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RECENT ADVANCES IN DOPING ANALYSIS (2)

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Determination of Free Methyltestosterone in Plasma Samples
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Determination of free methyltestosterone in plasma samples[1]

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Free methyltestosterone (17α-methylandrost-4-en-3-one-17β-ol) was measured in plasma samples provided by 19 male volunteers during the course of a double-blind study in which they were administered placebos (3 days), methyltestosterone, 40mg/day for 3 days, methyltestosterone, 240mg/day for 3 days, (total : 840mg).

The plasma samples analysed were collected during placebos administration, 13 hours after the last administered dose of methyltestosterone and three days after.

Methyltestosterone was measured by GC/MS in the SIM (selected ion monitoring) mode using mibolerone (7α,17α-dimethylestr-4-en-3-one-17β-ol) as internal standard. Steroids were isolated on Sep Pak C_{18} cartridges and analysed as their TMS-ether, TMS-enol derivatives. A limit of detection of 2ng/mL (6.6 nmole/L) for an initial volume of 200μL of plasma (S/N : 2.5) was obtained.

Calibration curves were established with authentic standards and verified with plasma samples spiked with known concentrations during the GC/MS analysis. The average methyltestosterone concentration was 63.4 $\pm$ 73.5 nmole/L (from 0 to 100 ng/mL, see table of results) 13 hours after the last dose. No methyltestosterone was detected during the placebo and withdrawal periods. Other methyltestosterone metabolites were identified in one plasma sample after the hydrolysis with β-glucuronidase from *E. coli* : epi methyltestosterone (free), 17α-methyl-5β-androstan-3α,17β-diol and its 17β-epimer.

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[1] This study was performed with plasma samples provided by T.Su *et al.*: Neuropsychiatric Effects of Anabolic Steroids in Male Normal Volunteers, J.A.M.A., 269, 2760 (1993), NIMH, Bethesda, Md. The whole protocol of administration, sample collection was determined by these authors.

In a typical experiment, 10ng of mibolerone was added to 200μL of plasma. After equilibration at 37°C for 1 hour, the sample was passed through a Sep Pak C_{18} cartridge (previously washed with methanol and water). After washing with 5mL of distilled water, steroids were eluted with 5 mL of methanol. The solvent was evaporated under a nitrogen stream, 1 mL of phosphate buffer (pH 6.9) was added and the extraction of free steroids was done with twice 5mL of diethyl ether. The TMS-ether, TMS-enol derivatives were obtained with MSTFA : TMSI : dithioerythritol (50μL, final volume).

After careful removal of residual diethyl ether, the conjugate steroids were isolated from a Sep Pak C_{18} cartridge and hydrolysed with β-glucuronidase (*E. coli*, type 1X-A) in 1mL of phosphate buffer (pH 6.9), at 55°C for 30 min. Extraction and derivatization were done as previously described.

The GC/MS (SIM) analysis (in duplicate) was carried out in the splitless mode by monitoring characteristic ions 446.4 and 301.3 amu.

Table 1 of results :

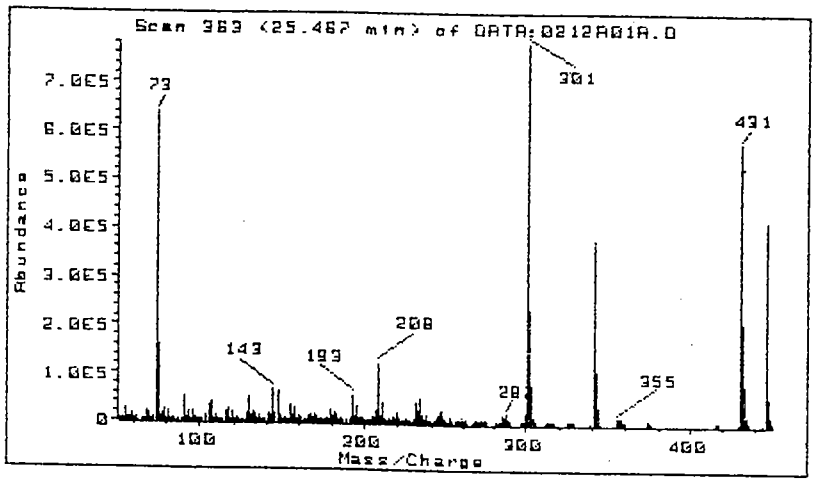
Plasma sample	Methyltestosterone concentration (ng/mL)
1B (D12)	30.39
2B (D12)	11.75
3B (D12)	49.49
4B (D12)	102.04
5B (D12)	7.83
6B (D12)	24.01
7B (D12)	5.64
8B (D12)	5.45
10B (D12)	26.99
11B (D12)	8.65
12B (D12)	9.74
13B (D12)	13.22
14B (D12)	9.98
15B (D12)	6.99
16B (D12)	7.54
17B (D12)	21.44
18B (D12)	20.27
19B (D12)	10.16
20B (D12)	9.78

