

Reprint from

RECENT ADVANCES
IN DOPING ANALYSIS
(5)

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Sport und Buch Strauß, Köln, 1998

D. THIEME, J. GROSSE, R.K. MUELLER, A. WAHL:
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Steroid Metabolites at pg Level in Blood and Urine
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in
doping analysis (5). Sport und Buch Strauß, Köln, (1998) 157-168

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Interpretation of high resolution and tandem MS data: two relevant cases of anabolic steroid metabolites at pg level in blood and urine

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Introduction

It is of special interest to deal with low concentrations of anabolic steroids in certain forensic cases. In spite of the recommendations concerning the interpretation of urinary metabolites detected at low concentrations in routine doping control, there are a number of cases where target concentrations below 1 ng/ml might be discussed.

The long time range between intake and sample collection and the necessity to analyse biological matrices other than urine may complicate for instance, to check whether:

- the intake of anabolics could have caused the death
- the person might have been under the influence of anabolics
- additional investigations permit to confirm or exclude a claimed urine sample mix-up.

In our first case, additional analytical effort was necessary to confirm the intake of methandienone in a case of an athlete, who claimed that a urine sample mix-up has caused the positive result in a doping control. A second urine sample collected several days later from the same athlete was negative in a routine screening (even with HRMS). Nevertheless, two methandienone metabolites could be confirmed after HPLC clean-up with HRMS or MS/MS.

The second case was related to a male bodybuilder, who died after an excessive long-term abuse of anabolics. He had stopped the intake of stanozolol, which is supposed to be the anabolic he

abused last, about ten days prior to death. There was no urine available for an anabolic screening, due to intense treatment in the hospital. Therefore, detection of stanozolol had to be carried out in blood, cerebrospinal fluid and liver.

The detection of 3'-OH-stanozolol was possible in all specimens investigated.

Experimental

An AutoSpec Ultima - Q (Micromass) high-resolution sectorfield hybride mass spectrometer, coupled to HP5890 gas chromatograph was applied for all analyses.

GC parameters

- 12.5 m Ultra-2 (0.2 mm ID, 0.33 μ m film thickness, crosslinked phenylmethylsilicone)
- carrier gas: helium
- head pressure: 10 psi
- injection mode: splitless, automatic
- injection volume: 1.5 μ l
- initial temperature: 150 deg C
- initial time: 0.5 min
- ramp: 12.5 deg C / min to 315 deg C
- final temperature: 315 deg C
- final time: 1 min

HR-MS parameters

- resolution 5 000 (SIR)
- ionisation mode: EI, 40 eV
- acquisition mode: SIR
- multiplier voltage: 300 V

MS-MS parameters

- acquisition mode multiple reaction monitoring (MRM-Q)
- collision energy 100
- resolution 1000 (magnet), 3 amu (quad)

Sample preparation

All urine samples were prepared by the conventional routine screening procedure for conjugated steroids:

2.5 ml of urine were hydrolysed with 20µl β-glucuronidase (1h at 50°C) and extracted with diethylether at pH9. The organic layer was evaporated to dryness, desiccated and derivatised with 40µl of a mixture of MSTFA/NH₄I.

All other specimens (blood, cerebrospinal fluid, homogenized liver) are prepared as follows:

1 ml of the sample was hydrolyzed with 20µl β-glucuronidase (1h at 50°C - this step was omitted for free fractions), pH adjusted with KOH to 9-10, extracted with diethylether and derivatised as described above.

HPLC clean-up

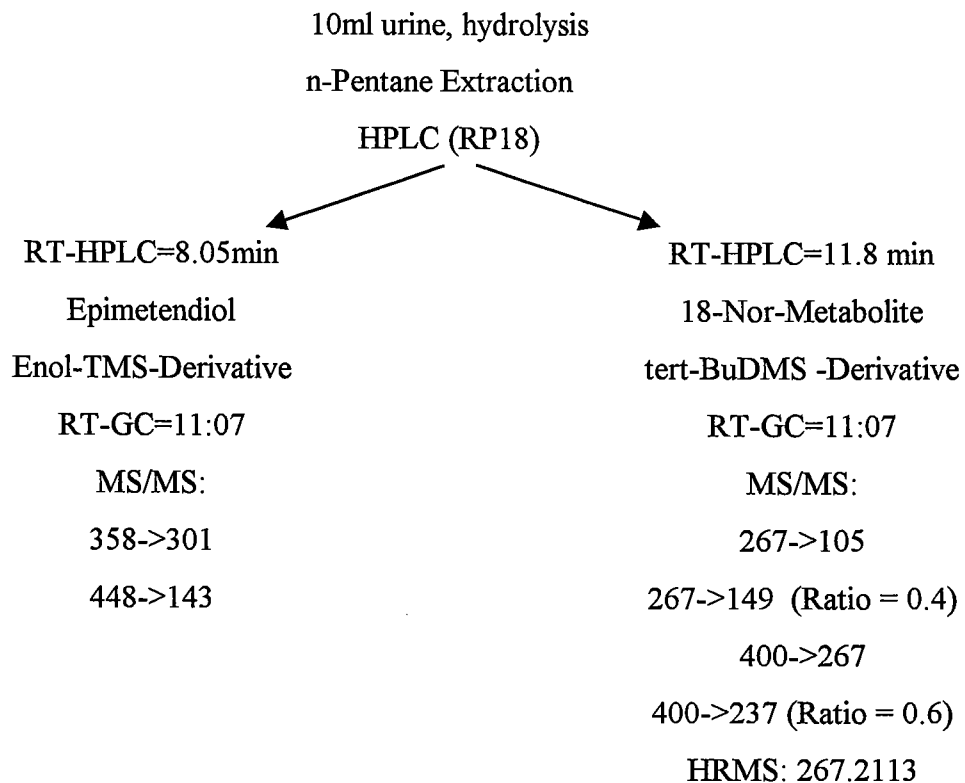
The urine extract was reconstituted with 40 µl acetonitril / water (5/95, v/v). An aliquot of 25 µl was injected onto HPLC system HP1090 using the following specifications:

