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RECENT ADVANCES IN DOPING ANALYSIS (7)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

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J. GROSSE, D. THIEME, R. LANG, R.K. MUELLER:
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Grosse J., D. Thieme, R. Lang, R. K. Mueller

Formebolone Detection

Institute of Doping Analysis and Sports Biochemistry,
Dresdner Straße 12, D-O1731 Kreischa (near Dresden) GERMANY

Introduction

The increasing number of substances to be included into the screening analysis of a doping control sample requires the rationalisation of the screening procedures employed. Concerning the anabolic steroids, one acceptable approach is the analysis of the combined steroid fraction. Giving up the information of a separate procedure for the free steroid fraction, the detection of the analytes of interest may be influenced positively *(p)* or negatively *(n)* by

- disturbing matrix interferences *(n)*
- the formation of other derivatives *(p/n)*
- an increased concentration in the case of an existing conjugated moiety *(p)*.

The signal of the main formebolone metabolite (2-hydroxymethyl-11α-hydroxy-metan-dienone) in the screening of the combined steroid fraction is strongly interfered by co-eluting corticosteroid metabolites, thus making the evaluation of the screening data unsatisfactory. The aim of this study was to find metabolites of formebolone, which are suitable for sensitive formebolone detection in the combined steroid fraction.

Experimental

Excretion Study

20 mg of formebolone (ESICLENE, 4 tablets of 5 mg) were administered to a male volunteer (43 years old, kaukasian race, 176 cm, 78 kg) as one single dose. Urine samples were collected after 2, 6, 8, 11, 20, 30, 35, 44, 52, 72, 96, 120, 144 and 168 hours.

Sample Preparation

The initial step was an XAD-2 extraction of 2.5-ml urine samples. In order to get information about the conjugation state different steroid fractions were collected:

1. free fraction (extraction by diethyl ether at pH 6)

2. glucuronide fraction (enzymatic hydrolysis by ß-glucuronidase from bovine liver in the residue of 1.)
3. combined fraction (enzymatic hydrolysis by ß-glucuronidase without previous isolation of the free steroid fraction)

Different derivatization procedures were used to get information about the functional groups of the metabolites:

1. "normal" trimethylsilylation by MSTFA/TMS-imidazol to derivatize all hydroxyl groups leaving keto groups unchanged.
2. "enhanced" trimethylsilylation by MSTFA/TMS-iodide to form TMS enol ethers from enolizable keto groups in addition.
3. formation of MO-TMS derivatives as a complementary method.

Instrumentation

1. GC/LR-MS

   All samples were analysed on a benchtop GC/MS (Hewlett-Packard 6890/5973 or GC 8000/MD 800 from Fisons Instruments, resp.).

   GC: 12.5 m Optima-1 MS column (0.2 mm ID, 0.20µm film) oven temperature programmed from 180 °C to 236 °C at a rate of 4 deg/min and then to 310 °C at 10 deg/min; column flow 0.8 ml/min (helium)

   MS: EI mode, 70 eV electron energy
2. GC/HR-MS

   Determination of the elemental composition using an Autospec Ultima Q (R= 3000)
3. HPLC

   Sample clean-up on a Zorbax C18 column using a Hewlett-Packard 1090 LC with fraction collector

Analytical Findings

Unconjugated fraction

As described in literature [1, 2], three major metabolites can be found in the free steroid fraction of a urine sample after administration of formebolone: M I (carboxy-formebolone) is formed by oxidation of the aldehyde group in position 2 and may undergo further reduction and decarboxylation to give M II (11α-hydroxy-methyltestosterone). The other way round, M III (2-hydroxymethyl-11α-hydroxy-metandienone) is the product formed by reduction of the formyl group *(fig. 1 and 5)*.

Glucuronide fraction

In the glucuro-conjugated fraction of the same urine sample M III can be detected in an amount equivalent to that in the free fraction. Obviously, glucuronidation occurs at the primary hydroxyl group of the 2-hydroxymethyl-side chain. In comparison with the blank urine sample before administration there is another distinct peak at RT 18.76 min that could be a characteristic metabolite *(fig. 2)*.

