Long Term Metabolites – Historical Aspects and the Cologne Strategy

Schänzer W, Fusshöller G, Guddat S, Geyer H, Piper T, Thevis M. Long Term Metabolites - Historical Aspects and the Cologne Strategy. Lecture held at the 16th Annual USADA Meeting in Orlando, USA on 1st October 2017.

Literature for this article and presentation:

Schänzer, W, Delahaut, P, Geyer, H, Machnik, M, Horning, S: Longterm detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography / high resoloution mass spectrometry (GC/HRMS). Journal of Chromatography B, 687 (1996) 93-108.

Schänzer, W, Geyer, H, Horning, S: Longterm determination of metandienone and mestanolone. In W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (5). Sport und Buch Strauß, Köln (1998) 13-26.

Schänzer W, Horning S,Opfermann G and Donike, M: GC/MS identification of longterm excreted metabolites of the anabolic steroid 4-chloro-1,2-dehydro-17α-methyltestosterone in human. J. Steroid Biochem. Molec. Biol., 57 (1996) 363-376.

Schänzer W, Geyer H, Fußhöller G, Halatcheva N, Kohler M, Parr MK, Guddat S, Thomas A, Thevis M: Mass Spectrometric Identification and Characterization of a New Long-Term Metabolite of Metandienone. Rapid Commun. Mass Spectrom. 10 (2006) 2252-2258.

Fußhöller G, Mareck U, Schmechel A, Schänzer W: Long-term detection of metandienone abuse by means of the new metabolite 17ß-hydroxymethyl-17α-methyl-18-norandrost-1,4,13-trien-3-one. In Schänzer W, Geyer H, Gotzmann A, Mareck U (eds.) Recent advances in doping analysis (15). Sportverlag Strauß, Köln (2007) 393-396.

Sobolevsky T, Rodchenkov G. Detection and mass spectrometric characterization of novel long-termdehydrochloromethyltestosterone metabolites in human urine. J Steroid Biochem Mol Biol. 2012 Feb;128(3-5):121-7.

Schänzer W, Guddat S, Thomas A, Opfermann G, Geyer H, Thevis M: Expanding analytical possibilities concerning the detection of stanozolol misuse by means of high resolution/high accuracy mass spectrometric detection of stanozolol glucuronides in human sports drug testing. Drug Test Anal. 2013, 5, 810-818.

Thevis M, Piper T, Horning S, Juchelka D, Schänzer W; Hydrogen isotope ratio mass spectrometry and high-resolution/high-accuracy mass spectrometry in metabolite identification studies: Detecting target compounds for sports drug testing. Rapid Commun. Mass Spectrom. 2013, 27, 1904–1912.

Long Term Metabolites – Historical Aspects and the Cologne Strategy

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Historically, the wording long term metabolite was first used in the Cologne laboratory in 1995. Unfortunately, this was the year when Manfred Donike, the head of the Cologne laboratory, director of the Institute of Biochemistry of the German Sports University and member of the IOC Medical Commission subgroup Doping and Biochemistry, unexpectedly died. He was the scientific "father" of the anti-doping laboratory in Cologne and published his first scientific articles – dope analysis based on GC separation techniques - in 1966. His respectable work in the fight against doping was a milestone. He untiringly advanced anti-doping science and improved analytical methods to detect doping substances in biological samples of athletes. We all in the Cologne laboratory and also scientists in other laboratories worldwide have learnt from Manfred Donike's ideas.
The wording long term metabolite is related to the detection of exogenous anabolic androgenic steroids (AAS). When in the beginning of the 1970ties the misuse of AAS became aware Manfred Donike and also the laboratory in Montreal focused their analytical investigations on an unambiguous identification of that group of substances by GC-MS. Beside of classical sector field MS systems such as the Atlas MAT CH-5, which was already used during the Olympic Games in Munich 1972, new and less expensive MS instruments became available at that time and in the 1980ties quadrupole separation techniques followed. These instruments were also called single bench top MS-Systems and connected to a gas chromatograph. The first methods could be developed to successfully identify AAS. But the problem remained with the testing itself as AAS were applied in the training period and doping tests were only conducted at competition events. This was less effective. Since 1988 with the introduction of out-of-competition testing the misuse of AAS could be controlled more effectively. But out-of-competition tests were not uniform and internationally not harmonized. That was one of the reasons why in Cologne Manfred Donike tried to improve the detection of AAS, even for long periods of time after their last administration.

1992 - High resolution mass spectrometry (HRMS)
One possibility to achieve longer detection times was the introduction of HRMS in anti-doping control analysis by Manfred Donike in 1992. With this HRMS technique it was feasible to improve the detection limits of AAS mainly by reducing the biological background compared to MS techniques with a single mass resolution.

1995 - Identification of long term excreted metabolites of AAS
The other possibility to improve detection times was to identify metabolites of AAS which were not so dominant at the beginning of the excretion period but showed prolonged half-lives and were excreted for a longer period of time.

17-Epimerization and 17,17-dimethyl-rearrangement process
In Cologne we mainly focused on 17α-methyl steroids and investigated their metabolism. A sulfate conjugation of the sterically hindered 17ß-hydroxy group became of particular interest. The 17ß-sulfate of 17α-methyl steroids once generated was instable and a 17-epimerization could be identified as a metabolic pathway. In 1989 this new metabolic pathway was described for metandienone in the metabolism of horses by Edlund et al. We investigated this process for a series of 17α-methyl steroids and could identify further water elimination products and rearrangement products with a 18-nor-17,17-dimethyl-13-ene C/D-ring structure.
Especially in the metabolism of metandienone we were able to identify a rearrangement product of metandienone with a 18-nor-17,17-dimethyl-13- ene structure which was further

reduced in the A-ring yielding 18-nor-17,17-dimethyl-5ß-androst-1,13-dien-3α-ol (also named 18-norepimetenediol or 18-nor-EMD). In the same excretion urines we could also confirm a 17- epimer 17ß-methyl-5ß-androst-1-ene-3α,17α -diol (epimetenediol, EMD). Both metabolites 18-nor-EMD and EMD could be detected longer than the classical, known metabolites of metandienone at that time.
The screening and confirmation of both of these long term metabolites in combination with the use of HRMS analysis led to an increase in the number of adverse analytical findings (AAFs) for metandienone. Furthermore, the detection of stanozolol metabolites was improved when using HRMS. In 1995 the Cologne laboratory could report additional 63 AAFs for metandienone (total number was 78) and additional 9 AAFs for stanozolol (total number was 22) only detected by HRMS. By means of low resolution MS alone we would have missed 80% of the metandienone and 40% of the stanozolol AAFs.

1996 – Long term excreted metabolite of dehydrochloromethyltestosterone (DHCMT)
At the same time, in 1995/96, we also concentrated on the metabolism of DHCMT and identified a long term excreted metabolite but with a 6,16-dihydroxy structure.
This was published in 1996 and especially in Germany we were able to report some AAFs on the basis of considering this metabolite. At that time we were of the opinion that DHCMT (Oral-Turinabol®) was only the classical, misused AAS in the East German Democratic Republic (GDR) and after the reunification of Germany we expected that some athletes would continue to misuse this anabolic steroid. About twenty years later, we see an extremely high number of misuse of this substance mainly in Eastern Europe countries.

2006 – Confirmation of a new long term metabolite of metandienone – 18-nor-17ß-hydroxymethyl-17α-methylandrost-1,4,13-trien-3-one (**NW**, “night watchman”)
The detection of this long term excreted metabolite of metandienone was more or less a discovery by chance. The routinely applied LC/ESI/MS/MS screening method for the detection of dihydroexemestane, the main metabolite of exemestane (a banned aromatase inhibitor), showed in some doping control urine samples an interfering signal for an unknown substance demonstrating a product ion at *m/z* 135 generated from the protonated molecular ion $(M+H)^+$ 299. Interestingly, in several samples with this unknown peak we could additionally identify EMD, a long term metabolite of metandienone (see above). Further investigations on this metabolite were successful and contributed to identify the interference as a new metabolite of metandienone with the structure 18-nor-17ß-hydroxymethyl-17α-methylandrost-1,4,13-trien-3-one (**NW**). This was unexpected and for the first time we confirmed that a rearrangement product with 18-nor-17,17-dimethyl-13-ene C/D-ring structure was hydroxylated at the 17ß-methl group. Based on the history of the discovery we abbreviated this metabolite with the naming “night watchman” (**NW**). Additionally, but with lower intensity, an epimer of the **NW** metabolite with the hydroxylation at the 17α-methyl group could be identified. With the introduction of this new metabolite in our screening methods for AAS in 2006, in the same year we were able to report about 53 additional AAFs for metandienone which before this new finding was discovered would have not been detected with the known metabolites.
The investigation of similar metabolic pathways producing long term excreted metabolites of other 17α-methyl steroids was successful and results were published also by other laboratories. I would like to mention two anabolic steroids, oxandrolone and DHCMT, for which the Cologne laboratory was able to report a high number of AAFs by introducing such long term excreted hydroxylated rearrangement metabolites into existing screening methods. For oxandrolone we could identify similar metabolites like for metandienone **NW** with 18-nor-17ß-hydroxymethyl-17α-methyl -13-ene structure and an 17-epimer with similar intensity. Comparable to the **NW** metabolite of metandienone the A-ring remains non-metabolized. The first AAFs for oxandrolone long term metabolites were reported in Cologne in 2012. The investigations confirming these new metabolites were published in 2013.

2013 Dehydrochloromethyltestosterone (DHCMT) - New long term metabolites identified by Sobolevsky and Rodchenkov.
A high number of AAFs could be reported for the anabolic androgenic steroid DHCMT when Tim Sobolevsky and Gregory Rodchenkov published several new long term metabolites of DHCMT in 2012 which were similarly metabolized at the C/D-ring as described for metandienone **NW** but subsequently the A-ring was partially and/or completely reduced. Among these new metabolites especially the fully A-ring-reduced metabolite could be detected for the longest period of time after the last application of DHCMT. Following a single excretion study of an oral application of 20 mg of DHCMT which was performed in Cologne in 2016 this new long term excreted metabolite of DHCMT could be detected for 250 days (approximately eight months).
Actually, this metabolite was successfully synthesized as reference material by the working group of Günter Gmeiner in Austria and the exact configuration of the A/B-ring, the 3-hydroxy- and 4-chloro groups will be published soon.
In 2013 we introduced this new metabolite into our screening methods for AAS and were able to report 61 AAFs for DHCMT in the same year compared to only one positive finding in the year before.

2012/2013 - Improved detection of stanozolol metabolites by HRMS orbitrap technology
At the same time, in 2012, we started the investigation to develop an improved method for the detection of stanozolol applying a new high resolution mass spectrometry technique called orbitrap technology. With this new sophisticated MS technique we were able to identify stanozolol and epistanozolol conjugated metabolites as N-glucuronides. The N-glucuronide of epistanozolol was detected up to four weeks after a single oral dose of 5 mg and was therefore confirmed as a long term excreted metabolite. When applying this method for routine doping control samples we increased our reports for stanozolol AFFs from about 12 in 2011 to 64 in 2012 (39 were only detected by the new orbitrap HRMS method) and to 150 AAFs in 2013 (146 were only detected by the new orbitrap HRMS method). However, the pattern of metabolites was changing only in a few samples to the long term metabolite of stanozolol. In most of the analysed samples we obtained a pattern with all metabolites. An explanation for this observation is still missing. Up to now it can only be speculated about the reasons, whether the substance is mainly administered by injection or given orally in low doses, which both may explain the results.