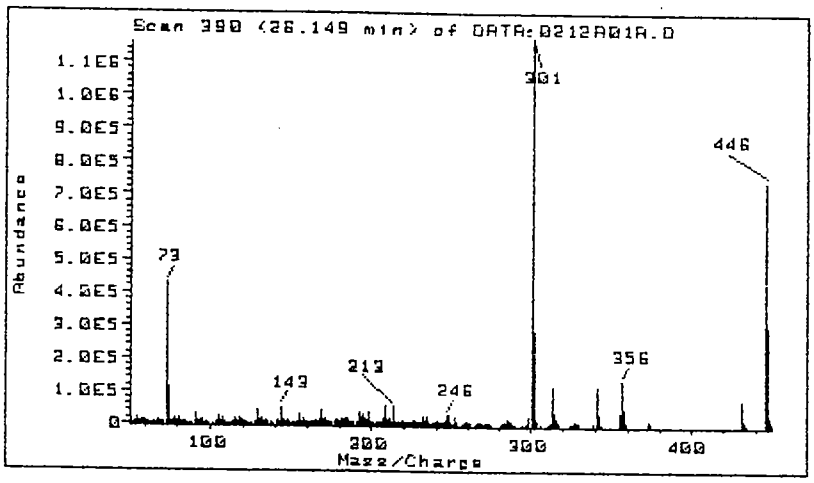
Procedure IV

GC/MS analyses in the SIM (selected ion monitoring) mode are carried out with Hewlett Packard HP 5890 gas chromatographs with direct coupling to HP-MSD 5970 quadrupolar filters. Systems are equipped with automatic samplers 7673 A and controlled by HP 59970 and Unix MS Chemstations. The injections are carried out in the splitless mode (1 μL) and the separation is achieved on HP-5 capillary columns.