column:	Hypersil ODS 100*2.1	
mobile phase:	water (A) + acetonitrile (B)	
flow	0.6 ml/min	
gradient	0 % B	0-1 min
	30 % B	2 min
	100 % B	16 min
Retention times	Epimetendiol	8.05 min
	18-nor-17,17-dimethyl-androst-1,13-dien-3-ol	11.8 min

Results and Discussion

Low concentrations of methandienone metabolites in urine

The detection of long term metabolites of methandienone in urine was described by Schänzer (1). The screening for 17 β -methyl-5 β -androst-1-ene-3,17-diol (epimetendiol) with HRMS permits a determination of methandienone abuse at low detection limits in screening sequences. However, the confirmation of low amounts requires special effort, due to co-elution of interfering substances. The application of Tandem-MS permits a higher specificity for this application, because a variety of fragmentation reactions exist with reasonable intensity. Especially the reaction 358 \rightarrow 301 has a very good selectivity and abundance (fig.1). Therefore, a straight-forward confirmation of suspicious screening results is accessible by this technique, without extra sample preparation or derivatisation. The second metabolite, 18-nor-17,17-dimethyl-androst-1,13-dien-3-ol is preferably analyzed as the tert-butyl-dimethylsilyl-derivative to avoid similar gas-chromatographic interferences. This derivative permits the formation of various intense product ions. The reactions 267 \rightarrow 105, 267 \rightarrow 149, 400 \rightarrow 267 and 400 \rightarrow 237 were chosen for confirmation analyses due to their high specificity and intensity (fig. 3). The application of an HPLC clean-up permits the decrease of biological background and enables the formation of different derivatives for both metabolites. The following procedure was applied to confirm methandienone metabolites at concentrations below 1ng/ml:



Stanozolol in post mortem material.

The knowledge about metabolism and pharmacokinetics in biological specimen other than urine is mainly limited to the detection of stanozolol in blood, serum (2) or hair (3) sample. Therefore it was uncertain, whether typical urinary long-term metabolites like 3'-OH-stanozolol can be detected in post mortem blood, liver and cerebrospinal fluid, 1-2 weeks after the presumed intake of huge amounts of stanozolol.

Based on GC-HRMS and MS/MS experiments, the identification of 3'-OH-stanozolol at a concentration about 1ng/ml (reliable quantification was impossible due to putrefaction) was possible (fig.4). The absence of 4-OH- or 16-OH-stanozolol at concentrations higher than 100pg/ml could be confirmed. Surprisingly, the concentration of stanozolol metabolite was much higher in hydrolyzed blood and cerebrospinal fluid samples, compared to the corresponding free fractions (fig.5). Both results show a high similarity to metabolism in urine at a late excretion state, but metabolic information obtained from this case are not reliable, because of the excessive long-term abuse of anabolics and a corresponding liver malfunction as the major cause of death.

The identification of 3'OH-stanozolol as a per-TMS derivative was confirmed by recording of a product ion spectrum of the substance (daughters of molecular ion 560, fig. 6). The biological matrices appeared to be unexpected clean (with respect to GC-MS analysis of anabolic steroids) in spite of putrefaction of the sample material.

Conclusions

The identification and confirmation of metabolites of methandienone in urine and of stanozolol in blood and cerebrospinal fluid was possible by application of HRMS and especially of tandem MS. Detection limits of 100pg/ml could be achieved for confirmation of the substances in several cases, where certain hints allowed to direct the analysis to few target substances. Detection and confirmation of two long term metabolites of methandienone were preferably carried out by MS/MS, mainly due to a higher number of undisturbed fragmentation reactions available.

The application of an HPLC clean-up permits the reduction of background and the formation of different derivatives for each metabolite.

The confirmation of 3'-OH-stanozolol in biological post mortem materials was possible after formation of per-TMS derivatives by HRMS or MS/MS. Only this metabolite could be detected in all specimen (blood, cerebrospinal fluid, liver) after hydrolysis.