2013 The new Cologne strategy for the detection of long term excreted metabolites of AAS using hydrogen isotope ratio mass spectrometry (HIRMS)
In 2013 my colleagues Mario Thevis and Thomas Piper started a project to identify long term excreted metabolites of AAS by use of hydrogen isotope ratio mass spectrometry (HIRMS) to calculate $^{2}H_1/^{1}H_1$. The isotope $^{2}H_1$ of hydrogen is also named deuterium (D) and, therefore, the isotope ratio of hydrogen can also be named D/H. As the natural occurrence of D is only 1 out of 6500 H atoms (0.015%), the use of deuterated compounds for excretion studies would substantially increase the signal intensities of the metabolites. As a result concentrations of a deuterium labeled metabolite in the lower ng/mL range will result in a detectable signal above the naturally present D-background in IRMS analyses. This idea was successfully tested by means of an elimination study using trideuterated metandienone (17α-$C^{2}H_3$-17ß-hydroxyandrosta-1,4-dien-3-one, D_3-metandienone). For the *gas chromatography / thermal decomposition-isotope ratio mass spectrometry (GC/TC-IRMS)* analysis selected urine samples were collected following the application (up to 477 h, approximately 20 days). The urine samples (20 ml) were concentrated via a SPE step, glucuronidated and sulfated metabolites were separated and hydrolyzed. All extracts were further cleaned up by HPLC fractionation and acetylated with acetic anhydride for analysis. After the Gparation of the different fractions the substances are thermally decomposed (TC) generating H_2 and DH and the GC/TC-IRMS is measuring the two masses *m/z* 2 and *m/z* 3 respectively. For hydrogen isotope ratio (HIR) measurements at natural abundance, the ion chromatogram at *m/z* 2 shows consistently higher intensities than the corresponding

chromatogram at *m/z* 3, although the abundance of *m/z* 3 is constantly amplified by a factor of
1000. When a deuterium labeled metabolite is eluting the GC-column the ratio of *m/z* 3 (DH) to *m/z* 2 (H_2) is obviously increased. This allows the detection of unknown metabolites. In the above-mentioned excretion study with D_3-metandienone the metabolite with the longest detection time measured by hydrogen IRMS was the known long term excreted metabolite of metandienone **NW** (see above). The sensitivity for the actual used method was about 0,25 ng/ml for a deuterium labeled metabolite.
The presented study demonstrates the value of GC/TC-IRMS in metabolism studies of long term excreted metabolites. In general this technique is not limited to AAS and it can be applied to other doping substances when the detection and identification of metabolites are required for a sensitive dope control.

Literature for this new strategy:
Thevis M, Piper T, Horning S, Juchelka D, SchänzerW. Hydrogen isotope ratio mass spectrometry and high-resolution/high-accuracy mass spectrometry in metabolite identification studies: Detecting target compounds for sports drug testing. Rapid Commun. Mass Spectrom. 2013, 27, 1904–1912

Long Term Metabolites - Historical Aspects and the Cologne Strategy

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Orlando – 1st October 2017

Manfred Donike

23.8 1933 – 21.8.1995

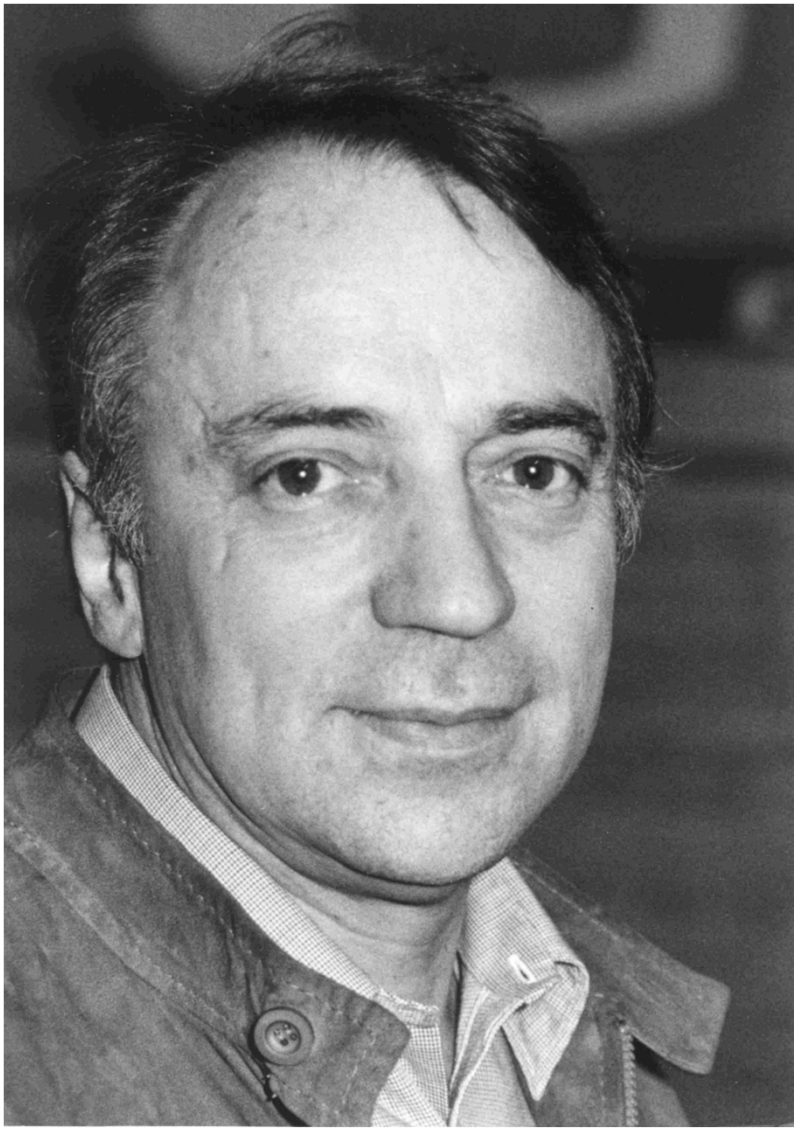
1966 First publication - dope analysis based on GC separation techniques

1969 Synthesis of MSTFA

1972 Dope testing Olympic Games Munich GC and MS

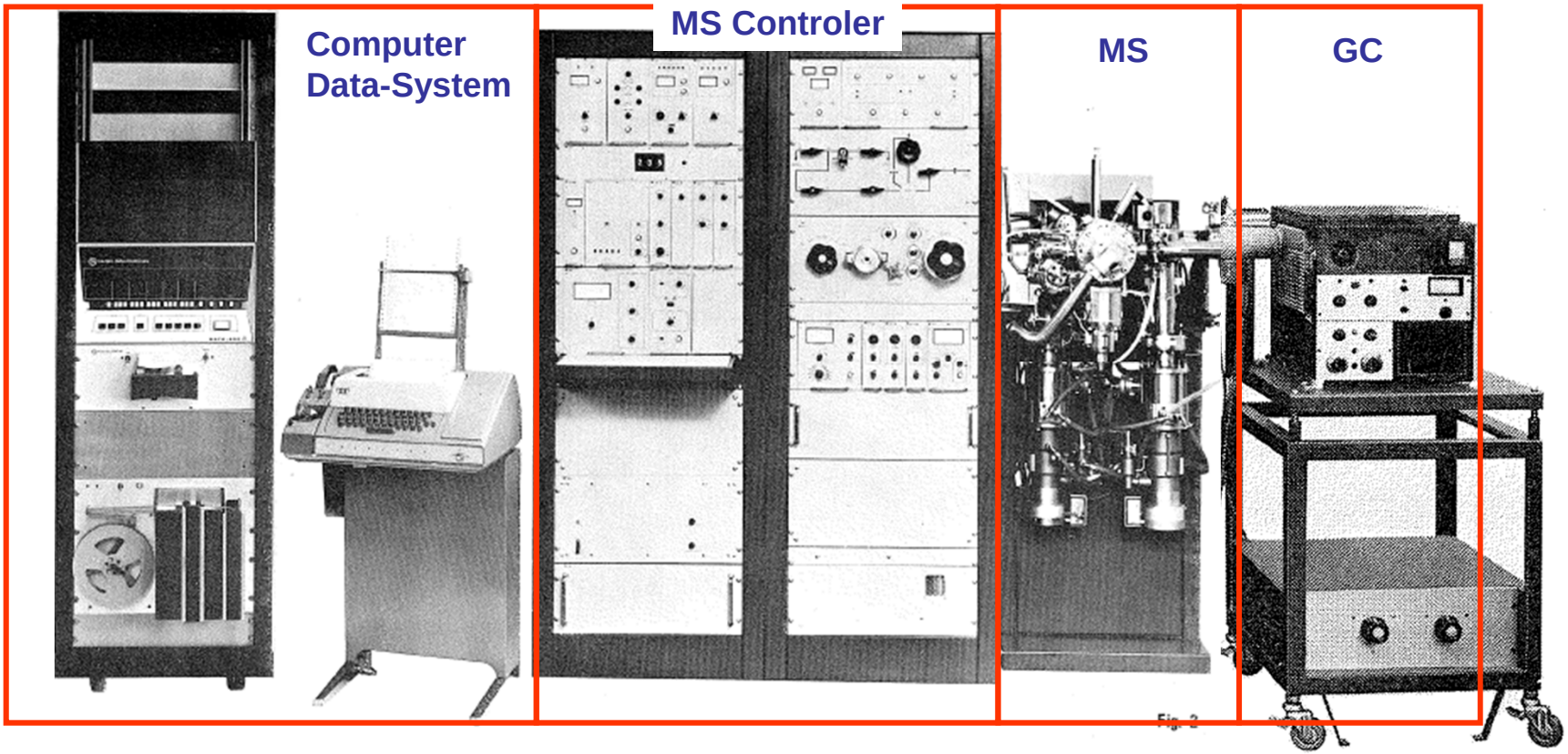
1977 Professor (appointment) Institute of Biochemistry German Sport University