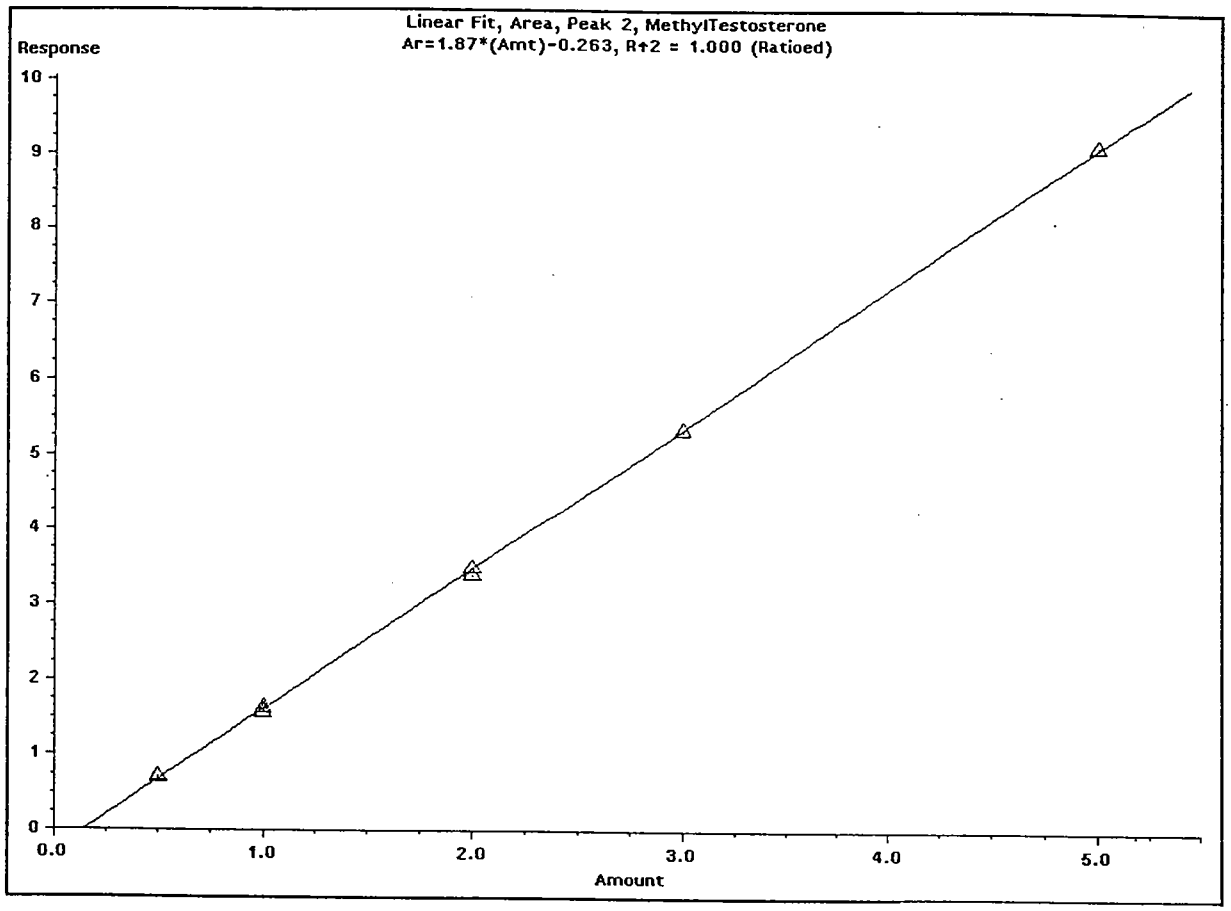
Chromatographic parameters

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



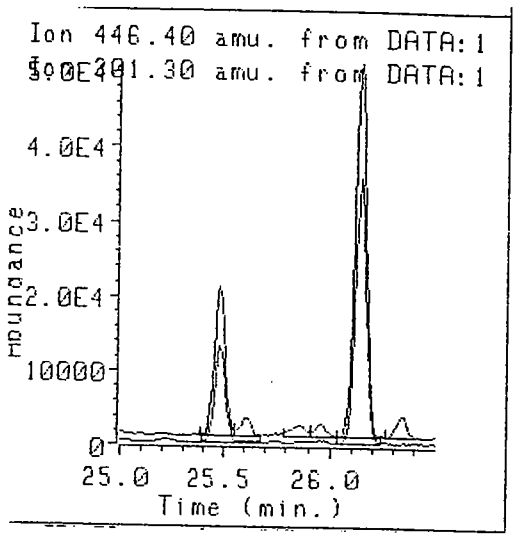


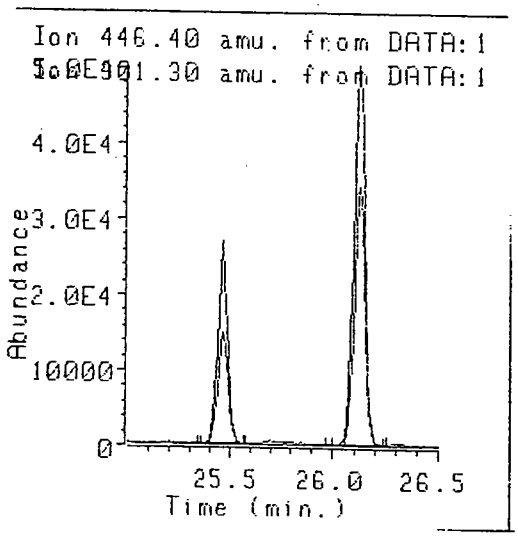
Mass spectra of TMS-ether, TMS-enol derivatives of mibolerone (upper) and methyltestosterone (10ng/μL injected).