Characterization of the novel metabolite

The EI mass spectrum exhibits a dominating base peak at m/z 707, which represents most likely the $[M-15]^+$; otherwise, it is rather poor of structural information *(fig. 3)*. With the hypothesis of a molecular ion at *m/z* 722, the empirical formula determined by HR-MS accurate mass measurement is $C_{36}H_{70}O_5Si_5$, suggesting a novel metabolite M IV formed by hydroxylation from M III. The study of the mass spectra of the keto-TMS and the MO-TMS derivatives of this metabolite allows a more detailed elucidation. In order to get non-interfered mass spectra the crude extract was purified by HPLC. The fraction containing the novel metabolite was collected for these studies. The obtained EI mass spectra of both the keto-TMS derivative and the MO-TMS derivative *(fig. 3)* support the assumption of a hydroxylated metabolite with one keto group and four hydroxyl groups. Moreover, the mass spectrum of the keto-TMS derivative provides useful structural information concerning the possible position of the introduced hydroxyl group. In comparison with the A/B ring fragments of the TMS derivatives of related hydroxylated metabolites of metandienone [3, 4] and dehydrochloromethyltestosterone [5, 6] the 6ß-position seems to be the most favoured one. The mass differences of the key fragments are correlating with the mass contribution of the A ring substituents, silylated hydroxymethyl side chain or chlorine, respectively *(fig. 4)*.
Hence, the metabolic pathway of formebolone can be supplemented by this hydroxylated and mainly glucuro-conjugated metabolite M IV *(fig. 5)*. GC/MS related data of the formebolone metabolites investigated are summarized in *table 1*.

Excretion profiles

The urinary excretion profiles of metabolites M I – M IV were recorded under routine conditions to evaluate the suitability of the hydroxylated metabolite for screening purposes *(fig. 6)*. Using LR-MS it can easily be detected as its enol-TMS derivative up to 72 hours after administration of 20 mg *(fig. 7)*, certainly longer by HR-MS or MS-MS.

Table 1: GC/MS data of formebolone metabolites

	M I acidic	**M II** decarboxylated	**M III** reduced	**M IV** hydroxylated
M	360	318	346	362
	TMS derivatives			
TMS groups	3	2	3	4
RT in min	19.58	15.79	18.78	19.01
RRT (ChP) *)	0.993	0.801	0.952	0.964
M	576	462	562	650
typical ions	561; 486; 471; **143**	372; 357; 316; **143**	472; 457; 382; 367; 222; **143**	635; 560; 470; 383; 311; **143**
	enol-TMS derivatives			
TMS groups	4	3	4	5
RT in min	19.23	15.65	18.04	18.76
RRT (ChP) *)	0.975	0.794	0.915	0.951
M	648	534	634	722
typical ions	633; 558; 543; 453; 413; **143**	**534**; 444; 429; 389; 143	544; 529; 439; 351;143; **103**	**707**, 619; 143; 103

*) retention time relative to cholesteryl propionate

Confirmation

Because of the few available diagnostic ions, the suitability of the enol-TMS derivative for the confirmation of this hydroxylated metabolite is limited, especially at low concentrations. In those cases other derivatization methods (keto-TMS, MO-TMS) should be applied for unequivocal identification, since the respective mass spectra contain much more structural information.

Summary

A novel hydroxylated metabolite of formebolone was detected. It is excreted in urine mainly as glucuronide. By comparison with the MS data of structurally related steroid metabolites a 6ß-hydroxylation can be proposed. As its enol-TMS derivative this metabolite is suitable for the sensitive detection of formebolone misuse in the screening procedure of the combined steroid fraction. For confirmation purposes other derivatives can be more useful to provide complementary information.

References

(1) Massé, R., Bi, H. and Du, P., Anal Chim Acta, 247 (1991), 211-221

(2) Cartoni, G.P., Ciardi, M., Giarrusso, A. and Rosati, F., J High Res Chrom, 14 (1991), 838-842

(3) Dürbeck, H.W. and Büker, I., Biomed Mass Spectr, 7 (1980), 437-445

(4) Schänzer, W., Geyer H. and Donike M., J Steroid Biochem Molec Biol, 38 (1991), 441-464