1980 Member of the IOC MC-subcommission „Doping and Biochemistry“

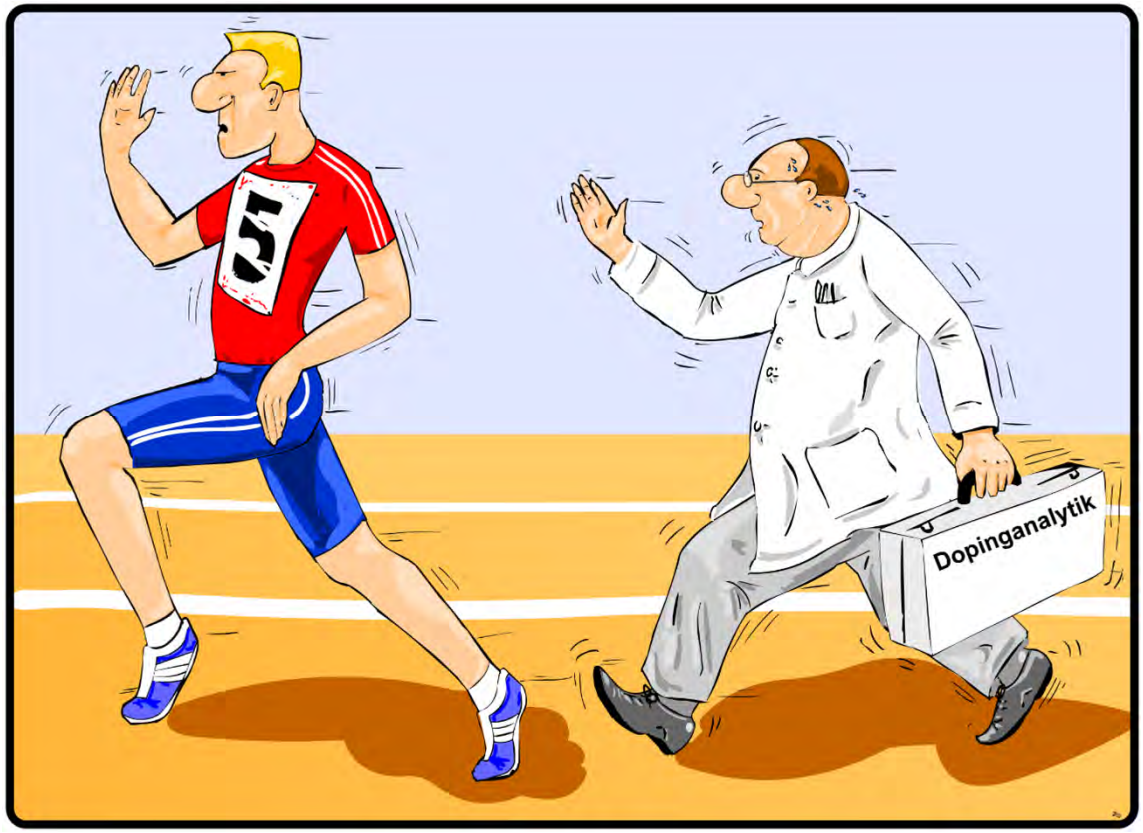


Dope Analyis 1972 – Olympic Games Munich

Mass Analyser with Gaschromatograph



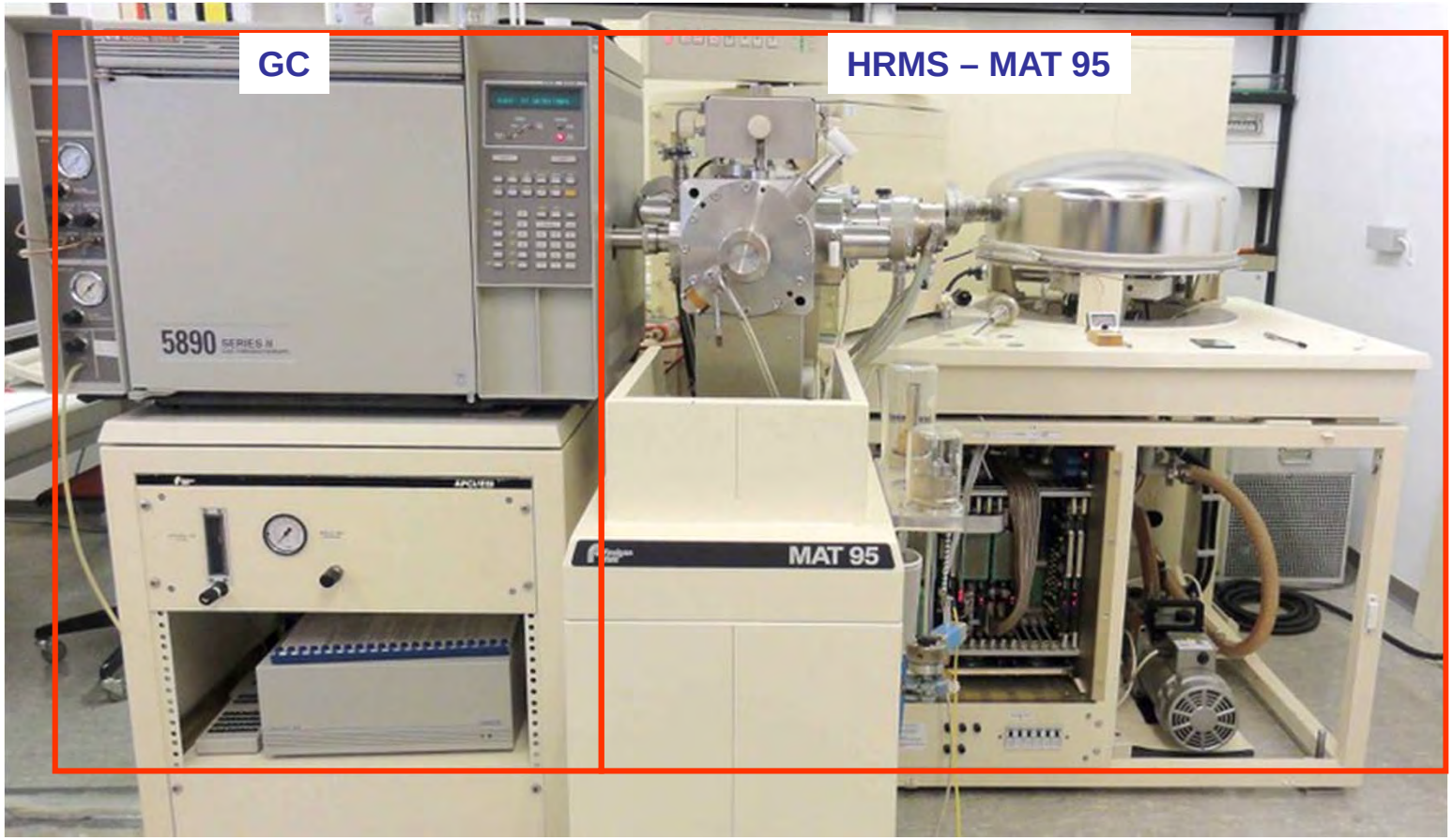
Atlas MAT CH-5 - with On-line-Data System (left) – Gaschromatograph (right)