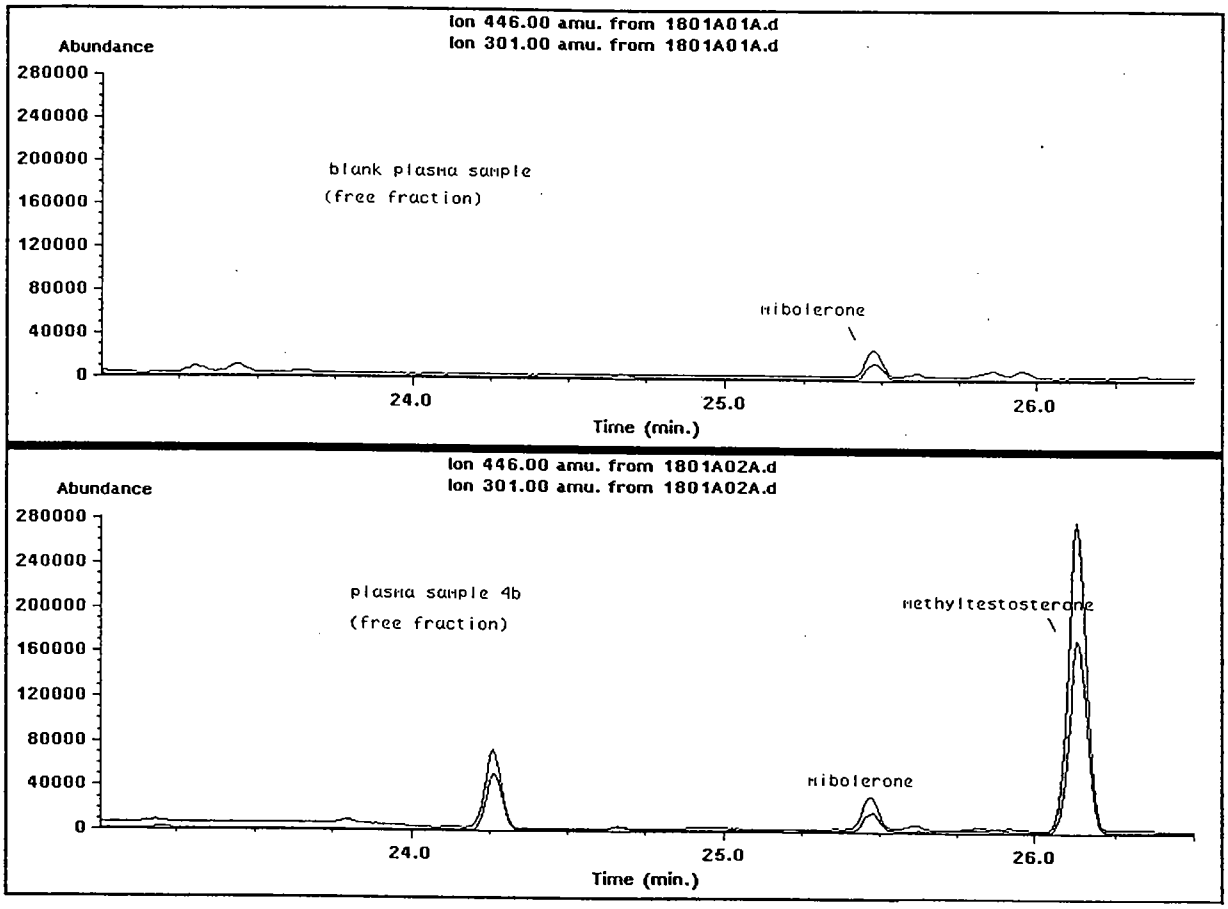
Ret Time	Pk#	Signal Descr	Amt ng/ul	Lvl	Area	Pk-Type	Partial Name
25.462	1	301.30 amu	0.2000000	10	744428	+I	Mibolerone
			0.2000000	7	749578		
			0.2000000	9	797201		
			0.2000000	8	827902		
			0.2000000	1	847274		
			0.2000000	3	848787		
			0.2000000	4	1002510		
			0.2000000	2	1032320		
26.120	2	301.30 amu	0.1000000	1	601599		MethylTestost
			0.1000000	2	738487		
			0.2000000	3	1376930		
			0.2000000	4	1579960		
			0.4000000	7	2614880		
			0.4000000	8	2803520		
			0.6000000	9	4256240		
			1.000000	10	6797970		