(5) Dürbeck, H.W., Büker, I. and Scheulen, B., J Chrom Sci, 21 (1983), 405-410

(6) Schänzer, W. and Donike M., Anal Chim Acta, 275 (1993), 23-48

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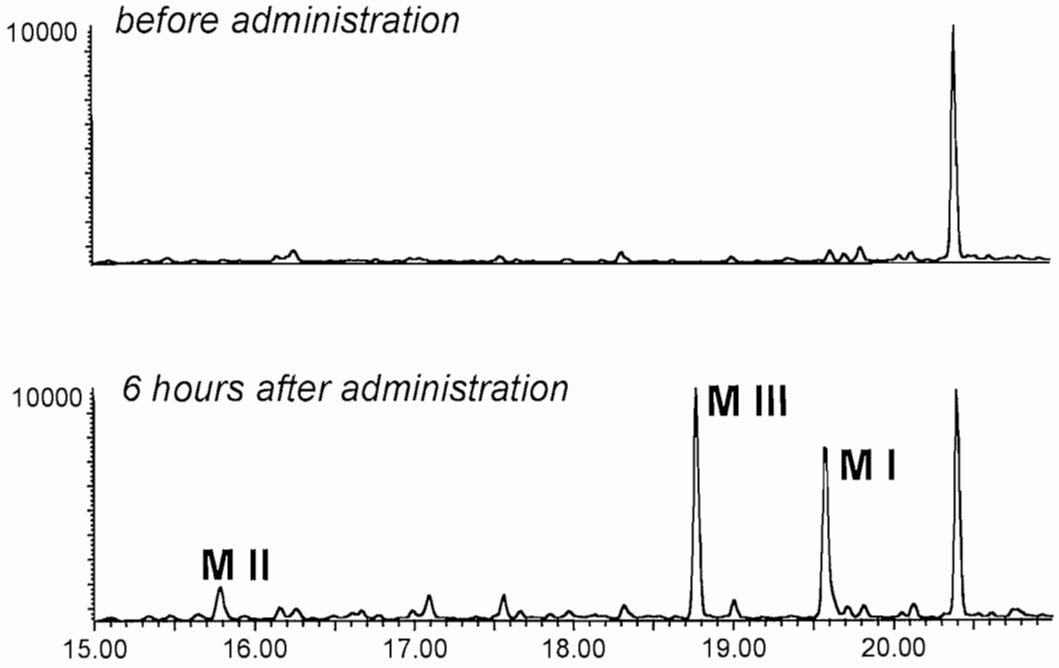

fig. 1: Formebolone metabolites detected in the free steroid fraction (keto-TMS derivatives)

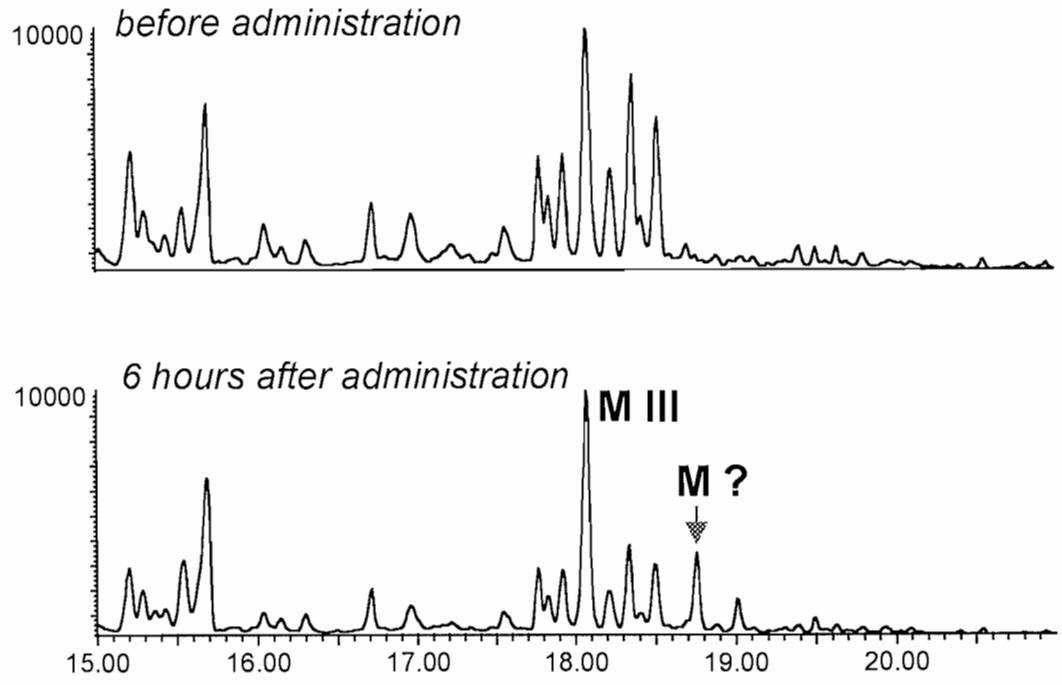

fig. 2: Formebolone metabolites detected in the glucuro-conjugated steroid fraction (enol-TMS derivatives)