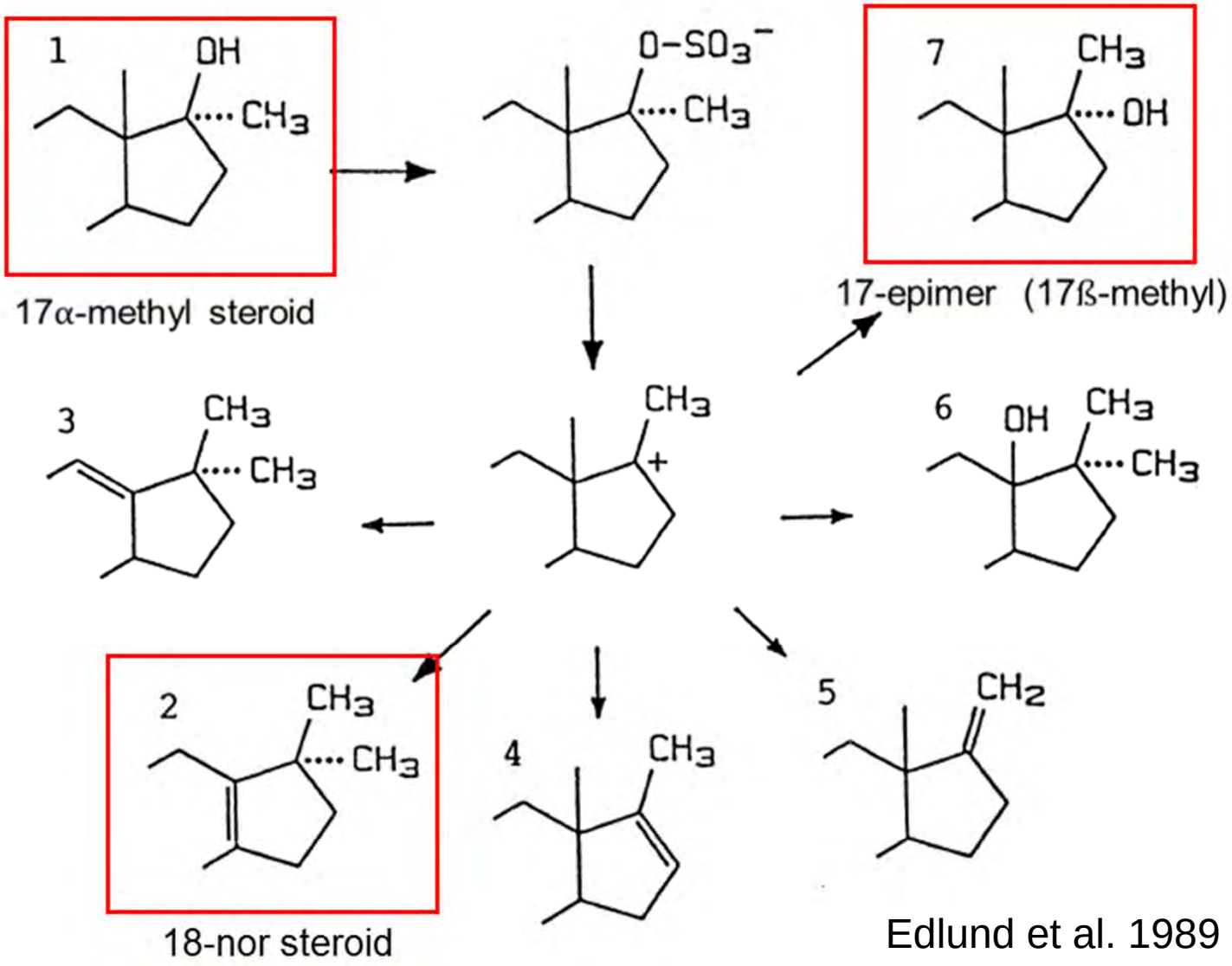


New Doping Methods Doping Control

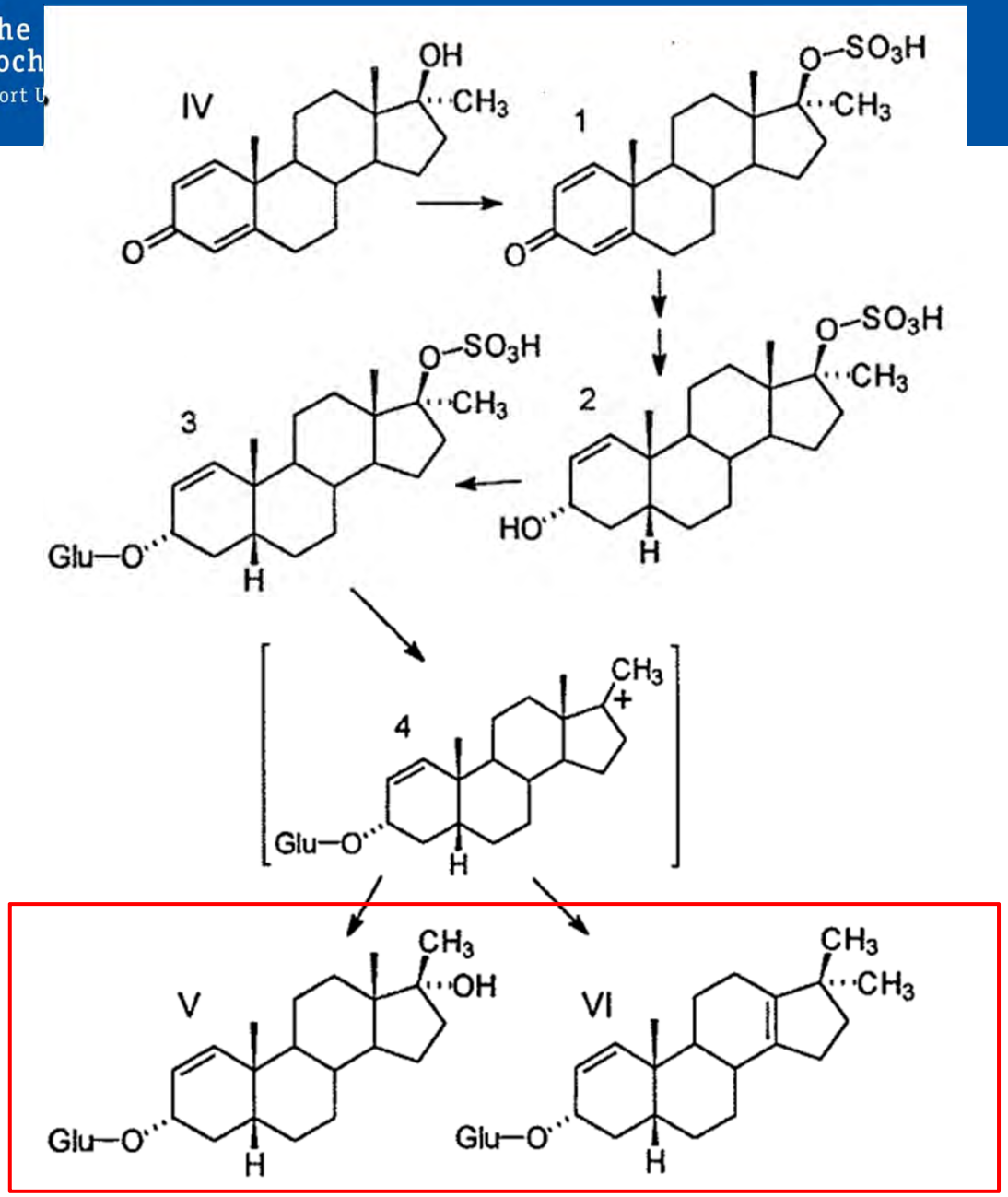
Dope Analyis 1995 – HRMS Instrument

Sektorfeld-MS: Finnigan MAT 95





Edlund et al. 1989


IV
OH
CH3
O
1
O–SO3H
CH3
O
2
O–SO3H
CH3
HO
H
3
O–SO3H
CH3
Glu–O
H
4
CH3
+
Glu–O
H
V
CH3
OH
Glu–O
H
VI
CH3
CH3
Glu–O
H

ELSEVIER

1996

Journal of Chromatography B, 687 (1996) 93–108

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography–high-resolution mass spectrometry

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Reasoning: *„Out of Competition testing is not uniform and international harmony has not been achieved, even for top level athletes.“*

1995

Improved Detection of Anabolic Androgenic Steroids (AAS)

- Improved sensitivity and selectivity by modern HRMS technology
- Identification of long term excreted metabolites

- 116 positive AAS in **1995** from 6700 urine control samples
- 41 AAFs with the conventional GC-MS technique
- 75 AAFs with HRMS (High Resolution Mass Spectrometry)

Table 1 Metandienone and Stanozolol positive A-samples in Cologne 1995 and 1996

Positive A-samples	**1995**	**1996**
All banned substances	155	100
Anabolic androgenic steroids	117	69
Metandienone total	78	28
LRMS	15	7
HRMS*	63	21
Stanozolol total	22	15
LRMS	13	13
HRMS*	9	2

* Metabolites were only detected in screening by HRMS

1996

Pergamon

J. Steroid Biochem. Molec. Biol. Vol. 57, No. 5/6, pp. 363–376, 1996

0960-0760(95)00276-6
0960-0760/96 $15.00 + 0.00

Gas Chromatography/Mass Spectrometry Identification of Long-term Excreted Metabolites of the Anabolic Steroid 4-Chloro-1,2-dehydro-17α-methyltestosterone in Humans

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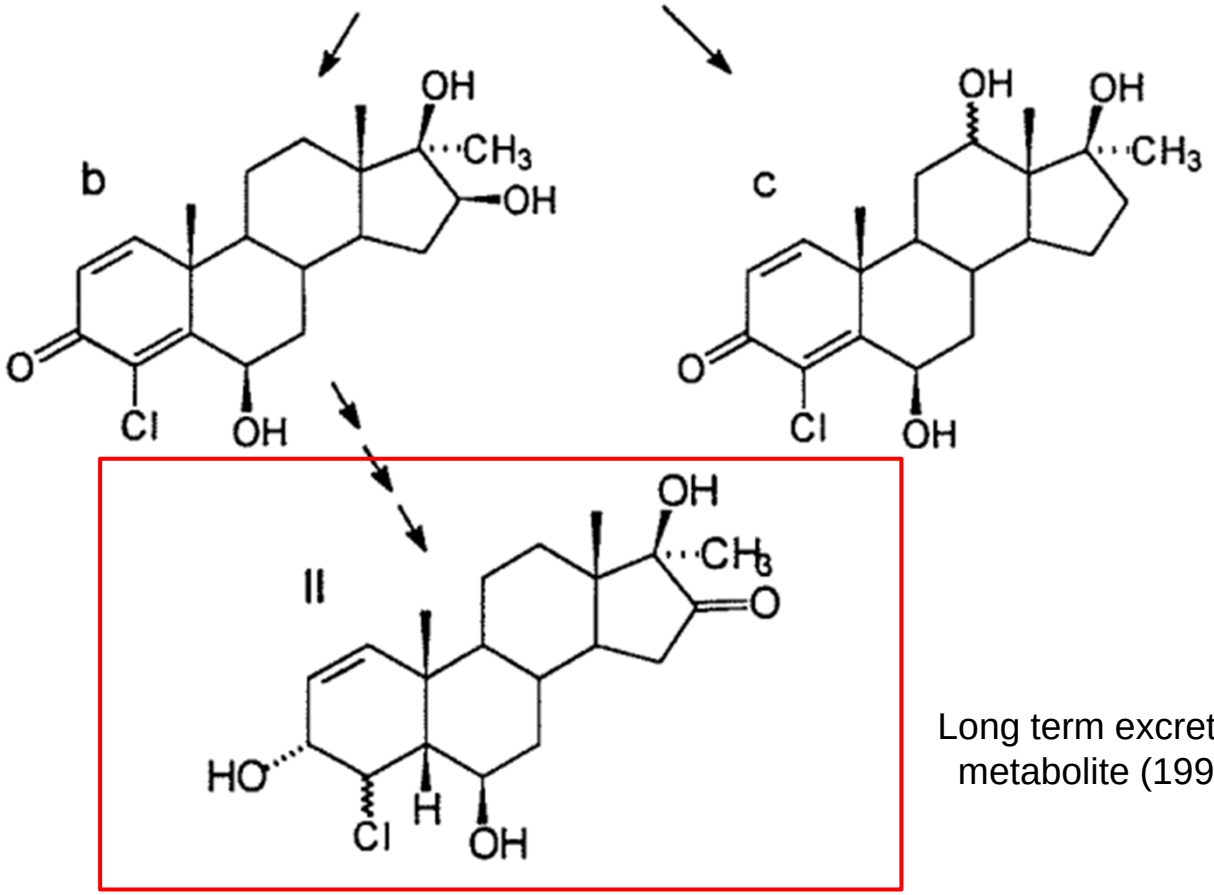


Long term excreted metabolite (1996)

2006

Identification of a Longterm Excreted Metabolite of Metandienone (anabolic steroid)

- **2006 Cologne Laboratory**

- **Additional reporting of about 53 cases for the abuse of metandienone**

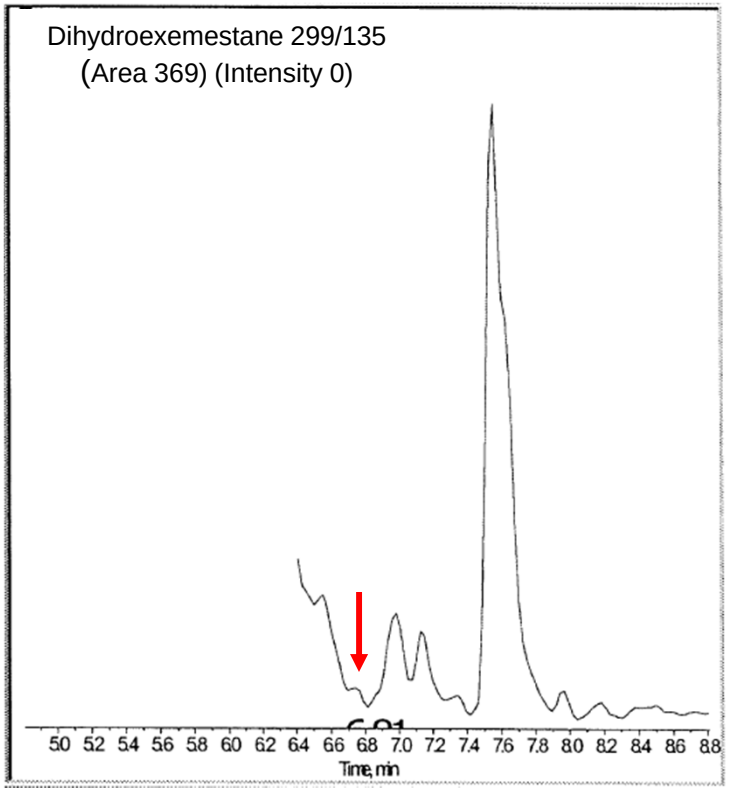
  (would have not been reported with the standard MS procedure)

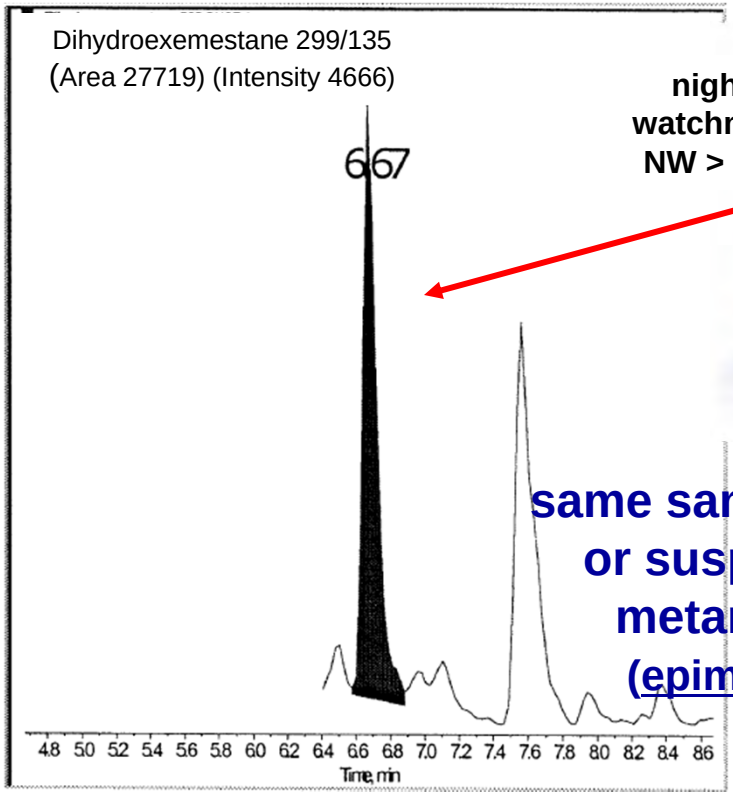
Dope Analysis 2006 – Routine Dope Control

How to detect a new metabolite?