Calibration curve : authentic standards of methyltestosterone (from 5 to 50 ng/mL, 16 to 160 nmole/L) and mibolerone.

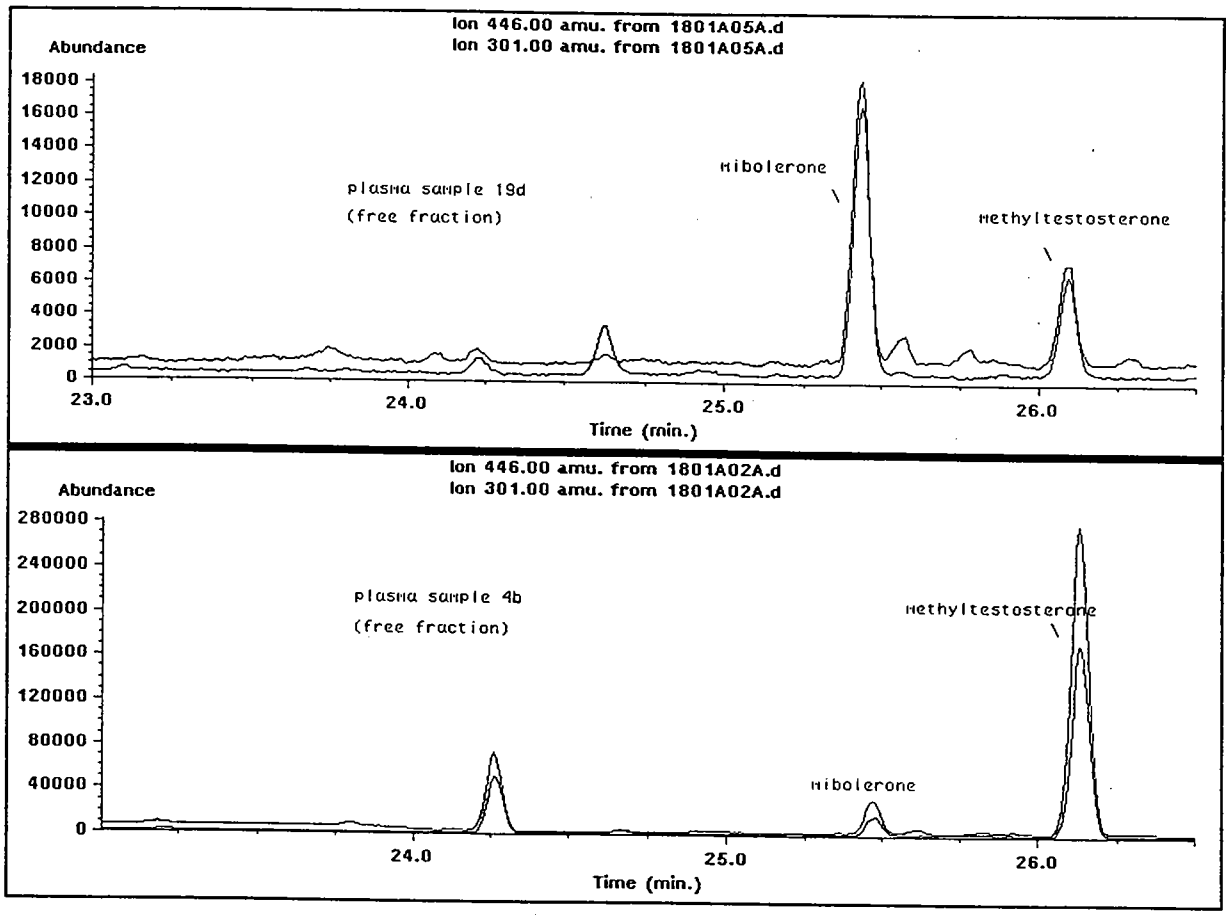