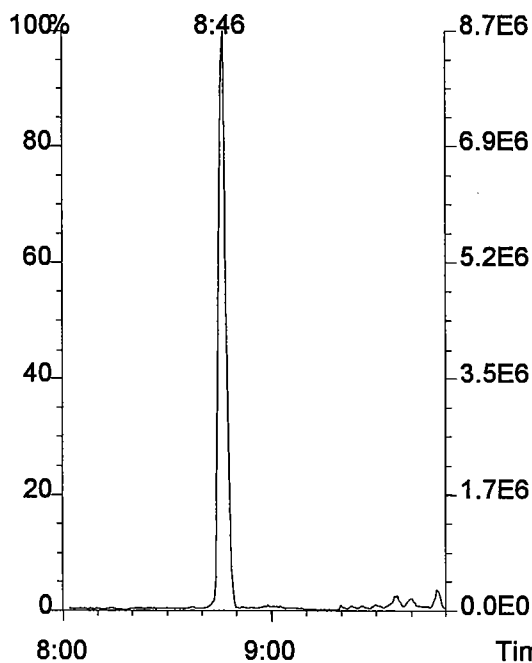
Signals in HRMS or MS/MS proved to have a much higher uniqueness, compared to conventional MS and allow to improve peak to noise ratios. But the possibility of interferences becomes more relevant with lower abundant signals for lower concentration ranges. The final decision whether peaks or spectra can be considered as 'undisturbed' and enable the identification of substances beyond reasonable doubts are still arbitrary.

References

- (1) Schänzer, W.; Delahaut, P.; Geyer, H.; Machnik, M.; Horning, S. *Journal of Chromatography B: Biomedical Applications* **1996**, *687*, 93-108.
- (2) Lampert, B. L.; Stewart, J. T. *J. Liq. Chromatogr., Dec* **1989**, *12*, 3231-3249.
- (3) Hold, K. M.; Wilkins, D. G.; Crouch, D. J.; Rollins, D. E.; Maes, R. A. *Journal of Analytical Toxicology* **1996**, *20*, 345-349.

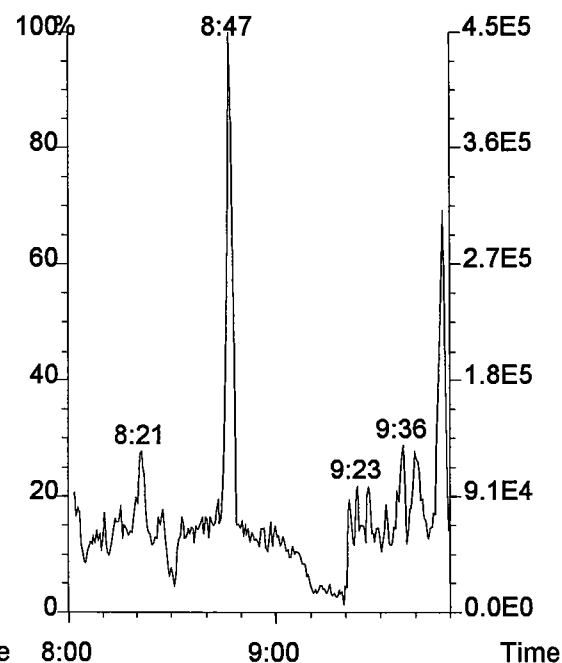
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Sample Text:QC 800