Screening LC/MS-MS for steroids and substances with anti-estrogenic activity

Exemestane > metabolite Dihydroexemestane (M+H)$^+$ 299 > 135



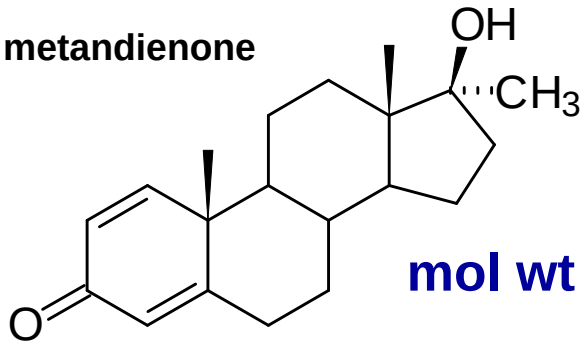


night watchman NW > NV

same sample positive or suspicious for metandienone (epimetenediol)

How to identify a new metabolite?

metandienone



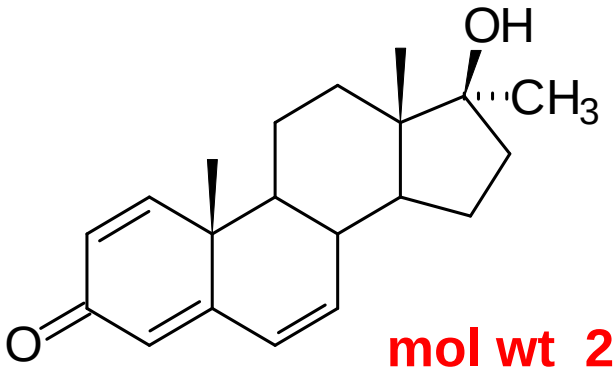
mol wt 300

ESI $(M+H)^+$ 301

↓

299

↓



mol wt 298

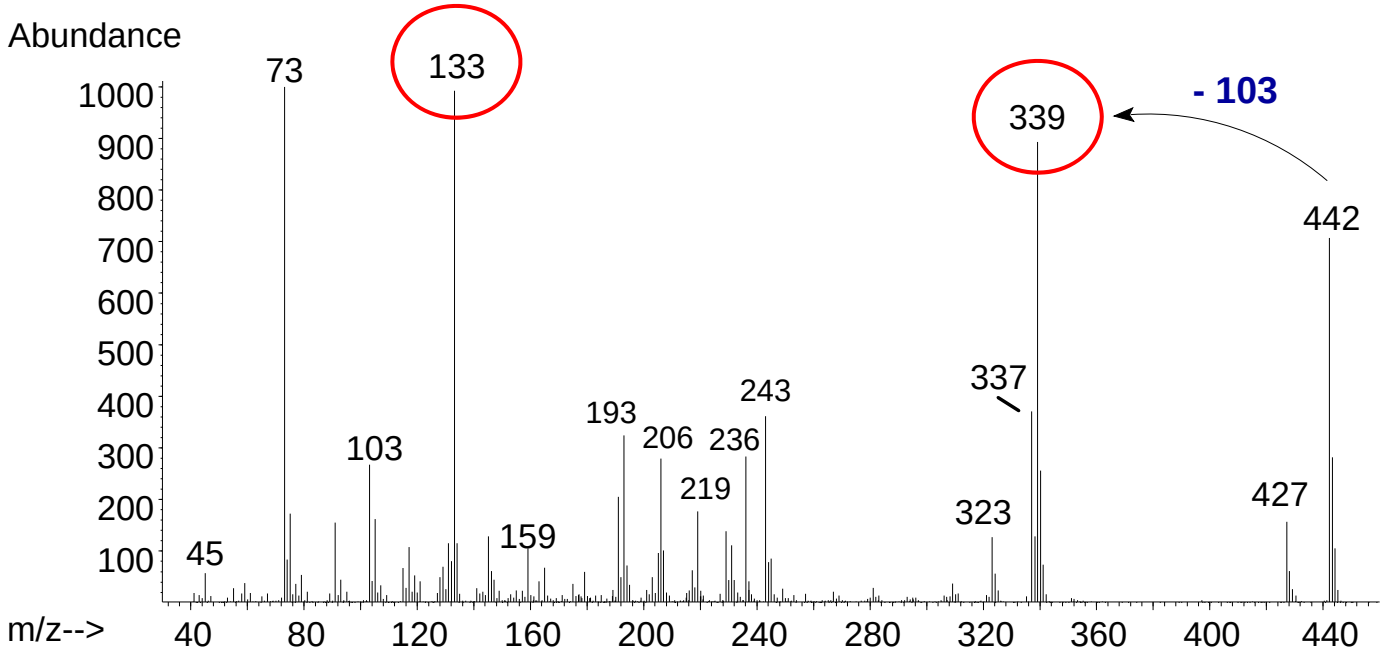
- **did not match with synthesized compounds (Mario Thevis) as 6-ene, 6-ene-17-epi, 9-ene**

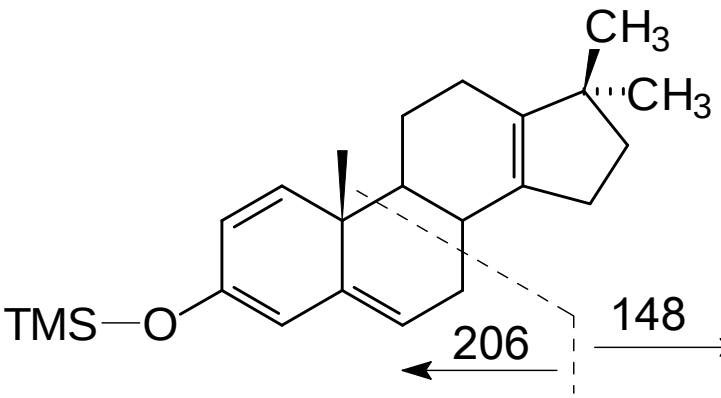
Isolation of 1 L of Urine from a metandienone excretion (20-50h)
- enzymatic hydrolysis
- silica gel
- liquid chromatography