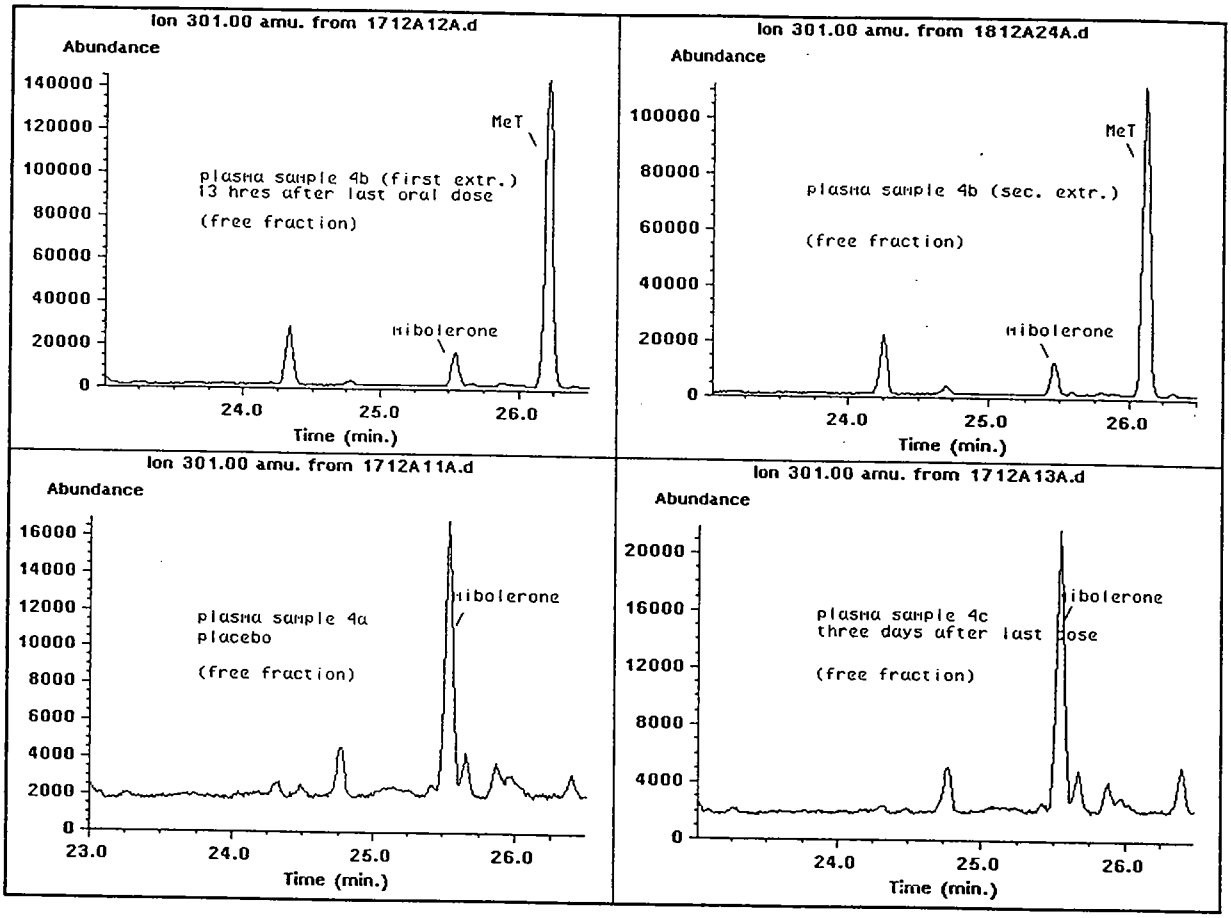
Mibolerone (0.2ng/μL) and methyltestosterone (0.3ng/μL) authentic standards and spiked and extracted in a plasma blank sample.