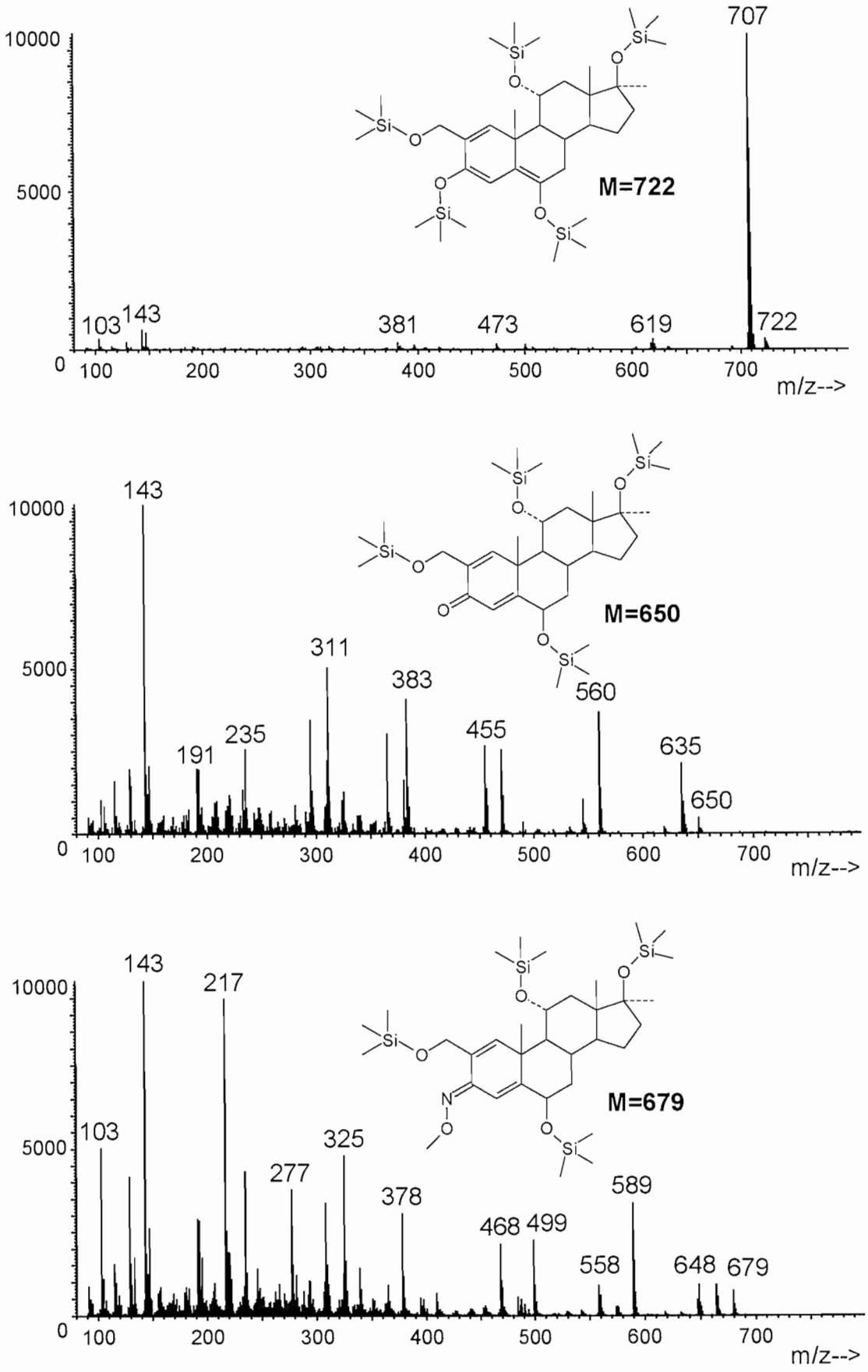

fig. 3: EI mass spectra of the novel formebolone metabolite: enol-TMS derivative, keto-TMS derivative and MO-TMS derivative