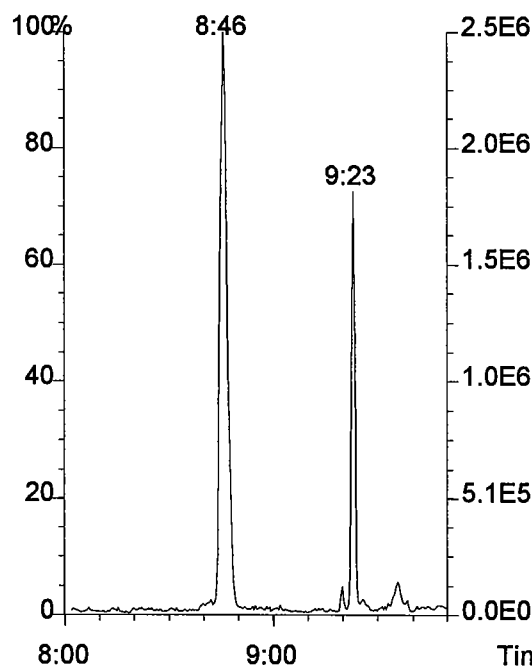
358.2692->301.2000 @90.0 S:6

Sample Text:WK 62564B



448.3193->143.0000 @90.0 S:3

Sample Text:QC 800



448.3193->143.0000 @90.0 S:6

Sample Text:WK 62564B

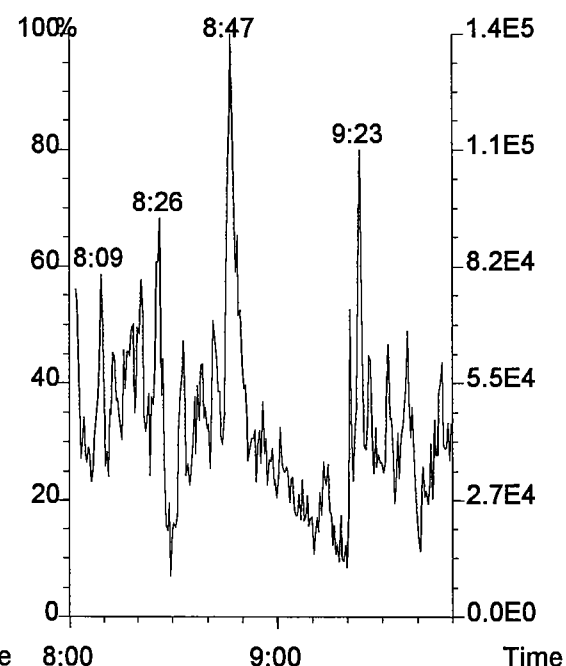


fig.1: Tandem MS screening for epimetendiol-bis-TMS in urine samples, prepared for routine steroid screening. Epimetendiol can be detected in a quality control sample (3ng/ml epimetendiol, left column) and in a suspicious sample (right column) without additional clean-up procedure. The fragmentation 358→301 proved to be the most specific and sensitive reaction to monitor epimetendiol.