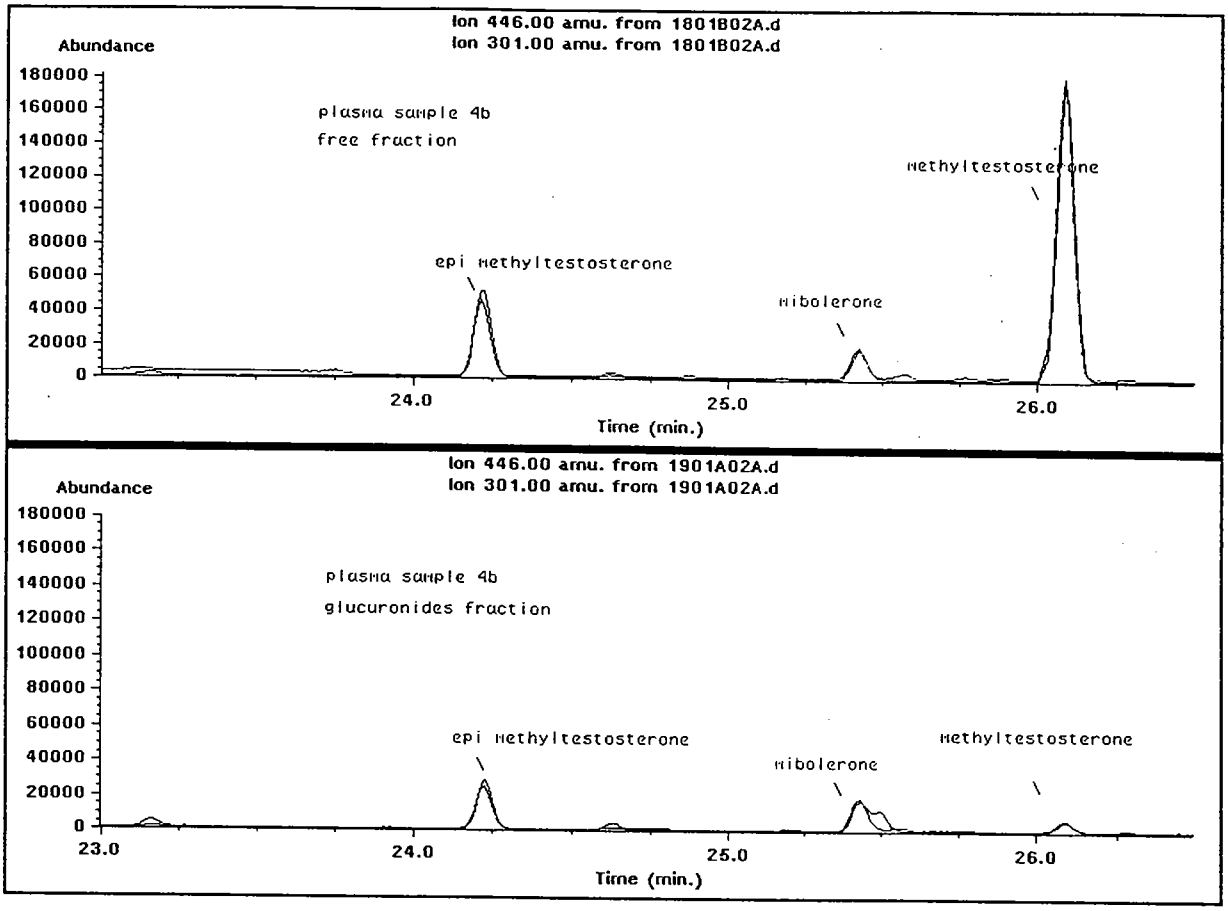
GC/MS analysis of the free fraction of a blank plasma sample (200μL) and plasma sample #4b (500μL).