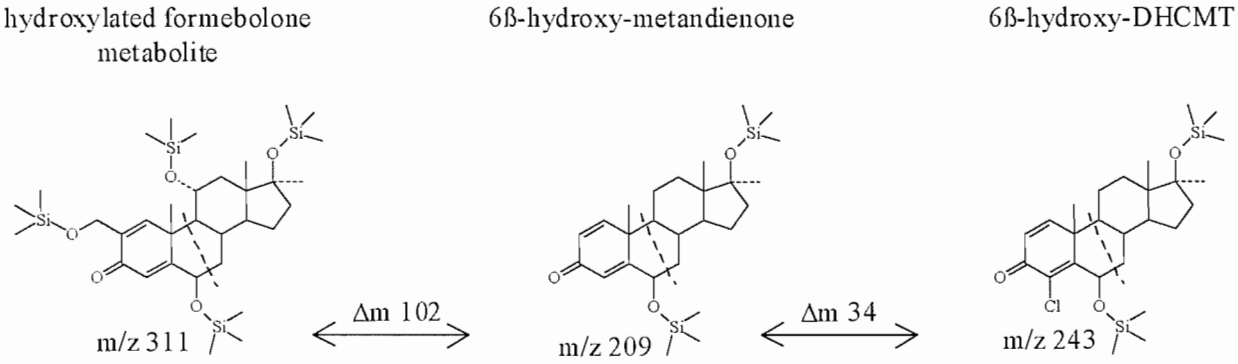

fig. 4: Comparison of A/B ring fragments of keto-TMS derivatives of related hydroxylated steroid metabolites