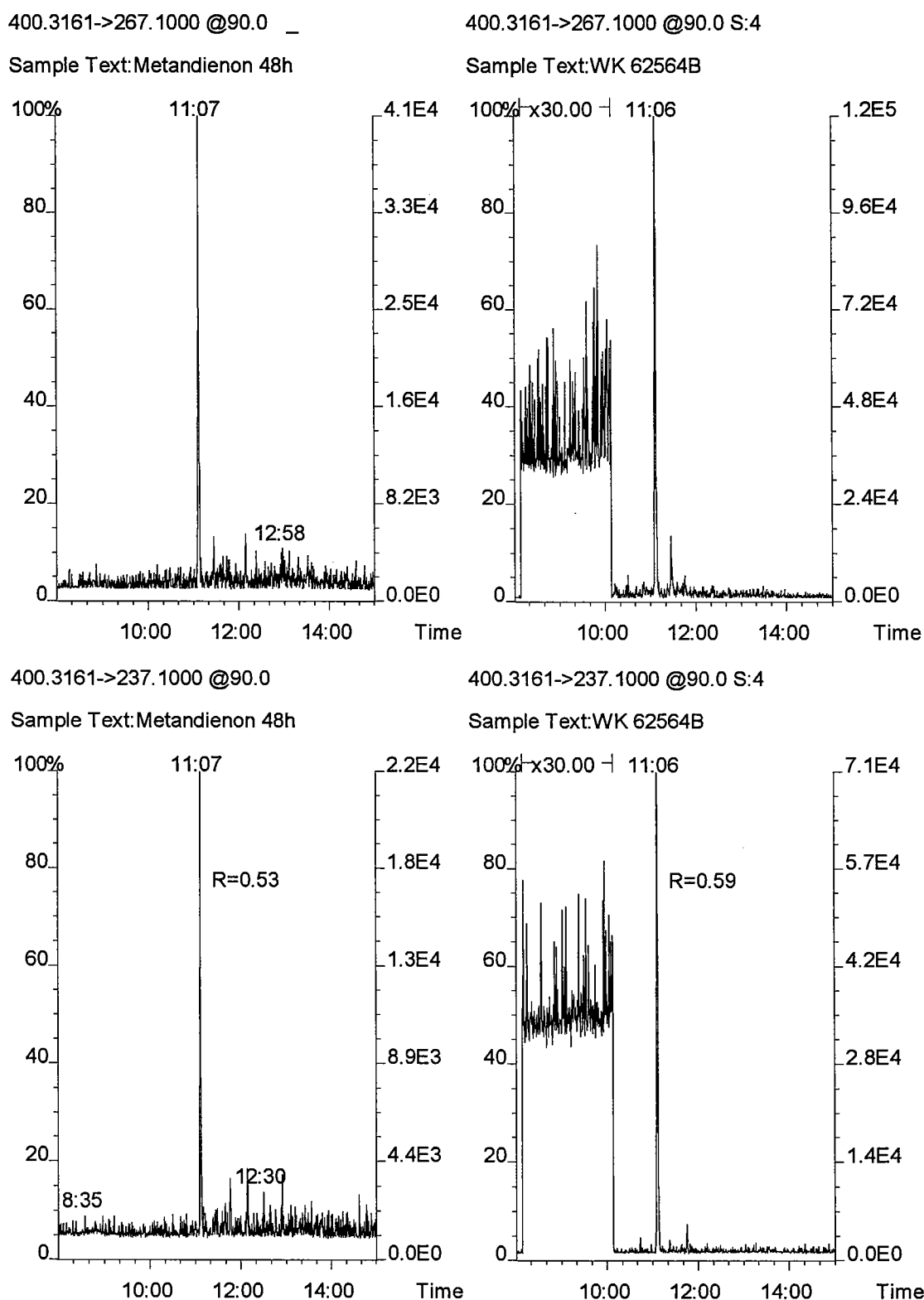


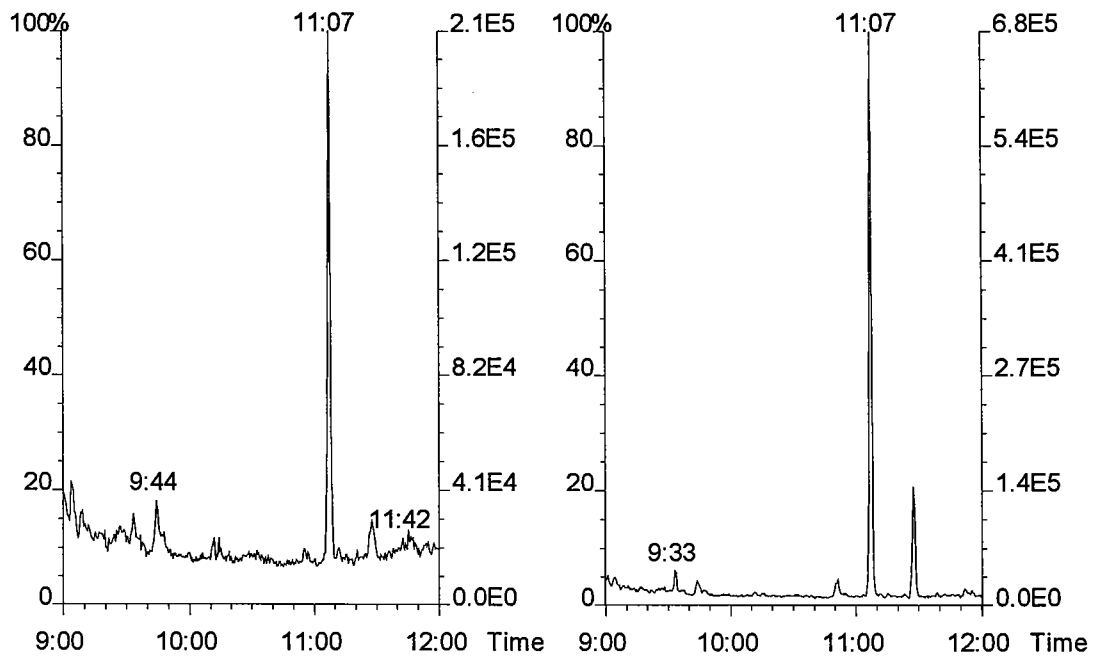
fig. 2: Additional information to confirm the intake of methandienone can be obtained by identification of 18-nor-17,17-dimethyl-androst-1,13-dien-3-ol (as tert-butyl-dimethylsilyl derivative) after HPLC clean-up. Selected ion recordings (10 000 resolution) of the base peak (above) and the molecular ion (below) are figured for a control urine (left column) and the suspicious sample (right column).