Maxie Kohler

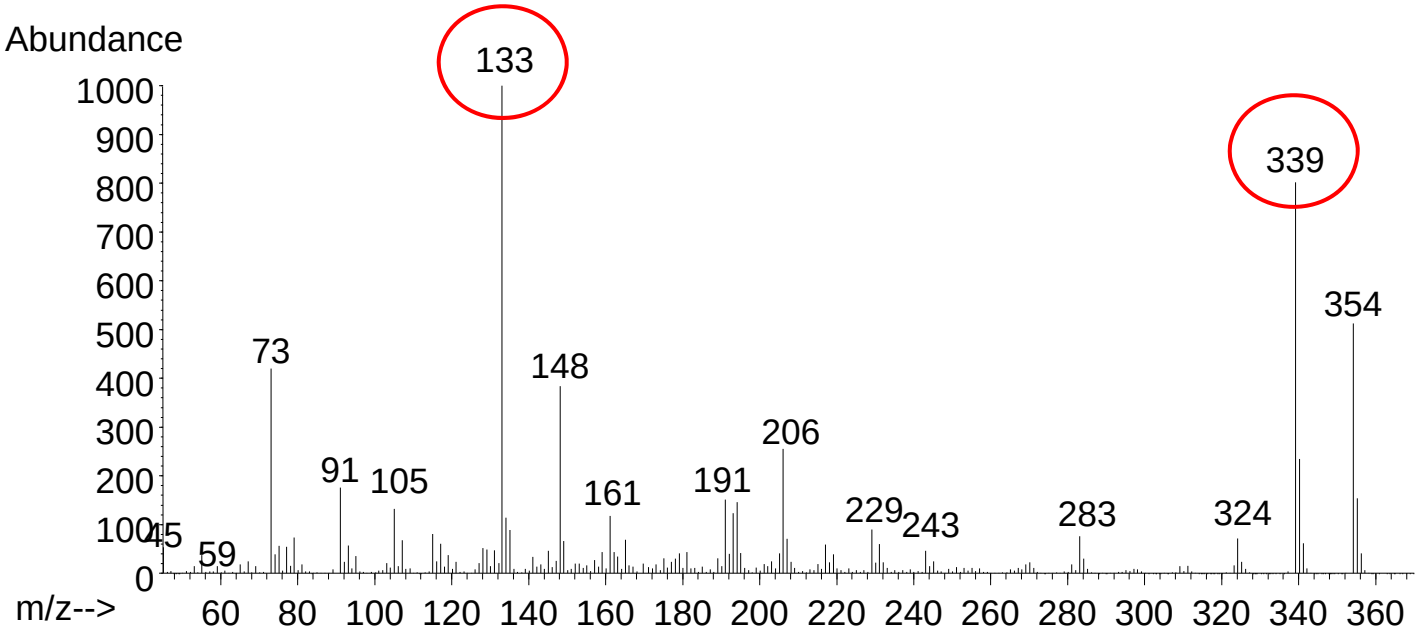
GC/MS-analysis as per-TMS derivative

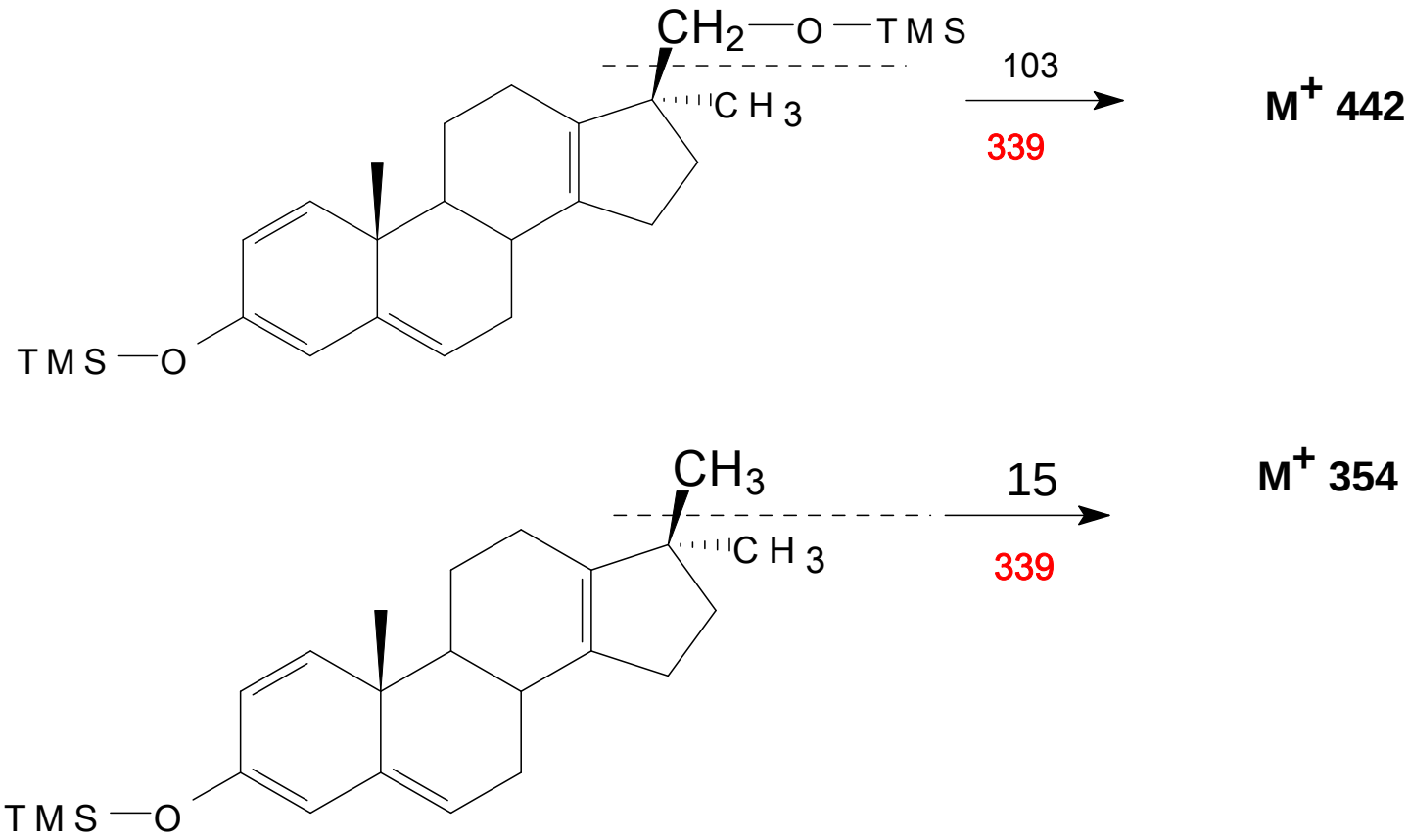
no m/z 143



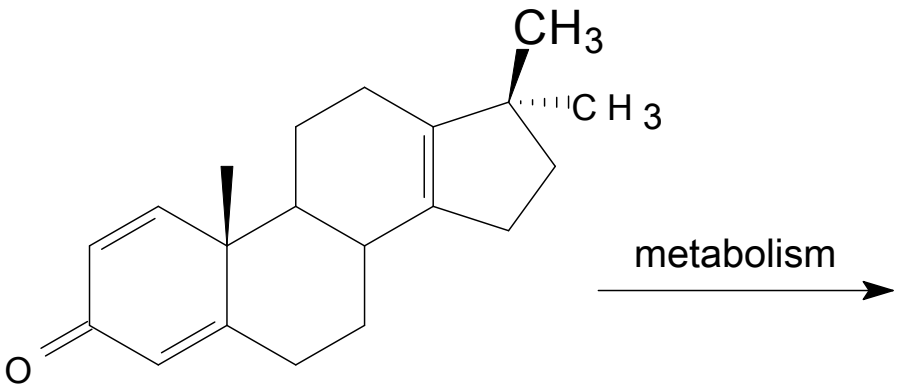


133 fragment of
18-nor-rearrangement product

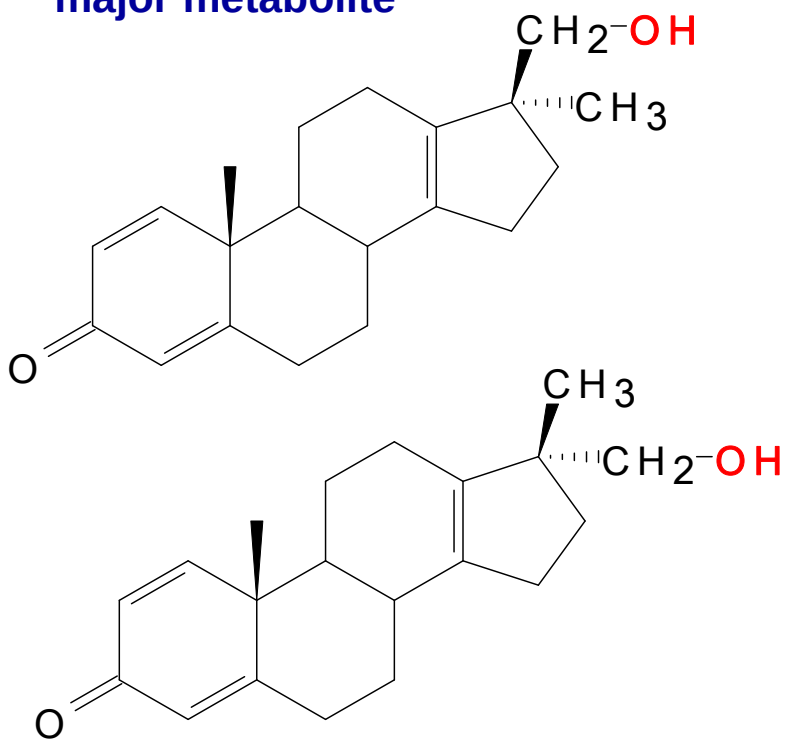



CH2—O—TMS
CH3
103
339
M+ 442
TMS—O
CH3
CH3
15
339
M+ 354
TMS—O

Ref.
Schänzer W, Geyer H, Fußhöller G, Halatcheva N, Kohler M, Parr MK, Guddat S, Thomas A, Thevis M.
***Mass Spectrometric Identification and Characterization of a New Long-Term Metabolite of Metandienone**. Rapid Commun. Mass Spectrom. 10 (2006) 2252-2258*

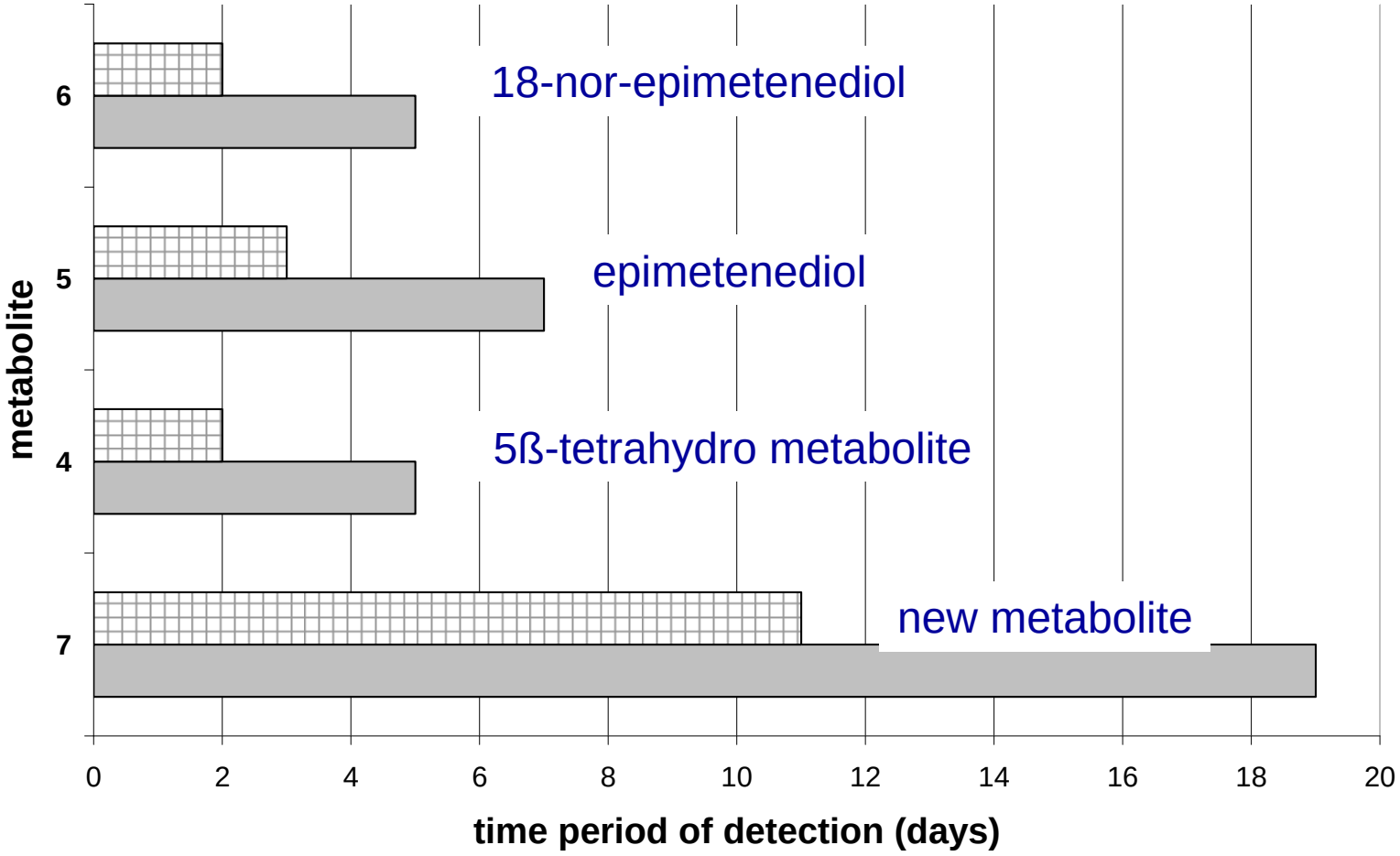


major metabolite



minor metabolite

oral application of 5mg metandienone, 2 volunteers



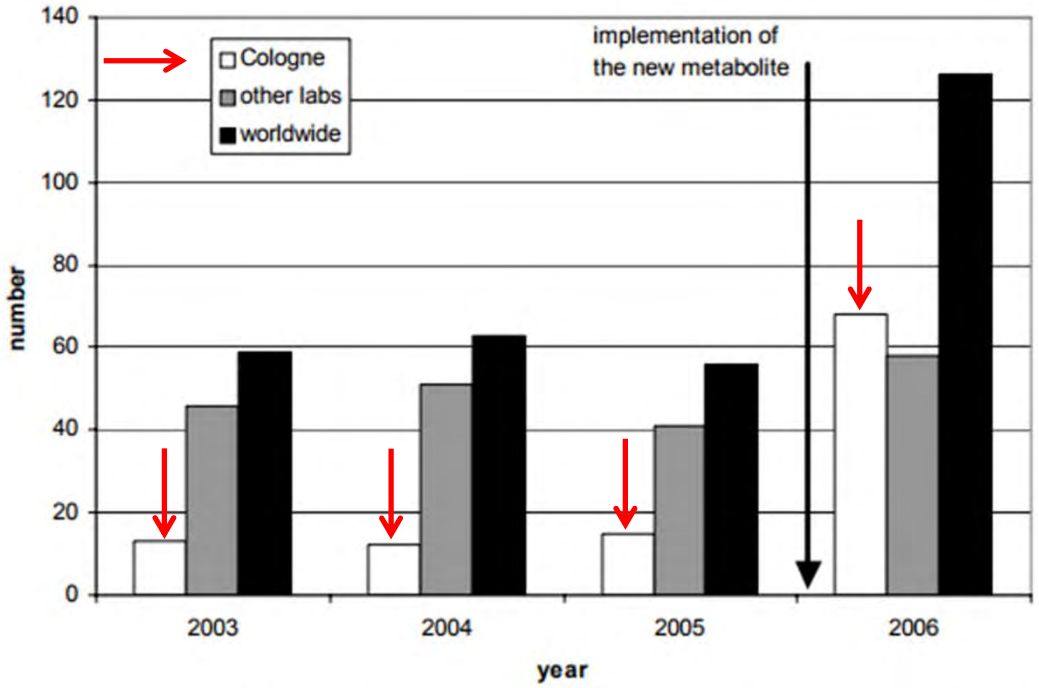


Figure 1: Comparison of the number of metandienone findings in Cologne laboratory with those of the other doping control laboratories.