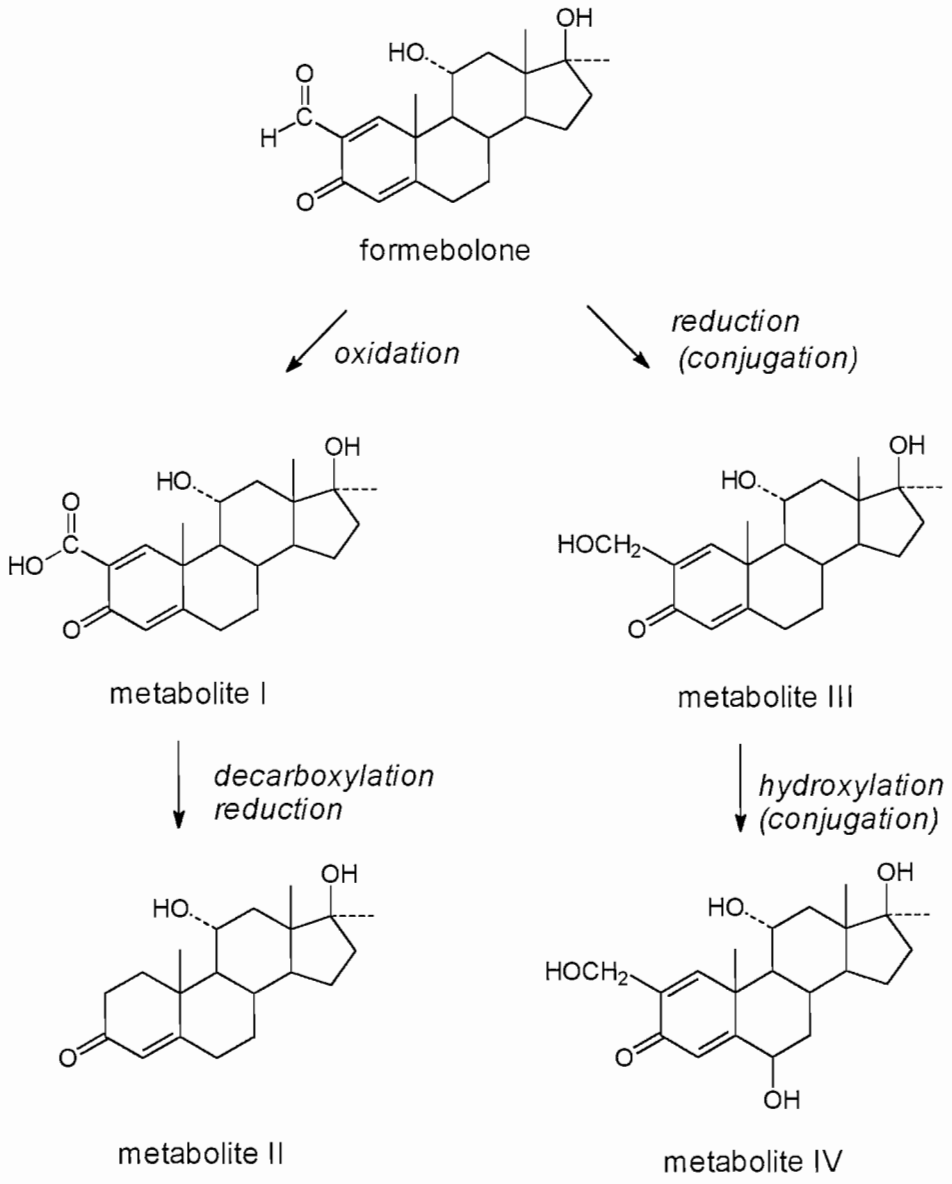

fig. 5: Proposal for the metabolic pathway of formebolone

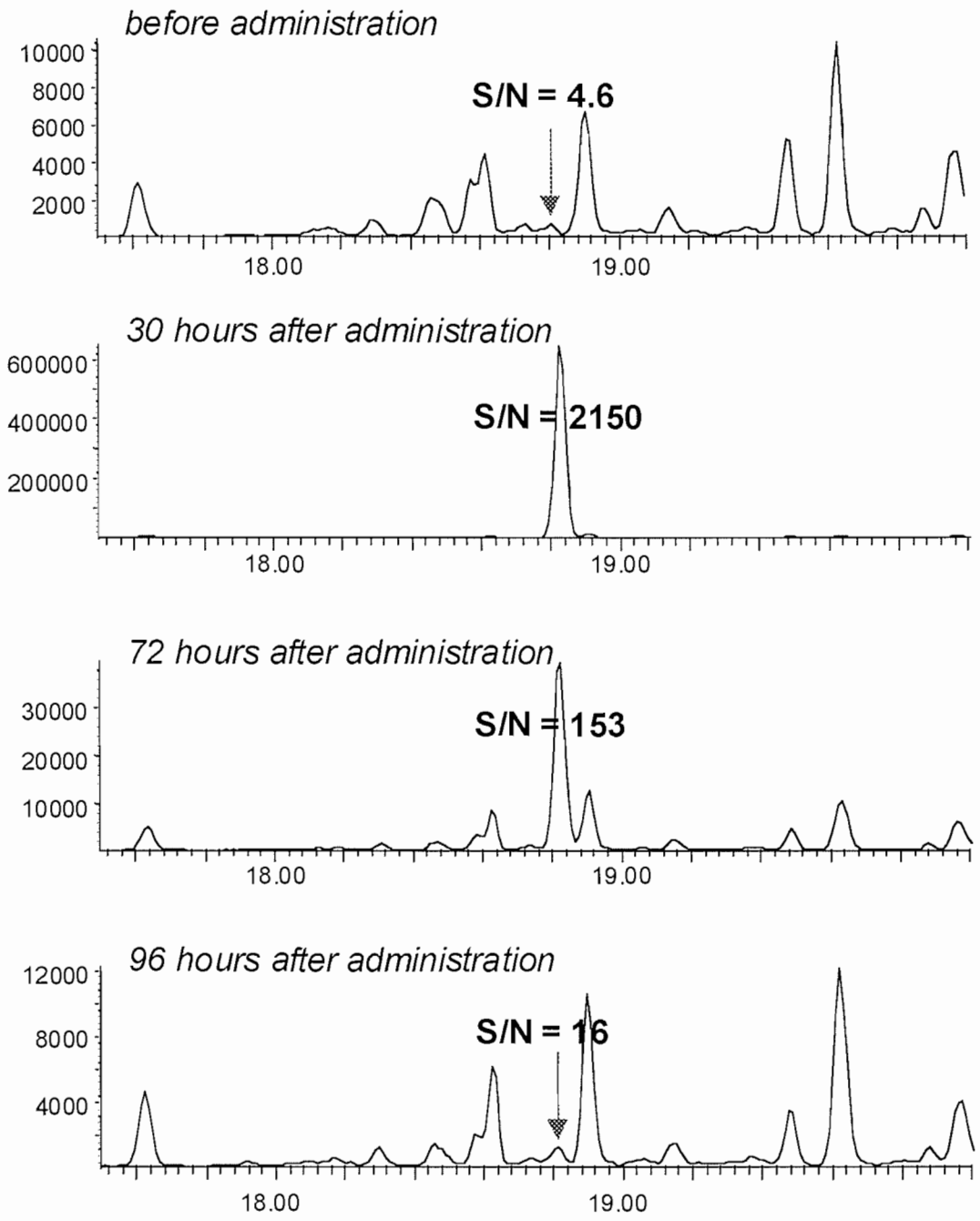

fig. 6: Screening of the combined steroid fraction: Monitoring of the hydroxylated formebolone metabolite as enol-TMS derivative at RT 18.8 min (signal m/z 707.4)

