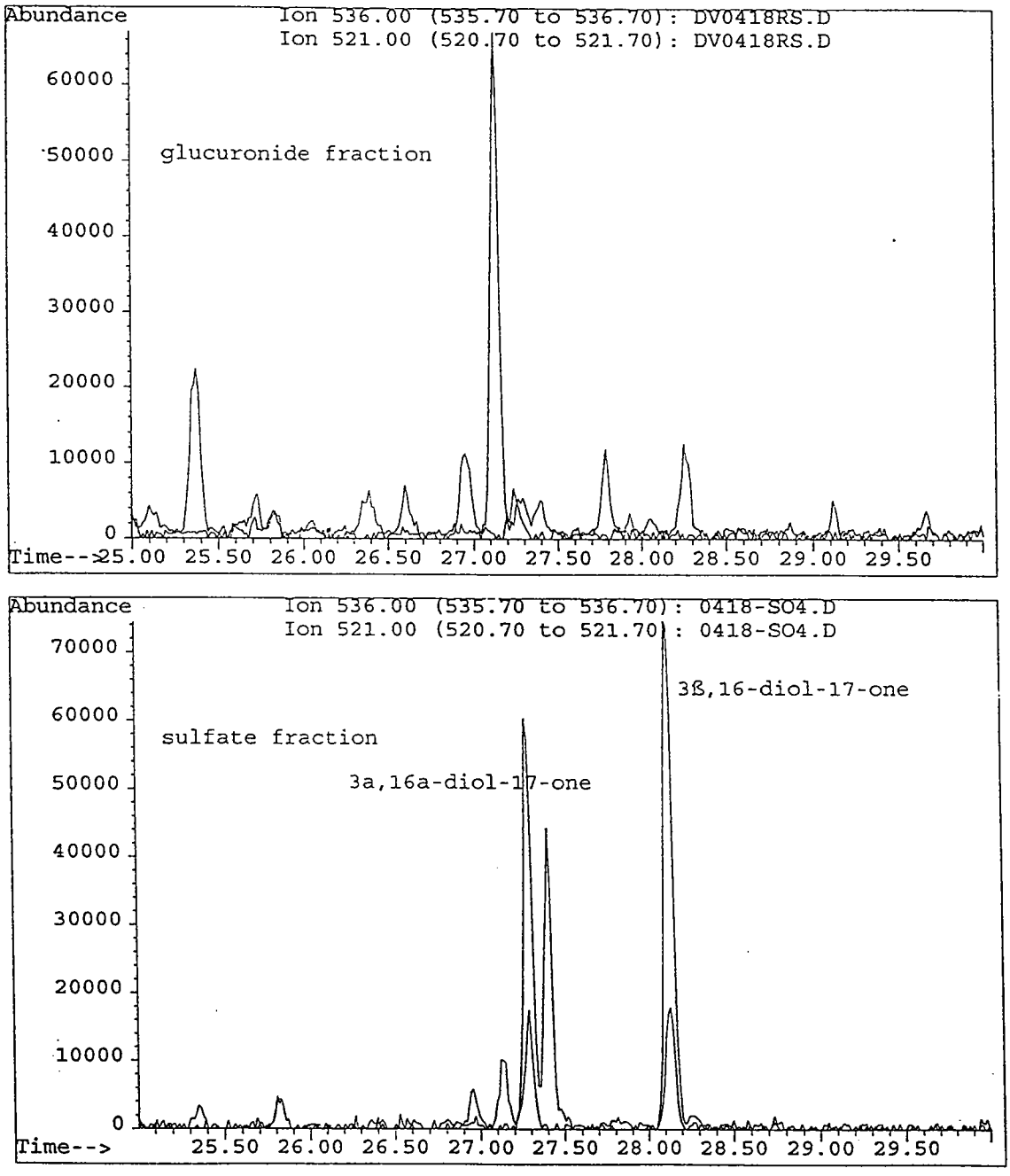

TIC of CRGG14.D
1.1E4
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
1
16
18
20
22
24
26

B


Abundance
TIC: DV0418RS.D
1.8e+07
1.6e+07
1.4e+07
1.2e+07
1e+07
8000000
6000000
4000000
2000000
0
A/E
(1)
Time-->
22.00
24.00
26.00
28.00
30.00
32.00



GC/MS analyses (full scan mode) of mesterolone control urine. (TMS-enol, TMS-ether derivatives)



GC/MS analyses (full scan mode) of mesterolone control urine. (TMS-enol, TMS-ether derivatives)