267.2113 S:2 Exp:18NOR_253_358

267.2113 S:4 Exp:18NOR_253_358

Sample Text:Metandienon 48h

Sample Text:WK 62564B



400.3161 S:2 Exp:18NOR_253_358

400.3161 S:4 Exp:18NOR_253_358

Sample Text:Metandienon 48h

Sample Text:WK 62564B

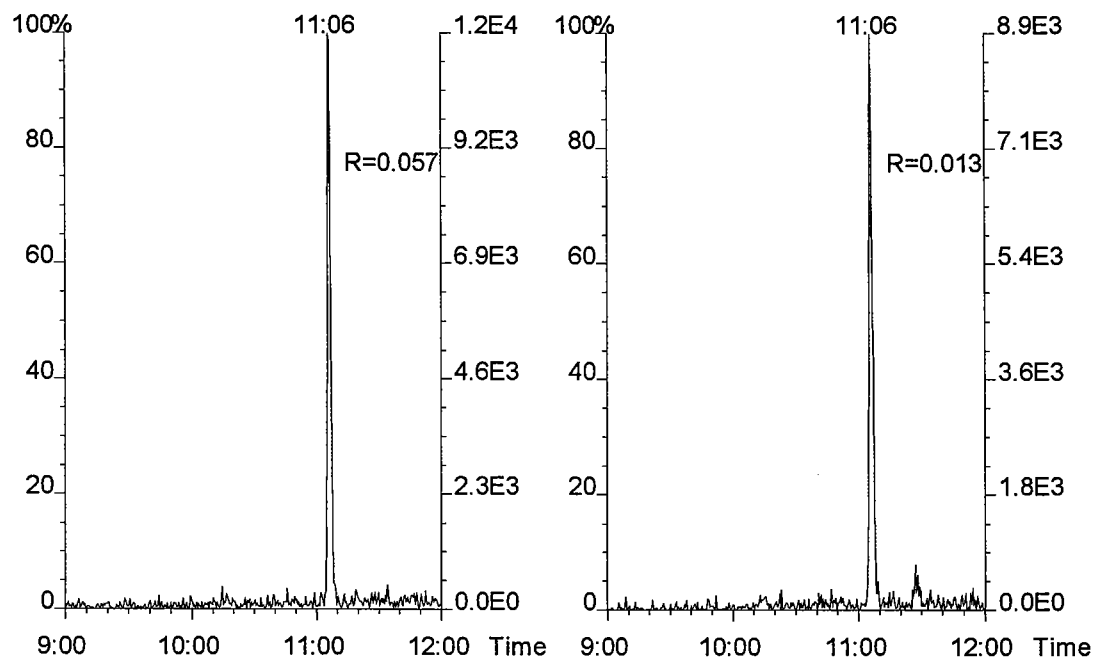


fig. 3: Additional information to confirm the intake of methandienone can be obtained by identification of 18-nor-17,17-dimethyl-androst-1,13-dien-3ol (as tert-butyl-dimethylsilyl derivative) after HPLC clean-up. Selected ion recordings (10 000 resolution) of the base peak (above) and the molecular ion (below) are figured for a control urine (left column) and the suspicious sample (right column).

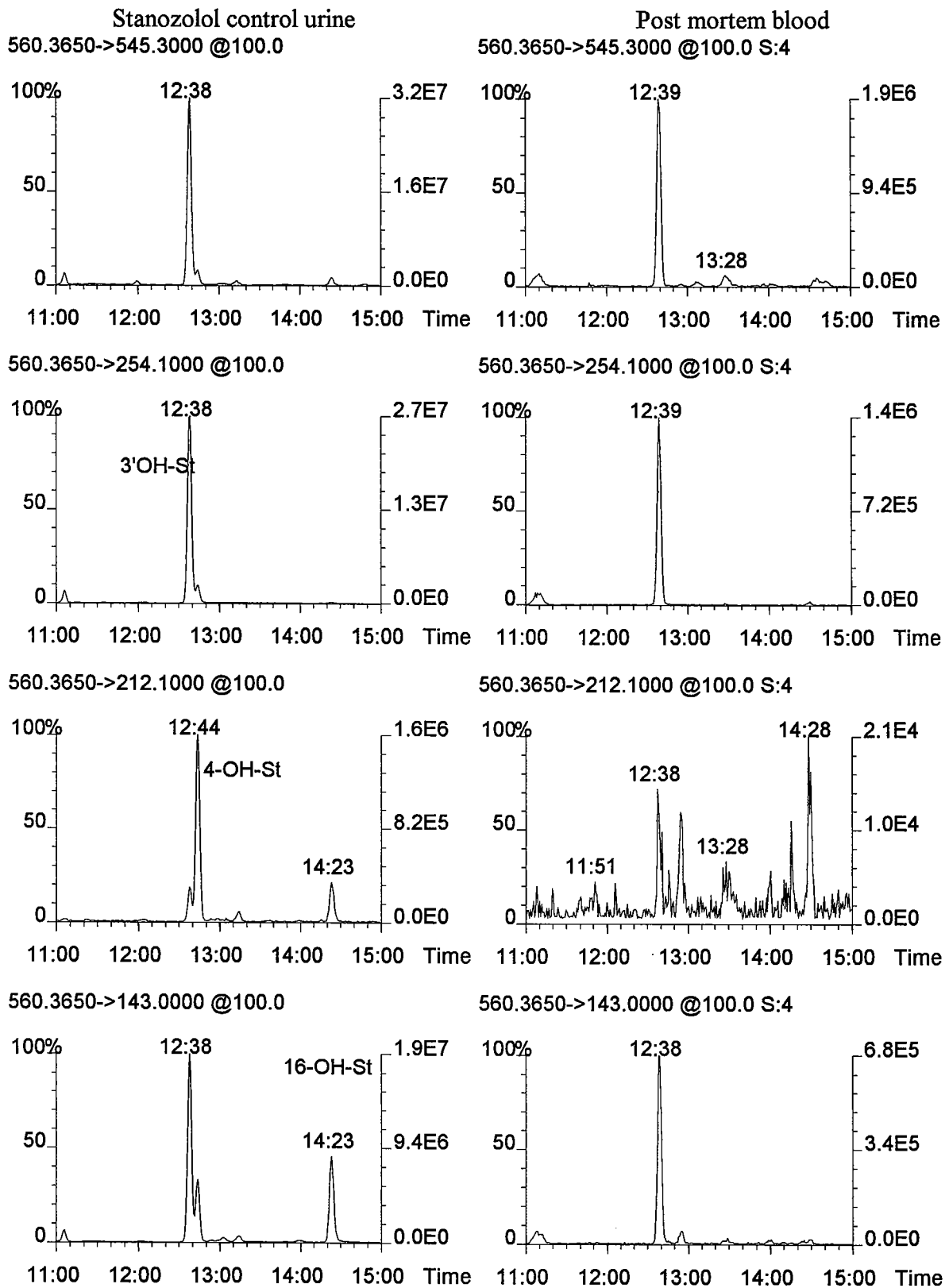
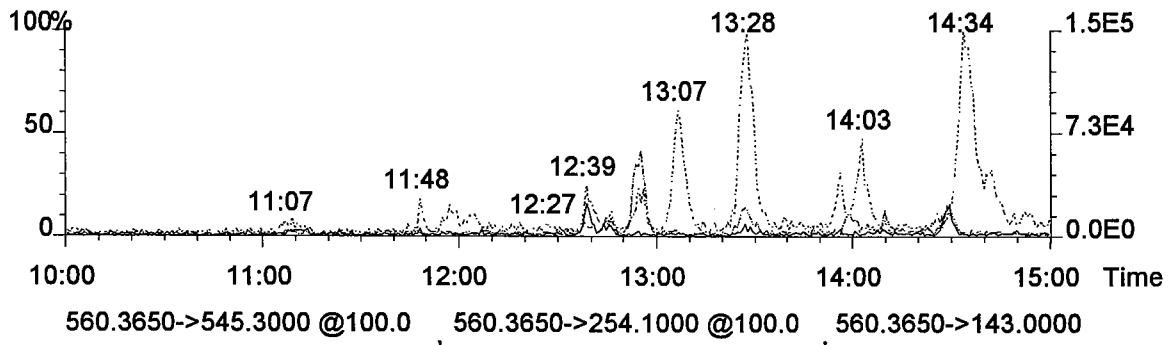


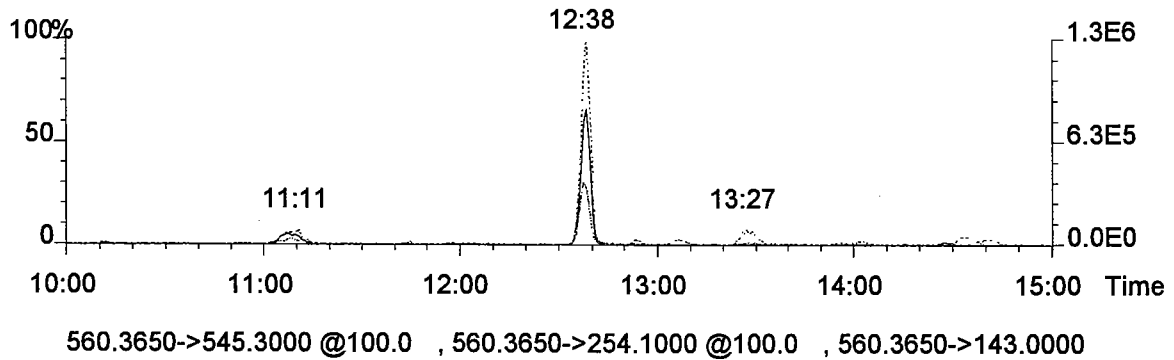
fig. 4: Tandem-MS screening for stanozolol metabolites in post mortem blood (left column, compared to a control urine obtained from an excretion study). Only traces of 3'-OH-stanozolol are detectable in the post mortem blood sample.