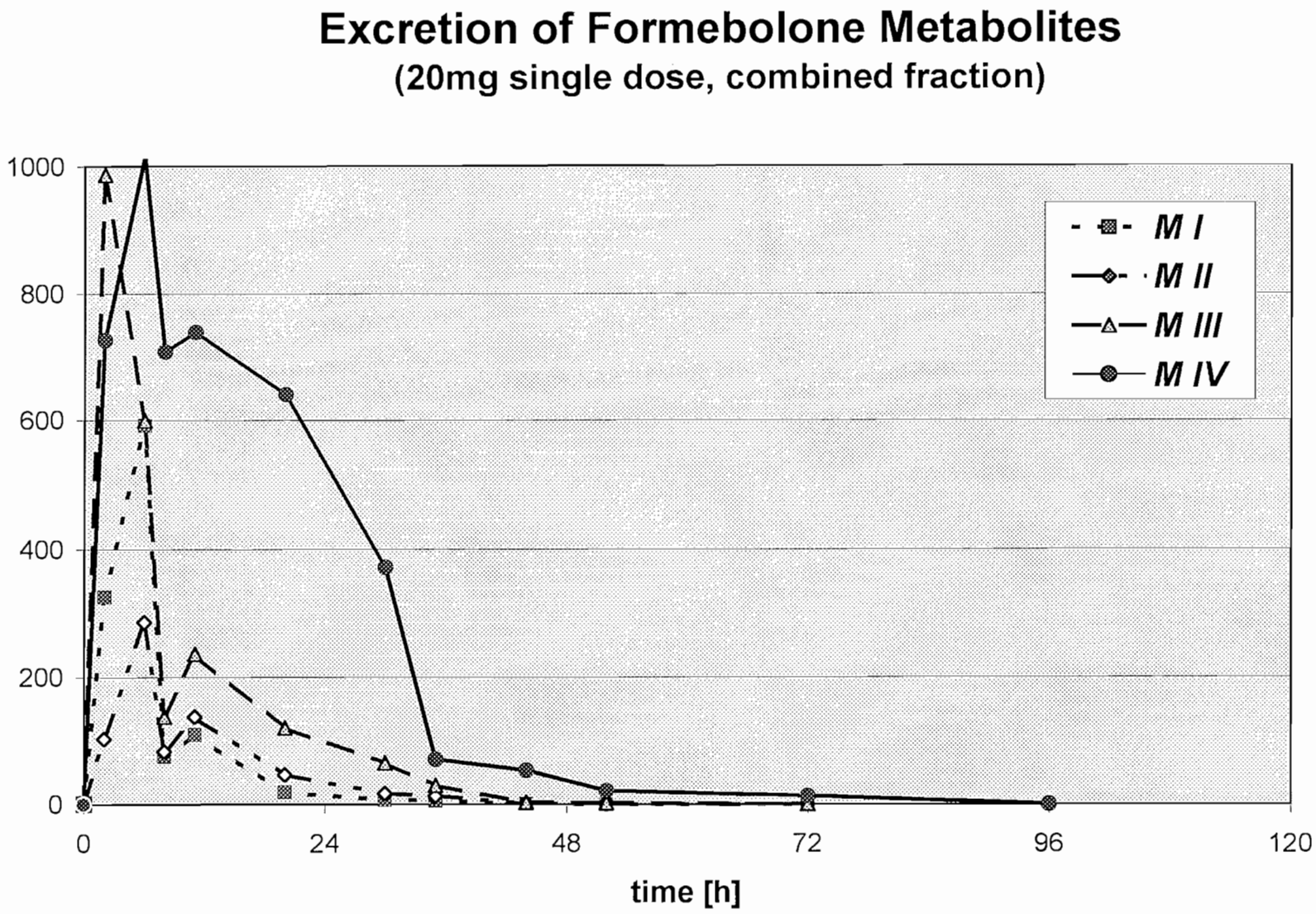

fig. 7: Urinary excretion profiles of formebolone metabolites