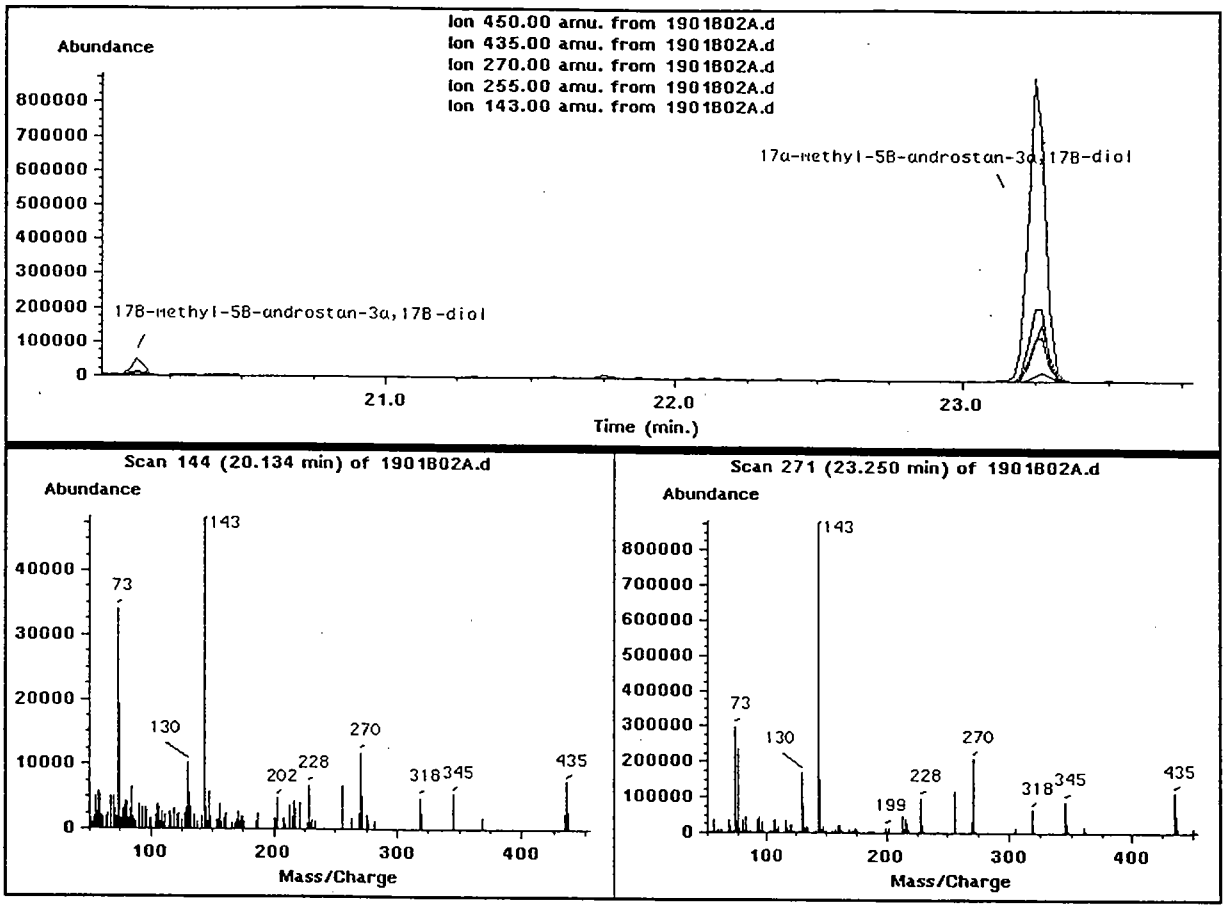
GC/MS (SIM mode) analysis of plasma samples #19d and #4b.



GC/MS (SIM mode) analysis of plasma samples #4a, b (duplicates), c.



GC/MS (SIM mode) analysis of plasma sample #4b, free and glucuronides fractions.



GC/MS (full scan mode) analysis of the glucuronides fractions of plasma sample #4b.