Ref. Fußhöller G, Mareck U, Schmechel A, Schänzer W Long-term detection of metandienone abuse by means of the new metabolite 17ß-hydroxymethyl-17?-methyl-18-norandrost-1,4,13-trien-3-one Schänzer W, Geyer H, Gotzmann A, Mareck U (eds.) Recent advances in doping analysis (15). Sportverlag Strauß, Köln (2007) 393-396

High Number of AAFs for other AAS

(following the same rearrangement process of 17α-methyl steroids and further hydroxylation)

- **Oxandrolone**
- **Dehydrochloromethyltestosterone (DHCMT)**

2012

Dehydrochloromethyltestosterone (DHCMT) Long term excreted metabolites

Journal of Steroid Biochemistry & Molecular Biology 128 (2012) 121–127

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

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Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine

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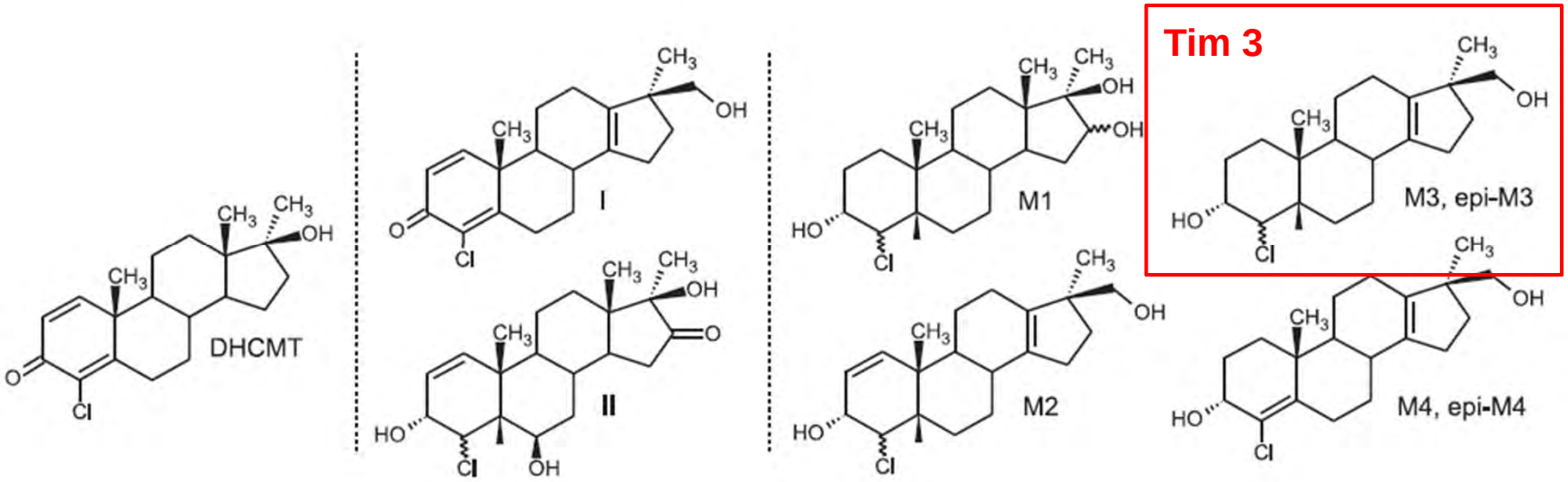
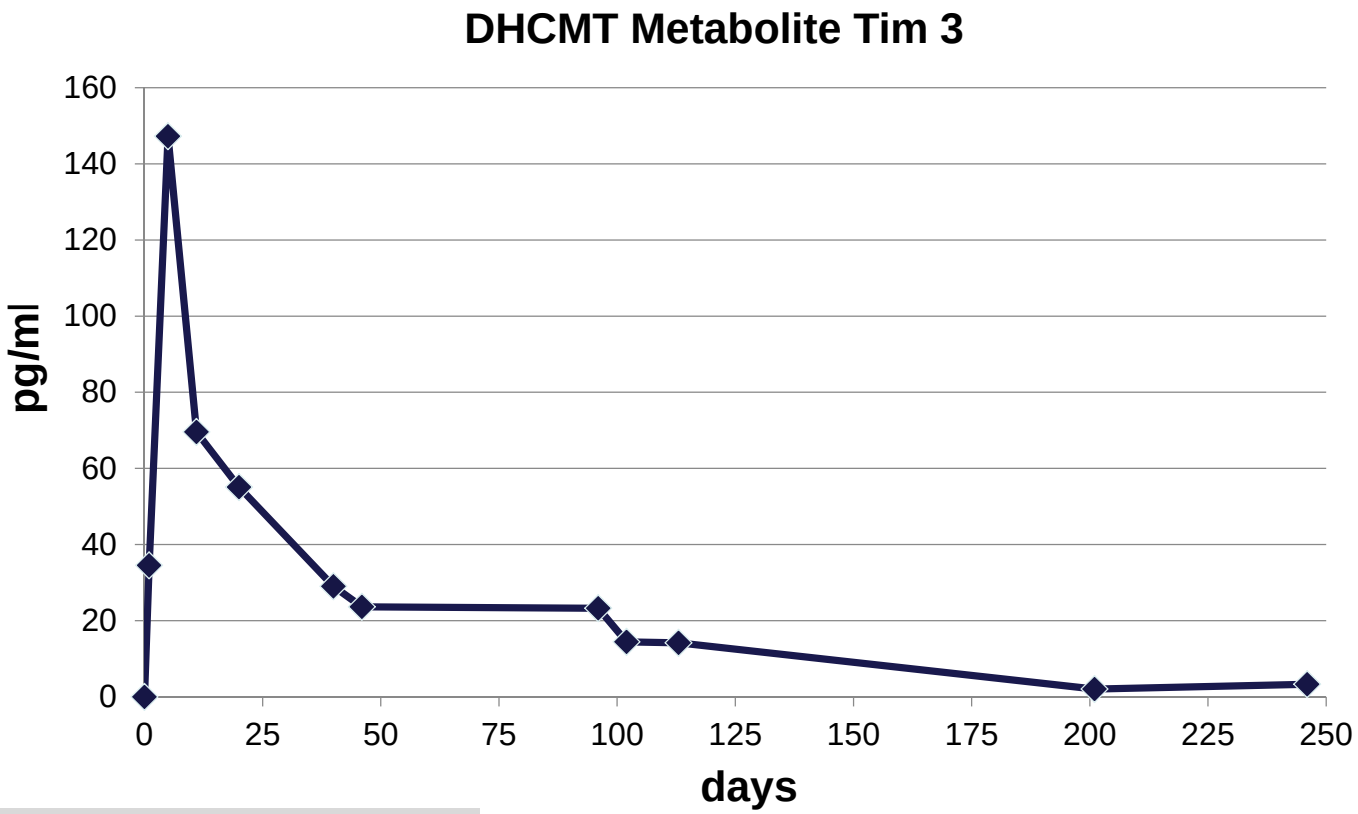


Fig. 2. Structures of DHCMT, its known long-term metabolites (**I** and **II**) and proposed structures of DHCMT metabolites **M1**–**M4** identified after HPLC fractionation of the pooled urine.

Excretion Study: Oral Application 20mg DHCMT



Other steroids	detection time
DHCMT parent	8 days
6ß-HO-DHCMT	3 days
16-Oxo-Metabolite II	18 days

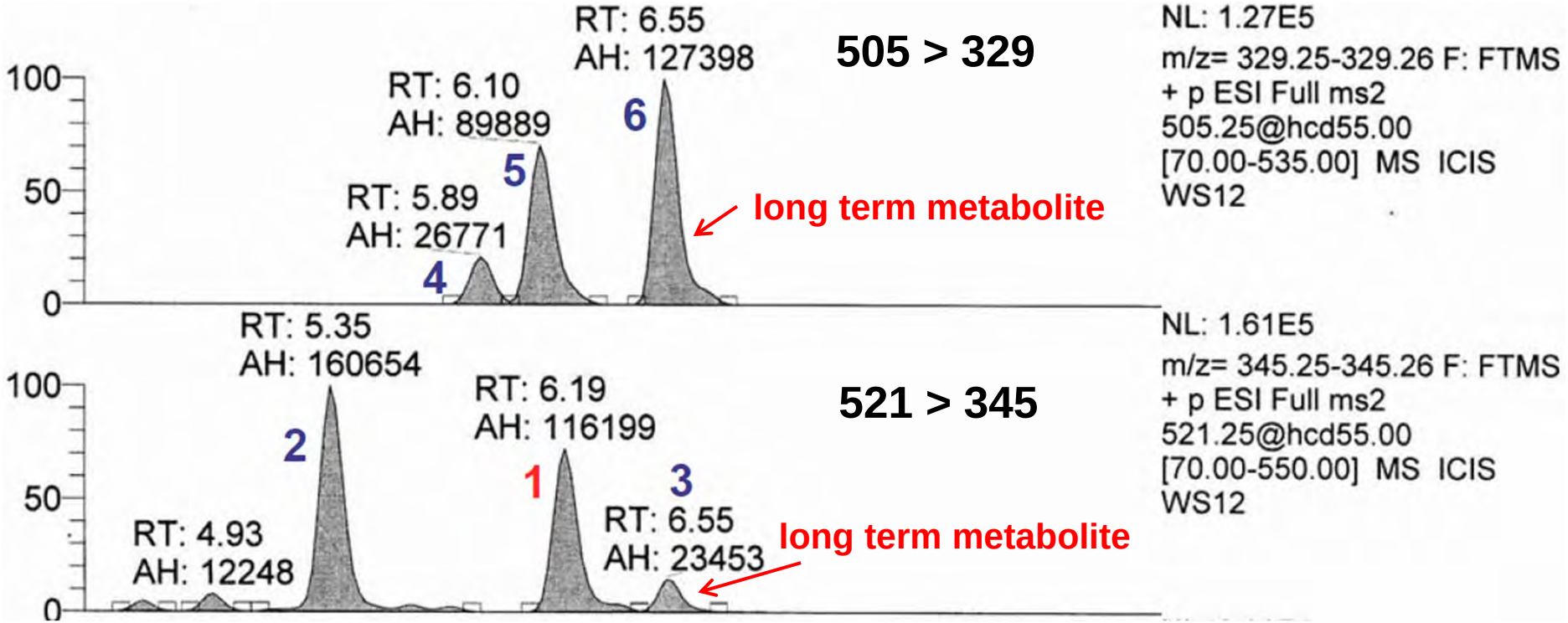
Positive Findings (AAFs) with DHCMT and Stanozolol in Cologne 2011-2014

Year	DHCMT*	LC-MS-MS Stanozolol**	GC-MS-MS Stanozolol
2011	0		12
2012	1	39	15
2013	61	146	4
2014	7	23	1

* Detection of long term metabolite Tim 3 since 2013

** Improvement using LC-MS-MS Q-Exactive with HRMS

Stanozolol – Pattern of Conjugated Metabolites (4 days after 5mg)



4 - Stanozolol-O-glucuronide
5 - Stanozolol-N-glucuronide
6 - Epistanozolol-N-glucuronide
1 - 3‘-Hydroxystanozolol-glucuronide
2 - 16ß-Hydroxystanozolol-glucuronide
3 - 3‘-Hydroxyepistanozolol-glucuronide

2013

The Cologne new strategy to detect long term excreted metabolites

- **GC/TC/IRMS** (TC = thermal conversion)
- **Isotope ratio MS of hydrogen $^{2}H/^{1}H$ (D/H)** (D = Deuterium)
- **Excretion studies with deuterated steroids**
- **The ratio D/H allows a sensitive detection of metabolites containing deuterium**

Research Article

Rapid Communications in Mass Spectrometry

Received: 18 April 2013 Revised: 31 May 2013 Accepted: 4 June 2013 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2013**, *27*, 1904–1912
(wileyonlinelibrary.com) DOI: 10.1002/rcm.6648

Hydrogen isotope ratio mass spectrometry and high-resolution/high-accuracy mass spectrometry in metabolite identification studies: Detecting target compounds for sports drug testing

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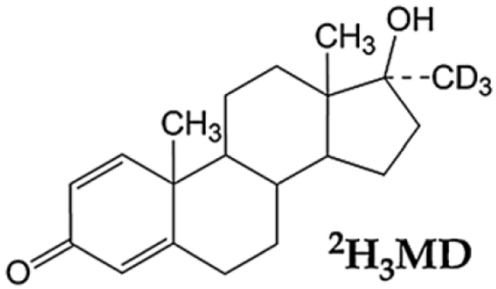
Isotope ratio MS of hydrogen $^{2}H/^{1}H$ (D/H)