560.3650->545.3000 @100.0 , 560.3650->254.1000 @100.0 , 560.3650->143.0000

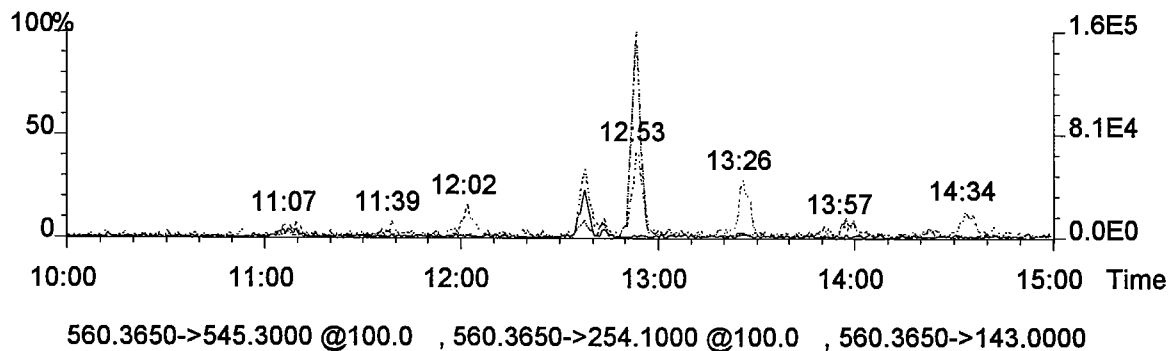
Sample Text:Blood -Hyd



Sample Text:Blood +Hyd



Sample Text:Cerebrosp.Fl. -H



Sample Text:Cerebrosp.Fl. +H

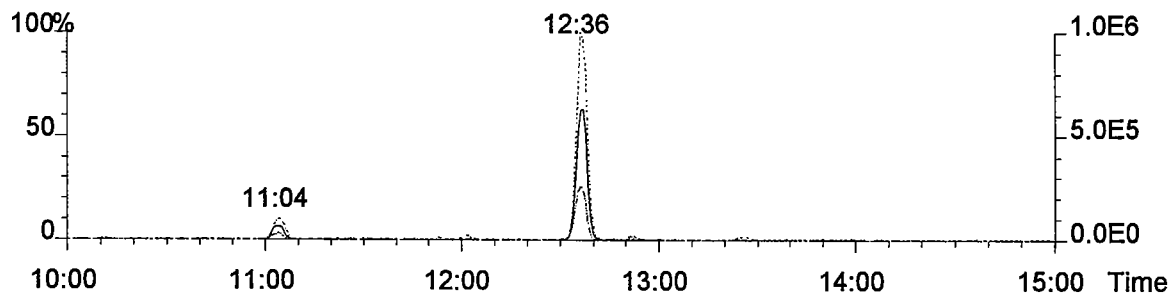


fig. 5: Comparison of 3'-OH-stanozolol in post mortem blood (upper two windows) and cerebrospinal fluid (below). A free (-Hyd, above) and a hydrolyzed fraction is figured for each of the biological specimen, indicating that the amount of metabolites increases after hydrolysis.

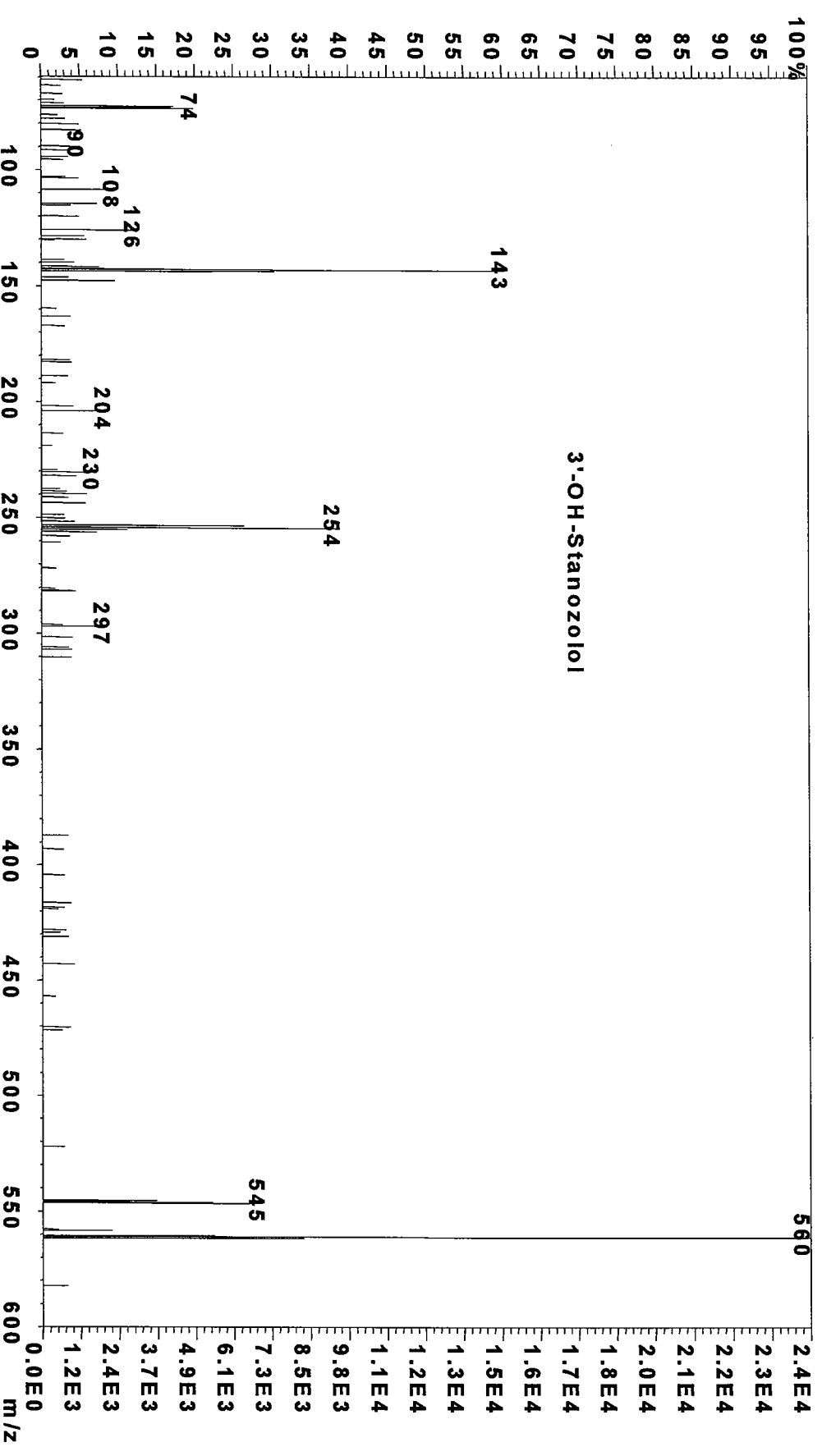


fig. 6: Full scan product ion spectrum obtained from a post mortem blood sample. The undisturbed spectrum permits an unequivocal identification of 3'-OH-stanozolol in a very dirty biological matrix without specific clean-up procedure