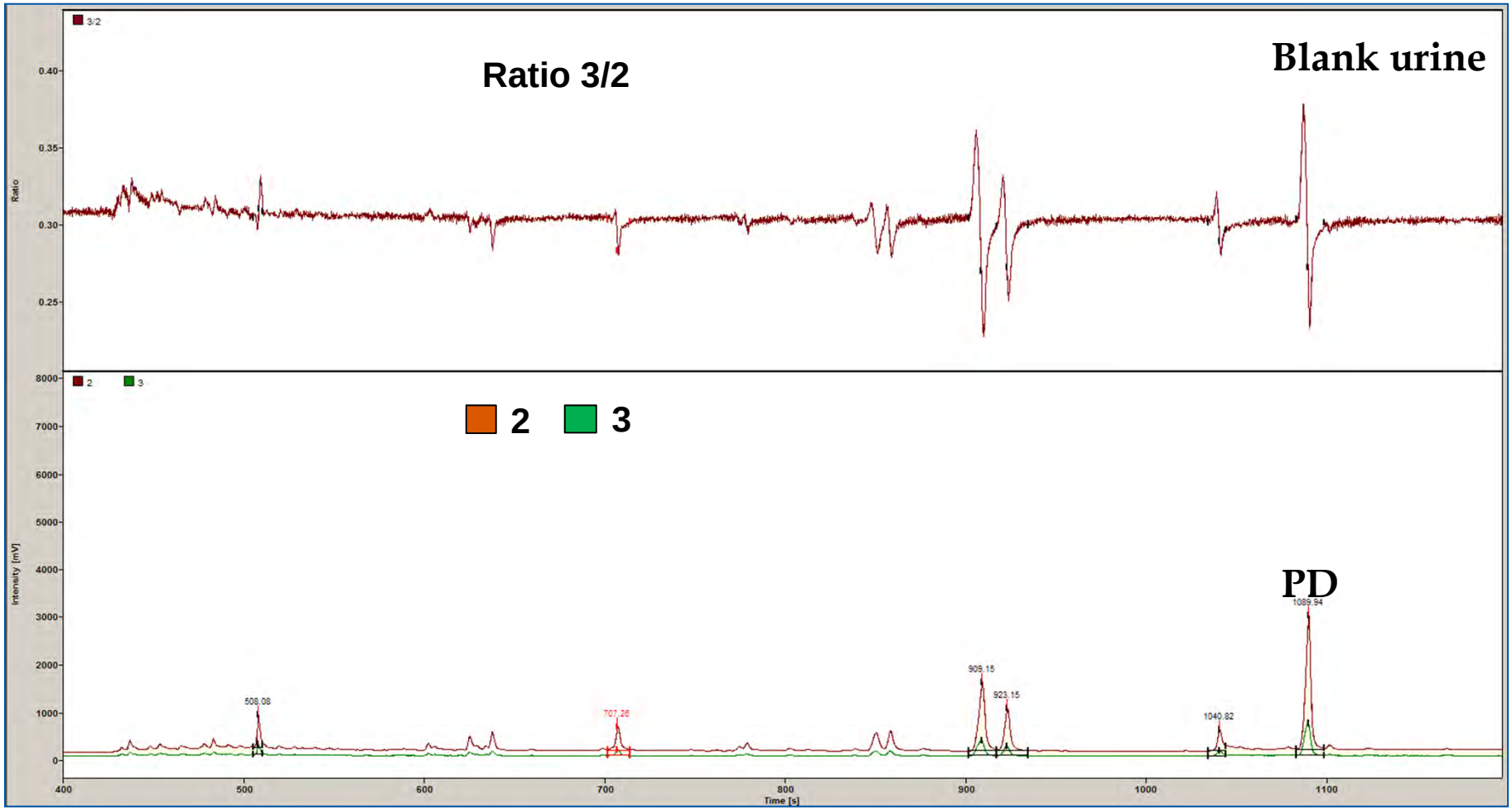
Thermal conversion
pyrolysis

H_2 + HD

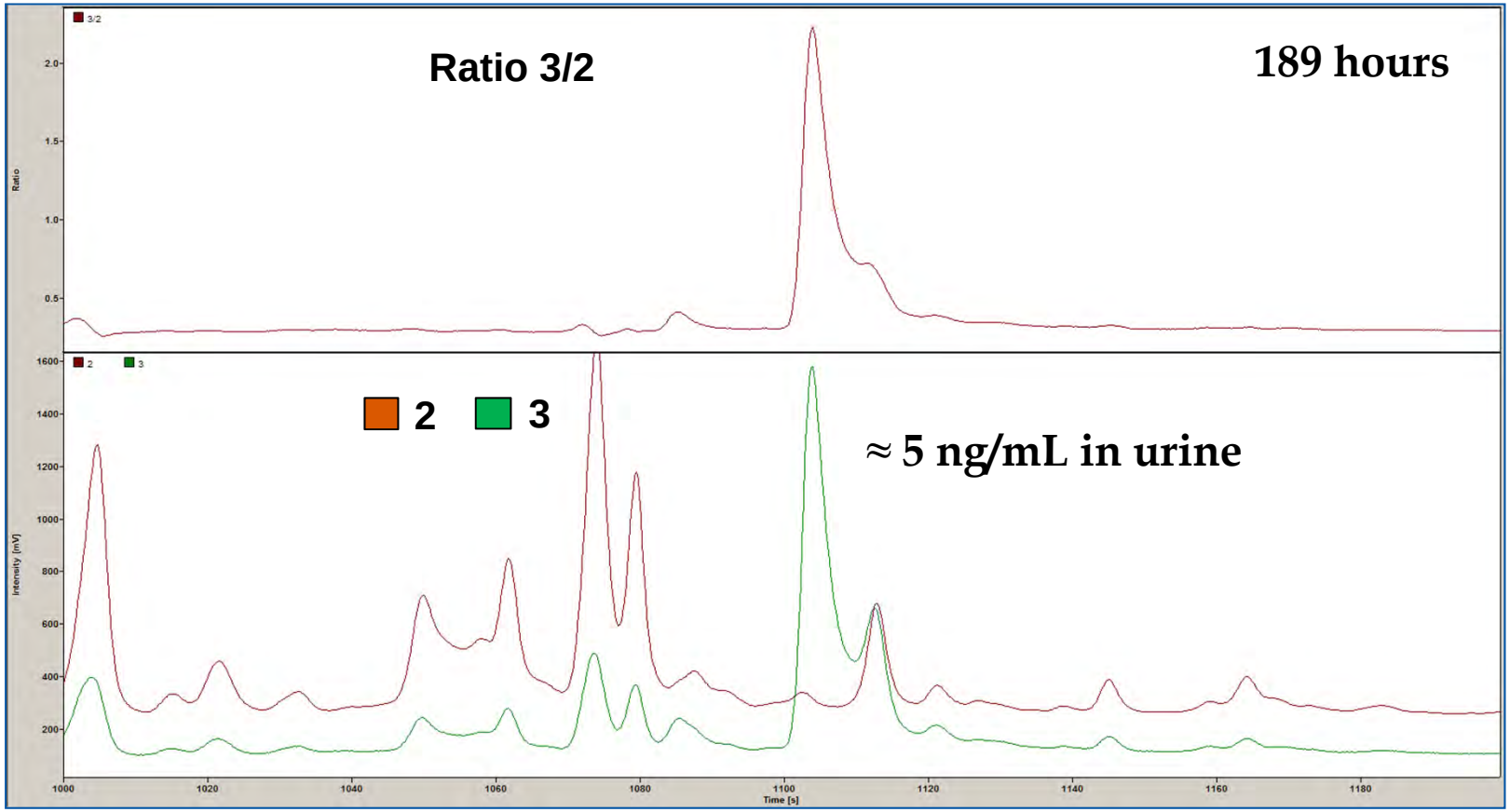
mass **2** mass **3** **ratio 3/2**

D/H ratio normally 1:6500 (0,015%)

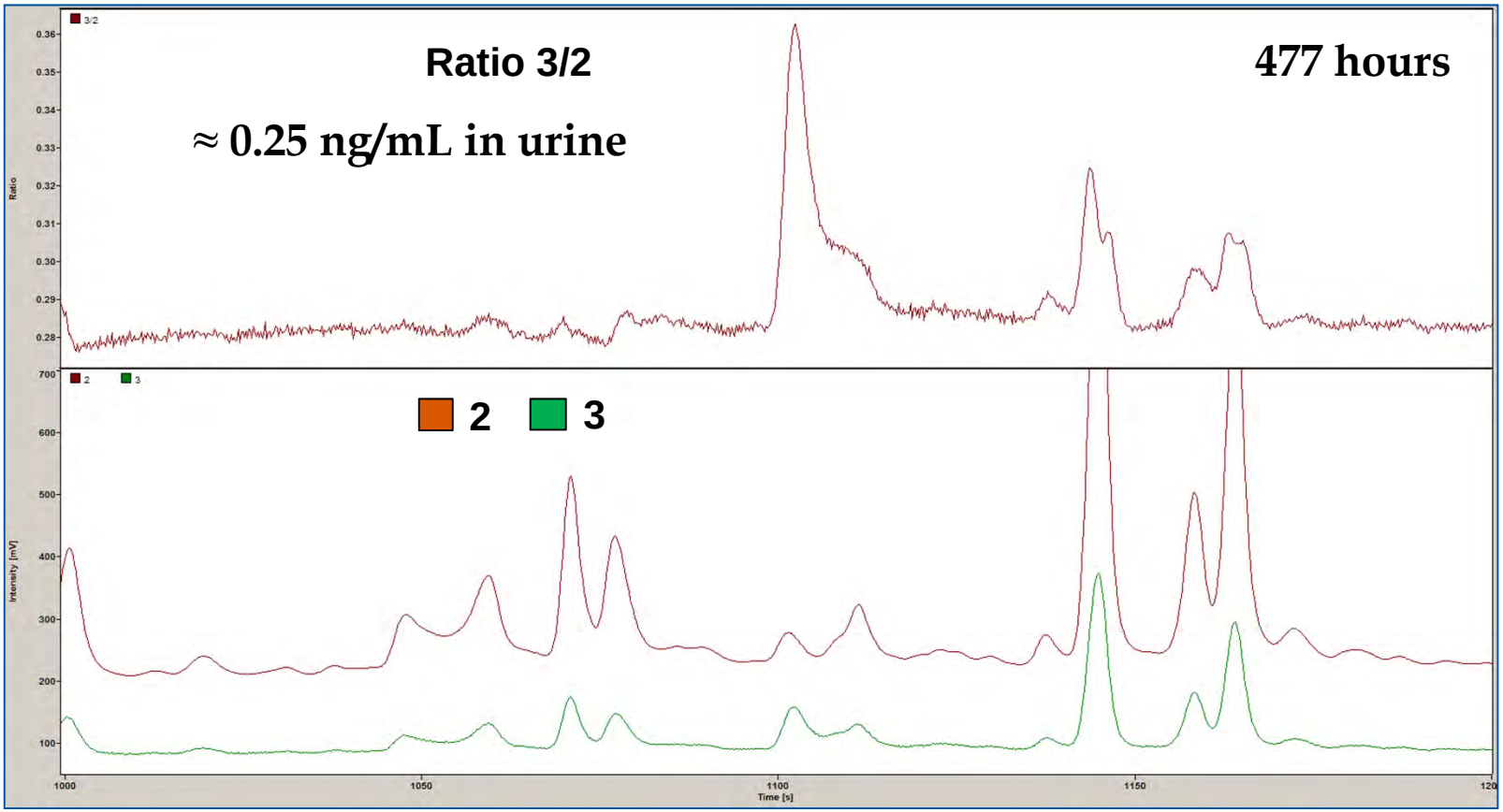
• IRMS results – fraction V



- **Nightwatchman (Gluc)**



- **Nightwatchman (Gluc)**



Summary

- Following the Cologne experience in dope control and anti-doping research we consider the situation (in Germany and several other countries) as follows: